REACTIONS OF ADULT MOUSE TISSUES AND EMBRYO ORGAN HOMOGRAFTS ON EXPOSURE TO CARCINOGENS.

by

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Submitted for the Degree of Doctor of Medicine, University of Glasgow.

December, 1964.

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ACKNOWLEDGMENTS.

The experimental work for this thesis was made possible by the receipt of a grant from the British Empire Cancer Campaign from 1960 to 1962. During this time I was a member of the Research Staff of the Royal Beatson Memorial Hospital, Glasgow.

I wish to thank Dr. A.D.T. Govan for his interest and support for continuing this work as a member of his staff in the Research Department of the Royal Maternity Hospital, Glasgow.

Finally, I would thank my colleague, Dr. G. Fell, who provided the samples of pure chemicals for testing.

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INTRODUCTION.

Many substances are known to be carcinogens. This knowledge is the result of a wide variety of observations and experiments. As a consequence considerable difficulty arises when attempts are made to compare one substance with another.

Much of the early work was based on careful clinical observations, which brought to light the association of occupation with the hazard of developing tumours of certain sites. Later investigations were directed towards incriminating crude substances such as Pitch or Shale Oil as the causative agents in these occupational cancers. In more recent times the search has been for more clearly defined carcinogenic agents such as pure chemicals or viruses. In parallel with these investigations, attempts were made to propagate spontaneous tumours and to induce tumours experimentally in laboratory animals.

While reading the literature on certain aspects of carcinogenisis a lack of uniformity was evident. Experiments carried out in various centres gave results which did not always fully support each other. In

some cases different interpretations could be drawn from similar experimental procedures. In part this was undoubtedly due to the variable circumstances governing each particular experiment.

An investigation into the factors influencing each of the methods of testing potential carcinogens was undertaken. In the course of this it was hoped to be able to clarify the apparent difficulties of reproducing similar experiments in different laboratories and perhaps evolve a simpler and more standardized procedure.

It was felt that the difficulties inherent in making comparisons from the literature might be considered sufficient reason to justify the need for a simple short term method of comparing one carcinogen with another. There is, however, a further and perhaps more urgent reason for requiring such a test method.

With the increasing exposure of the population to noxious substances in recent years, there has developed a growing awareness of the potentially dangerous sequellae. This type of risk can be occupational in origin or occur in the normal course of daily life. The fact of

exposure may be known but very often it is incurred unconsciously.

The recognition of these mounting hazards has led to an increasing interest in all forms of toxicity tests and of tests for potentially carcinogenic effects in particular. That there is a real need for a standardized test method, seems to follow naturally from this. No such method is at present in use as can be demonstrated by attempting to make direct comparisons from data already published.

Before doing so, however, it will be of help to make a survey of the events which have contributed to our present state of knowledge of carcinogenisis. In the following chronological survey the principle sources for the historical information up to the end of the 19th. century are: Woglom 1913; Ewing 1922; Menetrier 1926; Rousey 1929; Whitehead 1933; Haddow 1946; Maisin 1948; Oberling 1954; Willis 1955; Bett 1957. Wherever possible the original publications have also been consulted.

CHRONOLOGICAL SURVEY OF THE STUDY OF CARCINOGENESIS.

17th. CENTURY

Bergkrankheit. A high incidence of fatal lung disease in the miners of the Schneeberg mine of the Erzegebirge in Saxony and of the Joachmistahl mine in Bohemia was recognized at this time. The incidence being given as 75% for the Schneeberg mine and 50% for the Joachmistahl mine. It was later shown that the disease was pulmonary carcinoma and it would seem that this was the first recorded occupational cancer. Hesse and Harting (1897) published full clinical and pathological findings, resulting from their investigations into the disease. A high percentage of cobalt, nickel, and arsenic are present in the dust of the mines and the atmosphere has a high level of radioactivity principally Radon. It is this latter which is considered most likely to be the potent carcinogen which is at work in these mines.

18th. CENTURY

1775 Bernard Peyrilhe. The Academie des Sciences et Belles Lettres of Lyons, offered a prize for the best essay on the subject -

"Rechercher les causes du virus cancereux qui pourraient

nous conduire a connaitre sa nature, ses effets at les meilleuresmethodes pour l'eviter". Peyrelhe, in a prize winning entry entitled "Qu'est-ce que le cancer?" made the first systematic survey of the subject as a whole. He dealt with the nature of the disease, its manner of growth and treatment.

He also postulated the presence of a virus produced by the locally arising tumour while undergoing degeneration, which in turn he considered to be the source of the generalized cachexia of the malignant patient.

In an attempt to prove the theory he made an emulsion of human breast tumour tissue and injected it suboutaneously into a dog.

Not surprisingly an abscess arose at the injection site but no tumour, his servant drowned the dog, which terminated the experiment. However crude, this appears to be the first recorded attempt at the experimental passage of a spontaneous tumour.

1775 Percival Pott. Stimulated by the current climate of interest in the origin and behaviour of tumours, he made his classical clinical observation that there was a high incidence of scrotal cancer among chimney sweeps. This

he correctly attributed to constant exposure to soot.

19th. CENTURY

1801 Bichat published in France an 'Anatomie Generale' in which without the use of the microscope he distinguished between non-cancerous stroma and cancerous parenchyma in tumours.

1802 in London. The Society for the Investigation of the Nature and Cause of Cancer was formed in this year. It put forward the nature of the problem as a whole in a manner which is still applicable today. The Society was dissolved in 1806.

1808 Alibert, Fayet, Durand, Le Noble, Biett. These men tried to transmit human cancer by innoculating each other in the arm with exudate from a breast tumour, without success.

1807 Dupuytren considering cancer to be infectious in nature tried feeding tumour tissue to dogs. He also made intravenous innoculations of tumours into dogs but obtained only suppurating lesions.

1824 in Paris. In this year the first achromatic microscope was produced and opened up a new era of detailed microscopic study of tissue and tumour structure

by many workers.

1826 Raspail. As a result of his microscopic studies recognized that tissue growth was due to cell division.

1830 Sir Everard Home published a book "A short tract on the formation of tumours". This contained the first published drawings of the microscopic appearances of tumours.

1838 Schwann consolidating all that had been written about tissue growth and cell division described the nucleus and nucleolus.

1838 Müller made the first detailed study of the histology of Benign and Malignant tumours and confirmed that they had a cellular structure. This was proof of the error of the Gallenic theory of concentration of black bile as the cause of tumours. (Gallenic influence on medical thought was still evident at that time).

1851 Joseph Leidy of Philadelphia reported the first successful transplantation of spontaneous tumours in animals.

1853 Virchow consolidated the detailed work of the preceding half century and formulated his concept "omnis cellula e cellula". This finally cleared any

remnants of Gallenic theory from medical thought.

1854 Remak laid the foundation of present day classification of tissues by expressing the view that cells were type specific.

1865 Thiersh expanded Virchow's postulate as follows "omnis cellula e cellula ejuntem genesis" saying that a tumour is not only composed of cells but of cells similar to that of the tissue of origin.

1875 von Volkmann, a century after Pott described another occupational cancer finding cancer of the skin in Coal Tar workers at Halle in Saxony.

1876 Bell described skin tumours in workers employed in the shale oil industry in Scotland. A few years earlier Ogston (1871) had described a chronic dermatitis occurring in workers of the industry, but had not observed any tumours. His paper is very well illustrated.

1887 Yet another scrotal cancer of occupational origin was described. It occurred in the 'mule spinners' of the cotton industry in Lancashire. The workers used a rag to wipe mineral oil off a bar of the spinning machine over which they had to lean. This

was kept in their trouser pockets with the result that the anterior abdominal wall was permanently soaked in mineral oil from both sources.

1889 Hanau (a) Transplanted secondary nodules from a spontaneously occurring epidermoid tumour in a rat, into several other rats. Two of these, implanted in the tunica vaginalis, produced widespread peritoneal deposits within a few weeks. Histologically these were similar to the original.

(b) He also painted the scrotum of rats with tar but failed to produce any tumours. In this he was unfortunate because, had he used mice, he would have been successful.

His work was ignored by the medical world of his day, which must have added to the depression which made him eventually commit suicide.

1891 Morau made successful transplants of spontaneously occurring mammary tumours in mice into others of the same stock. However, he noted with surprise that the tumours often came up at a site remote from the implant, even across the mid-line, on the other flank.

Although he did not say so in so many words he

presumably thought in terms of tumour agent separate from the cells of the tumour because he tried feeding tumour to mice, and obtained subcutaneous nodules.

He made very clear drawings of all his tumours seen microscopically and they resemble strongly the virus induced adenocarcinomas of breast well known today.

There was an unfortunate end to his experiments which he records. While he was on holiday the mice ate holes in the wooden partition walls of their boxes and became irrevocably confused.

1894 Cazin was like Hanau, unfortunate in his choice of animal. He painted the ears of dogs for five months with tar without obtaining any tumours.

1895 Rehn described tumours of the urinary bladder in three workers in the aniline dye industry who were making magenta (fuchsin). After considerable investigation in later years β Naphthylamine which is an intermediary in the aniline dye production has been incriminated though it is possible there are other carcinogens also present.

It is worth noting that Perkin in 1856, while trying to synthesize QUININE accidentally discovered the aniline dyes from which the industry later developed. This gave a latent period of 15 to 20 years after the establishment of the industry before Rehn reported his cases.

This was later confirmed by the time lapse between the introduction of the industry into the United States and the appearance of the first tumours in the workers there.

1892 Roentgen discovered X-rays.

1896 Becquerel discovered radioactivity.

These two very important discoveries were not only tools of therapeutic value with treatment of cancer but also very potent carcinogens.

1896 Beatson The first person to describe and practice the use of Oophorectomy in the treatment of Breast Cancer. Although hormones were not known at this time he suspected some form of circulating ovarian control over breast tissue. Here one may note that John Hunter observed that castration produced progressive atrophy of the prostrate in the male.

20th. CENTURY

With the beginning of this century a change in the pattern of investigations occurred which resulted in the rapid expansion of laboratory investigations evolving into the experimental approach as we understand it today.

1903 Jensen successfully passaged a mouse tumour through 19 generations without any alteration of the histological appearance.

1906 Fischer injected Scarlet Red intradermally in the ears of rabbits. This dye is closely related to the azo-dyes. He obtained hyperplastic lesions but no histologically confirmed malignant tumours.

1908 Ellerman & Bang studying Leukaemia in fowls were able to transmit the disease to day-old chicks by injecting them intravenously and intraperitoneally with blood from affected birds. They obtained the same result using serum alone. Although they did not pursue this aspect of the problem further as it was still in doubt at that time that Leukaemia was a neoplastic disease, they were the first to show that a cell free extract could transmit a neoplastic condition.

1910 Rous reported the successful transmission of a

spontaneous sarcoma in a fowl by means of a cell free filtrate. This bird was a Plymouth Rock and the passage was made into others from the same stock source. This tumour which he referred to as chicken tumour No. 1 has become universally known as the Rous sarcoma. In the next two or three years a number of other filtrable tumours were described by Rous and his associates notably Murphy.

1910 Clunet by exposing rats to doses of x-rays produced areas of ulceration. These were prevented from healing by further irradiation. Eventually two animals developed sarcomas, one 9 months, the other 2 years, after the experiment began. This latter was successfully passaged for several generations.

This is the first record of an animal tumour experimentally induced.

1914 Fujinammi and Inamoto reported the successful passage of a fowl sarcoma by means of a cell free filtrate. This tumour was later also transmitted to ducks.

1912 Bayon injected gas tar into the ears of rabbits with resulting hyperplasia of the epithelium but no tumour formation. It is probable that he did not

continue the experiment long enough.

1913 Hagg applied soot to the ears and scrotum of rabbits also without success in producing tumours.

1913 Fibiger in the course of a study of tuberculosis found that three rats dying in the same cage on the same day had in addition to tuberculosis massive carcinomas of stomach. These originated at the cardia at the site of infestation with a Nematode.

He reconstructed one of the worms by serial sections but was unable to have it identified by any of the authorities in this field.

All attempts to propagate these tumours failed. After a long period of very careful research he found old reports from 1824 of "Blatta orientalis" as an intermediate host for rat nematodes. Careful examination of large numbers of rats and cockroaches from the warehouses of Copenhagen failed to demonstrate any parasites till finally samples from a sugar warehouse containing cane sugar from America were found to include Blatta americanus. In this warehouse the rats and cockroaches were nearly all infested with nematodes. Fibiger named this nematode Spiroptera neoplastica or

Gongylonema Neoplasticum and succeeded in infesting rats experimentally from the cockroaches of this warehouse with subsequent tumour development. At this point the factory burned down and the supply of material was destroyed. Histology of his material shows primary squamous carcinoma in tongue and fore-stomach with metastasis in a lymph node. So called metastases in the lungs were probably squamous metaplasia due to Vitamin A deficiency, and Bronchiectasis, the latter being common in adult rats. This was suggested by Passey, Leese and Knox (1935) who made a careful repetition of Fibiger's work and also re-examined his original material.

1915 Yamagiwa and Ichikawa reported the successful induction of skin tumours in rabbits as a result of painting with tar on the inner aspect of the ear.

1918 Tsutsui following up the work of Yamagiwa and Ichikawa induced a high incidence of tar tumours in mice both papillomas and carcinomas.

1922 Passey using an ether extract of soot induced carcinomas in mice after repeated skin painting.

1922 Leitch induced experimental tumours with 'mule spinners' oil by painting the skin of mice.

1924 Deelman was the first person to demonstrate the initiating and promoting stages of carcinogenisis. This he did by traumatizing the painted area and shortening the latent period of tumour production. This so called Deelman phenomenon has since been repeatedly confirmed.
1924 Choldin (Russia), Russell (England) independently induced sarcomas in fowls at the site of injection of tar.
1925 Murphy and Landsteiner transplanted tar sarcoma in fowls but could not demonstrate a virus.
1925 Gye and Barnard described a dual cause of cancer a 'virus' and a 'specific factor'. Their experimental evidence was however challenged by many workers and a split developed at about this time between those who adhered to a virus theory of causation of cancer and those who did not.
1924 Kennaway and Sampson made tar from a) Isoprene b) Acetylene heated in an atmosphere of Hydrogen. The resulting carcinogens could only be Hydrocarbons or carbon.
1927 Mayneord (see Rieger) observed the characteristic fluorescence spectra of carcinogenic oils. Mayneord noticed a similarity to the spectrum of 1:2.5:6 Dibenzanthracene. This had been described by Clar

without reference to carcinogenisis. This correlation of two unrelated pieces of work by Maynecord was a profound advance in the study of synthetic carcinogens.

1929 Martland described osteogenic sarcoma of the jaw in women painting luminous numbers of clock faces. They used radioactive material and were in the habit of pointing the brush in their mouth.

1930 Kennaway induced skin carcinomas in mice using 1:2 5:6 Dibenzanthracene. This was the first success with a pure synthetic carcinogen.

1932 Cook, Hewett and Hieger extracted 3:4 Benzopyrene from coal tar while looking for 1 2: 5 6 Dibenzopyrene. This they found to be an even more potent carcinogen for mice.

1932 Yoshida reported the production of hepatomas in rats with ortho-amino-azo-toluene. These animals were kept on a diet of polished rice, with supplements, which was later shown to be deficient in Riboflavine.

1932 Shope described a papillomatous lesion in the ear of cottontail rabbits due to a "filterable" virus.

1933 Peacock Induced transplantable non filterable sarcomas in fowls with (a) Tar (b) 1 2:5 6 Dibenzanthracene.

1933 Lacassagne induced mammary tumours in male mice using a 'follicular' hormone.

1936 Peacock Traced the metabolism of fluorescent carcinogenic Hydrocarbons in chicks, mice and rabbits.

1937 Kinosita found that Butter Yellow, a dye used to colour margarine (para-dimethyl-amino-azo-benzene) was more carcinogenic for the liver of rats than ortho-amino-azo-toluene. Kinosita and Rhoads showed by adding Riboflavine to a polished rice diet, and by using unpolished rice that considerable protection was given to the rats exposed to liver carcinogens of the azo-dye group.

1938 Hueper and Wolfe succeeded where many others had failed in using a carcinogen well known in man Naphthylamine, to induce tumours in dogs. They used bitches because of the ease with which they could be cystoscoped and obtained tumours of the bladder. In dogs the tumours are at the fundus of the bladder in contrast to man where they are in the trigone area. This suggests the possibility of a gravitational influence causing tumours to arise at the most dependent part of the bladder.

1937 Shear fed 2 amino-5 azo toluene to mice and obtained liver tumours.

1939 Roffo obtained tumours in the fore-stomach of rats fed on overheated fats and cholesterol.

1941 Wilson, de Eds and Cox while testing 2 acetylamino fluorene as a pesticide for fleas, bed-bugs, etc., found it to be carcinogenic. But for their work this substance might well have been used on a world wide scale as a pesticide.

1943 Nettleship and Henshaw produced a high incidence of lung tumours in mice using urethane. They injected pregnant mice and found that the offspring had a greater percentage incidence of lung tumours and leukaemia than did controls.

This survey shows the great diversity of the work from which our knowledge of carcinogens stems. The last twenty years have not been considered because of the great increase in the number of publications during this period. To have surveyed these would have added much to the size of the survey without adding emphasis to the point being made.

As this is a personal selection from the literature, it is acknowledged that it might well have been compiled differently by others.

Commentary on the difficulty of comparing data from different sources.

The preceding survey shows that with each advance in the study of cancer the scope of subsequent investigations has been widened. Since the turn of the century the rate of expansion of our state of knowledge has been particularly rapid so that there now exists a large volume of detailed clinical and experimental data. Attention was drawn in the introduction to the difficulty of comparing and correlating results from different sources. Numerous factors contribute to the confusion which can arise, and

one can list many variables which need to be considered in making direct comparisons of results from different sources, of which the following are some examples -

a) The wide variety of experimental animals in use.

b) The many different methods of exposure to the action of a carcinogen which can be employed.

c) The origin of the carcinogen, whether from a commerical source, or prepared in the laboratory.

In illustration of the problems of comparison Table I has been compiled. The information in an abbreviated form is taken from the following authors (Hartwell 1951; Steiner & Falk 1951; Steiner & Edgecombe 1952; Arbuzov & Grethkin 1952; Hadler & Darchun 1959).

The substances taken as examples have all been under test during the course of the personal experiments to be described later.

Table 1.

Comparison of Experimental Results with Certain Hydrocarbons. (Mainly after Hartwell).

Hydrocarbon Under Test.	No. of References given.	No. of species tested.	Total No. of animals used.	No. of test methods used.	Total No. of tumours.
Pyrene	9	2	157	5	3 Papilloma
1.2 Benzopyrene	1	1	10	1	1 Epithelioma
3.4 Benzopyrene	346	10	Many thousands	11	Multiple
1.2:3.4 Dibenzopyrene	1	1	2	1	1 Epithelioma
1.2:4.5 Dibenzopyrene		Contradictory Results.			
1.2:6.7 Dibenzopyrene		Not tested.			
3.4:8.9 Dibenzopyrene	15	2	467	10	Multiple
3.4:9.10 Dibenzopyrene	1	1	16	1	16
Anthracene	16	3	500	4	Nil
1.2 Benzanthracene	18	3	360	5	2 Epithelioma, Hepatomas
1.2:3.4 Dibenzanthracene	6	1	100	1	3 Epithelioma
1.2:5.6 Dibenzanthracene	229	12			Multiple
1.2:7.8 Dibenzanthracene	1	1	20	1	1 Epithelioma, 3 Papilloma
Triphenylene		Non-carcinogens.			
1.2:4.5:8.9 Tribenzopyrene		Non-carcinogens.			
2 Acetyl-Amino Fluorine	91	5	700	4	Multiple
Urethane	53	5		4	Multiple

If we consider the information which this table gives us in respect of 1.2 Benzopyrene, 3.4 Benzopyrene, and 1.2:3.4 Dibenzopyrene, we see that although all three are classed as carcinogens, the total information on which this is based varies considerably. For 3.4 Benzopyrene a very large volume of experimental evidence has been adduced, whereas in the other two cases only the induction of a single tumour by each had been reported at the time of Hartwell's compilation of published data.

A careful study of this table which covers only a small number of the substances known to have been tested will show other anomalies of a similar nature.

Attempts have been made from time to time to circumvent these difficulties by compiling scales of carcinogenicity. Each author has made arbitary standards against which the potency of a carcinogen is judged. It is inevitable however that personal variations in the observers should come into play in this kind of study and the results have not been entirely satisfactory in producing a universal standard.

A number of publications appearing in the period 1939 to 1959 have been abstracted to show how this personal variation can affect the grading of carcinogens against an arbitary scale. These are compared in Table 2. The substances taken as examples all appear in Table I also.

Table 2.

Comparison of Scales of Carcinogenicity After Various Authors.

Hydrocarbon Tested for Grading.	1939 Iball	1945 Berenblum	1948 Badger	1955 Pullman	1959 Wynder
1.2 Benzopyrene					+-
3.4 Benzopyrene	79	VIII	++++	++++	+++
1.2:3.4 Dibenzopyrene				+++	?
3.4:8.9 Dibenzopyrene	29			++++	
1.2 Benzanthracene	*2.6 to 18.5		0	?	+
1.2:5.6 Dibenzanthracene	26	VI	++	++	+++
1.2:7.8 Dibenzanthracene			Carcinogen but not graded.	+	

* According to Steiner (1952) who used 5 different samples to calculate the Index.

Notes on these Methods of Grading Carcinogens.

Iball used two measurable indications of carcinogenicity.

A. % tumour incidence.

B. latent period of induction in days.

He calculated the Index of carcinogenicity = $\frac{A}{B}$ x 100.

There was no differentiation made between benign and malignant tumours.

Berenblum determined tumour incidence at different dose levels and plotted them against latent period of induction to produce a graph giving a scale of carcinogenicity from I to XII.

Badger used the following scale -

Very marked carcinogenic activity	-	++++
Marked carcinogenic activity	-	+++
Moderate carcinogenic activity	-	++
Slight carcinogenic activity	-	+
Inactive	-	0

Allowing an error of plus or minus one symbol (+) in grading.

Pullman & Pullman do not give any indication of how they arrive at their scale but it is based on the concept of the correlation of the relationship of electronic structure with carcinogenic activity.

Wynder used the following scale -

High carcinogenic activity	+++
Moderate carcinogenic activity	++
Weak carcinogenic activity	+
Very weak carcinogenic activity	±
Negative	-
Not tested	?

Conclusion.

The degree of variation between these various scales is very striking not only in the grading of a given substance by different workers but also in the employment of each grading system vis-a-vis different substances.

From this commentary it is therefore clear that a great deal of difficulty exists in the correlation of current knowledge on the problem of carcinogenisis. It is further true to say that there is no universally accepted standard procedure for investigating and comparing the possible carcinogenic effects of various substances.

For these reasons this comparative study of the various methods of testing for carcinogenicity was undertaken with the object in view of evolving a simple

standardized procedure for the purposes already defined, and to explore the practical applications of any resulting techniques.

Preliminary Planning of the Investigation.

In any comparative study it is desirable to have all the factors other than the unknowns clearly defined. This is not entirely possible in biological systems but in so far as it was possible all the elements of this work were standardized before any full scale investigations were undertaken.

Criteria for a Standardized Routine Test for Carcinogenicity.

Most workers would agree that the ability to produce a recognizably malignant tumour is proof of carcinogenicity. This is in many instances a time-consuming procedure requiring many applications of the carcinogen followed by a long latent period before the production of a tumour.

It was felt that the ideal test would fulfil conditions evolved on a theoretical basis as follows -

1. The test should be easily performed and easily re-duplicated in any laboratory.
2. Very small quantities of test substance should be required as many new materials are only available in limited quantities.

3. A single application or procedure would be the 'ideal'.

4. A short latent period is necessary - a matter of weeks rather than of months. Sixteen weeks was selected as the maximum time which any test would be allowed to continue. It was felt that this gave ample time for any rapid results to develop and was still short enough to be substantially quicker than the majority of other procedures. It also permits the fulfilment of (6) below.

5. There should be sufficient adaptability to allow more than one type of tissue to be exposed to the test substance.

6. The animals should be young enough to be free of spontaneous tumours at the end of the test period.

These points were therefore used as guides to procedure whilst each of the test methods was being explored, with a view to assessing the value of any modifications which might be made.

Choice of Experimental Animal.

It became evident from an early stage in the planning of the investigations that large numbers of animal experiments would be necessary. For this reason from amongst the readily available laboratory animals

it was decided to use only mice for the following reasons.

1. They require less animal house space than any other animal.

2. They breed well all the year round.

3. They have a short life span.

4. They are known to be susceptible to a large number of carcinogens.

5. So that all the methods should be applied to the same strain, BALB/c mice were chosen for reasons connected with one of the experiments to be described (see page 122). This strain had the further advantage that the available colony was, at the time, free of Polyoma virus. Being a closely inbred strain the genetic pattern of each individual was also very similar.

Choice of Test Materials.

It was necessary to choose a number of substances whose characteristics as carcinogens were well established by previous investigations. This was to enable direct comparisons of various methods to be made, and also to allow comparison of the substances with each other, using any given method.

From the wide range of materials available a number of possible combinations were selected after discussion with my colleague Dr. G. Fell. I am indebted to Dr. Fell for making the final choice without telling me what the chemicals were, and thereafter preparing chemically pure samples for me to test. Until the experiments were completed they were known to me only by the code numbers that he had given them. As the experiments were thus a blind trial, they were a cross check both on the value of each technique and on the previously recorded behaviour of each substance.

The substances, which were derived from Pyrene and Anthracene, are shown in Tables 3 and 4. Two other substances each with a symmetrical configuration reminiscent of that of 1.2:6.7 Dibenzopyrene were also included. These were Triphenylene and 1.2:4.5:8.9 Tribenzopyrene and are shown in Table 5.

Their suitability for the preliminary investigations was determined by the following features.

1. All are solid in the pure state.

2. All fluoresce in ultra violet light, which makes their presence in the tissues easy to recognize.

3. All have clearly defined ultra violet spectra which permits their accurate identification.

4. The series contain known carcinogens and substances which though much tested have not proved to be carcinogens.

5. One substance 1.2:6.7 Dibenzopyrene had not been previously tested.

Table 3.

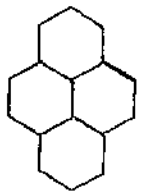
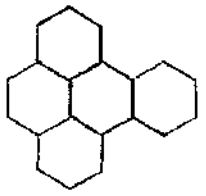
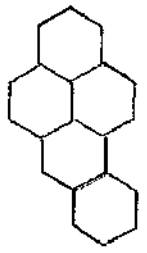
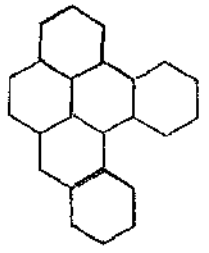
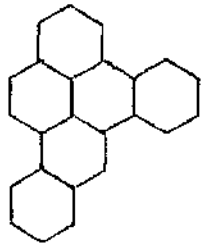
Code No.			Reported as Carcinogen.
1	Pyrene		No
2	1,2- Benzopyrene		No
3	3,4- Benzopyrene		Yes
4	1,2:3,4- Dibenzopyrene		Yes
5	1,2:4,5- Dibenzopyrene		Yes

Table 3 (contd.).

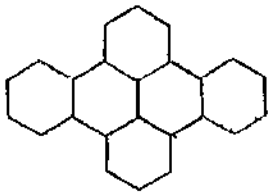
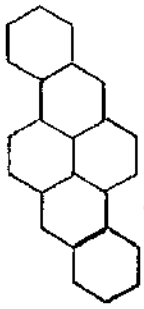
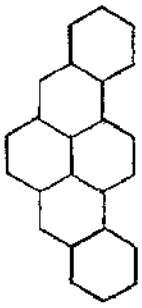
Code No.			Reported as Carcinogen.
6	1,2:6,7-Dibenzopyrene		Not tested.
7	3,4:8,9-Dibenzopyrene		Yes
8	3,4:9,10-Dibenzopyrene		Yes

Table 4.

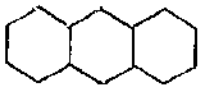
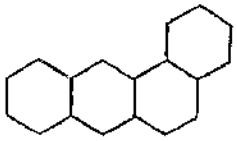
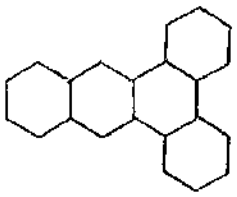
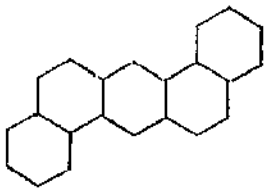
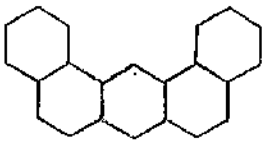
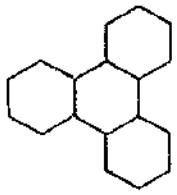
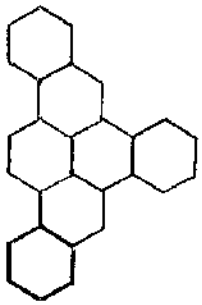
Code No.			Reported as Carcinogens.
9	Anthracene		No
10	1,2- Benzanthracene		Yes
11	1,2:3,4 - Dibenzanthracene		No
12	1,2:5,6- Dibenzanthracene		Yes
13	1,2:7,8- Dibenzanthracene		Yes

Table 5.

Code No.			Reported as Carcinogen.
14	Triphenylene		No
15	1,2:4,5:8,9-Tribenzopyrene		No

Testing Procedures in Common Use.

Testing Procedures in Common Use.

The evolution of the experimental approach to cancer research has resulted in a number of laboratory methods becoming accepted practice.

In regard to the testing of material for carcinogenic effect, the most frequently used over the past half century have been the following -

a. Feeding Experiments.

b. Skin Painting.

c. Inoculation.

d. Implantation.

Each of these four basic techniques was explored to determine their potential as a basis for the short term test for which the work was undertaken. Full scale investigations were undertaken under the last three headings, the results of which are reported separately.

It should be made clear at this point that all the experiments were in progress over the same period of time but independently of each other. The final analysis of results from each approach was made jointly. From these results a short term method of testing for carcinogenisis was evolved.

FEEDING EXPERIMENTS.

In some ways the simplest method of exposing an experimental animal to an unknown substance is to incorporate it into the diet. Although this method has the appeal of simplicity there are a number of problems arising out of it which require definition.

Animal Storage Space. Each animal needs to be in a separate cage as it would be impossible with many in one cage to tell if they were eating and drinking equal quantities of food and water. This greatly increases the animal house accommodation required.

Drinking water. If the substance is water soluble then an accurately measured amount can be given each day in the drinking water. There is however always some spilling in the cages by the animals. In order to calculate the daily dosage, the time consuming procedure of measuring and recording the residual volume at each refilling of the water bottles must be adhered to. The daily dosage given in this way is bound to vary considerably.

Solid Food. The even incorporation of a given amount of test material into the solid food is never easy and as modern practice is to have food in pellet form from

commercial suppliers it is virtually impossible under these circumstances. There is also always an unmeasurable amount of wastage of food by animals. The daily variation in dosage therefore also applies here as in the case of the drinking water.

Test Material. The wastage of food or water means that a very large amount of the test material would need to be available for this type of experiment.

Range of Organ Exposure. There is no control over which organ is exposed to the carcinogenic effect in a feeding experiment. So many factors can influence the site and degree of absorption of the ingested material and its subsequent metabolism that this tends to be a random technique.

Conclusion.

As in effect none of the theoretical features of an ideal test as proposed on page 29 are met by feeding experiments, these were excluded from practical testing as being unlikely to produce a reasonably acceptable standardized short term technique.

SKIN PAINTING EXPERIMENTS.

Introduction.

A survey of experiments based on skin painting as a test for carcinogenicity shows that many have the following features in common.

a. A need for multiple applications of the test substance.

b. A long latent period before tumours arise.

This remains true even when initiating and promoting factors come into play, although the effect of these may result in a shortening of the latent period or an alteration in the percentage tumour incidence.

A notable exception to this generalization was the work reported by Pullinger (1940) on the first effects of carcinogens on mouse skin. Acetone solutions of a number of polycyclic aromatic hydrocarbons were used to paint groups of mice. A histological study was then made of the changes in the skin at 24 hour intervals for the succeeding ten days. It was stated that the carcinogens in the series produced changes not shown by the application of the non-carcinogens.

This observation seemed a fruitful starting point

for a test based on skin painting as it met the following theoretical requirements -

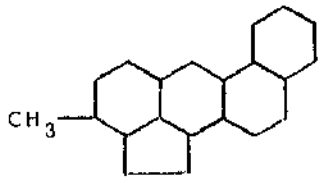
a. A single application of the test substance.

b. Only a small quantity of test substance was required.

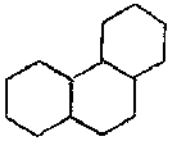
c. The latent period was short.

As a first step it was decided to reproduce, if possible, the experiment as described by Fullinger. None of the carcinogens in her series were amongst those chosen for this study.

It was necessary therefore to use 20-Methyl cholanthrene, the most readily available of her group of highly active carcinogens. This has the following graphic formula.



In common with many polycyclic aromatic hydrocarbons which are carcinogens it contains the phenanthrene nucleus.



(See Table 4).

Technique as described by Pullinger (1940).

a. Young mice 6-8 weeks old of mixed stock were chosen for their relative freedom from such factors as parasites, ringworm or bites from fighting, which might cause a hyperkeratotic lesion.

b. Cold acetone was used as a solvent as this causes no apparent histological changes in the skin by itself.

c. Hair was clipped from the area between the shoulder blades using curved scissors. At this time any mice cut by the scissors or showing parasites or bites were discarded. (Fig. I).

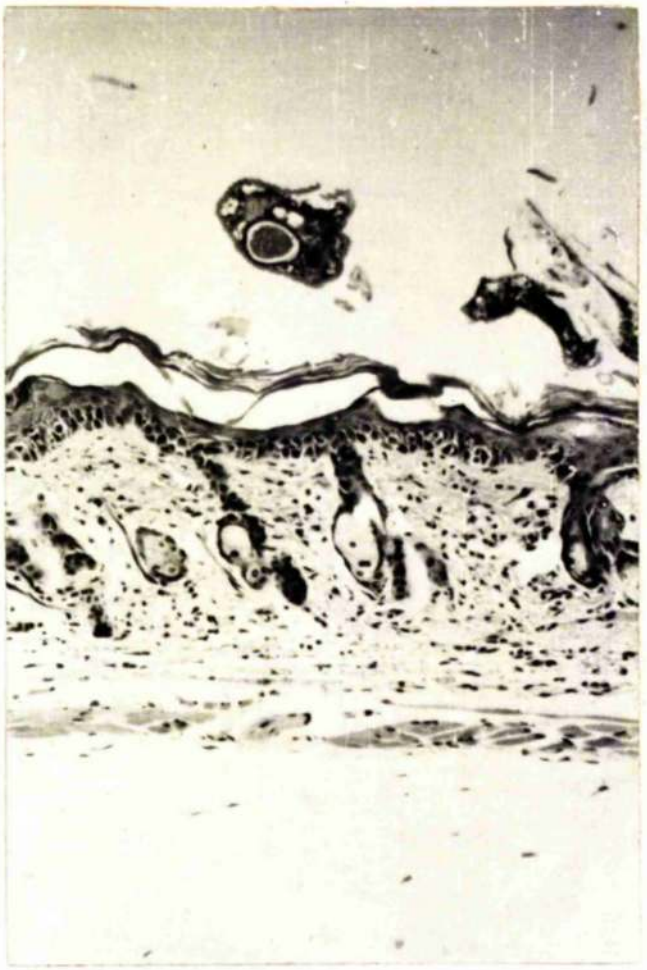
d. Using a pipette with a double bend (Fig. 2) which allows accurate control, an acetone solution of methyl cholanthrene was applied to the bare area. One drop of a 0.3% or 3 drops of a 0.1% solution were used. In the latter case each drop was allowed to evaporate before applying the next.

e. Sufficient mice were treated to allow 3 or 4 to be killed at daily intervals for four days and then on alternate days up to the 10th. or 12th. days.

When repeating Pullinger's experiment, they were

Figure 1.

Haematoxylin and Eosin. **X 120**



Untreated three months old mouse showing hyperkeratosis and thickening of epidermis due to infestation with parasites.

given code letters at post mortem examination as follows:-

First day.	A_1 A_2 A_3
Second day.	B_1 B_2 B_3
Third day.	C_1 C_2 C_3

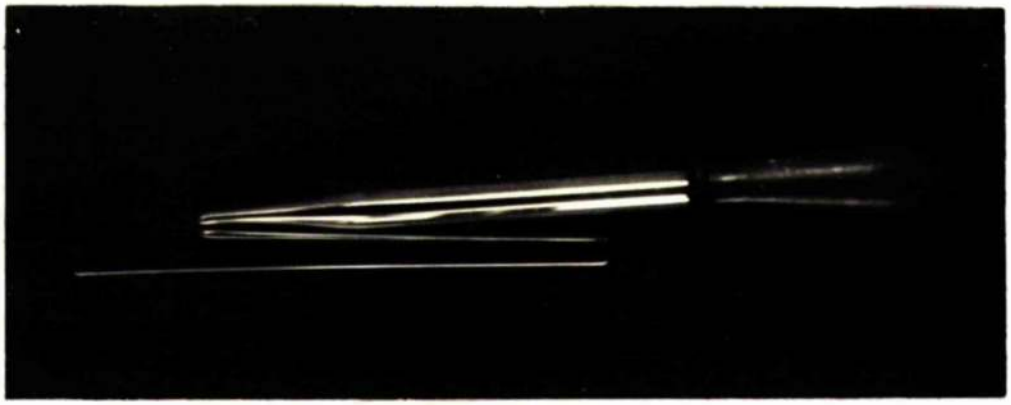
and so forth for succeeding days. This allowed accurate identification of each individual and was found more satisfactory than ear marking for this type of experiment.

f. After each mouse was killed the skin of the back was reflected by making two lateral incisions from the tail forward. The under surface of skin was then examined for any evidence of hyperaemia or inflammatory reaction (Fig. 3).

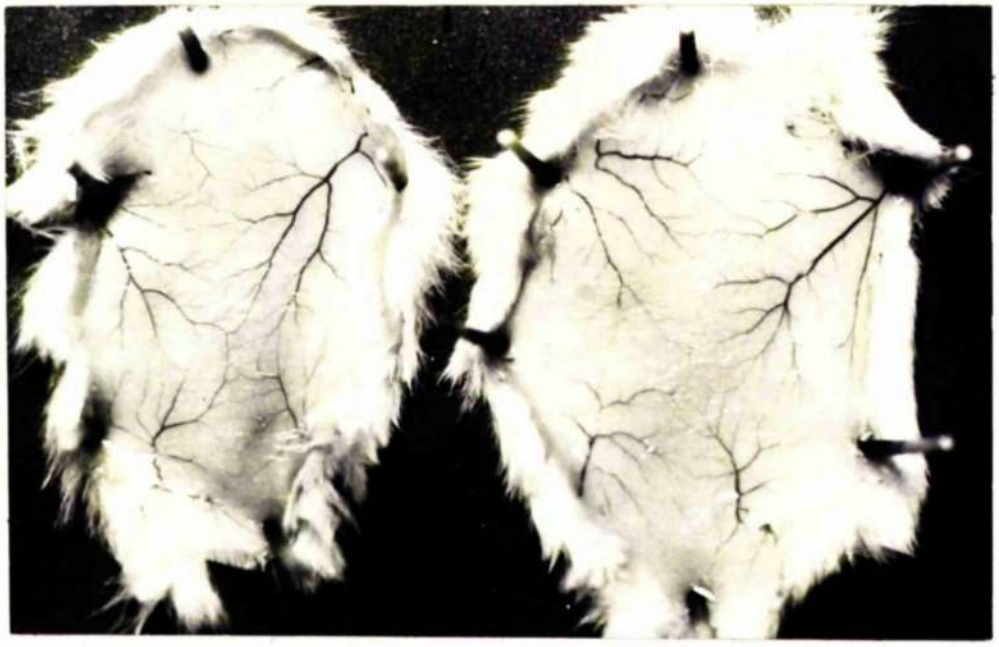
g. The painted area with some of the intact surrounding fur bearing skin was removed and pinned out hair side down on thin cork for fixation. It is important not to have the skin under tension when this is done. The use of hedgehog quills is recommended for pinning out as they do not corrode in the fixative.

h. After fixation for 24 hours in Zenker, a thin strip of skin was taken the full length of the area pinned out. As a routine this strip was taken just to one side of what was the mid line of the mouse's back. The area of

Figures 2 and 3.



Double bend capillary pipette used for application of acetone solutions.



The deep surface of the skin of the back from two mice 24 hours after painting with Methyl cholanthrene in acetone. Hyperaemia is evident with a dilated vascular network. In life the background is a deep red due to dilated capillaries.

greatest hyperaemia was included in this strip if possible.

i. These strips of skin were then processed according to the procedure set out in Appendix No. 1.

As a practical point when repeating Pullinger's experiment, it was found that in order to allow a steady progression of the tissues through the various stages of processing, a time-table covering a period of two weeks was necessary. The final form in which this was evolved is shown in Appendix No.2.

j. Each sequence of histological material prepared in the manner described was studied as a consecutive whole by Pullinger and characteristic appearances for each day were described. It was on the basis of the overall picture that a substance was determined to be carcinogenic or not.

Microscopic appearances described by Pullinger (1940).

