Guidelines for the testing of chemicals for mutagenicity / Committee on Mutagenicity of Chemicals in Food, Consumer Products, and the Environment.

Department of Health and Social Security

Report on Health and Social Subjects

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Preface

The awareness that some chemicals in the environment have the potential to bring about changes in human hereditary processes is relatively new although the fact that exposure to radiation or emissions from radioactive atoms can cause genetic changes has been known for half a century. Following the recognition of the potential effect of these chemicals on the health of subsequent generations it is now generally accepted that there is a need to diminish the risk of adding to the current burden of genetic disease.

Central Government is advised on mutagenicity by the Committee of Mutagenicity of Chemicals in Food, Consumer Products and the Environment under the Chairmanship of Professor Polani. Members of this Committee have prepared this report which deals essentially with the philosophy and methodology of testing mutagens. I am grateful to them for compiling this scholarly document which I think is an important contribution to the understanding of a difficult and complex subject.

H YELLOWLEES
Chief Medical Officer

Department of Health and Social Security
January 1981

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Contents

1. Summary

1.1 Mutagenesis refers to those processes that cause changes in the genetic material (mutations) in individuals or cells, spontaneously or by the actions of chemicals or radiation, whereby their successors differ in a consistent and heritable way from their predecessors. A proportion of such changes will produce deleterious effects in the offspring although some of these effects may not be manifested for many generations.

1.2 The first section of the guidelines consists of an elementary description of those genetic, cellular and molecular facts that are indispensable for the understanding of mutagen tests. In brief, the unit of genetic material is the gene, which consists chemically of deoxyribonucleic acid (DNA). This is a complex molecule in which sequences of purine (2 types, adenine, A, and guanine, G) and pyrimidine (thymine, T and cytosine, C) bases are linked up on a sugar phosphate backbone to form a long double helical structure with sequences of complementary base pairs (A-T or G-C) running between the sugar-phosphate molecules which form the sides of the double helix. The key element of this molecular arrangement is that the base sequence along one side of the helix, taken in triplets, forms the genetic code which when transcribed by a complex molecular system results in the incorporation of specific amino acids into polypeptides. The complete sequence carrying the information necessary for the synthesis of a specific product, eg an enzyme or a protein such as a globin, is one unit of genetic material, namely a gene, in these examples a structural gene. Other genes exercise controlling functions. DNA in a cell of a higher organism consists of genes in long strings and interposed repetitive sequences of bases, all of which in higher animals are organized into complex structures called chromosomes which are localized in the nucleus. Each chromosome is one very long thread-like DNA molecule which is coiled and supercoiled and is associated with special protein and enzyme systems to form the chromatin thread, which can easily be visualized, because of further compaction, in the special circumstances of a dividing cell. A mutation is a change in the number or types of nucleotide bases that make up a normal gene: this constitutes a point mutation. Thus the information necessary for the manufacture of a specific product is no longer adequate and a malfunctioning molecule may ultimately be synthesized, or possibly no synthesis occurs at all if parts of the coding sequence have been deleted or altered to nonsense. However, chemicals or radiation can cause many kinds of damage to genetic material, ranging from an alteration of a single base on the DNA molecule to removal or rearrangement of large sections of the chromatin thread in a chromosome, or in special circumstances, even an alteration in the number of the chromosomes themselves (chromosome mutations). The main importance of mutations is that they can be detrimental and can be transmitted from cell to cell during cell division or from individual to

individual via reproduction. The essence of reproduction is the handing on from one generation to another, via the ovum and spermatozoon, of the complete genetic information necessary to build a new individual resembling its 2 parents. This is achieved by accurate replication of DNA molecules and the passing of copies to the next generation by means of a special type of cell division called meiosis. Consequently, alterations or losses in the base sequence of the DNA molecule and some chromosomal changes may be transmitted as a mutation to succeeding generations. The implications for carcinogenesis are considered in Chapter 4.

1.3 In order to consider the possible untoward effects on human populations of an increase in mutation rates brought about by the presence of mutagens in the environment, it is necessary to establish the facts concerning the current burden of genetic disease, ie the existing background of known human detrimental mutations. Such changes in genetic material make a larger contribution than is generally realized to human malformation and disease. These may be manifest early in life but may also make their first appearance during young adulthood or even later. The total contribution which genetic change makes to human disease and disability is not easy to estimate, but it could affect somewhere in the region of one in 50 of the population. These matters are considered in Chapter 5 while Chapter 6 examines the problems of monitoring the human population and the environment in which they live, to ensure that individuals and groups of individuals are not being exposed to known or unknown mutagens, with consequent increase of the background mutation rates.

1.4 Chapters 7 and 8 deal with the Committee's recommendations. Many chemicals are known to possess mutagenic properties and the Committee accepts that response to these in humans might present a hazard to future generations. It is therefore necessary to identify and limit the spread of such chemicals into the human environment. The Committee also accepts that if substances have demonstrable mutagenic activity in several test systems this implies possible carcinogenic activity (it being accepted that mutation and malignancy may originate via similar processes), but advises that the mutagenic hazard should be considered in its own right.

1.5 The Committee recommends that initially all new chemicals and products to which human exposure would be extensive and difficult to avoid should be tested for mutagenicity, but subsequently there are two separate issues to consider. The first is to decide if the test results establish with reasonable certainty whether a chemical possesses mutagenic potential or not, and the second is the quite separate issue of evaluating the nature of the presumed hazard and its relevance to man.

1.6 The Committee considers (see para 7, 13) that in the light of present knowledge, the great majority of potentially hazardous chemicals can be detected by a combination of 4 test procedures designed to probe the hereditary machinery sequentially, at increasing levels of complexity. This *basic* package

of tests rests *firstly on the detection of point mutations in bacteria* (the bacteria autotrophy – prototrophy reversion test) both with and without the use of suitable metabolic activation. This is incontestably the most widely validated system in the field of genetic toxicology and it is believed to be the most sensitive. Once the activity of a chemical on the reversion system in microorganisms has been clearly established, all further testing should preferably be carried out using mammalian systems. As some genetic diseases result from demonstrable abnormalities of chromosome structure or number *the second test procedure recommended is the determination of the ability of a chemical to produce damage to the chromosomes of mammalian cells grown in vitro (metaphase analysis).* This procedure has a particular relevance in that human cells such as lymphocytes are as convenient as cells obtained from laboratory animals. *The third test procedure advocated is the induction of gene mutation in mammalian cells grown in culture* using techniques based on the detection of mutagenesis at, for example, the gene loci responsible for the activity of the enzymes hypoxanthine-guanine phosphoribosyl transferase and thymidine kinase, *or the induction of recessive lethals in Drosophila melanogaster.* These test allow for the fact that the path a chemical must take to reach the DNA complex in eukaryotic cells is quite different from that in bacterial cells. In the *in vitro* tests recommended above the possible metabolism of a chemical *(per se* non mutagenic) to mutagenic form in an intact animal can be partially simulated by the addition of microsomal preparations which may cause metabolic activation. *However no battery of tests can be considered complete without one in vivo test in a mammal to test the metabolic fate of the chemical in the intact animal. Although many different tests are available it seems that at present the preferred test should be either cytogenetic analysis of bone marrow in rodents or the dominant-lethal test in rodents.* It is the opinion of the Committee that the recommended basic package screening procedure should detect, if fully and properly exploited, the great majority of the potential mutagens among the chemicals entering the human environment. Any further improvement at the screening level would entail an expenditure of effort out of all proportion to the value of the additional information that might be gained. Although non-disjunction is an important cause of genetic disease the Committee considers that there is at present no suitable mammalian test available for a screening programme.

1.7 The test systems recommended by the Committee are directed at a selection of the possible targets in the hereditary apparatus. It is recognised that equivalent evidence derived from different tests could have equal validity and thus could be accepted as an alternative to part of the package. However, the onus would be on the applicant to prove that the evidence produced was at least as good as would be expected from the recommended tests. The Committee also recognises that there may be chemicals where the anticipated use is limited and the degree of human exposure small. In such instances a case can be made for accepting, as a first step, the first 2 test procedures, ie those for the detection of point mutations in bacteria and chromosomal damage in mammalian cells *(in vivo* or *in vitro).*

1.8 The evaluation of the hazard to man subsequent to the screening test is an issue which must take into account not only the results obtained in the tests but the whole toxicity profile of the chemical. This will involve the nature and properties of the chemical, its pharmacokinetics, the quantity manufactured, its proposed distribution and degree of human exposure, the age and reproductive structure of the potentially exposed population and any other relevant aspect that might bear upon the issue of human health. To this must be added considerations regarding the importance and advantages expected from the use of the chemical. The final decision or risk/benefit analysis will have to be made in the light of all these factors and may well call for supplementary mutagenicity tests to clarify the results obtained in the screening tests. Although at the screening level some constraint in the choice of tests has been recommended the choice of any supplementary tests, if needed, is left to be argued from the scientific facts resulting from the screening tests. The Committee has also recommended those supplementary tests which it judges to be valid. It is further proposed to add to this list or otherwise modify it as new knowledge appears. In addition the Committee has indicated those scientific advances which show promise of development into useful test procedures.

1.9 Chapter 8 considers the principles underlying the recommended basic package screening procedure. This sets out briefly the advantages and disadvantages of the recommended tests. The actual design, detailed protocol, technical procedures and precautions needed, together with the appropriate statistical treatment, are considered to be best dealt with by reference to accepted published work. Therefore a selected bibliography of the key literature for each test procedure is set out in Appendix 1.

2. General Introduction

2.1 It has long been known that ionizing radiation can induce hereditary changes in living organisms. It is now realized that a number of chemicals in our environment can do likewise. The purpose of these guidelines is to explain why there is now concern about this previously neglected hazard in the environment, what can be done to detect the chemicals concerned, and how they can be dealt with.

2.2 A section on human genetic diseases is included as a reminder of the wide range of harmful genetic changes which could increase in frequency as a result of the mutagenic action of chemicals. It is not possible as yet to know whether the current level of genetic disease has been affected by the levels of exposure to chemicals with mutagenic properties encountered so far. This is because it is only recently that some idea of the possible levels of hereditary defects in humans has emerged. Moreover the genetic variation in man is such that the cumulative effects of additional mutations may take many generations to produce a noticeable effect. The monitoring of populations for increased mutation rates in plasma enzyme systems or in somatic cells (already possible for chromosome aberrations in blood lymphocytes) is likely to give answers sooner, where suitable test systems have been developed to deal with the large numbers of samples involved in such surveys.

2.3 The new range of test procedures suggested in these guidelines, when combined with studies of exposure to and metabolism of the chemicals in question, should enable an evaluation of the genetic hazards of the use of chemicals to be made. This should eventually enable realistic assessments of risk and benefit to be made in order to minimize any potential increase in population levels of hereditary disease. As the rationale for these tests is not as straightforward as is the case in conventional toxicity testing, and as there are implications for many groups in the chemical and allied industries, such as administrators, lawyers, managers, technologists, trade unionists and scientists, it was felt that a more detailed consideration of the issue was justified than is usually presented in guidelines. This necessitates some consideration of the scientific basis underlying mutagenesis, which in turn calls for some knowledge of genetics and molecular biology. The science of genetics covers a wide spectrum ranging from plant and animal breeding programmes to the molecular arrangements in a single gene. In consequence, and of necessity, it has developed a technical language that is not readily understood by those outside the field. To assist understanding an attempt has been made to define each technical term when it first appears and for ease of subsequent reference the most important of these terms have been gathered in a glossary in Appendix 2.

3. The Role of DNA in Genetic Processes

3.1 The key to our understanding of how mutations can occur and how chemicals can interfere with genetic processes is knowledge of the role of deoxyribonucleic acid (DNA). Early work with bacteria showed the key role of DNA in inheritance (the fact that some viruses use ribonucleic acid (RNA) as their genetic material was discovered later), but it was the elucidation of the 3-dimensional helical double stranded structure of DNA and the appreciation of its significance by Watson and Crick (1953) that triggered the explosion of knowledge about the molecular basis of heredity.

3.2 In a topological sense, the DNA molecule is something like a ladder twisted around a central axis, where the sides of the ladder are a sequence of deoxyribose sugar molecules, with the 3 position of one sugar molecule linked to the 5 position of its neighbour in a systematic way by phosphate molecules. The rungs of the ladder are made up of complementary pairs of nitrogen-containing pyrimidine and purine bases. The remarkable feature of this simple basic structure is that there are just 2 complementary pairs of bases, viz adenine (A; a purine) and thymine (T; a pyrimidine), and guanine (G; a purine) and cytosine (C; a pyrimidine). These paired bases form a flat molecular complex whose aggregate structure in the DNA has been likened to a pile of coins spiralling around an imaginary central pillar. It is the sequence of these 4 bases taken in groups of 3 along one side of the ladder, as it were, that constitutes the genetic code for the amino acid sequences in the polypeptides of protein structures.

3.3 Four different bases taken 3 at a time give 64 possibilities, and as there are only about 22 amino acids involved in the make-up of proteins there are more than enough coding units (codons) for the amino acids in polypeptides. An important feature of the code is its universality; it is the same for all organisms. Thus a given amino acid has the same set of codons in a bacterium or a mammal. The codon AAA, for example, always codes for phenylalanine, and CCG for arginine.

3.4 In view of the faithfulness of the transmission of genetic information through the generations it is perhaps surprising to learn that DNA is itself not a particularly stable molecule and that it can be damaged relatively easily. The apparent stability of the system derives from the associated complex of proteins and enzymes, which not only subserve the basic functions of DNA (replication and transcription) but also have the capacity to repair various kinds of damage to the base sequence. This can happen when one of the 2 complementary strands is damaged at any one place. There are several known repair systems.

One of these is the system of enzymatic processes known as excision repair. A sequence of endonuclease, exonuclease and polymerase enzymes keeps the DNA molecule under constant surveillance, and if any small chemical alterations are detected on one of the strands the affected base or bases together with some unaffected adjacent bases are cut out and replaced, using the other strand as a template. The final sealing-in of the replaced sequence is accomplished by the synthesis of a phosphodiester bond by a specific ligase enzyme. Reference is often made to 2 other repair systems, viz enzymatic photoreactivation and post-replication repair. The manipulation of repair processes has become an important technique in mutation research.

3.5 The key to the hereditary mechanisms is the remarkable accuracy with which the DNA molecule is replicated, which in turn depends in part on the maintenance of the DNA base sequence by repair mechanisms. DNA replication occurs prior to somatic cell multiplication (mitosis) or during gametogenesis in preparation for meiosis. The process of replication is associated with a number of integrated enzyme systems. The DNA helix has to be exposed to the replicating enzyme system by a process of unwinding of the secondary coiling. At a specific point on the DNA molecule a splitting of the duplex occurs, thus creating a "replicating fork", so that a polymerase system can operate to build a complementary strand to each single strand. Using the ladder analogy, what happens is that the ladder is separated into 2 halves through the centre of each rung and as the separation process occurs each half of the ladder is reconstituted to make a new ladder, using the original portion as a template and building on rung by rung the complementary base — sugar-phosphate moiety. This results in the generation of 2 identical ladders, each one with half of the parent ladder material and half new material (semiconservative replication) but with the original code sequence precisely replicated. In this way an exact copy of the base pair sequence is prepared, containing all the genetic information.

3.6 In contrast to DNA replication, DNA transcription can occur throughout the life of the cell. It involves the transfer of information specifying the construction of polypeptides from the nuclear DNA to the ribosomes in the cytoplasm where the actual synthesis of proteins occurs. The duplex separates into single strands at the appropriate point of the DNA molecule. DNA directed RNA polymerase then organizes the synthesis of a polymer of nucleotides on a backbone of ribose-phosphate instead of deoxyribose-phosphate (hence RNA) using one strand of the DNA duplex as a template. This process is started and stopped by specific initiating and terminating codons. The resultant RNA molecule is single stranded and consists of a sequence of bases complementary to one of the 2 strands of the portion of the DNA molecule that acted as the template. However, in the RNA sequence, uracil (U) is substituted for thymine. After release from the DNA the complementary RNA, messenger RNA (mRNA), diffuses from the nucleus to the cell cytoplasm, where in association with ribosomal RNA (rRNA) and on delivery by transfer RNAs (tRNA) of appropriate amino acids, polypeptides are synthesized in accordance with the coded instructions.

3.7 A great deal is now known about the nature of the DNA code. The amino acid code is described as degenerate because more than one codon exists for some of the amino acids. In addition to codons for individual amino acids there are codons for "start translating" and "stop translating". Such codons have been derived by single base substitutions from normal codons specifying amino acids. UAG, one of the terminating codons, can, for example, be derived from the codons for serine (UCG), glutamine (CAG) or tyrosine (UAU or UAG) by a single base change, ie point mutation. As a result translation can be switched off in mid-message. The effect of such a mutation is usually far greater than that of a single base change producing a codon for a different amino acid. Termination codons are usually referred to as "nonsense" codons.

The organization of the genetic material — genes and chromosomes

3.8 The basic unit of the genetic material is the gene. As can be appreciated from the foregoing discussion, the physical form of a gene is a sequence of bases in a DNA molecule which contains the information for a functional unit of inheritance, such as for the synthesis of a polypeptide (a structural gene) or for a base sequence necessary for some controlling or regulatory gene function. The gene as a unit of function is sometimes known as a cistron. In all organisms the DNA is present in the cell as one or more giant filamentous molecules consisting of sequences of genes interspersed in a species specific way with repetitive sequences of bases, some of which in higher eukaryotes are relatively enormous. Such an arrangement of genes together with associated protein and enzymic systems is known as a chromosome. The composition, structure and cellular location of chromosomes differs between cells of prokaryotes (bacteria, blue-green algae) and eukaryotes (all higher organisms including man). In prokaryotes the chromosomes basically consist of the DNA of the gene string, which in *E. coli* for example is a single circular macromolecule of double stranded DNA consisting of about 4 million base pairs. This chromosome is organized with various proteins and other substances in a discrete "nuclear body" with a regular structure. In eukaryotes the chromosomes, usually many per cell, are more complex structures containing various proteins, enzyme systems and RNA in addition to DNA. Whereas in prokaryotes the nuclear body is free in the cytoplasm, in eukaryotes the chromosomes are enclosed in the nuclear membrane except at meiosis and mitosis. However, the essential features of DNA chemistry and gene replication are the same in both and there is good reason to believe that human DNA will react with the same compounds as bacterial DNA.

The organisation of the genetic material — the genome, ploidy, dominance, recessivity

3.9 All prokaryotes and some lower eukaryotes, eg certain fungi, are haploid, ie their cells contain only one set of genes, usually arranged as a single gene string of chromosome, or, if, the genes are divided into separate chromosomes, each chromosome consists of a different set of genes. A complete set of genes is

called a genome and thus designates the sum total of the genetic information contained in the individual (ie unpaired) genes in an organism.

3.10 Some lower and all higher eukaryotes are diploid, ie their somatic cells contain 2 copies of each chromosome. These matching pairs are called homologues. Many plants and some animals are polyploids, ie they have more than 2 sets of chromosomes, but this is not of interest in the present context. In sexually reproducing diploid species, one of the homologous chromosomes is derived from the father and the other from the mother. Each gene occupies a precise position or "locus" along the length of the DNA molecule of a given chromosome. Thus, with one important exception to be mentioned below, homologous chromosomes are functionally and spatially matched gene for gene along their entire length, even though one gene of the pair comes from the mother and the other from the father. These maternally and paternally contributed genes may be the same or they may differ. Different functional forms of the same gene are known as alleles, and it is important to note that they do not necessarily carry out their function in the same way. Thus, one allele of a gene may produce the normal type and amount of enzyme, while the other may produce less, or a temperature sensitive variety or none at all. Diploids are said to be homozygous for a given allele at a given locus when they carry identical versions — normal (wild type) or mutant — of this allele, and heterozygous when they carry different versions.

3.11 Heterozygosity leads to complications in gene expression. Sometimes, 2 different alleles express themselves independently of each other; this is called co-dominance, and is seen, for example, in the A&B pair of alleles of the AB blood group of man. Often, one of 2 alleles dominates development and function to the full or partial exclusion of the effects of the other allele. In these cases, the 2 alleles are called dominant (or partially dominant) and recessive (or partially recessive). An example of a recessive (or partially recessive) allele in man is the allele for phenylketonuria. The definition of dominance is somewhat ambiguous. A fully dominant allele of a gene should be equally well expressed in the heterozygous and in the homozygous states. This is true for many normal alleles in relation to fully recessive mutant ones (eg alleles for certain kinds of deafness). In man, however, dominant mutant genes are often only known in the heterozygotes because they are so rare in that homozygotes have never been seen, or because the heterozygotes do not produce offspring, or because the homozygotes die as early abortions. In these cases, especially when dealing with human abnormalities, one calls a mutant allele dominant when it manifests itself in the heterozygote although knowledge of the homozygote would probably show that it is only partially dominant. Examples of conditions caused by dominant alleles in man are brachydactyly and hereditary retinoblastoma.

3.12 The one exception to the rule that all genes in man are paired is that of the X-linked genes, ie the genes on the X chromosome, of which a male has only one and a female 2. Its partner in the male, the Y chromosome, is not known to carry any alleles for the X-borne genes. Thus every mutant X-borne gene, whether dominant or recessive, will manifest itself in the male as a sex-linked

(or X-linked) characteristic or disease. In females the situation is complicated by the fact that in different cells of somatic tissues either one or the other of the 2 X chromosomes, but never both, expresses its genes at the cell level.

3.13 Genetic mapping, namely arranging the genes in sequence along the length of a chromosome, is now a sophisticated branch of research and many details of gene arrangements and control are known, particularly in prokaryotes. Among eukaryotes the genetic maps of some fungi and of *Drosophila melanogaster* are especially precise and detailed. The gene map of the mouse is also known in considerable detail and mapping in man is progressing rapidly. Some idea of the size of a structural gene can be derived by considering the coding needs for a polypeptide of, say, 150 amino acids. This will be specified by a sequence of 450 bases, and as there are 10 base pairs per turn of the DNA helix this sequence will occupy some 45 turns or some 200-250 nm of the helical DNA molecule. In prokaryotes groups of genes subserving a common function may be subject to a set of controlling genes and be located close to each other in orderly sequence. Such an arrangement is known as an operon. The *lac* operon (named after the enzyme substrate lactose), which is a 3 enzyme system in *E. coli,* and the *his* operon (named after the product of its action - histidine), which is a 10 enzyme system in *Salmonella typhimurium,* are 2 operons which have been much exploited in mutation research.

