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CHEMICAL MODIFICATION OF NUCLEOSIDES AND CHEMICAL CROSS-LINKING OF POLYNUCLEOTIDES

By

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A thesis submitted for the degree of
Doctor of Philosophy of the
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To my parents

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DECLARATION

This dissertation is submitted to the University of Warwick in support of my application for admission to the degree of Doctor of Philosophy. It contains an account of my work carried out principally at the Department of Chemistry and Molecular Sciences of the University of Warwick during the period November 1976 to June 1980 under the general supervision of Dr. D. W. Hutchinson. No part of this dissertation has been used previously in a degree thesis submitted to this or any other university. The work described is the result of my own independent research except where specifically acknowledged in the text.

Cho Fat Hui

September 1980

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ABBREVIATIONS

A_{280}/A_{260}	Absorbance at 280 nm/absorbance at 260 nm
CD	Circular dichroism
DNA	Deoxyribonucleic acid
DEAE	Diethylaminoethyl
ds	Double stranded
dNDP etc.	Deoxy-nucleoside diphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
mRNA	Messenger ribonucleic acid
NDP	Nucleoside diphosphate
NMP	Nucleoside monophosphate
NTP	Nucleoside triphosphate
poly(I) etc.	Homopolyribonucleotides
poly(I).poly(C) etc.	Base paired hybrid between polyribonucleotides
poly(dI) etc.	Homopolydeoxyribonucleotides
poly(I-C)	Alternating copolyribonucleotide
polyd(I-C)	Alternating copolydeoxyribo-nucleotide
PBS	Phosphate-buffered saline
PNPase	Polynucleotide phosphorylase
RNA	Ribonucleic acid
RNase	Ribonuclease
SE	Sulphoethyl
ss	Single stranded
TCA	Trichloroacetic acid
T_m	Melting temperature
tRNA	Transfer ribonucleic acid

SUMMARY

The methylation of 5-hydroxycytidine with dimethyl sulphate or diazomethane in order to give 5-methoxycytidine was found to be unsuccessful. The methylation reactions gave two products, the 5-methoxycytidine and the dimethylated compound N^4-methyl-5-methoxycytidine. The overall yields of these reactions were low and the two methylated products were difficult to be separated. When an attempt was made to synthesise N^4-acetyl-5-hydroxycytidine, in order to protect the N-4 position from being methylated, it was found that N^4-acetyl C was very labile towards the conditions employed in the hydroxylation reaction. Therefore, another method was employed for the synthesis of 5-methoxycytidine. The first stage of the synthesis of mo^5C was the synthesis of 5-methoxyuracil, followed by the nucleoside synthesis. The next step after the nucleoside synthesis was the synthesis of 4-thio-5-methoxyuridine-2',3',5'-tri-O-benzoate, and finally, the conversion of the 4-thio group to the 4-amino group and the removal of the acyl protecting groups. The 5-methoxycytidine was phosphorylated to give 5-methoxycytidine-5'-diphosphate and polymerised with polynucleotide phosphorylase to give poly(mo^5C). Poly(mo^5C) formed a 1:1 complex with poly(I), and at neutral solution in the presence of 0.1 M sodium chlroide, the T_m value was 64.5°. However, this double-stranded complex was ineffective as an interferon inducer.

Attempts were made to synthesise alternating copoly(I-C) with polynucleotide phosphorylase, and were found to be unsuccessful. Alternating copoly(I-C) was synthesised with DNA-dependent RNA polymerase in the presence of template. Chemical cross-linking experiments of poly(I).poly(C) and alternating copoly(I-C), using a difunctional nitrogen mustard (methyl-1-N-bis(2-chloroethyl)amine hydrochloride) or 8-methoxypsoralen were carried out. In the case

of poly(I).poly(C), no cross-linking was observed in both cross-linking reagents. Alternating copoly(I-C) was cross-linked by 8-methoxypsoralen, and the cross-linked and non-cross-linked species were separated by hydroxylapatite chromatography at room temperature. The effectiveness of this cross-linked polynucleotide as an interferon inducer was found to have decreased.

CHAPTER 1

INTRODUCTION

1.1 Discovery of Polyribonucleotides as Interferon Inducers

Interferon is the name that was given to an antiviral substance produced by the cells of many vertebrates in response to virus infection (Isaacs and Lindenmann, 1957). Although the chemical structure of the interferon molecule has not been determined, it is generally believed to be a glycoprotein (De Clerq, 1974). It is species specific, that is, active only in the cells of the animal species in which it has been induced (Merigan, 1964). Gladsky et al. (1964) have demonstrated that crude interferon of the homologous cell species inhibits the RNA synthesizing activity of extracts of mammalian cells infected with virus. These studies indicate that the species specificity of interferon is not mediated by differences in absorption. However, other studies have suggested that the viral associated polymerase is coded on the viral genome. Hence it is puzzling how interferon can effect a host specific inhibition of this enzyme (Isaacs, 1963). Numerous early hypotheses have been put forward to account for the mechanism of action of interferon (Isaacs and Burke, 1958, Burke, 1961, Wagner, 1960), and De Somer, in 1962, suggested that interferon inhibited the synthesis of viral RNA. However, the actual mechanism of action of interferon has only recently come nearer to being elucidated, and this will be discussed later.

Another question which is of interest concerned the nature of the stimulus that induces cells to make interferon. Since the production of interferon can be initiated by RNA and DNA viruses, and in the absence of virus multiplication, it seems that viral

protein or viral nucleic acid must be the stimulus. Thus, a hypothesis was put forward that stated that the essential stimulus to make interferon might be a nucleic acid that was "foreign" to the cell. The induction of interferon by RNA in cell culture was first reported by Isaacs et al. in 1963, but large amounts of RNA were needed, and all the ribonucleic acids he tested were single-stranded. It was later demonstrated by Lampson et al., and Field et al., in 1967 that various polyribonucleotides, both synthetic and of viral origin, elicited interferon production, but DNA and single-stranded RNA were inactive. These two groups of workers discovered that double-stranded poly(I).poly(C) was highly active in inducing interferon and host resistance in vivo and in vitro, and they concluded that double-strandedness as well as polymer length was important to biological activity. These discoveries made it possible for the first time to study the process of interferon induction with chemically defined molecules in cell cultures. A great number of studies have analyzed the structural characteristics of polynucleotide molecules that act as interferon inducers (Torrence and De Clercq, 1977).

The production of interferon requires an interaction between viruses or polynucleotides and the cell, and a lag period is required both for the appearance of interferon and for the development of resistance. The viral-induced interferon was produced between hours 12-14, and the rate was maximal around hours 12-16 post-infection. Poly(I).poly(C)-stimulated interferon was produced much earlier, maximally 2-4 hours after stimulation (Tan et al., 1970). Therefore, there is a difference between interferon induction by viruses and by polynucleotides. It was further demonstrated by Atherton and Burke (1975) in work with a plant alkaloid, camptothecin, which blocks the formation of

ribosomes. This was used to inhibit interferon production induced by Newcastle disease virus (NDV) or U.V. irradiated NDV in chick and human cells, and by Sindbis virus in chick cells. Camptothecin had no effect on interferon production induced by poly(I).poly(C) in chick and human cells, and no effect of camptothecin could be detected on the multiplication of NDV. At the same time, Mozes and Vilcek (1975) found that in FS-4 human diploid foreskin cells, induced first with poly(I).poly(C) and then with NDV, the response to the latter inducer was clearly altered. Instead of the usual response peak at about hours 12-14, the interferon response to NDV in the doubly induced cells was biphasic, with an early response peaking at hour 5 and a much later response with maximum production between hours 24 and 48. These results suggest that NDV is capable of eliciting two types of interferon responses.

As mentioned previously, poly(dI).poly(dC) and naturally occurring double-stranded DNA are not active as interferon inducers, and poly(I), poly(dC), and poly(dI).poly(C) (Vilcek et al., 1968) have also been shown not to be interferon inducers. These differences in the antiviral activity and interferon inducing capacity among double-stranded RNA and DNA complexes cannot be accounted for by differences in the rates of polymer-cell binding or in the amount of polymer bound to the cells (De Clercq et al., 1972). Nor do differences in the antiviral activity of poly(I).poly(C) in different cell cultures correlate with the polymer-cell binding rates or amounts of polymer bound to these cells (De Clercq and De Somer, 1973). It has been shown in a number of reports that synthetic polynucleotides, whether single- or double-stranded polyribo- or polydeoxyribonucleotides, are bound to or taken up by the cells (Bausek and Merigan, 1969, De Clercq and De Somer, 1972, De Clercq and De Somer, 1973). A variety of radiolabelled

RNA and DNA polymers were studied with respect to the kinetics of their binding to cells and their persistence at the outer cell membrane. The kinetics of cell binding was monitored by the loss of acid-insoluble radioactivity from the supernatant fluid, and persistence of the polymer at the cell surface was determined by measuring the amount of cell bound radioactivity that was released upon treatment by nuclease. Although preincubation of the polymer at 37° increased the antiviral activity, the rate of cell binding and persistence at the cell surface of each individual polymer, when this was compared with their antiviral activities, no direct correlation could be found (De Clercq, 1974).

The first step in interferon production by double-stranded poly(I).poly(C) is the binding of the polymer to the cell surface. This has been demonstrated at 4° and at 37° by using radiolabelled poly(I).poly(C). A second, temperature-dependent step occurs rapidly after cell binding of poly(I).poly(C) and is followed by cellular resistance to virus infection and interferon production. These findings showed that the amount of cell-associated poly(I).poly(C) required for initiation of interferon production was not the same in different cell systems and much of the cellular uptake of poly(I).poly(C) was not essential for interferon production. After 1 hour of incubation at 4°, enough poly(I).poly(C) became cell-associated to mediate the subsequent production of significant amounts of interferon, and 20-fold more poly(I).poly(C) was associated with cells after incubation for 8 hours at 37°. At this temperature, a very small amount of cell-bound poly(I).poly(C) penetrated into the cell, and it was suggested (Bausek and Merigan, 1969) that this fraction might correspond to the active principle initiating interferon production inside the cell, but its magnitude would be small in comparison with the total amount of

cell-associated poly(I).poly(C). Autoradiography of human fibroblasts incubated for various intervals with this radiolabelled poly(I).poly(C) at 37° showed that poly(I).poly(C) was degraded in cells and that the fragments were utilized for cellular RNA synthesis. Such utilization could be blocked by treatment of cells with actinomycin D and cytidine without decreasing interferon production (Bausek and Merigan, 1969). Later, Pitha et al., in 1972, examined the effect of the length of time of exposure of cells to poly(I).poly(C) on interferon induction in order to clarify the nature of "triggering". The results showed that at 37°, exposure of cells for 1 minute to a given concentration of poly(I).poly(C) was sufficient to initiate the maximum protection against virus. When cells were exposed to poly(I).poly(C) at 4° for various times, then washed, and incubated in fresh medium at 37°, the percentage of cells resistant to virus was similar to that obtained at 37°, although the rate of the reaction was decreased, and maximum effects were not reached until 10 to 15 minutes. This delay was probably due to the temperature dependent diffusion of poly(I).poly(C) molecules to cell surfaces. Experiments using radiolabelled poly(I).poly(C) by these workers demonstrated that the attachment of poly(I).poly(C) to cells was sufficiently strong to be uninfluenced by its total removal from the medium within several minutes. More recently, Bradshaw et al. (1979) found that, after human osteogenic sarcoma cells (MG63) were treated with poly(I).poly(C) of high specific radioactivity at 4°, neither ribonuclease A and T_1 treatment nor salt washing were sufficient to prevent interferon formation even though a substantial proportion of the radioactivity was removed by these treatments (92% and 44% respectively). The production of interferon was associated with the presence of radioactivity within the cells. It was also found that a

non-inducing polynucleotide [poly(dI).poly(C)] and colloidal gold, which was known to be taken up by a micropinocytosis process, were rapidly taken up by MG63 at 4°. Thus, Bradshaw et al. (1979) concluded that the polynucleotides entered the cells by a non-specific process at 4°, and that poly(I).poly(C) was an active inducer of interferon because of a structurally specific process that followed uptake.

Prose et al., in 1970, attempted to elucidate the receptor site of polynucleotide in cells. The uptake of the tritiated poly(I).poly(C) by cultured rabbit kidney cells was observed by an electron microscope. The results indicated that the polyribonucleotide was phagocytized, and was distributed in the cytoplasm, nucleus, and nucleolus with no evidence of preferential localization. Vengris et al. (1975) attempted to solve the same problem by using antisera which were selectively reactive with the inducer poly(I).poly(C), the product (human fibroblast interferon), and the fibroblast cell surface. The anti-interferon serum completely neutralized the antiviral effect of human fibroblast interferon, and the antibodies specific to double-stranded RNA inhibit the antiviral activity of poly(I).poly(C). But, treatment with serum directed against the cell surface of human fibroblasts failed to inhibit the antiviral activity of poly(I).poly(C) or human interferon in these cells. Therefore, the results indicated that the antiviral effect of poly(I).poly(C) was interferon mediated, but failed to show that the receptor sites of poly(I).poly(C) and interferon were on the cell surface membrane.

Havell et al. in 1975 demonstrated that human cells could produce two different species of interferon. The rabbit antisera prepared against interferon produced in human fibroblast cell cultures stimulated with poly(I).poly(C) neutralized the activity

of interferon preparations produced in various human fibroblast cultures stimulated either with poly(I).poly(C) or with virus, but failed to neutralize activity of interferon produced in cultures of human leukocytes. On the other hand, a preparation of antiserum against leukocyte interferon was active against both leukocyte and fibroblast interferons, and the existence of two distinct antibody populations in this antiserum was shown by an affinity chromatographic technique. Therefore, they concluded that the heterologous neutralizing activity of sera from rabbit immunized with leukocyte interferon was likely to be due to the presence of two antigenic-distinct species of interferon. These findings were further studied by Cavalieri et al. in 1977 who isolated mRNA from induced human fibroblasts and lymphoblastoid cells and studied the products of translation in Xenopus laevis oöcytes. The mRNA from the respective cells yielded translation products in oöcytes, that was characteristic of the cells from which the mRNA was derived. It was concluded that human cells contained at least two structural genes for interferon, coding for polypeptides differing in primary sequence.

1.2 Structural Requirements for Interferon Induction by Polynucleotides

It is generally agreed that one of the most stringent requirements for interferon inducing capacity of polynucleotide inducers is double-strandedness (Field et al., 1968, Torrence and De Clercq, 1977). This definition is frequently accompanied by other operational criteria, namely, high molecular weight (Tytell et al., 1970, Mohr et al., 1972, Carter et al., 1972), high T_m (thermal stability) (Colby and Chamberlin, 1969, De Clercq et al., 1970), resistance to nuclease degradation (De Clercq and Stewart, 1974) and presence of 2'-hydroxyl groups (Colby and Chamberlin, 1969, Steward et al., 1972,

De Clercq, 1974, Ng and Vilcek, 1972). It is also known that triple stranded polynucleotides are not effective interferon inducers (De Clercq et al., 1974a, De Clercq et al., 1974b, Torrence and Witkop, 1975). It was suggested that poly(A) and poly(U) tended to rearrange to poly(A).2poly(U) under physiological conditions, and poly(A).poly(br^5U) behaved likewise, and they were totally inactive as interferon inducers (Torrence and De Clercq, 1977). Preincubation at 37° rendered various alternating ribonucleotide copolymers and homopolyribonucleotide complexes significantly more active in reducing vesicular stomatitis virus plaque formation in human skin fibroblasts (De Clercq et al., 1971). Poly(A-U) reduced plaque formation in this system at ≥ 1 µg/ml before incubation at 37° and at 4×10^{-6} to 10×10^{-6} µg/ml after preincubation at 37°. It was suggested (De Clercq et al., 1971) that the activation at 37° might have occurred through slippage of a branched helical structure to a longer unbranched structure. According to Scheffler et al. (1968) chain slippage (movement of one complementary strand relative to its partner) might occur by either complete unwinding of a single hairpin helix followed by a double straight-chain helix formation among two such unwound helices, or the reversal of branch formation. Therefore slippage might rearrange the base pairing so that the number of unpaired regions in the interior of the helix was reduced. Three analogues of poly(I).poly(C) were studied by Pitha and Pitha (1971). First, the poly(I).poly(CU), which had the helical structure of the poly(I).poly(C) complex, but interrupted by loops containing the uridylic residues, the antiviral activity of this complex is negligible. In the second analogue, poly(I).poly(C^{Ac}), approximately 30% of the amino groups of the cytosine residue had been acetylated. Such substitution did not interfere with base pairing, and the resulting helix was slightly more stable than that of

poly(I).poly(C), with the helical backbone not interrupted by any loops. The antiviral activity of this acetylated complex was only slightly decreased in comparison to that of poly(I).poly(C). In the third analogue, the poly(C) component had 2' → 5' phosphodiester linkages randomly distributed between the normal 3' → 5' linkage, and the antiviral activity of this complex poly(I).poly($C^{2' \rightarrow 5', 3' \rightarrow 5'}$) was negligible. All these experiments provide evidence for the importance of double-strandedness of polynucleotides, and the correct shape of the helix.

Interferon-inducing activity can be found with a variety of double-stranded helical polynucleotides, provided that all the sugar-residues are ribose (Colby and Chamberlin, 1969). It was later demonstrated by Steward et al. (1972) that replacement of 2'-hydroxyls with 2'-acetoxy groups on polyribonucleotides invariably resulted in loss of antiviral activity, despite the fact that 2'-O-acetylated poly(C) was found to be considerably more resistant to digestion with ribonuclease than its parent molecule with free 2'-hydroxyls. Therefore, they concluded that the 2'-hydroxyl group played an important role in determining the antiviral activity of polynucleotides. Similar results were obtained when the 2'-hydroxyl groups of poly(I).poly(C) were methylated (Merigan and Rottman, 1974), but they also found that the introduction of 2'-O-methyl groups in the poly(C) strand of the duplex impaired the interferon stimulation capacity more than a similar modification of the poly(I). Purified antibody to poly(A).poly(U) was used by Johnston et al. (1975) in quantitative microcomplement fixation assays to detect conformational variations among several double-stranded polynucleotide analogues of poly(A).poly(U) or poly(I).poly(C) that had been previously evaluated for their ability to induce interferon. It was found that modification at the ribose 2'-position of one or both strands resulted in a dramatic decrease

in serological reactivity, and most modification of the bases caused small serological changes, but no base modification caused complete loss of serological reactivity. This reaction pattern supported the conclusion that the structure of the ribose and the overall conformation of the helix are critical in the interferon-inducing ability of the polynucleotide (Johnston and Stollar, 1978). Also, it was found, by studying poly(2'-chloro-2'-deoxyA) and poly(2'-bromo-2'-deoxyA), that 2'-substituents exerted significant effects on the thermal stability of these polynucleotides, though the overall conformational structure was not greatly changed (Ikehara et al., 1977). In contrast to all the above findings, De Clercq et al., in 1978, found a particular analogue of poly(I).poly(C) in which the 2'-hydroxyls of the purine nucleotide strand were replaced by azido groups, poly(dIn_3).poly(C), to be highly effective in inducing interferon, while various other 2'-azido analogues of poly(I).poly(C) and poly(A).poly(U) were inactive as inducers of interferon. The most remarkable feature of the results reported here is the interferon-inducing ability of poly(dIn_3).poly(C) which equalled that of poly(I).poly(C), at least in human diploid cell cultures. Poly(dIn_3).poly(C) represents the first fully 2'-modified double-stranded RNA which has been found to be an effective interferon inducer. This finding indicates that the presence of free 2'-hydroxyl groups in both strands is not an absolute requirement for the interferon-inducing capacity of double-stranded RNA complexes and also the receptor site for interferon induction does not specifically recognize the 2'-hydroxyls per se, but rather the steric configuration conferred by the presence of them. Fukui et al. (1977) have showed that there is close resemblance in the Circular Dichroic spectra of poly(dIn_3).poly(C) and poly(I).poly(C), and the double-stranded character of poly(dIn_3).poly(C) was confirmed

by U.V. absorbance mixing curves and the monophasic melting profile, and was further ascertained by the reactivity of poly(dIn_3).poly(C) towards antibodies to double-stranded RNA. Therefore, it is apparent that the steric configuration resulting from substitution of 2'-azido for 2'-hydroxyl in the poly(I) strand of poly(I).poly(C) is also recognized by the interferon-induction receptor.

In order to study the importance of the intactness of the double-stranded complex in interferon induction, Carter et al. (1972) interrupted the poly(I) or poly(C) strand in the complex either by unpaired base (U or G) or by bond breakage, and looked at their effectiveness in interferon induction. They found that the imperfect complexes had T_m values substantially higher than 37° and could be protected from nucleases by complex formation with polylysine. The poly($I_{39}U$).poly(C) and poly($I_{21}U$).poly(C) complexes were found to have little induction ability, while poly(I).poly($C_{20}G$), poly(I).poly($C_{22}U$), poly(I).poly($C_{13}U$) and poly(I).poly(C_7U) were found to be active. Therefore they concluded that, for interferon induction, the structural requirements in strand continuity and base-pairing are apparently more stringent in the poly(I) strand than in the poly(C) strand. In fact, it has been shown by Pitha and Pitha (1971) that poly(C) may be replaced by a distantly related analogue poly(1-vinylcytosine) without significant loss of activity in vitro. This polymer has three major differences compared to poly(C). Firstly, the cytosine strand lacks the regular ribose sugar-phosphate backbone and does not carry any electric charge. Secondly, the complex contains four cytosine residues for each inosine residue, and therefore must display some looping in the poly(1-vinylcytosine) strand. Thirdly, the complex has a tendency to form large aggregates. It was postulated that the high activity was related to the reduction of the charge/mass ratio and to the

existence of this complex in an aggregate state, and these two factors enhanced the uptake of complexes by cells. Several other reports also pointed to a differential importance of poly(I).poly(C), in the process of interferon induction by poly(I).poly(C). When poly(I) and poly(C) were administered separately to the cell cultures *in vitro*, a significantly greater interferon response was obtained in cell-cultures exposed to poly(I) followed by poly(C) than in cell-cultures exposed to poly(C) followed by poly(I). Priming of cells with poly(I) followed by treatment with poly(C), gave a consistently greater antiviral activity than poly(I).poly(C) itself (De Clercq and De Somer, 1971), and similar results were obtained *in vivo*. It was suggested that poly(I) and poly(C) added successively to cell cultures did not act independently but reunited at the cellular level, most probably at the outer cell membrane (De Clercq and De Somer, 1972; De Clercq *et al*, 1973). Johnston *et al*. (1976) also found that human embryonic fibroblast produced interferon when incubated at 37° after being treated at 4° with poly(I).poly(C), either by addition of the double-stranded duplex or by sequential addition of poly(I) than poly(C). Cells which had been incubated with double-stranded poly(I).poly(C) could be prevented from forming interferon by washing the cells with high concentrations of salt, or by incubation of the cells with single-stranded polynucleotides, immediately after absorption of polynucleotide. This inhibition was probably due to the displacement of the inducing molecules from the cell surface. But, interferon production by cells treated sequentially with poly(I) and poly(C) was not inhibited by either of these treatments, and the polynucleotides were not easily displaced from the cell surface. When two treatments were employed together, only two-thirds of the labelled polynucleotide was displaced,

but the remaining polynucleotide was unable to induce the formation of interferon, presumably because it was bound to the surface sites which could not trigger interferon formation. A similar conclusion was reached by Pitha et al. (1974) who showed that the interferon response was destroyed by treatment of cells with either neuraminidase or phospholipase C without any significant effect on the binding of poly(I).poly(C)-DEAE-dextran complex.

Amongst the complementary polynucleotide duplexes studied so far, only poly(I).poly(C) behaves invariably as a potent inducer with poly(A).poly(U) definitely less effective, and there is considerable disagreement in the literature regarding the inducing ability of poly(G).poly(C) (Colby and Chamberlin, 1969, Novok-Latsky et al., 1975, Torrence and De Clercq, 1977). Other double stranded complexes such as poly(c^7A).poly(U), poly(c^7A).poly(rT), poly(c^7A).poly(br^5U), poly(Lys)poly(C) and poly(Lys)poly(br^5C) were found to be inactive in interferon induction, although these complexes partly or completely fulfilled the necessary criteria (De Clercq et al., 1974b, Torrence et al., 1975). On the other hand, Thang et al. (1977) reported that two preparations of poly(I) exhibited an unusually high interferon inducing activity both in vitro and in vivo. All batches of poly(I), inducers or not, had similar U.V. spectra and C.D. spectra, and these indicated that all the poly(I) samples examined could be considered as identical as far as overall structure is concerned. The only significant differences were observed in the recognition of the poly(I) samples by antibodies to either double-stranded RNA or to poly(I). The two particular poly(I) preparations which induced the formation of interferon did react with antibodies to double-stranded RNA, while non-inducer samples did not (Stollar et al., 1978); thus it was suggested that a portion of the poly(I) could be self-structured in a highly stable hairpin conformation.

Moreover, Marcus and Sekellick (1977) reported that a non-replicating defective interfering form of vesicular stomatitis virus (VSV) contained single-stranded mRNA with complementary sequences, which on deproteinisation, could self-anneal into a double-stranded hairpin structure, which was an extremely effective interferon inducer. Greene et al. (1978) found that the interferon inducing capabilities of copolymer duplexes poly(2'-O-methyl I-I).poly(2'-O-methyl C-C) of varying degrees of methylation and residue clustering correlated highly with the presence of clusters containing six or more consecutive ribosyl residues. Therefore, it was suggested (Greene et al., 1978) that interferon induction is dependent on the recognition of a particular spatial and steric organization of a double-stranded RNA, but the structural features that the interferon inducing ability of polynucleotides depend upon precisely, have not yet been established.

1.3 Mechanism of Interferon Induction by Polynucleotides

Suggestions made on the mechanism of interferon induction by polynucleotides were mainly based on results obtained from experiments with metabolic inhibitors. It was reported by Reich et al. (1961) that interferon induction by virus in rabbit spleen cell suspensions and in primary rabbit kidney cell cultures was inhibited by pretreatment of the cells with actinomycin D. Similar results were obtained by Finkelstein et al. (1968), but in addition they found that interferon production induced by poly(I).poly(C) was more resistant to actinomycin D than viral induced production. Youngner and Hallum (1968), observed that the blockade of protein synthesis by pretreatment of mice with cycloheximide did not prevent the appearance of interferon in the circulation after the injection of poly(I).poly(C). Therefore, they suggested that the interferon was present in a preformed

state. But this was disputed by Vilcek et al. (1968), who showed that interferon induction by poly(I).poly(C) in primary rabbit kidney and human leukocytes in cell cultures was inhibited by actinomycin D. Ho and Ke (1970) also found that interferon production by liver slices derived from rabbits injected with poly(I).poly(C) required protein synthesis, therefore, it did appear that at least in the case of poly(I).poly(C) stimulation of interferon, new protein synthesis was required, and that more than just "release" of "preformed" material was involved. A further detailed study (Vilcek and Ng, 1971) showed that low to moderate doses of cycloheximide had a stimulatory effect on interferon production in rabbit kidney cell cultures treated with double-stranded poly(I).poly(C), but higher doses of cycloheximide caused a shift in interferon release towards later intervals and a gradual decrease in the overall degree of stimulation. An even greater increase in the amount of interferon produced was observed if cells were treated with cycloheximide for only 3 to 4 hours immediately after their exposure to poly(I).poly(C), and under these conditions, a rapid burst of interferon production occurred after the reversal of cycloheximide action. Treatment with a high dose of actinomycin D before the reversal of cycloheximide action caused a further increase and a marked prolongation of interferon production, these types of treatments being known as super-induction. Thus, it was postulated that inhibitors of protein synthesis suppressed the accumulation of a cellular regulatory protein (repressor) which interacted with the interferon mRNA and thereby prevented its translation. Active interferon mRNA could apparently accumulate in rabbit kidney cells which, after exposure to poly(I).poly(C), were retained in the presence of an inhibitor of protein synthesis. Some of this accumulated interferon mRNA was translated during a

partial block of cellular protein synthesis, but its most efficient translation occurred after the reversal of the action of the protein synthesis inhibition (Tan et al., 1970; Vilcek and Ng, 1971; Vilcek and Havell, 1973).

Further evidence supporting the denovo synthesis of interferon came from De Maeyer-Guignard et al. (1972) who found that mouse interferon was produced by avian and simian cells preincubated with RNA extracted from interferon-producing mouse cells, and this RNA effect was inhibited by treatment of the receptor cells by cycloheximide but not by actinomycin D. Reynolds et al. (1975) also succeeded in isolating mRNA from poly(I).poly(C)-induced human fibroblasts and translated it in cell-free ribosomal systems and in Xenopus oocytes. This resulted in the production of biologically active proteins that had the properties of human fibroblast interferon. Similar results were obtained by Pestka et al. (1975) who isolated and purified interferon mRNA from human fibroblasts and translated this mRNA in a cell-free system from mouse cells. Cavalieri et al. (1977) assayed quantitatively polyadenylated interferon mRNA, obtained from induced human fibroblasts, by synthesis of biologically active human interferon in Xenopus laevis oocytes. The results demonstrated that, on induction with poly(I).poly(C), human fibroblasts accumulated interferon mRNA for 1-1.5 hours, after which time the mRNA was rapidly degraded with a half-life ($t_{\frac{1}{2}}$) of 18 minutes. Treatment of cells with cycloheximide prolonged the period of accumulation to 3 hours and decreased the rate of mRNA inactivation, and treatment with actinomycin D decreased the rate of inactivation still further. Thus, it was concluded that, on induction, the genes for interferon were activated to produce a transcript for a short time, and the super-inducing treatments prolonged the period of accumulation and decreased the rate of degradation of

this transcript. More recently, Atherton and Burke (1978) demonstrated that three different inhibitors of RNA synthesis, namely actinomycin D, camptothecin and α-amanitin, and five different inhibitors of protein synthesis, namely cycloheximide, puromycin, trichodermin, oxytetracycline and p-fluorophenylalanine, all caused superinduction in human diploid cells. It is a clear indication that both transcription and translation are required for superinduction.

U.V. irradiation was the second tool used for the study of the mechanism of interferon induction. Irradiation of cells prior to the exposure to virus resulted in a dose-dependent decrease in interferon production. The inhibition of total cellular RNA synthesis by U.V. irradiation in uninduced cultures was similar to the inactivation curve of interferon production in virus-induced cultures. In contract, the production of interferon with poly(I).poly(C) paradoxically was enhanced in cells irradiation with a wide range of U.V. doses (Long and Burke, 1971, Mozes and Vilcek, 1974).

It is generally regarded that both types of induction triggers cause the derepression of the cellular genome and leads to the transcription of the interferon gene and thus synthesis of interferon mRNA, so the induction of interferon is a true de novo synthesis. But there are numerous questions, concerning the mechanism of the induction of interferon, to be answered. Whether the induction process requires cellular uptake of the inducer or the induction is triggered on the cellular membrane is not at all clear as yet. The most obvious approach to this question is to attach a synthetic inducer (e.g. poly(I).poly(C)) to an insoluble matrix and to study the effect of this insolubilized polynucleotide on interferon induction. Hutchinson and Merigan (1975) found that stable, insolubilized poly(I).poly(C) did not induce interferon,

but no later workers have been able to obtain the same results. Pitha and Pitha (1973) attached either poly(I) or poly(C) to cyanogen bromide-activated Sephadex or cellophane and then complexed this with the complementary polynucleotide. The bound poly(I).poly(C) was aged in buffer until there was no further measurable loss of polynucleotide from the support before its use in the induction experiments. The insolubilized poly(I).poly(C) was active as an interferon inducer, but up to 10% of the bound polynucleotide was lost from the support during incubation with the cells. Therefore, the question mentioned above cannot be answered conclusively (Pitha and Hutchinson, 1977). No specific cellular receptor site for interferon inducers has been located, or extracted. It is also still not clear whether polynucleotide strands separation is necessary for the induction of interferon.

1.4 Recent studies in the Role of Interferon in Inducing the Antiviral State

Interferons are glycoproteins, which vary in their sugar components (Morser et al., 1978), secreted by dsRNA-induced or virus-infected cells which promote the establishment of an antiviral state in uninfected cells. Recent studies have been concentrated on the enzymatic differences between extracts of interferon-treated and control cells. Kerr et al. (1974) found that dsRNA was a potent inhibitor of protein synthesis in extracts of interferon treated cells, and it led to the discovery of two interferon-induced dsRNA-dependent enzymatic activities. Firstly, an oligonucleotide polymerase (designated 2,5A polymerase), which synthesized a series of oligonucleotides containing unusual 2',5'-phosphodiester bonds from ATP (Kerr and Brown, 1978), secondly, a protein kinase, which phosphorylated the small subunit

of initiation factor eIF-2 (Farrell et al., 1977). Furthermore, the product of the 2,5A polymerase, $ppp(2'p5'A)_n$ (designated 2,5A) was the activator of an endoribonuclease (Clemens and Williams, 1978). The increase in 2,5A polymerase activity upon exposure of cells to interferon has been studied by Minks et al. (1979). After an initial lag phase of 2-3 hours the enzymatic activity increased linearly for several hours, and levelled off after about 24 hours. This increase was dependent on RNA and protein synthesis, suggesting that mRNA for 2,5A polymerase was synthesized in cells exposed to interferon and that translation of this mRNA was necessary for the increase of this enzymatic activity. Baglioni et al. (1979) observed a correlation between the induction of 2,5A polymerase and the inhibition of viral RNA synthesis in encephalomyocarditis virus (EMCV)-infected HeLa cells. As 2,5A polymerase activity increased upon exposure of cells to interferon, synthesis of viral RNA progressively decreased.

The interferon induced dsRNA-dependent inhibitor of protein synthesis is the low molecular weight 2,5A. This inhibitor is made up of adenosines, with a terminal triphosphate, linked by a 2'-5' phosphodiester bond, and the number of adenosines is between 2 to 10, while the trinucleotide is the major component (Kerr and Brown, 1978). It has been suggested (Baglioni, 1979) that, in the presence of both dsRNA and ATP, 2,5A activates an endonuclease, and followed by cleavage of mRNA by the 2,5A-dependent endonuclease. The endonuclease cleaves both cellular and viral mRNA whether free or associated with polysomes, therefore the inhibition of protein synthesis by 2,5A is the result of endonuclease activation and subsequent degradation of mRNA (Clemens and Williams, 1978). It has been postulated (Baglioni et al., 1978) that the 2,5A polymerase

is activated by partially double-stranded replication intermediates (RI) of RNA viruses, and that the 2,5A synthesized may locally activate the 2,5A-dependent endonuclease. Viral mRNA would then be preferentially cleaved in this case. It has been demonstrated (Nilsen and Baglioni, 1979) that labelled RI, obtained from EMCV-infected cells, incubated with extracts of interferon-treated cells are degraded to a 20S "core" that are resistant to digestion with RNaseA. This shows that in interferon-treated cells there are enzymatic activities which can cleave the single-stranded nascent viral RNA chains from the double-stranded core of the replicative intermediate.

Farrell et al. (1977) discovered that the inhibition of initiation of protein synthesis in reticulocyte lysates involves a protein kinase which phosphorylates the smallest subunit (α) of initiation factor eIF-2 and concurrently inhibits the binding of initiator Methionyl-$tRNA_f$ to 40S native ribosomal subunits. The inhibition can be subdivided into two steps: activation, which requires both dsRNA and ATP, and results in phosphorylation of a protein (PI) of about 67,000 molecular weight, and phosphorylation of eIF-2α, which requires ATP but not dsRNA. Samuel et al. (1977) also obtained similar evidence for the involvement of a protein kinase in the enhanced phosphorylation of two polypeptides in their work on the effect of dsRNA on cell-free systems from interferon-treated cells. Jarvis et al. (1978) have studied the effect of dose and time of treatment with interferon on the induction of protein kinase activity, and found that in mouse 3T6 cells the level of protein kinase activity and the magnitude of the antiviral state were related to the log of the interferon concentration used. It was also found that the cytoplasmic extracts of untreated cultures of a virus-resistant

mutant of mouse 3T6 cells, designated 3T6-V^rB2, contained two dsRNA-activated enzyme activities associated with interferon action. It seems probable that if the protein kinase were activated, both viral and host protein synthesis would be inhibited, since all proteins are initiated via eIF-2. If accumulation of viral mRNA is not arrested by the combined action of the 2,5A polymerase and endonuclease, the kinase may block protein synthesis before structural viral proteins are produced in sufficient amounts for virus assembly. Thus, resulting from these studies on the relationships between the enzymatic activities and the establishment of the antiviral state by interferon, it seems probable that some explanation for the molecular basis of the antiviral state will be forthcoming in the near future. The interferon-induced effects and enzymatic activities in cells are summarized in Fig.1.1 (Morser and Burke, 1979, Baglioni, 1979).

1.5 Aims of the Present Investigation

5-Hydroxycytidine 5'-diphosphate has been synthesised by Eaton and Hutchinson (1973), and it was subsequently polymerised by polynucleotide phosphorylase to give poly(ho^5C). In contrast to poly(5-halogenocytidylic acids), poly(ho^5C) had little secondary structure in neutral or acid solution and did not form a complex with poly(I), poly(A) or poly(C). Stebbing *et al.* (1977a, 1977b) demonstrated that the protection of mice against encephalomyocarditis virus infection with a mixture of poly(I) and poly(ho^5C) copolymer was slightly greater than had previously been observed with sequential administration of poly(I) followed by poly(C) or the homopolymer form of poly(ho^5C). No interferon production was observed in these experiments, but the toxicity of a mixture of poly(I) and poly(ho^5C) copolymer was less than one tenth that of poly(I).poly(C). Therefore, it was of interest to prepare poly(mo^5C) in order to ascertain if

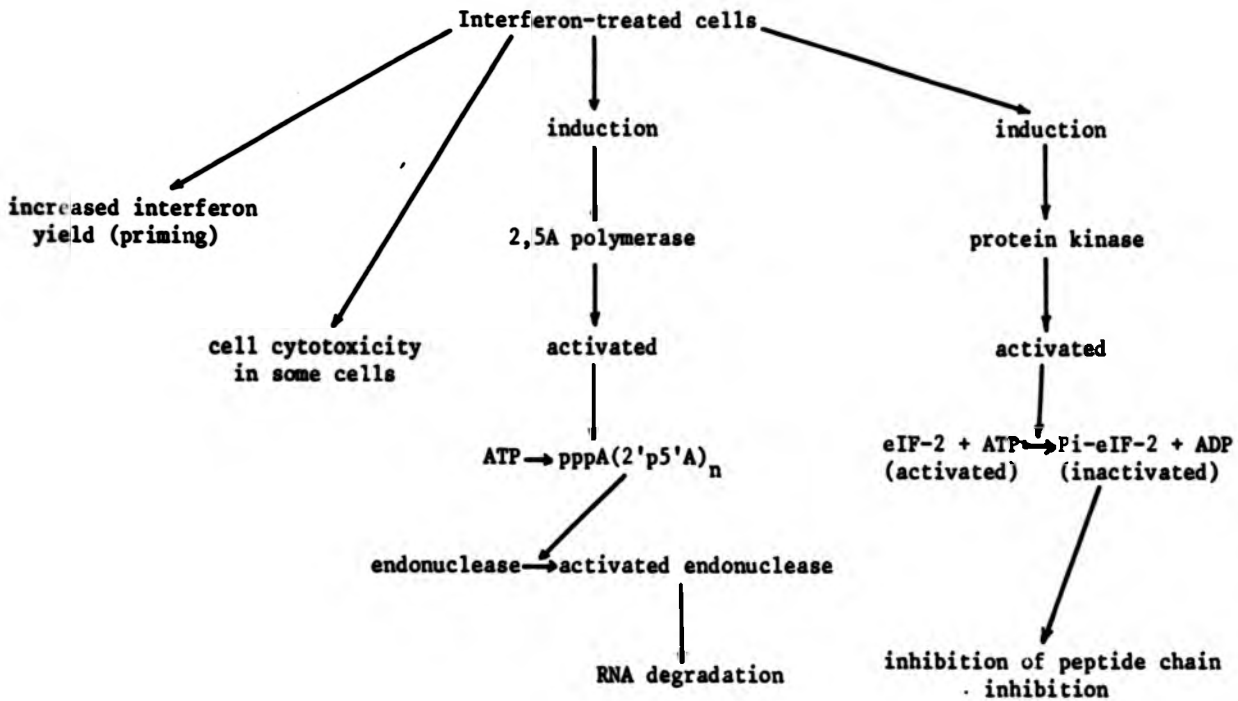


Fig. 1.1 Schematic presentation of interferon induced effects and enzymatic activities in cells (Morser and Burke, 1979, Baglioni, 1979)

it could form a complex with poly(I), and also if it were to possess any antiviral activity.

There are numerous questions, concerning the mechanism of the induction of interferon, to be answered. It is also still not clear whether polynucleotide strand separation is necessary for the induction of interferon. Therefore, the chemical cross-linking of two synthetic polynucleotides, poly(I).poly(C), and alternating copoly(I-C) was carried out. Two different types of cross-linking reagents were employed for this task, firstly, a difunctional nitrogen mustard (methyl-l-N-bis(2-chloroethyl)amine hydrochloride, HN2), and secondly, 8-methoxypsoralen. The interferon induction capability of the polynucleotides, before and after cross-linking could then be assessed.

CHAPTER 2

SYNTHESIS OF 5-METHOXYCYTIDINE 5'DIPHOSPHATE

2.1 Introduction

A consistently emerging problem, apparently inherent to the use of interferon inducers such as polynucleotides, even in animal species that do not respond by circulating interferon production, is toxicity (De Clercq, 1974). Niblack and McCreary (1971) studied how variations in the average molecular weight of the homopolymers comprising a polydisperse poly(I).poly(C) population affected the antiviral activity and acute parenteral toxicity of the double stranded polymer *in vitro*. They found that the acute toxicity of poly(I).poly(C) seemed to fall off with decreased homopolymer molecular weight at about the same rate as antiviral activity, thus it seemed unlikely that any significant separation of antiviral activity from toxicity could be made in poly(I).poly(C) by simple manipulation of homopolymer molecular weights. Similar results were obtained by Stewart and De Clercq (1974) who studied the molecular size of poly(I).poly(C) required for lysis of L cells, interferon-treated L cells, and the induction of interferon in these cells. Stewart *et al* (1972a) observed that concentrations of the poly(I).poly(C) that produced no detectable toxicity in normal L cells produced marked cytotoxicity in L cells treated with interferon. This increase in the susceptibility of cells to the toxicity of the polymer was also observed in human cells and secondary mouse embryo cells treated with homologous interferons before exposure to the polynucleotides. The degree of enhancement of toxicity was dependent on the concentration of interferon to which the cells were exposed. It is known that poly(I).poly(C) does not induce interferon production in L cells in the absence of DEAE-dextran

(Youngner and Hallum, 1969), and it has been suggested that DEAE-dextran potentiates interferon induction by rendering the inducer less susceptible to endonucleases (Pitha and Carter, 1971). Therefore, it was suggested (Pitha and Carter, 1971) that the ability of poly(I).poly(C) to induce interferon production in L cells treated with interferon in the absence of DEAE-dextran, and its increased toxicity, may be due to the decreased susceptibility of poly(I).poly(C) to enzymatic degradation in cells treated with interferon. This topic will be discussed in more detail later.

The toxic effects of poly(I).poly(C) in vivo had also been studied by Adamson and Fabro (1969). They observed that poly(I).poly(C) was embryotoxic, this effect, in pregnant rabbits, was evident when a dose of 1 mg/kg was used. Philips et al. (1971) studied the pathological effects of poly(I).poly(C) given in lethal or near lethal doses in different animals. They commented that one of the remarkable features of the toxic effects of poly(I).poly(C) was the precipitous and selective nature of the pathologic changes which were induced within a few hours after the administration of lethal doses. Also, because of the nonuniformity in the sites of major damage in these experimental species, it would be difficult to predict the lesions likely to be encountered in clinical trials. Finter (1973a) suggested that the toxic properties of double-stranded RNAs such as poly(I).poly(C) were reminiscent of those of bacterial endotoxins, which cause damage in cell membranes, particularly those of macrophages, and are pyrogenic. This also implied that its clinical use could be complicated by the effects which endotoxins produced, such as induction of abortion and metastasis of tumours. Finter (1973b) summarized by stating that poly(I).poly(C) produced no toxic effect in humans, even in high concentration, when administered intranasally, but fever and temporary suppression of erythropoiesis were observed when administered intravenously.

