

review article

Short term screening tests for carcinogens

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There are now short term tests with a high predictive value for mammalian carcinogens. Many of them are based on the ability to detect damage to DNA in bacteria or mammalian cells after metabolic activation by microsomal enzymes. Their introduction will enable provisional safety assessments to be made for the many thousands of industrial and environmental chemicals for which long-term animal testing cannot at present be considered.

It has been estimated¹ that if one could totally abolish human cancer it would add a mere two years to the average lifespan. Most cancer sufferers are past retiring age so that industrial production would be little affected by the abolition of cancer. The fight against cancer must instead be justified in terms of the cost of hospital services and of basic humanity; treatment of cancer, even when it is successful, is a miserable process. When it fails, as it so often does, one feels guilty of a double offence, not only the loss of the patient, but the imposition of heroic measures that themselves may cause considerable physical and mental suffering.

The International Agency for Research on Cancer holds it as a rule of thumb that around 80% of cancer has an environmental cause^{2,3}; others would give a higher figure⁴. The evidence is indirect, being based on differences in tumour incidence between genetically similar populations in different environments^{5,6}. Even if this estimate is only approximately correct it leads ineluctably to the conclusion that a substantial proportion of cancers, possibly a majority, are in principle preventable. In past decades those responsible for the disbursement of cancer research funds have tended either to look for a breakthrough in the area of curative treatments or to make a long term investment in basic biology in an attempt to understand the disease (or more properly diseases since "cancer" is but a general term for hundreds of different malignant conditions). Recently, however, these two essential approaches have been complemented by a third, the search for the specific environmental factors involved in carcinogenesis.

The nature of these environmental factors is not known in detail, but it seems likely that many of them are man-made or natural chemicals. Even factors such as diet or stress may act indirectly by altering the metabolism of chemicals in the gut or in the body itself. Of course, identification of environmental carcinogens does not necessarily lead to their removal but it does open the way to control so that the risk that they present is no more than is necessary when weighed against any benefits that they may give.

The most direct method of identifying environmental carcinogens for man is based on population studies, but unfortunately it is expensive and seems to have rather low resolving power. Only a handful of chemicals are known to be carcinogenic to man and most of these have been detected following the study of workers occupationally

exposed to chemicals capable of giving rise to specific and rather rare neoplasms. The classic case is soot which has been known for 200 years to produce scrotal cancer in young chimney sweeps⁷. More recent examples are 2-naphthylamine, vinyl chloride and asbestos which produce, respectively, rare cancers of the bladder, angiosarcomas of the liver, and mesotheliomas of the lung cavity. The problems involved in identifying two populations differing only in their exposure to one chemical are formidable and are further compounded if the chemical gives rise not to specific and otherwise rare tumours, but to a variety of common cancers. Population studies are thus likely to be of limited value in identifying environmental (as distinct from occupational) carcinogens but they will be indispensable in providing the basis for risk evaluation, particularly where dose-response data can be obtained.

The alternative is to screen chemicals to which man is exposed. The generally accepted method of doing this is to carry out long term carcinogenicity tests with laboratory mammals. Not only are those tests very demanding of resources but any extension of animal testing on such a wide scale would be vigorously opposed by a number of animal welfare lobbies. In practice, it is inconceivable that resources could be made available (either men, money or mice) on the necessary scale to screen all the tens of thousands of substances to which humans are exposed. Of necessity, therefore, testing with whole mammals will be restricted to certain groups of suspect substances, for example those suspect but already in use on a large scale, or those substances which it is proposed to administer on a large scale, as food additives or cosmetics, for instance.

If one is to screen for carcinogenic chemicals, therefore, one must use short term tests with a high predictive value. I propose to review a number of possible systems which have been suggested in recent years. As will become apparent, many of them are in fact systems for the detection of agents causing damage to DNA. Damage to DNA leading to heritable changes may be important to man not only because of carcinogenicity but because it may cause hereditary disease⁸⁻¹⁰. Moreover, DNA damage may conceivably be involved in ageing and diseases associated with ageing¹¹. I take it as self-evident that any agent likely to damage the DNA of man, whether in somatic or germ cells, is potentially hazardous.

Screening systems

The induction of cancer is but one aspect of long term toxicity and for the evaluation of such hazards a three-tier

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approach has been proposed^{10,11}. The first tier would consist of simple short term sub-mammalian tests with a high predictive value for the human effect ultimately of interest. As many substances as possible should be screened with these tests. Second-tier tests would be both short and long term, on mammals. Only selected, high priority substances would be screened by these tests in addition to the first-tier tests. Tests in the third tier are designed not to detect toxic agents but to evaluate as quantitatively as possible the hazards to man from agents shown to be potentially toxic. Only substances whose use or presence seems inescapable would be subject to the third tier of evaluation, the object of which would be to make a risk-benefit assessment and institute appropriate regulatory action. Tests in successive tiers show in principle increasing relevance to man but this is often accompanied by decreasing sensitivity and practicability.

Not all of the sub-mammalian tests depend upon the postulated electrophilic nature of the active forms of carcinogens and in particular on their ability to react with DNA. Williams and Rabin¹², for example, have proposed that substances might be screened using a test based on membrane-polysome association. They found that a number of carcinogens caused degranulation of rough endoplasmic reticulum (microsomal membranes) from male rat liver. This test has been further developed by Purchase and Lefèvre¹³ who have measured the loss of radioactive RNA from rough endoplasmic reticulum. Preliminary results (D. Anderson *et al.*, unpublished) with a large number of carcinogens and non-carcinogens indicate that the method predicts the activity of arylamines rather well (85% correct) although it is less successful with polycyclic hydrocarbons and direct acting alkylating agents.

The necessity for metabolic activation of many carcinogens by microsomal enzymes prompted the suggestion of McPherson *et al.*¹⁴ that the specific *in vitro* enhancement of biphenyl 2-hydroxylation activity in rat liver microsome preparation might be used as a screening test. They found that of eight known carcinogens, all caused an increase of around 100% in such activity, four compounds whose carcinogenicity is in doubt gave lower but significant increases, and eleven non-carcinogenic compounds gave no significant increase. This test system, like that of degranulation of ribosomes, is obviously promising and in need of a much more exhaustive validation on a scale similar to that used with some other systems.

Metabolic activation

There is a widespread belief among cancer workers that DNA damage is involved in the induction of cancer. That is the basis for the supposition that carcinogens might be detected by the consequences of DNA damage in simple systems. Two recent developments have enabled this possibility to be realised. First, it has become clear that many carcinogens are the products of metabolism of inactive chemicals by mixed function oxidases in the animal¹⁵, and that preparations of liver microsomes can be used *in vitro* to carry out this metabolic activation¹⁶⁻¹⁸. Second, ultra-sensitive bacterial systems, usually involving strains deficient in DNA repair, have been developed for the detection and characterisation of agents causing damage to DNA¹⁹⁻²².