3.14 Most of the genetic material of eukaryotes is located in the nucleus. There are small quantities of DNA outside the nucleus, for example in the mitochondria, and thus a distinction can be made between nuclear and organelle genomes. Likewise, in most bacteria there are additionally a number of small discrete elements of DNA known as plasmids. These may carry genes beneficial to the host bacterium (eg conferring resistance to various antibiotics) and often possess the ability to effect their transfer from one cell to another. The bacterial genome would be about 1mm in length if it were fully extended but in the cell it is packed into a volume of about one cubic micron, that is, about 40% of the cell volume. The extended *Drosophila* genome would be about 4cm long and the mammalian genome about 2m long.

The transmission of genetic information from one generation of cells or organisms to the next in eukaryotes

3.15 During growth the body cells (or "somatic" cells) divide by a process called mitosis. From the genetical point of view the relevant part of this is the precise replication of the chromosomal material in such a way that the nuclei of the daughter cells will have identical diploid genomes. This is achieved by the process of mitosis or cell division after chromosomal (DNA) replicative synthesis and it is concerned with the production of generations of particular somatic cells in a particular individual. However, early in the development of an embryo a group of cells, the germ cells, are set aside to be the precursors of the future gametes (ova or spermatozoa). Germ cell development, the process of gametogenesis, is quite distinct from the development of somatic tissues. A mature

gamete possesses a haploid set of chromosomes, and the special process of production of haploid gametes from diploid germ cells is called meiosis. The ultimate fusion of the female haploid gamete with the male haploid gamete produces the zygote, which possesses a diploid set of chromosomes and which by subsequent mitotic cell divisions produces the individual of the next generation.

3.16 During transcription into mRNA the nuclear chromosomal material in a eukaryotic cell is dispersed in its filamentous form throughout the nucleus and is not visible by conventional optical microscopical techniques. Prior to mitosis, the production of mRNA ceases and the process of DNA replication takes place. In the early stages of mitosis, ie prophase, the chromatin complex can be seen via optical microscopy to condense and form long and thin thread-like structures. These contract further into discrete elements which, because of their ease of staining and particulate nature, are called chromosomes ("stained bodies"). Each chromosome at this stage is in fact a pair of identical double helices of DNA compacted and condensed to form a pair of distinct chromatids linked together at a point (primary constriction) where a small body known as the centromere (spindle attachment) is organized. During the next stage, metaphase, the nuclear membrane disappears and the chromosomes move to the equatorial part of the cell, and a spindle apparatus develops between the centromeres and the polar regions of the cell. Colchicine inhibits the formation of the spindle and thus prevents the organization of the chromosomes in the equatorial region between the centromeres. They now become widely dispersed in the cell and thus individually more recognizable. It was by the use of techniques such as this that the characteristic number and shape of the chromosomal pattern for the cells of different animals was established. Man has 46 chromosomes ($2n = 46$), namely 22 pairs of homologous chromosomes, called autosomes, plus 2 sex chromosomes, X and Y in the male or XX in the female. The Chinese hamster, for example, has only 22 ($2n = 22$) and the cow 60 ($2n = 60$). At the next stage, anaphase, the centromere of each chromatid pair splits and the individual chromatids separate and move to opposite poles of the cell as new chromosomes. In the final phase, a partition is developed in the equatorial region which divides the cell into 2 halves, the chromosomes become diffuse, and 2 new nuclei are developed.

3.17 The process of meiosis differs from that of mitosis. At its beginning homologous chromosomes pair with each other and having exchanged genetic material (see para 3.21), separate from each other in the first meiotic division. Thus, in contrast to mitosis, the chromosome number of the daughter cells is half that of the mother cell and each chromosome still consists of 2 chromatids. Because of the exchange of genetic material in the first meiotic division, the 2 chromatids of a chromosome are usually no longer identical. The second meiotic divison resembles a mitotic one in that it splits the centromeres, separates the chromatids and does not change the number of chromosomes. Thus, in meiosis each diploid germ cell produces 4 haploid cells. In the male, these 4 cells will mature into 4 spermatozoa; in the female they will yield one ovum and the polar bodies.

The generation of genetic variability at meiosis and the implications for mutagenesis

3.18 *Introduction.* Thus far the basic elements of the hereditary processes have been outlined, from DNA replication and transcription to somatic and germ cell division, whereby a complete set of genes is transferred, either to the next generation of somatic cells or (after fetilization) to future individuals. However, these complex processes may go wrong, with the generation of hereditary mistakes or *mutations,* so that a functionally changed and often imperfect set of instructions is transmitted, from which defective cells or individuals may result. *Mutagenesis* is the name of this process of generating mutations, which may arise spontaneously or may be induced by chemical or physical agents, then described as *mutagenic.* The detection of chemicals that may be potential human mutagens is a rapidly expanding field of scientific endeavour that runs in parallel with a similar expansion of knowledge of the molecular biology of the genetic processes themselves. An added incentive for this interest is that apart from the serious implications for future generations if further genetic or partly genetic diseases are added to the far-from-negligible current burden, there is strong evidence that mutations in somatic cells may be associated with the production of malignancies (see Chapter 4). Before proceeding to more general considerations of mutagenesis there are some important aspects of meiosis that have considerable significance from the viewpoint of mutagenesis at the chromosomal level.

3.19 *Genetic variability: independent assortment: crossing-over.* Although all 4 products of meiosis contain a complete set of chromosomes, they are far from being genetically identical. Two processes are responsible for creating genetic variability at meiosis. They form the basis for suggested methods of assessing mutagenic hazard and hence need further consideration.

3.20 The first mechanism, called independent assortment by the earlier geneticists, rests on the fact that each pair of homologous chromosomes segregates independently of all the others. Thus, the daughter cells receive different combinations of maternally and paternally derived chromosomes. If the maternally derived chromosomes are indicated by the suffix m, and the paternally derived ones by the suffix p, a sexually produced individual has the chromosome set 1_m1_p; 2_m2_p; etc. His gametes have an equal chance of containing 1_m2_m or 1_m2_p or 1_p2_m or 1_p2_p. It will be seen that for the 23 chromosome pairs of man the variability created in this way is great. It is, however, not the only source of variability.

3.21 A second mechanism, called crossing-over, results in a mixture of paternal and maternal genes even within the same chromosome. At the beginning of meiosis when homologous chromosomes are closely paired, they exchange sections with each other in a precise manner. Each single cross-over takes place between 2 of the 4 chromatids, and successive exchanges (the number of which varies with the length of the chromosomes) may involve different chromatids. The detailed mechanism of exchange is not known but it

is possible that processes similar to DNA repair mechanisms are involved. The process, known as recombination, is clearly under strict genetic control and even though the molecular details of the process are not fully worked out there are cytological and genetical consequences. The cytological results can be observed just before first meiotic metaphase when previously paired homologous chromosomes begin to separate from each other. At this stage joins can be seen between 2 of the 4 chromatids so that some of the paired chromosomes take on the appearance of crosses. These joins are called chiasmata and each chiasma (= a cross) corresponds to a crossing-over. The genetical result, and indeed the fundamental outcome, of recombination is the reshuffling of alleles within the chromosomes which occurs at chromatid level. In this way individual chromatids may emerge from the meiotic process with different combinations of alleles. Thus a recombinant chromatid whose centromeric region, for example, is of paternal derivation, may carry in sequence next to it a stretch of DNA of maternal origin, and this in turn, may be followed by a further stretch of paternal homologues. This ensures that no 2 individuals, barring identical twins, are genetically alike and on this depends the vast amount of diversity in human populations.

3.22 A process that is related to recombination, called gene conversion, is used in mutagen testing in fungi. It occurs during mitosis as well as during meiosis but unlike crossing-over, which results in recombination of whole sequences of genes, it occurs within genes. It also differs from crossing-over in that it is not reciprocal, an allele in one chromosome being "converted" into that of the other without this other one undergoing a change. Tests for gene conversion can only be carried out in fungi and the correlation between the ability of a substance to cause gene conversion and to induce mutation appears to be high.

3.23 One of the steps in the process of crossing-over is breakage of single strands of DNA. This is probably the reason why many mutagens increase the frequency of genetic recombination, or induce it in cells in which it does not usually occur, eg the male germ cells of *Drosphila*. While meiotic recombination *per se* does not usually create a genetic risk, a substance that induces it is also likely to produce other types of chromosome damage that do constitute a hazard. These are the breakages of chromatids or chromosomes which are often followed by the formation of new "rearrangements" between the fragments (para 3.30). Since it is much easier to score recombination than chromosome breakage and rearrangement formation, the ability of a substance to produce the former is often taken as an indicator of its ability to produce the latter also. It should however be understood that crossing-over differs from the formation of chromosome rearrangements in several essential features. Rearrangements can be formed at random from any fragments that are close enough for rejoining; crossing-over occurs only between homologous chromosomes, and the points of breakage are identical in both. Rearrangements are formed without regard to the orientation of the chromosomal segment, hence the production of inversions. In crossing-over, the original orientation of the segment to the centromere is always preserved; thus the genes in the new strand are arranged in

exactly the same order as they were before crossing-over. Thus, although chromosome breakage and subsequent rearrangement and crossing-over may have one or more chemical steps in common, they clearly are not identical processes and caution has to be used in interpreting results of cross-over tests in terms of mutagenic potentialities. The same caution needs to be exercised in interpreting the sister chromatid exchanges (SCE) which have recently been introduced for mutagen testing. These are due to exchanges, not between homologous chromosomes, but between segments of sister chromatids of the same chromosome at mitosis. There are correlations between the ability of a treatment to produce SCEs and chromosome rearrangements, but again there are also differences.

Mutations and their induction

3.24 Knowledge of how changes in the DNA/chromatin system can be brought about has been growing for over 50 years as a result of extensive experiment with ionizing radiations of various kinds. It was the realization that chemicals could produce the same kinds of changes that initiated the present concern about the mutagenic hazards posed by many chemicals in common use. The growth of the chemical aspects of mutagenesis and its correlation with carcinogenesis has been so great that the literature is now much more extensive than that on radiation mutagenesis, yet it must not be forgotten that it is the fundamental knowledge gained from radiation studies that underlies our understanding of the way chemicals can act and in turn gives strong support to our realization of the potential dangers of chemical mutagens. No single chemical possesses a mutagenic data base that remotely approaches that available for X-rays.

3.25 When assessing the risks from chemical mutagens it will be necessary, as with radiation, to study quantitative aspects of their action, for example the relationship between the dose and the mutational response. However, it will also be necessary (which it is not with external radiation exposure) to investigate the metabolism of a mutagen so as to determine the extent to which it and its active metabolites are likely to reach the genetic material of somatic and germ-line cells, and what the effects in man may be. Only by a combination of mutational and pharmacokinetic studies is it possible to estimate likely genetic hazards at the low doses of human interest.

3.26 Broadly speaking, eukaryotic genetic defects stem from 3 categories of mutational events, which are (i) at the gene level, (ii) at the individual chromosomal level and (iii) at the level of the chromosomal set, ie a change in chromosomal number.

3.27 These result in dominant and recessive "point" mutations which may add to the number of dominant and recessive diseases. Some of these mutations, however, may stem from chromosomal changes. A distinction has to be made between the effects of dominant and recessive mutations. Dominant mutations affect any individual that has received the mutant genes from either the spermatozoon or the ovum. They therefore become manifest in the first generation. Recessive mutations affect only those individuals that have received the mutant gene from both ovum and spermatozoon. They can be carried in heterozygotes for many generations before a homozygous child who manifests the effects of the mutation is produced. In view of the generally very long latency of recessive genes they might be neglected in the calculations of genetic risk, were it not for the probability that, as found for X-rays applied to *Drosophila*, many of them have small but definite effects on survival in the heterozygous state. If this is so, they are likely to contribute an unknown but important component to the general genetic deterioration expected from an increased mutation frequency.

3.28 The damage leading to mutation must primarily affect DNA but these damaged sites are not themselves mutations. They must first become heritable changes of genetic information and this requires replication of DNA. Frequently repair is also involved. While attention was originally focussed mainly on the direct interaction of mutagens with DNA, it is now being directed to the great complexity of enzymes subserving the preservation of genetic information, in particular enzymes involved in replication and repair. Indeed, some mutagens produce mutations indirectly by inhibiting or mis-directing the action of these enzymes, eg some metal ions. The majority, however, act directly on the DNA of the chromosomes. Alkylating agents, for example, are known to produce these chemical changes in certain nucleotides. At the level of DNA base pair sequences there are some 20 known sites where these chemicals, particularly if strongly electrophilic, can react. At the next replication the altered base may select a "wrong" partner, eg an alkylated guanine may select thymine instead of cytosine and this will eventually result in a change from a GC base pair to an AT base pair. Alternatively the alkylated base may leave the chromosome, either by spontaneous or enzymatic hydrolysis, thus creating a gap which can be filled in by the cell's repair enzymes. These enzymes may be "error-proof", ie capable of putting in the correct base, or they may be "error-prone", ie prone to putting in the wrong base. The former enzymes remove potential mutations; the latter tend to enhance them. How much a base change mutation will affect the organism carrying it depends on many factors; its position in the nucleotide string of the gene, the type of "wrong" amino acid it codes for, and even the kind of nucleotides by which it is surrounded. There is however one kind of base change that always affects gene action drastically and usually abolishes it completely. This is the creation of a "nonsense" or chain-terminating codon (see para 3.7). In addition to base changes, mutagens may produce "frameshifts" by insertion or deletion of one base or several contiguous bases within a gene. Unless the number of these bases is a multiple of 3 this will

change the "reading frame" of 3 nucleotides per amino acid, eg a base sequence 123 456 789 will become 112 345 678 etc. Frameshifts can be produced either by covalent binding or by intercalation. Unless a frameshift occurs very near the end of a gene it leads to inactivation of the whole gene.

3.29 Most of our knowledge of mutagenic mechanisms comes from studies with lower organisms, particularly bacteria. Even with cultured mammalian cells, the systems are only now becoming well enough characterized to allow fundamental studies to begin. This raises the question of the extent to which results obtained with bacteria can be extrapolated to higher organisms, in particular to man. Certainly there are many major differences between the genetic systems of pro- and eukaryotes. The bacterial genome is not subdivided into several chromosomes. Bacteria have no meiosis, they are haploid and they have less DNA per gene than eukaryotes. This latter difference applies also to eukaryotes at different evolutionary stages. It is interesting to note that the difference in gene number between a yeast and a mammal is less than tenfold, while that between their respective contents of DNA is a millionfold. This shows that, gene for gene, the DNA content differs between lower and higher organisms. Moreover, mammalian cells contain a considerable amount of DNA that consists of a specific base sequence repeated many thousands or even millions of times, which is not transcribed. There is a need for far more knowledge in this area before it will become possible to extrapolate with some degree of confidence from the results obtained with micro-organisms to the expectations for man. Nonetheless it is true that the essential features of gene replication and the ultimate chemical interactions between mutagens and DNA are the same in all organisms, including bacteria. Thus there is reason to believe that a fair probability exists that tests for the production of gene mutations in bacteria will be of predictive value for man. This is not true, however, for chromosome breaks, which in prokaryotes are usually lethal and do not lead to the genetic consequences to be described for eukaryotes in para 3.30.

Damage at the level of individual chromosomes

3.30 Primary damage to individual chromosomes consists of breakage of chromatids which must result from a discontinuity of both strands of the DNA in a chromatid. The mechanisms whereby chemical mutagens produce chromosome breakage are not yet well understood, but various lesions in DNA, which are not in themselves discontinuities, will result in breakage of a chromatid as a consequence of their interference with the normal process of DNA replication. While in prokaryotes and haploid micro-organisms chromosome breaks are usually lethal, this is not so for diploid eukaryotes. In these latter, chromosome breaks may undergo 3 different fates, with quite different consequences for the cell.

3.30.1 They restitute in the old order, presumably as a result of an enzymatic repair mechanism. In this case no obvious cytological damage ensues.

3.30.2 They remain unrejoined as fragments. This may result in death of the cell at the next or one of the following mitoses. If unrejoined fragments are introduced into the zygote through a treated germ cell, the embryo may die at a very early stage from a so-called "dominant lethal". While there may be other causes of dominant lethality, its production by a chemical is a fairly good indication that this substance produced chromosome breaks.

3.30.3 They rejoin in a new order different from the original one, resulting in chromosome rearrangements. The most important of these and their genetic consequences are listed below.

3.30.3.1 *Translocations* remove segments from one chromosome to another. There are two main types of translocation, namely "Robertsonian" and reciprocal.

(a) *Robertsonian translocations* (centric fusions) are particularly noteworthy because they involve the joining together of 2 chromosomes, each of which has a centromere at, or very near, one end, to produce a single metacentric or submetacentric chromosome, that is, a chromosome with its centromere at or near the middle. Robertsonian translocations have played an important part in evolution. When they are produced *de novo* in a germ cell, they carry the danger that a resultant zygote will be abnormal because of a deficiency or excess of chromosomal material. This will usually result in the early death of the zygote, but in certain cases when the transposed chromosome is very small the embryo may develop into a functionally trisomic and abnormal individual (see later). This is called translocation trisomy. For example, if chromosome 21 is involved in the translocation, Down syndrome could result. Translocation trisomy has the added danger that, unlike non-disjunctional trisomy, the condition can be transmitted by entirely normal persons who carry the translocation in a balanced karyotype. Inherited Down syndrome is an example of this.

(b) *Reciprocal translocations* involve the exchange of chromosomal fragments between 2 chromosomes. Depending on the position of the centromeres in the rearranged chromosomes, this will lead to 2 quite different consequences. When one of the re-arranged chromosomes carries both centromeres while the other carries none, the zygote will usually die at an early stage. This type of translocation thus contributes to dominant lethality. If, on the contrary, each rearranged chromosome carries just one centromere, the zygote will develop into an apparently normal individual. When, however, such an individual forms germ cells at meiosis, the heterozygotes for reciprocal translocations behave in such a manner that about half the resultant gametes will be unbalanced, with duplications and deficiencies of chromosomal material. Nevertheless their chance of reaching the next generation is usually not reduced, but the usual consequences of such imbalances are death shortly before or after birth or congenital malformations in survivors.

3.30.3.2 *Inversions* result when a piece of a chromosome becomes detached and then replaced in its original position but the other way round, that is, with the order of the genes reversed. This does not necessarily constitute a genetic hazard but in some circumstances an unbalanced gamete could result and this could lead to fetal death or congenital malformations.

3.30.3.3 *Deletions or deficiencies* arise when 2 breaks occur in the same chromosome, the fragment between the breaks becomes detached, and the 2 ends of the chromosome join. The now unattached piece of the chromosome is likely to be lost at the next cell division. Very large deletions are believed to contribute to dominant lethality. Small deletions involving one or several contiguous genes are often difficult to distinguish from changes within a gene, ie point mutations. They are probably the most important genetic risk apart from non-disjunction (see para 3.31). Just as with some recessive mutations, deletions may uncover the deleterious effect of a pre-existing recessive gene. More important is the fact that even a small string of contiguous genes is likely to contain at least one gene that is essential for survival and is probably partially dominant in heterozygotes; deletion of such a gene would act as a lethal in homozygotes.

3.30.4 Chemicals differ widely in the kinds of damage they produce in chromosomes and in the proportion in which they may produce the effects mentioned above.

Changes at the level of the chromosomal set

3.31 Occasionally the mechanism by which homologous chromosomes segregate into different daughter cells goes wrong, so that both chromosomes move into the same daughter cell. This is called non-disjunction. If it occurs at meiosis it produces germ cells in which one particular chromosome is lacking, or conversely is present in duplicate. After fertilization these germ cells give rise to zygotes that are monosomic or trisomic for the chromosome in question. Among chromosomal changes non-disjunction undoubtedly produces the most drastic damage. It is also one of the commonest abnormalities observed in humans. Trisomy or monosomy for any of the larger chromosomes leads to early death of the embryo. Trisomy for a small chromosome may be compatible with life but the survivors are grossly abnormal, as they are for example in trisomy 21 or Down syndrome. The only monosomy found among survivors is sex-chromosome monosomy (XO), which is known as Turner syndrome. More than 2 sex-chromosomes can be tolerated but may result, however, in disturbances of sexual and mental development or behaviour, or both. Unlike translocation trisomy (para 3.30.3.1), trisomy due to non-disjunction is not transmitted by normal relatives.

4. Implications for carcinogenesis

4.1 This is an important and controversial subject which cannot be fully discussed in these guidelines. However, it is felt that some comment on the issues involved should be included, together with selected references. Boveri, (1914) reported on the chromosomal abnormalities seen in tumours, and implied a genetic origin of cancer. Fourteen years later Bauer (1928), formulated the somatic-mutation theory of cancer. The determination of the structure and genetic significance of DNA (Watson and Crick, 1953) did not immediately recall attention to the role of DNA in cancer, since at that time the "protein deletion theory" was strongly supported. This theory envisaged the induction of cancer by carcinogens which reacted with and eliminated a vital growth-controlling cytoplasmic protein.

4.2 The use of radioactively labelled carcinogens and the demonstration (Brookes and Lawley, 1964) that for a series of polycyclic hydrocarbons a correlation existed between carcinogenic potential and ability to bind covalently to the DNA of mouse skin, revived the somatic mutation theory. In the ensuing decade an ever increasing number of carcinogens were shown to react with the DNA of their target organ and the concept of the "ultimate carcinogen" as an electrophilic species generated by metabolism became widely accepted (Miller, 1970). Although the genetic or epigenetic origin of tumours is still a matter of debate it is clear that mutagenesis and carcinogenesis both involve heritable changes in cell phenotype. In the present context of mutagenicity guidelines evidence of genetic damage is sufficient to raise doubts as to the safety of a particular compound, but if the mutation theory of cancer is accepted then this same evidence would also be an indication of possible carcinogenic hazard. The role of mutation in cancer induction may be judged from the following brief summary of some relevant research areas.

