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1 INTRODUCTION

The project aims at a clarification of the capability of various molecular-biological investigations/techniques to contribute to a cancer risk assessment of environmental chemicals.

The design of a project with such purposes requires a number of definitions of concepts used and specifications as to wanted developments.

Cancer disease and cancer death may be defeated *either* by prevention of its origination ("genesis") *or* by cure of individuals that have contracted the disease.

For setting priorities with respect to allocation of resources for disease prevention it is primarily important to have knowledge of the risks associated with given exposures to environmental factors such as specific chemicals or mixtures of chemicals. The term risk is then used in the meaning : probability of contracting the disease (cf. ICRP, 1991). This *individual risk* concerns primarily an individual of average susceptibility. By integration over the exposed population the *collective risk*, can be calculated to indicate the expected number of cases of disease or death due to an exposure considered. It is obvious that, in dependence of the size of the exposed population, *either* the individual risk *or* the collective risk may be of decisive importance to risk-limiting intervention.

There are, further, a number of biochemical and molecular-biological methods that are able to tell whether an individual's susceptibility to carcinogens is above or below the population average and that may ultimately be able to quantify this deviation from the average. This kind of information may be useful for the individual's decision to avoid certain factors such as tobacco smoking or particular foods. It may, on the other hand, be less wanted if it is misused, e.g. in the selection of people for employments.

Some other observations may be taken to indicate that a person, at least in particular exposure situations, runs a cancer risk that approaches 100%.

The step is short from such observations to measurements that may be taken as preclinical indications of a developing tumour, i.e., in many cases with improved prognosis of curative measures.

The project has primarily dealt with the first-mentioned problem, viz., the estimation of average cancer risks in a population with given variation in susceptibility, problems of individual susceptibility being at this stage treated more summarily.

The following points (a-e) were considered essential in the planning and accomplishment of the work.

(a) Shortcomings of methods in use and need for improvements. - Disease-epidemiological methods are characterized by low power, the detection level corresponding in most cases to a 50-100 % increase above the background level (i.e., for a general initiator more than three orders of magnitude above the level where a risk might be considered acceptably low); by long latency times, years to decades for leukaemias and solid tumours, respectively (with the consequence that preventive measures, even if they are set in immediately after detection, are unable to prevent already progressing tumours from leading to disease); low ability to identify causative agents (because 'confounding factors' mostly obscure the exposure situation, the tumour as observed endpoint being unspecific to its cause); difficulties of correctly assessing the magnitude of the risks (at least for mutagens this would require that exposed populations are followed up for the rest of the life, as has been indicated to be the case for ionizing radiation[1]).

- Biomonitoring by genetic endpoints (mutation, chromosomal aberrations in somatic cells) is able to overcome the long latency times but suffers, like disease-epidemiology, from low power[2] and low ability of identifying causative factors. (In these respects mutational spectrometry by means of combined PCR - melting gradient gel electrophoresis under development[3] seems to

present a solution, however)

- Short-term tests are, in principle, able to detect genotoxic properties of a chemical, i.e. to disclose, with a high sensitivity, whether a chemical is able to act as a cancer initiator. Alone, such tests cannot, however, generate data able to form the basis of a risk quantification.

(b) Dose-response relationships. - For risk estimation, dose-response relationships for the actions of carcinogens have to be known. Particularly, this is important at the low—very low doses which often occur in the general environment, but where for statistical reasons informative observations cannot be obtained. In this dose/concentration range risks have to be estimated through extrapolation from observations at higher doses. Mathematical models for such extrapolation should be based on theory for the mechanisms involved. Although the knowledge of the mechanisms of carcinogenic processes is still incomplete, it may allow a description in operative terms including conclusions about the kinetics that are determinants of dose-response relationships[4].

(c) Environmental carcinogens have, in the past, been interpreted to mean carcinogenic agents, particularly man-made ones, to which humans are exposed via different routes. Since several carcinogens are generated endogenously[5], and since the origination of a tumour has to be understood as a consequence of interactions between exogenous and endogenous chemicals and pro- or anti-carcinogenic conditions, which may be heritable or acquired, in the tissues, environment has to be defined in a broad sense to comprise also dietary and other living habits that may affect these conditions[6].

(d) Risk assessment - a multipronged process.- It has further to be recognized that at present no single method is able, when used alone, to generate data for risk estimation adequate for decision-making. Risk assessment has to be a multi-pronged process, and the lack of recognition of this necessity, expressively described by Barr[7] as the 'overcompartmentalization of the risk assessment arena', is certainly a major cause of short-comings in efforts of estimating cancer risks from individual chemicals.

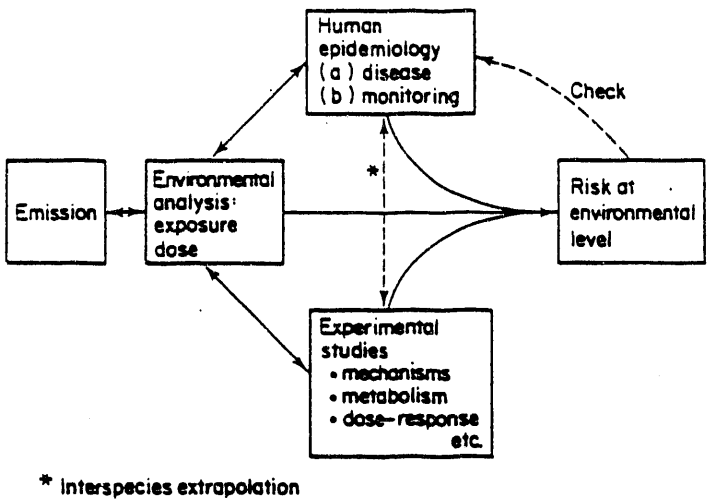


Fig. 1. Sources of information (cf. ref. 50)

Important cornerstones of the risk assessment process are sketched in Fig. 1. It should be noted that exposure assessment - and, for source apportioning, emission assessment - plays the twofold role as a basis for the epidemiological studies as well as for the exposure of the population of concern. Although the disease-epidemiological studies can only exceptionally generate data useful for risk estimation they may be applied to obtain an upper limit of a possible risk and thus serve as a check of the reasonableness of an estimated risk and contribute to the elimination of the impact of overestimates on the regulatory machinery.

(e) Mechanisms of carcinogenic processes. -As said above, knowledge of the

mechanisms of carcinogenic processes is essential, particularly for the proper modelling of dose-response and dose-risk relationships. For that reason research on mechanisms, which is at present mostly qualitative, should acquire a quantitative angle of approach, in exchange of experience with research aiming at risk assessment. Research on mechanisms is expected to continue for a long time generating data that successively will increase the reliability of procedures to risk estimation, in some cases simultaneously changing our interpretation of observed phenomena. Estimated risks should therefore not be considered final but should be left open for such improvements in accuracy and reliability.

2 DESIGN OF STUDY

The contact person at the Department of Energy, Dr. Paul Duhamel, expressed the wish that the usefulness of molecular/biochemical techniques for risk estimation of environmental chemicals should be evaluated. Besides a validation of the relative potency method based on radiation-dose equivalence of chemical dose (the 'rad-equivalence approach'), the possibilities of basing risk estimation on specific data for chemicals per se should be investigated.

By and large this project was suggested to be theoretical, with limited experimental input. This had consequences of two kinds, affecting the design of the study. First, the work would have to be directed towards an identification of problems, with an emphasis on the potential ability of molecular/biochemical methods to reach a solution, rather than aiming at solutions of the problems. Secondly, the work became dependent on certain experimental work within parallel projects. Initially, projects running at this laboratory were strongly tied up with practical matters, such as the development of monitoring methods for specific exposures, with limited resources for basic research. However, from 1990 a study on 'Validation of the rad-equivalence theory' is being sponsored by Shell Internationale Research Maatschappij B.V., The Hague, and from autumn 1991 a (small)

contribution from the European Community permits related work. Coordinator of the latter project, dealing with molecular dosimetry, is Prof. E. Vogel, Dept. of Radiation Genetics and Chemical Mutagenesis, University of Leiden, the Netherlands.

As sketched in the scientific report below (section 4) the meaningfulness of molecular/biochemical methods and their potential contribution to the problem of risk estimation has to be seen against a broad overview of this problem and current efforts to solve it. This overview, given as a brief summary in section 3, shows the necessity of combining different fields of research, holding them together by strictly quantitative aspects. For this reason it was essential to engage a mathematical statistician with the project (see further section 4.8).

In several of the papers and reports so far produced within the project (listed in section 5.2 below) this quantitative aspect may be seen as a major contribution from the present project. A few manuscripts dealing more directly with fundamental problems are still in preparation .

3 SCIENTIFIC BACKGROUND OF THE STUDY; BRIEF REVIEW, DEFINITION OF CONCEPTS

3.1 DOSE-RESPONSE RELATIONSHIPS AND INFLUENCE OF DOSE RATE

In this report the following denotations of dose ranges, as illustrated in Fig. 2, will be used (cf. ref. 4):

I Low doses: The range where, for statistical reasons, information on the response is not obtainable in studies of conventional scope.

II Intermediate doses: The range where the response (mutation frequency, tumour incidence) is mostly compatible with a linear dependence on dose.

III High doses: The range where the dose-response curves often bend upwards, with a power of the dose >1.

IV Very high doses: The range where dose-response curves often descend with increasing dose.

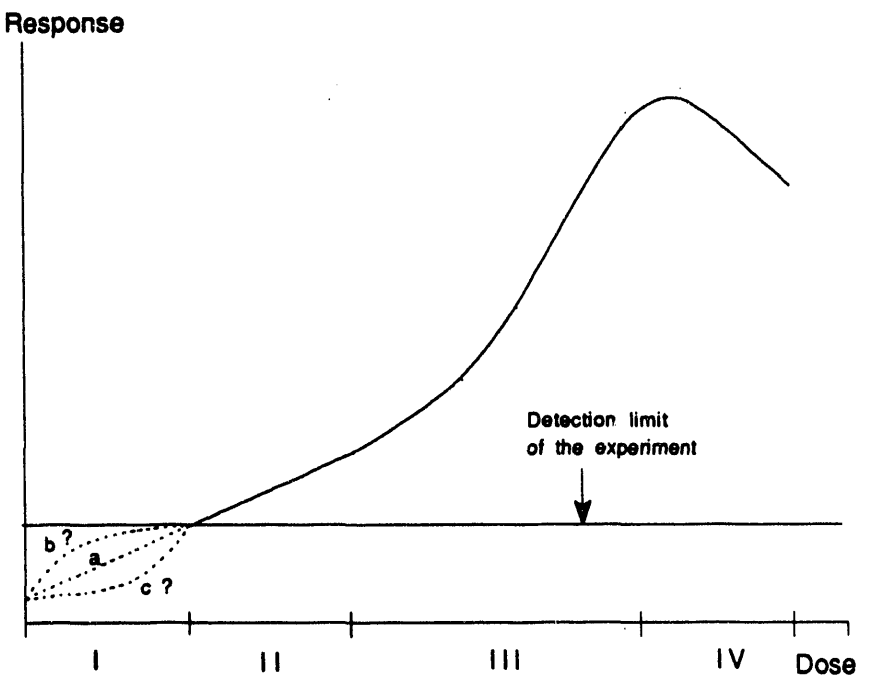


Fig. 2. Discussed regions of dose-response relationships

Most experimental and epidemiological data are obtained in the ranges II and III, whereas maybe the predominant number of exposures of humans occur at low doses (range I). The drop of the response in range IV may be described as a "curative" or "therapeutic" effect, mutated or pre-tumour cells being eliminated by the cell-killing effect at high doses.

