THE SYNTHESIS OF POLYNUCLEOTIDES

FOR INTERFERON INDUCTION

Dedicated to my wife
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ABSTRACT

Synthetic double-stranded ribopolynucleotides are inducers of interferon, a protein which increases the resistance of cells to virus attack.

This work describes the synthesis of poly (halogenated ribocytidylic acids) and their complex formation with both riboinosinic acid and deoxyriboinosinic acid. The RNA/RNA hybrids are potent interferon inducers with high thermal and nucleolytic stability, the DNA/RNA hybrids are, however, completely inactive as inducers of interferon.

The synthesis and physical properties of poly (5-hydroxycytidylic acid) are discussed. In basic solution this polymer undergoes a conformational change and can also bind magnesium ions in an unusual manner. The polymer does not hybridise with polyinosinic acid.
The discovery in 1967 that the complex between polyriboinosinic acid and polyribocytidylic acid could increase the resistance of cells to viral infection\textsuperscript{1} led to a great upsurge in the synthesis of such complexes. This resistance was later found to be due to the formation inside the cells of an anti-viral protein called interferon. The discovery of a synthetic interferon inducer was of great potential importance as a possible clinical treatment for viral infections and also for studies on the mechanism of interferon induction.

Interferon was discovered prior to this event in 1957 by Isaacs and Lindenmann who isolated a substance, which interfered with virus replication, from chicken eggs treated with heat inactivated influenza virus.\textsuperscript{2} The anti-viral activity was non-dialysable, sensitive to proteolytic digestion, insensitive to nucleases and heat stable. The first work on the purification of interferon was carried out by Burke\textsuperscript{3,4} who accomplished a limited purification of chick interferon using conventional protein-purification techniques. Merigan et al later purified interferon 20,000 fold which contained $10^6$ units/mg of protein\textsuperscript{5}. After some time it was accepted that interferon is a typical glycoprotein\textsuperscript{6} with a molecular weight between 25,000 and 35,000, isoelectric at pH 6.5 - 7.0 but stable from pH 2 - 10. It also contains some carbohydrate including glucosamine.

After virus infection interferon is transcribed from the host gene and synthesised from messenger RNA which can be seen from the reduction of interferon production on inhibition of host nucleic acid synthesis.\textsuperscript{7,8} Interferon induced in one cell type is biologically
active only on cells of the same species however it is highly non-specific against a range of viruses. The situation is more complex as big variations occur in the effectiveness of a virus to induce interferon in different cell lines. Viruses also vary in their susceptibility to interferon.

The mechanism of interferon induction is still very obscure although it is known that interferon does not inactivate the virus or inhibit its adsorption, penetration or uncoating. It appears that interferon causes a stimulation of an anti-viral protein which is as yet unknown. This protein presumably inhibits the translation of viral messenger RNA.

Initially all the inducers studied were viral RNA's but another class of 'inducer' was soon discovered which included microrganisms and polyanions. These were found not to induce interferon themselves but to release interferon pre-existing within the cell. The production of interferon by viruses is primarily an induction event, however, there is evidence that the release of preformed interferon does occur in some cases, although recently the idea of preformed interferon has lost favour.

Field et al in 1967 investigated interferon induction by poly-nucleotide complexes in rabbit spleen cell suspensions. He found that poly A, poly U and poly (I). (cpc)_n were active but to a lesser extent than poly (I). poly (C). The biological activity of poly (I). poly (C) is remarkable as it is active in concentrations far less than 1µg/ml in many systems.

Unfortunately the complex shows considerable toxicity to many cells which has reduced its potential as a clinical agent. An
additional problem arises as the endotoxicity of synthetic inducers seems inseparable from their interferon inducing ability which can be seen from their therapeutic indices.  

Poly (I). poly (C) in the cell may perhaps biologically resemble the double stranded replicative form of RNA which is believed to be the interferon inducing species in RNA viruses and some DNA viruses. However, as in the case of natural RNA's the possibility of the release of preformed interferon cannot totally be eliminated.

Following the initial discovery that synthetic double-stranded polyribonucleotides were extremely good inducers many investigators tested all the polymers available at that time. They found that many double-stranded polynucleotides containing sequences of A, U, G, X, I, C were active and so it was reasonable to suppose that there was no requirement for a specific base sequence. However there seemed to be a requirement for a stable secondary structure since single-stranded poly (I), poly (C), poly (A) and poly (U) were active at concentrations 10,000 times greater than poly (I). poly (C). Alternative single-stranded polynucleotides such as poly (A-U) and poly (I-C), where there is the possibility of interstrand hydrogen bonding, are very active. This also seems to suggest that a double-stranded inducer is vital for interferon production.

De Clerq et al were the first to suggest that there might be a relationship between interferon inducing ability and the thermal and ribonuclease stability of the double-stranded polynucleotides. They took the two alternating copolymers poly (A-U) and poly (8A-8U) (where there is a thiophosphate link between the nucleotides, see Fig. 1) which have the same Tm and found that there was a considerable
FIG. 1

The Structure of Poly(A-LU)
increase in ribonuclease resistance on sulphur substitution, together with a remarkable enhancement of interferon inducing ability.\textsuperscript{22,23} This result led De Clerq and others to postulate that ribonuclease resistance was essential for interferon induction, a theory against which there was considerable evidence. It is now thought likely that substitution of sulphur for oxygen may mean that poly (A\textsuperscript{2},U) has a greater affinity for the "intracellular site" which triggers interferon induction.

Another technique which was used to facilitate the uptake of polynucleotides by cells was to complex the polymer with DEAE Dextran.\textsuperscript{24,25,26} This technique rendered many assays considerably more sensitive. The idea of complexing polynucleotides with non-nucleic acid material became the basis of considerable work by Pitha, which will be discussed later.

An early parameter which was extensively investigated was the effect of polynucleotide chain length on interferon induction. Wacker showed that a minimum chain length of 12 - 15 nucleotides was necessary in either strand whilst a chain of 30 nucleotides or more in either strand gave a high induction of interferon.\textsuperscript{27} Tytell later produced polynucleotides by thermal denaturation and determined their \( S_{20,w} \) values.\textsuperscript{28} These results showed that reducing the chain length of the poly I strand in poly (I). poly (C) rapidly reduced its biological activity whilst reducing the molecular weight of the poly C strand as low as 23,000 gave no appreciable loss of activity. These findings were in conflict with those of Niblack who found that there was identical loss of activity on reducing the length of either the poly (I)
or the poly (C) strand. He found that above a molecular weight of 10,000 there was little increase in activity but below this the activity was markedly dependent on chain length in either strand. Another disappointing finding by Niblack was that the acute toxicity of poly (I). poly (C) seems to fall off with decrease in homopolymer molecular weights about as rapidly as the anti-viral activity falls off. This discovery that there is a minimum chain length required for interferon induction poses the problem as to how a small protein receptor can differentiate between short and long chains.

More recently Carter et al have studied the structural requirements of poly (I). poly (C) for interferon induction. They investigated the effect of mis-matching the bases (which may or may not cause looping out from the helix) and strand interruption. The results show that the interferon inducing activity of poly (I). poly (C) is lowered by these modifications to varying extents. The decrease is much more significant when the perturbation is in the poly (I) strand. The Tm's of the modified complexes are only slightly less than that of poly (I). poly (C) itself. Ternary complex formation with poly-L-Lysine increases the Tm's of the complexes and also their nuclease resistance, however despite this, they were still less active. Carter concluded that two of the requirements of poly (I). poly (C) to induce interferon in cell cultures i.e. high Tm and resistance to nuclease may not be valid.

Some DNA viruses induce interferon when they synthesise an RNA intermediate in the cell and it was a logical progression to test whether DNA's themselves will induce. At that time, only DNA from natural sources was available e.g. calf thymus DNA which was found to
be inactive. Later synthetic DNA's and DNA/RNA complexes were shown to be active only at very high concentrations, however many of these hybrids had low Tm's and may not have existed as double-stranded complexes at the cell surface. It can be postulated that the specificity of a protein receptor for a 2'-hydroxyl on the sugar can be overcome by a high concentration of deoxynucleotides. It is part of the present work to reinvestigate some of the low melting deoxy complexes and to redetermine their interferon inducing properties.

The theory that a double-stranded nucleic acid is essential for induction has been reinvestigated by Pitha and Pitha who showed that a complex of poly (I) with poly (I-vinyl cytosine) is highly active. It was postulated that the vinyl analogue boosts the activity of poly (I) in the same way as induction by poly (C) is boosted on treatment with DEAE dextran. Pitha concluded that DEAE dextran and poly (vinyl C) both act by aggregating the polynucleotide. Aggregation has two major effects, it markedly increases the rate of uptake of the polynucleotide by the cell and also increases the ribonuclease resistance, although this is probably a secondary factor.

The uptake of polynucleotides in mammalian cells has been the subject of much work by Schell, who unfortunately used a cell line which it is believed does not produce interferon. Schell showed, using Ehrlich ascites tumour cells, that different polynucleotide complexes are absorbed by the cell in different ways. In the case of poly (A), poly (U) both strands are transported into the cell whilst only the poly (I) strand of the poly (I). poly (C) complex enters the cell.
This early observation led many investigators to conclude that the poly (I) strand is the more important strand. There is considerable evidence that the poly (I) strand is much more sensitive to changes in molecular weight and chemical modification than the poly (C) strand. This effect might be explained by the disruption of the highly organised structure of poly (I) in solution.

Nearly all the synthetic polynucleotides studied have been analogues of poly (I). poly (C) mainly because this is one of the most active complexes. There are three positions in the polynucleotide chain where modifications can be carried out without destroying the structure. These are on the base, the sugar and the phosphate backbone.

PHOSPHATE BACKBONE MODIFICATIONS

Modification of the phosphate backbone is perhaps the most difficult as there are fewer possible analogues. Eckstein however has prepared the homopolymer poly (S-U) and the alternating copolymer poly (S-A-U) where the phosphate is replaced by a phosphorothioate group. The homopolymer was prepared from the corresponding pyrophosphate analogue using the enzyme polynucleotide phosphorylase from E. coli. The yields of polymer obtained were poor and were not improved by addition of the primer UpU. Polynucleotide phosphorylase from Micrococcus lysodeikticus failed to produce any poly (S-U) under the conditions used. The alternating copolymer was synthesised from pppS-U and pppS-A by DNA-dependent RNA-polymerase using poly (dA-dT) as template. Again the yields were low but this may be partly due to the
fact that the nucleoside 5′(ρ′-1-thio)riphosphates occur as two diastereoisomers and it is not known whether these are utilised equally by RNA polymerase. This may explain why it is impossible to polymerise \( \text{ppp}^S\text{-A} \) and \( \text{ppp}^S\text{-U} \) in more than 50% yield. Subsequently Eckstein prepared poly \( (^S\text{I-C}) \) and poly \( (^S\text{I}^S\text{C}) \).\(^{42}\) The introduction of phosphorothioate groups in the polymer backbone neither impaired the formation of a double-stranded complex or its thermal stability. However, when poly \( (\text{A-U}) \) and poly \( (\text{I-C}) \) and their substituted analogues were subjected to pancreatic ribonuclease treatment the sulphur analogues were significantly more resistant. Also more important still, the sulphur analogues were more active as interferon inducers than the unsubstituted polymers. The difficult question was whether the sulphur analogues were more effective inducers or whether it was due entirely to their increased resistance to nucleases. It will be shown in this thesis that nuclease resistance is a minor consideration in designing new inducers. The thiophosphate analogues have received considerable attention as they are better inducers, however it has been shown that they are more toxic with the net effect that their therapeutic index is the same as the unsubstituted analogues.\(^{41}\)

**SUGAR MODIFICATIONS**

The role played by the functional group at the 2′-position of the sugar ring of polynucleotides in determining the structure, function and stability of these compounds is not well understood. However, modifications at the 2′-position have given considerable information about the factors involved. In addition to polynucleotides
2 - modified polynucleotides

where $R = \text{OMe}$ 43,43,44
$\text{OEt}$ 45
$N_3$ 46,47
$\text{NH}_2$ 48
$F$ 49
$\text{Cl}$ 50,51
$\text{OCOC}_{\text{H}_3}$ 52
containing the natural ribose and deoxyribose residues homopolymers containing the moieties shown in Fig. 2 have been described.

The polymerisation of substrates with abnormal sugars has provided problems concerning which enzyme to use. Most polyribo-nucleotides are synthesised using polynucleotide phosphorylase because it is cheap, stable and requires no template or primer and the substrates are easier to prepare. Polynucleotide phosphorylase normally utilises ribonucleotide diphosphates in the presence of magnesium, but, with manganese present it will accept substrates with modifications at the 2'-position, and even deoxy substrates.\textsuperscript{5}

\(2'\text{-Deoxy-2'-amino uridine 5'-diphosphate}\) has been polymerised by polynucleotide phosphorylase, with an enzyme to substrate ratio of about 1:1, in the presence of manganese to give very low yields of polymer. This polymer showed a lower degree of ordering than poly (U) itself, which is shown by the circular dichroic spectrum. All attempts to form a double-stranded complex with this polymer failed probably because of an interaction between the phosphate backbone and the amino group on the sugar.

Eckstein \textit{et al.} have also synthesised uridine and cytidine polynucleotides containing 2'-chloro-2'-deoxyribose residues. In these polymers where the chlorine atom is of the same size as the hydroxyl group the polynucleotide should adopt a similar structure in solution and should be good interferon inducers if there is no requirement for a 2'-hydroxyl. Poly(U\textsubscript{Cl}) appears from its C.D. spectrum and hyperchromicity on enzyme digestion, to exhibit markedly more stacking of the uracil rings in solution at pH 7.5 than poly (U). In contrast to these results Eckstein found little difference in hyperchromicity
between poly(C) and poly(dC\textsubscript{1}) on digestion a result which was verified by their C.D. spectra. It appears therefore that a substituent at the 2'-position can exert different effects on stacking depending on the polymer.

The pKa's of cytidylic acid polymers are markedly affected by the substituent at the 2'-position. Poly(dC) has a pKa of 7.4 whilst poly(C) and poly(dC\textsubscript{Cl}) have pKa's at 5.7 and 5.5 respectively, which confirms that poly(C) and poly(dC\textsubscript{1}) have essentially the same structure at neutral pH. However, at pH 4.0 in 0.1M [Na\textsuperscript{+}] poly(C) has a Tm of 79\textdegree C whilst poly(dC\textsubscript{1}) does not melt below 92\textdegree C. Both poly(A). poly(dU\textsubscript{1}) and poly(I). poly(dC\textsubscript{1}) are inactive as interferon inducers despite their resistance to serum nucleases and their slightly greater thermal stability (about 2\textdegree C) over their ribose analogues.\textsuperscript{51}

On substitution of fluorine for chlorine Janik et al.\textsuperscript{49} found some unusual differences between poly(dU\textsubscript{1}) and poly(dUF). Poly(dUF) has apparently no secondary structure above 2.5\textdegree C even in the presence of Mg\textsuperscript{++} or spermidine. However, it forms a complex with poly(A) which melts 17\textdegree C higher in 0.1M [Na\textsuperscript{+}] than poly(A). poly(U) itself and which makes it similar to poly(A). poly(dCome) where the Tm is 11.5\textdegree C higher. It appears therefore that there is considerable variation in the secondary structures and complexing properties of polynucleotides containing modifications at the 2'-position. Since poly(dUome) readily forms a helical configuration whereas poly(dU) does not it follows that formation of a single-stranded stacked poly(U) helix is not dependent on the formation of intramolecular hydrogen bonds involving the 2'-OH as donor. However, despite the close structural similarities
of many of these 2'-modified polymers to their ribose analogues in solution none have been published which are active as interferon inducers. Part of the work presented here was to reinvestigate the interferon inducing properties of the poly(dI). poly(C) complex which is reported to be an inactive low melting complex.54

**BASE MODIFICATIONS**

The most extensive literature belonging to synthetic polynucleotides covers the area of base modifications. Nucleotide bases can act as nucleophiles and electrophiles and can therefore be attacked by a wide range of chemical modifying agents. Most of the work has been done on pyrimidine nucleotides as they are technically slightly easier to handle.

