A Study of Late Ventral Body Wall Degeneration in the Embryonic Chick, with Special Reference to the Cell Cycle.

Peter Barwell

Submitted for the degree of Doctor of Philosophy in Zoology at Victoria University of Wellington, New Zealand

1993

ABSTRACT

The cell kinetics and morphological changes during late ventral body wall development of the embryonic chick were studied, particularly midline degeneration and the medial migration of lateral tissues. An histological examination of these events was undertaken, along with autoradiography to determine the duration of the cell cycle, followed by teratological studies involving the prevention of differentiative events in the cell death pathway, using BrDU[1] and Janus B Green as agents. The effects of cell cycle blockade on rates of cell death were also examined, as was the tissues ability to express differentiative features *in vitro*.

Ventral body wall (VBW) cell death was classified as apoptosis, and was involved in two distinct events. Medial migration of lateral tissues began at day 5 of development, with widespread VBW apoptosis being seen by day 6, limited to the original mesoderm of the region. A later precise line of apoptosis (the VBL), involving both ectodermal cells of the midline ectodermal ruffle and the underlying mesodermal cells, was observed at day 7, spreading in a rostral to caudal fashion down the embryo, appearing as the migratory lateral tissues fused in the midline body wall.

Increases in the amount of cell death are matched by decreases in the MI, such that at its peak (day 7.5 of development) the cell death rate is sufficiently greater than both the cell proliferation and immigration rates that a state of negative tissue growth ensues.

---

[1] See Appendix for a fold-out glossary of abbreviations used in this thesis.

The histological half-life of the apoptotic bodies approximates 3.8 hours. The ability to undergo apoptosis at day 7 is dependent upon a differentiative event around day 4 of incubation, and involves signal mechanisms intrinsic to the VBW tissues.

BrDU application was found to inhibit apoptotic differentiation, in contrast to Janus B Green, which had a more generalised teratogenic effect on the region as a whole. Tissue culturing experiments revealed that an ectodermal-mesodermal interaction is important in regulating the extent of mesodermal apoptosis, the ectoderm playing a maintenance role for the mesoderm.

Dead cells derive from the cycling cell population, as shown by the occurrence of labelled dead cells after autoradiography, and by the prevention of apoptosis by a cell cycle blockade, and by the production of a semi-synchronised wave of apoptoses after release of this blockade. These cell blockading results further suggest that entry into the apoptotic death program requires cells to be in a particular cell cycle stage, and it seems most likely that the decision to die was made in early G1.

Tissue and cell growth rates, cell loss and death rates, cell birth rates and cell immigration rates were all determined for the VBW region throughout the time period studied.

TABLE OF CONTENTS

1. GENERAL INTRODUCTION

This thesis examines the morphogenic processes and kinetics of ventral body wall development. More specifically, it is about how cells die in the ventral body wall (VBW) of the developing chick.

Cell death is a process well worthy of study, as it is of universal importance to multicellular organisms (Umansky 1982, Wyllie 1987), and often under active genetic control (Buttyan et al 1989, Clem et al 1991, Crook et al 1993, Ellis and Horvitz 1986, Gregory et al 1991, Hockenberry et al 1990, Kenyon and Wang 1991, Schwartz et al 1990a,b). Cell death plays a fundamental role in morphogenesis (Glucksmann 1951, Lockshin 1981, Saunders 1966, Snow 1987, Tata 1966), neurogenesis (Cowan 1973, Cowan et al 1984, Oppenheim 1985, Oppenheim et al 1990, Truman 1984, van der Starre-van der Molen and Otten 1974) tissue and tumour homeostasis (Sarraf and Bowen 1986, Wyllie 1987), chemotherapy (Barry et al 1990, Eastman 1990, Kaufmann 1989, Webster and Gross 1970), the immune system (Christmas and Moore 1987, Cohen and Duke 1984, Duvall and Wyllie 1986, McConkey et al 1990) including its development and maintenance (Kawabe and Ochi 1991, Sambhara and Miller 1991, Sentman et al 1991, Shi et al 1991, Strasser et al 1991), teratogenics (Menkes et al 1964, 1970; Ritter et al 1973), and aging (Hayflick 1975, 1981, Lockshin and Zakeri 1990, Sheldrake 1974, Sinex 1977). It plays an important part in many birth defects (Alles and Sulik 1989, 1990; Sulik et al 1988) and in virtually all diseases (Trump et al 1981), including carcinogenesis (Basile et al 1973, Bursch et al 1984, Columbano et al 1985, Cooper et al 1975, Kerr and Searle 1972a,b, Wyllie 1985) and even leprosy (Cree et al 1986). Indeed, programmed cell death is

involved in multicellular life in a multitude of ways, beginning at gamete formation (Allan et al 1987, Gondos 1973), and continuing post mortem (Lovas 1986, El-Shennawy et al 1985).

The VBW was chosen as a suitable study area as it undergoes two distinct waves of degeneration during normal development (Fell 1939), while the chick embryo is both easily obtained and the subject of previous landmark studies on embryological cell death (see reviews by Greene and Pratt 1976, Saunders 1966, for example).

History and Classification of Cell Death.

Widespread interest in cell death has only arisen quite recently, despite its above-mentioned significance, probably because the phenomenon was first thought of as being solely due to injury, space competition, or phagocytic assassination, with the victim exerting no control (Virchow 1858).

The current realisation that cell death can be an actively controlled process has evolved through three basic lines of research (Wyllie et al 1980). The first involved the exposure of cells and tissues to violent environmental changes, which killed contiguous cell populations, so permitting biochemical analysis of the changes involved in their deaths (Farber 1981, Farber and El Mofty 1975, Sandritter and Riede 1975, Trump et al 1965, 1981). However it was soon realised that this non-physiological cell death might not involve the same mechanisms as naturally occurring cell death (Bessis 1964). The second line of research derived from the study of tissue growth kinetics, particularly of tumour growth, which led to the inference that cell death must be occurring, to help explain deficits between the growth rate expected from observed proliferation rates, and the actual growth rate seen. This brought

about a recognition of the kinetic importance of cell death in tissue homeostasis, even without physical study of the dying cells themselves (for examples see Iversen 1967, Norrby 1970, Norrby et al 1967, Shackney 1973, Steel 1968, Wright 1981, Wright and Allison 1984). The conceptual importance of thus relating the process of cell death to a positive biological purpose should not be underestimated. The final line of research leading to a reevaluation of cell death had its basis in developmental biology. The realisation that large scale cell death frequently and predictably occurred in larval metamorphosis, the removal of phylogenetic vestiges, and even in normal morphological development, and was histologically "quite distinct" from non-physiological cell death (Glucksmann 1951), led to active research on how and why the phenomenon occurred. That such death was spontaneous, predictable, and subject to genetic, hormonal and local tissue factors led to use of the term "programmed cell death" (Saunders et al 1962). However the mixture of dying and surviving cells within degenerating embryological tissue complicated biochemical comparison with non-physiological cell death (Wyllie et al 1980).

Initial attempts to classify types of cell death were by the circumstances in which death occurred (Bessis 1964) or by subjective interpretation of its biological function (Glucksmann 1951).

It was an important step forward when Kerr et al (1972) sought to unite the three early lines of research via a classification of cell death based upon the morphology of the affected cells themselves, beginning by the comparison of degenerating tumour cells with those described in non-physiological cell death (Kerr 1965, 1971). Kerr established that trauma-induced necrosis

differed from that observed in normal tissues, which involved isolation and shrinkage of separate cells with nuclear pyknosis and cellular fragmentation. Kerr initially referred to this physiological cell death as shrinkage necrosis (Kerr 1971), but later renamed it apoptosis to emphasise its perceived kinetic role opposing mitosis (Kerr et al 1972). Knowledge of the morphological similarity of cell death found in adult (Kerr et al 1972) and cancerous (Kerr and Searle 1972a,b) tissues to that in embryonic cell death (Bellairs 1961) formed a "crucial strut in the formation of the apoptosis concept" (Kerr and Searle 1980). 'Apoptosis' was able to replace the previous plethora of terms describing the types of dead cells in different tissues, and emphasised the similarity of death in many genera and under many different conditions (Wyllie et al 1980, Wyllie 1986). It is worth noting, however, that while many non-physiological stimuli can cause apoptosis, including damage by viruses, ionizing radiation, heat, cold shock, and exposure to toxins (Harmon et al 1990, Perotti et al 1990, Potten 1985, Savage et al 1985, Servomaa and Rytomaa 1990, Stephens et al 1986, Walker et al 1988), they still tend to induce apoptosis only in those subpopulations which were susceptible to it under physiological stimuli (Wyllie 1987).

Apoptosis in some cell lines (notably thymocytes and lymphocytes) was found to involve receptor-mediated internucleosomal cleavage of DNA by a Ca^{++}- and Mg^{++}-dependent endonuclease (Afanas'ev et al 1986, Arends et al 1990, Bursch et al 1990a, Compton 1991, Duke et al 1983, Lockshin et al 1981, Lockshin and Zakeri-Milovanovic 1984, Perotti et al 1990, Schwartzman and Cidlowski 1991, Wyllie 1980, Wyllie et al 1984b), possibly leading to NAD and ATP depletion by Poly(ADP)-ribose

synthesis (Berger et al 1985, Boobis et al 1990, Carson et al 1986, 1988, Thraves and Smulson 1982, Seto et al 1985). It should be noted, however, that chromatin cleavage was not noted in insect cells undergoing morphological apoptosis until very recently (Clem et al 1991), and internucleosomal cleavage is not essential to apoptosis (Tomei et al 1993).

Apoptosis also involves changes to cell surface sugars, allowing rapid macrophage recognition and binding (Duvall and Wyllie 1986, Duvall et al 1985, Morris et al 1984, Wyllie 1987).

Several genes are known to regulate apoptosis, the affects of their alterations being seen in mutants such as Epstein-Barr virus, bcl-2, and the P35 mutant virus whimsically named 'the Annihilator' (Clem et al 1991, Gregory et al 1991, Hengartner et al 1992, Sentman et al 1991).

Unfortunately the excitement generated by the simplicity and apparent universality of this apoptosis/necrosis classification (Searle et al 1982, Wyllie 1981) has led to the tendency to dismiss differing descriptions of cell death morphology as being of lesser importance, relating to "lower organisms", or even as a tendency to "miss-interpret" the evidence (see Wyllie et al 1980). Yet there is considerable evidence that some cells die, in a programmed way, showing a morphology that is not identical to apoptosis (Beaulaton and Lockshin 1982, Bowen and Davies 1971, Bowen and Ryder 1974, Clarke 1990, Schweichel and Merker 1973, Wyllie 1987), and it is also well documented that all cases of apoptosis do not involve precisely the same steps, since cells differ in what enzymes they may have already made (Lockshin and Zakeri 1990). Indeed, one of the best understood cases of genetically controlled cell death, that seen in *Caenorhabditis elegans* (Avery and Horvitz 1987, Ellis and

Horvitz 1986, Yuan and Horvitz 1990), has autolytic features that do not fit the apoptotic model (Wyllie 1987), despite some comments to the contrary (see for example Lynch et al 1986).

Given the disagreement which exists over the type of death occurring in some situations, such as that found in anuran tail degeneration [asserted by Kerr and Searle (1980) to be apoptosis, but by Clarke (1990) to be typical of non-lysosomal degeneration], it is disconcerting that many studies of biochemical events within dying cells fail to specify exactly what type of programmed cell death is occurring, as we may not be observing one universal process, but rather a few similar, but by no means mechanistically identical, ones. As an example of this tendency, the fascinating series of papers on specific gene expression seen in the prostate after castration (Buttyan et al 1988, 1989, Kyprianou and Isaacs 1988, Leger et al 1987, Martikainen and Isaacs 1990, Rennie et al 1988, Rouleau et al 1990) refer only to programmed cell death, although, to be fair, prostate involution has been identified as apoptosis elsewhere (Kerr and Searle 1973), which is implied in some of these papers. What is also disconcerting, however, is that this implication is solely based upon internucleosomal cleavage of DNA, which cannot be taken as being synonymous with apoptosis (D. Tomei, pers. comm.). A more blatant example is seen in Kyprianou et al (1991), who used internucleosomal laddering, and the presence of testosterone-repressed prostate-message-2 (TRPM-2, one of the genes expressed in castration induced prostate death), to positively identify the type of death induced in tumour cells by TNF (tumour necrosis factor) as apoptosis. Given that TNF can induce both apoptosis and necrosis, dependant upon the cell line (Larrick and Wright 1990, Laster et al 1988, Robaye et al 1991),

can operate in a calcium dependent or independent way (Hasegawa and Bonavida 1989), and indeed can induce either proliferation or death through different receptor pathways present in the same cells (Tartaglia et al 1991), identifying the type of cell death involved without recourse to the morphological criteria basic to the definition of apoptosis (Wyllie et al 1980) seems remarkably brave. It should also be noted that TRPM-2 expression, along with Polyubiquitin and specific protein expression, has been observed in *Manduca sexta* muscle degeneration after ecdysis (Schwartz et al 1990a,b, Wadewitz and Lockshin 1988), which has yet to be certainly identified as an example of apoptosis.

It is also of interest that at least two of the tissues classically studied in developmental programmed cell death, the Posterior Necrotic Zone (Fallon and Saunders 1965, 1968, Hincheliffe and Ede 1973, Pollack and Fallon 1974, 1976, Saunders et al 1962) and the ectodermal degeneration observed in palate formation (Greene and Pratt 1976), have since been classified as a non-apoptotic type of programmed cell death (Hurle and Hinchliffe 1978, Clarke 1990), in contradiction of Wyllie (1987). Since these systems are to a large extent models against which VBW degeneration can be compared, it has been decided to classify the nature of death in the region on morphological grounds, and, given the problems in cell death research caused by loose terminology (Alles et al 1991, Wolman 1985), the term PCD (programmed cell death) will be henceforth used to refer to physiological, but otherwise unspecified, cell death throughout this thesis.

Project aims:

The body of research represented in this thesis was designed to answer certain basic questions about PCD in the ventral body wall. The first goal was to determine the precise nature and classification of the types of cell death occurring in this tissue, using the morphological criteria described in the literature just reviewed. It was also hoped to use the histological material to determine the magnitude of VBW PCD; however the kinetic significance of the cell death in the region is also determined by the number of cells being produced, in the same way that the fullness of a glass of whisky is determined not only by the speed at which it is drunk, but also by the speed with which it is topped up. Because of this, autoradiographic studies were undertaken to quantify the amount of proliferation within the tissue, allowing this to be compared to the amount of PCD.

It was also intended that the timing and events involved in the induction of PCD in the VBW be determined, and it was for this reason that culturing and teratological experiments were undertaken. These also raised the possibility that the actual developmental significance of this cell death event could be evaluated, by studying the effects of increasing or, if possible, preventing its occurrence.

Finally, it was hoped that this information would suggest something about the nature of the PCD phenomenon itself, indicating possible avenues for future research into the molecular mechanisms of cell death, which I feel will be one of the most productive and interesting fields of inquiry for biologists over the next few decades.

1.1 HISTOLOGY OF VBW DEVELOPMENT

1.1.1 INTRODUCTION

The development of the VBW of the embryonic chick involves the initial fusion of the lateral body plates, followed by the degeneration of extant VBW tissue prior to the medial immigration of more lateral tissues, which meet and fuse in the medial midline, differentiating during this process to become the sternal plates, keel, pectoral muscles, and other structures of the chest wall as seen in the adult.

The occurrence of distinct waves of cell death during VBW development has been known since Fell's elegant experiments on avian sternum development (Fell 1939). She noted that, after an initial increase in cell proliferation, degeneration of the epithelium, mesothelium and mesenchyme of the mid-ventral line is required for the union of the body halves (Fell 1939, Glucksmann 1951). After the initial formation of the body-wall, a further generalised degeneration of the mid-ventral mesenchyme occurs, synchronised with the medial migration of lateral tissues (including nonspecialized cells, the developing pectoral muscles, connective tissue, and presumptive sternal anlages). This degeneration is in turn followed by further death, presumably of newly migrated cells, in a precise line from the skin to the pericardium, contemporaneous with the formation of a thickened ectodermal ridge or ruffle. This sharply defined, easily seen line of PCD (henceforth referred to as the 'ventral body line' or VBL) occurs when the lateral tissues meet, and Fell implied that it began in the ectodermal ridge, spreading to the underlying mesoderm. She considered that the downflow of migrating cells created a surplus in

the midline area, leading to ridge formation, and later to degeneration to remove this surplus.

Both epithelial ruffling and degeneration progress down the embryo in a rostral to caudal manner, and are most pronounced wherever the sternal plates are fusing (Fell 1939). However the VBL and ruffle extend beyond the sternal region both rostrally, and caudally to the yolk stalk.

Experimental manipulations in vitro showed that ventral degeneration is not essential for tissue migration towards the mid-ventral line, but it was considered necessary for ventral body-wall "shrinkage" (Fell 1939).

Fell's findings with the Budgerigar (with some reference to the chicken) were later found to apply to murine development as well (Chen 1952a,b, Chen 1953), and had an obvious effect on the development of Glucksmann's (1951) landmark classification of cell death. Glucksmann declared ventral body wall cell death to be morphogenetic, and "unrelated to pressure or other injurious effects" (Glucksmann 1951). However it has received no subsequent study.

Given the difficulty of accurately quantifying and classifying PCD by light microscopy prior to the use of methacrylate embedding (Bowen 1984, Sarraf and Bowen 1986, Del Vecchio et al 1991), it is perhaps unsurprising that no detailed account of cell proliferation and death rates in the VBW exists. It was decided to undertake such a survey, to allow assessment of any changes in cell density or overall tissue size, and to specify the timing of the major events in chick VBL development, providing the groundwork for a kinetic analysis of the region, and to provide clues to the inductive stimuli for the events observed.

1.1.2 HISTOLOGY METHODS

White Leghorn embryos (Golden Coast Hatcheries) were aged by H&H stages (Hamburger and Hamilton 1951), aided by the toe-length method of Tanaka and Kato (1982). A number of fixatives were tried (Carnoy's, cold formal-calcium, Bouin's, and Lillie's FAA), as were a number of stains (Methyl Green/Pyronin, Methyl Blue/Azure II in Borax, Toluidine Blue/Basic Fuchsin, Giemsa, Janus B Green, Masson's Trichrome, and Toluidine Blue/Safranine), to determine which treatment combination gave best fixative penetration with minimal shrinkage and sufficient stain darkness for ready cell identification without obscuring tissue details. Tissue was embedded either in Paraplast, to be sectioned at 5μm, or Methacrylate (Kulzer Technovit) to be sectioned at 3μm on a Kulzer Ultracut microtome.

Section thickness was checked by reimbedding, cross-sectioning, and measuring a range of section thicknesses from each microtome used.

Cell counting:

Counts were performed at high power (x400) on an Olympus microscope fitted with a Zeiss camera lucida. Every 5th section (from a randomly selected start) was assessed, to avoid the possibility of double counting cells. A labelled double lattice stereological counting grid (Weibel et al 1966) was superimposed upon the slide image, which was traced to allow exactly replicable counting, with counts being focused in the medial VBW (the site of the VBL at stage 34), so that a precisely located transect could be sampled in embryos of different ages. Transect quadrats were assessed for the total cell number, the cell density (number of cells

per fractional tissue area, i.e. cell number relative to how much of the quadrat was filled by the tissue being assessed), the number and phase of mitotic cells seen, the number of apoptotic cells, and of any cells undergoing other forms of PCD (as described in the references cited in section 1). The type of tissue being counted was also recorded (epithelial, or mesodermal, the later tissue being further described by its proximity to the ectoderm, ie 'near' 'mid-far' and 'far' mesoderm). Sections were further classified by their position within the embryo, as rostral 'neck' sections or caudal 'chest' sections, and were then more precisely defined for 'Order' within the embryo. This was done by subdividing the embryo into 3 easily identified regions (neck to top of shoulder-girdle, girdle to end of ribs, ribs to yolk stalk) which were still further subdivided into a total of 40 Order categories, using recognisable morphological landmarks (presence of specific ribs, vertebrae, organs, cross-sectional area). Order designations were checked by cross-reference against the sequential order of the serial sections being assessed. The 40 order categories began at the rostral 'neck' end of the embryo, and finished at the yolk stalk.

Between 10,000 and 70,000 cells were counted per embryo sampled, with from 3 to 9 embryos being counted for each age group.

Where more than one dead cell fragment was seen within the same cytoplasmic halo within a phagocyte, they were assumed to derive from the same dead cell (Del Vecchio et al 1991). Where necessary the number of dead cells was derived from the number of fragments by the Floderous correction formula (Marrible 1962, Hinsull et al 1977) and the methods of Abercrombie (1946).

MI (Mitotic Index) and DI (Death Index) were calculated as percentages of total cell number, not of viable cell number.

Area and volume assessment:

Standard stereological methodology allows for 3-dimensional quantitative estimates to be made from 2-dimensional information (eg microscope slides). By counting the number of particles, and the number hit by points and intersected by intercept lines of known length (regardless of what length is chosen), it is possible to estimate directly the actual number of particles existing, their average volume, and the volume of the overall tissue being assessed. The methods and principles used are not new -for instance Cavalieri's estimator for total reference volume date back to the 17th century- and they are well explained in reviews by Weibel et al (1966) and Gunderson et al (1988a,b), and so will not be further elaborated on here.

In addition to standard stereological techniques cell and tissue areas and volumes were also directly measured by a Koltron Mini-mop digitizer (Zeiss) via the camera lucida. VBW projected area measurements were taken from transverse sections at the level of the first costal rib, with 10 consecutive sections being measured and averaged.

Scanning Electron Microscopy (SEM):

Embryos were fixed with Lillie's FAA, trimmed with a sharp razor blade, critical point dried and prepared for viewing on a Phillips SEM 505 scanning electron microscope.

Statistical analyses:

Exact significance levels (p values) were obtained using the Kruskal-Wallis non-parametric test. Correlation tests used Spearmann's rank correlation coefficient on individual and pooled

data sets. Calculations were performed via SAS (Statistical Analysis Systems) version 6.06 (SAS Institute Inc.).

It should of course be remembered that statistical significance levels do not tell you anything about cell interactions, serving only to compare data samples (which is why correct sampling technique is so important, with proper randomisation of tissue and section orientation and so on). This is why, throughout this thesis, p values are quoted rather than explained in the results sections, to emphasise that they only indicate that a biological relationship *might* exist, worthy of later consideration in the discussion sections. Non-parametric tests were performed because it was not felt to be reasonable to asume that populations were normally distributed, indeed some indication of population skew aróse in section 1.1.3, where some standard deviations exceeded the mean value.

Summary:

Each section was thus assessed for cell density, cell type, the general region of the VBW involved, and for exact order along the rostro-caudal axis. Total cell number, and number of mitotic figures and cells undergoing PCD were recorded, along with the nature of that PCD. The phases of observed mitoses were also recorded.

10 or more equidistant sections were assessed from at least 3 embryos for each age group studied, with sufficient quadrats being counted to cross the VBW from ectoderm to endoderm (normally 9-12 quadrats per section, with 30-200 cells per quadrat). For no embryo was less than 10,000 cells sampled.

1.1.3: RESULTS

Body-wall development:

Body-wall fusion has been completed by day 4 of development (H&H stage 25).

The earliest sign of lateral body wall migration (of presumptive sternum and pectorals) occurs at stage 27 (day 5), while widespread VBW PCD begins at stage 29 (day 6), peaking at stage 30 (day 6.5), cell death being restricted to the mesoderm, and not in any precise line (see Plate 1). Lateral tissue movement becomes obvious at this time.

Lateral tissues meet at the anterior end of the ventral midline at stage 31 (day 7), with a precise line of PCD (the VBL) occurring upon contact (see Plate 2). VBL PCD peaks at stage 32 (day 7.5, see Fig.1a), as the sternal plates begin to rotate towards the vertical plane, and they will start to fuse anteriorly after the death wave has passed on posteriorly.

The thickened ridge or ruffle of midventral line ectoderm is clearly visible (see Plate 3) by stage 34 (8 days) as are two lateral ridges caused by developing rows of feather-germs.

Lateral tissue fusion and cell death occurs in a rostral to caudal fashion, so that caudal tissues of one stage show similarities to more rostral tissues of an earlier stage. Thus a series of rostral to caudal waves of cell division, increased population density, and cell death are seen to pass down the embryo with time, from neck to belly (see Fig 2a-c). Because of this, caudal tissues (order 35+) are still undergoing diffuse general body wall degeneration at stage 31, when the rostral body (order 10+) is undergoing VBL degeneration. VBL PCD does not disappear from the lower belly until stage 38 (12 days). Because of this, comparisons over time of rates of mitosis,

PCD and of cell density, have been made only over similar order ranges.

See Table 1 for a summary of MI, DI, and cell density for various ages of embryo.

Cell densities are higher in mesodermal tissues than in ectoderm for all ages of chick ($p<0.0001$). They are initially (stages 27 and 28) greater in rostral sections than caudal ($p<0.0001$), but this ceases to be clearly demarcated at stages 29 (cell density highest in orders 26, 28, and 34; $p=0.0091$) and 30 (higher in neck, but $p= 0.0102$). Two peaks in density are seen at stage 31 (orders 16-28 and 32-35; $p<0.0001$) but cell density peaks only in orders 33-40 by stage 34 ($p<0.0001$). It has returned to being greater in the rostral end of the body wall by stage 35 ($p=0.0183$).

PCD first appears predominantly in the ectoderm and the mesoderm farthest from it ($p<0.0001$) but by stage 32 is predominantly occurring in the ectoderm and the nearest mesoderm to it ($p<0.0001$), and is heaviest in the 'chest' region ($p=0.0006$).

While MI peaks at stage 30, cell density is constant from stage 27-30, only beginning to rise after the MI has dropped (Fig.1b,c). A subsequent rise in DI (stage 32) is followed by a decrease in cell density at stage 34 (Fig.1a,b).

The rostral and caudal ends of the VBW are always significantly different for cell density ($p=0.0320$ or less) and DI ($p=0.001$ or less), and often for MI ($p=0.0054$ to 0.0004 in 21 of 30 embryos), regardless of embryo age. Embryos of the same age (and similar order ranges) do not differ significantly for any of these traits (p ranging from 0.985 to 0.067).

The majority of mitotic cells seen were in metaphase (50%), followed by prophase (17.647%) then anaphase and telophase (11.765% each) and finally prometaphase (8.824%).

No significant correlation of mitoses to either PCD or cell density was seen (p ranging from 0.93547 to 0.2797), however cell density and PCD were positively correlated (p=0.0017 or less).

Nature of PCD:

Death affected isolated cells, sparing apparently similar neighbours. Dying cells occasionally showed nuclear pyknosis (also demonstrated by increased nuclear basophilia), with karyorrhexis and marginalisation of the chromatin. Cells exhibited cytoplasmic eosinophilia, and fragmented into small, mainly ovoid, bodies, both with and without nuclear material. On average 2.3 bodies were produced from each dying cell (cf. Bursch et al 1985, 1990b). These bodies were phagocytised, sometimes by neighbouring cells rather than specialist phagocytes, and were most often seen within vacuoles inside a surrounding cell (Plates 1 and 4). No sign of an inflammatory response was detected. Practically no pykno-mitotic figures were identified (3-4 in total out of over 800,000 cells sampled).

Under SEM rounded cell fragments were commonly seen, sometimes being engulfed by neighbouring cells (Plate 5), and sometimes appearing to have a rough or pitted exterior (Plate 6).

Area measurements:

The VBW projected area increases steadily with embryo age, then drops at stage 34 (see Table 2), indicating that shrinkage of the body wall occurs at the same time as a drop in cell density (seen in Fig.1b).

TABLE 1: MEAN CELL COUNT FOR EACH AGE OF EMBRYO.

