THE REACTIONS OF PYRIMIDINES AND PURINES WITH HYDROXYLAMINE

A dissertation submitted to the University of Cambridge for the degree of Doctor of Philosophy

by

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of

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The work described in this dissertation was carried out in the University Chemical Laboratory, Cambridge, under the supervision of Dr. D.M. Brown. The work is original and has not been submitted for a degree at any other university.

I should like to thank Dr. Brown for his encouragement and advice throughout the work, and other members of the research group for helpful discussions.

I wish to acknowledge the receipt of a Research Studentship from the Science Research Council.

M. Osborne.
PREFACE

The dissertation is divided into four chapters and an experimental section. The introduction describes the biological significance of the reaction of hydroxylamine with nucleic acid components. The following three chapters discuss the results of the author's work, and are virtually independent of each other. In chapter two, previous knowledge on the reaction of hydroxylamine with cytosine is extended to polymers containing cytosine, in an attempt to clarify the mechanism of hydroxylamine mutagenesis. Chapter three describes the lesser-known reactions with adenine and isoguanine. It is followed by a short chapter containing some initial observations on the reactions of other bases with hydroxylamine.

This work omits the effects of hydroxylamine on uracil and thymine which have already been adequately described (see, for instance, reference 14).

The system for numbering the rings of the natural bases is given below for reference, as it is highly confusing. It should be noted that representation of compounds in particular tautomeric forms does not imply knowledge of the true tautomeric forms.
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ABBREVIATIONS

The following abbreviations have been used in this work; other non-standard abbreviations are defined where they appear.

HA  hydroxylamine
MA  methoxyamine, CH₃ONH₂
CMA carboxymethoxyamine, HOOC·CH₂ONH₂
A,G,T,U,C,MC the bases adenine, guanine, thymine, uracil, cytosine and 5-methyl cytosine
CMP,AMP,IMP cytidylic, adenylic and inosinic acids
dAMP deoxyadenylic acid
HOCPMP N⁴-hydroxy cytidylic acid
poly C polycytidylic acid (poly CH⁺: protonated form)
poly G polyguanylic acid
poly I polyninosinic acid
HAP 6-hydroxylaminopurine
NOHA N⁶-hydroxy adenosine (riboside of HAP)
ANO adenosine N⁻oxide
µp molecular weight per phosphate group
mmₚ millimolar in phosphate groups
εₚ extinction coefficient referred to phosphate, i.e. optical density ÷ total nucleotide concentration.
ko rate constant of bimolecular reaction
k pseudounimolecular rate constant of bimolecular reaction, i.e. ko x reagent concentration.

Subscripted k's are defined in Fig. 2, page 14.
TO MY
MOTHER
AND
FATHER
CHAPTER 1

INTRODUCTION

Although the work described in this dissertation is entirely chemical, that described in Chapter 2, and to a lesser extent the content of Chapter 3, have biological implications. These will be outlined in this introduction.

Mutagenesis

A clone of genetically equivalent organisms does not stay constant when grown through several generations; variants arise owing to spontaneous changes in the genetic material (mutation). This phenomenon is observed most easily in micro-organisms such as the bacterium E. Coli and the bacteriophages T2 and T4 that live on it; most of the theory to be described here has arisen from work on these organisms. Some chemical substances, such as hydroxylamine or 5-bromouracil, accelerate mutation from this spontaneous rate to a much higher level; this action is known as chemical mutagenesis.

An explanation of this effect was only possible after the discovery of the base-paired structure of the DNA that contains the genetic information in each of these organisms: and of its role as a template for the synthesis of other coded molecules. DNA is a primer in its own synthesis (replication) and in the synthesis of messenger RNA (transcription), and mRNA is the template for protein
synthesis (translation). The universality of this sequence of events has become known as the central dogma of molecular biology.

With the announcement of the discovery of the structure of DNA Watson and Crick also proposed\(^2\) that correct replication of DNA depended on the bases taking up their usual tautomeric states (uracil having keto-groups, adenine an amino-group, and cytosine and guanine both). If a base took up the wrong tautomeric form during replication, a pairing error would occur, leading to an altered sequence in the new polymer. This hypothesis for the mechanism of spontaneous mutation was later expanded to embrace chemical mutagenesis.

For the purposes of this theory mutagens are divided into 3 classes.

(i) The true mutagens are small molecules that react with DNA, or which have \textit{in vivo} breakdown products that do. The reaction gives altered bases in the DNA which no longer hydrogen-bond readily with their normal partners in the other strand, but with another base. Thus, on replication of the DNA, a new base pair takes the place of the old, and this replication error is transmitted through the progeny (Fig.1).

(ii) Base analogue mutagens are heterocyclic substances that resemble the natural bases found in DNA. They only act as mutagens \textit{in vivo}, since in order to function they must be incorporated into the genetic material during its synthesis. An example is 5-bromouracil, which has characteristics both of thymine and of cytosine. It is
Transmission of a replication error in multiplying organisms.

Base pairing
weakly mutagenic in the sense that considerable proportions of it can be incorporated into the DNA of phage T₄ in the place of thymine, before mutagenesis becomes significant. Two mechanisms are conceivable for its mutagenic action; either (a) it occasionally poses as C and enters the DNA opposite G, then subsequently pairs with A during replication; or (b) it always enters DNA opposite A, as if it were T, but occasionally mis-pairs with G. It is believed that 5-bromouracil causes predominantly GC → AT changes, so presumably mechanism (a) is the more important.

These two classes of mutagens are known as point mutagens, because they cause the alteration of a single base pair which can be mapped to a single point on the genetic map of the organism. Mutant strains produced by them can be caused to revert back to the original strain by the action of other point mutagens, or even sometimes by the same substance.

(iii) It was first assumed that all mutagens acted in this way, but it soon became clear that another class of substances, including the acridines, gave mutants that were not revertible by the point mutagens. These mutations involve the insertion or deletion of base pairs in the genetic material; this class will be ignored for the purposes of this work.

It is evident that point mutagens should fall into two classes, those causing the substitution of one pyrimidine for the other (GC → AT, transition mutations) and those that cause the interchange
weakly mutagenic in the sense that considerable proportions of it can be incorporated into the DNA of phage T4 in the place of thymine, before mutagenesis becomes significant. Two mechanisms are conceivable for its mutagenic action; either (a) it occasionally poses as C and enters the DNA opposite G, then subsequently pairs with A during replication; or (b) it always enters DNA opposite A, as if it were T, but occasionally mis-pairs with G. It is believed that 5-bromouracil causes predominantly GC → AT changes, so presumably mechanism (a) is the more important.

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It is evident that point mutagens should fall into two classes, those causing the substitution of one pyrimidine for the other (GC → AT; transition mutations) and those that cause the interchange
of pyrimidines and purines (GC = CG = AT = TA = GC, transversion mutations). All the point mutagens so far discovered, however, cause predominantly transitions. A transversion mutagen would be very useful for genetic studies, and efforts have been made towards the synthesis of 3-glycosides of isoguanine which might be incorporated as adenosines and pair as cytidines.

Erroneous base-pairing in transition mutagenesis

Why should a modified base in genetic material pair differently from its parent? If the model suggested by Watson and Crick for normal base pairing is examined (Fig. 1, A and B) it will be seen that its specificity depends on certain groups in the pyrimidine rings of the bases. It depends on the tautomeric state of the group at C4 of the pyrimidine (C6 of the purine) and on whether or not N3 of the pyrimidine (N1 of the purine) is protonated. Thus any reaction on DNA which changes the tautomeric constant or pK of one of the bases may change its pairing properties. For instance, a dihydrocytosine has a lower tautomeric constant and a higher pK than its parent cytosine, i.e., it is more likely to take up an imino-form or to be protonated. It may therefore prefer to pair with adenine (Fig. 1, C or D) rather than with guanine (cf Fig. 1, B). Whether this actually happens will be further discussed in Chapter 2.

It has been noted that the CG pairs are theoretically more susceptible to changes in tautomeric constants and pK's than are
the AT pairs, and should be therefore more susceptible to mutagenesis. It is indeed found that the majority of transition mutagens cause mainly GC → AT changes, and no satisfactory specific AT → GC mutagen has yet been discovered. This preferred direction of mutation also probably explains why most natural DNA contains more AT pairs than GC pairs.

Difficulties in mutagenic theory

The foregoing argument has been pursued on a theoretical basis, with little reference to experimental fact; it might be referred to as the central dogma of mutagenesis. As with the dogma referred to earlier, it is not considered to be an expression of the detailed mechanism of point mutagenesis in every case, but merely a model with which experimentally obtained results are to be compared. And here, as often in biology, a clear theoretical picture is obscured by confusion in detail. A complete discussion of the difficulties and irregularities in the above theory does not belong here, but some of the difficulties will be briefly outlined.

(i) Except in the case of in vitro mutagenesis of bacteriophages, cellular processes will interfere with mutagenesis. One does not know the concentration of a substance inside a bacterial cell, or how fast it is being metabolised; often one cannot be sure whether it is the administered substance that is affecting the DNA, or whether it is one of its metabolic products that is the proximate mutagen.
(ii) The theory is built on results obtained from a variety of micro-organisms, under a variety of conditions. While this has helped to establish the generality of mutagenic theory, it has confused the embodiment of many experiments into it. The differences in reactivity between nucleic acids of different organisms has been stressed; the nucleoprotein surrounding DNA must play a large part in protecting it from chemical attack.

(iii) Given the classical structure of DNA and the general picture of mutagenic action, one would expect mutagenesis to be a random process, and that all loci on the genetic map corresponding to GC pairs (say) should be equally mutable. But this seems to be far from the case: some points ("hot spots") are highly susceptible to specific reagents, and many others have not been observed to mutate at all. This is the problem of mutability.

(iv) Nothing is known about the specificity of polynucleotide replicating enzymes, and it is often suspected that they play a part in ensuring that the sequence is normally replicated exactly.

(v) Not all alterations in the DNA sequence cause detectable alterations in the characteristics of the organism (phenotype). Most genetic changes involve either no change in the protein produced by the cistron, or one that does not affect its properties, and these mutations remain unobserved. The converse difficulty, that several different genetical changes can produce the same change in phenotype, caused much confusion in earlier work but can be eliminated by more refined genetical methods.
(vi) In order that a genetical change in an organism be detected, the organism must survive and replicate. In mutagenic experiments a proportion of the micro-organisms do not; processes removing them (inactivation) compete with those producing observable mutants (mutation). Mutagens are used under conditions which attempt to maximise the ratio of mutation to inactivation. The causes of inactivation are threefold.

(a) The mutagen interferes with other cellular components; in the well-investigated case of T4 phage with hydroxylamine, inactivation was believed to result from reaction of the reagent with the phage's tail fibres.

(b) Some reactions with the DNA, such as those causing cross linkage between bases or scission of the sugar phosphate chain, merely inactivate the polymer as a template without giving rise to mutation.

(c) Many mutations occur in sites essential to the organism's survival (lethal mutations). The use of the word inactivation to describe one or all of these effects gives rise to some confusion.

Hydroxylamine mutagenesis

The mutagenic action of hydroxylamine was discovered by Freese; his initial observations with T4 phage have been extended to many organisms. It has been established genetically that the reagent causes transitions, almost entirely in the direction GC → AT.
Assuming that hydroxylamine is itself the proximate mutagen, Freese studied its reaction with DNA components and discovered that of the four bases only cytosine was affected under the conditions used for mutagenesis. (The RNA base uracil is degraded by hydroxylamine at higher pH's but this reaction, as applied to RNA's, is believed to be inactivating rather than mutagenic). He stated that adenylic and guanylic acids were affected to less than 2% under the conditions of reaction (HNO at 37° for 60 hours) thus overlooking the reaction of adenine with hydroxylamine to give hydroxylaminopurine: this was discovered by Budowsky et al. eight years later (see Chapter 3). Though 6-hydroxylaminopurine and its nucleoside have been shown to act as mutagens it is unlikely that its formation in DNA in vivo has mutagenic consequences. But this conclusion cannot be made a priori just because the reaction is so much slower with adenines than with cytosines. For one thing, the reaction with the cytosine nucleus is much retarded in the polymers, and one cannot be sure that the reaction with adenines is equally retarded: for instance, their weaker hydrogen-bonding may render them relatively more reactive. And reactions important in mutagenesis need not be important chemically; a side reaction may be responsible for observed biological effects.

Carcinogenesis

The induction of tumours in higher animals by administration of certain substances, chemical carcinogenesis, bears at least
a superficial resemblance to the phenomenon of mutagenesis in lower forms of life. Though not intimately connected with the work described here, the subject will be briefly discussed.

Unfortunately the mechanism of carcinogenesis, even in outline, is less clear than that of mutagenesis; this is largely because we are dealing with more complex organisms. Several theories have been proposed concerning the mechanism of carcinogenesis; many are plausible for particular cases but none can accommodate all the available evidence. We shall only examine one idea that attempts to link carcinogenesis with mutagenesis.

The theory of somatic mutation envisages that when a substance which is mutagenic for micro-organisms acts on the DNA of cells forming an animal organ, the changes that caused mutation in the micro-organism may cause a neoplasm in the organ. This is an attractive theory as it partly explains the physiological aspects of cancer; the mutated cell will pass on the disease to its progeny. Also in its favour is the fact that many important mutagens are also carcinogens and vice versa, though the correspondence is not as close as might be expected. For instance, dimethyl sulphate is both mutagenic and moderately carcinogenic, and would appear to act by methylation of the guanine residues of DNA in each case. But there is no analogue in mutagenesis for the powerful carcinogenic effect of hydrocarbons such as benzpyrene. Hydroxylamine, familiar as a mutagen, is unimportant as a carcinogen. It has been suggested
that its carcinogenic action is related to its chromosome-damaging
effects rather than its action as a point mutagen. It has been
further suggested that all carcinogens might act in this way\textsuperscript{18},
but this seems unlikely. Sometimes the difference between carcino-
genesis and mutagenesis can be directly related to the difference
between mammals and micro-organisms. Thus dimethyl nitrosamine is
not mutagenic for bacteria, but, in rats, appears to be metabolised
in the liver to diazoate which then acts as a carcinogenic alkylating
agent\textsuperscript{19}.

It has recently been suggested\textsuperscript{20} that carcinogens differ from
mutagens in that they cause genetic changes giving rise to altered,
but still functional, proteins. Thus methyl methanesulphonate, a
mutagen causing chiefly GC \textrightarrow{} AT transitions, is not carcinogenic
(or weakly so). But nitroso methyl nitroguanidine (\text{MeN(NO)C(\textNH)\textNH.NO}_{2}),
a mutagen believed to cause mainly AT \textrightarrow{} GC transitions which cannot
give rise to nonsense sequences in DNA, is also a potent carcinogen\textsuperscript{20}.

\textit{N-oxidised derivatives in carcinogenesis}

Many compounds having N-O bonds, but nothing else in common,
are carcinogenic: these include hydroxylamine, C-nitroso compounds,
nitrosamines, and azoxy-compounds; and it is suspected that the
carcinogens \&- and \text{\textbeta}naphthylamine and 2-amino fluorene may act
through their N-hydroxy derivatives\textsuperscript{21}. It is therefore tempting to
look for ways in which these classes of compounds might be interconverted
\textit{in vivo}, or to find some common mechanism.
The hydroxylamine derivatives of cytosines described in Chapter 2 are believed to act as carcinogens\textsuperscript{22}, but the mutagenic hydroxylaminopurines seem to be inactive or nearly so\textsuperscript{23}. But their isomers the adenine 1-oxides, though not mutagenic, are carcinogenic like a number of other purine N-oxides\textsuperscript{23}. They also have some characteristics of mutagenic compounds: (a) the presence of adenine 1-oxide in DNA may upset its base-pairing\textsuperscript{24}, and (b) it can be formed in DNA \textit{in vivo} by the action of X-rays\textsuperscript{25}. It is not certain, however, that the oxides can be incorporated into DNA during replication\textsuperscript{23}. Though there is no obvious way in which these adenine 1-oxides and the hydroxylaminopurines can be interconverted \textit{in vivo}, this work shows that the isomerisation is at least feasible and should be suspected when one tries to explain the biological effects of the two groups of compounds.
CHAPTER 2

REACTION OF ALKOXYAMINES WITH POLYNUCLEOTIDES

(A) Reaction of hydroxylamine with cytosines

Hydroxylamine reacts with cytosine at neutral pH according to the reaction scheme given (Fig. 2). \(^{13,26,27,28,29}\) Hydrolysis to uracil is also possible as a side reaction but most authors agree that this is unimportant. However, the exact sequence of the reactions in Fig. 2 has been controversial. Since the presence of the intermediate adduct II cannot be detected in the reaction mixture, the reaction II → III must be very fast. This was also clear from studies on other 5,6-dihydrocytosines which also undergo rapid nucleophilic substitution at the 4-position: for instance the photo-induced water adduct of cytidine V has a half-life of about an hour in 0.24 M hydroxylamine at pH 5.5 at 0°C \(^{30}\) which suggests a \(k_2\) of the order of 100 at 39°C, the kind of value found in studies of dihydrocytosines \(^{31,32}\) and too high to measure accurately by normal kinetic methods. III and IV, the products found after the reaction, could be formed via either path from cytosine, but analysis of the kinetics has shown that III is formed from II \(^{33}\), IV is formed directly from I, \(^{34,35}\) and the equilibrium III ⇌ IV plays little part in the reaction.

