Contact address:

All correspondence for COM should be through the secretariat initially.

Khandu Mistry
Room 692D
Skipton House
80 London Road
LONDON SE1 6LH

Tel: +44 (0)20 7972 5020
Fax:+44 (0)20 7972 5156

Email: Khandu.Mistry@doh.gsi.gov.uk
Website: http://www.doh.gov.uk/com.htm

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Committee on MUTAGENICITY

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COM)

GUIDANCE ON A STRATEGY FOR TESTING OF CHEMICALS FOR MUTAGENICITY

CHAIR
Professor Jim M Parry BSc PhD DSc

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I. Preface

1. The Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) is an expert advisory committee whose members are appointed by the Chief Medical Officer for England following an appointments exercise involving public advertisement. Members serve in their own capacity as independent experts and observe a published code of practice including principles relating to the declaration of possible conflicting interests.

2. The remit of the committee is to advise the Department of Health, and other government departments and agencies with an interest in the safety of chemicals across various sectors, on all aspects of the mutagenicity of chemicals. The Secretariat is provided by the Department of Health (who lead) and the Food Standards Agency (FSA). Other government departments with an interest provide assessors to the Committee; these are specifically from the Department of Environment, Transport and the Regions (DETR), Health and Safety Executive (HSE), Pesticides Safety Directorate (PSD – a MAFF agency responsible for approval of pesticides), Veterinary Medicines Directorate (VMD - a MAFF agency responsible for the licensing of veterinary drugs) and the Medicines Control Agency (MCA - a DH agency responsible for the licensing of human medicines). In addition there are assessors from the devolved administrations (Scottish Executive, Welsh Assembly, Northern Ireland Executive).

3. The role of the COM is advisory. It has no regulatory status, although its advice may be provided to an agency that does have such a role (eg - HSE for occupational aspects and the EU new and existing substances regulations, MCAfor human medicines, PSD for pesticides etc). Its remit is to advise on all aspects of the mutagenicity of chemicals, and this may involve advice on a specific chemical, and also on testing strategies and research.

4. In the context of testing strategies the COM first published guidelines for the testing of chemicals for mutagenicity in 1981. These provided guidance to the relevant government departments and agencies on best practice for testing at that time. The need for guidelines to be periodically updated, to reflect advances in development and validation of methods, was recognised and revised guidelines were published in 1989. This new guidance continues this updating process. The strategy outlined is believed to be the most appropriate given the available methods and recognises the need to avoid use of live animals where practical and validated alternative methods are available. It is recognised that, as with the earlier guidelines, it will be some time before this strategy is reflected in the mandatory, regulatory guidelines of the various

agencies, and it is not, of course, intended that the COM guidance should be applied retrospectively.

5. The Committee believes that the approach outlined here will remain valid for several years and will encourage international recognition of the newer tests being recommended here for which there are, currently, no internationally harmonised guidelines.

II. Introduction

6. The Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment is an independent expert advisory committee appointed by the Chief Medical Officer (CMO) for England. The Committee advises the CMO and, through the CMO, the Government, on all matters related to the mutagenicity of chemicals. The COM also has a general remit to advise on important general principles or new scientific discoveries in connection with mutagenic hazard (the inherent mutagenic property of the substance) or risk (the likelihood of mutagenic effects occuring after a given exposure) and to present recommendations for mutagenicity testing. In practice the bulk of the work of the Committee relates to assessing mutagenic hazard.

7. The Committee last published guidance on a strategy for the testing of chemicals for mutagenic potential in 1989 (DH 1989). This provided advice on the application of methods which may be used to determine the ability of chemicals to induce point mutations or structural chromosome aberrations (clastogenicity). Since 1989 there has been a rapid growth in the published data available in this area and the development of many new methods. Thus, for example, the US Environmental Protection Agency and the IARC Genetic Activity Profile (GAP) Database lists results for over 90 different assay systems (Waters *et al* 1999). The Committee reaffirms its general advice published in 1989 that screening for mutagenicity should be based on a limited number of well validated and informative tests. This view is consistent with that reached by a meeting of international experts in 1995 convened by the International Programme on Chemical Safety (IPCS) (Ashby *et al* 1996). Major changes in the new strategy now being proposed are the consideration of the detection of the potential hazard of chemicals which may induce aneuploidy (numerical chromosome aberrations) and the application of *in-vivo* assays for tissues other than the bone marrow. It is the objective of this paper to set out a scientifically valid testing strategy comprising those methods which are believed to be the most informative and (when possible) are well validated. There is no discussion of those methods which experience has shown to have no place in the recommended mutagenicity testing strategy. Details of methodologies are not given since they are provided in the OECD test guidelines and in the extensive published literature (eg UKEMS 1989, 1990, 1993, McGregor *et al* 1999).

8. In this guidance document the term mutation refers to a permanent change in the amount or structure of the genetic material of an organism, which may result in a heritable change in the characteristics of the organism. These alterations may involve individual genes, blocks of genes, or whole chromosomes. Mutations involving single genes may be a consequence of

effects on single DNAbases (point mutations) or of larger changes, including deletions and rearrangements of DNA. Changes involving chromosomes as entities may be numerical or structural. Amutation in the germ cells of sexually reproducing organisms may be transmitted to the offspring, whereas a mutation that occurs in somatic cells may be transferred only to descendent daughter cells. Mutagenic chemicals may present a hazard to health since exposure to a mutagen carries the risk of inducing germ-line mutations, with the possibility of inherited disorders, and the risk of somatic mutations including those leading to cancer.

9. Modification by chemicals of the segregation of chromosomes during both mitotic and meiotic cell division can lead to malsegregation and thus to aneuploidy. This is a type of mutation which involves a change in chromosome number from the normal diploid or haploid status of a species, whereas polyploidy represents an increase in chromosome number which is an exact multiple of the haploid number, eg triploidy (3n) and tetraploidy (4n). Aneuploidy makes a major contribution to human embryonic loss and some birth defects such as Down Syndrome (trisomy of chromosome 21). Chemicals which induce aneuploidy as their predominant mutagenic effect are termed aneugens. A wide range of chemicals (primarily those which modify the spindle of the dividing cell) such as colchicine, benomyl, trichlorphon and griseofulvin have been shown to induce aneupoloidy in test systems ranging from *in-vitro* cultured mammalian cells and somatic tissue of intact animals, to germ cells of rodents (Aardema *et al* 1998). Currently, evidence for the carcinogenicity of aneugens is limited. However a large number of aneugens are inducers of malignant transformation in Syrian hamster cells *in vitro* (Gibson *et al* 1995, Oshimura and Barrett 1986, Parry and Sors 1993). Given the association between aneuploidy and heritable effects in germ cells, and potential carcinogenicity, the Committee concludes that the testing of chemicals for potential aneugenic activity should be included in genotoxicity testing strategies. Data from studies of induced aneuploidy have been used for the classification of chemicals in the EU and thus the advice provided here is timely.

10. It is therefore apparent that information on the three levels of mutation, namely gene, clastogenicity (ie structural chromosome aberrations) and aneuploidy (ie numerical chromosomal aberrations), is necessary to provide comprehensive coverage of the mutagenic potential of a chemical. This is also the case when assessing carcinogenic potential, since all three types of mutation have been shown to be associated with the activation and expression of oncogenes, and loss or inactivation of tumour suppressor genes and other classes of genes implicated in carcinogenesis.

11. Genotoxic (or genotoxicity) refers to agents which interact with the DNA and/or the cellular apparatus which regulates the fidelity of the genome, eg the spindle apparatus, and enzymes such as the topoisomerases. It is a broad term that includes mutation as well as damage to DNA or the production of DNAadducts, by the chemical itself or its metabolites. Genotoxic effects also include unscheduled DNAsynthesis (UDS), sister chromatid exchange (SCE) and mitotic recombination. However the detection of such effects does not in itself provide direct evidence of inherited mutations. The term "genotoxic carcinogen" as used by the Committee describes those chemicals that are carcinogenic and also give positive results in mutagenicity or genotoxicity tests *in vivo*.

12. The Committee reaffirms its view published in 1989 that there is currently no single validated test that can provide information on all three end-points, namely gene mutation, clastogenicity and aneuploidy and thus it is necessary to subject a given substance to several different assays. Arange of tests has been developed which employ a wide variety of organisms, including bacteria, yeasts and other eukaryotic micro-organisms, and mammalian cells studied *in vitro*, as well as whole mammals where effects in either somatic or germ cells can be measured. Anumber of different end-points can be used which may measure genetic changes or indicators for the potential to produce genetic change. Assays may be classified on the basis of these endpoints (eg gene mutation, clastogenicity, aneugenicity and tests for DNA damage) and by consideration of the different phylogenetic levels represented. The Committee is not aware of any substance giving clear positive results for the induction of gene mutations which does not also give, under appropriate conditions, positive results using *in-vitro* tests for clastogenicity. However the reverse is not true and there are some clastogens, eg inorganic arsenic compounds (IARC 1987), which do not give positive results in tests for gene mutation. In the case of aneugenic chemicals the detection of the induction of aneuploidy is dependent on the use of methods which allow the measurement of the malsegregation of chromosomes leading to chromosome loss and/or non-disjunction.

