VBr vs VCl

Benzyl
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# TRANS-SPECIES AND TRANS-TISSUE EXTRAPOLATION OF CARCINOGENICITY ASSAYS

Vinyl Chloride

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Mankind has from the earliest times endowed animals with specific human attributes. The language reflects many such attributes as, for example, "wise as an owl," "cunning as a fox," or "assinine." Soothsayers have used the detailed examination of the morphology of outbred animals to predict man's future. It is relevant to our present society that none of these attributions were scientific absolutes; they could be manipulated to suit the current political exigenices. Our current mysticism dictates that we regard all agents that induce cancer in animals as having the same effect in man. Although there is some evidence for this, and it represents a cautious view, this again is by no means a proven scientific absolute.

Today, it is prudent, that is, politically expedient, to believe that an agent which induces tumors in any species of animal will be a potent, and therefore, regulatable, carcinogen in man. It is my purpose to ask how we, as a society, might take apart the assumptions underlying this prudent concept

and replace it with something scientifically better defined.

Gross Differences Between Humans and Laboratory Animals

Slide #1 The first slide lists some of the more apparent differences between man and laboratory animals. Such properties tell us little about the likely relative potencies of an agent in different species. Longevity might lead us to suspect that man may be more sensitive to carcinogens because in a long-living animal, tumors have much longer to develop. However, the proportion of men and animals that develop tumors in old age is not all that dissimilar, and we have to look to differences in prereplicative DNA repair and the efficacy of immune processes to counteract the effects of species variations on longevity and tumor development. The effect of the size of man relative to rodents indicates that in man, many more cells are at risk; again, restorative processes must be invoked. The menstrual cycle in women and non-human primates might be predicted to affect hormone-responsive tumor development in a qualitatively or quantitatively different manner from the estrous cycle in rodents. There is no evidence for this, probably because so little work has so far been done on non-human primates. If the menstrual cycle can indeed be compared to psuedo-pregnancy in mice, maybe some more definite statements might be made.

Slide #2 Even when we consider the detail of the mechanisms of the chemical induction of cancer and compare: 1) metabolic activation of procarcinogens, 2) electrophile interaction

with critical targets, 3) DNA repair or replication to lock in chemically-induced lesions, as well as 4) the development of tumor progenitor cells to frank clinical cancer, we fail to observe many significant qualitative differences between species. The guinea pig, for example, is resistant to 2-FAA carcinogenesis and in vivo, fails to convert a significant proportion of this procarcinogen to its proximate form, N-hydroxy-2-FAA. Despite this series of observations being the key to the development of the current electrophilic activation theory of chemical carcinogens, the evidence on which both these pieces of evidence are based is by no means watertight. Although a total of 60 guinea pigs were used to test the lack of carcinogenicity of 2-FAA, the maximum survivals of 2.5 to 3.0 years may be somewhat less than desirable for a negative experiment. I have noticed recently a report by Takeishi in Mutation Research that perhaps the guinea pig can, after all, N-hydroxylate 2-FAA; it differs from other species in its pronounced ability to detoxify this metabolite in vivo. Even this one well documented example of a species difference in metabolism corresponding to a species difference in carcinogenesis must be regarded with some suspicion. I do not believe that such differences are, at this stage, likely to help in attaining meaningful criteria for trans-species extrapolation of carcinogenicity tests.

