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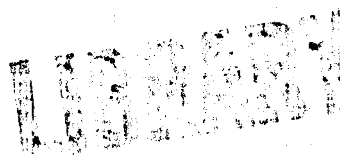

MOLECULAR AND RADIATION GENETICS

Final Report

1969

Work performed at the University of Leiden, Netherlands

Laboratories of Physiological Chemistry and Applied Enzymology and Radiobiology
and Department of Radiation Genetics

Association No. 052-65-1 BIAN

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ABSTRACT

Part A : Research activities of the Laboratories for Physiological Chemistry and Applied Enzymology and Radiobiology were distributed over six subjects :

1. *In vitro* induction of protein synthesis in general, the synthesis of immune antibodies in particular and the effect of radiation thereon;
2. Relation between structure and function of the DNA of viruses including bacterial and animal viruses and the modifying effects of radiation upon them;
3. Genetics of micro-organisms and the effect of radiation (UV and ionizing radiation) on such functions as viability, cell division and mutation of bacteria;
4. The effect of ionizing radiation on DNA;
5. Mechanism of action of enzymes and modification by irradiation;
6. Investigations of mutations that modify the biological activity of enzymes.

Part B : Research activities of the Department of Radiation Genetics were devided between the following three subjects :

1. Mutation studies with Drosophila;
2. Gene function, genetic recombination and replication of DNA of bacteriophage and the effect radiation on these processes;
3. Studies in regulatory mechanisms of somatic cells *in vitro*.

KEYWORDS

PROTEINS
BIOCHEMISTRY
GENETICS
BIOSYNTHESIS
IMMUNITY
ANTIBODIES
METABOLISM
ANIMAL CELLS
VIRUSES
BACTERIA
MICROORGANISMS
DROSOPHILA
BACTERIOPHAGES
DNA
SPERM
CHROMOSOMES
SEX
MITOSIS
MUTATIONS
ENZYMES
RADIATION EFFECTS
ULTRAVIOLET RADIATION
X RADIATION
NEUTRONS
RADIATION DOSES
OXYGEN
NITROGEN
NUCLEOSIDES
GLUCOSE
TRITIUM
GONADS
ELECTRON MICROSCOPY
RADIO AUTOGRAPHY
IN VITRO

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2) Induction of mutations by ionizing radiation
3) Physical chemistry of nucleic acids.

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Part B : Research activities of the Department of Radiation Genetics of the University of Leiden.

Introduction

Organization and Personnel

Research Activities :

Group I: Mutation studies with Drosophila

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16. Electronmicroscopical autoradiography of the *in vitro* incorporation of ^{3}H-5 uridine into pupal testis

Group II: Gene function, genetic recombination and replication of DNA of bacteriophage and the effect of radiation on these processes

1. The biological role of differential glucosylation of T-even bacteriophage DNA
2. The function of the exclusion gene
3. Radiation damage in bacteriophage and its repair

Group III: Studies in regulatory mechanisms of somatic cells in vitro

1. Cell size
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3. Chromosome studies

A. RESEARCH ACTIVITIES OF THE LABORATORIES FOR PHYSIOLOGICAL CHEMISTRY AND APPLIED ENZYMOLOGY AND RADIO-BIOLOGY OF THE UNIVERSITY OF LEIDEN

INTRODUCTION (*)

The present report covers the activities from January 1st 1965 - December 31st 1967 of the Laboratories for Physiological Chemistry and Applied Enzymology and Radiobiology of the University of Leiden, within the framework of the association EURATOM - University of Leiden, No. 052-64-1 BIAN. The activities described in this report were not only performed on the premises of the Leiden University in Leiden, but also on those of the Medical Biological Laboratory of the National Defence Research Organization TNO. The delay in the building program of the Leiden Laboratory was so considerable, that it remained necessary to keep a considerable part of the staff housed in Rijswijk stretching to the utmost the most generous hospitality of the Medical Biological Laboratory of the TNO Organization. (The outlook for the time at which the Leiden Laboratory will become available is not too bright. It seems certain that the new Laboratory shall not be ready for use before 1972 whereas the possibility remains that the event will be shifted to an even later date.) Also the Institute for Radiopathology and Radiation Protection (Leiden) took an active part in the program.

Many scientists from the Netherlands and from abroad visited the laboratories of the association during the period. Many of them gave lectures, seminars or lessons. A few spent longer periods (e.g. supported by fellowships) at the laboratories. Equally many scientists of the association travelled in their own country and abroad. Many gave lectures and a few stayed for a longer period abroad, usually in the United States supported by fellowships. It does not seem useful to give a detailed account of all these travels and lectures. Here it is only noted that scientists of the association played an important part in the International Symposium on the "Regulatory Mechanisms in Nucleic Acid- and Protein-Biosynthesis" at Lunteren, the Netherlands, June 5 - 10 1966, under the chairmanship of Prof. Dr. J.A. Cohen, and in the international EURATOM course on

(*) Manuscript received on 26 February 1969.

"Molecular and Radiation Biology", September 11 - October 20 1967 at Leiden and Rijswijk. This course was very succesful and 16 scientists from countries of the community were admitted. The course started with a week of introductory lectures in the field of molecular and radiation biology presented by staff members of the University and personnel of the Medical Biological Laboratory of the National Defence Research Organization TNO. Later in the course also lectures by distinguished foreign scientists were included. The next four weeks were devoted to laboratory exercises an physical and biological properties of DNA in four sections.

a) Biophysics of viral DNA

b) Biochemistry of DNA

c) Bacterial Genetics

d) Radiation Biology

In the sixth week of the course the class performed experiments connected with the genetics of drosophila in the laboratory of Prof. Dr. F.H. Sobels.

Comprehensive synopses of the practical courses and also of the lectures and seminars were issued by the Biological Service (EURATOM) of the General Directorate of Research and Training of the Commission of the European Community.

Before the onset of the contract period the groups involved had been active in the field of molecular biology for some time. Although the term "Molecular Biology" may be equivocal it is used here in the narrower sense and applied to work on proteins and nucleic acids and specially to studies of gene structure, replication and expression which give information about the molecular details of these processes. The field is then centered around the well-known scheme DNA ⟶ m-RNA ⟶ protein (with a circular arrow over DNA). In the biological sense it represents gene replication and expression, in the biochemical sense structure and synthesis of nucleic acids and proteins. It has been the purpose of the association to elaborate this field in particular in view of its relevance to radiobiology.

The present work constitutes of a partial realization of this intention. It will be necessary that the present program will be extented in the future so that it will be possible to fully reap the fruits of a number of interesting data and ideas which have emanated from the present work, so that the present investment will not be lost.

PERSONNEL

The whole operation stood under the general direction of Prof.Dr. J.A. Cohen, director of the Laboratories for Physiological Chemistry, Applied Enzymology and Radiobiology of the University of Leiden and the Medical Biological Laboratory of the National Defence Research Organization TNO, Rijswijk. The research activities reported were distributed over 6 sub-groups consisting of a number of scientists from the laboratories mentioned who devoted at least during a significant period a considerable part of their time to them.

The scientists mentioned in the following table are all those who at one time or another were associated with the respective projects. Many changes and replacements took place during the period among both senior and junior scientists. In the table the names have been listed independently of the period the scientists were involved in work on the relevant subject. In this connection we wish to limit ourselves to mentioning that during the last year of the contract period the leadership of group IV passed from Prof.Dr. Joh. Blok, who was appointed professor of biophysics of the Free University of Amsterdam, to Ir. J.F. Bleichrodt and that the appointment of Prof.Dr. H.S. Jansz to the chair of biochemistry of the University of Utrecht made it necessary to replace him by Dr. W.J.H.M. Möller and Dr. P.H. Pouwels in groups I and II respectively.

General director: Prof.Dr. J.A. Cohen

Group I - In vitro induction of protein synthesis in general, the synthesis of immune antibodies in particular and the effect of radiation thereon

Senior scientists: Prof.Dr. J.A. Cohen
Prof.Dr. H.S. Jansz
Dr. W.J.H.M. Möller

Scientists: Dr. B.N. Bachra
Ir. P.H.M. Lohman
Drs. S.O. Warnaar

Group II: Relation between structure and function of the DNA of viruses including bacterial and animal viruses and the modifying effects of radiation upon them

Senior scientists: Prof.Dr. J.A. Cohen
Prof.Dr. H.S. Jansz
Dr. P.H. Pouwels

Scientists: Drs. A.J. van der Eb
Dr. G. Veldhuisen

Group III: Genetics of micro-organisms and the effect of radiation (UV and ionizing radiation) on such functions as viability, cell division and mutation of bacteria

Senior scientist: Prof.Dr. A. Rörsch

Scientists: Miss Drs. I.E. Mattern
Drs. A. Hout
Dr. Ir. P. van de Putte
Ir. C.A. van Sluis
Drs. W.F. Stevens
Drs. H. Zwenk

Group IV: The effect of ionizing radiation on DNA

1) the biological significance of alterations in DNA induced by ionizing radiation
2) induction of mutations by ionizing radiation
3) physical chemistry of nucleic acids

Senior scientists: Prof.Dr. Joh. Blok
Ir. J.F. Bleichrodt
Dr. J.B.Th. Aten

Scientists: Drs. G.M. van der Ent
Ir. J.P. Goedbloed
Drs. J.J. van Hemmen
Drs. A.J. Hoff
Ir. L.H. Luthjens
Drs. G.P. van der Schans
Dr. M.G. Stern

Group V: Mechanism of action of enzymes and modifications by radiation

Senior scientist: Dr. R.A. Oosterbaan

Scientists: Dr. F. Berends

Prof.Dr. H.S. Jansz

Group VI: Investigation of mutations that modify the biological activity of enzymes

Senior scientist: Dr. A. de Waard

Scientists: Drs. H. van Ormondt

Drs. F.A.J. de Vries

RESEARCH ACTIVITIES

Group I - In vitro induction of protein synthesis in general, the synthesis of immune antibodies in particular and the effect of radiation thereon

Protein synthesis can be conceived in two major consequent steps. First the transcription of the information encompassed in DNA into RNA and next the translation of messenger RNA into protein proper. Most of the work during the period was devoted to the first step i.e. the transcription of DNA into RNA. To obtain more knowledge of this process the transcription was studied of the various forms in which the DNA of bacteriophage ΦX174 appears i.e. single strand circular phage DNA, double strand RF (replicative form) circular DNA (both in the form of RF-1 (21 S) and RF-11 (17 S))and super coil DNA obtained by denaturation of RF-1 in alkali. Base analysis and hybridization methods were applied to characterize the product RNA. The point of issue was the several contradictorary reports that had previously appeared in the literature on the question of whether in vitro both strands were copied into RNA or whether transcription was restricted to one strand of the priming DNA only. We decided to investigate this problem using the replicating form (RF) of DNA of phage ΦX174 which can be found in E.coli cells infected with this phage. The phage itself contains circular single strand DNA that is converted to a double stranded circular RF upon infection of the E.coli bacterium. This DNA offers certain obvious advantages. Its physical integrity can be rigorously demonstrated by analysing its sedimentation behaviour in the ultracentrifuge. It is biologically active (on spheroplasts) and it can be converted by a single strand break into RF-11 (S =17), a product that is still biologically active but differs from RF-1 in sedimentation behaviour and by its electronmicroscopical picture. The main results can be summarized as follows. The transcription using an intact double stranded RF primer proceeded in vitro from the same strand as in vivo results in plus RNA. A few breaks in the circles of RF-1 result in a primer which produces the same RNA of the plus-strand configuration and ΦX single stranded natural DNA hardly produces anything but minus-strand configuration RNA. It was also unequivocally demonstrated that linear T4-DNA is also transcribed asymmetrically. A useful spin-off of this work was the development of a hybridization technique to demonstrate homology between species of DNA.

The efforts invested in the production in vitro of immunological antibodies were unsuccesful. These attempts were finally abandoned and more attention was devoted to the production in vitro of collagen using a published method for RNA extraction from chicken embryo tissues; undegraded RNA preparations of messenger RNA were obtained. The extracted RNA was shown to stimulate the incorporation of proline into nondialysable polypeptides by preincubated bacterial cell free extracts. The synthesized polypeptides were partially susceptible to collagenase action, while similar polypeptides synthetized under the influence of MS_2 bacteriophage RNA were not. The role of collagen in tissue calcification was also studied as well as methods for isolating and assaying the enzyme protocollagen-proline-hydroxylase. The following results were obtained.

Although reconstituted fibers of acid-soluble rat tail tendon collagen are able to induce the crystallization of apatite in metastable solutions, they are a very inefficient nucleation catalyst. They require a very high degree of metastability and renewal of the buffer solution after one day is mandatory. Our results have shown that the renewal procedure causes a loss of CO_2. The resulting rise in the pH within the fibrous mass will facilitate nucleation catalysis and may further result in spontaneous precipitation within the material.

Collagen obtained from decalcified compact bone is a good catalyst for the nucleation of apatite. As rachitic epiphyseal rat bone cartilage, it does not require renewal of the buffer solution after one day. Our results indicate that the amount of mineral deposited in bone collagen in vitro is regulated by factors similar to those operating in biological calcification.

The effect of inhibitors of calcification of cartilage in vitro, like Mg'', Sr'' and F', is also studied in a model system consisting of bone collagen. The results obtained in such a system are similar to those obtained with the cartilage. The inhibitors affect both the phase of nucleation and that of crystal growth.

The remineralization of bone collagen in vitro is also studied by means of electromicroscopy, electron diffraction and X-ray diffraction in order to obtain information on the factors which cause the parallel orientation of the microcrystallites and the collagen fibers and on the factors which cause a considerable slow-down of crystal growth at a time when the buffer solution is still highly supersaturated (a situation similar to that in vivo).

