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THE ANTAGONISTIC EFFECT OF CARCINOGENIC
HYDROCARBONS ON MURINE VIRUS-INDUCED
LEUKEMIAS.**

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THE ANTIAGONISTIC EFFECT OF CARCINOGENIC
HYDROCARBONS ON MURINE VIRUS-INDUCED LEUKEMIAS

by
Alvin G. Fiscus

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GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my
direction by Alvin G. Fiscus
entitled The Antagonistic Effect of Carcinogenic
Hydrocarbons on Murine Virus-Induced Leukemias
be accepted as fulfilling the dissertation requirement of the
degree of Doctor of Philosophy

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TABLE OF CONTENTS

	Page
LIST OF ILLUSTRATIONS	vi
ABSTRACT	ix
INTRODUCTION	1
Previous Investigations	5
Shope Papilloma Virus	5
Shope Fibroma Virus	6
Rous Sarcoma Virus	6
Polyoma Virus	7
Bittner Virus	7
Herpes Simplex Virus	9
Fowl Pox Virus	10
Vaccinia Virus	10
Influenza Virus	11
West Nile Virus	12
Poliovirus 2	12
ECHO Virus 9	12
Coxsackie Virus B ₁₁	12
Possible Mechanisms of Interaction Between Chemical Carcinogen and Virus	13
OBJECTIVE	16
MATERIAL AND METHODS	17
The Host	17
The Viruses	18
Source	18
Preparation of Pools	18
The Chemical Carcinogens	19
Source	19
Methods to Study the Interaction of Friend and Rauscher Viruses with DMBA and 3-MC	19
Virus Injections	19
Chemical Treatment	20
Animal Observations	20
Tissue Culture	21
Source	21
Recovery Procedure	21
Medium	22
Procedure for Routine Passaging	22
Preservation Procedure	23

TABLE OF CONTENTS--Continued

	Page
Methods to Study the Effect of DMBA on the Cells of a Friend Virus-Induced Reticulum Cell Sarcoma Tissue Culture Injected into Mice	24
Cell Injections	24
Cell Titration	24
Chemical Treatment	25
Observation of Animals	26
RESULTS	27
Interaction of Friend Virus with DMBA and 3-MC	27
Control Animals	27
Gross Examination	27
Microscopic Examination	27
Experimental Animals and Results	31
Interaction of Rauscher Virus with DMBA and 3-MC	31
Control Animals	31
Gross Examination	31
Microscopic Examination	39
Experimental Animals and Results	39
The Effect of DMBA on the Cells of a Friend Virus-Induced Reticulum Cell Sarcoma Tissue Culture Injected into Mice	50
History and Characteristics of Tissue Culture	50
Results	51
Chemically Induced Neoplasms	57
Results of Injections	57
Results of Application to the Skin	57
DISCUSSION	65
The Inhibitory Effect	65
Data not Conforming to the Observed Trend and a Possible Explanation	65
Variation of Data	66
Possible Explanations which might Explain the Results	66
Polycyclic Aromatic Hydrocarbons as Antitumor Agents	66
Adrenal Cortical Insufficiency	68
Effect of Polycyclic Hydrocarbons on Spleen and Bone Marrow	68
Negative Nitrogen Balance	69
Interference with the Penetration and Proliferation of the Virus Itself	70
Interference with the Ability of the Cells to Respond to the Oncogenic Influence of the Virus	71
SUMMARY	72
REFERENCES	73

LIST OF ILLUSTRATIONS

Figure	Page
1. In Vivo Studies with Oncogenic Viruses	2
2. In Vitro Studies with Oncogenic Viruses	3
3. In Vivo Studies with Nononcogenic Viruses	4
4. The Abdominal Organs in Friend Virus Leukemia	28
5. Spleen in Fully Developed Friend Virus Leukemia x 125	29
6. Spleen in Fully Developed Friend Virus Leukemia x 550	29
7. Liver in Fully Developed Friend Virus Leukemia x 125	30
8. Liver in Fully Developed Friend Virus Leukemia x 550	30
9. Latent Periods (Friend Virus) for DMBA Treated Animals Expressed as Arithmetic Averages	32
10. Survival Times (Friend Virus) for DMBA Treated Animals Expressed as Arithmetic Averages	33
11. Spleen Weights (Friend Virus) for DMBA Treated Animals Expressed as Arithmetic Averages	34
12. Effect of DMBA on Friend Virus Leukemia	35
13. Latent Periods (Friend Virus) for 3-MC Treated Animals Expressed as Arithmetic Averages	36
14. Survival Times (Friend Virus) for 3-MC Treated Animals Expressed as Arithmetic Averages	37
15. Spleen Weights (Friend Virus) for 3-MC Treated Animals Expressed as Arithmetic Averages	38
16. Spleen in Fully Developed Rauscher Virus Leukemia x 125	40
17. Spleen in Fully Developed Rauscher Virus Leukemia x 550	40
18. Liver in Fully Developed Rauscher Virus Leukemia x 125	41
19. Liver in Fully Developed Rauscher Virus Leukemia x 550	41

LIST OF ILLUSTRATIONS--Continued

Figure	Page
20. Latent Periods (Rauscher Virus) for DMBA Treated Animals Expressed as Arithmetic Averages	42
21. Survival Times (Rauscher Virus) for DMBA Treated Animals Expressed as Arithmetic Averages	43
22. Spleen Weights (Rauscher Virus) for DMBA Treated Animals Expressed as Arithmetic Averages	44
23. Effect of DMBA on Rauscher Virus Leukemia	45
24. Latent Periods (Rauscher Virus) for 3-MC Treated Animals Expressed as Arithmetic Averages	46
25. Survival Times (Rauscher Virus) for 3-MC Treated Animals Expressed as Arithmetic Averages	47
26. Spleen Weights (Rauscher Virus) for 3-MC treated Animals Expressed as Arithmetic Averages	48
27. Effect of 3-MC on Rauscher Virus Leukemia	49
28. Disease Produced by Intraperitoneal Injection of the Cells of a Friend Virus-Induced Reticulum Cell Sarcoma Tissue Culture after 16 Days	52
29. Disease Produced by Intraperitoneal Injection of the Cells of a Friend Virus-Induced Reticulum Cell Sarcoma Tissue Culture after 16 Days	52
30. Spleen with Metastasis in Disease Produced by Intraperi- toneal Injection of the Cells of a Friend Virus-Induced Reticulum Cell Sarcoma Tissue Culture x 550	53
31. Liver with Metastasis in Disease Produced by Intraperi- toneal Injection of the Cells of a Friend Virus-Induced Reticulum Cell Sarcoma Tissue Culture x 550	54
32. Latent Periods Expressed as Arithmetic Averages (cells passaged 105 times in vitro)	55
33. Survival Times Expressed as Arithmetic Averages (cells passaged 105 times in vitro)	55
34. Latent Periods Expressed as Arithmetic Averages (cells passaged 86 times in vitro and 3 times in vivo) . . .	56

LIST OF ILLUSTRATIONS--Continued

Figure	Page
35. Survival Times Expressed as Arithmetic Averages (cells passaged 86 times in vitro and 3 times in vivo) . . .	56
36. Large Subcutaneous Sarcoma Induced by Injection of 3-MC after 88 Days - Dorsal View	58
37. Large Subcutaneous Sarcoma Induced by Injection of 3-MC after 88 Days - Ventral View	58
38. Sarcoma Produced by Subcutaneous Injection of 3-MC x 45	59
39. Sarcoma Produced by Subcutaneous Injection of 3-MC x 550	59
40. Skin Tumors Induced by Application of DMBA after 75 Days	60
41. Squamous Cell Papilloma Produced by Application of DMBA to Skin x 45	61
42. Squamous Cell Carcinoma Produced by Application of DMBA to Skin x 125	61
43. Epidermal Cyst with Squamous Cell Carcinoma Induced by Subcutaneous Injection of DMBA x 45	63
44. Epidermal Cyst with Squamous Cell Carcinoma Induced by Subcutaneous Injection of DMBA x 125	63
45. Epidermal Cyst with Squamous Cell Carcinoma Induced by Subcutaneous Injection of DMBA x 550	64

ABSTRACT

Previous studies using a combination of carcinogenic polycyclic aromatic hydrocarbons and oncogenic as well as nononcogenic viruses have always shown an enhancement of the disease process. In this investigation, using the polycyclic aromatic hydrocarbons 7,12-dimethylbenz(a)anthracene (DMBA) and 3-methylcholanthrene (3-MC) and the Friend and Rauscher murine leukemia viruses, the reverse result was obtained as indicated by increased latent periods, increased survival times and smaller spleen weights.

Tenfold dilutions of both the Friend and Rauscher viruses ranging from 10^{-1} to 10^{-7} were made from stock pools. The individual dilutions were inoculated intraperitoneally into three groups of five BALB/c mice each. The three groups of mice were treated in the following manner immediately after virus inoculation:

Group I - These animals received at weekly intervals subcutaneously 100 ug of DMBA or 100 ug of 3-MC dissolved in 0.1 ml. of corn oil.

Group II - 200 ug of DMBA or 3-MC dissolved in 0.05 ml. of reagent grade acetone were applied at weekly intervals to the hairless skin of each mouse.

Group III - These mice received only virus and acted as untreated virus controls.

The animals were observed daily, and latent periods, survival times and spleen weights were recorded. Portions of the spleen and liver, the

adrenals and the area treated with either chemical were saved and examined histologically. The experiments were terminated on the 100th day.

DMBA as described above produced no effect on the cells of a "Friend virus-induced reticulum cell sarcoma tissue culture" growing in the peritoneal cavity of mice. A certain number of these cells have to be injected intraperitoneally to produce growth of the sarcoma. In order to detect any possible effect that DMBA might have, two cell concentrations were used, and the number of cells injected was adjusted so that one hundred per cent of the animals receiving the largest number and fifty to sixty per cent of those receiving the smallest number of cells would develop sarcoma in a 50-day period. The desired number of cells was injected into each of seven mice intraperitoneally. Three treatments of DMBA at weekly intervals, as described previously, were given to each mouse before injection of the cells. Immediately after the cell injections the animals were again treated and weekly thereafter until the experiment was terminated on the 50th day. A control group was included and received only the tumor cells, but no DMBA. The animals were palpated at two-day intervals for peritoneal tumors. Latent periods and survival times were recorded and used to determine any possible effect.

INTRODUCTION

Ever since the carcinogenic effect of chemicals as well as viruses has been demonstrated, the interaction between these two has been studied. In all cases, the results have indicated varying degrees of enhancement. The subject of this paper is the interaction of the two carcinogens, 7,12-dimethylbenz(a)anthracene and 3-methylcholanthrene, with Friend and Rauscher murine leukemia viruses. The author believes it to be interesting and significant that the data given in this paper show a considerable divergence from those previously obtained. Instead of enhancing the disease process, the reverse was observed as measured by latent periods, survival times and spleen weights. These results suggest the possibility that the interaction between chemical carcinogen and virus is not a simple process of augmentation, but a composite of several reactions depending on the agents used. A survey of the work of previous investigators follows.

To the author's knowledge the first attempt to show the interaction between a virus and a chemical carcinogen was performed by Teague and Goodpasture in 1923 with Herpes simplex virus and tar. Since that time numerous investigations have been made on animals treated with chemical carcinogens and viruses, both oncogenic and nononcogenic.

The in vivo and in vitro experiments since 1923 dealing with the interactions of chemical carcinogens and oncogenic and nononcogenic viruses are listed in Figures 1, 2 and 3.

<u>Virus</u>	<u>Experimental Host</u>	<u>Chemical Carcinogen</u>
1. Shope papilloma	Guinea pig and rabbit	Tar Turpentine-acetone 3-Methylcholanthrene-scarlet red Benzene 3-Methylcholanthrene (X-rays) (Ultraviolet light)
2. Shope fibroma	Rabbit	Tar 3-Methylcholanthrene Benzo(a)pyrene (Cortisone)
3. Rous sarcoma	Chicken	3-Methylcholanthrene
4. Polyoma	Mouse	7,12-Dimethylbenz(a)anthracene- croton oil 7,12-Dimethylbenz(a)anthracene Croton oil Benzo(a)pyrene

Figure 1
In Vivo Studies with Oncogenic Viruses

<u>Virus</u>	<u>Tissue Culture</u>	<u>Chemical Carcinogen</u>
5. Bittner	Mouse and rat fibroblasts	3-Methylcholanthrene 3-Methylcholanthrene-chicken sarcoma nucleoprotein extract 3-Methylcholanthrene-rat sarcoma extract 3-Methylcholanthrene-human tumor extracts

Figure 2
In Vitro Studies with Oncogenic Viruses

<u>Virus</u>	<u>Experimental Host</u>	<u>Chemical Carcinogen</u>
6. Herpes simplex	Guinea pig and rabbit	Tar
7. Fowl pox	Chicken	3-Methylcholanthrene
8. Vaccinia	Mouse	3-Methylcholanthrene 7,12-Dimethylbenz(a)anthracene 2-Aminofluorene Dibenz(a,h)anthracene (Cortisone)
9. Influenza	Mouse	Urethane Ozonized gasoline
10. West Nile	Mouse	3-Methylcholanthrene Benzo(a)pyrene
11. Polio 2	Mouse	Dibenz(a,h)anthracene 2-Aminofluorene 7,12-Dimethylbenz(a)anthracene
12. ECHO 9	Mouse	Dibenz(a,h)anthracene 2-Aminofluorene 7,12-Dimethylbenz(a)anthracene
13. Coxsackie B ₄	Mouse	Dibenz(a,h)anthracene 2-Aminofluorene 7,12-Dimethylbenz(a)anthracene

Figure 3
In Vivo Studies with Nononcogenic Viruses

A brief summary of each experiment follows.

1. Shope Papilloma Virus

Rous and Kidd (1936, 1938) injected the papilloma virus intravenously into rabbits whose ears had previously been painted with tar. Approximately three weeks later many neoplasms were observed on the tarred ears. In many cases these became malignant and metastasized. No lesions were obtained when the virus was injected intravenously into untreated rabbits.

Friedewald (1942) applied the virus to the rabbit's skin by scarification and two days later the carcinogenic agent. He used tar, benzene, 3-methylcholanthrene, a mixture of turpentine and acetone, a combination of 3-methylcholanthrene and scarlet red, x-rays and ultraviolet light. He found shortening of the latent period, accelerated growth and an increased number of neoplasms. The infective titer of the virus was increased 10 to 100 times when the skin was treated with a mixture of turpentine and acetone. The greatest response was produced by treatment with 3-methylcholanthrene and the least with x-rays.

Rous and Friedewald (1944) reversed this procedure by applying first the virus and then the chemical. They used tar alone, 3-methylcholanthrene alone or a mixture of tar and 3-methylcholanthrene. In these experiments a greater number of papillomas changed into carcinomas and in a shorter period of time than in rabbits injected with virus only.

2. Shope Fibroma Virus

Ahlström and Andrewes (1938) injected tar subcutaneously or intramuscularly and at the same time the Shope fibroma virus intraperitoneally, intravenously or intracutaneously into domestic rabbits. Systemic inoculation of the virus alone did not induce any neoplasms. However the animals which received the intracutaneous injection of the virus developed large neoplasms which would progress until the death of the animal. It is interesting that the tar and viral injections were made on opposite sides of the animals.

Another group of animals received the tar subcutaneously and later an intravenous injection of the virus. Large neoplasms developed at the tar injection site where the virus localized. Eventually the disease progressed to generalized fibromatosis involving skin, mouth, eye lids, nose, peritoneal cavity, penis, testis, liver, and bone. Similar results were obtained when the virus was injected intraperitoneally and 3-methylcholanthrene and benzo(a)pyrene were used instead of tar.

Harel and Constantin (1954) injected the fibroma virus into the skin and testis of rabbits followed by massive doses of cortisone. The animals developed large neoplasms in 11 to 22 days some of which were invasive.

3. Rous Sarcoma Virus

Carr (1942) injected the virus into the breast and 3-methylcholanthrene into the leg of resistant inbred chickens. Due to the

resistance of the chickens only small, slow-growing tumors developed when the virus was injected alone. The birds which received the combined injections developed small neoplasms in the breast which regressed. While these neoplasms regressed "swellings" occurred in the 3-methylcholanthrene-injected legs. These "swellings" turned to "lumps" which continually regressed until the experiment was terminated on the 40th day. No neoplasms developed in birds treated with the 3-methylcholanthrene alone.

4. Polyoma Virus

Rawson et al. (1961) applied 7,12-dimethylbenz(a)anthracene alone or followed by 15 paintings with croton oil to the skin of polyoma virus-inoculated mice. He also used 15 paintings with croton oil alone or 40 paintings with benzo(a)pyrene alone. The results indicated a significant increase in tumor formation with all chemicals used. It is interesting that the polyoma virus was activated by tumor-initiating as well as tumor-promoting chemicals.

5. Bittner Virus

Benevolenskaya (1959) accomplished a malignant transformation of mouse fibroblasts in vitro by exposing them to 3-methylcholanthrene in combination with the Bittner virus. She (1959) also reported in vitro transformation of rat fibroblasts by using 3-methylcholanthrene together with a nucleo-protein extract taken from either a chicken sarcoma or a rat sarcoma. The transformations were characterized by

morphological changes and an accelerated multiplication rate. The cells exposed to the chemical carcinogen alone showed a tendency to aggregation and disintegration. No significant changes were observed when the virus or the extracts were used alone.

Mogila (1959) reported that a combination of human tumor extracts and 3-methylcholanthrene caused in vitro transformation of rat fibroblasts. Young rats inoculated with the transformed cells developed transplantable sarcomas in all cases.

F. Duran-Reynals (1940,1942) has attempted to show that there is no basic difference between oncogenic and nononcogenic viruses in the production of neoplasms. His experiments were based on his earlier observation and the observations of others that oncogenic viruses can induce a variety of non-neoplastic changes depending on such factors as age, species, dosage and route of inoculation. For example, the Rous and Fujiami sarcoma viruses produced in young susceptible chickens a lethal hemorrhagic disease whereas neoplasia was the result in the adult chicken. In young resistant chickens a latent infection was induced which developed into sarcoma later in life. He also reported that the dosage of the Shope papilloma virus can alter the outcome when inoculated into newborn rabbits: large amounts induced generalized degenerative lesions while small amounts produced tumors.

