BIOCHEMICAL STUDIES
ON
MAST CELL TUMOURS
IN CULTURE

by

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ABSTRACT

1. The major part of the life cycle of actively growing and dividing cells, often referred to as interphase, is the period during which all cell components are duplicated so that during the brief mitotic period, the cell can split into two similar daughters. This thesis describes an investigation into the processes of cell component duplication during the interphase of cultured neoplastic mast cells.

2. Chapter 1 sets out the concept of partitioning the cell cycle into 4 phases: G_1, S(DNA synthesis), G_2 and M(mitosis). It contains a survey of techniques used to study the life cycle of cells, concentrating especially on the synchronization of mammalian cells in culture. Chapter 2 describes the general experimental techniques used throughout the study.

3. Chapter 3 describes the development of gradient centrifugation as a method (i) to produce synchronously-growing cells and (ii) to obtain large numbers of suspension culture cells at specific stages of the cell cycle.

(a) Cells collected from an exponentially-growing culture were centrifuged on a Ficoll gradient. Fractions from various areas of the gradient incubated in fresh growth medium grew synchronously as judged by cell number, relative volume, mitotic index and thymidine incorporation.

Thus small cells from the upper region of the gradient had growth characteristics expected of G_1 cells while larger cells from the lower region behaved in the manner expected of G_2 cells. Cells taken after mixing the whole gradient showed an exponential/growth curve typical of unsynchronized cells. Thus gradient centrifugation does not subject cells to adverse physiological effects.

(b) Analysis of cells pre-labelled with thymidine and separated on the gradient confirmed that cells near the top were in G_1, cells near the bottom in G_2, and, in addition, showed that those in the middle were in the period of DNA synthesis (S).

(c) Limitations of 'conventional' gradient centrifugation, such as poor resolution at the S/G_2 region and low yield, were overcome by using a slow-speed zonal rotor.

4. Chapter 4 describes how gradient centrifugation has been applied to the study of the synthesis of phospholipids in relation to that of protein, RNA and DNA during the cell cycle.

(a) Analysis of cells pre-labelled with appropriate precursor and separated by conventional gradient centrifugation showed that protein, RNA and phospholipid synthesis were continuous throughout the cycle and that the rates of synthesis began to increase already during G_1. The pattern of phospholipid degradation followed that of synthesis.

(b) Analysis of pre-labelled cells separated by zonal centrifugation confirmed the results obtained by conventional centrifugation and in addition showed that the rates of protein, RNA and phospholipid synthesis reached a maximum in late S, decreasing again during G_2. The net amounts of protein, RNA and phospholipid, unlike that of DNA which increased relatively sharply, were found to increase continuously throughout the intermitotic period.

(c) These results show that phospholipid and macromolecular synthesis, and possibly membrane construction, are controlled by a mechanism other than gene dosage.

5. In Chapter 5, the work on the synthesis of macromolecules is extended by investigating changes in specific proteins, i.e. enzymes. The enzymes chosen were representative of different cell components so that an indication of duplication of intracellular organelles might be obtained.

(a) Two soluble cytoplasmic enzymes (lactate dehydrogenase and glucose-6-phosphate dehydrogenase), a microsomal enzyme (NADPH cytochrome c reductase) and two inner mitochondrial membrane enzymes (cytochrome c oxidase and succinate cytochrome c reductase) were found to show a rather similar variation in concentration (activity/cell) during the cycle, namely an increase starting in G_1, continuing through S and reaching a maximum in late S/G_2. Thus

they followed a pattern generally resembling net protein synthesis. Under certain circumstances, lactate dehydrogenase showed fluctuations superimposed upon a steady increase.

(b) A soluble enzyme of the mitochondrial matrix, glutamate dehydrogenase, showed a somewhat different pattern, the level remaining constant during G_1 and increasing only after DNA synthesis had started.

(c) Studies with fluorescent probes showed that the percentage of mitochondrial-electron-transport-protein relative to total protein remained constant during the cell cycle.

(d) It is concluded that the development during the cell cycle of intracellular structures such as mitochondrial membranes and microsomal membranes probably come under the same control (which is not that of gene dosage) but that glutamate dehydrogenase, located in the mitochondrial matrix, may be subject to a different mechanism.

(e) The scope of future work is discussed.

PUBLISHED WORK

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ABBREVIATIONS

As far as possible, abbreviations, symbols, conventions, definitions and general nomenclature used in this thesis are those recommended by the Biochemical Journal in 'Policy of the Journal and Instructions to Authors' *Biochem. J.* (1970). 116, 1.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 SCOPE OF PRESENT WORK

The work described in this thesis is concerned with the development and subsequent use of a tool to study the timing of events occurring during the life cycle of cultured mammalian cells. At some stage before division, all components of continuously growing and dividing cells have to be duplicated so that two similar functionally-independent daughter cells can be produced. How and when this happens and the systems of control that may be involved have become a major field of study in cell biochemistry.

The synthesis of bulk protein and RNA during the cell cycle is reasonably well documented, although agreement has not necessarily been reached (see Chapter 4). On the other hand, very little work has been done on enzyme synthesis, phospholipid synthesis and membrane construction. Only by examining this kind of event during the cell cycle will an insight eventually be gained into the complex control mechanisms that must exist for ordered cell proliferation to occur.

1.2 THE LIFE CYCLE OF CELLS

The completion of cell division is probably the most convenient means of marking the end of one cycle and the beginning of the next. In fact, for many years, mitosis - the actual splitting of the cell into two daughters - was the central point of interest in the study of the cell cycle. The time between successive divisions, known to be long compared to mitosis, was given little attention, being regarded as the 'resting' phase or interphase, when cell processes were relatively static (Hughes, 1952). But in the same year, Walker and Yates (1952) made one of the first observations on interphase events. They measured the optical density to ultraviolet light of individual living chick cells in culture and the Feulgen dye content of fixed cells and clearly showed that the synthesis of nucleic acids occurred during interphase, not early in mitosis as was generally thought.

At this time, two major experimental approaches were starting to be developed which have over the years shifted the whole emphasis of cell cycle study from mitosis to interphase. Firstly the use of autoradiographic techniques to detect and locate incorporated radioactively-labelled macromolecular precursors (Howard and Pelc, 1951) and secondly, the synchronization of cells in culture - unicellular Algae (Tamiya, Iwamura, Shibata, Hase and Nihei, 1953), Protozoa (Scherbaum and Zeuthen, 1954), and bacteria (Hotchkiss, 1954).

Howard and Pelc (1953) working with actively growing and dividing root meristem *in vivo* studied incorporation of ^{32}P into DNA at increasing length of exposure to the isotope. They prepared autoradiographs at each time stage and examined them for the % of labelled cells and the % of labelled mitoses; they found an interval of several hours between the time that the % of labelled cells started to increase rapidly (soon after exposure) and the time that the first labelled mitoses began to appear, i.e. there was a 'gap' between DNA synthesis and mitosis. Thus they demonstrated that not only did incorporation of ^{32}P into DNA (i.e. DNA synthesis) take place during interphase but that it was restricted to a discrete portion of interphase. This they termed the S (synthetic) phase. Initially (within 2 hr) 16% of the cells were labelled and thus the length of S was taken as approximately 16% of the total generation time. The period between the end of S and the start of mitosis (M) they termed $G(\text{'gap'})_2$ and the remaining 'gap' - deduced by subtraction of the length of S, G_2 and M from the total generation time of the cell - they termed G_1. This partitioning of the cell cycle into four periods, two of which, M and S, are distinct markers, was soon extended to mammalian cells *in vitro* (Lajtha, Oliver and Ellis, 1954), and today this concept is widely accepted, representing the four phases in terms of progression around a clock-face (Figure 1-1).

Nešković (1968) has suggested a further classification of these phases, especially G_1, into subphases on the basis of morphological characteristics of the cell at various times after division. This raises the question of when mitosis really starts (prophase) and finishes (telophase).

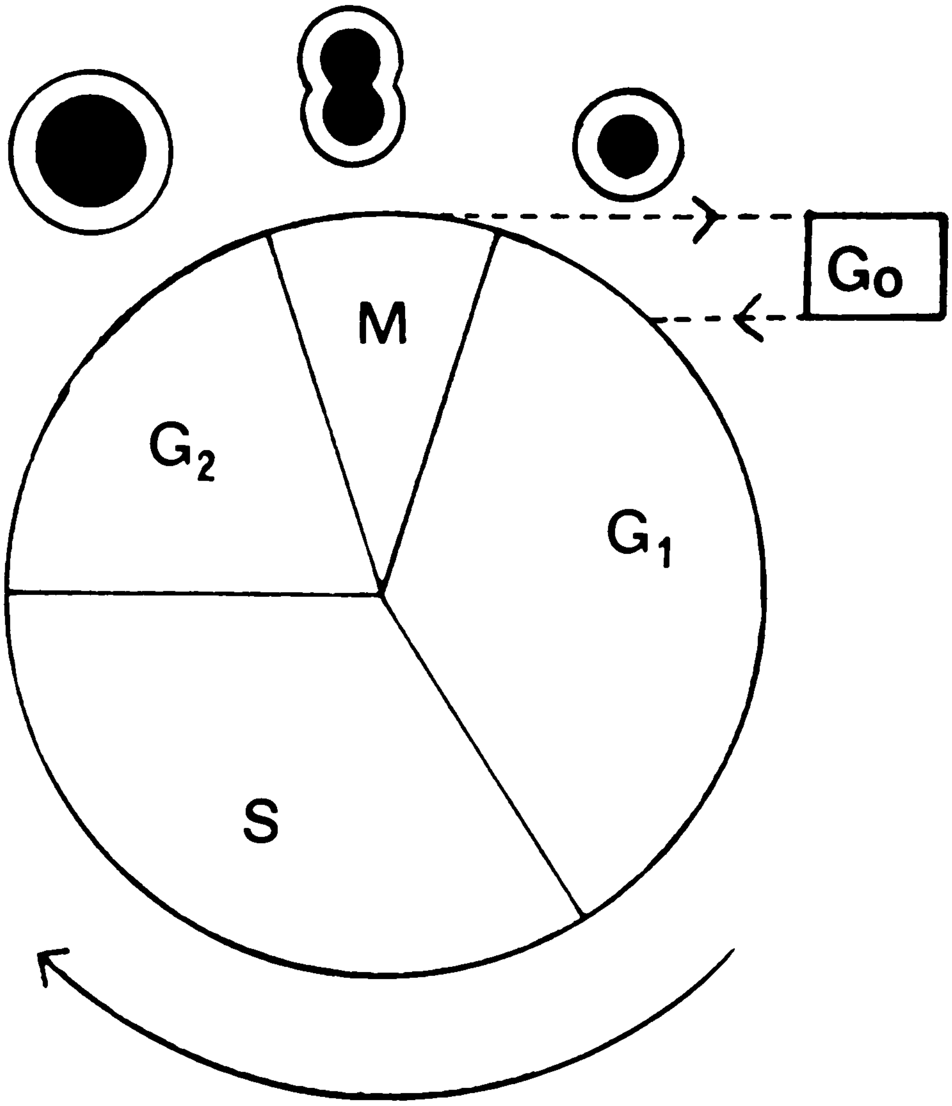


Figure 1-1 : The Cell Cycle

The boundaries are defined in terms of detection of chromosome condensation by light microscopy (Prescott, 1964a) and should not be considered as hard and fast. Moreover, we should really consider S not just in terms of total DNA duplication in the cell, but in terms of replication of individual chromosomes, or parts of chromosomes, each perhaps with its own S phase (Lajtha, 1963). However, boundaries have to be made for convenience; their limitations must be kept in mind.

One other phase must be mentioned in any discussion of the cell cycle: the 'G_0' phase. *In vivo*, some cells, e.g. epithelial cells lining the crypt of the small intestine, continuously divide and progress through the cycle. On the other hand, some cells, e.g. liver cells, rarely divide and are often considered to be in a true 'resting' state - G_0 - from which they can return to G_1 (Lajtha, 1963; Patt and Quastler, 1963).

1-3 METHODS FOR THE STUDY OF THE CELL CYCLE

The first step is an evaluation of the length of the various phases. Only then can various metabolic events be examined. A variety of methods exist for obtaining this sort of data, and have been summarised by Cleaver (1967, p. 108), Watanabe and Okada (1967) and Nachtwey and Cameron (1968). They will now be described briefly.

(i) Continuous labelling method

This has been described by Stanners and Till (1960) and is an extension of the Howard and Pelc technique previously mentioned. A population of cells is incubated with a labelled DNA precursor (usually [^{3}H] thymidine). The fraction of labelled and unlabelled mitoses, the fraction of labelled cells, and the average grain count per cell are determined from autoradiographs prepared at frequent intervals after isotope addition. The length of G_2 is the time until the appearance of labelled mitotic cells; S is the time for the average grain count to reach a constant level, and can also be measured by taking the % of labelled cells just after isotope addition as the % of the cell cycle occupied by S. M is obtained from the % of mitotic cells and G_1 then calculated by subtraction.

(ii) Pulse chase method

The cells are exposed to isotope for a short period only and then incubated in non-isotopic medium. Again, autoradiographs are prepared at frequent intervals and the fraction of unlabelled and labelled mitoses and the fraction of labelled cells determined. The cells labelled during the pulse will pass through mitosis as a wave of labelled mitotic figures. The length of G_2, as before, is the time until the appearance of labelled mitotic cells and S is the time between their appearance and disappearance. M and G_1

are calculated as described above.

Both these two methods can lead to large inaccuracies because they require the scoring of a very small number of cells (labelled or unlabelled mitoses) out of a large total. Frequent sampling over a long period (at least two generations) is also necessary. These disadvantages have been overcome using a mitotic inhibitor.

(iii) Mitotic inhibitor method

This was developed by Puck and Steffen (1963) and further described by Puck (1964a) and Puck, Sanders and Petersen (1964). It relies on the use of colchicine or colcemid, two closely related mitotic inhibitors, which interfere with spindle formation (Lushbaugh, 1956; Borisy and Taylor, 1967) thereby preventing chromosome separation and 'arresting' the cell in metaphase. Inhibitor and $[^3H]$ thymidine are added together to an exponentially-growing population of cells. Once more, the fraction of unlabelled and labelled mitoses and the fraction of labelled cells are determined at intervals from autoradiographs. This time, however, a far greater proportion of mitotic cells will apear with time. G_1 is the time required for the % of labelled cells to reach a plateau, S is given by the rate of accumulation of labelled cells (labelled collection function), and G_2 is the time between the accumulation of mitotic cells (mitotic collection function) and accumulation of labelled mitotic cells. Puck expresses the various accumulations in a series of equations which have recently been discussed by Bergeron (1969).

The durations of the phases of the cell cycle have been listed for a variety of mammalian cells *in vitro* by Cleaver (1967, p.126). The range of variation for the G_1 phase is greater than that for S or G_2, and in some cases, G_1 appears to be absent, e.g. Erlich ascites tumour cells (Baserga, 1963; Chinese hamster lung cells (Robbins and Scharff, 1967). The three methods mentioned so far yield information only on the lengths of the various cycle phases; the real problem, however, is to find out what is happening during the different phases. Again, the various approaches will be described.

(iv) Single cell studies

Perhaps the most obvious way to investigate the cell cycle is to make measurements, e.g. volume, mass, density etc., directly on a single growing cell (using interference microscopy, X-ray absorption or the Cartesian diver balance). This was the line of study developed by Prescott (1955) with Amoeba proteus and has more recently been reviewed by him (Prescott, 1964b).

(v) Time-lapse cinephotomicrography studies (TLCP)

Measurements on single cells in culture have been facilitated by the use of TLCP to delineate the sequence of morphological changes during interphase and division. For instance, using microspectrophotometric and micro-interferometric methods, Killander and Zetterberg (1965) followed the synthesis of DNA, RNA and increase in cell mass in fibroblast cells whose post-division age was identified by TLCP. Seed (1962) and Zetterberg and Killander (1965) coupled this technique with autoradiographic studies of labelled precursor incorporation to yield more precise information on rates of macromolecular synthesis. This type of experiment can also be used to determine the length of phases of the cell cycle (Sisken and Morasca, 1965) and even the pattern of DNA synthesis during S (Kozuka and Moore, 1966).

The methods described so far for investigating the cell cycle suffer from the severe limitation that they all depend on the microscopic selection of a cell known to be at a certain stage of the cycle (usually using mitosis as a marker) and making observations and measurements on those cells. To analyse the cycle further, to find the biochemical characteristics of G_1, S, G_2 and mitotic cells, requires large numbers of cells, all at a given stage of the cycle. Thus, a population of exponentially-growing cells, which will contain cells at all stages of the cycle, must be manipulated to provide cells all at the same stage. This manipulation - synchronization - provides a means of amplifying the events occurring in a single cell during the cycle.

1-4 CELL SYNCHRONY

Synchrony of cell division, when numerous cells divide together, has often been observed in natural systems such as cleaving marine eggs. It was an effort to imitate this sort of behaviour that provided the basis of early attempts to produce synchrony in the types of cell that could then be grown in culture. In the last fifteen years, nearly all varieties of cell in culture from bacteria to mammalian cells have been successfully synchronized and there are several reviews on this subject (Burns, 1961; Zeuthen, 1964; Cameron and Padilla, 1966; Prescott, 1968a).

In addition, cells that do not normally divide at all (i.e. in the G_0 phase - see 1.1) can, by means of appropriate stimuli, be induced to divide together and can thus be regarded as a synchronous system, e.g. regenerating liver after partial hepatactomy; phytohaemaglutt̊nin-stimulated lymphocytes. However, this type of system will not be discussed in any depth since the studies described in this thesis are restricted to a system of *in vitro* continuously-dividing mammalian cells. There follows a review of the techniques in existence for the synchronization of mammalian cells in culture (see Table 1-1).

Table 1-1: Synchrony methods for cultured mammalian cells.

	PHYSICAL	CHEMICAL
INDUCTION	Temperature changes	Specific inhibitors
SELECTION	Mitotic harvesting; Size	Selective killing

The distinction between synchronized and synchronous growth in cultures is often made (Abbo and Pardee, 1960) and can be used (James, 1966) to emphasize the difference between the induction of synchrony and synchrony by selection. Induction involves the treatment of an exponential culture so as to interrupt the flow of cells around the cycle. For this to achieve a build up of cells at one point, a process must be blocked which is discontinuous relative to the complete cycle, i.e. DNA synthesis or mitosis. Selection, on the other hand, involves the separation from an exponential culture of a few cells at the same stage.

(i) Induction of synchrony

(a) Temperature methods

Following from induction of synchrony in bacteria and Protozoa by temperature changes for the length of one growth cycle, similar methods were applied to mammalian cells. Wildy and Newton (1958), and Newton and Wildy (1959) reported partial synchrony in HeLa cells after chilling at

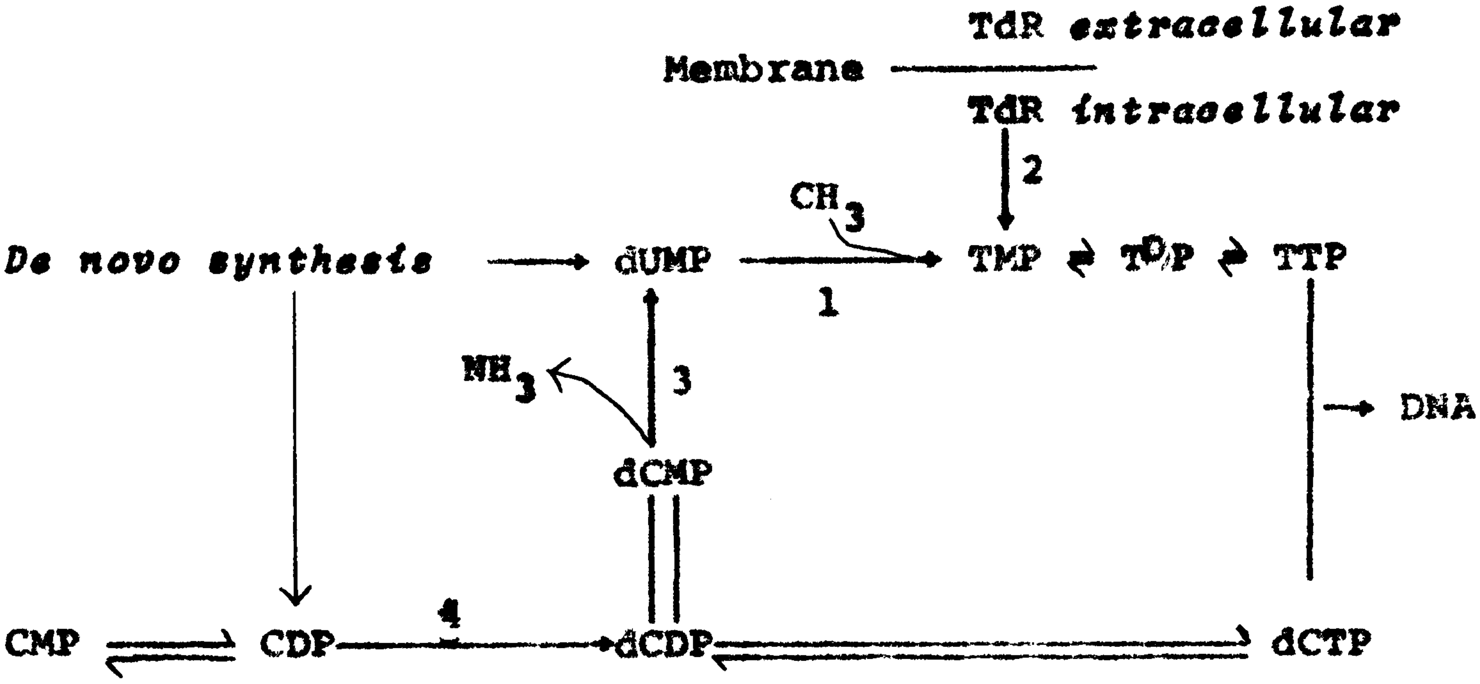
$4^{\circ}C$ for as little as one hour. Newton (1964) proposes possible mechanisms for this sort of induction, invoking the existence of a cold-labile enzyme system involved in DNA synthesis which might be normally renewed early on in the cycle. Deactivating this could rapidly bring all the cells to a 'telophase-like' state. Miura and Utakoji (1961) repeated the technique with human amnion cells but with very poor results. Rao and Engelberg (1966), in an extensive study of the effect of temperature on the cell cycle, showed that the most sensitive phase was mitosis and that G_1 was lengthened by temperature changes, but had little success in synchronizing cells by this means.

(b) Methods using specific inhibitors

Inhibition of DNA synthesis

Inducing synchrony by blocking DNA synthesis is most successful in cell lines in which the S phase takes up < 50% of the total generation time. To appreciate the action of the various blocking agents used, the biosynthetic pathways leading from nucleotides to DNA must be considered (Figure 1-2).

Rueckert and Mueller (1960) and Schindler (1963) used amethopterin (4 amino-N^{10}methyl folic acid), a folic acid analogue which inhibits the methylation of dUMP (Figure 1-2, site 1) by thymidylate synthetase (Mathias and Fischer, 1962). After exposing exponentially-growing HeLa cells to this



Inhibition sites

1. Thymidylate synthetase
2. Thymidine kinase
3. Deoxycytidylate deaminase
4. Cytidylate reductase

Abbreviations

TdR	thymidine
CMP	cytidine monophosphate
dCMP	deoxycytidine monophosphate
dUMP	deoxyuridine monophosphate
TMP	thymidine monophosphate
CDP	cytidine diphosphate
dCDP	deoxycytidine diphosphate
TDP	thymidine diphosphate
dCTP	deoxycytidine triphosphate
TTP	thymidine triphosphate

Figure 1-2: Pathways leading to DNA synthesis

(Adapted from Cleaver, 1967, p.44)

antimetabolite for 16-20 hours, so that > 90% of the cells accumulated in S, the effect was reversed by thymidine addition, resulting in a burst of cell division after 6-8 hours.

Another antimetabolite which also prevents the methylation of dUMP and whose action can be reversed by thymidine addition, has been used in a similar manner, namely 5-fluoro 2-deoxyuridine (FUdR), a deoxyuridine analogue (Littlefield, 1962; Till, Whitmore and Gulyas, 1963).

The concentration of thymidine used to reverse the effect is critical, because when it is present in excess quantities, DNA synthesis is inhibited anyway and thymidine can thus be used to induce synchrony (Xeros, 1962). In many ways, it is more satisfactory because it is a natural metabolite, although not on the normal pathway of *de novo* synthesis of DNA (Figure 1-2). Its inhibitory action has been explained by the fact that in the presence of excess thymidine, there is a high level of thymidine triphosphate which causes feedback inhibition on thymidine kinase (Figure 1-2, site 2), d-CMP aminase (site 3) and CDP reductase (site 4), (Ives, Morse and Potter, 1963).

Puck (1964a, 1964b) showed that excess thymidine can block cells throughout S, so that after exposure for one generation time, cells originally in G_2 and M will be at the G_1/S boundary, the remainder being spread through S.

Cells will thus still be at least several hours apart in time, depending on the length of S. This situation can be improved by the re-addition of thymidine (Bootsma, Budke and Vos, 1964; Petersen and Anderson, 1964; Puck, 1964a; Puck, 1964b). Cells from the first treatment are incubated in fresh medium for a time long enough for them all to progress out of S. Thus cells will now be in G_2, M or perhaps G_1, so that when thymidine is added again, they will all accumulate at the G_1/S boundary, showing a high degree of synchrony on release. Obviously, the longer the S phase, the more difficult this approach becomes, and a triple thymidine treatment has even been used (Galavazi, Schenk and Bootsma, 1966).

Inhibition of Mitosis

Various mitotic inhibitors can be added to exponentially-growing cells so that eventually they will all collect in mitosis. The inhibitor is then removed, with resulting synchrony. Rao (1968) applied nitrous oxide at high pressure as a mitotic block, but the mitotic inhibitors most commonly used are colchicine and colcemid [see 1.1(iii)] Stubblefield and Klevecz, 1965; Romsdahl, 1968), and vinblastine sulphate (Pfeiffer and Tolmach, 1967), which also interferes with spindle formation. Mitotic inhibitor methods are usually applied to monolayer cultures, when they can be used in conjunction with a selection method [see

1.4(ii)]. In fact, combination of various methods is often the best way to synchronize particular cell lines. Doida and Okada (1967), for instance, devised a method suitable for cell lines with a long S and short generation time (more practical than a triple thymidine treatment - see above) involving the thymidine block followed by a short colcemid block.

All inhibitor methods, however, have the major drawback that the cell is subjected to an unnatural chemical environment. The various antimetabolites may have all kinds of side reactions, while excess thymidine (and FUdR) can produce unbalanced growth, i.e. the continuation of RNA synthesis, protein synthesis and thus increase in mass in the absence of DNA synthesis and division. (Rueckert and Mueller, 1960; Till *et al.*, 1963; Studzinski and Lambert, 1968; 1969). Moreover, Firket and Mahieu (1967) working with HeLa cells, have shown that even a single thymidine block treatment shortens S and G_2 (with possible overlap) and lengthens mitosis.

(ii) Synchrony by selection

(a) Mitotic harvesting

In monolayer culture, cells in mitosis tend to round up and adhere far less than during interphase (Axelrad and McCulloch, 1958). During this loosely-bound period, these cells can be removed by gently washing or shaking the monolayer (Terasima and Tolmach, 1961 ;1963). The cells thus separated have been selected exclusively from a very narrow fraction of the cell cycle. Naturally the yield of cells is extremely low, a drawback of most selection methods.

Robbins and Marcus (1964) improved the yield of mitotic HeLa cells from monolayers by using calcium-free growth medium, but this may introduce metabolic alterations (Stubblefield, Klevecz and Deaven, 1967).