Age (days)	Mean DI (%)	+/- S.D.	Mean MI (%)	+/- S.D.	Mean Density	+/- S.D.	N
5	0.167	0.317	0.580	0.362	19.300	3.754	9
5.5	0.260	0.344	0.896	0.323	19.036	3.270	3
6	0.200	0.413	1.188	0.203	17.600	2.982	3
6.5	2.696	0.973	0.350	0.295	19.260	3.015	3
7	2.118	1.601	0.471	0.228	23.376	3.180	3
7.5	11.193	1.019	0.235	0.201	31.692	1.800	3
8	8.099	1.270	0.335	0.078	28.017	2.570	3
8-9	2.148	1.071	0.270	0.219	41.700	3.801	3

where N=number of embryos sampled, S.D.=standard deviation of the means.

TABLE 2: VBW AREA FOR EACH AGE OF EMBRYO.

AGE (days)	AREA (mm^2)	+/- S.D.	N
5	0.255	0.053	8
5.5	0.270	0.071	4
6	0.309	0.033	4
6.5	0.339	0.085	4
7	0.373	0.056	4
7.5	0.411	0.025	5
8	0.391	0.031	4

where N=number of embryos sampled, S.D.= standard deviation of the means.

Fig.1a: Mean percent PCD vs. embryo age.

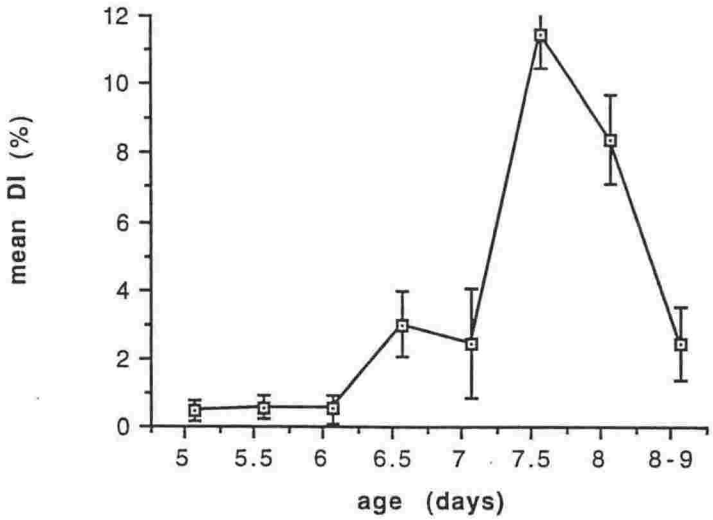

Fig.1b: Mean cell density vs. embryo age.

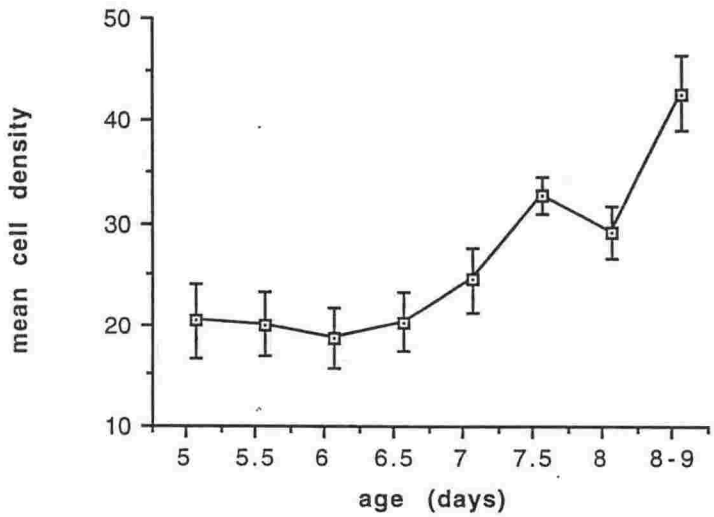

Fig.1c: Mean percent mitoses vs. embryo age.

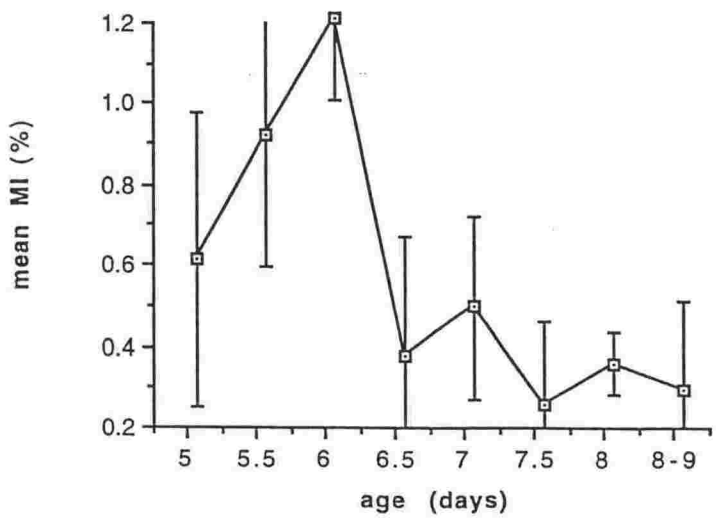

Fig.1a-c: Changes in cell count data with increasing embryo age.

Fig.2a: Cell density vs. positional order for the 5-day embryo.

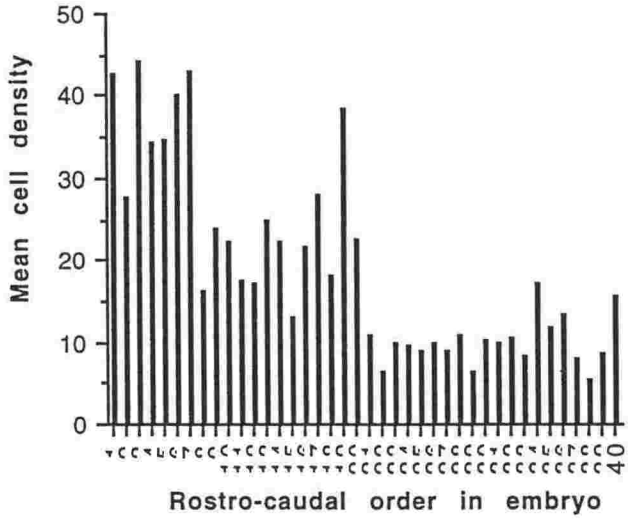

Fig.2b: Mean cell density vs. positional order for the 7-day embryo.

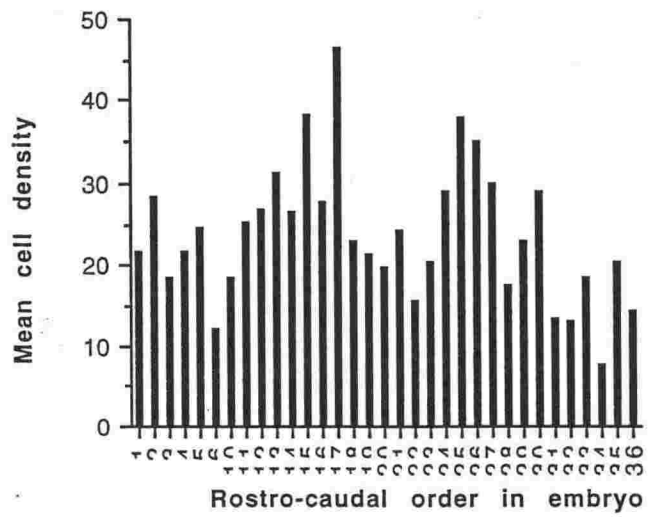

Fig.2c: Mean cell density vs. positional order for the 8-day embryo.

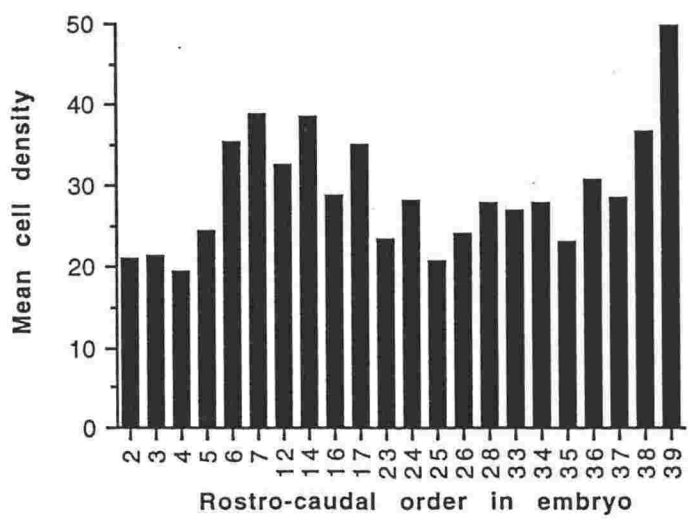

Fig.2a-c: A wave of increasing cell density passing rostro-caudally with increasing embryo age.

1.1.4: DISCUSSION

The histological description of cell death clearly indicates that the mechanism of PCD seen here is apoptosis (by the criteria of Bowen 1984, Bursch et al 1985, Columbano et al 1985, Del Vecchio et al 1991, Wyllie et al 1980), as is that seen in chick interdigital development (Wyllie 1987), but possibly not that seen in the fusing palate (Clarke 1990) or PNZ (Clarke 1990, Hurle and Hinchliffe 1978). VBW PCD will be referred to as apoptosis hereafter.

The fact that most apoptotic bodies, including all those undergoing severe degradation, were seen within other cells suggests that phagocytosis is both efficiently and swiftly carried out, as has been suggested in other situations (Duvall et al 1985, Wyllie et al 1980).

The extreme scarcity of pykno-mitotic figures (3-4 overall) suggests that cell death during mitosis is either rare, or hard to identify.

The standard deviations for some of the mean counts are quite high (see Table 1). However it is worth noting that significant differences exist between anterior and posterior regions of the VBW for all embryos at all ages, while no significant differences exists between embryos of the same developmental stage. This indicates that the major source of variation in the cell counts comes from within the embryos, rather than from between them, and suggests that the high standard deviations are largely due to order variations in the sections counted, such variations being impossible to fully avoid. It is for this reason that most counting effort was spent on getting full counts of each embryo (the major source of variation) rather than less representative counts of as many

embryos as possible. It is also why, in the later sections of this thesis, experimental treatments allowing only limited order ranges to be counted (organ cultures for instance) were compared to subsets of similar order range as well as to whole embryo counts.

It should also be realised, of course, that another potential source of variation between counts of the same developmental stage is the fact that such stages can represent long time periods (up to 12 hours in this study), so that embryos morphologically indistinguishable from each other may well not be in identical differentiative states.

The drop in cell density at stage 34 is interesting, since VBW area is also dropping at that time. This indicates not only that the apparent shrinkage of the VBW, commented on by Fell (1939), is a measurable phenomenon, but also that PCD in this area is more than able to remove the influx of cells one would expect if shrinkage is due to medial migration, suggesting apoptosis has a primary role in body wall shrinkage. Furthermore, since both area and cell number per area are dropping, cell death must be able to remove more cells than are entering the region through both immigration and proliferation combined, so that we would assume that the proliferation rate and immigration rate add to less than the cell loss rate for stages 32 to 34.

Previous researchers have shown that the perceived medial migration of lateral tissues is not primarily due to VBW shrinkage (Fell 1939, Chen 1952a,b; Chen 1953). That generalised VBW apoptosis is seen one stage after this migration is first detectable suggests that the converse may also be true. If VBW shrinkage is primarily due to medial migration of cells into a smaller area, cell density should increase as the VBW shrinks. However a significant

rise in cell density is not seen in the VBW until 36 hours after death is first detected, implying that tissue degeneration is at work prior to any migrational affect on regional size. This suggestion can be further examined by culturing VBW tissues before lateral migration is detectable (see section 1.2).

The later occurrence of VBL degeneration, however, does seem closely related to the meeting of migrating lateral tissues, as first reported by Fell (1939), based upon the visible correlation between the meeting of some easily-observed lateral tissues (the fusing sternal plates) and the initiation, peaking, and disappearance of VBL cell death. Any hypothesised inductive cause for VBL degeneration must be able to explain the exquisite precision of this medial line of apoptosis, as shown in Plate 2. The idea that cells making contact in the midline might induce each other to die is therefore quite attractive, in that it presupposes only that the migration speeds of cells will be similar.

Cell density and VBL DI were found to be positively correlated, indicating that increasing cell density is matched by increasing apoptosis.

While no significant correlation is seen between cell density and mitotic index, or mitotic index and death index, it is hard to see how increasing cell division, without any matching increase in cell death, would not show a relationship to increasing cell density. An inverse distribution of mitotic and PCD numbers did seem to exist within embryos, and between ages, and while it was not significant in this study, such an inverse relationship has also been reported in others (see Del Vecchio et al 1991, Ferguson and Anderson 1981, Forseberg 1967, Hinsull and Bellamy 1977, Poelmann 1980).

Mitotic indices have often been compared to PCD indices, because they are the nearest approximation to actual cell birth that can be obtained from static histological sections (Del Vecchio et al 1991). However, given the brief duration of mitosis relative to DNA synthesis, the autoradiographic labelling index is actually a more reliable measure of cell proliferation (Iversen 1967). Therefore it was decided to repeat the comparisons of apoptotic and proliferative indices using labelled cells as well as mitotic ones (see Section 2.1).

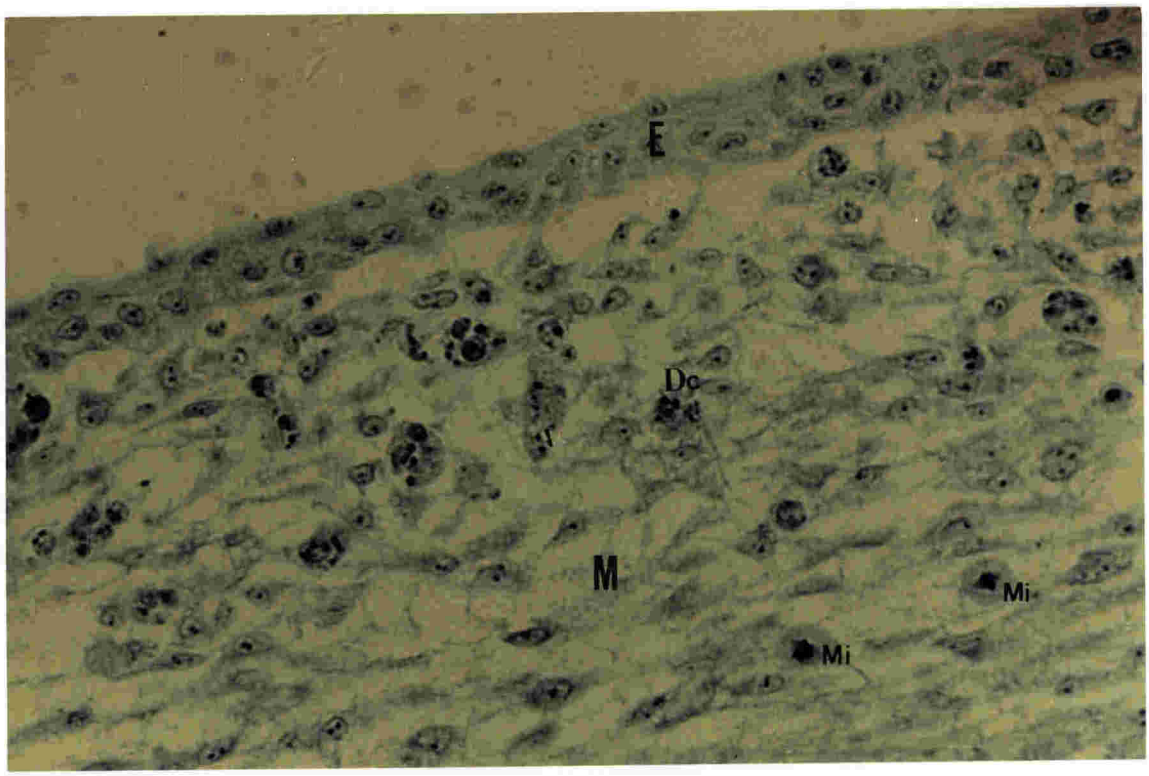

Plate 1: VBW section from a 6.5-day chick (x 50).
E= ectoderm, Dc= dead cell fragments, M= mesoderm, Mi= mitotic figure, P=phagocyte.

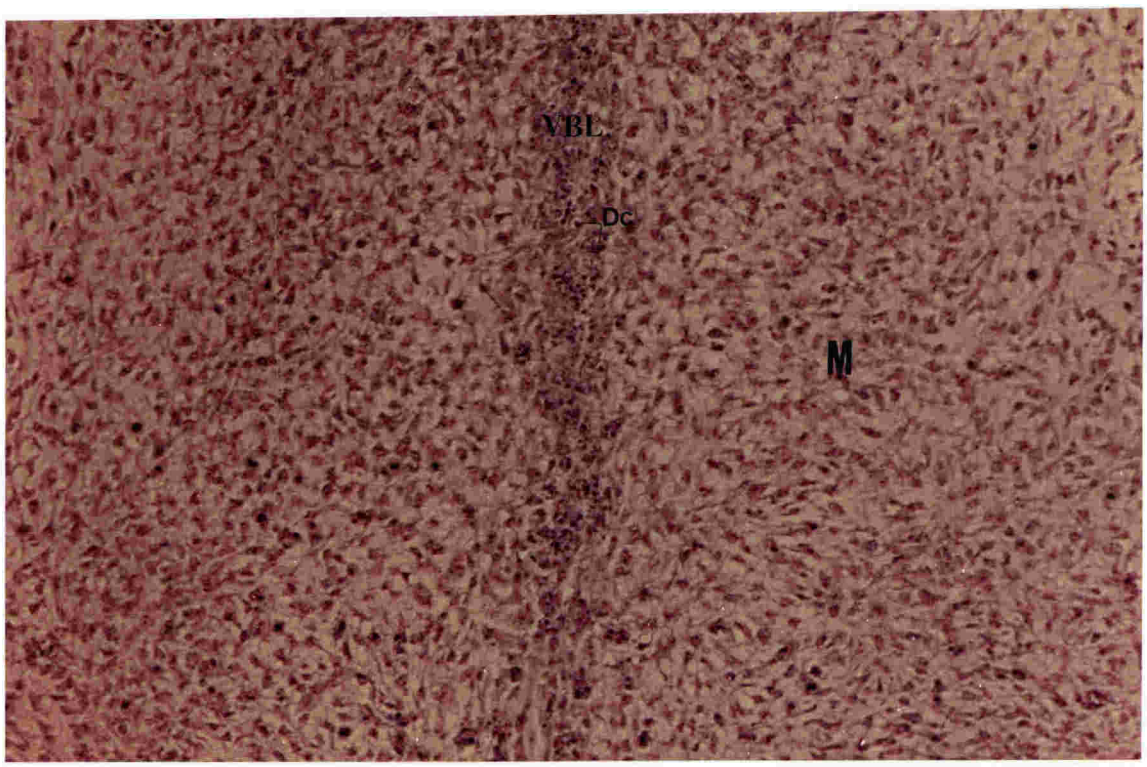

Plate 2: VBW section of 7-day chick mesoderm (x 25), showing a precise line of PCD (the VBL).
Dc= dead cell fragments, M= normal mesoderm.

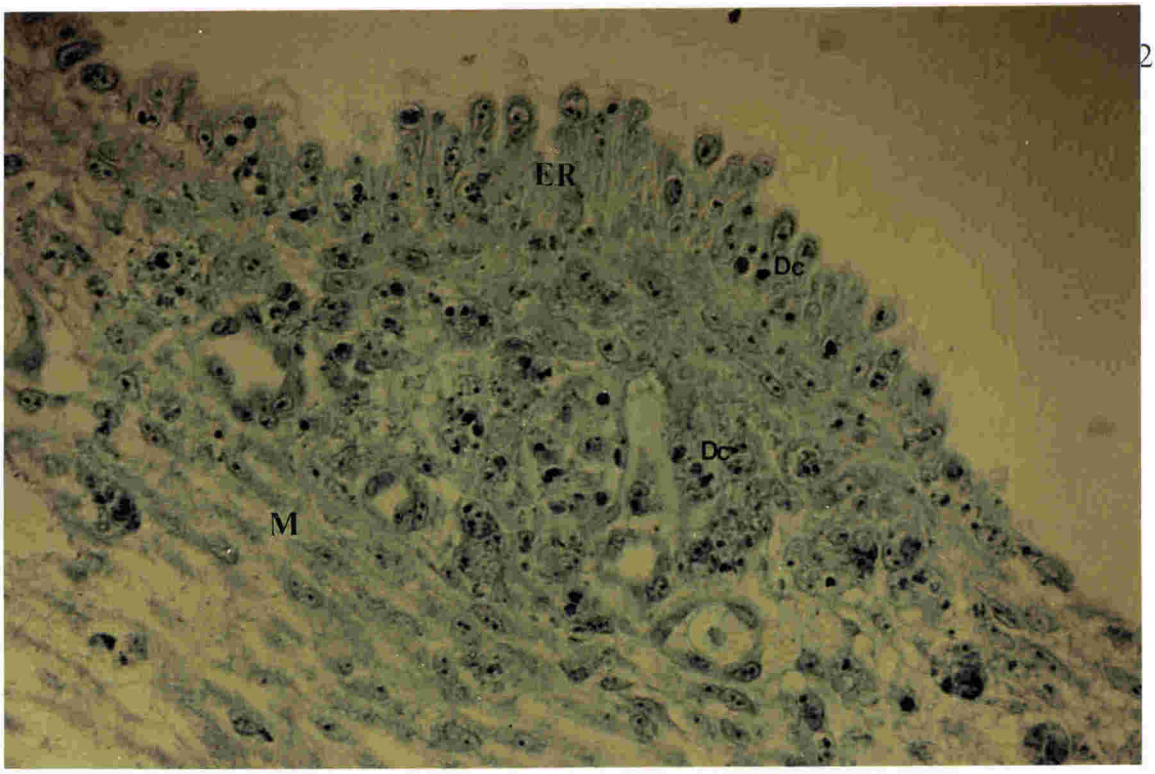

Plate 3: VBW section from an 8-day chick, showing the thickened ectodermal ruffle (x 50).

Dc= dead cell fragments, ER= ectodermal ruffle, M= mesoderm.

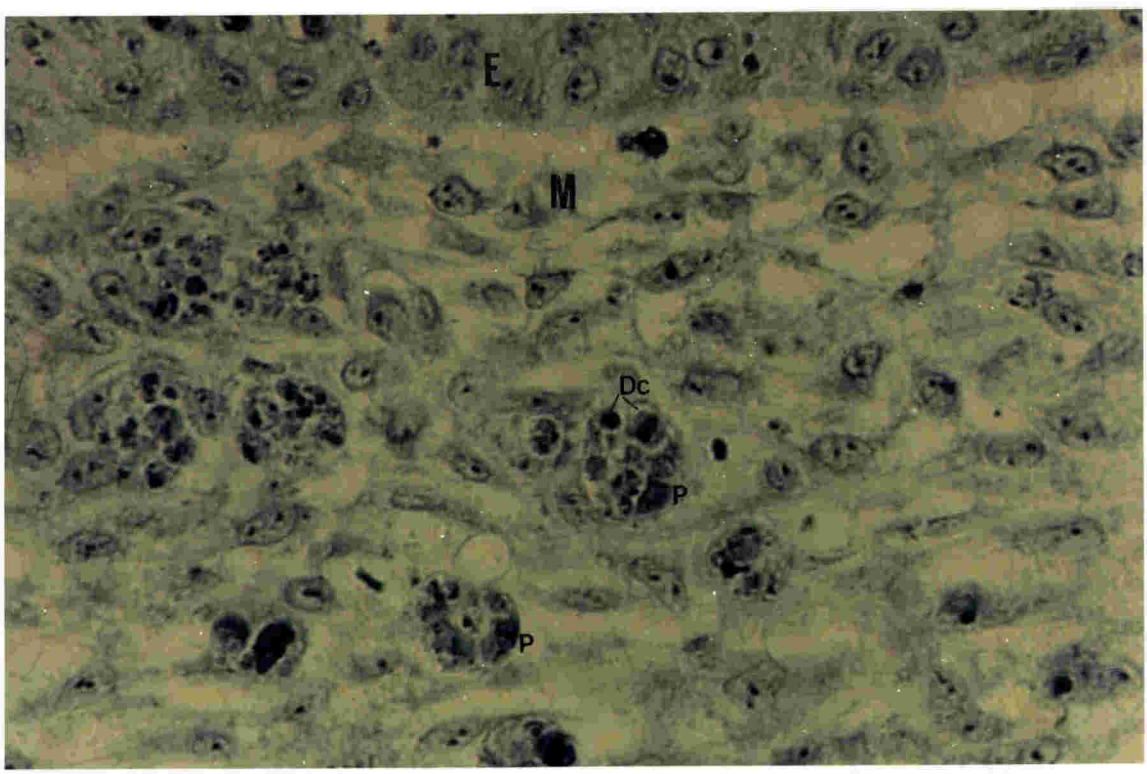

Plate 4: VBW section from a 6-day chick, showing dead cell fragments within phagocytes (x 100).

Dc= dead cell fragments, E= ectoderm, M= mesoderm, P= phagocyte.

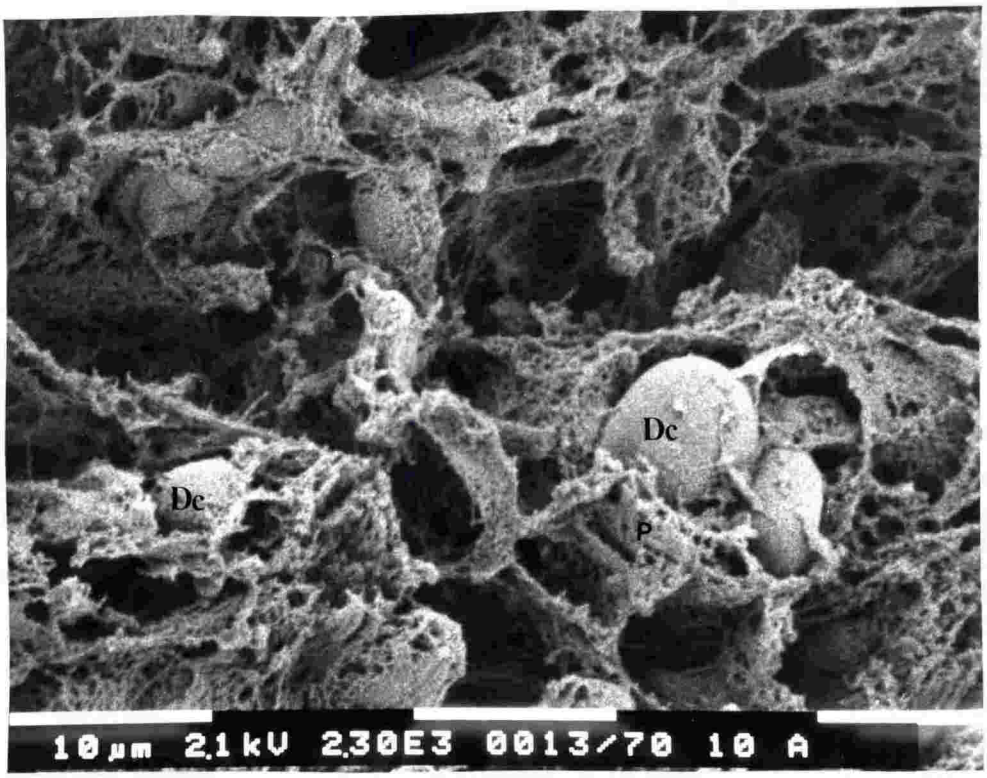

Plate 5: VBW mesoderm from an 8-day chick, under SEM. Dc= dead cell fragments, P= phagocytes.