With these simplifications it is possible to analyse the reaction curves and deduce the rate constants involved; those given in Table I
Fig. 2

\[
\begin{align*}
\text{I} & \quad \begin{array}{c}
\text{NH}_2 \\
\text{O} \\
\text{R} \\
\end{array} \\
\text{II} & \quad \begin{array}{c}
\text{NHOR} \\
\text{R} \\
\text{O} \\
\end{array} \\
\text{III} & \quad \begin{array}{c}
\text{NH} \\
\text{H} \\
\text{H} \\
\text{H} \\
\text{NHOR} \\
\text{R} \\
\end{array} \\
\text{IV} & \quad \begin{array}{c}
\text{OH} \\
\text{N} \\
\text{R} \\
\text{O} \\
\end{array} \\
\end{align*}
\]

\[k_{\text{net}} = \frac{k_1 k_2}{k_1 + k_2}\]

\[k_{\text{total}} = k_{\text{net}} + k_4\]

\(\begin{align*}
a: & \quad R=H, \ R'=H \\
b: & \quad R=\text{Me}, \ R'=H \\
c: & \quad R=H, \ R'=\text{Me} \\
d: & \quad R=H, \ R'=\text{ribofuranosyl} \\
e: & \quad R=\text{CH}_3\text{COO}^-, \ R'=\text{ribofuranosyl} \\
f: & \quad R=H, \ R'=\text{ribofuranosyl 5-phosphate} \\
\end{align*}\]
were calculated by Hewlins \textsuperscript{36}, ignoring the reversibility of I → II, i.e. his $k_1$ is our $k_1k_2/k_{-1}+k_2$. Budovski et al. go further than this and by mathematical analysis of the system derive a linear relationship between $(IV_d)/(III_d)$, the ratio of products at the end of reaction, and the reciprocal of the hydroxylamine concentration, from which a value of $k_{-1}/k_2$ can also be derived.\textsuperscript{37} The obvious objection to this is that after infinite time the ratio of these products represents simply the equilibrium between them, and one expects the plot of $(IV_d)/(III_d)$ against $1/(HA)$ to be a straight line through the origin, and the graphs they obtain look suspiciously like this. However, their experiments have been repeated with similar results\textsuperscript{38} and give more accurate rate constants in the case of methoxyamine as reagent. The picture one obtains is of a highly reversible initial addition at C6, with equilibrium very much in favour of cytidine; $III_d$ and $IV_d$ are then formed from this mixture by secondary reactions. This conclusion is corroborated by experiments on the cytosine derivative VI which can undergo the reaction I → II
intramolecularly: it exists largely in the form VI but undergoes reactions expected of a dihydrocytosine. Experiments using deuterated derivatives of the reagent or substrate have shown that the reaction of 1-methyl cytosine with hydroxylamine involves addition of a neutral reagent molecule to protonated cytosine followed by protonation from solution at C5, to give purely trans-adduct IIc. The reverse reaction involves removal of the proton from C5 and is base-catalysed. It was also shown that the enamine VIIc could be inserted into the reaction scheme of Fig. 2 as an intermediate of finite lifetime without upsetting the interpretation of the kinetic data.

(B) Reaction with other alkoxyamines

Methoxyamine is chemically similar to hydroxylamine, but a less powerful nucleophile; its reaction with cytosines is analogous to that of hydroxylamine but slower (k0 \text{ total} for cytidine estimated at 0.092 at pH 4.9, 28°C 37 and 0.082 at pH 5, 39°C 41) and with a lower pH optimum for disappearance of the cytosine (about 4.8, compared with 6 for hydroxylamine) due to the lower pH of the reagent. Reactions with methoxyamine are therefore carried out at a lower pH, resulting in increased ratios of IV to III.

The reaction of carboxymethoxyamine (CMA, NH2OCH2COOH) is of interest as Freese found it to be a mutagen of similar potency to methoxyamine for B. Subtilis transforming DNA. 42 In this work it
Fig. 3
Reaction of cytidine with
0.5 M carboxymethoxyamine, 39°

- pH 4.2
- pH 5.2
Fig. 4.
Reaction of cytidine with 0.5M carboxymethoxyamine, 39°

- pH 3.9
- pH 4.8
- pH 5.8
was found on reacting cytidine with 0.5 M CMA at 39°, and following
the progress of reaction by the UV absorbance of the solution at 269 nm
and 310 nm (see reaction curves, Figs. 3 and 4) that:

(1) The pH-rate profile was similar to that of methoxyamine +
cytidine, with a pH optimum of about 4.8. This is to be expected
as the reagent has a $pK_a$ of 4.67 similar to that of methoxyamine;
between here and its first $pK_a$ at 2.87 it presumably exists as the
unreactive zwitterion $\text{NH}_3^+\text{OCH}_2\text{COO}^-$.

(2) If the course of the reaction is similar to that proven for
hydroxylamine and methoxyamine, and the products are assumed to
have the same $\epsilon$-values as the methoxyamine derivatives, the ratio
of products IV to III can be estimated. The final mixture contained
about 80% IV which is similar to that found with methoxyamine under
these conditions.

(3) The reaction was faster than that with methoxyamine, having a
$k^0_{\text{total}}$ of 0.5 at pH 4.8. Hydroxylamine has a $k^0_{\text{total}}$ estimated at
0.43 in 3.5 M HCl, 39°, pH 6.5 and 0.37 at 30°, pH 6 from the data of
Hewlings and Budovski respectively.

There are several possible reasons for this acceleration in
rate caused by the carboxylate group. The inductive effect, if
any, should be deactivating; thus it was found that the ester,
ethyl 2-aminoxyacetate, at a similar pH reacts with cytidine at
about the same rate as methoxyamine does (Fig. 5). It is possible
to write mechanisms by which the carboxylate group acts catalytically
Fig. 5

Reactions of cytidine at 39°

- x with 0.5M NH₂OCH₂COOEt
- o with 0.5M NH₂OCH₂CH₂COOH
by removing a proton from one of the reacting molecules before or after addition but most of these involve 7-membered ring transition states rather than 5- or 6-. Moreover, it was also found that the homologue of \textit{CHA}, 3-aminoxy propionic acid, also reacts rapidly with cytidine (Fig. 5) so the effect is probably purely one of increased attraction between the reacting species because of their opposite charges.

\textbf{(C) Reaction of cytosine-containing polynucleotides with hydroxylamine}

It seems that the principal mutagenic reactions of hydroxylamine are those outlined above, and that the presence of the altered bases II, III and IV in the hydroxylamine-reacted genetic material causes mutations by mis-pairing. It now remains to be determined which one.

III has been ruled out as the mutagenic species, largely on the results of mutagenesis carried out using \textit{in vitro} replicating systems with poly C as a template. These experiments involve the synthesis of a new RNA strand using RNA polymerase with HA-reacted poly C as template, and measuring the uptake of labelled GTP and ATP into the new polymer. In this way it was found$^{13}$ that low hydroxylamine concentrations and short reaction times favoured mutagenesis (uptake of ATP), whereas more vigorous conditions for longer periods inactivated the polymer as a template. Such conditions also favour the formation of III rather than IV, so it appeared that III was inactivating to the polymer, and either II or IV was the mutagenic species. But
by removing a proton from one of the reacting molecules before or after addition but most of these involve 7-membered ring transition states rather than 5- or 6-: moreover, it was also found that the homologue of CMA, 3-amino8xy propionic acid, also reacts rapidly with cytidine (Fig. 5) so the effect is probably purely one of increased attraction between the reacting species because of their opposite charges.

(C) Reaction of cytosine-containing polynucleotides with hydroxylamine

It seems that the principal mutagenic reactions of hydroxylamine are those outlined above, and that the presence of the altered bases II, III and IV in the hydroxylamine-reacted genetic material causes mutations by mis-pairing. It now remains to be determined which one.

III has been ruled out as the mutagenic species, largely on the results of mutagenesis carried out using in vitro replicating systems with poly C as a template. These experiments involve the synthesis of a new RNA strand using RNA polymerase with HA-reacted poly C as template, and measuring the uptake of labelled GTP and ATP into the new polymer. In this way it was found \( \frac{43}{43} \) that low hydroxylamine concentrations and short reaction times favoured mutagenesis (uptake of ATP), whereas more vigorous conditions for longer periods inactivated the polymer as a template. Such conditions also favour the formation of III rather than IV, so it appeared that III was inactivating to the polymer, and either II or IV was the mutagenic species. But
other processes also act to inactivate the system, e.g. depolymerisation of the poly C, and the conclusion should be only tentative. It has been pointed out that mutation by HA has a lower pH optimum than inactivation, and that HA inactivates transforming DNA more than T4 phage. Since higher pH favours III over IV and the hydroxymethyl cytosine of T4 phage gives only substitution products with HA, these are arguments against III as a mutagenic species.

IV fulfils all the requirements to be a mutagenic base in a template polynucleotide. It is known to exist in a different tautomeric form from its parent cytosine, i.e. in the oximino form as shown, thus having an excuse to pair with the wrong base; and infra-red studies of the association of bases in non-aqueous solvents have shown that it does indeed pair with an adenine and not with a guanine. Synthetic poly N-hydroxycytidylic acid does not associate with poly I as poly C does; unfortunately it does not pair with poly A either. This may be simply because the new polymer forms a stable self-associated structure; as will be noted later, the synthetic polynucleotides do not associate very strongly with each other, unlike complementary strands of coded molecules like DNA which cannot easily arrange themselves in any other way. But experiments on the association of poly(U + IV) with poly A and poly(C + IV) with poly I confirmed that IV is unwilling to base-pair. The template properties of a copolymer of cytidylic and N-hydroxy cytidylic acids was tested in an in vitro replicating system, and the residues of
IV were found to direct the incorporation of adenylic acid into the new polymer with a high efficiency. It is fairly certain, too, that I → IV represents the mutagenic event in T-even phages as it appears to be the sole reaction in the case of 5-hydroxymethyl cytosine. Presumably steric factors disfavor the addition of hydroxylamine to the 5,6-double bond, as the inductive effect of the CH₂OH group should be favorable.

II is the species which has been advanced in this laboratory as that responsible for mutagenesis in vitro and in bacteria; its case has been championed¹⁴ and dismissed⁵,¹⁴ in recent reviews of mutagenesis. The debate revolves around three points.

(i) What are the likely base-pairing properties of II? Unfortunately derivatives of II itself cannot be prepared owing to their instability with respect to both I and IV. The tautomeric constants of some dihydrocytosines have been measured, and it seems likely that II has a tautomeric constant not far off unity in aqueous solution. It has been pointed out that the dihydrocytosines exist in the imino-form in non-aqueous solvents and that this may be the relevant fact to apply to the situation of an altered base in DNA where the interior of the molecule is a non-aqueous environment.¹⁴ The truth is that we know nothing about the situation of a base in DNA during the actual replication process, and all that we can say is that II would be more likely than cytosine to pair with the wrong base (adenine). Even if only half the II residues were in the
imino-form this would still be an efficient reaction by mutagenic standards.

(ii) If the adducts (II) have only a transient existence as monomers, why should they be fairly stable in polynucleotides? Obviously if II is in a double helical structure it will be H-bonded and thus protected from attack at $\mathrm{C}^4$, but it is more difficult to see why it should be particularly stable to elimination of hydroxylamine, even if the elimination has to be catalysed by attack of hydroxylamine on the $\mathrm{C}^5$ protons. For the $\mathrm{5-}$ and $\mathrm{6-}$ positions of cytosine are fairly exposed, even in DNA; in T4 DNA there is room for a $\mathrm{CH}_3\mathrm{OH}$ or even $\mathrm{CH}_2\mathrm{O}\,\text{glucose at the 5-position, an}$ and THV RNA can be mutagenically attacked by bromine to give 5-bromo derivatives, so it seems unlikely that the small base molecule $\text{NH}_2\text{OH}$ is hindered from reaching the 5-position. It must be considered, though, that the blocking effect of the $\text{NH}_2\text{OH}$ group already in the 6-position is probably considerable. If the enamine intermediate VII is brought into the picture, the idea of inaccessibility of the $\mathrm{C}^5$ position is even less clear than before, as the presence of a base/acid near $\mathrm{C}^5$ is essential for both the forward and backward reactions $\text{VII} \rightleftharpoons \text{II}$ and the relative ease of its approach should have no effect on the position of equilibrium. Meanwhile the decreased rate of reaction $\text{I} \rightleftharpoons \text{VII}$ will tend to make (II)/(I) even less than with the monomers. The above arguments are probably inapplicable to poly C, as whatever its exact structure is at neutral pH it is likely to be somewhat labile.
(iii) If the reaction $I \rightarrow II$ is mutagenic it does have parallels in other similar reactions on pyrimidine bases in DNA. Thus dipyrimidines have been blamed for hydrazine mutagenesis, and the recently discovered addition of bisulphite to uridine and cytidine$^{51,52}$ for sulphur dioxide mutagenesis, though this is a little premature as nothing is yet known about $SO_2$ as a mutagen. The best example is that provided by the photohydration of pyrimidines which is outlined here.

The principal reactions undergone by nucleic acids on irradiation by UV light are the dimerisation and water-addition of the pyrimidines. The photo-dimerisation reaction is believed to be inactivating to phage DNA$^{11}$ but in the dimeric state cytosine residues are liable to deamination and it has been suggested that this might be an important route for mutagenesis by UV light.$^{53}$ The photoaddition of water to uracil has been found to be potentially mutagenic: thus Grossman$^{54}$ found that poly (U+U) formed by the irradiation of poly U directed the incorporation of serine as well as phenylalanine in an in vitro protein synthesizing system, as would be expected for a U → C transition. This effect could be partly reversed by raising the pH, providing good evidence that the species $^*U$ is indeed the water adduct. But this observation is theoretically puzzling: the 5,6-water adduct of uracil should theoretically$^{55}$ be no more enolic than uracil, and unlikely to mis-pair; moreover, the presence of dipyrimidines in poly U has been found to
be template-inactivating and not mutagenic\(^5\)\(^6\). So if this experimental observation is correct (it has not been repeated\(^8\)) one must look further than tautomeric constants for an explanation.

The same experiment carried out on poly C using an in vitro replicating system gave a similar result\(^5\)\(^7\): here the theoretical basis of mutagenesis is clearer, as cytosine water adduct would be expected to have a low tautomeric constant. The possibility of a subsequent deamination to uracil being responsible for the result has been suggested\(^8\) but it seems unlikely: this experiment provides good evidence for the participation of a dihydrocytosine in mutagenesis. Moreover, the water adduct \(V\) is very similar to the hydroxylamine adduct \(II\), and if one induces mis-pairing then the other should. It cannot be argued, however, that \(II\) should show the same stability as \(V\) as an altered base in the polynucleotide because in the case of \(II\) we are considering stability in the presence of hydroxylamine and not just to unimolecular elimination.

(iv) Is hydroxylamine mutagenesis reversible, as the reaction \(I \rightarrow II\) is reversible? Experiments in this field\(^4\)\(^3\),\(^5\)\(^8\) have not given consistent results, and the answer seems to be: possibly, but other effects of hydroxylamine on the template irreversibly inactivate the replicating system (e.g., attack on the sugar phosphate chain) making biological reversibility difficult to prove.

(v) Can the presence of \(II\) in hydroxylamine-reacted polynucleotides be detected? This is the crux of the argument and will be discussed
at several points in the following sections. The difficulty is
that mutagenesis involves the alteration of just a few bases in
a million, which leads to a chemical dilemma: if the reaction is
carried out for a sufficient time for products to be isolable
(say 10% reaction) then the results are probably inapplicable to
mutagenesis. If the reaction is carried out for much shorter
periods, the accuracy of the experiment becomes suspect: and it
is largely on grounds of accuracy that these experiments have been
criticized or ignored.

