TUMOUR RESPONSE AND DISSEMINATION FOLLOWING HYPERTHERMIA

by

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SUMMARY

The following thesis describes an investigation into the use of hyperthermia as a remedial procedure using a new technique for treating animal tumours (Walker, 1980).

A brief historical review of the use of hyperthermia treatment is presented in Chapter 1. Current methods of treating local and metastatic disease are discussed, and the advantages (and problems) of using hyperthermia for the treatment of tumours which are resistant to chemo- and radiotherapy are outlined. Chapter 2 describes the biological properties of the C_3H mouse mammary carcinoma and presents reasons for the choice of this tumour system in this present research. The normal growth pattern is then presented. At early stages growth was measured indirectly by means of a dilution assay and directly by measurement after the tumour became palpable. In Chapter 3 the development and application of a new technique for administering hyperthermia treatment to animal tumours is described. The following chapter shows how treatment alters the normal growth rate. Regression, recurrence and cure are compared after treatment either with hyperthermia or with X-rays. After similar levels of treatment tumours were found to recur in a significantly shorter time after hyperthermia than after irradiation (Walker et al 1982). Possible reasons for this are discussed. Chapter 5 considers theories relating to metastasis and compares metastatic rates after hyperthermia, X-rays (Walker et al 1978) and surgery. The evidence suggests that inadequate treatment using hyperthermia may inadvertently promote metastasis.

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1.0 INTRODUCTION

1.1 Tumour Structure

A tumour or neoplasm has been defined as "... an abnormal mass of tissue, the growth of which increases and is uncoordinated with that of the normal tissues, and persists in the same excessive manner after cessation of the stimuli which evoked the change" (Willis, 1973).

This definition covers tumours which form discrete lumps, diffuse masses and such tumours as myloid leukaemias, where the mass is merely distributed diffusely through the marrow or lymphoid tissues. Tumours often show a resemblance to the normal tissue from which they have originated, though the cells may be more like the precursor cells from which the tissue has formed. In most tumours the neoplastic tissue consists of cells of a single strain. The connective tissue and blood vessels are merely the supporting and vascular framework of the tumour. The vascularisation has formed quickly and is less well organised than in normal tissues (see Section 1.4 [2]).

Tumours are divided into benign and malignant types. Benign tumours are limited in growth and do not normally invade surrounding tissue, while malignant tumours, if untreated, continue to grow and invade surrounding tissues. It is the infiltrative capacity which gives the name cancer (the crab). Normally the tumour edge is ill-defined although some rapidly growing tumours in a site that gives little constriction may appear encapsulated.

Nearly all malignant tumours metastasise. This may come about by

detachment of a group of tumour cells, or emboli, which could invade the lymphatic system and become lodged in a lymph node or may enter the blood stream and, on reaching a capillary network, become impacted, proliferate and thus develop into secondary tumours or metastases.

1.2 Treatment of Tumours and Reasons for Use of Hyperthermia

In choosing a method of treatment for cancer a primary consideration is whether the disease is localised or disseminated. Where a tumour is localised one can restrict treatment to that area so surgery, which can sometimes remove the whole tumour, and/or radiotherapy would be the preferred treatment. However, some tumours cannot be treated surgically either because of the resultant mutilation or, where a large amount of tissue would be involved, the loss of function of vital organs. Moreover, although conventional radiotherapy with X-rays can often destroy tumours with tolerable damage to the surrounding normal tissue, some tumours are radio-resistant. Where radiation therapy is appropriate it is normally used in treating regionally limited cancer, however systemic treatment by whole body irradiation is sometimes of value in the treatment of disseminated disease. Chemotherapy is, however, the most likely choice in this case. The drugs used disperse through the body, where they can attack the rapidly dividing cancer cells. Drugs are also used in combination with either surgery or radiotherapy where there exists the possibility that a primary tumour could have spread.

The failure, however, of conventional treatment in the management of some forms of cancer - particularly melanoma or bone tumours - has stimulated the search for improved methods of treatment, which

included using:

- neutrons instead of X-rays
- X-rays with unconventional fractionation schedules
- X-rays with radiosensitisers
- X-rays with chemotherapeutic drugs
- hyperthermia.

These treatment techniques have met with varying degrees of success. Some have merits in dealing with specific tumour types, but with the exception perhaps of neutron therapy, many fail to deal adequately with poorly vascularised, hypoxic, tumours. It is in this area in particular that hyperthermia seems (for reasons which will be discussed later) to be a most promising form of treatment, either on its own or in combination with radiotherapy or chemotherapy. Thus because of the probable merits of hyperthermia in dealing with difficult tumours, it deserves, and is receiving, much attention today. These possible advantages have stimulated the present study.

Claims have been made of the beneficient effects of hyperthermia on tumours over a long period of time. The first beneficial effects were found probably, as in many important discoveries, almost by accident in a case where a patient suffering from cancer also developed erysipelas and was cured from the malignancy. There is an early clinical report by Busch (1866) who found that a histologically confirmed case of sarcoma of the face disappeared after erysipelas associated with high fever. There are several other well documented clinical reports of cancer patients whose tumours regressed completely after attacks of erysipelas with concomitant high fevers (Coley, 1893). There are also reports of

patients who benefited from induced fevers, or from raising the temperature by hot baths, together with local heat application (Coley, 1893). Nauts, Fowler and Bogatko (1953) have reported on a series of 30 patients with inoperable tumours. The patients were treated with bacterial infections and bacterial products (Coley's toxins), which gave rise to very high temperatures. Twentyfive of the thirty survived for over ten years. Nauts (1978) in a review of this early form of hyperthermia treatment reported that the highest percentage of successes occurred in sarcoma of soft tissues and malignant lymphomas. In inoperable or metastatic carcinomas of the breast, malignant melanoma, neuroblastoma and various bone tumours complete or partial regressions were obtained. There were also remarkable remissions obtained in leukaemia after treatment with acute infection associated with raised body temperature.

Treatment with Coley's toxins was not always successful and even in those cases which were successfully treated the effect was attributed to stimulation of the immune system rather than to the hyperthermia (Nauts, 1978).

Since most of the early clinical observations were based on whole body hyperthermia, other mechanisms than hyperthermia could have been implicated. However, when regional hyperthermia was used successfully (Cavaliere et al, 1967) the effect was then attributed more readily to the high temperature. Thus it began to be appreciated that tumours could be 'cured' purely by elevation of temperature. These clinical observations thus prompted further investigation into the effects of heat on tumours using experimental animals and neoplastic cells *in vitro*.

'Cure' can be defined as no local regrowth of a tumour during a predetermined period, which is often related to the normal rate of growth of the tumour in question. With neoplastic cells *in vitro* cure or thermal cell inactivation can be defined as the inability of the heat treated cells to produce clones of cells. After treatment the cells may divide once or twice but the ability for unlimited division has been lost though the cells may not necessarily die immediately.

It was reported by Crile (1961) that tumours on experimental mice could be cured by immersing the tumours in water at temperatures of over 42°C. Crile found that the majority of the tumours were cured by heating for one hour at 43°C. It was found that for each degree that the temperature was raised the time of exposure required to obtain the same biological effect could be halved so that exposure of less than a minute at 49°C was equivalent to two hours at 42°C.

The effectiveness of hyperthermia was then further supported by studies on mammalian cells *in vitro* (Hahn, 1974). Loss of reproducibility was also found to take place above a critical temperature of approximately 42°C and Hahn also observed a similar relation between time, temperature and cure to that obtained by Crile. The exact temperature level which was required to inactivate different cells varied slightly with cell type. The results by Crile and Hahn confirmed that the clinically observed cures were probably based on the effect of heat alone.

1.3 Current Methods of Treating Tumours by Hyperthermia

1.3.1 Whole Body Hyperthermia

Hyperthermia may be applied locally or to the whole body. Whole body treatment is used today in cases of disseminated disease often after other treatments have failed. The major problem with whole body hyperthermia is that the body cannot tolerate temperatures above 41.8 - 42°C. Organs which limit treatment are the liver, which may be relatively sensitive to thermal damage, and the heart (Hand and ter Haar, 1981).

Various methods have been used to elevate whole body temperature. These include using Coley's toxins (Nauts et al, 1953), hot air and anaesthesia (Euler-Rolle et al, 1978; Blair and Levin, 1978; Pettigrew et al, 1978), microwaves (Pomp, 1977; Bolmsjö et al, 1979), warm water blanket (Larkin, 1979), radiofrequency heating (von Ardenne, 1978) and heated space suits (Bull et al, 1979). Body core temperature was elevated to not greater than 42°C for 1-2 hours in most cases, and various tumour types were treated. The methods, temperatures reached, tumour types and success rates, etc, where given, are summarised in Table 1.1.

Dickson and Muckle (1972) found total body heating in animals to be less successful than local heating, probably because animals died if a core temperature of above 40°C was maintained, while the intra-tumour temperature for local treatment was over 42°C. A further problem the results suggested was that the 40°C core temperature might have stimulated metastatic cells, although a different response of the immune system in the tumour-bearing animal to the two methods of

treatment could also have stimulated cells at metastatic sites after whole body treatment.

1.3.2 Local Hyperthermia

Since the work of Crile (1961), Hahn (1974) and others, it has been recognised that if the tumour temperature did not rise to about 42°C, then tumour cells would not be inactivated. This can be done more safely by means of local heat. Thus investigations into methods of applying localised hyperthermia to tumours, and into possible side-effects of this mode of treatment, are proving to be a most promising area of research. This is the main burden of the present study.

There are problems associated with localised treatment and these fall into two main categories (see Table 1.2).

[1] Technical problems:

- penetration is particularly difficult with deep tumours
- heat must be localised to the tumour in order to protect the surrounding normal tissue
- it is necessary to achieve and maintain predetermined temperatures at all points in the tumour. This is important since the therapeutic temperature is within a narrowly defined range
- continuous temperature monitoring is obligatory. Temperature measurement is especially a problem with microwave heating since conventional measuring equipment can cause burns in a microwave field.

[2] Biological problems:

- there may be differential heating when more than one tissue

is involved. For example with shortwave diathermy there is preferential heating of fat, so heating of sub-cutaneous fatty tissue may raise problems that could be unacceptable even with skin-cooling

- cooling of the heated volume by blood flow is probably the major problem. This modifies temperature to a greater or lesser extent depending on the vascularity of the tumour and on the temperature differential. Even within heated superficial tumours in the region of large blood vessels there can be cold spots which markedly affect cure (Robinson et al, 1978) because of the very strong dependence of thermal sensitivity on small changes in temperature. One way round this problem is to raise the whole body temperature to one which would not damage normal tissue. Then a local heat superimposed on this would be more readily maintained, and temperature gradients would be less - this may prove ultimately to be the most useful type of therapy. Not many clinical evaluations of this have yet been reported though von Ardenne and Kruger (1980) have described a heating technique where the whole body temperature was 40.5°C. Local hyperthermia was superimposed in the tumour area to give a tumour temperature of 42.5°C. This regime has been used in conjunction with tumour hyperacidification achieved by elevation of the blood glucose concentration, which lowers the pH in the tumour cells - making them more sensitive to heat (see 1.4[3]). Cancer Multistep Therapy, as this has been called, has given encouraging results. Tumours, however, often have a poorer blood supply than normal tissues and are not so readily cooled when heat is applied. Advantage can be taken of such vascular abnormalities to give selected heat to tumours.

There are four principal methods of delivering localised heat to tumours (see Table 1.2 - information contained in this Table is in Hand and ter Haar, 1981; Cetas and Connor, 1978; Strang and Patterson, 1980).

Direct heating is technically the simplest form of heating. Generally hot water is placed in thermal contact with the area to be heated, the temperature in the tumour rising due to conduction of heat from the water. It gives surface heating only and must be invasive for deep heating. A very considerable number of experimental studies have been carried out using waterbath heating. Fewer clinical reports are available on the use of localised direct hyperthermia alone, this normally being used in conjunction with other treatments such as chemo-therapy and X-rays. Treatment by regional perfusion which is really another form of conduction heating, has been carried out by Cavaliere et al (1967) as already described, and by Stehlin (1969, 1975 and 1980). Stehlin has treated patients with limb tumours by perfusing the affected limb with the drug Melphalan. In a large group of patients the perfused blood supply was pre-heated to temperatures of around 41-42°C and the survival rates compared favourably with that of patients who were treated similarly but with no elevation of temperature. Bladder tumours have been treated by irrigation using a type of cathetor arrangement (Ludgate et al, 1978), which is also a form of local surface heating. It was found that the response was limited because of the large heat gradient and the intensive cooling effect of the well vascularised bladder tissue.

Animal and human tumours can be successfully treated with microwaves by using specialised applicators in conjunction with different

frequencies for each particular situation. Penetration with microwaves is better than with conduction. It is a function of the frequency and of the properties of the tissue to be heated. Lower frequencies give better penetration but poorer localisation and vice versa so a compromise must be made. Penetration is better in tissues with a low water content (fat, bone, etc) than when the water content is high (muscle, skin, etc). Heat patterns are difficult to predict even in a homogeneous tissue and specialised temperature monitoring equipment must be used. Van Dijk et al (1979) and Luk (1980) have treated human tumours and found poor temperature distributions. Luk obtained no response at all when treating tumours at - what was supposed to be - 42.5°C. Results of treatment of some animal tumours using microwave and other methods is shown in Table 3.11. On the whole it appears that results of treatment with microwave are conflicting.

Radiofrequency heating has been used to treat animal and human tumours with some success. Penetration with radiofrequency heating is better than with other forms of heating if the tissue is uniform, when the heat pattern is also uniform. Radiofrequency waves cause oscillation of ions or changes in molecule orientation which is converted into heat. Penetration is dependent on the relationship between the geometry of the electrodes and the frequency; higher frequencies penetrate better but give poorer uniformity of heat pattern. Electrodes must be designed to give preferential heating to the periphery to overcome conduction of heat away from the tumour. Using specialised electrodes Harzmann et al (1979) found 'some measure of success' in treating carcinoma of the bladder in animals and in patients. In treating animal tumours Marmor, Hahn and Hahn (1977) found radiofrequency

to give a similar cell kill to waterbath heating, and van Dijk and Breur (1979) found it to be more successful. Storm et al (1979) have been able to treat large tumours at depth, if these displayed a different physiological adaptation to heat than the surrounding normal tissue, with little normal tissue damage. Dickson et al (1977) found it impossible to treat large tumours on rats, since all the animals died because of elevation of the core temperature. Short-wave diathermy, a form of radiofrequency heating, has been used by Overgaard and Overgaard (1972) to treat mammary tumours implanted in the feet of C_3H mice. The cure rate was poorer than that obtained by the treatment technique used in the present study and 20% of the mice died during anaesthesia and treatment.

Hand and ter Haar (1981) appear to favour ultrasound as a technique which provides a number of versatile ways in which soft tissue tumours may be heated selectively using multiple applicators. Ultrasonic hyperthermia offers advantages in localisation as well as focussing and penetration. In a homogeneous tissue the energy deposited as heat decreases exponentially. The acoustic absorption of skin and bone are significantly higher than other tissues. It is thus difficult to produce localised hyperthermia without causing a greater rise in the temperature of the overlying skin or underlying bone. If skin has to be spared cooling is essential. Ultrasound has been used clinically with some success by Marmor et al (1979) to treat superficial tumours, and Marmor et al (1978) have also treated animal tumours. The thermal effects of ultrasound have been found not to initiate metastases (Lele, 1976; Smachlo et al, 1979) and this makes ultrasound a valuable modality for tumours that readily metastasise. The instrumentation is available and inexpensive and similar equipment

could be used for tumour imaging and for treatment.

Strang and Patterson (1980) have compared different heating techniques using mathematical models. They calculated the effectiveness of waterbath or conduction heating of 'spherical' and 'cylindrical' tissue masses in the presence and absence of blood flow. It was found that, in heating small tumours $\lesssim$ 2cm diameter, if the heated volume approached a spherical shape conduction heating was as efficient as technically more difficult procedures such as microwave heating and gave only small temperature variations.

In the present study a form of conduction heating has been used and was applied to a tumour volume as near to spherical as could be obtained in order to obtain good thermal gradients (see 3.3.3). The form of heating used is suitable for small experimental tumours in animals or small cutaneous tumours in man.

1.4 Merits of Hyperthermia in Treatment

In reviewing hyperthermia in the treatment of cancer Field and Bleehen (1979) suggest that tumours may be intrinsically more vulnerable to heat damage than normal tissues because:

[1] neoplastic cells are intrinsically more heat sensitive

[2] tumours may have a poor blood supply and attain higher temperatures than surrounding tissues

[3] some tumour cells may be hypoxic, lacking in nutrients or at a low pH all of which may increase heat sensitivity

[4] cells in S-phase are sensitive to heat.

[1] It has been found in biochemical and in clinical studies that tumour cells *in vitro* and *in vivo* are selectively destroyed by heat and that there is a profound and selective toxic effect of elevated temperature on tumour cells. Various workers tested, by means of a clonogenic assay, for resultant malignancy in cells *in vitro* after treating the cells with hyperthermia. A summary of their results is given in Table 1.3. A positive sign under 'Differential' indicates where the malignant cells have been found to be more heat sensitive. Most of the comparisons of thermosentitivity relate to the temperature range 41-43°C. Above 43°C the differential sensitivity seems doubtful - perhaps some other mechanism becomes important which is not selective. This was also found by Overgaard and Overgaard (1972). Using as an assay the changes in oxygen uptake with increasing temperature Cavaliere et al (1967) found inhibition of respiration and a selective and irreversible damage to hepatoma cells after heating to 42°C for 90 minutes, while under identical conditions there was little damage to regenerating liver cells. The oxygen uptake of the tumour cells was reduced when the temperature was increased to 42°C while there was little reduction in comparable normal cells.

Dickson and Suzangar (1976) also found that respiration and/or glycolysis of some human tumours were often depressed or even irreversibly inhibited *in vitro* by exposure to 42°C while the metabolism of normal tissues remained relatively unaffected. The *in vitro* response of animal tumour cells to increased temperatures of around 42°C has proved to be a reliable guide to sensitivity of the tumour in the host animal to hyperthermia. In Yoshida rat sarcoma cells, after a 4 hour culture period at 42°C, there was an 80-90% inhibition of isotope precursor

uptake into DNA, RNA and protein. Even after 1 hour at 42°C, the tumour cells did not take on transplantation into rats. At this time isotope uptake was reduced by 70-80% (Dickson and Suzangar, 1974). This tumour at 2-3ml volume can be cured *in vivo* by heating to 42° for 2 hours (Dickson and Ellis, 1976). Mondovi et al (1969) investigated nucleic acid and protein synthesis during and after hyperthermic exposure of normal and neoplastic tissue of a rat, and of a human osteosarcoma. A depression of synthetic activity was observed in all the tumours tested including rat hepatoma tissue and osteosarcoma; however regenerating liver cells exposed to 43°C for two hours showed increased synthetic activity. Microscopic examination of various exerimental tumours, heated *in vitro* in the temperature range 41-43°C, showed that there was cell destruction specifically in malignant cells, without apparent damage to normal cells such as fibroblasts and endothelial cells (Overgaard and Overgaard, 1972). Overgaard (1976b) extended these observations in an electron microscope study. In this case it may however be that better vascularity of normal tissue allowed for better cooling than in tumour tissue and thus the greater heat sensitivity of the tumour cells observed by these workers was secondary to the poor blood supply of the tumour relative to that of normal tissue.

It thus seems reasonable to conclude from these results that malignant cells heated within the temperature range 41-43°C appear to be selectively destroyed.

[2] In 1966, Rubin and Casarett described how most tumours show less organisation than the tissue from which they have arisen and how, because they grow more rapidly than the normal tissue, there is a

rapid distortion in the network of blood vessels. Associated muscle and elastic tissue is also normally lacking. The vessels consist mainly of irregular incomplete channels with arterio-venous shunts. While it has been adequate to permit the cellular proliferation the circulation is sluggish. This appears not to be due to a lack of capillaries - the proliferation of which has been stimulated by, and has allowed for, the rapid early growth of the tumour. Such alterations in tumour circulation are probably due to rapid increase in numbers of tumour cells with focal compression of the capillaries and a fall in blood pressure, particularly in central vessels. Because of the poor circulation tumours cope less well than normal tissue with temperature regulation when heat is applied. On the contrary, however, recent work by Patterson (pers.comm.) suggests that in some tumours blood flow is better than in normal tissues.

When applying heat it is not normally possible to heat only a single tissue, thus the temperature achieved in each tissue will be expected to be a function of its blood flow. Patterson and Strang (1979) have calculated that if one tissue has twice the blood flow of a second tissue then on heating the temperature increase in the first will be only half of that attained in the second.

Differential rises in temperature as between tumour and normal tissue have been found in *in vivo* studies. Von Ardenne (1978) found that local hyperthermia decreased the blood supply to tumours by causing vasodilation of vessels in normal tissue, while in the tumour the cross section remained the same because of lack of elasticity. In a study of 97 human tumours Mäntylä (1979) found that the tumour blood flow was considerably lower than that in the surrounding normal tissue.

This would suggest that heating by conduction through the skin (as has been done in this study) would not cause excessive damage to the skin overlying the tumour. It has similarly been reported by Kim and Hahn (1979) that there is selective heating of superficial tumours as compared with adjacent tissues because the skin over the tumour was well vascularised. In experiments by Dickson and Calderwood (1980) when tumour heating was prolonged it was found that blood flow in the tumour was unaltered over a 1 hour period. When heating was continued beyond this time there was a progressive decrease in tumour blood flow to zero in 3 hours. In contrast the blood flow through the skin and other normal tissues increased 20 and 10-fold respectively. If applicable to tumours in general these findings could have important implications for heat therapy of cancer since they enable the tumour heating temperature to be reduced with time while still maintaining a therapeutic temperature yet giving protection to the surrounding normal tissues. This may be a valid argument for prolonging hyperthermia treatments, but must be offset against any possible risk of increasing metastases.

[3] There have been several studies that have shown that under hypoxic conditions tumour cells are more sensitive, or at least as sensitive, to hyperthermia destruction as under aerobic conditions (in contrast to the effect of X-rays on such cells). This has been shown *in vitro* by Gerweck, Gillette and Dewey (1974), Kim, Kim and Hahn (1975), Dewey, Thrall and Gillette (1977), Gerweck (1977), Bass and Moore (1978). The increase in sensitivity to heat may be due to insufficient nutrition. An appreciable fraction of Chinese hamster cells in culture were killed by heat in the absence of serum, when they lysed either during or shortly after heat exposure. The presence of serum

inhibits the lysis process. The role of oxygen and glucose appear less important; chronic or acute deprivation of oxygen has only minor influence on survival. Similar conclusions appear to be true for variations in available glucose concentrations (Hahn, 1974). Dewey et al (1977) found that heat eliminated the radioresistance of chronically hypoxic tumour cells in C_3H mouse mammary tumours implanted in the legs of mice. They did not find a selective thermal effect on acutely hypoxic tumour or skin cells, relative to oxygenated cells which, Dewey et al suggest, implies that the most important factor in increased thermal radiosensitisation is either low pH, as indicated in studies by Overgaard and Bichel (1977), or nutritional deficiency as found by Hahn.

Hill and Denekamp (1978) found that occlusion of the blood supply by a clamp during heating had a profound cytotoxic effect on tumours. The effect of increasing the clamping time before heating was studied and it was found that the proportion of tumours locally controlled increased from 33% if the clamp was applied immediately before heating, to 83% if the clamp was present for 60 minutes before heating commenced. No cures were observed for heat applied immediately before clamping or immediately after release of the clamp. This response could result from hypoxia, nutrient depletion, metabolite accumulation or pH changes, or to the loss of cooling effect of flowing blood. In experiments where heating took place at different times after clamping, there was an increasing effect as the pre-treatment under clamped conditions was lengthened. Thus heat dissipation or hypoxia is not as important as nutrient depletion, metabolite accumulation or pH changes. Poor vascularity gives rise to lactic acid production. This in turn produces a drop in pH in tumours because

of a deteriorating circulation. There is a markedly reduced viability in cells heated at low pH (Overgaard, 1976(a); Von Ardenne, 1978). Dickson (1978) however reported that a decrease in intracellular pH (pH_i) did not potentiate the destructive effect of heat at 42°C on tumour cells, as measured by their ability to replicate. Dickson suggests that sensitivity of heated cancer cells to decreased extra-cellular pH (pH_e) *in vitro* may involve damage to cell membranes or other components. Jackson and Dickson (1979) found that decrease in pH_e was not always accompanied by a decrease in pH_i, and that potentiation of the destructive effects of hyperthermia on tumours by low pH (pH_e is normally measured) is only effective when pH_e is lowered and this is not always associated with low pH_i.

[4] It is possible to manipulate a cell population *in vitro* so that the cells pass through the different phases of the cell cycle in synchrony. It has been found that these cells become progressively more radio-resistant as they progress through the DNA synthetic (S)-phase (Ben-Hur, Elkind and Riklis, 1977). This differs from the effect of hyperthermia to which all cells in S-phase appear to be sensitive (Westra and Dewey, 1971). A combination of the two modalities might thus be expected to be more effective than X-rays alone.

A further merit of hyperthermia is that there is some possible advantage in using heat with X-rays and drugs. This has been extensively studied by many people including Robinson, Wizenburg and McCready (1974), Yerushalmi and Har-Kedar (1974), Hill and Fowler (1977), Myers and Field (1977), Stewart and Denekamp (1978), Hume and Field (1978), Jansen, van der Schueren and Breur (1978), Hill, Denekamp and Travis

(1979) and Field (1980) for heat and X-rays, while Suzuki (1967), Hahn et al (1975), Dickson and Suzangar (1976), Stehlin et al (1979) and Arcangeli et al (1979) have investigated heat and drugs.

This has been reviewed by Field and Bleehen (1979) and the results indicate that this is a most controversial area. The timing of the heat dose in relation to other modalities, to obtain a therapeutic advantage, so that normal tissue damage is less than tumour damage, appears to be critical. To complicate matters, when heat is fractionated, as may be a recommended procedure when it is used in conjunction with fractionated radiation or drug therapy, one must take into account the fact that there is residual thermotolerance built up because of previous heat doses. This has also been investigated by many workers including Dewey et al (1977), Law and Hume (1979), Bicher, Sandhu and Hetzel (1979), Nielsen and Overgaard (1979) and Denekamp, Hill and Stewart (1980), and the results present a confusing picture.

The results of combined hyperthermia and X-rays are as a whole disappointing and it would appear that the situation still requires further evaluation. The optimal strategy for combination of heat and X-rays has yet to be determined, experimentally and clinically. Clinical data does suggest synergism between the effects of hyperthermia and some drugs, but the results are inconclusive.

1.5 Mode of Action of Hyperthermia

The mechanisms whereby hyperthermia modifies cell structure, composition and behaviour have been investigated and several theories advanced to explain what happens when tumours are treated with hyperthermia. Some

of the changes found are in:

[1] cell membrane structure and permeability

[2] lysosome stability

[3] respiratory depression.

[1] One possible mechanism of cell death by heat is damage to cell membranes. These are very sensitive to small changes in temperature and show sharp phase transitions (Wallach, 1978). The role of membrane lipids as the critical targets is receiving support from studies using unsaturated fatty-acid-requiring mutants (Yatvin and Dennis, 1977). These workers show that membrane fluidity is correlated with hyperthermic killing (local anaesthetics such as procaine, which fluidise membranes, increase sensitivity to hyperthermic cell killing (Yatvin, 1977)). Lipids at normal temperatures are in a liquid crystal state in which they retain their relative position, though can rotate. Proteins make up much of the membrane mass, and there is a complex protein-lipid configuration in the cell membrane. Wallach proposed that plasma membrane proteins of several cell types undergo major structural alterations in the physiological to hyperthermic temperature range and that these changes become irreversible above a critical temperature. Loshek (1976) plotted the logarithm of the 'cell inactivation' of cells *in vitro* against the reciprocal of the absolute temperature. From the results obtained from this procedure Loshek calculated that protein is a target for hyperthermia. He suggests that the protein disruption occurs at about a critical temperature of 42.5°C. Lett and Clark (1978) found also that above this critical temperature, which they found to vary slightly between different systems, enzymes and other proteins associated with DNA were

denatured or inactivated and remained so for extended intervals. This will lead to changes in membrane structure which may lead to changes in membrane permeability.

Even moderate temperature changes lead to modified permeability. Hahn, Braun and Har-Kedar (1975) found Adriamycin and Bleomycin more effective in chemotherapy treatment at 42-43°C than at normal body temperature and they postulated that the enhancement probably lies in the increased plasma membrane permeability. The reverse, however, appears to happen with vital dyes used to test cell viability. Dye exclusion tests reflect the competence of certain cellular membranes. Normally exclusion of vital dyes is regarded as a reliable method of assessing viability in cell suspensions under normal conditions (Dickson and Shah, 1972). The use of such stains as trypan blue after hyperthermia suggest a higher viability than was obtained by a direct assay of colony formation (Harris, 1966; Muckle and Dickson, 1971). Giovanella, Lohman and Heidelberger (1970) also found that after 2 hours at 43°C, 15% of L1210 leukaemia cells were unstained and thus appeared to be viable, yet injection of $1x10^4$ cells into mice caused no deaths, yet in this assay a single cell that is viable can cause death. Thus the exclusion of vital dyes as an indicator of viability cannot be used after hyperthermia.

[2] Increased lysosomal activity is also favoured as a mechanism for causing cell death during hyperthermia treatment. A few hours after hyperthermia treatment in the curative range there is a marked increase in the number of lysosomes and in lysosomal activity (Overgaard and Overgaard, 1972, 1975; Overgaard, 1976b). Lysosomes contain many hydrolytic enzymes capable of digesting cellular

components, so leakage into the cytoplasm through the lysosome membrane would cause extensive damage. Barratt, Keech and Wills (1978) also found that in mouse tumours subjected to temperatures of 41.5 - 43.5°C for 1 hour, there was immediate increased lysosomal activity, as measured by an increase in acid phosphatase. It returned to normal after 24 hours. Hume, Rogers and Field (1978) found that heating to temperatures below 42.5°C caused no observable change in lysosomal membrane permeability in normal tissue, although there was a transient increase in acid phosphatase activity. Heating to temperatures above 42.5°C resulted in an immediate increase in lysosomal membrane permeability which may be related to direct cell killing by hyperthermia. A less noticeable increase in lysosomal activity seems to occur in non-tumour cells (Overgaard, 1976), but as tumour cell lysosomes are more labile to heat than those of normal cells (Turano et al, 1970) the destructive capacity may be selectively enhanced in tumour cells. Turano et al investigated the thermal stability/lability of lysosomes isolated from normal and neoplastic tissues. The isolated normal lysosomes were labile at 38°C and incubation at 43° increased the rate of release of proteolytic enzymes. The lysosomes from Novikoff hepatoma cells were more sensitive than those from either normal or regenerating liver cells, both at 38°C and 43°C.

For example, the release of hydrolytic enzymes from the lysosomes was compared at 38°C and at 43°C, after freezing and thawing the lysosomes - a procedure which specifically disrupts cell lysosomes. From normal liver it was 8.3% at 38°C and 29% at 43°C, regenerating liver 12% and 33% while in Novikoff hepatoma cells it was 17% and 81% respectively. Thus from this study it appears that lysosomes are

the cause of the specific sensitivity of tumour cells to high temperatures.

Burger and Fahrman (1964) also found very little acid phosphatase accumulation in normal rat tissue at temperatures from 38°C to 42°C (though there was potassium leakage at these temperatures). Dickson and Shah (1972) found, however, leakage of lactate dehydrogenase in rat mammary tumour cells after heating for 3 hours at 42°C. Symonds (pers.comm.) found that the environment strongly influences cell sensitivity to heat. When L1210 leukaemic cells are in the ascites environment they are more resistant to heat than when they are in the marrow environment, when they are very sensitive to heat. This difference may possibly reflect leakage of lysosomal enzymes from damaged cells.

[3] Heat also induces respiratory depression, which appears to take place selectively in malignant cells (Cavaliere et al, 1967; Mondovi et al, 1969; Muckle and Dickson, 1971) which may in turn cause cells to become hypoxic with associated nutrient deficiency. These cells have been found to be more heat sensitive (Hill and Denekamp, 1978). Heat would thus have a two-fold effect. Muckle and Dickson found that O_2 uptake *in vitro* in cells from 8 different tumours was significantly depressed after heating for 1 hour at 42°C compared with the O_2 uptake from the same tumour cells kept at 37.5°C. They also found that normal rabbit liver, kidney and red blood cells showed no depression of O_2 uptake at 42°C. Cells heated *in vitro* at 42°C produced no tumours when injected into the host if the cells had been maintained at this temperature for more than 2 hours, even though 50% of the cell population was found to be viable at this time. Viability was assessed

by dye exclusion tests, however this test has been shown to give too high an apparent viability after hyperthermia because of alterations in the competence of certain cell membranes after hyperthermia, as already discussed.

Dickson and Shah (1972) found depression of respiration after heating rat mammary carcinoma cells for 3 hours at 42°C. They found anaerobic glycolysis however to be affected only by prolonged heating (6 hours at 42°C) and that restoration of the glycolysis and respiratory rates towards normal values after moderate amounts of heat paralleled the recovery of replicative ability of the cultured cells.

1.6 The Major Problem of Metastasis

The ability of malignant tumours to metastasise to other sites where their presence in vital organs can cause the patient's death, is a major problem. This can arise even when the local primary tumour has been eradicated. Thus when considering the method of treatment of the primary neoplasm attention must be given to the possibility of increasing the risk of metastasis. This is particularly important since two thirds of all patients with cancer die from metastatic disease rather than from local invasion.

Hyperthermia may cause metastases. Enhanced dissemination of the Yoshida sarcoma in rats was reported (Dickson and Ellis, 1974, 1976) as a result of local heating at temperatures which were not adequate for complete tumour destruction. Dickson and Ellis suggested that local hyperthermia may change the mobility of tumour cells because of some modification of cell surface, and that local active hyperaemia

may predispose to increased 'showering' of tumour cells into the blood stream. Van Dijk and Breur (1979), who have studied different heating techniques on mammary tumours situated on the backs and legs of mice, also found local heat to initiate metastases (pers.comm.). Yerushalmi (1976) failed to find promotion of metastases of the Lewis lung carcinoma by local heating, but noted earlier appearance of pulmonary metastases after total body hyperthermia, at a temperature which was obviously inadequate for complete tumour cell destruction. From the results quoted in the study by Yerushalmi however, it is difficult to draw any firm conclusions about metastatic spread after hyperthermia. Yerushalmi claimed that the appearance of metastases was observed sooner after local heating of tumours: however, the differences in times of appearance of metastases were only 0, 1 or 2 days and the numbers of animals in the trial was small, so it is doubtful if the results showed any significant difference in metastatic rates after the different types of heating. These results, however, taken along with the results of Dickson and Ellis do suggest that inadequate heating could be dangerous. Yerushalmi suggested that elevation of body temperature to a temperature below the critical temperature may lead to stimulation of premature activity of tumour cells already present at metastatic sites, or to changes in the mobilities of cells in the primary tumour. He also suggested that there may be alterations in properties of the tissue concerned in metastatic distribution of blood mediated metastases, or as Dickson and Ellis also proposed, that there may be a depression in the immune defence mechanism, leading to a more rapid spread of metastases. It is possible that the metastases from all these causes may have arisen because of poor temperature distribution within the primary tumour

during hyperthermia. The picture is still not clear as to what are the mechanisms of enhanced metastases by hyperthermia but it could be due to:

- inadequate heating
- increased shedding of tumour cells
- increased metabolism of shed cells
- changes in host immunity.