The structure of the acidic proteins present in 70S ribosomes (E.coli) is under study. The specificity of the interaction between purified ribosomal protein fractions and intact, protein free (16S and 23S) ribosomal RNA is now being investigated.

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Group II - Relation between structure and function of the DNA of viruses including bacterial and animal viruses and the modifying effects of radiation upon them

A method was developed by Veldhuisen and Cohen during the last years to assess the biological activity of DNA isolated from bacteriophage T4; the details were published together with a number of applications. The method involved infection of a host with a mutant phage, missing a particular character, along with DNA isolated from a wild type phage possessing this character resulting in the appearance of progeny phages with wild type characters. To this end it was necessary to remove the cell wall of the bacteria; this was done by converting the cells into spheroplasts by means of the penicillin method. The phage mutant to be transformed was converted, by treatment with urea, into a form that is able to infect spheroplasts (the so-called π-particles). A fraction of the progeny produced after 2 to 3 hours incubation of spheroplasts with π-particles and wild type DNA was composed of transformed (wild type) phages, i.e. the particles possessed one or more markers originally present in the isolated DNA. Improvements in this system were obtained by introducing modifications in the preparation of π-particles and spheroplasts. Eventually the following system was adopted:

E.coli B98, a variant that produced about 3-5 times more transformants than the original E.coli B, was used as a host. To 0.5 ml of a suspension of spheroplasts of E. coli B98, (containing about 8×10^7 spheroplasts/ml) was added 0.1 ml of a suspension of π-particles of the rII-mutant r_{51} (containing 2×10^{10} phage equivalents/ml). After incubation for 10 - 15 minutes at 37°, 0,5 ml T4rII$^+$-DNA (5 mu.g/ml) was added and the mixture was incubated at 37° during 2 - 3 hours; this resulted in the production of about 2×10^9 rII-mutants per ml and about 2×10^5 rII$^+$-phages (transformants) per ml. The efficiency (the number of transformants per DNA molecule) was $6 \times 10^{-5}/W$, in which W represents the mean burst size of the transformants.

In a number of experiments it was shown that the conditions that prevailed in the test system and the concentrations of π-particles and spheroplasts were optimal for the phage transformation process. The yield of the transformants is linear with the DNA concentration up to 10 - 20 mu.g/ml. At higher concentrations a saturation level is attained.

In many aspects the production of phages initiated by mixing π-particles with spheroplasts can be compared with the phage formation observed with intact phages and bacteria (a latent period of about 15 - 20 minutes and a burst size on the average of 240). The adsorption of π-particles on spheroplasts is slow compared with the adsorption of phages on bacteria (60 minutes after the addition of π-particles to spheroplasts as many infections will take place as shortly after the addition).

The low efficiency of the transformation experiments restricted the study to the transfer of genetic markers with low reversion frequencies and to those cases in which the transformants could be selected from a great excess of progeny. rII-mutants and amber-mutants proved satisfactory. By providing the transforming DNA with a marker affecting the plaque morphology (DNA isolated from an rI-mutant) it was possible to study the simultaneous transfer of two markers. In this case we had to distinguish between double transformants (transformants, resulting from the independent transfer of two markers from two different DNA molecules) and cotransformants (transformants resulting from the simultaneous transfer of two markers from one DNA molecule).

After developing the above-mentioned system, a study of the influence of some physico-chemical treatments on the biological properties of phage DNA was made.

The biological activity was not influenced by shearing the molecules to fragments of about 10% of the original length. Smaller fragments of about 1% of the original length showed a much reduced biological activity, if any. It was shown that shearing uncoupled the linkage between markers.

The biological activity of isolated T4-DNA was inactivated by UVirradiation and by nitrous acid treatment. The inactivation curves were compound; there was a relation between the slope of the second part of the curve and the length of the transferred marker. The shape of the inactivation curves could be explained as a special form of "marker rescue", a process based on genetic recombination.

Using the technique mentioned before for DNA-DNA hybridization and other techniques we investigated plant tumors induced by agrobacterium tumefaciens on tobacco plants. The so-called grown gall tumors of the transformed plant cells could be shown to contain bacterial genetic material.

The 31 types of human adeno viruses which have been identified up to now, may be classified in three groups according to the degree of oncogenicity for hamster. Three types are highly oncogenic, causing rapidly growing tumors in almost 100% of inoculated hamsters, five types are weakly oncogenic and most are nog (or very little) oncogenic. Detection of chemical and physical differences between the DNA (genetic material) of highly - and non - carcinogenic adenoviruses, could help to elucidate the mechanism of viral carcinogenesis. In view of the finding that all the members of the tumorigenic papova viruses contain circular molecules, experiments were undertaken to test whether adenovirus DNA's are linear of ring-shaped.

A study of the changes of the sedimentation coefficient of the DNA's of adenovirus types 5 (non-oncogenic), 12 and 18 (both highly oncogenic) as a function of pH and ionic strength indicates that the DNA molecules of all three types have a linear configuration. This indicates that circularity of the DNA and oncogenicity are not simply related.

Studies on heat-denaturation of adenovirus DNA in the presence of formaldehyde indicates that DNA molecules of non-oncogenic type 5 resist heat denaturation. This might be explained by the presence of guanine - and cytosine - rich sequences in the molecules (which have a high denaturation temperature).

The DNA's of oncogenic types 12 and 18 are less heat resistant, compared with type 5, suggesting that only smaller guanine- and cytosine-rich regions are available.

The molecular weight of adenovirus type 5 DNA was calculated using the sedimentation coefficient at DNA concentration =0, $S^{o}_{20,w}$, and the intrinsic viscosity $[\eta]$. It was found to be 23 - 24 x 10^6, contrary to a previously reported value of 5 x 10^6 by Smith. The molecular weights of oncogenic types 12 and 18 DNA are slightly lower ($\pm$ 20 x 10^6).

The ultraviolet sensitivity of double stranded and artificially prepared single-stranded polyoma virus DNA was measured. Both single- and double-stranded DNA molecules are infectious for primary mouse-embryo cells. It was found that double-stranded DNA is more resistant against UV irradiation than single-stranded DNA with respect to the plaque-forming ability (=infectivity) on mouse cells. This result suggests that a repair process, similar to the type observed in micro-organisms,

may operate in polyoma virus infected cells, although other explanations cannot be ruled out at the moment. This would be the first indication of a repair mechanism of UV-damage in mammalian cells.

Photoreactivation (repair of UV-damage by visible light) could not be demonstrated in mouse embryo cells, infected with UV-irradiated polyoma DNA, consistent with the results of others.

Double stranded ΦX-DNA which accumulates after infection with phage ΦX174 in the presence of chloramphenicol consists mainly of twisted circular double stranded DNA with no single strand breaks (component I) and of circular double stranded DNA, in which single strand breaks are present (component II). Component II in M NaCl is denatured with alkali within a sharp pH range, but component I is denatured over a wide pH range (pH 11.2 - 12.4). The midpoints of the transition in M NaCl are pH 11.3 and 11.9 respectively. Component I in 0,1 M phosphate is irreversibly denatured at pH 12.7 - 12.8 or higher, giving rise to a structure which sediments in neutral M NaCl with an $S_{20,w}$ =40. The configuration of denatured double stranded ΦX-DNA has been studied by means of velocity sedimentation, isopycnic centrifugation in CsCl, spectrophotometry and also the reactivity for formaldehyde has been determined. From the results it is concluded that denatured double stranded ΦX-DNA occurs as a highly twisted structure in which all or almost all hydrogen bonds have been disrupted. Denatured double stranded ΦX-DNA does not renature at high temperatures in low or high salt, but is converted spontaneously to native double stranded DNA after introduction of a single strand break by pancreatic DNAase.

The rapid renaturation of denatured double stranded DNA may be explained by assuming that the introduction of a single strand break generates a swivel which allows the unwinding of the tertiary twists. Because of the close proximity of the two complementary strands specific base pairing is facilitated and renaturation may proceed. Once a nucleus of paired bases is formed renaturation will proceed at low temperature.

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Biochim.Biophys.Acta

Group III - Genetics of micro-organisms and the effect of radiation (UV and ionizing radiation) on such functions as viability, cell division and mutation of bacteria

The sensivity of *Escherichia coli* to UV and X-irradiation is determined by several genetic loci. From strains in which one or more of these loci are altered by mutation, information can be obtained on the genetic control of radiation sensivity and the biochemical mechanisms involved.

The primary lesions brought about by ionizing or ultraviolet radiation need not necessarily be lethal because repair processes may occur. It is established that at least some of these processes are of enzymic nature, viz. photoreactivation and dark repair of UV-induced photochemical lesions in DNA. When enzymes are involved, there should be genes that code for such enzymes. When such genes exist, we should be able to alter or to destroy them by mutation. When the cell loses, partially or completely, its ability to repair the primary radiation lesions, its chance to proliferate will be reduced. Such mutations lead to the formation of ''radiation-sensitive mutants''.

So far seven different phenotypes can be distinguished among these mutants:

a) mutants in which the ability to divide after X- or UV-irradiation is reduced (indicated as DIR of FIL strains);
b) UV-sensitive mutants which have lost the ability to repair UV lesions in bacteriophage DNA (HCR strains);
c) UV-sensitive mutants which have not lost the ability to repair UV lesions in bacteriophage DNA (UVR strains);
d) UV-sensitive mutants which are in addition X-ray sensitive (EXR strains);
e) UV-sensitive mutants which are X-ray sensitive and in addition defective in genetic recombination (REC strains);
f) X-ray sensitive mutants, not sensitive to UV irradiation (ROR strains);
g) strains that lack the photoreactivating enzyme (PHR strains)

The phenotype and the genotype of these mutants were studied in detail during the past five years (see figure).

DIR mutants

Certain strains of E.coli, for example E.coli B wild type, show a marked inhibition of cell division after UV- or X-irradiation, without concomitant inhibition of growth. This results in the formation of filamentous cells. From such a strain a mutant can be derived that does not form filaments after irradiation, viz. the well-known mutant E.coli B/r isolated by Witkin in 1946. The nature of the mutation involved is still poorly understood.

In our laboratory a non-filament forming strain was isolated from E.coli B, similar to B/r, which was indicated E.coli $B_{II}fil^-$ (the wild-type strain was denoted fil^+). The synthesis of DNA, RNA and various cell wall components was studied in fil^+ and fil^- strains after irradiation but no significant differences were observed.

Besides the mutations that lead to the formation of non-filament-forming strains from filament-forming ones, the reverse mutations have also been observed. From an E.coli CR 34 strain that does not form filaments after irradiation, filament-forming mutants were derived, denoted as dir^- mutants. From crosses between Hfr strains (dir^+) and F^-dir^- strains it could be deduced that the dir^- mutation is located on the bacterial chromosome between the markers lac and gal which is in the same region as described by Howard-Flanders for a similar mutation denoted as lon^-.

Filamentous growth of E.coli fil^+ and dir^- strains is not only induced by UV- and X-irradiation, but also by ^{32}P decay and several agents like penicillin, novo biocin and crystalviolet. The induced filamentous growth, however, can be counteracted by pantoyl lactone. Moreover the chance to survive a certain UV dose is greatly enhanced for dir^- and fil^+ strains, if they are incubated after irradiation in a medium with pantoyl lactone. Therefore we isolated a number of pantoyl-lactone-requiring mutants but none of them showed filament formation during starvation for this substance.

To investigate whether dir^- mutations occur in the genes that control cell division, a large number of thermosensitive division mutants was isolated (fts^- strains), growing as normal cells at 28° C but in long filaments at 42° C. The chromosomal sites of the mutations involved were studied in 80 independently isolated mutants. All these mutants carried their fts mutation closely linked to

the leu marker except one, the mutation of which was located in the region between his and try. Thus the dir gene that influences cell division after irradiation is not linked to the genes that control cell division as such. Moreover pantoyl lactone which triggers cell division in irradiated fil^{+} or dir^{-} strains has no influence on the filament formation of fts^{-} strains. So the dir gene probably controls only processes which are required to restore normal cell division and is not directly concerned with cell division itself.

HCR mutants

The second type of UV-sensitive strain concerns the HCR mutants which, in comparison with the wild type are less able to propagate UV-irradiated bacteriophages such as T1, T3, lambda etc. This is because of a block in the repair process known as host cell reactivation.

According to the work of Howard;Flanders HCR mutations occur at three different regions on the chromosome, indicated as A, B and C (fig.) . All mutants isolated in our laboratory have a mutation in one of these linkage groups. The dar_5 mutation is located near his (C group) , the dar_1 and dar_6 mutations near gal (B group) , the dar_3 mutation near met_3 (A group) . The hcr_K mutation in the K12S strain isolated by Harm is located near met_3. Initially the HCR mutation syn in E. coli B was thougt to be located in a region between S' and ile. This conclusion was drawn from the results of $F^{+}XF^{-}$ crosses which however appeared to be unreliable. No co-transduction of the syn marker was observed with either S' or ile; with phage P1kc 10% co-transduction with his was found, thus indicating that the syn marker belongs to the C linkage group.

Mutants, carrying mutations at the various sites A, B and C, can be distinguished from each other only by the location of the mutation involved. Phenotypically only minor differences among the strains have been observed and it is questionable whether these differences have a bearing on the site of the mutations. It cannot yet be excluded that similar differences may occur between mutants that carry mutations belonging to the same linkage group.

UVR mutants

Besides the UV-sensitive strains mentioned above, other sensitive mutants were isolated that still show host cell reactivation (hcr^+), in contrast to HCR strains. These mutants, viz. dar_2, dar_4, dar_7, dar_8, dar_9, have high UV sensitivity, in common with the EXR and the REC mutants, but can be distinguished from them by their resistance to X-rays.