To summarize, III is inactivating, IV mutagenic in T4, and
II and/or IV mutagenic in other systems. The evidence for II lies
in experiments which by their nature are open to criticism. Some
authors are biased against II since they prefer the neatness of
a theory implicating I → IV as the mutagenic reaction in all organisms,
others see no reason why nature should prefer simplicity, and believe
that there is a multiplicity of mechanisms.

Use of synthetic RNA as models for DNA

Polycytidylic acid has been much used. At pH 5.5, it takes
the form of a double helix, probably stabilized by two H-bonds
between each pair of cytosine residues, one of them involving the
snare of a hydrogen ion between the two bases. Despite much
study the structure of poly C at pH 7 remains uncertain. It has
been envisaged as a highly ordered, base-stacked single-stranded
helix in which the cytosines are not H-bonded\textsuperscript{59} but held together by hydrophobic forces\textsuperscript{60}; and as a non-rigid, base-stacked random coil\textsuperscript{61}. But later studies indicated that the hypochromicity of poly C in aqueous solutions was entirely due to the adoption of double helical structures.\textsuperscript{62} Polyinosinic acid (in preference to the less available poly G) combines with poly C in aqueous solution to give a double helical structure, poly I·poly C; this interaction can be followed by increase in hypochromicity\textsuperscript{63}. This complex ought to be a perfect model for DNA; it has the advantage that it is hydrolysed under milder conditions.

Reactions followed by UV absorption

Phillips observed the progress of reaction of poly C in 2.5M HA by measuring the optical density at 269 nm\textsuperscript{33}. He obtained a sigmoidal reaction curve (Fig.6): an apparent induction period was observed which was not found with cytidine monomer or when the reaction with the polymer was carried out under denaturing conditions (85\% glycol as solvent). This was explained by supposing that during the initial stages of reaction, the loss in optical density due to reaction (II and III do not absorb at 269 nm, and IV less than I) is counterbalanced by increase in absorption of the polymer due to denaturation. He later obtained a similar result with methoxyamine as reagent,\textsuperscript{64} and confirmed the hypothesis by following the reaction in aqueous solution by measuring the UV absorption.
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Fig. 6

Reaction of polymers with hydroxylamine at 39°

- poly C + 2.5M HA (Phillips)
- poly I, poly C + 2.79M HA
Reactions of polymers with 1.8M methoxyamine at 39°

- poly C
- poly I, poly C
of aliquots of the reaction mixture both in water and in glycol. In the latter solvent, which destroys the polymer's secondary structure, the bases present had UV absorbances near those of the monomers, and so the true extent of reaction could be determined. An exponential curve was obtained by this method.

In the present work, the reaction of poly C with HA was repeated, and the reaction curves for poly I, poly C with HA and MA at 39° determined. These are given in Figs. 6 and 7. The UV absorption was read at 269 nm which is near the \( \lambda_{\text{max}} \) of the complex; this is largely due to the cytosine content (spectra are given by Davies and Rich). It is possible to estimate the absorption of the inosine content and subtract it from the total; this has been done for the curves given, and the absorption due to the cytosines expressed as a percentage of its original value.

(i) poly C + MA (pH 5.5). As in Phillips' experiment the reaction curve was not exponential, though no apparent induction period was observed.

(ii) poly I + poly C + HA (pH 6.5). The absorption of the mixture fell almost exponentially to that of the poly I alone. The experiment was repeated with a very low concentration of reagent (0.18M) to find whether the initial stages of reaction were similar at this concentration; the reaction curve was linear over the first 180 hours, with a rate constant about 1/27 that found with 2M reagent.
This confirms that the overall reaction is of first order in HA, although the product ratios are widely different between the two concentrations.

(iii) poly I. poly C + MA (pH 6.5). A marked induction period was obtained; virtually no change in optical density was recorded during the first five hours of reaction. Unfortunately this stage of the reaction, the one we are most interested in, is difficult to follow accurately as we are looking at small changes in a large quantity (the absorption of unchanged cytosines, of poly I, and of the reagent which was far from zero). The graph given combines the results from three experiments.

A Maximum rates of reaction. The following figures are the times that the observed falls in optical density would take to occur at the maximum observed rates, reciprocated and divided by the reagent concentration to correct to molar reagent (reaction is first order - see (ii) above).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rate (hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly C + HA</td>
<td>0.029</td>
</tr>
<tr>
<td>poly C + MA</td>
<td>0.040</td>
</tr>
<tr>
<td>poly I. poly C + HA</td>
<td>0.021</td>
</tr>
<tr>
<td>poly I. poly C + MA</td>
<td>0.022</td>
</tr>
</tbody>
</table>

These figures are approximations to \( k_{\text{total}}^0 \) and can be compared with the values for deoxycytidine (0.43 hr⁻¹ at 35°C with HA \( 35 \)) and cytidine (0.092 hr⁻¹ at 28°C with HA \( 37 \)). Why have the rates for HA and MA, different with the monomers, levelled out for the polymers?
The reaction of cytosine with HA, we have seen, is that of neutral hydroxylamine on the protonated form of cytosine. Since cytosine has a $pK_a$ of 4.6 and hydroxylamine 5.0, the reaction should theoretically proceed most rapidly at an intermediate pH where, although both molecules are largely in the wrong form for reaction, the "overlap" between the existence of protonated cytosine and free hydroxylamine is largest. The rate of reaction at optimum pH is then proportional to $(CH^+)(HA)$, where $(CH^+)$ and $(HA)$ are the concentrations of protonated cytosine and hydroxylamine respectively. Now the dissociation constants $K^C_a = (C)(H^+)/(CH^+)$ and $K^HA_a = (HA)(H^+)/HA.H^+$ so rate of reaction

$$\alpha (CH^+)(HA) = \frac{K^HA_a}{K^C_a} (C)(HA.H^+).$$

If $K^C_a >> K^HA_a$ then at optimum pH the cytosine will be largely free and the hydroxylamine largely protonated, and we can write

$$\text{rate of reaction } \sim K^HA_a \frac{K^C_a}{K^C_a} (\text{total } C) (\text{total } HA)$$

i.e. over a range of cytosines, the rate of reaction is approximately proportional to $1/K_a$ of the cytosine in question. Thus cytidine, with a $K_a$ 2.4 x that of cytosine, should react 2.4x slower than cytosine does in the reaction I $\rightarrow$ IV. This is indeed the case but there are obvious flaws to this argument. In the first place, the experimental optimum pH is somewhat higher than the 5.3 expected from the above, and secondly, there is a considerable electronic and
steric difference between cytosine and cytidine which must also affect the reaction rate. However, we shall apply the argument to the polymers to see where it leads. The pKₐ of cytosine in poly C is unfortunately unknown, but is probably different to that of the monomer. (The pKₐ of G in poly G has been estimated, and is found to be higher than that of the monomer in 0.1 M NaCl but probably lower at high salt concentration). If the pKₐ of cytosine in poly C is lower than that of the monomer under the conditions of reaction, it explains why poly C reacts more slowly with HA than C does: moreover, it would explain why this diminution in rate is less for MA than for HA, because MA has a pKₐ near that of the cytosines and the above approximation does not apply. It may also be that the change in structure in poly C between the pH's used for the HA and MA reactions is significant; if it takes the poly C• poly CH⁺ form in the NA reactions then all the cytosines are effectively protonated and able to undergo reaction.

B Later stages of reaction. These are not of great interest; nor are they of great accuracy, because after two days of incubation the solutions became a little turbid which masked the later readings. As with the cytidine reactions, lower reagent concentrations and lower pH's (as in the MA reactions) led to substitution (I → IV) and a high residual absorbance after reaction, and vice-versa.

C Initial stages of reaction. If there is a marked apparent induction period in the spectroscopic curve for the poly C + HA
reaction, then it is surprising that none was observed for the poly I. poly C + HA reaction, even at low HA concentration. But the induction period observed with the poly I. poly C + HA reaction is significant, and would appear to warrant further explanation as it would seem unlikely that reaction and denaturation of the polymer would coincidentally cancel each other out for so long to give a fortuitously flat curve. It must be that the lag period is real and that the rate of reaction of HA with the native double helix is in fact very low (cf. reaction with DNA, described later): then, after a small percentage of the cytosines have reacted, the double helix is broken down so that the reaction accelerates to a maximum rate approaching that of MA + poly C alone. Note in this connection that at pH 5 and below, poly I. poly C is thermodynamically unstable to break-up into poly I and poly C, though kinetically stable at normal temperatures. The conversion of a few of the cytosine residues to methoxyamine derivatives might well hasten this break-up. There are two ways of checking this conclusion.

(i) Dilution into glycol. It was found that glycol destroyed the secondary structure of poly I. poly C, as with other nucleic acids; thus the hypochromism of the complex was reduced by 95% by dilution into 80% glycol. As with poly C (above) this is a way of following the reaction, theoretically simple but practically clumsy; one difficulty is the limited solubility of the complex in aqueous glycol. The curve obtained (Fig. 8a) still showed a lag period.
**Fig. 8a**

Reaction of poly I-poly C with 1.8M methoxyamine, $39^\circ$, followed by dilution into glycol

**Fig. 8b**

Reactions of polymers with 0.5M carboxymethoxyamine, $39^\circ$

- poly C
- poly I-poly C
(ii) Uptake of radioactive methoxyamine. This experiment, to be described later, also showed a lag period thus verifying the conclusion above. To summarize, then: poly C reacts less than 1/10 as fast with HηA, and about 1/3 as fast with HηA, as cytidine does. The UV data give no indication that the reaction is in any way different from that with the monomer. The complex poly I-poly C has not been helpful as a model for RNA as it appears to break down under the reaction conditions, whereupon the reaction proceeds as with poly C alone.

The reaction of natural RNA with methoxyamine is perhaps more instructive. Thus E. Coli tRNA, a mixture of low molecular weight RNA's having both single-stranded and double-stranded regions, reacts like poly C does until 20% of the cytosines have reacted, and the reaction then slows down to 1/200 of its initial rate. This specificity of methoxyamine for non-hydrogen bonded cytosine residues was used to investigate the tertiary structure of E. Coli tRNA tyr.

The action of carboxymethoxyamine on RNA's has also been studied in the present work. The UV-followed reactions had to be done at low reagent concentration as its own UV absorption was considerable. When 1.43 mM p poly C was incubated with 0.5 M CMA at pH 5.8 and 39°C, the UV absorption of the solution at 310 nm rose by an amount corresponding to the conversion of some 80% of the I residues into IV (see Fig. 8b) with a t½ of about 50 hours.
This corresponds to a $k_4^0$ of $0.03 \text{ hr}^{-1}$, making the reaction somewhat faster than that with hydroxylamine.

When the same reagent was incubated with $1.84 \text{ mM}_{\text{p}}$ poly I, poly C at pH 5.5 and $39^\circ$, no change in the optical density of the mixture was seen over a period of 170 hours, either at 269 nm or 310 nm.

It seems that here the lag period is indefinite, i.e. that the polymer is reacting at the same rate that DNA would under these conditions. This would not have been detectable in this experiment. But since CMA is apparently unable to "unzip" the complex as HA does, it seems likely that it is not attacking the 4-positions of cytosine, being unable to reach them because of its size and/or charge. If, then, CMA is a bacterial mutagen, perhaps it has a case for candidature as one that acts by forming II residues. But the pH and other conditions in these experiments may be critical, and any study of CMA as a mutagen would have to include repetition of them under carefully controlled conditions.

Nature of the initial product in poly C + HA or MA

(A) by hydrolysis

In hydrolysing a reacted polymucleotide in order to determine what product is formed initially, we have the same difficulty as in UV experiments, that we are trying to detect a small quantity of product in the presence of much starting material. In experiments designed to prove that this product is II, which is converted back
to I by the hydrolysis procedure, we are trying to prove that no trace of product can be detected in the hydrolysate, which is even more difficult to do to the satisfaction of critics who require better evidence for the existence of II in a polymer.

(i) In RNA. Vervoerd treated E. Coli ribosomal RNA with $^4$H HA at 37°, then hydrolysed it (a) with RNase which produced no CMP and few C-terminated oligonucleotides, showing that the cytosines had been altered; and (b) with alkali which restored the original cytidylic acid. This observation, proving the reversibility of the initial reaction, does not appear to have been repeated or expanded.

(ii) In poly C. Phillips reacted poly C with 2.5M HA at 26°, taking aliquots at various times and submitting them to mild acid hydrolysis. This treatment hydrolysed the polymer to nucleotides and at the same time converted any acid-unstable II or III back to I or IV respectively. The mixture of products thus obtained was separated on ion-exchange columns into CMP, $^4$N-hydroxy CMP, UMP (none found), and free cytosines (traces only). He found a lag in the production of $^4$N-hydroxy CMP, indicating that no III or IV are formed in the polymer during the first 15 minutes of reaction, whereas cytosine is disappearing (by I → II) according to UV data. But the figures obtained hardly justify the conclusion; they deviate little from those expected for an exponential curve. Phillips states that the lag was more marked in another experiment using 1.5M HA, but does not give details.
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(iii) In poly I, poly C. An attempt was therefore made to repeat this result with a double-helical RNA which should if anything give a more pronounced result than with poly C. The method used was that of Phillips, but it is less accurate in this case as the anion-exchange resin used to separate the nucleotides did not completely separate IMP from IV, and the latter had to be estimated spectrophotometrically in the presence of the former. Yields were fairly good for the early analyses, but poorer in mid-reaction. The resulting reaction curve (Fig. 9) shows no obvious lag period in the production of $N^*-substituted$ derivatives: the rate of formation obtained ($k_{total}^0 = 0.02 \text{ hr}^{-1}$) is in good agreement with that found by spectroscopic methods.

(B) Uptake of labelled reagent

(i) By poly C. Phillips described two experiments with $^{14}C$-labelled methoxyamine. In the first, poly C was reacted with $2M$ reagent at $37^\circ$ and the total uptake of $^{14}C$ estimated by precipitation of the polymer and counting on filters. He showed that if the UV reaction represents $I \rightarrow II \rightarrow III$ then the radio-labelling reaction is $I \rightarrow III$, i.e. two moles of reagent are taken up. But the data are also consistent with the formation of a mixture of III and IV which is surely what one expects. In another experiment, in which poly C was treated with molar HA at $37^\circ$, the rate of substitution at C4 was also estimated by hydrolysis of the polymer and separation of labelled IV. The data were found to be consistent with an initial
Fig. 9
Reaction of poly I-poly C with 2.5M hydroxylamine, 39°C
formation of only II, followed by an increase in the rate of uptake as the stoichiometry of the reaction became $I + 2\text{MA} \rightarrow \text{III}$. The experiment has been criticized for its inaccuracy, but it remains one of the most important pieces of evidence in favour of the existence of II in a polymucleotide.

(ii) By poly I, poly C. This was originally carried out in this work in an attempt to extend the results of the above experiment to a double-helical polymucleotide, before it became clear from the UV experiments that nothing more could be learnt from the complex than from poly C itself. With $0.3\text{M}$ reagent at $37^\circ$, and using the method of Phillips to find the total uptake of radioactive methoxyamine by the polymer, a sigmoidal curve was obtained with an induction period of some 24 hours (Fig. 10a). The rate of uptake in molar terms was difficult to determine exactly for reasons outlined in the experimental section, but the maximum rate was about 0.003 moles of MA per mole of cytidylate residue per hour, which is the value one expects from the UV data. At this reagent concentration the reaction is almost entirely $I \rightarrow \text{IV}$: the induction period has been discussed in a previous section. Later readings on the curve became rather random, for reasons mentioned under the reaction with DNA, below.

Reaction of HA and MA with DNA

Theoretically, the ideal substrate for chemical investigation of mutagenic reactions is DNA itself, but practical difficulties
Fig. 10a
Reaction of poly I. poly C with 0.215M "C-methoxyamine, 37°

Fig. 10b
Reaction of DNA with 3.5M methoxyamine, 39°
have deterred people from using it. These problems are not insuperable, however.

(i) Followed by UV absorbance

Schell\(^6\) noted the decrease in UV absorbance at its \(\lambda_{\text{max}}\) of DNA incubated with 4M hydroxylamine, over a period of 6 hours at 21\(^0\) or of 1 hour at 50\(^0\). These times are extremely short compared with the following observations, so if the changes noted were not artefacts the polymer must have been in a less native state than that used here. When our calf thymus DNA was incubated with 5M HA, pH 6.5, for 50 hours at 39\(^0\), no change in UV absorbance was seen. However, after 150 hours at 60\(^0\) with the same reagent, the optical density at 257 nm fell slightly and the optical density at 310 nm rose from its initial very low value. This is what one expects if the reaction I \(\rightarrow\) IV is occurring in the polymer, and it is happening at the rate expected from the more quantitative experiments below, so it seems we are watching a real reaction. But use of HA cannot give an accurate result; the polymer becomes slightly denatured, and decomposition of the reagent (to give oxygen and nitrogen) gives rise to a background absorption.

