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PROCEEDINGS OF THE
FIRST NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

MAY 6-8, 1980

SHERATON/POTOMAC, ROCKVILLE, MARYLAND

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Appropriate portions of the discussions, working groups and plenary session were sent to the participants for editing. The style of editing varied, as could be expected. To the extent possible, we have attempted to arrive at a consistent format.

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Proceedings were developed from a workshop on the National Cancer Institute's, the Environmental Protection Agency's and the National Institute for Occupational Safety and Health's Collaborative Programs on Environmental and Occupational Carcinogenesis.

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First, I am going to talk about non-haired mice in general and work sponsored by the NCI/EPA agreement at Temple University directed by Dr. P. Donald Forbes. I am going to describe an outbred strain that is very common, the Skh-1 line, and an inbred derivative of that line, which I think will be very useful in photocarcinogenesis work in the future. I will talk about the photoresponses of these lines and of a number of other non-haired mouse lines. Then, I am going to talk about a contract at Emory University with Dr. Issac Willis, who is attempting to dissect the UV spectrum to show whether there are synergistic biological effects attributable to UVA and UVB light, again using the Skh-1 mouse. Lastly, I will talk about a postulated mechanism of UV carcinogenesis involving sterol derivatives.

Non-haired mice are not to be confused with nude (nu/nu) mice. I really want to emphasize this, because 90 percent of the people to whom I talked about hairless mice really think I am talking about the nude mice which are used commonly in immunological studies. The mice which I will be referring to are not nude (nu/nu) mice. They are not immunologically incompetent and they do not have the athymic condition that the nude mouse has. Furthermore, the term hairless is not always reserved for the genotype (hr/hr) which refers to a specific mutant, but is used interchangeably with the term non-haired.

Non-haired mice have been used in photocarcinogenesis studies as a convenience. In haired strains, tumors induced by UV irradiation are often confined to ears or non-haired extremities and are often of mesodermal rather than epidermal origin. Shaving or enzymatically removing hair from haired mice in photocarcinogenesis experiments is possible but is generally considered laborious. There are, however, some people who contend that the haired strains are intrinsically better models for photocarcinogenesis than the hairless mice or non-haired varieties.

Several mutations lead to the non-haired phenotype. The recessive gene hr is encountered most frequently. Non-haired mice, although convenient sources of bare skin for irradiation, have some peculiar problems on their own. Since mouse pigmentation is closely associated with the hair follicle and is most noticeably expressed as coat color or hair color rather than skin color, hairless mice are only weakly pigmented due to the disruption of hair follicles. A hairless mouse skin type comparable to negroid skin does not exist, as far as I know. Non-albino hairless mice are capable of a tanning reaction, however.

Another common problem with non-haired mice is that the young will not always accept a non-haired mother. This necessitates a heterozygote breeding program, which cuts the efficiency of animal production in half.

It was also suspected, and to some extent shown experimentally, that non-haired mice suffer from a variety of immunodeficiencies, not as striking as the athymic condition in nude (nu/nu) mice but, nevertheless, important in considering photocarcinogenesis. Photobiologists have addressed these problems in a variety of ways. The result has been the establishment of numerous colonies of outbred and inbred non-haired mice with fundamentally different characteristics. In the 1960's and 1970's, it was becoming more and more obvious that different responses to UV irradiation or to chemical treatment coupled with UV irradiation could be attributed in part to differences in the strains of mice that were being used.

I would like to digress here for a minute. I first became interested in photobiology about four years ago when I was given responsibility for monitoring a contract at Temple University. I was coming from the field of immunology. It appeared to me when I first reviewed some projects in photobiology as if the field were in a situation similar to that of histocompatibility antigen research prior to the discovery of inbred strains. There were two major variables that had not been standardized in photocarcinogenesis research. I think these two variables accounted for most of the discrepancies in data and for most of the disagreements between photobiologists. One was the differences that were prevalent in the mice that were being used. Everyone had his own non-haired strain of mouse reared in the basement breeding colony. Everyone assumed that he had the same mouse that others were working with. This was not the case at all. The other variable that was extremely significant to the outcome of photocarcinogenesis experiments was the light source used to irradiate animals. Different light sources had widely different spectral characteristics. They were roughly classified as UV-B emitters or UV-A emitters and were employed with and without filters. The spectra of light they emitted were quite different. As we shall see, this has a major effect on the outcome of a radiation experiment.

In a number of respects UV induced skin cancer as it is being studied in animals and as it is observed clinically is better understood than any other type of cancer. The relationship between UV exposure (dose or irradiance) and tumor incidence is understood in general terms. Molecular mechanisms of initiation of carcinogenesis have been proposed and various hypotheses are amenable to experimentation. Skin cancers, because they are surface cancers, can be observed readily, can be biopsied, and for these reasons, progression or regression can be scored very simply in both animals and in man. There is good correlation between the information we have about humans and the information we are generating in animal experiments. Hence the subject area, skin cancer, could serve well as a proving ground for ideas about human risk assessment based on an interpretation of animal data. Futhermore, there has been considerable progress in analyzing the various genetic factors that are responsible for susceptibility or resistance to skin cancer.

Fig. 1 shows the spectrum from a solar simulator. It is pertinent to all of the papers on photocarcinogenesis. I am going to use it here to indicate what the atmosphere does to protect the surface of the earth from ultraviolet radiation.

The top line in Fig. 1 represents the actual solar spectrum as it passes through space before reaching the earths atmosphere. UV light is divided arbitrarily into three categories, A,B and C. It is generally assumed that the UV-B component of ultraviolet light is the most active biologically and the most important type of light that reaches the surface of the earth. UV-C is also biologically important radiation, but it is effectively filtered by the ozone in the atmosphere. The biological role of

UVA is controversial. The bottom curve is the spectrum of the solar radiation (in this particular case experimentally simulated) as it reaches the earth.

In Figure 2, we can see that ozone does not act exclusively as a neutral density filter. When the effective thickness of the ozone layer changes, and this can be simulated in the laboratory by a series of Schott glass filters, there is a qualitative, as well as a quantitative shift in the UV spectrum. Filtration effectively eliminates most of the UVB that is biologically active. As the filtration decreases, either naturally (hypothetically) by cataclysmic loss of ozone from the atmosphere or, in the laboratory setting by decreasing the thickness of Schott glass filters, there is a disproportionate increase in incident irradiation in the biologically active region. For that reason, the potential changes in the atmosphere associated with ozone destruction are considered significant, because not only would they let in more UV light, but they would let in light at particularly active wavelengths.

Figure 2 gave an indication of the spectral quality of a solar simulator, which mimics very nicely what is called an effective ozone concentration. One of the very tedious jobs that was performed at the Temple University laboratory was to construct real ozone filters, and to measure filtration as a function of O_3 concentration. Dr. Forbes was able to show that the Schott glass filter system mimicked very nicely true ozone filtration with respect to the quality of the spectrum in the UV-B region.