Slide #3

Potency

The observation that there are few, if any, gross physiolo-

gical conditions that help us to explain, let alone predict, qualitative interspecies differences in carcinogenesis means that we must, if we are to succeed, make our judgments on a quantitative basis. That is to say, our task is to explain differences in potency of carcinogens in species A compared to Species B. First, we must define potency in a measurable and, as far as possible, meaningful way. As a start, let us examine the premise that:

Slide #4

$$\text{Potency of a Carcinogen} = \log_{10} D_{L50}$$

That is to say, using a standard experimental protocol, potency is the negative of the logarithm of the dose $D_{L50}$ (in micrograms/week/kg) required to induce a 50% incidence of tumors in a lifespan experiment. This definition has advantages. Furst, there is no need to extrapolate the dose requirement to one tumor in a population of $10^6$ or some other equally stupid figure. In many cases, it will be found that the figure for 50% tumor induction will be an interpolation of the data, rather than an extrapolation. Second, because we demand a full lifetime experiment, toxic doses which markedly shorten longevity will often be discarded using this definition. I have used log dose simply to compress the range of values and to avoid any undue emphasis on small differences. It will been seen to have other advantages later. I would have liked to use the mean plasma concentration rather than dose for systemic tumor induction, because that might allow a range of protocols to be compared. For standard carcinogens, plasma concentrations are seldom available. Therefore, the experimental

Slide #5

protocol used to assess potency must be stated. Typical potencies of experimental carcinogens fed to rats or mice are shown. These are very approximate, but serve, if nothing else, to indicate their wide range. (Slide 2)

Slide #6

Let us now see what is the potency of 2-naphthylamine in dog, hamster, rat, and mouse. (Slide 3). Where tests have only been done at one level, we can make a linear plot to zero or the control incidence of tumors and extrapolate or interpolate the data. I think these values, although only as accurate as my rough calculations and as the carcinogenicity assays which generated them, go some way to provide meaningful data for interspecies comparison. The model suggested can, of course, be modified for use with any series of standard assays.

Multistage Model of Carcinogenesis

Defining carcinogenic potency is only a very small part of the problem of interspecies extrapolation of carcinogenesis results. A chemical carcinogen possesses many properties relevant to carcinogenesis, each of which may vary independently, and possibly in an opposite direction to the other. Thus, the physical properties of a carcinogen may affect its distribution in the body and its plasma concentration and protein binding, that is to say, its availability. Physical properties may also affect its affinity for activating and deactivating metabolizing enzymes. The stability of the ultimate electrophile or its transport form may influence its ability to reach the critical receptors and its interaction. I visualize a site

on DNA as the most likely critical receptor. The configuration and placement of the carcinogen adduct on the DNA may affect the ability of DNA repair enzymes to act, while the original carcinogen or any of its metabolic products may be toxic to repair or other enzymes. Toxicity to the immune system, hormonal systems, proliferative systems and many others which act on the stages of tumor development may influence the ultimate tumor yield. Clearly, we can not, at this stage, summate each and every one of these biochemical or physiologic-biologic properties to achieve an exact explanation of potency differences between species. The amount of work required would be horrendous for even a single chemical and the weighting to be given to different factors would be the subject of seemingly endless and probably fruitless argument. We would be as well employed debating the number of angels that might dance on the eye of a needle as to attempt to line up our limited mechanistic knowledge in such a comprehensive way.

A simplification is clearly needed. That which is best established is the two-phase mechanism of carcinogenesis put forward for mouse skin by Berenblum and Shubik and now being experimentally tested in other tissues, such as liver by Peraino, bladder by Friedell and Cohen and others, pancreas by Pour, and so on. The interaction or initiation phase is a composite of all factors up to and including the DNA repair stage. The development or promotion phase includes all these factors that may influence tumor development.

Evaluation of Initiation and Developmental Potency

a) Initiation

For mouse skin and a few other model systems, we have whole animal data on the initiating ability of a few chemical carcinogens. However, in my opinion, whole animal experiments are not the most appropriate model for gaining a general insight into the initiating potency of chemical carcinogens which can be applied to trans-species extrapolation. I take this view because: (1) we have adequate models for only a few tissues in a very few species; (2) there are strong ethical objections to experimentation in man, the species of primary concern; and 3) the cost of whole animal experiments is often prohibitive.

