FINAL REPORT OF TWENTY-TWO YEARS OF AEC SUPPORT
1953 - 1975

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Summary of Accomplishments

Generous support by the AEC since 1953 has played an important role in the research and training programs of this investigator and of the Department of Biochemistry in this Institution. The support has permitted (a) the partial clarification of many aspects of genetic transformation in H. influenzae; (b) control of the many facets of the metabolic and genetic pathways of this organism; development of a totally defined medium for both growth and for competence. (c) The discovery, analysis of kinetics and purification of the photo-reactivating enzyme. The chromophor of this enzyme has been isolated and its structure is currently under study. (d) The formation of new genetic units ("heterozygotes") during renaturation of mixtures of transforming DNA and finally, (e) the generation of mutations in free wild type DNA by nitrous acid or by thymine hydroperoxide. The latter mutagen is formed in DNA exposed to ionizing radiation and explains the mutations induced in genetic material by ionizing radiation.

In addition to these research results, graduate students, post-doctorates and faculty have been trained in the molecular mechanisms of genetic processes through the study of transformation.
I. Historical Background

The interest of this investigator in self replicating units for the five years preceding 1953 when AEC support was begun had been focused on the nature of bacterial viruses, but the realization that viruses were more complex biochemically, being made up of both protein and nucleic acid, than the transforming "principle" which is only DNA, led to a shift in the simpler system.

Many laboratories were studying the pneumococcal system of transformation but its biochemistry was virtually unstudied whereas *E. coli* was the best understood of all bacteria. Transformation in *E. coli* had begun by Boivin in France (1947) but he lost the strain that had transformed. The AEC agreed to support my request to investigate transformation in the common strains of *E. coli* and Sol H. Goodgal, a recent graduate in genetics agreed to undertake this study with me. After a year of negative results we turned to the *H. influenzae* system, discovered only a few years earlier by Hattie Alexander and Grace Leidy at Columbia's College of Physicians and Surgeons. *H. influenzae* was chosen instead of the pneumococcal system because lysates of the latter contained damaging nucleases which *H. influenzae* did not. In addition there were fewer laboratories working on *H. influenzae* and so there would be less competition for students investigating this involved system.

When we began our studies, transformation frequencies were about 1 in 10,000. Today it is frequently as high as 1 in 20 and there are reasons for expecting this to be the maximum for unfractionated DNA.
I. Research Accomplishments

The material to follow is organized according to subject matter and not chronologically. Each topic is developed as far as our group was concerned. Reference to the work of others has been kept to a minimum and then only when necessary to indicate how our work relates to the larger picture. However, since in the early years support to the writer included support for associates - the results obtained by Drs. Goodgal, Rupert, Harm, Scocca and others during their stay here have been included.

A. Competence

Competence, as used in the present instance, is the capacity of cells to take in exogenous DNA. This capacity is the unique property in genetic transformation for the genetic processes are probably shared with other genetic processes of bacteria such as conjugation, transduction, and lysogenic viral infections. In \textit{H. influenzae} competence is a highly specific property for only homologous DNA or closely related para\textit{influenzae} DNA is recognized and taken up by competent \textit{H. influenzae} (Scocca \textit{et al.} 1973). Very little is understood in terms of molecular mechanisms about either competence development or how competent cells take up DNA. What is known for \textit{H. influenzae} will now be summarized.

1. The General Nature of Competence

Most transformable strains of bacteria are not competent as they are grown. Competence develops in a late phase of growth, i.e. as cells approach the stationary phase, or upon being transferred to a "step down" medium which directs their metabolic direction from growth and toward competence. In \textit{H. influenzae} competence develops in two phases; the growth phase and the competence development phase.
In the latter, (Phase II), growth or replication is inhibited.