According to Groupe, Rauscher and Bryan (1957) the route of inoculation of the Rous sarcoma virus played a great role in determining the response. Large amounts of virus inoculated intracerebrally into one-day-old chicks induced both hemorrhagic and degenerative lesions in the liver. On the other hand, lesions were not observed when (a) the

same amount of virus was inoculated subcutaneously, (b) when smaller amounts of virus were inoculated intracerebrally, or (c) when large amounts of virus were inoculated intracerebrally into six-day-old chicks.

Stewart and Eddy (1959) have shown that the polyoma virus can induce both degenerative and neoplastic lesions at the same time.

It is evident from these observations that at least some of the oncogenic viruses can act under certain conditions like nononcogenic viruses, i.e. inducing inflammatory and degenerative rather than neoplastic changes. It was for this reason that F. Duran-Reynals postulated that if oncogenic viruses could act like nononcogenic viruses, possibly the reverse might also be true under the proper set of conditions. One of these conditions might be the interaction between a nononcogenic virus and a chemical carcinogen.

6. Herpes Simplex Virus

This virus was used by Teague and Goodpasture (1923) in the first known attempt to show interaction between a virus and a chemical carcinogen. Levaditi and Nicolau (1922) had shown that Herpes simplex virus multiplied rapidly in actively growing neoplastic tissue. Since it was also known that tar painting induced skin tumors in rabbits, it was speculated that the initial hyperplasia induced by the tar might render the tissue more susceptible to the action of the virus.

The flanks of guinea pigs and rabbits were painted with tar and the Herpes simplex virus was injected simultaneously into the same

areas. Whereas the Herpes simplex virus induced only microscopic lesions in untaired animals, severe herpetic lesions developed at the site of inoculation in both guinea pigs and rabbits. Eventually the virus infected the central nervous system and the animals died in 8 to 14 days.

7. Fowl Pox Virus

F. Duran-Reynals and Bryan (1952) using naturally infected chickens without signs of the disease activated the virus by treating the animals with 3-methylcholanthrene. Typical malignant and benign epithelial neoplasms developed in the areas of painting. The virus was recovered from the neoplasms up to 15 months later. After this period, further 3-methylcholanthrene paintings could activate the virus again.

In another experiment, F. Duran-Reynals (1957) used a breed of chickens in which no fowl pox virus could be activated by 3-methylcholanthrene painting. The virus was inoculated into these chickens as 15-day embryos. The birds hatched showing typical fowl pox lesions which regressed. After regression the virus could no longer be isolated. When, however, four months after tumor regression these same animals were painted with 3-methylcholanthrene, neoplasms appeared in the painted areas and the virus was reactivated. The virus recovered from the neoplasms induced the typical disease, fowl pox.

8. Vaccinia Virus

Initial studies with the vaccinia virus in mice were difficult to carry out due to its lethal and highly contagious nature. These

problems were solved by F. Duran-Reynals (1957a) who observed that in cortisone-treated mice the lesions were strictly localized and not contagious. Mice received skin paintings with 3-methylcholanthrene followed by injections of cortisone and vaccinia virus into the painted area. Ulcers formed in approximately seven days and healed leaving scar tissue. Later, neoplasms developed at the site of scar tissue formation in 66 per cent of the animals, and one-half of these became malignant.

M. L. Duran-Reynals (1961) was able to show that tumor formation with these two agents depended more on the sequence in which they were used than on the quantity of the agents. When 3-methylcholanthrene was applied after virus inoculation, a higher incidence of tumors was observed with a smaller dose than when it was applied first.

Martin et al. (1961) obtained enhancement of tumor formation in mice injected with the vaccinia virus and treated simultaneously with 7,12-dimethylbenz(a)anthracene, 2-aminofluorene or dibenz(a,h)anthracene.

9. Influenza Virus

Askanazy (1919) and Winternitz, Wason, and McNamara (1920) reported that the damage induced by the influenza virus in humans leads to tissue proliferation resembling epithelial neoplasia. Straub (1937) and Steiner and Loosli (1950) made similar observations in the lungs of mice injected with influenza virus. McCordock and Muckenfuss (1933) pointed out similarities between the influenza virus-induced lesions and those induced by the vaccinia virus in rabbits. They stressed also the similarities of the proliferative lesions produced in man and mice

by the influenza virus with those in the lungs of sheep injected with the sheep pox virus as described by Borrell (1903).

Imagiwa, Yorhimori and Adams (1957) infected mice with the influenza virus intranasally and injected the animals later intraperitoneally with urethane. Mice which received both agents had a higher incidence of lung tumors than the animals receiving either agent alone.

Wisely et al. (1961) used C57BL mice, which are known to have a low incidence of spontaneous lung tumors, in an experiment to determine the interaction of the influenza virus and the carcinogen "ozonized gasoline". Animals infected with the virus and exposed to the inhalation of "ozonized gasoline" developed significantly more lung tumors than the control animals.

10. West Nile Virus

Tanaka and Southam (1962) injected the West Nile virus into mice which had been painted previously three times with either 3-methylcholanthrene or benzo(a)pyrene. Subsequently, these animals received another seven treatments with these chemicals. The result was an enhancement of tumor formation as indicated by greater numbers and shortened latent periods. Amount and time of virus recovery were similar in the experimental and control animals suggesting that the carcinogens had no effect on virus infectivity.

11-13. Poliovirus 2, ECHO Virus 9, and Coxsackie Virus B₄

Martin (1964) reported experiments in which virus-carcinogen pairs were formed from poliovirus 2, ECHO 9 and Coxsackie B₄ and

7,12-dimethylbenz(a)anthracene, 2-aminofluorene and dibenz(a,h)anthracene. Swiss mice were injected with a single dose of virus and a sub-threshold dose of chemical carcinogen at the same time. Malignant lymphomas, granulocytic leukemias, a reticulum cell sarcoma and a subcutaneous fibrosarcoma developed. These neoplasms do not arise ordinarily in Swiss mice.

Possible Mechanisms of Interaction Between
Chemical Carcinogen and Virus

Since the etiology of neoplasia is unknown, various theories have been proposed to explain the importance of the virus and the chemical carcinogen. M. L. Duran-Reynals (1963) has discussed the following two possibilities:

1. The chemical carcinogen enhances the action of the virus.
2. The virus enhances the action of the chemical carcinogen.

In favor of the first assumption is the fact that neoplasms produced by the Rous sarcoma, Shope papilloma, Shope fibroma and polyoma virus in conjunction with chemical carcinogens are of the same type as those produced by the virus alone. Furthermore, it has been shown that a chemical carcinogen activates the polyoma virus from a latent to a tumor-inducing state.

On the other hand, it is difficult to believe that the foregoing assumption applies to a nononcogenic virus, like the West Nile virus. Furthermore, essentially the same type of neoplasm develops in an animal when a chemical carcinogen is used alone as when a chemical carcinogen is combined with a nononcogenic virus like fowl pox,

vaccinia, influenza or West Nile virus. This would lend support to the second assumption. Martin (1964) has discussed the different mechanisms by which the mutual enhancement of chemical carcinogen and virus could be explained.

I. Virus and/or chemical carcinogen function at a distance from the target cell:

- (a) Virus infection impairs the normal systemic metabolism and excretion of the chemical carcinogen, increasing the local carcinogenic effect of the chemical.
- (b) Through the effect of a nononcogenic virus on host resistance, an oncogenic virus is activated.
- (c) The toxicity of the chemical carcinogen, in terms of depression of bone marrow, adrenal cortical stimulation, and inhibition of antibody formation, allows otherwise suppressed oncogenic viruses to emerge, propagate, and produce overt neoplasia.

II. Virus and chemical carcinogen act synergistically at the target-cell level:

- (a) Virus attack and injury enhance the permeability of cells to the chemical carcinogen.
- (b) Virus infection of the cell, though abortive, interferes with normal intracellular detoxification of the chemical carcinogen.
- (c) Cells proliferating or regenerating rapidly in the wake of the cytopathic effects of viral infection are more

likely to incorporate the chemical carcinogen than are relatively quiescent cells.

- (d) The chemical carcinogen inhibits the production of the cellular interferon and permits the emergence of a latent oncogenic virus.

III. There is direct virus-chemical carcinogen interaction:

- (a) The chemical carcinogen produces an oncogenic virus mutant in a population of otherwise benign virus particles.
- (b) The virus functions as a vector, facilitating entry of the chemical carcinogen into the cell, or delivering carcinogen directly to susceptible intranuclear chromosomal loci.

It is apparent that much of the information available on virus-chemical carcinogen interactions is fragmentary, and any pertinent theory at best could only be classified as presumptive. Nevertheless, it has served to direct the attention of the author to investigate the interactions of the murine leukemia viruses with polycyclic aromatic hydrocarbons.

OBJECTIVE

Previous investigations of the interaction between chemical carcinogens and oncogenic viruses have always demonstrated enhancement of the virus-induced neoplasms by the chemical carcinogen. Since these studies were only concerned with oncogenic viruses inducing solid neoplasms, it was decided to determine, if a similar effect could be obtained using leukemogenic viruses. The objective of this investigation was to study the interactions of two carcinogenic polycyclic aromatic hydrocarbons, 7,12-dimethylbenz(a)anthracene and 3-methylcholanthrene, and two murine leukemia viruses, Friend and Rauscher.

MATERIAL AND METHODS

The main investigation can be conveniently divided into three major experiments.

1. Interaction of Friend virus with 7,12-dimethylbenz(a)anthracene (DMBA) and 3-methylcholanthrene (3-MC).
2. Interaction of Rauscher virus with 7,12-dimethylbenz(a)-anthracene (DMBA) and 3-methylcholanthrene (3-MC).
3. The effect of DMBA on the cells of a Friend virus-induced reticulum cell sarcoma tissue culture, injected into mice.

The Host

The host animals employed throughout this investigation were inbred BALB/c mice which were obtained from a colony started and maintained at the University of Arizona. The original families were derived from the offsprings of BALB/c pregnant mice purchased from Simonsen's Laboratories, Gilroy, California. The animals were weaned not earlier than 21 days and not later than 25 days. Males and females were separated and used not younger than five weeks and not older than eight weeks of age. The families were fed Purina breeder chow pellets and the experimental mice Purina Laboratory chow pellets. The animal room was maintained at a temperature of 70° F. and a humidity of 10 to 15 per cent.

The Viruses

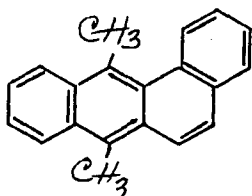
The Friend and Rauscher viruses were obtained from Dr. A. Howard Fieldsteel, Stanford Research Institute, Menlo Park, California, and were suspended in BALB/c mouse spleen homogenates. They were shipped in dry ice and were received in the frozen state. The vials were thawed quickly by gentle agitation in a 37° C. water bath and 0.2 ml. of the suspension were injected intraperitoneally into each of ten young adult BALB/c mice. The animals were sacrificed 21 days later and the infected tissue harvested. A pool was made by the following procedure:

1. The spleens were removed with sterile instruments. They were pooled, weighed and a 20 per cent suspension was prepared with a sucrose stabilizer devised by Bovarnick, Miller and Snyder (1950) but later modified by Fieldsteel (1964).
2. The whole spleens suspended in sucrose stabilizer were homogenized in a Virtis Homogenizer for two minutes, allowed to cool for two minutes and again homogenized for two minutes. The entire procedure was carried out in an ice bath.
3. The homogenate was dispensed into 5 ml. vials and quickly frozen in a dry ice bath. They were stored at -65° C. until required for study.
4. When needed the homogenate was thawed quickly at 37° C. and centrifuged in a refrigerated angle-head centrifuge at 1500 rpm for five minutes.
5. The supernatant was removed and employed as the virus inoculum. The Rauscher and Friend virus pools were titrated and calculated

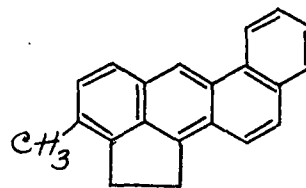
on the basis of histological examination by the Reed and Muench method. The Friend virus pool used in this study titered $10^{-5.3}$ ID₅₀/ml. and the Rauscher virus pool $10^{-4.8}$ ID₅₀/ml.

The Chemical Carcinogens

The chemical carcinogens, namely 7,12-dimethylbenz(a)anthracene and 3-methylcholanthrene, were obtained from Eastman Organic Chemicals, Rochester 3, New York.



7,12-Dimethylbenz(a)anthracene (DMBA)



3-Methylcholanthrene (3-MC)

The chemicals applied to the skin were dissolved in reagent grade acetone (Terracini 1960) and the chemicals used for injection were dissolved in corn oil (Fieldsteel 1964a). The acetone preparations were made weekly and the corn oil preparations were prepared only one time and stored at 2° C. to 5° C. They were used for the duration of the experiment.

Methods to Study the Interaction of

Friend and Rauscher Viruses with DMBA and 3-MC

Tenfold dilutions in cold sucrose stabilizer (ranging from 10^{-1} to 10^{-7}) were made from 20 per cent stock pools of both the Friend and Rauscher viruses. The individual dilutions were inoculated intraperitoneally into three groups of five mice each. The day before the inocu-

lations and weekly thereafter the hair was removed from the backs of the mice in Group II with a commercial depilatory ("Nair"). The three groups of mice were treated in the following manner immediately after the injection of the virus.

Group I - The animals received a subcutaneous injection in the region of the scapulae of 100 ug of DMBA or 100 ug 3-MC contained in 0.1 ml. of corn oil (Toth 1963).

Group II - A 0.2 ml. serological pipette and later a tuberculin syringe with an 18 gauge needle, which was easier to control, were used to apply over an area measuring approximately three square cm. 200 ug of DMBA or 3-MC contained in 0.05 ml. of reagent grade acetone to the hairless skin of each mouse. In order to prevent the material from running off, the skin was dried immediately after the application with a warm stream of air produced by a hair dryer.

Group III - The mice received only the virus and acted as the untreated virus controls.

In addition to the above groups the following controls were included:

1. Mice received only DMBA or 3-MC via the same routes indicated above.
2. Mice received sucrose stabilizer only.
3. Untreated and uninoculated mice.

Starting three days after the initial injection the animals were palpated every other day for the characteristic enlargement of the

spleen. When the spleen extended beyond the breast line the result was considered positive. The number of days from the day of inoculation up to this point was the latent period.

The cages were checked twice daily for dead animals. When found, they were necropsied and the adrenals, portions of the spleen, liver, and the area of the skin treated with either chemical were placed in ten per cent formalin. After fixation they were embedded in paraffin and sectioned. All sections were stained with hematoxylin and eosin and examined microscopically. The histological examination was used to verify the gross diagnosis of the disease.

The experiments were terminated on the 100th day and any animals living at that time were sacrificed and examined as indicated.

Tissue Culture

A frozen suspension of a Friend virus-induced reticulum cell sarcoma tissue culture was obtained from Dr. A. Howard Fieldsteel, Stanford Research Institute, Menlo Park, California. The cells were suspended in a preservative containing dimethyl sulfoxide (Porterfield 1962, Dougherty 1962). The cells were recovered employing the following procedure:

1. The material was thawed quickly at 37° C. and the suspension transferred to a conical centrifuge tube.
2. 3 ml. of "Medium 199" was added slowly down the side of the tube. The suspension was held five minutes at room temperature and centrifuged in an angle-head centrifuge at 1000 rpm for five minutes. The supernatant was discarded and the sediment resuspended. The procedure was repeated a second time.

3. The suspension was placed in a 4 oz. prescription bottle containing a medium devised by Fieldsteel (1964) which he designated as medium #1. Medium #1 was used to initiate growth and maintain the cells for routine passages. It was compounded as follows:

Medium 199	800 ml.
Lactalbumin hydrolysate	5 gms.
Glucose	3 gms.
Fetal bovine serum (inactivated)	200 ml.
Penicillin	100,000 units
Streptomycin	100,000 units
Neomycin	100,000 micrograms
Bacitracin	1,000 units

The procedure employed for routine passage of the cell line was as follows:

1. The cells were removed from the culture bottle and centrifuged at 1000 rpm for five minutes.
2. The supernatant was discarded and the cells were suspended in medium #1. Tenfold or less dilutions were made in order to obtain a "viable and dead cell count", using trypan blue (Fieldsteel 1964a) of approximately 100 cells per four squares of a wbc-hemocytometer.
3. The numbers of dead and viable cells per milliliter were calculated by taking the average number for the four squares and multiplying it by a factor calculated on the basis of the dilution used. The cell concentration was adjusted to the desired level with medium #1.

4. The per cent visibility was recorded and used as a guide to adjust the number of cells for future passages. The desired concentration of viable cells was that which would not require attention for a period of seven days. When the tissue culture was well established, a seeding of 2.0×10^6 cells seeded in a 16 oz. prescription bottle, containing 25 ml. of medium, was satisfactory.

Some passages were preserved by freezing and recovered when needed. The preservation procedure of Porterfield (1962) and Dougherty (1962) was followed.

1. Viable counts were performed using trypan blue. The cell concentration was adjusted with the following preservation mixture to approximately 10^7 cells/ml.

Medium #199	75%
Fetal bovine serum	15%
Dimethyl sulfoxide*	10%

2. Three ml. of the suspension were placed in 5 ml. ampules.
3. The ampules were sealed with a torch and set upright in 95% alcohol at room temperature for three to five minutes. Dry ice was added in small amounts to cause the temperature to drop 3° C. per minute. This was continued until -35° C. were reached. The temperature was then lowered to -70° as quickly as possible.

*Crown Zellerbach
Chemical Products Division
Camas, Washington

4. The ampules were stored at -65° C. and when needed the cells were recovered according to the procedure stated above. In the experiments to be described cells harvested from actively growing tissue culture and not preserved cells were used. In addition, cells harvested from the ascites fluid of mice in which the tissue culture cells had been passed were also used.

Methods to Study the Effect of DMBA on the
Cells of a Friend Virus-Induced Reticulum
Cell Sarcoma Tissue Culture Injected Into Mice

The following preliminary steps had to be accomplished before the effect of DMBA on the cells of a Friend virus-induced reticulum cell sarcoma tissue culture (Fieldsteel 1964) injected into mice could be determined.