When the more stable methoxyamine was used as reagent, a more reasonable curve was obtained (with 3.5M reagent at pH 5.6 at 39\(^0\)), the curve for absorbance at 310 nm was that shown in Fig. 10b). But it is not clear whether this really represents the reaction we are interested in. Several other processes contribute to rises
in optical density, and the rate of reaction suggested by the curve in Fig. 10b is twice that expected on the basis of experiments described below (though it is possible that IV_b has a greatly increased $\epsilon_{310}$ in DNA). It seems we must abandon UV spectrophotometry as a means of following these reactions with DNA.

(ii) Followed by hydrolysis of DNA and base analysis

(a) DNA + hydroxylamine

Schell 67 found that the hydrolysate of DNA which had been subjected to reaction with 4M HA at 21^0 for 7 days gave no blue coloration with FeCl$_3$ solution, which showed the absence of IV_a in the hydrolysate and therefore of III and IV in the polymer. Now the data given for DNA reactions in this work suggest that under the conditions given, only some 2% of the cytosines should have reacted. Even so, the sensitive FeCl$_3$ test should have detected this amount of product, if the test was working properly. This test is an important one and will be digressed upon.

Neutral FeCl$_3$ solution gives a blue colour with an aqueous solution of IV (R = H); the test works with N$_4^\text{H}$-hydroxycytosine and its nucleosides and 5-alkyl derivatives 67 but not with N$_4^\text{H}$-methoxylamin derivatives nor with 3-methyl derivatives, because it requires the hydroxyl group and N$_4^1$ as chelating sites. The colour slowly fades as the reagent oxidises the pyrimidine. A $\lambda_{\text{max}}$ of 608 nm and an $\epsilon$ of 900 have been estimated for the chelate with N$_4^\text{H}$-hydroxy cytosine, 67 but this is only an apparent $\epsilon$ for the
concentration of reagent used in that experiment (2-fold excess of reagent). If varying molar excesses of FeCl₃ are added to IVₐ and the absorbance measured, a real ε of 10640 and a Kᵣ of about 900 litre mole⁻¹ can be estimated. (Kᵣ = (chelate)/(free IVₐ)(ferric salt).) The optimum pH for the formation of the chelate is in the range 4 - 6: below pH 3 no reaction occurs (though the complex, once formed, is stable even at pH 2 67), and above pH 6.5 the Fe³⁺ combines preferentially with complexing anions present or gives Fe(OH)₃. This narrow pH range is one of the difficulties that make the test unreliable, and it sometimes fails even at the right pH.

This decreases our confidence in the observation on DNA above, as does the finding that when H₄N-hydroxycytosine was boiled with N HCl for an hour (conditions for DNA hydrolysis) and the solution neutralized only 1/5 of the expected blue coloration could be obtained with FeCl₃. This experiment, then, should be tried again with controls and standards, as its conclusion is important.

The dihydro-derivatives also give a purplish colour with FeCl₃, but with a lower ε and Kᵣ. With 5,6-dihydro N₄-hydroxy 1-methyl cytosine the Kᵣ was estimated at 100 but may have been lower. The dihydroxylamino derivatives (III, R=H) give a red colour with FeCl₃ by an oxidation mechanism. 67
(b) DNA + methoxyamine

The method used here is a standard one for DNA analysis. An aliquot of the reaction mixture is treated with ethanol to precipitate the DNA, which is transferred to a tube containing trifluoracetic acid. Heating this mixture at 156°C for an hour or more hydrolyses the polymer to a mixture of bases, phosphate and the charred remains of the sugar. The bases are then separated by paper chromatography and the spots eluted and estimated spectrophotometrically as described later. Hydrolysis can also be effected by perchloric acid but not by snake venom phosphodiesterase which hydrolyses DNA but apparently not MA-reacted DNA. Whether this was due to inhibition of the enzyme by the reagent or by mutated bases in the DNA was not clear, and the possibility of using enzymes for this work should be looked at again, as a sufficiently mild hydrolysing agent would yield III as well as IV. But the enzyme must hydrolyse the polymer completely, as an enzyme which preferentially leaves reacted sections of DNA intact will give spurious results.

The following disadvantages were found with the method. Firstly, that extensively reacted DNA gives low yields of bases, chiefly because the denatured DNA is more difficult to precipitate and much remains behind in solution (cf. Ref. 12). This should not lead to systematic errors in the result, however.

Secondly, the reagent tends to decompose during the long reaction times needed. This is probably unimportant with methoxyamine
but hydroxylamine is much less stable (see Chapter 3) and its reaction with DNA was not further studied, for this and the following reason. When $N^4$-hydroxycytosine is treated with trifluoracetic acid under the hydrolysis conditions, it is destroyed and two products are found. One of these is uracil, but the other has not been identified. Its $R_f$ and acid spectrum are those of cytosine, but it has a lower $\lambda_{\text{max}}$ in neutral solution. It gives no colour with ferric chloride and seems to be stable to reflux with $\text{NH}_3\text{Cl}$ for an hour. This product is not obtained if hydrolysis is carried out with perchloric acid, to which $N^4$-hydroxycytosine is stable. $N^4$-methoxycytosine was recovered unchanged after treatment with trifluoracetic acid at $156^\circ$ for 1 hour, except for a slight amount of hydrolysis to uracil ($<10\%$).

When DNA was subjected to base analysis by the method outlined above, base ratios were obtained in agreement with those reported. No spot corresponding to 5-methyl cytosine could be located on the chromatogram, but an area around $R_f 0.65$ was found to have a UV absorbance corresponding to about $5\%$ that of the cytosine. This may have been 5-methyl cytosine or an impurity. When methoxyaminereacted DNA was analysed, a spot could be seen at $R_f 0.65$ which slowly replaced that of cytosine, as shown in Fig. 11a, where the amount of product is expressed as a percentage of the cytosine originally present. The $3\%$ found at $t=0$ has been subtracted from the product
but hydroxylamine is much less stable (see Chapter 3) and its reaction
with DNA was not further studied, for this and the following reason.
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$\lambda_{\text{max}}$ in neutral solution. It gives no colour with ferric chloride
and seems to be stable to reflux with $\text{NH}_4\text{Cl}$ for an hour. This
product is not obtained if hydrolysis is carried out with perchloric
acid, to which $N^4$-hydroxycytosine is stable.\textsuperscript{70} $N^4$-methoxycytosine
was recovered unchanged after treatment with trifluoracetic acid at
$156^\circ$ for $1\frac{3}{4}$ hours, except for a slight amount of hydrolysis to
uracil (< 10%).

When DNA was subjected to base analysis by the method outlined
above, base ratios were obtained in agreement with those reported.\textsuperscript{6}
No spot corresponding to 5-methyl cytosine could be located on the
chromatogram, but an area around $R_f 0.65$ was found to have a UV
absorbance corresponding to about 5% that of the cytosine. This
may have been 5-methyl cytosine or an impurity. When methoxyamine-
reacted DNA was analysed, a spot could be seen at $R_f 0.65$ which
slowly replaced that of cytosine, as shown in Fig. 11a, where the
amount of product is expressed as a percentage of the cytosine originally
present. The 5% found at $t=0$ has been subtracted from the product
Fig. 11a
Reactions of DNA with
2.3M methoxynine, 39°

Fig. 11b
Reactions of DNA with
0.6M 14C-methoxynine, 37°
readings. The identity of the product we are watching with $N^4$-methoxycytosine was only established by its Rf and UV spectrum, but since IV$_b$ has been shown to be stable to the hydrolysis conditions, and the product is certainly not uracil, there can be little doubt that we are observing the formation of $N^4$-methoxycytosine.

If the reaction is of first order in methoxyamine, the reaction curve of Fig. 11a gives a $k_\text{total}^o$ of $2 \times 10^{-4}$ hr$^{-1}$, so the reaction is some 500 times slower than that with cytidine. Though consistent results were obtained with this particular sample of calf thymus DNA under the reaction conditions used, it cannot be said that DNA will always react at this rate; in particular a different way of preparing the reaction solution may well denature the polymer to a greater or lesser extent. But it can be said that in its physical condition and UV absorbance, the DNA after some 10% of reaction seemed to be still in good native condition: and no induction period, during which no $N^4$-substituted derivatives were being formed, was noted.

(iii) Followed by uptake of radioactive methoxyamine

The experiments described above for the synthetic RNA's were repeated with DNA. The principle is that the hydrolysis experiment above gives us the rate of formation of III + IV, and this one gives us the rate of formation of II + $2 \times$ III + IV. So if the overall reaction is I $\rightarrow$ IV we get the same reaction curve as in the above experiment: if it is I $\rightarrow$ III + IV we shall find a faster rate by this method, and if it is I $\rightarrow$ II a much faster one.
The difficulty is again practical. We are studying a reaction involving only 21% of the bases in DNA, and that to an extent of up to 10%, so the uptake of radioactivity, compared with the amount of background in the reaction mixture, is likely to be very small (using molar reagent, typically 0 - 200 cpm to be isolated from a background of 400,000 cpm). For this reason the reactions were carried out in the minimum volume of solution and with a low concentration of reagent, in order to maximise the uptake of \(^{14}\)C label.

The curve obtained in one experiment is shown in Fig. 11b, where the uptake of \(^{14}\)C is expressed as a percentage of that expected for the reaction of one mole of methoxyamine with one mole of cytosine residues in the DNA. It shows an initial rate of reaction similar to that obtained in the hydrolysis experiment (Fig. 11a) despite the lower concentration of reagent, i.e. \(k_{\text{total}} \) is about 4 times greater than expected. Since formation of III is negligible under these conditions (0.6M reagent) the conclusion is that II residues are being formed at 4 times the rate that IV residues are. The later readings are consistent with the hypothesis that II reaches an equilibrium concentration of some 6%, but not accurate enough to prove this conclusion; nor should much reliance be placed in the experiment as a whole. For other experiments under similar conditions gave considerably higher or lower rates of incorporation, and it was suspected that the filtering, washing and drying conditions were
critical. If species II is as labile as one might suppose, it might not survive the acid precipitation and washing treatments. And using milder treatment, it was found that radioactivity was being taken up into the polymer in other ways than by reaction. For instance, it was often found that counts were dissolved from the DNA and Millipore filter into the scintillation fluid used to count it.

To summarize: when HA or MA act on a polynucleotide, residues of type IV are formed; in the case of DNA, without any appreciable lag period but more slowly than earlier workers have realised. Experiments have been done to establish that II is being formed in the early stages of reaction at a much greater rate than IV, despite its theoretical instability; some of these can be dismissed but one or two have not been refuted. If the species II is as unstable as one is led to believe, then experiments designed to detect it are liable to give negative results (under vigorous conditions) or inconsistent results (under mild conditions). Theory does not require the presence of a high proportion of II in the material undergoing mutagenesis: if just one base in a thousand can exist as the hydroxylamine adduct under equilibrium conditions, this would be more than enough to make the reaction I → II mutagenic.

Are these experiments relevant to chemical mutagenesis? Are the polynucleotides being reacted under the same conditions
in chemical studies as in genetics? This seems so with the in vitro mutable material poly C, where the chemical and mutagenic studies can be paralleled, and with bacteriophages which are almost as reactive. Phillips' justifies the use of poly C as a model by suggesting that hydroxylamine mutagenesis might represent the attack of HA on certain exposed sites in the genetic material, as implied by the extreme variation in mutability between different sites on the same cistron: so poly C, with its bases all exposed, should be a good model. This is probably true for T4 phage, but more doubtful for transforming DNA, which usually has to be heated or otherwise partly denatured in order to make it react at all.

It has been estimated that transforming DNA is mutated by HA at only 1/1000 the rate that T4 is; this, on comparison with figures obtained above for DNA reactions, suggests that the DNA used there was at least ten times more reactive than transforming DNA. But the signs are that we are approaching the conditions of the mutagenesis we are trying to investigate.
CHAPTER 3

THE REACTIONS OF ADENINES WITH HYDROXYLAMINE

A. The hydroxylaminopurines

Practically all the work on this interesting group of compounds has been done by one group, that associated with A. Giner-Sorolla. The preparation of 6-hydroxylaminopurine (HAP) was reported in 1957.\textsuperscript{71} It was prepared by the action of hydroxylamine on 6-chloropurine.\textsuperscript{72}

Since then a number of these compounds have been prepared by the reaction of HA with the corresponding thio-, alkylthio-, chloro- and fluoropurines: thus were obtained a range of 9-substituted HAP's including N\textsubscript{6}-hydroxyadenosine (NOHA);\textsuperscript{73-76} \textsuperscript{2}-hydroxy HAP (\textsuperscript{6}-hydroxy isoquainine, VIII);\textsuperscript{76} 2,6-dihydroxylaminopurine;\textsuperscript{74} and a number of others with the NH\textsubscript{OH} group in other positions, including N\textsuperscript{2}-hydroxyguanine\textsuperscript{74} and its riboside.\textsuperscript{77} In this work we are only interested in the 6-hydroxylaminopurines. Physically and spectroscopically they resemble the parent adenines; three chemical properties are worth noting.

(i) Reduction to adenine derivatives, by dithionite or catalytically.\textsuperscript{74} This is the chief way of proving their structure.

(ii) The formation of a blue chelate with ferric chloride in aqueous solution. The considerations applying to the reaction of N\textsuperscript{4}-hydroxy cytosines with FeCl\textsubscript{3} also apply here; but the purines have also N\textsuperscript{7} available for chelation. The complex appears to be more stable
Fig. 12

VIII

IX

X

XI

XIIa

XIIb
than that with IV but the test is still not altogether reliable. The chelate is formed over the pH range 4–13, albeit slowly at alkaline pH, and best at neutral pH.

(iii) Reaction with dilute alkali: analogously to the reaction of N-phenyl hydroxylamine, HAP gives 6,6'-azoxypurine, \(^7_8\) for instance with 2N NaOH at room temperature, or with conc. NH3 over a period of 5 days. But the initial reaction with alkali is much faster than this. For instance, if NOHA is run on a paper chromatogram in ammonia solution, pH 10.1, as solvent, two spots are obtained: one is NOHA and the other an unknown product with a \(\lambda_{\text{max}}\) of 250 nm which is far from that of azoxypurine (which has 270 Å, & 385 at pH 7.8).

It would be interesting to know the tautomeric state of the hydroxylaminopurines; two factors suggest that it is the NHOH form.

(a) In its instability to alkali HAP resembles N-phenyl hydroxylamine rather than N4-hydroxycytosine which is an oxime and at least stable to ammonia if not to vigorous alkaline treatment.

(b) The more keto groups there are on a pyrimidine ring, the greater is the tautomeric constant in favour of a polyamide form. So conversely, if IV has a tautomeric constant of only 10 in favour of an oximino-form, \(^46\) then 4-hydroxylamino purine and HAP will probably prefer a hydroxylamino-form.

Such arguments are of limited value and it would be preferable to synthesise the fixed-tautomeric methylated derivatives IX and X (preferably the 9-alkylated derivatives, as X (R=H) can still
theoretically take up a hydroxylamino form) in order to determine the tautomeric constant as has been done for the N\textsuperscript{4}-hydroxycytosines.\textsuperscript{46} Though IX is presumably easily obtainable from 6-chloropurine, the synthesis of X presents difficulties. The corresponding chloro compound is unknown, so the compound must be sought by methylation of HAP or NOHA. If NOHA behaves like adenosine towards alkylation the product will be the N\textsuperscript{1}-substituted compound required, but if it resembles inosine then 7-methyl compound will be the main product. The reaction has been attempted here: thus on heating 0.05 gm NOHA with 1 ml MeI in 10 ml DMF for an hour at 70\textdegree, a product was seen on TLC of the reaction mixture but the only substance recoverable from the reaction had only end-absorption. The expected product X (R=ribofuranosyl) is likely to be subject to ready rearrangement, by reactions analogous to those discussed later.