2. Nontransferable Nature of Competence in H. influenzae

Contrary to the case of competence development in H. streptococci (Pakula 1963) and in pneumococci (Tomasz 1964) H. influenzae does not appear to secrete a transmissible factor which will accelerate the development of competence in noncompetent cells. In the other organisms, large molecular weight units have been found in filtrates of competent cultures which rapidly convert noncompetent cells into competent ones. Early studies (Goodgal and Herriott 1961) and later ones (Spencer & Herriott 1965) failed to reveal a transferable factor in filtrates of competent H. influenzae. This result was given strong support by kinetic studies (Herriott, Meyer & Vogt 1970). If a transferable factor existed, then the rate of competence development might be expected to be a function of the cell concentration. On the other hand, if competence development is totally an internal process, the rate of development should be independent of the cell concentration. Experiments in which cells at $10^7$, $10^8$ and $10^9$ per ml were allowed to develop competence, showed the same rate of development and hence eliminated any possibility of a transferable factor of the type found by Pakula and Tomasz. Since, as will be noted next, specific nutrients are required for competence development so conditions may be found in which filtrates from competent cells will accelerate development of noncompetent cells, but no such case has been observed in this laboratory.

3. Nutritional Requirements

It was not long after work with this organism was begun that we realized that if any progress in understanding of the genetic and metabolic processes was to be made, a defined growth and competence medium would be required, yet none had been reported before. A
growth medium was developed (Talmadge and Herriott 1960) and later improved, (Herriott, Meyer, Vogt and Modan 1970). During the interim it was found (Ranhand and Herriott 1966) that H. influenzae require inosine and lactate in the growth cycle (Phase I) if they were to develop competence in Phase II. Cells grow in the absence of inosine of lactate but they did not become competent when put through Phase II treatment.

The nutritional requirements of Phase II competence development were also examined and minimal (Spencer and Herriott 1965) and maximal requirements (Herriott, Meyer and Vogt 1970) were determined. It was obvious from all studies that a change in metabolic reactions was needed to develop competence. In a complex commercial medium, decreased aeration promoted competence development (Goodgal & Herriott 1961). When the defined minimal or maximal media were used, aeration was needed but in these instances growth was inhibited by the absence of factors essential for growth such as DPN, nucleosides, and sugars. Fumaric acid was included to supply the energy.

A few amino acids are essential for Phase II competence development. Arginine or citrulline, cystine and glutamic acid are the most important (Spencer & Herriott 1965). The aspartic acid of M-II can be replaced by 0.24M salt but as will be noted later the lag period seen in M-II is reduced sharply when a casein hydrolysate is added. That the observed lag in M-II is needed for adaptation to amino acid synthesis is suggested by the block of competence development in M-II if as little as 1 µg/ml of valine is added. This inhibition is quickly released by adding leucine or isoleucine. It has been concluded, as in all other transformation systems, that competence development requires protein synthesis. Scocca, in this laboratory,
is currently examining the competence-specific proteins found in the two membranes of this organism.

4. Measures of Competence

The level of competence of a culture may be measured in several ways. The simplest method and perhaps the most reliable, if less informative, is by determining the frequency of transformation when an excess of transforming DNA is used. In general this leads to a top figure of 5-10 per cent of the culture transformed to a given marker. That most of the other cells are competent but have taken up segments of the same DNA not carrying the marker which blocks marker DNA from entering was noted by Goodgal and Herriott (1957). In support of this explanation it was found that when DNA from cells containing a number of markers was used to transform cells each marker transformed to the maximum and as a result 30-40 per cent of the cells had been transformed to one or another of the markers.

In 1961 Goodgal and Herriott introduced a different method for evaluating competence based on the use of a mixture of two unlinked (independent) markers. Each marker-DNA could even be prepared from different cell cultures. In this method the frequency of double transformants equals the product of the frequencies of individual marker transformants only when all the cells are fully competent. When the culture is a mixture of competent and non-competent cells the observed frequency of double transformants exceeds the predicted. This follows from the expression:

\[
\frac{[T_1]}{f[C]} \times \frac{[T_2]}{f[C]} = \frac{[T_1T_2]}{f[C]} \quad \text{or} \quad \frac{[T_1]}{f[C]} \times \frac{[T_2]}{f[C]} = \frac{[T_1T_2]}{f[C]}
\]
Where \([T_1]\), \([T_2]\) and \([T_1T_2]\) are the number of individual and doubly transformed cells per unit volume, \([C]\) is the number of viable cells per unit volume and \(\phi\) is the fraction of cells that are competent.