One day after Painting. The epidermis may have increased up to twice normal thickness due to increase in size of individual cells. Nuclear diameters may have increased up to 1.4 times normal. Paired nuclei within cells are frequent but mitoses are few. There is hyperaemia and some polymorphonuclear infiltration. (Fig. 4).

Two days after Painting. Thickening of the epidermis is more marked and is due to -

a. considerable increase in size of individual cells,

b. increase in the number of cell layers of up to 4 or 5.

Variation in nuclear size is considerable and there are cytoplasmic vacuoles. There is a thick layer of keratin. Both macroscopically and microscopically there is an acute inflammatory reaction. (Fig. 6).

Three days after Painting. The epidermis is now anything up to 15 times thicker then normal and may be 5-6 cell layers deep. There is a striking increase in nuclear size up to 6 times normal. Mitoses are numerous. There is frequent cytoplasmic vacuolation. Evidence of cellular differentiation with intercellular bridges is to be seen with the appearance also of keratohyaline granules. There is a diffuse granularity of the cytoplasm. Keratin blocks many sebaceous follicles which show pressure atrophy. (Fig. 8).

Four days after Painting. There is a reduction in thickness of the epidermis with cells and nuclei now only 4 or 5 times larger than normal respectively. Vacuolation of the cytoplasm is less marked and there

is more evidence of normal differentiation. There is less keratin but no evidence of regeneration of sebaceous follicles is seen yet. Hyperaemia is less marked.(Fig.10). Subsequent days show a gradual return to normal and by the tenth day the picture of normality is completely restored.

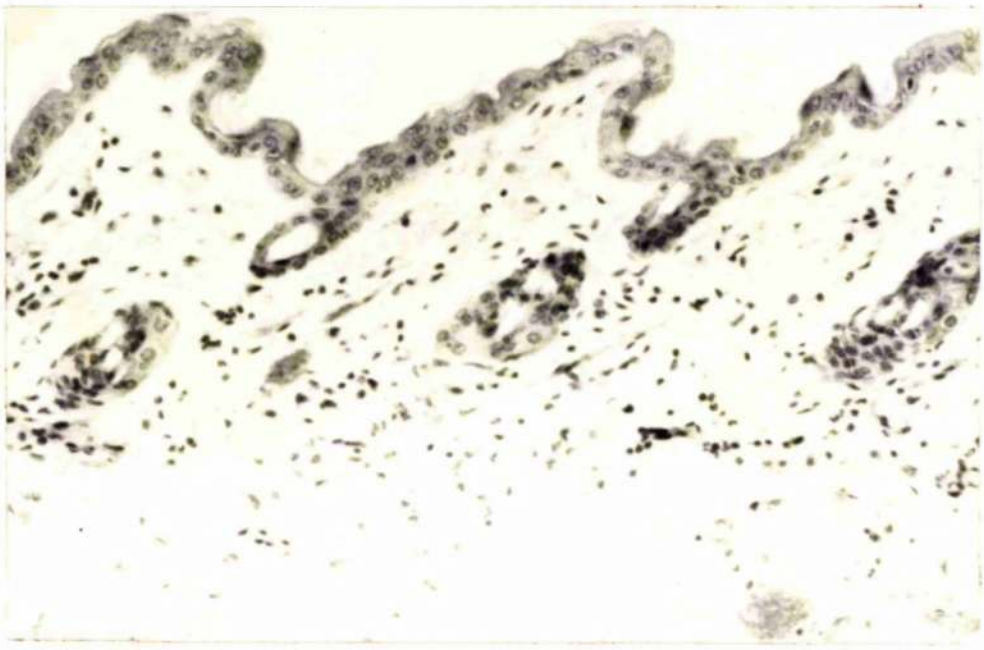
Personal Results of Skin Painting with an 0.3% Acetone Solution of Methyl Cholanthrene.

A group of 12 R III f hybrid mice were each painted with a single drop of solution. These were used as being the only mice of the correct age group available at the time.

Skin sections were prepared as has been described and the series was examined for comparison with the published work of Pullinger. The histological pattern seemed to accord well with that which she had described. As a confirmatory measure the sections were shown to Dr. Pullinger and she very kindly examined them. Not only did she agree that the reproduction of her experiment had been satisfactory (Figs. 5, 7, 9 and 11, and Table 6), she very generously gave me her original preparations to use as reference standards for these and future experiments. (Figs. 4, 6, 8, and 10).

Figure 4.

Haematoxylin and Eosin. X 120

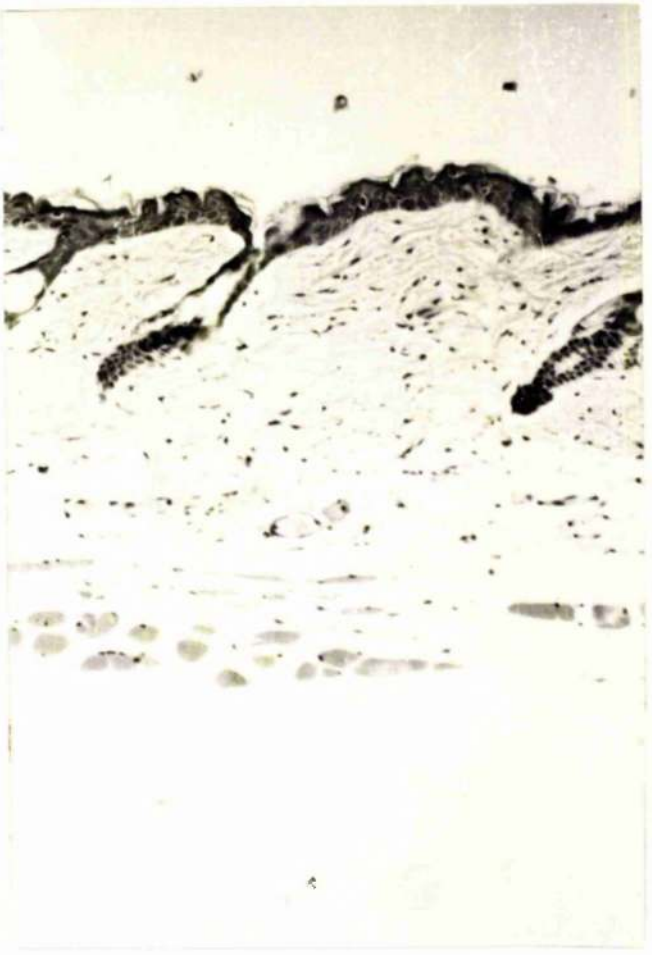


Pullinger's original preparation. 1st. day.

Increased epidermal thickness is clearly seen (compare with Fig. 12). Some paired nuclei are present. There is hyperaemia, as shown by dilated capillaries. A slight polymorph infiltration has occurred.

Figure 5.

Haematoxylin and Eosin. X 120.

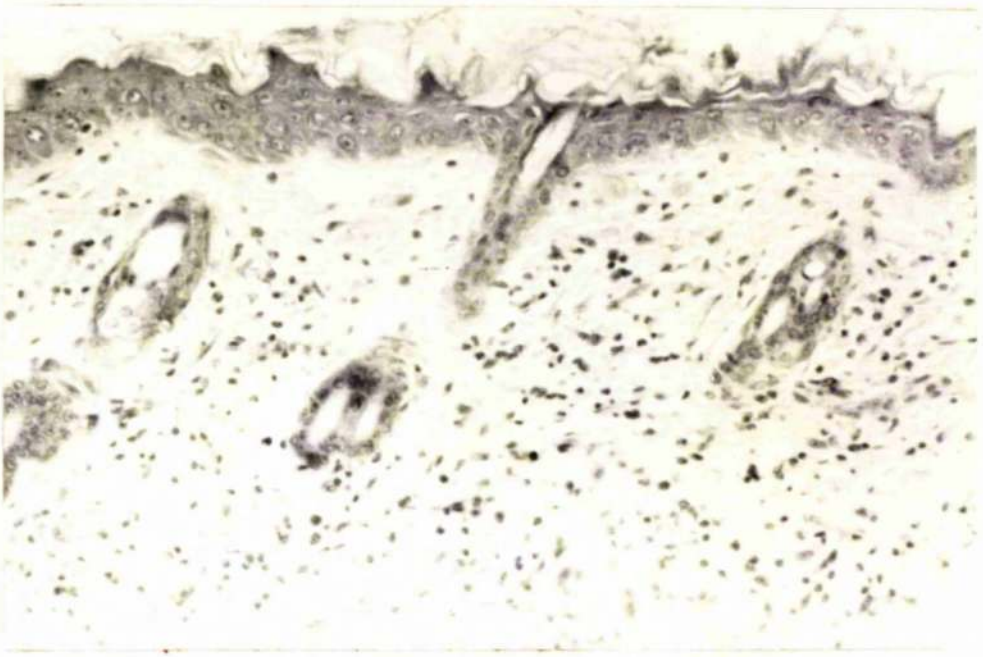


Personal repetition of Pullinger's experiment. 1st. day.

Increase in cell diameter is seen with consequent increase in epidermal thickness. Hyperaemia is less marked than in Fig. 4. Compare with Fig. 4.

Figure 6.

Haematoxylin and Eosin. X 120.

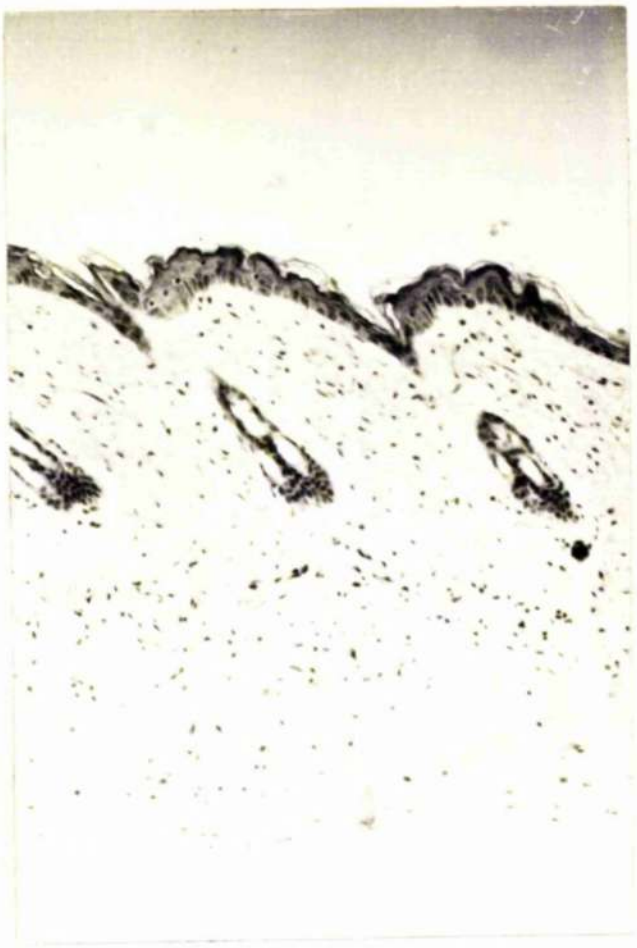


Pullinger's original preparation. 2nd. day.

Greater epidermal thickening is seen and hyperkeratosis. Early obstruction of a hair follicle by keratin is seen. The polymorph infiltration is more marked. Dilated lymphatics are evidence of oedema.

Figure 7.

Haematoxylin and Eosin. X 120.

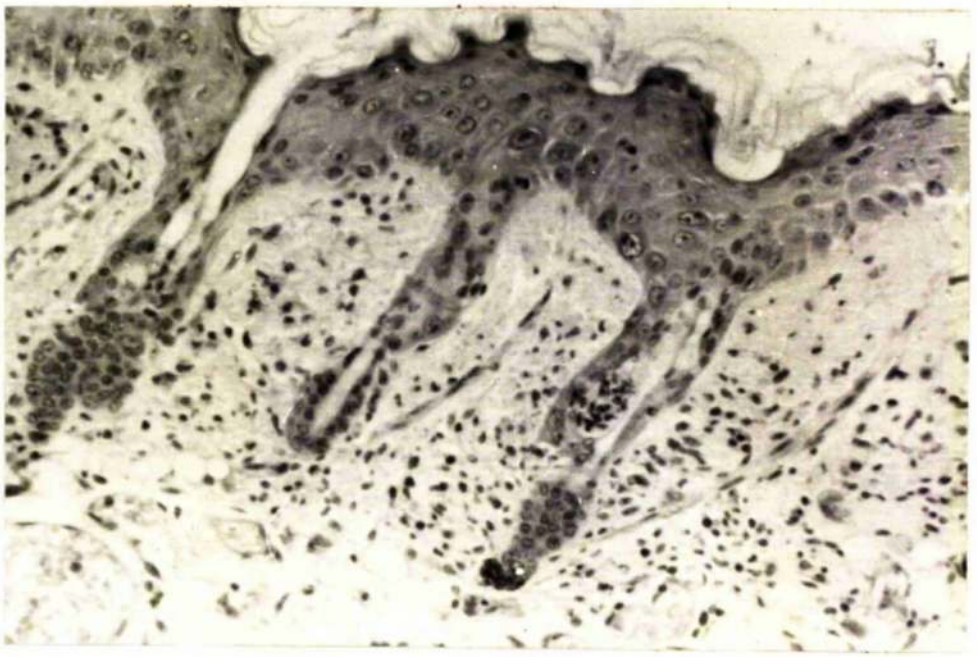


Personal repetition of Pullinger's experiment. 2nd. day.

The appearances are similar to those of Fig.6. The widely dilated capillaries are evidence of marked hyperaemia.
Compare with Fig. 6.

Figure 8.

Haematoxylin and Eosin. X 120

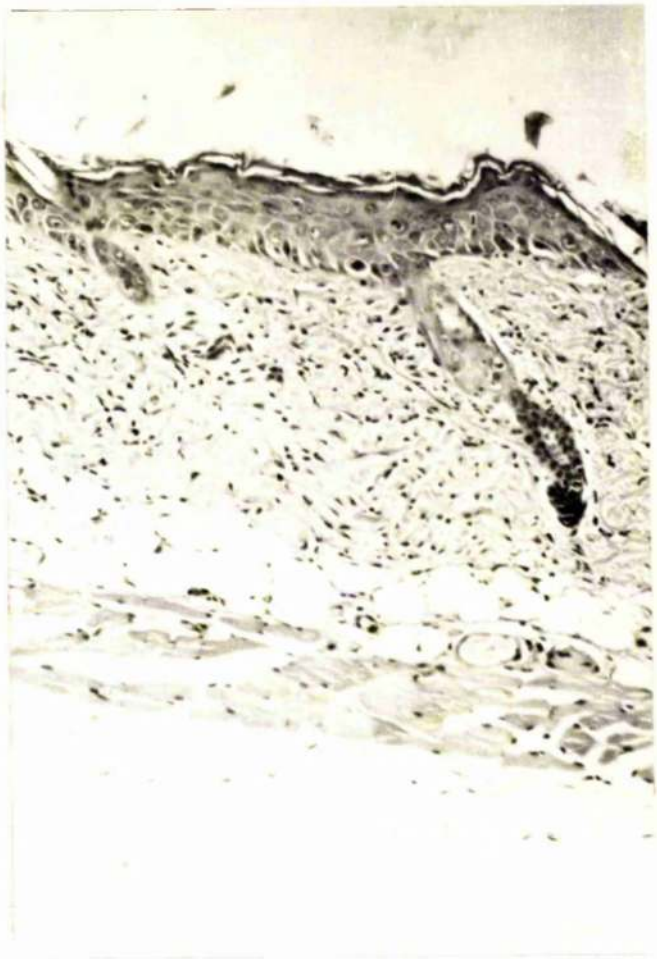


Pullinger's original preparation. 3rd. day.

A great increase in epidermal thickness is evident. There is variation in cell diameter and nuclear size. Mitoses are present and there is marked hyperaemia. Cytoplasmic vacuolation in many cells is seen. Hyperkeratosis is evident with obstruction of a hair follicle.

Figure 9.

Haematoxylin and Eosin. X 120.

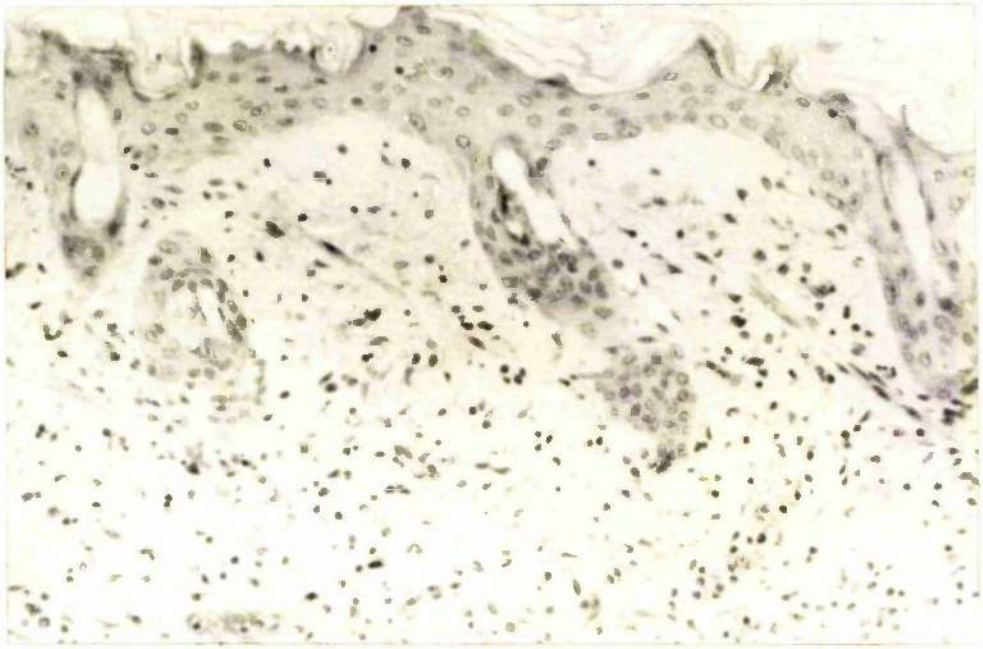


Personal repetition of Pullinger's experiment.
3rd. day.

All the features of the original description are present in this section.
Compare with Fig. 8.

Figure 10.

Haematoxylin and Eosin. X 120.

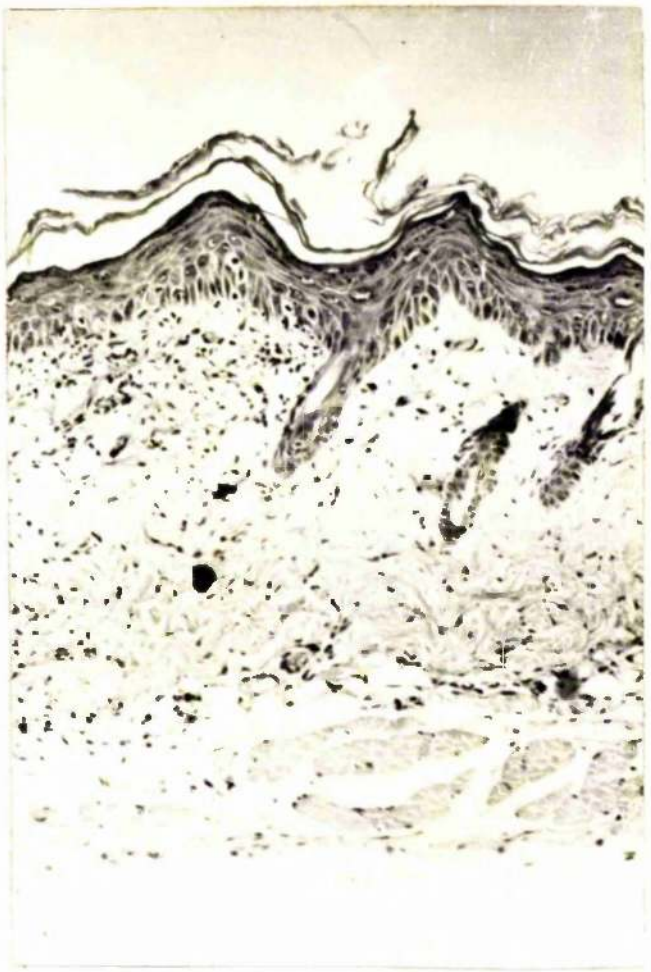


Pullinger's original preparation. 4th. day.

There is a general reduction in cell size and some reduction of epidermal thickness. Hyperaemia and oedema are absent and the polymorph reaction is less obvious. Hair follicles are still obstructed with keratin.

Figure 11.

Haematoxylin and Eosin. X 120.



Personal repetition of Pullinger's experiment. 4th. day.

Although cells show reduction in size the epidermis is still fairly deep. Keratohyaline granules are prominent near the surface. Hyperaemia is reduced but some oedema is still present.
Compare with Fig. 10.

Table 6.

Results of Exposure of R III f mice to a single drop of 0.3% Methyl cholanthrene in acetone.

Mouse No.	
A_1	No naked eye changes - characteristic changes microscopically.
A_2	No naked eye changes - no microscopic abnormality.
A_3	Mild Hyperaemia - characteristic changes microscopically. (Fig. 5).
B_1	Moderate Hyperaemia - characteristic changes microscopically.
B_2	Marked Hyperaemia - characteristic changes microscopically. (Fig. 7).
B_3	Moderate Hyperaemia - characteristic changes microscopically.
C_1	Moderate Hyperaemia - characteristic changes microscopically. (Fig. 9).
C_2	Moderate Hyperaemia - Unsuitable because of Tick bites.
C_3	Moderate Hyperaemia - characteristic changes microscopically.
D_1	Slight Hyperaemia - Reaction only focal in Epidermis.
D_2	Slight Hyperaemia - characteristic changes microscopically.
D_3	Moderate Hyperaemia - characteristic changes microscopically. (Fig. 11).

Factors Influencing Skin Painting Experiments.

Before proceeding with the comparison of the fifteen test hydrocarbons by this method a number of technical problems common to all skin painting experiments became evident. It was determined to clarify these first still using the acetone solution of Methyl Cholanthrene for painting. These and subsequent experiments were made on BALB/c mice.

Hair Cycle. (Reports from the Literature).

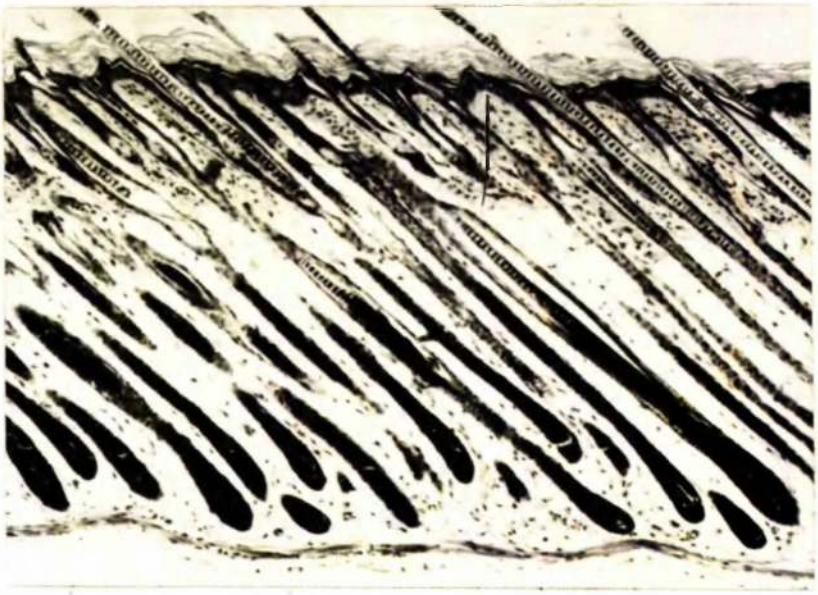
It has been reported Dry (1926), Borum (1954) that the hair of the mouse is shed and replaced throughout its life in a cyclical manner. Periods of active growth alternating with a resting phase each having a characteristic histological appearance.

In the growing phase the hair shafts extend to full thickness of the sub-cutis and are closely packed together. (Fig. 12). In contrast to this the resting phase shows small atrophic follicles which are more widely spaced and extend only about half the depth of the sub-cutis. (Fig. 13).

By various procedures such as bleaching and dyeing the hair one growth phase could be differentiated

Figures 12 and 13.

Haematoxylin and Eosin. X 48



Active phase of hair cycle in Balb/C mouse for comparison with Fig. 13 below which was taken from a litter mate 6 days previously.

Resting phase of hair cycle in Balb/C mouse.

from that preceding it and that following. In this way it was shown that the wave of growth begins at the head and travels posteriorly down the back and extends round the flanks on to the abdomen as it goes. A new wave starts at the head end before the previous one has reached completion. From this it follows that there are areas of active growth and resting phase alternating along the length of the mouse throughout its life.

Subsequent to these findings about the hair cycle it was observed by Andreasen and Engelbreth-Holm (1953) and Borum and Klinken-Rasmussen (1954-56) that the incidence of tumours resulting from skin painting experiments was greatly influenced by the stage of the cycle. The skin in the resting phase being much more susceptible than in the growing phase. Their results are summarised in Table 7.

These and similar confirmatory observations introduce a significant variability into the results which can be expected from skin painting experiments made on mice chosen at random from animal house stock.

Table 7.

Reported Effect of Hair Cycle on incidence of Papillomas after painting with 9.10 Dimethyl 1.2 Benzanthracene.

Phase of Cycle.	Males.	Females.	Authors.
Growth	3.3%	19.2%	Andreesen and
Resting	44%	64.7%	Engelbreth-Holm.
Growth	0%	0%	Berrum &
Resting	81%	96.3%	Klinken-Rasmussen.

Hair Cycle. (Personal observations).

In order to reduce these variations as much as possible the growth of the first hair coat of the mouse was plotted by killing a total of 27 animals at daily intervals from birth onwards; till the duration of the first resting phase was plotted. This was found to begin at the 17-18th. day and last till the 23rd. or 24th. day. It was assumed that this first resting phase would affect the mouse equally all over the body and this was confirmed by random biopsies. Therefore it would seem reasonable to suppose that the entire mouse is on this single occasion in its life equally susceptible over its whole surface to skin painting experiments.

A second repetition of the Pullinger experiment using 14 mice aged 18 days was made to test this hypothesis. These were killed at time intervals as follows -

1st. Day	3
2nd. Day	3
3rd. Day	3
4th. Day	3
5th. Day	2

Histologically the results were similar to those previously obtained giving a positive picture according to the criteria layed down by Pullinger.

Effect of Keratin. (Reports from the literature).

Twort & Twort (1936) showed that mice allowed to run free on a surface wetted by shale oil only developed tumours on the fur bearing parts of the body. The feet which were in closest contact with the carcinogen remained unaffected. Lacassagne (1945) irradiated new born mice with ultra violet light over a marked area of the skin which inhibited hair follicle development. He found that in this area methyl cholanthrene was not able to induce tumours. From this and other evidence in the literature, it would seem that only when hair follicles are present in the skin can tumours develop.

Effect of Keratin. (Personal observations).

To confirm this observation 24 new born mice were painted with the same methyl cholanthrene in acetone solution previously used and then killed at the following time intervals.

1st. Day	3
2nd. Day	4
3rd. Day	8
4th. Day	3

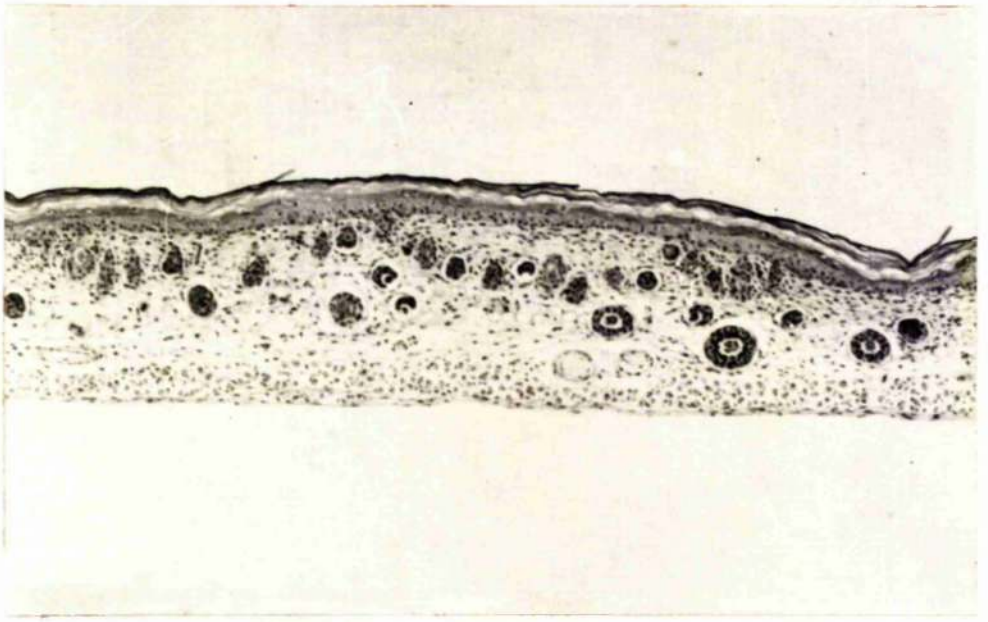
Six mice were eaten by their mothers and lost to the experiments. The rather uneven distribution over the four days is due to the abandonment of one litter by the mother on the third day.

The histology of the skins of these mice shows no abnormality. At birth the mouse has a thick layer of keratin not broken by erupted hair shafts. (Fig. 14). These negative findings were therefore in accord with the findings quoted in the preceding paragraph. The ability of methyl cholanthrene to induce tumours in other squamous epithelium such as the fore stomach, may be a function of exposure over a period of time.

Conclusion. It would seem from these personal observations and previously recorded work that the optimum time at which to carry out skin painting experiments is on the 17th. or 18th. day as this is the only time in the life of the mouse when the whole body surface is in the same receptive resting phase with regard to the hair cycle.

Figure 14.

Haematoxylin and Eosin. X 48.



Skin of normal mouse at birth. There is a thick layer of surface keratin. No hair shafts penetrate the surface of the skin but many rudimentary hair follicles are present.

Solvents.

It was originally intended to prepare solutions of each of the 15 test hydrocarbons in acetone. Each was to contain the equivalent molar concentration to that which was present in the 0.1% Methyl cholanthrene solution used by Dr. Fullinger.

When it came to making up the solutions it was found that those hydrocarbons with the largest molecules could not be made to go into solution in the appropriate concentration.

A search for alternative solvents, which would be capable of giving the appropriate solutions, but which were not in themselves irritants to the skin to the mouse, was made. The following substances were accordingly painted on to the skin of different groups of mice, and the effect studied histologically, over a period of six days at intervals of 24 hours. The numbers in parenthesis indicate the total number of mice in each sequence.

1. Tri-n-Butyrin (9)

2. Tri-n-Butyrin heated to 200°C. for thirty minutes and then cooled before application (8). (This procedure is necessary to enable some of the Hydrocarbons to go into solution).
3. Di-Methyl-Formamide (6)
4. Di-Methyl-Sulphoxide (6)
5. 1-Methyl-Naphthalene (6)
6. Trichloro Benzene (6)

Of these substances, Tri-n-Butyrin was found to be apparently completely non-irritant, and therefore the most promising alternative solvent.

To test Tri-n-Butyrin as a solvent, a 0.1% solution of Methyl-Cholanthrene was prepared and a trial painting experiment was made on a group of 12 mice, which were killed as follows -

1st. Day	2
2nd. Day	3
3rd. Day	4
4th. Day	3

No effects on the epithelium were seen in the histology of these animals.

This was an unexpected result and thus it became evident that there were some essential differences in properties between acetone and the above solvents.

The most obvious is that unlike acetone, they are all oily and non-volatile.

Viscosity and Volatility of Solvents.

When an acetone solution of a Hydrocarbon is painted on to the skin, it evaporates within a matter of seconds. If this is observed under ultra violet light, an area of fluorescence is seen on the skin where the acetone solution has deposited the solute. (Fig. 15).

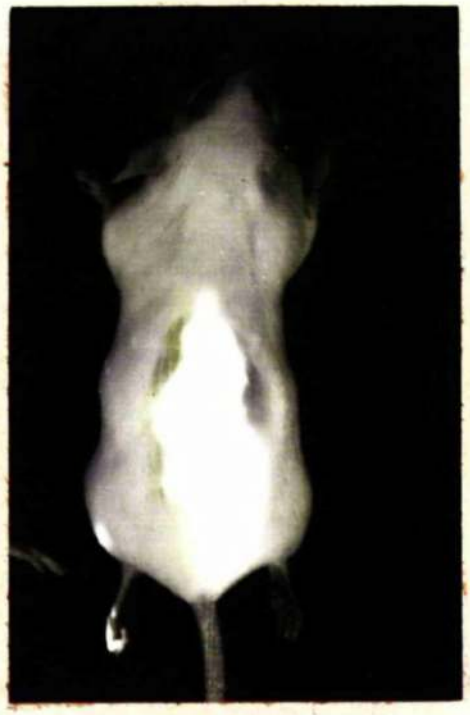
If the animal is killed and a frozen section is made of the painted area for examination under ultra violet light, fluorescence is seen. This is not only on the surface of the skin but also shows the solution tracking down the hair shaft into the hair follicle. This was described originally by Crammer and Simpson (1945). It seems reasonable to conclude that there is a capillary flow down the hair follicles, the hair shaft acting as a wick, with only a small fraction of the original dose penetrating the skin.

This would explain why painting the skin of new born mice, who have no hair, produces only negative results. Frozen sections of skin, which have been painted with the non-volatile solvents mentioned above, show no evidence of penetration down the hair shaft.

The prime factor in the failure of these solvents as vehicles for carcinogens in this procedure would seem to be their inability to penetrate the skinvia the hair follicle due to their physical properties.

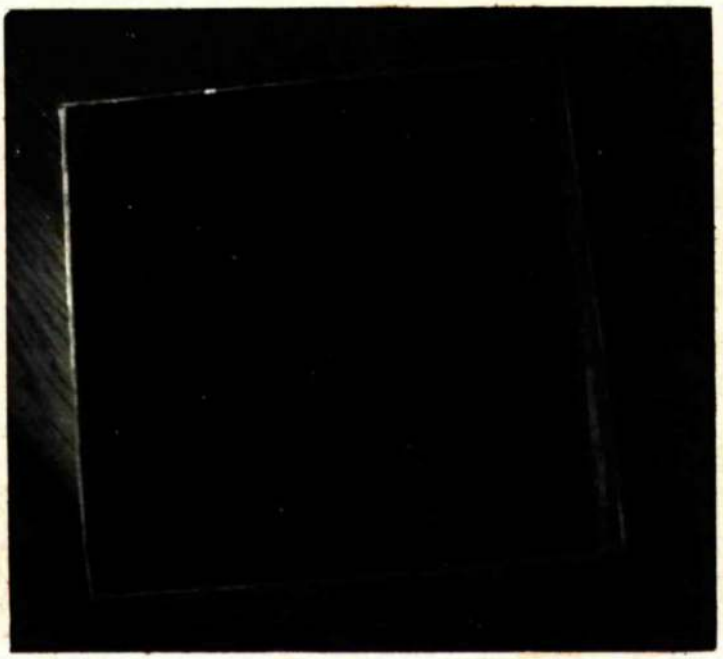
The stickiness and lack of volatility of these solvents have another unsatisfactory aspect. Even if one or two drops only are applied to the skin, they remain fluid for many hours, unlike acetone, which evaporates rapidly. This results in a widespread contamination of the cage and its contents within a very few minutes, as can easily be seen if this is examined under ultra violet light. (Fig. 16). The animal also in cleaning itself ingests a large quantity of the dose applied to the skin, as can be seen when a post mortem examination is made under ultra violet light a few hours after painting. (Fig. 17).

Figure 15.



Picture of a mouse taken by ultra-violet light. The area of fluorescence on the back after painting with three drops of an acetone solution of Pyrene is clearly seen.

Figure 16.



A picture of a standard mouse cage taken by ultra-violet light. Fine pin points of fluorescence are seen on the litter covering the bottom. This contamination occurred within ten minutes of the mouse shown in Fig. 15 being returned to its cage.

Figure 17.

A mouse shown under ultra-violet light half an hour after painting with a solution of Pyrene in Tri-n-Butyrin. Fluorescence is seen in the stomach and on the surrounding tissues where leaking occurred when the stomach was opened.

The Role of the Hair.

To see how much influence the projecting hair has on the passage of the solvent through the skin barrier, various methods of removing the hair prior to painting were tried.

a. Use of an epilatory agent, 7% Ba Sulphide, dissolves the hair, level with the skin surface, and leaves a follicle containing a hair shaft which does not project above the surface.(Fig. 18).

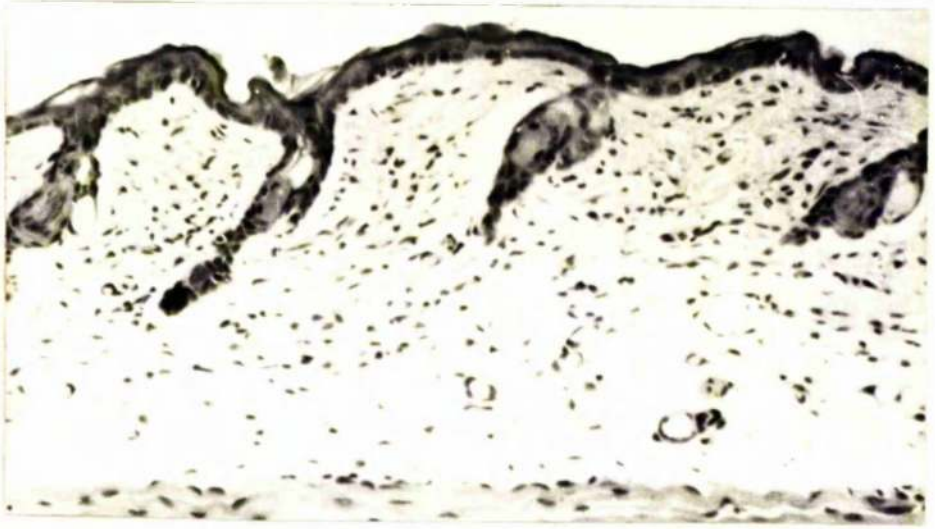
b. Shaving the skin which gives the nearest mechanical equivalent to the epilatory agent does not leave any projecting hair. (Fig. 19).

c. Clipping the hair with mechanical clippers leaves a short even amount of hair projecting from each hair follicle. (Fig. 20).

Clipping the hair with curved scissors, as was done by Pullinger, leaves an irregular length of hair projecting from the follicles, and carries some risk of cutting the skin, thereby causing a reaction which can confuse the subsequent histological picture. (Fig. 21).

Figures 18 and 19.

Haematoxylin and Eosin. X 120

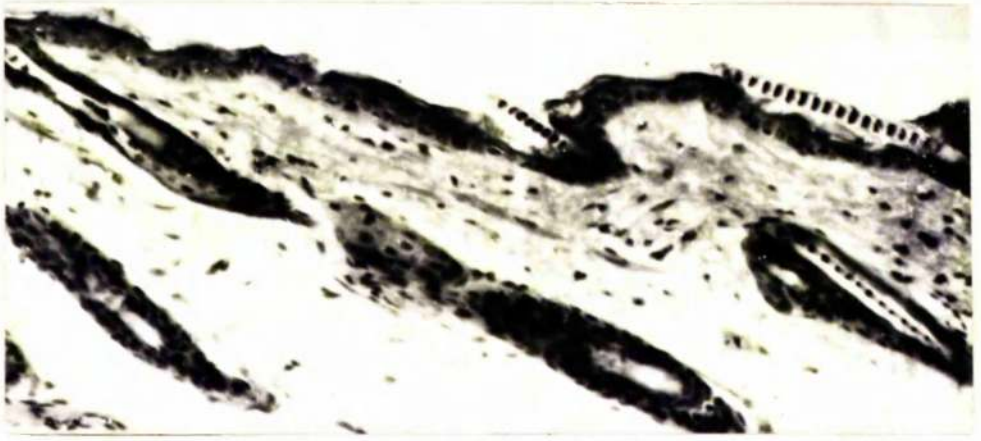


Hair removed from skin by 7% Ba Sulphide during resting phase of hair cycle - no hair projects above skin surface.

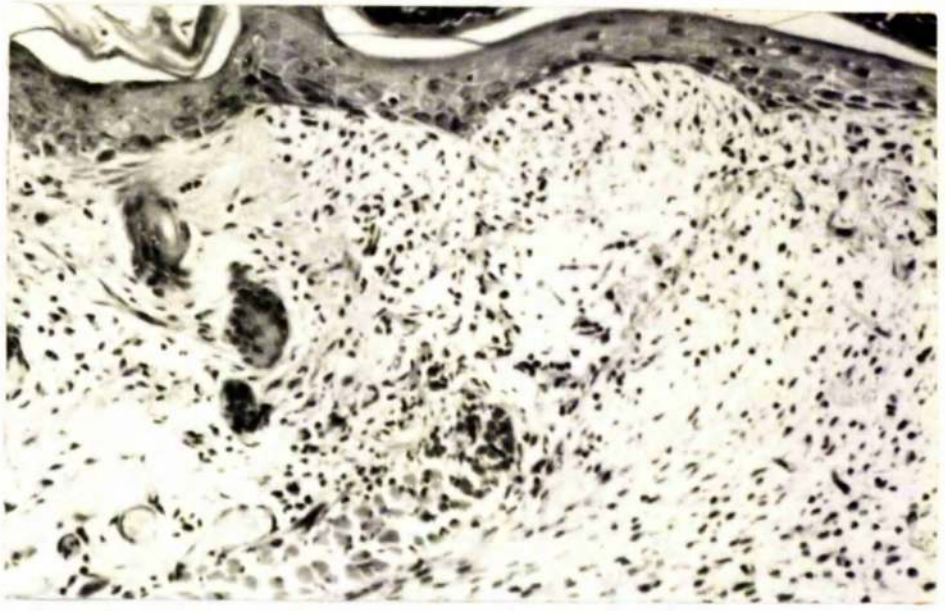
Hair removed by shaving during resting phase of hair cycle. No hair projecting above skin surface.

