BLOOD SUPPLY IN NEOPLASMS

A Thesis submitted for the Degree of

Doctor of Philosophy

in the Faculty of Science of the University of London

By

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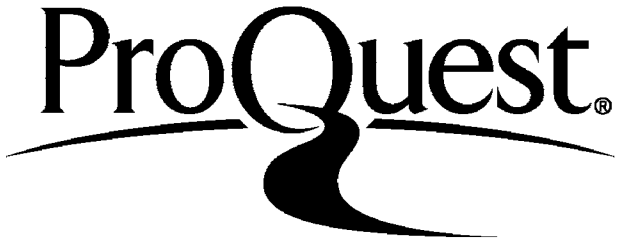

ABSTRACT

The present study is based on the finding that treatment with the new anticancer drug, ICRF 159, completely inhibited the formation of metastases in mice implanted with the Lewis lung carcinoma (3LL), at doses which had little effect on the growth rate of the primary tumour. The mode of action of the drug appeared to be on the development of the blood vessels of the primary tumour.

Studies were carried out to demonstrate the nature of the changes occurring in the structure and function of tumour blood vessels brought about by treatment with ICRF 159. X-ray angiography, carbon black (Pelikan ink) labelling and intravital staining with lissamine green have demonstrated that many of the characteristics of tumour blood vessels were absent in tumours treated with ICRF 159. Further, the blood vessels of treated tumours were relatively normal in structure and arrangement.

Any alteration of the morphology of tumour blood vessels may affect the rate of blood flow through the tumour. As this could be critical for most therapeutic modalities, the rate of blood flow through 3LL tumours treated with ICRF 159 was measured but was found not to be significantly different from control values.

It has been reported that tumour blood vessels are abnormally sensitive to serotonin at doses which have little effect on the blood vessels of normal tissue.

Although control 3LL tumours demonstrated a marked response to serotonin, no such effect was shown in tumours treated with ICRF 159.

Finally, it was demonstrated that ICRF 159 did not alter the viability of 3LL cells found in areas of the tumour which were devoid of functional blood vessels. These cells were capable of propagating the growth of new tumours.

The profound modification of the morphology and functional character of tumour blood vessels brought about by treatment with ICRF 159, termed "angiometamorphic", could be of considerable importance in the treatment of primary and secondary tumours.

CONTENTS

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SECTION I

General Introduction

Bergel (1970) stated, "The medical profession is almost powerless against the dissemination of malignant cells, leading to metastases or secondary deposits. This statement does not exclude short-term control of such growths by ionising radiation, adrenalectomy, other surgical interference with hormonally controlled organs or by chemotherapy of tumours of childhood. But on the whole, as the number of such favourable cases is relatively few, the problem of fighting metastases is one of the most urgent. The reason for this, well know to clinicians, is that although not infrequently primaries can be successfully attacked, the patient may succumb to the highly resistant secondaries".

It is well known that where cancer is diagnosed in its early stages, present day forms of therapy can prove highly effective. Unfortunately, where dissemination has taken place, surgery and radio-therapy can to a large extent be only palliative. It may be that chemotherapy will provide a potential remedy against the spread of cancer.

Dissemination of tumour cells by vascular channels and the establishment of distant metastases occurs during some stage in the natural history of many malignant tumours. Although most of the haematogenously spread cancer cells are probably destroyed within the confines of the blood vessels, the fact that 80% of terminal cancer patients have metastases in other organs suggests that some of the disseminated cancer cells,do, in fact, survive and proliferate (Roberts, 1961, Hellmann, 1971).

Tumour cell dissemination may be considered as occurring in four distinct phases:

1. Invasion of cancer cells into vascular channels or lymphatics.
2. Transportation from the primary tumour of single tumour cells or clumps of cells in the blood stream to distant parts of the body.
3. Implantation of the tumour cells in the arterioles and capillaries of the distant organs.
4. Growth of the secondary deposits.

Accordingly, any measures to prevent dissemination via the circulation should be aimed at preventing the invasion of tumour cells into the vascular channels (stage 1), as this would localise the neoplasm and facilitate therapy. The advantages of finding a suitable experimental model for studying the haematogenous spread of metastases are obvious, in that compounds could be tested specifically for inhibitory effects on tumour dissemination, but, the main problem for many years has been the discovery of such a test system.

Many workers have bypassed the problem by injecting tumour cells intravenously,but this expedient is not entirely satisfactory since important early stages in the process of metastatic spread are therefore also bypassed, i.e. the invasion and release of cancer cells into vascular channels. The assumption

that injected cells from a tumour mash are equivalent to malignant cells leaving a primary tumour cannot be justified.

The first attempts to investigate the mechanism of tumour dissemination were made by Levin and Sittenfield (1911), studying a number of rat sarcomas and carcinomas. However, it was not until the work of Ketcham et al (1966); Karrer et al (1967) and Wexler et al (1969), that an experimental tumour was described which metastasised spontaneously and consistently to the same organ. This tumour, the Lewis lung carcinoma (3LL), when implanted into the flanks on inbred C57Bl female mice, regularly metastasised to the lungs (Fig.1). The number and size of metastatic tumours in the lungs was directly related to the age of the primary tumour and the survival of the animal. This implied a continuous sloughing of tumour cells into the veins draining the primary tumour.

To simplify the identification of pulmonary metastases, the alveoli were filled with dilute Indian ink before fixation of the whole lung. This had the effect of showing the metastases as white nodules against the black substance of the lung, thus facilitating counting of the nodules (Fig.2). X-rays could also be used to identify pulmonary metastases. The lungs were filled with Micropaque instead of ink (Fig.3).

Using the Lewis lung carcinoma as a model for drug screening, Hellmann and Burrage (1969) showed for the first time that it was possible by means of the cytostatic agent ICRF 159 to obtain complete suppression of the formation of spontaneous metastases. The mode of action of ICRF 159 has been investigated in some detail and forms the basis for the present thesis.

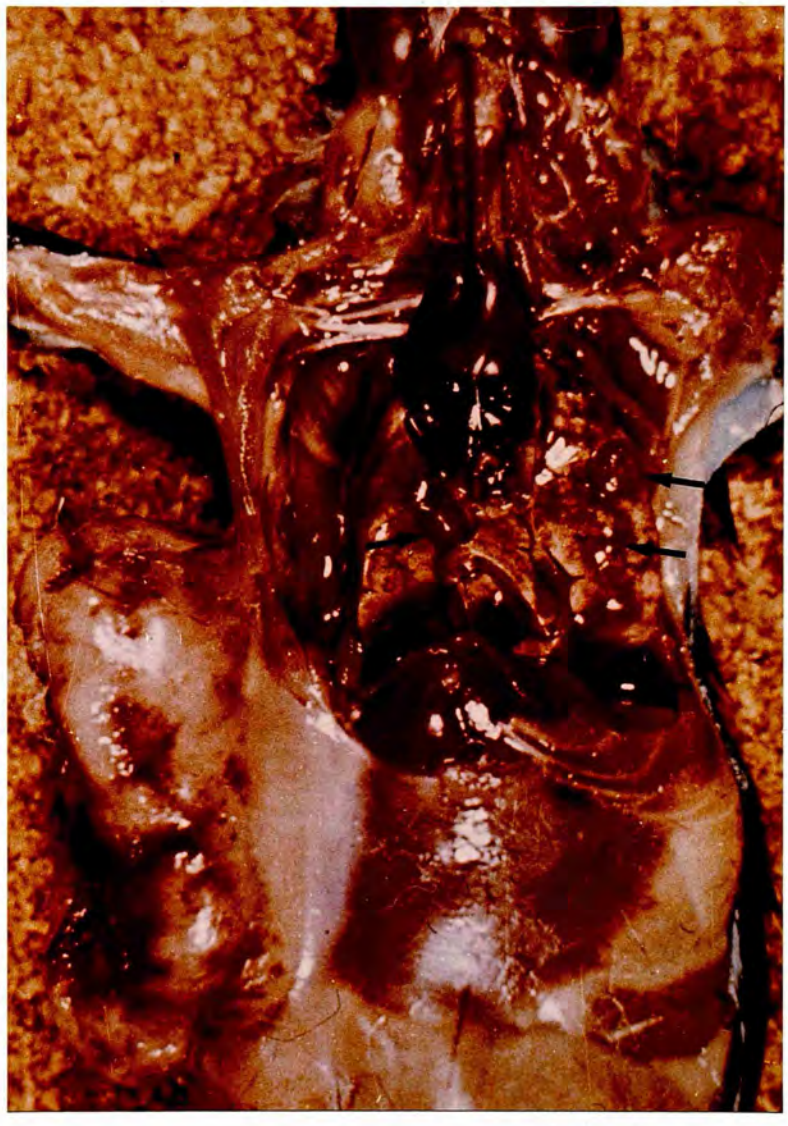


Fig. 1.

C57Bl mouse bearing a 21 day 3LL tumour in the flank. The front of the rib cage has been removed and the heart displaced to show the presence of secondaries or metastases in the lungs (arrowed).

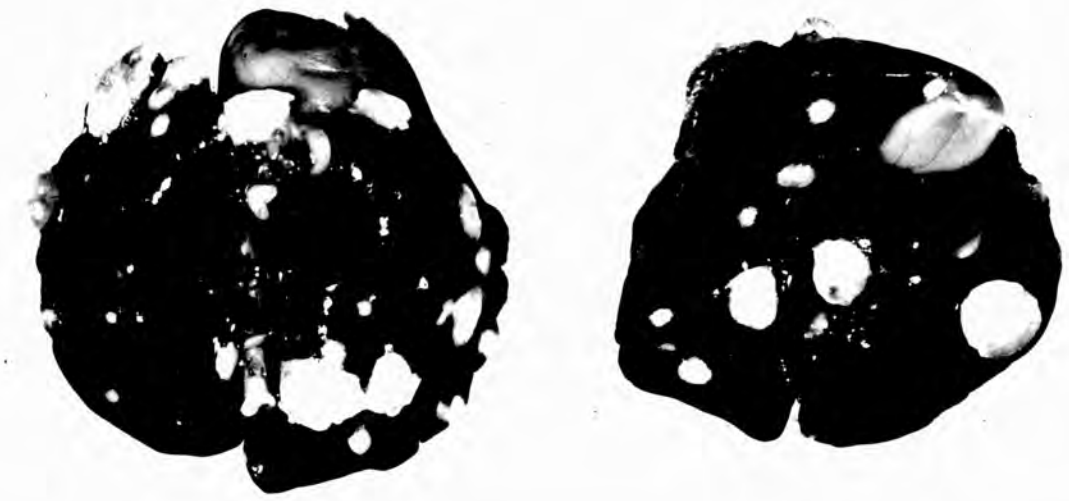


Fig. 2.
C57B1 mouse lungs inflated with Indian ink and then fixed, demonstrating metastases as white nodules.

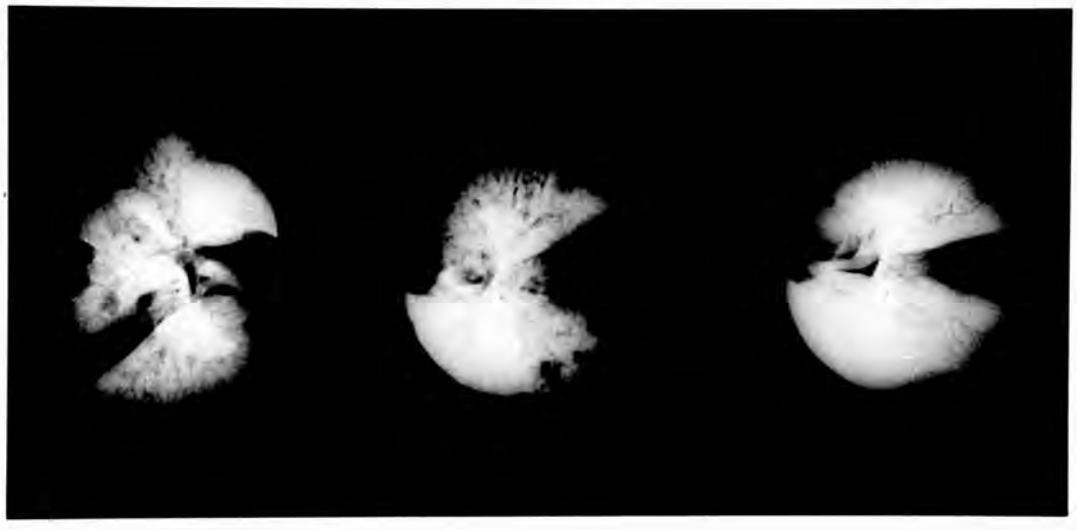


Fig. 3.
X-rays of C57B1 mouse lungs filled with Micropaque. Metastases are shown as black cut outs. Normal lungs are shown on right.

Inhibition of Metastatic Spread by ICRF 159

Salsbury et al (1970) demonstrated that treatment of the Lewis lung carcinoma with ICRF 159 inhibited pulmonary metastases resulting from blood borne dissemination of 3LL cells. Tumours were inoculated into the flanks of inbred C57B1 female mice (Day 0). The animals were killed 3 weeks later (Day 21), and the lungs examined for metastases. The lungs from animals treated with ICRF 159 showed no evidence of metastatic growth (Fig.4) in marked contrast to the control lungs which showed numerous metastatic nodules (Fig.5).

They suggested that the mode of action of ICRF 159 could have resulted from three different mechanisms:

1 The 3LL cells were killed by ICRF 159 during their carriage in the blood stream.
2 The 3LL cells failed to implant in the lungs or they implanted but did not grow.
3 The 3LL cells were not liberated from the primary tumour.

The last explanation was regarded as being the least likely of the alternatives.

Daily microscopical examination of blood concentrates and lung sections, however, showed the consistent presence from Day 10 onwards of circulating malignant cells in control animals and the complete absence of these cells (not even necrotic forms) in treated animals, thus ruling out the first two possibilities. The drug seemed, therefore to be acting in some way on the primary tumour. Gross observations of excised 3LL tumours revealed a marked

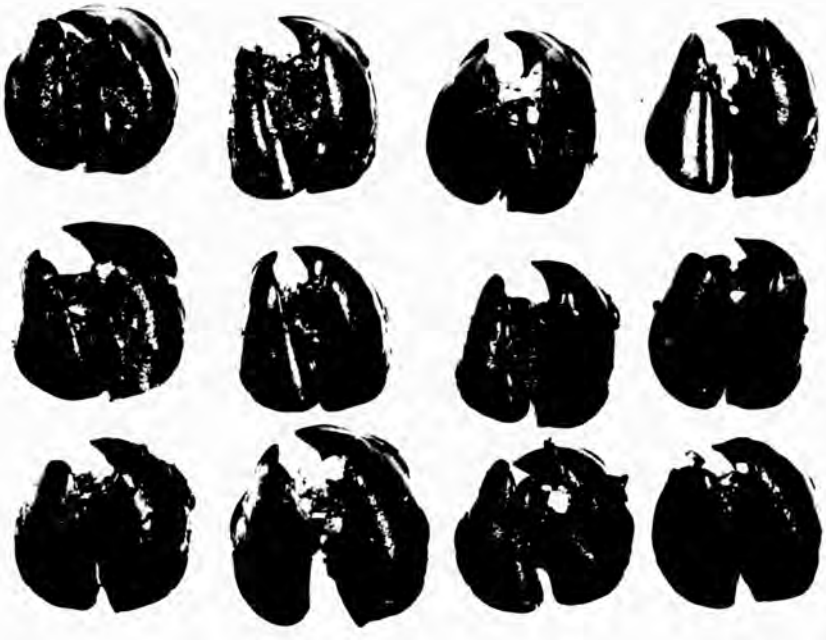


Fig. 4.
No metastases are seen in lungs from mice with 3LL tumours treated with ICRF 159.

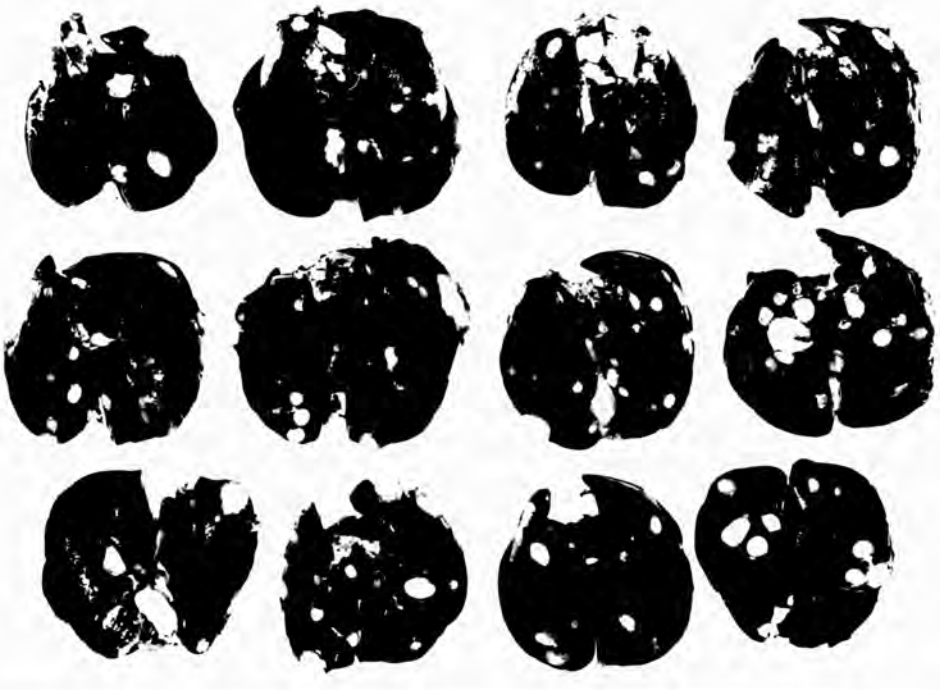


Fig. 5.
Lungs of control mice showing numerous metastatic nodules.

difference in colour, the control tumours being very red and haemorrhagic in contrast to the pale appearance of treated tumours.

The possible mechanism involved in the action of the drug was demonstrated by histological examination of the invading margins of the primary tumour. In control tumours the vasculature consisted largely of poorly defined sinusoids with red blood cells streaming between malignant cells. In many places malignant cells appeared to be in direct contact with blood cells, thus facilitating dissemination. On the other hand, treated tumours showed discrete well-formed blood vessels with malignant cells separated from the blood by a layer of endothelium.. These workers also pointed out that the growth rate of the primary implant was not significantly impeded by dosages of ICRF 159 required to produce this antimetastatic effect. Therefore retardation of growth of the primary tumour could not alone account for the inhibition of metastasis formation by ICRF 159. Comparable doses of cyclophosphamide had no effect on 3LL tumour vasculature and the mice still developed pulmonary metastases.

ICRF 159

Furst (1963) suggested that the majority of drugs which were known to inhibit tumour growth were, in fact, chelating agents. There were obviously many ways in which a chelating agent could interfere with cell growth providing the drug reached the appropriate site. EDTA, ethylene-diamine tetra acetic acid, one of the most powerful chelating agents had no significant antitumour activity, Leiter et al (1959).

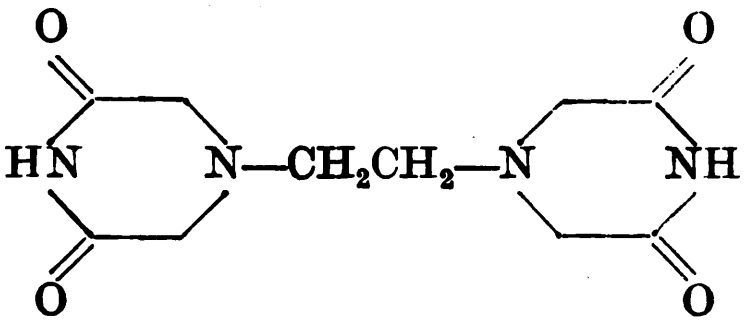
This result was not surprising since EDTA, being a highly polar molecule would not be expected to pass through biological membranes such as the cell wall. Creighton et al (1969) suggested that relatively non-polar derivatives of EDTA would be more likely to reach vulnerable sites, and once there, might break down to give potent chelating agents. Any selective antitumour action would depend on there being differences between normal and tumour cells; for instance, very small variations in trace metal, calcium or magnesium ion concentrations at specific sites might be sufficient to allow a differential effect.

Initial screening results with the first compound made, 1,2,bis(3,5-dioxopiperazin-1-yl) ethane (ICRF 154), were encouraging and further derivatives of EDTA were therefore made.

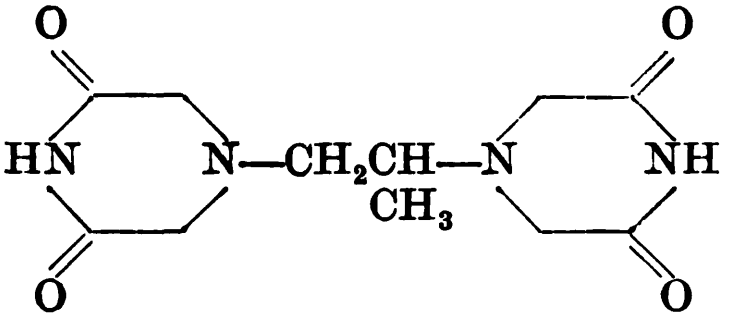
Antitumour activity was confined to a group of compounds very closely related to ICRF 154. Any lengthening of the central bridge or ring substitution resulted in complete loss of activity. It was retained however by methyl, though not ethyl substitution of the central bridge, viz. (±)-1,2,-bis (3,5 dioxopiperazin-1-yl) propane (ICRF 159).

The formulae of the two compounds are shown in Fig.6.

ICRF 159 is a potent antimitotic agent, rather than a general cytotoxic agent, in that it stops cell division only during a very brief period of the generation cycle as shown by Hellmann and Field (1970) using a quantitative cell culture technique with human epithelial (H Ep/2) cells. Further investigation by Sharpe et al (1970) revealed that mitotic figures in human lymphocytes grown in culture were



ICRF 154



ICRF 159

Fig. 6.

Formulae

drastically reduced by the drug and the cells arrested in early and late prophase. It was concluded that the drug acts on the cells at the end of G_2 phase or the early mitotic phases of the cell generation cycle.

The Present Study

In the present study an attempt has been made to demonstrate the nature of the changes occurring in the structure and function of 3LL blood vessels brought about by treatment with ICRF 159. The vascular morphology of the rat Walker tumour has also been studied following treatment with ICRF 159. This non-metastasising tumour was chosen, as Tucker (1970) had previously demonstrated that ICRF 159 produced a blanching of this neoplasm when it was grown on the chorio-allantoic membrane (CAM) of chick embryos. Changes in the vasculature of the tumour were demonstrated by X-ray angiography, carbon black (Pelikan ink) labelling, and intravital staining with lissamine green.

Any alteration of the morphology of tumour blood vessels may affect the rate of blood flow through the tumour. As this could be a critical factor in therapeutic considerations, the blood flow rates of 3LL tumours treated with ICRF 159 were measured.

Also the effect of serotonin on control and ICRF 159 treated 3LL tumours was tested, as it has been demonstrated that blood vessels in some tumours were abnormally sensitive to serotonin at doses which had little effect upon circulation in muscle tissue.

Finally, the vitality of 3LL cells in areas of control and ICRF 159 treated tumours devoid of functional blood vessels was demonstrated and discussed in relation to the poor results of chemotherapy and/or radiotherapy against large tumours.

This thesis is presented in the following Sections:-

Section II — The morphology of the blood vessels of 3LL and Walker tumours after treatment with ICRF 159.

Section III — The measurement of blood flow in 3LL tumours after treatment with ICRF 159.

Section IV — The effect of serotonin on 3LL tumours after treatment with ICRF 159.

Section V — The vitality of 3LL cells in areas of control and ICRF 159 treated tumours devoid of functional blood vessels.

Section VI — General discussion and conclusions.

SECTION II

The morphology of the blood vessels of 3LL and Walker tumours after treatment with ICRF 159

INTRODUCTION

Morphological studies

The vascular architecture of spontaneous and transplanted tumours has been studied intensively since the beginning of this century. Of the many historical accounts, those of Goldacre and Sylven (1962) and Urbach (1963) have been used extensively in this review of the literature.

Borst (1902), Ribbert (1904) and Apolant (1906) reported that most spontaneous malignant tumours were poorly and irregularly supplied with blood vessels especially in central regions. There were marked changes in existing vessels such as defective vessel walls, thrombosis, obliteration and dilation. However, the density of the vascular bed at the periphery of the tumour was generally increased.

One of the earliest descriptions of the anatomy of tumour blood vessels was presented in a series of papers by Goldmann (1911) using intra-arterial injections of Indian ink into living animals. His findings with transplanted mouse tumours may be summarised as follows:

1. There was marked proliferation of blood vessels at the periphery of the tumour. The vessels were usually dilated and spiral in appearance.
2. The architecture of tumour vessels was very irregular with numerous random anastomoses forming vascular networks. There were direct transitions from arteries to capillaries and then to veins with few vessels analogous to arterioles and venules.

3. As the tumours grew, the number of central blood vessels diminished and tumour cells invaded the pre-existing venules of host organs.