Since it has been shown that the poly(C) strand is less important than poly(I) (Carter et al., 1972), it may be the site where modification can be made without destroying its interferon induction ability but decreasing its toxicity. Indeed, it had been shown that by introducing unpaired bases (G or U) in the poly(C) strand, the antiviral activity and inteferon-inducing capacity of these modified poly(I).poly(C) were not affected, and yet its toxicity decreased to a marked extent (De Clercq, 1974). These findings suggested that the interferon-inducing and toxic properties of poly(I).poly(C) could be uncoupled by appropriate modifications in the molecule. Some other early chemical modifications involved the halogenation of pyrimidine and purine bases (Massoulie et al., 1966; Michelson and Monny, 1967). The reaction can be effected in water by bromine, and iodine. The bromination occurs at C-5 in the pyrimidine derivatives and at C-8 in guanine residues; adenine derivatives are slightly more resistant (Figure 2.1)(Ikehara and Uesugi, 1969; Cartwright, 1978; Brown, 1974). The halogenation of nucleosides was followed by phosphorylation to give the diphosphate and subsequent polymerisation (Massoulie et al., 1966; Michelson and Monny, 1967). The physical properties of poly(br^{5}C) and poly(iodo^{5}C) were studied, and found that both polymers formed a 1:1 complex with poly(I) with increased stability compared with poly(I).poly(C) (Howard et al., 1969; Ross et al., 1971). It was also found that poly(I).poly(br^{5}C) brought about the production of higher titers of interferon at lower concentrations than did poly(I).poly(C) in 'superinduced' primary rabbit kidney cells and human skin fibroblasts (Torrence et al., 1974). Since this same fibroblast 'superinduced' system is used for the production of human interferon for clinical trials, poly(I).poly(br^{5}C) or a similar behaving inducer could effect a significant reduction in the cost of interferon production (Torrence and De Clercq, 1977). Other chemically modified poly(C) derivatives

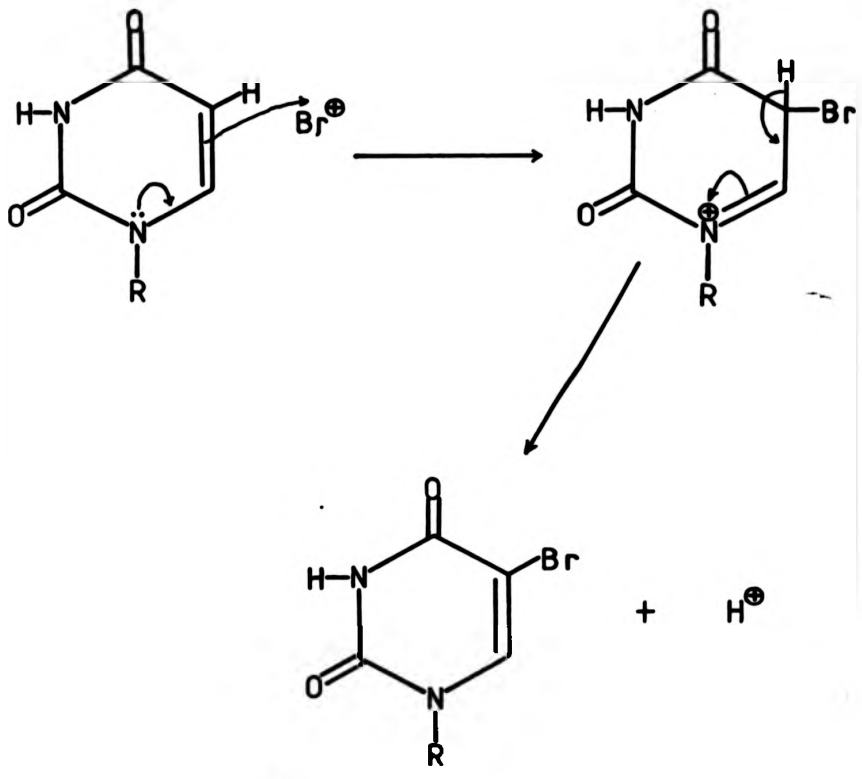


Figure 2.1: The possible reaction mechanism of bromination (halogenation) of a typical pyrimidine base

synthesized include poly(cl^{5}C) (Eaton and Hutchinson, 1972; Commerford, 1971), poly(fl^{5}C) (Folayan and Hutchinson, 1974), poly(N^4-acetyl C) (Michelson and Pochon, 1966; Pochon et al., 1968), poly (N^4-methyl C), poly(N^4,N^4-dimethyl C) (Brimacombe and Reese, 1966), and poly(s^{2}C) (Faerber et al., 1972). It is of interest to note that the T_m values of the hybrids of poly(I).poly(x^{5}C) varies, in the series x = H, F, Cl, Br, I, with the size of the substituent and not with its electronegativity. There appears to be a linear relationship between the T_m of the hybrid and the size of the substituent (as expressed by the cube of their van der Waals radii) (Figure 2.2)(Folayan and Hutchinson, 1974). Studies on the 5-substituted pyrimidine nucleosides and nucleotides were recently reviewed by Bradshaw and Hutchinson (1977) including their halogenation, hydroxymethylation, and mechanisms of reaction. None of the chemically modified poly(C) derivatives mentioned above, except poly(br^{5}C), when complexed with poly(I), has better therapeutic effect than poly(I).poly(C) (Torrence and De Clercq, 1977).

5-Hydroxyuridine diphosphate (ho^{5}UDP) has been prepared by the action of bromine in aqueous pyridine on UDP (Visser and Roy-Burman, 1968). Murao et al. (1976) have been successful in synthesizing 5-methoxyuridine (mo^{5}U) chemically by methylating 5-hydroxyuridine using dimethyl sulphate under alkali condition. This procedure gave a 25% yield of 5-methoxyuridine, 5% yield of 5-hydroxy-3-methyluridine, and 4% yield of 5-methoxy-3-methyluridine. 5-Methoxyuridine is the 'wobble' base (Crick, 1966) of tRNA^{Arg}, tRNA^{Val}, and tRNA^{Ser} from Bacillus subtilis (Murao et al., 1976; Albani et al., 1976). It presumably base pairs with A, G and, less effectively, with U in codon-anticodon type recognition. The altered ability of mo^{5}U to form hydrogen bonds, compared to U, has been explained by a shift in the keto-enol tautomerism of N(3)-C(4)-O(4) towards the enol form

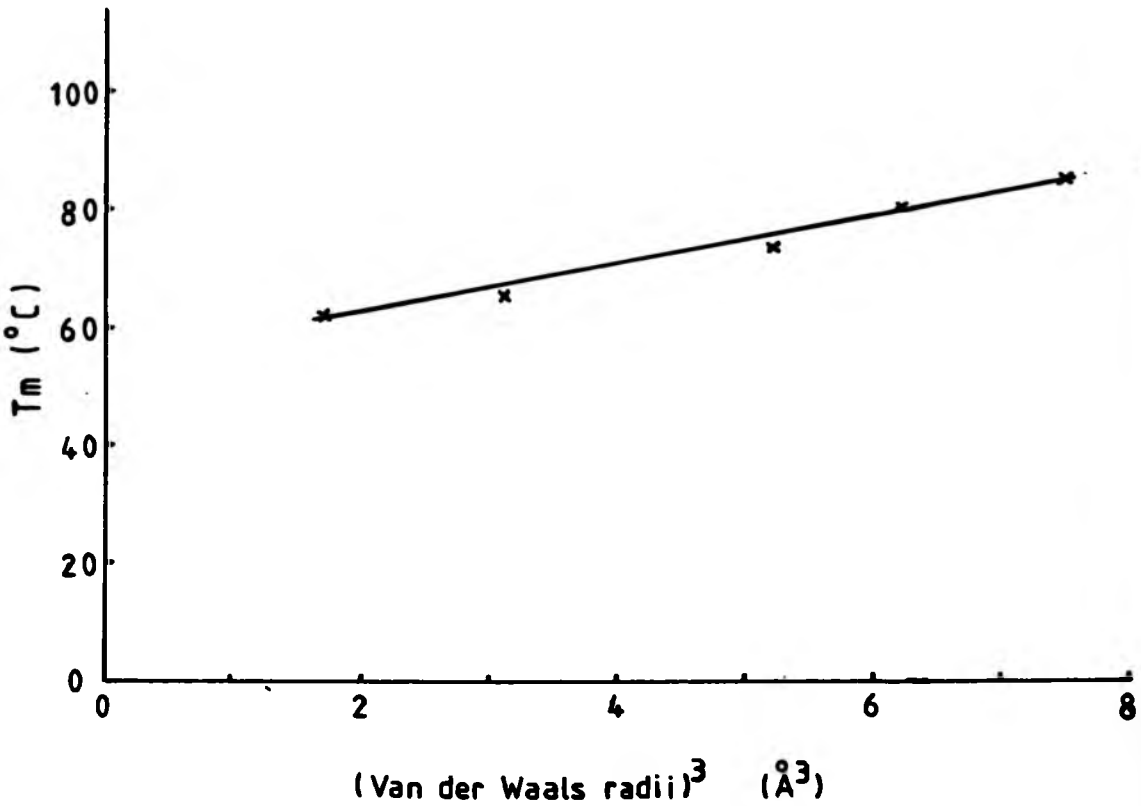


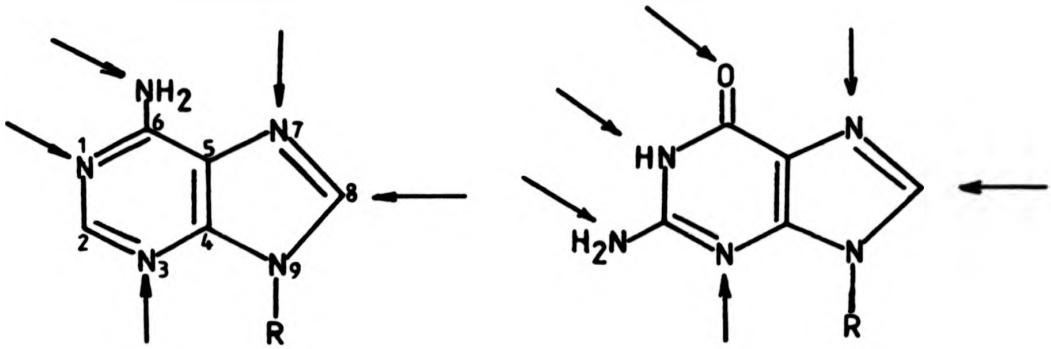
Figure 2.2: Relationship between melting temperatures (T_m) in 0.1 M Na^+ solution and the size of the 5'-substituent in the hybrids poly(I).poly(x^5C) (Folayan and Hutchinson, 1974).

(Hillel and Gassen, 1979). The synthesis of poly (mo^5U) has been carried out using polynucleotide phosphorylase, and the reaction required a high concentration (2.7 mg/ml, 150 units/mg) of PNPase as well as a long reaction time (48 hours) (Hillel and Gassen, 1979). The resulting polynucleotide had a chain length of approximately 100 nucleotides, and showed no indication of a stable secondary structure. When poly(mo^5U) was mixed with poly(A), a triple-stranded complex poly(A).2poly(mo^5U) was formed, and this complex had a melting temperature of 68.5 $\pm$ 0.5° at 150 mM Na^+ (Hillel and Gassen, 1979).

Eaton and Hutchinson (1973) found that if 2,4,6-collidine was used with bromine in place of pyridine reasonable yields of ho^5CDP were obtained and little deamination occurred if prolonged reaction times were avoided. The subsequent polymerisation of ho^5CDP by polynucleotide phosphorylase was found to be difficult and only took place in the presence of large amounts of the enzyme and with a $[Mg^{2+}]$:[substrate] ratio of 1:1. In contrast to poly(5-halogenocytidylic acids), poly(ho^5C) had little secondary structure in neutral or acid solution and did not form a complex with poly(I),poly(A) or poly(C). Stebbing *et al.* (1977a, 1977b) demonstrated that the protection of mice against encephalomyocarditis virus infection with mixtures of poly(I) and poly(ho^5C) copolymer was slightly greater than had previously been observed with sequential administration of poly(I) followed by poly(C) or the homopolymer form of poly(ho^5C). Evidence also was presented which showed that double-stranded complexes did not form when the poly(ho^5C) copolymers were mixed with poly(I), and 200 µg/mouse of a mixture of poly(I) and poly(ho^5C) copolymer was as protective as 60 µg/mouse of poly(I).poly(C) but the toxicity of the previous mixture was less than one tenth that of poly(I).poly(C). No interferon production was observed, and the mechanism of the protective effect with the mixtures of poly(I) and poly(ho^5C) was

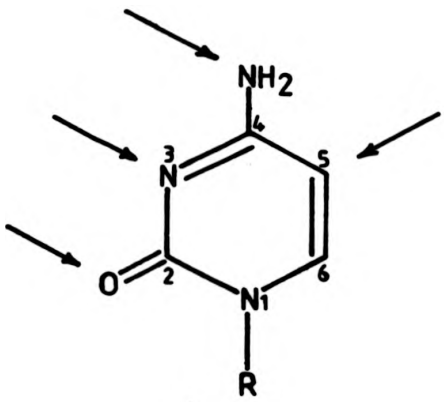
not known (Stebbing et al, 1977a, 1977b). Therefore, it was of interest to prepare poly(mo^5C) in order to ascertain if it could form a complex with poly(I), and also if it were to possess any antiviral activity.

For chemical methylation studies, two general types of reagents have been used, firstly, methyl esters of strong acids, such as dimethyl sulphate, and secondly, diazomethane (Griffin, 1967). There are so many potential sites in a nucleoside molecule which could be alkylated that, unless precautions are taken, a very complex mixture of products is obtained (Figure 2.3) (Brown, 1974). If the ring nitrogen atoms, which are available for alkylation in the purine and pyrimidine nucleosides, were considered, the order of basicity of these positions, as measured by pKa, is not directly related to their relative ease of alkylation. It has been pointed out (Brown, 1974) that protonation is an equilibrium process, whereas alkylation is irreversible and thus subject to kinetic control and the influence of steric factors. Thus, alkylation of nucleosides with diazomethane, or dimethyl sulphate gives a complex mixture of O'-, O-, ring N-, and exocyclic N-alkylated products, although in general the most reactive site towards esters of strong acids in guanosine is N-7, in adenosine is N-1, and in cytidine is N-3, whereas uridine reacts only very slowly (Brookes and Lawley, 1962; Brown, 1974) (Figure2.4). However, under strongly alkaline conditions with dimethyl sulphate, O'-alkylated products only are produced. This has been rationalized (Walker, 1979) in terms of the hardness and softness of the reactive centres and correlation of the reaction at a hard centre with an S_N1 type mechanism and at a soft centre with an S_N2 reaction. Strong aqueous alkaline reactions with alkylating agents are of the S_N1 type, which thus favours reaction at the hard O'-positions. There is kinetic evidence that methyl and

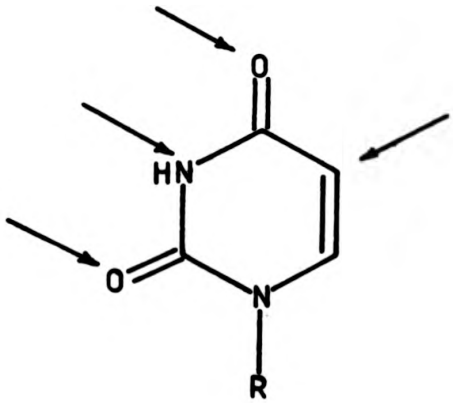


Adenine base

Guanine base



Cytosine base



Uracil base

Figure 2.3: The arrows indicate the positions of high electron density on these four heterocyclic bases, where electrophilic attack (halogenation, alkylation) may occur (Brown, 1974).

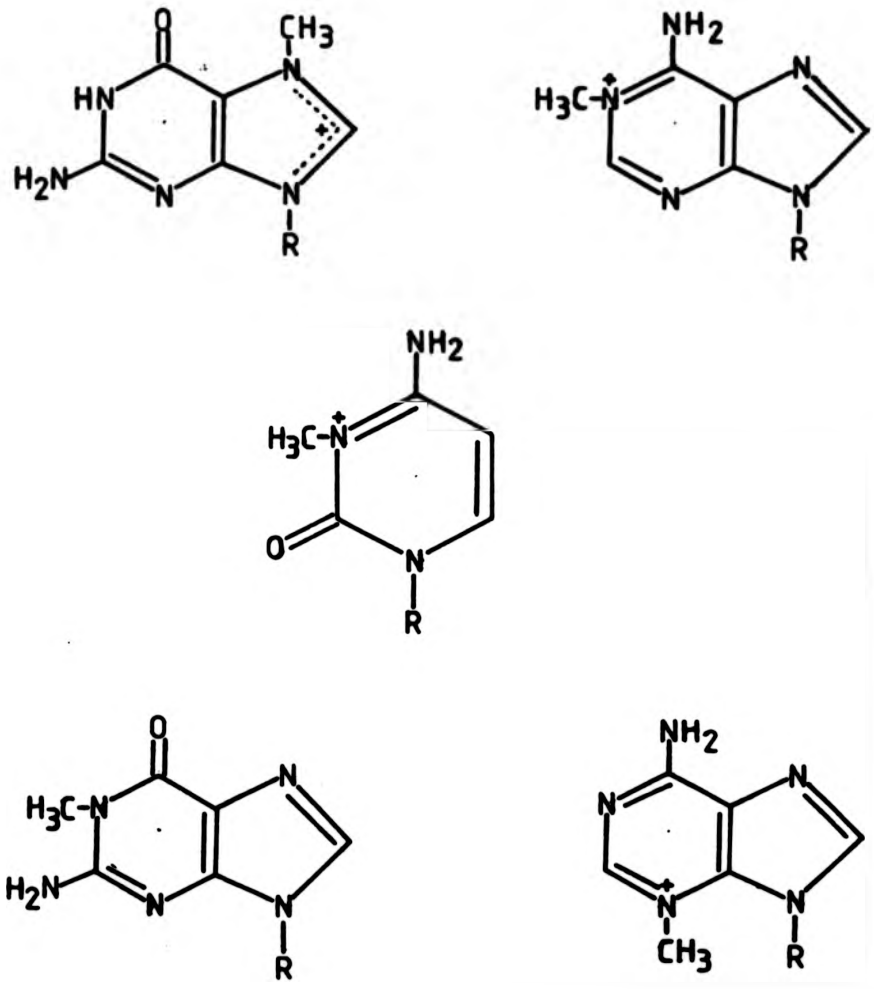


Figure2A: The corresponding major methylated products of guanine, adenine, and cytidine, using dimethyl sulphate or diazomethane in neutral aqueous systems (Brown, 1974).

ethylmethane sulphonates alkylate bases in DNA by an S_N2 process (Brown, 1974). The molecularity of substitution reactions depends on the structure of the alkylating agent, on the reactivity of the nucleophile and on the solvent, but detailed mechanistic interpretations are difficult to reach. Diazomethane is often reacted by shaking an ethereal solution of it with a buffered aqueous solution of the substrate - the order of reactivity is uridine > guanosine > cytidine > adenosine, with the attack at the site of the most acidic hydrogen (Griffin, 1967). The reaction mechanism of diazomethane presents a somewhat different picture. It does not react, per se, with nucleophiles (it is itself nucleophilic in character, $CH_2^-.N_2^+$), and alkylation of anions is therefore very slow. It is supposed (Brown, 1974) that an alkyldiazonium ion is the reactive species, generated by abstraction of an acidic proton (Figure 2.5).

Therefore, it should be possible to synthesize 5-methoxycytidine by direct methylation of 5-hydroxycytidine using either dimethyl sulphate in weak alkali conditions, or diazomethane, followed by phosphorylation to give 5-methoxycytidine 5'-diphosphate. However, our studies indicated that, indeed, a very complex mixture of products was obtained, and the amount of 5-methoxycytidine produced was too small to be isolated from the rest of the products. Thus, a second approach was required in order to synthesize 5-methoxycytidine. The starting compound, methyl methoxyacetate, was treated with ethyl formate, and sodium in toluene, the formylated ester was then treated with urea in ethanol under refluxing to give 2,4-dihydroxy-5-methoxy-pyrimidine (Chesterfield, 1960) (Figure 2.6). The next stage was a one step reaction which involved the prior silylation of the heterocyclic base, and potassium nonaflate ($C_4F_9SO_3K$, a Friedel-Crafts catalyst) by hexamethyldisilazane (HMDS) and trimethylchlorosilane (TCS), to the highly moisture sensitive silyl derivatives, then the reaction

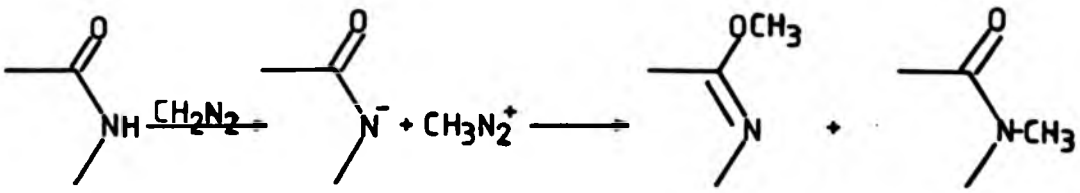


Figure 2.5: Reaction mechanism of methylation of heterocyclic bases of pyrimidine or purine by diazomethane (Brown, 1974).

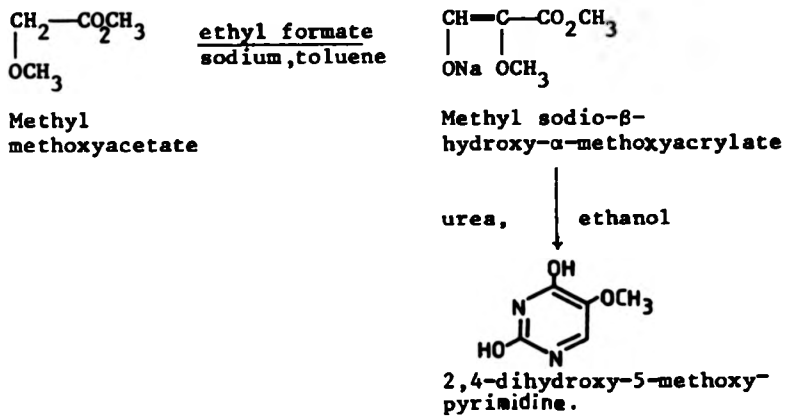


Figure 2.6

The synthesis of 2,4-dihydroxy-5-methoxypyrimidine

proceeded with the sugar, 1-O-acetyl-2,3,5,-tri-O-benzoyl-β-D-ribofuranose to give the pyrimidine nucleoside derivative 5-methoxyuridine-2',3',5'-tri-O-benzoate (Vorbrüggen and Bennua, 1978) (Figure27). The 5-methoxyuridine-2',3',5'-tri-O-benzoate was converted to 4-thio-5-methoxyuridine-2',3',5'-tri-O-benzoate by a simple refluxing with phosphorus pentasulphide in pyridine (Garrett, 1968) (Figure28). The 4-thio-5-methoxyuridine-2',3',5'-tri-O-benzoate was converted to the final product, 5-methoxycytidine by heating at 100° with alcoholic ammonia in a sealed Carius tube (Fox et al., 1959) (Figure29). The poly(mo^5C) was synthesized by the subsequent phosphorylation of 5-methoxycytidine to give 5-methoxycytidine 5'-diphosphate, and its polymerization by polynucleotide phosphorylase.

2.2 Materials and Methods

2.2.1 Materials

(a) Thin layer chromatography (t.l.c.) plates were either Kieselgel 60 F_{254} from E. Merck, Darmstadt; or Polygram Cel 300, PEI/UV_{254} (PEI:polyethyleneimine) from Macherey-Nagel, W. Germany. Paper chromatography was performed on Whatman 3 MM paper.

(b) N-methyl-N-nitroso-p-toluenesulphonamide was purchased from Aldrich Chemical Co. Ltd., Dorset, U.K. Dimethyl sulphate was purchased from May and Baker Ltd., Dagenham, U.K. 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranose (A grade) was purchased from Calbiochem-Behring Corporation, Herts, U.K. Potassium nonaflate ($C_4F_9SO_3K_6$) was a gift from Bayer A G Leverkusen, W. Germany.

(c) All other reagents were of analytical grade unless otherwise stated.

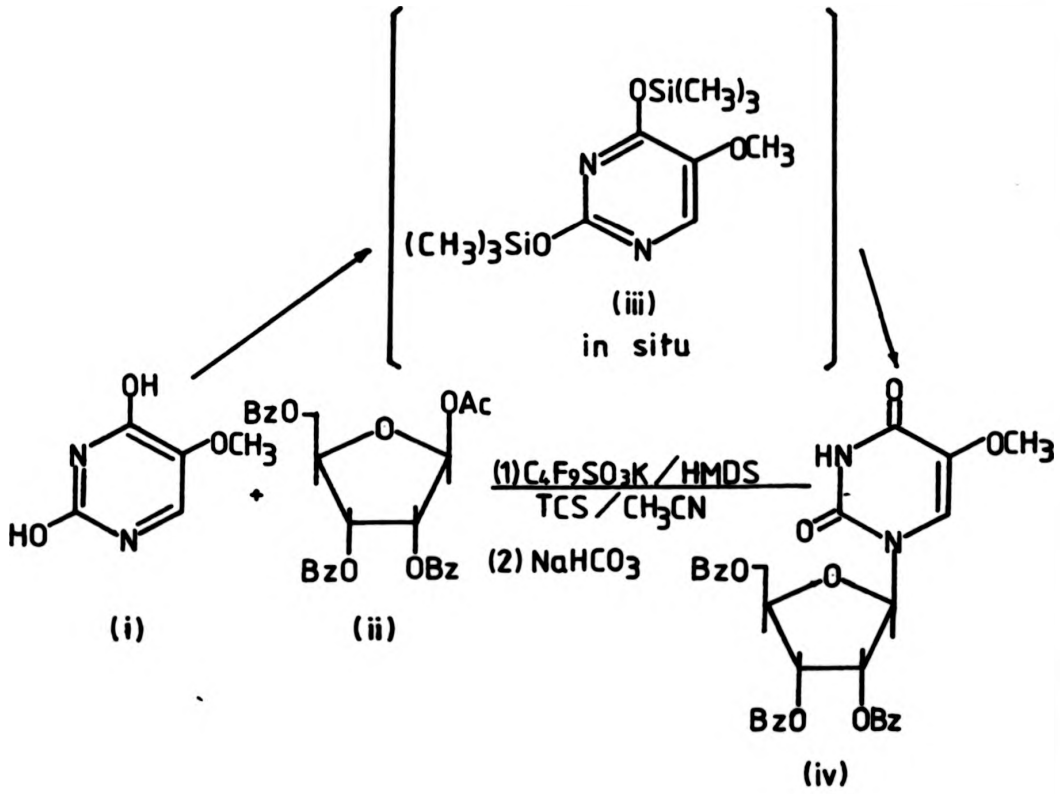
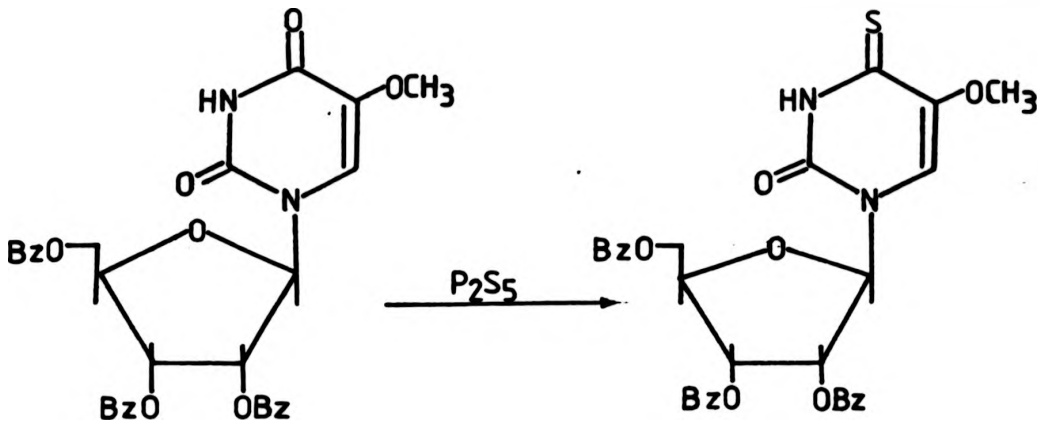


Figure 2.7: The synthesis of 5-methoxyuridine-2',3',5'-tri-O-benzoate

(i) 2,4-dihydroxy-5- methoxypyrimidine,
(ii) 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranose,
(iii) silylated pyrimidine derivative,
(iv) 5-methoxyuridine-2',3',5'-tri-O-benzoate.

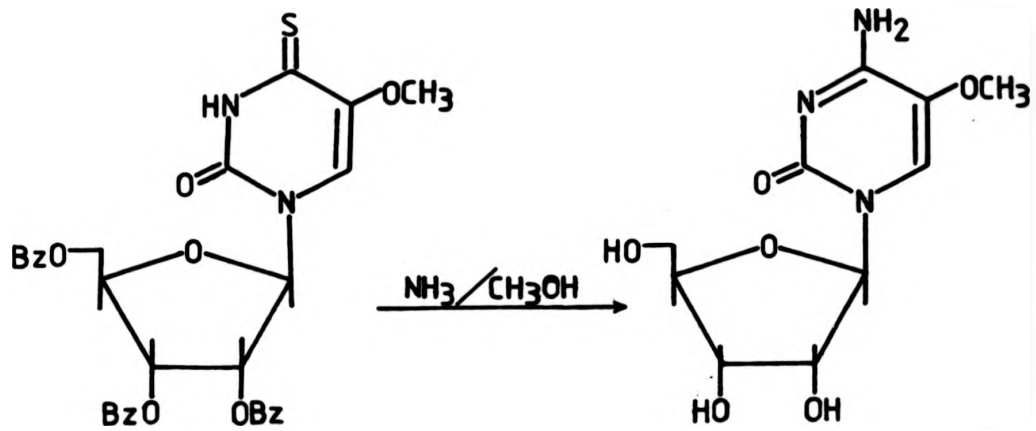
(Vorbrüggen and Benmua, 1978)



5-methoxyuridine-2',3',5'-tri-O-benzoate

4-thio-5-methoxyuridine-2',3',5'-tri-O-benzoate

Figure 2.8: The synthesis of 4-thio-5-methoxyuridine-2',3',5'-tri-O-benzoate (Garrett, 1968)



4-thio-5-methoxyuridine-2',3',5'-tri-O-benzoate

5-methoxycytidine

Figure 2.9: The synthesis of 5-methoxycytidine (Fox et al., 1959)

2.2.2 General Methods

(a) All U.V. measurements were performed either on a Unicam SP800 double beam U.V. spectrophotometer, or Cecil CE505 double beam U.V. spectrophotometer.

(b) Low resolution mass spectra were performed at P.C.M.U., Harwell, Berkshire, U.K. Other low resolution mass spectra were performed by the departmental mass spectrometry service on an AEI MS9 spectrometer.

(c) Microanalysis was performed at Butterworth Lab. Ltd., Middlesex, U.K.

(d) Chromatography was performed by upward development for t.l.c. plates and downward development for paper, using the following solvent systems.

		v/v
(a)	Isobutyric acid:0.5 N NH_4OH	5:3
(b)	Chloroform:methanol	1:1
(c)	Methanol:ethyl acetate	3:7
(d)	Ethyl acetate:hexane	4:6
(e)	t-Butanol: 1 M ammonium formate (pH 3.5)	1:1
(f)	Isopropanol:Conc. NH_4OH:H_2O	7:1:2
(g)	n-Propanol:Conc. NH_4OH:H_2O	11:7:2
(h)	0.1 M NaCl	
(i)	0.5 M Acetic acid:0.5 M lithium chloride	1:1

(j) Formic acid = 0.38 ml
Water = 2.12 ml
Isopropanol = 7.50 ml
Conc. NH_4OH = 0.025 ml

Spots were visualised by fluorescence under 254 nm light in the case of t.l.c. plates, or utilizing the phosphate spray in the case of paper as described below.

(e) Enol spray (ferric chloride-ferric cyanide spray) (Barton,

et al., 1952). Equal volumes of 1% ferric chloride and 1% potassium ferricyanide were mixed a few minutes before use. The presence of an enol group is indicated by the appearance of a blue spot. The blue spot was made permanent by washing the chromatogram with dilute hydrochloric acid and then water.

(f) cis-Diols spray (Baddiley et al., 1956).

A few drops of solution which contained the compound to be tested were applied on a piece of Whatman filter paper, and dried. The dried paper was sprayed with 1% aqueous sodium metaperiodate, and left for 10 min. It was treated with sulphur dioxide, and then sprayed with Schiffs' reagent (1% suspension of pararosaniline hydrochloride treated with sulphur dioxide until a pale-straw colour solution resulted). The treated paper was left in an oven at 50^{o} for 10 min. The presence of a cis-diol group is indicated by the appearance of a purple spot.

(g) Phosphate spray (Mann et al., 1979).

Sulphuric acid (3 M, 10 ml), ammonium molybdate (4%, 10 ml), and n-butanol (25 ml) were mixed and shaken several times in a separating funnel. The phases were allowed to separate and the butanol-rich phase was used. The plates or papers were sprayed but avoiding saturation, oven dried at 100^{o}, and then exposed under U.V. lamp or direct sunlight. The orthophosphate appeared as yellow spot, pyrophosphate as blue spot, and diphosphate ester as purple spot.

(h) Total phosphorus assay was based on the method described by Chen et al. (1956).

(i) ^{1}H Nuclear Magnetic Resonance spectra were obtained, using 30 mg/ml D_2O and DSS (3-(Trimethylsilyl)-1-propanesulphonic acid) as external standard, on a WH90 Bruker NMR spectrometer.

2.2.3 Synthesis of Diazomethane*

This synthesis was based on the method described by de Boer and Backer (1963) (Figure 2.10) It is important to note that no glassware with ground glass surface should be used.

Ethanol (95%, 25 ml) was added to a solution of potassium hydroxide (5 g) in water (8 ml) in a 100 ml distilling flask fitted with dropping funnel and an efficient condenser set downward for distillation. The condenser was connected to two receiving flasks in series, the second of which contained 20-30 ml ether. The inlet tube of the second receiver dipped below the surface of the ether, and both receivers were cooled to 0^{o} The flask containing the alkaline solution was heated in a water bath at 65^{o}, and a solution of N-methyl-N-nitroso-p-toluenesulphonamide (21.5 g, 0.1 mole) in about 200 ml of ether was added through the dropping funnel in about 25 min. The rate of distillation was approximately equal to the rate of addition. When the dropping funnel was empty, another 40 ml of ether was added slowly and the distillation was continued until the distilling ether was colourless. The combined ethereal distillate was titrated to determine the amount of diazomethane. The final volume of the diazomethane in ether was about 150 ml.

Titration of Ethereal Diazomethane

Ethereal diazomethane (10 ml) was added to an excess benzoic acid solution (50 ml, 8.2 m mole) in anhydrous ether. The resulting solution was colourless which showed that the benzoic acid solution was in excess. The excess benzoic acid was then titrated against potassium hydroxide solution using phenolphthalein as indicator. Total diazomethane in the ethereal solution (150 ml) was found to be 1.1 g (25.5 m mole).

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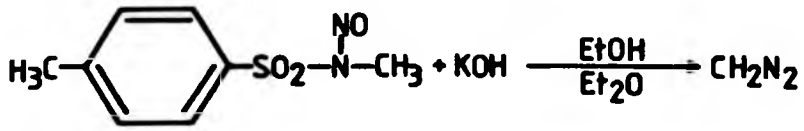
*THIS PREPARATION WAS CARRIED OUT BEHIND A SAFETY SCREEN IN A FUME-CUPBOARD.



N-methyl-N-nitroso-p-toluenesulphonamide

Diazomethane

Figure 2.10: The synthesis of diazomethane

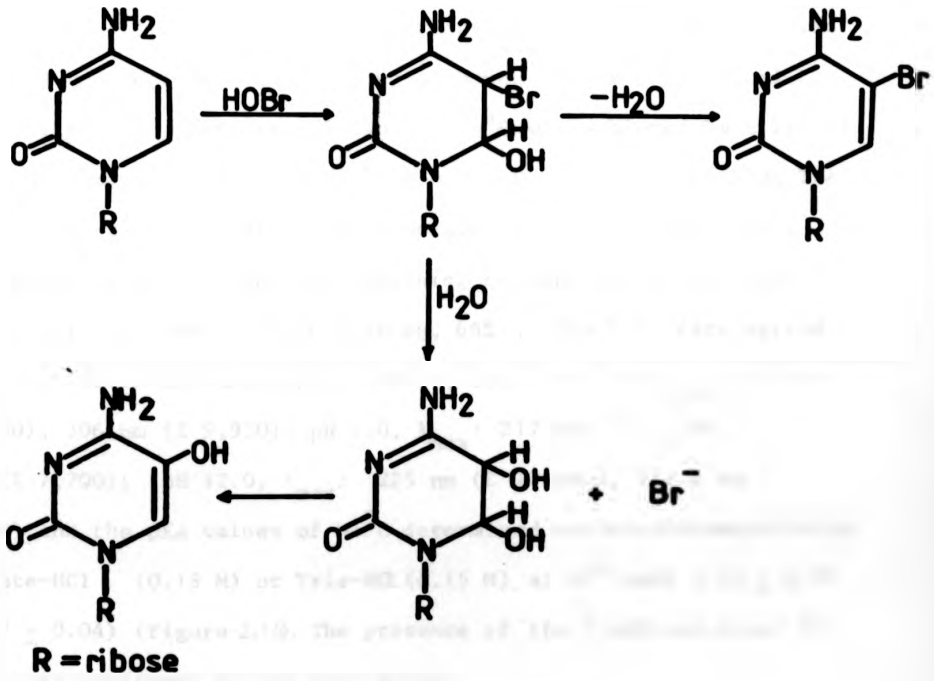


Figure 2.11: Possible mechanism for formation of ho^{5}C from C (Eaton and Hutchinson, 1973)

2.2.4 Synthesis of 5-Hydroxycytidine (ho^5C)

The improved method of the synthesis of 5-hydroxycytidine by Eaton and Hutchinson (1973) was employed (Figure 2.11).The reaction was monitored by silica t.l.c. in solvent A. After the reaction with 2,4,6-collidine, the reaction mixture was cooled and extracted with ether (4 x 4 ml), and the precipitate that appeared in the aqueous layer was redissolved by the addition of a few drops of dilute ammonium hydroxide solution. The solution was applied to a column (1.5 x 25 cm) of AG2-X8 (OH^- form). The column was washed with water until all U.V. absorbing impurities had been eluted. 2,4,6-Collidine and br^5C, detected by silica t.l.c. in solvent A, were eluted from the column immediately. The elution of ho^5C was achieved by a linear gradient of hydrochloric acid (0-0.1 M, 1 l) (Figure 2.12). The fractions of ho^5C were collected and applied to a column (1.5 x 25 cm) of Dowex 50 W x 4 (H^+ form). After washing with water, the elution of ho^5C was achieved by a linear gradient of ammonium hydroxide (0.01 M - 0.5 M, 1 l) (Figure 2.13). The ho^5C fractions were collected and evaporated to near dryness under reduced pressure at 40°, and the remaining residue was lyophilised to give a white product of ho^5C (220 mg, 68%). The U.V. data agreed with that of Eaton and Hutchinson (1973), lit.: pH 1.0, λ_{max}: 216 nm (Σ 13,200), 306 nm (Σ 9,950); pH 7.0, λ_{max}: 217 nm (Σ 14,200), 292 nm (Σ 7,700); pH 12.0, λ_{max}: 225 nm (Σ 17,600), 319.5 nm (Σ 7,550) and the pKa values of ho^5C determined spectrophotometrically in citrate-HCl (0.15 M) or Tris-HCl (0.15 M) at 20° were 3.80 $\pm$ 0.03 and 8.77 $\pm$ 0.04) (Figure 2.14). The presence of the 5-hydroxy group in the ho^5C was confirmed by the enol spray.

Figure 2.12: Elution profile from AG2-X8 column (OH^- form) in the purification of ho^5C, using a linear gradient of HCl.

(A) Collidine

(B) br^5C

(C) ho^5C

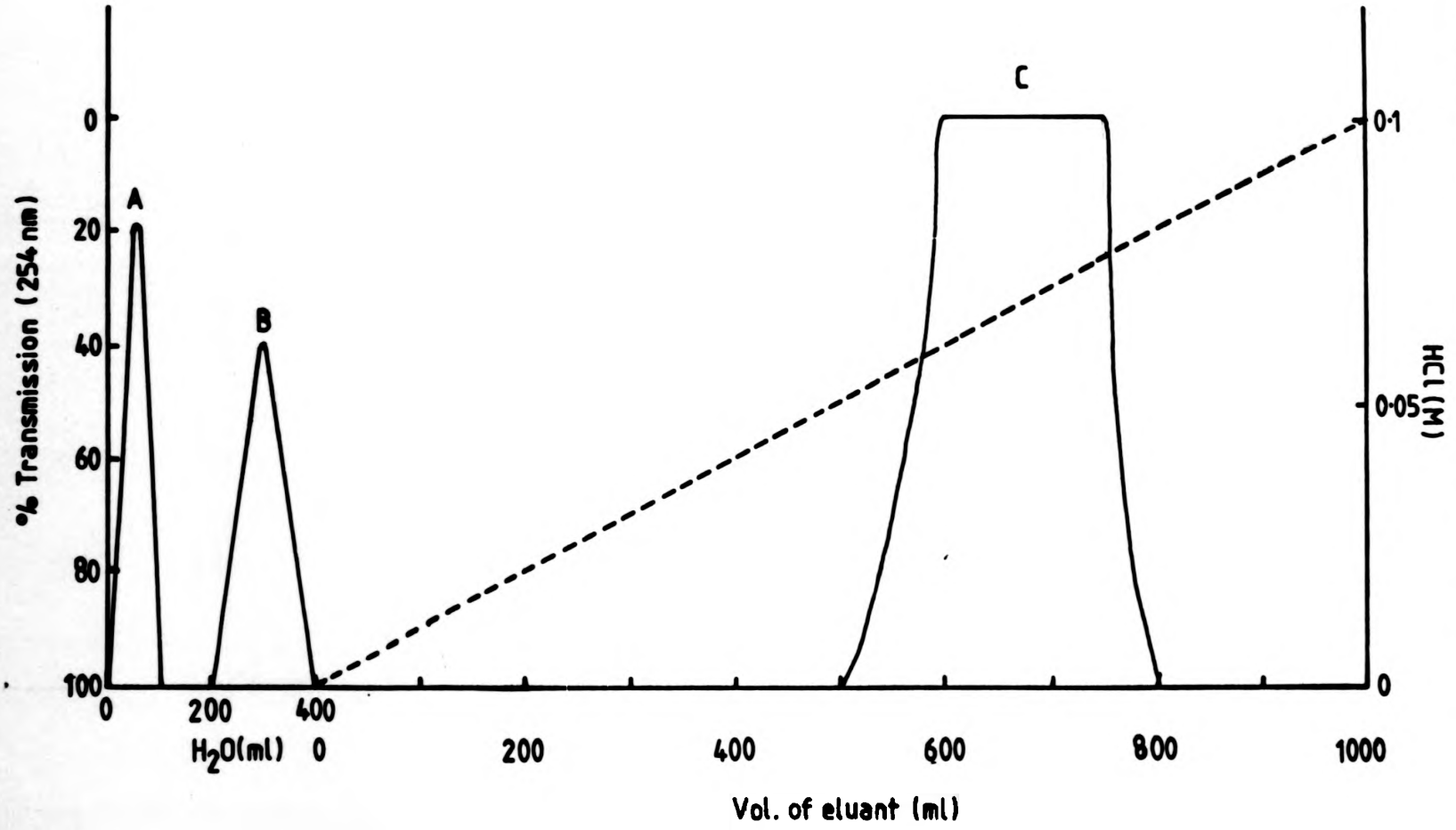

% Transmission (254 nm)
0
20
40
60
80
100
A
B
C
0
200
400
H_2O(ml)
0
200
400
600
800
1000
Vol. of eluant (ml)
0·1
0·05
0
HCl (M)

Figure 2.13: Elution profile from Dowex 50W x 4 column (H^+ form) in the purification of ho^5C, using a linear gradient of NH_4OH.