Whereas the dose, as said above, should be seen as the primary determinant of the response, the dose rate, i.e. the intensity factor (dose received per unit of time) may have modifying influences of different kinds. This has so far not been well studied for chemicals but the curve for the mutagenic action in mice of low-LET radiation (which will be discussed below) may serve as an illustration (Fig. 3). It is evident that below some low dose limit, the dose rates at which the doses are received will also be low.

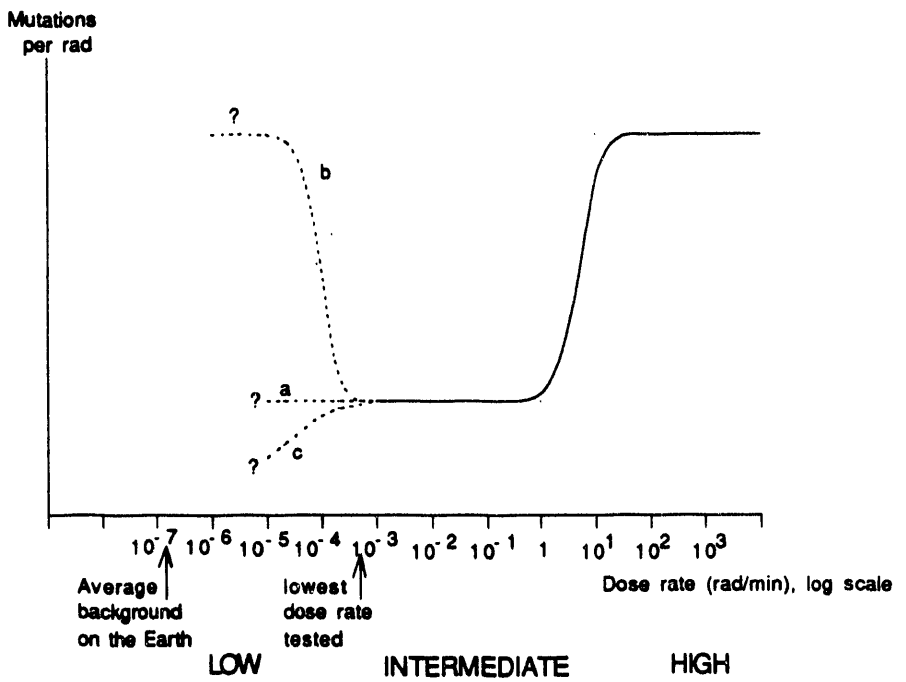


Fig. 3. Illustration of the effectiveness of low-LET radiation at different dose rates

In situations with intermittent (fractionated) exposure, the classification of the overall exposure becomes a question of the magnitude of dose and dose rate in the fractions.

Most radiobiological experiments, with doses in the range of 100-1000 rad (1-10 Gy) have for practical reasons been carried out at high dose rates.

At the lowest dose rate so far tested in mutation studies in mice, a dose of 100 rad requires 3 months of continuous irradiation.

Quite arbitrarily we may also subdivide the range of dose rates into low, intermediate and high. (Curiously enough very high dose rates, e.g. the prompt dose from an A-bomb explosion, with 100 rad delivered within 10^{-8}-10^{-7} sec, becomes less effective. This is, however, due to a mechanism — recombination of OH radicals[8] — unrelated to the drop in range IV of Fig. 2).

3.2 DEFINITION OF DOSE AND DOSE-RATE CONCEPTS

Dose - The definition of the dose of a reactive chemical or metabolite (here generally denoted RX) should be suitable for work aiming at risk assessment and translatable to various dose definitions in use (absorbed dose per kg body weight, exposure dose in air during worktime, etc.). As was early realized, and as was discussed in detail by Ehrenberg et al.[4], dose (D) is generally best defined by the integral over time of concentration, [RX], of reactive compound or metabolite (Eqn. 1, also illustrated in Fig. 4):

$$D = \int_t [RX](t)\, dt \qquad (1)$$

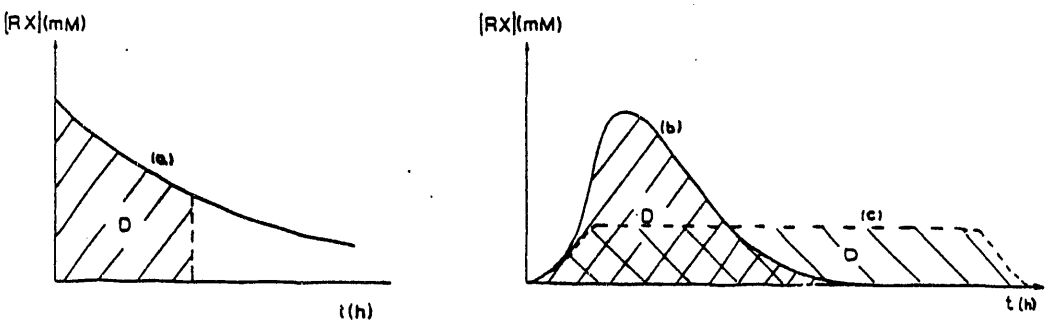


Fig. 4. Target dose of acute exposure to a reactive compound in an in vitro experiment (a); target dose in vivo of a reactive metabolite from a precursor (A): (b) acute exposure, (c) protracted exposure.

The dimension of dose will hence be concentration×time, in SI units

mol·dm^{-3}·s; Since experimental concentrations are mostly on the order of millimol per liter (millimolar, mM) and the duration of exposure on the order of hours, the unit millimolar-hour (mMh) was found to be a practical measure of dose.

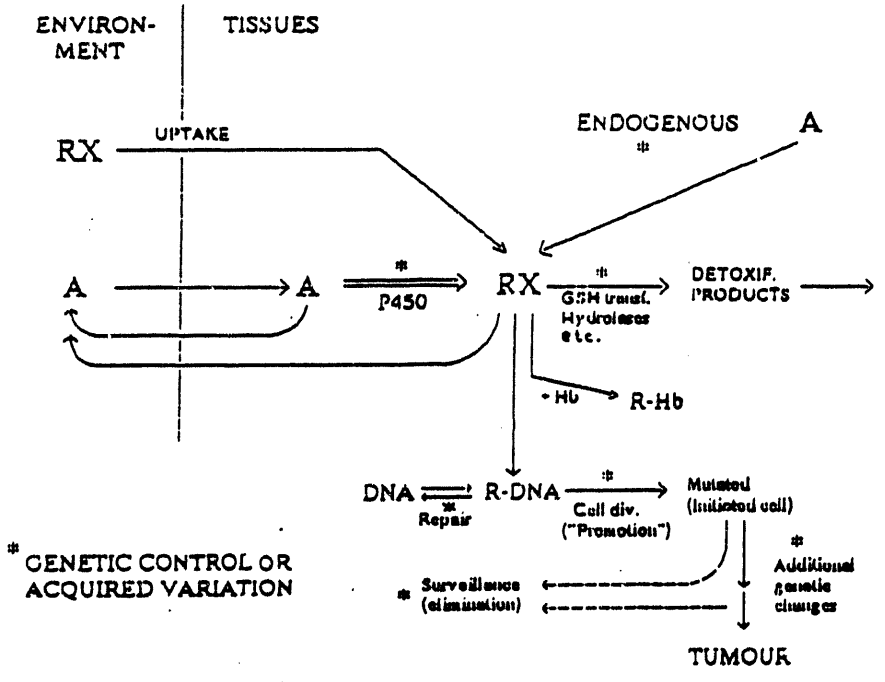


Fig. 5. Different mechanisms influencing the cancer risk.

Correspondingly, the exposure dose of an air pollutant may be given in ppm-hours (ppmh) or mg·m^{-3}·h. It can be shown[4] that, as long as the various steps in the metabolic machinery (Fig. 5) remain unchanged during the course of the exposure, a tissue dose, D_{tis}, as defined above and D_{exp} will be proportional:

$$D_{tis} = \alpha D_{exp}$$

Under the same conditions also an absorbed dose (e.g. following injection, ingestion, etc.), given in mg·(kg body weight.)$^{-1}$ or similar, will be

proportional to D_{tis}. Although this dose concept is, by definition, a concentration, it is so often used that it will be retained as a measure of dose.

When an electrophilically reactive compound or metabolite reacts with nucleophilic atoms (O, N, or S) in biomolecules,Y, according to

$$RX + Y \xrightarrow{k} RY + X \qquad (2)$$

the cumulative level, $[RY]_0/[Y]$, of the reaction product ("adduct") is simply the product of D and the (second-order) rate constant, k

$$\frac{[RY]_0}{[Y]} = k \cdot D_{tis} \qquad (3)$$

If Y and also RY is long-lived compared to the period of study, a measured adduct level, [RY]/[Y], will approach the cumulative value.

Dose rate - The dose rate, dD/dt, is equal to the concentration, [RX](t) (cf. Eqn. 1). This intensity factor varies with time in animal experiments and in vitro experiments with short-lived chemicals. In Fig. 4 one and the same cumulative concentration, $[RX]_0$, has been administered either by acute treatment (instantaneous i.p. injection) or by protracted exposure via inhaled air. In the former case the measured concentration, [RX], shortly after injection may approach $[RX]_0$, in the latter case a steady state concentration develops. It is of interest that in both cases the dose may be computed as

$$D = \frac{[RX]_0}{\lambda} \qquad (4)$$

where λ is the first-order rate constant for disappearance through enzyme detoxification, chemical reactions and diffusion.D has thus a simple relationship to "absorbed dose" discussed above (cf. ref. 4).

Target dose - Information from various sources has been collected to show that the main determinant of the frequency of mutations or tumours of a particular kind induced by genotoxic chemicals or ionizing radiation is the frequency of certain critical changes in DNA of a target organ under consideration. Indirect effects of chemical or radiation-chemical products formed in other organs may contribute only to a negligible extent, and modifying influences of effects in other organs, e.g. liver or immune system, are expected to occur only at high-very high doses and high dose rates.