The first published work in which the mutagenic activity of metabolites was detected after metabolic activation of carcinogens was by Malling¹⁹. The methodology of his quantitative liquid assay system has been recently described²⁰. Later, Ames *et al.*²¹ showed that microsomes could be added to the semi-solid agar overlay in a plate test, a procedure that is in some ways rather better for routine screening although it fails with a few compounds, for example, dimethylnitrosamine, possibly because the agar interferes with the diffusion of short-lived active metabolites.

Bacteria deficient in repair of DNA are killed more

easily by DNA-damaging agents than are wild type bacteria, and this is the basis for several simple tests. Bacteria deficient in excision repair have been used²³ but these are sensitive only to certain types of DNA damage. Much more useful have been bacteria lacking DNA polymerase I (Pol⁻)²⁴, or deficient in genetic recombination (Rec⁻)²⁵. These tests are usually conducted on the surface of agar plates but are also amenable to rather more quantitative procedures with liquid-phase treatment^{26,27}.

Another way of revealing the existence of DNA damage is to look for the repair that it usually initiates and this is the basis of a very useful test developed by Stich and his colleagues. It depends on estimating the amount of DNA synthesis involved in repair by measuring autoradiographically the uptake of tritiated thymine during the period immediately following exposure to the test chemical. The method has the advantage that it can be used with cultured human skin fibroblasts. To prevent normal DNA synthesis the cells are kept in an arginine deficient medium for 3 d before exposure.

In a report on 64 substances tested, Han and Stich²⁸ found that all directly acting carcinogens elicited unscheduled DNA synthesis whereas no repair synthesis was observed after treatment with 16 non-carcinogens. Most carcinogens known to need metabolic activation gave negative results although a few were active after prolonged exposure to high concentrations. More recent results²⁹ indicate that metabolic activation systems can be incorporated in this assay and make possible the detection of pro-carcinogens.

Mutation induction

Perhaps the most sensitive assay for DNA damage is the induction of mutations in bacteria, particularly if the bacterial strain carries a mutation rendering it unable to excise damage from DNA (Uvr⁻). Excision-proficient strains should always be included in any assay, however, because certain agents able to cross-link DNA are only mutagenic in such strains³⁰; presumably the mutational event occurs as an error during excision-initiated repair. Reversion to prototrophy is generally regarded as the most sensitive type of assay and the methodology has recently been reviewed^{31,32}. *Escherichia coli* WP2 is a tryptophan-requiring strain that responds to mutagens causing base-pair substitution mutations at both adenine:thymine and guanine:cytosine sites.

A more complete set of tester strains has been developed in *Salmonella typhimurium* by Ames and collaborators³³. Individual strains respond to base-pair substitution mutagens or to compounds causing various types of frameshift. The permeability of these *Salmonella* strains to some chemicals has been increased by the incorporation of a cell wall mutation ("deep rough")³⁴. From recent data one can calculate that these strains are capable of detecting mutagenic activity of between 61%³⁵ and 90% (D. Anderson, unpublished) of known carcinogens. In an attempt to detect the "false negatives" obtained with the deep rough strains, Ames's group developed a fourth generation set of strains containing the drug resistance plasmid pKM101³⁶. As suggested by MacPhee certain plasmids confer a mutator activity on their host cell which becomes more sensitive to many mutagens and carcinogens³⁷. The ability of these plasmid-containing strains to detect carcinogens as mutagens is impressive (see below). A word of caution is in order, however, since although the mechanism by which the plasmids act is still unknown, it is clear that they convert into mutations damage which would not be mutagenic in a normal cell. They may even act as amplifying systems and produce mutations at sites where no damage exists. The value of such strains lies in the correlation they show with carcinogenicity but there is at least a theoretical possibility of real "false positives".

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Group of compounds

Animal carcinogenicity and bacterial mutagenicity with and without metabolic activation (from McCann <i>et al.</i> ¹⁴)			
	Carcinogens detected as bacterial mutagens	Non-carcinogens not mutagenic to bacteria	Compounds of uncertain carcinogenicity detected as mutagens
Amines etc.	23/25	10/12	5/7
Alkaloids, etc.	17/20	1/3	1/1
Polycyclic aromatics	26/27	7/9	1/1
Esters, epoxides, carbamates, etc.	13/18	5/9	0/1
Nitro aromatics and heterocycles	28/28	1/4	0/2
F Miscellaneous organics	1/6	13/13	0/1
G Nitrosamines	20/21	2/2	1/1
H Fungal toxins and antibiotics	8/9	5/5	-
I Mixtures (cigarette smoke condensate)	1/1	-	-
J Miscellaneous heterocycles	1/4	7/7	-
K Miscellaneous nitrogen compounds	7/9	2/4	-
L Azo dyes and diazo compounds	11/11	2/3	3/3
M Common laboratory biochemicals	-	46/46	-
Total	157/178	101/117	11/17

tion is only as good as the confidence one has in the reliability of both parameters. Whereas positive and negative mutagenicity results can be both unambiguous and reproducible, the same is not true of carcinogenicity results where, as will be discussed below, there are several factors which could result in a failure to detect relatively weak carcinogens. As discussed by McCann and Ames¹⁵, there is good reason to believe that many of the "false" positive chemicals will eventually be shown to be carcinogenic. This has already happened with the food additive furoyl furamide which had been used for many years in Japan and had given negative results in two carcinogenicity trials¹⁶. After positive results had been obtained in *Bacillus subtilis* and *E. coli* systems for detecting DNA damage, it was re-examined and shown to produce a low but significant yield for tumours when given to foetal and young mice¹⁷. There is also the real possibility that some of the "false" negatives are genuine, that metabolism *in vivo* is different from that with isolated microsome preparations. Only further studies in depth can resolve this.

It is worth analysing the data of McCann *et al.*, further to see whether there is any particular type of mutational event (as detected by the *Salmonella*) that is correlated with carcinogenicity. It has been postulated¹⁸ that carcinogenicity is associated with the ability to produce specific types of frameshift mutation. This hypothesis does not hold up in any general application. As can be seen from Table 2, whereas most members of some groups of carcinogens (for example, aromatic amines, polycyclic aromatics and nitroaromatics) gave rise to both frameshifts and base-pair substitutions, others (for example, esters, epoxides and carbamates, nitrosamines, miscellaneous nitrogen compounds) gave rise exclusively to base-pair substitutions. There was no group that gave rise exclusively to frameshifts. Taken together, 45.2% of mutagenic carcinogens gave rise solely to base-pair substitutions, 14.8% solely to frameshifts, and 40% gave rise to both.

Rosenkranz (cited in ref. 62) using a Pol⁻ strain of *E. coli* together with the *Salmonella* set without plasmids, has obtained results as encouraging as those of McCann *et al.* with the plasmid-containing salmonellas. Of about 100 compounds tested, 85% of the known carcinogens were detected (91% of direct acting carcinogens, 72% of procarcinogens). The proportion of non-carcinogens detected as positive was rather high, 30%, but the figure is not comparable with the lower value derived from the data of McCann *et al.*¹⁴ since it did not include the 46 common laboratory biochemicals tested by the latter workers, none of which was positive.