Lewis (1927) studied the vascular patterns of transplanted rat tumours by intra-aortic injections of Indian ink into dead animals. He noted the considerable variations in tumour vascular patterns which could be encountered. Adenocarcinomas presented a highly irregular arrangement with large dilated sinusoidal vessels showing no differentiation into arterioles and venules, as opposed to adenofibromas of the breast which demonstrated a normal vascular pattern with large afferent and efferent vessels gradually breaking up into capillary plexuses. He suggested that the vessels were formed under the influence of the neoplasm and that the development of central necrosis was not necessarily a result of poor blood supply, but of other factors such as the continued multiplication of the tumour cells exerting undue pressure on the capillaries or preventing in some way the further penetration of the capillaries into the central areas.

Waters and Green (1959) studied the morphology of the vascular system of two transplantable granulosa-cell tumours of mice by means of histological sections and Indian ink-injected, cleared, whole mounts. The tumour vessels were always primitive sinusoids with relatively small amounts of connective tissue around them.

The development of the transparent ear chamber technique, Clarke *et al* (1931), made it possible to study the development of tumour vascularity *in vivo*. Thus Ide *et al* (1939) studied

the growth of explanted Brown-Pearce rabbit epitheliomas, and observed marked proliferation of vessels especially at the periphery of the tumour within 3-8 days following implantation. Tumour growth was grossly visible within 24 hours following the beginning of blood vessel growth. Blood vessel growth was orderly and characteristic, following a definite corona-like arrangement not observed in the normal tissues used as controls. All the normal mechanisms of blood vessel growth were found, such as buds, loops and anastomoses, but the speed of development was greatly increased over that of the controls. Control tissue transplants (liver, skin and kidney) were observed under the same conditions. Vascular development was observed 6 days after implantation of a piece of liver. This initial response did not develop and by the 14th day the liver fragment had been completely absorbed, so that all that remained was the pattern of the original stroma. Implants of skin and kidney also failed to elicit a sustained vascular development.

Though Goldmann, and Lewis, and Ide, Baker and Warren, had shown to the contrary, it was still thought that tumours had a poor blood supply. It was not until the significant works of Algire and Chalkley (1945) that tumours were thought to have a 'superior' blood supply in comparison to normal tissue. They used the transparent chamber technique to study tumour growth in vivo and compared it with the surrounding connective tissue, healing wounds, normal subcutaneous tissue, and liver tissue grafts. They observed that in transplanted sarcomas and mammary gland carcinomas, vascular proliferation occurred as early as 3 days after implantation compared with 6 days for wounds and subcutaneous tissue. Most of the newly-formed vessels consisted of endothelial channels of wide diameter with little or no differentiation into

arterioles or venules. The vascular density of the tumour implant increased rapidly to almost double that of the surrounding tissue and then remained fairly constant at this higher level. From such observations, the concept of "preferred nutritional status" of solid tumours arose.

They also suggested that the primary difference between normal and malignant cells was that the latter had an inherent capability of eliciting continued new capillary endothelium from the host. However, this premise was soon contradicted by Williams (1951) studying the V_2 carcinoma of Kidd and Rous grown in the rabbit ear chamber, where it was found that this tumour had no ability to stimulate the growth of vessels.

Many authors have reported progressive vaso-proliferation of neoplasms but the source and nature of the stimulus to the host's vessels are unknown. This gave rise to the hypothesis that humoral substances induce vasoproliferation (Chalkley, 1948; Williams, 1959; Urbach and Graham, 1962 and Warren and Shubik, 1966).

The first evidence for the humoral induction of tumour blood vessels was demonstrated by Greenblatt and Shubik (1968). Vasoproliferative activity was consistently seen in hamster cheek pouch stroma adjacent to tumour implants, despite separation of the stroma and tumour by a millipore filter which prevented the passage of cells. Similar results were reported by Erhmann and Knoth (1968) using choriocarcinoma and Warren et al (1972) with Fortner melanoma, both in the hamster cheek pouch. Folkman (1971) has recently isolated from human and animal tumour cells a humoral factor which is mitogenic for capillary endothelium.

This tumour-angiogenesis factor (TAF), when injected continuously into the dorsal air-sac of a rat, induced new capillary proliferation within 48 hours. TAF, consisting of protein (10%), ribonucleic acid (25%), carbohydrate (50%), and the rest lipoidal material, has not been isolated in normal tissue with the exception of human placenta.

These results gave rise to the possibility of inhibiting tumour growth by antagonising the action of TAF, a concept known as anti-angiogenesis.

Evidence was accumulated to show that neovascularization was a necessary condition for the growth of a solid tumour in two experimental conditions.

In the first case Greene (1941) transplanted heterologous tumours in guinea pig eyes and observed that some implants which did not vascularize, failed to grow for more than 1 year. However, when these dormant tumours were re-implanted into their original host, progressive vasoproliferation and tumour growth occurred. Similarly, when Gimbrone et al (1972) implanted small fragments of Brown-Pearce carcinoma directly on the iris in rabbits, the tumours vascularized and grew characteristically, destroying the eye within 2 weeks. In contrast, when implants were placed in the anterior chamber, they remained dormant for up to 6 weeks. When re-implanted on the iris, vascularization was followed by rapid invasive growth.

The second experimental situation involved isolated preparations of dog thyroid or intestinal segments which were perfused on average for 11 days with a mixture of human haemoglobin, calf serum and Eagle's medium. Folkman et al (1962 and 1963) demonstrated that these isolated perfused

preparations would accept a tumour from another species. Implants of mouse melanoma grew only to a small size (2-3mm diameter) but no further, as a result of failure of neovascularization. Histological studies invariably demonstrated degeneration of capillary endothelium in these perfused tissues - Gimbrone et al (1969) and Folkman et al (1966).

As a result of such observations, Folkman and his group are currently investigating methods of inhibiting the production of TAF and preventing tumour vascularization and therefore tumour growth.

A number of investigators have studied the vasculature of tumours by utilising either angiography or post-mortem injection of organs with radio-opaque dyes and subsequent radiography. Sampson (1912) injected radio-opaque lead and bismuth in his investigation of the blood supply of uterine myomata.

Kanno (1934) studied the effects of transplanting rabbit sarcoma on the blood vessels of various "host" organs in the rabbit, whilst Hasegawa (1934) concentrated on the blood supply of subcutaneous implants of sarcomat ous tissue also in the rabbit.

One of the most extensive angiographic studies was carried out by Shinkawa (1939) in which he reported a marked increase in arterial blood supply during the early stages of growth of transplanted fowl sarcomas. This paper also contains an extensive review of angiographic references from 1910 to 1932. In accordance with Braithwaite (1958) and Margulis et al (1961) these authors reported that

haemorrhagic zones, as manifested by leaks of radio-opaque material, were a constant and prominent feature especially near the centre of the tumour.

Angiography has been found to be extremely useful in the study of bone tumours in humans. (Farinas, 1937; Inclan, 1942; Dos Santos, 1950; Lasser and Schowingen, 1955; Schobinger et al, 1958.)

Billing and Lindgren (1944) and Lindgren (1945) combined radiography with histology to study the vascular supply of several human tumours. Tumour vessels were generally primitive, their walls lacking elastic lamellae. These workers also suggested that the capillary angioarchitecture of tumours was characteristic to a certain extent. This was especially true for highly differentiated tumours - e.g. hemangiomas. Margulis et al (1961) found similarly that various mouse tumours had characteristic angiographic appearances. For example, angiograms of sarcoma 37 showed many randomly arranged, dilated blood vessels interspersed with ill-defined 'lakes' filled with contrast medium. On the other hand, angiograms of squamous cell carcinomas showed only a few large vessels with no 'lakes'.

Rubin et al (1964) pointed out the discrepancies of microangiography as a technique, in that the vascular architecture of tumours was not demonstrated under physiological conditions. They also stressed the importance of standardising the variables of any angiographic technique, i.e. making sure that the contrast medium was injected at the same pressure and for the same duration if a comparison of tumour vascular patterns was to be made.

However, microangiography has been employed in the study of the microcirculation of tumours, Lagergren et al (1958); McAlister and Margulis (1963). Rubin and Casarett (1966) gave a detailed study of the Walker carcinosarcoma and the Murphy-Sturm lymphosarcoma tumours in rats. The vasculature in the Walker tumour was irregular and disorderly and confined to a thin outer cortex surrounding a central zone of necrosis. In contrast, lymphosarcomas showed little necrosis and a regular and orderly vasculature. They also suggested that there was an improvement of the tumour microcirculation following a course of radiation. Similar results were reported by Reinhold (1971) with the microcirculation of a mouse mammary tumour.

In summary, the blood vessels of transplanted tumours consist of the pre-existing vessels of the host and newly-formed vessels derived from them. In the host tissues, veins often suffer invasion and occlusion whilst the arteries long remain intact. As reviewed, the newly-formed tumour vessels have been described as tortuous, irregular and sinusoidal with a disorderly or chaotic branching arrangement. Newly-formed vessels in malignant tumours are usually small and often simple in structure, especially in the more rapidly growing tumours where the vessels consist of little more than irregular channels lined either by endothelium or by tumour cells alone. Many tumours, whilst growing in their peripheral regions, develop a central zone of necrosis, due to an inadequate growth of new blood vessels or a suicidal occlusion of the vessels already present.

MATERIALS AND METHODS

Lewis lung carcinoma (3LL)

This tumour originated spontaneously as a carcinoma of the lung of a C57B1 mouse in 1951. The tumour, as described by Sigiura and Stock (1955), is a very malignant type of epidermoid carcinoma (anaplastic carcinoma) which becomes extremely haemorrhagic. Subcutaneous injections of fresh blood of mice bearing the Lewis lung carcinoma into new hosts produce identical tumours. This tumour has been transplanted routinely in the Department of Cancer Chemotherapy since 1966.

Tumours were inoculated subcutaneously by means of a Bashford needle into the flanks of inbred C57B1 female mice weighing between 18 and 22g. Each implant was approximately 0.1ml in volume and weighed about 0.1gm. Aseptic precautions were observed.

Experimental mice received ICRF 159 30mg/kg suspended in 0.2ml of carboxymethyl cellulose (0.5%) in isotonic saline (CMC) given intraperitoneally. Control mice received 0.2ml of CMC alone intraperitoneally.

The first dose of the compound was administered 24 hours after implantation and the schedule of injections was that used routinely in the Department of Cancer Chemotherapy (Table 1).

TABLE 1

Days after implantation on which injections were given (inclusive)	Days after implantation on which primary tumours removed
1 - 4	7
1 - 4, 7 - 9	10
1 - 4, 7 - 11	14
1 - 4, 7 - 11, 14	21

Walker carcinosarcoma

This rapidly growing tumour developed spontaneously in the region of the mammary gland of a pregnant albino rat in 1928 in the laboratory of George Walker at the John Hopkins University School of Medicine. This tumour, as described by Stewart et al (1959), is an adenocarcinoma and is characterised by a large central necrotic zone surrounded by a thin viable zone usually only a few millimetres thick. The tumour has been maintained in this Department in an ascites form.

The tumour was maintained by weekly ascites transplantation to male Sprague-Dawley rats weighing between 150 and 200gms. When solid Walker tumours were required 0.3ml of ascites buffy coat (ascites fluid was centrifuged at 2,500 r.p.m. for 5 minutes) were injected subcutaneously into the flank. Tumours studied ranged from 4 to 10 days old.

Experimental rats received ICRF 159 50mg/kg suspended in 0.4ml of CMC solution given intraperitoneally daily. Controls received 0.4ml of CMC alone.

In all experiments food and water were given ad libitum throughout.

1 Radiographic procedure

Ninety-two C57Bl mice with 3LL tumours were used for angiographic studies of which sixty-seven angiograms were of sufficiently high standard to be used for evaluation.

Thirty Sprague-Dawley rats with Walker tumours were also used, of which sixteen angiograms were of a sufficiently high standard.

Various methods for the injection of contrast media in bromethol (Avertin) anaesthetised animals were tried, but retrograde intravenous injection proved most effective in demonstrating tumour blood supply. Micropaque (aqueous barium sulphate about 1oz/fl oz (55g/50ml)) was injected through a polythene catheter introduced into the inferior vena cava through the exposed right heart (Margulis et al, 1961). Care was taken to give the contrast medium in the same volume and identical manner each time.

Animals were placed in a supine position on a transparent tray with the X-ray tube 18in (46cm) above. X-ray film was placed on a shelf 18in (46cm) below and each radiograph was therefore twice life-size. Focal spot of the X-ray tube was 0.3mm and the exposure was usually:

Mice - 12 mAS 50 mA 42KV

Rats - 32 mAS 40 mA 48KV

Each film was developed for 6 minutes, overdeveloping by 1 minute to obtain contrast (Parish et al, 1964).

11 In vivo blood vessel labelling

Sixty-four C57Bl mice with 3LL tumours and eighteen Sprague-Dawley rats with Walker tumours were used in these experiments.

The Pelikan ink technique (Majno *et al*, 1961) was used to study the physiological state of tumour blood vessels *in vivo*.

A colloidal suspension of carbon black (Pelikan biological ink C11/143a) diluted 1:4 with 1% gelatine in sterile saline was used (Oswald and Cater, 1969). This suspension contains about 10mg/ml of carbon with an average particle size of 200Å; it is stabilised with 4.5% fish glue and contains 1.3% phenol as a preservative.

Mice (C57Bl) inoculated with 3LL tumour and treated with ICRF 159 or CMC received 0.12ml of the diluted ink per 20gm mouse into the lateral tail vein. 0.75ml(per 150gm Sprague-Dawley rat)of the diluted ink was similarly injected i.v. into rats inoculated with the Walker carcinosarcoma treated with ICRF 159 or CMC.

The animals were left for 1 hour by which time the ink was cleared from the circulation. The animals were then killed, the tumour and surrounding tissue were quickly removed, placed in 10% formol saline for 14 days and then washed in distilled water for 24 hours before being transferred to 12.5% gelatine at 37°C for 24 hours and then for another 24 hours into 25% gelatine also at 37°C. The blocks were embedded in 25% gelatine and stored in formol saline. Frozen sections, cut at 25-35μ were lightly

stained with alum carmine and mounted in Highman's mountant. At least eight frozen sections cut randomly from each tumour were examined.

Random areas of the 3LL primary tumours were examined with the electron microscope in order to locate the position of the ink within the blood vessels.

The tissues were fixed in 4% glutaraldehyde in phosphate buffer for 1 hour and then post-fixed for a further hour in phosphate buffered osmic acid, and embedded in Araldite.

Sections (about 400Å) were cut on a Porter-Blum microtome equipped with a glass knife and stained with 25% uranyl acetate in methanol for 10 minutes - washed in methanol and then further stained in lead citrate for 4 minutes and washed in distilled water. Sections were lightly coated with carbon and examined in Siemens Elmiskip 1 electron microscope.

Some 3LL tumours were excised 5 minutes after intravenous injections of ink, and thick cryostat sections, stained with neutral red, were examined.

111 Intravital staining with Lissamine Green

The technique described by Goldacre and Sylven (1959 and 1962) using the triphenyl methane dye, lissamine green, was employed for studying tumour blood supply.

The dye is non-toxic and non-reactive and can be tolerated by tumour bearing animals even when injected in large amounts.

The dye, which readily enters the interstitial fluid, colours the animal and the vascularized part of the tumour within seconds of i.v injection or within 30 minutes of i.p. injection.

Thirty-six mice inoculated with 3LL tumours and treated with ICRF 159 or CMC received 0.5-1ml of a 2% lissamine green solution, rapidly injected into the tail vein.

Up to 5 millilitres of 2% lissamine green solution was similarly injected into twenty rats inoculated with the Walker carcinosarcoma and treated with ICRF 159 or CMC. The animals were exsanguinated under ether 5 minutes after the injection of lissamine green. The tumours were bisected and examined.

RESULTS

X-ray angiography

Injection of Micropaque by the tail vein proved difficult and the radiographs were difficult to interpret because the contrast medium had circulated around the heart and thus into the arterial system (Fig.7).

Arteriograms taken after injecting Micropaque down the aorta demonstrated the larger arteries of the body but not those of the tumour (Fig.8).

Retrograde intravenous injections of Micropaque proved most effective in demonstrating the blood supply of control and ICRF 159 treated tumours.

The following observations were made on 3LL venograms:

Host vessels: No new vessels were seen to develop in response to the tumour, but host vessels draining the tumour were constantly dilated and some of these vessels became very tortuous (Fig.9). Identical appearances were seen in animals where the tumour was treated with ICRF 159. At the border of the tumour, angiographic appearances became very indistinct owing to vascular proliferation (Fig.10). It was not possible to distinguish whether this proliferation was in the host or the tumour tissues. Again the angiographic picture was the same for both control and treated tumours.

Tumour vessels: Control tumours showed large filling defects whose size and position varied from tumour to tumour. Such vascularization as there was appeared to be

Fig. 7.

Vascular system of a C57B1 mouse bearing a 10 day 3LL tumour. Micropaque injected into the tail vein circulated round the heart and into the arterial system.

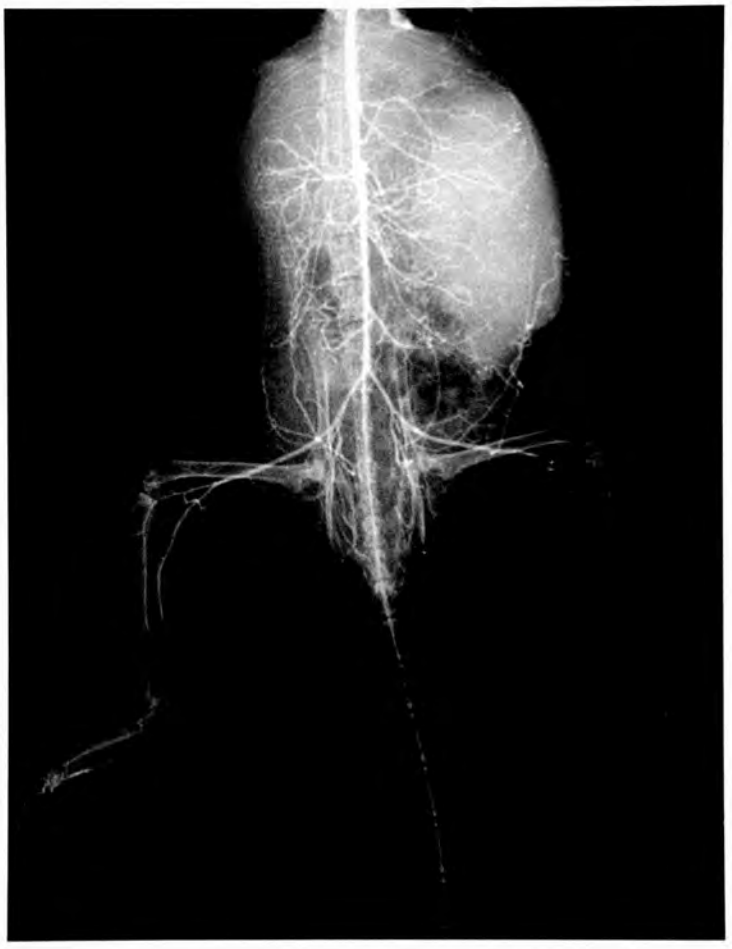


Fig. 8.

Arteriogram of a C57Bl mouse bearing a 14 day 3LL tumour. Little detail of the tumour vasculature can be seen.

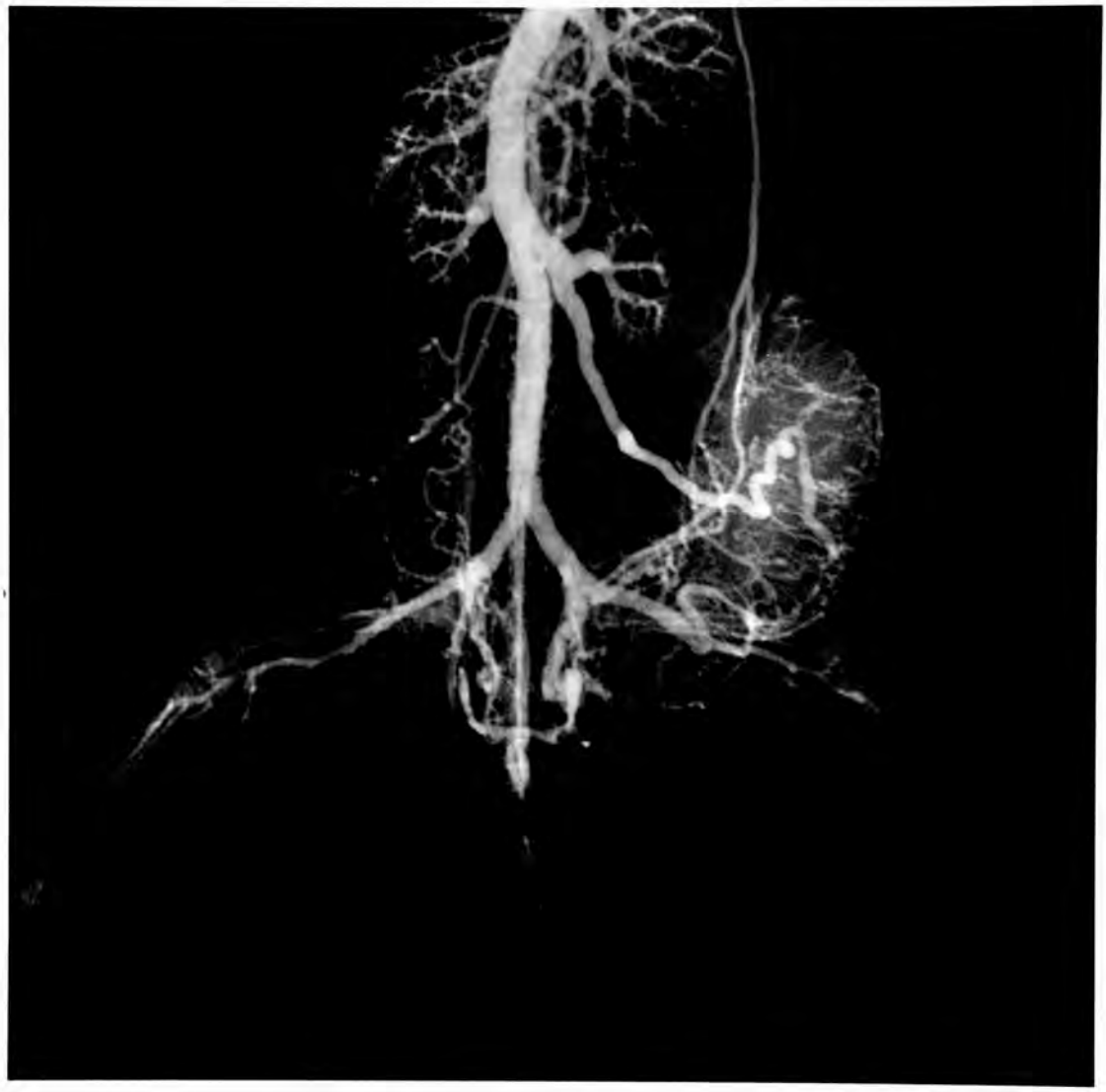


Fig. 9.

Venogram of a primary 3LL tumour 14 days after implantation and treated with ICRF 159. Note the dilated vessels draining the tumour and the tortuous appearance of one of these vessels.

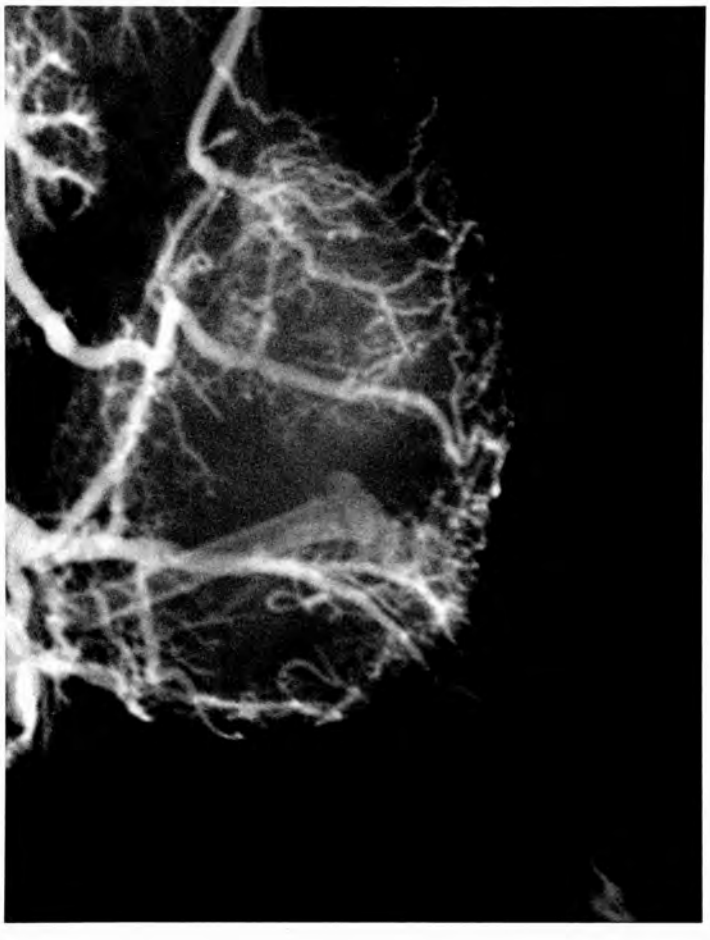


Fig. 10.