A certain number of these cells have to be injected intraperitoneally to induce the sarcoma. In order to detect any possible effect that DMBA might have, two cell concentrations were used and the number of cells injected was adjusted so that one hundred per cent of the animals receiving the largest number and fifty to sixty per cent of those receiving the smallest number of cells would develop sarcoma in a 50-day period. To obtain these values titrations were performed as follows:

1. The tumor cells were harvested from the growth bottles or, in the case of those passaged in vivo, from the ascites fluid. Cells were washed once with medium #1 and suspended in the same fresh medium.

2. The suspensions were then diluted until four hemocytometer squares contained approximately 100 cells.
3. The number of viable cells was determined using trypan blue. Their concentration was adjusted by the addition or removal of the suspending medium, so that the desired number would be contained in a 0.2 ml. volume. This was the volume routinely inoculated into the host throughout this experiment.
4. Once the cell concentration was adjusted a recount was performed as a check.
5. Tenfold dilutions of the adjusted cell suspensions were made in medium #1 ranging from 10^6 to 10^{-1} . Volumes of 0.2 ml. of each dilution were injected intraperitoneally into each of five BALB/c mice.
6. All dead animals were necropsied, and any that were living were sacrificed after 50 days. The observations were recorded as (+) or (-) depending on whether or not tumors were observed.
7. From the data collected the end point was calculated. This was defined as the minimal number of cells which would induce tumor formation within a 50-day period.

Prior to the injection of tumor cells, one group of mice received three injections of 100 ug DMBA at weekly intervals and another group was painted three times at weekly intervals with 200 ug DMBA. Then one-half of each group was injected with 5.1×10^3 and 5.1×10^4 cells passaged 105 times in vitro and the other half with 5.1×10^1 and 5.1×10^2 cells passaged 86 times in vitro and subsequently three times

in vivo. The desired number of cells was injected into each of seven mice intraperitoneally. Immediately after the cell injections the animals were again treated with the chemical as stated above and weekly thereafter until the experiment was terminated on the 50th day. A control group received only the tumor cells but no chemical carcinogen.

The following additional controls were included:

1. mice receiving medium #1 only,
2. mice receiving chemical carcinogen only,
3. mice without treatment

The animals were palpated at two-day intervals for peritoneal tumors. Latent periods and survival times were recorded. All animals were necropsied when found dead or when sacrificed at the end of the experiment.

RESULTS

Interaction of Friend Virus with DMBA and 3-MC

Control Animals

Gross Examination

In those animals receiving the Friend virus only, the disease process was the same as that described by Friend (1957), Metcalf and Furth (1959) and Dawson, Fieldsteel and Bostick (1963). The disease is characterized by a short latent period. The spleen enlarged rapidly and weighed 2 gms. or more by the third week as compared with an approximate weight of 0.5 gms. in normal spleens. It was not unusual to find spleens weighing as much as 5 gms. after the 35th day. Hemorrhagic infarction of the spleen was a common finding. In some animals the spleens looked like "bags filled with blood". However, in contrast to findings of the previous investigators, no cases of ruptured spleen and intraperitoneal hemorrhage were observed. If the animal survived the disease for an extended period, the liver became grossly involved weighing as much as 8 gms. compared to a normal weight of \pm 1 gm. (Fig. 4). No enlargement of the thymus or lymph nodes was observed.

Microscopic Examination

Initially the disease was characterized by the formation of reticulum cell nests originating in the red pulp and eventually spreading to the white pulp of the spleen. Later, massive infiltration of the spleen and liver with reticulum cells occurred (Fig. 5 through 8).

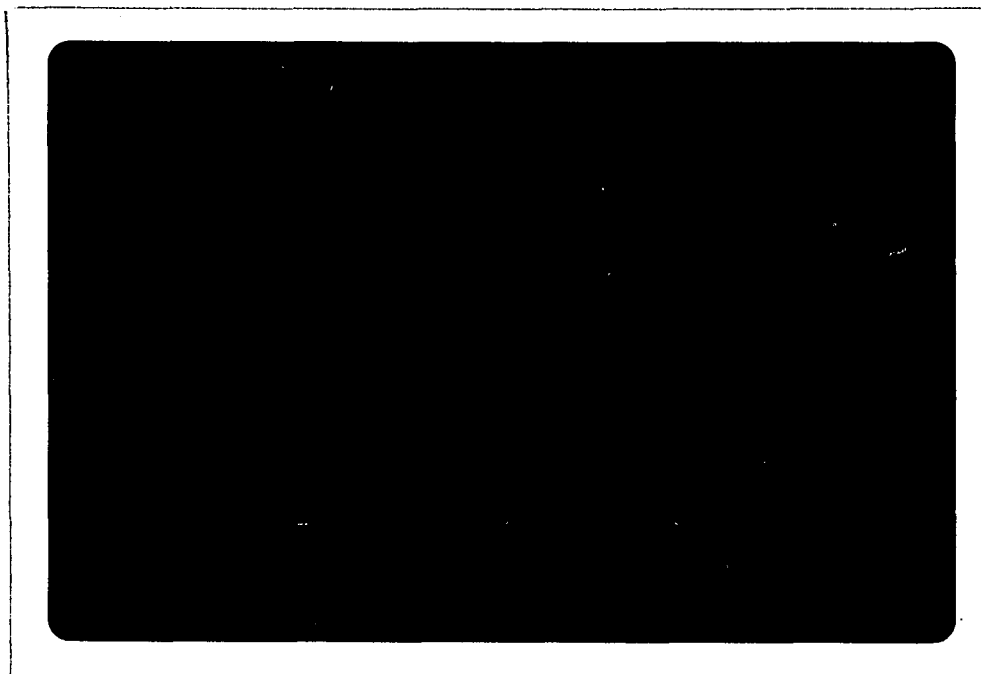


Figure 4
The Abdominal Organs in Friend Virus Leukemia

Left: Friend virus leukemia
Right: Normal mouse

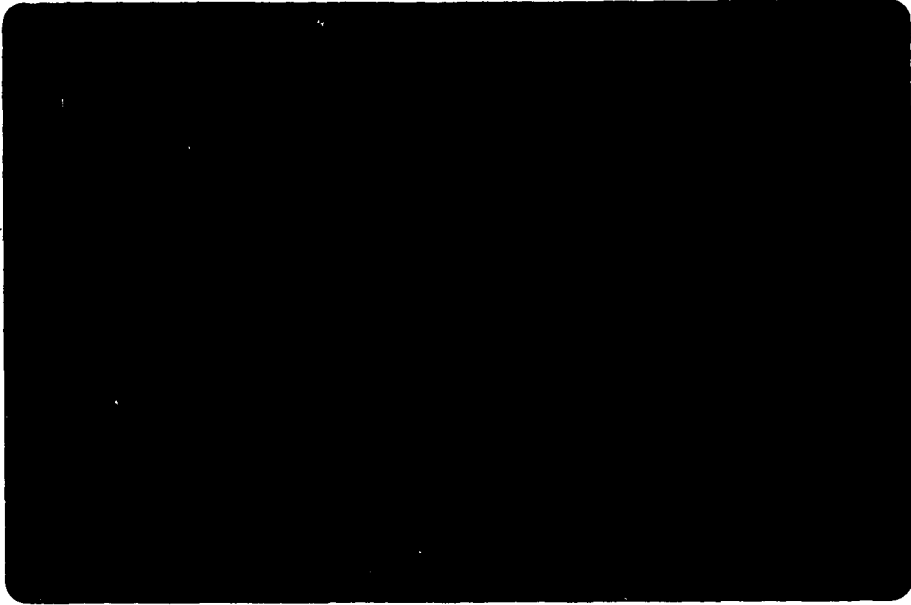


Figure 5
Spleen in Fully Developed Friend Virus Leukemia x 125

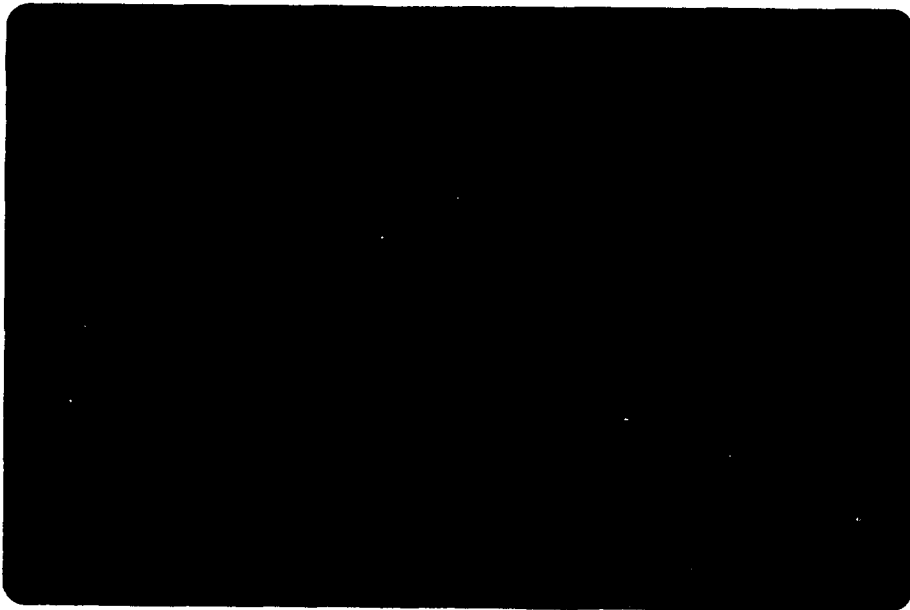


Figure 6
Spleen in Fully Developed Friend Virus Leukemia x 550



Figure 7
Liver in Fully Developed Friend Virus Leukemia x 125

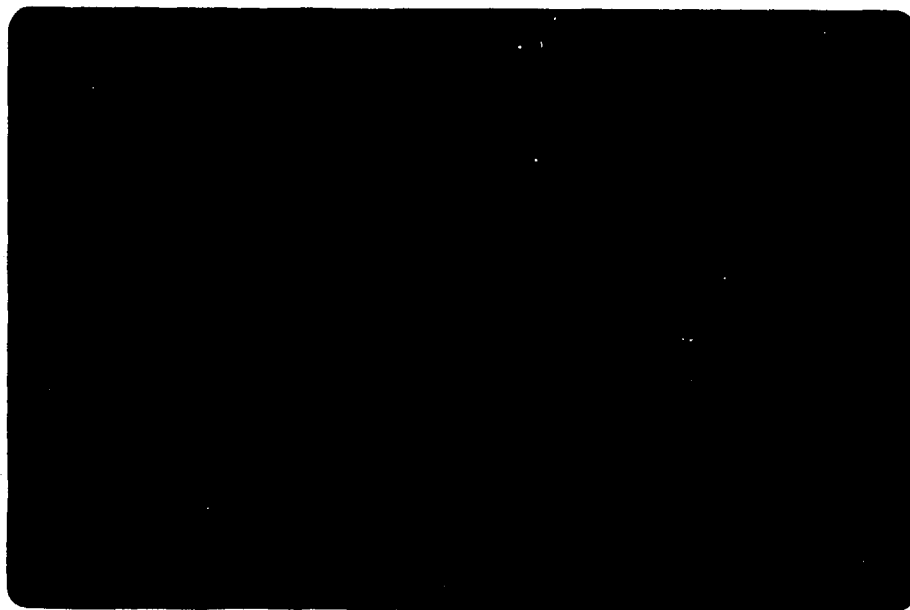


Figure 8
Liver in Fully Developed Friend Virus Leukemia x 550

Experimental Animals

Judging from gross and microscopic observations the major effect of the chemical carcinogens seemed to be a delay in the onset of the disease as shown by the longer latent periods and correspondingly increased survival times. This effect seemed to be mostly due to interference with one of the aspects of the Friend virus leukemia, namely engorgement and hemorrhagic infarction of the spleen. The treated animals seemed to show much less of this phenomenon. Whether or not the chemical carcinogens also interfere with the actual leukemic process will have to be determined by further investigations. Such interference could be an additional factor explaining the longer latent periods and survival times and the smaller spleen weights. (Fig. 9 through 15).

The number of animals developing leukemia sooner or later was essentially the same in both the treated and untreated animals.

A total of 175 animals was used. Four died accidentally, 84 died of leukemia, 30 were sacrificed after 100 days with leukemia, and 57 were sacrificed after 100 days without leukemia.

Interaction of Rauscher Virus with DMBA and 3-MC

Control Animals

Gross Examination

In the animals receiving the Rauscher virus only the disease process was the same as described by Rauscher (1962) and Boiron et al. (1965). The latent period between the virus injection and the appearance of palpable spleens was about two weeks. Survival times varied according to the dilution of the virus injected, but usually the animals

DAYS

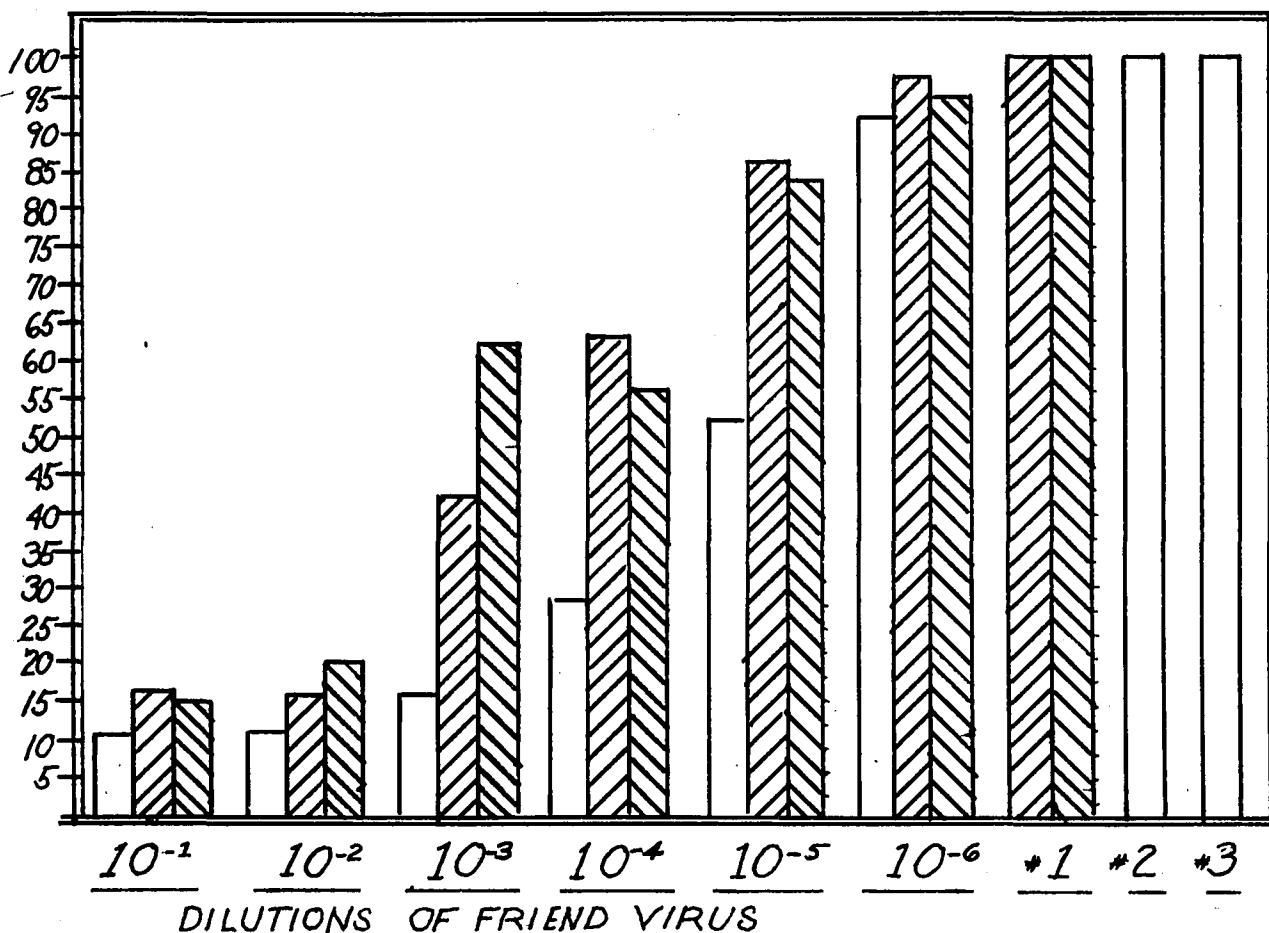


Figure 9
Latent Periods for DMBA Treated Animals
Expressed as Arithmetic Averages

□ = virus only

▨ = virus + DMBA (injected)

▩ = virus + DMBA (applied to skin)