III. General principles of testing strategy

13. The Committee recommends a three-stage testing strategy for the detection of mutagenic hazard. Initial screening for mutagenic activity at Stage 1 is based upon three [or two in those cases where little or no human exposure is expected eg industrial intermediates, some low production volume chemicals] *in-vitro* tests. Stage 2 consists of a number of *in-vivo* tests designed to investigate whether *in-vitro* mutagenic activity can be expressed in the whole animal. These two stages provide information on the mutagenic hazard of a substance. Stage 3 investigates, when necessary, whether any *in-vivo* mutagenic activity observed in Stage 2 can be expressed in the germ cells of mammals. Some consideration may also be given to assessment of risk of heritable effects at this stage. There is a clear strategy for planning tests within each stage and for progressing to the next stage (see Figs 1-3). Clear statements can be made regarding the *in-vitro* tests to be used in Stage 1 as these methods have been well studied. The strategy for Stages 2 and 3 is more complex, particularly with regard to investigating mutagenicity in target tissues other than the bone-marrow. Thus, some consideration of the current status of a number of alternative tests has been included here. Nevertheless, an overall strategy for Stages 2 and 3 is presented. Ashort overview of the rationale supporting the approach recommended by the Committee is given below, along with some brief comments on matters to consider before devising a testing strategy for a specific chemical.

14. It is recommended that the screening studies at Stage 1 should investigate the mutagenicity of the chemical using *in-vitro* tests. Few chemicals are active only *in-vivo* and, in such cases, this is usually due to limitations in the exogenous metabolism used in *in-vitro* test systems (Tweats and Gatehouse 1988, Ashby 1988). The available information confirms the Committee's view (expressed in 1989) that it is appropriate to concentrate on a relatively small number of assays, using validated, sensitive methods particularly chosen to avoid false negatives.

15. Under the strategy recommended by the Committee, the use of animals in mutagenicity testing is primarily required when it is necessary to investigate whether mutagenic activity detected *in vitro* is reproduced *in vivo*. Except in those cases where high, or moderate and prolonged human exposure is expected, (eg many human medicines) there is no justification for the routine use of animals for mutagenicity tests when there is no evidence for activity at Stage 1. All assays should be designed to provide the best chance of detecting potential activity, with respect to (a) the exogenous metabolic activation system; (b) the ability of the compound or its metabolite(s) to reach the target DNA and/or targets such as the cell division apparatus, and (c) the

ability of the genetic test system to detect the given type of mutational event. The assays should be carried out to internationally recognised protocols (eg OECD 1997).

16. The intrinsic chemical properties of the test substance must be considered before devising the mutagenicity testing programme. Whether the substance would be expected to have mutagenic potential can be assessed from its chemical structure, which may provide structural alerts for mutagenicity. A composite model structure has been devised indicating substituents or moieties associated with DNA-reactivity (Ashby and Paton 1993). This is a valuable tool for initially assessing the potential *in-vitro* mutagenicity of a novel chemical.

17. A number of commercial systems to investigate structure activity relationships (SAR) have also been developed (Zeiger *et al* 1996). These attempt to predict *in-vitro* mutagenicity by automated analyses of the statistical correlation between structure and mutagenic activity and/or programmed rules for prediction based on the available knowledge and expert judgement. Such systems can be useful when a large number of compounds require assessment and prioritisation for biological testing. However the commercial models currently available appear no better for predicting *in-vitro* mutagenic activity than an inspection of the chemical structure and the use of expert judgement.

18. The physico-chemical properties of the test chemical (for example, pH, solubility, and stability in solvents/vehicles) and its purity can affect the ease of conduct and results of tests. For example, the tolerance of cells to acidic chemicals can be enhanced by neutralisation but this may affect the inherent reactivity of substances to DNA (Hiramoto *et al* 1997). Alternatively, low solubility may limit the feasibility of undertaking some or all of the *in-vitro* mutagenicity tests recommended in this strategy. The toxic properties of test chemicals (such as acute toxicity, or irritancy/corrosivity in contact with skin or mucous membranes) and their toxicokinetics and metabolism will influence the choice of route of administration and the highest dose level achievable in *in-vivo* mutagenicity tests. Dose selection for *in-vivo* testing requires estimation of the maximum tolerated dose and consideration of tissue-specific effects. The strategy recommended in the following sections is concerned with investigating mutagenic activity of individual chemicals and no consideration is given in these guidelines to mixtures.

Stage 1: In-vitro tests

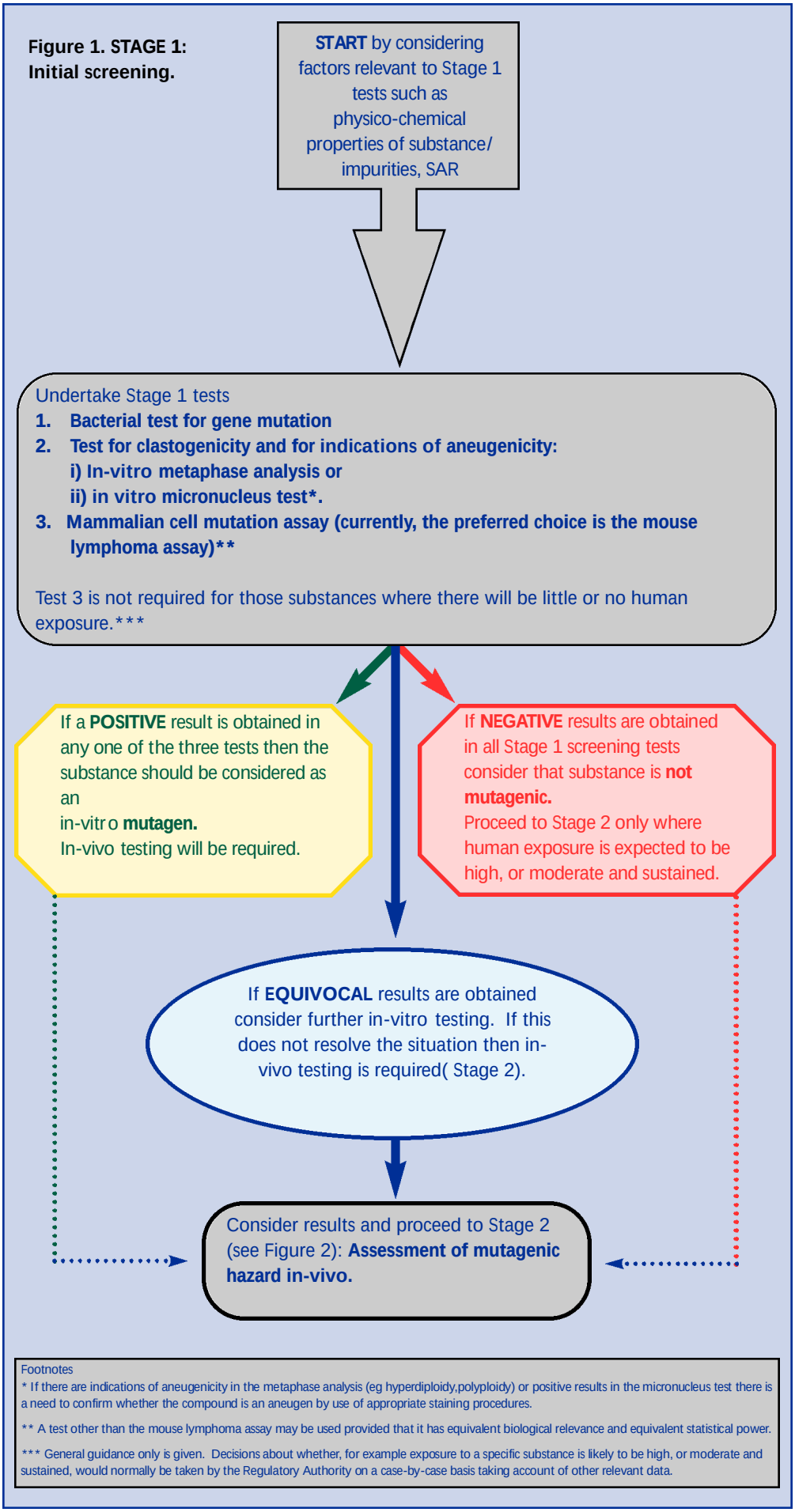
Introduction

19. As outlined above, Stage 1 involves screening tests for mutagenic activity using *in-vitro* methods and comprises three test-systems. A clearly positive result in any one of the three tests is sufficient to define the chemical as an *in-vitro* mutagen and the need to proceed to Stage 2. It is necessary to obtain clearly negative results in these tests in order to reach a conclusion that the chemical has no mutagenic activity. Usually data from all three tests will be necessary but in the case of those substances where there will be little or no human exposure, (eg industrial intermediates, some low production volume chemicals) results from only the first two tests will be necessary. Equivocal results should be investigated by further testing. If this does not resolve the situation then *in-vivo* testing is required (Stage 2). An outline of Stage 1 (initial screening) is given in Figure 1 and a description of the assays recommended is provided in the following paragraphs.