For chemicals, it has for these reasons been suggested to express a target dose, or molecular dose, in terms of level of DNA adducts[9]. Partly in view of the incompleteness of our present knowledge of the identity of premutagenic, or critical, DNA changes, partly because of the difficulties, due to variations in rate of repair, to measure dose-related levels of DNA adducts, we have preferred to retain the above concentration×time definition of target dose. This definition is sufficiently versatile, e.g. with access to rate constants and relative rates to permit calculation of (cumulative) adduct levels (Eqn. 3 above). (We feel that the concept "molecular dose" should be saved for levels of critical changes, e.g. integrated over time to adjust for efficiency of repair; cf. ref. 4.)

Defined in this way chemical target doses will also be easily compared with radiation doses which are given in units of absorbed energy per unit of mass ($J \cdot kg^{-1}$=Gy); for ionizing radiation it is at present not possible to use frequencies of specific changes in cellular DNA as a frame of reference.

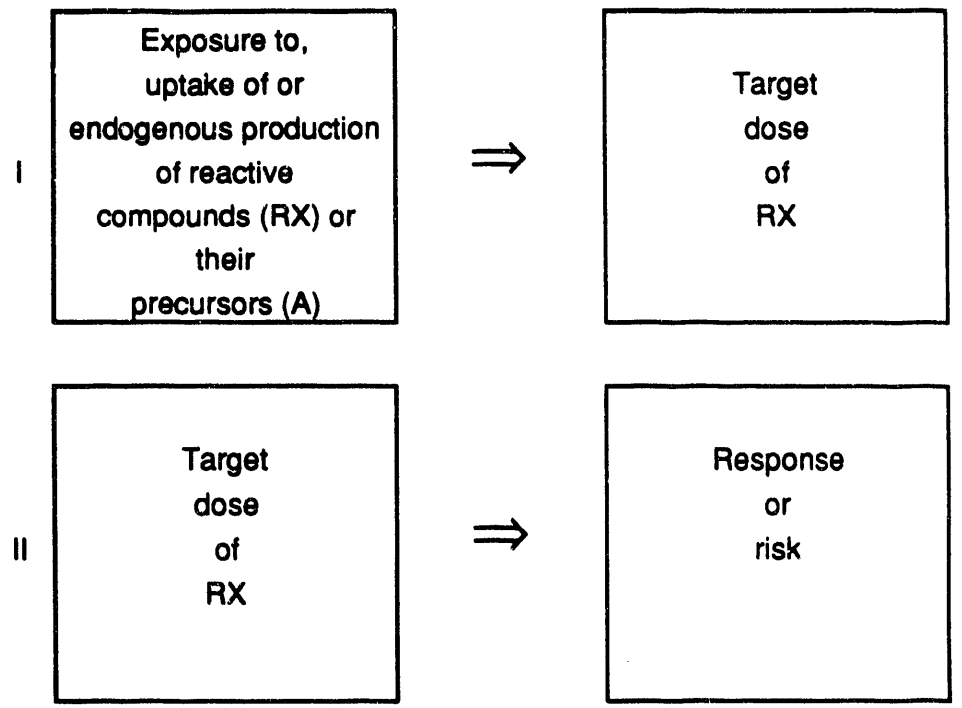
The target dose evidently takes, or should take, a central position in risk assessment schemes. In exposure to external radiation the target cells are directly receiving the dose, and the frequency of changes in their DNA is the determinant of the radiation risk. In exposure to genotoxic chemicals, all steps before the appearance of RX in the target cell, i.e. uptake, transport,

excretion, metabolism (bioactivation), detoxification (cf. Fig. 5), have to be treated as determinants of the dose, the risk being related to the fate of the changes in the DNA provoked by chemical reactions according to formula (2) above. Evidently, with this subdivision of the path of a reactive chemical or metabolic intermediate, repair of DNA damage will be part of this further fate, as it naturally is in the case of radiation damage. In certain cases the absolute value of the rate constant, k, is not easily accessible. The dose may then be expressed as adduct level, once the above definition of dose (Eqn. 1) is retained in principle.

Dose concepts at low doses. - The units of dose discussed, mMh for chemicals and Gy (rad=0.01 Gy) for radiation, are continuous. When these units are used at very low doses it should be remembered that, seen from the cell or cell nucleus, the effects of the doses cease to be continuous. E.g., 0.6 μMh ($=6 \cdot 10^{-4}$ mMh) of ethylene oxide will give rise to an average of 1 deoxyguanine-O^6 alkylation per cell, with a stochastic variation of cells with 0, 1 etc. alkylations. Similarly 3 mGy of γ-radiation is the average dose received by a human cell nucleus upon passage of an ionizing electron. These doses may thus be considered the lowest a cell nucleus can receive. At still lower average doses the number of cells without hits, i.e. with zero dose, will increase.

3.3 CHEMICAL (AND RADIOGENIC) CARCINOGENESIS: MECHANISMS AFFECTING DOSE AND DOSE-RESPONSE RELATIONSHIPS

According to the preceding it has been found practical to subdivide dose-response relationships of genotoxic chemicals into the steps I and II:



Factors that may influence any of these steps were discussed by Ehrenberg et al.[4]. These factors are, strongly simplified, illustrated in Fig. 5.

STEP I - Absorption from air is a relatively straightforward process, the absorbed amount per unit of time being proportional to the exposure concentration and the respiration rate (alveolar ventilation). A determinant is the solubility in the blood and at a low ratio between pulmonary blood flow and alveolar ventilation the rate of uptake may be decreased. The level (concentration) of RX in the target cells is determined by this rate of uptake, in the case of precursors (A) the rate of bioactivation (often by cytochromes P450), transport, diffusion, chemical reactions and rate of detoxification (by e.g. glutathione transferases or epoxide hydrolases). As long as these processes remain unaffected by the exposure, the relationship between exposure dose and target dose is expected to be linear[4]. (The role of inhaled particles will not be discussed here; cf. P12). — Uptake by injection, ingestion or via the skin, as well as endogenous production, may be treated in the same way. If a high-efficiency low capacity sink (chemical or biochemical) is

present, as is the case for phosphoric ester insecticides, the target dose will be lowered at low doses and then increase linearly with absorbed dose after consumption of the sink[10].

At the same time dose gradients may occur in the body. This concerns, particularly, very short-lived, i.e. highly reactive, compounds or metabolites which give higher doses at the site of uptake or tissue with fastest bioactivation. For instance, vinyl chloride and *N*-nitrosodialkylamines give the highest doses in the liver, with a gradient to other organs[11]. A long-lived compound or metabolite ($t_{1/2} \geq 1$ min) that is stable in both lipids and water, e.g. ethylene oxide, metabolite of ethene, will give approximately the same dose in all parts of the body. The same is found for *N*-alkyl-*N*-nitrosoureas which, following distribution all over the body, are bioactivated to a very short-lived RX by OH^- in equilibrium with water.

The enzymatic reactions of bioactivation (A —> RX) and detoxification follow Michaelis-Menten kinetics. Therefore, the D_{targ}/D_{exp} ratio tend to decrease with the dose of precursor, A, and to increase with the dose of reactive compound, RX, applied. It should be observed that these kinetics are valid strictly for the concentrations of a precursor or reactive compound, as exemplified in equation (5) for the rate of bioactivation.

$$\frac{d[RX]}{dt} = V_{max} \frac{[A]}{K_m + [A]} \qquad (5)$$

where K_m is the Michaelis constant. It affects, however, that the resultant dose in a similar way (cf. Eqn. 1).

If during an exposure enzymes for bioactivation or detoxification are induced to higher activities, the D_{targ}/D_{exp} ratio will exhibit a positive or negative, respectively, deviation from linearity with increasing dose. Little is known about the dose-response for such induction, particularly for protracted exposure at low dose rate. Our measurements indicate, however, the

induction of P450 isozymes is effective only above a threshold concentration (P13).

Step II - A large fraction of the DNA-changes from the target dose will be repaired. The non-repaired fraction of the changes may in following cell divisions become manifest and give rise to a mutated (initiated) cell. As long as a constant fraction of primary DNA lesions remains unrepaired (or erroneously repaired), the frequency of mutated cells is expected to depend linearly on D_{targ}. This presupposes, of course, that the rate of cell division is not affected, which may be true at low doses/dose rates, although the kinetics of mitotic delay needs further studies[4].

Like other enzymatic reactions, DNA repair is saturable, with Michaelis-Menten type kinetics. It is believed that the shift to higher mutation frequency per unit of radiation dose if doses of 100-500 rad are administered at > 1 rad/min (Fig. 3) is due to repair saturation, and this is to some extent confirmed by mathematical modelling (unpubl. data).

In interaction with existing promoters or promoting conditions (which favour cell division and reprogramming of cells) an initiated cell will develop into a pre-tumour and, during the ensuing progression, to a malignant tumour. During the promotion—progression additional genetical changes occur. Although exogenous exposure, particularly at protracted high doses in animal experiments, could certainly contribute to these additional mutations, required for malignanization, these changes are expected to be predominantly spontaneous, favoured by "genetic instability" in the promotion—progression phases .

As long as the promotive conditions, that exist or occur for reasons other than the exposure, remain unaffected by the exposure, the probability of tumour development will be proportional to the frequency of initiated cells and, consequently to D_{targ}. This is expected to hold true, in the general case, at

low—intermediate doses and dose rates. At high doses the initiator may act as a promoter, e.g. by unspecific killing of cells with stimulation of reparative growth in consequence, or by specific interaction with certain receptors. If a compound has a high receptor affinity, tumours of specific types may develop already at low—intermediate doses. When the initiator acts as a promoter, the dose-response curves will be non-linear, bending upwards from the doses where promotion occurs.

Certain support for this briefly summarized scheme is obtained from the experimental demonstration that administration of a promoter after the initiating treatment leads to a "linearized" response[12,13]; see further section 4.1.2.

3.4 DOSIMETRY AND "THE STOCKHOLM MODEL" FOR CANCER RISK ASSESSMENT

In this section methods for dosimetry by adduct measurement are summarized together with our model for risk estimation. This is because the needs for sensitive methods for detection, identification and quantification of mutagens/carcinogens emerged from results of kinetic studies indicating a potential risk model, and led to the original suggestion of the principle of dose monitoring by macromolecule adducts (Ehrenberg, 1974[14]; a paper at a conference in 1972 was never published).

The importance of, or necessity of, combining any work on adducts with aspects of associated risks, is discussed in section 4.4 below.

Reaction-kinetic studies. - In early efforts to identify determinants of mutagenic potency of alkylating agents, these compounds were characterized kinetically in terms of the linear free energy relationship (Eqn. 6) which had been employed by Swain and Scott[15]:

$$\log k_{i,n} = \log k_{i,0} + s_i \cdot n \qquad (6)$$

where $k_{i,n}$ and $k_{i,0}$ are the second-order rate constants for reaction of alkylating agents i with nucleophiles with strength n and 0, n=0 being by definition the nucleophilicity of water. s_i are the substrate (or selectivity) constants of the compounds. n varies in an arbitrary scale, with values increasing in the order O < N < S. Methyl bromide was used as standard alkylating agent with s=1.