Control #1 - DMBA without virus

Control #2 - sucrose stabilizer without
virus and without DMBA

Control #3 - normal control mice

DAYS

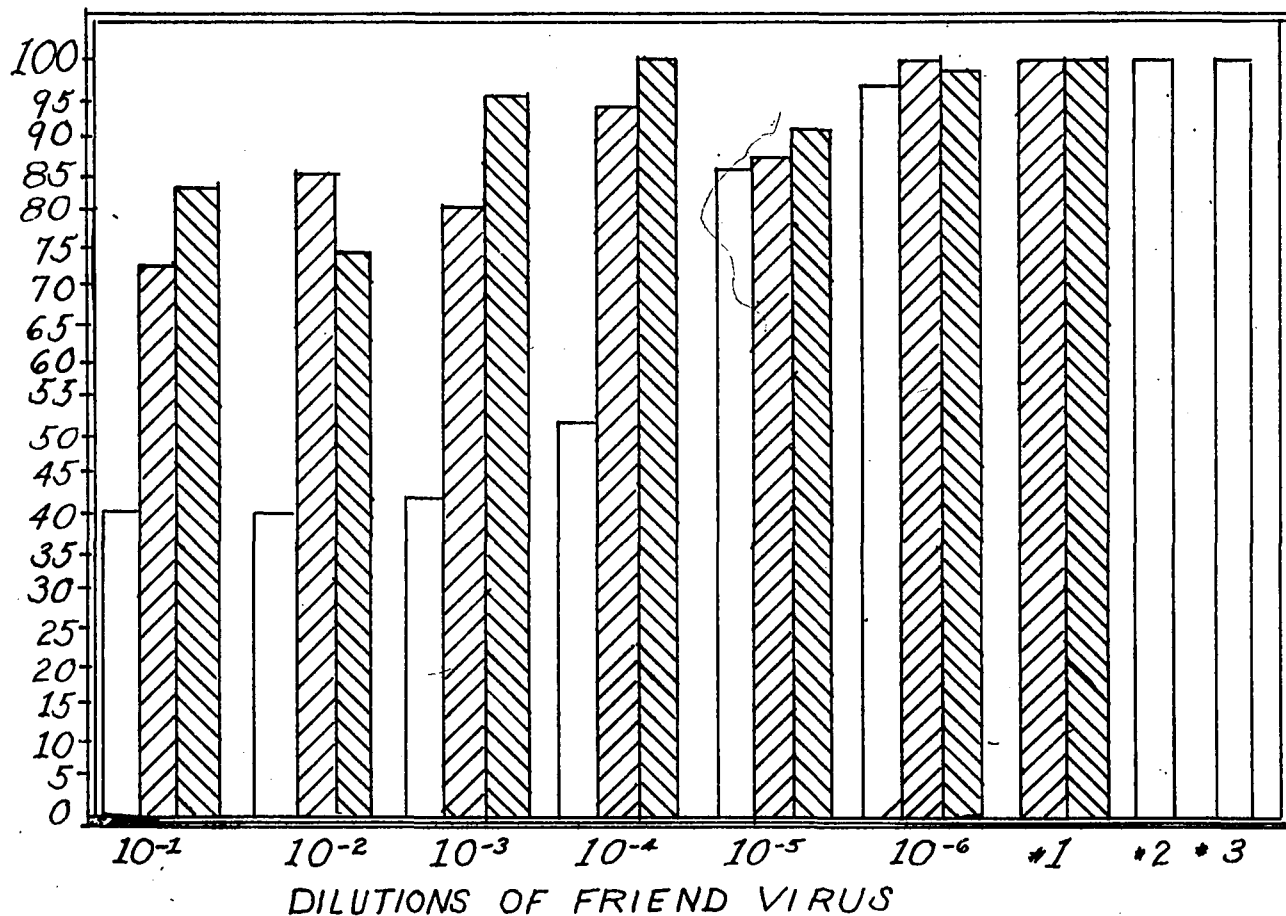


Figure 10
Survival Times for DMBA Treated Animals
Expressed as Arithmetic Averages

□ = virus only

▨ = virus + DMBA (injected)

▩ = virus + DMBA (applied to skin)

Control #1 - DMBA without virus

Control #2 - sucrose stabilizer without
virus and without DMBA

Control #3 - normal control mice

GRAMS

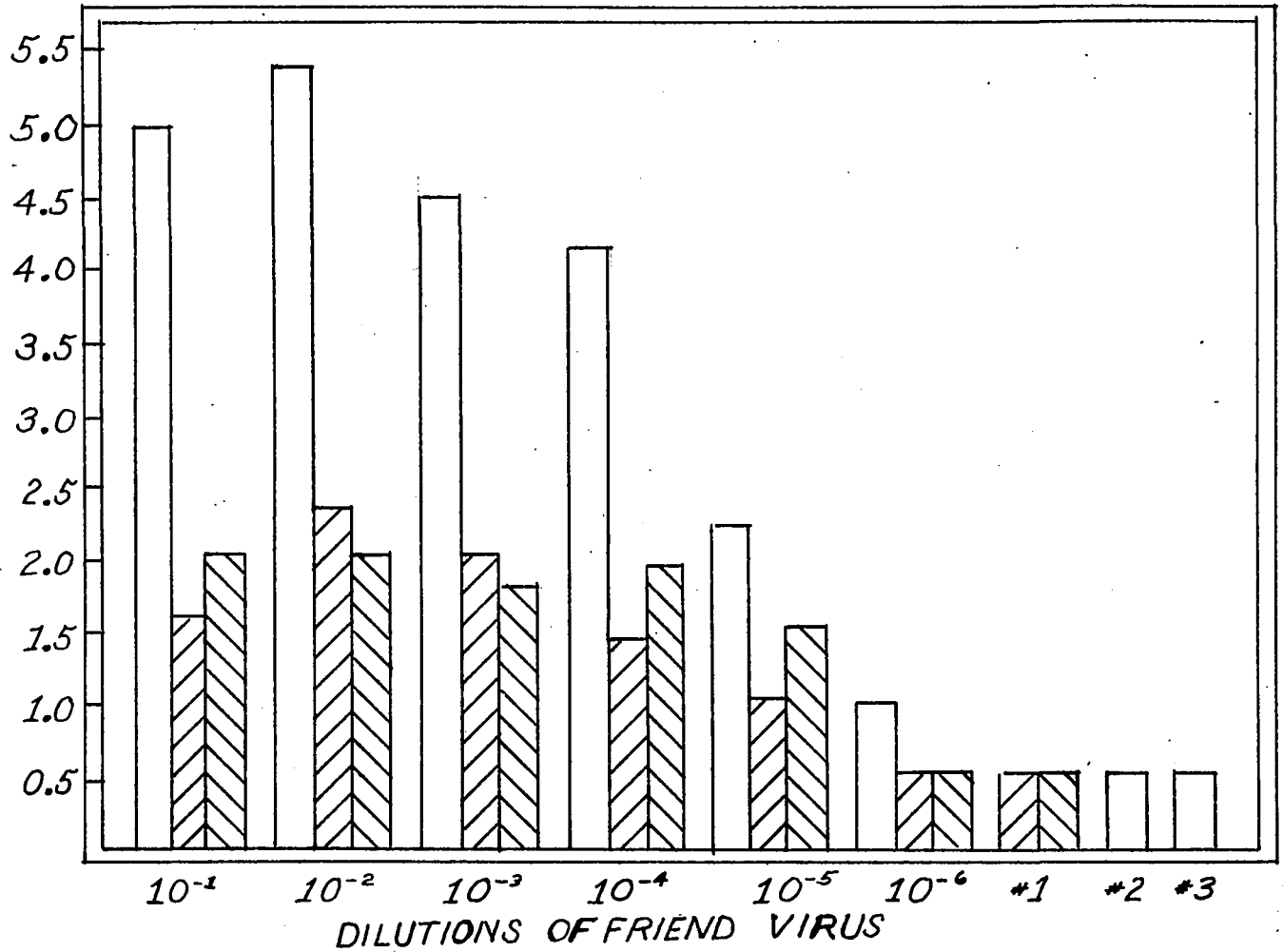


Figure 11
Spleen Weights for DMBA Treated Animals
Expressed as Arithmetic Averages

□ = virus only

▨ = virus + DMBA (injected)

▩ = virus + DMBA (applied to skin)

Control #1 - DMBA without virus

Control #2 - sucrose stabilizer without
virus and without DMBA

Control #3 - normal control mice

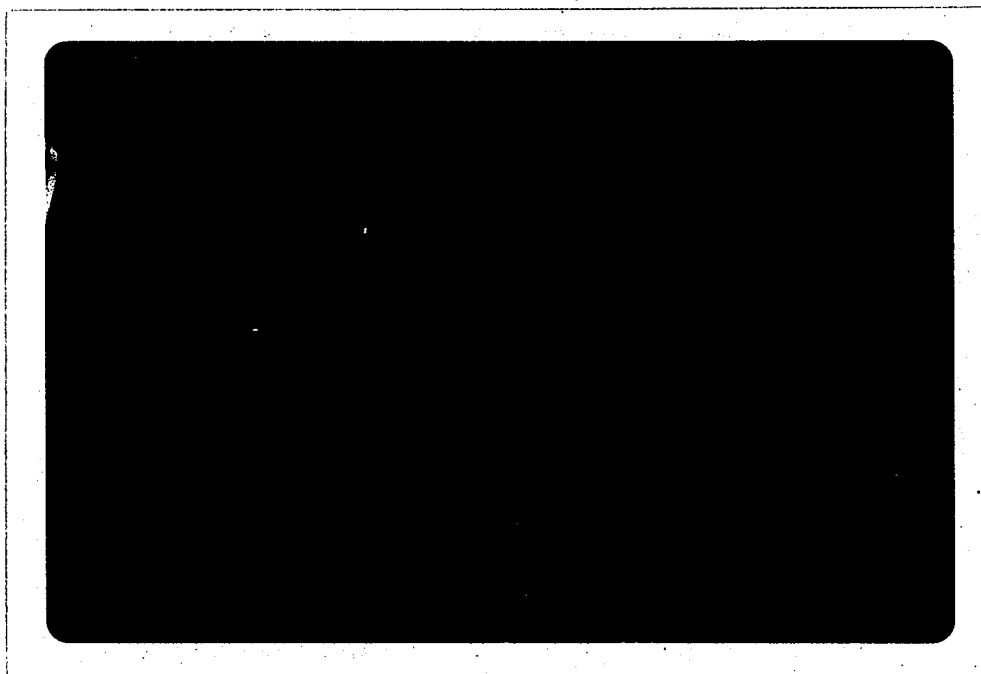


Figure 12
Effect of DMBA on Friend Virus Leukemia

From left to right:

1. Friend virus (10^{-5}) only after 70 days (note enlarged spleen and liver).
2. Friend virus (10^{-5}) plus DMBA (injected) after 70 days.
3. Friend virus (10^{-5}) plus DMBA (painted) after 70 days.
4. Normal mouse.

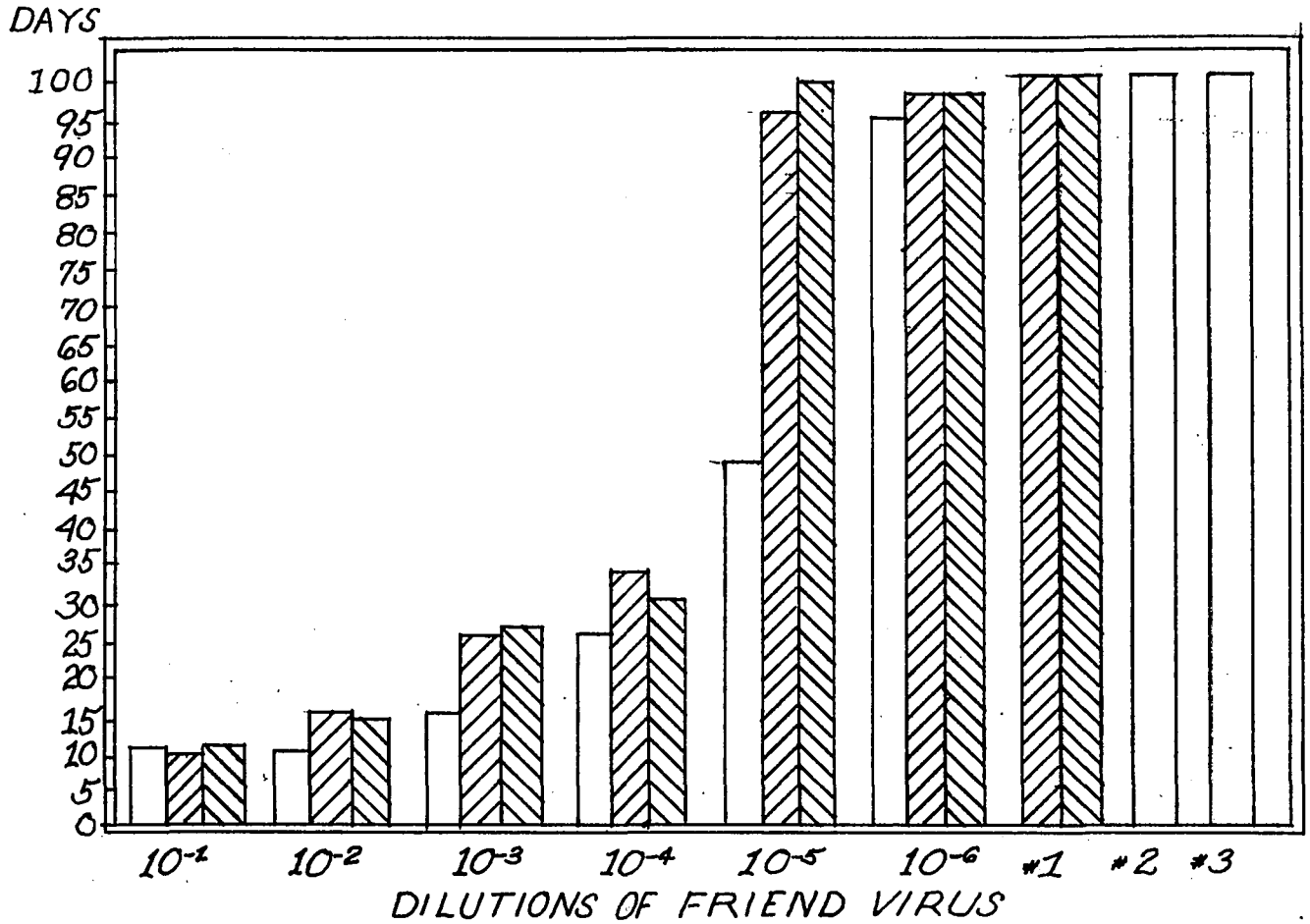


Figure 13
 Latent Periods for 3-MC Treated Animals
 Expressed as Arithmetic Averages

- = virus only
- ▨ = virus + 3-MC (injected)
- ▩ = virus + 3-MC (applied to skin)

Control #1 - 3-MC without virus
 Control #2 - sucrose stabilizer without virus and without 3-MC
 Control #3 - normal control mice

DAYS

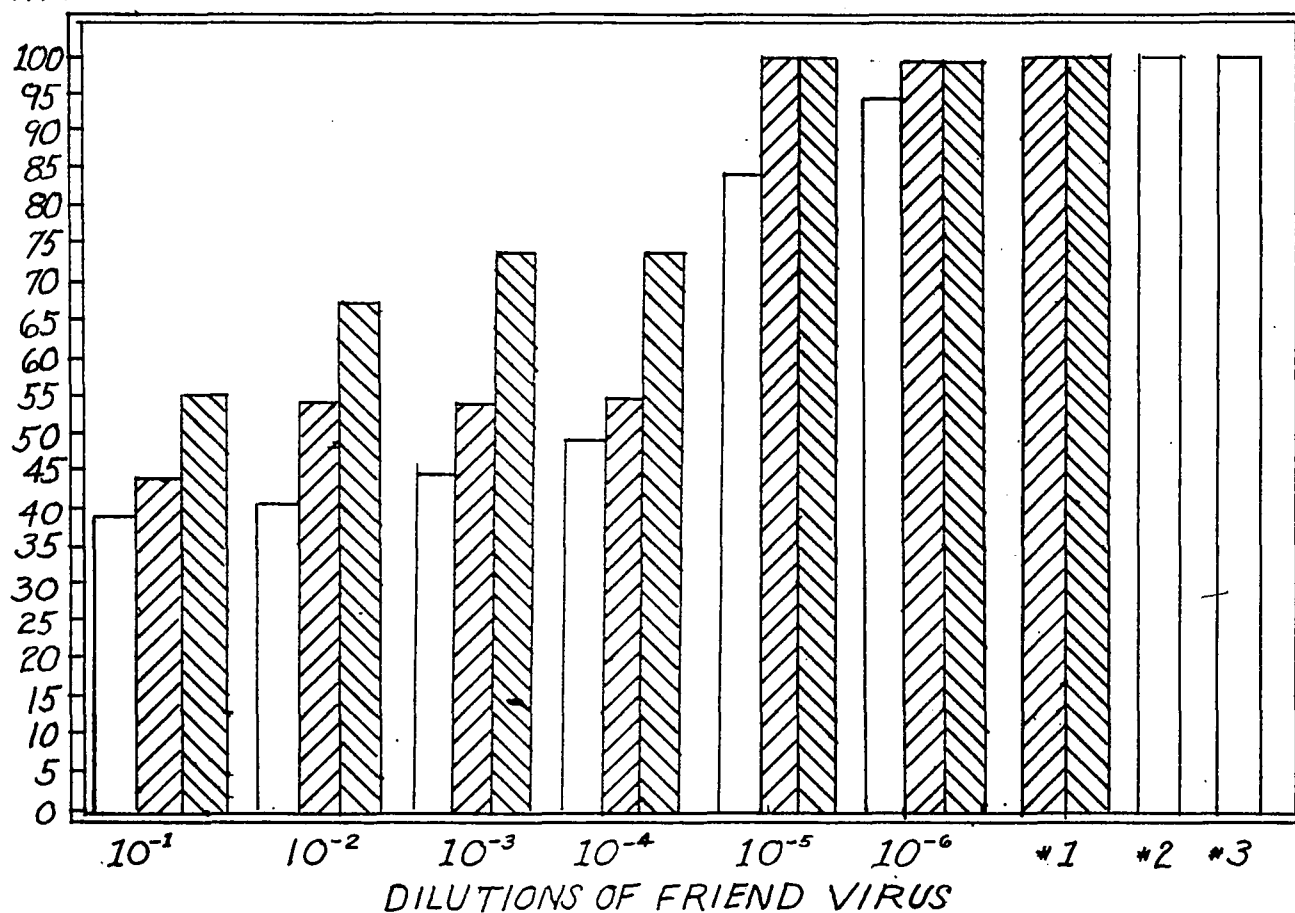


Figure 14
Survival Times for 3-MC Treated Animals
Expressed as Arithmetic Averages

□ = virus only

▨ = virus + 3-MC (injected)

▩ = virus + 3-MC (applied to skin)