The early results were compatible with this concept. Later work from this (Spencer and Herriott 1965) and other laboratories (Cahn and Fox 1968) (Hadden and Nester 1968) (Javor and Tomasz 1968) and (Porter and Guild 1969) did not fit with the initial proposal. Cahn and Fox (1968), pointed out an omission in the original assumptions which required a correction factor of 2 in the equation.

\[
\frac{[T_1] \times [T_2]}{2C} = \phi [T_1T_2]
\]

This correction brings most but not all cases into line. Spencer and Herriott had shown that cells develop competence gradually and there is a stage when they can take up only one piece of DNA and so could not be doubly transformed. This may account for deviations under some circumstances. A review of this subject contains more details on these points (Herriott, Meyer and Vogt 1970).

5. Kinetics of Competence Development

Competence development in minimal (M-II) medium (Spencer and Herriott 1965) proceeds with a 30-40 minute lag period after which there is a thousand fold rise in competence which follows a log-logarithmic course. The addition of leucine to the medium shortened the lag period and addition of a casein hydrolysate shortened it to just a few minutes. Other workers using other organisms have also noted the logarithmic rise but none have suggested an explanation for it.
B. **Transforming DNA**

1. **Purification:** Purification of transforming DNA has followed the procedure of Marmur (1961) based on an earlier procedure of Sevag which used chloroform to denature and emulsify protein without damaging the DNA. RNA is eliminated by treatment with RNAase and by subsequent precipitation of the DNA with isopropanol under specific ionic conditions which leaves RNA soluble.

2. **Molecular size of Isolated DNA**

   With only a minimum of care, Goodgal and Herriott (1957) prepared transforming DNA of $15 \times 10^6$ daltons -- the largest DNA at that time. This figure was obtained by direct measurement of both sedimentation and diffusion constants. Since then DNA of $30-100 \times 10^6$ daltons is readily obtained by taking precautions to open the cells in sucrose solution, avoid shearing stresses, and avoid introduction of nucleases. *H. influenzae* DNA of $400 \times 10^6$ was obtained by Berns (1965) by the use of redistilled phenol to extract the protein.

3. **Radiolabelling of DNA**

   a. **P³²:** Using either the defined growth medium (Herriott, Meyer, Vogt and Modan 1970) with reduced phosphate or brain heart infusion with its inorganic phosphate reduced by precipitation with ammonia and magnesium chloride, the nucleic acids of *H. influenzae* can be labelled with carrier-free $P^{32}$ phosphate. From such cells the DNA is isolated by the method noted in B-1.

   b. **Tritium (³H) or Carbon¹⁴ (¹⁴C)**

   Labelling of *H. influenzae* DNA with $³H$ or $¹⁴C$ labelled thymidine has been accomplished by growing the cells in the defined medium M-IC-citrulline to which is added labelled thymidine and some ribo- or deoxyribonucleosides (Carmody & Herriott 1970). In
MIC-Citrulline the arginine and uracil of MIC are replaced by citrulline. The nucleosides must be added to suppress the nucleosidase on the surface of the organism which will otherwise break down the thymidine -- and thymine is not taken up. DNA with greater than 50% of the thymine labelled has been prepared.

c. 5-Bromo deoxyuridine (5BUDR)

Incorporation of 5-bromodeoxyuridine into DNA with or without radiolabel has been accomplished by a slight modification of the Carmody method, (Roberts 1972). Ribo or deoxyribonucleosides must be added because of the nucleosidase. In addition, low levels (0.2-0.5 μg/ml) of fluorodeoxyuridine (FUDR) aid incorporation by inhibiting the endogenous synthesis of thymidine. Levels approaching 50% replacement of thymine with bromouracil were obtained when the medium level of 5BUDR was 20 μg/ml. DNA labelled with 5BUDR is highly sensitive to ultraviolet radiation. It is also readily separable from ordinary DNA due to its higher buoyant density.

