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CHAPTER 1

What is wrong with the way we test chemicals for carcinogenic activity?

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1. Introduction

If the concept of what you are looking for is wrong, then you may not recognise it when you find it.

In this chapter, I endeavour to show why carcinogenicity testing is in a mess and why present endeavours are unlikely to lead to any reduction in the overall human cancer burden. The main thrust of my thesis is that non-genotoxic mechanisms in carcinogenesis, including hormonally based mechanisms, need to be taken far more seriously, both in the design and in the interpretation of laboratory tests for carcinogenesis.

2. Historical background of present concepts of carcinogenesis

Not so very long ago, cancer was generally regarded as an 'Act of God' (or of His evil counterpart!). In the 1950s and early 1960s the main thrust of cancer research was to screen chemicals, not for carcinogenic activity, but for chemotherapeutic activity. The concept that human cancers might be prevented if one could identify and eliminate exposure to carcinogens in the environment has only been widely held during the last two or three decades. Of course, there was evidence that the concept was viable long before that. For instance, one may cite Sir Percivall Pott's (1775) observation of an association between the exposure of chimney sweepers to soot and their increased risk of developing cancer of the scrotum and other skin sites. Also, there was the evidence that mule spinners in the cotton industry were at increased scrotal cancer risk as a consequence of exposure to inadequately refined mineral oils (Henry, 1928). And there were several other well-known associations between particular occupations and increased risk of specific forms of cancer.

The first successful use of animals to demonstrate carcinogenic activity occurred only 72 years ago (Yamagiwa and Ichikawa, 1918) while it is only just over 50 years since the first demonstration of cancer induction by a pure chemical was reported (Cook *et al.*, 1933). The idea that it would be worthwhile empirically to screen chemicals to which man is exposed for carcinogenic activity is of much more recent origin still. Even, during the late 1950s and early 1960s, when I was recommending that chemicals in everyday use should be evaluated for carcinogenic potential, I seemed to be a voice crying in a wilderness in which vast sums of money were available for cancer chemotherapy screening, but hardly any for research aimed at preventing cancer.

Eventually, but only some 15–20 years ago, the idea that cancer-prevention

is better than cure and that the testing of environmental chemicals for carcinogenic activity was sensible caught hold and then, before we knew where we were, the flames got out of hand. Thus, in less than two decades the popular perception changed from cancer being an 'Act of God' to its being, in 80–90% of cases, a consequence of exposure to man-made chemicals in the environment. Of particular importance in bringing about this change in perception were the studies in migrants (e.g. from Japan to the USA and Hawaii—Haenszel, 1961) which indicated that patterns of cancer incidence are much more determined by environmental than by genetic factors.

In order for the idea that exposure to particular chemicals might cause cancers in man to be seen as plausible, it was necessary to be able to show that the chemicals in question could produce tumours in experimental animals. For various reasons, this demonstration was unduly delayed. Impatient investigators did not wait long enough for tumours to appear after animals had been exposed to putative carcinogens. Also, the animals available for them to use were generally full of infectious and parasitic diseases, with the consequence that they tended not to live long enough to develop neoplasms even when investigators exercised commendable patience. In parallel with these difficulties was the fact that there seemed to be no rhyme or reason in the results that were obtained in experiments involving the exposure of laboratory animals to chemicals. Some apparently chemically unreactive compounds were found to produce tumours, whilst other apparently equally unreactive compounds did not.

Although the structure of DNA was not known until the 1950s (Watson and Crick, 1953), long before then it was perceived that cancers might be the result of mutations in somatic cells, and that chemicals might produce cancers by giving rise to changes in the genetic information held within the nuclei of cells. However, the correlation between demonstrable carcinogenicity and demonstrable mutagenicity was relatively poor and full of anomalies. By demonstrating that chemically inert compounds could be converted within living organisms to chemically reactive (electrophilic) species as a result of 'metabolic activation', the Millers (Miller and Miller, 1976) vastly extended the horizons of research into carcinogenic mechanisms. The subsequent development, notably by Bruce Ames and his colleagues (Ames *et al.*, 1973), and also by many others, of relatively simple and cheap methods for screening chemicals for DNA-damaging, mutagenic and chromosome-damaging activity in the absence or presence of enzymes needed for metabolic activation provided sensitive new tools for exploration of the relationship between mutagenicity and *in vivo* carcinogenicity.