I feel that the present range of short-term prescreening tests for carcinogens in general are designed to reflect the initiating ability of a carcinogen. For quantitative results the Ames Salmonella test is, in my view, less than suitable because the rough treatment involved in the preparation of the S-9 metabolically activating fraction may damage or dissociate activating from deactivating enzymes, and because cell-free systems lack the membranes that may help to maintain enzyme co-factors at their proper levels. There are many examples of qualitatively correct, but quantitatively inexact, results from attempts to correlate results of these tests with the results of carcinogenesis assays. In addition, microbial mutation tests, such as the Ames test, appear insen-

sensitive to specific carcinogens, such as hydrazines, estrogens, possibly metals and even nitrosamines.

I feel that the mammalian cell mediated mammalian cell mutagenesis system as developed by Langenbach and Huberman may provide much more reliable answers. There is some safety in this suggestion since this approach has not been used or tested to the same extent as the Ames or other microbial mutation systems. This test consists of co-cultivating primary explants of cells from the chosen tissue as a metabolic system, with Chinese-hamster V-79 lung cells that are mutable to resistance to the lethal effects of drugs, such as azaguanine, thioguanine or ouabain. The use of primary explants of mammalian cells has several advantages over the S-9 fraction. There is no disturbance of metabolizing enzymes or intracellular membranes and therefore, there should be much greater similarity to in vivo metabolism. Primary explants appear to retain the metabolizing characteristics of the tissue of origin to a greater extent than continuously cultured cells. Cell suspension of many tissues may be utilized - preparation of S-9 fraction from some of these tissues may be difficult. Drs. Langenbach and Malick in the Eppley Institute have successfully exploited liver, kidney, bladder, epithelial, and lung tissue and shown that each metabolically activates major groups of carcinogens. Human cells from specific tissues urgenlty require to be ultilized.

Further work is required to illustrate fully the precision of this technique; however, in a preliminary series of studies,

Langenbach has found that when S-9 fraction from liver
de was used to metabolically activate a series of four pancreatic carcinogens, the results were almost inverseley related to the in vivo pancreatic carcinogenic potency; with the liver cell mediated assay, a good correlation was obtained. Such evidence is encouraging, but as yet, by no means conclusive. (Slide 5)

If this, or some of the other techniques, are used to obtain quantitative values for the initiating potency of a series of carcinogens, we might be able to establish a potency equation:

Slide #8

$$\text{Potency for Initiation} = -\log a d_m + R$$

where $d_m$ is the dose required to initiate a standard number of mutations or other events in the chosen system; a is a constant designed to line up the scale of potency with that in vivo studies; and R is a factor which reflects the ability of the particular tissue and species pre-replicatively to repair its DNA. The latter factor is introduced because the V-79 cell system does not reflect either tissue or species specific activity of DNA repair systems or the rate of replication occurring in a specific tissue in vivo.

b) Tumor Development

Such a wide variety of biological processes are presently believed to affect tumor development that proposals on how the potency of an agent for tumor development may be quantitated are exceedingly difficult. If we wish to include man in our trans-species extrapolation net, it is clear that, so

far as is possible, in vitro methods are needed. Presently available methods are limited to one species and one tissue. For example, phorbol esters appear to exert their major effects only in mouse skin; phenobarbital mainly in rat and mouse liver. Some attempts have been made to reproduce initiation-promotion in vitro, but to the best of my knowledge, no really general approaches have been devised. The reason for this is simply that we have not yet decided what we are looking for -- we have little idea of the mechanisms involved -- and, therefore, can not begin to judge whether any in vitro test is adequate.

At the risk of being accused of vastly oversimplifying a very complex problem, I will suggest that there are two major considerations underlying the tumor development phase of carcinogenesis. First, there is the effect of the overall proliferative stimulus the agent gives to the tissue, and second, the selective stress which the agent applies between untransformed cells and tumor progenitor cells.