Discussion of Stage 1 tests

20. The most widely used *in-vitro* test is the bacterial reverse mutation assay for gene mutations developed by Ames and his colleagues using *Salmonella typhimurium* (Gatehouse *et al* 1990). The very extensive database available for this assay justifies its inclusion in any initial testing package. Several strains of bacteria capable of detecting both base-pair and frame-shift mutations must be included, the best validated strains being TA1535, TA1537 (or TA97 or TA97a), TA98, TA100. These strains of *Salmonella typhimurium* may not detect some oxidising mutagens and cross linking agents and thus *Escherichia coli* WP2 (pKM101), WP2*uvrA* (pKM101) or *Salmonella* TA102 should also be used. Testing should be carried out both in the presence and absence of an appropriate exogenous metabolic activation system. However both the repair proficient and repair deficient strains of *E coli* should be used in those cases where the bacterial assay is the only mutagenicity test being carried out on a given substance, to ensure that cross linking agents are detected.

Figure 1. STAGE 1: Initial screening.



21. The Salmonella assay, whilst being an efficient primary screen for detecting compounds with inherent potential for inducing gene mutations, does not detect all compounds with mutagenic potential. Some compounds are clastogens but do not produce gene mutation in the Salmonella assay (eg inorganic arsenic compounds. IARC 1987). The second assay should therefore evaluate the potential of a chemical to produce both clastogenicity and aneugenicity, and it should use mammalian cells, either cell lines or primary human cultures such as fibroblasts or lymphocytes. The Committee notes that a major development since the publication of the previous guidelines has been the development of novel techniques (such as chromosomal painting) and methods (ie the *in-vitro* micronucleus assay) for the assessment of potential aneugenicity. It is now feasible to screen substances for their potential to induce aneuploidy in the initial testing stage.

22. One approach is the *in-vitro* cytogenetic assay for clastogenicity using metaphase analysis. Limited information can be obtained on potential aneugenicity by recording the incidence of hyperdiploidy, polyploidy and/or modification of mitotic index etc (Aardema *et al* 1998). If there are indicators of aneugenicity (eg induction of polyploidy) then this should be confirmed using appropriate staining procedures such as FISH (fluorescence *in-situ* hybridisation) or chromosome painting to highlight alterations in the number of copies of selected chromosomes (reviewed by Parry 1996). When cell lines are employed it is important that only those with a stable chromosome number are used. Reduced hypotonic treatment may be necessary to reduce artifactual changes in chromosome number. Only the detection of hyperploidy (gain in number) should be considered as a clear indication of induced aneuploidy.

23. Another procedure for the detection of both aneuploidy and clastogenicity is use of the *in-vitro* micronucleus assay. There have been considerable developments in deriving a suitable protocol for this assay (Doherty *et al* 1997). The Committee believes that the *in-vitro* micronucleus test has been adequately validated, but recognises that it will be some time before an internationally agreed OECD guideline would be available. The results from ongoing validation studies that are expected to be available shortly will facilitate this process.

24. In the case where the micronucleus test is used then kinetochore or centromeric staining should be incorporated to identify the nature of any micronuclei induced (ie to confirm whether the chemical is aneugenic). This will provide equivalent data to that obtained using the *in-vitro* metaphase analysis supplemented by chromosome painting to identify alterations in chromosome structure and number. A suitable procedure for the use of this assay to confirm aneugenicity is given in Appendix A.

25. The Committee reaffirms the view stated in the 1989 guidelines, that a combination of assays for gene mutation in bacteria and for chromosomal aberrations (plus aneuploidy) in mammalian cells may not detect a small proportion of agents with the potential for *in-vitro* mutagenicity. Thus a third assay, comprising an additional gene mutation assay in mammalian cells, should be used, except for compounds for which there is little or no human exposure. Certain mammalian cell gene mutation protocols that have been widely employed, particularly some of those involving the use of Chinese hamster cells, are now considered to be insufficiently sensitive, predominantly on statistical grounds (UKEMS 1989). Of the available systems, measuring mutations at the thymidine kinase (*tk)* locus in L5178Y mouse lymphoma cells has gained broad acceptance and has the advantage of detecting not only gene mutations but also various sizes of chromosome deletions.

26. The Committee, therefore, recommends the use of the mouse lymphoma assay (or an alternative test of equivalent statistical power) as the third *in-vitro* test in Stage 1. The use of the mouse lymphoma assay for the detection of all types of mutational end-point has been the subject of considerable debate, particularly by the International Conference on Harmonisation of the Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH 1997). The ICH considers that the mouse lymphoma assay can be used on a routine basis as an alternative to clastogenicity tests that employ metaphase analysis (Müller *et al* 1999).

27. The mouse lymphoma assay identifies substances which induce gene mutations. In addition, there are some data to justify the use of the mouse lymphoma assay to identify potential clastogens. In this regard the Committee considers this assay to be complementary, rather than equivalent to, metaphase analysis. It is felt that the use of extended treatment times to detect some clastogens needs further investigation. The Committee believes that there are insufficient data to assess the ability of the mouse lymphoma assay to detect potential aneugens. It is the view of the Committee that there are major advantages in using assays which primarily identify individual mechanisms of genetic damage, eg point mutations, clastogenicity and aneugenicity.

28. The Committee agrees that both the micro-well method and the soft agar versions of the mouse lymphoma assay are acceptable although it is noted there are methodological problems which may reduce the reliability of the latter method (Cole *et al* 1999). Poor growth conditions, particularly in Noble agar, can lead to inadequate detection of small colonies. For this, and other reasons given later in this paragraph, the Committee considers that appropriate use of positive controls and with colony sizing is an essential element in the quality control of mouse lymphoma assays (Moore *et al* 1999).

A bimodal distribution of colony sizes has been demonstrated in the mouse lymphoma assay. Small colonies grow slowly and have been shown, by microscopy, to generally contain visible chromosome aberrations. Large colonies which grow at the normal rate do not generally contain visible chromosomal changes, although some have been shown, by molecular analyses, to contain large deletions. In order to show that the assay is responding adequately, it is necessary to demonstrate that the cells are capable of producing both types of mutant colonies by the use of appropriate positive control chemicals.

29. Artifactual positive results, which do not reflect intrinsic mutagenicity, may be seen in mammalian cell assays. These effects can, for example, occur with exposures that involve low pH (Morita 1995) or low or high osmolarity. (Kalweit *et al* 1990, Nowak 1990). Variations in the concentrations of sodium, potassium and chloride ions have also been shown to significantly influence the outcome of mutagenicity tests in bacteria (Glatt *et al* 1994). These effects are not well characterised or understood, but their existence needs to be recognised.

30. In line with good scientific practice, the results of each *in-vitro* assay should be confirmed in an independent experiment. However, for mammalian cell assays this may not be necessary if the following rigorous criteria are met:

- there is no doubt as to the quality of the conduct of the test,
- the spacing and range of test substance concentrations leave no chance of missing a positive response,
- the result is not judged to be equivocal by statistical and biological criteria.

While it is accepted that there is no absolute requirement to repeat an *in-vitro* assay which has demonstrated a clearly positive result, there is a need to undertake further testing in an independent assay when an equivocal result is obtained. Further testing when negative results are obtained should be considered on a case-by-case basis. Where *in-vitro* screening tests are repeated in a further independent experiment it is not necessary to carry out the second study in an identical fashion to the initial experiment. Indeed it may be preferable to alter certain aspects of the study (eg concentration levels investigated) so as to obtain more useful data.