Comparison with biological data led to the conclusion that the potency, i.e. mutation frequency/D_{targ}, for induction of forward mutation at intermediate doses was proportional to the rate of reaction at $n \approx 2$ [16,17]. This value corresponds to the n of DNA oxygens, such as guanine-O^6. In other words, a number of compounds with different patterns and rates of reaction showed approximately the same mutagenic power at equal degree of alkylation of certain oxygens in the DNA, i.e. at equal values of the product $k_{i,n=2} \cdot D_{targ}$ (cf. Eqn. 3 above).

A comparison of the chemical data with the mutagenic potency of γ-radiation in the same systems led to the conclusion that the degree of alkylation at n=2 which is associated with the same forward mutation frequency as the one caused by 1 rad of γ-radiation, is $1 \cdot 10^{-7}$. Approximately the same value was obtained in various biological systems, including bacteria, plants and mammalian systems and would therefore probably be applicable to humans, once the target doses could be measured.

Risk estimation of chemicals i by a relative potency method is based on expressions

$$P(D_i) = k_{std} \cdot Q_i \cdot D_{targ,i} \qquad (7)$$

where k_{std} is a known risk coefficient for a standard agent (such as γ-radiation in the rad-equivalence approach), Q_i the relative potencies at the low—

intermediate doses where dose-response may be considered linear, and $D_{targ,i}$ the target dose as defined above (Eqn. 1).

During human exposures, which are mostly chronic or intermittent, steady-state levels of adducts are built up. The relationship of cumulative doses to the steady-state levels is determined by the ratio, k_-/k_+, of the rates of disappearance and formation, respectively, of the adducts (cf. P15). Due to the variation in rate of repair between chemicals , tissues, cells and chromosome regions, k_- for DNA adducts is unknown and doses cannot at present be calculated from steady-state DNA adduct levels.

In contrast, protein adducts are, at least at low levels, not subjected to "repair" and the value of k_- is thus well defined. Therefore, in the tissue which is generally most easily accessible, the blood, the haemoglobin (Hb) and to some extent serum albumin appear more useful than leukocyte DNA for in vivo dose monitoring. In the Stockholm group methods for measurement of Hb adducts have been developed. For the *N*-alkyl valine method which measures adducts to the N-termini, valines, of Hb, the analytical power reached was sufficient to cover the whole range where the associated cancer risk may be considered unacceptable[18].

Dose monitoring by adducts to blood proteins will give the doses in blood, $D_{bl,i}$. Possible gradients between doses in target organs and blood have so far been determined, from DNA adduct levels shortly after acute exposure, in organs of interest, and expressed as factors f_2. As expected, the rates of formation of adducts in tissue DNA and in blood proteins are proportional (review: ref. 19).

It is often wanted to express risk on the basis of exposure. This requires determination of the ratio $f_1=D_{bl}/D_{exp}$. Particularly with respect to air pollutants the determination of accurate exposure doses is a difficult problem which has been studied within the project (see P15 and 4.2.1 below). With these factors introduced, the risk equation (7) assumes the form

$$P(D_i) = k_{std} \cdot Q_i \cdot f_{1,i} \cdot f_{2,i} \cdot D_{exp,i} \qquad (8).$$

For applications of this model in risk estimation, see section 4.3.1 below and refs. P3, P12, P20, P21.

3.5 COMPARISONS WITH RADIATION-INDUCED CANCER

In human environments some 100,000 chemical compounds are spread through human activities and in addition a very great number of compounds occurs naturally. No single chemical has been subjected to experimental and epidemiological studies, aiming at risk assessment, to the same large extent as the ionizing radiations. This factor, particularly low-LET radiation, plays therefore naturally a role as model and standard in the whole complex of risk management activities. Comparisons with radiations seem particularly meaningful with respect to so-called genotoxic chemicals, treated in classical radiobiology under the name "radiomimetic compounds", because their effects, such as mutation, chromosomal aberrations and cancer mimic those of ionizing radiation. These effects of both genotoxic chemicals and ionizing radiation are stochastic, without (definable) no-effect thresholds, with specific consequences to the evaluation and control of risk, e.g. the "ALARA principle" (As Low As Reasonably Achievable; ICRP, 1977[20]). The value of rules developed for the management of radiation risks as a model in the corresponding evaluation and control of the corresponding chemical risks was therefore early recognized[21,22].

Since it is often believed that the comparison, at our laboratory, of chemical data with data for radiation effects is restricted to the use of the radiation-dose equivalents ('rad-equivalents') of chemical doses in risk estimation by a relative potency approach (Eqns. 7,8), the purposes of such

comparisons are summarized in Table 1:

TABLE 1

Purposes of comparisons of chemicals and ionizing radiation with respect to health effects

- Illustration, in terms of a known factor, of the detection level (power) of epidemiological studies or laboratory tests[11].
- Definition of "acceptable" risk, for instance as the required sensitivity of test procedures[18].
- Application of rules for radiological protection (dose optimization, ALARA principle) to chemical exposures.
- Using experience from epidemiological studies of irradiated populations to (testable) hypotheses of importance to risk estimation of genotoxic chemicals regarding expected effects (e.g. effects on IQ?); influences of dose-rate, age, background incidence, etc.
- Expression of chemical doses in radiation-dose equivalents (rad-equivalents) offers a promising principle for risk estimation (the rad-equivalence approach).
- By giving different exposures in a common unit, possibilities of comparison are gained, e.g. for solutions of technical problems (food preservation, energy sources[23]). Further, this permits common management of radiogenic and chemical cancer risks and may decrease the gap between estimated risk and perceived risk.

4 RESEARCH WITHIN THE PROJECT

As indicated in the background summary above (section 3), meaningful research on methods for cancer risk estimation requires a conjoint treatment of several, apparently disparate fields. Within the project the following areas, judged to be essential to the general problem, have been subjected to research

- Dose-response relationships
- Dosimetric problems
- Application of different models for risk estimation
- Biochemical and molecular epidemiology
- Structure-effectiveness relationships (a case study)
- Validation of the rad equivalence approach; possibilities of using other approaches.

Papers and reports produced within the project, listed in section 5.2 below, are referred to by numbers preceded by a "P". In this report other references are kept to a minimum.

4.1 ASPECTS OF DOSE-RESPONSE RELATIONSHIPS

Risk estimation has to be a mathematical operation on the basis of existing knowledge about dose-response relationships. Therefore, several studies within the project have dealt with the possibilities of mathematical modelling of biological mechanisms of carcinogenic processes. In particular, this has concerned the low doses (and/or low dose rates) where, for statistical reasons, informative observations are not obtainable in conventional experimental and epidemiological studies. As long as dose-response relationships can be considered linear down to dose zero, i.e. without no-effect threshold, risk estimation is relatively straightforward. If there are deviations, upwards or downwards (as indicated in Figs. 2,3), it is essential to

decide to which extent such deviations have to be considered in risk estimation, e.g. by estimating which uncertainty is introduced by disregarding them.

In this field there has to be a giving and taking between mathematical statistics and biological, particularly molecular-biological, research.

The various studies on dose-response relationships are classified under the sub-headlines 4.1.1-4.1.7.

4.1.1 MATHEMATICAL DOSE-RESPONSE MODELS FITTED TO EXPERIMENTAL CANCER DATA

A number of mathematical models, several of which have been suggested to be used for extrapolation to low doses, were fitted to some one-hundred experimental data sets for chemical carcinogenesis (P1, P2). These models, including multi-hit and multi-stage models, are based on the stochastic nature of definite "hits" by the ultimate carcinogen. In the published data, the doses as given by the authors were retained. This seemed correct in view of proportionality between administered dose and target dose, in some cases modified upward or downward by saturation of detoxification or bioactivation, respectively (equation 5 above). As for calculation of in vivo doses from experimental data, see 4.2 below.

Assuming that promotion and cocarcinogenic effects would rather be "deterministic" in nature, two "mixed two-stage models" were also tested in which the cancer incidence, $P_{can}(D)$, was seen as the product of the incidence of initiations, $P_{ini}(D)$, and promotion + cocarcinogenic effects, $P_{pro}(D)$:

$$P_{can}(D) = P_{ini}(D) \times P_{pro}(D), \qquad (9)$$

modelling initiation (mutation) by the one-hit kinetics

$$P_{ini}(D) = 1 - e^{-(a+bD)} \qquad (10)$$

and promotive etc. effects by the cumulative normal distribution:

$$P_{pro}(D) = \Phi(c+dD) \qquad (11)$$

Eqns. (9-11) could generally be fitted to dose-response data for, e.g. polycyclic aromatic hydrocarbons (PAH). The fact that in certain cases the upwards bend of the curve at higher doses is better modelled by a two-hit function

$$P_{can}(D) = 1 - e^{-(a+bD+cD^2)} \qquad (12)$$

is interpreted as a consequence of occasional circumstances. It has been argued that the sensitivity to promotion, in similarity with many toxic effects of chemicals, should rather be log-normally distributed,

$$P_{pro}(D) = \Phi(c+d \log D) \qquad (13)$$

i.e. the classical Mantel-Bryan model[24] for low-dose extrapolation. However, existing dose-response data for the phorbol ester TPA[25] prefer model (11) (P2).

Likewise the induction of cytochromes P450 by benzo[a]pyrene (BaP), an effect perceived as a cocarcinogenic action, follows the same kinetics (cf. P11). As a matter of fact strong indications have been collected to show that in the carcinogenic action of PAH the unmetabolized hydrocarbons act as promoters through interaction with the Ah receptor (cf. section 4.1.7 and P13, P14) at the same time as reactive metabolic intermediates act as initiators.

Due to these complex kinetics it has not been possible to study the dose-response of the promotive action of PAH such as BaP otherwise than by the parameter values of Eqn. (11) generated when the product Eqn. (9) is fitted to experimental data (P1, P2). Besides that, the convexity of dose-response curves may have causes other than promotion and P450 induction, such as involvement of true two-hit effects and saturation of repair.

It appears that in many animal tests a considerable promoter (etc.) action was exerted already at the lowest doses tested. This suggests that Eqns. (9-11) should not be used as an extrapolation model for risk estimation. This is also underlined by the observation that data for different chemicals are adaptable to different models. Some mathematical models not based on biological theory, like the polynomial model applied by U.S. EPA[26] are certainly generally adaptable to experimental data sets. Due to promotive and/or modifying effects, the validity of which to humans is not known, at the doses tested risk estimation by low-dose extrapolation can only be done with utmost care.

It should be added that the validity of Eqn. (11) at very low doses is not well defined. In principle the value of $dP_{pro}/dD > 0$ at any value of the parameter $c > -\infty$. Exposure to a particular promoter or cocarcinogen which by equality of mechanism acts additively to ongoing processes which have surpassed a no-effect threshold will lead to a risk increment that depends linearly on the dose[27]. This is modelled by the value of c which determines at which probability the action of the studied factor starts.

It deserves mentioning that in most cases all the models fitted generate a derivative, $dP/dD > 0$ at $D=0$, i.e. show data to be compatible with linearity at low doses.

