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ASSOCIATION
EUROPEAN ATOMIC ENERGY COMMUNITY – EURATOM
UNIVERSITY OF LEIDEN, NETHERLANDS

MOLECULAR AND RADIATION GENETICS

Annual Report 1966

1968

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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II. Relation between structure and function of the DNA of viruses and the effects of radiation modification.
III. Genetics of micro-organisms and the effects of radiation upon these.
IV. The effect of ionizing radiation on DNA.

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KEYWORDS

RADIATION EFFECTS
PROTEINS
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IN VITRO

VIRUSES
BACTERIOPHAGES
DNA
BIOCHEMISTRY
RADIATION EFFECTS
PHOSPHORUS 32
LABELLED COMPOUNDS
TRACER TECHNIQUES

ULTRAVIOLET RADIATION
IONIZING RADIATIONS
IRRADIATION
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MITOSIS
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MICROCOCCUS LYSODEIKTICUS

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INTRODUCTION

The research activities carried out in the period between January 1 and December 31, 1966 continue the program of research in molecular biology and radiobiology which has been described in the previous annual report (EUR 2983 e).

As before the work to be reported stood under the general supervision of Professor J.A. Cohen, director of the laboratories of Physiological Chemistry, Applied Enzymology and Radiobiology of the University of Leiden and the Medical Biological Laboratory RVO-TNO. During 1966 no significant changes were made in the general line of research reported previously. Six groups of research workers, each under supervision of a senior scientist, took part in the progress of the total program. The various subjects of research and the scientific personnel engaged therein are listed below.

Group I — *In vitro* induction of protein synthesis in general and 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
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 effects of radiation modification.

Senior Scientist:	Prof. Dr. H.S. Jansz
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

Manuscript received on December 11, 1967.

Scientists:	Miss Drs. I.E. Mattern
	Dr. 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
	Dr. J.B.Th. Aten
Scientists:	Ir. J..F Bleichrodt
	Ir. L.H. Luthjens
	Drs. G.P. van der Schans
	Drs. A.J. Hoff
	M.G. Stern, B.A.

Group V — Mechanism of action of enzymes and modifications by radiation.

Senior Scientist:	Dr. R.A. Oosterbaan
Scientist:	Dr. F. Berends

Group VI — Investigations of mutations that modify the biological activity of enzymes.

Senior Scientist:	Dr. A. de Waard
Scientists:	Drs. F.A.J. de Vries
	Ir. H. van Ormondt

CURRENT RESEARCH ACTIVITY

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

The work concerning the mechanism of strand selection in transcription from DNA to RNA using RNA-polymerase was continued, with various forms of ΦX174 DNA as templates. RF I (the double stranded ΦX174 DNA containing two separately continuous strands) is transcribed asymmetrically whereas RF II (containing 3-6 single strand breaks) is transcribed symmetrically.

The possibility that a single strand break gives a switch from asymmetric to symmetric transcription is under investigation.

Investigations concerning the presence of viral genomes in virus induced tumours using polyoma and adeno virus induced tumours was extended with plant tumours induced by agrobacterium tumefaciens in tobacco plants.

The latter subject was performed in collaboration with the Department of Biochemistry (Science Faculty). Preliminary evidence indicates that at least part of the bacterial genome is present in the plant tumour.

The formation of collagen in polysomes of cells of 7-16 days chicken embryo's and fibroblasts could be demonstrated after *in vivo* administration of C-14 proline on account of the presence of C-14 hydroxyproline in sucrose gradients of cell extracts. The bacterial cell free system for protein synthesis was used to study the stimulation of protein synthesis by RNA fractions extracted from embryo tissue and fibroblasts.

The biochemical study of the influence of RNA derived from spleen of sensitized mice on the induction of the antibody was stopped this year. Several methods were used for the extraction and fractionation of RNA and two different immunological test systems were employed. Although a good fractionation of RNA could be obtained it was not possible to induce *in vitro* antibody-formation in non-sensitized spleen cells.

Presently the induction of the immune response in intraperitoneally implanted diffusion chambers is studied at a cellular level. Spleen cells from non-primed mice could be induced to synthesis of 19S antibodies but not of 7S antibodies by sheep red blood cells.

Group II — Relation between structure and function of the DNA of viruses including bacterial and animal viruses and the effects of radiation modification

A new method could be developed which allows the purification of double stranded DNA (RF) of bacteriophage ΦX174 from phage infected cells in quantities of several milligrams.

Electron photomicrographs of RF I and RF II were obtained in cooperation with Dr. E. van Bruggen of the State University of Groningen. The results confirm earlier conclusion that RF I is a cyclic molecule which shows many tertiary twists. In RF II preparations most of the molecules are represented by open rings or slightly twisted rings. This difference in tertiary structure explains the difference in S value (21S for RF I and 17S for RF II) between the two forms of double stranded ΦX DNA.

Preliminary experiments suggest that a separation of the + and — strand of denatured RF II can be obtained using density (CsCl) centrifugation at alkaline pH and also that both strands are biologically active.

The T4-bacteriophage transformation system was used to continue studies on the relationship between the structure of DNA and its biological activity (G. Veldhuisen, Thesis "Genetic transformation of bacteriophage T4", Leiden).

The biological activity of DNA drops drastically when it is degraded to a molecular weight below 2×10^6.

The biological activity is not enhanced when the low molecular weight material has been annealed with DNA of (marker free) high molecular weight.

Hybridization experiments using ^{32}P labeled T4-DNA showed the possibility of the concentration of DNA fragments containing the (fragmented?) rII genome.

Efforts made to isolate biologically active fragments of DNA containing the rII genome were so far unsuccessful.

Studies on the structure and molecular weight of adenovirus DNA have been continued. The molecular weight of the DNA of non-tumorigenic adenovirus type 5 was found

to be 23 $\times$ 10^6, using $S^0_{20,w}$ and the intrinsic viscosity. This value agrees well with the molecular weight calculated from the length of DNA molecules on electron micrographs, made by Dr. E. van Bruggen.