Control #1 - 3-MC without virus

Control #2 - sucrose stabilizer without
virus and without 3-MC

Control #3 - normal control mice

DAYS

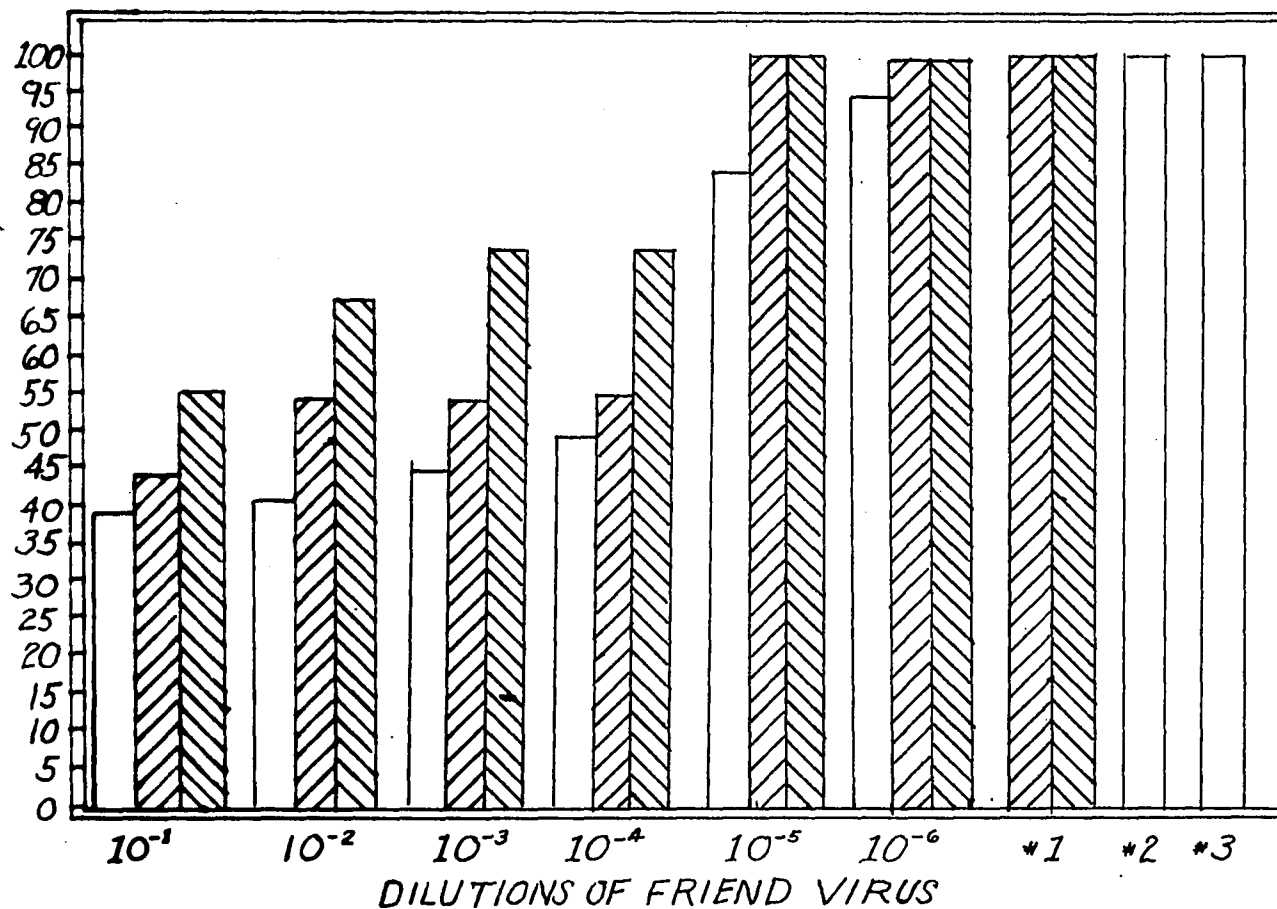


Figure 14
Survival Times for 3-MC Treated Animals
Expressed as Arithmetic Averages

- = virus only
- ▨ = virus + 3-MC (injected)
- ▩ = virus + 3-MC (applied to skin)

Control #1 - 3-MC without virus
Control #2 - sucrose stabilizer without virus and without 3-MC
Control #3 - normal control mice

GRAMS

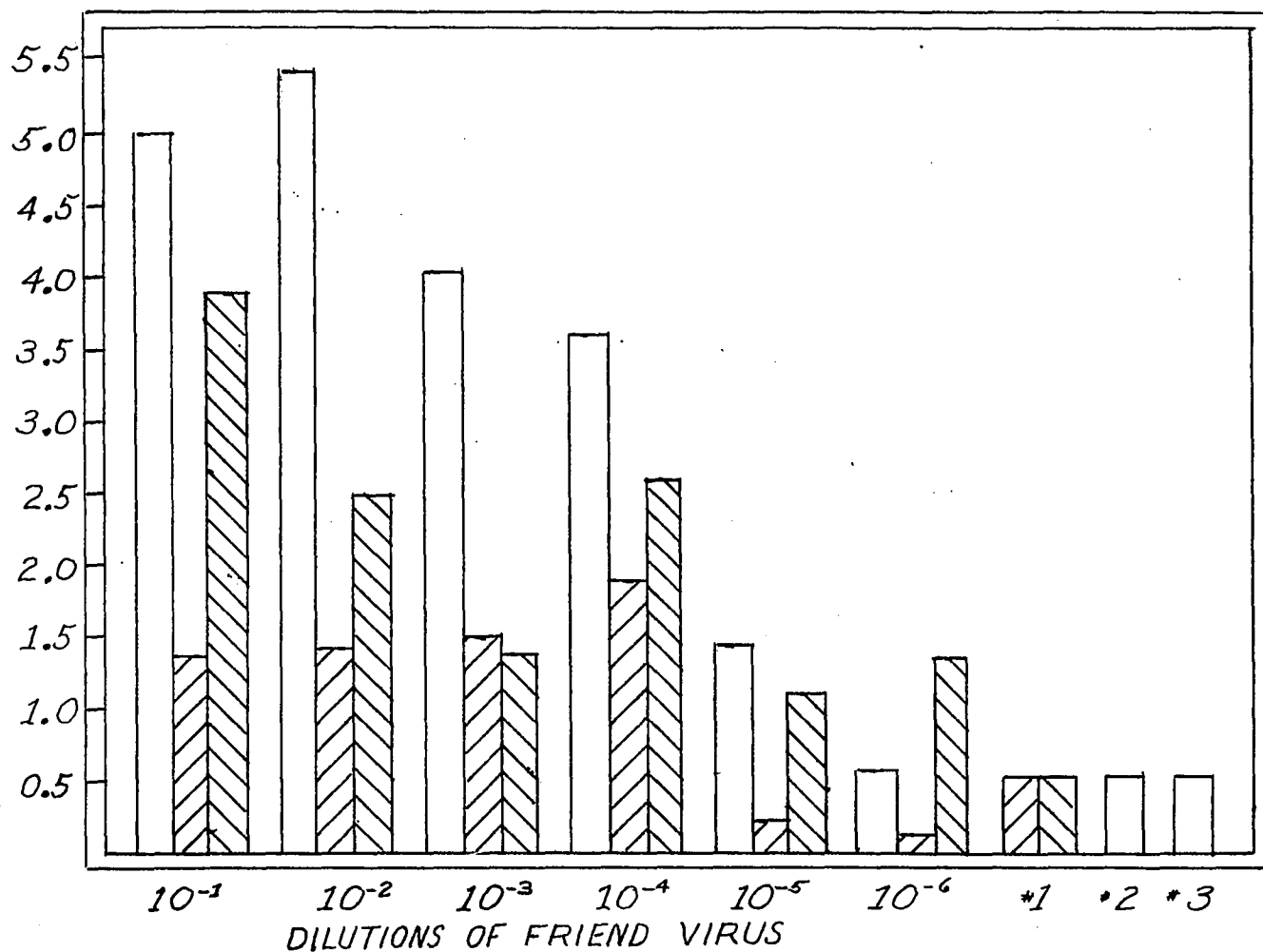


Figure 15
Spleen Weights for 3-MC Treated Animals
Expressed as Arithmetic Averages

- = virus only
- ▨ = virus + 3-MC (injected)
- ▩ = virus + 3-MC (applied to skin)

Control #1 - 3-MC without virus
 Control #2 - sucrose stabilizer without virus and without 3-MC
 Control #3 - normal control mice

receiving lower dilutions died between 30 and 35 days. At necropsy the gross picture was indistinguishable from that observed in Friend virus leukemia. Hemorrhagic infarction of the spleen was a common finding. In some animals the spleens looked like "bags filled with blood". No cases of ruptured spleen and intraperitoneal hemorrhage were observed. (See No. 1 animals in Fig. 23 and 27.) No enlargement of thymus or lymph nodes was observed.

Microscopic Examination

The picture was indistinguishable from that observed in Friend virus leukemia (Fig. 16 through 19).

Experimental Animals

Judging from gross and microscopic observations the actual disease process in the animals treated with DMBA and 3-MC was essentially the same as described for the Friend virus leukemia. (See page 27) The only difference was that the effect was less pronounced (Fig. 20 through 27).

The number of animals developing leukemia sooner or later was essentially the same in both the treated and untreated animals.

A total of 175 animals was used. Two died accidentally, 68 died of leukemia, 21 were sacrificed after 100 days with leukemia, and 84 were sacrificed after 100 days without leukemia.

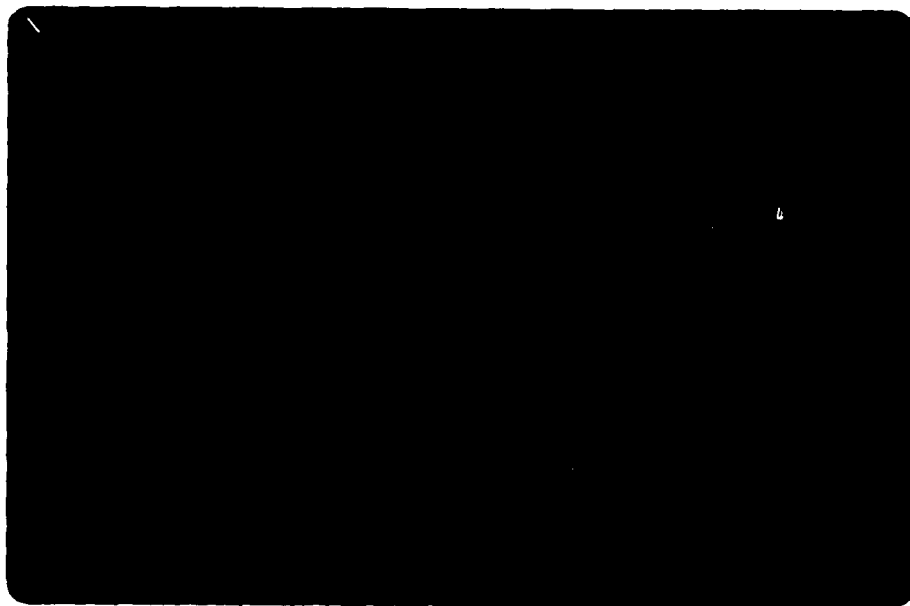


Figure 16
Spleen in Fully Developed Rauscher Virus Leukemia x 125

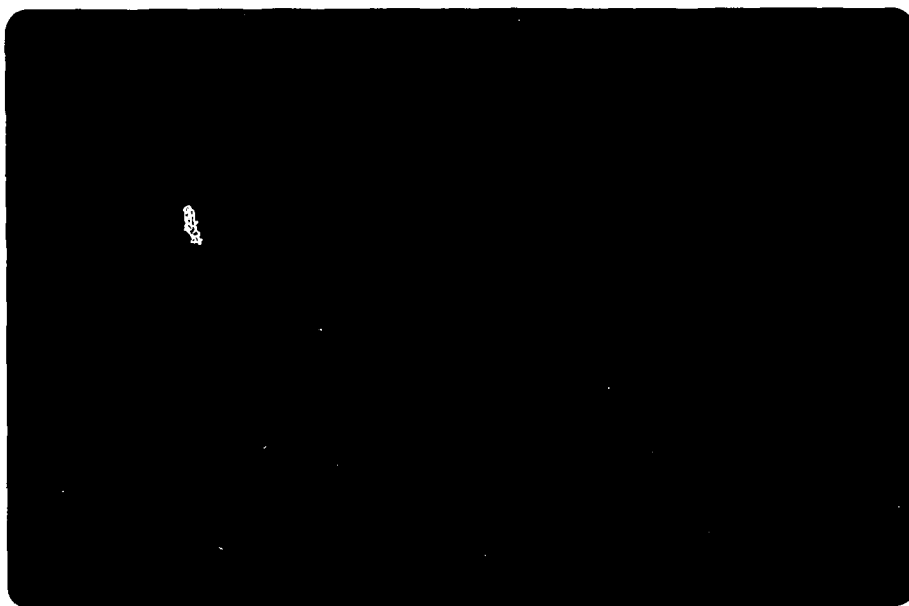


Figure 17
Spleen in Fully Developed Rauscher Virus Leukemia x 550

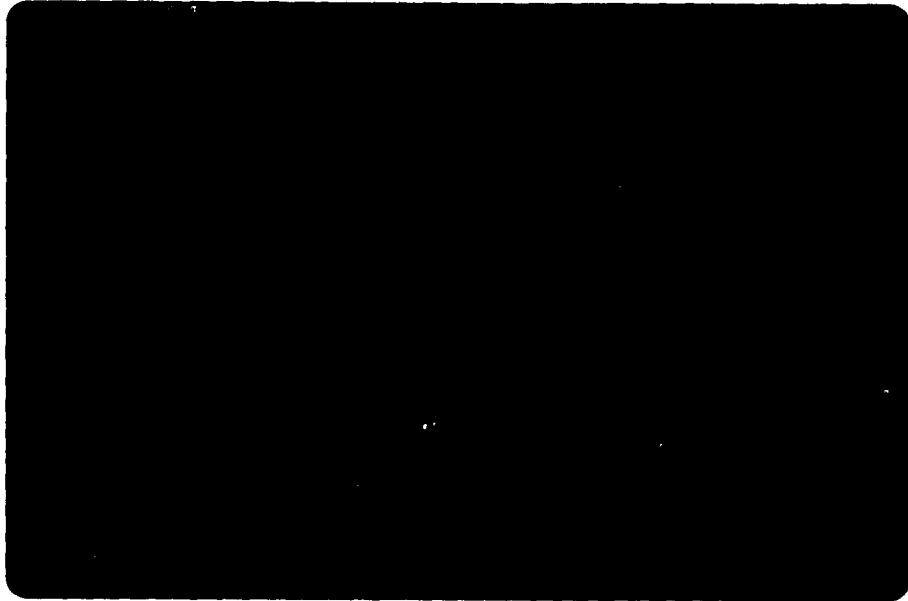


Figure 18
Liver in Fully Developed Rauscher Virus Leukemia x 125

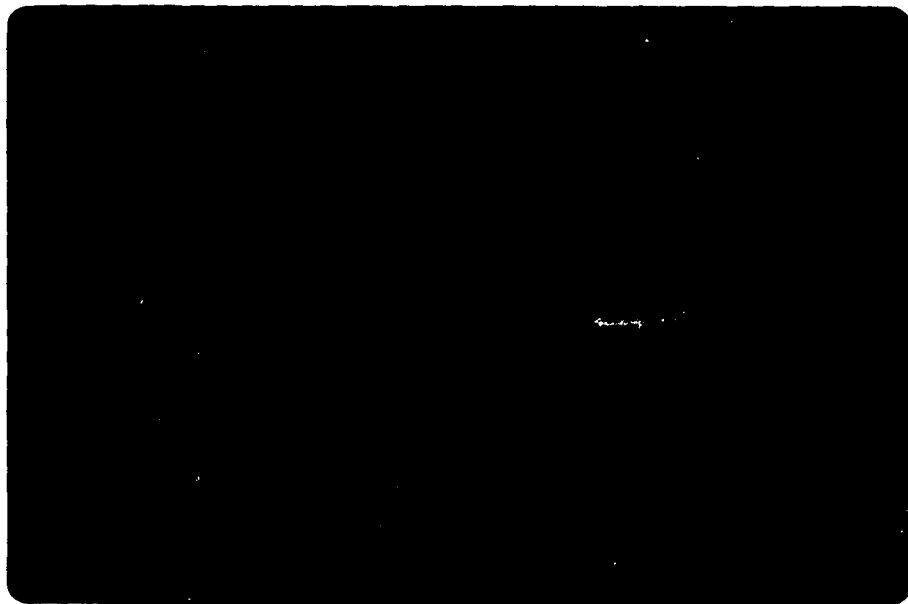


Figure 19
Liver in Fully Developed Rauscher Virus Leukemia x 550

DAYS

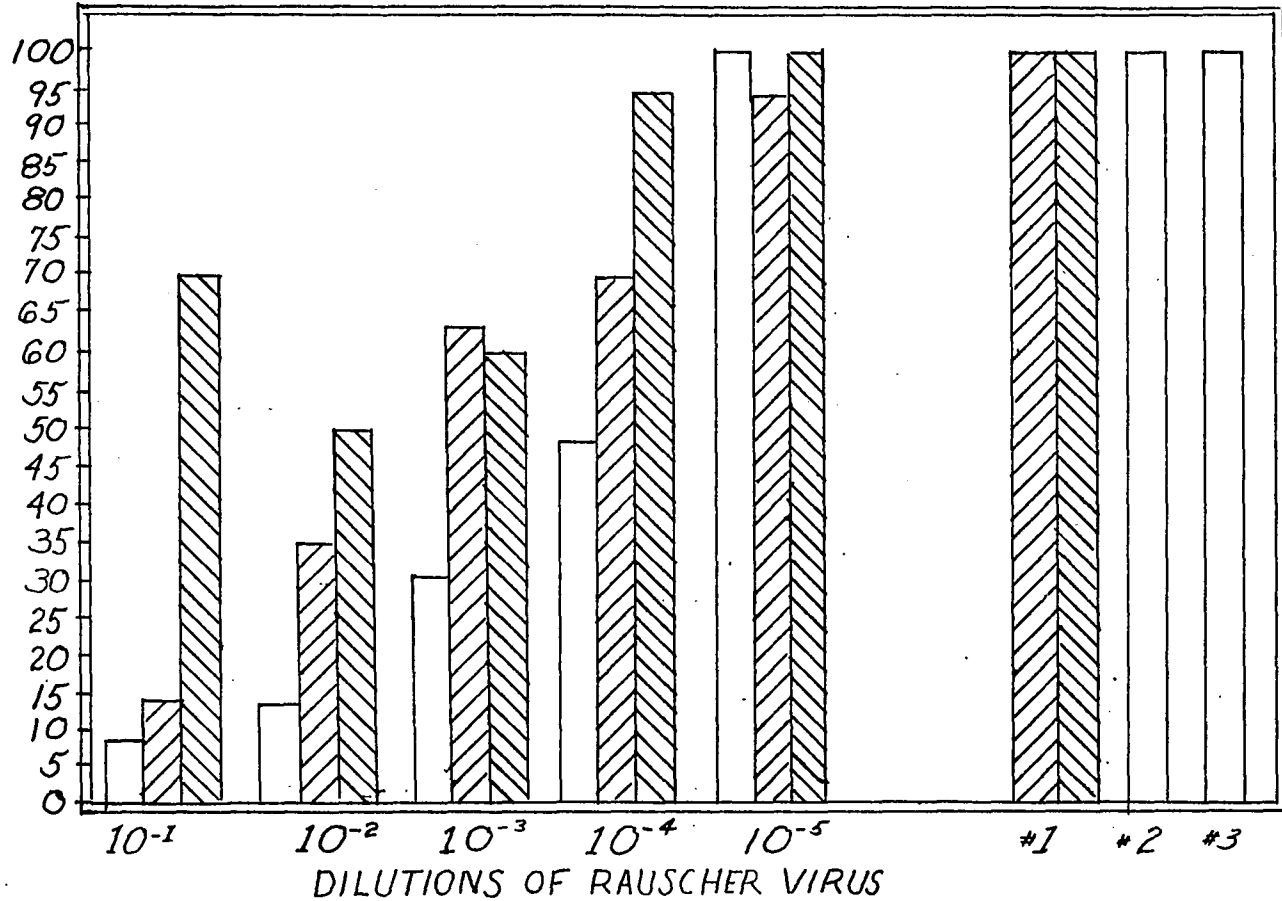


Figure 20
Latent Periods for DMBA Treated Animals
Expressed as Arithmetic Averages

