A Cytochemical and Immunological Study of Tumour Cells.

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This thesis is submitted in partial fulfillment of the requirements of the CNAA for the degree of Master of Philosophy, April, 1981.

Abstract

Initial tumour growth was found to be exponential following intraperitoneal inoculation of 5×10^6 Ehrlich ascites tumour cells into male BALB/c mice. After three days of this pattern the rate of growth became noticeably retarded and after 12 days a plateau phase ensued. The reduced growth rate was shown to be an immediate consequence of an increase in the duration of the cell cycle, in the fraction of non-cycling tumour cells and in cell loss. Evidence is presented to suggest that during plateau phase of tumour growth the G_o state does not represent a preferential pathway leading to cell death.

Repeat tritiated thymidine labelling was used to identify non-cycling cells during the plateau phase of tumour growth and to demonstrate their ability to return to rapid cyclic activity when 'Lebensraum' was increased. Subsequently, individual cell DNA, RNA and protein relative contents were determined microdensitometrically for non-cycling (G_o) and rapidly cycling tumour cells. Reduced protein and RNA levels were found to be a feature of G_o tumour cells.

Microdensitometric estimation of the DNA profile for the tumour cell population at various stages during tumour growth revealed that an increase in the proportion of heteroploid tumour cells was concomitant with tumour growth, a finding not supported by chromosome analysis. This factor may be involved in the reduction in tumour growth rate.

Following production of anti-Ehrlich ascites tumour antiserum, flow cytofluorimetric and combined immunofluorescent-autoradiographic analyses were carried out. These analyses failed to reveal gross antigenic changes associated with G_o phase.

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Acknowledgements

My grateful thanks to:

Dr. P. Fletcher, my supervisor, for all her help and encouragement.

Mr. J. E. Castro and Dr. D. J. Beadle for their advice and for reading the manuscript.

Professor K. Weinbren, Suzie Washington and Anias Corrison of the RPMS (London) for use of their microdensitometer and in addition for their kindness and friendship.

Dr. A. Edwards of the Clinical Research Centre, Northwick Park Hospital (Harrow) for FACS analyses.

Mrs. B. Hutchinson of the ICRF (London) for provision of extra tumours.

Dr. G. G. Steel, Institute of Cancer Research (Sutton, Surrey) for assistance with analysis of cell cycle data.

Mr. R. Lamb for assistance with photography.

Mrs. S. Southee for assistance with animal maintenance and handling.

My wife Sandy and Mrs. L. Lacey for assistance with preparation of the thesis.

All the staff of Thames Polytechnic who have contributed in various ways to this project.

Finally I acknowledge the financial support of the Science Research Council.

List of Abbreviations

ALS	Anti-lymphocyte serum.
AC	Acridine orange.
AU	Arbitrary units.
BDH	British drug houses.
BP-8	Benzpyrene-8 (carcinoma).
CI	Colour index.
cAMP	cyclic adenosine monophosphate.
cGMP	cyclic guanine monophosphate.
EAC	Ehrlich ascites carcinoma.
EAT	Ehrlich ascites tumour.
EL4	Dimethylbenzanthracene induced murine lymphoma.
ER	Endoplasmic reticulum.
f	Growth fraction.
FACS	Fluorescent activated cell sorter.
FCA	Freund's complete adjuvant.
FCM	Flow cytometry.
FDA	Fluorescein diacetate.
FIA	Freund's incomplete adjucant.
FITC	Fluorescein isothiocyanate.
G_+	Cell cycle progression (embracing all phases of cycle; G_1, G_2, S, M, but excluding G_o).

G_o	Cell cycle arrest.
G_1	The pre-replicative phase of cell cycle.
G_2	The post-replicative phase of cell cycle.
G_1/G_2	Interphase cell cycle excluding S phase.
GCA	Gallocyanin chrome alum.
^{3}HTdR	Tritiated thymidine.
HL-A	Human histocompatibility locus.
H-2	Murine histocompatibility locus.
ip	Intraperitoneal.
im	Intramuscular.
ICRF	Imperial Cancer Research Fund.
IgG	Immunoglobulin G.
k	cell loss factor.
L_{1210}	Leukaemia 1210.
LAC	Laboratory animal centre.
nd	Not determined.
NRS	Normal rabbit serum.
NYS	Naphthol yellow S.
OD	Optical density.
PBS	Phosphate buffered saline.
PLM	Percentage labelled mitoses.

S	The DNA synthetic or replicative phase of cell cycle.
S-180	Sarcoma 180.
sc	Subcutaneous
TAA	Tumour associated antigen.
T_c	Duration of cell cycle
TCA	Tricarboxylic acid.
TdR	Thymidine.
T_{dbl}	Doubling time.
T_{G1}	Duration of the G_1 phase.
T_{G2}	Duration of the G_2 phase.
TMP	Thymidine monophosphate.
T_s	Duration of S phase of cell cycle.
TTP	Thymidine triphosphate.
TSA	Tumour specific antigen.
UMP	Uridine monophosphate.
UV	Ultraviolet.
vs	Versus or against
N	Total cell number.

General Introduction

Surgery and radiotherapy of cancer are commonly employed as primary methods of treatment for most tumours, chemotherapy being used as an adjunct to such treatment. However, surgery and radiotherapy are relatively ineffective when the disease is widely disseminated, and it is in such a case that chemotherapy has a more prominent role (Ambrose and Roe, 1975 and Schabel, 1977).

The potential of chemotherapy was first recognised by Faber et al. (1948) when temporary remissions in acute leukaemia were achieved by the administration of aminopterin and methotrexate. Subsequent discovery and use of new chemotherapeutic agents was accompanied by reports of improved therapeutic response. Following work on experimental tumour systems by Schabel et al. (1965), Simpson-Herren and Lloyd (1970) and Skipper (1971), a relationship was shown to exist between tumour growth characteristics and response to treatment. However, drug effectiveness was still found to be limited by first order kinetics (Skipper et al., 1964) because most drugs are phase specific agents. This implies that the same percentage of the tumour cell population, and not the same number of tumour cells, are killed by administration of the highest dose of an effective drug conducive to host survival, irrespective of total tumour mass. Consequently, therapeutic schedules have been designed to obtain maximum cytotoxicity by referring to cell kinetic data. Thus the choice of appropriate anti-cancer drugs has been facilitated by determination of the tumour cell growth fraction, the intermitotic (cell cycle) time and cell cycle phase durations.

The most sophisticated therapies have involved attempts to 'recruit' out-of-cycle (G_0) tumour cells rendering them sensitive to drug action, or to 'synchronize' cells thereby increasing the percentage of the population occupying a given phase of cycle in which a particular drug is most effective. Further to this, considerable improvement in

the efficacy of chemotherapeutic agents has been achieved by the use of drugs in combination such that they act synergistically.

It cannot be disputed that recent advances in chemotherapy have improved the management of human cancer, and particularly human leukaemia, nevertheless, formidable problems remain. Damage to normal tissue, especially the intestinal mucosa and bone marrow is an inevitable result of chemotherapy. The latter tends to result in a reduction in immunocompetence leaving the patient at risk to the further possibility of fatal infection. Other unpleasant side-effects may accompany drug administration, these include vomiting, alopecia, and dermatitis, all of which serve to increase the general sufferings of the patient.

Perhaps the most serious problem is the existence of a subpopulation of G_o tumour cells which are less sensitive to chemotherapeutic agents but are capable of returning to cell cycle and so perpetuate the disease when growth conditions permit. Indeed, the presence of G_o cells has been described by Skipper and Schabel (1973) as the major limitation to 'cure' of advanced neoplastic disease by chemotherapy. Cavosto (1977) has shown that the use of recruitment procedures fails to eradicate human leukaemia due to: the presence of a large number of G_o cells, the persistence after treatment of G_o cells with the potentiality of leukaemic stem cells and the existence of a contrary flux of cells from a proliferating to a G_o compartment even during treatment. Thus, following a sustained period of complete remission, patients frequently relapse. It is evident that information concerning the nature of G_o cells is inadequate and no efficient practical techniques for their recruitment exist (Perry, 1976).

In view of these difficulties, the demonstration of tumour specific antigens (TSA) makes the possibility of immunotherapy very

attractive. However, results with this approach have been rather disappointing (Sinkovics, 1978 and Castro, 1978). The reasons for this remain unclear and research has been directed towards: removal of factors responsible for inhibiting potentially cytocidal conflict between the immune response and the tumour cell, amplification of the overall effectiveness of the immune response and sensitization of tumour cells to immunological attack. In this context, the immunological status of G_0 cells may be important, yet little, if any, relevant information exists.

It is probable then that more effective therapeutic methods will result from a better understanding of 'the underlying biochemical [1] *and immunological* basis of the G_0 state' (Baserga, 1978). Therefore, an attempt has been made here to answer the following questions: do differences exist between G_0 and rapidly cycling (G_+) tumour cells with respect to their RNA and protein contents, and are there detectable immunological differences between G_0 and G_+ cells?

For this investigation an experimental tumour system was required which could be shown to have a subpopulation of G_0 cells. In addition, owing to the difficulties encountered when considering G_0 cells it was felt essential that the experimental tumour system ought to be amenable to close control of experimental conditions, should be easily sampled and easily maintained. The Ehrlich ascites tumour (EAT) appeared to fulfil these important criteria and was, therefore, chosen.

Ascites tumours grow as a single cell suspension in peritoneal exudate. The first publication demonstrating the production and advocating the use of an ascites tumour was that of Hesse, which appeared in 1927. In 1932, Lowenthal and Jahn achieved the transformation of the solid Ehrlich mouse mammary carcinoma to that of an

[1] Italics mine

ascitic form, the Ehrlich ascites tumour. A major advantage in using a free cell neoplasm of this type is that an exact number of cells may be transferred from donor to recipient, and Lowenthal and Jahn discovered a correlation between the number of cells inoculated and the host survival time.

Initially, the tumour cells appear to act as a non-specific irritant within the peritoneal cavity (Hartveit, 1965 and Fastia, 1976) and thus promote a peritoneal exudate. The combined effects of increasing cell number and serosal effusion outstrip the drainage capacity of the lymphatic system which itself becomes progressively blocked (Coldie, 1956 and Bloomer et al., 1980). Thus fluid accumulates rapidly after a few days of cell growth. The exact cause of death of the host animal is conjectural; evidence exists to suggest that electrolyte imbalance due to the massive ascites, haemorrhage which may or may not be due to an Arthus or Schwartzmann type immune reaction and toxic products of tumour cell degeneration contribute to the demise of the host. The pathological nature of the EAT does not, therefore, represent a model for the study of malignant ascites in man where fluid accumulation is generally associated with a primary abdominal tumour or metastasis into body cavities. In this respect then the EAT is an artificial system which is comparable to some extent with <u>in vitro</u> tissue culture.

In conclusion, it is noteworthy that ascites tumours, because of their ease of use, have proved to be valuable model systems in the study of very many aspects of tumour biology.

Chapter One

Cell Kinetic Studies

Introduction

The study of cellular proliferation kinetics was revolutionised by the introduction of radioisotopic labels which become incorporated into DNA (Ottesen, 1948). Initial studies used ^{32}P and were concerned with the life span and developmental sequence of white and red blood cells. In 1953 Howard and Pelc, working with ^{32}P labelled bean root meristems, demonstrated the existence of an interval between the end of DNA synthesis and the beginning of mitosis; they called this phase the G_2 period. These workers were also able to infer the existence of the pre-DNA synthetic, post-mitotic interval which they termed the G_1 period.

The introduction of tritium (^3H) labelled thymidine (Taylor et al., 1957) greatly simplified the recognition of cells engaged in DNA synthesis (S-phase), since thymidine is a highly specific precursor of DNA. Thymidine is not, however, a normal component of the metabolic pathway leading to DNA. The formation of thymidine containing nucleotides usually results from the methylation of UMP to TMP. This is followed by the phosphorylation of TMP to TTP and ultimate incorporation into DNA. However, the existence of thymidine kinase, an enzyme which probably represents a nucleoside salvage mechanism, permits the incorporation of exogenous thymidine into the DNA synthetic pathway by phosphorylation of thymidine to TMP. There is much evidence to suggest that thymidine kinase is a universal constituent of proliferating tissue and therefore, thymidine incorporation provides a reliable indication of DNA synthesis (Brent et al., 1965 and Eker, 1965).

Quastler and Sherman (1959) made a classic contribution to kinetic analysis by their proposal of the technique of labelled mitoses. This method enables the determination of the cell cycle time and component phase (G_1, S, G_2) durations by monitoring the

proportion of labelled mitotic figures at various times following the administration of a single pulse of tritiated thymidine (^{3}HTdR).

The use of techniques involving radiolabelled precursors of DNA soon lead to the realization that the members of any given cell population display considerable heterogeneity with regard to cell cycle duration and proliferative status (Mendelsohn, 1960 and 1962). It was further concluded that within both normal and tumour tissues a considerable proportion of cells occupy a non-proliferating or G_0 compartment (Lajtha et al., 1962; Lajtha, 1963 and Gavosto et al., 1960 and 1964).

The nature and behaviour of G_0 cells has been the subject of considerable debate. It has proved extremely difficult to determine whether G_0 cells represent a very slowly proliferating compartment (Prescott, 1968 and Costlow and Baserga, 1973), a non-proliferating compartment via which cell death ultimately occurs (Lala, 1972b), or merely a dormant non-proliferating compartment (Lajtha, 1963).

The term G_0 was originally used to describe those liver cells which were not actively proliferating but which retained the capacity to divide under suitable conditions: thus 'a state of no cell cycle or a state of dormancy in respect of growth' (Lajtha et al., 1962). This definition is applied generally in the present work to any cell type which although not actively proliferating retains the capacity for cell division. Arguments involving the more elaborate aspects of cell cycle arrest, and which ultimately reduce to a matter of preferred definition are examined later in conjunction with cytochemical studies (Ch. 2). Identification and characterization of G_0 cells, therefore, necessitates the demonstration of their ability to return to rapid cyclic activity upon receipt of an appropriate stimulus. DeCosse and Gelfant (1966) reported the existence of slowly proliferating or non-proliferating cells in an S-180 ascites tumour. These workers

were able to show, by continuous thymidine labelling and cytophotometry, that cells were arrested in G_1 and G_2. Later (1968) DeCosse and Gelfant were able to demonstrate the recruitment of resting G_2 phase cells in an EAT using antilymphocyte serum as a mitogen. Lala and Patt (1968) presented evidence for the recruitment of resting G_1 cells in an EAT upon transplantation of the tumour cells. Subsequently, Bichel and Dombernowsky (1973) demonstrated recruitment of G_1 and G_2 arrested cells in the JB-1 ascites tumour.

The major difficulty associated with the study of G_o cells is the lack of a specific biochemical or morphological marker. At present, there are two possible methods available by which information concerning G_o cells can be obtained. Firstly, there is the method of flow cytofluorimetry (FCM) which has proved to be very valuable in the rapid assessment of the kinetic properties of G_o cells and the effect on them of mitogen recruitment in certain cell systems (Darzynkiewicz et al., 1976). The alternative method involves continuous, or repeat, ^{3}HTdR labelling. While the latter method is considerably more laborious than FCM and has inherent technical difficulties, it does offer an opportunity to gather information concerning G_o cells which is otherwise unobtainable at present.

The purpose of this kinetic study of an EAT was to fully characterize the tumour and subsequently determine the existence, or otherwise, of a sub-population of G_o cells. The main techniques used in this study consist of electronic cell counting, ^{3}HTdR labelling and autoradiography.

Materials and Methods

An EAT, obtained from the Chester Beatty Research Institute in London, was passaged every 7 - 10 days in 3-month-old male BALB/c mice by i p injection of 5×10^6 tumour cells. Donor ascites fluid was diluted with warm, sterile physiological saline where necessary in order to produce a suitable cell concentration.

The BALB/c mice, originally obtained from LAC (Laboratory Animal Centre), Carshalton, were maintained on a diet of FFC/m pellets (Dixon's Diets, Elstree, Herts.) and water ad libitum.

Cell Counts: Cell counts and cell size distribution analyses were made using a Coulter counter (model D) and Isoton II diluent (Coulter Electronics Ltd., Harpenden, Herts.).

Specific Growth Rate, R: The specific growth rate is given by the formula:

$$R = (dN/dt) / N_{pot}.$$

Where: dN/dt represents the rate of change in tumour cell number with time estimated from the slope of the tangent to the growth curve.

N_{pot} is the total tumour cell number estimated after time t when dN/dt is constant over the period dt (Andersson et al., 1977).

Chromosome Analysis: Chromosome analysis was performed according to the method of Moorhead et al. (1960). Tumour cells were incubated in a solution of colchicine (final concentration 1 µg/ml) in physiological saline for 90 min. and hypotonic saline for 30 min. The cells were then fixed in methanol: acetic acid 3:1 for 7 min., spread vigorously onto degreased microslides and stained with acetic orcein for 10 min. Chromosome number per cell for tumours of 2 and 13 days growth respectively, was estimated in each case by counting 60 - 70 well separated

metaphase spreads.

Viability Tests:

1) Nigrosine dye exclusion. Tumour cells were incubated for 15 min. at 37°C in a solution of sterile physiological saline containing 0.1% nigrosine. Following incubation, a small quantity of the cell suspension was introduced into a haemocytometer chamber, and cells were scored immediately under low power as being either viable (dye excluded), or non-viable (dye penetrated).

2) Trypan Blue dye exclusion. Tumour cells were incubated for 15 min. at 37°C in a solution of sterile phosphate buffered saline (PBS, pH 7.1 - 7.4) containing 0.5% Trypan Blue. Following incubation a small quantity of the cell suspension was analysed according to the method as indicated for the nigrosine test.

3) Fluorescein diacetate (FDA). FDA is a marker for non-specific esterase activity; therefore, viable cells convert non-fluorescent FDA into fluorescein which fluoresces when exposed to light of wavelength 450 - 500 nm. Tumour cells were incubated for 5 min. at 37°C in a solution of sterile PBS containing FDA at a final concentration of 200 µg/ml. Following incubation, the cells were washed three times with fresh PBS and a small quantity of the resulting cell suspension was analysed on a clean glass microslide using UV illumination. Fluorescing cells were scored as being viable, non-fluorescing cells were considered to be non-viable.

In all the viability tests duplicate counts of at least 500 cells were made for each tumour studied.

Autoradiography: Autoradiography employed Ilford K5 nuclear emulsion diluted 1:1 with distilled water. Fixed smears were rehydrated in running tap-water (30 min.) rinsed in distilled water and then dipped in the diluted emulsion at 50°C under an Ilford safety light, filter

No. S902. Autoradiographs were exposed for 2 - 4 weeks at 4°C before developing with Kodak D19b developer at 18°C for 4 min. and fixing with Ilfofix, diluted 1:4, for 10 min.

Tritiated thymidine (^{3}HTdR) (sp. act. 5Ci/mM. 6HT) was used in all radiolabelling experiments. Stock solution was obtained from the Radiochemical Centre, Amersham, and was diluted with sterile physiological saline to a concentration of 100 µCi/ml prior to use.

Staining Procedures: The various staining procedures used in conjunction with autoradiography are listed under 1 - 3 as follows:

1) Harris' modified haematoxylin. Rehydrated smears were stained post-autoradiography for 35 min. with a filtered 1:10 aqueous dilution of Harris' modified haematoxylin. The stock solution of stain was prepared to the following recipe: Haematoxylin 1gm dissolved in 10 ml 95% ethanol, potassium aluminium sulphate 20gm dissolved in 200 ml distilled water. The potash alum solution was heated to boiling then the two solutions were mixed and reboiled for 60 seconds. The solution was removed from the heat and 0.2gm of sodium iodate carefully added. The solution was allowed to cool and stored for up to 21 days in the dark. It was necessary to test each new batch of Harris' stain for its compatibility with autoradiography since some preparations of the stain oxidise and remove autoradiographic silver grains.

2) Giemsa. Rehydrated smears post-autoradiography were stained for 5 min. with a 30% solution of Giemsa (BDH Ltd.). The diluted stain was filtered just prior to use. Smears were washed for 5 - 10 min. in phosphate buffered water (pH 6.8) after staining.

3) Feulgen (Basic fuchsin). Rehydrated smears, (pre-autoradiography) were treated with 1N HCl at 60°C for 10 min., immersed in the Feulgen reagent for 1 hr. and washed in 3 metabisulphite baths for 10 min. per bath. Smears were then rinsed under a running tap for at least 30 min. before coating with Ilford K5 nuclear emulsion.

Feulgen reagent was prepared by stirring 3.5gm basic fuchsin (BDH) with 19gm sodium metabisulphite in 1l of 0.15N HCl. After stirring for 2 hr. the solution was decolourized by adding 5gm activated charcoal, then filtered and stored in the dark. The pH was adjusted to 2.0 just prior to use.

Metabisulphite baths consisted of 1 part 10% sodium metabisulphite, 1 part 1N HCl and 15 parts distilled water.

4) Acetic Orcein. This stain was used in conjunction with chromosome preparations only. Chromosome spreads were stained in a 2% solution of orcein in 50% glacial acetic acid for 10 min. and excess stain removed with methanol.

<u>In Vitro ^{3}HTdR Labelling Index</u>: The <u>in vitro</u> labelling index was determined at 6 - 12 hourly intervals over the first 4 days following a 5×10^{6} tumour cell injection and then daily up to the 16th day after cell injection. For each tumour between 0.1 and 0.2ml of ascites was diluted to a total volume of 2.0ml with physiological saline containing 10μCi ^{3}HTdR. This cell suspension was incubated at 37°C for 20 min. The cells were then washed 3 times in saline and recovered each time by centrifugation at $500g_{av}$ for 3 - 5 min. before being smeared onto grease-free microslides. Smears were air-dried and fixed in methanol for 10 min. prior to autoradiography. Following autoradiography smears were stained with Harris' modified haematoxylin and at least 1000 cells were counted for each tumour.

<u>Cell Cycle</u>: Cell cycle determinations were made according to the percentage labelled mitoses (PLM) technique of Quastler and Sherman (1959). In this experiment ^{3}HTdR is administered in the form of a single pulse; therefore, only those cells which are in S-phase of cell cycle become labelled. The fraction of labelled cells is then referred to as the 'labelled cohort'. It is possible to monitor the

progress of this labelled cohort as it moves round the cell cycle by using the morphologically distinct mitotic phase as a window. Thus, a series of samples is taken at various times after the single ^{3}HTdR pulse and the PLM at each point is determined. The parameters of the cell cycle are then defined by the shape of the PLM versus time curve.

The PLM rises from zero to one percent as the leading edge of the labelled cohort moves into mitosis. The time taken from ^{3}HTdR pulse to the appearance of the first mitotic figure is equal to the duration of the G_2 phase. The PLM then rises sharply to a theoretical 100% as the leading edge of the labelled cohort reaches the end of the mitotic phase. Thus the time interval between the appearance of the first mitotic figure and 100% labelled mitoses is equal to the duration of the mitotic phase. The PLM remains at 100 until the trailing edge of the labelled cohort just passes into mitosis. Following this there is a precipitous fall in PLM values and theoretically, zero PLM is reached over a period of time again equal to the mitotic interval.

The peak of labelled mitoses produced enables calculation of the duration of the S-phase. The duration of the S-phase is the time taken for the leading and trailing edges of the labelled cohort to pass a fixed point on the PLM curve. Usually, this fixed point is 50%. Thus the duration of the S-phase is taken as the time interval between 50% labelled mitoses on the ascending limb and 50% labelled mitoses on the descending limb of the curve; i.e. the width of the theoretical peak at half its height. Similarly, the duration of the complete cell cycle is given by the time interval between the 50% points on the ascending limbs of the first and second peaks, (the second peak being formed as the labelled cohort completes its second mitosis after original labelling). The duration of G_1 may then be obtained by subtraction.

Considerable differences are invariably evident between the theoretical and experimental PLM curves, and it is impractical to attempt to amass data which allow the precise interpretation of the PLM curve. Therefore, interpretation of the PLM data presented here was restricted as follows: the time interval between pulse labelling (zero time) and the 50% point on the ascending limb on the first peak is equal to G_2 phase plus half the mitotic phase ($T_{G2} + \frac{1}{2}m$). Similarly, after determination of S-phase duration (T_s) and total cell cycle duration (T_c) the pre-DNA-synthetic interval can only be defined as G_1 plus half the mitotic phase ($T_{G1} + \frac{1}{2}m$).

Animals bearing 2 day and 13 day tumours (log and plateau phase respectively) were given a single ^{3}HTdR injection of 0.5µCi/gm body weight. For the 2 day tumour 42 mice received ^{3}HTdR ip. Following injection 3 animals were sacrificed every 1 - 4 hours for preparation of smears, the final sample being taken 28 hr. after ^{3}HTdR injection. For the 13 day tumour 23 mice received ^{3}HTdR; these animals were sampled in batches of 3 or 4 at 2 - 12 hr. intervals over a 100 hr. period. Each tumour was sampled consecutively 3 times at most during the course of the experiment and less than 0.1ml of tumour fluid was aspirated for each sample. Ascites smears were air-dried, fixed in methanol for 10 min. and stained with Feulgen reagent (basic fuchsin), prior to autoradiography. Following autoradiography at least 100 mitoses were scored for each tumour.

Estimation of the Size of the Proliferating Fraction, (f):

Estimation of the size of the proliferating fraction of the tumour cell population was possible from the labelling index and cell cycle data by using the expression:

$$f = (Ns/N)/(Ns/Ng) \qquad \text{(Lala and Patt, 1966).}$$

Where: Ns is the number of cells in DNA synthesis.

N is the total number of cells (both in cell cycle and out-of-cycle).

Ng is the number of cells in cycle.

Thus: Ns/N is simply the ^{3}HTdR labelling index.

and: Ns/Ng represents the fraction of the proliferating cells in DNA synthesis. Computation of this factor is more difficult, but it can be derived from the data relating to the cell cycle (Cleaver, 1965).

Thus: $Ns/Ng = \left\{ e^{(T_{G2} + m)(1n2)/T_c} \right\} \left\{ e^{(T_s/T_c)(1n2) - 1} \right\}$

Where: T_c is the duration of the cell cycle.

T_s is the duration of the S-phase of cell cycle.

$T_{G2} + m$ is the combined duration of the post-DNA-synthetic period and mitotic phase of the cell cycle.

It must be appreciated that both the determination of cell cycle times and estimation of the growth fraction by the methods described above are subject to considerable error and values obtained are at best close approximations. The various reasons for these methodological inaccuracies are discussed later. The objectives, therefore, of the determination of the intermitotic time and subsequent estimation of the growth fraction were twofold; firstly, to postulate the existence or otherwise of a non-cycling or G_o cell population during log and plateau phase of tumour growth, and secondly, to enable the formulation of a repeat ^{3}HTdR labelling programme to ensure that all rapidly cycling (G_+) cells incorporate ^{3}HTdR during the course of the experiment.

<u>Computation of the Cell Loss Factor, k</u>: Computation of the cell loss factor (k) was possible from the predetermined factors T_c and f, thus:

$$k = (\ln\ 1 + f - (T_c/Tdbl)(1n2))/T_c$$

Where: Tdbl = population doubling time. (Lala and Patt, 1966).

<u>Repeat ^{3}HTdR Labelling Experiment</u>: Repeat ^{3}HTdR labelling experiments

were performed after 12 days of tumour growth. In each experiment batches of 6 animals received a series of ^{3}HTdR injections (0.5μCi/gm body weight) over a period greater than that equal to the length of the estimated cell cycle and at intervals less than the estimated length of the S-phase. In an initial experiment seven ^{3}HTdR injections were given at intervals of 10 hr. Twenty min. after each injection, (sufficient time for S-phase cells to incorporate ^{3}HTdR), a small quantity of ascites (<0.1ml) was removed for preparation of smears.

In a second experiment four ^{3}HTdR injections were given at intervals of 30 hr. and ascites smears prepared 20 min. after the final ^{3}HTdR injection. All smears were air-dried, fixed, stained and autoradiographed as previously described under <u>in vitro</u> labelling index method. At least 1000 cells were counted for each tumour and unlabelled cells were considered to be in G_0 phase. Some of these autoradiographs were exposed for as long as 3 months to ensure that lightly labelled cells became clearly positive.

<u>Experiment to Assess the Viability of the Non-Cycling Cells:</u>

Demonstration of the ability of the non or slowly cycling cells to recommence rapid cyclic activity was made by observing the appearance of unlabelled mitoses in repeatedly labelled tumours after both greatly reducing the tumour mass and retransplanting a fraction of the tumour mass. Initially, four 12 day tumours were labelled according to the second repeat labelling procedure then the bulk of the tumour mass was removed from each animal by percutaneous aspiration. Ten million cells from each of two of these repeatedly labelled tumours were then transplanted into batches of 25 new host mice (i.e. a total of 50 new host mice). Cell samples were obtained from tumours of reduced mass by repeated aspirations and from retransplanted tumours

by sacrificing the host and washing out the peritoneal cavity with physiological saline. Cell samples from both retransplanted tumours and donor tumours (the latter now of greatly reduced mass) were taken at various intervals up to ¾ hr. and 14 hr. respectively following the final ^{3}HTdR injection. After Feulgen staining and autoradiography PLM determination allowed the demonstration of those cells which had been non or very slowly cycling and which had returned to rapid cyclic activity (unlabelled mitoses).

Repeat labelling and retransplantation control experiment: As an adjunct to these repeat labelling experiments PLM determinations were made for 4 undisturbed 12 day tumours at various time intervals (20 min. and 10, 12, 25 and 50 hr.) after the fourth and final ^{3}HTdR injection. Less than 0.1ml of ascites was removed from every tumour at each sampling time and autoradiographs were prepared as outlined above.

The results of this experiment should indicate the extent to which the cycling cell population becomes labelled by the repeat labelling schedule employed, and the possibility of the recycling of G_0 cells in situ.

Results

Growth Pattern: Figure 1 indicates the increase in total tumour cell number with the passage of time following ip injection of 5×10^6 EAT cells. Four to 7 tumours were sacrificed each day for determination of total tumour cell number. There is no apparent lag phase following cell inoculation and initially tumour growth is exponential.

The maximum growth rate appears to lie between the first and third days of tumour growth. This observation is substantiated by determination of the specific growth rate (fig. 2). There then follows a progressive decline in the growth rate until after 12 days a plateau phase is reached. During the period of declining growth rate, 3 - 10 days, there appears to be a linear relationship between the cube root of the total cell number and tumour age (fig. 3).

Figure 4 shows variation in tumour cell concentration in the ascites with time following transplant. It is possible to extend this study into the earlier stages of tumour growth by the use of a dye dilution technique which allows estimation of the tumour volume before it is possible to aspirate (Lala and Patt, 1966). However, the present results were obtained by the application of two simple aspiration methods. The broken line joins values determined from counting the total number of cells in a volume of 0.1 ml of ascites, the solid line joins values determined from the total tumour cell number divided by total tumour volume. The results indicate that during the growth retardation and plateau phases of tumour growth there is a decrease in tumour cell concentration with increasing tumour age.

Chromosome Analysis: The distribution of tumour cell chromosome number was examined after 2 and 13 days of tumour growth (fig. 5). The results of this analysis show the EAT studied here to be hyper-

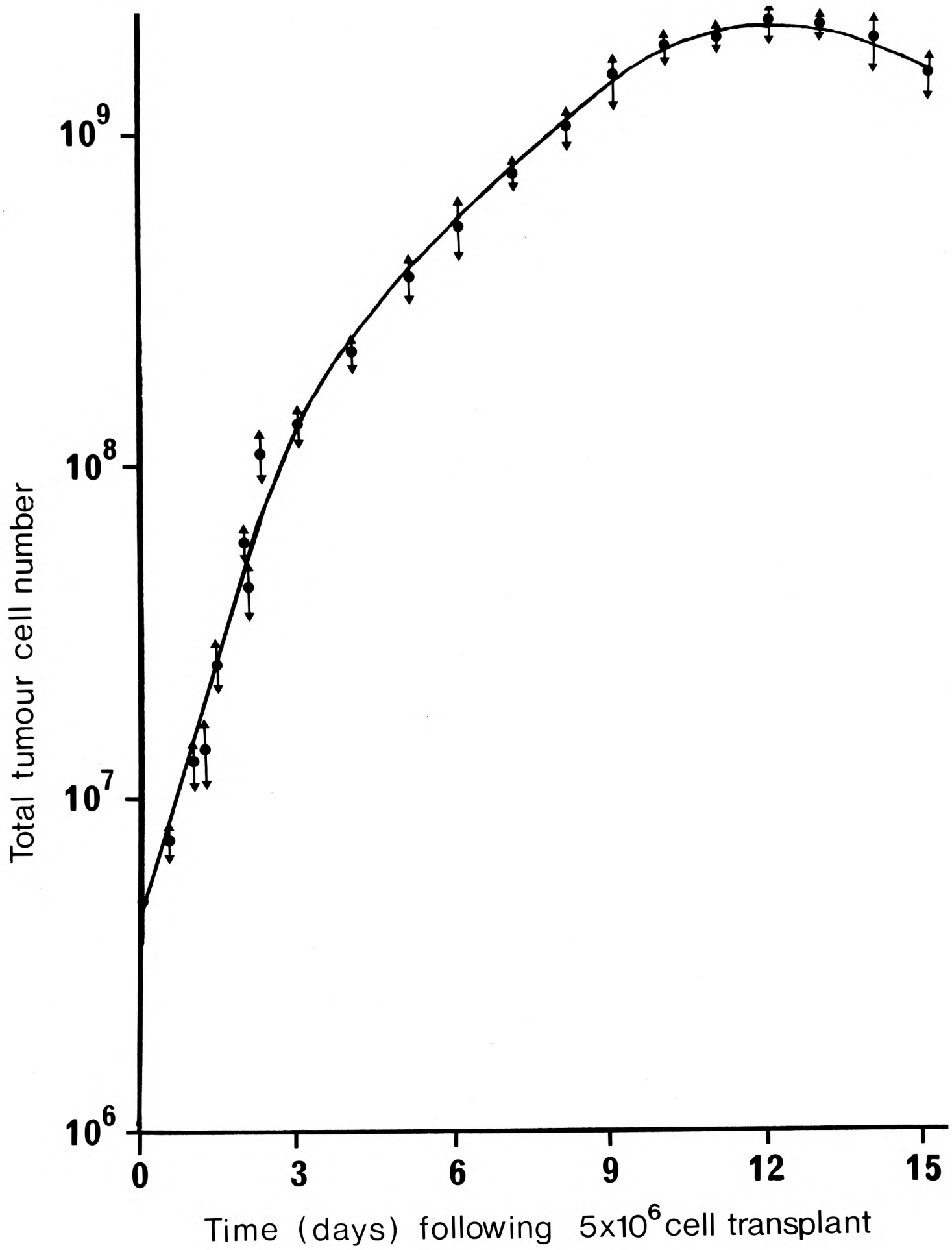

Fig. 1 Growth curve

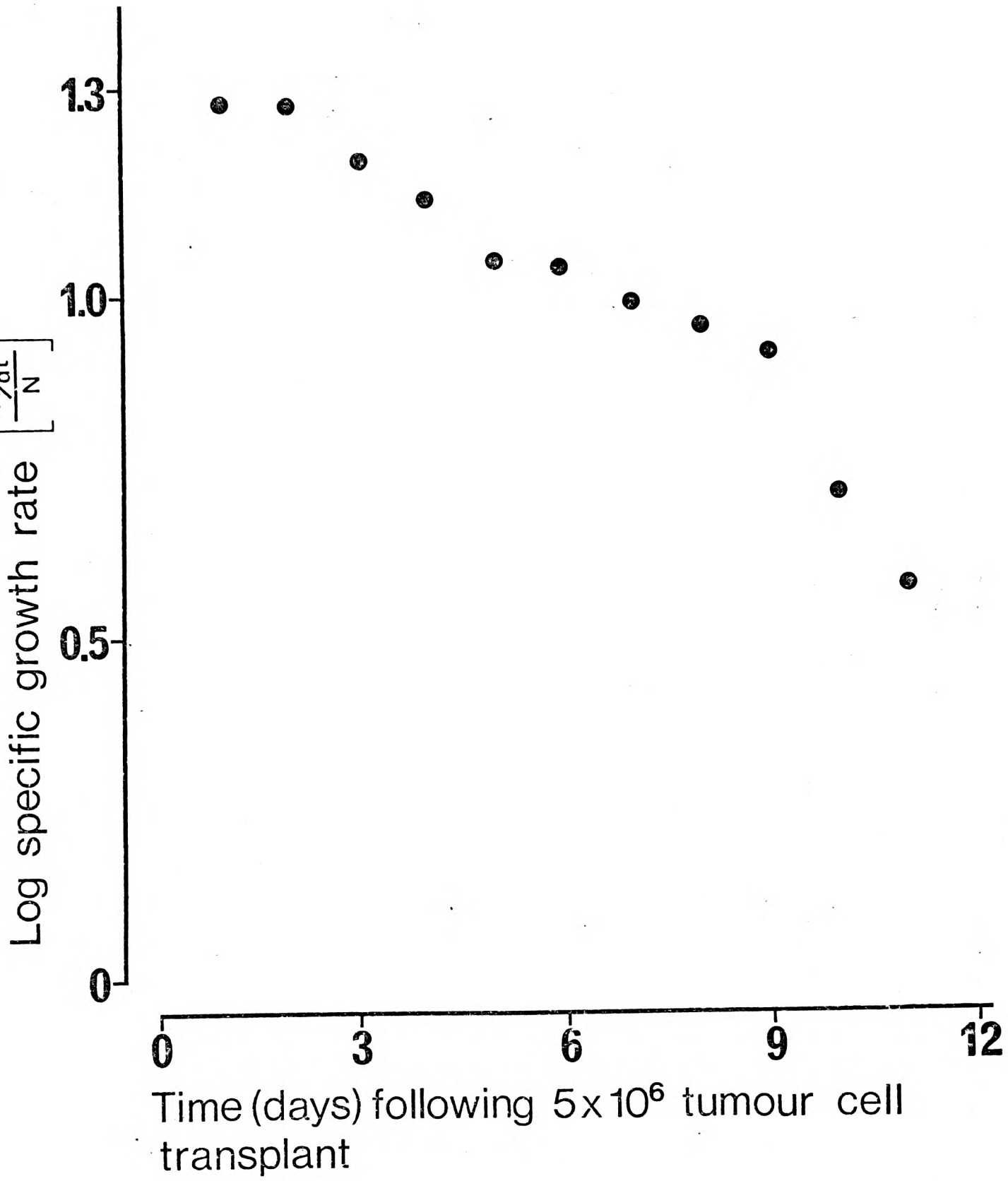

Fig 2 Variation in specific growth rate with increasing tumour age

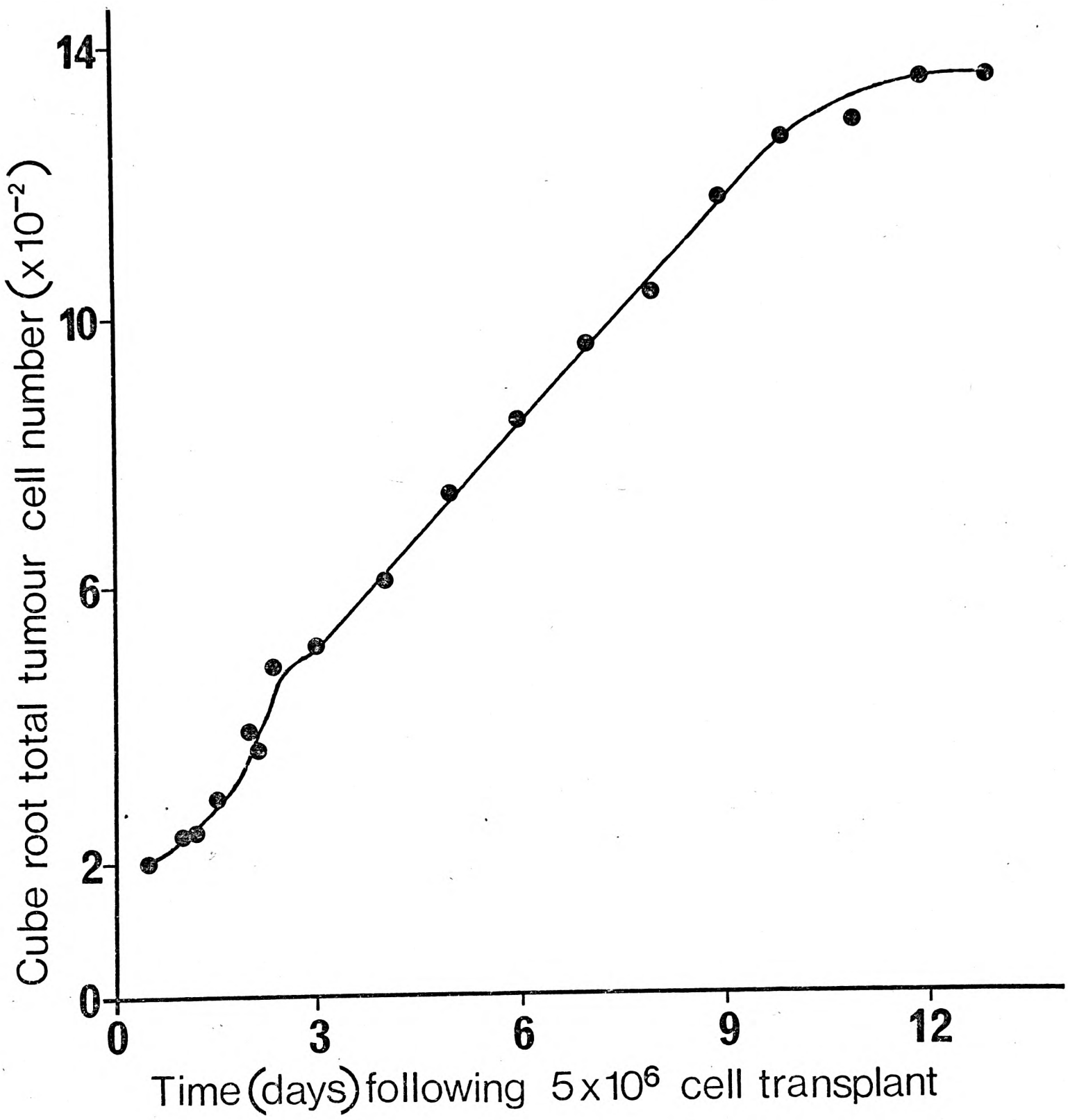

Fig3 Cube root total tumour cell number as a function of age of tumour.

