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The Chemical Production of Mutations

The effect of chemical mutagens on cells and their genetic material is discussed.

C. Auerbach

Twenty years ago, *Science* published an article with the above title (*1*). A few years earlier, the first potent chemical mutagens had been discovered, and this discovery started a vigorous and astonishingly successful search for more substances with mutagenic ability. The hopes which my colleagues and I set on the new field of chemical mutation research were expressed as follows. "If, as we assume, a mutation is a chemical process, then knowledge of the reagents capable of initiating this process should throw light not only on the reaction itself but also on the nature of the gene, the other partner in the reaction. Moreover, it could be hoped that among chemical mutagens there might be some with particular affinities for individual genes. Detection of such substances not only would be of high theoretical interest but would open up the long sought-for way to the production of directed mutations." It is interesting to look back and see how far these hopes of 20 years ago have been fulfilled.

Reactions between Mutagens and Genes

The chemical nature of the gene has not been elucidated by research on mutation but in entirely different ways. On the contrary, mutation research now starts from the presumption that "the gene, the other partner in the reaction" consists of DNA, or—in some viruses—of RNA. The DNA molecule is a duplex structure, consisting of two sugar-phosphate strands which are helically wound round each other and which carry attached sequences of the four nucleotide bases adenine, guanine, thymine, and cytosine. The two strands are held together by hydrogen bonds between opposite bases; since, for steric reasons, the purine adenine is always opposite the pyrimidine thymine and the purine guanine is opposite the pyrimidine cytosine, the whole structure is internally complementary. At replication, the two strands separate, and each constructs a new complementary strand. The genetic information is coded by the sequence of bases, and, if this has been changed by mutation, the same principle of complementarity that governed replication of the original sequence now leads to perpetuation of the mutated one. It appears that this structure of the genetic material is common to all living species, from viruses to man. Among viruses there are some exceptions, but they have retained the principles of coding by base sequence and of replication by complementarity. In some viruses, DNA is single-stranded when it is not engaged in replication, and in some it has been replaced by RNA. The RNA molecule is single-stranded except at replication; three of its nucleotide bases are the same as those in DNA, but thymine has been replaced by uracil. When speculating about the action of mutagens, we no longer ask whether they react with DNA, but how they react with it. We shall see that, as these questions were answered for a series of mutagens, specificities of reaction appeared at the level of nucleotides and nucleotide sequences and furnished valuable clues for the deciphering of the genetic code. Thus, chemical mutagens have, after all, proved important analytical tools for the study of the genetic material, but at a level of chemical structure that was still quite unsuspected 20 years ago.

Knowledge of the structure of DNA furnishes a framework for the classification of mutations. The least drastic alteration—which, however, may have drastic consequences for the organism—is replacement of one nucleotide base by another. The most frequently observed base changes are "transitions," in which a purine has been replaced by another purine (adenine by guanine or vice versa) or a pyrimidine by a pyrimidine (thymine by cytosine or vice versa). Much rarer are "transversions," in which a purine has been replaced by a pyrimidine or vice versa. Since the genetic code is read in triplets of bases, each triplet coding for one amino acid, mutations due to base changes—whether transitions or transversions—usually result in a protein in which one amino acid has been replaced by another. In which way this will affect the organism depends on the type of amino acid change and on the position in the protein in which it occurred. Occasionally, a base change results in a so-called "nonsense triplet" which, instead of coding for an amino acid, codes for premature termination of the growing polypeptide chain, so that only incomplete protein molecules can be formed. These mutations lead to complete loss of gene function. A mutant gene that arose through a base change may be reversed by another base change that restores the original

Dr. Auerbach holds a personal chair in the Department of Genetics at the University of Edinburgh and is a Fellow of the Royal Society of London.

code. Such mutations are called "reverse" mutations; they restore a gene function that has become altered or abolished by the first mutation. Since reverse mutations require a very precise chemical change, they are very rare even after mutagenic treatment and can be studied profitably only in material that permits work on very large numbers, such as bacteria or bacteriophages.