The contract, which I am going to describe now, is one that was awarded to the Temple University, School of Medicine, Skin and Cancer Hospital in Philadelphia. The principal investigator is P. Donald Forbes. The contract was initiated to examine the extent of strain variation in response to irradiation with a solar simulator and to develop criteria for selecting one strain of hairless mouse for large scale production. The initial objective was not to look at the significance of strain variation, but to find the ideal hairless mouse to put into production for all photobiology work. That concept proved somewhat naive, although Dr. Forbes has obtained information that has resulted in a special interest in the inbred strain designated HRA/Skh. This strain has good breeding characteristics and high UV sensitivity.

Table I outlines the strains that are being examined by the Temple University group and gives designations of their genotypes, their UV sensitivity, that is, whether they are highly susceptible to UV-B induced tumors or solar simulator induced tumors, their acceptance of a non-haired mother, which is a parameter of importance in developing a colony because it gives the production efficiency, and the breeding schedule for these strains. I am using inbred here to imply brother/sister mating and I am using the word outbred loosely to indicate any schedule that does not involve specifically brother/sister mating.

Figure 3 illustrates the production problems that are encountered with the animals that will not accept a non-haired mother. The figure shows rhino mice. One parent mouse is a homozygous male. It is the male, which is mated with the heterozygous female. Since the rhino gene is recessive, the heterozygous female will be haired. The offspring are either haired ($hr^{rh}/+$) or non-haired (hr^{rh}/hr^{rh}). This gives you an example a forced heterozygosis breeding schedule.

Table 1

Mouse Strain or Line Designations and Characteristics

Name	Genotype	UV Sensitivity	Non-haired Mother Accepted	Breeding Schedule
Skh:hairless-1	select hr/hr and c/c	H	+	Outbred, segregating c, b and a
Skh:hairless-2	select hr/hr and non-albino	M	+	Outbred, segregating c, b and a
Skh:crh	crh, c/c, a/a	L	-	Outbred, forced heterozygosis for crh
Balb/cSkh-ab	ab, c/c, b/b,	L	+	Inbred, forced heterozygosis for ab to maintain haired counterpart
HR/De/HfIcr	hr, br/br, p/p	H	-	Inbred, forced heterozygosis for hr
C3H/HeN-hr	hr	M	-	Inbred, forced heterozygosis for hr
HRS/J	hr, c/c b/b, d/d	M	-	Inbred, forced heterozygosis for hr
HRS/An1	hr/hr,c/c	M	+	Outbred
HRA/Skh	hr/hr, c/c	H	+	Inbred
Skh:(HRxRH)F_1	hr/hr^{rh}	?	?	Hybrid

H,M,L mean high, medium and low susceptibility to carcinogenesis induced by UV irradiation with a solar simulator.

Inbred implies a brother-sister mating schedule which may not have reached a 20th generation; outbred includes various schedules not specifying brother-sister mating.

Two of the offspring in Figure 3 are homozygous (hr^{rh}/hr^{rh}) rhinos that still have their juvenile hair coat. The mutation does not affect the juvenile hair coat; it only affects the adult hair coat. These mice are born haired and then they gradually lost their hair, starting from the face proceeding all the way down the back of the animal. Another one of the young in figure 3 is a heterozygote; it will be used in the subsequent breeding schedules if female. Most of them are discarded and this decreases the efficiency of the production of these mice.

Most of the mice in Table 1 which require a forced heterozygosis breeding schedule are bred homozygous male to heterozygous female. Fortunately, there are some strains that accept a non-haired mother. They can be bred directly by brother/sister mating. The most vigorous line is the HRA/Skh, which is bred as a homozygous (hr/hr) breeding pair on a brother/sister mating schedule. The pedigreed line is now approaching the F20 generation. It then will be designated an inbred strain. This strain has many useful characteristics, and has been designated for a large scale production and for use in the bioassay of psoralen derivatives. We suspect that this strain, the HRA/Skh, will replace the frequently used outbred strain Skh-hairless-1 from which it was derived.

DR. CAMERON: Can you give us the reasons for picking an inbred over an outbred?

DR. ORME: We want eventually to examine questions related to the genetic control of susceptibility to UV carcinogenesis. To do that, we needed the inbred lines. I think that in conducting a wide variety of biochemical experiments in which you are specifically looking for genetic control over various phenomena, there is no choice but to go the inbred route. I also do not see any advantage in continuing to use an outbred line when the breeding characteristics of a comparable inbred line are good. The only legitimate reason for using outbred lines is production ease. I think we have in the HRA/Skh line, an inbred line that has good breeding characteristics. They are not quite as good as those of the outbred Skh-1 line, but Dr. Stanley Mann who directs the breeding operations at Temple is getting satisfactory litter sizes and satisfactory viability at weaning. My general preference is to design carcinogenesis and toxicological experiments exclusively with the inbred line, because it allows repetition of experiments and the use of genetic tools in analyzing the observed phenomena.

The idea that outbred mice are better models of a heterogeneous human population does not lead to any worthwhile experiments. If, for instance, only 5 of 50 outbred mice irradiated with UVB were to develop tumors, it would be impossible to prove that these 5 are unusually susceptible for genetic reasons and impossible to reproduce their genotypes. If, in fact, only those 5 were susceptible because of genotype, the statistically meaningful group size for experimental design, would have been reduced from 50 to 5, and 100% of the mice of that particular genotype receiving UV treatment would have had to respond with tumors before an effect could be detected. The topic of genotype specific responses is important as we shall see. But meaningful experiments cannot be conducted with outbred mice. Artifical heterogeneity is the only approach, and this artificial heterogeneity in a mouse population can be constructed by using defined numbers of inbred mice selected from a variety of strains. The conclusion is simple. If genetic tools are to be used to analyze in vivo carcinogenesis, use inbred lines. A corollary might be that in vivo experiments not amendable to genetic analysis are primitive in conception.

I would now like to describe the solar simulator. The solar simulator system and its relationship to other models for solar light and ozone filtration has been described by Dr. Forbes (Figure 4). This unit contains a xenon arc lamp. Each of the panels contains a system of filters. These can be either neutral density filters, cut-off filters or the Schott glass filters, which are used to simulate various effective thicknesses of ozone. With this set-up, Dr. Forbes can simultaneously irradiate different racks of mice with qualitatively different light spectra depending upon the system of filters put in at the various windows.

Figure 5 is another picture showing the xenon arc lamp in the middle of a bank of racks. Each one of these racks is getting a qualitatively different type of UV, but they can be irradiated for the same period of time and reirradiated simultaneously.

Figure 6 shows what is called the Mouse Sheraton in contrast to some simpler arrangements which are called the Mouse Holiday Inns. You can see what is actually happening in the cages. In one cage, a mouse is trying to hide. But mice cannot escape irradiation. They do preferentially turn while they are being irradiated, so that most of the radiation falls on the back. There are left-handed and right-handed mice, as pertains to the side they prefer to present to the irradiation apparatus.

The most common response during the irradiation period is for mice to curl up and to go to sleep. I imagine that is part of their simulated nocturnal/diurnal cycle.

DR. CAMERON: Tom, do you know if there is any degree of blindness or retinal degeneration?

DR. ORME: These mice are albino. I do not know the answer to that, Tom. I do not know of any specific physical changes in the eye. Eye tumors, for instance, are not common. So, I would have to ask about any effects leading to blindness.