B. Purine N\textsuperscript{1}-oxides

The chemistry and biology of these compounds has been dealt with in a recent review;\textsuperscript{23} a few points will be noted here. Simple derivatives of adenine are easily N-oxidised by a mixture of H\textsubscript{2}O\textsubscript{2} and acetic acid at room temperature; thus adenine N\textsuperscript{1}-oxide and adenosine N\textsuperscript{1}-oxide (ANO) are obtained.\textsuperscript{79} 6-methyl purine reacts under more forcing conditions; isoguanine gives a mixture of products, one of which is believed to be the N\textsuperscript{1}-oxide, usually made by a longer route.\textsuperscript{23} NOHA, however, is converted into inosine in good yield after 5 days with the reagent at room temperature. This strange
result has already been reported by other workers\textsuperscript{76} for the action of pertrifluoracetic acid on HAP, but no mechanism was suggested.

6-methylaminopurine reacts very slowly with $\text{H}_2\text{O}_2 + \text{HOAc}$: after 7 days at 50\(^\circ\) only some 30\% of the starting material had reacted. One of the products may have been the required $N^1$-oxide XI (Fig. 12); it should be possible to separate this from the mixture, and indeed this seems the best way of making 6-methylaminopurine $N^1$-oxide, which would have been useful in the investigations to be described, because no other possible precursors are available.

On attempting to oxidise 6-methylaminopurine with the more reactive mixture of $\text{H}_2\text{O}_2$ with trifluoroacetic acid, the UV absorption of the purine shifted to higher wavelengths. This is probably due to attack of the reagent on the methyl amino group (cf. oxidation of anilines to nitrobenzenes by pertrifluoracetic acid\textsuperscript{80}).

The adenine $N^1$-oxides have a characteristic sharp UV absorption peak at around 232 nm with a high ε-value; this is useful for their detection and estimation. They give a reddish colour with ferric chloride, but the Fe\textsuperscript{+++} is not tightly bound in the complex. They are more labile to ring-opening than the parent adenines: thus on boiling with 3N HCl for 10 minutes adenine 1-oxide is converted into a salt of 4-aminomidazole 5-carboxamidoxime.\textsuperscript{79}
C. The reaction of adenosine with hydroxylamine

The effect of HA on adenine derivatives was first investigated by Freese who observed the action of low concentrations of HA on AMP. The effects of the reagent on DNA had already been studied: oligonucleotides, nucleotides, nucleosides and bases were liberated, and it was suggested that this resulted from breakdown of HA to give OH radicals which then attacked the sugar phosphate chain. This mode of action is stressed in Freese's work, and the stability of HA will now be digressed upon.

It may be noted that many of Freese's results are obtained at pH's greater than 7, and here the reagent becomes markedly less stable. For instance, at pH 8.5, 4M HA has a half-life of only 5 hours at 24° but at pH 6.5, 3M HA had a half-life of more than 7 days at 65°, both estimates made by KMnO₄ titration. Moreover in the pH range 7–8 the dependence of decomposition rate on pH is likely to be quite critical. This rate is also affected by catalysis by metals (thus EDTA protects T₄ phage from inactivation by HA), by oxygen (and therefore the decomposition is autocatalytic) and even, it has been suggested, by the bases in the genetic material undergoing mutagenesis. It seems unlikely, therefore, that consistent results will be obtained in this field. As mutagenesis is usually carried out below pH 7, and physiological pH's in bacteria should be lower than this, the alkaline region is also relatively unimportant. The rate of decomposition increases with increasing ionic strength.
and with increasing HA concentration, thus 0.3 M HA in the absence of salt was found to be fairly stable even at pH 9.

This seems at variance with findings that high concentrations of NaCl protect T4 phages from inactivation by HA; and that HA mutagenesis appears to be molecular at high HA concentration (i.e. by a I → II or IV mechanism) and radical at low concentration (i.e. by attack of OH on bases). But these experiments are done under varying conditions, and the findings of one need not apply to another. It would be useful to have knowledge of the precise breakdown behaviour under any conditions.

Returning to the action of hydroxylamine on AMP, it was found that apart from the expected fragmentation to give adenine, traces of other products could be observed, one of which was later identified as a 7-oxide. A similar reaction was observed with H2O2, where adenine gave adenine N7-oxide and smaller quantities of 8-hydroxy adenine and isoquamine, but no N1-oxide as might have been expected by analogy with the reaction in acetic acid (attack of OH+). It seems at first surprising that no N6-hydroxy derivatives were seen in the reaction with HA (see below) but under the conditions given the extent of formation of these products would have been of the order of only 1%. Moreover, NOHA is not entirely stable to the ammoniacal chromatographic solvent used.
Budowsky et al. reacted AMP with HA at pH 5 and \(40^\circ\) for 3 days and showed that the main product was \(\text{N}^6\)-hydroxyadenylic acid by enzymic dephosphorylation followed by comparison of the material obtained with authentic NOHA according to UV spectra and Rf's in paper chromatography. An earlier communication had reported the reaction to be as fast as that with cytidine but the reaction is in fact much slower as will be noted later. The reaction of methoxymamine with adenosine was reported to proceed analogously, but no details were given.

In view of the relative inaccessibility of 6-chloro nucleosides and especially deoxynucleosides it would be convenient to find a method for the isolation of NOHA derivatives in quantity from this reaction, but this has not proved possible. The reaction cannot be taken to completion, as further reaction occurs (see below). After performing the reaction with hydroxylamine acetate and subliming off most of the reagent in vacuo, there remains a mixture of NOHA, adenosine, reagent and traces of further products which must be separated. Ion-exchange resins failed owing to the instability of NOHA in alkali. Column chromatography (cellulose column, eluted with BuOH saturated with water; or Sephadex G10, eluted with water) yielded a product free from other purines though containing small amounts of hydroxylamine acetate. This, and the apparently pure product obtainable in small quantities from
Budowsky et al. reacted AMP with $\text{HA}$ at pH 5 and $40^\circ$ for 3 days and showed that the main product was $N^6$-hydroxyadenylic acid by enzymic dephosphorylation followed by comparison of the material obtained with authentic NOHA according to UV spectra and Rf's in paper chromatography. An earlier communication had reported the reaction to be as fast as that with cytidine but the reaction is in fact much slower as will be noted later. The reaction of methoxyamine with adenosine was reported to proceed analogously, but no details were given.

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TLC (cellulose plate; run in BuOH satd. with H₂O), gave solids when the solvent was removed but in each case the product decomposed before recrystallisation could be attempted. That the product isolated was indeed NOHA was shown by comparing its UV spectrum and Rf's in three solvent systems with those of authentic material.

If the reaction is carried out for a longer period the main isolable product is adenosine 1-oxide; this was identified by separation by paper chromatography, whereupon it gave the same UV spectra and orange coloration with FeCl₃ spray as authentic ANO; and also by separation on a Sephadex column to obtain a few mgs of fairly pure product. This gave the same Rf's in paper chromatography, and a similar mass spectrum, to authentic ANO; the mass spectrum had a molecular ion at m/e 283 and no trace at 299 which would be expected of another likely product (N⁵-hydroxy adenosine 1-oxide).

The reaction of adenosine with hydroxylamine was carried out at various hydroxylamine concentrations, pH's and temperatures; the reaction appeared to follow the same course in each case, to be of first order in hydroxylamine, and to have a pH-rate profile similar to that of cytidine, being fastest at pH 6 and slow outside the range 5-7. The concentration of NOHA, as estimated by the formation of the blue chelate with FeCl₃, rose to a maximum of about 60% of the purines present, then fell off to zero. The concentration of ANO, as estimated by the UV absorption at 233 nm,
rose more or less exponentially, without a visible lag period in its formation although the data are not accurate enough for one to be certain that there is none. Since the amount of ANO present at the end of reaction accounted for only 60% of the original adenosine, there must be a third product, and this can be seen on chromatograms of the reaction mixture; it is an unidentified substance with a $\lambda_{\text{max}}$ of ca. 205 nm and very little absorbance at 260 nm. Possible mechanisms for these reactions will be discussed later in the chapter after certain related reactions have been considered.

From the reaction curve for the formation of NOHA (Fig. 13b) can be estimated a $k_0$ of the order of 0.0005 hr$^{-1}$ at 39$^\circ$, 0.0045 at 65$^\circ$ and 0.08 hr$^{-1}$ at 100$^\circ$. This means that the initial reaction, presumably the attack of a neutral HA molecule on the adeninium cation, is some 200 times slower than the corresponding reaction with cytidine ($k_0 = 0.09$ 36,37). This difference is largely due to the electron-withdrawing effect of the keto-group in cytidine; unfortunately no figures for 4-aminopyrimidine are available for comparison, but the purine analogue of cytosine, isoguanine, has been studied (see below). For the formation of ANO, a $t_{1/2}$ of about 14 days with 1.5M reagent at 65$^\circ$ could be estimated, but this is not a meaningful quantity as the reaction is not a simple one.

Adenine reacts similarly with hydroxylamine; in this case the $N^6$-hydroxy derivative HAP is formed at a similar rate ($k_0$...
**Fig. 13a**

UV Spectra of adenosines at neutral pH

**Fig. 13b**

Reaction of adenosine with 15M HA, 65°
about 0.004 hr$^{-1}$ at 65°; see Fig. 14a) but its disappearance and
the formation of adenine 1-oxide were faster (formation of the oxide, 
t$\frac{1}{2}$ about 4 days with 2M reagent). Separation of the products by
paper chromatography confirmed the estimates of the products by
spectrophotometric methods.

D. Reaction of N$^6$-hydroxy adenosine with hydroxylamine

When NOHA was incubated with 4M HA at pH 6 at 65°, it was
transformed into ANO: by watching the reaction by UV spectrometry
and by the colour given with FeCl$_3$ (Fig. 14b) a t$\frac{1}{2}$ of about 5 days
was estimated. No transformation occurs in the absence of hydroxyl-
amine: for instance, the reaction takes place if NOHA is treated
with HA for 4 hours at 100°, but it appears to be quite stable in
water, in phosphate buffer of pH 7, or in acetate buffer of pH 3,
under the same conditions. (It has been reported that NOHA is
stable in water$^{72}$ but the free base HAP decomposes on boiling owing
to oxidation.$^{72}$) It is not clear whether the HA is here acting
a general base, as a general nucleophile, or specifically; so
the action of some other bases was tried.

Pyridine: 3M pyridine + HCl to pH 5.5, at 100° for 2 hours,
followed by paper chromatography in solvent B. The only spots
observed were pyridine, NOHA and a faint spot with end-absorption.

Morpholine: 4M morpholine (pK$_a$ = 8.3) + HCl to pH 7.5, at
100° for 5 hours, followed by UV spectra. Some increase in
absorption at 233 nm was seen, which might correspond to up to 2%
Fig. 14.a
Reaction of adenine with 2M hydroxylamine, 65°

Fig. 14.b
Reaction of N°-hydroxy adenosine with 4M hydroxylamine, 65°
of ANO in the reaction mixture. But when the incubation was carried out for longer periods all that happened was a shift of the $\lambda_{\text{max}}$ to 250 nm (see alkaline reaction under chemistry of HAP). This transformation takes place at near-neutral pH's in these experiments and may itself be general-base catalysed.

Methoxyamine: 4M MA$\cdot$HCl + KOH to pH 6, at 100$^\circ$ for 5 hours, followed by UV spectroscopy. No formation of ANO was observed.

N-methyl hydroxylamine: 3M MeNH$\cdot$HCl + KOH to pH 7, at 100$^\circ$ for 4.5 hours. This experiment was inconclusive owing to the high UV absorption and decomposition rate of the reagent (a 2M solution of it at 65$^\circ$ and pH 6.5 decomposed with a half-life of about 16 hours, turning yellow in the process).

Hydrazine: 2M N$_2$H$_4$ + HCl to pH 7.4, at 65$^\circ$ for 12 days. No reaction took place.

It seems, then, that the isomerisation of NOHA to ANO is not catalysed by a general base (e.g. pyridine, with a $pK_a$ near that of HA) and probably not by nucleophiles (MA, hydrazine). It would be interesting to know whether MeNH$\cdot$HCl can perform the reaction as this would provide a clue to the question of whether or not the oxide atom in ANO comes from the reagent.

The reaction of NOHA with HA was carried out at various pH's and at varying hydroxylamine concentrations at pH 5.7 (Fig. 15). The pH optimum was found to lie above pH 6, but above pH 7.5 the alkaline transformation reactions become more rapid than the conversion
of ANO in the reaction mixture. But when the incubation was carried out for longer periods all that happened was a shift of the $\lambda_{\text{max}}$ to 250 nm (see alkaline reaction under chemistry of HAP). This transformation takes place at near-neutral pH's in these experiments and may itself be general-base catalysed.

Methoxyamine: $4\text{M MA.HCl + KOH to pH 6, at } 100^\circ\text{C for 5 hours,}$ followed by UV spectroscopy. No formation of ANO was observed.

$N$-methyl hydroxylamine: $3\text{M MeNHOOH.HCl + KOH to pH 7, at } 100^\circ\text{C for 4.5 hours.}$ This experiment was conclusive owing to the high UV absorption and decomposition rate of the reagent (a 2M solution of it at $65^\circ$ and pH 6.5 decomposed with a half-life of about 16 hours, turning yellow in the process).

Hydrazine: $2\text{M N}_2\text{H}_4 + \text{HCl to pH 7.4, at } 65^\circ\text{C for 12 days.}$ No reaction took place.

It seems, then, that the isomerisation of NOHA to ANO is not catalysed by a general base (e.g., pyridine, with a $pK_a$ near that of HA) and probably not by nucleophiles (MA, hydrazine). It would be interesting to know whether MeNHOOH can perform the reaction as this would provide a clue to the question of whether or not the oxide atom in ANO comes from the reagent.

The reaction of NOHA with HA was carried out at various pH's and at varying hydroxylamine concentrations at pH 5.7 (Fig. 15). The pH optimum was found to lie above pH 6, but above pH 7.5 the alkaline transformation reactions become more rapid than the conversion.
Fig. 15

Reaction of $N^\circ$-hydroxy adenosine with hydroxylamine, 65°
to ANO. The rate depended on the HA concentration but the order of reaction in HA appeared to be less than unity (the graphs of Fig. 15 give an order of around 0.6). More experiments are needed to clarify this, but accuracy is difficult as the more rapid decomposition of reagent in the more concentrated solutions tends to lower the apparent reaction order.

E. Further reaction of adenine N₁-oxides

When ANO was incubated with 2M HA at pH 5.9 for 12 days at 65° no change was seen in the UV spectrum and it appears that ANO is stable under these conditions. But when the experiment is repeated with adenine 1-oxide (2M HA at pH 5.4 for 23 days at 65°) the optical density falls both at 231 nm and at 262 nm with a t₁/2 of about 4 days, and the final spectrum has no maxima. The final product may be similar to that noted above in the reaction of adenosine with HA, with only end-absorption: its formation must involve the dearomatisation of both rings and may conceivably arise by attack of the reagent at the 4,5-double bond analogously to what happens much more rapidly in cytosine. Decomposition of adenine 1-oxide occurs much more slowly, if at all, in the absence of HA (no change in UV spectrum in phosphate buffer, pH 5.9, over a period of 19 days at 65°).

This difference in stability between the base and nucleoside is the converse of the behaviour in alkali, where the base, stabilized by anion formation, is more stable to ring opening than the nucleoside.
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This difference in stability between the base and nucleoside is the converse of the behaviour in alkali, where the base, stabilized by anion formation, is more stable to ring opening than the nucleoside.
F. Reactions of adenines with methoxyamine

As was noted, it has been reported that MA reacts with adenosine to give \( \text{N}^6 \)-methoxy adenosine.\(^{85}\) The reaction is about 4 times slower than with hydroxylamine, as one expects by analogy with cytidine. Adenine also reacts; the presumed product, \( \text{6-methoxy-aminopurine} \), has been prepared\(^{74}\) but no spectra or Rf's were given. It would be interesting to know the effect of hydroxylamine on this molecule.

