

RADIOBIOLOGICAL STUDIES OF MALIGNANT
RAT CELLS IN VIVO AND IN VITRO

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by

NICOLAS JOHN McNALLY

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for the degree of Doctor of Philosophy

Medical Research Council,
Experimental Radiopathology Unit,
Hammersmith Hospital,
Ducane Road, London, W.12.

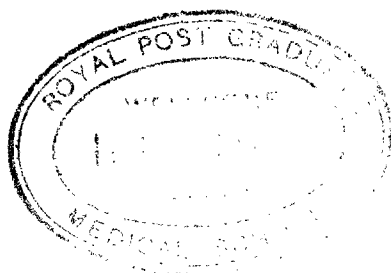
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ABSTRACT

A comparison has been made between the gross response of a fibrosarcoma growing in rats to radiation, and that of its cells. A permanent cell line has been established from this tumour and the radiobiological properties of these cultured cells, and of the tumours that resulted when they were reinjected into rats, have been studied.

The modifying effects of oxygen, the linear energy transfer of the radiation, and dose fractionation on tumour growth delay and cell survival have been studied. The properties of an in vitro model system of the non-proliferating cells in a tumour have been investigated.

The results indicate that studies in vitro may not be valid as a guide to the detailed radiobiological behaviour of cells in vivo, and that there may be qualitative differences between the types of cellular damage expressed following irradiations in vivo and in vitro.



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Chapter 1

INTRODUCTION

1.1. Tumours and Radiobiology.

The main characteristics of neoplasms have been described through detailed clinical observations over many centuries. Broadly, pathologists have divided neoplasms into benign or innocent tumours, and malignant tumours. Benign tumours are circumscribed and usually encapsulated lumps growing at a very slow rate, which do not tend to invade or destroy surrounding tissues. They are often curable by local excision and do not usually endanger life. In contrast, malignant tumours grow more quickly and more extensively. They invade and destroy neighbouring tissues and often recur after local excision. Probably their most dangerous characteristic is their ability to metastasise, to give rise to secondary growths of a similar kind in distant parts of the body due to discontinuous transport of cells from the original tumour to the appropriate site via the blood or lymphatic systems.

There are three basic methods available for the treatment of cancer: surgery, radiotherapy, and chemotherapy. At the present time many treatments fail to produce a cure, and the reasons for this are poorly understood. Unfortunately many patients present themselves with the disease at a fairly advanced stage, by which time metastases may have developed. It is possible that if more effort were put into ensuring early diagnosis in patients, the benefit in terms of cures produced would be greater than that due to the bulk of the research that has been carried out into the causes and treatment of cancer

(Eason, 1967). Even so, there would be failures. Suit (1969) has estimated that regrowth of the primary lesion following radiotherapy was a significant part of the terminal phase of the disease in about 36 percent of the patients dying of cancer in the United States in 1968. It is in part the concern of the tumour radiobiologist to explain these failures and to point to better methods of treatment by radiotherapy and, or chemotherapy.

1.2. Radiotherapy and Radiobiology.

The first report of successful treatment of cancer with radiation came from the United States in 1899, less than four years after Roöntgen published his first paper. The early radiotherapists attempted to administer the largest dose in the shortest time. In practice this meant submitting patients to single treatments lasting several hours. An alternative method consisted of single or short courses of treatment at intervals of a few weeks, for overall periods of several months. The cumulative character of the effects of radiation had quickly been noted, and the only rationale for this second approach was that it was more tolerable for the patient than that of a single massive dose. Nevertheless, clinically observed reactions in normal tissues were still severe.

In 1922 C.Regaud demonstrated that a single large dose, capable of producing necrosis of the scrotum, was unable to produce permanent sterilization of the testis of a ram, whereas the radiation delivered as four fractions within ten days did so without producing permanent skin necrosis. Regaud soon applied these results to radium treatments extending over periods of seven to ten days. H. Coutard extended Regaud's ideas to give daily doses

over periods of several weeks and demonstrated a considerable improvement in the results of treatment of cancer of the larynx and pharynx (Coutard, 1934). Subsequent developments in radiotherapy were essentially quantitative in nature. Ralston Paterson in 1952 maintained a statistical approach to the problem of optimum dosage in radiotherapy, to quote him "few radiotherapists will be able to justify their choice of dosage and treatment on really solid grounds" (Paterson, 1952). Fletcher (1969) has reviewed the progress of clinical research until the present time as it has been applied to considerations of the relationship between time and dose in radiotherapy. This research, though essentially empirical, has provided the framework for present radiotherapeutic practice. Developments in radiobiology may have seemed, in many instances, to be unrelated to this practice, and it is not yet possible to propose any rational optimum treatment regimes, however, these developments have made it possible to suggest reasons for success or failure in radiotherapy (Barendsen, 1969; Sheline et al, 1971), and it is to be hoped that current research will soon begin to have an impact on the planning of treatment regimes.

1.3. The Therapeutic Ratio.

In general radiotherapists limit their treatment not so much to that which will just destroy a tumour, but to that which will induce a reaction in the surrounding normal tissue which will just be tolerated. J.G. Stewart, studying the relation of dose to results in radiotherapy in two clinical trials, one for cancer of the mouth, and one for early carcinoma of the larynx, concluded that the optimum dose in both cases was the highest dose compatible

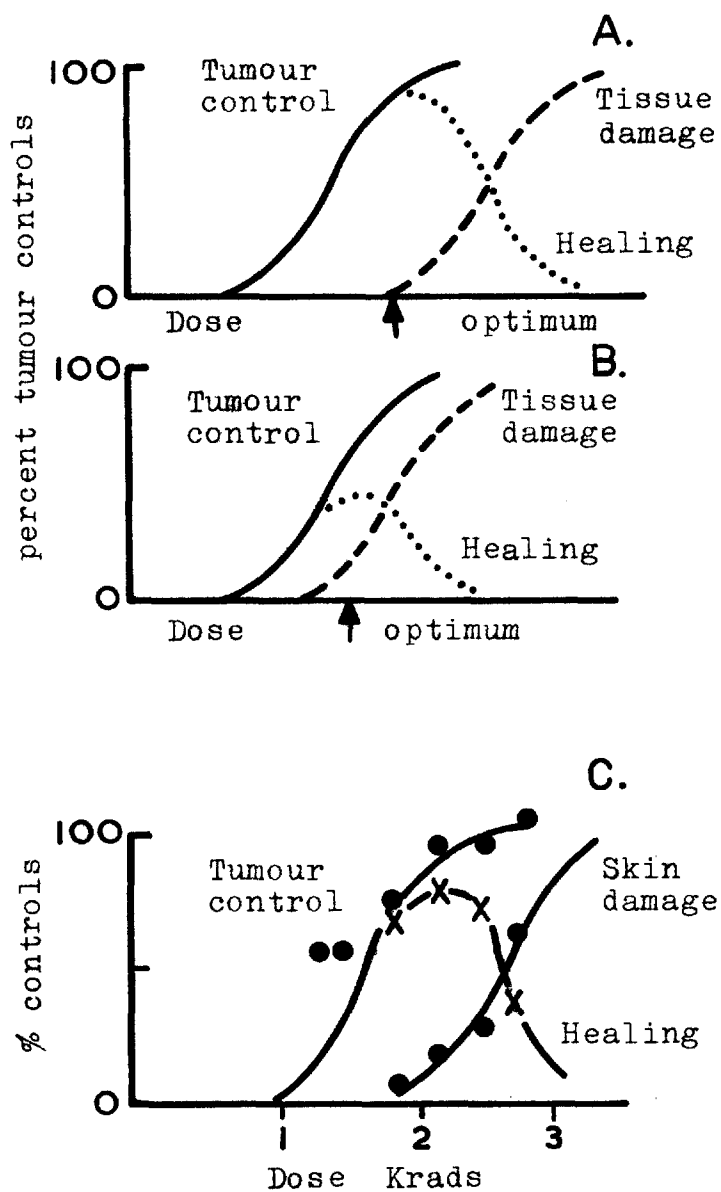


Figure 1

The therapeutic ratio

- A. A "radiosensitive" tumour with a high therapeutic ratio.
- B. A "radioresistant" tumour with a low therapeutic ratio.
- C. Data for cancer of the skin (Fletcher, 1969).

with acceptable necrosis risk (Stewart, 1964). This concern for the normal tissue has led to the concept of the therapeutic ratio as an aid in determining optimum dosage (figure 1). For a "radiosensitive" tumour having a high therapeutic ratio, complications increase slowly with increasing dose (figure 1A). For a "radioresistant" tumour, the dose-effect curves for normal and malignant tissues will be close together so that complications will increase quickly with increasing dose (figure 1B). Figure 1C shows actual curves plotted by Fletcher (1969) from data on skin cancer obtained by Strandqvist. The effectiveness of radiotherapy could be improved if the therapeutic ratio could be suitably manipulated, and it is one of the aims of radiobiology to provide information as to how this can be done.

1.4. Cell Survival.

In 1956 Puck and Marcus published the first directly measured mammalian cell survival curve. They used an in vitro cloning technique with cells derived from the lung of a Chinese hamster (Puck and Marcus, 1956). Soon afterwards Hewitt and Wilson (1959) showed that mouse leukaemia cells irradiated in vivo or in vitro but assayed in vivo had essentially the same relationship between dose and surviving fraction of cells as that measured by Puck and Marcus. Subsequent studies both in vivo and in vitro have shown that the survival curves for most cell lines have certain features in common, and these are illustrated in figure 2. The ordinate scale is a logarithmic one of surviving fraction, the dose scale is linear. Survival in this context refers to the specific ability of cells to undergo unlimited proliferation so as to form colonies

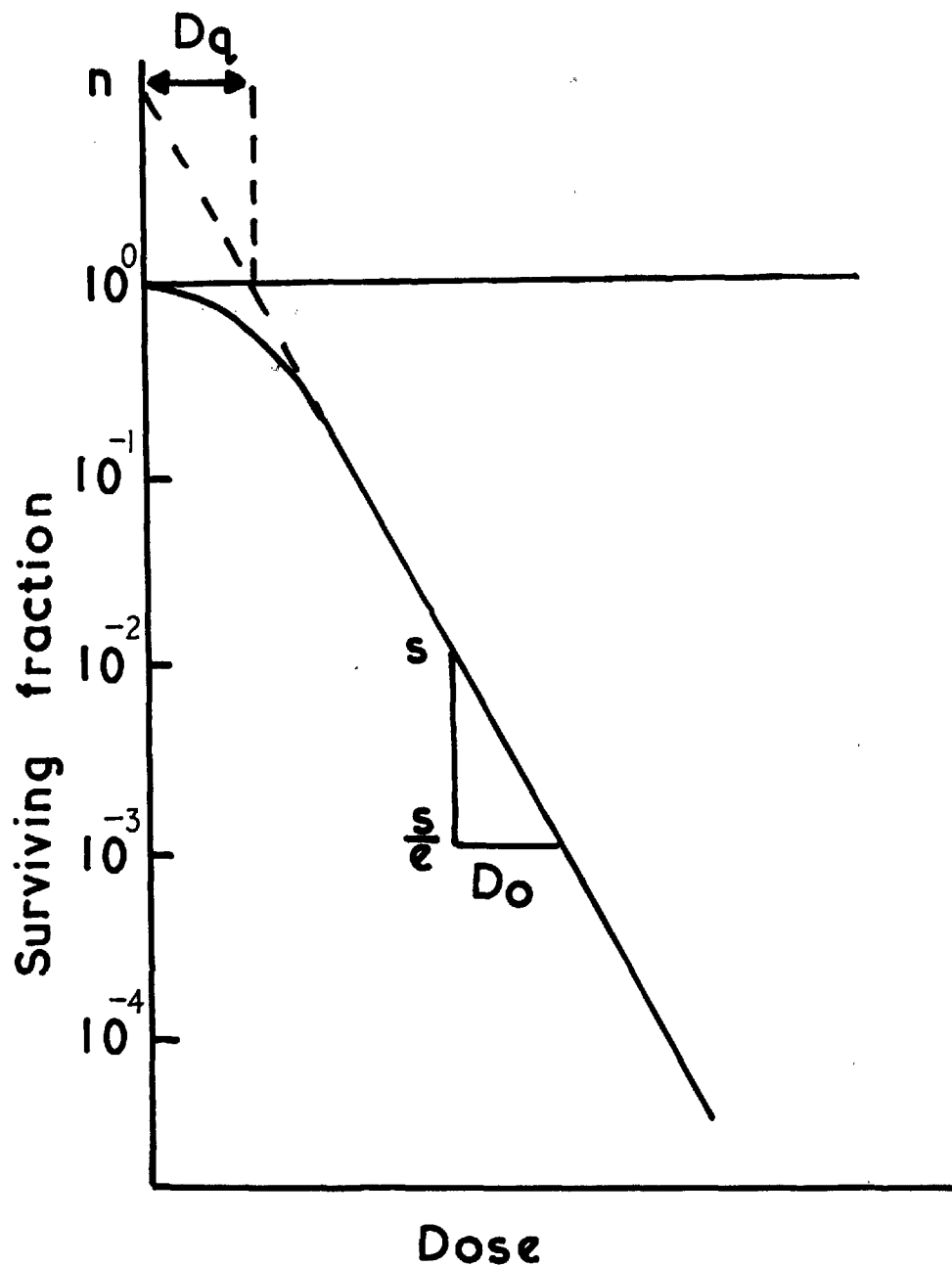


Figure 2

A C-type radiation survival curve. The ordinate is a logarithmic scale of surviving fraction, and the abscissa is a linear one of radiation dose.

on a petri dish, for example, or to cause a tumour.

The survival curve of figure 2 consists of an initial shoulder followed by a straight line corresponding to an exponential relation between dose and surviving fraction. (In practice, some cell lines have survival curves which continue to bend down at higher doses (Barendsen et al, 1960)). A survival curve of this form is characterised by any two of three parameters: D_0 , the extrapolation number n , and D_q . $(D_0)^{-1}$ is a measure of the slope of the exponential region of the survival curve, where D_0 is that dose which reduces the surviving fraction by a factor e^{-1} on the exponential part of the curve. It is sometimes called the mean lethal dose per sensitive site (Lea, 1962). The extrapolation number, n , is the intercept of the ordinate scale with the exponential portion of the survival curve extrapolated back to zero dose (Alper et al, 1960). The point at which this extrapolated line intersects the 100 percent survival level is defined as the quasi-threshold dose, D_q (Alper et al, 1962). These three parameters are related according to the expression $D_q = D_0 \log_e n$.

1.5. Tumour radiosensitivity.

Cellular radiosensitivity can be determined by measuring cell survival curves, and the radiosensitivity of individual cells can usefully be defined as the inverse of the average energy per lethal event, $1/D_0$ (Zimmer, 1961; Lea, 1962). According to this definition, a population with a uniform radiosensitivity will give rise to a survival curve with an exponential region, and changes in sensitivity will be reflected in changes in the slope of this exponential region. This definition of cellular radiosensitivity is precise and has units $(\text{dose})^{-1}$.

In contrast, clinical radiosensitivity as applied, for instance, to tumours, is not so well defined. As used in discussing the therapeutic ratio sensitivity refers to local curability, a sensitive tumour being one with a high therapeutic ratio. However, the term "radiosensitive" has also been applied to tumours which show rapid regression following a dose of radiation. Tumour radiosensitivity in this sense depends on other factors besides the radiosensitivity of the individual tumour cells. It will depend on their turnover rate and the rate of repopulation by surviving cells. A tumour with a rapid turnover rate may quickly decrease in size after irradiation, but if a sufficient number of cells survive, they will repopulate the tumour, so that to call it radiosensitive would be misleading. Unfortunately radiotherapists are not agreed about the meaning of the term "radiosensitive" as applied to tumours. Paterson uses the term to mean local curability without defining what is a cure (Paterson, 1948), while Easson describes a tumour as radiosensitive "if it is resolved rapidly in response to modest doses of x-rays" (Easson, 1967), and makes a distinction between sensitivity and curability. Such a definition depends in a complex way on the properties of the tumour, whereas that of Paterson is more directly associated with the survival of individual cells.

1.6. The Oxygen Effect.

The sensitivity of almost all types of cell to ionizing radiation for most types of damage is greater in the presence of oxygen than in its absence. This "oxygen effect" was described in radiotherapy almost as soon as radiation was used in the treatment of malignant

disease, although the reasons were not at that time understood.

The relationship between oxygen concentration and cell sensitivity was first accurately described for bacteria by Alper and Howard-Flanders (1956), and by Deschner and Gray (1959) showed that the same relationship held for the induction of chromosome aberrations in mammalian cells. This relationship can be represented by the equation

$$\frac{S}{S_N} = \frac{m [O] + k}{[O] + k}$$

where S_N and S are the sensitivities of the test system in the absence of oxygen and in the presence of a concentration $[O]$ of oxygen, and k is the concentration of oxygen which brings the sensitivity to half its maximum value m , the oxygen enhancement ratio (o.e.r.). The oxygen is said to be dose modifying, that is, if an effect is produced by a dose D of radiation in aerobic conditions, then the same effect is produced by a dose mD in the absence of oxygen.

There is now evidence to show that experimental tumours contain hypoxic cells (Gray et al, 1953; Powers and Tolmach, 1964; Thomlinson, 1965), and that these cells, which are protected from radiation injury, can repopulate the tumours (Powers and Tolmach, 1964; Thomlinson, 1968). As yet there is only indirect evidence to suggest that human tumours contain hypoxic cells (Churchill-Davidson, 1964). One would expect that this hypoxic component of the tumour cell population would determine the response of a tumour to large single doses, and this protection may be the reason that some tumours are not eradicated by such doses.

There are several possible methods of dealing with the problem of hypoxic cells in tumours (Fowler, 1966). In the present context only the use of radiations of high ionization density, of high linear energy transfer (LET) will be considered. The potential advantage of high LET radiation lies in the fact that the o.e.r. for damage by such radiation for most test systems is considerably lower than that for radiations of low LET. The ratio of the o.e.r. for low LET radiations to that for fast neutrons from the Medical Research Council cyclotron is in the range 1.5 to 1.7 for damage to mammalian cells (Hornsey and Silini, 1961; Berry, Bewley, and Parnell, 1965; Barendsen et al, 1966; McNally and Bewley, 1969). This factor represents the increase in effective dose to the hypoxic cells for high LET radiation over that for x-rays, and represents the chief reason for considering the possible use of neutrons in radiotherapy.

1.7. Shedding of Sublethal Injury.

The existence of a shoulder to the survival curve for irradiated cells (figure 2) implies that radiation damage has to be accumulated before it can express itself as lethal, in other words that cells can absorb some damage as sublethal. Elkind and Sutton (1959) showed that cells could recover from, or shed this sublethal damage. They showed that if cells in vitro were exposed to a dose of radiation and some time later were exposed to a series of second doses, then the survival curve shape was repeated, those cells that had survived the first dose behaved as if they had received no radiation, they had shed their sublethal damage. The time course for this recovery process has been studied both in vitro

(Elkind and Sinclair, 1965, for instance), and in vivo when the response of tissues was being measured (Hornsey and Vatistas, 1963; Fowler et al, 1965; Withers, 1967b; Withers and Elkind, 1969). Typically, for mammalian cells in vitro, as the time between the doses increases, there is an initial rapid recovery occurring within the first two to four hours, followed by a trough and subsequent increase in survival with time, reflecting changes in sensitivity due to the induction of partial synchrony by the first dose (Elkind and Sinclair, 1965).

The ability of cells to shed their sublethal damage is of great importance for radiotherapy. Fowler (1966) has discussed in some detail the relationship between what one might expect of a simple fractionation regime in terms of cell survival and the effects that are found in clinical practice. Again, the relative response of the normal to the tumour tissue is the crucial factor, and the size of the therapeutic ratio will depend not only on the different cellular radiosensitivities (if any) of the two tissues, but also on their repopulation kinetics, and their relative abilities to shed sublethal damage.

1.8. Aims of the study.

The developments in cellular radiobiology have been the principal stimulus to discussions of the scientific basis of radiotherapy (Fowler, 1966; Alper, 1967; Kaplan, 1970). The principal endpoints at present available to the radiotherapist are patient survival rates, and tumour growth and regression measurements. Nevertheless, in the final analysis it is probably radiation's ability to kill individual cells that will determine whether or not a tumour will be eradicated following radiotherapy. For

this reason cell cloning experiments are potentially of considerable importance. However, their possible relevance to the in vivo situation must be considered with some care.

In vitro experiments allow one to study the properties of clonogenic cells, both proliferating and non-proliferating, directly. The non-proliferating but clonogenic cells may be of particular importance since many tumours contain non-proliferating cells (Mendelsohn, 1960). A study has been made of the properties of a non-proliferating population in vitro both in terms of its response to radiation and in terms of its growth kinetics, and the relevance of such a model to tumour radiobiology will be discussed.

It is important to measure the radiobiological properties of clonogenic tumour cells directly, since the environment within a tumour will affect their response to radiation. One can do this using the type of assay first developed by Hewitt and Wilson (1959), or by the lung colony assay of Hill and Bruce (1969), or by measuring tumour cure rates (Suit, Shalek, and Wette, 1965).

However, all these methods require large numbers of animals and the assay period can be several months. A method which could measure the formation of colonies directly would be a considerable advantage. One way would be to culture the cells of the tumour of interest and to measure their properties in vitro. However, the characteristics of interest will almost certainly alter, since it can take many generations for cells to adapt to their in vitro environment (see chapter 3). A second method is to irradiate tumour cells in vivo and then to measure their ability to form colonies immediately upon transfer from the animal to the petri dish. With such a system, the environment of the cell at the time of irradiation is

the "true" environment, while the assay in vitro allows quick evaluation of results without the use of large numbers of animals. Ideally, a suitable system would be one which allowed movement from the in vivo to the in vitro state and vica versa. Such a system has been developed by Barendsen's group (Barendsen and Broerse, 1969), and I have developed a similar system using the transplantable fibrosarcoma RIB₅ as the starting point. This system will be described in detail in chapter two.

Studies of cells in vitro have shown the importance of three factors in considering the reponse of cells to radiation, the LET of the radiation, the sensitizing effect of oxygen, and the ability of cells to shed their sublethal damage. Each of these factors has been studied in terms of its effects on cell survival and on the gross response of a tumour to radiation. In addition, evidence will be presented to question the validity of studies in vitro as a guide to the detailed radiobiological response of cells and tissues in vivo.

Chapter 2

TECHNIQUES FOR STUDYING TUMOUR CELLS

IN VIVO AND IN VITRO

2.1. Methods for measuring the gross response of a tumour to radiation.

In any biological experimental system the effects of biological variability should be kept to a minimum. When measuring the gross response of a tumour to radiation, this is best done by using a transplantable tumour system in an inbred strain of animals, since spontaneous tumours, for instance the spontaneous mammary carcinoma in C₃H mice, tend to have a wide range of histological types and growth rates (Denekamp, 1968). The system I have used is one developed by Thomlinson (1960). John's strain of Wistar rats has been used, and most of the work to be reported has been done with the tumour RIB₅, a benzo-pyrene induced fibrosarcoma, or a modification of it.

The transplantation procedure is one that was developed to give approximately spherical tumours and more important, to reduce the tendency of the tumours to metastasise and cause the death of the animals before the effect of treatment on the primaries could be assessed. Tumours could be maintained by subcutaneous graft in animal flanks. Experimental tumours were obtained as follows. A tumour-bearing animal was killed by neck luxation and healthy tumour excised and finely minced with scissors in a cavity block. A measured quantity, free of lumps, was drawn into a syringe and transferred to a second block where it was mixed with a 4 percent solution of sodium alginate in the proportions three parts tumour to one of alginate. This tumour-alginate mixture was then drawn

into another syringe and dropped from a fine needle into a solution of 1 percent calcium chloride at room temperature. The tumour pellet so formed was removed to a beaker of normal saline after about 20-30 seconds, a time long enough for the calcium alginate gel to form a firm capsule around the tumour material. Meanwhile, a sausage skin was prepared by inverting the small intestine of a 3-5 week old rat and cleaning off the mucosa with a sterile gauze swab. The tumour pellet was then placed in this skin and tied off, producing an approximately spherical tumour sausage about 3 mm. in diameter. This was implanted subcutaneously into the left flank of a rat. In some experiments the sausage skin was omitted.

Depending on the type of tumour being studied, tumours reached measurable sizes one to four weeks after implantation, and could be measured along three diameters using vernier callipers. Growth curves were constructed by plotting the arithmetic mean of these diameters against time. Zero time was taken as the day of treatment which was when tumours had reached a standard size of 8-10 mm. in diameter. Tumours were measured five times a week until they had reached a mean diameter of about 30 mm., or until the animals could no longer tolerate their presence. At this time the animals were sacrificed and an autopsy performed.

The endpoint used was the time to grow from treatment size to 25 mm. in diameter after a dose of radiation, as compared with that for unirradiated tumours (Thomlinson, 1960).

2.2. Establishment of a permanent cell line from tumour tissue.

2.2.1. Introduction.

There are many techniques available for culturing tumour tissue both as organ cultures and as single cell cultures (see Paul, 1965). In the present context, only those methods that have been employed for the particular tumours available will be described. The basic problem was to obtain a permanent single cell culture from a tumour whose gross response to radiation was being studied. The particular tumour of interest was RIB₅. Initially two other tumours were also used, a butter yellow-induced liver tumour, and a spontaneous sarcoma of the orbit. The methods employed were the same for all tumours, but RIB₅ was the only one to be studied in any detail.

2.2.2. The primary culture.

A tumour-bearing animal was sacrificed by neck luxation and healthy tumour tissue excised under aseptic conditions. This was placed in a 5 cm. sterile plastic petri dish and finely minced with sharp pointed scissors. Some of the chunks from this mince were then placed in small Carrel flasks and attached to their inner surfaces by gently warming the flask bottoms. Appropriate amounts of complete medium were then pipetted into the flasks and, after exchanging the air with 5 percent CO₂ in air, the flasks were sealed and placed in a warm room at 37°C. Within a few days monolayer outgrowths could be seen. In practice these were not used further, their chief use being to check that the particular medium being used was satisfactory. The best medium was Eagle's minimum

essential, although Eagle's basal medium was also quite satisfactory. The complete medium used was:-

41 ml Eagle's minimum essential medium (x10)

40 ml NaHCO_3 , 2.8 percent solution in distilled water

50 ml foetal calf serum (Flow Laboratories)

Streptomycin, 0.5 mg/ml

Penicillin, 500 units/ml

Mycostatin, 100 units/ml

Glass distilled water to make up to 500 cc.

It was often an advantage in the early stages to use 20 percent serum rather than 10 percent.

The remaining tumour mince was placed in a 20 ml glass universal container, and 10 ml of 0.25 percent trypsin in tris-saline was added. In some cases this was then placed in a cold-room for 24 hours to allow the trypsin to penetrate the tissue. The container was then placed on a wrist-action shaker and shaken for 30 to 120 minutes in the warm room (37°C), and left to stand for 3 minutes to let the larger tumour chunks settle to the bottom. The supernatant was placed in a 10 ml centrifuge tube and centrifuged for 10 minutes at 800 r.p.m. in an M.S.E. centrifuge, and the trypsin was discarded. The cell pellet was washed once with whole medium, resuspended in medium and inoculated into 100 ml "medical flat" bottles containing 10 ml of medium in an atmosphere of 5 percent CO_2 in air. During the whole procedure the pH was maintained just acid (6.8 - 7.0). At this stage each bottle contained single cells, clumps of cells and tumour fragments.

There now followed a long adaptive phase during which the tumour cells gradually adapted themselves to their environment until finally a firmly attached monolayer

culture of clonogenic single cells was obtained. In practice this adaptive phase lasted for 8 to 12 months, and could be divided into a number of distinct periods.

Initially, during the first two weeks, many of the cells failed to attach to the glass surface and died. With the first change of medium this debris was removed leaving behind firmly attached cells and a few tumour fragments. Dividing cells could be seen, although not all of their daughters remained attached.

Unless within the fourth week, a reasonably healthy culture consisting of many lightly attached cells was obtained, the culture would never grow further. By the fourth week it was usually possible to make the first subculture. Unattached cells were transferred to a new bottle containing fresh medium, and the attached cells resuspended by gentle trypsinisation, and washed off in a few ml of medium. This suspension was then transferred to new bottles each containing 10 ml of medium, varying numbers of cells, never less than 10^5 , being placed in each bottle. If, within two days, many healthy attached cells were seen, then a successful culture could usually be established. From this stage on cells became progressively better adapted to their environment, until within four to six months a healthy monolayer culture was established.

It is difficult to define exactly what was required to ensure success. This method only worked with tumours that gave large numbers of single cells during the initial trypsinisation, and in general, the higher the initial concentration of cells in the bottle, the greater was the probability of success.

2.2.3. The permanent cell line.

Once a permanent monolayer culture had been established it was necessary to test the cells' ability to form colonies, or clones. A clone is a population of cells derived from a single parent, and cells capable of forming clones are said to be clonogenic.

Puck and his colleagues were the first to demonstrate the ability of mammalian cells to form clones in vitro (Puck and Marcus, 1955). They developed the technique of using a feeder layer of radiation-killed cells. Test cells were plated ontop of these feeder cells which though unable to divide more than once or twice, were still metabolically active, and "conditioned" the medium to allow single cells to divide and form macroscopically observable colonies. Subsequent work has shown that not all cell lines require a feeder layer, for instance the V-79 Chinese hamster cell line.

As a source of feeder layer cells one can use the line being tested, or an already established cell line. The number of feeder cells needed will depend on the cell line being tested. After four months in culture RIB₅ cells had a plating efficiency of 15 percent when plated onto a feeder layer of 4×10^5 Ehrlich ascites cells in a 5 cm petri dish. The plating efficiency is the fraction of single cells plated which are viable. A viable cell is arbitrarily defined as one which gives rise to a colony of at least 50 cells after ten days.

The highest value of the plating efficiency after six months in culture was 0.19, with 2×10^5 RIB₅ cells as a feeder layer. After a further four months the plating efficiency had risen to 48 percent, and it soon increased

to 60 - 80 percent, which has been the normal value ever since. As the plating efficiency increased, so the "quality" of the colonies improved. Initially they were very diffuse, with cells spread over a wide area, but by the time the culture was well established, colonies were rounded and compact.

During the period of adaptation of the cells to their culture conditions their characteristics changed. This could be most clearly demonstrated when cells were reinoculated into rats. The resulting tumour had a volume doubling time twice that of the original RIB₅ tumour (see chapter 4).

2.3. Assaying the survival of tumour cells irradiated in vivo.

2.3.1. Methods available.

The first method developed for assaying the survival of cells in vivo was the cell titration technique of Hewitt and Wilson (1959). A single cell suspension was obtained from the tumour of interest and the number of these cells required to cause a given number of animals to develop tumours was determined. The surviving fraction of cells was then the ratio of this number for untreated tumours to that for irradiated ones. The technique has been used by a number of workers to determine cell survival curves (Reinhold, 1965; van Putten and Kallman, 1968; for instance). Its chief disadvantages are that a large number of animals are needed, and it can take many months to be sure whether or not a tumour will develop. Also, there is a significant immune response from the host animal to many of the tumours that are used in laboratory

experiments, so that a large number of cells is needed to induce a tumour; with RIB₅ at least 10^3 cells have to be injected to ensure that a tumour will develop.

Barendsen and Broerse (1969) have described a system which allows the characteristics of tumour cells irradiated in vivo to be assayed in vitro, and I have developed a similar system using cultured RIB₅ cells and the tumours that result when they are injected into rats. These tumours have been called RIB₅C, to distinguish them from the original RIB₅ tumours.

2.3.2. Techniques with RIB₅C.

Tumours were obtained in the following way. RIB₅ cells in culture were grown almost to confluence in three 500 ml medical flat bottles. They were then trypsinised, suspended in medium, and centrifuged at 900 r.p.m. in an M.S.E. centrifuge for 10 minutes. The supernatant was discarded and the tumour cell pellet mixed with sodium alginate, and tumour sausages formed and implanted into rats as described in section 2.1. In experiments when tumours were to be excised immediately after irradiation, a suspension of $5 - 12 \times 10^5$ cells in 0.05 ml of medium was injected subcutaneously into a rat's left flank. Growth of the resulting tumours (RIB₅C) could be measured in the normal way. Cell survival was measured as follows. Immediately after irradiation, a tumour-bearing animal was sacrificed, and its tumour excised aseptically and placed in a sterile petri dish. The tumour was finely minced with scissors and transferred to a 25 ml "vortex-beaker". 12 ml of calcium free Hank's medium (Hank and Wallace, 1949), containing

0.5 mg/ml trypsin at a pH of 6.8 - 7.0, was added and the tumour further minced for 15 minutes in an M.S.E. homogeniser. This suspension was then filtered through a number 2 sterile hospital dressing (12 ply) into a centrifuge tube and centrifuged for 10 minutes at 900 r.p.m. The supernatant was discarded and the cells resuspended in 3 ml of medium and filtered once more. They were then counted in a Hawksley blood haemocytometer using phase contrast microscopy, and after appropriate dilutions were plated into petri dishes containing feeder layer cells, and incubated for 10 days at 37°C in an atmosphere of 5 percent CO₂ in air. Colonies were formed with a plating efficiency similar to that for the cultured cells from which the tumours had grown.

This particular tumour was used rather than the original RIB₅ tumour because not only did it yield a single cell suspension with ease, but these cells had a high plating efficiency when plated in vitro, unlike those of RIB₅.

Probably the most critical step in the assay procedure was the cell counting. Cells were counted if

- 1) they had a well defined shape (approximately circular)
- 2) they were not granular in appearance
- 3) they had a sharp halo surrounding the cell
- 4) they showed no sign of vacuolation or pyknosis.

Aggregates of cells were virtually never seen.

The chief disadvantage of enclosing pellets of cultured cells in pieces of small intestine was the low yield of tumours, 20 - 40 percent; this could be increased

to 40 - 60 percent by omitting the small intestine, but then the tumours tended to be sausage-shaped which rendered them unsatisfactory for growth measurements. The high yield of tumours (70 - 100 percent) following direct subcutaneous injection of cells was to a certain extent offset by the occurrence of broken-up tumours, probably due to seeding of cells along the needle track, and by non-uniformities in tumour shapes. Thus the high plating efficiency using RIB₅C has in part been offset by the low yield of suitable tumours. Nevertheless, the advantage of a quick and easy method for assaying cell survival is considerable.