Figure 7 was made before we introduced good laboratory practices. It shows the position and the multiplicity of the lesions observed. As I said, the entire back of this animal is a susceptible target, rather than just the ears as might be the case with a haired mouse. Maps or diagrams of the actual position of the various tumors are maintained on a weekly basis, so that the progression, regression or coalescence of various tumors can be followed precisely. One of the things I mentioned earlier is the uniqueness of this system for measuring time to tumor and following the progression of lesions. That coupled with the fact that these tumors arise in 24 weeks makes it a very useful experimental model.

Figure 8 shows some advanced tumors.

I think it is important to present Figure 9 to explain one of the major experimental variables. One of the reasons why plots of tumor incidence versus time differ from laboratory to laboratory so significantly is that people have not standardized the tumor scoring procedure. Figure 9 pertains to the same group of mice, but scoring is dependent on different diameters of tumor. If in fact you start the scoring with 0.5 millimeter tumors, which are barely perceptible red dots, you record an early incidence. Dr. Forbes thinks the tumors that can be scored with some degree of certainty are tumors one millimeter in diameter or larger. The time course changes very significantly according to what you consider a tumor of scorable size. Since most of the publications in experimental photocarcinogenesis do not specify this parameter, you can understand why discrepancies in published data exist.

The first major experimental variable that Dr. Forbes wanted to investigate in these studies was immunocompetence. Since there was a prevalent notion that non-haired mice were immunologically deficient, he set up a screen of immunological parameters looking at both cell mediated immunity and antibody formation. The conclusion is described in a publication that has just been submitted by Drs. Sharon Smith and Don Forbes, and it is that all the lines, as far as major responses such as the ability to form antibody and react with specific antigens and such as the ability to mount a T cell mediated immune response, are immunocompetent. There are quantitative differences in the various strains, but there was no deficiency that could be called a major immunodeficiency which could be responsible for differences in strain susceptibility to UV carcinogenesis. That does not mean that there are not specific subsets of the various basic immunological tests that are completely lacking. For instance, Dr. Smith did not break down T and B cell mediated functions into subfunctions that could be tested independently.

Then comes the meat of the matter. I find this fascinating.

Figure 10 shows the response of the various non-haired lines at 24 weeks. The names differ somewhat from those shown in Table 1. For instance, the HRS/J is called JAX in Figure 10, and the HRS/Argonne is called Anl. This is the response of the various lines to exactly the same irradiation conditions. The mice have been exposed five times a week to the solar simulator for a specified period of time. Those irradiation conditions are spelled out in the abstract.

Cryptothrix and absebia are the most resistant. They are mutations distinct from hairless (hr). They map at different sites and have quite a different physiology. The other mutations are all hairless (hr). Still, we can see a wide variation in the susceptibility with different backgrounds carrying the same mutation.

There is no correlation between susceptibility and the albino gene. For instance, Jax is an albino strain as is HRA/Skh. Their responses to the solar simulator differ significantly. There are major differences in the susceptibility even when the animals carry the albino gene. There is no association of susceptibility with the hairless (hr) gene.

The bar graph in the upper half of Figure 10 presents the percentage of the animals that have at least one tumor a millimeter in diameter at 24 weeks.

Not only is there a wide discrepancy in the number of mice or the percentage of mice affected, but the lower bar graph of Figure 10 shows the multiplicity of the tumors on the affected mice. Again, pronounced strain specific variation is indicated. The Skh-1 and the HRA showed multiplicities of tumors 40 and above within 24 weeks. What is being scored in Figure 10 are mainly papillomas, but a high percentage of them will progress to frank carcinomas.

That is really all I wanted to say about Dr. Forbes' work. Temple will exercise its option in a three-year incrementally funded contract to revise its work statement. Rather than screen more strains for susceptibility, they are going to start using the various differences that they have observed to analyze the mechanism of photocarcinogenesis.

A possible line of experimentation is to actually start making crosses between the inbred strains that are available to find out whether the susceptibility relationships are dominant or recessive. This could be done, for instance, with absebia, which is on a Balb/c inbred background. It could be crossed very easily with the inbred HRA line to look for different relationships there.

What I like about this series of experiments is that it is telling us something that we have known for a long time about humans but which we continue to ignore. Different people have different susceptibilities to irradiation. Probably the same could be said about chemical carcinogens. I use this to illustrate what I think is a great mistake in our approaches to human risk assessment.

We could have taken the data from any one of these lines, turned it over to a statistician and said give me a dose response curve that we can use to extrapolate by various fudge factors to the human population. Literally, we would have the full range of possibilities. What we really want to know when we make a human risk assessment is not the dose-response curve for or the probable susceptibility of the whole population; what we really want to know is the size of the population at high risk and why it is at high risk. I hope that this type of experiment is going to give us some insights into how to start looking at the human risk assessment problem with those considerations in mind.

I personally feel that this type of data shows the futility of applying mathematical modeling to the data that are obtained from one species or one strain of animal. It just may be totally atypical of the population at large. For this reason, I think we have to start building into our experimental designs for risk assessment some consideration of the variation in responses that we get.

DR. KELSEY: I just wanted to say that I am very excited about this area. I have been following the hairless mice story from a different point of view, from cholesterol metabolism. But showing the genetic susceptibility seems like an interesting way, as you have just mentioned. I am wondering how widespread would this type of analysis be in chemical carcinogenesis.

DR. ORME: Well, I have submitted a question for this afternoon's discussion which treats that. My feeling is that if you were to do the same thing with a chemical carcinogenesis screen, let's say test a carcinogen at the maximum tolerated dose, which I think overrides genetic resistance in some cases, in ten different strains, some might develop tumors and others not. I give you an example: C57 black mice are known to be resistant to most of the chlorinated hydrocarbons. They are not totally resistant, but you have to go a full two years before you start detecting carcinogenesis in the C57 black. On the other hand, with the C3H line, similar exposure conditions induce liver tumors rapidly.

With 2-AAF, the same pattern prevails:the C57 is resistant and will get tumors only very late, while the C3H gets tumors right away. In fact, if you give 2-AAF in the drinking water, a dose that kills C3H immediately is tolerated by C57 black for its full lifetime. So you see that there are major metabolic differences in these lines.

The problem, and this is the matter that I want to bring up this afternoon, is that we are not gearing our resources to multistrain testing. Current systems for production of animals are designed to make large numbers of Fischer 344 rats and B6C3F1 mice available and the use of a restricted number of strains is justified when

one considers animal health problems. The non-haired mice I have discussed are now in conventional facilities and they are susceptible to and are carrying a variety of undesirable viruses like Sendai and mouse hepatitis virus. We hope that those viruses are not influencing experimental results. But if we were to rederive and put into production all of these animals, the cost would be enormous. As I said, we have decided to do this with a single mouse line only so we are not getting much closer to multistrain testing.

DR. KELSEY: I guess my question would be particularly where you know have positive carcinogenic response from classic bioassays. Would it not then be a reasonable step to look at these effects in various other strains.

DR. ORME: Yes, I think that that ought to be done.

DR. KELSEY: How do you determine the metabolism? I realize that you may not be able to do a full blown bioassay.