1.7 Critique of Present State of Knowledge and Aims of the Present Study

Previous work by Crile (1961), Dickson and Muckle (1972), Thrall et al (1973), Dickson and Ellis (1974), Robinson et al (1975), Cetas et al (1977) and others (see Table 1.2) had established a solid core of knowledge on the response of animal tumours to heat, allowing a critical reassessment of some of the techniques which have been used in earlier studies. This overview showed that inappropriate tumour models had frequently been used - such as one of the tumour systems used by Crile, which was a poor one for human cancer in that the tumour often spontaneously regressed. It has also been pointed out that earlier studies had shown a close correlation between degree of cell killing and very small changes in temperature. It is therefore evident that several regularly used heating systems give far too large tumour temperature gradients (see for example Table 3.11) so that areas within the heated tumours may not reach a temperature where there would be cell killing. It was therefore necessary that any new study on the effect of hyperthermia on tumours must overcome these two difficulties by having:

- an appropriate tumour system for the particular question that was

being asked

- a very carefully controlled heating system which would permit a full investigation of the role of temperature and the duration of hyperthermia when applied to a localised tumour.

While some of the previous experimental work carried out was of a very high standard, and many aspects of tumour treatment using elevated temperatures had been investigated, it seemed that one very specific problem would be worth further study, using a technique with as few variables as possible. An important aspect of any treatment is whether or not it increases the possibility of metastases. The work of Kaae (1953), Kaplan and Murphy (1949) and Gulledge (1961) (see 4.1.2) for example had suggested that irradiation or surgical removal of tumours may increase metastases and the studies of Dickson and Ellis (1974, 1976) and of Yerushalmi (1976) had suggested that, under certain conditions, hyperthermia might also increase the risk of metastases while Smachlo et al (1979) have claimed that hyperthermia obtained by ultrasound (see 1.3.2) gave rise to no metastases. The reasons for this required consideration.

It is therefore evident, from the shortcomings of the available techniques at the time this study began, that for effective tumour treatment by heat the heating technique must be very rigidly controlled and that any required intra-tumour temperature must be reached within a given time. The system however must be flexible so that different heating regimes could be examined. The tumour system together with the heating technique must be one which has as few variables as possible and no factors which might make interpretation of results difficult (see 2.4.1).

It is evident that the problem of delivering heat to a tumour merits thorough investigation and the main lines of the investigation in this thesis were:

- to choose a tumour system which would be relevant to human cancer
- to devise a technique for giving localised uniform heating to this tumour, without any systemic hyperthermia, in order that the area of study could be limited to the effect of temperature on the tumour
- to measure the tumour growth rate before and after treatment and to use this information together with the growth delay after treatment to investigate cell kill. A comparison with growth delay after X-rays would then give information about the mechanisms governing regrowth after hyperthermia
- to investigate possible differences in metastatic rates after different regimes of localised hyperthermia and to compare this with metastasis after X-rays and surgery using the same tumour.

TABLE 1.1

COMPARISON OF RESULTS OF SOME OF THE DIFFERENT METHODS OF WHOLE BODY HEATING

Author	Technique	Core Temperature	Tumour Type	Result	Comment
Nauts et al (1953)	Coley's toxins		multiple metastases	25/30 patients survived > 10 years	
Nauts (1978)	Coley's toxins		leukaemia, sarcomas, lympho-sarcomas, multiple myeloma	regressions occurred	
Pettigrew et al (1974,1978)	hot air/ anaesthetic mixture with immersion in warm wax + chemotherapy	3 treatments of 41.8°/ 1 3/4 - 2 3/8 hours	multiple metastases	chemotherapy and hyperthermia potentiated each other if chemo-therapy given first	used alveolar surface of the lung, which has 20 times the area of the skin surface, as a heat exchanger
Euler-Rolle et al (1978)	hot air/ anaesthetic mixture	42°/2 hours	multiple metastases of carcinoma and sarcoma	can use 42°/2 hours if appropriate drugs to control heart rate and blood pressure given	no comment made on regressions, if any
Blair and Levin (1978)	hot air/ anaesthetic mixture with immersion in warm wax	> 41.8°/2½ hours	multiple metastases	some liver damage above 42°	temperature should be kept below 42°
Pomp (1978)	hot air chamber and microwaves	40-42°/1 hour - repeated	multiple metastases	regression in 23% and pain relief	fast heating up by this method. Head outside chamber therefore patient breathing cool air - no anaesthetic necessary
Bolmsjo et al (1979)	microwaves	any predetermined temperature ± 0.1°	deep seated rat tumours	none given	used microprocessor controlled system to maintain constant body temperature
Larkin (1979)	water blanket	42°/2 or more hours	advanced cancers progressing despite other therapy	43% response + 15% palliation	
Parks (1980)	extracorporeal systemic hyperthermia	41.5-42°C/6-9 hours	preterminal solid tumours	5/60 patients with stable regressions	this method gave precise control without haemolysis or thrombotic problems
Von Ardenne (1978) Von Ardenne and Kruger (1980)	RF heating and skin cooling and hyper-acidification of tumours	40.5°/50 minutes (42.5° to the tumour)	not given	remarkable regressions took place within 3 weeks	obtained irreversible occlusion of tumour vasculature at pH 6.5

TABLE 1.2

METHODS OF DELIVERING HEAT TO TUMOURS

Form of Heating	Direct or Conduction	Radiofrequency		Microwave	Ultrasound
		Shortwave Diathermy	Localised Current Field		
Practice	hot water or other fluids or gasses in direct contact with tumour.	radiofrequency current passed between electrodes or through coil round tumour.	radiofrequency current passed through electrodes superficial to, in direct contact with, or interstitically with the tumour.	radiating sources or direct contact application.	mechanical energy deposited as heat. Water or other bolus must be used.
Penetration	poor - as good as microwave with small tumours.	good - can heat deep tumours.	good especially when invasive procedure used.	quite good.	good.
Localisation	can localise only mechanically.	can localise using several applicators.	good localisation.	can be achieved with loss of penetration, also depends on tissue to be heated.	good - using focussing devices.
Advantages	simple, inexpensive, easy thermometry.	better for deep tumours than conduction or microwave.	better for deep tumours than conduction or microwave.	quite good penetration and localisation to sites accessible through body cavities.	possible enhancement of effect by non-thermal mechanism.
Disadvantages	poor penetration - no skin sparing.	heat patterns can be non-uniform and excessive heating of superficial and fatty tissues.	often invasive.	temperature measurement cannot be done by conventional methods although new types of measuring devices being produced.	problem of selectively heating skin and bone.
Small Animal	present method is adequate, waterbath alone gives poor temperature gradients.	can achieve relatively uniform heating using two or more electrodes round tumour.	better for large animal tumours.	no better than waterbath in tumours < 2cm. If tumour large get high core temperature. Results conflicting.	disadvantage of poor penetration in air makes complex set-up for animal studies.
Clinical Application	limited direct application - can be used with cathetor for bladder or heated blood for extremities.	can use superficial cooling to get skin sparing. Can improve localisation with multiple applicators.	can use in conjunction with implants.	if several applicators used can improve penetration and localisation.	useful in treating soft tissue tumours at depth.

TABLE 1.3

THERMOSENSITIVITY OF COMPARABLE NORMAL AND MALIGNANT CELLS ASSESSED BY CLONOGENIC ASSAY

Author	Normal Cell	Malignant Cell	Differential
Chen and Heidelberger (1969)	mouse prostate cell	transformed mouse prostate cell	+
Giovanella et al (1973)	mouse mesenchymal embryonic cells	mouse sarcoma cells	+
Stehlin et al (1975)	human embryonic melanocytes	human malignant melanocytes	+
Harisiadis et al (1975)	rat liver cells	rat hepatoma cells	0
Kase and Hahn (1975)	human embryonic fibroblasts	transformed human embryonic fibroblasts	+

2.0 PROPERTIES OF THE C_3H TUMOUR SYSTEM

2.1 Introduction - The C_3H Tumour As a Model to Test The Effects of Hyperthermia

A brief introduction to the various techniques used in this chapter will be given in order to show how these relate to the objects of the present study which were to investigate both the local and the systemic effects of hyperthermia. The choice of a suitable tumour system had to meet these experimental requirements, and various experiments were set up to test this suitability.

To study the local effect of treatment there had to be no artifactual immunological features which would affect the 'take' rate when tumour cells were injected, or would cause spontaneous tumour regression. Immunogenicity had thus to be tested for and this was carried out by 'pre-immunising' animals with lethally irradiated cells before inoculating with tumour cells of known growth potential.

The tumour selected had to transplant reliably using a technique which would give reproducible results, and to give easily measurable tumours. These had to show stable growth kinetics. In order to maintain reliable growth kinetics and histological characteristics over a period of time, selection had to be avoided in transplanting tumours. A reserve of tumour cells of known characteristics was maintained.

Because when tumours are very small the growth rate cannot be measured directly a limiting dilution assay was employed. From this the growth rate of a single tumour cell was inferred from different growth delay times after inoculation of serially diluted tumour cells. The growth rate was also measured when lethally irradiated cells were mixed with these diluted tumour cells. This simulates more nearly the

situation obtaining after treatment when tumours contain a mixture of viable and dead or dying cells. The dilution assay also permitted estimates of cell number and cell doubling times to be made at different periods in the tumour's history. At later stages tumours can be measured directly and those measurements were made regularly over the period of study to confirm that no change in growth rate was taking place.

To allow the investigation of the systemic effect of local treatment tumours should not metastasise too early or too frequently, and preliminary studies of the pathology of tumour-bearing animals were made to elucidate this. These studies suggested that the C_3H tumour would meet these requirements.

The various aspects of the techniques employed to investigate the suitability of the C_3H multi-generation mammary carcinoma, which have been outlined above, will now be dealt with more fully.

2.2 Methods

2.2.1 Animal Procedures

2.2.1.1 Care of animals

Normal, healthy virgin C_3H/He mice of either sex (as there had been no evidence that sex had affected the tumour growth rate or histology) were used in our experiments. The mice to be treated were aged between 8 and 12 weeks and their weight was 20-25 grams. Mice were bred in our own animal house by conventional inbred mating system. Mice were housed in polypropylene cages with a maximum of 10 mice per cage. They were fed on Diet 41 which is produced by the Angus Milling Company, Perth, and supplied by William Shearer and Company, Glasgow. Animals

were provided with tap water *ad libitum*. Sterile precautions were taken to reduce the risk of disease spreading and the state of health of the animals was under constant supervision. The precautions taken included autoclaving all cages and water bottles. The room where experimental animals were housed was kept at a constant temperature of 22°C with humidity of 50% and 12 hours light and 12 hours dark - light being 7 a.m. to 7 p.m. Animals were coded using a saturated solution of picric acid. This was applied to the fur with a paint brush and lasted for the length of time of observation and no skin irritation was ever noted.

Several criteria were considered in choosing a position on the mouse to site the tumour:

- tumours should be easily measurable: the skin on the back is very loose and there is easy palpation and measurement of three perpendicular diameters. Since there is little opposition to growth at this site tumours are discrete and non-invasive.
- the tumour should not be restricting, to gain access to food, as had been found when mice had tumours at other sites, such as the thigh and ventral aspect.
- the site chosen should be suitable for the type of heating techniques involved in this study which gave uniform dry heat to the tumour but did not cause distress to the animals, which did not thus require to be anaesthetised (see 4.4.1). Furthermore, the site should be suitable for testing other modalities, e.g. radiation and surgery, since comparison of effect of hyperthermia after different modalities was being investigated.

Animals which had been implanted with tumours were normally treated when

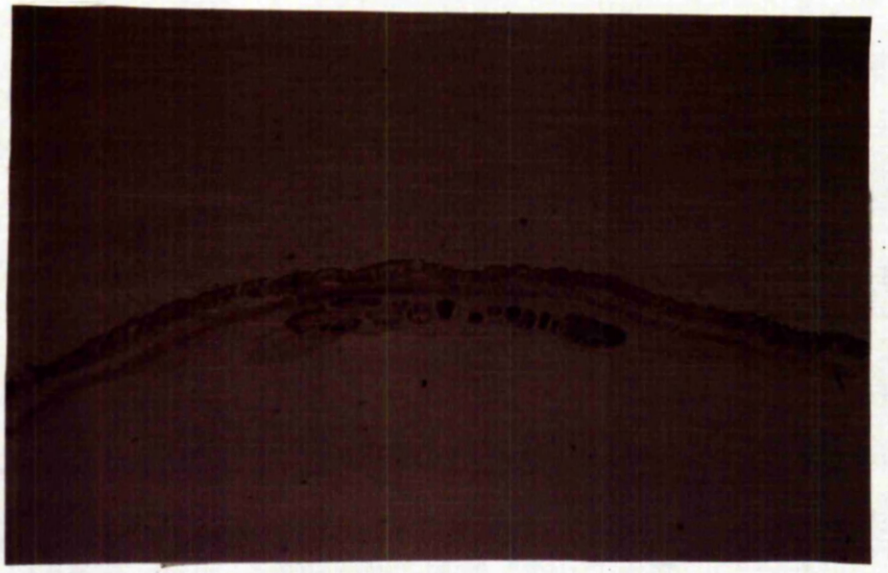


Figure 2.1

Small Tumour Showing Needle Track (Reproduced From x10)

Tumours were fixed in 10% formal saline, paraffin embedded and sectioned at 7μ. Staining was with haematoxylin and eosin.

the tumour measured 6-7mm in diameter, and tumours of this size were also used for transplantation. Where tumours regrew after treatment the animals with tumours were sacrificed when these were 10mm in diameter. Only in experiments where tumour growth was being investigated were tumours allowed to grow to larger sizes. Mice which appeared unwell were always sacrificed and were examined for cause of death. After treatment, animals were observed for 100 days, although regrowth after hyperthermia always occurred much earlier than this (see 4.3.1). No spontaneous tumours appeared during this period of observation.

2.2.1.2 *Tumour transplant technique*

Tumours were transplanted by aseptic removal in a flow cabinet from donor animals immediately after sacrifice. Tumours were selected which had grown at a normal growth rate (see 2.3.2) and which were not attached to muscle or underlying tissue. Occasionally tumours did not grow at the normal rate. These were never used for treatment or transplant.

The tumour was removed intact and freed from skin and a cell suspension prepared as will be described (see 2.2.2). -In the recipient animal a small area of skin, where the injection was to take place, had been clipped, and the tumour cell suspension was injected subcutaneously into the mid-line of the back of the animal at the level of the abdomen. Care had to be taken that the injection was given steadily in order to produce single tumours of approximately spheroidal shape. The needle track had to be short in order that any very small satellite tumours produced along the track (which occurred if there was any movement during inoculation) would be heated along with the tumour (Figure 2.1).Animals were initially given a brief anaesthetic, using an air/ether mixture, though it was found that by using two operators the anaesthetic was unnecessary.

According to Hewitt (1953), properly injected neoplastic cells in suspension should produce a tumour arising from a single focus. It was found that fewer deep tumours (i.e. tumours attached to underlying tissue and hence not usable for experiments) were produced if the needle aperture was directed upwards into a fold of skin held either with curved forceps or finger nails to form a 'tent' (Hewitt, pers.comm.). After retraction of the needle the skin was gently swabbed with alcohol to minimise the chance of satellite tumours at the skin surface - a problem in inexperienced hands. During the time of the injection procedure the cell suspension, which was kept cool, was gently shaken to prevent sedimentation and clumping of the tumour cells. The whole procedure with a transplant of 30 mice was timed to be completed in 20 minutes to minimise variability in viability as this falls off with time.

2.2.1.3 Tumour measurement by dilution assay

Early stages in tumour growth were measured indirectly by means of a dilution assay. To do this a tumour cell suspension of known numbers of viable cells was prepared and inoculi of varying numbers of cells obtained by serial dilution (see 2.2.2). These were then injected into recipient animals. It was considered essential that time taken from sacrifice of the donor to injection of the recipient should be the same as in normal transplants. The development of a tumour implied that at least one viable cell was present in the inoculum. There were different delay times for appearance of tumours depending on how many viable cells were present in any one inoculum, the longer delays occurring with lower numbers of viable cells.

When lethally irradiated (LI) cells (see 2.2.2) were added to the

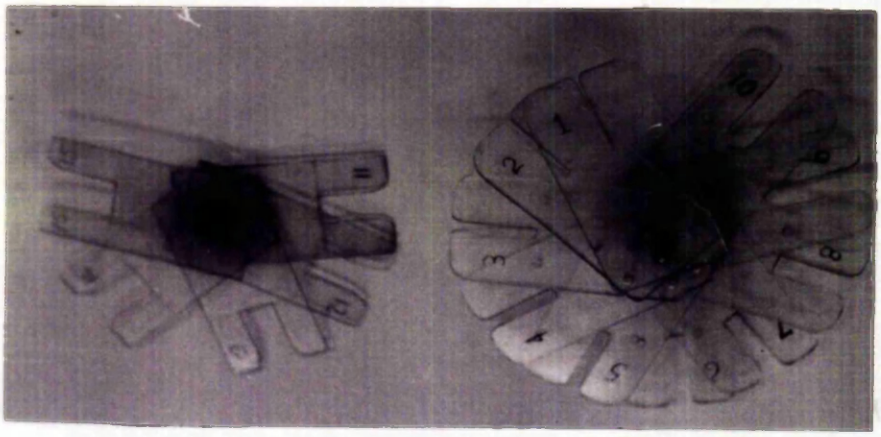


Figure 2.2

Tumour Measuring Device

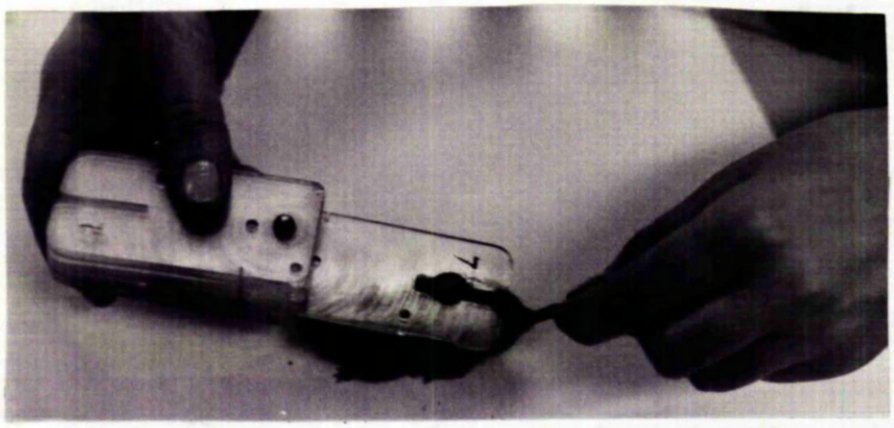


Figure 2.3

Tumour Measurement

diluted inoculum timing was again carefully controlled, the donor of the viable cells being sacrificed just prior to completion of irradiation of the LI cells. According to studies by Révész the addition of LI cells increases the 'take' rate or decreases the growth delay time.

2.2.1.4 Direct tumour measurement

Direct tumour measurement was carried out using a specially designed device with a series of slits of graded sizes (Figures 2.2 and 2.3). Mice implanted with tumour cells were palpated at intervals of 1-2 days and the day of definite appearance of a palpable tumour was noted. It is possible to detect tumours smaller than 1mm diameter but estimates of size are then subjective, though it was found that a '1mm' (diameter) tumour could be measured by someone with experience with a good degree of accuracy.

After appearance of a '1mm' tumour measurements were made daily. In preliminary studies three mutually perpendicular diameters were always measured - the diameters including skin thickness. This value was practically the same as the mean of two surface diameters, since in the size range used the tumour was nearly spherical and this measure was used in all comparative studies. 'Day 1' of any growth curve was always taken as the day before the tumour measured 2mm diameter.

After treatment (with either X-rays or hyperthermia) the first few days of regrowth were usually masked by some inflammation, so normally tumours could only be recorded from about size 4mm diameter. Measurements were hereafter recorded daily. Only two measurements were made, as taking a perpendicular diameter might have disturbed the

tissue round the tumour.

2.2.2 Preparation of Cells

2.2.2.1 Cells for tumour transplant, dilution assay and pre-immunisation

After the tumour was removed it was gently mashed through a fine stainless steel mesh (200/inch) and washed through with 2ml of solid tumour culture medium (see below). The crude suspension was aspirated gently through needles of serial sizes, the smallest being 0.18mm diameter, leaving the sediment which consisted of stromal tissue.

There are several methods of counting viable cells, some of which require a fairly lengthy procedure. In the procedure used here a cell count was made of the tumour cell suspension using a haemocytometer. The density of morphologically intact tumour cells was determined by counting with a phase contrast microscope. The criteria adopted for intact tumour cells were very similar to those used by Hewitt, Chan and Blake (1967) for squamous carcinoma cells:

- small round cells of diameter 8-12μ
- smooth outline
- yellowing tinge with a darker outline and surrounded by a narrow halo of light
- no clear cut distinction between nucleus and cytoplasm.

The final suspension contained approximately 90% single cells, with occasional small clumps of 2 to 5 cells. Normally the viability was approximately 60%. A final tumour cell suspension of 10^6 viable cells in 0.05ml was obtained by appropriate dilution with the solid tumour medium consisting of:

500ml MEM + Hank's salt solution

50ml Horse serum

5ml 200μM glutamine

3ml MEM non-essential amino acids

1.5ml N sodium hydroxide

The technique is simple and quick, which has the advantage that cells do not lose viability or have time to clump.

In order to confirm that the technique was accurate the viability was checked using the stain trypan blue. In this test the cell membrane of viable cells excludes the stain. It was found that approximately 60% of the cells were unstained, thus confirming the phase-contrast count. Cells for use in the dilution assay experiments were prepared as above, only appropriately diluted. The inoculum size was 0.1ml. In normal transplantation an inoculum size of 0.05ml had been used. It was decided to use the larger inoculum to improve accuracy. The growth rates using both sizes of inoculum were tested and no significant difference was found (see Table 2.1).

Two different assay systems can be used, either:

[i] all dilutions are made from one donor, or

[ii] only one dilution at a time is prepared.

Procedure [i] would seem preferable but this takes longer and loss of viability and clumping occurs with increasing time. Thus procedure [ii] was used and at least three assays using different mice were performed at each dilution.

As a check on the dilution procedure Coulter Counter measurements were made, as a haemocytometer is not accurate below concentrations of

$1x10^6$ cells per ml. Coulter Counter measurements are accurate to $1x10^4$ per ml.

Total Cells Counted (cells/ml)		Coulter Counter (counts/unit time)
Haemocytometer	1.1×10^6	28,000
By dilution	1×10^5	2,500
By dilution	1×10^4	258

Thus there is an agreement of ratios.

In preparing LI cells for dilution assay and pre-immunisation techniques tumour cells were irradiated *in vitro* at a dose rate of 5.7Gy per minute with γ-rays. The total dose was 100Gy. The cells were irradiated using a ^{60}Co source. Cells must be irradiated at a high dose rate or there is the possibility of repair taking place even with a high total dose. The cell suspension was held in a rotating jig and kept cool during irradiation to minimise clumping.

2.2.2.2 *Preparation of bank of cells*

A reserve of frozen tumour cells was always kept. To prepare cells for freezing 20% foetal calf serum was added to the normal solid tumour medium then all suspensions were prepared in the normal way. To the final suspension was added 10% dimethyl sulphoxide (DMSO) in solution. As DMSO is toxic to cells at temperatures above 4°C it was added just prior to cooling. The mixture, in 2ml ampules, was placed in the cooling chamber and cooling was carried out at a rate of 1°C per minute down to -30°C, when the ampules were transferred to liquid nitrogen. Recovered cells were thawed by shaking in a 37°C waterbath and used directly for inoculation. It is important to warm the cells rapidly or cells are disrupted by large ice crystals forming.

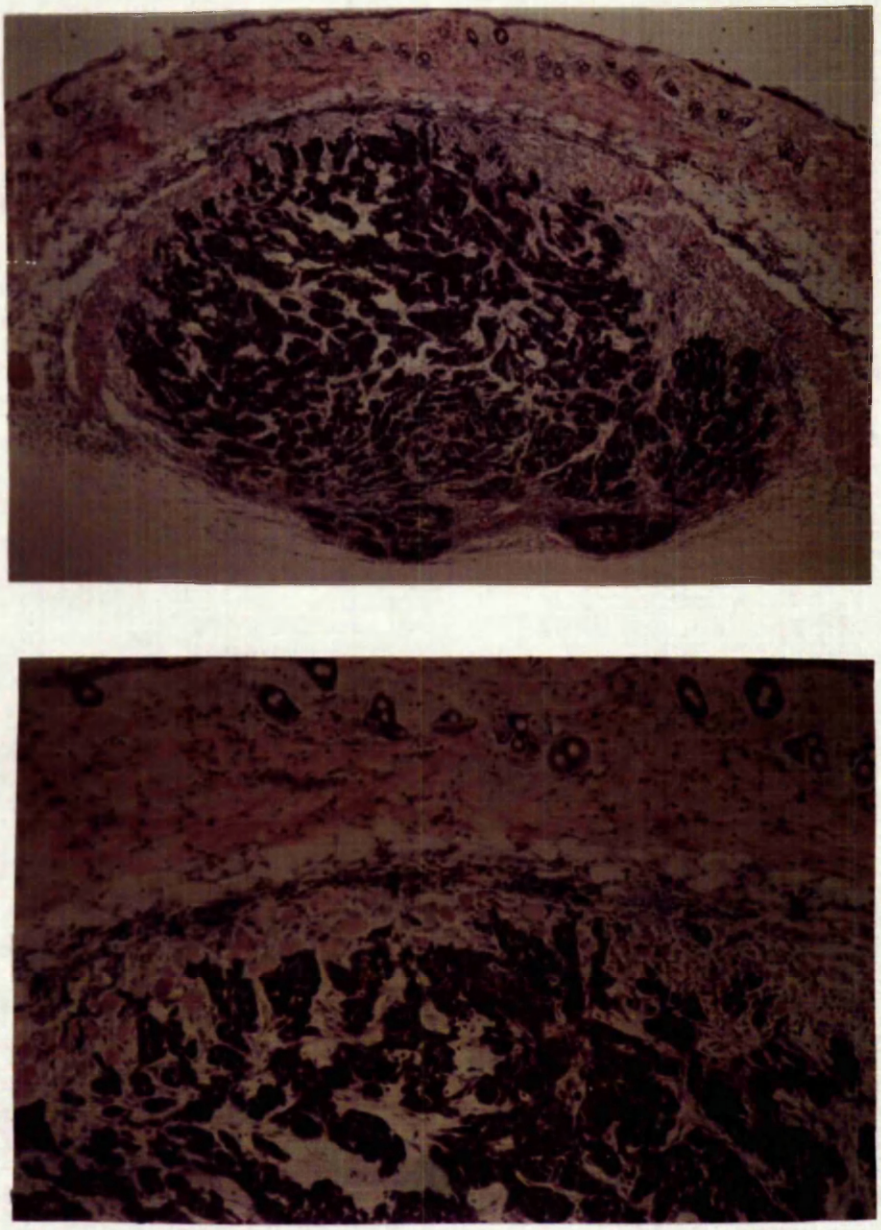


Figure 2.4

Photomicrographs of a 1mm tumour (reproduced from x40 and x100). This tumour is already vascularised and invading the overlying muscle. The tumour cells are in groups separated by septa of connective tissue with blood vessels. In some of the larger groups of tumour cells there is already some early degeneration of the central cells of the group. In the healthy zone many of the cells are in mitosis.

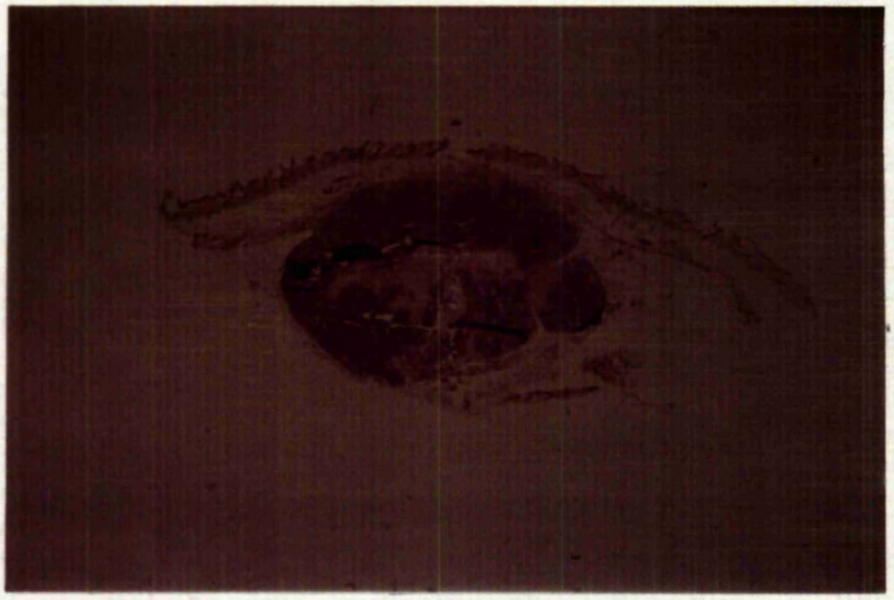


Figure 2.5

Photomicrograph of a 3mm tumour (reproduced from x10) showing a little necrosis. The panniculus muscle can be seen lying parallel to and below the epidermis.

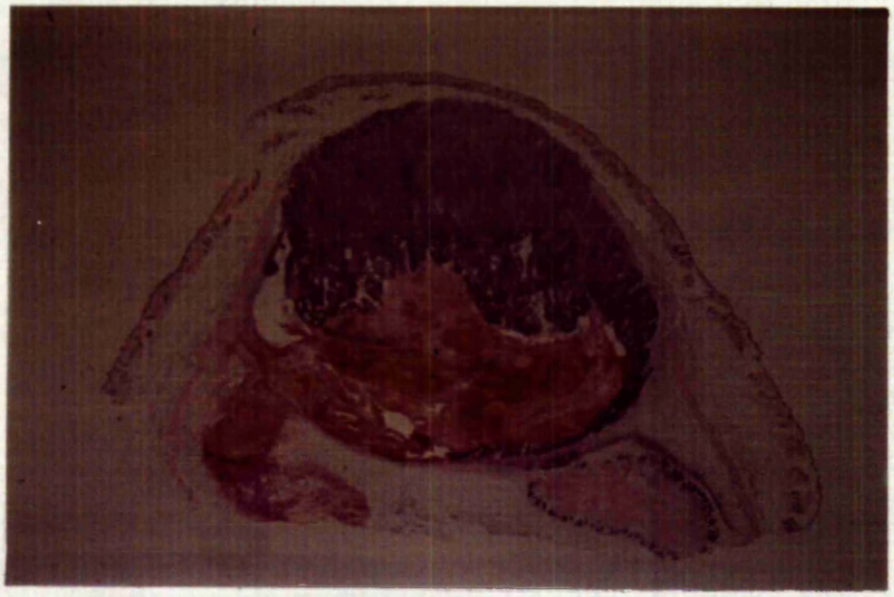


Figure 2.6

Photomicrograph of a 6mm tumour (reproduced from x10 and x250) showing a large necrotic zone towards the deeper end of the tumour. The tumour has invaded the panniculus muscle. There is a small satellite tumour seen close in to the main tumour.

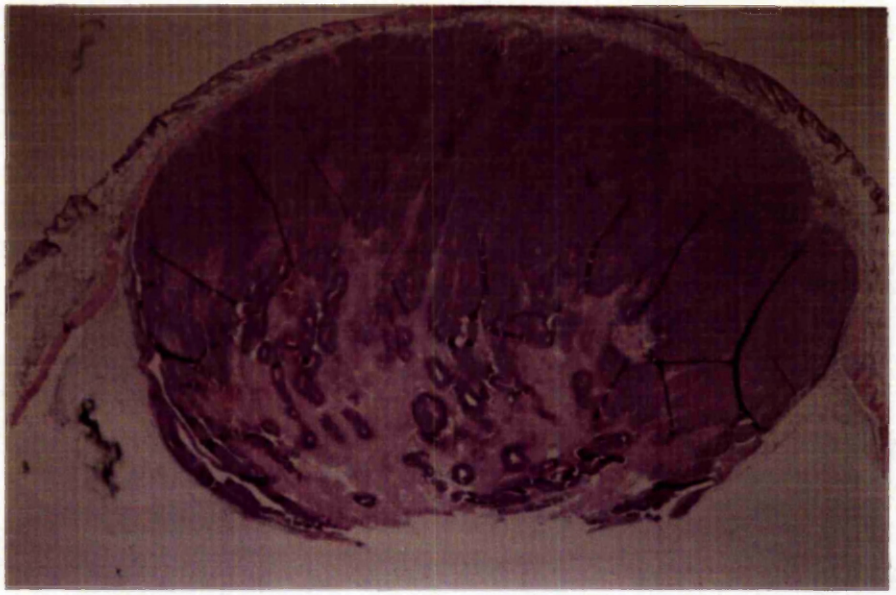


Figure 2.7

Photomicrograph of a 10mm tumour (reproduced from x10), showing a very large necrotic zone.

2.3 Results

2.3.1 Histology

An attempt was made to determine the optimum tumour size for an investigation into the effect of hyperthermia on tumours. A large proportion of hypoxic cells in the tumour was felt to be desirable since hypoxia is a common feature of many, or perhaps nearly all, human tumours treated clinically. The proportion of hypoxic cells becomes relatively larger as the tumour enlarges and therefore the most suitable tumour size was felt to be the largest that was compatible with the absence of any skin damage.

Even in the smallest tumour examined there was the beginning of some necrotic foci (Figure 2.4). In a 3mm tumour there was already some necrotic tissue (Figure 2.5). At the size used (6 ± 1mm diameter) there was 25-30% necrosis - especially in the lower centre of the tumour (Figure 2.6). The C_3H tumour is such a fast growing tumour that skin damage occurs earlier than in one whose growth rate is slower, owing to the rate of adaptation of the skin. In 10mm tumours the skin over the tumour was often involved (Figure 2.7). Thus a 6 ± 1mm diameter tumour was considered to be the optimal size for a study into the effect of localised hyperthermia.