Except for host cell reactivation, the UVR mutants are in many aspects similar to HCR mutants. For exemple, the induction patterns for lambda lysogenic derivatives of both types of strains are similar. The phage is induced at a much lower dose than in the wild-type strain and the capacity to propagate the induced phage is strongly reduced after the maximum of induction has been achieved.

The location on the chromosome of the mutations in the UVR strains dar_2, dar_4, dar_7, dar_8 and dar_9 was determined (fig.). All of them except one (dar_2) appeared to belong to one of the linkage groups B or C that were established for HCR mutations . Dar_4 is located near his (C), dar_7, dar_8 and dar_9 near gal (B). It was shown beyond doubt that the mutation dar_2 does not belong to the A linkage group; it is co-transducible with the markers met_2 and ile, but not with the marker met_3, to which the A mutations are linked.

EXR mutants

Not much is known about the properties of X-ray sensitive mutants.

The EXR mutation that occurs in the X-ray sensitive strain E.coli B_{s-2} is located in the A linkage group. It is co-transducible with met_3. A K12 strain with similar properties is the mutant AB 2494 lex^-, isolated by Howard-Flanders, in which the mutation responsible for the increased X-ray sensitivity is also located in the A linkage group.

REC mutants

The properties of four REC mutants (34, 35, 36 and 38), isolated in this laboratory were studied. The F^-rec^- strains are still able to conjugate with Hfr strains and chromosome transfer is not inhibited. Recombination, however, occurs only when the allelic rec^+ marker of the male strain enters the zygote. According to such ''complementation'' experiments it was initially thought that the mutations rec-35

and rec-38 must be located near thr. The rec$^+$ allel of rec-38 was, however, co-transducible with thy. The precise location of the mutations are still under investigation. It is considered likely that rec mutants carry in fact two mutations, one of the type ROR and an UVR like mutation, because X-ray sensitivity and UV-sensitivity can be separated by genetic crosses.

Remarkable differences among the various rec$^-$ strains were observed in the prophage induction patterns of their lambda lysogenic derivatives. The prophage induction pattern of the strains rec-35 and rec-38 resembles that of the wild-type, rec$^+$ strain. The induction pattern of the rec^-_{34} strain resembles most the pattern observed with the HCR and UVR mutants. In the lysogenic rec-36 mutant no prophage induction is observed.

ROR mutants

Several X-ray sensitive mutants were isolated that do not show an increased UV sensitivity. The mutation responsible for the increased X-ray sensibility appeared to be co-transducible with his.

Ultraviolet sensitive mutants of other bacterial species

Radiation sensitive mutants were also derived in this laboratory from Bacillus subtilis, Micrococcus lysodeikticus and Salmonella typhimurium. The various types described for E.coli could also be found with these strains.

Recovery of biological activity of UV-irradiated DNA in vitro

To demonstrate the repair from lethal radiation damage in a cell-free system, a biological active DNA is required as well as a recipient cell in which the acitivity of this DNA can be brought to expression. Jansz demonstrated that the UV-irradiated double-stranded form of bacteriphage ΦX174, the so-called replicative form (RF), is reactivated in spheroplasts from wild-type E.coli but not in spheroplasts of the radiation sensitive mutant E.coli K12S hcr$^-$.

We found that preincubation of UV-irradiated RF-DNA with an enzyme preparation from M. lysodeikticus leads to a marked increase of the biological activity of the DNA in hcr^-_K or dar^-_1 spheroplasts. Because of its low deoxyribonuclease content the enzyme preparation was obtained form M.lysodeikticus. It was found that the reaction leading to the recovery of the biological activity of the irradiated DNA

must be of enzymic nature; treatment of the extract with heat (5 min, 75° C) or trypsine destroys its repair capacity. When UV-irradiated DNA was treated with enzyme, no increase in biological activity could be detected with dar^+ spheroplasts as recipients. With dar^- spheroplasts 52% of the UV lesions were restored. This indicates that the reaction in vitro replaces or parallels the host cell reactivation capacity of the wild type. Repair of X-irradiated RF-DNA and repair of UV-irradiated single-stranded ΦX174 DNA could not be detected in our system. In this respect the repair in vitro resembles the characteristics of the repair, controlled by HCR genes, observed in vivo.

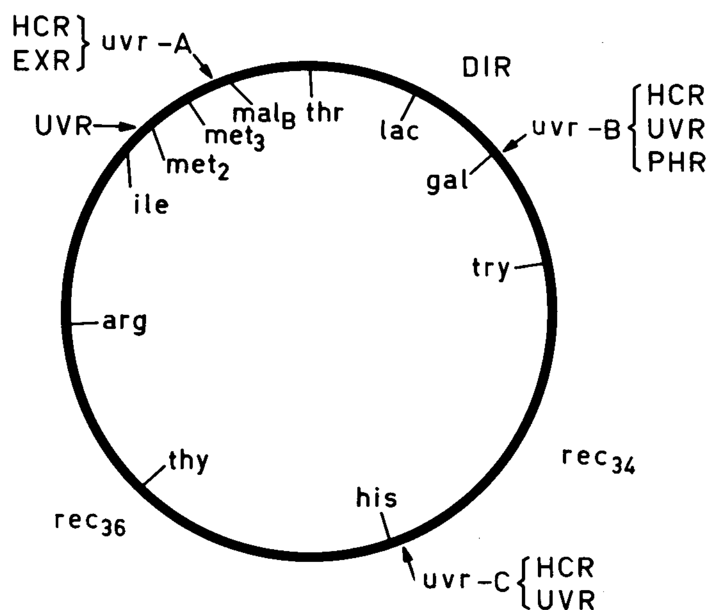
Using 5 to 10 times purified extracts we found no release of nucleotides or large DNA fragments from UV-irradiated RF-DNA. If the HCR spheroplasts lack enzymes that introduce a first clip near photochemically altered nucleotides, and the in vitro reaction is restricted to the introduction of these first clips, no release of nucleotides is to be expected. The spheroplasts may well be able to complete the rest of the repair process. This possibility was investigated in some detail with the RF system.

The RF, isolated from E.coli C, infected with phage ΦX174, consists of two components: one with a sedimentation coefficient of 21S (I), the other of 17 S(II). Component I can be transformed into II by treatment with a small amount of endonuclease. Pouwels and Jansz proved that the introduction of one break in either strand of component I converts it into II. Both components are equally infectious for spheroplasts, so the introduction of a single break does not inactivate the molecule. RF component I was irradiated with ultraviolet light and subsequently treated with the enzyme preparation, which was purified 10 times by DEAE chromatography. The RF-DNA was converted into component II concomitant with the recovery of its biological activity. Thus the enzyme preparation introduces breaks in the UV-irradiated DNA as was expected.

We should mention that other agents that convert component I into II, as for example small amounts of endonuclease I, have no effect on the recovery of biological activity of irradiated RF. Thus the simultaneously observed repair and conversion is specific for UV-irradiated DNA and for enzyme preparations from M. lysodeikticus.

Search for repair from X-ray damage in vitro

So far no recovery from lethal X-ray damage in vitro could be detected. If repair of X-ray damage does occur, our failure might be from the lack of a suitable test system. In this respect we mention that bacteriophages, such as lambda, T3 and ΦX174, show the same X-ray sensitivity when propagated either on wild-type strains or on X-ray-sensitive mutants of the EXR and REC type. It is possible that these strains are deficient in an enzyme, for instance a rejoining enzyme, for which the phage carries its own information, but more experimental work has to be done to elucidate this point.



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Group IV - The effect of ionizing radiation on DNA

1) The biological significance of alteration in DNA induced by ionizing radiation

The purpose of this work is to investigate which primary changes in DNA by ionizing radiation are biologically detrimental and which changes can be modified by environmental conditions. To this end the inactivation of the single stranded, biologically active DNA of the bacteriophage ΦX174 has been investigated.

By means of ultracentrifugation of irradiated DNA it was established that 30 - 50% of the inactivation by irradiation is due to chain breakage. This means that 50 - 70% of the inactivation should be ascribed to attack of free radicals on the purine and pyrimidine bases.

In dilute aqueous solutions under oxygen the radiation products H_2 and H_2O_2 are unreactive towards DNA. From the strong protective action of I^- which scavenges OH radicals it followed that also O_2^-, originating from the reaction of e^-_{aq} with O_2, is unreactive and that the biological activity is destroyed mainly by OH. Under nitrogen, however, reducing radicals contribute for about 50% to inactivation. This was concluded from the observation that I^- protected the DNA as well as O_2, whereas a combination of I^- and O_2 exerts an enormous protection by a factor of about 300.

In concentrated solutions of ΦX174 DNA 95% of the inactivation is due to OH, e^-_{aq} and H being responsible for the remaining 5%. The difference between the contribution of OH in dilute and in concentrated solutions is to be ascribed to the inhomogeneous distribution of nucleotided in the former. In dilute solutions there is a rather large intermolecular space between the molecules and radicals have ample time to react with impurity molecules which are always present in small amounts in DNA preparations. Apparently the OH radicals are scavenged more readily than e^-_{aq} and H. If in concentrated solutions e^-_{aq} is converted to OH by bubbling N_2O through the solution the sensitivity of the DNA is approximately doubled as expected (the yield of OH equals about that of e^-_{aq}). The low inactivating efficiency of e^-_{aq}, mentioned at the beginning of this paragraph, is surprising as it is known that electrons react very rapidly with nucleotides. It must be assumed that e^-_{aq} does not produce a permanent chemical change or that the altered bases are still able to function biologically.

In concentrated solutions of DNA oxygen exerts a slight sensitizing effect. This should probably be explained by reaction of peroxy radicals formed in the DNA with molecules still biologically active. Addition of thymidylic acid or deoxycytidylic acid to the DNA solution increases the sensitivity of the DNA during irradiation under oxygen. Deoxyguanylic and deoxyadenylic acid show no oxygen effect when added to the DNA. From the protective effect of nucleotides on DNA inactivation under N_2 it was concluded that free nucleotides react approximately five times faster with OH radicals than nucleotides bound in ΦX174 DNA

The protection of ΦX174 DNA by cysteamine has been found to be dependent on the pH of the solution, showing a maximum at pH 8. Thioglycol and cysteine protection are nearly independent of pH. It was investigated whether protection of bacteria from irradiation by these compounds was also dependent on pH. Both cysteamine and cysteine show a maximum in protection; cysteamine at pH 7.5 and cysteine at pH 8. pH had no effect on protection by glycerol. Thioglycol could not be investigated because of formation of a preciptate, presumably colleoidal sulfur, during irradiation at high pH in the presence of bacteria.

It was found that irradiation of bacteriphage T4 in buffer yields subviral particles which show biological activity on bacterial spheroplasts. These particles probably arose by indirect action of radiation on the protein coat. In the sucrose gradient they resemble the biologically active subviral particles obtained by treatment of T4 with ureau but not those formed by heat treatment of T4. The latter particles appeared to have a sedimentation coefficient of about 180 S in contrast to the other two, which show an S value of approximately 1000, which is also the value for viable phage. As the low S value of the particles obtained by heat suggests that they are smaller than the phage and therefore might be a biologically active phage fragment, possibly a DNA molecule with some protein molecules holding it in a compact structure, it was attempted to isolate and characterize these particles.

The 180 S particles are sensitive to deoxyribonuclease, to trypsin and to hydrodynamic shear stresses. These properties make isolation very difficult, as the preparations contain large amounts of inactive T4 DNA. Several procedures were tried to get rid of this DNA, but the biological activity of the samples was also lost. Attempts to improve the yield of the biologically active particles by changing the conditions of heating of the phage or by using a pH shock instead of heat had no success. It is estimated that the fraction of the phages yielding particles with biological activity on heating is of the order of 0.005.

Studies of the thermal inactivation of the particles showed that inactivation at temperatures above the melting point of T4DNA is not due to cooperative unwinding of the DNA strands.

2) Induction of mutations by ionizing radiation

It was shown that ionizing radiation induces mutations in bacteriophage ΦX174 and in its purified DNA. In the presence of oxygen the formation of mutants is lower than under nitrogen. This is explained by assuming secondary reactions under oxygen by which mutated DNA molecules are inactivated.

OH radicals are necessary for mutation induction in purified DNA. In the presence of I^- which scavenges the OH no significant production of mutants is observed. However, it was found that the presence of I^- did only reduce the biological activity of the DNA (phage production in spheroplasts) by only about 50%. This implies that reducing radicals are also capable of inactivating DNA.

Radiation induced mutants were found to have a much higher back ground mutation frequency than spontaneous mutants of the same genotype.

The presence of cysteamine and thioglycol increases the frequency of mutants by a factor of about 10 under nitrogen. There is no direct connection between this phenomenon and the strong protection afforded by these compounds. Comparison of mutation induction in the presence of SH compounds and in the presence of deoxyguanylic acid in experiments which were comparable with regard to protection, showed the increased mutation only for the former compounds.

These observations prompted on analogous investigation into the influence of SH compounds on mutation induction in bacteria. Mutation induction in *E.coli* B fil^- $citrul^-$ under nitrogen was not affected by the presence of cysteamine, S, 2-aminoethylisothiourea, thioglycol or cysteine. All compounds afforded protection but for a given survival treated and untreated bacteria displayed the same mutant frequency.

After irradiation of DNA solutions under nitrogen a characteristic fluorescence is observed that is absent before irradiation. Under oxygen the amount of fluorescence is very much weaker. Irradiation of solutions of the four nucleotides, nucleosides and of the bases showed that the fluorescence is mainly due to guanine. Two components are present in solutions of irradiated guanine dervatives. One (component A) with an excitation maximum at 315 nm and an emission meximum at 400 nm, the other (component B) with maxima at 360 and 450 nm respectively. Component B is formed predominantly by attack of guanine by OH radicals, whereas in the formation of A presumably both OH and H radicals are involved. Work was started on the isolation and characterization of A and B. The data obtained thus for indicate that both A and B are mixtures of a number of compounds probably differing in the structure of the sugar-phosphate group.

