THE PRODUCTION OF MUTATIONS IN *SALMONELLA TYPHIMURIUM* WITH N-METHYL-N'-NITRO-N-NITROSOGUANIDINE (NG) AND A LITERATURE STUDY OF THE ACTION OF NG ON LIVING CELLS

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INTRODUCTION

Until fairly recently, most of our knowledge about genetics has come from work with various multicellular organisms. Much of it was done in an attempt to improve crops and domestic animals. A great deal of detailed information about genetic processes has been learned from work, which was begun by T. H. Morgan in 1910, with Drosophila melanogaster, the fruit fly (Peters, 1959; Wagner and Mitchell, 1964). This organism offers the advantages of enabling geneticists to work with large numbers, propagate new generations rapidly, and control their environment fairly easily and inexpensively. Also, since this fly has large chromosomes in its salivary glands, genetic data can be correlated with structural positions that may be seen on the chromosome (Gardner, 1965).

Beginning about 1940, emphasis shifted to the genetics of bacteria and bacteriophages, since the first three advantages mentioned for Drosophila are even greater in them. Some bacteria reproduce in 20-30 minutes, resulting in many generations and millions of offspring in a matter of a few hours. Also, since most of them are haploid, one does not have a problem of recessive genes being masked by their dominant alleles in the expression of their phenotype. Since mutations are usually recessive, a haploid organism allows their immediate expression.

Discoveries began to focus research on the molecular biology of the chromosome. As early as 1871, Miescher had found nucleic acids in the nucleus of animal cells (Wagner and Mitchell, 1964; Sturtevant, 1965), but it was not until 1924, when Feulgen and Rossenbeck developed a stain that was relatively specific for DNA that it was learned that the DNA was contained in the chromosomes (Sturtevant, 1965).

In 1927, H. J. Miller, working with Drosophila, and Stadler, working with corn, discovered that mutants could be produced by X-ray treatment (Peters, 1959; Ravin, 1965). Shortly afterwards the mutagenic effects of other types of radiation were discovered, Ultra-violet light was discovered to be mutagenic by Promtov in 1938 (Hayes 1968).

Beadle and Tatum's 1941 discovery of mutants of Neurospora that were not able to produce certain needed chemicals in their metabolism resulted in the hypotheses that the production of each enzyme was controlled by one specific gene (Peters, 1959; Wagner and Mitchell, 1964; Watson, 1965; Wilson, 1966).

In 1946, Demerec and Latarjet began an intensive study of the mutagenesis of E. coli by uv light and Lederberg and Tatum found that about two bacteria in two million (in their first experiments) mated sexually. The result was the re-combination of genetic information (Hayes, 1968).

The following year, Muller suggested an explanation for the phenomenon of transformation. Griffith, in 1928, had found that a non-virulent, non-capsulated strain of Pneumococcus could be "transformed" into a virulent, encapsulated form if grown in the presence of an extract of the latter strain. Muller thought that fragments from the DNA of the latter had entered the non-virulent strain carrying the genetic information for virulence and encapsulation (Stainer, et al., 1963; Barry, 1964; Gardner, 1965; Hayes, 1968).

By 1952, work with bacteriophages of E. coli, started by Delbrück, resulted in the information that not only is phage genetic material DNA, but that some of these phages can transduce small parts of the host chromosome to a different strain and remain within the cell, apparently harmlessly, thus adding this bit of genetic information to the host cell (Hayes, 1968).

In 1953, Watson and Crick proposed the widely accepted model of the

molecule of DNA as a double helix of two nucleotide chains joined by hydrogen bonds between the bases; adenine bonded to thymine, and cytosine to guanine (Watson, 1965; Brink 1967).

With intensive study of bacterial mutants and their phages, it was realized that the definition of "gene," as previously accepted, was no longer valid because mutations had been discovered to occur within the gene. The accepted view now is that the gene is a segment of the chromosome that determines the production of a particular polyleptide chain (Watson, 1965).

If mutations are chemical changes in genes, the study of how various agents can produce these changes and how they affect the organism may inform us of the nature of the gene and possibly show us some way to control and correct genetic defects. For instance, if cancer is initiated by genetic defects, the use of chemicals that modify genetic material may lead to understanding of this serious medical problem.

The first really effective chemical mutagens were discovered during World War II. X-rays were known to produce mutations and since mustard gas /dichloroethyl sulfide, $S(CH_2CH_2Cl)_2$/ produced burns that resembled X-ray burns, it was postulated that it might also produce mutations. Mustard gas, nitrogen mustard /"NH_2" = $CH_3N(CH_2CH_2Cl)_2$/ and other "mustards" are alkylating agents due to their chloroethyl groups. There is a very strong correlation between their alkylating ability and their mutagenicity (Auerbach, 1967).

Mandell and Greenberg (1960) announced the discovery of a new chemical mutagen for bacteria: 1-methyl-3-nitro-1-nitrosoguanidine. This is also named N-methyl, N'-nitro-N-nitrosoguanidine and called nitrosoguanidine or just NG, which is the abbreviation that will be used in this paper. This compound has proven to be the most potent mutagenic agent yet discovered. It is reported to

induce at least one mutation/treated cell, while still permitting over 50% survival, with 50% auxotrophs resulting (Adelberg, et al., 1965).

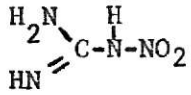
The mustards, azaserine, mitomycin C, nitrofurazone, proflavin, and NG, are called "radiomimetic" compounds because they all cause mutation, apparently by some attack on DNA. They share cross-resistance with ultra violet light and other radiations, and some of them induce filament production, as radiations do in E. coli (Terawaki and Greenberg, 1965; Auerbach, 1967).

The production of mutations in large numbers greatly enhances the study of genetics since it is through the study of mutations that knowledge is being gained about the replicating order of the genes, the control mechanisms of DNA replication, the mechanisms involved in protein production and in cancer cell development and control.

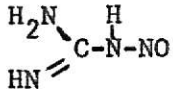
A REVIEW OF THE LITERATURE CONCERNING THE ACTION OF N-METHYL-N'-NITRO-N-NITROSOGUANIDINE ON LIVING CELLS

Early History of NG and Related Compounds

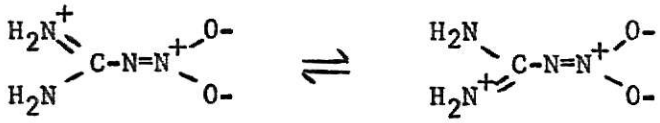
In 1877, Jousselin produced nitroguanidine,



thinking that it was nitrosoguanidine:

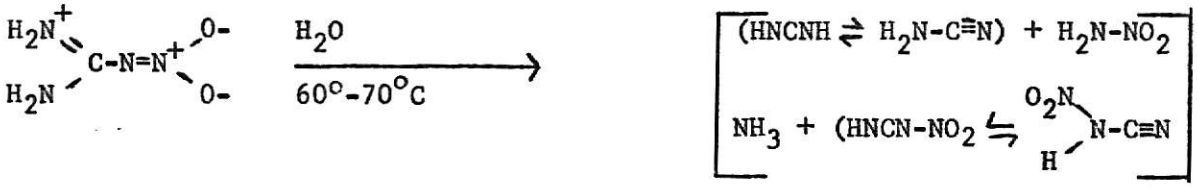


The latter compound was successfully produced by Thiele in 1892 (McKay, 1952). Uv studies suggested that nitroguanidine exists as Zwitterions:



(McKay, 1952).

Davis and Luce (1927) reported that nitroguanidine is unstable in an aqueous solution at 60°-70°C. and dearranges to form either cyanamide and nitroamide, or ammonia and nitrocyanamide:



They also found that its general reactions with primary aliphatic amines produced principally alkylnitroguanidines. They believed this to be the result of the amine reacting with the nitrocyanamide from the above dearrangement.

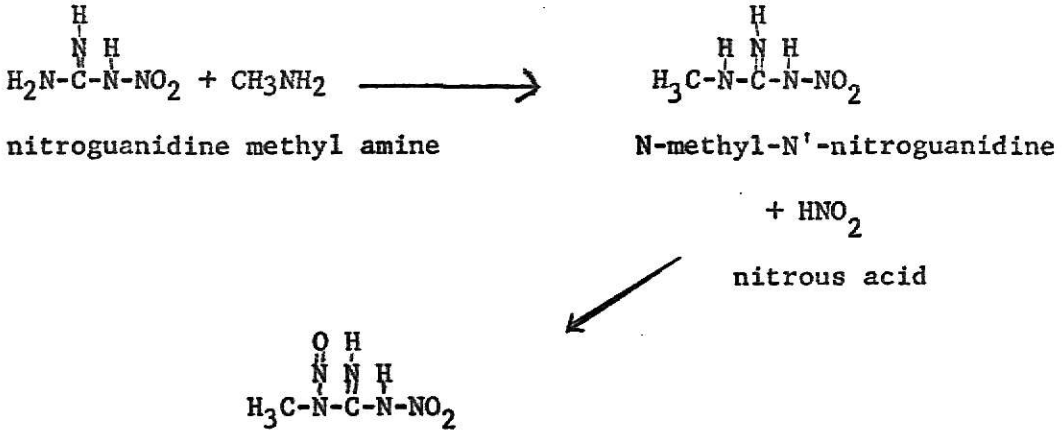
(Davis and Rosenquist, 1937).

Dimethylamine was the only secondary amine that would react in this way.

Since nitroguanidine was an important ingredient in flashless propellants, World War II stimulated a great deal of research with this and related compounds (McKay, 1949; Henry, 1950a and 1950b; McKay, *et al.*, 1951; McKay and Thomas, 1951; McKay and Milks, 1950).

The Discovery of N-Methyl-N'-Nitro-N-Nitrosoguanidine

McKay and Wright (1947) discovered a process for producing N-methyl-N-nitroso-N'-nitroguanidine (or N-methyl-N'-nitro-N-nitrosoguanidine), hereafter called NG in this paper. This was done by first methylating nitroguanidine and then reacting it with nitrous acid to give NG.

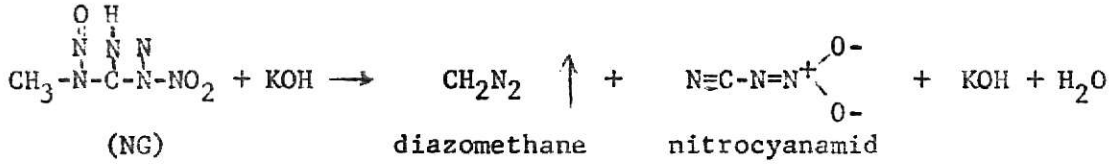


N-methyl-N'-nitro-N-nitrosoguanidine (NG)

The Physical and Chemical Characteristics of NG

Purified NG is an orange, crystalline material. It is relatively stable, but only if kept dark, dry, and in a freezer (Eisenstark, personal communication). In aqueous solution, it slowly decomposes. The rate of decomposition and its products depend upon the pH and the temperature of the solution. Mandell and Greenberg (1960) found that nitrous acid is produced as it decomposes at low pH. These authors found NG in aqueous solution to be most stable at pH 5.5. Above pH 5.5, the decomposition results in the formation of the solid nitrocyana-

mid, which remains in solution, and diazomethane which evolves as a gas. McKay (1948) was then able to produce diazomethane as follows:



(NG) diazomethane nitrocyanamid

Diazomethane was shown to be a powerful methylating agent and has been used in that capacity ever since.

McKay and Wright (1947) also discovered that NG would react with primary amines to produce N-alkyl-nitroguanidines. Davis and Rosenquist (1937) had found the same reaction with unsubstituted nitroguanidine.

(NG) primary amine N-alkyl-nitroguanidine

NG Discovered to be Mutagenic

Mandell and Greenberg (1960) were testing the resistence and cross-resistence of E. coli mutants to anti-cancer agents when they discovered unusually high numbers of mutants on their tryptone agar pH 5.5, containing 2ug/ml NG. This amount of NG was sufficient to inhibit the parent sensitive strain S, which led them to suspect that the NG was inducing the production of mutants resistant to itself, rather than just selecting spontaneously arising mutants. They ran fluctuation tests, as developed by Luria and Delbrück (1943) and used the replica plating technique of indirectly selecting bacterial mutants devised by Lederberg and Lederberg (1952). They found auxotrophs that needed amino acids, vitamins, purines, and pyrimidines. Many of their mutant clones were found to be mixtures of strains that differed in some growth requirement, colony size,

or colony morphology. (Confirmed by Eisenstark, et al., 1965). They also found that S/ng1 and S/ng2 (the first and second step mutants treated with NG) were equally sensitive with the parent strain S to nitrites in their growth medium. They concluded that the NG itself was the mutagenic agent, rather than the nitrous acid which was its decomposition product.

The Mutagenicity of NG

NG is the most mutagenic agent available and has been used with various types of organisms in diverse conditions.

Lopriano and Clark (1965) compared the mutagenicity of several mutagens on Schizosaccharomyces phombe. They found them to be effective in the following order of mutagenicity/lethality:

1. NG
2. Nitrosomethylurithane
3. Ultraviolet light
4. Nitrous acid.

Adelberg, et al. (1965) stated that NG "appears to be the most potent chemical mutagen yet discovered." (p. 178). They reported instances of over 50% survival with 40% auxotrophic mutants formed with E. coli K-12. There was shown to be a frequency of .2% valine resistance. By their calculations, this indicated that every cell received at least one mutation. They suggested that this might be a disadvantage in some kinds of studies because there is a possibility that every cell would be mutated at more than one site.