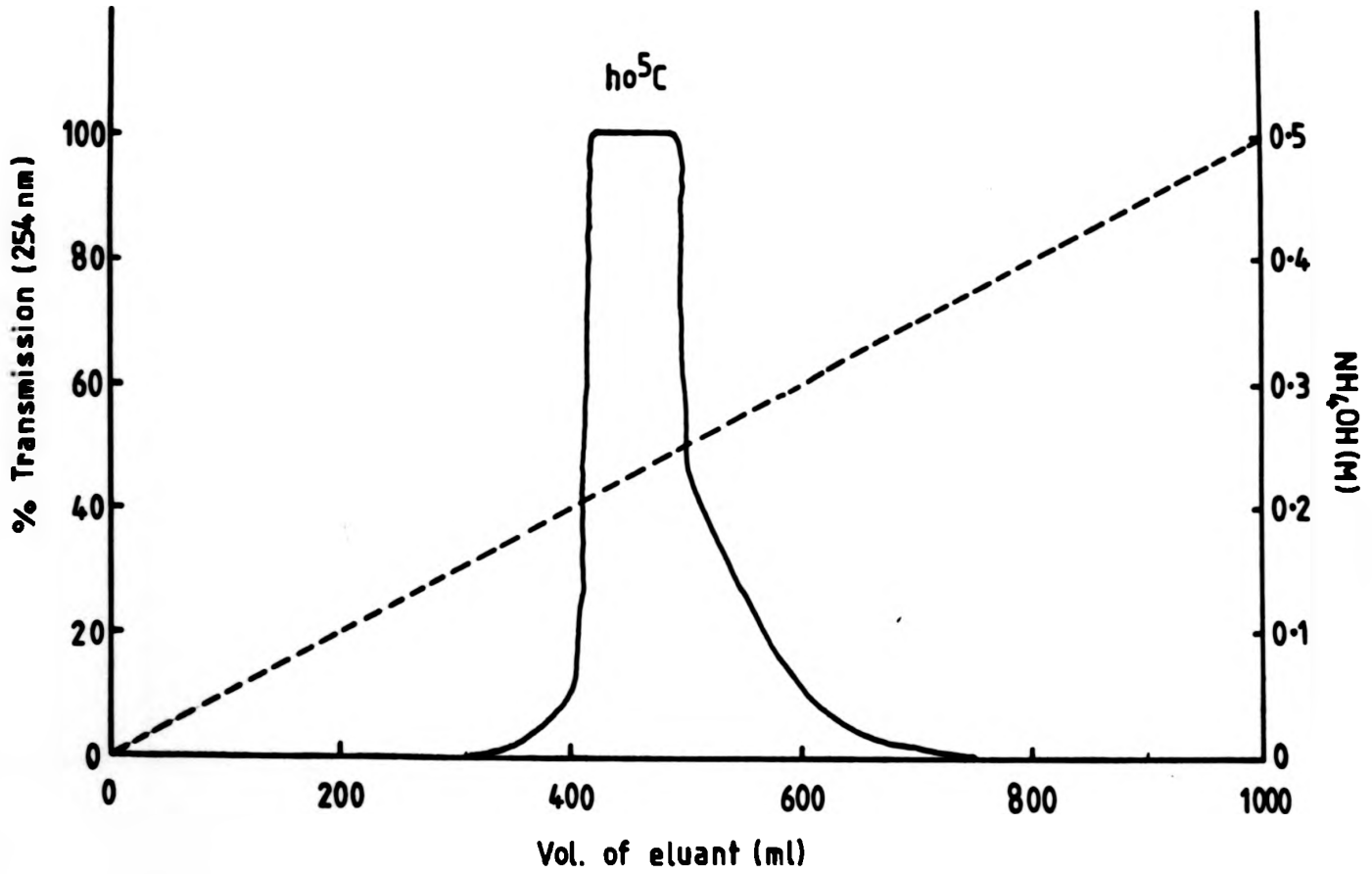
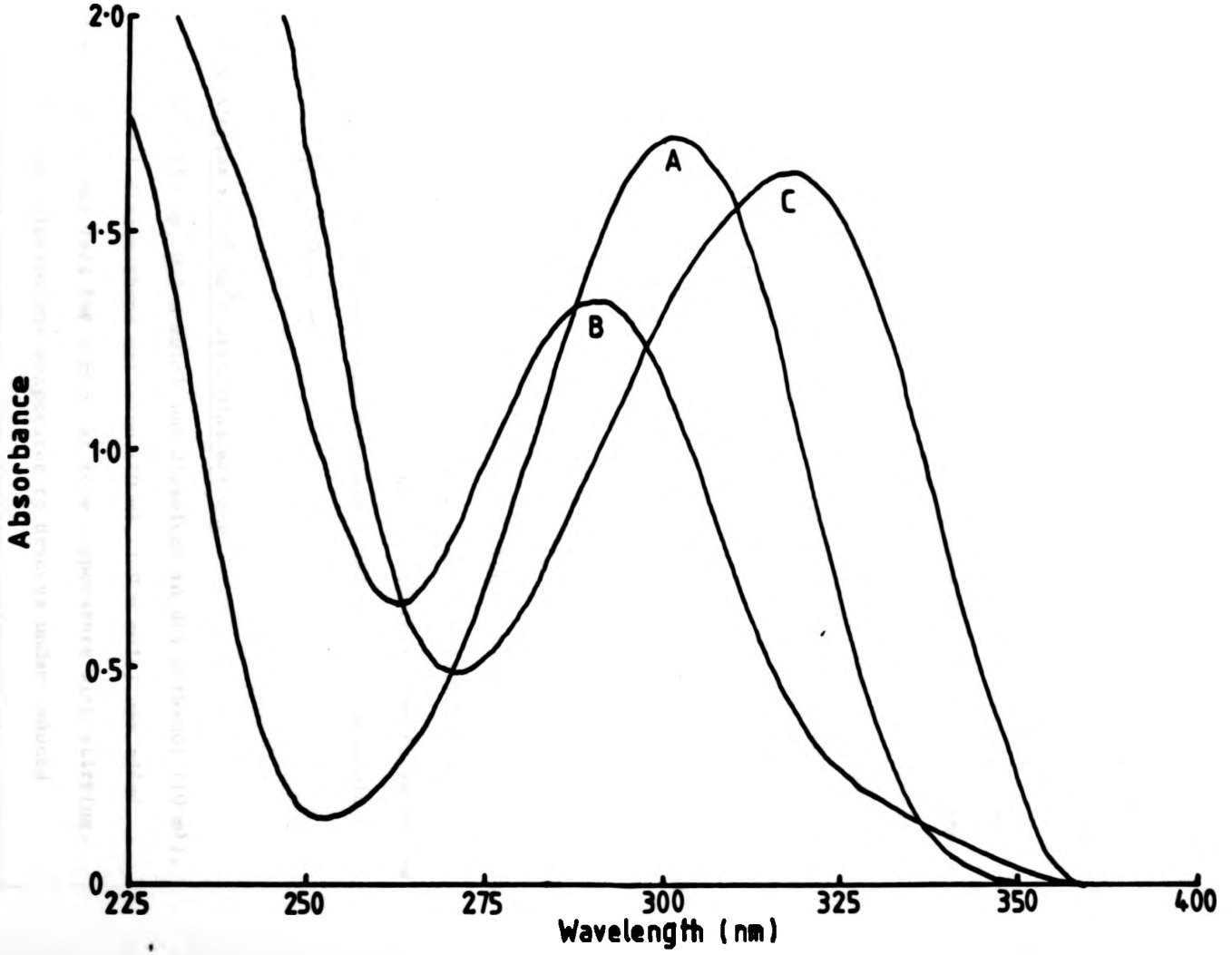

ho5C
% Transmission (254 nm)
100
80
60
40
20
0
0
200
400
600
800
1000
Vol. of eluant (ml)
0·5
0·4
0·3
0·2
0·1
0
NH4OH (M)

Figure 2.14: Ultra-violet spectra of ho^{5}C at (A) pH 1, (B) pH 6.5 and (C) pH 11.


2·0
1·5
1·0
0·5
0
Absorbance
A
B
C
225
250
275
300
325
350
400
Wavelength (nm)

2.2.5 Alkylation of 5-Hydroxycytidine with Dimethyl Sulphate

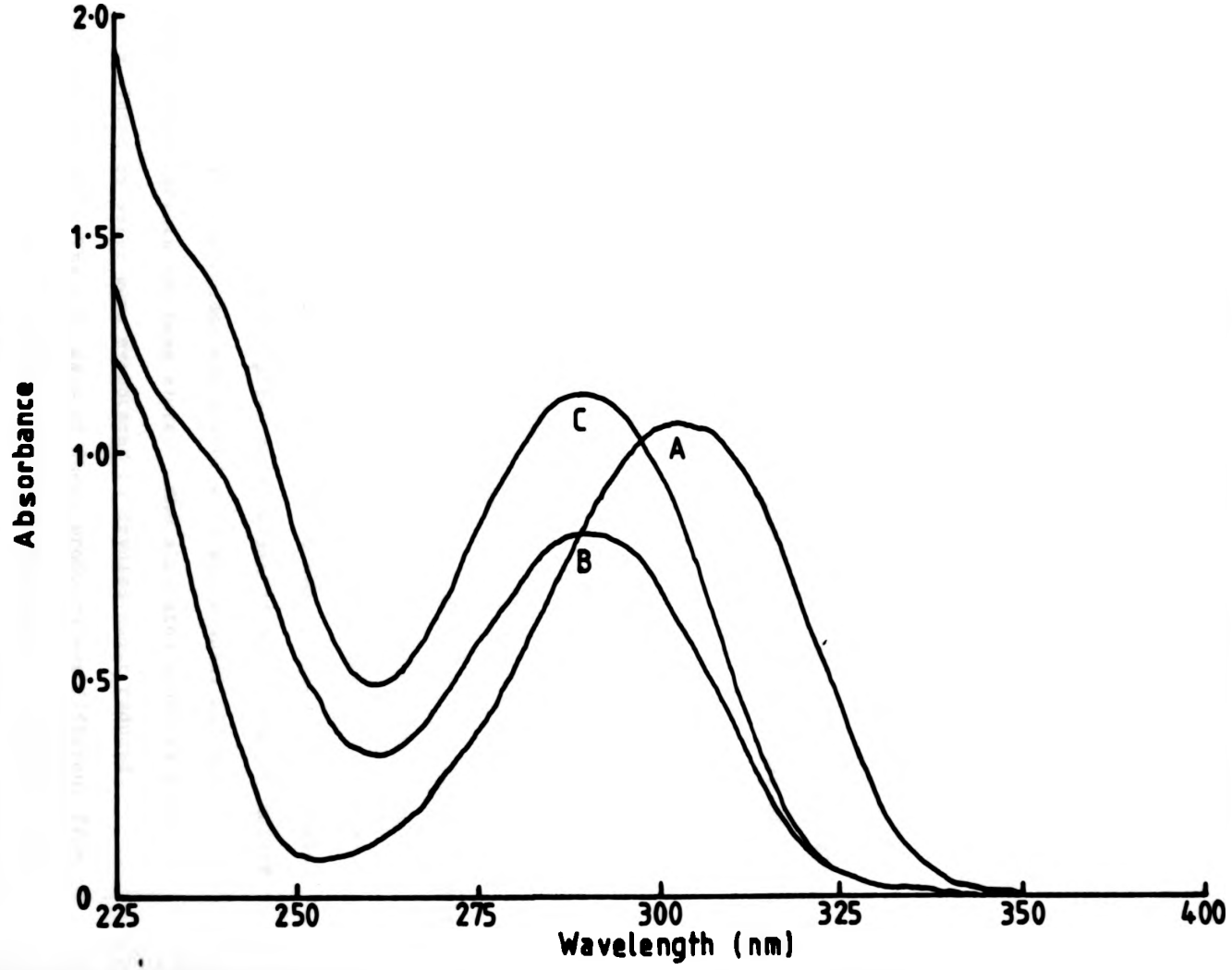
The alkylation method was based on that described by Murao et al (1976), but a number of modifications were introduced.

ho^5C (100 mg, 0.4 m mole) was dissolved in ammonium hydroxide solution (1 ml, 0.25 M), and the pH of the solution was adjusted to 9.5 by dropwise addition of ammonium hydroxide solution (0.5 M). Redistilled dimethyl sulphate (130 µl, 1.4 m mole) was added to the alkaline ho^5C solution in four equal portions at 30 min. intervals. The pH of the reacting solution was kept at 9.5 by dropwise addition of ammonium hydroxide solution (10 M), and constant stirring. This reaction was monitored by silica t.l.c. in solvent B. The resulting solution was applied to a column (1.5 x 25 cm) of AG2-X8 (OH^- form). The column was washed with water until all U.V. absorbing materials had been eluted. The alkylation products that were eluted by water were evaporated to dryness under reduced pressure at 40^o. The alkylation products were chromatographically homogeneous (silica t.l.c. in solvents A and B) and were enol negative. The alkylation products were tested for the presence of cis-diol group, and a positive response was observed, and the U.V. data were quite different from those of the ho^5C (Figure 2.15). Accurate measured mass: 287.111, and possible formula: $C_{11}H_{17}N_3O_6$.

2.2.6 Alkylation of ho^5C with Diazomethane

ho^5C (50 mg, 0.2 m mole) was dissolved in dry methanol (10 ml), and ethereal diazomethane solution (10 ml, 1.9 m mole) was added. The solution was left for 2 min. at room temperature with stirring. The resulting solution was evaporated to dryness under reduced pressure at room temperature. The alkylated nucleoside was redissolved in water, and the pH of the solution was adjusted to 9.0 with dilute ammonium hydroxide solution. The solution was applied to a column

Figure 2.15: Ultra-violet spectra of the products of the alkylation of 5-hydroxycytidine with dimethyl sulphate, at (A) pH 1, (B) pH 6.5, and (C) pH 11.


2·0
1·5
1·0
0·5
0
Absorbance
225
250
275
300
325
350
400
Wavelength (nm)
A
B
C

(1.5 x 25 cm) of AG2-X8 (OH^- form), and eluted with water until all U.V. absorbing materials had been eluted. The alkylation products that were eluted by water were evaporated to dryness under reduced pressure at 40°. Physical and chemical measurements of the alkylation products were very similar to the previous alkylation.

2.2.7 Synthesis of 5-Hydroxyuridine (ho^5U)

The synthesis of ho^5U was based on the method described by Ueda (1960).

The reaction yielded colourless crystals of ho^5U (0.36 g, 36%), m.p. 230-232° and the U.V. data substantially agreed with that of Ueda (1960), lit.: m.p. 230-232°; U.V. data, in H_2O, λ_{max}: 278 nm; pH 9, λ_{max}: 237 nm, 304 nm, λ_{min} 230 nm, 269 nm (Figure 2.16). The 5-hydroxy group of the product was confirmed by the enol spray.

2.2.8 Alkylation of ho^5U with Diazomethane

ho^5U (0.1 g, 0.38 m mole) was dissolved in dry methanol (10 ml), and ten molar excess of ethereal diazomethane solution (22 ml, 3.8 m mole) was added. The mixture was stirred for 15 min., and the solution was evaporated to dryness under reduced pressure. The residue was redissolved in water (10 ml), and the pH of the solution was adjusted to 8.0. The solution was applied to a column (1.5 x 25 cm) of AG2-X8 (OH^- form), and the column was eluted with water until all U.V. absorbing materials had been eluted. The alkylated products that were eluted by water were evaporated to dryness under reduced pressure at 40°. The U.V. data of these products was different from that of ho^5U (Figure 2.17). The alkylated products were separated into two spots (R_f: 0.37 and 0.52) by preparative silica t.l.c. in solvent C, and they were both enol negative. The two compounds were extracted separately with hot methanol and evaporated to dryness under reduced

Figure 2.16: Ultra-violet spectra of ho^5U at (A) pH 1, (B) pH 6.5, and (C) pH 11.

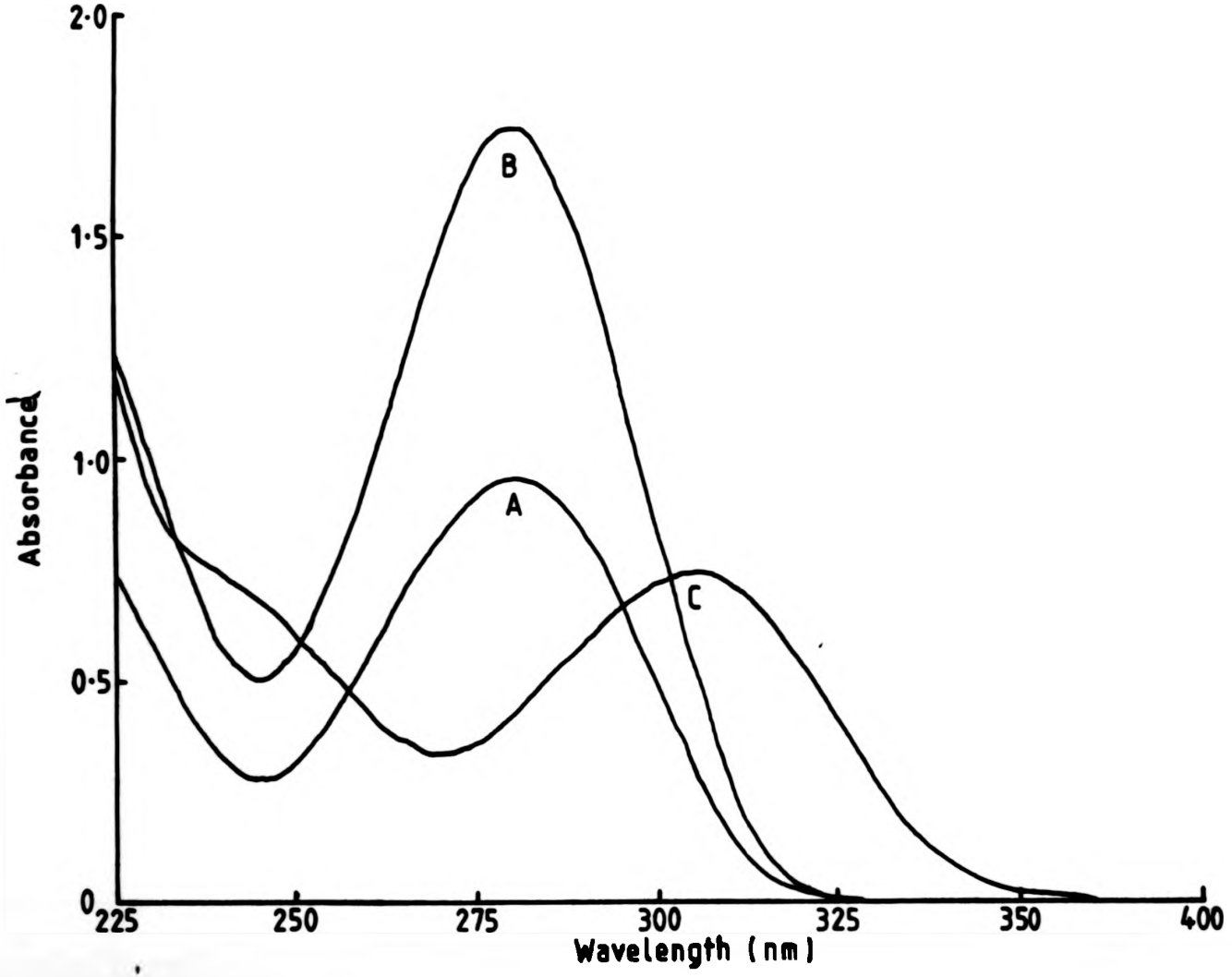

2·0
1·5
1·0
0·5
0
Absorbance
B
A
C
225
250
275
300
325
350
400
Wavelength (nm)

Figure 2.17: Ultra-violet spectra of the products of the alkylation of ho^{5}U with diazomethane at (A) pH 1, (B) pH 6.5, and (C) pH 11.

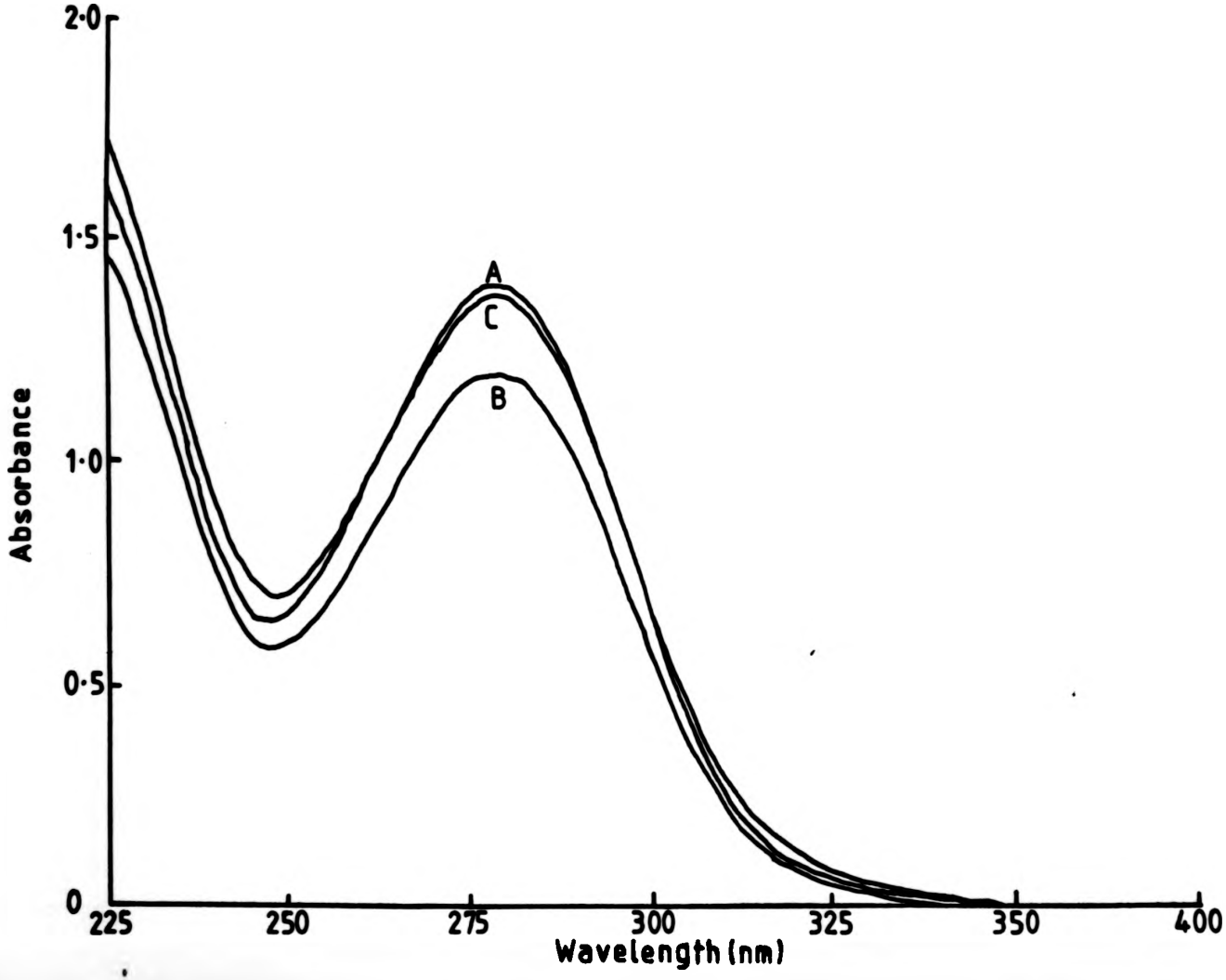

2·0
1·5
1·0
0·5
0
Absorbance
A
C
B
225
250
275
300
325
350
400
Wavelength (nm)

Figure 2.18: Ultra-violet spectra of one of the products of the alkylation of ho^5U with diazomethane at (A) pH 2, and (B) pH 11. This compound had a R_f of 0.37 when separated by preparative silica t.l.c. in solvent C.

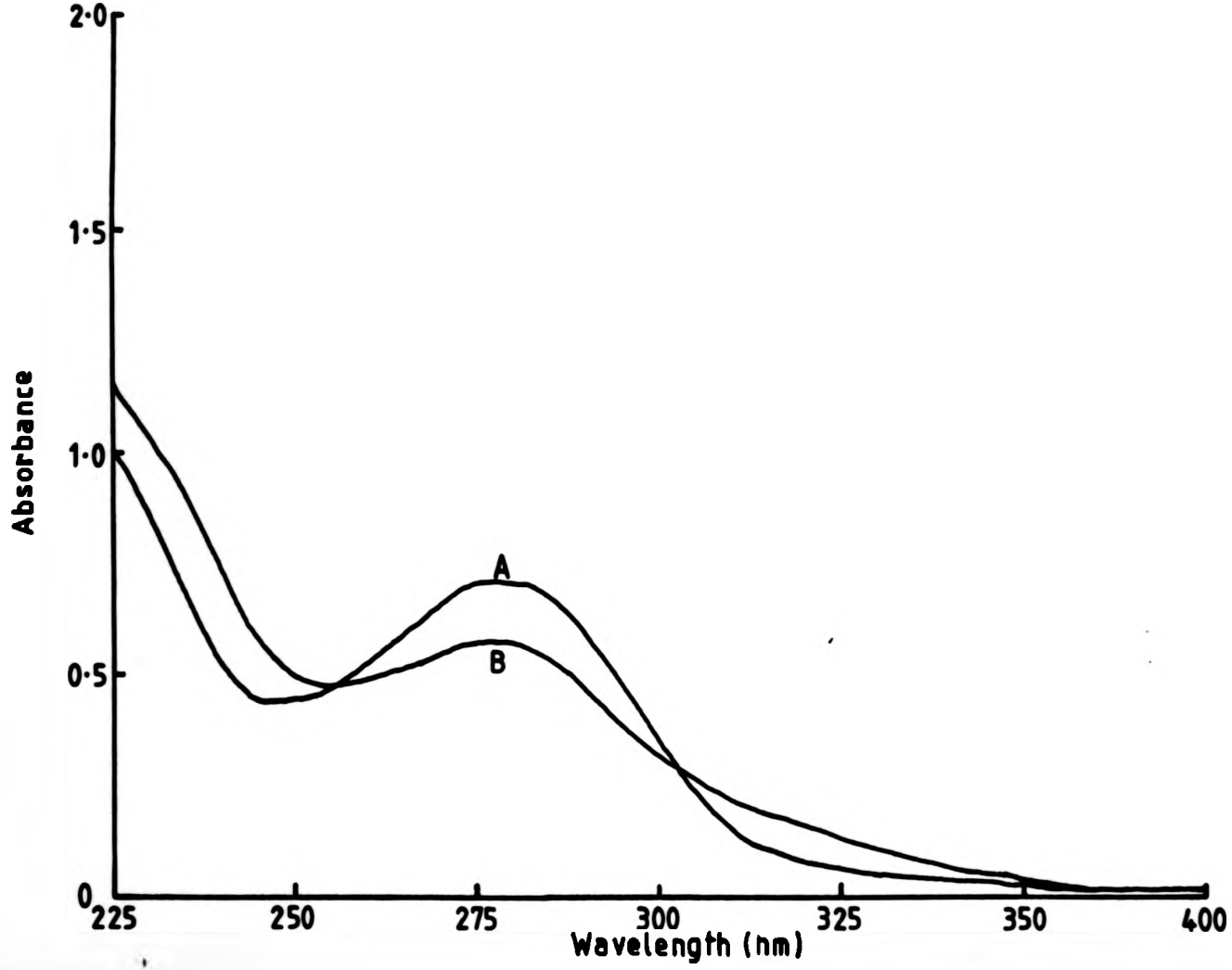
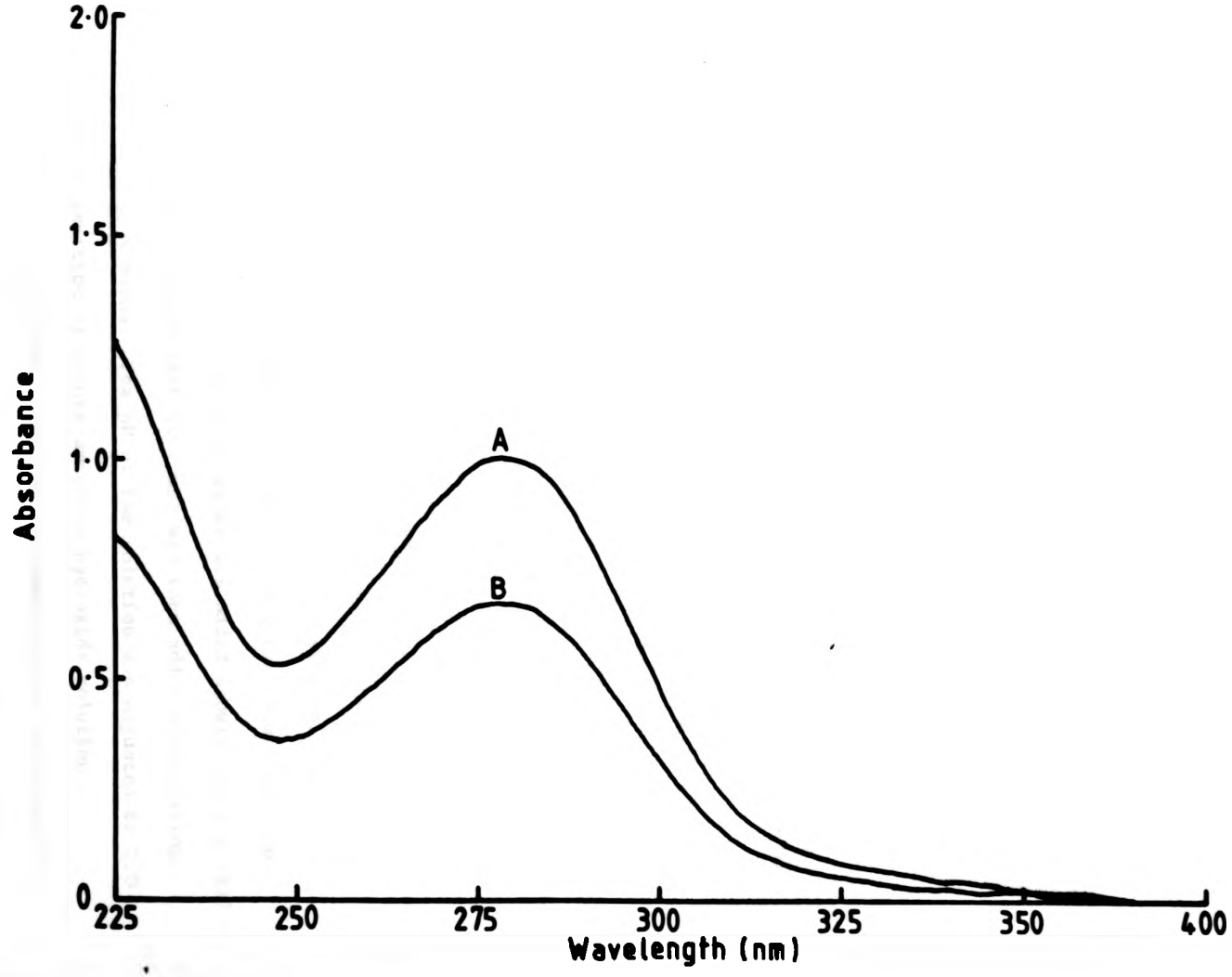

2·0
1·5
1·0
0·5
0
Absorbance
A
B
225
250
275
300
325
350
400
Wavelength (nm)

Figure 2.19: Ultra-violet spectra of one of the products of the alkylation of ho^5U with diazomethane at (A) pH 2, and (B) pH 11. This compound had a R_f of 0.52 when separated by preparative silica t.l.c. in solvent C.


2·0
1·5
1·0
0·5
0
Absorbance
A
B
225
250
275
300
325
350
400
Wavelength (nm)

pressure. The U.V. data of the compound with the low mobility (Figure 2.18) and that with the higher mobility (Figure 2.19) were similar.

2.2.9 Synthesis of N^4-acetylcytidine (N^4-acetyl C)

The synthesis of N^4-acetyl C was based on the method previously described by Folayan (1971).

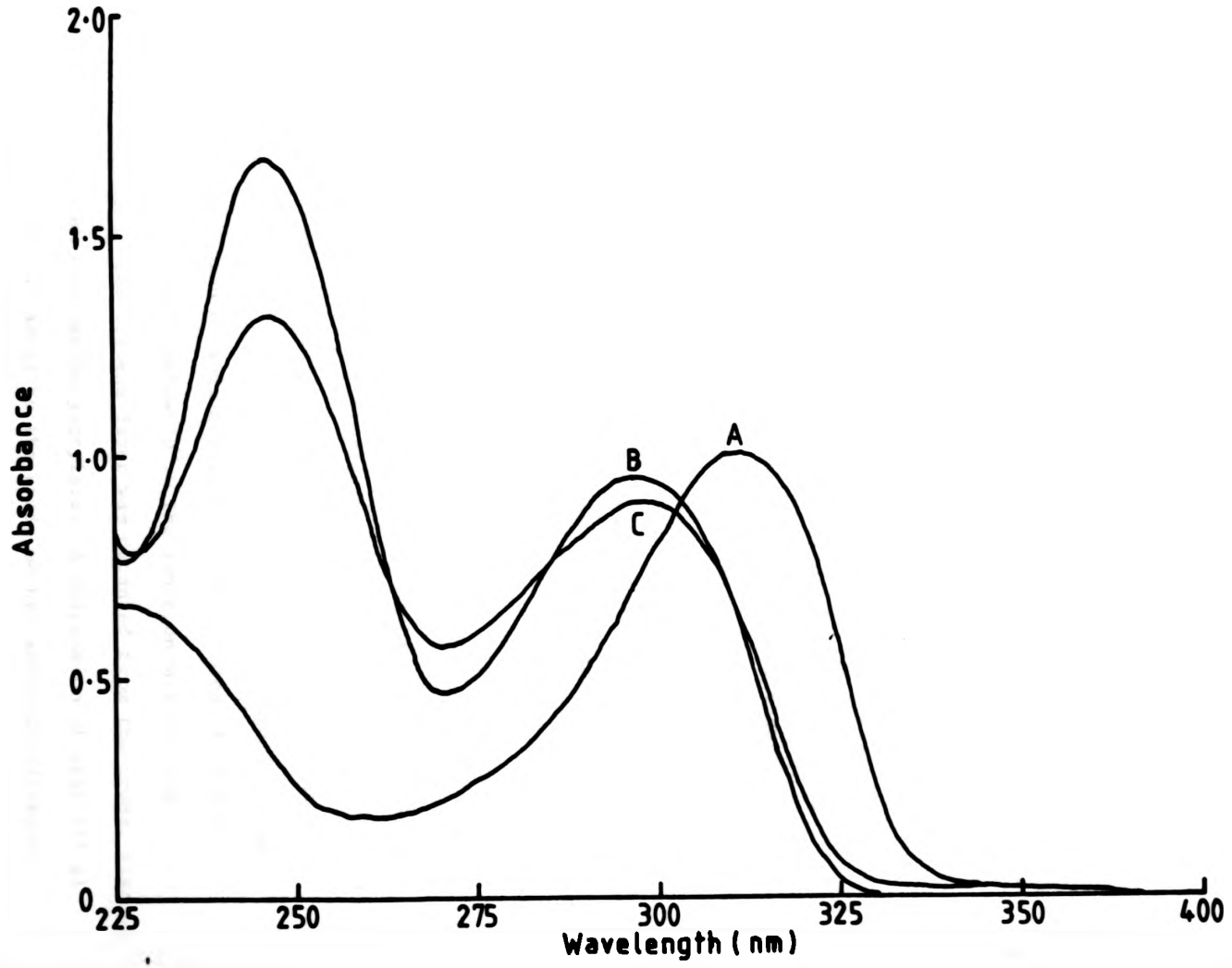
Cytidine (2 g) in anhydrous pyridine (340 ml) was stirred for 5 hr. at 50°. Thioacetic acid (6 ml) was added and stirring was continued for 18 hr. at 50°. The solution was evaporated to near-dryness under reduced pressure and washed with ether (4 x 40 ml). The resulting white precipitate was recrystallised in ethanol: water (90:10 v/v) at 0° (1.1 g, 44%). The U.V. data (Figure 2.20) (pH 6, λ_{max}: 247 nm, 297 nm, λ_{min}: 227.5 nm, 270 nm; pH 1, λ_{max}: 311 nm, λ_{min}: 260 nm; pH 11, λ_{max}: 246.5 nm, 298 nm, λ_{min}: 270 nm, 227.5 nm) agreed with that of Folayan (1971) (lit.: pH 7, λ_{max}: 247 nm, 297 nm, λ_{min}: 227.5 nm, 271 nm).

2.2.10 The Synthesis of N^4-acetyl-5-hydroxycytidine (N^4-acetyl ho^5C)

The synthesis of N^4-acetyl ho^5C was based on the method described by Eaton and Hutchinson (1973) for the synthesis of ho^5C.

N^4-acetyl C (0.1 g) was dissolved in hot water (5 ml) and the solution was cooled to 0°. Bromine water was added slowly until a yellow colour persisted. Cyclohexene (0.1 ml) was then added with shaking to remove excess bromine. The pH of the solution was adjusted to 7.0 with dropwise addition of dilute ammonium hydroxide solution. 2,4,6-collidine (0.5 ml) was added and the emulsion was incubated for 2 hr. at 40°. After cooling, the mixture was extracted with ether (2 x 4 ml). The aqueous layer was evaporated to dryness under reduced pressure. The remaining 2,4,6-collidine was removed by

Figure 2.20: Ultra-violet spectra of N^4-acetyl C at (A) pH 1, (B) pH 6 and (C) pH 11.


2·0
1·5
1·0
0·5
0
Absorbance
225
250
275
300
325
350
400
Wavelength (nm)
A
B
C

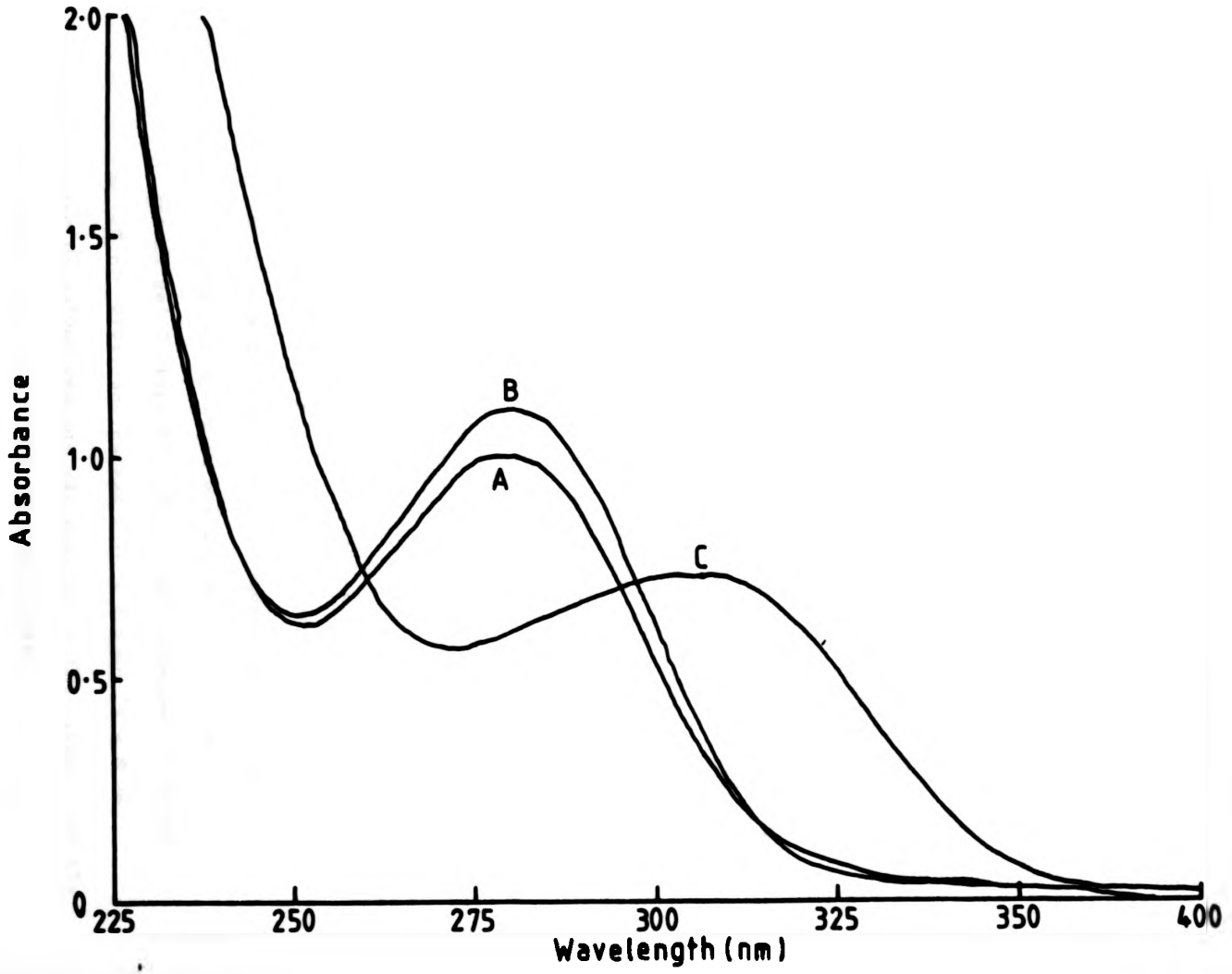
repeated addition and evaporation under reduced pressure of methanol. The residue was redissolved in water (10 ml) and applied to a column (1.5 x 25 cm) of AG1-X8 (OH^- form). The column was washed with water until all U.V. absorbing impurities had been eluted. The elution of the product was achieved by a linear gradient of tri-ethylammonium bicarbonate solution pH 7.5 (0-0.3 M, 1 l). The N^4-acetyl ho^5C was eluted at 0.1 M tri-ethylammonium bicarbonate concentration. The fractions were collected, and evaporated to dryness under reduced pressure at 40°. The last trace of tri-ethylammonium bicarbonate was removed by repeated addition and evaporation, under reduced pressure, of methanol, to give a pale yellow solid. Although this product gave a positive response to the enol spray, the U.V. data resembled that of the ho^5U. (Figure 2.21). Similar results were obtained when the purification by anion-exchange chromatography was replaced by recrystallisation by methanol:ether.

2.2.11 Synthesis of 5-Methoxy-pyrimidine

The synthesis of 5-methoxy-pyrimidine was based on the method described previously by Chesterfield et al. (1960).

Methyl methoxyacetate (25 g) and redistilled ethyl formate (16 g, 19 ml, b.p. 52-54°) in a dropping funnel, was added dropwise to a stirred suspension of sodium (5.5 g) in dry-toluene (100 ml), the temperature being kept below 30°. The reaction mixture was stirred overnight. The toluene layer was decanted from the crude, viscous methyl sodio-β-hydroxy-α-methoxyacrylate. A suspension of urea (15 g) in ethanol (100 ml) was added to the crude methyl sodio-β-hydroxy-α-methoxyacrylate, and the mixture was boiled under reflux for 5 hours, and after cooling was diluted with water (100 ml) and acidified with acetic acid. The precipitated 2,4-dihydroxy-5-methoxypyrimidine

Figure 2.21: Ultra-violet spectra of the product of the synthesis of N^4-acetyl ho^5C from N^4-acetyl C at (A) pH 1, (B) pH 6.5 and (C) pH 11.


2·0
1·5
1·0
0·5
0
Absorbance
A
B
C
225
250
275
300
325
350
400
Wavelength (nm)

was collected and washed with water and ethanol. The yield was 4.3 g (12.4%), m.p. 328-333° (decomp.) (same as lit.).

2.2.12 Synthesis of 5-Methoxyuridine-2',3',5'-tri-O-benzoate

The synthesis of 5-methoxyuridine-2',3',5'-tri-O-benzoate was based on the method described by Vorbrüggen and Bennua (1978).

To 5-methoxyuracil (1.26 g, 10 m mole), 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranose (5.14 g, 10 m mole) and potassium nonaflate (8.12 g, 24 m mole) in acetonitrile (140 ml), trichlorosilane (3.26 g, 3.78 ml, 30 m mole) and hexamethyldisilazane (1.14 g, 1.48 ml, 7 m mole) were added and the reaction mixture was refluxed for 20 hr. with the exclusion of moisture. After dilution of the reaction mixture with dichloromethane (100 ml), extraction with sodium bicarbonate (0.1 M) and sodium chloride (0.1 M) solution (150 ml), evaporation gave a brown residue, which was dissolved in minimal amount of boiling ethyl acetate, and any insoluble residue was filtered and discarded while the solution was still hot. Redistilled hexane (b.p. 68-69.5°) was added to the cooled filtrate until white precipitate started to appear. The ethyl acetate-hexane solution was kept at -20° overnight. The white precipitate was filtered off and washed with hot ethyl acetate-hexane (20:80, v/v). The dark brown filtrate was evaporated to dryness, and the dark brown residue was dissolved again in boiling ethyl acetate and then cooled. Silica gel G (type 60, 100 g) was suspended in ethyl acetate:hexane (40:60, v/v), and packed into a column (4 x 8 cm). The ethyl acetate dissolved residue was applied to the column, and the product was eluted with ethyl acetate:hexane (40:60, v/v). The rapid chromatography technique applied was based on the method described by Still et al. (1978). The yellowish solution eluted was collected and evaporated to dryness under reduced pressure at

40°. A white precipitate was obtained, and was combined to the white precipitate obtained previously. The yield was 4.1 g (71%), m.p. 206-208°. The silica t.l.c. in solvent D showed that the compound was homogeneous and had a R_f of 0.075.

2.2.13 Synthesis of 5-Methoxyuridine (mo^5U)

The synthesis of 5-methoxyuridine was based on the method described by Vorbrüggen and Bennua (1978).

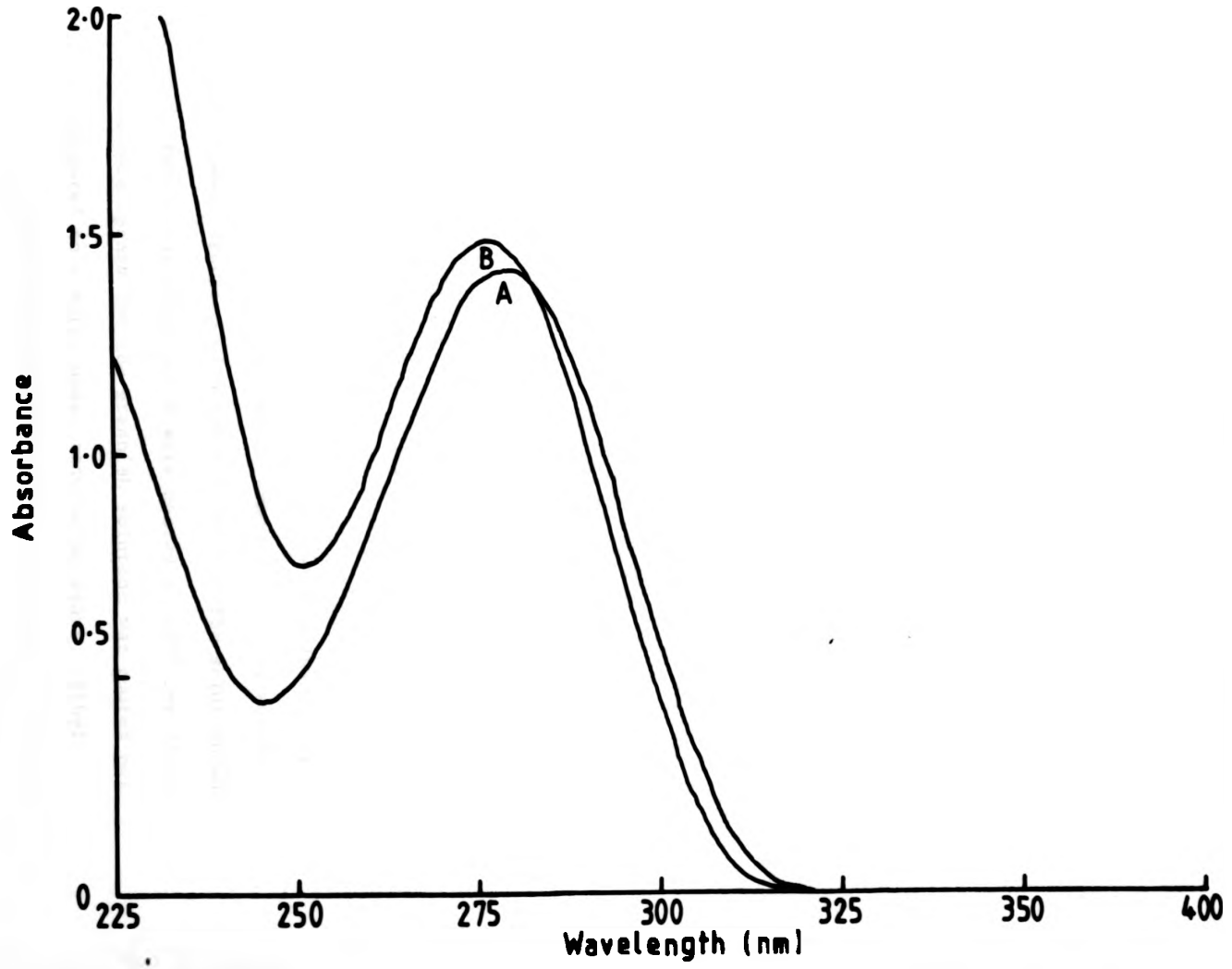
5-Methoxyuridine-2',3',5'-tri-O-benzoate (1 g) was dissolved in minimal amount of boiling methanol. After cooling to room temperature, gaseous ammonia was bubbled into the methanolic solution for 20 min. This solution was stoppered and incubated at 24° for 20 hr. The methanolic solution was evaporated to dryness under reduced pressure. The residue was dissolved in water (10 ml) and extracted with ether (10 ml). The aqueous layer was evaporated to dryness under reduced pressure. The crude product was recrystallized from methanol to yield in two crops pure 5-methoxyuridine (0.39 g, 82%), m.p. 214-216°. Silica t.l.c. in solvent E showed that the product has a R_f of 0.70, which agreed with that of Murao et al. (1976), and the U.V. data also agreed (Figure 2.22). Lit.: pH 2, λ_{max}: 279 nm; pH 12, λ_{max}: 277 nm. Proton NMR data: 1.31 p.p.m. (singlet, t-butanol); 3.79 p.p.m. (singlet, 5-methoxy hydrogens); 3.97 p.p.m. (multiplets, H-5'); 4.38-4.22 p.p.m. (multiplets, H-2',3',4'); 5.98-6.02 p.p.m. (singlet, H-1'); 7.67 p.p.m. (singlet, H-6).