Figures 20 and 21.

Haematoxylin and Eosin. X120



Hair cut with clippers showing short length of hair projecting above skin. Hair in growing phase of cycle.



Skin healed after cut from scissors. Note thickened epidermis and interrupted muscle layer. X 240

A further set of test paintings using Methyl cholanthrene in acetone were made on three groups of 12 mice. Each was painted immediately after having the skin prepared by one of the methods just described. In each group 3 animals were killed on the four consecutive days after painting. The results were poor with only occasional animals showing rather equivocal microscopic changes.

However on repeating the painting of three similar groups, first allowing 36 hours to elapse after preparing the skin, the results obtained were similar to those described by Pullinger.

Conclusions.

It would appear from the results obtained with these 72 mice that it is necessary for the hair to project to a certain minimum extent above the skin surface before successful reproduction of Pullinger's results can be obtained.

Conditions for the Basis of a Skin Painting Technique Derived from Personal Observations.

From these preliminary studies on the problems of skin painting experiments which involved a total of 202 mice, it would appear that the following factors need to be considered in standardizing a technique based on Pullinger's original observation.

a. Painting should begin on the first day of the first resting phase of the hair cycle. (17-18 day of life).

b. Hair should be cut with clippers 24 to 36 hours before painting takes place to allow minimum projection of the hair above the skin surface.

c. The solvent must be non irritant
volatile
of low viscosity.

It is important to remember that solvents which fulfil these criteria may not be capable of dissolving the test substance.

d. As the changes to be assessed are essentially cellular in nature, they may be easily obscured

by any inflammatory reaction in the skin and all possible precautions must be taken to select mice accordingly.

In addition to the risks of causing this already mentioned it must be remembered that parasites are often present in laboratory animals so that a careful campaign of disinfestation should precede the experiments. In practice it is found that the simplest method is to disinfest a pregnant female and isolate her in a sterilized box in which her litter is kept till the time of the experiment.

Personal Results obtained by Skin Painting with the test series of Hydrocarbons.

From the experience gained in the preliminary investigation acetone was chosen as the solvent. Because of the reportedly relatively weak carcinogenicity of some of the series, the concentrations were increased. Solutions equivalent to 10 times the molar concentration of 0.1% Methyl cholanthrene were prepared. To ensure an adequate penetration of the skin barrier the number of drops used to paint each animal was increased from 3 to 9 which is approximately equal to 0.2 ml. of solution. (This was applied with the double bend pipette illustrated in Fig. 2 on page 46).

As positive results occurred within the first four days in Pullinger's experiment, this was made the limit of the trial. If a promising pattern of results emerged a more extended sequence of painting was to be made.

Subject to the availability of stock 8 to 12 mice were painted with each solution.

A detailed post mortem examination was made of

each animal and the painted area examined microscopically. The results were later summarized on a chart as shown in Appendix No. 3. It was found that Hyperaemia of the subcutaneous tissues deep to the painted area was the only significant finding recorded on naked eye examination. In the assessing of the microscopic changes features which could not be directly attributed to the application of Hydrocarbons were frequently seen and these increased the difficulty of assessing a given sequence as to its specificity of reaction.

The results obtained with the pyrene and anthracene series of Hydrocarbons using 117 mice are summarized in Tables 8 and 9.

Table 8.

Skin Painting with Pyrene Series.

H/C No.	Nos. of mice killed. Day 1	Day 2	Day 3	Day 4	Degree of P.M. Hyperaemia.	Presence of characteristic histological changes.	Evaluation of Specificity of Sequence Reaction.	Hydrocarbon reported as Carcinogen.
1	2				Nil	Nil	Negative	No
		2			Nil	Nil		
			2		Nil	Nil		
				2	Nil	Nil		
2	3				Definite	Definite	Negative	No
		3			Definite	Definite		
			3		Slight	Doubtful		
				4	Nil	Nil		
3	2				Nil	Nil	Positive	Yes
		2			Slight	Definite		
			2		Definite	Definite		
				2	Definite	Definite		
4	2				Slight	Nil	Positive	Yes
		2			Definite	Definite		
			2		Definite	Definite		
				2	Slight	Definite		
5	3				Slight	Doubtful	Negative	Yes
		2			Slight	Doubtful		
			3		Definite	Definite		
				3	Nil	Doubtful		
6	2	2	2	2	All mice infested with lice.			Not tested.
7	2				Slight	Definite	Negative	Yes
		3			Slight	Definite		
			3		Slight	Doubtful		
				3	Nil	Doubtful		
8	3				Nil	Nil	Negative	Yes
		3			Slight	Doubtful		
			3		Nil	Doubtful		
				4	Nil	Doubtful		

Table 9.

Skin Painting with Anthracene Series.

E/C No.	Nos. of mice killed. Day 1	Day 2	Day 3	Day 4	Degree of P.M. Hyperaemia.	Presence of characteristic histological changes.	Evaluation of Specificity of Sequence Reaction.	Hydrocarbon reported as Carcinogen.
9	2				Nil	Doubtful	Negative	No
		2			Nil	Doubtful		
			2		Slight	Nil		
				2	Nil	Nil		
10	2				Nil	Doubtful	Negative	No
		2			Nil	Definite		
			2		Infected with lice.			
				3	Nil	Definite		
11	2				Nil	Definite	Positive	Yes
		2			Slight	Definite		
			2		Slight	Definite		
				2	Nil	Definite		
12	3				Nil	Doubtful	Doubtful Positive	Yes
		3			Definite	Doubtful		
			3		Slight	Doubtful		
				3	Nil	Doubtful		

Analysis of Results.

From these experiments the following points emerge.

a. No false positives were recorded.

b. Some animals showed definite histological changes due to factors other than exposure to the test solutions. This made interpretation of specific changes difficult in some instances.

c. Those substances graded as carcinogenic on the basis of specific microscopic changes were all known carcinogens.
The changes produced by this group of Hydrocarbons Nos. 3, 4, 11 are illustrated in Figs. 22, 23, 24, 25 for comparison with Figs. 4 to 11.
No. 12 which was very difficult to assess was possibly positive.

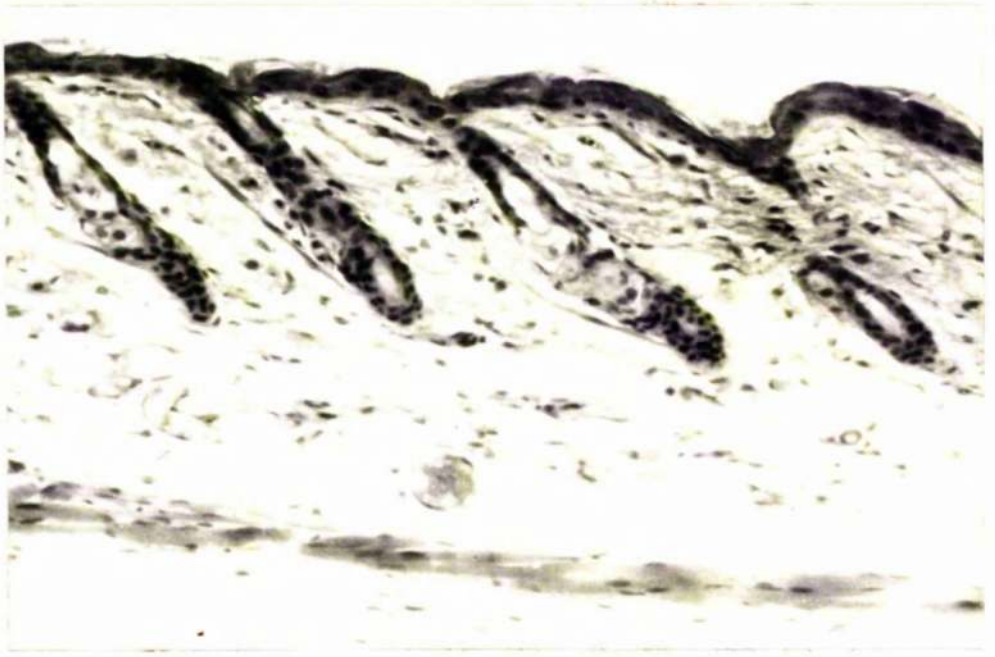
d. Comparison of the last two columns of Tables 7 and 8 shows that three false negatives were recorded.
These were with Hydrocarbon Nos. 5, 7, 8.
The latter is particularly surprising as it is a potent carcinogen under other circumstances.

In order to produce a test sequence which is satisfactory for examination many precautions required to be taken. Even so there was difficulty of interpretation in certain instances and false negatives occurred. For these reasons it is felt that the adaptation of the original observations of Pullinger to a short term method is not sufficiently reliable for routine use.

During the course of this part of the investigation much was clarified and systematized in regard to procedures applicable to all skin painting methods and it is felt that the principles proposed on page 79 should reduce the number of variables inherent in this type of study for whatever purpose it is undertaken.

Figure 22.

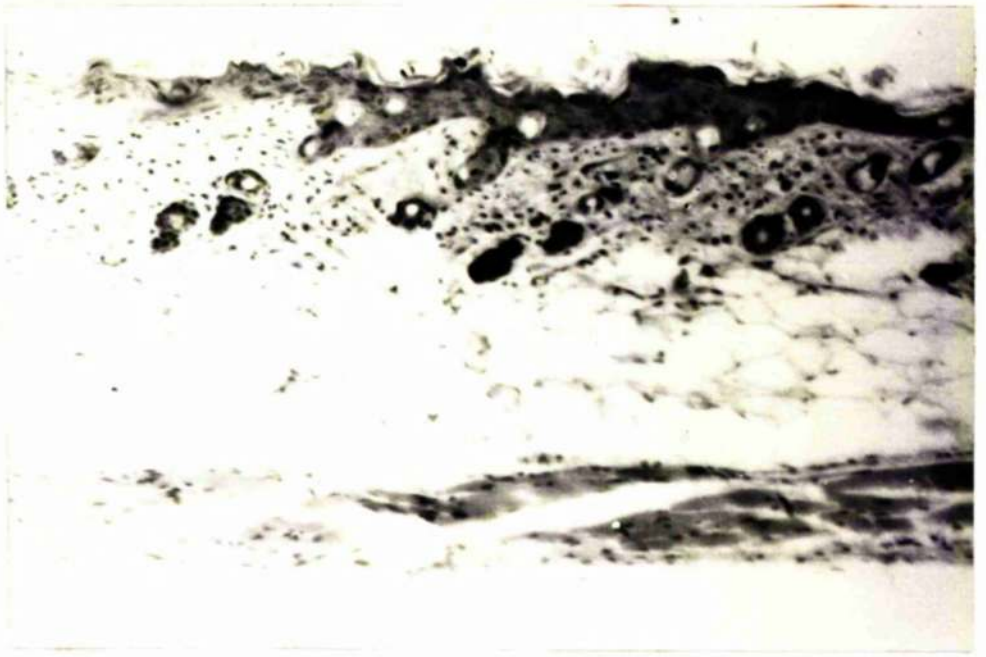
Haematoxylin and Eosin. X 120.



Skin of mouse 1 day after painting with a solution of 1.2:3.4 Dibenzanthracene in acetone. Epidermal thickening and hyperaemia can be seen clearly. There is also some increase in surface keratin. Compare with Figs. 4 and 5.

Figure 23.

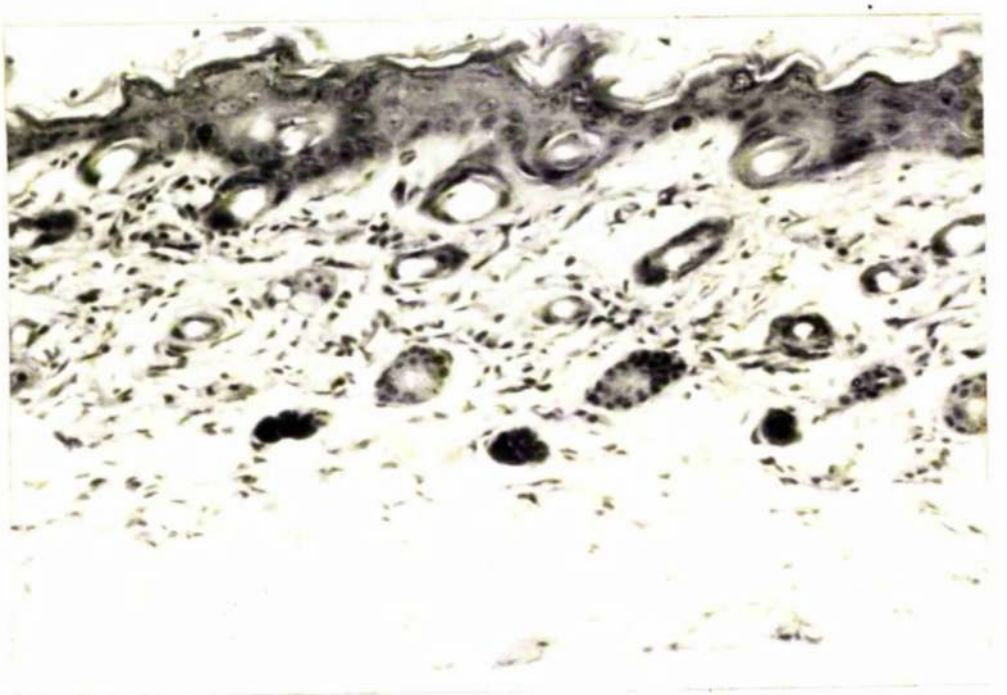
Haematoxylin and Eosin. X 120.



Skin of mouse 2 days after painting with a solution of 1.2:3.4 Dibenzopyrene in acetone. The epidermal changes are the most characteristic feature here. Hyperaemia is not very marked.
Compare with Figs. 6 and 7.

Figure 24.

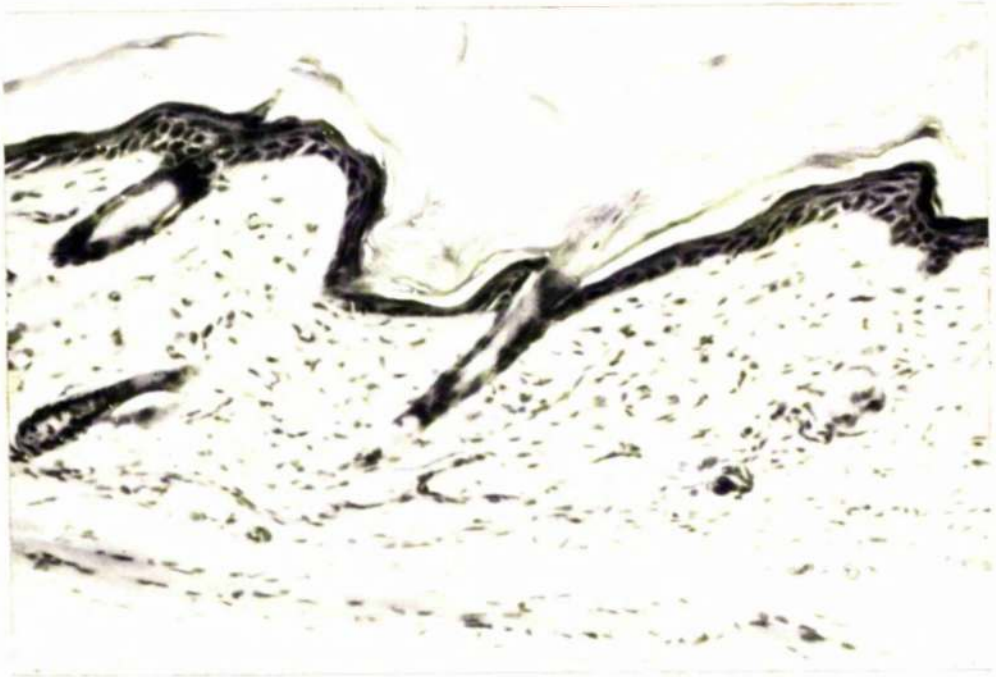
Haematoxylin and Eosin. X 120.



Skin of mouse 3 days after painting with a solution of 3.4 Benzopyrene in acetone.
The characteristic changes described for the third day after painting are seen in this specimen.
Compare with Figures 8 and 9.

Figure 25.

Haematoxylin and Eosin. X 120.



Skin of mouse 4 days after painting with a solution of 3.4 Benzopyrene in acetone. The changes here are focal in distribution. The mouth of a hair follicle is still plugged with keratin. This picture is not as clear cut as the original. Compare with Figures 10 and 11.

INOCULATION EXPERIMENTS.

Introduction.

The majority of inoculation techniques have made use of a non-toxic and non-irritant solvent as the vehicle for the test substance. A deliberate exception to this is the employment of the vehicle as a co-carcinogen or promoting agent. Most commonly the route of injection has been subcutaneous or intraperitoneal although under special conditions other routes have been used in attempts to induce tumours.

It has often been the case that the test substance, the vehicle, or both have rapidly diffused away from the injection site with subsequent metabolism or excretion within a few hours. This has meant that repetition of the injections has been necessary in order to maintain a local or systemic level of test material.

If a simple method of localizing the injected material were available the need for repeated injections would be eliminated.

Pietra, Spencer & Shubik (1959) described a method of preparing a colloidal suspension of Hydrocarbon in gelatin. This was done by injecting an acetone

solution of Hydrocarbon rapidly into a 1% aqueous gelatin solution in a tube kept at 56°C in a water bath and then driving off the acetone by passing nitrogen through the gelatin solution. With a solution of 3:4 Benzopyrene prepared in this manner they injected new born mice and produced a high incidence of Lymphoma after a period of about six months. Roe & Salaman (1961) followed up this work and reported multiple tumours in new born mice injected with a number of different hydrocarbons prepared in a similar manner.

Those results showed promise for further investigation as they satisfied the following theoretical criteria for a standardized test procedure -

a. A single application.

b. Small quantity of test material.

c. Simplicity of execution.

d. As the latent period was six months all the animals were still young enough to have a very low incidence of spontaneous tumours.

e. There is the further point that all the mice being under 24 hours old are relatively speaking "standard".

The only obvious disadvantage to be seen on examining the method was the fact that the 1% gelatin solution tends to set at room temperature and requires to be melted before injection. This introduces the theoretical risk of trauma at the injection site if the temperature of the inoculum is too high. There might also be a remote risk of protein shock from the 1% gelatin.

Alternative Colloid.

In order to circumvent the theoretical problem of protein shock it was decided to make use of Sodium Algenate in place of gelatin in preparing the solution. This is a carbohydrate derived mainly from sea weed which amongst its other properties has the ability to produce in 0.2% concentration the same physical effects as a 1% gelatin solution and it remains liquid at room temperature. This means that each injection would contain less colloid than when gelatin is used.

Preparation of Hydrocarbon in Algenate.

a. A 0.2% solution of Algenate in water was prepared using a motor driven stirring rod. This was necessary as the liquid is viscous during the dissolving of the algenate which takes about an hour. 100 ml. is a

convenient quantity to prepare at any one time for this reason. Such a solution will keep in the refrigerator for up to two weeks, beyond this time it sometimes becomes contaminated by a growth of mould.

b. 0.5 mgms. of Hydrocarbon were weighed out into a small piece of aluminium foil on the scale pan of the balance. The foil with the Hydrocarbon in it was then dropped into a tube containing 2 ml. of acetone.

c. 5 ml. of the 0.2% algenate solution was measured into a wide bore boiling tube and a mark made on the glass to record the fluid level.

d. The acetone solution of H/C was then added to the algenate as follows:- The acetone solution drawn into a double bend pipette (see Fig.2) was sprayed rapidly into the algenate solution from a distance of about 2 cms., the resulting mixture becoming turbid and the fluid level rising in the tube.

e. The boiling tube was sealed with a rubber bung through which two glass tubes pass as shown in Fig.26 and nitrogen bubbled slowly through the mixture making sure the tip of the delivery tube was well below the 5 ml. mark. In this way the acetone was driven off.

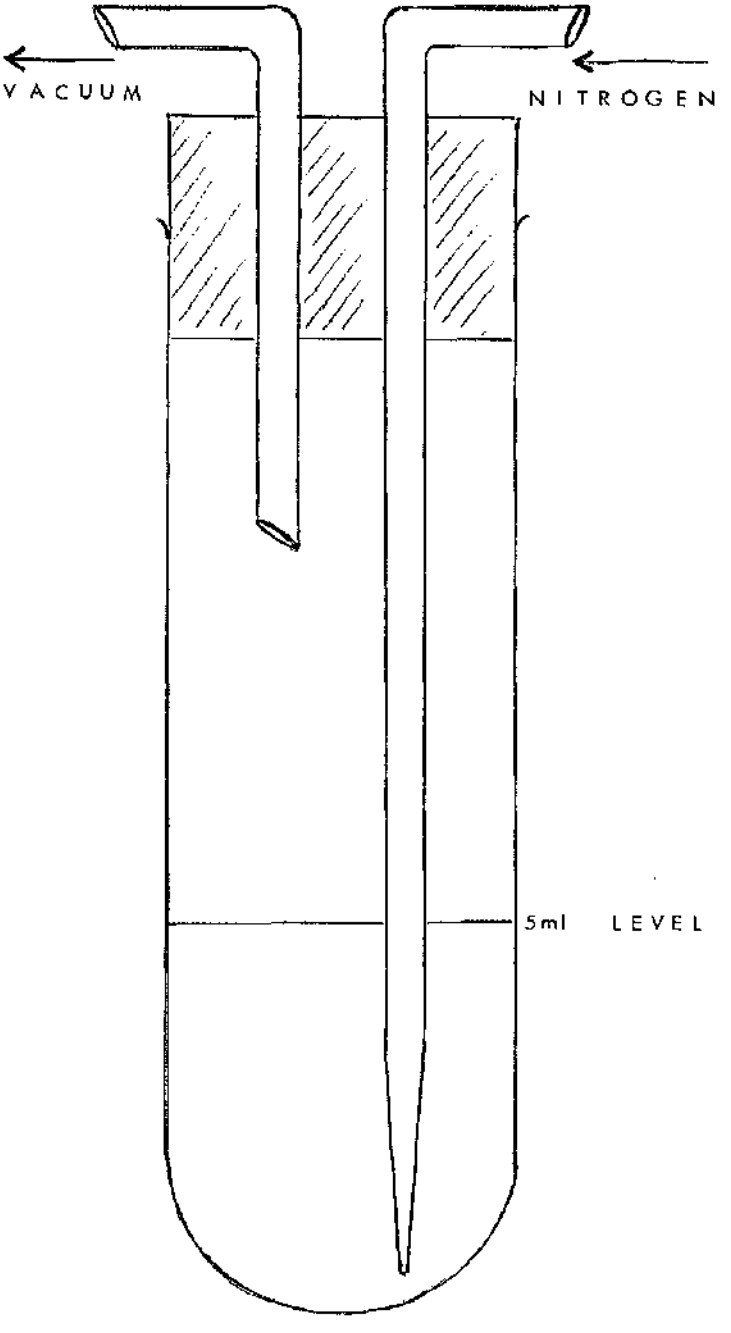
Light suction was applied to the outlet tube to remove the freed acetone. (See Fig. 26).

f. When the fluid level returned to the 5 ml. mark on the level of the tube, the acetone was held to have been completely driven off leaving behind a suspension of Hydrocarbon in algenate.

g. This algenate Hydrocarbon complex will keep in the refrigerator for up to ten days and only requires to be brought up to body temperature before injection. This means 102°F. for the mouse.

A dose of 0.5 ml. was calculated to be equivalent to 50 ugm. of Hydrocarbon. This volume though small is in fact quite a large one to give subcutaneously or intraperitoneally to a new born mouse.

Figure 26



Injection Experiments using an algenate suspension of Hydrocarbons.

Method.

An assistant wearing rubber gloves, to avoid any contamination from the Hydrocarbon solution, held the newborn mice for injection. This was made with a tuberculin syringe and using a No. 20 needle. The term newborn meaning mice under 24 hours old. It is however not a good principle to handle mice in the first 6 to 8 hours of life. Disturbing the nest at this time often means that the mother eats her litter.

a. Subcutaneous Injections.

The needle was passed under the skin of the back low down and the solution injected upwards between the shoulder blades. If the needle point is sufficiently far from the skin puncture then there is no loss of injected material due to back flow.

b. Intraperitoneal Injection.

The needle entered the abdominal cavity in the left iliac fossa and keeping the point well up under the anterior abdominal wall injection was made medially towards the liver, thus avoiding injury to the viscera.

Weaning.

After injection the baby mice were left with the mother till due for weaning. This was usually four weeks after birth. At this time they were sexed and segregated, also numbered by ear punching. It was often found that there had been a considerable wastage during these first few weeks due to deaths.

Duration of Experiments.

At 16 weeks all animals were killed with ether and a post mortem examination made. Any suspicious naked eye lesion was taken for histological examination. This included areas of residual fluorescence.

Post Mortem Procedure.

a. Subcutaneous Injections.

The skin of the back was reflected from the tail end, forward over the skull, by making two lateral incisions and any erythema of the subcutis or enlargement of lymph nodes noted. The animal was then examined under ultra violet light for evidence of fluorescence prior to completing the post mortem examination.

b. Intraperitoneal Injections.

The skin of the anterior aspect of the body was

reflected laterally, from a mid line incision, and examined under ultra violet light to be certain that the injection had not been accidentally made subcutaneously. The abdomen was then opened and any abnormality or residual fluorescence noted, care being taken not to damage the diaphragm.

c. Examination of the Thorax.

The trachea was exposed by a mid line incision in the neck, and a fine hypodermic needle introduced through the wall pointing towards the bronchi. A suture was passed under the trachea and tied firmly in a single knot around the needle. The lungs were then inflated with 2 to 3 ml. of Formol Saline. When fluid flowed back round the needle the suture was tightened, a second knot tied and the needle withdrawn. On opening the thorax it was now found that the lungs had taken up their normal relationship inside the chest cavity. (Fig. 27). Small tumours were then easily seenas white translucent areas, usually placed subpleurally at the periphery of the lobes.

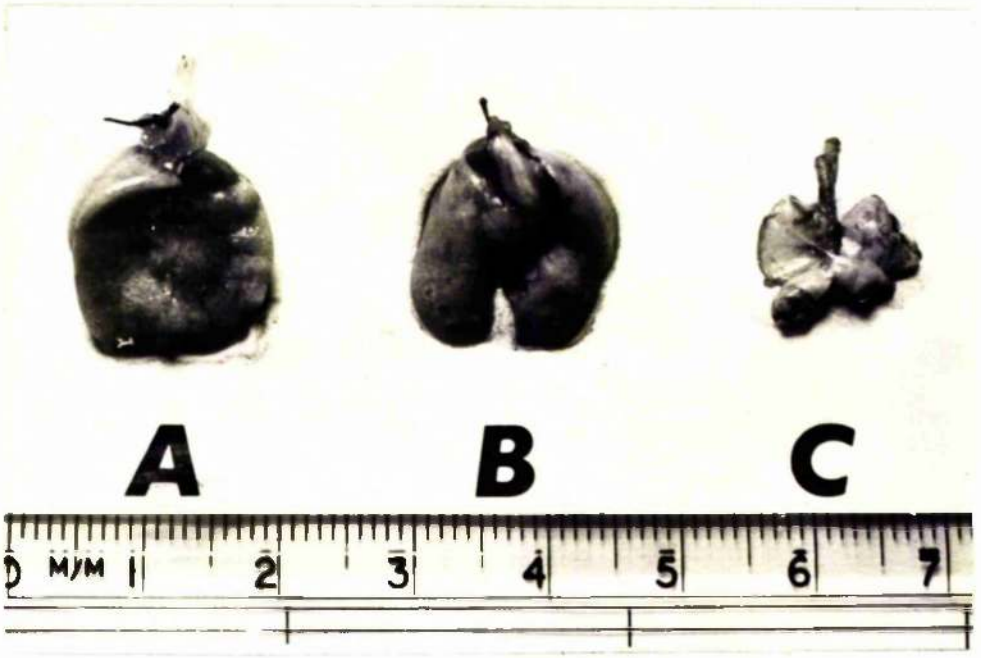
Distribution of Experimental Material.

A total of 282 mice were injected either subcutaneously or intraperitoneally. Of these only 195 survived to the age of weaning. This means that 87 of the animals under experiment were lost in the first four weeks (i.e. about 30%).

The reason for this was that the mothers either ate their litters or abandoned them and they died.

The numbers injected by each route with the different solutions of Hydrocarbon in algenate which survived to the end of the experiments is given in Tables10 and 11.

Figure 27.



The lungs of three different mice are shown.
A is the diaphragmatic surface of lungs inflated by formalin injection.
B shows the apical aspect of similarly injected pair of lungs.
C is a pair of lungs as normally seen within the chest at post mortem examination.
The greater ease of examination of the inflated lungs is seen from this comparison.

Table 10.

Subcutaneous Inoculation of Hydrocarbons in Algenat●.

Hydrocarbon No.	Animals per Group (F)	(M)	(Totals)	Fluorescence Postmortem	Histology Taken	Tumour Distribution. (S/C)	(Lung)	(Totals).
1	3	6	9	-	-			
2	4	2	6	2	-			
3	5	4	9	2	2		1A	1A
4	3	1	4	1	1			
5	1	3	4	4	2			
6	6	2	8	8	7			
7	3	5	8	7	8	7		7
8	4	3	7	1	5	5	1A	5+1A
9	2	1	3	-	-			
10	3	8	11	-	-			
11	3	1	4	1	1			
12	3	2	5	2	4		1A	1A
13	3	6	9	-	2			
*14	-	-	-	-	-			
15	3	3	6	5	6			
Totals	46	47	93	33	38	12	3A	12+3A

A = adenoma.
* = mice failed to survive till weaning.

Table 11.

Intraperitoneal Inoculation of Hydrocarbons in Algenate.

Hydrocarbon No.	Animals per Group (F)	(M)	(Totals).	Fluorescence Postmortem	Histology Taken	Tumour Distribution (S/C)	(Lung)	(Totals).
1	5	5	10	-	-			
2	3	4	7	-	2		1A	1A
3	3	4	7	-	1			
4	4	5	9	5	3			
5	1	2	3	3	3		1A	1A
*6	-	-	-	-	-			
7	6	3	9	5	6	2 spleen		2
8	4	4	8	-	3	1	1A	1+1A
9	2	2	4	-	-			
10	4	-	4	-	-			
11	7	2	9	1	1			
12	2	4	6	-	5		4A	4A
13	5	3	8	-	1		1A	1A
*14	-	-	-	-	-			
15	4	7	11	10	7			
Totals	50	45	95	24	32	3	8A	3+8A

A = adenoma
* = mice failed to survive till weaning.

Results of Hydrocarbon in Algenate Injections.

Both routes of injection show somewhat surprising results. Although the numbers involved for each individual hydrocarbon are not large the failure of several of the known carcinogens to produce tumours was unexpected. In the case of the subcutaneous experiments, frankly malignant lesions occurred at the inoculation site in 7 out of 8 animals using 3 4: 8 9 Dibenzopyrene and in 5 out of 7 animals using 3 4: 9 10 Dibenzopyrene, which indicates a good positive correlation with the known effects of these substances. However the failure of the 5 other known carcinogens in these series to induce similar tumours is not readily explained, especially in those cases where residual fluorescence was present at the inoculation site.

The presence of isolated animals with pulmonary adenomas is considered of some significance in view of the age of the animals in which they had developed.

When the intraperitoneal injection experiments are considered we again find the same two hydrocarbons responsible for the production of tumours at or near the site of inoculation.

The total number of pulmonary adenomas is greater in this group and the distribution is wider. 1 2: 5 6 Dibenzanthracene having the highest incidence in 4 out of 6 mice compared with 1 out of 5 for the subcutaneous route. This suggests that the absorption of the Hydrocarbon from the inoculum is more easily accomplished via the intraperitoneal route.

Histology.

a. All the subcutaneous tumours occurring at or near the sites of injection were of a poorly differentiated or anaplastic type often showing many mitoses. Invasion either of the deeper subcutaneous tissues or of the overlying squamous epithelium is evident. The general appearances resemble those of the type of sarcoma often experimentally induced in subcutaneous sites in animals. (Figs. 28, 29).

b. The two splenic tumours occurring with the intraperitoneal injection of 3 4: 9 10 Dibenzopyrene were probably due to the accidental direct injection of some of the solution into the spleen. These tumours appear to be sarcomas arising from the connective tissue of the spleen itself. (Figs. 30, 31).

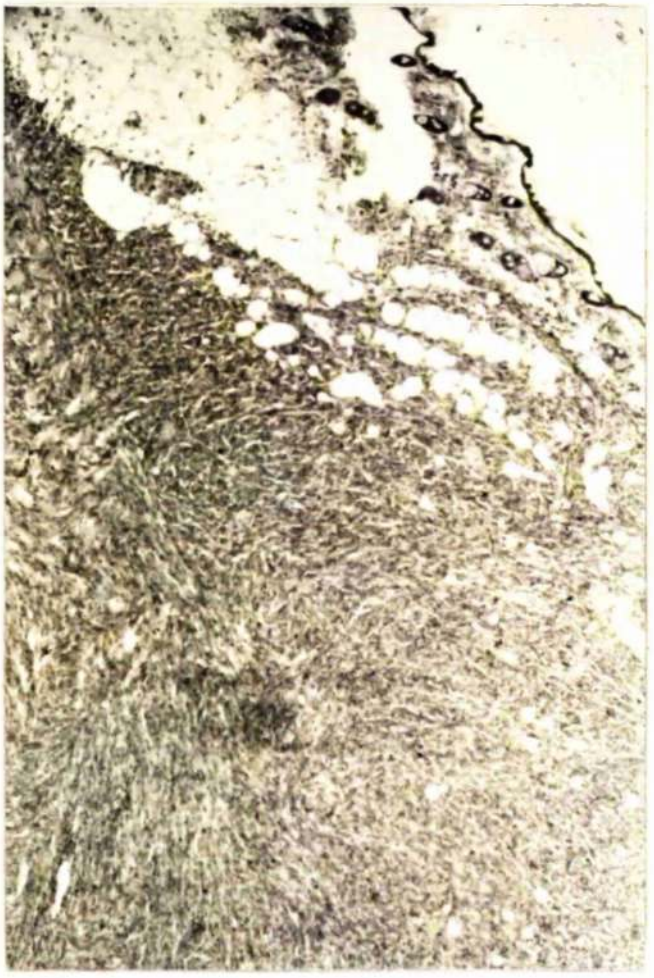
c. In each animal showing the presence of pulmonary adenomas these were found to be multiple. There was a variation in size from nodules just visible naked eye to lesions only visible microscopically. (Figs. 32, 33). There seem to be two principle sites of distribution, a subpleural peripheral nodule (Fig. 34) often rather solid and sometimes showing a papillary type of structure. (Fig. 35). The other is a centrally placed tumour which is often in close association with a small blood vessel and indeed often surrounds it. (Fig. 36). These seem in their early stages to be a less solid type of growth.

d. The histology of the fluorescent areas noted at post mortem in which no tumours were found all showed the presence of algenate surrounded by a simple fibrous capsule (Fig. 37). Viewed with an ultra violet light source this material fluoresced brightly in the microscopic preparations.

These observations held true both for areas of subcutaneous and intraperitoneal fluorescence seen at post mortem examination.

Figure 28.

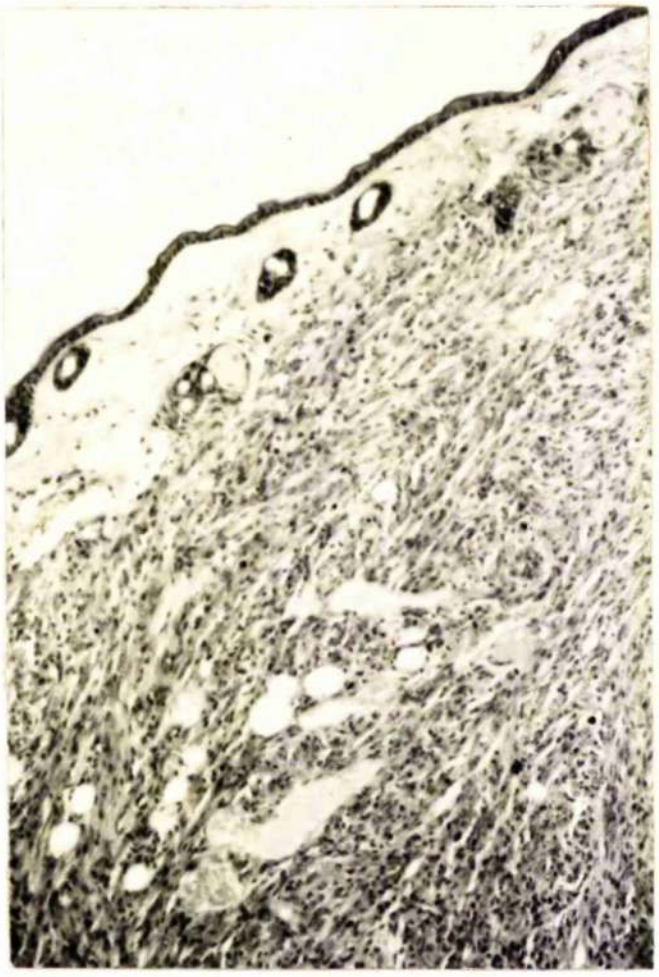
Haematoxylin and Eosin. X 48



Subcutaneous tumour at site of injection with 3.4:8.9 Dibenzopyrene in algenate. This is a sarcoma which is infiltrating widely in the subcutaneous tissues.

Figure 29.

Haematoxylin and Eosin. X 120



Part of Figure 28 magnified to show infiltration of subcutaneous tissues more clearly. The anaplastic nature of the tumour is well seen.

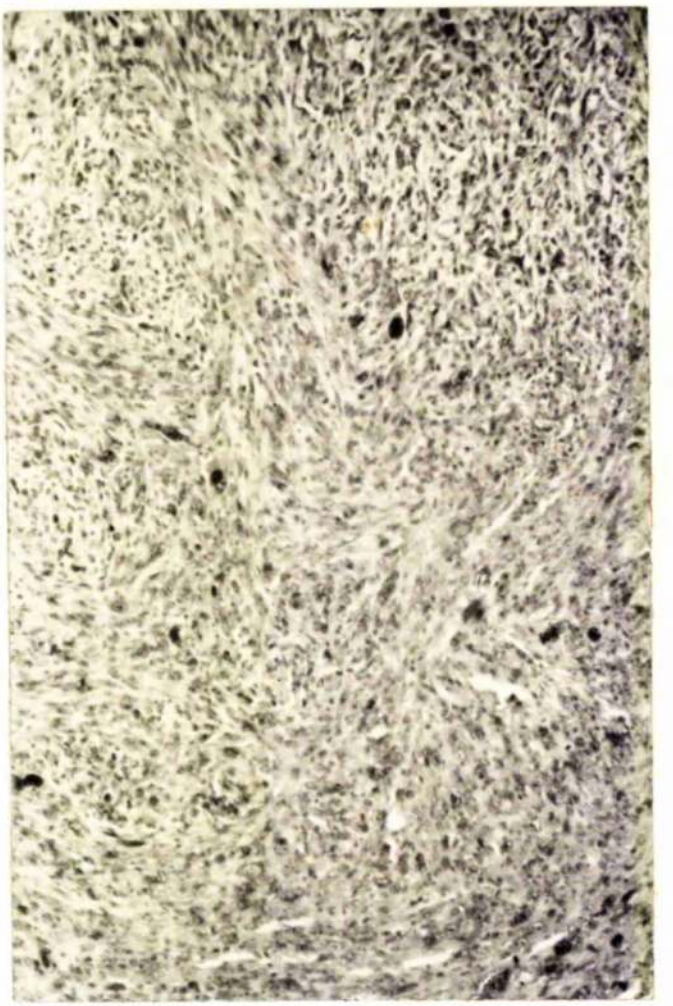
Figure 30.

Haematoxylin and Eosin. X 120

Spleen of mouse injected intraperitoneally with 3.4:8.9 Dibenzopyrene in algenate. A poorly differentiated tumour is seen arising within the substance of the spleen.

Figure 31.

