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RESEARCH PLANNING			
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UNSER ZEICHEN: 101-11/AHO/ila (0611) 7908428 TELEFON: 9. 1. 1968 DATUM:

## Use of the Rabbit in Multi-Site Skin-Painting Experiments

Dear Dr. Ayres,

During your last visit to Frankfurt in October 1968, we briefly discussed the above subject. We explained to you our interest in these problems and promised to study them carefully and, if possible, to propose some preliminary experiments. The objective of our considerations is outlined in your letters of 5th November, 1968. We arrived at the following result:

### 1. General Remarks

"There are no hard and fast rules for the testing of carcinogenic activity and for the extrapolation of the results of animal assays to humans. The testing of every chemical substance is to some extent a new problem which may require a specific assay procedure. The reasonable relevance of the results of testing to humans must be accordingly assessed, each time separately, in the light of the relative evolutionary closeness of the test species and the human species and of the dietary, environmental, and socioeconomic factors in the human population." These principles quoted from Arcos, Argus, and Wolf (1968) are placed at the beginning of our comments, although they will be well known to you. They are nevertheless a sound starting point because some of the problems referred to have to be briefly discussed in the following for reasons of completeness.

First, the problem of extrapolation of the results. Ideally, it would seem that our tests should be carried out in species most closely related to man, such as primates. In addition to

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this ideal requirement of close phylogenetic relationship, several authors (e.g. K.H. Beyer, 1966) emphasize the dietary and environmental conditions because of their effects on the metabolism of chemical substances in the organism. In the light of these considerations, the dog - man's companion for thousands of years - is gaining importance. However, tests with dogs must be limited in scope for practical reasons. Assays with primates or large mammals would be impracticable owing to the cost of the test. Hence, rats and mice are the most commonly used species because of their short life span, high reproductive rate, small size, and relatively high susceptibility to chemical carcinogens.

The relevance of the results of animal assays to humans is further complicated by the fact that the susceptibility of different species to a given carcinogen usually shows considerable variations.

This implies that parallel experiments with two species will increase the relevance of the tests. For this reason, it has become a rule to test drugs, food additives, etc. in several species.

The value of the skin-painting assays conducted under the Janus project would doubtless be increased by comparable tests in a second species.

Rabbits may be used in our opinion for the following reasons:

## 2. Rabbits and Skin-Painting

According to Wynder and Hoffmann (1967) the skin of rabbits was painted with tobacco "tar" as early as in 1911. Obviously for practical reasons, various authors have confined nearly all of their tests to the painting of ears. The results can hardly be compared with each other because of the many known variables involved in testing for the carcinogenicity of tobacco smoke condensates (for a review see Wynder and Hoffmann, 1964). Of the publications by Wynder and Wright (1957), Graham et al. (1957), and Wynder et al. (1958) quoted in document F 913, the two first-mentioned papers are actually comparable (strain of animals, type of condensate, and painting regimen similar; duration of experiment and sites of application comparable) and we fully agree to the conclusion of the author of this document. There is no doubt that cigarette smoke condensate has been demonstrated to be tumorigenic to the epidermis of rabbits.

On the other hand, the question of whether the skin of rabbits or of mice is more sensitive still remains to be clarified. Wynder and Wright/in 1957, "We shall subsequently stated

study their activity (fractions of tobacco tar) on various sites of different strains and species of animals", but in later years Wynder reported only once (1958) on tests with rabbits. In their review of 1964, Wynder and Hoffmann mentioned the rabbit as a test object with reference to a statement of the Food Protection Committee (1959) when they said, "... the more promising choice to test the carcinogenicity of tobacco smoke appears to be the repeated cutaneous application of the aerosols to mice and rabbits".

Another passage of this publication (and again in 1967 in "Tobacco and Tobacco Smoke") reads: "Perhaps because of the longer latent period and associated greater expense, relatively few investigators have utilized the rabbit as a test object. We see no particular advantage of using the rabbit instead of the mouse in these studies..."

Neither did Wynder and Hoffmann see any advantage in the method of rabbit multi-site painting employed first in 1956 by Hamer and Woodhouse (only three animals - five sites - painted with whole cigarette "tar"). Contrary to the comments by Wynder and Hoffmann (1964 and 1967), the rabbit tests (daily painting of both ears for six months) conducted by Gritsiute and Mironova (1969) were not negative, but five out of twelve rabbits developed multiple papillomata which, however, persisted only in two animals and which in no case showed signs of malignant growth. Condensate painting experiments on rabbits performed later than 1960 are not known to us.

Summarising we may establish that tobacco "tar" is doubtless tumorigenic to the skin of rabbits and that the susceptibility of rabbits is not substantially lower than that of mice. However, since experiments with rabbits may involve a longer latent period and higher expenses mice have been preferred, at least in tobacco research.