2.2.14 Synthesis of 4-Thio-5-methoxyuridine-2',3',5'-tri-O-benzoate

The synthesis of 4-thio-5-methoxyuridine-2',3',5'-tri-O-benzoate was based on the method described by Garrett (1968).

A mixture of 5-methoxyuridine-2',3',5'-tri-O-benzoate (3 g,

Figure 2.22: Ultra-violet spectra of mo^5U at (A) pH 2 and (B) pH 12.

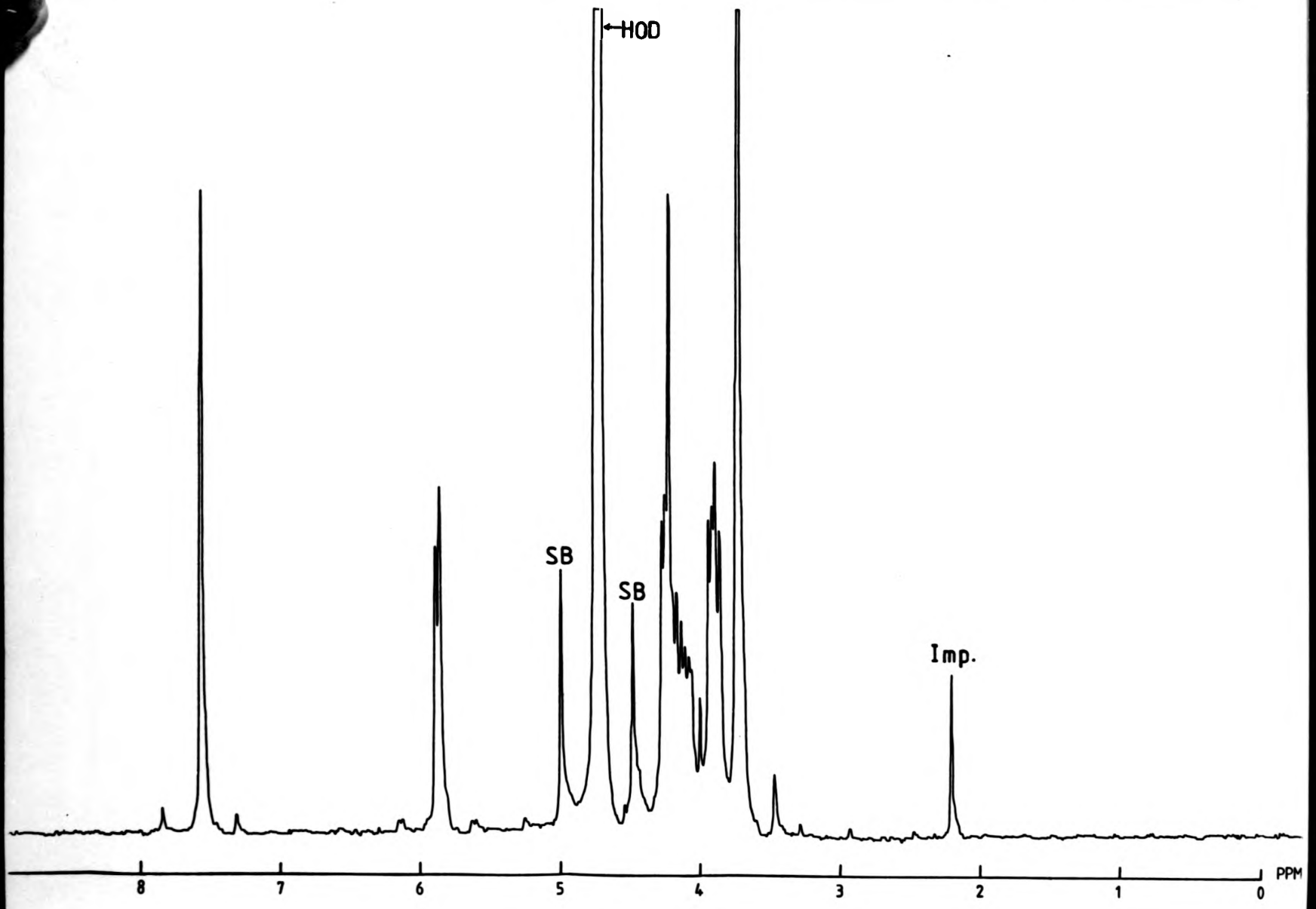

2·0
1·5
1·0
0·5
0
Absorbance
B
A
225
250
275
300
325
350
400
Wavelength (nm)

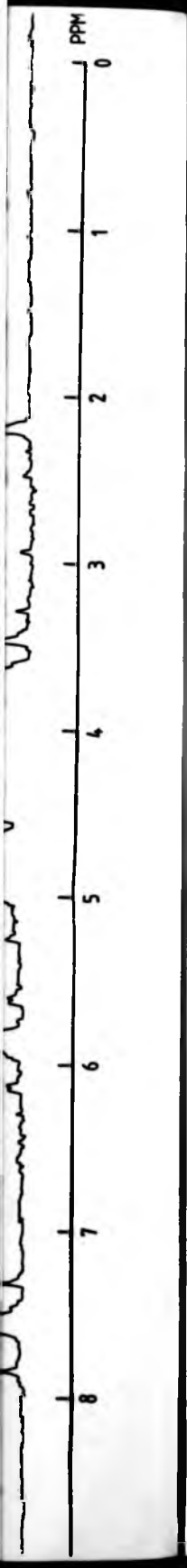
5.1 m mole), phosphorus pentasulphide (6 g, 34.2 m mole), which was purified by soxhlet extraction with carbon disulphide as solvent; and anhydrous pyridine (90 ml) was refluxed at 125° with stirring for 6 hr. After half of the pyridine was evaporated under reduced pressure at 40°, water was added, with vigorous stirring, to the residual brown mixture. Water was decanted from the residue, this was repeated until the water was colourless. The residue was dissolved in chloroform, and filtered. The filtrate was extracted twice with water, and the chloroform layer was dried with sodium sulphate, filtered, and evaporated to dryness under reduced pressure. The residue was dissolved in hot ethanol, filtered while hot and then cooled to room temperature. The light yellowish crystals were filtered off and dried under reduced pressure at 40°. The yield of 4-thio-5-methoxyuridine-2',3',5'-tri-O-benzoate was 1.5 g (50%), m.p. 220-224°, and the silica t.l.c. in solvent D showed that this compound had a R_f of 0.375.

2.2.15 Synthesis of 5-Methoxycytidine (mo^5C)

The synthesis of 5-methoxycytidine was based on the method described by Fox et al. (1959).

4-Thio-5-methoxyuridine-2',3',5'-tri-O-benzoate (1.2 g, 2 m mole) was dissolved in alcoholic ammonia (40 ml), which had previously been saturated with ammonia at 0°. The solution was placed in two Carius tubes which were heated at 100° for 24 hr. The tubes were opened and the greenish solution was poured out and concentrated to a syrup under reduced pressure. Ethyl benzoate was removed by distillation with water, until the water that came out was not oily. The aqueous solution was extracted twice with chloroform to remove benzamide. The aqueous layer was evaporated to dryness under reduced pressure at 40°. The syrupy


HOD
SB
SB
Imp.
8
7
6
5
4
3
2
1
0
PPM



residue was dissolved in methanol (5 ml) and then ethyl acetate was added until the precipitate started to appear. The mixture was then kept at -20° overnight. The precipitate was filtered and recrystallized with hot water. Colourless crystals which appeared on the bottom were washed with ice-cold water. The crystals were redissolved in water, and lyophilised. The yield of the compound 5-methoxycytidine was 0.39 g (72%), m.p. 112-116°. U.V. spectra (Figure 2.23); pH 2, λ_{max} 306 nm (Σ 10160), λ_{min} 254 nm; pH 7, λ_{max} 293 nm (Σ 7750), λ_{min} 262 nm; pH 12, λ_{max} 293 nm (Σ 7620), λ_{min} 263 nm. The pKa value of 5-methoxycytidine determined spectrophotometrically in HCl (0.1 M), or formic acid (0.01 M) + NaOH (1.0 M), or acetic acid (0.01 M) + NaOH (1.0 M) at 20° was 3.75 ± 0.17. Analysis calculated for $C_{10}H_{15}N_3O_6$:C, 43.90; H, 5.50; N, 15.40; O, 35.20%; Found C, 43.66; H, 5.61; N, 15.18; O, 34.90%. Proton NMR data: 3.72 p.p.m. (singlet, 5-methoxy hydrogens); 3.84-4.22 p.p.m. (multiplets, H-2',3',4',5'); 5.86 p.p.m. (doublet, H-1'); 7.59 p.p.m. (singlet, H-6) (NMR spectrum on facing page).

2.2.16 Studies on the Phosphorylation of Cytidine by Phosphoryl Chloride to Determine Optimum Condition

Cytidine (50 mg, 0.2 m mole) was suspended on trimethyl phosphate (various amounts) with or without water (various amounts) and stirred at 0° for 5 min. Phosphoryl chloride (various amounts) was added and the mixture stirred at 0° for 18 hr. (Table 2.1). The reactions were monitored with silica t.l.c. in solvent F, and PEI t.l.c. in solvent H. No new spots were detected for experiments from (1) to (7). The reaction was observed to have gone to completion in experiment (10).

Figure 2.23: Ultra-violet spectra of mo^5C at (A) pH 2, (B) pH 7, and (C) pH 12.

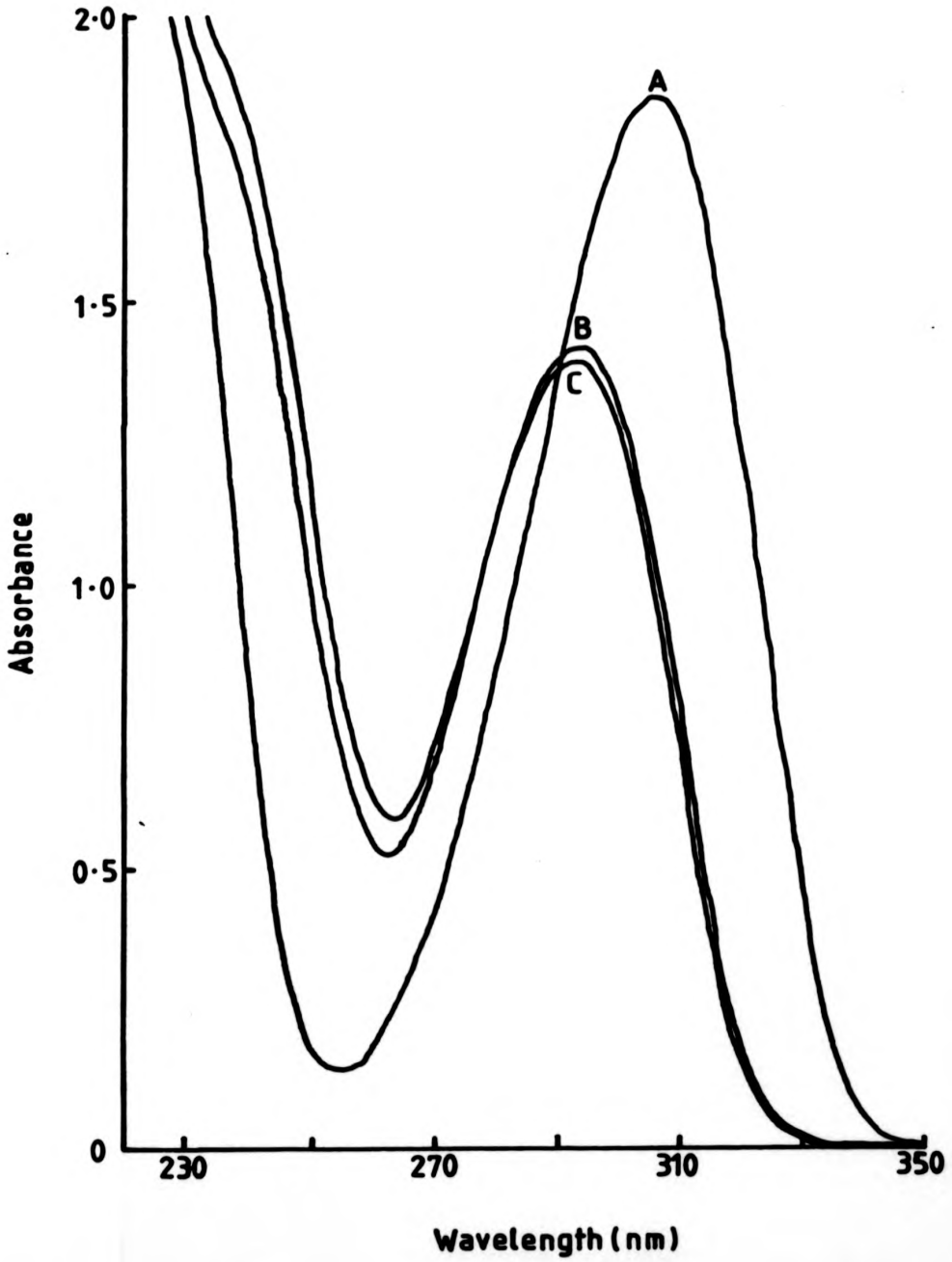

2·0
1·5
1·0
0·5
0
Absorbance
A
B
C
230
270
310
350
Wavelength (nm)

TABLE 2.1

	Nucleoside (cytidine) (mg)	Trimethyl phosphate (ml)	Phosphoryl chloride (µl)	Water (µl)
1[a]	↑	0.5	22, 0.24 m mole	
2[a]		0.5	100, 1.00 m mole	
3[a]		0.5	200, 2.00 m mole	
4[a]		0.5	40, 0.44 m mole	36, 0.20 m mole
5[a]	50, 0.2 m mole	0.5	100, 1.00 m mole	36, 0.20 m mole
6[a]		0.5	200, 2.00 m mole	36, 0.20 m mole
7[a]		2.0	100, 1.00 m mole	36, 0.20 m mole
8[b]		2.0	100 1.00 m mole	
9[b]		2.0	400, 4.40 m mole	
10[c]	↓	2.0	800, 8.80 m mole	

Studies on the phosphorylation of cytidine by phosphoryl chloride with and without water

a: no new spots were detected with silica t.l.c. in solvent F.
b: reactions were not complete.
c: complete conversion of cytidine to cytidine-5'-phosphorodichloridate was observed.

TABLE 2.1

	Nucleoside (cytidine) (mg)	Trimethyl phosphate (ml)	Phosphoryl chloride (µl)	Water (µl)
1[a]	↑	0.5	22, 0.24 m mole	
2[a]		0.5	100, 1.00 m mole	
3[a]		0.5	200, 2.00 m mole	
4[a]		0.5	40, 0.44 m mole	36, 0.20 m mole
5[a]	50, 0.2 m mole	0.5	100, 1.00 m mole	36, 0.20 m mole
6[a]		0.5	200, 2.00 m mole	36, 0.20 m mole
7[a]		2.0	100, 1.00 m mole	36, 0.20 m mole
8[b]		2.0	100 1.00 m mole	
9[b]		2.0	400, 4.40 m mole	
10[c]	↓	2.0	800, 8.80 m mole	

Studies on the phosphorylation of cytidine by phosphoryl chloride with and without water

a: no new spots were detected with silica t.l.c. in solvent F.
b: reactions were not complete.
c: complete conversion of cytidine to cytidine-5'-phosphorodichloridate was observed.

2.2.17 Synthesis of 5-Methoxycytidine 5'-phosphorodiamidate

The synthesis of 5-methoxycytidine 5'-phosphorodiamidate was based on the method described by Simoncsits and Tomasz (1975), but with the introduction of modification according to the findings in section 2.2.16.

5-Methoxycytidine (50 mg, 0.18 m mole) was suspended on trimethyl phosphate (2 ml) at 0° and stirred for 5 min. Phosphoryl chloride (0.8 ml, 8.80 m moles) was added to the 5-methoxycytidine suspension, and the suspension became a homogenous solution after a few min., and the solution was left stirring at 0° for 18 hr. The homogenous solution was poured into ammonium hydroxide solution (1:1, 40 ml) at below 10°. After standing for 10 min., the solution was evaporated to dryness under reduced pressure. The residue was resuspended in N,N-dimethylformamide (4 x 10 ml) and warmed at 45° with shaking and then filtered under suction. The filtrate was evaporated to dryness under reduced pressure at 40°. This compound, 5-methoxycytidine 5'-phosphoradiamidate, was not further purified (Figure 2.24).

2.2.18 Synthesis of 5-Methoxycytidine 5'-phosphoramidate

To a solution of 5-methoxycytidine 5'-phosphorodiamidate (from the above synthesis) in water (10 ml), was added hydrochloric acid (1 M, 4 ml) to adjust the pH to 1.7. After standing for 30 min. at 25°, the solution was neutralized with ammonium hydroxide solution (1 M). During this time the starting compound could be seen to have disappeared using silica t.l.c. in solvent F and PEI t.l.c. in solvent H, with the appearance of a new spot with a slightly lower mobility. The solution was evaporated to dryness under reduced pressure at 40°. The residue was redissolved in water (500 ml) and applied to a column (2.4 x 25 cm) of DEAE Sephadex A-25 (HCO_3^- form), which was

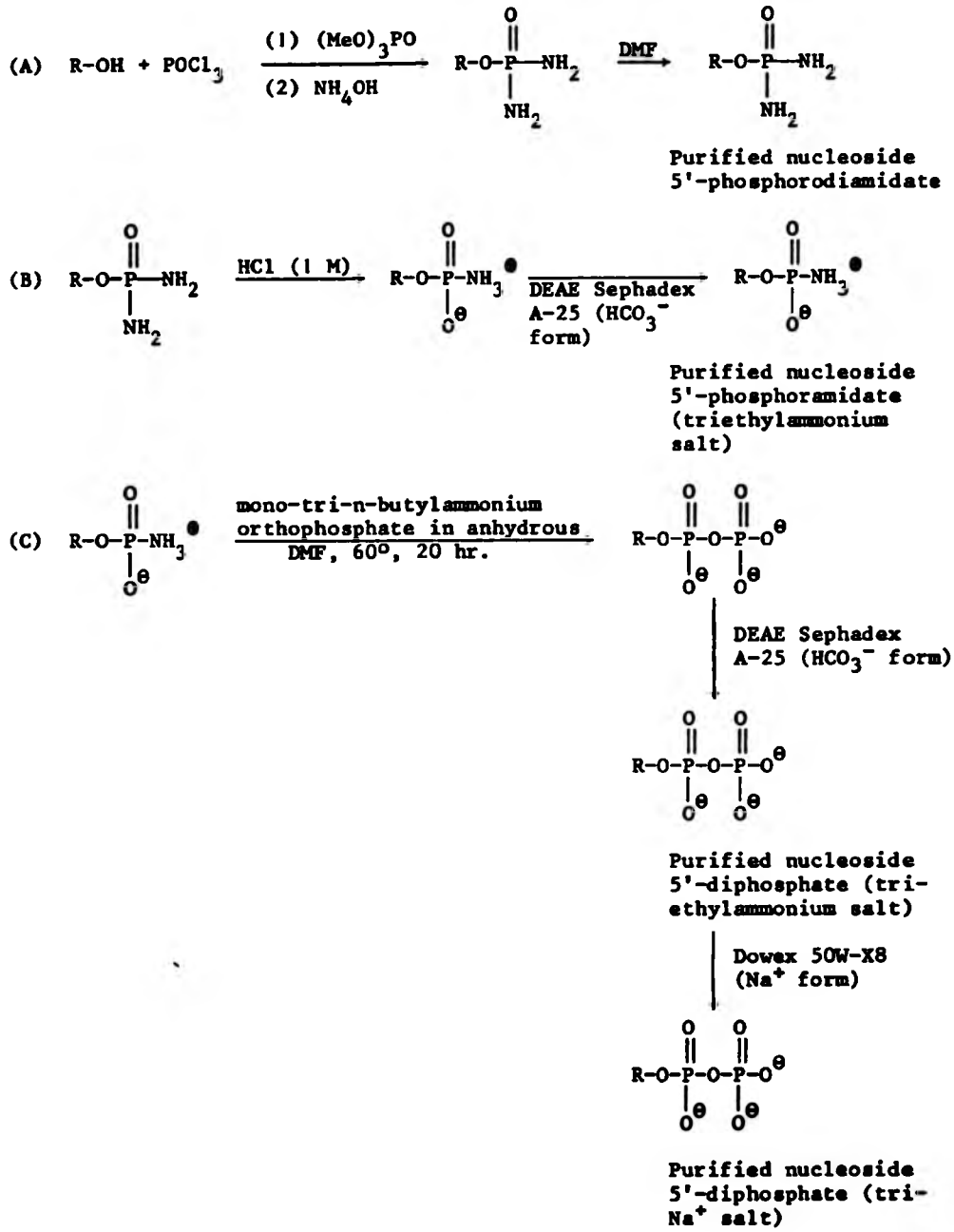


Figure 2.24: Synthesis of 5-methoxycytidine 5'-diphosphate from 5-methoxycytidine.

equilibrated with water. The column was washed with water until all U.V. absorbing materials had been eluted. The elution of 5-methoxycytidine 5'-phosphoramidate was achieved by a linear gradient of triethylammonium bicarbonate pH 8.9 (0-0.1 M, 1 l). The compound was eluted between 0.04-0.06 M triethylammonium bicarbonate solution. The fractions of 5-methoxycytidine 5'-phosphoramidate were collected and evaporated to dryness under reduced pressure at 40°, and the last trace of triethylammonium bicarbonate solution was removed by repeated addition and evaporation under reduced pressure of methanol. A further peak of U.V.absorbing material was observed to have been eluted from the column between 0.07-0.09 M triethylammonium bicarbonate solution. This product could be 5-methoxycytidine 5'-monophosphate, but it was not confirmed. A further linear gradient of triethylammonium bicarbonate pH 8.9 (0.1-0.2 M, 1 l) was applied to the column, and a third peak of U.V. absorbing material was observed to have been eluted between 0.18-0.20 M triethylammonium bicarbonate solution. This compound was confirmed to be 5-methoxycytidine 5'-diphosphate by silica t.l.c. in solvents F and G, and PEI t.l.c. in solvents H and I. Yields of 5-methoxycytidine 5'-phosphoramidate (triethylamonium salt) (in terms of O.D. units) = 23%, and 5-methoxycytidine 5'-diphosphate (triethylammonium salt) (in terms of O.D. units) = 27% (Figure 2.24).

2.2.19 Synthesis of 5-methoxycytidine 5'-diphosphate (mo^5CDP)

The synthesis of 5-methoxycytidine 5'-diphosphate was based on the method described by Tomasz et al. (1978) (Figure 2.24).

5-Methoxycytidine 5'-phosphoramidate (~ 0.04 m mole) was dissolved in mono-tri-n-butylammonium orthophosphate in anhydrous N,N-dimethylformamide (0.5 M, 25 ml, 12.5 m mole) with warming at 60°. The solution was incubated in a well-stoppered flask at

60° for 20 hr. The reaction mixture was cooled to room temperature and the N,N-dimethylformamide was evaporated to dryness under reduced pressure at 40°. The syrupy residue was dissolved in 0.1 M triethylammonium bicarbonate pH 8.9 solution (250 ml), and applied to a column (2.4 x 25 cm) of DEAE Sephadex A-25 (HCO_3^- form), which had been equilibrated with the same solution. The column was washed with the same solution until all U.V. absorbing impurities had been eluted. The elution of 5-methoxycytidine 5'-diphosphate was achieved by a linear gradient of triethylammonium bicarbonate pH 8.9 (0.1-0.2 M, 1 l). The fractions of the product, which were eluted between 0.18-0.20 M triethylammonium bicarbonate solution, were collected and treated as described before. The resulting white residue was dissolved in water (10 ml) and applied to a column (1.4 x 35 cm) of Dowex 50W x 8 (Na^+ form), and the product was eluted with water. The fractions were collected and evaporated to dryness under reduced pressure at 40° to give a white compound of 5-methoxycytidine 5'-diphosphate (tri-Na^+ salt). The combined products of 5-methoxycytidine 5'-diphosphate (tri-Na^+ salt) gave a total yield (in terms of O.D. units) of 42 mg (46%). The product was further purified by descending paper chromatography (Whatman 3 MM paper, 20 x 50 cm) in solvent J. The 5-methoxycytidine 5'-diphosphate that remained at the origin was cut off and extracted with water (3 x 10 ml), and then evaporated to dryness under reduced pressure at 40°. The product on the paper was made visible utilizing the phosphate spray. The final total yield of 5-methoxycytidine 5'-diphosphate was 34 mg (37%). The nucleoside to total phosphorus ratio was found to be 1:2, and the U.V. data remained unchanged.

The R_f values of all the nucleosides and nucleotides mentioned previously are tabulated in Table 2.2.

2.3 Results and Discussion

2.3.1 Alkylation of ho^5C and ho^5U

Dimethyl sulphate and diazomethane have been widely used in the alkylation of cytidine (Singer, 1975). It was discovered by Kusmierek et al. (1973) that at pH > 13, the ring nitrogen had resistance towards alkylation, but the ribose moiety was alkylated. Kusmierek and Shugar (1971) also found that alkylation of cytidine with dimethyl sulphate in 1 N NaOH gave almost exclusively products alkylated on the ribose moiety. In 1 N NaOH, with a 150-molar excess of dimethyl sulphate, the products included 1-5% N^4-methylcytidine; in 10 N NaOH, under the same conditions there appeared 10-20% N^4-methylcytidine and a few per cent of N^4-dimethylcytidine. Therefore, it was apparent that for the methylation of ho^5C with dimethyl sulphate, high temperature, pH > 13, and exhaustive methylation should be avoided. The condition of the methylation of ho^5C was comparatively mild, the pH was kept at 9.5, and only 3.5-molar excess of dimethyl sulphate was employed at room temperature. After the methylation, the attempt to separate the product from ho^5C using Dowex 50W was unsuccessful. Absorption chromatography using silica gel or Florisil was then tried, with chloroform with stepwise increase of percentage of methanol to elute the products, but the nucleosides were found to be unstable in silica gel and Florisil. The product was eventually separated from ho^5C by using AG2-X8 (OH^- form). This purified methylated product was chromatographically homogeneous (silica t.l.c. in solvents A and B). The negative enol spray response of the methylated product indicated that all the 5-hydroxy groups were converted to

TABLE 2.2

The R_f values of all the nucleosides and nucleotides mentioned in this chapter

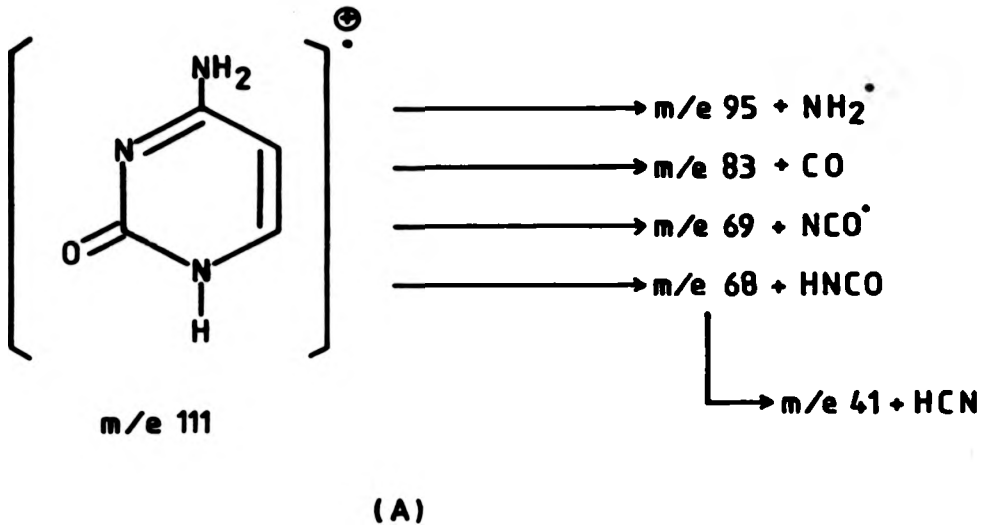
Nucleosides and Nucleotides	R_f									
	Solvents									
	A	B	C	D	E	F	G	H	I	J
Cytidine	0.67				0.71	0.68	0.48	0.77	0.77	
5-Bromocytidine	0.58									
5-Hydroxycytidine	0.67	0.29								
N^4-methyl-5-methoxy-cytidine	0.45	0.50								
N^3-methyl-5-methoxy-uridine			0.52							
5-Hydroxyuridine			0.20							
5-Methoxyuridine			0.37							
N^3-methyl-5-hydroxy-uridine			0.43							
5-Methoxyuridine-2',3',5'-tri-O-benzoate				0.075						
5-Methoxyuridine				0.00	0.70					
4-Thio-5-methoxyuridine-2',3',5'-tri-O-benzoate				0.375						

Table 2.2 Continued

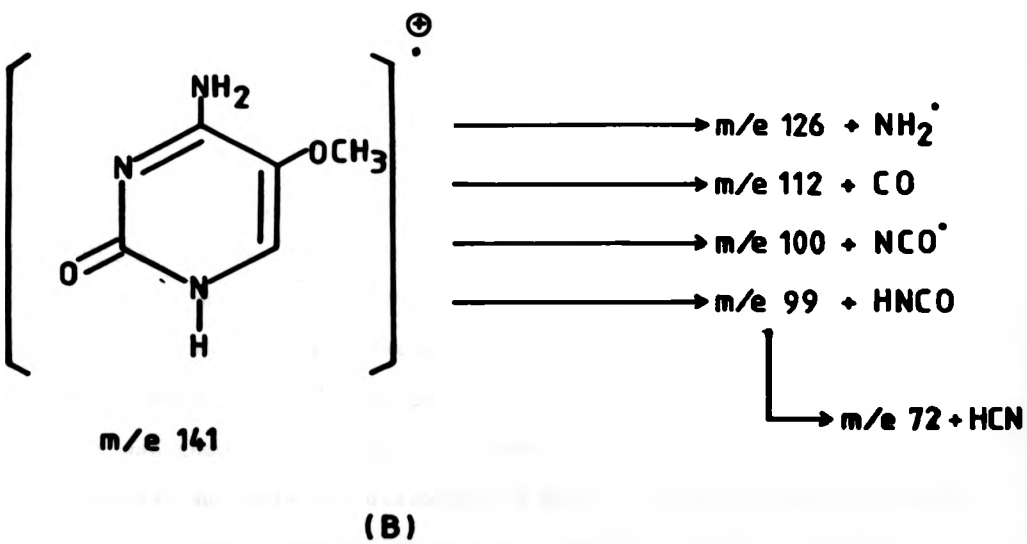
	A	B	C	D	E	F	G	H	I	J
5-Methoxycytidine					0.75	0.72	0.55	0.74	0.73	
5-Methoxycytidine-5'-phosphorodichloridate						0.33	0.00	0.36		
5-Methoxycytidine-5'-phosphorodiamidate						0.49	0.40	0.67		
5-Methoxycytidine-5'-phosphoramidate						0.46	0.40	0.37		
5-Methoxycytidine-5'-diphosphate						0.04	0.27	0.07	0.20	0.00
Cytidine-5'-diphosphate						0.03	0.24	0.09	0.28	0.00
Cytidine-5'-monophosphate							0.39			
Cytidine-2',3'-monophosphate							0.43			
Orthophosphoric Acid										0.29
Pyrophosphoric Acid										0.07

5-methoxy groups. The positive cis-diol spray response of the methylated product indicate that the 2' and 3'-hydroxy groups were not methylated. The U.V. data of the methylated product showed that the λ_{max} at pH 6.5 and pH 11 were both at 290 nm, and the λ_{max} at pH 1 remained at 302 nm as in the case of the ho^5C, but the shift of the λ_{max} to 318.5 nm at pH 11 in the ho^5C U.V. absorption spectrum did not appear in that of the methylated product (Figures 2.14 and 2.15). These results indicated that the 5-hydroxy groups had become 5-methoxy groups, and there was a good possibility that the product was mo^5C. However, the accurate mass measurement told another story. The accurate mass of the product was 287.111, which had a possible formula of $C_{11}H_{17}N_3O_6$, and it indicated that the product was dimethylated. Another low resolution mass spectrum showed that the mo^5C and its fragment peaks expected at m/e 141, 126, 112, 100, 99, and 72 were indeed present (Figure 2.25). The peaks at m/e 141, 126, 72 were very intense but two relatively small peaks at m/e 155, 154 were also observed. It indicated the presence of a dimethylated compound. No further experiment was carried out to identify the product. However, since the λ_{max} at pH 6.5 and pH 11 remained the same as in the case of ho^5C, it was more probable that the methylated compound was N^4-methyl-5-methoxycytidine.

Szer and Shugar (1966) found that cytidine did not react with diazomethane in non-aqueous solution, and direct alkylation of the cis- 2' and 3'-hydroxyl groups of cytidine with diazomethane could be achieved only at very high temperature (80°) and at very alkaline condition (Martin et al., 1968; Gin and Dekker, 1968). Therefore, methylation of ho^5C in non-aqueous solution, under mild alkaline condition at room temperature, should be possible to give mo^5C. However, the results obtained from this alkylation reaction of ho^5C were very similar to the previous one.



(A)



(B)

Figure 2.25: The possible fragmentation of (A) cytidine (Dejongh, 1968) and (B) 5-methoxycytidine in mass spectrometry

Murao et al. (1976) found that the methylation of ho^5U with dimethyl sulphate in alkaline condition gave a 25% yield of mo^5U. In addition to mo^5U and the raw material, the methylated products contained 5% 3-methyl-5-hydroxyuridine and 4% of 3-methyl-5-methoxyuridine. Their U.V. data is summarized in Table 2.3. In this study, diazomethane was used instead of dimethyl sulphate. After the reaction, three new spots were detected by silica t.l.c. in solvent C. The compound with a R_f of 0.43 gave a positive response to the enol spray test, thus it could be 3-methyl-5-hydroxyuridine. The other two compounds, which gave negative response to the enol spray test, with R_f of 0.37 and 0.52 could be 5-methoxyuridine and 3-methyl-5-methoxyuridine respectively (Table 2.2). Dowex-2 and AG-2 (OH^- form) were unable to separate the three methylated products and ho^5U satisfactorily. Therefore, preparative t.l.c. in solvent C was used to separate the four compounds. The two compounds with R_f of 0.37 and 0.52 were extracted with methanol and dried. Their U.V. data was similar, at pH 2 and pH 11; both λ_{max} were at 278 nm (Figures 2.18 and 2.19). At pH 2, λ_{max}: 281 nm, which was claimed to be the λ_{max} of 3-methyl-5-methoxyuridine at that pH by Murao et al. (1976), was not seen (Table 2.3). Both 5-methoxyuridine and 3-methyl-5-methoxyuridine gave a yield of well below 10%. Therefore, it was obvious that mo^5C could not be synthesized, via the synthesis of mo^5U, in good yield and quantity.

It was then thought that if indeed the methylation of ho^5C with dimethyl sulphate and diazomethane gave N^4-methyl-5-methoxycytidine, a protective group could be introduced in the N^4-position of cytidine before methylation. Therefore, the compound N^4-acetyl C was synthesized, and then converted to N^4-acetyl ho^5C as mentioned previously. However, the U.V. data of the product of the hydroxylation reaction (Figure 2.21) was similar to that of ho^5U. This was confirmed by the

Table 2.3: U.V. data of the three products from the methylation of ho^5U with dimethyl sulphate (Murao et al., 1976)

Nucleoside	λ_{max}	
	pH 2	pH 12
5-methoxyuridine	279 nm	277 nm
3-methyl-5-hydroxyuridine	280 nm	306 nm
3-methyl-5-methoxyuridine	281 nm	278 nm

mass spectrum, which showed that ho^5U and its fragment peaks at m/e 128, 100, 86, 85 and 58 were indeed present. It was concluded that N^4-acetyl C was very labile towards the conditions employed in the hydroxylation reaction, and no N^4-acetyl ho^5C was synthesized. No further methylation was carried out beyond this stage. Therefore, another method had to be used to synthesize mo^5C, as it is now clear that there are many sites in nucleosides which can be alkylated. These results agreed with some of the recent studies on the alkylation of DNA. Abbott and Saffhill (1979) methylated the alternating copolymer poly(dC-dG) with either dimethyl sulphate or N-methyl-N-nitrosourea, and found that in addition to the 3-methylcytosine, 3-methylguanine and 7-methylguanine, reaction with N-methyl-N-nitrosourea also yielded easily detectable amounts of O^6-methylguanine and phosphotriesters. These methylated polymers were then used as templates in an in vitro assay with E. coli DNA polymerase I measuring the incorporation of complementary (dCMP and dGMP) and non-complementary (dAMP and dTMP) nucleotides. When the N-methyl-N-nitrosourea-methylated polymer was used as template, there was a specific incorporation of dTMP but not of dAMP. Therefore, it was concluded (Abbott and Saffhill, 1979) that O^6-methylguanine was capable of miscoding with a DNA polymerase. Studies on the ethylation of poly(dA-dT) using ethylnitrosourea showed that the degrees of ethylation of phosphate group and the O^2-position on the thymine moiety were high. A low degree of ethylation also occurred at the O^4-position on thymine and the N^3-position on adenine. When methylnitrosourea was used, the degrees of methylation of phosphate group and the N^3-position on adenine were high. A low degree of methylation occurred at the O^2- and O^4-positions on thymine (Jensen and Reed, 1978; Jensen, 1978). Alkylation of DNA in vivo with dimethylnitrosamine or

diethylnitrosamine also induced the formation of phosphotriesters (Shooter, 1978; Shooter and Merrifield, 1978). It was found that the methyl or ethyl phosphotriesters were chemically stable and they were not eliminated by cellular DNA-repair systems. The short-term treatment in rat with high doses of dimethylnitrosamine had generally produced tumours of the kidney, whereas long-term treatment with low doses usually led to the appearance of tumours of the liver (Shooter, 1978; Shooter and Merrifield, 1978).

2.3.2 Synthesis of mo^5C

The first stage of the synthesis of mo^5C was the synthesis of 5-methoxyuracil. Relatively, it gave a very low yield (12.4%), but all the starting materials were easy to obtain and cheap. The second stage was the nucleoside synthesis, which was comparatively complicated in chemistry, yet interesting. The reaction can be broken up into three steps:

(A) Activation of the sugar moiety by the Friedel-Crafts-Type catalyst to give the electrophilic sugar cation.

(B) Reaction of the silylated base with the electrophilic sugar cation to form the nucleoside bond.

(C) Interaction of the catalyst with the silylated base.

In the first process (A), the catalyst $(CH_3)_3SiSO_3C_4F_9$ reacted with 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranose, the standard sugar building block, with formation of the 1,2-acyloxonium salt as the only electrophilic sugar moiety and concomitant formation of $C_4F_9SO_3^-$ and silylated acetic acid, $(CH_3)_3SiOCOCH_3$ (Figure 2.26A). In the next process (B), the nucleophilic silylated base could only attack the stable sugar cation from the top or β-side to afford exclusively the natural β-nucleoside. Simultaneously the activated α-tri-methylsilyloxy group in the heterocycle base reacted

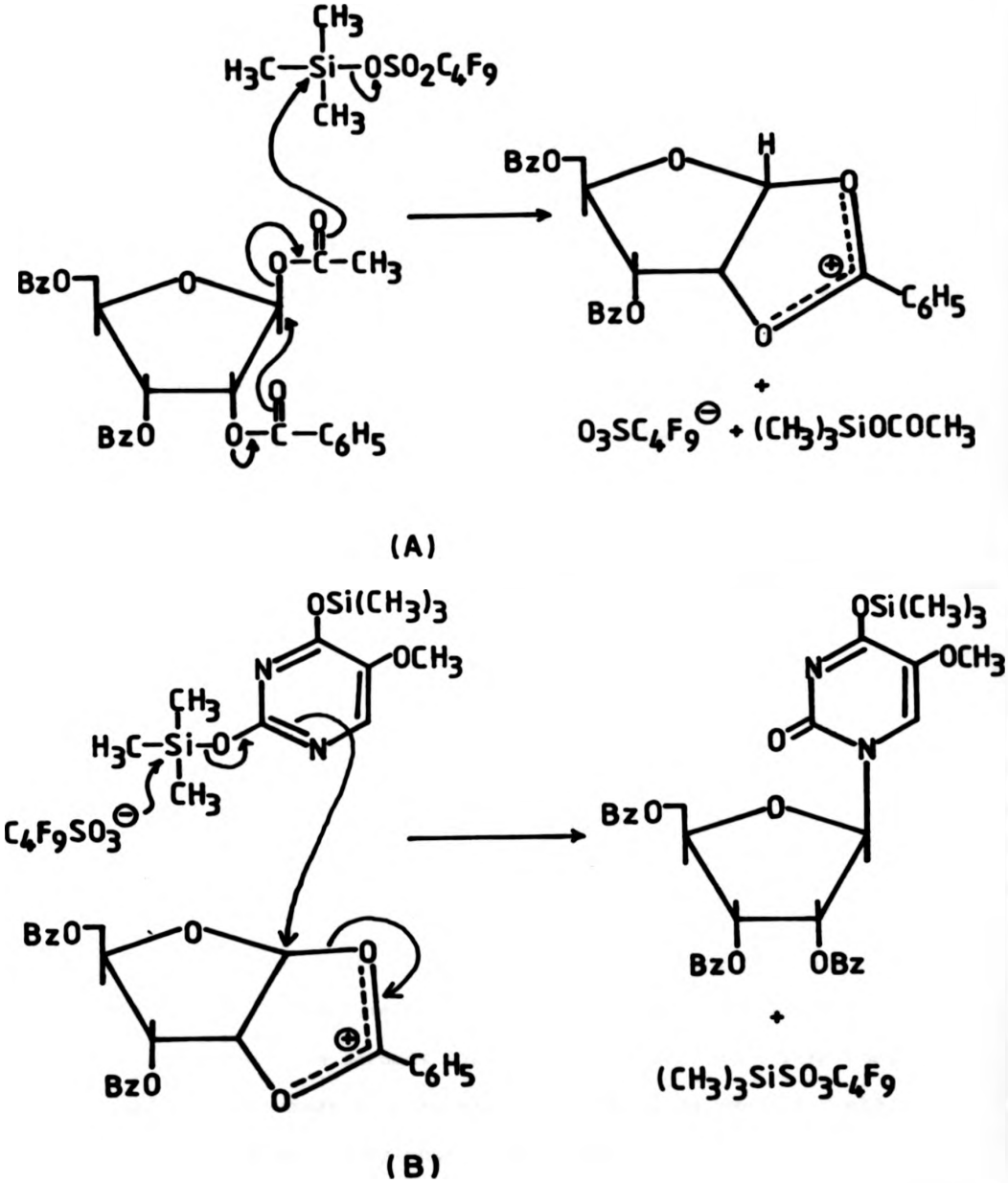


Figure 2.26: Reaction mechanism of the nucleoside synthesis with silylated base in the presence of Friedel-Crafts-Type catalyst (Vorbüggen, 1979).

with the nonaflate anion to regenerate the corresponding $(CH_3)_3SiSO_3C_4F_9$ (Figure 2.26B). Finally, in the third process (C), formation of the less reactive σ complex occurred between the silylated heterocyclic base and the Friedel-Crafts catalyst (Vorbrüggen, 1979). This reaction gave a high yield of 71%. In the synthesis of mo^5U, the next step was the removal of the acyl protecting groups by treating the acylated nucleoside with excess methanolic ammonia. This reaction also gave a high yield of 82%. The last two reactions gave an overall yield of over 58%, therefore mo^5U could be made on a gram scale, and the processes were far more effective than the method described by Murao et al. (1976). For the synthesis of mo^5C, the next step after the nucleoside synthesis was the synthesis of 4-thio-5-methoxyuridine-2',3',5'-tri-O-benzoate, which gave a yield of 49%. The final step of the synthesis of mo^5C was a single step reaction, which included the conversion of the 4-thio group to the 4-amino group and the removal of the acyl protecting groups. The U.V. data, NMR, and microanalysis results all indicated that the product was mo^5C. In the low resolution mass spectrum, the expected peaks at m/e 141 (B + H), 142 (B + 2H), and 170 (B + 30) were observed, but the D-ribose peak at m/e 133 and the peaks corresponding to the fragmentation of the base were not observed (Figure 2.27). The yield of this final stage was 72%. The advantages of using these methods for the synthesis of mo^5C were that the compound could be made in gram scale and the total overall yield from nucleoside synthetic step onwards was about 25%.