Vascular proliferation at the periphery of a 21 day 3LL control tumour.

randomly arranged with vessels often ending abruptly (Fig.11). This proved to be the constant picture in the control tumours. When the tumour was cut at apparently avascular areas, Micropaque flowed out from the cut surfaces. Tumours of the treated mice, however, showed numerous discrete blood vessels with some indication of a regular vascular arrangement (Fig.12).

Walker tumour host vessels: Large new vessels did not develop in response to the tumour. On the other hand, three main host vessels - the lateral thoracid-cutaneous, the ilio-lumbar, and the inferior epigastric veins - were always considerably dilated, presumably to meet the demands of outflow from the tumour (Fig.13). Essentially the same picture was seen in ICRF 159 treated and control tumours. At the margins of the tumour, however, there was considerable proliferation of blood vessels in both treated and control tumours (Fig.14).

Tumour vessels: In the tumours themselves, there was a marked contrast between treated and controls. The control tumours showed signs of pooling of the Micropaque; there were avascular areas of varying size and position. The dilated vessels were randomly arranged and irregular, all of which is characteristic of tumour blood vessels (Fig.15). The treated tumours, however, showed no signs of pooling and the blood vessels presented a more orderly arrangement. There was little evidence of any cork-screwing (Fig.16).

Pelikan ink labelling

3LL

In thick frozen sections of primary treated tumours

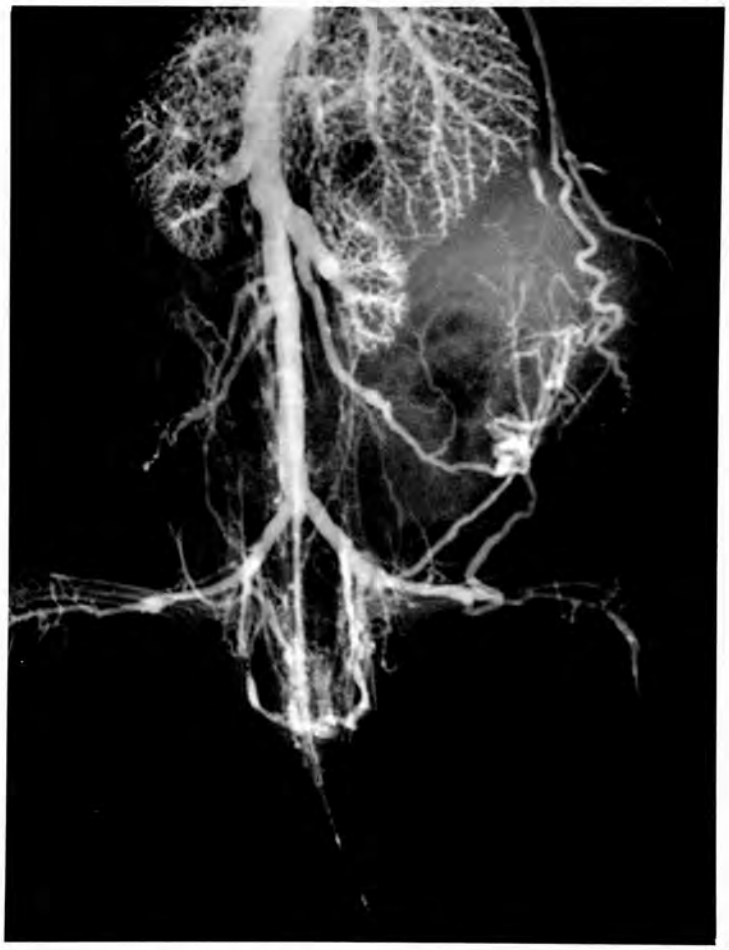
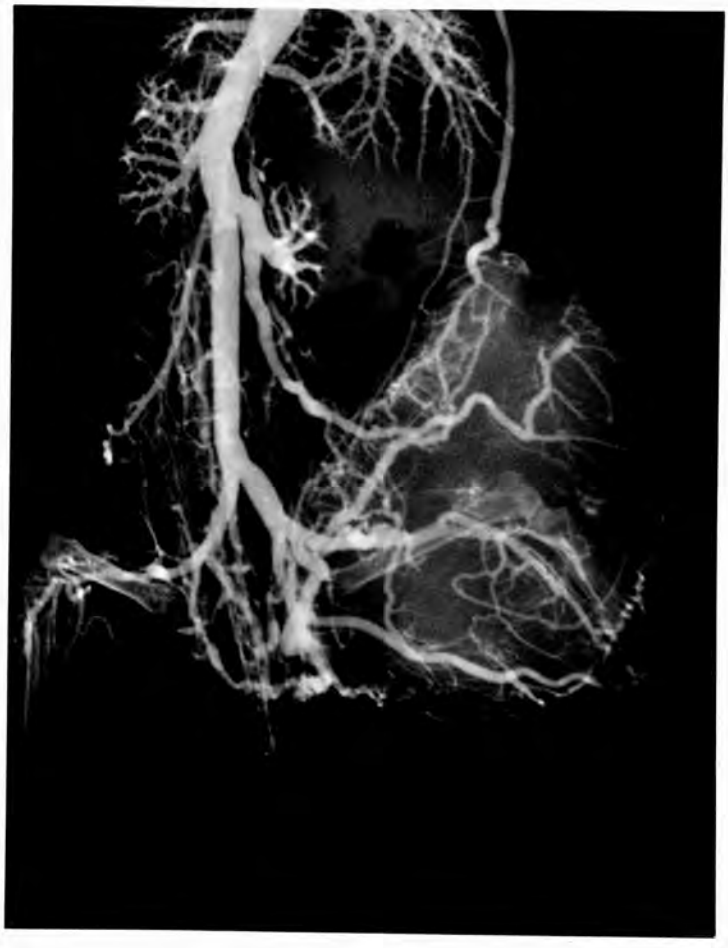


Fig. 11.

Venograms of control 3LL tumours.

14 day tumour

Note in both cases there are areas of the tumours which are apparently avascular. Also note that many of the tumour vessels end abruptly.



21 day tumour

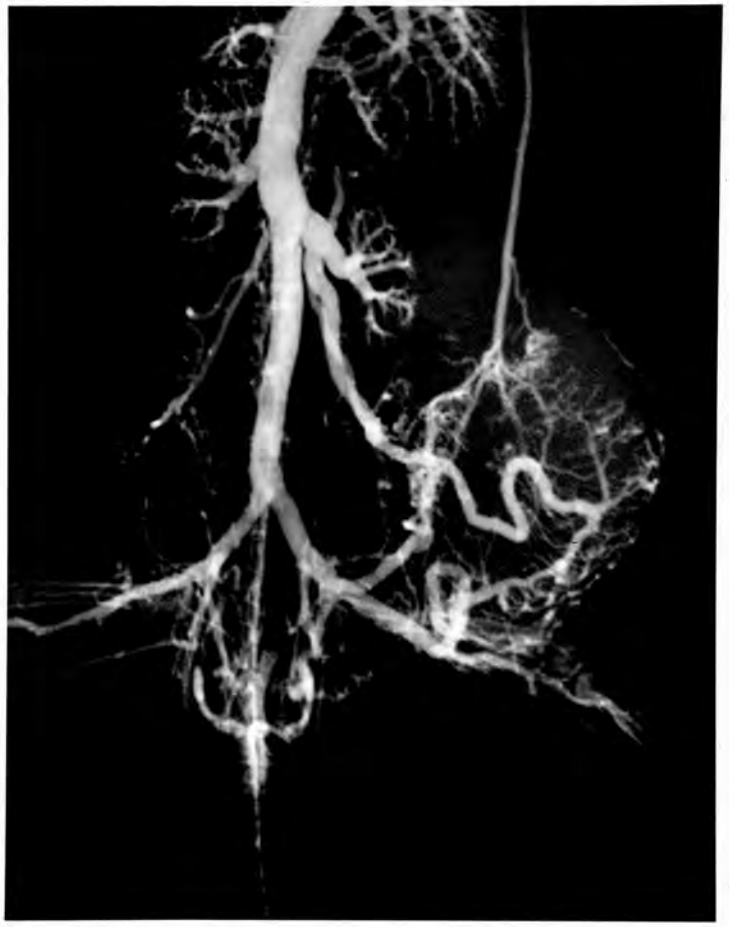
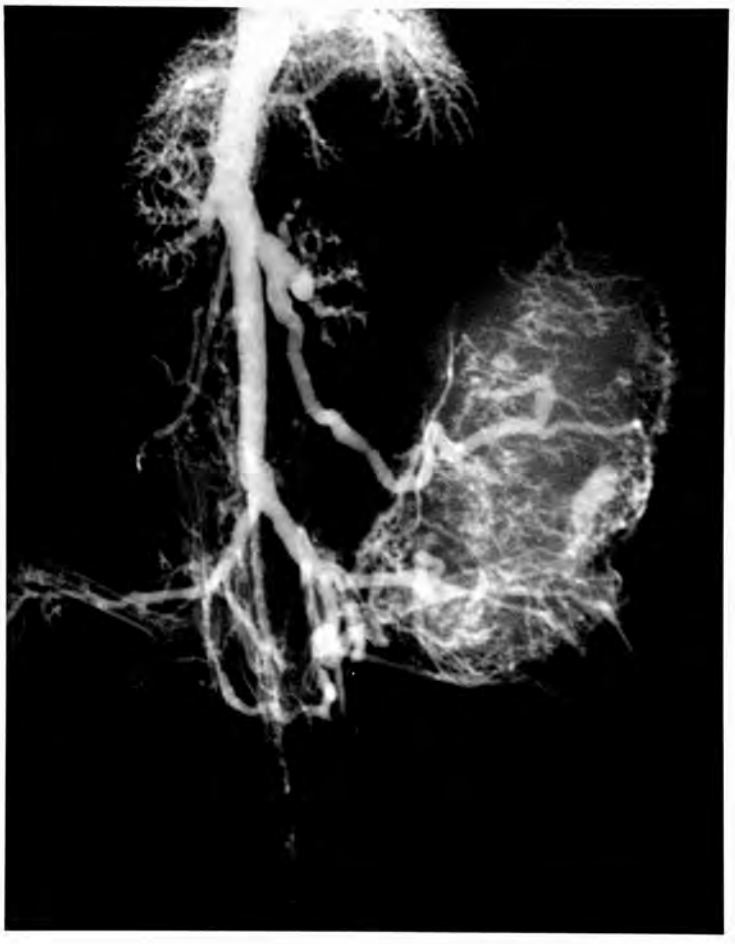


Fig. 12.

Venograms of ICRF 159 treated 3LL tumours.

14 day tumour

Note that the tumours are well vascularized with some indication in the 14 day tumour of a regular vessel arrangement.



21 day tumour

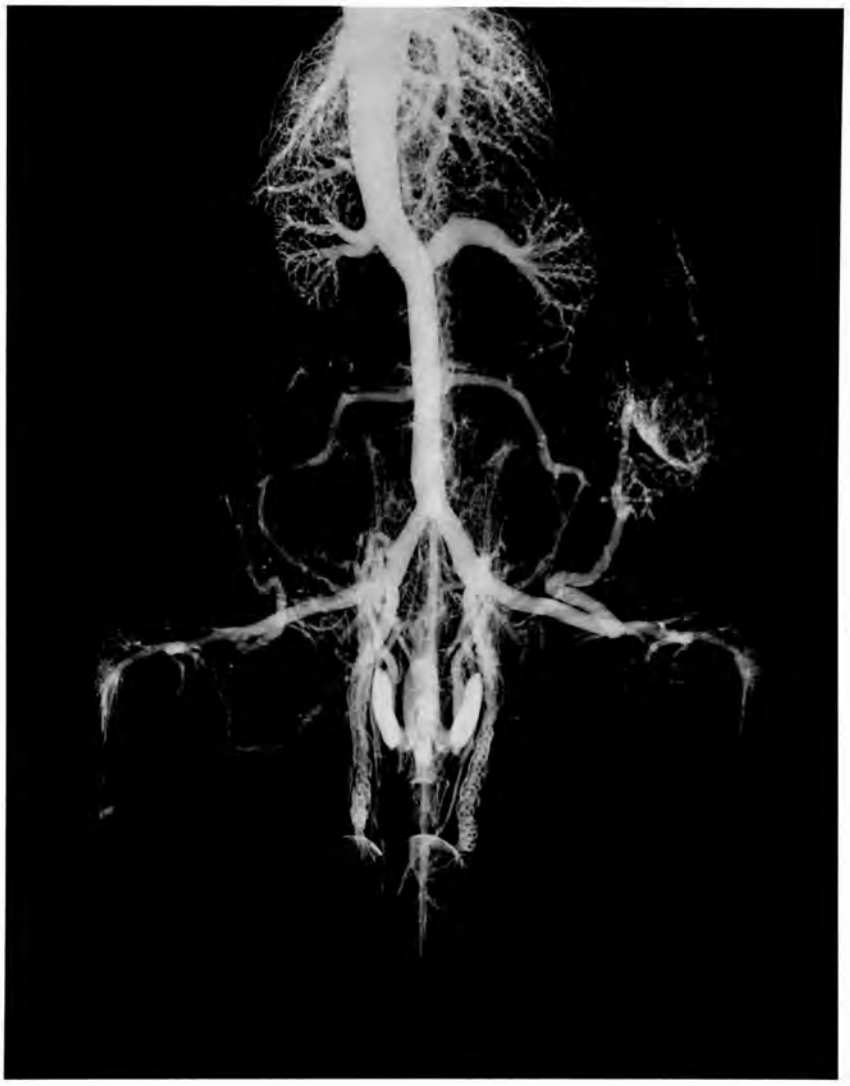


Fig. 13.

Venogram of a Sprague-Dawley rat bearing a 7 day control Walker tumour. Note again the three main vessels draining the tumour.

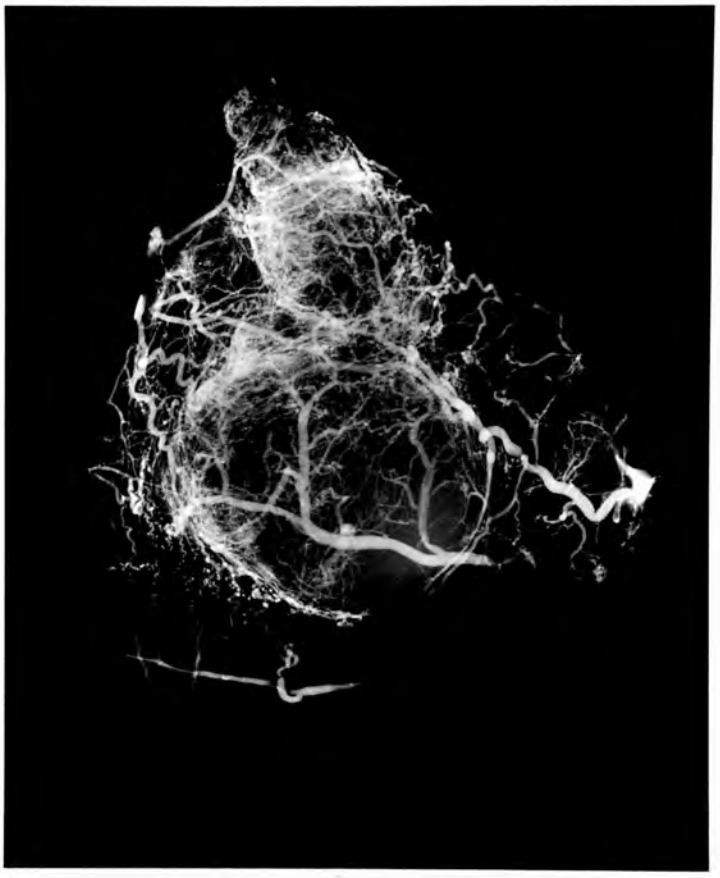


Fig. 14.

Marked proliferation of vessels at the periphery of an excised 8 day control Walker tumour.

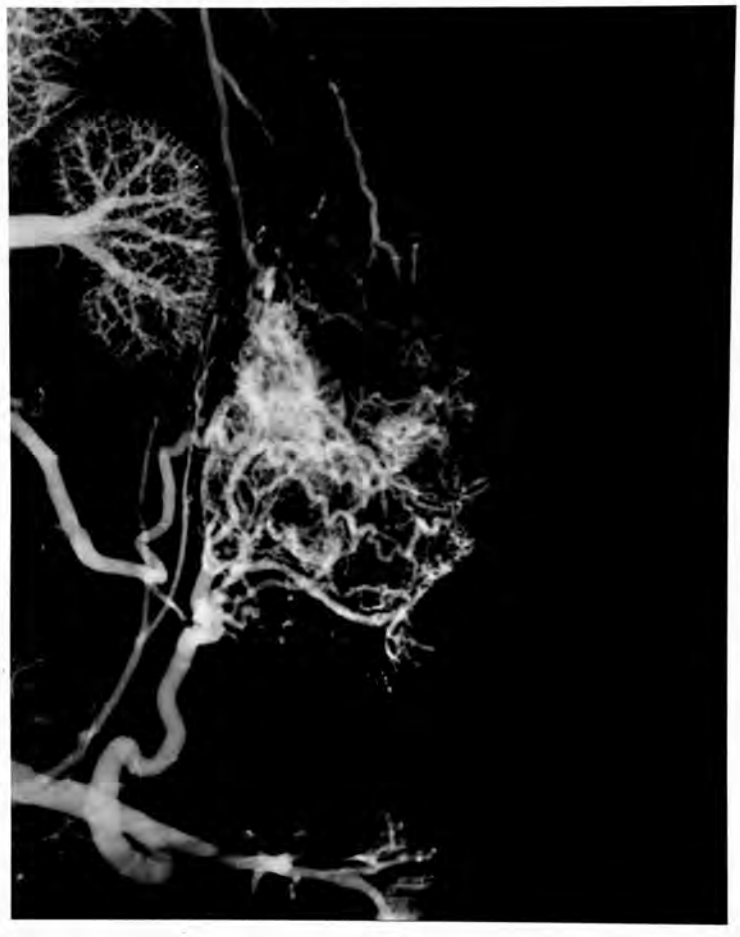


Fig. 15.

Venogram of a control Walker tumour 7 days after implantation. Note the large avascular areas and the pooling of Micropaque. Many of the vessels are cork-screw in appearance whilst others end abruptly.

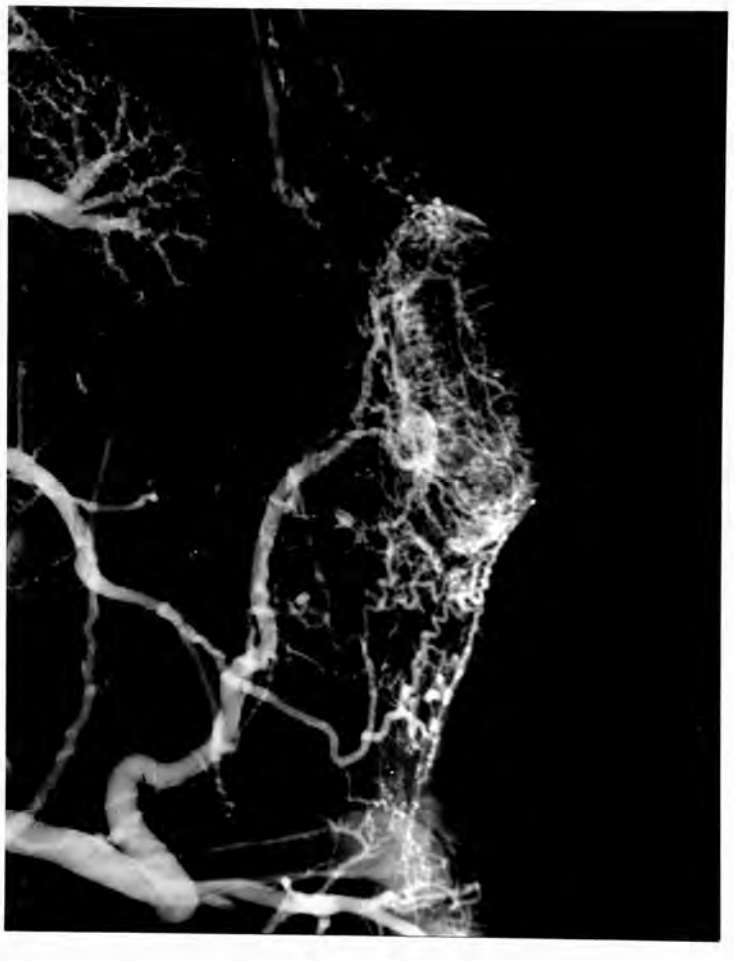


Fig. 16.

Venogram of an ICRF 159 treated Walker tumour 7 days after implantation. There are no avascular areas and no signs of pooling.

the ink was largely cleared thus showing little labelling (Fig.17) but in control tumours, vessels were clearly outlined with ink (Fig.18). With older and therefore more necrotic tumours (21 days) the differences between the groups became much less apparent (Figs.19 & 20).

Electron micrographs of control tumour vessels showed deposits of carbon trapped between basement membrane and an abnormal endothelium (Fig.21).

Walker tumour

With this tumour, treatment with ICRF 159 also reduced, but by no means abolished, the number of labelled vessels. Marked vascular labelling was always found in both control and treated tumours near areas of haemorrhage and necrosis. However, with this rapidly growing tumour, the results are more difficult to interpret because anatomical flaws in the labelled vessels are a real possibility due to their rapid formation. Fig.22 demonstrates the ink trapped round the periphery of the tumour blood vessels.

Intra-vital staining

3LL

Gross observations revealed no differences between control and treated tumours at similar ages after tumour implantation. As the tumour increased in size the peripheral vascularized zone became markedly reduced. There were no avascular, colourless, areas in the tumour periphery.

The youngest tumours (7days, approx.0.8cm across) were usually coloured throughout, whereas in the oldest

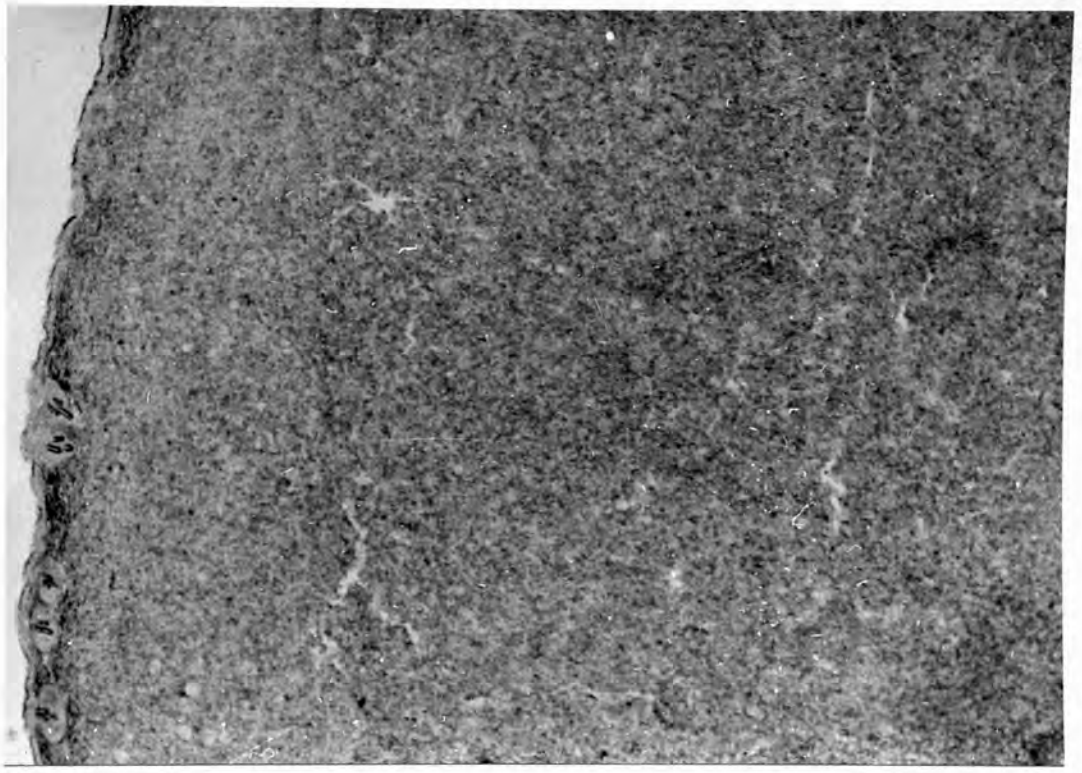


Fig. 17
Frozen section of a primary 3LL tumour 14 days after implantation and treated with ICRF 159. The tumour was excised 1 hour after i.v. injection of Pelikan ink. (Alum carmine x 50.) Note the absence of vascular labelling.