Hence the question arises whether these disadvantages inherent in rabbit tests can be compensated by multi-site painting. If multi-site painting is admissible by scientific standards and sufficiently reliable in practice, the use of rabbits has the advantage that comparable results are obtained from a second species of animals, even if their latent period is longer.

### 2.1 Multi-Site Painting

Before D.L. Woodhouse coauthored the paper on multi-site tobacco-tar painting in 1956, he and I. Hieger reported in 1952 on extensive multi-site painting experiments with petroleum fractions (Hieger and Woodhouse, 1952). The authors conducted the experiments parallel to experiments with mice.

They used the rabbit because of the advantages involved in the use of a second species and because "the possibility that other species (than the mouse) might prove more suitable under certain circumstances does not appear to have been sufficiently appreciated".

Under the conditions of these tests, the rabbits indeed proved more suitable because they were more sensitive. The results obtained by Hieger and Woodhouse were confirmed without reservation in the MRC Report on "The carcinogenic action of mineral oils- a chemical and biological study", which you kindly supplied to us.

Before accepting the reported higher sensitivity of the rabbit one should visualise the basis on which this statement was made. The following table contains as an example results which have been taken from Hieger's and Woodhouse's paper and from the MRC report.

Table 1: Comparison of the Tumour Incidence in Mice and Rabbits

Number of Animals		Number of Sites	Number of Tumours
Test in Birmingham			
Mice	900	900	33
Rabbits	105	705	61
Test in London			
Mice	900	900	6
Rabbits	105	705	39

The reported higher sensitivity of the rabbit in this and in numerous other experiments which gave similar results thus is based on the observation that in spite of a smaller number of sites (705 against 900) and of a smaller number of animals (105 against 900) the tumour incidence in rabbits was much larger than that in mice. The statement that the skin of the rabbit was seen to be distinctly more sensitive than that of the mouse hence applies only to multi-site painting. The seven sites painted as a rule in the above tests were later reduced to five sites because in this case "the animals were less liable to cross-contamination than when seven sites were used" (MRC Report).

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However, is multi-site painting admissible by scientific standards? The authors of the MRC Report do not discuss the question of a possible interaction between the five or seven different "substances" applied simultaneously. Interactions of this type cannot be excluded a priori because, for example, "malignant tumours often have a considerable influence on the metabolism of the whole organism even before a dissemination of the cells can be observed" (R.W. Begg, 1958) quoted from Arcos et al., 1968).

Questions of cocarcinogenesis were critically discussed by Berenblum in November 1967 (Berenblum in: Progress in Tumour Research, Basel, 1969). Defining cocarcinogenesis as all form of augmentation of tumour induction he points out, "Little need be said about the additive action of two potent carcinogens, or about synergistic action, the latter representing admission of ignorance as to why, in such cases, the combined effect is greater than expected. It should be noted that, in some cases, two carcinogens can actually interfere with each other's action". He refers to Hill et al. (1951) who demonstrated that DMEBA carcinogenesis is inhibited by PAH. Berenblum continues by saying, "Since the mechanism in multiple carcinogen action is not understood, there is no point in discussing it further".

It is also necessary to consider syncarcinogenesis as defined by K.H. Bauer, if multi-site painting is practised.

From the standpoint of modern tumour immunology there should be no objections to multi-site painting because "the antigens of chemically induced tumours are specific for each individual tumour. Even if two tumours are induced by the same carcinogen on the same site in genetically identical animals or even in one and the same animal, they bear different antigens, although these tumours cannot be distinguished from each other histologically" (Oettgen and Gallmeier, 1968).

This is all we can so far contribute to these problems. Numerous discussions with cancer specialists have shown that too little is known about this subject, because multi-site painting obviously was scarcely practised in the past. We are making intensive efforts at present to obtain additional information, especially by studying the relevant literature.

It should be noted, however, that several tumours, if observed in multi-site painting, were found only on the painted site (ear) and not in other areas. Papillomata were not formed on the backs of the rabbits when the test substance was applied to the ear. Metastases did occur, but they were restricted to regional lymph nodes or other glandular tissues (salivary gland) and did not involve other areas of the skin.

The significant differences between groups of rabbits often mentioned in the MRC Report have not been discussed by the authors. They may be associated, for example, with

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different stages of the hair cycle at the start and in the course of the treatment. Hence, the comprehensive report fails to give an answer to several questions of interest to us. Rabbit multi-site painting has definitely proved suitable for the comparative testing of mineral oil fractions. The questions left unanswered make it difficult, however, to assess its suitability for the testing of tobacco smoke condensates.