Examples of the importance of the general proliferative stimulus would be expected in tissues such as liver and bladder, which normally have a very low rate of proliferation. Peraino has demonstrated that phenobarbital stimulates rat liver cell proliferation and this may explain its promoting action on 2-FAA-initiated rats. My colleagues and I have over the years shown that bladder carcinogens fed continuously in the diet lead to hyperplasia, increased DNA synthesis and enhanced cell

proliferation. Similarly, physical irritation by stone or urothelial infection leads to these effects.

It is to be expected, although it has not yet been rigorously proved, that such stimulation is, in fact, regeneration in response to chemically-induced cell loss or necrosis. I assume, the induction of cell proliferation will be related in some way to the dose of the agent. I suspect for cells from tissues that have an inbuilt tendency to retain their mass or integrity within the whole animal, such as the liver or bladder, a good approximation to cell proliferation induction may be obtained from the level of cell killing induced by the agent.

Among the factors believed to influence tumor development, cell proliferation itself, however, induced, for example, by hormones and other humoral effects, may have an influence on the proliferative aspects of tumor development. Hormonal, other humoral, and possibly immunologic influences will play key roles in the selection process if the normal and transformed cells differ in either their receptor molecules or the efficiency with which they act.

Comparison of the toxicity of an agent to normal cells and those incubated in the presence of the agent itself may help quantify the selection pressure induced by the agent. For example, it was reported many years ago that cells from cultures maintained in the presence of carbon tetrachloride were no longer as responsive to the necrotizing effects of this agent, as were untreated cells. In a similar

vein, James and Elizabeth Miller determined that rat liver neoplasia induced by 4-dimethylaminoazobenzene had lost the ability to activate this carcinogen and was, therefore, "protected" against it. I suspect that both the proliferative and selection processes in any given tissue are likely to be dose-related. That is, we may postulate:

Slide #8

$$\text{Potency for Development} = -\log bd_p - \log cd_s$$

where b and c are sonstants, $d_p$ is the dose required to produce a defined proliferative stimulus and $d_s$ is the dose required to produce a given degree of selection. Unfortunately, we still need defined methods to determine $d_p$ and $d_s$.

Summation

Putting all our speculations together, we arrive at the following:

$$\text{Potency of a Carcinogen} = -\log_{10} d_{L50}$$

Slide #9

$$= \text{Potency of Initiation} + \text{Potency for Development}$$

$$= -\log ad_m + R - \log nd_p - \log cd_s + R$$

$$\text{"Potency" of a Carcinogen} = f(Rdm \cdot d_p \cdot d_s)$$

in which $d_{L50}$, dm, but not yet $d_p$ and $d_s$, can be measured by presently devisable techniques using either whole animals or cell culture.

This model assumes that both initiation and promotion are independently dose-related. In the initiation phase, dose determines the level of DNA change responsible for initiation in the tissue; in the development phase, dose determines the magnitude of the proliferative or selective processes. This

has some rather striking consequences that affect risk assessment. If we consider a "pure" initiator, that is, that tumor development is due only to natural body processes, we obtain a more linear-dose response model that is ~~so clearly~~ demonstrated by single dose irradiation experiments. Likewise, if we consider a "pure" tumor developer, we would also expect to obtain a more nearly linear relationship between dose and tumor response than we would if we consider a complete carcinogen possessing both initiating and promoting capabilities. The $ED_{01}$ experiment, sometimes known as the mega-mouse experiment, performed at NCTR, suggests this view may be correct.

The $ED_{01}$ Experiment

The $ED_{01}$ experiment examined the effect of low doses of 2-FAA on the incidence of liver and bladder tumors in BALB/c mice. In all, about 24,000 animals were used and at low doses, there appeared to be marked differences in the response of these two tissues. The bladder showed an apparent threshold (Slide 4), the liver did not (Slide 5). Various statistical tricks led to claims that this difference is more apparent than real, and that if the mice had liver for 6-10 years, this difference would have disappeared.