The tumour appeared encapsulated, though this was only a pseudo-capsule, being formed by a rim of fibroblasts several cells thick. These were slightly flattened by being pushed back by the rapidly expanding tumour. There was seen to be no apparent invasion of the surrounding connective tissue except in the panniculus carnosus muscle, which in the mouse lies just below the skin. Whether this is because the muscle provided some kind of resistance to the advancing tumour or not it can be seen in Figure 2.1 that even in a tumour of 1mm

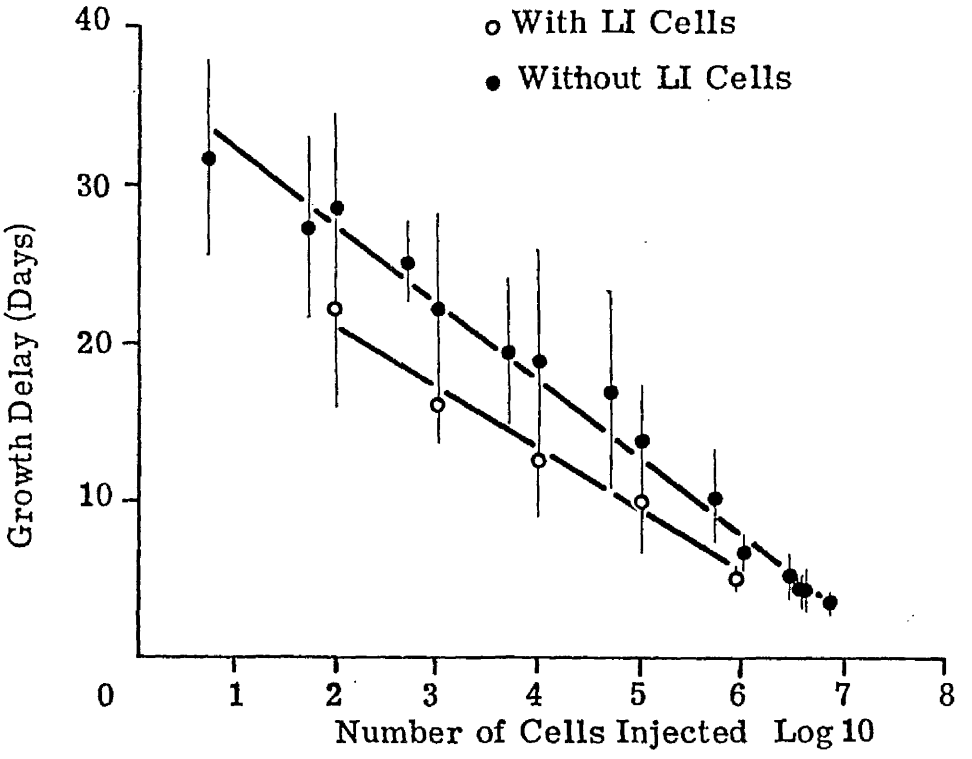


Figure 2.8

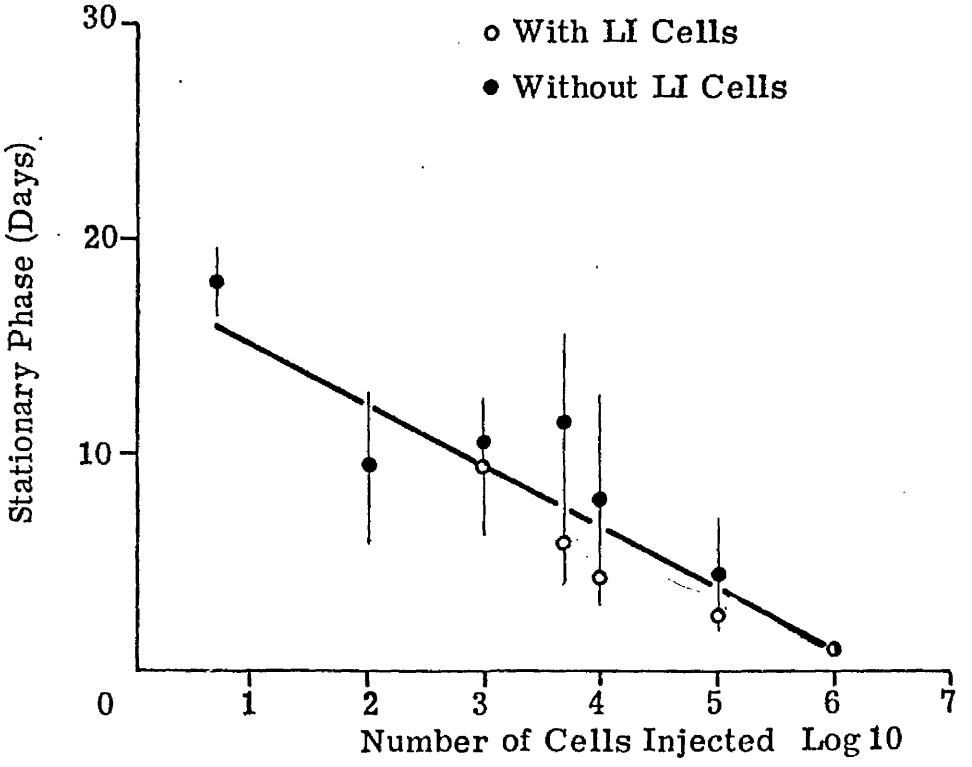


Figure 2.9

diameter the malignant cells had already begun to send finger-like processes into the muscle tissue. The vasculature round the superficial aspect of the tumour which arose from the well vascularised skin, was seen to be very good and there was no necrotic tissue here. Towards the base, or deep aspect of the tumour, where it was growing in the subcutaneous tissue there was seen a narrow rim of healthy tumour cells indicating that though less well vascularised than the area adjacent to the skin the vasculature here was adequate.

2.3.2 Growth Pattern

2.3.2.1 Microscopic growth

Results of the dilution assay are shown in Tables 2.1, 2.2 and 2.3 and in Figures 2.8 and 2.9. Best-fit lines were obtained by the method of weighted least squares. The results give times of appearance (latent period) for a '1mm' tumour versus the number of viable cells implanted with and without the addition of LI cells. Latent periods when LI cells were added to the inoculum were found to be significantly different ($P < 0.05$) from latent periods when no LI cells were added. This was found by establishing differences for the latent period at values of the number of cells injected between 10^2 and 10^5 with and without LI cells. (Breeze, Pers Comm).

At early stages in growth, just at palpability, there was found to be a period when the tumour appeared to stop growing. This period has been called the 'stationary phase' and its length was found to vary with inoculum size, but no significant difference was found in this stationary phase when LI cells were added with the inoculum. Grouping both sets of observations together a straight line has been drawn

through the two sets of points in Figure 2.9. It has been calculated that the 'stationary phase' when the tumour measured 128 cells was approximately 12 ± 1.7 days.

To establish an approximate growth rate with and without LI cells one could extrapolate to 1 cell the straight lines in Figure 2.8. This may not necessarily reflect the true biological situation so it was decided to look at the latent period at 128 cells (which is equivalent to 7 doublings) where extrapolation of the LI cell line seemed appropriate.

It was found that time to grow to a 1mm tumour from 128 cells was:

without LI cells 27.8 ± 1.1 days
with LI cells 20.8 ± 1.6 days

This period of growth included the 'stationary phase' which appeared to take place when the tumour was just under 1mm diameter. At 128 cells this was approximately 12 ± 1.7 days. The periods of growth from 128 cells to 10^6 cells is equivalent to 13 doublings and, according to estimates by Hewitt (1976), this is the number of cells in a '1mm' tumour (cf Steel (1977) who estimated that a '1mm' tumour $\equiv 7 \times 10^5$ cells).

From this information the doubling time without LI cells was just over one day and with LI cells was just under one day.

2.3.2.2 Macroscopic growth

Above a tumour size of '1mm' (diameter) the tumour was measured using the device described in 2.2.1. It took 6.8 ± 1.0 days (mean of over 100 measurements) for 10^6 viable tumour cells injected as described,

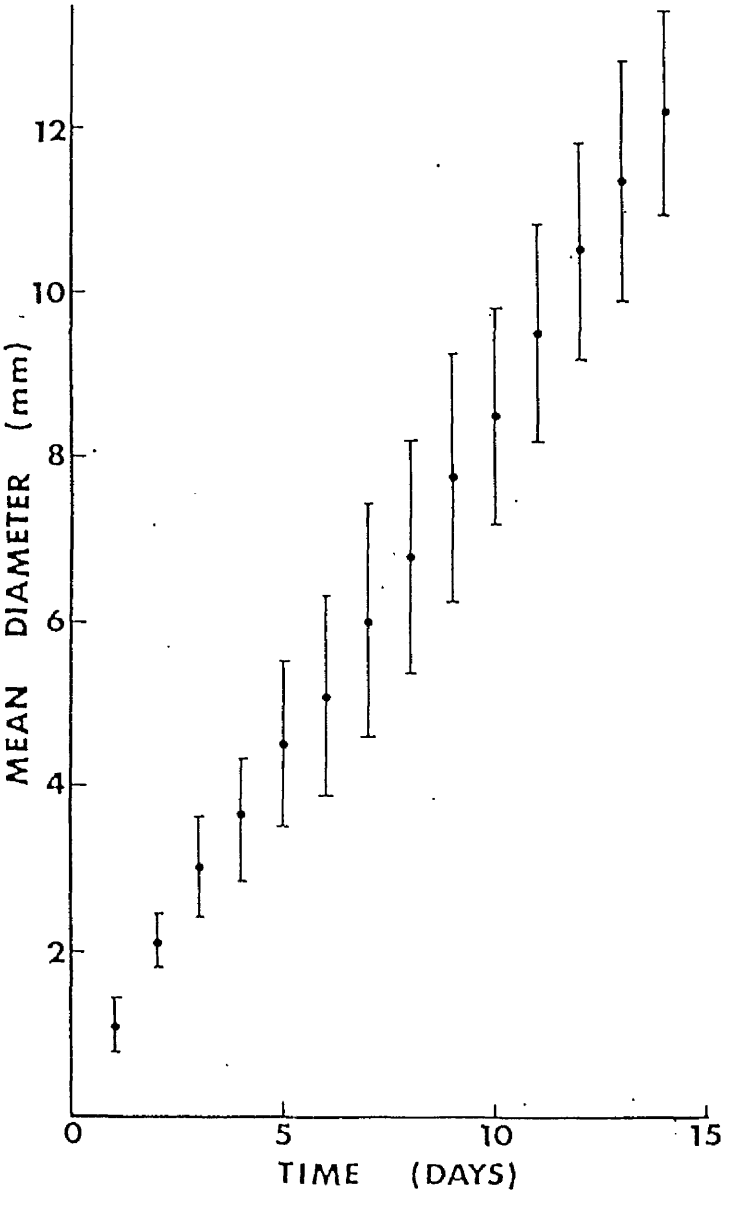


Figure 2.10

Mean tumour diameter (including skin thickness) versus time

to produce a '1mm' tumour. The diameter then increased by approximately 1mm per day which represents decelerating growth. The fact that the tumour became established and grew at a predetermined rate every time gave more confidence to growth delay measurements.

The mean diameter (of 22 tumours) versus time is shown in Figure 2.10. All best-fit lines have been found by the method of least squares. The measure used included skin thickness. Growth curves were measured and regularly compared over the period of study and no significant change in growth rate was found over this period ($P < 0.01$).

2.4 Discussion

2.4.1 Evaluation of the Tumour System

In this chapter the growth kinetics of the tumour has been investigated. It was necessary to ensure that the tumour system was a valid model of human cancer, and that it would be suitable for a study of tumour growth. Only thus could meaningful comparisons be made between

- normal growth, and
- regrowth after different degrees and types of treatment.

Results could then be used to help in understanding the complex mechanisms of regrowth after hyperthermia. Various aspects of the tumour system will now be discussed.

2.4.1.1 As a model for human cancer

Perhaps the most controversial condition of a transplanted animal tumour system in respect to its relevance to spontaneous cancer in man concerns the presence or absence of some degree of immunological reactivity of the host to the tumour. For an animal tumour system to

be an acceptable model of a particular form of human malignancy regard has to be paid not only to histological type, but also to avoidance of immunological features clearly attributable to laboratory artefacts associated with their induction or conditions of transplantation. Tumours produced by powerful carcinogens can exhibit a level of immunity rarely displayed by tumours of spontaneous origin (Klein, 1970). No chemical carcinogen was involved in the production of this tumour. Tumours of unknown origin or those transplanted out-with the inbred strain in which they arose are best avoided if there is any danger of rejection. There was no evidence of rejection response against this tumour which suggested that there was no artifactual immune response towards the tumour by the host.

The tumour arose as a spontaneous mammary adenocarcinoma in a C_3H mouse from the Gray Laboratory. The spontaneous tumour occurs in the milk line of over 90% retired breeders. It was known to be of viral origin (the Bittner factor) which is transferred to the young in the mother's milk. The tumour has been serially transplanted every 2-3 weeks into the dorsum of recipient animals, details of which procedure have been described. Care should be taken when using serially transplanted tumours that these are not transplanted outwith the sub-strain in which the original tumour arose, and in the present study the tumour has only been transplanted to isologous hosts (C_3H/He) thus avoiding the possibility of differential immunogenicity complicating the interpretation of results.

No spontaneous tumour regression has occurred during this study. Spontaneous regression suggests some host/tumour immune reaction. For example the results of Crile (1965) have been used as a base for

clinical treatment yet one of the tumours in this study regresses spontaneously in 40% of cases (Hewitt, 1976). This is not a common event in human cancer so such tumours are best avoided.

From histological examination of small tumours (see Figures 2.4 - 2.6) no significant cellular reaction was noted. Nor was this found round metastases and this suggested a low, if any, immunogenic response. A further confirmation of this came from the fact that the number of cells required for transplantation (to immunologically available sites) was small. A take rate of 29% was found with 5 viable cells (see Table 2.1).

One very sensitive test of immunogenicity was advocated by Hewitt (1976). In this animals were 'pre-immunised' with LI cells. Then the TD50 (the number of viable cells which must be injected to give progressively growing tumours in 50% of the injected sites) was tested for in paired experiments. According to Hewitt the TD50 should not be found to be higher in 'immunised' mice than in the untreated mice. A similar test was carried out in this study. In this case 10^6 tumour cells (the number used in normal transplants) were injected into the normal site on the dorsum of 'immunised' mice and the latent period of 7.2 ± 0.9 (mean of 20 immunised mice) days was found from transplantation to detection.* This agreed well with 6.8 ± 1.0 days after normal transplantation procedures suggesting that the immune response, if any, was low.

This result is in apparent conflict with the situation where LI cells were added along with the tumour cells in subcutaneous injections when the LI cells appeared to alter the growth rate of the tumour. In this situation, however, the mechanism of the LI cells was more of a

* Mice were immunised with LI cells - one injection per week for three weeks

physical one - the LI cells acting more like a mesh holding the viable cells in position (see 2.4.3).

As a check on the technique for sterilising cells by lethal irradiation (SEE 2.2.2.) the LI cells were injected as for a normal transplant and the mice observed for 100 days thereafter. No tumours arose from these cells thus confirming that the role of LI cells was not as malignant cells.

Thus from histological evidence, from the fact that no rejection or spontaneous regression had been found and that the number of cells required to transplant the tumour was small, together with the fact that pre-immunisation made no significant difference in the growth rate of the tumour, it can be seen that if there was any immunological artifact it was insignificant as far as this study was concerned.

From the human standpoint the tumour can be considered an appropriate model because the 1 day doubling time (see 2.3.2) in mice with a 2-year life span can be equated to the 40 day doubling time of a typical mammary tumour in women with a 70-year life expectancy. As far as the relative kinetic parameters are concerned, the values of this C_3H tumour for Growth Fraction and Cell Loss Factor are also similar to those in human carcinomas, at 30% and 70% respectively (Nias, Chamberlain and Abdelaal, 1980). Furthermore the syngeneic transplants of the C_3H tumour have vessels confined to the internodular tissues (McCredie, Inch and Sutherland, 1971) and simulate closely the conditions described by Thomlinson and Gray (1955) in some human carcinomas.

2.4.1.2 As a model for this study

The fact that the radiation response and growth rate were found not to

be sex-linked was an obvious practical advantage, as was the fast growth rate. The tumour is very radio-resistant, having a high degree of necrosis (see Table 2.4). The TCD_{50} (dose which cures 50% of tumours) is high, being 6.5Gy, when animals were treated without anaesthetic (Abdelaal, 1979). Indirect evidence from TCD_{50} and from growth delay data, and the efficiency of misonidazole in reducing the TCD_{50} value to 3.1Gy suggests that the fraction of clonogenic hypoxic tumour cells may be as high as 30% at 6mm diameter (Abdelaal and Nias, 1975). Histological examination showed the proportion of necrotic tissue to be about 25-30% at the same size (see 2.3.1). Spontaneous, early and multi-generation tumours have been compared in Table 2.4. Spontaneous tumours can be used but these have the disadvantage that one cannot plan the siting of tumours or the timing of experiments - also the growth rate is very slow. First or second generation tumours are frequently used and these have certain advantages because they are closer to a spontaneous tumour, yet can be used in more easily planned experiments. A fast growth rate has practical advantages and this is normally found to some extent in these and in multi-generation tumours, however the multi-generation tumour being less well differentiated and having more necrosis than early generation tumours, is a better model for many types of human cancer.

For an investigation into the effect of hyperthermia on the regrowth potential of malignant cells the tumour used had to fulfil certain criteria, the most important of which was that it should show stable growth kinetics, since time to regrow to a predetermined size after treatment (growth delay time) was being used as an assay of cell damage.

With this tumour system there was always a good supply of readily breeding animals, which 'took' a transplanted tumour of known characteristics, every time. Identical tumours were provided by the lack of selection in the transplantation technique and by having a bank of frozen cells available. By this means tumours could always be used that were at approximately the same stage in transplantation, in experiments which extended over a long period of time.

Thus in all respects this tumour has been found to be suitable for use for testing the effect of hyperthermia, both locally and systemically (see Chapter 5).

2.4.2 *Transplantation Technique - Advantages of the Method Used in This Study*

Tumours can be transplanted using three main methods. In one the tumour is finely divided and implanted in recipient animals using a trocar. The disadvantage of this method is that selection may take place.

One other technique which is regularly used and which is very convenient if one is working with a tough fibrous tumour is one which makes use of a disaggregating agent, such as trypsin. This can damage cells unless care is taken. In studies not reported in this thesis, where attempts were being made to find if there was any correlation between plating-efficiency and tumour take rate, trypsin was used to disaggregate the tumour. It was found that the plating efficiency and take rate were lower when trypsin was used than when using the simple mechanical dispersal technique used in this study. Hewitt (pers. comm.) however, performed an assay to find if there was a detrimental effect associated with the use of trypsin on viable cells *in vitro* and found

a TD_{50} (the number of cells required to produce tumours in 50% mice innoculated with tumour cells) of CBA Sarcoma F cells, produced by mincing only, to be 85, while the TD_{50} for cells released by trypsin was also 85, showing that in an assay, where the tumour may have been rather tougher than that used in the present study, results of mincing and trypsin disaggregation were equally good. The present tumour was very easily disaggregated requiring only to be pushed through a sieve with a rubber plunger and then mixed by aspirating as has been described. Thus, the method used, while only suitable for a soft tumour, gave reproducible results and since the whole tumour was used there was, as far as possible, no selection. Although this cannot be completely avoided, as some cells may withstand transplantation better than others, this was minimised by the fact that the method is quick, so there is little selective cell loss because of the time factor.

Tumours of 6 ± 1mm diameter - the size used routinely for transplants - contained 25-30% necrotic tissue (see 2.3.1) and the fraction of clonogenic hypoxic cells was approximately 30% (Nias et al., 1980). Thus the 60% viability obtained in cell suspensions by the present method shows that the technique is a good one for the type of tumour used in the present study.

2.4.3 Tumour Growth

2.4.3.1 Microscopic

The growth characteristics of tumours during the latent period, i.e. below palpability, when tumour cells are thought to be present and multiplying, are of considerable interest and importance. Metastases are often present as very small tumours when the primary presents

itself. Whether tumours grow exponentially or not is not merely of theoretical interest. It is clinically relevant since several authors, including Norton and Simon (1977), have made recommendations on treatment schedules based on particular growth patterns. It is possible to use information about growth obtained from dilution studies together with information about growth delay times after different treatments to postulate mechanisms of regrowth after treatment (Wheldon, 1980). After x-ray treatment, for example, regrowth is slower than expected, which gives information about the nature of radiation damage. The slow regrowth is known to be related to damage to the vasculature and to the fact that immediately after treatment the remaining cells will probably be hypoxic. The time for re-oxygenation is very variable and is (in part) related to the degree of damage.

2.4.3.1.1 Dilution assay technique

The dilution assay technique was first applied by Hewitt and Wilson in 1959 to lymphocytic leukaemia in mice. They found that by injecting diluted known numbers of malignant cells into the peritoneal cavity of recipient mice, a proportion of the mice subsequently died; the length of time of survival being a measure of the number of tumour cells. On average, leukaemia was found to be transmitted to 50% of the animals by injection of only 2 leukaemic cells. This procedure is useful as a method of determining the number of viable cells remaining in a tumour after different treatments.

The investigation of tumour growth at a microscopic stage by use of a dilution assay also gives information about the rate of growth of untreated cells during the latent period - subject however to certain assumptions (a) that the site of injection puts no limitation on growth,

(b) that one is dealing with a single population of clonogenic cells all growing at approximately the same rate, and (c) that the proportion of tumour cells in a palpable tumour is independent of inoculum size. If the cells are all growing at approximately the same rate then their growth from a single cell can be approximated.

2.4.3.1.2 Mechanism of action of LI cells

If a sufficient number of viable tumour cells is transplanted into an appropriate recipient animal a tumour will develop. Révész (1955) showed however that if an inoculum of tumour cells is accompanied by LI cells of the same tumour the resulting tumour develops and reaches a lethal size earlier than in the absence of these cells, as has been found in this study.

For this 'Révész effect' to occur there must be local association between the viable and the LI cells. Hewitt, Blake and Porter (1973) found an intense fibroblastic reaction associated with the LI cells whether these and the viable cells were injected, mixed or separately. This suggests that the effect of the LI cells is not exerted by modification of the host's cellular reaction to the inoculum.

LI cells do not necessarily die soon after irradiation; they may survive for certain periods of time. Their final disintegration usually ensues in connection with more or less unsuccessful attempts to divide. Before this happens they may conceivably stimulate a small viable cell fraction randomly intermixed within them by providing various usable metabolic products, cell organelles or specific growth hormones. Hewitt et al have made the suggestion that LI cells do not

play the role of 'feeder' cells because it has been shown that sonication destroys their capacity to exert this 'Révész effect'. One implication of this is that the effect must be exerted by structural elements considerably larger than those normally providing for the cells nutrition.

The initial inflammatory reaction is considered by Peters and Hewitt (1974) to be provoked by the fact that LI cells release thromboplastin. This (by the tissue fibrin it encourages) tends to retain cells in a site to which they are transplanted, or to enable the growth of viable cells left *in situ*. A large proportion of viable cells injected alone either die on site or after their emigration from the site; the addition of LI cells (and of some other additions) to the inocula ensures a sustained thromboplastic influence at the site. About 5 days after injection of LI cells Révész noted that the reaction changed to a fibroblastic proliferation, with signs of incipient vascularisation. LI cells from different tumours have a similar effect to those from the host tumour and when viable cells were injected mixed with thromboplastic materials such as brain extract the same effect occurs. O'Meara (1958) supports the view of Hewitt et al that LI cells release thromboplastic material which forms a fibrin lattice thus holding the viable cells in position. The fibrin lattice may also provide the conditions required for the lodgement of metastases (see 5.1.1).

2.4.3.1.3 Reason for use of LI cells

If tumour cells are killed by heat the post-heated tumour may be a mixture of still-viable and heat-killed cells. Any analysis of growth modification by heat treatment must consider the possibility that the

heat-killed cells will have some influence on the growth of the surviving viable cells. Information about the influence of heat-killed cells on associated viable cells is difficult to obtain because of a technical problem, which is that heat, in the temperature range concerned, does not always produce 100% kill (as does a high dose of radiation); thus a pure population of heat-killed cells may not be obtainable unless unrealistic temperatures are used. Révész (1955) investigated the effect of heat-killed cells and found them not to exhibit a similar function to LI cells - however he nearly boiled the cells and must have destroyed their ability to continue to metabolise even for a short period, as after lethal irradiation.

Peters and Hewitt (1974) showed that thromboplastin was released by dead cells which had died spontaneously in tumours. There is therefore no reason to believe that heat-killed cells would not also release thromboplastin, capable of influencing the growth of associated viable cells. Thus a consideration of the influence of LI cells on viable cells may very well shed some light on the effect of heat on tumours. Thus to compensate for lack of knowledge about the possible method of producing 100% heat-killed metabolising cells it seemed not unreasonable to use available information, and to seek further data, concerning the influence of LI cells in the fate of viable cells.

2.4.3.1.4 Stationary phase

In general, after inoculation of tumour cells into a suitable site, the cells begin to divide and form into spheroid or elipsoid aggregates, the size of which is limited by absorption of oxygen and nutrients, and by the disposal of wastes, which are exchanged by diffusion while the

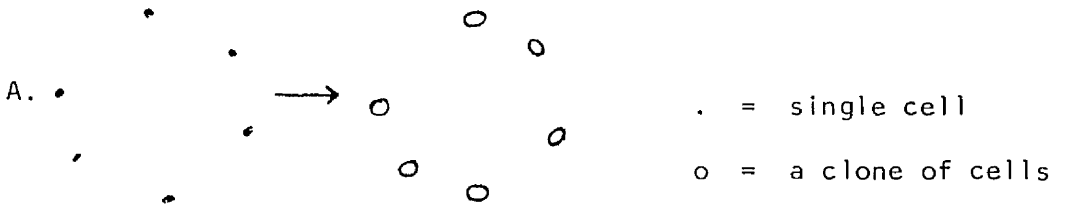
tumour is very small. There is thus a delay at this stage until new growth is promoted by stimulation of new capillaries which arise from the pre-existing vascular system of the host. The stimulus for this arises from what has been called the tumour angiogenesis factor (Folkman, 1974).

Capillary proliferation from the vascular system of the host can begin while the tumour colony is still very small. Wood, Baker and Marzocchi (1967) observed the formation of new capillary sprouts as early as 18 hours after lodgement of a colony of tumour cells in rabbit tissues.

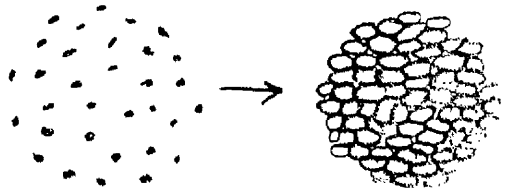
Hewitt (1953) has given a most convincing explanation of why tumours resulting from inoculated cell suspensions arise from a single focus, much smaller than the infiltration area produced. He found that inoculation brings together cells which have been dispersed in the original suspension. It has been found, however, by Hewitt, that injection of more dilute suspensions of cells do not give rise to a compact mass. This could be expected since the cells are distributed over the volume of tissue traumatised by the needle and are insufficient to fill it. In these circumstances the cells lie singly or in small aggregates within the traumatised connective tissue. Fidler, Gersten and Hart (1978) refer to evidence that cells from some tumours may be dependent upon one another for a stimulus to divide, and the fact that inoculation of small numbers of cells separates these cells, and this could determine in some tumour systems the minimum number required to transplant a tumour.

The results of the dilution assay indicate that the tumour cells appear to grow more quickly or have a better take rate when LI cells

are added. It seems reasonable to presume that the LI cells, acting as a framework for the widely separated tumour cells (from a small inoculum) act thus as a stimulus for them to divide because they in turn stimulate vascularity. From histological examination of very small tumours (of less than 1mm diameter) it was seen (Figure 4.9.) that these tumours which were produced from a low inoculum quickly assumed the form of a ring of small foci of rapidly dividing cells. Initiation of new capillaries had begun at the smallest size examined. The 'stationary phase' period seemed to coincide with this period of vascularisation. Another possible explanation for the variable 'stationary phase' is that with a low inoculum cells lie some distance apart as is shown in Figure A



These then begin to divide until their size is limited by nutrition etc. This may not happen abruptly and growth of 'mini' tumours will gradually slow down. Along with this decreased rate of growth the tumour cells may release a diffuse factor (tumour angiogenesis factor TAF). Folkman et al (1970) found that tumours which have reached a certain size released this TAF which is mitogenic for capillary endothelial cells and this was found to induce DNA synthesis in capillary endothelial cells as far as 3-5mm from the injection site within 24 hours. At this stage the vascularity will be stimulated and the tumour begin its exponential growth. With higher inoculi however, cells lie closer together. This is shown in Figure B.

B. 

They will thus suddenly reach the stage when there will be restriction of growth by lack of nutrients diffusing to all the dividing cells,

also large amounts of TAF will be produced, thus there may be a very short 'stationary phase'.

Another possible explanation of this effect is suggested by the work of Tannock (1968) who showed that the turnover time of the endothelial cell population in the system he was working with (50 hours) lagged behind the turnover time of its tumour cells (22 hours). As the tumour grew this led to increased intercapillary distance hence to decreased rate of growth as well as necrosis. It is possible that the turnover time of the microscopic tumour cells resulting from low inoculi could be very much more rapid than when these approached the situation when they tended to coalesce.

If the 'stationary phase' was caused by an immune response this would have been evident within 48 hours and signs of this would still be apparent at the '1mm' stage. There was only very slight evidence of any immune response and this was normally round the needle track which might have been expected as the response to a wound. This would not have been a variable response.

2.4.3.2 Macroscopic

At early stages tumour growth has been found to be exponential (see 2.3.2), a fact which is also true

in many human tumours (Spratt and Spratt, 1964; Hewitt, 1976; Steel , 1977). It might appear that tumour growth under ideal conditions and where there is no physical constraint, would be a simple exponential process, terminated by the exhaustion of the nutrient supply from the host. Most experimental tumours grow more slowly when large than when small. This kind of growth could arise if the active growth of a solid tumour were limited only to the outer layer of cells at the surface of the tumour (Mayneord, 1932). Here nutritional supply would be proportional to the surface area which may be the case when the supply is a network of very fine vessels rather than main arteries - as is often the case. However, tumours do not normally grow only at the surface. The reasons for growth limitation in larger tumours are probably multiple, however one reason is that as the tumour expands it outgrows its blood supply, since vessels within a tumour, lacking elastic tissue, cannot accommodate the increasing demands made by the constantly dividing cells. There is also physical pressure on the vessels causing them often to be occluded with the consequent build-up in the larger tumours of cytotoxic products of necrosis which may inhibit growth. This retarding effect could also be due to an increase in mean generation time (i.e. increase in cell doubling time) without a change in the proportion of reproducing cells or it may be due to a loss in reproductive cells without changes in mean generation time (the cell loss factor), or to a combination of these two processes. Necrosis is evident in most solid tumours, as in the C_3H tumour; the dead cells appear to remain in situ, and hence their contribution to tumour size remains (see 2.3.1). The only loss is in the proportion of reproductive cells contributing to later tumour growth, however

Laird (1964) has hypothesised that their loss cannot account for the shape of the curve representing observed tumour growth and thus it is likely that the observed deceleration is at least in part due to an actual increase in the mean generation time - i.e. a depression of the growth rate.

Folkman (1974) has hypothesised that angiogenesis contributes to growth limitation in two ways :

- by the inability of the endothelial cell proliferation to keep pace with tumour cell proliferation
- because endothelial cells in the depth of the tumour die.

All the features which have been described as typical of large tumours can be seen by examination of the histology of large C_3H tumours (see Figure 2.7), where one finds evidence of cells with pyknotic nuclei, and areas where cells are in a further state of autolysis. There are also cells with mitotic figures in well vascularised areas.

In the necrotic zone many remnants of capillaries were noted (Figure 2.6) which had ruptured, probably because the blood flow had become inadequate for the survival of most of the tumour cells, which had died and then swollen. One contribution to the increase in tumour size, apart from the rapidly dividing cells, is that which comes from dead cells. These tend to swell (as can be seen at high magnification) because the large molecules within them break down to give an increased number of smaller molecules which exert a higher osmotic pressure (cf what happens after hyperthermia treatment when the tumour becomes oedematous (see 4.3.1)). The swollen dead cells would then compress the capillaries so that no blood would get through them. At this stage even the live cells in the 100μ rim round the vessels would die.

The dead cells would begin to autolyse and so give no support to the vessel wall - which in tumour vessels is always thin. The vessels would then rupture and if there remained any connection with the main blood supply there would be haemorrhage. This describes many human tumours (McCallum, pers.comm.).

The suitability of the C_3H tumour system to test the effects of hyperthermia has now been well established. It now remains to describe how a heating system suited to this tumour was devised, and this will be described in Chapter 3.

TABLE 2.1

CELL DILUTION ASSAY - WITHOUT KILLED CELLS

Number of Cells Injected	Take Rate		Latent Period (± Standard Deviation)
7.5×10^6	10/10	100%	3.8 ± 0.4
4.0×10^6	21/21	100%	4.5 ± 1.2
3.75×10^6	22/22	100%	4.4 ± 1.1
3.6×10^6	3/3	100%	4.7 ± 0.6
3.0×10^6	31/31	100%	5.5 ± 1.4
1.0×10^6 (0.1ml)	40/40	100%	6.8 ± 0.9
1.0×10^6 (0.05ml)	29/29	100%	7.0 ± 0.8
5.0×10^5	36/38	95%	10.4 ± 3.00
1.0×10^5	34/38	94%	13.9 ± 3.6
5.0×10^4	32/35	91%	17.1 ± 6.3
1.0×10^4	28/32	88%	18.9 ± 7.0
5.0×10^3	44/49	89%	19.5 ± 4.5
1.0×10^3	32/40	78%	22.4 ± 6.0
5.0×10^2	22/33	67%	25.3 ± 2.5
1.0×10^2	15/37	41%	28.8 ± 5.9
5.0×10^1	23/64	36%	27.4 ± 5.8
5.0×10^0	14/49	29%	31.8 ± 6.0

TABLE 2.2

CELL DILUTION ASSAY - WITH KILLED CELLS

Number of Cells Injected	Take Rate		Latent Period (± Standard Deviation)
10^6	40/40	100%	6.8 ± 1.0
9×10^5	20/20	100%	5.2 ± 0.5
10^5	53/55	97%	10.0 ± 3.0
10^4	37/40	93%	12.7 ± 3.4
10^3	48/51	94%	16.2 ± 2.4
10^2	34/39	87%	22.3 ± 6.4

TABLE 2.3

'STATIONARY PHASE'

Number of Live Cells Injected	Stationary Phase With No Killed Cells (days) (± SD)	Stationary Phase With Killed Cells (days) (± SD)
10^6	1	1
10^5	4.5 ± 2.5	2.6 ± 0.5
10^4	8 ± 5	4.2 ± 0.9
5×10^3	11.5 ± 4	5.9 ± 2
10^3	10.5 ± 1.5	↑
10^2	9.5 ± 3.5	9.5 ± 3.2
5	18 ± 1.5	

TABLE 2.4

COMPARISON BETWEEN DIFFERENT GENERATIONS OF C3H TUMOURS

Author	Tumour Characteristic	Spontaneous	Early Generation	Multigeneration	Comment
Fowler et al (1975)	radiation response TCD50		4.7Gy♂ 3.6Gy♀		Difference attributed to higher degree of hypoxia in ♂
Abdelaal (1979)				6.5Gy ♂ and ♀	
Falk (1980)	Tissue organisation and vasculature	Vasculature only of capillary-like vessels following circuitous routes High degree of occlusion.			
McCredie et al (1971)		Relatively uniform distribution of blood supply, little necrosis. Cells well differentiated.	Peri-nodular distribution of blood vessels, centri-nodular necrosis. Cells poorly differentiated. More necrosis than spontaneous.	Similar to first generation but blood supply poorer. Necrosis more marked and cells less differentiated. 70% necrosis in large tumours.	
Abdelaal (1979)			Well differentiated, uniform vascularity, little necrosis.	Relative volume of necrotic tissue, 20-40% at 6mm diameter.	
Walker (this study)				Complete lack of organisation with necrosis even at 1mm diameter.	
Fowler et al (1975)	Cell doubling time at palpability		1.4 - 5.3 days*		These grew from trocar implants, hence the variability.
Walker (this study)				1 day	
McCredie (1971)	Volume doubling time at 10 days after palpability	Approximately 12 days	Approximately 5 days	Approximately 2.5 days	

3.0 DEVELOPMENT AND APPLICATION OF THE HEATING SYSTEM TO THE CHOSEN TUMOUR MODEL

3.1 Introduction - Criteria to be Satisfied

The aim of any heating technique is to raise the temperature within a tumour as quickly and uniformly as possible to the desired operating temperature, without significantly heating the surrounding normal tissue. In investigating the available methods of heating small animal tumours, no one method satisfied all the requirements which seemed necessary to this investigation. A new technique has thus been developed which satisfied the following criteria:

- the heating must be localised to the tumour without significantly raising the temperature of surrounding tissue. There must be no systemic hyperthermia.