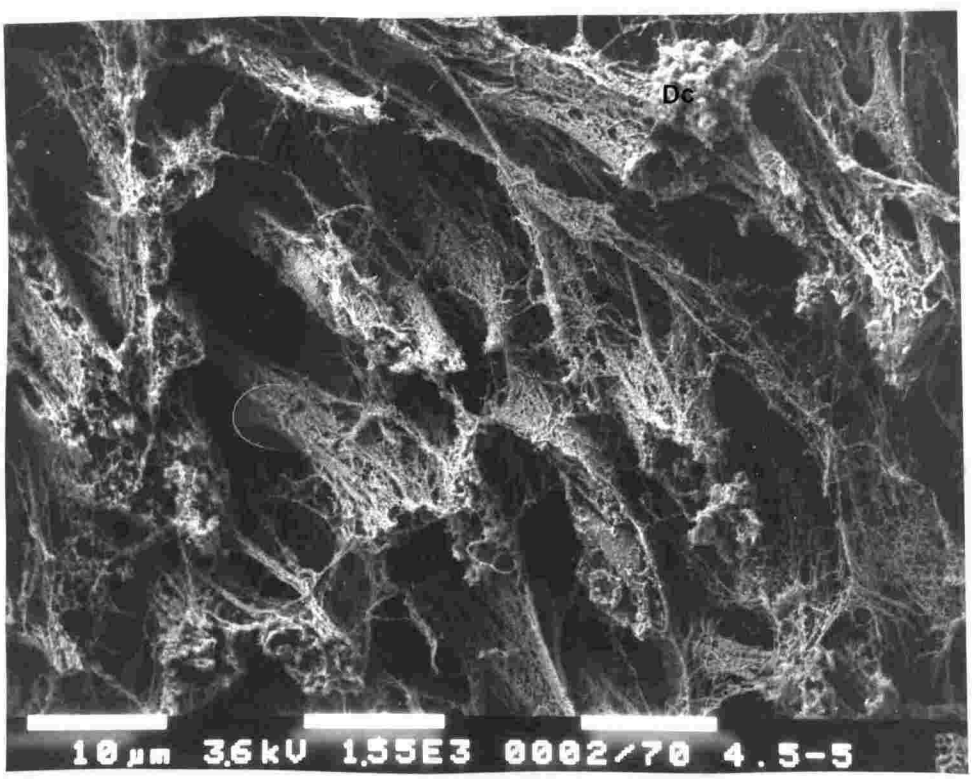

Plate 6: VBW mesoderm from an 8-day chick, under SEM. Dc= dead cell fragment.

1.2 CELL CULTURING

1.2.1 INTRODUCTION

Tissue culturing has long been used to determine whether embryological cell death is a programmed phenomenon, and to seek specific inductive events (see for example Saunders et al 1962, Mills and Bellairs 1989). The rationale is that by removing cells from the inductive (or inhibitive) environment prior to the first indications of death, the moment of irreversible commitment to that death can be found by whether the cells survive. Interest in *in vitro* models for PCD research has also arisen for ethical reasons, to avoid the use of laboratory animals (Perotti et al 1991).

However, some concern exists over the possible effects of serum upon these tissue cultures, given that it is a largely undefined media additive which may contain both mitogens and chalones (Barnes and Sato 1980a,b; Hamilton and Ham 1977; Maurer 1986; Rizzino et al 1979; Weiss et al 1980). Of particular concern is the fact that serum can induce apoptosis (Lucas et al 1991), or can influence anti-apoptotic factors in somecells, and can contain variable amounts of zinc, as zinc ions are known to have an inhibitive effect on the calcium and magnesium-dependent endonuclease fundamental to apoptosis (Cohen and Duke 1984, Cotter et al 1990, Walker et al 1988), although it can itself cause cell death (Waring et al 1991), along with many other biological effects (Bach 1981).

On the other hand, serum deprivation has serious effects on the growth and differentiation of cultured cells, and can be used to block cells in the G1 phase of the cell cycle (Adams 1980).

Because of these concerns it was decided to run a series of control cultures utilising serum-free media, enriched with selected additives (see Discussion, Section 1.2.4, for selection criteria).

Midline degeneration between approaching tissues is not an event restricted to VBW development (Glucksmann 1951). The mammalian secondary palate, for example, forms as lateral ridges, which meet in a midline seam over the tongue, with subsequent epithelial PCD (Greene and Pratt 1976). Failure of epithelial PCD leads to a cleft palate, like that seen in the chicken (Greene et al 1983). Interestingly, PCD is not essential for palate fusion in the reptile, as the alligator's epithelial cells simply migrate out of the way (Ferguson et al 1984), nor apparently for amphibian digit separation (Cameron and Fallon 1977), although it is for the lizard and turtle (Fallon and Cameron 1977). Midline cells are also known to exert a chemotaxic guidance effect in some systems (Klambt et al 1991).

It is worth noting that VBW development involves an ectodermal ridge, as interactions between a thickened apical ectodermal ridge (the AER) and the underlying mesoderm is known to be intimately related to PCD in the chick limb-bud (Amprino and Camosso 1959, Hinchliffe and Griffiths 1984), and indeed to the limbs entire development and polarity (Saunders et al 1957, Summerbell 1974a,b). A similar role in controlling mitosis and PCD has been proposed for a ventral midline ectodermal ridge in the developing human and chick tail (Mills and Bellairs 1989), which also undergoes programmed tissue degeneration (Schoenwolf 1981, Fallon and Simandl 1978).

Because organ culture methods allow for differentiative interactions between tissue layers to be maintained and

manipulated (Lasnitzki 1986), it was decided to use organ culturing of the original mesenchyme of the VBW, and of the downward-migrating replacement tissues, both with and without the presence of the ectodermal ridge and of heterogeneous lateral tissue, to test for patterns of development in the VBW similar to those mentioned above for other tissues.

1.2.2: METHODS

Serum-free media additives:

Transferrin (5µg/ml), insulin (5µg/ml), and selenite were added to DME media, which was then used as if already enriched with serum.

Selenite concentrations present in the culture reagents were first determined via the methylene blue reduction assay (Hamilton and Ham 1977), and additional selenite was added to give a total concentration of 100nM.

Gel cultures:

Nutrient or plain agar (Difco) was mixed to 3% in double strength HBSS (calcium and magnesium free), autoclaved, and kept liquid in a waterbath. 0.5ml agar was mixed with 0.1ml newborn calf serum and 0.4ml DME media (or 0.5ml serum-free media) kept at 37^0C. This mixture was poured into a 35mm x 10mm culture dish (Falcon) and allowed to cool. Gelling began at 36^0C and the gel was set at about 30^0C.

Embryos were aseptically removed from the egg, aged, then placed in sterile HBSS under a dissecting microscope. Required tissue areas were dissected free, moved to fresh HBSS (at 37.5^0C) in a new watchglass, inspected to ensure that only the desired tissue was present, then trimmed to 1.5mm or less per side. They were then transferred to the gel, with any excess moisture being removed by a fine pipette.

Sample cultures were assessed for handling injury by Trypan Blue uptake, with dissection techniques being modified until such damage was minimised.

Culture dishes were incubated at 37.5^{0}C in 5% CO_2 in oxygen, sitting on a wooden board (to prevent overheating the bottom of the dishes).

Liquid cultures:

Embryos were prepared as above, however explants were placed upon sterile strips of millipore filter (0.5µm. porosity) on a steel mesh support, then placed in a culture dish so as to just make contact with the surface of the media (DME plus 5% serum, or serum-free media).

Cell counts:

Cell counts were performed as detailed in the histology section (Section 1.1.2), with the exceptions that only those explants that seemed heathly and translucent, and showed no histological signs of necrosis, were analysed, and that due to small explant size only 1000-3000 cells were assessed per sample.

1.2.3. RESULTS

No observable difference in cell survival or epithelialisation resulted from liquid versus gel culturing, however liquid cultures proved more awkward to manipulate and were therefore discontinued in later experiments.

Tissue removed from the VBW exhibited apoptosis even when removed as early as stage 24 (4 days incubation). Furthermore, the levels of apoptosis are similar to those that would be expected *in vivo* at the age the tissue was cultured to (see Table 3). The only occasions where cell counts differ significantly from those in vivo are for cell density in explants from 2 of 5 stage 24 embryos, which were higher than expected ($p<0.0001$ and $p=0.0021$), and for mesoderm explanted without ectoderm. In the latter case, mesoderm cultured alone has a higher than expected DI, whether explanted at stage 24 ($p=0.0045$ or less) or stage 31 ($p<0.0001$).

Lateral tissues explanted at stage 24 did not undergo extensive apoptosis in culture, unless cultured in contact with VBW tissue, in which circumstance the lateral tissue's DI was not significantly different than that in the adjacent VBW tissue (p ranging from 0.615 to 0.0547). Lateral tissue MI and cell densities did not alter significantly when the tissues were cultured alone versus in contact with VBW tissues. Heterogeneous lateral tissue (from stage 24 embryos) cultured in contact did not show levels of PCD, mitosis, or cell density significantly different from those seen in lateral tissue cultured alone (p ranging from 0.063 to 0.572).

Serum-free culturing resulted in a very significant decline in apoptosis relative to similar cultures with serum ($p<0.0001$ in all cases). Mitotic levels were significantly higher in 2 stage 31 serum-

free cultures (p=0.002 and 0.015) and 1 from stage 24 (p=0.047), although any differences in cell densities were insignificant. The DI, MI, and cell densities were not significantly different from those seen in vivo when stage 24 and 27 cultures were made (p ranging from 0.957 to 0.084), but the DI in stage 31 cultures was significantly lower than that seen in vivo at that age (p=0.015 or less).

TABLE 3: MEAN CELL COUNTS AFTER TISSUE CULTURING.

culture type	age in culture	mean DI (%)	+/- S.D.	mean MI (%)	+/- S.D.	mean cell density	+/- S.D.	N
stage 24 tissue	stage 34	9.742	2.447	0.023	0.045	42.954	6.296	5
stage 24 ectoderm	stage 32	26.323	4.321	0.040	0.072	34.945	3.214	5
stage 24 mesoderm	stage 32	28.507	3.567	0.012	0.025	32.847	3.075	4
stage 24 lateral tissue	stage 34	0.050	0.017	0.014	0.038	32.148	3.672	5
stage 24 lateral tissue plus lateral tissue	stage 34	0.107	0.125	0.025	0.036	34.156	2.894	5
stage 24 lateral tissue plus VBW tissue	stage 34	8.793	3.046	0.018	0.036	37.380	4.325	5
stage 24 tissue, serum-free	stage 34	0.036	0.057	0.031	0.170	35.472	3.683	5
stage 27 tissue	stage 32	17.097	3.575	0.074	0.105	27.623	3.762	5
stage 27 tissue, serum-free	stage 34	0.053	0.129	0.051	0.103	30.526	2.940	4
stage 29 tissue	stage 32	11.31	2.175	0.018	0.032	31.326	4.302	4
stage 31 tissue	stage 34	6.314	0.084	0.398	0.167	36.606	3.836	5
stage 31 ectoderm	stage 34	6.374	0.092	0.280	0.084	33.894	2.987	5
stage 31 mesoderm	stage 34	25.166	3.572	0.061	0.084	45.542	4.931	5

where N=number of cultures sampled, S.D.= the standard deviation of the means.

1.2.4: DISCUSSION

Organ culture techniques were not selected because of any fear of dissaggregation injury to cells, as this can be controlled quite well (Waymouth 1974), at least relative to that level of cell injury unavoidable in any cell culture technique. Indeed, some preliminary monolayer cultures were established (using Trypsin/EDTA), with no significant increase in handling-induced necrosis, as observed by trypan blue exclusion. Organ cultures were used solely because they maintain the anatomical (and therefore functional) relationships of tissue components in culture (Lasnitzki 1986).

That apoptosis occurs in explanted tissue, even when taken at stage 24, and reaches levels similar to those seen later *in vivo*, implies that apoptosis is controlled by mechanisms intrinsic to the region explanted.

Lateral tissues explanted together or apart at stage 24 did not exhibit apoptosis, but did when explanted with VBW tissue. This implies that lateral tissues are not yet programmed to die, but have the capacity to become so, perhaps through the presence of other dead cells, or by some local, diffusible message as suggested by Lynch et al (1986). Whether lateral tissues become programmed to undergo apoptosis by stage 31 (when the VBL appears) is unknown, but seems quite possible given the continued occurrence of apoptosis in cultures from stages 29 and 31.

The possibility always exists that PCD in culture might be partly due to, or enhanced by, mild nutrient or energy deficiencies, as is seen in mild ischaemia (Boobis et al 1990, Wyllie et al 1984a). It is interesting that mesodermal tissue cultured alone shows a higher DI than when cultured with ectoderm, as it is hard to see

how removal of the ectodermal layer could have decreased the underlying mesoderm's access to medium components. Indeed the presence of at least some mitotic figures in most cultures suggests nutrient deficiencies are not responsible for the levels of apoptosis seen, as nutrient and growth factor scarcity would also affect the cell cycle (Paul et al 1978). Damage induced by stripping the ectoderm from the underlying mesoderm might also increase levels of apoptosis occurring; however no sign of increased handling damage to cells was detected with trypan blue exclusion, and it is hard to see why such mechanical trauma would not also have affected the ectoderm which was removed, yet no increase in apoptosis was detected in isolated ectodermal cultures.

It is probable therefore that VBW ectoderm has some limiting effect on cell death in the underlying mesoderm. This has indeed been shown to occur in chick limb-bud development, where the AER limits or suppresses underlying mesoderm degeneration, however the apical ectodermal ridge's survival is in turn dependent upon a mesodermally-produced maintenance factor (Amprino and Camosso 1959, Hinchliffe and Griffiths 1984, Saunders et al 1957, Summerbell 1974a,b, Wilson and Hinchliffe 1985). This was not evident here, given that ectoderm cultured alone does not show a greatly increased DI. It should however be noted that while complete ectoderm removal from the mesodermal tissue proved remarkably easy to ensure, it was harder to be certain that all mesodermal cells were removed from the ectodermal tissue. The possibility therefore exists that mesodermal cells might have contaminated some 'ectoderm only' cultures, perhaps masking any ectodermal dependence upon mesodermal maintenance factors.

It is puzzling that cell densities did not usually alter significantly in culture, given that the influx of migrating cells to the region is curtailed, but cell death is not, so that a decline in density might be expected. However, given the low levels of mitosis *in vivo* (see Table 1), it is quite possible that a higher level of mitosis existed at some time *in vitro*, perhaps not being detected because of the small sample sizes (often less than 3000 cells) involved. It might also be the case that explant tissue shrinkage due to VBW medial cell migration (Chen 1952b) resulted in denser packing of fewer cells.

Serum-free culturing:

Serum-free culturing seemed to have no adverse effects upon tissue survival, possibly because a very rich basal medium was used (after initial trials with MEM and Ham's MEM) for a short term culture.

DME/F-12 mixed 1:1 is a more commonly used serum-free basal medium, due to the extra vitamins and trace elements supplied from the F-12 medium (Barns and Sato 1980a). However F-12 unfortunately also contains zinc salts, which might have affected apoptosis rates, perhaps inhibiting the putative $Ca^{++}Mg^{++}$-dependent endonuclease (Cohen and Duke 1984). DME by itself, however, has proved to be a very suitable general medium for chick cells (Maurer 1986), and gave excellent results in this study.

The three additives used were selected for the serum-free medium because they were consistently important in most studies in the literature, which was deemed important given that heterogeneous cell populations were present in many of the cultures used. Insulin is essential as a promoter of glucose and amino acid uptake, and it appears that many of the growth factors

commonly added to serum-free media may work through binding to the insulin receptor (Hamilton and Ham 1977, Rizzino et al 1979, Weiss et al 1980). Transferrin is needed to bind Fe^{++} ions that can be toxic to cells (Maurer 1986).

Selenite was probably the most controversial additive chosen, for while it has been shown to be essential for some cells (Hamilton and Ham 1977), it has been reported to be toxic to others (Weiss et al 1980). It seems likely, however, that selenite in small quantities is essential both as a trace nutrient and for use in some enzyme pathways, most notably for activation of glutathione reductase, an enzyme needed for O_2 metabolism and therefore energy production (Maurer 1986). Selenite appears to be an active element in many other additives, most especially Thyroxine, and it seems likely that toxic results after selenite addition are due to its presence as a contaminant in many culture reagents (Hamilton and Ham 1977), giving a much greater total dosage than realised. To combat this all solutions used in serum-free media were checked for levels of selenite contamination, which was discovered in some salt mixtures and surprisingly in one batch of distilled water, and the amount added was altered accordingly.

The removal of serum from cultures had an obvious effect on apoptosis levels, which were all very significantly lower than in similar cultures with serum. This does not seem to be simply due to nutrient deficiencies, because the MI actually rose in some of these cultures, implying that more than sufficient nutrients for cell maintenance were available. Instead it appears that levels of apoptosis in serum-free cultures remained similar to those *in vivo* at the age explanted from, rather than achieving those of the age the tissue was cultured to. It could well be that only those cells

which are already committed to death will die, so that no statistical difference is detected relative to the low levels of death *in vivo* at stage 24 and 27, whereas the higher DI *in vivo* at stage 31 causes a statistically detectable decline in DI to be seen *in vitro*. Perhaps most notable is the virtual lack of apoptosis in explants of stage 24 tissue, when similar tissues will undergo apoptosis when cultured with serum. This suggests that while initial diffuse VBW degeneration is programmed by stage 24, it is not irreversibly programmed, and that some factor in serum is essential for continued signalling to die, or for the cells' response to that signal.

While it is tempting to speculate on the nature of serum factors that might affect the DI, considerable experimental work would be required to examine such speculations. For example, while calcium ions are known to be present in serum (Maurer 1986), and to be required for the function of the apoptotic endonuclease (Bursch et al 1990a, Cohen and Duke 1984, Waring et al 1991), merely adding Calcium to serum-free medium would not indicate that Ca^{++}-dependent endonuclease inhibition was occurring, even should apoptosis reappear. This is because we cannot take extracellular calcium levels as a measure of interstolic Ca^{++} levels, given the complexity of calcium receptor mechanisms (England 1986) and the cellular calcium pool (Irvine 1986), combined with the fact that calcium is fundamental to all forms of cell death in a number of different ways (Boobis et al 1990, Farber 1981, Perotti et al 1990, Waring et al 1991), due to its wide biological significance (Metcalfe et al 1986, Williams 1990) and the vulnerability of Ca^{++} homeostasis to diffusion gradient disruption and ATP depletion. Thus increasing calcium levels could affect apoptotic levels in a number of ways, perhaps in interaction with

other, unknown serum factors. Only an exhaustive series of substitution experiments, combined with an attempt to monitor interstolic mobilisation of the elements involved, can really begin to explore the nature of VBW apoptotic serum-dependence, and this is outside the scope of this project.

Nevertheless, that embryos already undergoing apoptosis show reduced levels of cell death in serum-free culture suggests that apoptosis in this tissue continues to be influenced by some external factor, unless apoptotic cells can be blocked from death long after being set on their differentiative pathway.

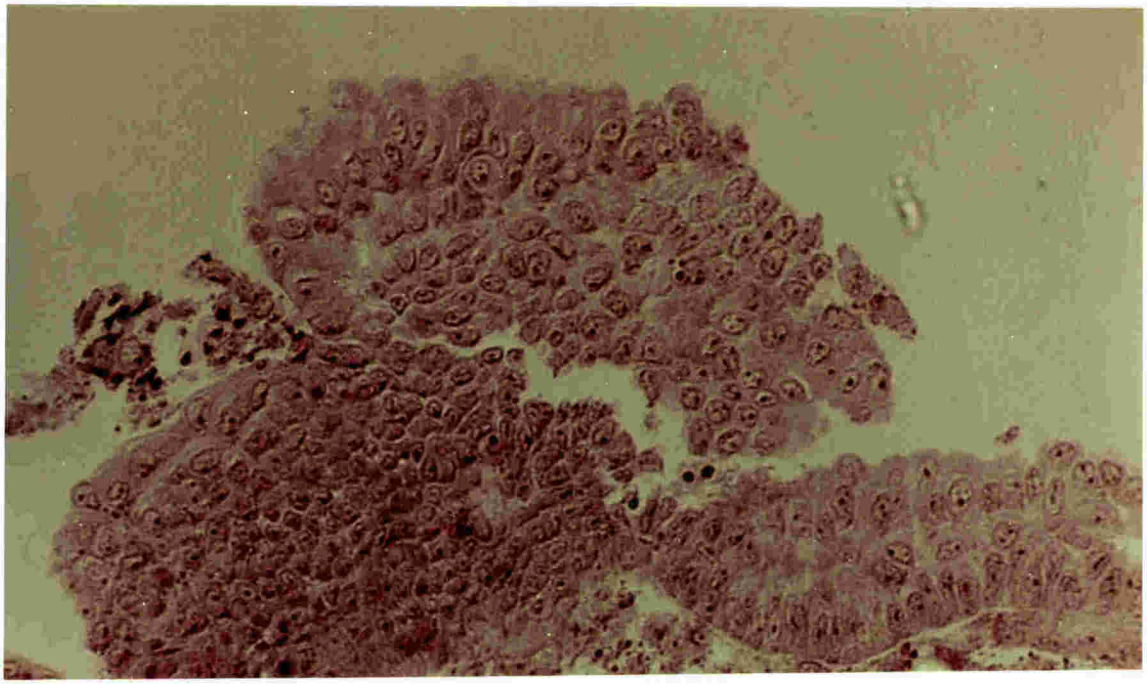

Plate 7: Ectoderm from a stage 24 embryo, cultured for 4 days without mesoderm (x 50).

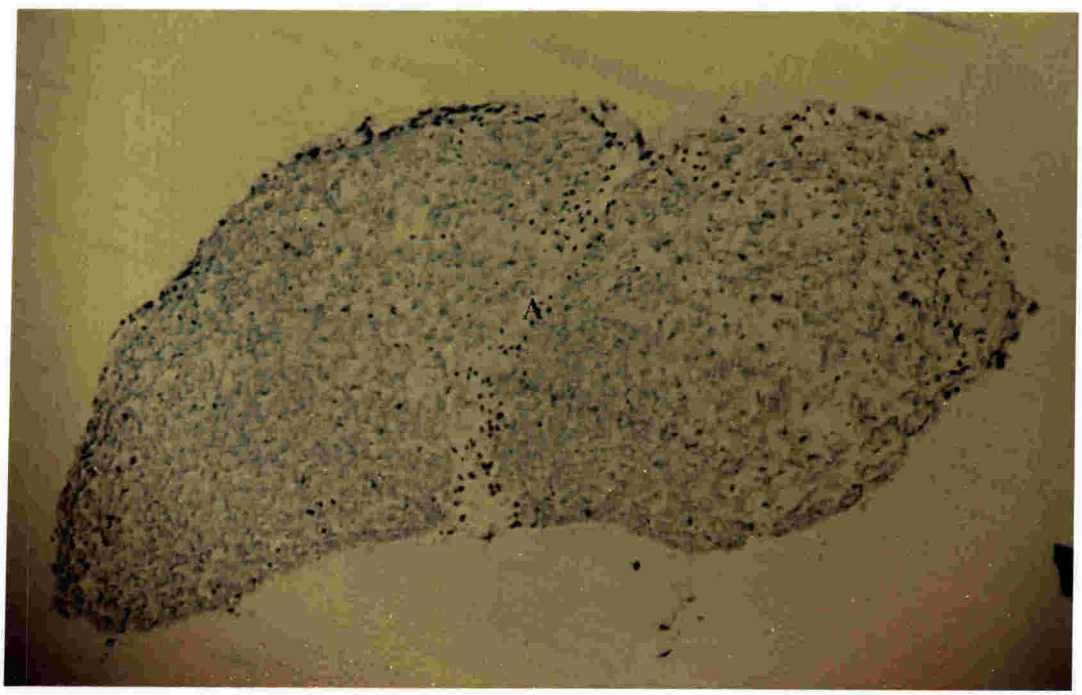

Plate 8: VBW tissue from a stage 24 embryo, cultured for 4 days. A= VBL apoptosis (x 20).

2: CELL CYCLE KINETICS

2.1 INTRODUCTION.

PCD, particularly apoptosis, has long been recognised to play a role complementary but opposite in effect to mitosis in the regulation of cell populations (Columbano et al 1985, Hinsull and Bellamy 1981, Hurle et al 1977, Kerr et al 1972, Rotello et al 1991, Steel 1968). The observation that degeneration occurs in or near areas of active proliferation (Columbano et al 1984, Hurle et al 1977, Kallen 1965, Forseberg 1967, Walker et al 1988) has not only led to the suggestion that it is density dependent, but also to attempts to explain this relationship by linking PCD to the cell cycle.

Similarity in the numbers of mitotic and pyknotic cells soon led to the idea that errors in mitosis (incomplete chromosome separation for instance) could lead to PCD (e.g. Kallen 1965), or even that the cytoplasmic changes involved in cell division could lead to organelle damage and death (Becker and Lane 1966, El-Labban and Osorio-Herrera 1986).

Given that PCD is inversely distributed to mitosis, usually peaking in numbers after mitosis has peaked and disappeared (Del Vecchio et al 1991, Ferguson and Anderson 1981, Forseberg 1967, Hinsull and Bellamy 1977, Poelmann 1980), and that growth promoters (mitogens and tumour promoting factors) inhibit apoptosis (Bursch et al 1984, 1985, 1990a,b; Schulte-Hermann et al 1990, Tomei et al 1988, Williams et al 1990, Wyllie et al 1980), it is unsurprising that non-proliferating cells have been proposed as being more susceptible to PCD (Bursch et al 1990a,b, Lala 1972). However, while quiescent cells have a lower repair rate for

damaged DNA (Harris 1985, Mitchell and Hartman 1990), they are also less susceptible to such damage (Epifanova 1977), which is fundamental to PCD (Eastman 1990, Strauss 1977, Wyllie et al 1980). This is why much of cancer therapy is aimed at getting as many target cells into the proliferating population as possible before treatment, as most chemotherapeutic agents [the majority of which induce apoptosis (Allan et al 1987, Eastman 1990, Kerr and Searle 1980, Searle et al 1975, Walker et al 1991)] are effective in the S and G_2-M phases of the cell cycle (Bontenbal et al 1990). It is therefore hard to see why non-proliferating cells should be primary targets for apoptosis, particularly since their removal would have less long-term kinetic effects (Kerr et al 1982). Indeed, Horn et al (1991) found that forcing tumour cells into G_0 removed their ability to undergo PCD after monocyte interaction, and hypothesised that processes essential to the lytic pathway could not take place in G_0.

Given the open chromatin configuration during DNA replication, one might expect cells in S phase to be more susceptible to DNA damage, and therefore apoptosis (Danno and Horio 1982). Transcriptionally active cells, however, are also better able to repair such damage (Epifanova 1977, Mitchell and Hartman 1990, Tobias et al 1980). While the cytotoxic effects of Cisplatin, an anticancer agent, are a function of how much DNA damage is taken into S phase (Pera et al 1981), this cytotoxicity is not dependent upon repair ability (Sorenson and Eastman 1988) and leads to cell arrest and death in G_2 (Eastman 1990, Sorenson et al 1990). Physiologically-induced cell death, however, more often seems to lead to postmitotic cell arrest, possibly in G1 (Eastman 1990, Lewis 1975, Sanderson and Glauert 1979, Vukmanovic and Zamoyska 1991, Watanabe and Okada 1967b).

It is also interesting that DNA repair, as well as proteolysis, (Finley and Varshavsky 1985) has been linked to ubiquitin (Mitchell and Hartman 1990) since polyubiquitin gene expression has been found to increase notably during PCD (Schwartz et al 1990a,b). Expression of *c-myc* and *c-fos* [proto-oncogenes involved with cell proliferation (Zelenka 1990)] have also been induced during PCD (Buttyan et al 1988, Rennie et al 1988), as indeed has increased transcriptional activity of mobile DNA in the LINE family (Servomaa and Rytomaa 1990).