The first modified polynucleotides were those of Michelson et al.55 who prepared uridine ribopolymers from nucleotide diphosphates containing the groups F, Cl, Br, I, OH, at their 5- position. He also prepared poly(BrC) but the sample was shown to be impure by Howard et al.56 who later fully characterised the polymer. Michelson also prepared poly(IodoC) by iodination of the nucleotide monophosphate, followed by phosphorylation to give the diphosphate and subsequent polymerisation.57 This polymer can also be prepared by direct iodination of poly(C),58 although it is questionable whether the polymer is pure. Most of the halogenated polynucleotides form considerably more stable complexes with their complementary homopolymers than their unsubstituted analogues. It can be seen that variation of the electronic structure of the base has a marked effect on the stability of the
hybrids formed. For instance the stabilities of the uridine polymers complexed with poly(A) follows the order IodoU > BrU > ClU > U > FU. This data is in agreement with other observations that the stability of polynucleotide complexes lies not in the hydrogen bonding but in direct interplanar interactions the nature of which are not fully understood. An exception to the general increase in hybrid stability on substitution at the 5-position is poly(5-hydroxyuridylic acid) where there appears to be a marked reduction in stability. This will be discussed later as the work presented here describes the synthesis of poly(5-hydroxycytidylic acid), an analogous compound.

Michelson has also described the synthesis of N-acetylpolycytidylic acid by direct acetylation of the polymer. This technique has been used by Pitha and Pitha who showed that modifications in the poly(C) strand of poly(I) poly(C) had little effect on its anti-viral protection. Insertion of a different base in the poly(I) strand has, however, been shown to greatly reduce the inducing properties.

Further modifications at the 5-position of cytidine have led to the synthesis of poly(CIC) and poly(MeC), both compounds when hybridised with poly(I), are highly active interferon inducers. One of the few other successful modifications of the cytidine ring has been that Scheit et al who synthesised poly(S²C) (where S²C = 2 thiocytidylate). The introduction of a thioketo group into poly(C) rather surprisingly gave it a high affinity for poly(I) forming a complex with Tm > 100°C in 0.025M [Na⁺]. In acid conditions poly(S²C) forms a much less stable helical structure than poly(C) probably as a
consequence of the N-H...S hydrogen bonding. It has been shown that substitution at the 5-position of CMP by halogens or a methyl group leads to an increase in stability of the corresponding hybrid and it seems that replacement of the 2-keto group by a 2-thioceto group is even more effective in this respect. S'UMP is a minor base in t-RNA and its presence could have a stabilising effect on the structure of the nucleic acid. The synthesis of compounds such as poly 4,4-dimethyl cytidylic acid, where the ability of the base to hydrogen bond has been reduced, led to hybrids with poly(I) which had low thermal stability.

Modifications at the 6-position have the effect of rotating the base from its normal anti-conformation to a syn-conformation. Nucleotide diphosphates with bases in abnormal conformation are polymerised by polynucleotide phosphorylase very slowly and poly-6-azacytidylic acid can only be prepared in low yield. Nucleotide diphosphates with abnormal conformations can often be induced to polymerise in the presence of normal substrates. A copolymer was formed in reasonable yield by polymerising 6-azido cytidine diphosphate with cytidine diphosphate but the 6-azido group destabilised the secondary structure of the resulting polymer, and inhibited complex formation.

Synthetic work on purine ribopolymers has been done largely by Ikehara and his co-workers. He found that there were few positions on the purine base which could be modified without destroying its hydrogen bonding properties. Substitution at the 8-position of poly(G) with bulky substituents like bromine makes the polymer adopt a syn conformation which then fails to form a complex with poly(C).
Ikehara has made a wide variety of modified purines with substitutions on the six membered ring which is involved in hydrogen bonding in nucleic acids. Using standard techniques he prepared poly(2,6-bis methylthio) purine ribonucleotides which only hybridises with poly(C) when prepared as a copolymer with GMP. Similar disappointing results were obtained with poly 2-methylinosinic acid and poly 2-methylthiinoosinic acid both of which failed to form stable double-stranded complexes with poly(C), poly(I) or poly(U). However rather surprisingly poly 2-methylthiinoosinic acid forms a stable 2:1 complex with poly(A) which melts above 75°C in 0.15M NaCl. More recently Ikehara has synthesised polynucleotides containing various ratios of N'-methylated guanylic acid in poly(A) and poly(I). He concluded that the introduction of odd purine bases into single-stranded polynucleotides had an inhibitory effect on the formation of double helices. However when the number of odd bases was relatively small he found that they did not form loops but could be retained in the double helical structure.

Frazier et al. have synthesised poly(2-dimethylaminoadenylic acid) and they showed that the introduction of the substituent into poly(A) prevented formation of normal Watson-Crick hydrogen bonding with Poly(U) and Poly(BrU) but allowed bonding at the N7-position. The interaction of poly(2-NMe₂A) with poly(U) and poly(BrU) has been investigated by I.R., U.V., and C.D. spectroscopy. These studies reveal a weak complex formation with poly(U) (Tm 8°C in 0.1M NaCl) and a stronger one with poly(BrU) (Tm 46°C in 0.1M NaCl) which is not of the normal hydrogen bonding structure.

Probably the most neglected interferon inducer known is the
poly(G)·poly(C) complex which because of its unusual properties\textsuperscript{75,76,77} has been little worked on. Poly(G) aggregates in solution making its preparation very difficult since the aggregates inhibit the polymerising enzyme. This phenomenon also causes considerable variation in complexes formed with different homopolymers since the product will be a mixture of poly(G)·poly(G) and poly(G)·poly(N) interactions. Despite the difficulty of forming double-stranded complexes with poly(G), the poly(G)·poly(C) complex has been shown to be as active as poly(I)·poly(C) in inducing interferon in chick embryo cells.\textsuperscript{78} Furthermore double-stranded RNA's extracted from viruses infecting \textit{Penicillium stoloniferum} have been shown to have a G-C content of 45\% or greater\textsuperscript{79} and are excellent inducers of interferon.\textsuperscript{80,81,82} Double-stranded RNA's with high G-C content have exceptional thermal stability usually greater than 100°C in 0.1M NaCl and this has to be explained by any theory of interferon induction. Poly (G) is virtually completely resistant to nucleases, even \textit{T}\textsubscript{1} ribonuclease attack is relatively slow on the homopolymer (the reaction has a half life of several hours) and complexing it with poly(C) renders it virtually resistant to attack.

Single-stranded poly(G), poly(I) and poly(X)\textsuperscript{79} have been tested as inducers and were slightly active whilst poly(C), poly(U) and poly(A) were completely inactive. The ability of these purine polymers to aggregate probably makes them more effective inducers.

One of the problems in interferon research attracting most attention at the present time is the way that polynucleotides are transported into the cell. The main questions asked are whether they are transported intact, into the cell, whether the strands separate and where the induction is switched on. These questions are difficult
to solve experimentally. The only experimental evidence about the transport of polynucleotides through cell walls has been the work of Schell\textsuperscript{36,37} who, using Ehrlich ascites tumour cells, found that they were taken up rapidly. He also found that the cell could differentiate between poly(I).poly(C), poly(A).poly 2(U) and poly(A).poly(U). Poly(A).poly(U) and poly(A).poly 2(U) enter the cell intact whilst only the poly(I) strand of poly(I).poly(C) enters. However no interferon production was studied during polynucleotide uptake and so the results may not be significant.

The possibility that the two strands of the complex may be taken into the cell independently was studied by De Clerq et al\textsuperscript{83} who showed that separate administration of poly(C) and poly(I) to cell cultures resulted in an anti-viral activity identical to or greater than that resulting from administration of the poly(I).poly(C) complex. Priming of cells with poly(I), followed by treatment (from one minute to one day later) with poly(C) gave slightly greater anti-viral activity than poly(I).poly(C) itself. These results can be explained by assuming either that the homopolymers form a double-stranded complex at the outer cell membrane or within the cell or that the two strands remain separate and act independently on the cell. The former explanation is probably the correct one since there seems to be no evidence for separate receptor sites for the two homopolymers. It seems likely that the interaction of polynucleotides with cells will be extensively investigated in the next few years. A preliminary publication\textsuperscript{84} has appeared describing the synthesis of a fluorescent poly(A) species which hybridises with poly(U). Such a hybrid may be of use in monitoring the environment of the polynucleotide when it is exposed to cells.
The early ideas that a high Tm and a high resistance to nucleases were vital for interferon induction have been replaced with the idea that 'double-strandedness' is essential only for transporting the RNA to the cell wall. At this point the synthetic RNA switches on interferon production within a few minutes and the RNA is then slowly degraded. A major pitfall in the design of synthetic polynucleotide inducers is their toxicity which could be due to their slow rate of enzymatic degradation compared with the fast process of interferon induction. Currently the search for better inducers has been centred on producing a double-stranded RNA with a Tm just above the incubation temperature so that strand separation would be reasonably easy. Such a polymer would arrive at the cell surface, switch on interferon production and then be quickly hydrolysed hence reducing its toxic effect.

A recent paper by De Clerq et al. dealt with the toxicity of poly(I)·poly(C). They showed that cells pretreated with interferon can be induced to produce interferon by concentrations of poly(I)·poly(C) that are too low to induce interferon in untreated cells. De Clerq speculates that poly(I)·poly(C) may be less susceptible to enzymatic degradation in cells treated with interferon. In L-cells poly(I)·poly(C) even in high concentrations is unable to induce interferon in the absence of DEAE dextran. However, if the cells are pretreated with interferon the complex will induce without any DEAE dextran. Since DEAE dextran is thought to render the polynucleotide complex less susceptible to endonucleases De Clerq concluded that pretreatment with interferon may act in the same way. An alternative explanation, suggested by De Clerq was that cells treated with
interferon are able to bind more polymer than untreated cells. Further work is necessary to investigate these findings more fully.

One of the severe limitations of poly(I). poly(C) as a chemotherapeutic agent is its toxicity which includes antibodies produced specifically against double-stranded RNA. Modifications in structure which increase the resistance of polynucleotide complexes to nucleases may also increase their toxicity, by prolonging their existence in the organism. Since the triggering of human interferon production has been shown to be rapid, a new type of inducer must be designed which is rapidly destroyed after the initial triggering event. The poly(C) strand apparently is less important than poly(I) and it may be here where modifications can be made which do not destroy the structure of the double-stranded helix but which will facilitate its rapid hydrolysis. Polymers such as polyformycin and 8-substituted purine polymers may be useful in this respect as the purine bases in these polymers exist in the syn-conformation. Purine nucleotides in this abnormal conformation are to pancreatic ribonuclease close analogues of the pyrimidinenucleotides and are thus degraded at a similar rate. By utilising such bases in the purine strand both strands should be attacked by ribonucleases which should reduce the toxicity of such complexes.
Poly (5-chlorocytidylic acid)

Introduction

Since the discovery that poly (I).poly(C) was an active inducer of interferon many investigators have studied the biological effect of chemically modifying the complex but they have been unable to draw any definite conclusions. The first section of this thesis deals with the synthesis of 5-halogenated cytidine polyribonucleotides, where an attempt is made for the first time to correlate structural changes with variation in biological activity.

Chemical modification of the pyrimidine bases can be readily accomplished as electrophilic attack by halogens occurs at the 5-position, to give the 5-halogeno nucleotides. Conversion of the latter to the pyrophosphates followed by polymerisation with polynucleotide phosphorylase lead to 5-halogeno pyrimidine polynucleotides. The other approach to halogenated polymers is by direct halogenation of the polymer. This approach was used by Means et al and Commerford who synthesised poly (BrC) and poly (IodoC). We studied this approach and abandoned it since the inevitable presence of water in the reactions aids the formation of adducts across the 5,6-double bond in pyrimidines, in addition to the substitution reaction. Such reactions mean that the resulting polymer is impure and more important still its ability to form complexes with poly(I) might be impaired.

Pure poly (BrC) has been prepared by polymerisation of the diphosphate by Howard et al, who showed that the introduction of an
halogen atom considerably stabilised any complex formed. Similarly poly (IodoC) has been prepared and its physical properties examined. Both poly (BrC) and poly (IodoC) form more stable complexes with poly (I) than poly (C) itself. The order of stability is poly (IodoC) > poly (BrC) >> poly (C). The nature of the stabilising influence was discussed by Howard et al and it was of interest to synthesise poly (C1C) so that more data could be available.

5-Substituted cytidine derivatives are known to be inducers of interferon and it is of interest to compare the inducing abilities of a series of halogenated polynucleotides. 5-Chlorocytidine has recently been found as a minor base in salmon sperm DNA and the present work may shed some light on its structural role in the nucleic acid.

Cytidine nucleosides have been chlorinated at the 5-position of the pyrimidine ring using N-chlorosuccinimide or chlorine together with ultraviolet irradiation. The yields of chlorinated nucleosides from these reactions are only moderate and if nucleotide polyphosphates are used hydrolysis occurs. Therefore, for the synthesis of 5-chlorocytidine nucleotides a reagent is required which will chlorinate the base under mild conditions. Preliminary results on cytidine and uridine showed that tetrabutylammonium iodo-tetrachloride was such a reagent. It had only been used previously to chlorinate olefins and phenols in fair yield. When tetrabutyl ammonium iodotetrachloride was reacted with cytidine in dimethylformamide and the reaction monitored by thin layer chromatography
(silica gel, eluted with methanol : ethyl acetate, 3 : 7) a faster moving band rapidly appeared which was identified as 5-chlorocytidine as below.

**EXPERIMENTAL**

**5-Chlorocytidine**

Tetrabutyl ammonium iodotetrachloride (1.5 gm) was added to a stirred solution of cytidine (500 mg) in dry dimethyl formamide (10 mls). The reaction was complete after 36 hrs at room temperature. The solution was evaporated to dryness and water (40 mls) added followed by shaking and filtration. The resulting solution was ether extracted (3 x 50 mls) and applied to a column (20 x 1 cm) of Dowex 50 x 8 - 100 (H+ form). The column was washed with water and finally with an ammonia gradient (up to 3%). The appropriate fractions were collected, evaporated to dryness, dissolved in methanol, ether precipitated and the product dried. Yield 425 mg (75%). M. p. 202° C (lit. M. pt. 175° C - 176° C). Ultraviolet spectra (H2O, pH 7.0) 

\[ \lambda_{\text{max}} 286 \text{ nm} (\log \varepsilon 3.87), \lambda_{\text{max}} 217 \text{ nm} (\log \varepsilon 4.07) \]

\[ ^1H \text{ NMR} (60MHz, D_2O) \]

1.82\( \tau \) (1Hs), 4.17\( \tau \) (1Hd), 5.78\( \tau \) (3Hm), 6.07\( \tau \) (2Hm).

Anal. calc. for C_{9}H_{13}N_{5}O_{5} : C, 38.92; H, 4.36; N, 15.13; Cl, 12.76.

Found: - C, 38.71; H, 4.77; N, 15.06; Cl, 12.71.