Alternatively, the yield can be improved by making use of a mitotic inhibitor (see above), but this introduces all the criticism applicable to chemically-induced synchronization. Yet another approach involves pooling mitotic cells collected over a period of several hours (Lindahl and Sörenby, 1966; Tobey, Anderson and Petersen, 1967; Petersen, Anderson and Tobey, 1968), but the cells must be kept chilled until enough have been obtained, again introducing possible metabolic disturbance [see 1.4(i)].

Despite these criticisms, some form of mitotic harvesting is probably the most widely used method at present, but of course, it is only feasible with monolayer cultures.

(b) Selection by size

Cell size and stage in the life cycle are obviously closely related (Prescott, 1964b). Just after division (early G_1) cells are small, whereas just prior to division (late G_2) they are twice the size, the result of doubling all components (Prescott, 1968b). Thus, selecting cells on a size basis should result in a synchronous population. Sinclair and Bishop (1965) separated small cells from an exponential culture by selecting the 5% that sedimented slowly, remaining in the top fraction after a short centrifugation on a sucrose gradient. On subsequent incubation they grew synchronously in a manner typical of G_1 cells. In the same year, Fulwyler (1965) used electronic selection to isolate groups of cells of a limited volume range; again, the yield was very low. Morris, Cramer and Reno (1967) extended the sedimentation technique by showing that cells towards the centre of the gradient were all in S. The development of gradient centrifugation techniques both to produce synchronous cells and to obtain large numbers of suspension culture cells at any given stage of the cell cycle (except mitosis) is described at length in this thesis.

(c) Selection by killing

To complete the survey of synchrony techniques for mammalian cells, this approach must be mentioned, but it is of very limited use. Lethal concentrations of hydroxyurea (Sinclair, 1965) or high specific activity [^{3}H] thymidine (Whitmore and Gulyas, 1966) are added to exponential cultures. Only cells in S are killed, and the remainder will eventually accumulate at the end of G_1.

Incubating in fresh medium results in synchronous growth, but there will always be considerable interference on any measurements made by the large quantities of dead cells present.

1-5 THE CELL LINE UNDER STUDY

The cell line used most extensively in the work described in this thesis has been a mouse mastocytoma, the P815 mast cell neoplasm, which originated in a DBAf/2 mouse after repeated application of methylcholanthracene (Dunn and Potter, 1957). This transplantable neoplasm was eventually grown in culture - cell line P815 strain Y (Schindler, Day and Fischer, 1959), and has been shown to remain relatively differentiated with respect to both biochemical and genetic characteristics. Thus histamine (Schindler *et al.*, 1959; Green and Day, 1960) heparin (Green and Day, 1960; Thomas, 1967) and serotonin (Green and Day, 1960) are produced in culture, and a chromosome count shows about the expected diploid number - i.e. 40 (Green and Day, 1960; M. Ashwood-Smith, personal communication). Therefore these lines of cultured cells are a better 'model cell' than many other neoplastic cells which tend to become rapidly dedifferentiated in culture.

Some of the work has been repeated with a similar transplantable mastocytoma in culture, the MC line, originally isolated from an irradiated LAF_1 strain mouse (Furth, Hagen and Hirsch, 1957).

Table 1-2 shows some characteristics of P815Y and HC neoplastic mast cells in culture.

Table 1-2: Characteristics of P815Y and HC neoplastic mast cells.

	P815Y	HC
Generation time	12-15 hours	15-17 hours
G_1	4.5 hours	3 hours
S	6.5 hours	9.3 hours
G_2	3 hours	2.4 hours
M	0.9 hours	0.6 hours
Mean cell volume (exponential growth)	1200 μm^3	1100 μm^3

P815Y cell cycle analysis - Bergeron (1969).

HC cell cycle analysis - R. Aisbitt and J. Raggatt (personal communication)

The mitotic inhibitor method [see 1.3(iii)] was used.

CHAPTER TWO

GENERAL EXPERIMENTAL

2.1 SOURCE OF MATERIALS

(i) Radiochemicals

(a) From the Radiochemical Centre, Amersham:

Isotope			Specific activity (mCi/m/mol)
[1-^{14}C] Na-acetate	CFA	13	50
[Me-^{14}C] choline chloride	CFA	138	32-54
[1-^{14}C] glucosamine-HCl	CFA	422	56
[1-^{14}C] palmitic acid	CFA	23	50
[U-^{14}C] L-proline	CFB	71	255
[2-^{14}C] thymidine	CFA	219	60
[2-^{14}C] uridine	CFA	315	58
[Me-^{3}H] choline chloride	TRA TRK	179 179	2000 15400
[G-^{3}H] L-proline	TRA	82	550
[Me-^{3}H] thymidine	TRA	120	5000
[5-^{3}H] uridine	TRA	178	5000

(b) From New England Nuclear Chemicals, Germany:

[2-^{3}H] *myo*-inositol	NET	114	3500

(ii) Other materials

Armour Pharamaceutical Co. Ltd.: crystallised bovine plasma albumin.

Boehringer Corporation (London) Ltd.: NADH, NADP, NADPH, ADP, cytochrome c, Na_2-glucose-6-phosphate, Na-oxoglutarate, Na-pyruvate.

Ciba (A.R.L.) Ltd.: scintillator butyl PBD.

Coulter Electronics Ltd.: Coulter counter calibration particles.

Eastman-Kodak Ltd., U.S.A.: nuclear track emulsion NTB 2.

Flow Laboratories, Scotland: 'membrane filtered horse serum'.

Glaxo Laboratories Ltd.: streptomycin sulphate, benzylpenicillin ('crystapen').

Grand Island Biological Co., U.S.A.: Fischer's medium for leukemic cells of mice ('instant' powder form).

Johnson's Ltd.: Johnson's Fixsol.

Kodak Ltd.: Kodirex X-ray film, D19b developer.

E. Merck AG., Germany: silica gel H.

Sigma (London) Ltd.: Ficoll, calf-thymus DNA (highly polymerised).

Tissue Culture Services: 'aseptic horse serum'.

Wellcome Research Laboratories: 'horse serum No. 5'.

All other chemical were obtained from British Drug Houses (B.D.H.) Ltd., and were AnalaR whenever possible.

P815Y and HC neoplastic mast cells in culture were a gift from Dr. G.A. Fischer; subsequently, P815Y cells were kindly donated by Dr. M. Fox.

2.2 CELL CULTURE METHODS

(i) Culture Medium

The culture medium used was that of Fischer and Sartorelli (1964) with 10% horse serum (v/v) adapted from Eagle (1955) and designed to satisfy the high folic acid requirement of these neoplastic mast cells in culture. Initially, the medium components (Thomas, 1967; Wheldrake, 1967; Bergeron, 1969) were weighed out and dissolved individually; for the majority of the work, however, the medium was obtained in powdered form [see 2.1(ii)] which required only the addition of antibiotics and bicarbonate.

The medium, except serum, was made up with doubly glass distilled water in 20 L batches, sterilized by filtration through a microporous porcelain filter, 0.3 μm absolute retention size (Selas Flowtronics Ltd.) and stored in sterile screw-cap bottles at 2-4°C until required, for up to 3 months.

Just before use, serum was added to the medium which contains phenol red and the pH was adjusted to approximately 7.2 by adding sufficient sterile Q1M-HCl to give a buff-straw colour.

Fresh batches of serum (and medium) were routinely tested. At first, Wellcome "Horse serum No. 5' was used; this became unsatisfactory and Flow 'Membrane-filtered horse serum' was tried instead; this too became unsatisfactory and was followed by Tissue Culture Services 'Aseptic horse serum', which is still currently in use.

Throughout this thesis, 'medium' is the Fischer's medium and acid as described above; 'growth medium' has serum added.

(ii) Sterilisation

Heat-stable materials were sterilised by autoclaving for at least 15 min at 15 p.s.i. Heat-labile materials (except for medium, see above) were sterilised by filtration through Oxoid Ltd. membrane filters (0.45 µm pore size) in a glass filter holder (negative pressure) or more efficiently, through Millipore (UK) Ltd. GS membrane filters (0.22 µm pore size) in a Millipore 'Swinnex-25' filter unit (positive pressure).

(iii) Growth of cells in culture

Cells were grown in suspension at 37°C (a) in 5 ml of growth medium in 15 ml screw-top culture tubes, (b) in 50 ml in 250 ml screw-top Erlenmeyer flasks, (c) in 250 ml in 1 L screw-top Erlenmeyer flasks, and (d) in up to 2 L in a spinner culture vessel (Figure 2-1) adapted from Paul (1965, p.262), the stirring rate being as slow as possible. Routine cell transfers (subcultures) were carried out every 2-5 days (when cells reached a concentration of about 1×10^6/ml) by centrifuging for 5-10 min at 1500 rev./min in

Figure 2-1: P815Y cells in spinner culture

an M.S.E. 'Mistral 6L' centrifuge (swing-out or fixed-angle head), decanting the supernatant and resuspending the cells in pre-warmed growth medium at a concentration of about 1 x 10^5 cells/ml.

Aseptic technique was maintained rigorously throughout. Contamination levels were monitored by frequent microscopic observation, at least every 3 days.

(iv) Cell cloning

The P815Y cells were cloned (Nias and Fox, 1968) half way through the three years taken up by these studies, under the kind supervision of Dr. M. Fox. The cloning procedure involved picking out and growing up colonies grown on agar from single cells.

(v) Storage of cells

As a reserve stock, cell cultures were frozen and stored at -70°C for periods of up to one year, when fresh stocks were prepared. The procedure (Paul, 1965, p.288) was to suspend the cells in medium containing 10% (v/v) dimethyl sulphoxide and seal the suspension in glass ampoules. These were then cooled slowly over 3-4 hr. to -20°C and then lowered into a Union Carbide LR 10 liquid nitrogen refrigerator (kindly provided by Dr. L.A. Stocken).

2.3 CELL COUNTING AND SIZING

An electronic counter, the Coulter counter model A, with a 100 μm aperture was used to count (Brecher, Schneiderman and Williams, 1956) and size (Brecher, Jakobek, Schneiderman, Williams and Schmidt, 1962) the cells.

(i) Counting

Cells (in the range 1 x 10^5/ml to 20 x 10^5/ml) were diluted 25-fold or 50-fold with 0.9% NaCl (w/v), to a total volume of 10 ml, and counted at a scale expansion setting(I) of 5 and threshold setting (T) of 50. [This combination of settings has been shown to be the optimal for the cell lines under study by comparison with haemocytometer counting (C.A. Pasternak, personal communication)].

Corrections were made for background and, when necessary, coincidence.

(ii) Sizing

Cells were diluted as above, but with phosphate-buffered saline, pH 7.4 (Krebs and Eggleston, 1940) or 50 mM-tris-HCl/0.9% NaCl (w/v) pH 7.4, to a total volume of 25 ml (sometimes 50 ml).

When cells traverse the aperture, they produce a voltage pulse proportional in size to the volume of the cell passing through. The threshold circuit in the counter enables counting of only those pulses at and above a given setting and thus of cells at and above a given size. Thus by counting at progressively higher threshold settings, more and more cells are screened out and an accumulative frequency curve can be constructed. Figure 2-2(a) shows a typical curve obtained with P815Y cells, with the scale expansion setting at 1. By plotting the difference between successive readings, a cell size frequency distribution curve is obtained [Figure 2-2(b)]. This is then subjected to group deviation analysis as described by Stanley (1963) to give the mean volume (M) of the population expressed in arbitrary units of threshold.

(iii) Counter calibration

To convert threshold units to units of volume (μm^3), the coulter counter was calibrated with materials of known diameter (Table 2-1). The mean volume of each sample in units of threshold was determined as described above so that a calibration curve of volume (calculated from diameter) against threshold setting could be constructed (Figure 2-3).

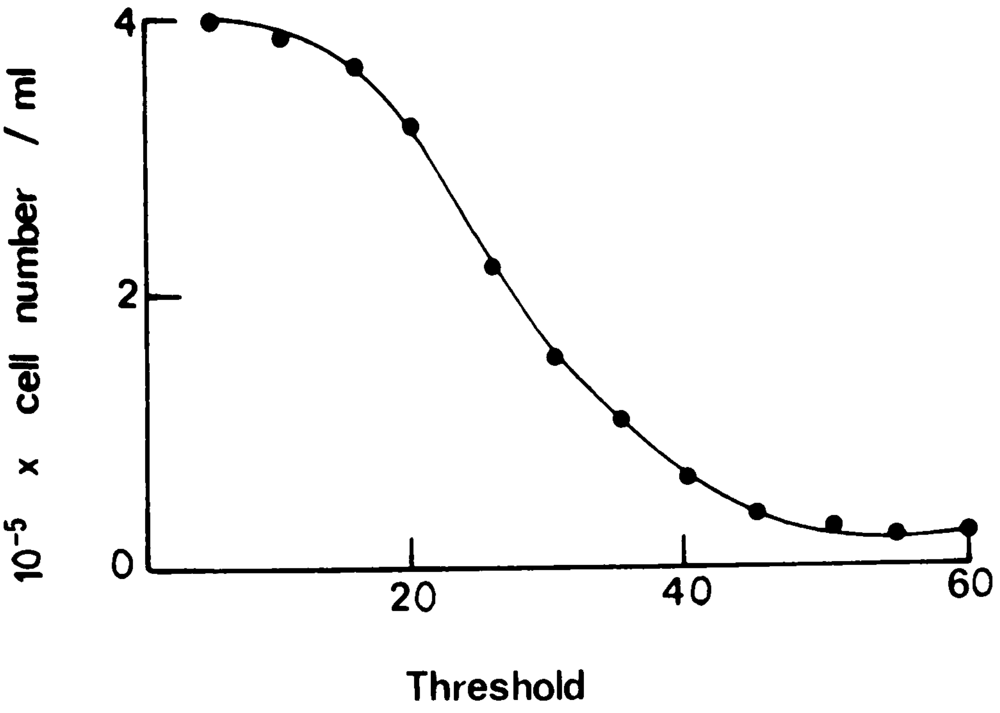
Table 2-1: Coulter Counter Calibration Materials

Calibration particle	Diameter (µm)
Puff ball spores	3.5
Polystyrene divinyl benzene latex beads	12.0
Paper mulberry pollen	13.5
Silver birch pollen	23.0

(iv) Rapid estimation of volume

The sizing procedure described is lengthy both in

(a) Cumulative frequency curve



(b) Frequency distribution histogram

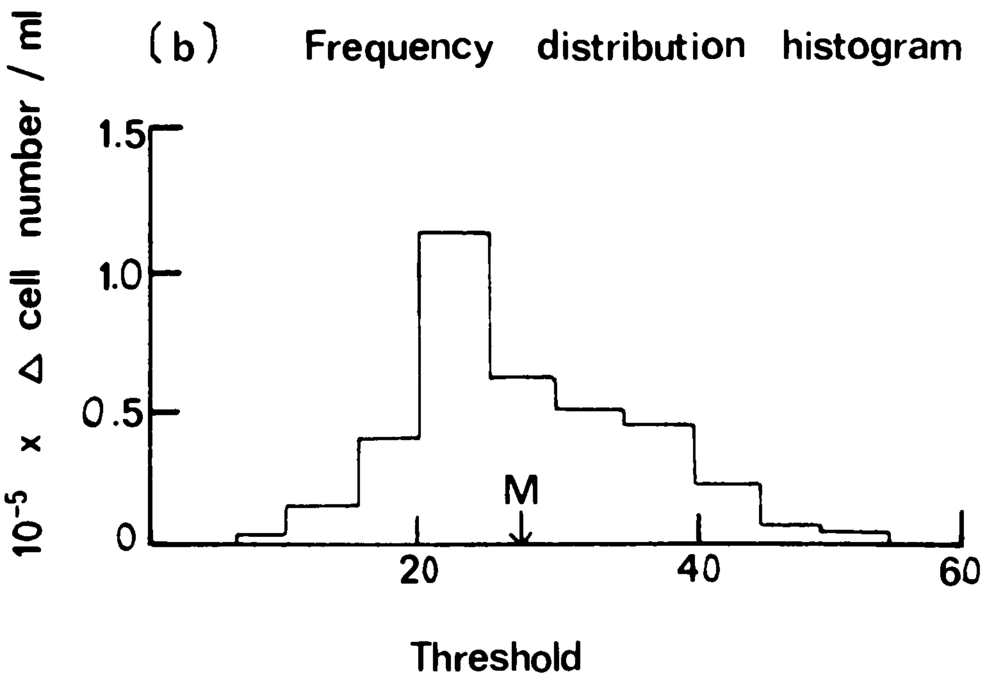


Figure 2-2 : The sizing of P815Y cells

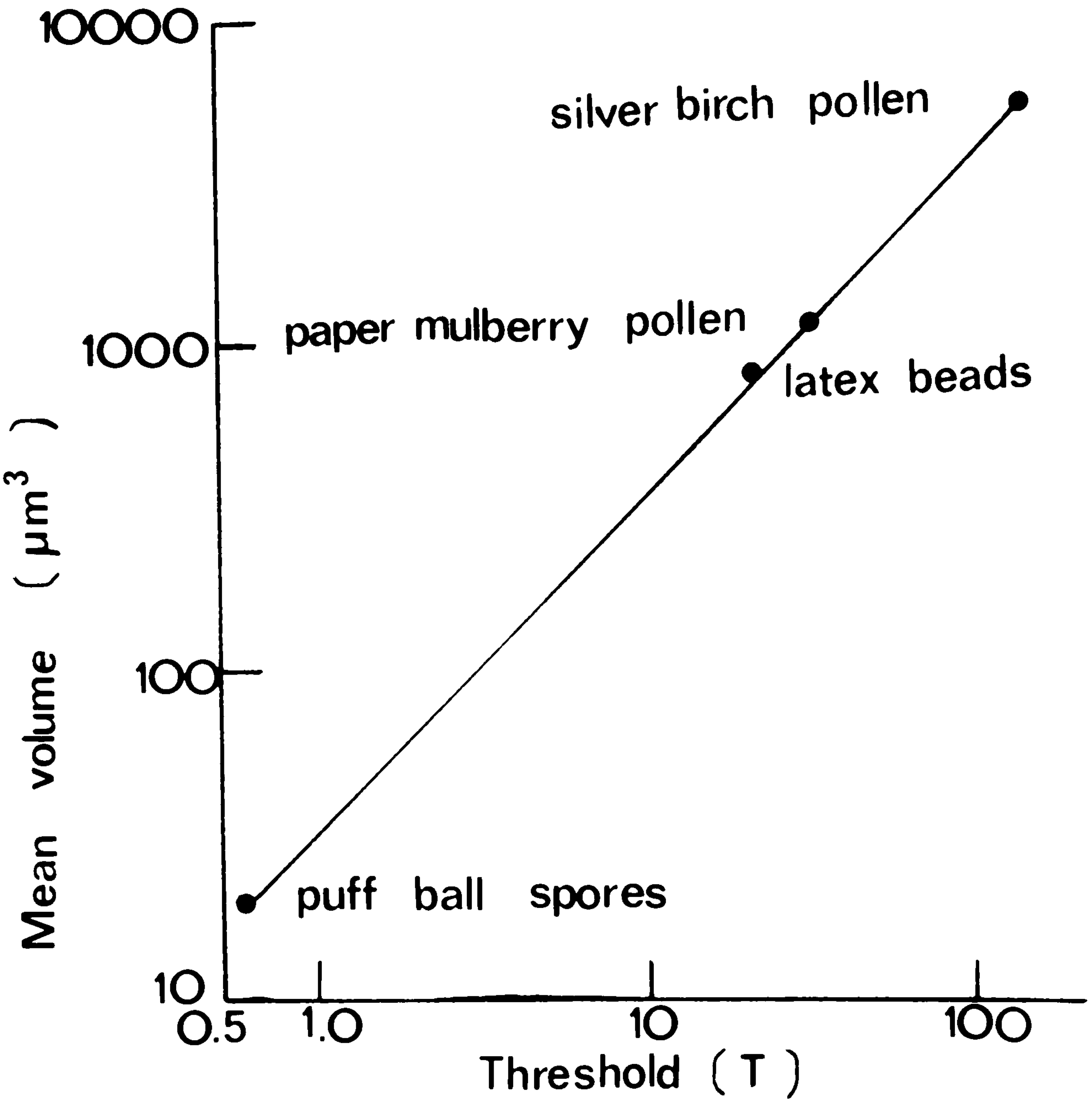


Figure 2-3: Coulter counter calibration curve

operation and subsequent calculation. A speedy determination of relative volume was routinely made by first determining the total number of cells (I = 5, T = 50 or I = 1, T = 5) followed by a count at a threshold (e.g. I = 1, T = 25) such that only larger cells in the population were recorded. The result was then expressed as the percentage of the total population at or above the volume corresponding to the threshold setting chosen. This has proved adequate for approximate volume comparisons.

2.4 MICROSCOPICAL TECHNIQUES

A Zeiss standard WL phase contrast microscope fitted with a Leica III camera for photomicrography was used throughout.

(i) Cell viability determination

The technique used was that of dye exclusion (Eaton, Scala and Jewell, 1959; Black and Berenbaum, 1964) exploiting the ability of living cells to exclude dyes such as nigrosin or eosin. 2 ml of cell suspension was mixed with 1 ml of 1% (v/v) eosin, concentrated by centrifugation if necessary, and transferred to a microscope slide. At least 500 cells and 20 fields were examined, scoring the total number of cells and the number that were eosin-stained (i.e. dead). Results are expressed as the percentage of cells that are dead which, in an exponentially-growing culture, was routinely < 10%.

(ii) Autoradiography of cells (including fixation and staining)

$[^3H]$ thymidine-labelled cells [see 2.7(i)] were washed with medium and resuspended in 0.3 ml phosphate-buffered saline. Drops of cell suspension were added to acid-washed microscope slides and, after standing for 5-10 min, the cells were fixed by immersion in Solvent Methanol (microscopical reagent, B.D.H.) for 5-10 min and dried in air. The slides were dipped in Kodak nuclear track emulsion NTB 2 at 43°C, dried in a stream of warm air for 2 hr and stored at 2-4°C in lightproof boxes. After exposure periods of up to 21 days, the slides were developed in Kodak developer D19 b for 4 min, fixed with Johnson's Fixsol (5-fold dilution) for 10 min and stained with Harris's haematoxylin [in conjunction with normal dehydration procedures for making permanent slide preparations (Pantin, 1946)]. A minimum of 500 cells and 20 fields per slide were examined, scoring cells with less than 5 grains as background. Results are expressed as the percentage of cells that are labelled.

(iii) Determination of mitotic index

The chromosome spreading procedure of Tobey, Petersen, Anderson and Puck (1966) was followed. Mitotic figures were prepared by swelling cells with cold hypotonic (0.1M sucrose, fixing with acetic acid: methanol (1:3 by vol.) and staining with haematoxylin. Metaphase cells could thus be easily distinguished, the proportion present being estimated and expressed as a fraction of 1 (mitotic index).

2.5 CHEMICAL FRACTIONATIONS AND ANALYSES

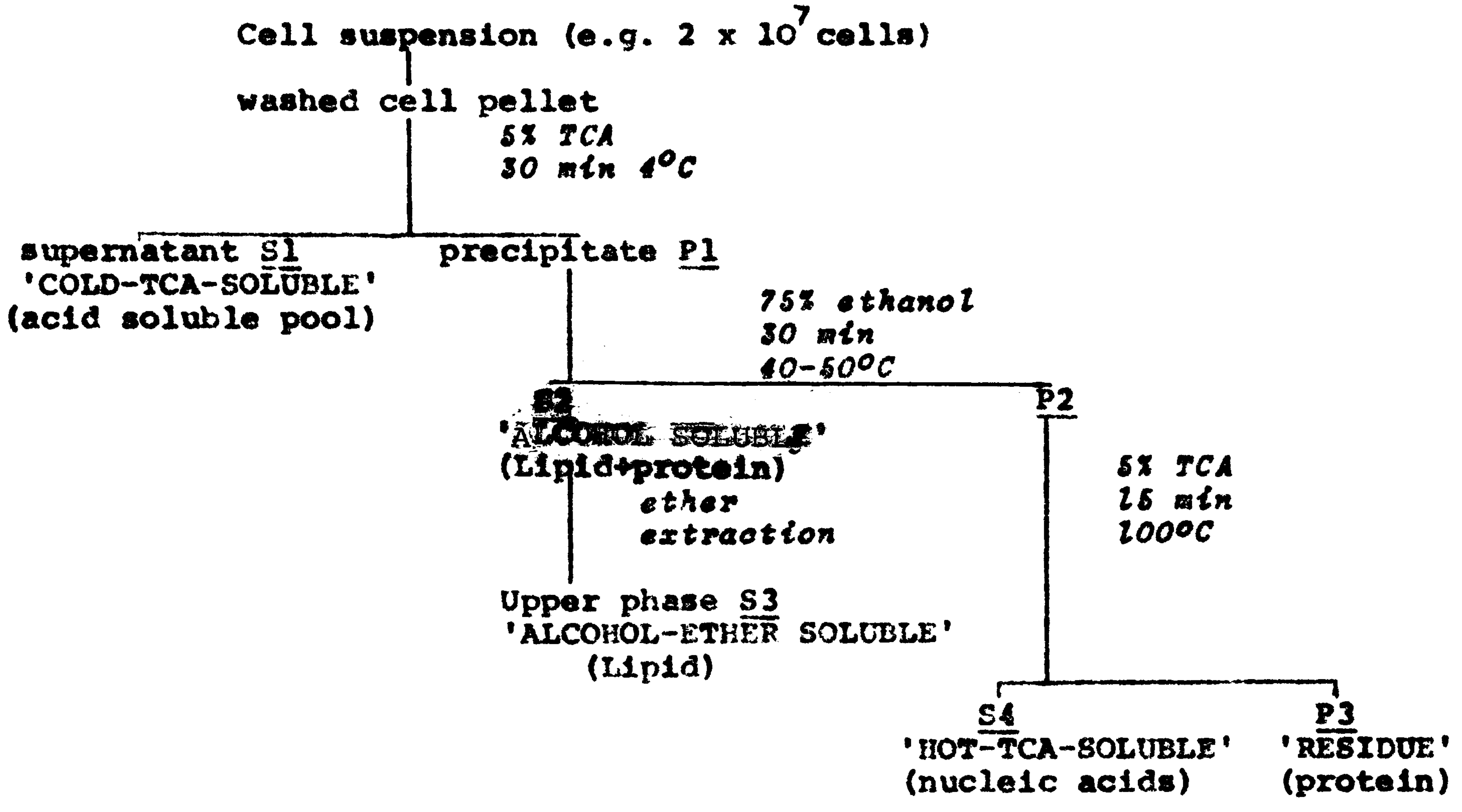
Figure 2-4 shows a typical scheme of chemical fractionation (adapted from Roberts, Abelson, Cowie, Bolton and Britten, 1957).

Alternatively, lipids were extracted by treating the cell pellets with chloroform: methanol (2:1 by vol.) and washing the lower (lipid) layer with 0.58% NaCl (w/v), (Folch, Lees and Sloane-Stanley, 1957).

All extinction determinations described in this section were made with a Unicam S.P.600 spectrophotometer.

(i) Analysis of the lipid fraction (S3)

To separate neutral lipids from phospholipids, the lipid extract (previously evaporated to dryness and dissolved in chloroform) was applied to the top of a silicic acid column, followed by elution first with chloroform (neutral lipid fraction) and then with methanol (phospholipids) as described by Bezman-Tarcher, Ottway and Robinson (1965). The phospholipid fraction was separated into individual phospholipids by ascending thin-layer chromatography (t.l.c.) on Silica Gel H, with a developing solvent of chloroform:



TCA - trichloroacetic acid.

Figure 2-4: Scheme of chemical fractionation methanol:acetic acid:water (46:29:6:3 by vol.) using iodine vapour to visualize the spots (Skipski, Peterson and Barclay, 1964).