A different class of mutations is due to deletions of one or more bases from the sequence of a gene. Since the code is read in base triplets from one end of the gene to the other, deletions of even one base will alter every triplet from the missing base to the end of the gene, and this in turn will alter every one of the corresponding amino acids. Insertion of an extra base has the same effect. Such mutations are often called "reading frame shifts"; they result in loss of gene function. They cannot be reversed by base changes, but, if a deletion and an insertion occur sufficiently close together, the original reading frame will be restored after the second change. If the portion between the two changes is relatively unimportant, this may result in a sufficiently normal protein to pass as a reversion.

Changes in genetic information that take place within the confines of a gene and affect the action of this gene only are called "gene mutations." On a grosser scale, we have to consider changes that affect the order of the genes on the chromosomes. In this article, I shall use the term "chromosome" for all linear structures that carry hereditary information in any organism whatsoever. This terminology is simple and suffices for our purposes, but it obscures the fact that in all organisms beyond the evolutionary level of bacteria the chromosomes are complex structures, containing not only DNA—often several replicas of it—but also proteins and RNA. The role of this complexity in the production of mutations is likely to be important, but hardly anything is known about it. Within the chromosome, there may be deletions of whole genes or sequences of genes. This can happen when a chromosome is broken into several fragments, one of which gets lost when the broken ends rejoin. Rejoining of chromosome fragments in the wrong order may also result in other types of "chromosome rearrangement," for example, a "translocation" in which two broken chromosomes have exchanged pieces. Some of these chromosome rearrangements resemble gene mutations in their effects on the organism and in their mode of hereditary transmission.

The Discovery of Chemical Mutagens

The first agent shown to produce mutations was x-irradiation, but the search for chemical mutagens had started already before this discovery and was continued after it. Although some of the substances tested in the 1930's, for example, iodide and copersulphate, seemed to have weakly mutagenic effects on the fly *Drosophila*, no clear positive results were obtained before the early years of World War II when, independently, the mutagenic action of mustard gas was discovered in Edinburgh and that of urethane was found in Germany. These first successes provided a strong stimulus for the testing of more substances, many of which proved mutagenic. By now, we know a large number of chemical mutagens, belonging to a variety of chemical classes. Very different principles have been used in the selection of chemicals for testing. Some of the most powerful mutagens were discovered on the basis of what we now know to be the wrong concept of the gene. Mutation research offers good cautionary examples against the belief that a successful experiment necessarily proves the hypothesis that inspired it. In the following, I shall briefly review the most important classes of mutagenic chemicals.

Alkylating Agents

This class contains some of the most potent mutagens, including mustard gas. The rationale for testing mustard gas was the pharmacological similarity between mustard-gas burns and x-ray burns, coupled with the knowledge that x-rays cause damage to chromosomes and genes. While this speculation was fully vindicated by the results, pharmacological observations are not always a reliable guide to the detection of chemical mutagens. This became clear very early when lewisite, like mustard gas, a potent vesicant war gas, proved quite ineffective in mutation tests. All the same, tests of pharmacologically active substances for mutagenicity have retained their value as part of the program for protecting man and his domestic animals and plants against genetic damage. Occasionally, this may lead to the discovery of a new group of strong mutagens. This happened with the pyrrolozidine alkaloids (for example, heliotrine) which were tested because they produce liver damage in sheep that ingest them in ragwort. The antibiotic streptonigrin has produced mutations in fungi and chromosome breaks and rearrangements in cells of plants and mammals. In mutation tests on *Drosophila*, streptomycin and the insecticide DDT were ineffective, and carcinogenic hydrocarbons gave, at best, doubtful results. It must be kept in mind, however, that negative results of mutation tests can rarely be considered as final. A chemical that fails to yield mutations in a particular type of experiment may be mutagenic under different conditions or for a different organism or cell type. Examples for this will be found in this article.

Chemically, mustard gas is dichloroethyl sulphide $S(CH_2CH_2Cl)_2$; the related and equally mutagenic nitrogen mustard "NH2" has the formula $CH_3N(CH_2CH_2Cl)_2$. These and other "mustards" owe their biological activity to their chloroethyl groups; they act by alkylating of biologically important macromolecules. In addition to mustards, many other compounds, in particular epoxides, ethylene imines, and alkylmethanesulphonates, have alkylating abilities, and many of them are mutagenic. Indeed, 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 nitrosocompounds and the pyrrolizidine alkaloids mentioned above.