**5-Chlorouridine**

To a stirred solution of uridine (500 mg) in dry dimethyl formamide (10 mls) was added 1.5 gms of tetrabutyl ammonium iodotetrachloride and the mixture stirred for 36 hrs. The solution was evaporated to dryness in vacuo and water (40 mls) was added. The
solution was filtered and the filtrate ether extracted (3 x 50 mls). Methanol (60 mls) was added to the aqueous layer and the solution applied to a Dowex 1 x 2-200 column (OH-form, 1.5 x 20 cms, pre-equilibrated in 60% methanol). The column was washed with 60% methanol and the product eluted with a linear gradient of triethylammonium bicarbonate in 60% methanol. After removing the bicarbonate by repeated evaporation the residue was dissolved in ethanol (10 mls) and ether added until the solution turned cloudy. The solution was left several days at 0°C after which time the solid was filtered off and dried. Yield 425 mgs (74%).

M. pt. 217°C (with dec.) (lit. m. pt. 217 - 217.5°C.)

UV spectrum (H2O) λmax 277 nm (log ε 4.11). 1H NMR spectrum (60 MHz) (D2O) 1.76τ (1Hs), 4.14τ (1Hd, J = 3Hz), 5.65 - 5.80 (3Hm), 6.10τ (2Hd, J = 4Hz)

Anal. calc. for C9H11N2O6Cl: C, 38.79; H, 3.98; N, 10.05; Cl, 12.72.
Found: - C, 38.67; H, 4.12; N, 9.93; Cl, 12.65.

5-chlorocytidine 5'-diphosphate (C1CDP)

A solution of tetrabutylammonium iodonitramine (300 mgs) in dry dimethyl formamide (1 ml) was rapidly added to a solution of CDP trisodium salt (100 mgs) in dry formamide (3 mls). The reaction mixture was stirred magnetically in a well-stoppered vessel until all the solid had dissolved and then left for 36 hrs in subdued light. The solution was poured into water (50 mls) which was then extracted with chloroform (3 x 50 mls). The aqueous layer was applied to a Whatman D.E. 23 cellulose column (1 x 20 cms, HCO3- form) and the column eluted with a linear gradient of triethylammonium bicarbonate.
FIG. 3

Ultraviolet spectra of C12DP in 0.1M NaCl, 0.005M Na cacodylate at 25 °C, pH 7.0 (--), pH 1.0 (-----)
ClCDP was eluted at a concentration of 0.15 M. The triethylammonium bicarbonate was removed by repeated evaporation in vacuo and the residue was dissolved in water and passed through a Dowex 50 column (Na⁺ form). The eluate was evaporated to an oil in vacuo below 40°C which followed by lyophilisation gave colourless trisodium 5-chlorocytidine-5'-diphosphate (92 mgs): UV spectrum (0.01 M ammonium acetate, pH 7.0) λmax 286.5 nm (θ 6460). (lit. value 96 for 5-chlorocytidine 287 nm) The pKa of ClCDP was determined spectrophotometrically in citrate-HCl buffer and was found to be 2.49 ± 0.05 at 18°C and the variation in absorbance of ClCDP with pH is shown in fig. 3.

Anal. calc. for C₉H₁₁ClN₃Na₃O₁₀P₂.2H₂O: C, 20.03; H, 2.80; Cl, 6.57; N, 7.78; P, 11.48.

Found: C, 20.22; H, 3.45; Cl, 6.85; N, 7.71; P, 11.47.

¹H NMR spectrum (D₂O 100MHz) 1.88τ (1Hs), 4.10τ (1Hd, J = 3Hz), 5.5 - 6.0τ (5Hm).

¹⁴C labelled ClCDP was prepared in an identical manner from [¹⁴C]CDP.

5-chlorouridine 5'-diphosphate (ClUDP)

ClUDP was prepared as the tripotassium salt in an analogous manner from UDP in 94% yield. UV spectra (H₂O, p.H. 7.0) λmax 277 nm (ε, 8300) lit. λmax (H₂O, p.H. 7.0) 276 nm.⁵⁵

¹H NMR spectrum (100 MHz, D₂O) 1.83τ (1Hs), 4.05τ (1Hd, J = 4Hz), 5.5 - 6.0τ (5Hm).

Anal. calc. for C₉H₁₀ClK₃N₂O₁₁P₂: C, 19.55; Cl, 6.41; H, 1.82; N, 5.07; P, 11.20.

Found: C, 19.75; H, 2.01; Cl, 6.23; N, 4.93; P, 10.92.
Dephosphorylation of ClCDP

To check that addition across the double bond in cytidine has not occurred during the reaction the nucleotide was dephosphorylated using snake venom.

$\left[ ^{14}C \right]$ ClCDP (10 μmoles) in 0.1 M tris-acetate buffer (0.1 ml, pH 8.0) was incubated with *Crotalus adamanteus* venom (100 μg) for 12 hrs at 37° C. Examination of the reaction mixture by descending paper chromatography (1-propanol-conc. ammonium hydroxide - H$_2$O, 6 : 3 : 1) showed only one radioactive compound with the same Rf as authentic 5-chlorocytidine.

Synthesis of poly (5-chlorocytidylic acid) [poly(ClC)]

The polymerisation of ClCDP by polynucleotide phosphorylase was followed both by release of inorganic phosphate$^{10}$ and by incorporation of $\left[ ^{14}C \right]$ into polymeric material over a range of substrate concentrations to determine the optimum conditions for polymerisation. In a typical case, the reaction medium contained 20 mM ClCDP, 1.25 Mg /m of polynucleotide phosphorylase (M. luteus, 30 u/mg), 10 mM MgCl$_2$ and 5 mM EDTA in 0.15 M Tris-chloride buffer (pH. 9.0), and this was incubated for 10 hrs at 37° C. After deproteinisation by repeated extraction with chloroform-isoamyl alcohol (5/2, V/V), the aqueous phase was desalted by dialysis over 36 hrs at 5° C against 0.1 M NaCl-0.001 M Na EDTA, 0.001 M EDTA and then twice against water. Lyophilisation at 0° C gave poly (ClC) in 53% yield (62% by release of inorganic phosphate).

Polymerisation reactions with $^{14}$C-labelled ClCDP (2 x 10$^4$ dpm/μmole) were monitored as follows: Aliquots were removed at intervals and
Variation of rate of polymerisation with substrate concentration in the polymerisation of $^{14}$C - C1CDP by polymucleotide phosphorylase. The reaction mixture contained 5 - 50 mM $^{14}$C - C1CDP, 5 - 50 mM MgCl$_2$, 2.5 - 25 mM EDTA, 0.15M Tris pH 9.0 and enzyme 0.75mg/ml. Incubation was at 37$^\circ$, aliquots were removed and assayed as described in the text.
Fig. 5

Variation in rates of polymerization with substrate concentration.
applied to Whatman 3 mm or Whatman No. 4 paper and the polymerisation stopped by the addition of a little acetone. The papers were developed in 0.5 M ammonium acetate in ethanol - H2O (1 : 1V/v). After drying, the polymer and monomer could be visualised under a U.V. lamp and appropriate sections of the paper were excised and their radioactivity measured. The polymerisation data is summarised in figs. 4 and 5 and from this Km and Vmax were estimated to be 5.0 mM and 0.95 μmole/hr. respectively.

Characterisation of poly (ClC)

Typical samples of poly (ClC) had S20W values of 3 - 6s determined by ultracentrifugation in an isokinetic sucrose gradient containing 0.1 M sodium acetate (pH 7.5). The chain length of the polymer was dependent on the time of incubation which in turn depends on the amount of enzyme used.

The U.V. maximum of poly (ClC) at 25°C in 0.01 M ammonium acetate (pH 7.0) was 286 nm (ε 5050 ± 50, maximum deviation of 5 measurements = 70), heating for ten minutes at 95°C caused a hypochromic change in absorption of 11.5%. (A95°C - A25°C)/A95°C. Pancreatic ribonuclease digestion of poly (ClC) gave a hypochromicity of 30%. (A monomer - A polymer)/A monomer. The UV spectra of poly (ClC) in neutral and acid solution are shown in fig. 8. Spectrophotometric filtration at 310 nm of poly (ClC) in 0.3 M NaCl showed an abrupt transition at pH 4.1 over a narrow range of 0.2 pH units (fig. 6). The Tm of the polymer was also dependent on pH in this region (fig. 7).
Spectrophotometric titration at 310nm of $1.85 \times 10^{-4}$ M solution of poly (Glu) (---o--) and CMBP (--o--) in 0.3 M NaCl at 25°C.
Temperature - ultraviolet absorption profiles at 310nm of poly (GCL) at different pH values.
Concentration of polymer $1.35 \times 10^{-4}$ M, buffer
$0.1$ M NaCl + $0.05$ M Na acetate
Ultraviolet spectra of poly (C10) at pH 7.0
(——) and pH 2.0 (———), and the 1:1 hybrid
poly (I) poly (C10) at pH 7.0 (---) in
6M NaCl, 0.005M Na acetate at 25°C.
Variation in ultraviolet absorption on mixing poly (ClC) and poly (I) in 0.1M NaCl, 0.005M sodium cacodylate pH 7.0 at 25°. Total polymer concentration 0.96x10^{-4} M. Readings were taken 6 hr. after mixing.
FIGS. 10 & 11

Mixing curves for poly (CIC) and poly (I).

Conditions as for fig. 9.
Ultraviolet melting curves of poly (ClC). poly (I) in 0.005M sodium cacodylate at differing Na$^+$
concentrations. Total polymer concentration 0.96x10$^{-4}$M.
Hydrolysis of Poly (C1C) by pancreatic ribonuclease

The polymer (0.2 μm) in 0.01 M ammonium acetate (pH.
(2 mls) was treated with 0.5 μg of ribonuclease. Under these
conditions poly (C) has $t_1 = 5$ secs, poly (C1C) has $t_1 = 5$;
poly BrC has $t_1 = 80$ secs and poly (IodoC) has $t_1 = 150$ se
values were determined spectrophotometrically by following
increase in optical density at the $\lambda_{max}$ of the polymer.

Preparation and Properties of a Poly(I):poly (C1C) H

Equimolar quantities of poly(I) ($S_{20 W} = 6.645$) and po
($S_{20 W} = 4.20$) were dissolved in 0.01 M sodium acetate (pH
at 37°C to give a 1 : 1 hybrid. The solution was left at
temperature for 2 hrs and then applied to a Sepharose 4B-2
Elution with acetate buffer gave double stranded poly (I).
in the void volume followed by a small amount of unanneale
The ultraviolet spectrum in 0.01 M sodium acetate (pH. 7( in
fig. 8 and the polymer has a $\lambda_{max}$ of 280 nm (ε = 6600)
(ε = 9500).

The stoichometry of the hybridisation was determined
method of continuous variations in 0.1 M NaCl - 0.005 
cacodylate (pH. 7.0) at 25°C (figs 9, 10, 11). At 245 nm
a break in the curve at 50% corresponding to the formatio
hybrid, at 286 nm no break can be seen.

The melting profiles of poly (I). poly(C1C) are summ
fig. 12. The Tm's in 0.01 M, 0.1 M, and 0.3 M Na⁺ are 65
and 82°C respectively. Poly(I). poly(BrC) has Tm values
approximately 65°C, 83°C and 88°C under the same conditions.
Poly(IodoC). poly(I) has a Tm of 92°C in 0.1 M NaCl.57

The circular dichroic spectra of poly(ClC) and poly(ClC).poly(I) are shown in fig. 13. The polymers were dissolved in 0.1 M sodium chloride - 0.005 M sodium cacodylate (pH 7.0).

Discussion

Polymerisation of ClCDP with polynucleotide phosphorylase gave poly(ClC) in high yield in a reaction in which little phosphorolysis appeared to occur. The reaction was left for ten hours at 37° C after which the polymer was isolated and found to have a typical S20,w value of 4.20. Poly(ClC) possessed considerable secondary structure in acid solution and a sharp change in absorption was observed at 310 nm in 0.3 M salt at about pH 4.1. The change in absorption of poly(ClC) occurred over a narrow pH range (0.2 pH unit). Furthermore the shape of the melting curve for poly(ClC) changes abruptly in shape in this pH range. This indicates that a co-operative phenomenon is taking place and the poly(ClC) has a helical structure in acid solution. Similar behaviour has been observed with poly (BrC)56 and poly(IodoC).57 The apparent pKa's of poly(C), poly(IodoC), poly(BrC) and poly(ClC) in 0.15 M NaCl are 5.82, 5.0 4.43 and 4.10 respectively. The reduction in the pKa is to be expected as the electronegativity of the substituent at the 5-posi has been increased.

It can be seen that poly(ClC) like poly(C), poly(BrC) and poly(IodoC) forms a structure in acid solution which involves a shifted proton (fig. 14) between two parallel chains103, 104,105 the stability of which is effected by the pKa of the amino group.
Circular dichroic spectra of poly (ClC) (—) and poly (I) • poly (ClC) (---) in 0.005M sodium cacodylate, 0.1M NaCl pH 7.0. Polymer concentrations were determined spectrophotometrically and were poly (ClC) 0.23mg/ml, poly (I) • poly (ClC) 0.4mg/ml.
FIG. 14

Acid structure of poly(C).
Halogenation of the cytidine ring renders the polymer more resistant to hydrolysis by pancreatic ribonuclease. This resistance increases proportionately with the size of the substituent and compounds such as poly 5-dimethylaminocytidylic acid, where the substituent is very large, the half life of hydrolysis is several hours compared to poly(IodoC) where the half life is three minutes under identical conditions. X-ray data shows that in the binding site of ribonuclease the 5-position of the pyrimidine ring is the most exposed and therefore modifications at this position should not have a major effect on the hydrolysis of polynucleotides by the enzyme. However the rate of hydrolysis is controlled by changes in hydrophobicity and bulk. Hydrogen bonding involving the 4-position can be eliminated since it does not appear to contribute to the normal binding of nucleotide to the enzyme.

The circular dichroic spectrum of poly(ClC) in neutral solution has a maximum at 296 nm ($\theta = 34,700$) and this resembles the C.D. spectrum of poly(C) which consists of a maximum at 277 nm ($\theta = 59,000$). The C.D. spectrum of the poly(ClC). poly(I) complex shows two principal maxima, one at 294 nm ($\theta = 14,000$) and the other at 244 nm ($\theta = 23,250$). This resembles the C.D. spectrum of poly(I). poly(C) which has maxima at 277 nm ($\theta = 15,550$) and at 245 nm ($\theta = 17,200$). It can therefore be said that base stacking in the two systems is very similar.

Poly(ClC) forms a 1 : 1 complex with poly(I) and like other 5-halogenated polycytidylic acids the thermal stability of the complex is greater than that of poly(I). poly(C) over a range of salt concentrations. It has been shown that substitution on the cytidine
with bromine produces a large enthalpic stabilisation of the complex with poly(I), probably because of the introduction of a large polarisable atom into the heterocyclic system. Since chlorine is less polarisable than bromine or iodine one would expect, if polarisability were an important feature in complex formation, that the stabilities would decrease in the order \text{poly(IodoC)} > \text{poly(BrC)} > \text{poly(ClC)} >> \text{poly(C)}. This is in fact the order observed for the Tm's and the creation of such a series of compounds enables the stabilising influence of these substituents to be investigated for the first time.