It is just at this point that things began to go wrong. Many interacting factors came into play and began to distort the picture. From Table 1, which lists some of these factors, it will be seen that distortion stemmed, not from scientifically demonstrated facts, but from concepts and beliefs that have insubstantial foundations.

Table 1. Seven concepts with insubstantial foundations which have distorted approaches to carcinogenicity testing

1. That all mutagens are carcinogens and all carcinogens are mutagens.
2. That the *in vitro* demonstration of mutagenicity implies cancer risk *in vivo*.
3. That carcinogenesis is a two-stage process that begins with *initiation* brought about by somatic mutation followed by *promotion* which involves the proliferation of mutant cells.
4. That most chemicals are non-mutagens and non-carcinogens and only a very few chemicals possess these activities.
5. That only man-made xenobiotic chemicals can cause cancer while all naturally occurring chemicals and endogenous substances lack carcinogenic potential.
6. That hormones, homeostatic regulators and simple physiological disturbances are irrelevant in relation to the risk of cancer development.
7. That, if there were no exposure to xenobiotic environmental carcinogens, there would be no risk of developing cancer.

3. *The relationship between mutagenicity and carcinogenicity*

Recognition of the importance of metabolic activation led some investigators to postulate that *all* chemicals that increased the incidence of any kind of tumour in laboratory animals did so by causing mutations in somatic cells, if not directly, then after metabolic activation. It followed that, if a chemical that had been shown to increase cancer risk *in vivo* gave negative results in tests for genotoxicity, then it was simply because the appropriate enzymes necessary for metabolic activation happened not to be present. Consequently, more and more elaborate test systems were developed with the aim of proving that what appear to be non-genotoxic carcinogens are in reality genotoxins.

The other side of this belief was the view that all mutagens are potential carcinogens, and that negative results in animal carcinogenicity tests, or in epidemiological cancer surveys, merely reflect the insensitivity of such methods for detecting carcinogenic activity (Tomatis, 1979).

It is particularly the latter concept which, by its very nature, can neither be proved or disproved, that has done more than anything else to impede progress towards understanding the factors and mechanisms that really contribute to the present human cancer burden.

Nowadays it is, of course, widely recognized that *in vitro* tests for genotoxicity cannot be used for ranking genotoxins in terms of potency. Also, in so far as some such systems purposely exclude DNA-repair processes, they may give rise to qualitatively false impressions. In real life exposure is rarely to single substances and much more often it is to mixtures. The fact that some substances can act as antimutagens and others as co-mutagens serves yet again to diminish the validity of extrapolating from *in vitro* test results to predict the *in vivo* carcinogenic response. In so far as many of the deficiencies of *in vitro* test systems for genotoxicity do not hold for *in vivo* systems involving realistic routes of exposure, positive results in *in vivo* tests for genotoxicity are

certainly more useful predictors of possible cancer risk. However, even here the cautious approach of assuming that the danger from untestably small doses of an agent is proportional, on a linear dose–response basis, to that of testable large doses has limited plausibility in many instances.

4. *The two-stage theory of carcinogenesis*

The evolution of the two-stage theory of carcinogenesis based on the experimental skin carcinogenesis studies of Peyton Rous and his colleagues (Rous and Kidd, 1941; MacKenzie and Rous, 1941) and Berenblum and Shubik (1947, 1949) has provided a great stimulus for research in carcinogenesis since the early 1940s. As a consequence a galaxy of new terms came into common use including: tumour initiation, tumour promotion, co-carcinogenesis, incomplete carcinogen. Unfortunately, many who use these terms today are largely unaware of the very limited factual basis on which they came into being. All the original studies related only to the skin. Peyton Rous and his colleagues showed that deep wound healing or the application of turpentine, which by themselves did not produce skin tumours in rabbits, could do so after animals had been painted with coal tar or a 'subcarcinogenic' dose of a known carcinogen. Berenblum and Shubik (1947, 1949) reported that croton oil could elicit skin tumours in mouse skin which had been previously treated with benzo[a]pyrene but not in previously untreated skin. Most, if not all, the tumours studied by both those sets of investigators were in fact benign warts and not true cancers. Furthermore, it later transpired that the so-called 'subcarcinogenic' doses of coal tar and benzo[a]pyrene used to *initiate* tumour formation were in fact within the carcinogenic range. Worse still, it eventually became clear that the putatively non-carcinogenic irritants, such as the croton oil used by Berenblum and Shubik to *promote* tumour development in mice, were in fact complete carcinogens if treatment and observation were continued for a long time. Even the excitement created by the suggestion that ethyl carbamate (urethane) might be a pure initiator (Graffi *et al.*, 1953; Salaman and Roe, 1953) was eventually shown to be unfounded when it was reported that skin tumours would develop in mice exposed only to urethane and without the subsequent application of a so-called tumour promoter (Deringer, 1962; Iversen and Astrup, 1984). It is probably still valid in the case of mouse skin to distinguish between potent carcinogens, which give rise to relatively little epidermal hyperplasia, and weak carcinogens, that cause pronounced and prolonged epidermal hyperplasia. However, it is no longer tenable to believe in the existence of 'pure initiators' and 'pure promoters'.