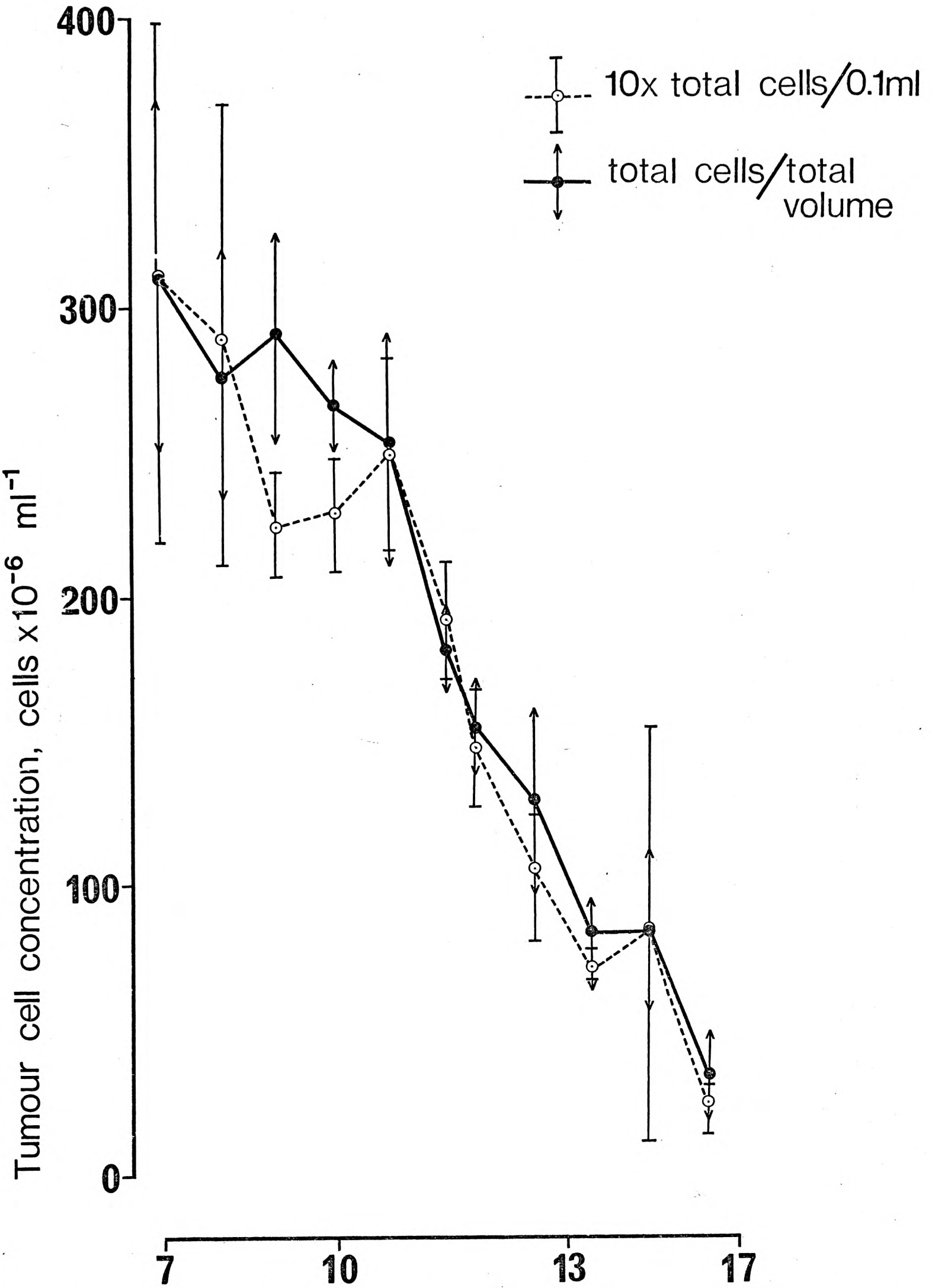

Fig 4 Time (days) following 5×10^6 tumour cell transplant

Variation in tumour cell concentration with tumour age

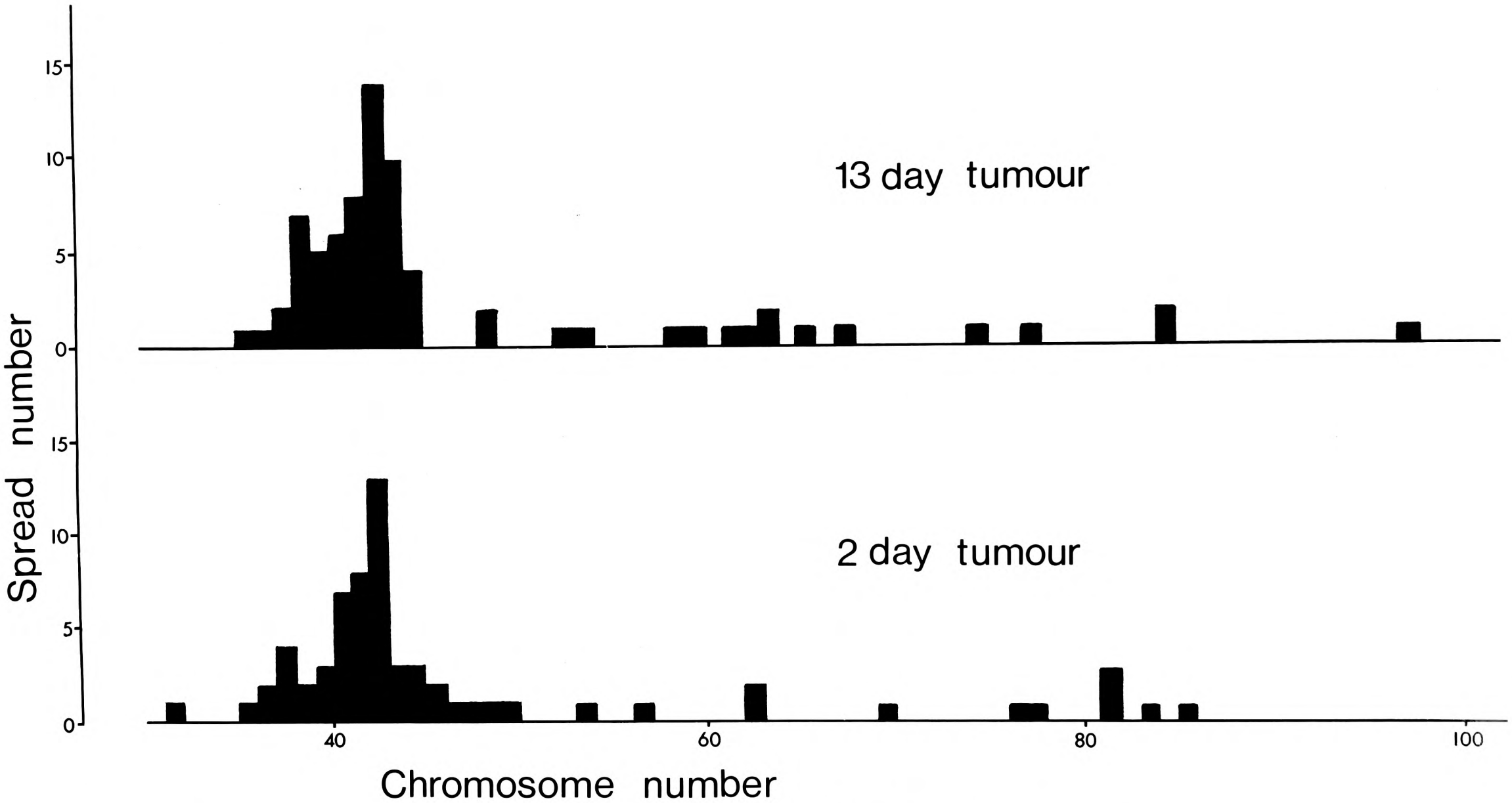

Fig 5 Chromosome distribution for the Ehrlich ascites tumour

diploid, with a modal chromosome number of 43. The chromosome distribution remained stable over the period of tumour growth from 2 to 13 days (variance analysis $P>0.1$).

Cell Size Distribution Analysis: The results of tumour cell size distribution analysis at 2, 13 and 16 days of tumour growth are given in fig. 6. The median cell diameter lies between 11 and 13 microns although the mean is probably in excess of this value since the distribution is skewed to the right. The data in fig. 6 indicate that no significant variation in cell size distribution occurs until the very late stages of tumour growth and that even then there is no variation in the modal cell size.

Viability Tests: An attempt was made to determine the percentage of non-viable tumour cells at various times following 5×10^6 tumour cell transplant. Several dye exclusion methods were tried. Some dyes, for example Evans blue, were found to have a deleterious effect on the cells thereby giving an erroneously high non-viable count. Such dyes were rejected. Ultimately, results, (table 1), were obtained from three methods, the nigrosine and trypan blue dye exclusion methods and the FDA method. Each value presented in table 1 represents the mean of the viability counts for at least three tumours. The general conclusion to be drawn from the data is that the percentage of non-viable cells is low until after or during the plateau phase of tumour growth when there appears to be a sharp increase in the number of non-viable cells.

Labelling Index Determination: The *in vitro* labelling index refers to the percentage of tumour cells which incorporate ^{3}HTdR and which are therefore assumed to be engaged in DNA synthesis (S-phase) at any one time. The labelling index therefore is taken as a measure of the proliferative activity of the tumour. Each point in fig. 7 represents

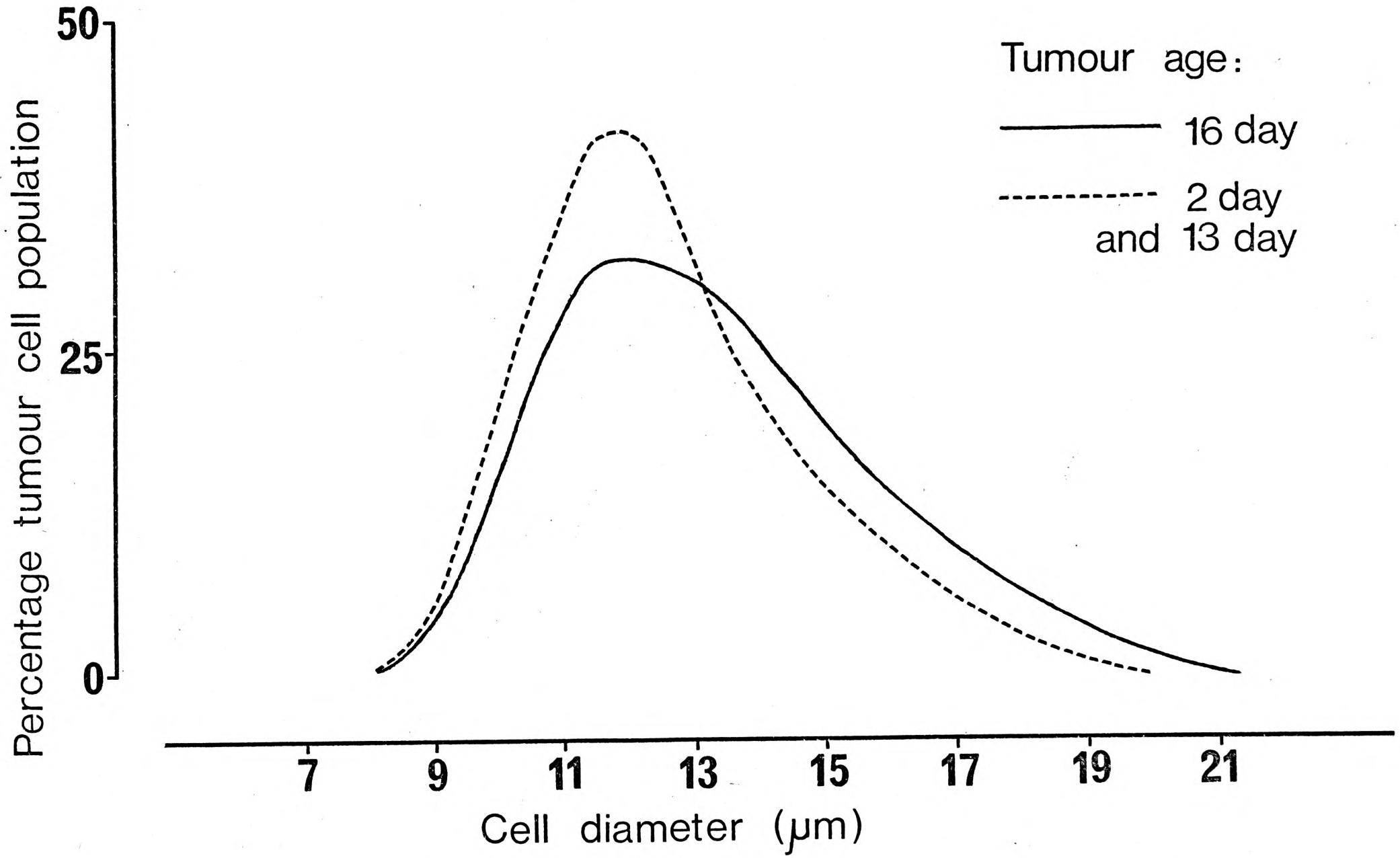

Fig 6 Tumour cell size distribution at various stages of tumour growth

Table 1

Viability Test

Days following 5×10^6 Transplant	% Non-Viable Cells		
	Trypan Blue	Nigrosine	FDA
3	nd	0.2	0.7 (± 0.1)
5	1.3 (± 1.1)	0.8 (± 0.1)	4.3 (± 1.3)
7	1.4 (± 0.1)	nd	nd
10	1.8 (± 0.8)	0.9 (± 0.4)	3.3 (± 0.6)
14	2.8 (± 0.7)	13.0 (± 2.8)	nd
17	2.5 (± 0.3)	19.0 (± 12.9)	15.0 (± 1.5)

nd = not determined

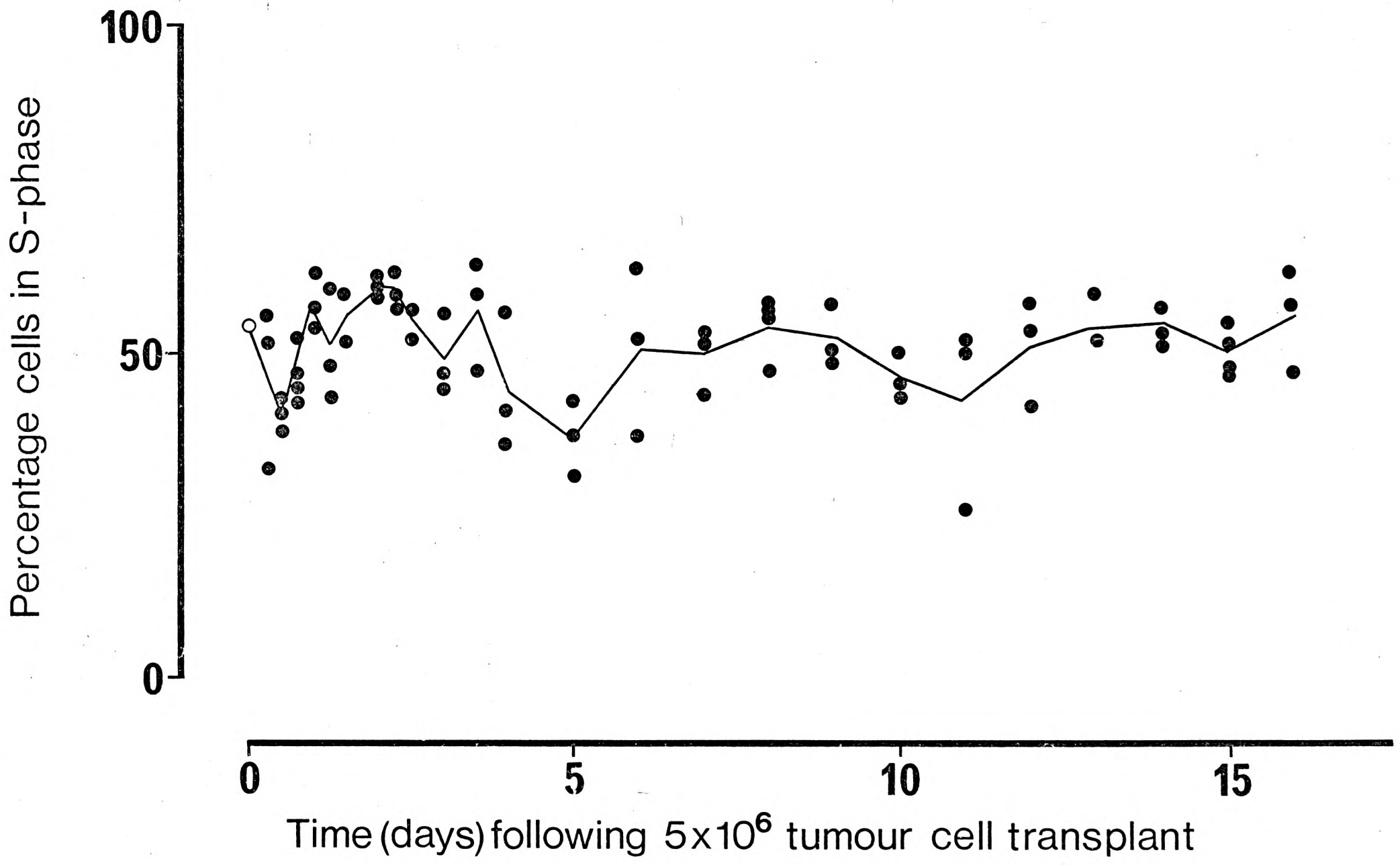

Fig 7 Variation in <u>in vitro</u> ^{3}HTdR labelling index during tumour growth

the labelling index for one tumour.

Following transplantation of 5×10^6 tumour cells there was an initial fall in the labelling index followed by a rise to the donor level by the twentieth hour. A peak value was seen at 48 hours following transplant, and after this a general pattern of decrease in labelling index was seen up to 5 days after transplant. Statistical analyses of the data presented in fig. 7 indicate that over the first 12 days of tumour growth and beyond, there is no significant trend. However, analysis of the first 5 days of tumour growth revealed a significant variation in labelling index with time at the one percent level ($P<0.01$).

Further to these results, it is interesting to note that <u>in vivo</u> labelling indices determined for 2 day and 13 day tumours were 59.1 and 32.8% respectively (table 2).

<u>Cell Cycle Determination</u>: The PLM curves for a 2 day tumour and a 13 day tumour are given in figs. 8 and 9 respectively. In fig. 8 each point represents the results of scoring 100 mitoses for a single tumour, each tumour being sampled once only. In fig. 9 each point similarly represents the results of scoring 100 mitoses for a single tumour; however, some counts were duplicated. A minimum of 3 tumours were sampled at each time point for the construction of fig. 9.

Superficial examination of the PLM curves in figs. 8 and 9 reveals that there is a substantial lengthening of the cell cycle and its constituent phases between the second and thirteenth day of tumour growth. The relative phase durations and the total cell cycle times have been estimated by inspection of the 50% labelled mitoses points. These values together with other relevant data are summarised in table 2.

The data contained in figs. 8 and 9 (excluding open circles) were

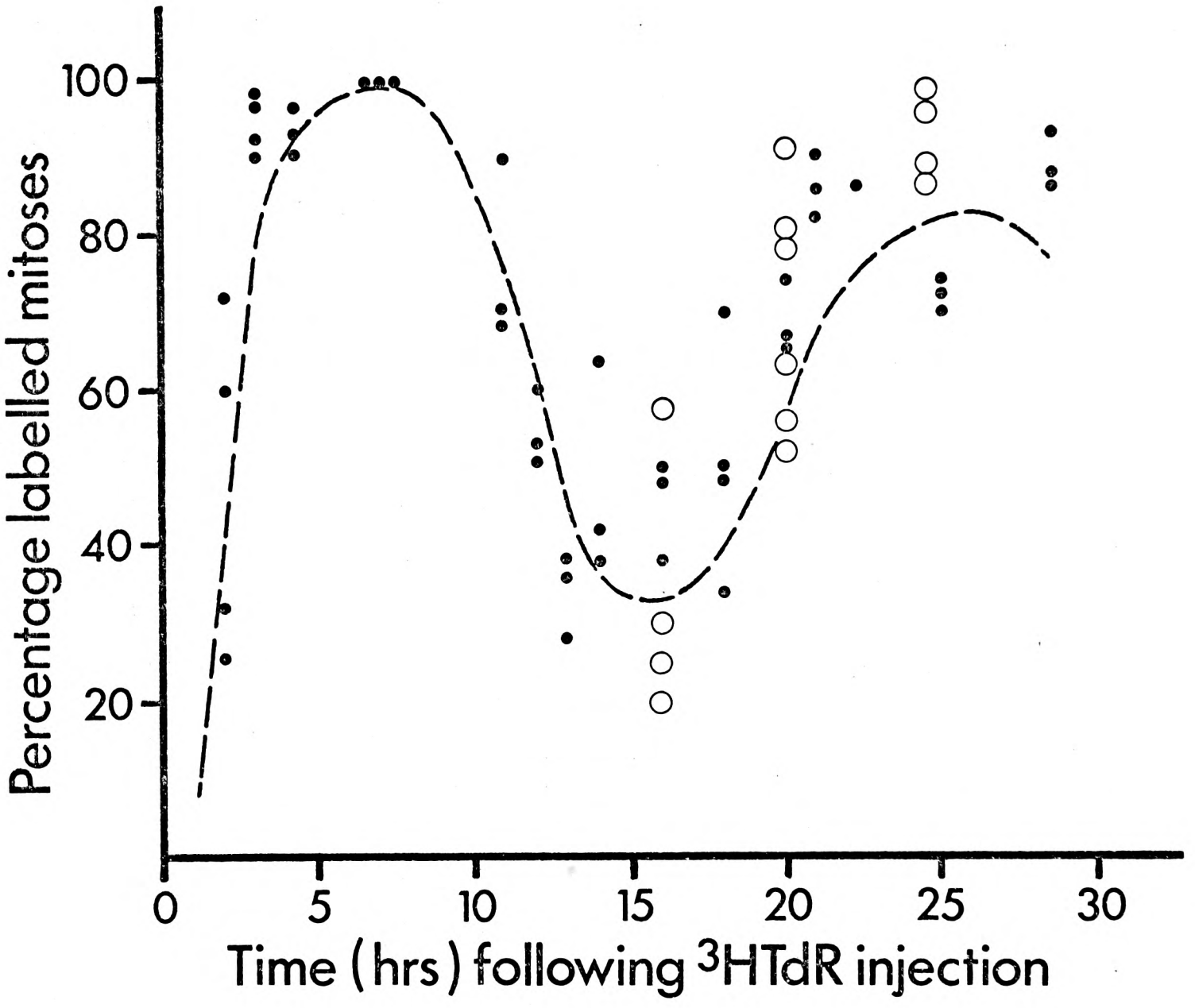

Fig 8 Cell cycle determination for a 2 day tumour (hand drawn)

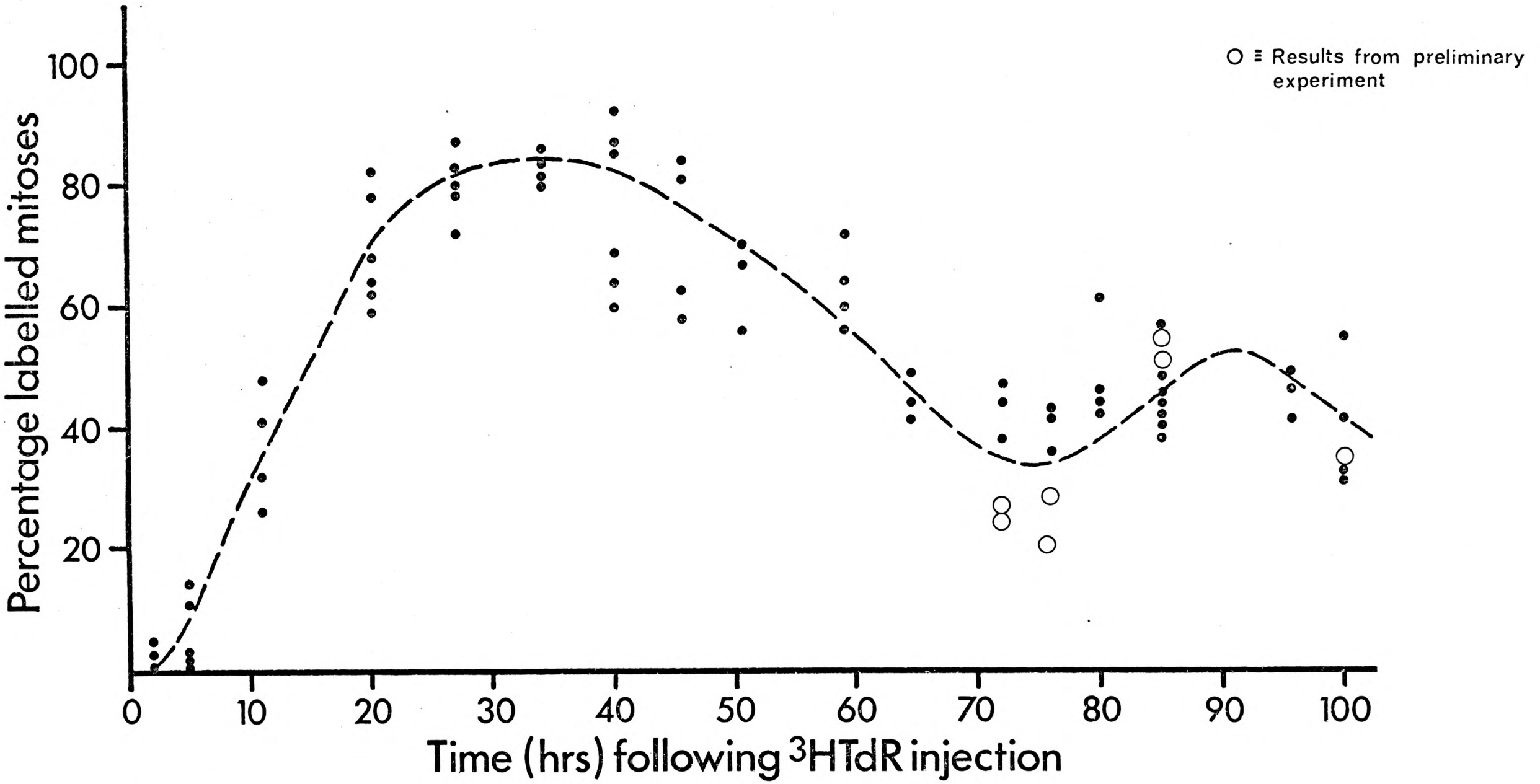

Fig 9 Cell cycle determination for a 13 day tumour (hand drawn)

Table 2

EAT growth parameters 2 and 13 days after inoculation of 5×10^6 tumour cells.

	Age of Tumour	
	2 day	13 day
Cell population (N) Total tumour cell number	6.1×10^7	2.5×10^9
Population (t dbl)† Doubling Time	18 hr.	∞
Duration of Cell Cycle (Tc)	17.5 hr.	75 hr.
S-phase (Ts)	10.5 hr.	50.5 hr.
G_1 + ½m phase (TG_1 + ½m)	4.5 hr.	10.0 hr.
G_2 + ½m phase (TG_2 + ½m)	2.5 hr.	14.5 hr.
<u>In Vivo</u> Labelling Index	59.1% (± 2.3)	32.8% (± 2.4)
<u>In Vitro</u> Labelling Index	60.1% (± 1.6)	52.7% (± 9.8)
Relative duration of S-phase *	0.567	0.692
Growth fraction (f*)	1.04	0.47
Cell loss factor (k*)	0.0002	0.02

†Estimated from the slopes of tangents drawn to growth curve.

*Determined according to the method of Lala and Patt (1966) utilizing the <u>in vivo</u> labelling index and cell cycle parameters estimated from the curves presented in figs. 8 and 9.

submitted to Dr. G. G. Steel for computer analysis according to the model proposed by Steel and Hanes (1971). The results of computer analysis are given in figs. 10 and 11. The computer fitted curves are ostensibly similar to the hand-drawn curves. Indeed, the computed durations for the cell cycle phases and complete cell cycle (table 3) do not differ substantially from those estimated from hand-drawn curves. However, the computer fitted curves have a poorly defined second peak which makes accurate cell cycle time determination impossible by this method.

The results summarised in table 2 indicate that after 2 days of tumour growth all cells are rapidly cycling and cell loss is extremely small. These observations are further supported by the estimated population doubling time of 18 hours, which is consistent with the total cell cycle duration. The calculated growth fraction after 13 days of tumour growth is 0.47. At this time the median cell cycle time is in excess of 70 hr. and the doubling time is infinite.

Repeat ^{3}HTdR Labelling Experiment: The results of the kinetic investigations presented above provide circumstantial evidence (discussed later) for the existence of a sub-population of non-cycling cells within the tumour cell population during the plateau phase of tumour growth. However, it is difficult to see how further useful information concerning the non-cycling tumour cells can be gleaned from these data. Therefore, a repeat ^{3}HTdR labelling experiment was devised, using as a basis the cell cycle model defined after 13 days, in an attempt to aid the visualization and further examination of this sub-population of non-cycling cells.

In an initial experiment ^{3}HTdR was administered to four 12 day tumours at 10 hourly intervals for 60 hours so that all rapidly cycling cells became labelled. Samples were taken 20 min. after each

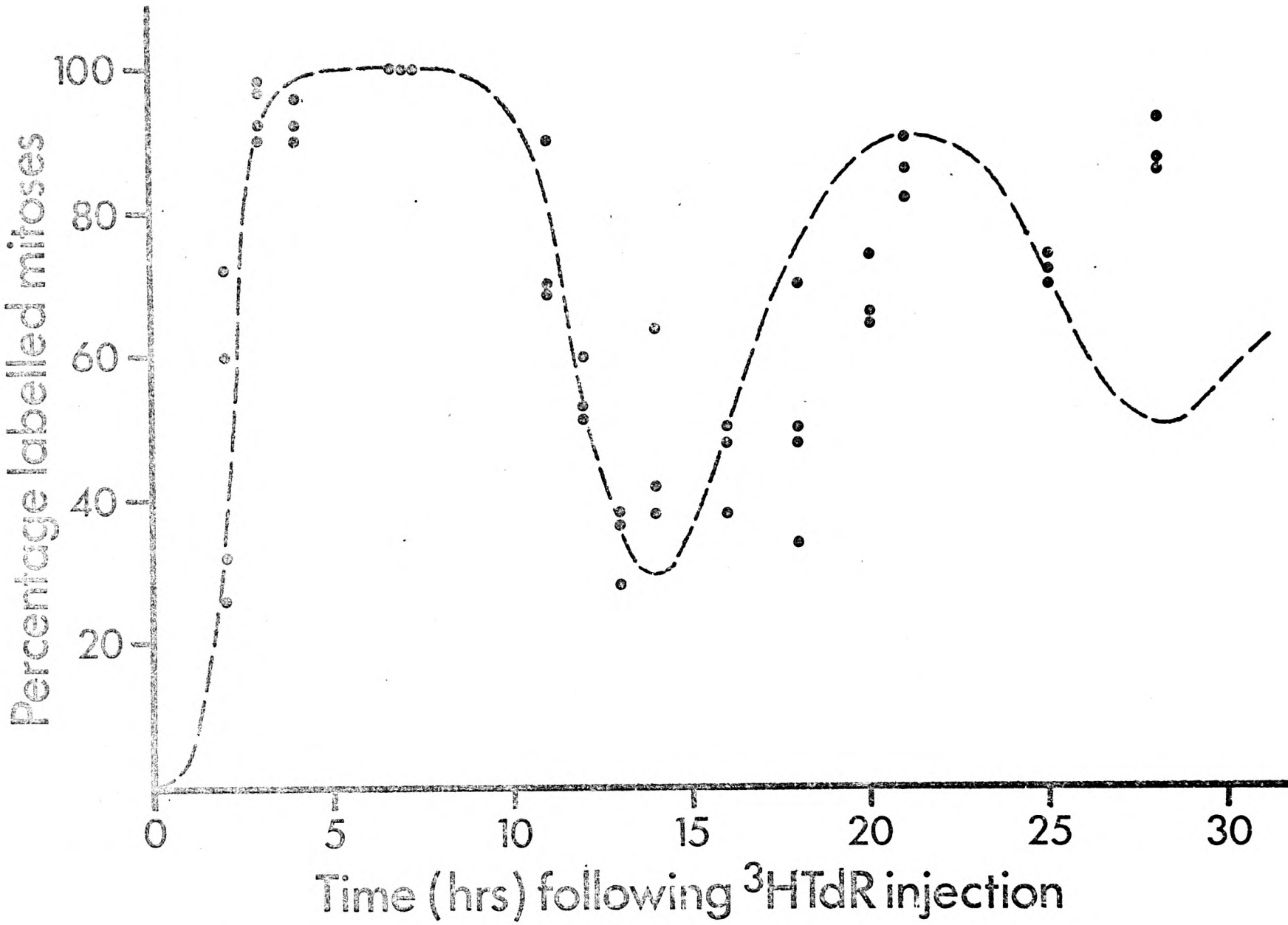

Fig 10 Cell cycle determination for a 2 day tumour (computer fitted)

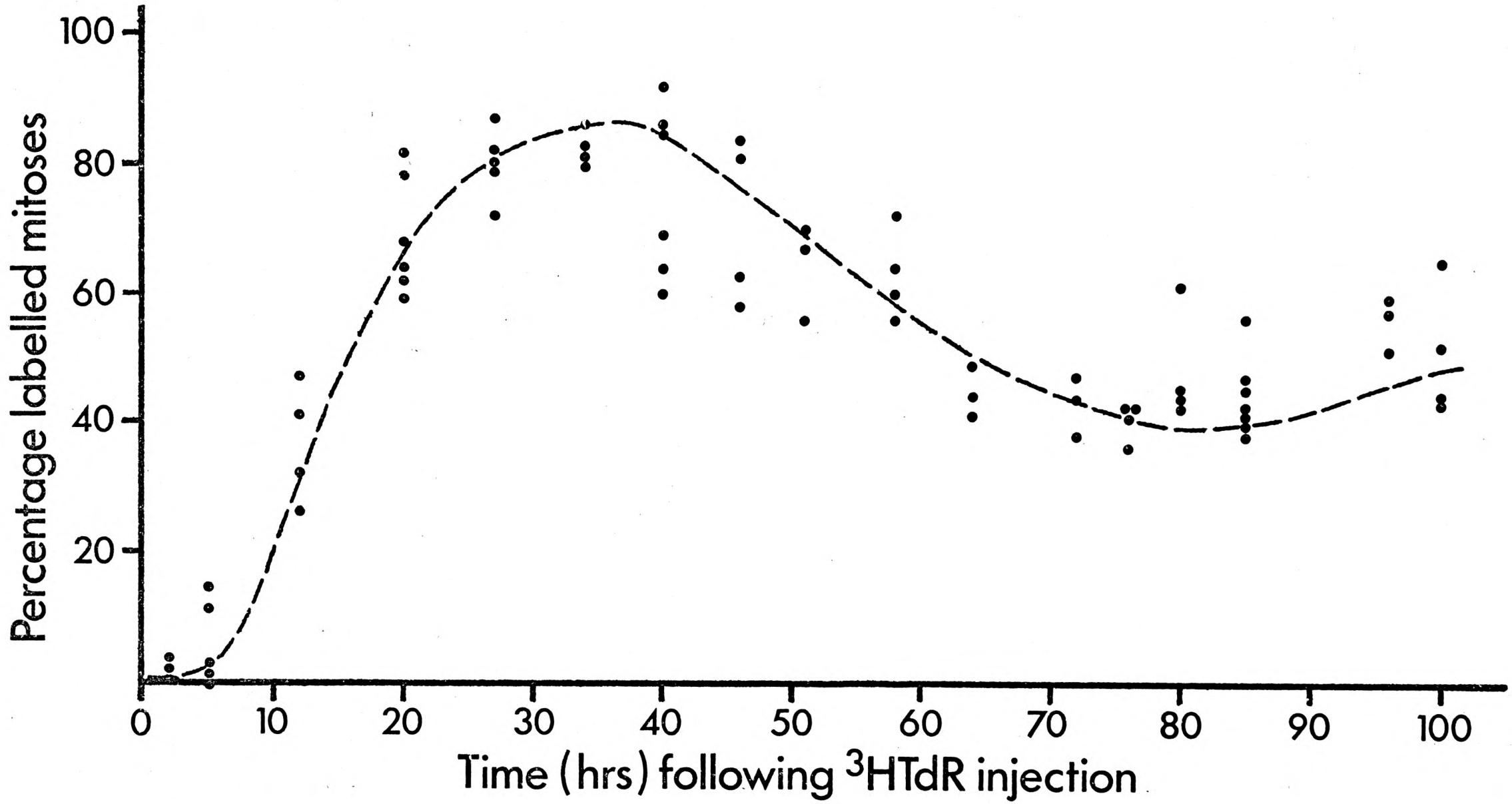

Fig 11 Cell cycle determination for a 13 day tumour (computer fitted)

Table 3

Cell cycle and composite phase duration by computer analysis of PLM data.

	Age of Tumour	
	2 day	13 day
Tc	14.7 hr*	89.1 hr*
Ts	10.2 hr	46.9 hr
TG_1	2.4 hr*	23.1 hr*
TG_2	2.1 hr	19.1 hr

* Unreliable values due to poorly defined second peak.

injection in order to follow the progress of the labelling procedure. The results of this experiment are presented in fig. 12. There is a continual increase in the percentage of labelled cells throughout the labelling procedure. Initially this increase in the percentage of labelled cells is rapid but after the injection at the fortieth hour the accumulation of labelled cells is much less rapid and the curve becomes shallow. After the final injection this experiment allowed the visualization of a small fraction, 15-17%, of the total cell population which can be considered to be out-of-cycle.

Further to this experiment it was felt prudent to extend the period of repeat labelling thus ensuring labelling of all rapidly cycling cells. In addition, the administration of 7 x 10 hourly injections of ^{3}HTdR seemed rather more than adequate to label all cells that pass through S-phase during the labelling period. Therefore, taking into account the possible wide distribution of cell cycle times, a repeat labelling experiment using 4 x 30 hourly injections was performed. This experiment resulted in the labelling of more than 98% of mitotic figures (fig. 15) and 87.6 $\pm$ 2.7% of tumour cells leaving some 12.5% of cells unlabelled and thus considered to be non or very slowly cycling.

Following the visualization of a fraction of a non or very slowly cycling tumour cell population, a question pertaining to their viability arises; are the observed non-cycling cells sterile end cells or are they resting cells which retain the capacity to return to rapid cyclic activity when stimulated, in the same way as a G_0 cell?

Experiment to Determine Viability of Non-cycling Cells: Figs. 13 and 14 are the results of PLM determination on repeatedly labelled tumours following retransplantation or great reduction of tumour mass. In the retransplantation experiment (fig. 13) two tumours A and B

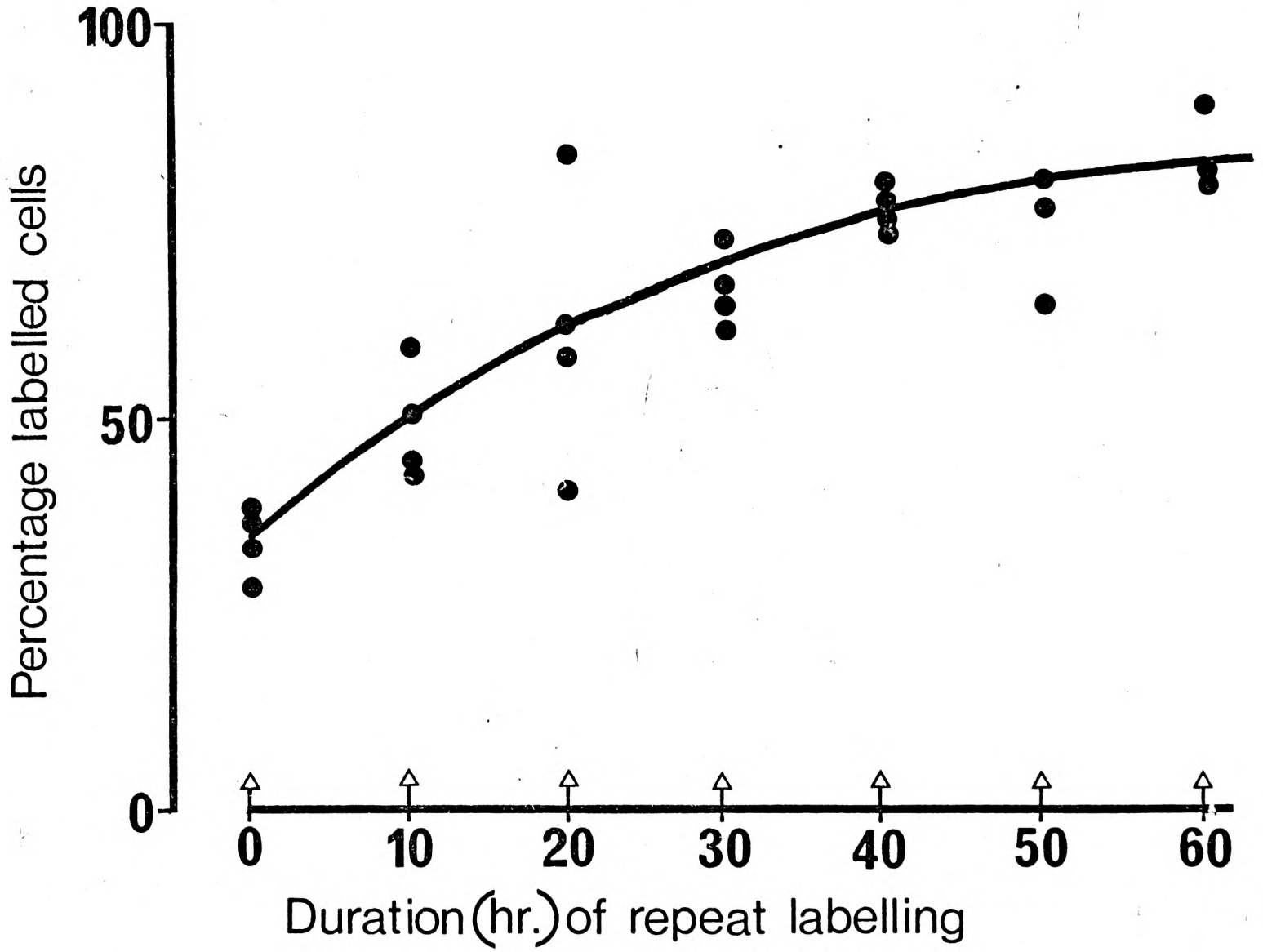

Fig 12 Increase in percentage labelled cells following repeat ^{3}HTdR injections, (↑).

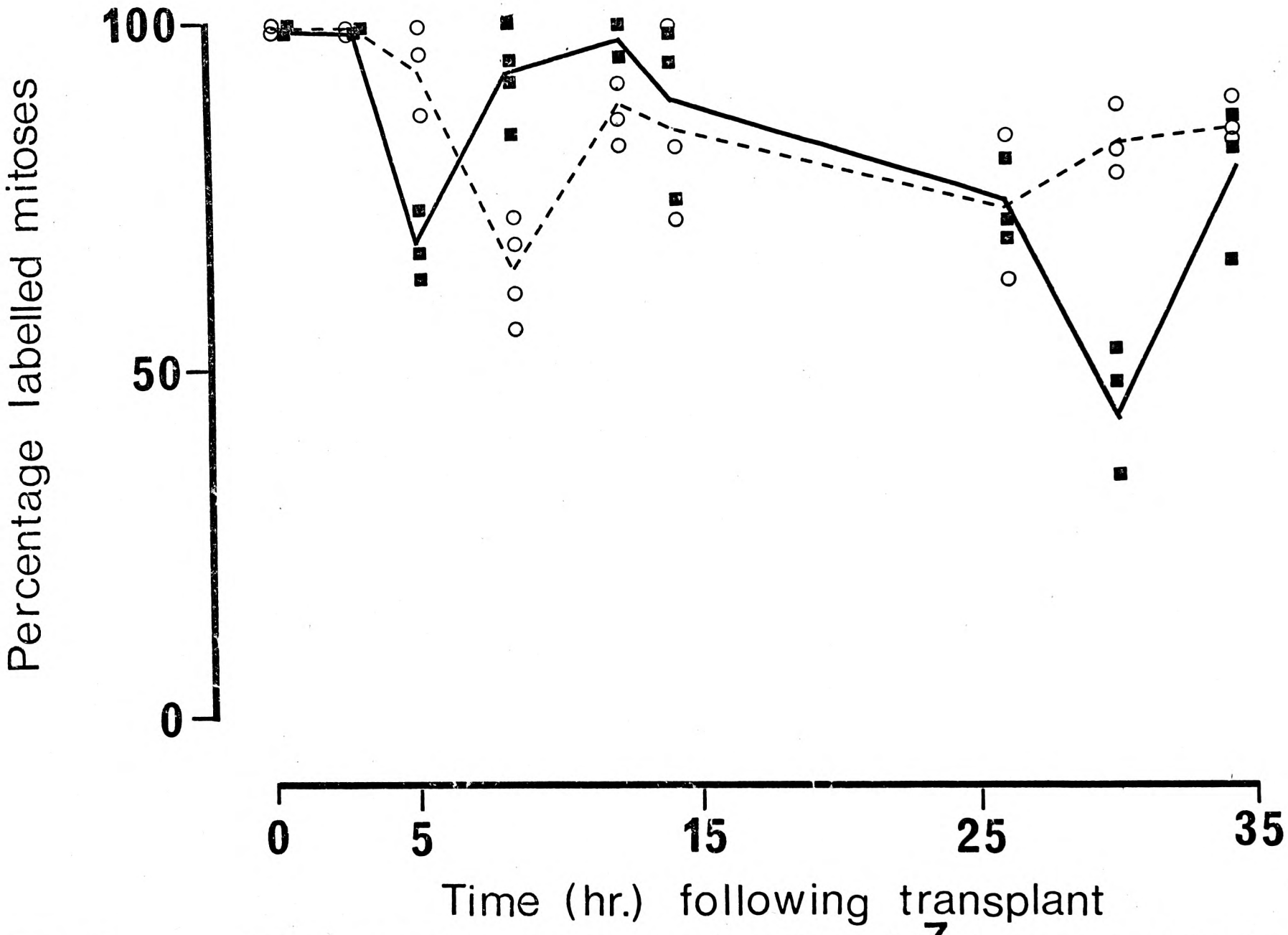

Fig 13 The results of transplanting 10^7 tumour cells from two mice (-o- and -▪-) after repeat ^{3}HTdR labelling

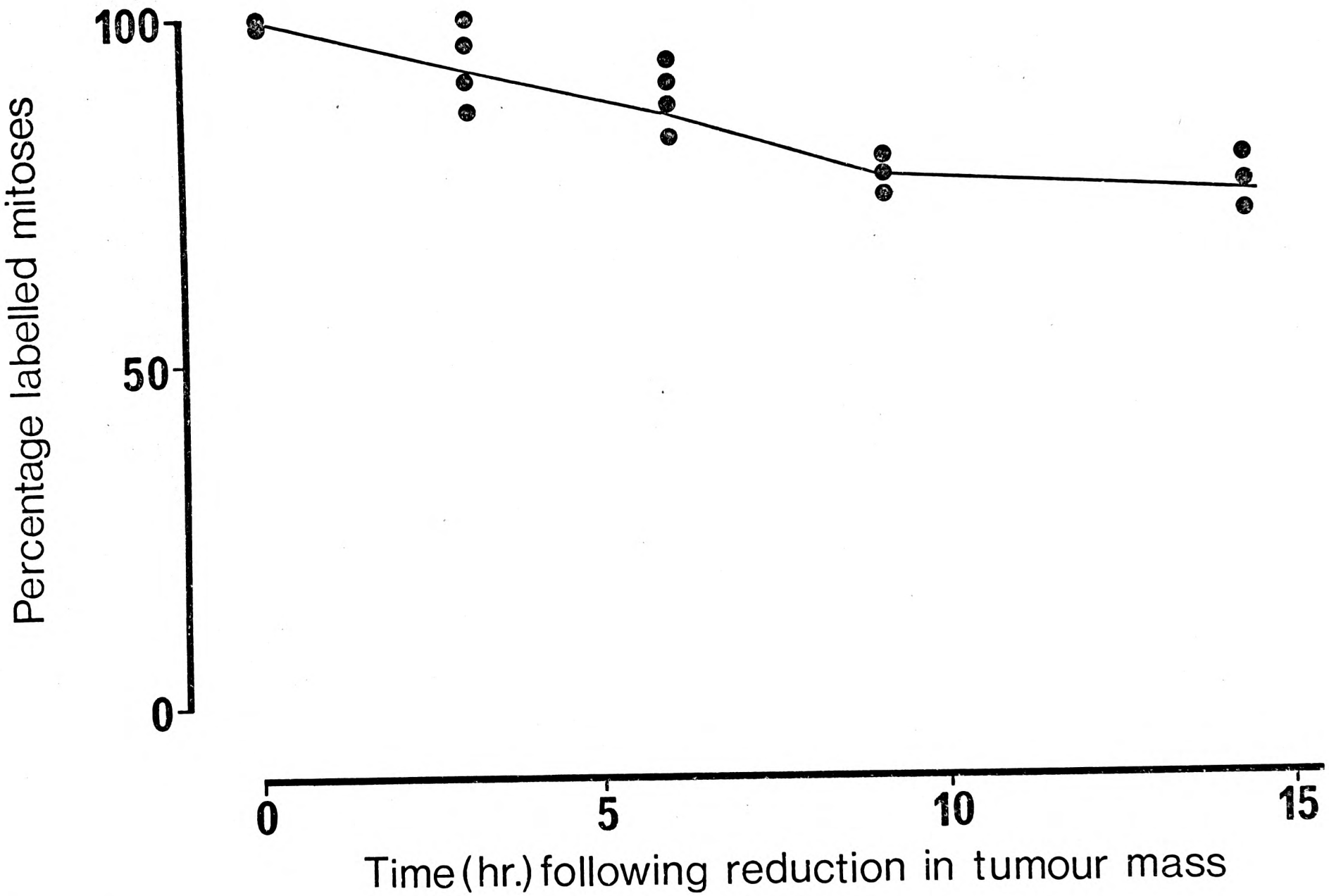

Fig 14 The appearance of unlabelled mitoses is shown after draining the ascites from mice given multiple injections of ^{3}HTdR

were repeatedly labelled with 4 x 30 hourly ^{3}HTdR injections. Fifty fresh host mice were inoculated in two batches of 25, the first batch received 10^7 cells from tumour A, the second batch received 10^7 cells from tumour B. Each open circle in fig. 13 represents the PLM for a retransplanted tumour in batch A and similarly each square represents the PLM for a retransplanted tumour in batch B.