The abilities of \text{poly(ClC). poly(I)}, \text{poly(ClC). poly(G)} and \text{poly(ClC). poly(dI)} complexes to induce interferon are discussed in the biological section of this thesis.
2. **Poly 5-Bromocytidylic acid [poly(BrC)]**

Poly(BrC) (S\textsubscript{20,w} 2 - 5S) was synthesised after the method of Howard et al.,\textsuperscript{56} with the minor modification that the BrCDP was chromatographed on DE 23 cellulose. The \textsuperscript{1}H NMR spectrum of BrCDP (D\textsubscript{2}O, 100 mHz) shows peaks at 1.84\tau (1Hs), 4.13\tau (1Hd, \textit{J} = 3Hz), 5.5 - 6.0\tau (5Hm). U.V. spectrum (H\textsubscript{2}O, pH 7.0) \lambda\text{max} 289 (\textit{E}, 7,100). The hydrolysis of poly(BrC) by pancreatic ribonuclease is discussed in the previous section on poly(C1C).

The interferon inducing properties of the poly(BrC). poly(I) complex are discussed in the biological section of this thesis.
Poly 5-Iodocytidylic acid [poly(IodoC)]

Poly(IodoC) was prepared by polymerisation of IodoCDP using standard techniques developed in this laboratory. \(^6\) 5-IodoCDP was prepared from CDP using iodine monochloride in dimethylacetamide.

5-IodoCDP

CDP (100 mg, sodium salt) was dissolved in 10 mls of dry dimethylacetamide and iodine monochloride (300 mgs) in dry dimethylacetamide (1 ml) was added. The reaction was left at room temperature for 36 hrs and then chromatographed as described for C1CDP. The final yield of IodoCDP was about 24%. Although the reaction was not extensively investigated it was certain that some addition across the double bond of the base did occur, probably because of traces of water in the reaction. \(^1^0^q, 1^0^o\)

U.V. spectra (H\(_2\)O, pH 7.0) \(\lambda\)max 295 nm, \(\lambda\)min 265 nm,
pH 2: \(\lambda\)max 310 nm, \(\lambda\)min 260 nm.

The hydrolysis of poly (IodoC) was discussed in the section on poly(C1C). The anti-viral activity of the poly(IodoC). poly(I) complex is discussed in the biological section of this thesis.
**Poly 5-hydroxycytidylic acid**

**Introduction**

Many polycytidylic acids have been synthesised with electron-withdrawing substituents at the 5-position and their properties described. The synthesis of cytidylic acids with electron-releasing groups at the 5-position is more difficult since electrophilic attack at the 5-position is the favoured reaction.

5-hydroxynuridine diphosphate (OHUDP) has been prepared by the action of bromine in aqueous pyridine on UDP. CDP has also been converted, in unstated yield, into 5-hydroxycytidine diphosphate (OHCDP) by the action of bromine followed by alkali treatment. However in our hands the yield of OHCDP was found to be low (<5%), the major product being OHUDP. Addition across the 5,6-double bond occurs in the reaction and it is known that 5,6-dihydrocytidylic acids readily deaminate in basic solution. While OHUDP is a substrate for polynucleotide phosphorylase, OHCDP has not yet been polymerised by this enzyme, only a copolymer of CMP and OHCMP of uncertain composition has been obtained.

The following section describes the synthesis of OHCDP in good yield and its subsequent polymerisation using polynucleotide phosphorylase. Preliminary investigations on cytidine were done using the basic ion exchange resin (Dowex 2 x 8 - 100, OH\(^{–}\) form) described by Means et al. 5-hydroxycytidine was produced in fair yield, but when the reaction was applied to CMP or CDP the prolonged time on the column led to the production of OHUMP and OHCDP. The reaction procedure of Visser et al was re-investigated and contrary
Elution profile of OHC6P from a TEAE column (1.5 x 40 cm, HCO₃⁻ form). The linear gradient was made by adding triethylammonium bicarbonate (0.1 M, 1 L, adjusted to pH 9.5 by the addition of triethylamine) to water (1 L).
to report that hydroxycytidine cannot be produced by the action of bromine and wet pyridine on the nucleoside, rapid formation of 5-hydroxycytidine was observed in two hours. 5-hydroxycytidine, after this time, was slowly hydrolysed until after 24 hrs (the conditions used by Visser) the major product was 5-hydroxyuridine.

The reaction was monitored by T.L.C. (silica, methanol : ethyl acetate 1 : 1 v/v) and it was found that 2, 4, 6-collidine is a more effective base than pyridine ($pK_a^{20}$ pyridine = 5.23, $pK_a^{20}$ 2, 4, 6-collidine = 7.59) and less likely to be involved in displacement reactions.

5-hydroxycytidine diphosphate

Bromine was slowly added to a solution of CDP trisodium salt (300 mgs) in water (3 mls) at 0°C until a yellow colour persisted. A few drops of cyclohexene were then added with shaking to remove excess bromine followed by the addition of 2, 4, 6-collidine (1.5 mls). The emulsion was incubated for two hours at 37°C. After cooling the mixture was ether extracted (4 x 4 mls) and the aqueous layer applied to a TEAE column (HCO$_3^-$ form, 1.5 x 40 cm) which was eluted with a linear gradient of triethylammonium bicarbonate. BrCDP(I) was eluted first followed by OHCDP(II) at about 0.1 M Et$_3$NHCO$_3$ (fig. 15). The fractions containing OHCDP were pooled, evaporated to dryness and excess bicarbonate removed by repeated addition and evaporation of methanol. The product was converted by means of a Dowex 50 column (K$^+$ form) into the pale yellow hygroscopic tripotassium 5-hydroxycytidine 5'-diphosphate (115 mgs, 34% yield). U.V. spectra (0.3 M NaCl, 25°C)
UV spectra of OHCP in 0.3 M NaCl at 25°:

pH 6.5 (---), pH 2.0 (— —), pH 11.0 (-----).
FIG. 17

Spectrophotometric titration at 310 nm of OHCDP (-----) and poly (OHC) (-----) in 0.3 M NaCl at 25°.
Variation of rate of polymerisation of OHCDP by PNPase with magnesium ion concentration. The \(^{14}\)C-OHCDP concentration was kept constant at 20 mM, other conditions as in text.
Variation in rate of polymerisation of OHCDP by FNPase with substrate concentration. The ratio of molar concentration of OHCDP to \( \text{MgCl}_2 \) was kept constant at 1:1, other conditions as in text.
FIG. 20

Mixing plot for poly(OHC) and poly(I) in 0.3 M NaCl, 0.005 M Na cacodylate, pH 6.5.
pH 1.0: \( \lambda_{\text{max}} \) 216 nm (6 9,200), 306 nm (6 9,950); pH 6.5, \( \lambda_{\text{max}} \) 217 nm (6 14,200), 292 nm (6 7,700); pH 11.0, \( \lambda_{\text{max}} \) 225 nm (6 17.600), 319 nm (6 7,550) (see fig. 16). The pKa's of OHCDP determined spectrophotometrically in citrate-HCl (0.15 M) or tris-HCl (0.15 M) at 20° C are 3.80 ± 0.03 and 8.77 ± 0.04.

The variation in absorbance with pH is shown in fig. 17. Anal. calc. for \( \text{C}_{9}\text{H}_{12}\text{N}_{3}\text{O}_{12}\text{P}_{2}\text{K}_{3}\text{H}_{2}\text{O} \): C, 19.60; H, 2.56; N, 7.62. Found: C, 19.46; H, 2.56; N, 7.92%. The \( ^{1}H \) NMR spectrum (100MHz, \( \text{D}_{2}\text{O} \)) had a signal at 2.37t (1Hs) \text{inter alia.} \[ ^{1}\text{C} \text{OHCDP} \]

was prepared in an analogous manner from \( ^{1}\text{C} \text{CDP} \).

Dephosphorylation of OH CDP

Alkaline phosphatase (0.1 mg, 3,000 u/mg) was added to \( ^{1}\text{C} \text{OHCDP} \) (10 mgs, 5 x 10\(^{6}\) cpmp/\( \mu \text{mole} \)) in 0.1 M glycine-NaOH buffer containing 1 mM MgCl\(_{2}\) (pH 10.5, 100\( \mu \text{l} \)) and the mixture incubated at 37° C for 6 hrs. Examination of the mixture by paper or thin layer chromatography showed that complete dephosphorylation had occurred. Only one radioactive compound was present and this had the same \( R_{f} \) as 5-hydroxycytidine. 5-Hydroxycytidines can be visualised on chromatograms using a ferric chloride/ferricyanide spray.\(^{17}\)

Polymerisation of 5-Hydroxycytidine diphosphate

The polymerisation of OHCDP by polynucleotide phosphorylase was followed by the incorporation of label from \( ^{1}\text{C} \text{OHCDP} \) into polymeric material over a range of substrate and magnesium concentrations to determine the optimum reaction conditions. The polymerisation was monitored as previously described for C1CDP.\(^{63}\) All the reactions were carried out in Tris-chloride buffer (6 15 M, pH 9.0) containing
2.5 mM sodium EDTA and 0.02% sodium azide. High enzyme concentrations (5 mgs polynucleotide phosphorylase per 50 mgs substrate) were required for the reaction to proceed at a reasonable rate. The rate of polymerisation is markedly dependent on magnesium ion concentration (fig. 18) and the maximum rate of polymerisation occurs with equimolar concentrations of substrate and magnesium ion. In a polymerisation reaction (0.25 ml) which contained PNPase (7.5 units) and a $\text{Mg}^{++}$/OHCDP ratio of 1 : 1 the approximate Km and Vmax were 4 mM and 0.175 umole/hr respectively. (fig. 19) It can be seen that the Km is similar to that for the polymerisation of CDP but the slower rate of chain elongation is reflected in the greatly reduced Vmax.

**Preparation of potassium poly(OHC)**

Tris-chloride buffer (0.15 M, pH. 9.0) containing 20 mM OH CDP, 22.5 mM MgCl$_2$, 2.5 mM potassium EDTA and polynucleotide phosphorylase (5 mgs [150units] per 50 mgs OHCDP) was incubated at 37° C for 10 hrs. After deproteinisation by repeated extraction with chloroform - isoamyl alcohol (5 : 2 V/v) the aqueous phase was desalted by dialysis over 24 hrs at 5° C against 0.2 MKCl, 0.02 M potassium EDTA (pH. 8.0 to avoid precipitation of the polymer), 0.02 M potassium EDTA and finally against two changes of water. Lyophilisation at 0° C gave pale yellow hygroscopic poly(OHC) in up to 25% yield.

**Characterisation of poly(OHC)**

Poly(OHC) had a $S_{20,w}$ value of 1.95 which was determined by ultracentrifugation in an isokinetic gradient of sucrose containing sodium acetate (pH. 7.0). The U.V. maximum of poly(OHC) at 20° C in 0.3 M NaCl, 0.01 M sodium cacodylate pH. 6.5 was 292 nm ($\varepsilon_p$, 7,000);
pH 11.0, 320 nm (E_p, 6,850). E_p values were determined by the
method of Chen et al. Spectrophotometric titration of poly(OHC)
at 320 nm in 0.3 M NaCl showed no abrupt changes. (fig. 17) Heating
the polymer from 5^0 to 95^0 in 0.3 M NaCl, 0.05 M sodium cacodylate,
pH 6.5 caused a small linear decrease in absorption at 292 nm due
to the loss of protons from the structure as has been observed with
poly(C).^{11a}

Hydrolysis of poly(OHC) by pancreatic ribonuclease

\[ 14^C \text{poly(OHC)} \text{ (0.2 \mu M)} \text{ in 0.01 M ammonium acetate, 1 mM}
\text{sodium EDTA (0.5 ml, pH 7.0) at 25^0 C was treated with pancreatic}
\text{RNase (0.125 \mu g) in ammonium acetate buffer. The amount of}
5-hydroxycytidine 3'-phosphate liberated was determined by paper
chromatography using 0.5 M ammonium acetate in ethanol/water (1 : 1\^v/v),
as developing solvent. Under these conditions the \( t_1 \) of the hydrolysis
of poly(OHC) was 11 hrs. Exhaustive degradation of poly(OHC) followed
spectrophotometrically gave a similar \( t_1 \) and a hypochromicity

\[ \frac{[\text{A monomer} - \text{A polymer}]}{\text{A monomer}} \] at 292 nm of 8.5%.

Attempted preparation of hybrids between poly(OHC)
and other polynucleotides

Equimolar solutions of poly(OHC) and poly(I), poly(G) and poly(A)
in 0.3 M NaCl, 0.01 M sodium cacodylate, pH 6.5, were mixed and the U.V.
spectra recorded after annealing at 37^0 C or 5^0 C for 12 hrs. In
every case the spectra were purely additive and no hybrid formation
could be detected; by the method of continuous variations,^{11b}
at any wavelength. No hybrids could be detected over the concentration range
0.01 - 0.3 M sodium chloride. (see fig. 20) Neither could a Tm be
seen. Equimolar quantities of poly(I) \((S_{20,w} = 12)\) and poly(OHC) \((S_{20,w} = 1.9)\) were mixed as before in 0.3 M NaCl, 0.01 M sodium cacodylate, pH 6.5 and passed through a sephadex G200 column. The column resolved the high and low molecular weight polynucleotides and no double-stranded material could be seen.

The rate of hydrolysis of poly(I) \((0.5 \text{ OD}_{248}^{248} \text{ units})\) in 0.1 M Tris-chloride, 0.3 M NaCl (0.5 ml) by T1 RNase (5 μl, 1,500 U) at 20°C was determined with and without added excess of poly(OHC). No difference in hyperchromicity could be detected at 248 nm between the two experiments. There was a 6.8% increase in five minutes and 26% in twelve hours.

Discussion

An interesting feature of the synthesis of OHCDP is that BrCDP is not an intermediate in its formation. This was shown by using BrCDP in place of CDP as starting material and the only products formed were non-u.v. absorbing nucleotides. The probable route to OHCDP is shown in (fig. 21), where initial attack by hypobromous acid on the cytidine ring of CDP leads to (I). Elimination of water from (I) can occur to give BrCDP or displacement of bromine can lead to a dihydroxy CDP(II). Elimination of water from the latter gives OHCDP. Both (I) and (II) should be readily deaminated if the reaction time was prolonged.

The crude reaction mixture is purified by chromatography on TEAE cellulose at pH 9.5. At this pH the phenolic group of OHCDP is ionised and the extra negative charge facilitates the separation of OHCDP from BrCDP. The dephosphorylation studies show that the product
Possible mechanism for formation of OHCDP from CDP.
<table>
<thead>
<tr>
<th>FOUND</th>
<th>SOLVENT</th>
<th>( \lambda_{\text{max}} ) (nm)</th>
<th>MOLAR ELLIPTICITY (c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDP</td>
<td>0.3M NaCl, 0.01M Na cacodylate, pH 6.5</td>
<td>292</td>
<td>41,000</td>
</tr>
<tr>
<td></td>
<td>0.15M Tris/HCl</td>
<td>228</td>
<td>-23,500</td>
</tr>
<tr>
<td></td>
<td>pH 9.0</td>
<td>291</td>
<td>27,700</td>
</tr>
<tr>
<td></td>
<td>0.15M Tris/HCl, 3 mM MgCl₂</td>
<td>no bands discernable</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 11.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.3M NaCl, 0.01M Na cacodylate, 3 mM MgCl₂</td>
<td>292</td>
<td>41,000</td>
</tr>
<tr>
<td></td>
<td>pH 6.5</td>
<td>228</td>
<td>-23,500</td>
</tr>
<tr>
<td></td>
<td>0.15M Tris/HCl, 3 mM MgCl₂</td>
<td>no bands discernable</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 9.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ly OHC</td>
<td>0.3M NaCl, 0.01M Na cacodylate, pH 6.5</td>
<td>300</td>
<td>53,500</td>
</tr>
<tr>
<td></td>
<td>0.15M Tris/HCl</td>
<td>240</td>
<td>-36,000</td>
</tr>
<tr>
<td></td>
<td>pH 9.0</td>
<td>307</td>
<td>59,500</td>
</tr>
<tr>
<td></td>
<td>0.15M Tris/HCl, 3 mM MgCl₂</td>
<td>295</td>
<td>14,500</td>
</tr>
<tr>
<td></td>
<td>pH 9.0</td>
<td>256</td>
<td>-23,000</td>
</tr>
</tbody>
</table>
is free of other nucleotides.