4. Physical distance between genetic markers

A method was developed (J.W. Bendler Ph.D. Thesis 1968) which permits an estimation of the physical distance between genetic markers. This method depends on a correlation between the change in number of transformants to both markers as the sedimentation constant falls from shear degradation. The minimum distance between markers obtained by extrapolation of values observed during shear degradation is sought. An expression describing this quantitatively is:

\[ f[DNA_{AB}] = 1 - \frac{M_{AB}}{M} \]

where
\[ f[\text{DNA}_{AB}] = \text{the fraction of the doubly marked transformants remaining after shearing; and M is the mass of the transforming DNA. } M_{AB} \text{ is the mass of the DNA that included the A and B markers.} \]

With this method a physical map of the \textit{H. influenzae} genome was prepared.

C. **Transformation Process**

1. **Uptake of DNA by Competent Cells**

Genetic transformation in bacteria can be described by an equation such as the following:

\[
\text{B}^* + n\text{D} \rightarrow \text{B}^* \cdot \text{Dn} \rightarrow \text{B-Dn}
\]

Where \( B^* \) are competent bacteria \( n = \) number of pieces of D, DNA

The first interaction is a random collision and the rate of forward direction is a function of concentration of components, pH salt concentration, temperature, and specificity of the DNA. The best conditions for interaction (Barnhart & Herriott 1963) are pH 7., 0.1M NaCl, 37°C and homologous DNA. The last was clearly established by Scocca \textit{et al.} (1974). This first interaction is reversible for the DNA can be washed off with 0.3N NaCl and the DNA is digestible by DNAase.

The first step is rapidly followed by the second if conditions are optimal. The DNA is rendered non-digestible by DNAase and it cannot be removed by washing. It is presumed to be taken into the cells but this may mean only into a membrane. This second step requires energy and was found to be blocked by such "uncouplers" of energy reactions as dinitrophenol, azide or iodoacetate (Barnhart and Herriott 1963).
There is no eclipse phase of the transforming DNA in *H. influenzae* as has been found for pneumococcal system (Fox 1960, Lacks 1962). A few minutes after cells are in contact with transforming DNA they can be washed free of external DNA and that DNA taken in, can be recovered in lysates (Goodgal and Herriott 1957).

2. **Genetic Linking**

Soon after uptake of DNA the introduced marker becomes associated irreversibly with the genome of the recipient cell and at a particular position on that genome. This was demonstrated by Voll and Goodgal (1961) with genetic markers. Later Steinhart and Herriott (1968) using radiolabelled materials confirmed the linkage and showed that a piece equivalent to half the donor DNA is liberated as small molecular weight fragments from the recipient cell genome soon after physical linkage takes place. The work of Notani and Goodgal (1966) indicated that a single strand of donor DNA becomes incorporated in the recipient and it seems plausible that the liberated recipient DNA seen by Steinhart and Herriott (1968) is the displaced single strand.

Attempts were made to identify an intermediate recombinant consisting of an alkali labile union of donor and recipient markers which could be made alkali stable by *in vitro* enzymatic (ligase) action. One hundredth molar cyanide was used to block the sealing process with ligase like enzymes. This project failed, however, and perhaps because a higher level of cyanide was not tested. Tomizawa (1967) found that intermediate recombinants of bacteria phage were observed using five one hundredths molar cyanide in *E. coli*.

Some preliminary studies (Alan Jacob) suggested that caffeine may inhibit the final stage of recombination.
3. Kinetics and Stoichiometry of Transformation

a. Kinetics

The interaction of DNA and competent cells follows second order kinetics. The rate of interaction is dependent on the concentration of each of these components. Fig. 1 contains a graph illustrating the effect of DNA concentration on the initial rate of transformation to streptomycin resistance when the competent cell concentration is constant at 1 x 10⁸/ml. A second order constant $K = 0.03$ was obtained. It has the dimension of transformations per minute per microgram of DNA and per cell (T/min/ug DNA/cell). The linear course of this curve at 45° on a log-log plot indicates that a single unit of DNA is responsible for the transformation event. On this same graph is a curve showing the number of transformants to streptomycin resistance after 30 minutes interaction with 1 x 10⁸/ml competent cells. The curve parallels the initial rate curve.