- temperature gradients within the tumour must be small since very small changes in temperature can have a large effect on cell kill (cf Crile, 1962 and Hahn, 1974).

- no anaesthetic should be used. Biological reasons for this are discussed in 4.4.1. Anaesthetics also introduce another variable to complicate analysis of results.

- stress must be minimised because there is evidence that stress lowers temperature in mice (Sheldon, Hill and Moulder, 1977).

- it must give results which will be clinically relevant. This has already been discussed in relation to the tumour system (see 2.4.1). A technique suitable for heating a small animal's tumour may be rather different from one designed to treat a human tumour. However, in considering the design, the basic concept was that it would be

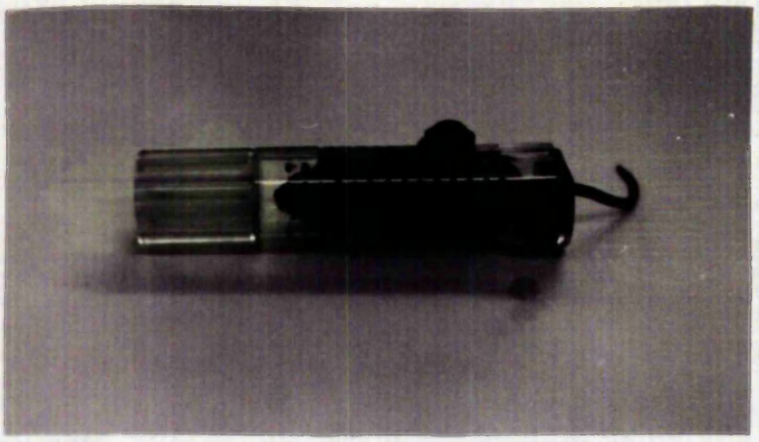


Figure 3.1

Constraining jig with animal inside - tumour is supported by collar

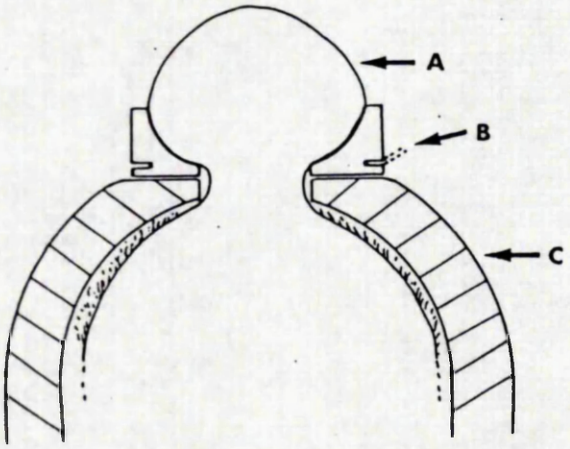


Figure 3.2

Tumour supported by collar.

A - tumour, B - collar, C - constraining jig

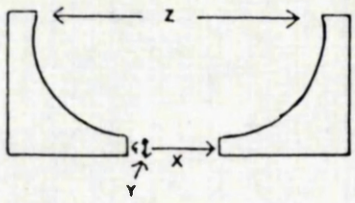


Figure 3.3

X - diameter of hole, Y - thickness of lead,

Z - diameter of top of collar ($\cong$ 7mm)

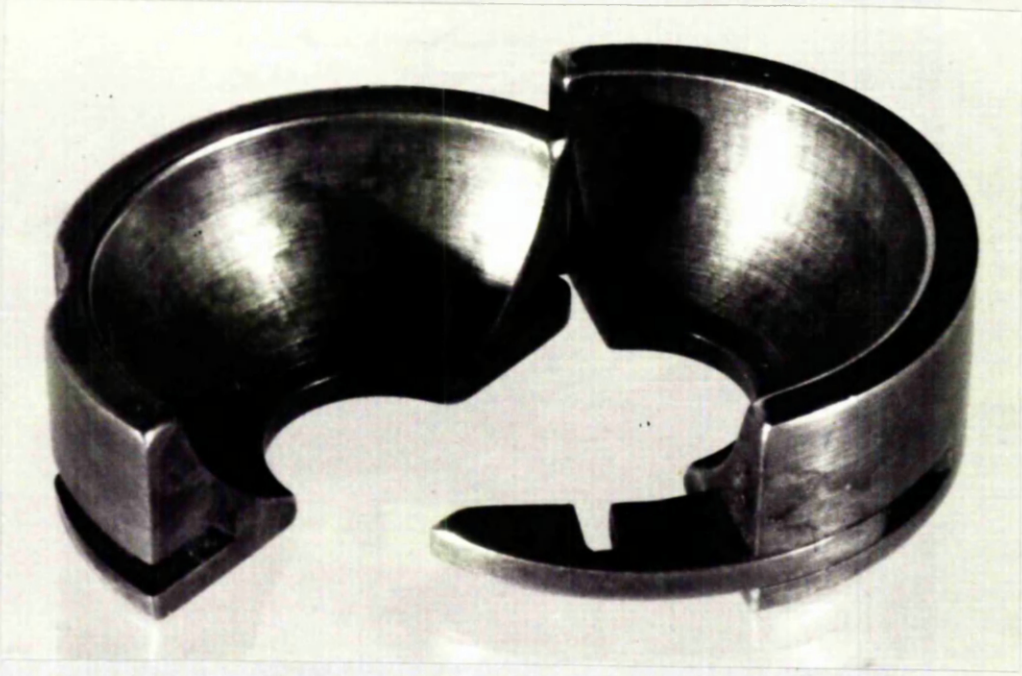


Figure 3.4

Collar

applicable clinically.

The local and systemic response to heat, to be described in this thesis, will be compared with the effects of surgery and of irradiation on the same animal tumour, carried out concurrently, and as far as the X-ray work is concerned, reported on more fully by Abdelaal (1979). The local responses will be compared as a means of helping to understand the complex mechanism of regrowth after treatment, while the systemic effects to be discussed in Chapter 5 give some further information on the major problems of metastases.

3.2 Methodology and Results of Calibration Techniques

3.2.1 Animal Supports

In all the hyperthermia experiments to be described animals were constrained in an upright position and were not anaesthetised. The tumour was retracted through a 6mm wide slit in a constraining jig (Figure 3.1). The dimensions of the jig permitted some freedom of movement and gave room for increased depth of breathing to occur (by which means core temperature in mice is regulated). The perspex construction allowed for the observation of animals during treatment. They settled down quickly and were under no apparent stress when treated at temperatures up to 45°C; thus no anaesthetic was required. Holes were drilled in the jig to help maintain normal body temperature. The design allowed tumours in slightly varying positions to be treated.

As unsupported tumours sagged only the top and sides of the tumours could be heated by the technique to be described, therefore a copper collar (Figures 3.2, 3.3 and 3.4) was designed both to provide support and to ensure maximum heat transfer to the base of the tumour. Various

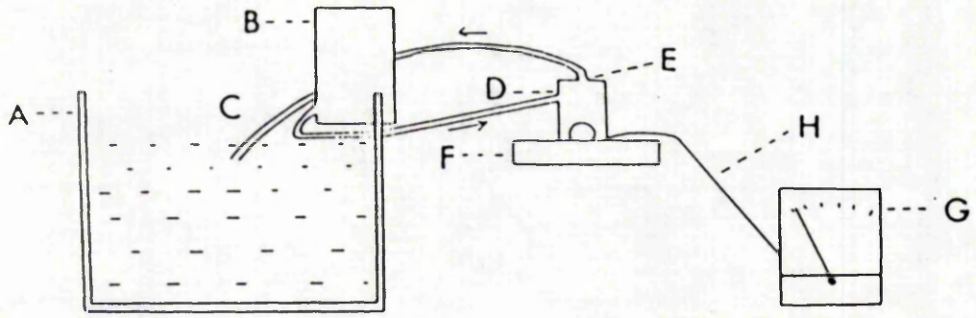


Figure 3.5

Schematic diagram of heating system.

A reservoir, B heat and pump unit, C outlet from heating unit, D inlet to heater applicator, E outlet from heater applicator, F constraining jig, G thermometer, H thermocouple lead

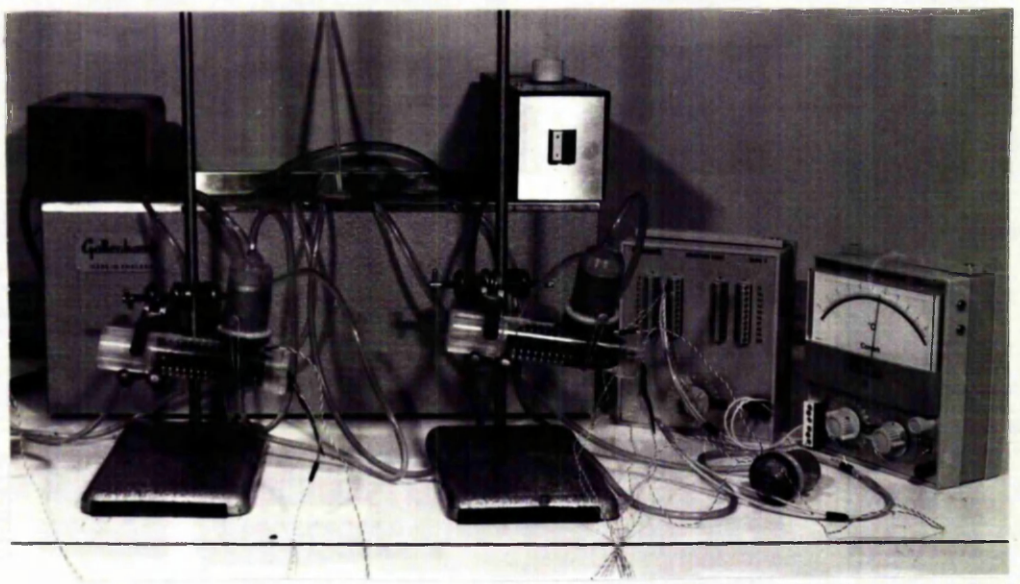


Figure 3.6

Heating set-up

sizes of collars were made (Figure 3.3) after taking into account the following various factors:

- tumours varied in size - 6.5 ± 0.5mm diameter
- mouse skin thickness, measured by a micrometer, varies with sex
  - male mice skin thickness approximately 0.75mm
  - female mice skin thickness approximately 0.62mm
- compression of blood vessels, which must arise purely by raising the tumour, should be reasonably comparable with that produced using the radiation jig. Since comparisons between the two treatments were to be made as it was decided that tumours should if possible be of similar hypoxic status
- the tumour was imbedded to approximately a third of its diameter and could be more efficiently heated if seated in the collar.

3.2.2 Heating System

This system is one where heated water was pumped through an applicator from a reservoir (see Figures 3.5 and 3.6) and heat was delivered from the water to the tumour through a membrane and collar. Tumour and animal temperatures were measured using thermocouples in conjunction with an electronic thermometer. The various elements of the heating system will now be described in more detail.

The thermostatic control unit (type SU6, Grant Instruments Ltd) is of the clip-on type which enables any suitable tank of over 10 litres capacity to be used as a reservoir, from which water can be pumped through other apparatus at a carefully controlled temperature. The liquid expansion thermostat gives temperature control to ± 0.05°C in tanks of over 10 litres. The temperature of the circulating water was monited by a thermistor as it left the pump unit. The maximum

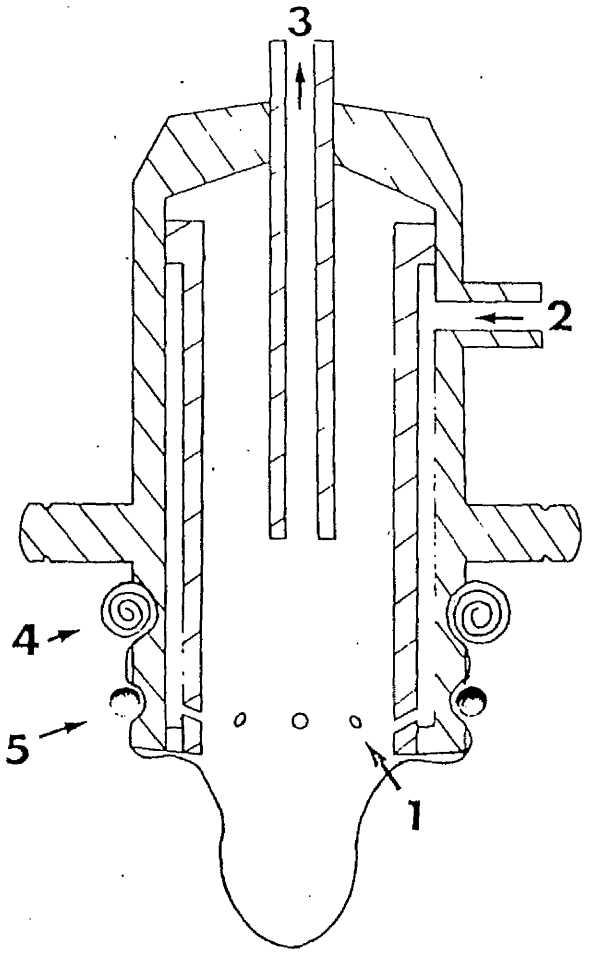


Figure 3.7

Diagram of water heater. Membrane is shown in convex position. It assumes a concave position when placed over tumour.

1 - inlet holes, 2 - inlet from reservoir,

3 - outlet to reservoir, 4 - membrane,

5 - O-ring

flow from the control unit was 11 litres per minute, and each heating device required just over 0.5 litres per minute to maintain the correct temperature; a by-pass valve was fitted to ensure the correct flow to each device. It was decided not to insulate the tubing carrying water to the individual heaters because of the space required to do this, but connections were, however, of constant length (45cms). The fall in temperature along each connecting tube was under 1°C and every mouse tumour temperature was always recorded.

3.2.3 Heat Applicator

Water pumped from the reservoir was circulated through the hollow wall of the perspex heater and entered the chamber above the tumour through holes drilled obliquely to the circumference. This produced an eddy flow round the tumour, which was heated through a membrane attached to the heater (see Figure 3.7). The height of water in the device was adjusted to give sufficient mixing and was just enough to hold the diaphragm in contact with the tumour. Introduction of coloured dye into the water showed that there was thorough mixing in the heater. The membrane was held in position by an O-ring, situated in a groove, which obviated leakage problems. The most suitable membrane for the particular tumour used in this study was the Horizon Stimula contraceptive sheath (G D Searle and Co Ltd, High Wycombe). To ensure good thermal contact between the membrane and the tumour, K-Y lubricating jelly (Johnson and Johnson Ltd, Slough), which is harmless to tissue and does not damage plastic or rubber, was routinely used. The temperature measured at the interface between the membrane and the tumour was designated surface temperature.

The heat applicator was placed over the tumour as is shown in Figure

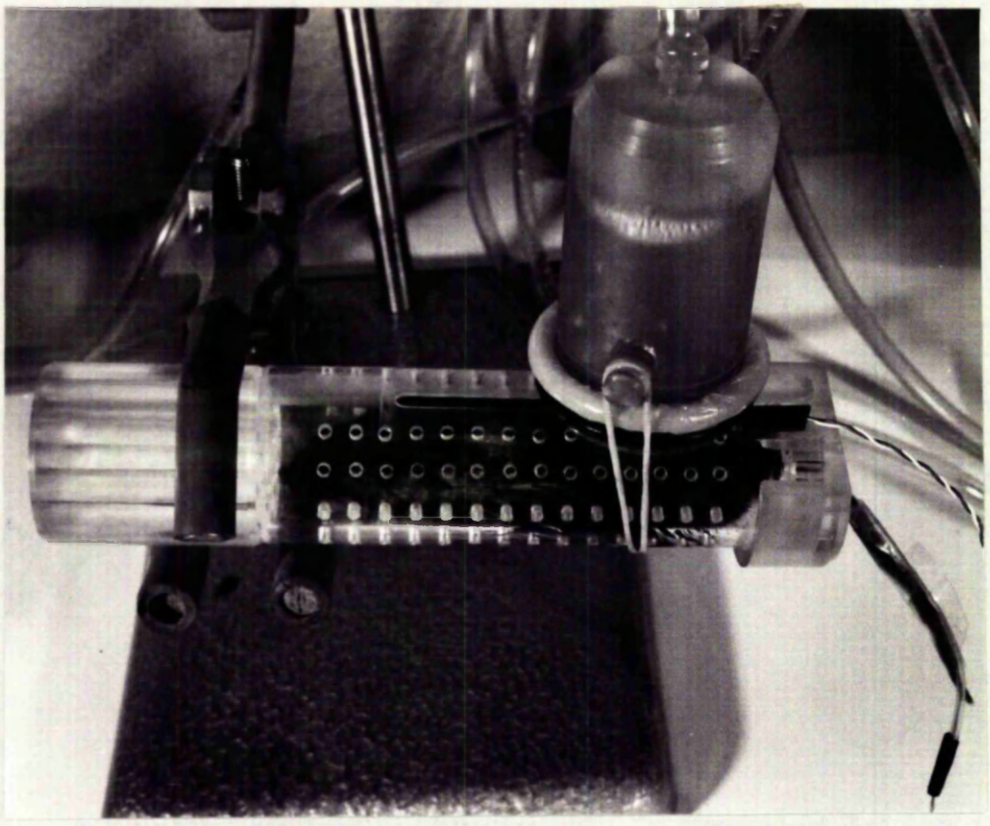


Figure 3.8

Heat applicator in position over a tumour

3.8. The sides of the heater were supported by the constraining jig and the membrane took up the shape of the tumour. The heater was held in position by an elastic band to allow the animal to move.

3.2.4 Temperature Measuring Equipment

3.2.4.1 Electronic thermometer

All temperature monitoring was carried out using copper-constantan thermocouples with the Comark electronic thermometer type 1624, in conjunction with Selector Unit type 1694, which enabled the thermocouples to be connected sequentially to the electronic thermometer. The thermometer was specially calibrated to give a precision claimed to be greater than ± 0.05°C in the temperature range 30-49°C. This thermometer has an automatic cold junction and it is claimed that over the range 10-30°C there is less than ± 0.5°C deviation. In practice ambient temperature was always 22 ± 1°C. The unit has a built-in compensation for changes in resistance due to differences in lengths of thermocouple leads, however in practice all leads were kept the same length.

3.2.4.2 Thermocouples

The thermocouples were made as follows:

Rectal probes were made of fine copper and constantan wires brazed at the junction. An 11cm length next to the junction was insulated with varnish, pulled through polythene sleeving (2mm internal diameter) and the polythene trimmed so that the thermocouple junction was just exposed. The remaining free ends were drawn separately through PVC sleeving and the junctions between these and the polythene sealed. The thermocouple junction was sealed using a globule of IS 12 adhesive which

when set was still spherical. This was achieved with Permabond Quick Setter (which speeds up hardening and helps bond with polythene) and gave a smooth flexible probe suitable for insertion into the rectum. It was well tolerated by the animal, allowing it to move with the probe in position.

Needle probes for measuring intra-tumour temperatures were made by drawing the insulated end of a thermocouple junction through a 21g needle (0.8mm diameter). A very fine junction had to be made. Only disposable needles had a large enough bore to allow the junction to be accommodated in the needle without damaging the insulation. The thermocouple junction was positioned just behind the needle tip. The main problem in construction was in sealing the needle tip without blunting the needle. A compromise in needle size was necessary, since some needles had to be strong enough to allow curvature and hold that curve to an arc, calculated so that the tip of the needle could be inserted into the centre of the tumour from below. Needles also had to be strong enough to withstand insertion to a distance of about 2cm under the surface of the skin in order to insulate them from the heater to prevent errors in temperature arising because of heat conduction along the needle.

Probes were made to measure tumour surface temperature. In making the surface probes a 5cm length from the junction was sealed in teflon and double-sided sellotape. This made very flexible thermocouples.

3.2.5 Calibration

Procedures to test certain criteria such as stability of heating system and calibration of heat appliances were more suitably performed using

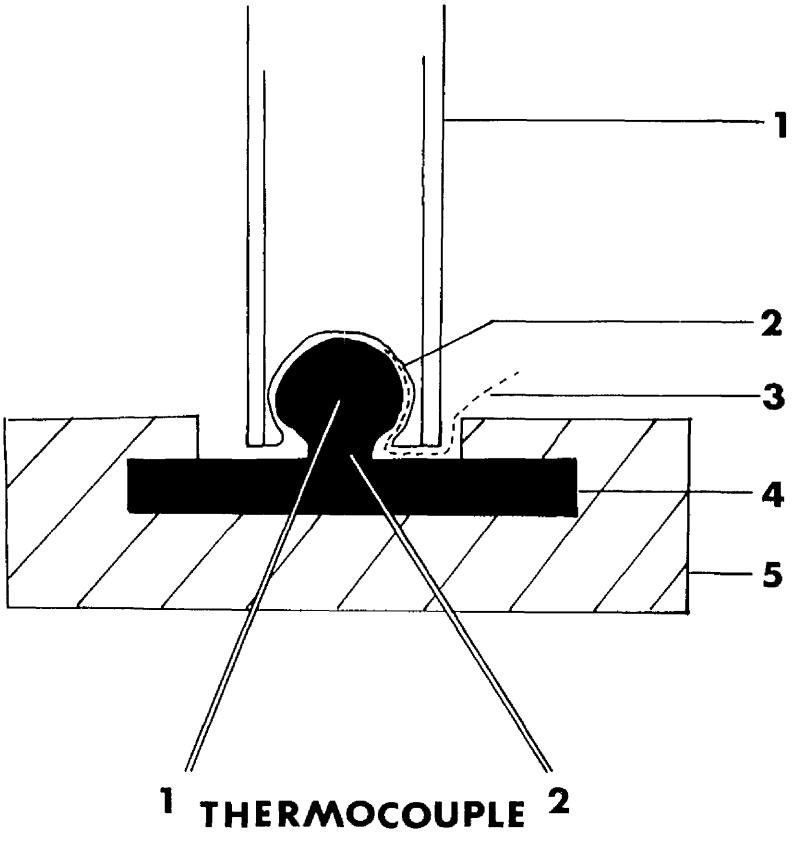


Figure 3.9

Cross-section of heater and phantom showing position of thermocouple probes - no collar is shown for clarity

1 - heat applicator, 2 - heater membrane, 3 - surface probe, 4 - phantom, 5 - insulation

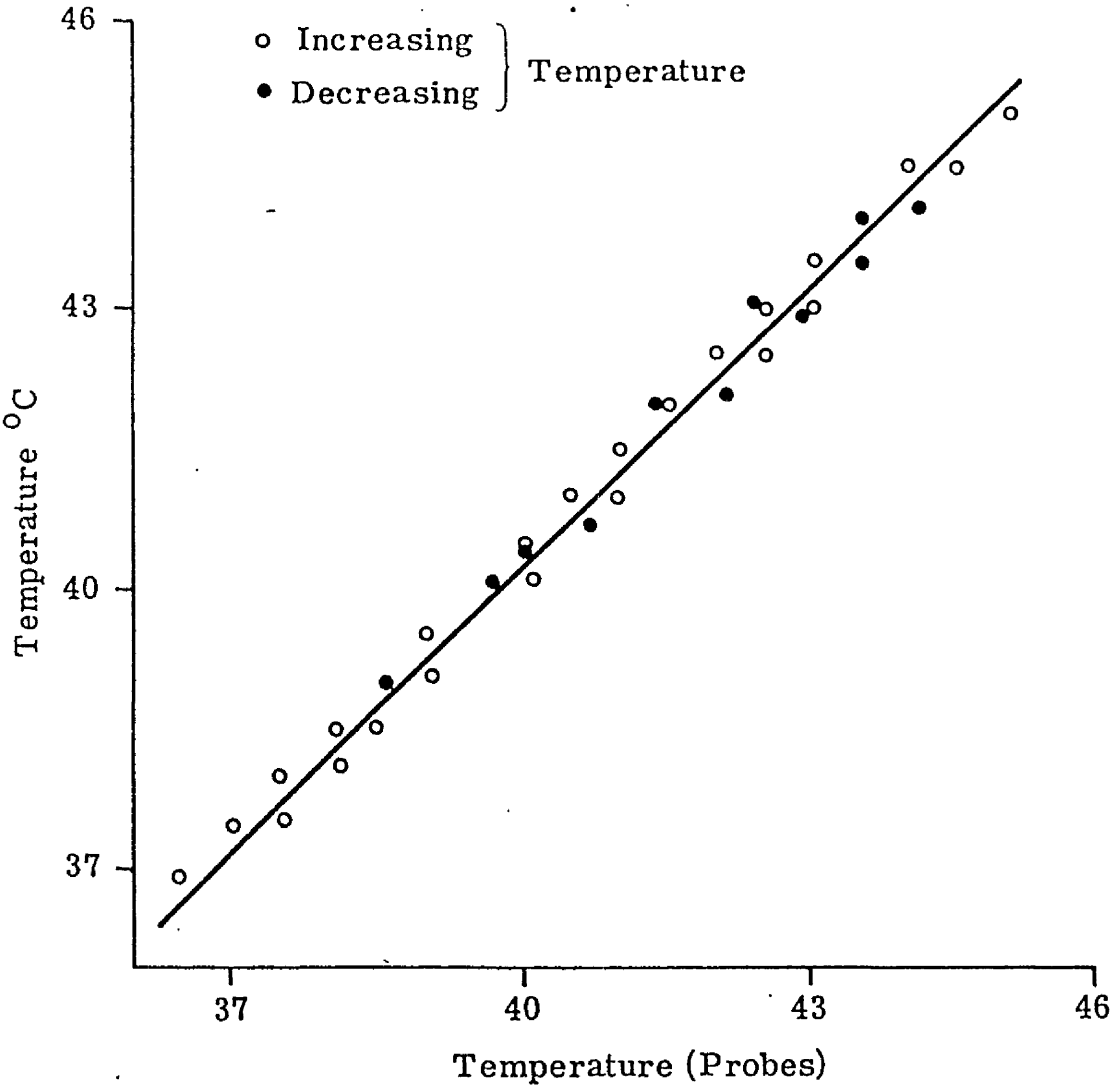


Figure 3.10

Typical calibration curve for thermocouples

a phantom, firstly since mice were saved and secondly because temperature variability, as was found in mice, was eliminated so performance of equipment was tested more accurately. The phantom, of dental wax, which has similar thermal properties to muscle (Hatfield and Pugh, 1951; Carter and Perry, 1977) was constructed as shown in Figure 3.9. The main slab of the phantom represented the mouse body, and a 'tumour' was shaped to simulate the mouse tumour of the size treated. The phantom was insulated with polystyrene.

Testing, calibration and pre-setting of equipment were normally performed using the phantom. In most of the calibrations the heat applicators were placed on top of the 'tumours' with thermocouples positioned as illustrated. Various calibrations were carried out.

- Thermocouple probes were calibrated routinely against a standard NPL mercury in glass thermometer. Temperature readings were made in steps of approximately 0.5°C with increasing then decreasing temperature. A mean deviation was recorded for every probe. Figure 3.10 shows typical thermocouple calibration curves for increasing and decreasing temperature. A best fit line was obtained by the method of least squares. Adjustments were always made to temperatures as measured by each probe so that corrected temperatures were recorded.
- To test for thermal stability of the heating system over the range of temperatures of interest measurements were made on the 'tumour' using only the surface probe as illustrated in Figure 3.9. Thermal stability of the heating system was demonstrated by constancy of temperature to ± 0.05°C as measured at intervals of 1°C over the temperature range 33-46°C after stabilising at each

temperature interval.

- The heating applicators were calibrated by placing surface probes at different positions on the 'tumour' surface - up to five being used simultaneously, to measure the variation in temperature over the 'tumour' surface, at different flow rates. The optimum flow rate gave minimum variation in temperature between different points on the 'tumour'/heater interface. Different flow rates were found by measuring the collected volumes of water, over a given period, after the water had flowed through the heating device. The smallest variation in temperature between probes was ± 0.05°C over the tumour surface. This was obtained with a flow rate of 0.5 litres per minute. A more rapid rate caused the applicator to lift and lose contact with the tumour, while a slower rate gave larger variations in temperature. With that flow rate 22 mice could be treated simultaneously since the flow rate from the pump unit was 11 litres/minute. The results of calibration of equipment showed that it was stable and capable of giving accurate and reproducible temperatures.
- Since tumours were to be heated at an exact predetermined temperature it was necessary to calibrate the heater so that it could be accurately pre-set to any temperature required. Stabilisation at a specified temperature took approximately 10 minutes therefore it was necessary always to carry out this calibration to pre-set the heater. A calibration curve was thus constructed from the temperatures recorded using a surface probe on a phantom and on an animal tumour, at varying pump/heating unit temperatures. This was called the phantom 'tumour'/mouse

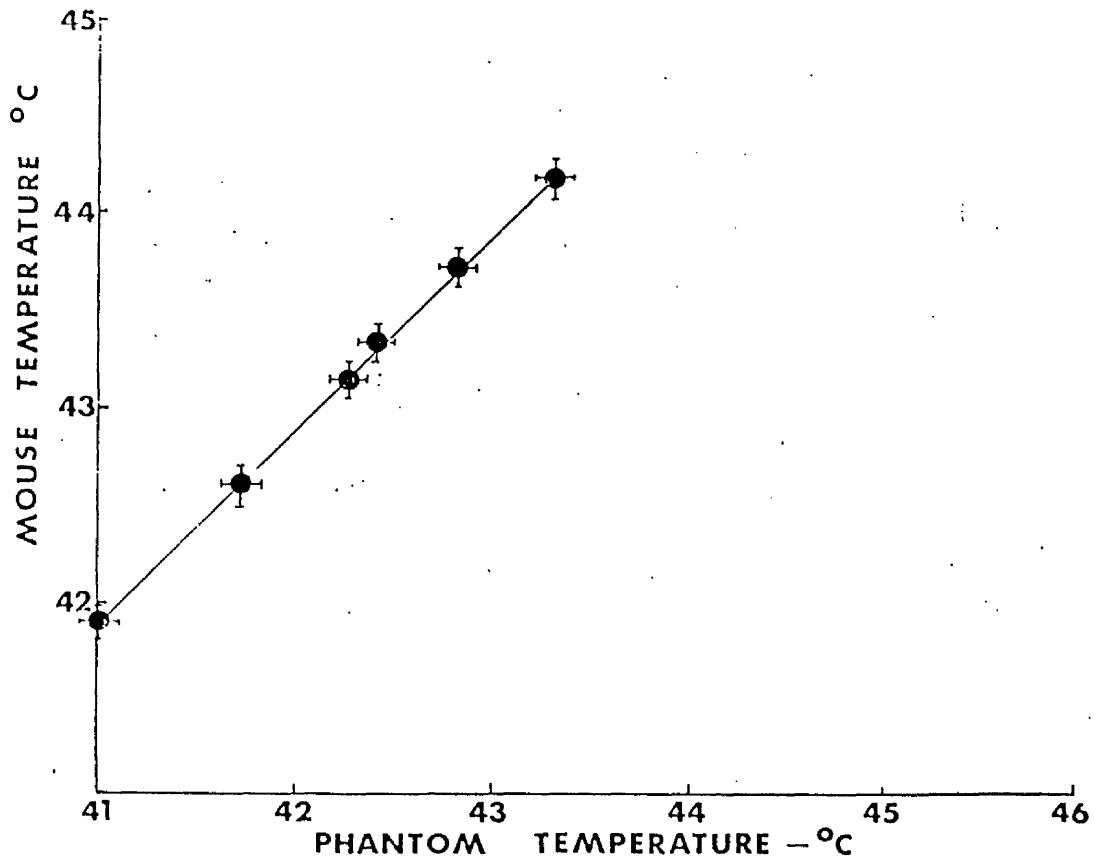


Figure 3.11

Phantom/mouse calibration

tumour calibration and was carried out for every surface thermocouple since thermocouples often varied slightly. Figure 3.11 shows a typical calibration curve of temperatures measured concurrently on phantom and animal tumours.

Although the phantom and animal tumours cannot be considered as exactly equivalent, as heat loss from the phantom 'tumour' by conduction was greater than from the animal tumour, this calibration was useful so that the required animal intra-tumour temperature could be reached in under 2 minutes from the start of a hyperthermia experiment.

Before the beginning of most hyperthermia experiments the heat applicator was placed over the phantom 'tumour' until the required equivalent temperature was reached, when it was moved - together with the surface probe - directly on to the animal tumour.

3.2.6 Tumour Temperature Gradients

3.2.6.1 Phantom

Methods of improving uniformity of temperature within the heated tumour were initially investigated using the phantom. To evaluate the advantage of collars being placed round the 'tumour' a series of experiments was performed using the set-up shown in Figure 3.9. For clarity the collar has not been shown in the diagram. The position occupied by the collar is shown in Figure 3.2.

Two different sets of experiments were carried out to see whether collars improved the temperature distribution in the 'tumour' when heat was applied.