A comparison of the efficiency of various microbial systems for detecting DNA damaging agents has been carried out by Shirasu *et al.*¹⁹. They found that the hypersensitivity of repair-deficient bacteria (Rec⁻ *B. subtilis*) was the most sensitive. Of 166 pesticides studied, 23 were posi-

tive in the Rec-assay (carried out without microsomal activation). Of the 143 negatives, none proved to be positive when tested with *E. coli* or *Salmonella* reverse mutation systems. Of the 23 positives 9 were positive in reverse mutation systems, and of these 9, 1 was not detected by the *E. coli* strains and 1 by the *Salmonella* strains. As far as base-pair substitution mutations are concerned, the non-plasmid *E. coli* strains were found to be preferable to the non-plasmid *Salmonella* strains at least with some groups such as nitrofurans. With other groups such as the organic phosphates a similar small proportion of mutagens was missed by both *S. typhimurium* and *E. coli* strains²⁰.

The only study in which a single laboratory has compared a number of different tests for predicting carcinogenicity appears to have been carried out by the Central Toxicology Laboratory of ICI (D. Anderson *et al.*, unpublished). The preliminary results with 120 chemicals point to the value of the bacterial mutation tests when metabolic activation is incorporated. The carcinogenicity, or non-carcinogenicity was accurately predicted for 90% of the chemicals by this test. Cell transformation *in vitro* came close with 83% accuracy. Rather less accurate was degranulation of endoplasmic reticulum, 72%, and morphological changes following subcutaneous implantation, 70% correctly predicted. Sebaceous gland suppression²¹ was good for polycyclic hydrocarbons (90%) but little better than random for other substances (52-62%). Tetrazolium reduction in mouse skin was also poor (62% overall). The authors conclude that some of these rapid tests are capable of distinguishing between carcinogens and non-carcinogens with sufficient accuracy to enable them to be used for selecting potential carcinogens. They also make the point that figures for successful prediction must be treated with

Table 2 Number of carcinogens detected as bacterial mutagens (with or without metabolic activation) classified as to type of mutation induced.

	Base-pair substitutions only	Frameshifts only	Both base-pair substitution and frameshift mutations
A	1	9	13
B	14	2	1
C*	7	5	14
D	13	0	0
E	5	0	23
F	1	0	0
G*	19	0	0
H	0	3	5
I	0	1	0
J	0	1	0
K	7	0	0
L	3	2	6
M	0	0	0
Total	70	23	62

* Data not available for one member.

Key for chemical groups as for Table 1. (From McCann *et al.*¹⁴)

It is possible to improve sensitivity by altering the methodology, and a modified fluctuation test has been proposed which achieves between ten and one hundredfold greater sensitivity than the conventional assay without the need for plasmid-containing strains¹⁷. There is also the possibility of developing a single tester strain that can be used to detect many different types of mutational events¹⁸. Although bacterial screening systems have proved very useful, there is still scope for further improvement.

As well as the bacterial tests that have now been extensively studied, a large number of other techniques can be used to detect DNA damaging activity and may perform a useful supplementary role, probing ambiguous or suspect results and characterising more fully the nature of the genetic damage. One may, for example, study the induction of mutations in cultured mammalian cells¹⁹⁻²¹. Mammalian cells may also be used for cytogenetic study of visible chromosome aberrations²². Recently developed staining techniques for demonstrating sister-chromatid exchanges show a greatly enhanced sensitivity²³ and their role in screening has been recently discussed²⁴. Sister-chromatid exchanges may now be detected in spermatogonia²⁵ and bone marrow cells²⁶ following exposure of the whole animal to carcinogens. It is already clear, however, that although the induction of sister-chromatid exchanges is a very sensitive response to some carcinogens it occurs hardly at all with others²⁷. Other eukaryotic mutation systems include fungi, yeasts and insects. All of these have their own advantages and disadvantages.

Malignant transformation in cultured cells

Rather than develop a model system depending on mutation or DNA repair, others have worked towards a screening method by which transformation to the malignant condition could be brought about and detected in cell culture. The only really valid criterion for malignant transformation is the ability of a cell to produce a tumour when inoculated into an appropriate host. There are, nevertheless, several secondary criteria (discussed by Freeman and Huebner²⁸), of which the most commonly used is the ability of cells to grow into clones in soft agar or, in the case of fibroblast cultures, to produce clones of piled-up cells when growing on a solid surface.

Most human cancers are carcinomas which are derived from epithelial cells. Relatively little work has, however, been done on the transformation *in vitro* of epithelial cells. Such cells are usually obtained from rat liver and are not easy to retain in culture in the differentiated state. Nevertheless they have been successfully transformed by 4-nitroquinoline-1-oxide²⁹, aflatoxin B₁, *N*-hydroxy-2-acetylaminofluorene, and 7,12-dimethylbenz[*a*]anthracene³⁰, dimethylnitrosamine and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine³¹, and *N*-acetoxy-2-acetylaminofluorene³². Epithelial cells transformed *in vitro* usually show no altered morphology although they may grow in soft agar.

Work on fibroblasts is considerably more advanced. Fibroblasts, when transformed, give rise to sarcomas, responsible for a minority of human malignant disease. They show, nevertheless, great promise as the basis of a potential screening system for chemical carcinogens. As with bacterial mutation systems, it has often been found necessary to supplement the fibroblast's relatively poor ability to metabolise carcinogens into their active form. This has been achieved, either by cocultivation with other cells capable of carrying out metabolic activation³³, or by isolating the cells from hamster embryos after treatment of the pregnant mother³⁴.

Much of the work on transformation has been carried out with treatment of mass cultures often for long periods of time and has been subject to some criticism. Transformation has, however, been reported with short treatments followed by cloning³⁵ and this would seem to be a

better approach to adopt in future. A noteworthy feature of much work with both fibroblast and epithelial systems has been the high spontaneous rates of transformation, sometimes considerably higher than one would expect for a gene mutation. Many workers regard this as the result of the artificial environment in which the cells are cultured, and have looked (often successfully) for conditions in which the spontaneous rate is lower. Nevertheless it is likely that the rate of transformation *in vivo* is higher than has been thought and that the body is normally able to deal effectively with the aberrant cells. If this were true the role of DNA-damaging carcinogens might be seen as increasing the already high spontaneous rate and thus overloading the ability of the natural defences of the body to cope with malignant cells.

Most of the work on transformation *in vitro* has concentrated on the development of systems that can be used as models for the study of carcinogenesis (for reviews see refs 55 and 56). To the uninvolved observer, a certain amount of contradiction and inconsistency is apparent. There is, notwithstanding, an impression that this technique may soon be a valued constituent of the battery of techniques for detecting carcinogenic and DNA-damaging substances. Two recent studies have shown successful prediction of carcinogenicity almost as good as that of the bacterial mutagenicity tests^{36,37}.

Validation

Some of the test procedures described above are very sensitive, but how good are they as predictors of carcinogenicity? Recent reports have presented³⁸ and discussed³⁹ carcinogenicity and mutagenicity data obtained for more than 300 chemicals. All the results come from the use of *Salmonella* strains developed by Ames. Where negative mutation results had been obtained with now-obsolete strains, the test was repeated with the latest plasmid-containing strains. Testing was done both without metabolic activation and (generally) with microsomes embedded together with the bacteria in soft agar. With some nitrosamines incubation was carried out with microsomes before plating. A summary of the results is given in Table 1. Since publication, one of the non-carcinogens, 5-hydroxy-2-acetylaminofluorene, has been shown to be mutagenic because of the presence of an impurity and has now been reclassified as non-mutagenic.