In Fig. 2 is a set of curves showing the variation in number of transformants to streptomycin resistance obtained when different cell concentrations are used - holding the DNA concentration constant. Each curve in Figure 2 is at a different DNA concentration as noted. In every instance interaction was allowed for 30 minutes at 37°C.

b. Stoichiometry: Efficiency of Transformation

One of the striking features of transformation is the small quantity of DNA needed to produce an observable effect. It will soon become clear that in fact the phenomenon is at the molecular or single segment level of DNA.

When more than 2 or 3 x 10⁸ fully competent H. influenzae per ml are mixed with 1 x 10⁻³ µg/ml of DNA, virtually all of the DNA is taken up by the cells and the DNA becomes incorporated into the
cells. At these concentrations of cells and DNA, very few cells have contacted or taken up more than one segment of DNA for the average distribution is the order 1 piece of DNA for 10 cells. Under these conditions 1-2 x 10^5 transformants is obtained from 1 x 10^{-3} \mu g/ml of DNA or 1-2 x 10^8 per \mu g of DNA. If novobiocin or erythomycin resistant markers are used the figure is more like 2-3 x 10^8 transformants/\mu g DNA. Since 1 \mu g DNA is the content of 5 x 10^8 donor cells the overall recovery from donor cell back to transformant is \frac{2-3 x 10^8}{5 x 10^8} or \sim 0.5. This indicates an overall efficiency of nearly 50 percent if the donor cell has but one copy of each marker being assayed. Staining techniques do suggest that H. influenzae may have two nuclei (Berns 1965). If this is true then the efficiency is 25% - still a remarkable figure considering the shearing forces during lysis the DNA undergoes and the possible damage to it during purification, uptake and incorporation by the cells.

D. Renaturation of DNA and Heterozygote Formation

Soon after renaturation of DNA was discovered by Marmur and Lane 1960, the notion was tested that hybrids or heterozygotes could be formed by annealing mixtures of denatured DNAs carrying linked markers, i.e. markers on separate pieces of DNA but closely linked when in the cell genome. Such units were found and they behaved as single physical units as shown by dilution studies (Herriott 1961). The rate of formation of these units followed second order kinetics very precisely.

The nature of these units was of considerable importance, for if the separate markers were on opposite (complementary) strands of the annealed unit as might be expected, it raised problems with the current dogma on how messenger RNA was complementary to only one strand of DNA. Experiments were designed to learn if the heterozygotes fit some of the
other possible models. Since there was no difference in expression time of an annealed unit carrying two markers and the homozygote carrying the same two markers it seemed unlikely that the marker could be on opposite strands in the annealed unit (Herriott 1965). This left the possibility that both marker DNAs were annealed to a common and complementary strand. This was given strong support by finding a physical unit carrying 3 markers which had been formed by annealing a mixture of 3 preparations of DNA each of which had a different but closely linked marker. Such a result fits best with the model pictured below

in which a marked or unmarked DNA pairs with the corresponding marker DNAs $a^-$, $b^-$ & $c^-$. No other model involving 3 strands of DNA made any sense.

The model pictured above resolves the dilemma about messenger RNA copying but one strand. It also indicates one way new markers get introduced into genomes.

E. Radiation, Photoreactivation and Lysogeny

1. UV Inactivation

In contrast to H. influenzae which are destroyed by ultra violet radiation by a typical logarithmic, 1 hit mechanism, free transforming DNA in solution follows a more complex curve (Rupert & Goodgal 1961). It follows an inverse square function of the dose. The expression

$$T = \frac{1}{(1 + CD)^2}$$

where $T$ & $T_0$ are transformations at any time of exposure and at zero time respectively; $C$ is a pro-
portionality constant and $D$ is the dose of radiation. This expression describes the case of the sensitive target decreasing in size as exposure increases. It has been suggested that the influence of the radiation lesion on introduction of the transforming DNA which involves two joining steps may be responsible for the second order effect.