Of particular interest in the control of both PCD and the cell cycle are the proto-oncogene bcl-2 (an apoptosis inhibitor in mammals homologous to the *Ced* genes in *C. elegans*) and the anti-oncogene p53, which are known to interact in formation of some lymphoma lines (see reviews by Merlino et al 1994, and Pittman et al 1994, for example). p53, amoung other things, appears to serve as a cell cycle control gene, acting as a G_1-M checkpoint preventing mitosis if DNA damage has occured. Overexpression of p53 can lead to either cell division or cell death.

The possibility that cells differentially respond to a supposed death signal according to their cell cycle stage, perhaps through the subversion or prevention of events that allow replication and mitosis to occur, is a very attractive one, in that it may help explain the way apoptosis is seen to affect scattered cells, but not their apparently identical neighbours. It would also have great impact for the study of tissue kinetics, not least of all because it might allow for indirect estimation of cell loss from proliferation parameters, since cell loss cannot be exactly assessed by any of the current methods (Wolman, 1985).

Because of this, autoradiographic studies were undertaken not only to assess cell production, so obtaining some understanding of the growth kinetics of the Ventral Body Wall, but also to attempt to correlate any stage of the cell cycle to the numbers and appearance of dead cells.

Unfortunately, most cell kinetics models neglect the immigration component to tissue growth, obviously an important aspect of VBW development. Thus it was decided to combine kinetic parameters taken from tissue cultures, to determine the kinetics of set cohorts of cells and the impact of immigration *in vivo*.

It should also be noted that a simple, circular model of the cell cycle, with consecutive, irreversable decision points, is almost certainly too simplistic to represent actual cell reproduction decisions. However given the likely simplicity of any model of PCD decision points that might be drawn from the data in this thesis, a more complex multi-descision probability state model of the cell cycle was considered unsuitable for comparison. It must also be remembered that the fore-mentioned circular cell cycle model is largely a product of autoradiography, but for that very reason is well suited for use with that technique and the kinetic formulae generated by it.

2.2: METHODS.

Autoradiography with tritiated thymidine (H^3tdr) was selected for cell cycle analysis because this isotope gives reliable quantitative results with light microscopy, particularly when combined with liquid emulsion techniques (Gude 1968, Rogers 1973).

Cell counts were made as per Section 1.1.3, with at least 100 mitoses being counted to determine the percent of labelled mitoses, and at least 200 apoptotic bodies being counted for the percent of labelled apoptotic cells.

2.2.1: AUTORADIOGRAPHY METHODS

All glassware was cleaned before use by soaking in a solution of 100g potassium bichromate in 850ml water plus 100ml conc. sulphuric acid, followed by a wash in several changes of distilled water overnight and a dip in 80% ethanol. It was then air-dried and stored dust free. Slides were subbed with 5g gelatin plus 0.5g chrome alum in 1L dist. water, made fresh and filtered, before being used for sections.

Thymidine application:

Tritiated thymidine, 10 μCi/embryo in 0.1 ml sterile PBS, was injected onto the chorioallantoic membrane (CAM) through a window in the egg, which was then resealed and incubated for the desired time. Continuously labelled embryos were reinjected every 3 hours (Tone et al 1983), while pulse labelled embryos were cold chased with 8 times the molar concentration of cold thymidine (Sigma) in PBS, then rinsed with sterile PBS, after a 30 minute pulse.

Treated embryos were removed from the egg, fixed in FAA, imbedded in methacrylate, then sectioned at 3μm.

Autoradiography:

NTB-2 emulsion (Kodak) was used under a safelight with a Kodak No.2 Filter, with extra masking. The darkroom was maintained at 20^0C and 40% humidity.

Slides were exposed in a light-tight slide box with a teaspoon of tissue-wrapped silica gel, for from 10 to 20 days. Hot (radioactive) and cold (non-radioactive) control slides were included with each batch, as was a light-fogged slide to detect negative chemography (as per Rogers 1973).

Exposed slides were developed in D19 developer (Kodak) diluted 2:1 with distilled water, for 2 minutes, washed for 10 seconds in distilled water plus 2 drops of D19, then fixed in Kodak fixer (1:1 with dist. water) for 10 minutes, or until emulsion is completely transparent. All solutions were kept at 18^0C and changed after 10 slides.

Slides were post-stained with a range of stains (see results, section 2.3), coverslipped with DPX mountant, then examined with both dark and lightfield microscopy, the former being used to check the accuracy of grain counts and to search for real or artefactual grain distributions.

Background grain counts were assessed for all sections used in quantitative analysis, and were adjusted for by the method of England and Miller (1970), and, where applicable, by comparison with grain counts over unlabelled metaphases.

2.3: RESULTS

Of the histological stains used, Giemsa stain, methyl-green/pyronin, and Feulgen's reagent were all too faint for easy cell identification, while Mayer's and Harris' Haematoxylin were too dark to allow easy silver grain identification. Best results were obtained with Methyl Blue/Basic Fuchsin, which gave clear cell detail allowing easy identification of mitotic and apoptotic figures, without obscuring grain counts (Plate 9a,b).

Emulsion thickness was found to extend above the range of the tritium particles on all areas used for counting, thus ensuring infinite thickness (beyond 3μm) was achieved, along with an adequate level of autoradiographic efficiency for quantitative studies to be performed (Rogers 1973).

No evidence of either positive or negative chemography was shown by any of the control slides.

Optimal grain counts versus background were achieved at 12 days exposure.

Labelled dead cells first appeared between 15 and 18 hours after pulse or continuous labelling (see Tables 4 and 5). No significant difference was found between the proportion of apoptotic bodies labelled in pulse versus continuous labelling, for the time period sampled (p ranging from 0.062 to 0.581). The mean percentage of labelled apoptotic cells (for both labelling methods combined) began at 5.153% (+/- 0.337) after 18 hours, rising to 69.231% (+/- 7.692) by 21 hours after the start of labelling.

There were significantly more labelled cells being found in the ectoderm and its closely underlying mesoderm than in mesoderm further away (p ranging from 0.025 downward), with the least labelling being seen in mesoderm equidistant from

ectoderm and endoderm (p=0.001 or less). No significant difference in the pattern of labelled cell distribution is seen in stage 34 embryos relative to those of stage 26 (p ranging from 0.068 to 0.427).

Cell density was found to be positively correlated to the Labelling Index (LI) (p=0.0002 or less) and DI (p=0.0058 or less). LI was also found to be negatively correlated to DI (p=0.0387 or less). MI was not significantly correlated to any of the above (p ranging from 0.9527 to 0.0843) with the exception of LI (p ranging from 0.0361 to 0.006).

The 5-day chick embryo ventral body has a 'flash LI' of 11% (ie 11% of the cells were labelled, despite being fixed immediately after treatment with tritiated thymidine, implying that 11% of the cells were in S-phase at that moment in time), and an average MI of 0.58%. The combined results from the pulse and continuous autoradiography experiments (see Fig.s 3 and 4) give an average cell cycle time (Tc) of 14 hours, with S-phase requiring 4.2 hours (Ts), the duration of G1 (T_{G1}) being 6.5 hours, and that of G2 (T_{G2}) being 3 hours. The time spent in mitosis (Tm) therefore equals Tc - (Ts + T_{G2} + T_{G1}), namely 0.3 hours.

The 8-day chick has a flash LI of 5.8%, an average MI of 0.34% and a maximum LI of 22.7%.

TABLE 4: Percent labelled mitoses and apoptoses after pulse labelling of 5-day embryos.

Hours after labelling	mean Labelled mitoses (%)	+/- S.D.	mean Labelled apoptoses (%)	+/- S.D.
0.3	0	0	0	0
3	44.200	6.791	0	0
6	100	0	0	0
9	12.822	5.246	0	0
12	7.882	3.215	0	0
15	6.969	2.187	0	0
18	100	0	4.978	0.398
21	98.533	2.540	65.741	3.264

where Number of samples=3 and S.D.=the standard deviation of the means.

TABLE 5: CELL COUNTS AFTER CONTINUOUS LABELLING OF 5-DAY EMBRYOS.

Hours continual labelling	mean LI (%)	+/- S.D.	mean LMI (%)	+/- S.D.	mean LDI (%)	+/- S.D.	mean grain count	+/- S.D.
0.3	11.077	0.384	0	0	0	0	4.992	1.156
3	18.884	1.125	49.836	1.377	0	0	17.883	2.987
6	29.217	1.469	100	0	0	0	24.407	4.959
9	41.077	4.530	100	0	0	0	21.818	3.768
12	41.254	5.635	100	0	0	0	24.657	3.711
15	41.753	4.572	99.965	0.061	0	0	24.712	4.541
18	40.479	4.306	100	0	5.327	0.247	23.762	3.108
21	42.082	4.494	100	0	71.455	5.427	24.644	4.329

where LMI= labelled mitotic figures, LDI= labelled apoptotic figures, number of samples= 3.

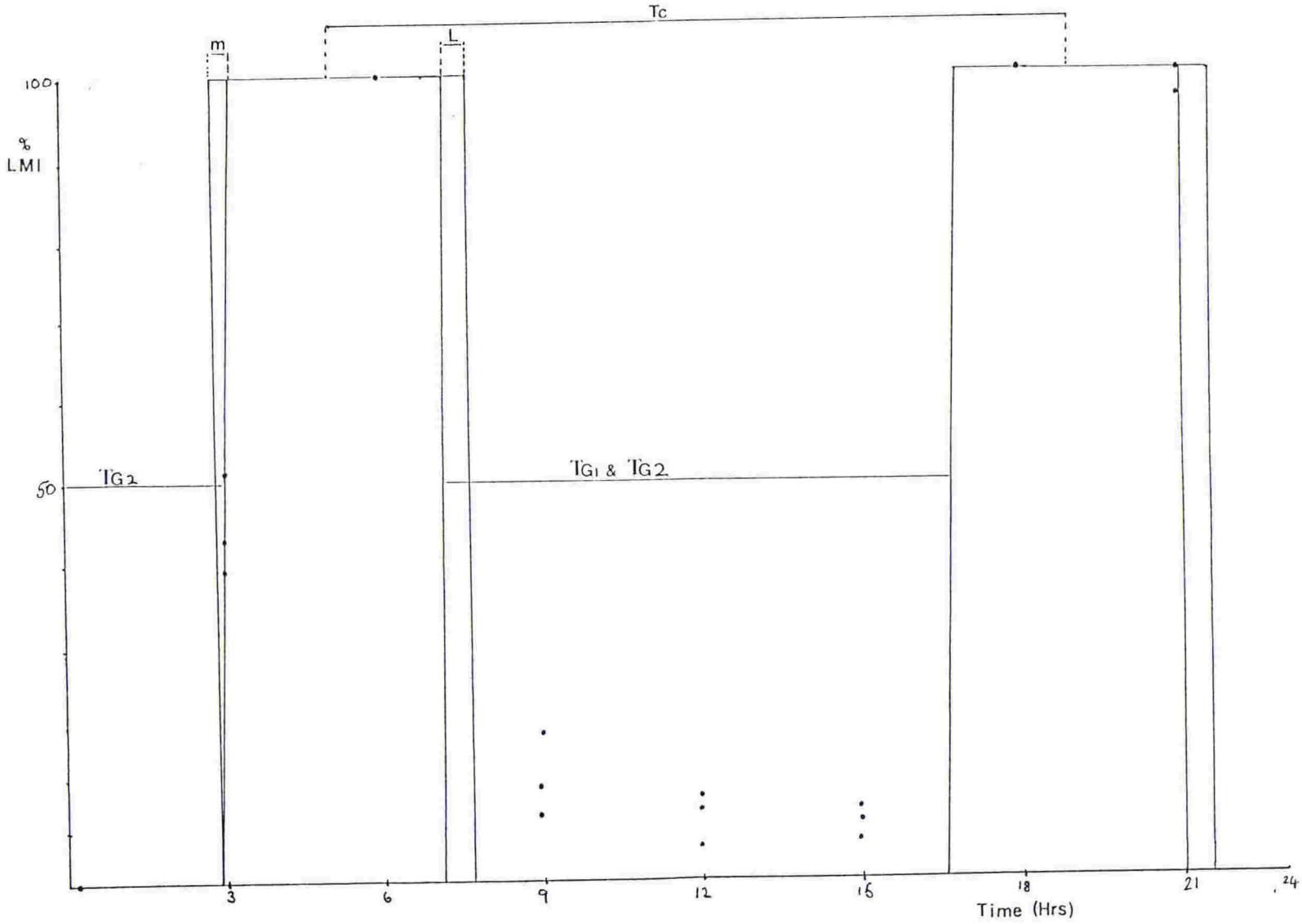

Fig.3: Cell cycle times derived from autoradiographic pulse labelling. = observed, lines = theoretical LMI.

Fig.4a: Percent labelled metaphases with continuous labelling.

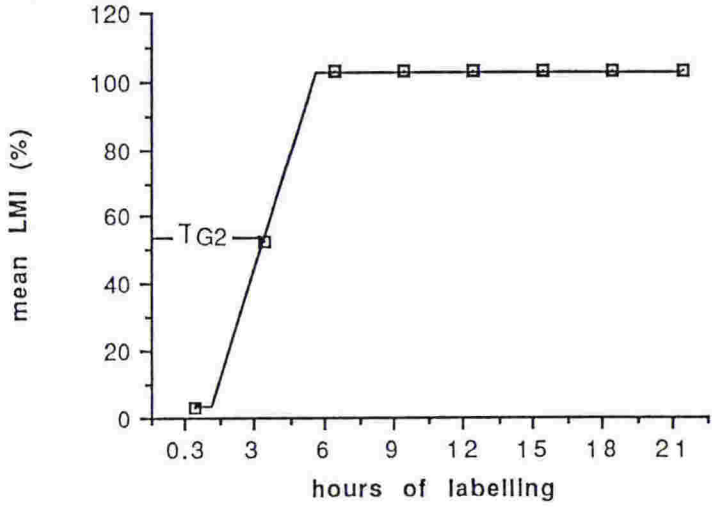

Fig.4b: Percent labelled cells with continuous labelling.

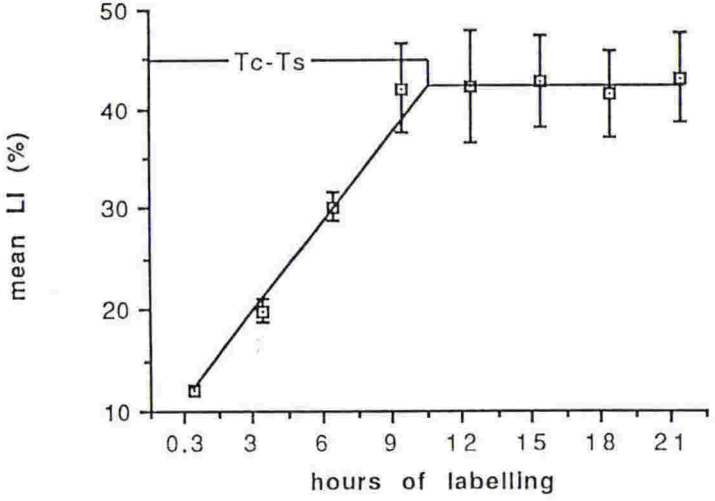

Fig.4c: Percent labelled apoptoses with continuous labelling.

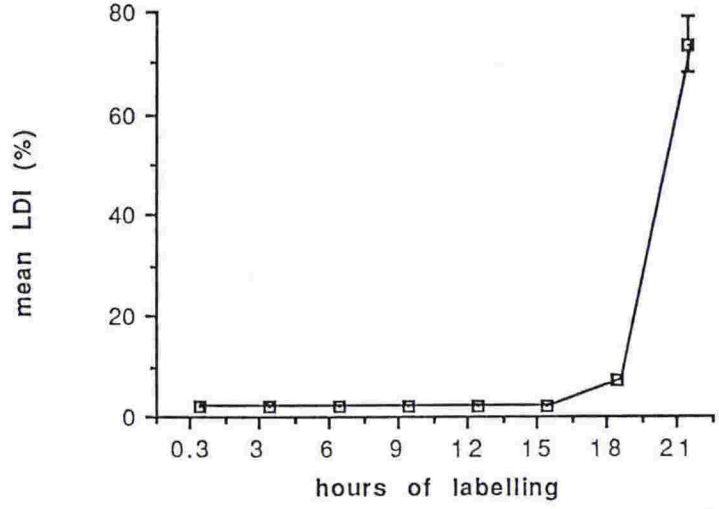

Fig.4d: Mean grain count with continuous labelling.

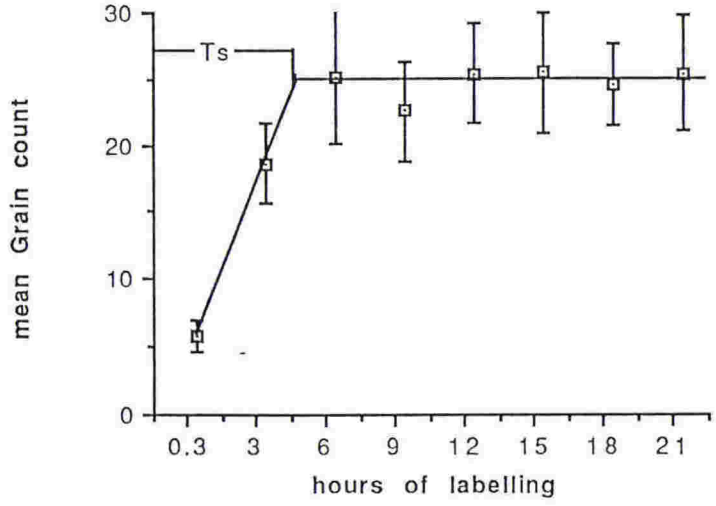

Fig.4a-d: Cell cycle times derived from continuous autoradiographic labelling of 5-day embryos.

2.1.4: DISCUSSION

The pulse labelled mitosis curve of Fig.3 departs from the idealised theoretical model in that labelling does not fall to zero in the trough of the curve. Data of this kind is referred to as 'Trough Limiting' (Steel 1977), and is due either to large variations in the length of either G1 and S or G2 and S, or by mistakenly scoring labelled cells as mitotic figures, the latter being quite likely, particularly when labelling is heavy (Steel 1977).

The appearance of labelled apoptotic bodies 18 hours after labelling commences demonstrates that at least some dead cells arise from the proliferating pool. Whether those cells have undergone mitosis before beginning to die is uncertain, as relative grain counts cannot be determined for apoptotic cells, because the number of apoptotic cells is estimated from the number of fragmented labelled apoptotic bodies. Obviously when lightly labelled cells are divided into bodies, not all of which contain nuclear material, and not all of which will be in section, it becomes improbable that all labelled cells will be recognised, so that undercounting is likely. This is exacerbated by the need to adjust for background labelling, for although grains over labelled bodies do tend to be noticeably bigger than background, this does not help where grains could have been contributed by a phagocytosing cell.

Because of this difficulty in accurately assessing apoptotic labelling indices, and the lack of longer term continuous labelling samples, it was not possible to ascertain whether all apoptotic cells would eventually have become labelled. However, that the labelled apoptotic population rose as high as 71% (Table 5) implies that a very large fraction indeed of the dying population was derived from the proliferating pool.

Note that the increase in number of pulse labelled apoptoses at 21 hours, versus 18, does not indicate that variation must exist in the timing to die, given that the length of S phase could account for the variation seen, combined with the likelihood that labelled bodies from 3 hours earlier would still remain histologically detectable, resulting in a cumulative effect.

Despite the above, if one is willing to assume that labelled cells die at a constant rate, dependent upon the rate of exiting mitosis for example, then one would expect that accumulation of labelled apoptotic bodies would follow a straight line relationship for as long as such bodies remain histologically detectable. If one is then foolhardy enough to extrapolate such a straight line relationship from the two points available to us, it estimates that the appearance of labelled dead cells began approximately 17.7 hours after labelling, and that 100% labelling of dead cells would be reached after a minimum of 22.5 hours (assuming all dead cells had been produced from the proliferating pool). While these estimates cannot be taken too seriously, they would help in the design of further labelling experiments to examine whether all dead cells had undergone synthesis prior to death.

Significant correlations were found between LI, DI and cell density using Spearmann's rank correlation coefficients. DI and cell density had already been found to be correlated (Section 1.1.3); however here LI was found to positively correlate to cell density where MI had not. This suggests that it is better to compare LI, rather than MI, to DI. This might be because the histological duration of DNA synthesis and apoptosis are more equivalent, allowing numerical similarity to be detected more easily. However MI is significantly correlated to LI, despite the difference in their

histological durations (although DI is more strongly correlated to LI than MI is). Both DI and LI are positively correlated to cell density, but they are negatively correlated to each other, showing that where LI is high PCD is low, and visa versa, as suggested in a number of other studies (Columbano et al 1985, Del Vecchio et al 1991, Ferguson and Anderson 1981, Forseberg 1967, Hinsull and Bellamy 1977, Poelmann 1980, Rotello et al 1991).

2.1.4.1: CELL CYCLE KINETICS.

The VBW was found not to be a homogeneously proliferating population, with more cell labelling in ectoderm and its proximal mesoderm than in other mesodermal regions, particularly mesoderm midway between ectoderm and endoderm. Despite this, calculations in the following cell kinetics section have been done for pooled tissue types. This is because of the difficulty of precisely identifying mesoderm subgroups, combined with the requirement for large sample counts to adjust for background in some grain count analyses. It should therefore be noted that the following kinetic analyses are for the VBW population as a whole, although some evidence does exist to suggest that regional variations are maintained over time, implying that the same proportions of cell gain and loss exist in different regions, given that similar distribution patterns of labelled cells per tissue type exist at stage 34 as was seen at stage 26.

Calculating the proportion of cells in each stage of the cell cycle depends upon the distribution of cells within that cycle, the two extremes being the Steady State distribution (approached when the growth fraction is low and cell loss occurs either in mitosis or in G0 after mitosis), and the more commonly assumed Exponential distribution (where the death rate and the proportion of cells in

each stage remain constant). In both these cases the Proliferation Fraction (Ip) is determined by the equation:

$$Ip = Nc/N = (Ns/N)/(Ns/Nc)$$

where Nc = the number of cycling cells, N = the total number of cells sampled, and Ns = the number of cells synthesising DNA (Steel 1968).

In the Steady State Ns/Nc is given by the ratio of time in synthesis to total cycle time (Lala 1972), so that for our 5-day results;

$$Ip = (Ns/N)/(Ts/Tc) = 0.11/0.3 = 0.36$$

In the exponential distribution Ns/Nc is given by the formula of Stanners and Till (1960):

$$Ns/Nc = (e^{Ts@}-1)e^{(TG2+Tm)@}$$

where @ = ln2/Tc, so that;

$$Ip = 0.11/0.276 = 0.4$$

Thus if the population is in Steady State one would expect an Ip of 36%, but if it is exponentially growing we would expect one of 40%, as was observed upon continuous labelling (Table 5, Fig.4b).

The 8-day embryo has a flash LI of 5.8%, and a MI of 0.34%, with a maximum LI of 22.7%.

If in steady state, one would therefore expect an Ip of 19.333%, in exponential growth, one of 21.333%. Thus the cell distribution still seems to fit the exponential growth model.

Maximal LI during continuous labelling tends to overestimate Ip, given that some labelled cells may move from a proliferating (P) to a nonproliferating (Q) state (Lala and Patt 1966). It is therefore noteworthy that at no time is maximal LI lower than 40% for the 5-day, and 22% for the 8-day embryo, even when combined with slightly sub-maximal indices from 9 hours labelling, and so the

assumption that the cycling population was exponentially distributed was felt to be justified. It is not unusual for a population experiencing cell loss, and with an Ip less than unity, to be exponentially growing (Lala and Patt 1966).

One can use the theoretical Ip to test whether cell cycle times have changed from day 5 to day 8, since:

$$Ns/N=[e@(Tm+T_{G2})(e^{@ts}-1)](Ip/N)$$

[adapted from Stanners and Till 1960]

so that, assuming an 8-day Ip of 21.33, we get an expected Ns/N of 5.8%, exactly the observed LI, implying that Tm, T_{G2}, and Ts are unchanged, as is Tc since this was required to calculate the Ip.

Given that Ip is less than unity, the cell production rate (Kp) of the population was calculated from the following equation (Wright 1981):

$$Kp = \ln(1+Ip)/Tc = \ln(1+0.4)/14 = 2.4\%/\text{hour}.$$

Kp is usually termed the cell birth rate, however it also contains an immigration component *in vivo*.

The potential doubling time (Tpd) for the 5-day population, assuming no cell loss, is given by:

$$Tpd = \ln 2/Kp = 28.8 \text{ hours (Steel 1968)}.$$

Given a Tc of 14hr. the birth rate (Kp) for the 8-day chick is 1.381%/hr, giving a potential doubling time of 50.192hr.

Both the graphical method of Okada (1967) and the eleven equations of Stanners and Till (1960) were employed to calculate the proportion of cells in any cycle stage.

Unfortunately both of these methods presuppose a growth fraction of unity (Watanabe and Okada 1967a). For example, according to Stanners and Till (1960),

$$Tm = (1/@)\ln(Nm/N+1)$$

so that with our data Tm = 0.1168 hours, underestimating that derived from Fig.s 3 and 4 (Section 2.1.3) by approximately 2.5. Thus a correction factor (Ip/N) for low Ip needs to be introduced:

$$Tm = (1/@)\ln[(Ip/N)Nm/N+1]$$

giving a Tm approximating 0.3hr, as observed.

The graphical method (Okada 1967) can be simply adapted either by substituting the %Ip for 100% on the ordinate (Fig.5), giving results equivalent to those calculated via Stanners and Till, or by multiplying MI and LI by the correction factor before use, allowing the proportion of cycling cells in any stage to be determined, rather than the proportion of the total population (Fig.6).

The proportion of cells in each cell-cycle stage at 5 and 8-days were as follows:

	5-DAY(%)	8-DAY(%)		BOTH[2] (%)
Ns/N	10.887	5.806	Ns/Nc	27.217
Nm/N	0.599	0.319	Nm/Nc	1.496
N_{G1}/N	22.014	11.740	N_{G1}/Nc	55.034
N_{G2}/N	6.501	3.467	N_{G2}/Nc	16.250

Because the ability to detect apoptosis histologically is affected by the rate of phagocytic degradation of apoptotic bodies as well as the number of apoptotic cells, the cell loss rate due to apoptosis (K_{la}) should be determined from both these factors (Bursch et al 1990b). Thus:

$$K_{la} = Nac/d.$$

where Nac=number of apoptotic cells, and d=their histological half-life.

---

[2] (N/Nc will be the same for each age as the exponential distribution is assumed for both).

Therefore, if we substitute the histological half-life of apoptotic fragments derived from section 2.2.3, we can determine the K_{la} for each embryonic stage from the DI given in Table 1 (see Table 6). This does assume that the histological half-life remains constant at all ages. While it is conceivable that at extreme levels of apoptosis 'phagocytic overload' might occur, with a subsequent prolongation of d, it was considered that the marked increase in numbers of phagocytes during such events [long used as a marker for the existence of PCD (Bowen 1981)], would be capable of handling the increase in apoptotic bodies without an increase in d. It is notable that nearly all apoptotic bodies seen were within phagocytes regardless as to the extent of PCD, suggesting that the increase in phagocyte numbers was capable of coping with the apoptotic bodies present. Since d was determined from 7-day embryos (section 2.2.2) when apoptosis is maximal, any error due to phagocytic overload will tend to underestimate K_{la}, not overestimate it.

The cell birth and apoptotic loss rates are proportional to the rate and quantity of mitosis and death histologically detectable, which is dependent upon their relative cell or fragment size and histological duration. Therefore;

$Kp/K_{la} = MI/DI[Ta(k+2ra)/Tm(k+2rm)]$ (Sarraf and Bowen 1986)

where Kp=birth rate, K_{la}=loss rate due to apoptosis, Ta=histological duration of apoptotic bodies (**not** their half-life), k=section thickness, and r=radius of apoptotic (a) or mitotic (m) nuclei.