It can be seen that of 179 compounds whose carcinogenic effect on animals is well documented, 157 (or 87.7%) were detected as positive in the bacterial test. This level of confirmation was obtained with essentially all types of compound and was also evident for the small group of compounds for which evidence exists for carcinogenicity in man. The proportion of compounds believed to be non-carcinogenic which gave negative results in the mutagenicity tests was also high: 101 out of 117 (or 86.3%). These included 46 common biochemicals all of which were negative. Seventeen compounds were tested for which carcinogenicity data are uncertain; of these 11 were positive in the mutagenicity test.

The apparent "false" positives and negatives have been discussed elsewhere⁴⁰. It is apparent that many of the latter damage DNA or cause mutations in other systems, or have mutagenic metabolites. Furthermore there is a very obvious limitation to the use of liver microsomes for activation: some chemicals may need reductive activation, or may be metabolised by the gut flora, by organs other than the liver, or by cell components other than microsomes. Indeed it is surprising that liver microsomes are as effective as they seem to be; certainly the method is capable of further improvement.

When one considers the "false" positives, that is the supposed non-carcinogens that register as mutagenic with bacteria, certain difficulties become apparent. Any correla-

some caution since they can be manipulated within wide limits by the choice of substances tested. As their substances include a large number of non-carcinogenic chemicals closely related to known carcinogens they feel that their results give a reasonably good indication of the likely value of the tests in practice.

DNA damage and human cancer

The correlation between mutagenicity and carcinogenicity is satisfying to those who believe in the somatic mutation theory of cancer²² and distressing to those who do not²³. I think the correlation can be more correctly described as being between DNA damaging ability and carcinogenicity. Gene mutation is but one consequence of DNA damage; others such as chromosomal structural rearrangements, virus integration and excision, and changes in gene expression, may well be important in the carcinogenic process. Non-genetic effects are also probably involved.

One could argue that detecting DNA damage is merely a very sensitive way of detecting electrophilic reagents, and that the actual target(s) may well be in other molecules as well as or instead of DNA. This is quite possible; but there is other evidence strongly implicating DNA damage as the rate-limiting step in many carcinogenic processes.

In man, for example, mutations in five complementation groups are known to reduce or abolish the ability of cells to remove ultraviolet photoproducts from their DNA²⁴. In all cases they enormously increase sensitivity to the carcinogenic effect of sunlight (resulting in the hereditary disease xeroderma pigmentosum). A further mutation causing the same symptoms has been shown to be associated with a deficiency in another DNA repair pathway active on newly synthesised DNA²⁵. Another human mutation responsible for the disease ataxia telangiectasia has been shown to block repair of ionising radiation damage²⁶ and also results in proneness to develop malignant disease²⁷. Thus, the human data strengthen our confidence in the reality of the observed correlation between DNA damaging ability and carcinogenicity. It must be emphasised, however, that even an empirical "litmus paper test", with no known theoretical basis, which gave an 80 to 90% predictiveness for carcinogenicity would be a powerful tool in the screening of chemicals for human toxicity.

The place of tests with mammals

No single test is adequate for a first-tier (sub-mammalian) screen: most authorities agree that a battery of tests must be used as false negatives may occur with any one test. The results of these tests would be used to assign priorities for further testing using mammalian systems. At one extreme, a substance with no apparent effect on sub-mammalian systems might be given a priority so low that no further tests would be considered unless a large human population exposure were to occur or be contemplated. At the other extreme a strongly active substance might well be regarded as hazardous without further testing if the population exposed were small. If it were, say, an industrial chemical, then production workers and users ought to treat it as if it were a known toxic agent or carcinogen, at least until such time as it became possible to carry out full scale animal tests.

The greatest problem in testing for carcinogenicity or mutagenicity with mammals is the insensitivity of most of the tests. This has led, for example, to difficulties in validating microbial carcinogenicity screening systems since many of the "false" positives obtained with these are based on animal experiments that may be inadequate²⁸. There have been, and still are, too many carcinogenicity tests with 20 or 30 animals per group.

Provided that the number of animals in each group is kept small, even a large increase in the frequency of neo-

plasms can fail to be statistically significant and enable a conclusion of "non-carcinogenic" to be drawn (see for example a recent study on the carcinogenicity of hair dyes²⁹). As long ago as 1954, Barnes and Denz³⁰ pointed out that to detect with a probability of 0.01 an effect occurring in 1% of the animals, one would need a group of at least 455 animals. If the effect also occurred spontaneously then the number of animals per group would have to be increased manyfold. Today, notwithstanding, carcinogenicity experiments with 100 animals per group are often regarded as "good" and those with 200 animals per group are extremely rare. But although "kilomouse" experiments are theoretically attractive there may be little to be gained from them in practice. Logistical problems dictate that such experiments be phased over many weeks and involve slightly varying conditions. "Spontaneous" rates of tumour occurrence unfortunately often vary in time and place, perhaps reflecting slight differences in diet, and it is often difficult to run an adequate control group. Errors in handling are also more likely in very large experiments.

Whereas a significant reproducible positive result in a mammalian test may be taken as indicating the existence of a potential hazard for man, a negative result taken should not necessarily be taken to indicate the absence of hazard, particularly if the human population to be exposed is very large, and the number of animals in the test small. We may take some comfort where the disparity in dose between the animal and human exposure is great. This is not always so. Anaesthetic gases, for example, are given to an appreciable fraction of the population in Western society at concentrations which are not far from the lethal level. One might well feel that a negative result in a screening test with a few dozen mice would be of little value.

It can be seen that mammalian tests are not wholly appropriate for the validation of sub-mammalian tests, and it is perhaps remarkable that they should show such good agreement. Validation of one type of test against another must not blind one to the real objective, which is to predict long term toxic effects in man. There are few proven human carcinogens and most of these can be detected by both mammalian and sub-mammalian tests. In man, carcinogens tend to be recognised only when the tumour is of a rare type and there is a sufficient cluster of cases to enable association with a particular occupation to be seen by an alert clinician. Genetic effects in man are even harder to detect retrospectively. There are several examples of somatic chromosome damage in lymphocytes of persons exposed to known mutagenic and carcinogenic substances (for example, vinyl chloride³¹, ozone³², benzene³³, toluene³⁴, cadmium^{35,36} and methyl mercury³⁷). Recent evidence for the possibility of dominant lethal damage in man by vinyl chloride (P. Infante, unpublished) and anaesthetic gases³⁸ is ominous but needs closer examination.