3) Physical chemistry of nucleic acid

The change of the extinction of DNA of the bacteriophage T4 after a sudden temperature increase (0.75^{o}) in the melting region as a function of time was investigated. Contrary to the theory developed by Crothers it was found that two processes, each with their own relaxationtime, are involved. The relaxation time of the slower process remains constant as a function of the initial temperature, i.e. the temperature before the temperature jump, up to a temperature where the hyperchromic effect is about 50%. At higher initial temperatures the relaxation time decreases. The fast process points to a similar behaviour, but the accuracy of the data is not very high.

Both relaxation times are almost independent of the average number of single strand breaks per molecule, but the fraction of the relaxation associated with the slower process decreases sharply with increasing number of single strand breaks.

For non-twisted, circular native DNA relations have been derived between the intrinsic viscosity, the sedimentation coefficient and the molecular weight, using the well-known relations for linear DNA and the theory developed by Bloomfield and Zimm about the hydrodynamic behaviour of ring-shaped macromolecules.

The theoretical s-M relation gives results which are in good agreement with the experimental values. The $[\eta]$-M relation has been checked, using intracellular DNA from bacteriophage ΦX174 (replicative form DNA, RF-DNA) and showed also experimental values that were in complete agreement with the theoretical results.

The relations are:

for linear DNA:

$$0.665 \log M = 2.863 + \log ([\eta] + 5)$$

$$0.445 \log M = 1.819 + \log (s - 2.7)$$

for circular DNA:

$$0.665 \log M = 3.086 + \log ([\eta] + 3)$$

$$0.445 \log M = 1.764 + \log (s - 3.1)$$

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Mutation Research

Group V - Mechanism of action of enzymes and modification by irradiation

The studies of the group on the mechanism of action of hydrolytic enzymes has been based mainly on the analysis of the active sites of enzymes which react with inhibitors of the organophosphate type like DFP. In all cases studied the inhibitor reacts with a hydroxylgroup of a serine residue. The amino-acid sequence around this residue has been determined for chymotrypsin, trypsin, butyrylcholinesterase, horse liver ali-esterase and subtilisin.

Much effort was spent on locating a histidine and methionine residue as additional functional group in subtilisin.

The efforts to prepare enzymatically active degradation products of subtilisin by chemical or proteolytic methods failed.

In a number of DFP-sensitive proteases two particularly located histidine residues participate in the hydrolytic process; so far no evidence has been found that such a particular structure prevails in horse liver ali-esterase, a model enzyme for the group of DFP-sensitive esterases.

It was found that exposure to X-ray irradiation of chymotrypsin was equally effective in repressing the esterolytic and proteolytic action of the enzyme and its capacity to react with organophosphates.

More recently the group became interested in an enzyme from a Pseudomonas strain, which is able to interact with atropine and atropine like substances.
The enzyme hydrolyzes the ester bond of atropine and can be inhibited by organophosphates. The enzyme could be purified and its physical and enzymological properties are under investigation. The M.W. is approximately 30.000; there is kinetic evidence for the presence of a complex active site containing (like acetylcholinesterase) an anionic next to an esteratic site.

It is hoped that the chemical analysis of altered enzymes isolated from mutants of Pseudomonas will reveal a relationship between the protein structure and the enzymological behaviour. A large number of mutants has recently been isolated.

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Group VI - Investigations of mutations that modify the biological activity of enzymes

During his sabbatical year (1964-1965) spent at Stanford University, Dr. de Waard was able to identify the structural gene for DNA polymerase in bacteriophage T4. This was accomplished by studying conditional lethal mutants of this bacteriophage which had been isolated by Epstein, Edgar and their coworkers. Amber mutants defective in gene 43 were shown to be incapable of inducing in restrictive hosts the phage induced DNA polymerase. Some temperature sensitive phage defective in the same gene appeared unable to produce DNA polymerase at 37°; a certain mutant was shown to synthesize a heat labile enzyme. This finding was interpreted to mean that the primary structure of the enzyme is affected by a mutation in gene 43.

Subsequently it was shown by Speyer that some of these gene 43 mutants are mutagenic. This investigator constructed double mutants carrying a mutation of the rII type and one in the polymerase gene. The reversion of the rII mutation to wild type in this double mutant occurred with a frequency thousand times greater than when the rII mutant was present single. Speyer made the suggestion that phage DNA polymerase is normally involved in the selection of the nucleotides to be incorporated.

In order to investigate whether these mutagenic polymerases make systematic or random errors in replication the following experiment was designed. A double mutant of the type ts L 56 (gene 43)/am H 36 was isolated. This amber mutation is known to reside on the structural gene for the head protein of bacteriophage T4; when the single amber mutant H36 infects a non permissive host like E.coli B, a fragment of the head protein is formed of the following structure:

ala.gly.val.phe.asp.phe

while the NH_2 terminal end of the wild type protein has the following amino-acid sequence: ala.gly.val.phe.asp.phe.gln.asp.pro.ile.asp.ile.arg.

We grew the ts L 56/am H36 mutant on a permissive strain of E.coli CR63 and then plated it on the non permissive strain of E.coli B. We selected plaques of the type ts L 56/am^{+} H36. Four independent amber revertants were then grown on ^{14}C labeled phenylalanine to facilitate the identification of the relevant peptide in a

trypsin digest of the head protein. The analysis of these digests has not been completed yet, but the results to be obtained should indicate into which triplet the original amber UAG symbal has been changed.

A similar approach is being made using mutants of T-even bacteriophages which are defective in the structural genes of the hydroxymethylcytosine α glucosyl transferases. In the case of T4 phage the mutant to be constructed is of the type ts L56 (gene 43)/ α gt ß gt which will only plate on non-restrictive hosts at 25°. The α gt ß mutant was made available to us by Dr. S.E. Luria (M.I.T.). Revertants in the α gt gene will plate on E.coli B and can be selected.

Attempts are being made to identify changes in the amino acid sequences of the α-transferase; Amberlite XE 64 and long Sephadex columns are used as well as preparative polyacryl-amide electrophoresis in trying to obtain the small basic protein in a homogeneous form.

In the case of T2 phage the mutant to start from has a simpler structure than in the case of T4. In the former case there is no hydroxymethylcytosine ß glucosyl transferase to compete with the α-enzyme for the same sites. Amber gt mutants have been made available to us by Dr. R.L. Russell at Cornell University.

The work on specificity differences between the T4-HMC-α and ß glucosyl transferases has been completed. The results can be summarized as follows:

The T4 specific hydroxymethylcytosine-α-glucosyltransferase is not capable of glucosylating HMC residues linked through their 5' carbon to a neighbouring HMC nucleotide, whereas the ß glucosyltransferase will react with such 5 hydroxymethyl-cytosine residues. HMC nucleotides in all the other sequences examined are susceptible to both enzymes. In the presence of both transferases the ratio of α and ß glucose residues introduced <u>in vitro</u> can be influenced by changing the Mg^{++} context of the incubation mixture. This situation applies to HMC residues generally and to HMC nucleotides present in some specific sequences. The proportion of α- and ß-glucosides in T4DNA produced <u>in vivo</u> has been found to remain constant irrespective of the Mg^{++} context of the growth medium.

The specificity of a DNA methylating enzyme from E.coli which will methylate cytosine residues in the DNA from M. phlei has been examined. Preliminary results would indicate that sequences of the type -T-C-Pu- are susceptible to this methylase.

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B. Research activities of the Department of Radiation Genetics of the University of Leiden.

INTRODUCTION

The scientific programme was carried out in the Department of Radiation Genetics of the University of Leiden with the active support or participation of a number of institutions and organizations, detailed in the foregoing summary.

The programme is mainly concerned with the effects of ionizing radiation on the genetic material of the fruitfly Drosophila melanogaster.
During the report period several series of investigations were carried out to study the influence of the following factors on radiation-induced mutations and chromosome aberrations. These factors are:

a. Differential radiosensitivity of the various stages of germ cell development
b. The effect of post-radiation repair processes
c. Modification of radiosensitivity or repair processes by pre-treatment with chemicals
d. The dose, rate of delivery and LET of the irradiation

Most of the studies were performed with male flies; lately, however, females were also included in the material.

The studies on morphological changes in the developing germ cells were continued. Techniques for electronmicroscopical autoradiography were considerably improved in close cooperation with the Department of Electron Microscopy, University of Leiden. Incorporation studies with labelled amino acids and nucleotides were performed in order to obtain basic information about the processes in the cell that are responsible for repair of radiation damage.

The phage work focussed on two aspects of genetics: first, the molecular aspect studied in the system of exclusion and modification by differential glucosylation of DNA of phage T4 and T2; second, the radiation aspect studied in phage crosses by means of marker rescue from heavily irradiated T4 by unirradiated T2.

The problems of regulatory mechanisms in somatic cells in vitro were pursued along the following lines:

1. Analysis of frequency distributions of cell sizes to characterize cell populations with respect to growth and contact inhibition
2. Cell-transformation experiments with X-rays to induce hereditary breakdown of the regulatory mechanisms
3. Chromosome studies to analyse the relationships between genetic material and control mechanisms and their breakdown

There was a close contact with numerous research workers from other laboratories, leading to exchange of visits and presentation of seminars on current research.

The Department of Radiation Genetics participated in the Euratom Course on Molecular and Radiation Biology, September - October 1967, at Leiden and Rijswijk. In the section on "Biochemistry of DNA" the visiting scientists identified unknown glucosyl transferases induced by recombinants from T4 by T2 crosses. The sixth week of the Course was devoted to Drosophila genetics. Demonstrations and lectures were given on fundamental principles of genetics, electron microscopy and paper chromatography of the eye pigments from eye-colour mutants. Further, sex-linked inheritance, radiation-induced recessive lethals, translocations and chromosome loss were studied in progeny from irradiated parental flies.

In this three-year period of research, some substantial progress has been made and the results are reported in the literature. Apart from the radiation studies sensu stricto, other investigations of a fundamental nature have been carried out and it is expected that these will serve as a basis for the further realization of our future research programme on radiation effects and repair by living material.

ORGANIZATION AND PERSONNEL

The reported studies were carried out under the general direction of Professor Dr. F.H. Sobels, Director of the Department of Radiation Genetics of the University of Leiden. During the period of this report, the new Transitorium for Medecine was completed. The Department moved to its new premises in the summer of 1965. The facilities of the new laboratories and the participation of a number of guest workers from abroad, led to a rapid development of the Drosophile project. In a later stage of the period, the phage work too, was considerably extended.

Some technicians were appointed during part of the period.

Some of the research workers left again before the end of the period.

All these details will not be presented in the following table of scientists concerned with the work of the three groups.

Group I - Mutation studies with Drosophila

Senior scientists:	Prof. Dr. F.H. Sobels
	Dr. K. Sankaranarayanan
Scientists:	Dr. N.G. Brink
	Drs. P. Kieft
	Dr. B. Leigh
	Dr. R.N. Mukherjee
	Mrs. U. Mukherjee, M.Sc.
	Dr. G. Olivieri
	Dr. A. Olivieri-Mancini
	Drs. A.D. Tates
	Dr. W.A.F. Watson

Group II - Gene function, genetic recombination and replication of DNA of bacteriophage and the effect of radiation on these processes

Senior scientist: Dr. B. de Groot

Scientists: Dr. V. Fučik
Drs. P. de Goede
Dr. B. Molholt
Dr. R.N. Mukherjee
Dr. E. Pees

Group III - Studies on regulatory mechanisms of somatic cells in vitro

Scientists: Drs. J.W.I.M. Simons
Drs. H. van Steenis
Mrs. K. Heyman-Zandstra, B.Sc.

RESEARCH ACTIVITIES

Group I - Mutation studies with Drosophila

1. The analysis of post-radiation recovery phenomena in mature sperm

Studies of the past few years in this laboratory have shown that after X-irradiation of fully mature spermatozoa in N_2, post-treatment with N_2 leads to a reduction of the mutation frequency relative to that observed after post-treatment with O_2.

Three alternative interpretations for the post-radiation recovery by nitrogen in sperm were considered, and tested experimentally: first, N_2 prevents development of a delayed oxygen effect due to interaction with radicals; second, N_2 post-treatment unfavourably affects restitution of chromosome breaks, resulting in the selective elimination of cells with recessive lethal-mutations and translocations; third, post-treatment with N_2 makes energy available for a repair process, which in the presence of O_2 is used up for sperm motility.

1.1 To investigate whether post-radiation enhancement by O_2 might be due to reaction with radicals, as in dry plant seeds or bacterial spores, the late Dr. L.H. Gray kindly offered the facilities available in the B.E.C.C. Unit in Radiobiology of Mount Vernon Hospital in Northwood. The flies were pre-treated with 10 Atm. of N_2 and exposed to 1.8 MeV pulsed electrons from the linear accelator, giving within 1 second. The next second the anoxically irradiated flies were subjected to post-treatment with 10 Atm. O_2. The effects of such very fast post-treatments with high pressure O_2 were compared to that of high pressure N_2. In parallel series pre-treatment and irradiation under 10 Atm. O_2 were followed by immediate post-treatment with N_2 or O_2.