In vitro, alkylation affects preferentially the guanine in DNA, and this appears to be so also in vivo, although in a bacteriophage with single-stranded DNA all four bases were attacked (*2*). Alkylation of guanine is thought to produce mutations mainly through the tendency of alkylated guanine to pair erroneously with thymine instead of cytosine. At the next replication, thymine will pair correctly with adenine, and the final result will be a transition from a guanine-cytosine to an adenine-thymine pair at the site of mutation. It has also been suggested that mutations may arise through the relative ease with which alkylated guanine detaches from the DNA back-

bone, leaving an "apurinic gap," which might be filled by a wrong base. This could lead to transversions as well as transitions, but so far there has been no clear evidence for the production of transversions by alkylation. The assumption that alkylation usually changes guanine-cytosine into adenine-thymine but only rarely adenine-thymine into guanine-cytosine agrees well with the fact that alkylating agents are not usually able to revert the mutations that they themselves have produced, while other mutagens—able to change adenine-thymine into guanine-cytosine (see below)—may do so.

In addition to gene mutations, alkylating agents also produce deletions and other types of chromosome rearrangement. Indeed, their genetical effects are so strikingly similar to those of x-rays that the term "radiomimetic substances" is often applied to them. Differences, however, also exist, and these are of special interest for an analysis of the mutation process. Two major differences from x-rays were found early and seem to be characteristic of all alkylating agents, possibly of most chemical mutagens. One is a relative shortage of chromosome rearrangements compared with gene mutations. This is found for all alkylating agents, but its magnitude varies from a moderately high number of rearrangements after treatment with ethylene imines to their almost complete absence after treatment with diethylsulphate. For mustard gas it has been shown that the shortage of rearrangements is not due to a shortage of chromosome breaks but to an inhibition of the process by which the broken ends rejoin into new arrangements. How far this factor contributes to the shortage of rearrangements after treatment with other alkylating agents has not been established.

The inhibition of reunion between chromosome fragments is mainly a consequence of the second and more fundamental peculiarity of alkylating agents and, possibly, of most other chemical mutagens. This is a tendency for injuries to the genetic material not to result directly in chromosome breakage and mutation but to remain latent over a period that may extend over many cell cycles. Since the formation of a chromosome rearrangement requires the simultaneous presence in the same cell of two chromosome breaks, while single unjoined breaks usually result in cell death, a potential rearrangement will be lost every time when the two breaks that might have given rise to it open in different cell cycles.

Although the phenomenon of delayed chromosome breakage and mutation was first observed and studied in *Drosophila*, it can be illustrated more easily by an example from experiments on fission yeast, *Schizosaccharomyces pombe*, in which mutations from red to white colony color can be scored. When cells of the red strain are exposed to the alkylating agent ethylmethanesulphonate and subsequently plated out, the majority grow again into red colonies, but a minority of mutated ones grow into white colonies. In addition, there are "mosaic" colonies that are partly white, partly red. In the early days of mutation research, this observation alone would have suggested that some mutations to white must have arisen with a delay of at least one division. This argument has become invalid with our realization of the duplex nature of DNA. If a chemical change affects only one strand of DNA—and the majority are likely to do so—the first division should result in one mutated and one nonmutated cell, and these should grow into a mosaic colony. Decisive proof for the delayed occurrence of mutations after treatment with ethylmethanesulphonate was obtained when cells from mosaic colonies were respread. As would be expected, the majority grew into either wholly red or wholly white colonies, but in most experiments some grew again into mosaics, and these, in turn, yielded some mosaics when respread. Moreover, although the mutations to white involved a number of different genes, in any particular line of mosaics it was always the same gene that gave rise to delayed mutations. The simplest way of describing these observations, and similar ones obtained in other systems and with other mutagens, is to say that treatment with alkylating agents, in addition to producing mutations immediately, may cause instabilities of individual genes that continue to give rise to mutations. Moreover, since in these lines each mosaic must have started with an instability, and since several cells from the same mosaic may again grow into mosaics, the instability must be able to replicate as such. No satisfactory explanation of these instabilities has so far been put forward. Their nature, in particular their ability to replicate in the unstable state, is difficult to fit into a molecular explanation at the level of DNA unless, as has been suggested very recently, an apurinic gap may replicate as such (*3*). Probably, different types of instability arise in different ways; this applies in particular to instabilities for gene mutations on the one hand, delayed chromosome breakage on the other.