2.4. Tumour irradiation techniques.

2.4.1. X-rays.

The apparatus and the techniques used to irradiate the tumours were those described by Thomlinson and Craddock (1967). A tumour bearing animal was anaesthetized with an intra-peritoneal injection of sodium amylobarbitone, and then placed on its side on a perspex couch in a special radiation vessel containing gas inlets and outlets, a pressure gauge, and a temperature control capable of maintaining the temperature at 28-30°C. The rat's position was arranged so that the tumour was located at the center of a one inch field defined by a thick ring of lead (figure 3). To reduce the incidence of diarrhoea, as little of the abdomen as possible was included in the field. The distance of the midpoint of the tumour to a fixed point of the vessel determined the exposure time necessary to deliver the particular dose of radiation. A hemispherical perspex lid was then placed over the rat

(figure 4) and the whole jig located in a standard position under the X-ray set head by means of a lead collimator fixed to the head. The vessel could then be flushed with the appropriate gas prior to and during irradiation. For aerobic irradiations, air was passed through the chamber at a flow rate of about 1 litre per minute. To render a tumour hypoxic, a U-shaped perspex clamp was applied 5 minutes before irradiation to occlude the blood supply to it. When irradiations were given with an animal breathing oxygen at high pressure, it was anaesthetised and placed in the vessel in the normal way. The lid was then bolted to the base and oxygen flushed through at 10 l/min. for 10 minutes. The internal pressure was then raised slowly to 10 lbs/sq. inch, to avoid any trauma to the rat, and in particular, to ensure that there was no movement. The pressure was then increased to a final value of 30 lbs/sq. inch and maintained for 30 minutes prior to irradiation. During all stages the respiration rate was monitored. Immediately before and after irradiating an unclamped tumour, X-ray films were exposed to check the position of the tumour in the beam. After irradiation, animals were given an intraperitoneal injection of megamide, and returned to their cages.

2.4.2. Neutrons.

The technique used was that described by Field, Jones and Thomlinson (1967). The neutrons were produced in the Medical Research Council Cyclotron by bombarding a beryllium target with 17 Mev deuterons. This gave a neutron beam with an energy spectrum of from 0 to 21 Mev

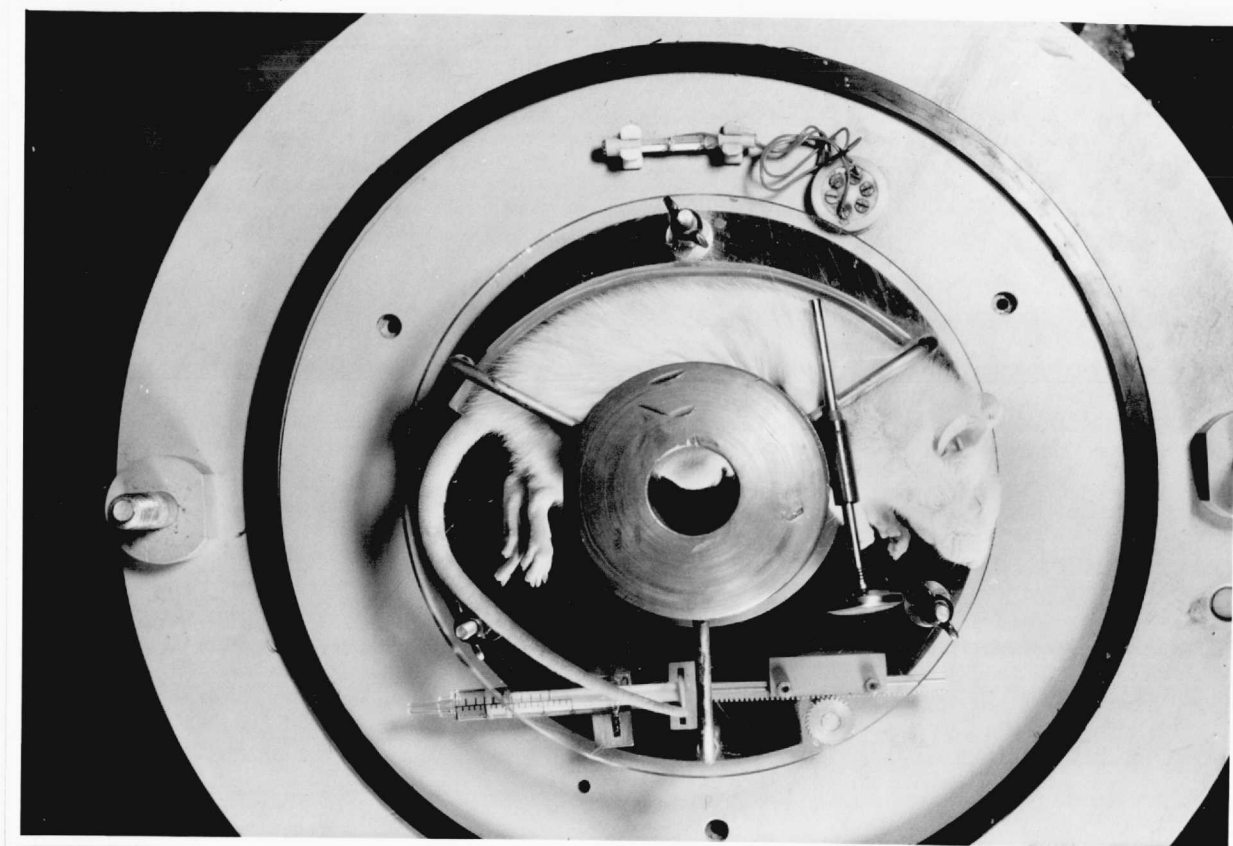


Figure 3

Pressure vessel showing tumour located beneath the lead collimator (from Thomlinson and Craddock, 1967).

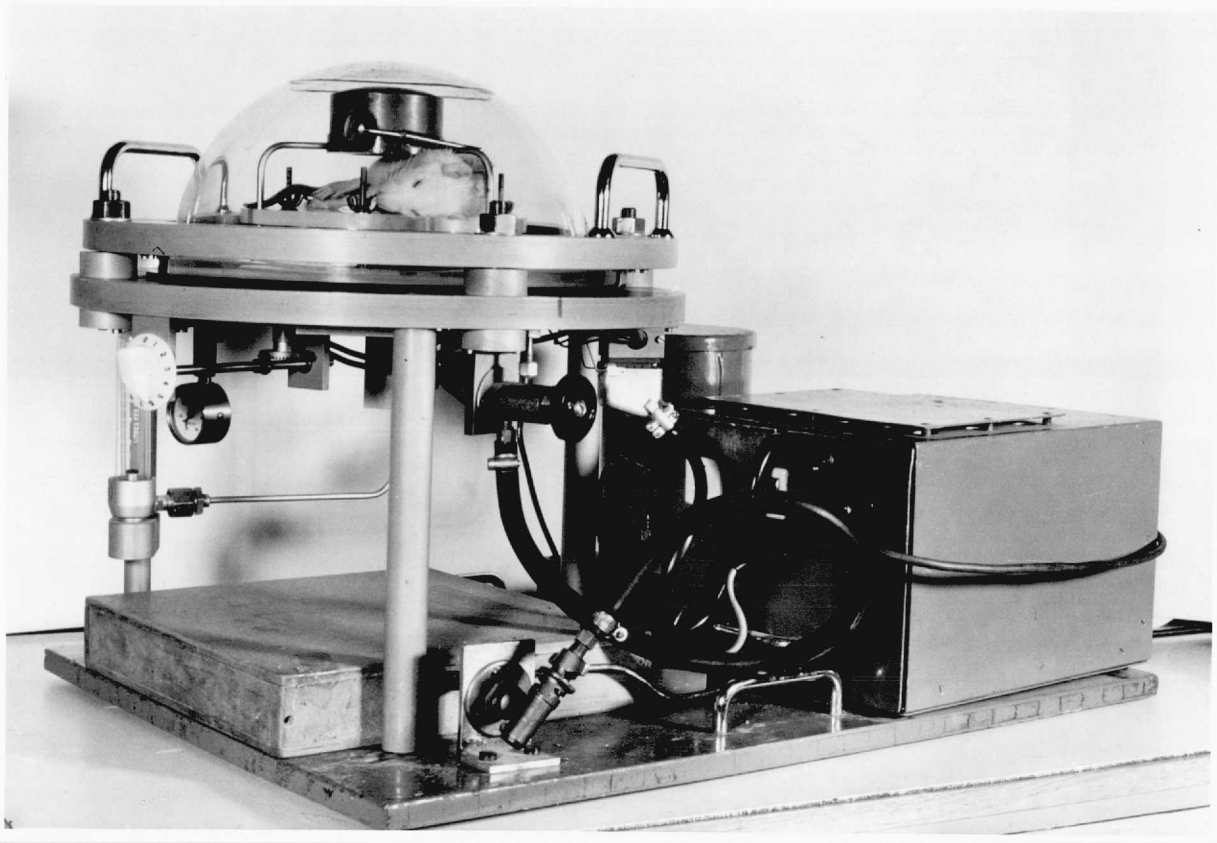


Figure 4

Pressure vessel with Perspex dome (from Thomlinson and Craddock, 1967).

with a modal value of 6 Mev. The neutron facility and dosimetry have been described by Bewley (1963) and by Bewley and Parnell (1969). The neutron dose rate was about 50 rads per minute and contamination due to X-rays was less than 5 percent.

The arrangement for tumour irradiations is illustrated in figure 5, and consists of a collimator designed to produce two divergent beams so that two tumours could be irradiated at the same time. The collimator was constructed of borated polythene containing two steel tubes having a half inch wall thickness and one inch internal diameter. The two rats were placed one above the other in front of the collimator at a distance of 75 cm. from the beryllium target. The tumours were aligned prior to treatment by placing the anaesthetized rats on couches on an aluminium base-plate which was then attached to a mock collimator. Any movement of the animals during irradiation was monitored by a T.V. camera.

2.5. Dosimetry.

2.5.1. Tumour irradiations.

A detailed description of the dosimetric techniques will not be given, as they have been described elsewhere. They can be considered in two parts, for X-rays and for neutrons.

A. X-rays. All tumour irradiations were performed using the arrangement described by Thomlinson (1960) and by Thomlinson and Craddock (1967). The source of radiation was a Marconi Industrial Model 250 Kv X-ray set, operated at 250 Kv and 15 ma., with filters of 0.5 mm. copper and 1 mm. of aluminium. The mid-point of the tumour was

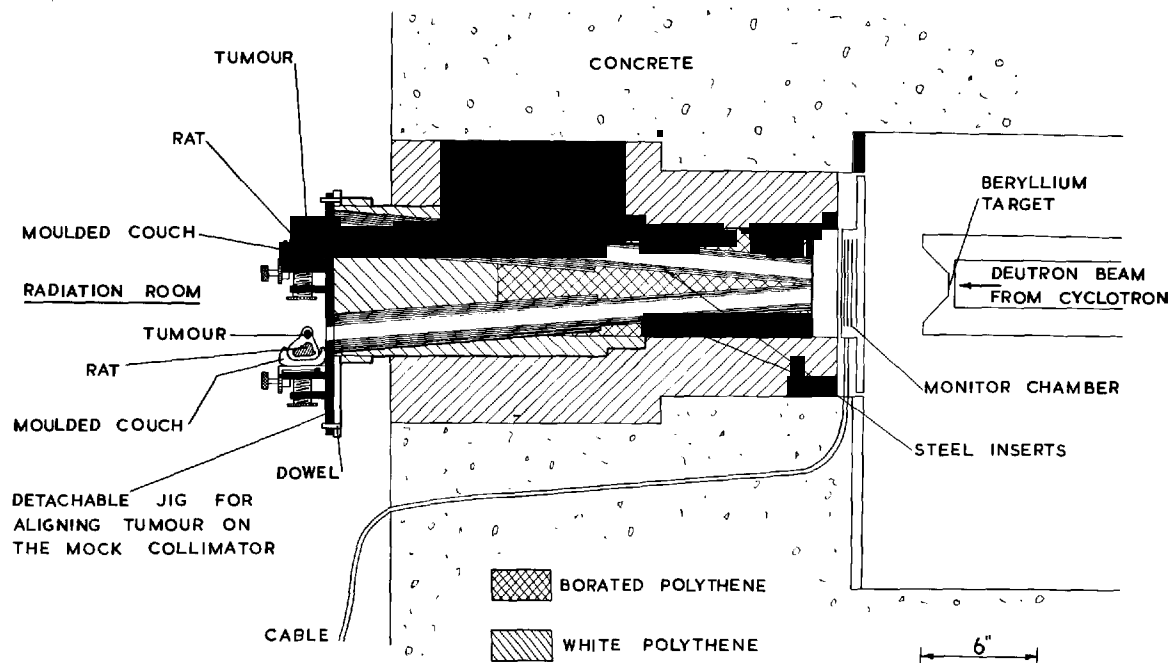


Figure 5

Vertical section through neutron target and collimator showing arrangement for irradiation of tumours with neutrons (from Field et al, 1967).

approximately 21 cm. from the X-ray tube target. The dose was monitored during irradiation by a parallel plate ionization chamber in front of the shutter, connected to an integrated current meter. The current was correlated with the dose received at the mid-point of the tumour as measured with a BD2 chamber calibrated against a sub-standard Baldwin Farmer Ionization Chamber. The stability of the X-ray set output and the monitor were checked at regular intervals of not more than three months. The divisions per minute recorded by the monitor gave a day-to-day check on the tube output.

B. Neutrons. The neutron dosimetry was determined by the Cyclotron operators and the techniques have been described by Bewley (1963), Parnell, Bewley, and Field (1967), and Bewley and Parnell (1969). Briefly, ionization chambers were used in pairs, one sensitive to both neutrons and X-rays by virtue of the presence of hydrogen in its wall, and the other with no hydrogen in its wall and a reduced sensitivity to neutrons. The X-ray contamination could be assessed more accurately using a small field and CaF_2 powder, or thin photographic sheets (Bewley and Parnell, 1969).

2.5.2. Irradiations in vitro.

All irradiations were with a Phillips Therapy Machine operated at 250 Kv and 15 ma., h.v.l. 0.3 mm. Cu. Irradiations were performed with petri dishes on trays at each of three levels in a special perspex box. This box had outer dimensions 18.5 x 18.5 x 19 cm. and was placed with its top surface 20 cm. from the filter housing of the X-ray set (figure 6). The dose was monitored by a parallel

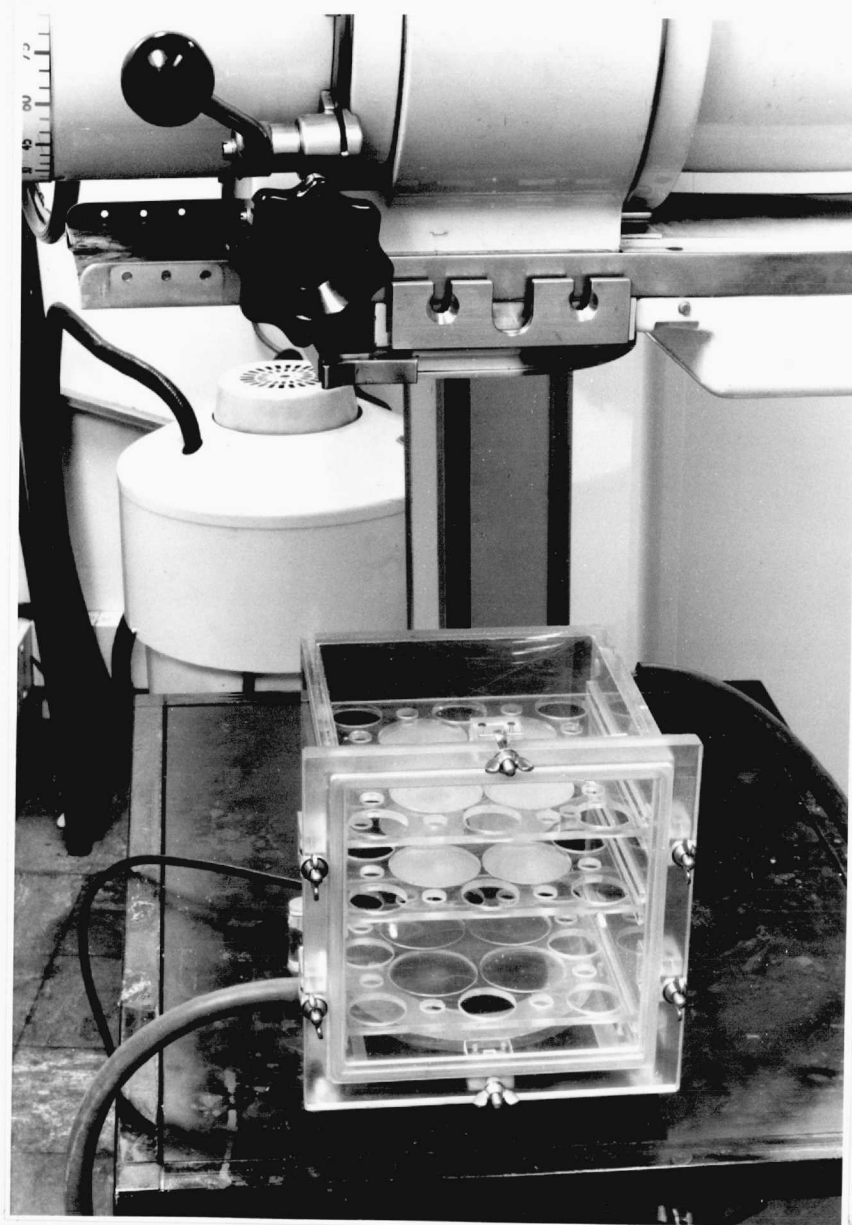


Figure 6

Perspex box for irradiation of cells in vitro
with 250 Kv X-rays.

plate ionization chamber in the base of the perspex box, connected to a ratemeter. The dose to the cells and the dose distribution across the field were determined using lithium fluoride capsules and BD2 chambers. The dose rate varied from 250 rads/min. on the top shelf, to 150 rads/min. on the bottom shelf.

Chapter 3

THE CHARACTERISTICS OF CULTURED RIB₅ TUMOUR CELLS AND THEIR RESPONSE TO RADIATION

3.1. The cell cycle.

3.1.1. Introduction.

On the basis of light microscope observations, prior to the use of radioactive precursors, the mammalian cell cycle was described as consisting of two main morphologically distinct phases, interphase, in which the cell spent most of its time and during which the cell nucleus was visible, and mitosis, a short phase during which the chromosomes were visible and cell division occurred. With the development of the Feulgen dye staining technique, Walker and Yates (1952) were able to show that DNA synthesis occurred during interphase. In 1953, Howard and Pelc, using P³²-labelled precursors and autoradiography, suggested that the cell cycle could be represented in terms of progression around a clock face, the phases of the cycle being represented as sectors on that face. This cycle is illustrated in figure 7. M and S represent the mitotic phase and the period of DNA synthesis, while G₁ and G₂ are gaps during which no DNA synthesis occurs.

3.1.2. The labelled mitosis curve.

The cell cycle can conveniently be studied by exposing cells to a brief pulse of tritiated thymidine (³HTdr) and measuring the appearance of labelled mitoses at various times after the pulse. If the period of labelling is brief, only those cells in the S-phase will incorporate the label into their DNA. As the culture grows, the

labelled cells will pass through mitosis as a wave of labelled mitotic figures. By measuring the percentage of mitoses labelled as a function of time after pulse labelling, the duration of the phases of the cell cycle can be measured (figure 8A). This technique is the basis for most measurements of the cell cycle both in vivo and in vitro.

3.1.3. Autoradiographic techniques.

The techniques employed with cultured cells were as follows. Between 2 and 4×10^5 cells from a culture in logarithmic growth were inoculated into each of a number of 5 cm. plastic petri dishes (Nunc) containing previously cleaned and sterilized glass cover-slips. After an appropriate period of growth in an atmosphere of 5 percent CO_2 in air at 37°C , pre-warmed medium containing $^3\text{HTdr}$, specific activity 5000 mCi/M, was added to give a final concentration of $0.1 \mu\text{Ci/ml}$ per dish. After 30 minutes incubation, this medium was removed, the plates were rinsed once with warm medium, 3 ml. of fresh medium was added, and they were returned to the incubator. The time of removal of the radioactive label was taken as time zero. At hourly intervals thereafter plates were removed from the incubator and the cover-slips placed in a solution of 3 parts methanol to 1 of glacial acetic acid for 15 minutes to fix the cells. The cover-slips were then attached to slides with D.P.X. mounting medium with the cells uppermost. When the slides were quite dry, the cells were stained with aceto orcein for 45 minutes at 37°C , rinsed in 45 percent acetic acid and allowed to dry. They were then ready for autoradiography.

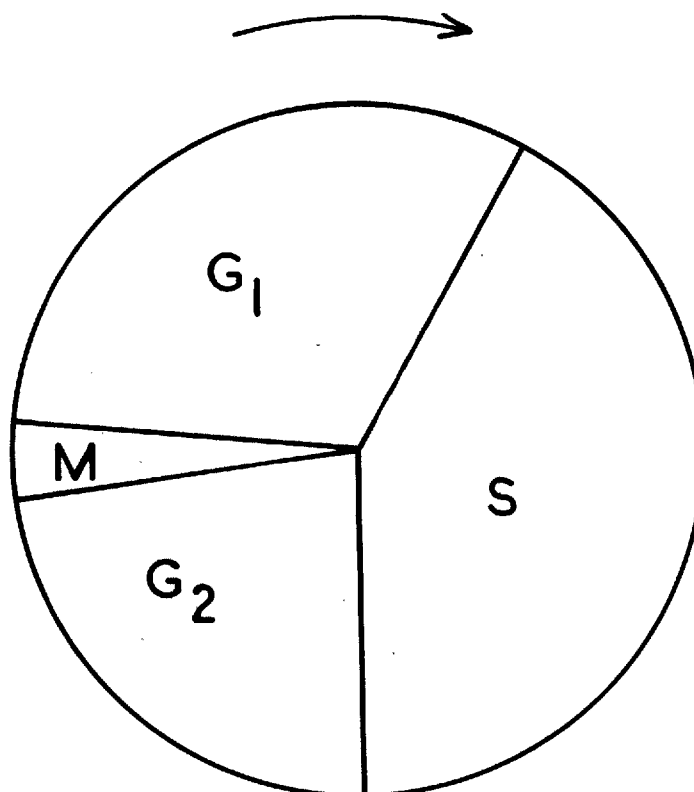


Figure 7

The cell cycle. The arrow indicates the direction cells move through the cycle.

The autoradiographic procedure, performed in the dark room using Wratten number 1 and 2 filters, was as follows:-

1. A water bath was set at 50°C and an appropriate amount of nuclear emulsion gel (Ilford K5, grain size 0.2 μ) placed in a 50 ml. beaker in it. After about 15 minutes, when the gel had melted, an equal volume of distilled water was added. The solution was stirred to ensure an even consistency and a clean slide dipped in, to test for the presence of air bubbles.

2. Slides were then dipped in and out of emulsion which had been poured into a special box big enough to admit a single slide, placed on a drying rack, and subsequently in a drying box containing a fan at one end.

3. When dry, the slides were placed in a light-tight box containing a small amount of silica gel to remove residual moisture, and stored at 4°C for a period of one to three weeks.

4. After the appropriate exposure time, the autoradiographs were developed in Kodak D19B developer at 16°C for 5 minutes, rinsed in tap water, and fixed in a 1 in 5 solution of Johnson's Fix-Sol in distilled water for a further 5 minutes, allowed to dry over-night, and then cover-slips were mounted on top with Euperal.

The Kodak D19B developer was made from the powder according to the manufacturer's directions and filtered before use. It was not kept longer than 2 days, and was discarded if any discolouration occurred.

The radioactive decay of tritium in labelled thymidine can cause similar effects to those caused by external radiation, ranging from division delay to cell killing

(see Whitmore and Gulyas, 1966, for example). The radiation effect depends quantitatively on the number of $^3\text{HTdr}$ molecules incorporated into the DNA, and this will be determined by the specific activity, the total amount of thymidine present, and the time of availability of the radioactive label. When short labelling periods are used, only those cells synthesising DNA will be affected. For instance, Drew and Painter (1962) found that a 30 minute pulse of $2\text{ }\mu\text{Ci/ml. }^3\text{HTdr}$, specific activity 1.9 Ci/mM , reduced the survival of HeLa cells by only about 1.2 percent. The specific activity used in the present studies was a factor 400 times less than this, and has been assumed to have had no effect on the cells.

3.1.4. Results.

Figure 8B shows the results of an experiment in which cells in logarithmic growth were exposed to a 30 minute pulse of $^3\text{HTdr}$, and the appearance of labelled mitoses was followed thereafter. Each point represents a count of at least 100 mitoses. The duration of the phases of the cell cycle were measured at the 50 percent mitoses level from this curve, and yielded values:-

$$T_C = 17 \text{ hours}$$

$$T_S = 8.5 \text{ "}$$

$$T_{G2+\frac{1}{2}M} = 3.5 \text{ "}$$

$$T_{G1+\frac{1}{2}M} = 5 \text{ "}$$

The growth fraction, that fraction of the population that was progressing through the cell cycle, was measured by exposing cells continuously to the radioactive label for periods up to 19 hours. At the appropriate times cells were removed from the label, rinsed and immediately

fixed. Autoradiographs were prepared and the fraction of cells labelled measured as a function of time exposed to the label. The results are shown in figure 9. Ideally, in a population in which all cells are in cycle with the same cell cycle time, the fraction of cells labelled should reach 100 percent in a time equal to $T_C - T_S$. From figure 8B this should be 8 - 10 hours; in fact it was nearer 16 hours suggesting that there was a significant spread in the cell cycle times of the individual cells.

Referring again to figure 8B, measurements at the 50 percent labelled mitoses level yield median values for the cell cycle parameters, and if the distribution of phase durations is not symmetrical, these will not be the same as the mean values. A plot of the slope of the labelled mitoses curve shows the rates of appearance and disappearance of labelled mitoses (figure 8C). The frequency distribution relating the number of cells with a given G2 period is indicated by the first rise and fall, and gives a most probable value of 3.5 hours (Stanners and Till, 1960). The difference in the widths of the first two peaks, showing that the initial rise in the labelled mitoses curve is faster than the subsequent fall, is a measure of the variation in the duration of the S-phase. The difference in the slopes of the labelled mitoses curve for the first fall and second rise, the difference in the heights of the second two peaks in figure 8C, can be interpreted as due to variations in G1 as well as G2. All these variations contribute to the departure of the experimental labelled mitoses curve shape from that of the theoretical one, and confirm the spread in cell cycle times.

Figure 8

The labelled mitoses curve. The scales are linear.

A. Theoretical curve. T_M =period of mitosis.

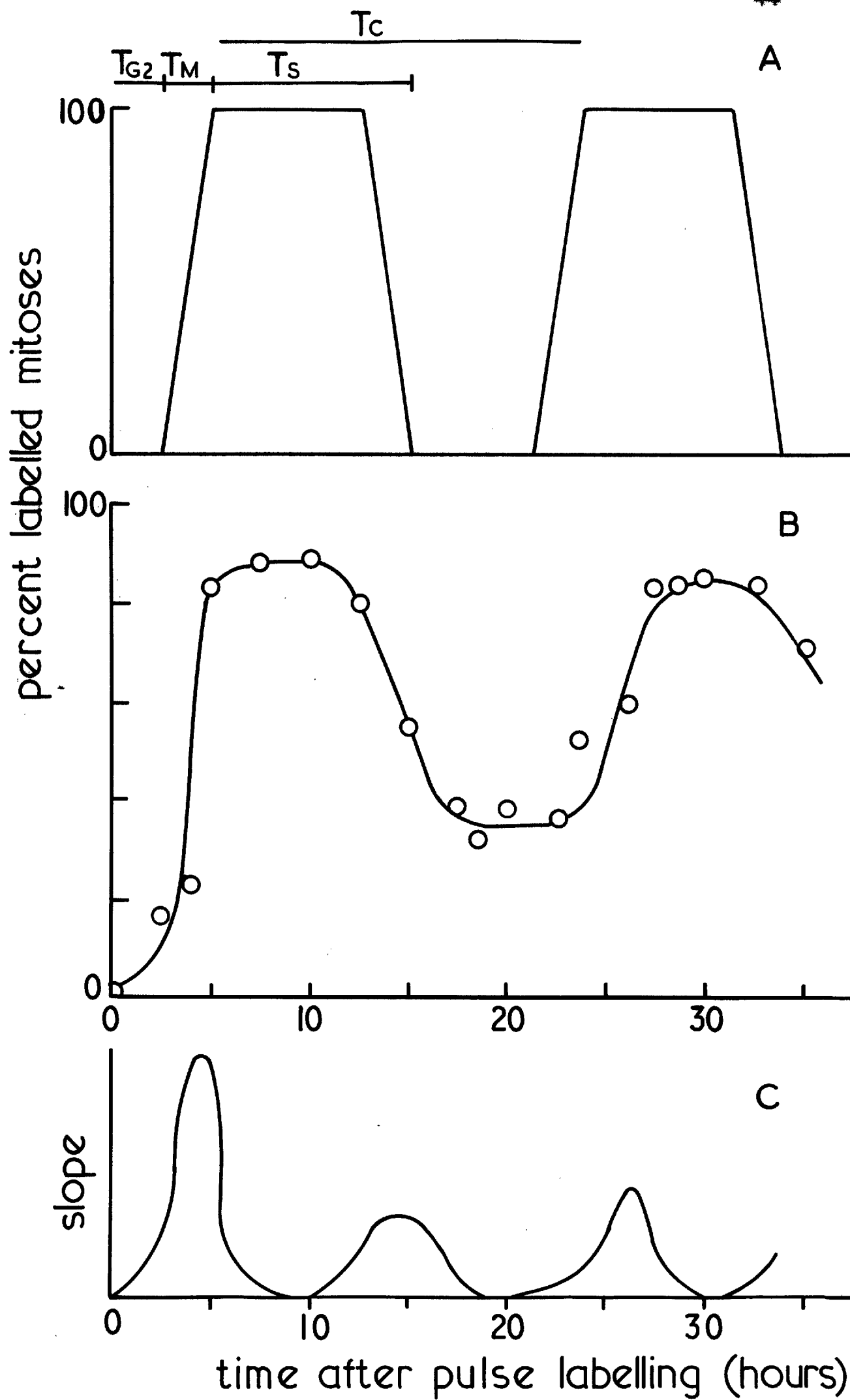
T_{G2} = period between DNA synthesis
and mitosis.

T_S =period of DNA synthesis.

T_C =cell cycle time.

B. The measured curve for cultured RIB₅ cells.

C. The slope of the measured labelled mitoses curve
as a function of time.



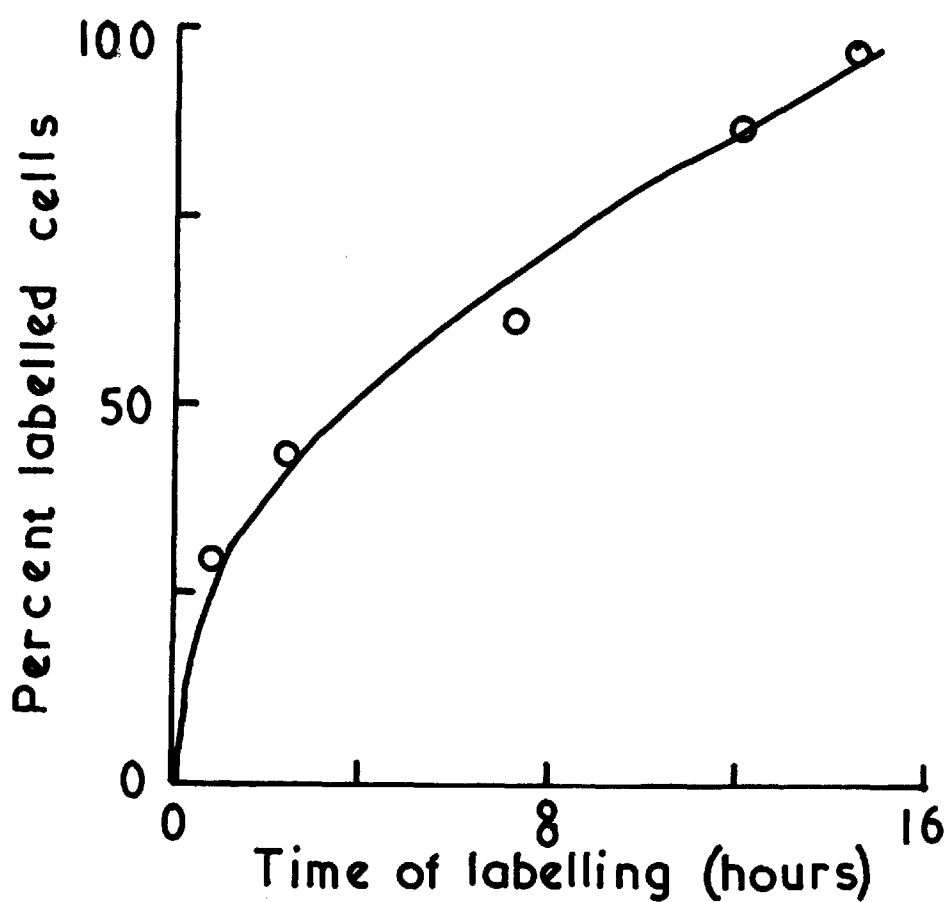


Figure 9

The fraction of cells labelled as a function of time of exposure to the label. Each point represents a count of at least 1000 cells.

3.2. The growth curve.

Perhaps the easiest parameter of growth to measure is the population doubling time T_D . It is most easily determined by measuring the growth curve of the population. A group of plates is inoculated with identical numbers of cells and at times after inoculation, individual plates are taken from the incubator, the cells removed by trypsinization and counted, and the number of cells per plate plotted as a function of time. For routine counting a haemocytometer is a convenient instrument to use, however, for the multiple counts required when measuring growth curves, a Model F Coulter Counter with a 100 μ aperture was used. The results of a typical experiment are shown in figure 10A. This curve is qualitatively similar to those obtained with many cell lines, and can be divided into three parts:-

1. The lag phase. This can be thought of as a period of adaptation of the cells to their environment, and the extent of the lag often depends on the number of cells plated per unit area. With RIB₅ cells, if less than 10^4 cells were inoculated into a 5 cm. petri dish, they had considerable difficulty in growing at all.

2. The period of logarithmic growth. Assuming the growth fraction remains constant, once past the lag phase, the population will grow exponentially according to the equation

$$N = N_0 \cdot e^{kt}$$

$$\text{where } k = \frac{\ln 2}{T_D}$$

The population doubling time, T_D , is not necessarily the same as the cell cycle time, T_C . The two parameters will only have the same value when the growth fraction is one

and there is no cell loss or death.

From figure 10A we obtain a value for T_D of 18 hours, which is about the same as the value of T_G measured from the labelled mitoses curve. One would expect the two parameters to be the same since the growth fraction was one (figure 9), and the absence of unattached cells in the culture medium indicated that there was little or no cell death.

3. The stationary phase. When the number of cells per dish exceeded 10^6 the growth rate decreased and the growth curve plateaued. This part of the curve is known as the stationary phase, and can result either through depletion of nutrients or a lack of space. The properties of cells in this phase will be discussed in more detail later (section 3.4.), however, it should be emphasised that in this context the term "stationary" only means that the overall population number does not increase, and perhaps a better term operationally would be "plateau phase".