Phospholipid-phosphorus estimation

Phospholipid-phosphorus in the chloroform/methanol extract lower-layer (Folch *et al.*, 1957) was estimated by the method of Bartlett (1959) which involved digestion with 70% perchloric acid and subsequent estimation of phosphorus released with the Fiske Subba-Row reagent. KH_2PO_4 was used as a standard, 1 μg phosphorus giving an extinction of approximately 0.25 at 830 nm.

(ii) Analysis of the nucleic acid fraction (S4)

The S4 fraction contains the nucleic acids (Schneider, 1945). In order to determine to what extent labelled thymidine is incorporated into DNA [see 2.6(i)], it was necessary to recover the label. This was achieved by hydrolysis with 70% perchloric acid (after removal of TCA with ether) at 100°C for 60 min to liberate thymine (Marshal and Vogel, 1951), followed by descending chromatography on Whatman No. 1 paper (Wyatt, 1951). The solvent was 65% isopropanol (v/v) 2M-HCl (final ctn.); the hydrochloric acid was subsequently removed by evaporation and the spots were visualised with a Locarte Ltd. UV lamp.

DNA estimation

DNA in the S4 fraction was estimated either by the method of Ceriotti (1952) in which indole forms a yellow colour with deoxyribose (liberated by acid hydrolysis) or by the method of Burton (1956) in which diphenylamine forms a blue colour with deoxyribose. Calf-thymus DNA (highly polymerised) was used as a standard, 5 μg giving an extinction of approximately 0.1 (at 490 nm - indole method; 600 nm - diphenylamine method).

RNA estimation

To estimate RNA, alkaline hydrolysis was carried out by incubating cell suspensions with an equal volume of 0.6M-NaOH for 2 hr at 37°C (Schmidt and Thannhauser, 1945) followed by addition of an equal volume of 1M-perchloric acid. Ribose released in the supernatant was measured by the orcinol reaction (Hurlbert, Schmitz, Brumm and Potter, 1954); ribose was used as a standard, 2 μg giving an extinction of approximately 0.25 at 665 nm.

(iii) Analysis of the protein fraction (F4)

Proteins in the F4 precipitate, or in a washed TCA-precipitate, were estimated either by the Biuret reaction (Gornall, Bardawill and David, 1949) or with the Folin reagent (Lowry, Rosebrough, Farr and Randall, 1951) in a solution obtained by dissolving the precipitate in 2M-NaOH. 'Crystallised bovine plasma albumin' (Armour) or 'crystallised plasma albumin' (B.D.H.) were used as standards, 1 mg giving an extinction of 0.2 at 550 nm (Biuret), 50 µg giving an extinction of 0.2 at 750 nm (Folin).

2.6 THE ASSAY OF RADIOACTIVITY

(i) General procedure

Radioactive solutions, filters (see below), or dried silica gel were added to 7 ml of a scintillation fluid consisting of 8g of butyl PBD [2-(4-tert.-butyl phenyl)-5-(4-biphenyl)-1-oxa-3, 4-diazole], and 80g of naphthalene in 1 litre of toluene-methyl cellosolve (3:2 by vol.), and assayed in a Beckman liquid scintillation spectrometer. The counting efficiency was 90% for ^{14}C and 40% for ^{3}H. Lowering of counting efficiency by quenching was corrected by means of standard curves for ^{14}C and ^{3}H, using the external standard calibration built into the spectrometer.

(ii) Assay of radioactivity incorporated into cells

Up to 1 ml of suspensions of radioactively-labelled cells were passed through Whatman GF/C glass fibre discs (2.1 cm diameter) in stainless steel filter holders (from Dr. D.G. Wild). The cells were then washed on the filter with 30 ml of 5% (w/v) TCA, the filter transferred to the scintillation fluid and radioactivity assayed as described above. Assaying incorporated counts in this way gave the same results as assaying counts in a washed TCA-precipitate, dissolved in 2M-NaOH. Therefore, counting on TCA-washed filters is taken as an estimate of counts in TCA-insoluble material, i.e. macromolecular.

2.7 CHOICE OF PRECURSORS

Macromolecular synthesis can be measured by following the incorporation of a radioactively-labelled precursor into the macromolecule concerned.

(i) DNA

Labelled thymidine was used as a precursor of DNA. In P815Y cells incubated for 48 hr (3-4 generations) with [2-^{14}C] or [Me-^{3}H] thymidine, > 95% of the label was in the hot-TCA-soluble fraction (Figure 2-4) After hydrolysis and co-chromatography with a thymine standard [see 2.5(ii)], the thymine spot was superimposable on a spot identified by autoradiography (Kodirex paper) and found to contain > 95% of the radioactivity applied to the paper.

(ii) RNA

Labelled uridine was used as a precursor of RNA. Although uridine is also incorporated into DNA (via conversion through TMP and CMP) this is not a serious drawback; Pfeiffer and Tolmach (1968) showed that only 2-6% of labelled uridine was incorporated into non-RNA components of exponentially-growing Hela cells, and even during S, this value rose only to 8-10%. Moreover, using [5-^{3}H] uridine, the label is lost completely during the methylation of dUMP to dTMP (Figure 1-2), leaving only the uridine to cytidine conversion as a possible path of incorporation into DNA. This has been shown to be only marginal with P815 cells (Schaer, Grieder, Heiniger and Schindler, 1969).

(iii) Protein

Labelled proline was used to follow protein synthesis. It was chosen for these studies because there is no proline in the medium and it is therefore taken up in substantial amounts by P815Y and MC cells.

(iv) Glycoproteins

[1-^{14}C] glucosamine was used to follow glycoprotein synthesis (Thomas, 1968).

(v) Phospholipids

Labelled choline is being used increasingly as a phospholipid precursor and thus perhaps to measure membrane biosynthesis, e.g. Penman, 1965; Arvidson, 1968; Plagemann, 1968; Rytter, Miller and Cornatzer, 1968. Nagley and Hallinan (1968) have shown choline to be incorporated specifically into lecithin (phosphatidyl choline) and moreover that it can be assayed by direct counting of TCA precipitates (confirmed by Bergeron, 1969).

60 -80% of labelled choline ($1-{}^{14}C$ or $Me-{}^{3}H$) incorporated by P815Y cells [or HC cells (Pasternak and Bergeron, 1970)] during growth for 2-3 generations was present as lipid soluble material. No radioactivity was found in the neutral fraction after silicic acid chromatography [2.5(i)]. Cells labelled with palmitate or acetate, on the other hand, contained 70-80% of total lipid radioactivity in the neutral fraction.

T.l.c. analysis of the phospholipid fraction showed that the label was predominantly in the lecithin area (80%). The same result was obtained with [$1.2-{}^{14}C$] choline (Pasternak and Bergeron, 1970).

[$2-{}^{3}H$] *myo*-inositol was also used as a phospholipid precursor. Like choline, 60 -80% was found to be lipid soluble and, of this, over 80% was present as phosphatidyl inositol (Pasternak and Bergeron, 1970).

CHAPTER THREE

GRADIENT CENTRIFUGATION

The development of a tool to investigate timing of events during the cell cycle

3.1 INTRODUCTION

At the time the work described in this thesis was initiated, thymidine blocking techniques [see 1.4(i)(b)] were already in use to synchronize P815Y cells (Bergeron, 1969). In order to confirm and extend results obtained by synchronization with excess thymidine on the timing of macromolecular syntheses during the cell cycle, a synchronization technique was sought for which involved the minimum of both chemical and physical disturbance to the cells.

Temperature shock synchrony [see 1.4(i)(a)] had been tried and found to be ineffective (Bergeron, 1969). Mitotic selection [see 1.4(ii)(a)] could not be used, since neither P815Y nor HC cells attach to glass. Therefore, selection by size seemed the most promising method.

Accordingly, the use of gradient centrifugation to separate mammalian cells of different size (Sinclair and Bishop, 1965) was subjected to an intensive study.

3.2 CONVENTIONAL GRADIENT CENTRIFUGATION

(i) Production of synchronous cells

(a) Experimental

Preliminary experiments with P815Y cells involved the centrifugation of cells (from an exponentially-growing culture) through a 5-15% (w/v) sucrose gradient. Samples from the upper and lower regions were removed with a Pasteur pipette and incubated in growth medium. They grew with some degree of synchrony as judged by cell number and size determination. However, when tested for tolerance to sucrose by exposing to solutions of 5%, 10% and 15% (w/v) sucrose for 15 min and 30 min and subsequently allowing to grow in fresh growth medium, it was found that growth was impaired in cells exposed to 15% sucrose for 30 min. Microscopic examination showed that the cells had shrunk.

Ficoll is a compact, highly branched, synthetic polysucrose, with a molecular weight of approximately 10^6 (Laurent and Pers[illegible],1964). It cannot penetrate membranes, and solutions of it have very low osmotic pressures compared to sucrose. Accordingly, in subsequent experiments, sucrose was replaced by Ficoll.

Centrifugation was carried out at room temperature in an M.S.E. 'Mistral 6L' centrifuge with a swing-out rotor (No. 62301). A linear gradient (40 ml) of 5-10% (w/v) Ficoll dissolved in medium was generated with an apparatus (Salo and Kouns, 1965) lent by Dr. M.R. Lunt. About 2 x 10^8 cells were collected by centrifugation from a culture in mid-exponential growth, resuspended in 1 ml of medium, layered onto the gradient (Figure 3-1) and stirred slightly to reduce streamer formation (Brakke, 1955). Centrifugation was for 3-5 min (depending on cell load) at 80 $g_{av.}$, after which fractions (1 ml) were collected by puncturing the tube at the bottom. They were then diluted 5-fold with warm medium, and the cells collected by centrifugation. Cells from various regions of the gradient were resuspended in warm growth medium and incubated at 37°C; samples were removed every 2 hr or less and analysed to assess the degree of synchrony, e.g. for cell number, relative volume and mitotic index. Incorporation of thymidine was also measured by exposing samples ($2-5 \times 10^5$ cells/ml) to radioactive precursor (1 μc of ^{3}H or ^{14}C/ml) for 30 min at 37°C. The entire operation was carried out aseptically.

(b) Results

Figure 3-1 shows the gradient and cells before and after centrifugation. The band of cells at the top of the tube (a) moved during centrifugation to give a broad, diffuse band around the centre of the tube (b). Cells from different areas of the gradient grew in synchronous fashion (Figure 3-2); those from the top of the gradient divided after 10-15 hr whereas those from the bottom began division within 5 hr. In each case, the generation time was 15 hr, the same as that of cells taken after mixing the whole gradient; mixed cells showed an exponential growth curve typical of unsynchronized cells. It would seem that cells near the top of the gradient are in G_1, those from near the bottom in G_2. Extent of synchrony was also judged on the

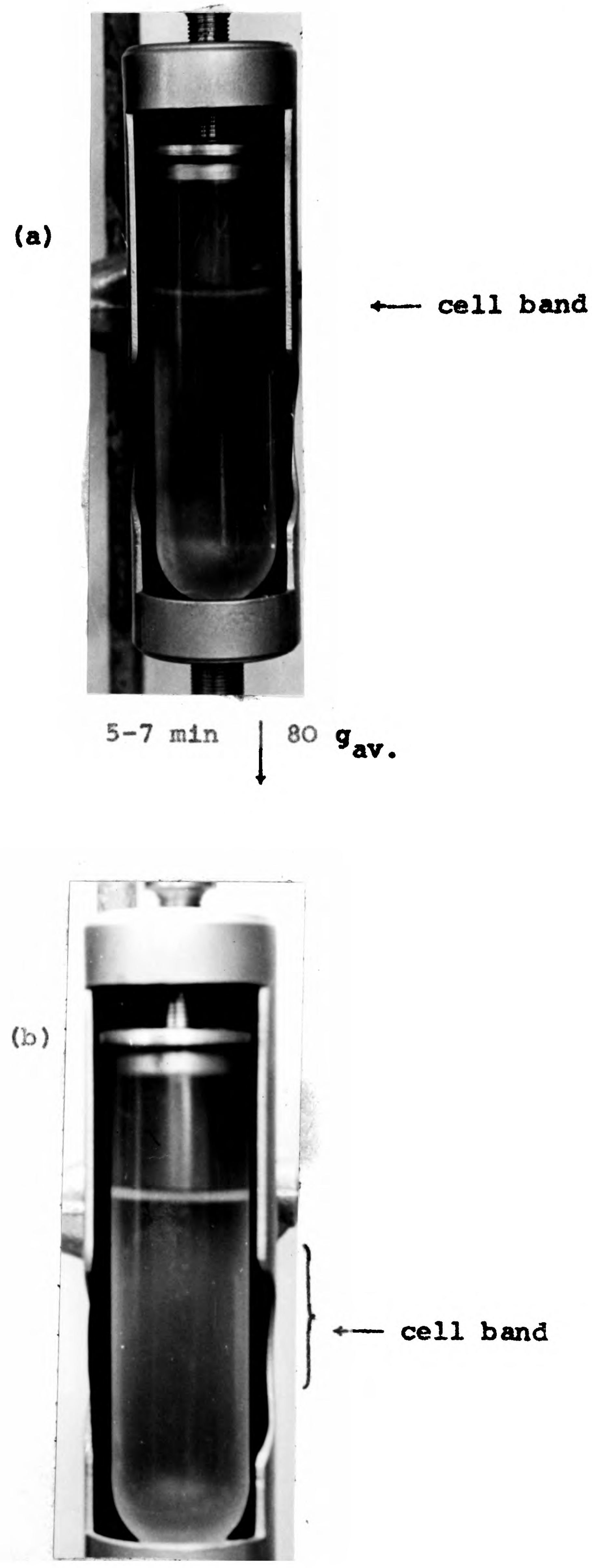


Figure 3-1: Conventional Gradient Centrifugation

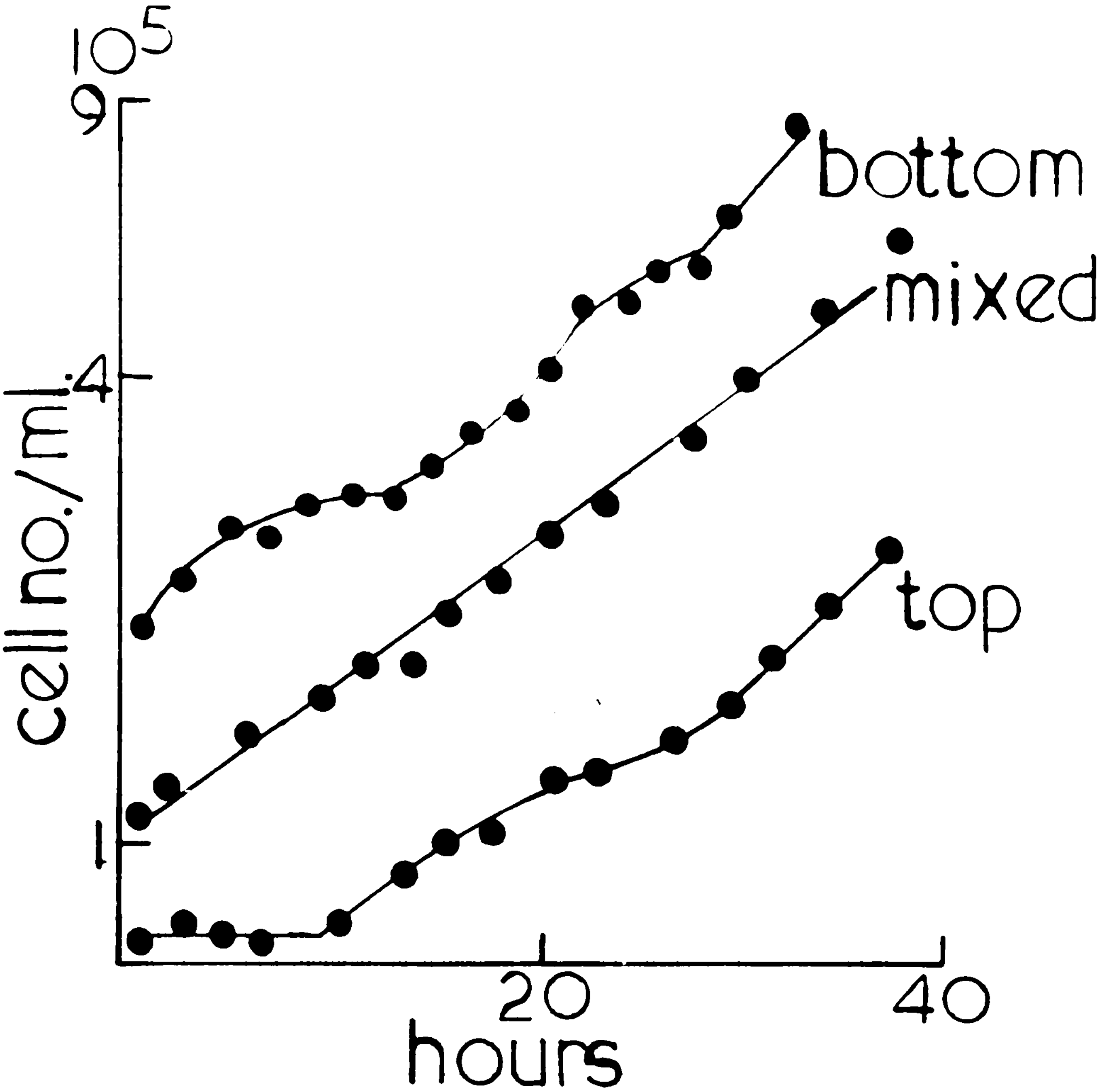


Figure 3-2: Synchronous growth of cells.
Cells (3×10^8) were separated by conventional gradient centrifugation and fractions taken from the upper region (18.7-19.0 cm from rotor centre), lower region (22.0-22.3 cm from rotor centre) and after mixing the gradient. They were treated, incubated at 37°C [see 3.2(i)(a)] and cell number determined at intervals [see 2.3(i)].

basis of thymidine incorporation; Figure 3-3 shows thymidine incorporation after 30 min at various stages during synchronous growth. Cells from the upper region of the gradient have the growth characteristics expected of G_1 cells, while those from the lower region behave in the manner expected of G_2 cells. Further confirmation was given by measuring thymidine incorporation autoradiographically, and by following mitotic index and relative cell size, as shown in Figure 3-4 (for convenience, results with cells from the lower region only are given; corresponding results were obtained with cells from the upper region). Mitotic index was at minimum when the percentage of thymidine-labelled cells was at a maximum, with a plateau in the growth curve. Just before division, the relative volume reached a peak and then fell, while mitotic index and cell number increased again.

(c) Discussion

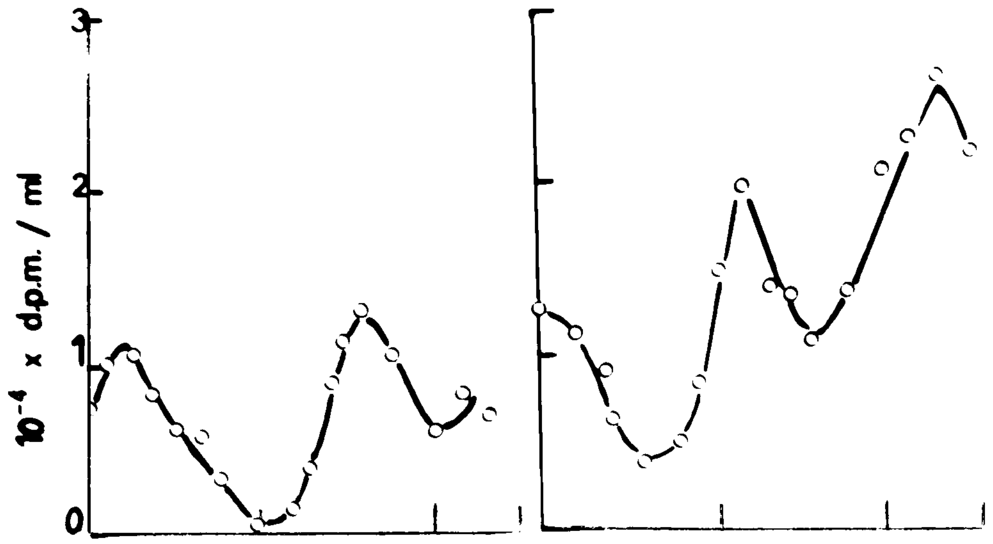
These results demonstrate that gradient centrifugation through Ficoll does not subject the cells to any adverse effects. Furthermore, cells can be separated on the basis of size into G_1 and G_2 populations, which grow synchronously. Concurrently with this work (Warmsley, Bergeron and Pasternak, 1969), similar results have been obtained with P388 cells (Ayad, Fox and Winstanley, 1969) who also demonstrated that viability and

Figure 3-3: **[^{3}H] thymidine incorporation by synchronous cells**

Cells (1.5 x 10^8) were separated by conventional gradient centrifugation and fractions from the upper region (18.7-19.0 cm from rotor centre) and lower region (22.0-22.3 cm from rotor centre) were treated and incubated at 37°C [see 3.2(i)(a)]. Samples (1 ml) were removed at intervals, exposed to 2 µCi of [^{3}H] thymidine for 30 min and cell number and incorporated radioactivity determined [see 2.3(i) and 2.6].

● cell number

○ [^{3}H] DNA



(a) upper region

(b) lower region

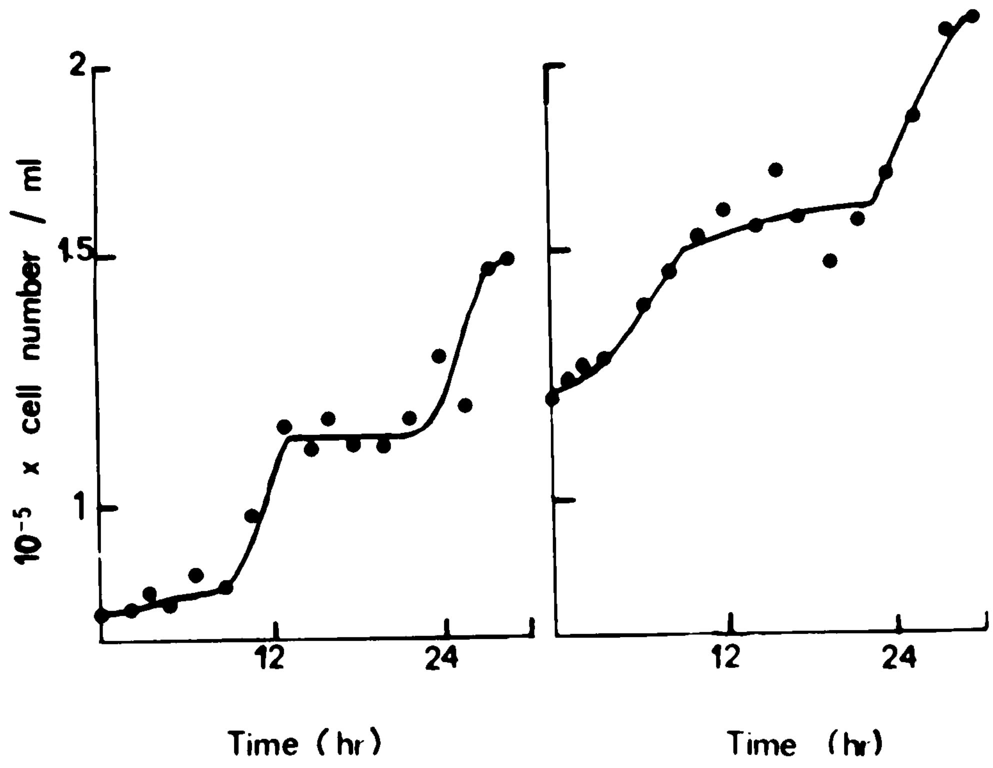
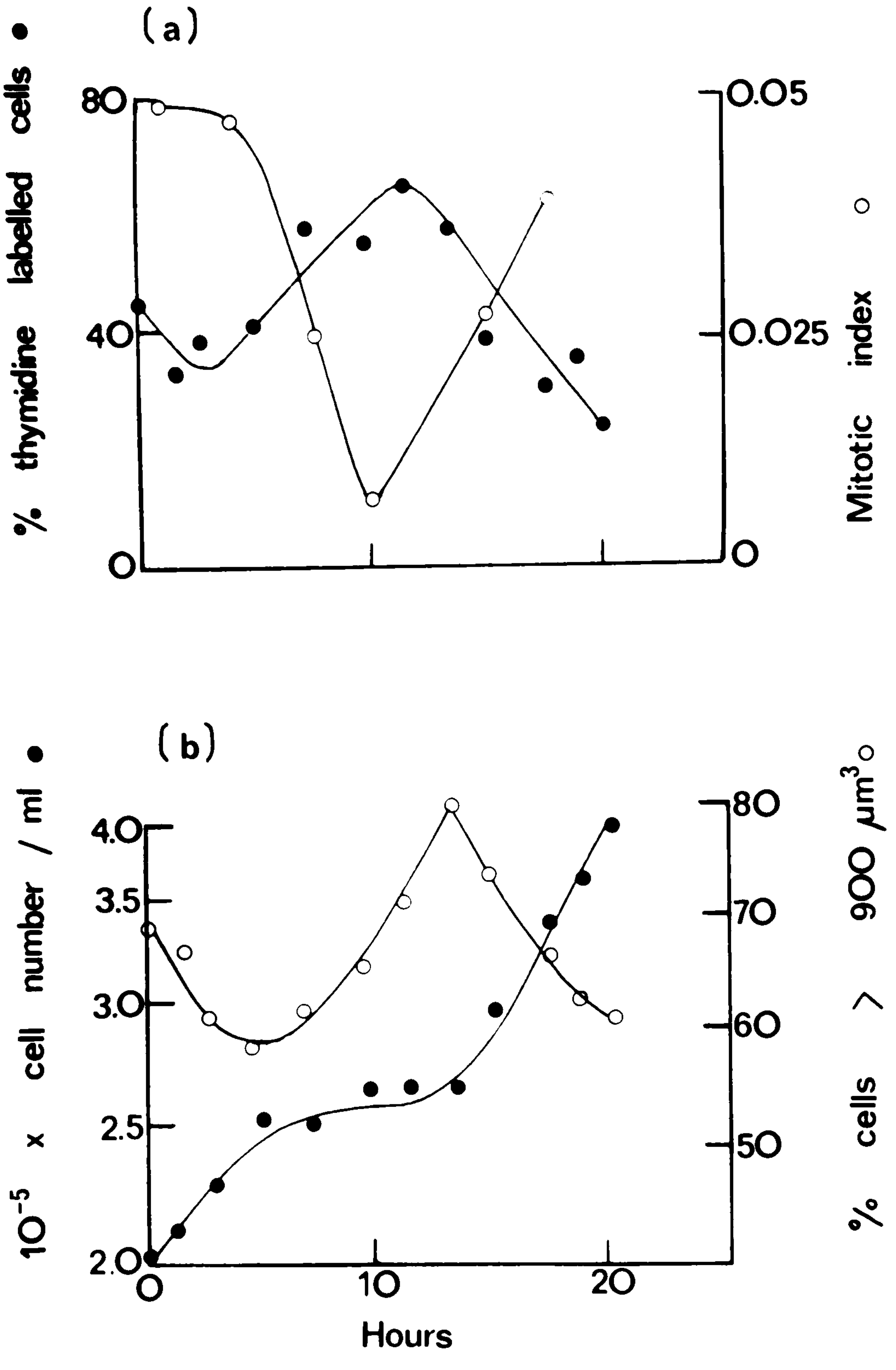


Figure 3-4: **Parameters of synchronous growth - lower region**

Cells (2.5 x 10^8) were separated by conventional gradient centrifugation and fractions from the lower region (22.0-23.3 cm from rotor centre) were treated and incubated at 37°C [see 3.2(i)(a)]. (a) % thymidine-labelled cells and mitotic index (see 2.4) and (b), cell number and relative volume (see 2.3) were determined at intervals.