Many alkylating mutagens are carcinostatic, and some have been found to be carcinogenic. This triad of effects is produced also by other agents, notably urethane and x-rays. The correlation between carcinogenicity and mutagenicity is obscure; it has been used in support of the somatic mutation theory of cancer. The carcinostatic action of mutagens is doubtless connected with their ability to break chromosomes; for chromosome breakage preferentially kills dividing cells such as are present in malignant tissue. Among alkylating agents, only those with two or more functional (for example, chloroethyl) groups are carcinostatic, while related ones with but one functional group are not. Yet many such agents not only produce high frequencies of gene mutations but are also efficient chromosome breakers. A clue to this discrepancy between oncological and genetical observations may be found in recent experiments on *Drosophila* in which pairs of closely related monofunctional and polyfunctional epoxides and ethylene imines were compared (*4*). When treated spermatozoa were utilized on the day following treatment, the ratios between chromosome rearrangements and gene mutations caused by mono- and polyfunctional members of the same pair were the same. This, however, was drastically changed when the treated spermatozoa were first stored for six or more days in the seminal receptacles of untreated females. During storage, the frequency of chromosome breaks and rearrangements, but not that of mutations, increased up to 15-fold after treatment with the polyfunctional compounds, while it did not change at all after treatment with the monofunctional ones. The superiority of polyfunctional compounds in cancer therapy may, therefore, be related to the fact that treatment allows the full effect of storage to manifest itself.

Urethane

Like x-rays and many alkylating agents, urethane is mutagenic, carcinostatic, and carcinogenic. In flowering plants, as well as in *Drosophila*, it

produces chromosome breaks and rearrangements; in *Drosophila*, the breaks produced by urethane resemble breaks caused by x-rays, at least to the extent that fragments produced by the two treatments given in succession combine as freely with each other as do fragments produced by only one of them. In *Drosophila*, urethane also produced gene mutations which appeared to be unconnected with chromosome breakage. Urethane is, however, a very "spotty" mutagen, acting strongly in some organisms and not at all in others. The fungus *Neurospora* was entirely recalcitrant to its action, even when the whole of the genome was tested for mutations and deletions by a special technique (*5*). Possibly, this organism specificity of urethane as mutagen is related to its even more striking organism specificity as a carcinogen. It produces lung cancers in some rodent species but not in others; the active principle in this case appears to be a metabolite of urethane which is produced in mice but not in guinea pigs (*6*).

Phenols

These are similarly "spotty" in their mutagenic effects. A number of phenols, for example, pyrogallol and hydroquinone, produced chromosome fragmentations in plants, although few rearrangements were formed. In *Drosophila*, exposure of the explanted and subsequently reimplanted larval ovary to phenol produced high frequencies of mutations (or small deletions) in some experiments and none in others, and, in spite of prolonged and determined efforts, the conditions for successful application could not be elucidated. These results have gained new significance through the finding of an increased frequency of chromosome rearrangements in lymphocytes of men that had been exposed to ambient benzene (*7*).

Formaldehyde

When formaldehyde is mixed into the food of *Drosophila*, it may act as a strong mutagen. The conditions for its action are, however, more stringent than for any other mutagen. Although, as shown by labeling experiments, formaldehyde penetrates into all germ cells of larvae and adults, mutations occur exclusively during one part of the cell cycle of one germ cell stage in one developmental phase of one sex, namely in early larval spermatocytes. Moreover, the frequency of mutations depends on the nutritional status of the larvae: any conditions that slow down development, including an excessive dose of formaldehyde, decrease the frequency of mutations, and under very poor growth conditions no mutations at all are recovered. On synthetic media, adenosine riboside is an indispensable adjuvant; whether it is involved in the production of a secondary product with mutagenic ability or whether it aids in the release of free formaldehyde from a reversibly bound form or acts in some other way could not so far be decided. Studies on the distribution of mutations along the chromosome have led to the conclusion that mutations are produced during the time of DNA replication and occur in close neighborhood to the point of replication. Casein that has been treated with formaldehyde is also mutagenic; this has raised some doubt about the advisability of feeding breeding pigs skim milk sterilized with formalin. Cytologically, formaldehyde produces few gross chromosome rearrangements but many small deletions and repeats, that is, duplications of small chromosome regions in tandem or reverse. Since duplications are generally assumed to have played an important part in evolution, it is of interest to see that they can be produced by a compound that is closely related to normal metabolic processes.