3.3. The response of lag and logarithmic phase cells to radiation.

3.3.1. Introduction.

In this section we shall be concerned primarily with the lethal effects of radiation on cells. The killing of a cell by radiation is a complex process involving first the absorption of energy and then the conversion of this energy into a molecular lesion which can express itself biologically. The basic theory which has been developed to explain the observed dependence of survival on dose depends on the concept of one or more discrete "targets"

which have to be "hit" one or more times in order that a cell shall be inactivated. This target theory, first described by Dessauer, Crowther and others, has been developed in some detail by Lea (1962) and by Zimmer (1961). Briefly, if we define the dose of radiation delivered (D) in terms of the number of inactivating events, or hits, per unit volume, and the sensitive volume (target size) as V, then the average number of hits per target is VD. On the assumption that the hits are distributed at random through the medium, the fraction of targets with exactly m hits is given by the Poisson formula, and is

$$\frac{e^{-VD}(VD)^m}{m!} \quad - - - - - 1.$$

If D_0 is the dose which on average gives one hit per target, $VD_0 = 1$ and equation 1 becomes

$$\frac{e^{-D/D_0}(D/D_0)^m}{m!}$$

If m hits are required in each of n targets to inactivate the cell, then the probability of survival is

$$S_{m,n} = 1 - (1 - B)^n \quad - - - - - 2.$$

where

$$B = e^{-D/D_0} \sum_{m=0}^{m-1} \frac{(D/D_0)^m}{m!}$$

This is the most general form of the equation for cell survival. The two most common simplifications are $m=n=1$, which gives the exponential relation, and $m=1, n=n$, the single-hit, multi-target form, giving

$$S_{1,n} = 1 - (1 - e^{-D/D_0})^n \quad - - - - - 3.$$

A plot of $\ln S$ against dose gives a straight line of slope $1/D_0$ at high doses. This straight line extrapolates back to $\ln n$ at zero dose.

The various models based on equation 2 have been

discussed by several people (Fowler, 1964; Kellerer, 1966; Zimmer, 1961). In practice it is probably not meaningful to try and distinguish between these models. Nevertheless, target theory provides a formal framework for handling the biological data.

3.3.2. Radiation and the growth curve.

The effect of single doses of radiation on the growth of RIB₅ cells is shown in figure 10B. Cells were grown as described in section 3.2., and about 24 hours after plating were exposed to the radiation, and growth measured thereafter. The data have been normalised to unity at the time of irradiation. Each point is the mean of the total population count for two plates.

There are three basic features of these curves. After relatively small doses of radiation there was a delay in cell division, but once this was overcome, cells continued to grow at the normal rate. As the dose increased, the division delay increased, the growth rate decreased, and the final plateau level of the population also decreased. A dose of 500 rads will kill about 95 percent of the population, so that the presence of dead cells might affect the growth of survivors. However, this was not the case, since the addition of 2×10^5 radiation killed cells to the growing population 24 hours after plating had no effect on the growth curve (figure 11)

Alterations in growth after irradiation, such as those illustrated in figure 10B, will affect the ability of cells to form colonies, and this will be discussed in the next section

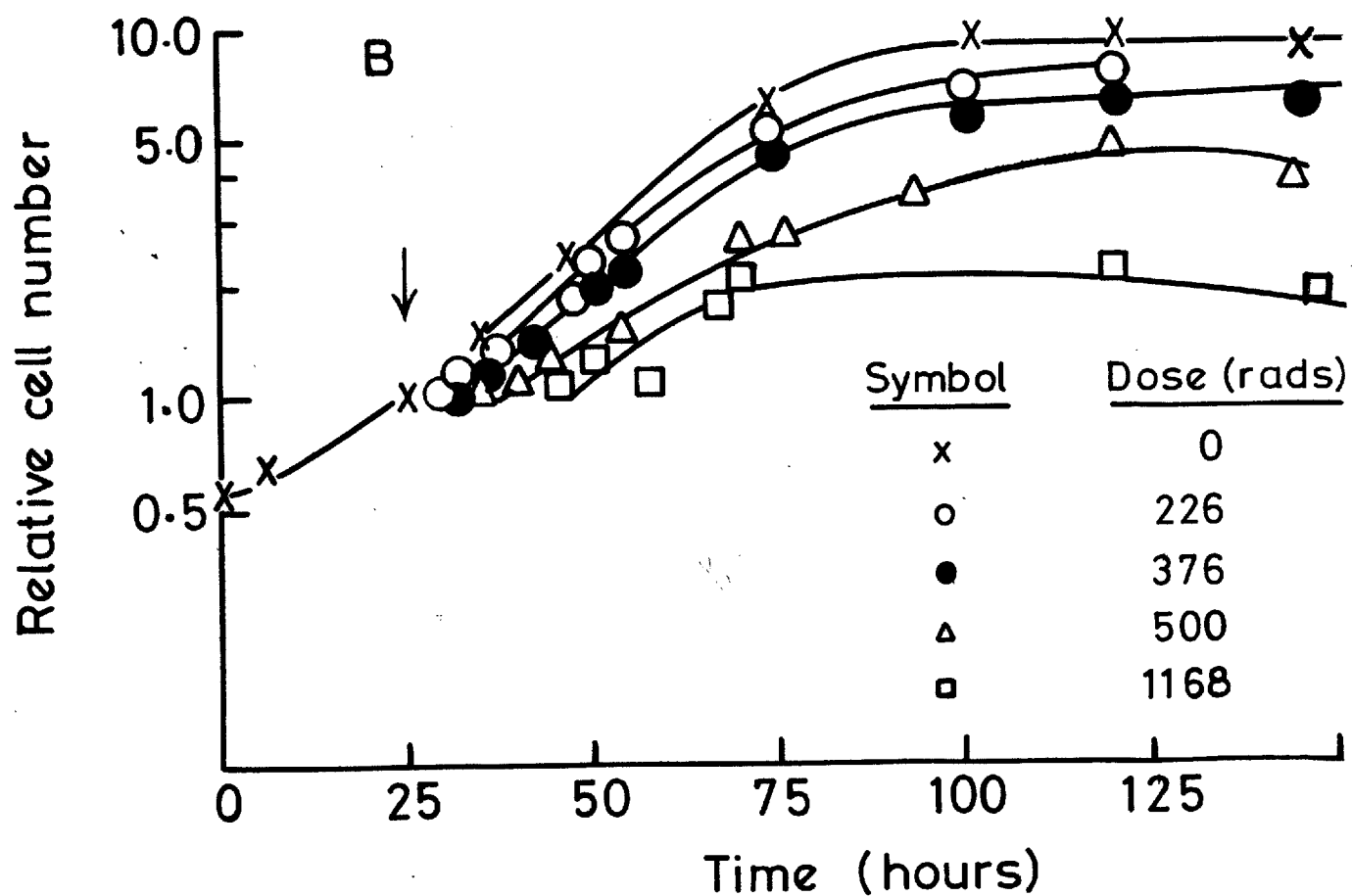
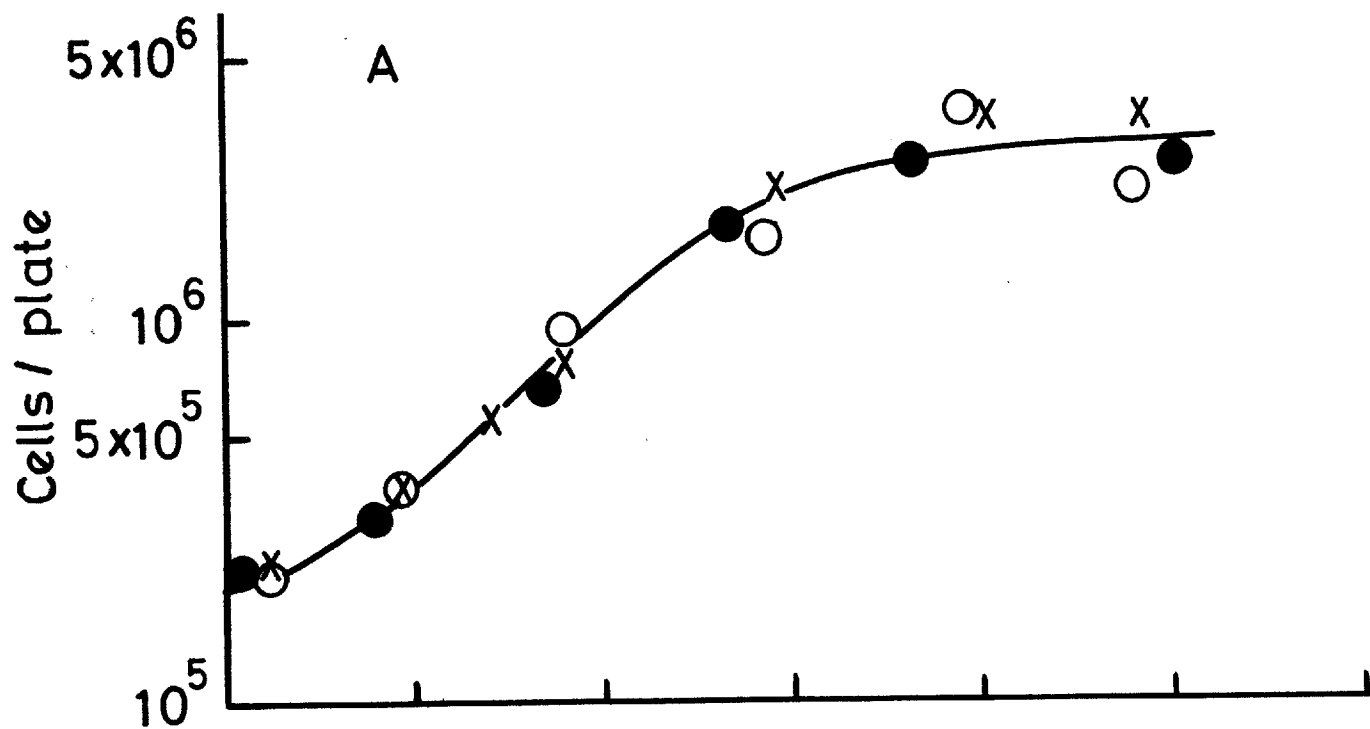
Figure 10

Growth of RIB₅ cells in vitro.

A. Growth curve for unirradiated cells.

B. Relative cell number following irradiation.

The arrow denotes the time of irradiation.



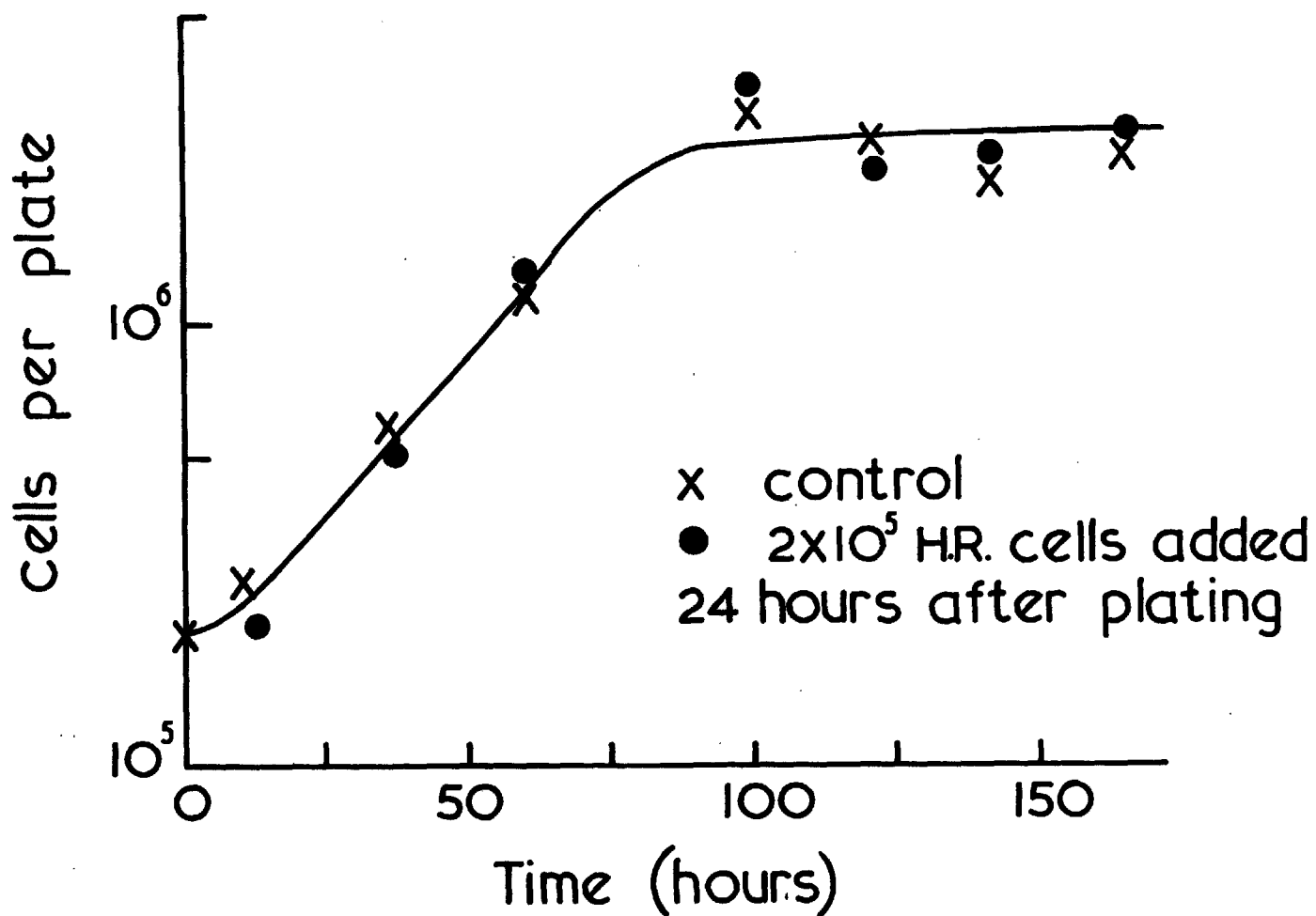


Figure 11

The effect of heavily irradiated cells
on growth of unirradiated cells.

3.3.3. Radiation and cell survival.

In this section we shall be concerned with logarithmic cells, or cells plated from a logarithmic population, and treated soon after. The experimental techniques were outlined in chapter 2. The standard adopted for cell viability was that a cell gave rise to a colony containing 50 or more cells ten days after plating. The requirement for 50 or more cells is arbitrary, since the number of cells per colony after irradiation will depend on the time at which the colonies are examined as well as on the dose of radiation. Figure 12 shows measurements of the diameters of colonies as a function of the dose, for cells fixed ten days after irradiation. As the dose of radiation increased, the colony size distribution broadened and the median diameter decreased, an effect to be expected on the basis of the effect of radiation on growth (figure 10). This induction of small colonies is an example of a non-lethal effect of radiation which is ignored when cell survival is measured by counting colonies (Sinclair, 1969).

Because the colony size distribution depends on the dose of radiation (figure 12), the time at which colonies are fixed and counted might affect the shape of the measured survival curve. Figure 13 shows that with RIB₅ cells, after eight days survival was independent of the time of scoring.

In addition to the factors already discussed, temperature, pH, changes of medium, etc., can all affect the shape of the survival curve (Sinclair, 1966), and this emphasises the need to standardise conditions. In the present experiments, plating procedures and irradiations were performed at room temperature; at all other times

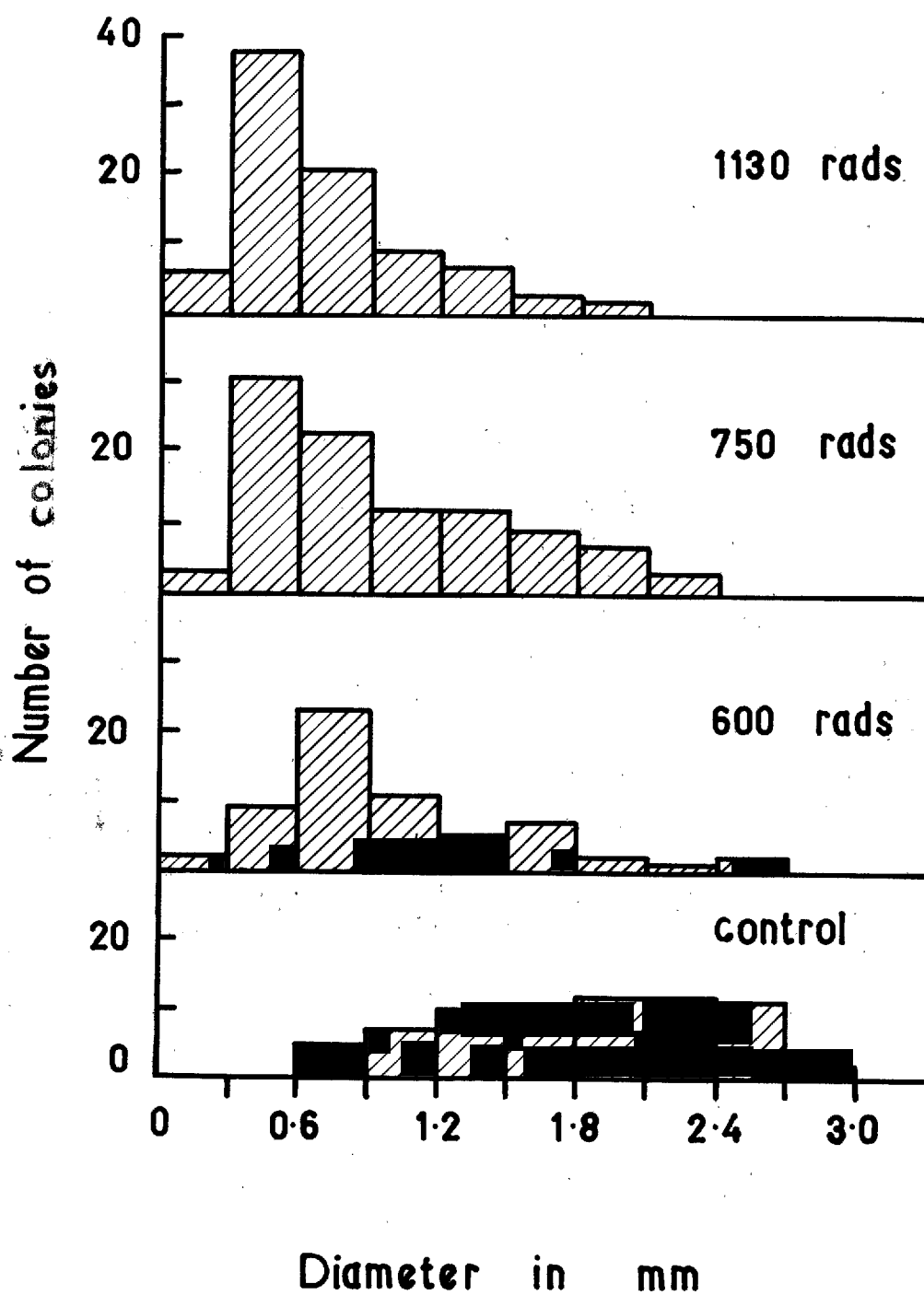


Figure 12

The effect of radiation on colony diameters.
Colonies were measured 10 days after irradiating
single cells.

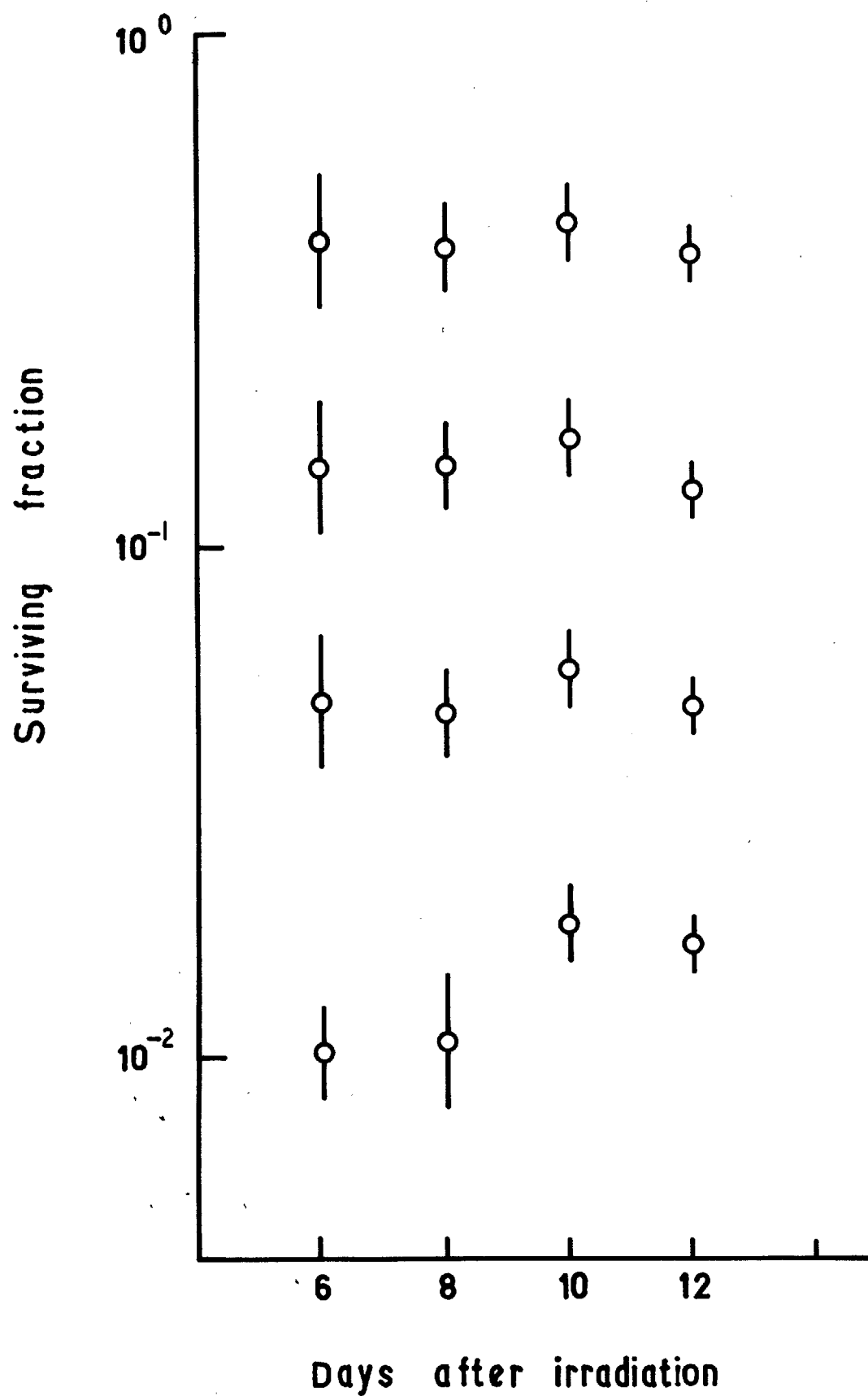


Figure 13

Surviving fraction of RIB₅ cells as a function of the time after irradiation that the colonies were fixed and stained.

cells were kept at 37°C in an atmosphere of 5 percent CO₂ in air.

Figure 14 shows survival curves for RIB₅ cells irradiated four hours after plating. The points represent the results of six separate experiments. Cells were counted in a haemocytometer and enough were plated to give about 100 colonies per dish. They were irradiated four hours later with the medium removed. After irradiation 7 ml. of fresh medium were added and the plates returned to the incubator. The two curves of figure 14 represent survival data for cells irradiated in aerobic and hypoxic conditions. In order to achieve a satisfactory level of hypoxia, "white spot" nitrogen (British Oxygen Co.) was passed through three wash bottles containing distilled water at 37°C and then into the irradiation box at a flow rate of 6 litres per minute for 8 minutes prior to irradiation, and at 1 litre per minute during irradiation. With these conditions, the effluent gas contained less than 20 parts per million of oxygen as measured with an Engelhard Mark 2 Hersch Cell. The lines of figure 14 are those calculated according to the method of Pike and Alper (1964), which considers the validity of two curves having a common extrapolation number. (Unless otherwise stated, all survival data to be presented have been treated according to this method). The survival curve parameters are shown in table 1.

Since these experiments were performed, it has been shown that polystyrene petri dishes can hold oxygen in solution which can be released slowly through the cells attached to the surface, maintaining their oxygen tension

(Chapman et al, 1970). Because of this, the measured oxygen enhancement ratio will be an underestimate of the true value. This effect is clearly demonstrated in figure 15 which shows survival curves for cells plated from the same stock culture onto both polystyrene and glass petri dishes, and then irradiated.

12 percent survival curves (table 1).

A dosimetric problem arises with experiments using glass petri dishes. The medium and low energy X-rays from a 250 Kv machine will give rise to a significant number of photoelectrons when they interact with the glass, and a proportion of these will be scattered backwards depositing energy in the cells attached to the glass. Because of the short range of the electrons, their contribution to the dose received by the cells will be difficult to measure. In the experiment of figure 15 the irradiation time for cells on glass was 0.69 times that for cells on polystyrene. This gave reasonable superposition of the aerobic survival curves.

The growth curves of figure 10B demonstrate a phenomenon that has not previously been clearly described in experiments on growth of cell following irradiation. The decrease in the value of N_s , the maximum cell number, with increasing dose of radiation appears to contradict the results of figure 12, showing that even after a dose of 1130 rads a significant proportion of the colonies developing from single surviving cells reached the same size as those from unirradiated cells. These results suggest that the behaviour of individual cells depends on whether or not they are surrounded by other dividing cells. This might have some bearing on the difference

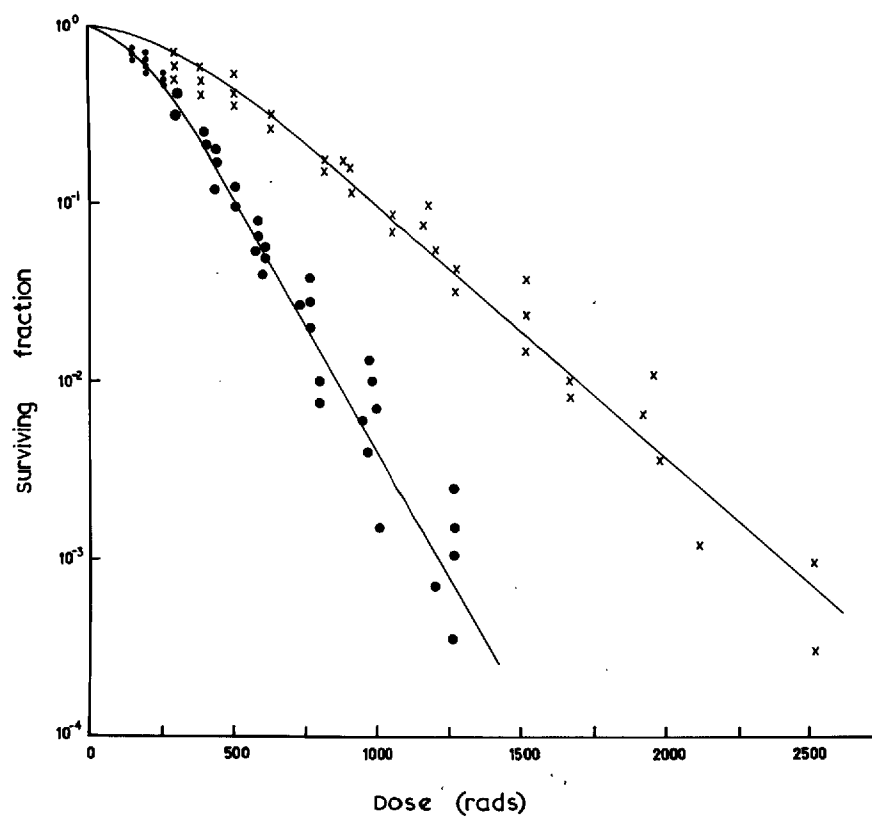


Figure 14

Survival curves for RIB₅ cells irradiated in aerobic (●) and hypoxic (x) conditions four hours after plating into plastic petri dishes from a population in logarithmic growth. The points represent the results of six separate experiments.

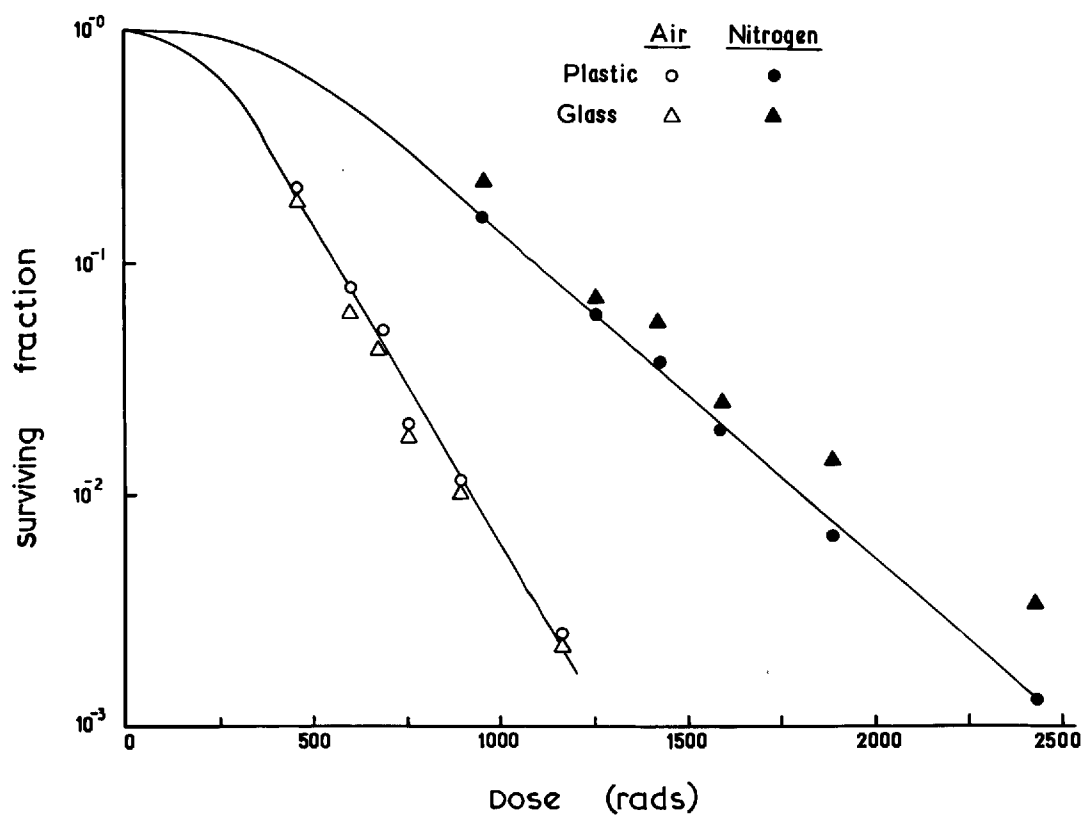


Figure 15

Survival curves for cells irradiated in glass and plastic dishes. Cells were plated from the same stock culture into both types of dish. The lines were drawn using only the results for cells in plastic dishes.

Conditions	n	Do (air), rads.	o.e.r.
Plastic (pooled results)	2.2 (1.8-2.7)	166 (157-175)	1.92 (1.83-2.02)
<u>Plastic v. Glass</u>			
Plastic	3.5 (2.5-4.8)	157 (147-168)	1.96 (1.89-2.03)
Glass	2.8 (1.8-4.3)	161 (147-178)	2.22 (2.11-2.30)

Table 1

Survival curve parameters with their 95% confidence limits for RIB₅ cells irradiated four hours after plating from a population in logarithmic growth.

between the response to radiation of a whole tumour and that of its individual cells, as assayed in vitro (see next chapter).

3.4. A comparison of the properties of logarithmic and stationary phase cells.

3.4.1. Introduction.

Models designed to explain the results of clinical radiotherapy using cells in culture have been based on results using cells in lag or early log phase (Elkind, 1960; Elkind, Withers, and Belli, 1968). However, many tumours contain cells in a non-proliferative state, resulting in a growth fraction of less than one (Mendelsohn, 1960, 1962; Demekamp, 1970). As a result, Hahn (1968), and Hahn, Stewart, Yang, and Parker (1968) have suggested that cultured cells in the stationary phase of their growth might be a better model of tumour cell behaviour. A study has therefore been made of the properties of stationary phase RIB₅ cells in vitro.

3.4.2. Theoretical considerations.

Figure 16A shows growth curves for two types of population, fed and unfed. Unfed cells were plated in 3 ml. of medium and counted at intervals; fed cells had their medium changed daily from the third day, resulting in a maximum number of cells per plate three times that for unfed cells, at a stage when no more space was available.

Consider the unfed cells. The maximum population number, N_s , will depend on such factors as nutrient supply, accumulation of toxic products, pH, etc. Consider the

case where the maximum cell number is determined by lack of nutrients only. Suppose growth continues until the concentration C of a given nutrient falls to zero. In general, growth will continue at the normal rate until C is almost zero, and is given by:-

$$\frac{dN}{dt} = kN$$

Let the average consumption rate per cell of the particular nutrient be r , then:-

$$\frac{dC}{dt} = -rN$$

$$C = C_0 - r \int_0^t N dt$$

When $C = 0$, $N = N_s$, the limiting value, so that neglecting the value of N at $t = 0$, we have:-

$$N_s = \frac{C_0 \cdot k}{r}$$

i.e. N_s is directly proportional to the initial concentration C_0 . This gives a means of distinguishing cessation of growth due to nutrient depletion from that due to accumulation of toxic products, since a plot of N_s against C_0 should give a straight line (figure 17A). Figures 17B and 17C show actual plots of N_s against concentrations of minimal essential medium and serum. The normal concentrations were 7.7 percent and 10 percent respectively, and figure 17 shows that unfed RIB₅ cells became stationary partly through lack of nutrients and partly through accumulation of toxic products. This phase was reversible, since even after a period of 50 hours in the stationary phase, unfed cells were still capable of division at the normal rate if the medium was then changed daily; they then grew to the same level as the fed cells (figure 16B).

Figure 16

Growth of RIB₅ cells.

- A. Fed (O) and unfed (X) cells.
- B. Unfed cells (X) fed daily from
the sixth day.