Slides #10 and #11

The first thing about these results that strikes me forcibly is that there is an appreciable incidence of liver neoplasia in untreated BALB/c mice, but there is an exceedingly low spontaneous incidence of bladder tumors (of between 0.1 and 0.5%). That is to say, liver tumor incidence might be increased, especially at low dose levels, just by enhancement.

The key question is now -- what happens if we repeat this experiment in a strain of mice that without treatment have an exceptionally low liver tumor incidence? I predict, probably without fear of contradiction, because nobody will wish to repeat the very costly $ED_{01}$ experiment, that the dose-tumor incidence curve may come to resemble that seen in the bladder in the $ED_{01}$ experiment. It it interesting that at the higher dose levels, the liver tumor rate appears to be exceeding its apparent linearity. This suggests that at these higher dosages, possibly both the initiating and promoting effects of 2-FAA come into play as in the bladder.

Pursuing this kind of agreement further may lead to some rather striking conclusions.

## Conclusions

I have discussed trans-species extrapolation in terms of practicalities. What can we measure that is relevant at this time; what else do we need to know? I believe that my approach is more likely to provide a meaningful insight into the problems posed by such extrapolations than is a detailed listing of differences between species that may or may not be relevant to carcinogenesis. The problem is, as I see it, a matter of obtaining quantitative parameters on which to base firm judgments, a mere listing of divergent properties between species will get nowhere. The detailed mechanistic interpretation of why A is a much weaker carcinogen than B can be more easily attempted when we know whether the difference is in the tumor initiation or tumor development stage.

Regulatory decisions to protect public health will probably be easier with broadly based, but quantitated information, than detailed mechanistic studies, which niether the public nor the regulators really understand.

Slide 1

Differences between Humans and Laboratory Animals

| Factor | Man | Mouse | Rat |
|---|---|---|---|
| Lifespan (years) | 70 | 2.5 | 3.0 |
| Gestation (days) | 270 | 21 | 21 |
| Weight (grams) | 70,000 | 25-40 | 100-500 |
| Estrous cycle | No | Yes | Yes |
| Menstrual cycle | Yes | No | No |
| Quadriped | No | Yes | Yes |
| Biped | Yes | No | No |
| Mammal | Yes | Yes | Yes |
| Gall bladder | Yes | Yes | No |
| Vitamin C Dependent | Yes | No | No |

Slide 3

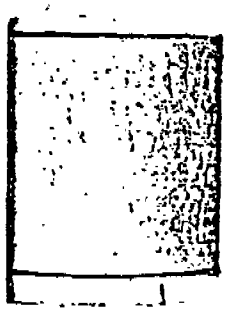
Potency of 2-Naphthylamine in Various Species (Oral Administration)

| | |
|---|---|
| Dog | -4.92 |
| Mouse | -5.33 |
| Rat | -6.42 |
| Hamster | -6.72 |

Slide 2

Approximate Potencies of Hepatocarcinogens Fed to Mice
Male Rats, Data Derived from Literature

| | |
|---|---|
| Trichloroethylene | -7.03 |
| Carbon Tetrachloride | <-5.27 |
| 2-Aminoanthraquinone | -6.72 |
| Dimethylnitrosamine | -4.90 |
| Michler's ketone | -4.88 |
| Aflatoxin $B_1$ | <-0.67 |
| Saccharin* | -8.15 |