31. All mutagenicity studies should as far as possible be carried out to internationally accepted protocols. The Committee recognised that there was currently no such guideline for the *in-vitro* micronucleus test. It was hoped

such a guideline would be agreed in the next year or two, and that the validation studies to be reported shortly will facilitate this process. Provided that the Stage 1 studies outlined in *Figure* 1 are done to a high standard the Committee felt there is little to be gained from further *in-vitro* testing. They do not recommend the routine use of other *in-vitro* tests such as assays for sister chromatid exchange or tests using fungi. If a positive result is obtained in any one of the *in-vitro* screening assays cited in *Figure* 1, there is a need for *in-vivo* testing. The Committee recommends that all appropriate tests in Stage 1 should be completed before undertaking any Stage 2 test. It is then necessary to investigate whether the intrinsic mutagenic properties of the compound detected *in vitro* can be expressed *in vivo* in mammals (ie Stage 2).

Summary Stage 1: In-vitro assays

32. The Committee's recommendations for Stage 1 testing are basically similar to those in the 1989 guidelines, the main change being the need at this stage to obtain information on aneugenicity in addition to gene mutation and clastogenicity. As in the earlier guidelines the initial testing is based on a small number of *in-vitro* tests conducted to a high standard. For most chemicals three tests are recommended. In those cases where little or no human exposure is predicted (eg chemical intermediates, or some low production volume chemicals) only the first two tests may be appropriate. Such decisions need to be taken on a case-by-case basis by the appropriate regulatory agency. When only two tests are considered necessary it is recommended that these consist of a bacterial assay for gene mutation and an *in-vitro* mammalian cell assay for clastogenicity which will also screen for aneugenicity. The Committee now believes that routine screening for aneugenicity and clastogenicity is possible using the *in-vitro* micronucleus test in interphase cells, with the use of kinetochore or centromeric probes to identify the nature of any micronuclei induced (whole chromosomes or fragments). Alternatively, an assay using metaphase analysis and appropriate staining procedures to highlight alterations in structure and number is acceptable. The Committee considers that these alternative cytogenetic approaches provide essentially equivalent information. The third assay recommended is an additional gene mutation assay in mammalian cells. The mouse lymphoma assay (or an alternative test of equivalent statistical power) is recommended. This will also provide additional information on clastogenicity; there are however insufficient data to assess the ability of this assay to detect potential aneugens. These three assays, if negative, will provide sufficient information for the assessment of most chemicals. However where high, or moderate and prolonged, levels of exposure are expected (eg most human medicines) an *in-vivo* assay is recommended to provide additional reassurance.

Stage 2: In-vivo assays in somatic cells

Introduction

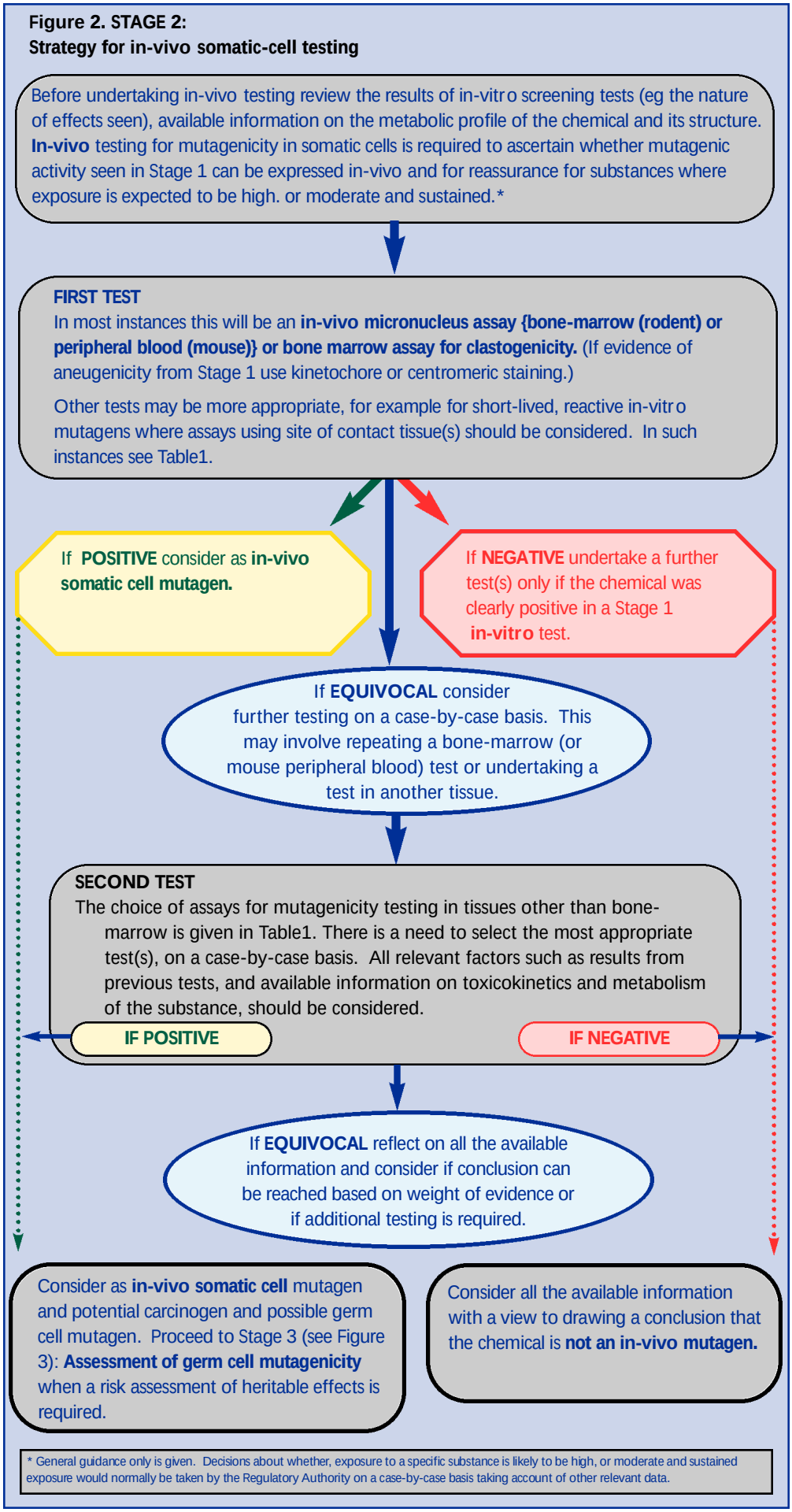
33. The second stage of the testing strategy involves an assessment of activity *in vivo* in somatic cells (see Figure 2). Stage 2 tests are needed for chemicals that are positive in any of the Stage 1 tests so as to ascertain whether mutagenic activity can be expressed *in vivo*. There are numerous reasons why activity shown *in vitro* may not be observed *in vivo* (for example, lack of absorption, inability of the active metabolite to reach DNA, rapid detoxication and elimination). Data from *in-vivo* experiments are therefore essential before any definite conclusions can be drawn regarding the potential mutagenic hazard to humans from chemicals which have given positive results in one or more *in-vitro* tests.

34. In addition, an *in-vivo* test may detect chemicals that only act *in vivo*, although experience has shown that such compounds are rare. Thus data from one *in-vivo* test is appropriate when additional reassurance is needed on the absence of mutagenic potential beyond that provided from the three *in-vitro* tests recommended in *Figure* 1. The Committee recommends that for chemicals where exposure is expected to be high, or moderate and sustained, (eg most human medicines) data from at least one *in-vivo* test are needed.

35. When considering any testing at Stage 2 it is important that a flexible approach is adopted. Consideration needs to be given to the nature of the chemical, the results obtained from initial tests and the available information on the toxicokinetic and metabolic profile of the chemical.

36. The primary objective of the *in-vivo* study is to assess whether the chemical is an *in-vivo* somatic cell mutagen. In the animal studies the routes of exposure should be appropriate to ensure that the substance reaches the target tissue. Thus routes unlikely to give rise to significant absorption in the test animal should be avoided.

Figure 2. STAGE 2:
Strategy for in-vivo somatic-cell testing



37. Most of the available *in-vivo* data on the mutagenicity of chemicals have been obtained from the rodent bone marrow micronucleus test. The bone marrow is readily accessible to chemicals that are present in the blood and a wide range of structurally diverse clastogens has been detected using this tissue. The micronucleus test indirectly detects clastogenicity by measuring micronuclei in newly formed cells in the bone marrow (or peripheral blood). It may be used to identify the induction of both structural and numerical aberrations. Micronuclei containing whole chromosomes (as opposed to fragments) should be identified by use of kinetochore or centromeric staining techniques. It should be noted that aneuploidy produced only by chromosome loss can be measured in the bone marrow micronucleus assay. Although most data are available from bone marrow assays, the use of peripheral blood is an alternative approach when mice are used and this is recognised in the relevant OECD guideline (OECD Test Guideline 474, 1997). The peripheral blood method is not a practical approach in the rat since the spleen removes micronucleated erythrocytes in this species.