The fall in the PLM shown in fig. 13 indicates that at least some of the non-cycling cells are capable of resuming rapid cyclic activity and can therefore be considered to be G_o cells. Further to this an attempt was made to determine whether the variation in PLM in fig. 13 represents a significant proportion of the non-cycling cells. For this a number of assumptions are necessary. Firstly, that the ratio of the area above the curve to the area below the curve for the duration of one cell cycle is representative of the previously non-cycling cells, (unlabelled cells), as a proportion of the total cell population. Secondly, that the curve eventually returns to 100% LM when all the unlabelled cells have completed their first cycle, and thirdly, that the time from transplantation to the point at which 100% LM is reached is equal to the duration of the cell cycle. The underlying basis of these assumptions and the subsequent implications for the adequacy with which estimation of the viable proportion of the non-cycling cells can be made is examined later.

The relevant calculations, based on the above assumptions, for curves A and B in fig. 13 are set out overleaf. These calculations reveal unlabelled cell populations amounting to 12.7% and 8.4% of the total population. These are to be compared with values of 7.5, 12.8, 13.3, 13.8 and 14.6% (mean 12.4 $\pm$ 2.8%) for the size of the unlabelled fraction determined after 4 x 30 hourly repeat ^{3}HTdR injections.

Estimation of the percentage of the total tumour cell population remaining unlabelled and viable after retransplantation. Calculations based on data presented in fig. 13.

Curve A

1) Estimated duration of cell cycle
by extrapolation to 100% — 14 hr.

2) Area above curve
between 0 and 14 hr. — 657 units*

3) Total area above and
below curve between 0 and 14 hr. — 5180 units*

Percentage of viable tumour cell population remaining unlabelled $= \frac{657}{5180} \% = \underline{12.7\%}$

Curve B

1) Estimated duration of cell cycle
by extrapolation to 100% — 14 hr.

2) Area above curve
between 0 and 14 hr. — 433 units*

3) Total area above and
below curve between 0 and 14 hr. — 5180 units*

Percentage of viable tumour cell population remaining unlabelled $= \frac{433}{5180} \% = \underline{8.4\%}$

* Arbitrary units of area determined by planimetry.

It is stressed at this point that these calculations are intended to serve as a guide to the level of viability of the G_o cells, and it is recognised that application of a method of this nature with complete confidence would require further extensive investigation.

The results presented in fig. 14 also indicate a return to rapid cyclic activity of the unlabelled fraction upon reduction of tumour mass. However, the results here give a more confused picture due in part to the premature termination of the experiment on death of the host animals.

Repeat labelling and retransplantation control experiment: The results of PLM determination on undisturbed, repeatedly labelled plateau phase tumours on completion of and at various time intervals following repeat labelling are shown in fig. 15. On completion of the repeat labelling procedure (4 x 30 hourly ^{3}HTdR injections), 98.5 $\pm$ 1.9% of mitoses were labelled. Further to this, analysis of variance of the data presented in fig. 15 indicates that no significant variation in PLM occurred over a 50 hr. period in the undisturbed tumour.

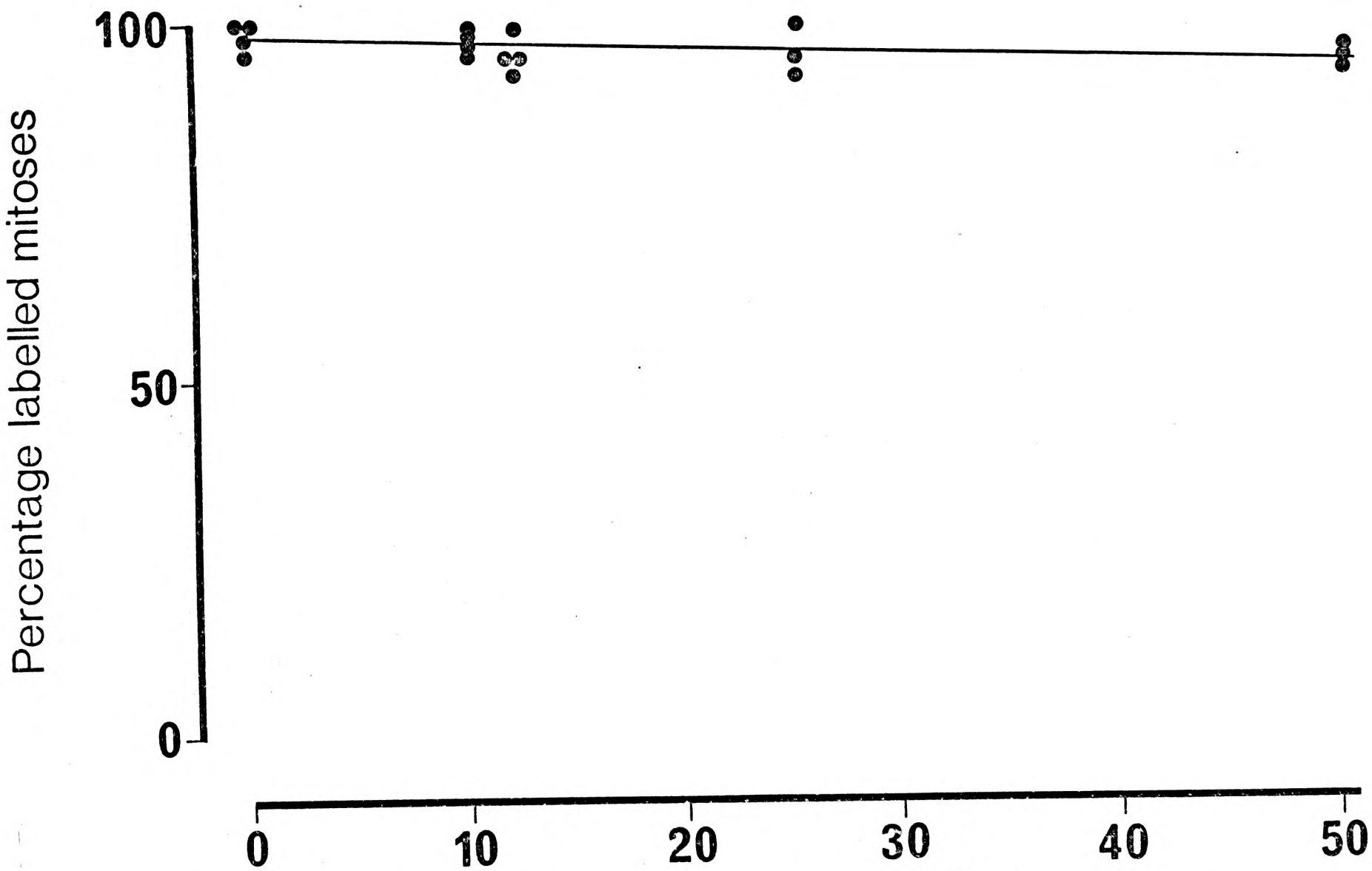

Fig 15 Time (hr.) following completion of repeat ^{3}HTdR labelling

Variation in P.L.M. in undisturbed tumours following multiple injections of ^{3}HTdR

Discussion

The growth curve indicates that there is no appreciable lag phase in tumour growth. There is an initial period of exponential growth which fades as the total tumour cell number rises above 10^8 between the second and third days after inoculation. During this initial period growth is unrestrained and the rate of cell production is constant and proportional to the total tumour cell number.

During the exponential phase of tumour growth there is good evidence from cell cycle, ^{3}HTdR labelling and viability studies to suggest that all cells are cycling rapidly and that cell loss is negligible. During this period, therefore, mean cell doubling time and mean cell cycle duration must be approximately equal and constant (table 2).

Subsequent to the latter phase, there follows a progressive retardation in the tumour growth rate which can be attributed to an increase in the duration of the cell cycle, a reduction in the growth fraction and an increase in the rate of cell loss. Eventually, the growth rate tends to zero and a plateau phase is reached. This general pattern of tumour growth is typical of murine ascites tumours (Lala and Patt, 1966; Fletcher, 1971; Frindel et al., 1969; Schiffer and Markoe, 1974, and Barfod and Barfod, 1980).

The rise in *in vitro* labelling index seen after 5 days (fig. 7) is rather curious and does not fit into the general pattern of declining tumour growth rate. Comparison of the ^{3}HTdR labelling index determined *in vivo* with that determined *in vitro* (table 2) indicates that for an older tumour rapid cyclic activity is more likely to be a feature of an *in vitro* system. This suggestion is supported by the conflicting reports of other researchers. Working *in vivo*, Cavosto et al. (1967, and 1977) and Clarkson et al. (1970) proposed the

existence of a subpopulation of out of cycle leukaemic blast cells. However, the *in vitro* studies of Minden et al. (1978) indicate that all blast cells in acute myeloblastic leukaemia are rapidly proliferating. The inference here is that *in vitro* conditions are more conducive to rapid cyclic activity.

No variation in modal cell size could be detected over the period of tumour growth studied. The distribution of tumour cell size is skewed to the right, possibly due to the existence of large polyploid cells for which there are few if any sub-diploid counterparts, or an unequal distribution of cells throughout the various phases of cell cycle. These results are at variance with those obtained by Peel and Fletcher (1969) who revealed significant variation in modal cell size between the log phase and plateau phase of growth of an EAT. It is unclear whether this discrepancy represents a genuine difference between the two tumours or shows that the present method was relatively insensitive. Increase in cell size is usually associated with the ageing process in animal cell culture (Greenberg et al., 1977).

During the period of declining growth rate the cube root of the total tumour cell number increases linearly with time. This cube root growth pattern is generally associated with restriction of cell proliferation to a spherical margin or shell of a solid tumour (Mayneord, 1932). However, cube root growth has also been observed in solid tumours in which proliferation is not confined to an external shell (Steel and Lamerton, 1966) and in ascites tumours (Klein and Revesz, 1953). It was proposed (Steel and Lamerton, 1966) that cube root growth could arise when nutrient supply to the tumour is proportional to the surface area, as may be the case when the external vasculature is composed of a network of fine vessels. This implies that the availability of nutrients and oxygen derived from the vasculature is

largely responsible for controlling the tumour growth rate. There is much evidence in the literature to suggest that cells are restrained from proliferating by essential nutrient deprivation (Stoker, 1972 and Baserga, 1976c). This mode of growth control would probably operate by restricting macromolecular synthesis when an essential component molecule, i.e. an amino acid, is unavailable (Melvin et al., 1979).

Macromolecular synthesis could also be limited when energy availability is reduced. If tumour cells in culture are deprived of a source of glucose then the energy supply (ATP) becomes limiting and cell proliferation stops (Live and Kaminskas, 1975). The most significant factor relating to glucose deprivation appears to be inhibition of protein synthesis (Van Venrooij et al., 1972). In the case of the EAT growing <u>in vivo</u> the host inability to supply sufficient nutrients would not appear to be a limiting factor (Lala, 1972a, and Burns, 1968), rather the availability of those nutrients to all parts of a large tumour may limit the rate of tumour growth (Hirst and Denekamp, 1979). Thus in old tumours, cells which lie adjacent to the vasculature are maintained in an environment of good nutrition and are able to proliferate. However, cells distant from the vasculature may be deprived of nutrients and restrained from synthesising DNA.

The decrease in proliferative activity with increasing tumour age may be the result of the low oxygen tension found in large ascites tumours (Warburg, 1966 and Del Monte, 1967 and 1969). Harris et al. (1970) in an attempt to explain the accumulation of cells in the G_1 period of the cell cycle, implicated general hypoxia and diminished frequency of random contact of cells with the peritoneum, where oxygen is transferred from capillaries into the ascites.

Increased aerobic glycolysis is a common feature of cell popu-

lations which have been stimulated to divide (Hulme and Weidemann, 1979). The glycolytic capacity of tumour cells, and particularly those of the ascitic variety is well known (Warburg, 1930 and Aisenberg, 1961). It appears that glycolysis is much more effective than mitochondrial oxidative phosphorylation in competing for the available ADP and inorganic phosphate and that a large fraction of the pyruvate formed is converted preferentially to lactate rather than oxidised via the TCA cycle.

The reasons for this metabolic behaviour are unclear. However, it is envisaged that malignant transformation or mitogenic stimulation cause a primary modification of plasma membrane function leading to altered glucose metabolism (Costlow and Baserga, 1973; Hatanaka, 1974; Gregory and Bose, 1977 and Lang and Weber, 1978). Alternatively, the mitochondria may have a limited capacity to oxidise the pyruvate formed, possibly a consequence of damaged or modified respiratory function (Weinhouse, 1955 and Warburg, 1956). It has been suggested (Gordon et al., 1967; Kovacevic, 1971 and La Noue et al., 1974) that mitochondrial mechanisms for reoxidation of the NADH generated during glycolysis are ineffective, and that this could account for excess lactate production. However, more recent studies (Dionisi et al., 1970 and 1974; Cederbaum et al., 1973; Eboli et al., 1976 and Greenhouse and Lehninger, 1976 and 1977) have indicated that one or more 'shuttle' systems operate, in highly glycolytic rapidly growing tumours at rates sufficiently great to reoxidise all of the NADH produced during glycolysis.

Ehrlich ascites tumour cells have nevertheless been shown to derive a significant proportion of their energy requirements from glycolysis (Aisenberg, 1961) and glycolytic and oxidative energy are used with equal efficiency by these cells for protein synthesis

(Quastel and Bickis, 1959). However, EAT cells are unable to grow under anaerobic conditions (Biczowa et al., 1968; Krause, 1973 and Loffler et al., 1978). There are several possible explanations for this. Lack of energy for metabolic processes might result from a reduction in oxygen tension, as production of oxidised cofactor is ultimately dependent upon oxygen availability, and enzyme inhibition would follow after exhaustion of oxidised cofactors (NAD and NADP) (Fletcher, 1972). Oxygen dependent biosynthetic processes, cholesterol or pyrimidine base synthesis, might have a prominent role in cessation of growth during anaerobiosis rather than reduced energy supply (Loffler et al., 1978 and 1980). In addition there is the possibility that lactic acid accumulation could cause a disturbance of intracellular pH and ultimately retard tumour growth, although lactate accumulation and disturbed intracellular pH in normal tissues has been proposed as a cancer promoting factor (Racker, 1972).

A variety of other factors have been considered to be responsible for the changing growth kinetics of Ehrlich and other ascites tumours.

The EAT is allotransplantable and can therefore be considered to be antigenically non-specific (Dux et al., 1967). However, occasional regression has been observed (Hauschka et al., 1953 and 1957), indicating that the vestiges of antigenic character remain. Host immune reactions directed against the EAT have received considerable attention (Litman et al., 1968, and Marusic, 1979). Hartveit (1965) considered the possibility that some cells may be precoated with donor antibody. Precoated cells would probably be susceptible to lysis when introduced into a medium of low protein content in the presence of complement. Janik (1969 and 1971) transplanted EAT cells into xenogenic hosts (rats) and noted that immunological rejection of the tumour was associated with prolongation of the mitotic and G_1 periods. However, no

appreciable lengthening of total cell cycle duration was seen. Choquet et al. (1970) examined the effects of transplanting the L1210 murine leukaemia into isogenic and allogenic hosts. These workers demonstrated an increase in cell doubling time in the primary stages of tumour growth and found this to be associated with allogenic inhibition. An additional line of evidence to support the role of immune factors in suppression of EAT growth is the observation of DeCosse and Gelfant (1968) that administration of antilymphocytic serum (ALS) to plateau phase EAT results in stimulation of resting phase G_2 cells. This might indicate that some form of immune response, abolished by ALS, has a role in reducing the tumour growth fraction.

Holmberg (1962) isolated a dialysable substance from ascites tumour fluid which was found to be toxic to L-cells in culture. Hartveit (1965) considered the possibility that increase in the duration of the cell cycle might result from altered chromosome number. Lala (1972a) theorised that an increase in ploidy could lead to an increase in the time required for duplication of the genome. This explanation is feasible in light of the demonstration of an accumulation of polyploid cells during ascites tumour growth (Andersson, 1977). The results obtained from chromosome analysis here and those obtained by Peel and Fletcher (1969) seem to indicate that no significant change in ploidy level occurs in association with increasing tumour age.

More recently it has been shown by the work of Russel (1970), Shield (1976) and Sunkara et al. (1979 a and b) that the biosynthesis of polyamines is a prerequisite for DNA replication and cell division. A reduction in tumour growth rate has been correlated with a reduction in the rate of intracellular polyamine synthesis (Andersson and Heby, 1977). It has been suggested that as the level of extracellular polyamine rises, due to release from dead, dying and possibly viable

cells, intracellular polyamine synthesis is inhibited via production of an antizyme (non-competitive protein inhibitor) which in turn inhibits ornithine decarboxylase, an enzyme important in polyamine synthesis (Gaugas and Dewey, 1979). It also appears that the enzyme polyamine oxidase is capable of interaction with exogenous polyamine resulting in inhibition of cellular proliferation by means of oxidised polyamine (Gaugas and Dewey, 1979). However, the exact nature of the latter inhibition mechanism remains obscure.

A more attractive concept which attempts to explain the correlation between polyamine synthesis and tumour growth rate involves the availability of pyridoxal phosphate, a cofactor required in polyamine synthesis. It is probable that a reduced energy supply, as previously suggested, would preclude the elaboration of compounds with a high energy of formation such as pyrodoxal phosphate. Consequently, a reduction in intracellular polyamine synthesis would be concomitant with a reduced growth rate if energy availability was a major limiting factor.

Cell coupling in normal cell cultures and in vivo has been shown to have a role in growth control (Lowenstein, 1973 and 1978). This mechanism in vitro is an example of contact inhibition of cell growth and is probably mediated by the movement of metabolic precursors (nucleotides) through cell to cell gap junctions (Lowenstein, 1975). Moreover, malignant transformation and loss of growth control has been widely associated with the breakdown of intercellular communication of this sort (Lowenstein, 1979 and Stoker et al. 1979). However, it is hard to see how cell to cell communication could have a consistent role in reducing the growth rate of the EAT since cell concentration in the ascites clearly diminishes with decreasing tumour growth rate after seven days growth. This latter finding is consistent with the

observations of Lala and Patt (1966) and Peel and Fletcher (1969).

Specific endogenous mitotic inhibitors, chalones, were originally postulated by Weiss (1952) to account for growth control in normal tissues. Saetren (1956) and Bullough et al. (1962, 1964 and 1967) then provided experimental evidence of epidermal chalones. Subsequently, chalone activity was claimed to account for normal growth control in a variety of tissues (Houck and Hennings, 1973). The latter workers concluded that cancer is the product of a breakdown or deterioration of an interaction between a chalone and its target cell. However, Burns (1969) put forward evidence to suggest that chalones were still active (to a degree) in cancerous tissues. He suggests that EAT cells stop growing, in vivo, due to the existence of a self-inhibiting growth mechanism dependent on the attainment and maintenance of a critical viable tumour cell protoplasmic mass. In support of this theory he cites Hauschka and Grinnell (1955) who demonstrated that maximum tumour cell number for a hyperdiploid EAT (approx. 2n DNA) was 2×10^9, which is double that for a hypertetraploid EAT (approx. 4n DNA). In addition to this Fletcher (1971) recorded a maximum tumour cell number of approximately 1×10^9 for a hypotetraploid EAT whereas the maximum tumour cell number attained using the hyperdiploid tumour studied here was 2.5×10^9. It is evident, within the limits of experimental error and on consideration that the host animals used in the latter two studies were drawn from separate stocks and would probably afford differing growth conditions, that some correlation between maximum tumour cell number and ploidy level exists. However, it is not clear whether this evidence is really indicative of chalone activity per se or is a reflection of increased competition for oxygen and nutrients.

The work of Bichel (1970) and Bichel and Dombernowsky (1973) has provided more direct evidence for the participation of a chalone in the

reduction of growth rate of the JB-1 murine ascites tumour. This group have extracted a component of the ascites fluid which inhibits tumour growth *in vivo* and *in vitro* and have been able to characterize this 'chalone' with respect to some of its biochemical properties (Bichel, 1971, and Barfod, 1977 and Barfod and Marcker, 1980).

Mathematical analysis indicates that the growth of an EAT is an exponential process limited by exponential retardation (Laird, 1964). This implies that the tumour growth pattern is fitted by a Compertzian function (Steel, 1977a, and Brunton and Wheldon, 1977). Burns (1969) therefore, suggested that the progressive deceleration in the growth rate could be attributed 'to some inhibitory process that was evident from the earliest stages of tumour growth and which increased with increasing numbers of EAT cells', (a chalone). Further to this it is possible to show that most experimental tumours, within limits, conform to the Compertzian model (Brunton and Wheldon, 1978).

In light of these studies, the results of the analysis of the growth curve data (fig. 2) are difficult to interpret. It can be seen from fig. 2 that there is a decline in the specific growth rate with time which can almost be fitted to an exponential. It would not be difficult to contend that the values obtained at 7, 8 and 9 days are slightly high due to experimental error, and in fact can be fitted to an exponential. Against this a very clear pattern of cube root growth can be seen to emerge (fig. 3) which is in agreement with Klein and Revesz (1953) who observed deviation from initial exponential growth when total tumour cell number reached a value between 5×10^7 and 2×10^8.

In addition to the above arguments it is worthwhile considering the view of Rutter (1973). In an article concerning the role of specific factors in the control of growth and differentiation Rutter

concluded, 'specific feedback inhibitors (chalones) have been poorly documented and, in most cases, seem more a conceptual possibility than an experimentally documented fact'.

The author shares the view that at present there is a lack of convincing evidence to support the chalone hypothesis. With particular reference to the EAT studied here it may be seen that any or all of the factors previously considered to have a possible role in the limitation of tumour growth rate could, in theory, constitute a 'chalone'

To reiterate; the results of detailed kinetic analysis of the EAT indicate that the tumour growth rate decreases between the second and thirteenth day after transplantation of 5×10^6 tumour cells. This reduction in tumour growth rate may be attributed directly to increase in cell cycle duration, reduction in growth fraction (increase in proportion of non-cycling cells) and increase in cell death rate. Additionally, this general phenomenon may be attributed to one or more possible factors, the most probable being a reduced availability of oxygen and other nutrients.

The next area warranting detailed discussion is that of the out-of-cycle or G_0 cell and its method of detection. A number of proposals concerning these cells are to be presented, but before disclosing them it is necessary to consider the cell cycle data more closely.

The interpretation of the theoretical PLM curve has been described previously (p. 17). However, PLM data rarely if ever conform to the theoretical curve. There are two major reasons for this. Firstly, most cell populations exhibit a degree of heterogeneity with regard to duration of cell cycle, consequently synchrony is lost soon after the thymidine administration. Secondly, as the cells divide the radio-label becomes diluted, thus an apparent cell loss occurs preferentially from the labelled cohort.

These problems were recognised by Quastler and Sherman (1959) who concluded that the method of PLM yielded attenuated data and in view of this only crude approximations to cell cycle and composite phase durations were possible. Following initial empirical analyses of cell cycle data (Quastler and Sherman, 1959 and Mendelsohn, 1965) there appeared a battery of theoretical cell cycle models (Barrett, 1966; Steel and Hanes, 1971 and Simon et al., 1972). These simulation methods are based on assumptions concerning the cell cycle phases and residence times of cells within each phase. Experimental data in these methods are usually fitted to a simulated curve by computer optimization.

It is recognised that incompatibility between the experimental data and the model exists when the model is ignorant of artefacts or special properties which are peculiar to a given experimental cell system. Furthermore, computer optimization is only preferable to curve selection by eye when the assumptions upon which the simulation is based are unquestionably correct (Steel, 1977c). Therefore, identification of discrepancies between experimental data and a given model can lead to a more complete understanding of the experimental system which is being studied.

The present data obtained from PLM determination for the FAT were analysed by both the empirical method of Quastler and Sherman (1959) and the simulation and computer optimization method of Steel and Hanes (1971). The results obtained from both methods leave no doubt that the cell cycle after 13 days of tumour growth is considerably longer in all phases than that seen after 2 days' growth. Similarly, growth fraction and cell loss calculations based on both sets of results are consistent. However, the cell cycle parameters for the log phase tumour are more compatible with the growth curve analysis

when estimated by the method of Quastler and Sherman (1959).

The most obvious difference between the results of the empirical analysis and the computer analysis concerns the length of the G_1 phase and consequently the overall cell cycle duration. The major discrepancy between the experimental data and the simulated curve for each phase of tumour growth relates to the positioning of a peak for the second wave of labelled mitoses.

In the case of the 2 day tumour most of the experimental points on the second peak lie above the theoretical curve. This phenomenon is known as 'enhancement'. Dr. G. G. Steel (personal communication) advised that the appropriate course of action was the redetermination of the PLM values between 15 and 30 hours. This advice was not taken since it was felt that the results of a pilot cell cycle experiment (open circles fig. 8) supported the position of the curve indicated in fig. 8. Thus, the hand drawn curve now encloses a larger area under the second peak implying a preferential accumulation of labelled cells.

The possible explanations for this include, reutilization of ^{3}HTdR normally associated with a substantial cell death rate. Alternatively preferential scoring of labelled cells could be a source of error particularly when samples are heavily labelled but lightly stained. Finally, if a rapidly proliferating stem cell pool exists which has a much higher labelling index than a more slowly proliferating fraction within the cell population, then enhancement would occur (Tannock, 1968).

In the case of the 13 day tumour the curve is damped heavily. The theoretical first peak fits well but beyond this the data fall below the theoretical curve. This phenomenon is known as fade and is one of the most common discrepancies encountered with analyses based on a conservative simulation model, that is, a model which

maintains the same area under each successive peak. Fade might be caused by the progressive loss of labelled proliferating cells and would occur if the ^{3}HTdR had a toxic effect upon the incorporating cell or if there was dilution of the radiolabel by cell division. The existence of a slowly proliferating stem cell pool could result in fade. Alternatively, the replacement, during the course of the experiment, of labelled cells in cycle with unlabelled and initially non-cycling cells could produce a similar effect. In any event it would have been preferable to extend the sampling period to beyond 100 hr. However, the sampling period was limited by the life span of the host animals.

The results of analysing the PLM data by empirical and simulation methods appear to correspond closely enough to allow the confident formulation of a repeat labelling experiment.

Based originally on hand drawn curves a programme of 7 x 10 hourly injections of ^{3}HTdR was used to label all cells with an estimated maximum cell cycle time of up to 110 hr. and an estimated minimum S-phase time of 10 hr. The reasoning behind this schedule made use of a knowledge of experimental design used by other workers (Peel and Fletcher, 1969; Dombernowsky and Hartmann, 1972 and Siracka and Pappova, 1978). However, it was felt prudent to extend the duration of the effective labelling period to 140 hr. in view of the results of computer analysis. With regard to the number of and interval between ^{3}HTdR injections required to label all rapidly cycling cells it appeared that one injection every 10 hr. was over-sufficient and likely to be cytotoxic (Cleaver, 1967 and Beck, 1980). Therefore, the number of injections was reduced to 4 at 30 hour intervals.

When compared with the number and timing of injections used by other workers for repeat labelling of experimental tumours (Lala,

1972b; Bichel and Dombernowsky, 1973; Simpson-Herren et al., 1974, Hartmann et al., 1976; and Siracka and Pappova, 1978) 4 x 30 hourly injections appears to be relatively sparse. However, repeat labelling in this case was performed on a truly plateau phase tumour with a relatively long cell cycle time. Other studies (Dombernowsky and Hartmann, 1972 and Simpson-Herren et al., 1974) have been conducted on tumours prior to the attainment of plateau phase and which consequently have a shorter cell cycle duration.

Weinstein and Mooney (1980) used a similar repeat labelling procedure to the one used here for an analysis of cellular proliferation kinetics in human hair roots. Their results indicated that 4 x 12 hourly injections of ^{3}HTdR were sufficient to label all proliferating cells; the cell cycle time of 39 hr. and S-phase time of 11 hr. were previously determined by the PLM method of Quastler and Sherman (1959). No recruitment of cells was observed during the repeat labelling procedure.

Determination of PLM for non-aspirated tumours following 4 x 30 hourly injections (fig. 15) indicates that in excess of 98% of cycling cells become labelled using this regime. Bichel (1973) achieved a maximum PLM of only 95% 24 hours from the start of continuous labelling and attributed this to a failure of some cells, traversing G_2 at the start of the experiment, to complete mitosis.

The probability that resting ascites tumour cells recycle <u>in situ</u> upon transient contact with the vasculature emphasises the artificiality of ascites tumours according to Steel (1977c). However, the slight decrease in PLM following the final ^{3}HTdR injection (fig. 15) is not significant and therefore precludes the possibility of G_0 cells recycling <u>in situ</u>. The present data confirm earlier work by Lala (1972b) which discounts the possibility that G_0 cells in EAT resume rapid cyclic

activity in the undisturbed tumour.

The result of repeat labelling (fig. 12) is the accumulation of labelled cells which progressively approaches 100%. This procedure has been advocated as a simple method of establishing the growth fraction (Yankee et al., 1967; Peel and Fletcher, 1969 and Terz et al., 1977). This is not the case (Steel, 1977b). However, use of this method to determine the growth fraction in certain human tumours, where the cell loss factor is small and the out-of-cycle population is large, may well be valid.

Steel (1977b) demonstrates how, given sufficiently precise continuous labelling data, it is possible to determine the nature of cell loss from a tumour. Thus a repeat labelling curve which asymptotically approaches 100% indicates cell loss occurring predominantly at the end of mitosis or non-preferentially from all phases of cell cycle, whereas a continuous labelling curve which quickly reaches 100% indicates a high degree of preferential cell loss from the non-proliferating compartment.

In the case of the EAT studied here, the initial sharp rise in percentage labelled cells (fig. 12) is largely due to labelling of rapidly cycling tumour cells. However, as the percentage of labelled cells rises above 70 the rate of accumulation of labelled cells falls. This is due to the gradual replacement of unlabelled non-proliferating cells with labelled non-proliferating cells which were rapidly cycling at the start of the experiment (Lala, 1972b, and Steel, 1977b).

The gradual approach of this curve (fig. 12) to 100% is suggestive of cell loss, non-preferentially, from all phases of cell cycle. This is contrary to the results obtained by Lala (1972b) and Dombernowsky (1972) who concluded that cell loss during the plateau phase of growth of an ascites tumour occurs preferentially from the non-cycling com-

partment. The discrepancies between these data may be explained by the fact, previously considered, that the present experiments involve a tumour which has reached plateau phase of growth, and during the course of the experiment a considerable decline in total tumour cell number is evident (fig. 1). However, the work of Lala (1972b) and Dombernowsky (1972) involved tumours which had barely reached plateau phase and which either increased or maintained total tumour cell number throughout the course of the repeat labelling procedure. It is proposed that the more even balance between cell loss from the non- and rapidly proliferating compartments demonstrated by the present data and as shown by those of Dombernowsky et al. (1974) and Siracka and Pappova (1978) is probably due to the increasing susceptibility of rapidly cycling cells to highly anoxic conditions, the latter being a feature of very advanced ascites tumours.

On consideration of all these factors it is proposed that the plateau phase tumour cells which remain unlabelled after 4 x 30 hourly ^{3}HTdR injections, and which have been shown to retain the ability to resume rapid cyclic activity, can be considered to be cell cycle arrested or extremely slowly cycling (Tc in excess of 140 hours) i.e. G_0 cells.

The results of retransplanting a repeatedly labelled tumour show a bimodal return to cycle of G_0 cells. A bimodal return to cycle would result if the G_0 cells resumed cell cycle in at least a partially synchronous fashion. Synchronous division on restimulation of out-of-cycle EAT cells in vitro has been previously demonstrated by Ioffler et al., (1978) and Merz and Schneider (1980). In addition, Belyaeva and Ivleva (1977) showed that retransplantation of terminal phase ascites hepatoma 22A cells resulted in an initial synchronous division of previously out-of-cycle cells and that the duration of the first cell cycle was independent of the time previously spent in G_0 phase.

This trend (fig. 13) is perhaps suggestive of an initial return to cycle of G_2 arrested cells approximately 5 hours after retransplantation, followed by the appearance in mitosis of previously G_1 arrested cells approximately 20 hours later. However, this latter possibility is unlikely due to the necessary timing of the first cell cycle after retransplantation. Cell cycle analysis indicates a cell cycle of approximately 18 hours for a two day tumour, and further kinetic analysis suggests that this is constant from transplant up to the third day of tumour growth. Thus if it can be accepted that the bimodal trend indicated in fig. 13 reflects a partially synchronous return to cell cycle of G_o cells then all G_o cells, feasibly arrested in G_1, would return to cell cycle within the first 18 hours from transplant. This timing of initial rapid cell cycle is consistent with that discovered by Peel and Fletcher (1969).

Comparison of the results obtained by retransplantation (fig. 13) with those obtained by greatly reducing tumour mass (fig. 14) does not help to resolve the problem concerning the identity of the out-of-cycle phase. The results of greatly reducing tumour mass indicate a smaller non-synchronous return to cell cycle of G_o cells. The difference between the curves shown in figs. 13 and 14 is a reflection of the conditions prevailing in the donor peritoneal cavity following tumour aspiration, which are probably not entirely conducive to rapid tumour cell growth. In addition, anaesthetic used during the percutaneous aspiration procedure could well have an effect on the rapid return to cell cycle of the G_o cells.

An attempt to estimate the extent to which recycling of G_o cells occurs on tumour retransplantation is described on page 45. The results of this analysis indicate that a majority of G_o cells remain viable. The model described is based on a partially synchronous return to cell

cycle of predominantly G_1 arrested cells. However, if the calculations are revised to allow consideration of the area above both troughs (fig. 13), the viability of a considerable majority of non-cycling cells is still indicated.

The validity of this method rests on certain assumptions which need qualification. The model requires that all cells are restimulated and maintain some degree of synchronous progress through the cell cycle for at least 15 hours. There are two reasons for suspecting that these conditions may not be satisfied. Rapid loss of synchrony with this cell system is a feature of cell cycle analysis and it is generally accepted that the G_o phase has 'depth', consequently, some G_o cells require more time to re-enter cell cycle than others (Augenlicht and Baserga, 1974). In addition, extrapolation of the curve back to 100% also assumes maintenance of a high degree of synchronous division.

Application of the above assumptions clearly indicates potential sources of error. Therefore, it is proposed that this model be adopted here only as a demonstration of the viability of a majority of non-cycling cells.

In conclusion, it is evident that the existence of a non- or slowly cycling sub-population of tumour cells contributes to a process of reduced tumour growth with increasing tumour age. The elaboration of the out-of-cycle cell population is most probably due to reduced oxygen and/or nutrient availability. The viability of the majority of the non-cycling tumour cell population has been demonstrated. The application of the repeat labelling procedure, herein described, permits the visualization of a proportion of G_o tumour cells in the plateau phase of tumour growth.

Chapter Two

Cytochemical Studies

Introduction

The concept of non-cycling or G_o cells, capable of undergoing mitosis following application of an appropriate stimulus, was the product of cell kinetic studies of the early 1960's (Mendelsohn, 1960 and 1962; Lajtha, 1963; Gelfant, 1963 and Cavosto et al., 1964). Since that time the most important questions raised have concerned the metabolic or biochemical status of G_o cells. In the first instance the problem that arises is whether apparently non-cycling cells can be classified as cells with an elongated G_1 phase or as being in a true G_o phase (Prescott, 1968; Fox and Pardee, 1971; Costlow and Baserga, 1973, and Baserga, Costlow and Rovera, 1973).

Non-cycling or G_o cells do not synthesize DNA and it can be appreciated that in this respect non-cycling cells are metabolically deficient. This is currently used as the criterion for the definition of non-cycling cells. The study of non-cycling cells is therefore highly problematical since cycle arrest almost invariably occurs in either the pre- or post-DNA replicative phase and such cells cannot easily be distinguished from cycling cells. The existence of the S-phase marker (radioactive TdR) would facilitate discrimination between S-phase cycling and S-phase arrested cells in normal cell populations at least (Baserga, 1976a and Steel, 1977d). This point emphasises the importance of the quest for an expedient biochemical marker for G_o (Sarna, 1974a and 1974b and Gaub, 1977).

Investigations into the nature of non-cycling cells forming part of an <u>in vivo</u> system have been largely restricted to the kinetic behaviour of the cells upon stimulation (Barka, 1965; Choie and Richter, 1973; and Hartmann et al., 1976). It is within <u>in vitro</u> cell systems that the metabolic behaviour of non-cycling cells has been more closely studied (Baserga, 1976c).

The information drawn from tissue culture systems has therefore proven invaluable to the current understanding of the metabolic status of non-cycling cells. However, there are several problems inherent in this approach. There is the major difficulty of extrapolating *in vitro* phenomena into an *in vivo* system. It is well recognised that cellular interactions observed *in vitro* may not reflect the events which occur *in vivo*.

Attempts to demonstrate possible biochemical differences between cycling and non-cycling cells have involved the equating of stationary phase cells in culture with G_0 cells. This approach may not be strictly valid since stationary phase cultures tend to retain a degree of cyclic activity (Van Venrooij et al., 1970 and Thomas, 1977) and the behaviour of stationary phase cultures is highly variable depending on cell type or culture conditions used (Baserga, 1976c). Nevertheless, comparative analysis *in vitro* has revealed both quantitative and qualitative variation between stationary phase and G_1 cells.

Becker et al. (1971) demonstrated reduced levels of protein and RNA for stationary phase Syrian hamster cells. In addition, electrophoretic analysis subsequently revealed that the protein complement of the stationary phase cells was qualitatively distinct from that of G_1 cells (Becker and Stanners, 1972). However, further examination of virally transformed cells in culture showed that stationary phase was associated with reduced protein synthesis and loss of viability but no reduction in RNA content (Stanners et al., 1979). Similar conclusions have been reached by many other researchers (Fox and Pardee, 1971; Salas and Green, 1971; Pardee and Dubrow, 1977, and Abelson et al., 1979). These findings suggest that virally transformed cells do not enter a true resting or G_0 phase, but must either "grow or die" (Stoker, 1972). In many other respects transformed cells do resemble tumour

cells (Stoker, 1972 and Ponten, 1976). Primarily, both systems do not show the degree of growth control and social behaviour shown by their normal counterparts.

The use of specific metabolic inhibitors to induce quiescence (cycle arrest) has often led to the conclusion that the inhibitory process employed has a natural role in cellular growth control (Hand, 1975; Melvin et al., 1979 and Loffler and Schneider, 1980). This may not be the case since specific inhibition of cellular protein synthesis, for example, would be expected to prevent DNA synthesis and division. Conversely, a naturally quiestcent cell may be capable of protein synthesis (Burns, 1968 and Epifanova et al., 1980). These methods do, however, provide a convenient approach to a difficult problem.

The aim of the present experimentation was to attempt to examine the metabolic status of non-cycling tumour cells subject to the constraints generally associated with <u>in vivo</u> physiological conditions. It was considered appropriate that such an examination be conducted on individual cells in order to achieve a precision not possible using, for example, biochemical extraction techniques.

Using the repeatedly ^{3}HTdR-labelled plateau phase EAT as a model system for the visualization of non-cycling tumour cells (Gelfant, 1977) analysis of their biochemical characteristics was made. This work parallels that of Bichel and Dombernowsky (1973), Peel and Fletcher (1969) and Gabutti et al. (1969) in which the DNA contents and kinetic properties of non-cycling tumour cells were studied. This method of visualizing non-cycling cells is not, however, without its limitations. The most serious problem encountered relates to the possibility that during the course of the repeat ^{3}HTdR labelling procedure a proportion of cells leave the cell cycle after becoming labelled. The implications of this for the interpretation of the results

obtained will become apparent. The current work advances a stage further the above mentioned experimentation of Bichel (1973), Peel and Fletcher (1969) and Gabutti et al. (1969) by using dual staining techniques and subsequent cytophotometric determination of individual cell DNA, RNA and protein contents.

The method of Kiefer et al. (1969) was used for microdensitometric estimation of individual cell DNA and RNA contents and that of Deitch (1955 and 1966) for microdensitometric estimation of individual cell DNA and total protein contents. These methods are preferable to alternative procedures using for example methyl green pyronin (this dye also interacts with non-histone protein, Deitch, 1966) or methylene blue or gallocyanin chrome alum, since the latter methods require sequential measurements to be made before and after extraction of one or other of the two nucleic acid types plus bound dye-stuff.

With regard to Feulgen cytophotometry (as applied here) it is recognised that the validity of this method has been rigorously questioned by many investigators (for reviews see Swift, 1955 and Leuchtenberger, 1958). The Feulgen reaction is now universally accepted as being a most reliable technique for the estimation of cellular DNA content. Furthermore, the Feulgen technique of DNA estimation affords a standard by which new methods are assessed (Filkuka et al., 1969 and Allison and Ridolpho, 1980). Nevertheless the results of separate experiments can only be compared if the staining conditions are carefully standardized (Fukuda et al., 1977).

Finally, the measurement of the optical density of microscopic biological material after specific staining has associated problems, the most serious of which so far encountered is that of 'distributional error' (Leuchtenberger, 1958 and Mendelsohn, 1966). When the stained material to be measured is optically non-homogeneous Beer's law cannot

be applied and serious error results. However, measurement of a large number of very small composite areas by scanning the sample with a small nucleus of light and subsequent integration of each measurement permits accurate microdensitometry (Pearse, 1972 and Fukuda et al., 1977). This scanning method was employed in the present microdensitometric analyses.