Now, if indeed the post-radiation enhancement by O_2 in the X-ray experiments had been brought about by a radiochemical after-effect of O_2, one would expect the considerably faster post-treatment with O_2, following pulsed electron-radiation in N_2, to be more effective in increasing the damage to the level obtained with irradiation-exposures in O_2. Four separate series of experiments at dose levels of approximately 1000, 2500, and 4000 rads were carried out. In none of these a delayed oxygen effect for either recessive lethals mutations or translocations has been observed. Since the possibility was considered that the extremely fast dose rates resulted in such an interaction of the radicals with each other that only few of the radiation products would be left over to react with oxygen, two more runs were carried out at a dose rate with a factor 60-100 lower than in the preceding exposures. Again, no post-treatment effect of oxygen could be seen.

Taken together, these results suggest that the post-radiation enhancement by O_2 following X-irradiation of mature sperm presumably is not a radiochemical effect arising from a reaction with radicals.

Throughout the whole dose range mutation frequencies increased linearly with dose, whereas for translocation a significant dose square component was observed. Since no other genetic data are available for pulsed electrons, this information by itself seems of certain value.

1.2 Another possible interpretation of the post-radiation recovery by N_2 has been proposed by Dr. S. Abrahamson from Madison, Wisc. (personal communication). He assumed that post-treatment with N_2 might unfavourably affect restitution of chromosome breaks which then could result in a selective elimination of cells with recessive lethal mutations and translocations. If this interpretation was correct, one would expect that post-treatment with N_2 results in a greater frequency of dominant lethals than that with O_2. An increase of the frequency of dominant lethals by post-treatment with N_2, was indeed first observed by Abrahamson, but could not be confirmed in later experiments (personal communication).

The same hypothesis was also tested extensively for the first sperm sample from 7-day old males from the stock that has been used in our studies. The results of eight extensive experiments with either nitrogen or oxygen post-treatment for different durations (25 min., 60 min., 90 min. and 120 min.) following irradiation (4000 R) of 7-day old males revealed no significant differences in the frequency of dominant lethals with the contrasting post-treatments. In addition these frequencies increased linearly with time ($Y = 0.6829 + 0.0055 X$), X being the time unit in terms of the successive 12-hr egg-collecting periods. The trend is independent of the different post-treatments, suggesting a "storage effect" on induced dominant lethality, with time.

Following prolonged post-treatments, a sharp increase in the proportion of unhatched eggs was noticed in experiments involving nitrogen exposures. Examination of the ventral receptacles and spermathecase of females inseminated by nitrogen treated males revealed that with nitrogen treatments (1) there occurred a drastic reduction in the number of sperm stored, the magnitude of reduction being dependent on the length of exposure to nitrogen and (2) there was a decrease with time in the number of sperm stored in the storage organs, with the effect being pronounced with 96 and 120 hrs of storage following insemination. These findings are explained on the basis of physiological damage to the nitrogen-treated sperm and by sperm exhaustion (ref. 6).

1.3 In view of the fact that spermatozoa are immobilized when flies are kept in nitrogen, it is thought that energy which is used up for sperm motility under post-treatment with oxygen, becomes available for an energy requiring repair process when nitrogen post-treatments are given. Since the capacity for sperm motility resides in the contractile tail fibres, one would have to postulate in addition, that energy can be transferred from its site of origin to the sperm nucleus. Clear indications for ATPase activity were obtained by Daems, Persijn and Tates (Experimental Cell Research 32, 1963 of 1964). Moreover, the available pictures on sperm morphology indicate that in Drosophila sperm, this energy transfer to the sperm nucleus may well be possible, because there is a region where tailfibres and sperm nucleus show an extensive overlap.

This interpretation would have the advantage that it brings the findings for mature spermatozoa in line with those in spermatids, where it could be shown that repair requires energy. The difference between the different types of cells would consist then, that repair in sperm makes use of energy derived from anaerobic pathways, whereas in spermatids and spermatocytes clearly oxidative respiration is required.

As it is known that glycolysis is the predominant respiratory pathway in mammalian sperm, the effects of inhibiting glycolytic enzymes on the frequency of radiation induced mutation have been studied. The following two inhibitors were used, iodoacetamide blocking triose phosphate dehydrogenase and sodium-fluoride blocking the enolase enzyme. At first pre-treatment, iodoacetamide showed a significant increase, but later replicas of these experiments showed somewhat more variable response, although altogether there is a tendency to increase the radiation induced mutation frequencies. A more consistent and highly significant radio-sensitization was obtained with sodiumfluoride pre-treatment. This effect has now extensively been confirmed for fully mature spermatozoa as sampled from the first ejaculate of three-day old males. It is considered improbable, that inhibition of glycolysis by NaF leads to an increase of the oxygen tension and thereby enhances the radiosensitivity in the treated cells, because no inhibition of oxi dative respiration has been observed in systems where this could be measured directly. Moreover, it was observed that a following pre-treatment and irradiation in oxygen, the radiosensitizing effect of sodium-fluoride persists. If indeed sodiumfluoride has acted by increasing the oxygen tension, no enhancing effect could have been expected under these conditions.

In conclusion then, it is thought that sodiumfluoride enhances the irradiation induced mutation frequency by depleting the source of energy, which is required for effective repair of pre-mutational damage.

A possible interaction on the repair system in mature sperm of pre-treatment with fluoride on one hand and post-treatment with oxygen versus that with nitrogen on the other, was extensively tested in experiments, in which flies were injected with either NaF or saline, pre-treated with and exposed to irradiation in N_2 (4000R) or O_2 (2000R) and then post-treated with either N_2 or O_2; only mature spermatozoa used for the first mating were sampled. It was observed that (1) irrespective of pre-treatment with N_2 or O_2, NaF enhanced the mutation frequency over that of the controls (the probability that the difference in mutation frequency observed would arise by chance is smaller than $1 . 10^{-5}$) (2) following irradiation under anoxia, post-treatment with N_2 reduced the mutation frequency below that observed with O_2 post-treatment even when the flies had been pre-treated with NaF. These additive effects of NaF pre-treatment and O_2 post-treatment have been taken as an indication that, even when glycolysis is inhibited by NaF, some energy is left which is still available for repair by post-radiation anoxia. This interpretation that the amount of repair in sperm depends on different levels of available energy is supported by the observation that NaF pre-treatment is still effective in increasing the mutation frequency over that in saline controls when N_2 was given before, during and after irradiation. Thus repair is maximal with NaCl-N_2-R-N_2, intermediate with NaF-N_2-R-N_2 and minimal with NaF-N_2-R-O_2.

1.4 To gain an insight into the nature of the energy-requiring repair process, experiments were started to study the effects of blocking the energy supply by the use of 2,4 - dinitrophenol (DNP) which is an uncoupling agent for oxidative phosphorylation and an inhibitor of ATP synthesis. Preliminary experiments with DNP, however, failed to show any modifying effect on the frequencies of radiation-induced sex-linked lethals in mature sperm. Experimental conditions were then altered in the subsequent series of tests.

The pH of a freshly prepared solution (10^{-3} M) of DNP in 0.7% NaCl is 3.5, whereas the optimum pH for action of DNP as an uncoupling agent for oxidative phosphorylation has been found to be 7.5. In order to test whether DNP could be effective at higher pH, the 10^{-3} M solution was adjusted to pH 7.5 by means of 1 mM solution of NaOH. Corresponding pH adjustments were also made in the NaCl solution. Three-days old males were irradiated with 2000 R of X-rays 90 minutes after injection with either DNP or NaCl. After collecting a sample of fully mature sperm from the first ejaculate, five successive two-day broods were sampled from the matings each male with six females. The results showed that there was some enhancement by DNP on sex-linked lethals in fully mature sperm samples in brood A and the effect was variable in other broods. The experiment was repeated with fully mature sperm and DNP at pH 7.5, but this time no enhancement was observed.

1.5 In other experiments the effects of pre-treatment with actinomycin-D, puromycin and ribonuclease have been studied. Although the results were somewhat variable, the over-all data for the various agents, considered together, lead us to conclude that inhibition of RNA- and/ or protein synthesis increase the radiation-induced mutation frequencies in mature sperm.

1.6 In has been shown (see above) that the amount of genetic damage induced in mature sperm, by X-irradiation in nitrogen, can be modified by varying the type of gas used as a post-treatment. One possible way, of extending this study, was to give irradiation in two fractions and compare the effects of different gases administered between irradiations. A series of 22 experiments were carries out, using males carrying a ring-X chromosome.

All irradiations were in nitrogen. Unfractionated exposures of 1.5 kR. The first fraction was given in nitrogen and then immediately followed by a one-hour post-treatment in nitrogen or oxygen, followed by 20 minutes in nitrogen

before the second irradiation. After irradiation the males were allowed to mate overnight, with one female per male. The progeny of four groups were tested for the induced frequencies of sex-linked lethals, while the progeny of all the other groups were tested for both sex-linked lethals and translocations.

In the unfractionated series, the results obtained are in accordance with those of Sobels (1964). The frequencies of sex-linked lethals were consistently lower in the nitrogen post-treated groups than in the oxygen post-treated groups. On the other hand, the translocations appeared to show an inverse effect with the frequencies being higher with N_2 post-treatment than with oxygen post-treatment.

Fractionation did not appear to have any effect upon the amount of induced genetic damage, nor was there any apparent effect of varying the gas treatments given between irradiations. However, the data are not sufficiently extensive to exclude the possibility that an inter-fraction treatment effect does exist.

1.7 In connection with problems concerning restitution of breaks and repair of potential breaks induced in mature sperm before or during zygote formation, experiments were aimed at the detection of translocations involving both the paternal and maternal autosomes.

Only three of such cases of paternal-maternal translocations have been described in the literature, but no systematic search for their occurrence has so far been made. Specially marked stocks which would permit the detection of translocations, involving paternal, maternal or both paternal-maternal exchanges were therefore constructed, and care was taken that only irradiated mature sperm and stage 14 oocytes were sampled.

Despite the scoring of a considerable number of progeny of such crosses, in which both male and female batches were irradiated, no single case of induced paternal-maternal translocation could be observed. In confirmation of observations of other authors translocation in oocytes was found to occur very infrequently. Apart from this, the fact that the paternal and maternal genomes in the zygote nucleus are merely juxtaposed without actual fusion may have been responsible for the negative outcome of these experiments.

2. The determination of RBE-values for fast neutrons in dependence of the degree of oxygenation of the irradiated sperm cells

It is well-known that damage, produced by fast neutrons, is less dependent on the presence of oxygen than that induced by X-irradiations. One would expect therefore, that RBE-values will depend on the degree of oxygenation of the irradiated cells. To test this hypothesis mutation- and translocation frequencies were determined for sperm cells from the first ejaculate of 7-day old males (old males) and 2-hour old males (young males), since previous work has shown that the higher mutation-frequencies in sperm cells from the old, than from the young males can be ascribed to differences in the degree of oxygenation between these two types of cells (Sobels1966). Originally the experiments were planned in collaboration with Dr. C.E. Purdom from Harwell. Since 1-2 MeV neutrons from the reactor were used for the experiments in Harwell, 15 MeV neutrons from the neutron generator of the Radiobiological Institute T.N.O. in Rijswijk were used for comparisons. Radiation exposures and dosimetry were carried out by Dr. J.J. Broerse, in collaboration with Dr. G.W. Barendsen. The dose rate of the 15 MeV neutrons was 54 rad/min. X-irradiation was given at 250 kV, 542 rad/min. with 0.25 Cu filtration (hvd 1.2 mm. Cu). So far, three large experiments have been carried out, in which old and young males were exposed to neutron- or X-irradiation in either air or nitrogen in the first two experiments, and to irradiation in air only in the third, 3000 rad. experiments.

When these experiments were already in progress, a paper by Dauch, Apitz, Catsch and Zimmer from Karlsruhe (Mutation Research 3 (1966) 185-193) appeared, in which RBE-values are given for sperm, utilized on the first- and second day following irradiation exposure of 3 - 5 day old males to fast neutrons produced by bombarding a lithium target with 2 MeV deuterons. The RBE-value recorded for first- and second day sperm are 1.16 and 2.21 for lethals and 2.30 and 3.27, respectively for translocations. Although the authors do not discuss the possible origin of these stage-specific differences, it is clear that differences in the degree of oxygenation are the most likely interpretation.

Although a more detailed discussion of our data has to wait until the statistical analysis of the dose effect curves has been completed, a few preliminary observations can be made.

a) For lethals, very low RBE-values are observed with the exception of sperm from young males, irradiated with 300 rads. It is only at 3000 rad. that the expected effect of a greater RBE in young, than in old males is observed. The reason is that the O_2-dependent sensitivity differences between "old" and "young" sperm are more pronounced at higher than at lower doses; the latter finding has also recently been recorded by Shiomi (Mutation Research, 1967). Moreover, 15 MeV neutrons have considerably greater energy, and in consequence, greater O_2-dependence than the neutrons applied by Dauch et al. Therefore only at the high dose of 3000 rads the difference in RBE between "old" and "young" sperm becomes manifest. Apart from these factors, the low RBE-values can be ascribed to the fact that lethals, associated with re-arrangements are eliminated from the ring-X which was used for these experiments, in contrast to the rod-X chromosome in the experiments by Dauch et al., and it is known that the RBE value for rearrangements is higher than for lethals.

b) The RBE-values for translocations for "old" and "young" males differ at all three dose levels, in the direction as expected, the effect being most pronounced at the highest dose of 3000 rads. Furthermore the RBE for translocations is found to decrease with increasing dose. This is to be expected on the different shapes of the dose effect curves for X-rays (3/2 power relation), and neutrons (linear relation), respectively.

In summary, these results then clearly indicate the greater mutagenic effectiveness of neutrons than of X-rays in cells which are by nature anoxic.