Preliminary investigations with the DNA of the tumorigenic adenovirus type 12 suggest that the molecule has a structure which is similar to that of type 5 DNA (i.e. linear). Further studies on the structure of the DNA's of type 12 and of tumorigenic type 18 are in progress.

The search for virus specific messengers RNA in polyoma and adenovirus tumours has been abandoned because American authors have demonstrated that these messengers are indeed present in those tumours. Efforts will be made to demonstrate the presence of viral DNA in tumour cells by DNA-RNA hybridization. For this purpose, polyoma virus DNA was freed from host cell nucleic acids and a method was found to extract chromosomal DNA from transformed hamster cells.

The study of the fate of infecting adenovirus DNA in human and hamster cells is in progress. ^{3}H-thymidine-labeled adenovirus type 5 has been grown, and a method has been developed to extract DNA from HeLa and hamster cells.

Preliminary studies have been made regarding the inactivation by ultraviolet irradiation of the infectivity of single and double stranded polyoma virus DNA.

The results obtained so far indicate that denatured, single stranded DNA is inactivated faster by ultraviolet irradiation than native, double stranded DNA.

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

It is known that the sensitivity of Escherichia coli to ultraviolet (UV) and X irradiation is determined by several genetic loci. Using mutant strains defective in one of these loci information has been gained on the genetic control of radiation sensitivity and the biochemical processes involved.

So far five different phenotypes can be distinguished among the mutants isolated:

a) mutants in which the ability to divide after X or UV irradiation is reduced (Fil mutants),

b) UV sensitive mutants which have lost the ability to repair UV lesions in bacteriophage DNA (Hcr mutants),

c) UV sensitive mutants which have *not* lost the ability to repair UV lesions in bacteriophage DNA (Uvr mutants),

d) UV sensitive mutants which are X ray sensitive as well (Exr mutants),

e) UV sensitive mutants which are X ray sensitive and in addition are defective in genetic recombination (Rec mutants).

Special attention has been given to a study of the properties of various Rec mutants and some Exr mutants. Mutations that lead to loss of the ability to recombine may occur at three different sites of the bacterial chromosome. The mutations *rec-35* and *rec-38* are located near *thr*, the mutation *rec-36* near *thy* and the mutation *rec-34* probably between *his* and *try*. The three genotypes of such mutants can also be distinguished from each other phenotypically on account of their prophage induction pattern. Biochemically the strains show differences in the excision of photoproducts from their DNA after UV irradiation.

Mutations of the type Exr appear to occur near mutations of the type Hcr and Ucr. The genetic relationship between the corresponding genes is studied by genetic fine structure analyses with the transducing phage Plkc. So far no definite proof could be given whether different genes are involved that belong to a single operon.

A new class of X ray sensitive mutants was found recently. All X ray sensitive strains, tested so far, were also UV sensitive. The members of the new class showed increased sensitivity towards X rays but were only slightly more sensitive to ultraviolet. Investigations to locate these mutations on the bacterial chromosome are in progress.

Several UV and X ray sensitive mutants of Salmonella typhimurium were isolated. Genetic studies to map these mutations are also under way (Prof. Dr. A. Eisenstark — Manhattan, Kansas — visiting scientist).

Extracts from Micrococcus lysodeikticus are capable of restoring *in vitro* damage caused by UV irradiation in double stranded ΦX174-RF DNA. Studies on the purification of this "repair system" resulted in a hundredfold purification of this enzymic entity in 30 % yield. Several Hcr-mutants of M. lysodeikticus were isolated and tested for their ability to repair UV lesions. These strains appear to be less capable than normal cells in excising thymine-dimers from their DNA after UV treatment. However, extracts prepared from these strains are still capable of repairing UV lesions *in vitro* as assayed with the ΦX174-RF DNA-E. coli spheroplast system. This indicates that a multiple-enzyme system is responsible for repair of UV damage in DNA.

From a Pseudomonas strain (isolated from soil), grown on the alkaloid atropine as the only carbon source, a thousand mutants were isolated which were blocked in the breakdown of atropine. From studies with these mutants it could be deduced that the atropine is first split into tropine and tropic acid by an esterase and that the tropic acid is converted into phenylacetic acid that is further metabolized. Two more enzymes, which are involved in the conversion of tropic acid into phenylacetic acid, were characterized and partially purified. The Pseudomonas strain shows a remarkable high mutation rate for the unability to use atropine as a carbon-source, even without irradiation or treatment with a mutagenic agent. In a growing culture less than 10^{-5} auxotrophic mutants are found. However, the number of "atropine-mutants" exceeds 10^{-2}. So far there are no indications that the metabolism of atropine is controlled by an episomal element. Alternative possibilities for the high mutation rate are under investigation.

Group IV — The effect of ionizing radiation on DNA

1. *The biological significance of alterations in DNA induced by ionizing radiation*

a) The radiation chemistry of DNA was further studied, using concentrated solutions in the range of 1-5 mg/ml. In order to minimize the amount of ΦX174 DNA necessary for the experiments, mixtures were used of ΦX174 DNA and denatured calf thymus DNA in a ratio 1 : 50. In the region 1-2 mg/ml such mixtures were exactly comparable to pure ΦX174 DNA as far as radiosentitivity is concerned. This work with concentrated solutions proved necessary because it appeared that at low concentrations too many free radicals were lost without reacting with DNA.

From measurements of the inactivation dose as a function of concentration it could be concluded that at zero dose 1.9 DNA molecules are inactivated per 100 eV of absorbed energy. This value is close to the yield of destruction of nucleotides in DNA as estimated from extinction measurements, but much smaller than the total yield of free radicals of

all types (OH, H and e^-_{aq}, total yield about 5). The explanation is that the inactivation at high DNA concentration is predominantly due to OH-radicals. This was demonstrated by the large protection afforded by iodide ions (which remove OH radicals only), by the twofold increase of radiosensitivity in the presence of N_2O (which converts e^-_{aq} into OH) and by the absence of a clearcut effect of O_2 (which scavenges H and e^-_{aq}).