In the first set only surface probes were used. Temperatures were

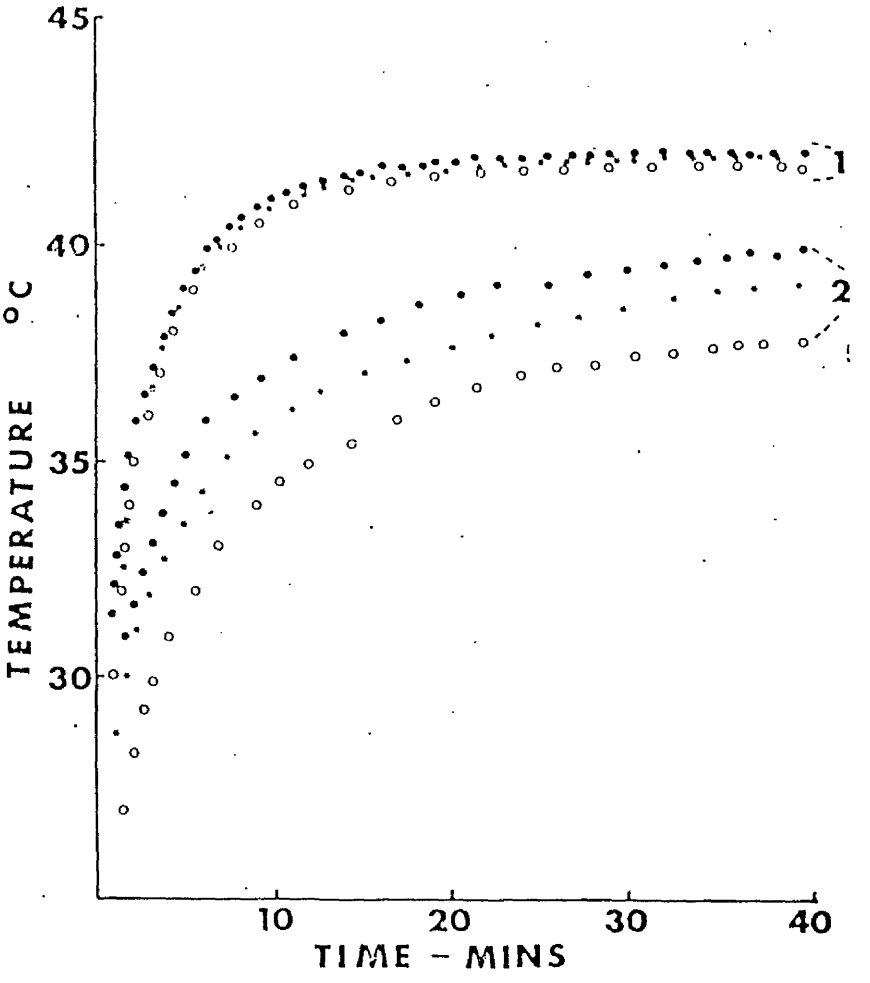


Figure 3.12

Evaluation of advantages of use of collar using phantom

o no collar, * collar, • collar + extra insulation

1 - needle in centre of tumour, 2 - needle at base of tumour

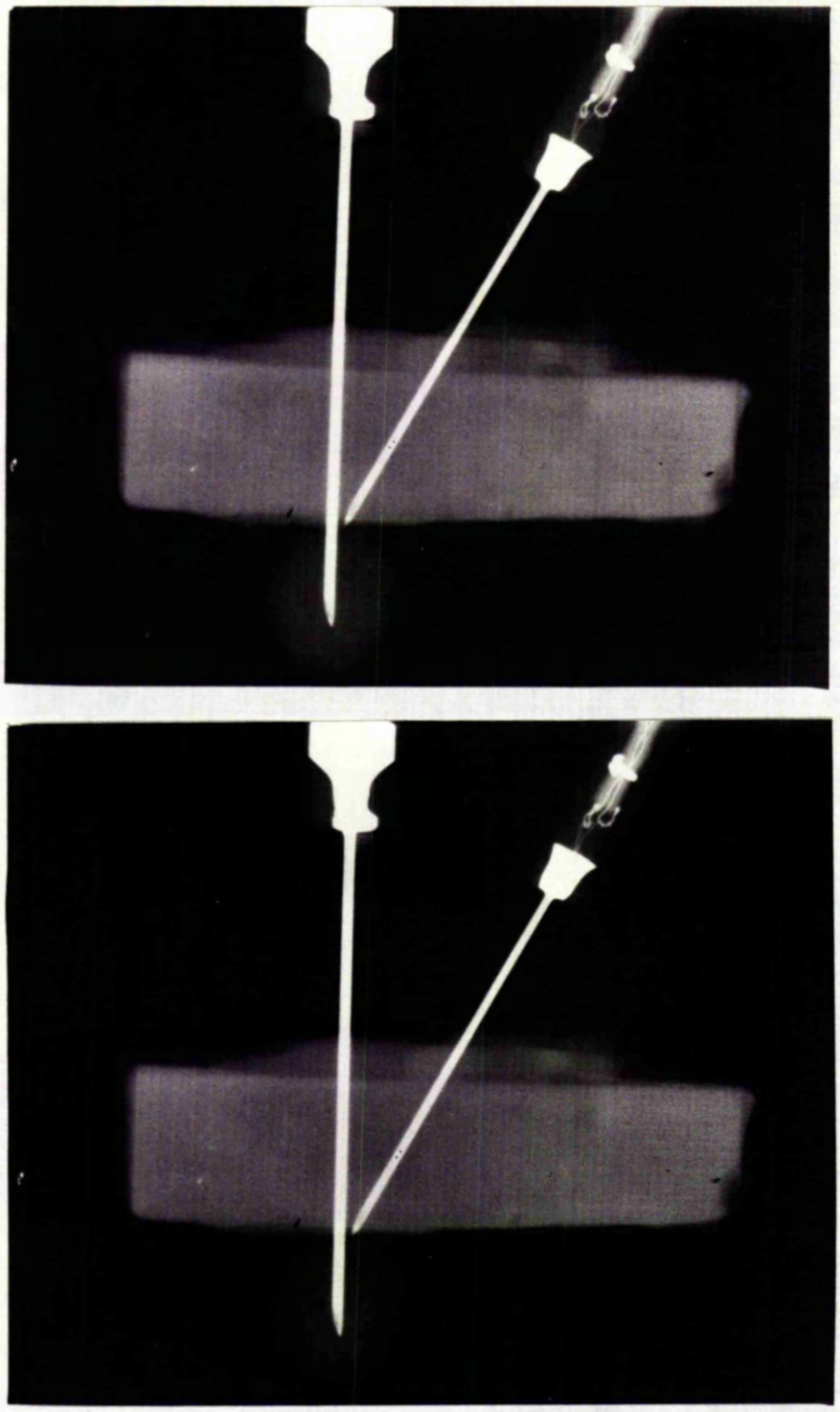


Figure 3.13

X-ray photographs of needle probes in phantom before and after hyperthermia

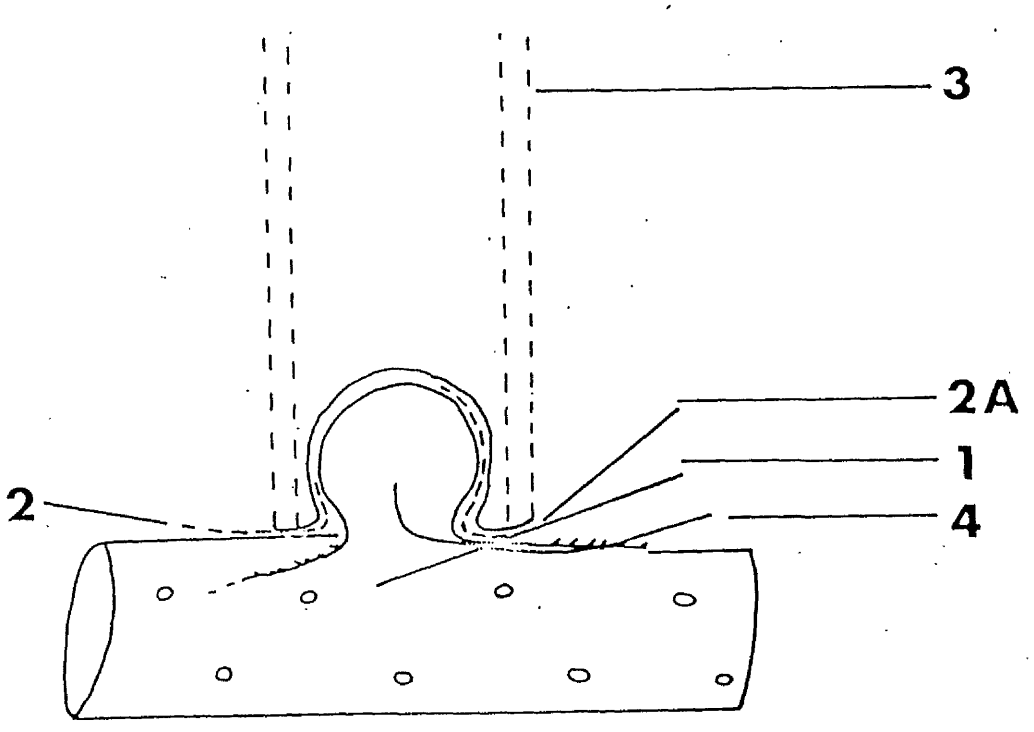


Figure 3.14

Cross-section of heater with tumour held above constraining jig. This figure shows positions of needle and surface probes. No collar is shown for clarity.

1 - straight needle probe, 2 & 2A - surface probes,

3 - heat applicator, 4 - curved needle probe

measured at different points over the 'tumour' surface with and without collars in position. The variation in temperature over the upper section of the 'tumour' surface was within the errors of measurement. Without a collar in position the temperature at the periphery of the 'tumour' was 0.4°C lower than when the collar was on. This in turn was approximately 0.1°C lower than that measured over the upper part of the 'tumour'.

In the second set of experiments temperature readings were made with the two needle probes. A surface temperature of 42.5°C was applied to the 'tumour'. One series of temperature readings was made with the collar in position, then another with the collar plus additional thermal insulation round the phantom. The collar was then removed and a third series of temperature readings taken. X-ray photographs of the set-up, with the collar in position, and after its removal, were taken to check if any relative movement of the needle probes had taken place when the collar was removed.

Figure 3.12 shows results of temperature measurements made within the 'tumour' by needle probes positioned as shown in Figure 3.9. X-ray photographs were taken (Figure 3.13) to check relative position of probes before and after collar was removed.

3.2.6.2 Animal

To investigate temperature gradients within animal tumours when local heat was applied several experiments were carried out. In most calibrations a tumour of 7mm diameter was used. In these experiments the surface probes were placed between the tumour and membrane as shown in Figure 3.14. The point of the straight needle probe was positioned by inserting approximately 2-3mm below the tumour as shown.

This was in the well vascularised zone below the tumour. The curved probe was introduced under the skin about 1cm from the tumour (in this way needle probes were insulated by the animal's skin and fur from the edge of the heater). The point of the curved probe was placed in different positions in different experiments. In all experiments temperature readings were taken when intra-tumour temperature had equilibrated with the surface temperature (i.e. in approximately 2 minutes).

As a preliminary study temperatures were measured in unheated tumours, using the curved probe, over a period of 30 minutes with and without collars. Animals were held in the jig during measurements. Similar measurements were made in ligatured tumours (see Figure 4.7).

The unheated intra-tumour temperature varied between mice, as is shown in Table 3.1, and was related to the tumour size ($P < 0.001$), but not to core temperature. Normally a large tumour had a low intra-tumour temperature.

Intra-tumour temperatures in unheated tumours supported (with collars) or unsupported fluctuated but fell gradually by 1-2°C over 30 minutes;

when the tumour was ligatured the temperature fell by approximately 3-4°C in 5-7 minutes then fell by a further 1-2°C over the 30 minutes (see Tables 3.2 and 3.3).

3.2.6.2.1 No collar - heated tumour

To check whether the surface temperature, as measured by the probe inserted between the heater membrane and the tumour, was recording tumour surface temperature the curved probe was inserted with the point just under the surface of the skin. Temperatures were recorded

simultaneously on this probe and on the surface probe.

Table 3.4 shows surface temperature, as measured by a surface probe and by a needle probe in the skin, just below the skin surface, to be very similar. The mean difference of 0.07°C is within the errors of measurement.

To measure thermal gradients within the tumour two methods were adopted. In the first method, the curved probe was positioned approximately 4mm from the tumour surface, and the temperature measured by this probe was compared with the surface temperature (see Table 3.5). The second measure of thermal gradient was made by careful positioning of the curved probe 2mm below the tumour surface. After allowing time for temperature equilibration, approximately 2 minutes, the needle was retracted 4mm, and a second temperature reading taken (see Table 3.6).

Results show that there was a thermal gradient of approximately 0.1°C/mm tumour tissue. In the second method of measuring this the gradient appeared slightly higher (see 3.3.1).

To measure the temperature in the well vascularised zone below the tumour at different surface tumour temperatures the straight needle probe was inserted as described. Temperatures were recorded simultaneously on this probe and on the surface probe. Table 3.7 shows that the mean difference between applied temperature and that measured in the well vascularised zone at about 2mm below the tumour was approximately 3°C.

Experiments to investigate thermal gradients in occluded tumours were performed by placing the curved probe in the centre of the tumour then ligaturing the tumour. Care had to be taken not to move the needle

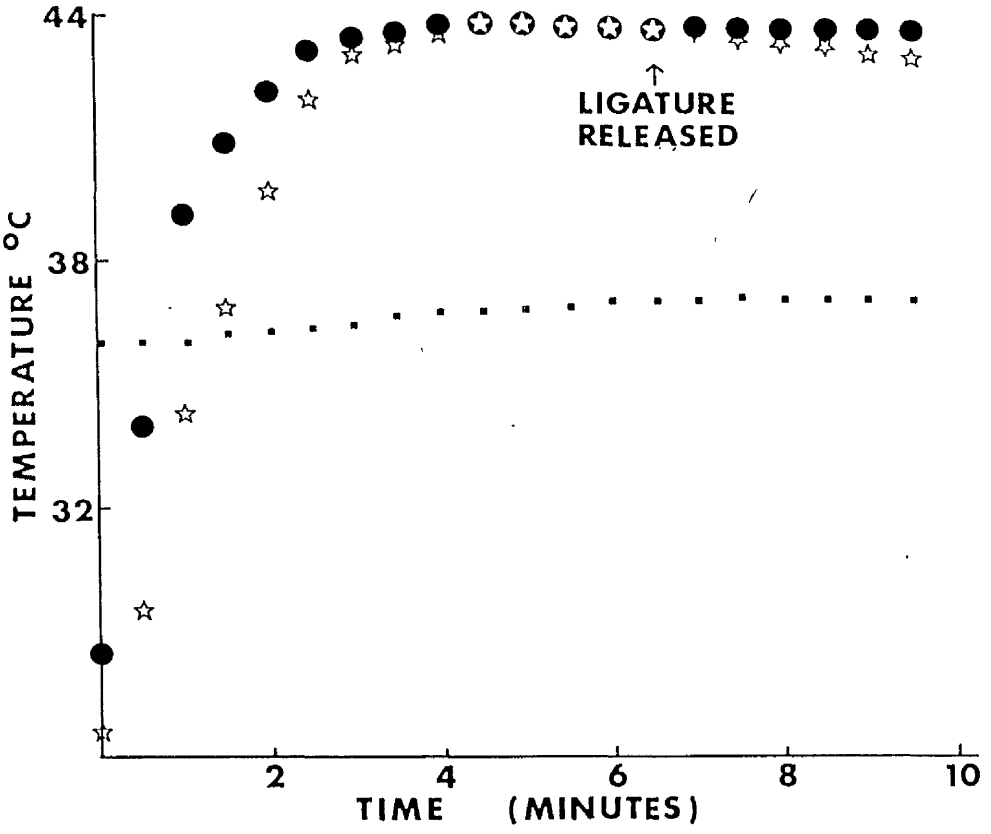


Figure 3.15

Temperature gradients within a ligatured tumour.

● surface temperature, ☆ intra-tumour temperature,

▪ rectal temperature

while ligaturing the tumour.

Table 3.8 shows that the temperature within a ligatured tumour was similar to the applied temperature. Figure 3.15 shows temperature measurements within a ligatured tumour during heat application. The thermal gradient was 0.05°C/4mm tissue. The temperature in the tumour did not fall immediately the ligature was released but fell gradually to approximately 0.3°C below surface temperature.

3.2.6.2.2 With collar

Three experiments were carried out to investigate the advantage of using collars round animal tumours.

Temperatures were measured over the tumour surface as was done with the phantom 'tumour' with and without a collar.

When the collar was used variation in temperature over the whole tumour surface was ± 0.05°C.

To measure the thermal gradients within a tumour with a collar on, a curved needle probe was positioned approximately 4mm from the tumour surface. The collar was then placed round the tumour base and the temperature measured on the probe was compared with the surface temperature.

Table 3.9 shows that the thermal gradient was approximately 0.04°C/mm tissue - this compares favourably with when there was no collar (Tables 3.5 and 3.6), and indicates that use of collars gave a temperature gradient nearly as good as that obtained when ligaturing tumour (Table 3.8), i.e. 0.04 versus 0.01°C/mm tissue.

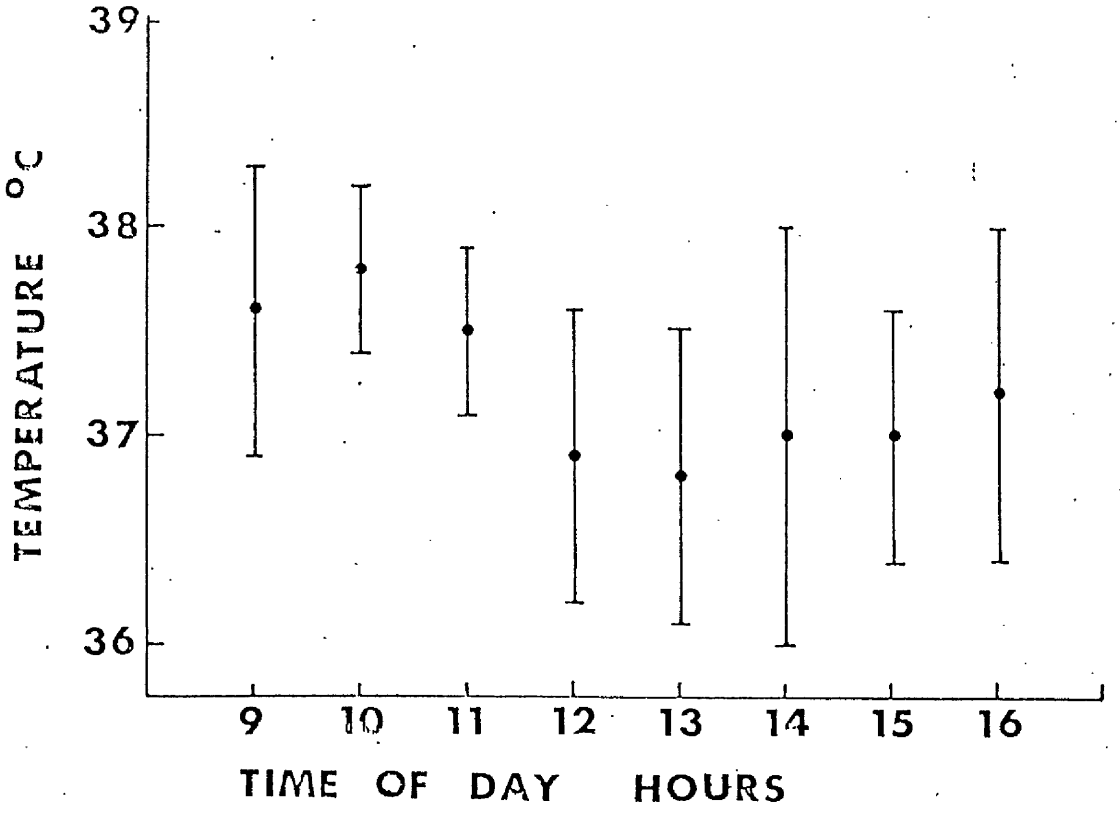


Figure 3.16

Temperature variation in mice over an 8 hour period

To measure the temperature in the well vascularised zone below the tumour a straight needle probe was placed below the collar.

Table 3.10 shows that the temperature differential between applied temperature and that in the well vascularised area below the tumour was approximately 1.6°C. This area was being heated more efficiently when the collar was in position than with no collar (Table 3.7).

3.2.7 Animal Rectal Temperature

3.2.7.1 Laboratory conditions

Rectal temperature was used as a measure of core temperature. It was monitored in all experiments.

To find the normal temperature of mice under laboratory conditions (ambient temperature 22 ± 1°C) the rectal temperatures of 10 mice were measured at hourly intervals over an 8 hour period.

Results are shown in Figure 3.16. The mean temperature was 37.2 ± 0.7°C and variation in temperature in one mouse over an 8 hour period was found to be as much as 2.3°C. Because there appeared to be a cyclical pattern in the rectal temperatures over the 8 hour period, all hyperthermia experiments were conducted at the same time of day to avoid any possible complications arising from this.

To find if constraining mice in the jig affected core temperature, rectal temperatures were measured in mice constrained in jigs for 30 minutes.

The rectal temperatures of mice constrained in the jig fell gradually over a period of 30 minutes to approximately 0.4°C below normal (see

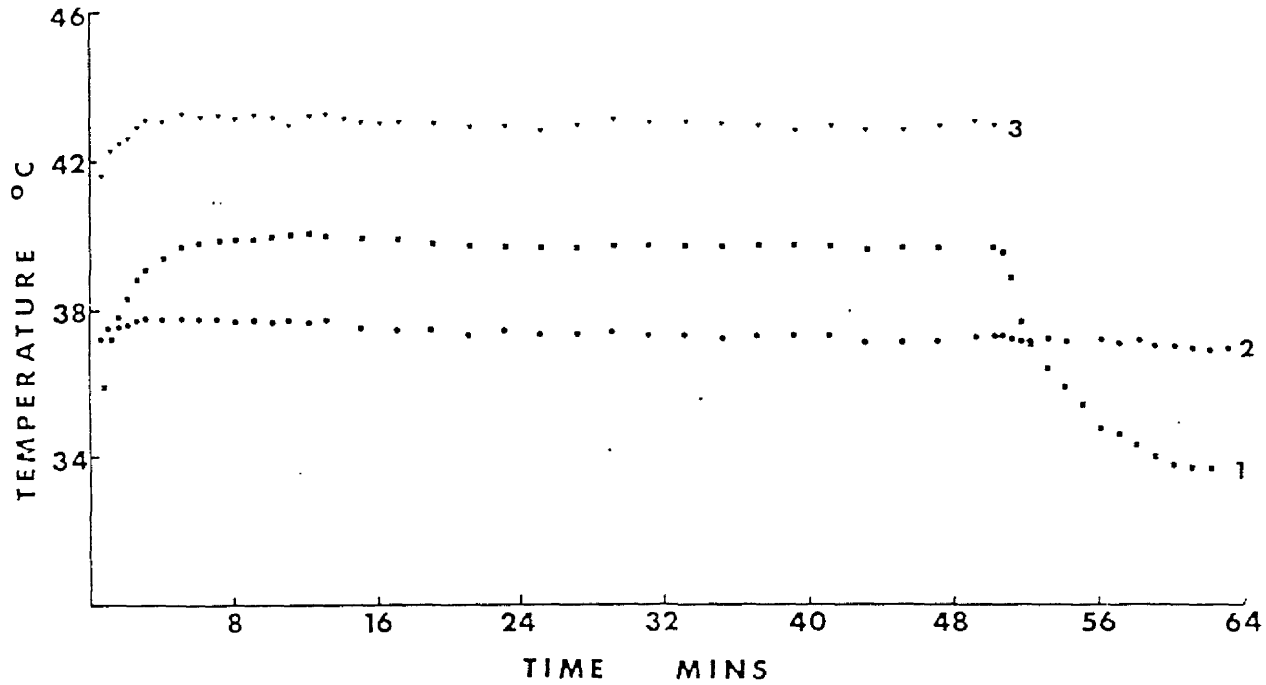


Figure 3.17

Temperature gradient between surface and base of tumour

1 - temperature at base of tumour, 2 - rectal temperature, 3 - surface temperature

Tables 3.2 and 3.3).

3.2.7.2 During treatment

The rectal temperatures of all mice given local hyperthermia were measured during treatment. The first 30 mice treated were kept in the jig for periods of up to 1 hour after treatment was completed in order to observe rectal temperatures. The temperatures were routinely taken daily for 3 days after treatment.

The rectal temperature rose, after heat had been applied, by approximately 1°C over a period of 5-10 minutes. It then fell gradually to approximately 0.5°C above normal and remained at that temperature until about 20 minutes after treatment had been completed (Figure 3.17). The rectal temperatures of 10 mice given local hyperthermia treatment, where the tail of the animal was insulated by wrapping with cotton wool, were taken during treatment and for 1 hour after treatment.

Where the tail was insulated during treatment the temperature rose to approximately 2.25°C above normal and remained at that level during the period of treatment and until about 40 minutes after treatment had been completed.

3.3 Discussion

3.3.1 Tumour Temperature Gradients

3.3.1.1 Phantom

Although phantoms have been of use in preliminary testing of equipment phantom experiments are of limited value since they give no information about the situation where there is blood flow, which has a major effect on modifying temperature distributions (Patterson and Strang, 1979).

Also although the phantom had similar thermal conductivity to muscle, and as such was useful to test thermal distributions when heat was applied, loss of heat by conduction from the heated 'tumour' to the 'body' of the phantom must have been greater than from a heated animal tumour to the animal body which would be at a higher temperature than the phantom. An attempt to compensate for this by putting extra insulation round the phantom lessened the temperature gradient. The information gained from this experiment to investigate thermal distributions in heated tumours with and without collars showed that the collar did improve the temperature distribution in the 'tumour'. No evaluation of the degree of improvement could be made.

3.3.1.2 *Animal*

The thermal gradients, as quoted in Tables 3.4 - 3.8, were as far as possible gradients along the direction of greatest thermal change, namely from the top of the tumour to its base. Gradients may be affected by the methods of measuring temperature and by the positioning of the probes, so great care was always taken in placing them. No more animals were used than was strictly necessary since an element of stress may have been inherent in the experimental procedure. It is for this reason that all preliminary testing of equipment and investigation of thermal gradients were carried out using a phantom.

In measuring tumour temperature gradients great care was taken to minimise disruption of tissues, since this would have resulted in changes in blood flow affecting temperature distribution. The needle probes were the finest that could be used considering the nature of the site and the fact that they had to be curved.

To investigate whether surface probes did measure skin temperature

rather than heater temperature, measurements of temperature were recorded from a needle probe positioned in the skin (Table 3.9). This was found to be 0.07°C below surface temperature. As the skin over the tumour is very well vascularised (Abdelaal, 1979) this could be responsible for the very slightly lower temperature. The difference was so small that it was concluded that the surface probe did provide a measure of skin surface temperature. The result also indicates that temperature measured on the skin (surface) can be used as equivalent of temperature just under the skin surface - i.e. the probe does not appear to heat selectively because it is in contact with the heated membrane.

Temperatures measured by surface probes over the tumour surface, with and without collars round the tumours, showed that the collar improved the level of heat delivered to the tumour periphery - the tumour being evenly heated over the whole surface area when the collar was on.

When the intra-tumour temperature gradient was being measured using the two methods described (3.2.6) the gradient was found to be 0.35°C/4mm tissue and 0.45°C/4mm tissue by the first and second method respectively. Since no collar was used in these experiments the slightly larger gradient in the lower part of the tumour (0.45°C/4mm) could have been expected. This technique of measuring thermal gradient by positioning a probe then withdrawing it a fixed distance to make comparative readings has been used also by Gibbs, Peck and Dethlefsen (1981). They measured temperature distributions in a C3H tumour situated on the flank and heated by a waterbath, and found temperature distributions similar to those in the present study when the collar was used - i.e. 0.16°C/4mm tissue (Table 3.8). The result

is interesting since Gibbs et al used one of the techniques evolved in the present study. A 'clip', which is a version of the collar, was used to hold the tumour out from the body. The 'clip' however does not act as a conductor of heat to the tumour base. This may not be necessary with waterbath heating, but the design does necessitate heating a larger area of skin round the tumour (also animals required to be anaesthetised in this study). It seems possible that the'clip' might occlude the blood supply to the tumour more than the collar, however Gibbs et al measured rate of Xenon-133 clearance when using the 'clip' and from this stated that the 'clip' did not significantly impair perfusion of the tumour. The rate of Xenon clearance was similarly measured in the present study, however the technique was not pursued since it was found to be difficult to differentiate between true tumour clearance and clearance through the needle hole made on injecting the Xenon. Rate of Xenon clearance has not always been found to be a reliable measure of tumour blood flow (Strang, pers.comm.).

There were several other experiments however that did suggest strongly that the collar did not impair the blood supply to the tumour. When the collar was used, the measured temperature in the well vascularised area below the tumour was higher than when no collar was used (Tables 3.10 and 3.7). This does suggest that heated blood was circulating to and from the tumour. This result is consistent with histological studies (see 2.3.1).

Further evidence to support this comes from the fact that when heating tumours supported by the collar, the thermal gradient of 0.16°C/4mm tissue (Table 3.9) was more than when the tumour was ligatured, in which

case the intra-tumour temperature was found to be (within the errors of measurement) the same as the surface temperature (Table 3.8). This implies that the collar did not occlude the tumour blood flow. The gradient of 0.16°C/4mm is less than that found when no collar was used (approximately 0.4°C/4mm). This however would have been expected from the results of phantom and other experiments which showed that the collar gave more even surface temperature and did improve distributions within the tumour so these results do not conflict with the evidence for blood flow to and from the tumour.

Perhaps the most convincing demonstration that the collar did not restrict tumour blood flow, comes from the fact that the intra-tumour temperature in untreated mice held in the jig for periods of 30 minutes were found to be similar whether collars were worn or not. The temperature fell in both cases by approximately 1°C over a period of 30 minutes, whereas in ligatured tumours it fell rapidly during the first 5 minutes by approximately 3°C then fell more slowly until it levelled out at approximately 2°C above room temperature (see Tables 3.2 and 3.3). This suggested that the ligature did not entirely restrict blood flow therefore the tumour was not made completely hypoxic by the ligature.

When heating ligatured tumours the intra-tumour temperature remained similar to the surface temperature and on removal of the ligature the temperature did not fall immediately. It fell gradually to the level found when the tumour was not ligatured presumably as the blood began to circulate (Figure 3.15).

3.3.1.3 Evaluation of collar

The collar changed the geometry of the tumour so that heat was applied to a volume of tissue as near to spherical as could be obtained. This was the shape advocated by Strang and Patterson (1980) (see 1.3.2).

No claim is made that the collar caused no constriction of the tumour blood flow, but as appropriate collars were matched to tumour size etc., the degree of constriction must have been minimal.

A further advantage in using the collar was that recurrence initially was found to take place just outside the heated area. On histological examination this was often shown to have originated at the site of injection from a small satellite tumour. The technique of subcutaneous injection was improved by using a very short needle, and by swabbing the clipped skin at the injection site with alcohol, as has been described. The tumours produced, together with any satellites, were then included in the heated volume when a collar was in position.

The use of collars was therefore considered to be a more acceptable compromise than the use of anaesthetics, the immersion of large areas round tumours in waterbaths or the use of other more sophisticated methods of heating small subcutaneous tumours.

3.3.2 Animal Rectal Temperature

That the rectal temperature first rose to $1^{o}C$ above normal then fell by $0.5^{o}C$ suggests that mice can regulate their body temperature unless too large an area is heated (cf when tumours on mice thighs were heated - Robinson et al, 1975). A rise of $0.5^{o}C$ in temperature is small when it is seen (3.2.7) that the temperature of a mouse can

fluctuate by as much as 2-3°C over a day. Thus the small rise in temperature probably indicated no major systemic effect. That the tail is well vascularised and acts as a temperature regulator in mice has been demonstrated by Hornsey, Myers and Andreozzi (1977). This was confirmed here in experiments where the tail was insulated during treatment. Thus the experimental technique used in this study does not materially raise the animal's core temperature. There is evidence that stress lowers temperature in mice (Sheldon, Hill and Moulder, 1977). This did not occur when animals were treated therefore there is no evidence to suggest that mice treated using the techniques described were stressed.

3.3.3 Evaluation of Technique Compared With Other Systems

The technique used is very simple yet it gave as good thermal gradients with small animal tumours as many more sophisticated systems (see 1.3.2). Table 3.11 summarises some of the techniques used in localised heating in studies where the temperature gradients across the tumours were quoted. Some assessment of the techniques are given under 'Comment'.

Microwave heating technique employing a liquid 'bolus' surrounding the tumour, as has been reported by Robinson et al (1978), gives similar though slightly less good thermal profiles to those obtained in this study. The systems are however nearly equivalent, because once the bolus reaches the target temperature it acts as a thermal reservoir similar to the heater in the present study - yet using a much more complicated system to obtain this. Robinson et al do not discuss whether or not an anaesthetic was used. Mendecki, Friendenthal and Botstein (1976) treated transplanted mammary tumours in C3H mice and

achieved treatment temperatures within 90 seconds - the centre of the 6mm tumour being 0.2°C higher than the surface. This gradient, in reverse, is similar to that obtained in the present study, however Mendecki et al found a rise in core temperature of more than 5°C during treatment, while there was only a very small systemic rise in temperature in the present system.

Radiofrequency heating was used by Dickson, Calderwood and Jasiewicz (1977) to treat small rat tumours of 1-1.5ml volume on flank or foot. With these the temperature rose quickly to the treatment temperature and remained stable, while there was considerable skin sparing - depending on the size of the tumour, skin temperature was 4-5°C below tumour temperature. However normal muscle tissue within the heated volume was 2-3°C higher than the tumour temperature. With this size of tumour rectal temperatures did not rise significantly, however when larger tumours were treated the rectal temperatures rose to approximately 41-42°C and all the rats died. It is difficult to compare these results with those of the present study. One good feature was the skin-sparing effect of the radiofrequency heating, but overheating of muscle tissue within the heated volume appears to be a major problem.

Radiofrequency and waterbath heating have been compared by Marmor, Hahn and Hahn (1977) and van Dijk and Breur (1979). Marmor et al investigated cell kill after treatment of mouse tumours in the temperature range 43-44°C and found it to be similar after both types of heating. Van Dijk and Breur found radiofrequency heating to be more effective than waterbath, at the same apparent temperature, when treating animal tumours. Their poor results with waterbath heating seem surprising. No cures were obtained with an apparent tumour

temperature of 44° for 50 minutes. One might suspect that the temperature gradient across the tumour must have been in excess of the quoted 0.5°C.

The present system appears from the results shown in Table 3.11 to be preferable to most waterbath heating systems, because where a low thermal tumour gradient has been obtained this has been at the expense of immersing large areas of normal tissue in the waterbath giving very high core temperatures in the mice and a large amount of normal tissue damage.