3. Differential radiosensitivity of spermatids, mature and almost mature sperm

From a comparison of the mutation frequencies in early spermatids and spermatozoa after irradiation in N_2 or O_2, followed by post-treatment with either N_2 or O_2, oxygen enhancement ratios for the induction of mutational damage in the two stages could be determined; these are 3.3 for spermatids and 1.8 to 2 for mature sperm. Radiosensitivity in the presence of O_2 is considerably greater in spermatids than in sperms. This finding also explains the pronounced differences in sensitivity between the two stages after radiation exposure in air (ref. 8).

To study the underlying causes for the decrease in radiosensitivity, as observed in successive ejaculates, from males which are repeatedly mated, fully mature sperm was obtained from the first mating of irradiated 7-day old males, and almost mature sperm by taking the first ejaculate from males which were irradiated when 1 - 2 hours old. Radiosensitivity of the latter sperm sample is comparable to that observed in the third mating of 3-day old males. Thus 7-day old and 1-2 hours old males were irradiated in either O_2, air or N_2, and this was followed by post-treatment with either N_2 or O_2. The results of various replica experiments showed that after radiation in N_2, radiosensitivity in mature sperm of 7-day old males is not significantly different from that of the almost mature sperm in the 1 - 2 hours old males, and a similar result was obtained after radiation in O_2, although here, of course, about twice the amount of damage was observed as after irradiation in N_2. Under comparable conditions of post-treatment the oxygen enhancement ratios for the two different stages of sperm development are not at all significantly different (only slightly higher ratios being obtained for fully mature sperm in the 7-day old males). Since only after irradiation in air, considerably higher ($P < 0.001$) mutation-frequencies were observed for sperm in the 7-day old, than for that in the 1-2 hour old males, it follows that these pronounced differences in radiosensitivity originate from a greater

availability of oxygen in the mature sperm of the old, than in the almost mature spermatozoa of the young males. The underlying cause for the differences in radiosensitivity between the different sperm samples thus is essentially different from that responsible for the higher radiosensitivity in spermatids. This was, as has been outlined above, due to a greater intrinsic sensitivity to radiation damage, induced in the presence of oxygen (ref. 9).

4. Oxygen-dependent repair in early spermatids

In early spermatids the effects of post-treatment with O_2 or N_2 are precisely reversed from those in sperm. That is, following X-irradiation at a high dose rate under anoxia, post-treatment with O_2 decreases the mutation-frequency in comparison to that obtained with N_2 post-treatment. This effect of oxygen on post-radiation recovery was observed in stages which were sampled in broods from 4-6 and from 6-8 days after irradiation of adult males. It is unlikely that oxygen-dependent repair in spermatids is a spurious effect resulting from shifts of germ cells with different radiosensitivities between the two groups of flies having received N_2 or O_2 post-treatments. This was indicated by the brood pattern of mutation frequencies and sterility in the two groups of flies which were differently post-treated.

For a further verification, the post-treatment effect was then studied in pure samples of early spermatids derived from irradiated pupae. Pre-treatment of 48-hour pupae for 25 min. with N_2 and post-treatment for 25 min. with either N_2 or O_2 failed to reveal any differential effect of the contrasting post-treatments. Since Falk (1962), in his work on the effects of nitrogen treatment on translocation frequencies, found that pupae can be kept for a considerable time under anoxia, the experiments were continued by Watson with more extended pre- and post-radiation exposures to the gases. Both 24-hour and 48-hour old pupae were pre-treated with N_2 for 6 hours, after which they were X-rayed with 1250 R, 2500 R and 3750 R at a dose rate of 46 R/sec.

Following irradiation they were post-treated with O_2 or N_2 for 2 hours. After eclosion, the frequency of recessive lethals was then determined for the first two one-day broods. A consistent and highly significant decrease following post-treatment with O_2, as compared to that with N_2 was observed in the first brood from 24-hour old pupae. If all results are considered together, the probability that this effect resulted from chance is smaller than one in a million.

When the effect of the contrasting post-treatments is tested on the formation of translocations, it was found that their frequency, like that of the recessive lethals, is significantly reduced by post-treatment with O_2. This observation is in line with the earlier observations that post-treatment with cyanide results in an increase of the translocation frequency in early spermatids. Since, according to findings by Oster (1957) and Falk (1962), restitution of breaks induced in spermatids requires up to 12 hours, it is thought that these post-radiation modifications of the translocation-frequencies may reflect an effect on repair of potential lesions rather than on restitution of breaks.

These data from 24-hour pupae (ref. 12, 13, 14) convincingly demonstrate that the similar results, obtained from earlier broods of adult flies (Sobels, 1964) did not originate from artefacts in the sampling technique. Selective elimination of cells with mutations by O_2-post-treatment can be ruled out both on the basis of the earlier data from irradiated adults (1965) and by the finding that the frequencies of dominant lethals, similar to those of recessive lethal mutations, are reduced by post-treatment with O_2.

From the earlier results on the enhancing effect by post-treatment with HCN and N_2 following irradiation in air, it had been proposed that there is a repair process, operating in early spermatids which requires oxidative respiration for its proper functioning. The present findings are in line with this hypothesis, since the reduction of the mutation-frequency by O_2 post-treatment in comparison to that with N_2, indicates that a repair-system which has been effectively inhibited by anoxia before and during irradiation, can be reversed again to normal functioning by post-radiation exposure to oxygen.

It is of interest that in the early spermatids, where O_2-dependent repair could be demonstrated, inhibition of RNA and/or protein synthesis by pre-treatment with chloramphenicol, ribonuclease, or actinomycin D leads to a reduction of the mutation frequency, and thus to a response which is opposite to that observed in sperm. Whether these agents act by giving more opportunity for repair to occur, is still a matter of speculation.

It is not yet known when mutation-fixation occurs and what process is involved in the terminal event when pre-mutational damage becomes irreversible. In early spermatids there is no DNA-synthesis, as measured by the incorporation of ^{3}H-thymidine (ref. 3). The possible role of RNA synthesis in the realization of mutation in spermatids was investigated (ref. 4) by studying the incorporation of ^{3}H-uridine in the various stages of spermatogenesis and it was found that young spermatocytes were the most advanced stage to incorporate the labelled nucleoside. Since both O_2-dependent repair and modification of radi-sensitivity by protein-synthesis inhibitors predominantly occurs in a later stage of sperm development, this observation offers no clue to a better understanding of these processes.

5. The effect of dose rate on O_2-dependent repair in early spermatids

The earlier studies with cyanide had shown that in early spermatids post-treatment with HCN modifies the mutation-frequency only, when X-irradiation was given at a relatively high dose rate of over 33.3 R/sec., but not at dose rates of 8.3 R/sec. or lower. We were interested therefore to see whether after irradiation of 24-hour pupae under anoxia, post-treatment with O_2 would still lower the mutation frequency as compared to that with N_2, when dose rates lower with a factor 5 (8,3 R/sec.) or 10 (4,2 R/sec.) were employed then in Watson's experiments where the dose rate was 46 R/sec.

The results indicate that after irradiation at the lower dose rates, post-radiation modification is no longer observed. That is, the mutation frequencies, recorded after post-treatment with either O_2 or N_2 are entirely similar to those obtained with N_2 post-treatment, following X-irradiation at the high dose rate of 46 R/sec. The difference between the mutation frequencies observed after post-treatment with O_2 at 46 R/sec. on the one hand, and with 8.4 or 4.3 R/sec., on the other, is, however, highly significant. In other words, following irradiation at the lower dose rates, post-treatment with O_2 is no longer effective in bringing about repair (ref. 5). A possible interpretation for this peculiar "inverse" dose rate effect is that the repair-process proceeds in such a short time that after longer irradiation exposures, post-treatment with O_2 becomes ineffective.

6. A study on the possible effect of dose rate on induced sex-ratio changes, chromosome loss and non-disjunction in spermatocytes

In earlier studies (Leigh, 1963, Ph.D. thesis 1965) indications had been obtained showing that the sex ratio shift in progeny derived from irradiated spermatocytes was dependent upon the rate at which the radiation had been delivered. An experiment was therefore designed to investigate the significance of this effect when larger numbers of progeny were obtained. Ring-X chromosome bearing males (X^{C2}, y B/sc^{8}·Y) were irradiated with 2,000 R, given at either 2,600 R/min. or 400 R/min. All irradiations were given in nitrogen, followed by either nitrogen or oxygen post-treatments. Following irradiation the males were mated for a series of five two-day broods, with sex tester females per male per day, and in each treatment the fifth brood was split into one-day sperm sampling periods.

The F_1 progeny were scored for sex ratio, chromosome loss, and non-disjunction. Neither the overall brood pattern nor the frequencies of XO males were affected by either dose rate or post-treatment. On the other hand, the sex ratio shifts

fluctuated to a greater extent than could be expected on a basis of random experimental variation, but these variations could not be correlated with either dose-rate or post-treatment.

7. Is there an oxygen enhancement effect on chromosome loss?

One-day old males, of the genetic constitution $X^{C2}, y\ B/sc^{8}\cdot Y$, were irradiated in either nitrogen or oxygen. The progeny were then scored for chromosome loss (XO males), recessive sex-linked lethals, and autosomal translocations. At a dose level of 2.000 R oxygen enhancement ratios (O.E.R.) for XO males was 1.02, for recessive lethals was 2.2, and for translocations was 3.6. At a dose level of 3,000 R the O.E.R.'s were 1.13, 2.2, and 4.5, respectively. It can therefore be concluded that the induction of XO males is not affected by whether oxygen is present or absent during irradiation. There are two ways in which this observation could have been spuriously produced. Firstly, if radiation did not affect the frequencies of XO males and the observed frequencies were thus spontaneous. This possibility can be excluded because the spontaneous frequencies of XO males were far lower than those found after irradiation. Secondly, the treatment gases might nog have penetrated the germ cells of the treated males. This explanation can be excluded, because both the frequencies of recessive lethals and translocations showed an oxygen enhancement effect.

By a consideration of the above data and those which are available in the literature, three patterns of XO male induction can be distinguished. Firstly, when ring-X males are irradiated there is a high sensitivity and no oxygen enhancement. Secondly, when males carrying the inverted In(1)EN chromosome are irradiated, there is a low sensitivity and no oxygen enhancement. Thirdly, when males with a regular non-inverted rod-X chromosome are irradiated there is a low sensitivity with an oxygen enhancement. This indicates that the refractivity to oxygen enhancement is dependent upon a structural feature which is common to the X^{C1}, X^{C2}, and In(1)EN chromosomes, possibly the distribution of heterochromatin about the centromere.

8. The effect of nitrogen and oxygen post-treatments on the frequency of mosaics induced by X-irradiation

In view of the results previously found by Watson, that an oxygen post-treatment reduced the frequency of sex-linked recessive lethals in spermatids compared with a nitrogen post-treatment following the irradiation of twenty-four hour old pupae, it seemed desirable to extend this study to mosaic mutations. Thereby, it was hoped to obtain further information on the repair process by comparing the relative frequencies of complete and mosaic mutations following the different post-irradiation treatments.

According to one hypothesis (the lethal hit hypothesis) complete mutations are formed from potential mosaics by the occurrence of a lethal hit in the complementary strand to the one carrying the mutant hit. On the basis of this hypothesis completes should increase relative to mosaics with increasing X-ray dose. This phenomena has been observed by Nakao (Mutation Research, 3, 1966, 268-272).

Using this result as a basis for the present experiments it was thought that oxygen post-treatment following irradiation in N_2 preferentially repairs the lethal hits thereby reducing the frequency of completes and producing a comparable increase in the yield of mosaics. Alternatively, lethal and mutant hits may be equally reparable and consequently the yield of mosaics as well as completes will be reduced.

To study this problem, extensive experiments were carried out to establish the ratio of complete to fractional mutations for recessive lethals. It was found that this system was not only cumbersome but also not sufficiently sensitive to detect differences of the expected magnitude. The work was therefore continued by using an other system, namely, the scoring of visible mutations at specific loci. Although the data collected by Dr. Brink are not extensive enough to draw definite conclusions they are consistent with the expectations based on the lethal-hit hypothesis-recovery of more fractionals than completes from the repaired than from the unrepaired group. The work is being continued further.

9. Y-suppressed lethals

Many mutation studies in Drosophila, including those on repair phenomena, have utilized the induction of recessive sex-linked lethals. These are expressed as inviability of XY males. Several years ago, Lindsley and Edington started to look for mutants on the X-chromosome which were viable in XY males, but inviable in XO males. This study revealed that such mutations were induced and could be placed in several classes. These classes could then be correlated with specific changes, such as deficiencies for the bobbled region of the X-chromosome, large re-arrangements, and position effects. It was therefore considered that this technique provided a means to make a detailed investigation of the radio-sensitivity of the ring-X chromosome in Drosophila and thus opening the way to a more specific study of repair phenomena.

Initial tests have revealed that Y-suppressed lethals are induced in ring-X chromosomes, but there are technical problems in the genetic testing system which will complicate further investigation.

10. Cytogenetic analysis of recessive lethals induced in the ring-X chromosome

Post radiation recovery phenomena have now clearly been demonstrated for both early spermatids and spermatozoa. For the recovery of the induced recessive sex-linked lethals, a ring-shaped X-chromosome has been used in all these studies, because this restricts the lethals to point mutations and possibly small deletions, whereas lethals associated with large rearrangements are eliminated by the formation of dicentrics. Earlier work by Oster (1958) using a specific-locus approach, indicates that deficiencies are also recovered with considerably lower frequencies from irradiated ring, than from normal rod-shaped X-chromosomes. This result would be expected in view of the fact that twisted restitution of the ring will also lead to dicentric formation, and hence to elimination of the irradiated chromosome.