As far as other tissues are concerned, there is limited experimental support for the concept that potent hyperplasia-producing agents may increase the risk of tumour development in animals previously exposed to a small dose of a

known carcinogen (Salaman and Roe, 1964). For example, benign tumours could be produced in the forestomach epithelium of mice by successively exposing them to a small dose of benzo[a]pyrene followed by repeated doses of an irritant essential oil, lime oil, (Pierce, 1961). Also, the implantation of roughened glass beads into the bladder of mice that had been previously exposed to ethyl carbamate led to the development of tumours of the bladder epithelium (Ball *et al.*, 1964). However, in both those instances, the hyperplasia-inducing irritant (i.e. lime oil in the case of the skin and the glass beads in the case of the bladder epithelium) proved capable by themselves of producing tumours of the same kinds at the same sites, albeit in lower incidence.

The fact is that less than adequate scientific rigour underlies most claims to have demonstrated two-stage carcinogenesis. According to the theory as propounded by Berenblum and Shubik, the *promoter* is non-carcinogenic and has no effect on tumour incidence if applied *before* the so-called *initiator*. In practice, many investigators who have used the term *promoter* in the interpretation of their experimental findings have never checked whether this condition is fulfilled.

This is particularly true in relation to liver carcinogenesis. Here many investigators blithely refer to non-genotoxic agents as liver tumour 'promoters' on the grounds that they enhance the effects of known liver carcinogens even though they have not checked the outcome of administering the two agents in question in the opposite order.

The attractiveness, simplicity and plausibility of the two-stage hypothesis have had the unfortunate effect of closing the minds of some investigators to alternative hypotheses. Moreover, once a substance has been branded as a 'tumour-promoter' strong resistance develops to accepting that it is, in reality, a complete carcinogen. This has happened in the case of TPA (12-O-tetradecanoylphorbol 13-acetate), the phorbol ester which has replaced croton oil as the main tool for two-stage carcinogenicity studies in mouse skin. TPA has, in fact, come to be seen as the archetype 'tumour promoter' and has been the subject of hundreds of studies in which the evidence has been ignored that it is, despite its lack of mutagenic activity, as much a complete carcinogen as, for example, benzo[a]pyrene or any of the carcinogenic nitrosamines. Those who continue to suffer from this delusion should read the reviews of Olav Iversen (Iversen and Astrup, 1984; Iversen, 1987).

5. *How common are DNA damage and somatic cell mutation?*

For many years it has been clear that the natural environment is hostile and not friendly. Its hostility is expressed *inter alia* as a continuous barrage of attacks on the DNA in cells of all creatures, including man. Such attacks are likely to

be registered as mutations in sensitive strains of the repair-deficient organisms used in *in vitro* tests for genotoxicity. However, under normal *in vivo* conditions, most of the daily burden of DNA damage is either (i) repaired or (ii) not repaired, but without dire consequence, because the cells which have suffered it lack the ability to become the progenitors of clones of altered cells because they are not stem cells, or because they have been fatally damaged. For these reasons, it is not surprising that there is no more then a very weak association between the occurrence of DNA damage and the enhancement of cancer risk secondary to DNA damage to somatic cells.

Clearly it is not DNA damage *per se* which is important but the identity of the cells which suffer the DNA damage, the nature of the damage, whether it is readily repaired, and whether the repair process is error-free or error-prone. In the case of some known potent carcinogens (e.g. certain nitrosamines) there is considerable knowledge about the precise nature of the DNA damage and the possibilities for its repair. However, in screening, important and biologically significant DNA damage is not distinguishable from less significant or insignificant forms of DNA damage.