4.3 *Correlation between mutagens and carcinogens.* The main reason for the loss of support for the mutation theory of cancer in the 1950s was the failure of a wide range of well known carcinogens, such as polycyclic hydrocarbons, aromatic amines and azo-dyes, to induce mutations in classical mutation systems such as phage, bacteria, yeast, etc. Burdette (1965) came out strongly against any relationship between mutagenesis and carcinogenesis. The recognition of *in vivo* carcinogen-DNA binding and the role of metabolism in generating the "ultimate carcinogens" totally changed this view when, with the use of a liver preparation plus micro-organisms, the DNA damaging activity of metabolites of potent carcinogens was first demonstrated (Malling, 1971; Garner, Miller and Miller, 1972). This idea was developed by Ames and his colleagues (Ames, Durston, Yamasaki and Lee, 1973). Two years later they reported the assay of 300 chemicals for mutagenicity in bacteria (McCann,

Chai, Yamasaki and Ames, 1975). In this and other more recent reports (Purchase *et al.*, 1976) a 90% correlation between carcinogenesis and mutagenesis was claimed. Much world-wide research effort is presently concerned with establishing the validity of this correlation using bacterial and other systems capable of testing for genetic damage (eg chromosomal aberrations, sister chromatid exchange, mutations in *Drosophila* and in a series of mammalian cells in culture). There are, however, other carcinogens such as asbestos and some hormones which are not generally considered to be mutagens.

4.4 It remains to be established what percentage of the more than 3000 known mutagens are also carcinogens. One example which will be followed with interest and concern is that of the food additive AF2 (trans-2-(2-furyl)-3-(nitro-2-furyl) acrylamide, which was widely used in Japan as a food preservative from 1964 until 1973, when it was withdrawn following the demonstration of its mutagenicity in bacteria and other test systems (Kada, 1973), and the demonstration of carcinogenicity in several animal species by further, more extensive, long term testing. Any carcinogenic risk to man would not yet be expected to have shown itself.

4.5 *Clonal origin of malignancy.* A mutational model for cancer would predict that any individual neoplasm would be derived from a single cell. Evidence that this is apparently the case for a variety of human tumours has been reviewed (Fialkow, 1976). The demonstration of monoclonality has included studies of chromosomal mosaicism and immunoglobin synthesis, but principally of X chromosome inactivation mosaicism. Study of this latter subject has mainly involved black women, among whom there is a high frequency of heterozygosity for an X-linked electrophoretic variant of glucose 6-phosphate dehydrogenase (G-6-PD).

4.6 As a result of random X chromosome inactivation the cells of such women may contain either the A or B form of G-6-PD, but any given cell contains only one form. The study of a wide variety of tumours occurring in these women has shown that individual tumours contain only one form of G-6-PD, while even small areas of adjacent normal tissue contain both enzyme variants. The neoplasms studied included chronic leukaemias, Burkitt lymphoma and carcinoma of many sites.

4.7 Unfortunately no suitable X-linked markers are available in laboratory animals, but this has been overcome by the production of experimental mouse chimaeras, dimorphic for the enzyme glucose phosphate isomerase. Skin tumours induced in such animals by hydrocarbons have been shown (Iannoccone, Gardner and Harris, 1978) to contain only one form of this enzyme, consistent with their being monoclonal.

4.8 *Epidemiological evidence.* Many dominantly inherited conditions which predispose to cancer in man are known. These include polyposis of the colon, nevoid basal cell carcinoma syndrome, Sipple disease (involving susceptibility to

medullary tumours of the adrenal and thyroid glands) and hereditary retinoblastoma. In a study of this latter condition, it was recognized that 40% of all cases of retinoblastoma were of the hereditary type, occurred at an earlier age, and were often bilateral and sometimes multi-focal within one eye. It was determined that the number of tumours per gene carrier was distributed in a Poisson fashion with a mean of 3 or 4 tumours. In contrast the incidence of retinoblastoma in the general population was one in 30,000, indicating that the predisposing gene increases the risk of developing the tumour by a factor of 10^5. This type of study led to the proposal (Knudson, Hethcote and Brown, 1975) that hereditary retinoblastoma results from a somatic mutational event in a cell already carrying a germinal, ie inherited, gene mutation.

4.9 A similar 2-mutation model for tumour induction was proposed (Armitage and Doll, 1957) as a result of studies of age-specific incidence of certain adult cancers; similar studies (Ashley, 1969a; 1969b) of polyposis coli also led to a multi-mutational model for colon cancer induction.

4.10 *DNA repair studies.* A recent review discussed in detail the role of faulty DNA repair in the induction of tumours (Paterson, 1977). Briefly, defective repair of damage induced in DNA by radiation or chemicals has been experimentally demonstrated in cells from patients suffering from the rare autosomal recessive disease known as xeroderma pigmentosum (XP), in which a predisposition to skin cancer is a common feature. Overall the data available from all sources strongly suggest that unrepaired or incorrectly repaired lesions in DNA lead to neoplastic transformation by increasing the frequency of somatic mutations. Direct evidence for this hypothesis comes from a study which showed that in the fish *Poecilia formosa* repair of u.v-induced lesions by photoreactivation protected against thyroid carcinoma (Hart and Setlow, 1975).

4.11 *Cell-fusion experiments.* In 1969 a collaborative study was started to analyse the genetics of malignancy by cell fusion studies. Hybrid cells were derived from the fusion of a series of mouse tumour cells in culture with non-malignant (or weakly malignant) cells. The hybrids were tested for malignancy by injection into X-irradiated newborn mice of the appropriate histo-compatibility group. In summary, this study demonstrated that malignancy could be suppressed by fusion of the tumour cell with a normal cell, but reappeared if the resulting hybrid lost certain chromosomes derived from the normal cell (Harris *et al.*, 1969). By an elegant series of experiments it was possible to show that suppression of malignancy was associated with the retention by the hybrid of a particular section of chromosome 4 of the normal mouse karyotype (Jonasson, Povey and Harris, 1977). Furthermore, since hybrids derived from fusion of different malignant cells did not complement each other (Weiner, Klein and Harris, 1974), it would seem that for these mouse tumours malignancy requires a recessive mutation at a single locus. Much further work is needed to substantiate this remarkable conclusion.

4.12 Further support for the recessive nature of malignancy was obtained through studies (Stanbridge, 1976), using HeLa cells and diploid human

fibroblasts. The human-human hybrids were tested for malignancy in immunosuppressed or nude mice. In contrast, evidence for the dominant nature of a malignant change has been provided by interspecific hybrid cells. Hybrids of non-malignant mouse cells and human fibrosarcoma cells are malignant in nude mice but lose their malignancy following the loss of human chromosomes. Similarly, hybrids formed by the fusion of human fibrosarcoma cells and normal human cells behave like tumour cells even though they retain the entire complement of chromosomes from both parental cells. In some cases malignancy in mammals may be a consequence of the presence of a virus, often integrated into the DNA of the host cell, and such virus-induced malignant states share many of the properties associated with a dominant change. For example, cell hybrids between virus transformed (malignant) human cells and normal mouse cells are malignant in nude mice and retain their virus-transformed state as long as the human chromosome bearing the virus is not lost from the cell (Koprowski and Croce, 1977).

4.13 Although there is considerable evidence implicating somatic mutation as well as virus involvement in the development of some malignant changes, there are, as has been mentioned, examples of carcinogenic agents (eg hormones and asbestos fibres) which do not appear to induce mutational changes. Thus it must be concluded that the somatic mutation theory of malignancy remains unproven, but the implication of the various lines of research outlined above is clear. The fact that a majority of known carcinogens, with certain exceptions, have been found to induce mutation is obviously important. The outstanding question concerns the carcinogenicity of the several thousand known mutagens. The final answer to this problem is likely to be delayed, but in the interim the pressure to use mutation-based tests as a pre-screen for potential carcinogens is increasing.

5. Genetic and partly genetic diseases of man: types, frequencies and mutation rates

Introduction

5.1 Before any untoward effects on human populations of increases of mutation rates which could be caused by environmental mutagens can be considered, it is necessary to have solid knowledge of the existing background of human detrimental mutations. It is the object of this chapter to review what is known of this background mutational load. Changes in genetic material, ie mutations, contribute significantly to human malformation and to disease manifest in early life, or that which makes its first appearance during early adulthood or later. For reasons that will be apparent the total contribution which genetic change, however it may originate and be maintained in the population, makes to human disease and disability is not precisely known, and in any case is variable in different populations, but is estimated to be of the order of 2%. However, this is a conservative estimate for the burden imposed by chromosome disorders and by diseases caused by single genes of large effect. If other diseases with a major genetic component are included the genetic contribution to frank disease can be estimated to be greater than 4% (see Table 5.10). In addition, however, there are many more subtle genetic influences that affect human characteristics, often clearly immunological or biochemical, which predispose those who carry such traits to a variety of untoward and pathological responses to environmental influences and thus to disease. It is at present not possible to quantify this part of the potential genetic contribution to ill-health but evidence suggests that it is substantial. However, there are some categories of genetic change that are more exactly documented than others and thus their contributions to human abnormality can be more accurately assessed. For this reason, as well as for reasons connected with the origins and maintenance in the population of the mutational changes, it is useful to consider these changes under 4 main categories:

a. Chromosomal abnormalities.

b. Defects and diseases due to single genes of large effect.

c. Congenital malformations with evidence of a sizeable genetic contribution to their origin.

d. Complex disorders in which genetic influences play a not indifferent role, and various other diseases in the origin of which there is a variable genetic influence.

5.2 In general, the influence of any increase in mutation rate will be uneven in these different categories. In (a) one is dealing with numerical and with structural chromosome mutations, and most are new mutations. The mechanism in the former case is generally non-disjunction, and its ultimate causation in man is unclear. It is maternal-age dependent (suggesting non-disjunction in oogenesis) but it is clear that meiotic non-disjunction can occur also in the male germ line, and may even involve both meiotic divisions sequentially. The mechanisms of structural chromosome mutations (rearrangements) are often, if not always, chromosome breaking events followed by misplaced reunion.

5.3 It has been estimated that at least 5%-10% of all human conceptions are chromosomally abnormal, and it is important to realize that a high proportion of the chromosome defects which result in genetic imbalance are lethal prenatally, and are a cause of spontaneous abortion (miscarriage), the exact frequency of which is not easily ascertained but which probably lies at between 15% and 20% of recognizable pregnancies. Thus the greatest proportion of these mutations remain hidden and this could make it difficult to detect moderate but still significant increments of these mutations. Among survivors at birth, mortality is high in some cases.

5.4 In (b) it can be assumed that point mutations are usually being dealt with but structural chromosome changes, such as deletions, that are undetectable (ie are beyond the limits of resolution of conventional microscopy) are a possibility in a proportion of cases. In this group it is in respect of point mutations especially, though also in respect of the other hypothetical changes, that it could be expected that clear and immediate effects of mutagens would be observed, for example, as an enhanced frequency of those detrimental dominant diseases and those conditions that are more obvious at or near birth. Naturally when considering germinal mutations, account would have to be taken of the long generation time of man, of the difficulties of monitoring human populations and of knowing accurately the baseline of existing detrimental mutations (see below).

5.5 In (c) and (d) the effects are often determined by the action of many genes and depend especially on strong interactions with the environment. It is difficult to predict what the influence of an increase of mutations might be on the incidence and prevalence of the various traits and disorders mentioned in these 2 categories. *Prima facie* the frequency of the conditions under consideration would appear insensitive to mutational changes unless they are of a very high order, or gradually accumulating over the generations. Recently, however, the contrasting view has been put forward that the impact resulting from mutational changes of genes which generally act in a multifactorial context — (c) and (d) above — may be far from negligible (Vogel, 1979).

a. Chromosome abnormalities

5.6 The normal chromosome complement of man consists of 46 chromosomes (ie 23 pairs), one in each pair of maternal and the other of paternal origin. Each chromosome can now be identified with precision using special staining methods which result in specific "banding". The number and different sizes of alternating dark and light bands which can be revealed give each chromosome a characteristic and individual appearance and define a number of segments within each chromosome. The chromosome complement, whose orderly arrangement is called a "karyotype", consists of 22 pairs of non-sex chromosomes (autosomes) and a pair of sex chromosomes. Normal females possess a medium-sized pair of sex chromosomes, called X chromosomes, while normal males possess one X chromosome and a much smaller sex chromosome named the Y chromosome. In normal circumstances sex development in the embryo depends on the presence (maleness) or absence (femaleness) of this Y chromosome, whose main function seems to be that of inducing testis development. By contrast the X chromosome carries a large number of genes which act on many aspects of somatic development and function ranging from, for example, blood clotting to intellectual function. Genes on the X chromosome are said to be X-linked; characteristics which they determine are called sex- (or X-)linked and are transmitted in a pattern which differs from that of autosomal genes. A further important characteristic of X chromosomes is that only one X functions when more than one, eg 2 in normal females, is present in the body cells. It follows that only one X chromosome reveals its genes at cell level. So in a normal female, some body cells show the activity of X-linked genes inherited from her mother, while others display those of paternal origin.

5.7 The autosome pairs are numbered sequentially from 1 to 22, from largest to smallest. When precise identification of individual chromosomes is not possible, it is customary to refer to them by "group lettering". Thus chromosomes 1, 2 and 3 together belong to Group A, 4 and 5 to Group B, 6 to 12 and the X Group C, 13 to 15 to Group D, 16 to 18 to Group E, 19 and 20 to Group F, and 21, 22 and the Y chromosome to Group G. It is an international convention to describe the chromosome complement by the total number of chromosomes, followed by the description of the sex elements. Thus a normal female is described as 46, XX and a normal male as 46, XY.

5.8 Chromosome anomalies are probably the most clear cut of all human mutations and account for at least 60 different conditions in survivors to birth. They are considered mutations in a genetic sense; more specifically they are genome and/or chromosome mutations, ie numerical and/or structural chromosome anomalies. The best known numerical anomalies are the autosomal and sex chromosomal trisomies which can cause the clinical syndromes of Down (mongolism, trisomy 21), Edwards (trisomy 18), Patau (trisomy 13), Klinefelter (sex chromosomes XXY), the XYY and XXX sex chromosome anomalies and the XO sex monosomic conditions (usually with Turner syndrome). In the XO (45, X) condition there is only one sex

chromosome, as a result of loss of either an X chromosome (maternal or paternal) or the paternal Y. Other numerical chromosome anomalies are known in survivors, some with more than one extra sex chromosome, for example 49, XXXXY or 49, XXXXX, or an extra autosome plus an extra sex chromosome, for example, trisomy 18 and XXY or XXX (symbolised by 48, XXY or XXX, +18). Most of the other chromosome abnormalities with imbalance are the result of structural chromosome changes which produce duplications (so-called partial trisomies) or deficiencies (so-called partial monosomies), or both together (duplication-deficiency). They are the derivatives of translocations and inversions, or result from simple deletions or from ring-or iso-chromosome formation. An iso-chromosome results from mis-division, transverse instead of longitudinal, at the centromere; it consists of a double short or long arm of a chromosome with the other arm missing.

5.9 In addition there are chromsome anomalies of structure in which no imbalance results, such as balanced reciprocal translocations (a special type of which are the centric fusions involving acrocentric autosomes, namely numbers 13, 14, 15, 21 and 22), and inversions of different types. The precise detection and classification of structural chromosome mutations has been made easier and more precise by the chromosome banding techniques which have been used since 1970 for specific chromosome identification.

5.10 Chromosome studies on some 56,000 newborn infants in many countries (Hook and Hamerton, 1977) detect a frequency of abnormalities of about one in 150 (Table 5.1 based on about 43,000 births), and most anomalies are numerical. Of these the commonest is trisomy 21 but sex chromosome trisomy (XXX, XXY or XYY), as a group, is somewhat more frequent than autosomal trisomy. Structural chromosome anomalies constitute about one third of the total (affecting about one in 500 newborn infants) and of these about 13% are unbalanced, though a somewhat higher figure is sometimes quoted. Of the structural anomalies, between 15% and 22% arise *de novo* (ie are new mutations) while the rest are inherited, somewhat more often from the mother than from the father.

5.11 Most autosomal anomalies are lethal prenatally or perinatally, as spontaneous abortions or as later fetal (stillbirths) and early neonatal deaths (grouped together as perinatal deaths). Some autosomal anomalies with imbalance are compatible with survival to birth but are generally lethal in early postnatal life, though trisomy 21 is a partial exception. Among neonatal deaths, chromosome anomalies are found in more than 5%; whilst among stillbirths the figure varies between 4.5% in fresh stillbirths and over 10% among the macerated. However, chromosome anomalies are particularly frequent in spontaneous abortons, 30% to 50% or more of which are chromosomally abnormal (see Table 5.2; Creasy, 1977; Boué and Boué, 1978). The variation reported in different studies depends largely on the gestation age of the abortuses. By pooling information from a number of major chromosome studies of spontaneous abortion data on almost 2000 chromosomally abnormal abortuses are available. The findings are shown in Table 5.3. A small

proportion of these were investigated by banding methods and allow some general conclusions to be reached about the distribution of the specific autosome trisomies.

5.12 From these data it is possible to arrive at approximate prevalence figures for various chromosome anomalies in early conceptions, based on the frequency of spontaneous abortion, on the proportion of chromosomally abnormal miscarried fetuses and on the proportion of the different chromosome anomalies found in them. The frequency of spontaneous abortion is estimated to lie at between 15% and 20% of all recognizable pregnancies and that of chromosomal abnormality among abortions is probably about 40%, though this figure is influenced by gestation age of the miscarriages studied.

5.13 On this basis, it seems probable that 6% or more of all early human conceptions which lead to a recognizable pregnancy are chromosomally abnormal. If very early conceptions are included this figure could be 8.5% or higher. The appropriateness of this estimate seems to receive confirmation from the chromosome findings in 500 abortions induced for socio-economic reasons, 7.4% of which were chromosomally abnormal. It can further be estimated that about 1.2% of all recognizable pregnancies could be 45, Xs, about 1% triploids and 3% autosomal trisomies (almost 1% trisomic for chromosome 16, and about 0.45% trisomic for chromosome 21 — the Down syndrome chromosome). Some 90% of chromosomally abnormal fetuses are rejected as spontaneous abortions, some (for example, triploids and 45, Xs) more frequently than others (eg trisomy 21 of which about two thirds abort and some are eliminated as stillbirths), and some hardly at all (sex chromosome trisomies XXX, XXY and XYY; some of these are lost as perinatal deaths). A point is worth making here which is relevant to monitoring of mutation in man. At present one class of mutations, namely the chromosomal ones and particularly among them the numerical ones, may seem to lend themselves reasonably well to monitoring at birth. Obviously there are problems with most trisomies because the frequency of these is related to the age structure of the population of breeding women, but this could be taken into account. However, it must also be remembered that chromosome anomalies are common among spontaneous abortions (40% or more). They are also quite common among perinatal deaths (5% to 6%) compared with survivors at birth (about 0.5%). In addition, compared with survivors they are more common in the middle trimester of pregnancy when diagnostic amniocentesis for chromosome disorders is done, generally on the grounds of maternal age. Taking those facts into account, chromosomally abnormal births in either direction because of its submerged variable buoyancy, the shifts of which could influence the incidence of chromosomally abnormal births in either direction because of its submerged part can be seen as a reservoir of chromosomally abnormal fetuses. It follows that differences in birth incidence of chromosome anomalies with imbalance and especially of autosomal trisomies, be they seasonal, secular or unpatterned, cannot be taken automatically to reflect differences in the frequency of non-disjunctional events occurring prior to conception; they could well be due to non-genetic factors affecting prenatal mortality. For this reason alone attempts to

monitor this type of mutation would be beset with difficult problems.

b. Defects due to single genes or large effect

5.14 Three main patterns of monogenic Mendelian modes of transmission are recognized; autosomal recessive, autosomal dominant and sex-linked (or X-linked), which may also be sub-divided into recessive and dominant. By 1975 altogether 1142 "proved" mutant phenotypes had been catalogued and there was an additional list of almost 1200 "probables". Of the former, 583 were classified as autosomal dominant, 466 as autosomal recessive and 93 as sex-linked, and the proportional distribution of the "probables" was similar (McKusick, 1975). This total number of diseases and conditions is thought to cover only a small fraction of the many relevant gene loci in man.

5.15 The range of gene (or point) mutations known to exist in man is best demonstrated by considering the haemoglobinopathies and the many haemoglobin variants that have been discovered, most of which have been carefully analysed in terms of their amino acid sequence and even more detailed chemical structure. Indeed even the sequences of some of the globin messenger RNAs are being determined. The various possible base substitutions and other rearrangements of the DNA of the genes coding for the various globin chains can be inferred from this work. Thus different mutant alleles of the different globin genes can be inferred to result from different types of changes of their DNA, ranging from transitions and transversions to frameshift changes due to base insertion or deletion, to alterations due to unequal crossing-over (an unusual type of mutational change). In addition mutants have been described in which a usually non-coding portion adjacent to a globin gene is apparently coded, either because of mutation of the termination code or because of its altered meaning resulting from a shift of reading frame. It is clear that all the amino acid substitutions in the globin molecule of man conform to alterations of the same genetic code that has been worked out on *E. coli.* and this is one of the facts that support the idea of the universality of the genetic code. It has been pointed out that it is remarkable that a bacterium, low in the organismic scale, should code for its protein with the identical codewords used by the cells of the Nobel prize winners who broke the code.

5.16 The incidence in the population of diseases and disorders apparently caused by single gene mutations has been estimated in different countries and with variable methods of ascertainment and is therefore imprecisely known. This accounts for some of the variability of the estimates of frequency. However, this variation is sometimes due to the actual variable frequency of some genetic diseases in different populations (Table 5.6; para 5.33). The main reasons for the variability of the estimates of the overall load of genetic disease are the inclusion or exclusion of disorders which cause relatively little inconvenience in early adult life, as can be the case with some autosomal dominant diseases, or of conditions which do not represent disease entities *per se* but nevertheless play a part in the origin of diseases that manifest mostly only in adult life, eg familial

hypercholesterolaemia and α_1-antitrypsin deficiency. These conditions have not been tabulated and are often considered as "traits", albeit potentially detrimental, rather than disease entities.

5.17 It must be stressed that most human genetic diseases are genetically heterogeneous (Harris, 1974). This may mean that in different families the same disease is caused by different alleles at one locus, or the heterogeneity may result from mutations at different gene loci which are normally responsible for the production of the same, or different, enzyme-proteins which subserve similar functions, and their alteration causes disorders which are not yet clinically distinguishable from each other. Thus "mimic" genes seem to be a feature of at least some human genetic diseases. Additionally, heterogeneity of genetic disease results from the activity of the whole genetic background on the loci under scrutiny and from the activities of environmental forces.