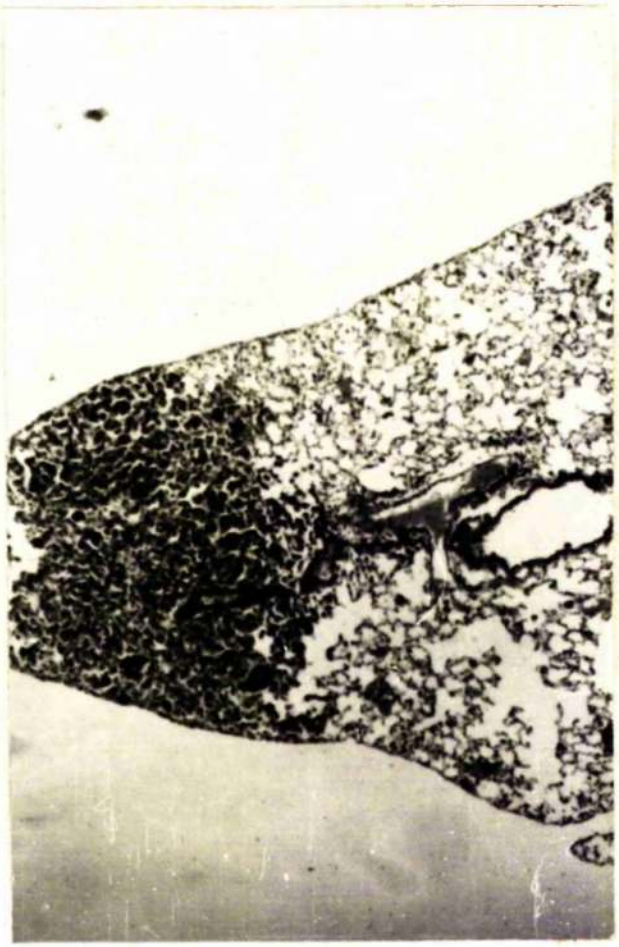
Haematoxylin and Eosin. X 480.



A magnified view of part of Figure 30 showing the sarcomatous appearance of the tumour with a number of mitotic figures.

Figure 32.

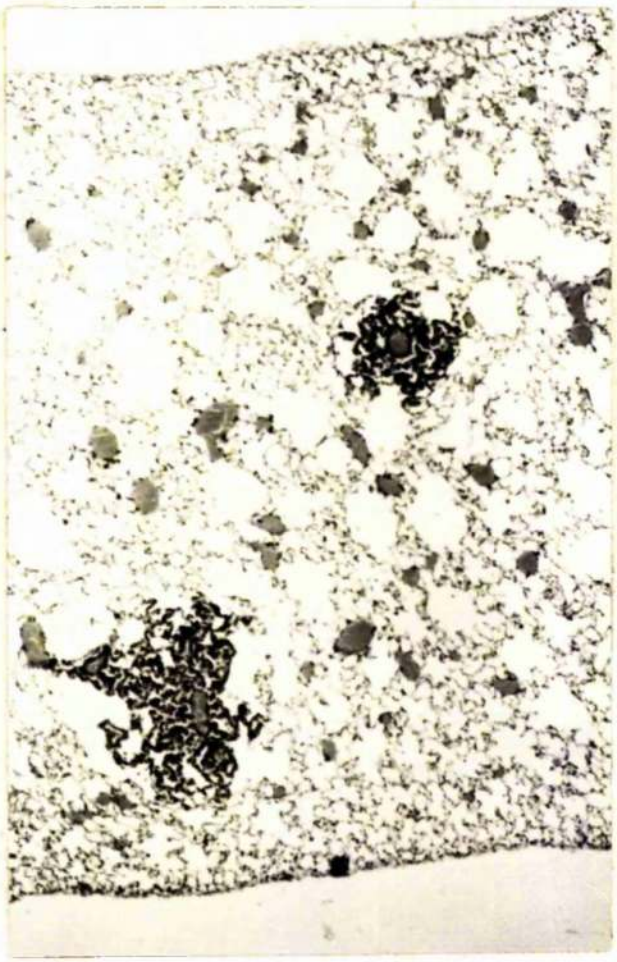
Haematoxylin and Eosin. X 48.



Adenoma of lung large enought to be visible naked eye. Arising in an animal injected subcutaneously with 1.2:5.6 Dibenzanthracene in algenate.

Figure 33.

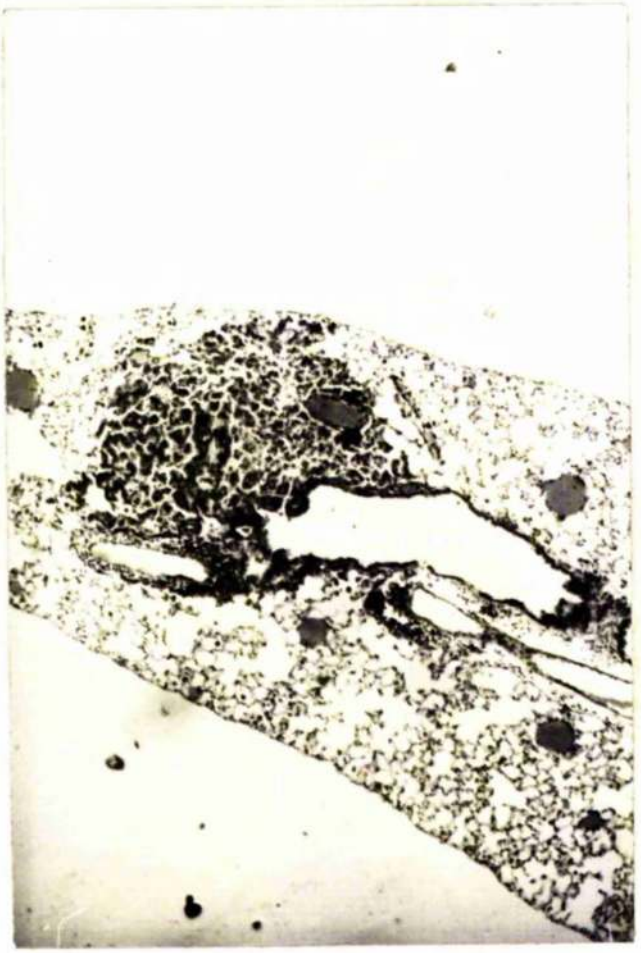
Haematoxylin and Eosin. X 48.



Microscopic centrally placed adenomas.
Arising in a mouse injected intraperitoneally
with 1.2:5.6 Dibenzanthracene.

Figure 34.

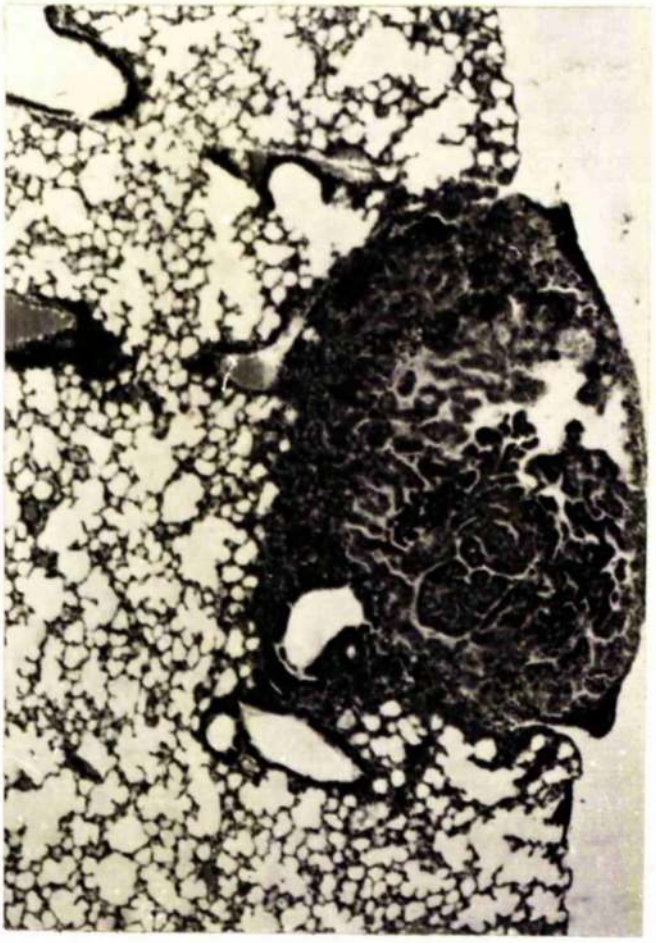
Haematoxylin and Eosin. X 48.



Subpleural type of adenoma in mouse injected intraperitoneally with 1.2:4.5 Dibenzopyrene. There is a suggestion of an early papillary arrangement in the tumour.

Figure 35.

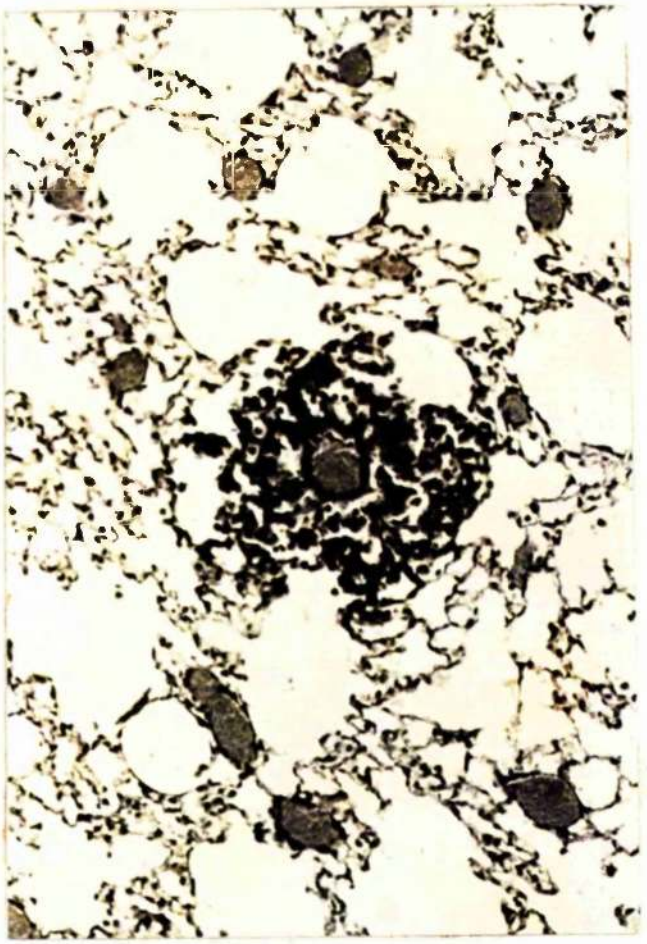
Haematoxylin and Eosin. X 120



Papillary type of subpleural adenoma arising in a mouse injected intraperitoneally with 1.2:7.8 Dibenzanthracene in algenate.

Figure 36.

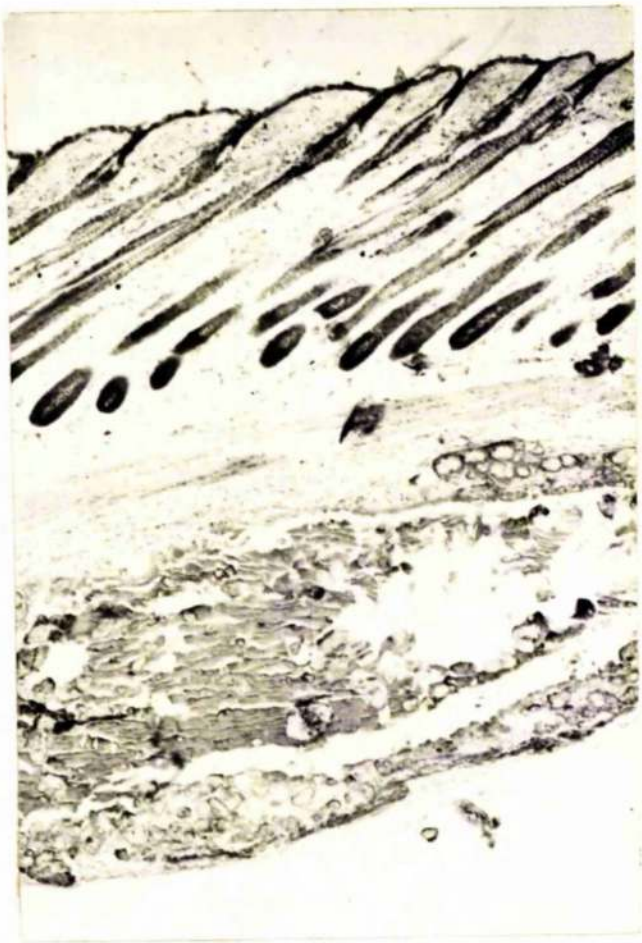
Haematoxylin and Eosin. X 120.



Early adenoma showing arrangement surrounding a small blood vessel. This was one of several similar tumours in a mouse injected intraperitoneally with 1.2:5.6 Dibenzanthracene. See Figure 33.

Figure 37.

Haematoxylin and Eosin. X 48.



Subcutaneous encapsulation of an inoculum containing 1.2:3.4 Dibenzanthracene in algenate. This nodule was brightly fluorescent at post mortem examination under ultra violet light.

Commentary.

From these experiments certain conclusions can be drawn.

a. There was a high wastage rate of animals in the early weeks after inoculation resulting in a considerable reduction in numbers available for final analysis. In order to obtain any given total number of results for an experiment of this type it is estimated that the injection of 30% in excess of the final number desired would be necessary to allow for this early loss.

b. As the mothers clean their babies after they are returned to the nest they run a high risk of ingesting some of the inoculum from leaking at the inoculation site. For this reason each mother has to be killed at the time of weaning the litter as she cannot be used for further breeding. The resulting strain on the breeding stock was at times very great.

c. The timing of experiments was dependent on the rate of breeding and days of birth of litters. The resulting limitation made for difficulties at times with regard to the preparation of the algenate solutions and other aspects of the experiments.

d. Only two of the known carcinogens gave a really satisfactory positive result to correlate with the known behaviour of the substances under test. These tumours were passaged through at least three further animal hosts and grew to transplantable size within three weeks of grafting.

e. The incidence of adenomas of lung in mice of this age group is of considerable interest as according to Shimkin 1955, spontaneous tumours of this kind are not recorded in mice under a year old. This would seem to imply some association between the experimental procedures and the incidence of these tumours, particularly in the case of 3 4: 9 10 Dibenzopyrene.

f. Although brilliantarees of fluorescence present at death indicate satisfactory localization of the inoculum the level of tumour incidence was disappointingly low, contrary to the reported behaviour of many of the hydrocarbons.

It may be that the algenate has a protective or inhibiting effect, or simply that it stimulates the production of a protective reaction around the injected material which is thus prevented from being absorbed and

metabolized.

Although this line of investigation would be of interest to pursue it was not within the scope of the present study and was not extended further.

This technique though simple and fulfilling many of the postulated requirements failed to produce a convincing degree of correlation in a sufficient number of cases to warrant further exploration of the method. No further trials have been made to explore the reasons for the apparently satisfactory behaviour of some of the test substances and the lack of response on the part of others.

IMPLANTATION EXPERIMENTS.

Review of Implantation Procedures.

It was considered that, in the context of the work being carried out in this study, the procedures for passaging experimentally induced tumours did not constitute a method of tumour induction, the commonly employed methods being the subcutaneous implantation of tumour fragments with a trocar and cannula, or the injection of tumour mince through a wide bore needle.

a. Human Tumour Explants.

A method of conditioning experimental animals by X-irradiation which made possible the successful explanting of human tumour material into the prepared hosts was described by Toolan 1951. Later the pre-treatment by X-irradiation was combined with cortisone injections. As a result of her work a number of human tumours became permanently established in successive generations of prepared animals.

From these strains, of which H Ep I and H Ep II are well known examples, successful establishment of tissue culture lines was made by Moore 1955. Many studies have been made on the factors influencing tumour

growth using both the solid tumours and the tissue culture cells derived from this human material.

It must be recognized however that this very important work does not fall into the classical mould of tumour induction by the application of a carcinogen.

b. The Use of an inert vehicle for a carcinogen.

In an attempt to obtain good localization of a carcinogen in the tissues with resulting long term exposure of a single site to the action of the substance Jull 1951 incorporated methyl cholanthrene in paraffin wax from which small pellets were made. These pellets were surgically implanted into the urinary bladder in mice. The post-operative mortality was rather high but the percent tumour induction in the surviving mice was good. He used 2% and 30% concentration of test material in his studies.

c. Variation in physical state of an inert material.

It was noticed by Oppenheimer 1956 that if an inert material such as plastic was introduced subcutaneously in a single sheet 2 x 1 cm. then a sarcoma arose around the implant site. If however a series of holes were made in the plastic or the plastic was

ground up prior to implantation no tumours arose.

Further any substance that was chemically active failed to induce sarcoma formation.

d. Implantation of tissues in conjunction with a carcinogen.

Rous & Smith 1945 made a fine mince of embryo skin and injected this deeply into the thigh muscle of adults of the same strain with the resulting formation of inclusion cysts lined by well differentiated skin.

These experiments were made using Balb/C mice a highly inbred strain as other work had shown that no growth of embryo implants occurred using mice of mixed strain origin. This strain is the Bagg albino C which was the result of 50 generations of brother and sister mating. It was the need to use this strain in these experiments that determined the use of Balb/C mice throughout the whole of this investigation.

When methyl cholanthrene dissolved in olive oil saturated with Scharlach R was included in the inoculum squamous carcinomas were induced in the implants.

Horning 1946 and 1947 used adult mouse tissue, both prostate and lung, in which he wrapped crystals of methyl cholanthrene prior to subcutaneous implantation. With both types of tissue there was successful induction of squamous tumours.

Whilst all these procedures were successful in achieving the object of the experimental conditions described, they were not in themselves sufficiently clearly defined that they could be modified to fulfil the requirements of a routine test procedure.

Before describing the implant technique used for these experiments it is appropriate at this point to explain the procedure which became necessary to provide an adequate supply of experimental animals.

Management of the Mouse Colony.

The Balb/C strain of mice was chosen at the beginning of this study because of the report that tumours had been induced in embryo implants of this strain.

When these implantation experiments were begun, the skin painting, and algenate solution injection experiments had already been in progress for some weeks. It was clear therefore that heavy demands for embryo tissue would rapidly deplete the breeding colony. For the embryo implant experiments alone two groups of animals would need to be constantly available.

I. Pregnant females as sources of Embryos.

II. Host animals of appropriate age for the implants.

In order to ensure that these demands as well as those of the other experiments could be met, the following routine was adopted.

a. Mice were mated two females and a male per box, this having been found to be the optimum ratio for this strain of mouse in the available colony.

b. Cages were examined daily and all pregnant mice segregated in single boxes. Some were set aside as sources of embryo material and others were kept for

increasing stock.

c. The date of birth of litters was recorded and they were weaned at four weeks. At this time they were sexed and segregated 6 to 8 per cage.

d. Mice between 8 and 9 weeks old were used as hosts for the implanting of embryo tissues. This age group was chosen for the following reasons.

1. They were almost adult in size and therefore easy to handle from an operative point of view.
2. They were too young to have spontaneous tumours.
3. After the 16 weeks of the experiment they were still only six months old and spontaneous tumour incidence would still be very low.

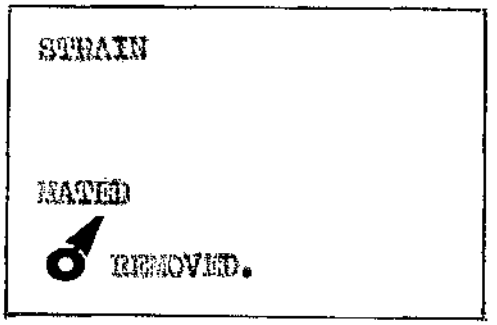
e. If mice passed the age of 9 weeks unused for inoculation experiments the females were all kept for future breeding and some males also to replace breeding males.

f. Each pregnant female killed to provide embryos was replaced by another female over 3 months old. To

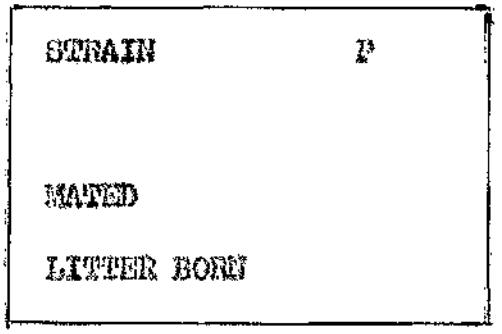
simplify the rapid assessment of the state of the colony at any time with regard to the availability of the various groups, the cage cards illustrated in Fig. 39 were designed and changed as appropriate with the passage of a group of mice from one stage to the next.

Figure 38.

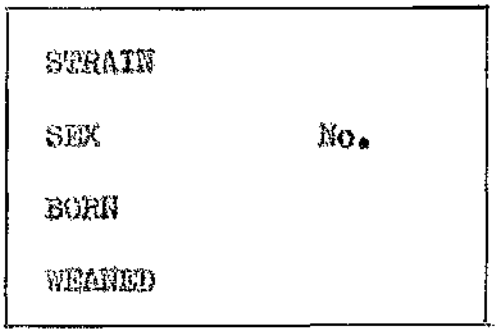
Card for Mating Cage.



Card for Isolation of Pregnant Mice.



Card for Weaned Litters.



Personal Embryo Tissue Implant Technique.

Personal Embryo Tissue Implant Technique.

It was felt that an open surgical technique would allow a more precise localization of the implanted embryo tissue than in previously described methods, and would also reduce the degree of tissue trauma in the host.

Selection of Embryo Material.

a. Pregnant females near term were killed by cervical dislocation. This method was chosen so as to avoid any possible effect on the embryo had an anaesthetic been used.

b. The abdomen was then opened and the intact uterine horns removed to a sterile Petri Dish. Plastic disposable dishes were used throughout these experiments. Sterile instruments were used to remove the uterus but no antiseptic was used on the skin to avoid contamination of the abdominal contents.

c. With fresh sterile instruments the foetuses were removed from the amniotic sacs and separated from the placentae. They were then stored in a second sterile Petri Dish containing sterile saline.

d. Each individual foetus was pinned on a clean sheet of paper on a cork mat for dissection, by passing a

steel pin into the mouth and through the skull. The tail end was immobilized by a pin through the root of the tail.

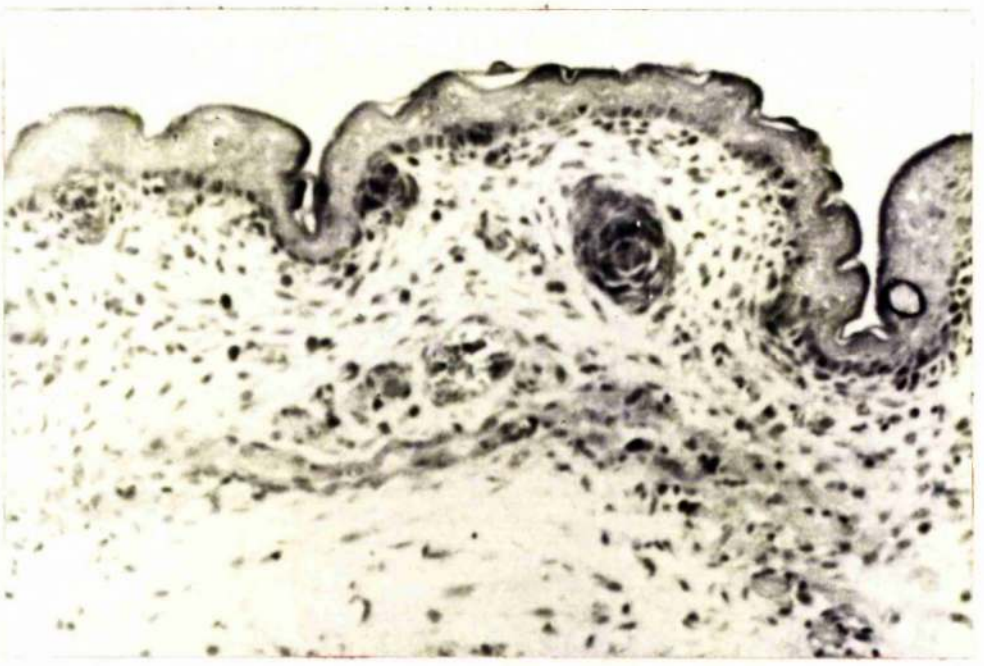
e. Using a magnifying lens on a head band it was found to be quite easy to identify individual organs. Fine instruments many of them designed for ophthalmic surgery were used to remove the tissues required.

f. The tissues were placed in sterile saline in previously labelled petri dishes. They could be kept in this manner at room temperature for up to an hour before implantation and still give satisfactory results.

The normal histological appearance of some of these tissues prior to implantation is shown in Figures 39 to 45.

Figure 39.

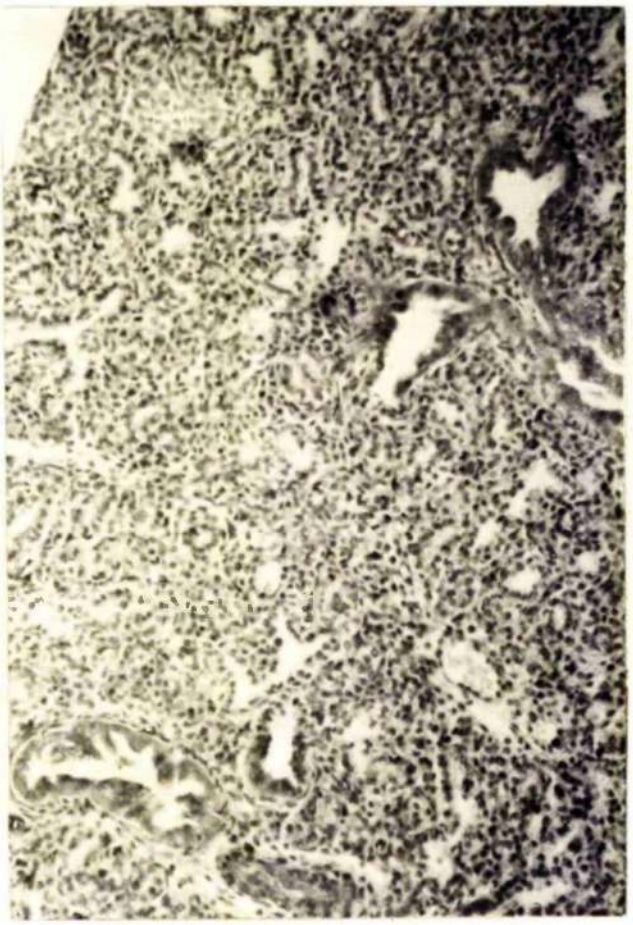
Haematoxylin and Eosin. X 120



Normal foetal skin near term. Overlying the squamous epithelium is a layer of keratin. No hair is present. Only occasional rudimentary hair follicles are seen.
Compare with Figure 14, page 67 and Figure 47, page 144.

Figure 40.

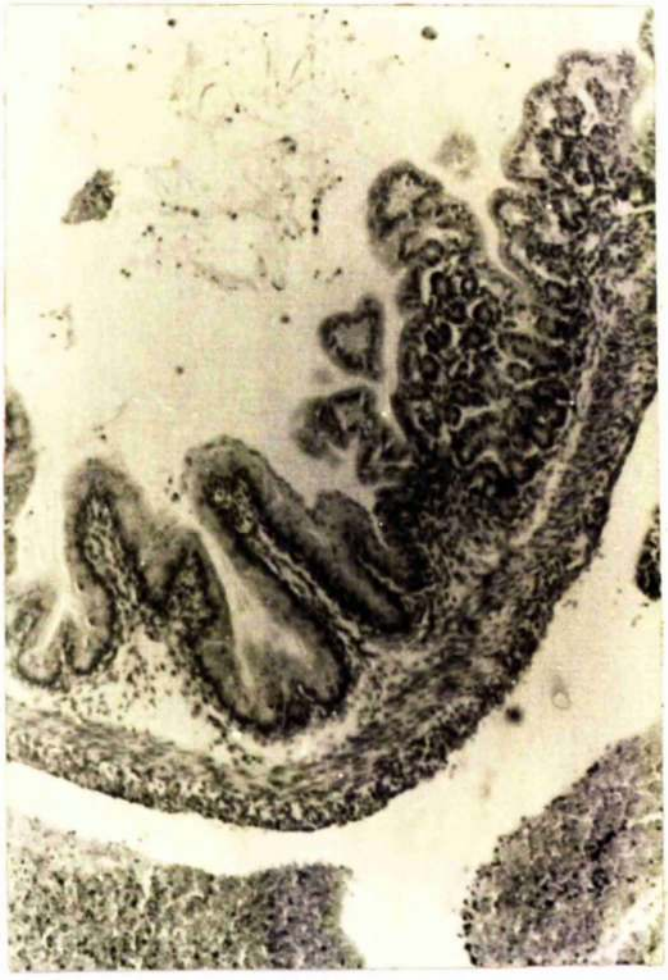
Haematoxylin and Eosin. X 120



Part of embryo lung from mouse foetus at term, showing prominent bronchi and unexpanded alveoli.
Compare with Figure 49.

Figure 41.

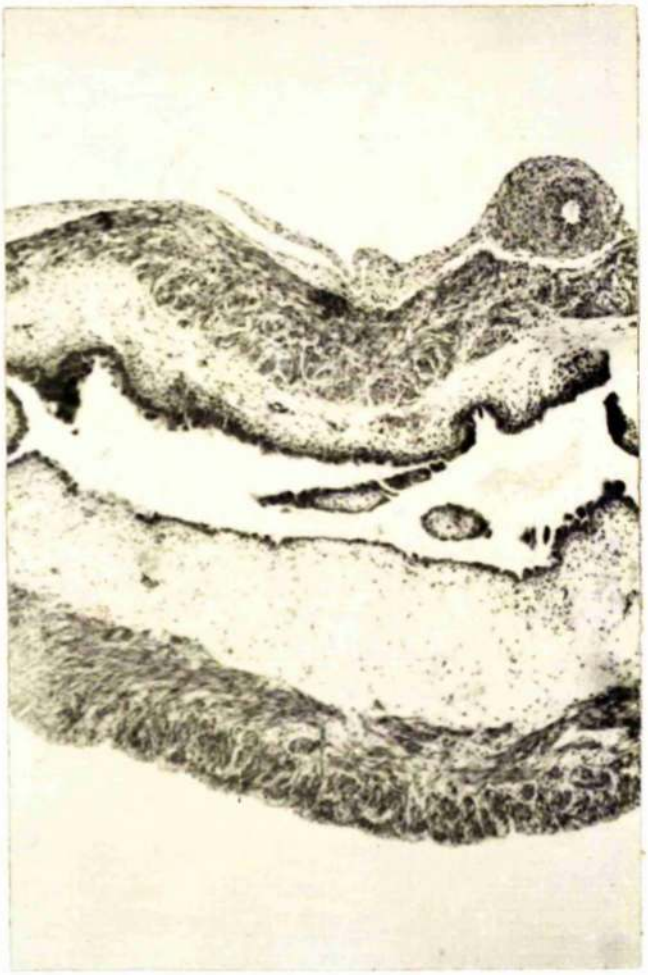
Haematoxylin and Eosin. X 48.



Part of a normal embryo mouse stomach showing the two types of epithelial lining. Squamous epithelium in the fore stomach and glandular epithelium in the body of the stomach.

Figure 42.

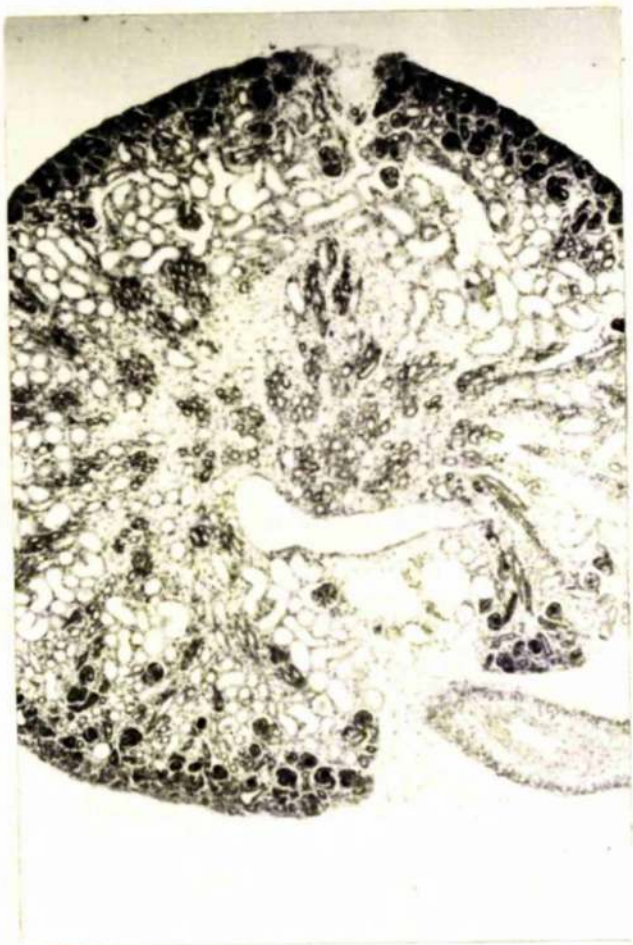
Haematoxylin and Eosin. X 48



Whole urinary bladder from mouse foetus at term including a cross section of ureter at the upper edge.
Compare with Figure 54.

Figure 43.

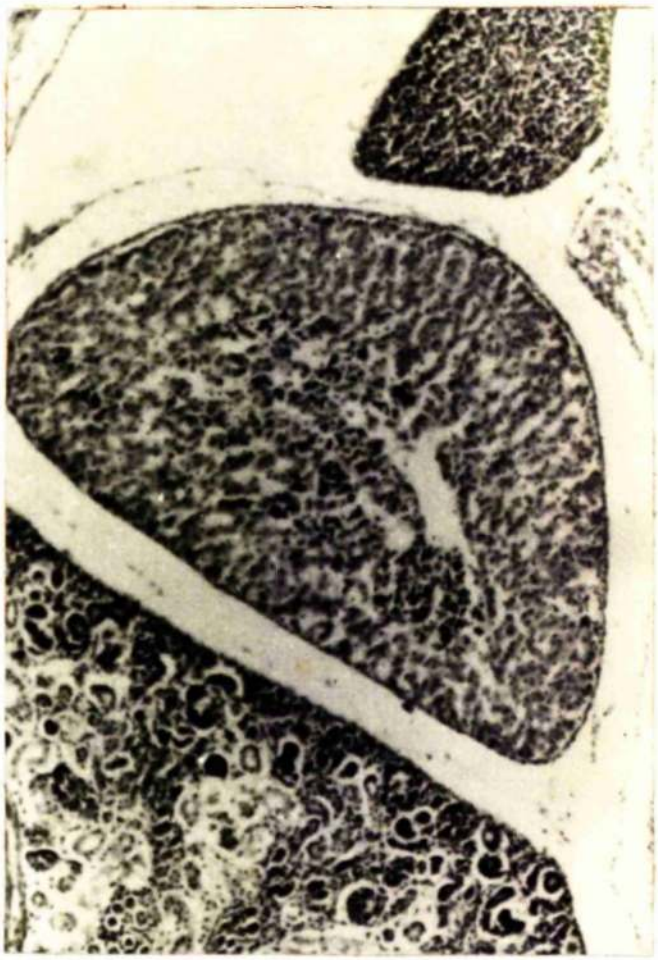
Haematoxylin and Eosin. X 48.



Whole embryo kidney from mouse foetus at term.
Compare with Figure 85.

Figure 44.

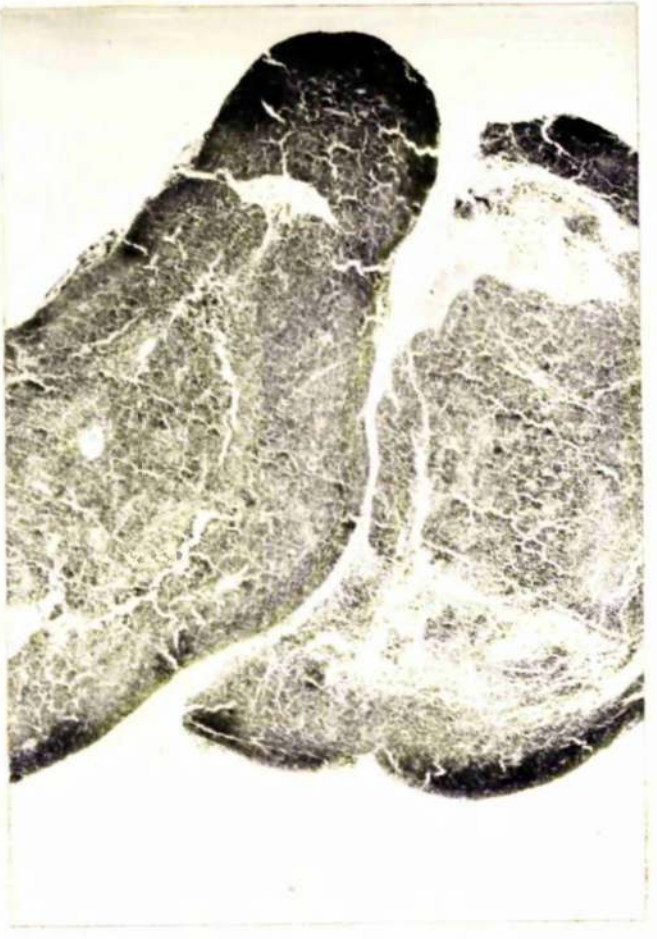
Haematoxylin and Eosin. X 48



Normal embryo adrenal of mouse. Small portions of spleen and kidney are seen on either side of the adrenal.

Figure 45.

Haematoxylin and Eosin. X 48.



Whole embryo thymus from mouse foetus at term.
Compare with Figure 59.

Method of Implantation of Embryo Tissue.

a. The outer aspects of both hind legs of the mice were clipped with electric clippers. Those used were the pattern supplied by Wahl Ltd. of London and called Clukes clippers which have a specially narrow head for use with small animals. With Balb/C mice this can be done without anaesthetizing the animals. Stock mice and some other strains will not stay still enough and quite severe skin cuts can result.

b. The mice were anaesthetized in a wide neck glass jar with a screw top. The best method is to have a small basket attached to the under surface of the lid in which cotton wool soaked in anaesthetic is placed. Putting the wool in the bottom of the jar results in the animals wetting the fur of the face area with consequent damage to eyes and mucus membranes.

Trichlor Ethylene BP (Trilene) was used as anaesthetic having been found by personal experience to be very suitable for maintaining anaesthesia in small animals for long periods of time with subsequent rapid recovery.

c. A small skin incision was made posterior to and parallel with the line of the femur using a No. 15

Swan-Morton blade on a number 5 handle.

d. The fibres of the thigh muscle were separated in the same line as the skin incision by stabbing a sharp pointed pair of scissors deeply into the muscle then opening the points. Carefully done this causes no bleeding, but if the scissors are too close to the femur the femoral vessels may be damaged with consequent haemorrhage.

e. The cavity so formed was held open with a pair of curved forceps, while the implant was placed deeply into the muscle with another pair. The muscle was then allowed to close over the implant. If this precaution is not taken movement of the leg can make the implant work loose from its intra muscular site and it may possibly be lost through the skin wound in the immediately post operative period.

f. The skin was closed with one or two interrupted stitches taking care not to stitch the muscle which would excite a foreign body reaction.

g. Each animal was numbered by ear punching.

This operative procedure can be carried out single handed but it is much more rapidly done if an assistant holds the animals and controls the anaesthetic. With practice it was found possible to make both implants in an animal in some three minutes.

A different type of tissue implant in each leg was found useful in helping to avoid possible confusion at a later date when tissues were processed and sections prepared for microscopic examination.

Care of animals after the Implantation procedure.

a. All animals were kept in standard mouse cages with a diet of pellet food No. 41 and water ad libidum.

b. Weekly examination of each animal was made to note localized or diffuse swelling in the hind legs. The week of first appearance of any such abnormality was noted. A few animals were killed before the 16 week period had elapsed due to ulceration of the skin over a swelling, or to the excessive size of the nodule.

In the majority of cases the presence of a large swelling was later found to be due to retention of Keratin or Secretion within a cyst. For this reason the presence of a palpable nodule cannot be taken as a

definite indication of the development of a neoplasm.

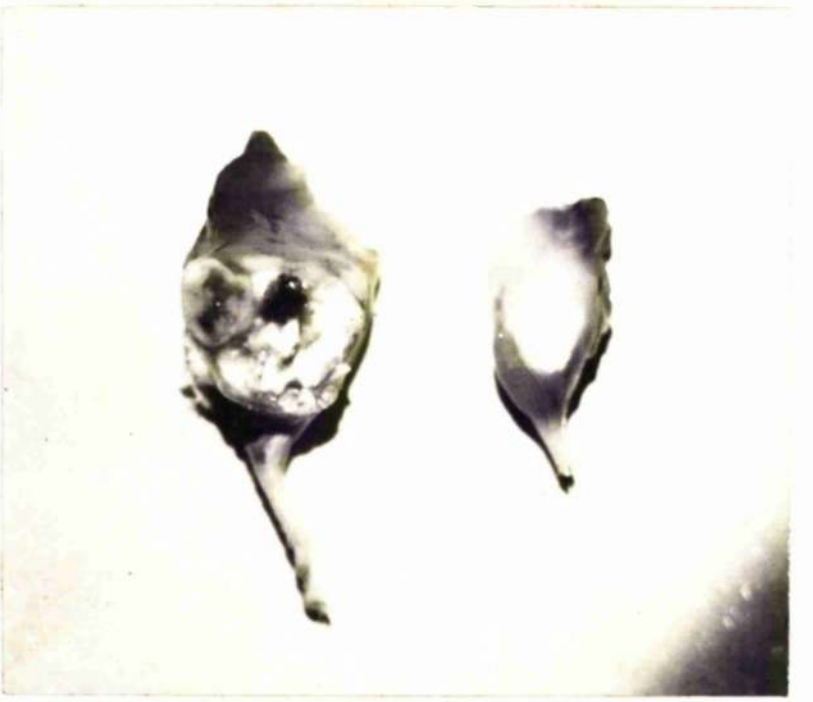
Post Mortem Procedure.

a. At the end of the 16th. week all animals were killed with ether.

b. The skin of the hind legs was dissected off and viewed under ultra violet light when some of those with tumours were found to be fluorescent. (Fig. 46). They were then disarticulated at the hip joint. They were placed in separate jars of fixative and labelled with the number of the mouse and the side from which they came. Further processing of the tissues was then carried out according to the Schedule detailed in Appendix No. 4.

c. The remainder of the animal was examined for any naked eye abnormality and suspicious lesions taken for histological examination.