2.3.3 Phosphorylation of Nucleoside

Current methods on phosphorylation of biological molecules have been reviewed by Slotin (1977). The most often used method on phosphorylation of nucleosides was described by Smith and Khorana

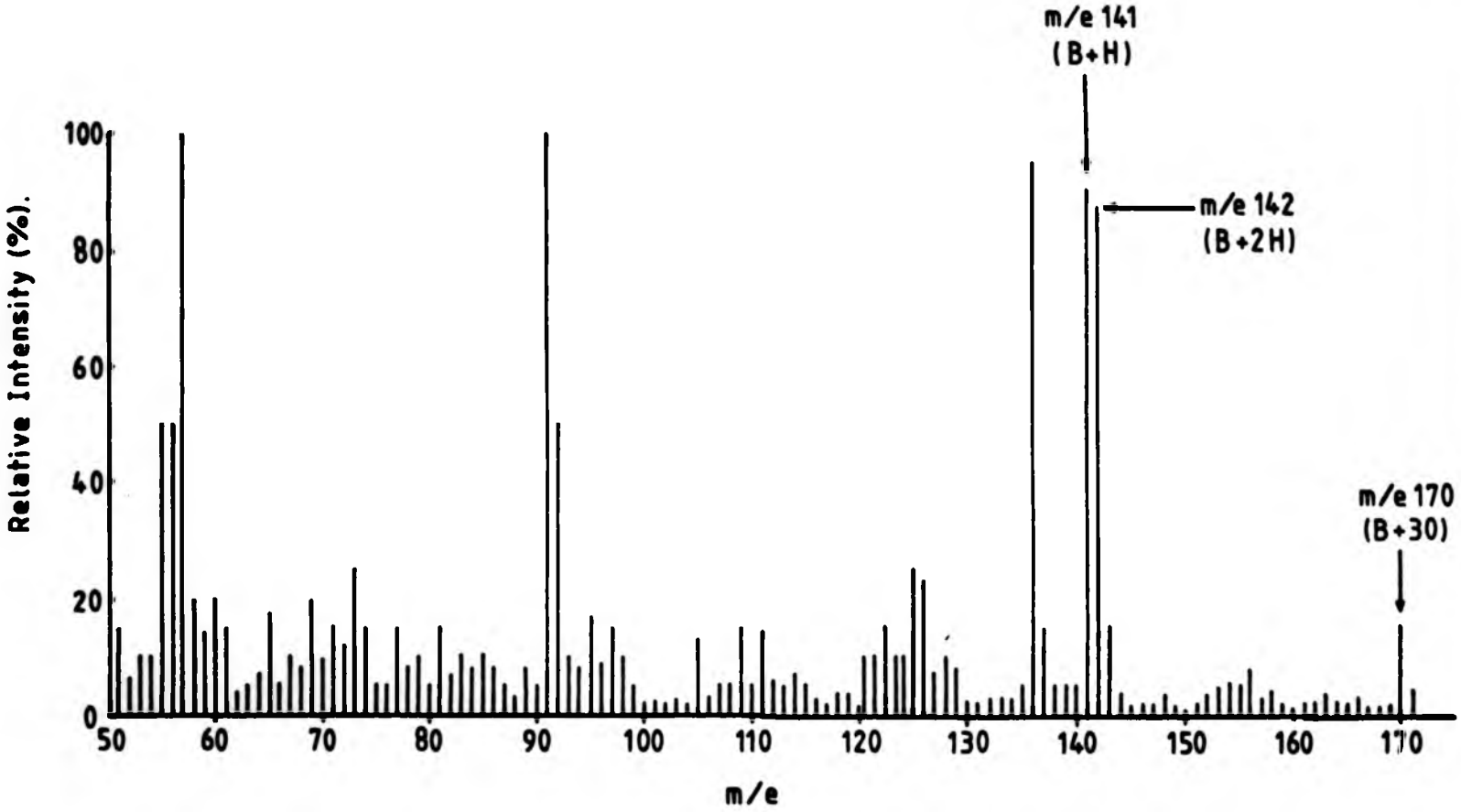


Figure 2.27: Mass spectrum of mo^5C.

(1963). Nucleosides were treated with p-nitrophenyl phosphorodichloridate, and the resulting nucleoside monophosphorodichloridate was then treated with dicyclohexylcarbodiimide and morpholine to give the nucleoside phosphoromorpholidate (Moffatt and Khorana, 1961). The N,N'-dicyclohexylcarboxamidinium nucleoside phosphoromorpholidate was treated with 85% orthophosphoric acid to give the nucleoside diphosphate. The nucleoside monophosphate also could be treated with 1,1'-carbonyldiimidazole and tri-n-butylammonium phosphate to give the nucleoside diphosphate (Hoard and Ott, 1965; Kozarich et al., 1973). Alternatively, phosphorylation could be carried out by treating the nucleoside with phosphoryl chloride. It was reported by Yoshikawa et al. (1967) and Yoshikawa et al (1969) that unprotected nucleoside could be phosphorylated by phosphoryl chloride and the reaction could be enhanced by using trialkyl phosphate as solvent yielding a main product which was nucleoside 5'-monophosphate. Recently, phosphoryl chloride was employed by Eckstein et al. (1975) to phosphorylate unprotected nucleoside, the amount of phosphoryl chloride used was only 1.2 molar excess. However, Simoncsits et al. (1975) used up to 2.2 molar excess of phosphoryl chloride to phosphorylate thymidine, and Tomasz et al. (1978) used up to 27 molar excess to phosphorylate dinucleotides. Furthermore, Yoshikawa et al. (1967) reported that the addition of a small amount of water enhanced the reaction to give a higher yield of the nucleoside 5'-monophosphate. This latter method was chosen for the phosphorylation of mo^5C (Figure 2.24). It was necessary to carry out a preliminary experiment with cytidine to find out the optimum conditions for the phosphorylation of mo^5C (Table 2.1). The results of this experiment showed that 44 molar excess of phosphoryl chloride gave a complete conversion from cytidine to cytidine-5'-phosphorodichloridate, and the presence of water did not enhance the reaction.

The phosphoryl chloride reaction, after the purification step with DEAE Sephadex A-25, gave 5-methoxycytidine 5'-phosphoramidate (23%), 5-methoxycytidine 5'-monophosphate (30%), 5-methoxycytidine 5'-diphosphate (27%), and 20% of the seemingly non-reactive-5-methoxycytidine 5'-phosphorodiamidate. The 5-methoxycytidine 5'-phosphoramidate was then treated with mono-tri-n-butylammonium orthophosphate to give 5-methoxycytidine 5'-diphosphate. This reaction was very effective, and gave a near-complete conversion, so that a total yield of 46% of the nucleoside diphosphate was obtained.

The product at this stage was found to be heavily contaminated with orthophosphate, and DEAE Sephadex A-25 or Bio-Rad P-2 were found to be ineffective for the purification of the product. Therefore, the paper chromatographic method in solvent J was employed with satisfactory results. The polymerisation of the purified 5-methoxycytidine 5'-diphosphate (tri Na^+ salt) by Micrococcus luteus PNPase is described in the next chapter.

CHAPTER 3

SYNTHESIS OF ALTERNATING CO-POLY(I-C) AND POLY(m^5C)

3.1 Introduction

3.1.1 Polynucleotide Phosphorylase

Polynucleotide phosphorylase (polyribonucleotide:orthophosphate nucleotidyltransferase, EC 2.7.7.8), designated PNPase, was discovered by Grunberg-Manago and Ochoa (1955), who found that extracts of Azotobacter vinelandii catalysed a rapid exchange of P^{32}-labelled orthophosphate with the terminal phosphate of ADP, IDP, UDP, CDP and less rapidly with GDP. There was no reaction with the corresponding nucleoside triphosphate or monophosphate, and the exchange was accompanied by the liberation of Pi and required Mg^{++}. There are four reactions catalysed by PNPase, namely, polymerisation, phosphorolysis, P^{32}-exchange, and arsenolysis, and the first one will be considered in detail. The reversible reaction catalysed by PNPase can be presented by the following equation:

$$nX\text{-}R\text{-}Pi\text{-}Pi \xrightarrow{Mg^{++}} (X\text{-}R\text{-}Pi)n + nPi$$

R = ribose

X = adenosine, hypoxanthine, inosine, uracil, cytosine, and guanosine

Pi-Pi = pyrophosphate

Pi = orthophosphate

In the presence of the enzyme, orthophosphate and Mg^{++}, the biosynthetic polynucleotides undergo phosphorolysis to yield the corresponding nucleoside 5'-diphosphate. In the direction of synthesis of the homopolymers (poly(A), poly(U), poly(C), poly(I)) or copolymers (poly(A-U), poly(A-G-U-C)) under standard conditions (temp. = 30°, pH = 8.1, 10^{-2}M $MgCl_2$) an apparent equilibrium is reached when the molar ratio of orthophosphate to nucleoside 5'-diphosphate attains

a ratio of 1.5 to 2, but this ratio decreases linearly as Mg^{++} concentration increases (Grunberg-Manago et al., 1956; Grunberg-Manago, 1963).

PNPases are widely distributed among aerobic or anaerobic bacteria, but the localization of the enzyme is uncertain. PNPase is usually found in the soluble portion of the cell. However, ribosomes of E. coli were found to contain up to 36% of the total PNPase (Wade and Lovett, 1961). Furthermore, in Streptococcus faecalis most of the enzyme was found in cell membrane ghosts prepared by metabolic lysis of its protoplasts (Abrams and McNamara, 1962). Experiments with a particular fraction prepared from the reproductive tract of Ascaris lumbricoides have suggested that the enzyme may also exist in animal tissues (Entner and Gonzalez, 1959). It has been suggested that the role in vivo of this enzyme may be in the synthesis and breakdown of RNA in the cell (Grunberg-Manago et al., 1956, Littauer and Kornberg, 1957). But, in view of the relatively high phosphate concentration in cells, it is quite unlikely that PNPase functions as a synthesising enzyme. Furthermore, it has no template specificity. It is, therefore, currently believed that PNPase functions as a degradative enzyme (Grunberg-Manago, 1963). The thermal stability of PNPases from Escherichia coli (Lucas and Grunberg-Manago, 1964), Micrococcus luteus (Brenneman and Singer, 1964), and Clostridium perfringens (Fitt et al., 1968) has been studied. Preparations of the enzyme from E. coli at any step of purification remain active for periods as long as one year if kept in the frozen state at -18°, although it loses activity on thawing, especially on repeated freezing and thawing. Also, this enzyme is stable up to 55° to 60° for 10 minutes, while the M. Luteus enzyme is only stable up to 36°. More recently, PNPase from Bacillus stearothermophilus has been purified to homogeneity, and the pure preparation has an

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optimal temperature of 69°, whilst a partial purification of PNPase from T. aquaticus functions optimally at 73° (Wood and Hutchinson, 1976).

In general, the phosphate-exchange reaction catalysed by PNPase has a sharp optimal pH at 8.1. In the polymerisation reaction in the presence of 0.01 M ADP and 5 mM $MgCl_2$, there is a plateau of optimal activity between pH 7.5 and 9, and the phosphorolysis reaction has a plateau between pH 8.1 and 10 (Litfauer and Kornberg, 1957). Furthermore, it has been reported that the pH optimum for the polymerisation reaction varies with the Mg^{++} and salt concentration (Beers, 1958). In order to study the metal ion requirement of PNPase, Babinet et al. (1965), have assayed a series of divalent metal compounds (Mn^{++}, Co^{++}, Ni^{++}, Cd^{++}, Cu^{++}, Zn^{++} and Ca^{++}) with sucrose gradient purified E. coli enzyme by both the polymerization and phosphorolysis reactions. With the exception of the calcium ion, all divalent metals tried are capable of replacing Mg^{++} in the phosphorolysis of poly(A), and four of them (Co^{++}, Mn^{++}, Zn^{++}, Cd^{++}) could replace Mg^{++} in the polymerization of ADP, but the efficiencies of the various ions are quite different. Mn^{++} is the most suitable metal for replacing Mg^{++}, since at optimal concentration of these two ions, and under conditions of substrate saturation, the amounts of polyA formed (ADP polymerization) or of ADP released (poly(A) phosphorolysis) are of the same order of magnitude. The polymerization of the other nucleoside 5'-diphosphates also shows no absolute requirement for Mg^{++}. The polymerization of all four common nucleoside 5'-diphosphates (ADP, IDP, UDP, CDP) proceeds in the presence of Mn^{++}. It is known that no polymerization of guanosine 5'-diphosphate occurs when PNPase and Mg^{++} are incubated with this compound alone, and the presence of an oligoribonucleotide containing an unsubstituted hydroxyl group at 3'position of the terminal nucleoside residue is needed for the reaction to proceed (Singer et al., 1960). But, in the

presence of Mn^{++}, at high temperature (60^{o}), high-molecular-weight poly(G) has been synthesized by PNPase isolated from E. coli (Thang et al., 1965), with the yield of the product comparable to that obtained for other polynucleotides. With E. coli enzyme, the optimal ratio of ADP/Mn^{++} is about 10 for the polymerization which indicates that the enzyme has a higher affinity for Mn^{++} than for Mg^{++} for which the nucleotide/metal ratio is 2 to 3. But it should be pointed out that at high concentrations Mn^{++} becomes inhibitory for the polymerization reaction.

It was believed (Grunberg-Manago, 1963), earlier, that the PNPase, which catalysed the polymerization reaction, is specific with respect to the nature of the sugar moiety of the nucleotide and the number of phosphates terminally esterified to the nucleoside. No reaction occurred with the nucleoside monophosphates or triphosphates (Littauer, and Kornberg, 1957), nor was there any reaction when the ribonucleoside diphosphates were replaced by their corresponding deoxyribose derivatives. Nucleoside diphosphates esterified at the 3'-OH position with phosphates which were not substrates. Some of the nucleotides, which have been polymerised by PNPase, are: ADP, CDP, UDP, IDP, GDP, N-MeUDP, S^{2}UDP, fl^{5}UDP, br^{5}UDP, cl^{5}UDP, $iodo^{5}$UDP, ribòsylthymine5'-diphosphate, xanthosine diphosphate and N^{6}-hydroxyethyl ADP (Grunberg-Manago, 1963). More recently, Rottman and Johnson (1969) was successful in synthesizing the homopolymer, poly(2'-O-methyl A) and the heteropolymers containing 2'-O-methyl A, 2'-O-methyl C, and nonmethylated nucleotides with PNPase from their corresponding nucleoside 5'-diphosphates. The distribution of 2'-O-methylnucleotides in these polymers was not completely random but occurred in pairs. The frequency of this paired incorporation indicated some form of cooperative polymerization of 2'-O-methylnucleotides in the presence of

nonmethylated nucleotides. Also, copolymers containing AMP and dAMP residues have been synthesized with PNPase from M. luteus (Chou and Singer, 1971). The rate of copolymerisation depended upon the input ratio of ADP to dADP, and also, depended in a nonlinear manner upon enzyme concentration. This copolymerization proceeded most readily when Mn^{++} replaced Mg^{++} and preparation of polymers in Mn^{++} permitted incorporation of a higher proportion of dAMP residues into the copolymer. It should be noted that the dADP alone was not polymerized. Therefore it appears that, in the presence of Mn^{++}, the substrate specificity of PNPase is not very rigid, and for preparative purposes Mn^{++} is the ion of choice.

The concentration of nucleoside diphosphates required to saturate the enzyme in the polymerization reaction appeared to be very high. At pH 8.1, saturating concentrations of ADP and IDP for their polymer formation was of the order of 0.10 and 0.05 M, respectively, for both E. coli and A. agilis enzyme. There was a complex relationship between the ADP and Mg^{++} concentrations and probably also to the pH. Both ADP and Mg^{++} were inhibitory when present in excess over the optimal ratio. At pH 8.1, optimum Mg^{++} levels were obtained at ADP/Mg or UDP/Mg ratios of 2 to 3. With saturating concentrations of the nucleotides, the rates of formation of poly(A), poly(U), poly(C) and poly (I) were essentially the same, while the rate of polymerisation of copolymers were slower (Grunberg-Manago, 1963). Studies on the base composition of copoly(A-G-U-C), prepared by PNPase using labelled nucleoside diphosphates, had shown that the four different nucleotide units were distributed randomly in the polynucleotide chains (Ortiz and Ochoa, 1959). But, base composition studies on copoly(A-U) had shown that its base ratios were dependent on the relative concentrations of the nucleoside diphosphates used in the preparation of the copolymer. However, the base ratio of the product was not always equal to the

ratio of the substrate used, for a reason which is not yet known. The copoly(A-U) formed was not completely random, but the alternating sequence (A-U) was predominant (Grunberg-Manago, 1959). Molecular weight determination of several different biosynthetic polynucleotides by physical methods yielded values from 30,000 to 1,000,000. Chemical and enzymatic degradations of these polymers showed that they were linear polymers composed of chains of ribonucleotide units linked by 3'-5' phosphodiester bridges.

The mechanism of the polymerization reaction catalysed by PNPase was thought to involve a "elongation reaction". Thang et al. (1970) found, among the polymerization products, that:

(a) the polymers formed were of high molecular weight and homogeneous in size,

(b) no polynucleotide with intermediate chain length.

These results suggested that the elongation process catalysed by the enzyme involved a mechanism of non-dissociation of the growing polynucleotide, which could be visualized as the reverse of phosphorolysis. Consequently, it was suggested that a "rolling on" mechanism was involved, i.e. a one-by-one addition of each nucleotide unit, the non-dissociation of the intermediates and the fixation of the growing polymer on the enzyme.

Although the function and detailed mechanism of action of PNPases remain somewhat obscure, these enzymes, isolated from various bacterial sources, have proved to be a great utility for the preparation of polyribonucleotides from the appropriate ribonucleoside 5'-diphosphates. Large quantities of polynucleotides can be prepared relatively easily, and, in general, primers are not usually needed, and the enzyme shows a very low substrate specificity with respect to the purine and pyrimidine base. A wide variety of polyribonucleotide analogues can thus be prepared

by the action of PNPase on the analogue ribonucleoside diphosphates (Godefray-Colburn and Grunberg-Manago, 1972). Therefore, it is possible that PNPase can be used to synthesise a copolymer from IDP and CDP and a homopolymer of poly(mo^5C) from mo^5CDP.

3.1.2 RNA Polymerase (E.C. 2.7.7.6)

It was first suggested by Weiss and Gladstone (1959) that the synthesis of RNA in the presence of all four nucleoside triphosphates as well as a DNA primer, which directed the assembly of complementary RNA chains, was catalysed by RNA polymerase rather than polynucleotide phosphorylase. Later, it was found that RNA polymerase from M. Luteus and A. vinelandii also could utilize plant viral RNA, poly(C), poly(A), and poly(U) as primers (Nakamoto and Weiss, 1962), though they were not as effective as DNA (Krakow and Ochoa, 1963). In terms of total polynucleotide material formed in this system, the relative efficiency of the primers in decreasing order is: native DNA, single-stranded DNA, RNA homopolymers, natural RNA, single-stranded RNA (Fox et al., 1964; Wood and Berg, 1964). Fox and Weiss (1964) also observed that polyamine stimulated the RNA synthesis by RNA polymerase, in the presence of four nucleoside triphosphates and native DNA as primer. However, this DNA-dependent RNA polymerase catalysed reaction was inhibited by poly(A), poly(U), poly(A).poly(U), and amino acid free soluble-RNA (Krakow and Ochoa, 1963; Tissieres et al., 1963). It was suggested that amino acid free soluble-RNA inhibited the reaction by combining with the enzyme. The DNA-dependent synthesis of complementary RNA catalysed by RNA polymerase required a divalent metal ion, and either Mn^{++}, Co^{++}, or Mg^{++} was able to satisfy this requirement. On the other hand, the reactions directed by poly(U), poly(A) and poly(C) required Mn^{++}, and the poly(U) directed reaction could be further stimulated

when Mg^{++} was added with the optimal Mn^{++} concentration (Fox and Weiss, 1964; Niyogi and Stevens, 1965). The DNA-dependent reaction was affected by the ionic strength of the reaction mixture (Maitra and Barash, 1969). At low ionic strength, the reaction soon ceased due to inhibition by accumulated RNA. In contrast, when this reaction was carried out in 10 mM Mg^{++} and 0.2 M KCl, RNA synthesis proceeded nearly linearly for hours, resulting in a marked increase in accumulated RNA.

Unlike the DNA-dependent reaction, the RNA-directed synthesis of RNA catalysed by RNA polymerase, was not enhanced by the presence of polyamines, and was not inhibited by deoxyribonuclease. The general requirements of this reaction were very similar to those of the DNA-dependent reaction. In priming with homopolymers, only a single complementary nucleoside triphosphate was required, and in priming with RNA copolymers, the presence of all the complementary nucleoside triphosphates are required (Fox et al., 1964). It was suggested that the RNA synthesizing reactions, with both DNA, and RNA were catalysed by the same enzyme, RNA polymerase (Krakow and Ochoa, 1963; Niyogi and Stevens, 1965), because the purification procedures used for RNA polymerase and electrophoresis studies had not resulted in the resolution of two separate enzymes. Also, both the DNA-dependent and the RNA-dependent reactions,catalysed by purified RNA polymerase were found to be stimulated by sulphydryl compounds.

RNA inhibited the DNA-dependent reaction by competing the same binding site in RNA polymerase, with the template DNA (Krakow and Ochoa, 1963). Fox et al. (1965), observed that both DNA and RNA were bound readily by the enzyme in the absence of added metal ions or nucleotide substrate. Under normal reaction conditions the interaction of polymerase with template DNA to form an enzyme-DNA

intermediate was irreversible, and the presence of RNA after this stage no longer affected the synthetic reaction. Jones and Berg (1966) demonstrated that a mixture of native T7 DNA and RNA polymerase were retained by a cellulose nitrate membrane filter, which was known to retain denatured DNA (Nygaard and Hall, 1963), while the native T7 DNA and the enzyme, separately, could pass through the membrane filter. Therefore, it was suggested that the binding of RNA polymerase to the DNA produced a collapse of the helical structure over a localized region, where transcription would be initiated. After the binding of the DNA template to the enzyme, the next step would be the initiation of chain elongation. It had been observed that the RNA chains formed contained nucleoside triphosphates at their starting points and these grew by the subsequent addition of nucleotides to the 3'-hydroxyl group of the nucleoside end (Maitra et al., 1965; Maitra and Hurwitz, 1965). It is now obvious that the function of DNA-dependent RNA polymerase in bacteria is for the transcription of its genetic sequences (Chamberlin, 1974).

RNA polymerase is also capable of a third kind of reaction: the synthesis of polyribonucleotides in the absence of an added template or primer. Two major products have been identified by Krakow and Karstadt (1967). Firstly, poly(A).poly(U) was formed when ATP and UTP were added as substrate, and secondly, the alternating copoly(I-C) was formed when ITP and CTP were employed as the substrate. The unprimed synthetic reactions appeared to be an intrinsic property of bacterial RNA polymerase and was not the result of contamination of the enzyme preparation (Chamberlin, 1974). These reactions required Mn^{++} as the divalent metal ion and usually showed a lag period of at least 30 min. Then, it was discovered by Sternbach and Eckstein (1970) that only the enzyme preparations, which were purified according to the purification method designed by

Zillig et al. (1966), were capable of the unprimed synthesis of the alternating copoly(I-C).

The bacteria RNApolymerases are large molecules, and the structural complexity of the holoenzyme is related to the presence of at least four subunits α_2, β, β' and σ, having a total molecular weight of about 500,000 daltons (Chamberlin, 1974). Present data indicates that the four subunits of the core enzyme (β, β', α_2) direct the catalytic activity whereas σ is dissociable and probably governs the selection of promoter sites (Okada et al., 1978). Bacterial RNA polymerases from different species appear to be closely related in subunit structure but show differences in the sizes of the subunits. The E. coli enzyme has been found to have four major subunits, α, β, β', and σ and a minor component ω (Burgess, 1969). The quaternary structure of E. coli RNA polymerase has been studied by cross-linking with a periodate-cleavable bis(imido ester), N,N'-bis(2-carboximidoethyl)tartaramide dimethyl ester dihydrochloride (CETD). The cross-linking holoenzyme gave a characteristic fine-band pattern after electrophoresis on sodium dodecyl sulphate polyacrylamide gels. The bands were (1) α β and α β', (2) σ β and σ β', (3) α σ β and α σ β', (4) β β', and (5) σ β β', and there were no bands corresponding to α α and α σ. On the basis of these observations, a model for the subunit arrangement of RNA polymerase was proposed (Coggins et al., 1977), which is as shown below (Figure 3.1).

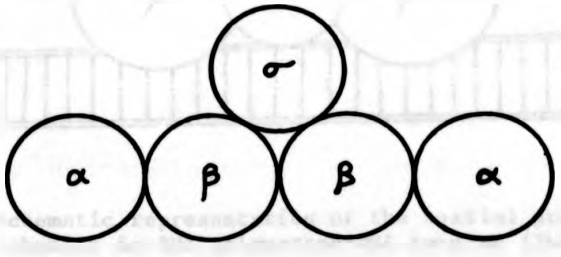


Figure 3.1: Schematic representation of the subunit arrangement of RNA polymerase proposed by Coggins et al. (1977)

Highly purified RNA polymerase could be dissociated into subunits with high ionic strength buffers, and they could be reassociated by dialysis into buffers with low ionic strength (Richardson, 1966). Also the reconstitution of E. coli RNA polymerase was found to be enhanced markedly by σ subunit (Ishihama et al., 1973), as well as DNA (Fukuda and Ishihama, 1974).

Recently, the subunits of E. coli RNA polymerase which are in close contact with the T7 phage DNA template have been identified using photochemical cross-linking. In nonspecific T7 DNA-enzyme complexes which occurred in all regions of the DNA, subunits σ, β, and β' were cross-linked to the DNA. In contrast, in specific binary complexes which presumably occurred at promoter sites, and in the initiation complex (holoenzyme + T7 DNA + initiator dinucleotides + three nucleoside triphosphates), only σ and β subunits were cross-linked to DNA, while cross-linking of β' could not be demonstrated. These results indicated that α subunit was not involved in the enzyme-template interaction, and the participation of σ subunit directly in promoter recognition (Hillel and Wu, 1978). Similar results were obtained by Okada et al. (1978), who proposed a new model for the subunits arrangement of RNA polymerase, which is as shown below (Figure 3.2).

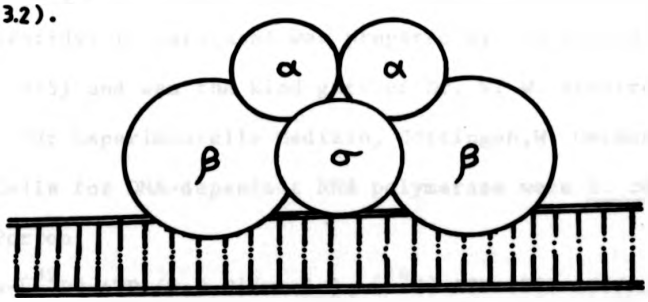


Figure 3.2: Schematic representation of the spatial arrangement of subunits in RNA polymerase-DNA complex (Ikada et al., 1978)

Similar experiments carried out by Simpson (1979) confirmed the participation of σ subunit directly in promoter recognition, and concluded that the β subunit forms at least part of the catalytic site, and the σ subunit plays a direct role in unwinding the DNA at the promoter.

The objective of this chapter is partially an attempt to synthesize the alternating copoly(I-C) using the unprimed and primed reactions of RNA polymerase.

3.2 Materials and Methods

3.2.1 Materials

(a) M. luteus polynucleotide phosphorylase (E.C.2.7.7.8) (5 mg, 30 units/mg at 37°, with UDP as substrate and GpU as primer), calf intestinal alkaline phosphatase (E.C.3.1.2.1) (750 μg/0.15 ml, 400 units) were purchased from Boehringer Mannheim, W. Germany. E. coli PNPase was prepared by Miss C. Chapman using the method of Wood and Hutchinson (1976).

(b) RNaseA, type XII-A (E.C.3.1.4.22), RNaseT (E.C.3.1.4.8) (1.86×10^5 units/ml), Crotalus adamenteus (E.C.3.1.4.1), yeast hexokinase (E.C.2.7.1.1) were products from Sigma (London) Chemical Co.

(c) DNA-dependent RNA polymerase (E.C.2.7.7.6 nucleoside triphosphate: RNA nucleotidyl transferase) was prepared by the method of Sternbach et al. (1975) and was the kind gift of Dr. V. W. Armstrong, Max-Planck Institüt für Experimentelle Medizin, Göttingen,W. Germany.

(d) Cells for DNA-dependent RNA polymerase were E. coli K12 from M.R.E. Porton.

(e) α-[^{32}P]-ATP (9.9 Ci/mmole), [^{14}C]-ATP (527 mCi/mmole) were obtained from the Radiochemical Centre, Amersham, U.K.

(f) Glass-fibre discs were Whatman GF/C (2.5 cm), supplied by Whatman Ltd., Maidstone, Kent, U.K.

3.2.2 General Methods

(a) Sedimentation coefficient of poly(mo^5C) was determined on a Beckman Model E analytical ultracentrifuge by Dr. C. S. Dow, Department of Biological Sciences, University of Warwick.

(b) Thermal transition profile determination of polynucleotides were carried out on a Gilford 250 series U.V. spectrophotometer coupled with an automatic 2527 thermoprogrammer. Thanks are due to Dr. R. J. Avery, Department of Biological Sciences, University of Warwick for permission to use this instrument. The polynucleotides were dissolved in 0.01 M sodium cacodylate, 0.1 M NaCl, pH 7.0. The polynucleotide solutions were heated at 70° for 10 minutes and cooled slowly to room temperature, then kept overnight at 4° before the melting profile determination was carried out.

(c) Circular dichroic spectra were obtained on a Cary 61 recording CD spectropolarimeter. Thanks are due to Dr. P. M. Scopes, Westfield College, University of London, for performing these.

(d) The scintillant used for the counting of radioactive samples from nearest neighbour analysis was ethoxy ethanol (400 ml), PPO (4 g), POPOP (0.2 g) and toluene (600 ml), and that for DNA-dependent RNA polymerase assay was PPO (4 g), POPOP (0.05 g) and toluene (made up to 1 l total).

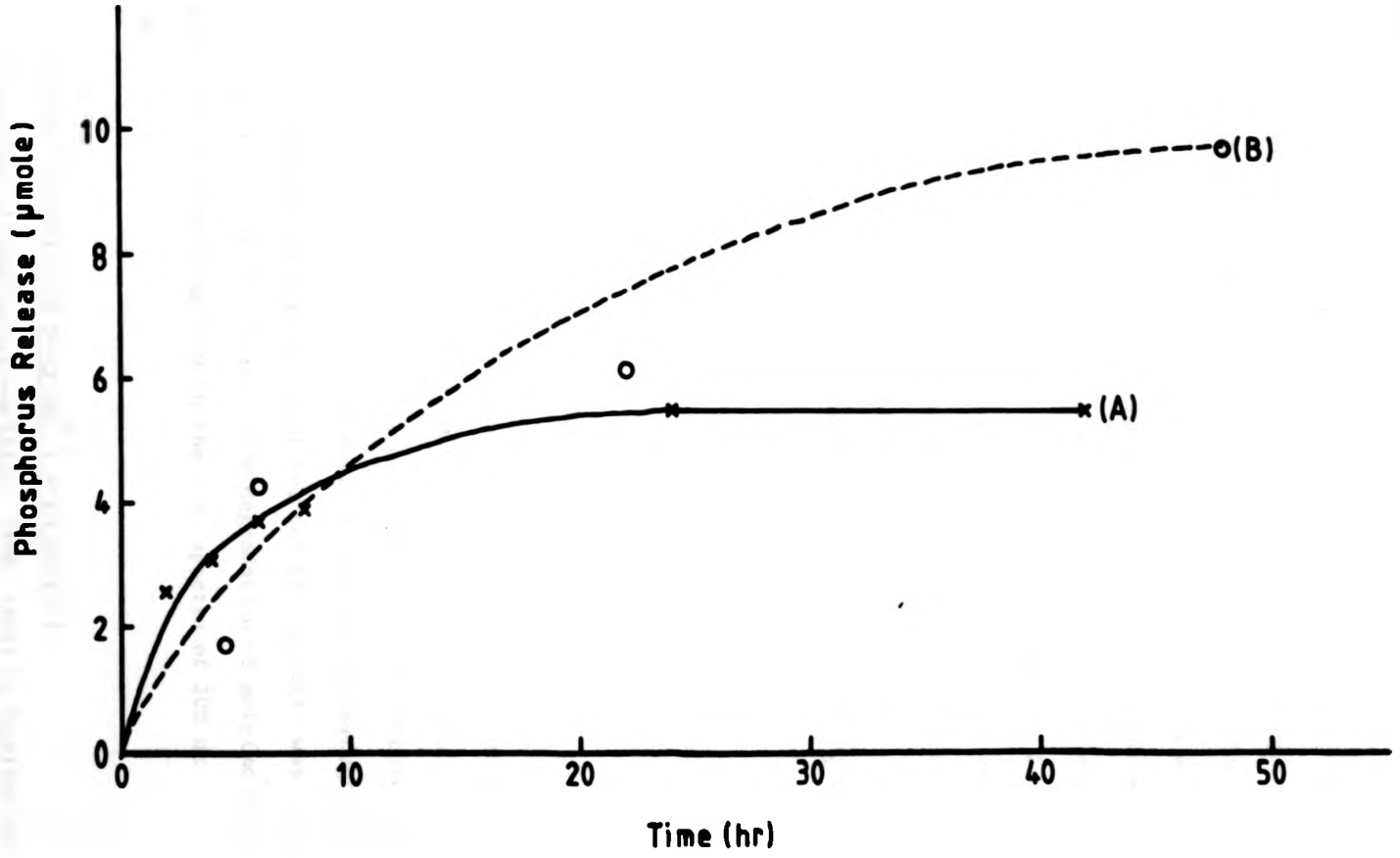
3.2.3 Polymerisation of mo^5CDP

The polymerisation mixture (1 ml) consisted of tris-HCl, pH 9.0 (0.05 M), mo^5CDP (0.044 M), $MgCl_2$ (0.02 M) (or 0.01 M $MnSO_4$), EDTA (0.025 M) and *M. Leuteus* PNPase (2.7 mg, 81 units). The polymerisation reaction at 37° was followed by the release of inorganic phosphorus (Fig.3.3). The reaction equilibrium was found to have been reached after 24 hours. The solution was extracted by

Figure 3.3: The release of inorganic phosphorus of the polymerisation of mo^5CDP by PNPase using different metals.

(A) Mg^{++}

(B) Mn^{++}


Phosphorus Release (µmole)
0
2
4
6
8
10
0
10
20
30
40
50
Time (hr)
(A)
(B)

shaking with an equal volume of iso-amyl alcohol/chloroform (3:1 v/v) followed by centrifugation at 7,000 rpm for 20 minutes to separate the phases. The top aqueous layer was withdrawn, the procedure was repeated until no precipitation of protein was observed. The top aqueous layer was applied to a Sepharose 4B column (1.5 x 55 cm), and eluted with double distilled water. The poly(mo^5C) was eluted at the void volume, and the eluant was concentrated down to 1 ml under reduced pressure at room temperature. The poly(mo^5C) was found to stay on the origin when chromatographed on PEI t.l.c. in solvent i. The concentrated poly(mo^5C) was lyophilised. The yield of poly(mo^5C) was 7.7% (1.7 mg) (15%. 3.3 mg when $MnSO_4$ was used). The U.V. absorption data of poly(mo^5C): pH 2, λ_{max} 306 nm (Σp 8300); pH 6.5, λ_{max} 294 nm (Σp 6200); pH 11, λ_{max} 288 nm (Σp 6300) (Fig. 3.4) (total phosphorus determination of polynucleotides was as described in section 4.2.6.A).

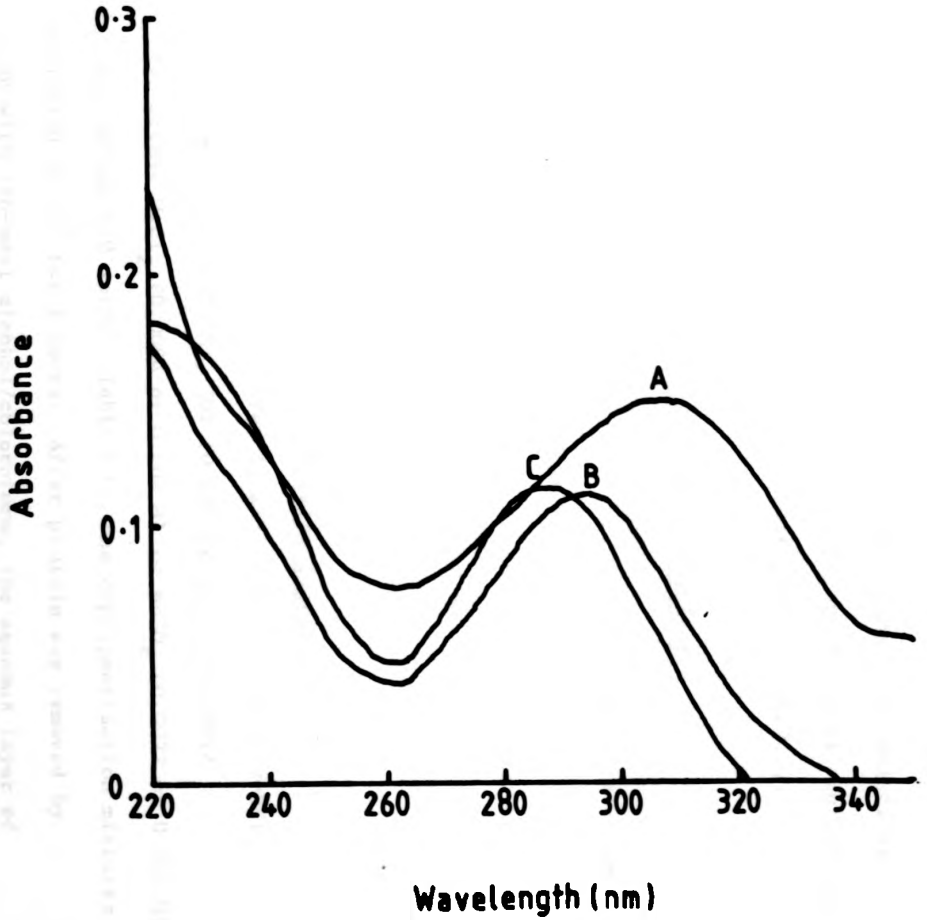
3.2.4 Hydrolysis of poly(mo^5C)

Poly(mo^5C) (0.1 mg) was dissolved in bicine buffer, pH 8.0 (0.02 M, 0.1 ml) containing $MgCl_2$ (0.01 M), RNaseA (0.1 mg), *C. adamanteus* phosphodiesterase (0.1 mg). The reaction mixture was incubated at 37°. The reaction was monitored by the chromatography system described in the previous section, and the reaction was found to reach completion after 6 hours. The degradation of poly(mo^5C) to monomers gave a hyperchromicity in the U.V. spectra of 20% at 294 nm.

3.2.5 Complex Formation of poly(mo^5C) with poly(I)

The method of continuous variation (Job, 1928) as applied to the determination of polynucleotide complex formation (Felsenfeld

Figure 3.4: Ultra-violet spectra of poly(mo^5C) at (A) pH 2, (B) pH 6.5 and (C) pH 11.


0·3
0·2
0·1
0
Absorbance
220
240
260
280
300
320
340
Wavelength (nm)
A
B
C

et al., 1957) was used to investigate the possible interaction between the synthetic polynucleotide poly(mo^5C) and poly(I).

A series of mixtures (0.1 ml) at fixed total nucleotide concentration were prepared with 0.01 M sodium cacodylate, 0.1 M NaCl, pH 7.0 as buffer. These were heated to 70° for 10 minutes and then allowed to cool slowly to room temperature. The solutions were stored at 4° for a minimum of 24 hours to ensure total equilibrium before observation of their spectral characteristic (Fig. 3.5).

3.2.6 Preparation of ds poly(mo^5C).poly(I) Sample for Interferon Induction

Equal molar quantities of poly(mo^5C) and poly(I) were dissolved in sterilized PBS (0.4 mg/0.4 ml). This solution was heated at 70° for 10 minutes and cooled slowly to room temperature, and then kept at 4° for 24 hours. The interferon inductions and assays were described in sections 4.2.10 and 4.2.11.

3.2.7 Copolymerisation of CDP and IDP using E.coli PNPase

The copolymerisation mixtures (2 ml) consisted of tris-HCl, pH 9.0 (0.05 M), CDP (0.005 M), IDP (0.005 to 0.012 M), EDTA (0.025 M), either $MgCl_2$ (0.0004 or 0.004 M) or $MnSO_4$ (0.0022 or 0.004 M), and E. coli PNPase (30 units) (Table 3.1). The copolymerisation mixtures were incubated at 45° for 8 hours. After protein was removed by extraction with iso-amyl alcohol/chloroform, the aqueous layer of each mixture was dialysed for 24 hours in Spectropore 2 tubing (M.W. cut-off 12,000-14,000; Raven Scientific, Suffolk, U.K.) against the following solutions (2 l each):

(i) 0.5 M NaCl, 0.01 M tris-HCl, 0.001 M EDTA, pH 7.2;

(ii) 0.5 M NaCl, 0.01 M tris-HCl, pH 7.2;

(iii) 0.1 M NaCl, 0.01 M tris-HCl, pH 7.2;

Figure 3.5 Complex formation studies on poly(I).poly(mo^5C).
Job plot of mixtures of poly(I) and poly(mo^5C)
in 0.01 M sodium cacodylate, 0.1 M sodium chloride,
pH 7.0.
240 nm (x——x) and 250 nm (o——o)

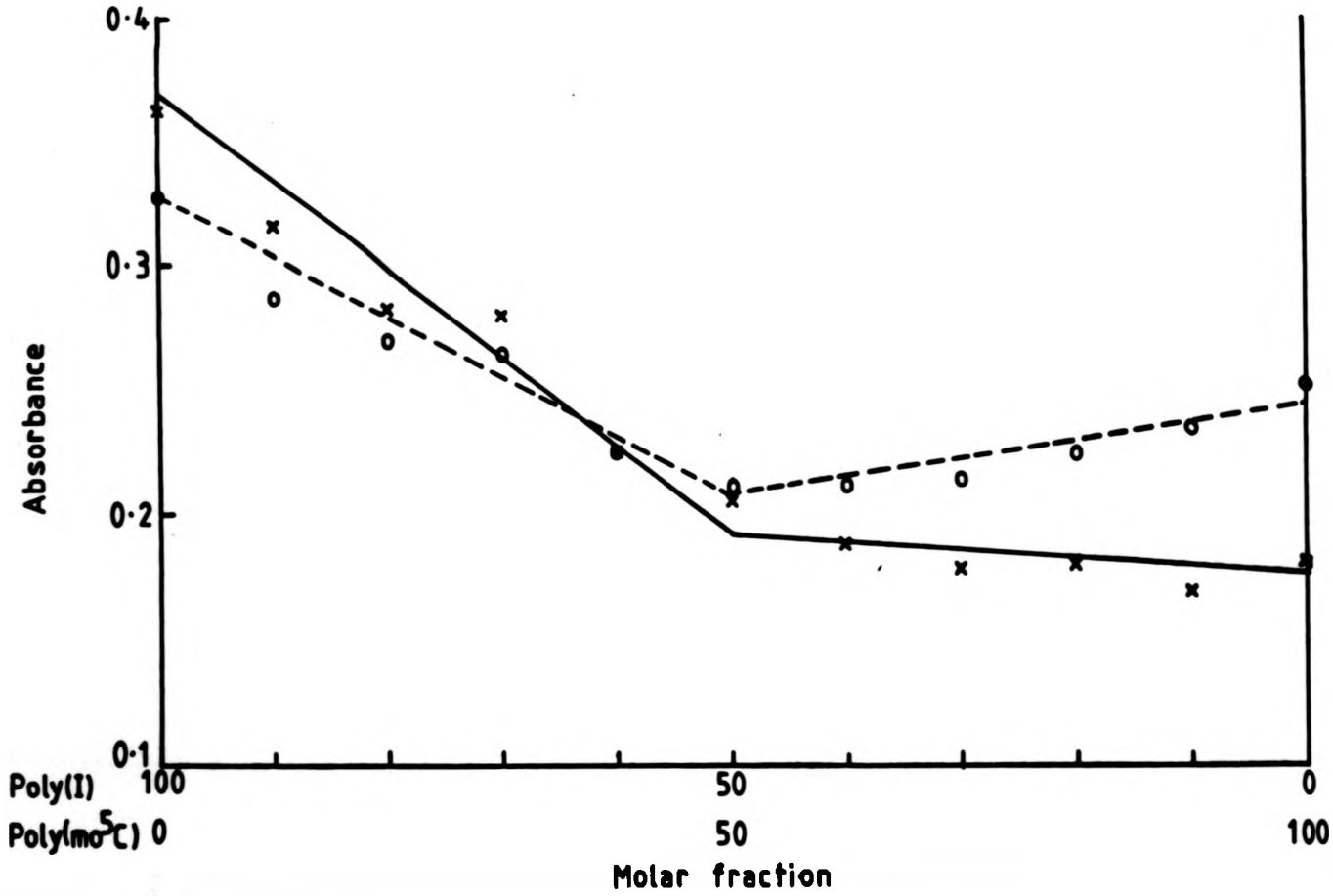

0·4
0·3
0·2
0·1
Absorbance
Poly(I) 100 50 0
Poly(mo5C) 0 50 100
Molar fraction

TABLE 3.1: The reaction solution (2 ml) which contained CDP (5 mM) in all cases and polynucleotide phosphorylase from E.coli was incubated at 45° for 8 hours

Experiment	IDP (mM)	Metal ion (mM)	Yield (mg)	C:I Ratio[a]
1	5	Mg^{++} 4	1.1	1:0.8
2	6	Mg^{++} 4	3.4	1:1.2
3	7	Mg^{++} 4	3.4	1:1.3
4	8	Mg^{++} 4	3.8	1:1.5
5	10	Mg^{++} 4	4.9	1:1.6
6	12	Mg^{++} 4	4.5	1:1.7
7	5	Mg^{++} 0.4	3.2	1:1.3
8	5	Mn^{++} 2.2	2.8	1:1
9	5	Mn^{++} 4	2.5	1:0.9

[a] The base ratios were determined by hydrolysing an aliquot of the copolymers with RNaseA, phosphodiesterase and alkaline phosphatase, separating the nucleosides by preparative silica plate and estimating the nucleosides by U.V. spectroscopy.