Ultimately quantitative risk assessments must be attempted, for we must face the unpalatable fact that man will almost certainly have to be exposed to some carcinogens and mutagens whose benefits cannot be dispensed with and others which it is impracticable to eliminate from the environment. Risk evaluations at the present time almost always require information which is not available, such as the nature of the dose-effect response at low doses. Nevertheless, approaches must be found that will lead eventually to risk-benefit evaluations based less on guesswork and more upon knowledge. In the meantime the use of short term tests would enable potentially carcinogenic substances to be identified among the many thousands for which long term animal testing cannot at present be contemplated. This in turn would open the door to provisional regulatory action to minimise human exposure. Taken seriously and on a large enough scale, there is good reason to believe that this approach would ultimately result in a reduction in the incidence of chemically induced cancer.

I thank Drs D. Anderson, E. J. Ashby, P. A. Lefèvre, E. Longstaff, I. F. H. Purchase, J. A. Styles and F. R. Westwood of ICI Central Toxicology Laboratory, and Dr Bruce Ames for allowing me to see their data before publication.

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articles

Isolation and N-terminal amino acid sequence of membrane-bound human HLA-A and HLA-B antigens

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Membrane-bound HLA-A and HLA-B antigens have been extensively purified in good yield. The sequences of the N-terminal 16 amino acids have been determined using about 1 nmol of protein eluted from polyacrylamide gel after electrophoresis in sodium dodecyl sulphate.

The major histocompatibility region of man (HLA) occupies at least 1 to 2 recombination units on chromosome 6 and probably contains a large number of genes involved in diverse immune and possibly other functions¹. The gene products that have so far been identified with this region comprise various

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MEDICHEM 4TH INTERNATIONAL CONFERENCE

HAIFA, ISRAEL

7-10 SEPTEMBER, 1976

Institutional Interactions in Problems of Occupational Health:

An Industry View on the Lessons from Vinyl Chloride

by

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Institutional Interactions in Problems of Occupational Health:
An Industry View on the Lessons from Vinyl Chloride

A W Barnes

I have been asked to speak this morning about "Institutional interactions in problems of occupational health". The views I want to express are personal ones: they derive solely from my personal experiences over the past 2½ years when I was closely involved with the vinyl chloride problem. My involvement, and that of my colleagues in the general management of the European PVC industry, was in part as a layman in a highly specialised field where the initial contributions had to be made by experts and professional people from universities, government and research associations. As industrial general managers, and with our trade union colleagues, our prime responsibility was to see that all possible steps were taken at the maximum speed to ensure the safety of the plant workers: ultimately though I believe we also made some contribution through our experience in bringing together the views of experts in different specialist fields, because, as generalists, the critical appraisal and pulling together of expert opinion is part of our normal daily jobs. Once our colleagues outside industry had found that our objective was precisely similar to theirs - i.e. the full safety of our workers - the result was an enormously fruitful co-operative effort involving everyone who could help. Indeed, if it were not for the personal tragedies which preceded and initiated all this effort, we would have good reason for feeling entirely pleased with the way groups of people from different disciplines and countries, from different institutions and with different responsibilities, came together to solve an immensely difficult problem.

Of course we had our difficulties in the early days. Industry seems nowadays automatically to be cast in the role of the villain of the piece by much of the media, and a suspicion that this might be true exists in the minds even of reasonable people. Our first task therefore was to convince people that we were just as concerned as they - perhaps more so - to see that our staff worked under safe conditions: but this was not too difficult since the statement was true, and the people we were involved with in government, universities and trade unions were willing to listen, and then to observe that our actions confirmed our words.

Another difficulty initially was the belief that this was a medical problem and that only doctors therefore could speak with authority, even when it came to framing legislation. But a doctor is not a lawyer nor yet an engineer: nor is he an expert on industrial control processes or analytical techniques; and rarely do we find, in any one doctor, an expert epidemiologist, toxicologist, oncologist, clinician and statistician combined! Yet all these and many other disciplines were necessary for the definition of the problem and for its resolution; and some of these resources industry could provide. The barriers between the medical profession and industry were real and potent because they prevented, initially, the dialogue which alone could lead to a full understanding of the situation: but with goodwill they were broken down and have led, I believe, to enhanced mutual respect and indeed to many lasting friendships.

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One of the bigger problems, which persisted throughout the period, was the effect of reports and comment from American sources. The character of some of these came as a total surprise and one of the major lessons many of us learnt from the vinyl chloride problem was that American information had to be treated with extreme care. America is enormously important because of its resources, and its influence throughout the world; but its method of debating matters of public concern is very different from the custom in Europe. The glare of publicity which attends these debates certainly seems to encourage some scientists to speak out with a certainty that is hardly justified by the quality of their information. Whatever the reasons, many American reports were of doubtful validity because they frequently gave information which was incomplete or unconfirmed or simply inadequately analysed. Their impact nevertheless was major: by heightening the feeling of alarm, they increased the pressure on European institutions to take up extreme positions, as in the USA, and this, if it had happened, would have made co-operation and rational analysis extremely difficult. Fortunately it didn't happen: medical institutes, government departments, trade unions and industry realised that, while taking full note of US information and comment, their proper task was to develop a European response, independently, to the problem. In this context it is interesting to note a remark by Dr Marcus Key, who was director of NIOSH at the height of the vinyl chloride crisis. Speaking at the Royal Society of Medicine Conference in September 1975 he commented that in Europe we seemed to have reached roughly the same end point as the USA without the tensions and trauma surrounding the generation of the OSHA regulations.

How did we achieve this, first nationally and then internationally in Europe; and how did representatives of institutions from many different nationalities and with widely different interests and experience learn to work together? At the national level, I can speak more knowledgeably of the UK, although similar processes, I know, developed in the rest of Europe.

At an early stage in the UK, the government set up a Tripartite Working Group composed of senior representatives from government, the trade unions and industry. It is important to note that, at no point, did this become a negotiating body made up of three factions. From the beginning, it was one group with a single common purpose: it owned the problem as a group and it was committed, as a group, to the solutions it developed. This attitude is well exemplified by one of its actions: a smaller tripartite group was formed from the main committee to visit all the UK PVC plants. The purpose of this was not to check that management was doing the right things - though we had the opportunity of seeing this too - but to allow everyone on the plant, from works manager to plant operator, the opportunity of checking that we, who ultimately had to recommend standards and codes of practice, were doing our job properly. On each visit an open forum was held to allow the works staff to fire questions at us so that they could determine to their own satisfaction that we knew what we were talking about. The standard of debate and of information throughout was at an extremely high level: and a very important result of the visits, was that the tripartite group and the workforce throughout the industry were very firmly united in a common endeavour.

Internationally, the European PVC industry formed a series of vinyl chloride committees under the auspices of CEFIC (the European Council of Chemical Manufacturers Federations); and within these committees a great deal of co-operative work was done in both the technical and medical fields. As a

result of this, representatives of the main committee were able to speak with authority for the whole of Europe whenever a contribution from industry was required. This was no mean achievement, for Badische and ICI and Solvay and Montedison are just as much institutions in their own right as is the University of Louvain or the University of Wurzburg or the Mario Negri Institute.