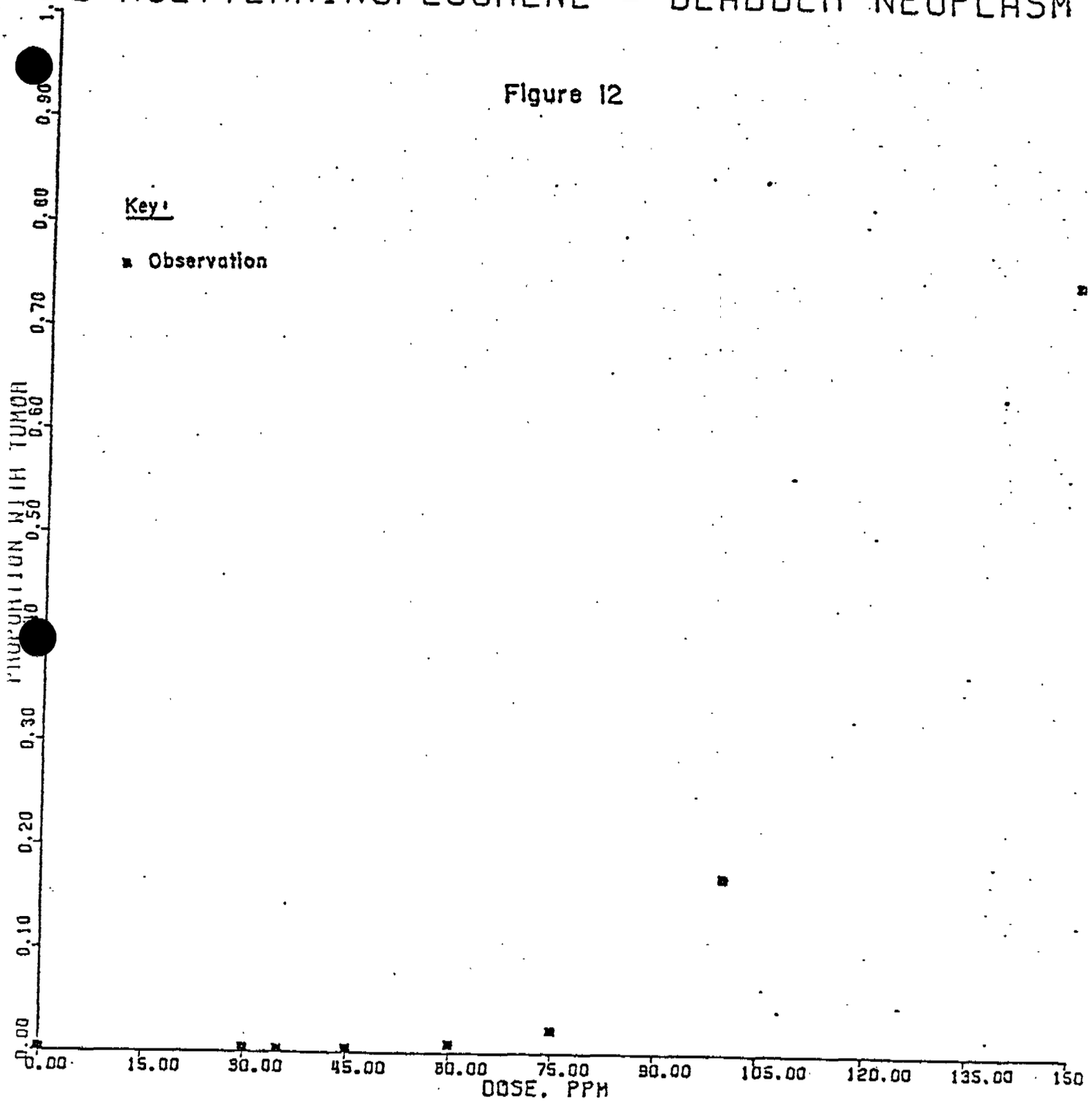


EC- 1961

slide4

# 2-ACETYLAMINOFLUORENE - BLADDER NEOPLASM

Figure 12



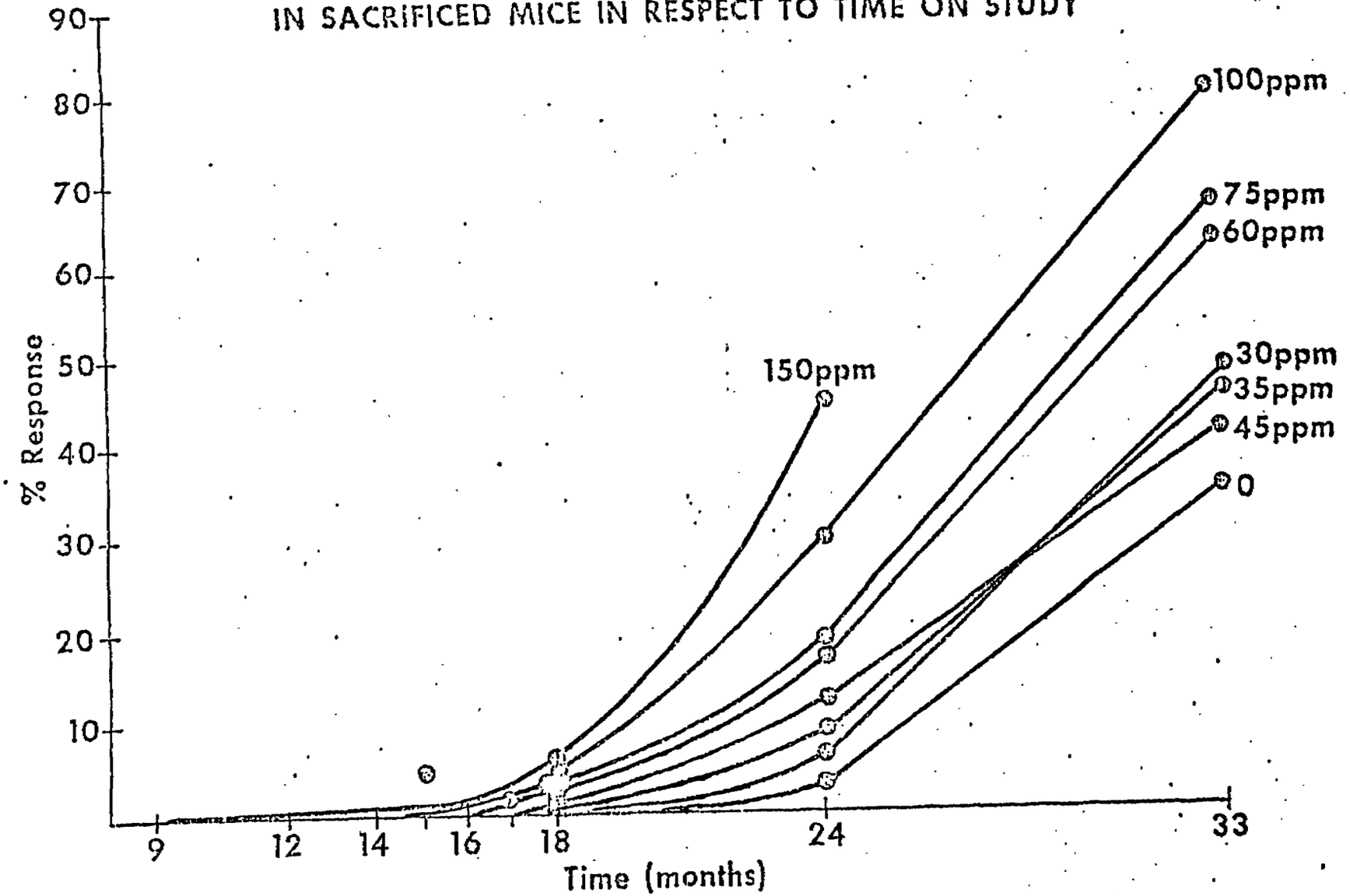

EC- 1962

(NCTR : ED01, Exp4)

Slide 5

Figure 3

THE PREVALENCE OF LIVER NEOPLASMS
IN SACRIFICED MICE IN RESPECT TO TIME ON STUDY




EC- 1963

(NCTR : ED01 Expt)