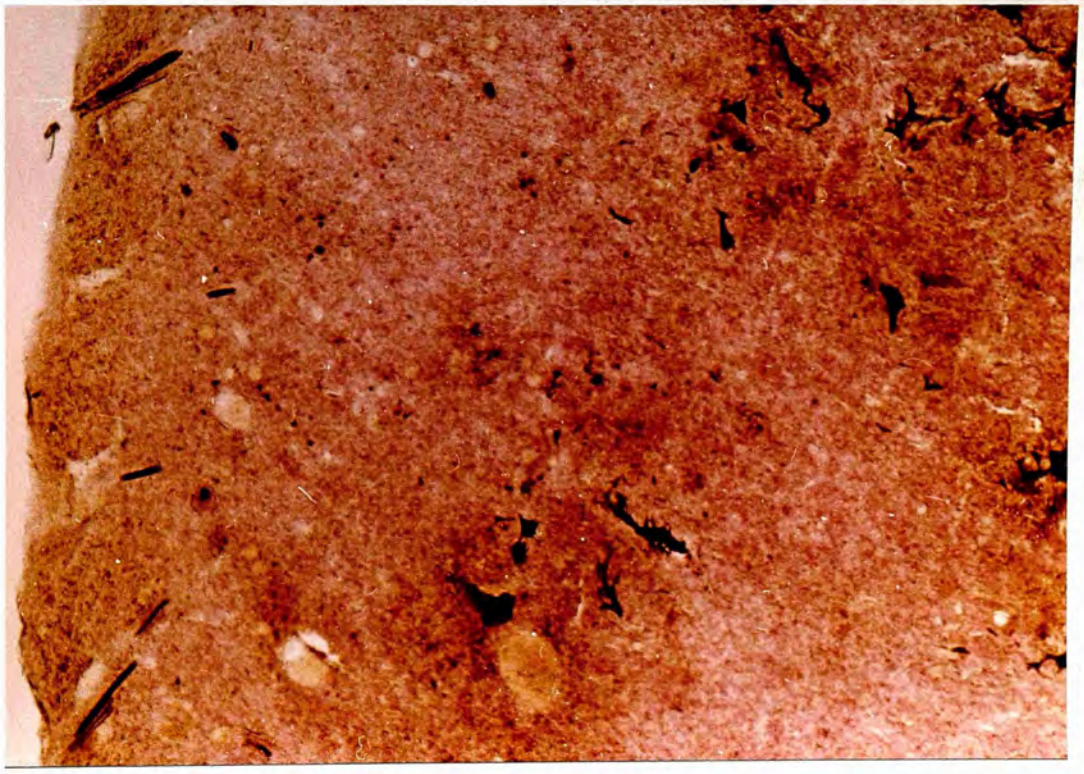


Fig. 18.
Frozen section of a primary 3LL control tumour 14 days after implantation. The tumour was excised 1 hour after i.v. injection of Pelikan ink. (Alum carmine x 50.) Many vessels are clearly outlined with ink.

Fig. 19
21 day treated 3LL tumour - some vessels are clearly outlined. (Alum carmine x 50.)

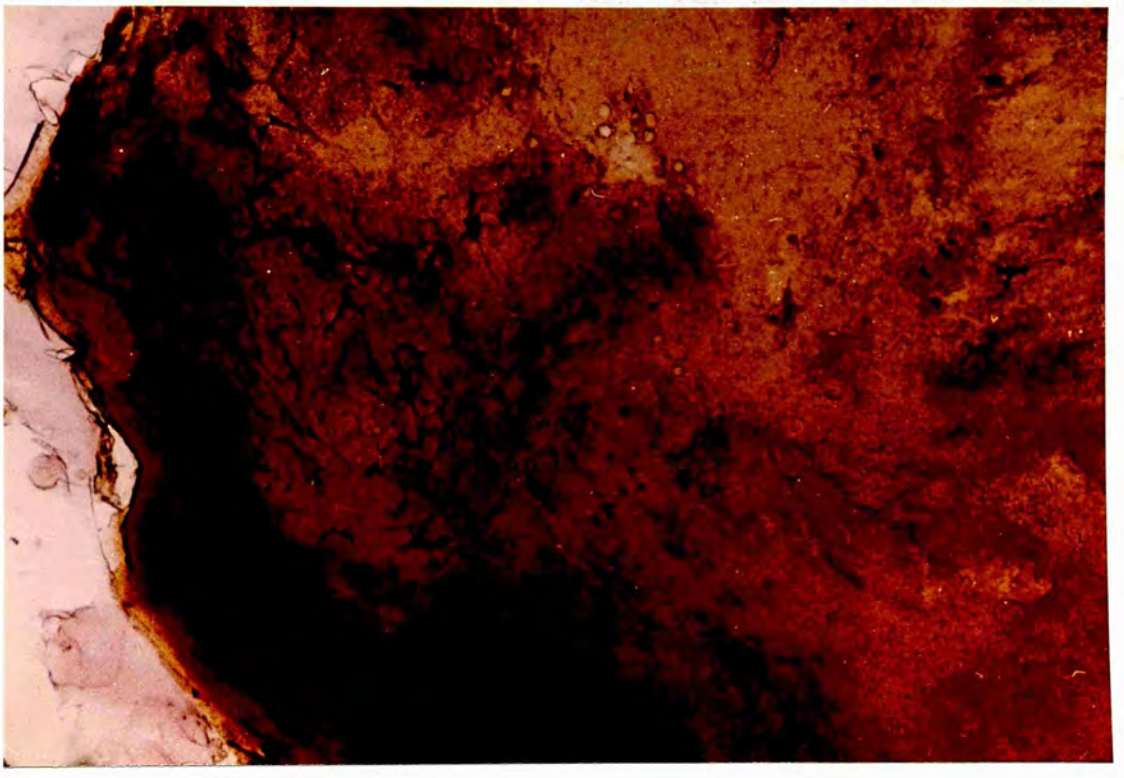


Fig. 20
Section from 21 day control 3LL tumour showing some labelled vessels and some extravascular carbon. (Alum carmine x 50.)

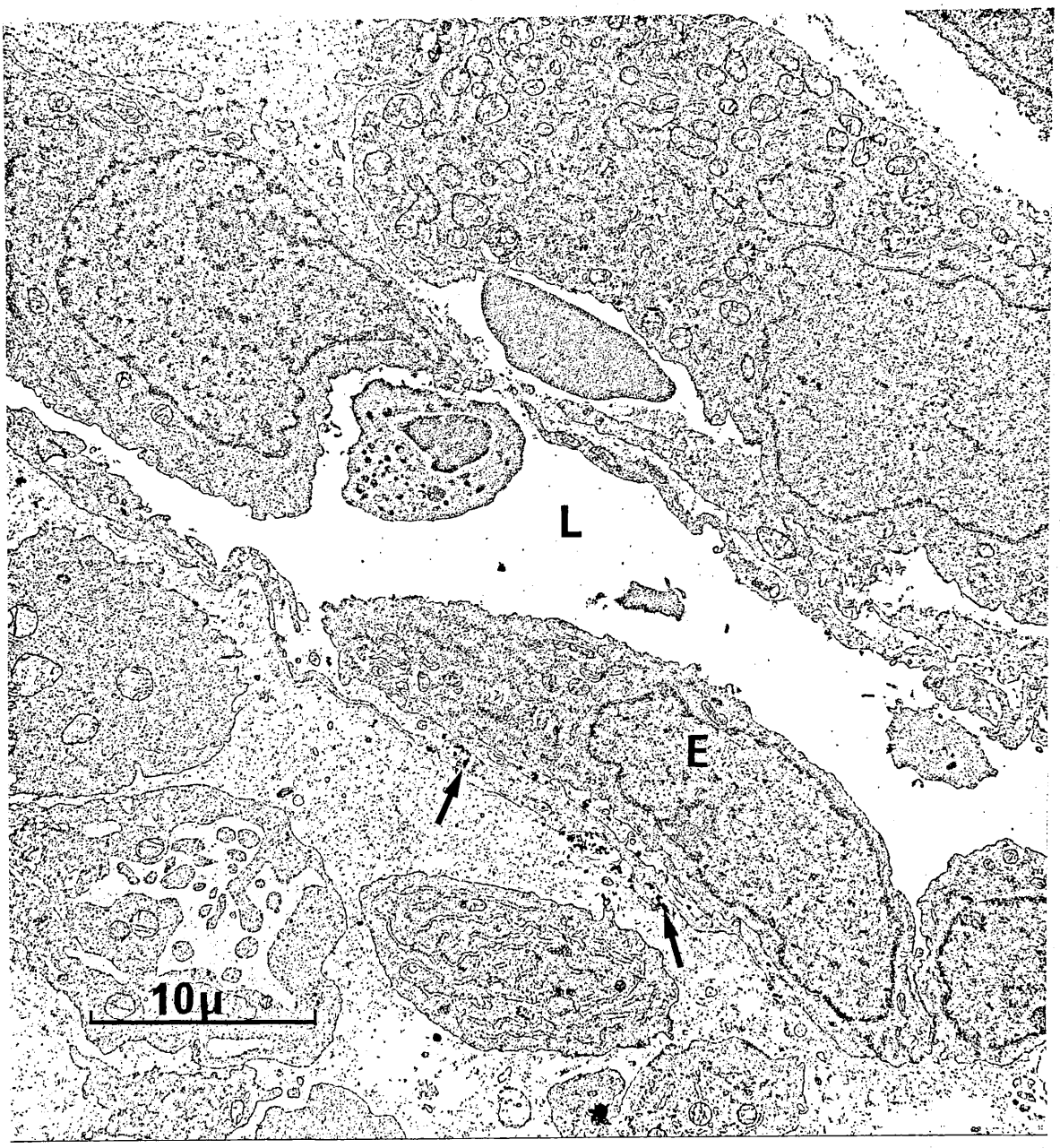


Fig. 21.

Carbon particles trapped between the basement membrane and an endothelial cell of a 14 day control 3LL tumour blood vessel.

e - endothelial cell

l - lumen of the blood vessel

Plate magnification = 1K

Print magnification = 3½K

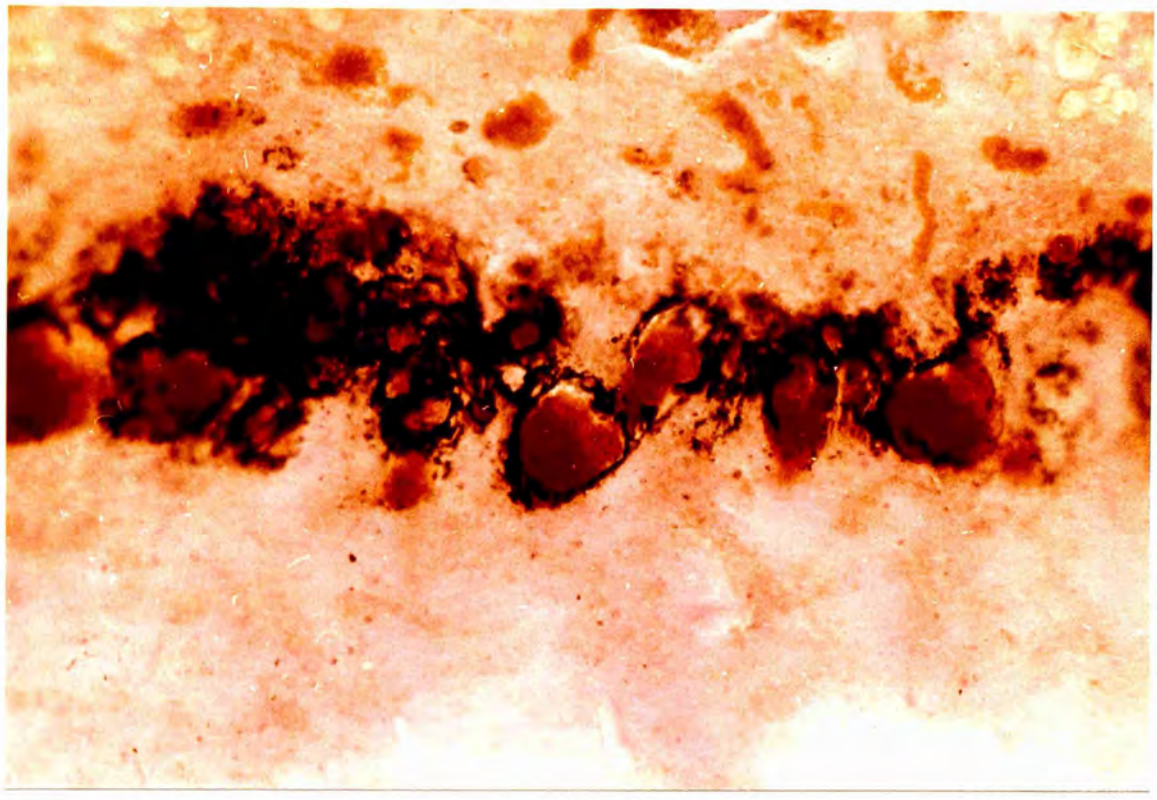


Fig. 22

This section is taken from a 10 day old control Walker tumour and demonstrates the carbon particles trapped around the periphery of a number of blood vessels cut in cross section. (Alum carmine x 100.)

tumours (21 days, approx.2.0cm across) the peripheral vascularized zone was only a few millimetres thick. There was a complete absence of dye from the central necrotic region (Fig.23).

Walker tumour

Again gross observations revealed no differences between control and treated tumours. In Walker tumours ranging from 4 to 10 days old there was an increase in the size of the necrotic centre until in the largest tumours, only a very thin peripheral vascularized zone remained (Fig.24).

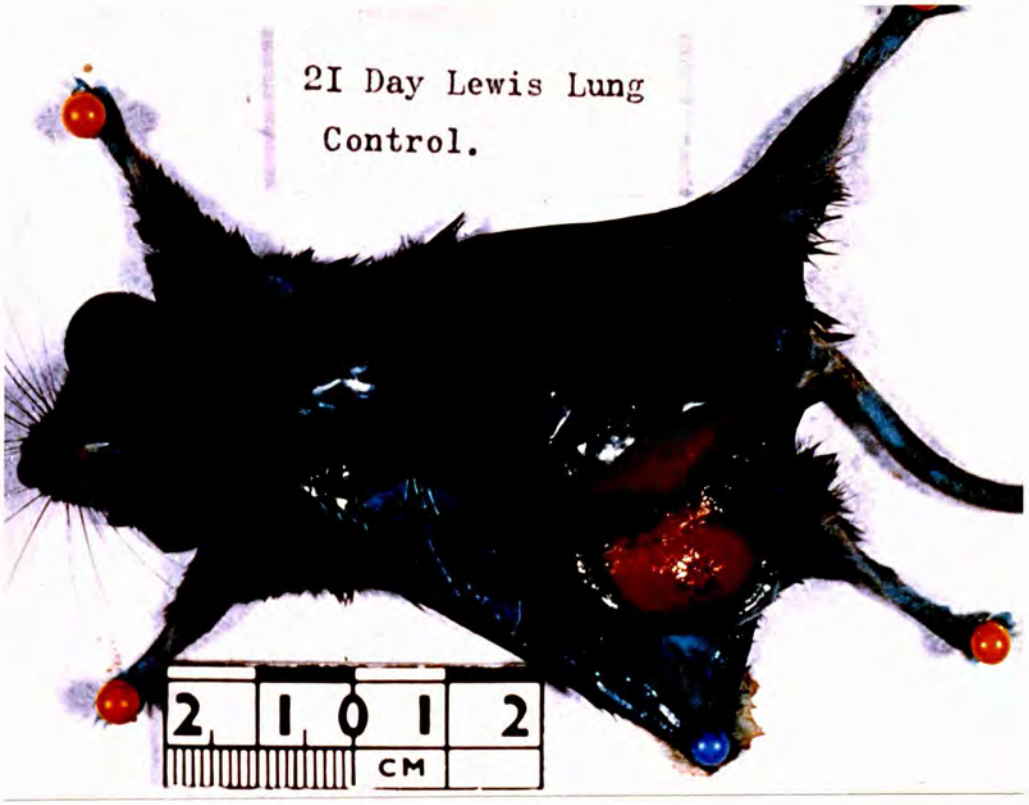


Fig. 23.

21 day control 3LL tumour showing a complete absence of dye from the central zone.

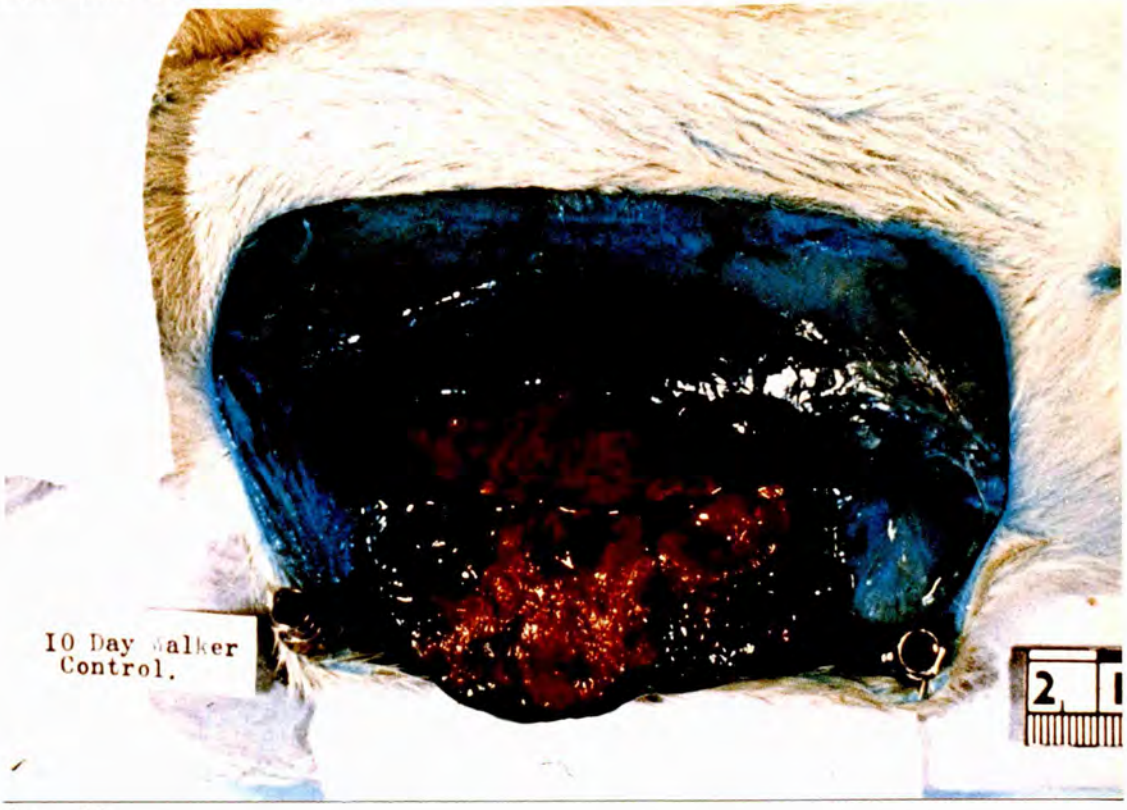


Fig. 24.

10 day control Walker tumour. Note the dye is confined only to a thin peripheral zone of the tumour.

DISCUSSION

Willis (1952) in his classic work on "The Spread of Tumours in the Human Body", points out that: "The structure of new-formed blood vessels in malignant tumours rarely approaches that of normal veins or arteries and the more rapidly growing the tumour, the more imperfect is the architecture of the vessels ..... in highly anaplastic growths, carcinomas as well as sarcomas, even the endothelium may be incomplete in places and the vascular channels lined in part by tumour cells". Clearly such imperfections of vascular structure must favour dissemination of malignant cells. Willis's statement applies with equal force to the Lewis lung carcinoma of the mouse (Salsbury et al, 1970).

Previous observations indicated however, that the structurally imperfect vascular architecture of the 3LL tumour can be improved by treatment with ICRF 159 (Salsbury et al, 1970), and the present results lend support to the assumption that treatment with this drug normalizes 3LL vasculature functionally as well as morphologically. They also strengthen the conclusion that treatment with ICRF 159 prevents pulmonary metastases, because malignant cells unable to penetrate the normalized blood vessels cannot reach the blood stream and are thus unable to escape from the primary tumour.

Although it seems clear that as in other tumours the 3LL and the Walker have an outer shell of rapidly proliferating tumour cells, permeated by a leash of imperfectly formed and randomly arranged vascular sinusoids, certain aspects of the angiograms call for comment, Le Serve (1971) and Le Serve and Hellmann (1972).

In 3LL control tumours, Micropaque diffuses into the wide spaces of the sinusoids in a "river-delta" effect, but because of the limitations of the resolution of the X-ray technique, radiopaque material cannot be demonstrated. The filling defects are therefore more apparent than real because if the tumours are sectioned at these "avascular" areas Micropaque flows out from the cut surfaces. In treated tumours, however, the Micropaque is confined to the discrete vascular channels and consequently can be seen on the angiograms. This difference in the tumour vascular pattern is more clearly seen with the larger Walker tumour.

In Pelikan ink studies also, where tumours were excised 5 minutes after i.v. injection, the ink distributed itself throughout extra and intravascular spaces in the controls, but remained confined to the vessels in the treated tumours. The lissamine green injections provide further confirmation that the filling defects in the control venograms reflect only inadequacies in the degree of fine resolution of the angiograms.

The lissamine green injections also reveal that in both treated and control tumours, vascularization is confined to an outer zone or cortex which encapsulates the tumours, but that the larger central and probably necrotic medulla appears not to be reached by the dye. It can be assumed therefore, that Micropaque is also confined to the peripheral zone and that the vessels seen on the angiograms are in the cortex. Thus a three dimensional picture of the tumour blood supply can be built up. The central, unstained part of these tumours is referred to as necrotic in this thesis, although, as demonstrated in Section V, this zone contains viable cells, despite an absence of functional blood vessels.

The abnormal character of tumour blood vessels and their functional normalization by ICRF 159 is indicated by the Pelikan ink experiments.

The Pelikan ink technique of Majno et al (1961) is used to outline inflamed vessels. The principle is that the ink injected i.v. will pass through gaps in the vascular endothelium caused by inflammatory agents, but will be retained by the intact basement membrane, thus outlining the vessels.

In this investigation as no inflammatory agents are administered, this technique gives an indication as to the "state" of the tumour blood vessels in vivo. Basically, there are four alternatives as to the fate of the ink:

1. In well-formed blood vessels unaffected by inflammatory agents etc., the ink will pass through and be cleared into the liver, spleen and kidney.
2. In vessels with a malformed endothelium, but an intact basement membrane, the ink will leak through any gaps and become trapped, thus labelling the vessel.
3. If the vessel has completely broken down with resulting haemorrhage, the ink will diffuse with the blood cells into the surrounding tissue.
4. Tumours and tumour vessels are in a constant state of pathological change. In the one hour allowed for the ink to clear from the blood system, some vessels will have been occluded and it is possible that ink could be trapped in that situation.

Goldacre and Sylven (1962) using injections of L.G., observed patent vessels containing intact red cells in

white tumour regions while the blood in the rest of the body was dark green - i.e. occluded but patent tumour vessels.

The E.M. study on the 3LL primary supported the second alternative for the ink's fate, showing the ink trapped between the intact basement membrane and the endothelial cells.

The venules have been found to be the main site of vascular leakage, even when the inflammatory agent is administered from the arterial side (Gabbiani et al, 1970). In these tumour vessels the labelling is non-specific. It is possible that the labelled vessels have anatomical flaws due to their rapid formation, but at the dose of ICRF 159 used in this experiment, there is little inhibition of tumour growth, so this would not explain the difference in the amounts of carbon found in control and treated tumours. It may be that the tumour vessels are inflamed by local effects of anoxia and necrosis. This would not account for the labelling to appear as early as Day 10 in the controls, that is, before the tumour has become very necrotic. ICRF 159 does not markedly decrease the degree of necrosis in the 3LL primary tumour anyway and so why is the labelling confined to the control tumour vessels? It is very important to distinguish between labelling in areas of actively dividing tumour cells and in necrotic areas. Labelling was noticed in the latter case, but was not thought to be of much significance because one would expect blood vessels to be in various stages of degradation when in close proximity to necrotic areas. It was the peripheral labelling that was thought to be functionally important.

The ink has been administered without inflammatory agents to give a guide as to the state of these tumour blood vessels *in vivo*. By means of histological evidence, we can see the differences caused by ICRF 159 on the blood vessels of the 3LL primary - that is, "the well-formed" endothelium (Salsbury *et al*, 1970), but there is no indication as to whether these vessels are well-formed in physiological terms.

This Pelikan ink test gives valuable information in that it tells us whether *in vivo*, the 159-treated tumour vessels are well-formed, or whether there are endothelial gaps, or whether endothelial breakdown is occurring. From this view, the lack of ink in the 159-treated tumours would suggest that the blood vessels are well-formed and that the ink has passed through the tumour, whereas in the controls there are real signs of disintegration of blood vessel structure. Similar results were reported by Dayal (1970) with the Gardner lymphosarcoma in which the absence of carbon labelling was linked with the normal appearance of the tumour blood vessels.