Mindlin and Churkina (1969) compared the mutagenic activity of N-alkyl-N-nitrosoureas and NG in E. coli K-12, insufficient B_1, threonine, and leucine. Various concentrations from .025-1% were used for 30-80 minutes. They found that NG gave the greatest mutagenic activity in a molar concentration that was

20-40 times lower than that of the nitrosoureas.

When about 300 stationary phase prototrophic Salmonella typhimurium were placed on minimal agar containing 2.ug/ml NG, 75-80% survived, and of these, 28% were auxotrophs. (Eisenstark, et al., 1965).

NG and nitrosomethylurea were also found to be the most effective mutagens for the induction of mutations in Streptomyces aureofaciens when compared with:

1. Radiation
2. Ultraviolet light
3. X-rays
4. Methanesulfonate
5. HNO_2
6. NH_2OH
7. HCHO
8. Combination of nitrogen mustard and ultraviolet light.

(Mracek, et al., 1969)

The comparative mutagenicity of NG on Arabidopsis seeds revealed it to be relatively low in toxicity but highly mutagenic, as is ethyl methane sulfonate. However, the latter compound had to be used in concentrations 50 times higher than NG to achieve the same degree of effectiveness. (Müller and Gichner, 1964).

The Effects of pH on the Mutagenicity of Nitrosoamides

Zimmerman, et al. (1965) determined the influence of pH on the mutagenicity of eight n-alkynitrosamides and nitrous acid in reverse mutation systems on the haploid adenine-requiring strain of Saccharomyces cerivisiae ad-6-45. All eight compounds tested were highly mutagenic down to pH 2, but there was a

greater induction of mutation by N,N'-dinitroso-N,N'-dimethyloxamide; N-nitroso-N-methylurea; 1-nitroso-2-imidazolidone; and N,N'-dinitroso-N,N'-dimethylterephthalamide at low pH. Induction of mutation by N-nitroso-N-methylacetamide and N-nitroso-N-methylurethan was greater at a high pH, while the mutagenic effects of NG were similar over the whole pH range from 2-8. NG, N-nitroso-N-methylurea and N-Nitroso-N-methylurethan caused the deamination of adenine to hypoxanthine. This was believed to have been caused by the nitrous acid, which is a decomposition product of N-methylnitrosamides at low pH. Nitrous acid is known to be a strong mutagen.

At high pH, these compounds decompose, yielding diazomethane, which is believed to be the agent responsible for mutagenesis.

The authors determined the effects of NG at pH 2, 4, 6, and 8 using 30 minutes exposure in a 1/15M phosphate buffer. pH 6 allowed a survival rate of 89% with a mutation frequency of $34/10^6$ survivors and 619 mutants counted. This was well above the other pH test comparisons. Spontaneous reverse mutants in this strain occur at a frequency of less than 1 in 10^7, and were therefore disregarded.

The fact that the relationship between the mutagenicity of NG to pH varies with the organism was shown by Cerda'-Olmedo (1967). Both the mutagenicity and lethality of NG on E. coli TAU-bar increases greatly above pH 5, and to a much lesser degree, as the pH drops below pH 5. However, there is no such increase in mutagenesis with either E. coli TAU-bar-N or E. coli TAU-bar-D, each of which is resistant to diazomethane-induced lethality.

Müller and Gichner (1964) were unable to detect any mutagenic effects from NG on Arabidopsis seeds treated in a boric acid buffer solution with a pH of 8.9. Therefore, they question whether diazomethane is involved in causing

the mutagenesis of NG.

The Effects of Temperature on the Mutagenicity of Nitrosoamides

The effects of temperature on the mutagenicity of these same compounds, using the same organism, is reported by Schwaier, et al. (1965). The treatment time and concentrations were the same, but all tests with the N-methylnitrosamides were carried on in the potassium buffer at pH 7. Action was stopped by dilution with ice water. Nitrous acid was tested in buffers at pH 4.5 and stopped with phosphate buffer at pH 7.5. All compounds tested were found to be strongly temperature dependent between 15°C and 25°C. The mutation frequency increased by a factor of about four for every 10°C.

Similar results with NG were obtained by Müller and Gichner (1964). They found that the mutant frequency was "drastically increased" (p. 1150) from 18-36°C in the treatment of Arabidopsis seeds.

Optimum conditions for the Production of Mutants with NG

In the production of mutants, what is considered optimum really depends upon the desired outcome. There have been several reports of optimum conditions for the production of bacterial mutants with NG. Adelberg, et al. (1965) found that the greatest numbers of mutants from strains of E. coli K-12 were obtained by removing the cells from a logarithmic phase, washing, and then treating them for 15-30 minutes with 100 ug/ml NG in buffer at pH 6. These cells were then cultured in broth to "permit segregation and phenotypic expression." (p. 792). There was a survival rate of 58% with 1.2 Val^R mutants/10^3 survivors. If this organism was treated during the early stationary phase, 85% survived, but there were only .27 Val^R mutants/10^3 survivors. Treatment during the late stationary phase resulted in 81% survival with only .12 mutants/10^3 survivors. There were

30% fewer mutants at pH 7 than at pH 6, and fewer cells were killed by treating them in the buffer than were killed by treating them in a broth. They concluded that growing cells were more susceptible to the lethal effects of NG.

As might be expected, lethality increased with the length of exposure to NG from 5-45 minutes. The peak production of Val^R mutants was obtained at 15 minutes, although beyond 20 minutes production never dropped below that found at 10 minutes exposure.

Cerda'-Olmedo (1967), working with E. coli TAU-bar, determined his optimum to be either pH 5 with 1mg NG/ml, or pH 5.5 with .1mg NG/ml, kept at 37°C. for 30 minutes in a buffer. He was primarily interested in high survival rates. The above conditions gave about equal mutagenesis with over 70% survival. There was about five times as much mutagenesis in cells which were treated at some stage of replication of DNA than in cells with the completed DNA circle.

Using E. coli, Suessmuth and Lingens (1968) found the mutation rate and uptake of NG-Me^{-3H} was similar between pH 4 and pH 8 with the maximum at pH 6.6. pH 6.6 gave a mutation rate of 10.9-11.3%. The addition of cysteine to the medium increased the mutation rate by increasing the methylation of DNA.

Optimums for phage mutation were found to vary with the host. Baker and Tessman (1968) preferred 5/ug/ml NG in broth cultures when using E. coli C600.1, and 10ug/ml with E. coli C as hosts for phage S13 at 37°C. S13 is a single-strand DNA phage. When phage T4 was grown in E. coli KB (λ), the optimum was 20ug NG/ml. These phages are known to differ in their responses to other mutagens as well.

The optimum for mutagenesis of Arabidopsis seeds was exposure to .5mM NG in distilled water at pH 4.2 for 18 hours at 24°C. (Müller and Gichner, 1964).

Basic Types of Mutations

In order to study the mutagenic effects of NG, the basic types of muta-

tions must be understood. These include:

Transition Mutations

In transition mutations, a pyrimidine is substituted for a pyrimidine, or a purine for a purine. There are several ways in which these mutations can be produced:

Production of transition mutations by base analogues:

Base analogues are chemicals which closely resemble the structure of some normal nucleic acid base. The enzyme which performs the task of adding that base mistakes the analogue and places it in the DNA chain instead of the normal base. For example 5-bromouracil and 5-bromo-deoxyuridine can be substituted for thymine and thymidine respectively. It has been suggested that the hydrogen atom at the #1 position is less firmly bonded than it is in thymine and may move up to the oxygen atom at the #6 position. *This tautomeric shift would allow this base to bond with guanine, thus causing an error in the replication of the DNA or in the production of m-RNA. If this shift in bases occurs in the significant position in the codon for an amino acid, the organism may exhibit some defect in the production of a protein. This type of shift often results in "leaky" mutants. (Watson, 1965; Conn and Stumpf, 1967). (Fig. 2 and 2a, page 15 & 16).

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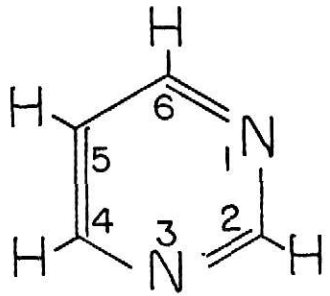
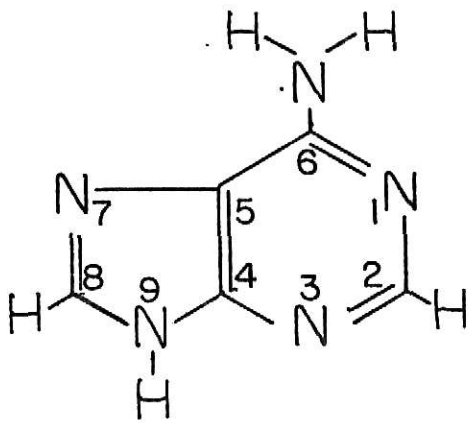
*There can be some confusion in studying these reactions due to the discrepancy in the numbering of the ring structures of the nucleic acid bases. Some of the older texts adopted the method of numbering these bases from the accepted numbering of the purine ring.

According to the biochemists, the proper method would be to start numbering at one of the hetero atoms of the ring and if there is a choice of direction, it must be numbered so that the other hetero atom has the smallest possible number. This rule is followed in numbering the pyrimidine ring, however, if a substituent is added, such as an NH at the top, as in the case of adenine, it, too, must have the lowest possible number. This would result in adenine being numbered as shown in Fig. 1 (Dr. Kenneth Burkhard, Kansas State Univ. personal communication.), page 14. Since the authors quoted have used the older method, of numbering, the same method will be used in this report.

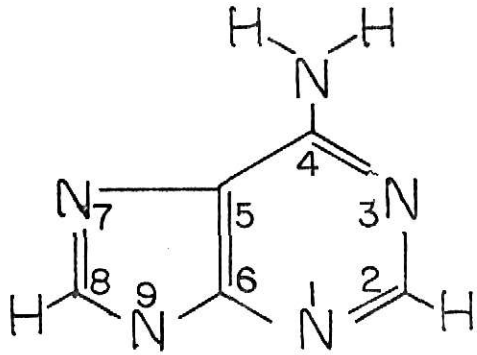


PYRIMIDINE

Correct numbering of the pyrimidine ring structure.



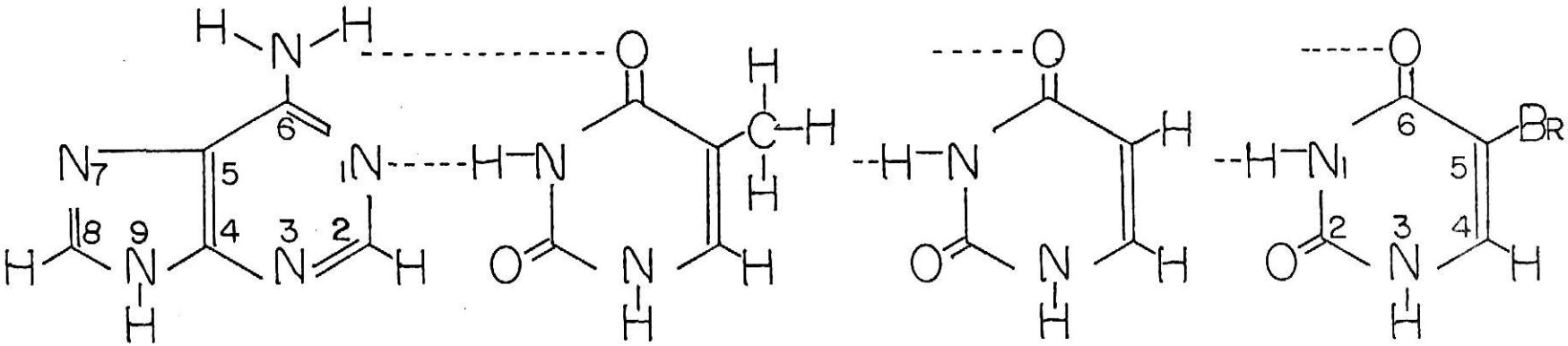
Older method of numbering following the method used in numbering pyrimidine. This is the method used in this report.



ADENINE

Preferred method of numbering according to biochemists' rules.

Fig. 1. The discrepancy in the numbering of the atoms in the ring structures of Pyrimidines and Purines.



Normal Hydrogen bonding between Adenine and Thymine in DNA and Between Adenine and Uracil in RNA.

The enzyme mistakes 5-Bromouracil, the base analogue of Thymine, for Thymine and incorporates it into DNA.

Fig. 2. Transition due to incorporation of a base analogue.