A further refinement to this is the two wavelength scanning method, devised by Fujita et al., (1972). This method combats the problem of light scatter (Fukuda, 1977). It was not possible to use this additional technique in the current work since all samples were dually stained.

Materials and Methods

All stains used were initially obtained in the form of a dry powder from E. Gurr Ltd. (London), unless otherwise stated. Preparation of staining solutions and other reagents used in quantitative cytochemical analysis was as follows:

Pararosanilin: One gram of pararosanilin (colour index, CI 42500) was dissolved with 19.0 gm sodium metabisulphite in a 0.15N solution of HCl by stirring in the dark for 2 hr. at room temperature. The final volume was made up to 1 litre with 0.15N HCl. The stain was decolourised by shaking briefly with 0.5 - 1.0 gm activated charcoal; the solution was clarified by filtration. The pH of the staining solution was adjusted to 2.0 just prior to use.

Coriphosphin O: Coriphosphin O solution (CI 46020) was prepared in similar fashion to that for pararosanilin, however, decolourisation was left uncompleted and the reagent remained pale yellow.

Gallocyanin Chrome Alum, (GCA): Five grams of chrome alum, analar grade, obtained from BDH Ltd. (Poole) was dissolved in 100 ml distilled water, and after the addition of 0.15 gm gallocyanin the solution was boiled gently for 5 min. The pH was then adjusted to 1.6. The stain was prepared and filtered just prior to use.

Naphthol Yellow S (NYS): Naphthol yellow S (CI 10315) was used at a concentration of 0.1% in a 1% solution of acetic acid. The pH was then adjusted to 2.8. The stain was prepared and filtered just prior to use.

DNase and RNase: Deoxyribonuclease - I and Ribonuclease - A, originally isolated from bovine pancreas, were obtained from the Sigma Chemical Co. Ltd. (Dorset, England). Each enzyme was made up separately to a concentration of 0.2 mg/ml in a 0.1M Tris/acetate buffer at pH 6.5

Trypsin: This enzyme, obtained from Sigma (U.K.) was prepared as a 0.1% solution in 0.2M Tris/acetate buffer at pH 7.0.

Feulgen Hydrolysis Curve: The optimum hydrolysis time was determined for the Schiff's reaction in EAT cells and normal mouse thymocytes. Smears of cellular material, fixed previously in absolute methanol for 10 min., were rehydrated in running tap-water, rinsed in distilled water, and in 1N HCl and then immersed in 1N HCl at 60°C for various periods of time from 2 min. to 18 min. Following hydrolysis, smears were rinsed briefly in cold 1N HCl and then stained with pararosanilin solution for 1 hr. at room temperature. Smears were then rinsed in 3 x 10 min. metabisulphite baths (prepared from 0.15N HCl : 10% sodium metabisulphite : H_2O ; 1:1:15) followed by running tap-water for 30 min. and finally dehydrated in a graded series of alcohols and cleared in xylene. All microdensitometric specimens were mounted in oil of identical refractive index to that used for the oil immersion objective employed.

Determination of DNA and RNA content within the same cell, Principle: Microdensitometric determination of single cell DNA and RNA contents was made according to the method of Kiefer et al. (1969). This method employed a basic dyestuff GCA (which interacts with both DNA and RNA) in conjunction with coriphosphin O, a 'Schiff type' base which interacts with DNA alone under the conditions used. It is possible to utilize these two dyes in the same specimen since their absorption maxima are sufficiently distant to allow independent assessments of each to be made. Light absorption for GCA and coriphosphin O was measured at 515 nm and 465 nm respectively on a Vickers M86 scanning microdensitometer.

This method required four separate preparations of tissue:

1) GCA stained only.
2) Coriphosphin stained only.
3) RNase pretreated and GCA and Coriphosphin stained.
4) GCA and Coriphosphin stained.

Integrated density measurements made on preparations 1, 2 and 3 were used to determine factors f1, f2 and f3 respectively. This was necessary since each stain either contributes to, or has an effect on, the total reading obtained at the absorbance maxima of the other. In this way, a measure of the relative RNA bound GCA and DNA bound coriphosphin O per cell was obtained from the GCA and coriphosphin O stained preparation (4), the final values being derived from manipulation of the density measurements made at both wavelengths using factors f1, f2 and f3 as follows:

$$f1 = \frac{OD_{GCA\ 515}}{OD_{GCA\ 465}} \qquad \text{Determined from preparation 1)}$$

$$f2 = \frac{OD_{Cori\ 465}}{OD_{Cori\ 515}} \qquad \text{Determined from preparation 2)}$$

Now, since each strain contributes to the total absorbance at both wavelengths, total absorbance for a dually stained preparation is:

$$OD_{TOTAL\ 515} = OD_{GCA\ 515} + OD_{Cori\ 515}$$

$$OD_{TOTAL\ 465} = OD_{Cori\ 465} + OD_{GCA\ 465}$$

A relative measure of the DNA bound Coriphosphin O ($OD_{Cori\ 465}$) per cell is then given by:

$$OD_{Cori\ 465} = \frac{(f2 \times OD_{TOTAL\ 515}) - (f1f2 \times OD_{TOTAL\ 465})}{1 - (f1 \times f2)}$$

Where the total absorbances ($OD_{TOTAL\ 515 \text{ and } 465}$) are measured from the dually stained preparation (4).

The determination of DNA and RNA bound GCA ($OD_{GCA\ 515}$) is made easier by virtue of having already calculated $OD_{Cori\ 465}$.

$$OD_{GCA\ 515} = OD_{TOTAL\ 515} - \frac{OD_{Cori\ 465}}{f2}$$

This value is now adjusted in order to obtain a measure of relative RNA bound GCA. This is possible since the quantity of DNA bound GCA is proportional to the coriphosphin O quantity and the DNA

bound GCA can be established by prior treatment of a comparable tissue preparation with RNase.

Therefore: $f3 = \frac{OD_{GCA\ (DNA)\ 515}}{OD_{Cori\ 465}}$ Determined from preparation 3).

and, $OD_{GCA\ (RNA)\ 515}$, the value for RNA bound GCA, is given by:

$$OD_{GCA\ (RNA)\ 515} = OD_{GCA\ 515} - (f3 \times OD_{Cori\ 465})$$

$$OD_{GCA\ (RNA)\ 515} = OD_{TOTAL\ 515} - (f3 + \frac{1}{f2})\ OD_{Cori\ 465}$$

Thus after measurements at each wavelength on tissue stained with both dyestuffs two values for each cell were calculated. These values were taken as relative measures of DNA bound coriphosphin O and RNA bound GCA and it can be seen that if the two dyestuffs behave in a stoichiometric fashion when binding to the DNA and RNA under the experimental conditions, then effectively this is a measure of the DNA and RNA content of the cell.

Naturally, Kiefer et al. (1969) were at pains to demonstrate the stoichiometry of these stains under the appropriate experimental conditions and hence the suitability of this method for cytophotometric estimation of DNA and RNA. However, in view of the difficulties concerned with control of staining conditions and reproducibility of results, added to the possibility that the origin, and perhaps the quality, of the staining reagents used in the present work could have been at considerable variance with those used by Kiefer, it was felt that the most prudent course of action was to test the coriphosphin O and GCA over the range of experimental conditions under which they were expected to function stoichiometrically. Therefore, the tests originally prescribed and performed by Kiefer were repeated, using EAT cells, normal mouse lymphocytes and rat thymocytes.

Staining procedures, (DNA and RNA contents determination): Smears of either EAT cells or normal mouse lymphocytes were air-dried and fixed

in absolute methanol for 10 min. The smears were rehydrated in running tap-water, rinsed in distilled water and treated according to one of 8 processes.

i) Rinsed in 1N HCl, hydrolysed in 1N HCl at 60°C for 10 min. and stained with Pararosanilin according to the Feulgen reaction scheme previously described.

ii) Hydrolysed as in (i) but stained with Coriphosphin O according to the Feulgen reaction scheme.

iii) Immersed in GCA stain and placed in a dark cupboard for 48 hrs. at room temperature. The smears were then rinsed in running tap-water for 30 min. dehydrated in graded alcohols and cleared in xylene.

iv) Immersed in GCA as in (iii) prior to washing and Feulgen treatment as in (ii).

v) Pretreated with RNase for 60 - 90 min. at 37°C followed by GCA treatment as given in process (iii).

vi) Pretreated with RNase as in (v) followed by dual staining as given in process (iv).

vii) Pretreatment with DNase for 45 - 75 min. at 37°C followed by GCA treatment as given in process (iii).

viii) Pretreatment with DNase as in (vii) followed by dual staining as given in process (iv).

Measurement of DNA and RNA contents of G_0 cells: In order to determine DNA and RNA values for G_0 cells it was necessary to stain ^{3}HTdR labelled preparations according to procedures (ii), (iii), (iv) and (vi) above, after removal of autoradiographic silver grains. Unstained autoradiographs, mounted in euparal, were initially photographed under low power (Mag. x 128) phase contrast. Re-examination of smears under high power enabled labelled and unlabelled cells to be indicated on photographic maps. The autoradiographic silver grains were then

removed according to the method of Bianchi et al. (1964) as follows: Mounted autoradiographs were warmed briefly so that the cover-slip could be removed easily, the autoradiographs were immersed in euparal essence for 2 min., followed by xylene 2 min., methanol 5 min., 50% alcohol 5 min., distilled water 2 x 5 min. and immersed in a 7.5% solution of potassium ferricyanide (K_3FeCN_6) for 3 min. The smears, now devoid of silver grains, were first washed in a 20% solution of sodium thiosulphate for 5 min. and then distilled water for 3 x 1 min. Finally, removal of the autoradiographic gelatin base was achieved by trypsinization of smears at 37°C for 10 - 15 min. Smears were then washed in running water for 10 min. before staining. Microdensitometric measurements were made of appropriate cells by referring to the photographic maps.

Determination of DNA and Protein content within the same cell: In this experiment, designed to determine the relative DNA and protein values for G_o and cycling cells the dual staining method of Deitch (1955 and 1966) was used.

Autoradiographs of pulse labelled 2 and 5 day tumours, pulse labelled 12 day tumours and the same 12 day tumours repeatedly labelled were stained with Feulgen reagent (pararosanalin) according to the reaction scheme already outlined and then immersed in NYS for 30 min., washed in 1.0% acetic acid for 1 min. and dehydrated in three changes of t-butyl alcohol and cleared in xylene. The smears were then mounted in immersion oil, DNA determination was made by direct measurement of DNA bound pararosanilin at 540 nm and total protein determination was made by direct measurement of protein bound NYS at 435 nm. Since these readings were performed on autoradiographs, only unlabelled cells were measured; such cells were, therefore, either G_1, G_2, G_o or mitotic cells.

Presentation of results of dual staining experiments: Explanation of

the presentation and analysis of the results of single cell DNA and RNA and DNA and protein determination has been incorporated into the results section (p. 90) where it is more immediately comprehensible.

Flow cytofluorometric (FCM) analysis of EAT cells with acridine orange (AO) staining:

(i) Acridine Orange: A stock solution of AO (Sigma), 1mg/ml in deionised water was diluted 1:20 before use.

(ii) Staining and Analysis: Staining was performed according to the method of Love (1979). Ten microlitres of the diluted AO stock solution was added to 2ml of a suspension of plateau phase (12 day) EAT cells in PBS, pH 7.0 - 7.4. The final cell suspension contained $2 - 3 \times 10^6$ cells/ml. The mixture was incubated at room temperature for 5 min. The stained cell sample was excited with UV light (488 nm) and readings were taken of emissions occurring at 515 - 575 nm (green fluorescence) and 600 - 650 nm (red fluorescence).

The displays presented (figs. 33a, b, c) represent the accumulated distribution of 10^4 cells.

Results

Feulgen hydrolysis curve; (1N HCl at 60°C): The results presented in fig. 16 are plots of mean absorbance per cell (arbitrary units, AU), determined at a wavelength of 540 nm for various cell types which previously had been subjected to treatment with 1N HCl at 60°C for various times prior to reaction with the Schiff's base. Each point on the curves represents a mean value for at least 25 interphase cells or 10 mitotic figures.

These results indicate that the optimum hydrolysis (1N HCl : 60°C) time for Feulgen staining of ascites tumour smear preparations is 10 min. In accordance with the staining procedure outlined by Swift (1955), Deitch (1966) and Fletcher (1971) and in the present methods section, p. 74 , an hydrolysis time of 10 min. in 1N HCl at 60°C was carefully observed.

Absorbance profiles: The absorbance profiles for ascites tumour cells stained with pararosanilin, coriphosphin O, GCA, and naphthol yellow S, are given in figs. 17, 18, 19, and 20 respectively. The absorbance profiles for dually stained tumour cell preparations, coriphosphin O - GCA, and pararosanilin - naphthol yellow S are given in figs. 21 and 22 respectively. The absorbance profiles for dually stained preparations appear not to vary significantly from corresponding profiles for singly stained preparations.

Arrows marked on the horizontal axes of figs. 17 - 22 indicate for each dye-stuff the wavelength (λ nm) at which subsequent measurements of optical density (OD) were made. It can be seen that the wavelengths selected in each case do not correspond precisely with the absorbance maxima. This tactic of measuring optical density just off the absorbance peak is designed to ensure increased transmittance, thereby reducing error due to non-random dye distribution (Leuchtenberger,

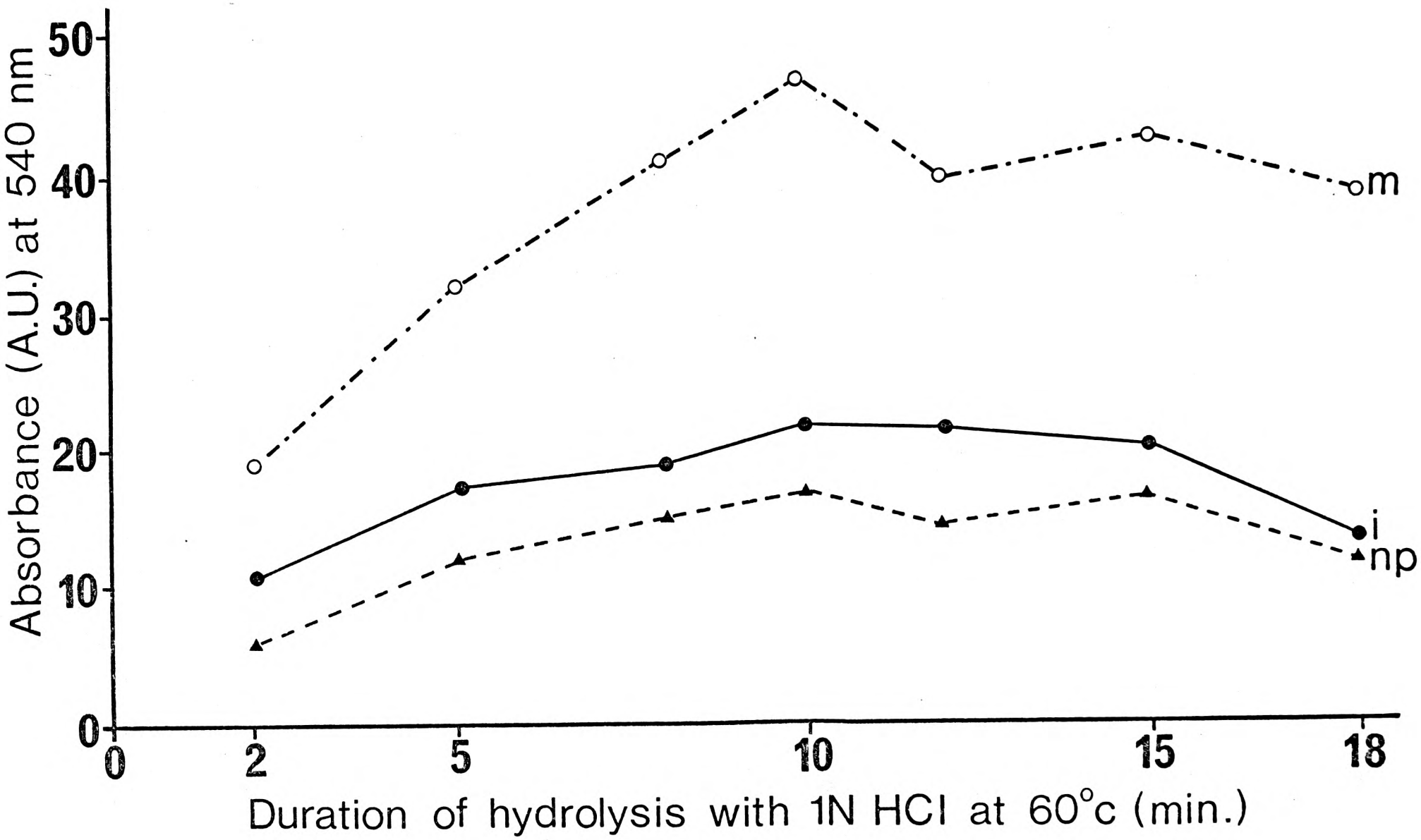

Fig 16 Feulgen hydrolysis curves, for various cell types found in the ascites: np ≡ normal peritoneal cells, i ≡ interphase tumour cells (2n DNA) and m ≡ mitotic figures.

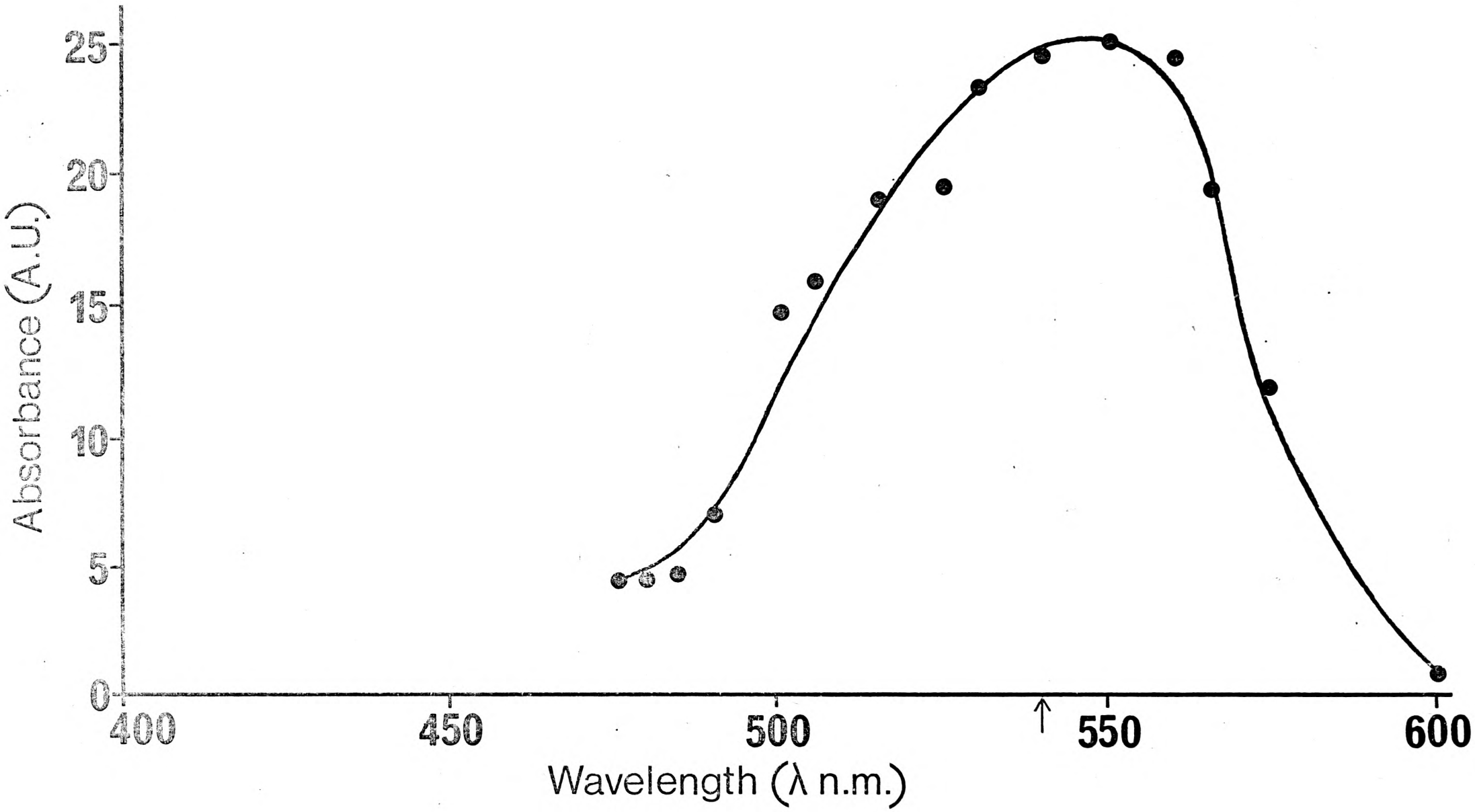

Fig 17 Absorbance profile for E.A.T. cells stained with Pararosanilin

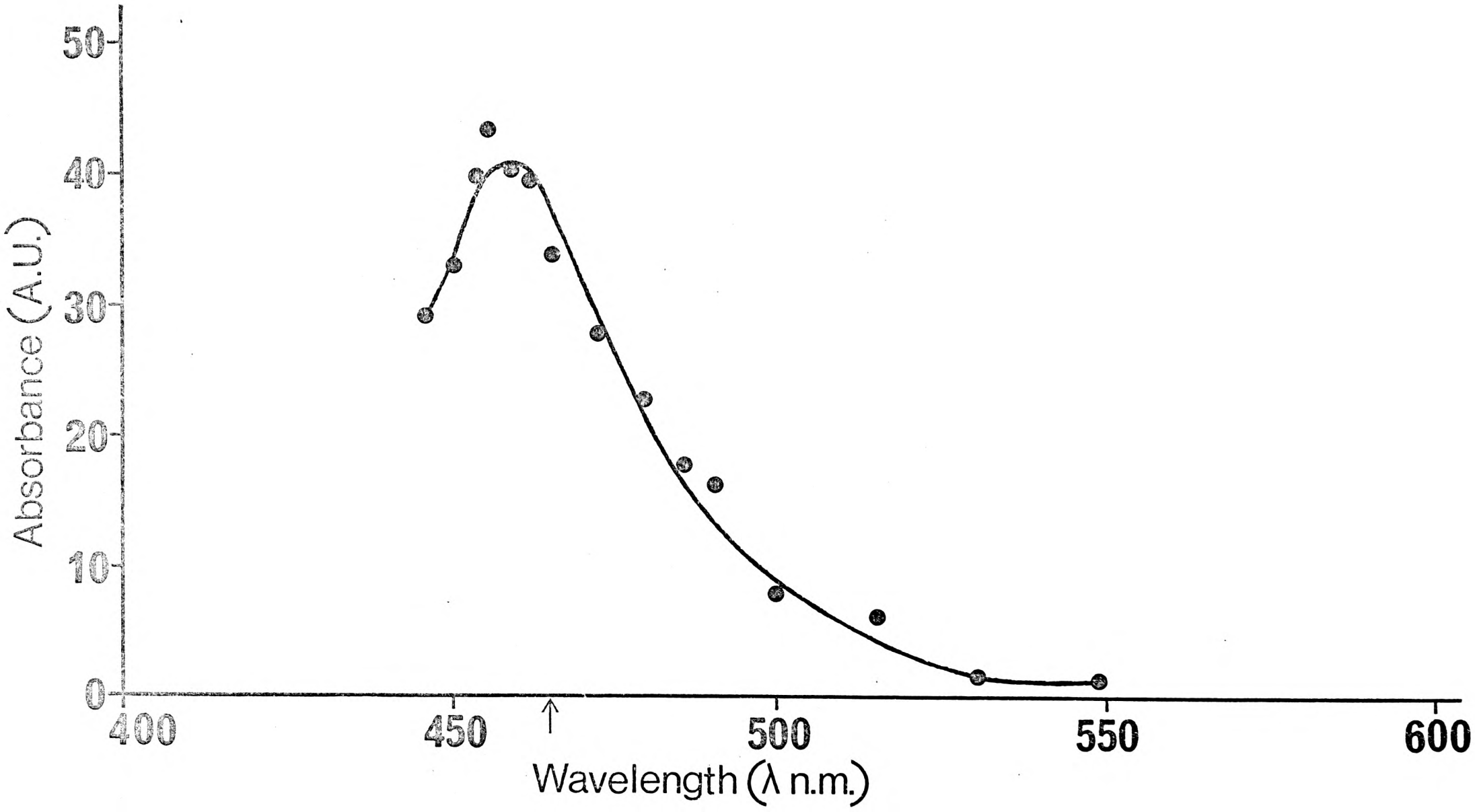

Fig 18 Absorbance profile for E.A.T. cells stained with Coriphosphin O

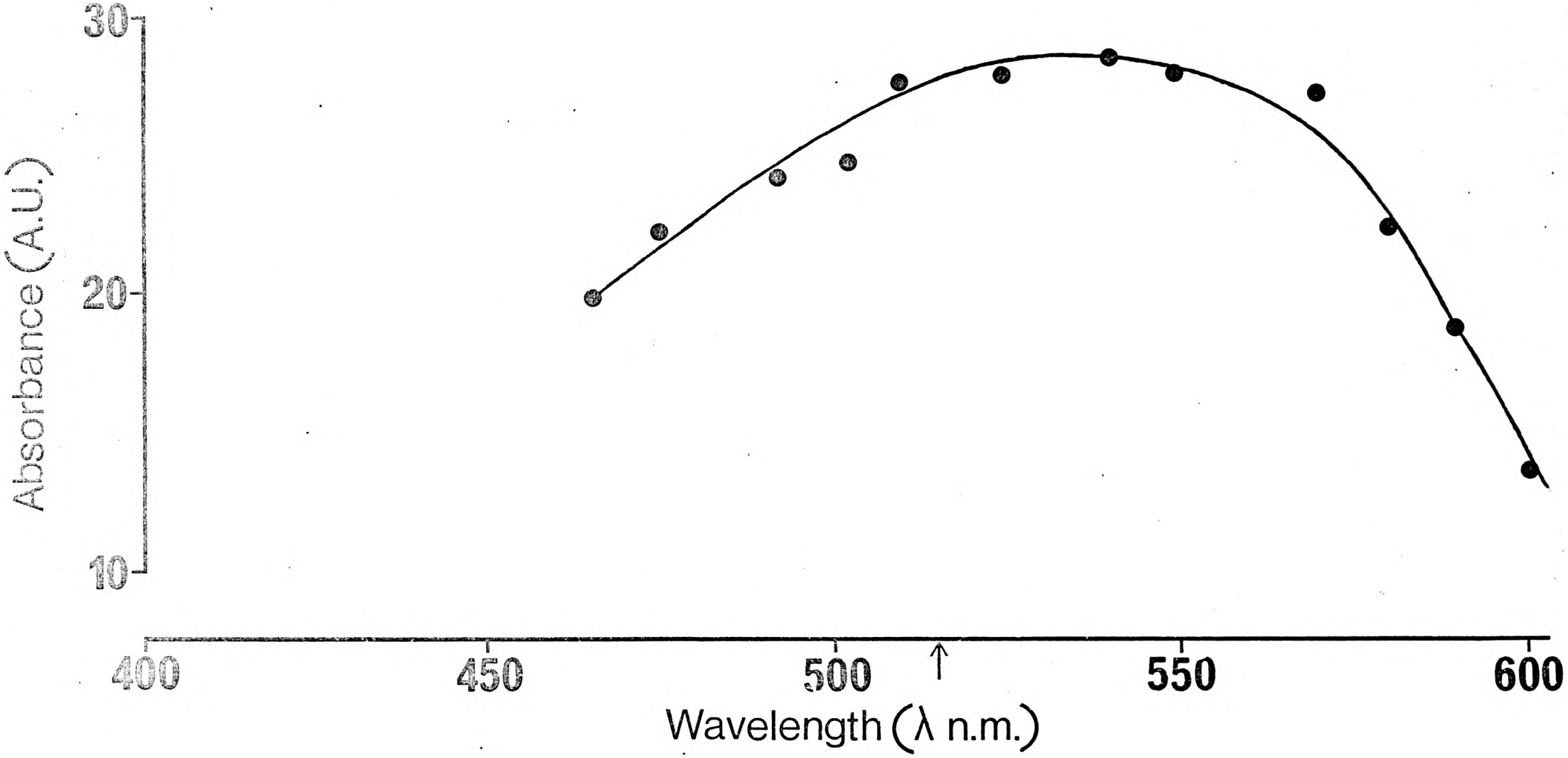

Fig 19 Absorbance profile for E.A.T. cells stained with Gallocyanin Chrome Alum

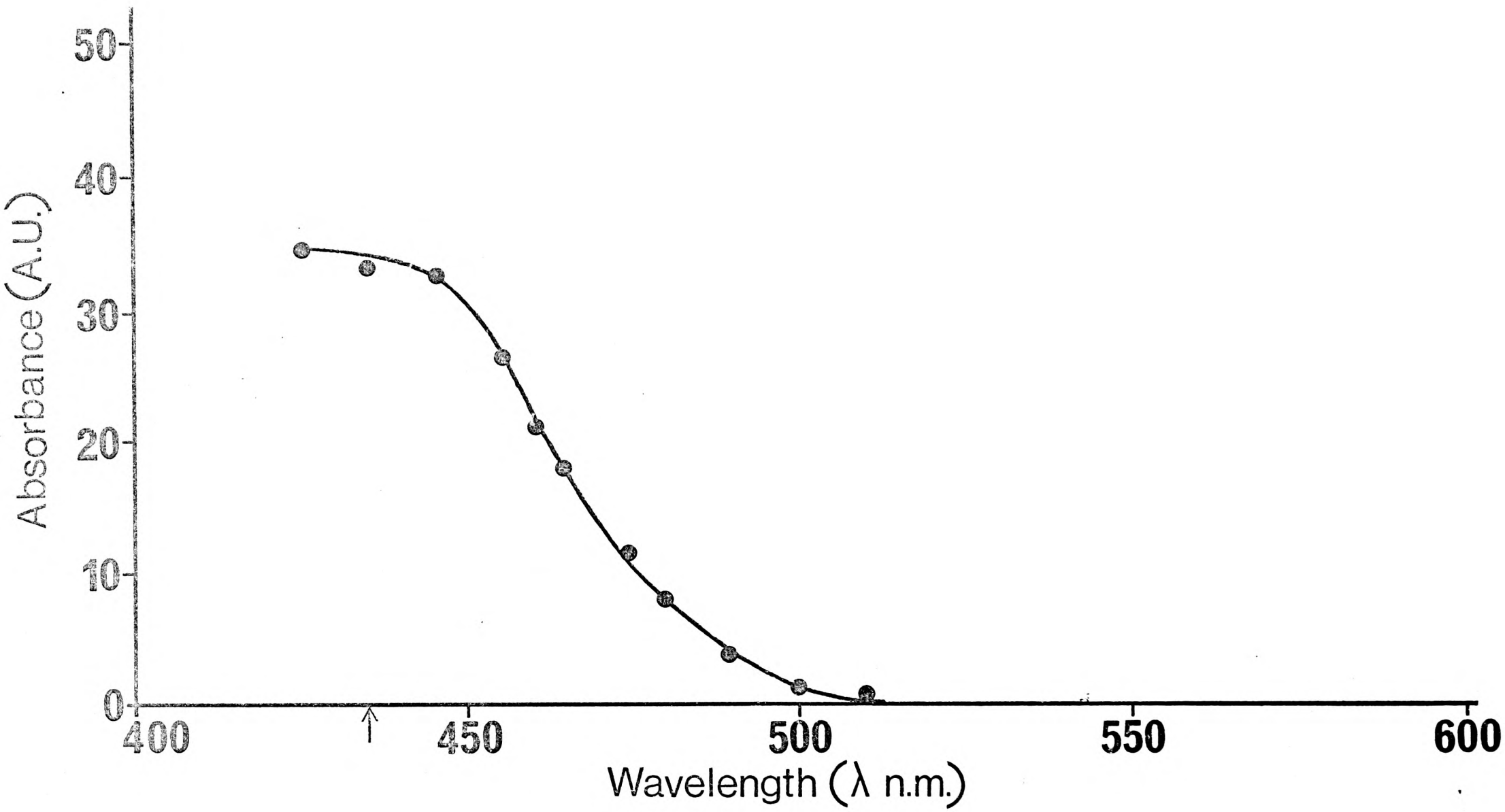

Fig 20 Absorbance profile for E.A.T. cells stained with Naphthol Yellow S

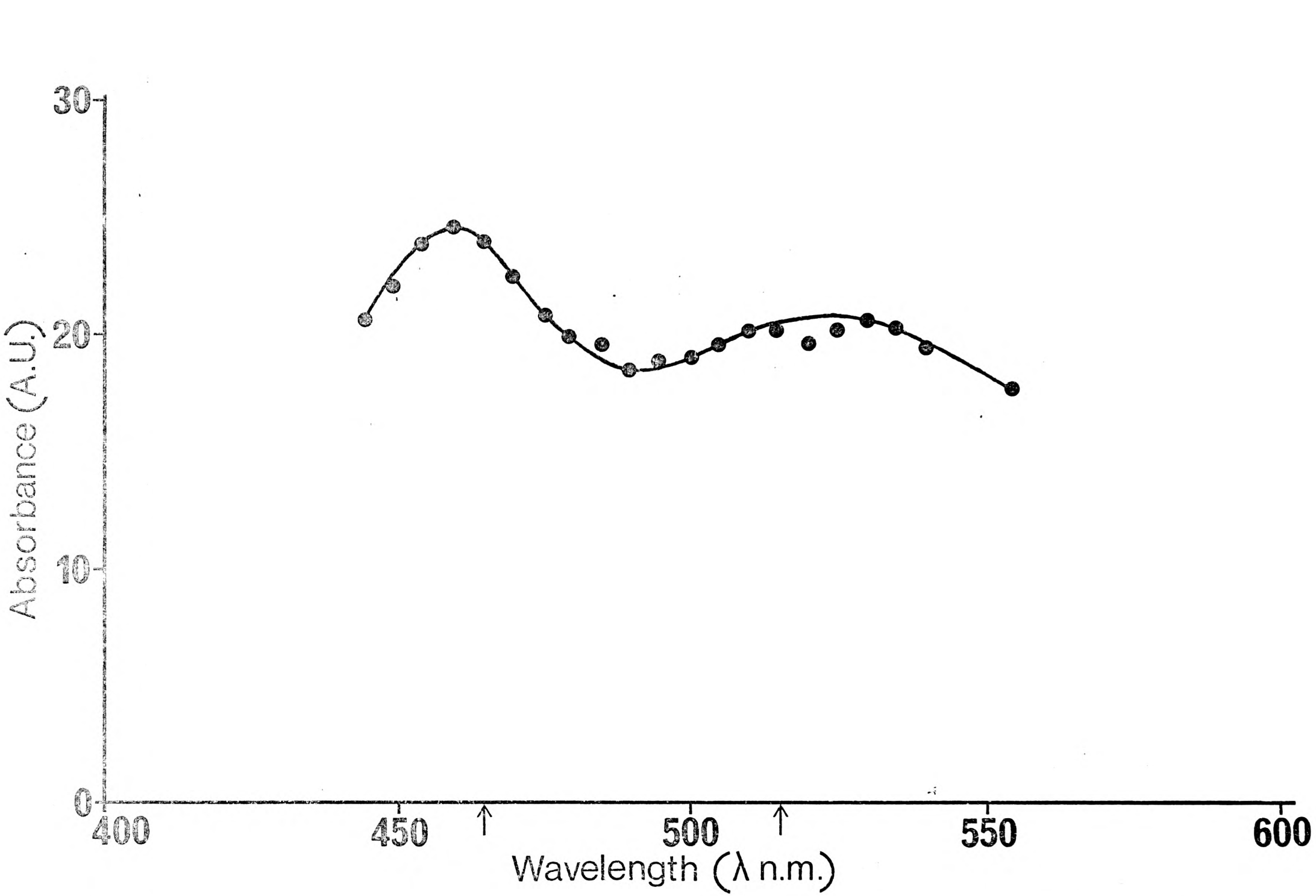

Fig 21 Absorbance profile for E.A.T. cells stained with Coriphosphin O and Gallocyanin Chrome Alum

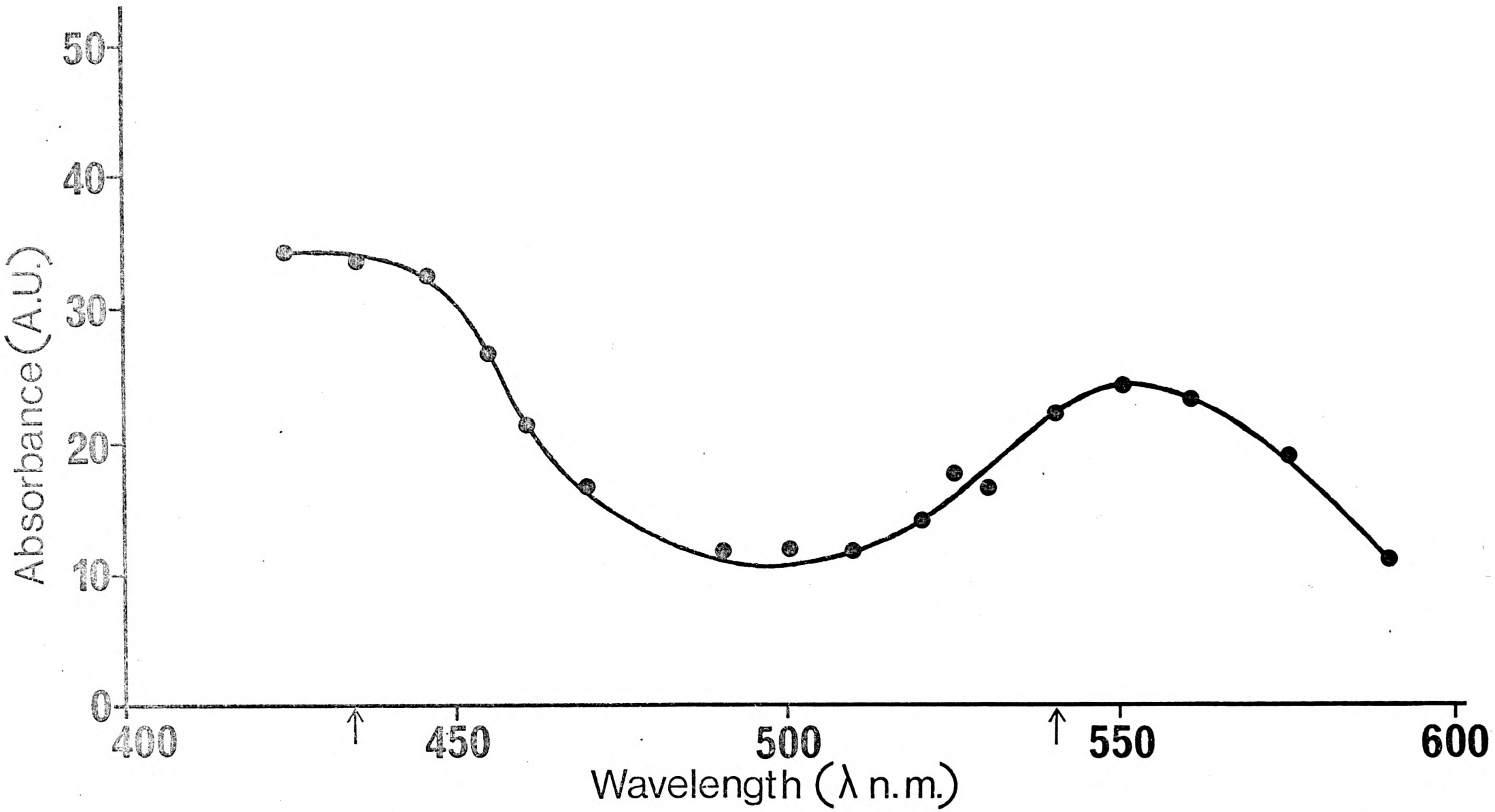

Fig 22 Absorbance profile for E.A.T. cells stained with Pararosaniline and Naphthol Yellow S

1958).

Estimation of individual cell DNA and RNA relative contents; system analysis: The factors f1, f2, and f3 represent the ratio of the optical density measured at the appropriate absorbance wavelength of GCA, coriphosphin O and GCA respectively to the optical density measured at the appropriate absorbance wavelength of coriphosphin O, GCA and coriphosphin O respectively. These ratios are for singly stained and in the case of f3, RNase treated and dually stained preparations, and are listed in table 4.

As expected, values obtained for the factors f1 and f3 show no tissue dependent variation (Kiefer et al. 1969) and accordingly the values indicated (table 4) have been applied consistently in all calculations.

The value for the factor f2 appears to be subject to variation. A significant difference between f2 values determined for normal peritoneal cells and EAT cells was observed. More importantly significant variation ($P<0.01$) in the factor f2 was observed between the different classes of tumour cells indicated. Discrimination between large and small tumour cells was made by a simple visual identification and in order to avoid bias, cell classification was recorded prior to performing optical density measurements.

On the basis that significant variation (Student's 't' test, $P<0.01$) exists between the mean f2 values determined for the groups indicated (table 4), application of the appropriate f2 value to calculations involving cells of a specific class or group was considered to be justified. Comparison of cells within the same class using appropriate f2 values should not present any special problems since the same factor has been applied throughout the said group. However, it should be noted that the results of trial calculations

Table 4

Determination of factors f1, f2 and f3 for computation of single cell DNA and RNA relative contents.

f1 = $\frac{OD\ 515}{OD\ 465}$	for Gallocyanin stained preparations	
f2 = $\frac{OD\ 465}{OD\ 515}$	for Coriphosphin O stained preparations	
f3 = $\frac{OD\ 515}{OD\ 465}$	for dually stained preparations, following RNase treatment.	
f1	(all cell types)	- 1.4 ± 0.1
f2	(mitoses, tumour)	- 5.7 ± 1.6
	(Small, tumour)	- 8.4 ± 0.8
	(Large, tumour)	- 7.4 ± 1.1
	(Normal peritoneal cells)	- 4.0 ± 0.8
f3	(all cell types)	- 0.7 ± 0.1

for the cell classes indicated, using a single average value for f2 across the board, varied from the presented results (fig. 25) by less than 2%.

Various cell types were stained with either coriphosphin O or pararosanilin using the Feulgen technique. The results of optical density measurements for comparable cell types are given in table 5. By comparison with pararosanilin staining the proposition is supported that total cellular coriphosphin O content provides a good measure of cellular DNA content.

Additional tests were made, similar to those devised by Kiefer et al. (1969) to compare the performance of coriphosphin O and GCA staining under single and dual staining conditions. The results of these tests are presented in tables 6a, 6b, 7, 8 and 9.

Tests pertaining to the performance of GCA staining (tables 7, 8 and 9) indicate a consistent reduction in optical density associated with DNA and RNA bound GCA under conditions of dual staining. The results of testing coriphosphin O under conditions of dual staining (table 6a) indicate a slightly variable performance. Subsequently, the relevant data for assessment of coriphosphin O performance under dual staining conditions were abstracted from a second experiment, the ultimate object of which was the measurement of DNA and RNA relative contents for cells labelled and unlabelled after repeat ^{3}HTdR labelling. The data derived from this second experiment, presented in table 6b, conform to the level of performance required from coriphosphin O under dual staining conditions.

Presentation of results of dual staining experiments: It is useful at this point to explain fully the format and rationale for the method of presentation and analysis of the cytophotometric data. The data presented in this chapter, excepting those which constitute a pre-

Preliminary microdensitometric analysis of dual staining method for determination of individual cell DNA and RNA contents

Table 5

Test 1: To assess the stoichiometric relationship between Coriphosphin O and DNA in the Feulgen reaction. Comparison with pararosanilin/Feulgen staining.

Cell type	Absorbance recorded at nm. AU.		
	Pararosanilin (λ 540nm)	Coriphosphin O (λ 465 nm)	OD540/OD 465
Normal mouse thymocytes	9.3	11.3	0.82
Normal peritoneal cells	9.3	11.6	0.80
Tumour cells:			
Mitotic figures	23.9	28.9	0.83
Interphase (2n)	12.1	15.6	0.78

Table 6a

Test 2: Coriphosphin O content for different cell types, with and without dual staining.