G. Reactions of 1-methyl adenine

1-methyl adenine, which eluded synthesis for many years, was obtained by hydrolysis of 1-methyl adenosine.\(^{87}\) It has a pK\(_a\) of 7.2 above which it has been said to exist in the imino form (XIIa, Fig. 12) rather than in the amino (XIIb) form\(^{87}\) though this has not been proved: the pK values of a compound, while providing useful evidence on the skeletal structure of a compound, are of little value in locating its hydrogen atoms. The 3-methyl isomer has been shown to have an amino-form.\(^{88}\) The pyrimidine ring of 1-methyl adenine can be opened by either acid or base; thus reflux in 6N HCl for 2 hours degrades it to an imidazole, and the action of conc. \( \text{NH}_3 \) for 18 hours at 100\(^\circ\) isomerises it to 6-methylaminopurine by a Dimroth rearrangement.\(^{87,89}\) This latter reaction can also be effected by aqueous alkali (t\(_1/2\) at pH 11.7, 37\(^\circ\) = 70 minutes).
On incubating 1-methyl adenine with hydroxylamine it is converted to adenine 1-oxide. The reaction curve in 2M HA, pH 6.0, at 65° (Fig. 16a) shows an initial rapid loss of optical density at 261 nm followed by a slower fall as the product is not stable under the reaction conditions (section E above). From data obtained using molar reagent at 65° followed by UV spectroscopy or chromatography (Fig. 16b) a k° of 0.17 hr⁻¹ at 65° was estimated, so this reaction is about 40 times faster than the reaction of HA with adenine to give hydroxylaminopurine. The product was obtained as a solid but could not be successfully recrystallised; the mass spectrum and UV spectra were in accord with those of authentic adenine 1-oxide, and the analysis figures, though not good, showed that the product was a C₂ compound and not a C₆ compound as one might have expected from the reaction. As we have seen, the 1-oxide of 6-methylnaminopurine was not available as a solid for comparison; the compound obtained in solution from oxidation of 6-methylnaminopurine could not be distinguished from adenine 1-oxide by its UV spectra or Rf in solvent B.

The mechanism one would have expected for the reaction of 1-methyl adenine with HA would have been attack at C₆, which one expects to be of the order of 100 times faster than with adenine owing to the increase in pKₐ but this would have given X (R=H) and XI which were not detected. (Traces of other products were seen
Fig. 16a
Reaction of 1-methyl adenine with 2M hydroxylamine, 65°

Fig. 16b
Reaction of 1-methyl adenine with M hydroxylamine, 65°
on chromatography, one of which gave a blue colour with FeCl₃ spray, but this may have been HAP arising from adenine impurity in the starting material. Presumably, then, this expected reaction is delayed by the steric effect of the 1-methyl group, though it has been noted that the change in reaction rate with HA in going from 3-methyl cytosine to 1,3-dimethylcytosine is relatively small.

The mechanism for the reaction observed cannot involve HAP as an intermediate as this would have been detected, and probably not X either, although this unknown substance may be quite unstable to HA and be a candidate as an intermediate. The suggested pathway (Fig. 17) involves HA-catalysed ring opening analogous to the base-catalysed ring opening of the Dimroth rearrangement (properties of 1-methyl adenine, above) to give XIII (R=H). This methyl amidine then undergoes displacement of the methylimino group by hydroxylamine, either intermolecularly (route a: analogous to the pathway suggested by Elion, see below) or intramolecularly (route b: this is the route we favour since it explains why formation of N-oxide is likely to be faster than route c, which is observed with methoxyamine — see below). Whichever route the reaction takes, it is not easy to see why methylamine is exclusively eliminated in preference to ammonia. Presumably elimination takes place from the protonated form of the trisamine XIV or XV (R=H), and then it will be the most basic of the three groups that is eliminated. But primary and
secondary amines have almost identical pK's and one expects a mixture of products. The cyclisation of hydroxylamines and oximes to heteroaromatic N-oxides is a well-known reaction, e.g. in the synthesis of the N-oxides of isoguanine, hypoxanthine and some pyrazines.

A similar rearrangement with loss of a methyl group was reported by Elion in attempting to prepare 1-methyl adenine from 1-methyl 6-thiopurine by the action of ammonia, she obtained, under increasingly vigorous reaction conditions, 4-aminoimidazole 5-carboxamide (by ring opening), 6-methylaminopurine (by Dimroth rearrangement) and adenine. For the formation of this last product she suggested two pathways (Fig. 18a); the one involving 6-methylaminopurine is not applicable to the reaction above as 1-methyl adenine reacts faster with HA than the 6-isomer, and the suggestion is that it is the other mechanism which is operative in this case also.

The action of nucleophiles on 1-methyl adenine promises to be an interesting field; two others have been tried here. The products of the action of M-methoxyamine, pH 5.5, at 65°C over 6 days were found to be similar quantities of starting material, 6-methylaminopurine (identified by Rf and UV spectra) and an unidentified compound with λ\text{max} in the 280 nm region. The reagent has thus acted as a catalyst in the Dimroth rearrangement (pathway c shown in Fig. 17), but the other product may be an
imidazole that cannot recyclise. 1-methyl adenine also reacts with hydrazine, and its half-life in 2M $N_2H_4$ at 65$^\circ$ is about 5 days. Several products are obtained; their identification has not been attempted.

**H. Mechanism of the reactions of adenosine with hydroxylamine**

The mechanism of formation of NOHA presents no difficulties; it is presumably that of isoguanine (below) or cytosine with HA. We need a mechanism for the reaction NOHA $\rightarrow$ ANO, and start by rejecting mechanisms involving 1,3-shifts of oxygen. The ionic mechanisms feasible for the acetic anhydride rearrangements mentioned later are also inapplicable here, and the mechanism cannot be radical since the attack of $\cdot$OH on adenosines gives 7-oxides: the reaction must involve ring opening as the first stage. As NOHA is not facilely ring-opened this step must also account for the observed kinetics: to account for the dependence of rate on HA concentration it must involve HA, and to account for the pH dependence it should involve the attack of neutral HA on the neutral NOHA molecule (or cation; but NOHA has a $pK_a$ of 3.1 so the cation is rare at neutral pH).

We have also to decide whether adenosine reacts with HA to give ANO directly. The kinetics seem to indicate that it does, because the formation of ANO is almost exponential and this can only be so if adenosine and NOHA are furnishing ANO at similar rates.
Theoretically it should; if 1-methyl adenine reacts to give the oxide, then the cation of adenosine should undergo the same reaction. Moreover adenosine is electronically very similar to NOHA (e.g. the pK's are similar) then at least the first step in the NOHA → ANO reaction should be undergone by adenosine also.

Finally, the suggested mechanism should explain why no NOHA N₁-oxide is observed among the products.

No mechanism has been found which will satisfy all the requirements. Fission of the 1,6-bond as has been suggested for a pteridine analogue (see below) would lead to NOHA N₁-oxide; a mechanism analogous to the one suggested for 1-methyl adenine seems most likely (Fig. 18b), but again it is difficult to see why the alternative product is not also seen. A mechanism like that proposed for a similar reaction in the quinazoline field (see below) involving successive attack at the 6- and 2-positions is also possible (Fig. 19a); it has no advantages over the one suggested but may in fact be an easier route (compare the original suggestion in the cytosine series, that the route I → II → III → IV may be faster than the direct route I → IV). Reaction route a of Fig. 18b envisages hydroxylamine as a specific catalyst for the rearrangement of NOHA, whereas route b leads one to expect methoxyamine, for instance, to perform the same reaction.
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Fig. 19a

HON\textsubscript{2}NH\textsubscript{2}NHX \rightleftharpoons HONH\textsubscript{2}NHX

\[ \xrightarrow{\text{elimination to XVI then as Fig. 18b}} \]

HONH\textsubscript{2}NHX

Fig. 19b

HON\textsubscript{2}NH\textsubscript{2}OH \rightleftharpoons O\textsubscript{N}H\textsubscript{O}H
I. Other rearrangements involving N-oxides

The action of HA or MA on 4-pteridone has been well investigated.\(^{92,93}\)

At pH 6 HA cleaves the pyrazine ring, but at pH 7.5 the pyrimidine ring is attacked, and an analogue of NOHA 1-oxide, 3-hydroxy 4-pteridone, is obtained: this can undergo further reaction, and in the case of methoxyamine the isolated product is an imidazole.

The mechanism that was suggested for the formation of 3-hydroxy 4-pteridone is noted in Fig. 19b.

Another related reaction was reported by Adachi\(^{94}\) and used in the preparation of quinazoline N\(^3\)-oxides. Thus a mixture of quinazoline and HA.HCl slowly dissolved in 2N NaOH at room temperature, and on standing a product was obtained which on heating with acetone furnished the 3-oxide XVII. A mechanism later proposed\(^{95}\) for the reaction is given in Fig. 20a. 4-methoxyquinazoline (XVIII) gave XIX, an analogue of ANO, on refluxing with HA in MeOH + H\(_2\)O.

This reaction may proceed (i) via 4-hydroxyaminoquinazoline, a compound which is unfortunately unknown; in this case the subsequent rearrangement is that of NOHA; or (ii) by ring-opening according to Hayashi's mechanism (Fig. 20a) which yields the benzenoid analogue of XVI (Fig. 18b) which then cyclises as shown there.

The product XVII is probably not HA-stable itself; just as hydrazine adds to it giving 4-hydrazinoquinazoline\(^{96}\), HA would be expected to give the 4-hydroxyamino compound, or its rearranged
derivative XIX. In fact the product obtained under more forcing conditions (quinazoline + HA at 125°) is the isomer of XIX, 2-aminoquinazoline 3-oxide; perhaps this is formed via 2-hydroxylamino quinazoline. It would be interesting to try to relate this field with that of the purines: for instance, considering the mild conditions under which quinazoline reacts with HA, purine, although rather less electrophilic, ought to react also.

A number of acetic anhydride catalysed rearrangements are known among the pyrimidines and purines. Thus 3-methyl cytosine and N4-methyl cytosine are interconverted in Ac2O; here the isomers are of equal thermodynamic stability and an equilibrium mixture results. With the N-oxides, however, the oxide is usually less stable than the product and reaction goes to completion. Thus XX (Fig. 20b) rearranges to a derivative of 6,8-dihydroxy purine, XXI to 6-acetoxy methyl purine, and XXII to N4-acetoxy cytosine. Despite the superficial resemblance of these reactions several mechanisms have been postulated: the rearrangements of XX and XXI may have a simple mechanism involving a double attack of OAc- on the acetylated oxide, or may involve an intramolecular shift of acetate (as with XXIII) or an intermolecular shift (as with XXIV). The rearrangement of XXII, which also occurs with isoquarine 1-oxide, is the most interesting from our point of view as it is the reverse of the NOHA-ANO rearrangement. The isocyanate XXV was postulated as an intermediate. When the same reaction was
Fig. 21

XXIII

XXIV

XXV

XXVI

XXVII

XXVIII

XXIX

XXX

XXXI

XXXII

XXXIII

XXXIV
attempted with adenine 1-oxide a mixture of imidazolyl oxadiazoles \( \text{XXVI}_a + \text{XXVI}_b \) was obtained: a mechanism can be written for this but it is not clear why the reaction does not occur in the cytosine series as well.

The rearrangement proceeds more straightforwardly with alkyl derivatives of the N-oxides: thus when 1-ethoxy 9-ethyl adenine hydriodide is neutralised and warmed it gives 6-ethoxysmino 9-ethyl purine, via an isolable ring-opened intermediate\(^{102}\); the reaction is of synthetic use. The reverse mechanism was proposed by Phillips\(^{66}\) for the action of acid on 1,3-dimethyl 6-hydroxylamino 5,6-dihydro-uracil 4-oxime, but the product was not clearly identified: according to Hewlins\(^{103}\) it was in fact simply the expected 1,3-dimethyl uracil 4-oxime.

**J. Related reactions**

Isoguanine and its nucleoside crotonoside also react with hydroxylamine. The difficulty in studying the reaction of isoguanine with HA was its very low solubility (1 in 16,000 at 20\(^\circ\)\(^\circ\))\(^{104}\) making the reaction preparatively useless and making kinetics and chromatographic separation difficult. The nucleoside is easier to study but only a small quantity was available.

The action of molar HA, \( \text{pH ca 6.5} \), on isoguanine at 39\(^\circ\)\(^\circ\) was followed by the decrease in optical density at 300 nm. A \( k^o \) of about 0.06 hr\(^{-1}\) was estimated: thus the reaction is 100 times faster than with adenine but 10 times slower than that of cytosine (\( k^o_4 = 0.35 \) at 35\(^\circ\))\(^{36}\). Since the \( pK \)'s of cytosine and isoguanine
are almost identical the factors affecting the difference in reactivity are the electron-donating effect of the imidazole ring and the steric effect of the N⁷ atom.

The product appears to be identical with N⁶-hydroxy isoguanine (VIII) prepared from 6-methylthioxanthine: it has a λ max of 272 nm in water, and spectroscopic pK's of about 1 and 9 (cf. isoguanine, 4,7 and 9,0), and mixtures of products from the two sources could not be separated on paper chromatography by either of the three solvent systems tried.

Methoxyamine also reacts with isoguanine, with a rate constant of the same order.

If the reaction of hydroxylamine with adenines gives hydroxylaminopurines, then the action of hydrazine should give hydrazinopurines. It has been reported that if deoxyadenylic acid is treated with anhydrous hydrazine at 60⁰, it is consumed with a half-life of about 20 hours;¹⁰⁵ no indication was given of the nature of the products. A similar result was obtained with DNA. It was claimed elsewhere¹⁰⁶ that adenosine reacted with 2,4-dinitrophenylhydrazine to give the corresponding hydrazinopurine, and cytidine and guanosine similarly; but this observation has been doubted in the case of guanosine at least.¹⁰⁷
CHAPTER 4

REACTIONS AT THE 2-POSITION

Pyrimidines substituted at C4, and the corresponding purines substituted at C6, are in general susceptible to nucleophilic attack at that position, and examples involving displacement of amino-groups have been discussed in the previous chapters. But the 2-position is much less reactive, both experimentally and theoretically (see, for instance, the localisation energy calculations on purines\textsuperscript{108}). Thus 4-chloro 2-ethylthiopyrimidine (XXVII) reacts with 1 mole of ammonia at 115\textdegree but with a second mole only at 190\textdegree \textsuperscript{109}. The presence of an oxo-group at C4 of the pyrimidine appears to activate the molecule slightly;\textsuperscript{109} this is also to be expected and is analogous to the effect of the 2-substituent in the reactions of cytosine and isoguanine with hydroxylamine. As with these reactions, too, the purine is less reactive than the pyrimidine: 6-methyl 2-methylthio 4-pyrimidine (XXVIII) can be reacted with p-chloroaniline at 130\textdegree \textsuperscript{109} whereas the corresponding purine needs 160\textdegree \textsuperscript{110}. These generalisations, however, are founded on very little data.
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Attempted preparation of uracil 2-oxime

This substance, which appears to be unknown, should result from the reaction of hydroxylamine with 2-chloro or 2-methylthio 4-pyrimidone. Analogies exist for this reaction: $N^2$-hydroxyguanosine was prepared by the reaction of 2-fluoro inosine with hydroxylamine; the corresponding fluoropyrimidone is not known. 2-methylthio 4-pyrimidone (XXIX) has been substituted with hydrazine and even with aniline after prior activation with $HgCl_2$, though this latter method is probably inapplicable to hydroxylamine.

When (XXIX) was refluxed for 5 hours with hydroxylamine in ethanol + butanol, however, no reaction occurred; only one spot was seen on a chromatogram in solvent A. The addition of a trace of hydroxylamine hydrochloride has been said to be catalytic for such reactions, but when the experiment was repeated thus (HA + HA.HCl in EtOH, reflux overnight) the main product was uracil, although a trace of the required product may also have been formed. Since the instability of hydroxylamine prohibits the use of more vigorous conditions, more reactive pyrimidines were tried. The nucleoside XXX (Fig. 21) was refluxed for 6 hours with 4M HA in ethanol without result, but the cyclouridine XXXI, prepared by the method of Varadarajan, appeared to react. After refluxing for 8 hours with 2M HA in ethanol containing a trace of HA.HCl, five spots were obtained on TLC; these probably
included starting material and nucleosides of uracil and of the required oxime. Further separation was not attempted.

Reactions of guanine and isocytosine with hydroxylamine

After incubating guanosine with 3M hydroxylamine, pH 6.5, for 26 days at 39° no change in its UV spectrum was seen, and no products could be detected by paper chromatography (solvent A). It was concluded that if reaction occurs to give the N2-hydroxy derivative it must have a k° of less than 3 x 10^-6 hr^-1.

The pyrimidine analogues of guanine, the isocytosines, react with hydroxylamine. The isocytidine XXXII was dissolved in 3M hydroxylamine solution, pH 6.7, and kept at 65°. The optical density of the solution at λmax (255 nm) fell to zero with a half-life of about 1.4 hours. This loss of optical density was not reversed by the action of acid as it is in the case of cytidine, so it seems unlikely that a simple addition to the 5,6-double bond is occurring; but the complete absence of this reaction with guanine suggests that this double bond must be involved.