DR. ORME: Yes, I think we get into a variety of problems by not doing more work in metabolism and pharmacokinetics. There are big loopholes in the basic theory of the bioassay. A good example pertains to the benzidine dyes that have been tested with the Fischer 344 rat. The benzidine dyes are very carcinogenic and metabolism studies have been done in the Fischer 344 rat showing that benzidine is excreted in the urine along with 4-amino-biphenyl. Unfortunately for the theory, 4-amino-biphenyl and benzidine have never been tested for carcinogenicity in the Fischer 344 rat but only in other strains of rats. So those people who are arguing that the measurement of urinary metabolites is presumptive evidence for the carcinogenicity of the parent benzidine dyes have one more experiment to do. And after this is done we will still wonder why the mouse is resistant to the benzidine dyes.

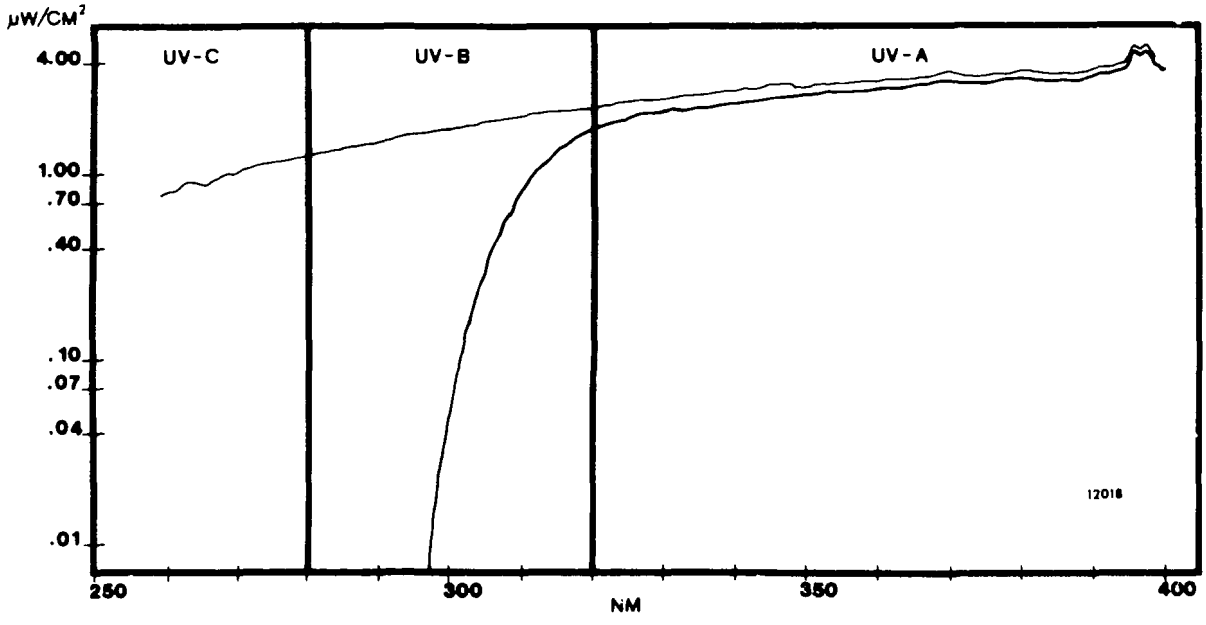

Figure 1

Solar Spectrum: Upper Line, in space; lower line; at sea level on Earth's Surface.

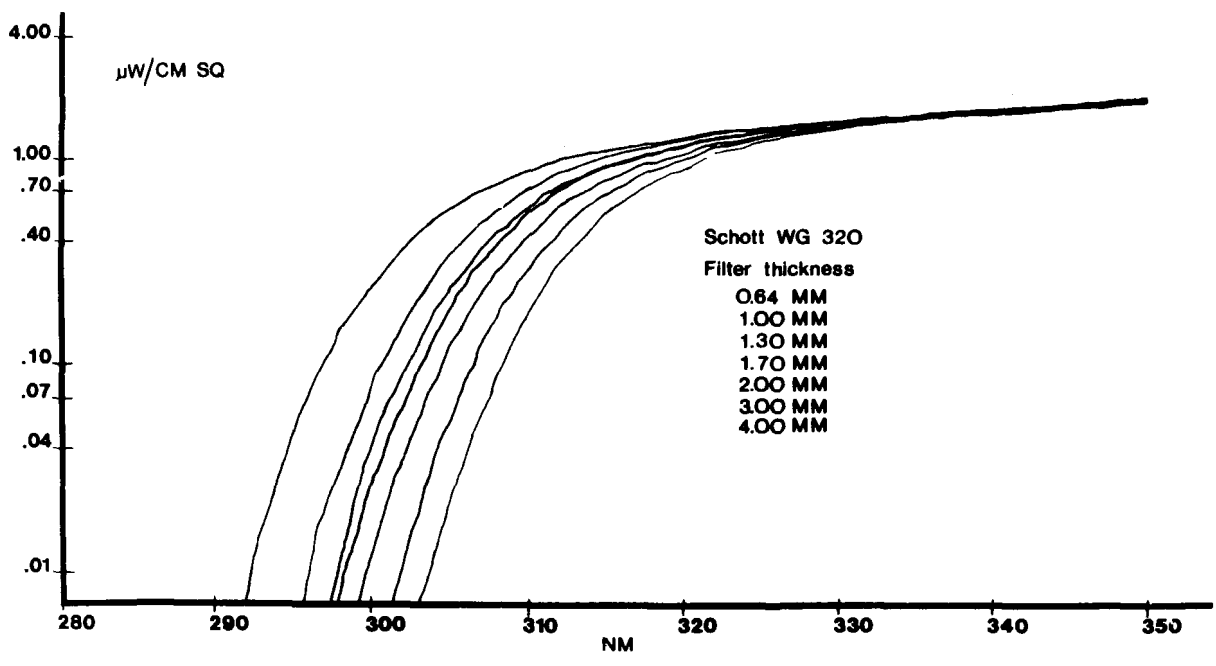

Figure 2

Modification of Solar Simulator Output by Schott glass filters of decreasing thickness. Simulation of Ozone filtration of solar irradiation.