For a proper interpretation of the observations on post-radiation repair it is considered important to determine what proportion of the lethals consists in fact of small deficiencies. More specifically, one would like to know whether, for example in the early spermatids, the reduction of the lethal frequency by O_2 involves repair of pre-mutational damage, or whether, alternatively, only those lethals originating from deficiencies are repaired. For, if the latter situation obtained, O_2 post-treatment would only affect restitution of broken chromosomes.

An investigation started on the genetic and cytological localisation of a sample of lethals derived from irradiated 24-hour pupae which were post-treated with either O_2 (repaired group) or N_2 (unrepaired group). Since single crossing - overs involving the ring-chromosome lead to the formation of dicentrics, only products from double cross overs can be used for the localisation of the lethals. A total of 50 lethals (19 from the O_2 group and 31 from the N_2 group) were localized genetically. The results showed no difference in localization of lethals recovered from a repaired and unrepaired group. These lethals (and a few others which could not be localized genetically because of the low double crossover frequencies) were then analysed cytologically using salivery gland chromosomes. It was found that in approximately 80% of the lethals examined this way (19/23 in the O_2 group and 33/38 in the N_2 group) no aberrations could be detected. Of the remaining four lethals in the O_2 group, two were associated with single salivary band deletions at the lethal site, one with a large deletion involving about ten bands and one with a big inversion. In the N_2 group, one lethal was associated with a big deletion of eight bands in the proximal heterochromatin and the other four were inversions of varying lengths. Thus despite the fact that the lethals were induced in a ring-X , some were associated with gross chromosomal aberrations.

In view of the relatively small sample of lethals analysed and in view of the fact, that similar studies on the rod X chromosome (with which the ring-X data should be compared) have not yet been done, no valid conclusions can be drawn regarding repair of premutational damage versus repair of chromosome breakage phenomena.

11. The mutagenic effects of tritiated uridine

The mutagenic effects of tritium-labeled uridine and thymidine were studied in the male germ cell stages of Drosophila melanogaster. Adult males were injected with the tritium-labeld nucleosides, and six successive two-day broods were scored for the induction of recessive lethal mutations. Maximum sensitivity to mutation induction was observed in the broods to spermatocytes and late spermatogonia. Changes in either specific activity (1.6-9.3 Ci/mM) or the total activity (250-1000 μCi/ml) of tritium in the labeled nucleosides did not result in significant changes of the rate of mutation induction. Uridine with tritium in the 5-position of the pyrimidine ring produced approximately twice as many lethals as an equivalent dose of either 6-^{3}H-uridine or 6-^{3}H-thymidine. This observation is considered as an indication for a possible transmutation effect at the site of tritium decay. With 5-^{3}H-uridine the ratio of the frequencies of second chromosome lethals to sex-linked recessive lethals is 2 to 1, as of the 2nd and X-chromosomes might be expected on the relative length. With the other tritiated nucleosides used, the X- and second chromosomes yielded identical lethal frequencies. Sex-linked recessive lethals are induced at a higher rate in the euchromatin than in the heterochromatin. Since in Drosophila only the X chromosome has a nucleolar organizer region, these findings make it unlikely that only nucleolar RNA is involved in the production of mutations by tritiated uridine.

12. Nucleic acid synthesis during spermatogenesis in Drosophila

A study was made on incorporation of ^{3}H-uridine and ^{3}H-thymidine in the Drosophila testis by means of autoradiography, at the light microscopical level. RNA synthesis was observed in spermatogonia and young spermatocytes (ref. 3).

At 30 min. after ^{3}H uridine injection only the nucleus and nucleolus appeared labelled; 2 - 4 hours post-injection labelling was very heavy in the cytoplasm and lighter in the nucleolus, while it disappeared altogether in the nucleus, indicating that a complete transport of labelled RNA from nucleus to cytoplasm had occurred as early as 30 min. after ^{3}H-thymidine injection. No conclusive evidence could be obtained for the incorporation of ^{3}H-thymidine in the spermatocyte stages.

13. A study of protein synthesis during spermatogenesis using lysine

In order to detect a possible role of protein synthesis in the post-irradiation recovery phenomena in Drosophila, several antibiotics which are known to affect protein synthesis in micro-organisms and which have also been found to modify the X-ray induced mutation frequency in Drosophila, have been used in conjunction with a study of protein synthesis during spermatogenesis using the incorporation of ^{3}H lysine as a measure of protein synthesis.

Previous autoradiographic studies by Olivieri using ^{3}H-thymidine and ^{3}H-uridine to investigate the pattern of DNA and RNA synthesis during spermatogenesis in Drosophila, showed that the incorporation pattern was stage specific. He detected no RNA synthesis in post-meiotic stages whilst DNA synthesis appeared to be confined to the nuclei of the spermatogonia.

On the other hand, the study of tritiated lysine incorporation indicated that protein synthesis is much less specific. Thus within 15 minutes of the injection of the isotope, label was present in all parts of the testis, but its heaviest concentration was in the pre-meiotic stages. Here label was present in the cytoplasm with a heavy concentration of grains over the nucleolar region. The nucleus was only lightly labelled after 15 minutes. The nutritive cells show little or no incorporation until about twenty-four hours after injection. Thereafter they become quite heavily labelled. At the present time the effect of pre-injection with actinomycin D on the level of lysine incorporation is being investigated.

14. Repair of radiation damage in stage 7 oocytes

A series of experiments designed to investigate the influence of oxygen and nitrogen post-treatments on the repair of radiation-induced genetic damage in stage 7 oocytes were performed. Attention was focussed on dominant lethals. 0-5 hr old virgin females of Oregon-K stock were pre-treated for 6' with N_2, irradiated under anoxia over a wide dose-range (1000 R-14, 000 R) and then post-treated with either N_2 or O_2 for 30'. The treated females were kept virgin for 48 hrs and then were mated to 3-4 day old males and allowed to lay eggs on appropriate medium. Only the first 24 eggs laid by any individual female were included in the calculations to restrict the sampling to stage 7 oocytes. The frequencies of unhatched eggs were scored following a 24 - 28 hr incubation at 25° C. Appropiate controls were maintained. While the data are not yet fully analysed it is clear that with O_2 post-treatment, there is a consistent and significant lowering of the frequencies of dominant lethals.

It is hoped that these and similar ones with irradiations in air and in O_2 which are currently underway, will shed some light on the mechanisms of dominant lethal induction and repair of this kind of damage in this germ cell stage.

15. Model experiments on electron-microscopical autoradiography (EMA)

The Ilford L4 photographic emulsion (AgBr crystal size $\pm$ 1300 A^{o}) has found wide application in EMA, because it is easy to handle. The photographic developers which have mostly been used in combination with the L4 emulsion (namely mikrodol-X and D 19b), have the serious disadvantage that the developed silver grains are 2 - 3 times larger than the original crystal. These unfavourably affect the resolution of autoradiographs. Salpeter and Bachmann developed a better method by using a Kodak NTE emulsion (Ag Br crystal size $\pm$ 500 A^{o}) in combination with a gold latensification-elon ascorbic acid developer (GEA developer). Since the NTE emulsion is not easy to manipulate for routine work, we worked out a new method which combines the specific advantages of the L4 emulsion and the GEA developer. This was done in close collaboration with Drs. E. Wisse from the Laboratory for Electron Microscopy at Leiden.

The method has fully been described in the 1967 Euratom Course in Molecular and Radiation Biology.

The qualitative merits of the L4 - GEA method were and are being analyzed by using tritiated parlodion films as a calibration source. Application of the method results in the formation of silver grains with a mean diameter of 300 A^{o}. The grains occur singly or in groups (2 - 16 grains) and positive staining of the emulsion's gelatin with phospho-tungstic acid revealed that most groups cover the surface of the original AgBr crystal. This finding makes it quite likely that the radius of the AgBr crystal determines the autoradiographic resolution.

As far as the quantitave aspects of the method are concerned, we now have preliminary data on the ratio of the number of developed silver grains to the number of radioactive disintegrations in the parlodion calibration source. In case the L4-GEA method is used, it is found that several silver grains may be developed on a AgBr crystal which is hit by only one beta-ray. Furthermore the data indicate that the grainyield (not corrected for the fact that a 30-40% of the silver grains occur in groups) for the L4-GEA methods is about 14 - 18 times higher than for the L4-Mikrodol method. At present the method has not yet reached the degree of perfection which is necessary for relating the observed silver grains in the emulsion to the disintegrations taking place in ultrathin sections of radioactive biological specimens.

16. The L4-GEA method applied to the in vitro incorporation of H^3 -5 uridine into pupal testis of Drosophila

For details of the technique the reader is referred to information presented in the Euratom Course in Molecular and Radiation Biology.

16.1 Localization of labelled uridine

When testes are incubated for 6-60 minutes in 500 muC/ml uridine and then fixed in osmiun tetroxide, the label (=silvergrains) is found in spermatogonia and young spermatocytes. Most silver grains are found over the nucleolus. Less grains are observed over the karyoplasm and an even smaller number of grains are overlaying the cytoplasm. The grains over the karyoplasm have no apparent localization. The label in the cytoplasm is not specifically bound to organelles, but in young spermatocytes a substantial part of the label is found in between the mitochondria and occasionally inside the mitochondria. More label is found after 60 minutes incubation than after 6 minutes, but the fine structural localization of the label seems to be the same in both cases.

The effect of incubation time on the amount of incorporated uridine was studied by liquid scintillation countings of individual testes. The data showed that testes of the 60 minutes series incorporate 4-5 times as much uridine as testes of the 6 minutes series. It is interesting to note that the increase in the amount of label, as reflected by graincounting of light microscopical autoradiographs, is of the same order of magnitude.

16.2 The effect of the type of histological fixation on the grainyield in light microscopical autoradiographs

Two types of fixation are widely used in electronmicroscopy:

a. Osmiumtetroxide (O).

b. Glutaraldehyde followed by Osmiumtetroxide (GO)

Preliminary studies indicate that more label is retained in GO fixed testes than in O fixed testes. This is particularly true for the number of grains found over spermatogonia and young spermatocytes. In contrast to the findings with O fixation of the testes, we observed small numbers of randomly distributed grains over old spermatocytes and spermatids of GO fixed testes. These findings do suggest that the extra amount of label in GO fixed testes is due to a specific binding of unincorporated uridine by glutaraldehyde. As a consequence of this, the label which is found over old spermatocytes and spermatids of GO fixed testes presumably does not indicate true RNA synthesis but the presence of an artefact.

16.3 The effect of a pre-treatment with actinomycin on the in vitro incorporation of H^3-5-uridine

A one-hour pre-treatment of dissected pupal testes with actinomycin D. (1 mu.g/ml or 100 mu.g/ml) drastically inhibited the incorporation (incubation time 6 or 60 minutes) of H^3-5-uridine into spermatogonia and young spermatocytes.

Thus far the effect of actinomycin has only been studied at the light microscopical level. The most striking effect of the actinomycin treatment is the strong reduction of uridine incorporation in the nucleolus, but there is also a considerable decrease of the number of silver grains in the cytoplasm and the karyplasm. The pre-treatment with actinomycin was found to have an effect on the morphology of the germ cells as well. The most pronounced effect involved a clumping or clotting of the "chromatin" in the nucleus of spermatogonia, young spermatocytes and early spermatids.

The outcome of the actinomycin experiment demonstrated that:

a. Our experimental set-up is such that it offers good possibilities for autoradiographic studies on the effect of specific metabolic inhibitors

b. The amount of the artefactual labelling in osmium fixed testes is possibly rather low, since the amount of residual labelling after a pre-treatment with actinomycin is small. For this conclusion to be corrected one has of course te presuppose that actinomycin does not affect the uptake of tritiated uridine.

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Group II - Gene function, genetic recombination and replication of bacteriophage DNA and the effect of radiation on these processes

1. The biological role of differential glucosylation of T-even phage DNA

Crosses between phages T4 and T2 produce progeny with unequal yields of T4 and T2 genes. The adsorption property of T2 is partially excluded in such crosses and its yield is only 5 to 10%. The role of T4 genes in the exclusion of T2 has been investigated with marker-rescue. UV-irradiated T4 was crossed with unirradiated T2 as a helper phage and marker-rescue progeny were selected with various bacterial strains. In this way, T2 like phages were found with T4 adsorption properties (ref. 1). They did not show exclusion of T2 and they were sensitive to exclusion like T2. There appeared to be no advantage for T4 over T2 adsorption, which might have led to a head start for T4 during intracellular development with exclusion of T2 as a result. Six progeny were selected for plating with large plaques on K (λ) like T4. Five of them were like T2 with respect to exclusion, but one was resistant to exclusion by T4. This strain (T2exr^{+4}) showed high yields of host range (h) and r markers of T2 (ref. 4, 6) and appeared to induce the T4 α- and ß-glucosyl transferases (gt) in the bacterial host. This is in contrast to the other five T2-like marker-rescue progeny which were T2-glucosylated. The possible relationship between resistance to exclusion and T4 glucosylation was further investigated in experiments designed to obtain strains with modified glucose content of the DNA.

1.1 Attempts were made to modify the glucose content of the DNA of phage T2 by means of superglucosylation. Deoxycytidine (dC) requiring mutants of T2 were found by means of density labelling with 5-BrdC and isolation from the front of a band of phage after centrifugation in CsCl. However, propagation of this mutant in a pyrimidine requiring E. coli B in the presence of glucosylated HMC was not succesful until now. Recently, genetically glucoseless T4 strains were found by Hosoda and Georgopoulos. These strains are used now to study exclusion with modified strains.

1.2 Recombinants between T4α- and T4 ß-glucosyltransferase were not found in crosses between T2 exr^{+4} and T2 αgt (a genetically glucoseless T2); it turned out that T2 exr^{+4} did not exclude the host-range property of the glucoseless T2, but still exclused the αgt gene. Therefore, the exclusion property is probably composed of more genes with specific targets.