2. Photoreactivation

a. Discovery

In vitro enzymatic photoreactivation was discovered in 1956 as a part of a class exercise. Goodgal and Rupert showed that $H.\ influenzae$ cells failed to recover on exposure to light after u.v. irradiation but that u.v. inactivated transforming DNA recovered when a lysate of $E.\ coli\ B$ was added and the mixture illuminated. (Goodgal, Rupert and Herriott 1957)

b. Kinetics

Rupert (1962) studied the action of the photo-enzyme from yeast on the uv'd transforming DNA. He found that the enzyme binds to the uv'd DNA in the dark but does not bind to unirradiated DNA. Repair and release of the DNA by the enzyme requires light of wavelength maximum at 380nm. The interaction of enzyme and substrate follows Michaelis-Menten kinetics closely with other uv'd DNAs competing for the enzyme. Competition depended on the concentration of lesions in the competing DNA relative to that in the transforming DNA.

Harm and Rupert (1968) developed an ingenious scheme using high intensity - short duration light-flash which repaired only the complexes formed prior to the flash. From such procedures they have obtained valuable information on the photo efficiency of the enzyme and the approximate concentration of enzyme in crude preparations.
c. The Nature of the Substrate (u.v. lesion)

Work in this laboratory began in 1961 to determine the structures produced in DNA by uv radiation. Dr. S.Y. Wang, who had earlier recognized thymine dimers in DNA, began this study but (Wulff & Rupert 1962) and J.K. and R.B. Setlow (1963) produced most of the evidence indicating that it was thymine dimer that was the specific lesion. To bind to the enzyme, the substrate must be an oligo-nucleotide of at least 9 nucleotides and for maximal action an 18 nucleotide unit containing the thymine dimer was required (J.K. Setlow and Bollum 1968). Final proof was supplied by Hayes, Rupert and Associates (1971) showing that a radioactive dimer placed in the center of a decamer polynucleotide was monomerized when the enzyme and light were allowed to act on it.

A crude analysis of the plot of surviving transforming DNA activity against dose of uv, before and after photoreactivation has indicated that 80-90 percent of the uv effect is enzymatically photoreversible (Rupert 1960). At least part of the nonreversible fraction is pyrimidine adducts discovered by Varghese and Wang (1968).

The enzyme - uv'd DNA complex is a perfect example of the Michael's "enzyme-substrate" unit [ES], which in most other systems has a very short half life. In the present case it is very stable and can be kept for days. In fact the enzyme is many times more stable to heat when complexed with the substrate than it is free or with unirradiated DNA (Rupert 1962).

d. Purification of the Yeast Photorepair Enzyme

Work initially with E. coli B as the source of enzyme was dropped in favor of yeast when it was found that the former contained a dialyzable component whereas the latter did not. Rupert carried
out the initial ammonium sulfate fractionation. Students and post-doctoral workers with the writer made progress in the isolation, but were anticipated in publications by others (Muhammed 1966, Minato and Werbin 1971). A method has been developed however which uses some of the features of Minato and Werbin and contains some steps that are unique. We have succeeded in purifying the enzyme at least a hundred thousand fold - a figure predicted would be needed by Harm and Rupert (1968).

As noted by Werbin, the enzyme fluoresces with an incidence maximum at 365 nm and a fluorescence maximum at 470nm. It has a weak absorption at 380nm.

e. **Chromophore Release and Isolation**

The chromophore is released slowly by heating the purified enzyme. Concentration and high pressure electrophoresis separates the main component from small quantities of other substances. Its structure is currently under study. The molecular weight appears to be low.

f. **Interest in the Photoenzyme**

Interest in this enzyme had been centered on its light absorbing property which permitted it to alter carbon-carbon bonds in the substrate that had developed by higher energy radiation. The enzyme takes on more interest since Betsy Sutherland has found it in human cells (1974) and R.B. Setlow has found that uv lesions in the tissues of a fish (Poecelia formosa) lead to malignancy unless they are illuminated with photoreactivating wavelength light (Setlow and Hart 1974).

g. **Lysogeny**

*H. influenzae* cells were found to be lysogenic and inducible by ultraviolet radiation. (Harm & Rupert 1963) They identified
Equally interesting is the observation that the phage in competent H. influenzae are induced by uptake of H. parainfluenzae DNA (J. K. Setlow/I-9-73T, and that explains the earlier observation that H. parainfluenzae DNA kills a large fraction of the recipient H. influenzae cells after uptake (Steinhart & Herriott 1968).