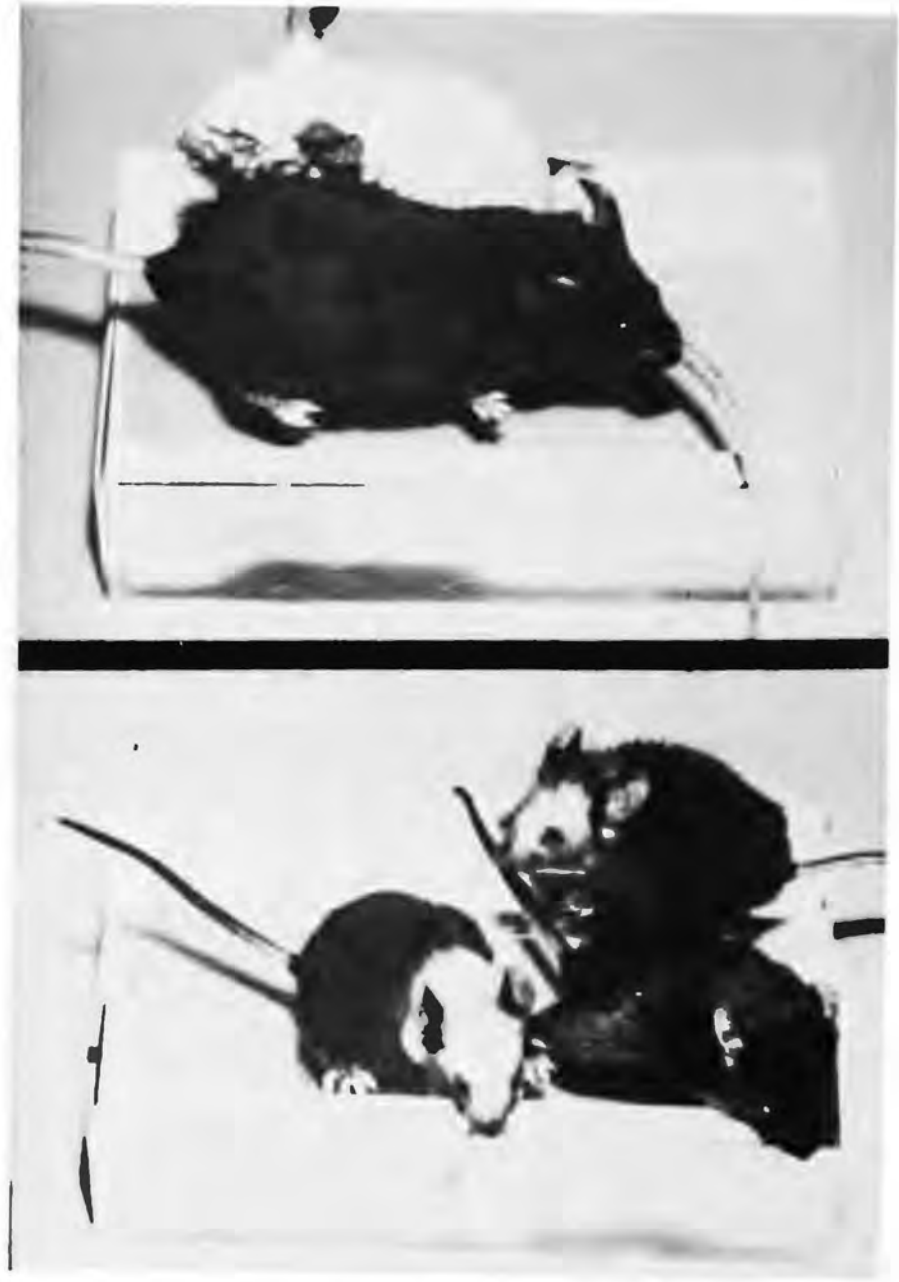

Figure 3

Heterozygous Breeding of Rhino Mice. (See text for explanation)