Thus, using data from the histology section, we can determine Ta for 5 and 8 day embryos, where Kp is known, using the Kla calculated previously:

for the 5-day embryo:

$2.4/0.044=0.58/0.17\{Ta[3+2(2.744)]/0.3[3+2(4.35)]\}$

so that Ta=6.6112hr.

for the 8-day embryo:

$1.381/2.09=0.347/8.099\{Ta[3+2(2.774)]/0.3[3+2(4.35)]\}$

so that Ta=6.6118hr, indicating that the duration of apoptosis is 6.61hrs for both ages, approximating the 6.8hrs estimated experimentally (for the 7-day embryo) in section 2.2.3.

Given this similarity in Tc calculated for 5-day (low death rate, pre death-wave), 8-day (high death rate, post death-wave) and 7-day (high death rate, peak death wave) embryos, it seems unlikely Tc would have varied between these sampled times. If we therefore assume the duration of apoptosis to be a constant, we can use the same formula to determine Kp for the other ages (see Table 6).

Since histological examination revealed signs of neither necrosis nor non-apoptotic forms of PCD, and given that the region is undergoing extensive cell immigration, with no apparent sign of cell emigration detected here or in similar studies (Chen 1952a, Fell 1939), it seems reasonable to assume that the rate of cell loss by apoptosis equals the overall cell loss rate (K_L).

The actual doubling time of the population (Td) differs from Tpd in that it includes reference to the cell loss rate and the immigration rate. Because of this:

$K_L=\ln 2/Tpd-\ln 2/Td$ (Wright 1984).

Since we know K_L, we can rearrange this equation to allow Td to be determined:

$Td=\ln 2/(\ln 2/Tpd-K_L)$;

and as $Tpd=\ln 2/Kp$ (Steel 1968):

$Td=\ln 2/(Kp-K_L)$.

Thus we can calculate theoretical values for Td and Tpd. Furthermore, since Td represents Tpd minus cell loss and plus further cell immigration, we can use Td, Tpd, and K_{Ia} to approximate Ir, the increase in immigration rate (see Table 6).

Unfortunately, it is not possible to use the cell rates in Table 6 to confidently determine changes in the VBW cell population from one developmental stage to another, given the possibility that diurnal or other circadian fluctuations exist (Laerum and Smaaland 1989, Bursch et al 1990b), and since each Kp and K_L calculation incorporates previous immigration and loss rates. However, given the increase in cell density (cell number per area) along with the increase in area itself (regardless of the units each is expressed in) we can calculate the actual increase in cell number (K_G) for the entire VBW. That is, the ratio of density from times a and b, times the ratio of VBW areas for the same times, will give the increase in cells over that period. Therefore:

$$K_{Gt(b-a)}=[(Db.Ab)/(Da.Aa)]-1$$

where t(b-a)=time from sample a to sample b, D=cell density at the sample time, and A=tissue area at the sample time.

Since density and area are known for each stage (see Tables 1 and 2), and each stage can be represented by an approximate time period with the exception of stage 35, K_G can be determined for any group of stages up to stage 35 (see Table 7).

TABLE 6: CALCULATED KINETIC PARAMETERS FOR EACH AGE OF EMBRYO.

AGE (days)	Kp (%/hr)	K_L (%/hr)	Tpd (hr)	Td (hr)	IR (%/hr)
5	2.4	0.044	28.881	29.421	0.049
5.5	3.768	0.068	18.396	18.735	0.072
6	4.938	0.520	14.0370	14.1860	0.054
6.5	1.445	0.696	47.969	92.543	1.142
7	1.319	0.343	52.551	71.019	0.528
7.5	0.969	2.889	71.532	-45.995	1.713
8	1.381	2.090	50.192	-97.764	0.610
8-9	1.113	0.554	62.277	123.997	1.171

TABLE 7: VBW GROWTH OVER DEVELOPMENTAL TIME PERIODS.

t(b-a) in days	K_G (%/hr)
5.5-5	0.370
6-5.5	0.484
6.5-6	1.671
7-6.5	2.795
7.5-7	4.116
8-7.5	-1.325

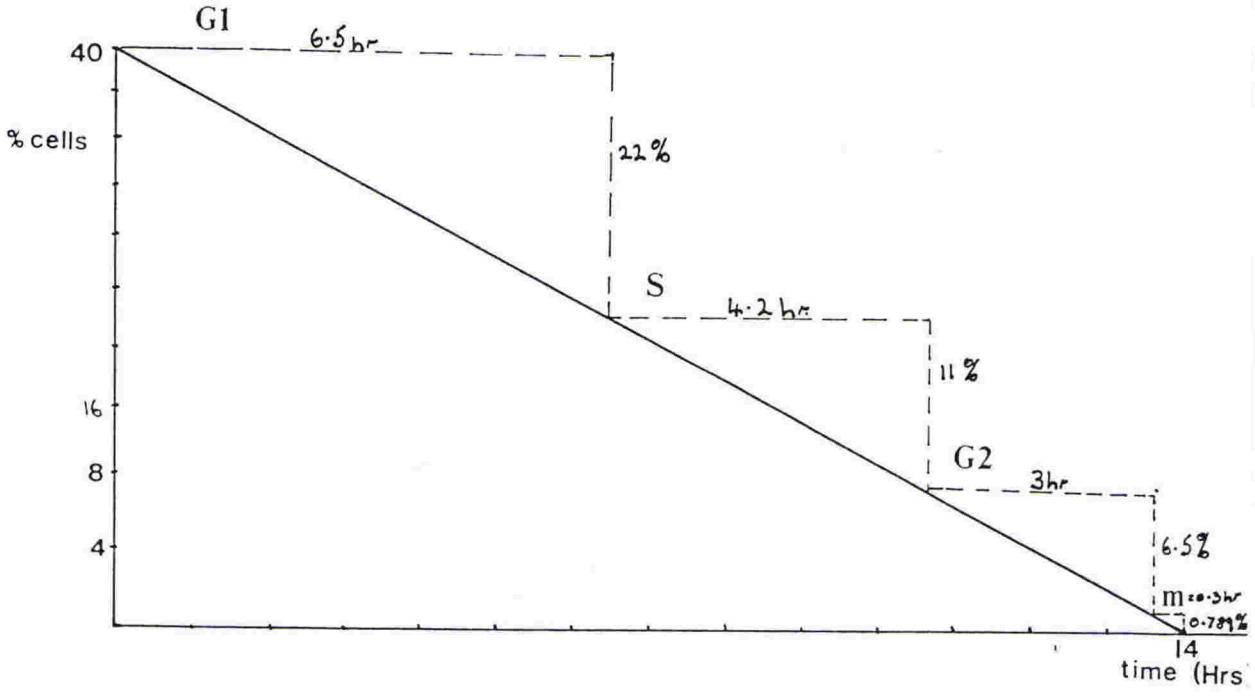

Fig.5: Cell cycle times and distribution of cells within stages, (Ip=40%).

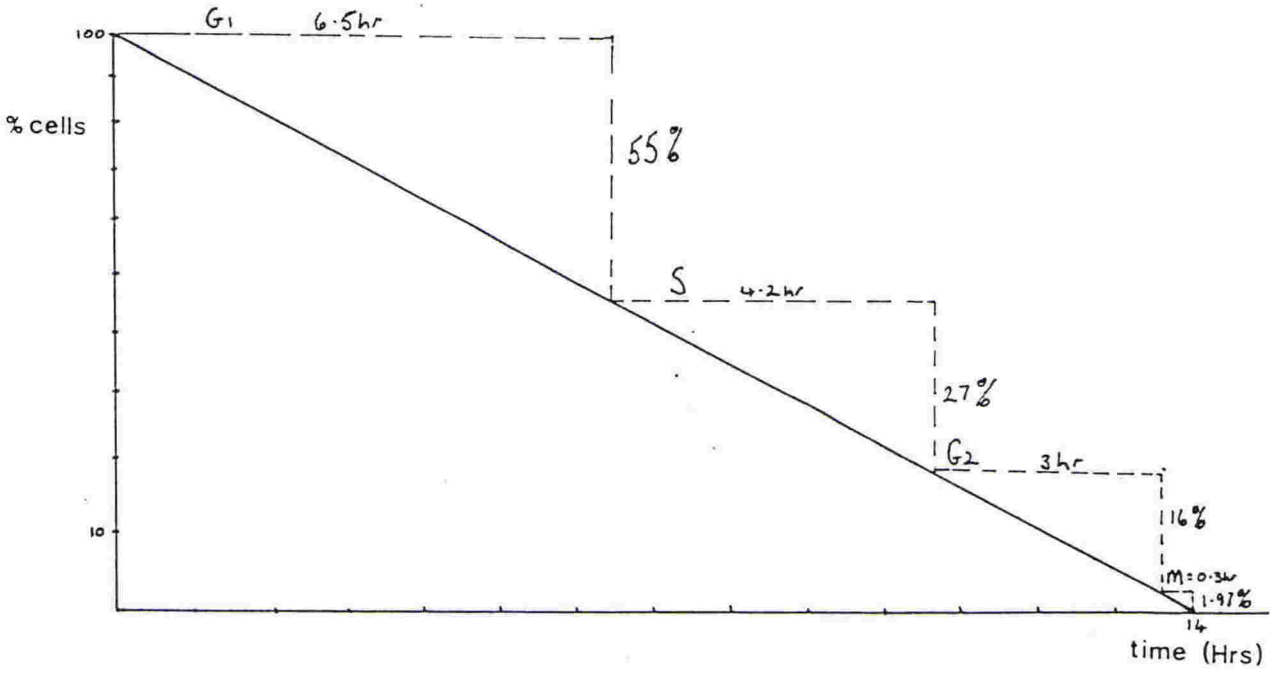

Fig.6: Cell cycle times and distribution of cells within stages, (Ip adjusted to 100%).

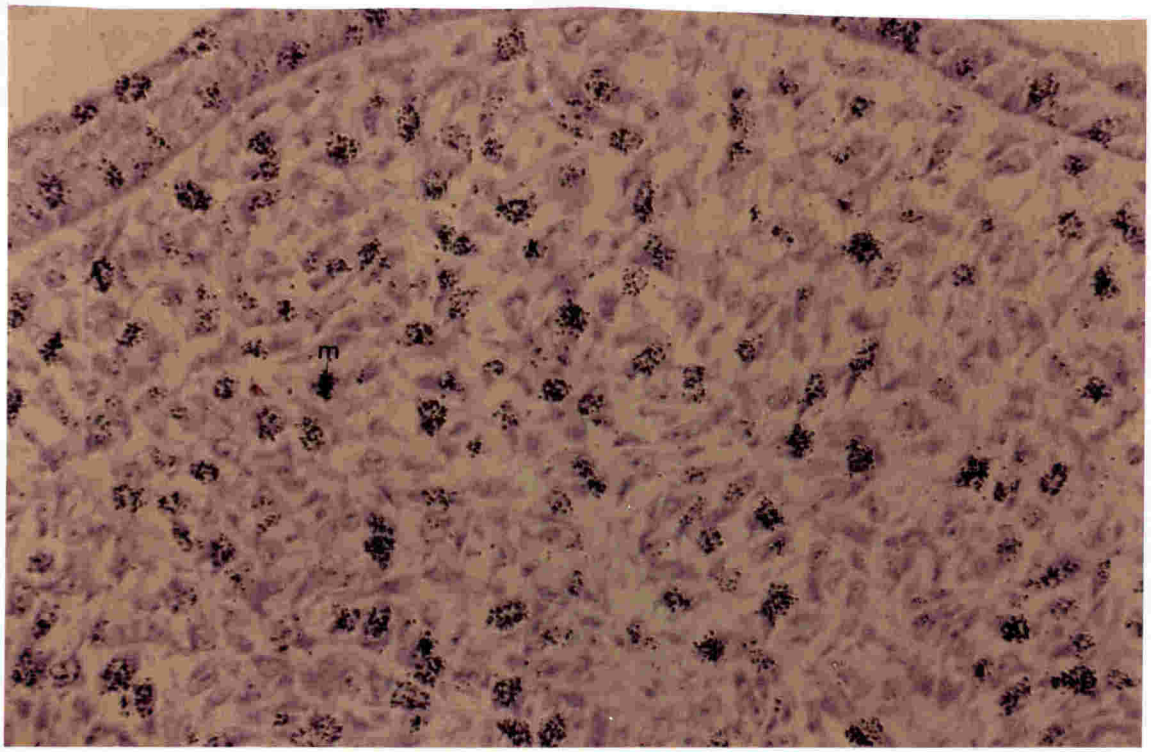

Plate 9a: 6 hours continuous exposure to tritiated thymidine (x 50).

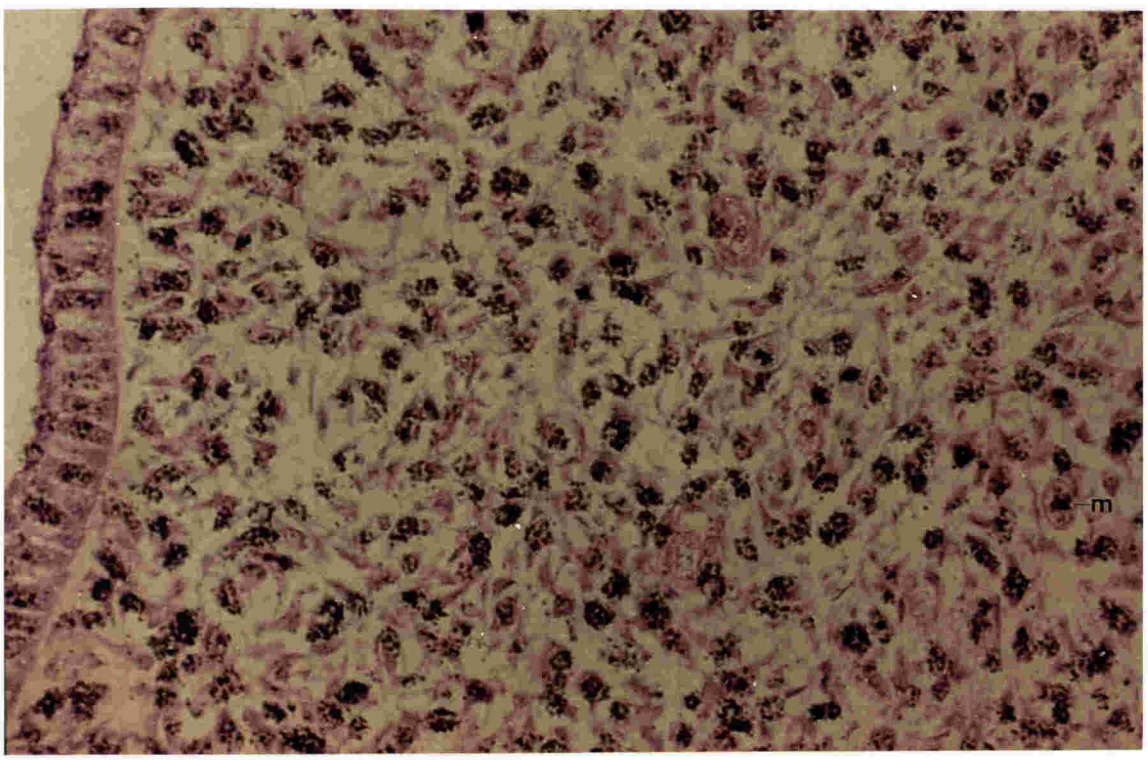

Plate 9b: 18 hours continuous exposure to tritiated thymidine (x 40).

Plates 9a and 9b: Autoradiographically labelled VBW sections after continuous administration of tritiated thymidine, stained with Methyl Blue/Basic Fuchsin. Note limited background labelling.
m= labelled mitotic figure.

2.2: CELL CYCLE BLOCKING

2.2.1: INTRODUCTION

If PCD is related to the cell cycle, then altering the cell cycle should produce alterations in the rate or quantity of PCD. For example, if cells receive the signal to die only in G2, but are blocked in the cell cycle prior to G2, then released in a semi-synchronous wave, one would expect to see a delayed synchronous wave of cell death occurring after the time required to reach the signal and respond, provided that the cell cycle blockage does not prevent either the signals transmission or the death response mechanism itself.

Furthermore, if blockading the cell cycle results in inhibition of PCD, we can use the speed of disappearance of dead cells in section to determine their histological half-life (Bursch et al 1990b). This can then be used, by manipulation of the formula of Sarraf and Bowen (1986), to determine the cell loss rate (due to PCD) in our embryos.

With the above in mind, it was decided to block the cell cycle prior to, and after, the signal to die had occurred, and to release some cells in a semi-synchronous wave, while monitoring their progress.

Given that cells could enter the G1 proliferating compartment from G0, cells were blocked at the G1/S boundary, so that such cells would not affect results. Excess thymidine was selected as the blocking agent, as it prevents DNA synthesis (by inhibiting Deoxycytidine triphosphate synthesis) with limited effect on protein and RNA synthesis, very limited toxicity, and good parasynchronisation of cells (Xeros 1962).

2.2.2: METHODS

Excess thymidine was administered to embryos in ovo, in a range of concentrations (37.5uM-10mM, in 0.1ml warm sterile PBS). Maximal inhibition of mitosis, with no teratological effects, was obtained with 2-3mM thymidine, thus this concentration was used hereafter, applied aseptically through a window in the egg, being reapplied every 2 hours. All embryos were aged *in ovo* before application.

Embryos were incubated *in ovo* for 5 or 5.5 days, treated with thymidine for 16 hours (greater than the Tc), rinsed with sterile PBS, left a further 2 hours to fully metabolise residual thymidine, then removed for fixation and sectioning every 4 hours for the next 28 hours.

A further series of embryos were incubated for 7 days, then exposed to excess thymidine for up to 32 hours, with some being sampled every 4 hours.

Control eggs were treated with warm sterile PBS (0.1ml) without thymidine.

2.2.3: RESULTS

Embryos continuously exposed to excess thymidine (see Table 8, Fig.7) showed levels of mitosis similar to controls after 4 hours (p ranging from 0.655 to 0.75). By 8 hours MI had become significantly lower than that of both control and untreated embryos, remaining so thereafter (p ranging from 0.001 to 0.0055). The number of dying cells, however, remained at control/untreated levels for the first 8 hours (p from 0.475 to 0.165), declining sharply by 12 hours exposure, remaining significantly lower than controls from then on ($p<0.0001$ in all cases).

PCD histologically disappears between 8 and 12 hours continuous exposure so that apoptotic bodies (the histological sign of dead cells) must have been phagocytically processed to an undetectable state by this time. Extrapolating the linear function from the percentage of cell fragments to total cells (Sarraf and Bowen 1986) gives a time to complete disappearance of 6.8hr. Since histological half-life equals the time to complete disappearance times 1.8 (Lewis 1975), here histological half-life was 3.8hr.

Embryos blocked at 5 days age, then released (see Table 9, Fig.8a,b), showed levels of Mitosis and PCD not significantly different from controls or untreated embryos, for 12 hours after release (p ranging from 0.4022 to 0.3133). After 16 hours release from block MI dropped below control/untreated levels ($p=0.001$ or less), while DI rose above these levels ($p= 0.0221$ to 0.001). Both MI and DI dropped to control levels by 18 hours, maintaining them hereafter (p ranging from 0.812 to 0.655).

Embryos blocked at 5.5 days, then released (see Table 10, Fig.9a,b) showed control/untreated levels of mitoses for the first 8 hours (p ranging from 0.057 to 0.156) and of apoptosis for the first

12 hours (p ranging from 0.01 down). After 12 hours MI peaks at a level higher than control/untreated embryos ($p=0.001$ or less), then drops to be significantly lower thereafter ($p=0.007$ or less). The DI reaches and maintains levels higher than controls or untreated 7-day embryos ($p<0.0001$ in all cases) after 16 hours from release, when MI has lowered. Interestingly, DI at these times is not significantly different to levels seen in untreated 7.5-day embryos in 8 of 12 cases (p ranging from 0.059 to 0.642) once section order is taken into account. Where significant differences occur experimental counts are lower in 2 cases ($p= 0.005$, 0.0125) and higher in 1 ($p= 0.001$).

At no time, in any sample, were control levels of mitosis or apoptosis significantly different from those of untreated embryos of the same age ($p=0.895$ to 0.0967).

TABLE 8: MEAN CELL COUNTS AFTER CELL CYCLE BLOCKADE BY CONTINUOUS EXPOSURE TO EXCESS THYMIDINE.

Hours in block	Age (days)	mean DI (%)	+/- S.D.	mean MI (%)	+/- S.D.	mean Control DI (%)	+/- S.D.	mean Control MI (%)	+/- S.D.	N
4	7	3.107	0.327	0.510	0.086	2.309	0.329	0.416	0.019	3
8	7.5	15.226	0.435	0.096	0.013	14.569	1.045	0.333	0.038	3
12	8	0.381	0.194	0.001	0.002	7.995	0.107	0.394	0.027	3
16	8	0.243	0.295	0.051	0.014	8.130	0.621	0.427	0.102	3
20	8-9	0.086	0.119	0.076	0.009	2.136	0.549	0.376	0.140	3
24	8-9	0.104	0.113	0.018	0.025	3.827	0.724	0.298	0.037	3
28	8-9	0.793	0.504	0.077	0.021	4.019	0.212	0.197	0.039	2
32	8-9	0.015	0.041	0.001	0.007	3.857	1.322	0.188	0.139	3

where S.D.=standard deviation, N=number of samples.

TABLE 9: MEAN CELL COUNTS AFTER RELEASE OF 5-DAY EMBRYOS FROM CELL CYCLE BLOCKADE.

Hours after block release	Age (days)	mean DI (%)	+/- S.D.	mean MI (%)	+/- S.D.	mean control DI (%)	+/- S.D.	mean control MI (%)	+/- S.D.	N
4	5.5	0.323	0.052	1.285	0.476	0.260	0.412	0.991	0.019	3
8	5.5	0.010	0.002	0.001	0.031	0.179	0.103	0.794	0.113	3
12	6	0.012	0.002	0.877	0.069	0.198	0.091	1.095	0.319	3
16	6	1.549	0.104	0.020	0.004	0.156	0.025	1.314	0.112	3
20	6	0.219	0.038	2.637	0.421	0.211	0.014	1.089	0.174	3
24	6	0.273	0.044	1.098	0.168	0.316	0.117	0.988	1.270	3
28	6.5	1.842	0.095	0.367	0.027	2.696	0.571	0.350	0.563	3

where S.D.=standard deviation of means, N=sample number.

TABLE 10: MEAN CELL COUNTS AFTER RELEASE OF BLOCKED 5.5-DAY EMBRYOS.

Hours after block removed	Age (days)	mean DI (%)	+/- S.D.	mean MI (%)	+/- S.D.	Mean control DI (%)	+/- S.D.	mean control MI (%)	+/- S.D.
4	6	0.001	0.040	2.053	0.162	0.236	0.025	1.266	0.539
8	6	0.011	0.062	2.174	1.537	0.194	0.101	1.330	0.577
12	6.5	0.301	0.132	3.360	0.488	2.742	0.886	0.487	0.215
16	6.5	14.924	2.379	0.001	0.001	2.467	0.303	0.299	0.131
20	7	21.720	6.584	0.000	0.002	2.522	0.316	0.882	0.451
24	7	13.597	4.388	0.037	0.042	2.779	0.655	0.338	0.056
28	7	20.667	3.591	0.073	0.005	2.010	0.325	0.448	0.033

where S.D.= the standard deviation of the means, number of samples=3.

Fig.7a: Mean percent MI vs. time of continued exposure to excess thymidine.

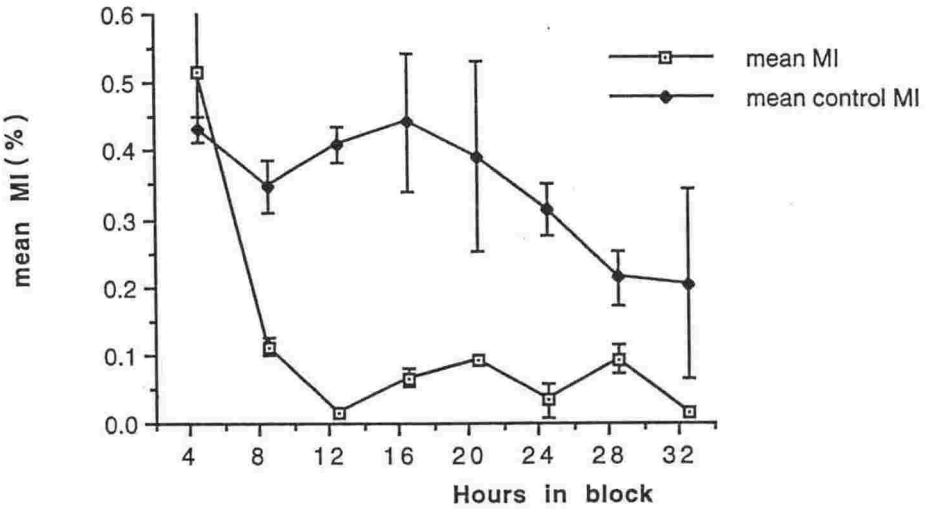

Fig.7b: Mean percent DI vs. time of continued exposure to excess thymidine.

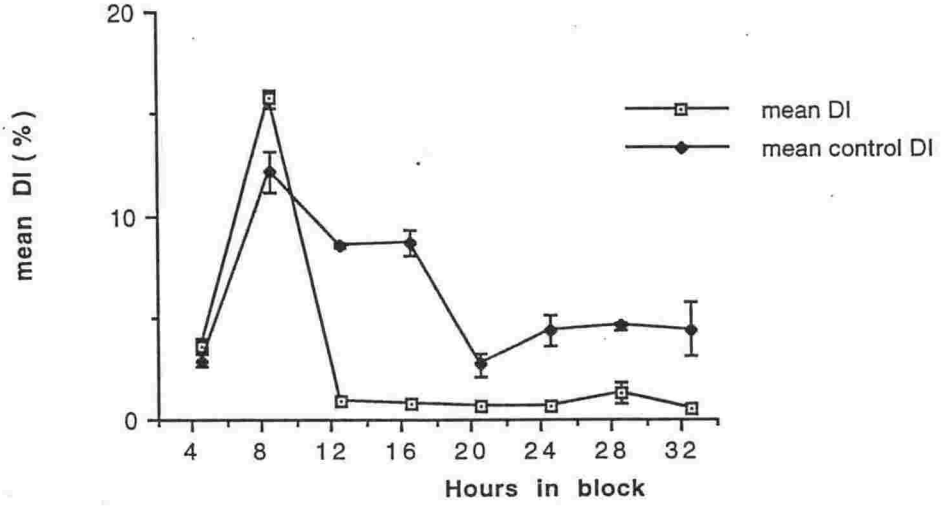

Fig. 7a,b: Cell counts after cell cycle blockade with excess thymidine.

Fig.8a: Mean percent MI vs. time after release from cell cycle blockade.

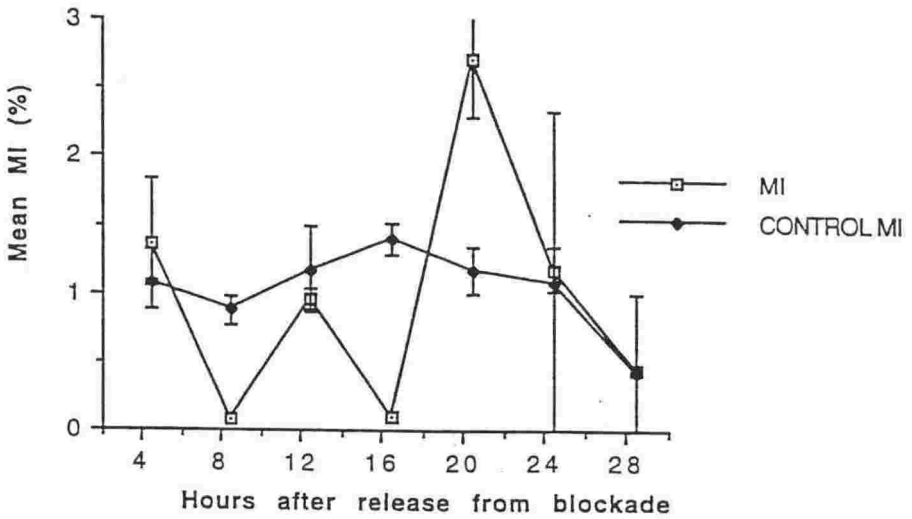

Fig.8a: MI versus time after release from blockade.