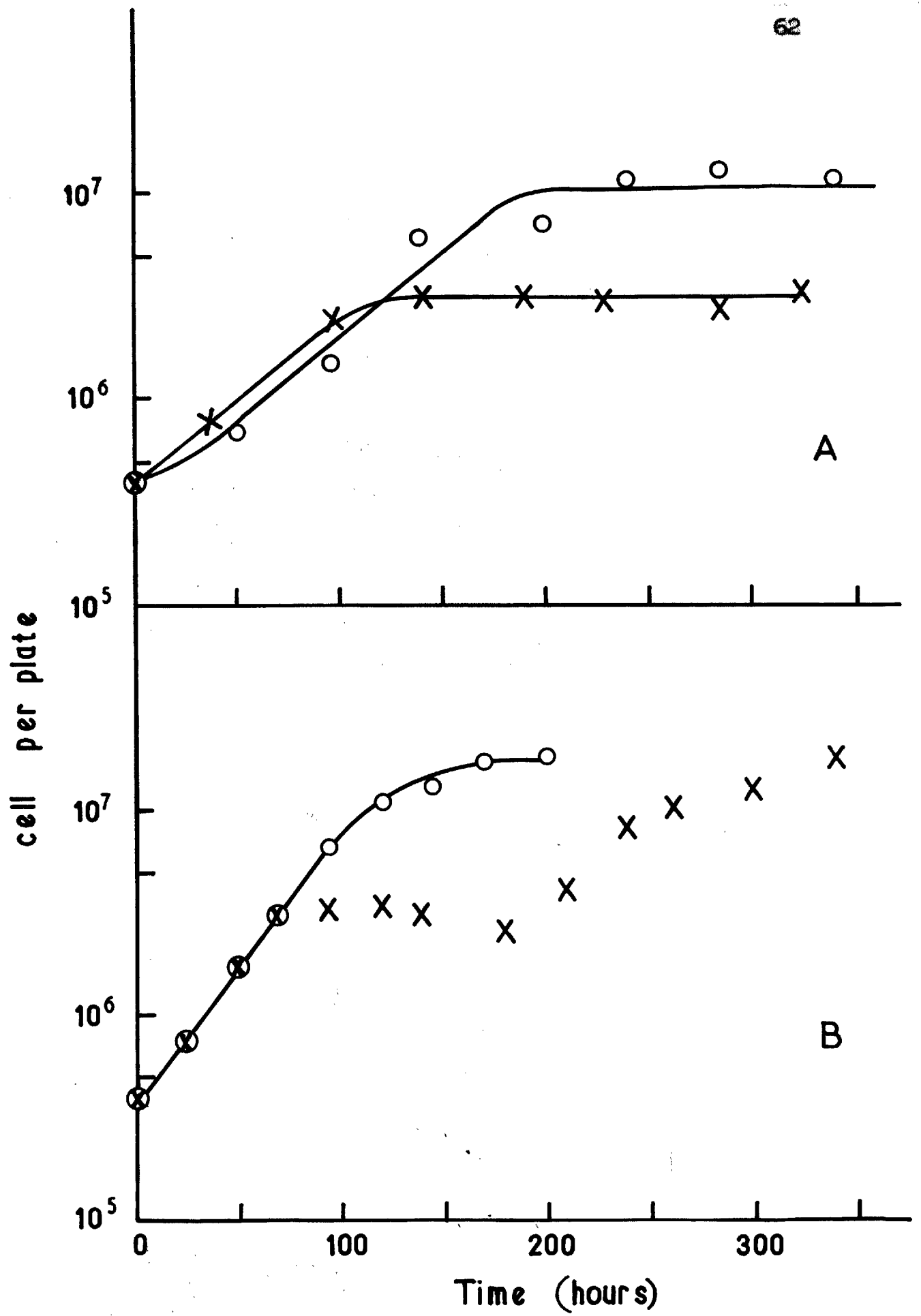
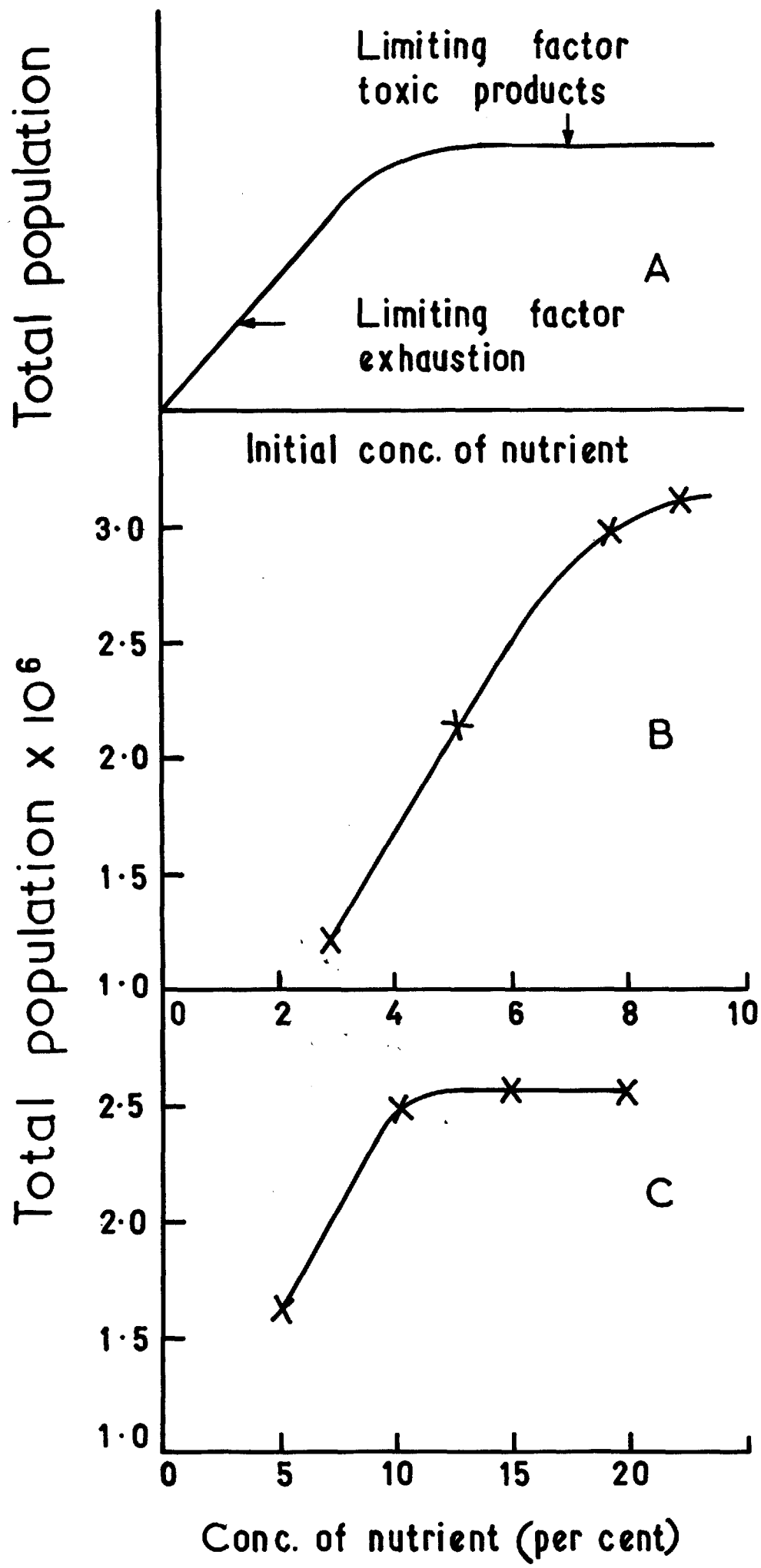


Figure 17

Dependence of the maximum population
number, N_s , on initial nutrient concentration.

- A. Theoretical curve.
- B. Dependence on M.E.M. concentration.
- C. Dependence on serum concentration.



3.4.3. Results.

Cells were irradiated either 30 hours after plating 4×10^5 cells, or after a period of about 50 hours in the stationary phase, corresponding to a period of growth of either 120 hours (unfed), or 160 hours (fed). They were irradiated in the plates in which they had grown but with the medium removed, and then trypsinised, counted and diluted, and plated into new dishes. The resulting survival curves are shown in figure 18. It is clear that the only survival curve parameter to change was the extrapolation number, which was reduced from 4 to unity. As the survival curves are drawn, n has a value less than 1 for the stationary phase cells, but in no case did it differ significantly from 1. There was no change in the values of the D_0 or the o.e.r. (table 2).

When the dose of radiation was given in two equal doses separated in time, the log phase cells were able to shed their sub-lethal damage (figure 19). Fed stationary phase cells showed no sparing effect of fractionation (figure 19). Unfed cells showed an apparent sparing effect. This was only apparent because if they were exposed to a single dose and then left for a period prior to replating into fresh medium, the same increase in survival was seen as when the dose was fractionated (figure 19). The cells were recovering from potentially lethal rather than sub-lethal damage.

The major difference between the two types of stationary phase population was in the distribution of cells through the cell cycle. Figure 20A shows the results of an experiment to measure the appearance of labelled mitoses in the two populations after a brief exposure to the radioactive

label. For comparison, the results for logarithmic cells are included. Since the radioactive thymidine had to be removed after 30 minutes, in order not to provide the unfed cells with fresh medium, this radioactive medium was replaced by "conditioned" medium which had been used to grow stock cultures, so that it was in the same nutrient-depleted state as the medium it was to replace.

These results show that fed cells encountered a block in G₁. Their growth fraction, as measured by continuous labelling, was only 8 percent (figure 20B). Presumably the reason that any mitotic figures were seen at all in the fed population was that as cells died, they became detached from the plate, and adjacent cells could then divide to fill the gap.

Unfed cells did not encounter a block at a particular stage of the cell cycle. The cycle increased due to a doubling of all its stages (figure 20A). In spite of this, there was no increase in cell number, due to a decrease in the growth fraction to 57 percent (figure 20B), together with an increase in the occurrence of pyknosis and cell degeneration.

3.4.4. Discussion.

Hahn (1968), and Hahn, Stewart, Yang, and Parker (1968), demonstrated a similar fall in the survival curve extrapolation number and a G₁ block in fed stationary phase Chinese hamster cells, and they suggested that the reason for the reduced extrapolation number is that fed cells are essentially a synchronised population in G₁, a phase in which Chinese hamster cells do show exponential survival (Hahn, 1970).

Figure 18

Survival curves for cells irradiated in aerobic (open symbols) and hypoxic (closed symbols) conditions.

- A. Log phase cells.
- B. Stationary phase cells, unfed.
- C. Stationary phase cells, fed.

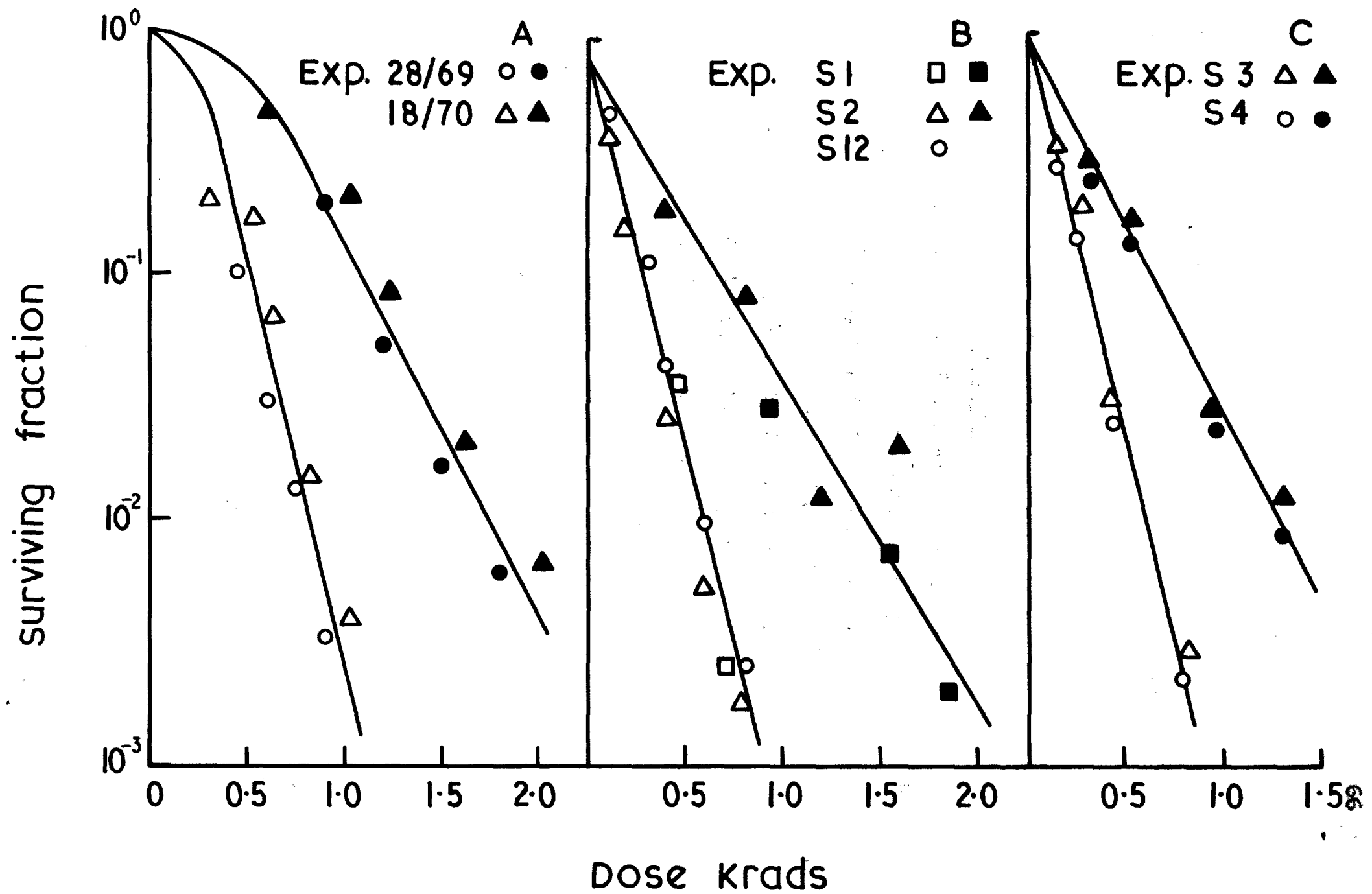


Figure 19

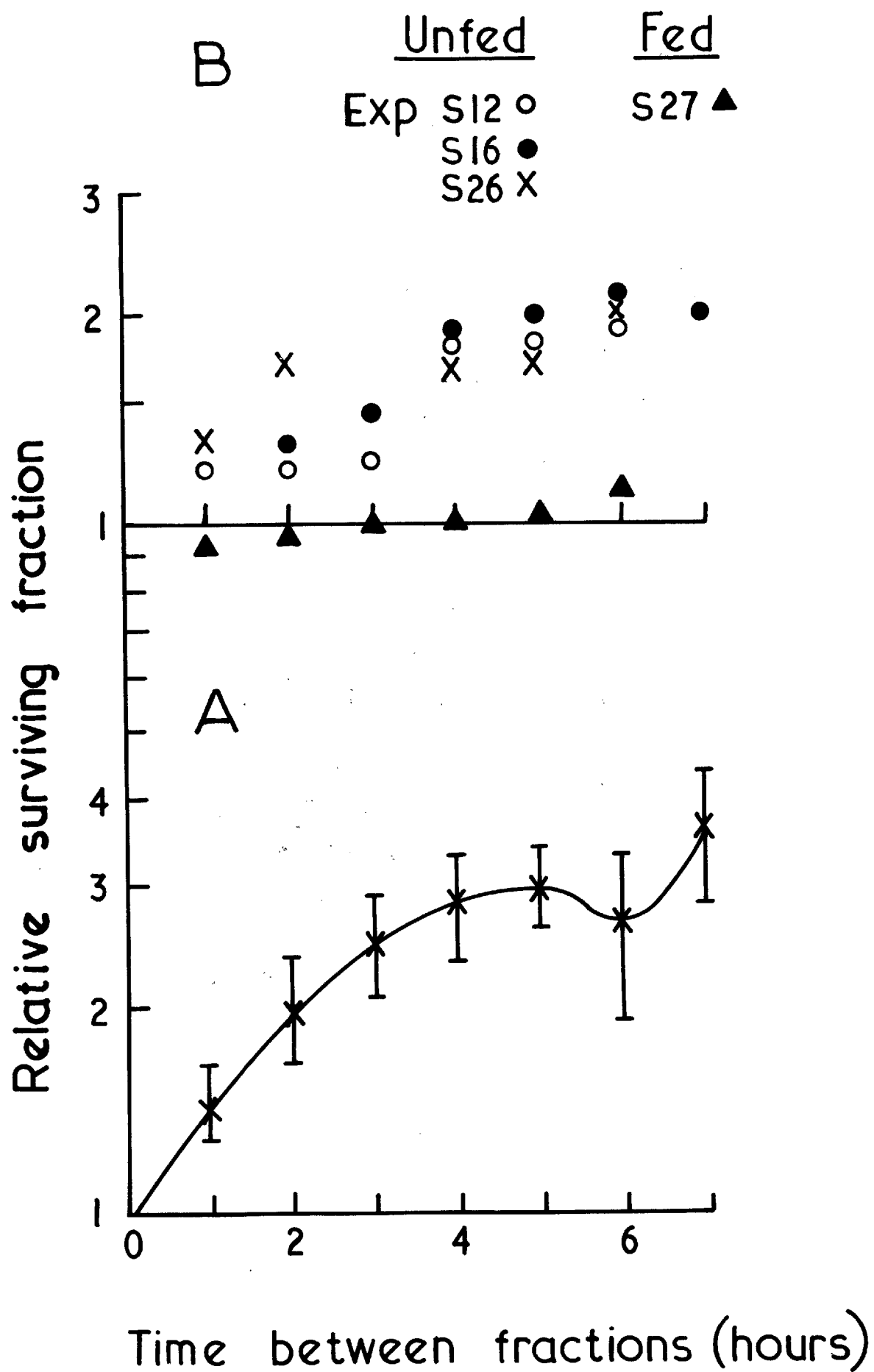
Shedding of sub-lethal and potentially lethal damage.

A. Logarithmic cells, 500r + 500r. Mean of three experiments.

B. Fed and unfed stationary phase cells.

Experiments S12, S16, S27, 500r + 500r.

Experiment S26, 500r and held prior to replating.



Phase	n	Do (air), rads.	o.e.r.
Log	4.5 (2.2-9.3)	134 (119-154)	2.12 (1.97-2.28)
Stationary fed	0.9 (0.7-1.1)	136 (128-146)	2.07 (1.95-2.21)
Stationary unfed	0.7 (0.4-1.3)	136 (120-157)	2.30 (2.12-2.67)

Table 2

Survival curve parameters with their 95% confidence limits for RIB₅ cells irradiated in the logarithmic and stationary phases.

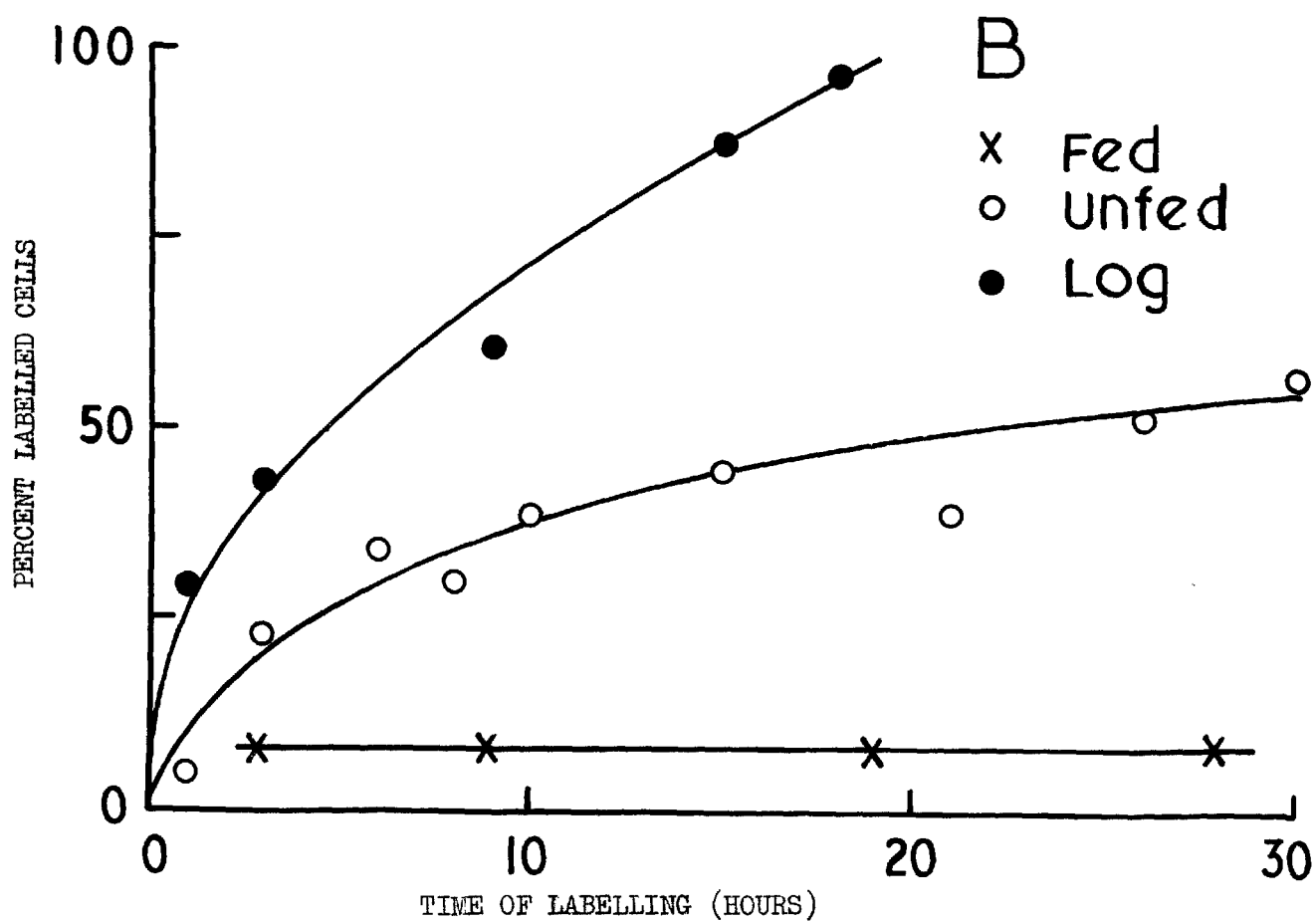
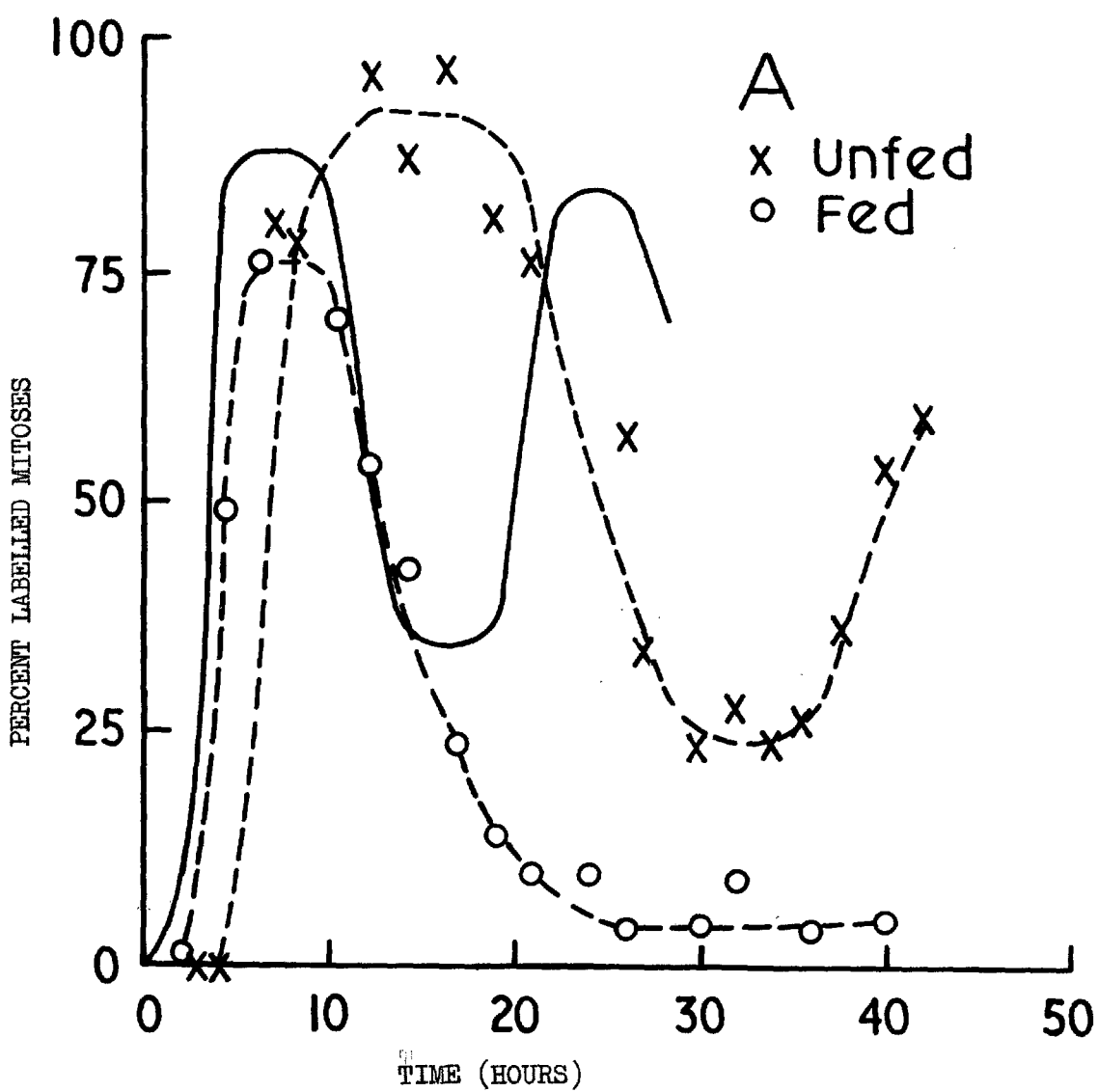
If synchrony were the explanation for the exponential survival seen when fed RIB₅ cells were irradiated, then one might expect that when cells were trypsinised and replated from the stationary phase into new plates, they would show some evidence of this synchrony. However, this was not the case. The change in the mitotic and labelling indices with time after replating fed stationary phase cells was essentially the same as that for replated log phase cells (figure 21), and there was no evidence of a synchronous movement out of the G1 block. However, the trypsinisation procedure may have obscured such a movement.

Unfed RIB₅ cells in the stationary phase are clearly not a synchronised population. Figure 20 shows that there was still a turnover of cells, and while those cells that did not take up the radioactive label during continuous exposure to it (figure 20) may have been in G1, they represented less than half the population and could not alone account for the occurrence of exponential survival to radiation. In fact it is unlikely that these cells were in a true G1 phase, since Stanners and Becker (1971) have shown that while such cells may have the DNA content characteristic of G1 cells, their polysome complement is quite different.

While these results on stationary phase RIB₅ cells agree well with those of Hahn (1968), and Hahn et al (1968), they differ from the results of other workers. Madoc-Jones (1964), using cells derived from a benzpyrene-induced sarcoma in CBR 1 rats, found marked changes in both the extrapolation number and D_0 . He found that the value of n increased from 2 to almost 100 as cells moved from the logarithmic to the stationary phase. Little (1969),

Figure 20

- A. Labelled mitoses curves for stationary phase cells. Each point represents a count of 100 mitoses. The curve for log. phase cells is included for comparison.
- B. The fraction of cells labelled as a function of the time of exposure to the label. 1000 cells counted per point.



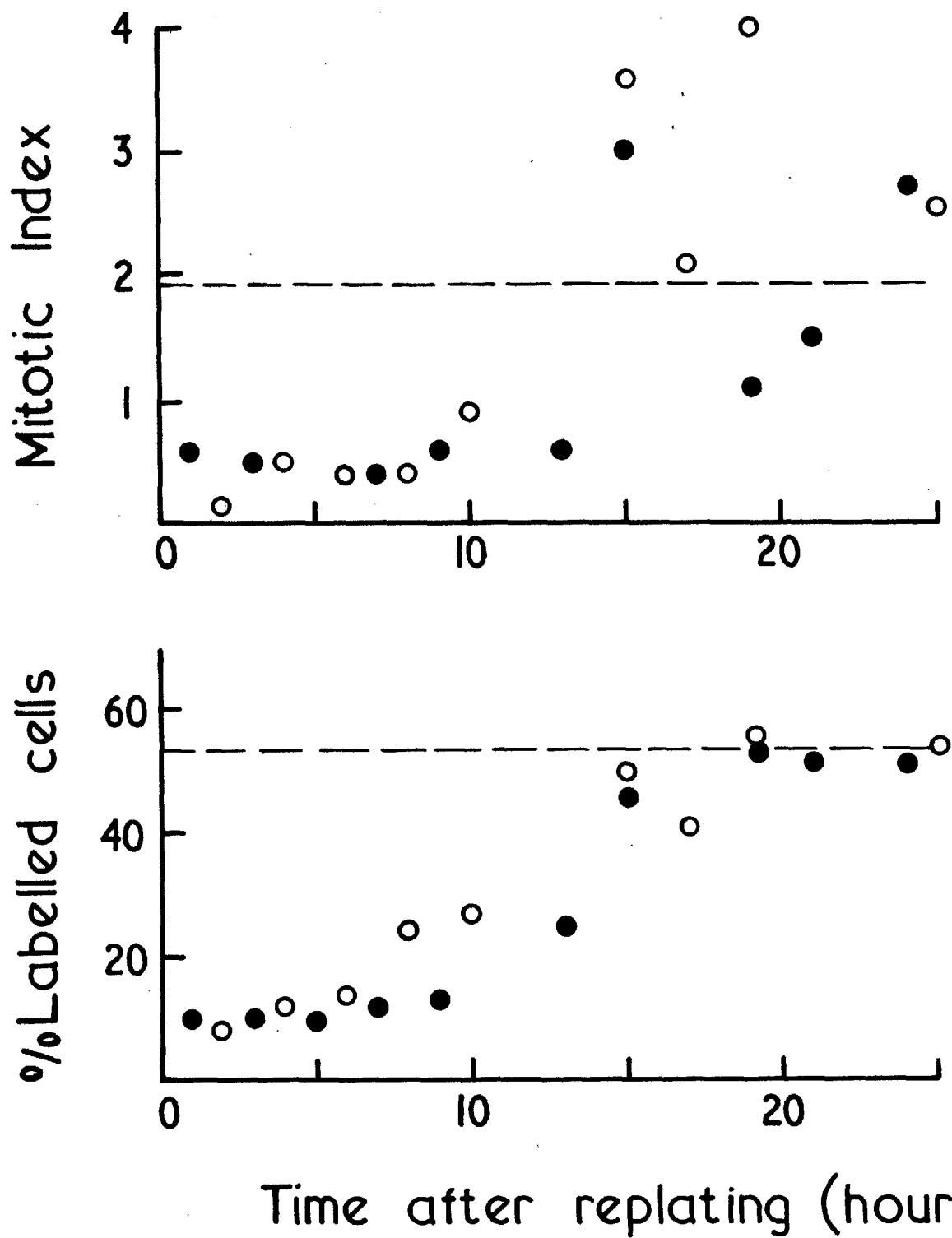


Figure 21

Growth of replated cells. Open points, cells plated from the logarithmic phase. Closed points, cells plated from the stationary phase (fed).

irradiating Chang liver cells, found a decrease in the D_0 and an increase in the extrapolation number. Berry, Hall, and Cavanagh (1970), irradiating HeLa and Chinese hamster cells, found a complex series of changes in D_0 and n depending on the medium in which the cells were grown. Both Hahn and Little were able to explain their results with fed cells in terms of a block in G_1 , since log phase cells synchronised in G_1 showed the same response as stationary phase cells. It may be a general property of fed cells that in the stationary phase they behave as a G_1 population, differences between cell lines reflecting differences in radiosensitivity through the cell cycle. It has not been possible to obtain a sufficiently well synchronised population of RIB₅ cells to test whether G_1 cells are killed exponentially.

It is interesting that two populations which became stationary for quite different reasons, lack of space in one case, lack of nutrient in the other, should have responded to radiation in the same way. Stanners and Becker (1971) have shown that Syrian hamster cells in the stationary phase (unfed) synthesised protein at about one third of the rate of those in log phase. This was due to a reduction in the number of ribosomes per cell, and an increase in the number of free ribosomes, leading to a decreased probability of attachment of ribosomes to messenger RNA. If these ribosomal changes are of importance in determining a cell's ability to absorb radiation-induced lesions as sub-lethal, the addition to log phase cultures, prior to irradiation, of a drug such as Puromycin, which releases ribosomes from the polysomes (Joklik and Becker, 1965), might simulate the conditions existing

in the stationary phase, and result in exponential survival. The actions of this, and other drugs, are the subject of further study.

Chapter 4

THE RESPONSE OF TUMOURS AND TUMOUR CELLS TO RADIATION

4.1. A cell survival curve for tumour RIB₅.

4.1.1. Methods.

The starting point for this work was the study made by Thomlinson (1960), and by Thomlinson and Craddock (1967), of the gross response of the fibrosarcoma RIB₅ to single doses of radiation. With their data as a base, an attempt was then made to measure the survival of RIB₅ cells directly.

The method of Hewitt and Wilson (1959), in which the TD₅₀ is measured as a function of dose, could not be used when cells were injected subcutaneously into rats, because at least 10^3 cells were needed to produce a tumour. However, when cells were injected into the brains of newly weaned rats, the TD₅₀ was reduced to less than 20 cells. It was then possible to construct survival curves for cells from tumours irradiated with the animals breathing either air at atmospheric pressure, or oxygen at a pressure of 3 atmospheres. Tumours could be rendered hypoxic by occluding the blood supply with a U-shaped clamp applied five minutes before irradiation (Thomlinson and Craddock, 1967). The irradiation techniques were as described in chapter 2.

After irradiation, tumours were excised and single cell suspensions prepared as previously described (section 2.4.). Known numbers of cells were then injected into rats' brains. Recently weaned rats had sufficiently soft skulls to allow a needle to go straight through. The formation of a tumour in the brain within 90 days was

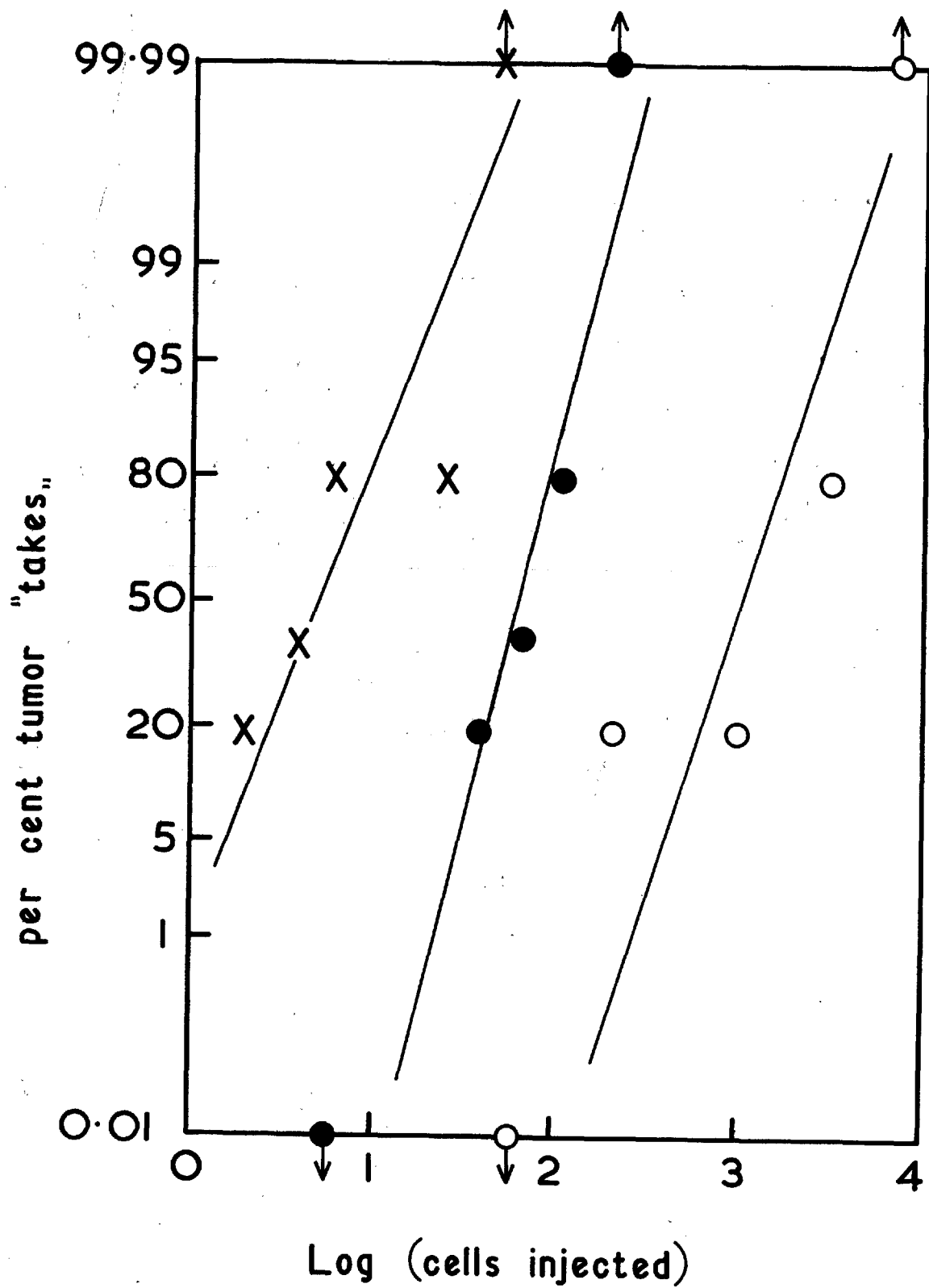


Figure 22

Percent tumour "takes" versus the number of $R1B_5$ cells injected into rat brains.