Isocytosine, prepared by published methods,113 reacted with hydroxylamine at a similar rate to XXXII, with an optimum pH around 6.5 - 7. A chromatogram of the reaction mixture (solvent A) gave an unidentified spot of λmax 288 nm, but no uracil, uracil 2-oxime or isoxazolone (tested for with diazotised sulphanilic acid26). In view of the difficult in preparing the oxime as noted above,
It seems unlikely that it is a primary product in this reaction. Possibly the first step is the attack of hydroxylamine at C6 of protonated isocytosine, followed by breakdown to non-absorbing products as in the case of uracil. But then one would expect the major product from the reaction of uracil, the isoxazolone XXXII, to be among the products here.
EXPERIMENTAL SECTION

Ultra-violet spectra were recorded on the Zeiss PMQ II spectrometer, the Unicam SP 500 or SP 800, or Cary, as indicated.

Descending paper chromatography was performed using the following solvent systems and Whatman No.1 paper.

Solvent A: 0.880 ammonia + water, 1:400 pH ≈ 10
Solvent B: n-butanol + acetic acid + water, 40:50:10
Solvent C: 7% ammonium chloride in water
Solvent P: isopropanol + ammonia + water, 7:1:2

Solvent B gives the best separations of purely organic mixtures, but in the presence of salts gives erratic Rf's; here solvent A and Wyatt's are more useful.

pH's given to 1 decimal place were those determined using a Pye pH meter. Approximate pH's were those shown by narrow-range pH paper.

A Packard 3375 instrument was used for liquid scintillation counting, with toluene-based (1 gm PPO in 250 ml AR toluene) or dioxan-based (1 gm PPO + 15 gm naphthalene in 200 ml dried and distilled dioxan) counting fluid. A background of about 20 cpm, as obtained with a blank solution, was subtracted from all readings, and the resulting figure divided by the automatic external standardisation
ratio, when available, to correct for slight variations in the counting efficiency.

Analyses were performed by the staff of the University Chemical Laboratory.

Isoguanine sulphate, polycytidylic acid, polyinosinic acid and calf thymus DNA (sodium salt, grade V) were purchased from the Sigma Chemical Corporation. \(N^6\)-hydroxy adenosine, adenosine \(N^1\)-oxide and \(N^6\)-hydroxy isoguanine were gifts from Dr A. Giner-Sorolla, to whom the author is very grateful. \(N^4\)-hydroxy cytosine was prepared by P. Schell. Free hydroxylamine was prepared from its hydrochloride by Brauer's second method. Free hydroxylamine was prepared from its hydrochloride by Brauer's second method. It was converted into the acetate by interaction with acetic acid in ethanol followed by evaporation and recrystallisation from ethanol (M. Pt 81\(^\circ\)).

**EXPERIMENTAL TO CHAPTER 2**

**Preparation of reagents**

Methoxyamine hydrochloride was obtained by the method of Hjeds and the hydrobromide by the method described below. Carboxyimethoxyamine hemi-hydrochloride was obtained as described in Organic Syntheses, and esterified according to Kornowski et al., the ester, ethyl 2-amino\(\ \text{Oxyacetate}, can also be prepared from ethyl bromoacetate using \(N\)-hydroxy phthalimide but the product is more difficult to purify.
Ethyl oxyiminoacetate was obtained by the method of
Knemutov;\(^{118}\) yields in the second step (ethyl iminoacetate +
hydroxylamine) were lower than that reported owing to alkaline
hydrolysis of the iminoacetate, even at -20\(^\circ\). A Michael reaction
takes place with methyl acrylate as described\(^{119}\) but more slowly
than was stated (it needs about 5 days at room temperature).
The product, CH\(_3\)C(OEt):NOCH\(_2\)CH\(_2\)COOCH\(_3\), was distilled and distinguished
from starting material by the loss of OH absorption in the infra-red
spectrum and the appearance of a carbonyl band at 1780 cm\(^{-1}\). This
substance was dissolved in conc. HCl, then diluted to 2N and refluxed
for 1 hour. The product, 3-aminooxypropionic acid hydrochloride,
had a melting point of 144-6\(^\circ\) dec. after two recrystallisations from
ethanol/ether. Found: C 25.8%, H 6.0%, N 10.1%. Calculated
for C\(_5\)H\(_6\)NO\(_2\)Cl: C 25.4%, H 5.7%, N 9.5%. Attempts to prepare the
ester by alkylation of N-hydroxy phthalimide sodium salt with ethyl
3-bromopropionate were unsuccessful as preferential elimination to
the acrylate took place.

N-hydroxy phthalimide was prepared by published methods.\(^{120}\)
The 0-methyl derivative has been described\(^{121}\) but the method of
preparation only gave 25% yield. Improved yields based on the
alkylating agent are obtained using DMF as solvent,\(^{122}\) and by the
use of pre-formed sodium salt. This cannot be prepared in good
yield as excess alkali converts N-hydroxy phthalimide to an
anthranilate.\(^{120}\)
To a mixture of 45 gm N-hydroxy phthalimide and 200 ml water is slowly added, with stirring, 11 gm NaOH in 20 ml water. The red mixture obtained is heated to 80°, whereupon the solid dissolves to a dark solution. On cooling to 0° the sodium salt is obtained as bright red hydrated crystals which, if kept as such, slowly decompose to anthranilate; but on drying in a desiccator a dark red powder is produced which is stable at room temperature. Yield 5%.

The methylation of this salt, followed by hydrolysis, seemed the best route to 14C-methoxylamine; other methods gave similar or inferior yields. Attempts were made to improve the yield in this method by varying the conditions, but the maximum yield obtained was 64% in each step (40% overall). The hydrolysis can be performed with NaOH,121 hydrazine,122 HBr/HOAc or methanolic ammonia.123

Preparation of labelled methoxylamine

1 mC of 14C methyl iodide (30 mC/mM = 4.7 mg MeI; purchased from the Radiochemical Centre) in a sealed vessel was attached to a vacuum line. The contents were allowed to distil into a receiver cooled in liquid nitrogen and containing 0.1 gm (0.54 mM) of N-hydroxy phthalimide sodium salt and a solution of 42.3 mg (0.33 mM) methyl iodide in 1 ml freshly distilled DMF. This mixture was then
To a mixture of 45 gm N-hydroxy phthalimide and 200 ml water is slowly added, with stirring, 11 gm NaOH in 20 ml water. The red mixture obtained is heated to 80°, whereupon the solid dissolves to a dark solution. On cooling to 0° the sodium salt is obtained as bright red hydrated crystals which, if kept as such, slowly decompose to anthranilic acid; but on drying in a desiccator a dark red powder is produced which is stable at room temperature.

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The methylation of this salt, followed by hydrolysis, seemed the best route to 14C-methoxyamine; other methods gave similar or inferior yields. Attempts were made to improve the yield in this method by varying the conditions, but the maximum yield obtained was 64% in each step (40% overall). The hydrolysis can be performed with NaOH, hydrazine, HBr/HOAc or methanolic ammonia.

Preparation of labelled methoxyamine

1 mC of 14C methyl iodide (30 mC/mM = 4.7 mg MeI; purchased from the Radiochemical Centre) in a sealed vessel was attached to a vacuum line. The contents were allowed to distil into a receiver cooled in liquid nitrogen and containing 0.1 gm (0.54 mM) of N-hydroxy phthalimide sodium salt and a solution of 42.3 mg (0.33 mM) methyl iodide in 1 ml freshly distilled DMF. This mixture was then
shaken overnight, and excess methyl iodide distilled off at room temperature (3% of the original counts). The remainder was centrifuged to remove excess sodium salt (3% of the counts), and the supernatant diluted to 12 ml with water and cooled to 0° for an hour. The white precipitate obtained was centrifuged off; this time the supernatant held most of the original radioactivity (58% of the counts; partly unprecipitated product, but also containing much volatile radioactivity). The solid product (crystalline N-methoxy phthalimide, XXIV (Fig. 21)) was boiled gently for 10 minutes with a mixture of two parts 48% HBr in H₂O and one part glacial HOAc, then cooled to 0° and seeded with phthalic acid. After an hour the solution was centrifuged to remove phthalic acid and the solvent evaporated on the vacuum line (60°, 2 hours at 1 mM). The distillate, HBr + HOAc, had 2% of the original counts and the product, crude ¹⁴C-methoxyamidine hydrobromide, about 32%. A solution of this in 3 ml of water was used in labelling experiments.

Reactions followed by UV spectroscopy

Cytidine + carboxymethoxyamine (Figs. 3 and 4)

The reaction mixtures were 12 ml of 0.5M CMA hemihydrochloride containing 0.8 mM cytidine, adjusted to the appropriate pH with KOH. These were incubated at 39° and 0.4 ml samples were taken
for dilution to 4 ml and optical density measurement at 269 nm and 310 nm on the Zeiss. The $\varepsilon_p$'s were obtained by subtracting blank readings of 0.022 at 269 nm and 0.010 at 310 nm and then dividing by $8 \times 10^{-4}$. (\( \varepsilon \) of cytidine = 9100 126). The reagent, when incubated alone, did not show any significant change in UV absorption.

Reactions with CMA at pH 3.7 and with 3-amino5xypionic acid at pH 4.8 were done similarly but using 0.7 mM cytidine.

With CMA at pH 3.7 a high background absorbance made the readings at 269 nm inaccurate but better results were obtained at 310 nm.

**Polymers + hydroxylamine and methoxamine**

The polymer solutions were prepared by dissolving the solids in N/10 NaCl. The complex poly I-poly C was prepared by mixing solutions of poly I and poly C in a molar ratio of 1:1:1; the increase in hypochromicity observed confirmed that interaction had occurred, though prior heating of the poly C sample has been recommended. 127 The polymer solution was then mixed with a solution of MA-HBr adjusted to pH 5.5 with NaOH, and incubated at 39°; the final concentration of MA was 1.8M. Similar results were obtained by direct reading of the solution in the Zeiss, or by diluting samples ten times for measurement; the graphs given (Figs. 6 and 7) combine results obtained by both methods. The molecular weight of the polymers per phosphate group ($M_p$) of the
polymers was not known, so literature values of \( \varepsilon_p \) were used in calculating the results. In the case of poly I, poly C, the complex is not hypochromic with respect to its constituents at 269 nm, i.e. the absorptions of poly I and poly C are additive at this wavelength. So to a good approximation subtraction of the \( \varepsilon_p \) of the poly I present (52% of 3260 = 1710) gives the absorption of the cytosine fraction. This has then been expressed as a percentage of its original value.

A solution of poly I, poly C in 2N NaCl at 39° showed no change in UV absorbance over a period of 120 hours; nor did a 1.6M solution of the reagent.

The reaction of poly I, poly C with hydroxylamine was carried out similarly (2.79M HA, pH 6.5; reagent absorption nil). It was repeated at low reagent concentration (0.18M HA).

The optical density of a poly I, poly C + methoxyamine reaction mixture was also measured by diluting 0.4 ml of reaction mixture (0.285 gpl. poly I, poly C + 1.82M HA.HBr adjusted to pH ca 5.5) to 4 ml with redistilled ethylene glycol. This had an optical density of 0.01 at 269 nm which has been subtracted from the readings obtained. The figures have then been converted to \( \varepsilon_p \) values and an approximate \( \varepsilon_p \) of 2000 for denatured polyinosinic acid subtracted. The results have then been expressed as a percentage of the original cytosine absorption (Fig. 8a).
Polymers + carboxymethoxyamine

Three tubes were incubated at 39°C, containing 0.5M CMA, 0.01M HCl adjusted to pH 5 with KOH, and (i) 0.54 gpl poly C, (ii) 0.39 gpl poly I + 0.33 gpl poly C, or (iii) nothing. Portions of 0.3 ml were diluted to 4 ml for reading in the Zeiss; the optical density of the blank tube remained constant. The readings at 269 nm changed very little; the expected slight decrease in optical density due to reaction was probably counterbalanced by some increase in background absorption of the reagent. The more informative readings at 310 nm are given in Fig. 8b as an approximate 6-value obtained as above, relative to this value at t=0.

Hydrolysis of hydroxylamine-reacted poly I, poly C.

The reaction solution in this experiment was similar to that used in the UV run (2.5M HA, pH 6.5, at 39°C; 0.58 gpl poly C + 0.71 gpl poly I). At intervals, 1.5 ml portions of this were treated with 1.5 ml of 3N HCl and hydrolysed at 60°C for a minute. These samples were left at 20°C for a day, then stored at 0°C. Each fraction was then analysed as follows.

The mixture of nucleotides obtained was freed from hydroxylamine by diluting the sample to 40 ml, passing down a Dowex 50 column (100 - 200 mesh, 13 x 1.4 cm), and eluting with N/20 HCl. The nucleotides were thus eluted, inosinic acid first: after their
collection the column was washed with N HCl. The pooled nucleotide fractions were then brought to neutrality with NaOH: the resulting solution, volume ca 140 ml, was then absorbed on to an anion-exchange resin (Bio-rad AGL-X2, 50-100 mesh, 12 x 1.42 cm, chloride form). Elution with N/400 HCl then gave CMP, and a N/200 - N/20 HCl gradient eluted N₄-hydroxy cytidylic acid (IV₄) and IMP. These nucleotide solutions obtained were each brought to pH 1 and estimated spectrophotometrically, using the values

\[
\text{CMP: } \epsilon_{280} = 12590 \quad \text{(Ref. 33)}
\]
\[
\text{N-OH CMP: } \epsilon_{280} = 12200, \epsilon_{248} = 4000 \quad \text{(Ref. 33)}
\]
\[
\text{IMP: } \epsilon_{280} = 600, \epsilon_{248} = 12300 \quad \text{(Ref. 128)}
\]

A typical result, obtained for the 3.6 hour sample, was as follows. The elution pattern from the anion-exchange resin was, to an arbitrary scale:
UV measurement.

**CMP fraction:** 85.1 OD's of CMP

**HO-CMP fraction:** 1.70 OD's of HO-CMP, 0.3 OD's of IMP

**Mixed fraction:** 1.93 OD's of HO-CMP, 1.9 OD's of IMP

**IMP fraction:** 1.65 OD's of HO-CMP, 24.0 OD's of IMP.

**Result:** 107% recovery of original cytosine, 22% as hydroxycytidylic acid; 98% recovery of IMP.

The results obtained are plotted in Fig.9 as the mean of CMP recovery % and 100 - (HO-CMP recovery) %. Total cytosines recoveries were 97 ± 8%.

**poly I + poly C + \(^{14}\)C-methoxyamine (Fig. 10a)**

4 ml of 0.215M MeONH<sub>2</sub>·HBr adjusted to pH ca 5.5 with NaOH and containing 0.34 gpl poly I + 0.27 gpl poly C and 0.4 ml of the \(^{14}\)C-methoxyamine preparation were incubated at 37°. At intervals 0.1 ml samples were withdrawn into 0.5 ml of ice-cold 10% TCA and stored frozen. The solution, containing precipitated polymer, was filtered on a Millipore filter, washed with 4 x 5 ml ice-cold 10% TCA containing hydroxylamine hydrochloride, and finally with ether. The filter was placed at the bottom of a liquid scintillation vial, dried at ca 60° and counted after the addition of 10 ml toluene scintillation fluid. The filter was then removed and the solution again counted. This gave a background reading which was
higher than the normal background (21 cpm): the counts obtained in solution were filterable and clearly not attached to the polymer and were therefore subtracted from the total. The value thus obtained was then corrected by means of the AES ratio (see above).

Unfortunately no labelled poly C was available for determination of the counting efficiency, so the following method was used. A 2μl sample was taken from the reaction solution, acidified, and adsorbed on to a Millipore filter. This was then dried at room temperature and counted as the other samples: a mean count of 1300 was obtained in this way. From this the theoretical labelling of each sample, if 1 mole of MA reacted with each mole of cytosine residues, could be deduced:

\[
\text{label per sample} = \frac{1300}{\text{AES ratio}} \times 0.1 \times \frac{0.27}{360} \times \frac{1}{0.215} \approx 220 \text{ cpm}
\]

since the experimental \( M_p \) of cytidylic acid in poly C was 360. This calculation is the most awkward part of the experiment, and its errors decrease the reliability of rates obtained by radioactive uptake.

Reactions of DNA

(i) A solution of 2.6 mg DNA in 5 ml 3.5M MeONH\(_2\), pH 5.6, was incubated at 39° and its absorption at 310 nm read at intervals on the Zeiss. The curve obtained is recorded in Fig. 10b.
(ii) Analysis of DNA. About 1 mg DNA was heated with 0.5 ml CF$_3$COOH in a sealed tube at 156° (boiling cyclohexanone) for an hour. The dark liquid obtained was spotted on chromatography paper (3 spots) and run in Wyatt's solvent overnight. Charred matter remained at the origin and UV photography of the paper revealed the four separated bases, of Rf 0.35, 0.45, 0.55 and 0.80; these Rf's are rather higher than those recorded by Wyatt. These spots were cut out and eluted by standing overnight in 4 ml of 0.1N HCl, and the UV absorbances of the resulting solutions measured using the SP 500. For each spot an area of paper of the same Rf was used as a blank. A spot was also cut out at Rf 0.65, though nothing was visible there.