RESULTS OF EXPERIMENTS WITHOUT COLLARS
(ALL TEMPERATURES IN DEGREES CENTIGRADE)

TABLE 3.1

Tumour Diameter	Intra-Tumour Temperature	Core Temperature
8	32.7	37.4
8	32.4	36.9
6	33.4	37.3
6	33.0	36.9
9	31.8	36.2
7	32.8	36.8
8	32.5	35.9
9	32.1	36.4

TABLE 3.2

FALL IN INTRA-TUMOUR TEMPERATURE IN DIFFERENT MICE OVER A 30 MINUTE PERIOD - MICE HELD IN JIG

Time	0	5	10	15	20	25	30
Tumour Temperature (no collar)	32.7	32.3	32.1	32.1	31.9	31.8	31.6
Rectal Temperature	37.4	37.3	37.4	37.2	37.1	37.1	37.1
Tumour Temperature (no collar)	32.4	32.2	32.0	32.0	31.7	31.2	31.0
Rectal Temperature	36.9	36.9	36.7	36.5	36.6	36.5	36.5
Tumour Temperature (with collar)	33.4	33.3	33.2	32.9	32.7	32.5	32.2
Rectal Temperature	37.3	37.2	37.3	37.1	37.0	36.9	36.8

TABLE 3.3

FALL IN INTRA-TUMOUR TEMPERATURE IN DIFFERENT MICE OVER A 30 MINUTE PERIOD - MICE HELD IN JIG AND AND TUMOUR LIGATURED

Time	0	5	10	15	20	25	30
Tumour Temperature	31.8	29.4	27.2	27.0	26.9	26.4	26.5
Rectal Temperature	36.8	36.5	36.6	36.6	36.4	36.2	36.3
Tumour Temperature	32.8	29.7	28.5	27.8	27.5	27.3	27.1
Rectal Temperature	36.8	36.7	36.7	36.2	36.4	36.3	36.2
Tumour Temperature	32.5	29.7	28.8	27.8	27.5	27.5	27.3
Rectal Temperature	35.9	35.9	35.7	35.6	35.7	35.3	35.3

TABLE 3.4

DIFFERENCE BETWEEN SURFACE TEMPERATURE AND THAT IN THE SKIN JUST BELOW THE SURFACE

Surface Temperature	Needle Temperature	Difference	Mean
45.5	45.4	0.1	
44.9	44.85	0.05	0.07
44.7	44.7	0	±0.04
43.35	43.25	0.1	
43.1	43.0	0.1	

TABLE 3.5

DIFFERENCE BETWEEN SURFACE TEMPERATURE AND THAT 4MM FROM SURFACE

Surface Temperature	Needle Temperature	Difference	Mean
45.1	45.0	0.1	
45.1	44.7	0.4	
44.8	44.25	0.55	
44.8	44.6	0.2	
44.6	44.0	0.6	
44.5	43.9	0.6	
44.4	44.1	0.3	
44.35	44.0	0.35	
44.3	44.2	0.1	0.35
44.2	44.1	0.1	±0.12
44.2	43.9	0.3	
44.1	43.85	0.25	
44.0	43.8	0.2	
43.9	43.4	0.5	
43.8	43.0	0.5	
43.6	43.3	0.3	
43.2	42.9	0.3	
42.7	42.3	0.4	

TABLE 3.6

DIFFERENCE IN TEMPERATURE BETWEEN TWO POINTS 4MM APART IN TUMOUR

Postion 1 (needle tip 2mm below surface)	Position 2 (needle tip 6mm below surface)	Difference	Mean
45.4	44.9	0.5	
45.0	44.5	0.5	
44.6	44.2	0.4	0.45
44.2	43.8	0.4	± 0.15
43.9	43.6	0.3	
43.6	43.0	0.6	
42.9	42.5	0.4	

TABLE 3.7

DIFFERENCE BETWEEN SURFACE TEMPERATURE AND THAT APPROXIMATELY 2/3MM BELOW BASE OF TUMOUR

Surface Temperature	Needle Temperature	Difference	Mean
45.9	42.5	3.4	
45.5	42.7	2.8	
45.0	41.5	3.5	3.0
44.6	41.4	3.2	±0.25
44.0	41.4	2.6	
43.9	40.9	3.0	
43.2	40.2	3.0	
43.0	39.8	3.2	

TABLE 3.8

DIFFERENCE BETWEEN SURFACE TEMPERATURE AND THAT AT APPROXIMATELY 4MM FROM THE SURFACE OF A LIGATURED TUMOUR

Surface Temperature	Needle Temperature	Difference	Mean
45.2	45.35	+0.15	
44.75	44.8	+0.05	0.05
44.65	44.75	+0.1	±0.05
43.25	43.25	0	
43.0	43.0	0	
42.8	42.8	0	

RESULTS OF EXPERIMENTS USING COLLARS

TABLE 3.9

DIFFERENCE BETWEEN SURFACE TEMPERATURE AND THAT 4MM FROM SURFACE OF TUMOUR

Surface Temperature	Needle Temperature	Difference	Mean
45.1	44.9	0.2	
44.4	44.3	0.1	
44.2	44.1	0.1	
44.1	44.0	0.1	0.16
43.9	43.7	0.2	± 0.03
43.8	43.6	0.1	
43.5	43.3	0.2	
43.0	43.0	0.05	

TABLE 3.10

DIFFERENCE BETWEEN SURFACE TEMPERATURE AND THAT APPROXIMATELY 2/3MM BELOW BASE OF TUMOUR

Surface Temperature	Needle Temperature	Difference	Mean
45.0	43.3	1.7	
44.5	42.5	2.0	
44.2	42.3	1.9	
43.9	43.2	0.7	1.6
43.8	43.0	0.8	± 0.6
43.5	41.0	2.5	
43.1	41.5	1.6	
42.8	41.5	1.3	

TABLE 3.11

Author	Heating Technique	Position of Tumour	Temperature Distribution Across Tumour (°C)	Comment
Thrall et al (1973)	wb	mouse leg (a)	0.4	leg immersed, only one probe used. Rectal temperature not measured.
Robinson et al (1975)	wb	mouse thigh (a)	approximately 1	temperature gradient measured with >1 probe. Surface of tumour immersed
Cetas et al (1977)	wb wb	mouse thigh (a) mouse thigh (a)	0 1	whole leg immersed. Core temperature rose to ≈ tumour temperature. Only upper surface of tumour heated.
Bleehen et al (1977)	wb	mouse leg (a)	approximately 0.5	temperature gradient measured with > 1 probe (probes insulated from wb. Diameter of probes 0.8mm - large if > 2 used). Leg immersed. Rectal temperature rose to 39°C.
Hill and Denekamp (1978)	wb	mouse ventral thorax (a)	0.3	only 1 intra-tumour temperature probe. Upper surface of tumour only heated.
	wb	mouse ventral thorax (a)	0 - 0.1	tumour occluded.
Hill et al (1979)	wb	different sub-cutaneous tumours	1.0 - 1.7	cold spots adjacent to muscle (1 probe).
Robinson et al (1978)	wb	mouse leg	> 1.0	large temperature gradient near major blood vessels and near bone.
	μW	mouse leg	0.4	tumour heated in liquid bolus.
Mendecki et al (1976)	μW	C_3H tumour - various sites (a)	0.2	centre of tumour hotter. Core temperature rose by 5°C.
Dickson et al (1977)	RF	rat foot (a)	0	skin 5° lower than IT temperature. > 1 probe used. 100% cure rate.
	RF	rat leg (a)	2 - 3	muscle 2-3° higher than tumour. Rectal temperature rose to > 42° - rats died.
Marmor et al (1977)	RF	mouse flank (a)	< 0.5	uniform temperature distribution in tumour and no systemic hyperthermia.
Van Dijk and Breur (1979)	RF	leg and back mice (?a)	not quoted	RF gave IT temperature of 44° (50% cure rate).
	wb	leg and back mice (?a)	0.5	surface temperature of 44.5° (no cures).
Walker (this study)	dry heat (conduction)	mouse back (Na)	0.16	probes used to check gradient at different points in tumour.
	dry heat (conduction)	mouse back (Na)	0	tumour occluded

wb = waterbath, μW = microwave, RF = radiofrequency, a = anaesthetic used, Na = no anaesthetic used,

IT = intra-tumour.

4.0 REGRESSION, RECURRENCE AND CURE

4.1 Introduction

Attention is increasingly being focussed on methods of determining the number of cells allowed to remain *in situ* after therapy - since this corresponds to the clinical situation of human cancer treatment by any modality. Also because *in vivo - in vitro* estimates of survival are not always comparable (Denekamp, 1980; Wheldon, 1980) other methods of estimating tumour response are necessary. Three methods can be used.

4.1.1 Regression Patterns

After treatment of tumours by (non-curative) hyperthermia the tumour may shrink completely because of rapid removal of dead cells by macrophages. This tells us only that comparatively few viable cells remain, but regrowth is determined by these few cells and the expression of how many viable cells remain is only shown by how long it takes for the tumour to regrow. Regression patterns tell us only about the ability of the animal to remove dead cells. This does not eliminate the possibility that knowledge of regression patterns may be of prognostic value. This has been found to be the case in fractionated X-ray treatment since here degree of shrinkage reflects reoxygenation, making tumours more sensitive to subsequent treatments with X-rays (Denekamp, 1977).

4.1.2 Regrowth Delay

Growth or regrowth delay is the difference in time taken by treated or untreated tumours to reach a given end-point. In this study 10mm diameter has been chosen. It is widely used as an indicator of

effectiveness of therapy. In some cases attempts have been made to relate the delay time to clonogenic survival by extrapolating backwards (to time of treatment) the visible regrowth curve, and the ordinate is interpreted as a measure of the number of surviving clonogens. This only applies if the following assumptions are made, viz:

- there is clearance of doomed cells and debris by time of visible regrowth
- there is rapid onset of proliferation of surviving clonogens
- there is a fair degree of synchrony of onset of proliferation between surviving clonogens
- the regrowth pattern is known (see Wheldon, 1980).

The technique of growth delay is simple and can be used as a method of comparing the response of tumours to different treatments. In this study it has been used to compare the effect of X-rays and hyperthermia. This, however, makes use of the relationship between delay and cell killing and there are problems in assessing cell kill by this method because:

- if too small an end-point is used, e.g. just above the treatment size, this could give an incorrectly small delay in a situation where the clearance rate is slow and where there may be oedema
- if too large an end-point is used there is an artefactually large delay related to normal tissue damage, e.g. after X-rays.

There is a slow shrinkage after treatment of the C_3H tumour with X-rays as compared with after hyperthermia so a small end-point can only be chosen if regrowth occurs some time after complete shrinkage, which it does at the TCD_{37} level for both X-rays and hyperthermia, hence

the suitability of using this level of treatment for comparison in these studies. The TCD_{37} level of treatment is one which gives a 37% cure level. For X-rays it is defined as a dose of X-rays, and with hyperthermia as a particular time/temperature combination.

Denekamp (1979), in a review of tumour assay systems, listed the advantages and disadvantages of regrowth delay as an assay.

Advantages
- covers wide dose range, i.e. can be used after sub-curative therapy
- fairly rapid assay (30-50 days) - in this study 20-100 days
- easy to plan experiments
- has clinical relevance

Disadvantages
- tumours must be discrete, accessible and measurable - as is the case in this study
- cannot assess prolonged schedules

The modification of growth kinetics, which occurs for tumours recurring after sub-curative therapy with large doses of radiation, has been recognised for many years (Hewitt and Blake, 1968). According to Brunton and Wheldon (1980) there are two distinct models of tumour response to a single radiation dose. The first assumes that repopulation occurs at a reduced and dose-dependent rate, while the second assumes that surviving cells experience a dose dependent delay before onset of repopulation at the untreated rate. The work of Abdelaal, Wheldon and Clarke (1980) and Begg (1980) confirms that normal tissue damage, i.e. tumour bed effect, is primarily responsible for the slowing of development of tumours irradiated *in vivo*. This conclusion is also supported by the observation of at least a partial

recovery of growth rate as the tumour extends beyond the original irradiated area. Because of the tumour bed effect and because the rate of regrowth to a given size after treatment is usually slower than normal growth, a large end-point at which to assess delay incorporates an artefactually larger error than a small end-point. The end-point chosen was the best compromise taking all the factors relating to regression time and regrowth delay into account.

4.1.3 Local Control or Cure

Before this study began it was not known if cure (with acceptable normal tissue damage) would be possible using heat alone. Thrall, Gillette and Bauman (1973) had found a similar tumour to be resistant to heat (see Table 4.6). Because metastatic rates after different treatments were being compared it was realised that the analysis of results would be simpler if cure by hyperthermia alone could be established as it had been after X-rays. Because metastatic rate had been linked to cure rate (Sheldon et al, 1974) it was hoped that a dose-dependent cure rate for hyperthermia could be found. There are several assumptions to be made in a cure analysis, viz:

- the tumour can regrow from a single cell
- a single species of clonogenic cell dominates cure
- cure dominating clonogens are uniformly sensitive
- there is the same number of dominant clonogens, i.e. tumours should be all the same volume (within 10%).

These are the normal assumptions made in a cure analysis after X-ray treatment (Wheldon, 1980) and are probably also applicable to cure after hyperthermia. Denekamp (1979) also compared the advantages and disadvantages of this assay.

Advantages - good resolution

- seems clinically relevant

Disadvantages - slow (90-180 days) - up to 100 days in this study

- expensive
- metastases may prevent its use
- restricted to high total doses.

In this chapter regrowth delay has been used as an assay of number of tumour cells damaged. This has been compared with the expected 'normal' growth rate of known numbers of tumour cells inoculated sub-cutaneously as has been described in Chapter 2 in order to give some estimate of cell damage for different levels of treatment.

4.2 Methods

4.2.1 Protocol for Hyperthermia Experiments

Tumours which were mobile and free of any skin or muscle involvement were selected at size 6.5 ± 0.5mm diameter. All tumours used had grown at the normal rate (see 2.3.2). The surrounding area of skin was clipped when the tumour measured 3mm diameter.

Mice were introduced into the contraining jig with tumour retracted as is shown in Figure 3.1. The rectal probe, dipped in liquid paraffin, was inserted up to the level of the pyrolic sphyncter. In this way rectal temperature closely approximated to core temperature and changes in this temperature very closely followed applied surface temperature changes. The probe was taped to the tail which was held outside the jig. A collar of suitable size was placed round the base of the tumour. Contact jelly was put on the tumour and allowed to penetrate inside the collar. A surface probe was placed on the tumour and both were covered with the perspex heat applicator (see Figure 3.8).

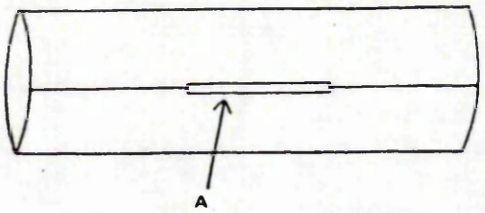


Figure 4.1

Irradiation jig. A - slit for tumour support

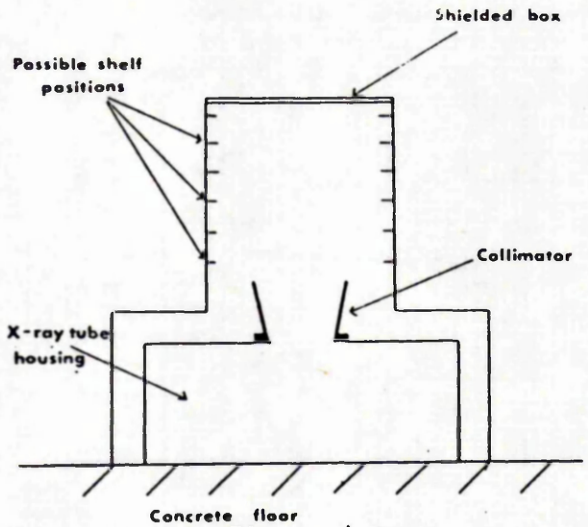


Figure 4.2

Schematic diagram of irradiation chamber

Figure 4.3

Photograph of radiation chamber showing mice in lead jigs in position for irradiation

For 'fast hyperthermia' the heat applicator was pre-heated using the phantom 'tumour'/mouse tumour calibration to obtain 44.2°C for surface tumour temperature. This surface temperature was approximately equivalent to an intra-tumour temperature of 44°C. The heating device was then placed over the tumour and a 'heat dose' of 44°C for 60 minutes was delivered to the tumour. It took approximately 2 minutes to reach the required temperature.

For 'slow hyperthermia' the heater was switched on and the heat applicator placed over the tumour. The temperature of the applicator rose to 43°C in approximately 10 minutes. At 43°C the time-clock was started and heating lasted for 60 minutes. It took another 60 seconds to reach the treatment temperature. This gave an approximately equivalent heat dose to that given in 'fast hyperthermia'.

After treatment (with either hyperthermia or X-rays) the first few days of regrowth were usually masked by oedema or scab, so normally tumours were only recorded from size 4mm diameter. Measurements were thereafter recorded daily until the tumour measured 10mm diameter. The animals were then sacrificed.

4.2.2 Protocol for X-Ray Experiments

A 2mm thick lead jig (Figure 4.1) was used for tumour irradiation and this was compatible, as far as constraining the animal and supporting the tumour, with the hyperthermia jig, while shielding the animal during irradiation. The tumour was retracted through a 1½mm wide slit for irradiation. While every effort was made to minimise constriction of blood flow (Abdelaal, 1979) it was felt that the design of the collar gave less distortion of tissue round the tumour, while still supporting it clear of the body by an equivalent distance.

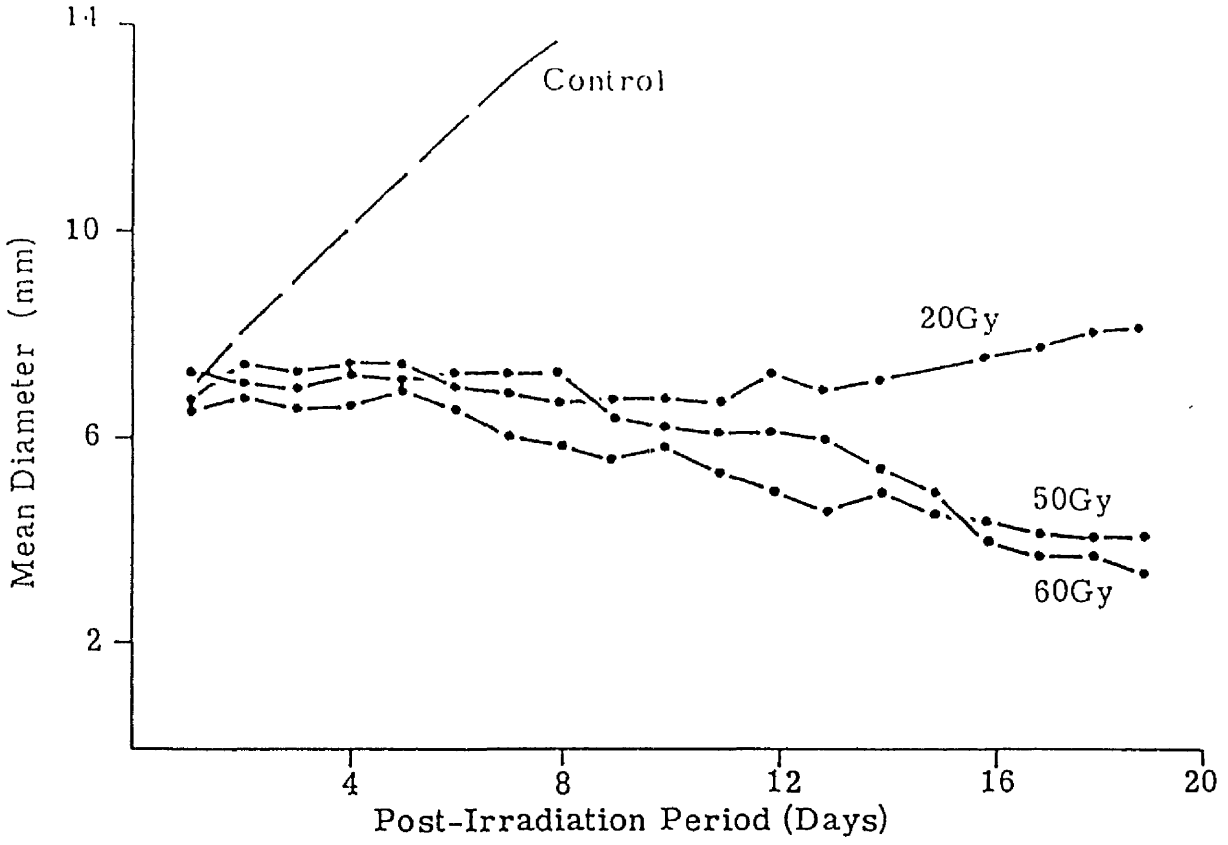


Figure 4.5

Composite regression patterns after single X-ray doses
After Abdelaal (1979)

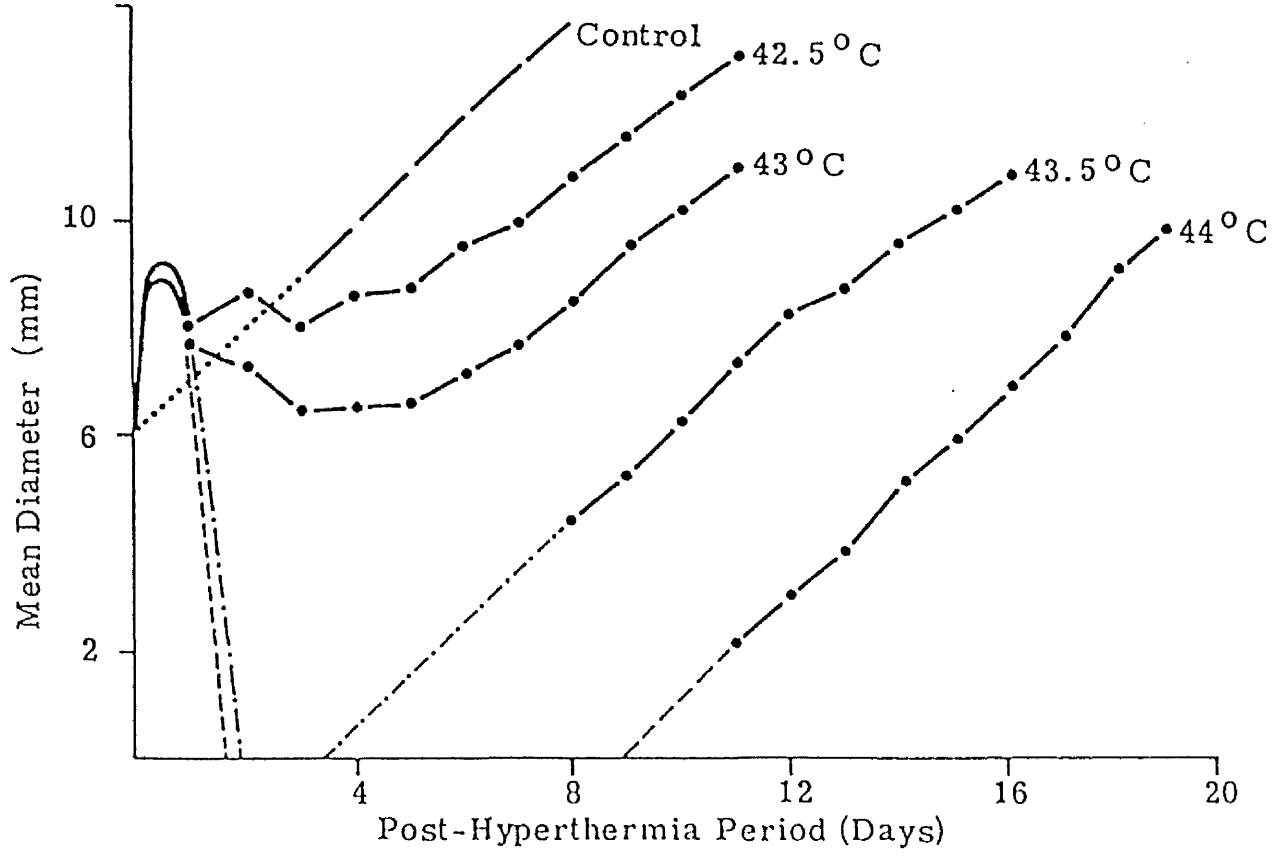


Figure 4.4

Typical regression and regrowth patterns after hyperthermia
given for 60 minutes

X-rays were delivered from a Siemens Stabilipan I unit, operating at 250kV and a filament current of 15mA, with an HVL of 1.8mm copper. The dose rate was 100 rad/minute. The X-ray tube was positioned at the base of a shielded box (Figures 4.2 and 4.3). Mice were carefully positioned to give an identical dose to all tumours. Because of the position of the tumour mice were lying on their sides during irradiation. All the dose rate measurements were made using a Farmer-Baldwin dosemeter which had been calibrated against a standard instrument. The dose rate was checked at weekly intervals. The dose profiles within the lead jig and the centre of the tumour were determined using a LiF thermo-luminescent dosemeter. Sachets of LiF - rods were placed within the jig which was filled with tissue equivalent material (standard bolus - $MgSO_4$/sucrose spheres) and in the centre of a tumour phantom made from the same material enclosed in a thin rubber covering. The dose determined by TLD dosimetry was in good agreement with the ionisation chamber measurement. The absorbed dose to the shielded parts of the mouse were found to be approximately 1% of the tumour dose (A M Perry, pers.comm.).

4.3 Results of Treatment

4.3.1 After Hyperthermia ('Fast' and 'Slow' were considered together)

4.3.1.1 Regression, recurrence and cure

Figure 4.4 shows typical regression and regrowth patterns after hyperthermia. The pattern of regression after a heat dose near to the TCD_{37} level (43°C for 1 hour is the nearest for hyperthermia) is given for comparison with that after X-rays (Figure 4.5). All regression patterns after curative levels of treatment were similar. The tumour first became oedematous (Table 4.1), the degree of which was dependent

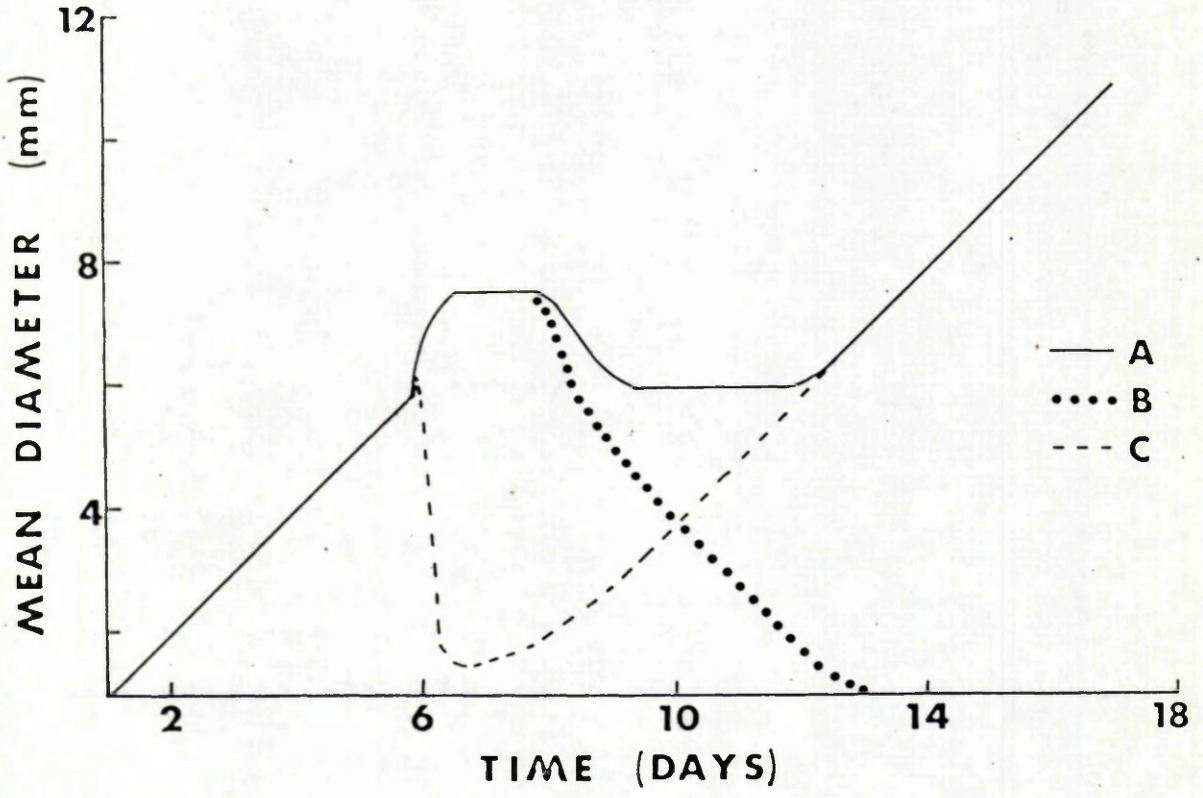


Figure 4.6

Effect of hyperthermia (42.5°/60 minutes) on tumour volume. Hyperthermia delivered at time when tumour reached 6mm diameter. A - regression and regrowth, B - clearance of dead cells (hypothetical curve), C - viable tumour cell component.

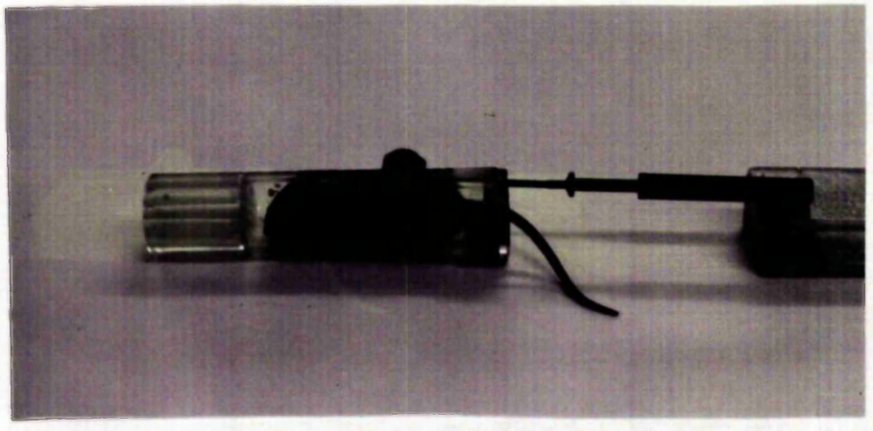


Figure 4.7

Ligature device

on the level of the heat dose. Tumours then shrank within one to two days. After sub-curative regimes the tumour continued to grow with no apparent change in growth rate. Near curative temperatures there was a slight delay, then normal growth continued. Figure 4.6 is a hypothetical regression and regrowth pattern after near-curative hyperthermia.

4.3.1.2 Cure rate versus time/temperature

Cure rate for (normoxic) tumours heated for one hour at different temperatures (temperature ≡ intra-tumour temperature) is shown in Table 4.2. For those tumours which were not cured regrowth time after treatment is given.

To evaluate the effects of occlusion of the blood supply to the tumours on cure rate, tumours were ligated, using a spring-loaded device (Figure 4.7). This was positioned round the tumour base and the tension adjusted by altering the load on the spring. Tumours were ligated for one hour before then during treatment.

Cure rates for normoxic and hypoxic tumours, heated for an hour, are shown in Table 4.3. From the results it would appear that hypoxia increases the incidence of cures to approximately the same extent as raising the temperature by 0.5°C. It is possible that with larger numbers in the hypoxic group the difference between the two groups might have been more significant, as has been found by others (Hill and Denekamp, 1978).

Since the tumour used in this study was already approximately 30% hypoxic (see 2.3.1) it was not surprising that clamping had no very marked effect. From temperature measurements (Table 3.8) within

ligatured tumours it can be seen that the intra-tumour temperature was approximately 0.1 - 0.2°C higher in ligatured tumours than in normoxic tumours during applied heat. This is not enough to explain the higher level of tumour cure found.

Control tumours were similarly constricted for 2 hours without heating. This appeared to have no marked effect on the growth rate. Time for tumours to grow from 6mm, when they were ligated, to 10mm was:

5 ± 1.1 days (25 mice) - ligated tumours

5.4 ± 1.3 days (20 mice) - normal growth rate

4.3.2 After X-Rays

4.3.2.1 Regression, recurrence and cure

Figure 4.5 shows composite regression patterns after single doses of X-rays. 60Gy is the treatment dose nearest to the TCD_{37} level. Tumour regression was only evident after single doses of 50Gy. Regression patterns above 50Gy were very similar, whether tumours were cured or not, since damage to the vascular system makes clearance of dead cells and debris difficult above this level (Abdelaal, 1979). At low sub-curative levels of treatment the tumour continued to grow, sometimes after a delay, at the control growth rate. After curative regimes the tumour became slightly oedematous for several days. Complete tumour shrinkage did not take place for approximately 2 weeks.

4.3.2.2 Cure rate versus dose

Cure rate for irradiated tumours is given in Table 4.4. For those tumours which were not cured regrowth times are given. Cure rate for 'clamped' and 'unclamped' tumours did not differ significantly in their response to radiation (Abdelaal, 1979). This might lead one to suspect

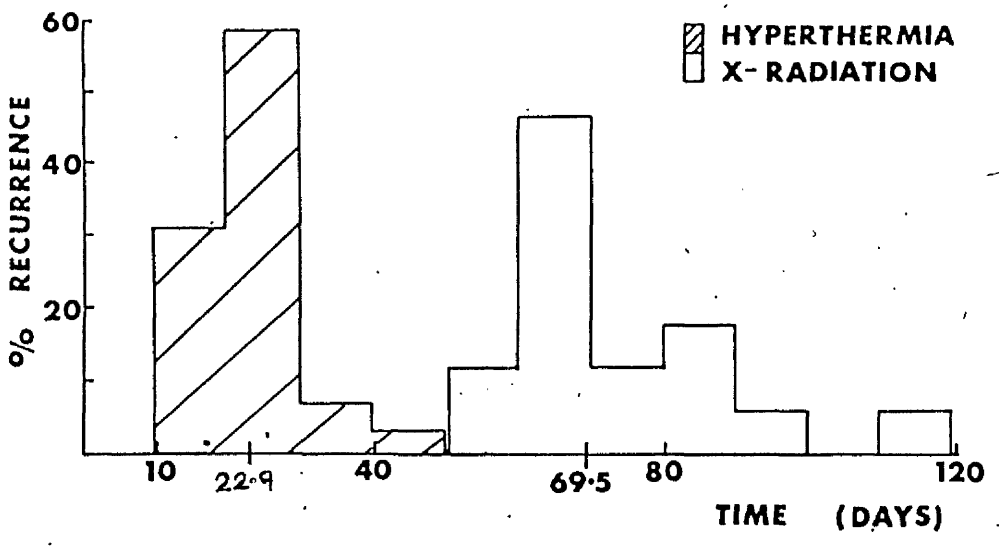


Figure 4.8

Distribution of growth delay time for tumours recurrent after X-irradiation or hyperthermia

that the tumours may not have been very effectively clamped. The tumour however is atleast 30% necrotic so the hypoxic status even of the 'unclamped' tumour would be quite high. This is in accordance with the fact that a high dose is required to cure this tumour.

4.3.3 Comparison of Results of Heat and X-Rays

Regrowth delay time has been compared in these studies at a near iso-cure level (i.e. the nearest to the TCD_{37} level for heat and for X-rays). Regrowth delay is here defined as the time taken from treatment for recurrent tumours to grow to 10mm diameter. These data are presented in Table 4.5 and Figure 4.8. The mean time for tumours to regrow to 10mm was 18.2 ± 6.7 days after hyperthermia and 69.5 ± 22.8 days after X-rays. Regrowth after hyperthermia at an iso-cure level is therefore much faster than after radiotherapy.

4.4 Discussion

4.4.1 Advantages of Not Using Anaesthetics

Mortality related to experimental procedures is not often quoted. In hyperthermia treatments there are several common causes of death. One is due to a rise in core temperature to above 42°C (Dickson, Calderwood and Jasiewicz 1977). Another cause is by infection of the tumour subsequent to treatment. Tumours sited on the back are less liable to infection than tumours in some other sites (Hewitt, pers. comm.). A third is related to the use of anaesthetics (Honess, pers. comm.; Wheldon, pers.comm.; Overgaard and Overgaard, 1972). In this study where over 1,000 mice have been treated with hyperthermia there was no treatment-related mortality.

Anaesthetics are widely used in animal experiments. In some experiments

they are essential. In others they complicate the interpretation of results to such a degree that modification of the experimental technique to obviate the necessity for anaesthesia may give more meaningful results.

The administration of anaesthetics has been reported to alter body core temperature (Johnson, Fowler and Zanelli, 1976). Some anaesthetics have the effect of depressing the rate and depth of breathing and, consequently, may reduce the oxygen tension in the tissues of the animal (Hornsey, Myers and Andreozzi, 1977) which would certainly affect cure rate after X-ray treatment. Barbiturates, which have been used routinely as a safe anaesthetic in animal experiments, are known to lower the blood pressure in man - though no documentation could be found on its physiological effect on mouse blood pressure and core temperature. Nembutal and urethane affect mouse blood pressure and tumour blood flow - nembutal causing a 5°C drop in both core and tumour temperature in 20 minutes. Urethane induced only a small drop in temperature. It initially caused a fall in blood pressure, followed by recovery, while nembutal caused a steady fall in blood pressure. The effect of nembutal on blood flow suggests that the lower blood pressure would decrease tumour perfusion, however changes in blood pressure could affect normal tissue to an even greater degree, since vessels in tumours lack elasticity and may not be as sensitive to pressure changes. Johnson (1978) has suggested that in mice total blood flow increases when the temperature rises from 38-41° and then may decrease. The initial increase is probably due to the opening of shunts and vaso-dilation from relaxation of arterial vessels and precapillary sphincters, controlled by the auto-regulatory system. Preliminary results from Johnson suggest that temperatures near 41°C

do not decrease tumour blood flow but what happens above that temperature is not clear. Thus the use of anaesthetics complicates the experimental procedure since allowance must be made to measure blood flow. On the whole the use of anaesthetics is, if possible, best avoided.

4.4.2 Cure Results After Different Methods of Heating C3H Tumours in Relation to Normal Tissue Damage

Different techniques of heating various small animal tumours have been already compared (see 3.3.3). A better assessment is obtained however if cure results after treating the same tumour are compared. Table 4.6 gives this for the C_3H tumour and some assessment of the results of treatment is given under 'Comment'.

When Overgaard and Overgaard (1972) treated a multi-generation tumour with radiofrequency heat they obtained a lower cure rate for an equivalent level of treatment than that found in the present study. Thus the present technique seems preferable. Theoretical calculations (see 1.3.2) do suggest however than one of the advantages of radio-frequency heating is in skin-sparing. This was found by Overgaard and Overgaard who could probably have used a higher heat dose and still spared the normal tissue while perhaps obtaining a higher cure rate. Van Dijk and Breur (1979), in treating multi-transplant tumours, sited on the backs and legs of mice, obtained a 50% cure rate at 44°C with radiofrequency heating (cf 65 ± 8% in this study). No cures were obtained with waterbath heating. Thus radiofrequency heating gave results perhaps slightly less good than those in this study while waterbath heating was much poorer - using an equivalent tumour system. This was especially bad as in the study by van Dijk and Breur, as also in that by Thrall,

Gillette and Bauman (1973) (cf also in the similar study by Crile, 1963) the normal tissue reaction at heat doses equivalent to those used in the present study gave rise to loss of leg or foot. There exists the possibility that any cures observed may only have been as a result of amputation rather than cell killing. Crile's results, for example, suggest a curative level of treatment as one where 50% or more of the mice lost their feet.