X control

● 1000r hypoxic

○ 1000r oxic

the endpoint scored. Because only a small number of animals was available, the survival values derived from this experiment must be regarded as preliminary, and for this reason no errors have been quoted. The results, expressed as percent tumour "takes" versus the logarithm of the number of cells injected, were plotted on probability paper, and the best straight line drawn by eye. Figure 22 shows such a plot. The number of cells to give 50 percent of the animals a tumour, the TD_{50} , was then read off, and the ratio of the TD_{50} for unirradiated cells to that for irradiated cells gave the surviving fraction.

If, instead of inoculating the cells into rats' brains, they were plated onto a feeder layer of cultured RIB₅ cells, then colonies were formed within 10 days with a plating efficiency of 8-45 percent. This enabled a second test of survival to be made. The counts were subject to larger errors than in conventional experiments because the colonies were diffuse.

4.1.2. Results.

The results of these two methods of assay are shown in figure 23A. Below a surviving fraction of 10^{-1} the survival curve for irradiations in aerobic conditions was parallel to that for the clamped tumours, and extrapolation of this line back to zero dose indicated an hypoxic fraction of about 15 percent. The lines of figure 23A were drawn using the data for survival assayed in vitro only. The survival curve parameters together with their 95 percent confidence limits are given in table 3. The confidence limits only represent errors associated with fitting the curves to the experimental points. They do

Figure 23

A. Survival curves for cells from tumour RIB₅. The tumours were irradiated and immediately excised, and the cells then either plated or injected into rats' brains.

<u>symbol</u>	<u>conditions</u>	<u>assay</u>
O	oxygen, 30 p.s.i.	in vitro
X	aerobic	"
●	tumour clamped	"
□	oxygen, 30 p.s.i.	brains
■	tumour clamped	"

B. Survival curves for cells from tumour RIB₅ irradiated four hours after plating into glass petri dished.

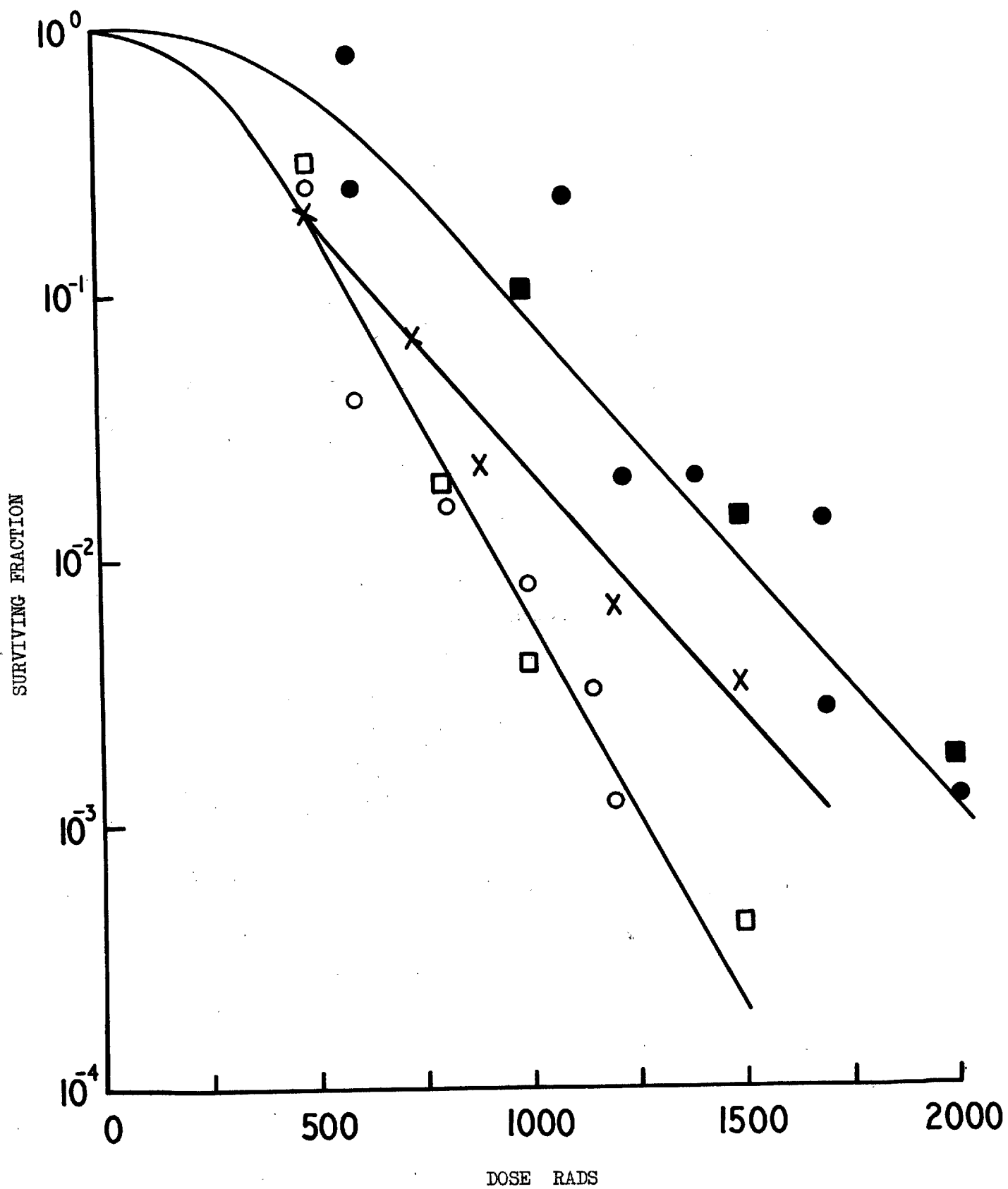


Figure 23A

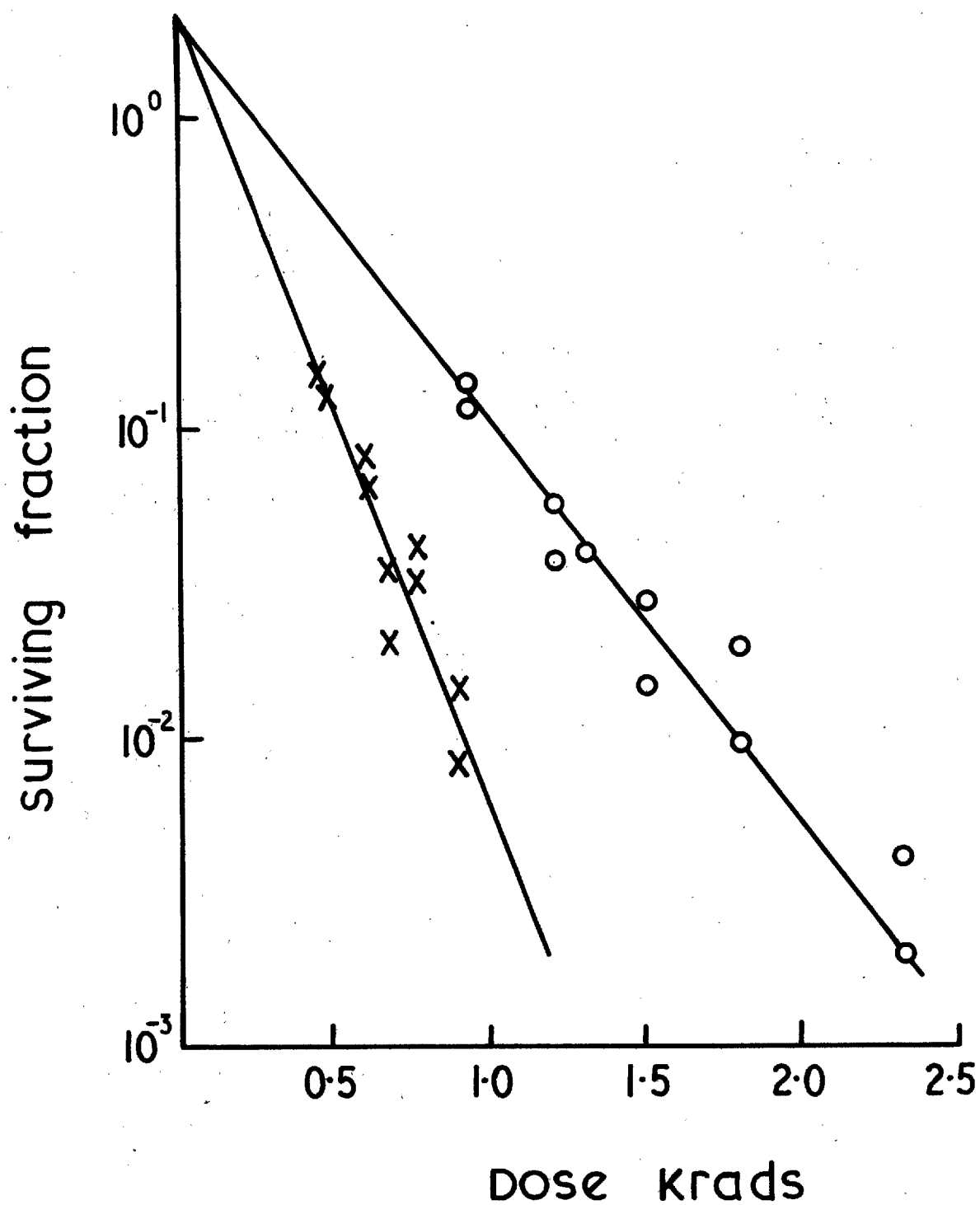


Figure 23B

Survival curves for cells from tumour RIB₅ irradiated four hours after plating into glass petri dishes.

X aerobic O hypoxic

Conditions	n	Do(oxic), rads	o.e.r.
Irradiated <u>in vivo</u> assayed <u>in vitro</u> .	5.8 (0.3-8.4)	145 (117-190)	1.63 (1.40-1.92)
Irradiated 4 hours after plating.	2.2 (1.3-4.4)	163 (145-184)	1.98 (1.48-2.19)

Table 3.

Survival curve parameters for the cells of tumour RIB₅
irradiated in vivo and in vitro and assayed in vitro.

not take into account the possible biological errors associated with the very low plating efficiency for the cells. However, the points from the brain inoculation experiments fit the calculated lines well (figure 23A), so that while there were large experimental errors associated with both methods of assay, the agreement between them suggests that the survival curve parameters of table 3 are a reasonable approximation to the true values.

A second pair of survival curves for the cells of tumour RIB₅ are shown in figure 23B. In this experiment cells were irradiated four hours after plating into glass petri dishes from a tumour. The calculated survival parameters are shown in table 3.

4.1.3. Discussion.

Perhaps the most striking feature of these results was the low value of the oxygen enhancement ratio. This value of less than two contrasts with the value of 3.4 measured for changes in tumour growth after irradiation (Thomlinson and Craddock, 1967). Thomlinson and Craddock constructed theoretical cell survival curves from their growth curves, and while calculations of cell survival based on growth measurements involve many assumptions (see section 4.2.3.), some general speculations as to the difference between the gross response and the measured survival curves are possible.

Most of the difference between the o.e.r.'s for the calculated and measured survival curves can be attributed to a shift in the hypoxic curve. It may be that the very

low plating efficiency of RIB₅ cells meant that a highly selected population was being tested, but the agreement with the results of the brain assay, in spite of their errors, would argue against this. Also, the o.e.r. for cells irradiated four hours after plating from a tumour had the same low value (table 3).

A second possibility is that the type of damage expressed when cells are left in situ, as in growth studies, may be qualitatively different to that when they are excised and plated in vitro. Alper (1963, 1969) has proposed a model of radiation damage which could be applied to these results. Briefly, she envisages at least two types of radiation damage that can lead to cell death: type O damage having an intrinsically high o.e.r., and type N damage having a low o.e.r. According to this model, we suppose that irradiated RIB₅ cells when left in situ after irradiation, suffer predominantly from type O damage, but that when they are plated in vitro there is a shift towards type N damage, resulting in a reduction in the o.e.r. This might be a true shift, or might be due to preferential reparability of type N damage in vivo, so that while it still occurred, it was less able to express itself than when the cells were plated into petri dishes. If this were the case, one might ask why the brain assay also gave a low o.e.r. Hornsey (1963) found a similar change in hypoxic D₀, and hence o.e.r., to a lower value when assaying the survival of Ehrlich ascites cells first subcutaneously in new-born mice, and then in mouse brains, suggesting that if the model is applicable, the brain is also a site where type N damage is preferentially expressed.

If it is valid to apply Alper's model to these results, they imply that the use of in vitro techniques for predicting detailed radiobiological responses in vivo may not be valid. If there is no qualitative difference between the types of damage expressed when cells are left in situ and when they are plated in vitro, these results indicate that the relationship between cell killing and tumour growth delay in RIB₅, at least as regards modifications by oxygen, is a more indirect one than has previously been thought to be the case.

4.2. The gross response of tumour RIB₅C to radiation.

4.2.1. General characteristics of the tumour.

As was explained in section 2.4., RIB₅ is not a particularly suitable tumour for studying the relationship between cell killing and changes in tumour growth. For this reason, the tumour which results when cultured RIB₅ cells are injected into rats, tumour RIB₅C, has been used (see chapter 2).

RIB₅C is histologically similar to RIB₅ except that it has a slightly greater degree of differentiation and grows more slowly, the time to grow from 9 mm. to 25 mm. in diameter being 12 days compared with 6 days for RIB₅.

The cell kinetic parameters of RIB₅C were measured by the use of standard autoradiographic techniques after labelling with tritiated thymidine, the principles involved being essentially the same as those for cultured cells. The techniques were those described by Denekamp (1968). The procedure was as follows. When tumours had

reached a diameter of 8 - 10 mm. the animals were injected intraperitoneally with $0.5 \mu\text{Ci/gm}$ body weight $^3\text{HTdr}$, specific activity 360 mCi/mM . It is assumed that the thymidine was available for incorporation into the cells only for a short time, so that a single injection represented a true pulse label (Denekamp, 1968). Animals were sacrificed at intervals of 1 to 40 hours after injection and the tumours excised, cut in half, fixed in about 20 ml of five percent formol saline for at least 48 hours, and then processed by standard methods in an Elliot-Shandon automatic tissue processor. After tumours had been blocked in Fibrowax, one tumour half was retained as a spare, and from the other, 4 micron sections were cut at approximately 0.8 mm. intervals through the tumour perpendicular to the cut surface. These sections were then mounted on slides, dewaxed and stained in aceto-orcein. Autoradiographs were prepared in the same way as for cultured cells (chapter 3). The exposure time varied from one to six weeks depending on the interval between injecting the animal and removal of the tumour.

Autoradiographs were scored without knowing beforehand the time interval between labelling and excising a tumour. Mitoses were scored as labelled if they contained 3 or more grains above background. The background varied from one batch of autoradiographs to the next, and also to a lesser extent from one side of a section to the other, an effect probably due to non-uniformity in the emulsion drying process, causing shearing stresses in it. Sections were scored at a magnification of x800, and 100 mitoses were counted on each slide. The tumour

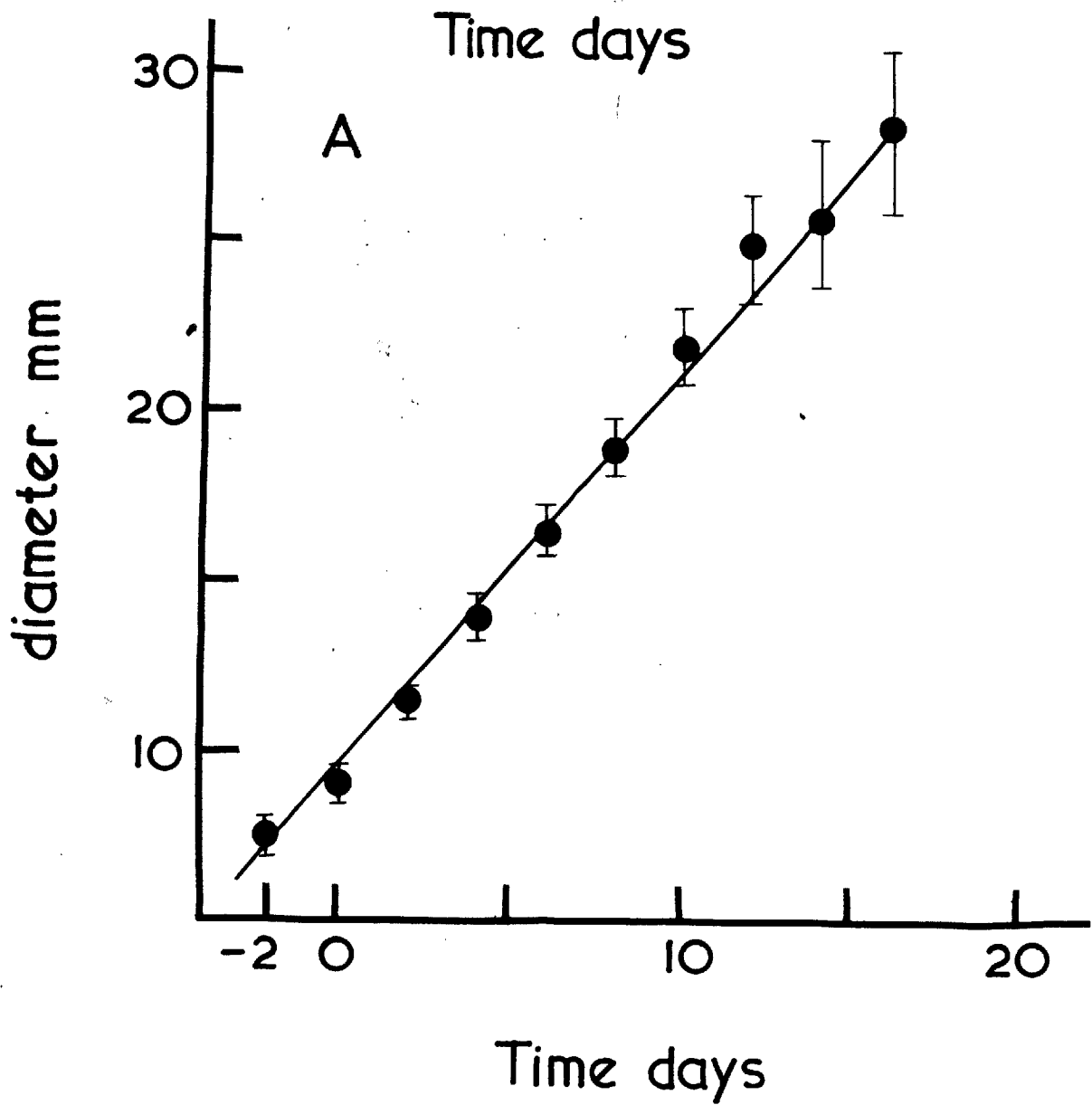
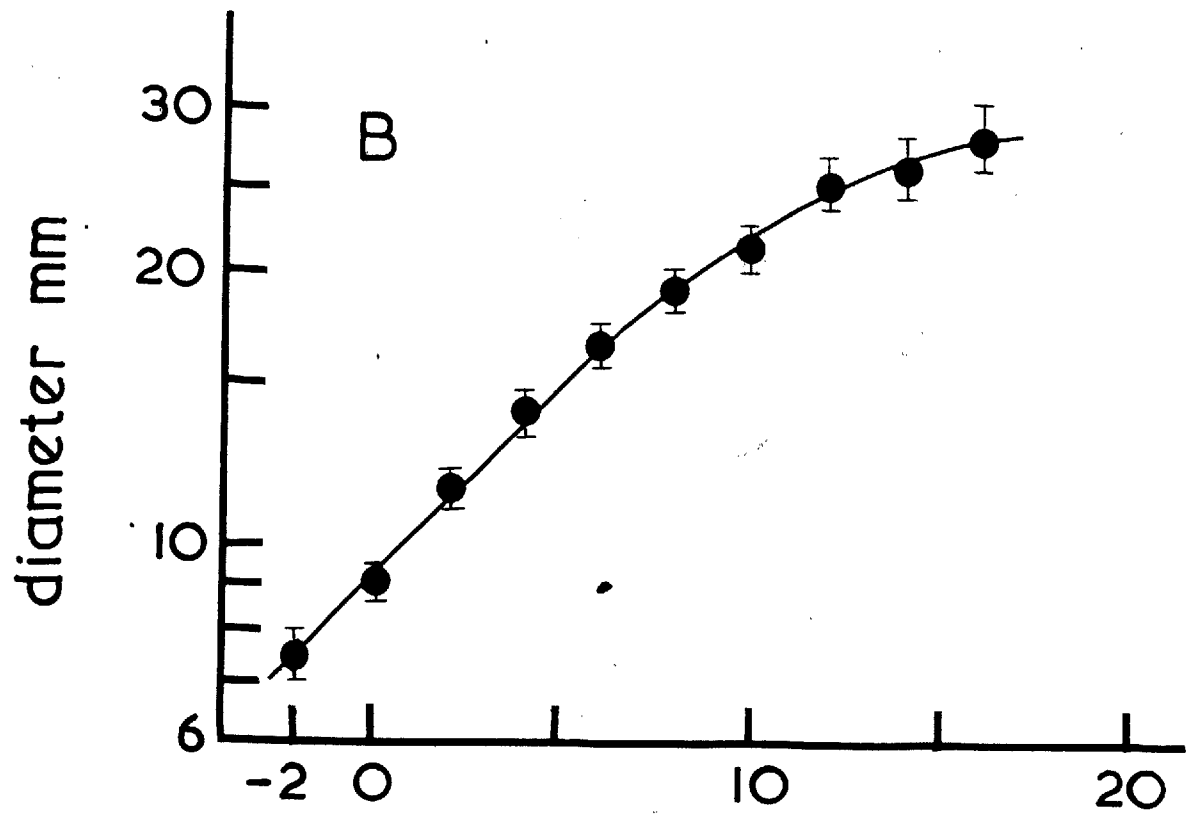
Figure 24

Growth curves for tumour RIB₅C.

- A. Linear scale of diameter versus time.
- B. Log scale of diameter versus time.

The lines represent the results of daily measurements on 18 tumours.

The error bars are the 95% confidence limits.



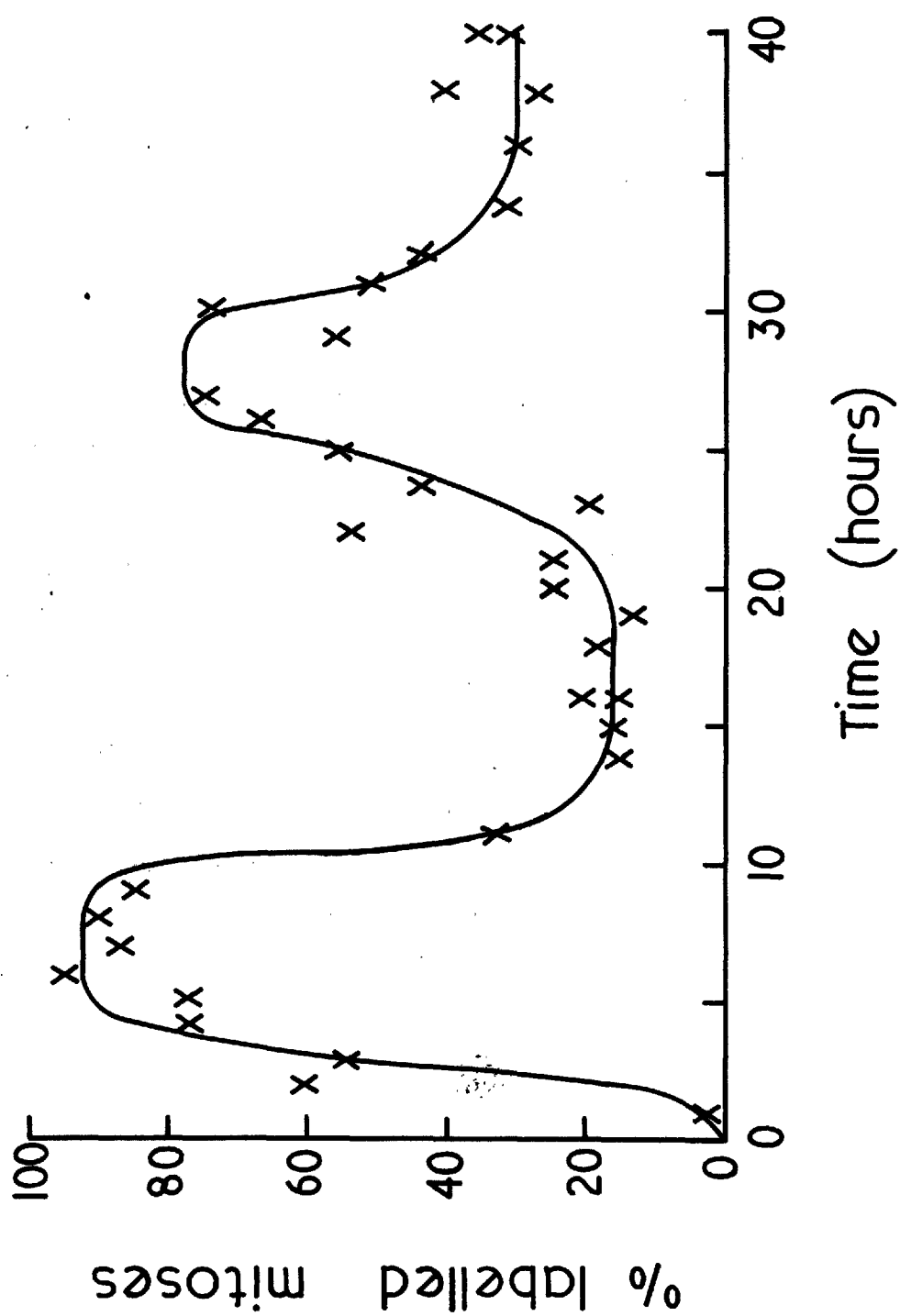


Figure 25

Labelled mitoses curve for tumour RIB₅C.

Figure 26

The relationship between necrosis and the pattern of labelling in RIB₅C. The left-hand ordinate is the number of intact cells per unit area (●), and the right-hand ordinate is the percent labelled cells per unit area (X). The unit area is $100\mu \times 100\mu$, and the tumour was scanned across the diameter indicated. It was excised one hour after injecting the $^3\text{HTdr}$.

excised one hour after injection was used to measure the labelling and mitotic indices and to look at the distribution of labelled cells across the tumour. The two indices were determined by counting a total of 4000 cells across the section, and had values of 19.5 and 0.93 per cent respectively.

Figure 25 shows the labelled mitoses curve for RIB₅C. The line has been drawn through the points by eye. The cell cycle parameters measured for RIB₅C from this curve were:-

$$\begin{aligned}T_C &= 22 \text{ hours} \\T_S &= 8 \text{ hours} \\T_{G2} &= 2.5 \text{ hours} \\T_{G1+M} &= 11.5 \text{ hours}\end{aligned}$$

Figure 26 shows the relationship between necrosis and the pattern of labelling. It was not possible with this tumour to relate the distribution of labeled cells to that of the blood capillaries, nevertheless it is apparent that the percentage labelled cells was at its highest in areas free of necrosis.

4.2.2. Effects of radiation on tumour growth.

The time for 18 unirradiated tumours to grow from 9 to 25 mm. in diameter was 12 ± 0.8 days. This time did not alter significantly during the period the tumour was studied. A graph of diameter plotted on a logarithmic scale against time (figure 24B) shows that growth was not exponential.

Growth curves for tumours irradiated with single doses of X-rays are shown in figure 27. As the dose of radiation increased, the variation in growth amongst

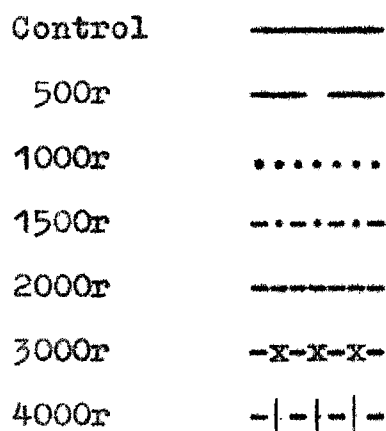
Figure 27

Growth of tumour RIB₅C after irradiation. Tumours were irradiated on day 0 when they had a diameter of 8-10mm.

A. Animals breathing oxygen at 30 p.s.i.

B. Animals breathing air.

C. Tumours clamped.



For clarity 95% confidence limits are shown only in one typical case, that following a dose of 1500r in oxygen.

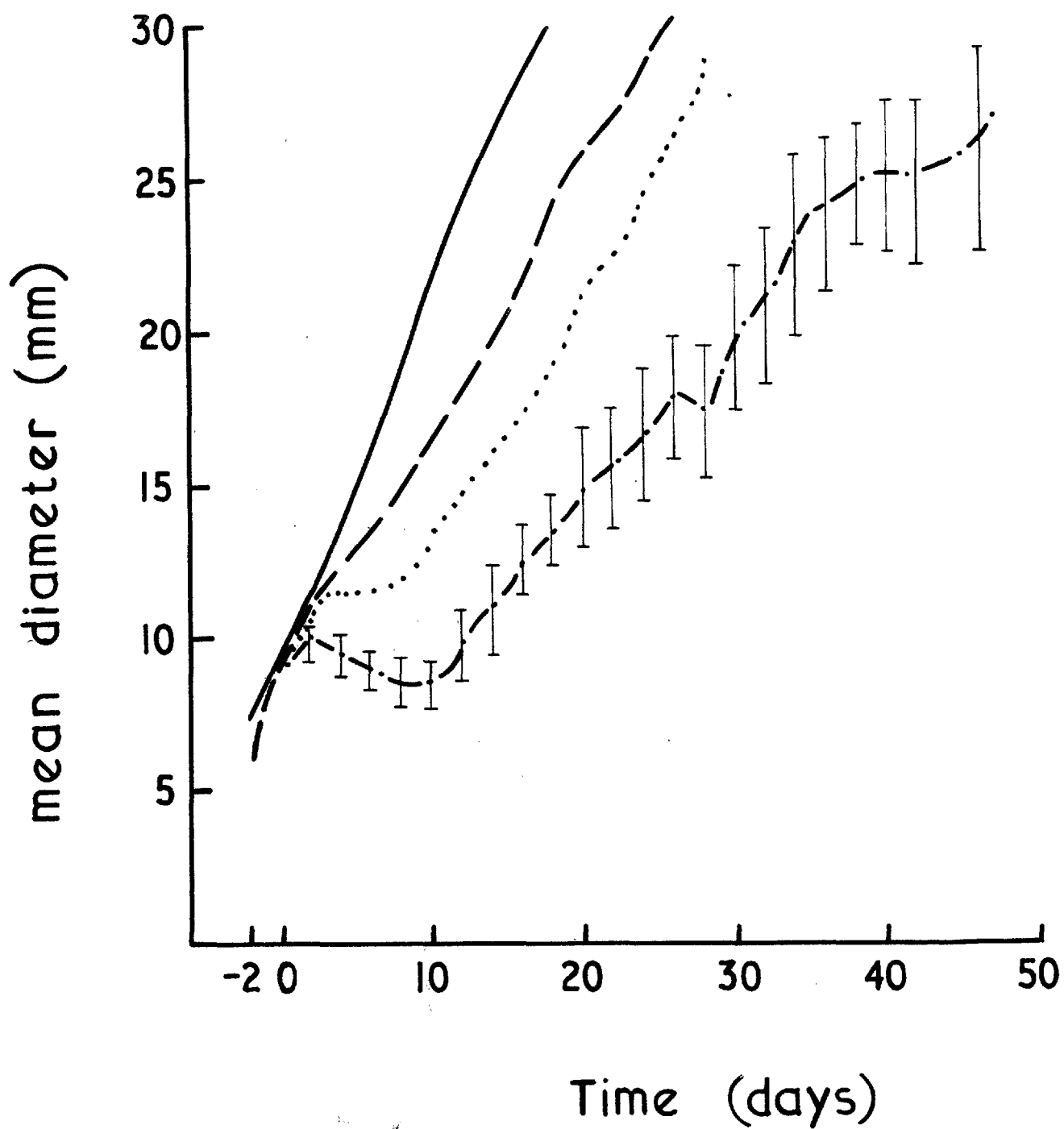
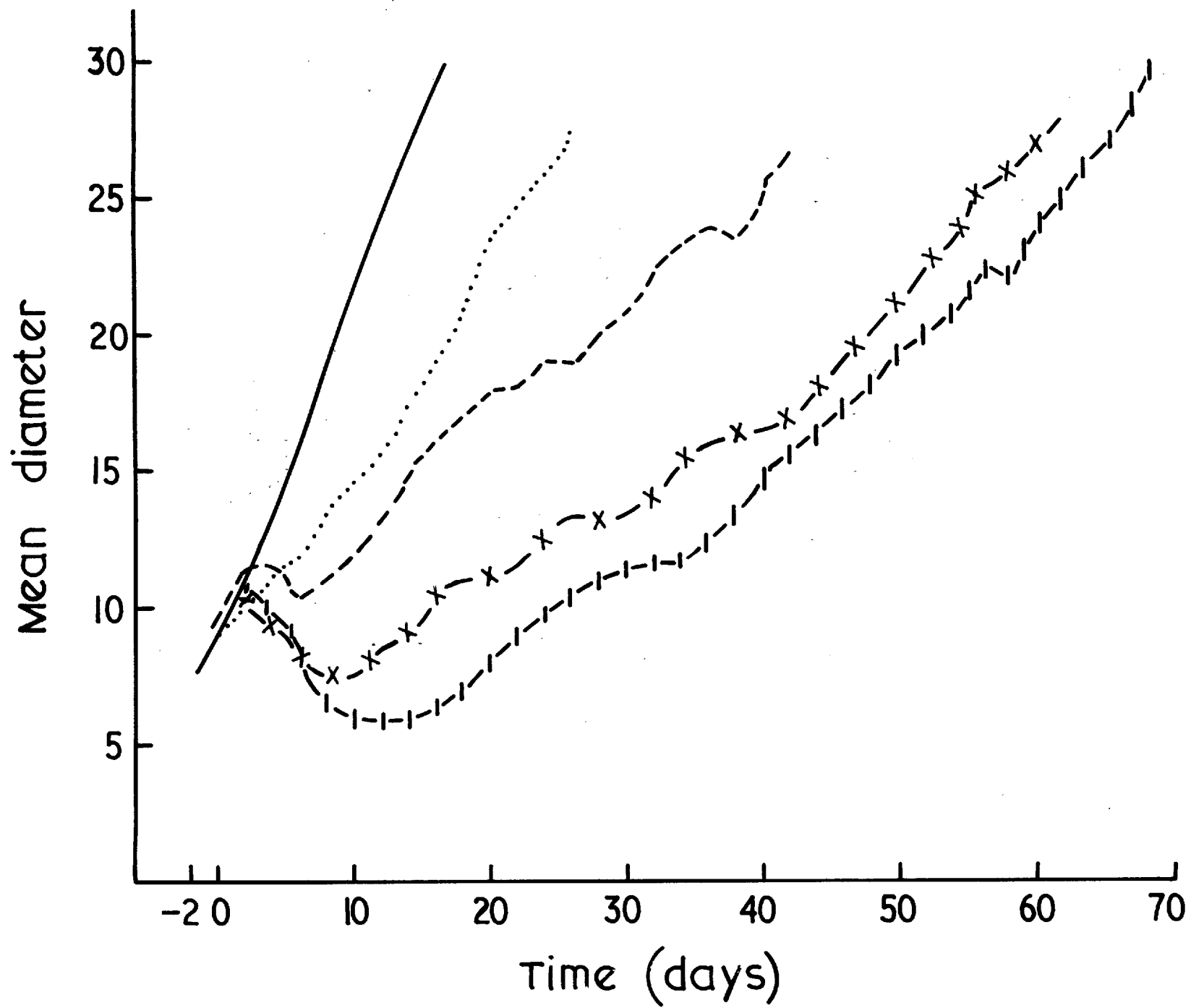


Figure 27A

Growth of RIB₅C after irradiation. Animals breathing oxygen at 30 p.s.i.