But one of the most constructive of European moves was the invitation to CEFIC from Directorate General V of the Commission to join with their Ad-Hoc Committee on MAK values in considering and advising on a possible Commission directive. This committee was composed largely of distinguished medical academics and the meetings provided the first opportunity for a dialogue between European industry and the European medical establishment as well as with the Commission itself. Of course there were some misunderstandings initially, for industry was talking the language of science and technology while our colleagues were talking the language of medicine. As an example may I quote the initial belief among our medical and commission colleagues that if a directive were to lay down a maximum of, say, 10 ppm (averaged over any 8-hour period) then industry would be able to operate at a constant level of 9.5 ppm. They had not appreciated that the products of industrial processes are rarely constant day in and day out, and that there is always a variation about a mean. Because of this, for 10 ppm never to be exceeded, plants must be operated, on average, well below 5 ppm and will frequently be producing results of 1 or 2 ppm! So a legal requirement for 10 ppm not to be exceeded, is very close to a medical requirement that average exposure should be below 5 ppm!

Fundamental difficulties of this type were relatively quickly sorted out however because of the willingness of members of the committee to meet and discuss the problems on an individual basis. For instance, Professor Maltoni from Italy and Professor Lauwerys from Belgium paid day-long visits to ICI's plants in the UK and Professor Foa arranged for one of his Italian colleagues to visit us also. Similar exchanges occurred in the other countries of Europe and, quite soon, we were all talking a common scientific language and progress was rapid.

Within DGVI of the Commission initial progress was not quite so positive. This Directorate-General, which was concerned with the control of PVC for packaging foodstuffs, also remitted the problem to its scientific advisory committee but unlike DGV made no formal invitation to industry, or other interested parties, to contribute. The committee, composed largely of toxicologists, had the impossible task of proposing control levels for vinyl chloride in foodstuffs, where the human exposure levels were already a million times lower than any level at which carcinogenic effects had been observed. Not surprisingly, their conclusions could owe little to toxicological data; and in an understandable effort to ensure the absolute maximum in safety while still, they believed, supplying a practicable solution, their first proposal was that "vinyl chloride should not be detectable in food or potable water by an agreed method. Attempts should be made to develop generally applicable analytical methods with a sensitivity of the order of 0.001-0.002 mg/kg."

An obvious philosophical objection to this early proposal was that it related the control level simply to the state of advancement of analytical science which of course is in no way related to the question of hazard from vinyl chloride. Of even more concern to industry, though, was that

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it was a totally impracticable solution since the problem of variation of results about a mean had again been overlooked; and the problem here was complicated further since vinyl chloride reaches a foodstuff by a slow diffusion process. In addition to the normal variation in any industrial process, which is to a degree controllable, a further factor therefore is the period of storage of the product, which is not. To ensure a non-detectable amount in any foodstuff at any time, it would be necessary to control at the factory or packing station at a level say of 1/10th of non-detectable! A moment's reflection will make it clear that this is somewhat difficult to measure! - and therefore impossible to ensure. Effective control of an industrial process demands the facility to measure key parameters of the system while they are still within specification, so that any trend away from the norm and towards the specification limit can be detected and then corrected.

Our concern, within industry, was such that the CEFIC committee produced a discussion paper which some of you may have seen. It is the only document I know which attempts to bring together, in an orderly fashion, all the facts and factors relevant to a particular aspect of the vinyl chloride story. Its purpose was to encourage debate on matters of judgement and, if necessary, to allow argument in areas of disagreement - but always disciplined by facts and figures. I believe it has proved of some value and that in this part of the vinyl chloride problem - as in that concerned with worker protection - real progress became possible once we had found a way for medical experts, government representatives and industry, to challenge and to educate one another in rational debate.

These are but a few examples of how, in Europe, we learnt to work together. In all they add up to a story of some considerable success: but before we get too complacent I think we have to ask the question "Did we do - have we done - all we could?". I'd like to spend the rest of this paper in explaining, as a layman in a medical field, why I believe we have not.

It seems to me that vinyl chloride is almost unique among known human carcinogens. Our knowledge of its effects on animals, through the distinguished researches of Professor Maltoni, is more extensive than for many, perhaps most, other carcinogens; and our knowledge of its effect on humans, because of the rarity of angiosarcoma of the liver, is in almost all cases precise and unambiguous. It must be rare to find so clear-cut an effect on a human population in conjunction with such thorough animal experimentation. I would have expected that the data on vinyl chloride would have been seized upon to see what more it could teach us about carcinogenesis in general. But what we have had is a flood of papers on epidemiology by epidemiologists, on toxicity by toxicologists and on metabolism, clinical treatment, immunology, chromosome aberration, etc. Any attempt at analysis of the human data on a world scale has been minimal and most references to Professor Maltoni's work have been limited to a superficial treatment of the results from his experiment BT1. It is indeed startling to observe that, from all of Professor Maltoni's massive research, the only conclusion that has actually been used in the field of industrial hygiene is the conclusion that vinyl chloride is a carcinogen. As an employee of one of the companies who sponsored Maltoni's work, I feel bound to ask whether the cost and effort, on our part and on his, was worthwhile if this is the only useful information we can extract.

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Why has the yield been so poor? Could it be that the medical profession itself is getting a little over-specialised and that vinyl chloride has been seized upon by the specialist as a useful vehicle for testing out theories and advancing knowledge in his own specialism? Is it possible that rather more interaction is required within the medical profession and that we need the interest of one or two good medical "generalists" who can pull together all the data and begin to answer some of the outstanding questions? Only you can say whether these are foolish questions or whether they are worthy of some consideration. But the fact remains that there are questions still to be answered.

General to the whole problem of carcinogenesis and toxicity is the relationship between the results of animal testing and the effect of the toxic substance on humans. How many times in the past 2½ years have I heard the remark that we don't know how to relate the one to the other; and while we remain in this ignorance the value of work like Professor Maltoni's is diminished. I cannot help feeling some surprise that there has been so little effort to analyse the human data on vinyl chloride and to relate this to Professor Maltoni's comprehensive findings. Of course any relationship we find for vinyl chloride will not provide the key for all future problems but it would at least be one more step along the road of understanding.

There remain two major questions, specific to vinyl chloride, which demand more application by all of us. The first asks whether there remains any risk to the consumer from food, wrapped in PVC, at the infinitesimally small levels of vinyl chloride which can still be detected: and if so, what the level of risk is. The problem for toxicologists is that there is no experimental animal data on vinyl chloride at the minute exposure levels we are talking about: but even if there could be, they would still be faced with the problem of relating rats to humans and this is a permanently insoluble problem if we confine our studies to evidence from ingestion. There would be no possibility of obtaining convincing human ingestion data even if the risk were a significant one - which is of course completely contrary to our belief. Yet, tantalisingly, there exists a great deal of data on the effect of inhaled vinyl chloride, on both rats and humans, which, as I've already indicated, could give us some pointers to the relative sensitivities of the two species. The need is to achieve some definition, albeit imprecise, of a risk; for in matters of public concern qualitative generalities have little - or too much - impact depending on the speaker. The problem is to find some way of bringing all the results together to bear upon the question.