- = virus only
- ▨ = virus + DMBA (injected)
- ▩ = virus + DMBA (applied to skin)

Control #1 - DMBA without virus
Control #2 - sucrose stabilizer without virus and without DMBA
Control #3 - normal control mice

DAYS

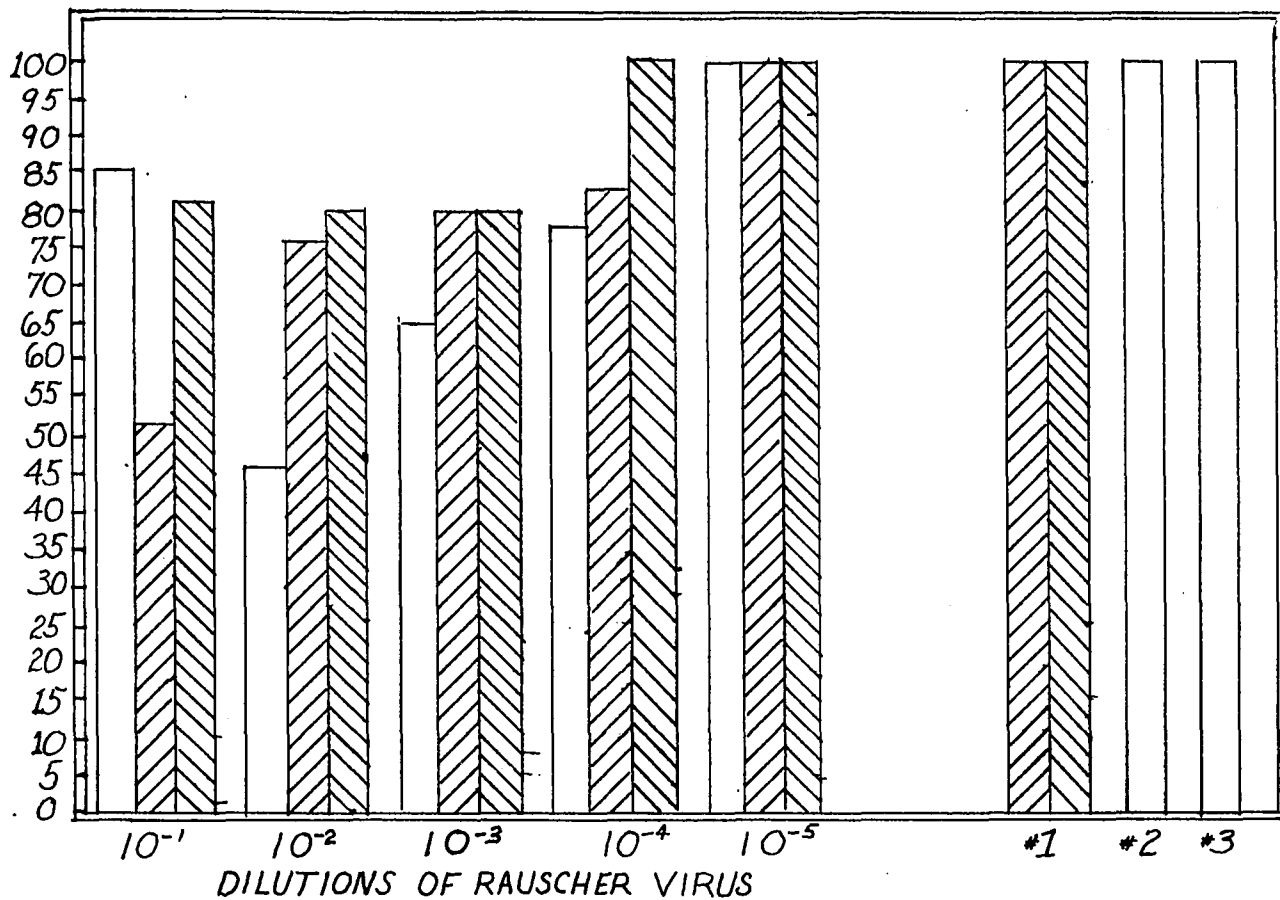


Figure 21.
Survival Times for DMBA Treated Animals
Expressed as Arithmetic Averages

- = virus only
- ▨ = virus + DMBA (injected)
- ▩ = virus + DMBA (applied to skin)

Control #1 - DMBA without virus
 Control #2 - sucrose stabilizer without virus and without DMBA
 Control #3 - normal control mice

GRAMS

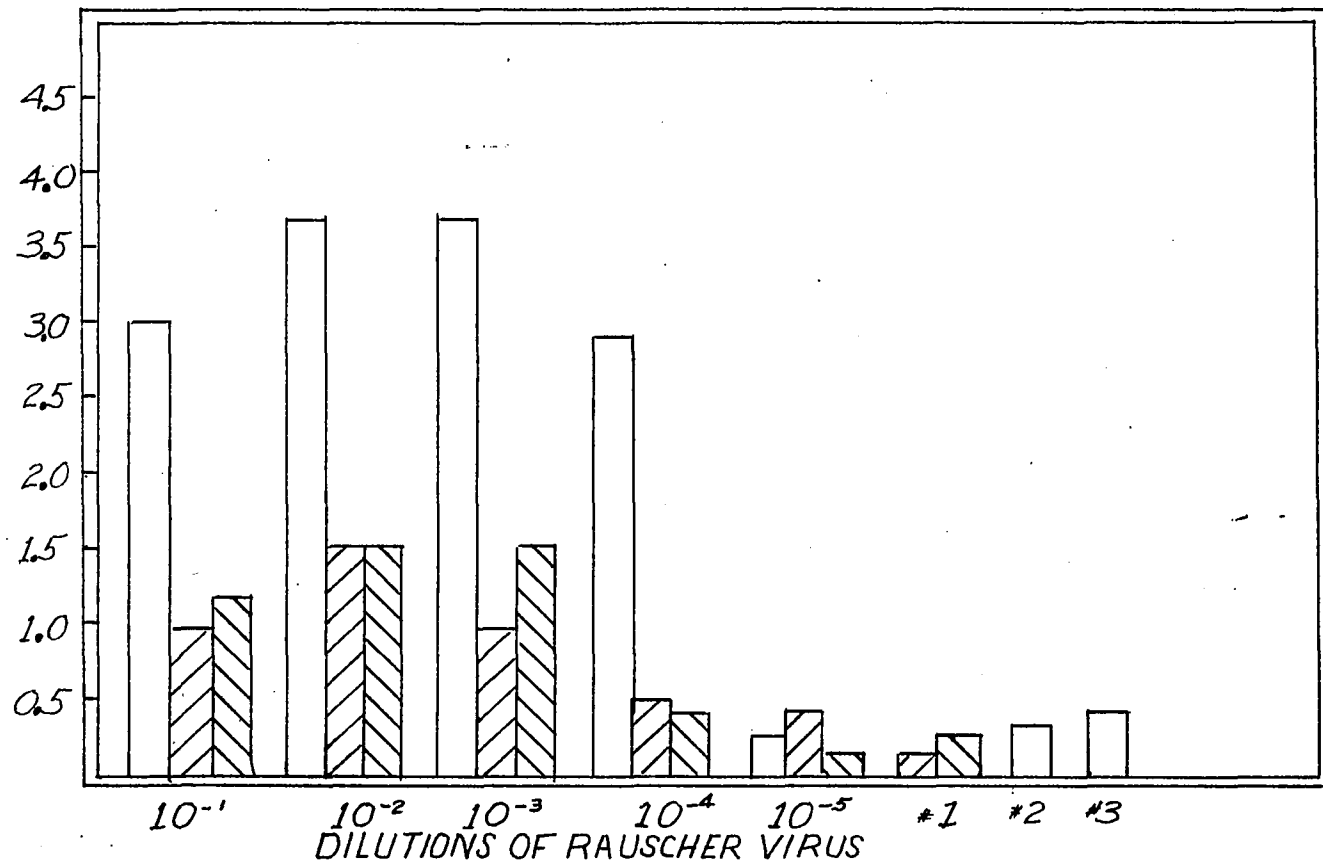


Figure 22
Spleen Weights for DMBA Treated Animals
Expressed as Arithmetic Averages

- = virus only
- ▨ = virus + DMBA (injected)
- ▩ = virus + DMBA (applied to skin)

Control #1 - DMBA without virus
 Control #2 - sucrose stabilizer without virus and without DMBA
 Control #3 - normal control mice

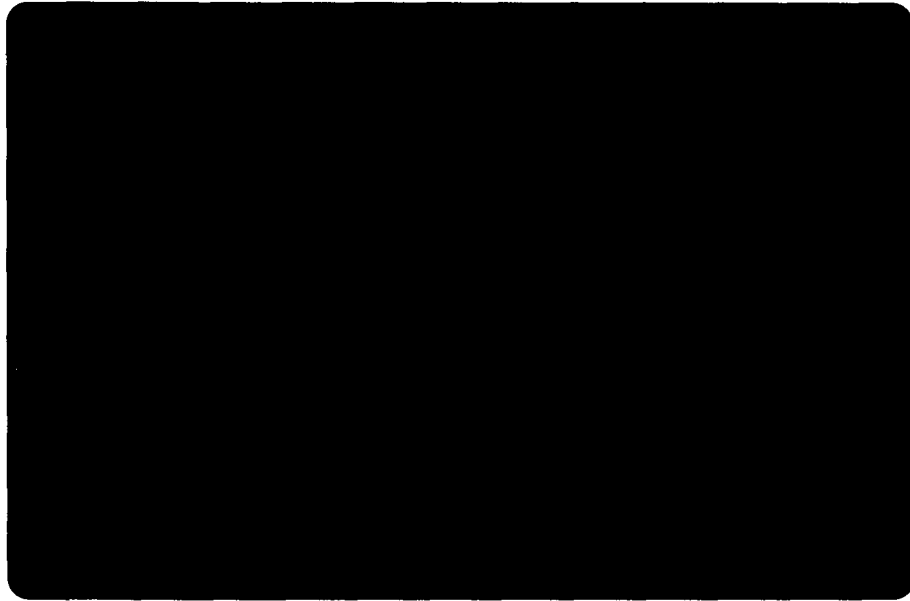


Figure 23
Effect of DMBA on Rauscher Virus Leukemia

From left to right:

1. Rauscher virus (10^{-2}) only after 50 days (note enlarged spleen and liver).
2. Rauscher virus (10^{-2}) plus DMBA (injected) after 50 days.
3. Rauscher virus (10^{-2}) plus DMBA (painted) after 50 days.

DAYS

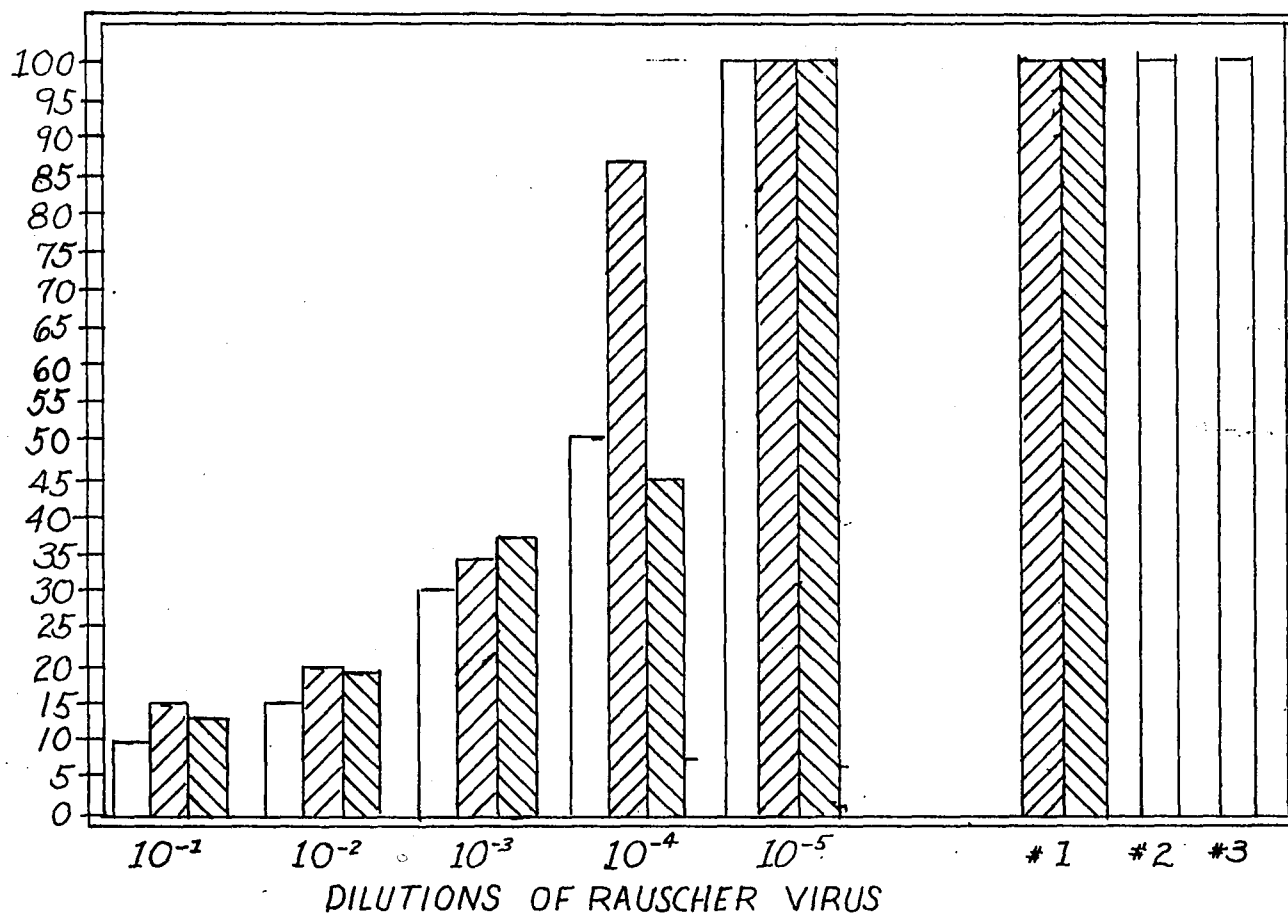


Figure 24
 Latent Periods for 3-MC Treated Animals
 Expressed as Arithmetic Averages

□ = virus only

▨ = virus + 3-MC (injected)

▩ = virus + 3-MC (applied to skin)