5.18 Table 5.4 is a conservative list of the incidence in Caucasian populations, related to 1,000 live births, of the major autosomal dominant diseases. The miscellaneous group "other" is likely to be an underestimate. It is worth noting that of these conditions neurofibromatosis, which according to some estimates appears to be relatively common, shows little evidence of genetic heterogeneity. Somatic mutation may be responsible for a proportion of cases of this condition which, incidentially, has nearly the highest estimate of germinal mutation rate of any of the known dominants (Table 5.12). The overall incidence of autosomal dominants is thus between 1.85 and 2.64 per thousand births if one includes the relatively common hereditary spherocytosis and bilateral polycystic disease of the kidney in adults. It is said that 75% of cases of this latter condition are not detected or recognized in life but that the anomaly is found in 0.2% of autopsies. Whilst in more recent times the cumulative population prevalence was estimated to reach 0.8 per thousand by 80 years, but at an earlier period only 0.5 per thousand (a difference presumably due to changes in diagnosti practice), by 45 years it was only 0.2 per thousand. However, of those cases that are recognized, over half present with urinary-tract symptoms and over one fifth with cardiovascular signs, and generally require renal transplantation or dialysis. If otosclerosis, adult-onset form (Morrison, 1967), which seems to be inherited as a Mendelian dominant in about one third of the cases, were added to the list the incidence of autosomal dominants would rise by a further 1.0-1.5 per thousand. Other conditions of lesser clinical importance could also be added. Conversely, it is clear that if hereditary spherocytosis and polycystic kidney were not included, the rate of autosomal dominant disorders would be between about 0.7 and 1.0 per thousand births. These points are made because the different estimates of the frequencies of genetic disorders found in the literature often depend on inclusions or exclusions of just this nature, and exclusions or inclusions are ultimately dictated by considerations of the detriment caused by the conditions, by uncertainties about their mode of inheritance or doubt about the method of ascertaining their frequencies.

5.19 It should be stressed again that some of the figures quoted are no more than rough estimates due to the difficulties of ascertainment, particularly of

those dominants with delayed manifestation in late adult life. This is the case, for example, with Huntington chorea, myotonia dystrophica, and polycystic renal disease.

5.20 It is important to note that some of these relatively common dominant diseases of delayed onset in late adult life are unlikely to be maintained at their high level of prevalence by a high mutation rate, but have been accumulated in the human population throughout evolutionary time. Technical reasons make estimation of the actual mutation rates (see Table 5.12) difficult and hence imprecise.

5.21 Deafness and blindness are comprehensive categories which, like severe mental retardation (Tables 5.5, 5.6 and 5.8), contain many conditions of varying genetic origin as well as many of environmental source. In Tables 5.5 and 5.6 attempts have been made to include only the genetic components. It has been estimated that there are at least 34 "mimic" genes for deafness (but there might be 100), and there may be more for blindness. For deafness, 5 is assumed to be the number of autosomal dominant genes, 15 to 25 are thought to be autosomal recessive and 4 to be X-linked. The corresponding figures for blindness "mimic" loci are 15, 23 to 35 and 9 (Cavalli-Sforza and Bodmer, 1971).

5.22 A condition not listed among the autosomal dominant disorders is familial hypercholesterolaemia (Fredrickson type II), which seems to be heterogeneous and has an estimated frequency of 0.1% to 0.5% in the UK population (Motulsky, 1976; Lancet, 1977). It is related to ischaemic heart disease, which tends to occur in the second decade of life in the homozygous mutants, whilst in half the heterozygotes some manifestation of coronary artery disease is present by 50 or 60 years of age, in this case clearly beyond the age of active reproductive life. Two other autosomal dominant disorders of lipid metabolism of this type seem to be even more common (familial hypertriglyceridaemia and combined hyperlipidaemia). A condition which is relatively common and clearly heterogeneous is gout (Wyngaarden and Kelley, 1972), with a population incidence of about 3 per thousand; its relationship to hyperuricaemia without gout seems clear and it is thought that it can result from the activity of different abnormal genes. Another relatively common state is α_1-antitrypsin deficiency, which is related to specific alleles present at the protease inhibitor (Pi) locus. It is estimated that in European populations between one in 1500 and one in 3000 newborn infants, or even more, may suffer from antitrypsin deficiency (Harris, 1975: Fagerhol, 1976). Some 10% to 20% of them develop a serious form of neonatal hepatitis. Later, as adults, some 15% to 30% of antitrypsin-deficient individuals develop serious pulmonary emphysema in response to environmental urban pollutants. Thus it can be seen that the addition of these and similar conditions and traits to the list of dominant diseases could increase considerably the estimate of total incidence.

5.23 Table 5.5 lists some of the commoner autosomal recessive diseases in Caucasians, and again the group "others" is certainly an understatement. As in

the case of dominant disease there is variation in the estimated frequencies of recessive diseases. This is not only the result of the sampling of different populations with different genetic backgrounds, or of variation due to sample size, but sometimes also the differences result from the methods used in arriving at frequency estimates. As an example, gene frequencies, and hence disease frequencies for rare recessive conditions, can be calculated by using an estimate based on the frequency of first cousin marriages. The method is only a coarse approximation, however, as it takes no account for example, of contributions of more than one locus, of more than one allele (in the case of abnormal compound heterozygotes), or of less strict degrees of consanguinity. The application of biochemical techniques to the study of autosomal recessive disorders has made their recognition easier in recent years (see below). These techniques have allowed the recognition of many examples of enzyme-protein variation, some very rare and others reaching polymorphic frequencies. Thus suxamethonium sensitivity, for example, which is found in about one in 2000 Europeans, is due to the presence of mutant alleles which result in the production of a serum cholinesterase with impaired activity (Harris, 1975).

5.24 By contrast, "silent" alleles at one of the 2 cholinesterase loci are very rare in most populations, though they are found very frequently in some (eg in 25% of Alaskan Eskimos). One should draw attention to the heterogeneity of autosomal recessive diseases even for conditions such as phenylketonuria. Also the special position of cystic fibrosis (fibrocystic disease of the pancreas) is worthy of comment. This serious lethal disease is relatively common in most Caucasian populations but 30 to 40 times rarer among Africans and Orientals (Table 5.6).

5.25 Similar ethnic variability is found for Tay-Sachs disease, which has a frequency of about one in 3000 in Ashkenazi Jews and about a thousand times less in other Jews and in Gentiles. These diseases must be maintained in the relevant populations at their frequency levels largely by means other than recurrent mutation. Ethnic variation is seen also in the incidence of phenylketonuria which tends to be somewhat more frequent in Scotland and again very rare in Africans and Orientals.

5.26 Table 5.7 lists the approximate incidence at birth of the commoner sex-linked recessive diseases. Many others are very rare; for example, Lesch-Nyhan disease, due to hypoxanthine-guanine phosphoribosyl transferase deficiency, is between one and two orders of magnitude rarer than some of those quoted in the Table (Seegmiller, 1976): yet even this disorder is genetically heterogeneous. Conversely the fairly high frequency of Duchenne muscular dystrophy should be noted. Secondly it should be remembered that the locus which specifies the enzyme glucose-6-phosphate dehydrogenase (G-6-PD) is X-linked. At this locus there may be more than 20 different alleles, many of which reduce G-6-PD activity (Harris, 1975; Kirkman, 1971) and 2 of which, one in Africans and the other in Mediterranean peoples, reach polymorphic frequencies, probably because the deficiency of the enzyme in the red cells acts as a protective mechanism for carriers of the gene in malarial zones. On the other hand, these

enzyme deficiencies underlie widespread sensitivity (with haemolysis) to many drugs (sulphonamides, primaquine, naphthalene) or to foods such as the fava bean (favism).

5.27 The queston of dominance or recessivity of sex-linked conditions is complex because of the random inactivation in cells of females of one X chromosome, with the consequent activity of its homologue only. Because of this intermediate female manifestations of many sex-linked "recessive" disorders are not unusual. In the few sex-linked abnormalities classified as dominant (incontinentia pigmenti, oro-facio-digital syndrome, etc) it seems that affected males die soon after conception, with consequent deficiency of males in the families where the abnormality segregates. Only genetically and phenotypically normal males survive and diseases are transmitted from mother to daughter. Mental retardation, like blindness or deafness, is a comprehensive category and the frequency of the occurrence of an X-linked variety is only an approximate estimate. It now seems that some types of X-linked mental retardation form a discrete group that will be specifically recognizable.

5.28 In summary, individual human single gene diseases have birth frequencies ranging from about 10^{-5} to about 10^{-6}. Conditions which result from mutant genes with a specific single base substitution may have frequencies of the order of 10^{-9}. Conversely, as we have seen, there are genetic conditions with birth frequencies around one in 1,000 or even higher. Taking an average frequency of single gene genetic disease of 10^{-5} to 10^{-6} births and relating this to the more than 1,000 genetic disorders so far catalogued with assurance, the overall incidence at birth of single gene diseases could lie between 1 and 0.1%, and within these limits lie the totals cited in Tables 5.4, 5.5 and 5.6. In the United Nations Scientific Committee's latest assessment of genetic risks from radiation (UN Scientific Committee, 1972), an estimate of 1% has been used as the combined incidence of serious dominant and sex-linked conditions. Naturally, as already stressed, these figures vary when different populations are considered, and some examples of the variable frequencies of genetic diseases are given in Table 5.6 while the ethnic variability of some malformations is instanced below. There are a number of possible reasons for the variation in Table 5.6, but one of them, when dealing with recessive conditions, may reside in a selective advantage possessed by heterozygous carriers, to which we have already referred when discussing X-linked G-6-PD (para 5.26).

5.29 In this situation the selective advantage enjoyed by the carriers in a particular environment leads to a persistence in the population of the recessive gene, which is detrimental in the homozygous state and indeed may lead initially to selective increment in gene frequency over a period of time. Such a selective advantage can operate only as along as the particular environmental conditions obtain, and a change in them may abolish or even reverse the advantage, which thus may be of a transient nature. A characteristic example is provided by the sickle-cell haemoglobin gene, which involves the production of a mutant β-globin. In double dose this causes a severe disease (sickle-cell anaemia) which results in a roughly 80% reduction in fitness compared to

persons with normal haemoglobin. However, heterozygotes for the gene (those with the sickle-cell trait) enjoy a selective advantage in a malarial environment where they are fitter (by about 25%) than those who are homozygous for the gene for normal β-globin production. For this reason the sickle haemoglobin gene frequency has become as high as 10% in certain populations in which malaria exercises a selective pressure.

5.30 Somewhat similar in respect of its widespread polymorphism and relationship to a malarial environment is the situation of the thalassaemias. These are heterogeneous disorders of synthesis of haemoglobin which result from the reduced rate of production of one or more of the globin chains, with consequent imbalance of the types of haemoglobin synthesized, and therefore with detrimental effects on blood production (Weatherall and Clegg, 1972). There are 2 main classes of thalassaemias, the α and the β, each with several distinct genetic types. The diseases which they produce can be relatively common in Mediterranean peoples (Italy, Greece, Turkey and Iran), where the heterozygous carrier state of thalassaemic genes (thalassaemia minor, thalassaemia trait) may reach frequencies of 10%-20%, and thus the numbers of those affected by the severe disease produced by the homozygous state of the abnormal gene may amount to up to 1% of all births. In Great Britain homozygotes for thalassaemia are found among those persons and their descendants whose country of origin, in more than half the recently detected cases, was Cyprus (Modell, Benson and Payling Wright, 1972).

c. Congenital malformations

5.31 The incidence of the commoner developmental anomalies is listed in Table 5.8 and is expressed per thousand births. In the context of this Table (as well as the previous ones) the meaning of "incidence" is, broadly, "proportion of persons ever affected". Relatively unimportant conditions like accessory auricles, "postural" foot deformities, or minor birth marks are excluded but glandular hypospadias, for example, is included.

5.32 A number of the listed conditions seem polygenically determined, often with an important environmental component. Support for this view is probably best for pyloric stenosis and congenital dislocation of the hip, and good for the major neural anomalies. Less good is the evidence, for example, for some types of cardiovascular abnormality.

5.33 For some of the conditions listed in Table 5.8 there is considerable regional or racial variation. For example both anencephaly and spina bifida in the British Isles have a low incidence in the South East (1.4 and 1.5 per thousand respectively), which is higher in the West and North of England; in Northern Ireland this rises to 4.2 and 4.5 per thousand respectively, and in Eire up to 6.0 per thousand (Carter, 1973; 1976). The incidence of talipes in Orientals is about half that among Caucasians, as given in the Table, whilst in Hawaiians and other Polynesians it seems to be 6 to 7 times as common.

Conversely cleft lip (with or without cleft palate) has a high incidence among Mongoloids and particularly among Amerindians (over 3.6 per thousand births), with a low frequency in Afro-Americans (0.4 per thousand births). Congenitally dislocated hips are commoner among Amerindians and Lapps and congenital pyloric stenosis among Northern Europeans. The prevalence of serious mental defect, 2.0 per thousand (Penrose, 1963; Polani, 1973) is based on estimates in school children, and excludes Down syndrome and the groups in Tables 5.5 and 5.6. Excluding mental defect resulting from severe infection, trauma etc, at least 12% of cases of severe mental defect could be attributed to the action of recessive genes (Tables 5.5 and 5.6); in addition some of the genetically determined cases will be due to dominant genes and some can be estimated to be of polygenic origin. Altogether it is thought that at least 350 autosomal loci are responsible for mental retardation, and that approximately one third of normal persons are heterozygous for gene(s) for severe mental retardation (Motulsky, 1976). Other points to note are the very high frequency at birth of "postural" foot anomalies, more than 10 times as common as the figure given in the Table, which refers only to the serious "non-postural" defects. Also congenital dislocation of the hip and instability is 5 to 20 times more common in the newborn than the estimate given in Table 5.8. There is evidence that the frequency of pyloric stenosis is falling. This condition is commoner in males than in females, and a much greater incidence of inguinal hernia and of course hypospadias are characteristic of the male sex (in the Table the frequencies are given for the 2 sexes together). Other conditions, for example congenital dislocation of the hip and idiopathic scoliosis, are commoner in girls (Wynne-Davies, 1970).

d. Other diseases in which there are genetic influences

5.34 Mental disease has a population prevalence of about 1.5% and with epilepsy represents a considerable burden of ill-health. Another relatively common condition is diabetes mellitus. Table 5.9 lists some conditions in which genetic factors seem to be important but play a variable role. To these could be added the apparent contribution of genetic influences to the origin of ischaemic heart disease (see para 5.22), of ankylosing spondylitis, rheumatoid arthritis, psoriasis, multiple sclerosis (see eg the relationship between these diseases and HLA haplotypes (Svejgaard *et al*, 1975) and cancer (eg xeroderma pigmentosum, Bloom disease, Faconi anaemia, ataxia telangiectasia and allegedly the carrier states of 2 of these at least (Polani, 1979).

5.35 As for the manner in which genes contribute to the origin of schizophrenia, various models have been proposed, from the simple monogenic to the multigenic. The monogenic model has 2 alternatives, autosomal dominant or recessive. A threshold for the origin of the disease in those carrying the relevant gene(s) is incorporated in some of the models. In all models a sizeable environmental component is accepted as a triggering factor of the illness (Slater and Cowie, 1971). The manner in which genes contribute to the origin of the affective psychoses (cyclothymia) is equally uncertain, though

again the reality of the existence of a relevant genetic component is not in doubt. Major autosomal single gene inheritance, particularly for the bipolar disorders, has been proposed by polygenic determination has also been considered an alternative in this condition (Slater and Cowie, 1971). Dominant X-linked inheritance has been proposed, at least for a group of patients with the bipolar illness (Mendlewicz, Fleiss and Fieve, 1972; Mendlewicz and Fleiss, 1974; Mendlewicz and Rainer, 1977), but this suggestion has met with criticism. Al already stated, it would be impossible to predict the effect on incidence which an increment of the mutation rate of the genes responsible for the genetic component of the psychoses and the other disorders in this heterogeneous group of conditions would have. Nevertheless it is inconceivable that any increment could be advantageous.

5.36 In conclusion it is possible to attempt to summarize (Table 5.10) the contribution of genetic influences to disease by considering together the contributions listed in Tables 5.1, 5.4, 5.5, 5.6 and 5.8. These figures can usefully be compared with those from 2 population surveys of genetic or part-genetic disorders conducted in 2 defined populations in the British Isles and in British Columbia. The first was published in 1959 and predates chromosome studies in man, while the second relates to a cohort of children born in 1964-1966 who were entered in a voluntary register or were identified through surveillance for congenital anomalies. Although the 3 sets of data in Tables 5.10 and 5.11 have been compiled and derived in different ways there is a degree of similarity of trends between them. The relative lack of chromosomal cases in the Canadian survey reflects the mode of ascertainment. The same is true for the paucity of dominants, due to exclusion of those with late manifestation. The method of ascertainment is likely to miss practically all sex-chromosome anomalies because phenotype changes suggesting the presence of these chromosome anomalies may not be apparent in early life, or indeed may escape notice altogether. It is also likely that this survey covers and includes trivial as well as more serious congenital malformations. However, the data in Table 5.8 (from which those in Table 5.10 are a derived proportion) and those in Table 5.11 agree quite well in respect of frequencies of congenital malformations.

e. Mutation rates in man

5.37 The calculation of gene mutation rates in man is particularly difficult because it has to be based on a number of parameters, measurements of which are often difficult to obtain and frequently are no more than approximations. One source of difficulty is in knowing exactly what some of the mutations really are at the molecular level. The detailed knowledge needed is not yet available and so it is impossible to be precise about the type of mutational process which is involved in most cases. Amongst other difficulties are the detection of rare phenotypes, the existence of genetic (as well as phenotypic) heterogeneity, and the necessity of estimating the fitness of the mutant phenotypes through which one may arrive at such estimates of the mutation rates as are possible in a non-experimental organism. For these reasons the most reliable methods are those

which rely on the detection of a clear phenotype in a specific population and on the identification by family studies of those who have directly inherited the defect and those who have not. This direct approach can only be applied to dominant conditions, and clearly is only really reliable when the penetrance of the condition is complete, its expression in the individual is full and relatively invariate, and when non-genetic phenocopies and genetic heterogeneity do not exist. Even then, as already stated, there is generally no information about the intricate nature and the molecular aspect of the mutational process at the chromosomal and genic levels. Furthermore, bias is introduced when selecting for study of the mutation rate the detrimental dominants that have been detected in man. Unavoidably the tendency is to select the commoner of these dominant conditions, so that it is not improbable that the mutation rate estimates may also be biased towards the higher values. However, under ideal conditions reliable estimates of mutation rates can be made and expressed on a per specific locus basis, per generation. But such ideal conditions are seldom met, so that calculations of human mutation rates tend to be made even more indirectly and to depend in general on many approximations.

5.38 Difficulties are particularly obvious when one is dealing with autosomal recessive conditions. It should be stressed that complete neutrality in heterozygous carriers, ie complete recessivity, of the more detrimental mutations which are responsible in the homozygous state for lethal conditions or for severe diseases, for example phenylketonuria, is unlikely. However, the possible detrimental effect in the carriers (if detriment exists) may be quite small and therefore may be difficult or in practice impossible to detect. Nevertheless, because of the relatively high frequency of these carriers in the population, a recessive disease with a population frequency of 1:40,000 will have a carrier frequency of about 1:100; even small detriments will result in a relatively prominent loss of the corresponding detrimental genes. For example, a recessive disease with a frequency of one in 40,000, which reduces viability of the homozygous mutants by 10%, even if it impaired the reproductive fitness of the carrier by only one per thousand, would lead to greater loss of the detrimental genes from the heterozygous carriers than from those affected by the disease. Yet it would be impossible to detect the small loss of fitness of the heterozygotes. Naturally equilibrium could only be maintained in this case if the mutation rate were proportionally higher when there is heterozygote disadvantage than when the heterozygotes are selectively neutral and the disadvantage resides only in the homozygous mutant state. The corollary of this is that an increased mutation rate for such a gene would introduce to the population new persons with a detriment, and so break the existing equilibrium between new mutation and elimination of the detrimental gene. In this view of things recessive mutations whose presence may remain unobtrusive for generations, that is until 2 such mutant genes happen to come together in the same individual, are of no small interest, as they might contribute substantially by force of numbers of carriers to detrimental effects.

5.39 It should be remembered that in general the maintenance of equilibrium frequencies of some detrimental genes in the population requires that gene

losses from the population be balanced by corresponding gains through mutation. In respect of recessives, because the losses of such genes from carriers are proportionally quite sizeable compared with the losses from the homozygous mutant, the influence of fitness of the heterozygotes on estimates of gene mutation rates is important. Therefore, because of the uncertainties of the effect of recessive detrimental genes in heterozygous carriers, which at times may actually be advantageous to them (see para 5.36), mutation rate estimates of recessive genes are likely to be very unreliable. For this reason Table 5.12, largely taken from a recent critical review of the problem, sets out the mutation rates for a number of autosomal dominant and sex-linked diseases as estimates only. In many instances, when more than one value is given by authors, the maximum and minimum estimates have been listed. It should be noted that recently attempts have been made to calculate the per locus mutation rates for conditions which are clearly heterogeneous clinically and genetically. For example, this has been done for "severe mental retardation" (2.4×10^{-5} per locus (Morton, 1960)) and for "dominant deafness" (4.7×10^{-5} per locus (Crow, 1961)). Granted the assumptions inherent in these estimates, they tend to fall in line with the values estimated more specifically. In the course of population and family studies on biochemical variation of a small number of specific enzymes in man, it has also been possible to put an upper limit to the estimate of mutation rate. For these loci this has turned out to be 2.24×10^{-5} per gene per generation. It can be seen that most mutation rates have values of the order of 10^{-5} to 10^{-6} per gamete per generation, and clearly these orders of frequencies also reflect the fact that the commoner types of disorders have been preferentially studied. It has been calculated that the median value for a fairly large number of X-linked mutations in man is 1.6×10^{-7}. Attempts have also been made, on the basis of data derived from the detailed biochemical resolution of mutant proteins like haemoglobin, to estimate the per codon mutation rate (specific base substitution in a codon), and values of the order of 10^{-9} have been obtained (Edwards, 1974).

5.40 Numerical and structural chromosome anomalies can be considered as dominant changes and calculation of their mutation rates, except for some of the structural chromosome mutations, is a relatively simple matter. The mutation rates for all chromosome anomalies taken together and relative to survivors at birth differ from the gene (or "point") mutation rates by about 2 to 4 orders of magnitude. It is to be noted that the great majority of numerical chromosome anomalies are new mutations and are only exceptionally inherited. Also one must note that mosaics represent mostly somatic mutations, though some probably follow on germinal mutation. Thus from Table 5.1 it can be concluded that numerical mutations detectable at birth have a mutation rate of the order of almost 2 per thousand gametes per generation. The total mutation rate for the structural chromosome anomalies, mostly centric (Robertsonian) fusions and translocations, is about 0.16-0.24 per thousand gametes per generation (Polani *et al.*, 1965; Jacobs, 1972).