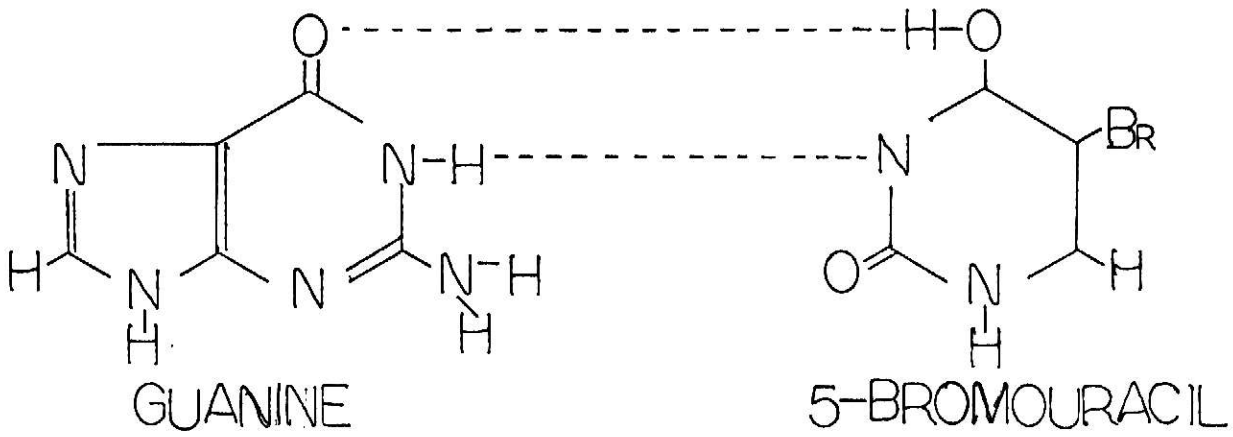


Fig. 2a. Base analogue causes an error in DNA replication resulting in a transition. The Hydrogen atom on the #1 Nitrogen is believed to be less firmly bonded than in Thymine. If there is a tautomeric shift of this Hydrogen of 5-Bromouracil from the #1 Nitrogen to the #6 Oxygen, 5-Bromouracil can Hydrogen bond with Guanine thus changing the base pairing from Adenine - Thymine to Guanine - Cytosine.

Transition mutations produced by chemical changes in the nucleic acid base:

1. Through oxidative deamination by nitrous acid:

Nitrous acid has been used for some time to induce mutants. At low pH it causes oxidative deamination of adenine to hypoxanthine by changing the NH_2 to a keto group (Zimmerman, et al., 1965). This prevents the normal hydrogen bonding of adenine to thymine but allows it to bond with cytosine, resulting in a transition from adenine-thymine pairing to cytosine-guanine pairing in the replication of DNA. (See Fig. 3, page 18).

Cytosine is deaminated to uracil, which then bonds with adenine. Here there is a transition from cytosine-guanine to adenine-thymine.

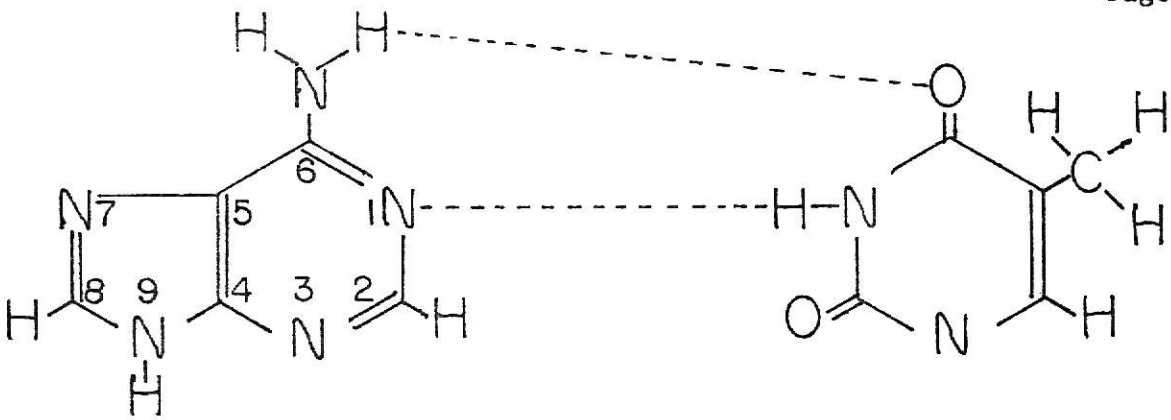
Similarly, guanine is deaminated to xanthine. It is still able to bond with cytosine, but only with two bonds instead of three. (Hayes, 1968). (Fig. 4, page 19).

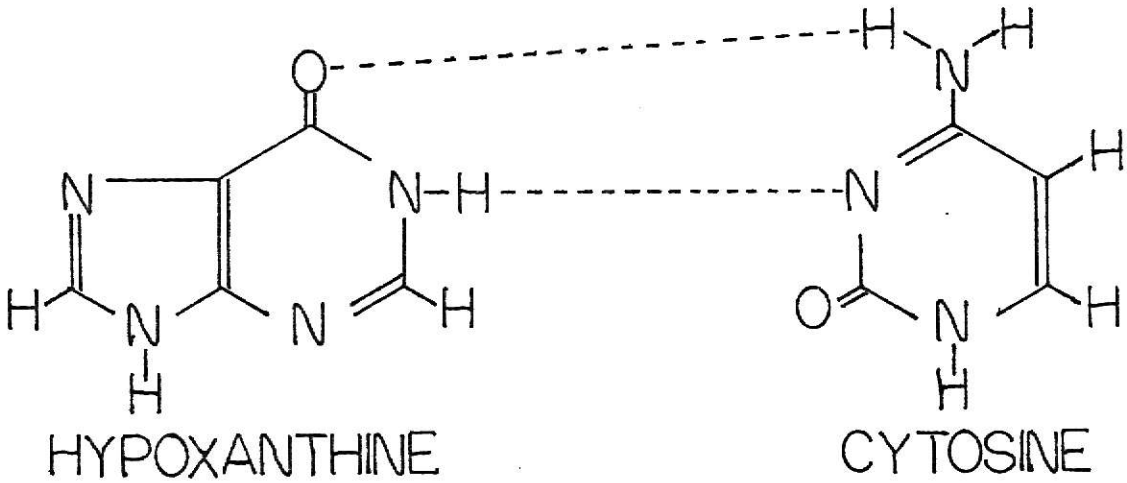
2. Alkylating the nucleic acid base:

The addition of a methyl group to the seven position in guanine is believed by Brooks and Lawley (1961) and Lawley and Brooks (1963) to increase the ionization of the proton on the N-1 position. This would allow guanine to form two hydrogen bonds with thymine. The result would be a transition in the base pairing from guanine-cytosine to adenine-thymine. (Fig. 5, page 20). Alkylation occurs to the greatest extent on this N-7 of guanine, but to a lesser extent (in order of decreasing activity) on N-3 of adenine, N-1 of adenine, and N-1 of cytosine (Lawley and Brooks, ibid.). (Brooks and Lawley, ibid.).

Translocation or Transversion Mutations.

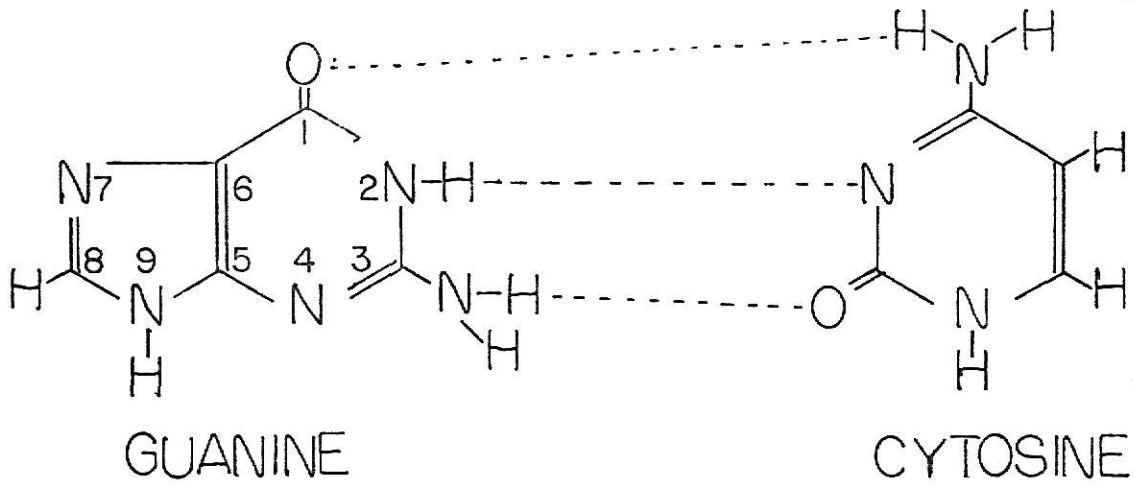
Translocation or transversion mutations involve the substitution of a



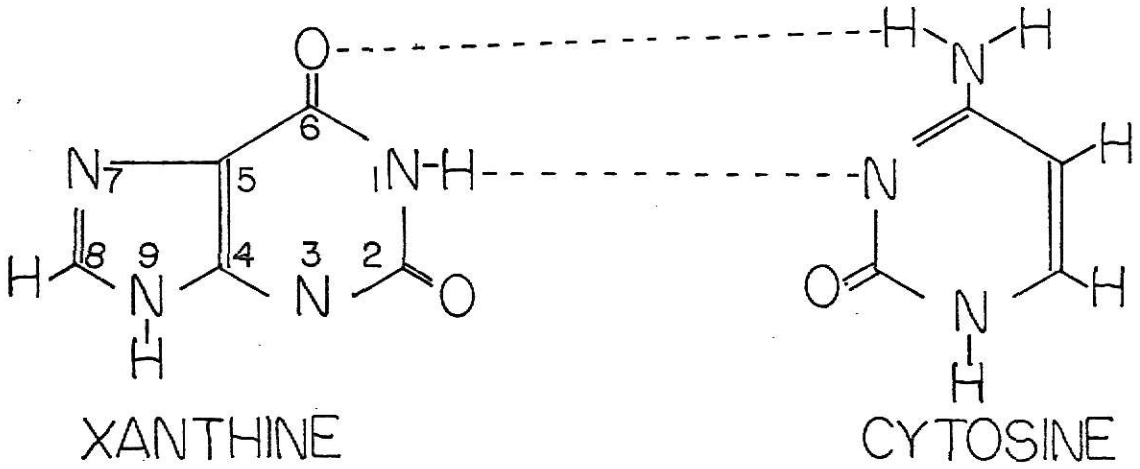


Base Transition due to Oxidative Deamination of Adenine to Hypoxanthine

Fig. 3. When Adenine is oxidatively deaminated by nitrous acid, the NH_2 group on the #6 carbon is replaced by oxygen. It can no longer bond to Thymine but bonds to Cytosine.

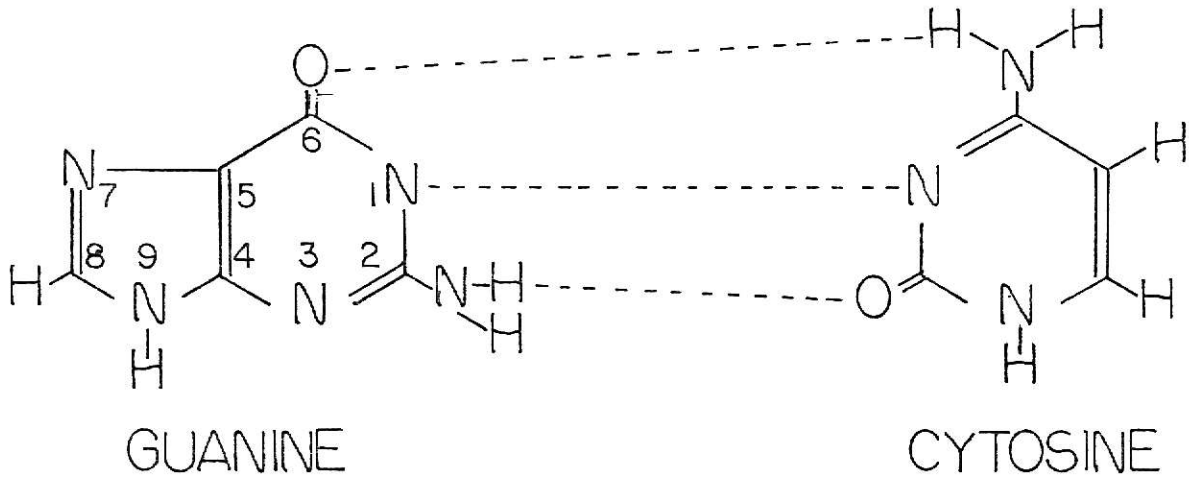


3 normal Hydrogen bonds between Guanine and Cytosine.