The use of tumour data on the level of time to event is generally better economizing with resources. The approach is, however, not uncomplicated since the endpoint of interest, the onset of tumour development, is not

observable. To overcome this problem, animals have to be sacrificed at different time points. Such a procedure is obviously quite an "animal consuming enterprise".

To be able to analyse time-to-event data with a parametric approach based on a foundation of biological mechanisms, different mathematical models have been suggested (e.g. Moolgavkar-Knudson-Venzon two-stage model; cf. ref. 28). These models consider the different steps in the carcinogenic process as if the cells are subjected to stochastic time dependent processes of exposure, DNA repair, proliferation, killing and promotion. Such models would often tend to include too many parameters to be able, without external input of parameter values, to distinguish between the influences of different mechanisms that yield the same effect on the observed endpoint.

Fitting mathematical models to animal test data has its greatest value in the illustration of hypothetical mechanisms, such as saturation of bioactivation (shown for vinyl chloride and benzo[a]pyrene) (P1, P2). Disagreement between model and, e.g. metabolic data, as for urethan, leads to the formulation of new, testable hypotheses.

4.1.2 ABSOLUTE OR RELATIVE RISK INCREMENTS?

Cancer risks from exposure to ionizing radiation or environmental genotoxic chemicals have long been computed in terms of absolute increments added to the background risk, P°_{can}. If the risk increment is assumed to depend linearly on dose, the relationship may be formulated

$$P_{can}(D_i) = P^{\circ}_{can} + k_i D_i \qquad (14)$$

where k_i and D_i are the risk coefficient and dose, respectively, of agent i.

In experimental studies of radiogenic cancer, particularly at the Oak Ridge National Laboratory[29], it has been shown that a multiplicative model gives for most cancer sites j a better description of the dose response:

$$P_{can,j}(D_i) = (1+\beta_j D_i)P^{\circ}_{can,j} \qquad (15)$$

where the risk coefficient β_j may assume approximately the same value for several sites j (cf. refs. 29, 30).

With longer follow-up times of human populations with radiation exposure (particularly A-bomb survivors and ankylosis spondylitis patients) it has become increasingly evident that the multiplicative model (15) is better fitted also to human data than the additive model (14). The National Research Council[1] has in the BEIR V report accordingly based its projections of lifetime risks from low-LET radiation on the multiplicative model (15), using empirically estimated values of β_j for site, sex and age.

An investigation of published experimental cancer incidence data for ethylene oxide, the compound chosen as a model in many of our studies, showed a significant correlation between the absolute increment of site-specific incidences and the corresponding background incidences, $P^{\circ}_{can,j}$ (P3).

This indicates that a multiplicative model should, at least to some extent, be included in risk estimation of chemical initiators as well. This conclusion has a bearing on the usefulness of epidemiological and experimental data for risk estimation. In both humans and laboratory animals there is a rapid increase of cancer incidence, per survivor, at higher ages. Irrespectively of the nature of the genotoxic agent, somatic mutations are irreversible and will remain in tissues (unless they are eliminated due to negative selection value). Due to long latency periods for solid tumours, too short follow-up times often lead to an underestimation of risks from exposure to initiators that lack

promoter activity. For related reasons animals should not be killed precociously, as is mostly done according to test protocols (cf. risk estimation, section 4.3 below).

Theoretically it may be noted that the interaction between initiating and promoting events is multiplicative (Eqn. 9). At low—intermediate doses (levels) of an initiator, the linear dose-response curve would be written

$$P_{can,j}(D_i) = (a + b_i D_i) P^{\circ}_{pro} \qquad (16)$$

In the multiplicative expression (15) above, $\beta_i = b_i/a$, i.e. indicates a relative increase of the initiation pressure, dP_{ini}/dD_i, as if this pressure had an approximately constant background (P2).

Support for the model (9-11) and its formulation (16) for low doses of an initiator is obtained from experimental data which show linearization of the dose-response curve after addition of a promoter[12,13].

4.1.3 RESPONSE (RISK) AT LOW DOSES AND DOSE RATES

In the above (section 3.1) arbitrary classification of dose and dose rates as low, intermediate and high, it was indicated that repair saturation is a probable mechanism for radiation being more effective at high dose rates. For doses on the order of 10^2-10^3 rad (1-10 Gy) of low-LET radiation, the change from intermediate to high dose rate occurs at about 1 rad (0.01 Gy) per min. For gene mutation induced in mouse spermatogonia intermediate dose rates are some 3 times less effective than high dose rates, and for cancer the corresponding factor may be somewhat larger (about 5). It would be expected that a single acute dose — i.e. by definition given at high dose rate —, if sufficiently small, would have a genotoxic effectiveness as if it were delivered at an intermediate dose rate.

Since most experimental studies are carried out within moderate limits of dose rate (cf. 3.1), the low dose rates are generally considered less effective[30], by a definite factor, e.g. 3, than high dose rates. However, experimental data (and also certain human data) indicate that at doses and dose rates below the detection level of conventional experiments, the genotoxic effectiveness is in many cases larger than at what we here call intermediate doses and dose rates. These data have been reviewed by Oftedal[31] and in P4, P5 of this project. By and large these data show that dose-response curves for induced mutation (and perhaps cancer) may exhibit positive deviations (b in Figs. 2,3) from the linear curve extrapolated from intermediate doses or dose rates, and that these deviations are of such magnitude (a factor 2-5) that they cannot be ignored in risk estimation.

A possible mechanism of this deviation, suggested in terms of our present knowledge, is an inducibility of error-free repair, with the consequence that a low dose will be more effective in uninduced cells than in induced cells. If now a low dose (e.g. a few hits) leads to induction, the following hits will occur in induced, less sensitive cells, provided that the time interval between hits is predominantly shorter than the duration of the induced condition. Conversely, if the time interval between hits is predominantly longer than the persistence of the induced condition, most hits will occur in uninduced and therefore more sensitive cells.

This hypothesis is supported by results of in vitro "adaptation" experiments in which a radiation dose corresponding to 1-2 hits per nucleus was shown to render the cells less sensitive to a later, high challenge dose[32]. For certain effects, an equivalent effect has been demonstrated in vivo in rats (collaboration with A.T. Natarajan, Leiden; to be published).

The kinetics of induction and its reversion are not well known. In some instances (e.g. P13) the duration of an induced condition (cytchromes P450) has been measured to ca. 1 day. Since a dose of 0.3 rad (3 mGy) causes

upon an average 1 hit per nucleus in mammalian cells, a dose rate $\leq 10^{-4}$ rad/min would be expected to show the raised effectiveness. This is by about one order of magnitude below the lowest dose rate studied in mouse spermatogonia (3.1 and Fig. 3). In an old study[33] of biochemical mutations induced by ^{90}Sr in the microspore generation of barley, the deviation was observed at dose rates 10^{-5}-10^{-4} rad/min. (This system showed a similar, although less significant, effect following exposure to ethylene oxide[4]; cf. P4).

Within the project a model experiment in which the dose-response curves following treatment of methyl transferase-inducible bacteria with a methylating mutagen (MMS) were investigated. The curves exhibit a significant superlinear "hump" at low doses followed by a flattening out or even negative slope of the curve with increasing dose (P6). In this paper an adaptable repair systems has also been modelled. The stochastic model resembles the time dependent processes of exposure and repair of a cell with a queue system, with the service rate corresponding to the rate of DNA repair and the arrival rate to the exposure. The proposed model allows the repair intensity to vary depending on the history of the individual cells. An adaptive repair system is modelled by letting a fixed number of damages be the induction event for a highly effective, but (in the case of alkyl transferase) consumable, repair system that is not initially present. By means of a simulation model the mean number of initiated cells at a certain time has been analysed as a function of dose (i.e. varying dose rate within a fixed time interval).

The resulting dose-response curves (exemplified in Fig. 6) can have varying shapes depending on the allowed input of parameters, — viz. repair rate of the adaptive systems, induction event, delay time to induction and capacity of the induced repair system. The common behaviour, of allowing this type of adaptable repair system, is a concavity of the dose-response curve. The slope of the response curve can be locally zero or negative for a

sufficiently powerful adaptive repair system. The result of this qualitative analysis is that the risk increment for a given dose increment varies with dose, yielding lesser effectiveness at higher doses than at lower doses.

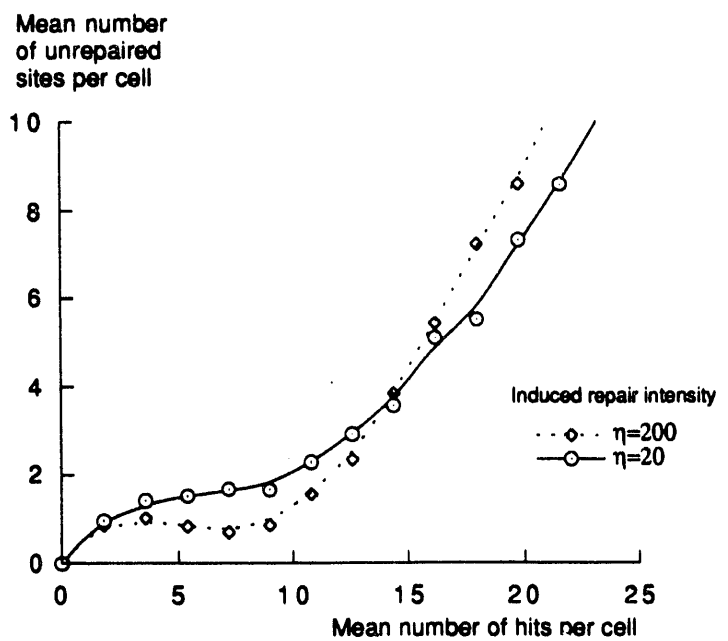


Fig. 6. An illustration of typical dose-response curves from the model simulating an adaptable repair system. The power of the induced repair state determines how accentuated the "hump" will be. η is the relative increase over the background repair intensity.

In bacteria also the reverse dose-response relationship for induced mutation, viz., a lower mutagenic effectiveness at the lowest dose has been shown to be caused by inducibility of error-prone repair (cf. curve c in Fig. 2, section 3.1): Damage of various kinds to the bacterial DNA activates the recA protein which proteolytically cleaves a repressor, lexA, for several genes the transcription of which is thereby initiated[34,35]. The induced functions comprise mutator functions as well as repair enzymes. For certain mutagens, particularly UV radiation, the mutator functions predominate, with the

consequence that UV light can induce mutation only after induction of this response.

Since this effect has been seen as a prevention of killing at the expense of mutation, it has been called SOS repair. In other cases, e.g. certain simple monofunctional alkylating agents, the simultaneous induction of functions for error-free repair leads to a somewhat lowered mutation frequency after induction of the RecA gene.