Cell type	Coriphosphin O content at 465 nm. AU.		
	*OD Cori O (465) (with GCA)	OD Cori O (465) (without GCA)	OD with/OD without
Normal mouse thymocytes	7.9	11.3	0.70
Normal peritoneal cells	7.2	11.6	0.62
Tumour cells			
Mitotic figures:	14.7	28.9	0.51
Interphase (2n)	7.6	15.6	0.49
Rat thymocytes	7.4	12.5	0.59

* calculated values

Table 6b

Test 2: Coriphosphin O content for different cell types, with and without dual staining. These data abstracted from experiment to determine DNA and RNA contents in repeating ^{3}HTdR labelled tumours.

Cell type	Coriphosphin O content at 465 nm. AU.		
	*OD Cori O (465)	OD Cori O (465)	OD with/OD without
Normal peritoneal cells	6.9	11.7	0.59
Tumour cells:			
Mitoses	20.4	31.4	0.65
Interphase (2n)	10.0	15.4	0.65

* calculated values.

Table 7

Test 3: DNA bound GCA content for different cell types with and without dual staining.

Cell type	Gallocyanin content at 515 nm. AU		
	*$OD_{GCA\ (515)}$ (with Cori O)	$OD_{GCA\ (515)}$ (without Cori O)	OD with/OD without
Normal mouse thymocytes	9.7	20.4	0.48
Normal peritoneal cells	10.1	22.4	0.45
Tumour cells:			
Mitotic figures	27.6	76.7	0.36
Interphase (2n)	13.4	41.9	0.32

* calculated values

Table 8

Test 4: RNA bound GCA content for different cell types with and without dual staining.

Cell type	Gallocyanin content at 515 nm. AU		
	*$OD_{GCA\ (515)}$ (with Cori O)	$OD_{GCA\ (515)}$ (without Cori O)	OD with/OD without
Normal mouse thymocytes	9.7	26.6	0.37
Rat thymocytes	14.4	54.2	0.27

In view of satisfactory correspondence obtained in test 5 and the fact that GCA is lost equally from DNA and RNA upon hydrolysis (Kiefer et al., 1969) tumour cells were not examined.

* calculated values

Table 9

Test 5: DNA and RNA bound GCA content for different cell types with and without dual staining.

Cell type	Gallocyanin content at 515 nm. AU		
	*$OD_{GCA\ (515)}$ (With Cori 0)	$OD_{GCA\ (515)}$ (without Cori 0)	$OD_{with}/OD_{without}$
Normal mouse thymocytes	8.2	27.8	0.29
Normal peritoneal cells	8.8	24.2	0.36
Tumour cells:			
Mitotic figures	24.0	103.3	0.23
Interphase (2n)	17.2	48.2	0.36

* calculated values.

liminary test, were derived from two basic experiments; determination of individual cell DNA and RNA and DNA and protein. In each case normal peritoneal cells found in the ascites were used as an internal standard. Those normal peritoneal cells which were located adjacent to a tumour cell of interest were measured and a sample of at least 15 normal peritoneal cells per slide was considered to be adequate. DNA, RNA and protein contents for individual tumour cells were subsequently normalized by an appropriate factor (Gill et al., 1978 and Raju et al., 1980).

This manipulation of raw data may seem to be an unnecessary complication. However, this procedure serves to enhance the accuracy of the technique. Cytochemical preparations are notoriously difficult to reproduce even when stained simultaneously, and variation in different parts of the same sample is not unknown. In the present experiments it was necessary to pool data from at least 5 different smear preparations for the RNA determinations and 11 preparations for the protein determination.

DNA data from all experiments are plotted in histogram form (figs. 23, 24, 26, 27, 29 and 31) and combined DNA and RNA and DNA and protein data are presented in scattergram form (figs. 25, 28, 30 and 32 respectively). Further conversion of the scattergram data to DNA/RNA and DNA/protein ratios was necessary to allow statistical analysis. In this way various cell groups were examined for the existence of variation between the observed DNA/RNA or DNA/protein ratios. Two types of statistical analysis were used for this: Student's 't' test and the Mann Whitney 'U' test, (Siegel, 1956 and Clarke, 1969a).

Determination of individual cell DNA and RNA contents for unlabelled cells, labelled cells and mitotic figures following repeat ^{3}HTdR labelling: The DNA distribution, determined by the method of Kiefer

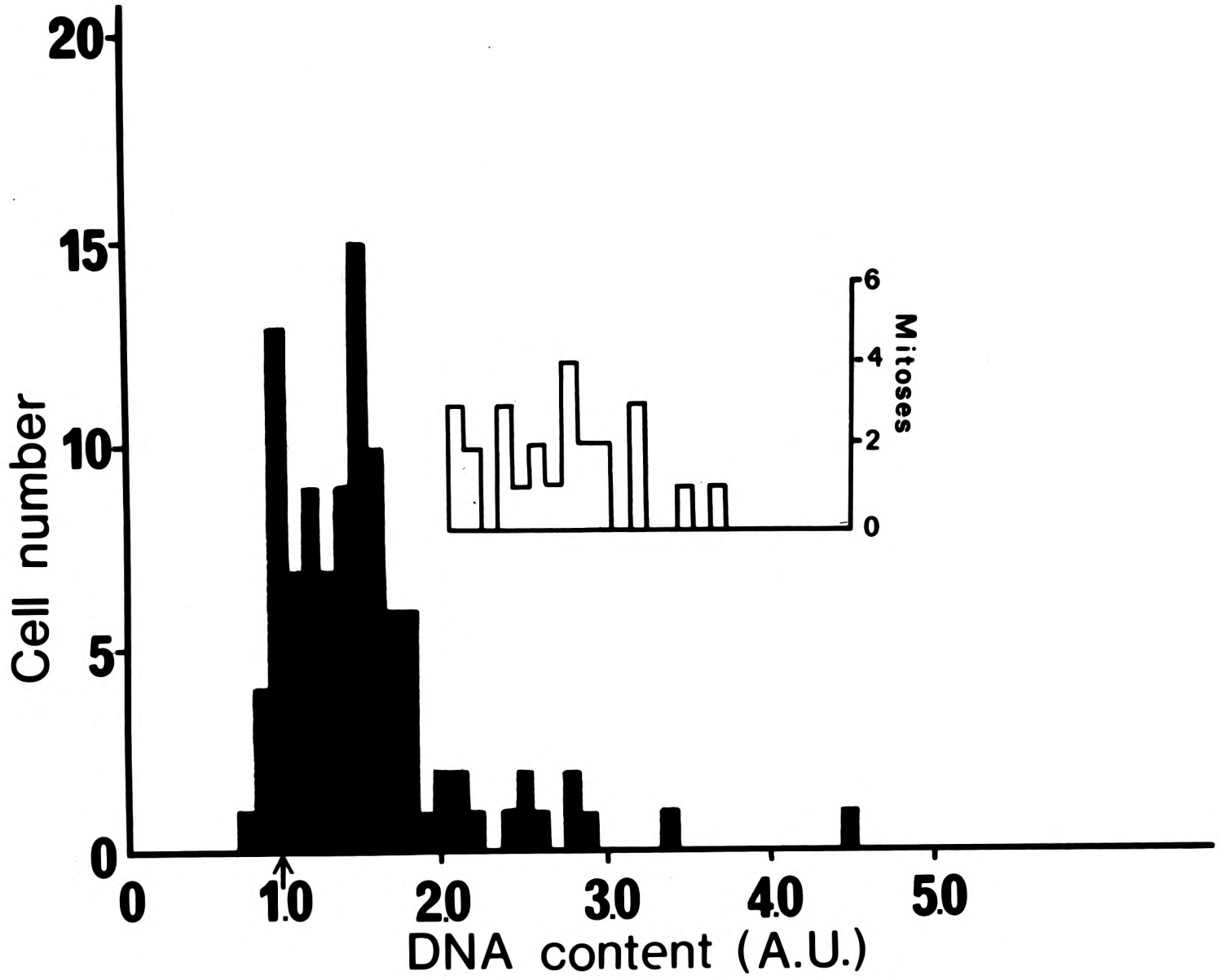

Fig 23 DNA distribution of 100 labelled tumour cells following repeat ^{3}HTdR labelling of a 12 day E.A.T. and DNA distribution of 25 mitoses from same tumour samples

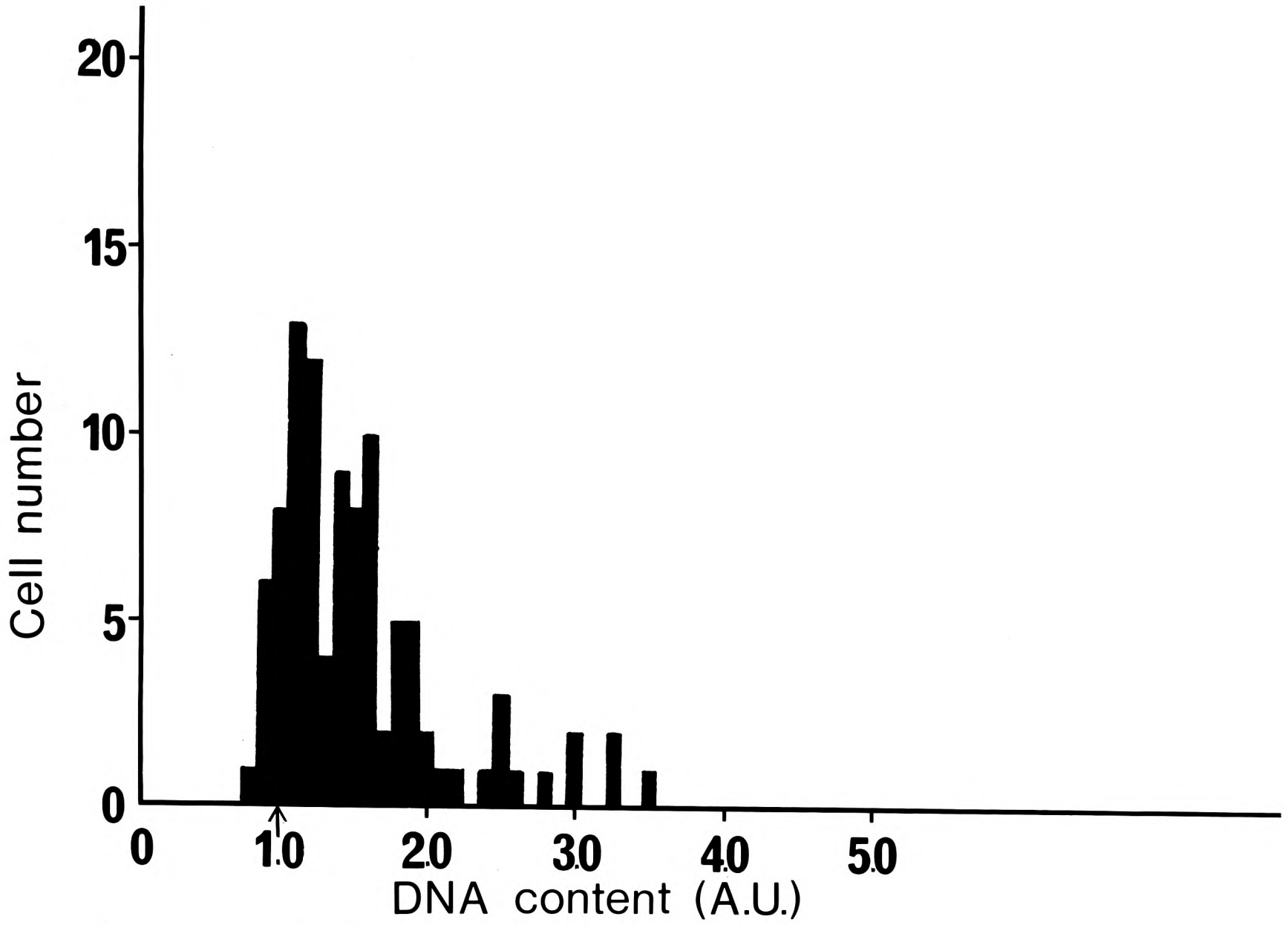

Fig 24 DNA distribution of 100 unlabelled tumour cells following repeat ^{3}HTdR labelling of a 12 day E.A.T.

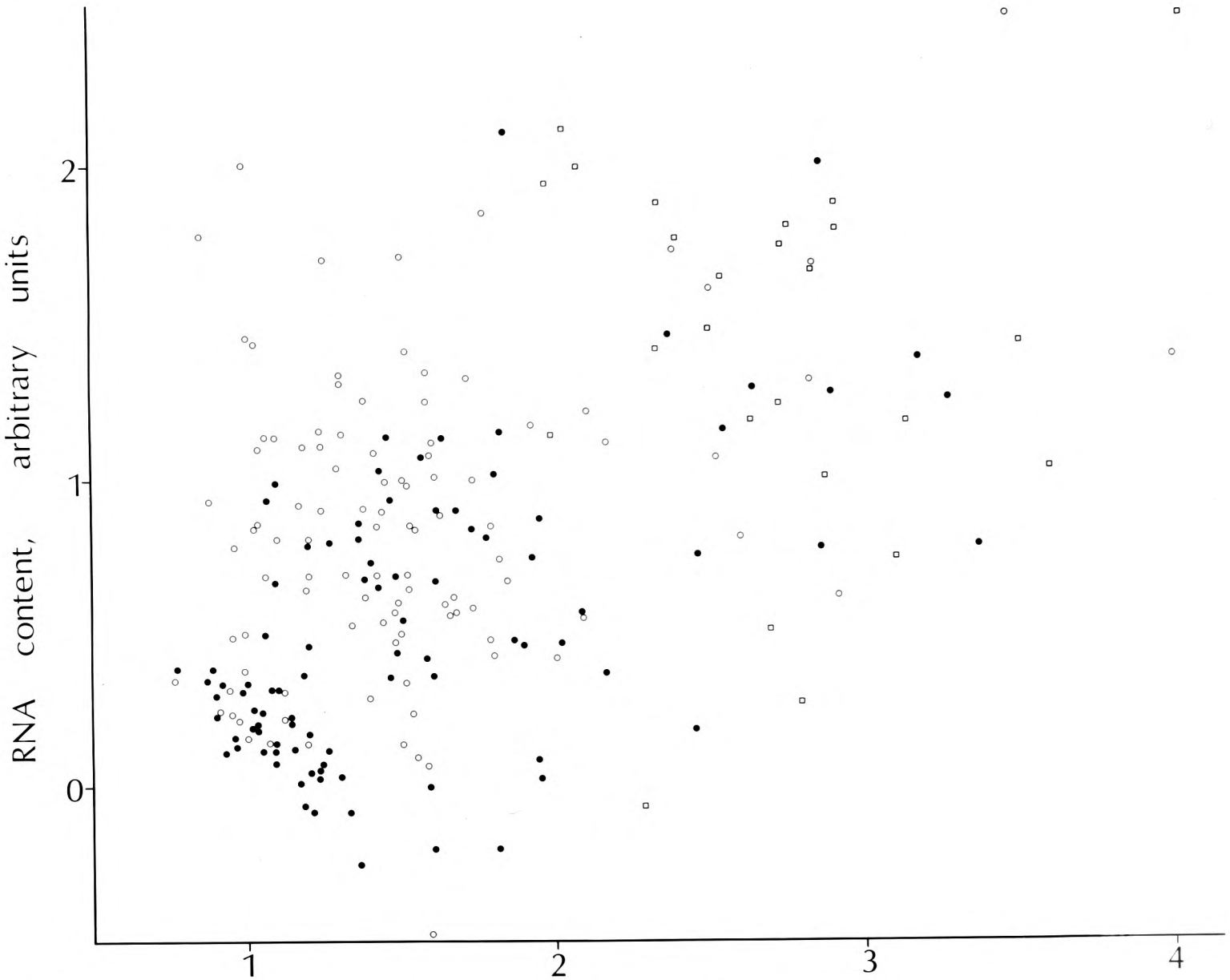

Fig. 25

Correlation of individual cell DNA and RNA content for G_o cells •, Labelled cells ◦, and Mitotic figures ▫.

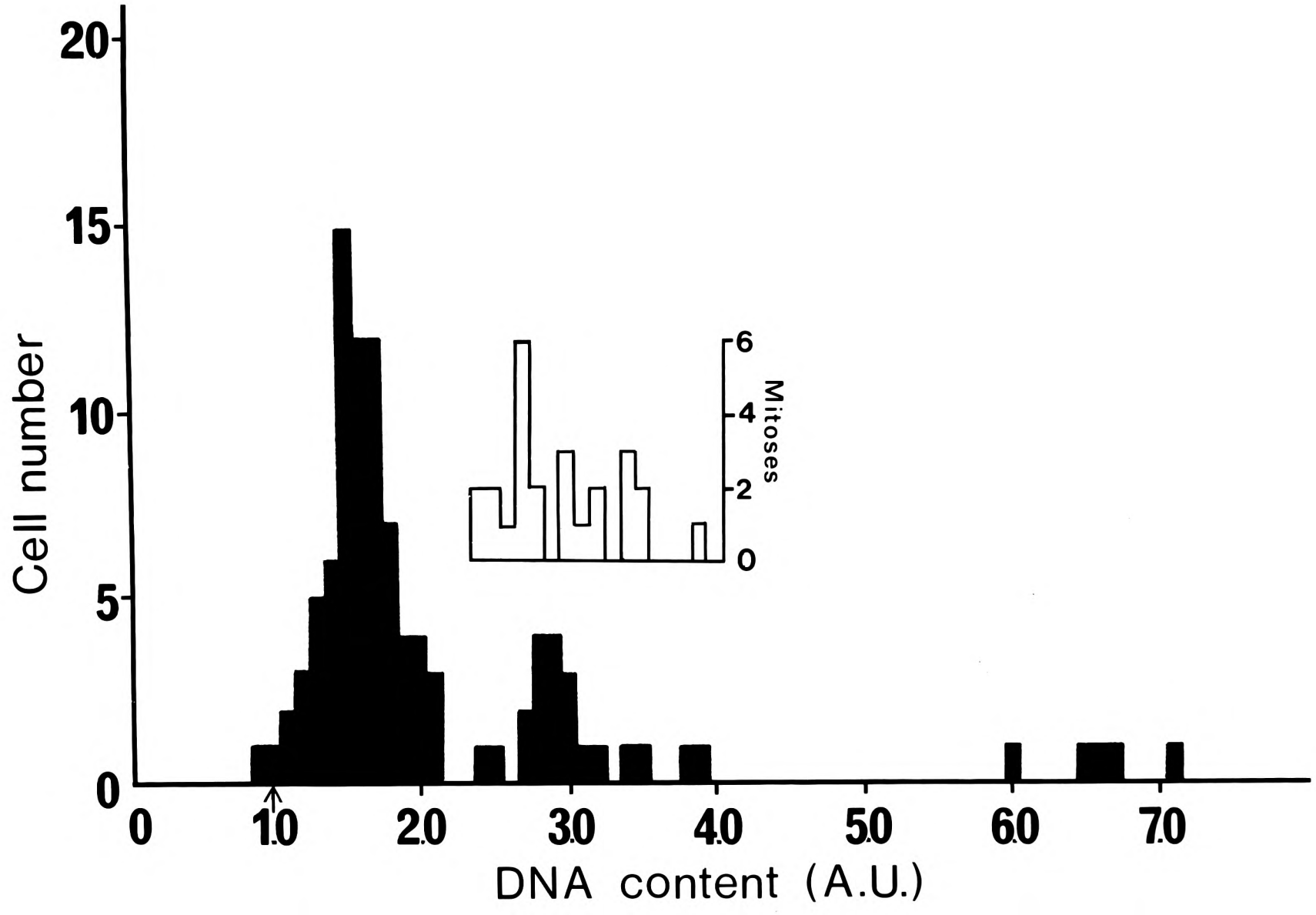

Fig 26 DNA distribution of 100 unlabelled tumour cells following pulse ^{3}HTdR labelling of a 12 day E.A.T. and DNA distribution of 25 mitoses from same tumour samples.

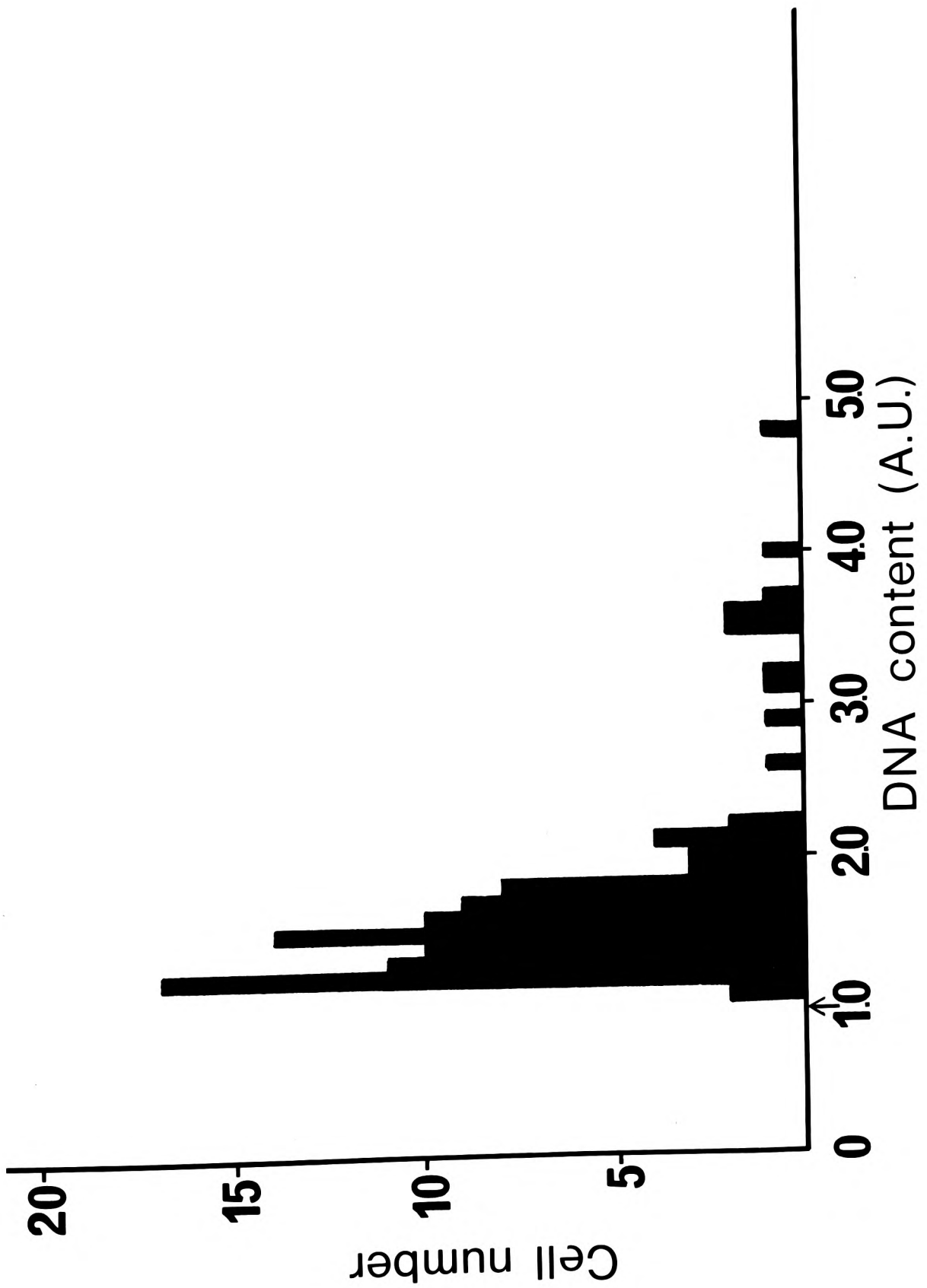

Fig 27 DNA distribution of 104 unlabelled tumour cells following repeat ^{3}HTdR labelling of a 12 day E.A.T.

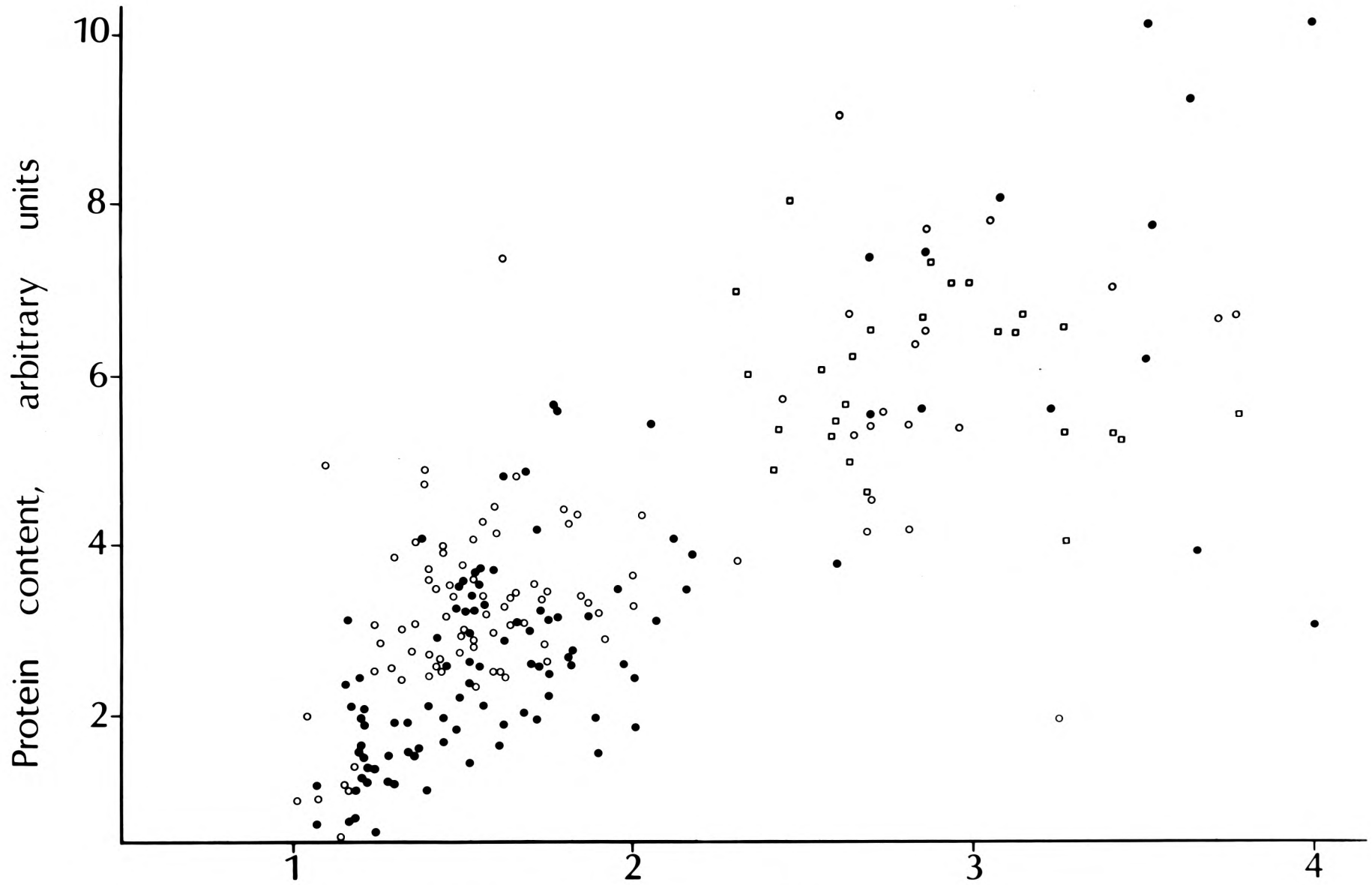

Fig. 28

Correlation of individual cell DNA and Protein content for G_0 cells •, Unlabelled interphase cells◦, and Mitotic figures ▫.

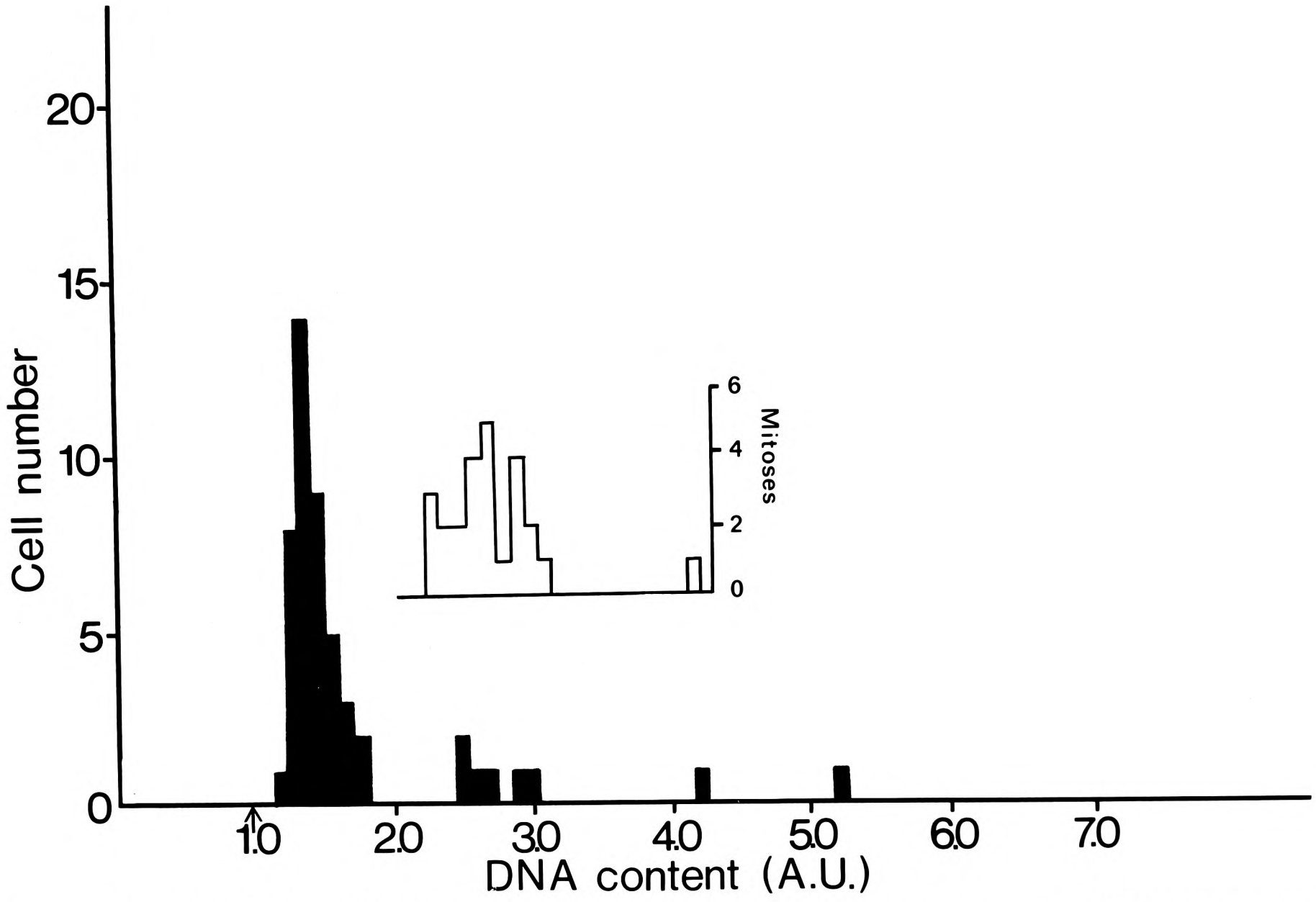

Fig 29 DNA distribution of 50 unlabelled tumour cells following pulse ^{3}HTdR labelling of a 5 day E.A.T. and DNA distribution of 25 mitoses from same tumour sample.

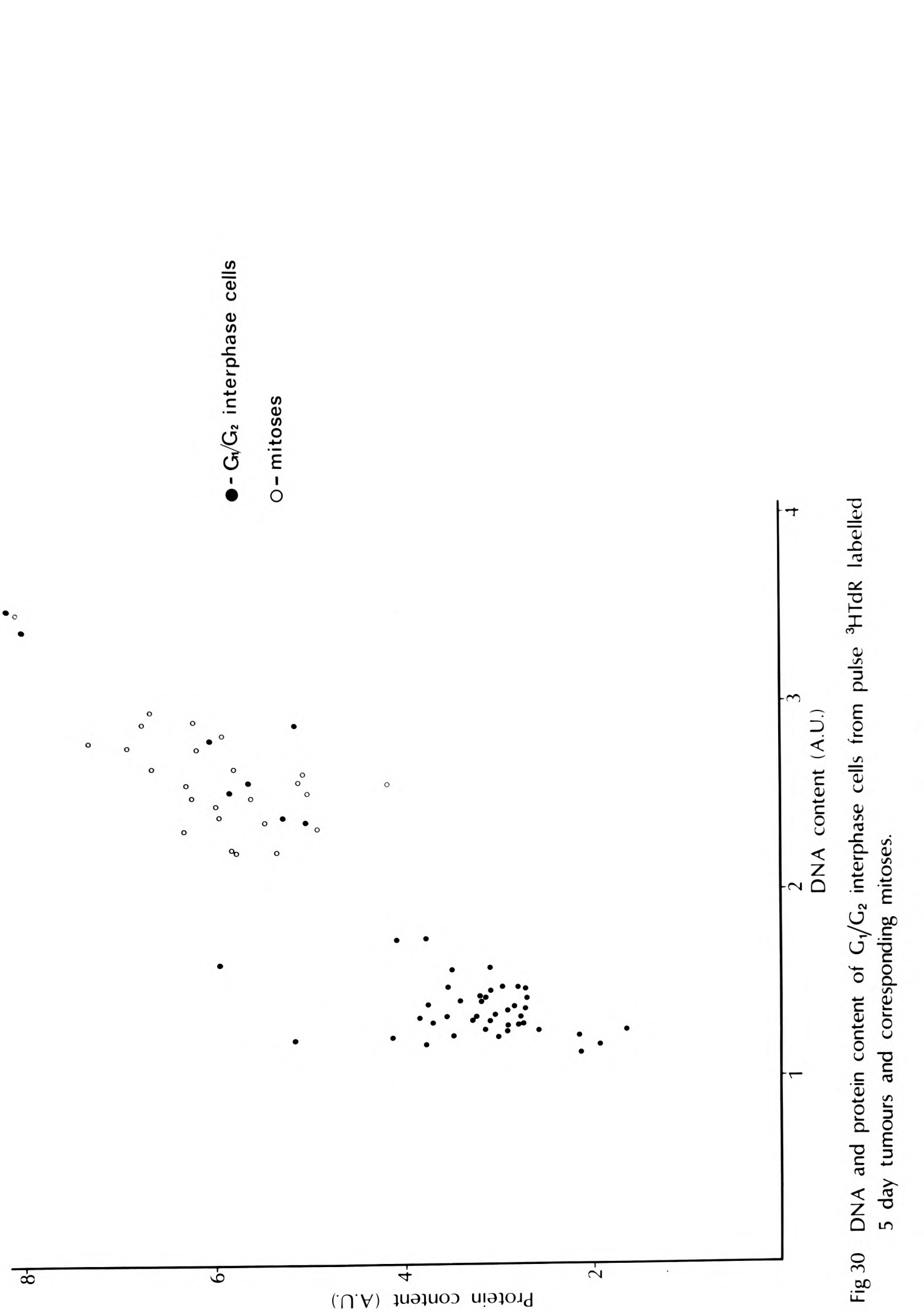

Fig 30 DNA and protein content of G_1/G_2 interphase cells from pulse ^{3}HTdR labelled 5 day tumours and corresponding mitoses.

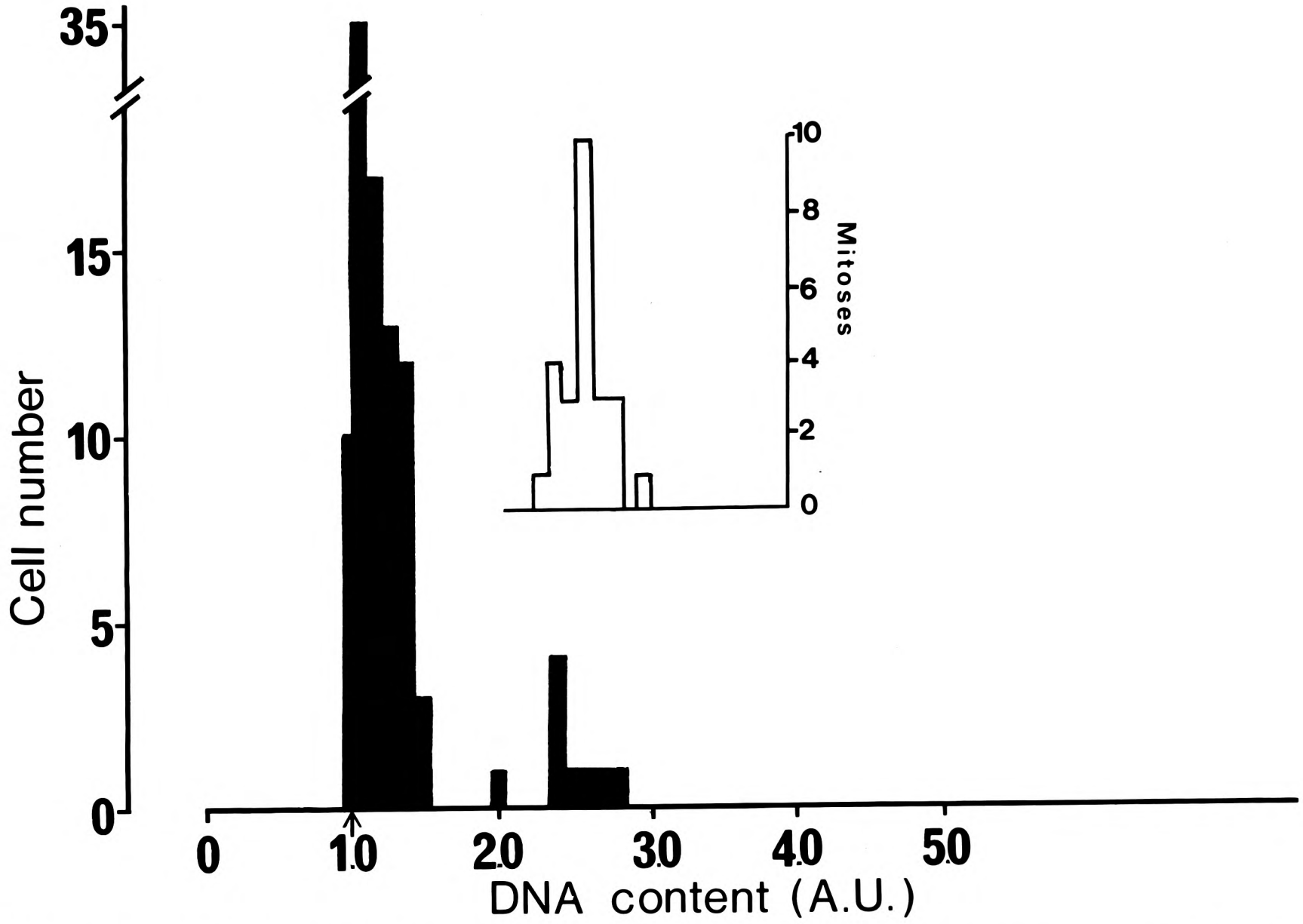

Fig 31 DNA distribution of 100 unlabelled tumour cells following pulse ^{3}HTdR labelling of a 2 day E.A.T. and DNA distribution of 25 mitoses from same tumour samples

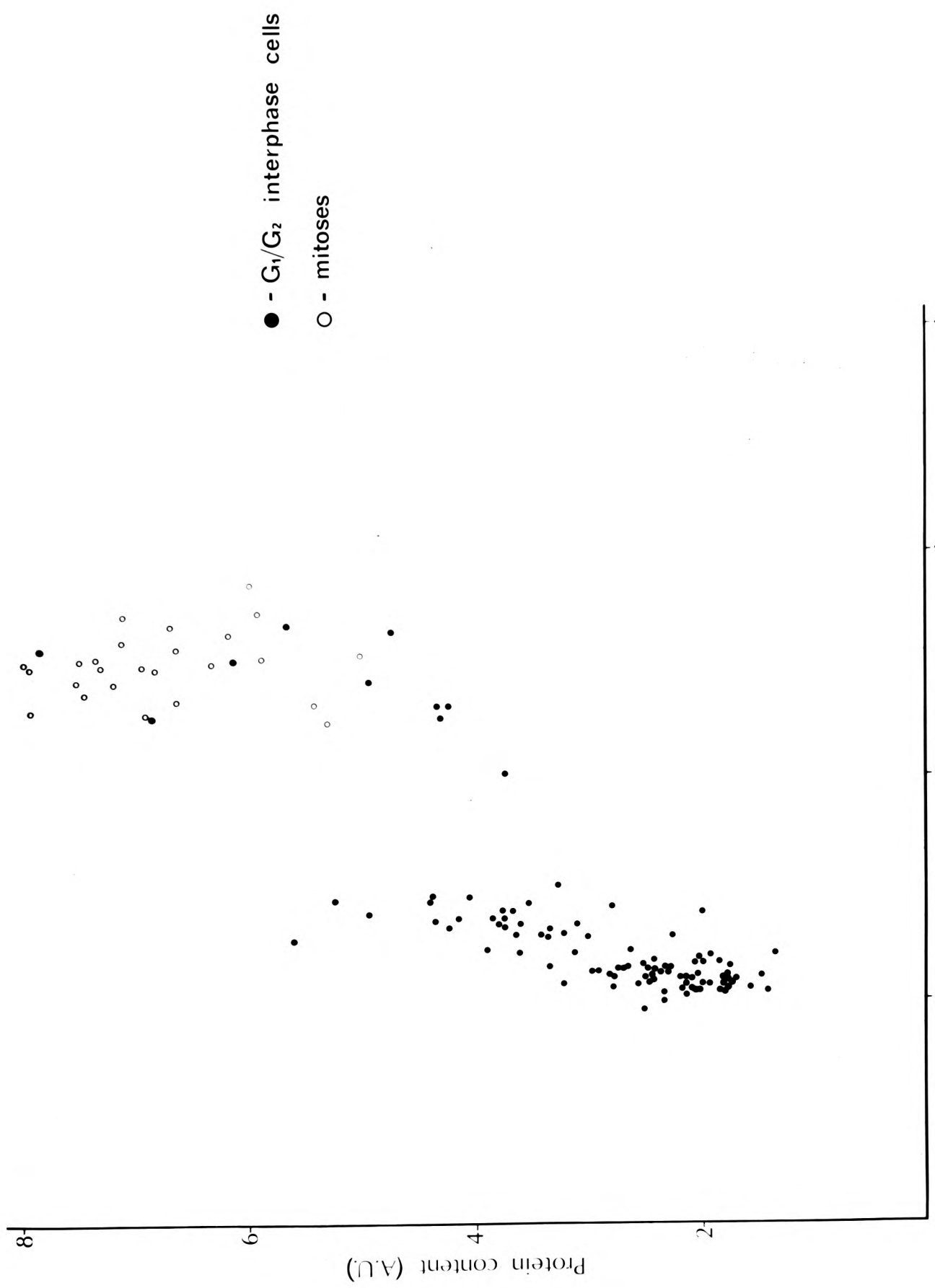

Fig 32 DNA and protein contents of G_1/G_2 interphase cells from pulse ^{3}HTdR labelled 2 day tumours and corresponding mitoses.

et al. (1969), for labelled cells and mitotic figures following repeat ^{3}HTdR labelling appears as a histogram (fig. 23). The DNA distribution here is broad, being representative of cells distributed throughout all phases of cell cycle (excluding mitosis), although a preponderance of cells appear to be of G_1 - phase DNA content. The DNA distribution for the corresponding unlabelled fraction of cells (fig. 24) all of which are G_o cells, also shows a considerable spread. However, the DNA distribution here is skewed further to the left than that for the labelled fraction. The mean DNA content for normal peritoneal cells is denoted by an arrow on the base line of figs. 23 and 24.

The combined DNA and RNA data for unlabelled cells (G_o), labelled cells (mostly cycling cells but all of which have synthesized DNA within the previous 90 hr.) and mitotic figures are presented as a scattergram of DNA vs RNA (fig. 25). This scattergram is the pooled measurements made for 5 repeat ^{3}HTdR labelled 12 day EATs. Each point on the scattergram represents the relative DNA and RNA contents of one cell. The G_o cells can be seen to occupy the lower area of this diagram; 59% of G_o cells have RNA values of less than 0.5 arbitrary units whereas only 25% of labelled cells fall into this zone. In addition, 37% of labelled cells have RNA contents of greater than 1.0 arbitrary units and only 15% of G_o cells (half of which are of G_2 DNA content) have as much RNA.

In order to facilitate assimilation of the data contained in fig. 25 and provide a system of valid comparisons, DNA/RNA ratios were derived for each cell. The mean ratios together with sample size are indicated in table 10. Naturally, no ratio was determined for cells which registered a negative RNA content, and such cells were therefore excluded from statistical analysis, table 12. Negative RNA values were

derived for 8 cells; 6 G_o, 1 labelled and 1 mitotic figure. If it were possible to include these 8 cells in the statistical analysis then enhancement of separation of the G_o cells from the rest of the cell population would result.