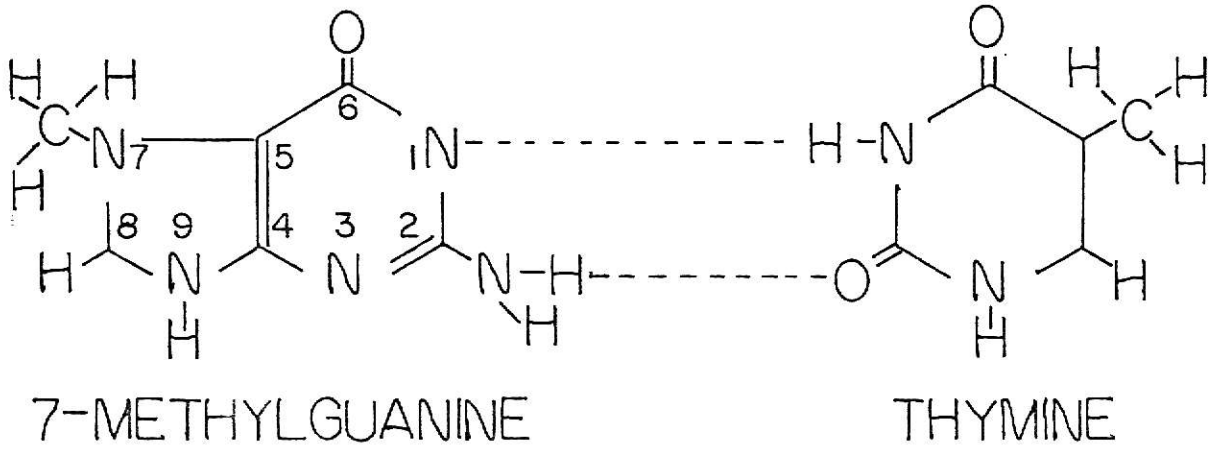


Transition due to Oxidative Deamination of Guanine to Xanthine

Fig. 4. Oxidative deamination of Guanine to Xanthine replaces the NH_2 group on the #2 carbon with an oxygen atom making it impossible to bond with the oxygen atom of Cytosine. However, the other two bonds can still be formed.



Normal Hydrogen Bonding.



Transition of Bases Resulting From Methylation of Guanine at N-7 Position

Fig. 5. Methylation of Guanine on the #7 position is believed to increase the ionization of the proton on the N-1 position. This would allow 7-methylguanine to form two hydrogen bonds with Thymine.

purine for a pyrimidine or vice versa. This type of mistake may occur when there is a deletion in the DNA of the parent cell. When the DNA replicates, the gap may be filled by the wrong class of base. This might occur if a methylating agent caused methylation of the phosphate group. The DNA chain could then be split at that position by hydrolysis. Brooks and Lawley (1961); Lawley and Brooks (1963) found that although alkylated RNA was stable in a neutral aqueous solution, alkylated DNA broke down slowly with the loss of alkylated guanine products (primarily the 7-alkyl guanines). The 3 alkyladenines were released more rapidly, but in much less quantity.

Mutation by permanent deletions of bases.

Mutations by permanent deletions of bases may occur from the methylation described above if the gap is not repaired during the DNA replication.

Mutation through addition of an extra base into the DNA chain.

Just as a transversion mutation might occur after a split in the DNA chain, additional bases could be incorporated before the chain is repaired.

The Effects of DNA Errors Upon an Organism

The Effects of Transition and Transversion Mutations.

The DNA genetic code for the production of proteins has been shown to be in triplets (Watson, 1965). Therefore, when DNA in which there has been a transition or transversion of the bases produces messenger RNA, the wrong amino acid will be placed into the protein, or none at all. Either change will make the protein defective. For instance, the mRNA code UGU (uracil-guanine-uracil) codes for cysteine. If guanine is replaced with adenine, it codes for tyrosine. CCU codes for proline, but ACU codes for threonine.

A classical example of this type of mistake in human genetics is sickle cell anemia, in which the only difference from normal hemoglobin is the substitution of a valine for a glutamic acid residue in the sixth amino acid position from the N terminus of the β peptide chain. Since normal hemoglobin is composed of two pairs of α and β chains, this means that only two amino acids are different from normal out of a total of 574 (Liener, 1965). If a person is heterozygous for sickle cell anemia, about half of his hemoglobin is normal, but the homozygous individual carries only the defective hemoglobin, which crystallizes whenever chilled, exposed to high altitudes, or anything that lowers the oxygen tension in the cells. This crystallization causes sickling of the cells, and they are destroyed by the spleen. The result is severe anemia. The condition is usually fatal in childhood (Hayes, 1968).

This is a case of "point mutation" of the transversion type, resulting in miscoding for one amino acid. The code triplets for glutamic acid are either GAA or GAG, whereas those for valine are GUA or GUG. If a similar mistake is made in the production of an enzyme, the enzyme might be rendered nonfunctional, thus stopping the reactions in the specific metabolic pathway in which it normally functions. The chemical on which it normally operates would then increase in concentration. This can cause irreparable damage and death. Also, the organism may be unable to produce an essential metabolic product. Survival is possible only if this product can be supplied in some other way.

The Effects of Permanent Deletions from or Additions to the DNA Chain.

Each of these errors would throw off the entire reading of the genetic code from that point to the end of the chain, unless a deletion occurs after an addition, or vice-versa. These errors are called "shift-frame" mutations (Hayes, 1968). If one occurs early in the DNA chain, or is not compensated-

for soon, the result is lethal because the organism cannot produce any of the proteins coded in the balance of the DNA chain.

Types of Mutations Caused by NG

Mutations in bacteria caused by NG

Much can be determined about the type of mutation induced by a mutagen by the study of the reversions it causes. Eisenstark, et al. (1965) found that NG brought about nearly the same reversions as diethyl sulfate in S. typhimurium, regardless of whether the mutants tested had been induced by ultraviolet light, 2-aminopurine, nitrous acid, X-rays, slow neutrons, or diethylsulfate. This indicates that NG and diethylsulfate have about the same specificity of mutagensis. It has also been shown that 2-aminopurine is able to induce both types of base transitions, but no transversions (Eisenstark, et al., 1965). When these authors compared the reversion activity of NG with 2-aminopurine, they found that not only could NG revert all 45 forward mutations caused by the 2-aminopurine, but that it could also cause reversions of mutations that could not be induced by the 2-aminopurine. These comparisons indicate that NG is capable of both types of base transitions and transversions.

Likewise, Cerda'-Olmedo (1967) found that NG can produce all transitions and transversions in E. coli, but does not induce frame-shift mutations. Most mutations were shown to be located at the point of DNA replications at the time of the treatment with NG. Voronia, et al. (1968) also found this to be true when treating synchronous cultures of E. coli with formaldehyde and hydroxylamine at different stages of replication. They reported that about one-half of the mutation correlated with the timing of the replication of the genetic make-up of the DNA as currently mapped.

Mutations in viruses caused by NG

Baker and Tessman (1968) found that NG reverted the transitions GC---→AT and AT---→GC about equally in phage S13. With phage T4, however, the transition from GC---→AT is in far greater proportion. The study compared NG with hydroxlamine, the specificity of which has been determined. These authors were unable to bring about reversions of the transversion type, unlike those in the case using Salmonella. They concluded that the mutagenic variation of NG in different organisms may be due to a difference in the "molecular environment at the replication point of the DNA, which should depend on the specific mechanism of replication, the nature of the DNA polymerase and the base composition of the DNA." (p. 446). There is also the possibility that enzymes in the cells of different organisms, or even different organs in the same organism, may cause NG to be metabolized in different ways. These differences affect the mutagenicity of NG and/or its break-down products. Baker and Tessman (1968) suggest the possibility that NG may be enzymatically converted to some active form, such as diazomethane, since they were able to induce mutants with NG in the phages S13 and T4 in vivo, but not in vitro. Similar results were experienced with TMV by Singer and Fraenkel-Conrat (1967) and Singer, et al. (1968). These authors believe it might be due to the conformation of the RNA in different environmental conditions.

The Nature of the Mutagenic Action of NG

The exact nature of the mutagenic action of NG is still a debatable question. However, there are some rather strong indications which are helpful in explaining its action:

From Nitrous acid, a break-down product in low pH.

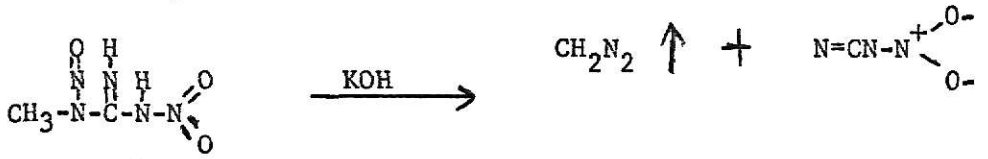
As mentioned, NG in acid solution decomposes to yield nitrous acid.

The mutagenic action of nitrous acid has already been described. Of course, for nitrous acid to be formed from NG, the pH must be low enough. If one is working in or near physiological pH 7, this acid could not be responsible for the mutations.

From Diazomethane; a break-down product in pH above 5.5

By alkylation of the bases in DNA

Above 5.5, NG slowly breaks down to form diazomethane, according to the following reaction:



(NG) diazomethane nitrocyanamid

(McKay, 1948 and 1952)

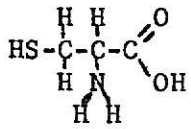
Diazomethane is a very strong methylating agent (Hultin, et al., 1960). Most researchers who have used it reason it is this methylating action on the DNA that causes the mutation. Auerbach (1967) says, "The correlation between alkylating and mutagenic abilities is so strong that a tendency has arisen to attribute alkylating reactions to mutagens whose mode of action is not yet understood, such as a number of nitroso compounds and the pyrrolizidine alkaloids" (p. 1143).

To show which decomposition product caused this methylation, McCalla (1968) used N-/^{14}C/-N'-nitro-N-nitrosoguanidine, or /Me-^{14}C/NG, similarly radioactively labeled N-/^{14}C/-methyl-N-nitroso-p-toluensulfonamide, or /Me-^{14}C/ MNTS and also N-methyl-N'-nitro-N-nitroso /^{14}C/ guanidine, or /Guanidine - ^{14}C/NG, with "highly polymerized" DNA in vitro (p. 115). He found the principal product to be 7-methylguanine. The radioactive label was in this methyl group. This indicates that the methylation was from the part of the

molecule of NG that forms the diazomethane upon decomposition. He also found that an increase in the OH^- concentration resulted in a greater amount of methylation of the guanine moiety of the DNA with this radioactive label. This would be suspected, if methylation is caused by the release of the diazomethane, since NG is most stable at pH 5.5 and the decomposition rate increases as the pH is increased.

This hypotheses was further supported by McCalla's finding that the addition of the label to the guanidine part of the NG molecule resulted in much lower amounts of the label being incorporated into the DNA. These results also correlate with the findings that a number of other N-methyl-N-nitroso compounds have produced 7-methylguanine and smaller quantities of other methylated bases in vivo (McCalla, 1967).

The addition of acid phosphate ions inhibited the methylation by both NG and MNTS. However, the presence of cysteine, which contains a thiol group, brought about a marked increase in the methylation with the radioactive label from /Me-^{14}C/NG.



Cysteine

The thiol groups is a very strong reducing agent (Dr. Kenneth Burkhard, personal communication). It reacts rapidly with NG to produce an evolution of N_2. This is believed to be from the diazomethane, which at the same time frees the radioactive methyl group to react with the DNA.

Unlike NG, MNTS is rapidly destroyed by cysteine with no evolution of gas. This leads McCalla to hypothesize that biologically inactive compounds rather than diazomethane are formed in this reaction.

Suessmuth and Lingens (1968) also found that the addition of cysteine to the system, in which they were treating E. coli with NG at pH 6, brought about a marked increase in methylation and an increased mutation rate.

Further evidence that NG and MNTS act in a similar manner on the DNA was quoted by McCalla (1967) from the work of Kilgore, et al. (1960). These authors showed that an NG resistant strain of E. coli was also cross-resistant to MNTS.

McCalla (ibid.) also referred to his work the previous year, in which both NG and MNTS caused the same "mass mutation of the Euglena chloroplast system" (p. 114), and an inhibition in the synthesis of macromolecules in this organism with MNTS. McCalla's findings are in contrast to reports by other authors that MNTS is not mutagenic (McCalla 1967). He suggests, however, that these contradictory results, involving work with different organisms and organelles, may be due to the fact that these N-methyl-N-nitroso compounds are strongly influenced by such environmental factors as the OH^- concentration and the presence of phosphates or thiols acting on these compounds to either release diazomethane or merely harmless substances.

In addition to the 7-methylguanine and 7-methylguanosine that resulted from treatment of salmon sperm DNA with NG (Lawley, 1968) and diazomethane (Kriek and Emmelot, 1964), smaller amounts of 3-methyladenine were reported in both instances. Ratios varied from about 9:1 of the 7-methylguanine:3-methyladenine (Lawley, 1968) to 3:1 (Kriek and Emmelot, 1964) depending upon the pH and other conditions of the experiment. The amount of 3-methylation of thymine or uracil residues, in dinucleosides also seems to be dependent upon the structure of the dinucleoside phosphate (Holy, et al., 1966). Lawley (1968) quoted Haines, Reese and Todd's hypotheses for their similar methylation results of

these guanine and adenine residues. Presumably, the existance of a rare tautomer containing an acidic $\equiv NH^{+}$ group at the N-7 position of guanine would allow methylation at this position. However, the N-7 of guanine and N-3 of adenine were shown by Pullman and Pullman to be the "chief nucleophilic centers in native DNA" (Lawley, 1968) (p. 581). Lawley believes, therefore, that these would naturally react with the methyl carbonium ion which could form from the decomposition of diazomethane, as suggested by Kriek and Emmelot (1964).

Unlike the above results, Friedman, et al. (1965) obtained deoxy-1-methylguanosine and deoxy-(0-6)-methylguanosine by treating polynucleotide chains, averaging eight nucleotide units with a large excess of diazomethane in an aqueous-methanol system at pH 6. They explained this as a possible methylation of a lactim tautomer of the deoxyguanosine. (See Fig. 6 and 6a, pages 29 & 30).