38. Clastogenicity may be measured by metaphase analysis in bone marrow of rodents as an alternative approach to the use of the micronucleus test.

39. The Committee considers that in most instances the bone marrow assay will be the appropriate initial *in-vivo* assay, and this should be used unless there is information to suggest otherwise. Either the bone marrow or peripheral blood micronucleus test, or a bone marrow metaphase analysis can be used; in both cases techniques for identification of whole chromosomes are appropriate if evidence of aneugenicity was found in Stage 1. In a few instances, however, the bone marrow assay may not be the most appropriate initial assay, for example with chemicals known to be short-lived reactive mutagens in the Stage 1 assays. In such cases an assay using the site of contact tissue may be more appropriate. The decision needs to be taken on a case-by-case basis having regard to all the relevant information.

40. Anegative result in the first *in-vivo* assay in somatic cells will provide sufficient reassurance for compounds that are negative in the three *in-vitro* assays in Stage 1, and which are being investigated *in-vivo* because of concerns about the extent of human exposure (because they are, for example, human medicines and involve direct human exposure to relatively high levels). In addition, a negative result may be sufficient for those compounds that were equivocal in Stage 1 and for which the *in-vivo* assay is being deployed to resolve this question. In the case of chemicals that are positive in any assay in Stage 1 a negative result in a single tissue will not provide sufficient data to conclude that the chemical is inactive in somatic cells *in-vivo*. Anumber of compounds that are active *in vitro* have been shown to give negative results in the bone marrow micronucleus test, but to give a

positive result on further testing *in vivo* in another tissue eg using the liver UDS assay. Examples are dimethylnitrosamine, 2-nitropropane, 2,4-dinitrotoluene, 3-methyldiaminobenzanthracene and dimethylaminophenylazobenzthiazole (Tweats, 1994). Thus further *in-vivo* data will be needed in somatic cells using different tissue(s).

41. The nature of the additional testing needed should be considered on a case-by-case basis taking into account all relevant information. Consideration needs to be given to the structure of the compound, its metabolism and toxicokinetics, the results from earlier studies and the availability of relevant expertise. There are no widely available routine methods for screening for gene mutagens *in vivo* in mammals. A number of approaches that may provide useful data should be considered. In most cases these have not been developed to a level where there is international agreement on methodologies, the one exception being the assay for unscheduled DNA synthesis (UDS) in the liver. Those assays that warrant consideration for further investigation of compounds negative in the initial *in-vivo* assay are discussed in the following paragraphs. A brief outline of these methods is given in Table 1.

42. The rodent liver UDS assay is an established approach for investigating genotoxic activity in the liver, and for which there is an OECD Guideline (No 486, OECD 1997); there is also advice from the UKEMS (Kennelly *et al* 1993). The endpoint measured is indicative of DNAdamage and subsequent repair in liver cells. Since the liver is usually the major site of metabolism of absorbed compounds this assay is particularly appropriate for investigating compounds that require metabolic activation to express genotoxic activity.

43. The comet (single cell gel electrophoresis) assay is a relatively simple procedure for detecting genotoxicity in any tissue (McGregor and Anderson 1999). The Committee's earlier concerns (COM Annual Report 1995) about the distinction between cytotoxic chemicals and genotoxins have now largely been resolved and much further work has been carried out on the development and validation of the assay. The method is of particular value in evaluating directly acting genotoxins at their initial site of action. Other DNAstrand breakage assays may however also be used as alternatives to the comet assay.

44. There are commercially available transgenic animal models that have the potential for measuring gene mutations *in vivo* in any tissue provided that sufficient DNAcan be isolated. Examples of these models are BigBlue^{TM} and Muta^{TM} Mouse (Schmezer and Eckert 1999). There has been relatively little published work to date on the validation of these assays and further work is needed on optimising methodology for particular tissues. Although the assays are not at the stage when they can be used routinely, they may

provide valuable information as supplementary tests, in particular in investigating mutagenic activity in specific tissues which are often the site of initial contact with the chemical (eg gastrointestinal tract, skin, respiratory tract).

45. In addition there are approaches based on measuring DNA adducts using either ^{32}P-postlabelling or covalent binding to DNA. These measure exposure, uptake and reactivity to DNA rather than mutagenicity, but they are useful in considering mechanisms *in vivo*, in combination with other data. These are considered below.

46. The ^{32}P-postlabelling assay is a sensitive method for measuring DNAadducts (which may or may not produce mutations) and it does not require the test compound to be radiolabelled (Phillips *et al* 1993). The method is complex involving numerous steps including digestion of DNAfollowed by ^{32}P-labelling of adduct nucleoside 3-monophosphates and detection of labelled adducts, for example by chromatography and autoradiography. The sensitivity of the assay may be increased by adduct enrichment techniques which remove normal nucleotides from the digest before ^{32}P-labelling. The choice of enrichment techniques needs justification; knowledge of the type of adduct produced allows tracking to be more sensitive.

47. Another method for measuring DNA adducts is to use radiolabelled compound and measure covalent binding to DNA(Martin *et al* 1993). This is a well established technique, but it does need radiolabelled compounds which are frequently not available for some chemical types. The significance of low level binding observed in this assay is often difficult to interpret since some low level activity measured may not be due to covalent binding to DNA.

48. Thus, there are a number of approaches that can be used when it is necessary to follow up negative results in the initial *in-vivo* somatic cell assay (see Table 1). Identification of the further testing necessary in a specific instance, and whether adequate data are available, will be helped by asking why activity seen *in vitro* was not expressed *in vivo*.

49. Other methods of detecting point mutations are at various stages of development and validation. Such approaches, based mainly on PCR technology, may be appropriate for specific chemicals and exposures (Huber *et al* 1998, Ward *et al* 1998, Jenkins *et al* 1999). Their use to provide supplementary data needs to be considered on a case-by-case basis.

Table 1: *In-vivo* assays for consideration in Stage 2 other than bone-marrow assays

(These assays may also be applicable to the initial evaluation for effects in germ cell)

Assay	Endpoint	OECD guideline	Main Attributes	Comments
Liver UDS	Thymidine incorporation outside S phase	Yes	Long history of use and acceptability by regulatory authorities	Limited use in tissues other than liver. Does not detect mutagenicity resulting from misrepair and non-repair.
Comet assay (or other DNA strand-breakage assays)	DNA strand breaks	No	Can be applied to a large range of tissues, including site-of-contact tissues. Relatively simple to undertake provided that a single cell suspension can be obtained.	May not detect some mutagens (such as those producing bulky adducts). Distinction between apoptosis, necrosis and genotoxicity requires expert judgement.
Transgenic animal models	Point mutations	No	Can be applied to all tissues provided that sufficient DNA can be extracted. Method measures mutations rather than interaction with DNA.	In general less sensitive than methods measuring DNA adducts. Need for further work to optimise protocols for specific tissues.
^{32}P-Postlabelling	DNA adducts	No	Can be applied to all tissues provided sufficient DNA can be extracted. Can be highly sensitive particularly with bulky adducts and if appropriate enrichment technique used.	Interpretation of results can be complex. Involves handling high-activity ^{32}P.
Covalent binding to DNA A variety of methods can be used such as those involving radioactive (eg ^{14}C-) or isotope measurements (eg Accelerator Mass Spectrometry AMS)	DNA adducts	No	Can be applied to all tissues. Some methods (AMS) are potentially very sensitive and can provide data on DNA binding at levels of exposure similar to low level environmental exposures.	Generally need radiolabelled compound (but very small amounts in the case of AMS). Interpretation of results can be complicated (eg by non-specific binding).

Summary: Stage 2 In-vivo assays in somatic cells

50. Stage 2 test(s) are required for compounds that are positive or equivocal in any of the Stage 1 tests to ascertain whether mutagenic activity can be expressed *in vivo*. In addition one appropriate *in-vivo* test is needed for all compounds where high, or moderate and prolonged, levels of exposure are expected, for example some human medicines.

51. It is important that a flexible approach is adopted for any testing strategy at this stage. Consideration needs to be given to the nature of the chemical, the results obtained in the initial tests, and also the available information on the metabolism of the chemical.

52. The most appropriate initial test will usually be a bone marrow micronucleus assay to measure clastogenicity and aneugenicity, unless initial considerations give an indication to the contrary. Techniques for the assessment of effects on whole chromosomes are appropriate if evidence of aneugenicity was found in Stage 1.