Specific alterations of tumour blood vessels could have considerable consequences for the treatment of tumours. This angiometamorphic effect may mean that in treated tumours the rate of blood flow is significantly higher than in control tumours and that consequently the availability of anticancer drugs might be increased. This consideration is examined in Section III of this thesis.

An attempt to grow two dimensional 3LL tumours

Many of the major works on the vascular architecture of tumours have involved the use of transparent chamber techniques, in which tumours grow as a sheet only a few millimetres thick. This makes it possible to observe microscopically the daily development of the tumour vascular system in the same animal. Also it permits the direct study of the tumour vessels under various physiological conditions.

With this in mind, experiments were set up in which attempts were made to grow thin 3LL tumours not in mice but on the highly vascular chorio-allantoic membrane (CAM) of chick embryos, as described by Tucker (1970). This would enable the vascular patterns of 3LL tumours to be established and to observe the daily effect of ICRF 159 on the development of the tumour vessels.

As the chick embryo is immunologically immature during the first two weeks of its development, heterologous tumours can be grown on the CAM without the problems of immunological rejection.

For the experiments, fertilised brown Leghorn eggs were incubated at 37.5^{o}C and candled on the eighth day to determine the position of a suitable vascular area of the CAM for tumour growth. Above this area of the CAM, a rectangle approximately 2.0cm x 1.5cm, was marked with a pencil and cut into the shell with a dental drill, taking great care not to damage the attached membrane. A small hole was ground in the centre of the rectangle through which a small drop of saline was gently manoeuvered. The

weight of the saline usually caused the membrane to drop away from the inside of the shell. If this did not occur, however, suction with a rubber teat, applied to a hole made in the air-sac end of the shell, aided the dropping of the CAM. The area of shell within the rectangle was then removed with sterile forceps leaving a window through which the required vascular area of the CAM was accessible. The windows in the shell were then sealed with Sellotape and the eggs put back in the incubator until required.

Various attempts were made using different techniques to grow two dimensional 3LL tumours.

A small volume (approximately $1mm^3$) of 3LL mash from 10 day old tumours was placed either directly on, or in close proximity to, various vessels of the CAM. Over the implant was placed a plexiglass diffusion chamber ring (14mm diameter and 2mm thick) with a coverslip cemented to the upper surface. This permitted direct observation of the tumour whilst limiting its upward growth (Fig.25).

In a number of eggs, 3LL tumours were grown on grids made from polytetrafluoroethylene mesh. This technique was the most successful used by Tucker in terms of reproducible tumour growth.

Finally, in other eggs, the tumour mash was simply placed on the CAM with no restrictive measures at all.

Over 150 eggs were used in an attempt to grow thin versions of the Lewis lung carcinoma. Unfortunately, there was little success in that as the tumours grew, the CAM gave way under the weight of the implant, thus allowing the normal spherical growth of the neoplasm to occur.

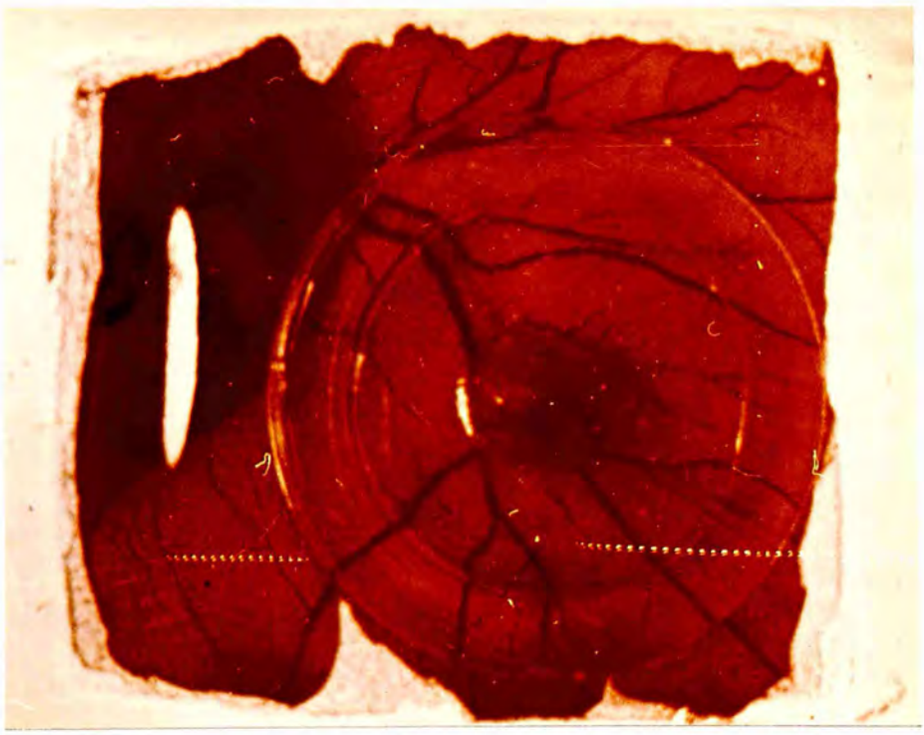


Fig. 25

Vascular bed of the CAM of a chick embryo through a window cut in the shell. A small volume of 3LL tumour mash was placed on, or close to, a vessel. Over the implant was placed a plexiglass diffusion chamber ring with a coverslip cemented to the upper surface.

Fig. 26

The vascular architecture of a 3LL tumour grown by this method.

Some degree of uniformity of tumour development could be achieved but not enough to permit conclusions concerning the vasculature of the Lewis lung carcinoma (Fig.26).

SECTION III

The measurement of blood flow in 3LL tumours after treatment with ICRF 159. The following two techniques were used:

1) Macroaggregates of human serum albumin - MAA I^{131} (Flohr (1968) and Flohr and Hoppe (1969))
2) The distribution of Rb^{86} (Sapirstein) (1958)

INTRODUCTION

Blood flow to tumours

Literature dealing with tumour blood flow and circulation was sparse until the advent of various isotope techniques.

Most of the early workers used techniques involving the systemic injection of dyes. In 1916 Weil noted that certain dyes, such as congo red, remained in the necrotic centre of the tumour after the dye had been excreted from the rest of the body.

Other workers, Karczag et al (1920) and Engel (1925) injected dyes, such as lissamine green and trypan blue, subcutaneously into tumour bearing animals and observed a slight colouration of the peripheral zone of the tumour. Duran-Reynals (1939) obtained similar results using Evans blue which was absent from the necrotic centre. This was also the case in some human tumours as reported by Brunschwig et al (1940).

A more careful study of the distribution of Evans Blue and other dyes was performed by Zahl and Waters (1941) using the S180 and a mammary carcinoma. They observed an initial diffuse staining of the peripheral stroma and a more intense staining of a thin layer of semi-necrotic cells on the border between the viable and necrotic zones of the tumour.

Goldacre and Sylven (1959 and 1962) and Owen (1960) used lissamine green to map out areas of tumours not supplied by blood. These regions suddenly appeared in tumours at a

critical age, which in the transplanted mouse tumours studied (mammary carcinoma, sarcoma 37, Ehrlich-landschutz solid ascites) was about 12 days, and in the rat Walker carcinosarcoma about 5 to 10 days depending on the type of host. These unstained zones contained living cells, as demonstrated by vital staining, tissue culture and particularly by transplantation into new hosts. In forty-one transplantations from these unstained zones, twenty-three typical tumours were produced.

Bierman et al (1951 and 1952), measuring the rate of blood flow to human tumours, showed that when fluorescent dyes were injected intra-arterially into patients with skin tumours the dyes appeared, under U.V. light, more rapidly in the primary skin tumours than in normal adjacent tissue. They also found that the oxygen content and saturation of the venous blood draining several human neoplasms was higher than the venous oxygen content obtained elsewhere, i.e. the differences in oxygen content between the afferent and efferent tumour vessels were smaller than the A-V differences found for normal tissues. They concluded that there was considerable A-V shunting within the tumour, and a greater blood flow through these tumours than in comparable normal tissue.

Other workers, however, have described a low tumour blood flow in comparison with normal tissue (Bergentz et al, 1963; Gullino and Grantham, 1961; Cataland et al, 1962; Peterson et al, 1969).

Gullino and Grantham (1961) devised an ingenious method to measure directly blood flow in rat tumours. An ovary or kidney was displaced into a subcutaneous position, leaving the vascular pedicle intact. A tumour was then implanted

into the organ which was enveloped in a paraffin sac and replaced in a pouch of subcutaneous tissue. The tumour in the host organ was thus completely isolated from the surrounding tissues. The blood flow was measured directly, after cannulating the single vein draining the tumour, which eventually destroyed the host organ. They also employed an indirect method based on the fractional distribution of radioactive potassium and rubidium. There was good correlation in the results from both methods. They concluded that the blood flow of the various rat tumours studied followed a uniform pattern independent of histological type, size of tumour and site of implantation, and that the blood flow of these tumours was unusually low, i.e. a hepatoma had a blood flow about one twentieth that of the host liver on a gram for gram basis.

Of all the techniques that have been used to study blood flow in various vascular beds, (Kramer, 1963), only indirect methods can be employed with small animal tumours unless one uses Gullino and Grantham's tissue-isolated tumours.

Sapirstein (1958) devised a method for measuring capillary blood flow by the fractional uptake of two highly diffusible ions, K^{42} and Rb^{86}, for which the cells act as a kind of "sink". Sapirstein demonstrated that after a single, rapid, intravenous injection of either $Rb^{86}Cl$ or $K^{42}Cl$, the isotope was distributed initially to the organs in proportion to their blood flow and then was carried away by the venous drainage. For a certain period, however, the amount of Rb^{86} or K^{42} removed by the venous drainage was negligible compared to that delivered by the artery. This period was longest for ions, such as Rb^{86}, which were rapidly transferred from the vascular system to the tissues and which had a large volume of distribution within the tissue.

After intravenous injection, the Rb^{86} or K^{42} concentration:time relationship for all organs except the brain showed a steep rise during the first 10 to 15 seconds, was steady between 20 and 60 seconds and was variable beyond a minute.

The plateau of the curve was interpreted as an indication that the amount of isotope found in each organ was approximately the amount brought by the blood flow. It was therefore possible to estimate the fractional distribution of the cardiac output by determining the amount of isotope in various organs, providing the animal was killed within 1 minute of the intravenous injection of isotope.

This technique was applied by Cataland et al (1962) for the measurement of blood flow in two transplanted mouse tumours, the mammary carcinoma 755 and sarcoma 180. They observed an inverse relationship between tumour blood flow and tumour size. This relationship was explained by the development of a central unvascularized necrotic zone in the tumour which increased the tumour mass but not the tumour capillary bed. Two disadvantages of this technique were that the cardiac output must be known in order to express flow in terms of ml/min and that the animals must be killed to determine tumour blood flow.

The clearance technique using radioactive inert gases to measure capillary blood flow avoided these disadvantages and has been employed to measure tumour blood flow. Gump and White (1968) used the clearance of radioactive krypton (^{85}Kr) to measure the blood flow of transplanted V_2 carcinomas in rabbits. Gelin et al (1968) measured the clearance of Xe^{133} from metastatic tumours in human livers.

Peterson et al (1969) used the clearance of two locally injected radioisotopes, ^{133}Xe and ^{24}Na, to measure capillary blood flow of two mouse tumours - mammary carcinoma and 20-methylcholanthrene sarcoma. A recent paper by Kallmann et al (1972) reported the measurement of blood flow in irradiated mouse KHT sarcomas by clearance of ^{133}Xe and found that anaesthesia significantly lowered the rate of tumour blood flow.

Many papers have described the use of radioactive particles, which do not pass through the systemic capillaries, for the measurement of regional blood flow (Grady et al, 1963; Neutze et al, 1968). The introduction of this type of indicator has made it possible to study the distribution of cardiac output directly and has facilitated the determination of blood flow to various organs and tumours. The principle was to inject a calibrated amount of indicator into the left ventricle, then the indicator was distributed throughout the body in proportion to the distribution of cardiac output, providing the indicator has dispersed homogenously in the aortic blood. Shibita and Maclean (1966) studied the blood flow of fourteen human malignant tumours using radioactive Yttrium^{90} microspheres (35 $\pm$ 5μ in diameter) and found poor blood flow to the tumours.

Muller and Rossier (1951) first devised a new approach using radioactive microspheres that can be localised at selected sites for tumour therapy thus minimising radiation damage in healthy tissue. The microspheres were injected on the arterial side of the "host" organ and were trapped in the capillaries of the organ and tumour. Variable results have been reported using this technique for tumour therapy (Grady et al, 1960, 1962 and 1963; Ariel, 1962; Lafave et al,

1963; Ya et al, 1961; Perry et al, 1962). Not all the microspheres will be trapped in the tumour. A small amount of radioactive material will pass through A-V shunts for recirculation (Prinzmetal et al, 1948; Flohr, 1968). Therefore, when specific doses of radiation are required for tumour treatment, an allowance must be made for the number of microspheres passing through A-V shunts.

Flohr (1968) and Flohr and Hoppe (1969) used Iodine^{131} labelled macroaggregates of serum albumin (5-50μ in diameter) in place of microspheres to determine blood flow within organs of cats, dogs and rabbits by means of auto-radiography. The radioactively labelled particles were injected into the left ventricle and mixed with arterial blood. The particles were carried via the systemic circulation to the various organs in proportion to the distribution of cardiac output. Any particles not trapped in the various capillary beds were transferred to the venous blood and eventually sequestered in the lungs. The animals were killed and various organs rapidly excised and frozen. Thick frozen sections were exposed on X-ray film.

Macroaggregates have provided a cheaper alternative to microspheres whilst exhibiting the same properties, i.e. there is a high extraction ratio of the macroaggregates as shown in rabbits, with only 2-3% being recirculated. Also, Tow et al (1966) showed that these particles dispersed homogenously in flowing blood, which was essential for the validity of this technique.

MATERIALS AND METHODS

In this section, two methods are used to measure rate of blood flow in control and ICRF 159 treated tumours.

1 Determination of blood flow in 3LL tumours using Iodine^{131} labelled macroaggregates of human serum albumin. (The technique of Flohr (1968) and Flohr and Hoppe (1969)).

Details of MAA (1^{131}):

Radioactive concentration	0.2m Ci/ml
Specific activity	$\sim$ 1m Ci/mg protein
Protein concentration	$\sim$ 0.2mg/ml
Half life	8 days

Sterile suspension of macroaggregates of iodinated human serum albumin containing 1% benzyl alcohol made isotonic with NaCl.

Diameter of particles 15-50 microns (up to 3-4% is less than 15μ in diameter).

Seventy mice with tumours ranging from 10-15 days were used in these determinations.

0.2ml MAA (1^{131}) was injected retrogradely down the ligated left carotid artery of control and treated mice anaesthetised with 0.2ml Avertin i.p.

The mice were kept warm, and all wounds were kept covered with cotton wool pads soaked in warm saline to minimise heat loss.

The mice were killed within 20 seconds of the injection and the tumours and kidneys were rapidly excised.

For quantitative evaluation the tumours and kidneys were counted with a well-type automatic crystal counter - Nuclear Enterprises NE8311.

As it was not possible to inject the same concentration of MAA (1^{131}) into each animal, the tumour macroaggregate concentration was expressed as a fraction of the kidney macroaggregate concentration. This assumed that the kidney blood flow remained fairly constant under experimental conditions.

A blood sample was taken from each animal to ensure the absence of circulating aggregates.

For qualitative evaluation both thick frozen sections (~1000μ) and thin histological sections (5μ) were cut from the tumours and kidneys. Sections were placed on dental X-ray film or Kodak X-ray film for varying exposure times. The films were developed and compared with the original sections.

(a) <u>To determine the effect of the anaesthetic - Avertin - on the blood pressure of C57Bl mice and its possible relevance to the MAA (1^{131}) technique</u>.

As will be discussed, any variation in blood pressure could be critical, when macroaggregates are used, for the measurement of tumour blood flow. Consequently, experiments were performed to see if the anaesthetic used in the macroaggregate technique caused a fall in blood pressure of the mice.

Sixteen C57Bl mice, weighing between 18 and 22 grams were anaesthetised with 0.2ml Avertin intraperitoneally. The left carotid artery was cannulated and the blood pressure was recorded with a Devices M2 pen recorder.

The cannula was filled with heparinized saline to prevent clotting. Blood pressure recordings were taken up to 1 hour after the injection of the anaesthetic.

The effect of Avertin on the blood pressure of C57Bl mice was compared with that of two other anaesthetics, Halothane (Fluothane) in 95% O_2, 5% CO_2 and Nembutal (Sodium pentobarbitone). 0.2ml 10% Nembutal was injected intraperitoneally into eight mice. The gas Fluothane was administered to four mice using a special nose piece. The blood pressure was recorded as before.

2 Determination of 3LL blood flow using the techniques of Sapirstein (1958) with $Rb^{86}Cl$.

For these studies, experimental mice received ICRF 159 20mg/kg suspended in 0.2ml of carboxymethyl cellulose (0.5%) in isotonic saline (CMC) given i.p. daily. Control mice received 0.2ml of CMC alone i.p. daily.

This minimised T/C values and produced the same angiometamorphic effect on the 3LL tumour blood vessels.

(i) Tissue concentration of isotope:time relationship in 3LL after intravenous injection of $Rb^{86}Cl$, and determination of killing time for part ii.

A dose of 2μc of $Rb^{86}Cl$ in 0.2ml saline was rapidly injected into the tail veins of 20 control and 19 treated mice bearing 10 day old 3LL tumours. Mice from each group were killed at 5, 10, 20, 40 and 60 seconds after injection by dropping them into liquid nitrogen. After thawing, the tumour, kidneys, spleen and liver were excised. The isotope content of the bodies and excised organs was measured with a well-type automatic crystal counter.

(ii) Blood flow measurements of control and treated 3LL tumours.

A dose of 2μc of $Rb^{86}Cl$ in 0.2ml saline was rapidly injected into the tail veins of 45 control mice and 43 treated mice bearing tumours ranging from 7-21 days old. All mice were killed at 40 seconds after injection by dropping them in liquid nitrogen. After thawing, the tumour, kidneys, spleen, liver, lungs, stomach and intestines, and the carcass were counted as before. The older tumours were divided into viable and necrotic zones and counted separately.

As the cardiac output has not been measured, the flow values cannot be expressed in terms of ml/minutes/gm of tumour. The values for the tumours and various organs were therefore expressed as percentage of injected dose.

RESULTS

MAA (1^{131})

The quantitative values of 3LL tumour blood flow using the technique of Flohr (1968) and Flohr and Hoppe (1969) were extremely variable for both control and treated animals. There was no significant difference in the values for control and treated tumours (Table 2).

There was virtually no activity in the blood samples taken from each animal, demonstrating the absence of particles in the circulating blood.

A significant finding was the difference in the macro-aggregate concentration found in the right and left kidneys from each animal. 81.5% of control and treated animals demonstrated a higher macroaggregate content in the right kidney. The magnitude of the difference between right and left kidney was as much as 3X.

Fig.27 shows the intratumoural particle distribution in six thick frozen sections, but because of the lack of detail and the difficulty of keeping the frozen sections on the dental X-ray films, thin sections were cut for most of the tumours.

Figs.28 & 29 show the particle distribution in thin histological sections of tumours and kidneys from control and treated animals.

Blood flow distribution was confined to the cortex of the kidneys. Flohr and Hoppe (1969), using this technique, were able to distinguish three distinct compartments of

TABLE 2 Blood flow values of control and treated 3LL tumours.

$$\text{Ratio} \quad \frac{\text{tumour activity (count sec.}^{-1}\text{ gm.}^{-1})}{\text{L + R kidney activity (count sec.}^{-1}\text{ gm.}^{-1})}$$

Age of tumour (days)	Number	Mean value ± S.E.		P*
		Control	Treated	
9	12	0.130 ± 0.033	0.320 ± 0.089	N.S.
10	30	0.138 ± 0.033	0.197 ± 0.049	N.S.
15	11	0.037 ± 0.006	0.189 ± 0.071	N.S.

P* - significance of difference (t test) from control value

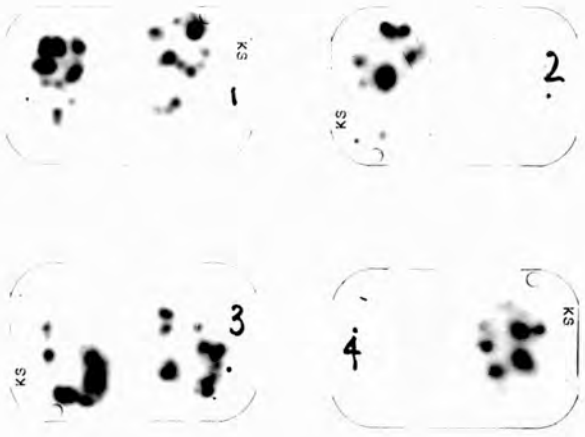


Fig. 27

Macroaggregate distribution in thick frozen sections of control (3 & 4) and treated (1 & 2) 3LL tumours.

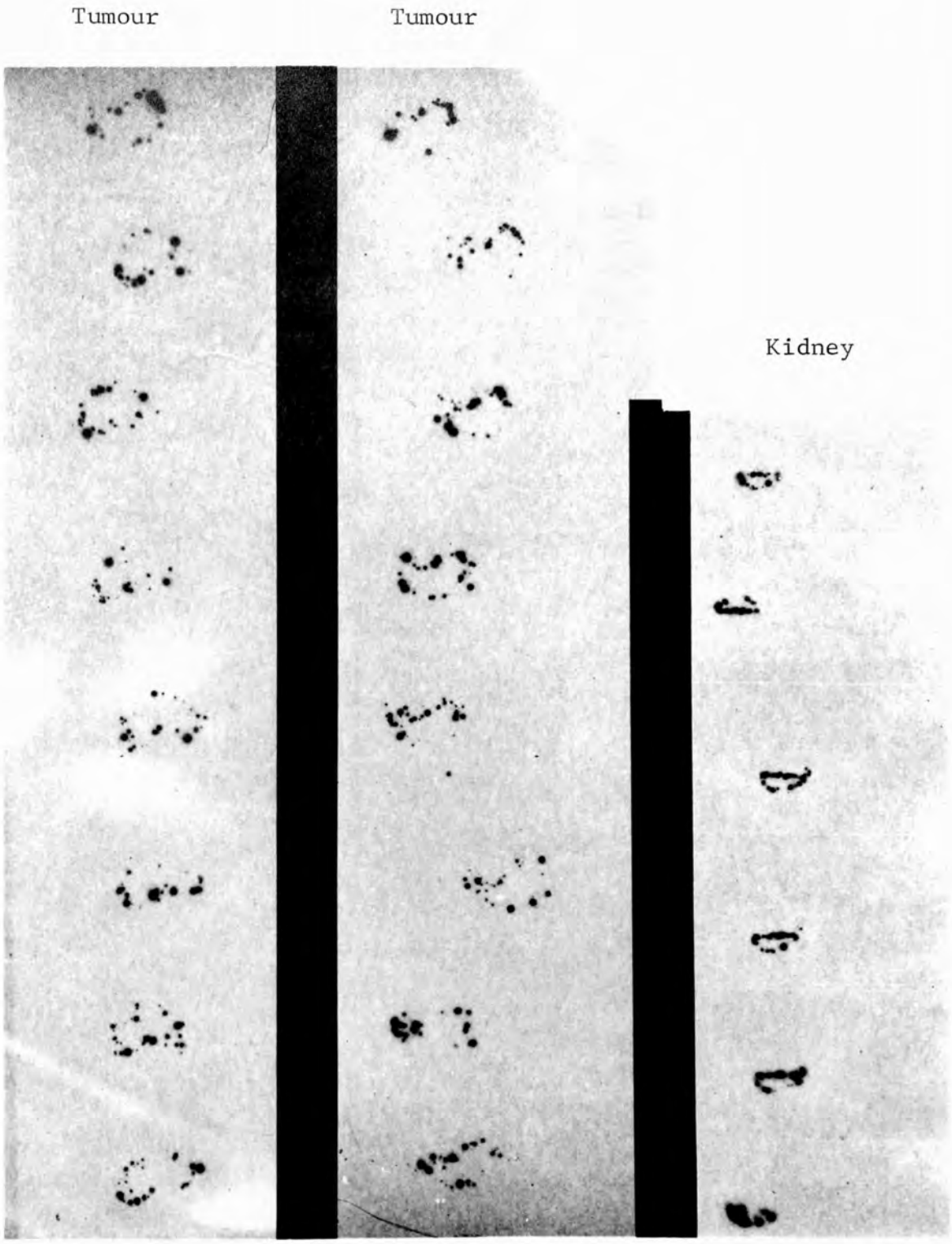


Fig. 28. Macroaggregate distribution in thin histological sections cut from the central segment of a large 11 day control 3LL tumour. Sections of the kidneys from the same animal are also shown. Sections were placed on Kodak X-ray film for an exposure time of 3 hours. Note that in both the tumour and kidney sections, the macroaggregate distribution is confined only to the outer cortex.