While this work was in progress, they report, the above-mentioned project of Haines, Reese and Todd was published. Friedman et al. (1965) explain that the difference in the products may be due to the difference in the experimental conditions. They were using an aqueous methanol or aqueous system while Haines, et al. were shaking an aqueous solution of DNA with an excess of diazomethane dissolved in ether, thus having a 2-phase system.

The reaction reported by Friedman et al. (ibid.) also produced 1-methylthymine and 1-methylthymidine. Meanwhile, he reports, Miles had also shown that diazomethane had formed 3-methylthymidine upon reacting with deoxythymidine.

In comparing these results of the methylation of nucleic acid bases with other alkylating mutagens such as diethyl sulfate, methyl sulfate and nitrogen mustards, Friedman et al. (ibid.) found that the latter two gave only alkylation of guanine on the 7-position, and none on the 1 and 0-6, in contrast to the

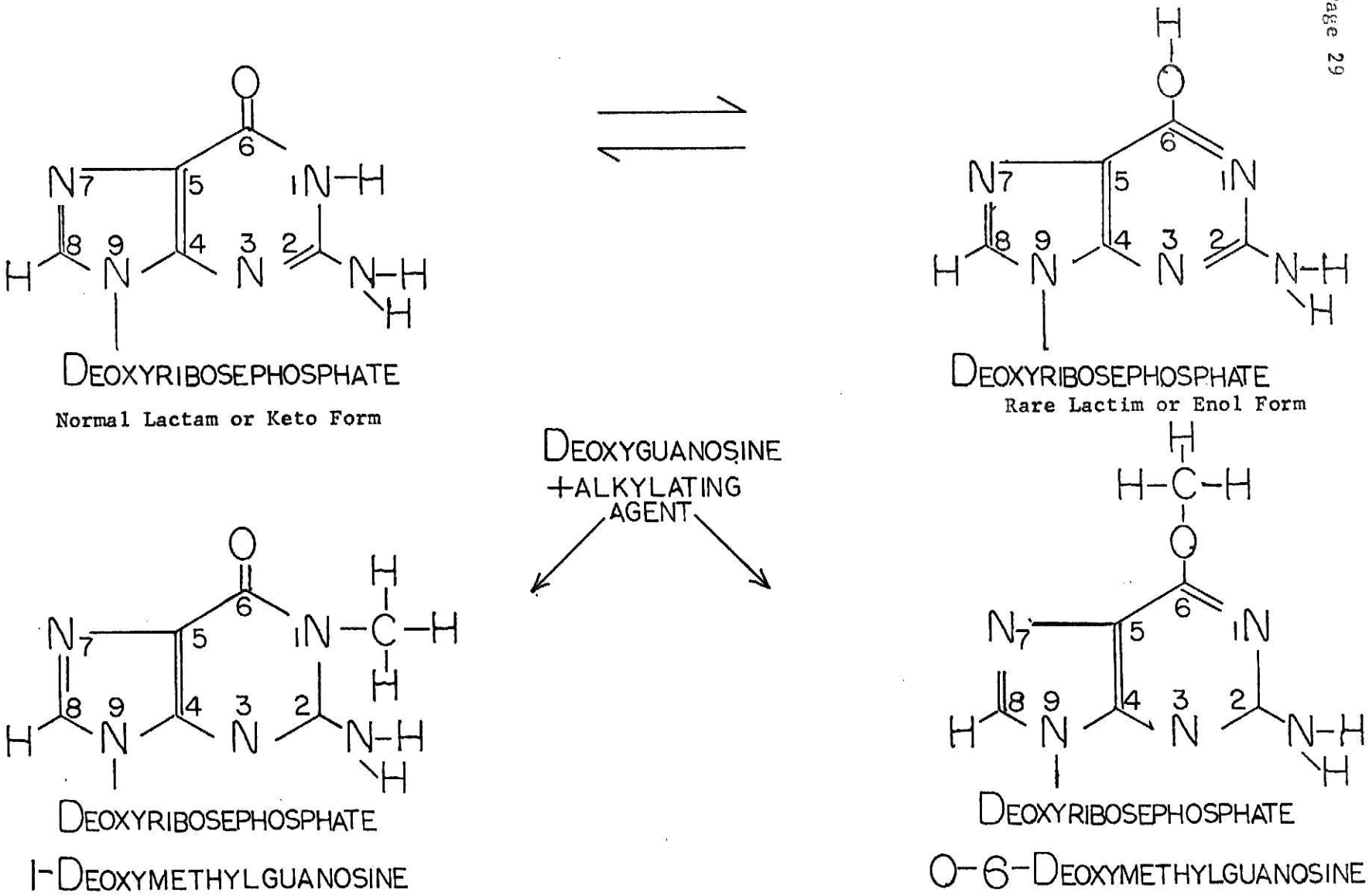
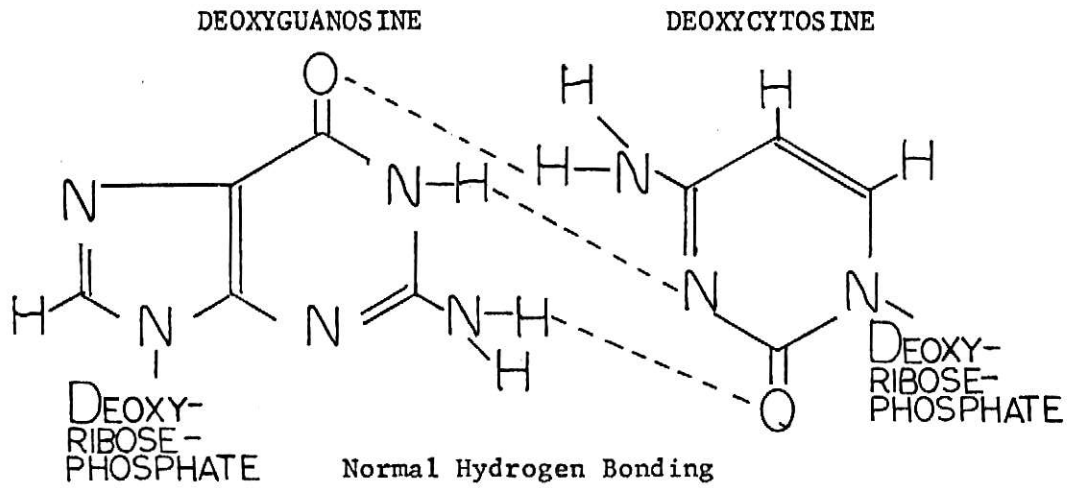
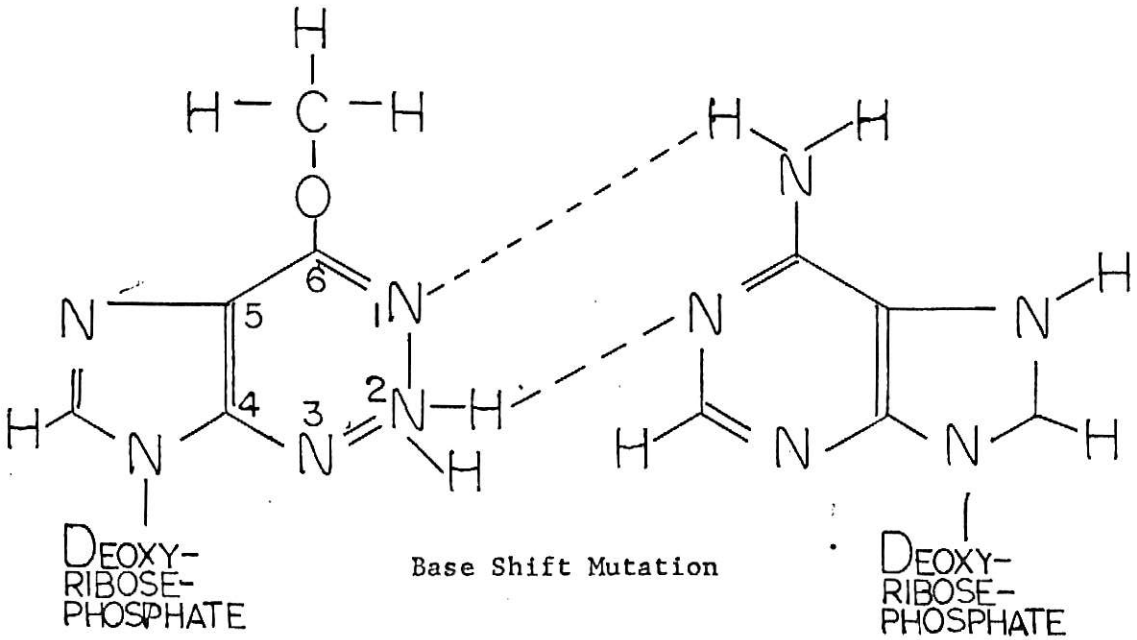


Fig. 6. 1-Deoxymethlguanosine and O-6-Deoxymethylguanosine result from methylation of both tautomers of Deoxyguanosine.



Normal Hydrogen Bonding



Base Shift Mutation

0-6-Deoxymethylguanosine Deoxymethyladenosine

TRANSVERSION:

Fig. 6a. Base shift mutation from Guanine - Cytosine bonding to Adenine - Thymine bonding resulting from methylation of Guanine at the 0-6 position when the DNA replicates.

diazomethane. Guanine was the most reactive in all cases. They did not find adenine and cytosine to be attacked by diazomethane, unlike their reactions with diethyl sulphate; and thymine was alkylated by diazomethane but not by dimethyl sulfate.

By Alkylation of the Phosphate moiety of DNA.

We find conflicting reports in this area. Because DNA has no OH group on the #2' C of the deoxyribose, it is relatively stable to alkaline hydrolysis, unlike RNA, (Conn and Stumpf, 1967). Friedman (1957) hypothesized that if the phosphate groups in dinucleosides could be methylated when the DNA nucleotides were in acid form, the resulting esters could then be hydrolyzed in alkali. His results showed that the primary and secondary phosphoryl groups were, apparently, quantitatively methylated, and he was able to carry out this hydrolysis.

Likewise, Brown, et al. (1955) had obtained uridine 3' dimethyl-phosphate from the treatment of uridine 3' phosphate with diazomethane in methanol until neutral.

Since the prior work of Brimacombe, et al. (1965), with methylation studies of 5' mononucleotides strongly indicated no esterification by diazomethane or dimethyl sulfate in neutral, aqueous solution, dinucleoside phosphates were treated with diazomethane in acetate-buffered, aqueous solution. They reasoned that these smaller units would react like the nucleic acids without the possible complications when using the macromolecules. The thymine residues of thymidylyl (3' 5')-thymidine were completely methylated by diazomethane, (Brimacombe, et al., 1965).

Effects of NG and Related Compounds on Proteins and Protein Synthesis:

The Effects of NG on Proteins

The proteins of the cell are attacked by NG and related compounds.

Heath and Dutton (1958) examined liver fractions for evidence of ^{14}C, ^{15}NO, and ^{15}NC labeling from dimethylnitrosamine (or N-nitrosodimethylamine) in rats and mice. They found that all the cell fractions were lightly labeled, and that the urine contained free bases and urea with heavily labeled nitrogen. The protein in the urine and plasma was lightly labeled. A great portion of the ^{14}C was respired as CO_2. They felt this indicated that the dimethylamino groups were oxidized to 1-carbon-atom intermediates and ammonia, and that the nitroso group was partly reduced to ammonia.

In 1962, Heath found that this decomposition of the dialkylnitrosamines in rats is primarily confined to the liver, which was also the only organ to be severely affected by these compounds. When he investigated the toxicities, metabolic products and other effects of four nitrosamines, he found that these compounds were not toxic in themselves, but that the toxicity was due to oxidation of these compounds to form carbonium ions or diazoalkanes. Of the compounds tested, those that would not be expected to metabolize to yield diazomethane did not cause serious hepatoxic damage.

Magee and Farber (1962) found that ^{14}C radioactively labeled dimethylnitrosamine caused a much greater amount of labeling in the proteins of the pancreas than in the liver, but could find no correlation between this labeling and the pathological lesions. At the dose level used, the liver was very acutely damaged, whereas there was little or no severe damage in the kidney, pancreas or spleen. Again, diazomethane was suggested to be the methylating agent.

The attack made by NG on the cell proteins was shown by the incorporation of the ^{14}C from /guanidine-^{14}C/NG while ^{14}C of the /Me-^{14}C/NG is incorpora-

ted into the DNA (Sugimura, et al., 1968). The more basic the proteins, the more label is bound, with histones binding the largest amounts. It might be noted that it is the histones that are closely hydrogen-bonded to the DNA of the normal cell. (Conn and Stumpf, 1967). How might this effect the chromosome?

Fifty three percent of the lysine in serum albumin, treated with a saturated solution of NG in phosphate buffer at pH 7, combined with the labeled nitroguanidino group of the NG according to McCalla and Reuvers (1968). They also found that for every five "guanido" groups incorporated into the proteins, about two methyl groups were bound. They pointed out that at pH 7 only a small proportion of the ε amino groups of lysine would be unprotonated. However, these could react with the imino carbon of NG, which carries a slight positive charge. As the reaction continues, more ε amino groups would become exposed resulting in a sort of "chain-reaction." An increase in the pH would increase the number of unprotonated amino groups and therefore increase the reaction rate. (See page 7).

In rats nitrosamines were believed to be converted into an active methylating agent which resulted in methylated histidine residues (Swan and Magee, 1968).