Typical results were (amounts recovered in 10$^{-9}$ moles):

\[
\begin{align*}
G & 283 \quad A & 358 \quad C & 260 \quad MC & 15 \quad T & 351 \\
G & 249 \quad A & 316 \quad C & 234 \quad MC & 12 \quad T & 315 \\
G & 178 \quad A & 311 \quad C & 230 \quad MC & 12 \quad T & 302
\end{align*}
\]

\[
\frac{A}{C + MC} = 1.28, \frac{MC}{C + MC} = 0.052
\]

(iii) Reaction with methoxygenine. DNA solution was prepared by leaving 11 mg DNA in 0.7 ml water overnight; this method prevents denaturation by dilution. Neutralised MA.HCl solution was then added to give 3.5 ml of 2.34 MA, pH 5.7 containing 3.15 gpl DNA; this was then incubated at 39°. 0.3 ml samples were removed and the DNA content precipitated by the slow addition of 5ml 95% ethanol
with stirring. The resulting fibrous precipitate was spun down, washed with ethanol and analysed as above. Typical results were:

\[
\begin{align*}
G & \quad A \quad 80 \quad C \quad 30 \quad X \quad 31 \quad T \quad 85 \quad \lambda / (C + X) = 1.32 \quad X / (C + X) = 0.51 \\
G & \quad A \quad 79 \quad C \quad 32 \quad X \quad 25 \quad T \quad 83 \quad \lambda / (C + X) = 1.38 \quad X / (C + X) = 0.44
\end{align*}
\]

thus the cytosine component is now about 48% X, of which 43% represents N-methoxy cytosine.

(iv) Effects of trifluoroacetic acid. When IVa was reacted with CF₃COOH for 1.5 hours at 156°C, no starting material remained (< 0.5% according to FeCl₃ test). Chromatography in Wyatts gave two spots, one having \( R_f 0.72 \) and \( \lambda_{\text{max}} \) 260, \( \lambda_{\text{min}} \) 229 at pH 1.4, as uracil; and the other having \( R_f 0.56 \) and \( \lambda_{\text{max}} \) 276, \( \lambda_{\text{min}} \) 240 at pH 1.4 and \( \lambda_{\text{max}} \) 268, \( \lambda_{\text{min}} \) 249 at pH 7.

IVb was prepared by allowing cytosine and methoxyamine to react for a long period at pH 4, then separated by chromatography in Wyatt's solvent. The material obtained was treated with CF₃COOH under the same conditions as above: this time the cytosine derivative was unchanged in \( R_f (0.57) \) and UV spectrum (\( \lambda_{\text{max}} \) 279 at pH 1, \( \lambda_{\text{max}} \) 276 and 237 at pH 7).

The hydrolysis product from the reaction of MA with DNA (X above) was shown to be \( N^\lambda \)-methoxyamino cytosine by comparison with the material obtained from cytosine. Both had an \( R_f \) of 1.16 relative to cytosine in Wyatts (uracil has 1.24) and a \( \lambda_{\text{max}} \) near 276 nm.
(v) Reaction with $^{14}$C-methoxyamine. The reaction mixture was 2 ml of 0.04 M MeONH$_2$·HBr adjusted to pH 5.6 with KOH and containing 4.7 gpl DNA and 0.3 ml of $^{14}$C-MeONH$_2$·HBr preparation. The reaction and counting of DNA were done as for poly I·poly C (above), but the 0.1 ml samples were diluted into strong NaCl solution and stored frozen. The DNA was precipitated, often visibly as a fibre, by addition of 5 ml 10% TCA, and after filtration as before the vials were dried at room temperature. A background of 420 cpm was subtracted from the readings, which were then expressed as a percentage of the theoretical labelling for a 1:1 reaction of methoxyamine with the cytosine residues. This quantity was calculated as for poly I·poly C:

$$\text{labelling per sample} = 13000 \times \frac{0.1 \times 20\% \times 0.37 \times 1}{0.002 \times 400 \times 0.4} \approx 3800$$

**Chelation between IV$_a$ and Fe$^{+++}$**

A 2% solution of FeCl$_3$ in 95% ethanol was added dropwise to a 0.85 mM solution of $^4$-hydroxy cytosine in water, and the absorption of the mixture measured on the SP 800. A graph of the apparent $\epsilon$ against the molar ratio of FeCl$_3$·IV$_a$ then gave $\epsilon$ by extrapolation and $K^f$ by evaluation at various points. Schell's value$^{67}$ lay on the curve.
The reaction was also carried out in the presence of hydroxylamine acetate buffer at various pH's:

<table>
<thead>
<tr>
<th>pH</th>
<th>2.0</th>
<th>2.5</th>
<th>3.1</th>
<th>3.9</th>
<th>4.9</th>
<th>6.0</th>
<th>8.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD 600</td>
<td>0.076</td>
<td>0.083</td>
<td>0.129</td>
<td>0.160</td>
<td>0.177</td>
<td>0.120*</td>
<td></td>
</tr>
</tbody>
</table>

* red colour (ferric acetate)

Experiments on other hydroxylamino-compounds were done similarly.

EXPERIMENTAL TO CHAPTER 3

1. N¹-oxidations

A small quantity of NOHA was dissolved in HOAc + 30% H₂O₂, 10:1 and left at room temperature for 8 days. Paper chromatography (solvent B) showed the mixture to contain small amounts of NOHA (RF 0.27) and a substance with end absorption (RF 0.53) but chiefly inosine (RF 0.36). This latter had λ max (acid) 249 and λ max (alkali) 252 nm. [Lit. 248 and 253].

0.1 gm 6-methylaminopurine was dissolved in 0.8 ml 30% H₂O₂ + 1.2 ml HOAc and kept at 50°, adding H₂O₂ periodically as it decomposed. Over a period of 5 days, the ratio OD 232/OD 264 rose from 0.4 to 1.3. Paper chromatography (solvent B) of the mixture showed spots at RF 0.72 (6-methylaminopurine), 0.47 (end-absorption) and 0.59. This latter had λ max (pH 1) 259, (water) 231 and 262, and (pH 13) 231 and 271 nm. Adenine N¹-oxide has (respectively) 258, 231 and 262, and 233 and 275, with similar OD ratios. 79
2. Reaction of adenosine with HA

(i) A mixture of about 50 mg of adenosine and 100 mg hydroxylamine acetate, a few mg of free hydroxylamine, and 2 ml water were heated at 100° in a sealed tube for 15 hours. After reaction the UV spectrum had $\lambda_{\text{max}}$ 233 and 263 nm with an OD ratio of 1.63:1.

The solvent was evaporated off and some of the reagent removed by sublimation at $10^{-3}$ mm, 70° for 1.5 hours. The remaining yellow solid was dissolved in water, applied to a Sephadex G10 column (40 x 120 mesh, 34 x 12 cm) and eluted with water. 4.6 ml fractions were collected as follows (schematic):

Fractions 22-30 were evaporated at 10 mm and at $10^{-3}$ mm and yielded a few mgs of a sticky white solid which, on standing, decomposed to a black tar. The solid was soluble in hot methanol but the solution refused to crystallise. The solution in water was spotted on chromatography paper and run in three solvents:

- Solvent B
  - Rf 0.29 = that of pure NOHA

- Wyatt's solvent
  - Rf 0.48 = that of pure NOHA

- Solvent P
  - Rf 0.49. Rf of pure NOHA = 0.53; Rf of mixture 0.52 (single spot only).
The spot at Rf 0.49 was cut out and eluted in water and its spectra taken. Water: $\lambda_{\text{max}}$ 265, pH 1 $\lambda_{\text{max}}$ 264, $\lambda_{\text{min}}$ 233.

pH 13: $\lambda_{\text{max}}$ initially 260 and 296, after 460 sec. 256 and 308 (see reactions of NOHA). Literature values: 265, 265 and 232, 252 and 310 respectively.

Fractions 12 - 18 from the Sephadex column, treated similarly, yielded a few mgs of adenosine 1-oxide.

Paper chromatography: Solvent B Rf 0.354 pure ANO has 0.34

Solvent P Rf 0.384 pure ANO has 0.37

UV spectrum in water: found $\lambda_{\text{max}}$ 232.5 and 261

Lit. $\lambda_{\text{max}}$ 232.5 and 260.

Mass spectrum. Found for product from adenosine + HA:

peaks at 268 (10), 268 (9), 267 (32), 250 (21), 238 (15), 237 (94), etc.

Found for pure adenosine 1-oxide:

peaks at 238 (40), 268 (6), 267 (24), 252 (22), 250 (20), 237 (56), etc.

In the mass spectrum of ANO, the oxygen atom appears to be lost to give adenosine (267) whose further breakdown pattern has been elucidated: 130 peaks are seen at 250 (loss of sugar OH) and 237 (loss of C5 as CH2O), and larger peaks at lower m/e (abundance scale above is arbitrary).

(ii) A 1.2M solution of HA at pH 5.8 and containing adenosine was kept at 65°, and 0.05 ml samples taken at intervals. These were
treated with 3 drops of 2% ferric chloride in ethanol and diluted to 4 ml for visible spectroscopy (SP 800) and then further diluted ten times for UV spectroscopy. The UV spectra (Fig. 13a) are not appreciably affected by the presence of FeCl₃. Two parameters were taken to follow the kinetics of the reaction: $OD^{232}/OD_{265}^{max}$ measures ANO and varies from 0.35 for adenosine to 4.4 for pure ANO. $OD^{550}/OD_{265}^{max}$ in the presence of FeCl₃ measures NOHA and rises to 0.086 for pure NOHA. These parameters are plotted in Fig. 13b: curves were similarly obtained for the reaction at 39° and at 100° (sealed tube). The half-lives of these curves were used to estimate approximate rate constants for the reaction. To show that there was no gross error in using UV curves in this way, the reaction was also followed chromatographically (solvent C): this confirmed the UV results.

3. Reaction of adenine with hydroxylamine

5 ml of 2M NH₂OH.HCl, adjusted to pH 6.4 with KOH, was saturated with adenine and incubated at 65°. 0.25 ml samples were diluted to 4 ml with water and examined by treatment with FeCl₃ and by UV spectroscopy as for adenosine (above). Chromatography of the reaction mixture in solvent B gave HAP (Rf 0.52), a faint spot (Rf 0.30), and a mixture of adenine and its N₁-oxide (Rf 0.61) which was resolvable by Wyatt's system.
4. Reaction of $N^6$-hydroxy adenosine with HA

$N\text{OHA}$ was dissolved in $4\text{M} \text{NH}_2\text{OH} \cdot \text{HCl}$ adjusted to pH 6.5 with KOH, and the mixture incubated at $65^\circ$. Samples were taken and examined as with adenosine (above). The curves obtained are given in Fig. 14a: the reaction was also carried out at $100^\circ$ with HA and with other reagents in sealed tubes, as stated in the text.

$N\text{OHA}$ was dissolved in $\text{NH}_2\text{OH} \cdot \text{HCl}$ solutions of various strengths, all adjusted to pH 5.7 with KOH, and the mixture incubated at $65^\circ$: samples were taken for dilution $\times 27$ for spectroscopy (SP 800) and calculation of the ratio of optical densities at 233 and 265 which is plotted in Fig. 15.

5. Reactions of 1-methyl adenine

1-methyl adenine was prepared by the method of Brookes and Lawley.

(i) A small quantity of the product was dissolved in 5 ml 2M $\text{NH}_2\text{OH} \cdot \text{HCl}$ brought to pH 6.0 with KOH, and the mixture was kept at $65^\circ$. 0.15 ml samples were diluted to 4 ml for UV spectroscopy (SP 800): the reagent cut out below 250 nm and only the absorption at 261 nm could be followed (Fig. 16a).

(ii) In another experiment using $\text{M} \text{NH}_2\text{OH} \cdot \text{HOMe}$ at $65^\circ$, the ratio OD 232/OD 260 could be followed as in the experiments above. The rate obtained by this method was confirmed as follows: the reaction
mixture was chromatographed using solvent B, and the spots cut out, eluted with water, and analysed by UV spectra. Assuming the N$_1$-oxide to have an $\epsilon_{\text{max}}$ of 3.15 x that of adenine, the ratio of product to starting material could be calculated. The figures obtained at three different reaction times are plotted in Fig. 16b.

(iii) 1 ml water containing 73 mg 1-methyl adenine and 93 mg hydroxylamine acetate was kept at 65° overnight. A white solid precipitated from the reaction mixture on cooling: it was filtered off but could not be purified by recrystallisation.

Analysis:  
C 35.5% H 4.9% N 40.3%. C$_3$H$_5$N$_5$O$_5$ requires  
C 33.6% H 4.5% N 39.1%.

UV spectra (Cary): pH 1, $\lambda_{\text{max}}$ 258 (\(\epsilon = 11540\)), $\lambda_{\text{min}}$ 230 (4610)  
\(\quad\) pH 7, $\lambda_{\text{max}}$ 262 (7700) and 231 (41500), $\lambda_{\text{min}}$ 249 (5850)  
\(\quad\) pH 13, $\lambda_{\text{max}}$ 273 (7310) and 232 (46600), $\lambda_{\text{min}}$ 251 (4600).

The $\lambda_{\text{max}}$ and $\epsilon_{\text{max}}$ values are close to those given by Stevens et al.\(^79\) for adenine N$^1$-oxide.

Mass spectrum: peaks at m/e 151 (11%), 149 (10%), 135 (100%), 119 (5%), 108 (3%), etc. Adenine N$^1$-oxide (prepared by the method of Stevens et al.\(^79\)) gave m/e 151  
\(\quad\) (M, 100%), 135 (M - O, 60%), 119 (M - NH$_2$, 17%),  
\(\quad\) 108 (M - CONH, 30%)

(iv) A small quantity of 1-methyl adenine was dissolved in M MeONH$_2$.HCl adjusted to pH ca 5.5 with KOH. After incubation at 65° for 6 days the UV absorption maximum had shifted. Chromatography in solvent B
gave three spots:

Rf 0.30, $\lambda_{\text{max}}$; (acid) 277, (neutral) 281, (alkali) 283, all approx.
Rf 0.33, $\lambda_{\text{max}}$; (acid) 259, (neutral) 260, (alkali) 270, as was also observed for 1-methyl adenine.
Rf 0.44, $\lambda_{\text{max}}$; (acid) 267, (alkali) 274 with slightly higher OD.

6-methylaminopurine has $\lambda_{\text{max}}$ (acid) 267, (alkali) 272 with slightly higher e-value.

The product and authentic 6-methylaminopurine had the same Rf (0.56) in Wyatt's solvent.

**Reaction of isoquamine with hydroxylamine**

Isoquamine was prepared from its sulphate by the method of Albert and Brown, and recrystallised from water.

(i) 8 ml of water were saturated with isoquamine at room temperature, then made molar in HA at pH 6.5 by the addition of hydroxylamine hydrochloride and KOH. The mixture was kept at 39°C, and samples of 0.6 ml diluted to 4 ml for UV absorbance measurement at 300 nm on the SP 500. The observed OD fell from 1.12 to 0.75 in 30 hours; blank solutions of M HA and of isoquamine in NaCl showed no change in absorption under these conditions.

(ii) Isoquamine was heated at 100°C with 2M HA acetate containing a trace of free HA for 6 hours. After removal of remaining solid the solution was evaporated and the reagent largely removed by
sublimation. The residue was extracted with hot water and the solution subjected to paper chromatography.

Solvent B: same Rf as N⁶-hydroxy isoguanine.

Solvent P: mixtures with authentic N⁶-hydroxy isoguanine gave Rf's ranging from 0.29 to 0.36 depending on salt concentration, but always single spots.

Wyatt's solvent: mixtures gave Rf's from 0.35 to 0.38, but always single spots which gave faint blue colours with ferric chloride spray. The spot obtained from the isoguanine + HA reaction was cut out and eluted for UV spectroscopy.

\[ \lambda_{\text{max}} \text{(water)} = 272 = \text{that of N}^6\text{-hydroxy isoguanine}. \]

Acid spectrum is unclear owing to small quantity of material (ca 1 OD) but not inconsistent with the \( \lambda_{\text{max}} \) of 285 observed with N⁶-hydroxy isoguanine at pH 0 (not 294 as given in the literature⁷⁶). The compound is unstable to alkali.
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