6. *The extent of exposure to genotoxins*

As Bruce Ames (1983) has pointed out, man lives in an inescapable sea of genotoxins. Apart from being bombarded by u.v. emissions from the sun, cosmic radiation and various forms of ionizing radiations from terrestial sources (e.g. radon, granite), he is inevitably exposed to numerous genotoxic substances that occur naturally in plants (Ames called them 'Nature's Pesticides'). Man consumes those genotoxins in his diet—destroying some but adding others by cooking. In addition, DNA-damaging electrophiles are generated endogenously during the metabolism of food ingredients. Notwithstanding these facts, the aim of carcinogenicity testing hitherto has been to detect and completely avoid the presence in food of minute traces of xenobiotic agents (either as food additives or contaminants) which have genotoxic potential, irrespective of the fact that many natural food constituents are, or may be, potent genotoxins.

7. *Quantification of carcinogenic risk*

Because concepts have been absurdly misdirected, carcinogenicity testing to date has been *qualitative* rather than *quantitative*. Xenobiotic substances have, for the most part, been tested one at a time at maximum tolerated doses

and, if animals exposed to them have developed more tumours than corresponding controls, their use has been restricted or banned. This has often happened even though positive results have only been obtained in response to dose levels that are orders of magnitude higher than those to which man is exposed. The assumptions have been made that genotoxicity is responsible for the enhancement of tumour risk, irrespective of the availability of supportive data from genotoxicity tests, and that some carcinogenic risk is likely to be associated even with very much lower levels of exposure.

If it had been true that most substances are non-carcinogenic and that very few are carcinogenic, then it might have been tenable to think in terms of banning or trying to ban all the carcinogenic ones irrespective of the mechanism by which they predispose to tumour risk. But this is not the position. The truth is that a very large number of environmental chemicals are both potentially genotoxic and carcinogenic under conditions of high dosage. Therefore, the emphasis must be on trying to distinguish truly hazardous exposure situations from circumstances of minimal or negligible hazard. If, for instance, we concentrated on identifying and then reducing man's exposure to, say, the ten most important environmental carcinogens, whether natural or man-made, and whether genotoxic or not, we would be much more likely to influence the present cancer risk from environmental carcinogens than simply by testing *ad nauseam* and banning or trying to ban a limited number of chemicals just because they are man-made.

During the last few years, Ames *et al.* (1987) have begun to try to introduce some order into the presently chaotic situation by ranking chemicals positive in animal tests for carcinogenicity according to their apparent potency and to the extent of human exposure to them. I applaud this as being a major step in the right direction, but it still falls short of overcoming all the problems. Its weakness is mainly that no attempt has been made to consider the mechanisms by which different agents may predispose to tumours of different sites and kinds.

8. The importance of hormones and non-genotoxic life-style factors as determinants of cancer risk in man and laboratory animals

Doll and Peto (1981), in their assessment of the factors contributing to death from cancer in the USA, considered that dietary factors were responsible for 35%, alcohol for 3%, reproductive and sexual behaviour for 7%, and infections of one kind or another for perhaps 10% of cancers in humans; altogether these factors accounted for about 55% of cancer deaths. At present virtually no carcinogenicity testing is directly concerned with investigating any

of these factors. Additionally, Doll and Peto (1981) considered that 30% of human cancers were attributable to the smoking, chewing or sniffing of tobacco in one form or another. Although much money and time is devoted to tobacco-related research, little of it comes within the category of carcinogenicity testing. Indeed, it has proved difficult, if not impossible, to produce lung tumours in laboratory animals by exposing them to smoke (Mori, 1964; Dalbey *et al.*, 1980; Dontenwill *et al.*, 1973; Davis *et al.*, 1975; Wehner *et al.*, 1981) although laryngeal neoplasms have been produced in hamsters (Dontenwill *et al.*, 1973), and skin tumours have been produced in mice (Wynder *et al.*, 1953). Adding the 30% to the 55%, in Doll and Peto's opinion 85% of human cancers stem from factors which are not presently the subject of carcinogenicity research. What of the other 15%? Doll and Peto suggest that 4% of human cancers may be due to occupational factors (although not necessarily exposure to carcinogenic chemicals), less than 1% to industrial products, 2% to pollution, 3% to geophysical factors and 1% to medicines and medicinal procedures, with 3% unknown. Current carcinogenicity testing concerns only the 4% of cancers attributed to occupation, the 2% to pollution and the 1% or $< 1\%$ contributions of food additives, medicines and industrial products. *Thus, carcinogenicity testing and the whole paraphenalia of national and international control of chemical carcinogens is probably relevant to less than 10% of all human cancers.*