Fig.8b: Mean percent DI vs. time after release from cell cycle blockade.

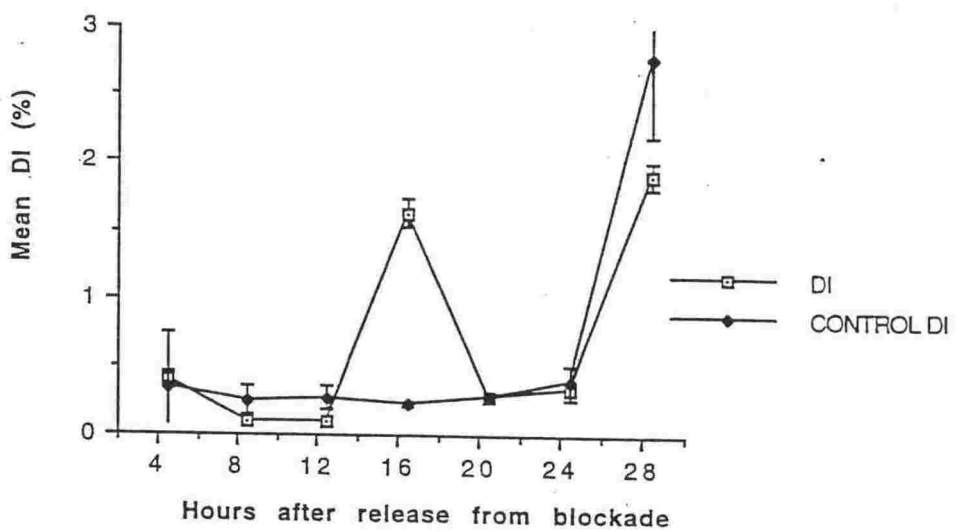

Fig.8b: DI versus time after release from blockade.

Fig.8a,b: Cell counts for 5-day embryos blocked in the cell cycle, then released.

Control= counts for embryos of the same age without blockade.

Fig.9a: Mean percent MI vs. time after release from cell cycle block.

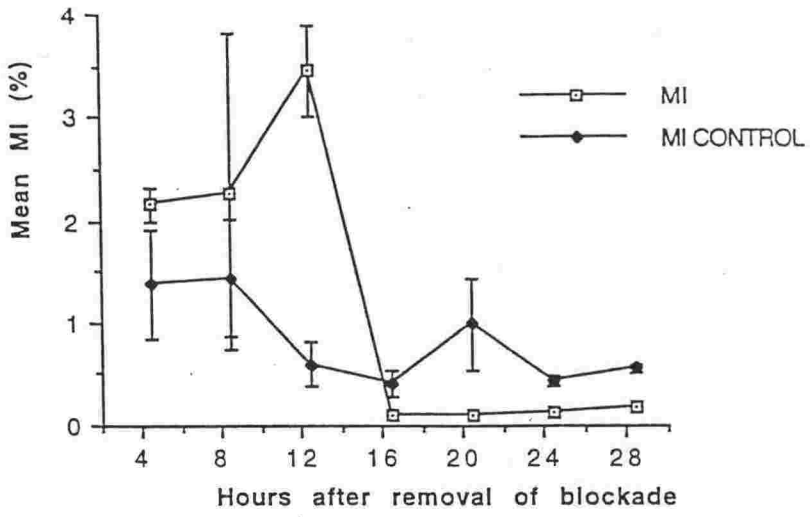

Fig.9a: MI versus time after release from blockade.

Fig.9b: Mean percent DI vs. time after release from cell cycle block.

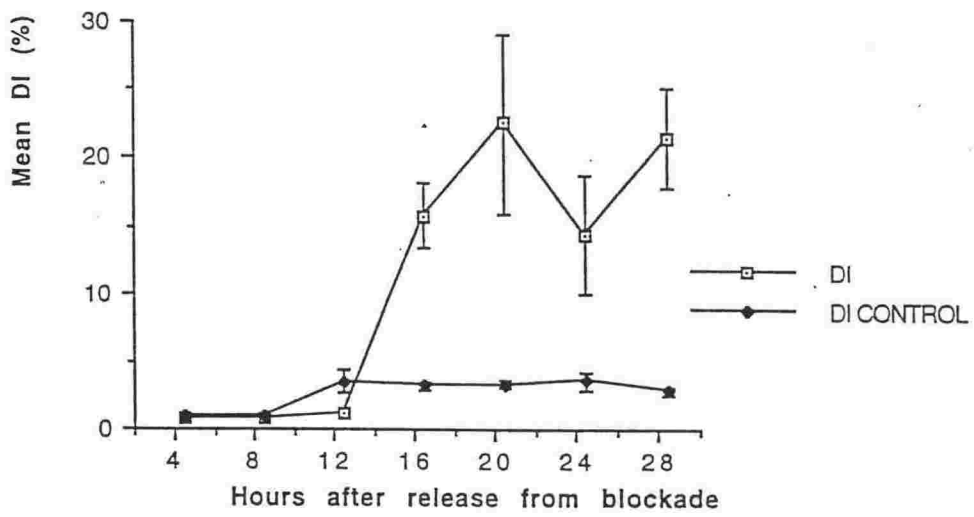

Fig9b: DI versus time after release from blockade.

Fig.9a,b: Cell counts for 5.5-day embryos blocked in the cell cycle, then released.

Control= counts from embryos of the same age, without blockade.

2.2.4: DISCUSSION

It is immediately apparent from these results that treating embryos with excess thymidine affects the apoptotic index as well as cell cycling.

Necrosis, which has been known to affect cells prevented from expressing PCD in some other situations (Wyllie et al 1980), was not detected histologically.

In all cases release from the cell cycle blockade seems to have occurred well within the first 4hrs without thymidine. This may indicate the efficiency of the saline washing used here, or suggest that Tone et al's (1983) estimate of 4hrs availability time for autoradiographic amounts of thymidine applies to excessive amounts as well. Indeed, given that $Ts+TG_2=7.2hr$, mitoses can only appear by the first sample period (a maximum of 6hrs after block removal) if they have escaped the blockade, or if synchronising cells at the G1/S boundary has allowed them to prepare for S or G2 while blocked, allowing faster progression once released. Interestingly Xeros (1962) also obtained a synchronised burst of mitoses only 4 hours after release from thymidine blockade, suggesting that fast resumption of cell cycling is not uncommon.

In both the 5 and 5.5 day chick, synchronisation of the cycling cell population results in a wave of mitoses, followed by a wave of apoptoses, with the latter featuring a contemporaneous decline in MI. This suggests that apoptosis may be a post-mitotic event. It is conceivable, however, that an increased DI follows an increased MI simply because apoptosis takes longer to become histologically detectable, rather than that the two events are sequential; however

this would also require that the contemporaneous decline in MI seen with the increase in DI be coincidental.

After release from blockade, both ages of embryo show a precocious peak in DI. For example, in the 5.5-day embryo, Death Indices after release from blockade seem more closely related to those seen in the 7.5 -day embryo than to 6.5 or 7 day embryos, as if cells had differentiated to die prematurely. It could be that blockaded cells are unable to express the death program, perhaps because of reduced protein and mRNA synthesis, but are able to prepare to do so upon release, resulting in a synchronised DI that does not occur *in vivo*. Synchronisation of cells in blockade might also lead to a synchronised susceptibility to any signal to die given before the 7-day period.

That cells were only semi-synchronised by the thymidine blockade was demonstrated by the high, but not significantly so, MI seen prior to the 'synchronous wave' seen after 12 hours.

Continuous blockading of the cell cycle in G1 leads to a sharp decline in MI between 4 to 8 hours later, as one would expect with a combined Ts and TG_2 of 7.2 hours, as it would take that long for cells already beyond the blockade point to cycle past mitosis. Apoptosis also declines however, between 8 and 12 hours after the block is applied. This demonstrates either that apoptosis is affected by cell cycle disruption (with an approximate 4 hour lag after MI is affected), or that excess thymidine incorporation prevents apoptosis directly, perhaps by preventing the production of some necessary protein, as has been proposed for excess-BrDU induced inhibition of PCD (Goz 1978, Tone et al 1983).

An histological half-life of 3.8hr is reminiscent of the 3.2hr determined for the toad eye by Hughes (1961), but a wide range of

times have been suggested in the few other studies to look at this, from 1.4hr (Flanagan 1969) to 10hr (Lewis 1975) or even 18-plus hours (Bursch et al 1990b, Ferguson and Anderson 1981, Sarraf and Bowen 1986), although half-lives for apoptotic bodies of under 9 hours are more common in light microscopy (Wyllie 1987, Wyllie et al 1980).

2.3: TERATOGENIC ALTERATIONS TO DEVELOPMENT.

2.3.1: INTRODUCTION

5-Bromo-2-Deoxyuridine (BrDU) is a thymidine analog, long known to have teratogenic, mutagenic and oncogenic effects (Goz 1978, Primmett et al 1989). More interestingly, perhaps, BrDU has an anti-differentiative effect, at levels well below cytotoxicity. Thus, if incorporated into the DNA at a specific time, it will prevent a specific future differentiative event, or reverse a current one (Goz 1978). This effect might be due to alteration of the transcriptional pattern (Tone et al 1983), possibly by BrDU increasing the binding affinity of regulatory DNA to proteins (Goz 1978). This latter hypothesis is interesting in that BrDU may preferentially incorporate into middle repetitive regions of DNA (Goz 1978), and it has been long suggested that these might have a regulatory role in transcription, but with little real evidence (Dowsett pers. com.).

BrDU's anti-differentiative effect is known to inhibit PCD in the PNZ (Beirne et al 1986), and in the interdigital spaces (Tone et al 1983, 1988), of the chick limb. However it has been noted in the latter case that BrDU might inhibit cell death simply by slowing the cell cycle (Wise and Scott 1982). Indeed, at high levels BrDU serves to block the cell cycle in S-phase (Primmett et al 1989), by inhibiting deoxycytidine synthesis, even more strongly than does excess thymidine (Goz 1978). Any effect on cell death due to slowing of the cell cycle would, of course, have interesting implications regarding the linking of PCD to the cell cycle. It also sounds a cautionary note in that BrDU is increasingly being used in immunohistology, to determine cell cycle kinetics without the

difficulties inherent in autoradiography (for example see Morstyn et al 1986). Such studies should bear in mind that possible alterations in cell cycle times and differentiative events may be associated with this substance.

Because BrDU inhibits PCD in other situations in the chick, and because it allows a specific differentiative S-phase to be linked with later death (Tone et al 1988), it was decided to study its effects on VBL degeneration.

BrDU is not the only teratogen to inhibit PCD, however it does seem unique in directly inhibiting the differentiation of the affected cells themselves. Janus B Green, for instance, prevents PCD when causing limb polydactyly in the embryonic chick (Menkes and Deleanu 1964), however this is due to its disruption of local tissue architecture. Janus B Green binds to the mitochondrial lipoproteins, affecting cell respiration, in the above case causing premature death of the apical ectodermal ridge (AER) which will later be required to induce normal mesodermal death (Fernandez-Terran and Hurle 1984, Hinchliffe and Thorogood 1974).

The effects of Janus B Green on VBL degeneration were also studied, for comparison with BrDU, and to see if ectodermal ridge formation would also be affected in the VBW, and whether this in turn would affect PCD, implying that an interaction between ectoderm and mesoderm might exist like that seen in the chick limb-bud.

2.3.2: METHODS

White leghorn chick embryos were incubated for various times, then aged through a window cut in the shell, under sterile conditions.

For the Janus B Green experiment, a total dosage of 10µg of sterile Janus B Green (Gurr's), in 0.1ml warm PBS, was injected through the chorioallantoic membrane (CAM) of embryos aged from 4 (stage 25) to 6.5 (stage 30) days old (Fernandez-Teran and Hurle 1984).

For the BrdU experiment, various dosages of sterile 5-bromo-2-deoxyuridine (Sigma) dissolved in warm PBS were injected through the CAM, 0.1ml/embryo, followed 1 hour later by an injection of sterile thymidine (Sigma) at 8 times the molar concentration. Dosages were adjusted to the average body weight of embryos at the given stage (Tone et al 1988), i.e. at stage 25, 0.1µM; stage 26, 0.172µM; stage 27, 0.375µM; stage 28, 0.655µM; stage 29, 0.98µM; stage 30, 1.375µM; stage 31, 1.74µM.

Controls were injected with equivalent moles of thymidine in PBS, and with PBS alone, in similar volumes.

All eggs were resealed and observed during incubation until stage 34, when they were removed either for fixation in FAA, followed by methacrylate imbedding, sectioning and cell counting, or for vital staining for macrophage activity with a solution of 1:10,000 Nile blue sulphate (Grubler's), or neutral red, in Ringer's saline (Fallon and Simandl 1978, Saunders et al 1962).

2.3.3: RESULTS

Morphological effects:

Treatment with excess BrDU resulted in severe, generalised developmental deformities, particularly when given at younger ages (see Plate 10 and Table 11).

Janus B Green had a similar, but lesser, effect, except when given at day 5 of incubation. It produced chest deformities when given at 5.5 and 6 days age, with abnormal separation between epithelial and mesodermal layers being seen histologically (Plate 11).

BrDU treatment (not in excess) produced chest abnormalities when given at 4.5 and 6 days incubation, however no disruption of epithelial-mesodermal connections were seen.

Types of chest deformity included shortening of the VBW (Plate 12) increased torsion of the upper body (Plate 13), and formation of two shelves of unfused tissue where the VBL should be (Plate 12).

Cell counts:

Injecting BrDU at day 4 or day 4.5 of incubation decreased the amount of apoptosis relative to control/untreated embryos (p ranging from 0.004 downwards) and raised the amount of mitosis (p=0.0175 to 0.0373). When given at 6 days incubation BrDU increased the amount of apoptosis (p=0.0071 or less) and decreased the amount of mitoses (p=0.0175 to 0.047) relative to control/untreated levels (see Table 12).

Janus B Green application (see Table 13) at days 5.5 and 6 also decreased the DI (5.5 day p=0.0088 or less; 6 day p=0.0283 to 0.004), but did not affect the MI (p=0.2608 to 0.6595), while BrDU

administered at days 5 or 5.5 affected neither (p=0.7897 to 0.1055).

Cell counts were affected in the above ways even when no morphological effect of drug treatment had been noticed in the unsectioned embryo's VBW, and were not significantly different from untreated embryos or controls (of similar order ranges) at other times (p ranging from 0.0818 to 0.7973).

Controls:

Thymidine controls appeared morphologically normal, and their mitotic and apoptotic indices did not differ significantly from those of untreated embryos (p ranging from 0.5987 to 0.0892). The one exception to the above had died at the time of treatment, suggesting damage due to the injection had occurred.

Vital staining failed to detect areas of known cell death.

TABLE 11: MORPHOLOGY OF TERATOGEN TREATED EMBRYOS.

Treatment regime	Age treated (days)					
	4	4.5	5	5.5	6	6.5
BrDU	N(9)	3L/7N (10)	N(10)	N(10)	8C/3N (11)	1T/9N (10)
Janus B Green	3G/6N (9)	5F/5N (10)	N(10)	8C/2N (10)	5L/7C/2N (8)	5L/5N (10)
Excess BrDU	D(10)	3C/7D (10)	3D/6G (9)	8C&L (10)	5L/3C/3N (10)	4C&L/4N (8)

where N=normal morphology, L=limb deformities, C=chest deformities, F=facial deformities, and D=dead from G=general body deformities. (No.)=total number of embryos sampled.

TABLE 12: MEAN CELL COUNTS AFTER EXPOSURE TO EXCESS BrDU AT VARIOUS AGES.

Age treated (days)	Age sampled (days)	Mean DI (%)	+/- S.D.	Mean MI (%)	+/- S.D.	Mean control DI (%)	+/- S.D.	Mean control MI (%)	+/- S.D.
4	8	1.288	0.805	0.396	0.173	10.837	1.986	0.433	0.088
4.5	8	3.376	2.144	0.250	0.103	8.991	0.672	0.339	0.047
5	8	8.657	1.988	0.164	0.132	7.999	1.367	0.237	0.054
5.5	8	5.615	1.239	0.062	0.233	8.367	1.295	0.118	0.059
6	8	15.447	3.764	0.813	0.303	9.989	2.870	1.255	0.068
6.5	8	8.674	1.392	0.165	0.054	8.886	0.935	0.384	0.100

where S.D.=standard deviation of the means, control=thymidine treated, number of samples=6.

TABLE 13: EFFECT OF JANUS B GREEN TREATMENT ON MEAN CELL COUNTS.

Age treated (days)	Age sampled (days)	Mean DI (%)	+/- S.D.	Mean MI (%)	+/- S.D.	N
4	8	5.652	0.981	0.011	0.025	5
4.5	8	3.376	1.045	0.253	0.059	6
5	8	9.849	1.992	0.160	0.127	5
5.5	8	0.362	0.113	0.181	0.056	6
6	8	8.400	1.832	0.389	0.160	6
6.5	8	9.971	1.080	1.861	0.450	6

where S.D.= the standard deviation of the means, N= number of embryos sampled.

2.3.4: DISCUSSION

The failure of vital staining to demonstrate cell death is puzzling, as it has often been used for this purpose (Bowen 1984), particularly since the work of Saunders et al (1962). However some other studies have also found this technique to be unreliable for different tissues (Fallon and Simandl 1978, Schoenwolf 1981).

It is important to note that BrDU administered to 4 and 4.5 day old embryos has an opposite effect to that given to 6-day embryos.

The reduction, near abolition, of PCD seen when BrDU is given to the younger embryos suggests that an S-phase around day 4 is proximal to the differentiative event which will enable cells to die 3.5 days later. The cell blocking experiments (section 2.2) have shown that cells which will die are still part of the cycling population after day 4, so that the programming event cannot involve immediate removal from the cell cycle. It is possible that whatever event is affected by BrDU treatment at days 4 to 4.5 somehow establishes the cells' ability to respond to a later signal to die, rather than starting the death program itself.

It is particularly interesting that, in contrast to the above, BrDU administration on day 6 increases the amount of apoptosis above control levels. There are several possible explanations for this. BrDU could be decreasing the size of the overall population, either by inhibiting immigration or by slowing the cell cycle, so increasing the relative proportion of dead cells seen. However, migrating cells are apparent in these embryos (tissues exhibiting a characteristic streaming pattern, with elongated cells, known to be actively migrating in normal embryos), and at least some of them develop a normal VBL ruffle, which has been assumed to be

produced by the influx of these immigrating cells (Fell 1939). Slowing the cell cycle might explain the concomitant decrease in observed MI, as Tm is unlikely to have been prolonged, and would thus be a smaller ratio of Tc, so that less cells will be in undergoing mitosis at any one time. However, if this is the case it is difficult to see why BrDU treatment at different ages would not have had a similar effect.

BrDU could be raising the DI by preventing some event or process which inhibits PCD in the controls. Also BrDU is known to increase transcription of some proteins and mRNA (Goz 1978), and thus could be up-regulating some component of the PCD pathway. In either of these two cases, apoptosis would have to effect the proliferating cell population, or else a relative increase in the MI would be seen. Indeed, for MI to be declining as DI increases death would have to be affecting the proliferating fraction more than the non-proliferating, unless DI is linked to MI through some other mechanism, such as the apoptosis monitoring compound hypothesised by Lynch (1986).

It is even possible that BrDU treatment may have semi-synchronised the cell population, with the apparent rise in DI and drop in MI resulting from this in the same way as that seen in the cell cycle blockade experiments (section 2.2). However since a single comparable dose of thymidine did not give the same results, BrDU would have to be a markedly better cell cycle blocker than is thymidine, although this has been suggested to be the case (Goz 1978). It seems unlikely, in any case, that such semi-synchronisation of cells could explain the results seen, given that no similar effect was observed for BrDU given at day 5 or day 5.5. It seems more likely that BrDU treatment is affecting a differentiative

event localised at day 4-4.5, and another at day 6, one leading to the initiation of apoptosis (at the expense of the MI), and the other inhibiting apoptosis (with a concomitant increase in MI). This latter differentiative event, if it exists, could explain the decline in DI seen by day 8 of incubation, implying that an event at day 6 can cause a decline in the number of apoptotic cells detected 2 days later.

That Janus B Green gives different results to BrDU further demonstrates that these two compounds act by different teratological mechanisms. That overall VBW architecture is greatly effected, with abnormal separation of the epithelia and mesoderm, on precisely those two days when Janus B Green administration affects the VBW DI, implies that some interaction between those two tissue layers is indeed important in regulating apoptosis, as was suggested by the cell culture experiments (section 1.2). It is interesting that here epithelial separation seems to have decreased the DI relative to controls, whereas in the culture experiments lack of epithelia increased the mesodermal DI. However not enough evidence exists to be sure that some change in epithelial-mesodermal communication is responsible for the decrease in DI seen.

It is also worth noting that while Janus B Green application at day 5.5 or 6 has decreased the VBW DI, it has not significantly affected the MI, so that while some cells may have been rescued from dying, they do not seem to have entered or remained in the cycling population, or else to have done so in such a ratio as to leave MI unchanged.

However, for both BrDU and Janus B Green experiments, it is important to bear in mind that large scale changes to the size, shape

and tissue makeup of the VBW have occurred, and that as such it is difficult to draw firm conclusions about changes to the apoptotic and mitotic indices, given that the overall population size may differ greatly between controls and treated embryos.

Despite this, it is certainly clear that administration of BrDU can affect the VBW's size, shape and the degree of midline fusion, and it is tempting to conclude that this demonstrates the kinetic importance of PCD rates in determining such morphological events.

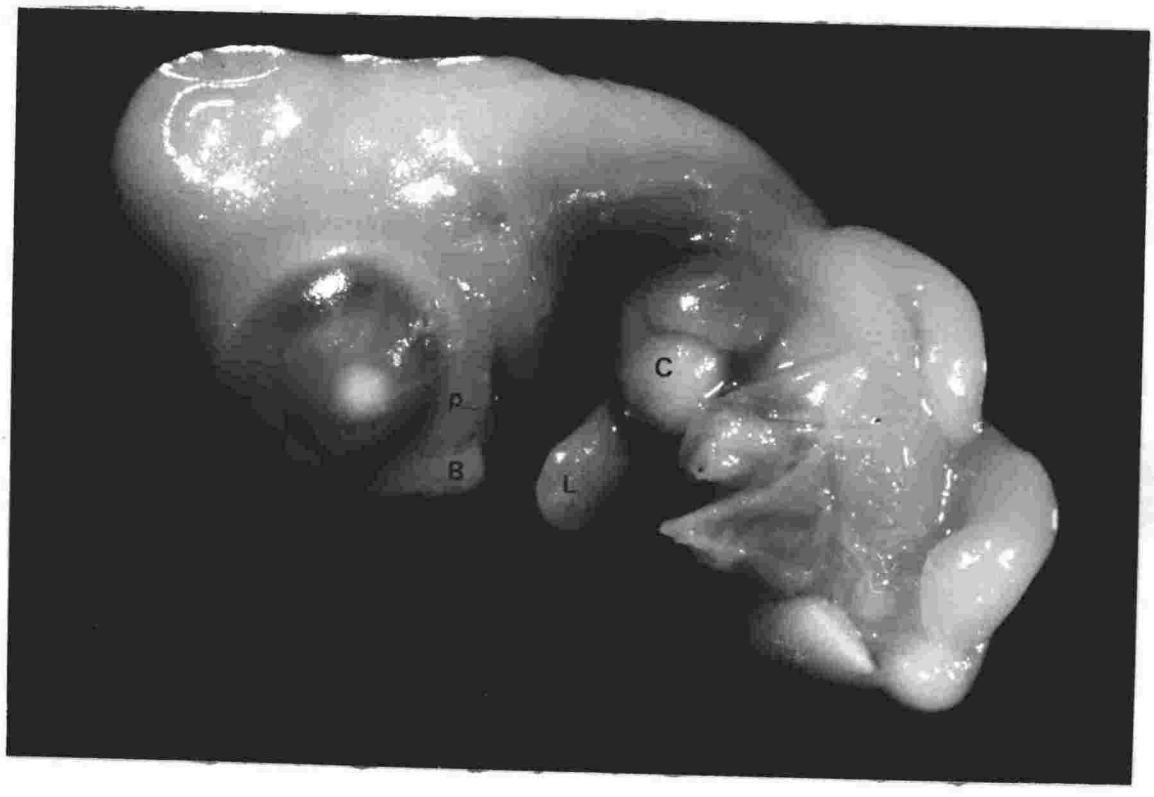

Plate 10: Generalised body deformities seen in a stage 34 embryo, treated with excess BrDU at stage 25 (x 5.6).

B= undeveloped beak, C= thin chest wall, L= lack of digits, P= cleft palate.

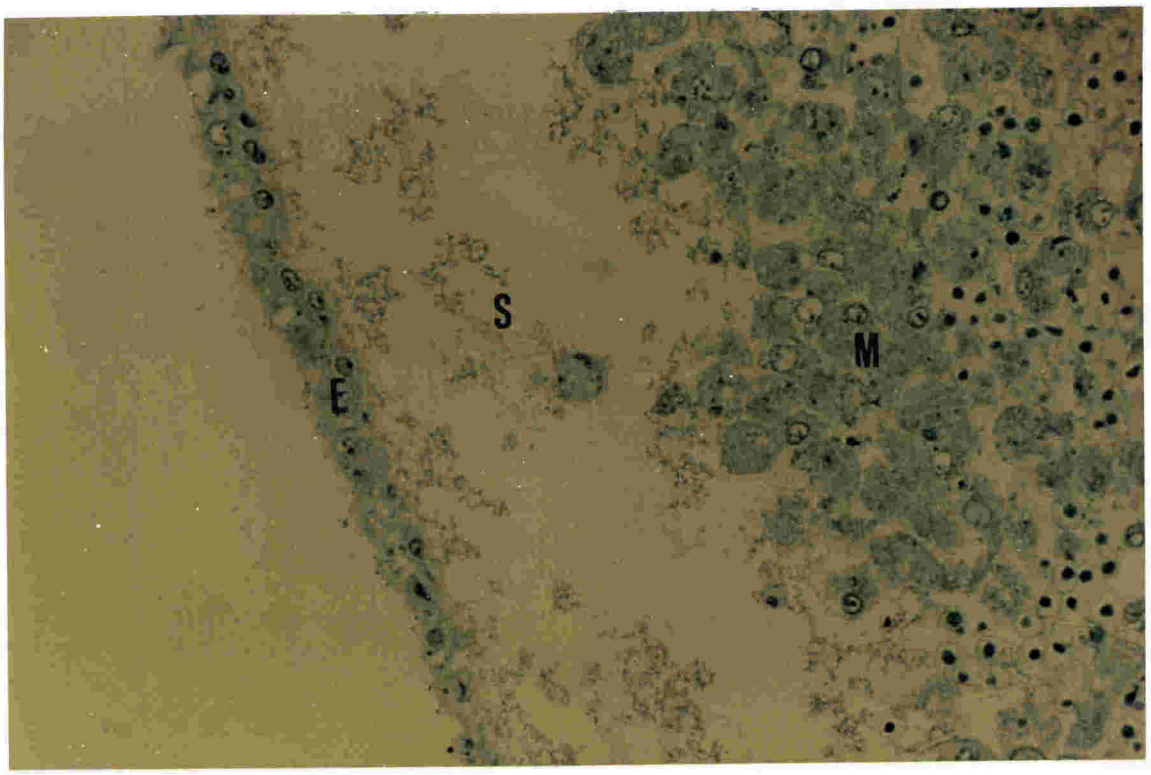

Plate 11: Separation of epithelial and mesodermal tissues seen in a VBW section of a stage 34 embryo treated with Janus B Green at stage 29 (x 50).