Figure 46.



The hind legs of a mouse 16 weeks after implantation with embryo tissue and 3.4:9.10 Dibenzopyrene. This photograph taken by ultra violet light shows fluorescence in the left leg at the centre of a tumour. In the right leg fluorescence shows through the undisturbed host leg muscle.

Results of Homologous Embryo Tissue Implants.

As a preliminary control investigation a wide variety of embryo tissues were implanted in order to determine if there was any selectivity in the ability of the Balb/C strain to accept embryo material. A few animals were killed at varying time intervals to see how soon it was possible to detect that the implants were viable. This was found to be as early as two weeks but little growth in size of the implants occurred under 6 weeks. The results for those implants which were allowed to survive for 16 weeks are shown in Table 12.

Table 12.

Embryo Tissue Implanted	No. of Implants made.	No of Implants growing at 16/52.
Skin	3	3
Lung	4	4
Stomach	3	3
Bladder	4	4
Kidney	5	2
Liver	4	2*
Thymus	3	3
Adrenal	3	2
Heart	1	0
Eye	2	1
Brain	3	0

* The surviving tissue appeared to be of Bile duct origin.

Two principle features were noted on the microscopic examination of these implanted tissues.

a. A complete lack of reaction around the implant on the part of the host animal which resulted in the appearance of a well tolerated but abnormally placed tissue growing in the leg muscles.

b. Active growth and differentiation on the part of the embryo tissues themselves with formation of an adult type of histological pattern only modified by the physical constraint of being in the deep intra muscular site.

The appearance of some of these untreated embryo tissue implants after 16 weeks in the host animals is shown in Figures 47 to 59.

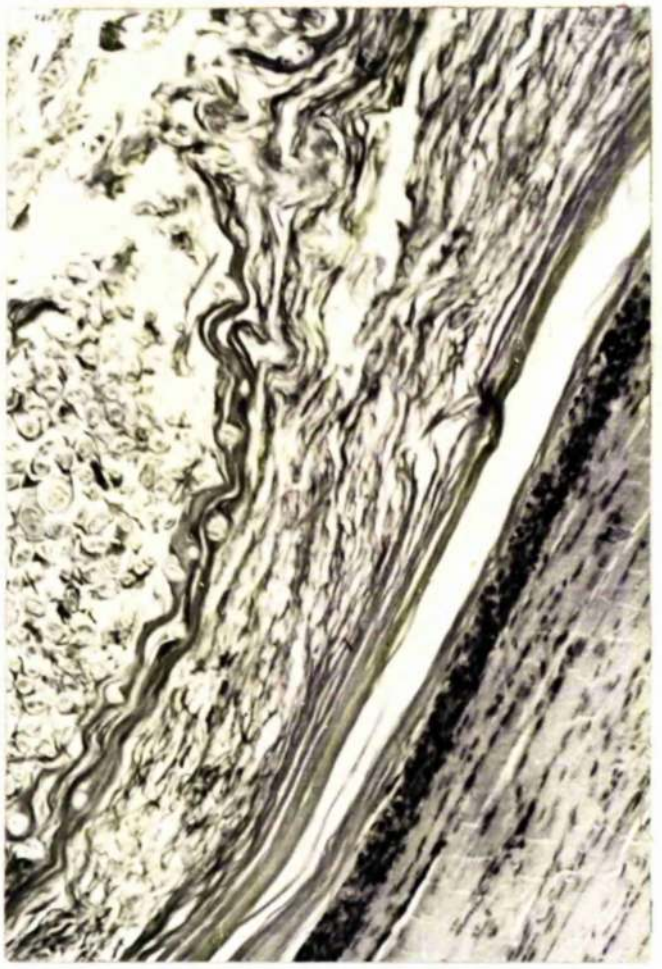
Figure 47.

Haematoxylin and Eosin. X 120

Control implant of skin showing differentiation from the foetal pattern to form hair and keratin. Note the absence of reaction to the skin on the part of the host tissues.

Figure 46.

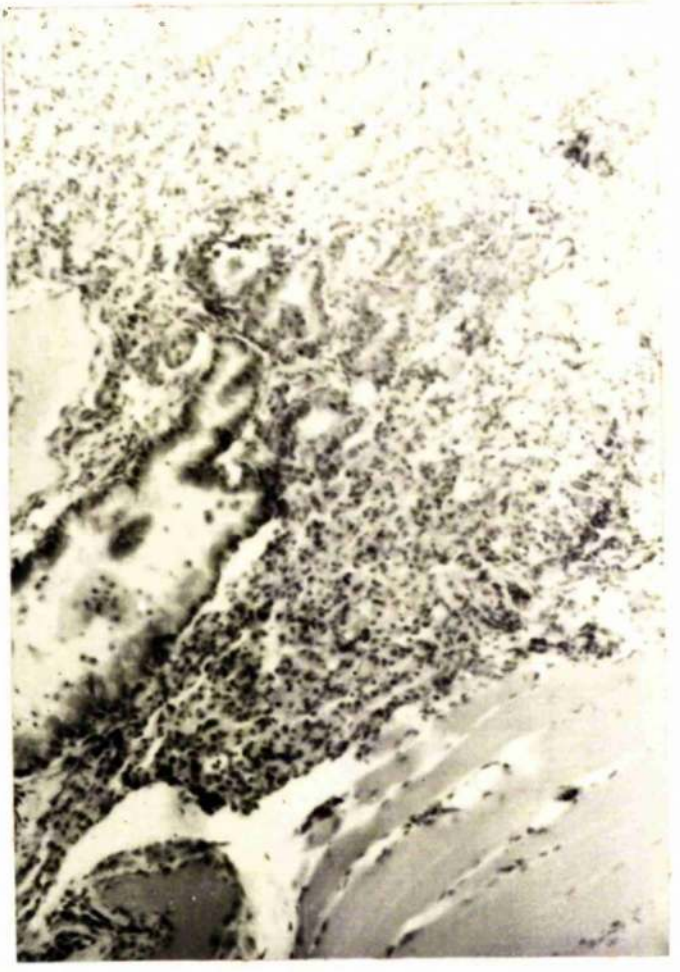
Haematoxylin and Eosin. X 120



Control embryo skin implant showing laminated keratin due to desquamation over 16 weeks to fill cyst cavity. Hair shafts in cross section are also seen deeper in the cyst.

Figure 49.

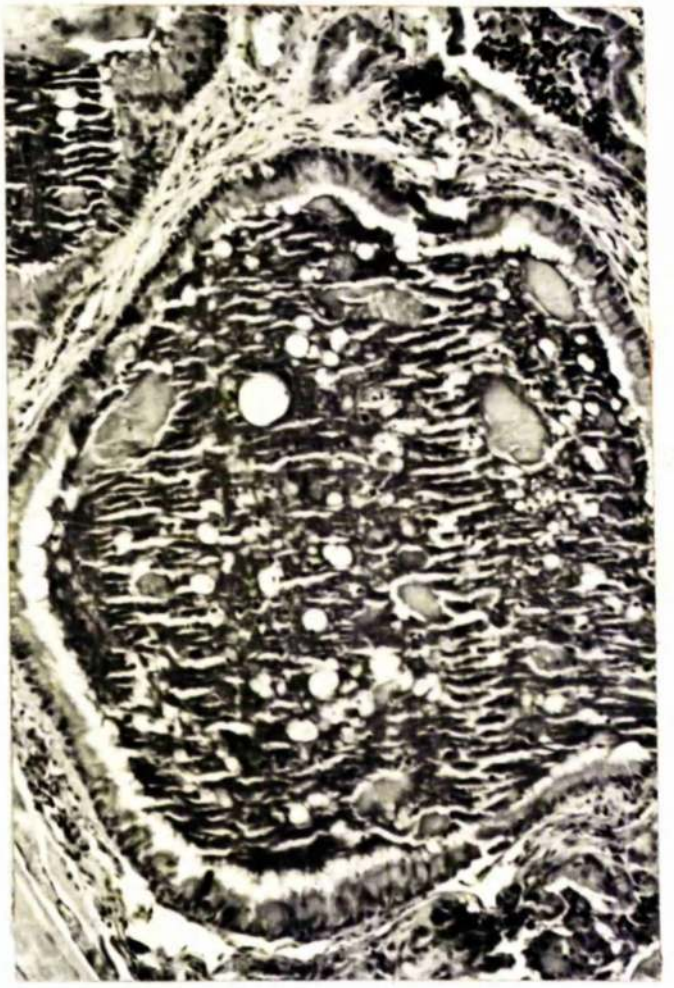
Haematoxylin and Eosin. X 120.



Control Embryo lung implant. Well formed bronchi are seen surrounded by unexpanded alveolar tissue.

Figure 30.

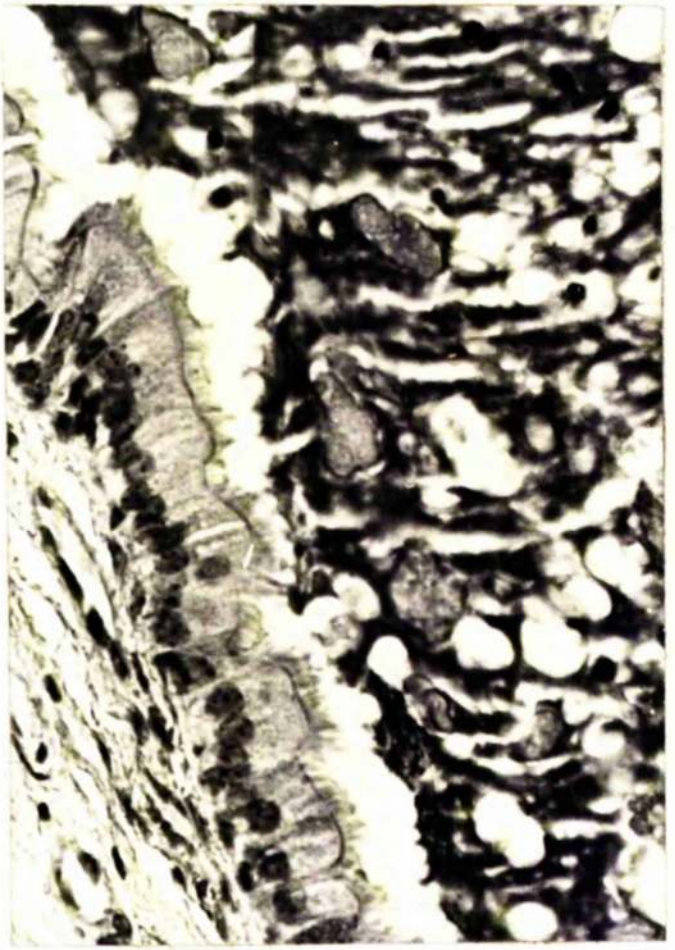
Haematoxylin and Eosin. X 120



Control embryo lung implant showing dilatation of a bronchus due to retained secretion.

Figure 51.

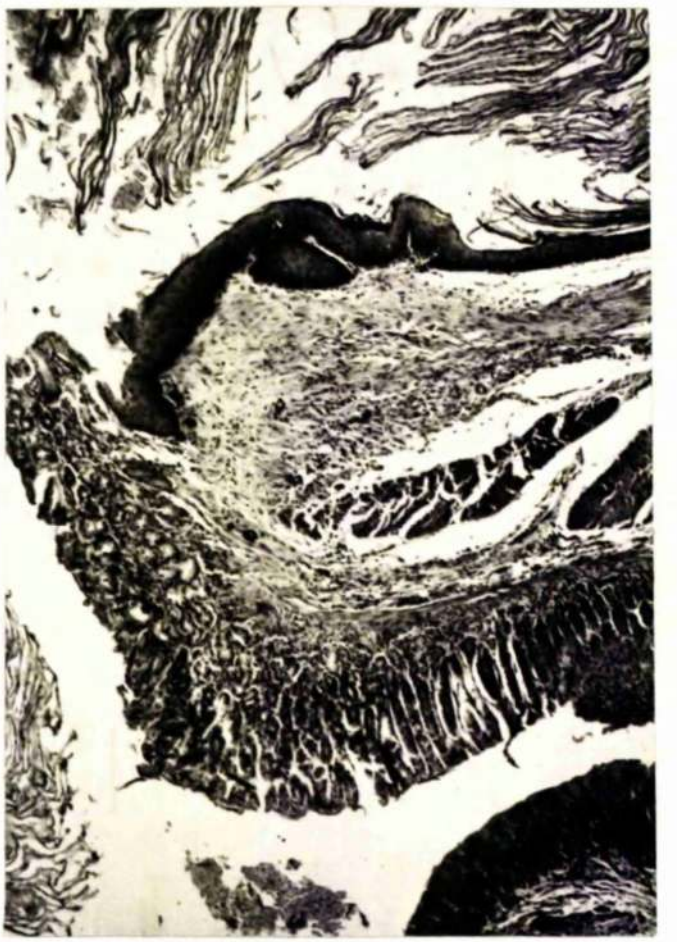
Haematoxylin and Eosin. X 480



Part of Figure 50 enlarged to show detail of ciliated columnar epithelium of bronchus. This demonstrates the retention of normal structure in these implants.

Figure 52.

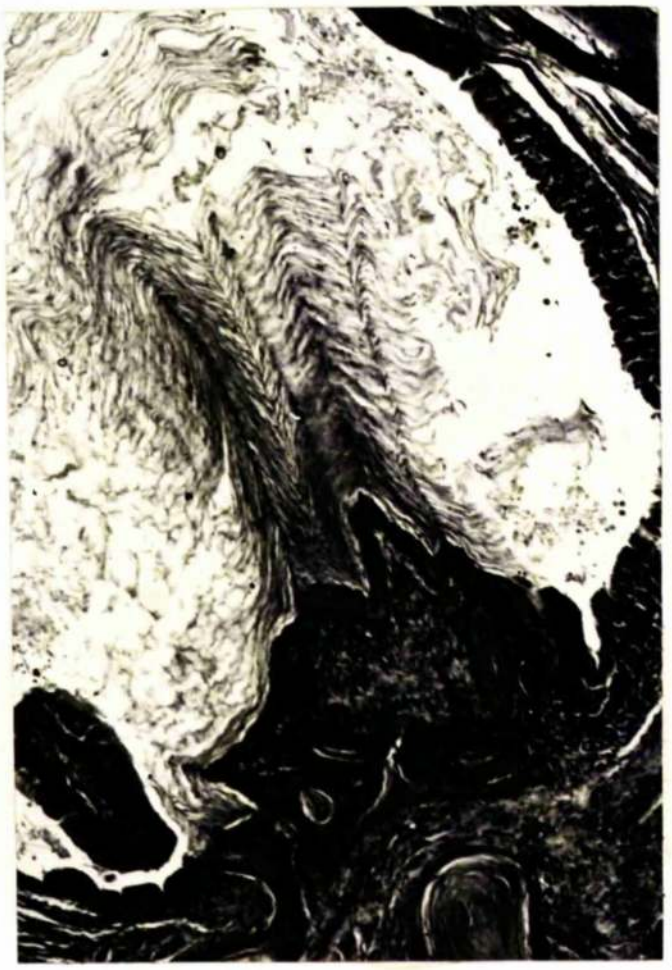
Haematoxylin and Eosin. X 48



Control stomach implant showing both types of gastric mucosa. The squamous epithelium of the fore stomach and the glandular epithelium of the body of the stomach.

Figure 53.

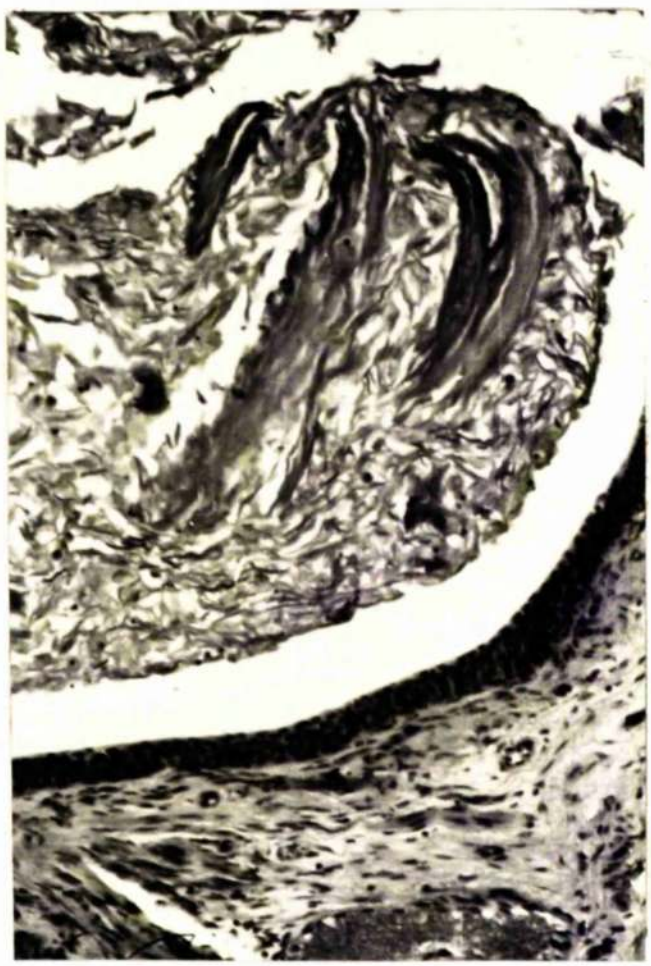
Haematoxylin and Eosin. X 48



Control stomach implant. With the passage of 16 weeks the successive layers of Keratin desquamated by the growing squamous epithelium have formed a laminated structure filling and dilating the cyst cavity.

Figure 54.

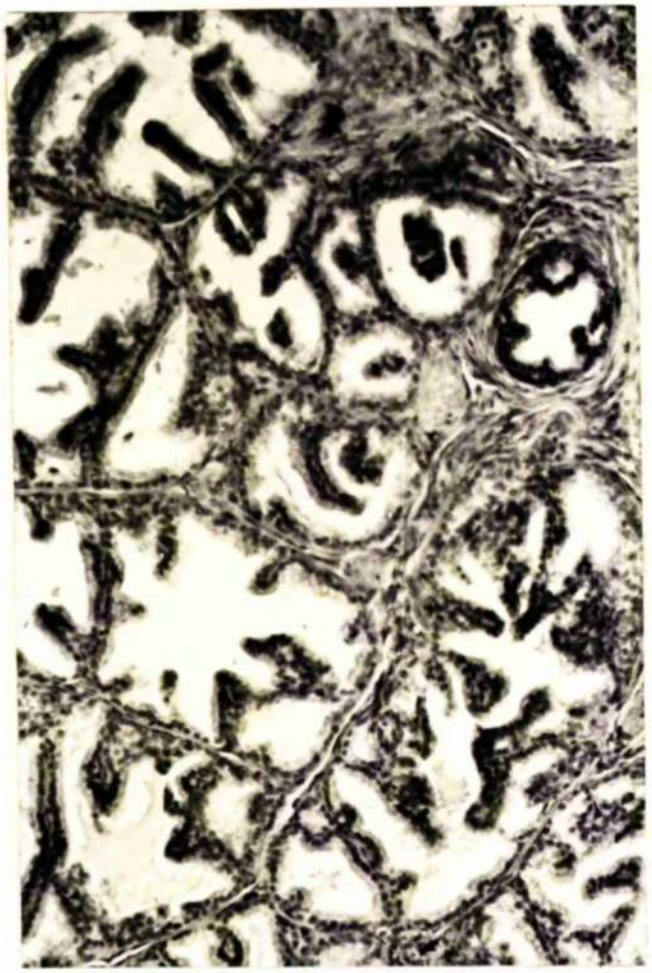
Haematoxylin and Eosin. X 48



Embryo bladder implant showing the filling of the cyst cavity with desquamated cellular debris. As with other implants there is no reaction on the part of the host tissues.

Figure 55.

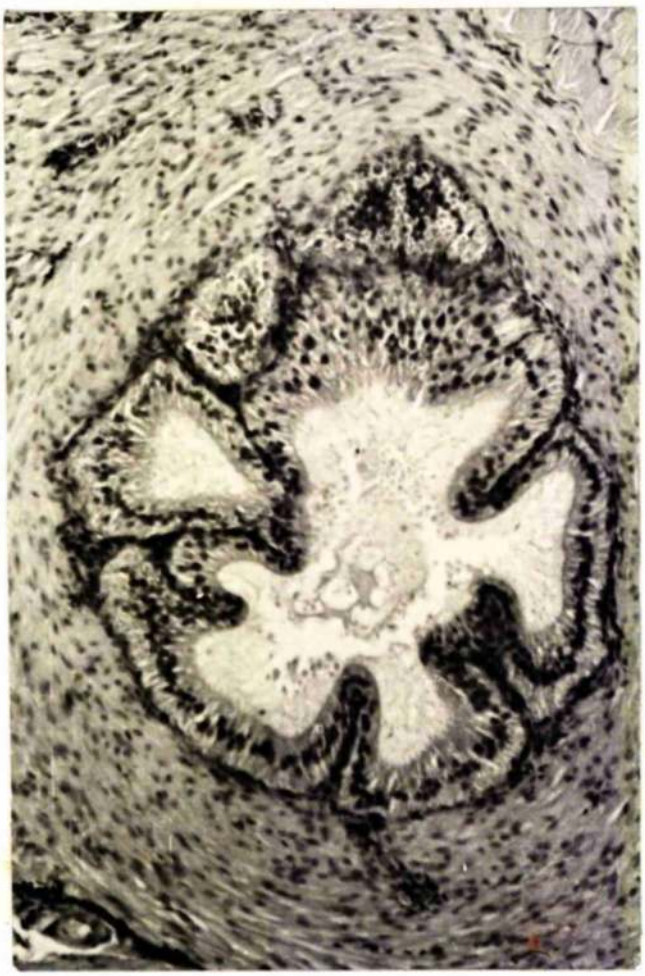
Haematoxylin and Eosin. X 120



Control embryo implant showing part of prostate gland implanted with a urinary bladder.

Figure 56.

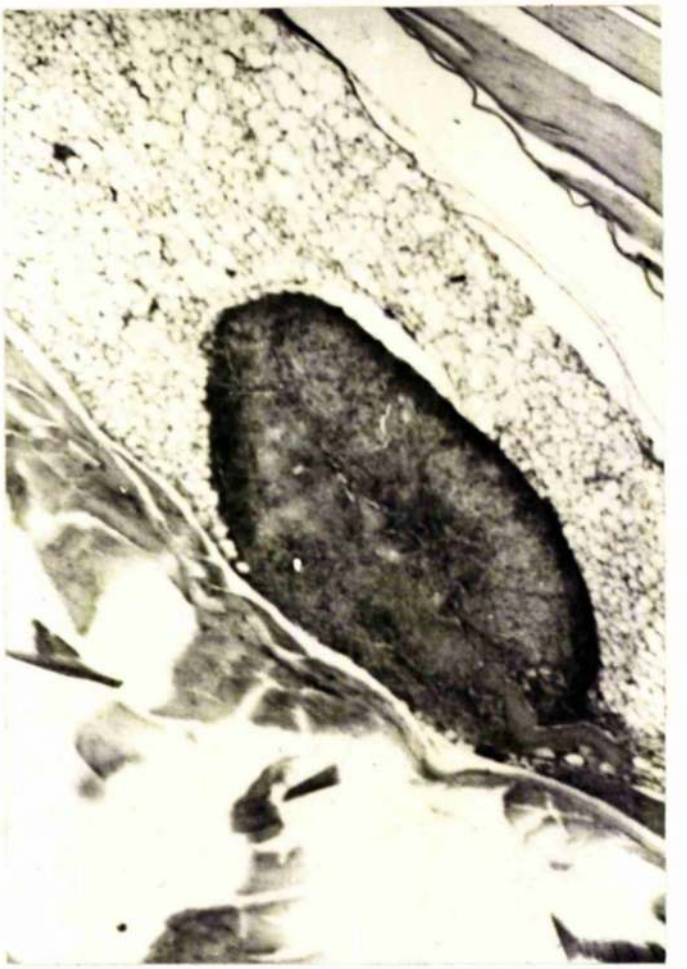
Haematoxylin and Eosin. X 120



Control embryo implant showing cross section of seminal vesicle found in association with a urinary bladder implant.

Figure 57.

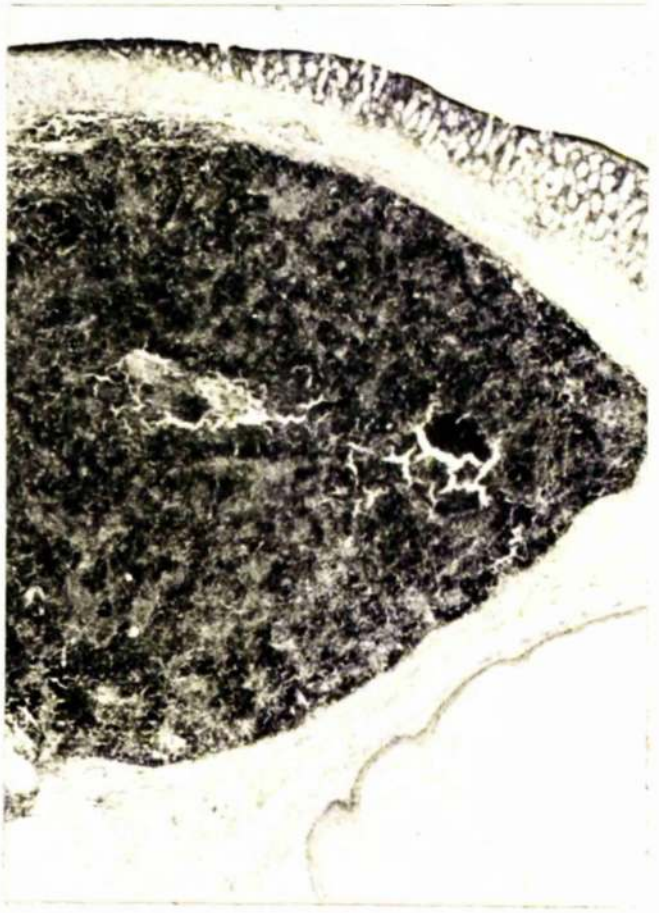
Haematoxylin and Eosin. X 120



Control embryo adrenal implant situated in fatty tissue between two muscle bundles of host. The structure of the gland is well maintained.

Figure 58.

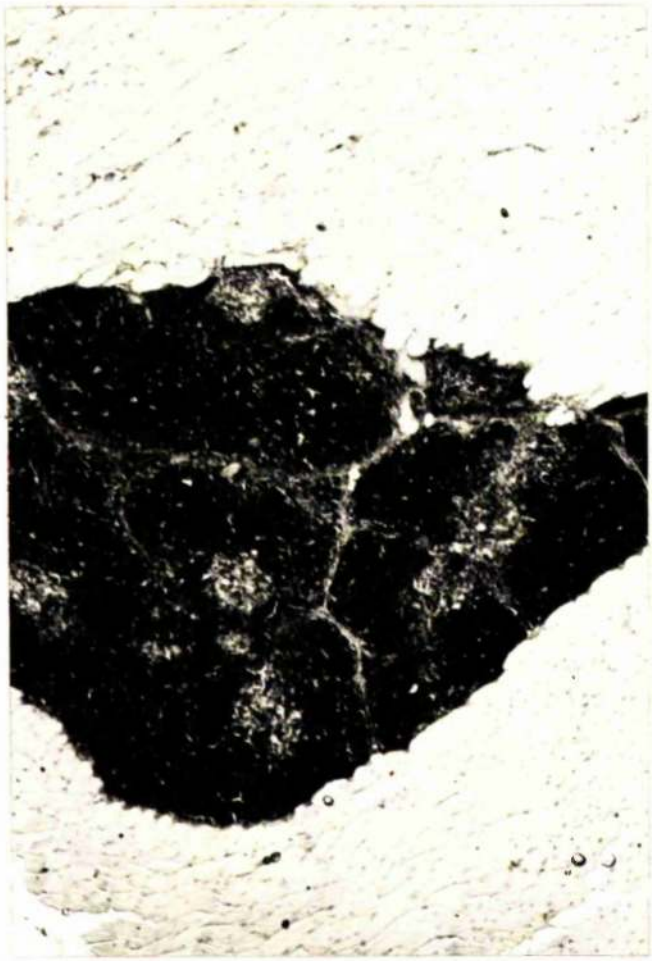
Haematoxylin and Eosin. X 120



Control embryo spleen implanted with stomach of which the glandular mucosa is seen above and the squamous lining below the spleen. Unless care is taken to remove the spleen it often accompanies stomach implants.

Figure 59.

Haematoxylin and Eosin. X 48



Control Embryo thymus implant showing well differentiated gland with normal architecture surrounded by host's muscle fibres.

Results of Heterologous Embryo Tissue Implants.

In view of the high percentage of successful implants with embryo tissues of Balb/C strain origin it was decided to test the possibility that this was due to some alteration of the immune mechanism in this strain which prevented the rejection of foreign tissue. This was done by making implants with embryos of other strains. In one group of 6 mice heterologous embryo implants were made in both hind legs.

In another group of 7 mice Balb/C embryo tissues were implanted on one side and heterologous embryo tissue in the other. Both experiments were terminated at six weeks as it had been previously established that viable implants would show evidence of differentiation by this time.

The results of these two experiments are summarized in Tables 13 and 14.

Table 13.

Implants of RIIIf/C_3H Hybrid Embryo Tissues alone.

Tissue.	No. of Implants made.	No. Surviving at 6 weeks.
Skin	3	Nil
Lung	3	Nil
Stomach	3	Nil
Bladder	3	Nil

Table 14.

Combined Implants of RIIIf/C_3H Hybrids and Balb/C Embryo Tissues.

Source of Embryo.	Tissues.	No. of Implants made.	No. Surviving at 6 weeks.	
Hybrid	Skin	2	Nil	Left legs.
"	Lung	2	Nil	
"	Stomach	2	Nil	
"	Bladder	1	Nil	
Balb/C	Skin	2	2	Right legs.
"	Lung	2	2	
"	Stomach	1	1	
"	Bladder	2	2	

Three conclusions can be drawn from these results.

a. Heterologous embryo tissues alone do not survive.

b. Heterologous embryo tissues do not survive in the presence of Homologous embryo tissue in the same host.

c. Heterologous embryo tissues do not inhibit the growth of Homologous embryo tissues in the same host.

Balb/C Embryo Tissue implants in Conjunction with the test series of polycyclic aromatic hydrocarbons.

The final form of the implant test method was that just described for the growth of control embryo implants with the following additional step.

Just prior to the implantation of the embryo tissue it was touched on to the surface of a single crystal or a small group of crystals of the hydrocarbon to be tested. Due to the residual surface moisture on the embryo tissue the crystals adhered closely to the implant and both could be inserted into the host leg muscle in close aposition to each other.

This technique was evolved when it was found that much smaller quantities of the hydrocarbon could be handled in this way than by trying to pick up crystals with fine forceps. By weighing it was estimated that the quantity of hydrocarbon used in each implant was never in excess of 150 ug. This is a much smaller quantity of material than is normally required for most accepted test methods.

In the first instance, Embryo Skin, Lung, Stomach and Bladder were exposed to each of the test hydrocarbons.

After the first series of experiments certain hydrocarbons were implanted in conjunction with other embryo tissues. 117 mice were used in these implant experiments. Of the 234 implants thus made, 38 were, for various reasons, not recovered at the termination of the experiments. The distribution of tumour incidence for the 196 successful implants is shown in Tables 15 and 16.

Table 15.

Code No.	Hydrocarbon.	Known Carcinogen.	Skin.	Lung.	Stomach.	Bladder.
1	Pyrene	No	0/3	1A/1 (1)	0/3 (1)	0/1
2	1.2 Benzopyrene	No	1/2	0/3 (1)	0/4	0/2
3	3.4 Benzopyrene	Yes	2/2 (1)	2/2 (1)	1/3	0/1 (2)
4	1.2:3.4 Dibenzopyrene	Yes	2/2	2A/3	1/3	1/3
5	1.2:4.5 Dibenzopyrene	Yes	1/5 (1)	1/2	0/1 (1)	0/4
6	1.2:6.7 Dibenzopyrene	Not tested	0/3	0/3	0/3	0/3
7	3.4:8.9 Dibenzopyrene	Yes	1/2	1A/2 (1)	0/2	0/1
8A	3.4:9.10 Dibenzopyrene	Yes	1/1 (2)	3(1A)/3	3/3	
8B	" "	"	3/3	3/3	3/3	2/2
9	Anthracene		0/4	0/3 (1)	0/2	0/1 (1)
10A	1.2 Benzanthracene	Doubtful	0/3	3A/3	0/2 (1)	0/3
10B	"	"	0/3	2A/3	0/2	0/1
10C	"	"	0/1 (2)	2A/2	0/2	0/2
11	1.2:3.4 Dibenzanthracene	No	0/2 (1)	3A/3 (1)	0/2	0/3
12	1.2:5.6 Dibenzanthracene	Yes	0/3	2+(3A)/5	5/5	2/5
13	1.2:7.8 Dibenzanthracene	Yes	0/1 (1)	2/2	0/3	(3)
14	Triphenylene	No	0/1 (1)	0/2	0/2	0/1 (1)
15	1.2:4.5:8.9 Tribenzopyrene	No	0/2 (1)	0/3	0/3	0/1 (2)

Figures give No. Implants with tumours / No. Implants recovered Numbers in () are of implants made but not recovered. A = Adenoma present.

(i) Three separate samples of H/C No.8 were tested only because there was some question as to whether the original specimen was free of impurities.

(ii) H/C No. 10 is an interesting substance in that it has been reported both as a carcinogen and as failing in attempts to produce tumours. It was felt that this might be due to the state of purity of the substance at the time of the test. To try and clarify this the three specimens used, were obtained as follows:

A) Pure material as obtained from a commercial source.
B) A re-purified sample of specimen A) used immediately to avoid oxidation.
C) Part of B) left to oxidise in the air on the bench in an unsealed container for 8 weeks.

It is of interest to note that all three specimens give comparable results.

Table 16.

Code No.	Hydrocarbon.	Known Carcinogen.	Kidney	Liver	Spleen	Thymus	Adrenal	Eye	Brain	Heart
6	1.2:6.7 Dibenzopyrene	Not tested	(1)	(1)		0/1		0/1		(1)
7	3.4:8.9 Dibenzopyrene	Yes	0/1	1/1	0/1		0/1			
8A	3.4:9.10 Dibenzopyrene	Yes							2/2 (1)	
8C	" "	Yes	2/2		1/1	1/1 (2)		1/2	1/2 (1)	(1)
10B	1.2 Benzanthracene	Doubtful	(1)							
10C	" "	"	0/3							
14	Triphenylene	No					0/1 (1)			
15	1.2:4.5:8.9 Tribenzopyrene	No	0/1					0/1		

Figures give No. Implants with tumours / No. Implants recovered.

Numbers in () are of implants made but not recovered.

Results.

Under the conditions of this test procedure there is exact correlation between the carcinogenic activity of the test series of hydrocarbons and their previously reported behaviour.

All the known carcinogens induced tumours in more than one type of embryo tissue, the non-carcinogens did not induce any tumours. There were therefore no false positive or false negative results. The previously untested 1 2: 6 7 Dibenzopyrene did not induce any tumours.

Positive results were recorded only when a histologically recognizable carcinoma or sarcoma was present, arising in the embryo implant. It will be remembered that at this time the hydrocarbons were only identifiable by a code number.

In some of the lung implants adenomas were present. These were recorded separately and not considered as proof of carcinogenic activity by the substance under test.

The distribution of tumours and adenomas in embryo implants with regard to exposure to each of the fifteen test hydrocarbons is shown in Table 17.

Table 17.

Incidence of Tumours in Embryo Implants Exposed to Hydrocarbons.

Hydrocarbon.	No. of Implants made.	No. of Implants recovered.	Tumour Incidence.
1	10	8	1A/8
2	12	11	0/11
3	12	8	5/8
4	11	11	6/11
5	14	12	2/12
6	18	15	0/15
7	12	11	3/8
8	35	28	26/28
9	12	10	0/10
10	34	30	7A/30
11	12	10	3A/10
12	20	19	9+3A/19
13	10	6	2/6
14	12	8	0/8
15	14	11	0/11

Histology:

a. Malignant Tumours. The majority of the tumours were squamous carcinomas showing variations in the degree of differentiation. The degree of differentiation did not seem to depend on the type of tissue implanted or the hydrocarbon under test. Invasion of surrounding host tissue was seen in most cases.

It is of particular interest that this type of tumour should arise in lung implants as it is not recorded among the spontaneously occurring tumours of lung in mice.

Examination of lung implants exposed to carcinogens, which have not developed tumours, shows alteration of the mucosa of the bronchi in some cases. There is a loss of the normal ciliated columnar epithelium which is replaced by a simpler type of epithelium of a squamous type. Although prickle cells were not clearly seen it can reasonably be said that the changes resemble those of squamous metaplasia as seen in human material.

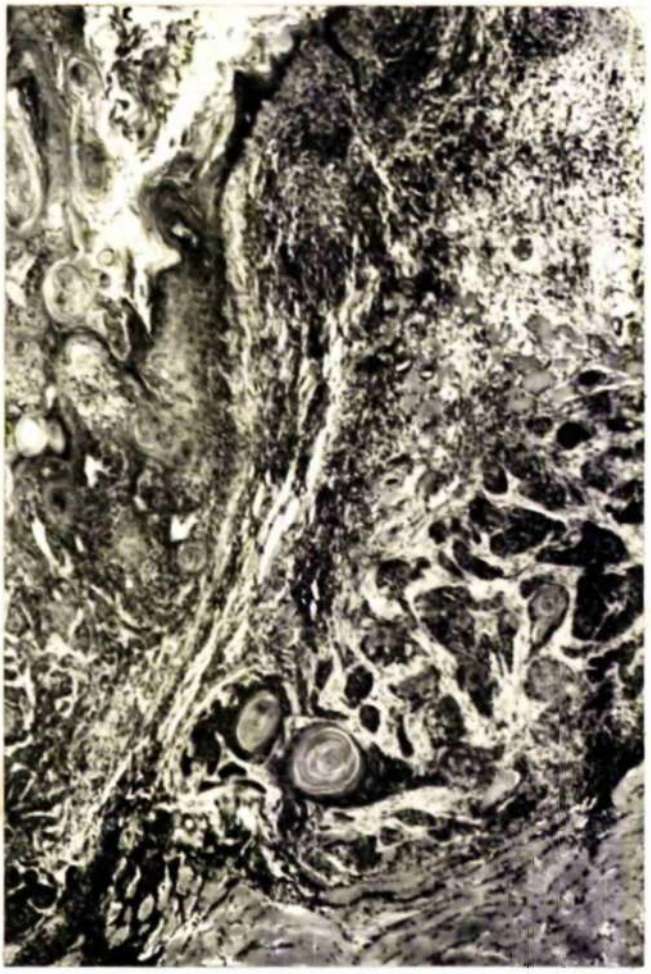
In lung implants with squamous carcinomas areas of metaplasia of this kind are seen (see Fig. 65) and it would seem that this is an intermediate stage in the development of these tumours in the embryo lung tissue.

In a small number of cases a spindle cell tumour was present, either alone or in conjunction with a squamous carcinoma. This type of tumour may be a sarcoma or a very anaplastic carcinoma. It is more likely that these are in fact sarcomas but it is not clear whether they arise solely in the connective tissue of the embryo implant or if the host connective tissue is also affected. The majority of these sarcomas occurred where a tumour was found at the implantation site but little or no survival of the normal embryo tissue implanted was seen.

Examples of the tumours induced by the various hydrocarbons in the different embryo implants are shown in Figures 60 to 75.

Figure 60.

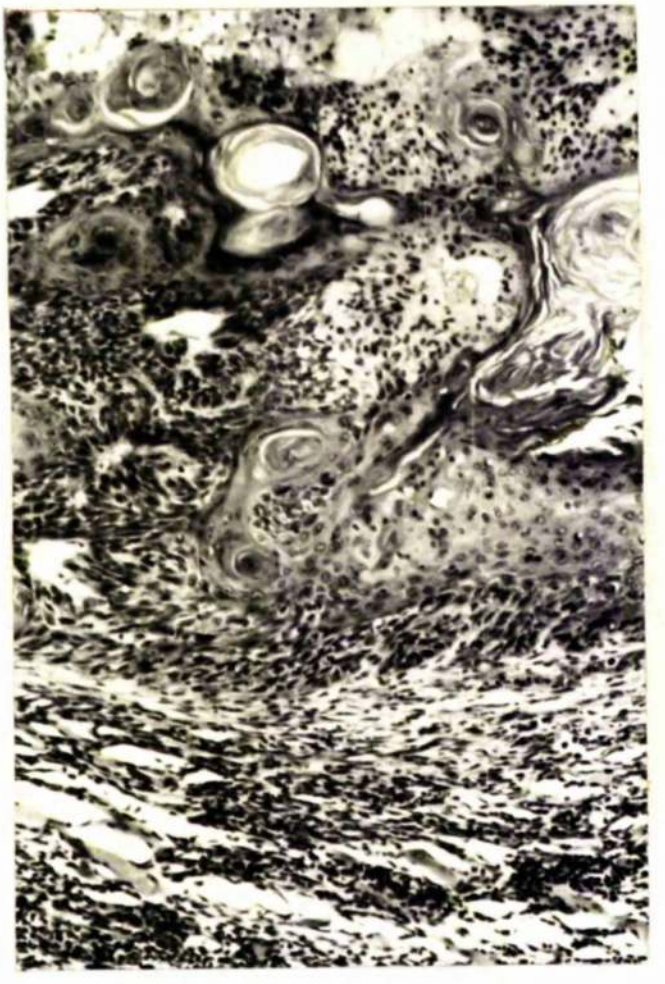
Haematoxylin and Eosin. X 48



Embryo skin implant showing an invasive squamous carcinoma infiltrating the host tissue. Implant exposed to 3.4:9.10 Dibenzopyrene.