Organic Peroxides and Irradiated Medium

Formaldehyde can also produce mutations when applied in aqueous solution to microorganisms or *Drosophila* spermatozoa. Applied in this way it is a very weak mutagen whose effectiveness, however, can be greatly enhanced by the addition of hydrogen peroxide, which by itself is hardly mutagenic. This suggests that a mutagenically active peroxide is formed and that small amounts of this substance can arise through reaction of formaldehyde with metabolically produced hydrogen peroxide. Indeed, the addition compound of formaldehyde and hydrogen peroxide, as well as other organic peroxides, are fairly good mutagens. They form a link with radiation mutagenesis, for peroxides have been made responsible for the mutagenic action of bacterial medium that has been exposed to heavy irradiation with ultraviolet light or ionizing radiation. In recent years, the sterilization of human food with very heavy x-ray doses has given concern about the possible introduction of mutagens into human consumption. Plant chromosomes can, indeed, be broken by heavily irradiated fruit juices or sugar solutions or by growing of the plants on irradiated potato medium (*8*). Mutation tests on *Drosophila*, on the other hand, have given negative results in some investigations and slightly, though significantly, positive ones in others (*9*). The bearing of these findings on human affairs is doubtful. Experiments on mice might give clearer evidence, although even extrapolation from mice to man is hazardous when one is dealing with slight genetical effects of chemicals that have been introduced to the germ cells by way of the food.

Inorganic Salts

A number of inorganic salts produce chromosome breaks and rearrangements in plant cells and mutations in bacteria. In particular, manganese chloride is an excellent mutagen for some bacterial strains, but its action depends strongly on ancillary conditions such as the presence of other salts before treatment. In fungi, attempts to produce mutations with $MnCl_2$ have been unsuccessful. It seems likely that the genetical effects of inorganic salts are not due to direct reactions with DNA, but to the creation of cellular conditions that favor the occurrence of "spontaneous" mutations and chromosome breaks.

Purine Derivatives

Certain alkylating agents, as well as formaldehyde, were first tested because of their known reaction with proteins. This was natural at a time when most geneticists believed that the specificity of the gene resided in its protein moiety. Yet even then, it was thought possible that reaction with the DNA moiety might produce mutations, and a number of purines and pyrimidines were tested for mutagenicity. While the results with pyrimidines were negative, various purines were found to produce chromosome breaks in plants and

mutations in fungi and bacteria. Special interest was aroused by the mutagenic action of caffeine because of the large amounts of it that civilized man consumes in tea or coffee. Tests on mice that had been given the highest tolerated dose of caffeine in their drinking water gave no evidence for the production of either chromosome breakage or mutation, but chromosome breaks, although no rearrangements, were found in human cell cultures that had been exposed to solutions of caffeine (*10*). Experiments on *Drosophila* gave contradictory results, but the latest evidence indicates that feeding or injection of caffeine has a weak mutagenic effect (*11*). As in the case of food sterilized with radiation, the application to human affairs is doubtful and hazardous. An interesting feature of the experiments on bacteria was the finding that adenosine riboside, which, as mentioned above, is required for the mutagenic action of formaldehyde, abolished that of caffeine and related purines. Moreover, it greatly reduced the frequency of spontaneously occurring mutations, suggesting that a proportion of them is due to ingested or metabolically produced mutagenic purines. The frequency of radiation-induced mutations was not reduced by adenosine riboside or related "antimutagens."