At low DNA concentration (5 to 50 μg/ml) the yield of inactivation is about 10 times lower. In this case only about half of the inactivations is due to OH-radicals and e^-_{aq} and H are responsible for the other 50 % of the radiosensitivity. At present the most likely explanation is that small amounts of impurities remove a large fraction of OH and a much smaller part of e^-_{aq} and H. In concentrated solutions such impurities are much less effective because the more homogeneous distribution of the nucleotides in DNA in the solution gives the DNA a better chance to compete with the small contaminating molecules.

It was shown that H_2O_2 does not react with DNA under the condition of the experiments. The speed of this reaction can be increased considerably by the presence of traces of metal ions but even then the reaction is too slow and the concentration of produced H_2O_2 too small to be of any importance as compared with inactivation by free radicals.

If the present results are compared with those obtained by irradiation of DNA in a solution of free nucleotides in the absence of oxygen, which were reported earlier, the conclusion is reached that the bound nucleotides in single-strand DNA react 5 times slower with OH radicals than free nucleotides.

b) The work on changes in T4 DNA after irradiation of whole phage was continued. The number of double-strand breaks per unit of dose in nitrogen appears to be constant provided that a sufficient amount of radical scavenger is present in the suspension to prevent separation of phage protein and nucleic acid during irradiation. The inactivation dose is dependent on character and amount of scavenger. Under conditions generally accepted as preventing indirect effects (e.g. broth) a comparatively large fraction of the biological damage is still due to other causes than double-strand breaks (up to 40 %). In the presence of a sufficient concentration of cysteamin (without O_2) a maximum value of the D_{37} is obtained of about 180 krad. In this case the number of double strand breaks per unit of dose is not significantly altered and nearly all the biological damage is accounted for by such breaks. If oxygen is introduced the number of double strand breaks becomes about twice as large but the inactivation dose increases approximately twofold, presumably because O_2 removes reducing radicals and prevents them from attacking the protein. Under these conditions the number of lethal hits is exactly equal to the number of double strand breaks.

These results seem to show that data in the literature on the radiosensitivity of this bacteriophage need reinterpretation. The fact that in broth effects of oxygen are small or absent seems to be due to the compensation of the increased double strand breakage by the scavenging of reducing radicals. The oxygen effect in the presence of cysteamin can nearly completely be accounted for by increased double strand breakage. The scavenging effect of oxygen is now small because cysteamin removes all types of radicals efficiently.

Single strand breaks are not very important from the point of view of inactivation because 10 to 20 of such breaks are produced per lethal hit.

c) In the previous report it was mentioned that subviral particles of T4 can be produced by irradiation of bacteriophage in buffer. Such particles are also made by treatment with urea and very efficiently by heating at 70° C.

The latter particles proved to be peculiar in that they showed a much lower sedimentation coefficient of about 200S. It was attempted to isolate them without loss of biological activity in sufficient amounts to examine their nature but these attempts have been unsuccessful so far.

d) After irradiation of calf thymus DNA solutions in the absence of oxygen a fluorescence was observed that was not found in unirradiated solutions. In the presence of oxygen this fluorescence was weaker by one or two orders of magnitude. By comparison with free nucleotides it could be shown that this fluorescence is due to radiation products of the purines, especially guanine. Two components are present, one with an excitation maximum at 315 nm and emission maximum at 400 nm and the other with the respective maxima at 360 and 450 nm.

e) The protective effect of sulfhydryl compounds against irradiation effects on ΦX174 DNA in solution was examined as a function of pH. Cysteamin clearly showed a maximum at pH 8. The protection by thioglycol and cysteine was independent of pH except that the protection decreased twofold at a pH value of approximately 10.

2. *Induction of mutations by ionizing radiation*

The enhancement of mutation induction in irradiated solution of ΦX174 DNA in the presence of sulfhydryl compounds and exclusion of oxygen was confirmed repeatedly. The bacteriophage mutants obtained behave in a irreproducible way. They are not true host range mutants but are characterized by a decreased efficiency of plating on the host bacterium which seems to get less easily infected. This decreased efficiency manifests itself only in the presence of glucose. Attempts to set up an improved mutant system were unsuccessful so far.

3. *Physical chemistry of nucleic acid*

a) The kinetics of the extinction change in the melting region was examined for DNA from the bacteriophage T4. In these experiments the response of the extinction to a sudden temperature change of about one degree was measured. Contrary to the theory developed by Crothers it was found that two processes, each with its own relaxation time, are involved. The relaxation times are independent of molecular weight but the ratio of the contributions of both processes to the hyperchromic effect depends on molecular weight or on the number of single strand breaks in the DNA molecule. Difficulties were encountered by the introduction of strand breaks due to the current in the heating spiral. Pyrophosphate proved very effective in the prevention of this effect. It is possible that the effect is due to free electrons originating from the heating spiral, because tests with dilute solutions of ΦX174 DNA showed that pyrophosphate protects against inactivation by hydrated electrons, but not against OH radicals and H radicals.

b) The study of T4 DNA by sedimentation was continued. The influence of concentration on the sedimentation coefficient appeared to be dependent on the ionic strength of the solution. From freshly prepared native T4 DNA a preparation of single strand molecules can be obtained by alkali denaturation that is nearly homogeneous with a sedimentation coefficient 160S. Such preparations are much more stable against single strand breakage than native DNA in which single strand breaks occur much faster.

The molecular weights of T4 and T2 DNA did not differ significantly. This was shown by measurements of sedimentation coefficient and viscosity. The possibility of such a difference was suggested by published length determinations with the electron microscope.

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

It has been postulated that the amino acid sequence his-cys-s-s-cys-x-his forms part of the active site of a number of DFP-sensitive proteases. Our investigations to establish whether this sequence also occurs in horse liver esterase (a model enzyme in the group of DFP-sensitive esterases) were continued. Using the "diagonal" electrophoresis technique the existence of several his+cys containing peptides could be isolated. Work is in progress to establish the amino acid sequence of those peptides.