53. If negative results are obtained in the initial *in-vivo* test, using compounds that were considered positive *in vitro*, then additional testing will be required using other tissue(s) before definite conclusions can be drawn regarding the absence of activity *in vivo*. [This will however not be the case if only equivocal results were obtained in the *in-vitro* assay, and the *in-vivo* assay is being used to resolve its mutagenic potential.] The type of study (or studies) necessary must be considered on a case-by-case basis having regard to the chemical structure, its metabolism, the expertise available and results from earlier tests. The process will be helped by a plausible explanation as to why activity seen *in vitro* may not be expressed in the whole animal. Anumber of types of study are available and these are listed below; the reasons for the choice of test in a given situation should be provided.

- **Measurement of induction of DNA lesions ie measure of exposure, uptake and reactivity to DNA**
  - Comet assay
  - ^{32}P-Postlabelling assay
  - Covalent binding to DNA

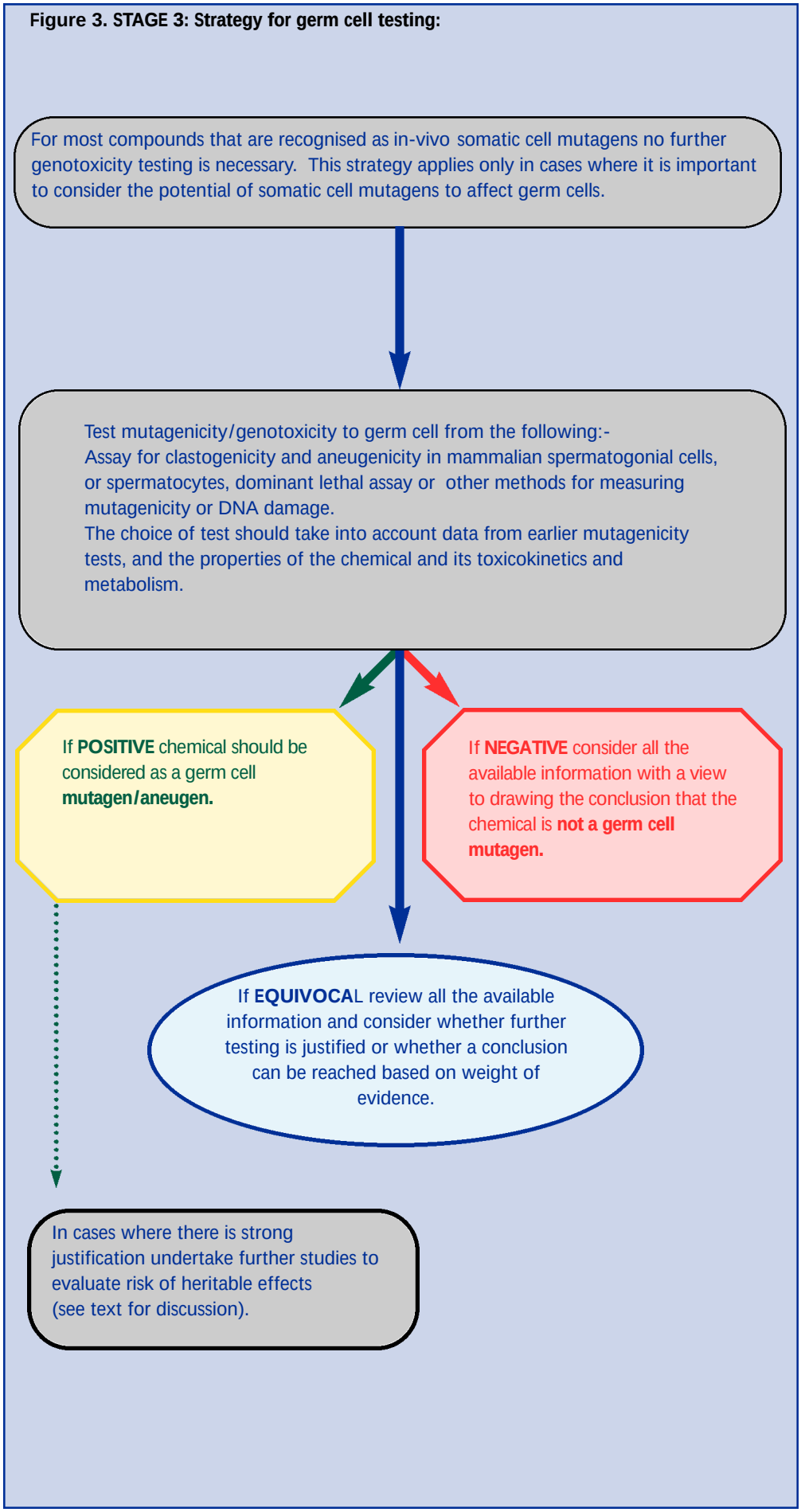
- **Measurement of the repair of DNA lesions**
  - Liver UDS
- **Measurement of induction of genetic changes**
  - Transgenic assay for point mutations
  - Chromosomal aberrations

Stage 3: Germ cell assays

Introduction

54. During the initial stages of investigating the mutagenic hazard posed by a chemical there is no need to screen for germ cell mutagens. Thus far all established germ cell mutagens have been shown also to produce positive results in bone marrow assays and there is no current evidence for germ-cell-specific mutagens (Shelby 1996). However, the reverse is not true. Not all somatic cell mutagens can be demonstrated to be germ cell mutagens. Data on the mutagenic effects in germ cell DNA are needed before any definite conclusions can be drawn relating to the mutagenic hazard of a chemical specifically to germ cells. However, no further genotoxicity testing will be needed for most compounds that are recognised as *in-vivo* somatic cell mutagens since they will be assumed to be both potential genotoxic carcinogens and potential germ cell mutagens. In some cases germ cell studies will be undertaken for the specific purpose of demonstrating whether an *in-vivo* somatic cell mutagen is, or is not, a germ cell mutagen. If it is important to consider the potential of somatic cell mutagens to affect germ cells a strategy for testing is outlined in Figure 3.

Figure 3. STAGE 3: Strategy for germ cell testing:



Studies to provide information on genotoxicity to germ cells

55. When identifying the most appropriate studies consideration should first be given to the type of genetic effect seen in the earlier studies namely point mutations, clastogenicity or aneugenicity. This will be important in identifying the most appropriate study (or studies) in a given instance. In general, the available methods involve measuring effects in the gonads of male rodents.

56. Methods for investigating clastogenicity in mammalian spermatogonial cells are well established. There is an internationally recognised guideline OECD No 483 for this approach (OECD 1997). The use of transgenic animals offers the potential for investigating mutagenic effects in germ cells. Aneugenic and clastogenic effects may be detected by measuring micronuclei induction in spermatocytes using appropriate staining methods. Information on the induction of DNA lesions in germ cell DNAmay be obtained from the methods considered in Stage 2 (^{32}P-postlabelling assays, comet assay, UDS, covalent binding to DNA).

57. The dominant lethal assay may also be used to investigate clastogenicity or aneugenicity in germ cells (Holstrom *et al* 1993). There is an internationally recognised guideline, OECD No 478, for this assay (OECD 1984). For this method the endpoint is the production of embryo-lethal genetic changes measured as death of the conceptus as a blastoma or soon afterwards. Dominant lethal mutations are believed to be primarily due to structural or numerical chromosome aberrations. There are essentially two different dosing regimes that may be used in this assay. In one case this involves repeated dosing of the males for a period covering spermatogenesis followed by mating with untreated females and examining the latter for dominant lethals after an appropriate period of gestation. In the other regime single dosing is followed by sequential mating of females. The latter provides information on the various stages of the germ cell cycle that may be affected but uses very many more animals and the need for this additional information is rarely justified.

Quantitative assessment of risk of heritable effects in future generations

58. The only methods available to provide data that allow such risk assessments to be carried out involve investigating effects in subsequent generations bred from treated animals. The methods available are the mouse heritable translocation test and the mouse specific locus test. In view of the very large number of animals that are needed these studies (particularly in the case of

the mouse specific locus test) are not a practical option and should only be used in exceptional cases. Furthermore neither of these assays has been carried out in the rat (nor is this possible in the case of the specific locus test). Currently no methods are available for investigating the induction of aneuploidy in offspring, following exposure of parental animals.

Summary Stage 3: Germ Cell Assays

59. The need to investigate effects in germ cells requires careful consideration. For most chemicals recognised as *in-vivo* somatic cell mutagens no further genotoxicity testing is necessary since they will be assumed to be potential genotoxic carcinogens and potential germ cell mutagens. However, in some specific cases germ cell studies may be undertaken to demonstrate whether a somatic cell mutagen is, or is not, not a germ cell mutagen. In those cases where it is important to obtain conclusive information on effects in germ cells the following approach should be followed.