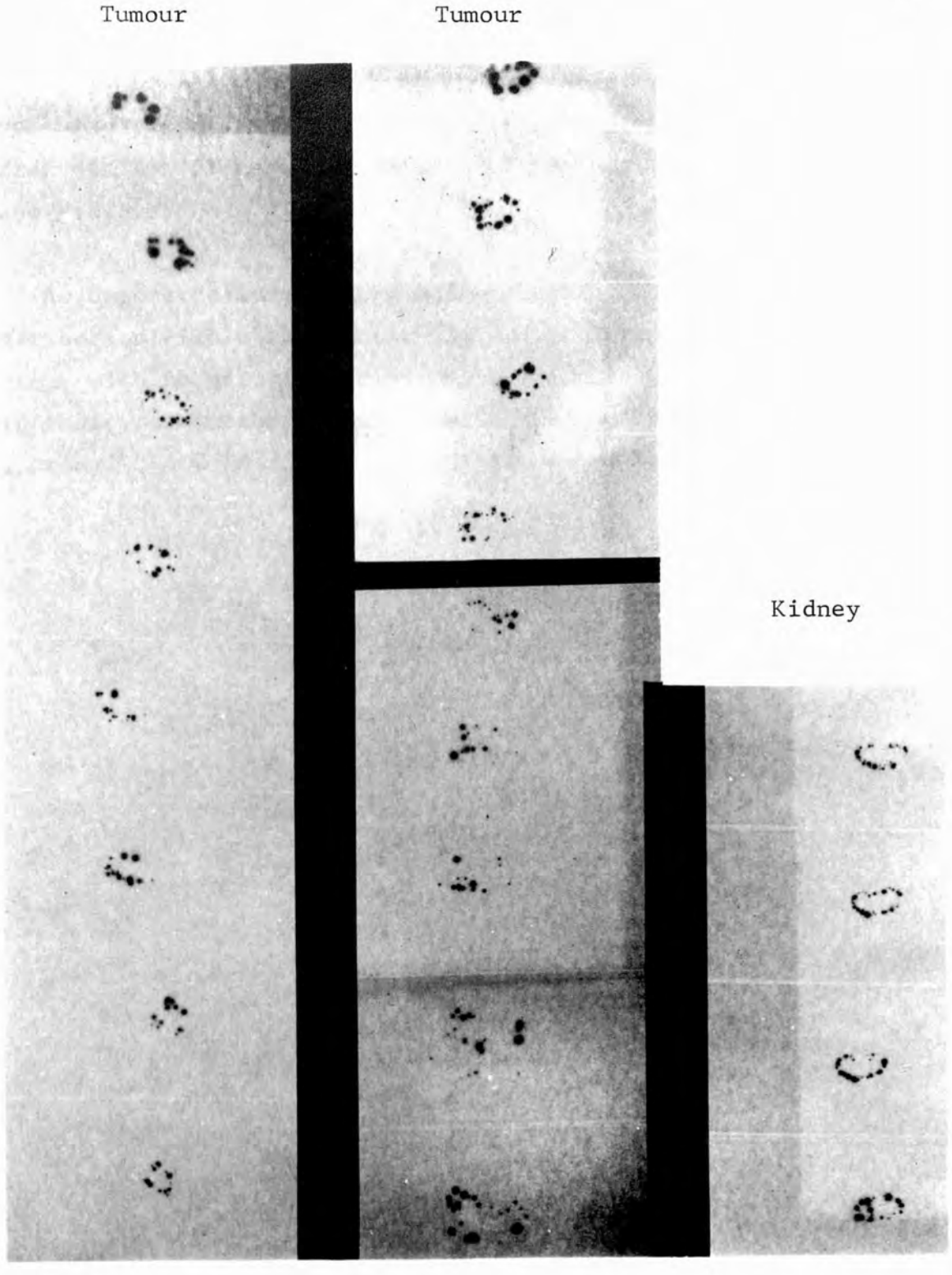


Fig. 29

Macroaggregate distribution in thin histological sections cut from some 11 day treated 3LL tumours. Sections of the kidneys from one mouse are also shown. Exposure time was 3 hours.

blood flow within the cortex of kidney sections taken from dogs. Any variation of particle distribution within the cortex was not discernible because of the size of the mouse kidneys.

As demonstrated with lissamine green, the particle distribution within the tumour was confined to an outer cortex, with no activity found in the middle of the tumour. This indicates an absence of functional blood vessels of the order 15-50μ in diameter within this region.

The effect of anaesthesia on the blood pressures and heart rates of C57Bl mice

Table 3 shows the effect of Avertin, Nembutal and Halothane on the heart rates and blood pressures of C57Bl mice.

TABLE 3

Anaesthetic - route administered		Mean heart rate and range - beats/min.	Mean b.p. and range mm.Hg.
Avertin	i.p.	450 (330-570)	77 (64-94)
Nembutal	i.p.	480 (420-540)	66 (57-76)
Halothane	inhalation	420 (360-570)	68 (57-89)

The values obtained for the heart rate and blood pressure were markedly lower than those quoted for unanaesthetised mice. (Table 4).

The blood pressure was recorded on average 15 minutes after injection of Avertin, i.e. a comparable state of anaesthesia existed in the mice injected with MAA (1^{131}).

TABLE 4

Blood pressures and pulse rates of unanaesthetised mice

Strain of mice	Pulse rate/min. (mean ± S.E.)	Systolic b.p. mm.Hg. (mean ± S.E.)	Reference
A	107.8 ± 1.8		Edwards and Reinecke (1953)
AJ male	83.9 ± 1.7	589 ± 11	Schlager (1965)
BALB/eJ male	104.9 ± 1.7	494 ± 11	" "
CBA/J male	97.4 ± 1.9	657 ± 8	" "
CBA male	83 ± 5		Henry et al (1963)
CBA male	103 ± 6		" " "
CBA male	99 ± 4		" " "
C3H	111 (95 to 138)		Wu and Visscher (1947)
C3H	136 (114 to 165)		" " "
C3H	151 (138 to 164)		" " "
C57/B1/6J male	93.3 ± 2.2	633 ± 11	Schlager (1965)
DBA/2J male	89.4 ± 2.0	614 ± 11	" "
RF/J male	96.0 ± 1.8	595 ± 11	" "
SJL/J male	96.0 ± 2.0	639 ± 14	" "
129/J male	88.7 ± 2.0	630 ± 8	" "

$Rb^{86}Cl$

(i) Table 5 shows the amount of Rb^{86} found in 3LL control and treated tumours and various organs of C57Bl mice at varying times after single intravenous injections of $Rb^{86}Cl$. Each value (% of injected dose) represents the average of 3-5 mice.

In general, the organs and tumours showed a continued accumulation of Rb^{86} for the first 5-20 seconds. The concentration of Rb^{86} then reached a plateau although there was some variation up to 60 seconds.

The killing time of 40 seconds after injection of the isotope was chosen as this fell midway along the Rb^{86} concentration plateau. The assumption was made that the percentage of injected material in the tumours and organs corresponded to the percentage of distribution of the cardiac output.

(ii) There was no significant difference between the Rb^{86} content per gram of control and treated tumours of different ages (Fig.30). However, there was a progressively lower fractional uptake of indicator per gram of tissue as the tumours increased in size (Fig.31). There was also a progressive diminution in the uptake of Rb^{86} per gram of tumour when the central necrotic tissue was removed and only the outer viable cortex counted. Removing the necrotic tissue made no difference to the blood flow values except in the oldest tumours (Fig.32). The percentage of injected dose of Rb^{86} (i.e. the percentage of the cardiac output) reaching control and treated tumours in toto increased from 1-5% between the 7th and 21st day (Table 6).

TABLE 5

Amount of Rb^{86} in the tumour and various organs of C57Bl mice at different intervals after a single, rapid, intravenous injection of $Rb^{86}Cl$.

Each value (% of injected dose) represents the average of 3 - 5 mice.

CONTROL

Time sec.	Tumour	Kidney	Spleen	Liver
5	2.15	2.90	1.27	8.76
10	2.21	3.95	1.49	9.50
20	1.73	6.30	1.72	8.60
40	1.94	4.80	1.77	8.31
60	1.50	3.39	1.19	7.47

EXPERIMENTAL

5	2.68	5.23	0.98	12.63
10	2.27	5.90	1.78	11.61
20	1.84	4.20	1.35	9.09
40	1.93	3.60	1.19	8.50
60	2.65	3.38	1.25	10.28

Fig 30

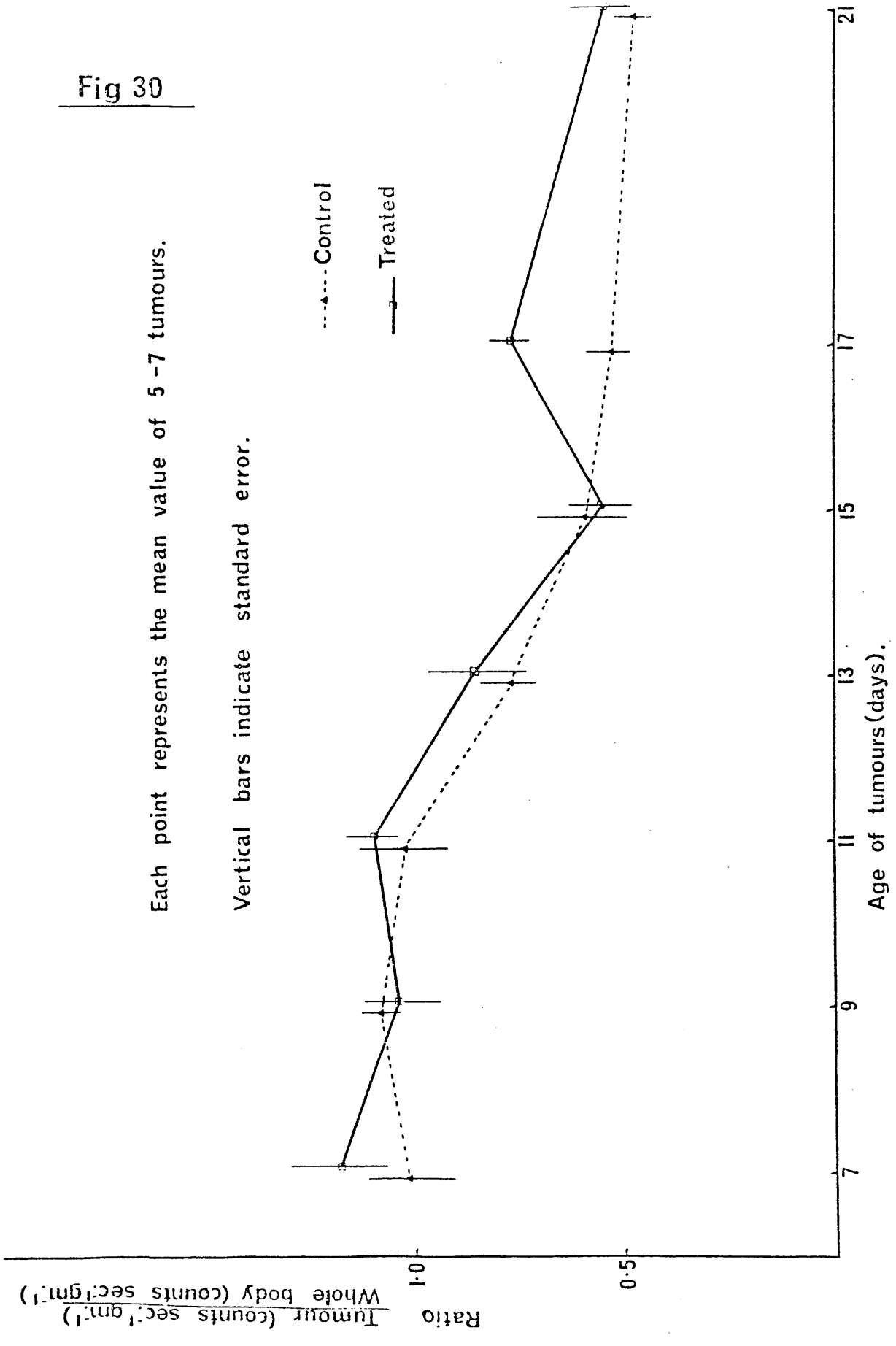

Each point represents the mean value of 5 -7 tumours.
Vertical bars indicate standard error.
Control
Treated
Ratio Tumour (counts sec⁻¹gm⁻¹) / Whole body (counts sec⁻¹gm⁻¹)
1·0
0·5
7
9
11
13
15
17
21
Age of tumours (days).

Fig 31

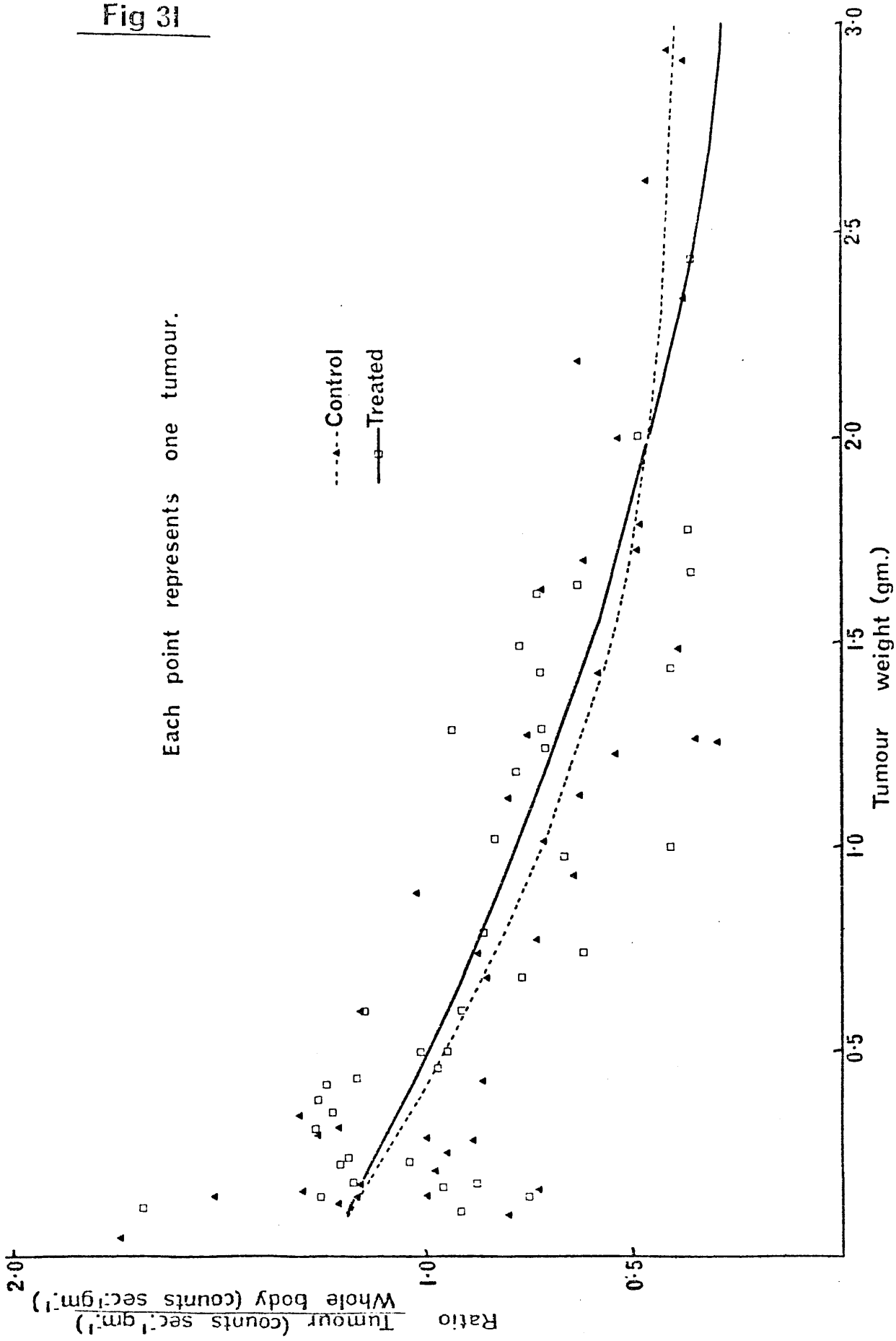

Each point represents one tumour.
Control
Treated
Ratio Tumour (counts sec⁻¹gm⁻¹) / Whole body (counts sec⁻¹gm⁻¹)
2·0
1·0
0·5
0·5
1·0
1·5
2·0
2·5
3·0
Tumour weight (gm.)

Fig 32

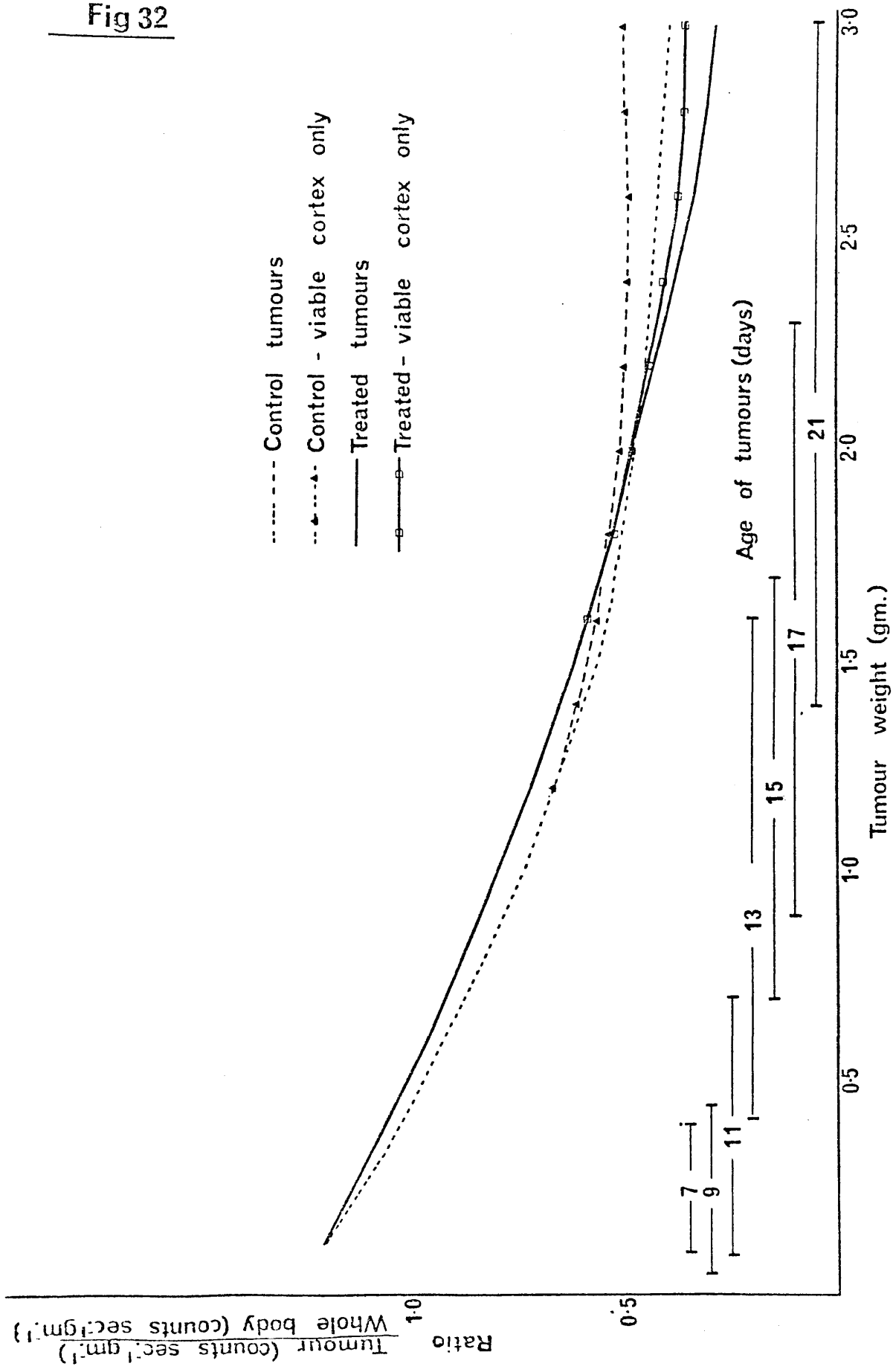

Control tumours
Control - viable cortex only
Treated tumours
Treated - viable cortex only
Age of tumours (days)
7
9
11
13
15
17
21
Ratio
Tumour (counts sec⁻¹ gm⁻¹) / Whole body (counts sec⁻¹ gm⁻¹)
0·5
1·0
0·5
1·0
1·5
2·0
2·5
3·0
Tumour weight (gm.)

TABLE 6

Amount of Rb^{86} in tumours of various ages and organs after a single, rapid, intravenous injection of $Rb^{86}Cl$.

Each value (% of injected dose) represents the average of 5 - 7 mice. All mice killed at 40 seconds after injection.

CONTROL

Age of Tumour Day	T	K	Sp	L	St & Int.
7	0.80	3.33	1.41	8.43	15.78
9	1.11	4.31	1.42	7.70	15.20
11	2.74	5.29	2.11	8.92	17.99
13	3.98	4.92	1.11	7.63	18.31
15	3.08	7.85	1.73	7.78	17.97
17	3.94	4.67	1.24	8.51	16.17
21	5.44	8.09	1.88	8.74	20.84

EXPERIMENTAL

7	0.91	3.26	1.25	8.85	16.71
9	1.33	2.53	1.01	8.42	13.78
11	2.53	4.67	1.37	8.55	16.93
13	3.02	7.60	1.45	7.89	20.50
15	3.38	6.32	1.53	9.81	20.68
17	4.65	5.17	1.03	8.63	17.21
21	4.86	4.66	1.65	8.59	19.43

The spleens, livers, and stomach and intestines of control and treated tumours showed a stable content of Rb^{86} throughout the whole experiment. Kidney values were low and extremely variable (Table 6).

DISCUSSION

Shibata and Maclean (1962) suggested that one of the main factors affecting chemotherapeutic treatment of cancer was the impaired blood flow to the primary tumours. Chemotherapeutic agents, to be effective against the tumour, have to be administered in ever increasing doses, which obviously affects detrimentally the host as well as the tumour.

Specific alterations in the morphology of tumour blood vessels could have considerable consequences for the treatment of neoplasms, in that the blood flow rates in tumours of animals treated with ICRF 159 may be higher than normal and consequently the availability of anticancer drugs might be increased. It was thought that this consideration might explain the greater effectiveness of other anticancer drugs when used in combination with ICRF 159 - Hellmann and Burrage (1970); Woodman et al (1971).

The present study however has failed to demonstrate a significant effect of ICRF 159 on 3LL tumour blood flow, as determined by the techniques of Sapirstein (1958) and Flohr (1968) and Flohr and Hoppe (1969).

Certain aspects of these results require clarification.

(1) The distribution of particles of MAA (I^{131}) within the tumours was detected by the autoradiographs and confirmed the earlier work with lissamine green, in showing that the blood flow distribution in both control and treated tumours was confined to an outer zone or cortex which encapsulated the tumours. There was no blood flow in necrotic centres of the tumours as demonstrated by these techniques.

The quantitative values for 3LL blood flow using MAA (1^{131}) are not easy to interpret as the results were extremely variable. No significant difference was observed between control and treated tumours. The technique, in principle is simple, and, as reviewed in the introduction, the method has been employed with great success for organ blood flow measurements in animals larger than mice. However, when one is dealing with mice, especially sick mice, the technique must be viewed with caution. The isolation of, and injection down the carotid artery is time consuming and difficult, and although the conditions are kept as stable as possible there must be great variations in the systemic blood pressure and blood flow to the various mouse organs.

(2) An essential prerequisite for the validity of the macroaggregate technique is that the particles are homogenously dispersed in the aortic blood so that distribution of the label will be in proportion to the cardiac output. The observation that 81.5% of control and treated animals demonstrated a higher macroaggregate content in the right kidney as opposed to the left, suggests that the injected particles are not dispersing homogenously in the blood leaving the heart.

(3) Another aspect of the technique that calls for comment is the effect of anaesthesia on the blood pressure of tumour bearing animals. Algire and Legallais (1951), using the transparent chamber technique, demonstrated that the tumour blood flow was decreased by lowering the peripheral blood pressure. The reduction of tumour circulation was directly correlated with the duration and degree of peripheral hypotension. These workers induced hypotension in several ways: by i.p. injections of histamine or hypertonic glucose,

by shock following the release of tourniquets, or by local mechanical obstructions to arterial flow.

Also, Kallmann et al (1972), using the xenon clearance method with KHT sarcomas in C3H mice, demonstrated that the state of anaesthesia lowered the systemic blood pressure and consequently decreased the rate of tumour blood flow.

The blood pressure values of C57Bl mice, as recorded from the carotid artery, were certainly lower than those obtained from the literature where indirect measuring techniques were used. All the three anaesthetics tried, produced a similar fall in blood pressure. Thus, from these results and from observations found in the literature, it would be expected that any agent or condition, such as the state of anaesthesia, that reduced the blood pressure in the arteries supplying a tumour would similarly interfere with the circulation within the tumour. Because of these findings, and the uncertainty of achieving reproducible levels of anaesthesia in the mice, a technique was used which involved no surgery and therefore no anaesthetic. Such a technique is that described by Sapirstein (1958).