Figure 61.

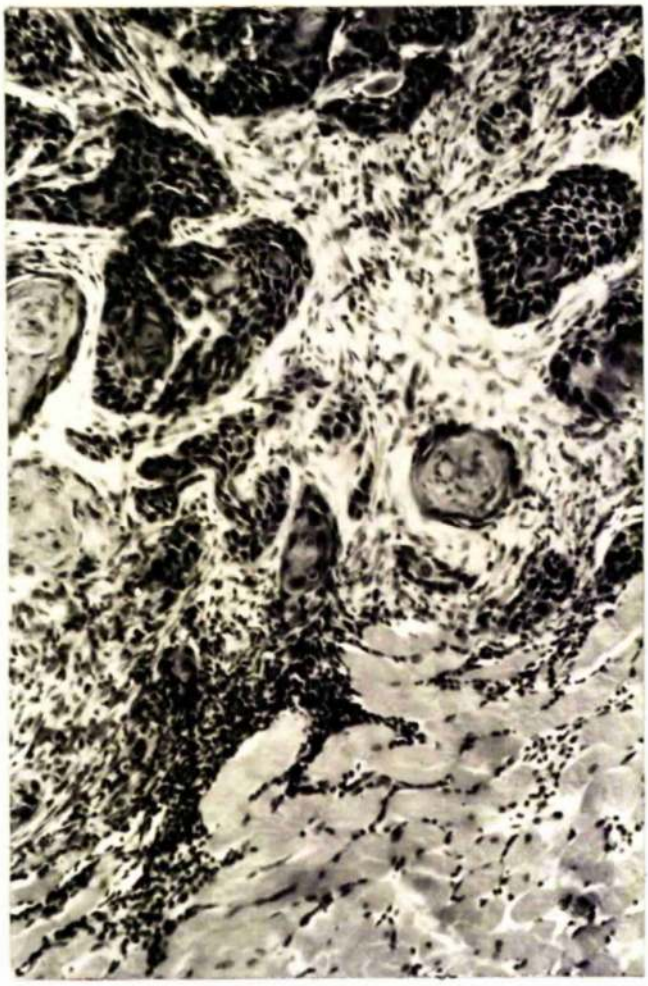
Haematoxylin and Eosin. X 120



Part of Figure 60 enlarged to show well formed epithelial pearls. The carcinoma is arising from the cyst lining which is seen at the centre of the picture.

Figure 62.

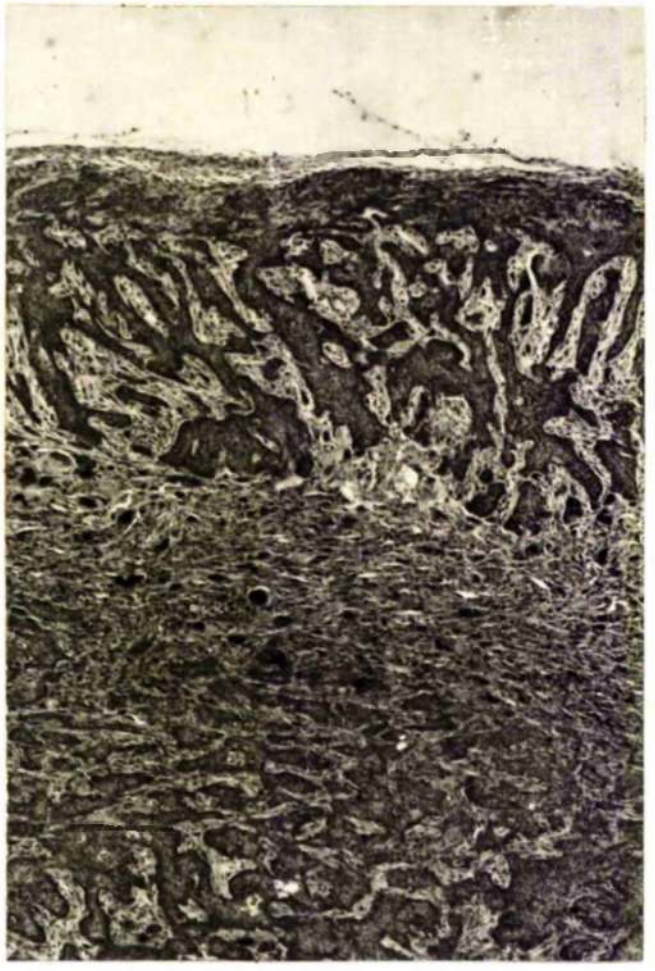
Haematoxylin and Eosin. X 120



Another field from Figure 60 enlarged to show early invasion of the host tissues. In this area the tumour is less well differentiated.

Figure 63.

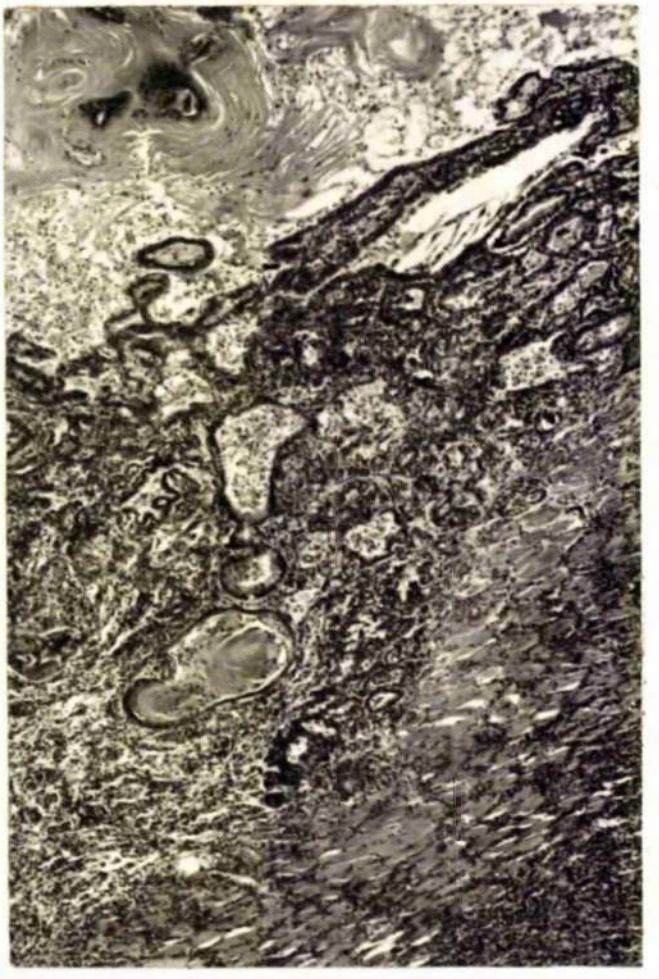
Haematoxylin and Eosin. X 48



Embryo skin implant showing a very extensively invading carcinoma arising from the epithelium of the cyst. In the mid zone there are many tumour giant cells present.
Implant exposed to 1.2:4.5 Dibenzopyrene.

Figure 64.

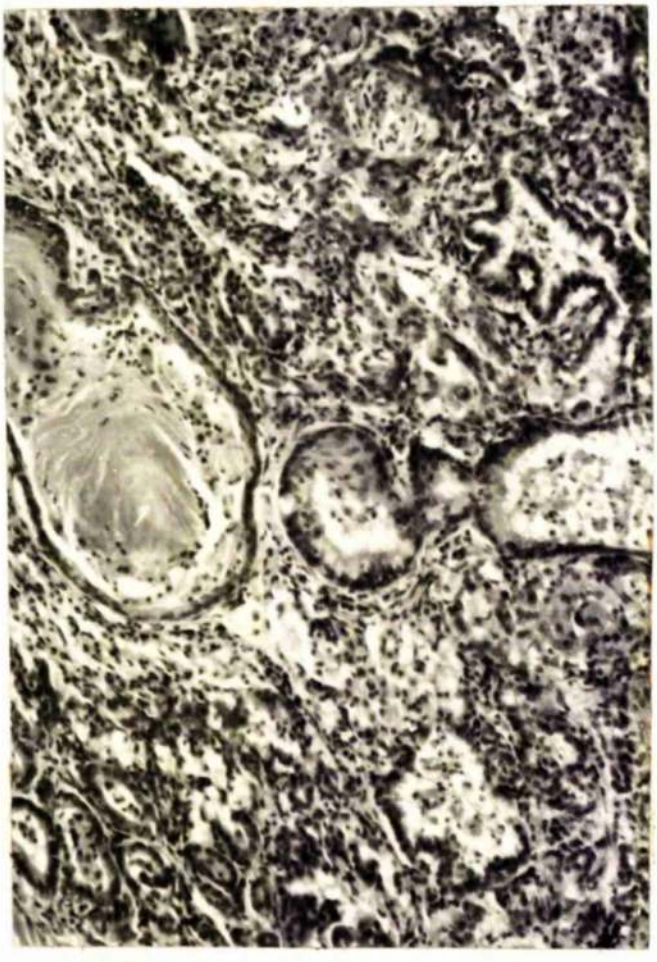
Haematoxylin and Eosin. X 48



Embryo lung implant showing a well differentiated carcinoma arising from clearly identifiable lung tissue and invading the host muscle.
Implant exposed to 3.4:9.10 Dibenzopyrene.

Figure 65.

Haematoxylin and Eosin. X 120



Part of Figure 64 enlarged to show three bronchi. In the central one there is squamous metaplasia affecting half the circumference. In the left hand one there is much accumulated keratin. This seems to be one stage in the development of squamous carcinoma in lung implants.

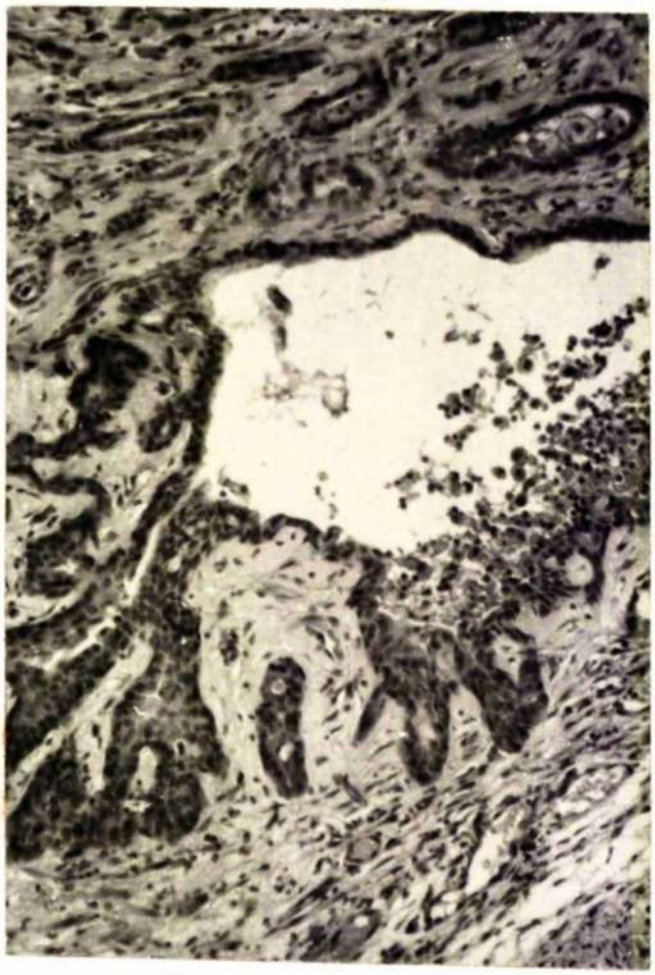
Figure 66.

Haematoxylin and Eosin. X 48

Embryo lung implant showing the origin of a carcinoma from the wall of a bronchus with local invasion. Normal lung is seen above adjacent to the host leg muscle. Implant exposed to 3.4:9.10 Dibenzopyrene.

Figure 67.

Haematoxylin and Eosin. X 120



Enlargement of part of Figure 66 to show the tumour arising from the wall of a bronchus.

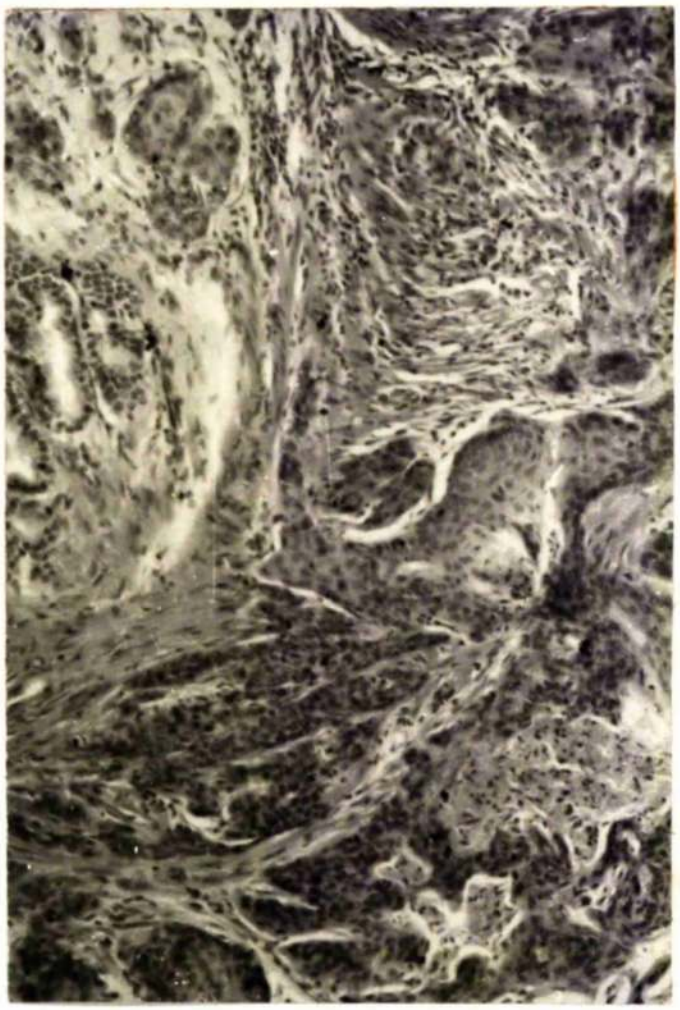
Figure 68.

Haematoxylin and Eosin. X 48.

Embryo stomach implant showing a moderately well differentiated squamous carcinoma. Normal epithelial lining of both types is seen at the edges of the tumour. Implant exposed to 1.2 : 5.6 dibenzanthracene.

Figure 69.

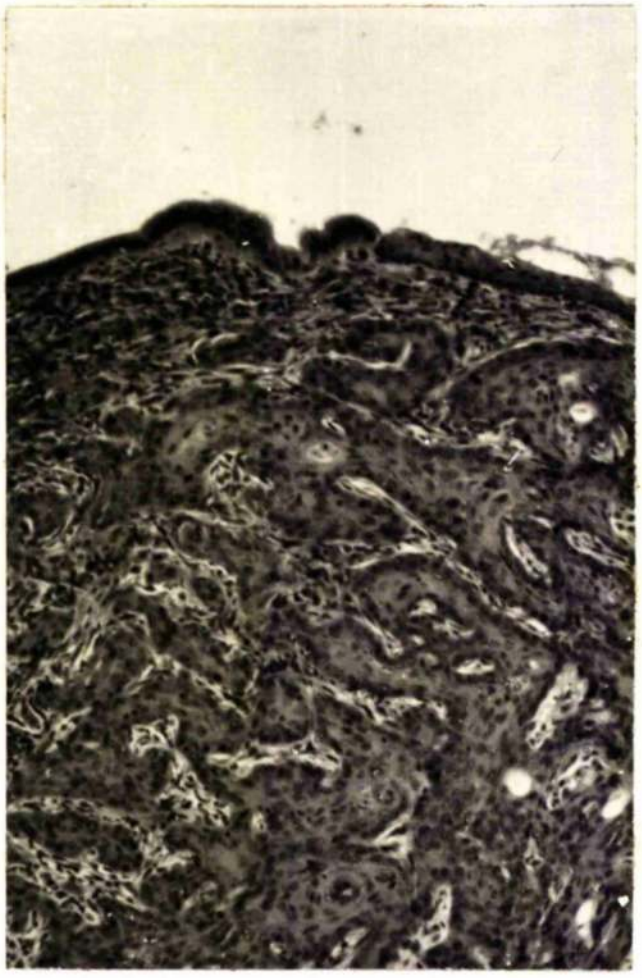
Haematoxylin and Eosin. X 120



Embryo stomach implant showing the presence of a squamous carcinoma with normal glandular mucosa present enclosed by columns of tumour cells.
Implant exposed to 1.2:5.6 Dibenzanthracene.

Figure 70.

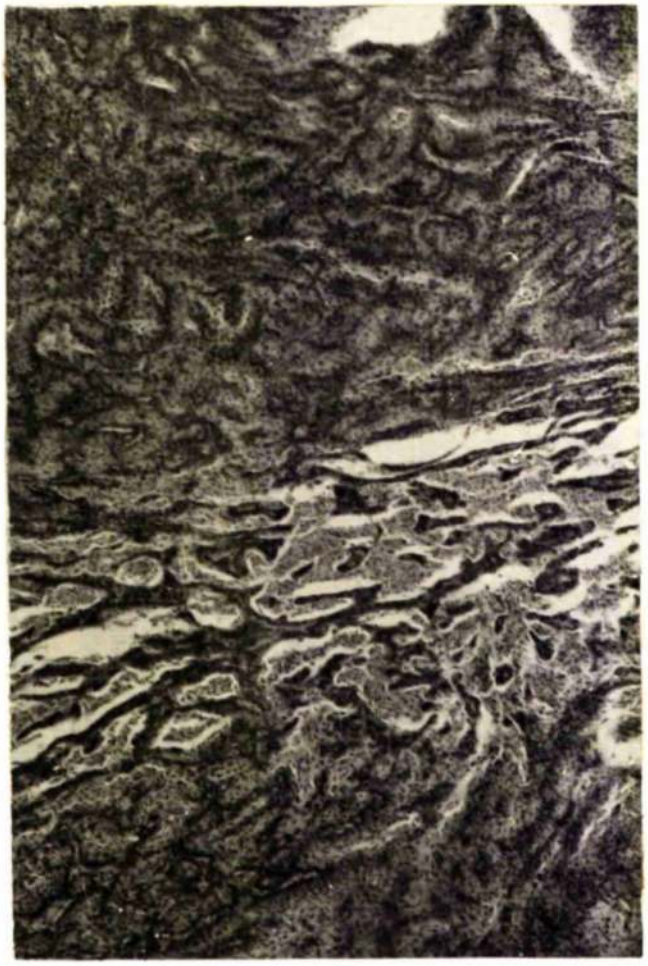
Haematoxylin and Eosin. X 120



Embryo stomach implant showing early squamous carcinoma invading the wall of the implant. The junction of squamous and glandular mucosa is clearly seen. Implant exposed to 3.4:9.10 Dibenzopyrene.

Figure 71.

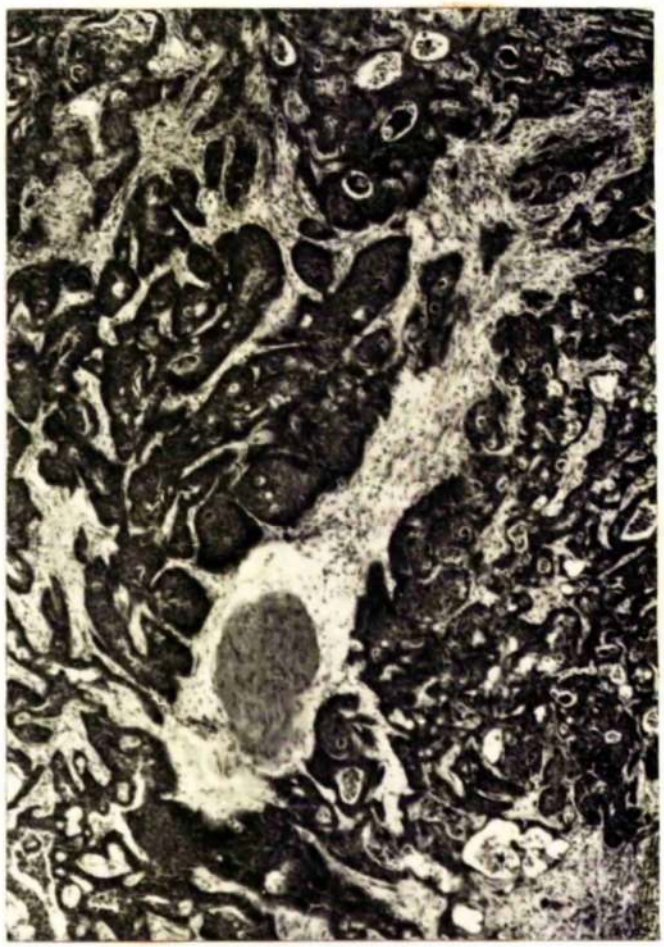
Haematoxylin and Eosin. X 48



Embryo bladder implant showing a rather solid adenocarcinoma which invaded widely into the host.
Implant exposed to 1.2:5.6 Dibenzanthracene.

Figure 72.

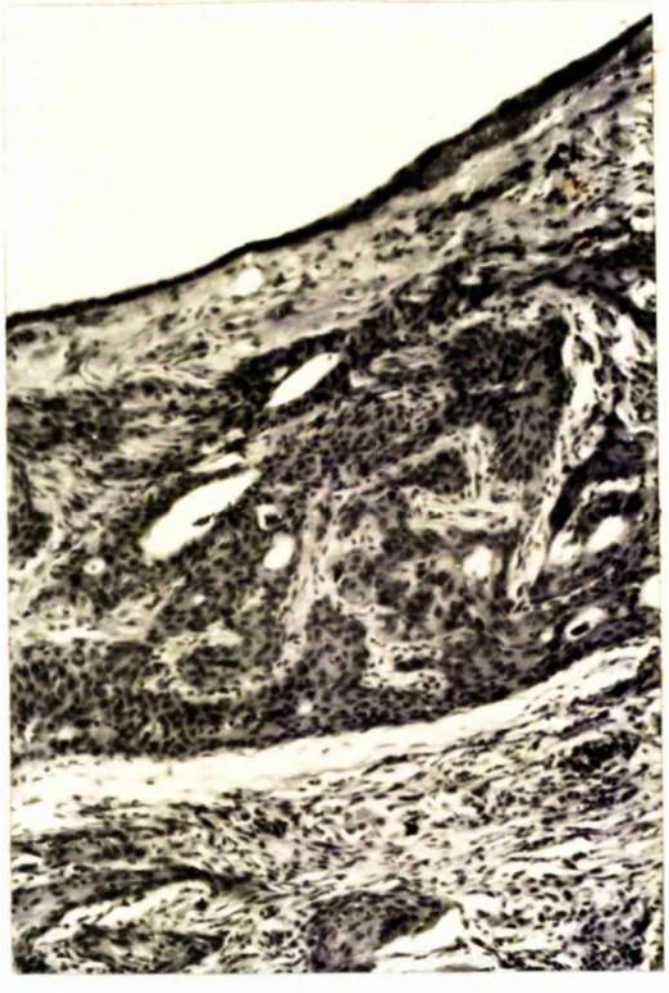
Haematoxylin and Eosin. X 48



Embryo bladder implant with a different type of tumour to that of Figure 71. This resembles more a poorly differentiated squamous carcinoma. It is in this tumour that the stromal changes shown in Figure 82 were seen. Implant exposed to 3.4:9.10 Dibenzopyrene.

Figure 73.

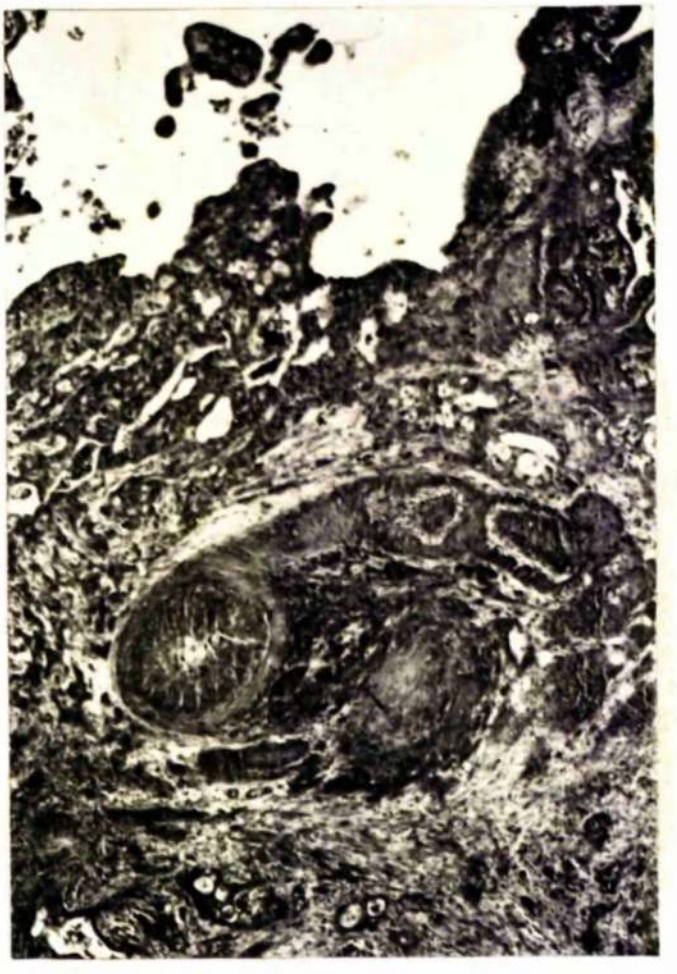
Haematoxylin and Eosin. X 120



Embryo bladder implant showing early carcinoma arising in the epithelium of the bladder and ivading locally.
Implant exposed to 3.4:9.10 Dibenzopyrene.

Figure 74.

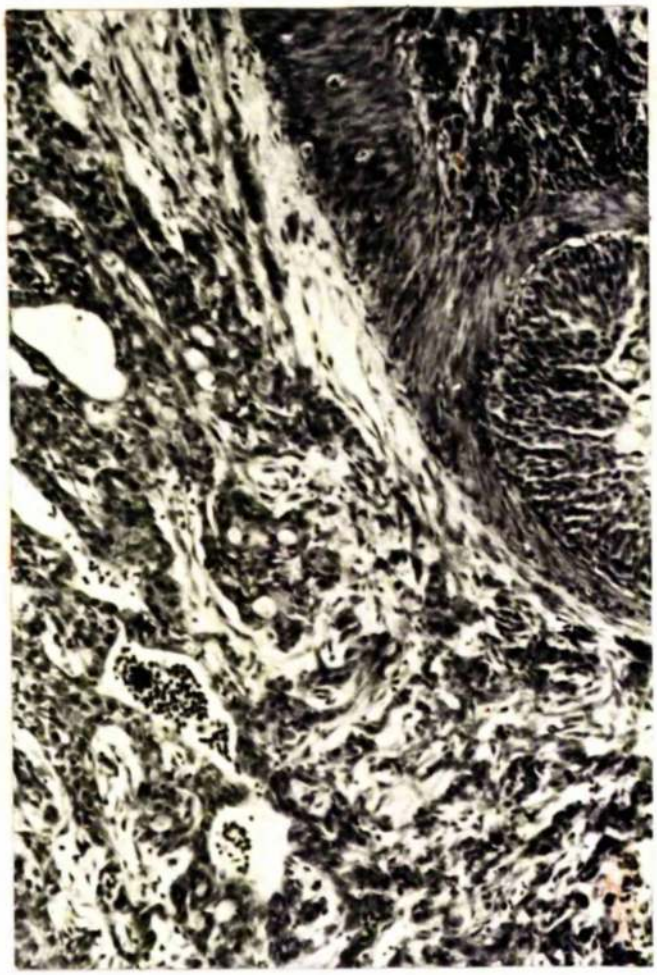
Haematoxylin and Eosin. X 48



Embryo kidney implant showing failure of survival of parenchyma but a rather anaplastic tumour developed from the ureteric pelvis is present and invading widely. The ureter is easily identified in cross section.
Implant exposed to 3.4:9.10 Dibenzopyrene.

Figure 75.

Haematoxylin and Eosin. X 120



Enlargement of part of Figure 74 showing poorly differentiated nature of the tumour and part of the ureter in which the mucosa appears hyperplastic.

b. Pulmonary Adenomas. Some of the lung implants showed the presence of adenomas of the type which occur spontaneously in adult mice. Both the solid and the papillary type of structure were seen. Figs. 76 and 77. Their appearance not being significantly altered by the confining intramuscular site, as will be seen by comparing these illustrations with Figures 32 to 36.

Adenomas occurred both as the only lesion in a lung implant and in conjunction with a squamous carcinoma. Because of their known spontaneous incidence in mice they were recorded separately.

It is of some interest that they should have arisen in lung tissue only six months old, as Shimkin (1955) states that they are not recorded in mice under a year old. In mice over that age he gives the incidence as between 10% and 30%, multiple lesions being infrequent.

That these tumours should have occurred in substantial numbers in the implantation experiment and also with injection of hydrocarbons in algenate suggests that they are related to the exposure of lung tissue to the action of the hydrocarbons.

However in neither set of experiments has their occurrence been included as positive evidence of carcinogenic activity.

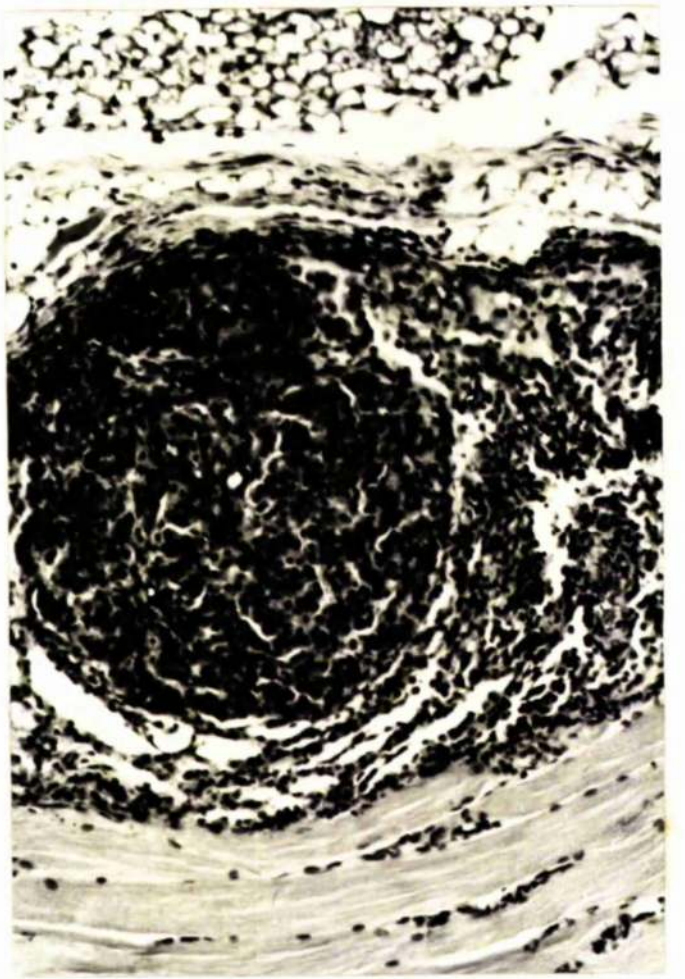
The distribution of adenomas in relation to the incidence of other tumours in lung implants is show in Table 18.

c. Papillomas. An occasional squamous papilloma was seen in some of the stomach implants. Fig. 78. In some instances this seems to have been a preliminary stage in the formation of a squamous carcinoma. Fig. 79.

d. Other Lesions. A stromal degenerative lesion was seen in some of the stomach and bladder implants (Figs. 80-82) which was not at first related to any other feature. It was then noticed that two bladder implants with well differentiated squamous carcinomas showed a similar appearance in small areas elsewhere in the implant. It would seem therefore that this may be a pre-malignant change although the exact nature of the lesion is not clear.

Figure 76.

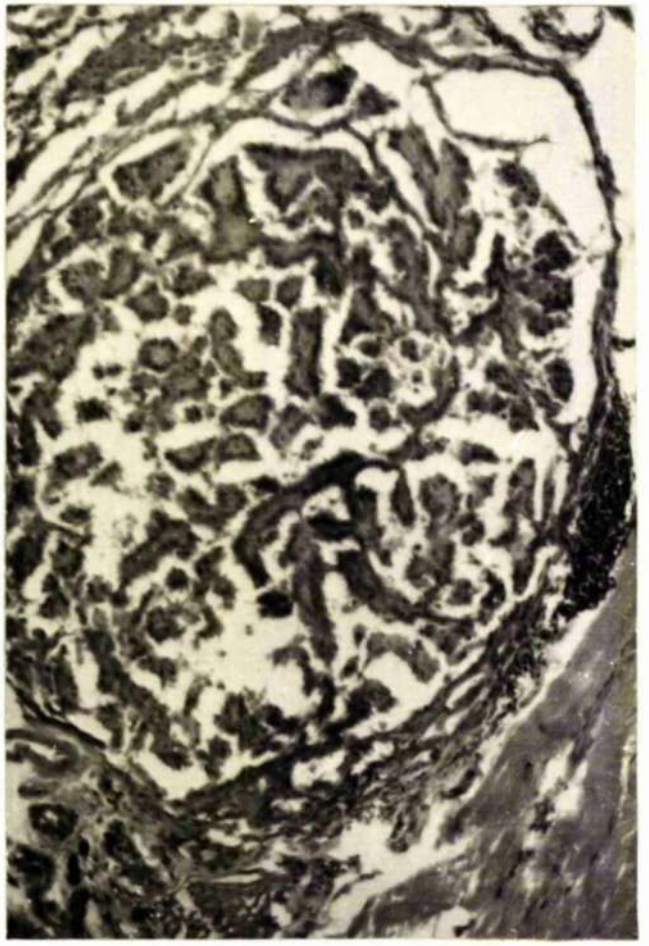
Haematoxylin and Eosin. X 48.



Lung embryo implant in which a solid type of adenoma has developed after exposure to 1.2:3.4 Dibenzanthracene.
Compare with Figures 32 and 34 (pages 111 and 113).

Figure 77.

Haematoxylin and Eosin. X 120



Lung implant showing a papillary type of adenoma after exposure to 1.2:3.4 Dibenzanthracene. Compare with Figure 35 (page 114).

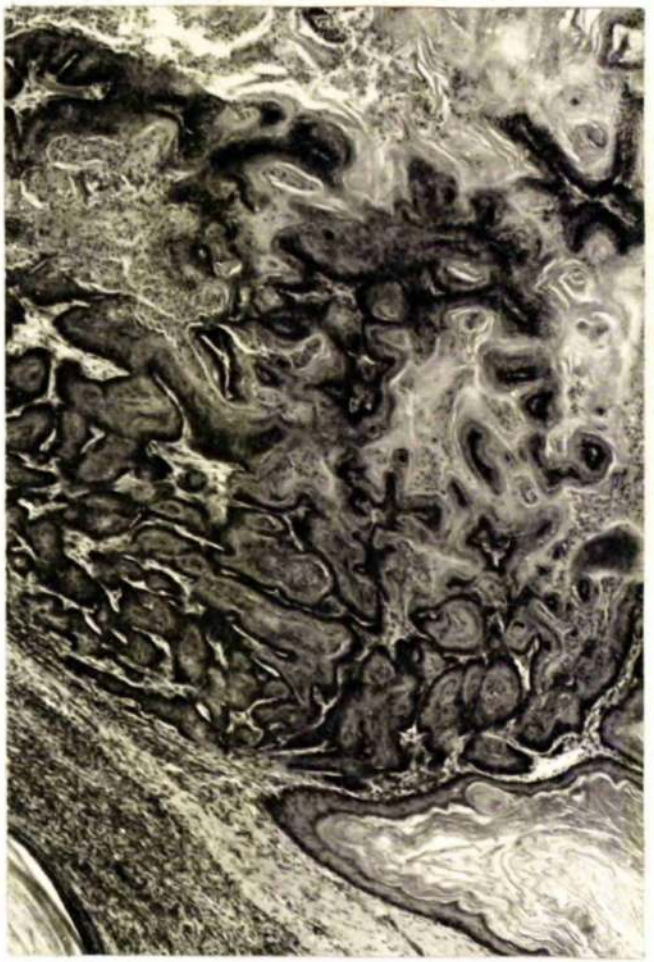
Table 18

Incidence of Adenomas in Embryo Lung Implants exposed to Hydrocarbons.

Hydrocarbon.	No. of Lung Implants.	No. of Implants with adenomas.	Incidence of adenomas.	Other tumours present.
1	1	1	1/1	No
4	3	2	2/3	Yes
7		1	1	Yes
8A	3	1	1/3	Yes
10A	3	3	3/3	No
10B	3 (8)	2 (7)	2/3 (7/8)	No
10C	2	2	2/2	No
11	3	3	3/3	No
12	6	3	3/6	No

Figure 78.

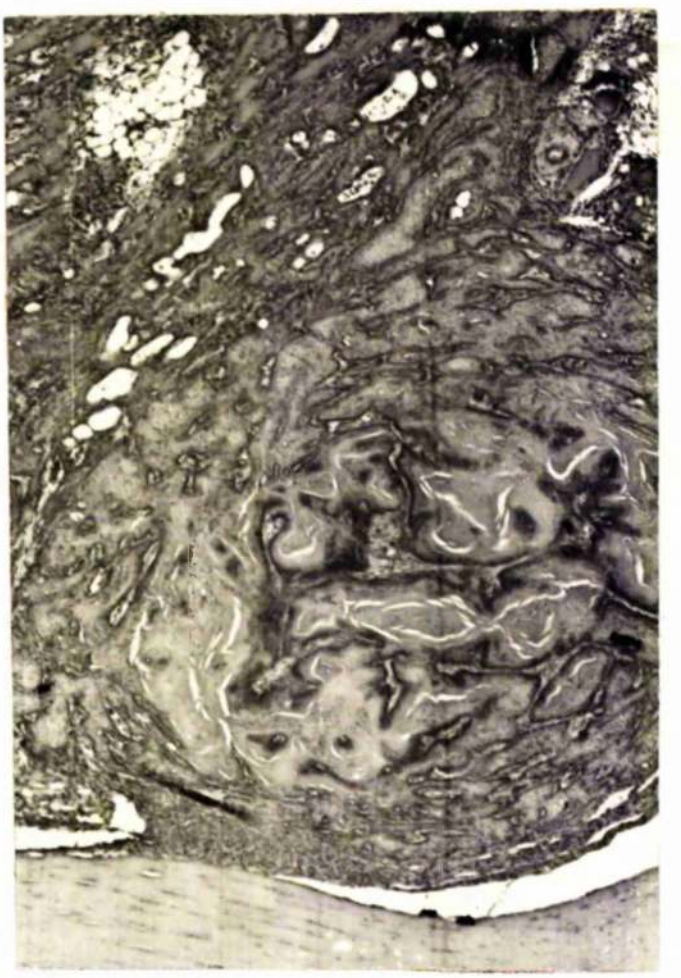
Haematoxylin and Eosin. X 48



Embryo stomach implant with a squamous papilloma arising from the fore stomach.
Normal squamous epithelium is present at the lower edge of the lesion.
Implant exposed to 3.4:9.10 Dibenzopyrene.
This implant also had a completely separate squamous carcinoma present.
See Figure 70.

Figure 79.