As pointed out in our introductory remarks, new problems may require specific assay procedures. The testing of tobacco smoke, which unfortunately has to be carried out via the condensate anyway, appears to be a problem which justifies the application of specific assay procedures such as multi-site painting, provided that the following requirement is satisfied: the condensates to be tested have to be balanced properly with regard to their tumorigenic potency and general toxicity. The reasons are obvious, and may be found in the MRC Report: "It should be mentioned also that practical considerations dictate that very strong and very weak carcinogens should not be assessed in the same series; if they were, the tumours induced by the strong carcinogen solution would tend to be far advanced and liable to cause symptoms in the test animal before sufficient time had elapsed for the weak solutions to have exerted measurable effects." We are of the opinion that the above requirements can be satisfied in co-operation with you. Summarising we may say that the suitability of rabbit multi-site painting should be examined in preliminary experiments. The advantages pointed out in document F 913 are also expected by us. We therefore propose the following assay procedures:

3. Preliminary Experiments

Table 2: Design of Tests in Rabbits

Painting Site	Ear left	Ear right	Inter-scapular	Abdomen left	Abdomen right
Designation in MRC Report	I	II	VII	V	VI
1 - 10	d	f	a	e	c
11 - 20	b	a	d	f	e
21 - 30	c	d	b	a	f
31 - 40	a	e	f	c	b
41 - 50	f	c	e	b	d
51 - 60	e	b	c	d	a

a = 9,10-dimethyl-1,2-benzanthracene  
b = acetone/water 9:1  
c - f = four different types of condensate

### Number of Animals and Duration of Tests

We suggest the fairly large number of ten rabbits per group because the experiments will most probably require a longer period of time than the testing of the mineral oil fractions. This suggestion has been based on the assumption that the majority of the expected tumours will not become manifest before the first of, say, two test years has elapsed. On the other hand, the literature contains ample data from which a shorter latent period and hence a shorter test period may be expected.

### Explanation of 6 x 5 Square Design

The tests have been so designed that the groups only differ from each other in the omission of one "substance" or condensate. Possible differences in the tumour yield between the groups can thus be examined by analysis of variance, in order to determine which of the "substances" is responsible for the difference.

### DMBA as Reference Carcinogen

9,10-dimethyl-1,2-benzanthracene has been selected because it was the reference carcinogen used in the assay of the mineral oil fractions. At two applications per week, the maximum dose level was 600  $\mu$ /3 cm<sup>2</sup>. During a period of six years, the average tumour probability was 0.71, in the majority of the tests nearly 0.8. The corresponding probability of tumours for 150  $\mu$ /3 cm<sup>2</sup> was 0.3. Our dose level will be fixed when the types of condensate are known.

### Selection of Condensates

The condensates have to be selected together with you on the basis of scientific and practical considerations (e.g. capacity of smoking machines, availability of cigarette samples).

### Dose Level and Painting Regimen

Analogous to the above-cited mineral oil tests, the painted areas should have a size of 3 cm<sup>2</sup>. The dose level cannot be fixed until the type of condensate is known. The animals should be painted three times per week. The duration of the treatment will depend on the tumorigenicity of the condensates. If possible, the animals should therefore be painted with condensates whose activity is known from experiments with mice or can be assessed from current experiments.

Quality of Test Animals

The test, of course, requires animals of excellent constitution which have to be free, to the highest possible extent, from endo- and ectoparasites. The animals will be bought from a renowned breeder whose therapeutic and prophylactic measures will be supervised by us.

The often debated question whether random-bred stock animals or an inbred strain should be used may be decided in the present case as follows:

If we consider the preliminary experiments to be a feasibility study aimed at a high tumour yield (at least in the test of the most active substance) we have to use random-bred stock animals, because the genetic make-up within an animal species is known to influence the development of spontaneous tumours and of chemically induced tumours. For this reason, the probability of tumours is much higher in genetically heterogeneous random-bred stock animals than in any arbitrarily selected inbred strain.

If the preliminary experiments were conducted for comparison of several condensates, inbred animals would be preferred. It should be noted, however, that an inbred strain is actually of value only if its spontaneous tumour rate is known. A strain which meets this requirement is not available as yet. In a recently published paper Löliger (1968) stated that rabbits are suitable for the experimental cancer research, but that suitable strains for these investigations are not yet known.

Keeping of the Rabbits

Only male rabbits will be used for the tests. They will be accommodated in an air-conditioned room in individual brand-new metal cages provided with a perforated bottom plate. A standard pellet diet poor in microorganisms and tap water will be allowed ad libitum.

Start of Test

The test can be started as soon as animals are available which meet the above requirements. Since the breeding has to be supervised by us and since the animals should be about four months old at the start of the treatment, a period of about eight weeks will be required after we have been advised start the experiment.

Yours sincerely,  
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Biology Department



Dr. A. Hofmann

For references see the attached appendix

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