(iv) double distilled water (twice).

The products were lyophilized.

3.2.8 Base Ratio Analysis of copoly(I-C) by Nuclease Hydrolysis

The cytidine:inosine base ratios of the copoly(I-C) samples prepared in the previous section were analysed by the total degradation of the polynucleotide to its nucleoside components with RNaseA, phosphodiesterase and alkaline phosphatase. The reaction mixtures were the same as described in section 3.2.4 with the addition of alkaline phosphatase (10 units) to degrade the polynucleotides to the nucleoside components. The reaction mixtures were incubated at 37°, and monitored by silica t.l.c. with butanol:acetic acid:water (4:1:5 v/v). In the preparation of this solvent system, all three components were mixed and after the two phases had settled, only the top layer was taken off and used in the chromatography) as solvent (R_f = cytidine (0.17), inosine (0.25)). The reaction was observed to have reached completion after 6 hours. The samples were chromatographed on preparative silica plates with the same solvent system, and dried at 80° for 3 hours. The cytidine and inosine spots were extracted with 0.1 M phosphate buffer, pH 7.0 (10 ml), and the silica gel was removed by centrifugation at 4,000 rpm for 10 minutes. The quantities of cytidine and inosine in each copoly(I-C) sample were determined by U.V. spectroscopy from the knowledge of their respective extinction coefficients at the given wavelengths (Table 3.1).

3.2.9 Preparation of $\alpha[^{32}P]$-adenosine diphosphate

The preparation of $\alpha[^{32}P]$-ADP was based on the method devised by Cartwright and Hutchinson (1977). $\alpha[^{32}P]$-ATP (12.2 mCi) was added to the aqueous unlabelled ATP (15 mg, 28.5 μmole) solution, and the mixture was lyophilized. The following reagents were

added in the order: sodium phosphate buffer, pH 7.5 (0.05 M, 21.84 ml), $MgCl_2$ (0.1 M, 2.01 ml) and glucose (0.5 M) in sodium phosphate buffer, pH 7.5 (0.05 M, 6 ml). After incubation at 25° for 5 minutes yeast hexokinase (240 units, 0.15 ml) was added and the incubation continued for an hour. Silica t.l.c. with isobutyric acid:conc. NH_4OH:H_2O (66:1:33 v/v) as solvent (R_f: ATP (0.08), ADP (0.16)) showed that complete conversion to the diphosphate had occurred. Ethanol (30 ml) was added and the precipitated protein was removed by centrifugation at 7,000 rpm for 20 minutes. The supernatant was concentrated to 10 ml under reduced pressure, and applied to a DEAE Sephadex A-25 column (HCO_3^- form, 1.1 x 25 cm), and then washed with water. The elution was achieved by a linear gradient of triethylammonium bicarbonate, pH 8.9 (0.05-0.35 M). The fractions containing the $\alpha[^{32}P]$-ADP was eluted around 0.24 triethylammonium bicarbonate. The product $\alpha[^{32}P]$-ADP was worked up as described for 5-methoxycytidine-5'-phosphoramidate. The lyophilization of the eluate gave $\alpha[^{32}P]$-ADP in 80% yield. The product was stored at -20°.

3.2.10 Preparation of $\alpha[^{32}P]$-inosine diphosphate

The deamination of adenosine presented here was taken from Eaton (1973), whose method was based on the original work by Holmes and Robins (1964). $\alpha[^{32}P]$-ADP (triethylammonium salt, 22.8 μmole) was dissolved in 10% acetic acid (0.75 ml), and the solution was cooled to 0°. Aqueous sodium nitrite solution (0.22 g, 0.9 ml) was added dropwise over a period of 2 hours and the mixture was left at 4° for 12 hours. The reaction was observed to reach completion with PEI t.l.c. in solvent i (R_f: ADP(0.26), IDP(0.13). The resulting solution was diluted with water (10 ml), applied to

a column (1.1 x 25 cm) of DEAE Sephadex A-25 (HCO_3^- form), and then washed with water. Elution of the product was the same as mentioned in the previous section. The fractions containing α-[^{32}P]-IDP were eluted at 0.27 M triethylammonium bicarbonate. The α-[^{32}P]-IDP (triethylammonium salt) was dissolved in water (10 ml) and applied to a column (1.1 x 25 cm) of SE-Sephadex C-25 (Na^+ form), and then washed with water. The fractions containing α-[^{32}P]-IDP (sodium salt) was lyophilized, and gave a yield of 73% (specific activity: 0.28 mCi/μmole). This product was found to have its U.V. spectrum identical to that of IDP with λ_{max} at 249 nm.

3.2.11 Nearest Neighbour Analysis

Results from section 3.2.8 indicated that when the input ratio of CDP and IDP, in the copolymerisation reaction, was 1:1, the ratio of C and I incorporated was also near to 1:1. Therefore, this CDP and IDP input ratio was employed in this experiment. Reagents and reaction conditions were the same as described in section 3.2.7 and α [^{32}P]-IDP (5 mM, 0.52 mCi) was used instead of the unlabelled nucleotide.

The resulting copoly(I-C) (0.5 mg) was degraded to its mononucleotides as described in section 3.2.4, except that RNaseA (0.1 mg) and RNaseT, (930 units, 0.005 ml) were used. After 6 hours incubation at 37°, the resulting CMP and IMP were separated with PEI t.l.c. in solvent i (R_f: CMP (0.60), IMP (0.47)). The CMP and IMP were extracted with sodium phosphate, pH 7.0 (0.1 M, 10 ml). Radioactivity was found to have been incorporated into both CMP (7.92 μCi) and IMP (4.93 μCi). Therefore, this copoly(I-C) was not alternating.

3.2.12 Sodium Bisulphite Conversion of Exposed Cytidine Residues in Polynucleotides

Polynucleotide (4.4 μmole of nucleotide) was dissolved in 0.006 M sodium phosphate, 0.15 M NaCl, pH 7.0 buffer (1 ml) (Table 2.2), annealing procedure was carried out on samples 3 and 5 as described in section 3.2.5. A solution of 1.76 M sodium bisulphite,

0.01 M $MgCl_2$, pH 6.0 (1 ml) was added to the polynucleotide solution and incubated at 25° for 24 hours. Dialysis as described in section 3.2.7 was carried out on this reaction mixture. Conversion of the 5,6-dihydrouracil-6-sulphonate residues in the dialysed polynucleotide to uracil residues was followed by the increase in U.V. absorbance at 260 nm upon incubation in 0.1 M tris-HCl, pH 9.0 (1 ml) at 37°. Complete conversion of modified poly(C) required 5 hours of incubation. The polynucleotide solution was neutralized and desalted by dialysis against water for 24 hours, and the dialysate was lyophilized.

Degradation of the modified polynucleotide was carrried out as described in section 3.2.8. The reaction was monitored by silica t.l.c. with methanol:ethyl acetate:formic acid (30:70:2 v/v) as solvent (R_f = C(0.1), I(0.19), U(0.44)).

The results indicated that all the cytidine residues of poly(C), copoly(I-C) were converted completely to uridine, while the untreated poly(C), ds poly(I).poly(C) were unaffected (Table 3.2). Therefore, the copoly(I-C) was a single stranded species with and without going through the annealing procedure.

3.2.13 DNA-dependent RNA Polymerase Assay

The polymerisation mixture (0.1 ml) consisted of 0.2 M tris-HCl, 0.05 M $MgCl_2$, 0.25 M KCl, pH 8.0 buffer (20 µl), DTT (0.005 M), poly d(A-T) (0.1 O.D.), UTP (0.001 M), [^{14}C]-ATP (0.001 M, 17,600 cpm/nmole) and water (sufficient to bring volume to 0.1 ml when enzyme was added). Before the addition of DNA-dependent RNA polymerase (2 µg, 0.5 µl), the reaction mixture was incubated at 37° for 5 minutes, and then continued for 1 hour after the enzyme was added. Aliquots (10 µl) of the assay solution

TABLE 3.2: Sodium bisulphite conversion of exposed cytidine residues in polynucleotides

Sample	Polynucleotide	Annealed before treatment with sodium bisulphate	Treatment with sodium bisulphate	Conversion to uridine
1	poly(C)	-	-	-
2	poly(C)	-	+	complete
3	poly(I).poly(C)	+	+	-
4[(a)]	copoly(I-C)	-	+	complete
5[(a)]	copoly(I-C)	+	+	complete

[(a)] The copoly(I-C) used had the C:I ratio near to 1:1.

were taken at 10 minute intervals, applied to a GF/C disc and dried under an IR lamp. The disc was suspended in 5% TCA and stirred for 15 minutes to precipitate the polynucleotide and washed off nucleotides. A blank disc was included in this procedure to serve as a control. This procedure was repeated twice, the discs were then washed with ethanol for 5 minutes and diethyl ether for 5 minutes and dried under the lamp. The discs were counted separately in toluene scintillant (10 ml) (Fig.3.6). The DNA-dependent RNA polymerase obtained from Dr. V. W. Armstrong was found to have a specific activity of 860 units/mg. One unit of this enzyme was defined as 1 nmole of AMP incorporated during 10 minutes of incubation at 37^{o}.

3.2.14 Preparation of [^{14}C]inosine Triphosphate

The method of deamination of [^{14}C]-ATP to give [^{14}C]-ITP was the same as described in section 3.2.10.

The lyophilized [^{14}C]-ITP (sodium salt, specific activity: 740 cpm/nmole) gave a yield of 76%.

3.2.15 Extraction and Purification of DNA-dependent RNA polymerase (I)

The following extraction up to the ammonium sulphate fractionation step was based on the method by Burgess (1969a).

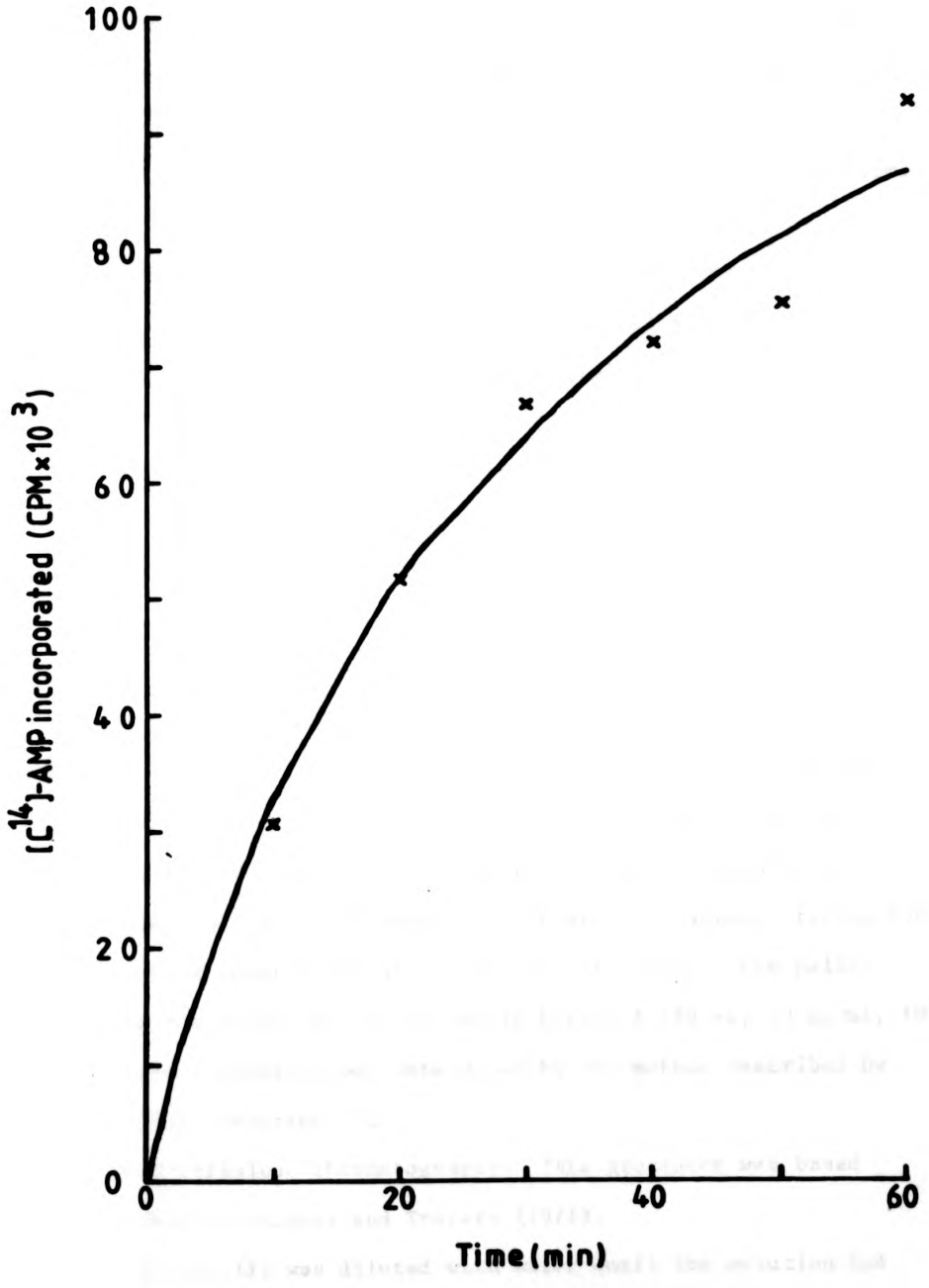
(A) Buffer solutions:

Buffer G: 0.05 M tris-HCl, 0.01 M $MgCl_2$, 0.2 M KCl, 0.001 M DTT, 0.001 M EDTA, pH 7.5, 5% glycerol (v/v).

Buffer A: 0.01 M tris-HCl, 0.01 M $MgCl_2$, 0.001 M EDTA, 0.001M DTT, pH 7.9, 5% glycerol (v/v).

Buffer C: 0.05 M tris-HCl, 0.001 M EDTA, 0.001 M DTT, pH 7.9, 5% glycerol (v/v).

Figure 3.6: DNA-dependent RNA polymerase assay.
Synthesis of poly(A-U) with UTP, [^{14}C]-ATP as
substrate and poly d(A-T) as template.


100
80
60
40
20
0
0
20
40
60
Time (min)
[C14]-AMP incorporated (CPM×10 3)

Storage Buffer: 0.01 M tris-HCl, 0.01 M $MgCl_2$, 0.1 M KCl 0.001 M EDTA, 0.001 M DTT, pH 7.9, 50% glycerol (v/v).

(B) Grinding and DNase treatment: The grinding of the frozen pellet (200 g) was carried out in buffer G at 4^{o}, and DNase I (2 ml, 1 mg/ml) was added with mixing. The supernatant (400 ml) was collected (Fraction I). All procedures that followed were performed at 4^{o}.

(C) High speed centrifugation: Fraction I was centrifuges at 30,000 rpm for 2 hours at 4^{o}. This removed cell debris and ribosomes in one step. The clear amber supernatant (320 ml) was collected (Fraction II).

(D) Ammonium sulphate fractionation: Ammonium sulphate (21 g/ 100 ml) was added to fraction II to give 30% saturation, the pH of this solution was kept above 7 by addition of 1 M NaOH. The solution was stirred for 30 minutes and the precipitate removed by centrifugation at 9,000 rpm for 30 minutes. The salt concentration of the supernatant was increased to 65% ammonium sulphate (24.5 g/ 100 ml) saturation. The resultant precipitate contained the RNA polymerase and was stirred for 30 minutes, then centrifuged at 18,000 rpm for 30 minutes. The precipitate was resuspended in buffer A (260 ml) with 60% ammonium sulphate saturation, stirred for 45 minutes and centrifuged at 18,000 rpm for 1 hour. The pellet containing the enzyme was dissolved in buffer A (88 ml, 13 mg/ml, 10% nucleic acid contamination, determined by the method described by Layne (1963)) (Fraction III).

(E) DEAE-cellulose chromatography: This procedure was based on the method by Burgess and Travers (1971).

Fraction III was diluted with water until the solution had

a conductivity equal to or just less than buffer A + 0.13 M KCl. This fraction was applied to a column (2.2 x 25 cm) of DEAE-cellulose, which was equilibrated with buffer A, at a flow rate of 30 ml/hr., and washed with buffer A + 0.13 M KCl (300 ml). The elution of the enzyme was achieved by a linear gradient of buffer A + 0.13 M KCl to buffer A + 0.30 M KCl (1 l total) (Fig.3.7). The peak of the RNA polymerase activity was eluted at 0.20 M KCl, just before a great deal of nucleic acid was eluted. The fractions were collected and were brought to 60% ammonium sulphate saturation, stirred for 30 minutes and centrifuged at 18,000 rpm for 1 hour. The pellet was dissolved in buffer C (10 ml, 4.8 mg/ml, 7% nucleic acid contamination) (Fraction IV).

(F) Agarose gel filtration at low salt: Fraction IV was dialysed against buffer C for 24 hours, and applied to a column (2 x 65 cm) of Sepharose 4B, which was equilibrated with buffer C, and then eluted with buffer C at a flow rate of 30 ml/hr. Two fractions with RNA polymerase activity (a) and (b) were eluted, but fraction (b) was heavily contaminated with nucleic acid (14%) (Fig.3.8). Therefore, only fraction (a) was collected and worked up as described in section 3.2.15 (E). The pellet was dissolved in buffer A (1 ml) and dialysed against the same buffer for 24 hours. Equal volume of glycerol was added to the dialysed protein solution (1.5 ml), and this purified RNA polymerase solution (3 ml, 5.7 mg/ml, 0.25% nucleic acid contamination) was stored at -20°.

3.2.16 Studies on the Properties of DNA-dependent RNA Polymerase (Purified by the Method of Burgess (1969a, 1971)) Towards Different Metals, Templates and Substrates

The procedures and conditions of these studies were the same as described in section 3.2.13. Each assay consisted of

Figure 3.7: Elution profile of the purification of DNA-dependent RNA polymerase by DEAE cellulose chromatography.

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aphy.

Figure 3.8: Elution profile of the purification of DNA-dependent RNA polymerase by Sepharose 4B filtration chromatography.

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tris-HCl, pH 8.0 (4 µmole), $MgSO_4$ (1 µmole) or $MnSO_4$ (0.2 µmole), DTT (0.5 µmole), substrates (100 nmole each), KCl (5 µmole) (this was omitted when $MnSO_4$ was used), enzyme (5.7 µg) and template (0.01 or 0.1 O.D.). The results were presented in Table 3.3.

3.2.17 Extraction and Purification of DNA-dependent RNA Polymerase (II)

In this extraction and purification of the enzyme, the method by Burgess (1969a, 1971) was followed in the first four stages (section 3.2.15 B-E). In the final stage of purification, the agarose gel filtration was not carried out, instead a linear sucrose density gradient centrifugation, as described by Zillig et al. (1966) was employed.

(A) Buffer solution: 0.01 M tris-HCl, 0.022 M NH_4Cl, 0.01 M $MgCl_2$, 0.0001 M DTT, pH 7.9.

(B) Linear sucrose gradient centrigugation: The enzyme fractions eluted from DEAE-cellulose chromatography were worked up as described in section 3.2.15 D. The precipitated protein was dissolved in the buffer solution (2 ml) and dialysed against the same buffer solution for 24 hours. A Spinco SW 25-2 tube was filled with a linear sucrose gradient ranging from 30 to 10% (W/v) sucrose in the buffer solution (144 ml). The dialysed protein solution was layered on top of the linear gradient of sucrose and centrifuged at 23,000 rpm for 20 hours. Fractions were collected with a needle dipped into the bottom of the centrifuged tube, and the U.V. absorbance at 254 nm was followed by a LKB-Uvicord recorder. The fractions containing the enzyme activity were collected, and the enzyme was precipitated as described in section 3.2.15 D (Fig. 3.9). The enzyme was dissolved in the buffer solution (2 ml) and dialysed against the same buffer for 24 hours. Equal volume of glycerol was added to the dialysed protein solution

TABLE 3.3: Studies on the properties of DNA-dependent RNA polymerase (purified by the method of Burgess (1969a, 1971)) towards different metals, templates and substrates

	Substrates[a]	Template	Template Concentration (O.D.)	Metal	nmole of [^{14}C]XMP incorporated
1	ATP, UTP	↑	0.1	Mg^{++}	1.77
2	ATP, UTP	Polyd(A-T)	0.01	Mg^{++}	1.10
3	ATP, UTP	↓	0.1	Mn^{++}	0.45
4	ITP, CTP	↑	0.1	Mg^{++}	0.45
5	ITP, CTP	Polyd(I-C)	0.01	Mg^{++}	0.13
6	ITP, CTP	↓	0.1	Mn^{++}	5.10
7	ITP, CTP	↓	0.01	Mn^{++}	5.00
8	ITP, CTP	-	-	Mg^{++}	1.01
9	ITP, CTP	-	-	Mn^{++}	0.39
10	ATP, UTP	-	-	Mg^{++}	1.29
11	ATP, UTP	-	-	Mn^{++}	0.76

[a] The substrates ATP and ITP used were the labelled nucleotide triphosphates [^{14}C]-ATP (1760 c.p.m./nmole) and [^{14}C]-ITP (740 c.p.m./nmole).

Figure 3.9: Elution profile of the purification of DNA-dependent RNA polymerase by linear sucrose gradient centrifugation.

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entrifuga-

(4 ml) and this purified RNA polymerase solution (8 ml, 15.1 mg/ml, 0.25% nucleic acid contamination) was stored at -20°.

3.2.18 Studies on the Properties of DNA-dependent RNA polymerase (Purified by the Method of Zillig et al. (1966)) Towards Different Metals, Templates and Substrates

The procedures, conditions, reagents and reagent concentrations were the same as described in section 3.2.16. The results were presented in Table 3.4.

3.2.19 Synthesis of copoly(I-C) using DNA-dependent RNA Polymerase Purified by the Two Different Methods

Each polymerisation mixture (10 ml) consisted of:

(i) 0.2 M tris-HCl, 0.05 M $MgCl_2$, 0.25 M KCl, pH 8.0 (2 ml). When $MnSO_4$ was used to replace $MgCl_2$, 0.25 M tris-HCl, 0.01 M $MnSO_4$, pH 8.0 (2 ml) was used as buffer:

(ii) 0.01 M ITP (1 ml), 0.01 M CTP (1 ml);

(iii) 0.1 M DTT (0.5 ml);

(iv) with or without poly d(I-C) (0.1 O.D.);

(v) enzyme (1.4 mg) from purification method by Burgess (1969a, 1971), or that by Zillig et al. (1966);

(vi) water (sufficient to bring final volume to 10 ml when enzyme added).

Therefore, a total of eight sets of polymerisation were carried out. The reaction mixtures were incubated at 37° for 2 hours. DNase (1.5 mg) was added to those reaction mixtures containing poly d(I-C), and incubation continued for a further 20 minutes. Protein was extracted by addition of iso-amyl alcohol/chloroform as described in section 3.2.3. Ethanol (2-3 volumes) was added to the final protein-free aqueous solution, and kept at -20° for 24 hours. The precipitated copoly(I-C) was collected by centrifugation at 10,000 rpm for 10 minutes. The polynucleotide was dissolved in water (1 ml) and dialysed against

TABLE 3.4: Studies on the properties of DNA-dependent RNA polymerase (purified by the method of Zillig et al. (1966) towards different metals, templates and substrates

	Substrates[a]	Template	Template Concentration (O.D.)	Metal	nmole of [^{14}C]XMP incorporated
1	↑	↑	0.1	Mg^{++}	1.90
2	ATP, UTP	Polyd(A-T)	0.01	Mg^{++}	1.12
3	↓	↓	0.1	Mn^{++}	0.63
4	↑	↑	0.1	Mg^{++}	1.43
5	ITP,CTP	Polyd(I-C)	0.01	Mg^{++}	1.35
6	↓	↓	0.1	Mn^{++}	0.50
7	↓	↓	0.01	Mn^{++}	0.37
8	ITP, CTP	-	-	Mg^{++}	1.29
9	↓		-	Mn^{++}	0.37
10	ATP, UTP		-	Mg^{++}	2.17
11	↓		-	Mn^{++}	1.21

[a] The substrates ATP and ITP used were the labelled nucleoside triphosphates, [^{14}C]-ATP (1760 c.p.m./nmole) and [^{14}C]-ITP (740 c.p.m./nmole).

water for 24 hours. The dialysate was lyophilized and stored at -20°. One more polymerisation was carried out using the DNA-dependent RNA polymerase (400 µg) obtained as a gift from Dr. V. W. Armstrong, with Mg^{++} as the metal ion and a higher concentration of poly d(I-C)(10 O.D. in 10 ml of polymerisation mixture). Other reagents, procedures, and conditions were identical as described in this section. The lyophilized copoly(I-C) gave a yield of 28% (3 mg).

3.3 Results and Discussion

3.3.1 Polymerisation of mo^5CDP

Hillel and Gassen (1979) reported that a high concentration (2.7 mg/ml, 150 units/mg) of PNPase as well as a long reaction time (48 hours) were required to synthesize poly(mo^5U), in the presence of Mg^{++} at 37°. The polymerization of mo^5CDP with M. luteus PNPase was achieved in the presence of a moderate concentration (2.7 mg/ml, 30 units/mg) of the enzyme. However, in the presence of Mg^{++}, the yield of poly(mo^5C) was very low (7.7%), and the equilibrium of the reaction was reached after 24 hours. In the presence of Mn^{++}, the yield of poly(mo^5C) was observed to have increased (15.5%), and the equilibrium of the reaction was reached after 48 hours. The poly(mo^5C) obtained had a S_W^{20} value of 18.2, and the degradation of poly(mo^5C) to its monomers by ribonuclease and phosphodiesterase gave a hyperchromicity in the U.V. spectra of 20% at 294 nm.

The method of continuous variation (Job, 1928) as applied to the determination of polynucleotide complex formation (Felsenfeld et al., 1957) was used to investigate the possible interaction between the synthetic polynucleotide poly(mo^5C) and poly(I). When the U.V. absorbance at 240 nm and 250 nm of the polynucleotide

solutions with various molar fractions of poly(mo^5C) and poly(I) were plotted as shown in Fig.3.5 a break was observed at the molar fraction of 50:50. Therefore, at neutral solution and 0.1 M salt concentration, poly(mo^5C) can form a 1:1 complex with poly(I), and the U.V. absorption spectrum was shown in Fig.3.10.This 1:1 complex formation was confirmed by the thermal transition profile studies on poly(I).poly(mo^5C). In neutral solution in the presence of 0.1 M NaCl, poly(I).poly(mo^5C) has a T_m value of 64.5° (Fig.3.11) which is slightly higher than that of poly(I).poly(C) (57°), and a hyperchromicity of 39%. Earlier similar results were obtained by Kulikowski and Shugar (1974), who polymerized 5-ethylcytidine 5'-diphosphate with PNPase from E. coli and M. luteus, and found that, in neutral solution, poly(5-ethylC) formed a 1:1 helical complex with poly(I). The T_m of this complex was slightly higher than that of poly(I).poly(C), but considerably lower than that of poly(I). poly(5-methylC). Sagi et al. (1979), have studied the thermal transition of poly(d(A-r^5U)) polydeoxynucleotides, where r was a hydrogen atom, or a methyl, ethyl, n-propyl, n-butyl or n-pentyl group, by measuring the derivative melting profiles of the polymers in the range of 0.01-0.36M K^+, at pH 6.8. At a given salt concentration, T_m of the alkyl analogues decreased as the number of carbon atoms in the r substituent of poly(d(A-r^5U)) increased. It was suggested that the change in the hydrophobic character of modified polydeoxynucleotides is mainly responsible for the decrease in stability, due to the change in the ion-solvating capacity of phosphate anions. This may not be the case in poly(mo^5C).poly(I), but it is quite conceivable that as the bulkiness of the 5-substituents increase, the T_m value of the helical complex with poly(I) will decrease, and consequently the interferon-inducing ability of the double stranded polynucleotide will decrease.

The results of interferon induction and assay by poly(I).poly(mo^5C) is presented in Table 3.5. Surprisingly, poly(I).poly(mo^5C) induced

Figure 3.10: Ultra-violet spectrum of double-stranded poly(I). poly(mo^5C) in 0.01 M sodium cacodylate, 0.1 M sodium chloride, pH 7.0.

ly(I).

M

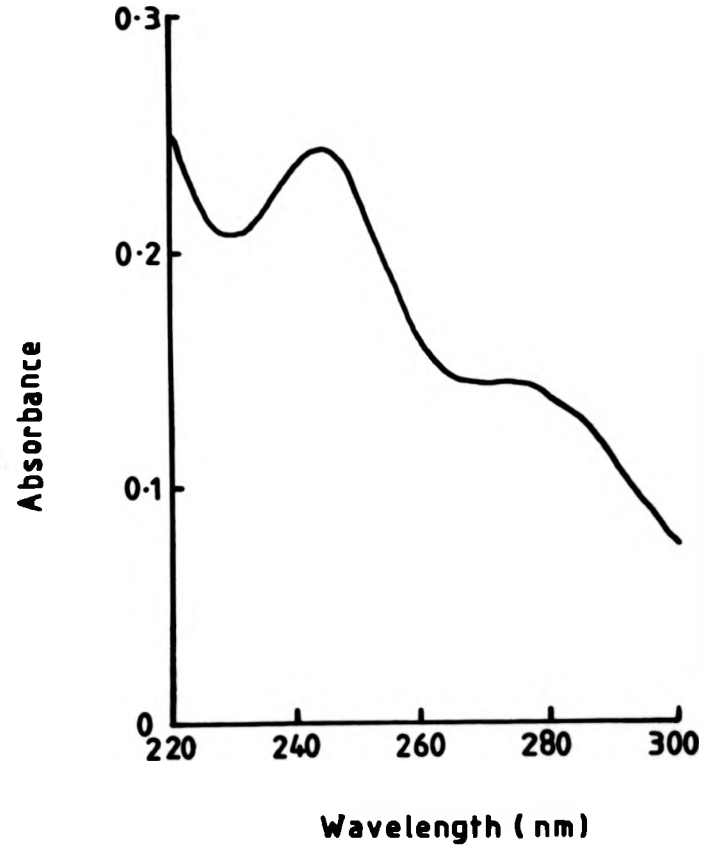


Figure 3.11: Thermal transition of a 1:1 mixture of poly(I) and poly(mo^5C) recorded in 0.01 M sodium cacodylate, 0.1 M sodium chloride, pH 7.0, at 250 nm.

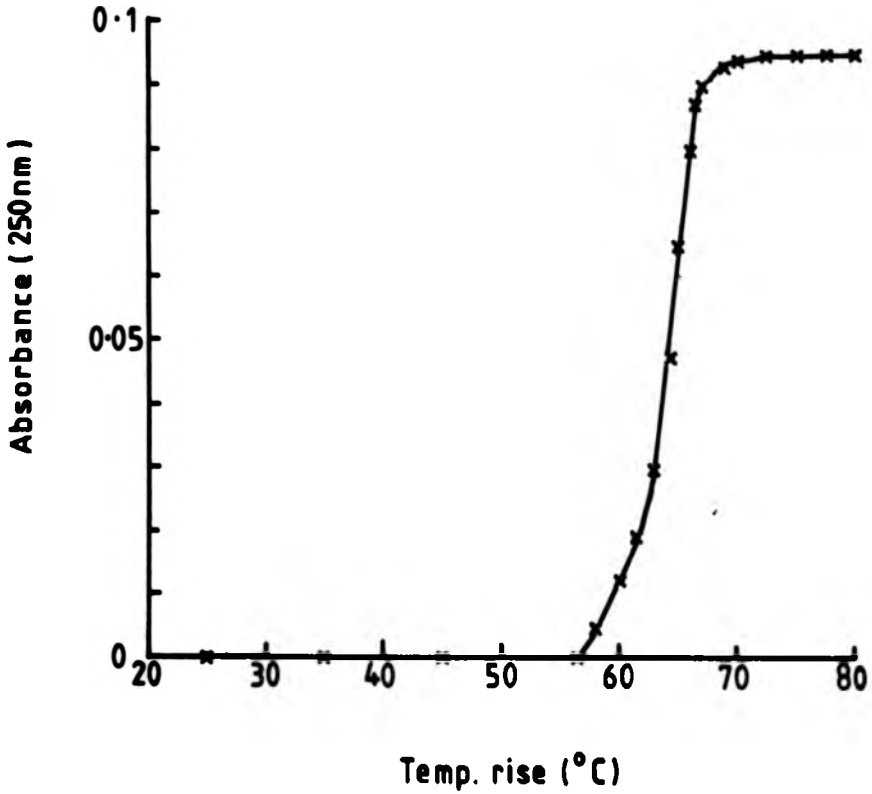

0·1
0·05
0
Absorbance (250nm)
20
30
40
50
60
70
80
Temp. rise (°C)

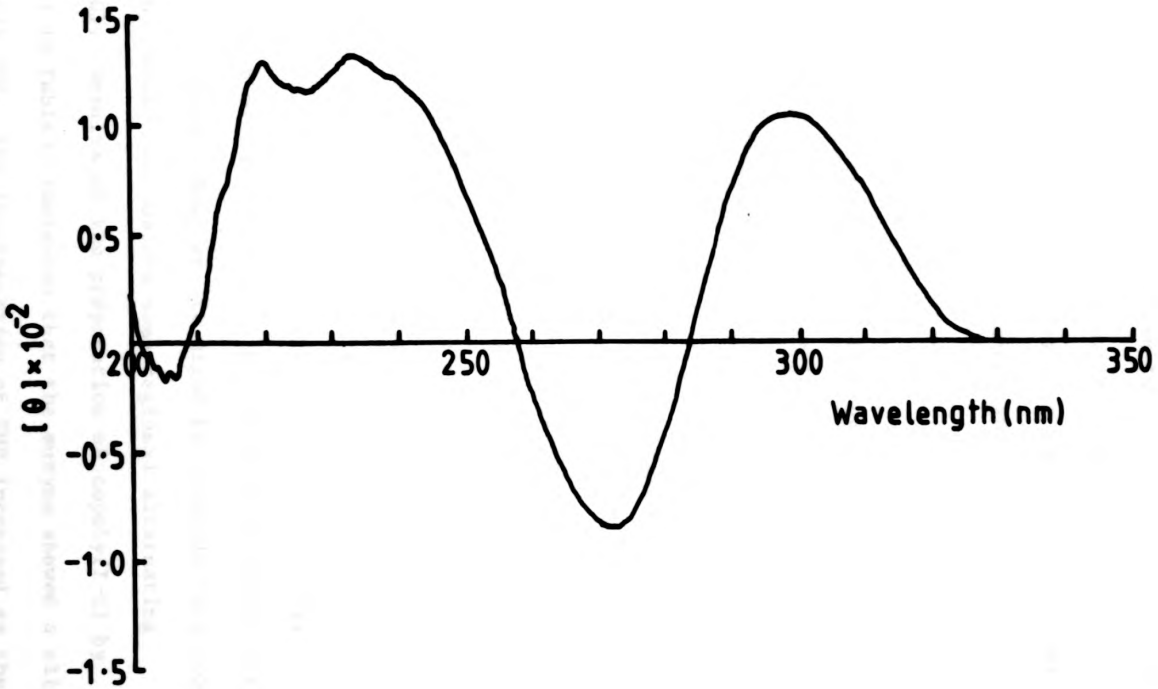
TABLE 3.5: Interferon induction by poly(I).poly(C) and poly(I).poly(mo^{5}C) with and without DEAE-dextran in MG63 cells

	Concentration in each induction (μg/ml)	DEAE-dextran	Interferon titre (log $_{10}$U/10^6 cells)
poly(I).poly(C)	50	-	2.0
poly(I).poly(C)	50	+	4.1
poly(I).poly(mo^{5}C)	50	-	<0.6
poly(I).poly(mo^{5}C)	100	-	<0.6
poly(I).poly(mo^{5}C)	50	+	0.8
poly(I).poly(mo^{5}C)	100	+	1.1

All interferon inductions and assays were carried out in duplicate.

interferon very poorly, although admittedly the interferon titre induced by poly(I).poly(C) was comparatively lower than usual in these experiments. One factor may be due to the abnormally large size of poly(mo^5C), which might hinder the uptake of the poly(I).poly(mo^5C) by the cells. However, in the presence of DEAE-dextran which facilitates the uptake of polynucleotides by cells, the interferon induction ability of poly(I).poly(mo^5C) had increased slightly. Hence, size is probably not an important factor. The CD spectrum of poly(I).poly(mo^5C) was examined (Fig. 3.12). It was found to be different from the CD spectra of poly(I).poly(C) and copoly(I-C) as shown in the following chapter. CD spectrum of poly(I).poly(mo^5C) showed a broad positive Cotton effect with two small peaks at 220 nm and 233 nm, and another sharper positive Cotton effect with a peak at 298 nm, but there was also a prominent negative Cotton effect with a peak at 273 nm. However, this CD spectrum was similar to that of poly(I).poly(br^5C) (Bobst et al., 1976). Bobst et al. (1976) concluded that, according to their CD data, a pyrimidine substitution at the 5-position with a methyl or bromine group does not strongly affect the base tilt within a particular duplex series. However, a possible twist variation cannot be excluded, because twist changes cannot be readily detected by CD. It was also suggested that a 5-pyrimidine substitution did not substantially affect the interferon inducing ability, unless the thermal stability of the analogue became critical. In the case of poly(I).poly(mo^5C), the T_m value was 64.5^o. Therefore, the overall conformation of poly(I).poly(mo^5C) should be very similar to that of poly(I).poly(br^5C) and the T_m value was certainly high enough, yet it did not induce interferon. It can only be suggested that due to the bulkiness of the 5-methoxy substituent, which has a Van der Waals radius of >3.50 (Van der Waal radii of

Figure 3.12: Circular dichroic spectrum of double-stranded poly(I).poly(mo^{5}C) in 0.01 M sodium cacodylate, 0.1 M sodium chloride, pH 7.0, at 20^o.


1·5
1·0
0·5
0
-0·5
-1·0
-1·5
200
250
300
350
Wavelength (nm)
[θ]×10⁻²

C-O, C-H bonds were 1.42 and 1.09 respectively), the conformation of the poly(I).poly(mo^5C) was changed slightly. The conformational change may be due to base tilting or base twisting which cannot be detected by CD spectrum. However, the notion that interferon induction is dependent on the recognition of a particular spatial and steric organization of a double stranded RNA still holds true (Torrence and De Clercq, 1977).

3.3.2 Copolymerisation of IDP and CDP with PNPase

Studies on the base composition of copoly(A-G-U-C), prepared by PNPase using labelled nucleoside diphosphates, had shown that the four different nucleotide units were distributed randomly in the polynucleotide chain (Ortiz and Ochoa, 1959). However, base composition studies on copoly(A-U) had shown that the polynucleotide was not completely random, but the alternating sequence (A-U) was predominant (Grunberg-Manago, 1959). Its base ratios were dependent on the relative concentrations of the nucleoside diphosphates used in the preparation of the copolymer, but the base ratio of the product was not always equal to the ratio of the substrate used, for a reason which is not yet known. Therefore, it is unlikely that a completely alternating copolymer of IMP and CMP can be prepared by PNPase with IDP and CDP as substrates. However, it might be possible that copoly (I-C) prepared by PNPase can possess some regional alternating sequence of (I-C). Results of the preparation of copoly(I-C) by PNPase presented in Table3.1 indicated that the enzyme showed a slight preference towards CDP. The incorporation of IMP increased as the IDP input was increased. When the input substrate ratio was 1:1, the base ratios of the copoly(I-C) was close to 1:1 with either Mg^{++} or Mn^{++} as metal ions. Therefore, both Mg^{++} and Mn^{++} can be used to

produce copoly(I-C) with a base ratio of 1:1. However, in the case of Mg^{++} as metal ion, the yield of the copoly(I-C) seemed to increase as the input of IDP was increased, but this might only be due to the slight increase in the total input of substrates.

The alternating sequence of the nucleotides in a copolymer can be established by [^{32}P] transfer studies, using as substrates a α-[^{32}P]-labelled nucleotide and a non-labelled one, and measuring the distribution of [^{32}P] in the alkaline hydrolysis product or the ribonuclease hydrolysis product (Chamberlin et al., 1963). Sternbach and Eckstein (1970) polymerized ITP and α-[^{32}P]-CTP with poly d(I-C) as the template using DNA-dependent RNA polymerase from E.coli. In the nearest neighbour analysis, it was found that all the radioactivity was associated with IMP, therefore the copoly(I-C) was strictly alternating. RNaseA and RNaseT, were used in the nearest neighbour analysis mentioned in section 3.2.11. Ribonucleases catalyse the reaction shown in Fig. 3.13. The first step consists in the cleavage of the phosphodiester bond between the 3'- and 5'-positions of the ribose moieties in the RNA chain with the formation of oligonucleotides terminating in 2',3'-cyclic phosphate derivatives. In the subsequent steps, these terminal groups are split off as free mononucleotide cyclic phosphates, which are then hydrolysed with the formation of the corresponding nucleoside 3'-phosphates. The results of the nearest neighbour analysis on the copoly(I-C) with a base ratio of 1:1, prepared by PNPase with CDP and α-[^{32}P]-IDP as substrates, showed that the radioactivity was associated with both CMP (7.92 μCi) and IMP (4.93 μCi). Therefore, the copoly(I-C) was not strictly alternating, however, the radioactivity associated with CMP was higher than that of IMP, and it might still be possible that there were regions with alternating sequence of (I-C).

The next step on the characterization of the copoly(I-C)

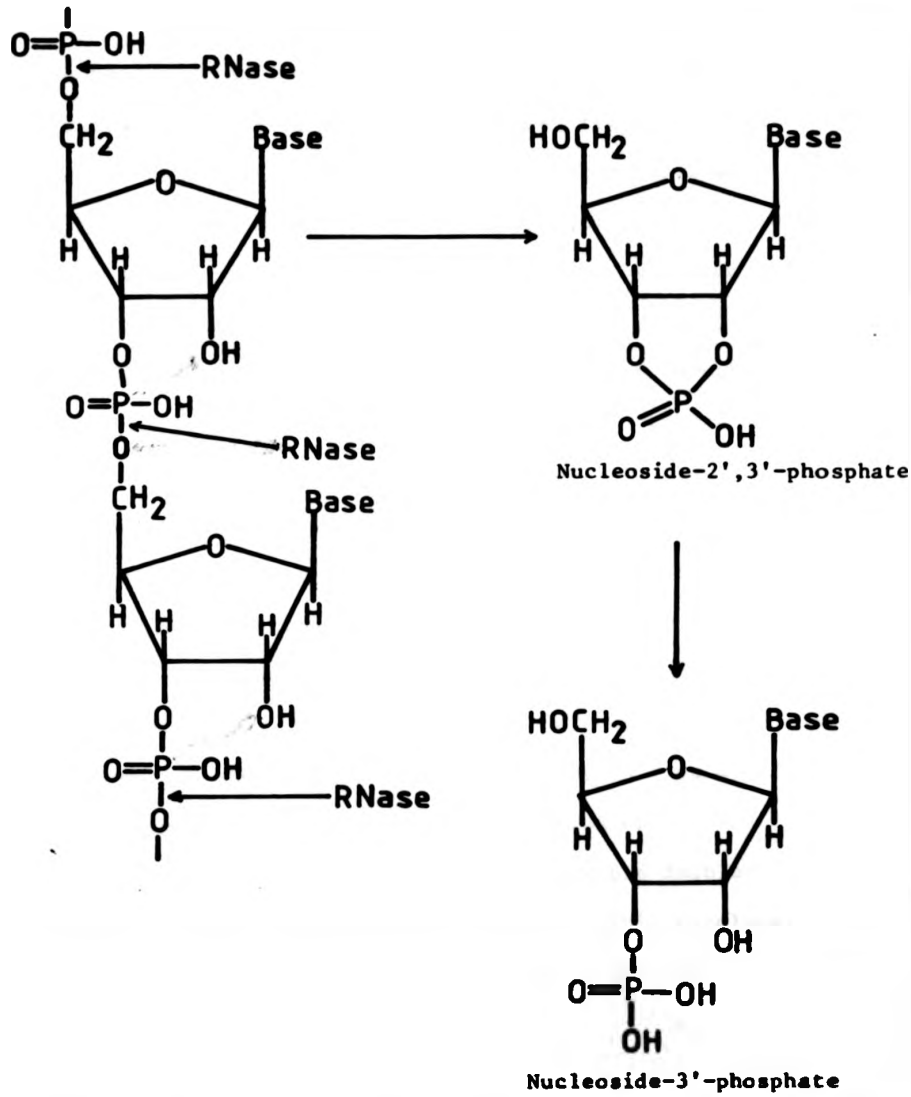


Figure 3.13: General scheme of the mechanism of action of ribonuclease (Anfinsen and White (1961))

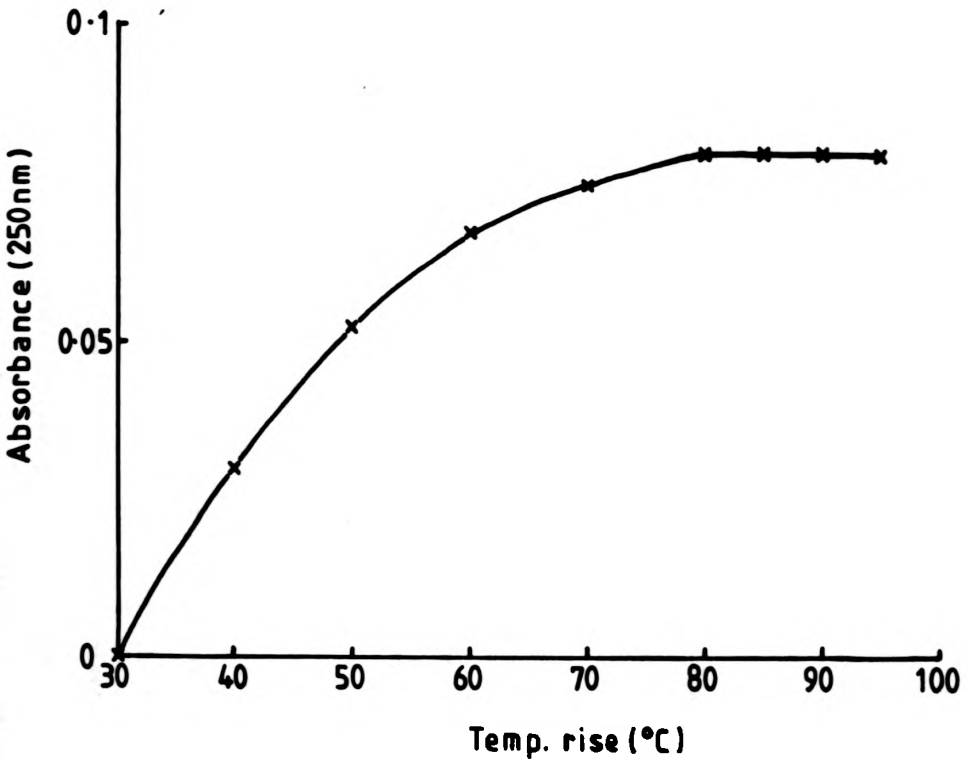
prepared by PNPase was to establish whether the polynucleotide was double-stranded or if regions of double-strandedness existed. In polynucleotides with cytidine as a component, if any cytidines were not involved in the hydrogen bonding formation, these exposed cytidines can be converted to 5,6-dihydrouracil-6-sulphonate residues by sodium bisulphite, and in hydrolysis, these residues will be converted to uridine. Results of these experiments were presented in Table 3.2 and it was found that the cytidines of poly(C) and copoly(I-C) were converted completely to uridine by sodium bisulphite, while the untreated poly(C) and the ds poly(I).poly(C) were completely unaffected. The results were unaltered even though the annealing procedure was carried out on the copoly(I-C) before treatment with sodium bisulphite. Therefore, it can be concluded that the copoly(I-C) does not possess any double strandedness. Also, when the thermal transition profile determination was carried out on this copolymer, no sharp melting point was observed. The increase in O.D. upon rising temperature started at 30^{o} and then continued gently until 75^{o} (Fig. 3.14). Therefore, it can be concluded that PNPase polymerizes IDP and CDP, with either Mg^{++} or Mn^{++} as metal ions, to give a copolymer with a random distribution of IMP and CMP, and the copolymer does not contain any appreciable double-stranded regions with and without prior annealing. This copolymer was not used in the cross-linking experiments which will be mentioned in the following chapter.