Our interest into the interaction of atropine and atropine-like substances with biological receptors leads to the investigation of the mode of action of an enzyme which is able to hydrolyze the ester bond of atropine. It was found that such an enzyme was produced by a Pseudomonas strain when grown under certain conditions. The enzyme exhibits a great specificity towards (—) atropine. The tropic acid part as well as the tropine part of the ester are required for maximal activity. The enzyme shows the phenomenon of substrate inhibition. This and other evidence suggest that this enzyme (like acetyl-cholinesterase) contains an anionic site next to an esteratic site. Moreover the enzyme is inhibited by organophosphates due to phosphorylation of a serine hydroxyl group.

Work is in progress to extend our knowledge on the substrate specificity, on the mode of action and on the active site of the enzyme.

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

1. *Bacteriophage T4-induced DNA polymerase*

In the previous annual report a description was given of the experiments leading to the identification of the structural gene for T4 specific DNA polymerase. In view of the work of Speyer, who showed that some mutants defective in this gene are mutagenic, an investigation of the mutagenic site of the enzyme was planned. Since it was suspected that the T4 induced DNA polymerase consists of sub-units, studies were undertaken to show intragenic complementation. Following unpublished suggestions by Edgar, several pairs of temperature sensitive mutants in gene 43 were screened for intra-allelic complementation by comparing their ability to produce viable phage at 42° C at mixed infection compared with single infection. The results listed below show that intra-allelic complementation in this gene indeed occurs.

Percent complementation among temperature-sensitive mutants (gene 43) of bacteriophage T4D

	burstsize 42° C		*burstsize 42° C as percent of wildtype at the same temperature*
1. T4D wild	200	260	100 %
2. P26 × G40	0.50		0.25
3. L88 × G43	0.2		0.1
4. L88 × CB79	0.4		0.2
5. L56 × P26	17.6		8.8
6. L56 × G40	6.5		3.7
7. L56		0.11	0.04
8. P26		0.08	0.03
9. G40		0.06	0.02
10. L56 × L159 *	77.4		38.2

* Inter-allelic complementation: L159 is a mutant in gene 45.

In our hypothesis it is assumed that the mutagenic site of the enzyme resides on a distinct sub-unit. If this is so, mutagenic mutants (in gene 43) should belong to a common complementation group. Our studies on intra-allelic complementation will be extended with mutagenic mutants other than L56.

In order to establish by chemical means whether the lesions in mutagenic mutants are confined to a certain region of the enzyme molecule attempts are being made to obtain the T4 induced DNA polymerase in a homogeneous form.

2. *Specificity of bacteriophage T4-induced DNA-glycosyl transferases*

Our studies on this subject have been carried out along the lines mentioned in the previous report and are near their completion. It was shown that the relative amount of α and β glucose molecules introduced *in vitro* into HMC-DNA depends on the Mg-ion concentration of the incubation medium and is independent of the nucleotide sequence with one exception. This single case concerns sequences with adjacent hydroxymethyldeoxycytidine residues. α-Glucose is only introduced into -glcH-H-. The final results on the distribution of β-glucose will soon become available; it is almost certainly present in both H residues of -H-H- sequences.

The ratio of α and β glucose introduced into T4 DNA *in vivo* was shown to be independent of the Mg-ion concentration of the growth medium. A manuscript of a scientific publication on this subject is in preparation (De Waard, Ubbink and Beukman).

3. *Characterization and function of enzymes capable of methylating ribosomal RNA*

The presence of a variety of such enzymes was shown in extracts of normal ribosomes of E. coli. These extracts also contain submethylated rRNA (probably present in still unfinished ribosomes). The enzymes different from two of these (described already by Hurwitz *et al.*) will be characterized. It is intended to establish the role of rRNA methylation in the maturation of the ribosomal particle.

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INTRODUCTION

The research activities carried out in the period between January 1 and December 31, 1966 are a logical continuation of the program of research which has been outlined in the previous annual report (EUR 2983 e).

As before the work to be reported was carried out under the general direction of Professor F.H. Sobels, Director of the Department of Radiation Genetics of the University of Leiden. The work was divided between the following three sub-groups in which the scientists who contributed are listed:

Group I — Mutation Studies with Drosophila

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

Group II — The effect of ultraviolet and ionizing radiation on gene function, genetic recombination and replication of bacteriophage DNA.

Senior Scientist:	Dr. B. de Groot
Scientist:	Dr. E. Pees

Group III — Studies in regulatory mechanisms of somatic cells *in vitro*.

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

1. Mutation Studies with Drosophila

1.1. *Modification of radiation-induced mutation frequencies by RNA or protein synthesis inhibitors (F.H. Sobels)*

In earlier experiments it was observed that pre-treatment with chloramphenicol or ribonuclease lowered the radiation-induced mutation frequency in spermatids, but enhanced

that in sperm cells sampled during the first two days after radiation exposure. The same was found after pre-treatment with actinomycin D. by G. and A. Olivieri, and the reduction of radiosensitivity in spermatids by this agent has now been extensively confirmed in further experiments.

To verify whether inhibition of RNA and/or protein synthesis increases the radiation-induced mutation frequencies in fully mature sperm, a number of experiments has been carried out. Following pre-treatment with ribonuclease (0.05 mg/ml) this has indeed been found for sperm sampled from both males and inseminated females, and in sperm used for the second ejaculate. After an initial observation of radiosensitization by puromycin, inconsistent results were obtained in later experiments.

The effect of actinomycin on mature sperm has now been studied in six replica experiments, but no consistent data have been obtained.

1.2. *The effects of pre-treatment with sodium fluoride or dinitrophenol (R.N. Mukherjee)*

Further data have been obtained on the effects of sodium fluoride under different conditions of pre- and post-treatment with either N_2 or O_2 in mature sperm sampled from the first ejaculate of treated males. The results of these experiments are similar to those reported earlier. They confirm the significant radio-potentiating effect of sodium fluoride, irrespective of the conditions of pre- or post-treatment with N_2 or O_2. Furthermore, following sodium fluoride pre-treatment and irradiation in N_2, post-treatment with N_2 favours repair in mature sperm. In other words, N_2 dependent repair in mature sperm is not affected by sodium fluoride pre-treatment. This effect has been consistently found in all the three experiments carried out. The mechanisms underlying this interesting interaction of sodium fluoride and N_2 are being further explored.