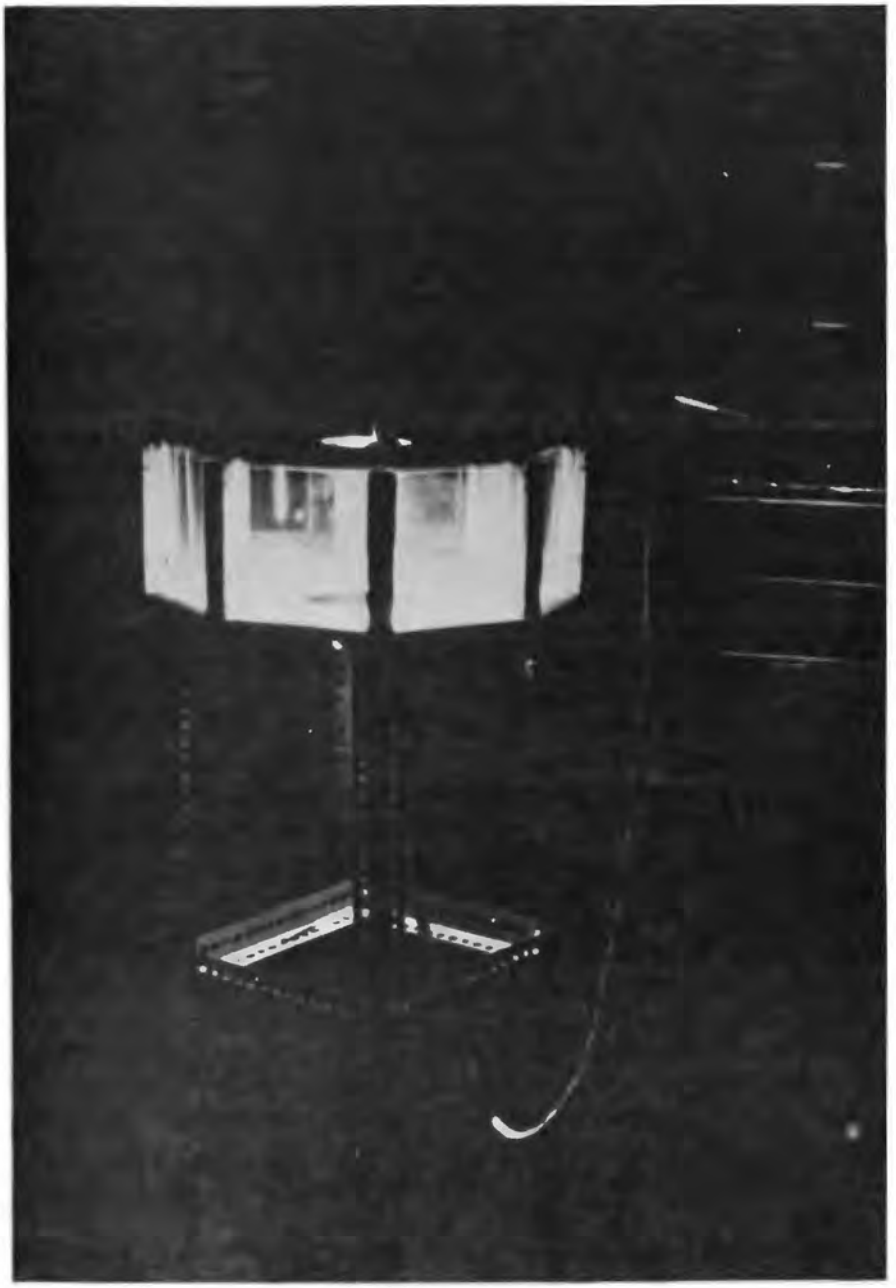

Figure 4

Solar Simulator

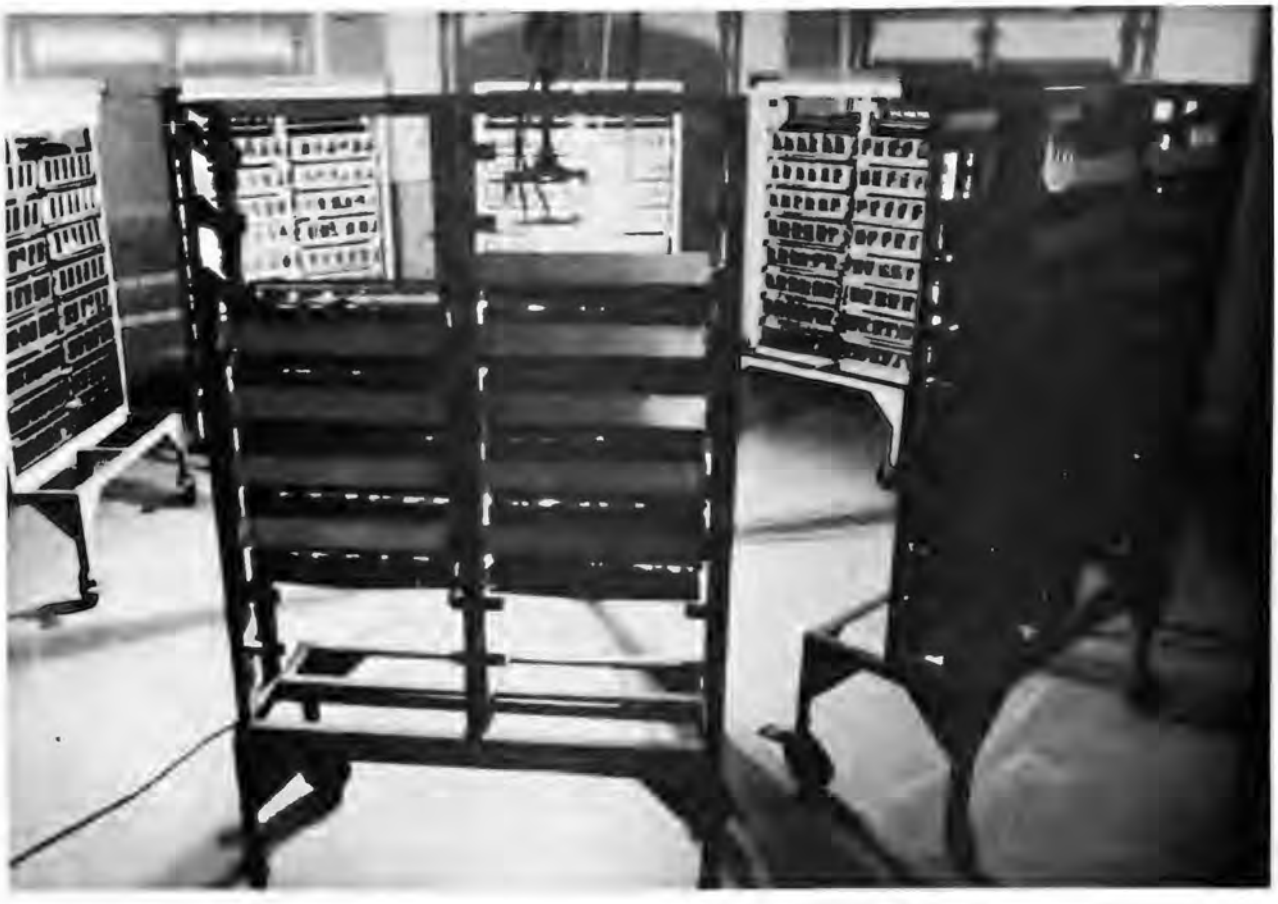

Figure 5

Solar Simulator in bank of racks.