E= epithelia, M= mesoderm, S= abnormal separating space.

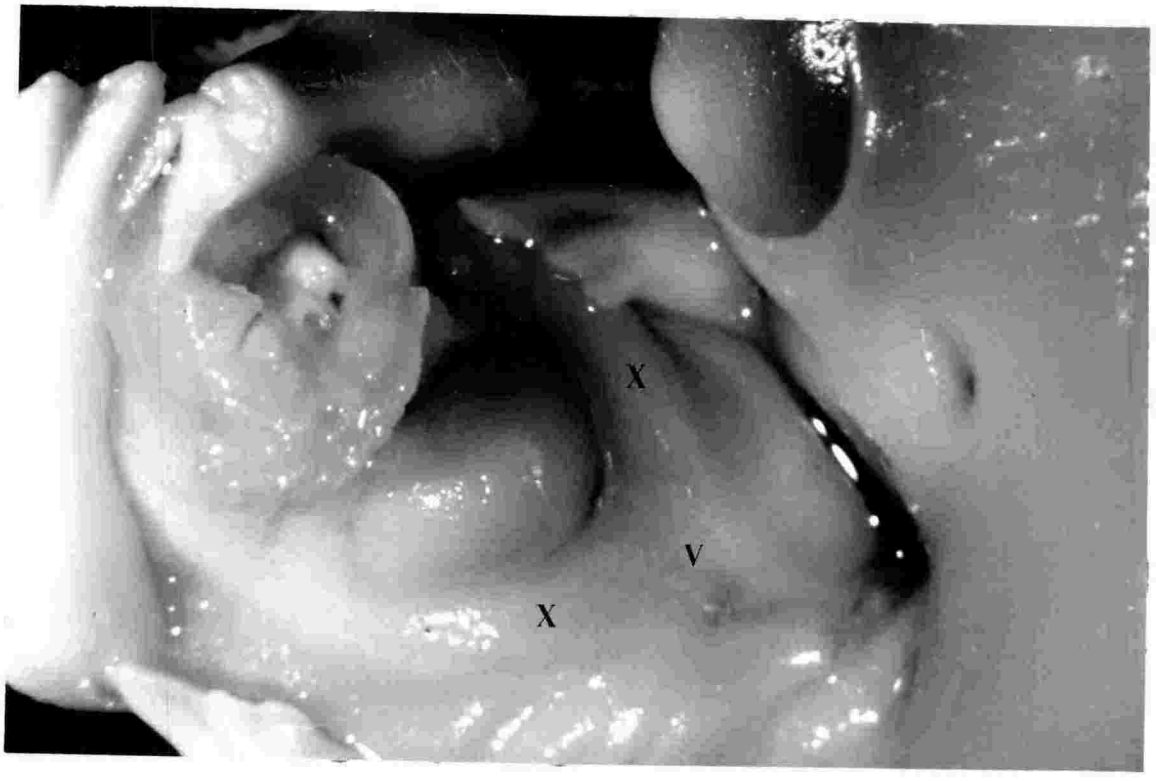

Plate 12: Chest deformities in a stage 34 embryo treated with excess BrDU at stage 29 (x 7).

V= shortened VBW, X= unfused lateral tissue shelf.

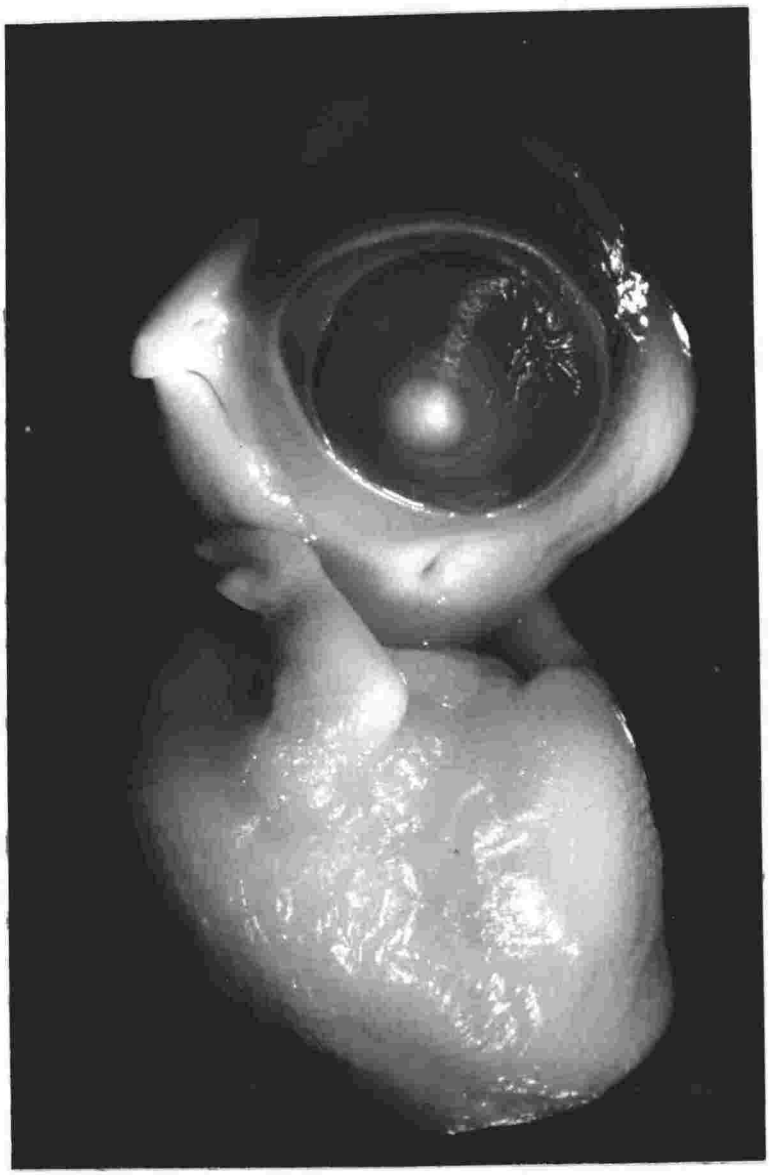

Plate 13: Torsion of upper body seen in a stage 34 embryo after treatment with excess BrDU at stage 29 (x 5.5).

3: SUMMARY AND CONCLUSIONS

The period of chick development, here referred to as 'late body wall development', consists of large-scale tissue migrations and remodellings, and involves the fusion and establishment of important adult structures, such as the sternum and pectoral muscles, ribs and abdominal wall. It includes the ventral migration of lateral tissues into the midline region (after initial body fusion is complete), and the subsequent degenerations required to allow these tissues to meet. As such it runs from the beginning of Day 5 of development (H&H stage 27) until the end of the 12th day (stage 38).

During the period described, a dramatic (10 fold) initial increase in growth rates is seen, yet increases in the MI are followed by increases in DI, such that the growth rates actually decline in the period from day 7.5 to day 8. These events demonstrate that the role of PCD in cell kinetics should not be underestimated, yet sadly it sometimes has been. It was because of such previous neglect that the fundamental role of cell death during VBW development was so closely followed in this study.

Induction of apoptosis:

Culturing experiments (section 1.2) and BrDU administration (section 2.3) revealed that apoptosis of the original VBW mesenchyme of the body wall has been programmed by day 4.5 of development, after body wall fusion is complete, but that the lateral cells which will die later, during VBL degeneration, are not yet programmed to do so. This original VBW programming is not irreversible at day 4.5, however, requiring some factor present in serum media additives to occur *in vitro*.

Early, generalised apoptosis of the VBW is not primarily induced by migrating lateral tissues, as immigration has yet to seriously affect the region, and VBW tissue will exhibit apoptosis even when removed to culture prior to the start of immigration.

VBL apoptosis, on the other hand, does seem closely related to lateral tissue migration, so that it would be quite possible that lateral tissue contact somehow induces VBL cell death. However, lateral tissue cells have not become competent to die without external influences by the time they begin to migrate, and after this stage they are so intermixed with other VBW tissues that they cannot be separated to allow detection of any change in such competence. Lateral tissues are competent to undergo apoptosis upon contact with other dying cells, however, and so VBL degeneration may involve their exposure to remnants of the original VBW mesoderm, which undergoes widespread degeneration at day 6.

Culturing (section 1.2) and Janus B Green experiments (section 2.3) demonstrated that ectodermal tissue exerts a modifying (possibly inhibitory) effect on mesodermal apoptosis in the VBW. However given the precision of the VBL it is understandable if current theories about unknown diffusible signals (Saunders et al 1962) seem somewhat unsatisfactory.

BrDU administration also revealed that a second differentiative event may occur, around day 6 of development, which prevents further cell death. It could well be that VBW cells are all capable of undergoing apoptosis, but are prevented from doing so by some local maintenance factor, like that involved in preventing AER degeneration in the chick limb-bud (Hinchliffe and Griffiths 1984), or like that seen in *C. elegans* Ced-9 gene activation

(Raff 1992), or in ACTH maintenance of the adrenal cortex (Wyllie 1980). It is notable that VBW degeneration does not spread to the more specialised lateral tissues, allowing instead the medial migration and fusion of sternal plates and pectoral musculature. It is a point to remember, when debating potential inductive stimuli for PCD, that often the question should not be 'Why did these cells die?', but rather 'Why didn't they earlier', or 'why don't these other cells die?'.

Apoptosis and the cell cycle:

It is clear, from the occurrence of autoradiographically labelled apoptotic bodies (section 2.1), that at least some dead cells arise from the proliferating pool. Indeed the number of labelled dead cells is likely to have been underestimated, given that a labelled cell may fragment into bodies, some of which appear to be unlabelled. Therefore, that the labelled apoptotic population rose as high as 71% (Table 5) implies that a very large fraction indeed of the dying cells were derived from the proliferating pool. Indeed, given that Ip is greater than DI (see Table 6), it is numerically quite possible that all dead cells came from the proliferating fraction.

While the DI and MI are not significantly related by either the Kruskal-Wallis or the Spearmann's rank correlation test, DI and LI are, suggesting that the low count sizes for mitotic cells was all that prevented a correlation being seen (since LI and MI are, of course, related).

These points support previous suggestions that apoptosis is functionally related to the cell cycle, with a role in balancing increased cell proliferation (Kerr et al 1972, Hinsull and Bellamy 1977). The easiest way cell proliferation and apoptosis could be

linked, it would seem, is by proliferating cells themselves being the population that is preferentially affected by cell death.

Cotter et al (1990) proposed that the phase of the cell cycle cells were in had a direct bearing on their readiness to undergo apoptosis. However, which stages of the cell cycle labelled dead VBW cells may have derived from is difficult to answer. Given a mean Tc of 14hrs, and a time to appearance of labelled apoptotic cells about 21 hours (certainly of more than 15 hours), it is quite possible for labelled cells to reach any stage of the cell cycle before beginning to die, dependent upon the length of time the death program itself requires. Cells might even have entered a G0 state before undergoing apoptosis. Interestingly, Tone et al (1988), when working on chick interdigital PCD, also found a time to first appearance of labelled cell death of approximately 20 hours.

However it is of particular kinetic significance whether cells leave the cycle in G2, prior to undergoing mitosis, as this would have an equivalent effect to removing 2 cells from G1 (Lewis 1975). If all cycling cells due to die were to leave the cell cycle from G2, without undergoing mitosis, one would expect to see a decline in the MI predating any increase in DI. Such a decline should be detected histologically, since the time until labelled apoptotic bodies appear after labelling exceeds T_{G2} by more than 12hrs, and thus a decline in MI should occur one H&H stage prior to any increase in DI, and would certainly be detectable with the 4 hour sampling employed for the continuous labelling experiments. Yet, while decreases in MI are associated with increases in DI, these are contemporaneous events throughout most of this thesis, not fitting the pattern predicted if apoptosis had occurred from G2.

Given also that few abnormal mitoses were seen, it is unlikely that cells die during mitosis itself, and we can therefore assume that cells which die after having been labelled reach G1 before doing so.

When cells are blockaded at the G1-S boundary by exposure to excess thymidine, cell death continues to be seen for at least 12 hours, but then declines and disappears (see section 2.2). This indicates that cells can undergo apoptosis if prevented from entering S-phase, but that it is necessary to do so, and traverse the cell cycle, for further apoptosis to be induced. The relationship between cell cycle progression and apoptosis can also be clearly seen by the production of an increased DI after release of a semi-synchronised wave of cycling cells upon blockade removal. It is notable that increases in MI in the above experiments predate similar increases in DI, again implying that apoptosis is a post-mitotic event in the VBW.

However, if death occurs from G1, the ability to undergo apoptosis must be lost by the end of G1, since viable cells blocked at late G1 do not die until they have again traversed the cell cycle. This indicates that the decision to die is able to be made only during a brief 'window' in the cell cycle, and that this window may be situated in early G1.

G1 would seem to be a logical time for cells to initiate the cell death pathway, as it is early in this stage that decision points on whether to enter G0, differentiate, or become committed to a new cell cycle, are also reached (Broek et al 1991, Draetta 1990, Pardee 1989, Whittaker and Patel 1990). Cells use early G1 as an opportunity to interact with their environment, which influences

these decisions (Epifanova 1977, Pardee 1989), just as the cells environment affects the decision to begin apoptosis.

Horn et al (1991) found that the decision either to die or enter G0 was made by prostate cells in G1, after being challenged with TNF. They further suggested that similar signal transduction pathways for PCD and proliferation might exist, given that prostate PCD is associated with the expression of growth-related genes (such as *c-fos* and *c-myc*). The numerical relationship of dying and dividing cells revealed in this thesis further supports Horn et al's feeling that the general homeostatic principles of both processes are similar, an idea also suggested by others (for examples see Buttyan et al 1988, Eastman 1990, Walker et al 1988).

It is interesting, in this light, that the apoptotic endonuclease is Ca^{++}-dependent (Wyllie et al 1984b), and that cytosolic Ca^{++} levels are important in glucocorticoid induced apoptosis (McConkey et al 1989), given that calcium ion transients are important control factors in cell cycle decision sites like START, affecting cyclin synthesis and thus the ability to begin a new cell cycle (Hunter and Pines 1991, Whittaker and Patel 1990). Indeed, Ca^{++} transients serve as a pacemaker regulating the cell cycle control proteins (P34 and cyclin) by both translational and postranslational mechanisms, for example leading to cyclin phosphorylation at ENTRY, so allowing entry to mitosis (Broek et al 1991, Draetta 1990, Whittaker and Patel 1990).

Whether or not Cyclin or P34 phosphorylation states are related to a cell's willingness to undergo PCD has yet to be investigated, although some suggestion has been made that the phosphorylation of protein kinase C seen during some steps of the cell cycle may influence the initiation of PCD (Perotti et al 1990).

Munck and Crabtree (1981) also proposed that glucocorticoid-induced PCD may be confined to G1, based largely on the work of Harmon et al (1979) on dexamethesone induced G1 arrest and death.

Cytotoxic T cells are also reported to kill cells in G1 faster than other cells (Sanderson and Glauert 1979).

However these suggestions contradict the work of Eastman and his associates (Ciccarelli et al 1986, Eastman 1990, Sorenson et al 1990, Sorenson and Eastman 1988) with cisplatin, the cancer therapeutic drug. They found that tumour cells exposed to cisplatin progress to and arrest in G2, sometimes for several days, prior to death. This cell death involves inappropriate chromatin condensation, which may involve a premature rise in Ca^{++} levels, linking the PCD seen with cyclin/kinase activation and chromatin condensation normally seen at the G2-M boundary (Eastman 1990, Sorenson et al 1990). Other cancer therapeutic agents may also arrest cells in G2 (Kimler et al 1978).

It is notable, in support of this, that most cancer therapeutic drugs seem to work in S or G2-M phase (Bontenbal et al 1990, Drewinko et al 1983, Eastman 1990, Sorenson et al 1990).

However such drugs may cause such extreme damage that cells are prevented from progressing through the cell cycle to undergo PCD in G1, as they otherwise might have. It is also of interest that a less preferred decision point to leave the cell cycle and enter G0 exists in early G2 for at least some cells (Epifanova 1977). Thus it is possible that severely wounded cells are employing a G2 'differentiative window' in the cell cycle that is subsidiary to that more commonly used in G1.

It is hoped that this project has demonstrated the kinetic significance of PCD, and provided further evidence that cycling and dying cells are numerically related. A more detailed discussion of the mechanisms by which such a relationship exists awaits further research; however it is further hoped that this final discussion has expressed some of the excitement involved with future work on the molecular genetics of cell cycle and PCD mutants, given that a mechanistic link between these pathways would provide a direct, almost poetically sparse, explanation for the kinetic correlations here described.

REFERENCES

Abercrombie, M. 1946. Estimation of Nuclear Population from Microtome Sections. *Anatomical Records,* **94**:239-247.

Adams, R.L.P. 1980. Cell Culture for Biochemists. *Laboratory Techniques in Biochemistry and Molecular Biology* Ed. Work, T.S., and Burdon, R.H. Elsevier/North-Holland. Biomedical Press, Amsterdam.

Afanas'ev, V.N., Korol', B.A., Mantsygin, Y.A., Nelipovich, P.A., Pechatnikov, V.A., and Umansky, S.R. 1986. Flow Cytometry and Biochemical Analysis of DNA Degradation Characteristic of Two Types of Cell Death. *FEBS Letters,* **194(2)**: 347-350.

Allan, D.J., Harmon, B.V., and Kerr, J.F.R. 1987. Cell Death in Spermatogenesis. Pp.229-258 in *Perspectives on Mammalian Cell Death.* Ed., Potten, C.S. Oxford University Press.

Alles, A., Alley, K., Barrett, J.C., Buttyan, R., Columbano, A., Cope, F.O., Copelan, E.A., Duke, R.C., Farel, P.B., Gershenson, L.E., Goldgaber, D., Green, D.R., Honn, K.V., Hully, J., Isaacs, J.T., Kerr, J.F.R., Krammer, P.H., Lockshin, R.A., Martin, D.P., McConkey, D.J., Michaelson, J., Schulte-Hermann, R., Server, A.C., Szende, B., Tomei, L.D., Tritton, T.R., Umansky, S.R., Valerie, K., and Warner, H.R. 1991. Apoptosis: A General Comment. *FASEB Journal,* **5**: 2127-2128.

Alles, A.J., and Sulik, K.K. 1989. Retinoic-acid-induced Limb-reduction Defects: Peturbation of Zones of Programmed Cell Death as a Pathogenetic Mechanism. *Teratology,* **40**: 163-171.

Alles, A.J., and Sulik, K.K. 1990. Retinoic Acid-induced Spina Bifida: evidence for a Pathogenetic Mechanism. *Development,* **108**: 73-81.

Amprino, R., and Camosso, M. 1959. On the Role of the "Apical Ridge" in the Development of the Chick Embryo Limb Bud. *Acta Anatomica,* **38**: 280-288.

Arends, M.J., Morris, R.G., and Wyllie, A.H. 1990. Apoptosis: the Role of the Endonuclease. *American Journal of Pathology,* **136(3)**: 593-608.

Avery, L., and Horvitz, H.R. 1987. A Cell that Dies During Wild-Type *C. elegans* Development Can Function as a Neuron in a *ced*-3 Mutant. *Cell*, **51**: 1071-1078.

Bach, J-F. 1981. The Multi-faceted Zinc Dependency of the Immune System. *Immunology Today*, **November**: 225-227.

Barns, D., and Sato, G. 1980a. Methods for Growth of Cultured Cells in Serum-free Medium. *Analytical Biochemistry,* **102**: 255-270.

Barns, D., and Sato, G. 1980b. Serum-free Cell Culture: a Unifying Approach. *Cell,* **22**: 649-655.

Barry, M.A., Behnke, C.A., and Eastman, A. 1990. Activation of Programmed Cell Death (Apoptosis) by Cisplatin, Other Anticancer Drugs, Toxins and Hyperthermia. *Biochemical Pharmacology*, **40(10)**: 2353-2362.

Basile, D.V., Wood, H.N., and Braun, A.C. 1973. Programming of Cells for Death Under Defined Experimental Conditions: Relevance to the Tumor Problem. *Proceedings of the National Academy of Sciences, USA*, **70(11)**: 3055-3059.

Beaulaton, J., and Lockshin, R.A. 1982. The Relationship of Programmed Cell Death to Development and Reproduction:

Comparative Studies and an Attempt at Classification. *International Review of Cytology*, **79**: 215-235.

Becker, F.F., and Lane, B.P. 1966. Regeneration of the Mammalian Liver IV. Evidence on the Role of Cytoplasmic Alterations in Preparation for Mitosis. *American Journal of Pathology*, **49**: 227-237.

Beirne, E.M., Lanser, M.E., and Fallon, J.F. 1986. Thymidine Analog 5-Bromo-2-deoxyuridine (BRDU) Inhibits Programmed Cell Death of the Posterior Necrotic Zone (PNZ) in the Chick Wing Bud. *Clinical Research*, **34(4)**: 994A.

Bellairs, R. 1961. Cell Death in Chick Embryos as Studied by Electron Microscopy. *Journal of Anatomy*, **95**: 54-60.

Berger, N.A. 1985. Poly(ADP-ribose) in the Cellular Response to DNA Damage. *Radiation Research*, **101**: 4-15.

Bessis, M. 1964. Studies on Cell Agony and Death: An Attempt at Classification. *Cellular Injury, Ciba Foundation Symposium.* Ed. de Rueck, A.V.S., and Knight, J. J& A Churchill, London.

Bontenbal, M., Sieuwerts, A.M., Peters, H.A., Sonneveld, P., Foekins, J.A., and Klijn, J.G.M. 1990. Manipulation of Cell Cycle Kinetics: Influence on the Cytotoxicity of Doxorubicin in Human Breast Cancer Cells. *Journal of Steroid Biochemistry and Molecular Biology*, **37(6)**: 1097-1101.

Boobis, A.R., Fawthrop, D.J., and Davies, D.S. 1990. Mechanisms of Cell Toxicity. *Current Opinion in Cell Biology*, **2**: 231-237.

Bowen, I.D. 1981. Techniques for Demonstrating Cell Death. Pp.379-446 in *Cell Death in Biology and Pathology*. Ed. Bowen, I.D., and Lockshin, R.A. Chapman and Hall, New York.

Bowen, I.D. 1984. Laboratory Techniques for Demonstrating Cell Death. Pp.5-40 in *Cell Aging and Cell Death.* Ed. Davies, I., and Sigee, D.C. Cambridge University Press.

Bowen, I.D., and Davies, P. 1971. The Fine Structural Distribution of Acid Phosphatase in the Digestive Gland of *Arion hortensis* (Fer.). *Protoplasma,* **73**: 73-81.

Bowen, I.D., and Ryder, T.A. 1974. Cell Autolysis and Deletion in the Planarian *Polycelis tenuis* Iijima. *Cell and Tissue Research,* **154**: 265-274.

Broek, D., Bartlett, R., Crawford, K., and Nurse, P. 1991. Involvement of $p34^{cdc2}$ in Establishing the Dependency of S Phase on Mitosis. *Nature,* **349**: 388-393.

Bursch, W., Kleine, L., and Tenniswood, M. 1990a. The Biochemistry of Cell Death by Apoptosis. *Biochemical Cell Biology*, **68**:1071-1074.

Bursch, W., Lauer, B., Timmermann-Trosiener, I., Barthel, G., Schuppler, J., and Schulte-Hermann, R. 1984. Controlled Death (Apoptosis) of Normal and Putative Preneoplastic Cells in Rat Liver Following Withdrawal of Tumor Promoters. *Carcinogenesis,* **5(4)**: 453-458.

Bursch, W., Paffe, S., Putz, B., Barthel, G., and Shulte-Hermann, R. 1990b. Determination of the Length of the Histological Stages of Apoptosis in Normal and in Altered Hepatic Foci of Rats. *Carcinogenesis,* **11(5)**: 847-853.

Bursch, W., Taper, H.S., Lauer, B., and Schulte-Hermann, R. 1985. Quantitative Histological and Histochemical Studies on the Occurrence and Stages of Controlled Cell Death (Apoptosis) During Regression of Rat Liver Hyperplasia. *Virchows Archives of Cell Pathology,* **50**: 153-166.

Buttyan, R., Olsson, C.A., Pintar, J., Chang, C., Bandyk, M., Ng, P-Y., Sawczuk, I.S. 1989. Induction of the *TRPM-2* Gene in Cells Undergoing Programmed Cell Death. *Molecular and Cellular Biology*, **9 (8)**: 3473-3481.

Buttyan, R., Zakeri, Z., Lockshin, R., and Wolgemuth, D. 1988. Cascade Induction of c-*fos*, c-*myc*, and Heat Shock 70K Transcripts During Regression of the Rat Ventral Prostate Gland. *Molecular Endocrinology*, **2(7)**: 650-657.

Cameron, J., and Fallon, J.F. 1977. The Absense of Cell Death During Development of Free Digits in Amphibians. *Developmental Biology,* **55**: 331-338.

Carson, D.A., Carrera, C.J., Wasson, D.B., and Yamanaka, H. 1988. Programmed Cell Death and Adenine Deoxynucleotide Metabolism in Human Lymphocytes. *Advances in Enzyme Regulation,* **27**: 395-404.

Carson, D.A., Seto, S., Wasson, D.B., and Carrera, C.J. 1986. DNA Strand Breaks, NAD Metabolism, and Programmed Cell Death. *Experimental Cell Research,* **164**: 273-281.

Chen, J.M. 1952a. Studies on the Morphogenesis of the Mouse Sternum.I: Normal Embryonic Development. *Journal of Anatomy, London,* **86**: 373-386.

Chen, J.M. 1952b. Studies on the Morphogenesis of the Mouse Sternum.II: Experiments on the Origin of the Sternum and its Capacity for Self-differentiation *in-vitro*. *Journal of Anatomy, London,* **86**: 387-401.

Chen, J. M. 1953. Studies on the Morphogenesis of the Mouse Sternum III: Experiments on the Closure and Segmentation of the Sternal Bands. *Journal of Anatomy, London,* **87**: 130-151.

Christmas, S.E., and Moore, M. 1987. Immunologically-mediated Cell Death. Pp. 259-294 in *Perspectives on Mammalian Cell Death.* Ed. Potten, C.S. Oxford University Press, Oxford.

Ciccarelli, R.B., Solomon, M.J., Varshavsky, A., and Lippard, S.J. 1985. *In Vivo* Effects of *cis*- and *trans*-Diamminedichloroplatinum(II) on SV40 Chromosomes: Differential Repair, DNA-Protein Cross-Linking, and Inhibition of Replication. *Biochemistry*, **24**: 7533-7540.

Clarke, P.G.H. 1990. Developmental Cell Death: Morphological Diversity and Multiple Mechanisms. *Anatomy and Embryology.* **181**: 195-213.

Clem, R.J., Fechheimer, M., and Miller, L.K. 1991. Prevention of Apoptosis by a Baculovirus Gene during Infection of Insect Cells. *Science,* **254**: 1388-1390.

Cohen, J.J., and Duke, R.C. 1984. Glucocorticoid Activation of a Calcium-Dependent Endonuclease in Thymocyte Nuclei Leads to Cell Death. *The Journal of Immunology*, **132(1)**: 38-42.