Synchronous growth - lower region

plating efficiency were unimpaired, with P815-X2 cells (Schindler, Ramseier, Schaer and Grieder, 1970) and with Chinese hamster ovary cells (Fox and Pardee, 1970).

(ii) Analysis of pre-labelled cells

(a) Introduction

The experiments described so far show that cells separated by gradient centrifugation differ physiologically according to their size. However, the synchrony decays fairly rapidly. An alternative method is to look more closely at the fractions obtained immediately after separation on the gradient. This was the approach of Morris *et al.* (1967); they exposed cells to radioactive thymidine for a short period before separation so that only S cells would be labelled. By subsequently examining the fractions obtained after separation, it was possible to locate the S cells in relation to G_1 and G_2 cells.

The approach of pre-labelling cells and analysing them after separation is both more accurate and more convenient than attempting to grow up cultures of synchronous cells. By using appropriate isotopic precursors, the timing of the synthesis of various macromolecules can be examined. In every separation it is possible to set up an internal control by using thymidine as one of the precursors in a mixed isotope experiment. Work of this sort is described in Chapter 4.

(b) Experimental

Before centrifugation through the gradient, the cells (collected as before from an exponentially-growing culture) were resuspended (1 x 10^7/ml) in 10 ml of warm growth medium and incubated for 30 min at 37°C with radioactive precursor (1-5 µCi of ^{3}H/ml, 0.5-1 µCi of ^{14}C/ml). The suspension was then washed twice with chilled medium (containing the appropriate unlabelled compounds), resuspended in 1 ml of medium and layered onto the gradient.

The general procedure was very similar to that described above [see 3.2(i)(a)] except that the entire operation was carried out at 4°C, that strict aseptic technique was no longer required, and that the cell load was decreased (5-10 x 10^7 cells) to achieve better separation, with a corresponding increase in centrifugation time (5-7 min).

Fractions were assayed directly for cell number, mean cell volume and incorporated radioactivity.

(c) Results

The distribution of cells through the gradient showed a broad peak towards the centre (Figure 3-5). The thymidine incorporated (calculated per cell) was low at the top, rising sharply towards the centre, giving a broad peak just below the cell peak. An identical pattern was

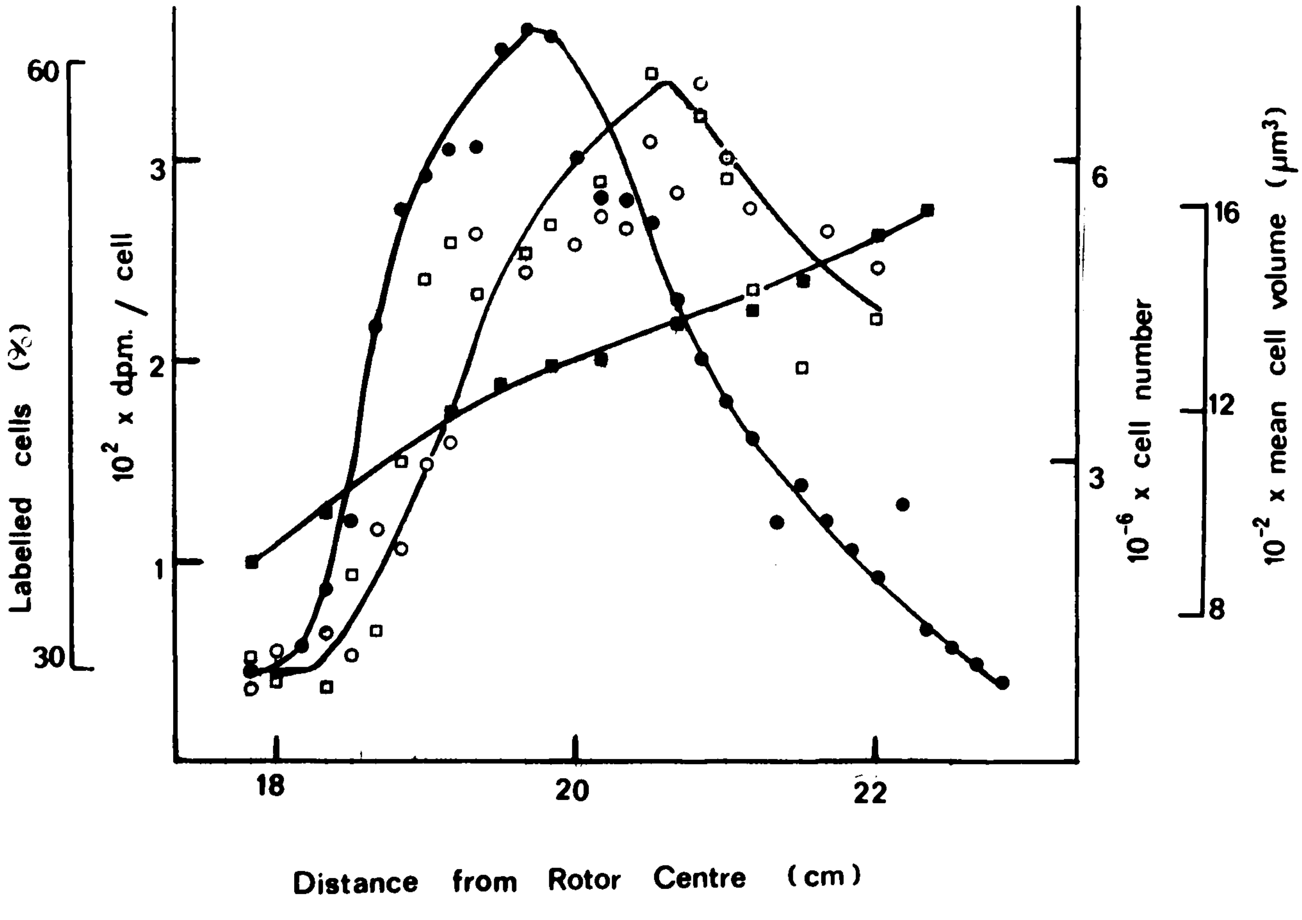


Figure 3-5: Analysis of thymidine-labelled cells separated by conventional gradient centrifugation.

Cells (1×10^8 in 10 ml) exposed to 10 µ i of $[^3H]$ thymidine for 30 min at 37^{o}C were separated and analysed [see 3.2(ii)(b 2.3, 2.4 and 2.6].

● cell number

■ mean cell volume

○ $[^3H]$ DNA

□ % $[^3H]$ thymidine-labelled cells determined autoradiographically.

obtained by autoradiographic determination. The mean cell volume rose steadily from top to bottom, with a near 2-fold increase.

To examine net DNA content of the cells through the gradient in relation to rate of synthesis, cells were grown for 60 hr (4-5 generations) in the presence of [^{14}C] thymidine, then exposed for 15 min to [^{3}H] thymidine and separated on the gradient. A difference in the two incorporation curves is apparent (Figure 3-6), with the cells showing a gradual near 2-fold increase in net DNA content. Subsequent measurement of total DNA by chemical estimation [see 4.2(ii) and Figure 4-8(d)] confirmed and extended this result.

(d) Discussion

These results confirm those obtained by Morris *et al.* (1967) and together with the synchrony work (see previous section) show clearly that cells can be separated by gradient centrifugation into populations representing different phases of the life cycle. Essentially similar results have been obtained with HC cells.

Limitations

A closer look at thymidine incorporation after a pulse (Figures 3-5, 3-6) reveals that separation of cells was not very good, especially at the lower end. If sep-

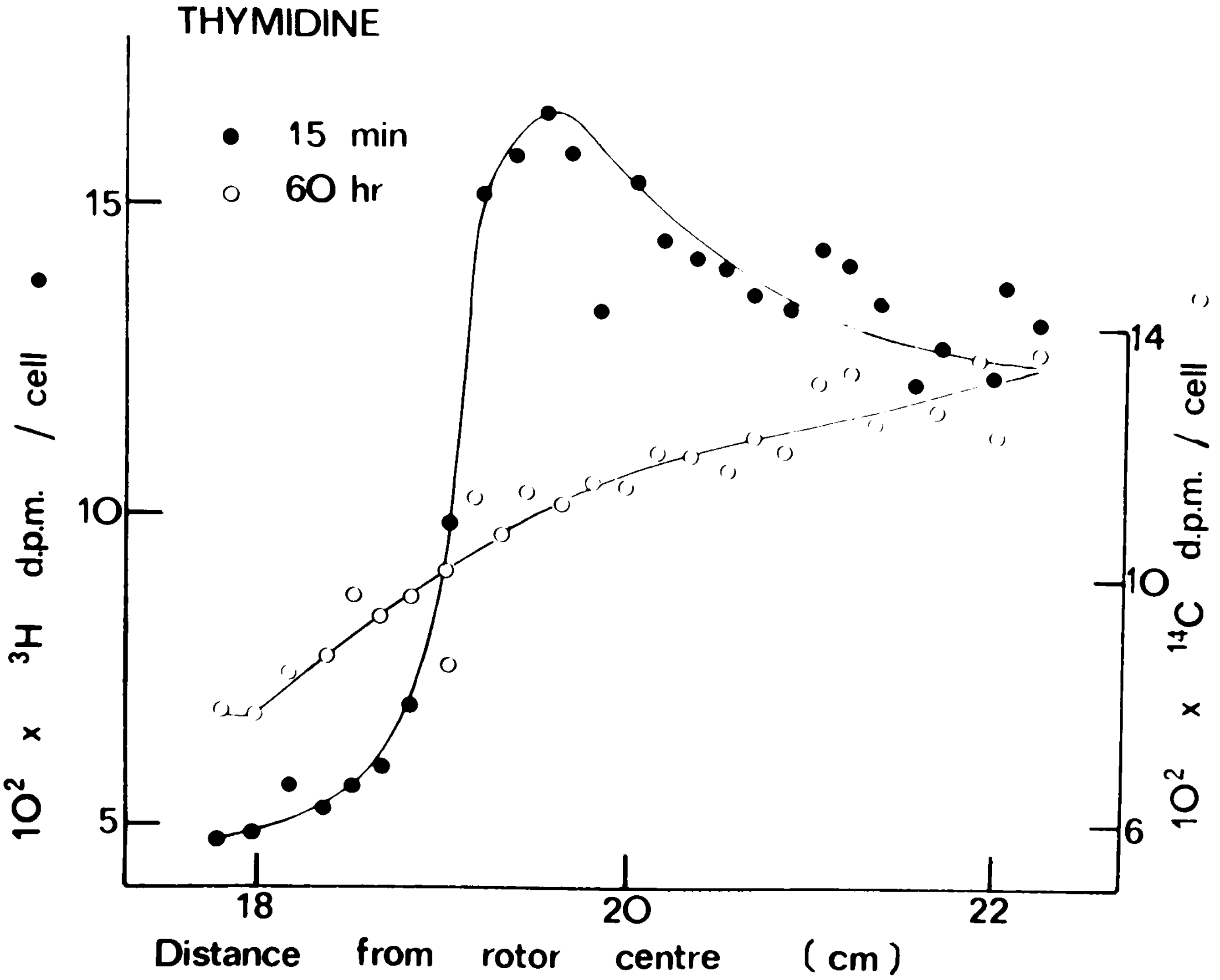


Figure 3-6: Analysis of short- and long-term thymidine-labelled cells, separated by conventional gradient centrifugation.

Cells which had been grown in the presence of [^{14}C] thymidine (0.05 μCi/ml) for 60 hr were exposed (1 x 10^8 cells in 10 ml) to 100 μCi of [^{3}H] thymidine for 15 min at 37°C, and then separated and analysed [see 3.2(ii)(b), 2.3 and 2.6].

aration of pure G_2 cells had been achieved, the incorporation/cell in the lower region of the gradient might be expected to decrease much further than it does. In fact, in some experiments, it hardly drops at all (e.g. Figure 4-3). Thus there is a poor resolution towards the bottom of the gradient, resulting in a mixture of S and G_2 cells. This helps to explain why a higher degree of synchrony was not achieved and is confirmed in Figure 3-3 which shows that at zero time, cells from the lower region are still incorporating thymidine at a relatively high rate. (Similarly, cells from the upper region have already begun to incorporate thymidine). There must therefore be a mixture of G_1 and early S cells at the top end, and, to a greater extent, late S and G_2 cells at the lower end.

To attempt to overcome this limitation of resolution, and also the relatively low yield of cells, zonal centrifugation techniques were adapted, tested and found successful.

3-3 ZONAL GRADIENT CENTRIFUGATION

(i) Introduction

A number of factors contribute towards poor resolution in any conventional gradient centrifugation system, e.g. hydrodynamic effects, streaming, swirling and wall effects (Anderson, 1955). Wall effects, especially, enable smaller particles to progress further down the gradient than they otherwise would. Thus resolution deteriorates progressively from top to bottom of the gradient. In view of this, and bearing in mind that with P815Y cells only approximately 15% of an exponentially-growing population will be in G_2 (Bergeron, 1969), it is perhaps not surprising that good separation of G_2 cells could not be achieved.

Some of the adverse effects listed by Anderson (1955) and mentioned above were partially overcome with the use of sector tubes (Anderson, 1956), but it was the development of the zonal rotor (Anderson, 1966) that has eliminated them almost completely, and at the same time allowed for a massive scale-up of the entire centrifugation process. The design permits introduction and recovery of both gradient and sample during rotation, ensuring maximum stability. High speed zonal centrifugation is now routinely used for subcellular fractionations. A low speed rotor - the 'A' series (Anderson, Barringer, Cho, Nunley, Babelay, Canning and Rankin, 1966) has been developed for the separation of larger particles, visible under the light microscope. The 'A' rotor has already been used to separate heterogeneous mixtures of mammalian cells [HeLa cells and thymocytes (Boone, Harell, and Bond, 1968)] and liver nuclei of different sizes (Johnston, Mathias, Pennington and Ridge, 1968).

The following work describes the use of the 'A' rotor to separate a homogeneous population of mammalian cells into cells at different stages of the cell cycle.

(ii) Experimental

Preliminary separation experiments were performed at the Department of Biochemistry, University College, London, and I gratefully acknowledge the advice and help of Dr. A.P. Mathias and Mr. D. Ridge. It was established that successful separations with respect to cell size were being achieved, and subsequently, a zonal rotor in the Department of Forestry, University of Oxford, was made available by Dr. T.W. Tinsley. I am extremely grateful to Mr. J. Longworth and Mrs. K. Coppin of the Department of Forestry, whose experience and help in zonal centrifugation techniques made subsequent work possible.

All operations were carried out at 4°C in an M.S.E. type 'A' zonal rotor, run in an M.S.E. 'Mistral 6L' centrifuge. A gradient (1 litre) of 2-10% (w/v) Ficoll dissolved in 50 mM-tris-HCl buffer, pH 7.4, was generated with an M.S.E. fixed-profile gradient former. Figure 3-7 shows a general view of the apparatus. The gradient was pumped in at 50 ml/min while the rotor was spinning at

Figure 3-7: The M.S.E. type 'A' Zonal Rotor

300 rev./min and was followed by an underlay of 15% (w/v) sucrose. Exponentially-growing cells were pooled (5-10 x 10^8 in 25 ml) labelled with appropriate radioactive precursor and washed, as described previously [see 3.2(ii)(b)]. The cells were then suspended in 25 ml of chilled 1% (w/v) Ficoll and introduced into the centre of the rotor followed by an overlay of 80 ml of tris-buffered saline so that the sample band reached a distance of about 2-3 cm from the rotor centre [Figure 3-8(a)]. Centrifugation was carried out for 12-15 min (depending on cell load) at 500 rev./min, producing a diffuse, barely visible, band of cells towards the middle of the gradient [Figure 3-8(b)]. The rotor speed was then decreased to 300 rev./min and fractions (25 ml) collected by pumping more underlay at 70 ml/min to the outer edge of the rotor. Fractions were analysed for cell number, mean cell volume and extent of isotopic incorporation

(iii) Results and Discussion

A good separation of G_1, S and G_2 cells, judged by thymidine incorporation, was achieved (Figure 3-9). The same pattern of incorporation was observed whether TCA-precipitates on filters [see 2.6(ii)] or hot TCA-extracts [see 2.5(ii)] were examined.

Mean cell volume increased gradually from approximately 800-1600 μm^3, and there was less spread of

(a)

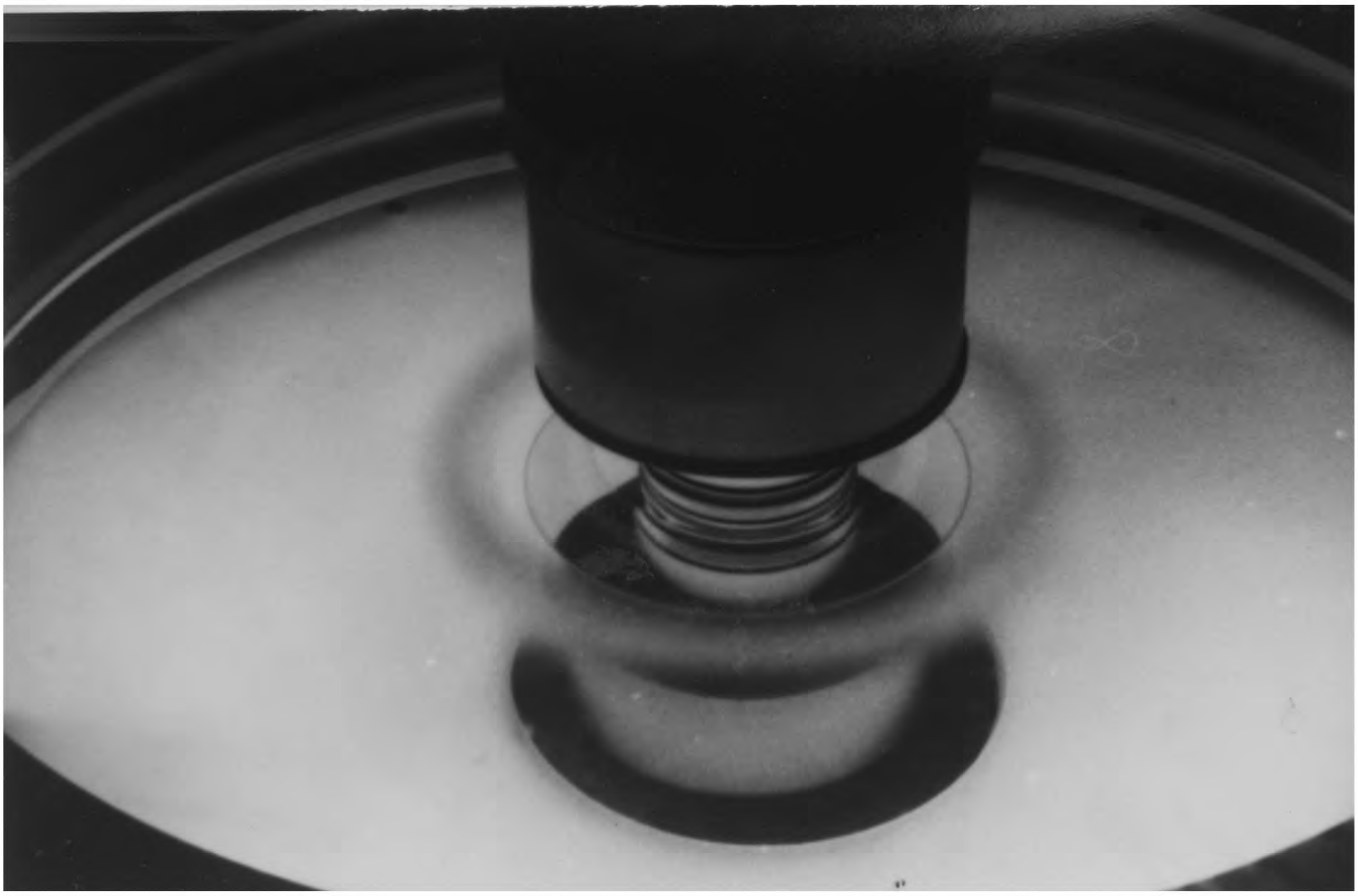


Figure 3-8:
Zonal gradient centrifugation

15 min ↓ 500 rev./min

(b)

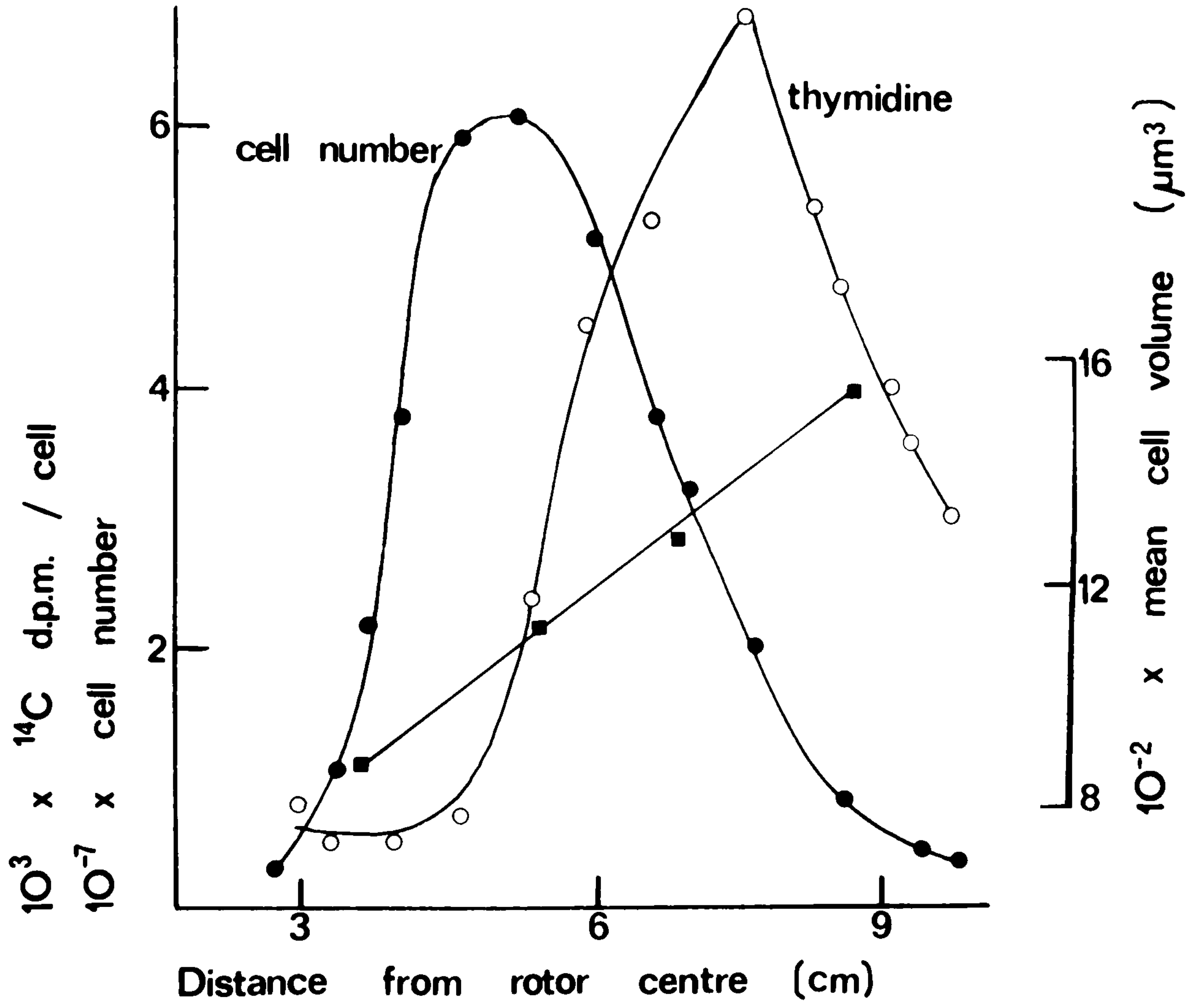


Figure 3-9: Analysis of thymidine-labelled cells separated by zonal gradient centrifugation.

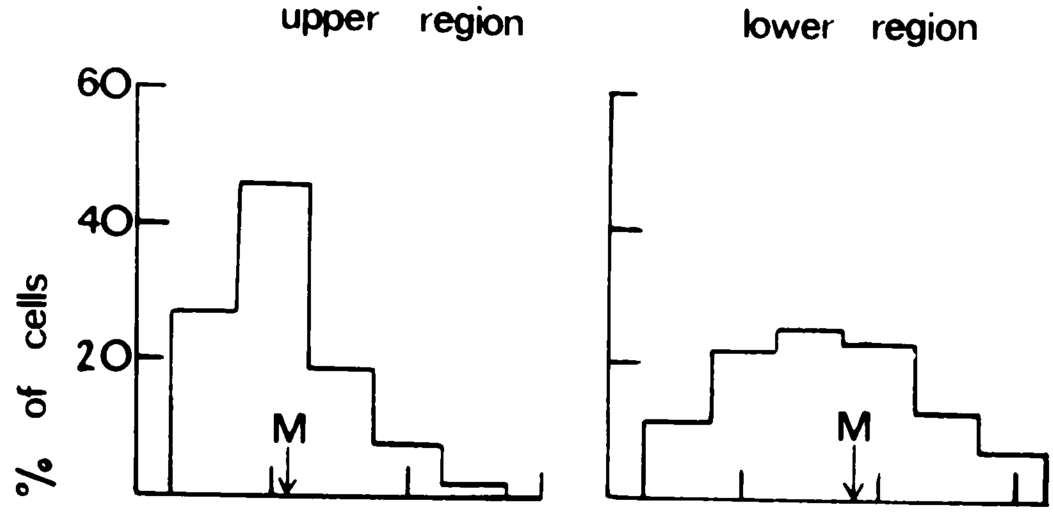
Cells (1 x 10^9 in 25 ml) exposed to 20 μCi [^{14}C] thymidine for 30 min at 37°C were separated and analysed [see 3.3(ii), 2.3 and 2.6].

● cell number
■ mean cell volume
○ [^{14}C] DNA

smaller and larger cells around the mean, especially at lower regions of the gradient, than in the case of conventional gradient centrifugation (Figure 3-10).

Essentially similar results have been obtained with MC cells. The success of the separation indicates that so far as neoplastic mast cells are concerned, the problem of lack of correlation between volume and age (Petersen *et al.*, 1969) cannot be a serious one.

(a) Conventional gradient separation



(b) Zonal gradient separation

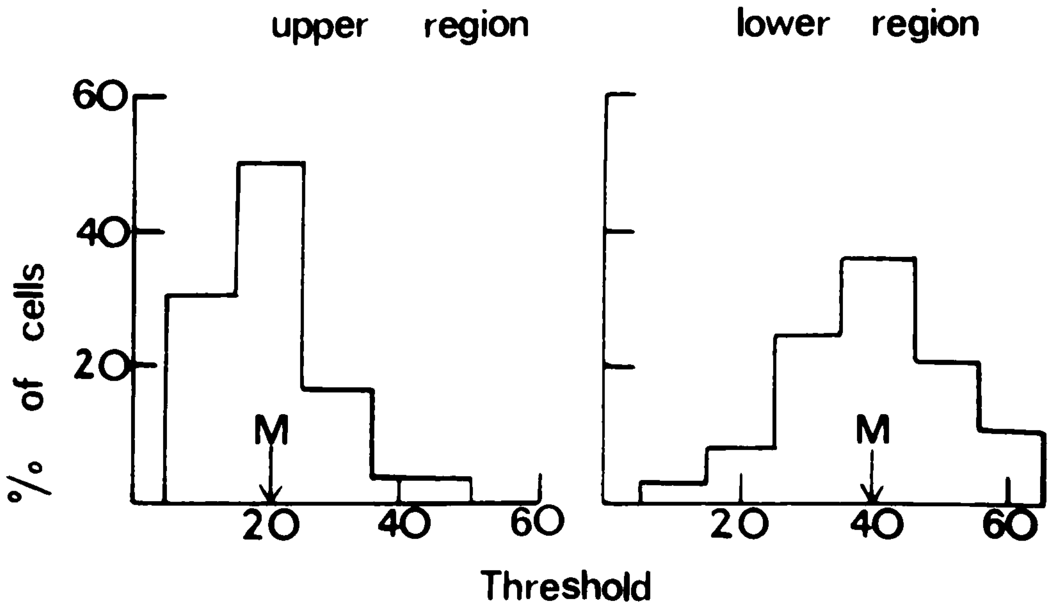


Figure 3-10 : Cell size distributions [see 2.3(ii)]

CHAPTER FOUR

THE SYNTHESIS OF MACROMOLECULES AND PHOSPHOLIPIDS DURING THE CELL CYCLE

4-1 INTRODUCTION

The biochemistry of the cell cycle

There have been several recent reviews on the biochemistry of the cell cycle (Baserga, 1968; Prescott, 1969; Mueller, 1969; Padilla, Whitson and Cameron, 1969). RNA synthesis has received closest attention to date. Quite a lot of information exists on the timing of protein synthesis and in the last two years or so, information has slowly begun to appear on the synthesis of specific proteins during the cell cycle. But virtually no information, except what has appeared in the last few months [see 4-3(ii)], exists on phospholipid synthesis and membrane construction at various stages of the cell cycle.