(4) The distribution of blood flow in mice was measured by the fractionation technique for tumours and organs using Rb^{86}. This method depends on the fact that tumours and all organs, other than the brain, have virtually the same extraction ratio of Rb^{86} as the whole body during the first minute after a single intravenous injection of Rb^{86}. Thus, the uptake of the isotope by any organ during the first minute will be related to the total body uptake as is that organ's blood flow to the cardiac output.

To apply Sapirstein's method to the determination of control and treated 3LL blood flow, it was first necessary to determine whether the concentration:time relationship in the neoplastic tissue reached a plateau as in normal organs, as had been shown by Sapirstein (1958); Gullino and Grantham (1961) and Fowler et al (1972). These workers confirmed the existence of a plateau in Rb^{86} uptake between 15 and 60 seconds, indicating that the amount of isotope found in each organ was approximately that brought by the blood flow.

In a pilot study, the time:concentration relationships for 3LL tumours and various organs showed agreement with the results obtained by the above workers, and so a similar killing time of 40 seconds was chosen as this fell in the middle of the concentration:time plateau. The assumption was made that the amount of isotope found in the various organs and tumours at 40 seconds corresponded to the distribution of the cardiac output.

Although there was no significant difference between the amounts of Rb^{86} found in control and treated 3LL tumours, there was fair agreement with the values obtained for various mouse tumours, as reported by Gullino and Grantham (1961) - Table 7.

Also, despite great variance in species, size of different animals, tissues studied and experimental techniques, the values obtained for blood flow to the various organs were similar to those found in other reports - Table 8.

Only the values obtained for kidney blood flow were markedly different from expected physiological values. Gullino and Grantham (1961) suggested that anaemia probably

TABLE 7

Amounts of Rb^{86} found in mouse tumours

Tumour	Av. wt. gm.	Range gm.	% injected dose of Rb^{86} Av. value	Range
Hepatoma	2.5	0.3 - 5.2	3.00	0.36 - 6.24
Osteogenic sarcoma	2.8	0.5 - 3.5	2.24	0.40 - 2.80
Histiocytoma	1.5	0.2 - 2.5	3.75	0.50 - 6.25
3LL control	1.0	0.1 - 2.9	3.01	0.43 - 8.51
3LL treated	0.9	0.1 - 2.4	2.95	0.47 - 7.17

TABLE 8

Amounts of Rb^{86} found in various organs

Animals used	Killing time sec.	with or without tumours	% injected dose of Rb^{86}				Reference
			Liver	Kidney	Spleen	Stomach & Intestines	
Rats	32	-	7.7	15.8	22.4		Sapirstein
Rats	40	-	5.1	14.8			Sapirstein
Dogs	20 - 49	-	10.1	10.6	1.1	15.6	Sheppard
Mice	30	+	6.3	11.6	22.9		Gullino and Grantham
Mice (control)	40	+	8.2	5.5	1.6	17.5	
Mice (treated)	40	+	8.7	4.9	1.3	17.9	

caused the low values for kidney blood flow in their experiments and cite two references, Bradley and Bradley (1947) and Whitaker (1956), in support of this premise.

With the Lewis lung carcinoma, however, two considerations have to be made with regard to kidney blood flow. Firstly, the mice used in these experiments - C57Bl - are susceptible to hydronephrosis, Cotchin and Roe (1967). Although the kidneys of control and treated mice appeared normal in size and shape, it is possible that early stages of this pathological condition exist, with a resulting decrease in kidney blood flow. Secondly, X-ray arteriography has indicated that one of the vessels supplying the 3LL tumour arises from the area of the renal artery. Whether this vessel acts as a shunt for some of the renal blood flow is debatable. As Sapirstein concluded, there is no explanation for the low kidney values.

(5) One of the major differences between these experiments and those previously reviewed, is the technique for killing experimental mice. Sapirstein (1958) and Gullino and Grantham (1961) killed the animals by cutting through the thorax just below the axillae with a mallet driven axe. The timing was accurate to $\pm$ 1 second. Cataland *et al* (1962) decapitated the animals. Both techniques however, would not stop blood flow immediately as the heart would still pump out blood for many seconds. This could be critical for concentration:time curves especially when the readings were taken at 3, 6, 9 and 12 seconds in Sapirstein's paper; at 2, 5 and 10 seconds in Gullino and Grantham's paper; and at 20 seconds in Cataland's paper. The killing technique employed in these experiments, i.e. dropping the animals into liquid nitrogen, is likely to arrest the circulation sufficiently rapidly to avoid the above criticism.

(6) Many workers, using a variety of tumours and techniques, have demonstrated an inverse relationship between tumour blood flow and tumour size - Gullino and Grantham (1961); Cataland et al (1962); Summers (1966); Vogel and Haynes (1966); Robert et al (1967); Rogers et al (1969); Song and Levitt (1971); Kallman et al (1972) and Fowler et al (1972). This observation was explained in the papers of Cataland et al (1962) and Rogers et al (1969), by the development of central unvascularized necroses during tumour growth. The growth of the tumour vascular bed did not keep pace with tumour growth as a whole, giving rise to an inverse relationship between blood flow and tumour size. This is not the case with the 3LL tumours because there is still a decrease in blood flow even if the necrotic centre is removed and only the outer viable cortex is counted. Also, as Jirtle and Clifton (1973) suggested, the inclusion of any non-viable tissue, especially in large tumours, is likely to be a fundamental error in any calculations of tumour blood flow.

(7) It is important to note that both the macroaggregate and Rb^{86} techniques measure only functional rather than total blood flow to the tumour. Blood flow through arteriovenous shunts and other non-exchanging areas is not measured. Thus, any isotope carried by the shunted blood will be redistributed, and in the case of Rb^{86}, to the exchanging areas giving slightly higher values for the fractional uptake of indicator than their true fraction of the cardiac output. Any macro-aggregate passing through arteriovenous shunts will be trapped in the capillary beds of the lungs. The amount of shunting that occurs in 3LL tumours has not, as yet, been measured.

SECTION IV

The effect of serotonin on 3LL tumours after treatment with ICRF 159.

INTRODUCTION

Cater et al (1962) and Cater et al (1966) measured blood flow changes in rat and mouse tumours by means of oxygen tension measurements and the response to oxygen inhalation after the injection of various vasoactive drugs. Tumour oxygen tensions were measured with microelectrodes inserted into the tumours. They found that tumour blood flow varied directly with systemic blood pressures except after the injection of physiological amines, in particular serotonin. These workers demonstrated that serotonin profoundly decreased tumour blood flow at doses which had little or no effect on muscle oxygen tensions and produced only a slight fall in the systemic blood pressure. If the systemic blood pressure was low, serotonin tended to raise it, and in spite of this, the tumour oxygen tension still fell. They pointed out that serotonin had a specific action on tumours, as anti-serotonin drugs (e.g. 1-methyl-lysergic acid butanolamide) given prior to serotonin reduced or prevented the effect on tumour blood flow. When anti-serotonin drugs were administered after serotonin, the effect was reversed.

Using the same technique, Cater et al (1963) demonstrated that serotonin actually stopped tumour blood flow, since the inhalation of oxygen at 5 atmospheres absolute failed to produce a rise in tumour oxygen tension.

Cater and Taylor (1966) suggested several possible explanations for the abnormal sensitivity of tumour blood vessels to serotonin:-

1. Newly-formed tumour vessels could simply be particularly sensitive to serotonin as is the case with the tips of new capillary buds, Schoefl (1964). Alternatively the sensitivity of tumour vessels to serotonin could be due to anatomical defects, perhaps produced as a direct result of their rapid formation.

2. The poor blood flow of tumours might account for the effect in that this would allow time for the serotonin to become localised. Also, a sluggish blood flow would result in low oxygen tensions, which in turn would inhibit the activity of the enzyme - mono-amine oxidase - and consequently lead to an accumulation of serotonin within the tumour.

3. It is possible that many of the tumour blood vessels are already partially inflamed because of their close proximity to necrotic areas, and therefore only very small doses of serotonin need be injected to affect these vessels. Cater and Taylor (1966) demonstrated this point by injecting Pelikan ink (colloidal carbon which labels inflamed vessels as described in Section II) i.v. into rats transplanted with hepatomas followed by an injection i.p. of either serotonin or saline. The tumours of rats injected with saline showed labelled vessels near zones of necrosis, whereas in rats injected with serotonin not only was there an increase in the labelling of vessels near necrotic areas, but a number of vessels in the viable parts of the tumour were also labelled.

4. It is known that subcutaneous injections of serotonin will cause marked local oedema. Cater et al (1962) thought that tumours with their higher than normal tissue fluid pressures would be abnormally sensitive to serotonin in that it might increase tissue oedema to the point of blocking the circulation.

The effect of serotonin on tumours was further investigated by Goldacre and Dayal (1969) and Dayal (1970) using lissamine green to map out tumour zones not reached by blood-borne dyes. Serotonin, injected i.p., caused existing uncoloured zones in large tumours to extend even further in transplanted Walker, Crocker and ADJ-PC-6A tumours, so that only a very thin cortex at the periphery of the tumours was coloured green. One tumour, however, the Gardner lymphosarcoma, was unaffected by serotonin, even after multiple injections of the agent - the tumour appearing green throughout. These workers suggested that the difference in response to serotonin was due to the vascular arrangements within these tumours. The vessels of the Walker, Crocker and ADJ-PC-6A appeared to be newly-formed, whereas the Gardner lymphosarcoma appeared to be vascularized by the appropriation of pre-existing normal vessels.

On the basis of these results, the effect of serotonin can be used as a test for the normal 'character' of tumour blood vessels. Consequently, the effect of serotonin on control and ICRF 159 treated 3LL tumours was tested and the results discussed in relation to the theoretical mechanisms of the action of serotonin.

MATERIALS AND METHODS

Forty C57B1 mice bearing tumours ranging from 10 - 15 days old were used in these experiments. Control or treated mice were injected either with 0.1ml saline or 0.1ml serotonin (5mg base/kg of 5 hydroxytryptamine creatine sulphate in saline) intraperitoneally, Cater et al (1962).

After 20 minutes, 0.3ml of lissamine green was injected into the tail vein. The mice were killed 5 minutes later and the tumours bisected.

With some mice the tumours were exposed after i.p. injection of serotonin. Then Indian ink instead of lissamine green was injected i.v. and the tumours observed under a dissecting microscope.

RESULTS

The action of serotonin on control and ICRF 159 treated 3LL tumours is demonstrated in Figs.33 & 34.

Areas of control tumours not reached by lissamine green were much larger after the injection of a single dose of serotonin. The unstained areas extended over most of the tumour leaving only a very thin green zone at the periphery of the tumour (Fig.33). If saline instead of serotonin was injected i.p., only the necrotic area of the tumour remained uncoloured, as previously described in Section II.

With treated tumours, however, no serotonin effect was shown (Fig.34), even if a second dose of the agent was injected 30 minutes after the first. The tumours were coloured throughout, except for the central necrotic areas. This was also the picture for treated tumours when saline was injected in place of serotonin.

When Indian ink was injected in place of lissamine green, the tumours demonstrated the same response to serotonin. The ink filled the host vessels surrounding control tumours, but not until the effect of serotonin started to wear off (10 to 20 mins. after injection of serotonin) did the ink penetrate the tumour vessels. After 1 hour all the control tumour vessels were filled with ink.

Serotonin had no effect on treated tumours in that all the vessels appeared black straight after the ink was injected. This also occurred in control and treated tumours where saline was administered as a control for serotonin.

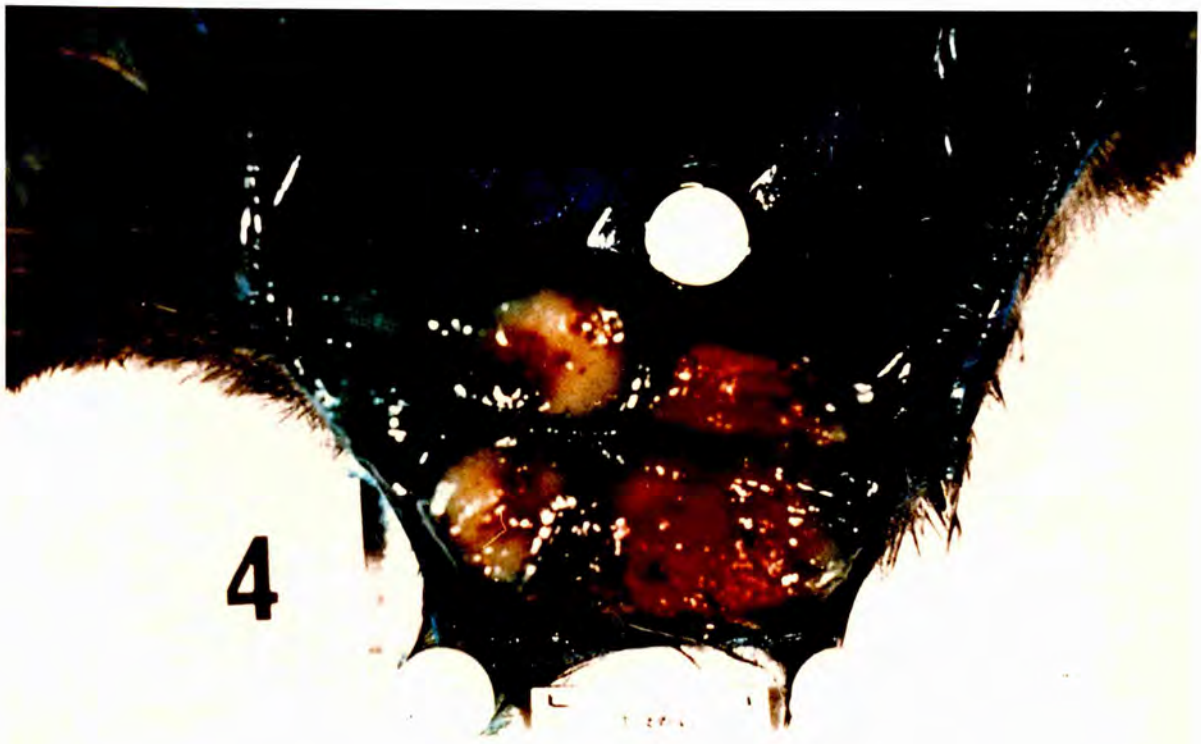


Fig. 33
Effect of serotonin on 10 day control 3LL tumour. Note the large unstained zone due to serotonin.

Fig. 34
No serotonin effect on a 10 day 3LL tumour after treatment with ICRF 159.

DISCUSSION

Dayal (1970) and Day (1964) have both suggested that the difference in the response of certain tumours to serotonin lies in the manner of their vascularization. Tumours with newly-formed vessels (e.g. Walker, Crocker and ADJ-PC-6A) were found to be abnormally sensitive to serotonin compared with tumours which were vascularized by the appropriation of pre-existing normal vessels (e.g. Gardner lymphosarcoma). On this basis, the observation that serotonin did not affect ICRF 159 treated tumours suggests that the blood vessels are more akin to normal blood vessels than newly-formed tumour blood vessels. Further, these results support the hypothesis of Cater and Taylor (1966) that the sensitivity of tumour blood vessels could be due to anatomical defects in their structure. The other possible mechanisms of action of serotonin as described by Cater and Taylor (1966) could be the direct result of the abnormal structure of tumour blood vessels.

The premise that only newly-formed tumour blood vessels are affected by serotonin cannot apply to the present results as both control and treated tumours are mainly vascularized by newly-formed blood vessels and therefore should both show a positive serotonin effect.

In Section III it was demonstrated that there was no significant difference in the blood flow values for control and treated 3LL tumours. Therefore the theory that a sluggish tumour blood flow results in a build up of serotonin again cannot account for the present situation. If this were so, then the blood vessels of treated 3LL tumours would similarly be affected by serotonin. Also, the blood flow results indicate that the theory of inhibiting the action of mono-amine oxidase must be viewed with caution.

SECTION V

The vitality of 3LL cells in areas of control and ICRF 159 treated tumours devoid of functional blood vessels.

INTRODUCTION

The subject of necrosis was reviewed in detail by Dayal (1970). One of the most interesting points discussed was the vitality of tumour cells found in necrotic areas and its relevance to the poor results of chemotherapy and/or radiotherapy against large tumours.

Many workers have demonstrated an inverse relationship between size of tumour and the curative effect of chemotherapy. Shapiro and Fugmann (1957); Martin and Fugmann (1960) and Martin (1961) found that prior surgical reduction of large tumours significantly aided their response to chemotherapy. Surgery was also suggested by Larionov (1959) in his study of the effect of alkylating agents on different human neoplastic diseases. He concluded that "the degree of the antitumour effect is inversely proportional to the mass of the tumour tissue".

Radiotherapy has also been found more effective against small tumours. Suit et al (1960), using a mammary carcinoma in C3H mice, demonstrated that radiation in high pressures of oxygen produced an enhanced "cure rate" with small tumours (7-10mm diameter) compared with larger tumours (10-15mm diameter). This was also the conclusion of Scott (1953) using the Ehrlich ascites tumour, and the quoted results of Goldfeder (1960) using an adenocarcinoma.

There are several accounts as to why treatment, whether it be chemotherapy and/or radiotherapy, is more successful against small tumours. Lamerton (1972 and 1973) suggested that as the tumours increased in size, tumour cells would develop a critical resistance to the chemotherapeutic drug.

Also, as the tumour grew, there was an increase in the number of slowly dividing or resting tumour "stem" cells. It is well known that the cytotoxic action of many chemotherapeutic agents is directly dependent upon the rate of tumour cell division. Thus any phase specific or cycle dependent drugs would be completely ineffective against these resting tumour cells.

Martin et al (1962 and 1964) suggested that with the larger tumours, it was the sheer number of tumour cells "blocking" the host defence mechanisms that diminished chemotherapeutic measures. Consequently, more encouraging results could be produced by first reducing the size of the tumour mass and then stimulating the defence mechanism of the host.

Goldacre and Sylven (1962) and Goldacre and Whisson (1966) suggested that it was the number of viable cells respiring anaerobically in necrotic areas that aided the re-growth of tumours following chemotherapy. These central necrotic regions, which were largely inaccessible to chemotherapeutic agents because of an absence of functional vessels, contained living cells as shown by transplantations into new hosts. These workers reported that of forty-one transplantations of necrotic material from sarcoma 37 and Walker tumours, twenty-three typical tumours were produced.

Dayal and Goldacre (1969) demonstrated that the central necrotic areas of the Walker tumours contained living cells that were not affected by the anti-tumour drug, melphalan. Tissue from both the peripheral well-vascularized zones and the central necrotic zones of Walker tumours was transplanted into new hosts after the donor animals had been

treated with various doses of melphalan. The percentage of takes from the peripheral zones decreased with increasing concentrations of melphalan, whereas, the percentage of takes from the central zones of the same animals remained constant, even at toxic doses of the antitumour drug.

Similar conclusions were reached by Thomlinson (1960), in his paper on the synergistic effect of oxygen in the radiotherapy of tumours. After irradiation of large tumours in aerobic conditions, some tumour cells may survive in necrotic areas because of the protective effect of hypoxia and subsequently regenerate the tumour after the death and absorption of the radiosensitive cells. Thus, the extra oxygen used in radiotherapy experiments, only increased the radiosensitivity of tumour cells within close proximity of functional tumour blood vessels.

The vitality of 3LL regions from control and treated mice was tested using the technique of Goldacre and Whisson (1966) in which systemically injected lissamine green was used to map out the necrotic areas of the tumours. The results are discussed with reference to the autoradiographs of tumour blood supply from Section III.

METHODS AND MATERIALS

Thirty C57B1 mice (fifteen control and fifteen treated) bearing tumours ranging from 10-20 days in age were injected i.v. with 0.3ml lissamine green. The mice were left for 1 hour, then the tumours were excised and divided into stained and unstained regions. Unstained tissue within 1mm approximately of the viable border of the tumour was not included for transplantation. This excluded any viable cells within the limits of diffusion of essential metabolites from the vessels in the peripheral zone of the tumour.

Only the unstained tissue was implanted subcutaneously by Bashford needle into new hosts of the same strain. The mice were observed for positive takes. Any tumours which arose, were excised when approximately equivalent in size to 21 day tumours of the routine tumour line. The lungs were examined for metastases.

RESULTS

Table 9 gives the results of forty-three transplants of necrotic material from control and treated 3LL tumours.

TABLE 9

Age of donor tumour (days) Control or Treated		Positive takes/ Number implants	Days after implantation when tumours removed
10	Control	3/3	30
	Treated	1/1	30
13	Control	1/5	54
	Treated	3/4	28
15	Control	2/5	52
	Treated	5/5	27
17	Control	4/5	50
	Treated	1/5	50
20	Control	1/5	48
	Treated	2/5	48

All but one (17 day old, 159 treated) of the mice with positive tumour transplants had numerous metastases in the lungs.

DISCUSSION

If a tumour is to be treated with chemotherapeutic agents, it is essential that the drugs reach all the viable tumour cells. This ideal is not achieved in the case of the Lewis lung carcinoma. The results demonstrated that the central necrotic zones of control and treated 3LL tumours contain viable cells capable of propagating growth of new tumours. These central areas, however, are devoid of functional blood vessels as shown by: (i) autoradiography with labelled macroaggregates, and (ii) intravital staining with lissamine green. Consequently, viable cells in the necrotic centre are inaccessible to chemotherapeutic agents administered via the systemic circulation, unless a slow diffusion of the drug occurs from the viable cortex of the tumour. These results are in agreement with those of Goldacre and Sylven (1962) and Goldacre and Whisson (1966) as described in the introduction.

Steel (personal communication) has similarly demonstrated that transplants of necrotic material from 3LL tumours have resulted in positive takes but the interpretations differ widely. He describes the existence of a 'cord-like' arrangement, as demonstrated in mouse tumours by Tannock (1968), existing in the central areas of 3LL tumours. That is 'cords' (radii 60-120μ) of viable tissue arranged cylindrically about tumour blood vessels within large areas of necrotic tissue. Clearly, transplants from these supposedly necrotic areas into new hosts would result in re-growth of the neoplasm. The discrepancy between the two interpretations can be resolved however, in that Steel only allows the tumours to grow to 1cm in diameter before transplanting central tissue into new hosts. In these

laboratories, 3LL tumours of 1cm diameter (about 8 days old) are not necrotic and one would expect functional blood vessels and viable cells to be present in the central zone. At this size, if lissamine green is injected i.v. the tumours are coloured throughout.

Whether the viable cells found in the necrotic areas of 3LL tumours are near incipient necrosis, ("B" type cells) of Caspersson and Santesson (1942), has not been demonstrated. These cells, which are found in areas devoid of functional blood vessels, present a serious problem for therapy in that they are inaccessible to chemotherapeutic drugs and probably protected from radiation by hypoxia.

As Goldacre and Sylven (1962) conclude "the necrotic centre is an uneven dispersion of living tumour cells surviving almost anaerobically in a medium of autolysed tumour tissue, which has no blood supply and exchanges material only very slowly with the external living tissue".

SECTION VI

General Discussion

GENERAL DISCUSSION

In a recent paper, Reinhold (1972) stated: "Blood vessels and microcirculation are probably not essential to the cause of cancer, but they are involved in so many aspects of tumour growth and therapy that they certainly deserve all our attention.

Imagine that we could influence the vascularization of tumours, what would we wish to do? There are two changes that may benefit the patient, but they are in some ways contradictory.

(a) During therapy we would like to see an improvement in the blood supply, so that all tumour cells become better oxygenated and therefore more radio-sensitive. In the case of chemotherapy, this would mean that all cells would become exposed to the drug.

(b) If no active therapy is performed, we would like to inhibit tumour growth in so far as is possible. If we could prohibit the induction of the vascular supply, then this would also arrest tumour growth". An example of this latter approach to tumour therapy is the work of Folkman and his group with the concept of anti-angiogenesis, as described in Section II.

However, it is statement (a) which is directly relevant to the present study for it explicitly supports the current reasoning behind the utilization of ICRF 159, in that if the drug could improve the vascularization of the tumour, this would facilitate both chemotherapy and radiotherapy. Although no significant difference was demonstrated between the blood flow values of control and treated 3LL tumours, the improvement in the morphology and character of the tumour blood

vessels may, in part, explain the greater effectiveness of other anticancer drugs when used in combination with ICRF 159, Hellmann and Burrage (1970); Woodman et al (1971). Similarly, this normalisation in the vasculature may account for the improved results of radiotherapy when given in combination with ICRF 159, Hellmann and Murkin (personal communication).

It seems unlikely that ICRF 159 is unique in its angiometamorphic effect on tumour blood vessels, although a search of the literature has failed to reveal any such agents. More likely, research workers have been concerned with simply inhibiting tumour growth and one wonders how many of the apparently ineffectual cytostatic agents thrown out by drug-screening programmers, would have proved highly active as angiometamorphic drugs. Consequently, using the 3LL as a model of tumour dissemination, a number of anticancer agents have been tested for their antimetastatic effect and their action, if any, on the vasculature of the primary implants, Hellmann et al (1973).