Within industry, we were greatly indebted to my colleague Dr K S Williamson who pointed out that all known results, inhalation, ingestion, human and animal, could be examined in one table by defining exposures in mgm/kgm of body weight; and that, when this was done, a degree of consistency could be observed across all the results. Starting from these unified results, and with the help of published work by Schneiderman, Mantel and Bryan and the late Dr Leo Friedman (this last incidentally in a very relevant WHO report which has received surprisingly little attention) the CEFIC discussion document was able to suggest a value for the maximum residual risk from foodstuffs of the order of 1×10^{-10} , or some 80,000 times lower than the risk, calculated by Friedman, arising from the consumption of 70 gms of "charcoal-broiled" steak daily. The analysis we have done is an elementary and quite unsophisticated one: our answer is certainly imprecise - how could it be otherwise at these levels? - but wherever we

have had to make assumptions of our own, rather than drawing on the published work of specialists in the field, we have tried always to be conservative. What is disturbing to me is that I know of no other published attempt to determine the order of magnitude of the risk. I recognise the immense difficulty of being able to speak with certainty when results have to be extrapolated over at least six orders of magnitude. But where the need is great, some effort should be made: in its absence the discussion is left wide open for pure speculation undisciplined by any facts.

So far I have discussed two problems which require us to apply much more thought to the relationship between Maltoni's animal results and the known human data. A third problem exists which is, for industry, much more important than the previous two and which concentrates our attention exclusively on better analysis of human information.

Throughout the past 2½ years one group of people has shown a maturity and a sense of responsibility and of determination which is an example to the rest of us. This is the industrial workforce actually engaged on producing PVC. Clearly all of us - medicals, engineers, chemists, legislators - had to establish conditions which were safe for work, but, with that achieved, our obligation to the work people is not yet fully discharged. We, and they, know that, in the future, there will be further cases of angiosarcoma of the liver among vinyl chloride workers, deriving from the higher exposures of earlier years. When these occur, there will be an inevitable diminution in their sense of security; and doubts will naturally arise, for them and their wives and families, about the effectiveness of the new safety measures. If this happens, we who helped to devise the regulations will not have done our duty if we can only reproduce the same qualitative reassurances that we were using in 1973 and 1974. With the finalising of regulations around the world the job - for doctors, administrators, statisticians and general managers - is not finished. We must be in a position to reassure those most directly concerned that we have done our job well; and this requires us to pursue our research and our analyses further and further towards a quantitative conclusion.

What, you may ask, can be done? I'd like to devote the last section of this paper, to a brief review of what has been done on data analysis and then to suggest a scheme for further work. These latter proposals derive from many discussions I have had with my colleagues Dr Williamson and Dr Paddle of ICI who would be much better equipped to present them today: I must present them in simple layman's terms for that is the only way I know.

Until early 1976, the only formal document which surveyed the world data on the effect of vinyl chloride on humans was the NIOSH list of angiosarcoma cases among vinyl chloride workers. But this was only a list; no analysis of any kind was attempted.

In April 1976, the CEFIC document, to which I have already referred, made a first step in classifying the human data. It listed the cases according to the particular plant on which they occurred; and it summarised its findings in the following (up-dated) table.

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TABLE 1

<u>Number of A/S Deaths/Plant</u>	<u>Number of Plants</u>	<u>Location of Plant</u>
10	1	USA
9	1	Canada
3 each	2	USA
2 each	6	Germany, Sweden, France + E Europe
1 each	13	USA + Europe
—	—	
50	23	
—	—	
No cases reported	~20 plants with >20 years life	

Many of us believe that this rather puzzling distribution can only be explained on the basis of differing exposure levels, many years ago, from one plant to another and that this is the first indication that some sort of dose-response relationship might be deduced for man. If it does - and Maltoni has certainly demonstrated one for rats - then we should be doing all we can to determine it. Before considering how this might be done, let us see whether the crude data can tell us anything else.

If the deaths are tabulated, by year of death (where we know this) we get the following information.

TABLE 2

<u>Years of Deaths</u>	<u>Numbers of Deaths in Period</u>	<u>Average Number of Deaths/Year</u>
Before 1961	0	0
1961-65	2	0.4
1966-70	10	2.0
1971-75	24	4.8
1976 so far	4	>4.0
	—	
	40	
	—	

At first sight this is alarming since the number of deaths/year appear to be on a rapidly ascending curve; but before we proceed, it is worth looking at another classification, which I have not seen published elsewhere. If we group the deaths according to the year of first exposure, we get results which I have summarised in Table 3.

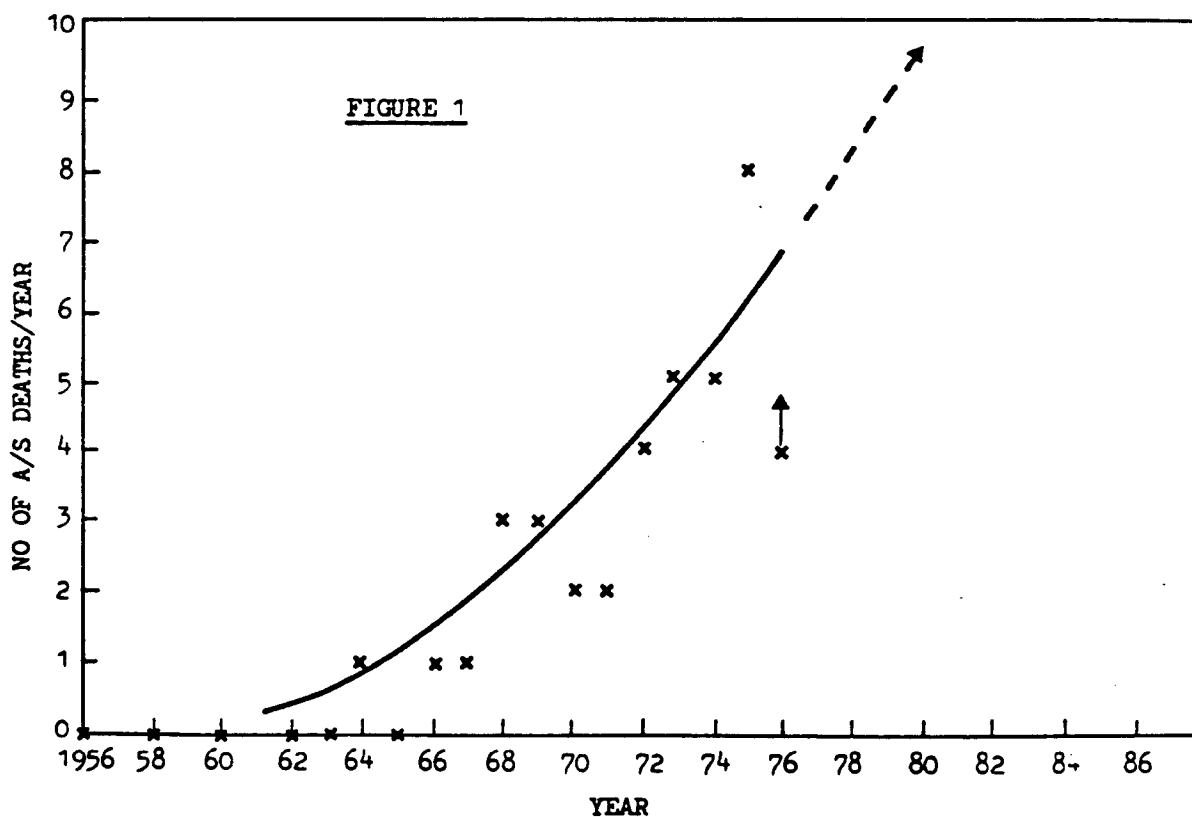
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TABLE 3