Growth of RIB₅₀ after irradiation. Animals breathing air.

Figure 27B



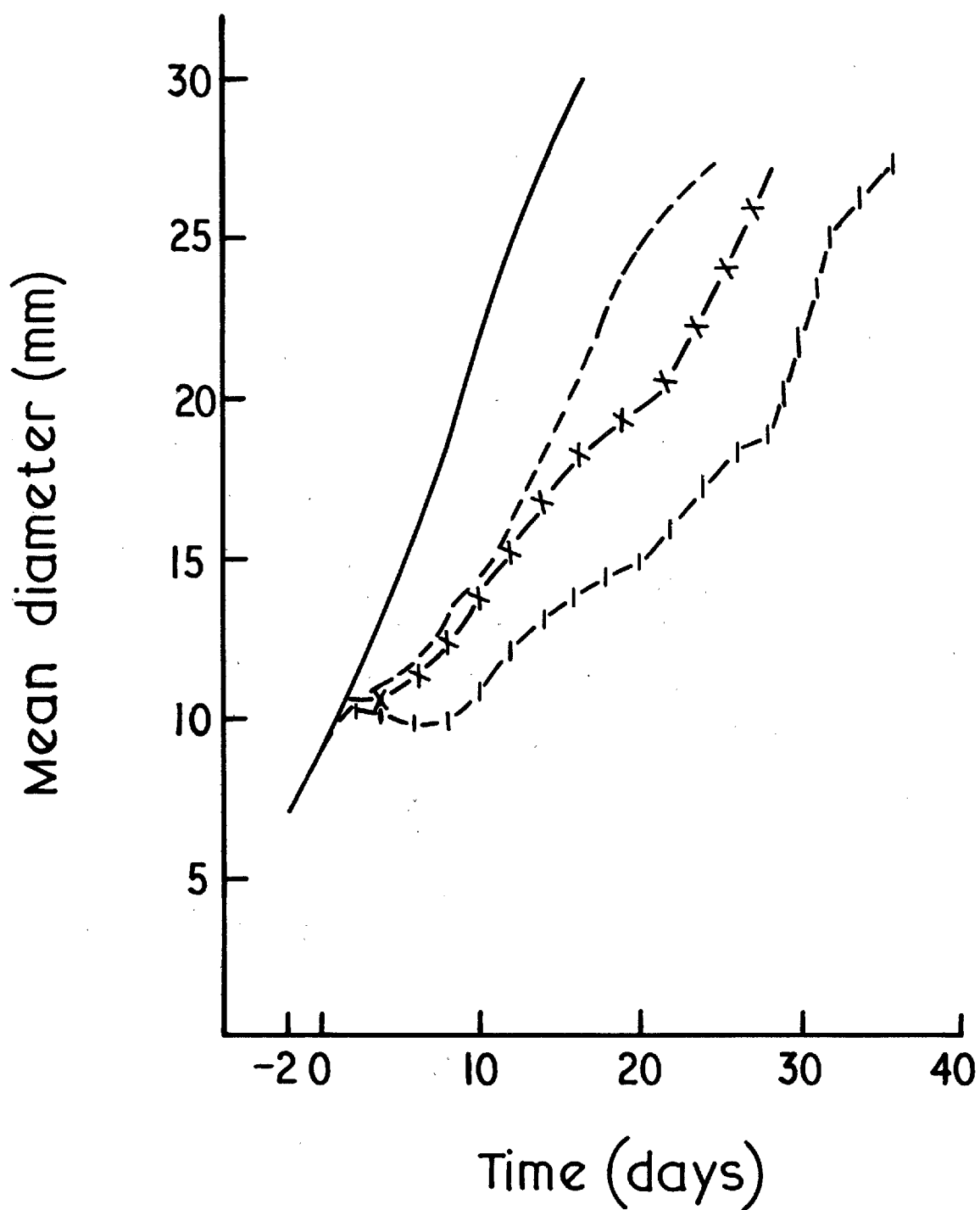


Figure 27C

Growth of RIB₅C after irradiation. Tumours clamped.

individual tumours exposed to the same dose increased, and this is reflected in the standard errors for the time to grow to 25 mm. (table 4). The standard errors of the mean diameters from which the growth curves were constructed are shown in a typical case, that for irradiation with 1500 rads in well oxygenated conditions (figure 27). All tumours grew at the normal rate for the first 20-30 hours after irradiation; there was then a decrease in growth rate, and as the dose increased, tumours shrank, the level they shrank to and the time they started to grow again depending on the dose of radiation (figure 27). The growth rate after irradiation always remained less than that of the controls.

A comparison of the effects of different treatments can be made by plotting the time taken for tumours to grow from 9 to 25 mm. in diameter as a function of dose (figure 28). On the assumption that the parameter "time to grow from 9 to 25 mm." is a measure of the increase in cell number, the biphasic shape of the aerobic curve of figure 28 would indicate the presence of a significant proportion of hypoxic cells in the tumour. The dashed line in figure 28 is the dose-effect curve for clamped tumours displaced upwards; the highest aerobic points fit this line well, suggesting that they do reflect the response of an hypoxic component. The triangles of figure 28 were obtained by multiplying the dose scale for tumours with animals breathing oxygen by 3. That they fall on the hypoxic curve implies that oxygen was dose-modifying with an o.e.r. of 3.

Tumours were also exposed to single doses of neutrons

Figure 28

Dose-effect curves for tumour RIB₅C. The effect is the time to grow from the diameter when irradiated (9mm) to 25mm. The vertical lines indicate the 95% confidence limits.

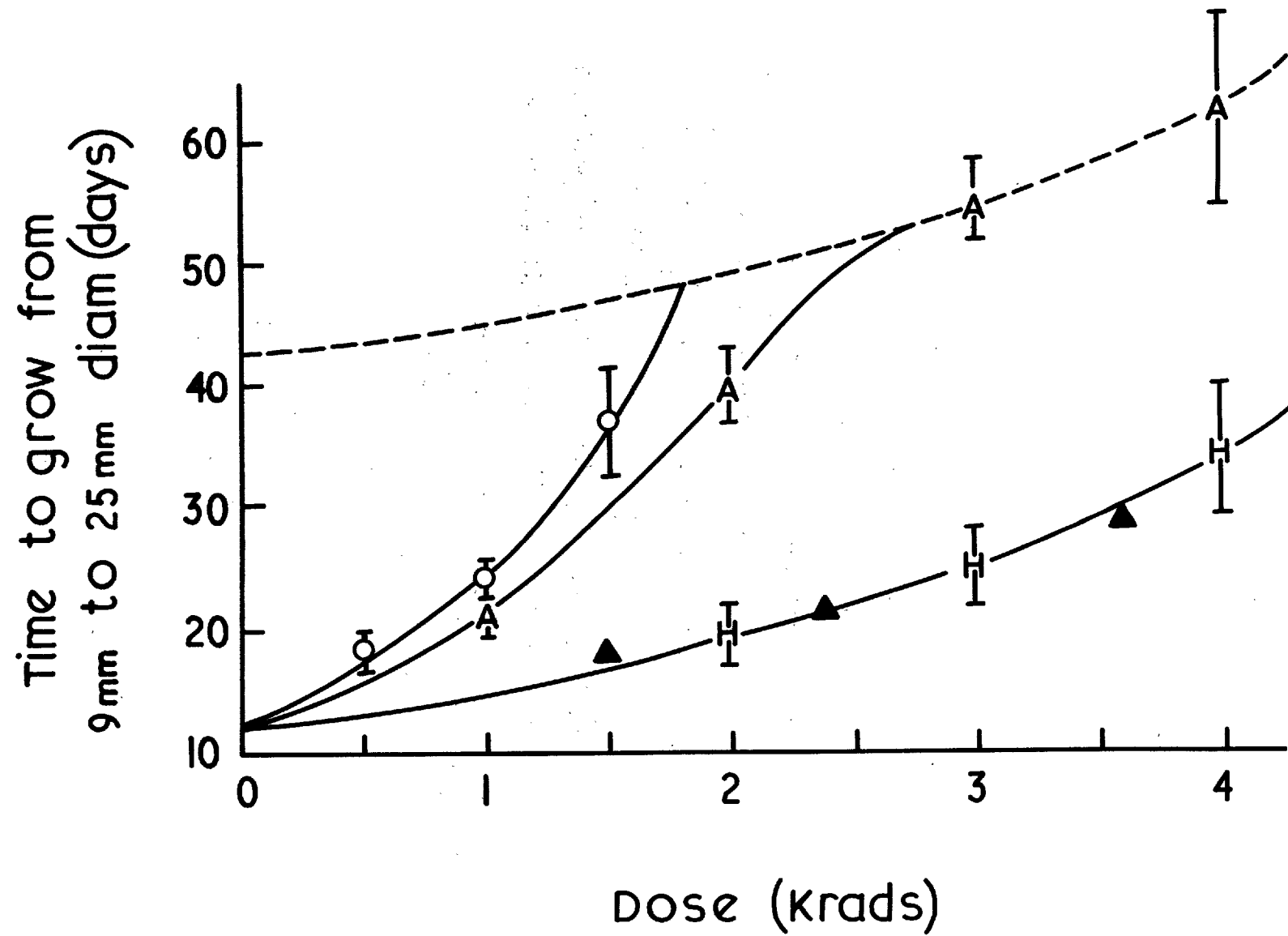
The dotted line is the line through the hypoxic points drawn at a higher level.

The triangles were obtained by multiplying the dose scale for the oxie points by 3.

O Animals breathing oxygen.

A Animals breathing air.

H Tumours clamped.



Irradiation conditions	Dose Krads	No of animals	Time to grow from 9 to 25 mm. days	95% c.l.
Controls		18	12.0	± 0.8
<u>X-rays</u>				
Hypoxic	2.0	6	19.6	± 2.5
	3.0	6	24.8	± 3.3
	4.0	5	34.7	± 7.0
Aerobic	1.0	5	21.8	± 2.4
	2.0	6	40.0	± 3.0
	3.0	6	55.0	± 3.2
	4.0	5	62.0	± 8.0
Oxic	0.5	7	18.4	± 1.0
	1.0	6	24.0	± 1.4
	1.5	5	37.1	± 4.8
<u>Neutrons</u>				
Aerobic	0.3	6	20.5	± 1.6
	0.6	6	31.8	± 4.2
	0.8	5	50.0	± 8.0

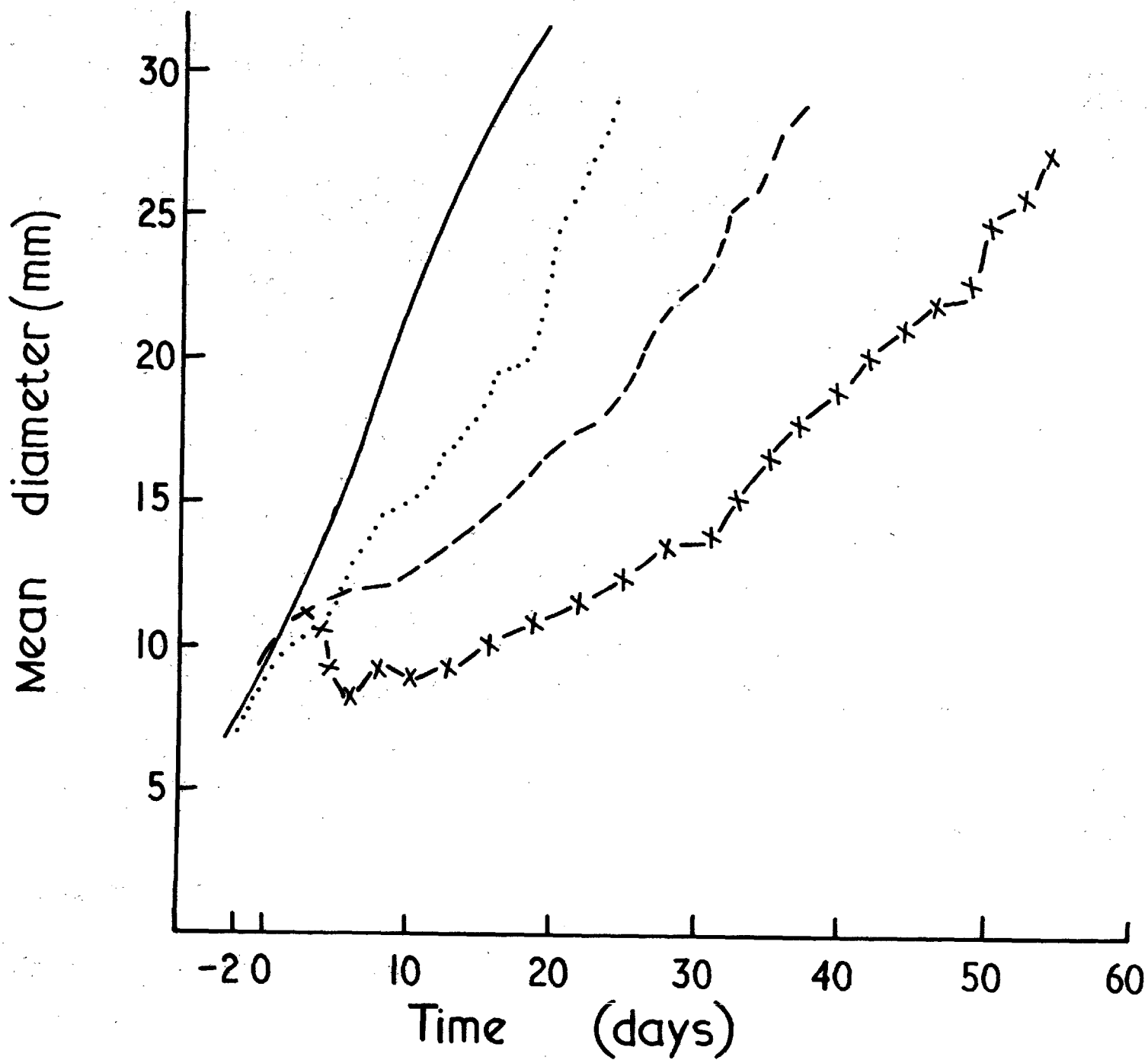
Table 4Growth of RIB₅C after irradiation.

from the Medical Research Council Cyclotron. Growth curves after irradiation of unclamped tumours with animals breathing air are shown in figure 29. The pattern of delay followed by regrowth at a slower rate was similar to that following X-irradiation. The times to grow from 9 to 25 mm. (table 4) are plotted in figure 30. The lines for X-irradiation are included for comparison. There was no obvious break-away in the dose-effect curve.

4.2.3. Discussion.

If one could assume that the effect of radiation on tumour growth reflected only changes in cell survival it would be possible to construct survival curves from the growth curves. Consider the curve for tumours exposed to 1500 rads of X-rays in oxygen. The time to grow to 20 mm. was 30 days (figure 27). The time for an unirradiated tumour was 8 days (figure 24). If we extrapolate the control growth curve back 30 days from the 20 mm. point, we obtain a hypothetical untreated tumour the ratio of whose size to that of the treated tumour at the time of irradiation gives a measure of the cells surviving the treatment (Thomlinson and Craddock, 1967). By calculating such volumes, survival curves were constructed for the different conditions of irradiation with X-rays (figure 31). There are many assumptions involved in the calculations of these curves; not all cells in the tumour will be proliferating, nor will tumour cells represent the whole of the tumour mass. Growth delay, alterations in growth rate after irradiation and effects on the vasculature have also been ignored.

Growth of tumour KB₅O after neutron irradiation. Animals
 breathing air.
 Control —————
 300r
 600r ————
 800r -X-X-X-



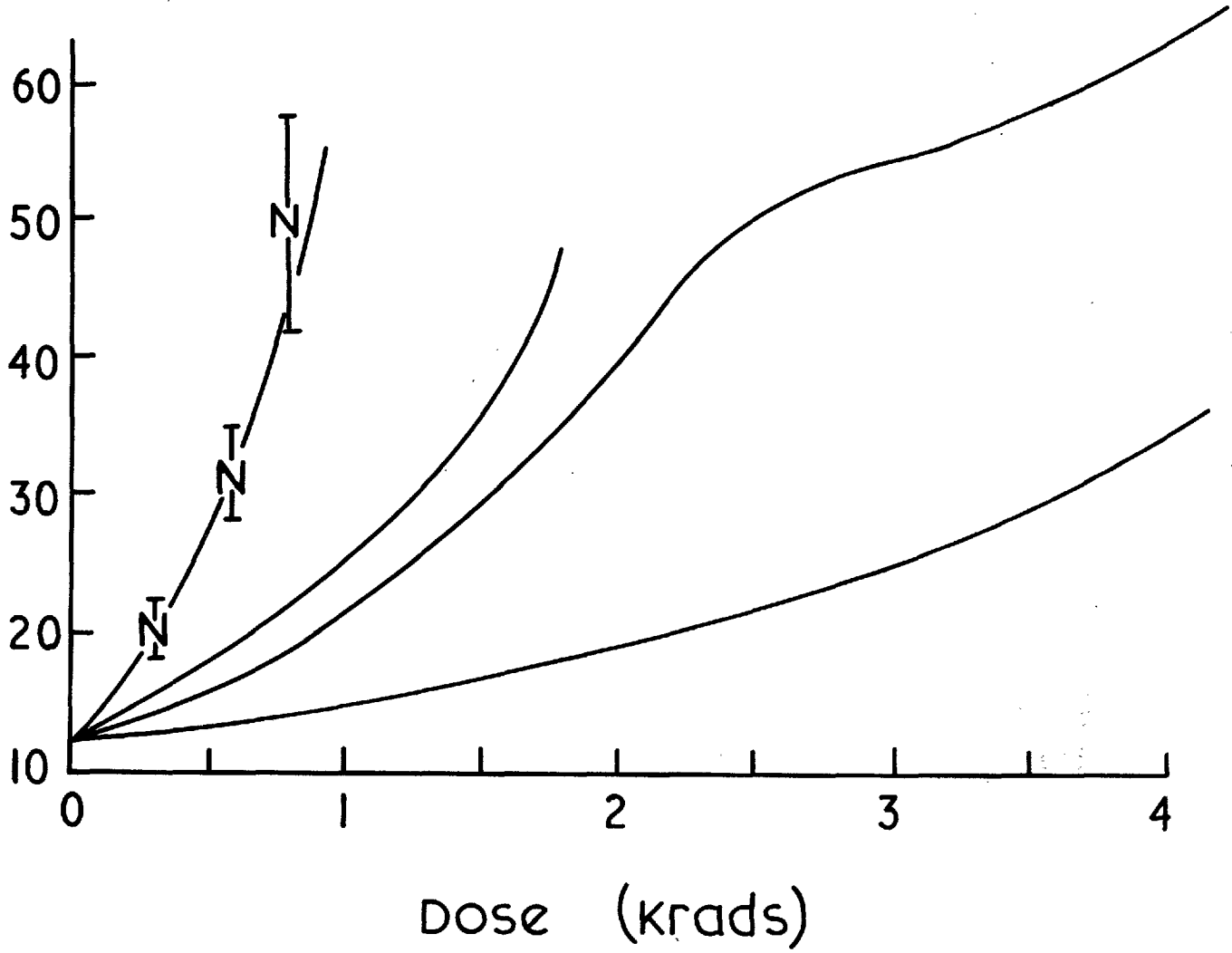


Figure 30

Dose-effect curves for tumour RIB₅C following neutron irradiation in air. The vertical lines indicate the 95% confidence limits. The curves for X-irradiation are included for comparison.

Time to grow from 9 to 25 mm (days)	Neutron dose (rads)	X-ray dose (rads)	RBE
13	40	130	3.25
14	80	240	3.0
15	120	340	2.83
20	300	710	2.36
25	440	1200	2.73
30	570	1500	2.63
35	660	1760	2.67
40	750	2000	2.67
50	830	2500	3.10

Table 5

RBE for growth delay in tumour RIB₅C.

The dotted lines in figure 33 represent the survival curves obtained by irradiating cells in vivo and then plating them in vitro, and will be discussed later (section 4.3.), but it is clear that qualitatively the calculated curves are not too different from these.

If the radiobiological equivalent dose of neutrons, the RBE, is defined as the ratio of the dose of X-rays to that of neutrons to produce the same effect, then comparing the growth curves following irradiation in aerobic conditions gives the dependence of RBE on dose (table 5). The dotted line in table 5 separates the low dose values calculated using the X-ray curves for irradiation in oxygen from those using the curve for aerobic irradiation (see figure 28). The RBE initially fell with increasing dose. After a dose of about 2000 rads of X-rays there was an inflection in the dose-effect curve suggesting that the response of the tumour was now governed by that of hypoxic cells. There was no inflection in the neutron curve, with the result that the RBE now increased with increasing dose in a manner similar to that found by Field, Jones, and Thomlinson (1967) for tumour RIB₅.

4.3. RIB₅C tumour cell survival.

4.3.1. Single doses.

The advantages of tumour RIB₅C over RIB₅ and the methods whereby single cell survival is measured have already been discussed in chapter 2.

Survival curves for cells from tumours irradiated with either X-rays or neutrons, and then immediately

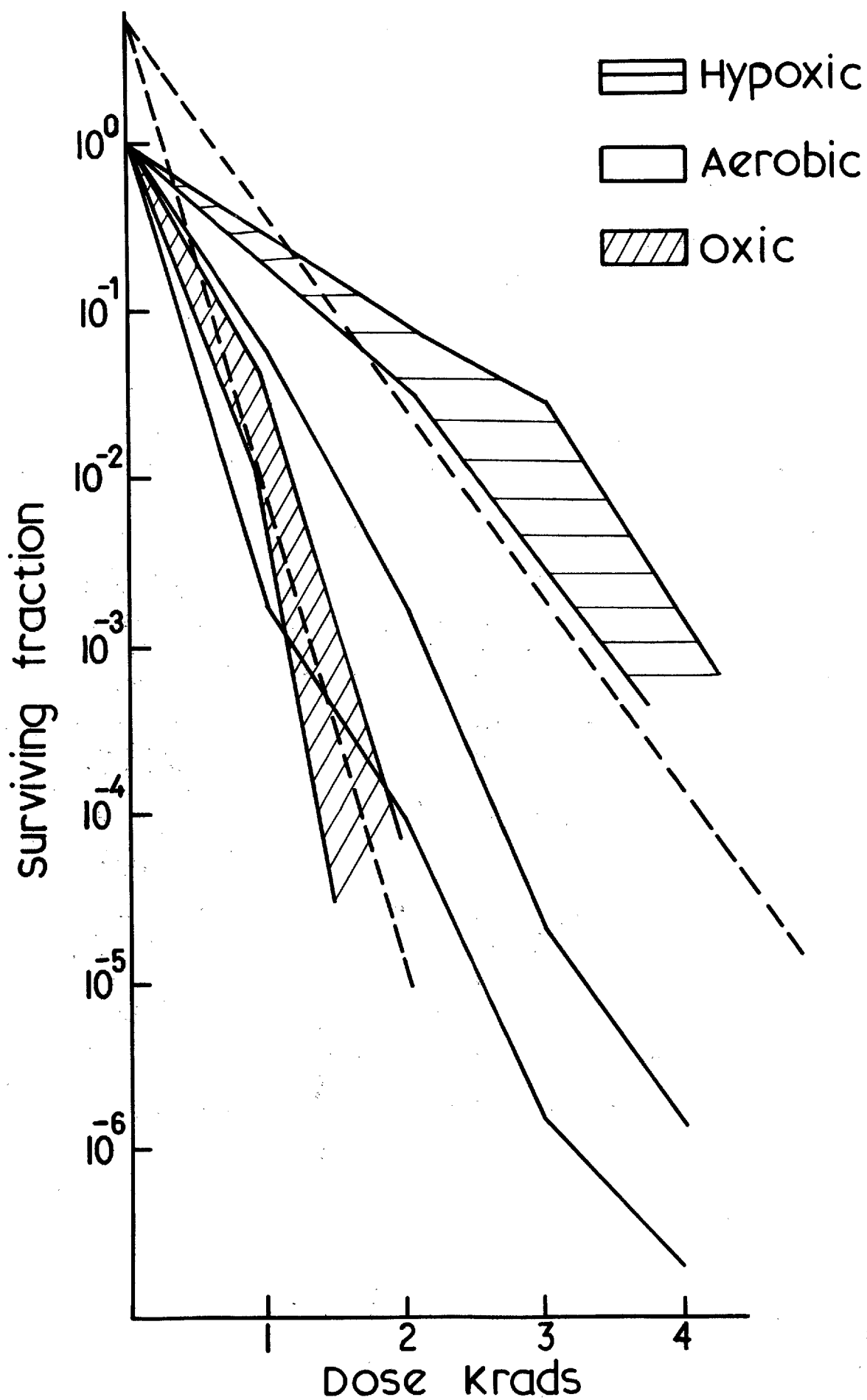


Figure 31

Calculated cell survival curves for RIB₅C (see text)

The dashed lines are the measured survival curves.

excised and plated, are shown in figure 32. The curve for aerobic irradiations with X-rays had the biphasic shape characteristic of a mixed population of well oxygenated and hypoxic cells. Because of this, only survival values below 10^{-1} were used in calculating the survival curve. Extrapolation of the curve back to zero dose indicated an hypoxic proportion of 15 - 20 percent. The survival curve parameters together with their 95 percent confidence limits are shown in table 6.

The neutron RBE's as a function of surviving fraction are listed in table 7. As in the case of growth delay (table 5), there was a decrease in RBE with increasing dose, followed by a subsequent increase due to the influence of hypoxic cells. These values of the RBE are all greater than 3, in contrast to the ratio of the values of the D_0 for oxic cells which was 1.65.

If, instead of irradiating the tumours and then excising them, a tumour was excised, the cells plated, and then irradiated four hours later, the resulting survival curve for oxygenated cells agreed well with that for irradiations in vivo. However, the points for anoxic irradiations were consistently below those for in vivo irradiations in hypoxic conditions (figure 33). In fact the points for irradiations in vitro fit a pair of survival curves with a lower extrapolation number than that shown; nevertheless, the chief difference between the two sets of data was a change in the response of hypoxic cells, resulting in a reduced o.e.r. when cells were irradiated four hours after plating.

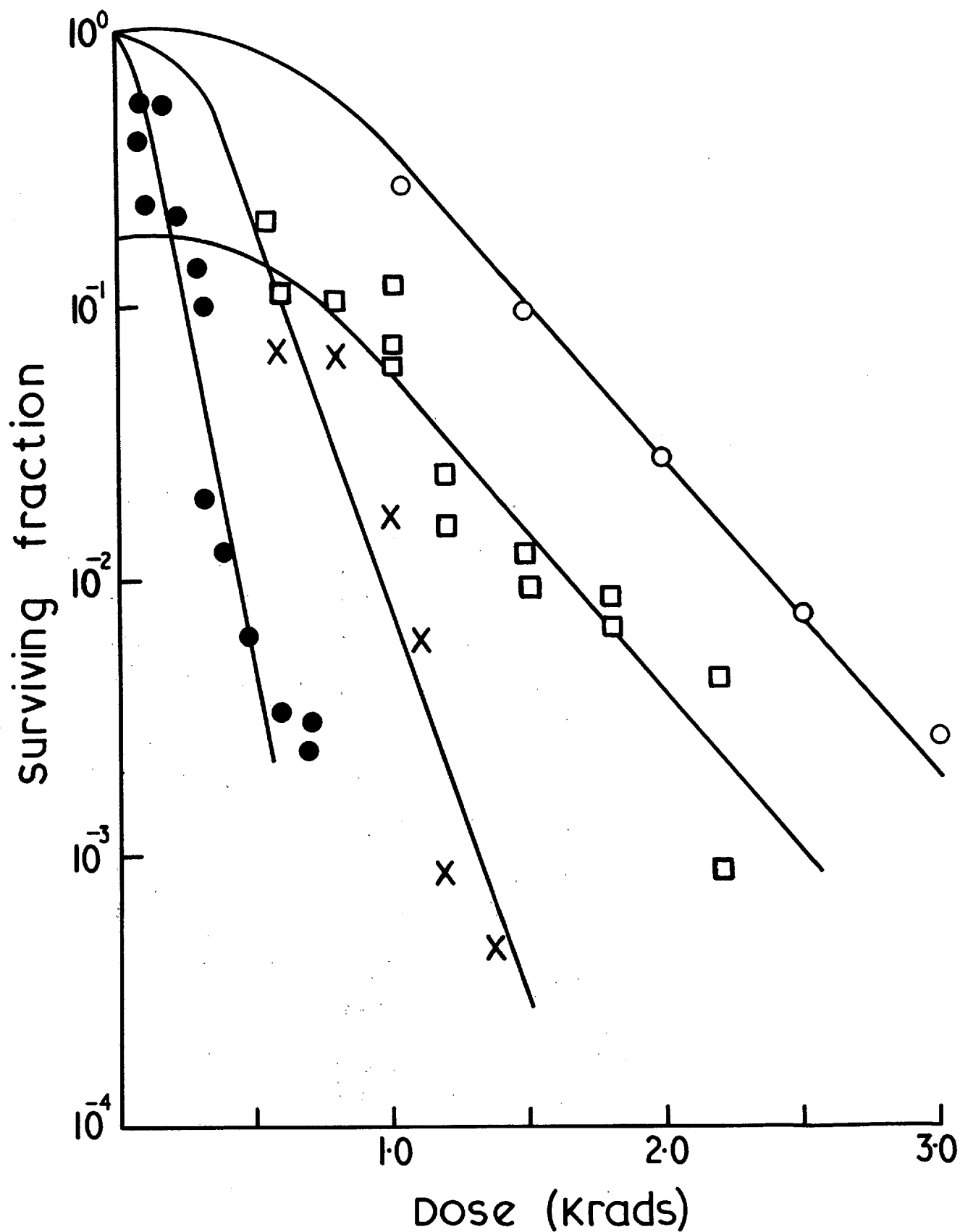


Figure 32

Survival curves for cells of RIB₅C irradiated in vivo and assayed in vitro. X animals breathing oxygen at 30 p.s.i. □ animals breathing air. ○ tumours clamped. ● neutron irradiation, animals breathing air.

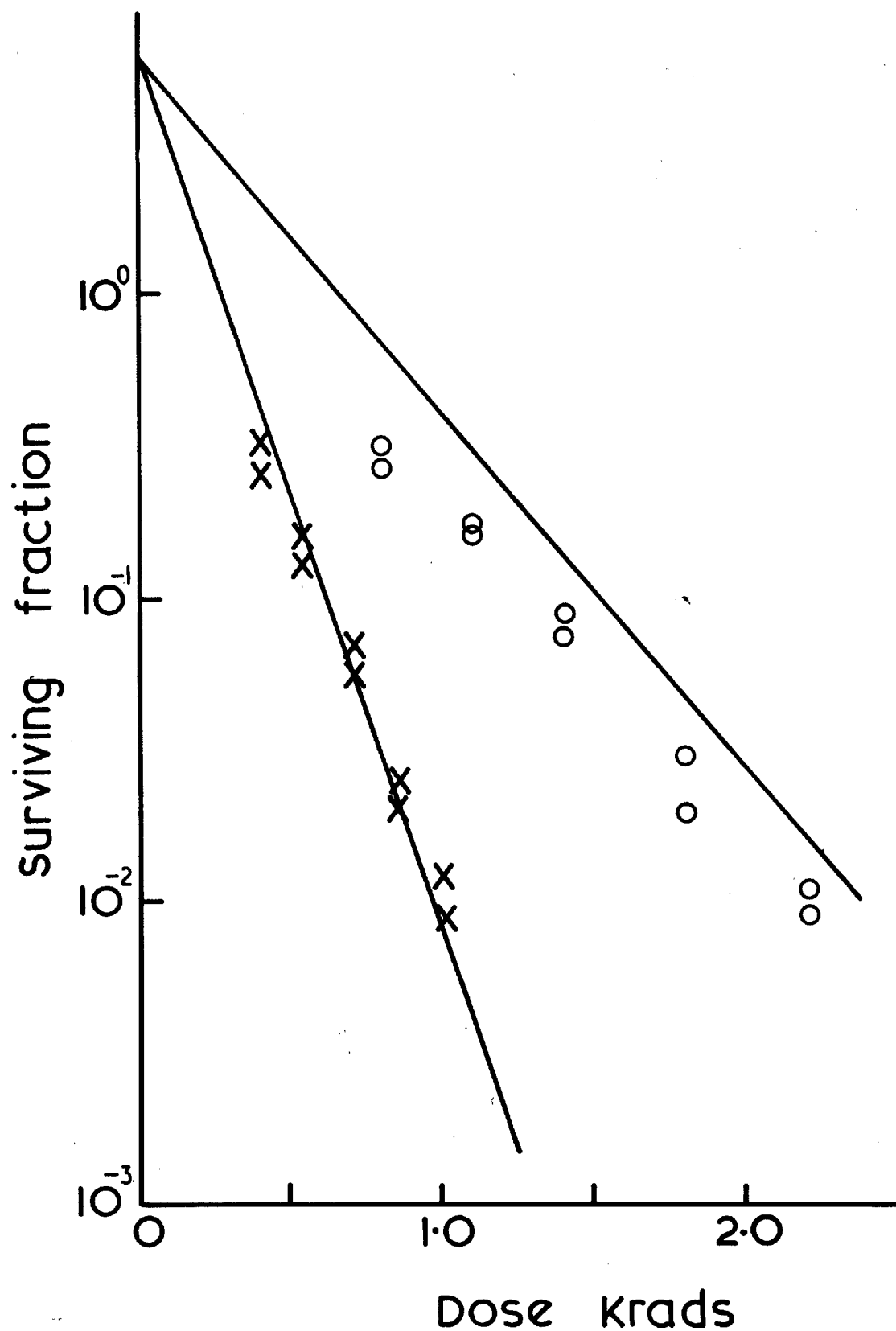


Figure 33

Survival curves for the cells of RIB₅₀ irradiated four hours after plating into glass petri dishes. The lines are those for irradiations in vivo (figure 32).