Terawaki and Greenberg (1965) reported that 10 ug/ml NG caused inactivation of transforming DNA. McCalla and Reuvers (1968) found that the same concentration of NG would "not cause the formation of nitroguanido groups in any significant proportion of protein molecules" (P 1414). Therefore, they believe that the protein damage caused by NG is not its primary effect. They suggest that the reaction described above increases the rate of formation of diazomethane which then reacts primarily with the DNA.

β-galactosidase in E. coli is inactivated by NG (Cerda'-Olmedo and Hanawalt, 1967). If groups from NG were added onto either an active or an allosteric site of an enzyme, or if it changed the charge on the active site, it would impair the enzymatic activity. McCalla and Reuvers (1968) suggest that the above reaction of NG with protein may be the explanation for this inactivation.

The chemical alteration of DNA-polymerase by NG might result in abnormal replication of DNA, a possibility which needs further exploration (Sugimura, et al., 1968).

Modification of the protein coat of TMV seems to have some effect on the infectivity of the virus, since intact, treated TMV slowly lost infectivity. When NG-treated TMV-RNA was reconstituted with untreated protein coat, it was about twice as infective as the treated whole virus. Yet there had been about an equally high level of mutation produced in both of them. (Singer and Fraenkel-Conrat, 1967).

Effects of NG on Protein Synthesis

Not only are proteins affected directly by NG, but Cerda'-Olmedo and Hanawalt (1967) found that protein synthesis and RNA synthesis are reduced and that the rate of protein synthesis is slowed down in E. coli.

Hultin, et al., (1960) felt that the various damages observed in rat liver when using dimethylnitrosamine were due to the release of an alkylating agent, probably diazomethane, near the sites of active amino acid incorporation into the proteins. This implies more direct interference with protein synthesis than is implied in an alteration of the DNA.

Similarly, when S. Vila-Treviño (1967) injected 30 mg/KG of body weight of dimethylnitrosamine intraperitoneally into rats, he found that the protein synthesis in the liver was progressively decreased. Effects were noted

as early as 15 minutes after injection with the maximum effect occurring between the 5th and 7th hour. This could be the result of accelerated destruction or defective function of mRNA due to methylation. This author quoted the work of Wilhelm and Ludlum in Science 153; 1403, 1966, which demonstrated that methylated co-polymers of UMP and GMP had greatly decreased template activity for synthesis of polypeptides in vivo.

McCalla and Reuvers (1968) also suggest that NG may inactivate the ribosomes by reacting with their proteins, which could then interfere with protein synthesis.

Effects of NG on RNA

Lawley and Brooks (1963) had compared the rates and extents of alkylation of both DNA and RNA in a neutral aqueous solution at 37°C. They found that for RNA, the sites of alkylation, in order of their decreasing activity are:- N-7 of guanine, N-1 of adenine, N-1 of cytosine and N-3 of adenine. They also reported that rearrangements occurred slowly, with the 1-alkyladenine moieties yielding 6-methylaminopurine moieties and 1-alkylcytosines yielding 1-alkyluracil.

Magee and Farber (1962) have been shown that there was a distinct correlation between ^{14}C labeling of RNA by dimethylnitrosamine in rats liver, kidney and pancreas and the pathological conditions found.

Brimacombe, et al., (1965) showed that uracil residues of polyuridylic acids were extensively methylated by diazomethane. There was some evidence of polymeric breakdown. However, they were able to methylate considerable amounts of polycytidylic acid and polyadenylic acid without any appreciable degredation. Both the adenine and uracil of adenylyl-3'5'-uridine were methylated without any esterification of the internucleotidic linkage. Kriek and Emmelot (1963)

working with both microsomal and soluble RNA from rat liver, found that 1,7-dimethylguanine was the principal product of the methylation caused by diazomethane. They also found that there was methylation of the secondary phosphate triesters, some of which hydrolyze readily in water. Because of the 2'-OH group on the ribose moiety, RNA that is methylated by diazomethane breaks down in aqueous solution, unlike DNA, which lacks this group.

Work done by Lark, et al., (1963) seems to indicate that protein and RNA synthesis are necessary for the initiation and completion of a new cycle of DNA synthesis if the new DNA is to replicate normally. These authors and also Doudney (1969) quote work by Hanawalt, et al., J. Mol. Biol 3, 1961, 156 and Maaloe et al., J. Bol. Biol. 3, 1961, 144 to support this hypothesis.

Repair of DNA Damage Caused by NG:

After uv treatment, some bacteria can repair part of the damage done if they are grown in the dark. Cerda'-Olmedo and Hanawalt (1967c) found that this was also true of NG damage. A comparison was made, using two strains of E. coli TAU-bar and B_s-1 leu^-thy^-. The latter organism is sensitive to uv and cannot repair damage caused by it. This strain was also found to be much more sensitive to NG than TAU-bar. They concluded that the mechanism for repairing uv damage to DNA is the same as, or similar to, that used to repair NG, and may be a very general type of repair mechanism which is independent of the actual mutational effects of NG which cannot be recognized and repaired by the cell.

Significance of the Methylating Effect of NG on Nucleic Acids Questioned:

Singer, et al. (1968) could find no evidence that the mutagenic effect of NG on TMV was due to the methylation of either the RNA or the protein coat,

although the changes brought about in the protein coat did seem to reduce its infectivity. Unlike previously reported results of increased rates of mutagenicity of bacterial DNA with increased pH, Singer, et al. (1967) observed decreased mutagenicity when the pH was increased from 5.5 to 6.5 and the mutagenicity was quite low at pH 7.5. In a saturated solution of NG at pH 5.5, TMV showed a high level of mutagenesis comparable to that produced by nitrous acid. This was about ten times more mutagenesis than resulted from the direct treatment of TMV-RNA with NG. The authors found the greatest numbers of mutants when TMV-RNA was treated with NG in formamide. However, in these cases there were no base changes detected. These findings suggest the possibility that the effect of NG on TMV "may be due to the structure of the TMV particles" Singer, et al. (1968) (P 1236), and that deamination by NG needs to be studied.

Methylation of both RNA and DNA occurs normally in the cells of various organisms. A summary of the work that has been done on this subject was published by Borek and Srinivasan, 1966). Ten different enzymes have been discovered that methylate nucleic acids. The methyl groups all come from methionine. These authors refer to work by Hurwitz, et al. (1964), in which they found six enzymes, each having specific sites for methylation of tRNA for uracil, cytosine and adenine, one enzyme for the 7-position of guanine and two for the 1-position on guanine. Others showed that amino-acid-activating enzymes from E. coli will operate equally well whether the rRNA is methylated or not. In the case of yeast, however, these enzymes do not attach the amino acids to non-methylated rRNA (Borek and Srinivasan, ibid.).

It has also been shown that the position of the methyl groups has an apparant species specificity, since fully methylated tRNA from E. coli K12 would not be methylated further by homologous enzymes, but was extensively

methylated by enzymes from other sources (Borek and Srinivasan, ibid.).

Methylation appears to confer greater stability on RNA, as shown by its being more highly structured, with a higher melting point, and its greater resistance to alkali and nucleases. In fact, there is a virus-like particle, called "rhapidosomes," isolated from lysates of Saprospira grandis, a marine alga, resembling blue-green algae, which is highly resistant to hydrolysis by alkali or pancreatic ribonuclease. It has been shown to have 80% of the ribose sugar of the RNA substituted at the 2' position with OCH_3 groups, showing that not only are the bases methylated, but the sugar moiety as well. (Borek and Srinivasan, ibid.).

Similarly, methylation is found to be a normal condition of DNA. These same authors suggest that the individuality of the methylated DNA sturcture of an organism might somehow protect the cell from having a foreign organism's DNA becoming incorporated into the host's DNA during infection. It might also protect the DNA from deoxyribonucleases. Another suggested reason for the methylation concerns the replication or non-replication of the strands of the DNA. Much study will be needed to determine the answers to these questions. It is because of this normal methylation of the nucleic acids that some authors feel the mutational effect of NG is not dependent on its methylating ability.

EXPERIMENTAL

Materials used in Producing New Mutants

Parent Organism used:

The mutant strain Salmonella typhimurium #3599 obtained from the stock of Dr. Demerec, and needing histidine, methionine and tryptophan was used. It was also resistant to Streptomycin and uses xylose. These two latter mutations were not considered in this study. The method used to prepare the above mutant is not known, but is assumed to have been developed by the use of some radiation.

2X-Minimal Salts:

Ingredients for 10X Minimal Salts

1. 63 gm K_2HPO_4
2. 27 gm KH_2PO_4
3. 6 gm $(NH_4)_2SO_4$
4. 3 gm. Na-citrate
5. 600 ml. Dist. H_2O
6. 0.3 gm $MgSO_4.7H_2O$./10ml Dist. H_2O

Method: #1-5 were heated in a steamer 10 min. When clear, #6 was added. This was then sterilized in 400 ml. lots. When needed, 1 part 10X salts was added to 4 parts sterile, distilled water. Eight milliliters of 2 mg/ml Dist. H_2O concentration of each of the "needed components" were then added. (Histidine, methionine, tryptophan and each additional amino acid, pyrimidine or purine required by the new mutant for growth.) Also 8 ml 40% sterile glucose were added.

Alternate method: 2X Minimal Salts plus "parent mutant's" "needed components":

10X salts immediately diluted to 2X salts, bottled in 400 ml lots and

8ml histidine, 8ml. methionine, 8ml tryptophan and 8ml of the additional needed component or components were all mixed and sterilized. As new components were needed, a proportional amount of distilled water was omitted to prevent excessive dilution of the salts and a softening of the minimal agar prepared from it.

Minimal Agar:

2X agar was first prepared by dissolving 18 gm. Ionager per liter of distilled water and sterilizing in 400 ml. lots. To prepare the Minimal Agar, 1 bottle of 2X minimal salts plus the "needed components," mentioned above, and 8ml. 40% sterile glucose were added to 1 bottle of melted 2X agar and poured immediately. The glucose should not be added to the minimal medium before sterilization. To do so results in a browning reaction.

Single-Enriched Agar (For picking mutants)

400 ml melted 2X agar

400 ml 2X salt solution plus "needed components"

8 ml. sterile 40% glucose

10 ml. nutrient broth

Nutrient Broth:

8 gm nutrient broth powder

5 gm NaCl

1 liter distilled water

Nutrient Agar:

13.5 gms Ionagar

8 gms. nutrient broth powder

5 gms Na Cl

1 liter distilled water

Phosphate Buffer Solution (PBS) Stock Solution

Prepare separately:

A. $Na_2HPO_4.7H_2O$--- 268 gm in 2 liters H_2O

B. $NaH_2PO_4.H_2O$--- 138 gm in 2 liters H_2O

Stock Solution:

386ml A

114ml B

500 ml. distilled water. Sterilize and refrigerate.

PBS: (2 liters);

80ml stock solution

17 gm. NaCl

1,920 ml H_2O

Sterilize

Amino Acid, Purine-Pyrimidine, and Vitamin Pools

(2mg/ml H_2O of each component.)

Pool 1	Pool 2	Pool 3
l Cysteine	Dl-Leucine	Dl-Tryptophan
Dl-Methionine	Dl-Isoleucine	Dl-Phenylalanine
L-Arginine	Dl-Valine	l-Tyrosine
Dl-Lysine		

Pool 4	Pool 5	Purine-Pyrimidine Pool
l-Histidine HCl	Serine	Guanine
Dl-Threonine	Glycine	Thymine
l-Proline	Dl-Alanine	Uracil
Dl-Glutamic acid	Dl-Aspartic acid	Adenine

Vitamin Pool

1. Ascorbic acid
2. Vit. B6 HCl
3. Calcium Pantothanate (dextro-rotatory)
4. I.V.C. (Sodium PABA)
5. Nicotinic acid
6. Riboflavin
7. Thiamine HCl

Method For New Mutant Production

Fifty percent serial dilutions of NG in 2ml of nutrient broth were prepared in a series of 8 tubes plus a control tube with no NG. These were numbered from 1-10 including the saturated NG tube. Fresh, sterile applicator sticks were used for innoculating each tube, from a broth culture, of the test organism. Salmonella Typhimurium #3599 was the original parent organism in this study. The series was then incubated 24 hours. All cultures were incubated at 37°C.

A culture was selected that had a fair amount of growth but less growth than the control. Usually this was found to be the 1:16 or 1:32 dilution. One tenth of a milliliter of this culture was then serially diluted in 3 tubes of 10 ml. phosphate buffer solution to a final concentration of 1:1 million. One tenth ml. of this diluted suspension of cells, was then spread onto each of several sterile plates of single enriched agar containing the parent organism's needed components. To do this an "L" shaped sterile glass rod was used, while the plate was spun on a rotating platform. These plates were then incubated 48-72 hours. Any new mutants would have been unable to grow on the minimal

agar, but the presence of approximately 1% broth allowed them to form minute colonies. The unaffected bacteria would form larger colonies and revertants would be larger and also cream colored.

The minute, mutant colonies were picked with sterile tooth picks, and transferred onto a plate of nutrient agar placed over a numbered grid. The untreated mutant was placed on the #14 space for a control.