The position of substitution in the cytidine ring was confirmed by $^1$H NMR spectroscopy. The signal due to $H_5$ which normally appears as a doublet at $3.95\,\tau$ in CDP was absent and the signal due to $H_6$ appears as a singlet at $2.37\,\tau$.

Polymerisation of OHCDP with polynucleotide phosphorylase gave poly(OHC) in a reaction which was markedly dependent on magnesium ion concentration. The activity of polynucleotide phosphorylase normally requires the presence of traces (2 - 3 mM) of magnesium ions, however during the polymerisation of OHCDP the maximum rate of polymerisation occurred at $a[Mg^{2+}]/[\text{substrate}]$ ratio of 1/1. At pH. 9.0 (the pH. of the polymerisation medium) the hydroxyl group of OHCDP should be partially ionised therefore it is an attractive idea to postulate that the magnesium neutralises negative charges on the hydroxyl group and on the nearby phosphate residue. Evidence of this is shown by the C.D. data summarised in Table 1. From this it can be seen that the amplitude of the longest wavelength band in the C.D. spectra of OHCDP is pH. dependent near the pKa, the amplitude being zero at pH. 11.0 where the hydroxyl group is fully ionised. Repulsion between the ionised phenolic hydroxyl group and the phosphoryl oxygens may cause the relative orientations of the pyrimidine ring and the ribose residues to alter. At pH. 9.0 there is a reduction in the amplitude of the C.D. maxima from the neutral spectrum and this peak is totally abolished by the addition of 3 mM magnesium chloride, an effect which is not noticed at neutral pH. During the polymerisation,
therefore, it seems likely that there is a complex formed between the pyrophosphate group, a magnesium ion and the phenolic hydroxyl of OHCDP.

The C.D. spectrum of poly(OHC) shows little change going from pH. 7.0 to pH. 9.0 which may show that the repulsive forces which move the base in OHCDP from its normal anti-conformation are counter-balanced by stacking in the polymer. The C.D. maximum of the polymer at pH. 9.0 however shows a marked reduction in the presence of magnesium ions indicating that a change in conformation must occur, an effect which is not observed with poly(C).

Poly(OHC) was very resistant to hydrolysis by pancreatic ribonuclease, the half life being 11 hrs under conditions where the half life for poly(C) was about 5 secs. This resistance to hydrolysis could be due to the nucleotide base being in a syn conformation since nucleotides such as 6-MeU 2', 3'-cyclic phosphate which adopt this conformation are not degraded. However it seems unlikely from the C.D. data that this is true and it is also unlikely that there is a steric reason why the hydrolysis is slow as the analogous chloro compound \( \text{Cl} \) \( r_{\text{covalent}} = 0.99\text{Å}, \text{OH} \) \( r_{\text{covalent}} = 1.02\text{Å} \) is hydrolysed in 50 secs. Perhaps the answer may lie in the change in the hydrophobicity on introducing a polar hydroxyl group which my interact with the solvent near the active site.

Poly(OHC) has little secondary structure in solution with a hyperchromicity of 8.5%. It does not form a duplex at low pH. like poly(C1C). The reason why poly(OHC) does not form a hybrid with poly(I) or poly(G) is difficult to explain although the factors which govern a
stable hybrid are not well known. It has however been observed that the presence of a hydroxyl at the 5-position of poly(U) weakens the formation of the poly(A)·poly(U) complex.
**Poly (deoxyinosinic acid)**

**Introduction**

Colby and Chamberlain, in a series of experiments using chick embryo cells, showed that the double-stranded deoxyribonucleotide homopolymers poly(dA). poly(dT), poly(dI). poly(dC) and poly(dG). poly(dC) were inactive as interferon inducers as was double-stranded coliphage DNA.\textsuperscript{20} De Clerq later showed that these compounds had anti-viral activity at very high concentrations.\textsuperscript{92}

Field et al. postulated that a special replicative form of the DNA or a viral DNA.RNA complex might be responsible for interferon induction in cells infected with DNA viruses.\textsuperscript{19} If protection from ribonucleases was an important factor in induction then single-stranded polyribonucleotides hybridised with deoxynucleotides should be very good inducers. The following DNA.DNA and DNA.RNA complexes have been synthesised but so far none have been found with anti-viral activity approaching that of poly(I). poly(C):

<table>
<thead>
<tr>
<th>Complex</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly(dG). poly(dC)</td>
<td>20</td>
</tr>
<tr>
<td>poly(dI). poly(dC)</td>
<td>20, 54, 34, 122, 123</td>
</tr>
<tr>
<td>poly(dI). poly(rC)</td>
<td>54</td>
</tr>
<tr>
<td>poly(dI). poly(dC)</td>
<td>54</td>
</tr>
<tr>
<td>poly(dI). poly(dBrC)</td>
<td>123</td>
</tr>
<tr>
<td>poly(dI-dC). (alternating)</td>
<td>124, 125</td>
</tr>
<tr>
<td>poly(dG - dC). (alternating)</td>
<td>124</td>
</tr>
<tr>
<td>poly(5MrdC). poly(dI)</td>
<td>126</td>
</tr>
<tr>
<td>poly(dA). poly(dT)</td>
<td>20</td>
</tr>
</tbody>
</table>
Although these complexes had little anti-viral activity (generally 10^4 less than poly(I). poly(C) many of them had low Tm's and may not have existed as double-stranded complexes at the temperature of the assay incubation medium. Chamberlain et al. have made the following complexes:

<table>
<thead>
<tr>
<th>Complex</th>
<th>Tm in 0.01 M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly(dI). poly(rC)</td>
<td>10.0 C</td>
</tr>
<tr>
<td>poly(dI). poly(dC)</td>
<td>27.5 C</td>
</tr>
<tr>
<td>poly(rI). poly(dC)</td>
<td>34.8 C</td>
</tr>
<tr>
<td>poly(rI). poly(rC)</td>
<td>41.5 C</td>
</tr>
</tbody>
</table>

In particular the poly(dI). poly(rC) hybrid is very unstable and it would be of interest to increase the Tm of this complex so that a fairer estimation of the abilities of DNA-RNA complexes to induce interferon might be made. The following sections describe the synthesis of poly(dI) and its hybridisation with poly(C1C) to form a complex with a Tm of 38.0 C in 0.01 M NaCl.

The synthesis of polydeoxynucleotides is technically difficult because of the absence of an efficient enzyme. In order to synthesise polydeoxynucleotides one must use DNA polymerase, terminal deoxynucleotidyl transferase or reverse transcriptase. Reverse transcriptase is very costly and difficult to prepare for routine polymer synthesis and DNA polymers are usually synthesised using DNA polymerase. This enzyme requires two complementary nucleotide triphosphates and yields a double-stranded polymer. The resultant hybrid can unfortunately be either a complex of two homopolymers, an alternating copolymer or an intermediate complex. For example, the polymerisation of dITP and
dCTP can yield either poly(dI). poly(dC) or poly(dI - dC) depending on the composition of the polymerisation medium and its pH.\textsuperscript{125} The structure of a polymer made in this way would need to be verified by nearest neighbour analysis. If a single-stranded deoxyhomopolymer is required this step has to be followed by a buoyant density separation of the two strands.

One of the few enzymes known to synthesise single-stranded deoxyhomopolymers is terminal deoxynucleotidyl transferase. (An enzyme which utilises dNDP's to synthesise deoxypolymers has recently been isolated from E. Coli but its properties are not well known as yet.)\textsuperscript{127} Terminal transferase isolated from calf thymus gland\textsuperscript{128}, \textsuperscript{129} can be used to prepare single-stranded polydeoxy-nucleotides according to the following reaction.

\[
d(pN)_m + n\text{dNTP}^{Mg^{++}} \rightarrow d(pN)_m(pN)_n + np\Pi
\]

where \( m \geq 3 \), \( n \geq 50 \).

The resulting polymers are graft polymers and the discovery that the enzyme can use ribonucleotide triphosphates in a limited manner has enabled it to be used for the sequence analysis of oligodeoxynucleotides.\textsuperscript{130} The problems associated with this enzyme are its primer requirement, its requirement for deoxynucleotide triphosphates and its low yields of polymer.

Terminal deoxynucleotidyl transferase is still a relatively unknown enzyme which is not commercially available in any quantity. The purification procedure described here is a relatively quick and fairly easy method for preparing large quantities of highly active enzyme at the cost of some loss in purity. Terminal transferase is technically
a difficult enzyme to handle as it aggregates readily, is thermally unstable and is inactivated by certain divalent metal ions.\textsuperscript{131} Deproteinisation of the polymer product is also a problem since the enzyme operates at 1 mM substrate concentration which means the concentration of product is also very low. The primer requirement is also a problem since until recently oligodeoxynucleotides were not commercially available. An alternative to the use of deoxy primers is to prime the reaction with ribonucleotides.\textsuperscript{132} The ribo polymer can later be readily hydrolysed with ribonuclease.

Since the preparation of a DNA.RNA complex was to be attempted and a single-stranded deoxyhomopolymer was required, terminal deoxynucleotidyl transferase was the logical choice of polymerising enzyme. The primer was generated by the hydrolysis of several O.D units of commercial poly(dI) and by the use of d(pT)\textsubscript{3}.

**EXPERIMENTAL**

Polydeoxyinosinic acid was synthesised using terminal deoxynucleotidyl transferase \textsuperscript{133} (Bollum's enzyme). The enzyme was prepared as follows:–

**Terminal deoxynucleotidyl transferase (TDTase)**

TDTase was isolated by Bollum et al as an impurity in calf thymus DNA polymerase\textsuperscript{128} and it was subsequently purified to give a homogeneous enzyme which consisted of two sub units.\textsuperscript{129} The enzyme isolation procedure of Bollum et al was used with modifications to reduce the preparation time. Although the resulting enzyme was not homogeneous, it was a highly active preparation suitable for synthesising polynucleotides and it could be prepared in just over a week.
dATP was purchased from Boeringer Corp., d(pT)₃ from P-L Biochemicals Inc. and sodium cacodylate from Sigma Chemical Corp. The sodium cacodylate was further purified by recrystallisation from methanol/ether.

Sodium azide is routinely used in the polymerisation medium of polynucleotide phosphorylase and in subsequent buffers to maintain sterility. However TDTase is reversibly inhibited by azide in solution at 0.02% w/v and care must be taken that all columns used are washed free of azide to avoid spurious assay results. Sterility was maintained throughout the enzyme preparation and in later preparative reactions by using sterile equipment, working at 5°C as far as was possible and using cacodylate in buffers as a bactericide.

**TDTase assay**

Terminal transferase activity was measured by the polymerisation of dATP into acid-insoluble material in the presence of d(pT)₃ as initiator. Deoxyadenosine triphosphate polymerisation was carried out by incubating the enzyme at 35°C in 0.2 M sodium cacodylate (pH 7.2), 4 mM MgCl₂, 1 mM 2-mercaptoethanol, 10 μM d(pT)₃ and 1 mM[^1H]dATP. (S.A., 1.2 x 10⁶ cpm/μmole.) The reaction was followed by measuring the amount of product formed as a function of time. The product was precipitated by TCA on glass fibre discs¹³³ and the amount determined by scintillation counting, as previously described.⁶³ The specific activity of the enzyme is expressed as enzyme units per mg of protein. One unit of enzyme activity is defined as that which produces 1 nanomole of dNTP incorporation per hour at 35°C.
Routine assays were carried out by addition of enzyme (10 μl) to the reaction mixture (50 μl) in a melting point tube and incubating at 35°C for one hour. The ends of the melting point tubes were sealed with paraffin wax prior to use and after the incubation the contents were absorbed onto the glass fibre disc by inverting the tube and piercing the wax plug with a hot needle.

The source of the terminal transferase was calf thymus gland which was frozen immediately after removal from the animal and which had not been kept for more than one month. The preparation was carried out on a 2.5 Kg scale. During the preparation where possible 1 mM EDTA was included in all the solutions to prevent aggregation and denaturation of proteins by contamination with traces of metal ions.

**Purification procedure**

The thymus glands were thawed ready for use and the extraneous tissues and fat were removed. The glands were then finely chopped and placed in a Waring blender together with the cold extracting solution (12 litres containing 0.499 moles NaCl, 0.095 moles KH₂PO₄, and 0.405 moles K₂HPO₄, pH 7.3 - 7.4). The turbid solution obtained was adjusted to pH 6.5 by careful addition of 10% acetic acid and then allowed to settle overnight. The resulting precipitate was spun down (2,000 r.p.m.) and the supernatant filtered through cheese cloth. To the crude extract was added a slurry of phosphocellulose (equivalent to 250 gms dry weight) and the pH adjusted to 6.5 if necessary.

The suspension was mixed slowly but thoroughly for one hour
during which time protein absorption can be detected by the watermelon red colour of the phosphocellulose. The red colour follows the enzyme throughout the purification and is a useful indication of the location of the enzyme. The phosphocellulose was allowed to settle for one hour and the supernatant discarded. The phosphocellulose slurry which contains 85% - 95% of the enzyme activity was washed twice with 10 litres of 0.05 M phosphate buffer, pH 6.5 containing 1 mM mercaptoethanol and then it was sedimented by centrifugation at 2,000 r.p.m. The enzyme was eluted from the cellulose by extracting with 0.2 M phosphate buffer pH 7.2, (2 x 1 litre) followed by centrifugation. The supernatant was passed through a DEAE cellulose column to remove residual nucleic acids. The resulting solution was desalted, either by the dilution method described by Bollum or by dialysis until the buffer is 0.05 M phosphate, pH 7.2. A quicker alternative procedure is to dilute the enzyme solution to 0.03 M phosphate pH 7.2 and then to continue the purification immediately by the addition of a phosphocellulose slurry (equivalent to 100 gm dry material). The transferase readily absorbs onto the cellulose and after stirring for one hour the cellulose was sedimented by centrifugation. The enzyme was eluted with 0.2 M phosphate buffer pH 7.2 (2 x 500 mls).

The protein was concentrated by precipitation with ammonium sulphate (80%), the 55% saturation described in the literature was found to be insufficient to precipitate most of the transferase. The extract at this point contained considerable DNA polymerase activity which is totally destroyed in the acid treatment of the next step.
The protein solution (70 mgs protein/ml, 20 mls in 0.1 M KH₂PO₄) from the ammonium sulphate fractionation was diluted to 200 mls with 0.01M KH₂PO₄ and the pH. adjusted to 4.5 with phosphoric acid. The solution was then allowed to stand at 5°C for three days and the resulting precipitate removed by centrifugation. The supernatant was concentrated by precipitation with ammonium sulphate at 80% saturation. The precipitate was dissolved in 100 mls of 0.1 M KH₂PO₄ and clarified by centrifugation. The enzyme was stored in this buffer and appeared to lose no activity over several months.

The number of enzyme units recovered from 2.5 Kgs calf thymus was 2 x 10⁵ in 300 mgs of protein i.e. a specific activity of 666 units/mg. Pure TDTase prepared by Bollum had a specific activity of 99,787 units/mg, however a high specific activity is not essential for synthetic purposes.