Nor were Doll and Peto alone in providing the basis for such a conclusion. Higginson and Muir (1979) concluded that 30% of cancers in men and 63% in women were attributable to what they called 'life-style' factors, by which they meant factors 'such as lack of dietary fibre, excess fat and caloric intake and possibly hormonal carcinogenesis'. Earlier, Wynder and Gori (1977) had concluded that dietary factors contributed 40% of cancers in men and 57% in women.

Despite the conclusions of these eminent epidemiologists, laboratory investigators working in the field of experimental carcinogenesis and carcinogenicity testing have, to a large extent, ignored dietary and life-style factors, and have turned a blind eye to the fact that a large number of the tumours seen in untreated animals as well as in animals exposed to test substances arise from endocrine glands or hormonally influenced tissues. In many cases, experimentalists involved in this area of laboratory research have allowed themselves to be seen as mere 'lump-counters', while biologically untrained statisticians have taken over the key role of deciding which substances are carcinogens and which are not, and no-one makes a serious attempt to distinguish real from theoretical carcinogenic hazards.

The simple fact is that, in laboratory animals in long-term tests for carcinogenicity, as in humans, life-style factors rather than the particular chemical under investigation are the major determinants both of how many tumours develop and of their types. Furthermore, among the array of life-style factors, quantity and composition of diet seem to be the most important.

Table 2. Percentage of human[a] and rat[b] tumours that are (a) endocrine/hormonal, (b) epithelial non-endocrine, or (c) non-epithelial non-endocrine

	Human		Rat	
	Male	Female	Male	Female
Endocrine/hormonal	8·4	35·0	75·6	94·8
Epithelial non-endocrine	91·6	65·0	15·1	3·5
Non-epithelial non-endocrine			9·3	1·8

a Based on cancer registry data (Waterhouse, 1974)
b based on benign and malignant tumours seen in 86 control male and 86 control female rats in a study reported by Kociba *et al.* (1979).

Elsewhere, I have pointed out that overfeeding of rats profoundly influences the incidence particularly of endocrine tumours (Roe, 1981, 1987 a, 1987 b). Furthermore, endocrine tumours and tumours of tissues, such as the breast and uterus, which are very directly under sex-hormone control, constitute a very high proportion of the tumours observed in most carcinogenicity studies in rats. Table 2 compares the incidences of tumours in these categories in men and women with those in male and female rats. The human data used are based on cancer registry data (Waterhouse, 1974) and the rat tumour data on figures published by Kociba *et al.* (1979) for untreated control Sprague-Dawley rats (86 of each sex) in a carcinogenicity study on 2, 4, 5-T. Needless to say, 'like' is not being compared with 'like' in this table. However, to achieve this is not possible. Tumour incidence data for laboratory animals are compiled from information collected from full-scale detailed necropsies to which all animals are subjected, whereas cancer registry data in the main represent just those cancers which are evident during life plus a few that are discovered incidentally at necropsy. Complete necropsies are performed only on a small minority of humans, with the result that many small, clinically silent neoplasms in humans are not recorded in cancer registry data.

Even though the data for rats and humans summarized in Table 2 are not comparable, the differences between the two species are sufficiently striking to make one wonder how appropriate the laboratory rat is as a model for man in terms of the spectra of tumours to which they are prone.