Aerobic X

Hypoxic O

Conditions	Radiation	n	Do(air)	o.e.r.
Irradiated <u>in vivo</u> .	X-rays	5.5 (1.4-20.0)	153r (127-195)	2.5 (2.2-2.9)
Irradiated 4 hours after plating.	"	3.8 (2.5-5.8)	170r (154-190)	2.0 (1.9-2.2)
Irradiated <u>in vivo</u> .	Neutrons	1.8 (0.4-8.2)	93r (61-197)	

Table 6

Survival curve parameters for the cells of tumour RIB₅C
irradiated in vivo and in vitro and assayed in vitro.

Surviving fraction	5×10^{-1}	10^{-1}	5×10^{-2}	10^{-2}	5×10^{-3}
RBE	3.36	3.10	3.50	3.78	3.90

Table 7

The RBE for cell survival for tumour RIB₅C.

4.3.2. Fractionated doses.

The survival curves of figure 32 show that tumour RIB₅C contains 15-20 percent hypoxic cells as measured in terms of the ability of cells to form colonies in vitro. Since in clinical practice tumours are usually treated by fractionation, it is important to know whether or not these hypoxic cells can shed their sub-lethal damage.

To test the ability of hypoxic cells to shed sub-lethal damage, tumours were exposed to two equal doses of 1000 rads of X-rays separated in time, while animals were breathing air. These doses were sufficient to ensure that the population being tested had been hypoxic at the time of the first dose, since even if the well-oxygenated cells were capable of shedding all their sub-lethal damage, but the hypoxic cells shed none, the ratio of hypoxic to oxie survivors would be greater than 20 to 1 after the second dose. The results are shown in figure 34 (circles). There was a prompt increase in survival, characteristic of a population capable of shedding its sub-lethal damage (Elkind et al, 1965). The fluctuations in survival seen after two hours imply that the first dose induced a partial synchrony in cells that were in cycle (Elkind and Sinclair, 1965). The solid triangles of figure 34 refer to tumours that were exposed to a single dose of 1000 rads and then left for a period before excision. There was no significant change in survival with time up to six hours.

A complication in the interpretation of fractionation experiments using tumours containing hypoxic cells is the

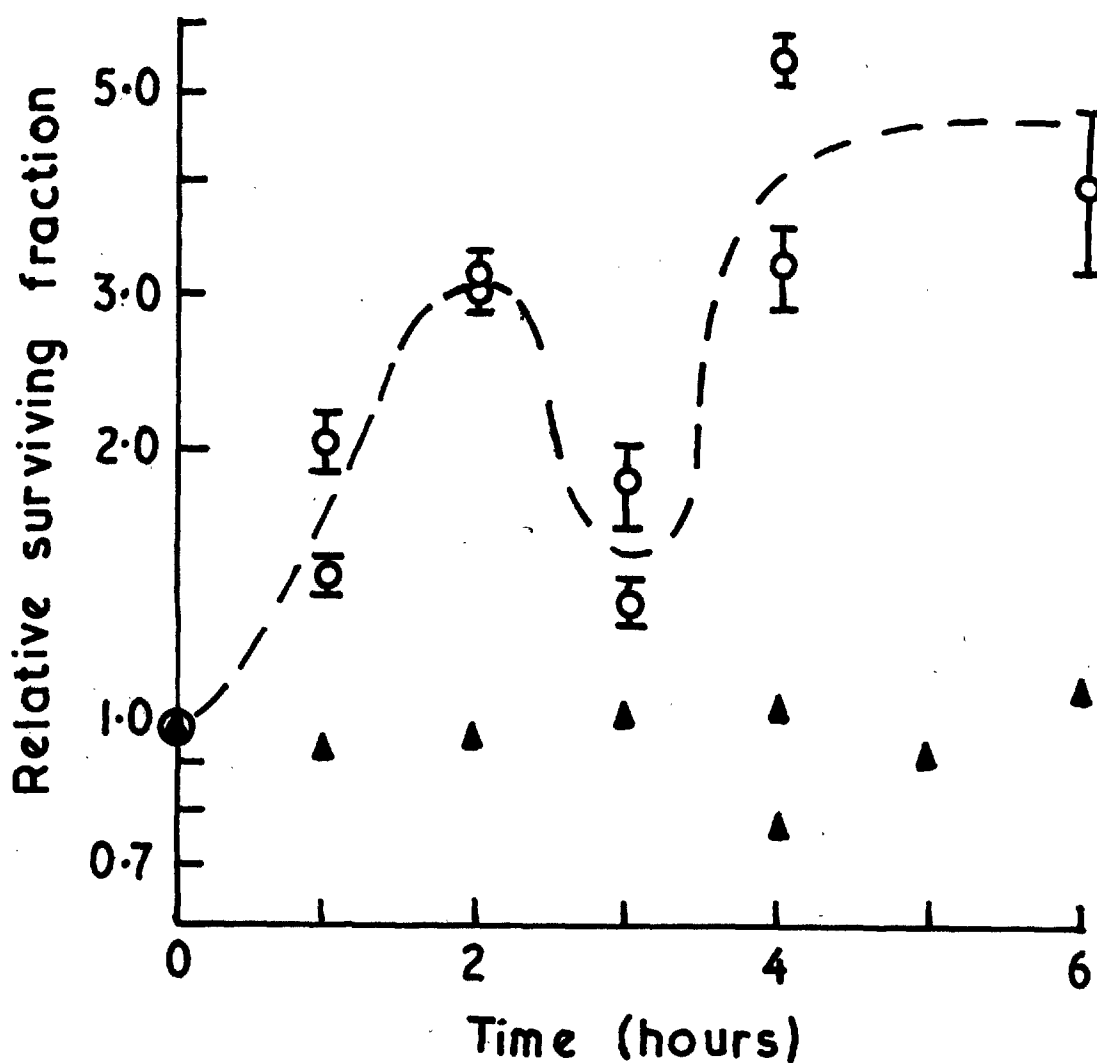


Figure 34

Relative surviving fraction of RIB₅₀ cells irradiated in vivo, as a function of the time between two doses of 1000 rads. Animals breathing air. Standard errors were calculated from the variation in the number of colonies on each of four plates. Triangles, tumours exposed to a single dose of 1000 rads, and excised at various times thereafter.

possibility of reoxygenation (van Putten and Kallman, 1968; Thomlinson, 1968; Howes, 1969). It is possible that cells that were hypoxic at the time of the first dose of radiation became reoxygenated in the time interval before the second dose. Consideration of the shapes of survival curves shows that the results shown in figure 34 were due to effects on hypoxic cells which remained hypoxic between irradiations, and not to reoxygenation.

Figure 35 shows hypothetical survival curves for oxygenated (AC) and hypoxic (AB) cells having an extrapolation number n , oxygen enhancement ratio m , and aerobic sensitivity $1/D_0$. Consider the hypoxic curve. After a dose D_1 the surviving fraction is given by point B (figure 35). If there is no recovery or reoxygenation, the surviving fraction after a second dose will be given by the line BG. If there is complete reoxygenation and shedding of sub-lethal damage, survival will follow line BPE with the shoulder, which is curve AC displaced to start from B. Clearly if there is reoxygenation but no recovery, survival will be below this line. In what circumstances will survival be above that given by the line BG? If the second dose is less than D_2 (figure 35) this can occur if there is reoxygenation and recovery. However, for doses greater than D_2 this can only occur if hypoxic cells shed their sub-lethal damage and remain hypoxic between doses. The value of D_2 can be calculated by considering the similar triangles HBK and HJF (figure 35). From these we have:-

$$\frac{HB}{BK} = \frac{HJ}{JF} \quad - - - - - 1$$

Now $HB = \log n$ (natural logarithms)

$$BK = D_0 \log n$$

$$HJ = HB + BJ = \log n + \frac{D_2}{mD_0}$$

$$JF = D_2$$

Substituting in 1

$$\frac{\log n}{D_0 \log n} = \frac{1}{D_2} \left\{ \log n + \frac{D_2}{mD_0} \right\}$$

Solving this for D_2 gives

$$D_2 = \frac{mD_0 \log n}{m - 1}$$

and this is independent of the initial dose D_1 . For the survival curves of figure 32 the value of D_2 was 430 rads. Since the second dose in the fractionation experiment was more than twice this, we must conclude that the hypoxic cells of this tumour shed their sub-lethal damage and remained hypoxic for the period during which this process took place.

This is confirmed by the results illustrated in figure 36, which shows a survival curve for cells from tumours exposed to a first dose of 1000 rads in air and a series of second doses five hours later. The experimental points agree well with the calculated curve for full shedding of sub-lethal damage by hypoxic cells, the dashed curve of figure 36.

This shedding of sub-lethal damage by hypoxic cells was reflected in the decreased time for tumours to grow from 9 to 25 mm. following two doses of 1000 rads 5 hours apart, as compared with that following a single dose of 2000 rads. For the single dose the time was 40 ± 3 days.

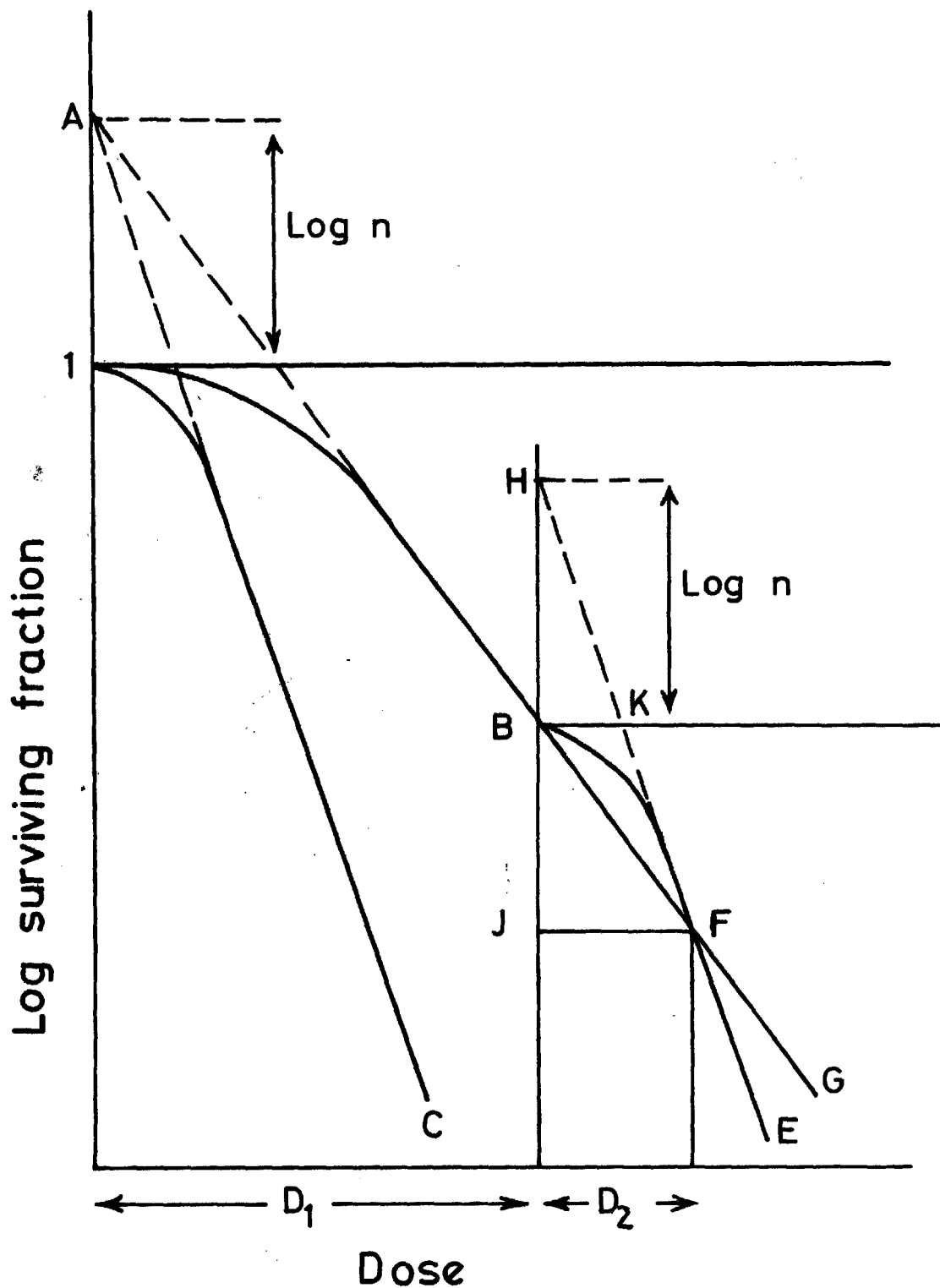


Figure 35

Theoretical survival curves for aerobic and hypoxic cells. The ordinate is the natural logarithm of the surviving fraction.

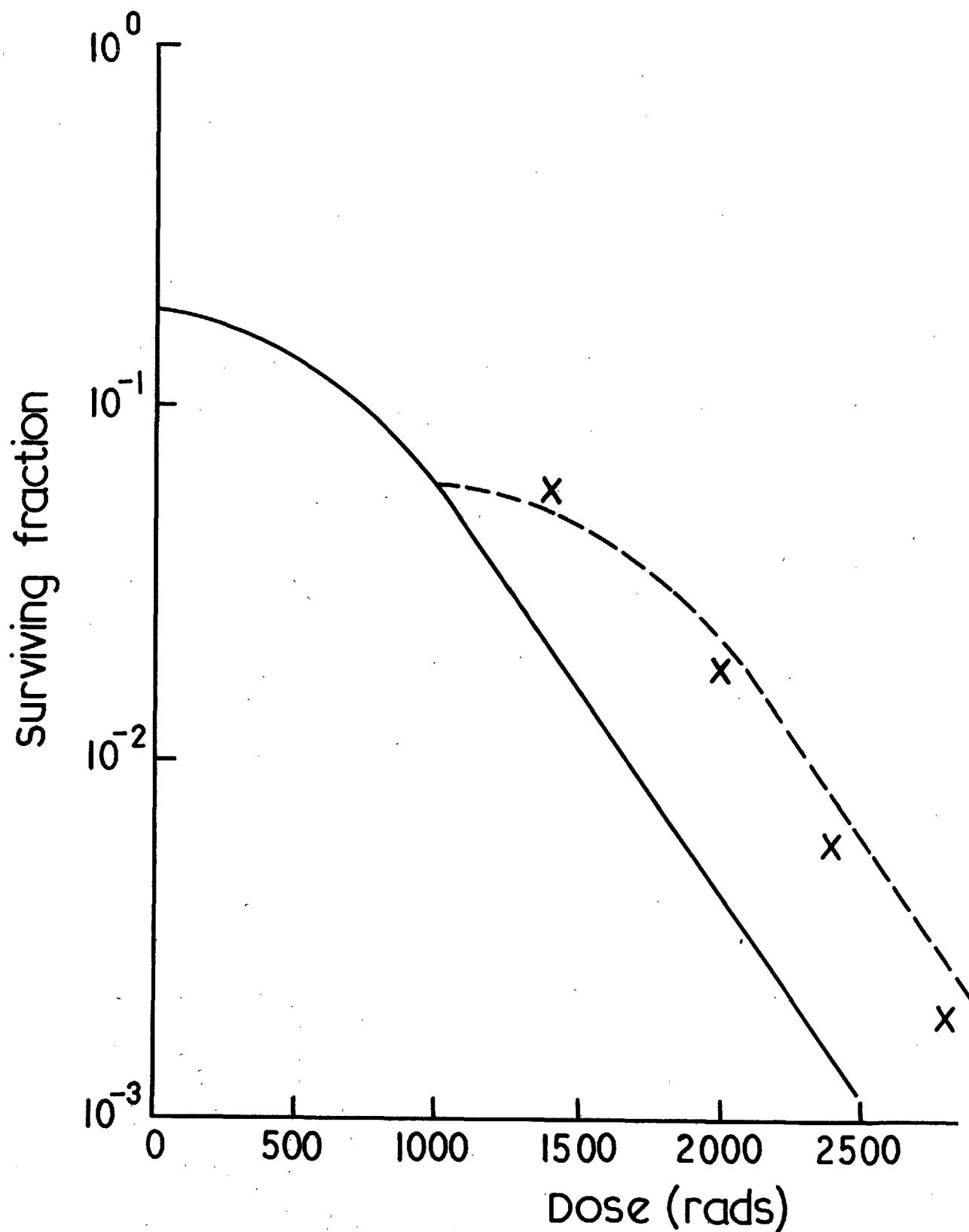


Figure 36

Fractionated survival curve for irradiation of RIB₅C tumours with an initial dose of 1000 rads, and graded second doses five hours later. The solid line is the hypoxic tail of the single dose survival curve for cells from tumours irradiated in air, and the dashed line is the calculated curve for full shedding of sub-lethal damage by hypoxic cells.

For the fractionated dose, with the second dose given to unclamped or clamped tumours, the times were 32.6 ± 2.5 days, and 29.1 ± 2.7 days respectively, implying that there was shedding of sub-lethal damage between the doses by an hypoxic population.

4.3.3. Discussion.

It is possible to compare the survival curves actually measured for the cells of tumour RIB₅C irradiated in vivo and assayed in vitro with those calculated on the basis of the gross response of the tumour. This comparison is made in figure 31, in which the dotted lines represent the actual survival curves and the shaded areas the calculated ones.

The difference between the o.e.r. for cell survival and that for changes in tumour growth is reflected in the difference between the measured and calculated survival curves. The difference in the o.e.r.'s could be attributed to a difference in the response of hypoxic rather than oxidic cells (figure 31). In section 4.1.3. a similar difference between the calculated and measured survival curves for the hypoxic cells of tumour RIB₅ was described. The discussion on the types of damage expressed in vivo and in vitro following irradiation of cells in vivo as outlined in that section applies equally to the results following irradiation of RIB₅C, and will not be repeated here.

Table 8 lists the survival curve parameters together with their errors for the three conditions in which the

Conditions	n	Do(oxic)	o.e.r.
RIB ₅ C irradiated <u>in vivo</u> , assayed <u>in vitro</u> .	5.5 (1.4-20.0)	153r (127-195)	2.5 (2.2-2.9)
RIB ₅ C irradiated 4 hours after plating.	3.8 (2.5-5.8)	170r (154-190)	2.0 (1.9-2.2)
RIB ₅ cultured cells.	2.8 (1.8-4.3)	161r (147-178)	2.2 (2.1-2.3)
RIB ₅ C tumour growth delay.			3.0

Table 8

Survival curve parameters for cultured RIB₅ cells
and cells from tumour RIB₅C.

cells of RIB₅C were irradiated:- 1) irradiated in vivo and then plated; 2) grown as a tumour in vivo, plated and then irradiated; 3) grown in vitro and irradiated as a monolayer culture. The decreasing o.e.r. with the time the cells were in culture was due to changes in the response of hypoxic rather than oxic cells; there was also possibly a decrease in the survival curve extrapolation number, although the confidence limits do overlap (table 8).

Barendsen and Broerse (1969), irradiating rhabdomyosarcoma cells, have obtained the opposite result. When the survival of cells irradiated and assayed in vitro was compared with that of cells irradiated in vivo and then plated, there was no difference in the survival curves for hypoxic cells, but the o.e.r. was decreased from 2.4 ± 0.2 for cultured cells to about 1.9 for cells irradiated in vivo and assayed in vitro. They did not irradiate tumours while animals breathed oxygen, so that the o.e.r. following irradiation in vivo was measured from the low dose part of the survival curve for cells irradiated in air-breathing animals. It is possible that even at low doses the cells being assayed were not radiobiologically well oxygenated, but the biphasic shape of the survival curve, and the observation that the slope of the terminal portion of this curve was parallel to that for survival of tumour cells irradiated in dead animals, would suggest that they were.

It is not known to what extent the oxygen concentration is reduced in radiobiologically hypoxic cells which are still viable, since radiosensitivity may be much reduced

at oxygen concentrations which are high compared with those at which respiration rates are reduced (Alper and Howard-Flanders, 1956; Froese, 1962; Dewey, 1963). The fractionation experiment illustrated in figure 34 provides some information as to the lower limit of the pO_2 of these viable hypoxic tumour cells. Revesz and Littbrand (1964) reported that when the pO_2 was 3 p.p.m. damage was no longer accumulated in Chinese hamster cells in sub-lethal form. Other authors have found oxygen to be fully dose-modifying, so that proportionately the same amount of damage was accumulated in sub-lethal form in hypoxic as in well oxygenated cells (Elkind et al, 1965; Dewey, 1963; Howard, 1968; Bryant, 1970), and Elkind et al found that Chinese hamster cells were able to shed sub-lethal damage when the pO_2 between doses was 15 p.p.m. The ability of hypoxic RIB₅C tumour cells to shed their sub-lethal damage (figure 34) suggests that hypoxic conditions such as those in which Chinese hamster cells show no sparing effect of fractionation (Revesz and Littbrand, 1967) are unlikely to be of clinical importance, and that the oxygen concentration of these hypoxic clonogenic cells is probably greater than 3 p.p.m.

Chapter 5

GENERAL DISCUSSION

5.1. Cell proliferation in vivo and in vitro.

The volume doubling time of tumour RIB₅C was twice that of RIB₅, 48 hours compared with 24 hours for RIB₅. One might have expected that during the period of adaptation of RIB₅ cells to their in vitro culture conditions there would have been selection for cells with short cell-cycle times, which in turn would have been reflected in faster growth of the tumour after reimplantation into rats. In fact the cell cycle was longer for cells in vitro than for those in the original tumour, and this was increased further when they were inoculated into rats (table 9).

The difference in growth of the two tumours can be better understood if a specific model of tumour growth is considered. The model is one developed by Steel (1968), according to which there are two main processes which can give rise to a difference between the cell cycle time and the population doubling time, such as is observed with both tumour RIB₅ and RIB₅C. One process is a decrease in the growth fraction due to the movement of cells into a non-proliferative state (Mendelsohn, 1962). The other is the continuous loss of cells from the whole population (Steel, 1968). On the basis of this model, a potential doubling time, T , is defined, which is the expected population doubling time in the absence of cell loss. T may be calculated from the frequency in the population of cells in any recognisable phase of the cell cycle, and

the duration of that phase. For instance, for a steady state population

$$T = \frac{T_S}{LI} \quad - - - - - 1.$$

where T_S is the duration of the DNA-synthetic phase, and LI is the labelling index, the fraction of cells in that phase.

In an expanding population this relation has to be modified to take account of the non-uniform distribution of cells in the phases of the cell cycle

$$T = \frac{\lambda T_S}{LI} \quad - - - - - 2.$$

Steel (1968) has shown that this correction factor, λ , is given by the equation

$$\lambda = \frac{T}{T_S} \left\{ \exp \frac{\ln 2}{T} (T_{G2} + T_S) - \exp \frac{\ln 2}{T} \cdot T_{G2} \right\} - - - 3.$$

T_S and T_{G2} can be measured from a labeled mitoses curve, and using equations 2 and 3, T and λ can be calculated by a series of successive approximations.

T will be greater than T_C , the cell cycle time, in a population with a growth fraction of less than one, and Steel has shown that the growth fraction, f , is given by the equation

$$\ln(1 + f) = \frac{T_C}{T} \ln 2 \quad - - - - - 4.$$

Steel has interpreted any difference between T and the actual doubling time, T_D , in terms of loss of cells from the tumour volume. This cell loss may be due to removal of dead cells, or migration of viable cells away from the tumour to other sites in the body. Assuming that the exponential model of cell growth is

valid, and that there is no change in mean cell volume, the cell loss can be described in terms of a factor, ϕ , which is the fraction of additional cells formed at each division which are lost from the tumour, and which is given by

$$\phi = 1 - \frac{T}{T_D} \quad - - - - - 5.$$

These growth parameters have been calculated for RIB₅C to compare with the values obtained by Denekamp (1970) for RIB₅, and are shown in table 9. The increased cell cycle for the cells of RIB₅C compared with those of RIB₅ was due mainly to a large increase in the G1 phase, from 2.4 hours to 11.7 hours. This, in part, explains the decreased growth rate of RIB₅C. The other main difference between the two tumours was the considerable amount of cell loss occurring in RIB₅C, 35 percent compared with none in RIB₅. Why there should be these large differences between two such closely related tumours is not clear. Denekamp (1970) has reviewed the available data on a number of tumours, and the values of the kinetic parameters for RIB₅C fall within the general range she found.

5.2. Are stationary phase cells in vitro a reasonable model of the non-proliferating cells in a tumour?

About 36 percent of the cells in tumour RIB₅C are not engaged in active proliferation (table 9). While a proportion of these cells may no longer be able to divide, those that still can, probably represent a significant proportion of the clonogenic population. The question arises do they respond to radiation in the

<u>Measured parameters</u>	Symbol	Units	Tumour RIB ₅	Cultured cells	Tumour RIB ₅ C
Volume doubling time	T _D	Hours	24	17	48
Labelling index	L.I.		0.30	0.53	0.19
Cell-cycle time	T _C	Hours	13.8	16.5	22
Pre-synthetic time	T _{G1}	Hours	2.4	4.0	11.7
DNA-synthetic time	T _S	Hours	9.0	9.0	7.5
Post-synthetic time	T _{G2}	Hours	2.0	3.5	2.8
Mitotic index	M.I.		0.010	0.018	0.009
<u>Derived parameters</u>					
Potential doubling time	T	Hours	24.2	16.6	31
Proportionality constant	λ		0.83	0.98	0.81
Growth fraction	f		0.45	0.99	0.64
Cell loss factor	ϕ		0.0	0.02	0.36

Table 9

Population kinetics parameters for tumours RIB₅ and RIB₅C, and for cultured RIB₅ cells.

same way as the actively proliferating cells? In chapter 3 the properties of a non-proliferating but still clonogenic population were described, and the relevance of this stationary phase population in vitro as a model of the non-proliferating cells in a tumour will now be considered.

It is unlikely that cells in a tumour would stop dividing because of a lack of space, whereas nutrient depletion due to failure of the vascular supply, for instance, may be a common occurrence (Thomlinson, personal communication). For this reason, unfed rather than fed cells should be considered as the possible model system.

Unfed cultured RIB₅ cells in the stationary phase are characterised by a decreased growth fraction and an increase in all stages of the cell cycle (figure 20). How well does this behaviour mimic that of non-proliferating tumour cells? Denekamp (1968) and Tannock (1968) observed that non-dividing tumour cells were often located furthest from the blood supply. Considerations of the diffusion of metabolites through tissues lead Thomlinson and Gray (1955) to suggest that cells may remain dormant through lack of nutrients for a considerable time without losing their capacity for proliferation, and Tannock's work supports this hypothesis. However, results of labelled mitoses experiments in vivo have yielded conflicting results. Hermens and Barendsen (1969), using an experimental rhabdomyosarcoma in rats, demonstrated an increased cell cycle and possibly a decreased growth fraction in the central region compared with the outer region. Tannock (1968) studied tumour cells in relation to their distance from capillaries, and showed that as this distance increased, the growth fraction fell, but

there was no change in the cell cycle.

With ascites tumour cells, a number of workers have found the cell cycle time to increase with tumour age in a manner qualitatively similar to that observed for unfed RIB₅ cells in vitro (table 10). This might suggest that cells in vitro which have become stationary because of nutrient depletion are a better model of ascites than of solid tumours. In that case one might expect the extrapolation number of the survival curve for ascites tumour cells to decrease with increasing tumour age. Belli, Dicus, and Bonte (1967) irradiating P-388 lymphoma cells in DBA/2 mice, found just such a decrease. One day after inoculation of 10^6 cells the extrapolation number was 8.5 and the D_0 value was 131 rads. After six days the tumour had become hypoxic and the values were 1.6 and 458 rads. I have done similar experiments using diploid Ehrlich ascites tumour cells. Figure 37A shows a growth curve for these cells growing in T0 mice. Tumours were irradiated $1\frac{1}{2}$ and 10 days after injecting 4.5×10^6 cells. In order to overcome problems of hypoxia in the older tumours, all tumours were irradiated 5 minutes after sacrificing the animals, a time long enough to render the tumour cells hypoxic regardless of tumour age (Hornsey, personal communication). Immediately after irradiation, cells were removed from the peritoneal cavity, counted, diluted and plated in vitro, and then assayed for their colony forming ability 10 days later. There was the same fall in extrapolation number with no change in D_0 as was found for stationary phase RIB₅ cells (figure 37B). If tumours were exposed to a single

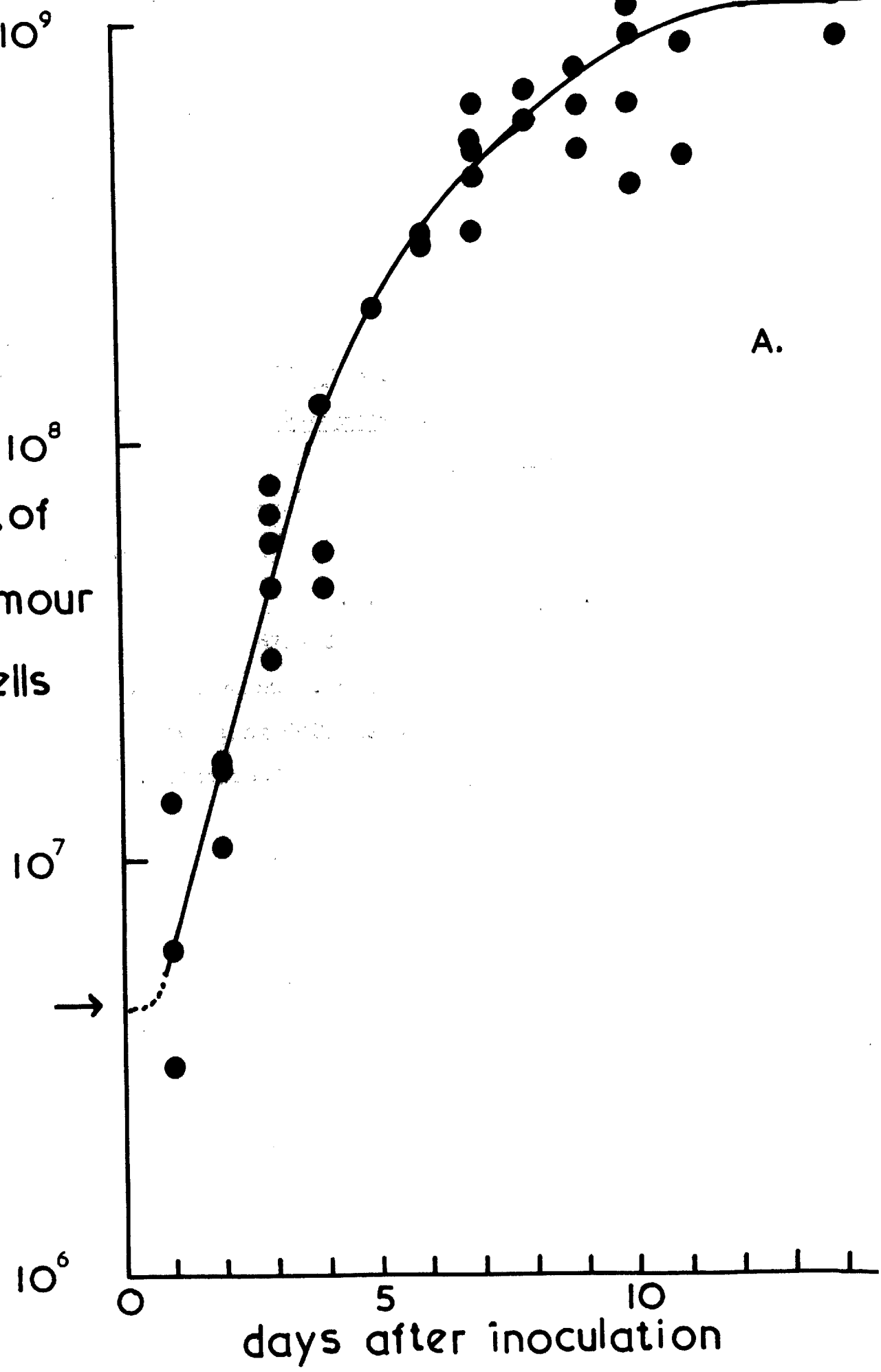
Figure 37A

Growth of Ehrlich ascites tumour cells in
TO mice.