Table 5.1: *Chromosome anomalies in live births, per thousand**

Numerical				
	45, X	0.07		
Sex chromosome trisomics	47,XXX	0.54		
	47,XXY	0.61	1.64	
	47,XYY	0.49		
	Other sex chromosome anomalies (mostly mosaics)	0.96		4.14
Autosomal trisomics	47, +D	0.12		
	47, +E	0.19	1.45	
	47, +G	1.14		
	Triploidy	0.02		
Structural				
	Balanced D/D centric fusions	0.79		
	Balanced D/G centric fusions	0.21	1.93	
	Balanced translocations: Inversions	0.93		2.21
	Unbalanced translocations, inversions and deletions	0.28		
Unclassified		0.51		
Total		6.86	(1 in 146)	

*References: Bochkov *et al.*, 1974; Friedrich and Nielsen, 1973; Hamerton *et al.*, 1975; Jacobs *et al.*, 1974; Lubs and Ruddle, 1970; Nielsen and Sillesen, 1975; Sergovich *et al.*, 1969

Table 5.2: *The prevalence of chromosome anomalies in spontaneous abortions, perinatal deaths and live births* (All per 1,000)*

Type of anomaly	Spontaneous abortions			Perinatal deaths (N = 500)	Live births (N = 43558)
	<12 weeks (N = 1498)	<18 weeks (N = 225)	<28 weeks (N = 941)		
Tetraploidy	38.05	47.06	12.75	0	0
Triploidy	122.16	54.90	40.38	2.00	0.13
Autosomal trisomy	330.44	250.98	151.98	28.00	1.24
Monosomy X	93.46	156.86	72.26	2.00	0.046
Other	30.70	39.22	27.62	24.00	4.43
Total all anomalies	614.81	549.02	304.99	56.00	6.26

References: Boué *et al.*, 1975
Creasy *et al.*, 1976
Evans, 1977
Lauritsen, 1976
Machin, 1974

Table 5.3: *A. Chromosome anomalies among spontaneous abortions (per cent)**

Autosomal trisomy	45,X	Tri-ploidy	Tetra-ploidy	Structural anomaly	Mosaic-ism	Double trisomy	Autosomal monosomy	Sex trisomy	Others
50.6	19.6	16.1	5.3	3.7	2.7	1.6	0.2	0.2	0.3

B. Specific autosomal trisomies as a percentage of all trisomic spontaneous abortions

Specific chromosomes involved	16	21, 22	14, 15	2, 7, 8, 18	4, 9, 10, 13	20	3, 5, 6, 11, 12, 19	1, 17
Incidence, %	30	20	7.5	5	2.5	1.5	0.5	Nil

*Creasy, 1977

Table 5.4: *Estimated incidence at birth of some relatively common and better known autosomal dominant diseases.*

Condition	Frequency per thousand	References
Huntington chorea	0.1-0.22	Baraitser *et al,* 1977; Bodmer and Cavalli-Sforza, 1976; Skokeir, 1975
Neurofibromatosis	0.07-0.4	Verschuer, 1962
Myotonia dystrophica	0.12	Grimm, 1975
Epiloia	0.006-0.03	Penrose, 1963; Borberg 1951; Verschuer, 1962
Retinoblastoma	0.041	Vogel, 1961
Achondroplasia (classical form)	0.015-0.10	Rimoin, 1975
Diaphyseal aclasis (Multiple exostoses)	0.05-0.1	Murken, 1963a; Murken, 1963b
Marfan syndrome	0.015-0.04	Lynas, 1958; McKusick, 1972
Multiple intestinal polyposis	0.042-0.12	Reed and Neel, 1955; Veale, 1965
Gardner syndrome	0.07	Pierce et al, 1970
Bilateral polycystic disease of the kidney (adult form)	0.8	Dalgaard, 1957
Profound childhood deafness	0.07-0.12	Cavalli-Sforza and Bodmer, 1971; Chung, Robinson and Morton, 1959; Fraser, 1976; Nance and McConnell, 1973
Waardenburg syndrome	0.005-0.025	Fraser, 1976
Hereditary spherocytosis	0.22	Morton, *et al,* 1962
Acute intermittent porphyria	0.015-0.024	Stanbury, Wyngaarden and Fredrickson, 1972; Tschudy, 1973; Waldenstrom and Haeger-Aronsen, 1967
Porphyria variegata	0.01	
Others	?0.2	Bodmer and Cavalli-Sforza, 1976
Total	1.85-2.64	

Table 5.5: *Estimated incidence at birth of some relatively common and better known autosomal recessive diseases*

Condition	Frequency per thousand	References
Cystic fibrosis	0.4-0.5	Bodmer and Cavalli-Sforza, 1976
Severe mental retardation (excluding PKU and other inborn errors listed below)	0.8	Bodmer and Cavalli-Sforza, 1976
Phenylketonuria (PKU)	0.09	Harris, 1975; Levy, 1973
Galactosaemia	0.013	
Other aminoacidurias	0.28	
Mucopolysaccharidoses	0.043	McKusick, 1972
Tay-Sachs disease	0.001-0.003	Harris, 1975; Myrianthopoulos and Aronson, 1966
Severe childhood deafness	0.22-0.37	Cavalli-Sforza and Bodmer, 1971; Chung Robeson and Morton, 1959; Nance and McConnell, 1973
Childhood blindness	0.1	Cavalli-Sforza and Bodmer, 1971
Acute spinal muscular atrophy (Werdnig Hoffman)	0.039	Pearn, 1973; Pearn and Wilson, 1973
Oculo-cutaneous albinism	0.04-0.1	Witkop, 1971
Adrenogenital syndromes	0.1	Bodmer and Cavalli-Sforza, 1976
Others	?0.1	Bodmer and Cavalli-Sforza, 1976
Total	2.23-2.54	

Table 5.6: *Some genetic diseases that show generally marked regional variation*

Condition	Frequency per 100,000	Region	References
Porphyria variegata	300	South African Whites	Brock, 1972
	1.0	Caucasians generally	Tschudy, 1973
Myotonia dystrophica	90	New Zealand	Brock, 1972
	5.5	Germany	Grimm, 1975
Huntington Chorea	17	Tasmania	Brock, 1972
	8.4	Canada	Shokeir, 1975
	3.0	Japan	Kishimoto, Nakamura and Sotokawa, 1959
Adrenogenital syndromes	200	Yupik Eskimos, Alaska	Brock, 1972
	20	Switzerland	New and Levine, 1973
	2.5	North America	Polani 1973
Cystic fibrosis	40.0-50.0	North Europeans	Polani, 1973; Bodmer and Cavalli-Sforza, 1976
		Victoria, Canada	Brock, 1972
	1.0	Afro-Americans and Orientals (Chinese) Hawaiians (non-Caucasoid)	Bodmer and Cavalli-Sforza, 1976 Wright and Morton, 1968
Tay-Sachs disease	17.0-40.0	Ashkenazi Jews	Harris, 1975
	0.1-0.3	Gentiles; Sephardi Jews	Harris, 1975; Polani, 1973 Myrianthopoulos and Aronson, 1966 Brock, 1972
Gaucher disease (adult type)	5.0-50	Ashkenazi Jews-Israel	Stanbury, Wyngaarden and Fredrickson, 1972; Brock, 1972
	"seems very rare"	Other Caucasians	Stanbury, Wyngaarden and Fredrickson, 1972
Hereditary tyrosinaemia	15.0-18.0	Chicontim Region of North East Quebec, Canada	Brock, 1972
	0.4	North East USA and East Canada	Polani, 1973
	0.07	Caucasians generally	Levy, 1973
Phenylketonuria (PKU)	16.7-22.2	Scotland, Ireland	
	8.5	Caucasians generally	Levy, 1973
	"virtually absent"	Ashkenazi Jews	
Cystinuria (I and II)	96.0	Sweden	Bostrom and Hambraeus, 1964
	2.5	England	Knox, 1958
Duchenne muscular dystrophy	41.0	Western Australia	Brooks and Emery, 1977
	21.2	Caucasians generally	
Thalassaemia	1000	Mediterranean peoples, etc	Polani, 1973
Sickle cell anaemia	1000-2000	Africans	Polani, 1973

Table 5.7: *Estimated incidence at birth in males of the commoner sex-linked diseases*

Condition	Frequency per thousand *males*	References
Duchenne muscular dystrophy	0.20-0.33	Brooks and Emery, 1977; Stevenson and Kerr, 1967
Becker muscular dystrophy	0.009-0.012	Stevenson and Kerr, 1967
Haemophilia A	0.10-0.12	Stevenson and Kerr, 1967
Haemophilia B	0.02-0.03	Stevenson and Kerr, 1967
Ichthyosis	0.20	Stevenson and Kerr, 1967
Non-specific mental retardation	0.08	Stevenson and Kerr, 1967
Severe childhood deafness	0.025-0.915	Stevenson and Kerr, 1967
Childhood Blindness	0.020	Cavalli-Sforza and Bodmer, 1971; Bodmer and Cavalli-Sforza 1976 Fraser, 1966
Anhydrotic ectodermal dysplasia	0.01	Stevenson and Kerr, 1967
Testicular feminization	0.016	Jagiello and Atwell, 1962
Others	?0.1-0.25	
Total	0.780-1.983	

Table 5.8: *Frequencies in the population (mostly in Great Britain) of the commoner anomalies of development expressed per 1,000 births**

Conditions	Frequency per 1,000 births	References
Anencephaly with or without spina bifida	2.0-30	Polani, 1973
Spina bifida cystica without anencephaly	3.0	Polani, 1973
Malformations of heart and great vessels	6.5-8.0 (+4.00**)	Polani, 1973; Kenna, Smithells and Fielding, 1975
Congenital hydrocephaly (without spina bifida)	0.5-1.4	Leck, 1974a; 1974b.
Hypertrophic pyloric stenosis	2.0-4.0	Leck, 1976
Idipathic scoliosis	2.2	Polani, 1973
Talipes equinovarus	1.0	Leck, 1976; Wynne-Davies, 1973
Congenital dislocation of the hip	0.7-0.9	Leck, 1974a
Cleft lip with or without cleft palate	1.0-1.4	Leck, 1974a
Cleft palate only	0.4	Polani, 1973
Hirschsprung disease	0.2	Polani, 1973
Inguinal hernia	10.0	Polani, 1973; Jagiello and Atwell, 1962
Hypospadias	0.9-1.2	Leck, 1974a
Poly- and syn-dactyly	1.4-1.8	Leck, 1974a
Total	37.8-44.5	

* Excluding conditions like renal agenesis and other congenital renal anomalies, rectal and other intestinal atresia, etc.

** Bicuspid valve: included in totals

Table 5.9: *Relatively common conditions in which genetic factors play a variable role**

Condition	Approximate population frequency (per thousand)
Schizophrenia	8-10
Cyclothymia	4
Epilepsy	5
Diabetes mellitus	3-10
Total	20-29

*Polani, 1973

Table 5.10: *The overall influence of genetic factors on human disease*

Type of condition	Frequency per thousand	
Chromosome abnormalities	6.86	Table 5.1
Defects caused by single genes of major effects:		
Autosomal Dominant	1.85-2.64	Table 5.4
Autosomal Recessive	2.23-2.54	Table 5.5
Sex-linked	0.78-1.99	Table 5.7
Sub-total	11.72-14.03	
Congenital malformations*	(19.00-22.00)	Table 5.8
Complex disorders**	(7.00-10.00)	Table 5.9
Other diseases	?	
Total genetic contribution (approximately)	(38.00-46.00)	

* Conditions listed in Table 5.8; genetic contribution, say, half

** Conditions listed in Table 5.9; genetic contribution, say, one third

Table 5.11: *Frequencies of genetic and part-genetic disorders in two population studies, per 1,000 livebirths*

Type of condition	Stevenson, 1959	Trimble & Doughty, 1974
Autosomal Dominant	33.2*	0.8
Autosomal Recessive	2.1	1.1
Sex-linked	0.4	0.4
Chromosome abnormalities	—	2.0**
Congenital malformations	14.1	42.8
Other multifactorial disorders	14.8	47.3
Unknown origin	—	27.0

* Half of the anomalies included are of trivial import, and there are conditions not now considered autosomal dominants. A corrected value could give a frequency of about one order of magnitude lower.

** 97% are Down syndrome.

Table 5.12: *Mutation rates for some genetic defects*

Condition	Mutation rate per gamete per generation	References
Autosomal dominant mutations		
Huntington Chorea	$1\text{-}5 \times 10^{-6}$	Becker, 1966
Neurofibromatosis	$50\text{-}100 \times 10^{-6}$	Vogel and Rathenberg, 1975
Myotonia dystrophica	$8\text{-}11 \times 10^{-6}$	Vogel and Rathenberg, 1975
Epiloia	$6\text{-}10 \times 10^{-6}$	Vogel and Rathenberg, 1975
Retinoblastoma	$6\text{-}12 \times 10^{-6}$	Vogel and Rathenberg, 1975
Achondroplasia	$6\text{-}10 \times 10^{-6}$	Vogel and Rathenberg, 1975
Diaphyseal aclasis	$6\text{-}9 \times 10^{-6}$	Vogel and Rathenberg, 1975
Marfan syndrome	$4\text{-}6 \times 10^{-6}$	Vogel and Rathenberg, 1975
Multiple intestinal polyposis	$4\text{-}13 \times 10^{-6}$	Veale, 1965; Vogel and Rathenberg, 1975
Polycystic disease of the kidney	$65\text{-}120 \times 10^{-6}$	Vogel and Rathenberg, 1975
Hereditary spherocytosis	22×10^{-6}	Morton *et al*, 1962
Acrocephalosyndactyly	$3\text{-}4 \times 10^{-6}$	Vogel and Rathenberg, 1975
Nail-patella syndrome	2×10^{-6}	Renwick and Izatt, 1965
Sex-linked mutations		
Duchenne muscular dystrophy	$43\text{-}105 \times 10^{-6}$	Vogel and Rathenberg, 1975
Becker muscular dystrophy	$1\text{-}2 \times 10^{-6}$	Stevenson and Kerr, 1967
Haemophilia A	$32\text{-}57 \times 10^{-6}$	Vogel and Rathenberg, 1975
Haemophilia B	$2\text{-}3 \times 10^{-6}$	Vogel and Rathenberg, 1975
Ichthyosis	1×10^{-6}	Stevenson and Kerr, 1967
Testicular feminization	5×10^{-6}	Jagiello and Atwell, 1962
Incontinentia pigmenti	$6\text{-}20 \times 10^{-6}$	Vogel and Rathenberg, 1975
Oro-facio-digital syndrome	5×10^{-6}	Vogel and Rathenberg, 1975

6. The monitoring of human populations for mutational changes

6.1 Accepting the overwhelming direct evidence that mutational changes in man are responsible for a significant proportion of genetically determined disease states and congenital malformations, and the indirect evidence that exposure to mutagens will increase the incidence of such mutations, it is important to ask whether it is possible to monitor populations and the environment in which they live to ensure that individuals and groups of individuals are not being exposed to known or unknown mutagens.

6.2 The principle of monitoring for the presence of toxic agents or pathogens in the general environment, or in a variety of specialized environments associated with particular types of industry, has long been established, and is practised by Public Health Authorities or similar bodies. Monitoring in these contexts is carried out at two levels. Firstly, there is the continuous surveillance of the environment to detect the presence of physical, chemical or biological agents which may produce specific effects on individuals who are "over-exposed" (ie on the coalminer's canary principle). Secondly, there is the surveillance of individuals who are known to be at risk of exposure, for example to a particular toxin, to ensure that they do not manifest symptoms of over-exposure. Under certain circumstances the first type of monitoring may be prospective and could ensure the prevention of future exposure; the second is necessarily retrospective, detecting exposure after it has occurred, but enabling steps to be taken to ensure that no further exposure is possible. In principle, similar monitoring for exposure to mutagens is possible but there are special problems.

6.3 Agents that are mutagenic may be identified individually or collectively by use of mutagen testing systems so that, once identified, monitoring by physical or chemical means is possible. This may be particularly relevant in cases where individuals may be exposed to a chemical agent of known mutagenicity during the course of their work, so that the levels of this agent in the working environment may be continuously monitored to ensure that no over-exposure occurs. However, in the general environment, populations may be exposed to a very wide variety of mutagens with a range of potencies. Moreover, in many cases individuals may well be exposed unknowingly to significant levels of substances which are not yet known to be mutagenic. Most important is the fact that over-exposure to mutagens does not result in one specific and easily observed clinical manifestation as it does, for example in the case of over-exposure to a bacterial toxin, a specific virus or metals such as lead or cadmium. However, the consequences of induced mutation may be manifest in

later life and in a variety of ways, in the descendants of affected individuals. The question of the possibility of monitoring populations for the presence of induced mutations then arises.

6.4 In addition to sampling for the presence of known mutagens by chemical or physical means, it is theoretically possible to monitor for unknown mutagens present in at least some components of the environment by introducing them into bacterial and other mutagen test systems. Routine monitoring in this way presents formidable problems, particularly in the case of large populations. For this reason more attention has been paid to the possibility of undertaking retrospective epidemiological studies on populations and developing methods for detecting mutations in somatic cells of individuals in populations.

6.5 Increases in mutation rate over and above background could be detected if we could easily and accurately identify dominant or X chromosome recessive mutations. The possibilities of monitoring for changes in sex ratio, which would detect sex-linked recessive lethal mutations, and in the incidence of genetically determined disease states, have also been considered. One possibility would be to look for mutations involving serum proteins; another, which would be a measure of non-disjunction, would be to monitor the incidence of Down syndrome in the population and to look for any change in incidence; however, as has been pointed out, this is not easy (see para 4.13). Monitoring for changes in the so-called "sentinel phenotypes", or for increases in spontaneous abortions and stillbirths, would involve a considerable amount of organization and would be heavily dependent upon the maintenance and collation of accurate records. Moreover, background estimates of mutation rates are at present inexact and differences may exist in different populations and different areas. This lack of accurate baseline data means that at present even fairly large changes induced by exposure to mutagens would be difficult to detect and, equally, false positive results could be obtained. Thus, at the present time, such an epidemiological approach does not appear to be feasible as a practical measure for monitoring the general population for exposure to mutagens. A combination of the gross incidence of spontaneous abortion with cytological analysis of abortuses would, however, be a much more sensitive system. Changes in the spectrum of the chromosomal abnormalities found, especially combined with changes in the gross incidence of abortion, would indicate changes in the responsible factors, mutagenic or otherwise. Using the technique of electrophoresis in one dimension to separate proteins of different electric charges (Neel, Tiffany and Anderson, 1963), it has been suggested that mutational changes could be monitored by screening plasma for the products of some 20 genetic loci. If one assumes a mutation rate/locus/generation of 5×10^{-5}, then 2 samples of 6,000,000 observations (ie 2 lots of 300,000 separate blood samples) are necessary to detect a 50% increase in mutation rate (Neel, Tiffany and Anderson, 1963), a somewhat daunting possibility. Recently, however, techniques for separating proteins in 2 dimensions have brought the possibility of monitoring for changes in proteins as an index of mutation rate a considerable step nearer. Two-dimensional separation can be performed by first subjecting samples to isoelectric focussing, to separate on the basis of electric

charge (O'Farrell, 1975), and then to electrophoresis, to separate on the basis of size. Using this approach (Anderson and Anderson, 1977), it has been shown that it is possible to monitor the protein products of 60-100 genetic loci from plasma and possibly up to 200 proteins by using blood cells, with some 80 sample being processed per day by a team of 3 people. If 200 gene products could be assayed easily using semi-automated 2-dimensional separation procedures then 2 successive blood samples of 30,000 each, and the running of 120,000 gels, should detect a 50% increase in mutation rate in a human population.

6.6 An alternative approach is to look for mutagenic changes in somatic cells and possibly also in sperm samples from individuals known to be at risk. The exposure of certain strains of mice to high levels of chemical mutagens can be detected by an increase in the incidence of morphologically abnormal sperm. Unfortunately, the background incidence of morphologically abnormal sperm in healthy fertile human males is quite high, so that small increases due to exposure to mutagens would be difficult to detect. This approach has had limited success so far in man but it may be capable of further exploitation. Encouraging results are being obtained in attempts to reveal the chromosome constitutions of mammalian sperm by *in vitro* fertilization, and such an approach, using human sperm, might provide a means for monitoring chromosomal changes in human male germ cells. The possibility of detecting induced gene mutation by searching for altered gene products in somatic cells is currently under investigation, and methods suitable for screening of small populations are beginning to appear. An approach which is currently better established involves the examination of somatic cells (peripheral blood lymphocytes) for the presence of mutagen-induced chromosome aberrations. In this context considerable research is being undertaken to analyse chromosome damage in individuals known to be exposed to mutagens at low levels and various techniques, such as those that identify sister chromatid exchanges, are being used. Indeed, these cytological methods are successfully used to monitor exposure in personnel handling ionizing radiation. They are also beginning to be utilized to monitor personnel who may be exposed to known chemical mutagens in the course of their work, and may well be applicable eventually to studies of larger populations. If preliminary studies have indicated the presence of DNA damage in the exposed individuals, it may be possible to survey for fetal loss or congenital abnormalities. Such studies would be of great value in the calibration of human response to exposure to mutagens.

6.7 At the present time, therefore, although one might consider the investigation of groups specifically known to be exposed to mutagens, the general monitoring of populations for genetic damage is not a practical possibility. A number of approaches are under active investigation and there is some hope that eventually some relatively simple and efficient systems will become available for monitoring fairly large populations.

7. Recommended test systems for mutagenicity

Introduction

7.1 From the preceding Chapters it can be appreciated why the mutagenic possibilities of chemicals are now recognized as a potential health hazard. Many Governments have accepted the need to try to prevent the current incidence of genetic disease from rising still further. There is the obvious danger of the unwitting exposure of a sizeable susceptible population to a powerful mutagen, such as may already have happened in Japan with the food preservative AF2 (2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide), but a more serious aspect of the problem may be the insidious genetic effect of continuous low level exposure of a large number of people to a range of chemical mutagens. There has been little research on the possibilities of synergism between mutagens.