An intense search for a related system in mammalian cells has mostly given equivocal results. However, recent studies seem to show the fos gene to play a role in eukaryots as a switch by which a number of genes are controlled in a "DNA damage response"[36]. In model induction-challenge studies an effect of this kind has been shown for radiation-induced mutation in vivo in the rat (collaboration with A.T. Natarajan, Leiden; to be published). (Similar experiments with chemical mutagens are planned.)

4.1.4 DOSE-RESPONSE AND THRESHOLDS

As long as the question whether no-effect thresholds do exist is not settled, there remains a lingering suspicion (which can be used in biased attacks on figures for estimated risks) that the risks at very low doses are over-estimated. In efforts to arrive at scientifically true dose-risk relationships (which need not to be identical with relationships applied for practical administrative purposes) it was found necessary to deal with the threshold problem. As a first approach to this problem it was considered essential to find methods to estimate upper confidence limits of possible thresholds.

The common definition of a no-effect threshold is that the response above the background is zero up to a certain threshold dose.

From a cell's point of view there can certainly exist a threshold if the first "hit" is completely ineffective, as in the case of "error-prone" repair where the first hit opens a pathway for the following hits to be effective. From the point of

view of an organ, "a collective of cells", the presence of "error-prone" repair leads to a purely quadratic dose-response relationship at low doses if no background is present, assuming the Poisson distribution of hits and that $1+e^{x} \approx x$ when x is small:

$$P(D) = 1 - e^{-(bD)} - (bD)e^{-(bD)} \approx (bD)^2$$

This could be interpreted as a threshold since $P'(0)=0$.

However, with a background level, a, of "hits" present (i.e. there exist cells where a pathway for effective hits is already open) the linear component dominates the dose-response curve at low doses:

$$P(D) = 1 - e^{-(a+bD)} - (a + bD)e^{-(a+bD)} \approx (a + bD)^2 = a^2 + 2abD + b^2D^2$$

As can be seen from the latter expression the size of the background determines the linear component and if it is sufficiently small it can, in practice, be impossible to perceive the linear component in the analysis of dose-response data at very low doses.

As described in section 4.1.3 (cf. Fig. 6) the presence of a highly effective inducible repair system can lead to responses which, if observed at certain fixed doses, could very well be perceived as a dose-response curve with a no-effect threshold.

4.1.5 STATISTICAL METHOD FOR THRESHOLD ESTIMATION

Within this project a statistical method for the estimation and testing the location of a threshold in dose-response experiments has been developed (P7-P10). The model is formulated as having no increase of risk up to a certain dose whereafter the risk increases linearly, but it can also be applied

to a response that initially increases linearly whereafter a plateau phase follows.

The study considers the statistical behaviour of the threshold estimate in an experimental design, typical of dose-response experiments, where the response is relatively well determined in a few dose points. The relevance of this model in the case of mutation and cancer experiments lies in its ability to detect deviations from linearity in the low-dose region. The rejection of linearity in favour of a threshold or plateau model suggests the existence of mechanisms depending on dose that alter the effectiveness of the studied agent, e.g. the above-mentioned adaptive systems (cf. 4.1.3). The location of the shift-point suggests within which range of doses further examination is needed.

The method also suggests a procedure to estimate the upper confidence limit of the threshold that, if sufficiently low, carries some information about the non-existence of a threshold (P8) and in general it reveals the information value of a certain experiment concerning its ability to detect a threshold if it exists.

It can be argued that the model is synthetic, suggesting an abrupt shift in response, and this might be true. However, a typical dose-response experiment conducted only with a fixed number of dose points does not allow great power for discrimination between different models (cf. ref. 37). Threshold-like shapes can be achieved in a number of models (e.g. logit, probit) but the suggested model has the advantage of expressing the threshold as an explicit parameter allowing direct inference of its location.

Statistical properties of the threshold estimate. - The statistical problem can be characterized as maximum likelihood estimation under non-standard conditions (P7, P10). The threshold estimate also reveals undesirable statistical properties. Through, to our knowledge new, derivation of the statistical distribution of the estimator these problems are understood. The

undesirable properties of the point estimate of the threshold consist in built-in bias and lack of translational invariance of the error distribution of the estimator. In spite of this the proposed method for interval estimation (i.e. confidence intervals, CI), at least at the commonly used confidence levels (95% and higher), seems to have desirable covering properties, i.e. 95% CI's for the threshold estimate fails to cover the true threshold value in only 5% of the cases.

4.1.6 DOSE-RESPONSE OF SOMATIC MUTATIONS IN VIVO

In epidemiological and experimental studies aiming at a validation of approaches to estimate risks on the basis of measurements of D_{targ}, the measurement of genetic endpoints in somatic cells has been employed as an economically advantageous substitute for disease-epidemiological data (current work supported from other sources).

In studies of induced HPRT mutation in vitro and in vivo[2] the large scattering of mutant frequencies called for an analysis of causes and an improvement of the statistical treatment of mutant data. In the determination of the response to an exposure, the number of mutants per cell (M) observed on selective medium is corrected for cloning efficiency (CE) determined on non-selective medium. Human in vivo data indicate that this procedure is erroneous, part of M being independent of CE. This suggests an improved method of data analysis now tested on large human data sets (P11, collaboration with British and Dutch investigators).

4.1.7 POLYCYCLIC AROMATIC HYDROCARBONS (PAH)

Benzo[a]pyrene (BaP) is by ca. 2 orders of magnitude a more effective carcinogen than fluoranthene (FA), although in conventional in vitro mutagenicity tests with mammalian cells the two compounds are about equally effective. Since FA is a common environmental pollutant — it is, e.g. a

predominant PAH in diesel exhaust (P12, P13) — this constitutes a key question with regard to the relevance of mutagenic potency to cancer risk estimation. Since FA becomes carcinogenic in the presence of BaP[38], it was investigated whether the metabolism of FA is inducible by BaP. In this context the dose-response for P450 IA induction was investigated and shown to have a broad no-effect threshold. FA does not induce P450, and is not bioactivated by the enzyme(s) induced by BaP (P13).

A theoretical analysis of data (P14) indicates that the above difference is due to BaP but not FA possessing promoter activity. Comparing different PAH shows a strong correlation between affinity to the Ah receptor and carcinogenic potency, FA and other PAH that lack Ah affinity being mostly judged, e.g. by IARC, to be non-carcinogenic. These and other data are compatible with the Ah receptor interaction of the PAH itself being the event which releases, through the ensuing enzyme induction, the promotion (cf. P13, P14).

Since thus the promotion and initiation are caused by different chemical species, an increase of the rate of PAH metabolism including bioactivation may increase or decrease the response, in dependence of whether the initiation or promotion is rate limiting in the carcinogenic process (P14). These kinds of kinetics is partly due to the difference in the dose-response curves between initiation and promotion (cf. 4.1.1 above and P1,P2).

4.2 DOSIMETRIC PROBLEMS

Various theoretical problems concerned with calculation of target dose (and hence risk estimation) from observed adduct levels have been analysed.

4.2.1 ADDUCT LEVELS FROM VARIABLE EXPOSURE

This problem has been given a general solution considering monitor molecules which decay in zeroth-order and first-order kinetics, such as

haemoglobin (Hb) and serum albumin (SA), respectively (P15; cf. ref. 39). Also the influence of adduct instability on adduct levels have been analysed.

This study also considers the establishment of the relationship between target dose and exposure dose, of importance in risk control. The zeroth-order decay, which introduces a "memory" of the exposure pattern during the 4 months before the adduct level determination, complicates the establishment of the D_{targ}/D_{exp} ratio in the case of Hb adducts. An optimized procedure for the determination of this ratio, e.g. in work environments, has been suggested.

4.2.2 APPLICATION TO UNSTABLE ADDUCTS

The procedure reported under 4.2.1 has been applied to doses of malonaldehyde (MA) in mice and humans. This aldehyde forms unstable Hb adducts from the levels of endogenously formed or experimentally administered doses. From the Hb adduct levels, individual blood doses could be calculated, partly as a basis for the determination of the kinetics of DNA adduct formation (P16).

4.3 APPLICATION OF DIFFERENT MODELS FOR RISK ASSESSMENT

4.3.1 RISK ESTIMATION BY THE RAD-EQUIVALENCE APPROACH

This approach, which is still under development, has been applied to a few compounds (P12, P20). It is briefly summarized here with ethylene oxide and its precursor, ethene, as example (for details, see refs. P12, P20, P21, 4, 41).

In work environments with relatively well characterized exposure concentration of ethylene oxide, the steady-state level of the adduct, hydroxyethylvaline, to N-termini of Hb, was determined to 2400 pmol/g Hb at 1 ppm, 40h/week. From the rate constant for adduct formation, $5 \cdot 10^{-5} \cdot l \cdot g^{-1} \cdot h^{-1}$,

and considering that the steady-state level is equal to the level accumulated during ca. 9 weeks (360 working hours), i.e. one-half of the erythrocyte life span, this gives the ratio, f_1, between blood dose and exposure dose (Eqn. 8 in section 3.4):

$$f_1 = 1.3 \cdot 10^{-4} \text{ (mM·h)(ppm·h)}^{-1}$$

From the approximately even distribution of the dose in model animals, it is concluded that

$$f_2 = 1.$$

For radiation-dose equivalent of the ethylene oxide dose, a mean value from measurements in various biological systems,

$$Q = 80 \text{ rad-equ. (mM·h)}^{-1}$$

is adopted[4,41].

For cancer mortality we have applied a risk coefficient

$$k_\gamma = 2 \cdot 10^{-4} \text{ rad}^{-1} \text{ or rad-equ}^{-1}.$$

For cancer morbidity the risk would be approximately two times larger. These values allow for risks being ca. 5 times lower at low dose[41] rates compared to the value, $k_\gamma = 10 \cdot 10^{-4}$ rad^{-1} valid at high doses and high dose rates[1].

Occupational exposure to ethylene oxide for 40 h/week, 46 weeks/year (1840 h/year), at the present Swedish TLV, 1 ppm, would accordingly lead to an annual target dose

$$D_{targ,ann} = 0.245 \text{ mMh}$$

The annual rad-equivalent dose is thus ca. 20 rad-equ. At low dose rate this is expected, according to Eqns. 7, 8 above to be associated with a risk

$$P = 20 \cdot 2 \cdot 10^{-4} = 4 \cdot 10^{-3}$$

of dying later in life from cancer due to one year's average occupational exposure to ethylene oxide at a concentration of 1 ppm.

This means that in a population of 100,000 with the exposure discussed, 400 are expected to die from cancer

Employment of the multiplicative model (Eqn 15 in section 4.1.2 above) leads to approximately the same value. For low-LET radiation and ethylene oxide the relative increase in cancer risk was estimated to 0.1% per rad or per rad-equ., respectively (P3). In a given population of 100,000 it is expected that 20,000 will die from cancer (cf. ref. 1). The expectation from the multiplicative model is thus

$$20 \text{ rad-equ./year} \cdot 0.1\%(\text{rad-equ})^{-1} \cdot 20{,}000 = 400$$

For ethene the risk per inhaled amount (in molar units) is about 5% of the values calculated for ethylene oxide. This is due to the low rate of conversion to ethylene oxide in vivo (P12, P20, P21 and ref. 41).