Control #1 - 3-MC without virus

Control #2 - sucrose stabilizer without virus and without 3-MC

Control #3 - normal control mice

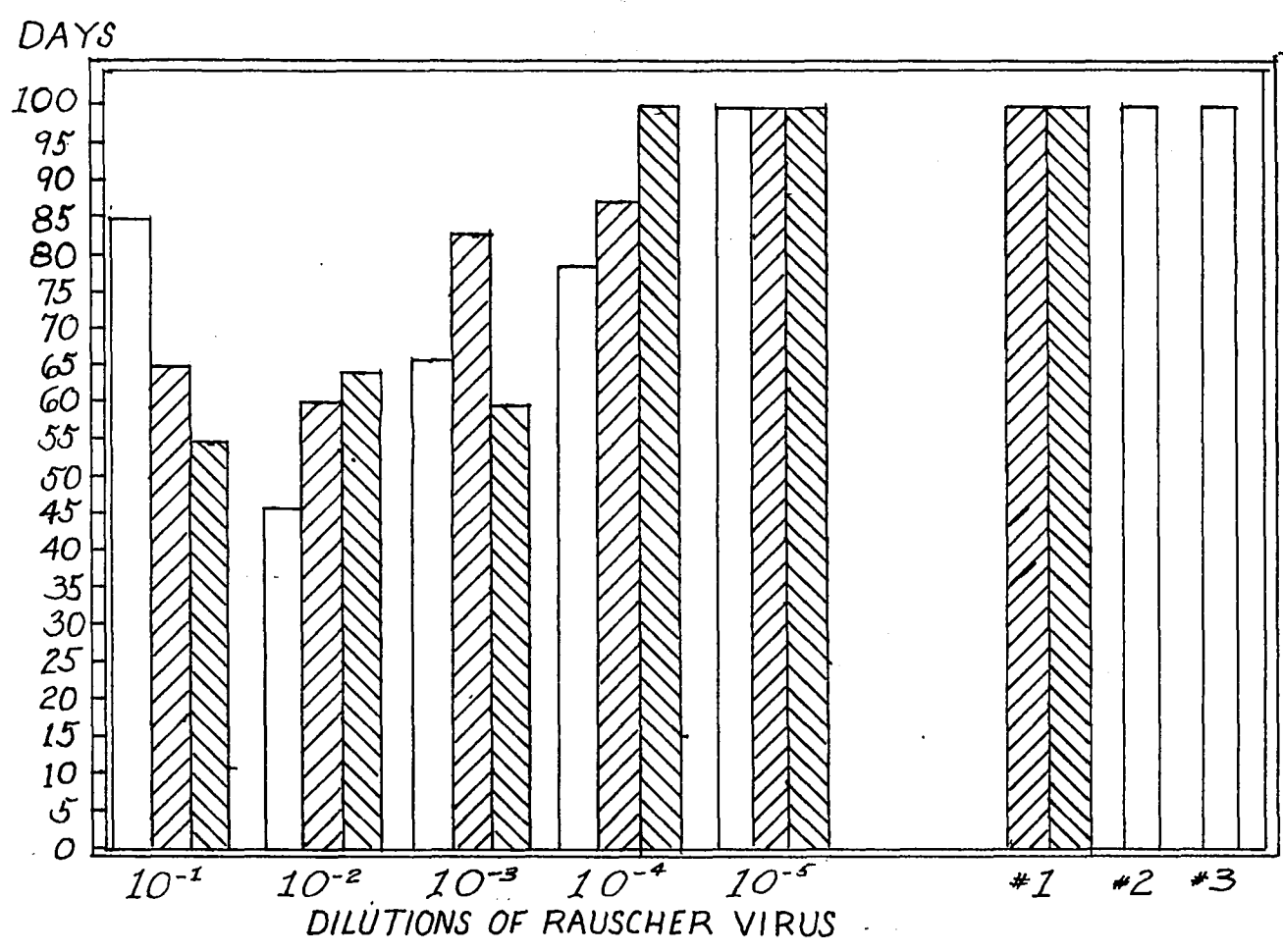


Figure 25
Survival Times for 3-MC Treated Animals
Expressed as Arithmetic Averages

- = virus only
- ▨ = virus + 3-MC (injected)
- ▩ = virus + 3-MC (applied to skin)

Control #1 - 3-MC without virus
Control #2 - sucrose stabilizer without virus and without 3-MC
Control #3 - normal control mice

GRAMS

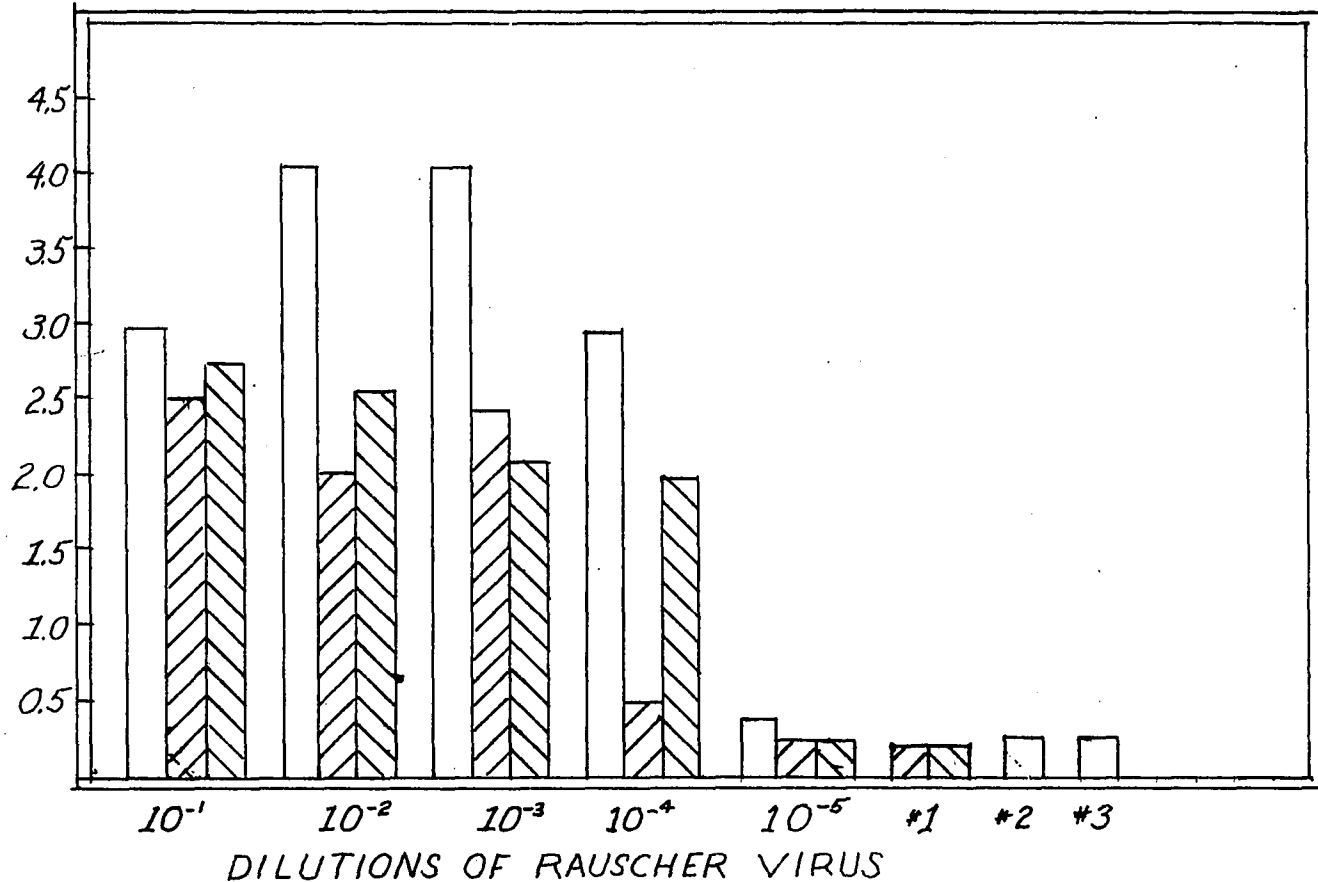


Figure 26
Spleen Weights for 3-MC Treated Animals
Expressed as Arithmetic Averages

□ = virus only

▨ = virus + 3-MC (injected)

▩ = virus + 3-MC (applied to skin)

Control #1 - 3-MC without virus

Control #2 - sucrose stabilizer without
virus and without 3-MC

Control #3 - normal control mice

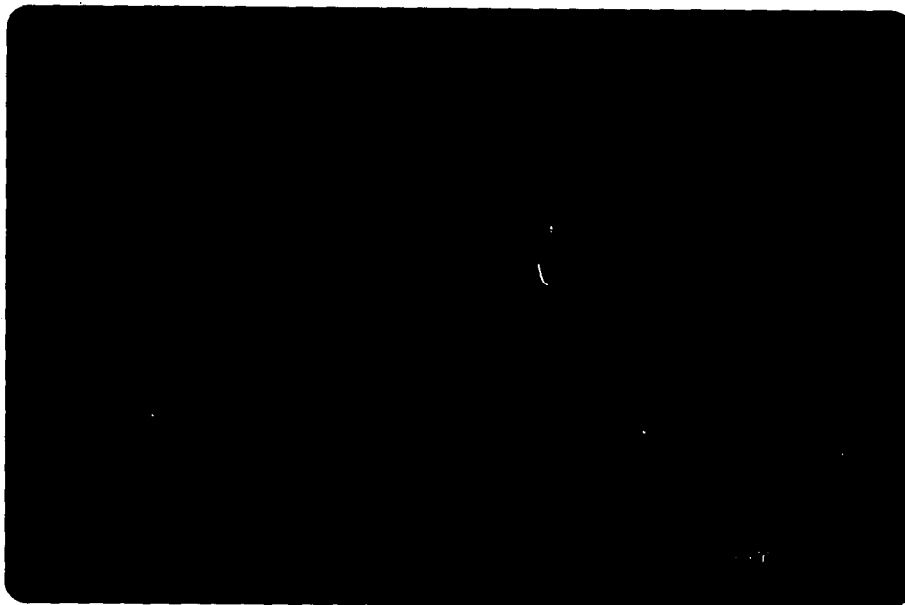


Figure 27

Lack of Effect of 3-MC on Rauscher Virus Leukemia

From left to right:

1. Rauscher virus (10^{-2}) only after 50 days.
2. Rauscher virus (10^{-2}) plus 3-MC (injected) after 50 days.
3. Rauscher virus (10^{-2}) plus 3-MC (painted) after 50 days.

(Note enlarged spleens and livers in all animals.)

The Effect of DMBA on the Cells of
a Friend Virus-Induced Reticulum Cell
Sarcoma Tissue Culture Injected into Mice

Although it is difficult to obtain transplantable variants of liver and spleen cells of Friend virus leukemia, this has been accomplished by several investigators (Buffet and Furth 1959, Friend and Haddad 1960). Dawson, Fieldsteel and Bostick (1963) developed two separate tumor line variants in BALB/c mice. They transmitted Friend virus leukemia from Swiss mice to BALB/c mice. From one of the BALB/c mice two tumor lines were obtained. One was derived from spleen and the other from liver. The latter is of particular interest since it was the origin of the tissue used by Fieldsteel (1964) to initiate the tissue culture line used in this study.

The liver cell-derived tumor was passed subcutaneously four times as a solid tumor and followed by 32 intraperitoneal passages in the ascites form. The ascites tumor produced typical Friend virus leukemia in addition to formation of variable amounts of a thick, yellow to bloody ascites. This was accompanied by large numbers of shot-sized solid tumors on the omentum and metastatic foci in spleen and liver. Mortality was one hundred per cent, usually by the third week after inoculation.

After the 32nd intraperitoneal passage, the ascites cells were successfully grown in tissue culture. These cells do not attach to the wall of the culture flasks, but remain suspended in the overlying medium maintaining their original in vivo morphology. This is an interesting behavior since only Fischer (1958) has described serial passages of

leukemic cells in tissue culture with similar characteristics. These, however, were not virus-associated. During serial passage the number of cells necessary to initiate growth was progressively diminishing. Presently, at the 100th passage, a seeding of 2.0×10^6 cells suspended in approximately 25 ml. of medium in a 16 oz. prescription bottle, will yield a 25 to 40 fold increase of cells within a week's time. The generation time ranged from 24 to 48 hours.

According to Fieldsteel (1964) the tissue culture cells after 22 passages in vitro maintained their specificity for BALB/c mice as shown by histocompatibility experiments. Infectious virus could no longer be detected in the tissue culture cells after 32 passages. Intraperitoneal inoculation of the tissue culture cells into BALB/c mice did not produce typical Friend virus leukemia (Fig. 28 and 29). Furthermore, lysates of tissue culture cells produced neither Friend virus leukemia nor intraperitoneal tumors. However, 1.4×10^3 viable tissue culture cells produced tumors in vivo that were indistinguishable from the original tumors (Fig. 30 and 31).

Since in our experiment DMBA had been shown to inhibit Friend virus leukemia, it appeared logical to determine whether a similar effect using the same concentrations could be shown on the Friend virus-induced reticulum cell sarcoma in vivo. As shown in figures 32 through 35 no such effect was obtained. A total of 84 animals was used. One died accidentally, 56 died of sarcoma, 12 were sacrificed after 50 days with sarcoma and 15 were sacrificed after 50 days without sarcoma.

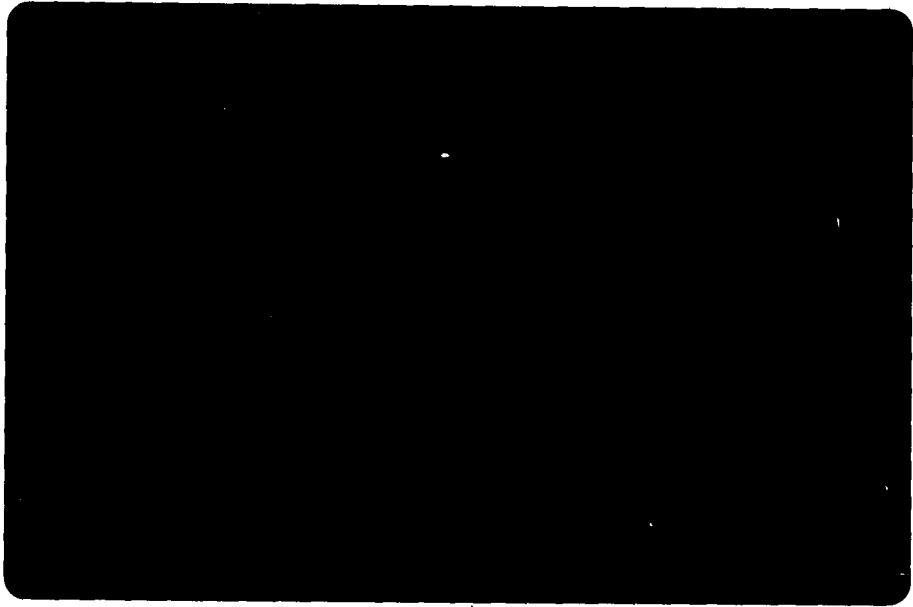


Figure 28
Disease Produced by Intraperitoneal Injection of the Cells of a Friend
Virus-Induced Reticulum Cell Sarcoma Tissue Culture after 16 Days

Left: normal mouse
Right: diseased mouse



Figure 29
Disease Produced by Intraperitoneal Injection of the Cells of a Friend
Virus-Induced Reticulum Cell Sarcoma Tissue Culture after 16 Days

Left: normal mouse
Right: diseased mouse (Note tumor nodules in peritoneal
cavity and syringe with ascites.)



Figure 30
Spleen with Metastasis in Disease Produced by Intraperitoneal
Injection of the Cells of a Friend Virus-Induced
Reticulum Cell Sarcoma Tissue Culture x 550

(Note sarcoma cells occupying the left half of the picture.)



Figure 31
Liver with Metastasis in Disease Produced by Intraperitoneal
Injection of the Cells of a Friend Virus-Induced
Reticulum Cell Sarcoma Tissue Culture x 550

(Note neoplastic giant cell in center of picture.)

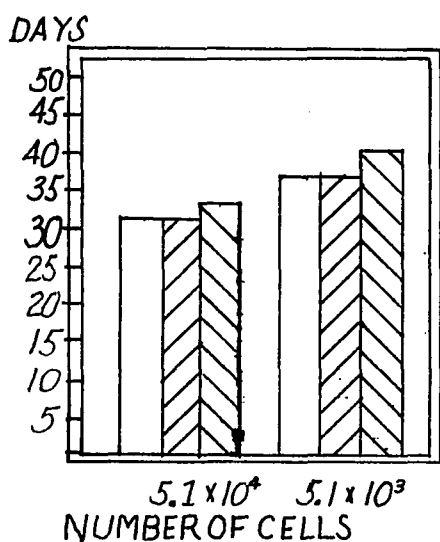


Figure 32
Latent Periods Expressed
as Arithmetic Averages

(Cells passaged 105 times
in vitro.)

- = cells only
- = cells +
DMBA (injected)
- = cells +
DMBA (applied to skin)

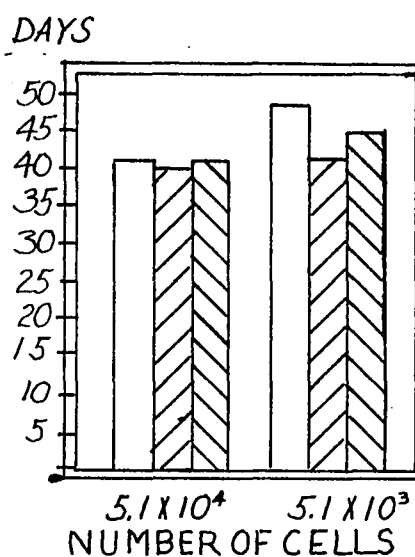


Figure 33
Survival Times Expressed
as Arithmetic Averages

(Cells passaged 105 times
in vitro.)

- = cells only
- = cells +
DMBA (injected)
- = cells +
DMBA (applied to skin)

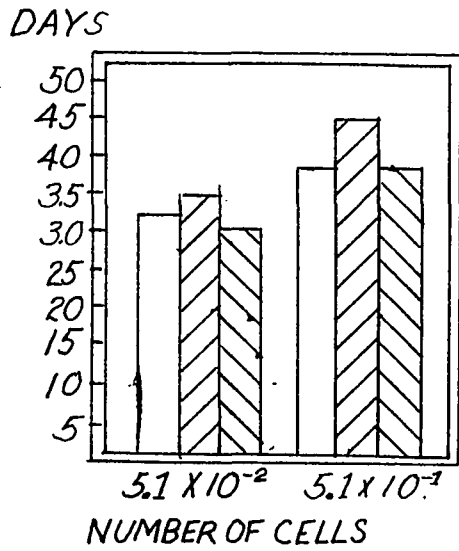


Figure 34
Latent Periods Expressed
as Arithmetic Averages

(Cells passaged 86 times
in vitro and 3 times in vivo.)

- = cells only
- = cells +
DMBA (injected)
- = cells +
DMBA (applied to skin)

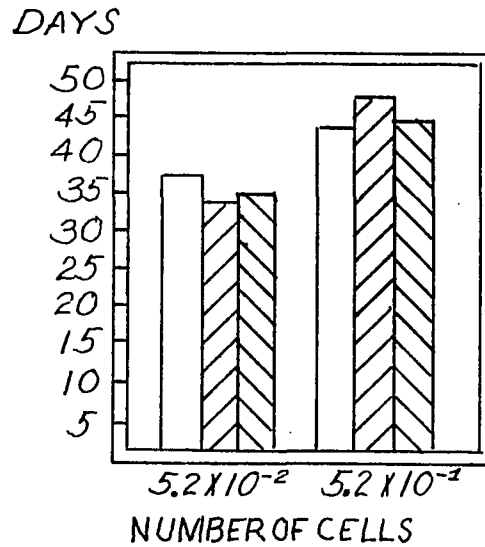


Figure 35
Survival Times Expressed
as Arithmetic Averages

(Cells passaged 86 times
in vitro and 3 times in vivo.)