In order to try to obtain data for rats that are more comparable with cancer registry data and, at the same time, study the effects of diet restriction on cancer risk, my colleagues and I undertook a study (Salmon *et al.*, 1988) comparing animals fed for 24 hours/day on a standard laboratory diet with animals given access to the same diet but for only 6·5 hours/day. The former visited their food basket for repeated little snacks on 15–20 occasions during the 12 hours of darkness and refrained from eating during the daytime. By contrast, the restricted animals, which only had access to food between 9 a.m.

and 3.30 a.m. (i.e. during the daytime) helped themselves to one very large meal when the food basket was provided and another very large meal just before the food basket was removed. In addition, some animals took a third meal halfway between the other two. The method of diet restriction reduced food consumption and body weight gain to approximately 80% of the values for 24 hour/day-fed rats. Very dramatic differences were observed between the 24 hour/day-fed 6·5 hour/day-fed rats in longevity, in death before 2 years because of malignant neoplasia (Table 3), in the incidence of non-fatal neoplasms found incidentally in decendents (Table 3), and in benign and malignant neoplasms found incidentally in terminally killed rats (Table 4).

Table 3. Deaths from malignant neoplasia and incidences of neoplasms found incidentally in decendents before 2 years

	24 hr/day-fed		6·5 hr/day-fed	
	Male	Female	Male	Female
No. of rats	60	40	20	20
No. (%) of deaths	26 (43·3)	13 (33·3)	2 (10)	4 (20)
No. (%) of deaths from malignant neoplasm	10 (16·7)	6 (15)	0 (0)	1 (5)
No. (%) of deaths from malignancy in the two sexes combined	6/100 (16)		1/40 (2·5)	
No. of benign tumours found incidentally in premature decendents				
— Pituitary	6	9	1	1
— Other endocrine	4	0	0	0
— Non-endocrine	2	0	0	0

Table 4. Tumour incidence in terminally-killed rats at 2 years

	24 hr/day-fed		6·5 hr/day-fed	
	Male	Female	Male	Female
No. of rats killed at 2 years	34	27	18	16
No. (%) with malignant neoplasm	3 (8·8)	3 (11·1)	0 (0)	0 (0)
No. (%) with benign neoplasm				
— Pituitary	11 (32·4)	12 (44·4)	3 (16·7)	4 (25)
— Other endocrine	11 (32·4)	6 (22·2)	5 (27·8)	0 (0)
— Non-endocrine	9 (26·5)	2 (7·4)	2 (11·1)	2 (12·5)
Total number of neoplasms	34	23	10	6
Mean number of neoplasms/rat	1·0	0·85	0·56	0·38
Mean number of neoplasms/rat for the sexes combined	57/61 (0·93)		16/34 (0·47)	

Not only were the effects of time-restricted access to food dramatic and highly statistically significant, but of special interest is the broad spectrum of malignant tumours which occurred in the 24 hour/day-fed animals as compared with 6·5 hour/day-fed rats (see Table 5). Whereas only 1 out of 40 diet-restricted rats developed a malignant tumour (a cholangiocarcinoma) before 2 years, no less than 22 of 100 rats fed 24 hr/day developed malignant tumours, of which 16 were fatal before 2 years. Furthermore, the kinds of malignant tumours which these animals developed constituted a wide histopathological spectrum. Whereas most of the benign tumours seen in the overfed rats (59/72 = 81·9%) were of endocrine glands, most of the malignant tumours (5/9 = 55·6%) in those animals did not originate either in endocrine glands or in obviously sex-hormone-controlled tissues (e.g. mammary gland, uterus).

Despite the publicity given during recent years to the reduction in background tumour incidence that can be achieved in carcinogenicity tests by diet

Table 5. The lists of malignant neoplasms which developed in 24 hr/day-fed and 6·5 hr/day-fed rats

Males	24 hr/day-fed (60 rats)	6·5 hr/day-fed (20 rats)
Before 24 months	1 Transitional-cell carcinoma of renal pelvis 3 Zymbal gland carcinomas 1 Ependymoma of brain 1 Glioma of brain 1 Thyroid follicular carcinoma 1 Sarcoma arising next to spinal column 1 Abdominal myxosarcoma 1 Generalised lymphosarcoma	None
Found in rats killed at 24 months	1 Squamouse carcinoma of mouth 1 Anaplastic carcinoma of hind limb 1 Mesothelioma	None
Females	**24 hr/day-fed (40 rats)**	**6·5 hr/day-fed (20 rats)**
Before 24 months	1 Squamous carcinoma of tongue 1 Squamous carcinoma of cheek 2 Mammary adenocarcinomas 1 Ependymoma of brain 1 Thymic sarcoma	1 Cholangiocarcinoma
Found in rats killed at 24 months	1 Carcinoma of nose 1 Mammary adenocarcinoma 1 Adenocarcinoma of uterus	None

restriction, there has been very little movement towards the routine use of diet restriction in carcinogenicity testing. Belief that regulatory authorities would not accept the results of studies conducted under conditions involving dietary restriction is the principal reason; devaluation of (in my view, almost worthless) historical control data is the second reason. The third reason often given is that diet restriction does no more than delay the development of neoplasia so that, to be meaningful, tests conducted in diet-restricted animals would have to be continued for a longer period than in overfed animals (e.g. 2·5 or 3 years instead of 2 in the case of rats) or that, if tests were stopped after only 2 years, they would be far less sensitive than if overfed animals were used.