The mean DNA/RNA ratio for G_o cells is considerably greater than that for either the labelled fraction or the mitotic cells. Reference to table 11 indicates a highly significant difference between the G_o population and the other groups and no significant difference exists between the labelled fraction and the mitotic cells. Further comparison of G_o cells, of DNA content of less than 1.35 arbitrary units with labelled cells of the same DNA content again reveals a highly significant difference between the mean DNA/RNA ratio determined for these groups. Even when all G_o cells which have DNA/RNA ratios of greater than 10 are excluded from the analysis, the remaining G_o cells retain an average DNA/RNA ratio of 4.4 ($\pm$ 2.4) which is significantly greater than that for the other groups ($P<0.01$).

Determination of individual cell DNA and protein contents for cells of differing proliferative status: This second cytophotometric analysis examines DNA and protein relative contents for 7 cell types after either pulse or repeat ^{3}HTdR labelling. These cell types are: rapidly cycling cells with G_1 and G_2 DNA contents (unlabelled after pulse labelling at 2 days of tumour growth), two populations containing different proportions of cycling G_+ and G_o cells with G_1 and G_2 DNA contents (unlabelled after pulse labelling at 5 and 12 days of tumour growth), a population of pure G_o cells (unlabelled after repeat ^{3}HTdR labelling at 12 days of tumour growth) and mitotic figures from each pulse labelled preparation.

The DNA distribution, determined according to the dual staining method of Deitch (1966) for unlabelled cells and mitotic figures after

Table 10

Summary of data pertaining to DNA/RNA ratios for tumour cells after repeat ^{3}HTdR labelling at 12 days.

Cell type	DNA/RNA ratio (±SE)	Sample size (Cell No.)
Unlabelled (G_c)	6.60 (±10.9)	85
Unlabelled (DNA content <1.35 AU)	8.79 (±11.6)	41
Labelled	2.75 (±3.3)	102
Labelled (DNA content <1.35 AU)	2.21 (±2.0)	41
Mitoses	2.47 (±2.1)	25

Table 11

Statistical analysis of data presented in table 10.

Comparison of mean DNA/RNA ratios for the following cell types:	Result of statistical analysis	
	Mann Whitney 'U' test	Student's 't' test
G_o vs. Labelled	$P < 0.001$	$P < 0.001$
G_o (DNA content < 1.35 AU) vs. Labelled (DNA content < 1.35 AU)	$P < 0.001$	$P < 0.001$
Labelled vs. Mitoses	*$P > 0.05$	*$P > 0.1$
G_o vs. Mitoses	$P < 0.001$	$P < 0.001$

* No reason to reject null hypothesis

pulse labelling of 12 day tumours are given in fig. 26. The corresponding DNA distribution for G_o cells is given in fig. 27. The features of note here include a broad distribution of DNA contents associated with the aneuploid state; the DNA distribution for G_o cells is slightly skewed to the left relative to fig. 26 but the pulse labelled 12 day distribution includes a higher proportion of G_2 cells. Arrows on the base lines of figs. 26 and 27 show the mean DNA content of normal peritoneal cells.

Combined DNA and protein data for the groups of cells defined in the histograms (figs. 26 and 27) are expressed in fig. 28 as a DNA vs protein scattergram. Each point in fig. 28 represents the DNA and protein content for a single cell. This scattergram contains the pooled data from 5 tumours. In this case protein values determined for G_o cells are clearly lower in comparison with those determined for 12 day interphase (G_1/G_2) cells (a proportion of which is likely to be in G_o) and mitotic figures.

The DNA distribution for unlabelled cells and mitotic figures after pulse labelling of three 5 day tumours is shown in fig. 29. There is a general bimodal trend indicating a broad distribution of cells with G_1 DNA and rather fewer cells which correlate with the mitotic DNA level and are most probably G_2 cells. The mitoses in particular demonstrate a cell system with a high degree of aneuploidy. The narrowed G_1 peak here as compared with that of the corresponding 12 day tumour is possibly the result of a reduced sample size.

The DNA distribution for unlabelled cells and mitoses after pulse labelling of three 2 day tumours is shown in fig. 31. There is a bimodal distribution of cells. The major peak lies at the hyperdiploid DNA level and the minor peak corresponds to the DNA level observed for mitotic figures. This indicates that the population of G_1 cells is

larger than that of G_2 cells. The width of both the G_1 and mitotic peaks is indicative of aneuploidy. However, the distribution of DNA contents observed for the 2 day tumour (fig. 31) is considerably less than that observed for the 5 day tumour or the 12 day tumour.

The DNA data presented in figs. 29 and 31 are combined with corresponding protein data as scattergrams (figs. 30 and 32). Each scattergram contains the pooled data from either three 2 day or three 5 day, pulse labelled tumours, each point being representative of the DNA and protein content of one cell. The points marked for the 2 day tumour are skewed to the left as demonstrated by the corresponding DNA histograms, (figs. 29 and 31). The mean protein content for 2 day tumour cells ($3.0^{+}_{-}1.2$) is less than that for 5 day tumour cells ($3.7^{+}_{-}1.6$). However, because of the skewed DNA distributions measured for 2 day tumour cells there is no apparent variation in the DNA/protein ratios for 2 and 5 day interphase cells (table 12). The values recorded for the protein contents of mitotic figures in the 2 day tumour are greater than those for the 5 day tumour. This is evident from the mean DNA/protein ratios (table 12).

DNA/protein ratios were determined for the cells represented in figs. 28, 30 and 32. The mean DNA/protein ratios for 2, 5 and 12 day G_1/G_2 cells, mitotic figures and G_o cells are presented in table 12. There appears to be little variation between the DNA/protein ratios obtained for G_1/G_2 cells at various stages of tumour growth. This is confirmed by the results of statistical analysis presented in table 13. The DNA/protein ratios obtained for mitotic figures increase with increasing tumour age and significant variation between 2 day mitoses and all other cell classes is apparent. The DNA/protein ratios for 5 day and 12 day mitoses are not significantly different from any other class of interphase cell, with the possible exception of

Table 12

Summary of data pertaining to DNA/protein ratios for tumour cells after repeat ^{3}HTdR labelling at 12 days and pulse ^{3}HTdR labelling at 12, 5 and 2 days.

Cell group	DNA/protein ratio (±SE)	Sample size (Cell No.)
G_o (Unlabelled after repeat labelling at 12 days)	0.70 (± .32)	104
Unlabelled (after pulse labelling at 12 days)	0.57 (± .34)	100
Mitoses 12 day	0.50 (± .13)	25
Unlabelled (after pulse labelling at 5 days)	0.45 (± .09)	50
Mitoses 5 day	0.44 (± .06)	25
Unlabelled (after pulse labelling at 2 days)	0.47 (±0.12)	100
Mitoses 2 day	0.37 (±0.06)	25

Table 13

Statistical analysis of data presented in table 12

Statistical comparisons of the listed cell types (DNA/protein ratios)	G_o	G_1/G_2 (2)	G_1/G_2 (5)	G_1/G_2 (12)	Mitoses (2)	Mitoses (5)	Mitoses (12)
G_o		P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001
G_1/G_1 (2)	P<0.001		*P>0.1	*P>0.02	P<0.001	*P>0.1	*P>0.1
G_1/G_2 (5)	P<0.001	*P>0.1		*P>0.02	P<0.001	*P>0.1	*P>0.08
G_1/G_2 (12)	P<0.001	P<0.01	*P>0.02		P<0.001	P<0.002	*P>0.1
Mitoses (2)	P<0.001	P<0.001	P<0.001	P<0.001		P<0.001	P<0.001
Mitoses (5)	P<0.001	*P>0.1	*P>0.1	*P>0.05	P<0.001		*P>0.03
Mitoses (12)	P<0.001	*P>0.1	*P>0.1	*P>0.1	P<0.001	*P>0.05	

Top right hand side represents results of Mann Whitney 'U' test.

Bottom left hand side represents results of Student's 't' test.

* No reason to reject null hypothesis.

G_1/G_2 ≡ Cells unlabelled after pulse ^{3}HTdR labelling of a 2, 5 or 12 day tumour as indicated.

variation between 5 day mitoses and 12 day G_1/G_2 cells. The highest DNA/protein ratio of 0.70 ($\pm$0.32) was obtained for G_o cells. This value is significantly greater ($P<0.001$) than the DNA/protein ratios determined for G_1/G_2 cells or mitotic figures at any stage of tumour growth.

The results of variance analysis of the data shown in figs. 26, 29 and 31, which depict the DNA distributions of G_1 interphase cells and mitotic figures, are given in table 14. Comparison of the DNA distributions for 2, 5 and 12 day tumours indicates an accumulation of cells with heteroploid DNA content in the plateau phase of tumour growth.

The results presented in table 15 were calculated from figs. 26 and 31. The distribution of cells within the various compartments of cell cycle are shown for a log and a plateau phase tumour. An accumulation of cells within the G_1 and G_2 phases of cell cycle during the plateau phase of tumour growth, at the expense of the S-phase and mitotic phase populations is evident. However, reference to the histograms (figs. 26 and 31) tends to suggest that there is sometimes difficulty in ascribing a cell to the G_1 or G_2 phase owing to the large heteroploid presence, particularly in the 12 day tumour.

Flow cytofluorimetric analysis of acridine orange stained plateau phase EAT cells: Figures 33a, 33b, and 33c represent light scatter vs. green, light scatter vs. red and green vs. red fluorescence respectively. There appears to be a broad distribution of DNA and RNA contents, and some suggestion of groupings. However, none of these data indicate existence within this tumour of a sub-population of cells which are biochemically distinct.

Table 14

Determination of variance ratios to compare the DNA distributions of G_1 interphase cells and mitotic figures at 2, 5 and 12 days of tumour growth. (Data contained in figs. 26, 29 and 31).

Comparison of variance between DNA distributions for G_1 interphase cells derived from 2, 5 and 12 day tumours.

2	vs	5 day	*P>0.1
5	vs	12 day	P<0.005
2	vs	12 day	P<0.005

Comparison of variance between DNA distribution for mitotic figures derived from 2, 5 and 12 day tumours.

2	vs	5 day	*P>0.1
5	vs	12 day	P<0.005
2	vs	12 day	P<0.005

* No reason to reject null hypothesis.

Table 15

Distribution of cells in various compartments of cell cycle.

Tumour age	% Cells			
	G_1	G_2	S	M
2 day	34.4	3.8	59.1	2.7
13 day	50.8	14.9	32.8	1.5

Values for S are given in vivo labelling indices.

Values for M are given by in vivo mitotic indices.

Values for G_1 and G_2 are given by Feulgen microdensitometric estimation of single cell DNA contents.

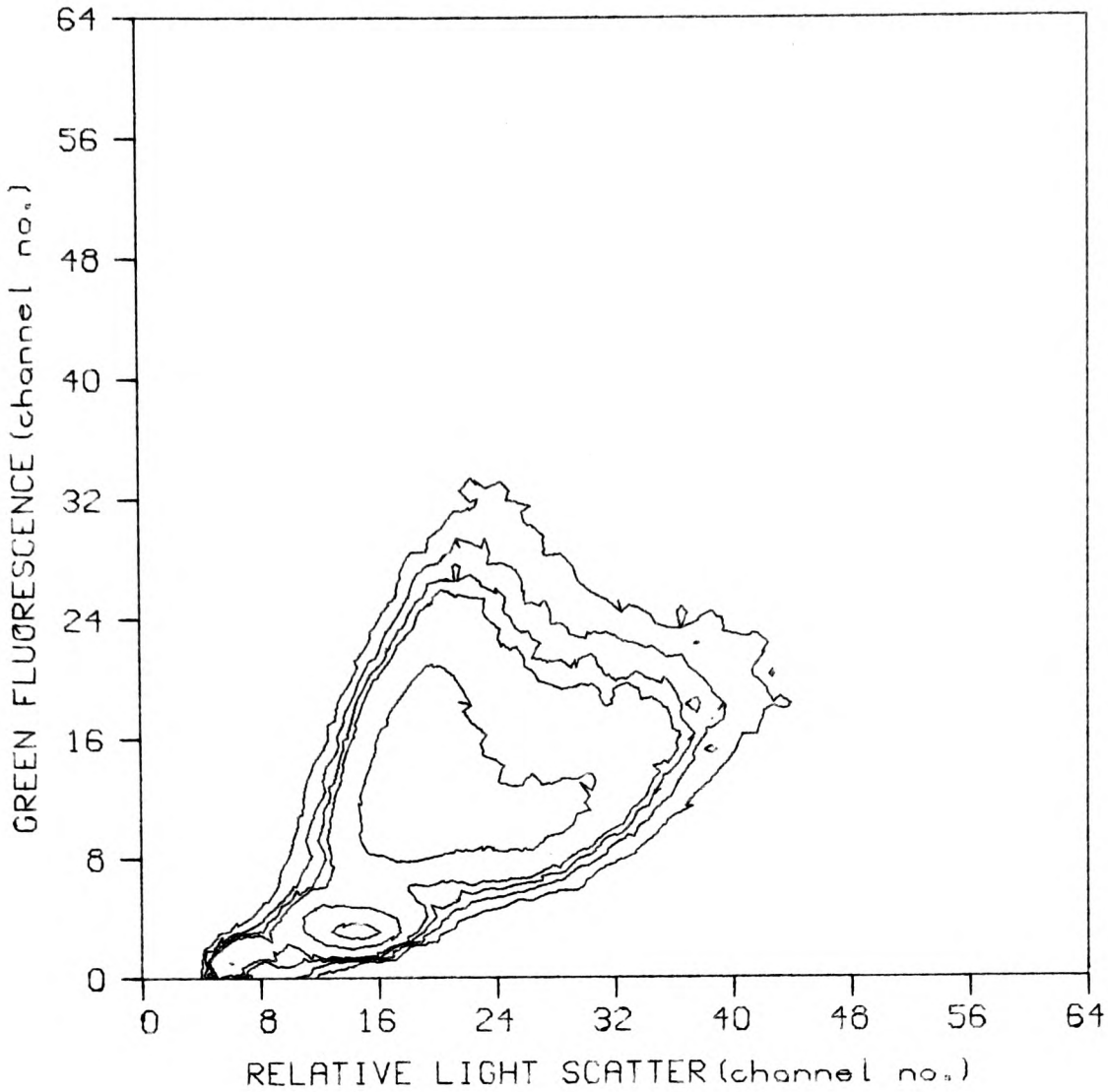

Fig. 33a **Contour display of an acridine orange stained sample of EAT cells. The contour levels range from 25 cells (outer trace) to 500 cells (inner trace). Green fluorescence (measured at 515 - 575 nm) is plotted as a function of light scatter (cell size).**

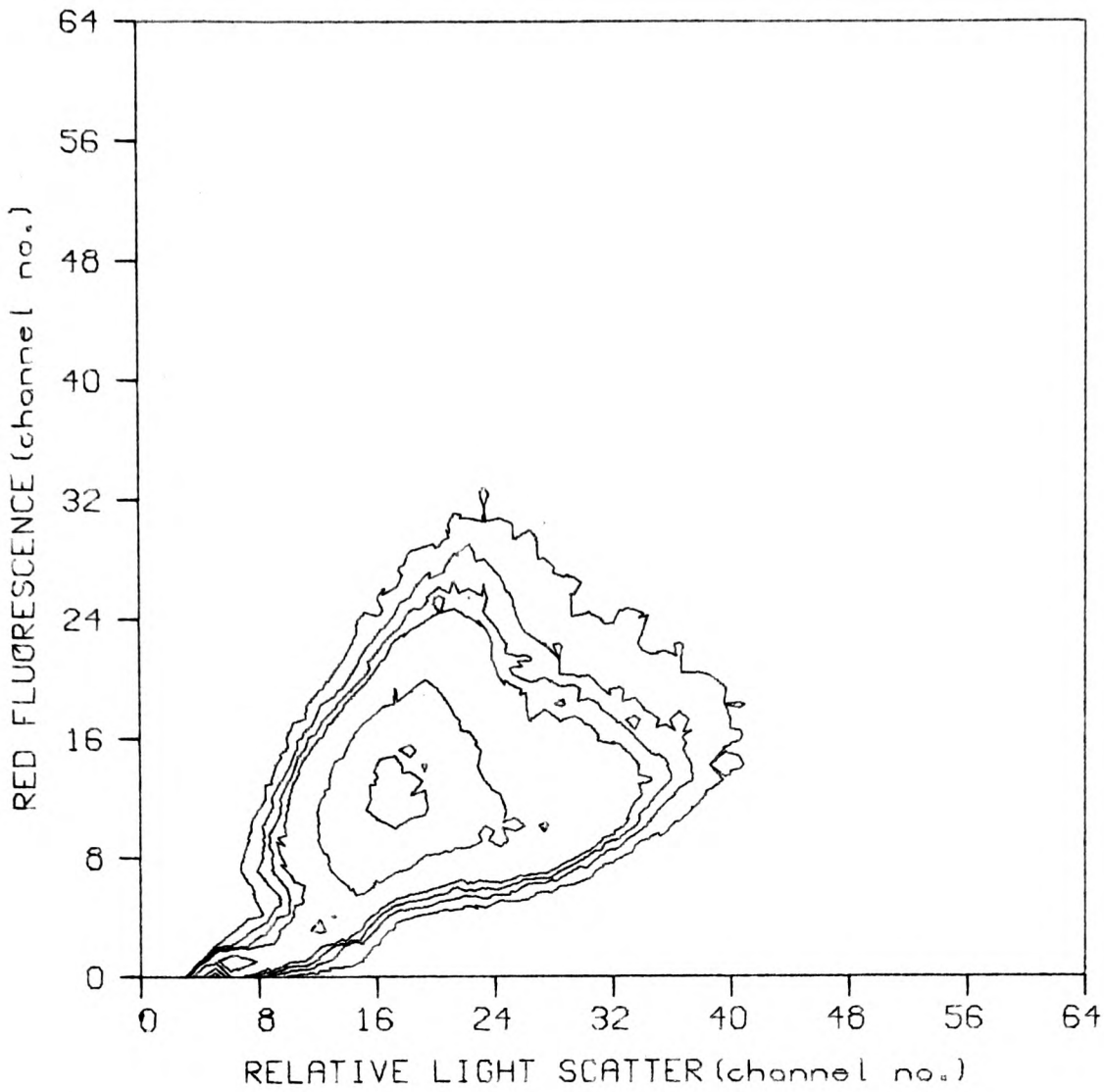

Fig. 33b Contour display of an acridine orange stained sample of FAT cells. The contour levels range from 25 cells (outer trace) to 500 cells (inner trace). Red fluorescence (measured at 600 - 650 nm) is plotted as a function of light scatter (cell size).

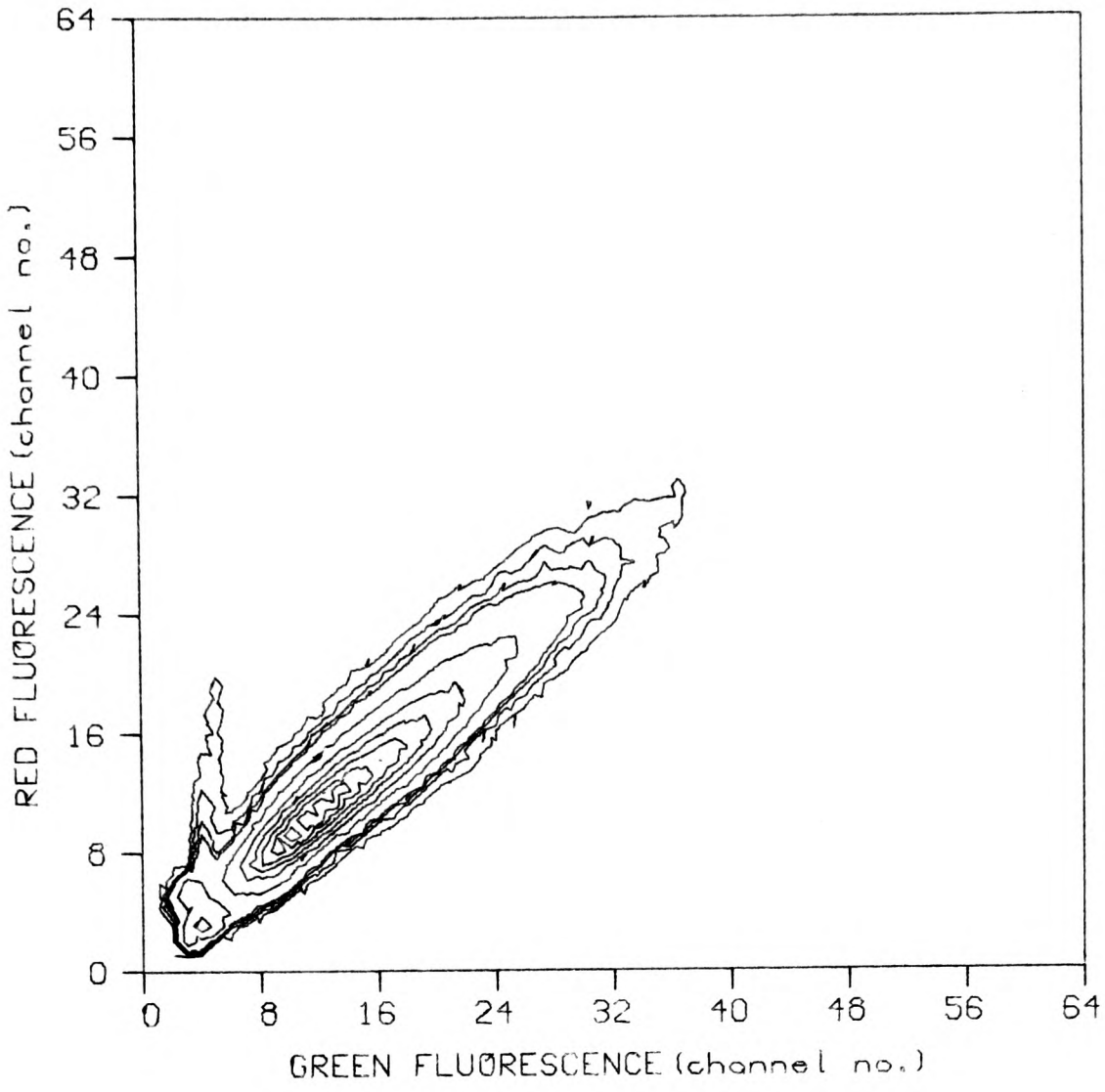

Fig. 33c Contour display of an acridine orange stained sample of EAT cells. The contour levels range from 25 cells (outer trace) to 1500 cells (inner trace). Red fluorescence (measured at 600 - 650 nm) is plotted as a function of green fluorescence (measured at 515 - 575 nm).

Discussion

The aneuploid character of tumour cell populations, with respect to both chromosome number and DNA content, poses a problem in Feulgen cytophotometric analysis. This is because some G_1 tumour cells will thus have the same level of DNA as some S-phase tumour cells. The combination of cytophotometric analysis with autoradiography is therefore mandatory in order to eliminate confusion between aneuploid and S-phase cells.

The problem of aneuploidy impinges upon the test results for the DNA : RNA dual staining method presented here. No error due to variation in DNA content would be expected for the normal cell types included in the study and these results provide the most useful indication of the reliability of the method. However, the valid comparison of two different treatments of the tumour cell population clearly assumes the measurements of optical densities for two equivalent groups of cells. This point might help to explain why the results of testing this dual staining method correlate poorly compared with the results obtained by Kiefer et al. (1969). In spite of this, the stoichiometric relationship between coriphosphin O and DNA as compared with that between pararosanilin and DNA appears to be remarkably good.

Some significance must be attached to the variability in f2 values obtained for the different cell categories indicated previously. It is difficult without further investigation to produce a satisfactory explanation for the discrepancy seen here. The results obtained might be associated with variations in chromatin condensation states of different cell types. This phenomenon has been recognised previously (Ringertz and Blound, 1969; Ringertz et al., 1969 and 1970, and Darzynkiewicz et al., 1969 and 1979a) and has been connected with chromatin variation

in actively proliferating cell populations (Nicolini, 1977, and Millet, 1979). This explanation implies that non-homogeneous dye distributional effects are responsible for the variation seen in the factor f2. However, this is unlikely since the use of the scanning procedure for microdensitometric analysis should eliminate such discrepancies. Leuchtenberger (1958) points out that optical density measurements carried out too far from the absorbance peak may introduce errors other than non-random distributional errors. These include unspecific light loss and amplification of noise disturbances in the photomultiplier tube leading to increased percentage error.

The inconsistency in f2 values between various cell types was not observed by Kiefer et al. (1969) and these workers applied a single f2 value of 5.64, determined on a lymphocyte population, to all calculations irrespective of cell type. The reasons for variation in the factor f2 remain obscure, however, application of the appropriate f2 factor for a given cell type would seem appropriate, whereas application of a single f2 factor across the board would not.

The wide distribution of DNA contents observed for pulse labelled tumours and mitotic figures is characteristic of a heterogeneous cell population. This result is in agreement with the chromosome analysis presented earlier. The modal DNA content estimated by Feulgen cytophotometry is clearly hyperdiploid; this also corroborates the results of chromosome analysis. Absolute correlation between cytophotometrically determined DNA content and chromosome number in tumour cell populations is not possible. This is due to the existence of abnormally sized chromosomes in tumour cell populations (Bohm and Sandritter, 1975). Therefore, the results presented here are taken as an indication that the EAT studied exhibits considerable aneuploidy around hyperdiploid modality with respect to both DNA content and chromosome number.

The broad distribution of DNA contents seen here may be typical of ascites tumours. Other workers (Bichel and Dombernowsky, 1973; Andersson and Kjellstrand, 1974 and Loffler et al., 1978) have also observed considerable heterogeneity in DNA contents determined for G_1-phase ascites tumour cells. In addition, Peel and Fletcher (1969) observed marked heterogeneity with respect to the chromosome number and cytophotometrically determined single cell DNA contents of a hypo-tetraploid EAT.

The DNA distribution for G_o cells suggests that cycle arrest occurs at some point or points within the G_1-phase. A small percentage of the non-cycling cells have G_2 DNA contents, but these could either be arrested in the G_2 of a diploid cell cycle or the G_1 of a tetraploid cell cycle. The further possibility exists that some of the non-cycling cells are arrested in S-phase; Collste et al. (1969) reached a similar conclusion following FCM analysis of leukaemia and urothelioma cells, although these workers did not consider the possibility of aneuploidy. It is difficult to envisage S-phase arrest in view of the current ideology which insists that once DNA synthesis has been initiated a cell is irreversibly committed to complete the division cycle (Baserga et al., 1965a and b, and 1976b); the exception to this being G_2-phase arrest (Gelfant and Smith, 1972; Prescott, 1976 and Gelfant, 1977).

The DNA distributions for non-cycling cells represented by the histograms in figs. 24 and 27 differ from those for G_1/G_2 cells following pulse ^{3}HTdR labelling. With particular reference to those cells considered to be of G_1-phase DNA content, the non-cycling cells no longer show the unimodal trend previously considered to be a feature of the G_1 interphase cell population. This feature might be attributable to the following: normal peritoneal cell contamination of the measured tumour cell sample, contamination of the measured cell sample with non-

viable tumour cells, the presence of some G_2 arrested cells or the measurement of a group of tumour cells which have adopted an altered chromatin configuration and consequently have registered an apparently reduced DNA content. An alternative explanation might be possible following reappraisal of the Feulgen DNA distributions for pulse labelled cell populations from tumours of various ages. This appraisal is presented below.

It appears from determination of tumour cell DNA distributions at 2, 5 and 12 days (figs. 26, 29 and 31) that there is an increase in heterogeneity concomitant with tumour age. This observation is made with particular reference to the peaks which are representative of the G_1-phase DNA contents for 2, 5 and 12 day tumours. Excluding the possibility of a cytophotometric artefact, the increase in tumour cell heterogeneity is postulated to represent an accumulation with increasing tumour age of cells with heteroploid DNA contents predominantly in the G_1-phase of cell cycle.

An increase in tumour cell heterogeneity, on the basis of chromosome analysis, has previously been discounted as being responsible for the decrease in growth rate of the FAT. There are several explanations which account for the apparent discrepancy between the cytophotometric data and chromosome analysis; there is either a selective or general increase in chromosome size which could feasibly be demonstrated by karyotyping; a change in the chromosome number does occur but was not detected by the chromosome analysis; or the newly selected or accumulated heteroploid cells have a reduced capacity to divide. This third alternative could not preclude the possibility that the accumulated heteroploid cells participate in the cell cycle since analysis of mitotic figure DNA distributions also showed an increase in heterogeneity concomitant with tumour age. In any case, it has been shown (Tribukait

and Skog, 1980) that an increase in the size of the genome does not result in an increase in the duration of the S-phase since families of genes undergo simultaneous replication (Prescott, 1976). This contradicts the earlier theory of Lala (1972a), referred to in the discussion in chapter 1, that an increase in ploidy could lead to an increase in S-phase duration.

The G_1 DNA distributions determined for non-cycling cells and shown in figs. 24 and 27 no longer show the unimodal trend previously considered to be associated with the G_1 interphase cell population. This is now presented as evidence for the accumulation of non-cycling cells which are members of both the 'stem cell' line (Bohm and Sandritter, 1975; Bosman et al., 1979 and Sandberg, 1980a) and newly selected heteroploid cells. Further evidence for the existence of both a 'stem cell' and an accumulated or selected heteroploid tumour cell population comes from kinetic studies. The PLM determination for the 2 day tumour shows enhancement which could be explained by the existence of a rapidly dividing 'stem cell' population. The possible biphasic re-entry of non-cycling cells following the retransplantation of a repeatedly labelled plateau phase tumour (fig. 14) is also possible evidence for two or more sub-populations of non-cycling cells. Similar conclusions have been reached by Wiebel and Baserga (1968), DeCosse and Gelfant (1968) and Gelfant (1977) following kinetic analysis of EAT cells <u>in vivo</u>.

Any explanation of the change in DNA distributions seen during the course of tumour growth must ultimately account for the restoration of the relatively narrow DNA distributions associated with the 2 day log phase tumour. Within an heterogeneous cell population the proliferative abilities of various entities or sub-populations could be different. If the proliferative abilities of these sub-populations were

dependent on the microenvironmental conditions, then variations in these conditions could act to change the proliferative balance. It might be envisaged that the existence of a sub-population of heteroploid cells is the result of aberrant mitosis (asymmetrical division is a common observation). The possibility that these cells have a reduced capacity to divide and enter a non-cycling phase could lead to the conclusion that the non-proliferative compartment of EAT cells is a preferential pathway to cell death (Lala, 1972b). Upon transplantation of the plateau phase tumour the 'stem cell' population would probably out-grow the less rapidly dividing heteroploid cells; for example, it is well known that if a cell culture is contaminated with rapidly dividing fibroblasts or HeLa cells, the more slowly dividing cells become overwhelmed and disappear from the culture. Furthermore, it follows, that as a cell becomes larger (a phenomenon associated with increased DNA content) the surface to volume ratio decreases. If molecular traffic across the plasma membrane is retarded as a result of decreased surface to volume ratio, then larger cells are at a nutritional disadvantage compared with smaller cells (Hauschka et al., 1957). Thus smaller, near diploid, cells would tend to outgrow larger heteroploid cells. Alternatively, the accumulated heteroploid cells may not have completed their first division cycle at this time (2 days). Consequently, they could be engaged in DNA synthesis and therefore not accessible to the cytophotometric analysis of a 2 day EAT as described on p 118.

It can be appreciated that the heterogeneity of the EAT cell population with regard to DNA content, chromosome number and proliferative status is complex and poses problems in cytophotometric analysis. However, analysis of non-cycling cells in a heteroploid/aneuploid tumour cell system is probably more closely allied to the fundamental understanding of tumour cell proliferative characteristics than analysis

of stationary cells in culture. This is not least because increased DNA contents and aneuploidy are clearly associated with malignancy (Bohm and Sandritter, 1975, and Sandberg, 1980b).

Interpretation of the DNA and RNA and DNA and protein data requires the comparison of DNA/RNA and DNA/protein ratios. The reasons for this are threefold; it is difficult without reference to some form of simple numerical aid to assimilate the data presented in figs. 25, 28, 30 and 32 since no clear groupings are evident. Formulation of a ratio for individual cells allows statistical analysis and thirdly, a reference point is required in order to compare the RNA and protein contents of cells with different DNA contents. It is difficult to envisage related cells of different DNA contents possessing RNA and protein complements totally unrelated to the respective genomes.

The results of determining DNA/RNA and DNA/protein ratios have been analysed using two statistical techniques, the Mann Whitney 'U' test and the Student's 't' test. Both tests are employed to assess the extent to which any observed variation between two groups of experimental data could have arisen by chance. For analysis of the data presented, the 't' test is suspect because the distribution of ratios is heavily skewed. The 't' test is however, robust (Clarke, 1969b) and should give reliable information in the majority of cases. The Mann Whitney 'U' test is the non-parametric equivalent of the 't' test and is applicable where the data are non-normally distributed. The Mann Whitney 'U' test is more appropriate in view of the skewed distribution and the 't' test is included for comparison purposes. The fact that the results of the 't' test and 'U' test correspond so closely (tables 11 and 13) tends to suggest that variation, where indicated, is real.

The results of microdensitometric analysis provide good evidence that the total protein content of tumour cells in the non-proliferative

phase is reduced. It can be inferred from this that protein accumulation must occur before progress through cell cycle and DNA synthesis can occur. Similarly, it is apparent that the RNA content of non-cycling cells is reduced. This observation could account for the reduced protein content seen in these non-cycling cells and supports the observations made by Frindel et al. (1970) and Benz and Cadman (1981) on whole tumour extracts.

It can be concluded that the overall metabolic status of non-cycling cells is reduced and that activation of some process or processes operational at the transcriptional level is necessary before re-entry into cell cycle can be said to have occurred.

The demonstrable inequality between non-cycling and cycling cells appears to be more clearly apparent in the case of protein content rather than that of RNA content. This phenomenon is possibly related to the fact that as with many biochemical processes, initial variations become greatly augmented as a metabolic pathway is followed. A single molecule of mRNA for example is read several times prior to its destruction. However, the connection between protein and RNA levels in this way could be seriously influenced by dissimilarities between the procedures used for their determination.

The observation that total mitotic protein content is elevated over that of interphase cells suggests that there is some cyclic variation. This cannot be explained by the presence of non-cycling cells within the measured interphase group since this disparity is most obvious for the 2 day tumour where the growth fraction tends to unity. However, it is known (Zylber and Penman, 1971 and Miroslaw and Baglioni, 1979) that protein synthesis falls dramatically during mitosis, and some catabolism probably occurs before the start of the G_1-phase.

Gaub (1977) has indicated that nuclear non-histone protein content is reduced in G_0 tumour cells and has concluded that this indicates a post-mitotic series arrangement of the G_0 - G_1 phases. That is, the non-proliferative phase exists as an entity which is distinct from and occurs prior to the G_1-phase (Smith and Martin, 1973, and Brooks, 1977). This view is contrary to that of Baserga (1976c) and Stanners et al. (1979) which states that only normal cells enter a true G_0 phase, and tumour cells become arrested in G_1 possibly due to having RNA which is relatively more stable (Leibhaber et al., 1978). An alternative view is that certain restriction points may be situated within or towards the end of G_1-phase (Prescott, 1976; Pardee, 1974 and Pardee and Dubrow, 1977). It is possible to see that cells arrested adjacent to the G_1/S boundary would already have a full complement of the relevant RNA species, enzymes and other macromolecules necessary for the initiation of the S-phase. In this case cells would probably be restricted from entering S-phase via a mechanism involving cyclic nucleotides (cAMP, cGMP), protein phosphorylation and specific gene repression (Rasmussen, 1970; Burger et al., 1972; Goldberg and Haddox, 1977 and Busch, 1979). Briefly, receptor molecules located on the cell surface respond to contact inhibition, chalones, hormones and other exogenous stimuli. Subsequently, membrane associated enzymes such as adenyl cyclase are activated or deactivated. The resulting alteration in the balance of cyclic nucleotides initiates the cytoplasmic response (Cuatrecasas, 1974) which ultimately coordinates metabolic repression or derepression at either the enzyme or gene level.

There is good evidence from other kinetic and biochemical studies to suggest that cell cycle arrest has more than one form and the mechanism of cycle arrest is dependent on cell type or environmental factors (Gelfant, 1977 and Melvin et al., 1979). Gelfant (1977) has

further suggested the possible coexistence of two or more independent non-proliferative states. However, this theory could merely represent a contortion of the already established concept that G_o phase has depth (Augenlicht and Baserga, 1974; Gunther et al., 1974, and Merz and Schneider, 1980).

The non-cycling cells of the EAT studied here would appear to be arrested in a biochemically distinct G_o phase. There is some evidence to suggest that these cells do not form a homogeneous population. However, the method of their detection (repeat ^{3}HTdR labelling) only allows visualization of those cells which have not synthesised DNA for more than 90 hours. To conclude the existence of a broad spectrum of G_o cells, arrested at various levels of G_o according to the length of time they have been residing in G_o, would require the examination of a wider range of G_o cells than is possible by this method.

It has been postulated (Van Venrooij et al., 1970) that processes concerned with cycle arrest induced by nutrient deficiency initially operate at the translational level. Thus, Van Venrooij et al., (1970 and 1972) consider that some process inhibits protein synthesis rather than RNA synthesis. Miroslaw and Baglioni (1980) and Becker et al. (1971 and 1972) reach broadly similar conclusions indicating inhibition of polyribosome formation. The results presented here could be taken to indicate that G_o cells cannot enter cell cycle before RNA synthesis occurs, suggesting that inhibitory processes ultimately operate at the transcriptional level. There are two possible reasons for the apparent disparity between the conclusions reached here and those reached by Van Venrooij et al. (1970 and 1972), Becker et al. (1971 and 1972) and Miroslaw and Baglioni, (1978). Firstly, the mode of G_o induction could be significantly different in the tumour system studied here and

this could be related to cell type or environmental factors. Secondly, and perhaps more likely, following a drop in protein synthesis, RNA synthesis would be reduced due to a deficit of relevant enzymes. In this way the relative RNA and protein levels would be related to the length of time spent in G_o; since the EAT cells studied here spent at least 90 hours in G_o and the cells studied by Van Venrooij, Miroslaw and Becker spent less than 24 hours in G_o, this factor could account for the disparity.

The biochemical analysis of G_o cells supports the general conclusion that decline in proliferative activity of the EAT is associated with a decline in cellular metabolic activity. Fletcher (1972) demonstrated that a decline in the activity of thymidine kinase, a marker for proliferation, and Glucose - 6 phosphate dehydrogenase was closely associated with reduced proliferative activity and an increase in the size of a subpopulation of non-cycling tumour cells. These results were presented as being suggestive of inhibition of cellular metabolism due to reduced oxygen availability. Harris et al. (1970) were unable to demonstrate any change in TdF kinase activity or the activity of 5 other enzymes during the course of ascites tumour growth. However, these workers did indicate that reduced protein synthesis in the later stages of tumour growth was probably due to inefficient ATP production as the tumour cells were heavily reliant on anaerobic metabolism at this stage.

The discrepancy between the conclusions reached by Harris et al. (1970) and those reached by Fletcher (1972) may be explained by the fact that Harris et al. presented results of biochemical analysis following extraction of 10^8 disrupted tumour cells, whereas Fletcher presented results obtained using cytochemical procedures and examination of individual cells. Merz and Schneider (1980) have indicated that EAT cells enter a G_o phase when oxygen is not available and that the

duration of the lag phase extending from the application of a stimulus to proliferate to initiation of DNA synthesis is proportional to the duration of anaerobiosis. These results presumably infer that a lag phase is necessary for the accumulation of proteins and other macromolecules required for cell cycle progression.

Another approach to the problem of distinguishing between G_o and G_+ cells is concerned with individual cell DNA and RNA determinations using FCM. Darzynkiewicz et al. (1976) demonstrated that RNA accumulation was necessary in G_o lymphocytes before DNA synthesis could be initiated. This technique has subsequently been applied to examination of tumour cell populations (Kurland et al., 1978; Collste et al., 1979; Darzynkiewicz, 1979b). However, no clear distinction between G_o and G_+ tumour cells was possible, mainly due to the existence of aneuploid cells. Additional problems lie in the use of acridine orange as a differential stain, that is its ability to display metachromasia depending on the intra-cellular components with which it interacts. The basis for distinction between DNA and RNA lies in the propensity of acridine orange for monomeric intercalation with a double helix and polymeric association with single stranded RNA. Considerable debate still exists as to the exact nature of the AO - nucleic acid interaction (Nishia and Ishizaki, 1979; Frankfurt, 1980, and Darzynkiewicz, 1975 and 1979a).

The Acridine Orange staining profile for a plateau phase EAT gives a confused picture (figs. 33a, 33b and 33c) that can be related to a cell system which exhibits considerable heteroploidy. There is evidence to suggest groupings and two major peaks can be identified which probably relate to tumour cell G_1 and G_2-phase DNA contents. However, there is no evidence to indicate the existence of a sub-population of cells which are biochemically distinct. The failure of FCM to reveal a sub-population of G_o tumour cells which are deficient in RNA could indicate

that no definite G_+ - G_o cut-off point exists. That is to say the $G_+ \longleftrightarrow G_o$ transition is a continuum with no clear distinctions. It can be seen that without the benefit of ^{3}HTdR labelling and statistical analysis the reduced RNA content of G_o EAT cells might have remained hidden from any FCM or cytophotometric analysis.

The present results clearly indicate the existence of a sub-population of tumour cells arrested in a biochemically distinct G_o phase. The fact that there is a lack of homogeneity within the G_o population with regard to RNA and protein content might be indicative of cells being located at various 'depths' of the G_o phase. This idea is also consistent with the mode of entry into cell cycle of G_o cells following stimulation (figs. 13 and 14). It is envisaged that cycle arrest is the result of a general alteration in metabolic activity due mainly to reduced availability of oxygen and other nutrients. The biochemical deficiency seen in G_o EAT cells could therefore reflect a shift in the balance between anabolic and catabolic processes during the $G_+ \longrightarrow G_o$ transition (Live and Kaminskas, 1975 and Epifanova, 1980).

Chapter Three

Immunological Studies

Introduction

The experimental results detailed in chapters 1 and 2 can be taken to demonstrate the reduced metabolic capacity of G_0 tumour cells. This third section represents an attempt to extend previous experimentation in order to embrace an analysis of the antigenic properties of the G_0 cells. The rationale for this approach stems from consideration of the cell membrane as a functional cellular organelle and not merely as an inert boundary separating the cytosol from the extracellular milieu. A greater awareness of this general concept can be gained from an appreciation of the extent to which the plasma membrane is involved with and integrated into the general metabolic processes of the cell.

The cell membrane occupies a unique position as the mediator of any interaction between the cell and its external environment. The role of the plasma membrane as a sensory device capable of receiving and translating certain extrinsic stimuli, then transferring the relevant information onto the cytoplasm is now established.

There is a considerable body of evidence to suggest that the endoplasmic reticulum (ER) is the principal site of initial plasma membrane assembly. For example, the synthesis and insertion of the majority of membrane proteins is associated with ribosome dense regions such as rough ER (Harrison and Lunt, 1980). Glycosyltransferases are closely associated with the ER and Golgi apparatus and account for the glycosylation of the protein and lipid components of the plasma membrane (Shur and Roth, 1975), although their precise function at this site is not yet fully understood.

This membrane flow hypothesis also embraces cellular antigenicity since antigens arise as products of this process. Indeed, the demonstration that the ER and the plasma membrane share some common antigenic determinants (Hoelzl-Wallach and Vlahavic, 1967) lends support to the

membrane flow hypothesis.

Thus, it can be seen that plasma membrane components are produced by cytoplasmic processes which are probably influenced by the general metabolic status of the cell. On further consideration of a link between membrane flow and cellular antigenicity, the possibility exists that antigenicity alters with variation in general cellular metabolic status. Naturally, the possibility of antigenic variation between cycling and non-cycling tumour cells (of reduced metabolic status) impinges on the host's ability to detect and subsequently destroy neoplastic cells.

The immunobiology of cancer received considerable attention in early studies involving tumour xenografts. However, it was not until the advent of inbred strains of laboratory animals that the role of histocompatibility antigens in tumour rejection following xenografting was realised (Leob and Wright, 1927; Woglom, 1929 and Medawar, 1946). Subsequently, the presence of neo-antigens on the surface of tumour cells was demonstrated (Foley, 1953 and Prehn and Main, 1957). Neo-antigens may take a variety of forms, these include: modification of existing isoantigens, the expression of viral antigens following virus infection in a permissive cell and expression of latent oncofoetal antigens.