Preparation of primer

TDTase is a primer dependent enzyme which can be primed by large or small polydeoxynucleotide fragments and even under some circumstances by RNA. Poly(dI) is commercially available, but very expensive, however oligo(dI) is not available. Oligodeoxynucleotides are usually made by the method of Bollum⁴ which involves exhaustive treatment of poly(dI) with DNase I followed by chromatographic isolation of the oligomers. Removal of the enzyme is essential if the oligomers are to be used as primers for polydeoxynucleotide synthesis. The method described here was to insolubilise DNase I.

Insoluble DNase I

DNase I has been bound to glass⁵ but not to sepharose which was
the support chosen. The general procedure of Axén and Ernback was used. 136

A solution of cyanogen bromide in water (100 mgs in 4 mls) was prepared and sepharose 4B (500 mgs) was added. The solution was stirred vigorously and the pH. maintained constant at 11.0 by addition of 2 M NaOH. After 6 - 8 mins. the reaction was complete and the polymer was washed onto a porous glass filter. The activated sepharose was washed for 5 - 8 mins. with cold 0.1 M NaHCO₃ (300 mls, pH. 8.5) and was then ready for coupling with the enzyme.

To the activated sepharose (6 mls, 500 mgs dry weight) was added 1 ml of coupling buffer (0.1 M NaHCO₃, pH. 8.5) containing 16 mgs DNase I and the mixture then stirred for 16 hrs at 4°C. The preparation was washed with 0.1 M NaHCO₃, 1 M NaCl and water successively and then stored in 0.14 M NaCl, 0.01 M Tris/Acetate, 1mM MgCl₂, pH. 7.0. containing 0.02% NaN₃. The bound enzyme, unlike soluble DNase, can be stored for several months at 4°C without detectable loss of activity.

**Protein estimation**

Protein binding to sepharose was assayed by the method of Crook et al. 137 The insolubilised enzyme was lyophilised, 100 mgs placed in a hydrolysis tube containing 6 N HCl and heated at 110°C for 24 hrs. A control was set up containing 1 mg DNase I and approximately the same amount of dry sepharose. After the hydrolysis was complete the contents were filtered and lyophilised. The residue was made up to 10 mls with water. A series of standards were prepared containing 0.01 + 1 ml of the control solution. The aliquots were made up to 1.0 ml with water and to each sample was added 0.5 mls 0.1 M citrate,
FIG. 22

3% polyacrylamide gel scans of calf thymus DNA before (----) and after (.....) passage through a DNase-sepharose column as described in the text.
Tm of the poly (dI).poly (C1C) complex in phosphate buffer pH 7.8, 0.01M Na⁺ (●), 0.10M Na⁺ (▲), 1.00M Na⁺ (■).

% CHANGE IN ABSORBANCE AT 246 nm
pH. 5.0, 0.2 ml ninhydrin (5% w/v in 2-methoxyethanol) and 1 ml KCN solution (2% v/v of 0.01 M aqueous KCN in 2-methoxyethanol). The samples were heated at 100°C for 15 mins, cooled and the absorbance read at 570 nm, diluting if necessary with 60% ethanol. A standard curve was constructed. Using the same procedure the 100 mgs of sample was found to contain 1.5 mgs of protein and therefore the original 500 mgs (dry weight) of sepharose had 7.5 mgs of protein bound. 16 mgs DNase I had originally been applied to the sepharose and therefore 47% was bound to it.

The sepharose was shown to be enzymatically active by the rise in O.D 260 on repeated cycling of calf thymus DNA through the column (1 x 6 cms). 10 O.D 260 units of DNA in 0.14 M NaCl, 0.01 M TRIS/acetate, 1 mM MgCl₂ (pH 7.0), were washed through the column in 10 mins. The hydrolysis of DNA was shown to occur by applying samples of the eluant and DNA itself to polyacrylamide gels (3%). The electrophoresis was carried out at 5 mA/gel for five and a half hours and the resulting gels scanned at 260 nm. The results are shown in fig. 22.

Oligodeoxyinosinic acid primer was made by passing 5 O.D 260 units of poly (dI) through the column in 0.5 mls of buffer (as described above) over a period of one hour. The resulting solution was used as a primer for the synthesis of poly (dI).

**Synthesis of deoxyinosine triphosphate**

dITP is an extremely expensive nucleotide and in order to be able to study its rate of polymerisation radioactive dITP is required, which is even more expensive. However, dATP is relatively cheap and there are several methods described in the literature for the
deamination of adenosine to inosine. The method of Sigel et al.\textsuperscript{130}, in which AMP is treated with nitrosyl chloride in DMF in our hands failed to give IMP, although the product has a $\lambda_{\text{max}}$ at 248 nm. The method of Inman and Baldwin\textsuperscript{130} for converting ATP to ITP is difficult and only gives moderate yields. The following method is an extremely mild method for the deamination of adenosine derivatives and produces dITP from dATP in almost quantitative yield. Dephosphorylation of the dITP produced yielded only deoxyinosine.

Sodium nitrite (300 mgs) was added slowly with cooling to dATP (100 mgs) in 10% acetic acid (3 mls) at 0\degree C. The reaction mixture was stored in ice for 24 hrs. and then lyophilised before applying to a DEAE cellulose column (HCO\textsubscript{3}\textsuperscript{-} form, 1 x 40 cms). The column was eluted with a gradient of triethylammonium bicarbonate buffer. The product was lyophilised and converted to the sodium salt by dissolving the nucleotide in methanol and precipitating with sodium iodide in acetone. After washing with methanol the yield was found to be quantitative.\textsuperscript{[14C]}-dITP (5 x 10$^6$ dpm/m.mole) was made by the same procedure.

Preparative synthesis of polydeoxyinosinic acid

Poly(dI) was prepared in a reaction mixture which contained: 40 mM sodium cacodylate, pH. 6.8, 1 mM 2-mercaptoethanol, 8 mM MgCl\textsubscript{2}, 10 $\mu$M oligodeoxyinosinic acid (estimated as trimer), 1 - 3 mM dITP, 4,000 U/ml of TDTase, 0.1 $\mu$g/ml of yeast inorganic pyrophosphatase. The reaction was started by the addition of terminal transferase to the polymerisation mixture incubating at 35\degree C. The reaction was monitored by using \textsuperscript{[14C]}-dITP as substrate. The yield of polymer after a 24 hr.
Variation in ultraviolet absorption on mixing poly (dI) and poly (dC) in 0.14M sodium phosphate buffer, pH 7.8 at 25°. Readings were taken 6hr. after mixing.
FIG. 25

Ultraviolet spectra of poly (dI) (.....),
poly (ClC) (-----), poly (dI)\(\cdot\)poly (ClC) (———),
and the summation spectra poly (dI) + poly (ClC)(--.--)
All spectra recorded in 0.1M sodium phosphate
pH 7.8 at 25°.
incubation was found to be 14%.

The polymer was deproteinised by extraction with chloroform/isoamyl alcohol (5.2 V/v) and can be further purified by gel filtration. The resulting polymer was desalted by dialysis against 0.01 M EDTA and twice against water before lyophilisation. The overall yield from a 10 ml incubation was 1 mg poly(dI).

**Characterisation of poly(dI)**

Poly(dI) had a $S_{20,w}$ of 5 determined by ultracentrifugation in an isokinetic gradient of sucrose containing 0.1 M sodium acetate (pH 7.8) at 30°C. The U.V. maximum of poly(dI) at 25°C in 0.14 M NaCl, 0.01 M Na$_2$HPO$_4$ (pH 7.0) was 246 nm ($\Theta(p) = 9.5 \times 10^3$).

**Preparation and properties of hybrids**

Poly(dI) was hybridised with poly(C) and poly(C1C). The poly(dI). poly(C) complex has been made by Chamberlain and Patterson and the Tm's of the hybrid in 0.01 M, 0.1 M and 1.0 M sodium phosphate buffer, pH 7.8, were found to be 10.1°, 35.4° and 52.6° respectively. The poly(dI). poly(C) complex made in this laboratory by hybridising poly(C) ($S_{20,w} = 6.2$) with poly(dI) ($S_{20,w} = 12.55$) in phosphate buffer, pH 7.8 containing sodium chloride agreed with the above data. In order to increase the Tm of this DNA/RNA complex the poly(dI) was hybridised with poly(C1C) ($S_{20,w} = 5.45s$) and under identical conditions the Tm's in 0.01 M, 0.1 M and 1.0 M [Na$^+$] were found to be 38°, 57° and 87° respectively. (fig. 23)

There is evidence that at salt concentrations greater than 0.6 M [Na$^+$] the poly(dI). poly(C) complex can disproportionate to form
the triple-stranded poly(dI). [poly(dI). poly(C)] complex. To check if this occurs under physiological ionic strength the stoichiometry of the hybridisation in 0.1 M Na\(^+\), pH 7.8 was investigated by the method of continuous variation. (fig. 24) This showed at 245 nm a break in the curve at 50% corresponding to the formation of a 1 : 1 hybrid. The summation U.V. spectrum of poly(dI) and poly(C1C) are shown in fig. 25 together with the spectrum of the poly(dI). poly(C1C) complex.

The poly(dI). poly(C1C) and the poly(dI). poly(C) complexes were tested as inducers of interferon and the results are given in the biological section of this thesis.
BIOLOGICAL RESULTS

Antiviral activity of poly (5-halogenated cytidylic acid). poly (Inosinic acid) complexes.

These compounds were tested by Dr. T. Cartwright of G.D. Searle & Co. Ltd., and his findings are listed in his preliminary report which is shown below.

INTERFERON INDUCTION BY HALOGENATED POLYNUCLEOTIDES

Samples of substituted poly C were dissolved in tissue culture medium (I = 0.15 pH 7.2) and hybridized at 37° for 16 hours with commercial poly I (Sigma). The optimum hybridization proportions were determined by separate experiment.

All testing of interferon inducing capacity was performed in vivo and three different systems were employed as follows:-

1. Intra-venous injection of polynucleotides in rabbit followed by titration of serum interferon levels.
2. The same in mice.
3. Measurement of the degree of protection afforded by intracerebral injection of polynucleotide followed by intracerebral injection of Semliki Forest virus in suckling mice.

In the first two systems it has not yet been possible to demonstrate any significant difference between the interferon producing capacity of Miles poly (IC) and the chlorinated and brominated derivatives. All three compounds behave uniformly as inducers of high potency giving interferon titres in the serum in excess of 2000 over a relatively wide dose range.

In the suckling mice system it appears that poly I : 5 Cl C is somewhat less effective than unmodified poly (IC) or the brominated derivative. Three separate experiments have all produced this result although the experimental variation is such that more runs need to be performed. Table II shows results from one such experiment.
Table II

Prolongation (hrs) of mean death time of suckling mice inoculated intracerebrally with polynucleotide before challenge by the same route with Semliki Forest Virus. Mean death times in control mice were 35 hours at virus 32 LD$_{50}$ and 40 hours at 1 LD$_{50}$.

<table>
<thead>
<tr>
<th>Dose (μg/mouse)</th>
<th>Poly I:C</th>
<th>Poly I:5 Br C</th>
<th>Poly I:5 Cl C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 LD$<em>{50}$ 32 LD$</em>{50}$</td>
<td>1 LD$<em>{50}$ 32 LD$</em>{50}$</td>
<td>1 LD$<em>{50}$ 32 LD$</em>{50}$</td>
</tr>
<tr>
<td>2.5</td>
<td>7.5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>5.0</td>
<td>9</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>10.0</td>
<td>9</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>25.0</td>
<td>13</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>62.5</td>
<td>-</td>
<td>-</td>
<td>11</td>
</tr>
<tr>
<td>125.0</td>
<td>30</td>
<td>30</td>
<td>13</td>
</tr>
</tbody>
</table>

Since this report poly (ClC) poly (BrC) and poly (iodo C) complexed with poly (I) have been shown to be as active as poly (I). poly (Cl) in a protection assay using human fibroblast cells challenged with sindbis virus. The polynucleotides were left in contact with the cells for one hour at 37°C. The cells were then washed and reincubated for a further twenty four hours after which they were challenged with virus and the cytopathic effect (c.p.e.) measured. poly (ClC). poly G and poly (Cl). poly (G) were also assayed.

The minimum inhibitory concentrations were as follows:-
It appears that in the systems tested the halogenated cytidylic acid complexes are only as effective as poly (I).poly (C) despite the fact that they are thermally more stable and also more resistant to nucleolytic attack.

**Interferon induction by poly (5-halogenocytidylic).poly(deoxyinosinic acid) complexes**

The polynucleotide hybrids shown below were dissolved in phosphate buffered saline (P.B.S.) to give a concentration of 50 μg/ml and were tested for their ability to induce interferon in human embryo fibroblast cells. The interferon assay, using the same cell line and semliki forest virus as challenge, was an adaptation of the neutral red dye uptake method. The assays were performed by Dr. M. Johnston, of the School of Biological Sciences, The University of Warwick.

<table>
<thead>
<tr>
<th>Polynucleotide hybrid</th>
<th>Tm(0.1M phosphate pH 7.8)</th>
<th>Hyperchromicity %</th>
<th>Interferon Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly (dI).poly (rC)</td>
<td>350</td>
<td>46</td>
<td>&lt;2</td>
</tr>
<tr>
<td>poly (dI).poly (ClC)</td>
<td>570</td>
<td>20</td>
<td>&lt;2</td>
</tr>
<tr>
<td>poly (dI).poly (BrC)</td>
<td>660</td>
<td>15</td>
<td>&lt;2</td>
</tr>
<tr>
<td>poly (dI).poly (IodoC)</td>
<td>680</td>
<td>27</td>
<td>&lt;2</td>
</tr>
<tr>
<td>poly (rI).poly (rC)</td>
<td>610</td>
<td>48</td>
<td>55</td>
</tr>
</tbody>
</table>

These results, which have since been repeated by Dr. T. Cartwright using a superinduction assay, confirm earlier findings that a 2'-hydroxyl
group is required in both strands of the complex.

**Antiviral activity of poly(5-hydroxy cytidylic acid)**

Poly (OHC) and poly (I) although they apparently fail to form a detectable hybrid were mixed 1:1 and tested for antiviral activity. The results of the protection assay are summarised below:

<table>
<thead>
<tr>
<th>M.I.C. (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly (OHC/poly (I))</td>
</tr>
<tr>
<td>poly (OHC)</td>
</tr>
</tbody>
</table>

At the present time it is uncertain whether poly (OHC)/poly I induces interferon or not, however, it appears to be highly active in the cell protection assay!
DISCUSSION

Although in 1973 it seems that we are no closer to understanding the mechanism of interferon induction and how it exerts its antiviral effect, a considerable number of questions have been answered since its discovery. The vast numbers of publications on the mechanism of interferon induction have been the subject of many excellent reviews and these clearly show the problems involved.

Burke, mainly from his work with inhibitors of protein synthesis and nucleic acid synthesis, has divided the process of interferon production by viruses into three stages.  

a) Virus invasion, which is presumably followed by the dissolution of the virion and release of the substance which induces interferon formation.

b) Interaction of the inducer, either directly or indirectly, with the host cell genome, which then leads, by a process similar to de-repression, to synthesis of interferon messenger RNA.

c) This m-RNA then directs the synthesis of interferon and any other proteins which might be involved in its production.

Burke's hypothetical scheme, show in fig.26, accommodates all the available evidence and without speculations it is probably as much as one can say at the present time.