For an analysis of the radio-potentiating effect of NaF, dominant lethal frequencies were studied in irradiated mature sperm samples, which were pre-treated with either NaF or saline. The results show no significant difference in the frequencies of dominant lethals induced by either treatment. The same treated males were tested for the induction of sex-linked recessive lethal mutations and then the NaF treated group showed a significantly higher mutation frequency as compared to that of the control group which had received saline pre-treatment. This seems to indicate that NaF does not affect the initial radiosensitivity, and that its potentiating effect on radiation-induced mutation frequency is presumably brought about by the inhibition of a repair process.

To gain an insight into the nature of the energy-requiring repair process, experiments are under way to study the effects of blocking the energy supply by the use of 2,4-dinitrophenol which is an uncoupling agent for oxidative phosphorylation and an inhibitor of ATP synthesis. Several preliminary experiments have been done to gather information regarding optimal conditions for future experimentation. The results so far obtained show no effect on the induction of recessive lethals in mature sperm.

1.3. *The effects of oxygen and nitrogen post-treatments on the frequencies of X-ray-induced dominant lethals and on the physiology of the sperm (K. Sankaranarayanan)*

The rationale of this work can be traced to a personal communication from Dr. S. Abrahamson in which he raised the possibility that the post-radiation reduction of mutation and translocation frequencies in mature sperm by nitrogen is persumably an artefact arising as a result of selection process. If it is assumed that nitrogen post-treatment un-

favorably affects the restitution or reunion of chromosome breaks this might result in the conversion of potential recessive lethals and translocations into dominant lethals and hence lead to their selective elimination. If this explanation were correct, one would expect nitrogen post-treatment to result in a greater frequency of dominant lethals. Abrahamson (unpublished data) indeed found this to be the case for sperm sampled from the first ejaculate of 7-day-old males (Oregon-R) but only after post-treatment lasting 60 minutes and not after post-treatments lasting only 30 minutes. It must be mentioned that all the earlier results on post-radiation modification of the frequencies of recessive lethals and translocations were obtained with nitrogen post-treatments which did not exceed 25-30 minutes or for inseminated females lasted only 15-20 minutes. A verification of Abrahamson's hypothesis with the stocks used in earlier studies was nevertheless considered important since if it were true, the concept of repair processes operating in mature sperm would need to be re-examined. It was later learnt that Abrahamson would not confirm his results in subsequent experiments. However, the experiments were carried through to completion, since besides demonstrating the lack of validity of Abrahamson's hypothesis, these studies exposed certain other aspects of radiation and physiological damage in mature sperm.

The results of eight extensive experiments with either nitrogen or oxygen post-treatments for different durations (25 min., 50 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 ($Y = 0.6329 + 0.0056X$, 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 spermathecae 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) the number of sperm stored in the storage organs decreased with time, 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.

1.4. *The effects of immediate post-treatment with 10 Atm.* O_2 *or* N_2 *on the mutation frequencies induced by exposures to pulsed electrons in* N_2 *or* O_2 *(F.H. Sobels)*

To test whether the post-radiation enhancement by O_2 in mature sperm (after irradiation under anoxia) is due to reaction with radicals, a number of experiments were carried out with 1.8 MeV pulsed electrons at the BECC Unit in Radiobiology of Mount Vernon Hospital, Northwood (Middlesex). Radiation exposure lasted no longer than approximately 100 msec. Before and during irradiation the flies were kept under 10 Atm. of N_2. Immediately after irradiation the flies were post-treated with 10 Atm. O_2, and this was compared then with the effect of high-pressure N_2 post-treatment. Four separate series of experiments at dose levels of approximately 1000, 2500 and 4000 rad have so far been carried out. In none of these a delayed oxygen effect for either recessive lethal 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 a remarkable proportionality of mutation frequencies to dose could be observed. Since no other genetic data are available for pulsed electrons, this information by itself seems of certain value.

1.5. *Dose-fractionation studies on mature sperm* (B. Leigh)

It has been shown by Sobels and co-workers 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 carried out using males carrying a ring-X chromosome.

All irradiations were in nitrogen. Unfractionated exposures were 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. 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.

1.6 *A study into the occurrence of translocations involving both the paternal and maternal chromosomes (F.H. Sobels)*

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 to see 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.

1.7. *The determination of RBE-values for fast neutrons in dependence of the degree of oxygenation of the irradiated sperm cells (F.H. Sobels)*

It is well-known that damage, produced by fast neutrons, is less dependent on the presence of oxygen than that induced by X-irradiation. 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 had 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 (Sobels 1966). 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 (HVT 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. The RBE-values 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.

1. For lethals, very low RBE values are observed with the exception of sperm from young males, irradiated with 3000 rad. 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:

a) 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*, in the press).

b) 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 rad 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 rearrangements 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.

2. 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 rad. 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.

The experiments are being continued at the highest dose level.

1.8. *A study on the possible effect of dose rate on induced sex ratio changes, chromosome loss and non-disjunction in spermatocytes (B. Leigh)*

In earlier studies (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 2000 R, given at either 2600 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 six tester females per male per day, and in each treatment the fifth brood was split into two 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. These variations could not be correlated with either dose rate or post-treatment.

1.9. *Oxygen-dependent repair in early spermatids (W.A.F. Watson)*

Following the finding (see previous report) that an oxygen-dependent repair system exists in early spermatids sampled from Drosophila pupae, further experiments have been carried out to investigate the phenomenon in more detail.

Firstly, cytological studies of the pupal testis at the time of irradiation showed that the germ cells in which the repair occurs are in fact early spermatids. Secondly, using the same techniques as mentioned in the previous report, data have been collected concerning the effect of different doses, and the production of dominant lethals and translocations.