- = cells only
- = cells +
DMBA (injected)
- = cells +
DMBA (applied to skin)

Chemically Induced Neoplasms

Since DMBA and 3-MC are known to be carcinogenic, it was not surprising to observe the formation of neoplasms at the chemically treated site.

Of the animals surviving the experiment, 49 per cent which had received DMBA subcutaneously and 90 per cent which had received 3-MC subcutaneously developed sarcomas (Fig. 36 through 39). These neoplasms usually appeared between 60 and 80 days, and, except for two cases, were not considered to be the cause of death of the animals which did not survive the entire experiment.

Of the animals painted with DMBA and surviving the experiment one hundred per cent had from one to fifteen neoplasms in the area where the chemical was applied (Fig. 40 through 42). These appeared as early as four weeks after the initial application. Histologically, these neoplasms ranged from small areas of hypertrophy and hyperkeratosis of the epidermis to markedly hyperkeratotic squamous cell papillomas. Only occasionally were there signs of carcinoma in situ or, even rarer, signs of early invading squamous cell carcinoma. In no instance were they considered to be the cause of death of the animals which did not survive the experiment. In contrast to DMBA, less than one per cent of the animals painted with 3-MC developed neoplasms of the skin, in all instances squamous cell papillomas.

A consequence of the subcutaneous injections of both carcinogens was a development of epidermal cysts in the subepidermal tissue in close proximity to the sarcoma in a number of animals. Apparently these epidermal cysts, which were lined with squamous epithelium and filled with



Figure 36
Large Subcutaneous Sarcoma Induced by Injection of 3-MC after 88 Days
Dorsal View



Figure 37
Large Subcutaneous Sarcoma Induced by Injection of 3-MC after 88 Days
Ventral View

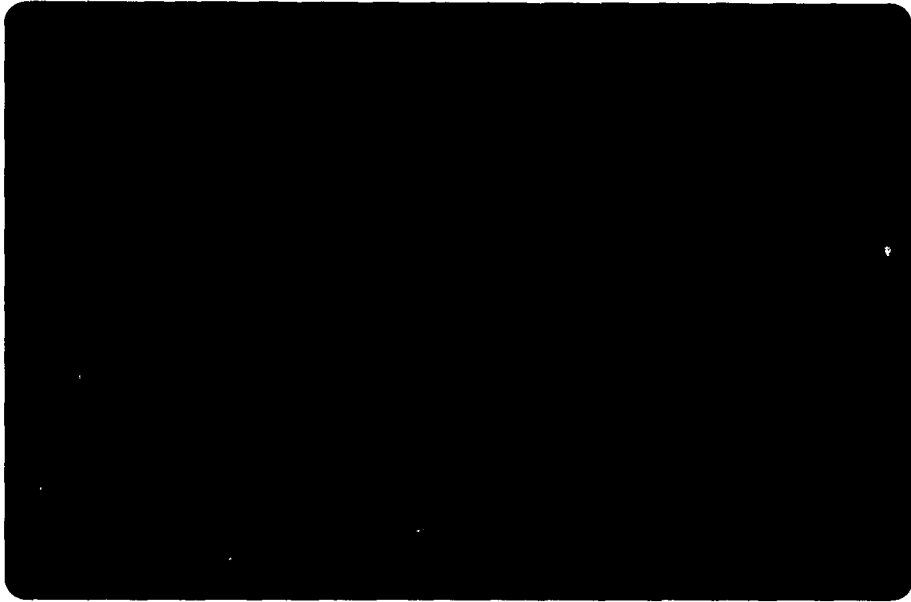


Figure 38
Sarcoma Produced by Subcutaneous Injection of 3-MC x 45

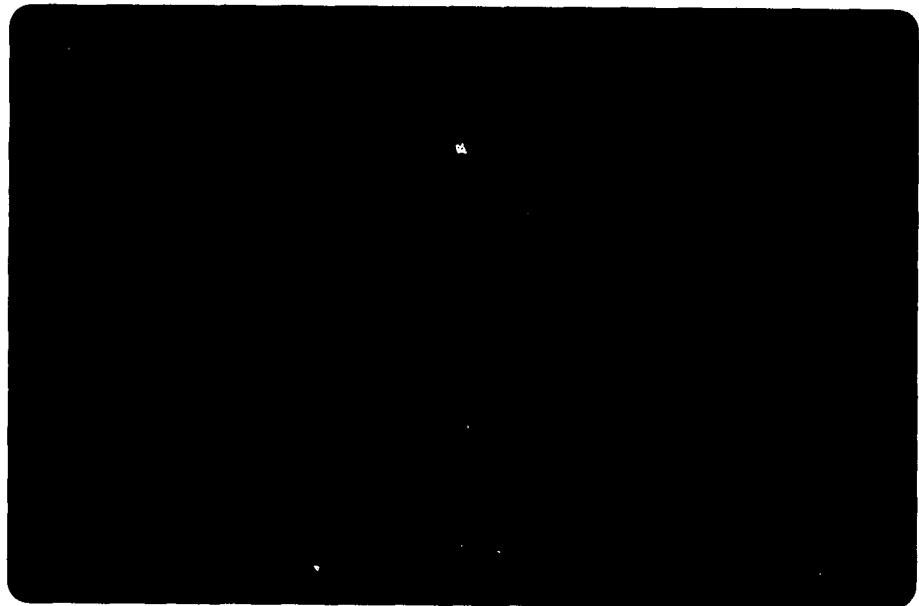


Figure 39
Sarcoma Produced by Subcutaneous Injection of 3-MC x 550

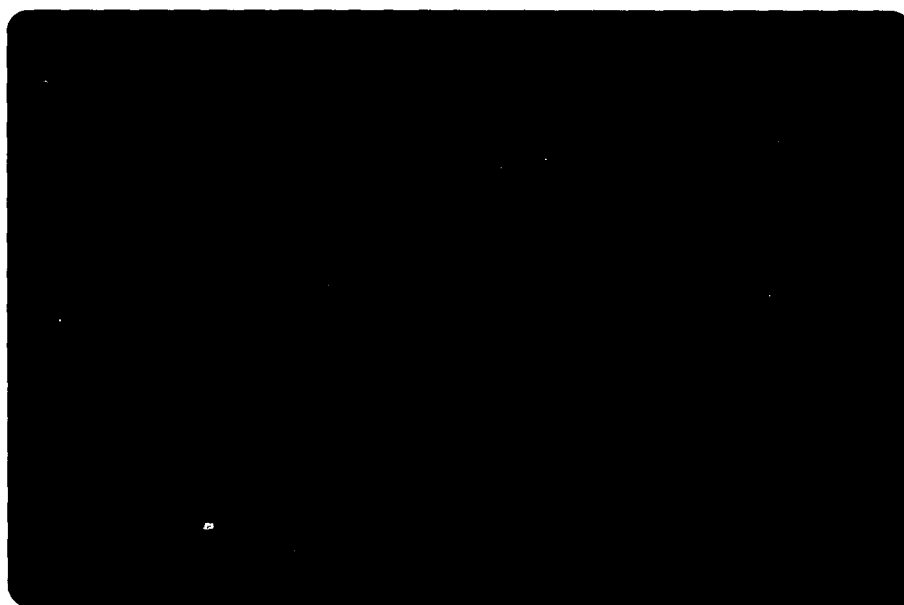


Figure 40
Skin Tumors Induced by Application of DMBA after 75 Days



Figure 41
Squamous Cell Papilloma of the Skin Induced by Application of DMBA x 45



Figure 42
Squamous Cell Carcinoma of the Skin Induced by Application of DMBA x 125

keratin, developed from pieces of epidermis which were buried in the subepidermal tissue during the trauma of injection. Interestingly enough, a greater number of frank squamous cell carcinomas developed from these epidermal cysts, both after injection of DMBA and 3-MC, than from the squamous cell papillomas arising on the surface of the skin. The explanation might be a more sustained contact of the carcinogen with the squamous epithelium of the cyst. (Fig. 43 through 45).



Figure 43
Epidermal Cyst with Squamous Cell Carcinoma
Induced by Subcutaneous Injection of DMBA x 45



Figure 44
Epidermal Cyst with Squamous Cell Carcinoma
Induced by Subcutaneous Injection of DMBA x 125



Figure 45
Epidermal Cyst with Squamous Cell Carcinoma
Induced by Subcutaneous Injection of DMBA x 550

DISCUSSION

Previous investigations dealing with interactions between viruses and chemical carcinogens have always shown enhancement of the disease process. The results presented in this paper, however, show an inhibition when the chemicals (DMBA and 3-MC) were either injected or applied to the hairless skin as measured by latent periods, survival times and spleen weights. The inhibitory effect was most evident using the combination of Friend virus and DMBA while other pairs, namely Friend virus and 3-MC, Rauscher virus and DMBA, and Rauscher virus and 3-MC, produced a less striking result. With the Friend virus and DMBA there were no results which were not consistent with, or divergent from, the observed trend of inhibition. However, this was not so with all other pairs. For example, as shown in Figure 25, animals receiving large amounts (10^{-1}) of Rauscher virus lived as long or even longer than those receiving smaller amounts. It is tempting to speculate that this might be analogous to the "incomplete virus phenomenon" for the influenza virus, also an RNA virus, observed by Henle and Henle (1949) and Hoyle (1948) in embryonated eggs, Daniels, Eaton and Perry (1952) in tissue culture, and Ginsberg (1953) in mice. When influenza virus was used in high concentrations, both infectious complete and noninfectious incomplete virus were formed. It has been suggested by von Magnus (1952) that the formation of noninfectious particles may result from partial interference by incomplete virus with the growth cycle. The hypothesis has been proposed that with large amounts both kinds of particles will

infect cells. When the noninfectious particles start to multiply, they inhibit the conversion of the newly formed (immature) virus into fully active forms. More investigation will have to be done in order to determine if a similar mechanism is applicable to the Rauscher virus.

The data between individual mice within a given group varied considerably. This was particularly true for those animals receiving higher dilutions of virus alone or in combination with the chemical carcinogens. For example, the values for animals inoculated with 10^{-1} dilution of virus were more uniform than those injected with 10^{-4} . This variation was least noticeable in spleen weight values. It should be emphasized that even though the values varied, there was very little overlapping between the chemically treated and untreated series.

The development, or lack of development, of epithelial neoplasms and sarcomas was entirely unrelated to the results of our experiment. In other words, there was no relationship between carcinogenic activity of the chemical carcinogens and the interference with the development of Friend and Rauscher virus leukemias.

Polycyclic Aromatic Hydrocarbons as Antitumor Agents

It is somewhat surprising, considering that so many approaches to the problem of cancer chemotherapy have been tried, that the polycyclic aromatic hydrocarbons have not been more thoroughly investigated as potential antitumor agents.

Haddow (1935) reported the inhibition of the growth of transplanted Jensen rat sarcoma by daily intraperitoneal injections of polycyclic aromatic hydrocarbons. This investigation was extended by Haddow

and Robinson (1937, 1938) to other transplantable rat tumors. Haddow, Scott and Scott (1937) concluded by using litter-mate controls that the tumor inhibition was part of the general inhibition of somatic growth. Green (1954) found in numerous experiments that only transplanted tumors were inhibited by polycyclic aromatic hydrocarbons, but not tumors spontaneously arising or induced in the same animal.

Carr (1942) obtained regression of established Rous No. 1 sarcomas in chickens remote from the site of 3-MC injections. Huggins and McCarthy (1957) inhibited transplanted hormone-dependent mammary tumors in rats by using 3-MC. The inhibition was considered to be due to a depression of the level of pituitary gonadotrophins and direct effects on the tumor cells.

Bauer (1940) was the first to introduce carcinogenic polycyclic aromatic hydrocarbons into human cancer therapy. He injected benzo(a)-pyrene into the skin cancers of 22 patients and reported clinical cure in seven. Later, Engelbreth-Holm and Stamer (1947) injected suspensions of DMBA intravenously into patients suffering from multiple myeloma and acute and chronic leukemia. Only one case of chronic leukemia showed remission which was still maintained 18 months after the last treatment.

Since it had been shown in our experiments that DMBA had an inhibitory effect on Friend virus leukemia, the question arose whether or not it has a similar effect on already established Friend virus-induced tumor cells. To investigate this possibility tumors were produced by intraperitoneal injection of the cells of a Friend virus-induced reticulum cell sarcoma tissue culture in BALB/c mice and were treated with DMBA as described earlier. The result was completely negative. (See Figures 32 through 35.)

It has been shown that the administration of carcinogenic polycyclic aromatic hydrocarbons to animals will produce profound disturbances. Buu-Hoi (1963) suggested that these disturbances might interfere with the growth of certain tissues, normal and malignant. The disturbances referred to by him and other authors are the following:

- (a) destruction of the adrenocortical function in rats by DMBA and 3-MC,
- (b) retardation of growth due to a negative nitrogen balance,
- (c) histological and functional modifications of lymphoid tissue with a decreased immune response.

The following discussion pertains to the possible mechanisms producing the inhibitory effect of chemical carcinogens on murine virus leukemias.

Adrenal Cortical Insufficiency

Buu-Hoi and Ratsimamanga (1947) and Huggins and Morie (1961) reported that a large single dose of DMBA destroys the zona fasciculata and reticularis of the adrenal cortex in the adult rat. Our histological examinations did not show a similar effect in BALB/c mice. Therefore, adrenal cortical insufficiency does not seem to play a role.

Effect of Polycyclic Hydrocarbons

On Spleen and Bone Marrow

Since polycyclic aromatic hydrocarbons are benzene derivatives, it is obvious that they should cause a systemic in addition to a carcinogenic effect. Picard and Laduron (1934) injected emulsions of

benzo(a)pyrene into mice and observed atrophy of the spleen and bone marrow. This would suggest that polycyclic aromatic hydrocarbons might have a therapeutic value in diseases resulting in splenomegaly.

Buu-Hoi et al. (1962) reported this to be the case. They treated a series of patients with daily injections of 6-aminochrysene and obtained a therapeutic effect on splenomegalies due to malaria and granulocytic leukemia. Payet et al. (1963), using 6-aminochrysene, observed a reduction in six out of nine patients with splenomegaly due to portal hypertension. The question arises whether or not these observations can be used to explain the results of our experiments.

Both the Friend and Rauscher viruses produce a leukemia which is in part characterized by increased erythropoiesis. Mirand et al. (1961) speculated that the Friend virus had a direct stimulating effect on the erythropoiesis in the spleen itself. On the other hand, Huggins and Pollice (1958) reported DMBA and 3-MC to exert a depressive effect on the erythropoiesis. Therefore, in our experiments, we might be dealing with two competing mechanisms: stimulation of erythropoiesis by the virus and depression of erythropoiesis by the chemical carcinogen. How this interaction will manifest itself will have to be determined in further experiments investigating the hematological picture of these mice from beginning to end. It does not seem likely at the present time that these hematological changes were responsible for the results of our experiments.

Negative Nitrogen Balance

Hadow, Scott and Scott (1937), as mentioned before, concluded that tumor inhibition by polycyclic aromatic hydrocarbons was part of

the general inhibition of somatic growth. Similarly, Elson and Warren (1947) reported that treatment of rats with dibenz(a,h)anthracene, a polycyclic aromatic hydrocarbon, retarded their growth. He stated that this was due to a negative nitrogen balance resulting in decreased protein synthesis. Recently, Sidwell et al. (1965) reported that caloric restriction markedly affects the extent of the splenomegaly in Swiss, DAB/2, and BALB/c mice with Friend virus leukemia. Since these results might also apply to our experiments, it had to be shown that a retardation of growth also occurred with DMBA and 3-MC. When animals weighing 15 gms. instead of the usual 25 gms. were treated with DMBA or 3-MC by the routes previously described, they showed a pronounced retardation of body growth compared to the untreated control group. Since no tests were performed to determine the existence of a negative nitrogen balance, it cannot be stated, that this was the mechanism responsible for retardation of growth. It should be emphasized that neoplastic cells, once established, are completely independent in their growth of the normal cells of the body. Therefore, retardation of growth seems to be an unlikely explanation of our results.

Interference with the Penetration and Proliferation of the Virus Itself

DeMaeyer and Demaeyer-Guignard (1964) reported that the carcinogenic polycyclic aromatic hydrocarbons benzo(a)pyrene and DMBA inhibit plaque formation of two DNA viruses, namely Herpes simplex and vaccinia, but not the RNA Sindbis virus. Martin et al. (1961) reported a significant uptake of DMBA-C¹⁴ in vitro by the DNA polio virus. It might well be possible that DMBA and 3-MC in some way interfere with penetration

and/or proliferation of the RNA Friend and Rauscher viruses. To investigate this question, the virus content of the spleen could be determined by titration at various time intervals.

Interference with the Ability of the Cells to Respond to the Oncogenic Influence of the Virus

Another possibility would be the interference of DMBA and 3-MC with the ability of the reticulo-endothelial cells to respond to the virus by neoplastic transformation. To answer this question, the functional capacity of the reticulo-endothelial cells could be determined by phagocytosis experiments. The answers to these questions will be obtained by further research.

SUMMARY

The antagonistic effect on Friend virus and Rauscher virus leukemia of the carcinogenic polycyclic aromatic hydrocarbons, DMBA and 3-MC, has been described.

When either chemical was injected subcutaneously or applied to the hairless skin of BALB/c mice which had been injected with virus, the leukemic process was markedly delayed. This observation was especially interesting since all other studies using combinations of chemical carcinogens and viruses have shown an enhancement of the effect. The effect observed in our experiments was most apparent using Friend virus and DMBA and less noticeable using Friend virus and 3-MC, Rauscher virus and DMBA, and Rauscher virus and 3-MC. Judging from gross and histological findings the leukemias did not seem to be altered otherwise.

No effect was observed, using DMBA, on the cells of a Friend virus-induced reticulum cell sarcoma tissue culture growing in the peritoneal cavity of BALB/c mice.

The possible mechanisms which might explain the findings are discussed.

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