MS 7591

9

10^9
no. of
tumour
cells

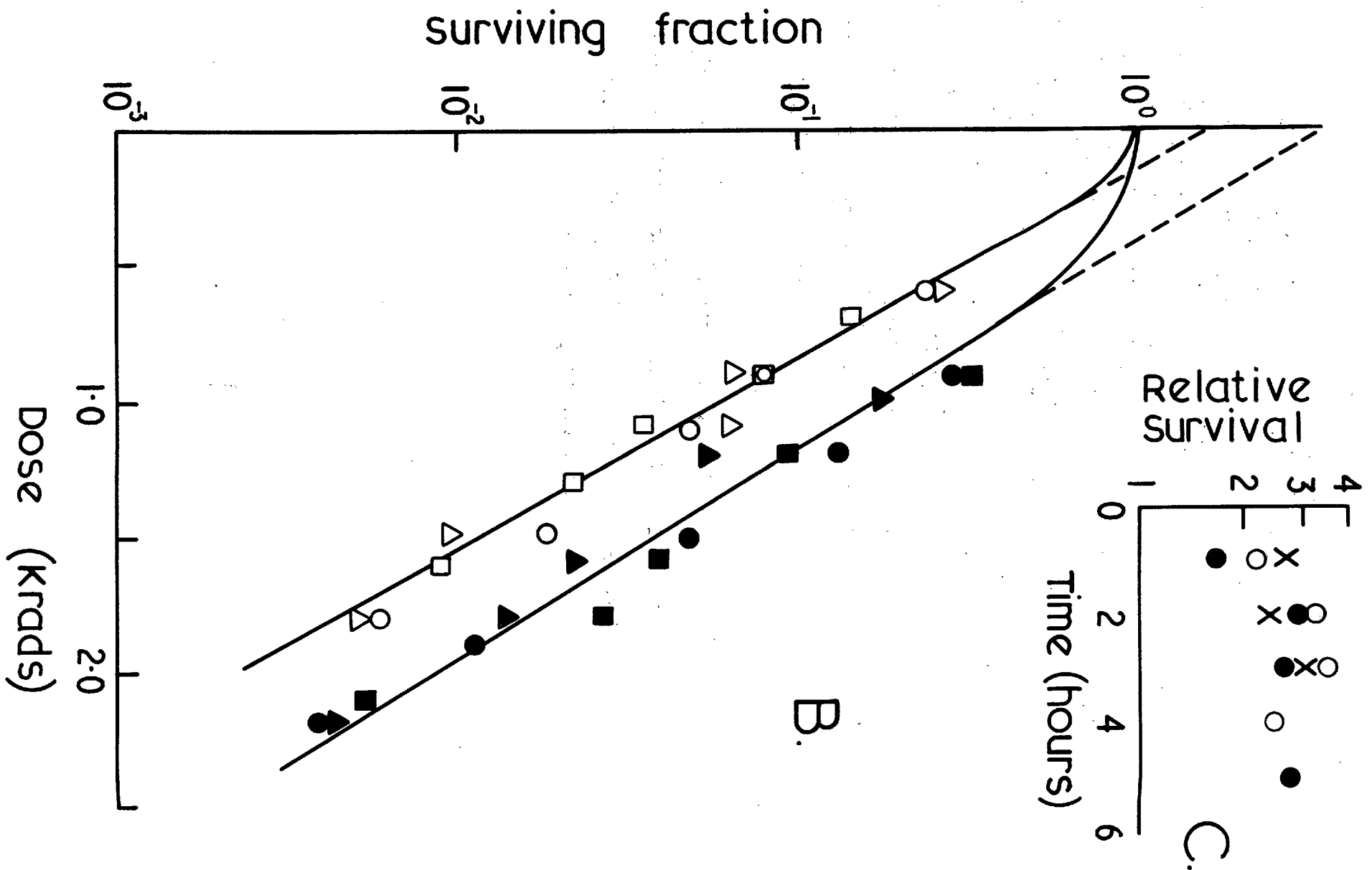


A.

Figure 37

B. Survival curves for ascites tumour cells irradiated in mice $1\frac{1}{2}$ days (closed symbols) and 10 days (open symbols) after injecting 4.5×10^6 cells i.p.

C. Recovery from potentially lethal damage in 10 day old tumours. Tumours were exposed to a single dose of 1500 rads, and cells plated at various times thereafter.



System		T _C hours	T _S hours	T _{G1} hours	T _{G2} hours	G.F. %
RIB ₅ <u>in vitro</u>	log.	17	9	4	3.5	99
	stat. fed	-	9	7.2	3	8
	stat. unfed	33	18	8	7	56
E.A.C. Lala, 1968	1 day	8	6	-	2	82
	7 days	22	18	-	4	53
E.A.C. Wiebel & Baserga 1968	5 days	47	26	14	5.5	
	1 day	21	16	0.5	4	
N.C.T.C. Frindel et al, 1969	4 days	17	9	6.4	1.6	74-86
	12 days	40	21	15	4.5	75

Table 10

Cell cycle parameters for cultured RIB₅ cells
and ascites tumours of different ages.

dose of 1500 rads and then left for a period before removing cells, there was a similar recovery from potentially lethal damage as occurred with RIB₅ cells in vitro (figure 37C).

It has yet to be demonstrated whether stationary phase cells in vitro are a reasonable model of non-proliferating cells in solid tumours. These results together with the results using cultured RIB₅ cells (derived from a solid tumour), suggest that in the stationary phase, cultured cells behave in a similar manner to cells from large ascites tumours. However, results of Berry, Hall and Cavanagh (1970) with HeLa cells show that the response can depend not only on the state of the culture, but also on the nature of the medium in which the cells are growing, so that the general validity of the model, even for ascites tumours, must be in doubt.

5.3. Cell survival and tumour growth delay.

Measurements of tumour growth delay following single doses of radiation, and its modification by oxygen and high LET radiation, suggest that to a first approximation the response of the whole tumour can be described in terms of that of single cells. Oxygen was found to be dose modifying for growth delay with an o.e.r. of 3 (figure 28). The biphasic nature of the growth delay curve for irradiations with animals breathing air (figure 28) can be understood in terms of the existence of two populations of cells, one well oxygenated and the other hypoxic. The fall in RBE with increasing dose for well

oxygenated tumours was similar to that for cell survival. Each of these observations would suggest a correlation between cell killing and tumour growth delay.

However, the difference in the o.e.r. for growth delay and that for cell survival found for both the tumours studied implies that the relationship between the two endpoints is not a direct one. The techniques for measuring cell survival were indirect insofar as it was not possible to measure the formation of clones of proliferating cells in situ as can be done with the gut (Withers and Elkind, 1970; Hornsey, 1970), skin (Withers, 1967; Emery, Denekamp, and Ball, 1970), cartilage (Kember, 1965), and with haemopoietic tissue (McCulloch and Till, 1962; Hornsey, 1971). Nevertheless, the agreement between the brain assay and the in vitro plating technique used with RIB₅, and the correlation between the dose needed to produce a 90 percent cure rate in an experimental tumour, and that calculated from cell survival data (Barendsen and Broerse, 1969), would suggest that cellular radiosensitivity as expressed in terms of the ability of individual cells to regrow a tumour, can be measured by the cloning technique.

Possible reasons for the different o.e.r.'s for the two endpoints were discussed in chapter 4 in terms of the way cells express their damage in different growth conditions. In addition, factors such as mitotic delay, alterations in proliferation kinetics of surviving cells (Barendsen and Broerse, 1969), damage to stroma and blood vessels, will all contribute to the pattern of regrowth of a tumour, and will have their own inherent

radiosensitivities and o.e.r.'s. Siracka, Littbrand, and Revesz (1969), irradiating Chinese hamster cells, found a higher o.e.r. for formation of small colonies than for cell survival. Thomlinson and Craddock (1967) have discussed the possible importance of small colony formation in the regrowth of a tumour, and if alterations in the growth properties of surviving cells have a higher o.e.r. than cell survival, this could contribute to the higher o.e.r. for growth delay.

The correlation between tumour growth delay and cell survival can be tested by plotting one parameter as a function of the other for various conditions of irradiation. Such a plot is shown in figure 38. It may be seen that the delay induced for a given level of survival was greater when tumours were irradiated in well oxygenated conditions than when they were clamped, reflecting the smaller o.e.r. for cell killing than for growth delay. Another feature of interest is the difference between the curve for aerobic irradiation (unclamped tumours) and those for tumours irradiated when either hypoxic or well oxygenated. The curve for X-irradiation of unclamped tumours follows the oxic curve down to a surviving fraction of 10^{-1} and then diverges. Below a surviving fraction of 10^{-1} the surviving cells were those that had been hypoxic at the time of irradiation (figure 32), so that this displacement may have reflected the greater delay in repopulating the tumour by cells that were initially hypoxic as compared with those that were well oxygenated. A similar explanation could apply to the curve following neutron irradiation (figure 38); in this case the displacement from the oxic curve occurred

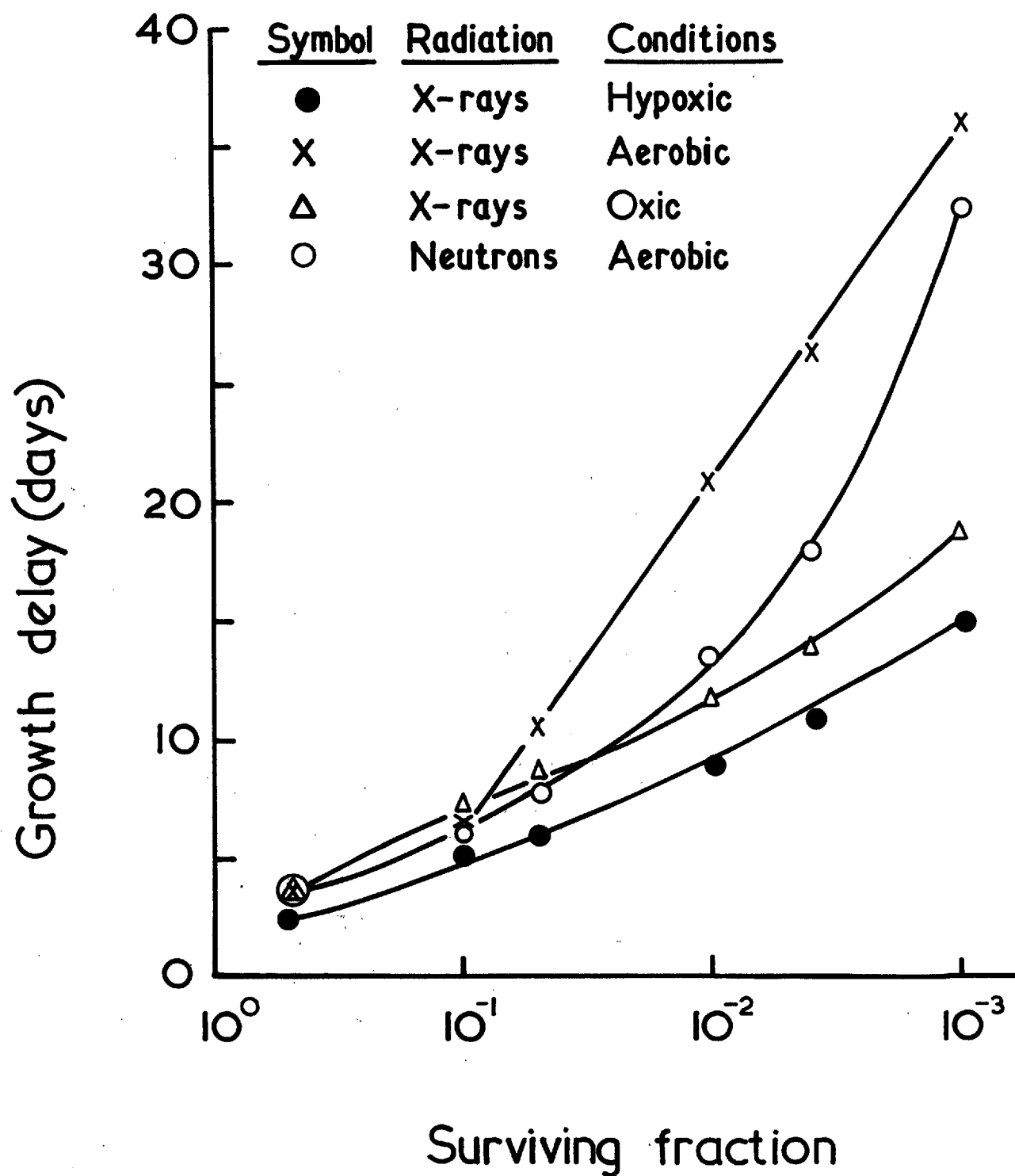


Figure 38

Growth delay induced in tumour RIB₅C as a function of the surviving fraction as assayed in vitro, for different conditions of irradiation.

at a lower level of survival due to the greater cell sensitivity and lower o.e.r. following neutron irradiation.

The increased time to repopulate a tumour for a given level of survival (as measured in vitro) by cells that were hypoxic rather than well oxygenated may have been because the "effective" hypoxic fraction was less for cells left in situ than that measured by plating them in vitro, which "rescued" cells that would otherwise have died due to the hypoxia.

If death due to hypoxia were the only cause of the greater growth delay following irradiation in air (figure 38), then for a surviving fraction of 10^{-2} as assayed in vitro the greater delay induced for irradiation in aerobic conditions over that in oxygenated conditions corresponds to a decrease in surviving fraction to about 10^{-3} (figure 38), so that 90 percent of the cells that survived the radiation must have died because of the hypoxia. If this were the case, one might expect that there would be a fall in the surviving fraction of cells as assayed in vitro with time after irradiation that the tumour was excised. There was no such change with time up to six hours after exposure of RIB₅C to 1000 rads (figure 34). However, this dose may not have been sufficient to ensure that all survivors had been hypoxic at the time of irradiation, and the time interval may not have been long enough. The effects of larger doses and time intervals are at present being studied. Hermans and Barendsen (1969) exposed the R-1 rhabdomyosarcoma to 2000 rads, a dose sufficient to ensure that all the

surviving cells had been hypoxic at the time of irradiation. They measured survival at daily intervals thereafter; the fraction of clonogenic cells remained constant for four days and then increased due to repopulation of the tumour by surviving cells. It would therefore appear that death of hypoxic cells cannot alone explain the increased delay in growth following irradiation in aerobic conditions. There must also be differences between the repopulation kinetics of surviving cells that were initially hypoxic and of those that were oxygenated.

Fractionation experiments such as that described in section 4.3.2. provide information on the shedding of sub-lethal damage by hypoxic cells, but also demonstrate the complexity of the tumour response to radiation. Figure 39 shows the dose-effect curves for tumour regrowth following irradiation in aerobic and hypoxic conditions redrawn from figure 28. The experimental points represent the delay induced by two doses of 1000 rads five hours apart; in one case the second dose was delivered to clamped tumours, and in the other to unclamped tumours. There was no significant difference between these points, indicating that the second dose was being delivered to an hypoxic population. Since reoxygenation was not occurring in this time interval (section 4.3.2.), the expected amount of delay should be given by the hypoxic dose-effect curve displaced to start from the 1000 rads point on the aerobic curve if there was a direct correlation between cell killing

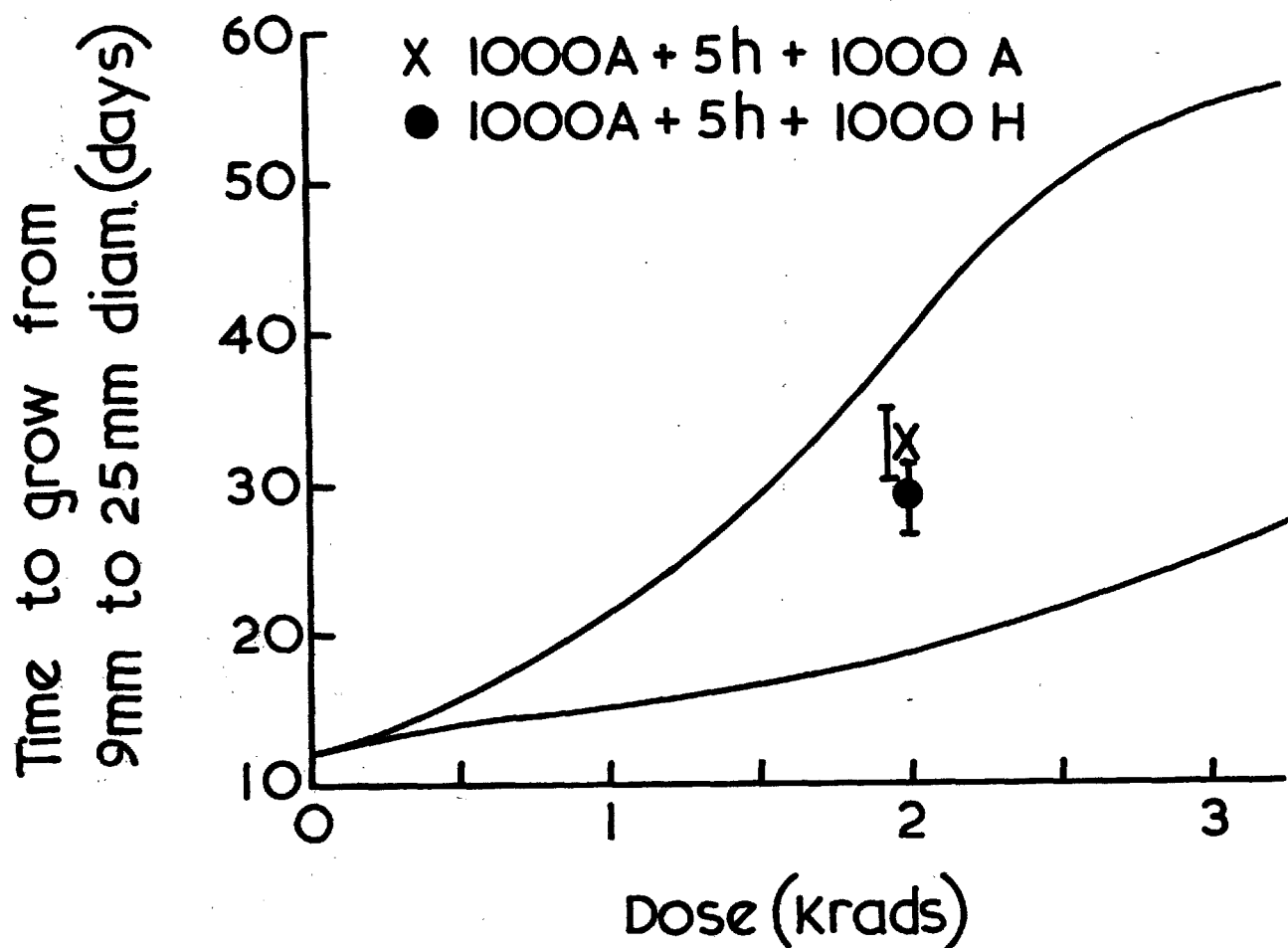


Figure 39

The effect of two doses of 1000 rads five hours apart, on growth of RIB₅C. Each dose was given with animals breathing air. In one group the tumours were clamped for the second dose (●), and in the other they were unclamped (X). The single-dose growth-delay curves for irradiation in aerobic and hypoxic conditions are included for comparison.

and growth delay. In fact the points fit the aerobic curve redrawn from this point rather than the hypoxic curve. This could be accounted for if cells were dying because of hypoxia in the time interval before the second dose. However, figure 34 shows that this did not occur, at least up to six hours after the first dose.

5.4. The RBE's for cell survival and growth delay.

From the curves of figures 28, 30, and 31 a comparison can be made between the effects of neutrons and X-rays on cell survival and tumour growth delay. Figure 40A shows a plot of the RBE's for these two endpoints as a function of neutron dose, for irradiations with animals breathing air. Above a dose of 250 rads the RBE for cell survival was consistently above that for growth delay. The components of these curves can be separated more clearly by plotting the RBE as a function of the inverse of the neutron dose (Alper, 1972). Such a plot is shown in figure 40B. For cell survival curves having a shoulder followed by an exponential region, the RBE depends on the neutron dose according to the relationship

$$\text{RBE} = \frac{1}{D_N} (L^D_q - R_s N^D_q) + R_s$$

once the exponential region of survival has been reached. R_s is the ratio of the slopes of the exponential regions of the two survival curves, and is the RBE at infinite dose. L^D_q and N^D_q are the quasi-threshold doses for

the low LET and neutron curves (Alper, 1972) and D_N is the neutron dose. At low doses the RBE depends on the shapes of the shoulder regions. For a mixed population of oxygenated and hypoxic cells, there will be a sharp increase in the RBE in the region where the response is controlled by the hypoxic cells, i.e. when effectively all the ~~oxic~~ cells have been killed. Figure 40B shows that this occurred at a much lower dose for cell survival than for tumour growth delay. The curve for tumour RIB_5 calculated from the results of Field, Jones, and Thomlinson, (1967), is shown for comparison. The main difference between the two tumours was the larger slope of the straight portion of the curve for RIB_5 , and the smaller value of R_s , 1.2 for RIB_5 , and 2.7 for RIB_5C . A value of R_s close to unity implies that differences in the shoulder regions of the dose-effect curves are of prime importance in determining the RBE. Alper (1972) has shown that a number of different tissues have values of R_s close to unity, and in this respect RIB_5C is exceptional.

An alternative method for visualising the comparative effects of X-rays and neutrons is to compare the growth delay induced by doses of the two radiations which give the same level of survival. Figure 41 shows a plot of the ratio of the delay induced by X-rays to that induced by neutrons as a function of the surviving fraction. As the surviving fraction decreased from 1 to 10^{-1} the X-rays became progressively more effective in delaying growth. For lower levels of survival the ratio decreased,

although not until the surviving fraction was less than 10^{-3} did neutrons become more effective than X-rays.

Barendsen and Broerse (1969), irradiating the R-1 tumour in rats, found that for a given level of survival assayed in the same way as for RIB₅C, 15 Mev neutrons caused a greater amount of delay in tumour growth than did 300 Kv X-rays. Their data has been replotted in figure 41 for comparison. Below a surviving fraction of 10^{-1} X-rays always caused less growth delay than neutrons. They were able to show that the time interval between irradiation and the onset of rapid proliferation by surviving cells repopulating the tumour was greater for neutrons than for X-rays. A similar explanation may also apply in the case of RIB₅C, although in that case X-rays would have to cause a greater delay than neutrons, the opposite effect to that found by Barendsen and Broerse.

The peak in the curve for RIB₅C in figure 41 (and that for R-1) can be understood by relating it to the cell survival curves of figure 32. Below a surviving fraction of 10^{-1} , with X-rays the response of hypoxic cells was being measured. With neutrons, the hypoxic cells did not become important until the surviving fraction was below about 5×10^{-3} . Below a surviving fraction of 10^{-3} hypoxic cells alone were the determining factor with both types of radiation. Thus the increase in delay induced by X-rays relative to neutrons for surviving fractions between 10^{-1} and 10^{-2} reflects the different oxygen status of the survivors immediately after irradiation, hypoxic for X-rays and oxia for neutrons. Below a

Figure 40

A. The dependence of the RBE for cell survival and tumour growth delay on neutron dose for tumour RIB₅C.

B. The RBE as a function of the inverse of the neutron dose.

X RIB₅C cell survival.

O RIB₅C growth delay.

● RIB₅ growth delay.

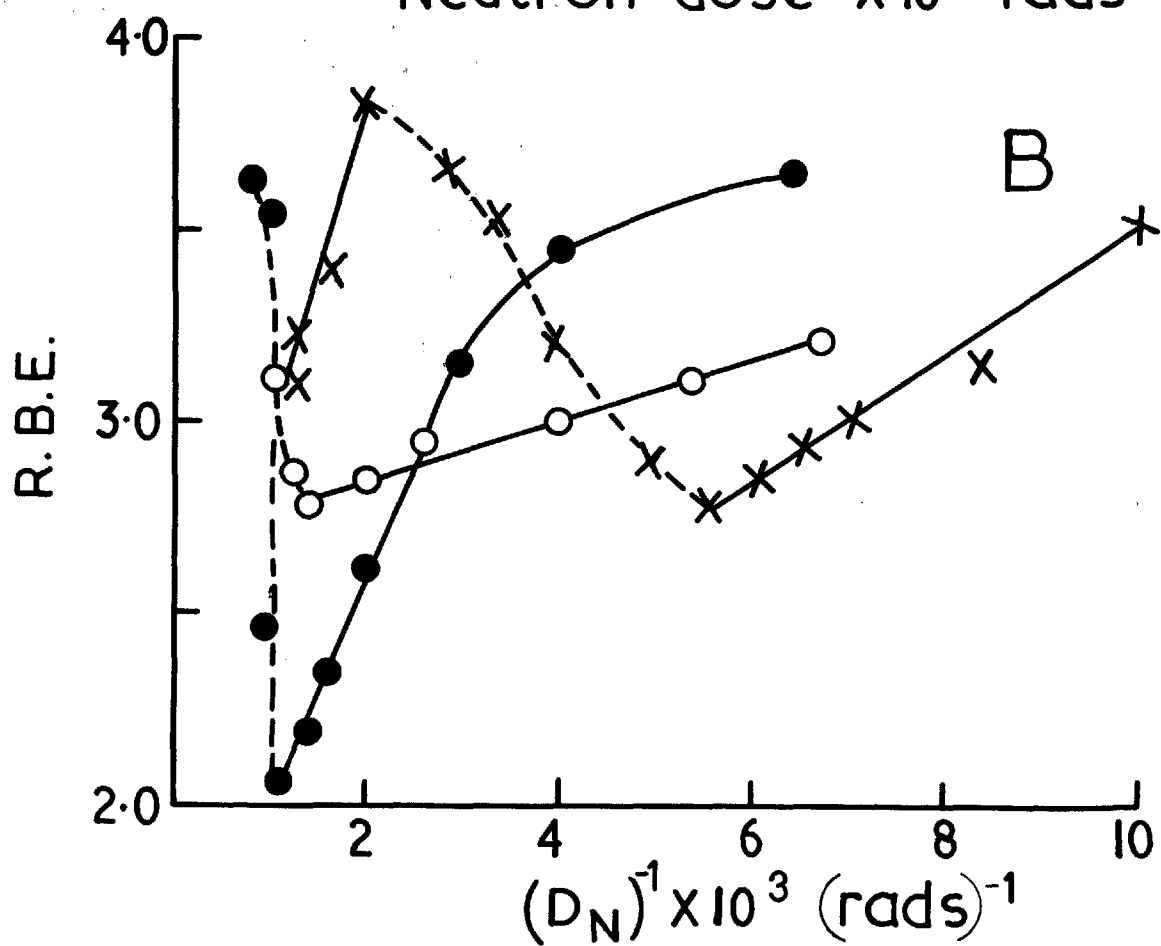
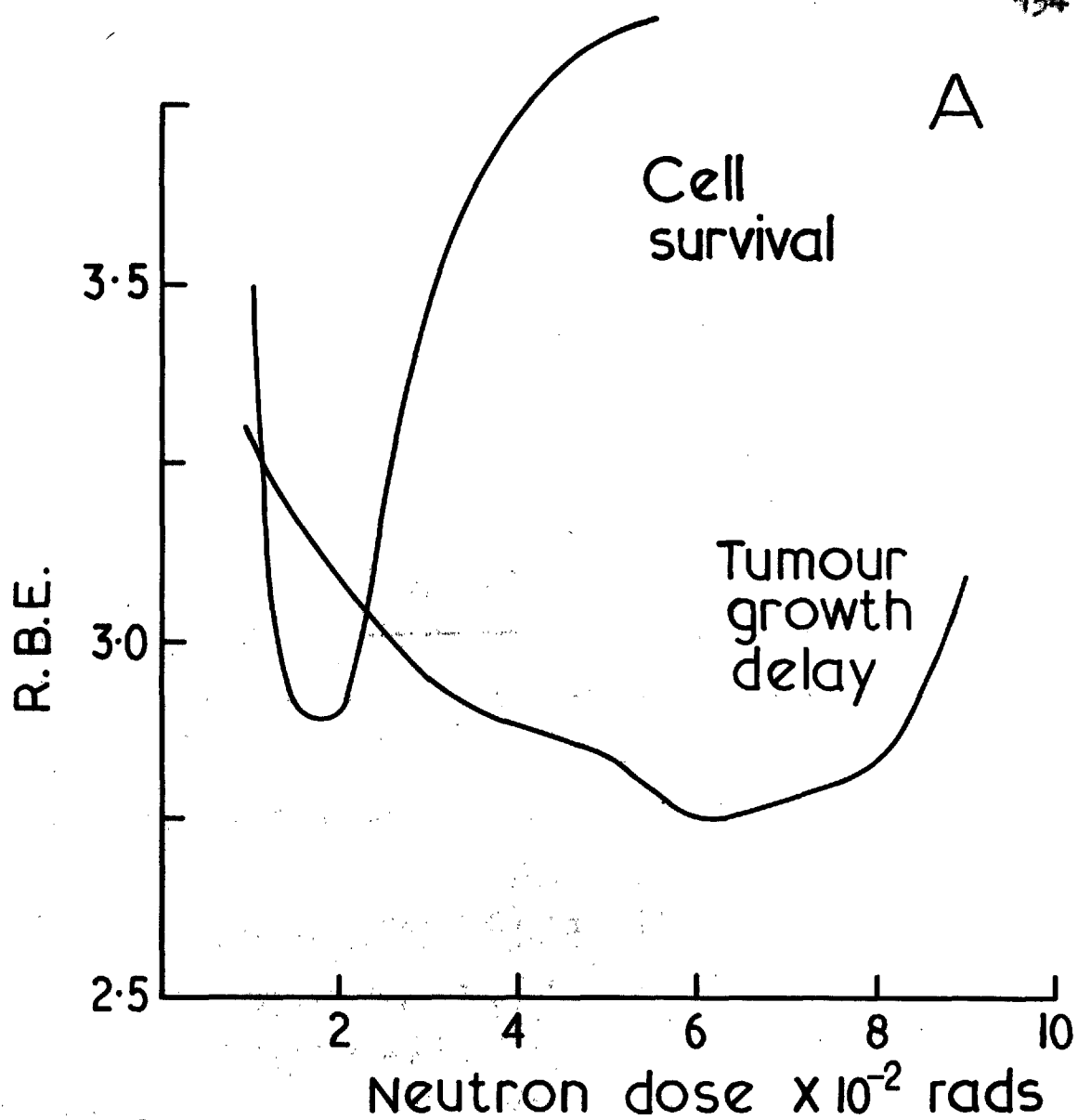


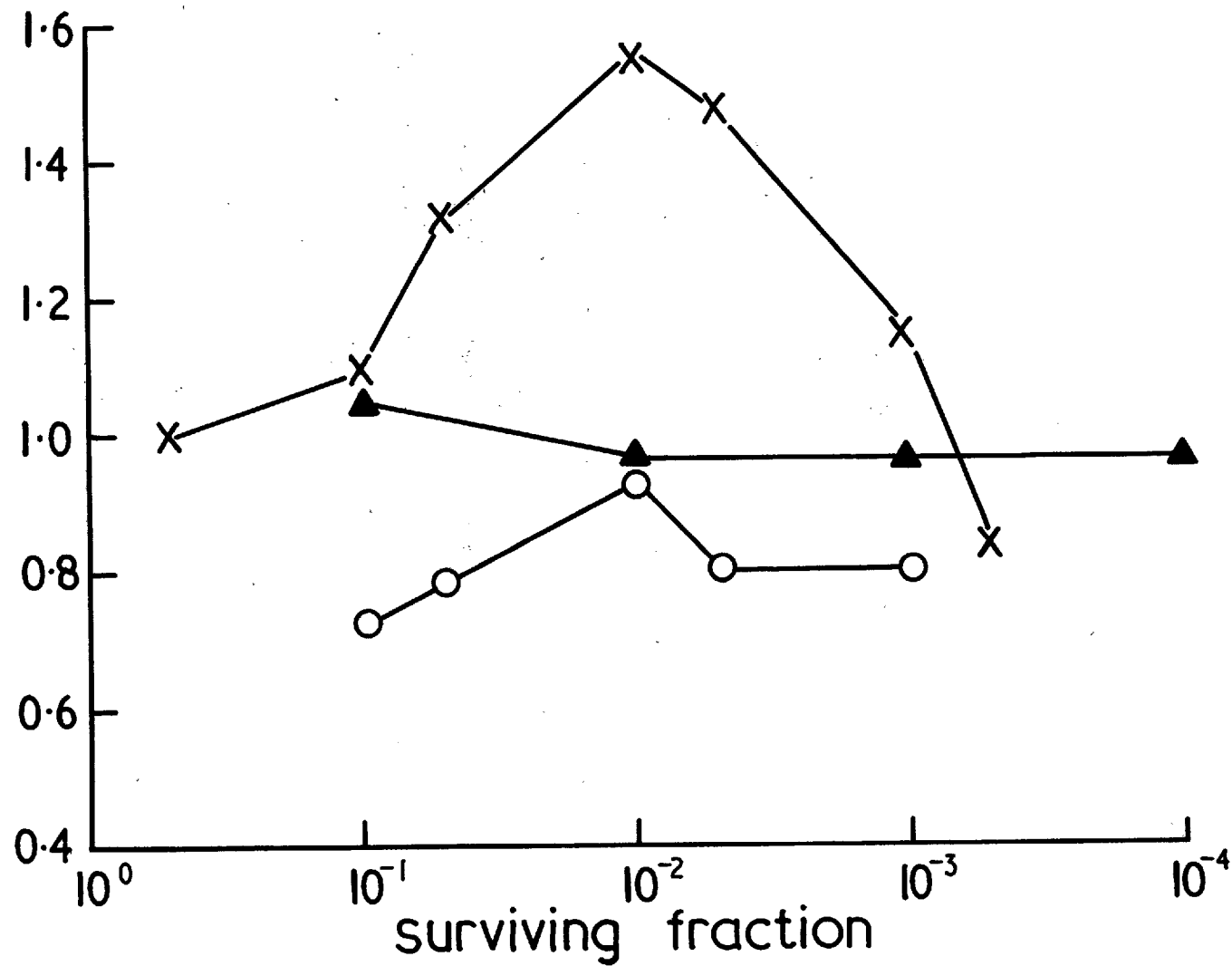
Figure 41

The relative delay induced by X-rays and neutrons as a function of the surviving fraction.

- X Tumour RIB₅C
- O Tumour R-1 (redrawn from Barendsen and Broerse, 1969).
- ▲ Mitotic delay induced in Chinese hamster cells (redrawn from Schneider and Whitmore, 1963).

Delay induced by X-rays

<u>Delay induced by</u>	x-rays
" "	" Neutrons



surviving fraction of about 5×10^{-3} hypoxic survivors predominated for both types of radiation.

Schneider and Whitmore (1963) have compared the effects of X-rays and neutrons on cell survival and the induction of mitotic delay in cultured Chinese hamster cells, and their results have been replotted in figure 41 for comparison with the tumour data. For a given level of survival below 10^{-1} , neutrons were only slightly more effective than X-rays in causing mitotic delay.

5.5. Conclusions.

The results presented in this thesis have demonstrated that the relationship between the gross response of a tumour and that of its cells to radiation is neither a direct one nor a unique one. It depends on the type of radiation used as well as on the level of cell survival obtained; it also depends on the type of tumour under investigation (figure 40). The difference in the o.e.r.'s for growth delay and cell survival for tumour RIB₅ and RIB₅C also demonstrates the complex nature of the relationship between the two endpoints. These differences, together with the change in the o.e.r. for survival of the cells of RIB₅C depending on whether they were irradiated in vivo or in vitro before assaying in vitro (table 8), suggest that deductions based on observations made in vitro may not be valid for predicting the detailed radiobiological response of cells in vivo. As was discussed in chapter 4, this might be due to qualitative differences in the types of damage expressed in vivo and in vitro.

Studies of tumour cell kinetics have demonstrated the existence of non-proliferating cells in tumours. For instance, the growth fraction of RIB₅C is 60 percent. Little is known about the properties of these non-proliferating cells. Studies in vitro (section 3.4.) have shown that non-proliferating cultured RIB₅ cells differ from proliferating ones in their response to radiation. However, it has yet to be demonstrated that such cells are a reasonable model of non-dividing cells in solid tumours.

In a fractionated course of radiotherapy, repopulation by surviving cells between doses may be an important factor. Tubiana (1971), using data of Barendsen and Broerse (1970), has shown that during a course of treatment of the R-1 rhabdomyosarcoma there was a gradual decrease in the doubling time of the surviving cells from 4 days before treatment to 2 days after the end of treatment. These calculations were possible only because the clonogenic fraction could be determined by plating cells in vitro. This emphasises the value of a tumour system in which an estimate of cell survival can be made directly, even if such an estimate may not accurately reflect the in vivo radiosensitivity. Similar studies of repopulation kinetics with RIB₅C should help to explain the differences in growth delay for a given level of survival following irradiation in different conditions (figure 38), as well as the difference in the RBE's for cell survival and growth delay (figure 40).

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