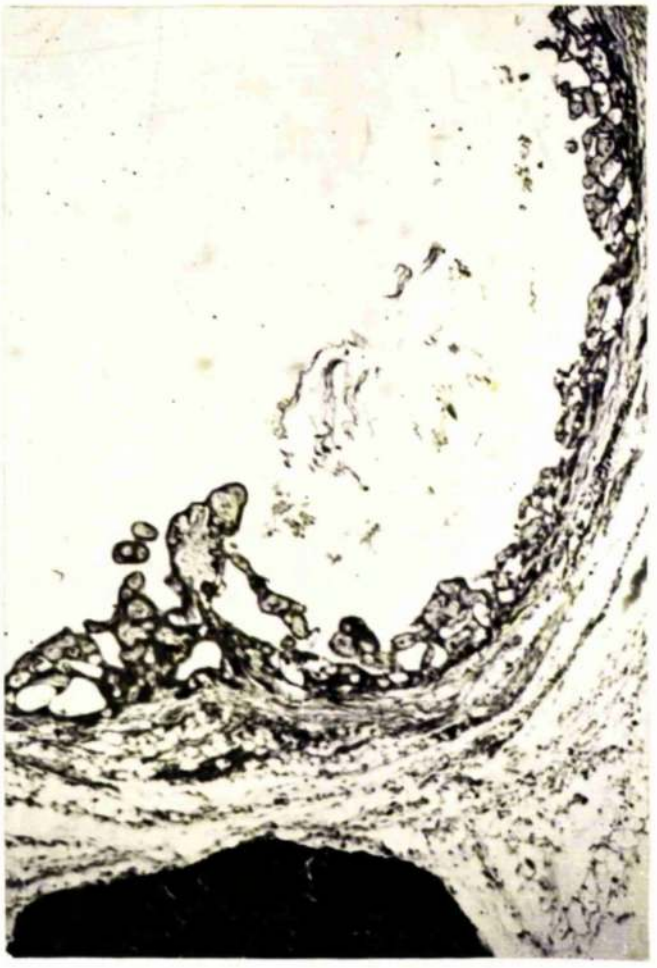
Haematoxylin and Eosin. X 48



This is a similar lesion to that in Figure 78 but in this case there has been further progression to form a frankly invasive squamous carcinoma as can be seen at the top of the picture. This implant was exposed to 3.4 Benzopyrene.

Figure 80.

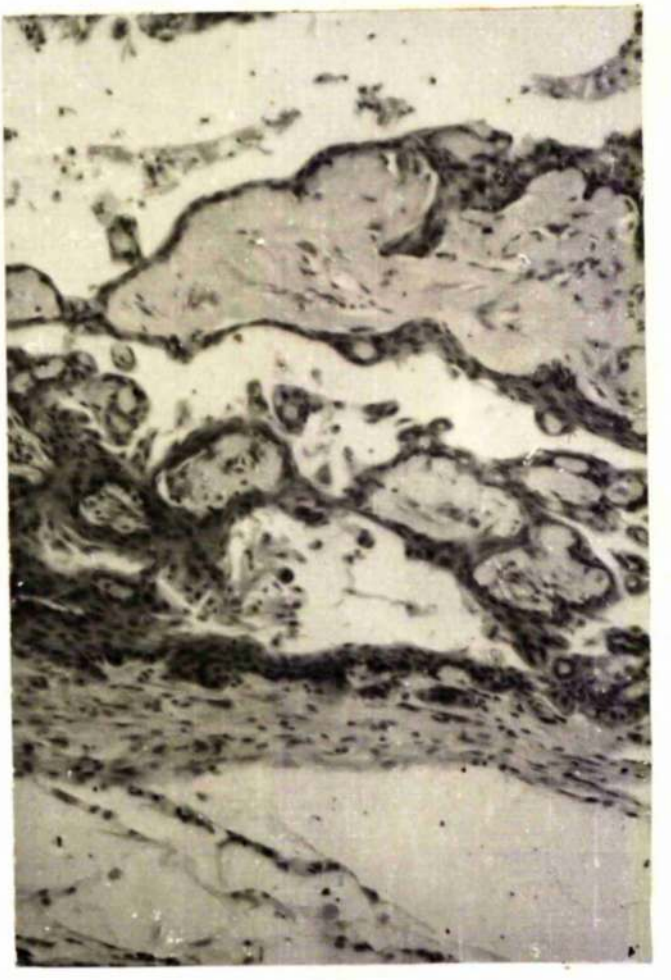
Haematoxylin and Eosin. X 48



Alteration in the squamous epithelium of a stomach implant exposed to 1.2:5.6 Dibenzanthracene. This appearance affected the whole of the fore stomach. Similar changes were seen in some implants which had developed a squamous carcinoma.

Figure 81.

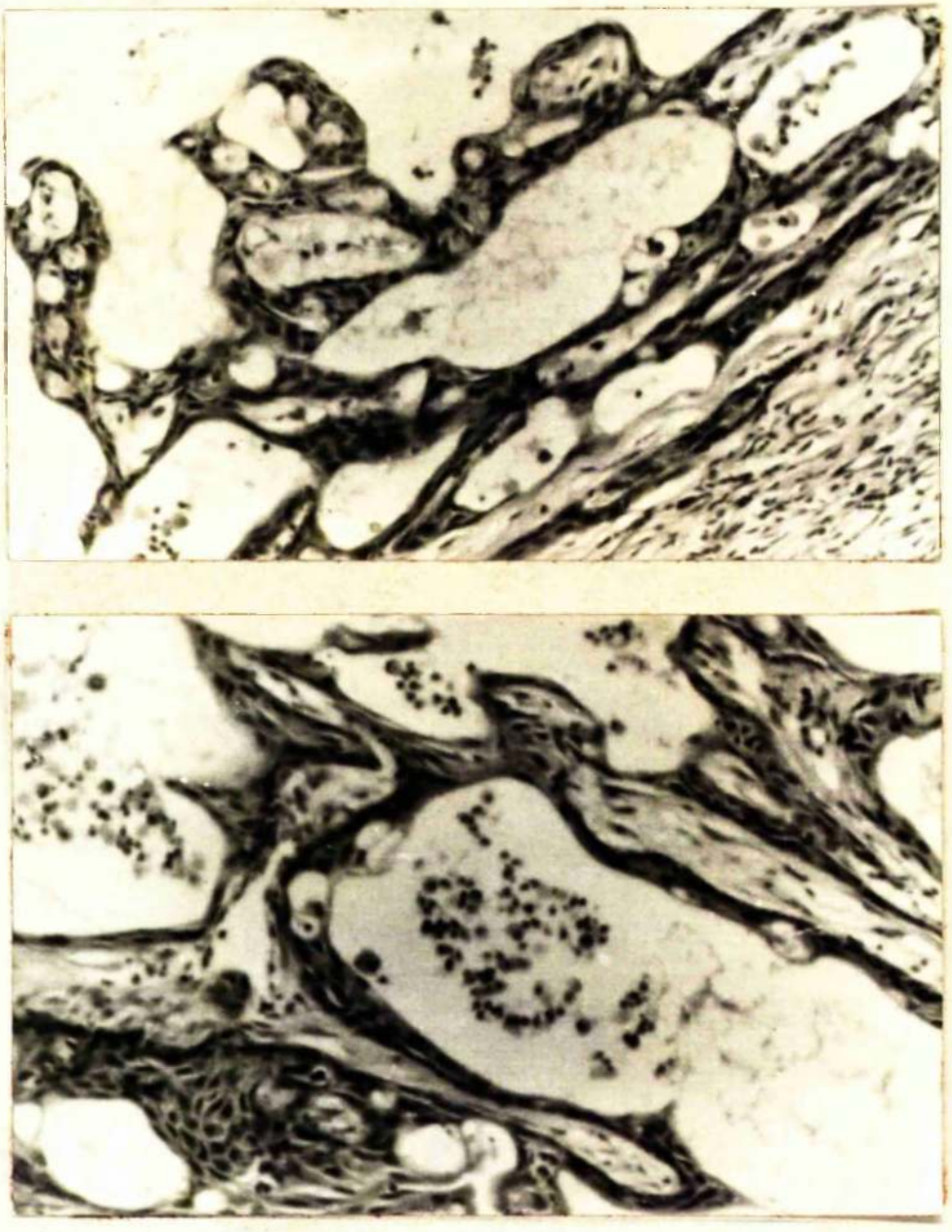
Haematoxylin and Eosin. X 120



An enlarged view of part of the same specimen as Figure 80 showing that the appearance seems to be due to an alteration of the supporting stroma rather than in the squamous epithelium itself.

Figure 82.

Haematoxylin and Eosin. X 120



Two separate fields are shown from an embryo bladder implant exposed to 3.4:9.10 Dibenzopyrene. The appearances are similar to those seen in Figs. 80 and 81.

Site of Action of Implanted Carcinogens.

Where a squamous carcinoma developed in these experiments there can be no doubt that the tissue from which the tumour arose was that of the embryo tissue implant. In the case of the anaplastic undifferentiated tumours there was the possibility that some at least were sarcomas arising from the host's connective tissue.

In order to test this hypothesis, hydrocarbon crystals alone were implanted into a number of host animals.

The substances used were 3 4: 9 10 Dibenzopyrene (No.8) which had shown itself to be the most potent carcinogen under the conditions of this experimental procedure and 1 2: 7 8 Dibenzanthracene (No. 13) which was weakly carcinogenic.

Later a non carcinogen 1 2: 4 5: 8 9 Tribenzopyrene (No. 15) was also implanted by itself for control purposes. Table 19 shows the results of these implants.

Table 19.

Hydrocarbon No.	No. of Implants.	No. of Tumours.
8	6	6
13	6	Nil
15	6	Nil

These results show that a potent carcinogen implanted for 16 weeks can induce a sarcoma in the host animal. Fig. 83. The weak carcinogen and the non-carcinogen both stimulated a foreign body giant cell reaction which in the case of Hydrocarbon 15 was particularly marked. Fig. 84.

The presence of embryo tissue is therefore an essential part of the test procedure as without it a false negative result was obtained with Hydrocarbon No. 13.

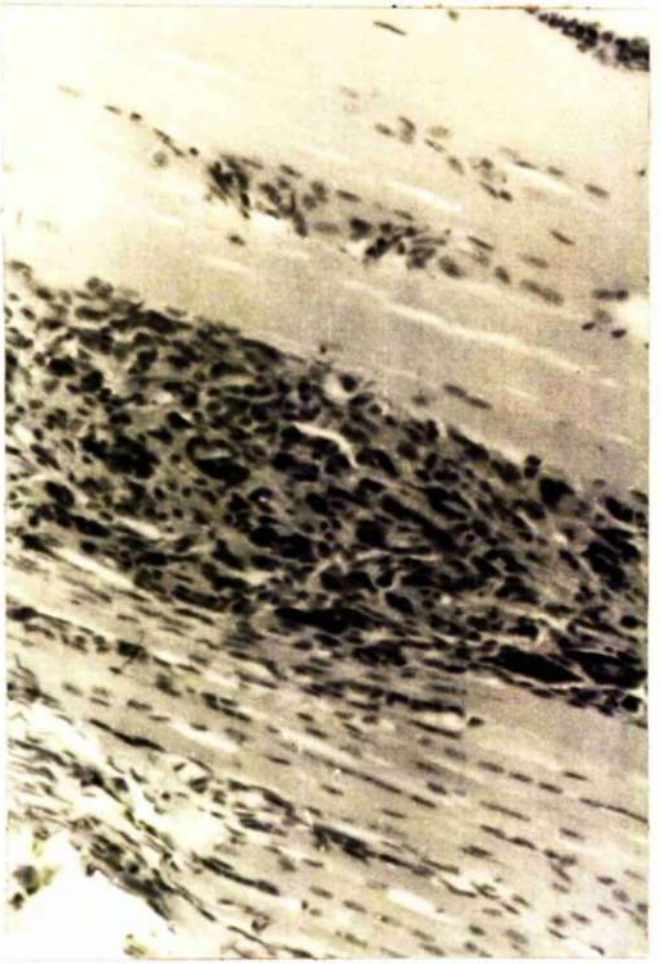
Figure 83.

Haematoxylin and Eosin. X 120.

Site of implantation of crystals of 3.4:9.10 Dibenzopyrene alone.
An anaplastic sarcoma is seen invading the host muscle.

Figure 84.

Haematoxylin and Eosin. X 120.



Site of implantation of crystals of 1.2:4.5:8.9 Tribenzopyrene.
There is a foreign body giant cell reaction at the implant site.

CONCLUSION

This standardized implantation technique fulfils the postulate proposed for a theoretically ideal test procedure given on pages 29 and 30 which may be summarized as follows -

a. A simple procedure easily performed and easily reduplicated.

b. A short latent period - 16 weeks.

c. Adaptability to several types of tissue.

d. Freedom of animals from spontaneous tumours.

e. Small quantity of test material required - less than 150 ug. (in this context it should be noted that the test material is used pure and there is no solvent or other 'vehicle' involved whose action on the tissues must also be considered).

The use of this method for the blind testing of the fifteen Hydrocarbons gave results which correlated exactly with their known behaviour as carcinogens with no false positive or false negative results.

Thus the first objective of this study was achieved. A new short term method of testing for carcinogenicity has been evolved from the consideration of the factors

influencing the results obtained by currently accepted methods.

An account of the method and the results obtained was presented at the 8th. International Cancer Congress in Moscow in July of 1962 and subsequently published in the British Journal of Cancer. (Peacock, 1962).

Applications of the Embryo Implant Technique.

Arising out of the successful completion of the first part of this study were a number of problems requiring further experimental investigation.

In broad outline these were -

a. Could a quantitative assessment be made of the amount of a given carcinogen required to induce a tumour.

b. Where a substance had given equivocal evidence of carcinogenic action, could more positive results be obtained by this method.

c. Some carcinogens have a long latent period, might this method shorten the time required to produce similar results.

d. Because some substances only accumulate slowly in the body there is a long time interval till toxicity effects are built up. The constant exposure of this method might demonstrate toxic effects sooner than other techniques.

e. Is one particular tissue more sensitive than any other in reacting to carcinogenic effects.

A series of experiments were designed to try to

answer these questions and in addition some straightforward toxicity tests were carried out on various substances for some of my colleagues to supplement experimental work in which they were engaged and on other carcinogenic agents not related to the Hydrocarbon series.

Quantitative Study of the Effect of a known Carcinogen.

Having shown that very small quantities of a carcinogen could induce tumours by direct weighing methods, a quantitative study was undertaken as the logical extension of this observation.

3:4 Benzopyrene, Hydrocarbon No. 3 of the test series, was chosen as it had given evidence of carcinogenic effect in most types of embryo implant.

The procedure was as follows -

a. A small piece of aluminium foil was weighed and then re-weighed with a small crystal of the Hydrocarbon. The Hydrocarbon was handled on the foil till the time of implantation. The amounts used were deliberately varied and all were larger than previously used to increase ease and accuracy of measurements.

b. An embryo implant was made incorporating the weighed amount of Hydrocarbon. Only lung tissue was used and six implants were made each into a different mouse.

c. The empty foil was then placed in a measured amount of acetone and the instruments rinsed in

the same acetone to recover any traces of hydrocarbon present.

d. The amount of hydrocarbon if any in the acetone was estimated using a recording spectrophotometer. In one case only was a very small trace measured.

e. After 16 weeks the animals were killed and a small fragment of the tumour present was taken for histology in order to confirm the action of the carcinogen.

f. At post mortem it was noted that in four cases the crystals were still present and fluorescent. These were labelled and placed in individual containers.

g. The leg used for the test was amputated and skin and foot removed. The remaining tissue was emulsified and extracted with benzene.

h. The amount of hydrocarbon remaining in the leg tissues was estimated as in (d) using the recording spectrophotometer and where applicable the amount of the residual crystal was also determined separately.

i. The difference between final figure of recovered hydrocarbon and that originally implanted was

held to be the maximum amount which could have been metabolized in the process of inducing each particular tumour. See Table 20.

Table 20.

Results of a quantitative implantation experiment using 3:4 Benzopyrene.

Tissue Implanted.	Sample No.	Weight of Sample.	% Recovery of sample.
Lung	1	7.0 mgs.	7.3%
Lung	2	24.5 mgs.	11.6%
Lung	3	39.2 mgs.	7.14%
Lung	4	5.3 mgs.	21.2%
Lung	5	2.4 mgs.	7.9%
Lung	6	13.5 mgs.	7.4%

The procedure for a qualitative approach was thus successfully established and the previous conclusion that very small quantities of material were sufficient to induce tumours confirmed.

There is no evidence that the total unrecovered amounts were in fact necessary to induce these tumours. A titration type of experiment would be necessary in order to determine a lower limit for the amount which could be carcinogenic under these circumstances.

This would be quite an extensive study and at the present time has not been undertaken.

It is probable that the major part of the carcinogen was absorbed by the host and excreted unaltered or as a metabolite.

This probability would need to be taken into account in designing further experiments of a quantitative nature.

Embryo implants with "Imferon".

Imferon is an iron preparation for intramuscular injection used in the treatment of iron deficiency anaemia. It was reported by Richmond (1957) and Haddow & Horning (1960) that this substance could induce sarcomas in experimental animals. The tumours arose at the subcutaneous site of injection.

When the embryo implant method was presented at the Cancer Congress in Moscow one of the questions asked was if embryo implants had been exposed to Imferon. At that time this had not been done, but subsequently it was felt that it would be of interest to make such a series of implants.

As Imferon is a liquid the embryo tissues were immersed in the fluid before implantation so that there was a close contact between the tissue and the test material and fairly high dose levels for the size of tissue ensured.

Altogether 48 implants of various embryo tissues were made the distribution of which is shown in Table 21.

The column headed "Lesions present" refers to any variation in microscopic findings from the normal, as

judged by comparison with the control series of implants. A similar heading is used in later tables.

Table 21.

Embryo Implants made with Imferon.

Tissue	Implants made.	Implants Recovered.	Lesions found.	Totals.
Skin	10	6	Nil	0/6
Lung	11	9	5A	5A/9
Stomach	11	10	1	1/11
Bladder	9	7	Nil	0/7
Kidney	7	5	Nil	0/5

A = adenoma.

Histology.

The principal microscopic findings were as follows:-

a. A high incidence of adenomas in the lung implants. These were similar in appearance to the solid adenomas recorded in previous experiments.

b. A single stomach implant showed stromal changes of the type that were described on page 184.

c. All the tissues examined showed macrophages laden

with pigment around the implantation site. In some of these the concentration was considerable. Even when the embryo tissue had failed to survive the site could be identified by these pigment laden cells.

d. Several kidney implants were made to see if this tissue could survive. In three cases there was recognisable parenchymal tissue present after 16 weeks. (Fig. 85). This was rather surprising as previous attempts at implanting whole kidneys only showed evidence that the ureteric epithelium could survive.

e. Skin, which normally gives a satisfactory implant, showed a very poor survival rate in conjunction with Imferon.

Conclusions.

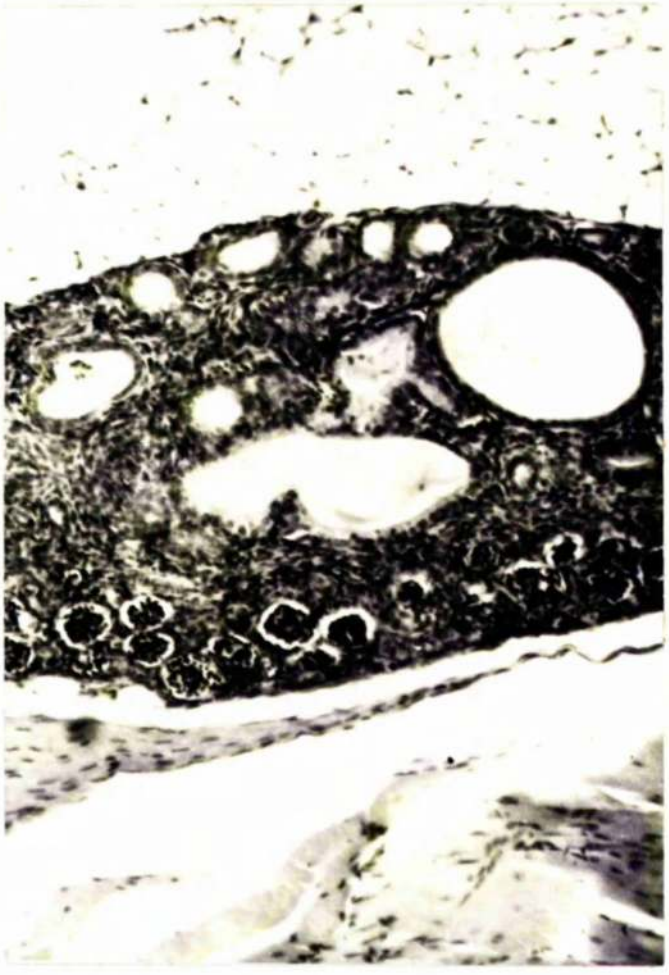
A high incidence of pulmonary adenomas is recorded and a poor survival rate for most of the tissues compared with controls, this is most marked in the case of the skin.

These findings seem to indicate a degree of toxicity on the part of Imferon towards the embryo implants. Other than the possibly acceptable lung adenomas no evidence of tumour induction is seen. This may be explained by the great disparity in dosage between this

series of experiments and those of Richmond & Haddow suggesting that the level of Interon which needs to be reached before a lesion occurs may in fact be fairly high.

Figure 85.

Haematoxylin and Eosin. X 120



Embryo kidney implanted after exposure to Imferon. There is a recognizable cortical area present with well formed glomeruli. The large spaces in the implant are presumably due to dilatation of tubules or parts of the ureteric pelvis.

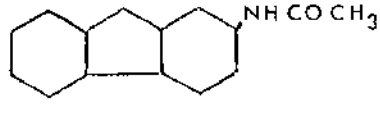
Embryo Implants with Acetyl-Amino-Fluorine.

There are substances which are carcinogenic but which require many applications over a period of time to produce their effects. This may be due to the interaction of three factors.

a. The substance may be either rapidly metabolized or slowly absorbed. The interplay of these two means that it takes a long time for a toxic level to be reached and then maintained in the body.

b. The third factor is the latent period of induction of a given tissue once it is exposed to a toxic dose level of a given carcinogen.

Acetyl-Amino-Fluorine (A.A.F.) a chemical with the graphic formula



was found to be a very satisfactory pesticide and in the course of routine toxicity tests Wilson, DeEds & Cox (1941) found that it was also a potent carcinogen. The experiments involved feeding large quantities daily mixed in the diet of the experimental animals.

All subsequent reports of tumour induction by A.A.F. have made use of high dosage, frequently repeated. This

has probably been necessary due to the fairly rapid rate of metabolism of A.A.F. by the body.

As the implant method ensures localization of the target tissue and the test substance in close contact it was felt that adequate exposure to A.A.F. might be obtained over a shorter period of time than previously recorded. Further it was possible that the rate of metabolism in this site might be reduced so that the single dose given at the time of implantation might exert its action over a number of weeks.

In all 56 implants were made, their distribution being shown in Table 22.

Table 22.

Embryo Implants made with Acetyl-Amino-Fluorine.

Tissue	Implants made.	Implants recovered.	Lesion present.	Totals.
Skin	13	13	Nil	0/13
Lung	13	12	5A	5A/12
Stomach	15	14	Nil	0/14
Small Intestine	3	3	Nil	0/3
Bladder	12	11	Nil	0/11

A = adenoma

Histology.

The pulmonary implant adenomas were of the same type as previously recorded. They were all smaller than in any of the other implant experiments suggesting perhaps a lower level of stimulation of the embryo tissue by A.A.F. than was obtained by other substances under test.

All types of embryo tissue showed a normal pattern of growth and no other microscopic abnormalities were seen.

Conclusion.

This substance which is known to be rapidly metabolized and to require a high dose level to produce its effect failed to create any significant changes in the embryo tissues under the conditions of this experiment.

From this one would conclude that implantation of a similar substance with embryo tissue is not capable of producing a sufficient degree of stimulus as a result of a single exposure.

Embryo Implants with Urethane.

It was shown by Nettleship & Henshaw in 1943 that the offspring of pregnant mice, injected near term with urethane, had a high incidence of Leukaemia and pulmonary tumours. These conditions only manifest themselves in the adult life of the mice affected in utero, so there was an interval of up to two years before these results were obtained.

This group of implants was made to see if the influence of urethane injections on foetal tissue in utero could be demonstrated in a shorter period of time. The procedure adopted was as follows. Pregnant Balb/C mice were injected intraperitoneally with 0.2 ml. of a 1.0% solution of urethane. The injections were made within 48 hours of death and were sometimes single, sometimes multiple. At the chosen time interval after injection the mothers were killed and the foetuses removed for dissection in the usual way. Only lung tissue was taken for implantation and the implants were made without any further treatment. After 16 weeks the host animals were killed and the implants examined microscopically. The time intervals for the injections of the pregnant females were as

follows -

a. 48 hours and 24 hours before death.

b. 24 hours before death.

c. 24 hours and 6 hours before death.

The results of the implants made from the embryos of these groups of treated females are shown in Table 23.

Table 23.

Embryo lung implants exposed to urethane in utero.

Injection Times.	Implants made.	Implants Recovered.	Lesions Present.	Totals.
48 hours and 24 hours.	14	11	5	5/11
24 hours.	8	7	Nil	0/7
24 hours and 6 hours.	14	13	8	8/13

Histology.

Three main features were present in these embryo lung implants.

a. A diffuse but heavy infiltration of the implant by cells which have the appearance of small lymphocytes. Sometimes this is a focal infiltration leaving zones of normal alveolar lung tissue. Figure 86 illustrates

this type of lesion.

b. Often quite large amounts of thymic tissue were seen in association with these implants. Fig. 87. The thymus in these cases having been accidentally implanted with the embryo lung. This had been noted in other experiments but only in this series have such large amounts of tissue been seen, which suggests it may be under the stimulus of the urethane. This tissue may be the source of the cells infiltrating the lung implants, though no direct correlation could be established.

c. A compact mass of pale staining rather poorly differentiated cells forming a distinct nodule. The precise origin of these cells has been difficult to determine. They resemble in many ways smooth muscle fibres and it may be that they are of origin from the smooth muscle of the bronchi. This appears to be a benign lesion. It has not been observed in any other type of implant experiment. The appearances are illustrated in Fig. 88 and Fig. 89.

Conclusion.

It would seem from a study of Table 23 that more than one injection needs to be given to the mother before any

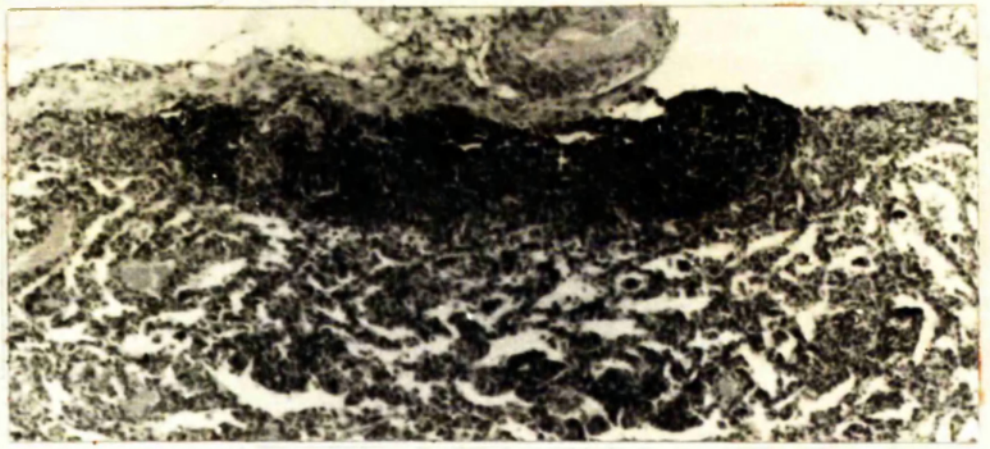
lesion is seen in the subsequent embryo tissue implant. The exact timing of these injections appears to be immaterial.

As all the implants survived equally well in each group, failure of survival does not account for the lack of lesions occurring in those only exposed to one maternal injection.

With regard to the type of changes taking place in the implants these seem to be benign in so far as they do not affect the host. However, as they are characteristic only of embryo lung exposed to urethane it seems reasonable to suppose that they are early evidence of potential later changes of the type first described by Nettleship et al. The object of demonstrating evidence of this change in a shorter period than the normal life span of the affected foetus is held to have been successful.

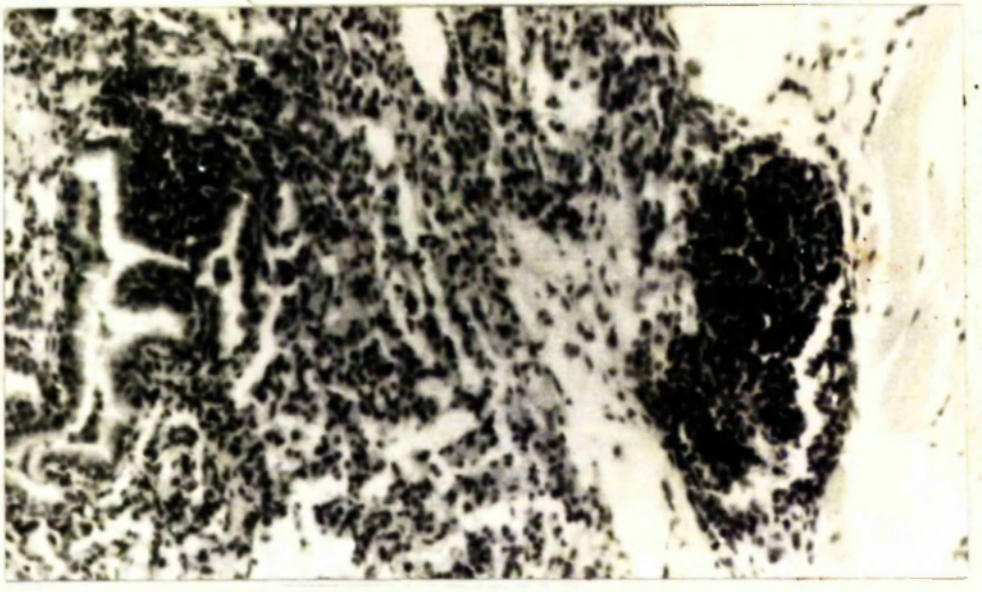
Figure 86.

Haematoxylin and Eosin. X 48



Implanted lung exposed to Urethane showing an area of diffuse round cell infiltration.

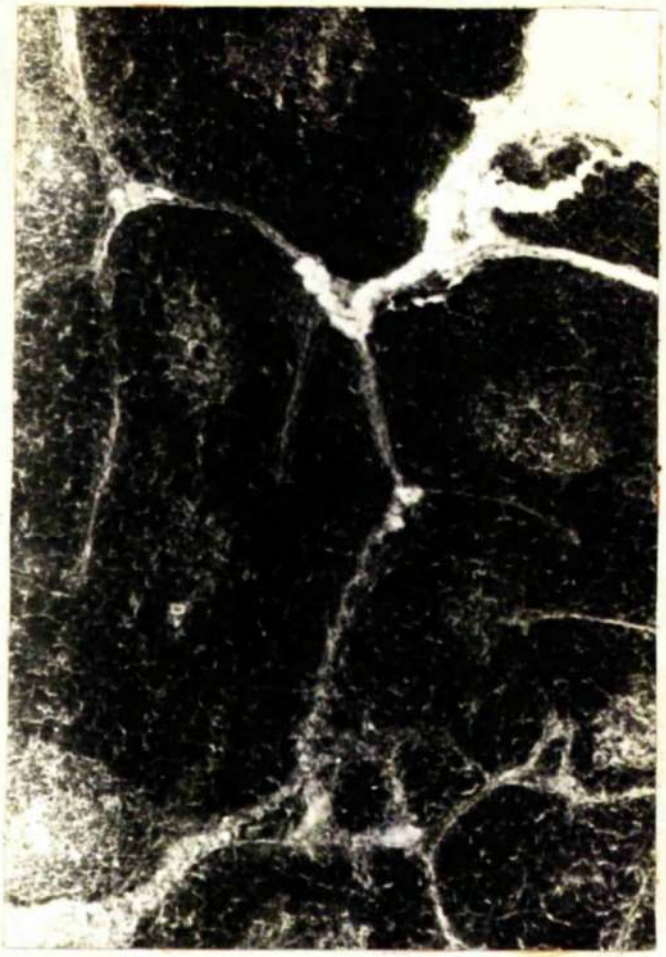
Haematoxylin and Eosin. X 120



Higher power view of another lung showing the focal type of round cell infiltration.

Figure 87.

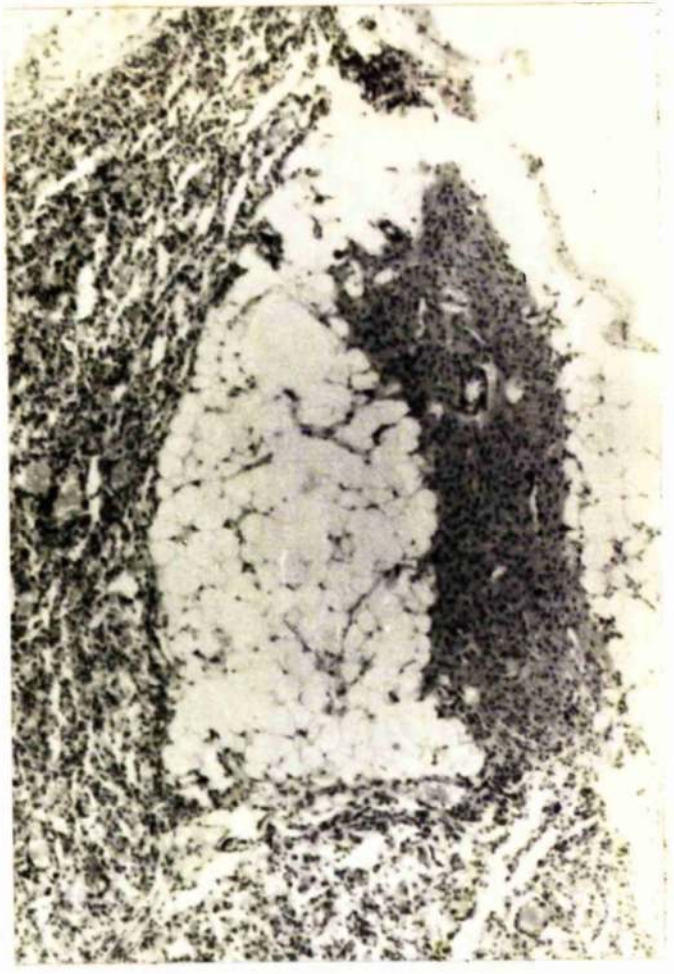
Haematoxylin and Eosin. X 48



Embryo thymus implant exposed to Urethane. The great increase in size due to hyperplasia is seen when compared with Fig. 59, page 156, which is at the same magnification.

Figure 86.

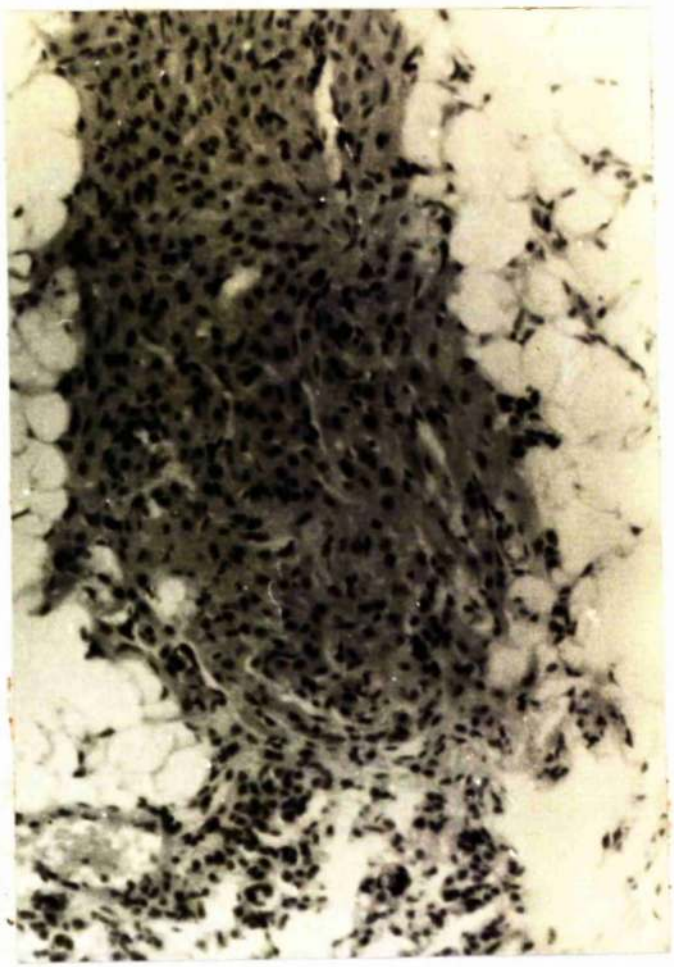
Haematoxylin and Eosin. X 48.



Lung implant exposed to urethane showing an associated nodule of smooth muscle origin. The continuity with the lung tissue is clearly seen.

Figure 89

Haematoxylin and Eosin. X 120



An enlarged view of part of figure 88 showing the structure of the smooth muscle nodule. There is clear evidence of continuity with the embryo lung.

Embryo Implants with Isoniazid.

In 1957 it was reported that large doses of isoniazid (INH) injected intraperitoneally induced lung tumours in mice, Juhasz, Balo & Kendrey, 1957. As this substance is a well established drug in the treatment of tuberculosis the report was of considerable significance. Reports from other workers soon confirmed these findings, Mori, Yasuno, Matsumoto 1960 and Bianchifiori 1961.

Embryo lung can be influenced in utero by a carcinogen as we know from the experiments of injecting urethane into pregnant mice.

It seemed reasonable as INH had been shown to be a carcinogen for the adult mouse lung that it might also affect embryo lung tissue in utero.

To test this hypothesis a number of pregnant Balb/C mice were injected with a solution of INH. A similar concentration to that used by Juhasz et al was employed. 100 mg/Kg. of body weight. It should be noted that the therapeutic dose in man is of the order of 3-8 mg/Kg. body weight. This solution proved to be very toxic given intraperitoneally. 0.5 ml. causing abortion in many mothers within 6 to 8 hours. It was finally established

that 0.2 ml. was the maximum dose which could be given in 24 hours without causing abortion.

For comparative purposes the same injection schedules were used as in the urethane experiments described in the preceding section. The numbers of implants made from each group of maternal injections is shown in Table 24.

Table 24.

Embryo lung implants exposed to isoniazid in utero.

Injection times.	Implants made.	Implants Recovered.	Lesions Present.	Totals.
48 hours and 24 hours.	28	26	1+4A	1+4A/26
24 hours.	28	25	4+1A	4+1A/26
24 hours and 6 hours.	22	22	3+3A	3+3A/22

A = adenoma

This table shows that there is a good survival rate in all the group of implants. In contrast to the urethane series a single maternal injection produced histological changes.

Histology.

a. As in the case of some other substances tested a number of adenomas of lung occurred. One of these showed an unusual appearance growing into the lumen of a small bronchus in a polypoidal manner. (Fig. 90).

b. In two implants from the single injection group there were areas of intra bronchial keratinization characteristic of squamous metaplasia. (Figs. 91 and 92).

c. Nodules of smooth muscle origin of the type first seen in the urethane series occurred in all three injection groups. Altogether there were six of these nodules. Specially stained sections confirmed their origin from smooth muscle.

The fact that they were not seen alone in the host tissues but always in close association with embryo lung strongly suggests a foetal origin and not an origin from the host. (Figs. 93 and 94).

d. Many of the implants showed marked alveolar dilatation as their only microscopic abnormality. This was due mainly to the retention of secretion. In some cases active secretion by the lining cells could be seen (Figs. 95, 96 and 97).

From the experience of examining over 800 implants of various tissues it can be stated that this type of appearance had not previously been recorded. A definite increase in the secretory activity of these lung implants had occurred.

e. Pigment laden macrophages were present in many of the implants. This pigment was shown by special stains to be a lipofuscin.

Conclusion.

A definite effect on the subsequent growth pattern of embryo lung implants is evident as a result of injecting the pregnant mouse with INH.

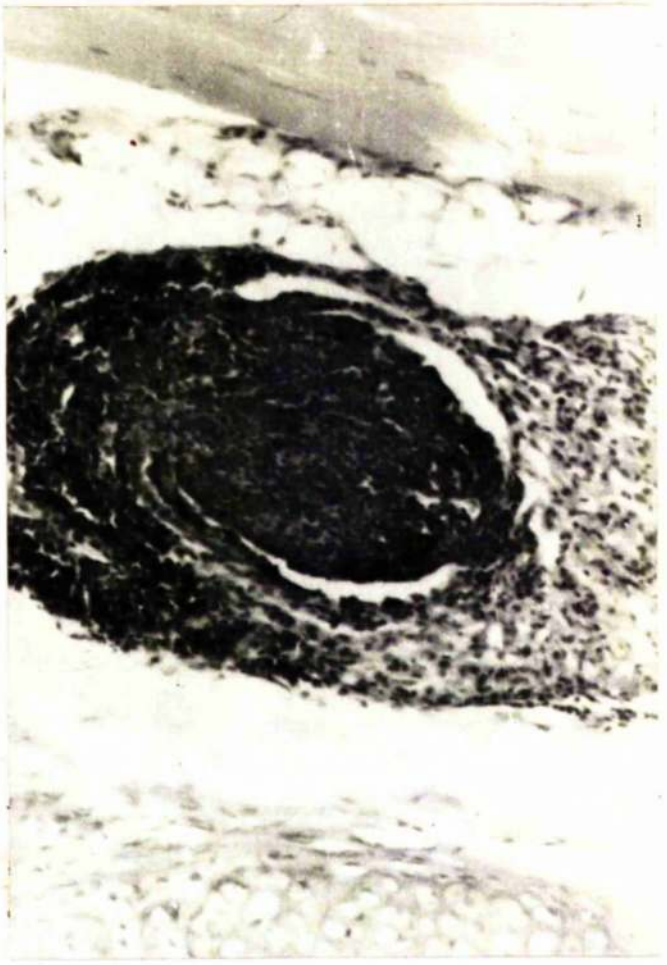
The principle feature common to the changes observed was a stimulus of growth or activity by a particular cell type. It is of interest that this type of reaction was seen only in the two series of experiments where exposure of the foetal tissues occurred in utero. The tissues were at least five or six days younger at the time of exposure by this method than if implanted in conjunction with a test substance. This difference in tissue may have been the deciding factor in this group of implants.