Substances Tested because of Their Reactivity with DNA

Base analogs. With the recognition of DNA as the essential genetic material, the search for mutagens became directed towards substances that are known to react with DNA or may be presumed to do so. The first of these to be tested were analogs of the purine and pyrimidine bases in DNA. It was thought that these analogs, by being mistakenly incorporated into DNA, might misdirect the subsequent incorporation of the natural bases at the time of replication, leading to transitions from adenine-thymine to guanine-cytosine or from guanine-cytosine to adenine-thymine. Indeed, the pyrimidine 5-bromouracil which, under appropriate conditions, may replace most of the thymine in the DNA of bacteria and viruses, proved to be an excellent mutagen; so, however, did the adenine analog 2-aminopurine of which only traces are incorporated. Thus, although incorporation into DNA almost certainly is a prerequisite for the mutagenic action of base analogs, their mutagenic efficiency does not necessarily depend on the chemically detectable degree of incorporation. Since then, evidence has been obtained for assuming that 5-bromouracil, when incorporated instead of thymine, is only weakly mutagenic, presumably because its pairing preference for adenine is almost as high as that of thymine. However, in the rare and chemically not yet detected instances in which it is incorporated instead of cytosine, this same strong pairing preference for adenine leads to the eventual replacement of a guanine-cytosine pair by an adenine-thymine pair. It is in agreement with this assumption—and, in fact, has been used in formulating it—that mutations which are easily reversed by alkylating agents are also easily reversed by 5-bromouracil. 2-Aminopurine, on the contrary, preferentially reverts mutations that do not respond to alkylating agents, including those produced by them. This is taken as evidence that 2-aminopurine usually acts by incorporating instead of adenine, pairing erroneously with cytosine, and finally changing an adenine-thymine pair into a guanine-cytosine pair. Base analogs have also proved mutagenic in some fungi, and they are able to break chromosomes in human cell cultures. The use of one of them, iododeoxyuridine, for the treatment of herpes lesions in the cornea has caused some concern for possible genetical consequences on the patient; but the chance that this strictly localized treatment will allow appreciable amounts to penetrate to the gonads seems negligible.

Acridines. These have long been known to react with nucleic acids, and instances of chromosome breakage and mutation by substances such as acridine orange or proflavine have been reported repeatedly in the past. In most of these experiments, however, visible light had not been excluded, and the effects might have been due to the so-called photodynamic action of the dyes, that is, their ability to sensitize other molecules to the action of visible light. A different and more interesting mechanism of mutagenesis by acridines was suggested by the finding that in vitro acridine molecules intercalate between the nucleotides of DNA. If this should happen also in vivo, replication of the affected strand would be disturbed, and this, it was thought, might lead to the insertion or deletion of a base in the normal sequence. In other words, mutations produced by acridines should be reading-frame shifts and should be characterized by three properties: (i) complete absence of gene function, (ii) lack of reversibility by agents producing only base changes and (iii) ability of being at least partially reversed by other reading-frame shifts of the complementary type that had occurred close enough to the first one to give a functional protein. Experiments with bacteriophage confirmed all these predictions: acridine-induced mutations resulted in completely inactive genes; they could not be reversed by base analogs, which are assumed to cause mutations exclusively by transitions; they could be reversed, or partially so, by additional acridine mutations in their neighborhood. The final proof for the hypothesis was brought by amino acid analysis of the enzyme lysozyme in a certain type of bacteriophage (*12*). The enzyme was completely absent from two strains with acridine-induced mutations, and was partially active in a strain combining these two mutations. As predicted, the enzyme in the doubly mutant strain had a short stretch of faulty amino acids between the sites of the two mutations but was normal beyond it. Moreover, the faulty amino acids were of just the types expected from a reading frame shift applied to the original base sequence. Acridines, sometimes in the form of a so-called "acridine mustard" with a mustard as well as an acridine moiety, have produced mutations also in fungi and *Drosophila*. There is evidence that these mutations, too, are due to reading-frame shifts.

Nitrous acid and hydroxylamine. Nitrous acid was tested many years ago because of its known reactions with proteins. These experiments gave suggestive results, but they were not followed up. More recently, new experiments were stimulated by the fact that nitrous acid in vitro deaminates three of the bases of DNA and RNA: adenine, guanine, and cytosine. Deamination of adenine produces hypoxanthine, whose pairing properties resemble those of guanine, so that the process in vivo might lead to a transition from adenine to guanine (in double-stranded DNA from adenine-thymine to guanine-cytosine). Deamination of guanine yields xanthine, whose pairing properties resemble those of guanine, so that no mutational change is expected. Finally, deamination of cytosine yields uracil. In RNA, this change