Thrall et al (1973) and Gillette and Ensley (1979) treated C_3H tumours, situated on mouse legs, using waterbath heating. The tumour used was a 3rd generation tumour, which is better vascularised than the present tumour so one might have expected a rather lower cure level because of heat loss. No cures at all were obtained however, even using a surface temperature of 44.5°C for 1 hour, which was supposed to be equivalent to 44.1°C intra-tumour temperature. This heat 'dose' gave a better than 60% cure rate in the present study. It is possible that the temperature distribution in the tumour treated by Thrall et al was affected by the large blood vessels in the leg (see Robinson et al, 1978). Even assuming this, one might have expected to obtain some cures with the heat dose used. In addition approximately 6-7% of all mice treated died within 24 hours of treatment. Reason for death is not given and no core temperatures were quoted in these studies, so death could have been caused by excessive normal tissue damage or by systemic effects of elevated core temperature.

It is of interest that the best results obtained have been with microwave, about which there are such conflicting reports. Mendecki et al (1976) obtained 100% cure rate with intra-tumour temperatures of 43.5°C and with skin sparing. This result is particularly interesting

as the tumours were implanted in the leg - a site which normally gives poor thermal distributions. The only problem associated with this particular treatment was the high core temperature, and it was considered that this was best avoided as has been discussed.

In considering the alternative forms of heating systems to use in the present study the results shown in Table 4.6 were considered along with the theoretical calculations by Strang and Patterson (1980)(see Chapter 3). These confirmed that waterbath heating was less satisfactory than radiofrequency or microwave heating, and that a form of conduction heating would be appropriate for the type of tumour to be treated.

In the present study it is of interest that after hyperthermia treatments, at temperatures of $\leq$ 45°C for 1 or 2 hours, healing appeared to follow the same time course, whatever the heat dose. If the heat dose was curative a dry scab formed over the treated area within 3-4 days and this remained until healing under the scab was complete. Thus cure took place at a heat dose that was below normal tissue tolerance since healing always accompanied cure. When the tumour was not controlled the scab became moist round the area of regrowth, which thus appeared to interfere with the process of healing. The time for healing to take place after treatment was the same regardless of the hypoxic status of the tumour treated.

Tumours treated for 2 hours - 18 ± 6.7 days

Tumours treated for 1 hour - 19 ± 6.8 days

Ligatured tumours treated for 1 hour - 18 ± 5.9 days

This time may be related to the maturation period of epithelial cells from the basal cells which would be stimulated to replace the damaged

skin cells.

In the clinical situation skin healing takes place at different rates in different areas of the body. Generally epithelial cells from round the wound migrate quickly across the wound to give a quick cover of normal epithelial cells. The dermis never completely recovers and is replaced more slowly by fibrous wound tissue. The timescale for this depends on the extent of the wound and so the similar timescale for the different treatments may reflect the physical size of the damaged area.

In studies on the effect of wet heat on tumours no comparable data on healing time has been found so results of this study cannot be directly compared, however it does appear that from the studies of Thrall et al and others that wet heat is more directly damaging to skin than is dry heat. This result has also been found by Hopewell (pers.comm.) in studies on pig skin.

The pessimistic view of the value of heat as an adjunct to radiotherapy taken by Stewart and Denekamp (1977), Field and Law (1978) and Gillette and Ensley (1979) may be, to some degree, because in these studies on combined treatment 'wet heat' was used, thus giving more normal tissue damage than might have been observed using other heating techniques. Also the effective tumour temperature could have been overstated because of perhaps unrecognised thermal gradients within the tumour (cf with waterbath heating by van Dijk and Breur, 1979) or because of slowly equilibrating intra-tumour temperatures. Time to reach thermal equilibrium is not always quoted and must be taken into account when stating a heat dose.

4.4.3 Use of the Results as a Measure of Tumour Response

4.4.3.1 Local control or cure

Local control or cure has been taken in these studies as no local recurrence after 100 days. This is a reasonable length of time owing to the rapid growth and regrowth after treatment of this tumour. Only one tumour out of several hundred treated by X-rays (at all doses up to 85Gy) did recur after 100 days, and after hyperthermia, only one recurred later than 40 days after treatment (for all different time and temperature combinations up to 46° for 60 minutes). One problem of the use of cure as an end-point is that metastases can end the normal life-span of the host before local cure can be assessed. This is unlikely with hyperthermia since local recurrence was prompt, and indeed any mice with metastases were always investigated for local recurrence. Because of the rapidity of local recurrence this could always be determined, so cure as an end-point is a suitable index of tumour response after hyperthermia.

4.4.3.2 Growth delay and cell kill following hyperthermia

From the results shown in Figure 4.8 the growth delay distributions are very different for the two modalities. X-radiation and hyperthermia, at similar treatment levels, have identical effects in terms of cure, but grossly unequal effects as judged by regrowth delay, therefore comparison of growth delay after X-rays and hyperthermia cannot provide a direct measure of cell kill by these two modalities. After radiotherapy the tumour bed and its associated vascular tissues are normally damaged when the dose given has been in the curative range.

Hyperthermia appears to cause no equivalent effect to this 'tumour bed effect' (TBE). Pre-transplantation hyperthermia on C_3H normal skin

gave no retardation of tumour growth, whereas the concurrent experiment on pre-transplantation irradiation produced marked retardation of tumour growth (Urano and Cunningham, 1980). Abdelaal (1979) reported also altered growth kinetics after X-rays of the C_3H tumour used in this study Growth delay after hyperthermia however is so markedly different from that observed after X-rays, that the difference cannot be due entirely to the TBE or the slight alteration in growth kinetics after X-rays.

One interpretation of this discrepancy could be in terms of a differential rate of repopulation (or onset of repopulation) of surviving clonogenic cells following treatment. Because of vascular cooling, nutritional status, etc, the cells surviving hyperthermia treatment are probably oxic cells which are capable of prompt division: also since damage to the vasculature is minimal the surrounding tissue with its framework of connective and vascular tissues is in a position to react, as any normal tissue would, to heat injury. Part of the cellular response to increased temperature is to give a reaction similar to any inflammatory condition, which results in stimulation of a fibrinous exudate. This could have a similar effect to lethally irradiated cells in promoting an increased rate of repopulation of the surviving tumour cells. After X-rays the surviving cells are probably hypoxic, awaiting re-oxygenation.

There exist, however, some alternative explanations for the rapid regrowth after hyperthermia. Such an effect can, in principle, result from poor control of intra-tumour temperature (akin to poor radiation distribution in clinical radiotherapy) but it is then very surprising that only a single tumour that regrew exceeded the single-cell growth time for untreated cells. In any case, the control of intra-tumour

temperature achieved in this study is no worse than that achieved by other workers (Table 3.11) suggesting that if non-uniformity of temperature be responsible for this effect, then the effect may be quite generally encountered in hyperthermia research.

A more speculative explanation for the observed discrepancy could be that tumour cure by hyperthermia conforms to a 'cooperative' process whereby either all cells are killed or substantial numbers survive. This could be related to the degree of oedema. Initially, after treatment, there was considerable oedema (Table 4.1) so cell kill may have been accelerated by the oedema, inflammation and resultant toxins released as a by-product of this.

Whatever the explanation, a deeper understanding of tumour response *in situ* is required before regrowth delay as an end-point of tumour response can be confidently accepted as providing a valid measure of the magnitude of tumour cell kill achieved by alternative modalities.

Other curative regimes appear to have had a different mechanism of cell kill.

Calderwood and Dickson (1980) have suggested that cells pass through several mitoses after hyperthermia, as they do following X-ray therapy, before expressing lethal damage. With their heating technique (42°C surface temperature for 2 hours) which was curative for the Yoshida sarcoma implanted on rat feet, proliferating cells were found 6 days after treatment. However, using the present system, in which tumours were heated to temperatures of 42-46°C for 1 hour, they shrank within 48 hours of treatment in curative regimes, following the oedema. Where no oedema occurred (at sub-curative temperatures) there was little

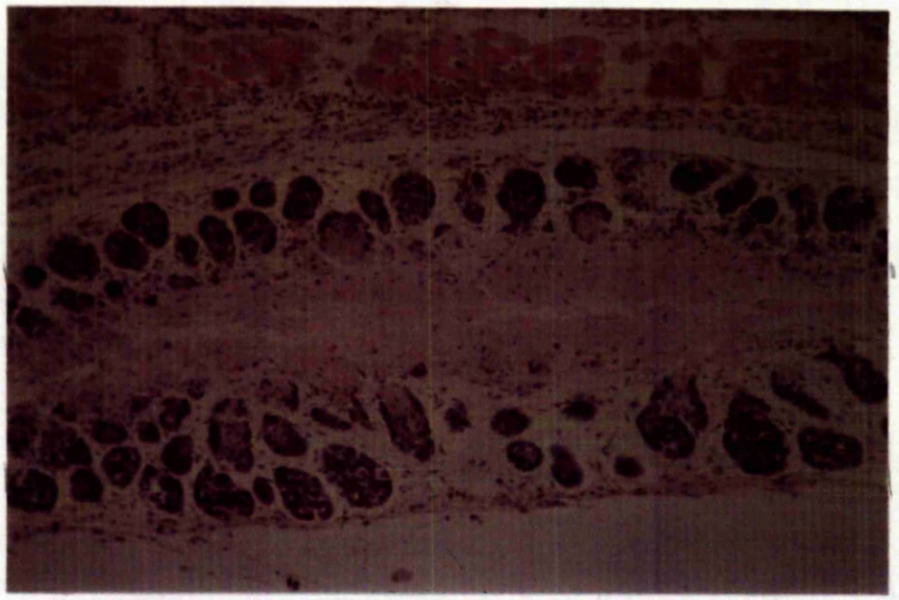


Figure 4.9

Photomicrograph of a just palpable tumour

(reproduced from x100)

alteration in growth rate and no cure.

Regrowth after hyperthermia took place within a similar time span to that which might have been anticipated from the results of the dilution assay studies if one considers that regrowth has taken place from only a few remaining cells surrounded by dead and dying cells (the equivalent of the LI cells in the assay). These studies have shown a 'stationary phase' during early stages in tumour growth, which appears to have been related to inoculum size, but which is also associated with a variable time lag before vascularisation took place, as has been postulated by others (Folkman, 1974). From examination of the histology of very small tumours during this 'stationary phase' it appeared that these tumours arose from a ring of foci of rapidly dividing cells which soon had a necrotic centre (see Figure 4.9). It has been hypothesised that necrosis stimulates initiation of vascularity (see 2.4.3) and after hyperthermia there is much necrosis. Thus the stimulation for vascularity would take place very quickly after hyperthermia. There may be no delay in growth similar to a 'stationary phase' during regrowth after treatment with hyperthermia.

All this would indicate accelerated regrowth after inadequate therapy. Thus in the clinical situation hyperthermia must be used with great care.

TABLE 4.1

TUMOUR DIAMETER (IN MM) AFTER HYPERTHERMIA
44°C/60 MINUTES (SEPARATE ANIMALS)

Before Treatment	Immediately After	2 Hours Later	4 Hours Later	8 Hours Later	24 Hours Later
7	7.5	8	9	9	~3
7	7.5	8	8	8	0
7	7.5	9	9	9	~4
6	8	9	8	8	0
6	7.5	8	9	9	~2
6	8	8	8	8	~3
6	8	9	9	9	0

TABLE 4.2

TEMPERATURE CURE STATISTICS FOR TUMOURS HEATED FOR ONE HOUR

Temperature °C	Total Number of Tumours Treated	% Cure	Days to Regrow to 10mm
42	13	0	7 ± 2
42.5	21	0	9 ± 1.6
43	40	32	14.5 ± 3
43.5	64	42	18 ± 5
44	309	65	19 ± 5
44.5	31	65	20 ± 5
45	169	85	20 ± 3
45.5	8	88	25

TCD_{37} Level = 43.3°C

TABLE 4.3

Temperature °C	Hypoxic Tumours		Normoxic Tumours	
	No Treated	% Cure	No Treated	% Cure
41	4	0	10	0
41.5	4	0	10	0
42	4	25	13	0
42.5	17	35	21	0
43	10	40	40	32
43.5	9	55	64	42
44	6	83	309	65
44.5	9	88	31	65
45	6	100	169	85
45.5	4	100	8	88

TABLE 4.4

DOSE CURE STATISTICS FOR X-IRRADIATED TUMOURS
(ABDELAAL, 1979)

Dose	Total Number of Tumours Treated	% Cure	Days to Regrow to 10mm
50Gy	63	0	38.5±3
55	15	6	42.2±4.4
60	32	22	66.5±3.8
65	32	44	75.8±5.3
70	34	71	87.4±12.4
75	24	79	-
80	10	100	-

TCD_{37} Level = 63.5Gy

TABLE 4.5

STATISTICS OF GROWTH DELAY FOR TUMOURS RECURRENT AFTER X-IRRADIATION AND HYPERTHERMIA AT OR NEAR THE TCD_{37} TREATMENT LEVEL

	X-Radiation	Hyperthermia
TCD_{37} treatment level	63.5Gy	43.3°C for 1 hour
Treatment group closest to TCD_{37} level	65Gy	43.5°C for 1 hour
Number of tumours recurrent in closest treatment group	18/32	38/64
Mean growth delay	69.5 days	18.2 days
Standard deviation of growth delay	22.8 days	6.7 days
Absolute range of growth delay	50-120 days	10-49 days

Note

As clamped and unclamped tumours did not differ significantly in their response to irradiation (Abdelaal, 1979) they are considered together. Only unclamped tumours are included in the hyperthermia results.

TABLE 4.6

COMPARISON OF CURE RESULTS BY LOCAL HEATING OF C_3H MAMMARY TUMOURS

Author	Technique	Heat Dose (°C)	Position of Tumour	% Cure	Comment
Overgaard and Overgaard (1972)	radiofrequency	41.5°/4 hours 43°/45 minutes 43.5°/1 hour	flank	40 20 26	sites are reasonably comparable as far as vascularity is concerned. Treated tumours examined histologically - some effect seen at 41°C/1 hour
Walker (this study)	conduction	43.5°/1 hour 44.1°/1 hour	back	42 60	though no cures found below 41.5°/3 hours.
Van Dijk and Breur (1979)	radiofrequency	44°/1 hour (intra-tumour)	back and leg	50	waterbath gave poorer heat penetration (see 3.3.3).
	waterbath	44.5°/1 hour (surface)	back and leg	0	
Thrall et al (1973)	waterbath	44.1°/1 hour	leg	0	used better vascularised 3rd generation tumours. Also in leg large vessels act as cooling pipes giving cold spots (Robinson et al, 1978).
Mendecki et al (1976)	microwave	43°/1 hour (surface) 43.5°/1 hour (intra-tumour)	leg	100	only person to obtain 100% cure.

5.0 COMPARISON OF METASTATIC RATES AFTER DIFFERENT TREATMENTS

5.1 Introduction

5.1.1 Cellular Mechanisms Related to Metastatic Spread

The work of Fidler, Gersten and Hart (1978) suggests that a neoplasm may consist of heterogeneous populations of cells with different capabilities of invasion and metastases. Only some of them may be capable of invasion, and of these even fewer may survive the hostile environment of the bloodstream to be arrested in small vessels, as in the lung, extravasate into the parenchyma, escape host defence mechanisms, and finally grow into metastases. Metastases may come about by several mechanisms; some of these may be deduced from histology, or from cell surface changes that can be seen in neoplastic transformed cells cultured *in vivo* (Poste and Fidler, 1980), or by following patterns of circulation of labelled tumour cells (Glaves and Weiss, 1976).

Sugarbaker and Ketcham (1977) suggested that gaps in the endothelium caused by the rapid turnover and shedding of endothelial cells could facilitate tumour cell migration out of the capillaries. Intercellular junctions of endothelium may open up during cell division (Sherwin, 1976) also tumour associated vessels are defective and therefore perhaps susceptible to penetration. Tumour cells with increased deformability (Sato and Suzuki, 1976) can take advantage of small gaps in blood vessel endothelium and through increased motility pass through vessel walls (Wood, Baker and Marzocchi, 1967). The adhesion of tumour cells to endothelium may lead to damage to the vessel wall, perhaps associated with release of proteolytic enzymes, such as collagenase, from the tumour cells, or the aggregation of

platelets at the site of lodgement may release mediators that may contribute to vascular spasm, thus inducing permeability and motility of tumour cells (Gasic et al, 1973).

There appear to be major biochemical differences and different enzyme surface properties between tumour cell lines that metastasise readily and those that do not (Fidler, 1973a). These may be related to tumour cell surface charges, level of surface enzymes, level of fibrin surrounding the cell, host blood clotting mechanisms and endothelial wall properties (Fidler, 1976).

The malignant properties of tumour cells have been attributed in part to their reduced adhesiveness. Coman (1944,1953) showed that cells could be detached more easily from certain carcinomas than from corresponding normal tissues, and suggested that the mutual adhesiveness of malignant cells in general was in some way defective, hence their tendency to metastasise. The view of Coman has been questioned because some of the controls in his experimental procedure may not have been adequate. However the rounding up of mitotic cells in culture, and their decreased attachment to other cells is a well known fact, so because there are always cells in mitosis in tumours, this could be one of the factors that facilitate shedding.

As the primary tumour mass grows in size it exerts mechanical pressure on the surrounding normal tissue. Eaves (1973) proposed that the rapidly proliferating tumour cells, possessing a higher intracellular osmotic pressure than the normal cell, force their way along lines of weakness in host tissues. Spreading by infiltration along lines of weakness may not be completely different from metastatic spread. A

higher osmotic pressure may also facilitate shedding of tumour cells. Similarly one may find isolated tumour cells near tumours which are frequently shown by serial sections to have no continuity with the neighbouring tumour (Strauli and Weiss, 1977). This appears not to be the case with the C_3H tumour, since surgical excision resulted in so few regrowths, so from this it would appear that even a pseudo-capsule would make surgery a treatment of choice.

The final growth of secondary tumours represents the end point of many destructive events from which few tumour cells probably survive. The presence of tumour cells in the blood is in itself no positive indication of distant metastases, however from the results of a lung colony assay where different numbers of tumour cells were injected intravenously (see 5.2.2) it appears that the metastasising potential is directly related to the numbers of tumour cells circulating at any one time. It has been found, however, that large tumour emboli are more effective at implantation than smaller ones, when given by intravenous injection (Fidler, 1973b). Thompson (1974) found that single cells from spontaneous animal tumours did not give rise to lung metastases, however aggregates did in proportion to the size of the aggregates. It is of interest to note, in this work by Thompson, that single cells from passaged tumours did give rise to lung colonies. This may merely be an indicator of the higher proportion of clonogenic cells in transplanted tumours, or may indicate also a change in metastases-forming ability during transplantation, perhaps because of changes in cell surface properties.

Tumour cells often enter the lymphatic system and travel to regional lymph nodes. This is the most common route of dissemination of

carcinomas, while sarcomas tend to spread via the blood stream. This is the reverse of the position in the present tumour which shows blood-borne spread (see 5.2.3). Cells are not always trapped by the first obstacle met. Various factors can aid in the arrest of tumour cells.

Many tumour cells are highly thromboplastic and elicit fibrin formation (Chew, Josephson and Wallace, 1976). This coating of fibrin may alter their eventual arrest in the capillaries (Warren, 1973). Active attachment of tumour cells to the vascular endothelium must thus be distinguished from passive lodgement. The attachment is probably facilitated by several factors. The relationship of fibrin and its effect on arrest and survival has been extensively studied and fibrin appears to facilitate lodgement since anti-coagulants administered before IV injection of tumour cells can reduce the incidence of experimental metastases. The effect of these anti-coagulants is to reduce the level of fibrin. Warfarin has been used on C3H mice implanted with KHT sarcoma which metastasises spontaneously to the lung. There is evidence that this anti-coagulant reduces the number of lung deposits by acting on the host's clotting mechanism (Brown, 1973).

An association between the growth, metastasising potential of tumours and the formation of fibrin has been the subject of many publications. O'Meara (1958) suggested that growth of a tumour is conditional on the prior formation of a fibrin lattice in the adjacent tumour bed, and that this formation is maintained by the tumour cells themselves. Fibrin formation may be stimulated by treatment of tumours with radiotherapy or hyperthermia (see 5.1.2).

Another factor which may be involved in metastatic spread is the tumour angiogenesis factor (TAF). It is responsible for tumour growth after a solid tumour reaches a size, where further growth is limited by absorption of oxygen and nutrients and the disposal of wastes. This limitation is overcome by connection of the neoplasm to a bed of capillary blood vessels. These do not originate in the tumour but are elicited from the host by the apparently diffusible TAF (Folkman, 1974). This has been extracted from a variety of tumours by Gimbrone et al (1972). These workers deliberately caused the arrest at a small size of a homologous solid tumour, by preventing neovascularisation. This was observed by implanting tumours in the vascular iris and in the avascular anterior chamber of the eye of a rabbit. Only neoplastic cells were able to elicit the stimulation of vascularisation which could be observed if the implant of neoplastic cells was placed within 2mm of the iris. Thus the TAF was able to diffuse over 2mm to cause the vascularisation.

Coman (1944,1953) showed that a loss of adhesion that has been found in neoplastic cells was accompanied by an increase in 'stickiness',i.e. tendency to adhere to other types of cells, which was found to be organ specific. Organ distribution in metastases shows that tumour cells play an important role in determining their ultimate fate. Organ specificity has been shown in experiments where pieces of lung and other organs have been implanted, subcutaneously, and tumour cells normally arrested in the lung did lodge preferentially in this lung implant (Sugarbaker, Cohen and Ketcham, 1971). The present tumour has also shown some organ specificity when it metastasised after treatment (see 5.2.3).

5.1.2 *Investigations into Treatment Techniques Which Might Stimulate Metastatic Spread*

Promotion of metastases by radiotherapy has been investigated in various animal studies. Hewitt and Blake (1977) found that pre-irradiation of recipient mice with sub-lethal whole-body irradiation gave increased metastases in regional lymph nodes and lungs and other systemic metastases. From tests carried out by Hewitt and Blake this enhancement was considered unlikely to be caused by immunosuppression. The tumour used was non-immunogenic and the effect persisted long after the expected time of recovery of immune reactivity. Hewitt and Blake gave good reasons to suggest that the result may relate to stored lethal radiation damage to migrating cells of slow turn-over tissues perhaps giving rise to inflammation in the lungs, and have also postulated that inflammation or congestion of the lungs caused by pre-irradiation of the lungs of experimental animals gave rise to increased metastases. This has also been found by Fidler and Zeidman (1972) and van den Brenk and Kelly (1974), after local irradiation of the lung. In these studies arrest may have resulted purely because of an increased mechanical lodgement caused by the local inflammation and oedema, or because inflammation is normally associated with increased local presence of fibrin, which can form a matrix advantageous for the tumour cells' arrest and growth.

There have been several reports of increased metastatic rate in mice after treating local tumours with X-rays. Krebbs (1929) noted pulmonary metastases in 24% of control tumour-bearing animals as compared with 37% of those with locally irradiated tumours. He however ascribed this difference to the slightly longer average survival time of the latter group rather than to the effect of the irradiation. Increase

in metastatic rate of mammary carcinoma in mice after local tumour irradiation has been reported both in spontaneous (Kaae, 1953) and transplanted tumours (Kaplan and Murphy, 1949; von Essen and Kaplan, 1950; Suit, Sedlacet and Gillette, 1970; Sheldon et al, 1974) - see Table 5.0. While Kaae found 22% pulmonary metastases after radiotherapy was significantly different from 5.4% in controls, after biopsy he found 11.4% which was not found to be significantly different from controls. Sheldon et al found the incidence of metastases after treatment with X-rays and surgery were similarly dependent on whether the local tumour was cured or not.

After successful treatment of the local tumour the metastases rate was found to be 8%, while the rate when there was no local cure was 35%. They found a peak time of incidence of metastases which was independent of the time during the tumour's history that the treatment was carried out.

Von Essen and Kaplan, as a result of experiments where there was irradiation at a non-tumour site, or irradiation of tumour cells *in vitro* with subsequent implantation, considered that the X-rays probably facilitated entry of tumour cells into blood vessels.

In studies of combined chemotherapy and radiotherapy on primary tumours Moore and Dixon (1977) found that the extent of dissemination of metastases in rats was greater where the treatment included cyclophosphamide. This chemical is a potent immunosuppressive agent, in the dose range used (Harris et al, 1976) so this could have potentiated the metastases by reducing the immune surveillance in the animals so treated, allowing tumour cells to lodge and clone.

Host immunity against neoplasms might be expected to destroy malignant cells before or during metastatic spread. By stimulating immunity Baldwin and Pimm (1974) found enhanced protection against IV injection of tumour cells, however manipulation of host immunity can have quite the opposite effect, resulting in tumour enhancement (Fidler, 1974).

It has been suggested that immunogenicity of tumour cells is enhanced by heating (Mondovi et al, 1972). This might account for the observation that a heat treatment which is inadequate to sterilise all the cells in a tumour, when this is tested by transplantation of the tumour, may sterilise the tumour if left *in situ* (Suit, 1977; Marmor, Hahn and Hahn, 1977). Shah and Dickson (1978) have indicated that immunogenic tumours are more readily cured by heat than non-immunogenic tumours. Heat in some ways appears to stimulate the immune system. It has been demonstrated by Shah and Dickson that regression of the VX2 carcinoma in the rabbit after local hyperthermia, was accompanied by stimulation of a host anti-tumour immune response. It is not clear, however, whether this is applicable to non-immunogenic tumours and of course many human tumours are non-immunogenic. Dickson and Ellis (1974) suggested that after local hyperthermia reabsorption of dead cells leads to potentiation of the immune response with destruction of metastases which did not happen after radiotherapy; however the tumour system used in Dickson's study has an artifactual immunity which, according to Hewitt (1976), makes it unsuitable to investigate normal immune responses. Cell damage, by whatever means, gives rise to some kind of immune response but this is hard to quantify. There are contradictory results from different studies into the involvement of host immunity with metastases so the situation is far from resolved.

Peters (1975) set up a system to test whether or not manipulation of tumours increased the metastatic rate, and found no significant increase after normal handling procedures. However, although great care was taken in the present study to avoid undue manipulation when measuring tumours it was realised that the findings of Peters would not necessarily hold for this particular tumour. Kaplan and Murphy also investigated the effect of handling,during their investigation into the effect of X-rays, by having a control group which was handled identically to those in the trial. Since no metastases were observed in this group (0 out of 13) they concluded that the manipulation was not a significant factor in the formation of metastases in their system. However, numbers were too small to substantiate their claim that handling caused no metastases.

Information concerning the effect of treatment on metastases can be obtained by comparison of the incidence in untreated animals with that in treated animals. This information is not always available when the growth rate of the primary tumour is so fast as to necessitate killing the animal (for humane reasons) before the time that the metastases will become evident. The problem as to whether a particular treatment actually causes metastases is very difficult to resolve unless a base-line metastatic rate can be found and often the best one can do is to find a comparative metastatic rate for different modalities.

In this thesis the effect of hyperthermia using different temperature regimes on a local tumour has been compared with that of radiotherapy and surgery. The possibility of handling causing an increased metastatic rate has been considered and all handling procedures were standardised where practicable. Other factors which could have made

interpretation of results difficult (see 2.4.1) have been considered and excluded where possible.

5.2 Methodology and Results of Experimental Trials

5.2.1 Surgery and Anaesthetic Techniques

5.2.1.1 Mouse anaesthesia

For surgical resection of tumours a combination of a sedative analgesic and a tranquiliser was used to produce central nervous depression approaching general anaesthesia - neuroleptanalgesia (Green, 1975). The analgesic used was Hypnorm which is a solution of 0.315mg per ml fentanyl citrate and 10mg per ml fluanisone. The sedative was Valium which is 5mg per ml diazepam. These techniques were adapted from Green.

Dosage:

(i) Hypnorm: The recommended dose is 1ml per 2.0kg body weight. The average weight of mice used in experiments was 25g. Hypnorm was diluted 1:10 and the dose then is 0.125ml. By experience it was found that the best dose was 0.2ml. This was injected intra-peritoneally.

(ii) Valium: The recommended dose is 5mg per kg. Valium must be diluted to a minimum of 1:25 otherwise separation occurs. It can of course be used without dilution but for the small amounts required it must be diluted to obtain accuracy. The calculated dosage is 0.625ml per mouse. The findings in this work were that 0.3ml of the diluted drug was sufficient, though for slightly larger mice 0.4ml was required. This was also injected intra-peritoneally immediately following the injection of Hypnorm.

Surgical Technique:

The animal was ready for surgery in 10-15 minutes after injections. This was tested by noting eye reflex. Mice are still mobile and make 'paddling' movements with their limbs so must be taped down for surgery. To prepare the animal hair was removed and skin swabbed with chlorhexidene (5% in 70% EtOH). Using scissors skin was incised round the tumour. An elliptical piece of skin was removed making the wound easier to close. Tumour and skin were carefully freed from underlying connective tissue. The wound was closed with clips. The tumour and attached skin were kept for histology to see if a correlation between area of skin removed and recurrence could be found. It was thought that recurrences might arise from satellite tumours at the original site of injection. No correlation was found. There was only 1 recurrence out of 64 animals treated.

The mouse must be kept warm during the recovery period in an airy box. Respiratory depression does occur and if this becomes serious a reversing agent can be used. Neonatal Narcan (0.02mg malonone hydrochloride per ml). Dosage is 0.01mg per kg. Dose given was Narcan diluted 1:10 and 0.125ml was the correct dosage for the size of animal used. In practice 0.2ml was given. All dilutions were made in normal saline. The Narcan may be given at 15-30 minute intervals until symptoms are relieved.

5.2.2 Lung Colony Assay

A lung colony assay was carried out to determine whether there was a relationship between the number of tumour cells circulating in the blood and the number of lung metastases. To carry out this assay a single cell suspension was prepared as for a normal transplant and diluted

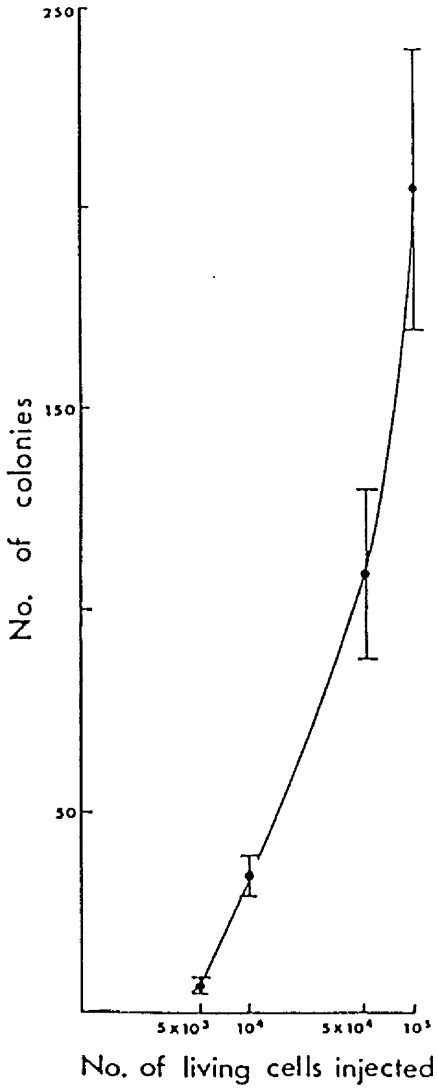


Figure 5.1

Inoculum size versus number of lung tumours

appropriately as in a dilution assay. 0.2ml of the final suspension was injected intravenously into the large vein at the top of the tail. In order to facilitate this, and because the vein is difficult to see, the animals were placed in a supporting jig with the tail held outside. The tail was warmed by shining a lamp on it during the time the cell suspension was being prepared. The syringe was inserted along the vein and the suspension slowly released. Animals were killed 14 days later. The lungs were removed, fixed in Bouin's solution then cleared by placing in 70, 90 then 100% alcohol then xylol. The lobes of the lung were separated and the total number of colonies counted. These appeared white against the pink lung tissue. The results of this are shown in Figure 5.1. Within the range of inoculum size used an increase in the number of tumour cells injected gave an increased number of lung metastases. With a larger inoculum numbers of lung tumours were uncountable as the tumours had coalesced.

The lung colony assay results indicated that the system provided a reasonably sensitive test for metastases. Colonies were observed as early as 14 days after injection of $5x10^3$ tumour cells (as a single cell suspension). This assay would be more sensitive if:

- this time period were extended to 37 days (the mean time for metastases to become apparent)
- LI cells were injected along with the tumour cells as was carried out by Hill and Bush (1969)
- shed tumour cells had a higher metabolic rate than inoculated cells
- cells were not shed singly, since large emboli have been shown to be more effective than single cells in producing metastases (Fidler, 1973b).