No malignant lesions were seen but the difference in dose levels of exposure by this method and that of Juhasz et al was considerable.

It is felt that this experiment approximated more nearly to the levels given therapeutically in man.

Figure 90

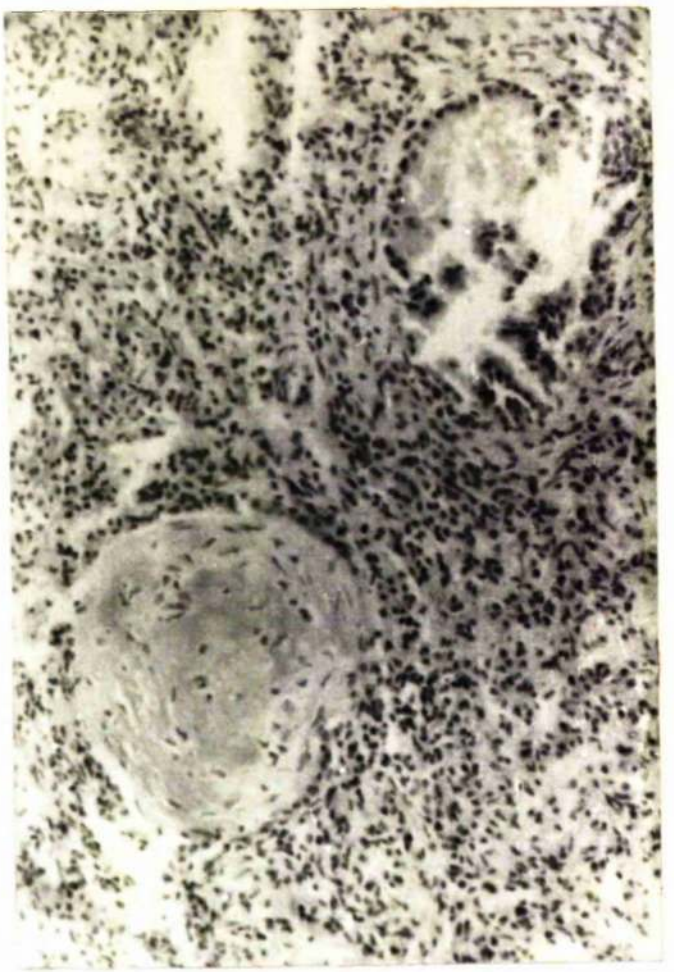
Haematoxylin and Eosin. X 120



Embryo lung implant exposed to isoniazid showing an atypical adenoma. The adenoma has grown into the lumen of a bronchus giving a polypoidal appearance.

Figure 91

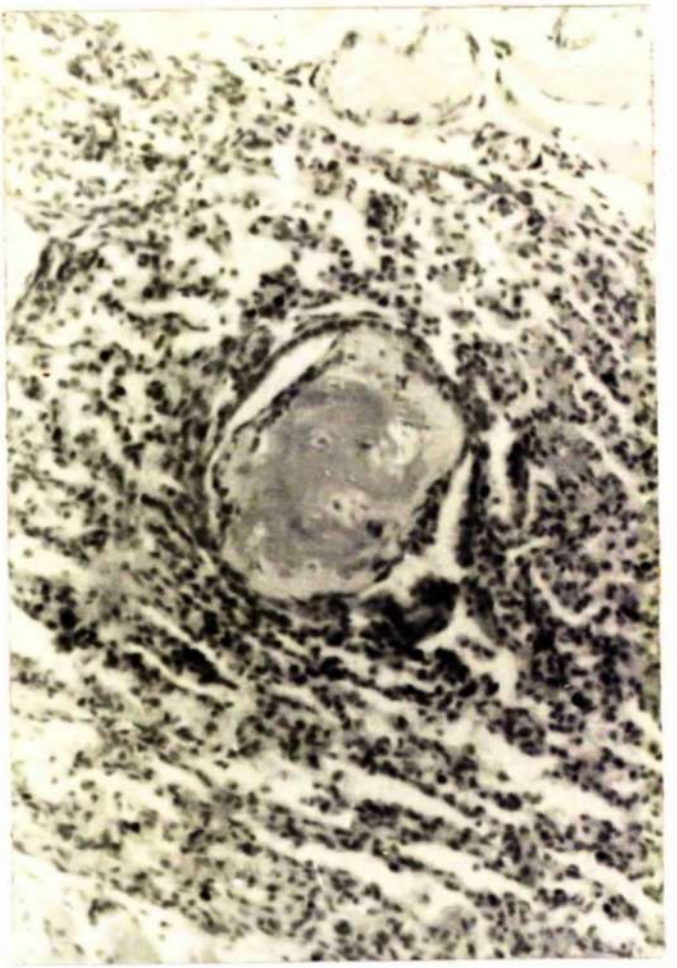
Haematoxylin and Eosin. X 120



Embryo Lung Implants exposed to isoniazid showing squamous metaplasia in a small bronchus.

Figure 92

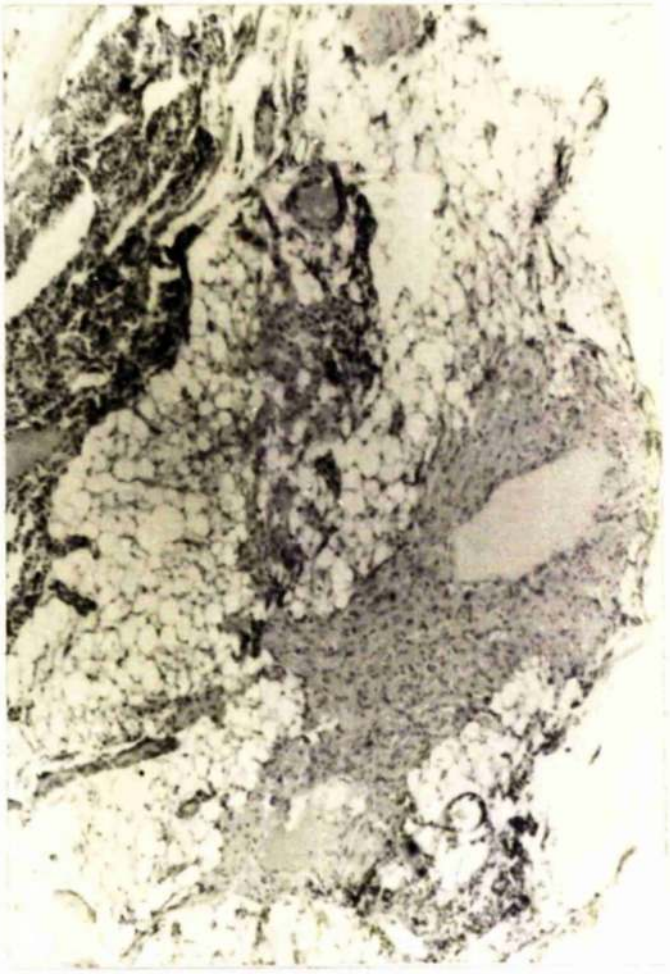
Haematoxylin and Eosin. X 120



Embryo lung implant after exposure to isoniazid. Squamous metaplasia is seen in a bronchus. Compare with preceeding illustration and figure 64, page 172.

Figure 93

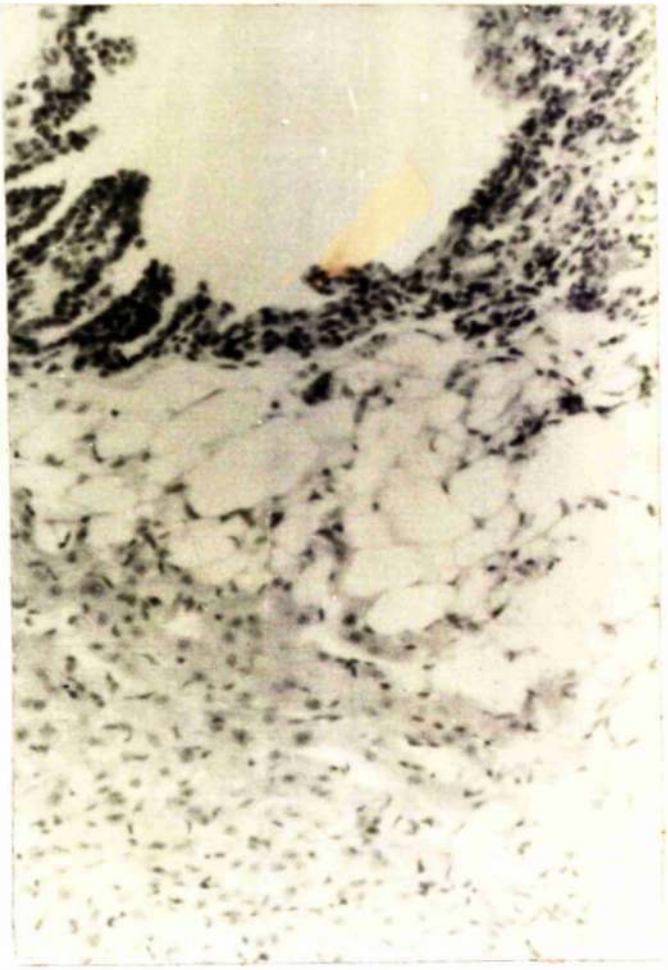
Haematoxylin and Eosin. X 48



Lung implant exposed to isoniazid showing a smooth muscle tumour similar to that shown in figure 88.

Figure 94

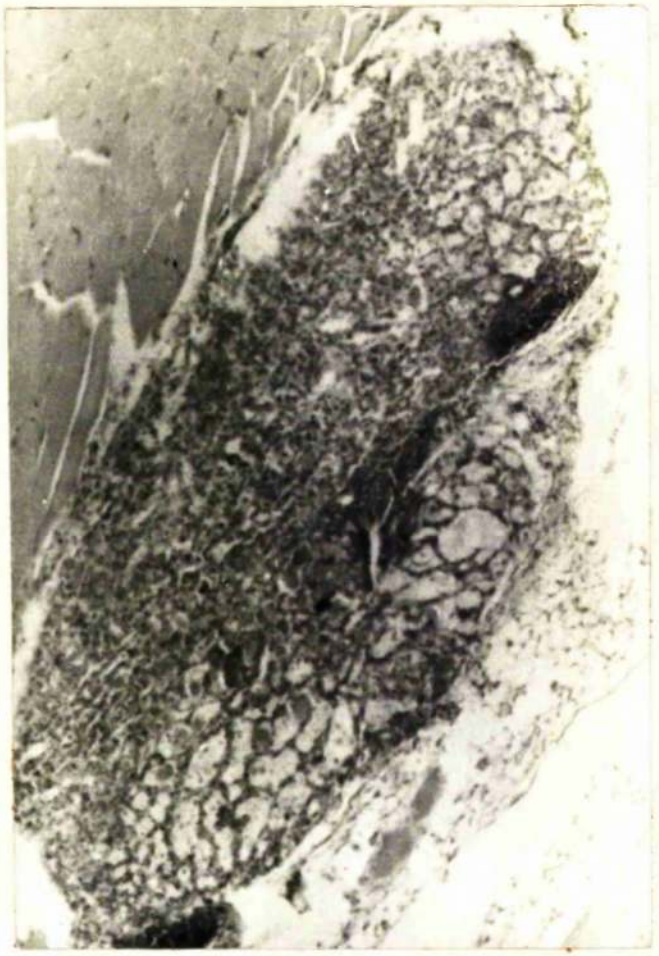
Haematoxylin and Eosin. X 120



An enlarged view of part of figure 93 showing the arrangement of smooth muscle fibres. Compare with figure 89, page 221.

Figure 95.

Haematoxylin and Eosin. X 48



Embryo lung implant exposed to isoniazid.
There is very obvious dilatation of many of the alveoli which contain eosinophilic material.
Focal round cell infiltration is also seen.

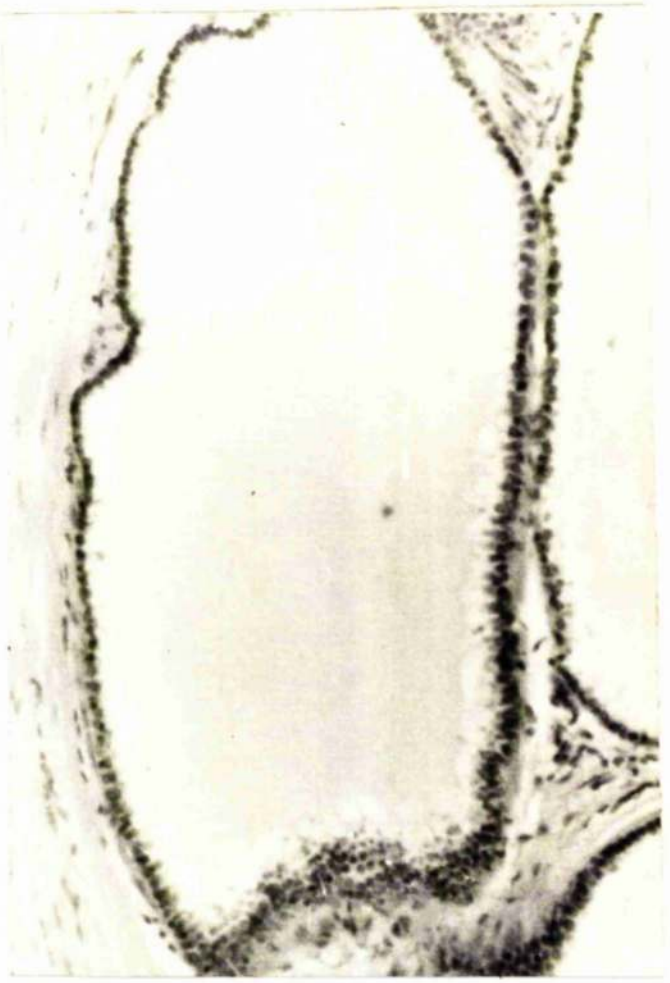
Figure 96.

Haematoxylin and Eosin. X 48

Embryo lung implant exposed to isoniazid. Some alveoli are dilated but the striking feature in this case is dilatation of terminal bronchi.

Figure 97

Haematoxylin and Eosin. X 48



Dilated alveoli in lung implant treated with isoniazid showing active secretion by the lining cells.

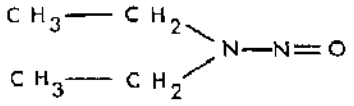
Embryo Implants with Nitrosamine.

The nitrosamines are a group of substances which have been reported to be highly toxic under experimental conditions. Tumours of various sites have been induced in mice including lung and liver. Drukrey 1962.

There is also evidence to suggest that where they are used in certain industrial processes the operatives handling them are at risk. A significant association between exposure to these chemicals and pulmonary tumours appears to exist. Drukrey personal communication.

The nitrosamines are oily volatile liquids. Several samples of them were obtained from Professor Drukrey after the 8th. International Cancer Congress in 1962.

A high incidence of pulmonary tumours had been obtained with N. nitroso Diethylamine.



This member of the group was used for the following toxicity studies on embryo tissue implants.

Because of the volatile nature of the substance

special precautions were taken in carrying out the implant procedure.

a. Both operator and assistant wore rubber gloves which were disposed of after each implanting session and surgical masks with a cellophane insert.

b. A perspex hood with an exhaust fan in the outlet vent was installed over the operating table. This allowed all the escaped vapour to be extracted along the duct to the outside atmosphere.

c. The tissues for implantation were placed around the periphery of a wide diameter petri dish. At the centre of the dish was placed a small watch glass. The nitrosamine was put in the watch glass and the petri dish covered. This exposed the tissues to a concentrated vapour of nitrosamine without direct contact with the liquid.

Exposure was for 10 minutes before implantation and 0.5 ml. of nitrosamine was used.

d. Earlier implants were made using tissues dipped into the nitrosamine but these were found to undergo rapid necrosis and within 48 hours were no longer viable.

The distribution of embryo implants made after

exposure to the vapour of nitrosamine is given in Table 25.

Table 25.

Results of Embryo Tissue Implants exposed to Nitrosamine Vapour.

Tissue Implanted.	No. of Implants made.	No. of Implants Recovered.	Necrosis Present.	Hyperplasia Present.
Skin	12	10	1	3
Lung	24	19	7	1
Stomach	13	10	4	2
Bladder	10	8	1	3

Histology.

Varying degrees of necrosis were noted in these implants. In some cases there was a nearly normal tissue mass present, in others small islands of viable cells were seen surrounded by necrosed tissue.

Damage did not extend to the host tissues but where there was massive necrosis of the implant a chronic inflammatory reaction was sometimes seen around the embryo tissue.

a. Lung was the most sensitive tissue with an incidence

of necrosis twice that of any other. Only a few implants recovered were in a generally good state of preservation and evidence of normal function such as mucous secretion was not found. Fig. 98.

b. The glandular part of the stomach showed a somewhat similar sensitivity to that of the lung. Quite wide zones of necrosis separated areas of viable mucosa. Fig. 99.

c. The most striking feature where there was good survival of embryo tissues was in squamous epithelium. Alteration of the pattern of keratinization was seen both in skin and the squamous part of the stomach. The cells of all levels remained nucleated and there were prominent kerato-hyaline granules. The thickness of the epithelium was also increased in a number of areas. Figs. 100 and 101.

Conclusion.

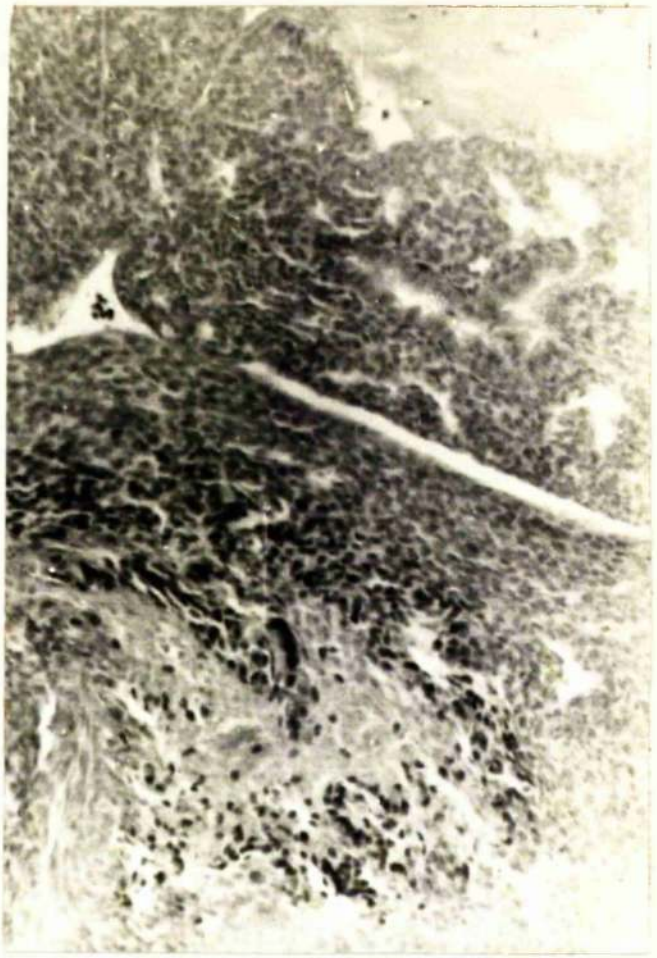
The marked degree of toxicity of N. nitroso Diethylamine for more than one type of tissue was confirmed.

The sensitivity of this method of toxicity testing was underlined by the method of exposure to the

nitrosamine. There was no physical contact between the tissues and the nitrosamine except as a concentrated vapour in a confined atmosphere for a measured time before implantation.

Figure 98.

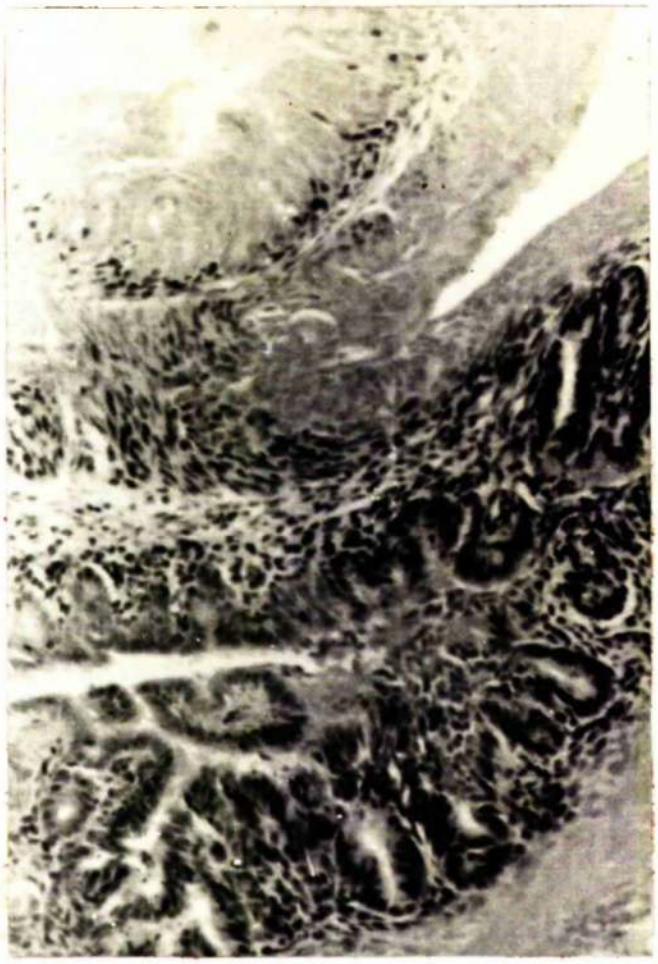
Haematoxylin and Eosin. X 120



Embryo lung implant exposed to nitrosamine. Much of the tissue is necrosed and only small groups of cells seem to be viable.

Figure 99.

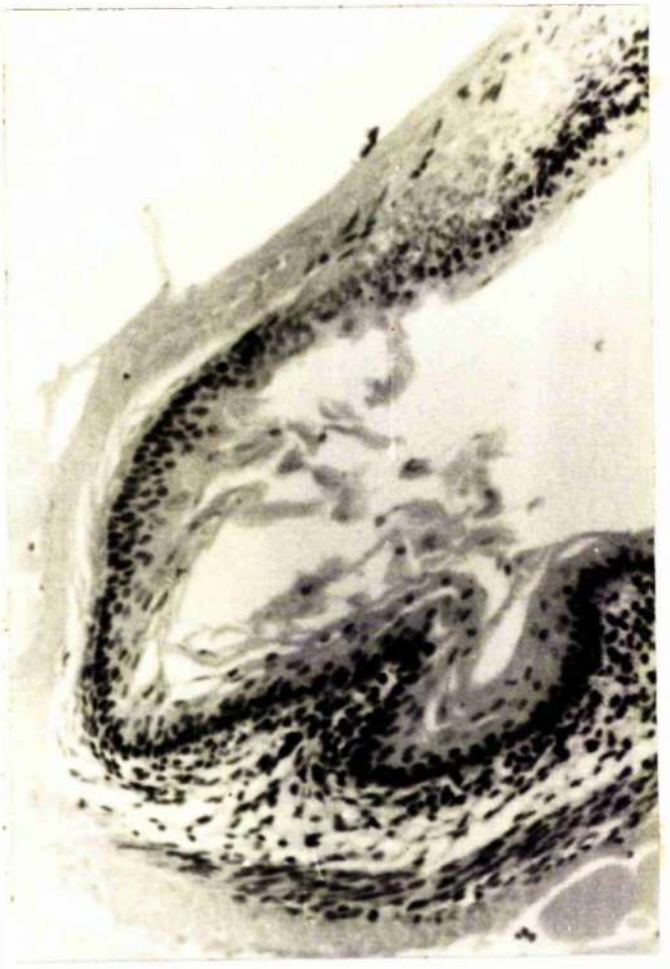
Haematoxylin and Eosin. X 120



Embryo stomach implant exposed to nitrosamine. Glandular mucosa showing marked necrosis with only the bases of the glands appearing to be viable.

Figure 100.

Haematoxylin and Eosin. X 120



Embryo stomach implant exposed to nitrosamine. Squamous epithelium showing alteration of keratinization pattern and necrosis.

Figure 101.

Haematoxylin and Eosin. X 120

Embryo skin implant exposed to nitrosamine. Marked alteration in the keratinization pattern is seen and only some of the tissue remains viable.

Embryo Implants with Tobacco leaf derivatives.

As part of a general study of the association of tobacco with the development of carcinoma my colleague Dr. Fell was studying Indian Tobacco leaf. This leaf is usually used for chewing rather than smoking and is made up into a 'wad' containing a number of constituents which gives an alkaline solution when masticated. In an attempt to simulate this, an alkaline aqueous extract was prepared which gave a brown amorphous residue on evaporation. This extract was free of known carcinogens of the polycyclic aromatic hydrocarbon type.

Toxicity tests using the implant method were made using this tobacco extract.

Some implants were also made using tobacco extract alone.

The distribution of these implants is shown in Table 26.

It is of interest to note that within a few minutes of the implantation of tobacco on one side, the host mouse became deeply unconscious quite unlike the normal state of anaesthesia. The respiratory rate dropped and long periods of apnoea occurred with intervening episodes

of Cheyne-Stokes type of respiration. If the second side was implanted also the host failed to regain consciousness and died in about 15 minutes. This was possibly due to a high nicotine content in the tobacco as the symptoms closely resembled those of nicotine poisoning. In practice it was found possible to use each host for only one implant in which case consciousness was regained in about 10 minutes instead of the usual 3 to 4 minutes.

Table 26.

Embryo Implants with Tobacco Extract.

Tissue.	Implants made.	Implants Recovered.	Lesions present.	Total.
Skin	9	3	0	0/3
Lung	21	17	1+4A	1+4A/17
Stomach	16	16	1	1/16
Bladder	5	5	1	1/5
Tobacco alone.	15	13	0	0/13

Histology.

a. Where tobacco alone had been implanted the site was easily identified by golden brown pigment free in the tissues and also in macrophage. Fig. 102. No lesions of any other kind than a mild inflammatory reaction were seen in this group.

b. A few of the lung implants showed the presence of adenomas, of the type seen in other experiments, with no distinguishing features.

c. One lung implant had a hyalinized area present in a bronchus similar to others seen with squamous metaplasia. Fig. 103. In this case no transition zone from glandular to squamous epithelium was seen in a bronchus.

d. One bladder implant had a very marked papilloma present. A change in the bladder epithelium which had not been seen in any other experiment. Fig. 104. This appears to be a benign lesion.

e. One stomach implant showed a degenerative condition affecting only the glandular mucosa. This is in contrast to the changes in the squamous epithelium described on page 194. Nearly all the cells appear to be in a degenerative state with the exception of a few of the

parietal cells. Figs. 105 and 106.

Conclusion.

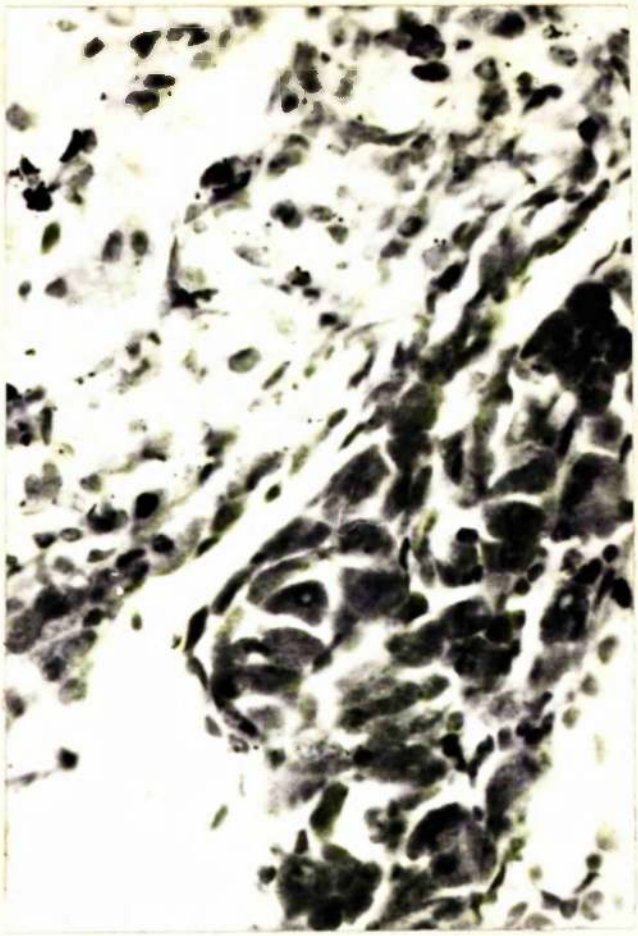
An immediate toxic effect on the host was seen at the time of operation. With regard to the implanted embryo tissue there has been a selective toxic effect on the skin which is the only tissue showing a poor survival for the period of the experiment. Three isolated implants each of a different tissue showed distinct abnormalities which could be attributed to the exposure to tobacco extract. As in other experiments a number of adenomas of lung were observed.

These findings point to the presence of a toxic element in this fraction of the tobacco but no positive proof of a carcinogenic effect was seen.

Further investigation to determine the nature of the toxic substance or substances would require a detailed analysis of the composition of this possibly complex aqueous extract followed by separate toxicity tests for each component.

Figure 102.

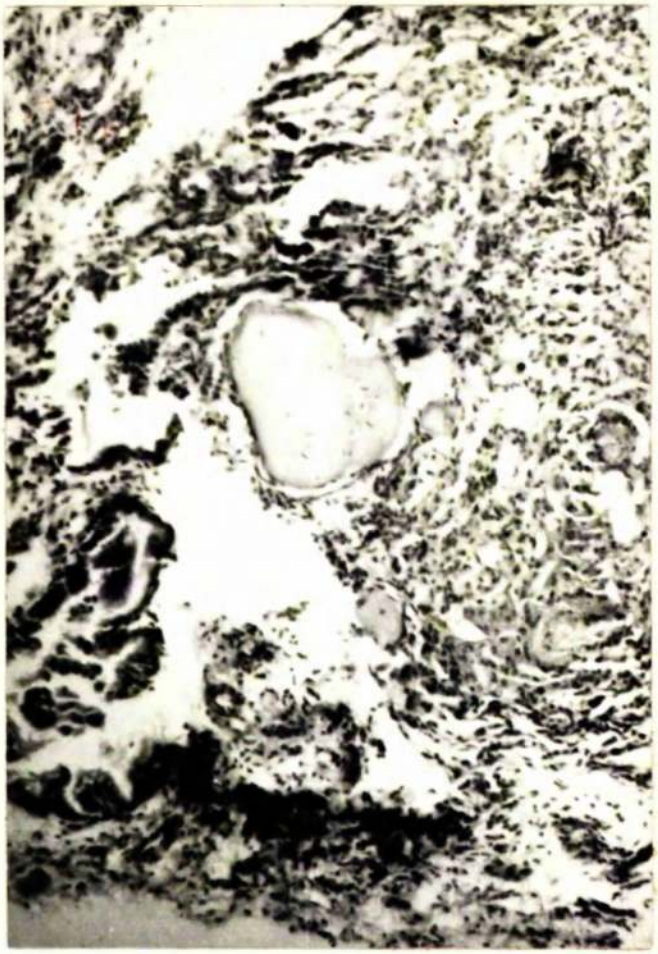
Haematoxylin and Eosin. X 480



Pigment laden cells from the site of implantation of tobacco alone.

Figure 103.

Haematoxylin and Eosin. X 120



Embryo lung implant exposed to tobacco extract. Hyaline material is present, an appearance previously seen in association with squamous metaplasia in the bronchi. Compare with Fig. 65, page 172 and Figs. 91, 92, pages 228 and 229.

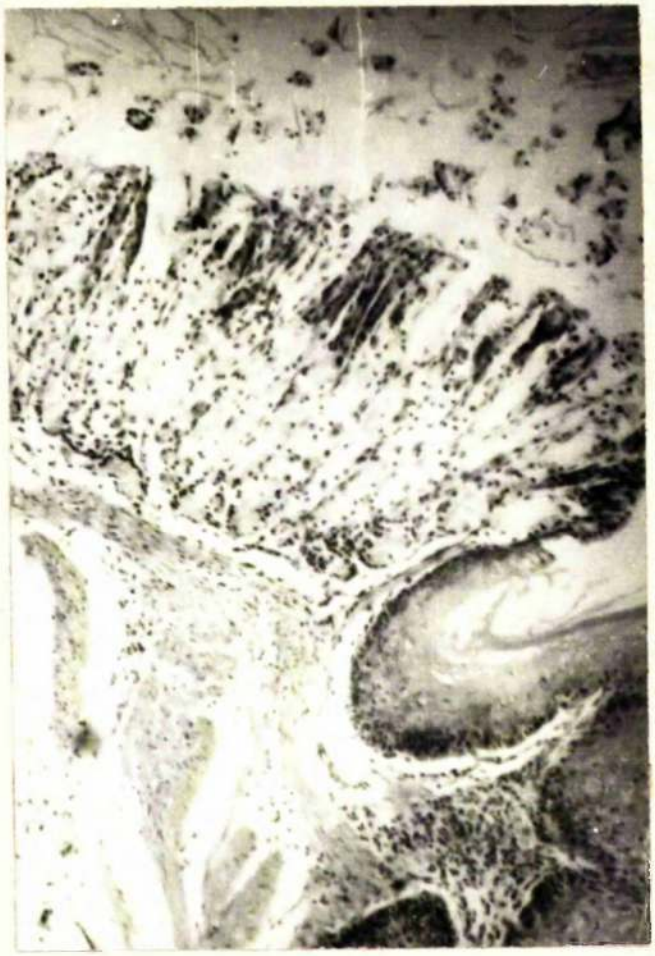
Figure 104.

Haematoxylin and Eosin. X 48

Embryo Bladder implant exposed to Tobacco Extract showing a papillomatous lesion of the bladder wall.

Figure 105.

Haematoxylin and Eosin. X 1

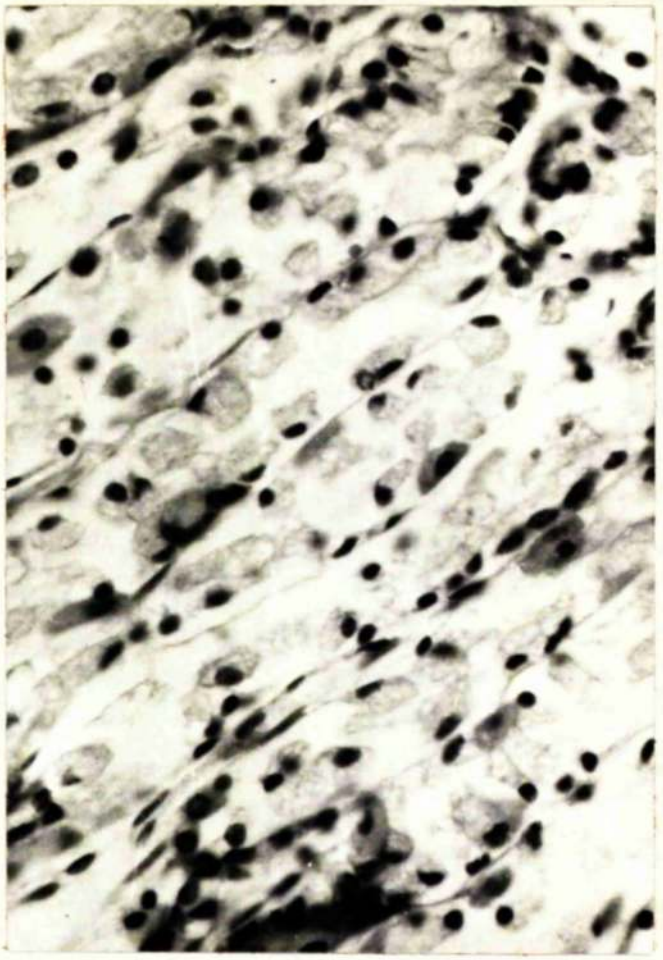


Embryo Stomach Implant exposed to Tobacco Extract.

A degenerative lesion of the glandular mucosa is present, the squamous epithelium being unaffected.

Figure 106.

Haematoxylin and Eosin. X 480



Part of Fig. 105 enlarged to show the changes in the glandular mucosa. Only an occasional dark staining parietal cell appears to be viable.

Discussion of the Embryo Implant Method.

This surgical procedure has been found very adaptable while carrying out the experiments which have been described in the preceding sections.

In the course of evolving and applying the method over 800 implants have been made and studied microscopically.

From this experience certain conclusions have been drawn.

a. Untreated embryo tissue shows no microscopic abnormalities of development after 3 months in the host. Normal differentiation takes place and certain evidence of functional activity is seen.

b. Exposure to non-toxic or non-carcinogenic substances at the implant site does not produce any lesions in the implanted tissues.

c. Where a substance known to be toxic or carcinogenic has been implanted in association with embryo tissue, microscopical lesions are seen. These vary in degree from mild hyperplasias to frank malignancy with invasion of the host tissues.

d. Involvement of the host tissues in these reactions does not occur directly except in the case of one or two

potent carcinogens.

e. More than one route of exposure of the implant to the test substance was used to produce microscopically recognizeable changes. Each has been in its own way equally successful.

Subsequent to the publication of a paper describing the method and some of the results obtained (Peacock 1962) many requests for reprints were received.

It was also a privilege to receive requests from a number of Institutes to train members of their staff to practice the embryo implant technique. In particular it was an honour that some of these visitors should have come from other countries for this express purpose.

The possible applications in the fields of physiology and pharmacology have been discussed with those interested in these fields. It is a method of providing isolated normal tissues free of the rest of the organism yet potentially they can be reached via the blood supply of the host, or where cysts form can be directly approached by inoculation of test substances.

SUMMARY.

The accepted standard methods of testing substances for their toxic properties were examined under the following general headings:- Skin Painting, Injection Methods, Implantation Methods.

From published work it was evident that many factors influenced the results obtainable by any given procedure.

Animal experiments were therefore devised to investigate the influence of these factors. These studies involved large numbers of animals from which considerable histological material was examined. The results so obtained made it possible to systematize and standardize many of the methods to a degree previously unrecorded.

Thereafter comparative studies were made of the improved techniques as tests for carcinogenicity. As a series of test substances fifteen polycyclic aromatic hydrocarbons were used. These were tested blind and only identified after completion of the experiments.

It was found that the implant method personally evolved gave the best results as a test for carcinogens.

Exploration of the potential of this method of embryo organ homografting has shown that in each case the objectives of the application have been fulfilled.

It is felt that this work has produced a very adaptable and sensitive experimental procedure for which there are many applications.

An account of the method has been published prior to the writing of this thesis. It was intended to include a reprint with the thesis but these were all exhausted by requests within three months of publication.

APPENDIX No.1.

Procedure for Processing Skin after Zenker Fixation.

a. Wash in water for 24 hours minimum.

b. 70% alcohol by day.

c. 90% alcohol overnight.

d. Absolute alcohol I 2 hrs.

Absolute alcohol II 2 hrs.

Xylol: absolute alcohol 50:50 1½ hrs.

Xylol I 2 hrs.

Xylol II 2 hrs.

e. Paraffin Wax: Xylol 50:50 overnight at 36°C.

f. Paraffin Wax I 20 mins.

Paraffin Wax II 20 mins.

Paraffin Wax III 20 mins.

g. Block in Paraffin Wax of melting point 56°C.

APPENDIX No.2.

First week.

Monday	Paint all animals.	
Tuesday	Fix Group A.	
Wednesday	Fix Group B	Wash Group A.
Thursday	Fix Group C	Wash Group B.
Friday	Fix Group D	Wash Group C.
Saturday		Wash Group D.
Sunday	Leave Group A & B in 70% alcohol Group C & D washing.	

Second week.

Monday -

Group A & B 90% alcohol	Group C & D 70% alcohol.

Tuesday -

Group A & B Absolute Alcohol-Xylol	Group C & D 90% alcohol.

Wednesday -

Group A & B Through Paraffin & Embed.	Group C & D Absolute Alcohol - Xylol.

Thursday -

	Group C & D Through Paraffin & Embed.

APPENDIX No. 3.

Summary of Results of Mouse Painting Experiments.

Ref. No.

Strain of Mouse: Age in Days:

Date of Painting: Painted With:

* Number of Drops: Killed After:

Naked Eye Appearances:

Skin fluorescence.

Skin damage.

Deep surface fluorescence.

Deep surface hyperaemia.

Microscopic Appearances:

Stage of hair cycle.

Hyperkeratosis.

Epithelial hyperplasia.

Cellular infiltration.

State of vessels.

Oedema.

*Ten drops = 0.2 ml. (approx.).

APPENDIX NO. 4.

Tissue Processing Procedure.

a. Fixation was for a minimum of 48 hrs. in 10% Formol Saline.

b. Decalcification was then carried out for 5 to 7 days in a mixture of 5% Formic acid and cation exchange resin. The resin used was Zeokarb 225 manufactured by the Permutit Co.

c. After decalcification the legs were bisected in the sagittal plane giving two halves to be called A and B. These were then processed in an automatic tissue processing machine to the stage of embedding in wax.

d. If the implant was visible the tissue was embedded so as to place it on the face to be cut first.

e. Serial sections were cut and every tenth section was mounted and stained with Haematoxylin and Eosin till the implant was identified. Then a short ribbon of 5 or 6 sequential sections was mounted and stained for examination.

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