In principal, the expression of tumour associated neo-antigens should result in immunological recognition of non-self and consequent rejection by the host of the tumour. The incidence of cancer in immunocompetent hosts seems to discredit this theory (Cochran, 1978a). The frequency with which a clinically detectable tumour results from a single aberrant cell or microscopic tumour nodule is unknown and the central issues of the 'immunological surveillance' theory (Burnett, 1965) remain a matter of conjecture (Cochrane, 1978a).

The persistence of tumour growth in an immunocompetent individual

could be the result of failure of either the afferent (detective) or efferent (destructive) arm of the immune response. Much emphasis has been placed on the possible mechanisms by which tumour cells are able to avoid or resist lethal immunological attack. Rapidly growing tumours may simply outstrip the capacity of the immune system to respond, and immune paralysis could in some cases result. The phenomenon of 'sneaking through' (Old et al., 1962) would appear to depend on a balance between growth rate and the speed of invocation of an effective response. Enhancement of tumour growth can occur in the face of the host immune response if non-cytotoxic anti-tumour antibody impedes subsequent host anti-tumour action (Baldwin, 1973), or if antigens are shed and subsequently complex with free anti-tumour antibody (Currie and Basham, 1972). The formation of antigen-antibody complexes can also result in the diversion of effector cells from the tumour target (Currie and Alexander, 1974). Tumour cells which are antigenically unobtrusive or weakly immunogenic are more likely to avoid immune detection and ultimate destruction. Fenyo et al. (1968) have shown that selection of an immunoresistant cell line of a murine lymphoma (YCR) by repeated passage was associated with reduced antigenicity. Similarly, Law (1980) demonstrated the emergence, by continuous tumour grafting, of a tumour specific transplantation antigen (TSTA)-negative sarcoma cell population from what was originally a dimorphic ($TSTA^+$ and $TSTA^-$) cell population.

The above mechanisms of immunological evasion by tumours have been well documented and are the subject of reviews by Currie (1974b) and Cochran (1978b). However, a further potential mechanism by which tumours could avoid immunological detection and destruction is antigenic modulation or modification. Although this mechanism has previously received some attention (Boyse and Old, 1969; Currie, 1974b and Cochran, 1978b) it has been less intensively researched.

Evidence exists for cell cycle phase related variation in antigenic expression. Most workers have been concerned with cell cycle dependent antibody mediated cytotoxicity (Cikes and Klien, 1972a and Leibson et al., 1980), and the antigenic species on which most attention has been focused are those of the H-2 and HL-A type for mouse and human neoplastic cell lines in culture (Pasternak et al., 1971 and Pellegrino et al., 1974); H-2 and viral antigens arising on cultured murine lymphoma cells (Cikes, 1970a and Cikes and Frieberg, 1971) and the blood group antigen H expressed by Hela cells (Kuhns and Bramson, 1968). The validity of this use of cytotoxicity or other secondary membrane associated phenomena has been challenged (Pellegrino et al., 1974). The question that arises is how closely does the secondary event (cytotoxicity) relate to a particular primary phenomenon? That is, how much do the physical properties of the membrane: fragility, fluidity, variation in electrical potential, cell surface architecture and altered membrane repair function contribute to the observed cytotoxicity? Thus Pellegrino et al., (1974) in an attempt to measure degree of antigenic expression obtained dissimilar results using an indirect method (cytotoxicity) and a direct method (indirect immunofluorescence). Similarly, Segerling et al., (1975) discovered cell cycle phase related variation in antibody mediated cytotoxicity for ascites hepatoma cells in vitro, the cells being partially synchronized by treatment with cytotoxic drugs. However, the cell cycle related cytotoxicity could not be attributed to variation in antigenic expression. This might well apply to all studies involving drug induced synchrony and cytotoxicity (Pellegrino et al., 1972 and 1974; Ferrone et al., 1973 and Leibson et al., 1978) because of possible membrane disturbances, caused by such drugs, subsequently influencing the cytotoxic event. Consequently, some workers have been induced to supplement cytotoxicity studies with indirect immunofluorescent analysis (Cikes et al., 1970b

and 1972b and Leibson et al., 1978).

Essentially, the evidence previously documented by other workers gives no absolutely clear picture of phase related antigenic variation. Lerner et al. (1971) and Cikes et al. (1972b) demonstrated G_1 cells to be more susceptible to antibody mediated cytolysis in vitro, and consequently concluded increased antigenicity to be associated with the G_1 phase of cell cycle. Pellegrino et al. (1974) found G_1 cells to be relatively resistant to antibody mediated cytolysis but could not substantiate variation in antigenicity with cell cycle phase as being an important factor. Ferrone et al. (1973) and Leibson et al. (1980) were unable to detect any phase related variation in antigenicity throughout the cell cycle.

Very little information exists on the possible antigenic variation between cycling and non-cycling tumour cells. Cikes (1970b) concluded that reduced antigenic expression was associated with both small cells from plateau phase lymphoma cells in culture and with rapidly dividing (log phase) G_1 cells from the same cultures. However, it is not clear from the latter work whether the small cells derived from the non-growing cultures represent a rapidly cycling sub-population or a resting phase population. Thomas (1971) using an immunofluorescent technique demonstrated variation in expression of the B and H blood group antigens of cultured human lymphocytes. Cells in S-phase, G_2 and mitosis were found to be B^+, H^-; cells in G_1 and G_o were found to be B^-, H^+. Later (1973) Thomas and Phillips studied T and B lymphocyte blast associated antigens. These antigens were lost on movement of the cells into a quiescent phase; it is unclear whether this loss is associated with the lymphocyte differentiation and ageing process or a true resting phase.

Evidence from biochemical analysis suggests cell cycle phase related variation in the composition of the plasma membrane (Warren and

Glick, 1968 and Bosmann and Winston, 1970). These workers demonstrated altered patterns of protein, lipid and carbohydrate synthesis to be associated with the resting phase. However, the significance of these observations in relation to cellular antigenicity remains to be established.

It is possible to envisage that any antigenic modulation associated with the reduced proliferative and metabolic activity of G_o tumour cells could protect such cells from lethal interaction with the host immune system. The possibility that such antigenic modulation exists has important implications for the treatment of cancer by various combinations of cytotoxic drugs and immunotherapeutic reagents, combinations of which have more recently been advocated (Newlands et al., 1976; Ghose and Blair, 1978 and Goodnight and Morton, 1980). Possibly a cytotoxic drug which induces cycle arrest within or adjacent to a phase of cell cycle involved with associated antigenic modulation could render those cells resistant to subsequent action of immunotherapy. Secondly, attempts to enhance cytotoxic specificity by administration of antibody-drug conjugates (Ghose and Guclu, 1974) would be futile in the absence of expression of the antigens to which the antibodies were raised.

The present work is an attempt to compare the overall antigenicities of rapidly cycling and G_o tumour cells. This study is based on the binding of fluorescent antibodies to EAT cells after treatment with xenogenic anti-EAT antiserum. The antiserum, raised in rabbits, could not be rendered specific for a particular antigen, although some antibodies directed against isologous mouse antigens were eliminated from the whole antiserum (see discussion) prior to tumour cell treatment and staining. The implications of this procedure are discussed in relation to the results presented. In one experiment a combination of immunofluorescence and autoradiography was used. Such a combination was first

described by McDevitt et al. (1966). In a second experiment a comparison was made of the fluorescence profiles of rapidly growing tumours (5 - 6 days old) with those of plateau phase tumours (12 - 13 days old). This analysis of fluorescence profiles of tumour cell populations following sequential incubation with rabbit anti-FAT then fluorescent anti-rabbit IgG antisera, was carried out using a fluorescence activated cell sorter (FACS).

Materials and Methods

Immunization of rabbits against EAT cells: Three immunization procedures were tried. The first two were based on the method of Chose et al. (1967) and the third was based on the method of Jamasbi et al. (1978). A total of 6 female, half-lop cross rabbits ('Hop Rabbits', Canterbury, Kent) 2 months old, were bled from the marginal ear vein to provide normal rabbit serum (NRS) before immunization, which was carried out as follows:

(i) Four of the rabbits received intramuscular (im) injections of 5×10^7 twice washed (PBS) viable EAT cells suspended in an emulsion of sterile PBS: Freund's complete adjuvant (1:1) total volume 0.5 ml. After 6 weeks each rabbit received a similar injection, except that the complete adjuvant was replaced by Freund's incomplete adjuvant. Three weeks later each rabbit received an im booster injection of 0.5 ml containing 5×10^7 twice washed viable EAT cells in sterile PBS alone. An identical second booster injection was administered 7 days after the first and all the rabbits were bled from the marginal ear vein 7 - 10 days later.

(ii) Two of the rabbits involved in the complete immunization procedure outlined above were subsequently reboosted 3 months later. This consisted of giving two im injections, separated by an interval of 7 days, of 0.5 ml containing 5×10^7 twice washed viable EAT cells suspended in sterile PBS. The rabbits were again bled from the marginal ear vein 7 - 10 days later.

(iii) Two previously untreated rabbits received im injections of 0.5 ml containing 5×10^7 twice washed viable EAT cells in sterile PBS emulsified with Freund's complete adjuvant. This inoculation was repeated after 7 days. Both rabbits were then boosted 7 days later with im injections of 0.5 ml of 5×10^7 twice washed viable EAT cells suspended

in sterile PBS. This booster injection was repeated 7 days later and the rabbits were bled 7 - 10 days after the final injection.

For summary of immunization procedures see Table 16.

Absorption procedures: After collection, the blood was allowed to clot at room temperature for 1 - 2 hr. Following this the majority of the serum was collected and stored overnight at 4°C. The clot was similarly stored overnight before residual serum was collected.

Complement activity was destroyed by incubating the serum at 56°C for 30 min.

Mixed homogenates of liver, kidney, spleen, thymus and lymph node tissue from BALB/c mice were prepared. These homogenates were washed at least 10 times in cold (4°C) PBS by centrifugation at 3000 rpm for 5 min. The homogenate was considered to be satisfactory for use when the supernatant became clear after the centrifugation procedure.

Initially, antisera were absorbed with a 1/8 - 1/5 volume of packed cells for 3 periods of 2 hr. each. Subsequently the duration of the absorbing procedure was increased to 8 periods of 2 hr. each. These absorptions were carried out at 4°C in 2 ml tubes strapped onto a slowly rotating wheel.

On completion of the absorption period antisera were spun at 30,000 rpm and 4°C for 30 min. to remove any remaining immune complexes.

Extra tumours: Sarcoma - 180 (S - 180) ascites tumour, carried in Swiss mice; S - 180 solid tumour, carried on the flank of Swiss mice and BP - 8 ascites tumour carried in C57B mice were all obtained from the Imperial Cancer Research Fund Laboratories (ICRF), Lincoln's Inn Fields, London. Solid forms of the EAT grown in BALB/c hosts were obtained by sc flank injection of 10^7 EAT cells.

Indirect Immunofluorescent analysis: Fluorescent antibody staining was performed according to the method outlined by Weir (1973). All tissues

Table 16

IMMUNIZATION SCHEDULE	RABBITS USED	INJECTION NUMBER 1	2	3	4	5	6	RABBITS BLED (DAY)
(i)	1,2,3,4	day 0: $5x10^7$ EAT cells emulsified in 0.5 ml PBS : FCA (1:1)	day 42: $5x10^7$ EAT cells emulsified in 0.5 ml PBS : FIA (1:1)	day 63: $5x10^7$ EAT cells suspended in 0.5 ml PBS	day 70: $5x10^7$ EAT cells suspended in 0.5 ml PBS	day -	day -	77-80
(ii)	3, 4	day 0: $5x10^7$ EAT cells emulsified in 0.5 ml PBS : FCA (1:1)	day 42: $5x10^7$ EAT cells emulsified in 0.5 ml PBS : FIA (1:1)	day 63: $5x10^7$ EAT cells suspended in 0.5 ml PBS	day 70: $5x10^7$ EAT cells suspended in 0.5 ml PBS	day 160: $5x10^7$ EAT cells suspended in 0.5 ml PBS	day 167: $5x10^7$ EAT cells suspended in 0.5 ml PBS	174-177
(iii)	5, 6	day 0: $5x10^7$ EAT cells emulsified in 0.5 ml PBS : FCA (1:1)	day 7: $5x10^7$ EAT cells emulsified in 0.5 ml PBS : FIA (1:1)	day 14: $5x10^7$ EAT cells suspended in 0.5 ml PBS	day 21: $5x10^7$ EAT cells suspended in 0.5 ml PBS	day -	day -	28-31

FCA = Freund's complete adjuvant } obtained from Difco
FIA = Freund's incomplete adjuvant } (Detroit, U.S.A.)

All injections were im.

stained by this method were either single cell suspensions or, in the case of the solid tumour tissue, after preparation of 10 μm thick cryostat sections.

Single cell suspensions (approx. 5×10^6 cells/ml) were washed 2 - 3 times in cold ($4^{\circ}C$) sterile PBS. The cells were resuspended in cold PBS prior to the addition of antiserum. The cell suspensions were incubated on ice in the presence of antiserum for 30 min. with periodic agitation. Following this incubation the antiserum was diluted *in situ* by a massive excess of cold PBS. Cells were then washed twice with fresh PBS and centrifugation at 1000 rpm ($4^{\circ}C$). Cells were resuspended in cold PBS prior to the addition of sheep anti-rabbit IgG - FITC (fluorescein isothiocyanate) conjugate (Wellcome, Beckenham, England) at a final working fluorescent antiserum dilution of 1:20 in PBS. Such cell suspensions were incubated on ice for 30 min. with periodic agitation. Following the second incubation the cells were rewashed as above and mounted in 30% Glycerol in PBS pH 7. Coverslips were sealed with hot wax.

Stained preparations were excited with light of wavelength 450 - 490 nm and examined using a Reichert Biopan epifluorescent microscope fitted with a barrier filter to prevent light of <500 nm and >600 nm from reaching the eye.

Solid tumour tissue was cut into pieces of approximately 1 - 2 mm^3. These small pieces of tissue were quenched in n-hexane for 60 seconds at $-65^{\circ}C$. A freezing mixture of dry ice in acetone was used to chill the hexane. The tissue was removed from the hexane and allowed to drain on a block of dry ice. A small piece of quenched tissue was frozen onto the top of a drop of water which had been placed on a chuck surrounded with dry ice. The completed chuck was placed in the cryostat and 10 μm thick sections of tissue were cut. The tissue sections

were carefully transferred to subbed microscope slides and were maintained at -25°C until ready for staining with antibody. These cryostat sections were subsequently incubated at 4°C in the presence of diluted antiserum. After 30 min. the antiserum was flushed from the slide with cold PBS. The tissue sections were carefully dried by absorbing as much moisture as possible from around the section with the corner of a paper towel. Subsequently, the tissue sections were incubated at 4°C for 30 min. with fluorescent antibody. The tissue sections were again flushed with cold PBS and mounted in 30% Glycerol in PBS prior to examination under the UV microscope.

Combination of immunofluorescence and autoradiography: Preparations were made according to the methods outlined by Henderson and Smithyman (1974) and Smithyman (1977), and as originally described by McDevitt et al. (1966). Briefly, 12 day EAT cells were repeat-labelled *in vivo* with ^{3}HTdR as described previously. A sample of the radiolabelled cells was then stained according to the method presented for indirect immunofluorescent analysis. In this case anti-EAT antiserum was used at a dilution of 1:8 with PBS. Following fluorescent antibody staining and final PBS washes the cells were smeared onto degreased microslides, air-dried and fixed in 95% methanol for 10 min. (Smithyman, 1977 and Leong et al., 1979).

Fixed immunofluorescent smears were dipped in autoradiographic emulsion (Ilford K5) as previously described. Initially, tests for latent image fading indicated that the presence of the FITC conjugate on the cell surface in some way interfered with latent image formation. Therefore, prior to dipping in the autoradiographic emulsion, immunofluorescent smears were rapidly dipped in a dilute aqueous solution (1%) of gelatine at 60°C. This procedure was designed to provide a barrier between the FITC conjugate and the autoradiographic emulsion,

without reducing the efficiency of the autoradiographic process. The immunofluorescent - autoradiographs were exposed for 2 - 4 weeks prior to developing and fixing as previously described.

In order to maintain impartiality in scoring, photographic maps of the immunofluorescent - autoradiographs were prepared under phase contrast using a Zeiss light microscope. Mapped cells were recorded under the light microscope as being either radiolabelled or not, then the same cells were examined under the UV microscope and an estimation of relative fluorescence for individual cells made. Cells were therefore scored as being positive or negative with regard to ^{3}HTdR incorporation and as being either: (+++) very brightly fluorescent, (++) brightly fluorescent, (+) weakly fluorescent or (-) non-fluorescent.

FACS analysis of an EAT cell population in different phases of growth following indirect immunofluorescent staining: Two batches of the rabbit anti-EAT antiserum were used in this analysis; one was absorbed with BALB/c mouse tissue only, the other was in addition absorbed with S-180 ascites tumour cells.

Ehrlich ascites tumour cells ($2\text{-}3 \times 10^{6}$) from a 12 day tumour were stained as previously described by indirect immunofluorescence using a range of antiserum dilutions (fluorescent antibody dilution being constant at 1:20). The stained cells were resuspended in 2 ml PBS, introduced into the FACS and examined using laser light of wavelength 488 nm. Light scatter and green fluorescence (emissions between 500 and 550 nm) were measured.

Cytotoxic assay: Cytotoxicity assays were conducted according to the method outlined by Boyle et al. (1976). Approximately 10^{6} tumour cells were suspended in 75 µl NRS or an alternative source of complement (pure rabbit complement. Behring, W. Germany, guinea-pig complement, Sera Laboratories Ltd., Crawley Down, Sussex, or normal mouse serum),

To this suspension 75 µl complement deactivated anti-EAT antiserum was added. The suspension was mixed and incubated at 37°C for 60 - 90 min. Following incubation 10 µl of the cell suspension was added to 190 µl of trypan blue solution (0.1% in PBS). Differential counts of stained (non-viable) and unstained (viable) cells were made 10 min. later with the aid of a haemocytometer.

Results

Detection of anti-EAT antibodies (Ig G) in immune rabbit sera: Indirect immunofluorescence was used to determine the presence of anti-EAT antibodies in the sera of rabbits following immunization against whole viable tumour cells. Specific cell-staining, the result of an antigen : antibody interaction (Goldman, 1968) was marked by bright peripheral fluorescence and plates 1, 2 and 3 show fluorescent antibody staining of EAT cells after treatment with 8 times absorbed antiserum at a dilution of 1:10. Plate 4 is the result of staining with fluorescent antibody following treatment with pre-immune serum (NRS); note that there is a small amount of non-specific fluorescence (arrow) which can arise to varying degrees through: non-immunological protein-antibody interactions (charge effects), penetration of non-viable cells by FITC conjugates or possible internalization of plasma membrane components following surface antigen : antibody interaction.

Using this technique it was possible to detect fluorescent antibody staining of both EAT cells and normal mouse cells (thymus, splenic and lymph node lymphocytes) after treatment with whole antiserum at a dilution of 1:50. Following 3 x 2 hr. absorptions with mixed homogenates of normal BALB/c mouse tissues the minimum effective dilution of anti-tumour antiserum for production of fluorescent antibody staining of tumour cells was reduced to 1:20. In addition, some residual anti-normal BALB/c mouse lymphocyte activity persisted.

Anti-EAT activity was still present at an antiserum dilution of 1:20 following 8 x 2 hr. absorptions against normal BALB/c mouse tissue homogenates, whereas anti-normal BALB/c mouse lymphocyte activity was abolished. These results, determined after institution of the original immunization procedure, (i) (Methods section p.145), are summarized in tables 17a and 17b.

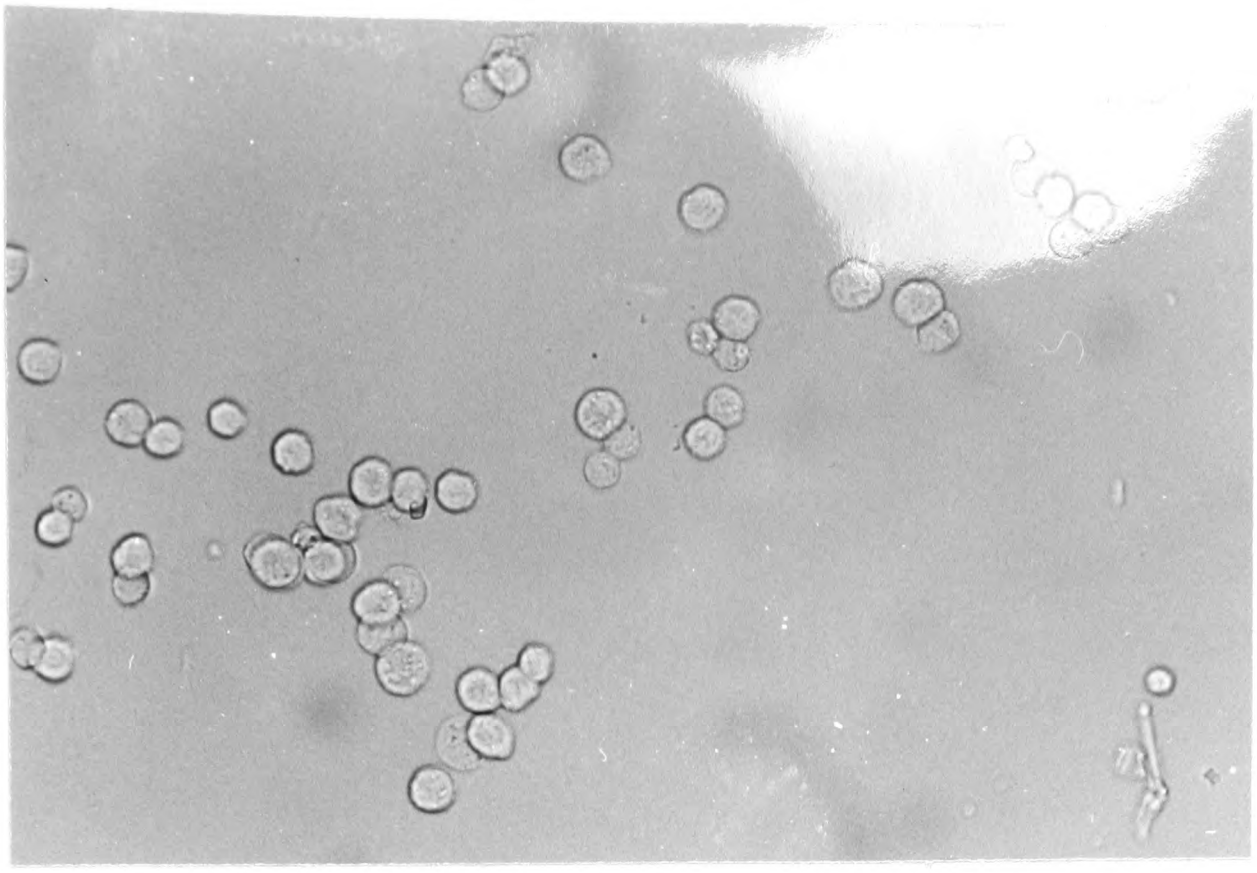

Plate 1a EAT cells treated with rabbit anti-EAT antiserum and stained with sheep anti-rabbit FITC conjugate. Phase contrast, 320 x mag.

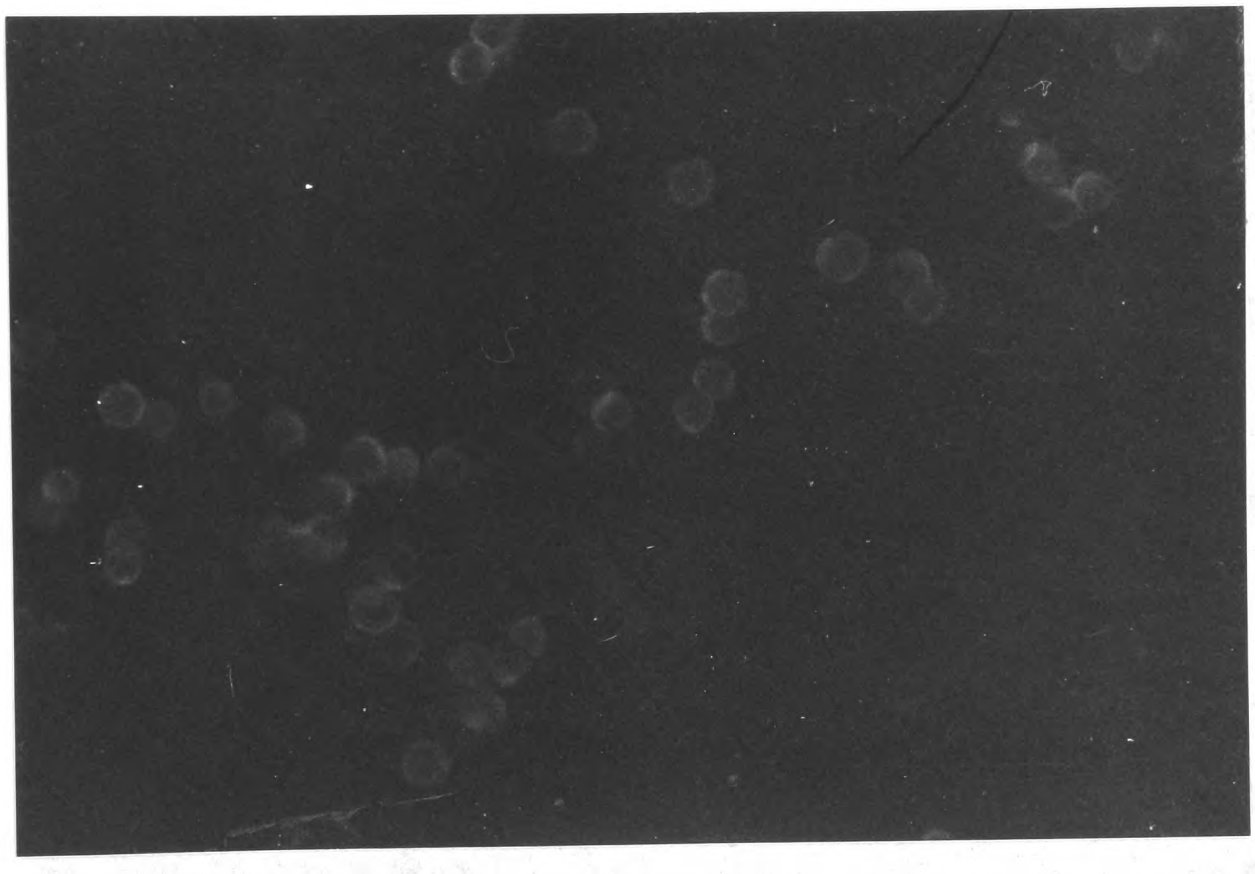

Plate 1b Fluorescence micrograph of cells shown in plate 1a.

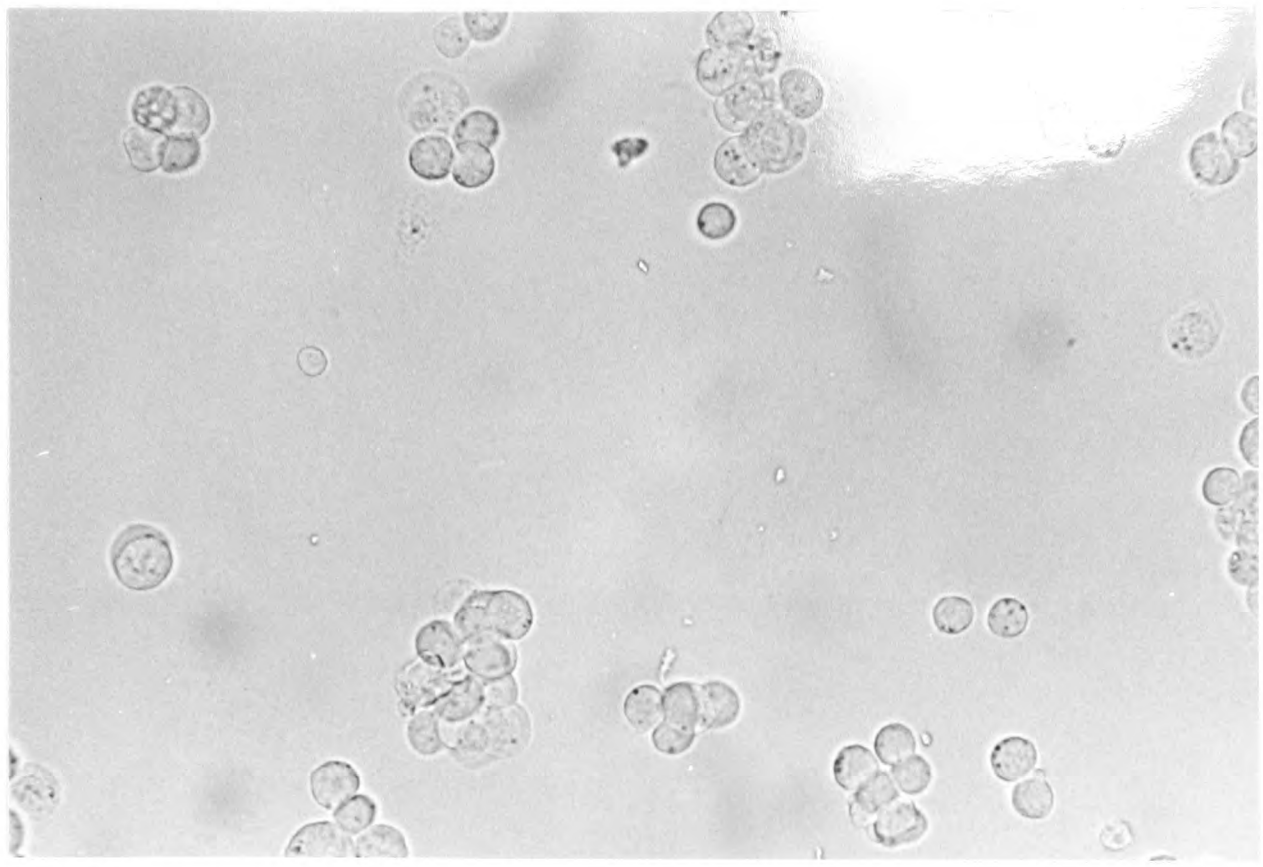

Plate 2a EAT cells treated with rabbit anti-EAT antiserum and stained with sheep anti-rabbit FITC conjugate. Phase contrast, 320 x mag.

Plate 2b Fluorescence micrograph of cells shown in plate 2a.

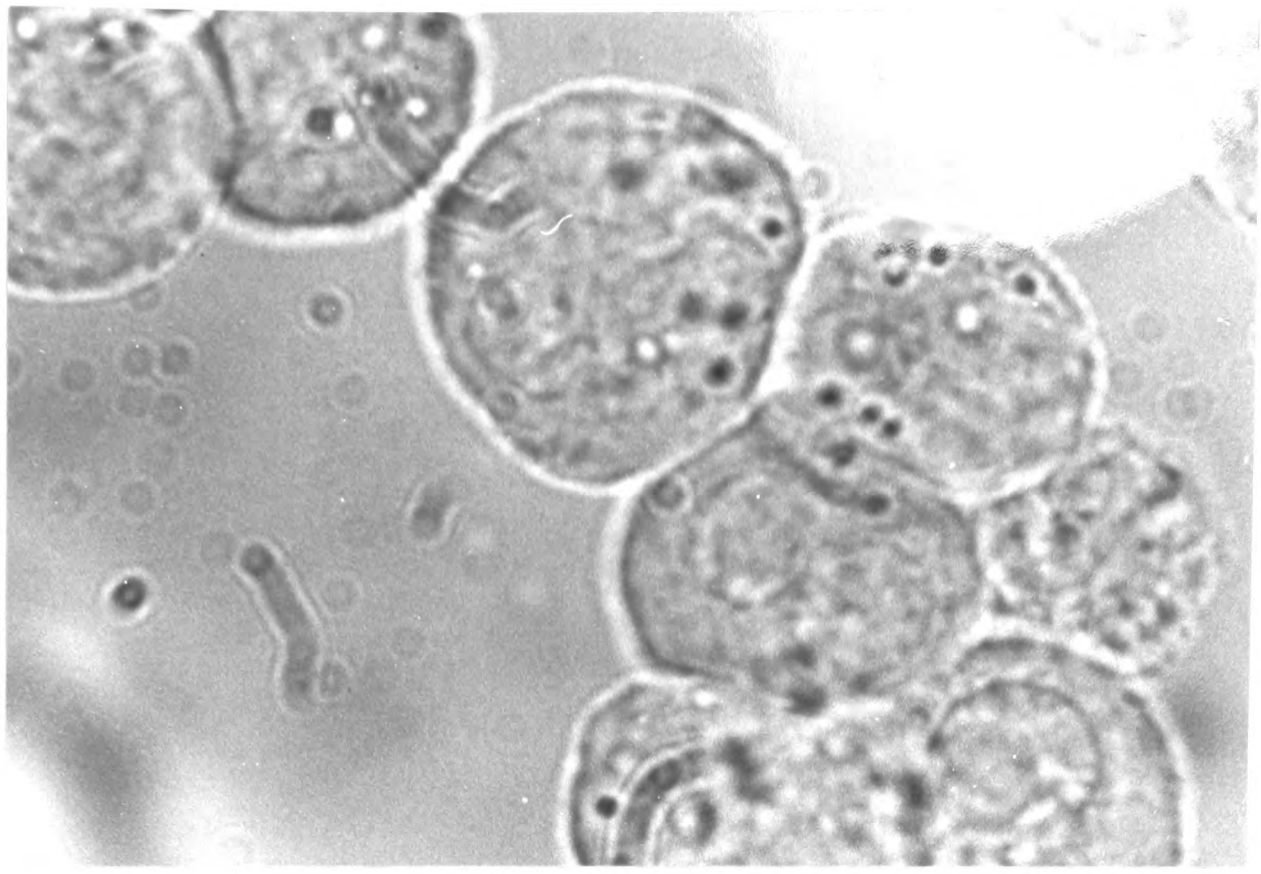

Plate 3a EAT cells treated with rabbit anti-EAT antiserum and stained with sheep anti-rabbit FITC conjugate. Phase contrast, 800 x mag.

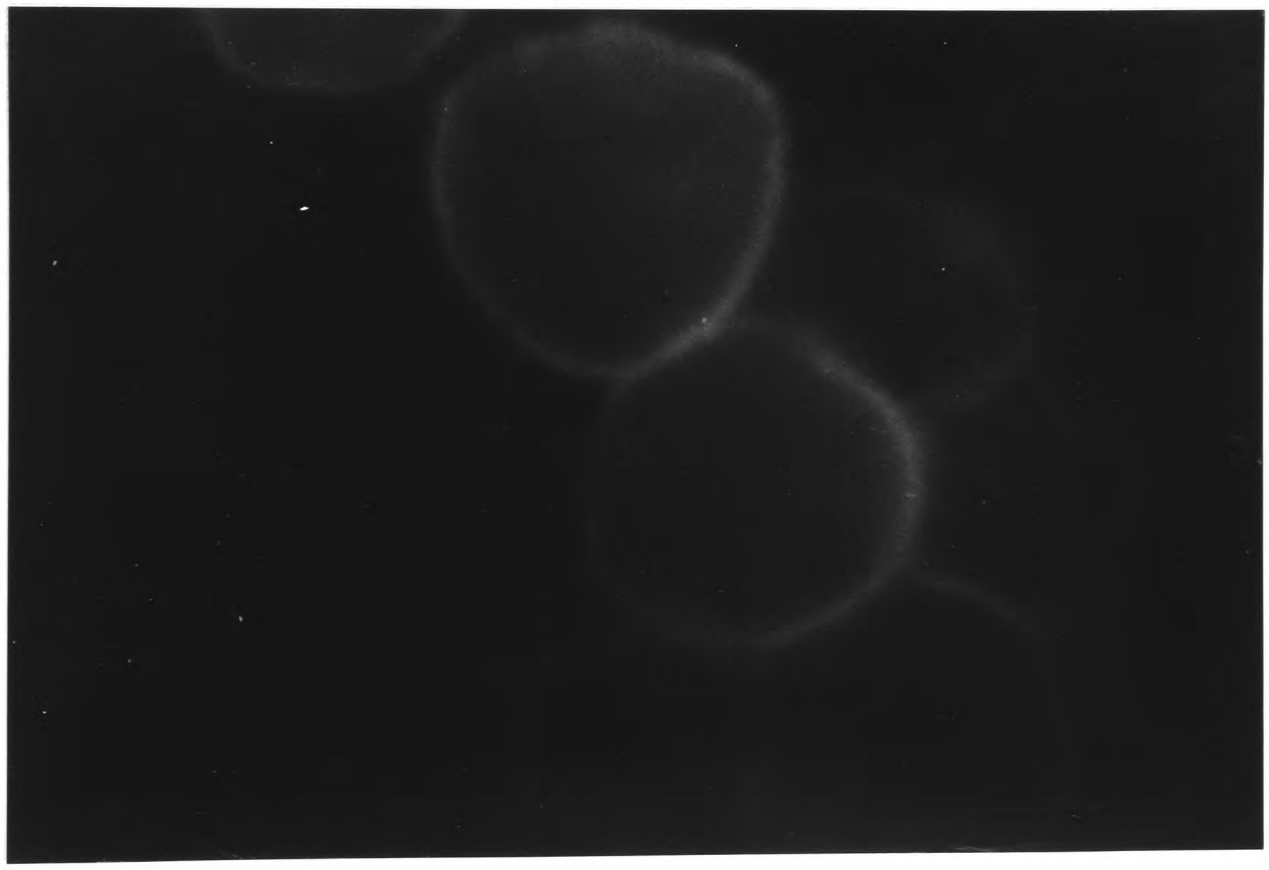

Plate 3b Fluorescence micrograph of cells shown in plate 3a.

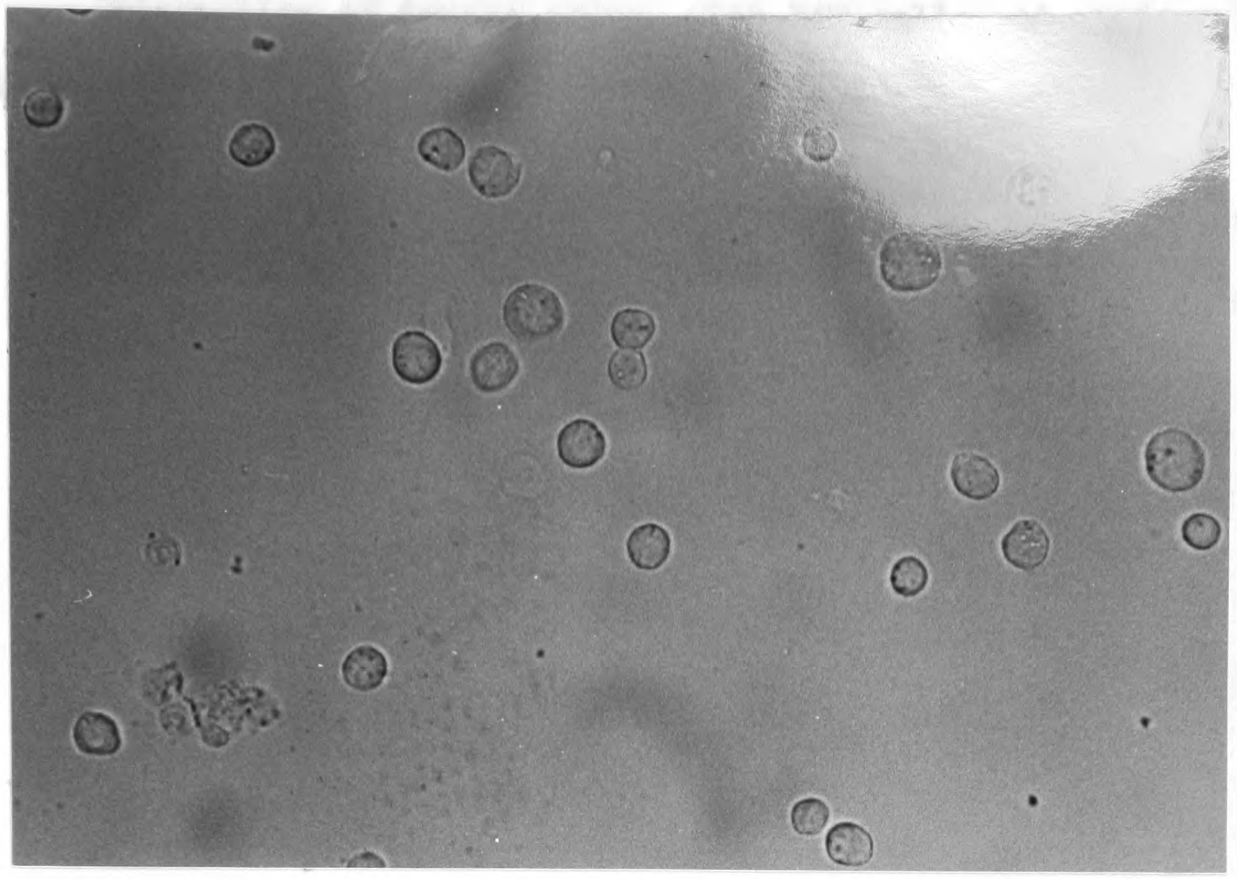

Plate 4a EAT cells treated with NRS and sheep anti-rabbit FITC conjugate. Phase contrast, 320 x mag.

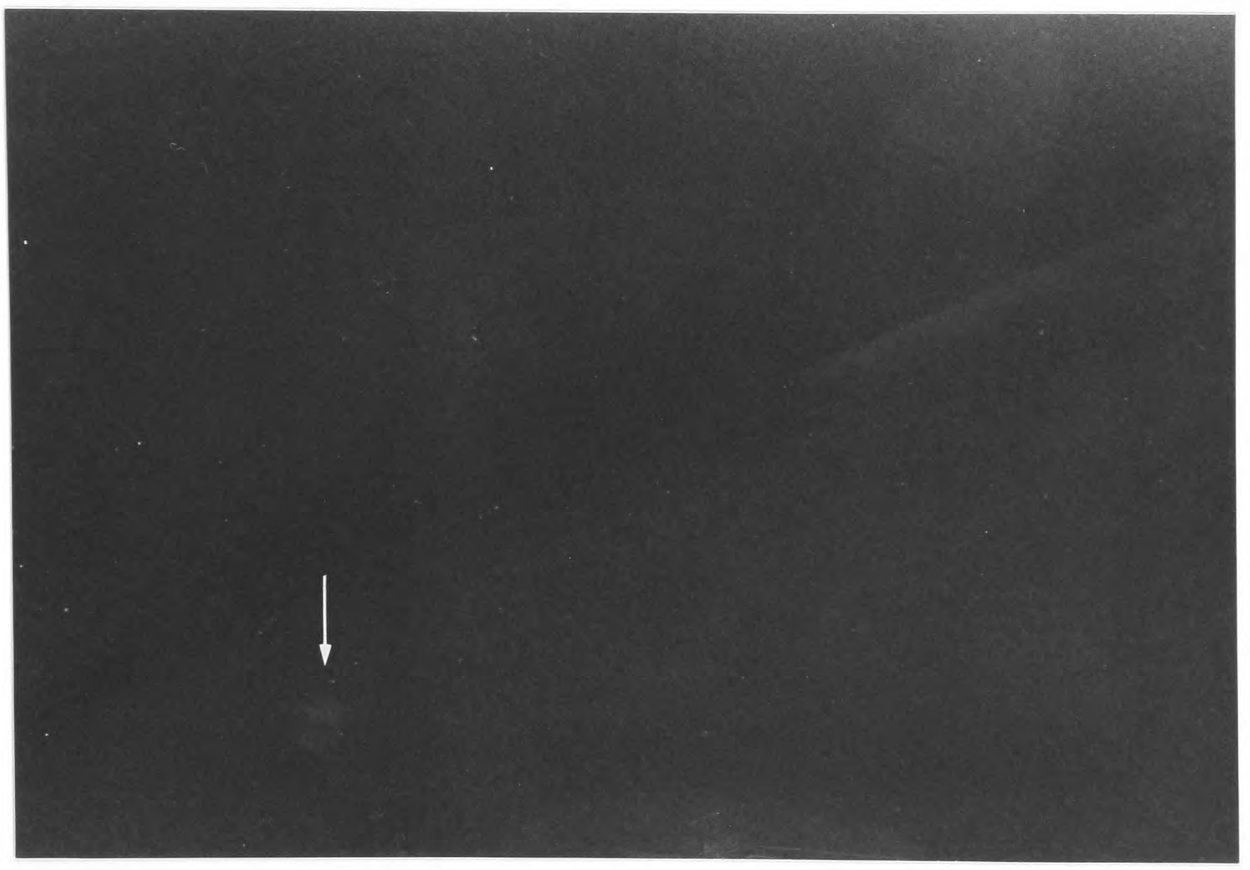

Plate 4b Fluorescence micrograph of cells shown in plate 4a.