7.2 For the purposes of discussion on the practical issues of testing for mutagenicity it is convenient to define chemical mutagenicity as "the capacity of a chemical to bring about a heritable change in the genetic material by whatever means". Such a definition would cover, for example, interference with the spindle apparatus which could produce non-disjunction and hence a genetic change by a mechanism which has not directly involved an interaction with DNA itself. Implicit in all these considerations is the view that all chemical mutagens are potentially a health hazard to man and that if it can be proved that there is a high probability of a mutagenic chemical reaching human gonadal tissue, or producing deleterious somatic mutations, steps should be taken to regulate the use of such a chemical. It is the purpose of these guidelines to set out the first practical steps towards the implementation of such preventive measures.

7.3 It must be conceded at the outset that there are formidable difficulties ahead not only in devising a relevant test programme but also in dealing with the many tens of thousands of chemicals which under ideal circumstances should be tested. The introduction of a consistent and logical strategy of testing is an urgent necessity, as it will at least begin the process of setting up in a systematic way the body of knowledge of chemicals and processes which will be essential if realistic judgements of the potential hazards of chemicals are to be made.

7.4 Judgement will always be particularly difficult to exercise in questions of mutagenicity as, in common with other toxicological procedures, extrapolation from any test organism to man will always involve considerable uncertainty. To

some extent, a multiplicity of tests would be expected to reduce the area of uncertainty. Given the current empirical nature of some of the tests such expectations would seem unduly optimistic. It will be some time before a practical test programme and risk evaluation procedure will be available such that the problem of mutagenic hazard can be considered at the levels of probability acceptable in other areas of preventive medicine. Thus, as with other toxicity problems and medical procedures (eg vaccination and other environmental hazards), society should be prepared to accept a certain degree of risk. It has taken about 50 years to arrive at a concept of acceptable risk for radiation and as the actions of mutagenic chemicals are much more complex it would seem unlikely that there will be much agreement on an equivalent acceptable risk level for mutagens for some years.

7.5 The techniques of conventional toxicology can frequently establish the potential hazard to man of a chemical with a high degree of certainty, particularly at the biochemical level, based on the results obtained from studies in animals alone. The understanding of the underlying toxic mechanism can be such that extrapolation to man can be made with confidence. Even in the case of carcinogens, where there can be doubts as to the relevance of, for example, tumours induced in rodents by high doses, there are a growing number of instances where the parallel between cancers induced in animals by treatment and the cancers observed in humans exposed to the chemicals is clear cut. This lends strength to the argument that if an agent is clearly carcinogenic in 2 species of animals then the risk that it will prove to be carcinogenic in man should be accepted as being high; thus the question of human exposure requires careful evaluation. In genetic toxicology, however, with an inherent time scale of generations and a "noisy" background of spontaneous genetic disease, it may be many years before the mutagenic effect of chemicals would be manifested. Thus for humans the effect to be avoided lies at present in the realm of probability rather than of demonstrable mutagenic effect. However, from a knowledge of the mechanism of action of the particular chemicals in a variety of test situations it is believed that it is possible to infer the likelihood of hazard with some confidence. It follows from this that it is essential from the outset to obtain as much information as possible from any test systems. Thus the most satisfactory systems will theoretically be those which give information about specific interactions with the genetic systems in developing germ cells of mammals. Unfortunately, in the practical situation it is not easy to fulfil these conditions at present. There is a further important dimension to the problem of chemical mutagenesis which is lacking in radiation mutagenesis. There is no doubt that if tissues are irradiated all the cells in that tissue are involved, but with chemicals there is no such certainty. Thus the metabolic fate of the administered chemical, and whether or not it or an active metabolite can reach the mammalian germ cells, may become a matter of crucial importance in decision making. Such detailed pharmacokinetic studies are as yet in their infancy. Research in this area may well show that this approach might provide important information in less time and at less cost than long-term genetic studies in mammals.

7.6 Proposals for mutagenicity testing can now be viewed against an impressive background of basic scientific knowledge of the genetic mechanisms and also the development of a wide range of experimental procedures with the potential for use as test systems. To date several thousand chemicals, some of them in common use (eg some food additives and hair dyes), have been shown to be mutagenic in at least one test system. As has been mentioned, over a hundred chemical animal carcinogens, including 20 or so of the known human carcinogens, have been shown to be mutagenic as well.

7.7 There is now an extensive literature on the various test procedures and the practical difficulties associated with them. It is not the purpose of this document to discuss this aspect in any detail, but rather to set out the principles of those tests which are believed to be the most informative and best validated and hence likely to be most helpful in a legislative context.

7.8 The Committee has tried to identify those test systems which have, in its opinion, proved themselves internationally and for which academic back-up exists in Britain. Scrutiny of test systems will be a continuing function of the Committee and the list of recommended systems will be modified as and when necessary. The Committee will also indicate what it considers to be the most promising developments as far as new tests are concerned, and which tests it feels will be acceptable additions to the test repertory after further validation. Within such a repertory it should be possible to devise a flexible strategy for each class of chemical. Furthermore, agreement on what can adequately be tested for should encourage a coordinated effort to develop more acceptable test procedures in those important areas at present not adequately covered (eg no-disjunction in mammals).

7.9 Before considering individual test systems, there are some general comments which apply to all tests, some of which arise because of the unique problem of decision making in this new area of toxicology. Substances do not always fall conveniently into 2 categories: mutagen and non-mutagen. The mutagenicity of some substances can be detected with ease, and of other substances only with difficulty. Thus the problem arises of how exhaustive the testing should be in dubious cases. This becomes a matter of judgement and whatever test system is used the test must be so constructed that the maximum of information can be extracted. Thus the results must be statistically meaningful and, wherever the method permits, a dose response curve should be obtained, and, finally, a detailed account should be available if required. It will be the responsibility of the laboratory carrying out the test to make sure that its facilities are adequate to meet well established international criteria for the particular techniques used. Wherever possible, some form of consultation or collaboration should be available with a University or Research Institute Laboratory which is carrying out basic research in the appropriate disciplines underlying the particular test systems in use.

7.10 In setting out recommendations for test systems the Committee do not wish to provide a rigid schedule of tests, as in a rapidly advancing subject this is

likely to waste limited resources without achieving the desired end. There is however some advantage in suggesting a limited number of test systems as the current "best buys" in a difficult situation. By concentrating on a few tests it should be possible to establish the true worth of each test more rapidly. With few exceptions, even those tests which have an excellent theoretical rationale have not been used so extensively that proper inter-test comparisons can be made.

7.11 From the discussion in Chapter 3 it can be appreciated that the objectives of testing are to discover whether a chemical can interact with the human genome at any of the three basic levels of mutation: the gene itself, or stuctural or numerical changes in the chromosomes. No single test yet available can provide this information. Thus at present there is no alternative to the subjecting of each chemical to a series of tests. There is no agreement internationally as to what particular combination of tests is most appropriate, and thus it is felt that recommendations should be modest, soundly based and the best compromise between what is realistic and what is desirable.

A strategy for mutagenicity testing

7.12 From all previous considerations it follows that before any assessment of a chemical can be made in terms of mutagenic hazard, it is essential to establish as a basis whether or not it can interact at the gene level in prokaryotes or eurkaryotes (ie produce "point" mutations), and whether or not it can produce chromosomal damage in mammalian cells. Thus there is what may be termed a minimal package of tests for any chemical which will be introduced into the human environment in any appreciable quantity. This minimal test programme (or "screen", or "first tier") of tests will be followed by supplementary tests which will be dictated by the results already obtained, the kind of human exposure expected, the pharmacokinetics of the chemical and similar considerations. Just how much of the proposed test programme should be completed for any product can be a complex issue. In the chemical industry it might be that the demonstration of a positive result in a bacterial test system would be sufficient to warrant abandoning the development of that particular chemical in favour of a similar chemical which was negative in that particular test. On the other hand there are situations in medical therapy where the mutagenic property of the drug is considered to be a negligible matter which is far outweighed by therapeutic advantages in a particular situation, for example in geriatric medicine. Various Government Departments and Statutory Bodies have regulatory responsibilities for specific classes of chemicals and products. In many instances several authorities could be involved in various aspects of the production, distribution and use of the same product. There will be a need for specific consultations with the responsible authorities, industries and other interested bodies on the extent of testing warranted for the various classes of products, and in the interpretation of the findings and the development of an appropriate risk/benefit evaluation procedure. For the present, however, the Committee feels that the method of establishing the scientific facts is the first issue to be resolved. What is needed at this juncture is general agreement on a realistic programme of tests to establish whether or not a particular chemical,

in its particular environmental context, is likely to constitute a genetic hazard to humans. It is also important that whatever test procedures are proposed should have a reasonable prospect of being of use for at least 5 years. Once the repertory of test procedures is agreed the Committee envisage that it will be possible to offer specific advice on how the system could be used to best advantage for the various classes of products, such as industrial chemicals, food additives, hair dyes and cosmetics, medicines, domestic chemicals and the like. Once a testing scheme is proceeding and the "data base" grows it will become easier to establish risk evaluation procedures and the monitoring of the population for the presence of an increase in mutation.

The recommended testing procedure

7.13 The following scheme summarizes the recommended test strategy for the screening of chemicals for mutagenic properties. These four test procedures are designed to probe the hereditary machinery sequentially, at increasing levels of complexity, and to take account of the passage of the chemical to the genetic target. This minimal package of tests constitutes the basic screen for all new

Recommended tests in the "basic package" of 4 test screening procedures for mutagenic properties of chemicals.

TEST 1

7.13.1 A test designed to demonstrate the induction of point mutations (base pair change and frameshift mutations) in established bacterial test systems such as *Salmonella typhimurium*, *Escherichia coli* or *Bacillus subtilis*. The tests should be conducted with and without the use of appropriate metabolic activation systems.

TEST 2

7.13.2 A test designed to demonstrate the production of chromosome damage in appropriate mammalian cells grown *in vitro* with and without the use of appropriate metabolic activation systems.

TEST 3

7.13.3.1 The induction of mutations in mammalian cells grown *in vitro*.

or

7.13.3.2 Tests designed to induce recessive lethals in *Drosophila melanogaster*.

TEST 4

7.13.4.1 A test designed to demonstrate the induction of chromosomal damage in the intact animal using either the micronucleus test or, preferably, the metaphase analysis of bone marrow or other proliferative cells.

or

7.13.4.2 The induction of germ cell damage as demonstrated by the dominant-lethal test in the rat or mouse.

chemicals and products to which human exposure will be extensive and difficult to avoid, for example food additives, cosmetic preparations, domestic chemicals or medicines. If for any reason suspicion arises about any chemical already in wide use it should also be subjected to these testing procedures. However, there may be circumstances where a chemical is not proposed for human ingestion and where it is expected that its use will be limited and the degree of human exposure small or containable. In such a situation a case could be made for accepting the first 2 test procedures, that is, point mutations in bacteria and chromosomal damage in mammalian cells *in vitro* (an *in vivo* procedure could be considered if testing is planned in parallel with acute toxicity testing).

7.13.5 The principles underlying these tests are discussed in Chapter 7, and references to specific test, their recommended methodologies, their difficulties and their interpretation, including advice on appropriate statistical techniques, are set out in Appendix 1. The Committee acknowledge that in this rapidly developing area the state of knowledge is such that, because each test is directed at a limited aspect of the hereditary process, equivalent evidence derived from other test systems with different genetic endpoints could be accepted as an alternative to part of the basic package. However, the onus would have to be placed on the applicant to prove that the evidence produced was at least as good as would be expected from the tests recommended above.

Supplementary tests

7.14 These tests may be useful in providing clarification where unexpected or equivocal results are obtained in the basic test programme. They may help to elucidate the mechanism of action and spectrum of effects if a substance shown to be positive in the basic tests is proposed for a risk-benefit analysis . Also, where negative results are obtained in the basic test programme, further negative results in one or more supplementary tests would allow greater confidence. In the selection of supplementary tests some of the tests in the basic package are repeated because the alternative to the chosen test is acceptable as a supplementary test.

7.14.1 Prokaryotic tests in repair deficient strains (Rec, Pol and further mutagenicity tests).

7.14.2 Tests in sub-mammalian eukaryotic systems such as yeasts, fungi and *Drosophila melanogaster.*

7.14.3 Test for mutations in mammalian cells in culture, for example at the hypoxanthine — guanine phosphoribosyl transferase or thymidine kinase (HGPRT or TK) loci or oubain resistance.

7.14.4 Tests for the induction of chromosomal damage in mammalian somatic cells *in vivo;* for example, the micronucleus test and marrow metaphase analysis.

7.14.5 Unscheduled DNA synthesis in cultured mammalian cells.

7.14.6 Host-mediated assay, and developments from this such as analysis of blood and urine from treated animals for evidence of the *in vivo* formation of substances with mutagenic properties.

7.14.7 Dominant-lethal test in the mouse or rat.

7.14.8 Sister chromatid exchanges.

7.14.9 *In vivo* somatic mutation in the mouse (coat colour recessive spot test).

In vivo tests in the intact mammalian germ cell system (in addition to the dominant-lethal test)

7.15 This is a difficult area as some of the tests are particularly demanding either in numbers of animals or experimenters' time or both, even though it is a crucial area of knowledge as far as extrapolation to humans is concerned. The Committee regard these tests as giving information which might be called for where a known or suspected mutagen is to be proposed for a full risk benefit evaluation.

7.15.1 Mouse specific locus test.

7.15.2 Heritable translocation test in the mouse.

7.15.3 Cytogenetic studies on spermatocytes and oocytes, specifically for the detection of translocations.

7.15.4 Non-disjunction tests in the mouse. The most relevant method at present is the cytological examination of 10-day embryos for trisomy after parental treatment. However, considerable technical expertise is required.

Tests in the progress of validation which show promise

7.16 While the following tests have yet to be fully validated, the Committee welcome their use, since this will assist in their eventual evaluation. Many of these test procedures could be incorporated at little extra cost into conventional whole animal toxicity testing programmes.

7.16.1 Sperm morphology.

7.16.2 Chromosomal analysis of sperm used for *in vitro* fertilization of hamster eggs. Theoretically this procedure could be used not only for the study of meiotic non-disjunction in experimental animals but also for monitoring human males.

7.17 In the above repertory of tests there is a serious deficiency of tests for non-disjunction. It is however likely that new advances in this field will soon lead to practical tests which will remedy this situation.

7.18 Specific references for the supplementary tests are set out in Appendix 1 Part 2, and for the tests which show promise in Part 3.

8. The principles of recommended test systems

Bacterial test systems

8.1 At first there were doubts about the usefulness of bacterial test systems because it was known that many chemicals are carcinogenic only after a metabolic conversion in mammalian cells, particularly liver cells. As the bacterial cell is deficient in the necessary enzyme systems it was believed that many potentially hazardous chemicals would be missed. This situation was transformed when it was discovered that microsomal preparations of liver cells with adjuvants could supply the mixed-function oxidases necessary for most of the metabolic conversions. Metabolic activation, mainly by tissue microsomal preparations, is now an integral part of many test procedures both in prokaryotes and eukaryotes (see Appendix 1). Microsomal preparations of livers from animals challenged by various chemicals, such as barbiturates, polychlorinated-biphenyl mixtures or 3-methylcholanthrene, have induced mutagenically active metabolites in a surprisingly wide range of chemicals, despite the fact that the microsomal preparations cannot fully represent the range of enzymic activities of all cells. However there are other routes of activation, such as the intestinal flora which are necessary for the conversion of substances such as cycasin, and the use of whole liver cells may give a more realistic picture than that afforded by microsomes. There are also technical problems both in the preparation and use of microsomal systems. Thus it is necessary to emphasize that despite the importance of this procedure it has its limitations. However, the general approach of *in vitro* activation clearly has great potential.

8.2 The bacterial test systems fall into 3 main classes, namely those that detect backward mutations, those that detect forward mutations, and those that rely on a DNA-repair deficiency. The most widely used backward-mutation systems are those developed in *Escherichia coli* and *Salmonella typhimurium*. The principle of these systems may be illustrated by reference to the strain *E. coli* WP2. This strain contains an alteration in a gene involved in tryptophan synthesis, so that the organism can only grow if exogenous tryptophan is supplied. The genetic alteration can be precisely identified as a sequence (termed an ochre, or nonsense triplet) of 3 adenine:thymine base pairs (Bridges, Dennis and Munson, 1967). This sequence (which generates the sequence uracil-adenine-adenine in the mRNA) does not specify an amino acid and therefore results in an incomplete gene product. The organism may regain the ability to synthesize tryptophan for itself if a mutation occurs such that either a different pair are substituted for one or more of these base pairs, or one or more

base pairs (including guanine:cytosine pairs) are replaced in a tRNA so that it acquires the ability to read the nonsense codon, thereby allowing the gene product to be completed. Such a mutated bacterium is now able to grow in the absence of tryptophan and its presence can be noted in about 48 hours by the presence of a colony or clone on the surface of a tryptophan-free agar plate which was originally treated with up to 10^9 tryptophan-requiring bacteria. The presence of chemically induced mutants can be detected in this way, either by adding the chemical to the agar plate or by treating the bacterial culture before plating. Each colony represents a reverted bacterium and a comparison of treated with control agar plates gives an estimate of the number of mutants induced by the chemical. The property of reversion from dependence on a specific metabolite is referred to as a change from auxotrophy to prototrophy. In the *Salmonella* system the properties of the *his* operon have been utilized and a series of mutant strains that have lost the ability to synthesize the amino acid histidine have been developed (Ames, 1971). In addition to a strain capable of reverting by base pair substitution, others revert by addition or deletion of a base pair. This is because they already carry such a deletion or addition, which causes the read-out of the sequence from that position to the end of the coded instructions to be totally wrong (frameshift mutation). These mutations often occur at what are known as "hot spots" and in the *his* operon they are associated with a long repetitive sequence of base pairs such as GC. A deletion causes a strain with a base addition to revert and vice versa.

8.3 In addition to specific auxotrophy, the sensitivity of the various test strains of bacteria has been greatly increased by inducing in them a variety of other properties. One example is the introduction into *E. coli* WP2 of a deficiency at an excision-repair gene *(uvrA)* (Hill, 1965). This and other repair-deficient strains of *E. coli,* have proved useful in detecting and characterizing mutagens (Igali *et al.*, 1970; Bridges, *et al.*, 1972). In *Salmonella typhimurium* a deletion was introduced into an excision repair gene *(uvrB),* followed by further deletions through the galactose operon *(gal),* biotin operon *(bio),* and the genes for resistence to chlorate *(chl),* and for a cell envelope lipopolysaccharide resulting in a deep rough strain *(rfa)* which has a more permeable cell wall. By such manipulations the well-established test strains TA 1535 (base substitution) and TA 1538 (frameshift) were created and are thus designated *his gal bio uvrB rfa* strains. However, as most of these changes have been brought about by deletions it is only the histidine operon target that is available for reversion.

8.4 In a similar way, well-established auxotrophic strains have been created in *Bacillus subtilis* as well as in many other micro-organisms and cells, but by far the greatest information has come from *S. typhimurium* and *E. coli.* A further recent modification has been the addition of plasmids to these organisms (MacPhee, 1973). These can add to the cell properties such as resistance to antibiotics, but, in a way that is not yet fully understood, some plasmids carry genes that not only elevate the spontaneous mutation rate but greatly increase the sensitivity of the bacterial strain to the mutagenic action of known carcinogens, apparently by changing the priority of the repair systems (for further details of specific strains see Appendix 1). The use of plasmid-containing *Salmonella*

strains has enabled a remarkable correlation to be demonstrated between the carcinogenicity of substances in animals and their ability to induce mutations in such plasmid-containing strains (McCann *et al.*, 1975). This correlation inevitably gives these strains a valuable place as a screen for substances that might have carcinogenic potential. Their use as indicators of potential genetic risk is, however, less certain, firstly because the way in which the plasmids enhance the mutation rates (both spontaneous and induced) of the bacteria is as yet unknown; secondly because, at least in some cases, they seem effectively to cause DNA damage, normally repaired without concomitant production of mutations, to be repaired in such a way that mutations are produced, ie to result in what could be regarded as false-positive effects. It is possible that research might soon resolve these areas of uncertainty but for the present it would seem desirable to examine the response to both plasmid- and non-plasmid-containing strains, although the latter effect may justifiably be regarded as an indicator of possible carcinogenicity.

8.5 Two strains have been widely used for detecting the induction of base pair substitution mutations, *E. coli* WP2 and *S. typhimurium TA 1535.* In general, both show a very similar spectrum of substances detected, although with both there is a small proportion that may be missed by one and detected more readily by the other. Both strains are available with the plasmid pkM 101 (strains CM891 and TA 100 respectively).

8.6 For the detection of frameshift mutagens, TA 1537 and 1538 have been most widely used, together with TA 98 which is a plasmid-containing derivative of TA 1537. In *E. coli* the *lac* (from lactose) and *trp* (from tryptophan) operons are as well studied as the *his* operon in *Salmonella.* A number of *lac* and *trp* frameshift strains have been used for mutagenicity testing, although not widely. There is, however, at least one instance of a mutagen being readily detected with the *lac* strain ND 160 when it was missed or detected only with difficulty in *Salmonella* strains.

8.7 Using a selection of such reverse-mutation tester strains, there is an extremely high probability of detecting the ability of a substance to mutate bacteria. A substance with such activity would also be expected to mutate mammalian germ cells if the active form was able to reach the DNA in significant quantity. This latter qualification is important and must always be borne in mind when interpreting the results of bacterial tests. Thus a positive result in such a test should not by itself be regarded as indicating a significant hazard to man. It must also be noted that bacterial tests may fail to detect mutagenic activity on the part of some agents, eg certain metals which are active in eukaryote cells.

8.8 Reverse-mutation tests in bacteria have proved themselves to be extremely efficient screens for mutagenic activity, and easily handled in the laboratory. In addition to these, a number of workers have developed bacterial systems in which forward-mutations resulting from inactivation of gene function are scored. Theoretically they should possess the advantage of being able to respond

to a wider range of genetic alterations than reversion systems, but in principle this must be accompanied by a reduced ability to respond to a weak and highly specific mutagen, since the spontaneous frequency will tend to be dominated by the most common changes and may mask an otherwise significant increase in the frequency of a rare, highly specific, change. In practice it has yet to be shown whether forward-mutation systems in bacteria are superior to, or even the equal of, reversion systems. They are therefore presently acceptable only as supplementary tests.