It is not possible on the basis of the data in Tables 3–5 to predict what the eventual tumour incidence would have been in the diet-restricted animals had they been allowed to live for much longer. However, it is reasonable to question whether a test system in which the end points used are subject to so much uncontrolled variation (i.e. animals are free to overeat and give themselves cancers prematurely or not as they wish) is suitable for detecting and measuring the carcinogenic potential of chemicals.

There are, of course, many life-style factors other than caloric intake which await evaluation in rodents. What effect does exercise have? What effect does freedom to indulge in sexual activity have? Are uniparous or multiparous females at the same risk of cancer development as life-long virgins? Experiments that throw some light on the answers to these questions are currently in progress. My impression of the preliminary results of these studies is that none of these variables influences the risk of tumour development to anything like the same extent as caloric intake.

9. *How does diet-restriction work? Are life-style factors genotoxic or non-genotoxic?*

I can only guess the answers to these questions. My guess is first, that mutations (probably several in the same cell) *are* essential for neoplasia. However, a mutation is not necessarily the first event, as suggested by the two-stage hypothesis. Where cancers develop in the wake of obvious hormonal disturbance, it is suggested that hormone-stimulated increases in cellular proliferation, cell turnover and/or metabolic activity render tissues more susceptible to unrepaired genotoxic damage than hormone-unstimulated cells. Further, in this context we need to consider not only the limited number of classical hormones known to be secreted by endocrine glands, but also the ever-increasing number of homeostatic regulatory peptides and neurotransmitters. It may well be that prolonged disturbance of homeostasis underlies the risk of development of many kinds of neoplasia by creating a situation in which cells are more likely to undergo cancerous mutation in response to

exogenous and endogenous DNA-damaging electrophiles, of which there is certainly no shortage in the background environment.

If this view were correct, then it can be deduced that the overfeeding of laboratory animals, which renders them obese and prone to a wide variety of endocrine disturbances, also makes them more susceptible to the adverse effects of exogenous and endogenous mutagens.

If overfed, endocrine-disturbed animals are exposed to potent carcinogens, then adverse treatment-dependent effects on tumour incidence may still be seen against the background of huge numbers of endocrine and other spontaneous neoplasms. However, such a background is just not suitable for detecting a weak carcinogenic effect. Furthermore, since exposure to exogenous chemicals in high doses can interfere with tissue homeostasis in many different ways, it is not surprising that essentially non-genotoxic substances (e.g. lactose) can be readily shown to predispose to tumour development (Roe and Baer, 1985).

One further pitfall must be mentioned. Not only is the correlation between genotoxicity and *in vivo* carcinogenicity poor for the many reasons discussed above, but it is probably even weaker than is sometimes suspected. Non-genotoxic carcinogenesis is not rare, but common. Thus, many drugs which have been shown to lack genotoxicity in an impressive array of tests nevertheless produce one or other form of tumour in animals exposed to high doses for prolonged periods. In other cases, an isolated positive result among many tests for genotoxicity may be seen as in some way 'confirming' an isolated positive result in an *in vivo* test, whereas the truth of the matter is that the positive result in the genotoxicity test is a misleading artefact and the positive finding in the *in vivo* test is dependent on a non-genotoxic mechanism.

10. Conclusions

In the light of the above considerations I suggest:

(1) More research should be devoted to the investigation of life-style and hormonal factors which influence tumour incidence in rodents.
(2) Pending the availability of the results of this further research, all carcinogenicity tests in rodents should be conducted under conditions of dietary restriction, even though this would require trials to be continued for longer than at present.
(3) Far more attention should be paid to the importance of non-genotoxic mechanisms in carcinogenesis, both in the design and interpretation of carcinogenicity tests in rodents.

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