Lung	Pleural Cavity	Heart	Thorax	Brown Fat	Kidney	Spleen	Lymph Node	Liver
46	8	1	3	6	8	1	1*	1*

* invading from surrounding tissue

Figure 5.2

Anatomical distribution of metastases after hyperthermia

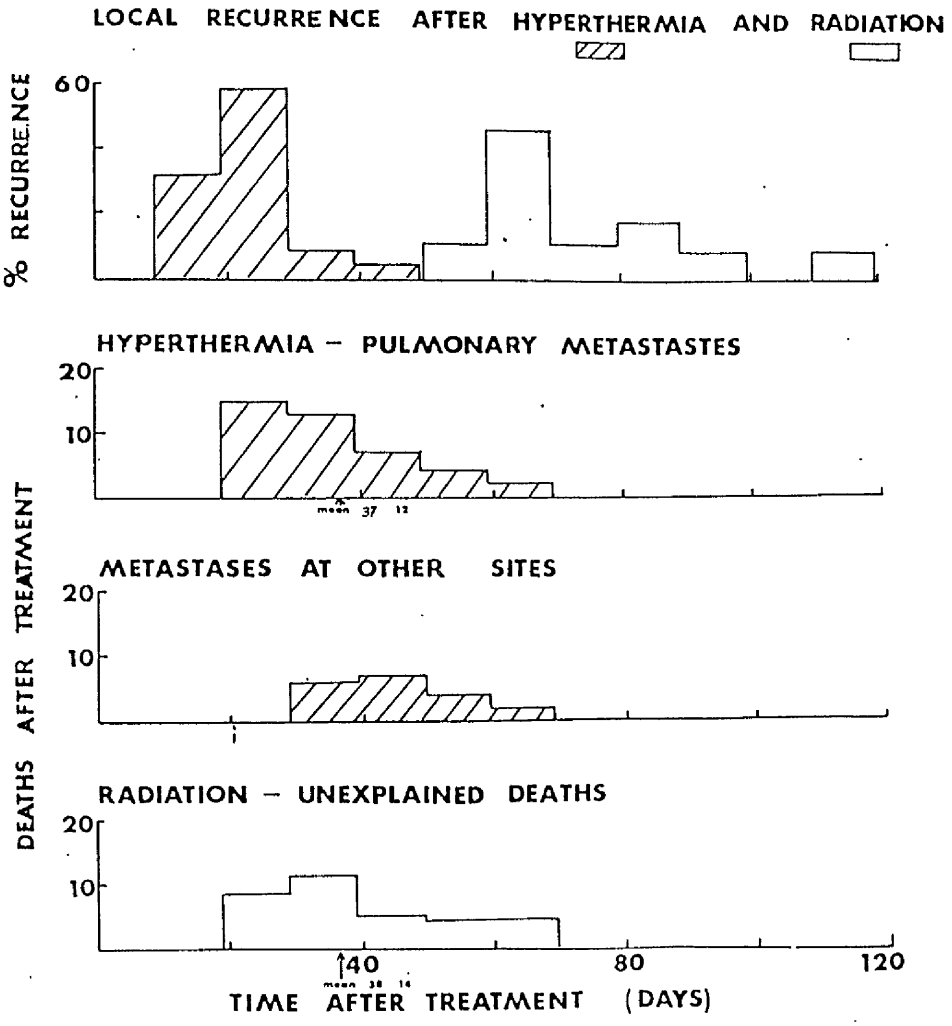


Figure 5.3

Times of appearance of tumours after treatment

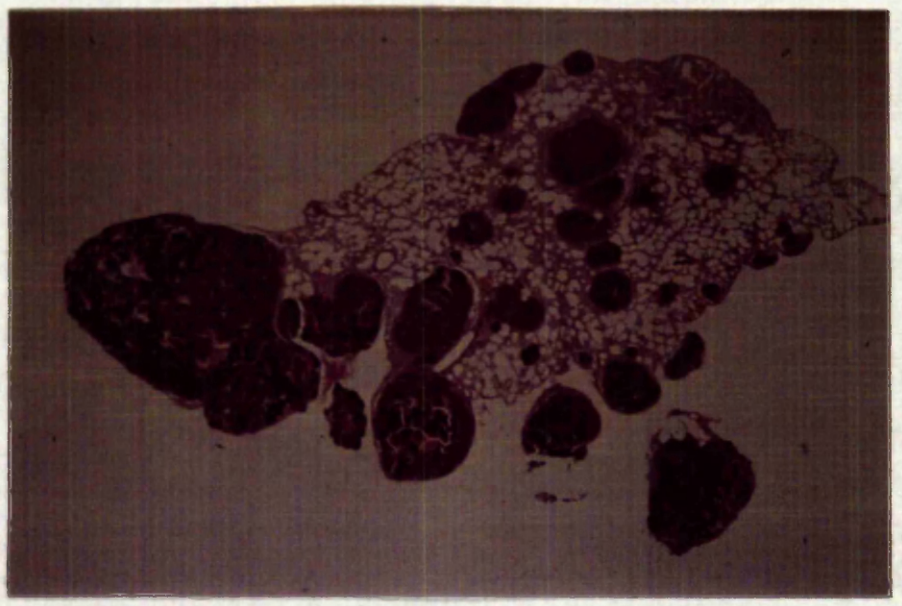


Figure 5.4

Photomicrograph of lung tumours (reproduced from x10)

5.2.3 Pathological Investigation

Autopsies were performed to establish whether mice had metastatic tumours. A search was first made for macroscopic metastases in all 5 lobes of the lungs and in other organs. These were then excised, fixed, sectioned, stained with haemotoxylin and eosin. Two or three sections were cut through different planes of each lobe of the lung and through other organs. Animals were not recorded as negative until careful study of all sections had been completed.

Sites and times of appearance of metastases are shown in Figures 5.2 and 5.3. All mice with metastases had lung metastases. Metastases killed mice by approximately 37 days after treatment of the primary tumour. By this time the largest lung tumours were over 3mm diameter.

The size distribution of lung metastases was difficult to ascertain since in some cases tumours had coalesced. By 28 days after treatment lungs had tumours of 1, 2 and 3mm diameter, together with many other small tumours (see Figure 5.4).

5.2.4 Experimental Trials

Trials were instigated to compare the systemic effect of hyperthermia, radiotherapy and surgery on the local tumour. After treatment, mice were examined daily for 100 days after treatment. If the primary tumour recurred these mice were excluded from the trial, although a note was kept of the cure rate since previous work had shown a relationship between this and metastatic rate (Sheldon et al, 1974). Autopsies were performed on all mice that died, appeared sick or had recurrent tumours. Metastatic rate was scored as the number of histologically proven metastases in mice whose local tumours had been

controlled, at 100 days or time of death, or sacrifice if earlier.

5.2.4.1 Trial 1 (table 5.1)

Trial 1 was a retrospective study. In this the effects of hyperthermia and radiotherapy over a range of dose levels were compared.

After hyperthermia the metastases rate was very high. Out of 52 cured mice, 7 died unexpectedly prior to routine autopsy, and 14 were autopsied and found to have in all cases pulmonary and in some cases other metastases. The cure rate was only 10% as the heating technique was inefficient and no collars were used. For a comparison of cure rates versus metastatic rates in all trials see Table 5.5.

After radiotherapy 14 apparently cured mice died unexpectedly and were not autopsied.

Taking only proven metastases, the minimum rate for hyperthermia was 14/52 or 27%, which may be contrasted with a maximum rate of metastases (taking all unexpected deaths after successful treatment with X-rays of 14/131, or 11%). Hyperthermia appeared to give rise to significantly more metastases than radiotherapy ($P < 0.02$). For a comparison of the significance levels of differences in metastatic rates after different modalities see Table 5.4.

5.2.4.2 Trial 2 (table 5.2)

This was a prospective study, comparing again the effects of hyperthermia (slow heat) and radiotherapy. A dose level of 44°/1 hour was chosen to give a reasonable cure rate.

In the first hyperthermia series (Group A), mice were treated using developmental collars which were not as efficient as those used in the

later series (Group B). Again hyperthermia appeared to give rise to significantly more metastases than radiotherapy ($P < 0.02$).

Groups A and B have been considered together. A larger metastatic rate would have been expected from Group A than from Group B. It was therefore considered justifiable to use the combined number of metastases since Group B would only make the metastatic rate less, so would make the difference less significant.

5.2.4.3 Trial 3 (table 5.3)

In this prospective trial the effects of 'fast' and 'slow' hyperthermia were compared with surgery.

All hyperthermia treatments were carried out at 44°C for 1 hour (or equivalent - see 3.2.6). Since 44 of the cured mice from Trial 2 were treated using identical procedures to the 'slow heat' series in Trial 3 these results have been incorporated with those of Trial 3.

'Slow' hyperthermia gave rise to significantly more metastases than 'fast' hyperthermia ($P < 0.01$). The metastatic rate after surgery was not significantly different from after other treatments (see Table 5.4). Perhaps with larger numbers in the trials significantly different results might have been found for 'slow' heat and surgery.

5.3 Discussion

5.3.1 Comparative Metastatic Rates After Treatment

The results of the three trials show that 'slow' hyperthermia appears to increase the possibility of metastases more than some other treatments. The trials will however be considered in turn.

5.3.1.1 Trial 1 (table 5.1)

At the beginning of this study all available evidence pointed to the fact that the C_3H multi-generation tumour did not regularly metastasise. It was not even known whether this tumour could be cured by heat alone, since the early-generation C_3H tumour was not found to be controlled by heat (Thrall et al, 1973). Thus the cure rate after hyperthermia (and after other modalities) had to be established before any evaluation of the metastatic rate after heat could be considered.

The results of this trial seemed to prove that hyperthermia caused more metastases than did X-rays but the trial had too many variables - treatment had been carried out at a variety of temperatures which gave widely different cure rates. The results were also compared with historical results of the X-ray series where the animals which died during the period of observation after treatment were not examined for metastases.

If all the mice that were not autopsied in the hyperthermia group had been considered, as in the radiotherapy group, as having died of metastatic disease the evidence for hyperthermia increasing the metastatic rate would have been very strong. As it was the results were strong enough to warrant a more controlled prospective investigation.

5.3.1.2 Trial 2 (table 5.2)

The results of this trial showed that 'slow' hyperthermia gave more metastases than radiotherapy ($P < 0.02$). Thus it would seem that time at temperatures below the critical temperature are dangerous. The

increased metastatic rate could have been caused by stimulation of the tumour cells during this time when the cells would have received inadequate therapy.

There is an alternative explanation. There was a 100% cure rate after radiotherapy and studies by Sheldon et al (1974), Peters (1975) and Moore and Dixon (1977) have correlated metastatic rate with cure rate thus one might on this basis have expected a low metastatic rate after radiotherapy.

The results of Trial 2 were therefore inconclusive.

5.3.1.3 *Trial 3 (table 5.3)*

Hyperthermia given with a fast heating-up time was used as one of the arms of this trial because stimulation of dissemination of tumour cells by inadequate heating had been suggested by the results of Trial 2 and by results of earlier workers (Dickson and Muckle, 1972; Smachlo et al, 1979). This was compared with a group of animals treated as in Trial 2 (hyperthermia, Group B) and another group where the tumour was removed surgically. The surgery was selected not only to give an overall comparison between different treatments but because of the earlier studies that had equated metastatic rate with cure rate. Because the tumour was apparently encapsulated and showed no signs of infiltrating surrounding tissue a high local cure rate was anticipated which, it was realised, would make a useful comparison with the radiotherapy group in Trial 2.

The results of this trial, when the Group B mice from Trial 2 were included in the analysis, showed clearly that the metastatic rate after 'slow' hyperthermia was greater than after 'fast' hyperthermia

($P < 0.01$). The metastatic rate after surgery was not found to be significantly different from that found after 'fast' hyperthermia.

The overall results of the 3 trials show that the metastatic rates after radiotherapy, surgery and 'fast' hyperthermia were not significantly different. 'Slow' hyperthermia was found to give rise to more metastases than radiotherapy. The most important finding was however that 'fast' hyperthermia gave a significantly lower metastatic rate than 'slow' hyperthermia.

As can be seen in Table 5.5 there are very different cure rates associated with different treatments. Broadly speaking the metastatic rate increased with decreasing cure rate, as had been found by Sheldon et al (1974) and others. There are however other factors that must be considered.

It appears from Table 5.5 that radiotherapy (Trial 1) had a similar cure rate and metastatic rate to the slow heat groups in Trial 2 (Group B) and Trial 3. However the average time for regrowth of the primary tumour after radiotherapy was approximately 69.5 days (cf after hyperthermia it was approximately 18 days) while the time of appearance of metastases was only 38 ± 14 days after both modalities. Thus some of the metastases found after X-ray treatment would be in mice where the primary tumour might still have recurred. Thus the metastatic rate given may well be artificially high. Sheldon et al, Peters (1975) and Moore and Dixon (1977) have all found that regimes which did not sterilise, but only produced an increased delay in regrowth of the primary permitted an increased incidence of metastases. Moore and Dixon found that to be the case irrespective of the mode of treatment (which did not include hyperthermia).

In studies to elucidate the effect of occluding the blood supply to a mouse tumour when treating with local heat or X-rays Hill and Denekamp (1978) found 50% of animals with pulmonary metastases after heat treatment. This suggested that the heat could have increased the incidence of metastases, however this was not supported in a retrospective analysis when the incidence of lung metastases was found to be 35% after X-rays alone, which was not significantly different from the 50% rate. Hill and Denekamp speculated that the apparent increase in metastases could have become obvious since animals survived longer to develop clinical symptoms.

It is preferable therefore to compare metastatic rates after treatments which give a similar time for tumour regrowth. The results of comparison of metastatic rates after 'slow' and 'fast' hyperthermia can therefore be viewed with more confidence since both cure rates and regrowth times after both treatments were similar. Therefore the results show clearly that heating up the tumour slowly causes an increase in metastatic spread.

5.3.2 Base-Line Metastatic Rate

From histological evidence obtained before this study began it appeared that the base-line metastatic rate might be low. No metastases were found in lungs or other tissues of tumour-bearing animals. However because the primary tumour grew so quickly animals had to be sacrificed by 20 days after inoculation of tumour cells, while metastases were not normally apparent until 37 ± 13 days after treatment, so it is not surprising that no metastases were found in mice with untreated tumours. This negative result may only mean that spontaneous metastases do not appear at an early stage in tumour

growth, however it does suggest that spontaneous metastatic rate might not be very high.

It is of interest that Gulledge (1961), in a study into the factors influencing metastases of different spontaneous mammary carcinomas in mice, found a 50% metastatic rate in one strain of the C_3H mice with untreated tumours. He found no correlation between the number, location or the structure of the primary tumour and lung tumours. Surgical removal of tumours in some mice (not C_3H) increased the incidence of metastases. No figures were quoted for C_3H mice, perhaps because with such a high spontaneous rate results might have been difficult to analyse without using very large numbers of mice. It is possible that the very high incidence of metastases may merely reflect the slow growth rate of the primary tumour allowing a long period during which seeding could take place.

The reason for trying to obtain a base-line metastatic rate by giving 'fast' hyperthermia was prompted by two sets of experimental results.

If tumours were heated in the temperature range 37.5 - 40.0°C it was found that there was stimulation of the metabolic rate of the tumour cells, such that at 40°C the rate was found to be double or more control value. This feature was demonstrated by Dickson and Ellis (1974) who found that tumours treated inadequately at 42°C exhibited both enhanced spread and metastases. This was probably because of large temperature gradients within the tumour so that some cells were only heated to about 40.0°C, so that there was stimulation of growth and metastases.

Studies by Smachlo et al (1979) showed that ultrasonic treatment of

tumours resulted in an absence of metastases in a system where tumours could be produced following inoculation of as few as 30 tumour cells. In the present treatment, as in that by Smachlo et al, the intra-tumour temperature rose to a therapeutic temperature in 1-1½ minutes. It thus seemed reasonable that the quick elevation of temperature could have been a factor in the absence of metastases.

Sheldon et al (1974) found that it was not possible to obtain a base-line metastatic rate using a first generation C_3H tumour because, as in the present study, the fast growth rate of the primary tumour necessitated sacrifice of the animals before metastases became evident. The tumour, however, was found to metastasise to a significantly different extent after different regimes, thus, although a base-line rate was not known the tumour system was still useful in comparison of treatments. It was initially decided that the C_3H multi-generation tumour could be used in the same way. However, the results of this study are not merely comparative. No metastases, or at most a very low rate, was found after 'fast' heat and this has been taken to be the base-line rate.

5.3.3 Possible Mechanisms of Metastatic Spread Suggested by the Results of this Study

5.3.3.1 Sites and times of appearance of metastases

From the anatomical distribution of sites shown in Figure 5.2 the pattern suggests blood-borne rather than lymphatic spread, because the one lymph node affected was embedded in metastatic tumour in the brown fat, and only the peripheral sinus was involved. The distribution of metastases may merely reflect the fact that the fine capillaries of the lungs are the first obstacles met, or there may be a tissue specificity since no mice that were found with metastases up to 35 days

after treatment had metastases in any site other than lung. All lobes of the lungs were equally affected by tumour. Other tumours could have seeded from the lung. The consistent peak of time of appearance of metastases, after hyperthermia and X-rays (see Figure 5.3), indicates that treatment causes metastases since the experiments using 'fast' hyperthermia have established that there is a low basic metastatic rate. Thus the data reflects wholly, or mainly dissemination of clonogenic cells during or after treatment.

5.3.3.2 Correlation with depression of immunity

Depression of the immune system can result in enhanced metastases (Fidler, 1974) and after treatment of the C_3H tumour with X-rays there was a general depression of the immune status of the mice and it remained depressed for several weeks before gradually returning to normal (Chamberlain, pers.comm.). This compared with a similar fall in immune response after hyperthermia where there was however a much more rapid return to normality, except in mice which were subsequently found to have metastases (Nias, Chamberlain and Abdelaal, 1980). Thus if lowering of the immune status was responsible for increasing the metastatic rate, it should have been higher after radiotherapy and this was not the case which suggests that changes in immune status do not appear to have been responsible for the enhanced metastatic rate after hyperthermia, at least in this practically non-immunogenic tumour system. This appears to contradict the findings of Shah and Dickson (1978) who found heat to stimulate a host anti-tumour immune response, however this was found in an immunogenic tumour (see 5.1.2).

5.3.3.3 Tumour and cellular response to hyperthermia

Hyperthermia alone has been found to cure a tumour which is very resistant to X-radiation; thus it is possible that hyperthermia may provide an alternative treatment for tumours that have been found to be resistant to other treatment modalities. There is rapid tumour regression, and with this there appears to be rapid re-oxygenation since the tumour, where there is no cure, regrows promptly. This should make pre-treatment with hyperthermia useful in increasing tumours sensitivity to X-rays.

Hypoxic tumours have been found to be more sensitive to hyperthermia than near normoxic tumours - in this, confirming the results of other workers. Hyperthermia is thus of value to use in association with radio or chemotherapy to which hypoxic tumours are resistant, since most tumours, especially when they are large, are to some degree hypoxic.

Earlier studies, already described, have established that there are two specific regions of hyperthermic cellular response which are separated by a critical temperature. When cells are heated above physiological temperature macromolecules are excited and chemical reactions are accelerated: above the critical temperature enzymes and proteins associated with DNA are denatured and inactivated. Thus if there is slow shedding of tumour cells at physiological temperatures then it seems reasonable to presume that this, like other reactions, will be speeded up. Thus heating in the temperature range above this but below the lethal temperature must be very dangerous, as has been demonstrated.

High metastatic rates after treatment with hyperthermia have been found in studies where a slow heating-up period of 5-15 minutes has been described. In the study by Hill and Denekamp (1978) it was not described as a factor in the high metastatic rate but it may have been one component in producing this high (50%) rate. Another related factor could have been large temperature gradients within the tumour which was heated by immersion in a waterbath so that some cells were inadequately treated. This was certainly described in the study by Dickson and Ellis (1974) as a possible contributory factor into increased metastatic spread. However in that study this interpretation was not the only one since the whole body temperature of the animals, treated with local hyperthermia, was elevated. This could have led to

- changes in the biological properties of the site tissues
- stimulation of metastatic cells already at distant sites
- depression of the immune defence mechanism of the host.

In the present study these other interpretations are irrelevant because there was no elevation in body temperature and because there appeared to be no preferential immune response against hyperthermia as against other modalities.

Thus all the available evidence points to the fact that inadequate treatment with hyperthermia is very dangerous and that if hyperthermia is going to be used to treat tumours, either alone or in conjunction with other modalities, it is essential that the heating technique is very efficient and gives a very rapid rise in temperature to all parts of the tumour as quickly as possible. Only thus can hyperthermia be used as a clinically relevant treatment.

TABLE 5.0

EFFECT OF LOCAL TUMOUR IRRADIATION ON MOUSE MAMMARY CARCINOMA

Year	Authors	Tumour System Tested	Result
1949	Kaplan and Murphy	transplanted	fourfold increase in lung metastases
1950	von Essen and Kaplan	transplanted	fourfold increase in lung metastases
1953	Kaae	spontaneous	fourfold increase in lung metastases
1970	Suit et al	transplanted	"increased metastases in tumours not ablated"
1974	Sheldon et al	transplanted	increased metastases in non-cured mice

TABLE 5.1

TRIAL 1

	Hyperthermia	Radiotherapy (Abdelaal, 1979)
Treatment Critique	various temperatures from 42-46°C. Poor intra-tumour temperature gradients	various doses from 55-80Gy
Cured mice	52	131
Proven metastases	14	0
Unexpected deaths	7	14
Proven metastatic rate	27	0
Maximum metastatic rate (if unexpected deaths included)	40	11

TABLE 5.2

TRIAL 2

	Hyperthermia (slow heat)			Radiotherapy (Abdelaal, 1979)
Treatment critique	intra-tumour temperature of 44°C for 1 hour			85Gy
Cured mice	Group A Group B	33 44	77	32
Proven metastases	Group A Group B	8 6	14	0
Metastatic rate	18			0

TABLE 5.3

TRIAL 3 (INCLUDING RESULTS FROM TRIAL 2)

Treatment critique	Fast Heat	Slow Heat	Surgery
Cured mice	64	96(including 44 from trial 2)	63
Proven metastases	0	12(including 6 from trial 2)	3
Metastatic rate	0	13	5

TABLE 5.4

COMPARISON OF METASTATIC RATES

Trial	Comparison	Significance (chi-squared test)
1	retrospective X-radiation versus hyperthermia (various conditions)	$P < 0.02$
2	prospective X-radiation versus hyperthermia	$P < 0.02$
2&3	fast heat versus slow heat	$P < 0.01$
3	fast heat versus surgery	not significantly different
3	slow heat versus surgery	$P < 0.5$
2:3	radiotherapy versus surgery	not significantly different

TABLE 5.5

CURE RATE VERSUS METASTATIC RATE

Trial	Modality		% Cure Rate	% Metastatic Rate
1	Hyperthermia		52/523 = 10	14/52 = 27
	Radiotherapy		131/~210 = approximately 64	14/131 = 11
2	Hyperthermia	(Group A	33/66 = 50	8/33 = 24
		(Group B (slow heat)	44/65 = 68	6/44 = 14
	Radiotherapy		approximately 100	0/32 = 0
3	Hyperthermia	(Fast heat	64/99 = 65	0/64 = 0
		(Slow heat	96/150 = 64	12/96 = 13
	Surgery		63/64 = 98	3/63 = 5

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CONTRASTING REGROWTH DELAY RESPONSES OF A MURINE TUMOUR TO ISO-CURATIVE HYPERTHERMIA OR X-IRRADIATION

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The most commonly used end-points of tumour response in situ are tumour regrowth delay and long-term cure of treated animals. Each of these end-points is generally regarded as providing a measure of the degree of tumour cell kill achieved by the treatment, and their use to estimate 'enhancement ratios' or to compare the relative efficacy of different forms of treatment, depends on this being (at least approximately) true. Here we report the in situ response to hyperthermia and to X-irradiation of a C3H mouse mammary carcinoma and show that, at treatment levels which are iso-effective in terms of cure, the regrowth delay responses are very different for these two modalities.

The properties of the tumour and methods of treatment have been described previously (Abdelaal & Nias 1978; Walker 1980) and will be presented only briefly. The tumour, a poorly differentiated adenocarcinoma of spontaneous origin has been propagated by serial transplation in C3H/He mice for several years. Tumour cells were prepared as a cytosieve suspension, implanted subcutaneously by needle beneath dorsal skin and allowed to grow to 5-7 mm mean diameter before being exposed to X-irradiation or hyperthermia treatment. Irradiation was carried out using 300 kV X rays and with conscious animals immobilized as described previously (Abdelaal & Nias 1978). Hyperthermia treatment was carried out by immobilizing conscious animals in a different jig and treatment administered using a 'dry' water-heater, heat being conducted to the tumour via a thin membrane (Walker 1980). Tumours assigned to treatment were exposed to graded doses of X rays or to hyperthermia treatments consisting of 60 min heating at water-bath temperatures ranging from $42^{o}C$ to $45^{o}C$. Tumour responses were assessed as proportion of animals 'cured' (i.e. tumours non-recurrent by 100 days) or as time to regrow to 10mm mean diameter for recurrent tumours. In the experiments reported here, a total of 199 mice received X-irradiation, whilst hyperthermia treatment was administered to 412 animals.

Figure (1) shows the dependence of proportion of animals cured on the severity of treatment, for hyperthermia and for irradiation. These graphs permit estimates to be made of the TCD-37 treatment level (i.e. the treatment level giving 37% cure) for each of these two modalities. For hyperthermia, the TCD-37 level is 43.5^{o}C (for one hour) and for X rays a single dose of 63.4 Gy. At the TCD-37 level, or the treatment level closest to it in the experimental design, approximately 63% of the tumours will recur and statistical distributions of regrowth delay may be recorded. Comparison of these regrowth delay distributions, at an 'iso-cure' level of treatment, provides a measure of the consistency of the end-points of cure or growth delay for X rays and hyperthermia. These data are presented in table I and, as histograms of regrowth delay, in figure (2). At the TCD-37 level, the mean regrowth delay of recurrent tumours, is 69.5 days for X-irradiation and 22.9 days for hyperthermia - a more than three-fold difference. The distribution is also broader for X-irradiation than for hyperthermia, and the two histograms do not overlap. Statistical testing is scarcely necessary to quantify so dramatic a difference; however, the Wilcox-Mann-Whitney 'U-test' confirms the difference between the means of the two distributions, at a very high level of significance ($p < 0.001$).

Since hyperthermia and X-irradiation have identical effects in terms of cure, but very different effects in terms of regrowth delay, these two end-points cannot provide equally valid measures of tumour cell kill achieved by treatment. Moreover, since the treatments were iso-curative, the discrepancy cannot be removed by the inclusion of cure data amongst the regrowth delay data, by any of the several methods which have been proposed (Denekamp 1980; Fowler et al. 1980; Wheldon and Brunton 1982). These disparate growth delay responses are not confined to the TCD-37 level; for all iso-curative treatments recurrent tumours grew back more quickly after hyperthermia than after X-irradiation. The TCD-37 level is however the statistically preferable level at which to make such

comparisons, on account of the degree of reliability with which this level can be estimated (Porter 1980). A possible explanation for the discrepancy is provided by the 'tumour bed effect' which, in the case of X-irradiation, gives rise to a slower growth rate of tumours growing (or regrowing) in irradiated, relative to unirradiated, normal tissues (Thomlinson and Craddock 1967; Hewitt and Blake 1968; Urano and Suit 1971; Jirtle et al. 1978). Recently, however, the 'tumour bed effect' has been reported not to occur for hyperthermia, with tumours in pre-heated tissue, or recurring after hyperthermia treatment in situ, growing at the same rate as controls (Urano and Cunningham 1980; Wheldon and Hingston 1982). In the present studies, tumour regrowth after hyperthermia was not well-quantified, owing to post-treatment oedema, but the C3H mammary carcinoma does show a pronounced tumour bed effect for X-irradiation, the regrowth rate of recurrent tumours being halved (for all doses above 20 Gy) relative to controls (Abdelaal et al. 1980). It seems possible that a significant tumour bed effect for X-irradiation, but not hyperthermia, could account for the relatively rapid regrowth following hyperthermia.

An alternative possibility is that different cells repopulate the tumour following X-irradiation or heat. After single-dose X-irradiation, the surviving cells are likely to be hypoxic and may have to await re-oxygenation during tumour shrinkage before they are able to initiate repopulation. In this tumour system, complete regression following irradiation takes 2-3 weeks (Abdelaal and Nias 1978) and reoxygenation may occur over a similar time-scale. For hyperthermia, by contrast, selective survival of hypoxic cells is unlikely and it is probable that most surviving cells will be well-oxygenated and well-nourished and perhaps capable of relatively prompt initiation of repopulation.

Each of these explanations invokes a mechanism leading to slower repopulation after X-irradiation than would be anticipated on the basis of cell survival. Some indication that this may be correct is provided by latent period studies using different numbers of implanted 'viable' cells

for which the upper-limit of time to grow to 10 mm (the time to grow from one single clonogenic cell) is estimated to be 45 days (Brunton, G.F., Walker, A. and Wheldon, T.E.: Unpublished). For a homogeneous tumour system, it may be predicted from Poisson statistics that (on average) a single clonogenic cell survives at the TCD-37 treatment level, so that the regrowth time at the TCD-37 level should approximate to the single-cell growth time, as estimated from latent period studies. Heterogeneity, however, leads to the TCD-37 regrowth time being less than the single-cell growth time (Wheldon and Brunton 1982), and this is indeed the case for the heat-treated tumours, whilst for X-irradiation the regrowth-delay times are displaced to longer times than the single-cell growth time (Figure (2)). These data are consistent with a moderately heterogeneous response to hyperthermia, but with a relatively rapid initiation of repopulation which then proceeds with normal growth rate. X-irradiated tumours appear subject to a significant additional delay not explicable by tumour cell kill alone.

Whatever the mechanisms responsible, the contrasting responses described could lead to a systematic under-valuing of hyperthermia, relative to X-irradiation, as a cytocidal agent. Combined end-point studies, as here, may be necessary to ensure that systematic biases do not occur, when in situ tumour responses are used to gauge the relative efficacies of different treatment modalities.

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TABLE I

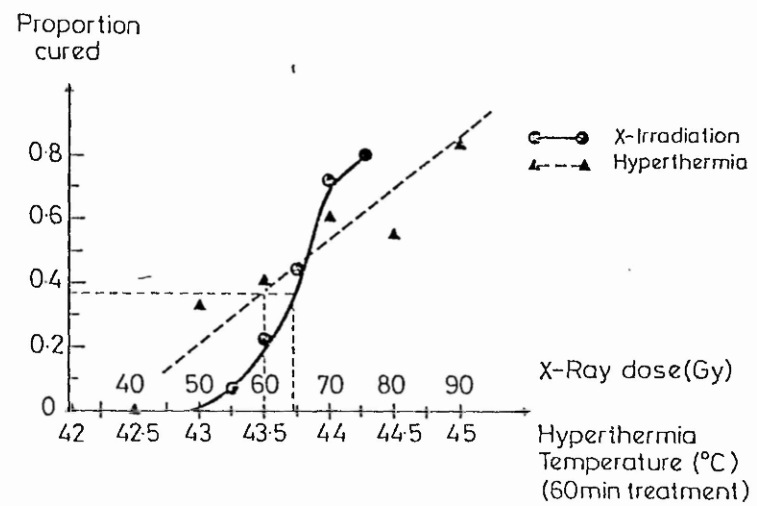
STATISTICS OF GROWTH DELAY FOR TUMOURS RECURRENT AFTER HYPERTHERMIA OR X-IRRADIATION AT OR NEAR THE TCD-37 TREATMENT LEVEL

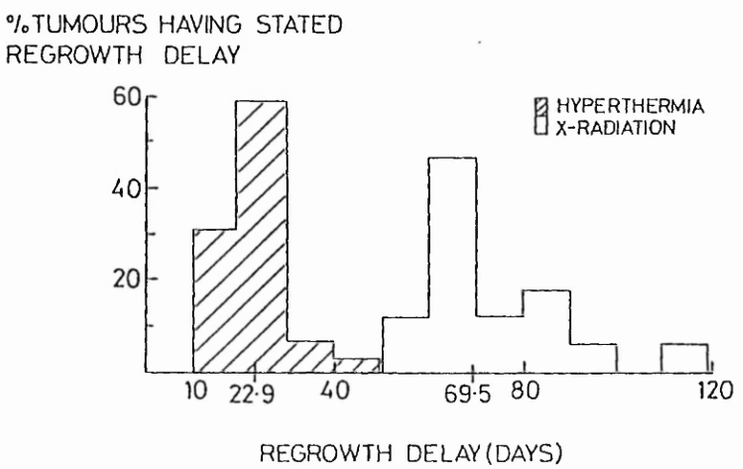
	X-irradiation	Hyperthermia
TCD-37 treatment level	64 Gy	43.5°C for one hour
Treatment group closest to TCD-37 level	65 Gy	43.5°C for one hour
Number of tumours recurrent in closest treatment group	19	29
Mean growth delay	69.5 days	22.9 days
Standard deviation of growth delay distribution	22.8 days	6.7 days
Absolute range of growth delay	50-120 days	10 - 49 days

FIGURE LEGENDS

Figure (1) : Proportion of animals 'cured' (i.e. tumours non-recurrent by 100 days) by X-irradiation or hyperthermia.

Figure (2) : Histograms of regrowth delay times (time to regrow to 10 mm mean diameter) for tumours recurrent after X-irradiation or hyperthermia administered at the TCD-37 treatment level.


Proportion cured
0·8
0·6
0·4
0·2
0
40
50
60
70
80
90
X-Ray dose(Gy)
42
42·5
43
43·5
44
44·5
45
Hyperthermia Temperature (°C) (60min treatment)
X-Irradiation
Hyperthermia


%TUMOURS HAVING STATED
REGROWTH DELAY
60
40
20
HYPERTHERMIA
X-RADIATION
10
22·9
40
69·5
80
120
REGROWTH DELAY (DAYS)

ORAL PAPERS

METASTASIS FOLLOWING HYPERTHERMIA OR X-RAYS.

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We have examined the response of the C3H mouse mammary carcinoma, dorsally implanted, to X-rays and to local hyperthermia. We found locally curative X-ray dosages to lie in the region 5000–8000 rad and locally curative hyperthermia to lie in the temperature region 42–45°C and the time range 1–2 h. Hyperthermia was administered to unanaesthetized animals by water pumped through a heating device, using a fine membrane as interface, the temperature being constantly monitored.

During the course of the hyperthermia studies, we noted a high incidence of unexpected deaths in locally cured mice, and this observation prompted the instigation of necropsy, not hitherto routine. All mice examined by necropsy because of early death or visible deterioration in absence of local tumour proved to have distant metastases, usually in the lungs, but occasionally in kidney, brown fat or other sites. The incidence of proven metastases (14/52 or 27%) in hyperthermia-cured mice may be contrasted with the maximum incidence of metastases (14/131 or 11%) inferred from early death of mice locally cured by X-rays. By the χ^2-test this difference is significant ($P < 0.02$). A controlled prospective trial has now confirmed a significant ($P < 0.01$) increase in frequency of metastases after hyperthermia (14/77 or 18%) as compared to X-rays (0/32).

Pending confirmation of this phenomenon, and of investigation of the causal mechanisms and exploration of possible ways of obviating it (*e.g.* by pre-irradiation) clinical trials involving treatment with local hyperthermia alone of human tumours of known metastatic potential should proceed with caution.

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METASTASES AND IMMUNOLOGICAL STUDIES IN C3H MICE AFTER HYPERTHERMIA

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We have examined the response to hyperthermia of a carcinoma in C3H mice and report a dramatic promotion of metastasis by local heating administered in time-temperature combinations sufficient to eradicate the primary tumour (*e.g.* one hour at 43.5°C).

The carcinoma originated as a spontaneous mammary tumour in 1974 and has been serially transplanted ever since. The volume doubling time is about one day at the size of 6 mm diameter when treatments are given. The tumour is poorly differentiated and contains many foci of necrosis. The very high radiation TCD_{50} value of 67.5 Gy is compatible with this evidence of extensive hypoxia.

The cell-mediated immunological response of the mice has been assessed using the PHA response of splenic lymphocytes. The PHA index was significantly reduced in tumour-bearing mice but returned towards a normal value after successful treatment of the primary tumour by either surgical excision, radiotherapy or hyperthermia. The PHA index remained low, however, in a number of the mice which had to be sacrificed earlier than 100 days after hyperthermia with the tumour locally controlled. Histological examination revealed pulmonary and other blood borne metastases in these mice.

The incidence of metastasis was 25% after local hyperthermia, significantly higher than the 10% rate after local radiotherapy ($P = < 0.04$).

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ABSTRACT

METASTASIS OF C3H MOUSE MAMMARY CARCINOMA FOLLOWING LOCALLY CURATIVE X-RAYS OR HYPERTHERMIA

by

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We have examined the response of the C3H mouse mammary carcinoma to X-rays and to hyperthermia. We found locally curative X-ray dosages to lie in the region 5000–7000 rad and locally curative hyperthermia regimes to lie in the temperature region 42–46°C and the time domain 1–2 hours. Local hyperthermia was administered by water pumped through a heating device, using a fine membrane as interface – the temperature being constantly monitored.

During the course of the hyperthermia studies, we noted a high incidence of unexpected deaths in locally cured mice, and this observation prompted the instigation of autopsy, not hitherto routine. All mice autopsied because of early death or visible deterioration in absence of local tumour proved to have distant metastases, usually in the lungs, but occasionally in kidney, brown fat or other sites. The incidence of proven metastases (14/52 or 27%) in hyperthermia-cured mice may be contrasted with the maximum incidence of metastases (14/131 or 11%) inferred from early death of mice locally cured by X-rays. By the χ^2-test, this difference is significant at the 95% confidence level ($p < 0.02$).

Pending confirmation of this phenomenon, and of possible ways of obviating it (e.g. by pre-irradiation), clinical trials involving treatment with local hyperthermia alone of human tumours of known metastatic potential should proceed with caution.