60. Information as to whether the compound is genotoxic in germ cells can be obtained from a number of assays. These include metaphase analysis of spermatogonia (for clastogenicity) or micronuclei induction in spermatocytes (clastogenicity and aneugenicity) and the dominant lethal assay (clastogenicity and aneugenicity). Alternatively the transgenic animal models may be used to investigate mutations in germ cells. Information on exposure, uptake and reactivity to germ cell DNA may be provided by investigating DNAdamage or adduct formation using various approaches (as described for the Stage 2 studies). Consideration of the types of mutation seen in the initial tests will be important when deciding on an appropriate assay in a given instance. None of these assays provide conclusive information as to whether the effects seen are heritable in future generations.

61. The only approaches that provide data that allow estimates of risks of heritable effects are of the mouse specific locus test and the mouse heritable translocation test. In view of the very large number of animals used, these are not a practical options and should only be used in exceptional circumstances.

OVERALL SUMMARY

The strategy being recommended, as in the Committee's earlier guidance, is based on three progressive stages.

Stage 1 (initial screening – see Figure 1) is based on *in-vitro* tests. For most chemicals three tests are recommended, but for those where little or no human exposure is expected (eg industrial intermediates, some low production volume industrial chemicals) two tests may be appropriate, namely a bacterial assay for gene mutation and an *in-vitro* mammalian cell assay for clastogenicity and aneugenicity. The Committee believes that screening for both clastogenicity and aneugenicity is now possible in the initial (Stage 1) tests. The second test may be metaphase analysis, with consideration of hyperdiploidy, polyploidy and effects on mitotic indices as indicators of possible aneugenicity; if these suggest potential aneugenicity this needs to be confirmed by use of appropriate staining procedures, such as FISH and chromosome painting. Alternatively an *in-vitro* micronucleus test may be used. If a positive result is obtained, kinetochore or centromeric staining should be employed to ascertain the nature of the micronuclei induced (ie whether induction is due to clastogenicity or aneugenicity). The third assay is an additional gene mutation assay in mammalian cells, the mouse lymphoma assay being recommended. These three assays, if negative, will provide sufficient information for the assessment of most chemicals. However where high, or moderate and prolonged, levels of exposure are expected (eg most human medicines) an *in-vivo* assay is recommended to provide additional reassurance. Decisions on the extent of testing appropriate for given exposure levels of specific chemicals need to be taken by the relevant regulatory authority on a case-by-case basis.

Stage 2 (see Figure 2) involves an assessment of whether genotoxic activity seen in any of the *in-vitro* tests can be expressed in somatic cells *in vivo*. In addition, one appropriate *in-vivo* test is needed for all chemicals for which human exposure is expected to be high, or moderate and prolonged. Aflexible approach is needed with consideration of the nature of the chemical, its metabolism and results obtained in the initial *in-vitro* tests. The most appropriate initial test will be a bone marrow micronucleus assay unless the initial considerations give an indication to the contrary. Techniques for the assessment of whole chromosomes are appropriate if there is evidence of aneugenicity. If negative results are obtained in this assay additional testing in other tissue(s) will be required for all compounds that are positive *in-vitro*, to provide adequate reassurance for the absence of activity *in-vivo*. The type of study (or studies) needs to be considered on a case-by-case basis having regard to the available information on the compound including the results from earlier tests. Studies that may be appropriate include the liver UDS assay, comet assay, ^{32}P-postlabelling assay, covalent binding to DNA and assays using transgenic animals; the reasons for the choice of assay in a given situation should be given.

Stage 3 (see *Figure* 3) consists of assays in germ cells. The need for such studies requires careful consideration. In most cases chemicals that are recognised as *in-vivo* somatic cell mutagens will be assumed to be both potential genotoxic carcinogens and potential germ cell mutagens, and no further genotoxicity testing is necessary. However, in some cases germ cell studies may be undertaken to demonstrate that a somatic cell mutagen is not a germ cell mutagen. Information on whether a compound is genotoxic in germ cells may be obtained from a number of assays (eg metaphase analysis in spermatogonia or micronuclei induction in spermatocytes, the dominant lethal assay and mutation assays in transgenic animals). Information on the induction of DNAlesions in germ cells may be obtained using the various approaches listed for phase 2. Consideration of the type of mutation produced in earlier studies is important when selecting the appropriate assay in a given instance. None of these assays provide conclusive information as to whether effects will be seen in future generations, and the only methods on which risk estimates for the effects can be based are the heritable translocation test and the mouse specific locus test. These are not practical options in view of the very large number of animals needed. Currently there are no routine methods available for investigating the induction of aneuploidy in offspring following exposure of parental animals.

APPENDIX A

Asuitable procedure for use of the *in-vitro* micronucleus test for detection of clastogenicity and aneugenicity

i) Undertake an *in-vitro* micronucleus assay in which binucleate cells are produced by treatment with the actin inhibitor Cytochalasin B in interphase cells of a type with a stable karyotype. Prepare duplicate cell suspensions.

ii) Score binucleate cells for the induction of micronuclei: if negative then consider the test substance as non-clastogenic and non-aneugenic in this test: if positive consider as potential clastogen or aneugen.

iii) If positive in (ii) stain second set of cells for presence of centromeric DNAor kinetochore proteins. If there is an increase in centromeric negative micronuclei then the compound is considered a clastogen. If there is an increase in centromeric positive micronuclei then the compound is considered an aneugen.

In most cases no further testing will be necessary. However, if the investigation relates to the identification of thresholds (rather than just identifying the chemical as an aneugen) then it is advisable that the dose response for the induction of non-disjunction is determined as aneugenic chemicals may induce non-disjunction at concentrations lower than those which induce chromosome loss. In these circumstances a further modification of the suggested *in-vitro* micronucleus assay may be undertaken as follows:

Treat a third set of cells with chromosome specific centromere probes. Analyse the distribution of chromosomes in binucleate cells to quantify the frequency of chromosome non disjunction where the sum of the signals for each chromosome equals 4.

References

Aardema MJ, Albertini S, Arni P, Henderson LM, Kirch-Volders M, Mackay JM, Sarrif DA, Stringer DA, Taalman RDF (1998). Aneuploidy: a report of an ECETOC task force. Mutation Research **410**, 3-79.

Ashby J (1988). The continuing search for an *in-vivo* mutagen which is non genotoxic *in vitro*. Mutagenesis **3**, 103-104.

Ashby J and Paton D (1993). The influence of chemical structure on the extent and sites of carcinogenesis for 522 rodent carcinogens and 55 different human carcinogen exposures. Mutation Research **286**, 3-74.

Ashby J, Waters MD, Preston J, Adler ID, Douglas GR, Fielder RJ, Shelby MD, Anderson D, Sofuni T, Gopalan HNB, Becking G, Sonich-Mullin C (1996). IPCS harmonisation of methods for the prediction and quantification of human carcinogenic/mutagenic hazard, and for indicating the probable mechanism of action of carcinogens. Current Issues in Mutagenesis and Carcinogenesis, No 67. Mutation Research **352**, 153-157.

Cole J, Harrington-Brock K and Moore M (1999). The mouse lymphoma assay in the wake of ICH4 – where are we now? Mutagenesis **14**, 265-270.

COM Annual Report (1995). The Comet Assay, Department of Health, London pp 39.

Doherty AT, Ellard S, Parry EM and Parry JM (1997). A study of the aneugenic activity of trichlorfon detected by centromere-specific probes in human lymphoblastoid cell lines. Mutation Research **372**, 221-232.

DH (1989). Report on Health and Social Subjects, 35. Guidelines for the testing of chemicals for mutagenicity. Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment. London, HMSO.

Gatehouse DG, Rowland IR, Wilcox P, Calander RD and Forster R (1990). Bacterial Mutation Assays in Basic Mutagenicity Tests. UKEMS Recommended Procedures. D Kirkland (ed) UKEMS Report. Cambridge University Press pp 13-61.

Gibson DP, Aardema MJ, Kerckaert GA, Carr GJ, Brauninger RM, Le Boeuf RA (1995). Detection of aneuploidy-inducing carcinogens in the Syrian hamster embryo (SHE) cell transformation assay. Mutation Research **343**, 7-24.

Glatt H, Staffa-Piée A, Enders N, Baidossi W, Blum J (1994). The presence of KCl in the exposure medium strongly influences the mutagenicity of metabolites of polycyclic aromatic hydrocarbons in *Escherichia coli*. Mutation Research **324**, 111-114.

Hiramoto K, Nasuhara A, Michikoshi K, Kato T, Kikugawa K (1997). DNA strand-breaking activity and mutagenicity of 2,3-dihydro-3,5-dihydroxy-6-methyl-4-H-pyran-4-one (DDMP), a Maillard reaction product of glucose and glycine. Mutation Research **395**, 47-56.