results in an immediate change of code; in DNA it might be expected to do so after replication, because uracil tends to pair with adenine rather than with guanine. Genetic experiments, first on tobacco mosaic virus (containing RNA), then on bacteria and fungi, showed that nitrous acid is an excellent mutagen, provided the *p*H is kept low. The results with tobacco mosaic virus were of special importance because of their contribution to the decoding of the nucleotide base triplets. Coding triplets were derived from the correlation between the chemically predicted base changes and the observed amino acid changes in the mutant proteins, and they agreed remarkably well with those derived in other ways. In addition to base changes, nitrous acid can produce deletions; this may be related to its ability to form crosslinks between the strands of DNA. Nitrous acid was the first agent to cause mutations when applied to free nucleic acid in vitro, either to the RNA of tobacco mosaic virus or to the DNA of bacteria. The induced mutations could be detected because RNA, like the virus from which it has been isolated, infects tobacco leaves, while bacterial DNA can enter other bacterial cells and become integrated into their chromosomes by a process called transformation.

Hydroxylamine, too, can produce mutations in free bacterial DNA. In vitro, it reacts preferentially with cytosine. The *p*H dependence of this reaction parallels that of mutation frequency in extracellularly treated bacteriophage, suggesting that mutations are produced exclusively by reaction with cytosine, presumably followed by a transition from guanine-cytosine to adenine-thymine. This high chemical specificity has made hydroxylamine into a standard mutagen for deriving the chemical specificities of others. Thus, the fact that 5-bromouracil tends to revert the same mutations that are also reverted by hydroxylamine, while 2-aminopurine rarely does so, is the mainstay for the assumption that the former preferentially changes guanine-cytosine into adenine-thymine; the latter, changes adenine-thymine into guanine-cytosine. It is, however, important to realize that this simple picture is valid only for bacteriophage treated outside the bacterial cell. Applied to phage inside its host cell, hydroxylamine produces mutations by acting on all four bases (*2*). This should be taken as a warning against the frequently made assumption that the genes of cellular organisms, which are unavoidably treated inside their "host cell," will necessarily undergo the same reactions with mutagens as does DNA treated in the test tube or bacteriophage treated outside its host.

Directed Mutation and the Mutation Process

What has become of the hope that chemical mutagens might be a tool for producing directed mutations by reacting selectively with certain genes? Since the vast majority of randomly produced mutations are harmful, means for directing mutation into desirable channels would be of immense importance for "mutation breeding." At a time when we conceived of genes as complex nucleoprotein molecules with highly specific overall composition and structure, the hope of finding selectively acting chemicals did not seem unreasonable. Nowadays, when we conceive of genes as linear sequences made up of the same four nucleotides, it seems a forlorn hope. It is true that, in the nucleotide bases, mutations do not occur at random but tend to attack preferential sites, whose positions within a given gene depend on the mutagen. The nature of these "hot spots" is obscure. They cannot be due to specific reactions between a given chemical and one out of four nucleotides, every one of which occurs many times within the gene. They have been tentatively attributed to the effect of neighboring nucleotides on the chemical reactivity of a given base. Since, however, the same short nucleotide sequence that produces a hot spot in one gene is likely to recur in many or most other genes, the phenomenon of hot spots, however interesting in its own right, holds out no promise for the production of directed mutations.

Does this mean that we have to give up all hope of achieving at least a modicum of control over the direction of mutation? I do not think it does. This defeatist conclusion could be drawn only if numbers and types of mutation were determined wholly by the reaction between DNA and mutagen, and this is most certainly not so. It is true that a change in the information carried by DNA is a necessary condition for mutation, but it is not a sufficient one. It is preceded as well as followed by secondary steps, and these act as so many sieves determining whether a change in DNA will take place and whether, once it has taken place, it will give rise to an observable mutation, that is, to a population of cells with a new type of genetic information. It is at the level of these sieves that specificities may be expected, and have already been found (*13*).