<u>Years of First Exposure</u>	<u>Number of Cases</u>	<u>Latency Period from First Exposure</u>	
		(a) Range (years)	(b) Number of Cases With <15 Years Latency
1943-54	28	15->32	0
1955-66	14	9-20	8

In the second of the two 12-year periods the fact that we have no latency periods > 20 years is not of course surprising. The elapsed time has not yet been sufficient for longer latencies to appear; and regrettably we must expect further cases to arise. What is interesting, however, is that 8 out of the 14 cases in this period have latencies less than 15 years whereas, in the first period, no latencies as short as this have been reported. It seems difficult to believe they did not exist: indeed this analysis seems to provide the clearest evidence yet in support of the suggestion that, prior to 1961 and possibly later, deaths from angiosarcoma were under-recorded.

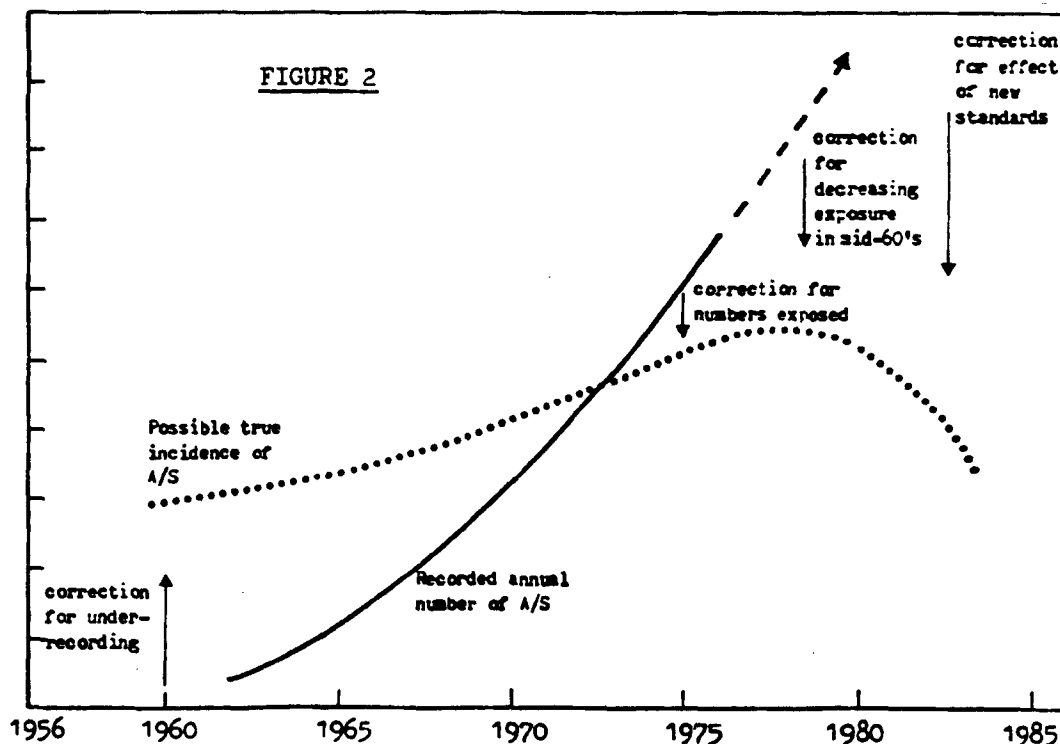
We can best see where this takes us by looking at some simplified graphs. If we plot actual numbers of deaths per year against the year, we get the following picture (Figure 1), which is disturbing if true.



SPI- 12414

However, we have seen that early deaths are probably under-recorded, so the real line has, almost certainly, a lower slope than this. Further, the industry, and the numbers of people becoming at risk each year, grew rapidly in the 1950's and 1960's so that if we were to plot the incidence of deaths, i.e. the number of deaths each year as a proportion of the population at risk, say 20 years earlier, then we would expect the righthand side of the graph - and its extrapolation - to decrease relative to the earlier years. A further factor still is that exposure levels have steadily decreased over the past 20-30 years, from values of possibly 1000 ppm in the 40's and 50's to values below 200 ppm in 1973, immediately before the recognition of vinyl chloride as a human carcinogen. Since we believe there is a dose-response relationship, these changes should result in a decreasing incidence by the late 1970's. By 1985, the effect of the latest standards should be quite marked; we must expect, still, some 30 year latencies dating from 1955 (but what about Maltoni's BT3 results?) but there ought to be no 12 year latencies dating from 1973, and I hope there will be few 20 year latencies dating from 1965.

Figure 2 shows the original line of Figure 1 with arrows suggesting likely changes because of the factors listed above. A very notional revision is indicated by the dotted line.



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Can we get a better idea of where this new line really is? As an amateur who doesn't therefore see the difficulties may I suggest

- (a) that our statisticians might be able to give some estimate of the number of missing cases in the early years and
- (b) that industry, with some difficulty, ought to be able to estimate the yearly populations at risk.

To estimate the effect of reduced exposures, we need to know what the exposures have been over the whole period and we need to know the dose-response effect for humans. These are much the most difficult problems; but indications of exposures have been given by industry spokesmen and some refinement of these must be possible. With these available, the human mortality data may be susceptible to analysis. In addition, we need a detailed development of a dose-response relationship for rats, from Maltoni's data. At best this relationship may be cohesive with and supportive of any tenuous relation we develop from human information: at worst we may have to use it alone, possibly with arbitrary factors applied, since the crude data does seem to suggest that rats may be more sensitive than man to vinyl chloride.

A dose-response relationship is essential for the last correction which requires the calculation of the risk of getting angiosarcoma in 1935 after exposure to only a few ppm of vinyl chloride. This may be difficult, but we have a duty to the people in the industry to attempt it. Perhaps we do not have to be overprecise, for if we are correct in believing our new standards to give a large margin of safety, the calculation of extremely low risk at these exposures may not be too sensitive to errors in the extrapolation.

All this then needs to be done. What is being done? Within ICI, my colleagues Dr Williamson and Dr Paddle are working on the problem. but it is difficult within one company to achieve everything, for the data available to us in ICI may not be representative of the world situation. Nevertheless I believe they will be able to publish some preliminary observations shortly, and I know they would welcome comment, suggestions, or involvement with them, on the subject.

The word "involvement" brings me back to my subject of "interactions". When the task - of devising safe operations - was clear, many institutions in Europe interacted well and achieved much. I've tried to show that much remains to be done: so let interaction, co-operation and contribution flourish still. As another of my colleagues, Dr John Stafford, has put it "the sooner we address and apply ourselves ... unreservedly to ... the task ..., the sooner we shall have really well-founded data to satisfy ourselves, our neighbours, customers, critics etc that the VCM health problem has not only been satisfactorily contained but finally solved."