1.3 Marker-rescue of T4 glucosyltransferase into glucoseless T2 αgt led to progeny with unexpected properties. There was a significant yield of glucosylated phage due to rescue of gt^{+}; however, the rescue products appeared to posses the T2 α-glucosylating property. This may be interpreted as the result of rescue of a very small homologous part of the αgt^{+} gene from T4 into T2. No ß-gt^{+} activity was recovered in this experiment.

Now, double mutants of T2 αgt are used in this type of investigations of rescue larger fragments of the T4 αgt^{+} gene into glucoseless T2 αgt. In this way, the location of T4 specificity within the αgt^{+} gene is open to investigation. For this purpose, fifteen hydroxylamine induced T2 αgt mutants were isolated; six of them were amber gt, which is a remarkably high proportion.

1.4 A temperature-sensitive glucosyltransferase (tsgt) from a T2 mutant (isolated by B. Molholt, Stockholm) was studied by means of its capability of in vitro incorporation of radioactive glucose in phage DNA. The following results were obtained:

The activity of the tsgt enzyme was the same as that of standard-type enzyme at 24.5° C. At elevated temperatures, the initial incorporation rate was greater than at 24.5° C, but it rapidly levelled off due to inactivation of the enzyme. The DNA of T2 tsgt phage grown at 30° C and 39° C appeared to have 41% and 6% of its HMC groups glucosylated, respectively. The 41%-glucose-HMC DNA could be further in vitro glucosylated by tsgt enzyme, isolated from the culture at 30° C that had produced the 41% glucose-HMC DNA. Hence, maturation of phage and lysis of the culture had interfered with the completion of glucosylation by the mutant enzyme. Tsgt enzyme was 4 times more sensitive

to $(NH_4)_2SO_4$ than standard-type glucosyltransferase. Pre-incubation of tsgt enzyme at 39° C, or elevation of temperature to 39° C during in vitro incubation led to irreversible inactivation of the enzyme with a residual activity of 5% (ref. 7).

2. The function of the exclusion gene

2.1 Strain T2 exr^{+4}, mentioned above, was crossed with T4. In the progeny, the gene for host-range specificity of T2 showed frequencies which were high enough (0.25-0.43) to locate the exclusion gene (ex^{+4}) of T4; it was located between host-range (h9 and (r) at the beginning of the map segment for early functions (ref. 2). Further confirmation that the exclusion property is an early function, was obtained in the following experiment:
An early amber mutant of T4 was crossed with standard-type T2 in a non-permissive host. There was no net growth of phage and if there was some complementation, it was very weak, at least for the amber function concerned. Nevertheless, the am^{+2} and h^{+2} genes of T2 were nearly eliminated from the progeny. Measurements of DNA synthesis showed a decrease of DNA content in the cross in the non-permissive bacterial host. The results can only be explained if the exclusion is a function, expressed immediately upon infection of the host (ref. 2).

2.2 The yield of T2 genes in the progeny from crosses of T2 exr^{+4} with T4 was unequal and showed a non-random distribution over the map. Polarity was observed of low T2-gene frequencies near the host-range locus to higher frequencies over the map segment for the early functions (fig.). In the region for the late functions, the T2 gene frequencies were higher than 0.4, which is next to equilibrium (ref. 5). Crosses of T4 with UV-irradiated T2 exr^{+4} revealed a lower yield of T2 genes, but polarity over the segment for early functions was still observed.

2.3 In order to study polarized T2 gene frequencies in T2 <u>exr</u>$^{+4}$ by T4 crosses with more markers, 260 <u>amber</u> mutants were isolated from hydroxylamine treated T2 <u>exr</u>$^{+4}$. The characterization was carried out with <u>ambers</u> in standard-type T4 and T2. T2 <u>exr</u>$^{+4}$ <u>am</u> gene 32 did not complement glucoseless T2 <u>agt</u>; this was also found for T4 <u>am</u> gene 32, but T2 <u>am</u> gene 32 did show complementation. Approximately the same result was found for gene 56. Obviously, T2 <u>exr</u>$^{+4}$ contains more T4 genes than might be expected from a marker-rescue product. Crosses between T2 <u>exr</u>$^{+4}$ and T2 led to the isolation of a T2-like recombinant which was excluded by T4 in gene 32, host-range, <u>r</u> and gene 39, but not in gene 56. This result with gene 56 was in contrast to the control crosses between standard type T2 and T4, where exclusion of gene 56 of T2 was observed.

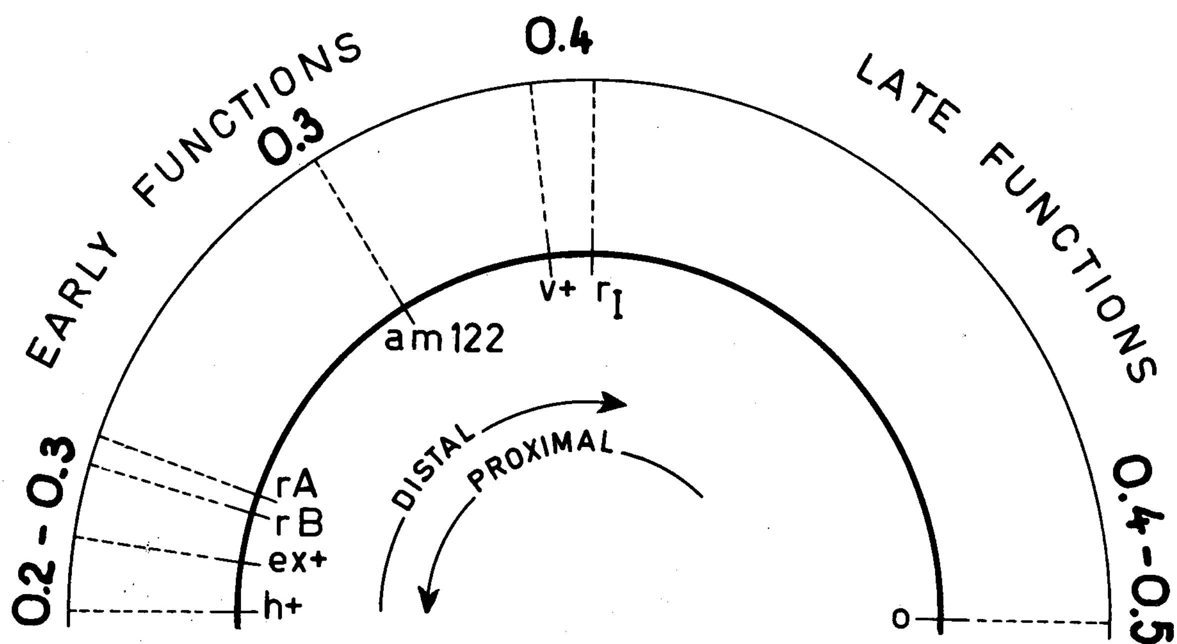
2.4 The fate of parental T2 <u>exr</u>$^{+4}$ DNA in crosses of T2 <u>exr</u>$^{+4}$ with T4 might be studied in crosses with heavily labelled T2 <u>exr</u>$^{+4}$ and density gradient centrifugation of the progeny. Control runs, however, showed different positions of the bands of unlabelled T2 <u>exr</u>$^{+4}$ and T4 after centrifugation in $CsSO_4$.

Moreover, osmotic-shock resistant strains of both parents appeared to band at higher densities than the shock-sensitive standard-type. A prerequisite for the experiment is the isolation of strains of both parents which show the same banding in $CsSO_4$ before and after crossing. This problem is under further investigation.

3. <u>Radiation damage in bacteriophage and its repair</u>

Crosses of T2 and UV irradiated T4 reveal multiple mutant progeny (ref. 1). One mutant was very unstable upon dilution and appeared to be osmotic-shock hypersensitive. Electron-microscopic pictures were made through the courtesy of Dr. W.Th. Daems (Department of Electron Microscopy, University of Leiden). They showed a majority of swollen phage heads with some penetrated phosphotungstic acid.

Crosses between standard-type T2 and UV-irradiated T4 or T2 exr^{+4} showed that progeny selected for T4 characteristics are like T2 or mutant T2. Ten isolations tested for T4 glucosyltransferase turned out to be T2-like. One of the ten was a weak enzyme but it was capable to glucosylate in vivo its DNA to the full extent. A second demonstrated about 10% of its a-activity versus 2% in the control, when tested under the condition for ß-glucosyltransferase activity. In another marker-rescue experiment where selection was made for the T4 host-range property, a significant number of T2 host-range mutants was found. The results may be explained as rescue from UV T4 of a very small region of DNA homologous to that of T2 which does not govern information specific for T4 adsorption. There appears to be a high frequency of mutants among the progeny selected for marker rescue.



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Group III - Studies in regulatory mechanisms of somatic cells in vitro

1. Cell size

Measurements of cells under the microscope revealed that the frequency distributions of the cell diameters of diploid human skin fibroblasts changed during cultivation in vitro, the distributions became broader and polymodal. The modes of the distributions indicate that in these cell populations three cell types can be distinguished. In human aneuploid cell lines the same modes occur, indicating that gross abnormal nuclear contents are not strongly reflected in cell size. When the modes of diploid cells in phase II, the phase of active growth, are compared with the modes in phase III, the phase of cell degeneration, there appears to be a shift of the modes. Also under poor growth conditions changes occur in the frequency distributions of cell diameters.

As this way of experimentation provided useful information regarding organization of cells and their response to changes in their environment a coulter counter was introduced, which allows more accurate and faster measurements. A shearing apparatus was constructed to provide truly unicellular cell suspensions, and a salt solution had been developed, which prevents cells from attaching to the glassware without killing them.
It is possible to store the cell suspension in the cold room for a period of several days without major changes in cell volume and cell number, which is important, when an experiment has to be carried out in which a lot of volume distributions have to be determined.
The equipment could be calibrated by comparing measurements of the coulter counter with measurements under the microscope.
The influence of temperature on the measurements proved to be slight.

Each complete measurement gives information about modus, mean, dispersion, skewness and cell density.

Present work is oriented towards an understanding of the interrelationship of these parameters in the BSC cell line under conditions of growth and contact inhibition and under suboptimal conditions.

Thus far, evidence has been obtained for the existence of two stable types of contact inhibition, a finding which will allow us to define contact inhibition more clearly. In addition, it is found that the effects of unfavourable conditions are mainly reflected in enhanced skewness and dispersion of the distribution. It is hoped that this will enable us to quantitate maladjustment of the cells to the culture conditions and to handle this criterion as a factor in experiments on contact inhibition.

2. Cell transformation

Activities have centered on acquiring a system for the induction of cell transformation with X-rays. BSC cells could be transformed and the transformed state proved to be hereditary. However, the variation in cell-transformation frequency is high and the occurrence of many partly transformed clones indicates a delayed effect of X-irradiation.

3. Chromosome studies

Chromosomes of diploid human cells and of an aneuploid cell line have been measured. From these measurements it appeared that it is possible to distinguish the groups of chromosomes; however, only the best metaphases can be used for this purpose. As only a few metaphases were obtained from cells in the later phases of growth and dicentric and ring chromosomes are not easy to classify by measurements, the cells are now analysed by karyotyping. A survey of the pertinent literature revealed that it is possible to identify some characteristics of tumors by analysis of karyotypes metaphases. Statistical analysis of metaphases of a tumor cell population provided some evidence for the idea that all the cells of a tumor cell population could have developed out of one

parental cell with an abnormal genome. This might lend support to the theory of a relationship between a specific chromosome and malignant cell-transformation. In other analysis, evidence was obtained for a preferential translocation in tumor cell populations of D with G chromosomes, which resulted in an excess of C chromosomes.

From cells in the later phases of growth it is difficult to obtain metaphases. Some experiments have been carried out to increase the number of metaphases. Cells have been treated with a high concentration of colcemid and with cold shock... Both treatments, however, have adverse effects on the cells, and consequently this method has to be abandoned. The chromosomal changes of two human fibroblast cellstrains have been analysed.
From one cellstrain metaphases of the last subculture were obtained. The cells were found to be completely aneuploid. Many dicentrics and some ring chromosomes were present. The cells can be grouped into three categories, one with a mode of 39 chromosomes, one with a mode of 77 chromosomes and one with a mode of 150 chromosomes. From the second cellstrain some preparations were made from the 8th, 10th, 14th, 16th and 17th subculture. This cellstrain was in culture for about three years. The cells of the 8th subculture showed a normal chromosomal distribution. From then on, an increase in tetraploid cells occurred and, finally, the cells became aneuploid. Dicentric chromosomes and rings were present. At the 17th subculture the cell population resembled that of the first cellstrain.
A third cellstrain is currently under investigation.

In view of the fact that human chromosomes are not individually recognizable these are less ideally suited for studies on chromosomal changes. However, Potorous tridactylis, the Tasmanian rat kangaroo, offers great advantage for this purpose. The chromosomes of this animal can be individually recognized. In 1966 the first cellstrain was started. It survived only three passages and was still normal. In 1967 several strains have been started and most of them are growing satisfactorily. Some of them were in the 18th passage by the end of 1967.
Normal cells are still in the majority, but there are a great many of polyploid cells and the frequency of dead cells in the preparation increases slowly.

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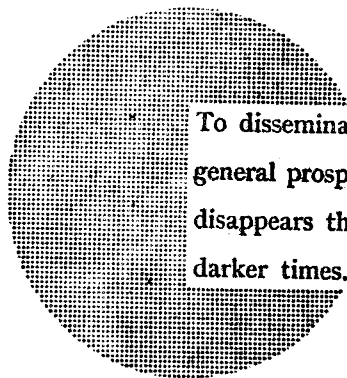

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Alfred Nobel

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