8.9 When the DNA of cells of all types is damaged by a mutagen several different repair pathways act to restore the DNA to a functional state. In bacteria a number of different repair-deficient strains have been isolated, which are unable to carry out certain types of repair, and these strains tend as a result to be more easily killed by agents producing such DNA damage. Comparison of the sensitivity of a pair of repair-deficient and repair-proficient strains to the cytotoxic action of a substance may therefore be used to determine whether it produces DNA damage. Two types of repair-deficient strains have been widely used, one deficient in DNA polymerase 1, and the other deficient in recombination ability. Tests with these organisms will be referred to respectively as the Pol and Rec tests. They have proved to be useful in so far as they are even easier to perform than bacterial mutation tests and sometimes detect activity that the latter do not. There are, however, good theoretical reasons why certain types of mutagen might be missed by the Pol and Rec tests. For these reasons they are regarded as supplementary rather than primary screening tests. Another bacterial response to DNA damage is the induction of prophage in lysogenic strains. A lysogenic strain carries the chromosome (prophage) of a bacterial virus (bacteriophage) integrated into its own chromosome. When this chromosome is damaged the prophage genes may become active, resulting in the production of large numbers of new bacteriophages which are released from the bacterium, and which are easily detectable. This response is extremely sensitive to cetain types of DNA damage, although not to all, and there are, as with the Pol and Rec tests, some classes of mutagen which are not effective. The prophage induction test, usually carried out with bacteriophage 2, is a useful supplementary test. At present the data base does not allow the relative efficiencies of the Rec, Pol and prophage induction tests to be compared.

8.10 It is not the intention of these guidelines to lay down rigid protocols for the performance of tests; acceptable protocols may be found in the references cited in Appendix 1. Nevertheless there are a number of minimal criteria that should be observed, the importance of many of them having been recognized during a recent international collabortaive programme (De Serres and Ashby, 1980). They may be stated briefly as follows: (1) all experiments should be repeated at least once; (2) substances should be tested up to the limit of toxicity or solubility; (3) below this limit, doses should not be spaced at levels differing by more than a factor of 5; 3-fold differences are preferable; (4) raw data should always be given in addition to mutation frequencies or other transformations; (5) activation by systems other than rat liver microsomes may be necessary, for example where unusual metabolism by gut flora is suspected; (6) it is recognized

that bacterial mutagenicity tests may not be appropriate for substances with high bactericidal or bacteriostatic activity.

Test systems using mammalian somatic cells in culture

Introduction

8.11 In a basic package screening system a well conducted bacterial test for mutagenesis is an essential primary component. However, further testing beyond the reversion system using micro-organisms should be directed to mammalian systems. A key element in any mammalian *in vitro* screening system is the use of tissue culture techniques coupled with metabolic activation systems. Individual somatic mammalian cells taken straight from a donor carry within their nuclei the complete information for the construction of the whole animal. They can grow and reproduce in a defined environment which can be modified by subtraction or addition of nutrients, physical agents and potential mutagens. These latter can be used when necessary at dose levels which would be lethal in the intact animal. Cells grown in tissue culture can be manipulated in ways which superficially resemble the way bacterial cultures can be exploited. Thus they can be plated out into suitable media in Petri dishes or culture flasks, incubated, subcultured by dilution and on solid media, they can form single-cell-derived colonies or clones similar to those formed by bacteria. Their use for the testing of mutagens is in part a substitute for the testing of germinal cells.

8.12 An important special technique is the use of short-term culture of cells sampled from animals or man, particularly lymphocytes. Lymphocytes in the blood are in what is called the G_0 condition; that is, they are in a non-cycling or non-multiplying state. One of the circulating lymphocyte types, so called T (thymus-derived) lymphocytes, can be stimulated *in vitro* to undergo mitosis by various substances, especially phytohaemagglutinin (PHA), an extract of the bean *Phaseolus*. The technique is to withdraw a few millilitres of blood, stimulate the T lymphocyte population with PHA, and then add the substance to be tested for mutagenicity. The stimulated lymphocytes transform into lymphoblasts and undergo mitosis, thus outgrowing other unstimulated white cells, and in due course can be analysed for chromosome aberrations as described later. Other cells can be used; in the main these are usually fibroblasts, with epithelial cells and lymphoblasts being less frequently utilized.

8.13 Long-term cell cultures only superficially resemble bacterial cultures. Bacteria are normally autotrophic, which means that all they require for growth are water, oxygen, minerals and an energy source such as sugar. By contrast, mammalian cells, like the animals from which they originated, require a range of amino acids, usually derived from proteins, constant warmth (37°C), oxygen (20%) and a controlled pH, which they previously obtained from the buffer systems in the host serum which bathed them. They also require some extra factors, not yet specified, which are present in animal serum, not necessarily of the same animal species as the original donor of the cells. Bovine serum is

usually added to supply these factors, but some progress has been made towards the replacement of this serum by specific substances, with the aim of producing a fully man-made and defined medium. Fibroblasts and epithelial cells can only grow in association with a surface (glass, plastic or collagen) to which they can adhere. These cell types are thought to be relatively non-specialized. Although there are many specialized cell types so far only a few of them have been exploited successfully with current tissue culture techniques.

8.14 The cells that grow out of fragments of tissue explanted into a culture medium are usually fibroblasts, and they form a "primary culture". Their genotype is identical to that of the donor, and their growth rate is slow and density dependent; to maintain growth they are usually plated out at near-confluent densities and furthermore they need a high concentration of serum in the medium. Cells that grow on a surface, such as fibroblasts and epithelial cells, are also contact inhibited. This means that they grow over the available surface until a complete monolayer sheet is formed and then growth ceases. Providing high densities are maintained, these cells can be subcultured many times when provided with all their known requirements. After an interval depending upon the species, growth ceases and the cells die unless a process called "transformation" occurs whereby a new kind of cell type appears with a variety of new properties.

8.15 The "transformed" cells almost invariably have an abnormal karyotype compared with the cells of origin. Such cells are said to be *aneuploid*. Further useful changes are that they usually have a fast growth rate with a cell cycle of 12-24 hours, independent of cell density. As a result single cells can be plated out and these may multiply to form clones. Other features are a lower serum requirement, a disorientated growth pattern and a loss of contact inhibition which results in cells growing over each other and piling up. If such transformed cells are injected into an animal genetically identical or immunologically neutral to the one from which they came, they frequently develop into tumours. Transformed lines are often referred to as "established" lines, and are virtually immortal as far as numbers of subcultures are concerned. This cell transformation can occur spontaneously with increasing frequency towards the end of the natural life span of a diploid culture, especially in cells derived from rodents, but of considerable importance, in the present context, is the fact that it can be induced by radiation, oncogenic viruses or carcinogenic chemicals.

8.16 Thus there are 2 basic types of cell in tissue culture systems, primary or mortal cell cultures and established or immortal cell lines. Both have their own uses in experimental biology. Primary cell cultures are most suitable for experiments in which the cells are required to have a stable phenotype and genotype similar to that of the *in situ* cells of origin. This is essential for the study of the transformation process. Established lines by contrast are more convenient in cytotoxicology and cell genetics because of their ease of manipulation. Allowances have to be made however for the inherent instability which is a characteristic of these lines. Once the stability of a primary culture has been disrupted by transformation it cannot be restored. For the purposes of

mutagenicity screening chromosome aberrations are best studied using the stable karotype of diploid cultures. Gene mutations where mutation rates of around 10^{-4} need to be detected require cells with a high plating efficiency of discrete colonies rather than a stable karotype; hence established lines are more convenient

Chromosome structural changes

8.17 As has been discussed in para 3.16, chromosomes are the highly condensed form of the chromatin threads which appear during the mitotic process. They have a strong affinity for basic dyes such as haematoxylin, but classically orcein, and more recently the Giemsa stain, have proved to be most useful for chromosome studies. As discussed, chromosomes are at their most condensed state at metaphase when they are congregated in the equatorial plane of the spindle apparatus, just prior to their chromatids being drawn to the opposite poles of a dividing cell by the contraction of the centromerically attached spindle fibres. Unfortunately, even in a population of cells all of which are actively dividing, only between 1 and 5% of the cells will be found in metaphase at any one time; in other words mitosis is asynchronous. The use of drugs such as colchicine, or a less toxic derivative, inhibits spindle formation an hence the chromosomes cannot be organized into a central region but are free to disperse throughout the cytoplasm, making it much easier to observe individual chromosomes. If colchicine is allowed to act for some hours this gives an opportunity for a number of cells to enter mitosis and accumulate at metaphase, thus enabling relatively large numbers of chromosomal complements to be analysed under optimal conditions. However, colchicine has the disadvantage of causing excessive chromosomal contraction which may hinder the definition of the cross-banding of chromosomes which is revealed by special staining techniques.

8.18 Chromosomal aberrations induced by chemical and physical agents are often lethal to the daughter cells to which they move because of the resulting imbalance of genetic material. It is the non-lethal changes brought about by the presence of a damaged chromosome which constitute the long-term hazard. Thus if all the aberrations which have been induced by a chemical are to be observed it is necessary to examine the cells at the first metaphase after treatment, usually not more than 48 hours later. As has been stated in Chapter 3, the chromosomes at metaphase are linearly double structures, the result of DNA replication during the S phase. The paired structures are held together at a modified region, the centromere, whose division permits the attached spindle fibres to draw the chromatids apart to the daughter cells. It is an appreciation of this sequence of events that facilitates an understanding of the time at which the various kinds of structural damage occur. Common types of structural change are breaks and gaps which affect both chromatids at identical points, or iso-chromatid breaks and gaps, if the change was induced during the first part of the interphase (ie G_1 and hence before S) in an unreplicated chromosome. By contrast, if the lesion was induced after replication (ie after S, in G_2) only one chromatid may be affected. There is considerable argument over the genetic

significance of the gross structural discontinuities or gaps that can be induced in chromatids. One definition is to designate a gap as any discontinuity that appears in the length of a chromatid such that the space (or non-staining area) is no larger than the width of the chromatid and the 2 parts are in alignment. If however the 2 parts are well separated and are generally unaligned it is designated as a break (ie the 2 fragments are independent of each other). Such fragments are the basis of the rods or rings, or, if they "stick" to another chromosome end on, unbalanced translocations; if it is an iso-chromatid break, they may form so-called dicentrics by joining end on to another chromatid pair. All these aberrations are observable at metaphase.

8.19 The essentials of the techniques used in screening for mutagens are to expose the growing cells to the test substances and to follow this by colchicine treatment for 1-2 hours to accumulate cells in metaphase. At the appropriate time, depending on the cell type, the cells are treated with a trypsin preparation to detach them from surfaces and so bring them into a suspension of separate cells. They are then treated with hypotonic saline to swell and soften the cells, followed by fixation in acetic-alcohol (ratio 1:3), concentrated by gentle centrifugation into a dense suspension which is dropped onto a clean slide and air dried, when the cells will flatten and adhere to the slide as the fixative evaporates. When dry they are stained and examined under a light microscope and scored for aberrations of various kinds. These are recorded as percentage of cells with aberrations and aberrations per cell. Cultured cells have a limited capacity for metabolic activation but, as with bacterial test systems, the metabolic activation that occurs in the whole animals can be mimicked to some extent by the addition of preparations of tissue microsomes as described in para 8.1. A liver microsomal preparation known as S9-mix is frequently used but, as has been mentioned, there are limitations and problems associated with the use of metabolic activation systems. It is important in setting up the experiment to include adequate controls. Thus, in addition to, say, S9-mix and test substance, there should be included S9-mix alone, test substance alone, neither ingredient and S9-mix with a known positive control; a total of 4 controls.

8.20 A much older and easier technique is to grow the cells on coverslips immersed in the medium. These can be removed and the cells fixed and stained *in situ*, thus avoiding the trypsinization step and the use of colchicine. The cells are examined a few hours after treatment for anaphase bridges between daughter nuclei. Anaphase is the stage immediately following metaphase (see para 3.16). The centromeres split and the spindle fibres contract and draw the individual chromatids, now daughter chromosomes, to their respective poles. Some of the dicentric rod or ring chromosomes will be seen at this stage as a strand of chromatin joining the forming daughter nuclei (called a "chromatin bridge"). The other visible signs of structural change are chromatin fragments lagging behind the daughter nuclei because they have no attachment to a spindle-fibre. These fragments are the origin of the so-called micronuclei seen in the daughter cells. A disadvantage of this anaphase technique is that anaphases cannot be accumulated by the use of colchicine in the way that metaphases can. Thus at any one time only a small proportion of actively

dividing cells will be in anaphase. It follows that in the presence of colchicine there can be no anaphase analysis.

Gene mutation in cultured cells

8.21 As stated above, every cell in culture potentially contains the complete gene complement of the whole animal. All these genes are subject to mutation in culture. However, only a relatively basic form of life is possible for cells growing in culture, so very few of the mutations will be detectable. Cell biologists have however devised methods of detecting specific forward and reverse mutations which confer drug resistance on the mutant cells. The 2 most commonly used are involved in nucleic acid metabolism. The possibility of exploiting such systems arose partly from the study of the rare sex-linked disease known as the Lesch-Nyhan syndrome, in which there is a failure to metabolize purines adequately.

8.22 The great majority of purines and pyrimidines needed in cellular metabolism are synthesized *de novo* in the cell from simple precursors. However, cells have evolved enzyme systems for re-cycling these bases when they are liberated into the system by the degradation of DNA and RNA. Hence the system is known as a salvage pathway. Thymidine is salvaged by the enzyme thymidine kinase (TK), coded by an autosomal gene, and hypoxanthine and guanine by the dual-purpose hypoxanthine-guanine phosphoribosyl transferase (HGPRT), coded by a gene on the X chromosome. However, the presence of such enzymes makes cells liable to poisoning by drugs such as bromodeoxyuridine (an analogue of thymidine) and 6-thioguanine (an analogue of guanine), which can be incorporated into nucleic acids and severely impair their functions. Loss or damage of either of these enzymes, that is, mutation from TK+ to TK− or from HGPRT+ to HGPRT−, makes the cells resistant to the respective base analogues. Because they are only salvage pathways, the mutant cells can grow perfectly normally by *de novo* synthesis of the necessary bases. However, such synthesis is dependent upon folic acid and if a folic acid antagonist such as methotrexate is used this *de novo* synthesis is blocked. Normal cells, however, are resistant to methotrexate as long as they are provided with sufficient thymidine and hypoxanthine, which mutant cells (TK− or HGPRT−) can no longer utilize. Thus, by manipulating the *de novo* or salvage pathways by the use of a simple selective procedure, which rests on the choice of an appropriate culture medium, either forward or reverse mutations can be detected using survival of the cells in the presence of the base analogues as the indicators.

8.23 Reverse mutations (eg TK− to TK+) are more specific than forward mutations, in the same way that it is easier to break something than to repair it, but for that same reason reverse mutations are much less probable. At present, forward mutations (eg TK+ to TK−) are the more convenient to use. Cell lines which have been found useful in these tests are CHO and V79 (Chinese hamster fibroblasts) for HGPRT− mutants, and L5158Y (mouse lymphoma) for HGPRT− and TK− mutants (see Appendix 1).

8.24 The general procedure is to expose cells to the mutagen for a short time and then return them to a non-selective medium for a period known as the "expression time", which must be determined by experiment for each cell line and for each mutagen. This is the time from induction of the mutation to its expression by the cell. This delay occurs because each cell will have some enzyme remaining from the premutated condition which must be used up before the enzymeless phenotype can be manifested. The cells are then trypsinized into a suspension, diluted and plated out, one sample at low density in normal medium to determine their viability, and a further sample at high density in selective medium in which only the specific mutants will grow. Most mutagens are also toxic to cells in these systems at the concentrations used, hence it is important to express the mutation rate relative to the number of surviving cells rather than the number of treated cells. It is also important to replate the cells in selective medium rather than leave them in the original vessel. This is because in the latter case small clones of cells develop containing both mutant and non-mutant cells. The enzymes from the non-mutant cells can diffuse into the mutant ones (metabolic cooperation) restoring their drug sensitivity, and thus bringing about an underestimate of the mutation rate. A further source of misinterpretation can arise if the concentration of the selective drug is not sufficiently high. At low drug levels it has been found that, presumably owing to the instability of established cell lines, apparent mutations, which revert to wild type in the absence of selection and cannot therefore be genuine, are selected.

8.25 In conclusion, it is necessary to consider why it is possible to select out mutations at all, since the mutations are recessive to the wild type, as has been demonstrated by experiments in cell hybrids. The HGPRT gene is on the X chromosome and in consequence male cells have one copy of the gene, while the female cells have only one active gene as the other one is on the inactive X. Thus in any female heterozygous cell line all the cells will manifest only one allele, the other being inactive. In contrast, the TK gene is on an autosome, so that it is normally present in duplicate, and the TK mutation is only detectable in cell lines in which one of the 2 genes is already mutant, or in which one of the chromosomes carrying the gene, or the gene-carrying segment of that chromosome, has been lost.

Test systems using in vivo methods

Introduction

8.26 Thus far a sequence of *in vitro* test procedures has been discussed which started with the interaction of a chemical with DNA in its simplest and possibly most vulnerable situation, namely the bacterial genome. This was followed by consideration of test systems involving macroscopic damage to DNA in its most complex form, namely the chromatin fibre organized into the chromosome in mammalian cells. Damage to the macroscopic organization of the chromosome may be associated with human disease via unbalanced genetic material. Thus

test procedures on somatic mammalian cells demonstrate the ability of a chemical or an intermediate metabolite, if metabolic activation has been used, to enter the cell and reach a vulnerable site of the target. This applies also to those tests designed to reveal gene (as opposed to chromosome) mutations in cultured cells. Logically any basic screening system must include one procedure in the whole animal, to meet the criticism that metabolic activation systems used *in vitro* fall short of the range of metabolic possibilities of the whole animal. The most convenient relevant test is probably the analysis of cells in metaphase in the bone marrow of the mouse. Alternatively micronuclei are easier to score, but the test may be less sensitive and the results are less informative, although effects on the spindle apparatus might be seen. Thirdly, although the dominant-lethal test in the mouse or rat is generally considered to be less sensitive than others it is one of the few that is directly relevant to germ cells. Finally, in considering any *in vivo* mutagenicity test procedure, serious consideration must be given to results obtained from the use of *Drosophila melanogaster*, even though this is a non-mammalian system. It is the most intensively studied eukaryote animal whose metabolism, as far as converting chemicals to active mutagenic metabolites is concerned, is surprisingly similar to the mammal. Though the system is relatively insensitive, the convenience of the technique and the significance of a positive result must commend it as one possible alternative in the recommended basic test sequence.

In vivo metaphase analysis

8.27 The basic idea is to treat the animal and after the appropriate interval to sample a rapidly dividing tissue, the most convenient for experimental purposes being marrow. Alternatively, lymphocytes from the treated animal can be removed in a blood sample and then prepared exactly as described for *in vitro* treated cells (paras 8.12 and 8.19 above). For marrow examination it is necessary to treat the animals with colchicine some hours after administration of the mutagen (about 22 hours in the rat), to facilitate the accumulation of marrow cells in metaphase. The animals are killed 2 hours later and the marrow is collected from femurs (thigh bones) into suitable culture fluid, and thereafter handled in suspension and resuspension after centrifugation in much the same way as described for tissue culture cells.

In vivo micronucleus test

8.28 The micronucleus test utilizes a less exacting technique but correspondingly it is less critical. For a long time it has been known that a small proportion of normal circulating red blood cells contain small chromatin bodies, called Howell-Jolly bodies after their discoverers. The micronucleus test utilizes an enhancement of this phenomenon by clastogens, that is, chromosome-breaking agents. A micronucleus owes its origin to a chromosomal fragment which has lagged behind at anaphase, as described earlier, and its relationship to Howell-Jolly bodies has only recently been appreciated. Such a fragment, or fragments

may fail to be included in either of the daughter nuclei, and then it appears as a micronucleus. One cell type is particularly suitable for observing this phenomenon, namely the newly formed erythrocyte, which stains differently from the mature erythrocyte in that it exhibits polychromasia for a period of 24 hours or so. At the last maturation division of an erythrocyte precursor (erythroblast), the nucleus is extruded, but for some reason, probably mechanical, any chromatin fragments present remain behind and persist as small chromatin bodies in an otherwise chromatin-free cell, and thus are conspicuous in spite of their small size. The technique of the test is to examine the polychromatic erythrocytes in marrow smears at a critical interval after injection of the chemical, allowing for micronucleus formation. It is necessary to use a series of sampling times in order to include the right interval, and the inclusion of positive controls is especially important. From their origin it will be appreciated that the relation of micronuclei to chromosome aberrations is derivative; in the same way this applies to anaphase bridges.

Dominant-lethal mutation in a rodent

8.29 As has been mentioned, among the many mutagenicity tests available few are directly relevant to germ cells. The dominant-lethal test is one of these and is therefore frequently used, although the test does not indicate the precise genetic nature of each apparent mutation. The test is often considered to be less sensitive than some others but a review of past results suggests that scrupulous attention to design and details might lead to a change of this view.

8.30 A dominant-lethal mutation is one occurring in a germ cell, which does not cause dysfunction of the gamete but kills the fertilized egg. Such an embryo may fail to implant, but it can be distinguished from an unfertilized egg only by microscopical examination of pre-implantation ova, and this is not practical except in a research context. However, if the embryo dies soon after implantation the result is an aborted implantation site. In rodents this persists to full term as a deciduoma or "mole" which coexists with the live embryos. Dominant-lethal mutations are generally scored in mid-term pregnancies, the optimal time for distinguishing live embryos from deciduomata, but this can be done unambiguously from 10 days to full term (19 days) in the mouse and from 11 days to full term (21 days) in the rat.

8.31 It is usual to expose males to the drug and then to mate them to virgin females. The incidence of dead implants is therefore a measure of mutations induced in male gametes, scored in females. Spermatogenesis is a long and complex process and it has been shown that the different stages, from precursor cell (spermatogonium) to spermatozoon, can vary widely in their sensitivity to mutagens. Usually, but not invariably, the maturing post-meiotic spermatids or spermatozoa are the most sensitive to mutation induction. Because of this uncertainty it is necessary to sample the response of cells to the test substance at every stage of gametogenesis. There are 2 ways in which this can be accomplished. In the first the male mouse is injected once with the test substance and

then mated to a succession of females over a period of 8 weeks. During successive weeks matings will represent in temporal order the response of epididymal sperm, late spermatids, early spermatids, late and early spermatocytes, and different generations of spermatogonia. The second method is to treat the male mouse with the test substance continuously for 8 weeks and then mate it for 1 or 2 weeks. The mature sperm produced for these matings will have been exposed throughout its development, but if there is a response there is no way to determine at what germ cell stage the chemical acted. The 2 methods should have the same sensitivity providing that the dosage levels are equivalent at all stages.