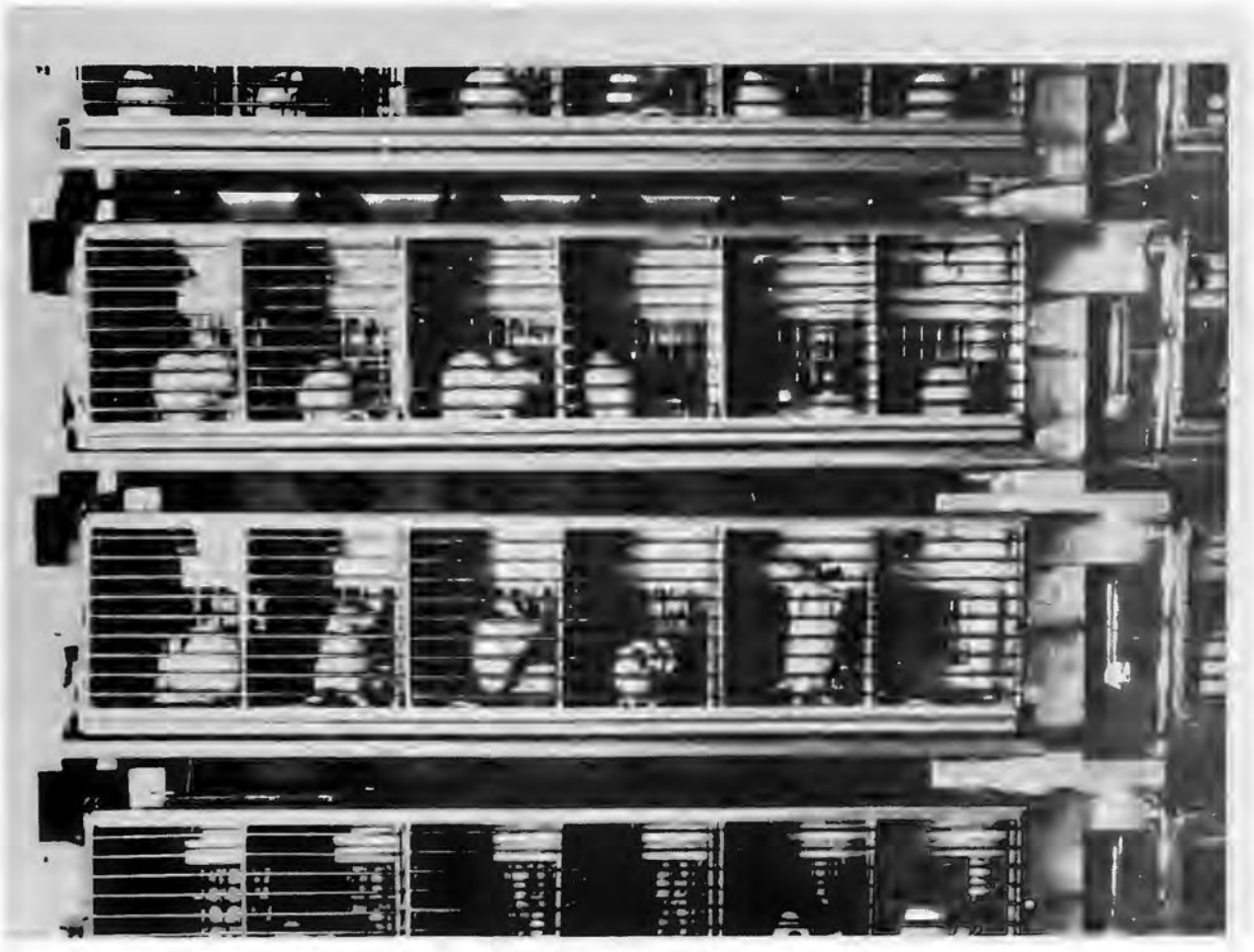

Figure 6

Irradiation Rack

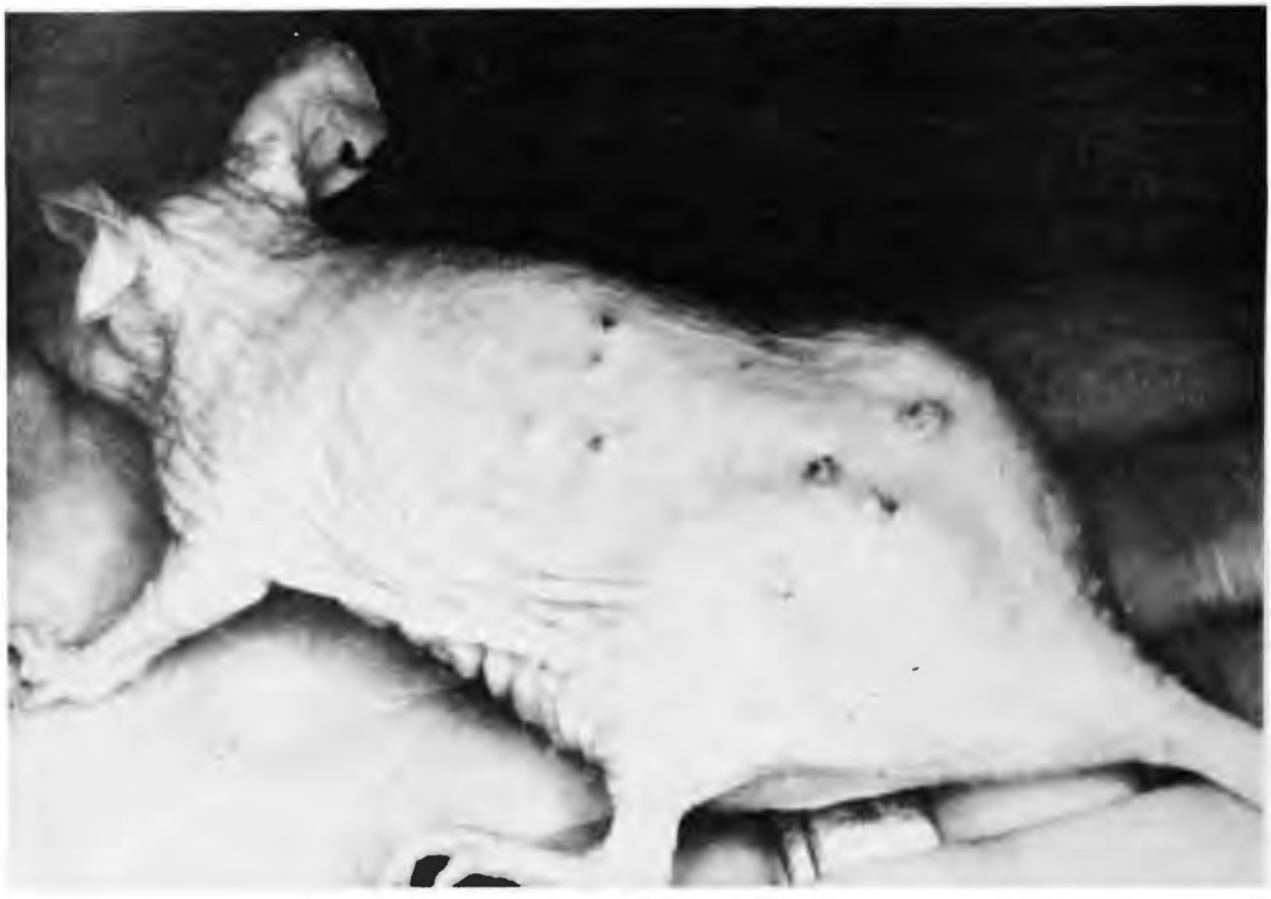

Figure 7

Early tumors

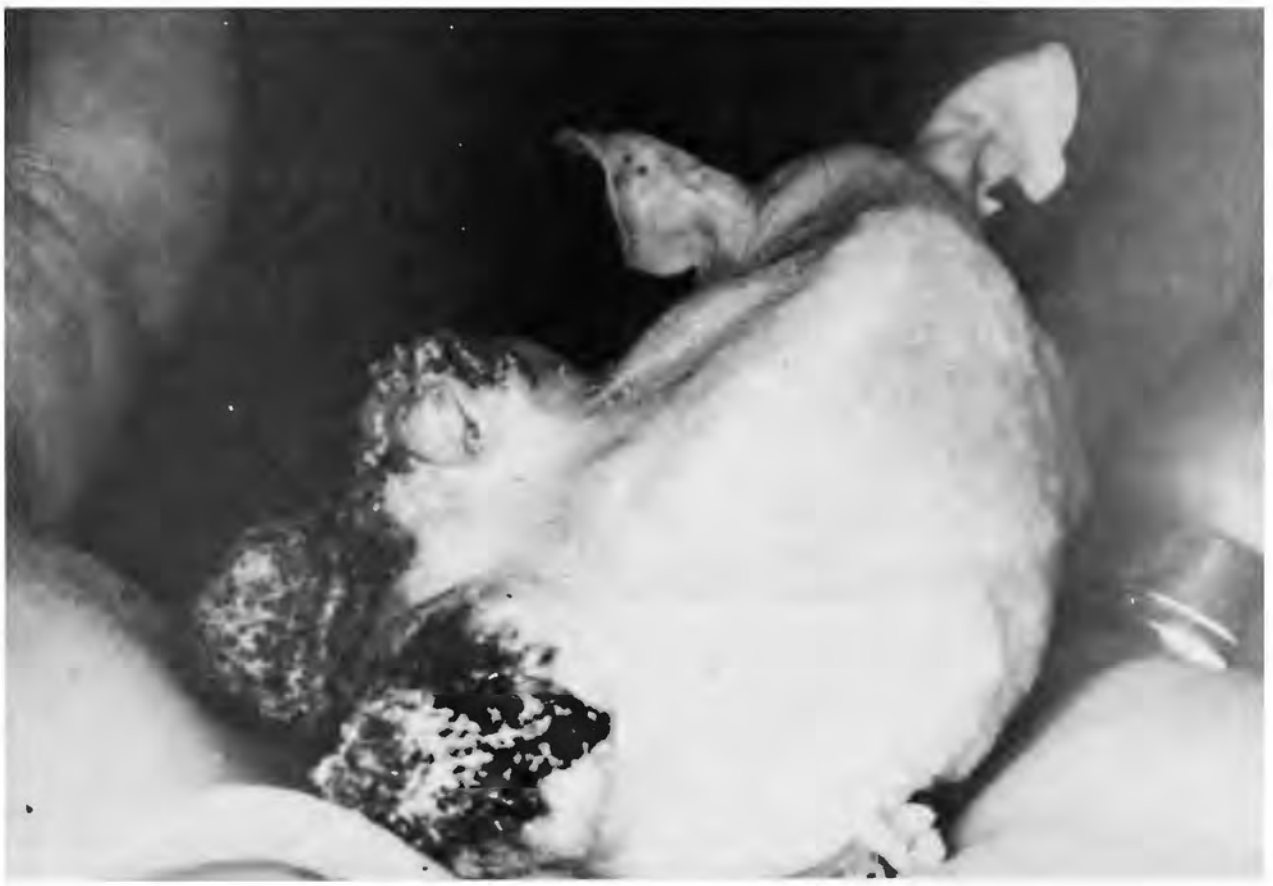

Figure 8