3.3.3 Copolymerization of ITP and CTP with DNA-dependent RNA Polymerase

Sternbach and Eckstein (1970) reported that the unprimed synthesis of the alternating copoly(I-C) could be obtained using DNA-dependent RNA polymerase only when the enzyme was purified

Figure 3.14: Thermal transition of copoly(I-C), synthesized by PNPase, in 0.01 M sodium cacodylate, 0.1 M sodium chloride, pH 7.0, at 250 nm.

ed by
odium



according to Zillig et al. (1966). The Zillig et al. (1966) purification method included grinding and DNase treatment of cells, high speed centrifugation, ammonium sulphate fractionation, DEAE-cellulose chromatography, and linear sucrose gradient centrifugation. This enzyme preparation contained only part of the σ-factor. When the two Burgess (1969a) purification methods were used, a polymer with T_m = 57° (poly(I).poly(C)) was obtained. The first enzyme preparation contained no σ-factor (designated PC-enzyme, the first four steps of purification was the same as described by Zillig et al. (1966), but the final two steps involved phosphocellulose chromatography and agarose gel filtration chromatography), and the second enzyme preparation contained σ-factor (designated GG-enzyme, the first four steps of purification were the same as PC-enzyme, but the final step was a glycerol gradient centrifugation). When large concentrations of substrates were used with the PC- and GG-enzymes as recommended by Krakow and Karstadt (1967), the polymeric material obtained exhibited two T_m-values, 47° and 57°, respectively, which suggested the presence of both the alternating copoly(I-C) as well as poly(I).poly(C). Since the PC- as well as the GG-enzyme catalysed the synthesis of poly(I).poly(C), the σ-factor apparently was not involved in this reaction. Therefore, Sternbach and Eckstein (1970) suggested that it was unlikely that the amount of σ-factor contained in the Zillig et al. (1966) enzyme preparation was responsible for the synthesis of the alternating copoly(I-C). There must be other differences between these enzyme preparations which directed the synthesis of either the alternating copolymer or the homopolymer pair. There is no explanation so far for the fact that at high substrate concentrations, even the PC- and GG-enzymes can synthesize some of the alternating polymer.

Due to the above observation by Sternbach and Eckstein (1970), the Zillig et al. (1966) (designated Zillig enzyme) and Burgess (1969a) (designated Burgess enzyme) purification methods of DNA-dependent RNA polymerase were carried out in this chapter. Both the Zillig and Burgess enzymes had a ratio of $A_{280}/A_{260} > 1.6$ indicating that the enzymes were fairly free of nucleic acid contamination. Results on the studies on the properties of the Burgess enzyme (Table 3.3)indicated that this enzyme preparation, in the presence of templates, with Mg^{++}, had a high preference towards ATP, UTP than ITP, CTP. The enzyme activity, in the absence of template, was higher with Mg^{++} than Mn^{++} in both substrate systems, but the overall activity was very low. However, with ITP and CTP as substrates, in the presence of template and Mn^{++} as metal ion, there was a drastic increase in enzyme activity. According to these observations, the unprimed synthesis of copoly(I-C) by this enzyme was unlikely to produce adequate amount of the copolymer. Results on the studies on the properties of the Zillig enzyme (Table3.4) indicated that the enzyme activities were low, in the presence of Mn^{++}, in all cases. With Mg^{++} as metal ion, the enzyme activities in the primed and unprimed reactions were very similar in both substrate systems.

When copoly(I-C) synthesis was carried out with both enzymes, in the presence of low concentration of template (0.1 O.D. in 10 ml reaction mixture) or without template, it was found that the polymeric materials obtained had a thermal transition profile similar to that synthesized by PNPase (Fig. 3.14). The polymeric material with a T_m-value = 49.5° (Fig.3.15)was obtained only when the template concentration was increased ten fold. However, the overall yields by both enzymes were very low (< 10%). Therefore, the DNA-dependent

Figure 3.15: Thermal transition of alternating copoly(I-C), synthesized by DNA-dependent RNA polymerase with poly d(I-C) as template, in 0.01 M sodium cacodylate, 0.1 M sodium chloride, pH 7.0, at 250 nm.

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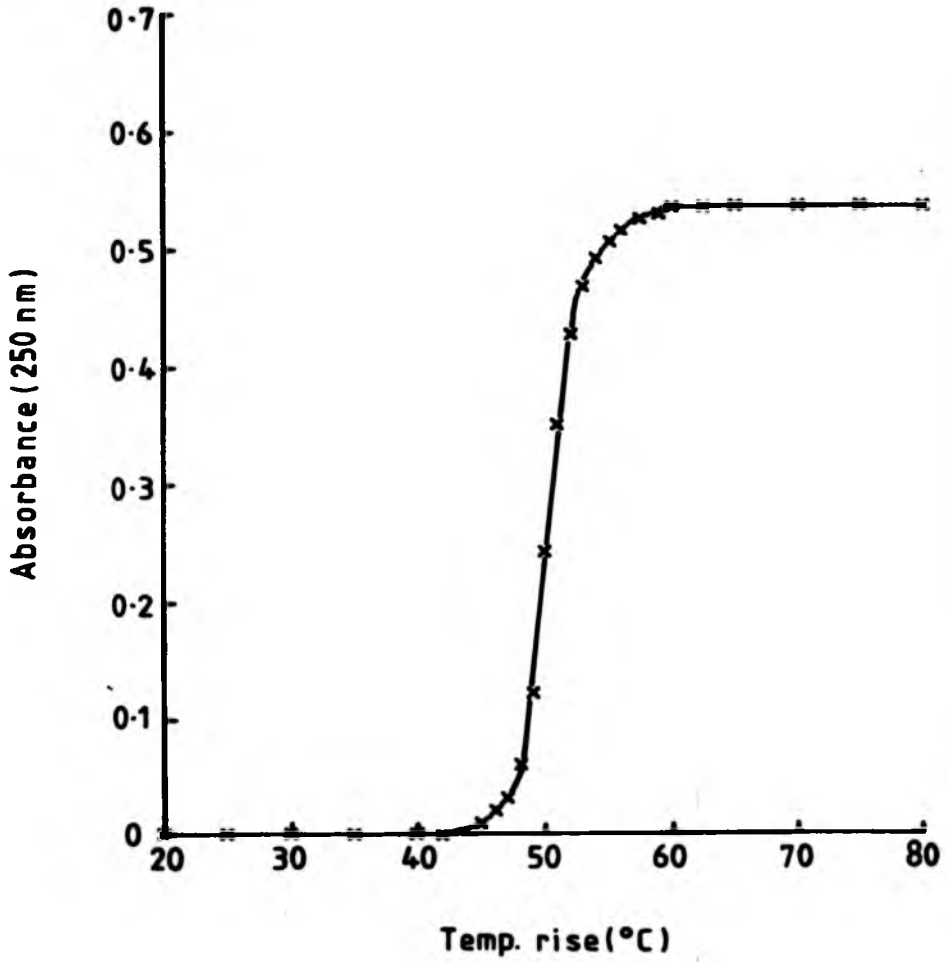
with

codylate,



RNA polymerase obtained from Dr. V. W. Armstrong as a gift was used, and the yield of the alternating copoly(I-C) was increased to 28%. The T_m-value (49.5°) of this polymeric material was the same as that obtained by Karstadt and Kradow (1970). Also, the immuno-diffusion experiments for double-strandedness on this polymeric material carried out by Dr. M. Leng (Centre de Biophysique Moléculaire, Avenue de la Recherche Scientifique, France) (Fig. 3.16)showed that it reacted similarly as poly(I).poly(C), but the random copoly(I-C) reacted much less than the other polynucleotides. With these observations, it was concluded that this polymeric material was a double-stranded copoly(I-C) with a alternating sequence of (I-C), and was used in the cross-linking experiments in the following chapter. (This polymeric material was designated poly(I-C) in the following chapter.)

Figure 3.16: Immuno-diffusion experiments for double-strandedness on (A) alternating copoly(I-C), (B) and (E) double-stranded poly(I).poly(C), (C) random copoly(I-C), and (D) being the blank. The polynucleotides were dissolved in 0.2 M Na_2SO_4, and the gel was 1% agarose (solvent: 0.1 M NaCl, 0.005 M tris-HCl, pH 7.5).

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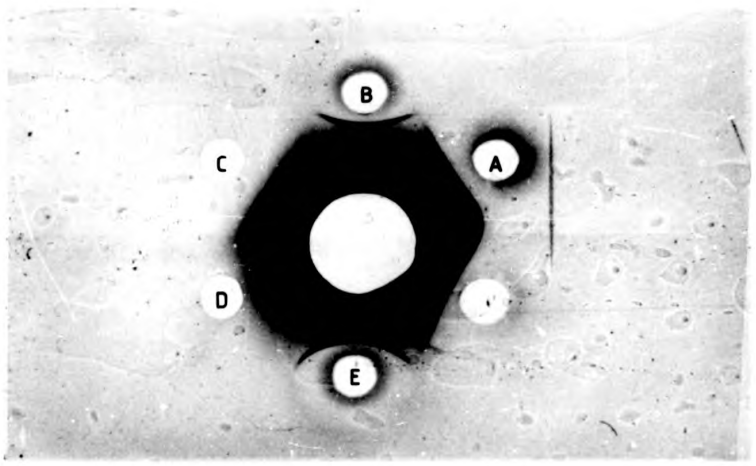

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-HCl,

CHAPTER 4

CHEMICAL CROSS-LINKING OF SYNTHETIC POLYNUCLEOTIDES

4.1 Introduction

This chapter is concerned with the chemical cross-linking of two synthetic polynucleotides: poly(I).poly(C), and alternating copoly(I-C). Two different types of cross-linking reagents were employed for this task, firstly, a difunctional nitrogen mustard (methyl-1-N-bis(2-chloroethyl)amine hydrochloride, HN2), and secondly, 8-methoxypsoralen (Figure 4.4). The interferon induction capability of the polynucleotide, before and after cross-linking, could then be assessed.

4.1.1 The Difunctional Nitrogen Mustard, HN2 (Figure 4.1)

Difunctional nitrogen mustard belongs to a group of biological alkylating agents, which include the monofunctional nitrogen mustard, and the mono-and difunctional sulphur mustards. At the time when the reaction of these agents with nucleic acids was being investigated their role as carcinogens, and their potential value in cancer chemotherapy was in question (Brookes, 1975). Early studies on the action of the nitrogen mustard HN1, HN2, dimethyl sulphate and diethyl sulphate on DNA from calf-thymus showed that the purines were more reactive than the pyrimidines, and the guanine moiety was most susceptible to alkylation (Lawley and Wallick, 1957; Reiner and Zamenhof, 1957). To summarise, the principal biological alkylating agents were shown to react with the ring nitrogen atoms of the nucleic acid bases, guanine, adenine, and cytosine and in particular with the N-7 position of guanine moieties (Figure 4.2)(Brookes, 1975). Lawley and Brookes (1963)

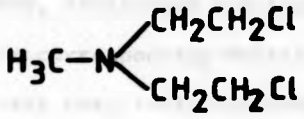


Figure 4.1: Molecular structure of the difunctional nitrogen mustard methyl-1-N-bis(2-chloroethyl)amine.

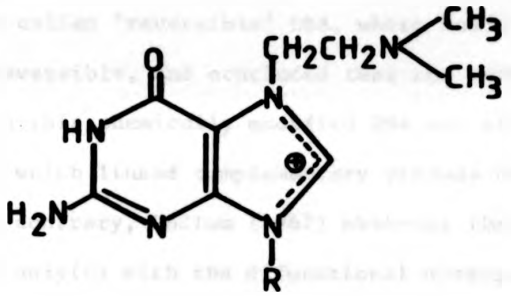


Figure 4.2: The guanyl derivative which was produced by the alkylation action of monofunctional nitrogen mustard at the N-7 position.

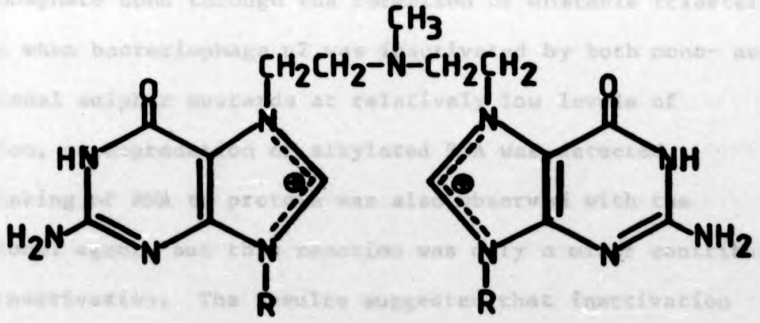


Figure 4.3: The diguanyl derivative which was produced by the action of difunctional nitrogen mustard at the N-7 positions.

observed that in all cases, alkylation resulted in destabilization of the nucleosides or the corresponding moieties in the nucleic acids. At neutral pH, with DNA, 7-alkylguanines and 3-alkyladenines were slowly liberated by hydrolysis, the latter at a greater rate, whereas the RNA, slow rearrangements occurred, with 1-alkyladenine moieties yielding 6-methylaminopurine moieties and 3-alkylcytosines giving the corresponding 3-alkyluracils. Geiduschek (1961) successfully reacted DNA with the difunctional nitrogen mustard, methyl-1-N-bis(2-chloroethyl)amine hydrochloride, at pH 7.1, to produce the so called 'reversible' DNA, whose helix-coil transitions were totally reversible, and concluded that the reversibility of denaturation in this chemically modified DNA was controlled by covalent bonds which linked complementary strands of the double helix. On the contrary, Ludlum (1967) observed that the reaction of poly(A) and poly(U) with the difunctional nitrogen mustard resulted in degradation of both synthetic polynucleotides, as shown by the marked decreases in molecular weight and specific viscosity, thus, the chain scission obscured any cross-linking reactions which might occur. It was suggested (Ludlum, 1967) that this degradation probably resulted from a direct attack on the sugar-phosphate bond through the formation of unstable triesters. However, when bacteriophage μ2 was inactivated by both mono- and difunctional sulphur mustards at relatively low levels of alkylation, no degradation of alkylated RNA was detected. Cross-linking of RNA to protein was also observed with the difunctional agent, but this reaction was only a minor contribution to the inactivation. The results suggested that inactivation resulted from the mono-alkylation of adenine or cytosine, and in experiments with the difunctional agent cross-linking of RNA bases or of RNA to protein also prevented replication (Shooter *et al.*,

1971). In the case of the inactivation of bacteriophage T7 following treatment with mono- and difunctional sulphur mustards, the major lethal event was the formation of diguaninyl product (Figure 4.3) which covalently cross-linked the two DNA strands, and blocked the replication of DNA (Lawley et al., 1969). It has been suggested (Ötvös and Elekes, 1975a, 1975b) that the cross-links, which formed with difunctional alkylating agents wherein the two functional groups were separated by five or six atoms, would have a zig-zag conformation if the distance between the two reaction centres was suitably short.

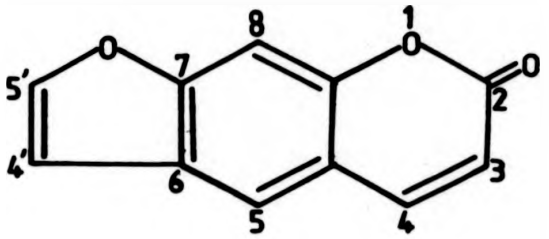
Alkylation of poly(G-C), poly(G).poly(C), and poly(I).poly(C) with HN1 and HN2 nitrogen mustard have been studied, and their antiviral activities before and after alkylation were compared (Eaton, 1974). Alkylation of poly(G-C) occurred readily, and on reaction with HN2, the percentage hyperchromicity of this synthetic polymer dropped, and the T_m was claimed to be higher and with a broadened melting profile. All the HN2 treated samples were shown to reanneal immediately. All these effects were not observed with HN1 treated polynucleotides; in this case the hyperchromicity remained unchanged but the T_m fell slightly indicating some destabilization. The alkaline hydrolysis of alkylated poly(G-C), with HN2, showed that most of the alkylated guanines were present as diguanyl derivatives. Antiviral activity studies of the alkylated poly(G-C) samples showed that increasing monofunctional alkylation caused some loss in antiviral activity whilst difunctional alkylation up to about 15% caused a slight increase in antiviral activity. The studies on the alkylation of poly(I).poly(C) indicated that the alkylation made the molecule less stable. Nevertheless, the antiviral activity of poly(I).poly(C) with 15% of the inosine rings alkylated at N-7 was retained. The

antiviral activity of the alkylated poly(G).poly(C) with HN2 also increased slightly. It was suggested (Eaton, 1974) that the increase in interferon inducing ability of the alkylated poly(G-C) and poly(G).poly(C) was due to the cross-linking of the polymers which became more stable.

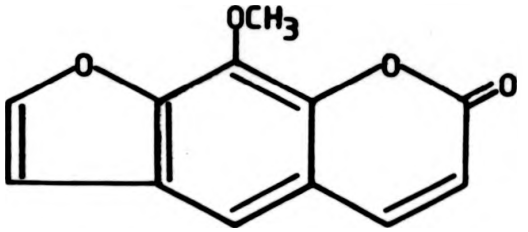
4.1.2 8-Methoxypsoralen

The linear isomers of the furocoumarin family known as psoralens (Figure 4.4)were first recognized as potent dermal photosensitizing agents by the ancient Egyptians who employed print and seed extracts containing them for centuries in the treatment of vitiligo. More recently, psoralens have drawn attention as photochemotherapeutic agents used in the treatment of psoriasis (Isaacs et al., 1977). Coumarins, (Figure 4.5)particularly psoralens, upon topical application and subsequent irradiation with near-U.V. (320-400 nm) light, cause skin photosensitization in rodent, an augmented erythema reaction (Pathak et al., 1959), and potential skin cancer in mice and guinea pigs, though skin cancer in man is not unequivocally established. The skin-sensitizing potency of psoralens has been correlated with their photoreactivity toward pyrimidine bases of DNA via cycloaddition (Mantulin and Song, 1973).

Coumarins are well known in photobiology as photosensitizing agents in photodynamic actions. Coumarin itself is also known to show interesting photochemical behaviour, particularly dimerization in polar and non-polar solvents. Early spectroscopic studies and theoretical calculations of the electronic structures of the excited states of coumarin indicated that the excitation is substantially localized in the pyrone moiety (Figure 4.6)(Song and Gordon, 1970). The lowest excited singlet and triplet states of coumarin, and psoralen have been assigned to the (π, π^*) type



(a)



(b)

Figure 4.4: (a) Molecular structure of psoralen and its numbering system (Song et al., 1971).

(b) Molecular structure of 8-methoxypsoralen.

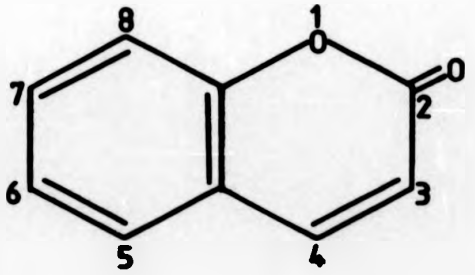


Figure 4.5: Molecular structure of coumarin (1,2-benzopyrone) and its numbering system (Song and Gordon, 1970)

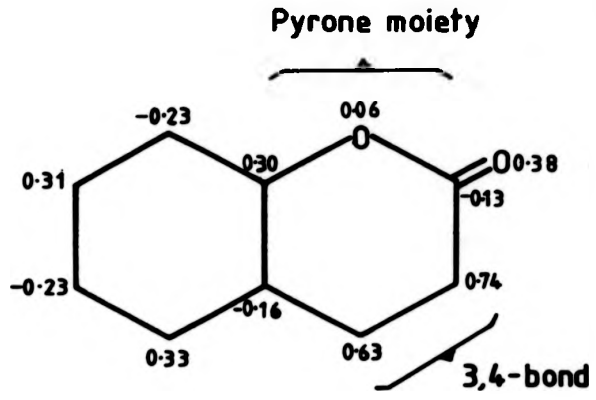


Figure 4.6: Spin densities in the excited triplet (π, π^*) coumarin without annihilation, note the high spin densities at the 3,4-pyrone double bond position (Song and Gordon, 1970).

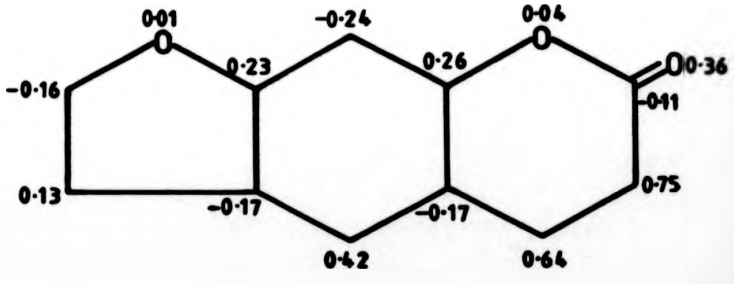
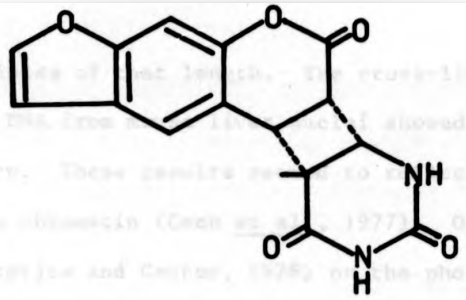


Figure 4.7: Spin densities in the excited triplet (π, π^*) psoralen without annihilation (Song et al., 1971).

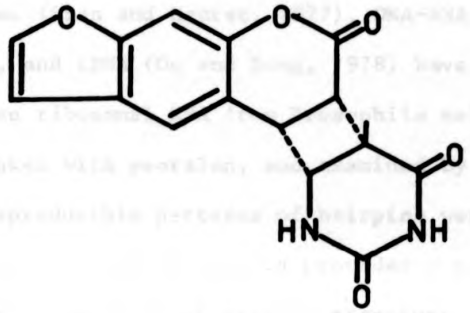
on the basis of the luminescence spectroscopy and Molecular Orbital calculations. The mechanism of photocycloaddition of coumarin and psoralen to thymine has been described in terms of the perturbational Molecular Orbital model and Molecular Orbital reactivity indices. In the excited triplet state, the 3,4-bond of coumarin, 3,4-bond of psoralen, and the 4',5'-bond of psoralen are very reactive, and this is thought to be due to the high localized spin density at positions 3,4-bond of the pyrone moiety and the furo C=C group (position 4',5') (Figure 4.7). Four possible "four-centre" mechanisms for photocycloaddition between psoralen and thymine have been suggested (Figure 4.8) (Song et al., 1971). It has been suggested (Mantulin and Song, 1973) that the excited triplet state of coumarin and psoralen undergo cycloaddition at the 3,4- and 4',5'-double bonds with pyrimidine bases only, and a light induced interstrand cross-linking, that involves the excited psoralen molecules and pyrimidine bases in opposite strands of DNA and RNA duplexes, can occur (Cole, 1972). However, not all psoralens exhibit the same potency as skin photosensitizers, for example, 5-hydroxypsoralen and 8-hydroxypsoralen have been found to be inactive, whereas 5-methoxypsoralen and 8-methoxypsoralen are very active (Song et al., 1975).

The basic repeating subunit of chromatin, the nucleosome, contains 8 histone proteins and about 200 base pairs of DNA (Kornberg, 1974). Nucleosomes serve the structural role of packing DNA into a chromatin fiber. Psoralen cross-linking reactions have been used to map the positions of nucleosomes of DNA (Cech et al., 1977). DNA purified from mouseliver nuclei, before deproteinization, was photoreacted with tritiated 4,5',8-trimethylpsoralen, and cross-links in this DNA preparation were found to be separated by intervals of 190 base pairs, or by

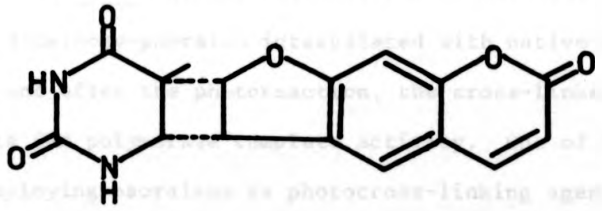
(1)



(2)



(3)



(4)

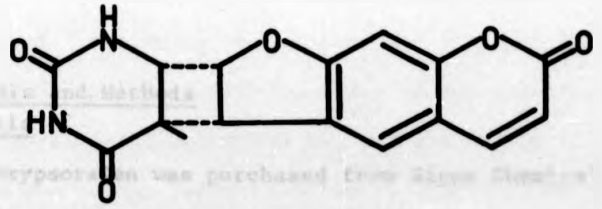


Figure 4.8: The four possible "four-centre" mechanisms for photocycloaddition between psoralen and thymine (Song et al., 1971).

integral multiples of that length. The cross-links in the deproteinized DNA from mouse liver nuclei showed no such regular pattern. These results seemed to reflect the periodicity of proteins in chromatin (Cech et al., 1977). Other similar studies (Chatterjee and Cantor, 1978) on the photocross-linking of Drosophila melanogaster nuclei (Wiesehahn et al., 1977), simian virus 40 genome (Shen and Hearst, 1977), DNA-RNA helices (Shen et al., 1977), and tRNA (Ou and Song, 1978) have been carried out recently. When ribosomal RNA from Drosophila melanogaster was photocross-linked with psoralen, and examined by electron microscopy, reproducible patterns of hairpins were found in both the 26s and 18s RNA. These results provided a good confirmation on the frequent occurrence of hairpin structure in RNA (Wollenzien et al., 1978). Ou et al. (1978) recently found that even at its ground state, 8-methoxy-psoralen intercalated with native double-stranded DNA, and after the photoreaction, the cross-linked DNA lost 97% of its DNA polymerase template activity. One of the problems in employing psoralens as photocross-linking agents is its insolubility in aqueous solution, thus Isaacs et al. (1977) attempted to synthesize new psoralen derivatives, and 4'-amino-methyl-4,5',8-trimethylpsoralen was found to be the most soluble one.

4.2 Materials and Methods

4.2.1 Materials

(a) 8-Methoxypsoralen was purchased from Sigma Chemical Company, St. Louis, U.S.A. Diethanolamine was purchased from Hopkin and Williams, Romford, U.K.

(b) Tritiated water (5 ci/ml) and [^{14}C]formaldehyde (1-3% aqueous solution, 1-5 mCi/mmole) were purchased from Amersham, U.K.

(c) Hydroxylapatite (Bio-Gel HTP) was purchased from Bio-rad Lab., California, U.S.A. Spectropore dialysis tubing (mol. wt. cut-off 10,000 to 12,000) was purchased from Spectrum Medical Ind. Inc., Los Angeles, U.S.A..

(d) Poly(I) and poly(C) were purchased from Miles Biochemicals, Slough, U.K. and poly(I-C) was synthesized as described previously in Chapter 3.

(e) Human cell lines used were: human foreskin fibroblasts (HFF) and osteosarcoma cell line, MG63.

4.2.2 General Methods

(a) The scintillant used for counting of radioactive samples was: toluene (600 ml), 2-ethoxyethanol (400 ml), PPO (4 g), POPOP (0.2 g).

(b) Reannealing of polynucleotides were achieved by dissolving the polynucleotides in PPN buffer (0.01 M potassium phosphate pH 6.8, 0.1 M NaCl) and heating at 70° for 10 minutes. The solution was cooled very slowly to room temperature and then left at 4° overnight.

(c) Photolysis experiments were performed with a 100 W high pressure mercury compact arc lamp (Hanovia Lamps Ltd., Slough, Bucks., U.K.). The light beam was focussed by means of a quartz lens (8 cm local length), on to a 1 cm quartz U.V. cuvette containing the solution to be photolysed. Filtering of the lamp was provided by a 10% $CuSO_4$ solution (Ou et al., 1978) in order to cut out the high energy wavelengths (wavelengths below 300 nm was cut out by this solution together with the perspex cuvette holder). The cuvette was kept at 2° when necessary by passing a stream of ice-water from a peristaltic pump over it.

4.2.3 Tritiation of 8-methoxypsoralen ([^{3}H]8MOP)

The basis of the method used was that due to Isaacs et al. (1977).

8-methoxypsoralen (43.2 mg, 0.2 mmole) was dissolved in dioxan (10 ml). Diluted tritiated water (160 µl, 0.425 Ci) mixed with conc. sulphuric acid (0.3 ml) was then added and the reaction mixture refluxed at 110° for 4 hours. After this time the solution was cooled to room temperature, water (10 ml) was added, and the mixture was extracted with ether (2 x 20 ml). The ethereal layers were collected, dried over anhydrous potassium carbonate, filtered and evaporated to dryness under reduced pressure. The [^{3}H]8MOP was dissolved in a small amount of acetone and applied on a preparative silica t.l.c. plate, and chromatographed with chloroform: ethanol (98:2, v/v) as solvent. The region containing [^{3}H]8MOP was cut out, extracted with ether, filtered and evaporated to dryness under reduced pressure. The yield of [^{3}H]8MOP was 35.2 mg (determined spectrophotometrically, 81%; 3.82 ± 0.14 mCi/mmole, 0.14% incorporation of radioactivity). The U.V. data (ethanol): λ_{max}: 301 nm (Σ 1.01 x 10^4) (Figure 4.9), lit.: $\lambda_{max}^{H_2O}$: 301 nm (Σ 1.24 x 10^4) (Isaacs et al., 1977).

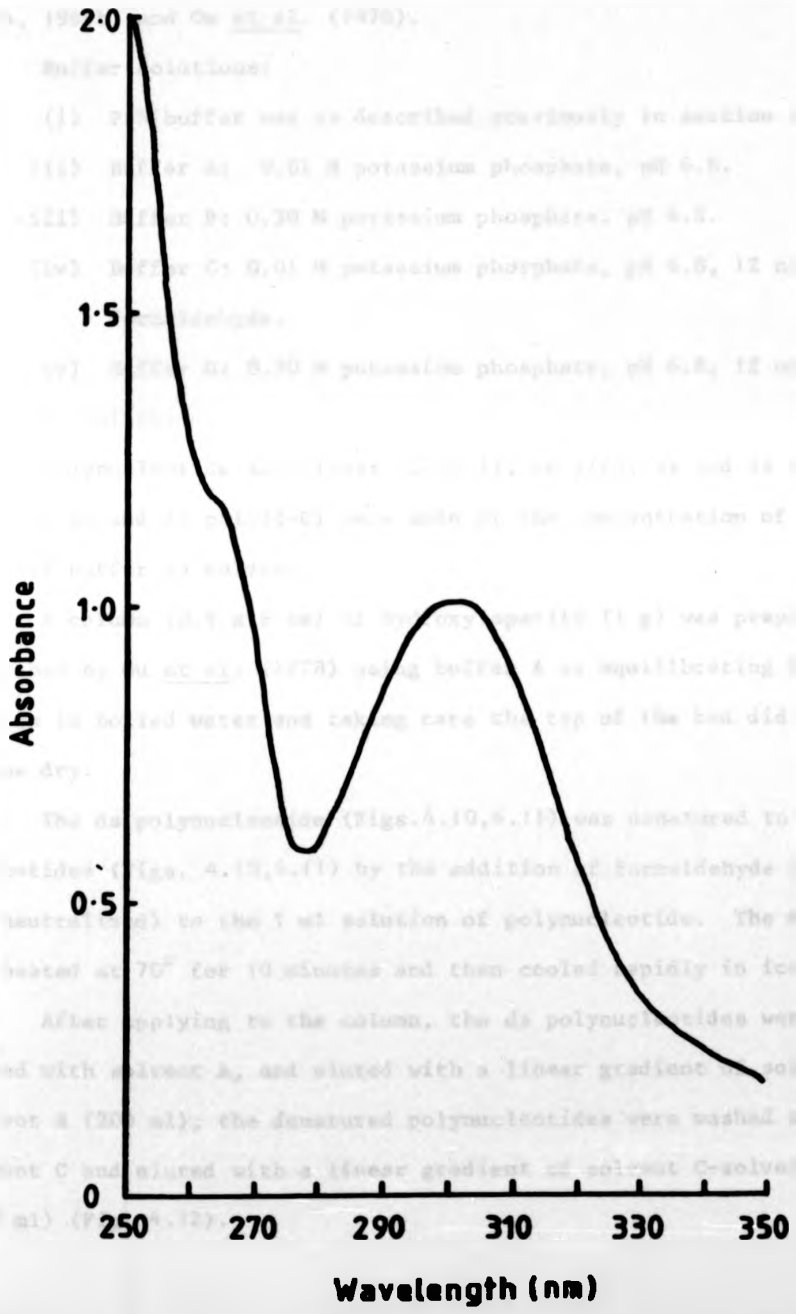
4.2.4 Preparation of [^{14}C]-methyl-N-bis(2-chloroethyl)amine hydrochloride ([^{14}C]-HN_2)

The preparation was based on that described previously by Eaton (1974).

The white solid [^{14}C]-HN_2 (0.25 g, 27%) had a specific activity of 18.87 ± 0.66 µCi/mmole, 59% incorporation of radioactivity); m.p. 110°, lit.: 110° (Kohn et al., 1966).

2.0
1.5
1.0
0.5
0
250
270
290
310
330
350
Wavelength (nm)

Figure 4.9: Ultra-violet spectrum of [^{3}H]-8-methoxypsoralen in ethanol.


2·0
1·5
1·0
0·5
0
Absorbance
250
270
290
310
330
350
Wavelength (nm)

4.2.5 Chromatography of ds and ss poly(I).poly(C) and poly(I-C) on Hydroxylapatite

The method was based on that described by Bernardi (1965, 1969a, 1969b) and Ou et al. (1978).

(A) Buffer solutions:

(i) PPN buffer was as described previously in section 4.2.2b.

(ii) Buffer A: 0.01 M potassium phosphate, pH 6.8.

(iii) Buffer B: 0.30 M potassium phosphate, pH 6.8.

(iv) Buffer C: 0.01 M potassium phosphate, pH 6.8, 1% neutralized formaldehyde.

(v) Buffer D: 0.30 M potassium phosphate, pH 6.8, 1% neutralized formaldehyde.

(B) Polynucleotide solutions: (poly(I), poly(C), ss and ds poly(I).poly(C), ss and ds poly(I-C) were made at the concentration of 1 mg/ml with PPN buffer as solvent.

(C) A column (0.9 x 6 cm) of hydroxylapatite (1 g) was prepared as described by Ou et al. (1978) using buffer A as equilibrating buffer made up in boiled water and taking care the top of the bed did not become dry.

(D) The ds polynucleotide (Figs.4.10,4.11) was denatured to the ss polynucleotides (Figs. 4.10,4.11) by the addition of formaldehyde (38%, 26µl not neutralized) to the 1 ml solution of polynucleotide. The mixture was heated at 70° for 10 minutes and then cooled rapidly in ice-water.

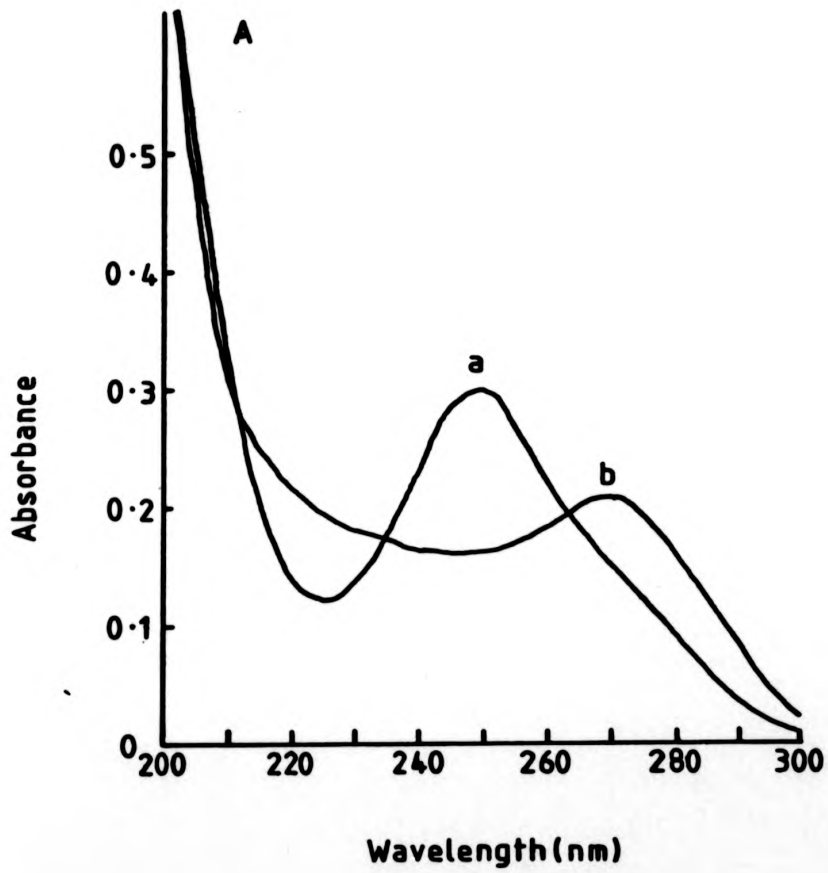
(E) After applying to the column, the ds polynucleotides were washed with solvent A, and eluted with a linear gradient of solvent A-solvent B (200 ml); the denatured polynucleotides were washed with solvent C and eluted with a linear gradient of solvent C-solvent D (200 ml) (Fig. 4.12).

Figure 4.10: (A) Ultra-violet spectra of (a) poly(I) and (b) poly(C) in 0.01 M sodium phosphate, 0.1 M sodium chloride, pH 6.8.

(B) Ultra-violet spectra of (a) single-stranded poly(I).poly(C) and (b) double-stranded poly(I).poly(C), in 0.01 M sodium phosphate, 0.1 sodium chloride, pH 6.8.

(b)
ium

ded
I).poly(C),
de,



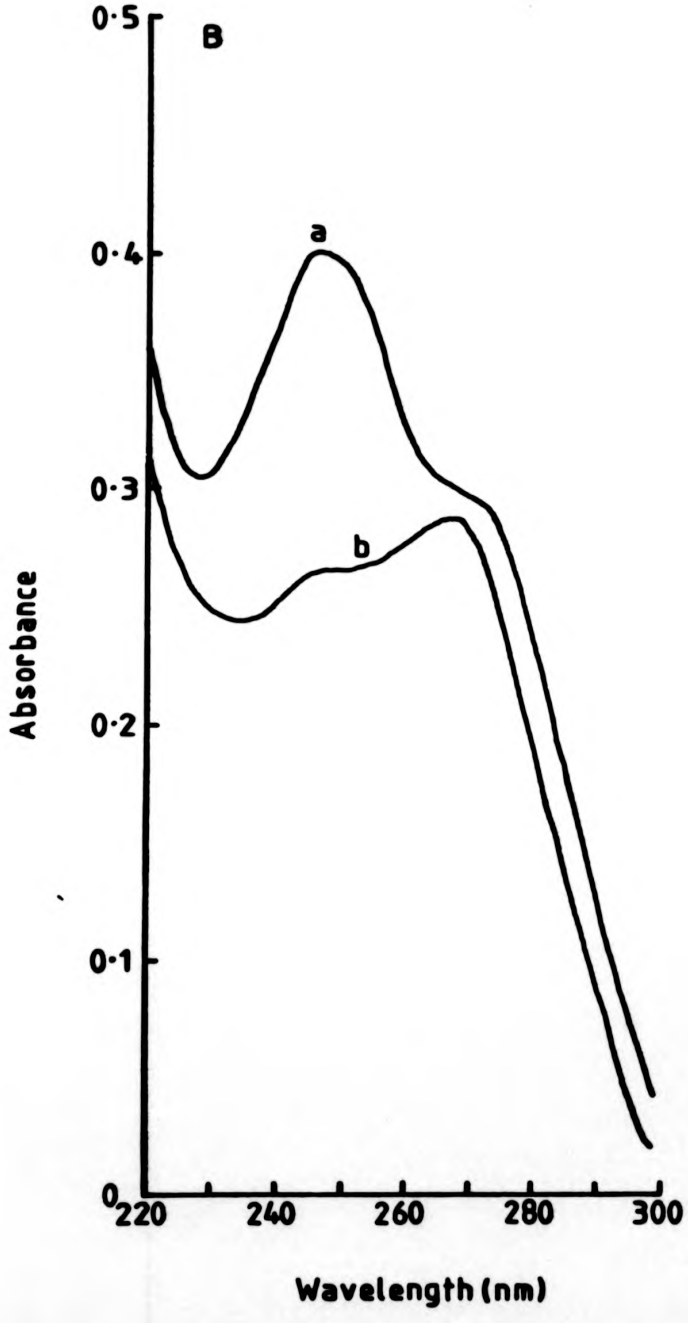

B
0·5
0·4
0·3
0·2
0·1
0
a
b
Absorbance
220
240
260
280
300
Wavelength (nm)

Figure 4.11: Ultra-violet spectra of (a) single-stranded poly(I-C), in 0.01 M potassium phosphate, 0.1 M sodium chloride, pH 6.8, 1% formaldehyde, and (b) double-stranded poly(I-C) in 0.01 M potassium phosphate, 0.1 M sodium chloride, pH 6.8.

oly(I-C),
hloride,
ded
M sodium

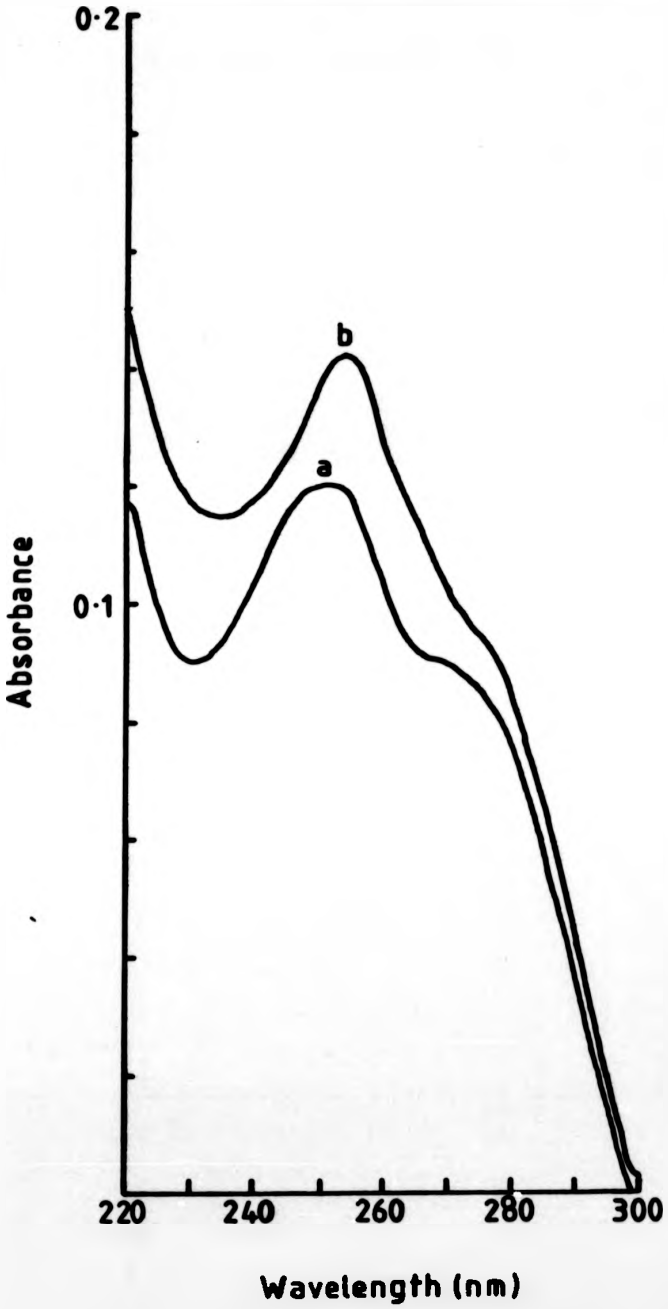
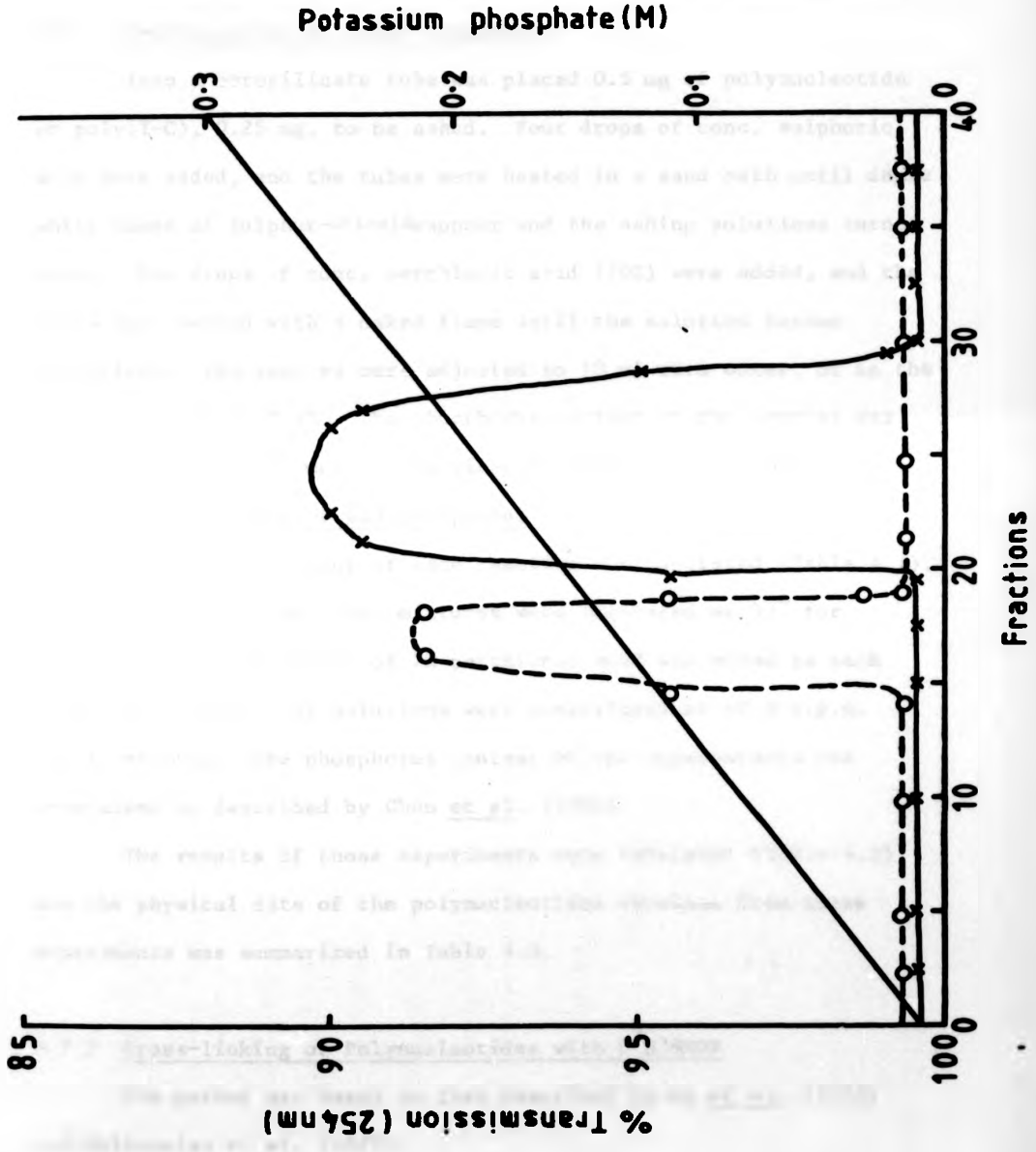


Figure 4.12: Elution profile of the separation of single-stranded and double-stranded poly(I-C) by hydroxylapatite chromatography at room temperature.

ss poly(I-C) (o---o)

ds poly(I-C) (x——x)


Potassium phosphate (M)
0·3
0·2
0·1
0
40
30
20
10
0
Fractions
% Transmission (254nm)
85
90
95
100

4.2.6 Chain Length Determination of poly(I).poly(C) and poly(I-C) by the Measurement of End to Total Phosphorus

The method used was based on that described by Seaman (1968).