After the virus infects the cell the virion causes the synthesis of interferon m-RNA by a de-repression mechanism. This is shown by the action of low doses of actinomycin. Although the control mechanisms of bacterial protein synthesis have been explained in terms of the Jacob-Monod de-repression model, little is known about mammalian control mechanisms. The interaction of the viral RNA with the host genome is therefore a matter of speculation. However, Kleinschmidt has designed a model which explains interferon induction in terms of a de-repression mechanism.
Current concepts about interferon synthesis and action. Shown in the scheme are the events following the penetration of an RNA-virus (1) into a susceptible cell (Cell 1). The viral RNA which is released from its protein capsule (2) combines with cell ribosomes into a polyribosome (3). Such polyribosomes produce virus-specific proteins (4), including one or more enzymes necessary for the replication of viral RNA. A double-stranded, replicative form of viral RNA is known to be formed (5), followed by the synthesis of progeny viral RNA (6). Some of the newly synthesized viral RNA will combine with cell ribosomes and produce more virus-specific proteins, including the capsid protein (7). The latter assembles with the newly formed viral RNA into new virus particles (8). The virus which penetrates into the cell, before or after uncoating, or perhaps newly synthesized virus particles or intermediary products of viral synthesis, activate the locus in the cell DNA which carries the information for the synthesis of interferon (9). (See Fig. 2 for possible mechanisms of this particular step.) Messenger RNA for interferon (10) and the interferon protein itself (11) are synthesized. Most of the newly synthesized interferon is rapidly released from the cell, but some will activate another locus of the cell DNA (12) to make messenger RNA (13) for the translation inhibitory protein (TIP) (14) which represents the proper antiviral molecule. It is believed that this protein reacts in some way with cell ribosomes (15). The ribosomes then either cannot form polyribosomes with the viral RNA at all or form non-functional viral polyribosomes. The same ribosomes can probably function normally with cellular messenger RNA. The heavy interrupted line points to the two possible steps inhibited by TIP. Since step 13 will usually not be completed until quite late in the cycle of virus infection, interferon may have relatively little effect on the course of the original virus infection shown in Cell 1 of the scheme. However, Cell 1 will be resistant to superinfection with another virus. Interferon released from Cell 1 may reach another cell (Cell 2) in which it will induce the synthesis of TIP (steps 14-15). Cell 2 will then be resistant to viral infection.

Diagrams are reproduced from ref 141-143.
and this is shown in fig. 27.

In this model de-repression of the host genome occurs by the inducer neutralising the repressor which then permits the coding of a m-RNA for the interferon molecule. Kleinschmidt postulates that the inducer must be of a polyanionic nature and that the repressor could possibly be a histone. This hypothesis does not however explain the remarkable selectivity for a double-stranded ribopolymer which is required for interferon induction by synthetic polynucleotides.

Another observation which has puzzled many workers is the phenomenon of super induction. If actinomycin, which inhibits DNA directed RNA synthesis, is added at the time of induction it can completely inhibit interferon formation. However, if actinomycin is added after the cell has made interferon m-RNA it can actually stimulate an increase in the production of interferon in some cells. This phenomenon, termed super induction, can be interpreted in terms of a protein interferon inhibitor, which is transcribed by the nucleus after the synthesis of interferon m-RNA and which cannot be formed in the presence of actinomycin. There seems to be evidence from the work of Vilcek\textsuperscript{145} and others\textsuperscript{146,147} that two inhibitors of interferon are present, one which operates at a genetic level (the repressor) and controls the synthesis of interferon m-RNA and the other a post-transcriptional control protein (see fig. 27).

Some cells, after the addition of an inducer, enter a refractory period and during this time addition of more inducer does not produce more interferon. This refractory period could be explained by a high cellular concentration of interferon inhibitor which blocks the synthesis of interferon at a post-transcriptional level. Kleinschmidt has suggested that interferon in an oligomeric form may act as a super-repressor of interferon synthesis and cause the hyporeactive state. However, there is no evidence for such a mechanism apart from the observation that interferon can aggregate.
The evidence from experiments using DNA transcription inhibitors shows that interferon production is probably controlled by the host genome. At an actinomycin concentration of 0.06 μg/ml in the chick cell, DNA transcription is completely inhibited and interferon inducing capacity is lost. Other inhibitors of RNA synthesis produce the same effect. The argument that actinomycin inhibits the movement of m-RNA from the nucleus to the cytoplasm is a valid one as the drug has a marked effect on the nucleolus. Protein inhibitors such as puromycin and cycloheximide also prevent interferon production whilst DNA synthesis inhibitors have no effect.

Further genetic evidence about interferon is coming from cell fusion experiments where hybrids of mouse and human cells produce both types of interferon. Also De Maeyer has recently provided direct evidence for interferon m-RNA in mouse embryo fibroblasts. The RNA was extracted from mouse embryo fibroblast cells infected with Newcastle disease virus and put into chick embryo cells which in turn produced mouse interferon.
CONCLUSIONS

The mechanism by which inducers trigger the formation of interferon has been studied extensively although good biological probes have yet to be discovered. Modified polynucleotides have however provided some useful information about the requirements for induction. For example, cross linked mycophahe RNA is as active if not more so than the natural RNA.\textsuperscript{15} This interesting result shows that strand separation probably does not occur at the cell wall, and supports the idea of a receptor protein for double-stranded RNA.

Whilst the necessity for the 2'-hydroxyl group on the sugar in the poly (C) strand of poly (I).poly (C) is well established few studies have been performed with poly (I) modified at the 2' position. Poly (dI).poly (rC) is inactive as an interferon inducer, however, the lack of antiviral activity could be due to strand separation as the Tm in 0.1M Na\textsuperscript{+} is only 35°. This work describes the synthesis of poly (dI).poly r(5-halogenocytidylic acids) which have Tm's in the region of 60°C and these hybrids are still inactive as interferon inducers. This confirms the requirement for a free 2'-hydroxyl group in both the poly (I) and the poly (C) strands of poly (I).poly (C).

Poly (5-halogenated cytidylic acids) when hybridised with poly (I) or poly (C) form strong complexes which are more thermally stable and more resistant to nucleases than the unmodified derivatives.

Interferon production \textit{in vitro} is not enhanced by these modifications, and it seems that the Tm of a poly ribonucleotide is unimportant provided it is well above the incubation temperature. Increased resistance to nucleases appears to have had little effect on the amount of interferon produced, but it could be an unfavourable characteristic as it may increase the cytotoxicity of the complex.
Poly (5-hydroxycytidylic acid) is unusual in that it fails to hybridise with poly (I) and yet possesses antiviral activity. This feature is currently being investigated as it may have an important bearing on the mechanism of interferon induction.
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Poly(5-chlorocytidylic acid)†
M. A. W. Eaton and D. W. Hutchinson*

ABSTRACT: 5-Chlorocytidine diphosphate has been obtained in high yield by the chlorination of cytidine diphosphate using tetrabutylammonium iodotetrachloride. 5-Chlorocytidine diphosphate has been polymerized with polynucleotide phosphorylase and the resultant poly(5-chlorocytidylic acid) has been characterized. Poly(5-chlorocytidylic acid), which is more stable toward hydrolysis by pancreatic ribonuclease than the unchlorinated polynucleotide, possesses considerable secondary structure in acid solution and forms a 1:1 hybrid with poly(I). The thermal stability of the hybrid is considerably higher than that of poly(I)·poly(C).

The discovery that double-stranded polynucleotides, e.g., poly(I)·poly(C) can induce the formation of interferon (Field et al., 1967; Colby, 1971) has encouraged the synthesis of polynucleotides which have been modified in the base (Michelson and Monny, 1967), the sugar (Hobbs et al., 1971), and the phosphate backbone (Eckstein, 1970). Little systematic work has, however, been carried out on the relationship between structure and biological activity in these compounds.

Chemical modification of the pyrimidine bases in the polynucleotides can be readily accomplished as electrophilic attack occurs at the 5 position in pyrimidines and, for example, halogenation of pyrimidine nucleotides gives the 5-halogenonucleotides. Conversion of the latter to the pyrophosphates followed by polymerization with polynucleotide phosphorylase leads to 5-halogenopyrimidine polynucleotides (Michelson and Monny, 1967). The direct bromination of poly(C) to poly(5BrC) has been reported (Means et al., 1969). Introduction of a halogen atom in the 5 position of a pyrimidine has a pronounced effect on the physical properties of the polynucleotide and, for example, poly(5BrC) and poly(5IodoC) possess considerable secondary structure in acid solution (Michelson and Monny, 1967). 5-Substituted cytidine polyribonucleotides form stable hybrids with poly(I) (Ross et al., 1971) and these are inducers of interferon (De Clercq et al., 1970; Colby and Chamberlin, 1969). In the present investigation the hitherto unknown poly(5ClC) has been prepared and the physical properties of the polynucleotide and its complex with poly(I) have been investigated. Biological studies on the poly(5ClC)·poly(I) complex are in progress.

Materials and Methods

Materials. CDP was synthesized from the phosphoromorpholidate (Moffatt and Khorana, 1961) and was converted by ion-exchange chromatography into the trisodium salt before use. UDP, pancreatic ribonuclease type IA (EC 2.7.7.-16), and Crotonus adamantus venom were purchased from Sigma Chemical Corp. Polynucleotide phosphorylase (poly-
ribonucleotide orthophosphate: nucleotidyl transferase, EC 2.7.7.8) from *Micrococcus luteus* was purchased from Boehringer Corp. Polyinosinic acid was purchased from P-L Biochemicals Inc. Tetrabutylammonium iodotetrachloride was prepared from the corresponding iodide (Popov and Backles, 1957) and stored in the dark at 0°.

14C-Labeled CDP was purchased from the Radiochemical Centre, Amersham. The 14C content of samples was measured using a toluene-based scintillation medium containing 2,5-diphenyloxazole (5 g/l.) and 1,4-di(2-(4-methyl-5-phenylloxazoyl))benzene (0.2 g/l.).

Circular dichroic spectra were recorded by Dr. P. M. Scopes, Westfield College, London, on a Russel-Jouan Dichrograph 185 and the values obtained are expressed in terms of molecular ellipticity θ (Green and Mahler, 1970). Ultraviolet spectra were recorded on a Cary 14 spectrophotometer. Molar extinction coefficients of polynucleotides are based on the molecular weights of the monomers.

5-Chlorocytidine 5'-Diphosphate (CICDP). To a solution of CDP trisodium salt (100 mg) in dry formamide (3 ml) was rapidly added a solution of tetrabutylammonium iodotetrachloride (300 mg) in dry dimethylformamide (1 ml). The reaction mixture was stirred magnetically in a well-stoppered reaction vessel until all solid has dissolved and then left 36 hr in subdued light. The solution was poured into water (50 ml) which was then extracted with chloroform (three 50-ml portions). The aqueous layer was applied to a Whatman DE-23 cellulose column (1 x 20 cm, HCO3- form) and the column eluted with a linear gradient of triethylammonium bicarbonate, CICDP being eluted at a concentration of 0.15 M. The triethylammonium bicarbonate was removed by repeated evaporation in vacuo, and the residue was dissolved in water and passed through a Dowex 50 column (Na+ form). The eluate was evaporated to an oil in vacuo below 40°, lyophilization of which gave colorless trisodium 5-chlorocytidine 5'-diphosphate (92 mg): ultraviolet (uv) spectra (0.01 M NH4Cl, pH 7.0) λmax 286.5 nm (ε 6460). 5-Chlorocytidine has a λmax of 287 nm (Fukuhara and Visser, 1955). The pKₐ of CICDP determined spectrophotometrically in citrate–HCl buffer was 2.49 ± 0.05 at 18° and the variation in absorbance of CICDP with pH is shown in Figure 2. Anal. Calcd for C₉H₈ClKaN₂O₁₀P₂: C, 20.03; H, 1.14; Cl, 6.57; N, 7.78; P, 11.48; Found: C, 20.22; H, 1.24; Cl, 6.85; N, 7.71; P, 11.47. The nuclear magnetic resonance (nmr) (100 MHz, D₂O) showed 8.06 ppm (1 H s) inter alia. 14C-Labeled CICDP was prepared in an identical manner from [14C]CDP.

5-Chlorouridine 5'-Diphosphate. CICDP was prepared as the tripotassium salt in an analogous manner from UDP in 94% yield: uv spectra (H₂O, pH 7.0) λmax 277 nm (ε 8300), lit. (Massoulid et al., 1966) λmax (H₂O, pH 7.0) 276 nm; nmr spectra (100 MHz, D₂O) 8.09 ppm (1 H s) inter alia. Anal. Calcd for C₉H₈ClK₃N₂O₁₀P₂: C, 19.55; Cl, 6.41; H, 1.82; N, 5.07; P, 11.20; Found: C, 19.75; H, 2.01; Cl, 6.23; N, 4.93; P, 10.92.

Dephosphorylation of CICDP. [14C]CICDP (10 μmoles) in 0.1 M Tris-acetate buffer (pH 8.0) was incubated with *Crotalus adamanteus* venom (100 μg) for 12 hr at 37°. Examination of the reaction mixture by paper chromatography (Howard et al., 1969) showed only one radioactive compound with the same Rₚ as authentic 5-chlorocytidine.

Synthesis of Poly(5-chlorocytidylic acid) (Poly(CIC)). The polymerization of CICDP by polynucleotide phosphorylase was followed both by release of inorganic phosphate (Fiske and Subbarow, 1925) and incorporation of 14C into polymeric material over a range of substrate concentrations to determine the optimum conditions for polymerization. In a typical case, the reaction medium which contained 20 mM CICDP, 1.24 mg/m mole of polynucleotide phosphorylase, 10 mM MgCl₂, and 5 mM EDTA in 0.15 M Tris-chloride buffer (pH 9.0) was incubated for 10 hr at 37°. After deproteinization by repeated extraction with chloroform–isooamyl alcohol (5:2, v/v) (Scheit and Gacrtner, 1969), the aqueous phase was desalted by dialysis over 36 hr at 5° against 0.1 M NaCl–0.001 M NaEDTA, 0.001 M NaEDTA, and then twice against water. Lyophilization at 0° gave pool (CIC) in 53% yield (62% by release of phosphate).

Polymerization reactions with 14C-labeled CICDP (2 x 10⁶ dpm/μmole) were monitored as follows. Aliquots were removed at different times applied to strips of Whatman No.
FIGURE 3: Spectrophotometric titration at 310 nm of 1.85 × 10⁻⁴ M solution of poly(CIC) (O) and ClCDP (●) in 0.3 M NaCl at 25°.

FIGURE 4: Temperature-ultraviolet absorption profiles at 310 nm of poly(CIC) at different pH values. Concentration of polymer, 1.35 × 10⁻⁴ M; buffer 0.1 M NaCl-0.05 M sodium acetate.

Characterization of Poly(CIC). Poly(CIC) has a S₀₂₀,₅ of 4.20 S determined by ultracentrifugation in an isokinetic gradient of sucrose containing 0.1 M sodium acetate (pH 7.5). The UV maximum of poly(CIC) at 25° in 0.01 M ammonium acetate (pH 7.0) was 286 nm (ε 5050 ± 50), heating for 10 min at 95° caused a hypochromic change in absorption of 11.5% ((A₂₈₆ - A₃₈₆)/A₂₈₆). Pancreatic RNase digestion of poly(CIC) gave a hypochromicity of 30% ((ν₀₃₈₆ - ν₀₃₈₆).
absorption in transition in the region of pH 4 (Figure 3) and the ApOlr-1-ADlD0mer which agrees well with the value 27.5% calculated using $A_{max}$ in place of $A_{monomer}$. The ultraviolet absorption spectra of poly(CIC) in acid and in neutral solutions are shown in Figure 2. Spectrophotometric titration at 310 nm of poly(CIC) in 0.3 M NaCl solution showed an abrupt transition in the region of pH 4 (Figure 3) and the $T_m$ of the polymer was also dependent on pH in this region (Figure 4).