After over night incubation, this plate was velveteen-replicated onto minimal agar which included the parent mutant's needed components, but no broth; and also onto a nutrient agar plate. Mutants that grew on the nutrient agar but not on the minimal agar were picked with sterile applicator sticks and placed into tubes of 2ml nutrient broth and numbered. They were then incubated over night.

Method for Testing the New Mutants:

These mutants were then tested by spreading them onto sectors of plates of minimal medium (plus the parent mutant's needs) and adding 0.1ml of an amino acid pool, (purine-pyrimidine pool or vitamin pool) to each sector. When a mutant grew on one sector, another set of similar tests was set up, using the individual members of the pool for testing to identify the defect added to the treated mutant. Where two pools were needed for growth, the various combinations of components in both pools were similarly tested.

These final tests were confirmed by innoculating this new mutant into a tube containing 2ml of minimal salts, plus glucose, and the components needed by the parent mutant and the new component. A control tube lacking the new component was similarly prepared. Growth in the 1st and not 2nd tube was considered "confirmatory."

This new, identified mutant was then given a new mutant number and the whole process described above was repeated.

Results of Mutant Production

Step 1: From the NG Treatment of Salmonella Typhimurium #3599 (Table I)

There were 20 mutants picked onto each of these 8 plates, plus the control colonies of #3599. Of these, 77 were selected for testing. The rest were "leaky mutants," or revertants since they grew on the minimal medium after velveteen replication. Of those selected, 29 had to be discarded, after the first screening test with the pools because they, too, turned out to be revertants. There was also one slow-growing bradytroph. Ten of the remainder gave rather confusing results on further testing. They appeared to be multiple mutants, since they did not grow until several pools were combined. The combinations contained different pools, possibly indicating some were using alternate routes of metabolism. Time did not permit checking them out.

The remaining 18 mutants did not grow in any of the single pools. A number of them were tested by adding additional pools, but still did not grow or showed only slight traces of growth. Time did not permit further testing.

Step 2: From the NG Treatment of MSK-1 (Arginine) (Table II)

Of the 20 mutants picked, 18 grew on the minimal agar. One appeared to be a revertant. One needed threonine for growth. It was renumbered MSK-9.

Step 2: From NG Treatment of MSK-2 (Isoleucine); (Table II)

Thirteen colonies grew on the minimal plate. Two of the seven tested were slowly-growing bradytropha. Three were revertants. One gave negative results after repeated testing with pool combinations. The remaining one gave

MUTANTS - TABLE I

Step 1: From NG Treatment of Salmonella typhimurium #3599

Total # picked	Grew on minimal	Total # tested	Bradytrophs Revertants	Conflicting Results	Consistantly negative	Identified Mutants
160	83	77	28R 1B	10	18	20

*New #--given to mutants to be saved for re-treatment. "Sister mutants" kept, but not re-numbered. (*) Vitamin Pool mutants not further checked to determine specific vitamin needed. Those underlined were used for the next NG treatment.

Plate	Colony	Component needed	New #*
A	12	Vitamin Pool (*)	
A	13	Vitamin Pool	
A	19	Adenine & Guanine	MSK-3
A	23	Isoleucine, Val.	MSK-8
B	6	Threonine	MSK-4
B-	7	Arginine	
B	13	Isoleuc. (large amt) OR "+Valine	MSK-2
D	5	Arginine	MSK-1
D	10	Arginine	
D	12	Threonine	
E	6	Arg. & Guanine	MSK-7
E	17	Threonine	
G	22	Adenine	
J	2	Uracil	MSK-6
H	10	Adenine OR .2ml Guanine	MSK-21
W	4	Isoleuc. +Val.	
W	7	Leucine (slow, needs extra amount)	MSK-5
W	10	Cysteine + Arg.	MSK-19
W	13	Arg. OR .2ml Guan.	
W	14	Arginine	

conflicting results.

Step 2: From NG Treatment of MSK-4 (Threonine) (Table II)

Thirty colonies grew on minimal plates. Three of these proved to be "leaky," and four were not completely checked out. One mutant needed lysine for growth on minimal agar. One needed isoleucine and valine. A third grew slowly if uracil was present.

Step 2: From NG Treatment of MSK-6 (Uracil) (Table II)

Three of the 26 mutants tested were discarded as revertants. One grew so poorly in broth that it was not tested. Another was repeatedly negative, and four additional mutants gave confusing results upon further testing. One of them grew in either Pool 2 or Pool 3. Another grew in either Pool 4 or 5. Neither was checked out. The remaining 13 colonies grew on the minimal plates, so were discarded.

Step 2: From NG Treatment of MSK-7 (Arginine and Guanine)
(A Double Mutant from Step 1) (Table III)

The first NG tube with visible growth was the 1:30 dilution, which had only a trace of growth. There was only moderate growth in the 1:64 and 1:128 dilutions. The control broth had a slightly heavier growth, but it, too, had only a moderate growth. The 1:128 dilution was selected for dilution and spreading. Four plates were spread with the 1:1 million PBS dilution of cells, but there was no growth of mutant colonies on the single enriched agar. The next five plates were spread, four from the 1:10,000 PBS dilution of cells, and one from the NG 1:64 dilution directly, .03ml used. The colonies were extremely small (even after 72 hours of incubation) on the single enriched medium. One hundred and forty were picked and replicated. However, nearly all of them

MUTANTS - TABLE II

Step 2: From NG Treatment of MSK-1 (needing Arginine)

Total # picked	Grew on minimal	Total # tested	Bradytrophs Revertants	Conflicting results	Consistantly negative	Identified mutants
20	18	2	1 R	0	0	1

Plate	Colony	Component needed	New #
A	1	Threonine	MSK-9

Step 2: From NG Treatment of MSK-2 (Isoleucine)

20	13	7	3R 2B	1	1	

Step 2: From NG Treatment of MSK-4 (Threonine)

40	30	10	3B	0	4	3

A	2	Uracil (slow growing)	
B	3	Isoleucine + Valine	MSK-10
B	5	Lysine	MSK-11

Step 2: From NG Treatment of MSK-6 (Uracil)

40*	14	26**	3	4	1	16

A	3	Arginine	MSK-13
A	5	Proline	
A	6	Glycine (slow .2ml needed)	
A	7	Arginine	MSK-17
A	8	Arginine	
A	9	Arginine	
A	10	Cysteine	
A	11	Cysteine	
A	12	Arginine	
G	1	Isoleucine & Valine	MSK-16
G	2	Arginine	
G	3	Arg. (or slight growth on lysine)	
G	6	Arg. (or slight growth on lysine)	
G	8	Isoleucine	MSK-12
G	11	Arginine	
G	12	Lysine	MSK-14

*MSK-6-G-5 discarded: very little growth in nutrient broth.

**One grew in Pool 2 and also in Pool 3 (not checked out.)
One grew in Pool 4 and also in Pool 5 (not checked out.)

MUTANTS - TABLE III

Step 2: From NG Treatment of MSK-7 (Arginine + Guanine) (A double mutant from Step 1)

Total # picked	Grew on minimal	Total # tested	Bradytrophs Revertants	Conflicting results	Consistantly negative	Identified mutants
40	27	13	4B	0	9 (only very slight growths in Pool 1 & 5)	

Step 2: From NG Treatment of MSK-8 (Isoleucine & Valine) (A Double mutant from Step 1)

160	137	23	2B 1R	1*	8	5

Plate	Colony	Component needed	New #
A	2	Glycine or Threonine (sl. growth)	MSK-20
E	2	Aspartic acid or Glutamic Acid	
E	9	Lysine	MSK-15
E	1	Vitamin Pool	
H	8	Guanine	MSK-16

*Additional colonies (incomplete information)

Two grew in Pools 1, 2, or 3. Not checked further.

One grew in Pools 2, Vitamin and slightly in Pool 1--not checked further.

MSK-8-E-6 grew poorly in broth, but did grow on the minimal agar with Pool 2 & PP Pool.

MSK-8-E-7 grew in Pools 4 or 5 but did not grow in Aspartic Acid or Glutamic Acid--not checked further.

MSK-8-H-9 grew in Pool 1 but not in any of its individual components--not checked further.

grew slowly, some not showing up for six days. Five of the nine were tested and showed slight growth in pools 1 and 5. They grew lightly in nutrient broth. None were checked out.

Step 2: From NG Treatment of MSK-8 (Isoleucine & Valine)
(A Double Mutant from Step 1) (Table III)

Of 160 mutants picked, 136 grew on minimal agar. Twenty-four were tested. Of these, two were leaky, and one was revertant. Eight were consistantly negative, and one gave conflicting results, six grew in more than one pool, but were not checked out. Five mutants were identified, and two renumbered for further studies.

Step 3: From MSK-9 Treatment with NG (Arginine and Threonine) (Table IV)

Steps 3 and 4 gave nothing but trouble. MSK-9, needing Arginine & Threonine when treated with NG, did not grow through the 1:16 dilution of NG. Growth in the 1:32 NG dilution appeared only slightly less turbid than the control tube. All the colonies that grew when this was spread on the single-enriched medium were very small, and nearly all appeared to be mutants. Many were extremely small, about the size of a pin prick.

When these were replicated, they grew on the nutrient agar, four grew on the minimal medium plates for MSK-9.

Fifteen mutants were tested. Five proved to be slow bradytrophs. One showed slight growth in the Vitamin Pool, and although the other seven were tested and retested with one pool after another added to the test plate, none of them ever checked out.

Step 3: From NG Treatment of MSK-10 (Threonine, Isoleucine & Valine.) (Table IV)

There was no growth in the NG 1:8 dilution, but slight growth in the

MUTANTS - TABLE IV

Step 3: From the NG Treatment of MSK-9 (Arginine & Threonine)

Total # picked	Grew on minimal	Total # tested	"Leaky" mutants	Conflicting results	Consistantly negative	Identified mutants
100	4	15	5 + 1 revertant	1	7	1

Plate	Colony	Component needed	New #
E	2	Vitamin (but grew poorly)	

Step 3: From the NG Treatment of MSK-10 (Threonine, Isoleucine & Valine)

100	77	23	8 + 4 revertants	3	6	1

Plate	Colony	Component needed	New #
B	21	Leucine	MSK-22

Step 4: From NG Treatment of MSK-15 (Isoleucine, Valine & Lysine)

20	11	9	2	5	0	1

Plate	Colony	Component needed	New #
A	1	.6ml Leucine	MSK-23

if extra (.2ml) MSK-15 needs also added, grew sl with .2ml Arg + .2ml Leucine, grew well with .2ml Cysteine + .2ml Lysine, + .2ml Leucine + .2ml Arginine.

1:16 dilution. The 1:32 dilution was slightly less turbid than the control. It was chosen for use.

Unlike MSK-9, these colonies were more varied in size, with more of the "normal" sized colonies, and few tiny ones. Twenty-three of the "mutants" were tested. Twelve grew on all pools, so were found not to be new mutants. Eight never checked out, although they had as many as four or five pools added, in some cases, with only light growth resulting. One gave confusing results and the last one was identified to require Leucine. (Renumbered MSK-22).

Both MSK-10 and MSK-22 seem to need an extra heavy amount of their respective amino acid requirements to grow.

Step 4: From NG Treatment of MSK-15 (Isoleucine, Valine & Lysine) (Table IV)

The 20 mutants picked from MSK-15 were very tiny, but 11 grew on minimal medium. Of the nine tested, three were extremely tiny, and were not in a droplet shape. They were flattened like water on a scowered slide. Two of these, MSK-15-5 and MSK-15-6, grew in Pool 1 and in the Vit. pool. The other one, MSK-15-7, grew lightly when pools 3, 4, 5, & PP were all combined. MSK-15-8 and MSK-15-9 became slowly positive after Pools 3, 5, & PP had all been added. These five were never checked out, since further tests showed conflicting results. (#9 was a very tiny colony with a "hazy" growth on the single enriched medium.)

MSK-15-2 and MSK-15-3 grew well on three pools and slightly on one or two others. MSK-15-4 gave inconsistent results. MSK-15-1 was worked with a great deal. It tested positive for pools 1, 2, & 3 separately, but inconsistently. It grew slightly on MSK-15 minimal agar with .2ml Arginine plus .2ml Leucine and grew well on it with .2 ml Cysteine, .2 ml Leucine, .2 ml. Arginine and an additional .22mml Lysine. It was named MSK-23.

Step 4: From NG Treatment of MSK-16 (Isoleucine, Valine & Guanine) (Table V)

Both MSK-15 and MSK-16 were mutants resulting from the NG treatment of MSK-8. This organism was shown by Elsie Johnson, of the K-State Viral-Genetics Lab, to be very stable, but many revertants resulted from the treatment with NG crystals on minimal agar.

MSK-16 was treated with NG on four separate occasions. The colonies on the single enriched medium, were very tiny. Some could scarcely be seen. Yet out of a total of 420 colonies picked, all but 28 grew on minimal medium, at least faintly, as confirmed with a binocular disecting microscope. Of these 28, 23 proved to be slow-growing bradytrophs and five were never completely checked out, since they gave confusing results. None of these colonies were revertants to the original parent organism. They were of mutant type colony morphology and some grew very poorly even in nutrient broth.

Step 4: From NG Treatment of MSK-22 (Threonine, Isoleucine, Valine & Leucine) (Actually Step 2 Resulted in Double Mutation.) (Table V)

Although the colonies, on the single enriched agar, were tiny and clear all grew on minimal agar. The growth was very faint. However, seven were tested. Of these, five were "leaky," one remained negative, and the other was tested and retested with mainly negative, but several conflicting results. Some grew poorly in nutrient broth.