(A) Determination of total phosphorus

Into a borosilicate tube was placed 0.5 mg of polynucleotide or poly(I-C), 0.25 mg, to be ashed. Four drops of conc. sulphuric acid were added, and the tubes were heated in a sand bath until dense white fumes of sulphur-trioxide appear and the ashing solutions turned brown. Two drops of conc. perchloric acid (70%) were added, and the tubes were heated with a naked flame until the solution became colourless. The samples were adjusted to 10 ml with water, or in the case of poly(I-C) 5 ml. The phosphorus content of the samples was determined using the method described by Chen et al. (1956).

(B) Determination of end phosphorus

The material input of each reaction was tabulated (Table 4.1).

The enzymic reaction mixtures were incubated at 37° for 4 hours. An equal volume of 2% perchloric acid was added to each reaction mixture. The solutions were centrifuged at 10 K r.p.m. for 15 minutes. The phosphorus content of the supernatants was determined as described by Chen et al. (1956).

The results of those experiments were tabulated (Table 4.2) and the physical data of the polynucleotides obtained from these experiments was summarized in Table 4.3.

4.2.7 Cross-linking of Polynucleotides with [^{3}H]8MOP

The method was based on that described by Ou et al. (1978) and Wollenzien et al. (1978).

The ds polynucleotide (250 µg in 0.25 ml PPN buffer) was mixed with a solution of [^{3}H]8MOP (114 µg, 4.47 x 10^6 d.p.m., in 3 ml of PPN buffer). The reaction mixture was photolysed at 2°

TABLE 4.1

The material-input for the enzymic end phosphorus determination reaction

Sample	Sample input	Tris-HCl[(a)] buffer (ml)	Enzyme[(b)] (μg)	Enzyme-solution (ml)	H_2O (ml)	Total vol. (ml)
poly(I)	0.5 mg/0.5 ml	0.10	50	0.010	0.390	1.0
poly(C)	0.5 mg/0.5 ml	0.10	50	0.010	0.390	1.0
poly(I-C)	0.25 mg/0.25 ml	0.05	25	0.005	0.195	0.5
CMP	0.5 mg/0.5 ml	0.10	50	0.010	0.390	1.0

(a) 1 M Tris-HCl Buffer, pH 8.3

(b) Alkaline phosphatase (750 μg/0.15 ml, 400 units)

TABLE 4.2: Results of the end and total phosphorus determination experiments

	Sample	Sample in-put (mg)	Phosphorus content in samples (µg)
End phosphorus determination	poly(C)	0.50	0.12
	poly(I)	0.50	0.24
	poly(I-C)	0.25	0.24
	CMP	0.50	36.40
Total phosphorus determination	poly(C)	0.50	34.00
	poly(I)	0.50	31.00
	poly(I-C)	0.25	22.00
	CMP	0.50	38.00

TABLE 4.3: Physical data of the polynucleotides obtained from the end and total phosphorus experiments

Sample	Ratio of end to total phosphorus	Molecular weight	Σp [a]
poly(I) [b]	258	9.0×10^4	1.50×10^4
poly(C) [b]	560	1.8×10^5	9.57×10^3
poly(I-C) [c]	275	9.2×10^4	6.11×10^3
CMP	1	-	-

[a] Σp data was calculated with data from Table 4.3 and data from Figs. 4.2 & 4.3. Σp of poly(I) was determined at 250 nm, poly(C) was at 270 nm, and ss poly(I-C) at 250 nm.

[b] Σp of ss poly(I).poly(C) determined at 260 nm is 1.55×10^4, and that of ds poly(I).poly(C) at 260 nm is 1.29×10^4.

[c] Σp of ds poly(I-C) determined at 253 nm is 5.00×10^3, lit.: Σp at 253 nm, of ds poly(I-C) is 4.90×10^3 (Karstadt and Krakow, 1970).

for 3 hours, with constant stirring, as described previously in section 4.2.2.C. This photolysed solution was then mixed with ethanol (2-3 volumes), and left at -20° for 12 hours. The ethanolic solution was centrifuged at 7 K r.p.m. for 20 minutes. The supernatant was discarded. The precipitate was redissolved in PPN buffer (1 ml), and dialysed at 4° against one change of PPN buffer (1 l) and two changes of water (2 x 1 l) for 24 hours each. The dialysate was lyophilized. The resulting polynucleotide was redissolved in buffer A (1 ml), and aliquots (100 µl) of this solution was counted for the incorporation of radioactivity. The results were summarized in Table 4.4. It can be seen that only sample 3 shows any incorporation of [^{3}H]8MOP after the photolysis experiment, therefore the purification of sample 3 with hydroxylapatite was carried out.

The denaturation procedure as described previously in section 4.2.5.D was carried out on sample 3, and the sample then applied to a hydroxylapatite column, washed with buffer C, and eluted with a linear gradient of buffer C-buffer D (200 ml). The ds poly(I-C) was eluted between 0.15-0.20 M potassium phosphate. The fractions were collected, and dialysed at 4° against one change of solvent A (1 l) followed by two changes of water (2 x 1 l) for 24 hours each. The dialysate was lyophilized. The ds poly(I-C) was redissolved in water (1 ml) and aliquots (100 µl) were taken for counting of radioactivity incorporation. The amount of ds poly(I-C) recovered was 180 µg (determined spectrophotometrically, 0.98 nmole) and the amount of [^{3}H]8MOP incorporated into this polynucleotide was 1.6 ± 0.1 µg (6.22 ± 0.30 x 10^4 d.p.m., 7.4 nmole, 36 base-pairs of poly(I-C) per molecule of [^{3}H]8MOP).

TABLE 4.4

The results of the counting of radioactivity incorporated into the ds polynucleotides after photolysed in the presence of [^{3}H]8MOP

Sample	Polynucleotide ds	Quantity input (µg)	[^{3}H]8MOP (µg)	Photolysis	[^{3}H]8MOP incorporated in d.p.m.
1	poly(I).poly(C)	250	114	+	0
2	poly(I).poly(C)	250	114	-	0
3	poly(I-C)	250	114	+	$1.79 \pm 0.09 \times 10^5$
4	poly(I-C)	250	114	-	0

4.2.8 Alkylation of ds Polynucleotides with [^{14}C]HN_2

The alkylation method was based on that described previously by Eaton (1974).

The ss polynucleotides (250 µg) was dissolved in PPN buffer (0.5 ml) and reannealed as described previously. A solution of [^{14}C]HN_2 in solvent A (100 µg, 0.5 µmole, 2.1 x 10^4 d.p.m.) was added to the ds polynucleotide solution, and the reaction mixture was incubated at 37° for 4 hours (Table4.5). After cooling to room temperature, the reaction mixture was dialysed at 4° as described previously in section 4.2.7. The dialysate was lyophilized and redissolved in water (0.5 ml) and aliquots (100 µl) were taken for counting to determine radioactivity incorporation. No radioactivity was found to have been incorporated into the ds polynucleotides, therefore the experiments were repeated with different reaction conditions. Various amounts of [^{14}C]HN_2 (0.5 to 5.0 mg) were added to the ds polynucleotide solution (250 µg, 0.5 ml). The buffer solution used in these experiments was 0.01 M Tris-HCl, pH 8.0. The reaction mixtures were incubated at 37° for 6 hours. After cooling to room temperature, the reaction mixtures were dialysed at 4° against one change of 0.01 M Tris-HCl, pH 8.0 (1 l) and two changes of water (2 x 1 l) for 24 hours each. The dialysates were lyophilized and redissolved in water (1 ml) and aliquots (100 µl) were taken for counting to determine radioactivity incorporation. No radioactivity was found to have incorporated into the ds polynucleotides. After the denaturation procedure as described in section 4.2.5.D was carried out on these ds polynucleotides, the U.V. absorption spectra showed that they had been denatured to the ss species. Therefore, no alkylation or cross-linking was observed.

TABLE 4.5: The alkylation of ds polynucleotides with $[^{14}C]HN_2$

Sample	Polynucleotides ds	Quantity input (µg)	$[^{14}C]HN_2$ (µg)	Incubation at 37° for 4 hours
1	poly(I).poly(C)	250	100	+
2	poly(I).poly(C)	250	100	-
3	poly(I-C)	250	100	+
4	poly(I-C)	250	100	-

4.2.9 Preparation of 8MOP Treated poly(I).poly(C) and poly(I-C) for Interferon Induction

Preparation of buffers, ds polynucleotide solutions and experimental procedures were the same as that described in section 4.2.7, except that non-radiolabelled 8MOP was used, and the samples were not chromatographed on hydroxylapatite. Samples 3 from section 4.2.7 and samples for control experiments were included (Table 4.6).

4.2.10 Interferon inductions

These were performed in HFF cells and MG63 cells, and were based on the method described by Atherton and Burke (1975). Two different interferon inductions were carried out.

(A) Induction of interferon by poly(I-C), and studies on the effects of heat treatment (37^{o} for 1 hour) prior to the induction, and the presence of DEAE-dextran, on the interferon induction capability of this polynucleotide (Table 4.7).

(B) Induction of interferon by polynucleotide samples described in section 4.2.9 (Table 4.6) and results were tabulated (Table 4.8).

4.2.11 Interferon Assays

These were performed in HFF cells and MG63 cells and were based on the method of inhibition of nucleic acid synthesis as described previously by Atkins et al. (1971). The titres are given in research reference units. Interferon inductions and assays were performed by Dr. A. Meager and Mrs. J. Flint, Department of Biological Sciences, University of Warwick.

TABLE 4.6: Samples of 8MOP treated ds polynucleotides for interferon induction

Sample	ds polynucleotide	8MOP	Photolysis	Yield (mg)	8MOP incorporation
1[a]	poly(I).poly(C)	-	-	0.50	-
2[a]	poly(I).poly(C)	-	+	0.50	-
3[a]	poly(I).poly(C)	+	+	0.50	-
4[a]	poly(I-C)	-	-	0.30	-
5[a]	poly(I-C)	-	+	0.20	-
6[a]	poly(I-C)	+	+	0.30	+
7[b]	poly(I-C)	+	+	0.18	+

(a) 8MOP used in these preparations was non-radiolabelled, and no chromatography on hydroxylapatite was carried out.

(b) This was the sample 3 from section 4.2.7 preparation where [^{3}H]8MOP was used and chromatography on hydroxylapatite to separate the cross-linked poly(I-C) and the ss species was carried out.

TABLE 4.7

Interferon titres of MG63 and HFF cells after induction by poly(I).poly(C) or poly(I-C) at different conditions, as described in section 4.2.10.A

Inducer[(a)]	Cell Types	Heating at 37° for 1 hour prior to induction	DEAE-dextran	Interferon titre (log_{10}U/10^6cells)
poly(I).poly(C)	MG63	-	-	3.20
poly(I).poly(C)	MG63	-	+	3.95
↑	↑	+	+	>2.50
↑	MG63	+	-	1.20
↑	↓	-	+	>3.00
poly(I-C)	↑	-	-	<0.50
↓	↑	+	+	2.50
↓	HFF	+	-	<0.50
↓	↓	-	+	2.65
↓	↓	-	-	<0.50

[(a)] Concentrations of all polynucleotides used in the induction were 50 µg/ml

TABLE 4.8

Interferon titres of MG63 cells after induction by poly(I).poly(C) or poly(I-C) at different conditions as described in section 4.2.10.B (Table 4.6

Sample	Inducer	Concentration (μg/ml)	8MOP	Photolysis	Interferon titre ($log_{10}U/10^6$ cells)
1	poly(I).poly(C)	50	-	-	3.95
2			-	+	3.95
3			+	+	4.20
4	poly(I-C)		-	-	3.30
5			-	+	3.60
6			+	+	2.15
7			+	+	1.95

4.3 Results and Discussion

4.3.1 Chromatography of ds and ss poly(I).poly(C) and poly(I-C) on Hydroxylapatite

Chromatography on hydroxylapatite (calcium phosphate) was introduced as a very useful tool in protein chemistry by Tiselius et al. (1956). Work began in 1959 by Bernardi (1965, 1971), as a development of previous investigations on the chromatography of phosphoprotein on hydroxylapatite columns, led to the recognition that hydroxylapatite at room temperature, could discriminate nucleic acids endowed with different secondary structures, rigid, ordered structures having higher affinity for hydroxylapatite than flexible, disordered ones. It was found (Bernardi, 1965) that heat-denatured DNA had a lower affinity for hydroxylapatite than native DNA, also results obtained by running salmon sperm DNA, cross-linked by mustard gas, and then denatured, indicated that a much larger amount of DNA was eluted at the molarity of native DNA in the cross-linked samples compared to the untreated samples (Bernardi, 1971). Using the stepwise elution technique, native DNA was found to be eluted by 0.20 M and 0.25 M potassium phosphate buffer, pH 6.8 (Bernardi, 1969a), while high-molecular-weight RNA from Ehrlich ascites tumour cells, tobacco mosaic virus, and turnip yellow mosaic virus were all eluted in two peaks at 0.15 M (ss RNA) and 0.20 M (ds RNA) phosphate. When using gradient elution, soluble RNA from E.coli on yeast was eluted at about 0.13 M phosphate, and the undialysable residue left after digestion of RNA by pancreatic RNase was eluted at 0.01 M phosphate, which also eluted DNA oligonucleotide. Synthetic polyribonucleotides like poly(U), a polymer devoid of any organized secondary structure under the condition used for chromatography, was eluted at 0.10 M phosphate, while, poly (A), which has some helical structure at room temperature, was eluted in a broad peak at 0.20 M phosphate.

The ds poly(A).poly(U) complex was eluted at a molarity only slightly higher than poly(A), and the ts 2poly(U).poly(A) complex was eluted at the much higher molarity of 0.45 M to 0.50 M phosphate (Bernardi, 1965). The ds poly(I).poly(C) complex was eluted at a molarity close to 0.20 M phosphate. In the presence of formaldehyde, and after heat-denaturation, all ss polyribonucleotides were eluted a molarity below 0.20 M (poly(U) at 0.05 M, poly(C) at 0.12 M, poly(I) at 0.16 M, poly(A) at 0.17 M) phosphate, and the recoveries were complete in all cases. However, in the absence of formaldehyde, the recovery of poly(A) was only between 60 to 85% and poly(I) gave very irregular results and its behaviour was characterized by extremely low recovery.

It was suggested (Bernardi, 1965, Kawasaki, 1978) that the absorption of polynucleotides on hydroxylapatite took place because of the electrostatic interaction between the negative phosphate groups of the nucleic acids and the positive calcium ions of the hydroxylapatite crystals. Increasing the molarity of the eluting phosphate buffer progressively reduced this interaction to zero, at which point desorpton occurred. The decrease in the interaction appeared to be due to a specific competition between the phosphate ions of the eluting buffer and the phosphate groups of polynucleotides for the calcium ions of hydroxylapatite, and not simply to increase ionic strnegth. In fact, if elution was performed at constant ionic strength, with a linear gradient between 0.001 M potassium phosphate buffer pH 6.8 and 0.5 M potassium phosphate buffer pH 6.8, in the presence of 1 M potassium chloride, native DNA was desorbed essentially at the same phosphate molarity as in the absence of potassium chloride. This suggestion was supported by the finding that treatment of hydroxylapatite with dilute chelating agents, e.g. EDTA or citrate reduced the absorption capacity of hydroxylapatite for polynucleotide. Furthermore, an

investigation on nucleoside mono-, di-, tri-, tetra-, and polyphosphates and phosphorylated coenzyme derivatives showed that their absorption on hydroxylapatite was only related to the ionization of the phosphate groups, with no interference by the organic moieties of the molecules. Since the charge density of denatured DNA and its electrophoretic mobility were lower than those of native DNA, this suggestion of the mechanisms of hydroxylapatite chromatography of nucleoside phosphates were certainly valid (Bernardi, 1965).

The results of the experiments mentioned in section 4.2.5 indicated that with chromatography using hydroxylapatite the ds poly(I-C) eluted between 0.15 M to 0.22 M phosphate. In the presence of 1% formaldehyde, and after heat-denaturation, the ss poly(I-C) was eluted between 0.12 M to 0.14 M phosphate (Fig. 4.12).The recoveries of the ds and ss poly(I-C) were between 70 to 75%. However, the results obtained from ds and ss poly(I).poly(C) were irregular. They both gave essentially two broad peaks with molarity of 0.09 M to 0.12 M and 0.14 M to 0.20 M phosphate. Therefore, hydroxylapatite chromatography can certainly be used to separate ds and ss poly(I-C), but in the case of ds and ss poly(I).poly(C), the results were not conclusive enough for the chromatography to be meaningful.

4.3.2 Cross-linking of Polynucleotides

When the photoreactive [^{3}H]8MOP was photoreacted with ds poly(I-C) as mentioned in section 4.2.7, radioactivity was found to have been incorporated into the ds polynucleotide. The non-cross-linked poly(I-C), if it were present at all, was separated from the cross-linked poly(I-C) in the presence of formaldehyde under denaturing conditions, by hydroxylapatite chromatography. The recovery of the cross-linked poly(I-C) was around 70%. The results from the

recounting of radioactivity incorporated into this ds polynucleotide coupled with the results from the total and end phosphate determination of the polynucleotide and its Σp values showed that every 36 base-pairs of poly(I-C) were cross-linked by one molecule of [^{3}H]8MOP. It can be concluded that the ds poly(I-C) can successfully be cross-linked covalently by the photoreactive 8MOP. This is because, as the results indicated in the previous section, if the poly(I-C) were not cross-linked covalently, after the denaturation procedure as described previously in section 4.2.5.D was carried out on the ds poly (I-C) and under the denaturing conditions of the hydroxylapatite chromatography, the poly(I-C) should have been eluted between 0.12 M to 0.14 M phosphate as a ss species. The U.V. absorption spectra of this cross-linked polynucleotide, before and after hydroxylapatite chromatography, also resemble that of the ds poly(I-C), with its peak at 253 nm. However, the same results did not apply in the case of ds poly(I).poly(C), because no radioactivity was found to have been incorporated into the ds polynucleotide after treatment with [^{3}H]8MOP. When this [^{3}H]8MOP treated ds polynucleotide was subjected to the denaturation procedure mentioned in section 4.2.5.D, the polynucleotide became single stranded, indicated by its U.V. absorption spectrum. These results are consistent with the findings by Mantulin and Song (1973) and Cole (1972) that psoralen is pyrimidine specific. When [^{14}C]HN_2 was used to react with ds poly(I-C) and ds poly(I).poly(C), no radioactivity was found to have been incorporated into the ds polynucleotide. Experiments were repeated again without incorporation of radio-activity, no further studies were carried out to find out the reason for its failure.

When the T_m determination experiment was carried out on the cross-linked poly(I-C), it was found that there was no sharp melting from the double-stranded to the random coil poly(I-C) occurring

any more. The O.D. increase was a very gradual one, which started from 35° to 60° (Fig.4.13). This might be due to the fact that the poly(I-C) was cross-linked covalently, and the strands could no longer separate. However, strands on the two ends of the cross-linked species still could come apart from each other, and cause the slight hyperchromacity. It could also suggest that the secondary structure of the poly(I-C) had been altered.

It is well established that circular dichroism (CD) characteristics of polynucleotides are useful for obtaining insight into their conformational properties (Bobst et al., 1976). However, it is difficult to draw any direct conclusions on polynucleotide geometry from CD data alone. Therefore, an attempt was made to assign qualitatively the conformational difference between the cross-linked and the non-cross-linked poly(I-C). The CD spectra of ds poly(I).poly(C), ds poly(I-C) and cross-linked ds poly(I-C) treated with different amounts of 8MOP, were shown in Fig.4.14. The CD spectrum of ds poly(I). poly(C) showed two positive Cotton effects with their peaks at 243 nm, 276 nm and a shoulder at 222 nm, and a small negative Cotton effect peaked at 260 nm. While, the CD spectrum of ds poly(I-C) showed three positive Cotton effects with the peak at 243 nm remaining and the other two at 213 nm and 266 nm. The CD spectra of the cross-linked poly(I-C) were essentially the same as that of the non-cross-linked material, apart from a slight increase in the intensities of the maxima. Therefore, it can be concluded that no gross conformational changes can be detected by the CD spectra.

4.3.3 Studies on Cross-linked poly(I-C) as an Interferon Inducer

Investigation was carried out on the capacility of ds poly(I-C) under different conditions and in different cell types, as an interferon

Figure 4.13: Thermal transition of the cross-linked poly(I-C) by 8-methoxypsoralen recorded in 0.01 M sodium cacodylate, 0.1 M sodium chloride, pH 7.0, at 253 nm.

253 nm.

-C)

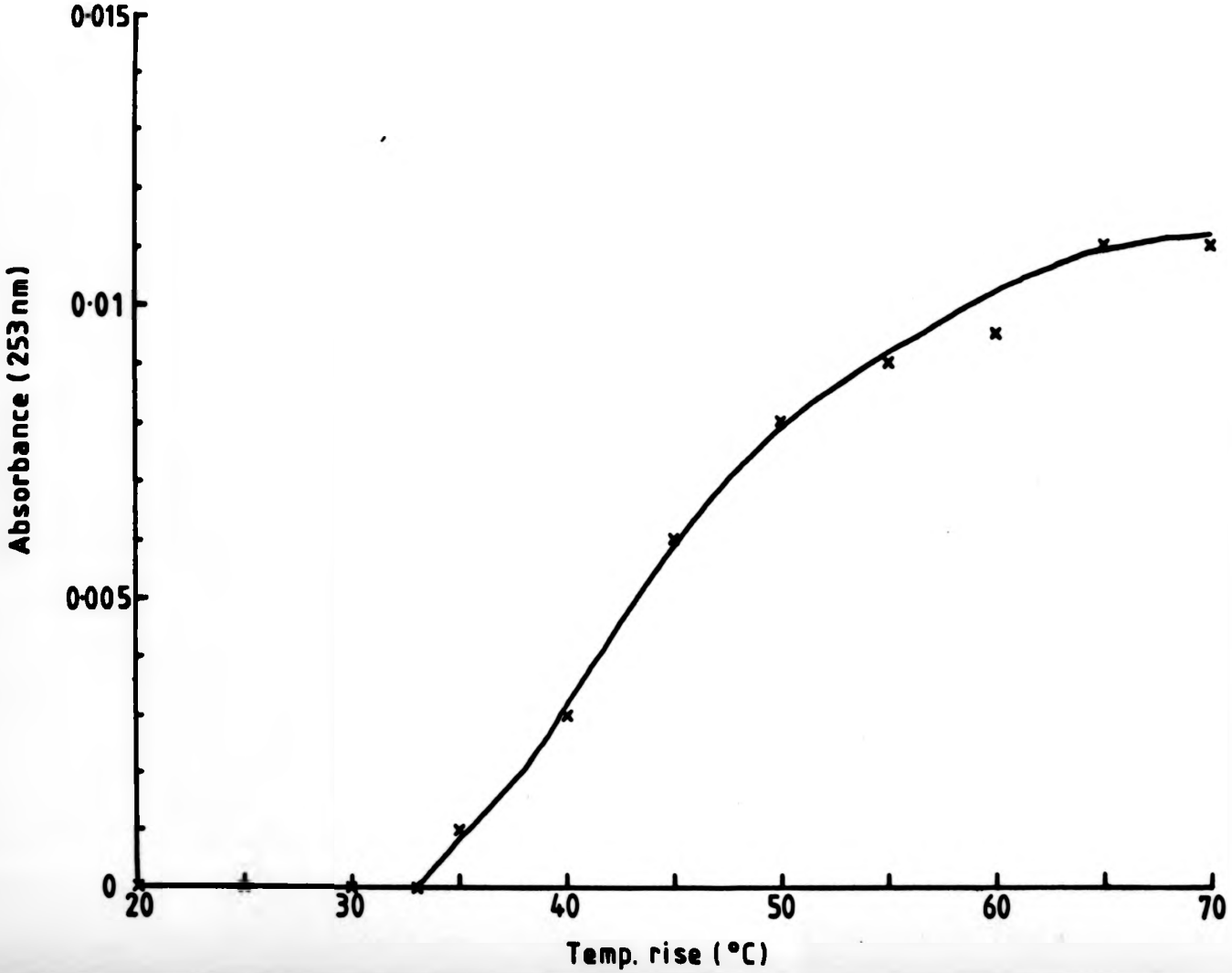
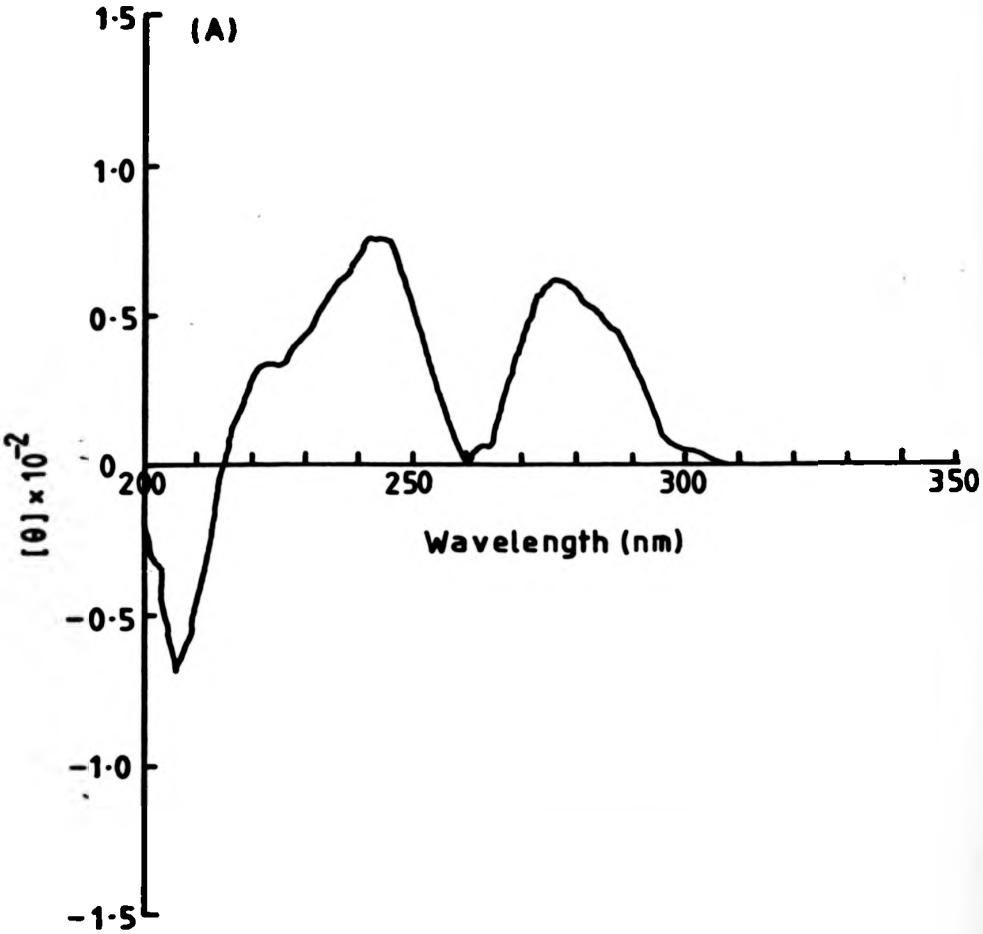
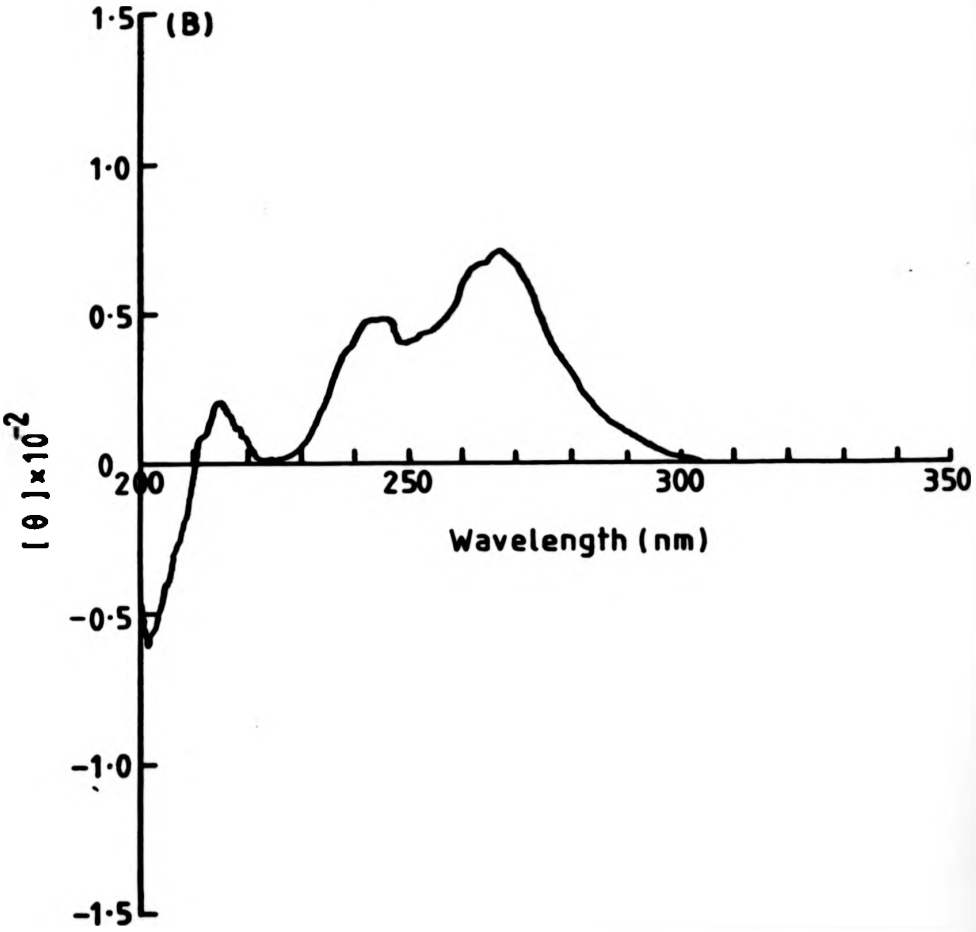
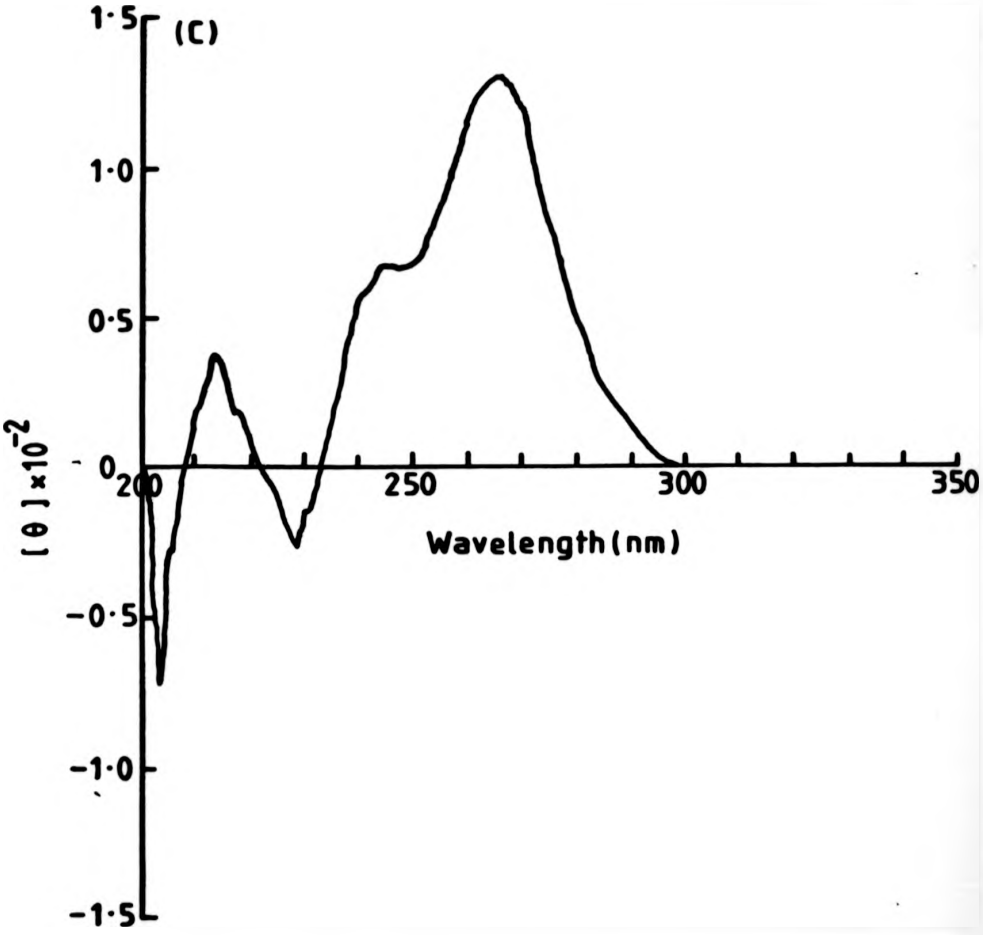
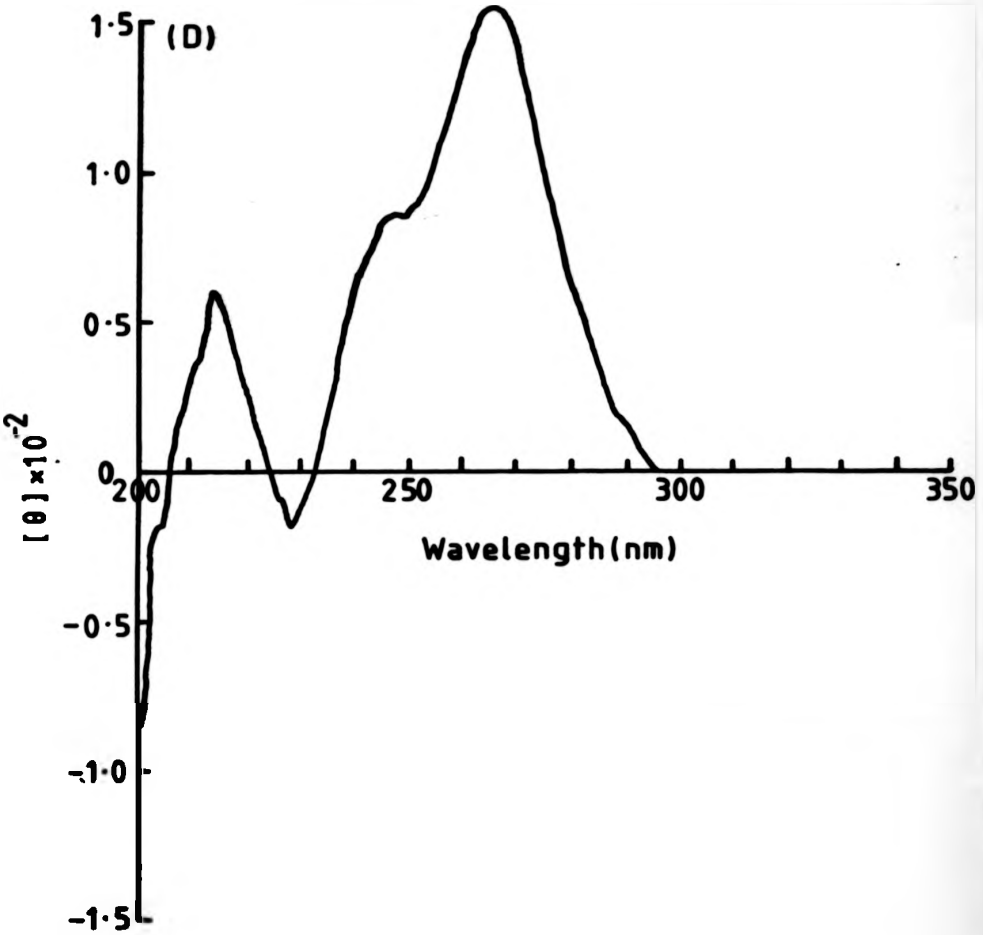


Figure 4.14: Circular dichroic spectra of (A) double-stranded poly(I).poly(C), (B) double-stranded poly(I-C), (C) cross-linked poly(I-C) by 8-methoxypsoralen (60 µg) and (D) cross-linked poly(I-C) by 8-methoxypsoralen (120 µg), in 0.01 M sodium cacodylate, 0.1 M sodium chloride, pH 7.0, at 20°.


(A)
1·5
1·0
0·5
0
−0·5
−1·0
−1·5
200
250
300
350
Wavelength (nm)
[θ] × 10⁻²


(B)
1·5
1·0
0·5
0
−0·5
−1·0
−1·5
200
250
300
350
Wavelength (nm)
[θ] × 10⁻²


(C)
1·5
1·0
0·5
0
−0·5
−1·0
−1·5
200
250
300
350
Wavelength (nm)
[θ] ×10⁻²


1·5
(D)
1·0
0·5
0
-0·5
-1·0
-1·5
[θ]×10⁻²
200
250
300
350
Wavelength(nm)

inducer (Table 4.7). The results indicated that the induction was successful in both cell types, MG63 and HFF cells. It was found that the heat-treatment at 37° for 1 hour prior to induction was not necessary, but unlike ds poly(I).poly(C), the presence of DEAE-dextran was crucial. In the absence of DEAE-dextran, ds poly(I-C) induced interferon in a very low titre of below 0.5, but in the presence of DEAE-dextran, the interferon titres was above 3.00, which was as high as that induced by ds poly(I).poly(C). Therefore, studies on the effectiveness of cross-linked ds poly(I-C) as an interferon inducer were carried out in MG63 cells and in the presence of DEAE-dextran. The results were shown in Table 4.8. Before treatment with 8MOP, the interferon titres induced by ds poly(I-C) were between 3.30 to 3.60, but after cross-linking, the interferon titres induced were around 2.00. There was a drop in activity of cross-linked ds poly(I-C) as an interferon inducer, but it could be observed clearly that the cross-linked species was still capable of inducing interferon. Therefore, it can be concluded that strand separation is not necessary for the ds polynucleotide during the induction of interferon. The drop of activity is probably due to some localized conformational changes which occur in the cross-linked ds polynucleotide due to the chemical modification of their bases. The CD spectra of the cross-linked ds polynucleotide may not be sensitive enough to show these localized conformational changes, while the biological system of interferon induction is sensitive enough. In the case of ds poly(I).poly(C), both the 8MOP treated and untreated species induced interferon to give around 4.00 interferon titres, but they, of course, were not cross-linked as shown in the previous section.

Meager et al. (1978) found that soluble calcium salts enhanced the interferon yield from poly(I).poly(C) induced human cells. The

maximal amounts of interferon were produced when $CaCl_2$ at 12 mM was present continuously until 20 hours after induction when the interferon was harvested. When cells were induced with poly(I).poly([5-^{203}Hg]C) more radioactivity was found to be associated with the cells when 12 mM $CaCl_2$ was present than when the normal medium level of 2 mM $CaCl_2$ was present. Therefore, the enhanced interferon yield might be due to the increase in uptake of poly(I).poly(C). Borden et al. (1978) found that the production of interferon by poly(I).poly(C) and poly(I).poly(C)-DEAE-dextran in L929 cells was enhanced from 10 to 100 times by polyene macrolides, including amphotericin B (AmB), AmB ester, nystatin, and filipin. AmB and its methyl ester were the most effective, but interferon induction by NDV was not enhanced by AmB. The kinetics of interferon production were not markedly altered by AmB. Polyene and poly(I).poly(C)-DEAE-dextran did not need to be present on cells simultaneously to enhance interferon production. Pretreatment with polymer was as effective as simultaneous addition, and even treatment of washed cells several hours after removal of poly(I).poly(C)-DEAE-dextran, enhanced interferon production. The production of interferon was delayed as though the actual induction had occurred at the time of the addition of the polyene rather than the poly(I).poly(C). This suggested that the polyene did not enhance interferon release but were potentiating the effects of previously cell-associated poly(I).poly(C), also, because AmB did not enhance interferon production induced by NDV, the polyenes must have affected an initial step in the interferon induction process not shared by the two inducers. It was observed that AmB did not form a macromolecular complex with poly(I).poly(C), because neither the U.V. absorption spectrum nor the m.p. of poly(I).poly(C) were altered by mixing with AmB. AmB, also, was

found not to enhance binding of poly(I).poly(C) to cells. It was known that membrane permeability alterations, induced by polyenes, could enhance cell penetration by anionic compounds and could potentiate intracellular action of metabolic inhibitors. Therefore, it was suggested (Borden et al., 1978) that the enhancement of interferon production by polyenes was due to the increase cell penetration of poly(I).poly(C). More recently, Bradshaw et al. (1979) found that when MG63 cells were treated with poly(I).poly([5-^{125}I]C) at 4° and then either washed with salt at 4° or treated with ribonuclease A and T_1 immediately after warming to 37°, interferon was produced, and this production was associated with the presence of radioactivity within the cells. It was also found that both a non-inducing poly(dI).poly(C) and colloidal gold were taken up at 4°, therefore, it was suggested (Bradshaw et al., 1979) that the polynucleotides entered the cells by a non-specific process, like micropinocytosis at 4°. These recent findings substantiated well with the results presented in this section, since they established the facts that strand separation of ds polynucleotides was not necessary for penetration of the cell membrane, and also whatever or wherever the binding-sites of polynucleotides for the induction of interferon are in the cells, strand separation is not necessary for the binding process and the induction of interferon.

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