(i) Mitosis

Earlier work to elucidate biochemical events during the cell cycle was mainly autoradiographic, coupled with single cell studies and time-lapse cinephotomicrography as described previously [see 1.3(iv) and (v)]. Often, delineation of the various phases of the cycle was limited to comparing mitosis to interphase. It is fairly widely accepted today that during mitosis, biosynthetic activity is at a minimum. Feinendegen and Bond (1963) showed that in HeLa cells, RNA synthesis was virtually switched off from late prophase to telophase, and this was confirmed by Kim and Perez (1965), Robbins and Scharff (1966) and King and Barnhisel (1967). Similarly, protein synthesis was found to be at a minimum during mitosis (Prescott and Bender, 1962; Robbins and Scharff, 1966), and the incorporation of $^{32}PO_4$ into phospholipids was also found to be depressed during mitosis (Robbins and Scharff, 1966). It has been observed in HeLa cells that polyribosomes disaggregate during mitosis (Scharff and Robbins, 1966; Steward, Schaeffer and Humphrey, 1968) and this may well account for the depressed rate of synthesis at this time.

(ii) Interphase

Killander and Zetterberg (1965) and Zetterberg and Killander (1965)[see 1.3(v)] showed that RNA and mass, unlike DNA, increased continuously throughout interphase. Data on changes occurring in rates of synthesis, however, varies widely. There seems to be general agreement that at some stage between G_1 and G_2 there is an increase in rate, but many different patterns have been reported. Thus, for RNA synthesis, a continuous increase has been found in HeLa cells (Scharff and Robbins, 1966) and Chinese hamster cells (Enger, Tobey and Saponara, 1968; Enger and Tobey, 1969); on the other hand, Klevecz (1965) and Kasten and Strasser (1966) with Chinese hamster cells and human adenocarcinoma cells respectively, found a depression of synthesis during S, while a gene-dosage pattern, i.e. doubling in rate of synthesis following the replication of DNA during S, has often been observed, e.g. Klevecz and Stubblefield (1967) with Chinese hamster cells; Pfeiffer and Tolmach (1968) with HeLa cells; Martin, Tomkins and Granner (1969) with hepatoma cells. A similar state of confusion exists in the case of protein synthesis; both a continuous increase (e.g. Robbins and Scharff, 1966) and gene-dosage (e.g. Martin *et al.*, 1969) having been reported. Reasons for such differences are discussed later [see 4.3(i)].

This chapter sets out to describe how gradient centrifugation techniques have been used to elucidate the timing of phospholipid synthesis in relation to that of DNA, protein and RNA in P815Y and HC neoplastic mast cells. The methods used depend mainly on measuring the extent of incorporation of isotopic precursor during 30 min [and hence the rate of incorporation, which is linear for up to 1 hr (Bergeron, 1969)]. Since the results of such rate measurements and of chemical determination of net changes [see 4.2(ii)] agree, it seems valid to equate incorporation with synthesis.

In the case of phospholipids, there is the added complication of turnover [see 4.2(i)(a)]. However, using both thymidine-synchronized P815Y cells [Bergeron *et al.* (1970] and P815Y cells separated by gradient centrifugation [see 4.2(i)(a)], it has been shown that the timing of the synthesis of those phospholipids that turn over coincides with that of total labelled phospholipid, and moreover that net phospholipid increases at the same time. Thus despite turnover, choline incorporation is a useful measure of phospholipid formation.

4-2 EXPERIMENTAL AND RESULTS

(i) *Conventional gradient centrifugation*

(a) Analysis of pre-labelled cells

Cells which had been exposed to appropriate labelled precursor were separated and analysed using the procedure described previously [see 3.2(ii)(b)].

The rate of proline (2 experiments), glucosamine (1 experiment) and uridine (2 experiments) incorporation increased 2-3 fold between G_1 and S-G_2 (Figures 4-1, 4-2 and 4-5), that of choline (4 experiments) and inositol (1 experiment) 3-4 fold, (Figures 4-3 and 4-4), while thymidine incorporation increased more than 6 fold. Essentially similar results in the case of proline and choline were obtained with HC cells.

The distribution of label in the gradient following a 15 min pulse of labelled uridine or choline was identical to that obtained with cells incubated for 60-70 hr (5 generations) in the presence of labelled precursor (Figures 4-5 and 4-6), a completely different result from that obtained

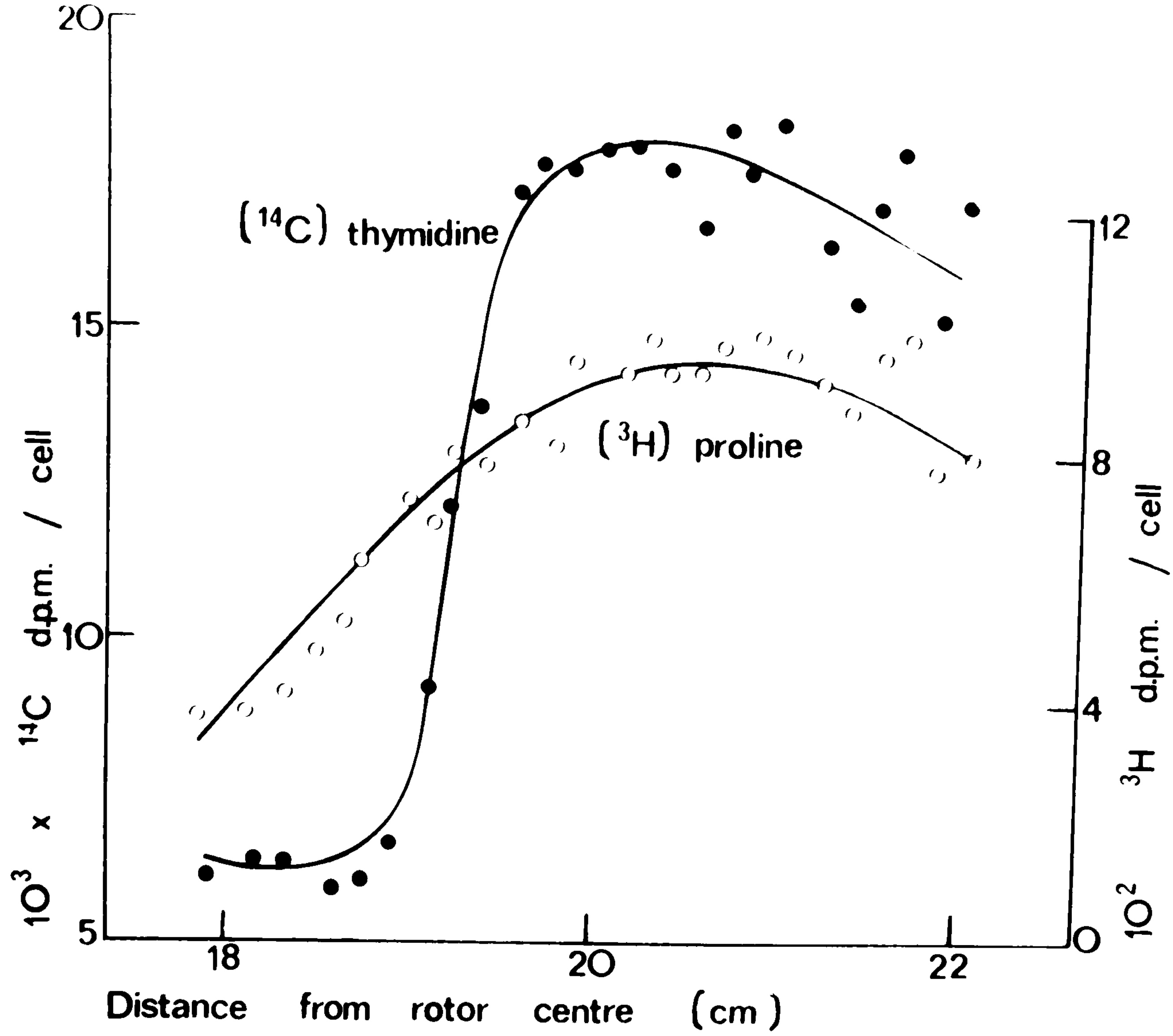


Figure 4-1: Analysis of proline-labelled cells separated by conventional gradient centrifugation

Cells (5×10^7 in 10 ml) exposed to 10 μCi of [^{14}C] thymidine and 40 μCi of [^{3}H] proline for 30 min at 37°C were separated and analysed [see 3.2(ii)(b), 2.3 and 2.6].

● [^{14}C] DNA

o [^{3}H] protein

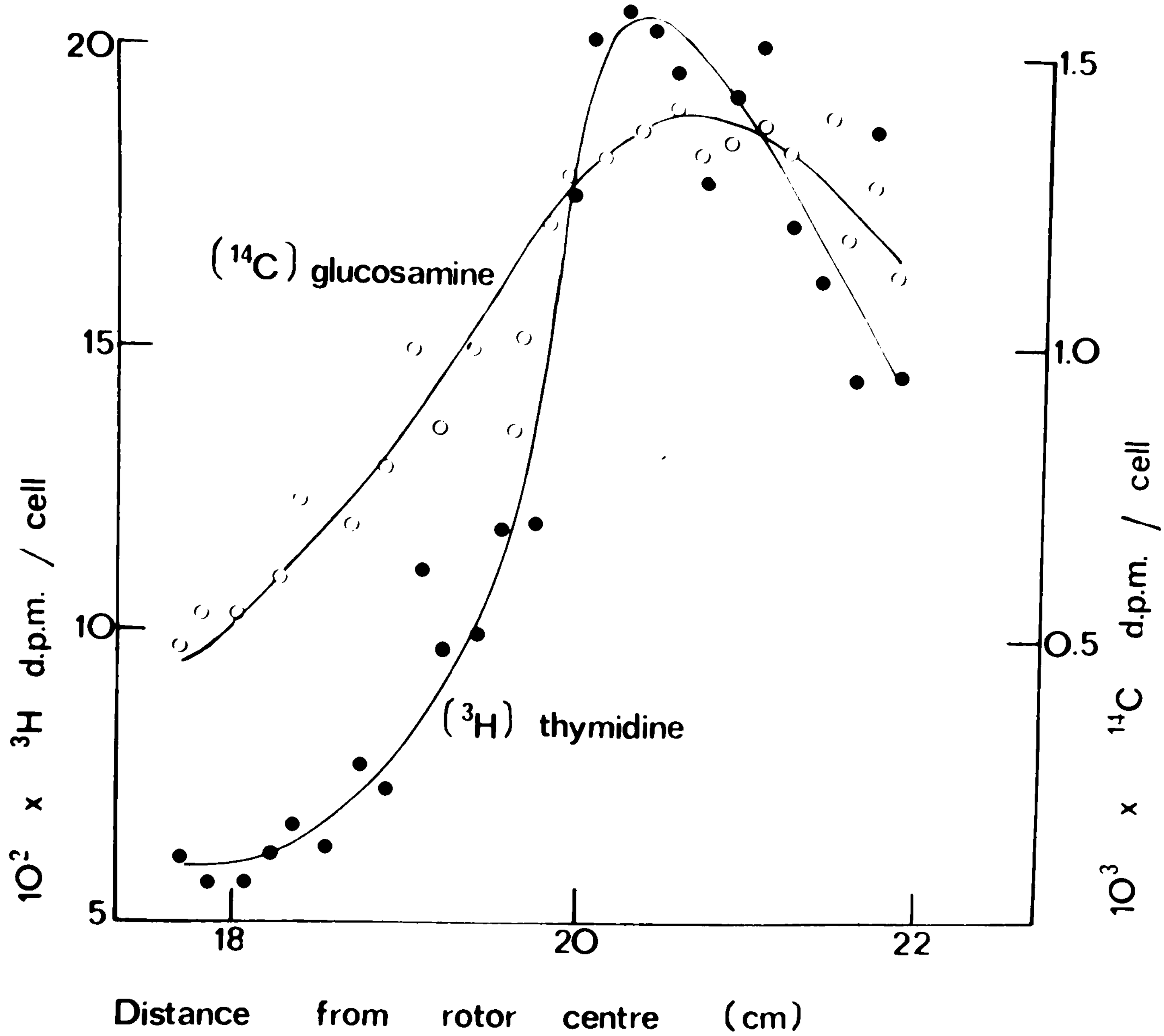


Figure 4-2: Analysis of glucosamine-labelled cells separated by conventional gradient centrifugation

Cells (5 x 10^7 in 10 ml) exposed to 20 μCi of [^{14}C] glucosamine and 60 μCi of [^{3}H] thymidine for 30 min at 37°C were separated and analysed [see 3.2(ii)(b), 2.3 and 2.6].

● [^{3}H] DNA

○ [^{14}C] glycoprotein

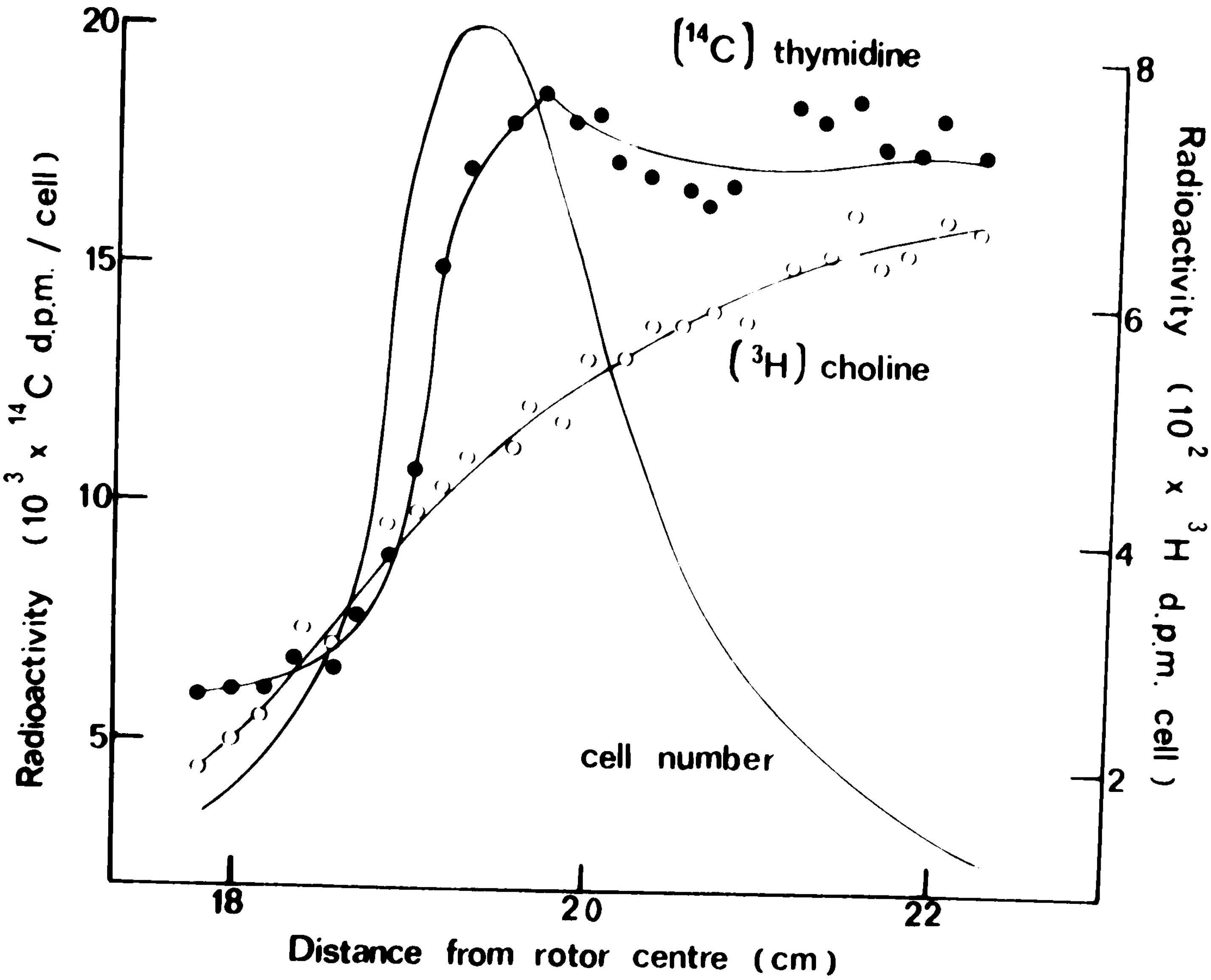


Figure 4-3: Analysis of choline-labelled cells separated by conventional centrifugation

Cells (5 x 10^7 in 10 ml) exposed to 10 μCi of [^{14}C] thymidine and 80 μCi of [^{3}H] choline for 30 min at 37°C were separated and analysed [see 3.2(ii)(b), 2.3 and 2.6].

● [^{14}C] DNA

○ [^{3}H] choline-labelled phospholipid

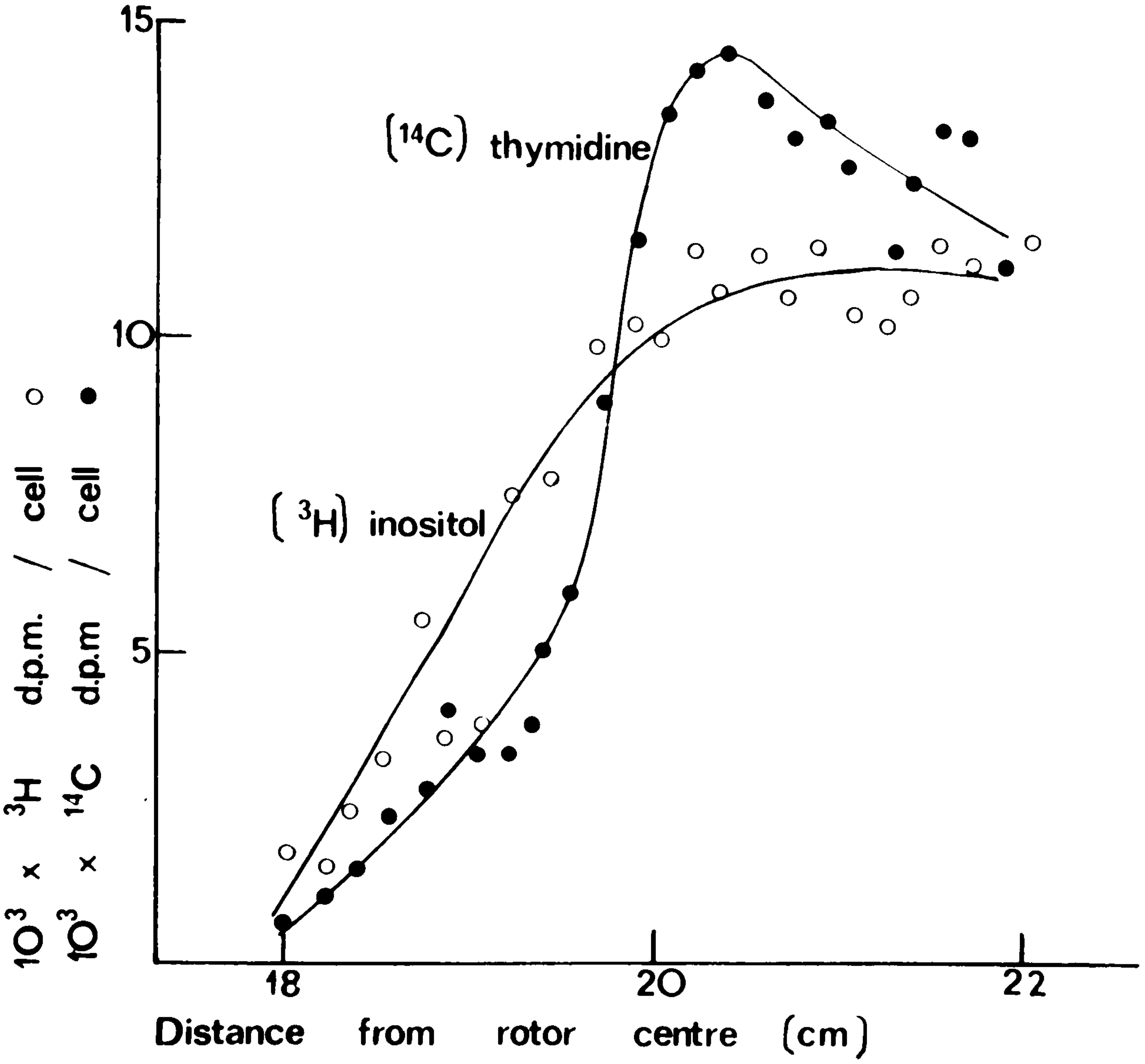


Figure 4-4: Analysis of inositol-labelled cells separated by conventional gradient centrifugation

Cells (5 x 10^7 in 10 ml) exposed to 10 μCi of [^{14}C] thymidine and 60 μCi of [^{3}H] inositol for 30 min at 37°C were separated and analysed [see 3.2(ii)(b), 2.3 and 2.6].

● [^{14}C] DNA

○ [^{3}H] inositol-labelled phospholipid

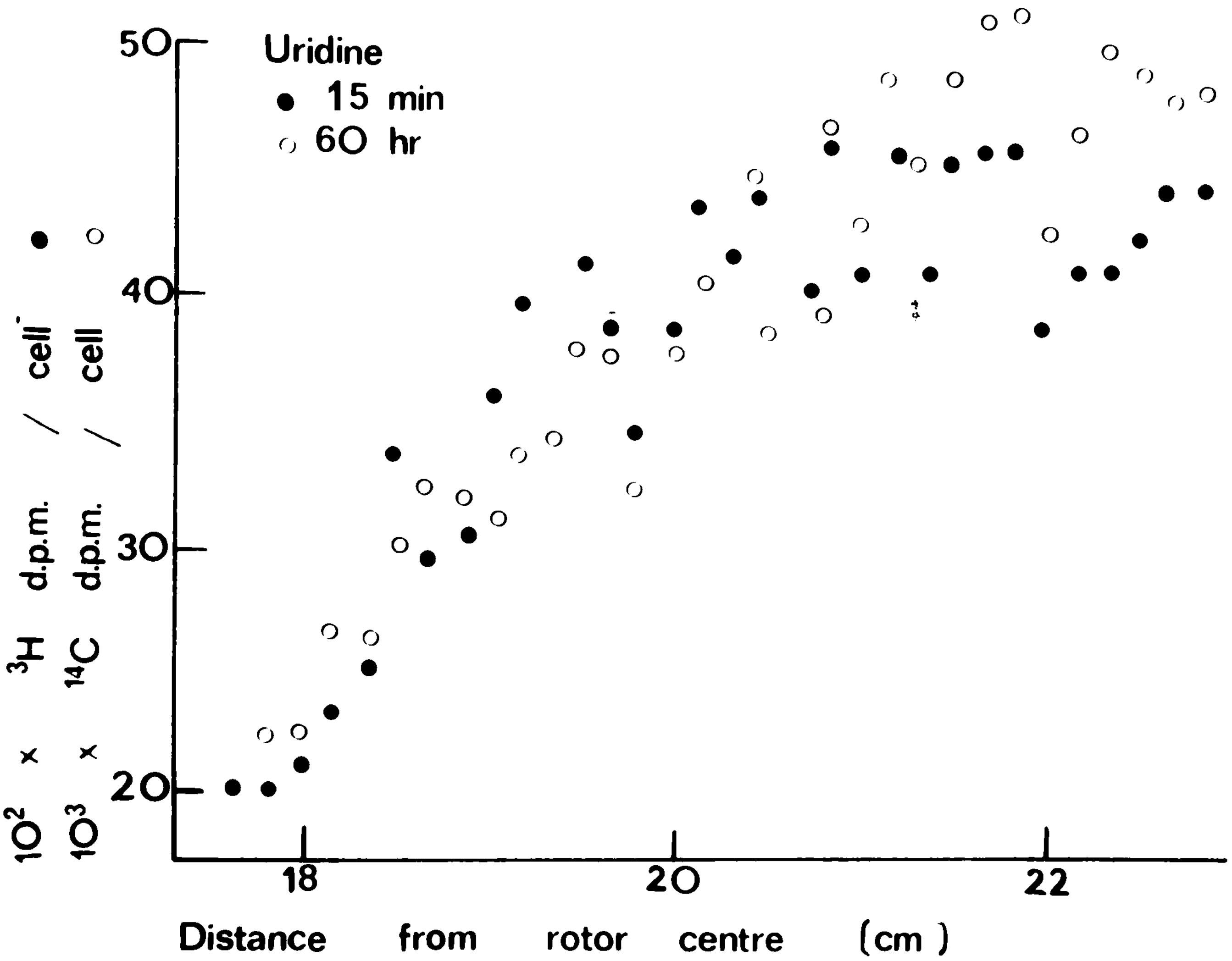


Figure 4-5: Analysis of short- and long-term uridine-labelled cells separated by conventional gradient centtifugation

Cells which had been grown in the presence of $[^{14}C]$ uridine (0.01 μCi/ml) for 60 hr were exposed (5×10^7 cells in 10 ml) to 100 μCi of $[^3H]$ uridine for 15 min at 37°C and then separated and analysed [see 3.2(ii)(b), 2.3 and 2.6].