8.32 Dominant-lethal mutations (as pragmatically defined in para 8.30 above) occur in untreated controls at a rate of about 5% of all implantations. At the highest tolerable doses, the proportion of moles present should be at least double that in controls and sufficient matings should be carried out to detect this. The tolerable dose is defined for this purpose as that dose permitting fertile matings, as clearly without these the test cannot be made. If a substance produces complete sterility, this may reflect 100% dominant-lethal mutation, and lower doses of the test substance should be investigated. The incidence of dominant-lethals is best expressed as a percentage of total implantations per pregnancy. It should be noted that there are difficulties inherent in the statistical evaluation of test data from dominant-lethal tests, and it is important to consider these in the design of the test (see Appendix 1).

8.33 The strain of mouse chosen should be one with a regular spontaneous dominant-lethal incidence of about 5%, as mentioned above. Higher or erratic rates indicate the segregation of a sterility factor, for example one of the embryo-lethal "t" alleles, the presence of which will interfere with the significance of the test results. The dominant-lethal test can also be used to detect mutations in females. Unfortunately, the embryos are then growing in an exposed animal, which may have indirect effects on the developing embryos, namely effects which do not depend only on the action of chemicals on the ova.

Induction of sex-linked lethals in Drosophila melanogaster

8.34 This is a test for heritable mutations induced in the germ cells of an animal. The arguments justfiying the use of *Drosophila melanogaster* as an important test animal have been mentioned before and can be summarized briefly as follows. All eukaryotes have their DNA organized into chromosomes in a basically similar way. It has, furthermore, been shown that *Drosophila* metabolizes mutagenic chemicals in a similar manner to mammals. *Drosophila* has been used intensively in genetic research, in population and in biological studies, as well as in mutation research, over a period of 50 years, and a particularly large fund of basic knowledge is available. At the practical level it is well suited to routine laboratory use as it requires a minimum of simple, basic, laboratory equipment such as an incubator, small vials (3×1 inch in size), a means of preparing a simple medium for the larvae, a means of etherizing the

flies in order to handle them, and a low-power dissecting microscope. The mode of treatment can be adapted to the manner in which humans are exposed to the substance under test. Thus, food additives can be added to the drinking water of the flies; occasionally, it may be useful to mix them with food of the larvae. For airborne substances, the flies are exposed to vapour in closed containers. Intraperitoneal injections are feasible, but require some skill and experience; they are most important in studies of basic problems than in routine tests, where they are hardly ever used.

8.35 The only important routine test is that for sex-linked recessive lethals, ie for viability genes carried on the X chromosome. A mutation in one of these genes kills the males but can be carried in females that are heterozygous for it. The test consists of mating treated males to virgin females with an X chromosome that is recognizable by a dominant marker and carries an inversion for the prevention of crossing-over. The most frequently used chromosome of this type is called Muller-5, and the test is often referred to as the Muller-5 test. Every daughter of a treated male with a tester female carries one treated X chromosome. If this chromosome bears a lethal mutation, there will be no males with the treated X in the next generation. Since the absence of a whole class of males from a culture is not easily missed, the personal error is very low. No previous genetical knowledge is required; any competent laboratory worker can learn the test in a few weeks under the guidance of a geneticist. Subsequently, a geneticist, not necessarily in the same institution as the technician, is required only for advice in case of difficulties and for the interpretation of doubtful areas.

8.36 A special advantage of the test lies in the fact that germ cells of all stages from spermatogonia to spermatozoa can be tested separately by mating the treated males to a succession of females. The first brood will then have been produced by spermatozoa that were treated as mature germ cells, the last by spermatozoa that were treated as spermatogonia, and the intermediate ones from sperm treated at intermediate stages.
treated at intermediate stages.

8.37 Since sex-linked lethals are recognized as abnormal segregations within a population (one vial) of flies, they are of necessity due to nuclear changes, and the uncertainty as to the nuclear or cytoplasmic nature of mutations found in micro-organisms or cell cultures is avoided. Sex-linked lethals may be due to changes within the gene (base changes or frameshifts), or to deletions of a small string of genes. It is the latter that are an especially important genetic risk, and their detection in the sex-linked lethal test is one of the greatest advantages of this test. In prokaryotes, deletions are usually lethal and kill before detection; even those that can be detected probably arise by a mechanism that is different from the one that produces deletions in eukaryotes.

8.38 In tests on micro-organisms or cell cultures, negative controls for detecting pre-existing "background" mutations are mandatory. The *Drosophila* sex-linked lethal test is carried out on males that are alive and thus

cannot carry a pre-existing lethal. The only lethals that can be found in untreated males are those that arose in the males themselves during their development from egg to adult fly. This is the so-called "spontaneous" mutation frequency of sex-linked lethals. A vast body of data on spontaneous lethal frequency is available. If the data collected over the past 20 years from different laboratories are compared, a remarkable consistency emerges. Regardless of strain, most values cluster around 0.1-0.2% and they rarely reach or exceed 0.4%. The practical consequence of this is that simulataneous negative controls are an unnecessary complication in routine analyses provided an annual test is made to ensure that the spontaneous mutation rate of the strain is as expected. It is necessary, however, to fix a baseline for comparison before a mutagen can be identified; a suitable one would be 0.4%. The tests for mutagenicity must be performed at least twice, but need not be on a large scale. 1000 chromosomes per replicate will normally suffice, but the numbers can be adjusted in the light of the results of the first trial. If the replicates show that there is a 95% probability that the true mean of the treated population exceeds 0.4%, the substance should be regarded as a mutagen.

Substances may just fail to reach significance because they produce a preponderance of one-stranded or otherwise delayed mutations. These would be missed in the F_2 of the standard test but they may be detected if the test is extended to the F_3. This is done by mating an F_2 female from each non-mutant culture vial with one of her brothers and looking for lethals among her sons in the F_3. Hydroxylamine is a chemical which is detected as a mutagen in *Drosophila* in this way (Shukla and Auerbach, 1979). The application of this extension would not be routine, however, being dependent on the F_2 results, and would be at the discretion of the geneticist conducting the tests.

8.39 Substances which are positive in *Drosophila* must be regarded as highly suspect mutagens, even if they are negative in the Ames test (eg heliotrine and formaldehyde). Substances which are negative or doubtful in the routine test as described may be subjected to more rigorous tests in *Drosophila*. These involve not only larger numbers but also simultaneous controls in which all known unwanted differences between treated and control samples are either eliminated or their significance evaluated, eg crowding, or sperm utilization rates (Kilbey et al., 1980). These tests can only be done in laboratories with special facilities. In most cases it will be preferable to base the assessment of mutagenicity on the routine *Drosophila* results in relation to those obtained with other tests.

8.40 The generation time of *Drosophila* is about 2 weeks; the basic test for sex-linked lethals (carried to the F_2) thus requires 4 weeks for tests on mature spermatozoa and 2 weeks more if younger germ cell stages are to be tested or if the test on mature sperm is carried on to F_3. The main time-consuming work is the mating of the F_1 and, where necessary, of the F_2. An experienced technician can mate 1000 vials in one day. Several substances can be tested simultaneously even by one technician. The achievement of 2 technicians is more than twice that of one because the scheme will be less easily interrupted by holidays or illness. At a conservative estimate, 2 technicians, plus one laboratory helper for making media and washing glassware, can deal with at least 22 substances per year.

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Appendix 1

A selected bibliography of test procedures

PART 1: "BASIC PACKAGE" MUTAGENICITY TEST SCREEN (para 7.13)

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PART 2: SUPPLEMENTARY TESTS WHICH MAY BE USEFUL IN PROVIDING CLARIFICATION WHERE UNEXPECTED OR EQUIVOCAL RESULTS HAVE BEEN OBTAINED IN THE MINIMAL TEST SCREENING PROGRAMME (para 7.14).

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APPENDIX 2: Glossary*

ALLELE One of 2 or more alternate forms of a gene at a specific locus on a particular chromosome. In diploid organisms the corresponding alleles on homologous chromosomes may be identical or different (see HETEROZYGOTE).

ANEUPLOID(Y) Cells or individuals with one or more chromosomes of the euploid (see DIPLOID) set of chromosomes absent from or additional to this set. In the case of addition to a basically diploid set of 2 (or more) homologues (see HETEROZYGOTE), the extra chromosome causes trisomy (tetrasomy, polysomy) of that chromosome. If one chromosome of a pair is missing, the result is *monosomy* of that particular chromosome (absence of the pair is called *nullisomy*). It is customary to describe cells or individuals with extra or missing chromosomes as hyperploid or hypoploid.

BASE PAIRS The characteristic hydrogen-bonded purine-pyrimidine pairs of bases, located sequentially like the rungs of a ladder along deoxyribose-phosphate strands of the DNA helix. There are only 2 complementary base pairs involved in DNA (q.v.), adenine with thymine and guanine with cytosine. In RNA (q.v.) uracil-thymine is substituted for adenine-thymine. The nitrogenous bases adenine and guanine are purines, while thymine, cytosine and uracil are pyrimidines. The compound formed by a base and the 5-carbon sugar (deoxyribose or ribose) is termed a nucleoside, and when this is bound to a phosphate the compound is termed a nucleotide (either a ribonucleotide or a deoxyribonucleotide depending on the sugar molecule).

CENTROMERE (KINETOCHORE) The characteristic region of each chromosome with which the spindle fibre becomes associated during cell division. When a chromosome has replicated it consists of 2 chromatids joined together at the centromere.

CHIASMA In meiosis (q.v.), the visible site of exchange between homologous chromatids. The process of exchange of genetic material between homologous chromatids is known as crossing-over, and chiasmata are accepted as the visible expression of this event.

*For more detailed definitions, the interested reader is referred to Rieger, R., Michaelis, A. and Green, M. M., 1976. *A Glossary of Genetics and Cytogenetics — classical and molecular*, 4th ed. Berlin, Heidelberg, New York: Springer Verlag.

CHROMOSOME, CHROMATID, CHROMATIN A chromosome consists of a single condensed, coiled and supercoiled giant molecule of DNA (q.v.) associated with a complex of protein, enzymes and RNA (q.v.), known as *chromatin*. Chromosomes are most easily visualized after DNA replication, at the metaphase (q.v.) of cell division when each chromosome consists of 2 (sister) chromatids joined at the centromere.

CODON A group (triplet) of any 3 adjacent bases on one of the 2 DNA strands of the double DNA helix.

DEOXYRIBOSE (2-deoxy-D-ribose). A 5-carbon sugar differing from *ribose* by the absence of an oxygen atom at carbon 2.

DIPLOID(Y) In general the body cells of higher organisms are diploid, ie they have 2 homologous sets of chromosomes, one derived from the father and one from the mother. Homologues are similar in size and genetic make-up. The exception is the XY pair in the male, which is unequal in size. The general term "ploidy" refers to the number of chromosome sets per cell. The term "euploid(y)" is often used to describe cells (or individuals) with one complete chromosome set, or with whole multiples of the basic chromosome number which is characteristic of the species (see also ANEUPLOID(Y)). Different euploid complements are described by the terms *haploid(y)* (q.v.) — one set of chromosomes, or *polyploid(y)* — more than 2 sets (specifically *triploid(y), tetraploid(y), pentaploid(y),* etc).

DNA Deoxyribonucleic acid, the genetic material of all cells, is a giant polymer of basic nucleotides (see BASE PAIRS), generally arranged in a double helix (double stranded DNA). It contains the genetic information which is coded for by sequences of bases (triplets) along the helix (see CODON).

EUKARYOTES Eukaryote organisms, in contrast to *prokaryotes,* are those higher plants and animals whose cells have a nucleus in which the DNA is organized into characteristic sets of chromosomes. Cell division occurs by the process of mitosis; or, in the case of gamete formation, by meiosis, when "germ cells" (q.v.) undergo reduction division.

GAMETOGENESIS A series of processes leading to the production of mature gametes, which are essential for sexual reproduction. In mammals, the gametes are the spermatozoa and the ova. These are formed by division and maturation from the "germ cells" (gonocytes). During this process the chromosomal complement is halved by meiosis (q.v.), giving rise to haploid cells. At fertilization the 2 gametes fuse and the resulting *zygote* thus has a full complement of chromosomes, consisting of a set from each parent; it is therefore diploid.

GENE May be defined as a functional unit of inheritance, and is a specific sequence of bases along the DNA molecule. There are at least 3 different classes of genes: those which code for polypeptides through transcription into messenger RNA (mRNA) (structural genes), those which are *transcribed* into

RNA which is not *translated* into proteins (transfer RNA (tRNA); ribosomal RNA (rRNA)), and those whose function may not require transcription.

GENOME The sum total of the genes of an organism. In eukaryotes the basic genome is considered to be equivalent to the haploid set of chromosomes.

GERM CELLS A group of primordial cells which are set aside during early embryonic development. They are the precursors of the future gametes (ova or spermatozoa). Alternative names are "primordial germ cells", "ancestral cells", "gonocytes" and sometimes "gonia".

HAPLOID The cells of an individual, including the primordial germ cells, possess 2 sets of chromosomes, one from each parent, and are therefore diploid. In gametogenesis, the germ cells undergo meiotic division, whereby the double set of chromosomes is reduced to one set (haploid state) in the gametes.

HETEROZYGOTE In diploid eukaryotes each cell (gametes excepted) has 2 complete sets of chromosomes, one from each parent. The pairs of equivalent chromosomes are called *homologous,* and are taken to be structurally the same. At precisely equivalent locations *(loci)* on the DNA molecules of the homologues, alleles (allelic forms) of a gene occur, which subserve the same function. Often these pairs of alleles are not identical. If so, one allele might express itself dominantly, at the expense of the other, which acts recessively. However, this is not necessarily so. When the alleles are not identical, the individual is described as *heterozygous* for the particular gene at the given locus. If the alleles are identical, he is described as *homozygous,* ie he is a *homozygote.*

HOMOLOGOUS, (HOMOLOGUE) See HETEROZYGOTE.

HOMOZYGOTE See HETEROZYGOTE

MEIOSIS The term used to describe that special sequence of cell divisions, and the concomitant behaviour of the diploid set of chromosomes, which leads to the formation of haploid gametes. During meiosis there are 2 cell divisions which follow each other, but only one round of replication of the chromosomal DNA. This precedes the first meiotic division and is similar to the replication that occurs before mitosis. Subsequently, during the meiotic prophase (see MITOSIS), homologous chromosomes associate as pairs, and crossing-over occurs between *homologous chromatids,* namely at the *four-strand stage;* thus there is recombination (q.v.) of *linked* genes (which results from an exact exchange of genetic material between homologous chromatids). At first meiotic division, which follows, the previously paired homologues separate from each other, each chromosome consisting of 2 (sister) chromatids. Where recombination has occurred, the 2 chromatids (although originally genetically identical) differ genetically. Second meiotic division then follows. This is similar to a mitotic division but differs from it in that the dividing cell has only a haploid set of chromosomes. At this division the chromatids of each

chromosome separate from each other. Between them the 2 divisions give rise to 4 products of meiosis, all haploid, and all potentially genetically different from each other. In the male these 4 haploid products mature into sperm. In the female, only one of them matures into the oocyte; the others form polar bodies.

METAPHASE A stage in cell division, either meiotic or mitotic, when the nuclear membrane has disappeared, and the chromosomes, in their most condensed form, are arrayed across the equatorial region of the cell, on the fibrous spindle. The function of the spindle is to separate from each other the sister chromatids of each chromosome, at mitotic metaphase or at second meiotic division (or the homologous chromosomes at first meiotic division). If the formation and function of the spindle fibres are inhibited at metaphase, the 2 chromatids remain attached to each other, and condensed, and this is especially favourable for microscopic study. The substance most commonly employed for deliberately blocking chromosomes (ie chromatid separation) at metaphase is colchicine.

MITOSIS The process of cell division whereby the sister chromatids of each replicated chromosome separate from each other and migrate to opposite poles of the dividing cell. The sister chromatids are exact copies of each other and their segregation ensures the transmission of genetic information from mother cell to daughter cells. Mitosis is divided into a number of phases, of which metaphase is of special practical importance. Metaphase is preceded by *prophase,* and is followed by *anaphase,* during which the spindle draws one set of chromatids to each pole, where they form the chromosomes within the nuclei of the 2 daughter cells *(telophase).*

MUTATION Viewed broadly, mutation is any observable heritable change (either spontaneous or induced) whereby an individual offspring (or cell) differs unexpectedly in some property from its parent (in the case of simple organisms) or parents. This implies that there has been a failure to transmit correctly the chromosomes and/or other genetic information encoded in some part or parts of the DNA molecule. Events giving rise to mutations range from single base changes in the DNA to structural changes in the chromosomes, or alterations in their number.

NON-DISJUNCTION Abnormal chromosome separation at cell division, which causes 2 chromatids to fail to separate from each other at mitotic or at second meiotic division, or the homologous chromosomes of a pair to fail to separate from each other at first meiotic division. The result of non-disjunction is that one of the 2 products of division will contain the extra chromosome, which the other will lack. It is possible for non-disjunction to affect more than one pair of chromatids (or chromosomes) at the same division. After non-disjunction occurs at either first or second meiotic division, the zygote formed by the non-disjunctional gametes is generally inviable, with some exceptions. In man, for example, one of the exceptions is non-disjunction of chromosome 21, which produces trisomy of that chromosome, and Down syndrome (or, exceptionally, its monosomy). Another example compatible with survival is

trisomy of the X chromosome (triplo-X female). Non-disjunction of the sex chromosomes in man can also result in a viable individual with only one sex chromosome, an X (45, X or XO Turner Syndrome).

PHENOTYPE The observable properties, characteristics, or traits of an individual (or cell) produced by the interaction between the individual's genetic potential (its genotype) and the environment.

PLASMIDS Small elements of DNA found in bacteria, which are separate from the single, circular, bacterial chromosome. They determine properties such as resistance to drugs and bacterial conjugation, and can transfer from one cell to another during bacterial division and independently of the bacterial chromosome.

POLYMERASES Enzymes which catalyse the ordered assembly (polymerization) of mononucleotides to reproduce DNA or RNA. They use one of the 2 strands of the DNA duplex as a template, in the process of genetic transcription to RNA (RNA polymerases) or DNA replication or repair (DNA polymerases). RNA may also act as a template for generating DNA (reverse transcription).

PROKARYOTES The simplest of living organisms, namely viruses, bacteria, and some blue-green algae, in which the genetic material is arranged into one chromosomal complex consisting of a single circular molecule of DNA (or RNA in some viruses). There is no nuclear organization and mitosis and meiosis do not occur, although nucleotide polymerization (chromonema) replication takes place, and division and multiplication follow.

RECOMBINATION In higher organisms, the events during meiotic prophase (when homologous chromosomes pair, and are in close and precise alignment with each other) leading to an exchange of maternal and paternal genetic (chromosomal) material between homologues (crossing-over). As a result of recombination, individual chromatids contain a different combination of alleles compared with their original, pre-meiotic, make-up, and are called recombinant chromatids. Recombination is, therefore, the process which gives rise to cells or individuals in which genes are linked in different combinations, and it thus introduces an important element of genetic variability into progeny.

REPLICATION The process whereby the DNA molecule is copied precisely, in preparation for the handing on of genetic information to the offspring of an individual, or from one somatic cell to its daughter cells. During DNA replication, the chromosome is uncoiled under enzyme and protein control, exposing the 2 strands of the chromosomal DNA to a number of highly integrated enzyme and protein systems. At specific points, the 2 strands of the DNA helix unzip where bases pair. The points where the unzipping first occurs are called *replication forks,* and here the polymerase system of enzymes, using each of the 2 strands as a template, synthesizes 2 copies of DNA polymers, each complementary to a strand of the original duplex. In this way, 2 identical DNA

duplexes are generated, each one consisting half of parental and half of newly assembled material (semi-conservative replication).

RIBOSE See DEOXYRIBOSE.

RNA Ribonucleic acid is a polymer of nucleotides in which the linking strand is a ribose-phosphate chain. The bases attached to the ribose are adenine, guanine, uracil and cytosine. RNA molecules are single stranded, as in messenger RNA (mRNA), but complex molecular forms also occur. Ribonucleic acids function in many synthetic processes, particularly in the extra-nuclear synthesis of enzymes and other proteins. Messenger RNA (mRNA) is synthesized on one strand of the DNA duplex at a designated locus, so that its base sequence is a complement of the DNA base sequence at that position *(transcription)*. The mRNA then diffuses out of the nucleus to appropriate *ribosomes* in the cell cytoplasm where, in association with ribosomal RNA (rRNA), and upon delivery by transfer RNAs (tRNA) of appropriate amino acids, polypeptides are synthesized *(translation)* in accordance with instructions encoded at the DNA locus.

TRANSCRIPTION, TRANSLATION See RNA.

TRANSLOCATION Refers to a change in the position of a section of a standard chromosome following its breakage and reunion. A translocation may occur within a chromosome, between one chromosome and its homologue, or between different, non-homologous chromosomes. In practice, when translocations occur between 2 chromosomes, they may be reciprocal, if there is swapping of segments of equal or unequal size. A special type of translocation are the Robertsonian translocations (also called centric fusions), which occur between acrocentric chromosomes (with a subterminal centromere); they are characterized by the fact that the breaks and reunions occur close to the centromeres of the acrocentrics involved in the translocation. The chromosomes which result from a reciprocal Robertsonian exchange often consist of a large compound chromosome which in practice is made up of almost the entire long arms of two acrocentric chromosomes, and a very small element resulting from the fusion of their 2 short arms. It is not unusual for these short arms to be eliminated during subsequent cell division. On occasions, both breaks in the 2 acrocentric chromosomes seem to occur in the short arms, and the resulting bi-armed chromosome is then a *dicentric,* namely, it possesses 2 centromeres. Within the given cell in which a translocation occurs, the chromosomal complement remains *balanced,* but especially during meiosis chromosome segregation may lead to the formation of *unbalanced* products of meiotic division. It is for this reason that translocations, both reciprocal and non-reciprocal, both Robertsonian and non-Robertsonian, can lead to the production of chromosomally unbalanced zygotes. A characteristic example of this in man is provided by so-called "translocation Down syndrome".

TRISOMY See NON-DISJUNCTION.

ZYGOTE See GAMETOGENESIS.

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