F. Mutagenesis

1. With Nitrous Acid

One of the primary interests the writer had initially in transforming DNA was the potential it had for teaching us how mutagens change DNA. A number of experiments were carried out earlier using transforming DNA with nitrous acid when Litman and Ephrussi-Taylor (1959) reported obtaining mutations with nitrous acid in pneumococcal transforming DNA. Our earlier experiments were repeated with the same negative results. In trying to explain this difference it was recalled that pneumococcal lysates contain nucleases. If nitrous acid acts at breaks in the strands, it was hypothesized, it should be even more effective in transforming DNA than in transforming DNA. Since the strands of DNA separate in vivo during replication at the "growing fork", our experiments suggested that at such a point they might be especially sensitive to nitrous acid and perhaps other mutagens.

Nitrous acid has been seen previously. Since the strands of DNA separate in vivo and Herriott 1962. This marked difference in mutagenesis had not previously been seen. The DNA was renatured at pH 7 and nitrous acid at pH 4.6 and then the DNA was renatured at pH 7. Nitrous acid acts at breaks in the strands if nitrous acid is added to the denatured form with resistance markers following treatment of the denatured form with nitrous acid. A large fraction of the wild type DNA was converted to several antibiotic resistant mutants, as suggested were immediately successful. A large fraction after renaturation.

Tests for mutations could be performed on single stranded (denatured) DNA. If nitrous acid was hypohesited to act on the strands, it should be even more effective if nitrous acid acts at breaks in the strands. It was suggested that pneumococcal lysates contain nucleases not found in H. influenzae lysates. If nitrous acid acts at breaks in the strands, it was hypothesized, it should be even more effective in transforming DNA than in transforming DNA. Since the strands of DNA separate in vivo during replication at the "growing fork", our experiments suggested that at such a point they might be especially sensitive to nitrous acid and perhaps other mutagens. When Litman and Ephrussi-Taylor (1959) reported obtaining mutations with nitrous acid in pneumococcal transforming DNA, our earlier experiments were repeated with the same negative results. In trying to explain this difference it was recalled that pneumococcal lysates contain nucleases.
2. With Thymine Hydroperoxide

Ever since Muller discovered mutagenesis by X-rays in 1928, investigators have sought to offer explanations for the mutations at the molecular level. Even after the radiation is stopped lethal and mutagenic changes continue for a period (Wyss et al. 1948, Watson 1952). These effects have usually been attributed to peroxides but little work has appeared to support it. Hydrogen peroxide is not appreciably mutagenic on transforming DNA but succinyl peroxide is (Freese et al. 1967).

Thymine hydroperoxide was found as a component of DNA after X-irradiation (Weiss and Wheeler 1956) and was found to be mutagenic for E. coli (Chevalier and Luzzati 1960) but no one has paid attention to this latter effect. In attempting to clarify this picture the action of thymine hydroperoxide was allowed to act on transforming DNA - a relatively simple system compared to treatment of whole cells. Synthesized 6-peroxy thymine hydroperoxide was found to produce antibiotic mutations when in the presence of transition metal ions. There was a net increase in mutants formed. The rate of mutagenesis was directly proportional to the thymine hydroperoxide, to the cupric ion (the most effective metal). Chelation of the metal ion with EDTA blocked both the lethal and mutagenic actions of thymine hydroperoxide.

Catalase which blocks the lethal action of hydrogen peroxide had no effect on the action of thymine hydroperoxide. This shows that the thymine hydroperoxide does not act through hydrogen peroxide as an intermediate breakdown product.

These results show clearly that thymine hydroperoxide can act directly on DNA. Since it is produced in situ in cellular DNA (Weiss and Scholes 1956) and may be free in the nucleotide pool -
it is probably able to modify neighboring bases in the DNA. Even the excision of thymine hydroperoxide as reported by Hariharan and Cerutti (1972) does not prevent its action as a mutagen.

Work is in progress to determine which of the several types of mutations this agent produces, i.e. point mutations, deletions or frame shift.

It is also contemplated that we can separate the direct mutagenic effect, if any, of radiation from the indirect or after effect, for the latter appears to be stopped by chelation of any metal ions.

This should decide whether there are multiple mechanisms of mutagenesis by ionizing radiation.
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