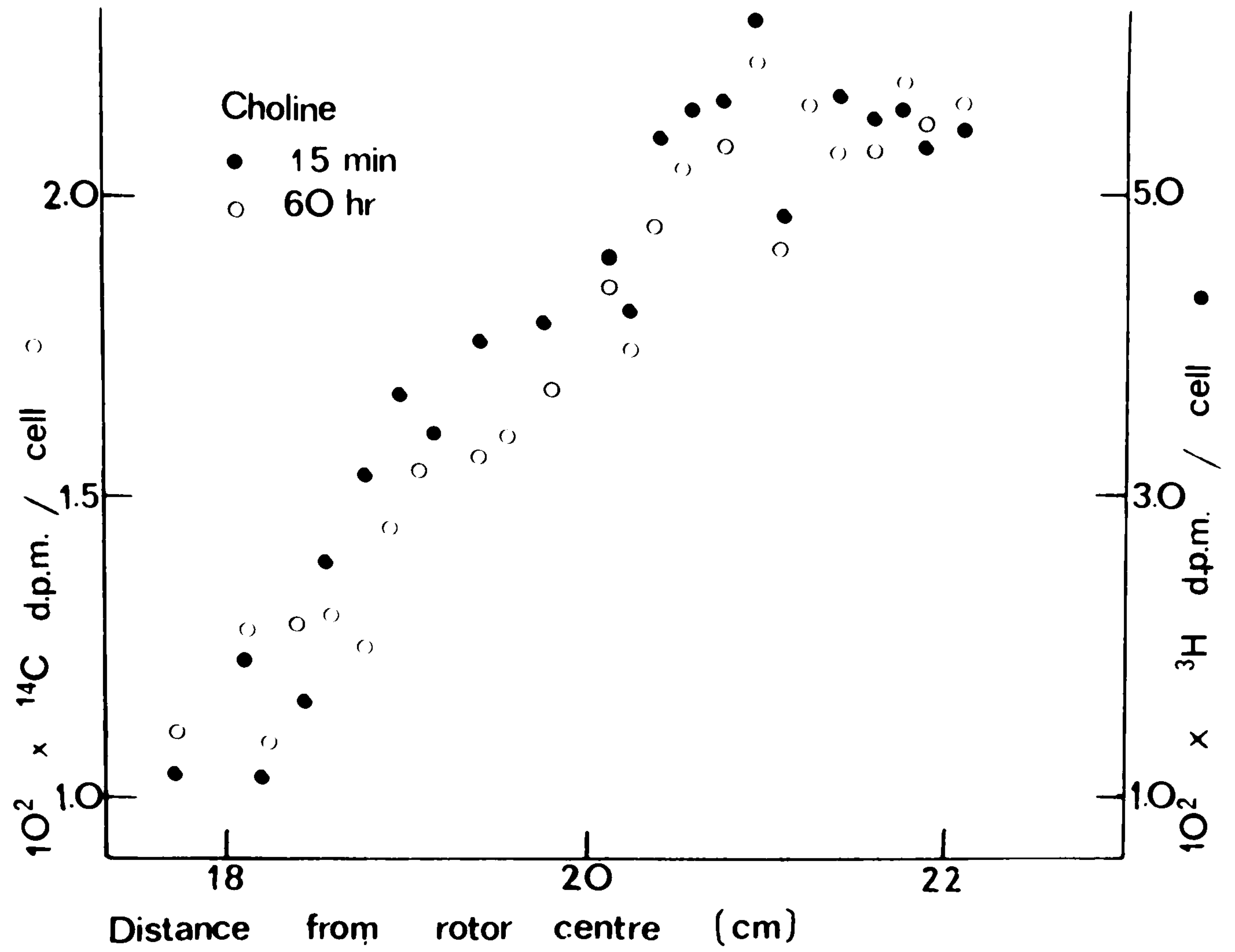


Figure 4-6: Analysis of short- and long-term choline-labelled cells, separated by conventional gradient centrifugation

Cells which had been grown in the presence of [^{14}C] choline (0.02 μCi/ml) for 60 hr were exposed (7×10^7 cells in 10 ml) to 100 μCi of [^{3}H] choline for 15 min at 37°C and then separated and analysed [see 3.2(ii)(b), 2.3 and 2.6].

in a similar experiment with labelled thymidine (Figure 3-6). This is indicative of a gradual and continuous increase in the total content of RNA and phospholipid, and is confirmed by chemical estimation [see 4.2(ii)].

Phospholipid turnover

Choline- and inositol-labelled phospholipids of exponentially-growing (or static) P815Y and EC cells were shown by Pasternak (1969) and Pasternak and Bergeron (1970) to turn over, by degradation and resynthesis of the whole molecule. Turnover followed a biphasic pattern, the 'unstable', fast component accounting for 60-80% of the labelled phospholipid. The residual 'stable' component showed a similar rate of turnover to protein and DNA.

The biphasic pattern of phospholipid turnover was seen at all stages of the cell cycle of P815Y cells (Figure 4-7); the rate of turnover fell to that of DNA, used as a control, by 40-50 hr. The amount of stable phospholipid remaining over this time varied from 15% to 35% of the total, being lowest in S cells when incorporation was maximal, and highest in G_1 cells, when incorporation was minimal. This was the result obtained by Bergeron *et al.* (1970), and makes it unlikely that penetration of isotope (Stone, Miller and Prescott, 1965) is a determining factor in the synthesis of phospholipid during the cell cycle.

Figure 4-7: **Phospholipid turnover in choline-labelled cells separated by conventional gradient centrifugation.**

Cells (6 x 10^7 in 10 ml) were exposed to 10 µCi of [^{14}C] thymidine and 100 µCi of [^{3}H] choline and; separated [see 3.2(ii)(b)]. Fractions from all parts of the gradient were diluted 5-fold with warm medium, centrifuged, resuspended (approx. 1 x 10^5/ml) in fresh growth medium containing 20 mM choline and incubated at 37°C. Samples were removed for radioactive assay [see 2.6].

(a) 17.7-18.2 cm from rotor centre;
(b) 18.5-18.8 cm;
(c) 19.2 cm;
(d) 19.6 cm;
(e) 20.1 cm;
(f) 20.6 cm;

■ [^{3}H] choline-labelled phospholipid
○ [^{14}C] DNA.

The % of stable [^{3}H] phospholipid in each fraction is indicated.

[cont'd. overleaf]

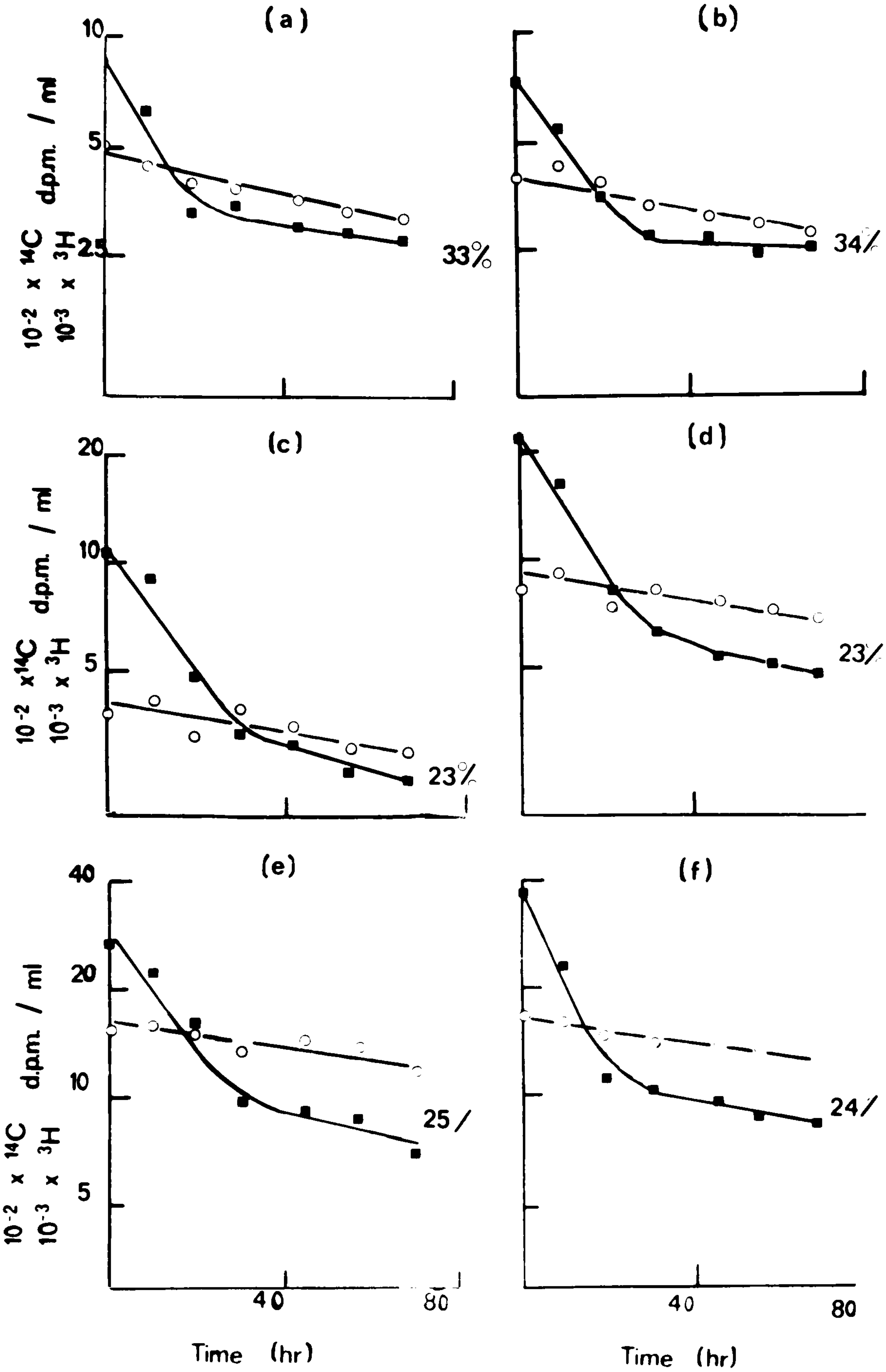

(a)
10
5
2.5
33%
(b)
34%
(c)
20
10
5
23%
(d)
23%
(e)
40
20
10
5
25%
(f)
24%
10⁻² x ¹⁴C d.p.m. / ml
10⁻³ x ³H
40
80
40
80
Time (hr)
Time (hr)

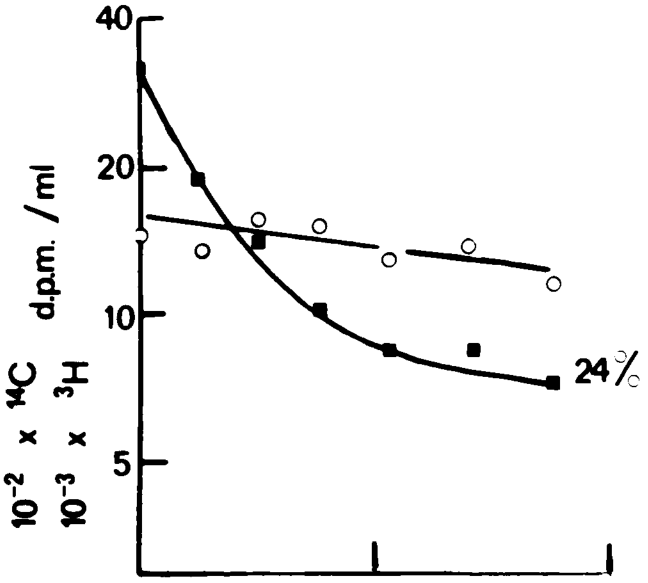
Figure 4-7: Phospholipid turnover (cont'd.)

(g) 21.1 cm from rotor centre;
(h) 21.6 cm;
(i) 22.1 cm;
(j) 22.5 cm;

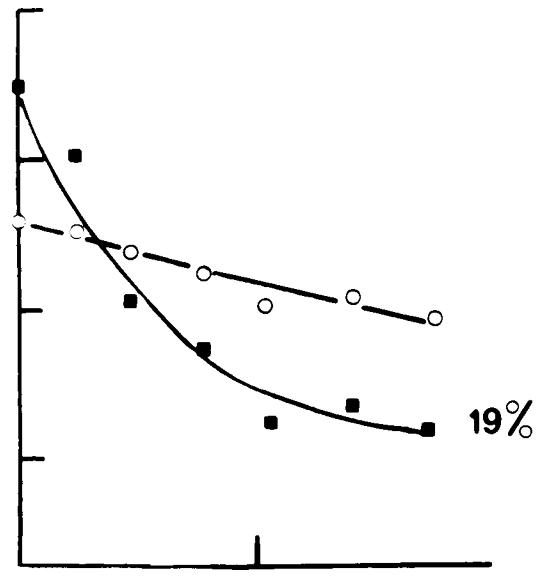
■ [^{3}H] choline-labelled phospholipid
○ [^{14}C] DNA

The % of stable [^{3}H] phospholipid in each fraction is indicated.

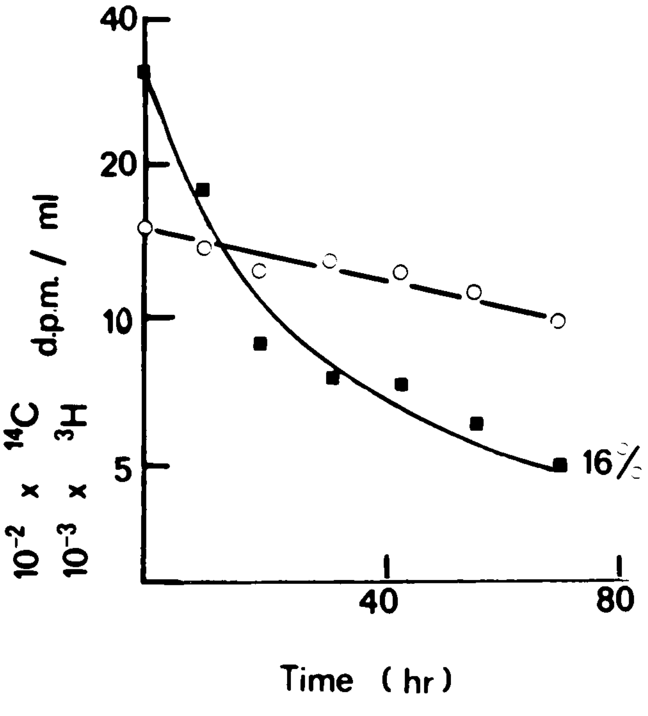
(g)


40
20
10
5
10⁻² x ¹⁴C d.p.m. /ml
10⁻³ x ³H
24%

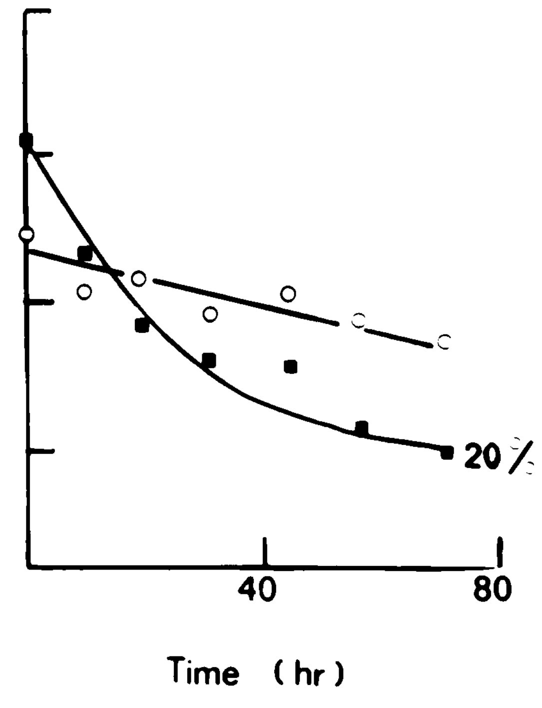
(h)


19%

(i)


40
20
10
5
10⁻² x ¹⁴C d.p.m. / ml
10⁻³ x ³H
16%
40
80
Time (hr)

(j)


20%
40
80
Time (hr)

(b) Synchronously-growing cells

Incorporation of choline during a 30 min pulse by synchronous cultures of P815Y cells selected by gradient centrifugation as described previously [see 3.2(i)(a)] followed a pattern similar to that of thymidine (Figure 3-3) in the same way as in cells synchronised chemically (Bergeron *et al.*, 1969, 1970). Note that the rate of choline incorporation varied 3-4 fold compared with > 8 fold in the case of thymidine. While this result substantiates the thymidine method for synchronizing cells, the rather sharp decrease in rate of incorporation in G_2 is surprising in view of the results with pre-labelled cells [see 4.2(i)(a), 4.2(ii)]. Perhaps the dramatic decrease during G_2 in growing cells is due to contamination with mitotic cells in which biosynthetic activity is at a minimum [see 4.1(i)]. Certainly, the phases of the cell cycle are more closely defined in cells isolated from a gradient.

(ii) Zonal centrifugation - Analysis of pre-labelled cells

Cells which had been exposed to appropriate isotopic precursor were separated and analysed using the procedure described previously [see 3.3(ii)].

Incorporation of precursor increased 2-3 fold with proline and uridine [Figure 4-8(b)], 2-4 fold with choline and inositol [Figure 4-8(c)] and more than 10 fold with

Figure 4-8: Analysis of pre-labelled cells separated by zonal centrifugation

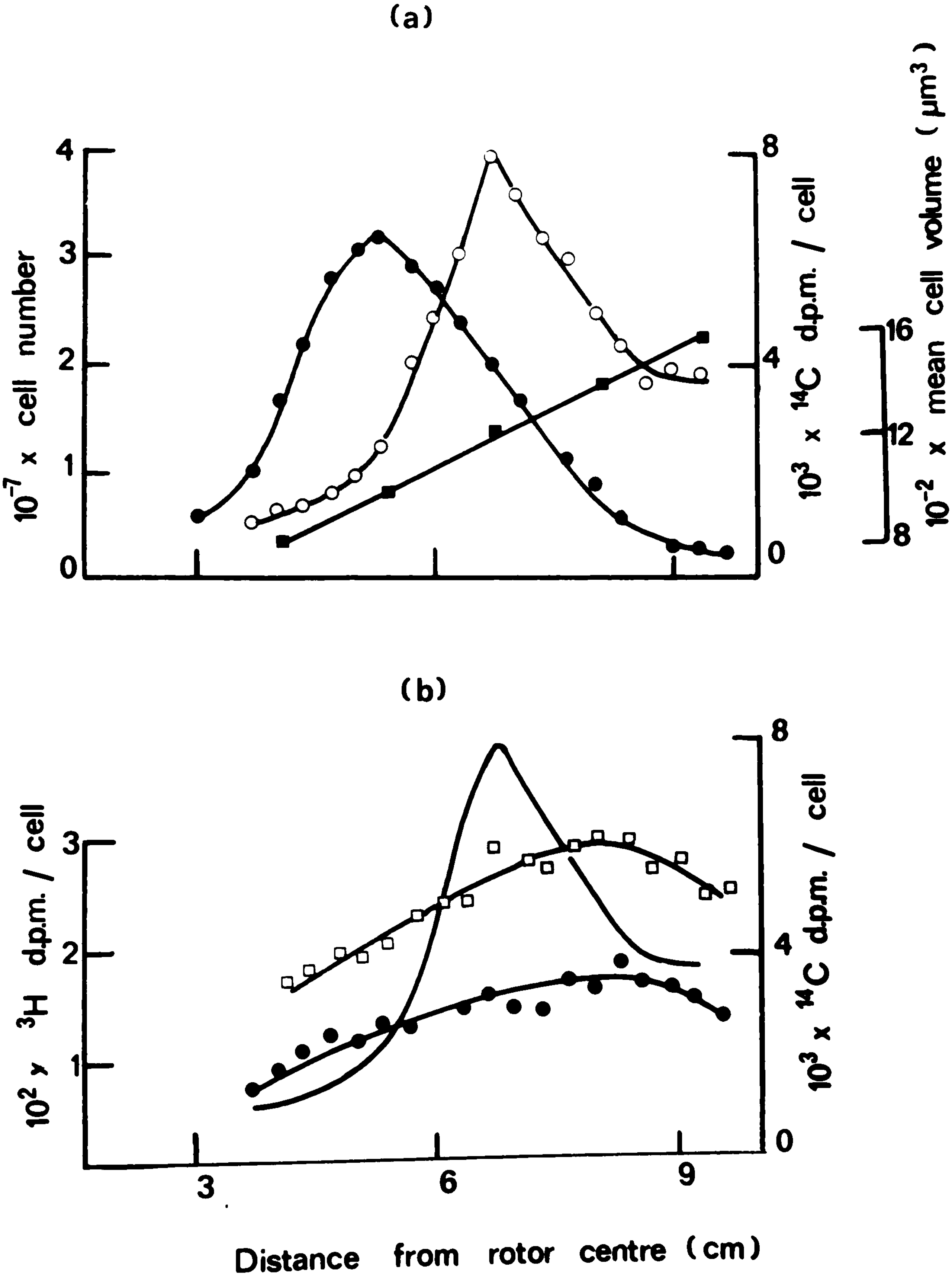
Cells (1×10^9 in 25 ml) were exposed to 20 µCi of $[^{14}C]$ thymidine and either 60 Ci of $[^{3}H]$ proline, 40 µCi of $[^{3}H]$ uridine, 40 µCi of $[^{3}H]$ choline, or 40 µCi of $[^{3}H]$ inositol, and fractionated and analysed [see 3.3(ii), 2.3, 2.5 and 2.6].

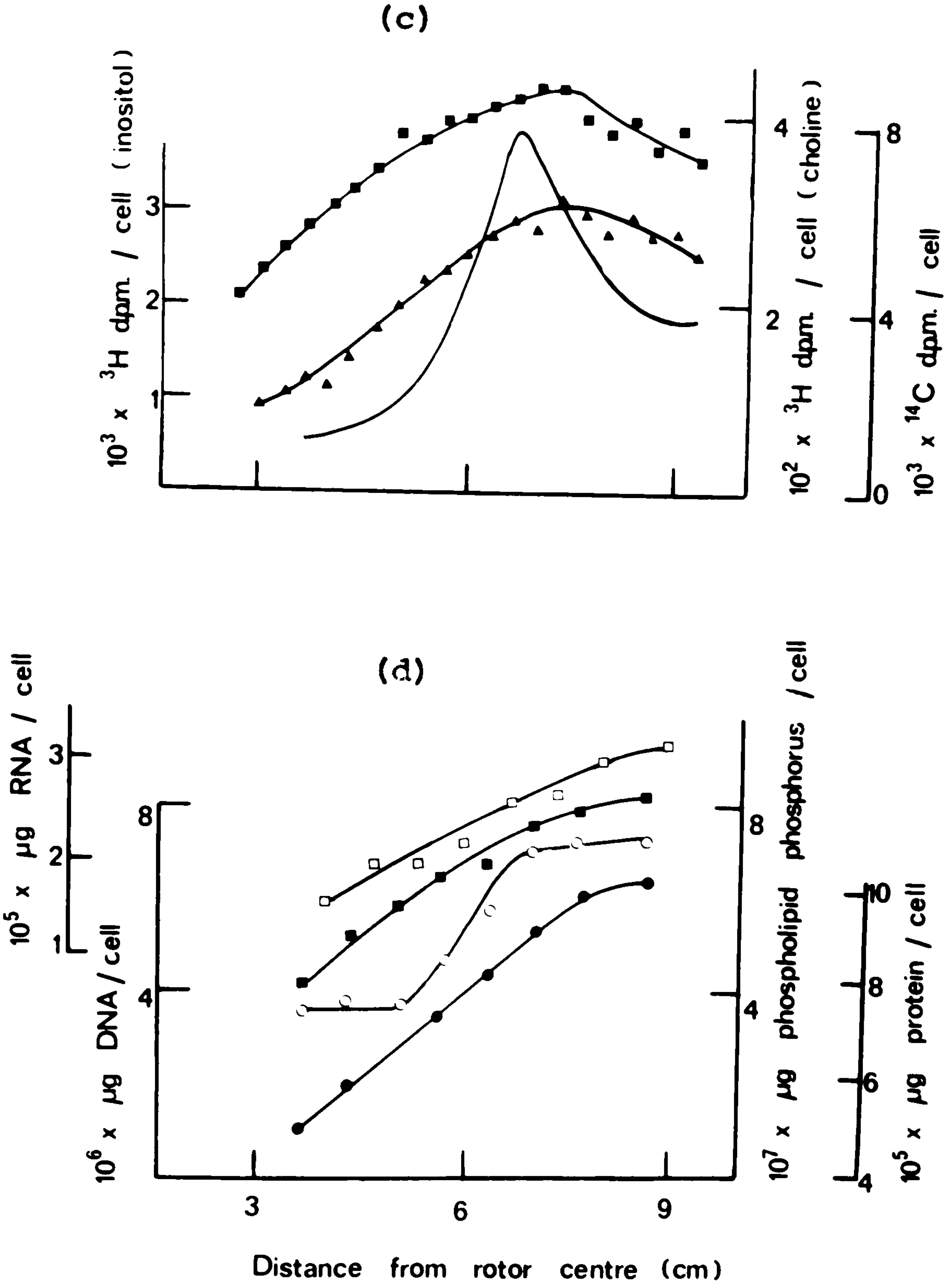
The results of four separations, reproducible with respect to cell number, mean cell volume and $[^{14}C]$ thymidine incorporation, are presented.

(a) ● cell number
■ mean cell volume
○ $[^{14}C]$ DNA
(b) ● $[^{3}H]$ protein
□ $[^{3}H]$ RNA

The pattern of $[^{14}C]$ DNA synthesis shown in (a) is repeated as a continuous line in (b) and (c).

[cont'd. overleaf]


(a)
10^{-7} x cell number
0
1
2
3
4
10^3 x ^{14}C d.p.m. / cell
0
4
8
10^{-2} x mean cell volume (μm^3)
8
12
16
(b)
10^2 x ^{3}H d.p.m. / cell
1
2
3
10^3 x ^{14}C d.p.m. / cell
0
4
8
3
6
9
Distance from rotor centre (cm)



<u>Figure 4-8</u>: <u>Analysis of pre-labelled cells separated by zonal centrifugation</u> (cont'd.)

(c) ■ [^{3}H] choline-labelled phospholipid

▲ [^{3}H] inositol-labelled phospholipid

(d) ○ total DNA

● total protein

□ total RNA

■ total phospholipid

The pattern of [^{14}C] DNA synthesis shown in (a) is repeated as a continuous line in (c).

thymidine. The same pattern of incorporation was observed with choline and inositol whether TCA-precipitates on filters [see 2.6(ii)] or chloroform-methanol extracts (lower layer) [see 2.5(i)] were examined.

Incorporation showed a distinct decrease toward the lower end of the gradient, while cell volume was still increasing [Figure 4-8(a)]. The increase in volume and the incorporation of thymidine and choline by BC cells followed a pattern similar to that of P815Y cells.

Because more cells could be separated in the zonal rotor, it was possible to determine net protein, RNA, phospholipid and DNA chemically (see 2.5). The results confirmed those obtained by 'long-term' labelling [see 3.2(ii)(c) and 4.2(i)(a)] and showed that the net content of protein, RNA and phospholipid per cell doubled more gradually than that of DNA [Figure 4-8(d)] which itself increased more gradually than the rate of its synthesis.

4-3 DISCUSSION

(i) Phospholipid synthesis in relation to protein, RNA, and DNA synthesis during the cell cycle

Analysis of pre-labelled cells separated by conventional gradient centrifugation indicates that incorporation of proline, glucosamine, uridine, choline and inositol into protein, glycoprotein, RNA and phospholipids respectively is not limited to a specific portion of the cell cycle, nor is the rate of incorporation constant throughout. Rather, incorporation appears to increase gradually from a low rate in G_1 to a higher rate in $S-G_2$ (Figures 4-1 to 4-6).

The improved resolution at the $S-G_2$ region gained with zonal centrifugation shows there to be a decreased incorporation of precursor into protein, RNA and phospholipid during G_2 (Figure 4-8).

Thus the application of gradient centrifugation techniques to the study of phospholipid metabolism during the cell cycle has confirmed and extended some of the results obtained with cells synchronized by treatment with excess thymidine (Bergeron *et al.*, 1969, 1970).

The increase observed in rate of synthesis of protein, RNA and phospholipid during G_1 is similar to results obtained by Enger and Tobey (1969) for RNA synthesis in Chinese hamster cells, whereas Crippa (1966), Pfeiffer and Tolmach (1968) and Martin *et al.* (1969) find little increase in RNA or protein synthesis during G_1 in Chinese hamster, HeLa or hepatoma cells respectively.

The fact that the rates of protein, RNA and phospholipid synthesis decrease in G_2, taken in conjunction with the increase observed during G_1, indicate that a gene-dosage effect of the kind observed by Crippa (1966), Pfeiffer and Tolmach (1968), and Martin *et al.* (1969) is not operative in P815Y and HC cells.