Advanced Tumors

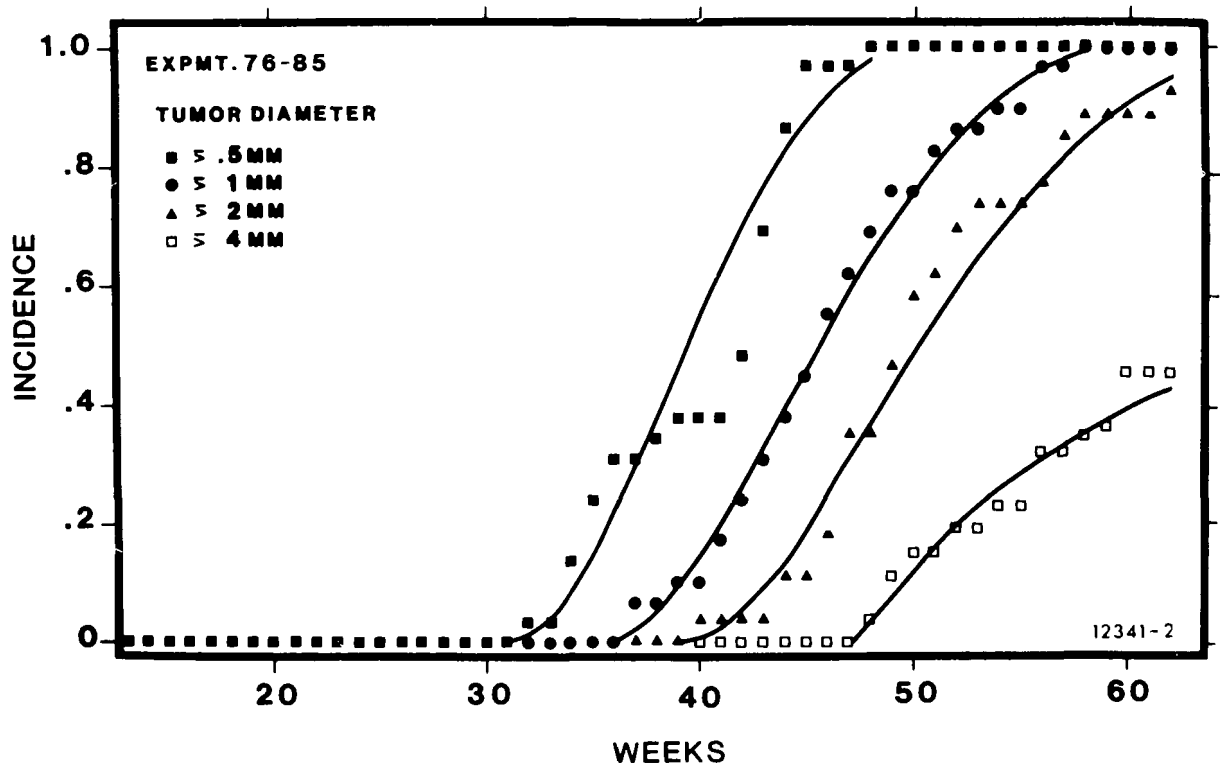

Figure 9

Tumor Incidence vs. Minimal Size for Scoring.

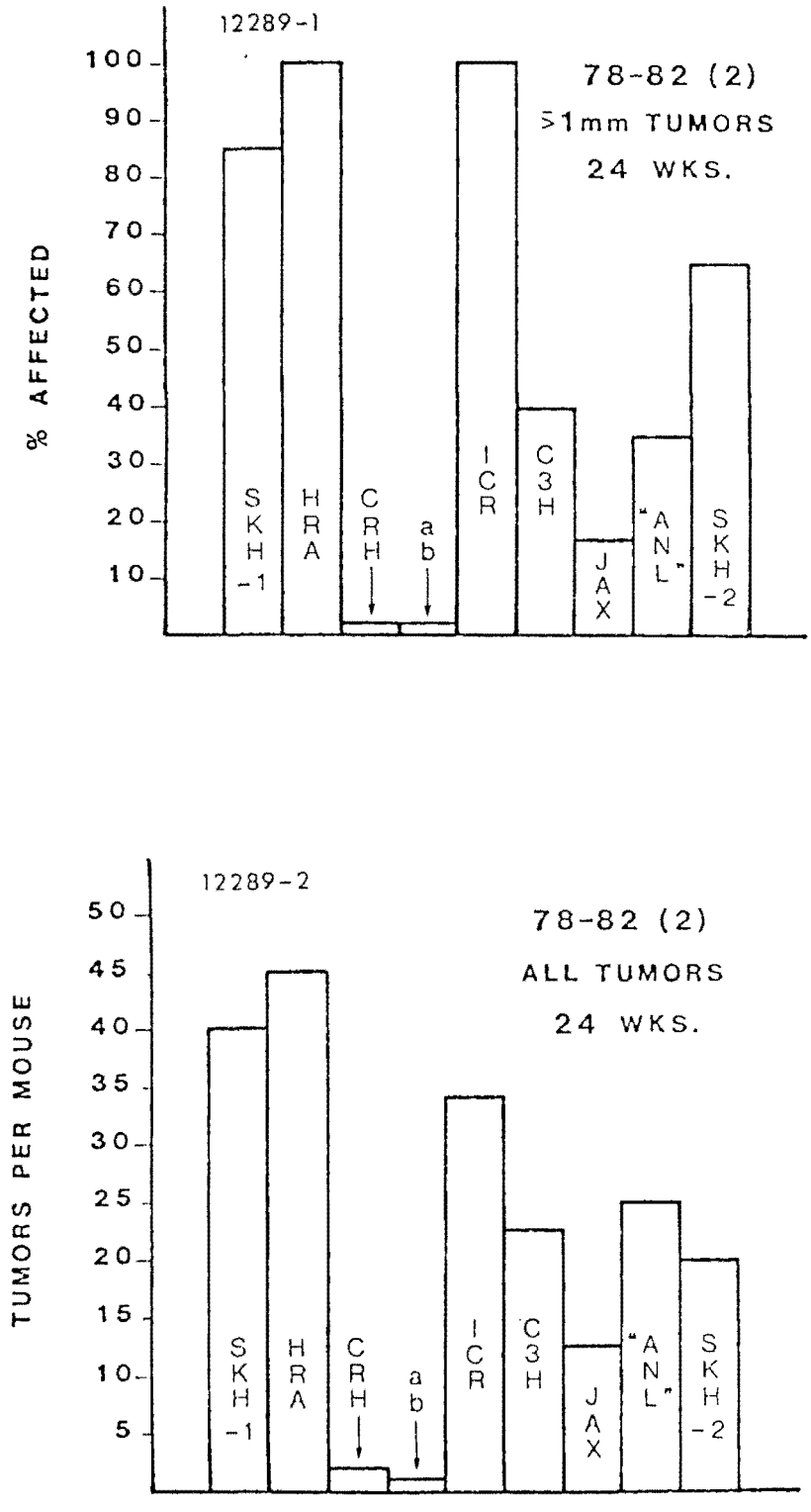

Figure 10. Strain Variation in Tumor Incidence