Step 5: From NG Treatment of MSK-23 (Isoleucine, Valine, & Leucine.)

One hundred tiny colonies were picked and replicated, but all of them grew on the minimal medium.

MUTANTS - TABLE V

Step 4: From Treatment of MSK-16 (Isoleucine, Valine & Guanine)

Total # picked	Grew on minimal	Total # tested	"Leaky" mutants	Conflicting results	Consistantly negative	Identified mutants
420	392	28	23	5	0	0
Step 4: From Treatment of MSK-22 (Threonine, Isoleucine, Valine & Leucine)* *(Actually step 2 resulted in double mutation of Isoleucine & Valine)						
40	40	7	5	1	1	--
Step 5: From Treatment of MSK-23 (Isoleucine, Valine, Guanine & Leucine)						
100	100	0	--	--	--	--

DISCUSSION OF RESULTS

In this investigation, an attempt was made to determine how many identifiable, consecutive defects could be added to the genetic make-up of Salmonella typhimurium, #3599. This strain, originally obtained from Dr. Demerec, already had the following alterations in the wild type DNA:- needed: methionine, histidine and tryotophan, and resistant to: streptomycin and uses xylose. This investigation was complicated by the fact that NG is so highly mutagenic that it frequently causes multiple damage. The phenotypic effect depends upon not only the extent of this damage but also upon its location. There could be a number of mutations of the bases of which one would not be aware. A base change could be made, for instance, on the 3rd letter of the genetic code for the production of an amino acid and not affect the proper selection of the amino acid for the construction of a particular protein. For example:-

The mRNA code for Arginine, is any one of the following: CGU, CGC, CGA, CGG, AGA, AGG (Watson, 1965) (Conn and Stumpf, 1967). The DNA code would have been: GCA, GCG, GCT, GCC, TCT, TCC. As can be seen, the DNA code for the 3rd letter could have been any one of the bases for the 1st 4 codes. However, if a transition occurred in the DNA of the 5th and 6th codes. The resulting mRNA codons would be AGU and AGC, both of which code for serine. (Watson, 1965). As was mentioned, in the literature review, some authors believe that the effect which a mutagen has on the DNA is partly determined by the neighboring groups around a DNA base which might be attacked by the mutagen. Therefore, these "hidden defects" might play a role during further exposure to the mutagen.

If a base were changed that codes for a different amino acid, it might produce a protein which is defective in its function, but not completely inert. If this should be an enzyme needed for the production of an essential metabolic intermediate of an amino acid, the result might be to produce it in very small

amounts, compared to normal. This could result in a bradytroph or "leaky mutant." It would grow slowly and possibly poorly. The defective enzyme might even be so inefficient in the attack on the substrate, that the substrate would accumulate until its concentration became toxic----unless there were some feedback control to slow down its production this would possibly slow down the cell's growth even more.

Wagner and Berquist (1960) pointed this out in their work with eight known precursors of isoleucine-valine in mutants of Salmonella. There are rather critical concentrations of some of these precursors. Armstrong and Wagner (1964) refer to a previous work in 1961 with S. typhimurium in which they show that the precursor of isoleucine is ten times more reactive with the enzyme reducto-isomerase, in the metabolic pathway leading to the production of isoleucine, than is the precursor of valine. Therefore, if enough valine is available, a mutant, in which this enzyme is defective, could produce enough isoleucine to enable it to grow, though weakly.

Since leucine, isoleucine and valine use some of the same enzymes in their production, even though their precursors are not the same, one mutation could result in the organism's needing two or even all three of these amino acids. (Glanville and Demerec, 1960). This author obtained one of these mutants. It had acquired the need for threonine on the first, isoleucine and valine on the second, and leucine on the third treatment with NG. This could indicate damages to just two of the enzymes in the isoleucine, valine, leucine pathways.

Many mutants that had been picked for testing would not grow on any of the supplemental pools added to the minimal medium. Even adding combinations of several pools produced no growth. Possibly this was due to the fact that

certain amino acids act as inhibitors if their concentration is out of balance with some other amino acid. For example, Rowbury et al. (1969) found that cysteine and homo-cysteine act as inhibitors to some of the methionine-regulated enzymes in the metabolism of precursors of methionine.

Another consideration is multiple mutation, possibly even coupled with making an enzyme inoperable early in the common pathway of the production of several amino acids. If such an event happened to prevent the production of shikimic acid, the organism would be unable to produce phenylalanine, tyrosine, tryptophan and Vitamin K, among those requirements being tested, plus ubiquinone, not tested for, and enzymes for other metabolic reactions. Refer to Gibson and Pittard (1968) and Hayes (1968) for the outline of these pathways.

There have been E. coli mutants reported that have the following requirements:

1. threonine,
2. threonine or homoserine (believed to be a common metabolic intermediate for both threonine and methionine),
3. threonine plus methionine,
4. threonine plus methionine or homoserine.

(Cohn and Hirsch, 1954; quoted by Glanville and Demerec, 1960). The latter authors stated that mutants requiring both threonine and methionine had not been detected in Salmonella. However, such mutants were found in the study by this author. There were three "sister mutants" in the first step treatment with NG and one with the second step treatment. The latter had acquired the need for arginine from the first step treatment. Glanville and Demerec (1960) also found that some Salmonella mutants are unable to grow in L-threonine, which is the normal form of the amino acid in nature, but were able to grow slowly on D-threonine.

This extra test was not made in this experiment.

Nester (1968) found that the production of Hydroxyphenyl Pyruate, an enzyme in the synthesis of tyrosine, is inhibited by histidine and by phenylalanine, to a lesser extent, in B. subtilis. This inhibits the growth of bradytrophs deficient in this enzyme and also those with defective hydroquinate synthetase, which is an early enzyme in the metabolic pathway of tyrosine, phenylalanine and tryptophan. Similarly, tyrosine inhibits the growth of bradytrophs deficient in the production of histidine. These mutants will, however, grow in the presence of their needed amino acids, in spite of the inhibitory action of the other amino acids.

The extent of actual mutation could not be determined without further testing, since, as Nishioka et al., (1967) pointed out, single one-step mutations can produce different phenotypes of these aromatic mutants depending on what part of the aromatic A gene is changed. One section would result in the organism requiring tyrosine and phenylalanine. A different site could result in these two and the additional requirement of tryptophan, plus one, two or possibly three intermediate substances. If all of these sites on the gene were defective, the phenotype would not indicate it since there are two quadruple-requiring sites, one triple and one double-requiring site. If a mutant developed that needed five of these requirements, we would not know the actual extent of the damage, or its location. The test pools do not contain any of the metabolic intermediates.

There is a suppressor locus near the tryptophan operon, (Bauerle and Margolin, 1968). If this locus is deleted, it suppresses the leucine auxotrophy of the S. typhimurium strain leu-500, thus "reverting" the phenotypic expression of this mutant. Therefore, if a leucine auxotroph were treated with NG and this

suppressor locus were mutated, it would not show up in testing, since the test medium would already contain leucine, and growth on that medium would be expected if there were no new auxotrophy developed.

There appear to be fourteen enzymes responsible for the production of the purine nucleotides. Westby and Gots (1969) studied the role of the first three of these enzymes on four of the twelve genetic groups of S. typhimurium affected by these enzymes. They found these four groups of purine-requiring organisms, purD, purF, purG and purI, to be phenotypically identical they could not be separated except by recombination and transduction studies, since they all were indiscriminate in their choice of purines for growth. The purG strain has been found to be deficient in the fourth enzyme of the sequence, and the purI, is deficient in the fifth. The other two groups have not been determined. In these cases, several of these enzyme sites could have been damaged, and with the present author's method of testing one would not be able to detect which of these sites had been mutated.

This author isolated and identified twenty-three mutants and sixteen "sister mutants," in addition to a number of mutants which needed some vitamin. The latter were not tested further to determine which vitamin or combination of vitamins was needed. Twenty of the identified mutants were from the first step treatment with NG. Several of these were carried on to the second, and third steps. One was carried to the 4th step. The latter produced a rather "temperamental" mutant (MSK-23) that may have been very sensitive to the concentrations of the added amino acids. It grew slightly on the MSK-15 minimal agar containing isoleucine, valine and lysine, plus 0.2ml arginine and 0.2ml leucine solutions, and grew well on MSK-15 minimal medium plus .2ml cysteine, .2ml leucine, .2ml arginine and an additional .2ml lysine.

It appeared that the location of the damage in one step had some effect upon how many identifiable mutants could be obtained from an organism in the next step. However, it is rather presumptuous to make this statement, since time did not allow sufficient numbers of mutants to be compared to give valid results. Also, the particular mutant selected could have had one or more of the "hidden defects" mentioned above, which would make a big difference in the further treatments with NG.

CONCLUSIONS PERTAINING TO RESEARCH BY THIS AUTHOR

The first and second step NG treatments of Salmonella typhimurium #3599 by overnight culture in NG-serially diluted in nutrient broth resulted in 26-30% identifiable mutants needing one or two additional amino acids, purines, pyrimidines or vitamins. There was an abrupt drop on the third step treatment to an average of .06% identifiable mutants. Subsequent NG treatments only resulted in consistantly larger numbers of bradytrophs and revertants and no identifiable mutants.

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THE PRODUCTION OF MUTATIONS IN *SALMONELLA TYPHIMURIUM* WITH N-METHYL-N'-NITRO-N-NITROSOGUANIDINE (NG) AND A LITERATURE STUDY OF THE ACTION OF NG ON LIVING CELLS

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ABSTRACT

Salmonella typhimurium 3599 was cultured in serial dilutions of N-methyl-N'-nitro-N-nitrosoguanidine (NG) in nutrient broth for 24 hours to produce successive, identifiable mutants needing additional amino acids, purines and/or pyrimidines. Twenty three were identified, plus sixteen "sister mutants." Several were carried to the third and fourth step and one identified after the fourth step.

Literature review reveals:-

1. NG is considered the most mutagenic chemical agent discovered (Adelberg, et al., 1965 ; Lopriano and Clark, 1965). Lower concentrations produce more mutants and an estimated mutation rate of at least one mutation per cell has been found (Eisenstark et al., 1965; Mindlin and Churkina, 1969; Mracek, et al., 1969; Müller and Gichner, 1964).

2. NG causes all types of transitions and transversions but no deletions or additions (Eisenstark, et al., 1965; Cerda'-Olmedo, 1967; Baker and Tessman, 1968).

3. NG breaks down in low pH to produce nitrous acid, a known mutagenic agent, causing deamination of nucleic acid bases (Mandell and Greenberg, 1960; Zimmerman, et al., 1965).

4. In high pH, NG yields diazomethane (McKay, 1948 and 1952), an active methylating agent (Hultin, et al., 1960), believed by some to be the cause of mutations above pH 5.5 (Auerbach 1967; Heath, 1962; Hultin, et al., 1960; McCalla, 1967; McCalla and Reuvers, 1968). This hypothesis is supported by mutants caused by the use of diazomethane (Kriek and Emmelot, 1964; Friedman, et al., 1965).

5. This hypotheses is questioned since no correlation has been shown

between the high mutagenicity of TMV and the low methylation (Singer, et al., 1968) and because cells normally possess enzymes that methylate the nucleic acids (Borek and Srinivasan, 1966).

6. Most mutations appear to occur at the point of replication (Cerda - Olmedo, 1967).

7. Differences in mutagenicity of NG in different organisms may be due to differences in the "molecular environment" at the replication point of DNA, including the specific mechanism of replication of the organism, the nature of the DNA polymerase, the base composition of the DNA, the OH^- concentration, presence of phosphates or thiols and other compounds, and the conditions of the experiment (Friedman, et al., 1965; Holy and Scheit, 1966; Lawley, 1968; Singer, et al., 1968).

8. Various enzymes, in different cells, may metabolize NG in different ways, which would result in different break-down products and therefore affect its mutagenicity (Swan and Magee, 1968).

9. NG interfers with protein production (Cerda -Olmedo and Hanawalt, 1967; Villa-Treviño, 1967) by the incorporation of ^{14}C, from the guanidino part of the NG molecule into an amino acid, (Magee and Farber, 1962; McCalla and Reuvers, 1968; Sugemura, et al., 1968).

10. The more basic the protein, the more label is bound (Sugemura, et al., 1968).

11. If this occurs in the active or the allosteric site of an enzyme, it would impair the enzymatic activity (McCalla and Reuvers, 1968). If this were DNA polymerase, it might result in abnormal replication of DNA (Sugemura, et al., 1968).

12. NG does inactivate β galactosidase (Cerda'-Olmedo and Hanawalt,

1967).

13. RNA synthesis is inhibited to some extent, (Cerda'-Olmedo and Hanawalt, 1967).

14. If the proteins of ribosomes are altered, it might inactivate them, thus inhibiting protein production, (McCalla and Reuvers, 1968).

15. There is some delay before mutations show up, during which time there may be some repair, similar to the dark repair after uv damage (Cerda'-Olmedo and Hanawalt, 1967).

16. The phosphate moiety of the nucleic acids is methylated by NG, (Brown, et al., 1955; Friedman, 1957).

It is evident that much more work must be done before the exact nature of the mechanism of mutagenesis of NG will be understood.