Holmstrom LM, Palmer AK, Favor J (1993). The rodent dominant lethal assay. In Supplementary Mutagenicity Tests. UKEMS Report, Cambridge University Press pp 129-156.

Huber KB, Bittner J, Bauer K, Trumper L, Sek P, Sebesta C, Rosen H, Tagl KH (1998). Restriction digest PCR (RD-PCR) for the analysis of gene mutations. Application to *K-ras*. Clinical Chemical Laboratory Medicine **36**, 593-595.

IARC (1987). International Agency for Research in Cancer (IARC) monographs on the evaluation of the carcinogenic risks to humans. Supplement 6. Genetic and related effects: an updating of selected IARC monographs from volume 1-42. Arsenic and Arsenic Compounds, IARC, Lyon pp 71-76.

ICH International Conference on Harmonisation of technical requirements for the registration of pharmaceuticals for human use (1997). Genotoxicity. Astandard battery for genotoxicity testing of pharmaceuticals In, Proceedings of the Fourth International Conference on Harmonisation (ICH), D'Arcy PF, Harron DWG (eds) Brussels 1997 Queens University Press Belfast pp 987-997.

Jenkins GJS , Suzen HS, Sueiro RAand Parry JM (1999). The restriction site mutation assay: a review of the methodology development and the current status of the technique. Mutagenesis **14**, 439-448.

Kalweit S, Nowak C, Obe G (1990). Hypotonic treatment leads to chromosomal aberrations but not to sister chromatid exchanges in human lymphocytes. Mutation Research **245**, 5-9.

Kennelly JC, Water R, Ashby J, Lefevre PA, Burlinson B, Benford DJ, Dean SW, Mitchell I deG (1993). *In-vivo* rat liver UDS assay. In Supplementary Mutagenicity Tests. UKEMS Report. Cambridge University Press pp 52-77.

Martin CN, Lawley PD, Phillips D, Venitt S, Waters R (1993). Measurement of covalent binding to DNA *in vivo*. In Supplementary Mutagenicity Tests. UKEMS Report, Cambridge University Press pp 78-104.

McGregor DB, Rice JM and Venitt S (eds) (1999). The use of short and medium term tests for carcinogens and data on genetic effects in carcinogenic hazard evaluation. IARC Scientific Publication No. 146, (536 pages) Lyon.

McGregor DB and Anderson D (1999). DNA damage and repair in mammalian cells *in vitro* and *in vivo* as indicators of exposure to carcinogens. In, The use of short and medium term tests for carcinogens and data on genetic effects in carcinogenic hazard evaluation. McGregor DB, Rice JM, Venitt S (eds) IARC Scientific Publication No. 146, Lyon pp 309-354.

Moore MM, Collard DD and Harrington-Brock K (1999). Failure to adequately use positive control data leads to poor quality mouse lymphoma data assssments. Mutagenesis **14**, 261-263.

Morita T (1995). Low pH leads to sister chromatid exchanges and chromosomal aberrations, and its clastogenicity is S-dependent. Mutation Research **334**, 301-308.

Müller L, Kikuchi Y, Probst G, Schechtman L, Shimada H, Sofuni T and Tweats D (1999). ICH-Harmonised guidances on genotoxicity testing of pharmaceuticals: evolution, reasoning and impact. Mutation Research **436**, 195-225.

Nowak C (1990). Chromosomal aberrations in V79 hamster cells induced by hyperosmotic solutions of sodium chloride. Mutation Research **230**, 227-234.

OECD (1984). Organisation for Economic Co-operation and Development. Guidelines for Testing Chemicals No 478. The Dominant Lethal Assay, Paris.

OECD (1997). Organisation for Economic Co-operation and Development. Ninth addendum to the OECD guidelines for the testing of chemicals. Update of Section 4, Health Effects. Revised guidelines 471, 473, 474, 475, 476, 483, 486. Paris.

Oshimura M and Barrett JC (1986). Chemically induced aneuploidy in mammalian cells. Mechanisms of biological significance in cancer. Environmental Molecular Mutation **8**, 129-158.

Parry JM and Sors A (1993). The detection and assessment of the aneugenic potential of environmental chemicals: the European Community Aneuploidy Project. Mutation Research **287**, 3-15.

Parry JM (ed) (1996). Molecular Cytogenetics. Mutation Research Special Issue **372**, 151-294.

Phillips DH, Castegnaro M, Bartsh H (eds) (1993). Postlabelling methods for detection of DNA adducts. IARC Scientific Publication No 124, (396 pages) Lyon.

Schmezer P, Eckert C (1999). Induction of mutations in transgenic animal models. BigBlue™ and Muta™ Mouse. In, The use of short and medium term tests for carcinogens and data on genetic effects in carcinogens hazard evaluation. McGregor DB, Rice JM, Venitt S (eds) IARC Scientific Publication No. 146, Lyon, pp 367-394.

Shelby MD (1996). Selecting chemicals and assays for assessing mammalian germ cell mutagenicity. Mutation Research **352**, 159-169.

Tweats DJ (1994). Follow up testing for *in-vitro* positives. In Proceedings of the Second International Conference on Harmonisation (ICH). D'Arcy PF, Harron DWG (eds), Orlando 1993, Queens University Press, Belfast, pp 240-244.

Tweats DJ and Gatehouse DG (1988). Further debate on testing strategies. Mutagenesis **3**, 95-102.

UKEMS (1989). Statistical evaluation of mutagenicity test data. UKEMS sub-committee on guidelines for mutagenicity testing. Report. Part III. Kirkland DJ. (ed) Cambridge University Press.

UKEMS (1990). Basic mutagenicity tests: UKEMS recommended procedures. UKEMS sub-committee on guidelines for mutagenicity testing. Report. Part I revised. Kirkland DJ. (ed) Cambridge University Press.

UKEMS (1993). Supplementary mutagenicity tests: UKEMS recommended procedures. UKEMS sub-committee on guidelines for mutagenicity testing. Report. Part II revised. Kirkland DJ and Fox M. (eds) Cambridge University Press.

Waters MD, Stack HF and Jackson MA(1999). Short term tests for defining mutagenic carcinogens. In: McGregor DB, Rice JM, Venitt S (eds.) The use of short- and medium-term tests for carcinogens and data on genetic effects in carcinogenic hazard evaluation. IARC Scientific Publications No. 146. IARC, Lyon, pp 499-536.

Ward R, Hawkins N, O'Grady R, Sheehan C, O'Conner T, Impey H, Roberts N, Fuery C, Todd A (1998). Restriction endonuclease-mediated selective polymerase chain reaction. A novel assay for the detection of *K-ras* mutations in clinical samples. American Journal of Pathology **153**, 363-366.

Zeiger E, Ashby J, Bakale G, Enslein K, Klopman G, Rosenkranz HS (1996). Prediction of Salmonella mutagenicity. Mutagenesis **11**, 471-484.

APPENDIX B

List of Members

Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM)

Appointments are for a three year period from 1st April 2000 to 31st March 2003.

CHAIR

Professor Jim M Parry BSc PhD DSc
Professor of Genetics. University of Wales Swansea
(reappointed till 24.10.01)

MEMBERS

Professor John Ashby BSc PhD CChem FRCS
Senior Research Associate. Mutagenesis, Carcinogenesis and endocrine disruption.
Syngenta, Central Toxicology Laboratory

Dr Julie Clements BSc PhD
Head of Molecular Toxicology, Covance

Professor Colin Cooper BSc PhD DSc
Head of Molecular Carcinogenesis Section.
Institute of Cancer Research, Haddow Laboratories

Professor Peter B Farmer MADPhil C.Chem FRSC
Professor of Chemistry, MRC Toxicology Unit

Dr Nigel J Gooderham PhD C.Chem FRSC
Senior Lecturer in Molecular Toxicology,
Division of Biomedical Sciences,
Imperial College of Science, Technology and Medicine

Ms Margaret Langley BA
Lay member

Dr Ian Mitchell BAPhD
Consultant in Genetic and Molecular Toxicology,
Kelvin Toxicology Associates

Professor David H Phillips BAPhD DSc FRCPath
Professor of Environmental Carcinogenesis,
Institute of Cancer Research

Professor David J Tweats BSc PhD CBiol FIBiol FRCPath
Director of Preclinical Safety Sciences.
Glaxo Wellcome Research & Development Ltd

SECRETARIAT

R J Fielder BSc PhD Dip RCPath (Scientific)
K N Mistry (Administrative)
J M Battershill BSc MSc (Scientific)