4.3.2 RISK ESTIMATION: COMPARISON OF PROCEDURES

Within a project mainly sponsored from other sources, different approaches for cancer risk estimation have been intercompared. Particularly, this has concerned urban air pollutants with emphasis on ethene, which is metabolized to ethylene oxide (P12, P20, P21).

By and large the rad-equivalence approach leads to risk estimates that are higher, by about one order of magnitude, than the estimates reached by extrapolation from animal data or (for air pollution) by the relative potency approach developed by J. Lewtas[40]. We believe that these apparent discrepancies could have the following explanations:

It should first be realized that the recent increase of the radiation-risk coefficient for cancer mortality from 2% to ca. 10% per Sv (at high dose, high dose rate) was counteracted by a reduction of the same magnitude to account for the release from repair saturation at intermediate doses and dose rates (for dose-rate effects of chemicals see P3). For this reason the previously used, lower coefficient was retained[41]. This coefficient agrees with a relative risk (β in Eqn. 15 above) of 0.1 % of the background cancer mortality per rad or rad-equivalent (cf. preceding section).

In several approaches to extrapolate from animal test data the linear coefficient b of the fitted polynomial model

$$P(D) = 1 - e^{-(a+bD+cD^2+dD^3+....)}$$

is calculated and assumed to be valid at low to intermediate exposure of humans. Here, however, D is given in terms of mg/(kg body weight)·day, i.e. a dose rate. If, instead, the lifetime dose more correctly reflects the risk (as is expected for an irreversible effect such as initiation by mutation; cf. 4.1.2 above), this would lead to an underestimation of the human risk by the ratio of the life lengths of the species, a factor about 35 (70 years/2 years). In the approach adopted by U.S. EPA[26] this underestimation happens to become reduced by the recalculation of doses from mg/(kg body weight)·day, to mg/(m^2 body area)·day. This recalculation, which has the purpose of allowing for differences in metabolic rate, renders man 6 or 14 times more sensitive than the rat or mouse, respectively. One reason for the responsible authorities not to abandon this expression of dose in mg/kg·day or mg/m^2·day, which can be shown to be biologically incorrect, at least for cancer initiators, is the compatibility of the risks predicted by these extrapolation procedures and the risks observed in exposed human populations[42]. There are strong indications, however, that in such intercomparisons of predicted and found risks, the latter are mostly underestimated because of observation times being too short, i.e. a parallel of the increase of the radiation-risk coefficients after prolongation to higher ages of the follow-up of irradiated populations. Another reason for the mg/kg·day or mg/m^2·day approach to appear applicable might be a contribution from a promoter action and other side effects at the high experimental doses. (For promoter action by interaction with receptors or by cell killing the concentration — i.e. the dose rate — is certainly important.) The possibilities of correctly extrapolating from animal data are further

disturbed by the use of testing protocols that exclude the youngest (and perhaps more sensitive) ages and prescribe precocious killing of the animals.

The uncertainties could be eliminated by a proper in vivo dose monitoring provided the rad-equivalence approach (or some similar relative potency method) could be shown to give correct estimates.

With respect to follow-up we are, in the case of the model compound, ethylene oxide (EO), facing the question whether this compound is exclusively a leukemogen in humans (as many tend to believe) or whether also solid tumours are to be expected. We give preference to the second of these alternatives for three reasons:

- EO gives a dose in all organs;
- EO induces cancer of different types in animals;
- The situation is similar to the studies of the A-bomb survivors where in the first 15-20 years, significant increments of solid tumours were not obtained (in recent risk estimates[1] the total cancer risk is about 8 times the leukaemia risk).

Our risk estimates of the other urban air pollutants are higher than those of J. Lewtas for the related reason that her reference standard (occupational exposure in coke-oven and similar work) considers lung cancer only, whereas we have tried to estimate the total cancer risk.

4.3.3 RISK IDENTIFICATION: BACKGROUND ADDUCTS AND ASSOCIATED RISKS

In measurements of various low-molecular weight adducts to Hb, background levels have in many cases been encountered in control animals and in knowingly unexposed humans. Hydroxyethyl adducts seem to originate from ethylene oxide as the first metabolite of endogenously produced ethene, to the formation of which dietary factors and intestinal flora contribute[43]. The

role of hereditary factors, as demonstrated for methylations, is being investigated.

In view of the linearity of dose-response curves and the randomness of individual substitution reactions the observation of and clarification of the origin of the adducts should be seen as an identification of risk factors[11].

This raises the question to which extent somatic mutation from endogenous alkylators and other electrophiles contribute to the background cancer incidence[44], which is by far the largest part of the total cancer risk, or to what extent other mechanisms (e.g. miscoding in DNA synthesis[45], changed methylation pattern, etc.) could operate. The background hydroxyethylations occur at a level of ca. 20 pmol of the adduct to N-termini (valines) per g Hb. The dose corresponding to this level is calculated to the order of $2 \cdot 10^{-3}$ mMh/year or ca. 0.18 rad equivalents per year (P19). The associated cancer risk contribution is therefore expected to be similar to that which is ascribed to the background radiation, on the order of 1% of the total cancer risk.

Background levels of several other compounds, including a number of aldehydes, have been encountered in this work (P16,P17).

4.4 STRUCTURE-EFFECTIVENESS RELATIONSHIPS OF ELECTROPHILIC REAGENTS

For the applicability of reaction-kinetic data (cf. below 4.6) for the risk estimation of electrophilically reactive chemicals and metabolites in general, sterical factors, charge, etc. will have to be considered.

Within the project, and with support from other sources for the experimental work, a different question has been taken up, that is of relevance to the properties of the compound, ethylene oxide (EO), used as a model in many of our studies (P22). At a microscopic scale alkylating agents vary with respect to the ratio of chromosomal aberrations to point mutations

induced, and for EO and other 2-hydroxyethylating agents this ratio is relatively high, e.g. compared to the higher homologue, propylene oxide (PO). If the chromosomal aberrations play a role in carcinogenesis, estimation of the cancer risk of EO would have to take this effect into consideration.

It was early shown in our lab that hydroxyethylation of DNA leads to strand breaks, a possible mechanism of the clastogenic action. It was hypothesized — and later shown — that the breaks were due to alkylation of DNA phosphate-*O*, followed by interaction of the hydroxyethyl oxygen with the phosphorus atom with formation of a configuration with pentavalent P, followed by breakage of one of the phosphate-deoxyribose bonds (for refs. see P22). Certain chemical data indicate that this reaction is less frequent with PO, a possible explanation of why EO is a much more effective inducer than PO of sister-chromatid exchanges in vivo. An analysis of this problem has been initiated with chemical model experiments (ongoing) and biological experiments (P22). The latter illustrate that at equal target dose, effects involving recombination are more effectively induced by EO than by PO whereas for induction of point mutation they are equally effective. (Curiously enough this concerns also induction of SCE in vitro in contrast to the long-term in vivo situation, a problem that is studied further.)

If genotoxic potency is referred to alkylations at n=2, two-(and tri-)-functional electrophiles such as diepoxybutane(s) are, per alkylation at n=2, more effective mutagens and, probably, carcinogens by one to two orders of magnitude[48,49].

This high effectiveness seems to be due to the formation of DNA-base dimers, e.g. by bridging two neighbour guanines. In bacteria this lesion seems to be repaired in the same way as UV-induced pyrimidine dimers. The high carcinogenic potency, particularly in mice, of 1,3-butadiene has certainly to be ascribed to diepoxybutane(s), the dosimetry of which is thus essential to the risk estimation of this compound (cf. P12).

4.5 VALIDATION OF THE RAD-EQUIVALENCE APPROACH FOR THE ESTIMATION OF RISK, AND OTHER APPROACHES BASED ON "MOLECULAR" METHODS

Validation of a procedure for risk estimation has to be carried out by the verification of predicted risks. Projects with this aim have been initiated along the following lines, in several cases using ethylene oxide as a model. (This compound was employed as a model in various studies, mainly because it occurs as the sole electrophile/mutagen in several human exposure situations, with the consequence that unequivocal conclusions from epidemiological data seem to be attainable.)

a) In exposed humans, by epidemiological follow-up of cohorts with a known exposure and with the establishment of the corresponding target dose. For leukaemias the risk was early predicted and confirmed to the order of magnitude in a small group of sterilization workers (with 2 or 3 cases), and a large cohort (700) with a 10-fold excess incidence (8 cases against 0.8 expected) is now studied carefully with respect to received doses during a mean employment time of 10 years. Since also solid tumours, with longer latencies, are expected (cf. 4.3.1), this cohort is followed up. The relative excess of non-leukaemia cancer is, however, much smaller (cf. cancer in A-bomb survivors), and large statistical variation is expected.

b) Observation of somatic mutations and other cytogenic endpoints in human cohorts with known exposure. In an East-German group studied[2] the observed increase in HPRT mutation frequency is compatible with measured blood doses, which however, only cover the last few months of several years of exposure.

c) Measurement of HPRT mutation and other cytogenetic endpoints in exposed animals are going on with ethylene oxide and a series of other compounds.

d) A study at this institute of initiation-promotion induction in mice of skin and liver tumours induced by EO or X-rays + TPA or + CCl_4, respectively, has given results compatible with theory. It has been statistically investigated within this project and will be published (P24).

e) In cooperation with GSF-München (Germany) liver foci induced in young rats by X-rays and EO showed the effectiveness of EO to be some 4-5 times lower than expected. However, X-radiation was given as acute doses (at high dose rate) and EO chronically during three weeks at dose rates < 0.1 rad-equ./min, i.e. at intermediate dose rate. This result thus agrees with both EO and X-ray effects showing the same influence of dose rate (cf. P3). This result will be published (P25).

(Non-toxic doses of EO can hardly be administered at high dose rate, in the meaning rate of alkylation of critical sites in DNA. For studies of the influence of dose rate of hydroxyethylations, experiments with *N*-hydroxethyl-*N*-nitrosourea, which decays more rapidly, have been initiated.)

f) A comparison of monofunctional alkylating agents and low-LET radiation that are equivalent with respect to frequency of induced mutation, has been carried out at the microdosimetric level (P3). At one radiation hit per nucleus, corresponding to a radiation dose of 0.3 (3 mGy), about 6 ionizations occur in the DNA and at 0.3 rad-equivalents of chemical dose (e.g. 3.75 µMh of ethylene oxide) approximately the same number of guanine-O^6 alkylations occur in the DNA. It is investigated further whether this agreement has a scientific meaning or whether it is fortuitous.

g) Regrettably, animal studies of the carcinogenic action of radiation and chemicals have nearly exclusively been carried out in different animal strains. However, studies of published animal cancer test data indicate a high predictive value of the hypothetical degree of alkylation of DNA oxygens, i.e. the type of kinetic studies that were discussed above (section 3.4). It seems

that for monofunctional alkylating agents a risk estimate could be based on a simple expression of the kind:

$$P(D) = a + k_{n=2} \cdot \frac{"D_{abs}"}{\lambda} \cdot F \qquad (17)$$

where $k_{n=2}$ = the 2nd-order rate constant for reaction at n=2,

"D_{abs}" = the absorbed dose in mol/kg body weight (Since D_{abs} is a concentration, it should be called $[RX]_0$ or similar, as in Eqn. 4),

λ = the 1st-order rate constant for elimination through detoxification, chemical reaction and excretion, and

F = constant conversion factor. (In some cases F should include a factor for dose distribution.)