Hydrolysis of Poly(CIC) by Pancreatic RNase. The polymer (0.2 μl) in 0.1 M ammonium acetate (pH 7.0) (2 ml) was treated with 0.5 μg of RNase (Massoulié et al., 1966). Under these conditions poly(C) has $t_{1/2} = 5$ sec, poly(CIC) has $t_{1/2} = 50$ sec, and poly(BrC) (Howard et al., 1969) has $t_{1/2} = 80$ sec determined spectrophotometrically at room temperature.

Preparation and Properties of a Poly(I)-Poly(CIC) Hybrid. Equimolar quantities of poly(I) (s20,w = 6.64 S) and poly(CIC) were dissolved in 0.01 M sodium acetate (pH 7.0) at 37°C to give a 1:1 hybrid. The solution was left at room temperature for 2 hr and then applied to a Sepharose 4B 200 column. Elution of the column with acetate buffer gave double-stranded poly(I)-poly(CIC) in the void volume followed by a small amount of unannealed material. The ultraviolet spectrum in 0.01 M sodium acetate (pH 7.0) is shown in Figure 5 and the polymer has a $A_{max}$ of 280 (ε 6600) and 245nm (9500).

The stoichiometry of the hybridization was determined by the method of continuous variations (Job, 1928) in 0.1 M NaCl-0.005 M sodium cacodylate (pH 7.0) at 25°C (Figure 6). At 245 nm there is a break in the curve at 50% corresponding to the formation of a 1:1 hybrid, at 286 nm no break can be observed. The melting profiles of poly(I)-poly(CIC) are summarized in Figure 7, the $T_m$'s in 0.01, 0.1, and 0.3 M NaCl are 65, 75, and 82°C, respectively. Poly(I)-poly(BrC) has $T_m$ values of approximately 65, 83, and 88°C under the same conditions (Howard et al., 1969).

The circular dichroic spectra of poly(CIC) and its 1:1 hybrid with poly(I) are shown in Figure 8. The polymers were dissolved in 0.1 M sodium chloride-0.005 M sodium cacodylate (pH 7).

Discussion

Cytidine nucleosides have been chlorinated in the 5 position of the pyrimidine ring using N-chlorosuccinimide (British Patent, 1969) or chlorine and ultraviolet irradiation (Fukui and Visser, 1955; Frisch and Visser, 1959). The yields of chlorinated nucleosides are only moderate and we have found that when these methods are applied to the chlorination of CDP, decomposition of the pyrophosphate residue occurs. Tetrabutylammonium iodotetrachloride has been used to chlorinate olefins and phenols under mild conditions (Buckles and Knaack, 1960), and we have found that this reagent will chlorinate CDP and UDP in virtually quantitative yield. An advantage of this method is the ease with which excess iodotetrachloride can be removed from the reaction mixture by extraction with chloroform. Diphosphorylation of 14C-labeled CICDP by Crotalus adamanteus venom indicated that chlorination of the pyrimidine was complete. The only radioactive material which could be detected by paper chromatography was 5-chlorocytidine. The position of chlorination in the cytidine ring was confirmed by $^{1}H$ nmr spectroscopy, as the signal due to H1 which appears as a doublet at 6.05 ppm in CDP was absent. The signal due to H1 in CICDP appeared as a singlet at 6.06 ppm. Similarly, in the nmr spectrum of CIUDP the signal due to H2 of the uridine was absent while the signal due to H2 appeared at 8.09 ppm. Polycondensation of CICDP with polynucleotide phosphorylase gave poly(CIC). Little phosphorolysis appeared to occur and the polymerization was allowed to proceed for 10 hr after which time poly(CIC) was isolated and found to have $s_{20,w} = 4.20$ S. Poly(CIC) possessed considerable secondary structure in acid solution, and a sharp change in uv absorption was observed at 310 nm around pH 4 in 0.3 M salt solution. In contrast to the gradual change in the spectrum of CICDP with pH (Figure 2) the change in absorption of poly(CIC) occurred over a narrow pH range (ca. 0.2 pH unit). Furthermore the shape of the melting curve for poly(CIC) changes abruptly in shape in this pH range. This indicates that a cooperative phenomenon is taking place and that poly(CIC) has a helical structure in acid solution. Similar behavior has been observed with poly(BrC) (Michelson and Monny, 1967) and poly(BrC) (Inman, 1964).

Halogenation of the cytidine ring renders the polymer more resistant to hydrolysis by pancreatic ribonuclease. At pH 7.0 in the presence of an excess of enzyme the half-life for poly(CIC) hydrolysis was approximately ten times greater than for poly(I).

![Figure 5: Ultraviolet spectra of poly(CIC) at pH 7.0 (---) and pH 2.0 (----), and the 1:1 hybrid poly(I)-poly(CIC) at pH 7.0 (---) in 0.1 M NaCl-0.005 M sodium cacodylate at 25°C.](image)

![Figure 6: Variation in ultraviolet absorption on mixing poly(CIC) and poly(I) in 0.1 M NaCl-0.005 M sodium cacodylate (pH 7.0) at 25°C. Total polymer concentration 0.96 × 10^{-4} M. Readings were taken 6 hr after mixing.](image)
than the half-life for poly(C) hydrolysis while poly(BrC) was even more stable under the same conditions.

The circular dichroism (CD) spectrum of poly(CIC) in neutral solution consists of a maximum at 296 nm (θ 34,700), and thus resembles the CD spectrum of poly(C) which consists of a maximum at 277 nm (θ 59,000) (Green and Mahler, 1970). The CD spectrum of the 1:1 complex of poly(CIC) with poly(I) shows two principal maxima, one at 294 nm (θ 14,000) the other at 244 nm (θ 23,250). This resembles the CD spectrum of poly(I)-poly(C) which consists of two maxima one at 277 nm (θ 15,550) the other at 245 nm (θ 17,200). It has been shown (Brahms and Sadron, 1966) that the CD spectrum of poly(I) consists of a maximum at 248 nm. Hence it appears that the CD spectra of poly(I)-poly(C) and poly(I)-poly(CIC) are made up of components due to the purine and pyrimidine residues which is not the case for poly(A)·poly(U), where the CD spectrum consists of a single maximum at 262–265 nm (Brahms, 1965).

Poly(CIC) forms a 1:1 complex with poly(I) and like other 5-substituted polycytidylic acids the thermal stability of this complex is greater than that of poly(I)·poly(C) over a range of salt concentrations. It has been shown (Ross et al., 1971) that substitution in the cytidine ring by bromine produces a large enthalpic stabilization of the complex with poly(I) probably due to the introduction of the polarizable bromine atom into the heterocyclic system. Chlorine is less polarizable than bromine and if polarizability were an important feature of the stabilization forces, poly(CIC) should form a less stable complex with poly(I) than poly(BrC). We have found that at a given salt concentration the melting temperatures of poly(I)-poly(CIC) complexes are slightly lower than those of poly(I)-poly(BrC) complexes. Furthermore, it has been shown (Michelson and Monny, 1967) that melting temperatures of polyinosinic acid·polyiodocytidylic acid complex is higher than the poly(I)-poly(BrC) complex.

Acknowledgment

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References

KINETICS OF BOVINE AND HUMAN PLASMINS

ISOCOUMARINS FROM *STREPTOMYCES MOBARAENSIS*

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Isocoumarins are of less widespread natural occurrence than coumarins. Some have been obtained from micro-organisms (1-3) and 3,4-dihydroisocoumarins have been isolated from corn (4) and bitter carrots (5). We wish to report the isolation from *Streptomyces mobaraensis* of a series of isocoumarins (I-IV) which differ only in the substitution of the aromatic ring.

\[
\begin{array}{c c c c}
R^1 & R^2 \\
I & OMe & H \\
II & OMe & OMe \\
III & OH & OMe \\
IV & OH & OH \\
\end{array}
\]

*S. mobaraensis*, a micro-organism which produces the Piericidins (6), was grown aerobically for four days at 27°C in a medium which contained glucose (2%), peptone (0.7%), dipotassium hydrogen phosphate (0.2%) and sodium chloride (0.2%). The cells were separated from the growth medium (20 l) by centrifugation, extracted with acetone (4 x 1 l), the extracts dried and evaporated to dryness in vacuo (Extract A). The growth medium after removal of the cells was brought to pH 5 with sulphuric acid and extracted with ethyl acetate (3 x 1 l). The ethyl acetate extracts were dried and evaporated to dryness in vacuo (Extract B).

Extracts A and B were then subjected to column chromatography on silicic acid. The chromatograms were eluted with benzene containing ethyl acetate (1-20%) to give the isocoumarins (I-IV) as well as Piericidin A and B.

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Evaporation of the eluants gave the enolic isocoumarins which were obtained as colourless crystalline solids after trituration with hexane. Compounds (III) and (IV) were not completely separated by the initial chromatographic procedure. Separation was finally achieved by tlc on silica using 2% methanol in chloroform (R_f 0.60, (IV), R_f 0.34). The isocoumarins could be further purified by recrystallisation (ethanol) or by sublimation in vacuo.

Examination of the spectroscopic properties of (I)-(IV) suggested that they were probably oxygenated isocoumarins; e.g. their u.v. spectra were very similar to that of 6,8-dihydroxy-7-methoxy-3-methylisocoumarin(2). Their i.r. spectra contained bands in the region of 3400 and 1680 cm\(^{-1}\) indicating the presence of a hydroxyl group hydrogen-bonded to a carbonyl group. On methylation, the band at 3400 cm\(^{-1}\) disappeared while the band at 1680 cm\(^{-1}\) was displaced to 1720 cm\(^{-1}\). The n.m.r. spectra of (I-IV) were entirely consistent with their structures, in particular all spectra showed signals in the region of -1.0 (due to the hydrogen-bonded proton) and 7.7\(\tau\) (due to the 3-methyl group).

The presence of the 3-methyl group which was suggested by biosynthetic considerations (1), was confirmed by the alkaline hydrolysis of (II). The resulting ketone (V) had an n.m.r. spectrum with singlets at 8.20 (2H) and 6.18\(\tau\) (2H) due to the exocyclic methyl and the methylene groups.

**TABLE**

<table>
<thead>
<tr>
<th>Compound and order of elution</th>
<th>Amount Present (mg) in 20 l medium</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Extract A</td>
</tr>
<tr>
<td>(I)</td>
<td>20</td>
</tr>
<tr>
<td>Piericidin B</td>
<td>20</td>
</tr>
<tr>
<td>(II)</td>
<td>200</td>
</tr>
<tr>
<td>Piericidin A</td>
<td>1000</td>
</tr>
<tr>
<td>(III)</td>
<td>-</td>
</tr>
<tr>
<td>(IV)</td>
<td>-</td>
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</tbody>
</table>
The substitution pattern in the aromatic ring of the isocoumarins was demonstrated by methylation of (II) with diazomethane followed by oxidation of the product with permanganate to 3,4,5-trimethoxyphthalic acid which was then dehydrated by sublimation to 3,4,5-trimethoxyphthalic anhydride (VI) (7,8). The single aromatic proton appeared at much lower field in the n.m.r. spectrum of (VI) than in the spectrum of (II). Hence the proton is in position 5 and is deshielded in the anhydride (9).

The mass spectra of compounds (I-IV) were in agreement with the proposed structures. For example, the mass spectrum of (II) showed peaks at: 236 M(100%), 221 M-CH₃ (94), 193 M-CH₃-CO(51), 165 M-CH₂-2 CO(5), 150 M-2CH₂-200(16).

8-Hydroxy-6-methoxy-3-methylisocoumarin (II): m.p. 129°. λ_max (MeOH) 244 nm, (log ε 4.62), 277 (3.77), 326 (3.72). ν_max (CHCl₃) 1685 cm⁻¹. NMR 60 MHz (CDCl₃)τ -1.12 (1Hs), 3.51 (1Hd, J=2.6 Hz) 3.67 (1Hd, J=2.6 Hz), 6.15 (3Hs), 7.76 (3Hs). Mass spectrum M⁺ 206.0567, C₁₁H₁₀O₄ requires 206.0579.

8-Hydroxy-6,7-dimethoxy-3-methylisocoumarin (III): m.p. 199°. λ_max (MeOH) 243 nm (log ε 4.61) 278 (3.84), 335 (3.69). ν_max (KCl) 3400, 1678 cm⁻¹. NMR (CDCl₃)τ -1.42 (1Hs), 3.64 (4Hs), 3.80 (4Hs) 6.02 (3Hs), 6.07 (3Hs) 7.72 (3Hs). Mass Spectrum M⁺ 236.0687, C₁₂H₁₄O₅ requires 236.0685.

6,8-Dihydroxy-7-methoxy-3-methylisocoumarin (IV): m.p. 194°. λ_max (MeOH) 245 nm (log ε 4.68), 278 (3.86), 330 (3.76). ν_max (nujol) 3435, 1679 cm⁻¹. NMR (CDCl₃)τ -1.10 (1Hs), 3.53 (2Hs broad), 6.19 (3Hs), 7.77 (3Hs). Mass spectrum M⁺ 222.0525, C₁₁H₉O₅ requires 222.0528.

Methyl ester of (II): (II) (35 mg) was kept at -15° for 7 days in ether/CHCl₃ (1:1) containing a 10 fold excess of diazomethane. The methyl ester (35 mg, m.p. 119°) was isolated by tlc on silica using 10% ethyl acetate in benzene. λ_max (MeOH) 245 nm (log ε 4.66), 276 (3.83), 327 (3.60), 337 (3.57). ν_max (CHCl₃) 1725 cm⁻¹. NMR (CDCl₃)τ -1.04 (1Hs), 3.88 (1Hs) 6.00 (3Hs), 6.05 (3Hs), 6.09 (3Hs), 7.77 (3Hs). Mass spectrum M⁺ 250.0846, C₁₃H₁₄O₅ requires 250.0844. Found C, 62.3, H, 5.48, O, 31.8%. C₁₃H₁₄O₅ requires C, 62.4, H, 5.64; O 32.0%.
Alkaline Hydrolysis of (II): (II) (30 mg) was heated under reflux for 1 hr. in 2N NaOH (15 ml). The cooled solution was acidified and continuously extracted with chloroform for 12 hr. The extracts were dried, evaporated to dryness, and the product (17 mg, m.p. 180°) was recrystallized from methanol. $v_{\text{max}}$ (nujol) 1730 cm$^{-1}$ NMR (CDCl$_3$) $\delta$ 3.61 (1Hs), 6.02 (3Hs), 6.07 (3Hs), 6.79 (2Hs), 8.20 (3Hs). Mass spectrum $M^+$ 254.0802, C$_{12}$H$_{14}$O$_6$ requires 254.0790.

3,4,5-Trimethoxyphthalic anhydride. The methyl ether of II (30 mg.) was oxidized with alkaline potassium permanganate (10) and the acidified solution, after removal of manganese dioxide with bisulphite, extracted with chloroform for 12 hr. The chloroform extract was dried, evaporated to dryness and the residue heated in vacuo, 3,4,5-trimethoxy phthalic anhydride sublimed as long colourless needles (16 mg, m.p. 144°). Lit. m.p. 144-5° (7), 147° (8). $v_{\text{max}}$ (nujol) 1838, 1770 cm$^{-1}$ NMR (100 MHz, CD$_3$SOCD$_2$) $\delta$ 2.55 (1Hs), 5.93 (3Hs) 6.00 (3Hs), 6.15 (3Hs). Mass spectrum $M^+$ 238.0474, C$_{11}$H$_{10}$O$_6$ requires 238.0477.

References