1.9.1. *Dose-effect relationship*

When doses of 1250 R and 3750 R were given at the same dose rate of 46 R per second, as had been used in first experiments using 2500 R, the same consistent reduction of the sex-linked lethal frequencies was observed. When the results of these experiments were combined with those of the 2500 R series by means of 2×2 contingency tables, the probability of the reduction by oxygen being due to chance is less than one in a million. The most interesting feature of the results was that the absolute reduction of the mutation frequency by oxygen was the same at all three doses. This suggests that the repair system becomes saturated at high doses, and can cope with only a limited amount of damage, as at the low dose level the repair is much more effective, approximately 75 % of the damage being repaired at 1250 R as compared to 15-20 % at 3750 R. This is similar to

Russell's explanation on the effectof dose and dose rate in the mouse. It is hoped that the use of still lower doses will support this idea.

1.9.2. *Translocation and dominant lethal studies*

Tests for translocations, carried out simultaneously with those for lethals, showed that they behaved in exactly the same manner as the lethals, i.e. being reduced in frequency by oxygen post-treatment as compared to that with nitrogen. As Oster and Falk had proposed that up to 12 hours were required for restitution of breaks induced in spermatids, it is possible that the effect on translocations is due to the repair of potential breaks.

The possibility also existed that the "repair" was actually an artefact caused by the selective elimination by the oxygen post-treatment of cells carrying mutations. If this were so, then one would expect that the frequency of dominant lethals would be greater in the oxygen post-treated group. A dominant lethal test was therefore carried out, and again the frequency of lethals was lower following oxygen post-treatment, which rules out the above argument.

1.10. *Dose-rate effect in the repair of radiation damage in spermatids (K. Sankaranarayanan)*

Earlier work has shown that in spermatids post-treatment with HCN was effective in increasing the mutation frequency only if X-irradiation was administered at a relatively high dose-rate of over 33 R/sec but not at dose-rates of 8.3 R/sec or lower. It was therefore considered of interest to investigate the effect of low dose-rates on repair of radiation damage in spermatids sampled from 24-hr-old pupae. A series of experiments paralleling the high dose-rate experiments of Watson (46 R/sec) were carried out at two exposure levels (2500 R and 1250 R) with four different dose-rates (16.6 R/sec, 8.3 R/sec, 4.2 R/sec and 2.1 R/sec). The experimental material and methods are basically the same as those used by Watson. The results indicate that the frequencies of sex-linked lethals obtained at both the levels of exposure and at the four dose-rates studied are far from being significantly different between the oxygen and the nitrogen post-treated groups. These results are thus in striking contrast to those of Watson who found a consistent and significant reduction in the mutation frequencies in the oxygen post-treated series, relative to the nitrogen post-treated ones. Furthermore, with low dose-rate irradiation, the mutation frequencies obtained with nitrogen and oxygen post-treatments are similar to those at high dose rate exposures with nitrogen post-treatment. In other words, following radiation exposures at low dose-rates, post-treatment with oxygen was no longer effective in bringing about repair. A possible explanation for this "inverse" dose-rate effect is that the repair process in spermatids proceeds in such a short time that after longer irradiation exposures, post-treatment with oxygen becomes ineffective. This hypothesis is currently being tested by means of delayed post-treatment after irradiation at high dose-rate. Preliminary data suggest that even after a five-minute delay, repair is no longer possible. Further work is in progress.

1.11. *The effect of nitrogen and oxygen post-treatments on the frequency of mosaics induced by X-irradiation (N.G. Brink)*

In view of the results previously found by Watson, that an oxygen post-treatment reduces 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 phenomenon has been observed by Nakao (*Mut. Res.*, 3, 1966, 268-272).

Using this result as a basis for the present experiments it is postulated that oxygen post-treatment following irradiation 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. The latter alternative may also occur if completes and mosaics, although arising by different mechanisms, are subjected to the same repair process following irradiation.

Although preliminary experiments using mosaic sex-linked lethals did not provide a clearcut answer to these alternatives, a specific locus test using forward mutations at the *y w sn; dp b* and *bw* loci is at present being carried out to test these alternative hypotheses.

1.12. *Y-suppressed lethals (B. Leigh)*

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 bobbed region of the X-chromosome, large rearrangements, and position effects. It was therefore considered that this technique provided a means to make a detailed investigation of the radiosensitivity 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.

1.13. *Cytogenetic analysis of recessive lethals induced in the ring-X chromosome (U. Mukherjee)*

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 throughout 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 was obtained, O_2 post-treatment would only affect restitution of broken chromosomes.

An investigation has therefore been 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.

The results so far obtained for a total of 55 lethals, show no difference in localisation of lethals recovered from a repaired and an unrepaired group. To determine what proportion of these lethals possibly consists of small deletions, cytological analysis using salivary gland chromosomes are being carried out. As a basis for this work, standardization of the band-pattern of salivary chromosomes of untreated ring-X chromosomes has now been completed.

1.14. *A study of protein synthesis during spermatogenesis using tritiated lysine (N.G. Brink)*

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 ^3H lysine as a measure of protein synthesis.

Previous auto-radiographic studies by Olivieri using ^3H thymidine and ^3H 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 stage 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 labeled after 15 minutes. The nutritive cells show little or no incorporation until about twenty-four hours after injection. Thereafter they become quite heavily labeled. At the present time the effect of pre-injection with Actinomycin D on the level of lysine incorporation is being investigated.

1.15. *The mutagenic effects of tritiated uridine (P. Kieft)*

Two hypotheses can be proposed to explain the mechanism underlying the mutagenic effect of tritiated uridine: 1. uridine may be converted to thymidylic acid; 2. uridine may be aminated by glutamine and converted to cytidine nucleotides; it is possible therefore that tritiated uridine labels both RNA and DNA.