$k_{n=2}$ is in most cases easily determined experimentally and so is λ in animals. For λ in humans, values have to be determined by various approaches, including relative values, following certain adjustment for chemical structure (e.g. sterical factors and charge of the ultimate reagent).

Since the factor F has a general value this result indicates a possibility, primarily for absorbed reactive compounds, to estimate cancer risks without using the risks from ionizing radiation as a reference standard. In equation (17), absorbed amount/λ is an expression for the dose (target dose), which has to be known. Accordingly, for precarcinogens, the human metabolism has to be determined, e.g. in work with biopsy material.

4.6 BIOCHEMICAL AND MOLECULAR EPIDEMIOLOGY

It has long been known that the metabolism of drugs is subjected to genetic control, and from various epidemiological studies it has been shown convincingly that this is the case with xenobiotics in general. This is certainly valid also for the endogenous production of mutagens (P17). Fig. 5 above indicates (by *) a number of points (particularly bioactivation, detoxification,

DNA repair) where a heritable polymorphism could lead to changes in the metabolism of carcinogens and precarcinogens. Since these functions are inducible, the same metabolic steps are also subjected to acquired variation, influenced by, for instance, tobacco smoking.

Various enzymes involved in the metabolism of carcinogens have been measured in epidemiological investigations and correlations have been demonstrated between high rate of bioactivation or deficient detoxification, e.g. of some GSH-transferase, and increased cancer risk[46]. For aromatic amines the polymorphism in N-transacetylase plays a similar role.

Monitoring of such enzyme activities may tell whether a person will develop a high or a low dose from exposure to a xenobiotic or to what extent he is able to repair DNA damage, of great value as a way to understand mechanisms of variations in in vivo doses of exogenous or endogenous carcinogens, with variations in individual risk in consequence. Such measurements seem to identify smokers with excessive P450 IA activity as running a very high risk of lung cancer[47]. In most other cases the levels of these enzymes tend to modify the cancer risk around the population mean value.

It seems that the inherited spectrum of oncogen activities will play a more decisive role for the probability that an initiated cell will lead to a tumour. Apart from rare diseases or conditions with a dramatically high probability of tumour development, the susceptibility to carcinogens is certainly subjected to continuous modification, probably with a polygenic basis. Advances in molecular biology have now offered a number of techniques which can be used to clarify genetic polymorphism etc. of relevance to susceptibility[51]. Current research in this field will undoubtedly lead to the identification of persons with a cancer-prone genotype, irrespectively of whether such knowledge is wanted (cf. P19 and section 1 above).

In order to avoid misunderstanding it should be stressed that the measurement of dose (via adducts), of metabolic characteristics (via enzyme activities) or genetic constitution, has nothing to do with a developing tumour in a studied donor. These measurements are only able to give information on an average susceptibility and the ensuing probability, within a group with the same data, of contracting a cancer. An exception may be the above-mentioned combination of P450A1 with smoking, where the development of lung cancer is highly probable[47].

Measurement, by immunochemical techniques, of products (oncoproteins) of activated oncogens[52] may, in contrast to the measurements so far discussed, be an indicator of early preclinical stages of a developing tumour.

4.7 DISCUSSION: SUMMARY AND LOOK AHEAD

If compared with an investigation limited to a well-defined specific question the present study may make a scattered impression. It was, however, found necessary, in view of the question raised by DOE, to employ a sufficiently broad approach to identify weak points in methods currently used for risk assessment and to analyse the ability of "molecular" research and techniques to eliminate question-marks that were encountered. Holding the various aspects together within a qualitative framework, a coherent picture seems the emerge from the apparent "scatteredness". In fact, the general picture arrived at is sufficiently coherent to justify a publication in monograph form, by and large with the contents of the present report. An enterprise of this kind will, however, be dependent on the availability of certain resources.

In order to facilitate communication and mutual understanding between scientists involved in risk assessment, scientists studying cancer with respect to mechanisms and people involved, at the administrative and political level, in the evaluation and control of risks, it was found necessary to distinguish

doses and dose rates in the classes low, intermediate and high (Figs. 2,3). The limits between these classes are still arbitrary, "low" referring to the doses (and dose rates) where, in experiments according to generally applied protocols, the statistical power is insufficient to generate informative data for biological endpoints, "intermediate" is the dose region where data are mostly compatible with a linear dose-response without threshold, and "high" refers to a region where the response bends upwards, e.g. due to repair saturation. What is called here "intermediate" and "high" is often referred to as "low" and "high", respectively. Human exposures mostly occur at low doses and dose rates, and reaches incidentally the "intermediate" dose region, where disease-epidemiological studies are able to generate data that are informative on cancer risks.

A dose monitoring by macromolecule adduct solves, in principle two kinds of problems, viz.,

- by introducing the high power of chemical analysis it permits detection (e.g. of a priori unknown reactive intermediates) and measurement of exposure-related events within the whole range of low doses where risk may be considered unacceptable;
- it generates data for the calculation of target doses and risk estimation.

Since, adduct measurement is able to generate risk estimates (according to 4.3) that are presumably more reliable than those which can be obtained from long-term animal tests, it has the further advantage that experiments with animals can be reduced considerably. As a matter of fact, the remaining need for such experiments can, after demonstration of genotoxicity in biological or biochemical in vitro tests, be limited to short-term tests with a few animals to clarify the in vivo metabolism to genctoxic products. As far as possible human exposure situations should be utilized to determine the ratio between in vivo dose and exposure dose or absorbed dose.

A disadvantage of risk assessment methods based on adduct measurements or other endpoints related to genotoxicity is that they are in principle not applicable to non-genotoxic carcinogens, such as promoters. For that reason it is important to study dose-response relationships for promoters, particularly with regard to additivity and the existence of no-effect thresholds.

For the purpose of dose monitoring, blood proteins, particularly haemoglobin (Hb), are at present preferred. For DNA as a dose monitor, the kinetics of repair has to be better characterized and methods for quantification and identification of chemical structure have to be developed.

Risk estimation from measured target doses has so far been carried out by a relative potency approach, using risk coefficients for radiation-induced cancer as reference standard. It appears, however, from intercomparisons of chemicals that compound-specific data could be used directly for risk estimation.

The estimated risks would primarily be valid for intermediate doses and dose rates, where linear dose-response relationships may be presumed. Special studies have repeatedly indicated superlinearity of the response in the low dose/low dose rate region, at the same time as sublinear responses and even no-effect thresholds below some very low dose, although so far not encountered in experiments, cannot be excluded. If, therefore, the risk/dose curve is extrapolated linearly into the low-dose region, the uncertainty due to underestimation or, possibly, overestimation has to be calculated from the magnitude of the deviations (in experiments a factor 2-5).

For a solution of this problem, permitting risk estimation based on the true slope of the dose-response curve at low doses/dose rates, the dose-response for a relevant genetical endpoint has to be measured in humans or at least rodents. It appears that "mutational spectrometry" by a combination of PCR and denaturing gradient gel electrophoresis as employed by W. Thilly[3] has the potential of reaching sufficient sensitivity for a determination of the

true dose-response curves. Since the mutation spectra at the DNA level are to a high degree specific to the causative agent, this technique is indispensable also for efforts to understand the determinants of initiations in background cancer incidence, particularly with regard to the role of electrophiles involved in the formation of the observed background adducts.

Important in this context are also the studies of inducible reprogramming of the cells, particularly as conducted by P. Herrlich[36] and co-workers. It appears that a determination of the kinetics of these effects would be informative to the question of dose-response at low doses and dose rates. Possibly, measurable criteria for reprogramming could be used for a monitoring also of promoters.

4.7.1 RECOMMENDATIONS FOR FUTURE RESEARCH

DNA adducts: For long-term dose monitoring, clarification of relevant repair kinetics is an urgent issue (P21) and development of methods (MS/MS?) for simultaneous identification and quantification of adducts is required. The methods at present applied for DNA-adduct monitoring (^{32}P-postlabeling, immunochemical methods, fluorescence methods) are not sufficiently compound-specific for safe identification of chemical structure. This could be done by MS techniques, the continued development towards identification-quantification of DNA adducts is foreseen[53].

Inducible functions with influence on dose-response relationships (primarily bioactivation; detoxification; "error-free repair"; reprogrammation via the fos gene, possibly including "error-prone" repair or mutator genes): clarification of relevant kinetics of induction and persistence of induced conditions. This concerns not only the kinetics following acute administration but also chronic exposure at low level (dose rate)

and with regard to additivity of factors (e.g. components of tobacco smoke). Correlations between induced conditions.

"Induction status": There is a need for methods to determine to what extent inducible functions are induced in tissues in investigated individuals and populations.

Research on mechanisms: Quantitative aspects should be included to an increased degree.

Long-term animal experiments: If and when such experiments are carried out, samples of blood proteins, white blood cells, liver and, when judged important other organs should be collected. Levels of adducts to proteins and DNA should be determined in specialized laboratories and activities of relevant enzymes (such as P450, GSH-transferases) should be measured. This would lead to a data base of great value for research on risk assessment.

Comparison with radiogenic cancer. At a suitable laboratory an animal strain (e.g. B6CRF$_1$ mice used by NTP) used for studies of chemical carcinogenesis should also be exposed to (chronic and acute) doses of low-LET radiation for determination of dose-response relationships for induced tumours.

4.8 TRAINING WITHIN THE PROJECT

The greatest gain from the project has been the employment of a mathematical-statistician (F. Granath) throughout the project period. This has made it possible for him to become acquainted with biological and chemical work within the general problem of risk research, and to develop into a good

biostatistician, able to collaborate effectively in research planning and evaluation of and statistical inference from data. Besides him scientists in the fields of biochemistry and radiobiology (M. Törnqvist, C. Vaca, D. Segerbäck) have worked within the project, for certain periods with full-time employment.

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5.2 PUBLICATIONS WITHIN THE PROJECT

(Appended to this report under the numbers given. Mss. in preparation, denoted *, will be sent after publication has been accepted.)

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*P3. Ehrenberg, L., Törnqvist, M. and Vaca, C. Cancer risks from low doses of ionizing radiation and electrophilic chemicals: similarities and differences (ms. in preparation).

*P4. Ehrenberg, L. Dose-response relationships at low doses (ms. in preparation).

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