A decreased rate of RNA and protein synthesis in G_2 has also been observed with Chinese hamster cells (Sinclair, 1967) and with monkey kidney cells (Showacre, Cooper and Prescott, 1967). Moreover, the decrease of non-nucleolar RNA synthesis during G_2 is sharper in monkey kidney cell nuclei than in HeLa nuclei (Seed, 1963). It might therefore appear that the neoplastic mast cells under study have more in common with normal than with cancer cells. While it is true that P815Y and HC cells remain relatively differentiated in culture with respect to both biochemical and chromosomal characteristics (see 1.5) it is also the case that the pattern of net synthesis of protein, RNA and phospholipid is more akin to the increase in nuclear dry mass in HeLa and mouse L cells than that in monkey kidney cells or embryonic fibroblasts (Seed, 1962, 1963). Moreover, Baserga (1962) finds a decrease in protein synthesis in G_2 in Ehrlich ascites tumour cells. Limitations of present methodology and differences in culture conditions, rather than genuine differences between cell types, are more likely to account for such conflicting results.

(ii) Membrane synthesis during the cell cycle

Phospholipids are a characteristic constituent of cellular membranes, and it appears that only little phospholipid exists free within cells (e.g. Getz, Bartley, Stirpe, Notton, Renshaw and Robinson, 1961; Kuff, Hymer, Shelton and Roberts (1966). Thus the timing of phospholipid synthesis probably reflects the timing of the synthesis of cellular membranes.

Assuming that incorporation of choline, and hence synthesis of phospholipid, is a measure of membrane formation [see 2.5(v)] it follows that construction of membranes must be continuous during the intermitotic period, with a maximum rate in late S. Moreover, the fact that the mean cell volume changes in concert with net phospholipid [Figure 4-8(a) and (d)] is compatible with the view that the surface (plasma) membrane in particular is laid down continuously throughout the cell cycle. This result is in general accord with observations (Warren, 1969) on the incorporation of labelled leucine and glucosamine into the surface membrane of KB cells. Bosmann and Winston (1970), on the other hand, report cellular lipid and glycolipid to be synthesized almost exclusively in G_2 and M of L5178Y cells, and suggest that addition of lipid components occurs only <u>after</u> the integration of protein and glycoprotein into membrane (which occurs maximally during S).

However, many aspects of the work described by Bosmann and Winston (1970) are surprising. Thus (a) the pattern obtained for total incorporation of choline by L5178Y cells during the cell cycle is very similar to the pattern of incorporation into either TCA-insoluble material or into chloroform-methanol extract (lower layer) [see 4.2(ii)] of P815Y and MC cells. Yet on calculating total cellular incorporation from the data presented by Bosmannand Winston (1970) and comparing this to incorporation intc chloroform-soluble (lipid) material quoted by them, it would appear that as little as 5% of the choline is incorporated into lipid material of L5178Y cells after 30 min. With P815Y or MC cells, at least 50% is incorporated in this time (Pasternak and Bergeron, 1970). Furthermore (b) it is claimed that secretion (release) of choline-labelled lipid occurs in L5178Y cells (during G_2 and M), whereas with P815Y and MC cells, no radioactive chloroform-soluble material can be found in the medium during growth (Pasternak and Bergeron, 1970). Thirdly (c), Bosmannand Winston (1970) do not consider phospholipid turnover, although this is a property not only of cultured cells (Pasternak and Bergeron, 1970; Pasternak and Friedrichs, 1970), but of most cellular phospholipids in general (Dawson, 1966). Lastly (d), in view of the fact that mitosis (M) is a period of minimal biochemical activity [see 4.1(i)], it is rather difficult to believe that this is the exclusive period of cellular phospholipid and glycolipid synthesis.

Other ways of following the construction of membranes during the cell cycle involve examining specific membrane-bound enzymes or indeed total membrane protein. This approach is described in the next chapter.

CHAPTER FIVE

THE SYNTHESIS OF ENZYMES
DURING
THE CELL CYCLE

5-1 INTRODUCTION

This chapter extends the work described so far by investigating the timing of the synthesis of specific proteins (i.e. enzymes) during the cell cycle of P815Y cells. A brief review of studies to date follows. As mentioned previously when discussing protein, RNA and phospholipid synthesis [see 4.3(i)] any comparisons between different patterns of synthesis must be made with caution because of the many differences in methodology and culture conditions.

(i) *The synthesis of non-enzymic proteins during the cell cycle*

Table 5-1 sets out the present knowledge on this aspect of cell cycle studies. The rate of synthesis and accumulation of histones closely parallels the progress of DNA replication. Immunoglobulins, too, seem to be synthesized maximally in S, while collagen, on the other hand, follows net protein synthesis, although in this case (Davies *et al.*, 1968) it is not clear how net protein does, in fact, behave.

(ii) *The synthesis of enzymes during the cell cycle*

Mitchison (1969) reviewed this topic; Table 5-2 summarises present knowledge with cultured mammalian cells. Initially enzymes concerned with DNA synthesis came under the most study. Lately, other enzymes have been looked at.

TABLE 5-1:

The synthesis of non-enzymic proteins during the cell cycle

Protein	Cell Line	Timing of synthesis	Reference
Histones	HeLa	Mainly in S	Spalding, Kajiwara and Mueller (1966) Robbins & Borun (1967) Mueller (1969)
	Chinese hamster ovary	Mainly in S	Gurley & Hardin (1968)
Immunoglobulins IgG and IgM	Human lymphoid WiL2	Maximally in S	Buell & Fahey (1969)
$\gamma G_2 a$	Mouse myeloma	Maximally in S	Byars & Kidson (1970)
Collagen	Mouse 3T6	Follows net protein synthesis	Davies, Priest & Priest (1968).

TABLE 5-2:

The synthesis of enzymes during the cell cycle

Enzyme	Cell Line	Timing of increase in activity/cell	Reference
Thymidine kinase	HeLa	During S	Stubblefield, & Mueller (1965)
		Peak in G_2	Brent, Butler & Crathorn (1965)
	Mouse fibroblasts	Gene-dosage pattern	Littlefield (1966)
	Chinese hamster fibroblasts	G_1 to G_2	Stubblefield & Murphree (1967)
	Mouse L	S to G_2	Mittermayer, Bosselmann & Bremerskov (1968)
Thymidylate synthetase; Lactate dehydrogenase	Chinese hamster	Specific activity peak in S, falls, then rises in G_2	Hooper (1967)
Deoxycytidylate deaminase	Mouse L	S to G_2	Mittermayer *et al.* (1968)
	HeLa	Peak in G_2	Gelbard, Kim & Perez (1969)
Ribonucleotide reductase	Mouse L	G_1 to G_2	Turner, Abrams & Lieberman (1968)
	Chinese hamster fibroblasts	G_1 through S	Murphree, Stubblefield & Moore (1969)
DNA polymerase	Mouse L	Low in S	Gold & Helleiner (1964)
		G_1 to G_2	Turner *et al.* (1968)

TABLE 5-2 (cont'd)

Enzyme	Cell Line	Timing of increase in activity/cell	Reference
Alkaline phosphatase	Human Henle, HeLa	Peak in G_2	Melynkovych, Bishop & Swayze (1967)
Ornithine transaminase	Rat hepatoma	Constant specific activity (inducible in late G_1 and S only)	Martin *et al.* (1969)
Lactate dehydrogenase, alcohol dehydrogenase and glucose-6-phosphate dehydrogenase	Rat hepatoma	Constant specific activity	Martin *et al.* (1969)
Lactate dehydrogenase and glucose-6-phosphate dehydrogenase	Chinese hamster	Peaks in G_1, S and G_2	Klevecz & Ruddle (1968)
UDPase, esterase and 5'-nucleotidase	L5178Y lymphoma	Peak in S	Bosmann (1970)

In general (Table 5-2) there seems to be an increase in activity/cell during S, reaching a peak in G_2, prior to division. Confusion arises because in some cases, specific activity rather than activity/cell is quoted, without showing the actual pattern of increase of net protein (e.g. Martin *et al.*, 1969). Also, it is often not clear what happens at each end of the cycle, (i.e. in G_1 and G_2) usually because of poor synchrony, or lack of sufficient samples taken during the synchrony. It is seen that membrane enzymes have not yet come under study except for the work of Bosmann (1970), which appeared while this thesis was being written and which is discussed more fully later [see 5.4(ii)].

(iii) Choice of enzymes

An investigation into the timing of the synthesis of enzymes during the cell cycle can reveal information of two types.

(a) Linear reading

If genes are transcribed for only a restricted portion of the cell cycle, the order of transcription may be the same as their linear sequence on the chromosome. This sort of mechanism has been suggested for some bacterial enzymes, e.g. aspartate transcarbamylase, ornithine transcarbamylase, dehydroquinase and histidase (Masters and Pardee, 1965) but not acid phosphatase (Masters and Donachie,

1966) and also in yeasts, e.g. aspartate transcarbamylase and tryptophan synthetase, but not sucrase, acid phosphatase or alkaline phosphatase (Mitchison and Creanor, 1969). Information on the order of genes along mammalian chromosomes could thus possibly be obtained as well as information on the sequence of duplication of individual chromosomes.

(b) Duplication of cell structures and organelles

Certain enzymes or enzyme systems are known to be restricted to specific structures or organelles within the cell and are widely used for the identification and analysis of subcellular fractions. Table 5-3 lists some typical marker enzymes and shows their location in the cell. Thus, following the increase in activity of such marker enzymes during the cell cycle may yield information on the timing of duplication of the corresponding organelle.

5-2 EXPERIMENTAL

(i) Enzymes studied

Lactate dehydrogenase - L-Lactate:NAD oxidoreductase [E.C.1.1.1.27]

Glucose-6-phosphate dehydrogenase - D-Glucose-6-phosphate:NADP oxidoreductase [E.C.1.1.1.49]

Isocitrate dehydrogenase (NAD) - Threo-D_s-Isocitrate:NAD oxidoreductase (decarboxylating) [E.C.1.1.1.41]

Succinate cytochrome c reductase - Succinate:(acceptor) oxidoreductase [E.C.1.3.99.1]

Glutamate dehydrogenase - L-Glutamate:NAD oxidoreductase (deaminating) [E.C.1.4.1.2]

TABLE 5-3:

The location of some marker enzymes

Enzyme	Location	Reference
Glucose-6-phosphatase	Microsomes	Mahler & Cordes (1966, p.398)
NADPH cytochrome c reductase	Microsomes	Dallner, Siekevitz & Palade (1966)
UDPase	Smooth endoplasmic reticulum	Novikoff & Goldfischer (1961)
Esterase	Rough endoplasmic reticulum	Lansing, Belkhode & Lieberman (1967)
5'-nucleotidase	Plasma membrane	Coleman & Finean (1967)
Monoamine oxidase	Outer mitochondrial membrane	Schnaitman, Erwin & Greenwalt (1967)
Cytochrome c oxidase	Inner mitochondrial membrane	Schnaitman *et al.* (1967)
Succinate cytochrome c reductase	Inner mitochondrial membrane	Sottocasa, Kuylenstierna, Ernster & Bergstrand (1967)
Glutamate dehydrogenase	Mitochondrial matrix	Munn (1969)
Isocitrate dehydrogenase (NAD)	Mitochondrial matrix	Mahler & Cordes (1966, p.398)
Lactate dehydrgenase	Soluble (cytoplasmic)	Mahler & Cordes (1966, p.398)
Glucose-6-phosphate dehydrogenase	Soluble (cytoplasmic)	
Acid phosphatase	Lysosomes	Appelmans, Wattiaux & De Duve (1955)

Monoamine oxidase - Monoamine:oxygen oxidoreductase (deaminating) [E.C.1.4.3.4]

NADPH cytochrome c reductase - Reduced NADP:ferricytochrome c (b_5) oxidoreductase [E.C.1.6.2.a]

Cytochrome c oxidase - Ferrocytochrome c:oxygen oxidoreductase [E.C.1.9.3.1]

5'-Nucleotidase - 5'-Ribonucleotide phosphohydrolase [E.C.3.1.3.5]

Glucose-6-phosphatase - D-Glucose-6-phosphate phosphohydrolase [E.C.3.1.3.9]

(ii) Preparation of cell extracts

Exponentially-growing cells were separated by zonal centrifugation as previously described [see 3.3(ii)]. Cells from the various fractions were collected by centrifuging and resuspended in 2 ml cold 50 mM-tris-buffered saline, pH 7.5; when necessary, cells from two or more fractions were pooled, so that each 2 ml final fraction contained at least 1.5-2 x 10^7 cells.

Cells were disrupted by sonic oscillation using an M.S.E. Ultrasonic Disintegrator (100 watt) fitted with a titanium probe (end-diameter, 9.5 mm), tuned for maximum output. A sonication time of 60s was used; this is in excess of the time needed to disrupt plasma membranes but was chosen in order to ensure rupture of mitochondrial membranes (G.K. Radda, personal communication). During sonication, the cell suspension was kept chilled.

Assays were carried out on the crude sonicated extracts within 48 hr of disruption (6 hr in the case of glucose-6-phosphate dehydrogenase). In certain cases, as indicated in 5.3, subcellular fractionation was carried out as well.

(iii) Enzyme assay methods

All spectrophotometric assays were carried out in a final volume of 1 ml in semi-micro cells of 1 cm light path in a Beckman D.B.6 spectrophotometer fitted with a constant temperature device and coupled to a Sargent S.R. recorder.

Activity is given as μmol substrate transformed (or product formed)/min (U), either per cell (when it is quoted as % of maximum) or per mg protein (specific activity).

The molar extinction coefficient for NADH (NADPH) was taken as 6.2×10^6 $lmol^{-1}$ cm^{-1} (340 nm) and for cytochrome c as 18.5×10^6 $lmol^{-1}$ cm^{-1} (550 nm). All assays were carried out at 2 enzyme concentrations such that rate was proportional to concentration.

Lactate dehydrogenase

Activity at 30°C was measured by following the decrease in extinction at 340 nm due to the oxidation of NADH during the reduction of pyruvate to lactate (Bergmeyer, Bernt and Hess, 1963). The reaction mixture contained 0.1 μmol of NADH, 45-50 μmol of tris-HCl buffer, pH 7.5, and 0.005-0.02 ml of sonicated extract. The reaction was started by the addition of 0.3 μmol of Na-pyruvate.

Glucose-6-phosphate dehydrogenase

Activity at 30°C was measured by following the increase in extinction at 340 nm due to the reduction of NADP during the oxidation of glucose-6-phosphate to 6-phosphogluconate (Löhr and Waller, 1963). The reaction mixture contained 0.6 μmol of NADP, 30-50 μmol of tris-HCl buffer, pH 7.5, and 0.1-0.4 ml of sonicated extract. The reaction was started by the addition of 0.8 μmol of glucose-6-phosphate.

Glutamate dehydrogenase

Activity at 37°C was measured by following the decrease in extinction at 340 nm due to the oxidation of NADH during the reductive amination of 2-oxoglutarate (Schmidt, 1963). The reaction mixture contained 0.1 μmol of NADH, 120 μmol of ammonium acetate, 1 μmol of EDTA, 30-50 μmol of tris-HCl buffer, pH 8.0, 0.1-0.3 ml of sonicated extract and 1.5 μmol of ADP to activate the enzyme (Williamson, Lund and Krebs, 1967). After waiting 2-3 min for any endogenous reaction to finish (usually negligible) the reaction was started by the addition of 8 μmol of 2-oxoglutarate.

Cytochrome c oxidase

Activity was measured at 37°C by following the decrease in extinction at 550 nm due to the oxidation of reduced cytochrome c (Appelmans, Wattiaux and De Duve, 1955). The reaction mixture contained 40-50 μmol of Na-phosphate buffer, pH 7.5 and 0.05 μmol of cytochrome c (the stock solution was previously reduced by the addition of solid $Na_2S_2O_4$) and the reaction was started by the addition of 0.01-0.05 ml of sonicated extract.

NADPH cytochrome c reductase and succinate cytochrome c reductase

Activity was measured at 37°C by following the increase in extinction at 550 nm due to the reduction of cytochrome c during the oxidation of NADPH or succinate (Sottocasa, Kuylenstierna, Ernster and Bergstrand, 1967). The reaction mixture contained 40-50 µmol of Na-phosphate buffer, pH 7.5, 0.3 µmol of KCN, 0.1 µmol of NADPH or 2.5 µmol of succinate and 0.05-0.2 ml of sonicated extract. The reaction was started by the addition of 0.1 µmol of cytochrome c.

Isocitrate dehydrogenase (NAD), monoamine oxidase, 5'-nucleotidase and glucose-6-phosphatase

The above enzymes showed negligible or undetectable activity at the highest concentrations feasible and are not quoted in the results section.

Isocitrate dehydrogenase (NAD) was assayed by measuring the reduction of 0.3 µmol of NAD during the oxidation of 1 µmol of isocitrate, in the presence of 5 µmol of citrate, 2 µmol of ADP, 1 µmol of $MnSO_4$ and 2 µmol of $MgCl_2$ at pH 6.5 (E.A. Newsholme, personal communication). The assay was carried out immediately after sonication; the presence of ADP during sonication had no effect on the negligible level of activity.

Monoamine oxidase was assayed by the method of Schnaitman *et al.* (1967) by following the decrease in extinction at 250 nm during the formation of benzylamine from 2.5 μmol of benzaldehyde. No activity could be detected.

5'-nucleotidase was assayed by the method of Michell and Hawthorne (1965) by measuring the phosphate released (King, 1932) from 5 μmol of AMP in the presence of 100 μmol of KCl, 10 μmol of $MgCl_2$ and 10 μmol of NaK tartrate (to inhibit acid phosphatase) at pH 7.4. No activity was detected. A more sensitive method was employed (Murray and Friedrichs, 1969) by using 0.1 μmol of $[^3H]$ CMP (0.025 μCi) as the substrate and measuring the release of $[^3H]$ cytidine (separated by paper chromatography). Again, no activity was detected.

Glucose-6-phosphatase was assayed by the method of Hübscher and West (1965) by measuring the phosphate released (King, 1932 or Chen, Toribara and Warner, 1956) from 20 μmol of glucose-6-phosphate, in the presence of 3 μmol of EDTA and 1.5 μmol of KF (to inhibit alkaline and acid phosphatase respectively) at pH 6.0. No activity could be detected. This was not due to masking by the rather high level of phosphate initially present in the glucose-6-phosphate solution since no glucose release (Bergmeyer and Bernt, 1963) could be detected either. Using the same assay system, rapid phosphate release was observed with a mouse-liver homogenate, known to contain high levels of glucose-6-phosphatase.

(iv) Fluorescent probe studies

To determine the percentage of 'mitochondrial-electron-transport-protein' present in the sonicated extracts, fluorescent probe techniques developed by Dr. G.K. Radda (Brocklehurst, Freedman, Hancock and Radda, 1970) were applied. The work that follows was carried out in collaboration with J.R. Brocklehurst.

The reaction mixture, 1 ml final volume, contained 225 μmol of mannitol, 75 μmol of sucrose, sonicated extract corresponding to 0.1 mg of protein and 20 μmol of tris-HCl-buffer, pH 7.4. 32.5 μmol of 1-anilinonaphthalene-8-sulphonate (ANS) were then added, and the resulting fluorescence due to protein binding was measured with a Zeiss spectrofluorimeter. Excitation was at 390 nm and emission read at 490 nm. Addition of 50 μmol of succinate resulted in an enhancement of fluorescence. This was reversed by adding a specific uncoupler of electron transport, carbonyl cyanide p-trifluoromethoxyphenyl -hydrazone(FCCP). The decrease in fluorescence thus obtained was expressed as a percentage of the total fluorescence obtained with ANS and is thus a measure of the percentage of succinate-dependent, FCCP-sensitive, mitochondrial-electron-transport-protein, relative to total protein.

5-3 RESULTS

The two soluble cytoplasmic enzymes, lactate dehydrogenase (4 experiments) and glucose-6-phosphate dehydrogenase (3 experiments) showed a gradual doubling in activity/cell in relation to net DNA content [Figure 5-1(a)], following a pattern similar to that of net protein [Figure 4-8(d)]. This is seen more clearly by the negligible variation in specific activity [Figure 5-1(b)]. The same pattern was obtained when high-speed supernatant fractions prepared from the crude sonicated extracts were analysed. > 95% of the total lactate dehydrogenase activity was found in the supernatant fraction.

The microsomal marker enzyme, NADPH cytochrome c reductase (3 experiments) showed a similar variation in activity, also following the pattern of net protein synthesis (Figure 5-2).

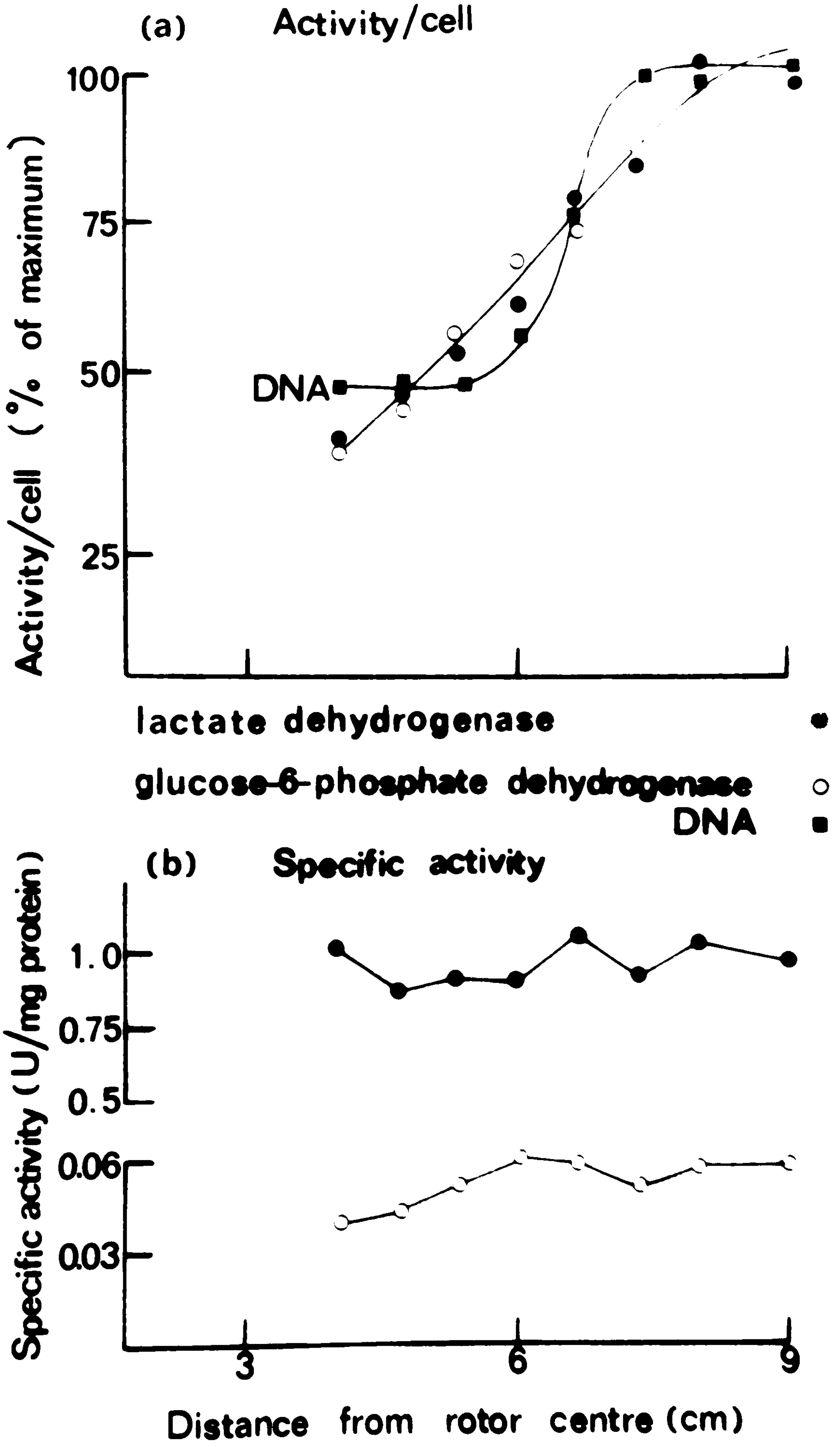
The two mitochondrial inner membrane marker enzymes, cytochrome c oxidase (4 experiments) and succinate cytochrome c reductase (2 experiments) again followed the same pattern (Figure 5-3). Essentially similar results in the case of cytochrome c oxidase were obtained when mitochondrial fractions [prepared by sub-cellular fractionation (Mahler and Cordes, 1966, p.394) of a cell suspension disrupted with a Potter-Elvejhem glass homogeniser] were analysed. > 95% of the total cytochrome c oxidase activity was found in the mitochondrial fraction.

On the other hand, glutamate dehydrogenase (4 experiments), a soluble enzyme of the mitochondrial matrix, showed a different pattern. There was no major increase until after DNA had begun to double [Figure 5-4(a)]. The initial sharp decrease in specific activity [Figure 5-4(b)] confirmed that this enzyme was indeed following a pattern of duplication different to that of net protein. The same pattern was

Figure 5-1: **Lactate dehydrogenase and glucose-6-phosphate dehydrogenase activities in cells separated by zonal gradient centrifugation**

Cells (1×10^9) were separated and their number and mean volume determined [see 3.2(ii)]. Sonicated extracts [see 5.2(ii)] were assayed for enzyme activity [see 5.2(iii)] and for total DNA [see 2.5(ii)].

Separations were reproducible with respect to cell number, mean cell volume, total protein and total DNA. The pattern of DNA increase shown is repeated as a continuous line in Figures 5-2, 5-3 and 5-4.


(a) Activity/cell
Activity/cell (% of maximum)
100
75
50
25
DNA
lactate dehydrogenase ●
glucose-6-phosphate dehydrogenase ○
DNA ■
(b) Specific activity
Specific activity (U/mg protein)
1.0
0.75
0.5
0.06
0.03
3
6
9
Distance from rotor centre (cm)

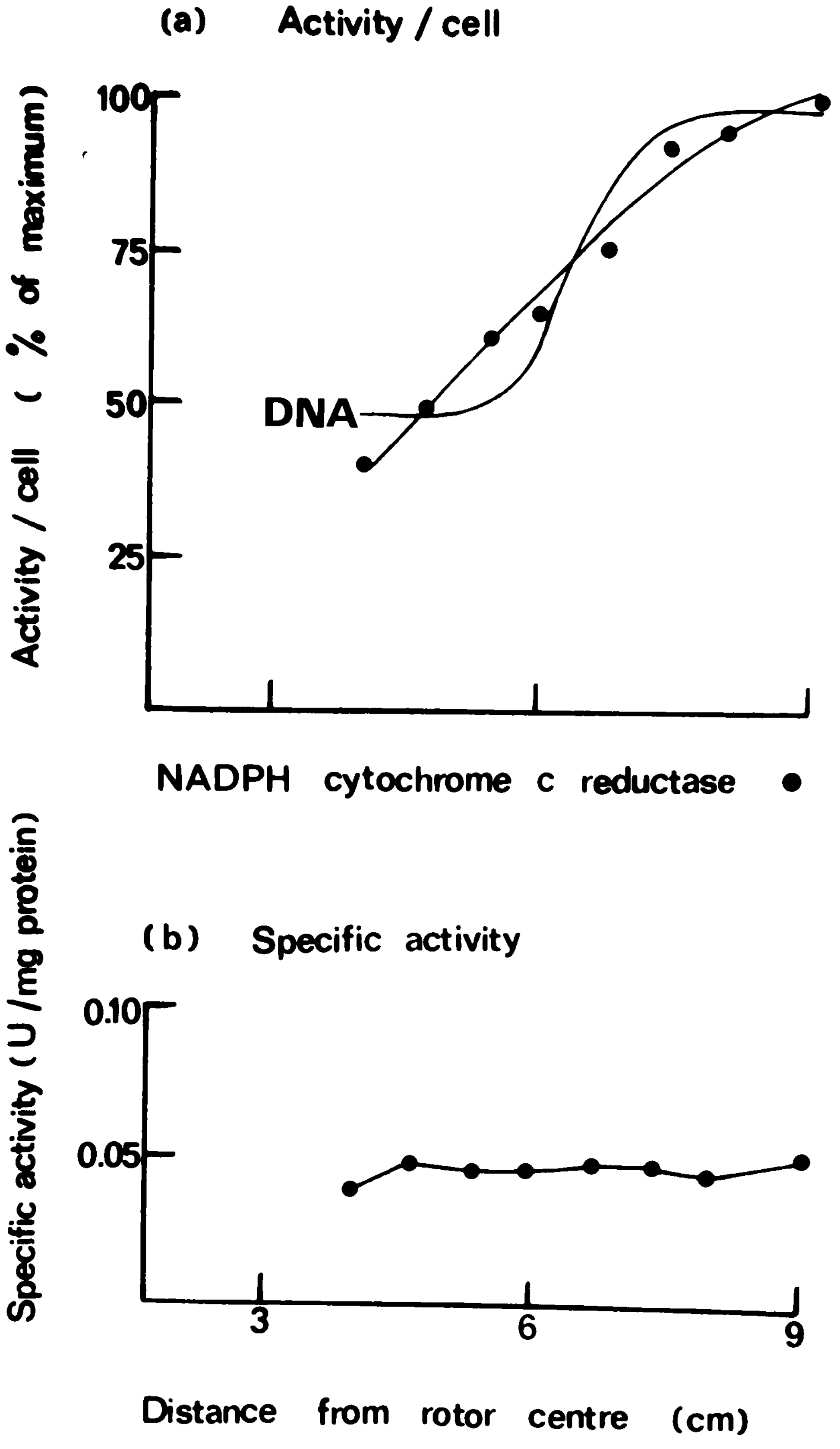


Figure 5-2: NADPH cytochrome c reductase activity in cells separated by zonal gradient centrifugation

[Procedure - see Figure 5-1].