Uridine, generally labeled with tritium (specific activity 2.18 C/mM) was injected into one day old 1) Oregon-K males (two nucleolar organizer regions on the X and Y chromosome) and into 2) males with the genetic constitution In(I) $sc^{S1L,4R}$, sc^{S1} yB/B^{S}Y, (three nucleolar organizer regions, two on the X and one the Y chromosome). The Oregon-K stock was used to find out whether uridine is incorporated in RNA or DNA, and the stock with the duplication in the X chromosome was used to find out if there is any differential incorporation of uridine. Four hours after injection the testes were dissected and washed in 0.7 % NaCl, 50 % of each group of testes was treated with RNAase or DNAase. The radioactivity of the testes and enzymes was measured separately in a "Tricarb" liquid scintillation counter. Both RNAase and DNAase showed tritium labeling; less label, however, was retained in the testes after incubation with RNAase compared with DNAase-incubated testes. This would support the idea that uridine was mainly incorporated into RNA. A comparison of the tritium activity in the testes and enzymes of the two stocks showed no difference in uridine incorporation. In order to find out whether the damage scored as sex-linked recessive lethals results from beta-rays emitted by the tritium atom, or from transmutation of tritium to a helium isotope, three types of tritiated uridine (against a control of cold uridine) were used:

1. Uridine-T (G): uridine labeled on the 5th as well as the 6th C-atom of the pyrimidine ring with tritium, specific activity 2.18 C/mM.

2. Uridine-5-T: uridine labeled only on the 5th C-atom of the pyrimidine ring with tritium, specific activity 5 C/mM.

3. Uridine-6-T: uridine labeled only on the 6th C-atom of the pyrimidine ring with tritium, specific activity 6.55 C/mM.

The label of the first and the third type of uridine may be incorporated into DNA as thymidylic acid and/or as cytidylic acid.

The label of the second type may be incorporated only as cytidylic acid into DNA, but the label is lost when uridine is converted to thymidylic acid.

Four groups of one day old Oregon-K males were injected with one type of tritiated uridine (total activity 1000 μC/ml), and crossed with virgin females. Sex-linked recessive lethals and second chromosome recessive lethals were scored in six successive broods. Uridine-5-T gave the highest mutation frequencies in broods A (0.89 %) and E (0.55 %) for the X chromosome, and in brood D (0.80 %) for the second chromosome. Uridine-6-T produced in broods D, E, and F respectively 1.11 %, 2.56 % and 1.19 % sex-linked recessive lethals, and for the second chromosome respectively 1.69 %, 1.08 % and 1.00 %.

Uridine-T (G) caused maxima in brood D (2.07 % sex-linked recessive lethals, and 3.65 % second chromosome recessive lethals).

The group injected with cold uridine showed a recessive lethal frequency not exceeding the spontaneous mutation rate.

These results suggest that 1) uridine-5-T is less effective in producing mutations than uridine-6-T; and 2) uridine-T (G) is not more effective than uridine-6-T. These differences could perhaps be explained as resulting from unequal specific activities.

To check this possibility two groups of flies were injected with uridine-6-T with a total activity of 250 μC/ml, while the specific activity in one group was 1.64 C/mM, and in the other 9.34 C/mM.

Twice as many sex-linked recessive lethals were observed in broods E and F in the group injected with 9.34 C/mM; this finding suggests an effect of specific activity on the

mutation rate. When males of the stock carrying two nucleolar organizer regions were injected with 1000 μC/ml tritiated uridine (6-T), and tested for sex-linked recessive lethals, it was observed that no recessive lethals could be detected as compared with the situation in Oregon-K males. This finding was not expected.

To gather information on the localization of the lethals on the X chromosome the so called S-5 technique was used (cf. Lindsley *et al.*, *Genetics* Vol. 45; 1960, p. 1649). Orthodox and Y-suppressed sex-linked recessive lethals were scored following tritiated uridine injection. Most of the orthodox lethals appeared to be euchromatic the Y-suppressed lethals were unstable when sub-cultured. The greatest effect was found in treated spermatogonia and spermatocytes.

2. The effect of ultraviolet and ionizing radiation on gene function, genetic recombination and replication of bacteriophage DNA

Senior Scientist: Dr. B. de Groot
Scientist: Dr. E. Pees

2.1. *The biological role of glucosylation of T-even phage DNA*

a) The isolation of genetically glucoseless or glucose-poor mutants of phage T4 was continued by using phage T2 exr^{+4}. This T2-like phage strain cannot exclude standard-type T2, shows partial resistance to exclusion by T4 and induces T4α-and T4β-glucosyltransferase in the bacterial host. With this strain, complementation tests with T2 can be carried out without the interference of exclusion of the reference phage T2. Two out of 300 *amber* mutants of T2 exr^{+4} did not show complementation with glucoseless T2, and their glucosylating properties in *amber* suppressing and non-suppressing bacterial hosts are under further investigation. Contrary to reports in the literature, glucoseless T2 showed complementation with an amber mutant of gene 44 in T4 which, accordingly, was capable of inducing T4α- and β-glucosyltransferase in the non-suppressing *E. coli B*.

b) Previously, several recombinants between T2 and T4 were isolated which showed coincidence of T4 glucosylation and resistance to exclusion by T4. The possible identity of the two properties, in the sense that additional glucosylation according to the T4 pattern protects against the exclusion factor of T4, was further investigated in superglucosylation experiments. The design is to have more glucose substituted on the T2 DNA than usual and to examine the degree of exclusion by T4. This might be achieved by interfering with the synthesis of deoxycytidine (dC) during the propagation of phage T2 and supplying glucosylated hydroxymethylcytidine (Gluc HMC). The isolation of a dC-requiring T2 mutant was carried out as follows: T2 was treated with nitrous acid and propagated in a suspension of *E. coli* B pyr^- in minimal medium supplemented with dC and BdC(5-bromodeoxycytidine). The dC-requiring mutants of phage T2 were expected to incorporate more heavy BdC since they should not be capable of synthesizing dC. After density-gradient centrifugation, four particles were found in front of the band of phage. One of them did not grow on *E. coli* B pyr^- without supplementary dC. An attempt to superglucosylate this strain in *E. coli* B pyr^- supplemented with a digest of T4 DNA was unsuccessful.