Table 17a

Interaction of immune serum, with EAT cells, at various stages of absorption with normal BALB/c tissue homogenates.

	Antiserum dilution				
	1:2	1:10	1:20	1:50	1:100
Unabsorbed	+	+	+	+	-
Absorbed 3 x 2 hr.	+	+	+	-	-
Absorbed 8 x 2 hr.	+	+	+	-	-

Table 17b

Interaction of immune serum, with normal BALB/c mouse lymphocytes, at various stages of absorption with normal BALB/c tissue homogenates.

	Antiserum dilution				
	1:2	1:10	1:20	1:50	1:100
Unabsorbed	+	+	+	+	-
Absorbed 3 x 2 hr.	+	+	+	-	-
Absorbed 8 x 2 hr.	-	-	-	-	-

The antibody titre is apparently low in comparison with some reports found in the literature (Smith et al., 1974, and Ghose et al., 1975). However, these latter studies pertain to other types of cell and the present results are comparable with those reported by Ghose et al. (1967). Further attempts to immunize (immunization procedures ii and iii, Methods section p. 145) failed to improve the antibody titres. Therefore, pooled serum from rabbits immunized according to the method of Ghose et al. (1967) (schedule - i) was used exclusively in further investigations.

It is of note, however, that antisera from different rabbits tended to agglutinate tumour cells to varying degrees, although this aspect of antigen:antibody interaction was not pursued.

Testing antiserum for cross-reactivity against other mouse tumours:

Washed, single cell suspensions of the S-180 ascites and the BP-8 ascites tumours were treated with anti-EAT antiserum (previously absorbed 8 times with normal BALB/c mouse tissue homogenates) prior to immunofluorescent staining. The results of this test indicated that the anti-EAT antiserum was active against the S-180 ascites tumour cells at a dilution of 1:8. No cross-reactivity against the BP-8 ascites tumour was seen.

Further investigation entailed treatment of cryostat sections of a solid S-180 tumour with anti-EAT antiserum prior to immunofluorescent staining. However, no fluorescent staining was seen indicating that cross-reactivity was apparently confined to the ascitic form of this tumour. By comparison a solid form of the EAT used in this study showed fluorescent staining after treatment with anti-EAT antiserum and FITC anti-rabbit conjugate.

In addition it is of interest that absorbing the anti-EAT antiserum with 1/8 - 1/5 volume of S-180 cells for 3 x 2 hr. abolished

Table 18

Interaction of immune serum with various tumour cell types after absorption with BALB/c mouse tissue homogenates or BALB/c mouse tissue homogenates and S-180 ascites tumour cells.

	Tumour type				
Antiserum Preparation	EAT (ascites)	EAT (solid)	S-180 (ascites)	S-180 (solid)	BP-8 (ascites)
Anti-EAT serum 8 x absorbed with BALB/c tissues. Dilution 1:8	+	+	+	-	-
Anti-EAT serum 8 x absorbed with BALB/c tissues and 3 x absorbed with S-180 cells. Dilution 1:8	+	nd	-	nd	nd

nd = not determined

activity against S-180 ascites tumour cells. Anti-EAT antiserum absorbed with S-180 cells remained active against EAT cells. These results are summarised in table 18.

Analysis of immunofluorescent-autoradiographs: Immunofluorescent-autoradiographs were prepared following repeat ^{3}HTdR labelling and immunofluorescent staining which had followed treatment with anti-EAT serum at a 1:8 dilution. Estimations of relative fluorescent intensities were made as previously described. Table 19 contains the pooled results of the analysis of immunofluorescent-autoradiographs prepared for six plateau phase tumours.

Chi-square (χ^2) analysis of these data reveals that there is no reason to reject the null hypothesis; i.e. no significant difference exists between the level of fluorescent labelling between the ^{3}H labelled and unlabelled cell groups.

As an adjunct to this experiment the labelling indices of 3 of the repeat labelled tumours were determined in order to establish the extent to which precoating the smears with gelatin as described interferes with autoradiographic efficiency. Labelling indices of 89.7, 87.3 and 84.5% were recorded. These do not appear to vary substantially from the value of 87.6 $\pm$ 2.7% previously determined after repeat labelling of a 12 day EAT (Ch. 1, p. 41). Perhaps the best method of assessing autoradiographic efficiency is by grain counting. However, this method is usually applied in conjunction with stripping film autoradiography. Since an uneven grain distribution is likely to occur between autoradiographs prepared by the dipping technique, assessment of autoradiographic efficiency by grain counting is devalued for the latter technique.

FACS analysis of fluorescent antibody stained EAT cell populations in different phases of tumour growth: An initial experiment (i) was

Table 19

Percentage distribution of fluorescent intensities for ^{3}HTdR labelled and unlabelled cells after repeat ^{3}HTdR injections and treatment with anti-FAT antiserum and fluorescent antibody staining.

Cell group	Estimated relative intensity				Total cells in group
	-	+	++	+++	
G_o	18	31	47	4	181
Labelled after repeat ^{3}HTdR labelling	12	29	55	5	500

conducted in order to determine a suitable antiserum dilution for use in a second experiment (ii). The second experiment (ii) was an attempt to examine the binding of anti-EAT antibodies to EAT cells at different stages of tumour growth.

(i) Antisera titrations: Plates 5a and 5b indicate cell or particle number (ordinate) against total fluorescence (abscissa) for 6 day populations of EAT cells stained with fluorescent antibody, after treatment with various dilutions of anti-EAT antiserum. Thus, plates 5a and 5b are representative of the fluorescence profiles for tumour cell samples treated with anti-EAT antiserum (at dilutions of 1:2, 1:5, 1:10, 1:30) and with NRS control. The cell populations represented by the results shown in plate 5a had been treated with anti-EAT antiserum which had previously been absorbed with both normal BALB/c tissue homogenates and S-180 tumour cells; whereas those represented by the results shown in plate 5b had been treated with anti-EAT antiserum which had previously been absorbed with normal BALB/c tissue homogenates only.

There is considerable variation in the range of total fluorescence observed for these cells. The range of fluorescence can be seen to increase with decreasing antiserum dilution. This is shown by a flattening of the peak with decreasing dilution (i.e. increasing antibody concentration). Below an antiserum dilution of 1:5 the readings obtained for fluorescence begin to increase again. This increase is associated with a reduction in the readings obtained for cell or particle number at any given fluorescent value, and is probably a result of cellular agglutination. However, a plateau appears to be reached between antiserum dilutions of 1:5 and 1:30.

There is a clear difference between the titration profiles in plate 5a and those in plate 5b which may be related to the qualitative nature of the antisera following absorption with S-180 ascites tumour

cells.

Subsequently, antisera were diluted 1:10 for preparation of light scatter versus fluorescence profiles for tumours in different phases of growth.

(ii) Fluorescence profiles of tumours in different phases of growth: Plates 6a and 6b indicate light scatter versus fluorescence for EAT cells, from tumours of 5 days and 12 days growth respectively, after treatment with S-180 absorbed anti-EAT antiserum and fluorescent antibody. Plate 4c indicates light scatter versus fluorescence for a 5 day tumour cell population after treatment with NRS and fluorescent antibody.

Ostensibly the light scatter and fluorescent characteristics of these two EAT cell populations were similar. The accumulation of small cells on the fluorescence base line (arrow), particularly in the case of the 12 day tumour, probably represents unstained normal peritoneal cells.

This experiment was repeated for 6 day and 13 day tumours using anti-EAT antiserum absorbed against normal BALB/c tissue homogenates only (results given in plates 7a and 7b); this comparison was made in order to reveal possible differences in fluorescent profiles associated with variation in the qualitative nature of the antiserum brought about by the different absorption procedures. There are no gross differences between the fluorescence profiles for the 6 and 13 day EAT cell populations after treatments with anti-EAT antiserum and fluorescent antibody. The existence of a subpopulation of small unstained cells probably normal peritoneal cells, corresponds with the analysis of 5 and 12 day EAT cell populations (Plates 6a and b).

There is perhaps a suggestion of a wider range of fluorescence and associated broader range of light scatter properties for EAT cells after staining with S-180 absorbed antiserum (comparison of plates 6a, b with plates 7a, b).

Plate 5a Analysis of the fluorescence distribution of 10^4 EAT cells following treatment with various dilutions of anti-EAT antiserum (absorbed with normal BALB/c tissue homogenates and S-180 ascites tumour cells) and fluorescent antibodies.

Ordinate: Total fluorescence (gain 1.0).
Abscissa: Cell number.

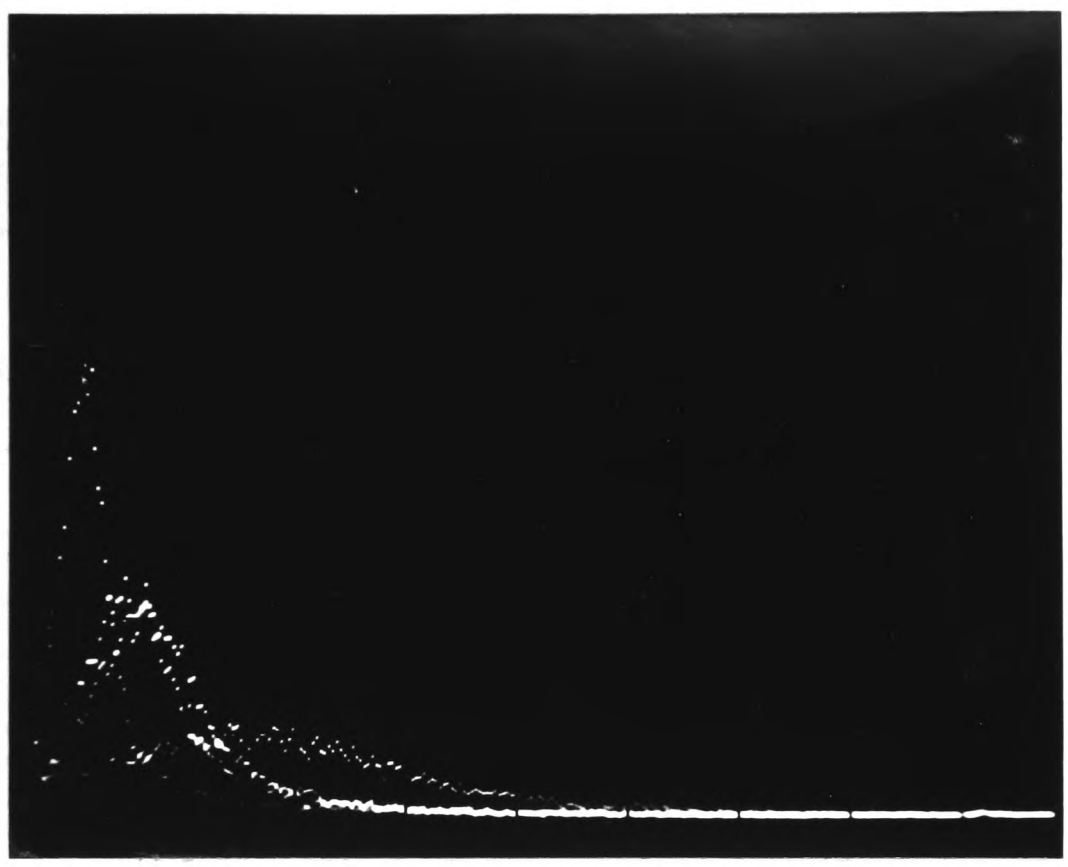

Plate 5b Analysis of the fluorescence distribution of 10^4 EAT cells following treatment with various dilutions of anti-EAT antiserum (absorbed with normal BALB/c tissue homogenates only) and fluorescent antibodies.

Ordinate: Total fluorescence (gain 1.0).
Abscissa: Cell number.

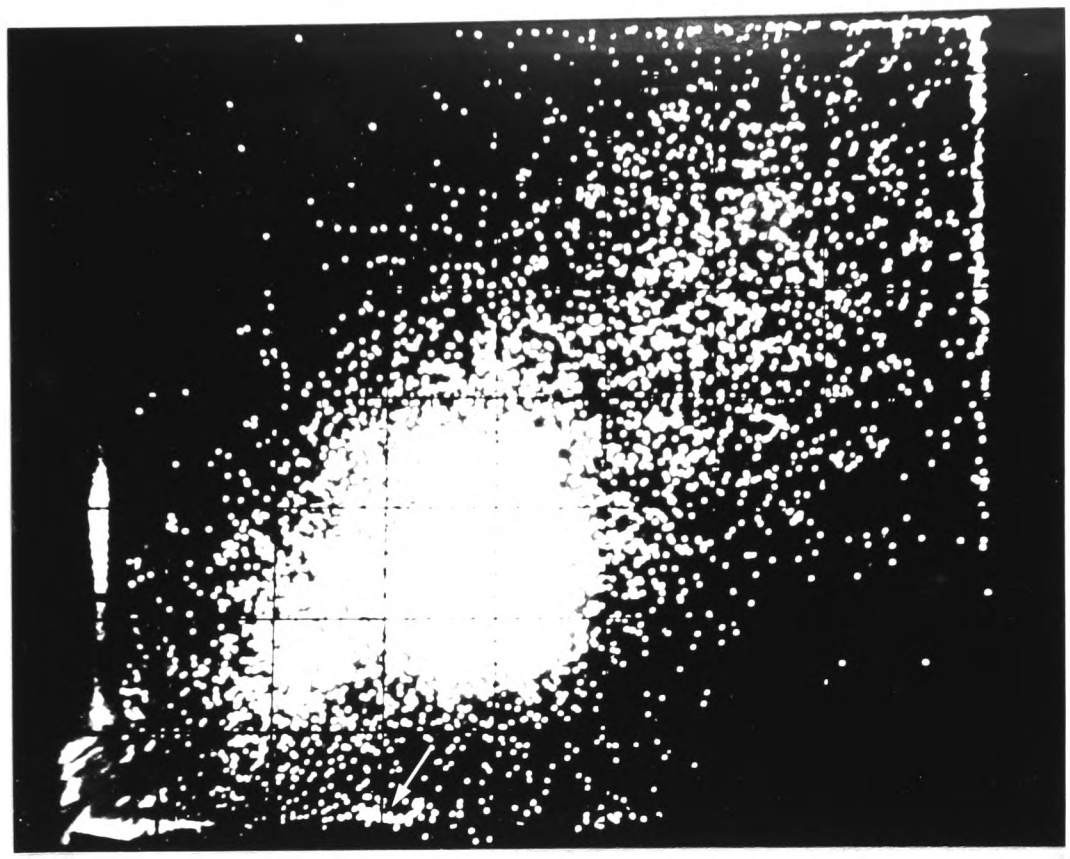

Plate 6a Fluorescence profile of a 5 day EAT following treatment with anti-EAT antiserum (S-180 absorbed) and fluorescent antibodies.

Ordinate: Light scatter (cell size) (gain 1.0).
Abscissa: Total fluorescence.

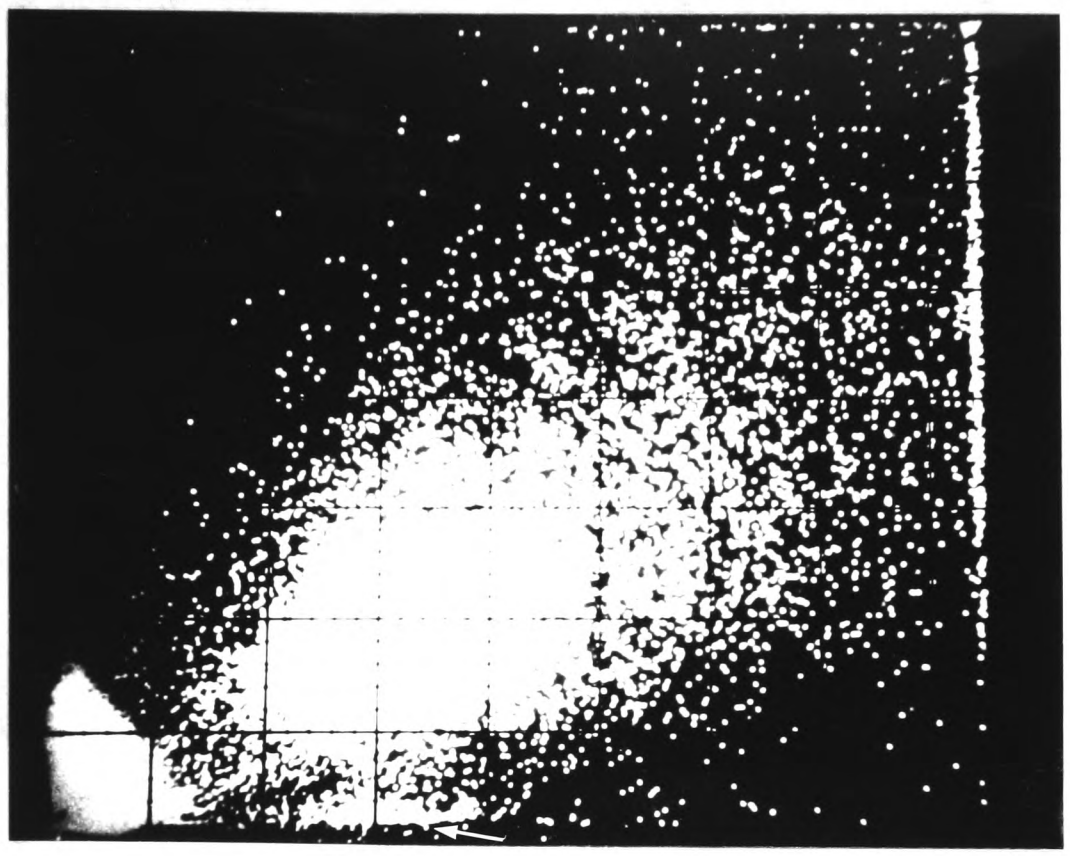

Plate 6b Fluorescence profile of a 12 day EAT following treatment with anti-EAT antiserum (S-180 absorbed) and fluorescent antibodies.

Ordinate: Light scatter (cell size) (gain 1.0).
Abscissa: Total fluorescence.

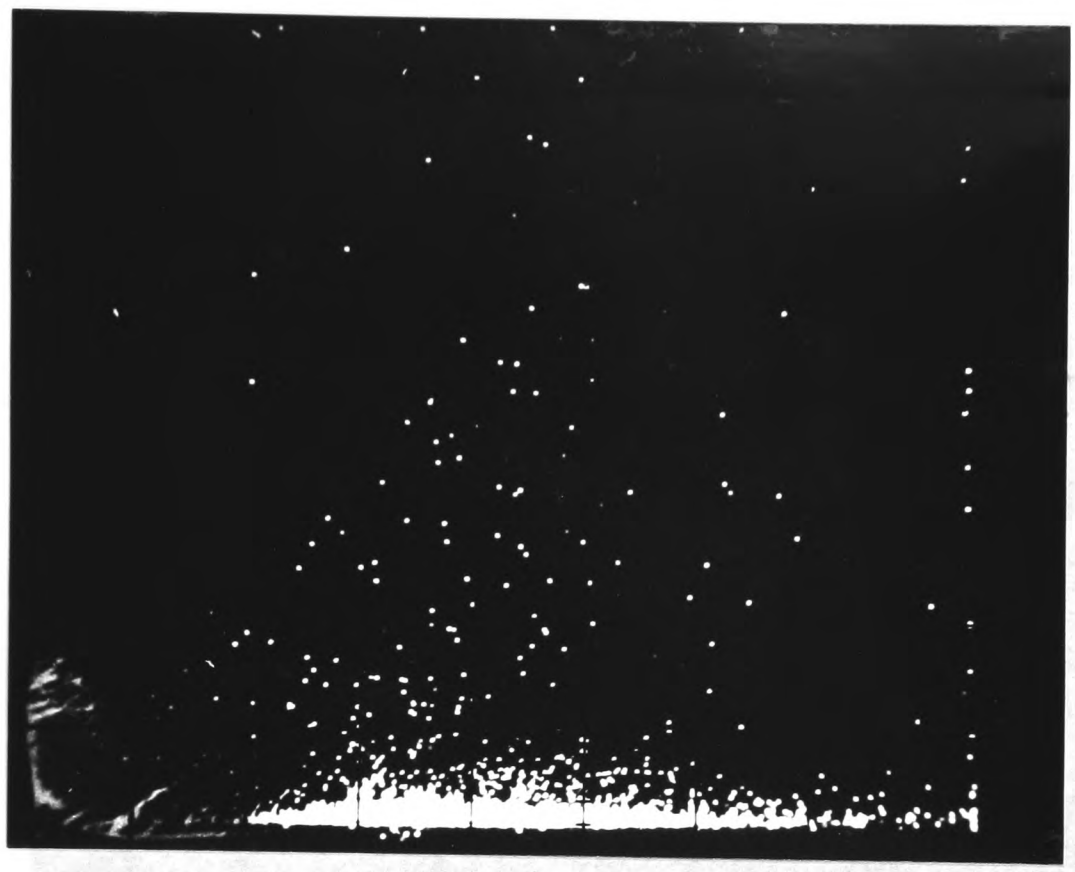

Plate 6c Control tumour treated with NRS in place of anti-EAT antiserum.

Ordinate: Light scatter (cell size) (gain 1.0).
Abscissa: Total fluorescence.

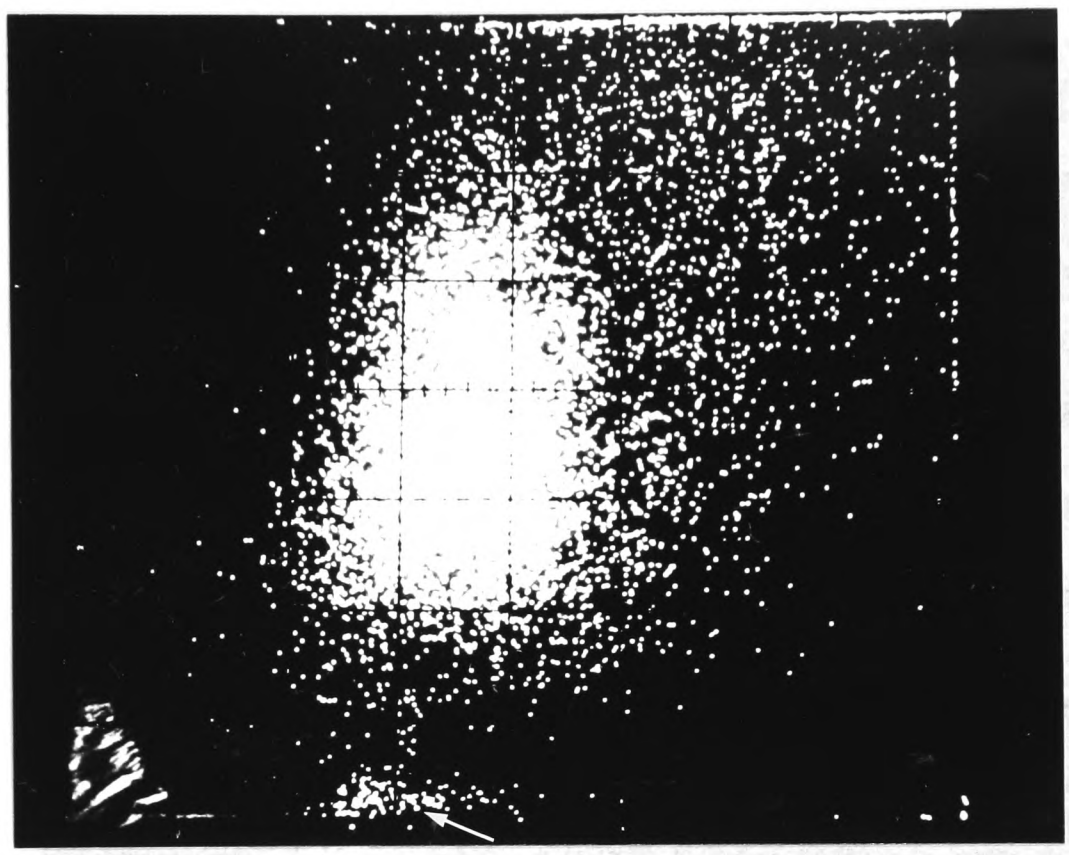

Plate 7a Fluorescence profile of a 6 day EAT following treatment with anti-EAT antiserum (normal BALB/c tissue absorbed) and fluorescent antibodies.

Ordinate: Light scatter (cell size) (gain 1.0).
Abscissa: Total fluorescence.

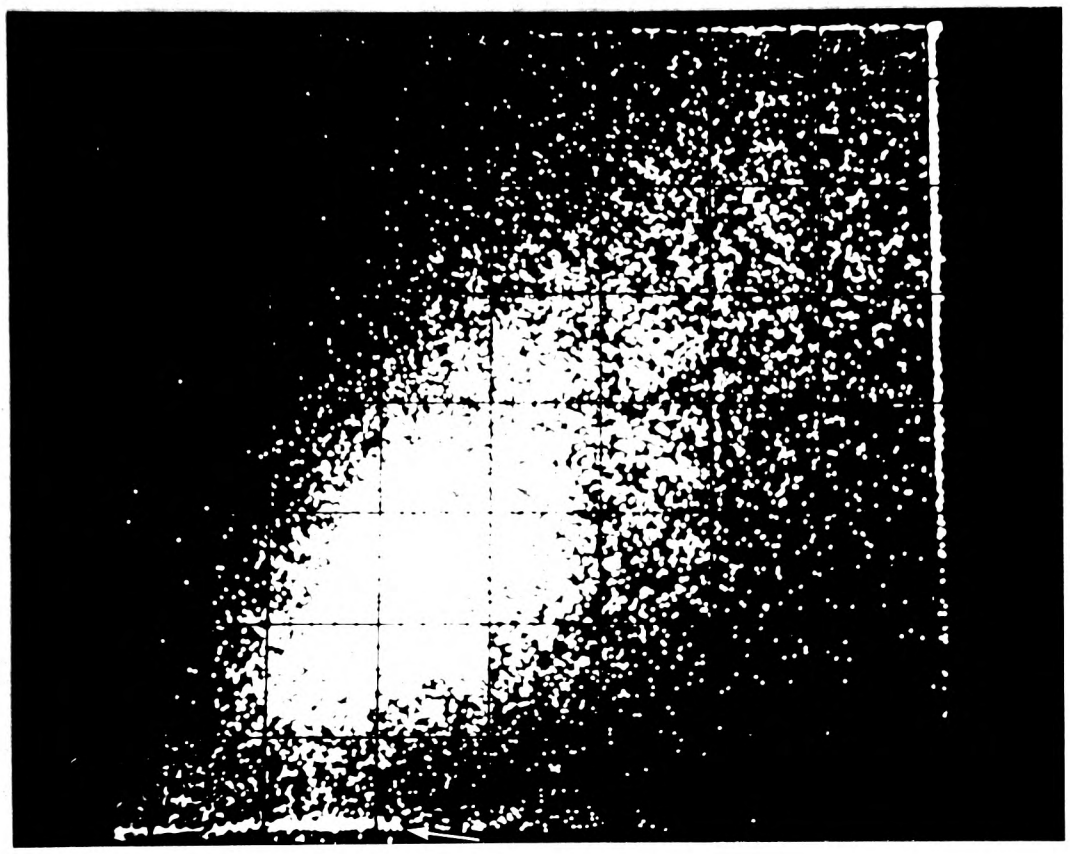

Plate 7b Fluorescence profile of a 13 day EAT following treatment with anti-EAT antiserum (normal BALB/c tissue absorbed) and fluorescent antibodies.

Ordinate: Light scatter (cell size) (gain 1.0).
Abscissa: Total fluorescence.

Plate 7c Control tumour treated with NRS in place of anti-EAT antiserum.

Ordinate: Light scatter (cell size) (gain 1.0).
Abscissa: Total fluorescence.

Cytotoxicity assay: None of the antisera produced according to the immunization procedures outlined on p.148 could be shown to have cytotoxic activity. Cytotoxic activity was apparently lacking irrespective of the source of complement since attempts to induce cytotoxicity included the use of rabbit, guinea-pig and mouse complement.

Several batches of NRS exhibited strong non-specific (antibody independent) cytotoxicity against FAT cells prior to complement fixation.

Discussion

The result of immunizing rabbits against the EAT was the production of xenogenic antisera with relatively low titres of anti-EAT antibodies (1:20 after absorbing with normal BALB/c mouse tissue homogenates). Naturally, this could be attributed to a partial failure of the immunization protocols used here to promote an optimal immune response. Alternatively, this may be due to the antigenic nature of the immunizing agent, namely the EAT cells. For example, it is considered that EAT cells are of generally low antigenicity (Wang and Halliday, 1967). The nature of immune mechanisms in EAT bearing mice is poorly understood (Marusic, 1979), although immunity to this tumour can be induced in some strains of mice (Donaldson and Mitchell, 1959; Apffel et al., 1966, and Turcotte, 1979). There is evidence to suggest that the immunological reactivity against the EAT in tumour bearing mice is not directed against H-2 antigens (murine major histocompatibility complex antigens) (Thunold, 1968), and yet further evidence to suggest the complete absence of these antigens in EAT cells (Chen and Watkins, 1970). It seems highly probable that serial passage of the EAT over a long period (possibly in excess of 50 years) has resulted in a progressive immunoselection (Hauschka et al., 1956; Vaage, 1978 and Chow and Greenberg, 1980). Such a process would lead to the production of a weakly antigenic tumour cell line by elimination of those cells bearing strong antigens. Hence the allotransplantability of EAT cells (Dux et al., 1967) could be seen to be a consequence of the loss of those cells bearing H-2 type and other antigens.

Chose et al. (1967) raised antisera,in rabbits against EAT cells, which were of low titre (1:8) and the antigen:antibody interaction was detected by a gel diffusion method which is known to be less sensitive

than indirect immunofluorescence. Subsequently, Chose and Cuclu (1974) raised xenogenic antiserum to EL4 murine sarcoma cells. This antiserum was absorbed more extensively than the anti-EAT antiserum had been and with normal mouse tissue (C 57 BL/6J), which is probably more closely related genetically to the EL4 than the BALB/c is to the EAT (host used in the present work). The antiserum so produced was found to have a titre in excess of 1:64 by indirect immunofluorescence. Later Chose et al. (1979) raised antiserum against renal cell carcinoma; this antiserum was absorbed extensively (8 x 2 hr., with normal human tissue homogenates). Ultimately, the antiserum was active against antigens associated with human renal cell carcinoma. In this case no precipitin arcs were detected by a gel diffusion technique, and antibodies directed against tumour associated antigens (TAA) were only detectable by indirect immunofluorescence.

The results of work presented by Chose et al. (1967, 1974 and 1979) further illustrate the difficulty of demonstrating high titre antisera to weak antigens. The additional possibility exists that whole cell inoculation in rabbits confronts the animal with a mixture of antigens which causes antigen induced suppression (Pross and Eidinger, 1974).

Further differences between the natures of the anti-EAT antiserum produced here and that produced by Chose et al. (1967) concern other less direct anti-tumour cell activities. Chose et al, (1967) reported that anti-EAT antiserum was cytotoxic to EAT cells in the presence of xenogenic complement as evidenced by indirect immunofluorescence after fixing of target cells in acetone at -10°C. In contrast the antiserum produced here was not cytotoxic. Xenogenic anti-tumour antisera are generally reported to be cytotoxic <u>in vitro</u> in the presence of xenogenic

complement, however, resistant cell types are not unknown (Currie, 1974a and Ghose et al., 1974), and such behaviour may be more closely allied to anti-tumour mechanisms in vivo (Currie and Basham, 1972, and Baldwin, 1973). Ghose et al. (1967) demonstrated cytoplasmic staining after treatment of EAT cells with specific antiserum and fluorescent antibodies. In contradiction to this earlier work, indirect immunofluorescent staining here was largely confined to the peripheral membrane, diffuse cytoplasmic staining being considered to be non-specific and associated only with non-viable cells (Goldmann 1968, and Gikes, 1970b). This discrepancy is likely to arise from the fixing procedure used by Ghose et al. which was not used here.

The agglutinability of the present antiserum is demonstrated in plates 1 and 2, and is discussed later in relation to FCM analysis.

The precise genetic origin of the EAT is not known. It is probable, therefore, that this tumour is non-syngeneic with respect to the BALB/c hosts used here and indeed with the majority if not all strains of laboratory mice. The implications are that xenogenic antibodies directed against murine isoantigens, viral antigens, and oncofoetal antigens in addition to tumour specific antigens could remain in the anti-EAT antiserum following absorption of whole antiserum with normal BALB/c tissue homogenates. Thus the specific immunofluorescence staining observed here could be accounted for by antigen:antibody interactions involving any, or a combination of any of these antigen types.

The cross-reactivity of the anti-EAT antiserum with S-180 ascites tumour cells but not with solid S-180 tumour cells is rather curious. The nature of the observed cross-reactivity is by no means certain and it is appreciated that such cross-reactivity could be the result of the expression of common isoantigens, viral antigens or oncofoetal antigens.

However, this result infers that either preferential expression of a common antigen is restricted to the ascites form of the S-180 tumour or that a contaminating virus is common to the EAT studied here and the ascitic form of the S-180 tumour obtained from ICRF. This latter suggestion is valid since the solid and ascitic forms of the S-180 are maintained separately whereas the solid form of the EAT was produced here by subcutaneous inoculation with the corresponding ascites tumour cells.

The cross-reactivity of anti-EAT antiserum with the S-180 ascites tumour confirms a report by Littman et al. (1968) who observed that immunization of mice against lethally irradiated S-180 ascites tumour cells afforded some protection against subsequent sc challenge with EAT cells.

The result of absorbing anti-EAT antiserum with S-180 ascites tumour cells was the production of an antiserum containing antibodies directed against antigens more specifically related to the EAT. This conclusion was reached since absorbing anti-EAT antiserum with S-180 ascites tumour cells abolished anti-S-180 activity but not anti-EAT activity. However, on the basis of the FACS analyses involving these different antisera it was impossible to conclude the existence of any qualitative or quantitative variation between them.

Immunofluorescent analysis of labelled and unlabelled tumour cells following repeat ^{3}HTdR injections to the donor tumour revealed no overall antigenic variation between G_0 cells and the labelled fraction. This conclusion was derived from the observations that the two tumour cell groups studied (labelled and unlabelled after repeat ^{3}HTdR injections) contained cells which could be ascribed to all grades of fluorescence and in similar proportions. This result was supported by results of preliminary experiments using the FACS. No gross antigenic

variation was observed between rapidly proliferating tumour cell populations after 5 or 6 days of tumour growth and plateau phase tumour cell populations after 12 or 13 days of tumour growth. This, by implication, is evidence that the G_0 tumour cell population which exists in plateau phase is not antigenically distinguishable from rapidly cycling tumour cells.

These conclusions cannot go unqualified since certain objections concerning the experimental techniques can justifiably be raised. Pertaining specifically to the repeat labelling analysis, it might be argued that any variation in antigenicity between G_0 and G_+ cells would be masked by the existence within the labelled fraction of some G_0 cells, a problem which has already been discussed in relation to RNA analysis. Lack of specificity of anti-EAT antiserum could have a serious effect on immunofluorescent analysis. Despite the fact that the EAT is weakly antigenic and following the elimination of some antibodies directed against murine isoantigens, the possibility exists that residual antibodies directed against non-BALB/c related isoantigens consequently reduce the specificity of the anti-tumour antiserum and ultimately affect the sensitivity of immunofluorescent analysis. Thus analysis both by FACS and by correlation of fluorescence with ^{3}HTdR autoradiography may be insensitive to minor variations in degree of antigenicity. However, even after the antiserum was absorbed against the S-180 ascites tumour, eliminating more of the less specific antibodies to the EAT, no antigenic variation between 5 day and 12 day tumour cell populations was seen. The relatively good conditions of oxygenation <u>in vitro</u> could also have an effect on antigenic expression. The possibility that <u>in vitro</u> incubation could have a stimulatory effect on EAT cells has previously been discussed, (also see Loffler et al., 1978 and Merz and Schneider, 1980). If such a stimulatory effect was characterized by early cell surface changes, as has previously been suggested (Costlow and Baserga, 1973), it is possible that any

variation in antigenic expression occurring *in vivo* would be masked following the *in vitro* manipulations imposed on the tumour cells that are necessary for FCM and immunofluorescent/autoradiographic analysis. This effect could explain the disparity between antibody mediated tumour cell cytotoxicity *in vitro* and tumour presistence *in vivo* which is commonly observed (Currie, 1974a).

The results of immunofluorescent analyses, as far as they can be taken to be a measure of the relative antigenicities of G_0 and rapidly cycling tumour cells, in this case provide no evidence for antigenic variation between the two cell groups. These results are in agreement with data provided by Pellegrino et al. (1974) who found no antigenic variation to be associated with the phases of cell cycle in cultured mammalian neoplastic cells. These results could be explicable on the basis that antigenic variation which results in immunoresistance is not related to cell cycle events per se, but arises spontaneously in tumour cell populations, possibly as a consequence of gene suppression or a mutational event. This implies that immunological or antigenic modification is part of the process of immunoselection, and that the host, therefore, has a role in the progression or development of a tumour. Metastases are known to be generally less antigenic than primary tumours (Cochran, 1978c and Fogel et al., 1979) (facilitating circumvention of the immune response) and they might arise through an immunoselection process similar to that described above.

General Discussion

The overall growth characteristics of the EAT studied here serve as a general confirmation of the previous studies of Klein and Revesz (1953), Lala and Patt (1966) and Peel and Fletcher (1969). The rate of tumour growth was found to be exponential over the first 2-3 days following 5×10^6 tumour cell inoculation. This growth pattern gave way to a period of cube root growth and ultimately a plateau phase ensued. The reduction in tumour growth rate was shown to be an immediate consequence of an increase in the duration of the cell cycle, a reduction in the growth fraction and an increase in cell loss. In addition the cube root growth pattern was highly indicative of growth limitation due to restricted nutrient and oxygen availability.

A feature of ascites tumours, which is fundamental to a study of G_o cells, is the possibility of transient contact of cells with the vasculature (a source of nutrients) and consequent recycling of G_o cells in situ. The present results, and those obtained by Lala (1972b) indicate that this recycling phenomenon does not occur to any detectable extent during the plateau phase of EAT growth.

The apparent evolution of increasing heteroploidy during growth of the present tumour, as shown by microdensitometric analysis but not revealed by chromosome analysis, cannot be discounted as being contributory in part to the declining tumour growth rate (see discussion, ch. 2, p. 126).

The concept of the G_o tumour cell, although long considered to be a major problem in cancer chemotherapy (Perry, 1976), does not appear to have gained universal acceptance, particularly in the sphere of tissue culture where various studies have indicated that transformed cells are incapable of entering a G_o phase (Baserga, 1976c). This disparity may be seen as a genuine difference between the growth conditions encountered in vitro and those found in vivo (Boffa et al.,

1981). It has been shown that experimental tumour cells in culture can undergo a period of proliferative inactivity before being re-stimulated (a G_o phase) (Merz and Schneider, 1980). However, it is not clear whether the G_o phase is a preferential pathway leading to cell death. In the present work and that of Siracka and Pappova (1978) the result of repeat ^{3}HTdR administration during the plateau phase of tumour growth was a gradual, possibly asymptotic, approach of the labelling index to 100%. Thus it was concluded that the G_o state does not represent a preferential pathway leading to cell death (Steel, 1977d). Additionally, the finding that the non-cycling tumour cells have reduced RNA and protein contents is further evidence for the existence of tumour cells arrested in a genuine G_o phase.

Analyses of the cell cycle data and of the kinetics of re-entry of G_o cells into cell cycle upon restimulation, together with cyto-photometric investigation, suggest the coexistence of distinct sub-populations of G_o tumour cells similar to those envisaged by Gelfant (1977). However, a lack of sufficiently detailed kinetic information makes it impossible to distinguish between near diploid G_o cells arrested adjacent to the G_2 phase and near tetraploid G_o cells arrested adjacent to the G_1 phase.

A theory such as that of Gelfant suggesting sub-groups within the G_o population can be viewed as an extension of the proposition of Augenlicht and Baserga (1974) for depth within the G_o state.

These observations and conclusions serve to emphasize the importance of a better understanding of the nature of G_o tumour cells.

In the context of the above discussion the EAT would seem to possess other advantageous features as a model system. Growth occurs as a single cell suspension facilitating sampling, maintenance and experimental control. The rapid growth of the tumour although uncharac-

teristic of most if not all human tumours, is a necessary feature for easy experimental design. Other features of this tumour; its heterogeneity with respect to chromosome number and its low immunogenicity, correspond generally with the characteristics of human tumours. On the other hand the use of a syngeneic ascites tumour would probably represent an improvement with regard to the immunological studies.

It was stated previously (p. 9) that the Ehrlich and other ascites tumours have been widely used in many areas of cancer research. Despite this and the versatility of ascites tumours, their use has not been without criticism (Hewitt, 1978). The most important criticism concerns the use of allografted tumours (where the precise genetic origin of a tumour is undefined and dissimilar to the host strain used to bear the tumour) in cancer therapy studies. It is highly questionable whether the results of such therapy studies have any relevance for human cancer therapy since it is generally accepted that human tumours are rarely immunogenic to any degree in the autochthonous host (Hewitt and Blake, 1978). In addition a general problem encountered in the use of any transplantable tumour or cell line in culture is the inadvertant introduction of viral infection, possibly altering immunological and metabolic parameters and cellular proliferation kinetics. From this view-point the EAT parallels any other comparable system. Notwithstanding such criticisms present results would appear to indicate that G_o tumour cells are not antigenically distinct from rapidly cycling tumour cells. Consequently G_o tumour cells are probably equally sensitive to immunological attack as are rapidly cycling tumour cells. This observation provides a rational basis for continuing efforts to improve immunotherapeutic protocols since the G_o cells, by virtue of their reduced metabolic status, are resistant to chemotherapy and radiotherapy.

In broader context, timing in the planning of cancer treatment, is of well recognized importance. Surgery, radiotherapy or any other relevant treatment needs to be instituted as early as possible in the treatment of primary tumours. On the basis of the relevant kinetic characteristics of a given tumour, major advances have been made in development of chemotherapy utilizing sequential drug administration schedules. As far as immunotherapy is concerned the major limitation is that its efficiency is only manifest in the presence of residual tumour masses. It is important, therefore, to use immunotherapeutic techniques in conjunction with other therapies and obviously there is the need for appropriate timing (Eckhardt, 1978).

With regard to the above mentioned factors it is worthwhile considering some of the wider aspects of tumour biology which might be classified as host mediated influences, and which it is envisaged might ultimately have considerable bearing on tumour growth, development and therapy.

There is a great deal of evidence in the literature to suggest the existence in man and animals of diurnal or circadian rhythms. Indeed, circadian rhythms have been demonstrated in various types of tissue (Burns and Scheving, 1975 and Clausen et al., 1979) including tumour tissue. Brown and Burns (1973) and Brown (1974a) demonstrated measurable circadian variation in both the exponential phase and near plateau phase EAT. Subsequently, Brown (1974b) advanced evidence suggesting the involvement of the host immune response in the establishment of circadian stereotypy after transplantation of EAT. These results lead to the conclusion that tumour development is probably under the influence of host circadian rhythms in a process mediated by the immune system. If this is the case it is interesting to speculate that a general feature of tumours may be the non-existence of intrinsic

circadian rhythm.

Stress may also be involved in adverse modulation of immune mechanisms. It has been documented that the imposition of stress leads to a depletion of cholesterol and ascorbic acid in the adrenal gland resulting from an increase in corticosteroid output, (Tepperman, 1973). This reaction is likely to suppress the host immune response (Ratte et al., 1973). Further to this, the general well-being of the cancer patient is now considered to be an important facet of successful therapy (Stoll, 1979).

Indirect hormonal involvement in tumour development (that is in such instances where the hormones are neither promoters nor pre-requisites for tumour growth) might also be mediated via an immune response. Hartveit (1965) indicated that female mice generally display greater immune resistance to tumour growth than do male mice. In accordance with the above mentioned factors most biological investigations involving whole animals, such as with the present investigation, are performed on male animals, are standardized chronologically (as far as is practicable) and an attempt is made to minimize stress on the animals.

In view of the evidence in the literature it is logical to associate circadian rhythm, stress and hormonal considerations with optimization of cancer therapy. Moreover, experimental data exist in support of this view. The survival and cure of L1210 inoculated mice after treatment with cyclophosphamide and cytosine arabinoside have been shown to be dependent on administration of the drugs at an appropriate circadian stage (Scheving, 1977). These points represent a wider view of probable host influences on tumour development which could potentially be manipulated to enhance the beneficial effects of therapeutic protocols. It would be difficult, however, to appreciate fully

the role of stress factors, circadian rhythms, indirect hormonal effects and other subtle influencing factors until some of the more fundamental aspects of tumour biology are comprehended.

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