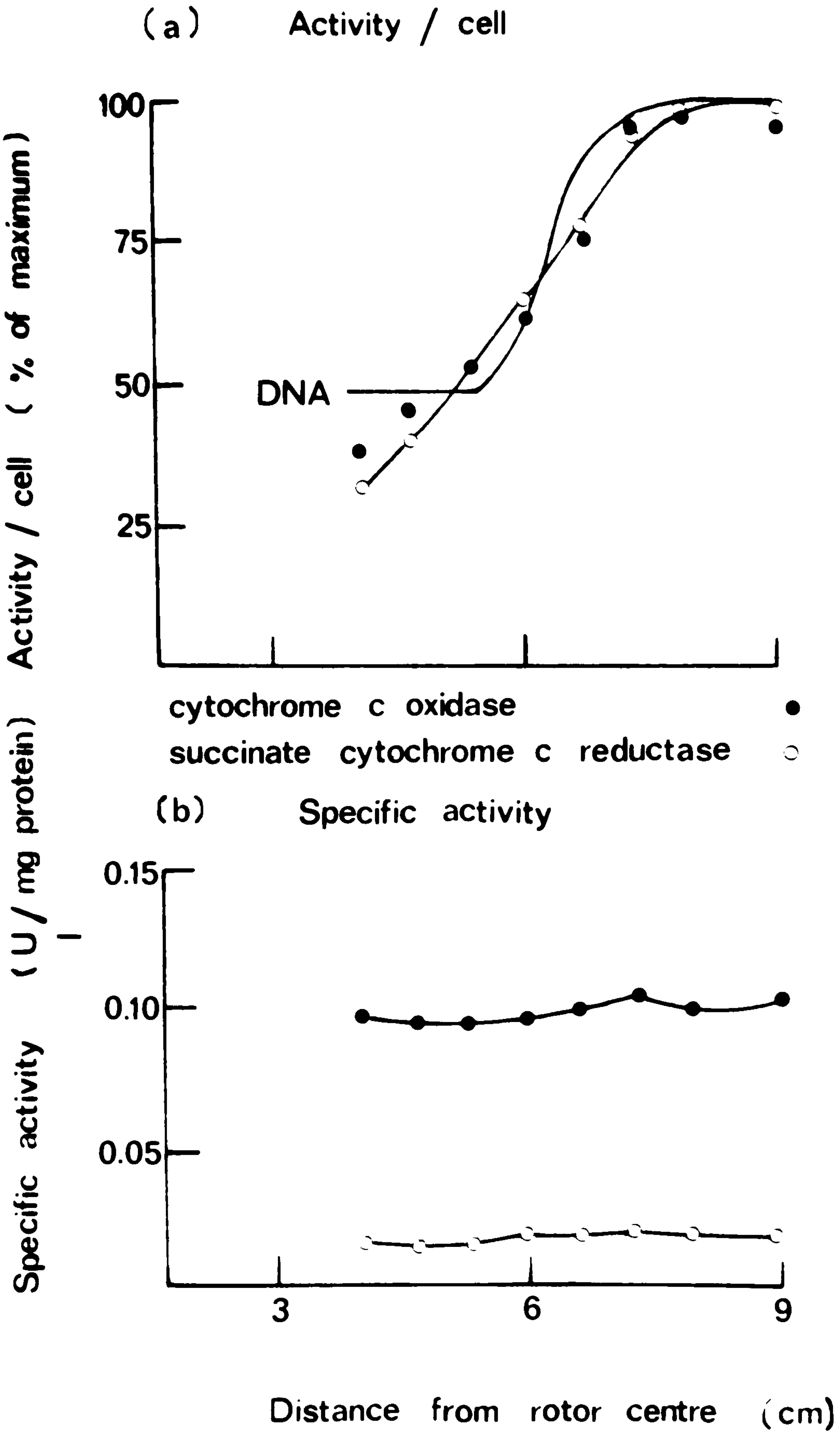
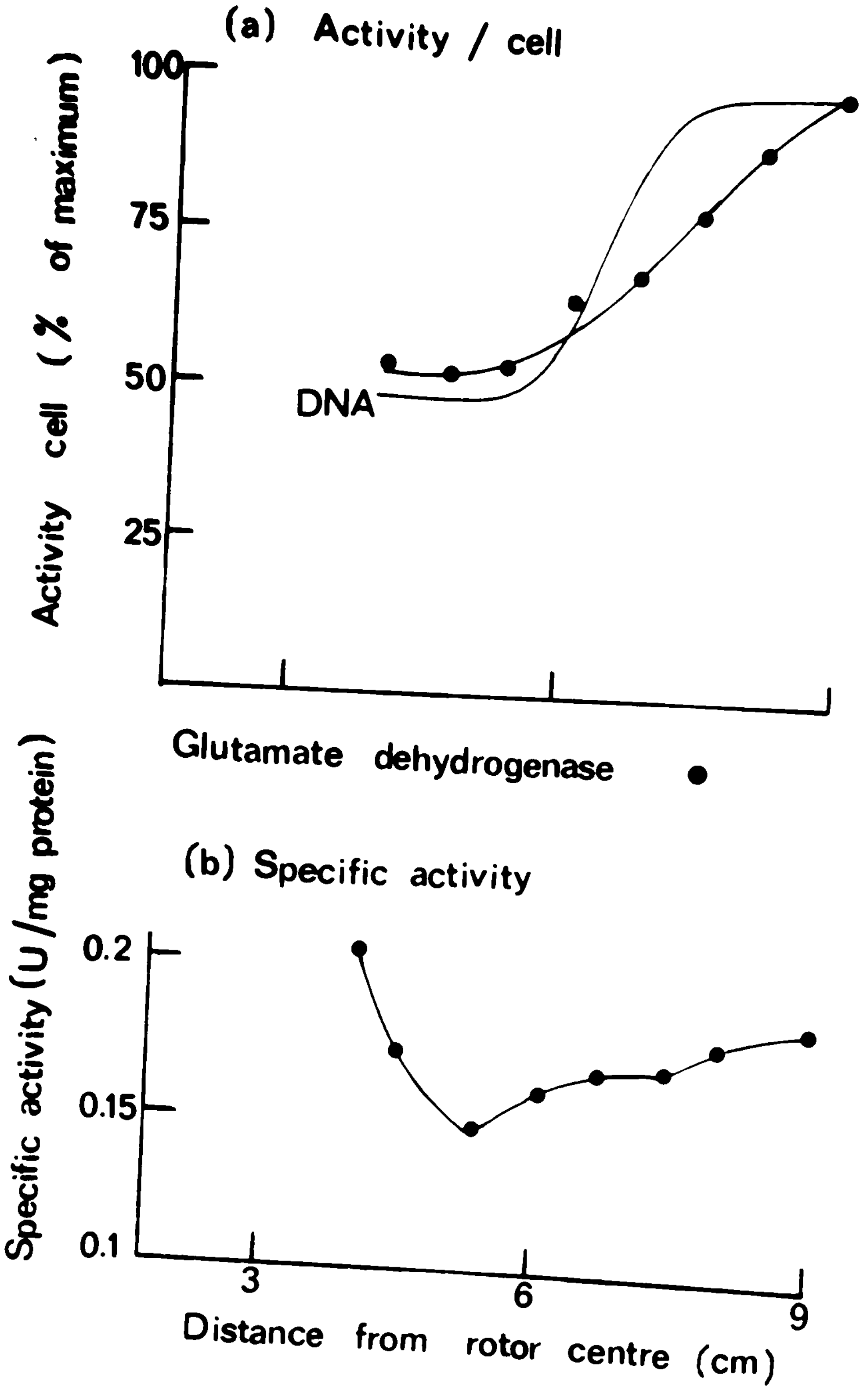


Figure 5-3: **Cytochrome c oxidase and succinate cytochrome c reductase activities in cells separated by zonal gradient centrifugation.**

[Procedure - see Figure 5-1].



<u>Figure 5-4</u>: <u>Glutamate dehydrogenase activity in cells separated by zonal gradient centrifugation</u>

[Procedure - see Figure 5-1].

obtained when high-speed supernatant fractions, prepared after sonicating (60s) mitochondria [prepared from sonicated (15s) extracts by differential centrifugation (Mahler and Cordes, 1966, p.390)] were analysed. This also gave a > 20 fold increase in specific activity.

The results of the fluorescence studies are shown in Table 5-4. There was little change during the cell cycle in the percentage of mitochondrial-electron-transport-protein present, which thus followed a pattern resembling that of net protein synthesis.

Table 5-4:

The Percentage of mitochondrial-electron-transport-protein present in cells separated by zonal centrifugation and measured by fluorescent probe techniques. [see 5.2(iv)].

Distance from rotor centre. (cm)	% of total protein
3.3	15.2
4.0	15.1
4.7	16.0
5.3	14.5
6.0	13.5
6.9	15.3
8.0	15.1

5-4 DISCUSSION

Results with enzyme assay experiments must be interpreted with caution. Only changes in activity were being measured; it is often difficult to distinguish the cause of the change, e.g. inhibition/activation, induction/repression, synthesis/turnover.

(i) Lactate dehydrogenase and glucose-6-phosphate dehydrogenase

The patterns of change in activity of these two enzymes during the cell cycle of P815Y cells resembles the results of Martin *et al.* (1969) with hepatoma cells (Table 1-2) in that there is no significant variation in specific activity. Klevecz and Ruddle (1968) on the other hand, found three peaks of activity during the cycle of Chinese hamster cells and further work suggests this to hold true for total enzyme protein purified immunologically (Klevecz, 1969). In two experiments with P815Y cells, not mentioned previously, lactate dehydrogenase activity did, in fact, show fluctuation, superimposed upon a steady increase, although not as pronounced as those obtained by Klevecz and Ruddle (1968). Mixing samples of high activity and low activity gave the mean values expected; it is therefore unlikely that an activation or inhibition of activity is occurring. Hooper (1967), working with Chinese hamster cells, observed variation in specific activity of lactate dehydrogenase during exponential growth, from low early on to high as the cells reached stationary phase. It should be noted that in the two cases where fluctuations were observed with P815Y cells, the cells had been harvested earlier on in exponential growth (3-4 x 10^5/ml) as compared to 6-8 x 10^5/ml in all the other cases, Perhaps the stage in exponential growth of the cells prior to size separation must be taken into account.

(ii) NADPH cytochrome c reductase, cytochrome c oxidase and succinate cytochrome c reductase

The activities of the microsomal marker and the two inner mitochrondrial membrane markers all follow similar patterns, again resembling that of net protein. This may indicate that microsomal and mitochondrial membranes are duplicated in concert and would agree with previous results [see 4.3(i)] that total protein, phospholipid, RNA and cell volume all increase together. The only other work on the synthesis of membrane enzymes is that of Bosmann (1970) who examined changes in activity of UDPase, esterase, and 5'nucleotidase (all membrane markers - see Tables 5-2 and 5-3) during the cell cycle of L5178Y cells.

He found a distinct peak of activity in S for all three marker enzymes. This was followed by a sharp decline from which he concluded that synthesis was followed by turnover during the cell cycle. UDPase has now been examined in P815Y cells separated by zonal centrifugation, and no such sharp decline in activity during G_2 has been observed (C.A. Pasternak, personal communication).

(iii) Mitochondrial-electron-transport-protein

The negligible variation in the percentage of this particular mitochondrial component during the cell cycle seems to confirm that mitochondrial membranes are duplicated in a pattern similar to total protein, RNA, phospholipid and cell volume, that is by a control other than gene-dosage.

(iv) Glutamate dehydrogenase

This enzyme shows an increase of activity different to that of net protein, and hence to the other five enzymes mentioned so far. Perhaps this difference is correlated with its location in the mitochondrial matrix. It is interesting to note that Koch and Stokstad (1967) found that in human liver cells, incorporation of thymidine into mitochondrial DNA occurred only after incorporation into nuclear DNA. It may be the case that glutamate dehydrogenase synthesis is controlled by mitochondrial DNA rather than nuclear DNA, although the bulk of evidence at the moment suggests that mitochondrial DNA codes for a very limited number of proteins, most of them structural (Borst and Kroon, 1969).

Alternatively, the locus for glutamate dehydrogenase may be on a nuclear chromosome which is duplicated late in S.

Finally, the late rise in activity may reflect the timing of synthesis and subsequent transport of the various subunits of the enzyme into the mitochondria, and their construction into a functional unit.

5-5 SCOPE OF FUTURE WORK

Experiments are currently in progress to investigate more thoroughly the duplication of cell structures and organelles during the cell cycle. To achieve this, a complete quantitative sub-cellular fractionation of cells at each stage of the cycle (separated by zonal centrifugation) is being carried out. This enables total mitochondrial protein and total microsomal protein to be measured. Preliminary results indicate that mitochondrial protein, at least, shows a pattern of increase resembling that of net protein, as might be expected from the results of the fluorescent probe studies [see 5.4(iii)]. Many aspects of the biogenesis of mitochondria, the biogenesis of membranes and the controlling systems involved might be examined with this type of system.

Another project for which the zonal centrifugation system should prove useful is the study of surface antigen production during the cell cycle. Preliminary experiments are in progress.

Changes in the nucleus and nuclear components other than DNA during the cell cycle are currently attracting much attention, and zonal centrifugation should prove useful for obtaining nuclei at different stages of the cell cycle. M. Cross is currently investigating histone modifications during the cell cycle of P815Y cells.

Finally, it would be interesting to study the effects of various inhibitors with the zonal centrifugation system. Tobey, Anderson and Petersen (1966), for instance, applied cycloheximide and actinomycin D at various times during synchronous growth and were able to demonstrate a requirement for RNA and protein synthesis for mitosis; the timing of this requirement was also investigated. This type of approach has led to the idea that progression through the cell cycle may be the result of a series of sequential inductions (e.g. Prescott, 1968c). Perhaps by applying various inhibitors for a brief period to exponentially-growing cells, separating them by zonal centrifugation and studying changes in the concentration of various enzymes and other proteins, more may be learned about such induction processes. Ultimately, perhaps, the mechanism whereby discontinuous processes of the cell cycle, such as mitosis and DNA synthesis, are 'triggered' may be elucidated.

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The Use of Gradient Centrifugation to Study Events in the Life Cycle of Cultured Cells

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Exponentially growing P815Y neoplastic mast cells (Schindler, Day & Fischer, 1959) were briefly exposed to radioactive precursors, collected by centrifugation, washed and layered on to 40ml. of a gradient (5–10%, w/v) of Ficoll in growth medium (Morris, Cramer & Reno, 1967). After centrifugation at $80g_{av.}$ for 7min. the tube was punctured and samples were collected. The number of cells in each sample, and their mean volume, was measured with a Coulter counter. Precursor incorporated into cells was determined by filtering a portion of each sample, washing with trichloroacetic acid and measuring the radioactivity.

The distribution of cells through the gradient showed a broad peak near the centre. The mean volume of cells near the top was about half that at the bottom. When thymidine was used as a precursor, the incorporated radioactivity/cell showed a broad maximum just below the cell peak, in agreement with the results of Morris *et al.* (1967). When proline, uridine or choline was used, the incorporated radioactivity/cell increased from the top to the bottom of the gradient.

When cells that had been separated by gradient centrifugation were resuspended in medium, they grew with a certain degree of synchrony; those from the top of the gradient divided after 10–15hr. (mean generation time 15hr.) whereas those from the bottom began division within 5hr. Hence cells near the top of the gradient appear to be early in their life cycle (phase G1), and cells near the bottom at the end of the cycle (phase G2). The experiment with labelled thymidine shows that cells near the middle of the gradient are in the period of DNA synthesis (phase S).

These results, in confirmation of other work (Bergeron, Warmsley & Pasternak, 1969), indicate that incorporation of proline, uridine and choline into protein, RNA and phospholipid respectively is not limited to a specific portion of the cell cycle (as is the case with thymidine), nor is the rate of incorporation constant throughout the cell cycle. Rather incorporation appears to increase from a low rate in phase G1 to a higher rate in phase G2. Such an increase has been observed by using other techniques in the case of RNA and protein synthesis (e.g. Robbins & Scharff, 1966; Pfeiffer & Tolmach, 1968; Martin, Tomkins & Granner, 1969).

A.M.H.W. is a Medical Research Council Scholar. J.J.M.B. is a Rhodes Scholar.

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THE TIMING OF PHOSPHOLIPID SYNTHESIS IN NEOPLASTIC MAST CELLS

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1. Introduction

It is known that DNA synthesis occurs at a discrete time (the S phase) during the life cycle of eukaryotic cells [1,2] whereas RNA and protein are synthesized throughout the intermitotic period, the rate of synthesis increasing during S [3–5]. The timing of phospholipid synthesis is of interest in so far as the event is a prerequisite for the construction of new membranes. Using choline as a specific precursor, we have now investigated phospholipid synthesis in neoplastic mast cells.

2. Materials and methods

P815Y neoplastic mast cells, kindly donated by Dr. G.A.Fischer, were grown as previously described [6]. Cell number and mean cell volume were measured with a Coulter model A counter [6].

Cells were synchronised by the addition of 2 mM thymidine [7] to an exponentially growing culture for 15 hr, which is the mean generation time. Cells were washed free of inhibitor, allowed to recover for approximately 7 hr and thymidine added for a further 15 hr. On removal of inhibitor, synchronous growth commences (fig. 1).

Radioactive precursors (Radiochemical Centre, Amersham, England) were added to cell suspensions (2–5 × 10^5 cells/ml) during exponential or synchronous growth. A sample (0.1–0.3 ml) was filtered (Whatman GF/C paper), washed with 5% trichloroacetic acid and incorporated radioactivity assayed in 7 ml of a solution containing 8 g Scintillator Butyl-PBD (CIBA) and 80 g naphthalene in 1 l of toluene-methyl Cellosolve (3:2, v/v) in a Beckman liquid scintillation spectrometer. Radioactive choline was incorporated solely into phospholipid, as determined by chemical analysis of cells exposed to [1,2-^{14}C] choline and [methyl-^{14}C] choline (unpublished observations).

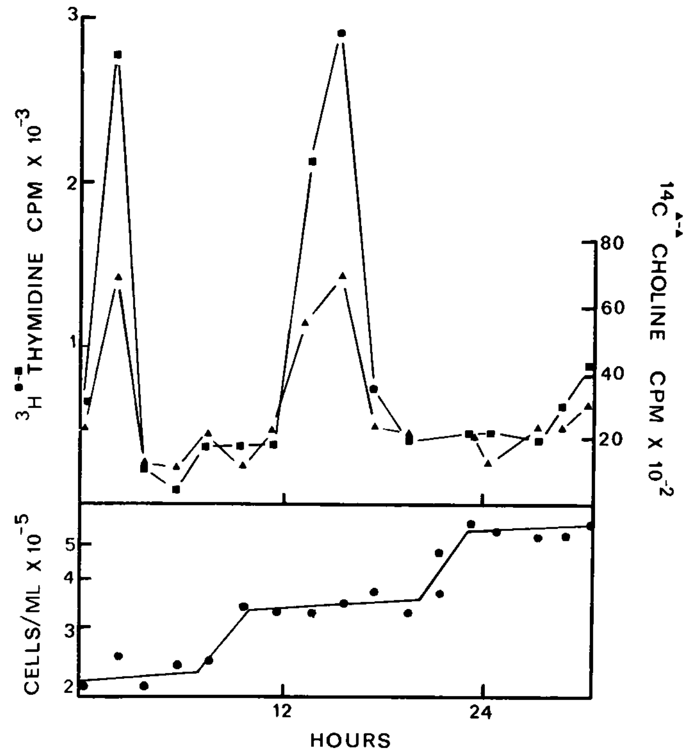

Fig. 1. Rate of DNA and phospholipid synthesis in synchronous P815Y cells. Samples were removed at intervals and exposed to a pulse of [^{3}H] thymidine and [^{14}C] choline for 30 min and analysed as described in Materials and Methods.

The percentage of exponentially growing cells incorporating radioactive precursors during a 15 min pulse (60 min in the case of choline) was determined by conventional autoradiography. Cells which had been exposed to a pulse of [^{3}H] choline were fixed in osmium tetroxide [8].

Gradient centrifugation through Ficoll was used to separate cells according to size and position in the cell cycle [9].

3. Results and discussion

Autoradiography of cells exposed to a pulse of [^{3}H] thymidine showed that 40% of the cells had incorporated the isotope. When exposed to a pulse of [^{14}C] uridine, [^{14}C] valine or [^{3}H] choline, 100% of the cells became labelled, clearly indicating that RNA, protein and phospholipid synthesis are continuous throughout the cell cycle. This result does not distinguish between different rates of synthesis, though some cells were definitely more heavily labelled than others in the case of choline.

In order to examine rates of synthesis, synchronously growing cells were exposed to a pulse of [^{3}H] thymidine, [^{3}H] uridine, [^{3}H] proline or [^{14}C] choline at intervals. Fig. 1 shows that choline uptake increases and decreases at approximately the same time as does thymidine incorporation. However in the case of thymidine the lowest points are not significantly above background whereas with choline they represent true incorporation. Thus the variation in rate is more than 8-fold with thymidine but only 3–4-fold with choline. Uridine and proline incorporation vary 2–3-fold, though the timing again coincides with thymidine uptake. That choline incorporation fluctuates less markedly than thymidine incorporation is confirmed by an experiment in which isotope was present throughout synchronous growth (fig. 2).

If phospholipid synthesis and membrane construction are coupled, an increase in choline incorporation might result in an increase in cell size. It is therefore interesting to note that the mean cell volume increased throughout the intermitotic period of synchronously growing cells.

Results obtained with cells which have been synchronised by thymidine treatment are open to the criticism that the cells pass through an unphysiological milieu, even though recovery from inhibition is rapid. An alternative method of studying the timing of macromolecular synthesis, namely the separation of cells by gradient centrifugation, was therefore used. Fig. 3 shows that cells near the top of the gradient have incorporated less isotope than those near the bottom, and that the increase in incorporation is sharper for [^{14}C] thymidine than for [^{3}H] choline. It may be wondered why the rates of incorporation do not decrease towards the bottom of the gradient, in view of the results of fig. 1. The reason is probably that G 2 cells are not well separated from S cells and

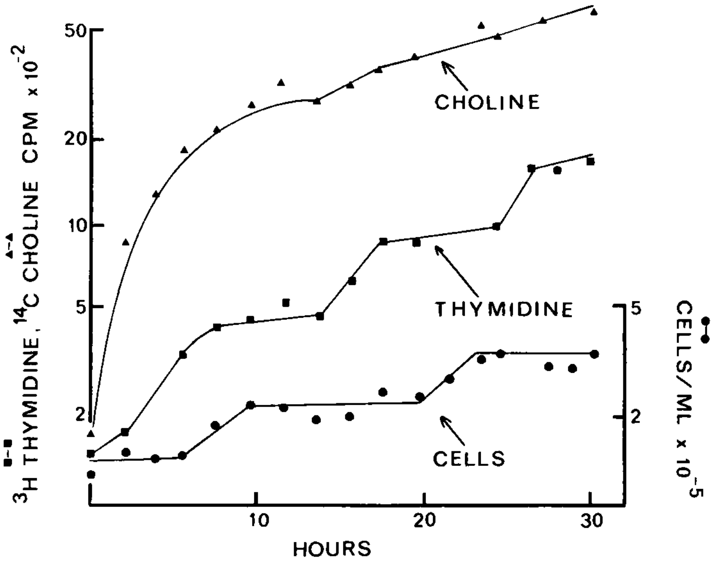

Fig. 2. DNA and phospholipid synthesis in synchronous P815Y cells. [^{3}H] thymidine and [^{14}C] choline were added at 0 time and samples were removed at intervals and analysed as described in Materials and Methods.

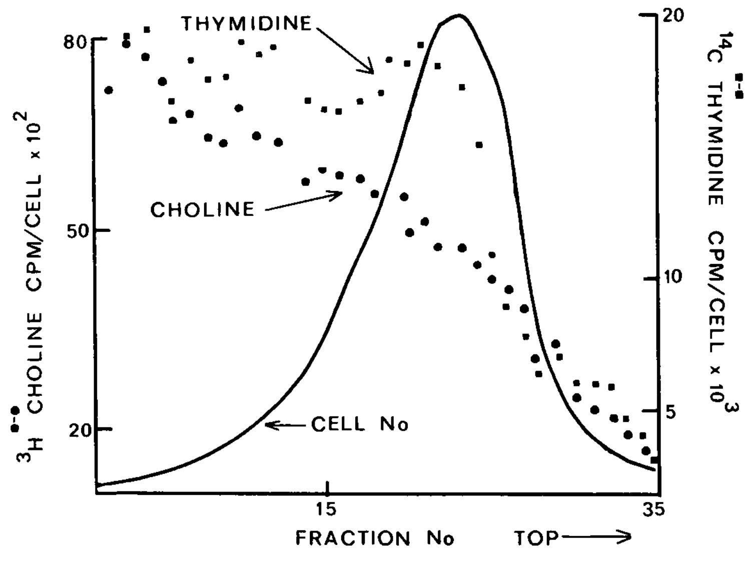

Fig. 3. Gradient centrifugation of P815Y cells. Exponentially growing cells were exposed to a pulse of [^{14}C] thymidine and [^{3}H] choline for 30 min and separated by centrifugation through a 5–10% gradient of Ficoll in growth medium at 80 X g for 7 min in an M.S.E. Mistral Swing-out Rotor (No. 62301) at 0°.

that the values obtained – at least in the case of thymidine – may therefore represent a mean of high incoporation (by S cells) and low incorporation (by G 2 cells). The pattern obtained with [^{3}H] uridine and [^{3}H] proline was similar to that of choline. The mean cell volume also increased gradually, like choline incorporation, from the top to the bottom of the gradient, suggesting that phospholipid synthesis and plasma membrane formation may indeed be related.

In general, the results of the experiments with cells separated by gradient centrifugation are in accord with those obtained by autoradiography and with synchronously grown cells. That is, the synthesis of phospholipid, like that of RNA and protein, is continuous throughout interphase, the rate increasing from a low value in G1 to a maximum prior to division.

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