Cyclophosphamide inhibited 3LL metastasis though only to an extent proportional to its inhibition of the growth of the primary implant; it seemed to have no angiometamorphic effect (Salsbury et al, 1970).

Methotrexate, even at maximum tolerated doses, did not affect primary or secondary tumours. Similar results were demonstrated with Vincristine.

Prednisolone markedly inhibited the growth of the primary implants but did not prevent the appearance of secondaries in the lungs.

5-Fluoruracil, like ICRF 159, almost completely inhibited the formation of metastases without affecting the growth of the primary implant. Consequently, this agent is under further investigation in these laboratories.

Using the same test system, Franchi et al (1971) demonstrated that Triton WR 1339, a formaldehyde polymer of polyoxyethylene ether of octylphenol, inhibited the formation of pulmonary metastases at doses which caused no reduction of weight of the primary tumour. As to its mode of action however, there is no similarity with ICRF 159, since Triton WR 1339 fails to prevent the release of 3LL cells into the circulation. The possible explanations for the mode of anti-metastatic action of the agent were twofold: (i) the drug directly inhibited the movement of cancer cells by altering their adhesiveness and increasing their volume, and (ii) the drug enhanced the host's defence mechanisms to the circulating cancer cells. However, these results have been criticised by Hellmann (1973) who showed that Triton WR 1339 did not in fact inhibit metastasis. Further, Carter et al (1971) found that this agent actually aided the spread of a spontaneously disseminating hamster lymphoma.

One of the main criticisms levelled at the introduction of new anticancer agents is that although they are highly effective against experimental tumours, their usefulness is limited against human neoplasms. ICRF 159 is currently undergoing clinical trials, but it is too early yet for evaluation.

From the present study, two approaches to tumour therapy can be made: (i) At presentation many tumours have already metastasised. With some patients, although clinically no metastases can be detected, secondaries are found when more

sensitive techniques are employed. An example of this is that patients with early stage carcinoma of the breast often show metastases when the ^{18}F isotope bone scanning method is used, Sklaroff and Charkes (1968); Galasko et al (1968) and Galasko and Doyle (1972). In these cases it is too late to test for the antimetastatic effect of ICRF 159. However, the angiometamorphic effect on the primary and secondaries with subsequent radiotherapy or chemotherapy could be evaluated. (ii) The second approach concerns patients with tumours which are non-resectable by surgical criteria but are still in a relatively early stage with no metastases e.g. some patients with carcinoma of the bronchus. In this case, ICRF 159 could be tested for both its antimetastatic effect as well as its angiometamorphic effect.

When these approaches to therapy may be tried is a matter for speculation, partly because of the lack of suitable cases and partly because too many clinicians are too set in their ways. As Hellmann (1971) put it: "Naturally, untried drugs must go to the end of the queue, so that when the patient is finally given the new drug, the conditions for its clinical trial are severe. With an increase in the number of compounds hopefully awaiting clinical trial, the organization of such trials should be considered carefully on a national level, if useful drugs are not to be discarded".

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Metastases and the Normalization of Tumour Blood Vessels by ICRF 159 : A New Type of Drug Action

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Summary

Profound modification of the structure and arrangement of the blood vessels has been shown in tumours after treatment with ICRF 159. *X*-ray angiography, carbon black (Pelikan ink) labelling, and intravital staining with lissamine green were used to demonstrate the changes. Alteration of the morphology of the blood vessels at the edge of a tumour may affect the escape of malignant cells and the rate of blood flow (and thus the concentration of anticancer drugs) through the tumour.

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Introduction

Treatment of the Lewis lung carcinoma (3LL) with ICRF 159 ((±)-1, 2-bis(3, 5-dioxopiperazin-1-yl) propane) inhibits the pulmonary metastases resulting from bloodborne dissemination of 3LL cells (Hellmann and Burrage, 1969). The growth rate of the primary implant is, however, not significantly impeded by dosages required to produce this effect.

Histological examination of the possible mechanisms in-

205/72

volved showed that the inhibition brought about by ICRF 159 is probably due to normalization of the developing blood vessels at the invading margins of the primary tumours (Salsbury, Burrage, and Hellmann, 1970). In this region an ill-defined leash of poorly formed vascular sinusoids with frequent areas of haemorrhage is replaced, in treated tumours, by vessels of relatively normal appearance and character.

Such modification of the growth of tumour blood vessel structure appears to be a new type of drug action and one which could be of considerable importance, not only for prevention of metastases but also in the treatment of primary and secondary tumours. It seemed desirable, therefore, to study this "angio-metamorphic" effect more closely, particularly by means of *x*-ray angiography.

Materials and Methods

Lewis Lung Carcinoma (3LL).—Tumours were inoculated subcutaneously into the flanks of inbred C57/B1 female mice weighing between 18 and 22 g. Aseptic precautions were observed. Experimental mice received ICRF 159 30 mg/kg suspended in 0·2 ml of carboxymethyl cellulose (0·5%) in isotonic saline (CMC) given daily intraperitoneally. Control mice received 0·2 ml of CMC alone intraperitoneally daily.

Walker 256 Carcinosarcoma.—In order to see differences in tumour vascular pattern more clearly the rat Walker tumour grown in the flanks of Sprague-Dawley rats (150-200 g) was studied. Experimental rats received ICRF 159 50 mg/kg suspended in 0·4 ml of CMC solution given intraperitoneally daily. Controls received 0·4 ml of CMC alone.

Radiographic Procedure

Ninety-two C57/B1 mice with 3LL tumours were used for angiographic studies, of which 67 angiograms were of sufficiently high standard to be used for evaluation. Twenty Sprague-Dawley rats with Walker tumours were also used, of which 16 angiograms were of a sufficiently high standard.

Various methods for the injection of contrast media into bromethol (Avertin) anaesthetized animals were tried, but retrograde intravenous injection proved most effective. Micropaque (aqueous barium sulphate about 1 oz/fl. oz (55g/50ml)) was injected through a polyethylene catheter introduced into the inferior vena cava through the exposed right heart (Margulis, Carlsson, and McAlister, 1961).

Animals were placed in the supine position on a transparent tray with the *x*-ray tube 18 in (46 cm) above. *X*-ray film was placed on a shelf 18 in (46 cm) below and each radiograph was therefore twice life-size. Focal spot of the *x*-ray tube was 0·3 mm (Parish, Akester, and Gregg, 1964). Care was taken to give the contrast medium in the same volume and identical manner each time.

IN VIVO BLOOD VESSEL LABELLING

Sixty-four C57/B1 mice with 3LL tumours and 18 rats with Walker tumours were used in these experiments. A colloidal suspension of carbon black (Pelikan biological ink C11/1431a) diluted 1:4 with 1% gelatin in sterile saline was used (Oswald and Cater, 1969). This suspension contains about 10 mg of carbon per ml with an average particle size of 200 Å; it is stabilized with 4·5% fish glue and contains 1·3% phenol as a preservative.

Mice (C57/B1) inoculated with 3LL tumour and treated with ICRF 159 or CMC received 0·12 ml of the diluted ink per 20 g mouse into the lateral tail vein; 0·75 ml per 150 g Sprague-Dawley rat of the diluted ink was similarly injected intravenously into rats inoculated with the Walker carcinosarcoma treated with ICRF 159 or CMC. The animals were left for one hour and then killed, the tumour and surrounding tissue were quickly removed, placed in 10% formol saline for 14 days, and then washed in distilled water for 24 hours before being transferred to 12·5% gelatin at 37°C for 24 hours and then for another 24 hours into 25% gelatin also at 37°C. The blocks were embedded in 25% gelatin and stored in formol saline. Frozen sections cut at 25-35 μm were lightly stained with alum carmine and mounted in Highman's mountant. At least eight frozen sections cut randomly from each tumour were examined. Random areas of the 3LL primary tumours were examined with the electron microscope in order to locate the position of the ink within the blood vessels.

The tissues were fixed in 4% glutaraldehyde in phosphate buffer for one hour and then post-fixed for a further hour in phosphate buffered osmic acid, and embedded in Araldite.

Sections (about 400 Å) were cut on a Porter-Blum microtome equipped with a glass knife and stained with 25% uranyl acetate in methanol for 10 minutes, washed in methanol, and then further stained in lead citrate for four minutes and washed in distilled water. Sections were lightly coated with carbon and examined in the Siemens Elmiskop 1 electron microscope.

Some 3LL tumours were excised five minutes after intravenous injections of ink, and thick cryostat sections, stained with neutral red, were examined.

INTRAVITAL STAINING WITH LISSAMINE GREEN

Thirty-six C57/B1 mice inoculated with 3LL tumour and treated with ICRF 159 or CMC received 0·5-1 ml of a 2% lissamine green solution (Gurr) rapidly into the tail vein (Goldacre and Sylvén, 1962). Five millilitres of 2% lissamine green solution was similarly injected into rats inoculated with the Walker carcinosarcoma and treated with ICRF 159 or CMC. The animals were exsanguinated under ether five minutes after the injections of lissamine green. The tumours were bisected and examined.

Results

ANGIOGRAPHY

3LL Tumour

Host Vessels.—No new vessels were seen to develop in response to the tumour, but host vessels draining the tumour were constantly dilated and some of these vessels became very

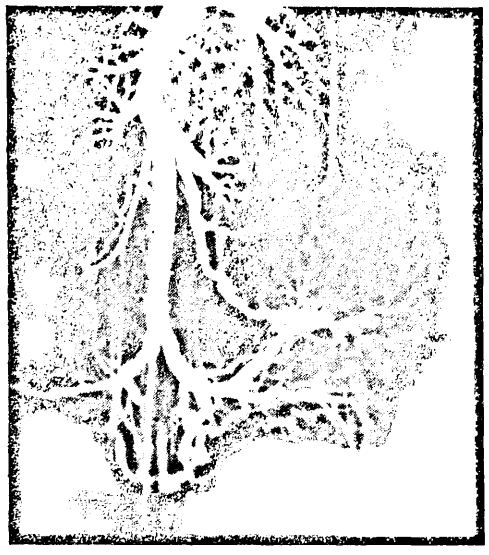

FIG. 1—Venogram of a primary 3LL tumour 14 days after implantation and treated with CMC only. (× 1·4.)

tortuous. Identical appearances were seen in animals where the tumour was treated with ICRF 159. At the border of the tumour angiographic appearances became very indistinct owing to vascular proliferation. It was not possible to distinguish whether this proliferation was in the host or the tumour tissues. Again the angiographic picture was the same for both control and treated tumours.

Tumour Vessels.—Control tumours showed large filling defects whose size and position varied from tumour to tumour. Such vascularization as there was appeared to be randomly arranged with vessels often ending abruptly (Fig. 1). This proved to be the constant picture in the control tumours. When the tumour was cut Micropaque flowed out from the cut surfaces. Tumours of the treated mice, however, showed numerous discrete blood vessels with some indication of a regular vascular arrangement (Fig. 2).

FIG. 2—Venogram of a primary 3LL tumour 14 days after implantation and treated with ICRF 159 (30 mg/kg intraperitoneally. Days 1-4, 7-11, and 14). (× 1·7.)

FIG. 3—Venogram of a Walker carcinosarcoma seven days after implantation and treated daily with CMC. (× 1·5.)

Walker Tumour

Host Vessels.—Large new vessels did not develop in response to the tumour. On the other hand, three main host vessels—the lateral thoracic-cutaneous, the ilio-lumbar, and the inferior epigastric veins—were always considerably dilated, presumably to meet the demands of outflow from the tumour. Essentially the same picture was seen in ICRF 159 treated and control tumours. At the margins of the tumour, however, there was considerable proliferation of blood vessels in both treated and control tumours.

Tumour Vessels.—In the tumours themselves there was again a marked contrast between treated and controls. The control tumours showed signs of pooling of the Micropaque; there were avascular areas of varying size and position and commonly "corkscrewing" of dilated vessels. The vessels were also randomly arranged and irregular, all of which is characteristic of tumour blood vessels (Fig. 3). The treated tumours, however, showed no signs of pooling and the blood vessels presented a more orderly arrangement. There was little evidence of any corkscrewing (Fig. 4).

FIG. 4—Venogram of a Walker carcinosarcoma seven days after implantation and treated daily with ICRF 159 (50 mg/kg intraperitoneally). (× 1·5.)

BLOOD VESSEL LABELLING

3LL Tumour

In thick frozen sections of the primary treated tumours the ink was largely cleared, thus showing little labelling (Fig. 5), but in control tumours many vessels were clearly outlined (Fig. 6). With older and therefore more necrotic tumours the differences between the groups became much less apparent. Electron micrographs of control tumours showed the carbon trapped between basement membrane and an abnormal endothelium.

Walker Tumour

With this tumour treatment with ICRF 159 also reduced, but by no means abolished, the number of labelled vessels. Labelling in the treated tumours was found only near areas of haemorrhage and necrosis.

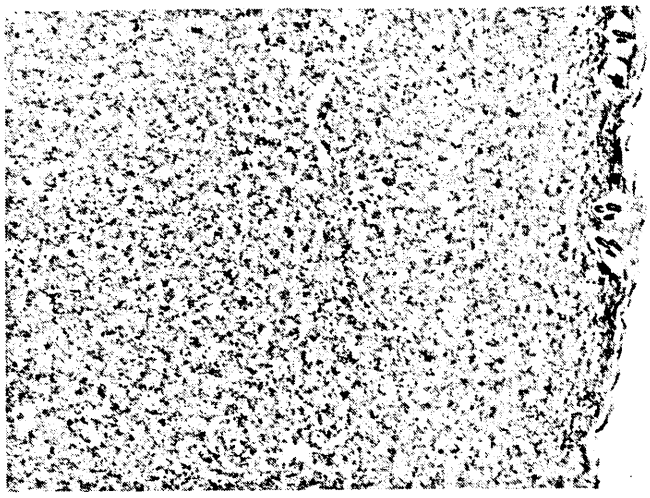

FIG. 5—Frozen section of a primary 3LL tumour 14 days after implantation and treated with ICRF 159 (30 mg/kg intraperitoneally. Days 1-4, 7-11, and 14). Tumour excised one hour after intravenous injection of Pelikan ink. (Alum carmine. × 29.)

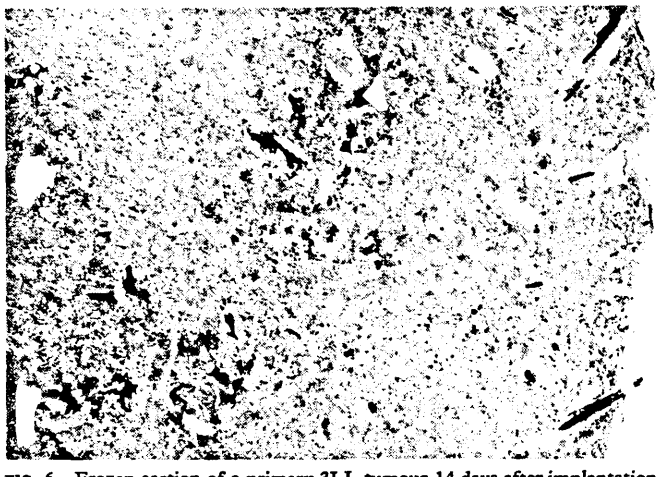

FIG. 6—Frozen section of a primary 3LL tumour 14 days after implantation and treated with carboxymethyl cellulose. Tumour excised one hour after intravenous injection of Pelikan ink. (Alum carmine. × 29.)

INTRAVITAL STAINING

3LL Tumour

Gross observations revealed no differences between control and treated tumours at similar ages after tumour implantation. As the tumour increased in size the peripheral non-ischaemic zone became markedly reduced. There were no avascular, colourless areas in the tumour periphery. The youngest tumours (seven days, about 0·8 cm across) were usually coloured throughout, whereas in the oldest tumours (21 days, about 2·0 cm across) the peripheral vascularized zone was only a few millimetres thick.

Walker Tumour

Again gross observations revealed no differences between control and treated tumours. In Walker tumours ranging from 4 to 10 days old there was an increase in the size of the necrotic centre until in the largest tumours only a very thin peripheral vascularized zone remained.

Discussion

Willis (1952) pointed out that "the structure of the new-formed blood vessels in malignant tumours rarely approaches that of normal veins or arteries, and the more rapidly growing the tumour, the more imperfect is the architecture of its vessels . . . in highly anaplastic growths, carcinomas as well as sarcomas, even the endothelium may be incomplete in places and the vascular channels lined in part by tumour cells." Clearly such imperfections of vascular structure must favour dissemination of malignant cells. Willis's statement applies with equal force to the Lewis lung carcinoma of the mouse (Salsbury *et al.*, 1970).

Previous observations indicated, however, that the structurally imperfect vascular architecture of the 3LL tumour can be improved by treatment with ICRF 159 (Salsbury *et al.*, 1970) and the present results lend considerable support to the assumption that treatment with this drug normalizes 3LL vasculature functionally as well as morphologically. They also strengthen the conclusion that treatment with ICRF 159 prevents pulmonary metastases because malignant cells do not line, nor do they penetrate, the normalized blood vessels and therefore cannot reach the blood stream. Consequently, they are unable to escape from the primary tumour.

Although it seems clear that as in other tumours the 3LL and the Walker have an outer shell of rapidly proliferating tumour cells permeated by a leash of imperfectly formed and randomly arranged vascular sinusoids, certain aspects of the angiograms call for comment.

In 3LL control tumours Micropaque diffuses into the wide spaces of the sinusoids in a "river-delta" effect, but because of the limitations of the resolution of the *x*-ray technique, radio-opaque material cannot be demonstrated. The filling defects are therefore more apparent than real, and if the tumours are bisected Micropaque flows out from the cut surfaces. In treated tumours, however, the Micropaque is confined to the discrete vascular channels and consequently can be seen on the angiograms.

In Pelikan ink studies also, where tumours were excised five minutes after intravenous injection, the ink distributed itself throughout extravascular and intravascular spaces in the controls, but remained confined to the vessels in the treated tumours. The lissamine green injections provide further confirmation that the filling defects in the control venograms reflect only the insufficiently fine resolution of the angiograms.

The lissamine green injections also show that in both treated and control tumours vascularization is confined to an outer zone or cortex which encapsulates the tumours, but that the larger central and probably necrotic medulla appears not to be reached by the dye. It can be assumed, therefore, that Micropaque is also confined to the peripheral zone and that the vessels seen on the angiograms are in the cortex. It seems probable that the blood flow rate in the wide diffuse spaces of the sinusoids in the "avascular" areas of the peripheral zone of the controls is very much lower than in the discrete vascular channels of the cortex of the treated tumours.

Recent histological examination of other tumours treated with ICRF 159 have shown that the blood vessel changes are not confined to the 3LL tumour (Salsbury, Burrage, and Hellmann, to be published). It seems possible, therefore, that the drug affects the fundamental biochemical processes by which a tumour organizes a vascular supply to and into itself. These processes could well be similar in most tumours and have little or no parallel among normal tissues, thereby providing a selective target for cancer chemotherapy.

The abnormal character of tumour blood vessels and their functional normalization by ICRF 159 is well shown by the Pelikan ink experiments. When Pelikan biological ink is injected intravenously it passes rapidly through normal vessels, but is trapped between the basement membrane and the endothelium if they are inflamed or otherwise damaged (Cater and Taylor, 1966; Majno, Palade, and Schoefl, 1961). The considerable degree of vascular labelling in the control Walker and 3LL tumours is greatly diminished on treatment, but less so in the rapidly proliferating Walker tumour than in the more slowly growing 3LL. This bears out Willis's statement that the more rapidly growing the tumour, the more abnormal its vessels.

Specific alterations of tumour blood vessels could have considerable consequences for the treatment of tumours. The use of vasoactive drugs to impair selectively the blood supply of tumours has been proposed on occasions—for example, Cater, Grigson, and Watkinson (1962) and Dayal and Goldacre (1969)—but the possibility that drugs might be used to alter blood vessel structure so as to prevent the escape of malignant cells from the primary tumours has not, so far as we are aware, been previously entertained or observed. This angiometamorphic effect may, however, also mean that blood flow rates in treated tumours are higher and that consequently the availability of anticancer drugs might be increased. This consideration may, in part, explain the greater effectiveness of other anticancer drugs when used in combination with ICRF 159 (Hellmann and Burrage, 1970; Woodman, Venditti, Schepartz, and Kline, 1971). Although ICRF 159 is the first compound to be shown to exhibit an angiometamorphic effect, it is unlikely to be unique in this respect, indeed other cytostatic bisdioxopiperazines are known to be active (Burrage, Hellmann, and Salsbury, 1970).

The antimetastatic effect of a number of well-known anticancer agents has recently been investigated (Hellmann and Burrage, in preparation) but only cyclophosphamide has been studied in any detail. Cyclophosphamide inhibits 3LL metastasis, though only to an extent proportional to its inhibition of the growth of the primary implant; it seems to have no angiometamorphic effect (Salsbury *et al.*, 1970). 5-Fluorouracil, like ICRF 159, inhibited metastasis without overt effect on the primary implant. Prednisolone, on the other hand, had a pronounced inhibitory effect on the primary, but did not prevent metastasis. Whether these variations of response are related to changes in the tumour vasculature is being studied.

The profound differences in the mode of action of anticancer drugs as exemplified by these results emphasizes once again that selective therapeutic activity among anticancer agents is more likely to be obtained by closer study of their pharmacological activities than by analysis of their non-specific inhibition of cell division.

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(Schöpf, Gänshirt, Hutzler, Klug & Reinshagen, 1969). Although the (−)-isomers of these racemic compounds have the essential structure thought to be associated with biological potency, they are inactive in the rat liver protein synthesizing system *in vivo* and *in vitro*. This leads us to support the idea first put forward by Lietman (1971) that structural subtleties among emetine derivatives not originally envisaged by Grollman (1966) may emerge as different protein synthesizing systems are methodically studied.

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Vascular changes in tumours after treatment with ICRF 159

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Mice implanted subcutaneously with the Lewis lung (3LL) carcinoma regularly develop pulmonary metastases, which can be prevented by treatment with [(±)-1,2-bis (3,5-dioxopiperazin-1-yl) propane] (ICRF 159) (Hellman & Burrage, 1969). The poorly defined sinusoids which act as vascular channels in the periphery of control primary tumours are replaced by well formed discrete blood vessels in tumours treated with ICRF 159 (Burrage, Hellmann & Salsbury, 1970). These changes in the structure of the blood vessels are probably responsible for preventing the escape of malignant cells from the primary tumour into the circulation.

The distribution and character of these 3LL blood vessels has now been further investigated by means of X-ray angiography and by a colloidal carbon technique which specifically outlines damaged and inflamed blood vessels (Majno, Palade & Schoefl, 1961). A comparison has also been made of the effects of ICRF 159 treatment of rats inoculated with the Walker carcinosarcoma.

A polythene catheter was inserted into the inferior vena cava through the exposed right heart of animals anaesthetized with Avertin. Micropaque was introduced slowly by retrograde injection and care was taken that introduction of contrast medium was performed in exactly the same way each time.

For venograms, the animals were placed in a supine position on a transparent tray with the X-ray tube 45 cm above them. The X-ray film was placed on another shelf 45 cm below. Thus each radiograph was twice life size. Focal spot of the tube was 0·3 mm. Venograms of control tumours showed avascular areas, pooling of Micropaque and an irregular vascular network with many tortuous vessels. In treated tumours the venous pattern was discrete and well organized.

The colloidal suspension used was carbon black in the form of dilute Pelikan biological ink CII/143Ia. Animals were left for 1 h after intravenous injections of the dilute ink (0·12 mg/20 g mouse and 0·75 ml/150 g rat) after which the tumours were quickly removed and placed in 10% formol saline for 14 days. Frozen sections cut at 25–35 μm were lightly stained with Mayer's Carmalum. Virtually no carbon labelling was found in the treated Lewis lung tumours, whereas controls showed vessels outlined with carbon in zones of growing tumour. The Walker tumours of control rats showed areas of carbon labelling both peripherally and centrally near necrotic and haemorrhagic zones. Treatment with ICRF 159 reduced but did not abolish the labelling of vessels.

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Influence of phenobarbitone on liver regeneration and microsomal N-demethylating activity in partially hepatectomized rats

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Normal rat liver is capable of remarkable adaptations to meet the functional demands upon it. For example, the low resting mitotic rate increases dramatically when part of the liver is exised, the magnitude of the response being inversely related to the amount of liver remaining. In addition, oxidative enzymic activity in the intact liver can be greatly increased by drugs. For example, phenobarbitone increases the synthesis of the microsomal enzymes responsible for its metabolism. Each of these processes involves the hepatocyte in considerable reorganization and in synthesizing new protein for such specialized structures as the mitotic spindle or the endoplasmic reticulum. These two processes represent quite different types of adaptation, since regeneration involves a less differentiated activity (mitosis) than the synthesis of drug metabolizing enzymes. It is therefore of interest to know if both processes can occur simultaneously or whether one has priority.

This study investigated these possibilities in partially hepatectomized and sham operated male Wistar rats (250–350 g), pretreated 12 h before surgery with phenobarbitone (80 mg/kg i.p.) and 1 h before death with metaphase inhibitor colchicine