The sieves that precede the reaction between mutagen and DNA are concerned with the chemical changes that a mutagen may undergo before reaching the gene, and with the accessibility of the gene. They are influenced by strain, cell type, and metabolic state and probably depend on the degree of coiling of chromosomes and chromosome regions and on the amount and type of the chromosomal components other than DNA. A possibility that has been considered but not yet adequately tested is that active genes are more accessible to certain mutagens than repressed ones. If this were true, it would offer a means of selecting genes for mutagen attack. There is also the more remote possibility that one might maneuver a mutagenic group, say a chloroethyl or ethylene imine group, into close neighborhood of repressed genes by attaching it to a repressor substance. This should give a complementary response pattern to the above, and the pattern should be similar if similar mutagenic groups were attached to the same type of repressor substance.

The sieves following the reaction between mutagen and gene determine which of the changes in DNA will eventually appear as observable mutations. The first sieve is repair which, in one form or another, seems capable of reversing chemical changes in DNA after treatment with most or all mutagens. Repair processes are presently under intensive study (*14*). They involve enzymes, and their efficiency depends on the time available between the production of the chemical change and the next replication of DNA. The latter factor certainly is influenced by mutagenic treatment, the former probably is so in many cases. This is one of several ways in which a mutagen may act as a screening agent for the potential mutations that it has itself induced. There has as yet been no systematic search for repair processes

that are specific to certain genes; research on *Neurospora* and bacteria suggests that they may exist.

Once the mutational change in DNA has become stabilized it has to be transcribed by messenger RNA. This particular sieve may be clogged by substances like fluorouracil, which specifically inhibit the manifestation of certain mutational changes (*15*). Then follows a series of sieves concerned with translation; they include all processes by which a mutated cell is formed under the influence of the new messenger RNA. When the mutation leads to the formation of a new enzyme or other protein, ribosomes and transfer RNA are involved; other steps have to be carried through for the formation of a new type of transfer, or ribosomal, RNA. Again, many factors including the mutagen itself may affect the action of these sieves, and some may do so specifically. Thus, streptomycin and neomycin specifically prevent the manifestation of certain types of mutation by misdirecting translation on the ribosomes (*16*). Finally, the mutated cell has to grow into a population of mutant cells. In mutation experiments on microorganisms, this last sieve has often to be passed in competition with a vast majority of nonmutant cells. Moreover, in the most frequently used type of experiment, the screening for mutations that render the mutant cells resistant to conditions by which the nonmutant ones are killed, there is a race between death and the completion of the mutation process, and all sieves have to be passed within a strict time limit.

For many years, the remarkable successes in the molecular analysis of mutagenesis at the DNA level have channeled most mutation research into this line of approach, and a study of mutation as biological rather than chemical process has hardly begun. There are, however, already a fair number of cases which point to the importance of cellular events for making or marring potential mutations. In some of them, the observed effects were directed ones in the sense that the proportion between different types of mutation could be profoundly altered by conditions such as temperature, *p*H, visible light, plating medium, type and dose of mutagen, treatment before or after with mutagenic or nonmutagenic chemicals, or the introduction of new genes into the genetic background of those to be screened for mutation (*17*). In my opinion, it is along these lines that progress toward a direction of mutation is to be expected. Moreover, this approach will help us to understand one of the most interesting biological processes, by which a change in the information carried in DNA leads to the emergence of a population of cells with altered hereditary properties.

Summary

Since the discovery of the first potent mutagens over 20 years ago, progress in mutation research has been rapid. Many new mutagens, belonging to a variety of chemical classes, have been discovered, and for some of them the reaction with DNA in vitro has been established. It seems that the findings of these chemical investigations usually also apply to viruses which are treated outside the cell. This has made chemical mutagens into an important tool for the analysis of the genetic code. When DNA is treated inside the cell, its reactions would not be expected to be always identical with those observed in vitro; in one case they have, indeed, been found to be different.

A chemical change in DNA is a necessary but not a sufficient condition for the production of an observable mutation. Intercalated between this primary change and the emergence of a population of cells with a new hereditary property is a whole series of cellular events, including a variety of repair mechanisms, transcription and translation of the new information, and growth of the mutant cell into a mutant population, often in the face of severe competition from nonmutant cells. These events act as so many sieves that screen out a proportion of potential mutations for realization. The study of mutation as cellular process has hardly begun, but it already shows the importance of these cellular events for the numbers and types of mutation produced. In addition to its theoretical interest, this approach is the only one likely to lead to the production of directed mutations for "mutation breeding."

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