2.2. *Polarized restriction of phage T2 by T4*

Premature-lysis experiments designed to investigate restriction and escape from restriction during the intrabacterial development, require a set of suitable mutants in T2

exr^{+4}. A series of 300 *amber* mutants were isolated from hydroxylamine-treated T2 *exr*$^{+4}$ (vide 2. 1a)). The genes in which the mutations were induced were determined by complementation tests with well-known *ambers* in T4. In this way, *ambers* were found for gene 1 and 2, gene 32 to 45, for lysozyme and possibly for glucosyltransferase.

3. Studies in regulatory mechanisms of somatic cells *in vitro*

Scientists: J.W.I.M. Simons
H. van Steenis
Klara Heyman-Zandstra

3.1. *Cell size*

It had been found that deterioration of cell strains in tissue culture was connected with changes in the frequency distribution of cell diameters. Polymodality of the distribution, cell hypertrophy of the large cells and cell hypotrophy of the small cells occurred. To investigate whether this process had been induced by the medium in which the cells had been cultured or whether this process is an inevitable consequence of the cultivation of normal cells *in vitro* (senescence theory), an experiment has been carried out in which six cell lineages of one cell strain of human skin fibroblasts were cultured in three different media, one medium with autologous serum, a second with homologous serum and a third with heterologous serum. Diameter measurements were taken from passage 3 until passage 8. The frequency distributions of the four cell lineages in medium with autologous and homologous serum were very much alike each other, but the cells in heterologous medium showed hypertrophy. This cell hypertrophy in heterologous medium increases in the course of time.

In comparison with the cell lineages in autologous and homologous serum the two cell lineages in medium with heterologous serum show a marked reduction as well in total time in culture as in the number of passages. The same reduction in life potential was seen for three other cell strains. This finding supports the idea that the changes of normal cells in culture are caused by inadequate culture conditions. However, the fact that no differences were observed between cell lineages kept with autologous or homologous serum for either the total-time in culture or the number of passages, is rather in favour of the senescence theory.

Measuring of cell volumes with the coulter counter has been continued. Data have been collected with respect to the reliability of the measuring of cells and to the influence of some milieu factors on the frequency distributions of cell volumes. The reliability of the method has been tested by counting the number of cells present before and after measuring and by checking whether there is a shift of the peak in the frequency distribution during the measurement. Changes in the number of cells can be due to cell lysis, cell aggregation or attachment of cells to the glass. Shifts of the peak of the frequency distribution can be due to cell swelling or cell shrinking. As these changes did not occur, the way of measuring is reliable. There is, however, some heterogeneity between the frequency distributions of different cell populations, cultured under the same conditions. Moreover, by the production of large amounts of dead cells, certain cell strains are less suited for this type of measuring. As, during 1966, J.W.I.M. Simons was on leave of absence at the Weizmann Institute in Israel, and returned only at the end of the year, the analysis of the collected data did not yet make enough progress to permit conclusions.

3.2. *Cell transformation*

A system for the detection of cell transformation has been developed. Using BSC-1 cells, (a monkey kidney cell line) a clone was isolated, characterized by complete contact inhibition. Cell transformation could be induced by X-rays in these cells, the cells then growing in an irregular pattern. An approximately linear dose-response relationship was observed. As this cell line was lost a new one with the same characteristics has to be developed.

3.3. *Chromosome studies*

The karyotype analysis of two human cell strains of female diploid fibroblasts during subsequent subcultures has been completed. The first cell strain was in culture for about one year and a half. Chromosome preparations were only obtained from the 15th and last subculture; 77 cells were karyotyped. There were three groups, one with a mode of 39 chromosomes, one with a mode of 80 chromosomes, and one with a mode of 160 chromosomes. In all three groups abnormal chromosomes were seen, dicentrics, rings, fragments. The second cell strain was in culture for about three years. During this period 19 passages were performed. Chromosome preparations were obtained at the 8th, 10th, 15th and 16th passage. At the 8th passage the cell population was still normal, only 3 % of the cells being tetraploid. The chromosome preparations of the 10th subculture showed a marked increase in tetraploid cells (19 %). At this time the culture stopped growing for two months. After the cells had resumed growth, an abnormal chromosomal distribution was observed in preparations of the 14th subculture: 94 % of the cells were aneuploid, 60 % of the cells had chromosome numbers between 78 and 90. From the 16th passage 107 cells were karyotyped. There were two groups, one with a mode of 45 chromosomes, and one with a mode of 88 chromosomes. In both groups dicentrics and rings were present. The conclusion of these findings is that first there is a build-up of a tetraploid population and that then the cells begin to loose chromosomes at random, which leads to aneuploidy. The analysis of another human fibroblast strain during subsequent subcultures is started.

T-1 cells, an aneuploid cell-line of human kidney is used in some experiments with the aim to find an easy method for accumulation of cells in metaphase. From these experiments it appeared that treatment of the cells with cold, 15 hours at 4° C, is very harmful, resulting in an accumulation of dead cells to a percentage of about 70, ten hours after treatment.

The third attempt to start cell cultures from different tissues of the Tasmanian rat kangaroo *(Potorous tridactylis)* has been more successful than previous ones. Cultures of heart and muscle grew well for two subcultures and chromosome preparations were obtained which showed a normal female complement. Some cells of the third subculture of heart cells are still alive.

An investigation is started with the aim to find a correlation between ^{131}I treatment and chromosomal damage. Peripheral blood samples of four patients who had received ^{131}I treatment for thyroid carcinoma are investigated for chromosome aberrations. No abnormalities have so far been found in these patients who have received the last treatment at least four months before the blood was sampled. This work is carried out in collaboration with the clinic for Metabolic disease.

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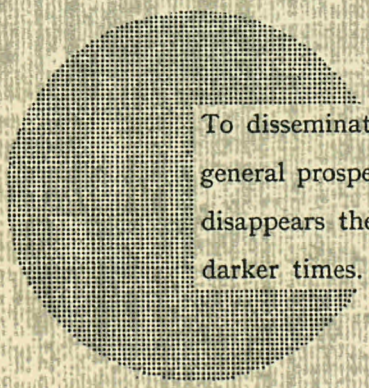

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Alfred Nobel

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