Columbano, A., Ledda-Columbano, G.M., Coni, P.P., Faa, G., Liguori, C., Santa Cruz, G. and Pani, P. 1985. Occurrence of Cell Death (Apoptosis) During the Involution of Liver Hyperplasia. *Laboratory Investigation*, **52(6)**: 670-675.

Columbano, A., Ledda-Columbano, G.M., Rao, P.M., Rajalakshmi, S., and Sarma, S.R. 1984. Occurrence of Cell Death (Apoptosis) in Preneoplastic and Neoplastic Liver Cells: a Sequential Study. *American Journal of Pathology,* **116(3)**: 441-446.

Compton, M.M. 1991. Laboratory Methods: Development of an Apoptosis Endonuclease Assay. *DNA and Cell Biology*, **10(2)**: 133-141.

Cooper, E.H., Bedford, A.J., and Kenny, T.E. 1975. Cell Death in Normal and Malignant Tissues. Pp. 59-120 in *Advances in Cancer Research v.21*, Ed. Klein,G. and Weinhouse, S. Academic Press, New York.

Cotter, T.G., Lennon, S.V., Glynn, J.G., and Martin, S.J. 1990. Cell Death *via* Apoptosis and its Relationship to Growth and Differentiation of both Tumour and Normal Cells. *Anticancer Research,* **10**: 1153-1160.

Cowan, W.M. 1973. Neuronal Death as a Regulative Mechanism in the Control of Cell Number in the Nervous System. Pp.19-41 in *Development and Aging in the Nervous System,* Ed.Rockstein, M. Academic Press Inc., New York.

Cowan, W.M., Fawcett, J.W., O'Leary, D.D.M., and Stanfield, B.B. 1984. Regressive Events in Neurogenesis. *Science,* **225**: 1258-1265.

Cree, I.A., Gardiner, C.A., Beck, J.S., and Mehta, J. 1986. Studies of Cell Death (Apoptosis) and Cell Division in Leprosy Granulomas. *International Journal of Leprosy,* **54(4)**: 607-613.

Crook, N.E., Clem, R.J., and Miller, L.K. 1993. An Apoptosis-inhibiting Baculovirus with a Zinc-finger like Motif. *Journal of Virology,* **67(4)**: 2168-2174.

Danno, K., and Horio, T. 1982. Formation of UV-induced Apoptosis Relates to the Cell Cycle. *British Journal of Dermatology,* **107**: 423-428.

Del Vecchio, M.T., Leoncini, L., Buerki, K., Kraft, R., Megha, T., Barbini, P., Tosi, P., and Cottier, H. 1991. Diffuse Centrocytic and/or Centroblastic Malignant Non-Hodgkin's Lymphomas: Comparison of Mitotic and Pyknotic (Apoptotic) Indices. *International Journal of Cancer,* **47**: 38-43.

Draetta, G. 1990. Cell Cycle Control in Eukaryotes: Molecular Mechanisms of *cdc2* Activation. *Trends in Biological Sciences,* **October**: 378-383.

Drewinko, B., Yang, L-Y., Barlogie, B., and Trujillo, J.M. 1983. Comparative Cytotoxicity of Bisantrene, Mitoxantrone, Ametantrone, Dihydroxyanthracenedione, Dihydroxy-anthracenedione Diacetate, and Doxorubicin on Human Cells *in Vitro*. *Cancer Research*, **43**: 2648-2653.

Duke, R.C., Chervenak, R., and Cohen, J.J. 1983. Endogenous Endonuclease-induced DNA Fragmentation: an Early Event in Cell-mediated Cytolysis. *Proceedings of the National Acadamy of Science, USA,* **80**: 6361-6365.

Duvall, E., and Wyllie, A.H. 1986. Death and the Cell. *Immunology Today,* **7(4)**: 115-119.

Duvall, E., Wyllie, A.H., Morris, R.G. 1985. Macrophage Recognition of Cells Undergoing Programmed Cell Death (Apoptosis). *Immunology,* **56**: 351-358.

Eastman, A. 1990. Activation of Programmed Cell Death by Anti-cancer Agents: Cisplastin as a Model System. *Cancer Cells,* **2(8 & 9)**: 275-280.

El-Labban, N.G., and Osorio-Herrera, E. 1986. Apoptotic Bodies and Abnormally Dividing Epithelial Cells in Squamous Cell Carcinoma. *Histopathology,* **10**: 921-931.

El-Shennawy, I.E., Gee, D.J., and Aparicio, S.R. 1985. Renal Tubular Epithelia Ultrastructure in Autolysis. *Journal of Pathology*, **147**: 13-21.

Ellis, H.M., and Horvitz, H.R. 1986. Genetic Control of Programmed Cell Death in the Nematode *C. Elegans. Cell,* **44**: 817-829.

England, P.J. 1986. Intracellular Calcium Receptor Mechanisms. *British Medical Bulletin*, **42(4)**: 375-383.

England, J.M., and Miller, R.G. 1970. The Statistical Analysis of Autoradiographs II: Theoretical Aspects Including Methods for Optimal Allocation of Measurement Effort. *Journal of Microscopy,* **92**: 167-177.

Epifanova, O.I. 1977. Mechanisms Underlying the Differential Sensitivity of Proliferating and Resting Cells to External Factors. *International Review of Cytology Supplement,* **5**: 303-328.

Fallon, J.F., and Cameron, J. 1977. Interdigital Cell Death During Limb Development of the Turtle and Lizard with an Interpretation of Evolutionary Significance. *Journal of Embryology and Experimental Morphology,* **40**: 285-289.

Fallon, J.F., and Saunders,J.W.,Jr. 1965. *In Vitro* Analysis of the Control of Morphogenetic Cell Death in the Wing Bud of the Chick Embryo. *American Society of Zoologists*, **5**: 213-214.

Fallon, J.F., and Saunders, J.W.,Jr. 1968. In Vitro Analysis of the Control of Cell Death in a Zone of Prospective Necrosis from the Chick Wing Bud. *Developmental Biology,* **18**: 553-570.

Fallon, J.F., and Simandl, B.K. 1978. Evidence of a Role for Cell Death in the Disappearance of the Embryonic Human Tail. *American Journal of Anatomy*, **152**: 111-130.

Farber, J.L. 1981. The Role of Calcium in Cell Death. *Life Sciences*, **29**: 1289-1295.

Farber, J.L., and El-Mofty, S.K. 1975. The Biochemical Pathology of Liver Cell Necrosis. *American Journal of Pathology*, **81(1)**: 237-250.

Fell, H.B. 1939. The Origin and Developmental Mechanics of the Avian Sternum. *Philosophical Transactions of the Royal Society of London, Series B*, **229**: 407-463.

Ferguson, D.J.P., and Anderson, T.J. 1981. Morphological Evaluation of Cell Turnover in Relation to the Menstrual Cycle in the "Resting" Human Breast. *British Journal of Cancer*, **44**: 177-181.

Ferguson, M.W.J., Honig, L.S., and Slavkin, H.C. 1984. Differentiation of Cultured Palatal Shelves From Alligator, Chick, and Mouse Embryos. *The Anatomical Record*, **209**: 231-249.

Fernandez-Teran, M.A., and Hurle, J.M. 1984. Syndactyly Induced by Janus Green B in the Embryonic Chick Leg Bud: a Reexamination. *Journal of Embryology and Experimental Morphology*, **84**: 159-175.

Finley, D., and Varshavsky, A. 1985. The Ubiquitin System: Functions and Mechanisms. *Trends In Biological Sciences, **September***: 343-347.

Flanagan, A.E.H. 1969. Differentiation and Degeneration in the Motor Column of Foetal Mouse. *Journal of Morphology*, **129**: 281-305.

Forseberg, J-G. 1967. Studies on the Cell Degeneration Rate During the Differentiation of the Epithelium in the Uterine Cervix and Mullerian Vagina of the Mouse. *Journal of Embryology and Experimental Morphology,* **17**: 433-440.

Glucksmann, A. 1951. Cell Deaths in Normal Vertebrate Ontogeny. Biological *Revue of the Cambridge Philosophical Society,* **28**: 59-98.

Gondos, B. 1973. Germ Cell Degeneration and Intercellular Bridges in the Human Fetal Ovary. *Z Zellforsch Mikrosk Anat,* **138**: 23-30.

Goz, B. 1978. The Effects of Incorporation of 5-Halogenated Deoxyuridines into the DNA of Eukaryotic Cells. *Pharmacological Reviews,* **29(4)**: 249-272.

Greene, R.M., and Pratt, R.M. 1976. Developmental Aspects of Secondary Palate Formation. *Journal of Embryology and Experimental Morphology,* **36(2)**: 225-245.

Greene, R.M., Shah, R.M., Lloyd, M.R., Crawford, B.J., Shanfeild, J.L., and Davidovitch, Z. 1983. Differentiation of the Avian Secondary Palate. *The Journal of Experimental Zoology,* **225**: 43-52.

Gregory, C.D., Dive, C., Henderson, S., Smith, C.A., Williams, G.T., Gordon, J., and Rickinson, A.B. 1991. Activation of Epstein-Barr Virus Latent Genes protects Human B Cells From Death by Apoptosis. *Nature,* **349:** 612-614.

Gude, W.D. 1968. *Autoradiographic Techniques: Localization of Radioisotopes in Biological Material.* Prentice-Hall Inc., New Jersey.

Gundersen, H.J.G., Bagger, P., Bendtsen, T.F., Evans, S.M., Korbo, L., Marcussen, N., Moller, A., Nielsen, K.,

Nyengaard, J.R., Pakkenberg, B., Sorensen, F.B., Vesterby, A., and West, M.J. 1988a. The New Stereological Tools: Disector, Fractionator, Nucleator and Point Sampled Intercepts and Their Use in Pathological Research and Diagnosis. *APMIS*, **96**: 857-881.

Gundersen, H.J.G., Bendtsen, T.F., Korbo, L., Marcussen, N., Moller, A., Nielsen, K., Nyengaard, J.R., Pakkenberg, B., Sorensen, F.B., Vesterby, A., and West, M.J. 1988b. Some New, Simple and Efficient Stereological Methods and Their Use in Pathological Research and Diagnosis. *APMIS*, **96**: 379-394.

Hamburger, V., and Hamilton, H.L. 1951. A Series of Normal Stages in the Development of the Chick Embryo. *Journal of Morphology*, **88**: 49-92.

Hamilton, W.G., and Ham, R.G. 1977. Clonal Growth of Chinese Hamster Cell Lines in Protein-free Media. *In Vitro*, **13(9)**: 537-547.

Harmon, B.V., Corder, A.M., Collins, R.J., Gobe, G.C., Allen, J., Allans, D.J., and Kerr, J.F.R. 1990. Cell Death Induced in a Murine Mastocytoma by 42-47°C Heating *In Vitro*: Evidence that the Form of Death Changes from Apoptosis to Necrosis Above a Critical Heat Load. *International Journal of Radiation Biology*, **58(5)**: 845-858.

Harmon, J.M., Norman, M.R., Fowlkes, B.J., and Thompson, E.B. 1979. Dexamethanose Induces Irreversible G_1 Arrest and Death of a Human Lymphoid Cell Line. *Journal of Cell Physiology*, **98**: 267-278.

Harris, A.L. 1985. DNA Repair: Relationship to Drug and Radiation Resistance, Metastasis and Growth Factors. *International Journal of Radiation Biology,* **48(5)**: 675-690.

Hasegawa, Y., and Bonavida, B. 1989. Calcium-Independent Pathway of Tumor Necrosis Factor-Mediated Lysis of Target Cells. *The Journal of Immunology,* **142(8)**: 2670-2676.

Hayflick, L. 1975. Cell Biology of Aging. *Bioscience,* **25**: 629-637.

Hayflick, L. 1981. Cell Death in Vitro. Pp.243-286 in *Cell Death in Biology and Pathology.* Ed. Bowen, I.D., and Lockshin, R.A. Chapman and Hall, London.

Hengartner, M.O., Ellis, R.E., and Horvitz, H.R. 1992. *Caenorhabditus elegans* Gene *ced-2* Protects Cells from Programmed Cell Death. *Nature,* **356**:494-499.

Hinchliffe, J.R. and Ede, D.A. 1973. Cell Death and the Development of Limb Form and Skeletal Pattern in Normal and *wingless* (*ws*) Chick Embryos. *Journal of Embryology and Experimental Morphology,* **30(3)**: 753-772.

Hinchliffe, J.R., and Griffiths, P.J. 1984. Experimental Analysis of the Control of Cell Death in Chick Limb Bud Development. Pp. 223-242 in *Cell Aging and Cell Death.* Ed. Davies, I., and Sigee, D.C. Cambridge University Press, Cambridge.

Hinchliffe, J.R. and Thorogood, P.V. 1974. Genetic Inhibition of Mesenchymal Cell Death and the Development of Form and Skeletal Pattern in the Limbs of *talpid*^{3} (*ta*^{3}) Mutant Chick Embryos. *Journal of Embryology and Experimental Morphology,* **31(3)**: 747-760.

Hinsull S.M., and Bellamy, D. 1981. Tissue Homeostasis and Cell Death. Pp.123-143 in *Cell Death in Biology and Pathology.* Ed. Bowen, I.D., and Lockshin, R.A. Chapman and Hall Ltd., London.

Hinsull, S.M., and Bellamy, D. 1977. The Relationship Between Cell Density and Cellular Proliferation in Normal and Neoplastic Tissue. *Proceedings Royal Microscopic Society*,**12**: 217.

Hinsull, S.M., Bellamy, D. and Franklin, A. 1977. A Quantitative Histological Assessment of Cellular Death, in Relation to Mitosis, in Rat Thymus During Growth and Age Involution. *Age and Ageing,* **6**: 77-84.

Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R.D., and Korsmeyer, S.J. 1990. *Bcl*-2 is an Inner Mitochondrial Membrane Protein that Blocks Programmed Cell Death. *Nature,* **348**: 334-336.

Horn, D., van der Bosch, J., Ruller, S., and Schlaak, M. 1991. Suppression of Tumor Cell Susceptability to Monocyte-induced Cell Death by Growth-inhibitory Signals Generated During Monocyte/Tumor Cell Interaction. *Journal of Cellular Biochemistry,* **45**: 213-223.

Hughes, A. 1961. Cell Degeneration in the Larval Ventral Horn of *Xenopus laevis* (Daudin). *Journal of Embryology and Experimenatl Morphology,* **9**: 269-285.

Hunter, T., and Pines, J. 1991. Cyclins and Cancer. *Cell,* **66**: 1071-1074.

Hurle, J., and Hinchliffe, J.R. 1978. Cell Death in the Posterior Necrotic Zone (PNZ) of the Chick Wing-bud: a Stereoscan and Ultrastructural Survey of Autolysis and Cell Fragmentation. *Journal of Embryology and Experimental Morphology,* **43**: 123-136.

Hurle, J.M., Lafarga, M., and Ojeda, J.L. 1977. Cytological and Cytochemical Studies of the Necrotic Area of the Bulbus of the

Chick Embryo Heart: Phagocytosis by Developing Myocardial Cells. *Journal of Embryology and Experimental Morphology,* **41**: 161-170.

Irvine, R.F. 1986. Calcium Transients: Mobilisation of Intracellular Ca^{2+}. *British Medical Bulletin,* **42(4)**: 369-374.

Iversen, O.H. 1967. Kinetics of Cellular Proliferation and Cell Loss in Human Carcinomas: a Discussion of Methods Availiable for *in Vivo* Studies. *European Journal of Cancer,* **3**: 389-394.

Kallen, B. 1965. Degeneration and Regeneration in the Vertebrate Central Nervous System During Embryogenesis. *Progress in Brain Research,* **14**: 77-96

Kaufmann, S.H. 1989. Induction of Endonucleolytic DNA Cleavage in Human Acute Myelogenous Leukemia Cells by Etoposide, Camptothecin, and Other Cytotoxic Anticancer Drugs: A Cautionary Note. *Cancer Research,* **49**: 5870-5878.

Kawabe, Y., and Ochi, A. 1991. Programmed Cell Death and Extrathymic Reduction of $VB8^{+}$ $CD4^{+}$ T Cells in Mice Tolerant to *Staphylococcus aureus* Enterotoxin B. *Nature,* **349**: 245-248.

Kenyon, C., and Wang, B. 1991. A Cluster of *Antennapaedia*-Class Homeobox genes in a Nonsegmented Animal. *Trends in Genetics,* **7**: 516-517.

Kerr, J.F.R. 1965. A Histochemical Study of Hypertrophy and Ischaemic Injury of Rat Liver with Special Reference to Changes in Lysosomes. *Journal of Pathology and Bacteriology,* **90**: 419-455.

Kerr, J.F.R. 1971. Shrinkage Necrosis: A Distinct Mode of Cellular Death. *Journal of Pathology*, **105**: 13-20.

Kerr, J.F.R., Bishop, C.J., and Searle, J. 1982. Apoptosis. *Recent Advances in Histopathology*, **12**: 1-15.

Kerr, J.F.R., and Searle, J. 1972a. A Suggested Explanation for the Paradoxically Slow Growth Rate of Basal-cell Carcinomas that Contain Numerous Mitotic Figures. *Journal of Pathology,* **107**: 41-44.

Kerr, J.F.R., and Searle, J. 1972b. The Digestion of Cellular Fragments Within Phagolysosomes in Carcinoma Cells. *Journal of Pathology,* **108**: 55-88.

Kerr, J.F.R., and Searle, J. 1973. Deletion of Cells by Apoptosis During Castration-Induced Involution of the Rat Prostate. *Virchows Archives of Cell Pathology,* **13**: 87-102.

Kerr, J.F.R., and Searle, J. 1980. Apoptosis: Its Nature and Kinetic Role. Pp.367-384 in *Radiation Biology in Cancer Research,* Ed. Meyn, R.E., and Withers, H.R. Raven Press, New York.

Kerr, J.F.R., Wyllie, A. H., and Currie, A.R. 1972. Apoptosis: A Basic Biological Phenomenon with Wide-Ranging Implications in Tissue Kinetics. *British Journal of Cancer,* **26**: 239-257.

Kimler, B.F., Schneiderman, M.H., and Leeper, D.B. 1978. Induction of Concentration-dependent Blockade in the G_2 Phase of the Cell Cycle by Cancer Chemotherapeutic Agents. *Cancer Research,* **38**: 809-814.

Klambt, C., Jacobs, J.R., and Goodman, C.S. 1991. The Midline of the *Drosophila* Central Nervous System: A Model for the Genetic Analysis of Cell Fate, Cell Migration, and Growth Cone Guidance. *Cell*, **64**: 801-815.

Kyprianou, N., Alexander, R.B., and Isaacs, J.T. 1991. Activation of Programmed Cell Death by Recombinant Human

Tumor Necrosis Factor Plus Topoisomerase II-Targeted Drugs in L929 Tumor Cells. *Journal of the National Cancer Institute*, **83(5)**: 346-350.

Kyprianou, N., and Isaacs, J.T. 1988. Activation of Programmed Cell Death in the Rat Ventral Prostate After Castration. *Endocrinology*,**122(2)**: 552-562.

Laerum, O.D., and Smaaland, R. 1989. Circadian and Infradian Aspects of the Cell Cycle: from Past to Future. *Chronobiologia*, **16**: 441-453.

Lala, P.K. 1972. Evaluation of the Mode of Cell Death in Ehrlich Ascites Tumor. *Cancer*, **29**: 261-266.

Lala, P.K, and Patt, H.M. 1966. Cytokinetic Analysis of Tumor Growth. *Proceedings of the National Acadamy of Science, USA*, **56**: 1735-1742.

Larrick, J.W., and Wright, S.C. 1990. Cytotoxic Mechanism of Tumor Necrosis Factor-α. *FASEB Journal*, **4**: 3215-3223.

Lasnitzki, I. 1986. Organ Culture. Pp.149-181 in *Animal Cell Culture: a Practical Approach.* Ed. Freshney, R.I. IRL Press Ltd., Oxford.

Laster, S.M., Wood, J.G., and Gooding, L.R. 1988. Tumor Necrosis Factor Can Induce Both Apoptotic and Necrotic Forms of Cell Lysis. *The Journal of Immunology*, **141(8)**: 2629-2634.

Leger, J.G., Montpetit, M.L., and Tenniswood, M.P. 1987. Charaterization and Cloning of Androgen-Repressed mRNAs From Rat Ventral Prostate. *Biochemical and Biophysical Research Communications*, **147(1)**: 196-203.

Lewis, P.D. 1975. Cell Death in the Germinal Layers of the Postnatal Rat Brain. *Neuropathology and Applied Neurobiology*, **1**: 21-29.

Lockshin, R.A. 1981. Cell Death in Metamorphosis. Pp.79-122 in *Cell Death in Biology and Pathology*. Ed., Bowen, I.D., and Lockshin, R.A. Chapman and Hall, New York.

Lockshin, R.A., Royston, M., Joesten, M., and Carter, T. 1981. Nucleic Acids in Cell Death. Pp.287-293 in *Cell Death in Biology and Pathology*. Ed. Bowen, I.D., and Lockshin, R.A. Chapman and Hall, New York.

Lockshin, R.A., and Zakeri, Z.F. 1990. Programmed Cell Death: New Thoughts and Relevance to Aging. *Journal of Gerontology,* **45(5):** B135-140.

Lockshin, R.A., and Zakeri-Milovanovic, Z. 1984. Nucleic Acids in Cell Death. Pp.243-268 in *Cell Ageing and Cell Death*, Ed. Davies, I., and Sigee, C.D. Cambridge University Press.

Lovas, J.G.L. 1986. Apoptosis in Human Epidermis: A Postmortem Study by Light and Electron Microscopy. *Australian Journal of Dermatology*, **27**: 1-5.

Lucas, M., Solano, F., and Sanz, A. 1991. Induction of Programmed Cell Death (Apoptosis) in Mature Lymphocytes. *Federation of European Biochemical Societies,* **1**: 19-20.

Lynch, M.P., Nawaz, S., and Gerschenson, L.E. 1986. Evidence for Soluble Factors Regulating Cell Death and Cell Proliferation in Primary Cultures of Rabbit Endometrial Cells Grown on Collagen. *Proceedings of the National Academy of Science, USA,* **83**: 4784-4788.

Martikainen, P., and Isaacs, J. 1990. Role of Calcium in the Programmed Death of Rat Prostatic Glandular Cells. *Prostate,* **17**: 175-187.

Maurer, H.R. 1986. Towards Chemically-defined, Serum-free Media for Mammalian Cell Culture. Pp. 13-31 in *Animal Cell*

Culture: a Practical Approach. Ed. Freshney, R.I., IRL Print Ltd., Oxford.

McConkey, D.J., Nicotera, P., Hartzell, P., Bellomo, G. Wyllie, A.H., and Orrenius, S. 1989. Glucocorticoids Activate a Suicide Process in Thymocytes Through an Elevation of Cytosolic Ca^{2+} Concentration. *Archives of Biochemistry and Biophysics,* **269(1)**: 365-370.

McConkey, D.J., Orrenius, S., and Jondal, M. 1990. Cellular Signalling in Programmed Cell Death (Apoptosis). *Immunology Today*, **11(4)**: 120-121.

Menkes, B., and Deleanu, M. 1964. Leg Differentiation and Experimental Syndactyly in Chick Embryo. Part II. Experimental Syndactyly in Chick Embryo. *Revue Roumaine D'Embryologie et de Cytologie,* **1**: 69-77.

Menkes, B., Litvac, B., and Ilies, A. 1964. Spontaneous and Induced Cell Degenerance in Relation to Teratogenesis. *Revue Roumaine D'Embryologie et de Cytologie,* **1**: 47-60.

Menkes, B., Sandor, S., and Ilies, A. 1970. Cell Death in Teratogenesis. Pp. 170-206 in *Advances in Teratology,* Vol.4. Ed. Woollam, D.H.M. Academic Press, New York.

Merlino, G. 1994. Regulatory Imbalances in Cell Proliferation and Cell Death During Oncogenesis in Transgenic Mice. *Seminars in Cancer Biology,* **5(1)**:13-20.

Metcalfe, J.C., Moore, J.P., Smith, G.A., and Hesketh, T.R. 1986. Calcium and Cell Proliferation. *British Medical Bulletin*, **42(4)**: 405-412.

Mills, C.L., and Bellairs, R. 1989. Mitosis and Cell Death in the Tail of the Chick Embryo. *Anatomy and Embryology,* **180**: 301-308.

Mitchell, D.L., and Hartman, P.S., 1990. The Regulation of DNA Repair During Development. *BioEssays,* **12(2)**: 74-79.

Morris, R.G., Hargreaves, A.D., Duvall, E., and Wyllie, A.H. 1984. Hormone Induced Cell Death 2. Surface Changes in Thymocytes Undergoing Apoptosis. *American Journal of Pathology,* **115**: 426-436.

Morstyn, G., Pyke, K., Gardner, J., Ashcroft, R., de Fazio, A., and Bhathal, P. 1986. Immunohistochemical Identification of Prolifferating Cells in Organ Culture Using Bromodeoxyuridine and a Monoclonal Antibody. *The Journal of Histochemistry and Cytochemistry,* **34(6)**: 697-701.

Munck, A., and Crabtree, G.R. 1981. Glucocorticoid-induced Lymphocyte Death. Pp.329-362. in *Cell Death in Biology and Pathology.* Ed. Bowen, I.D., and Lockshin, R.A. Chapman and Hall, London.

Norrby, K. 1970. Population Kinetics of Normal, Transforming and Neoplastic Cell Lines. *Acta Pathologica et Microbiologica Scandinavia,* **70(suppl. 214)**: 1-50.

Norrby, K., Johannison, G., and Mellgren, J. 1967. Prolifferation in an Established Cell Line: an Analysis of Birth, Death and Growth Rates. *Experimental Cell Research,* **48**: 582-591.

Okada, S. 1967. A Simple Graphical Method of Computing the Parameters of the Life Cycle of Cultured Mammalian Cells in the Exponential Growth Phase. *Journal of Cell Biology,* **34**: 915-916.

Oppenheim, R.W., 1985. Naturally Occurring Cell Death During Neural Development. *Trends in Neuroscience,* **8(11)**: 487-493.

Oppenheim, R.W., Prevette, D., Tytell, M., and Homma, S. 1990. Naturally Occurring and Induced Neuronal Death in the Chick Embryo *in Vivo* Requires Protein and RNA Synthesis: Evidence for the Role of Cell Death Genes. *Developmental Biology,* **138**: 104-113.

Pardee, A.B. 1989. G_1 Events and Regulation of Cell Proliferation. *Science,* **246**: 603-608.

Paul, D., Brown, K.D., Rupniak, H.T., Ristow, H.J. 1978. Cell Cycle Regulation by Growth Factors and Nutrients in Normal and Transformed Cells. *In Vitro,* **14(1)**: 76-84.

Pera, M.F. Jr., Rawlings, C.J., Roberts, J.J. 1981. The Role of DNA Repair in the Recovery of Human Cells from Cisplatin Toxicity. *Chemical and Biological Interactions,* **37**: 245-261.

Perotti, M., Taddei, F., Mirabelli, M., Bellomo, G. 1991. *In Vitro* Models for Biochemical Investigations on Programmed Cell Death. *Alternatives to Laboratory Animals,* **19(1)**: 71-77.

Perotti, M., Taddei, F., Mirabelli, M., Vairetti, M., Bellomo, G., McConkey, D.J., and Orrenius, S. 1990. Calcium-dependent DNA Fragmentation in Human Synovial Cells Exposed to Cold Shock. *FEBS Letters,* **259(2)**: 331-334.

Pittman, R.N., Mills, J.C., DiBenedetto, A.J., Hynicka, U.P., and Wang, S. 1994. Neuronal Cell Death: Searching for the Smoking Gun. *Current Opinion in Neurobiology,* **4(1)**: 87-94.

Poelmann, R.E. 1980. Differential Mitosis and Degeneration Patterns in Relation to the Alterations in the Shape of the Embryonic Ectoderm of Early Post-implantation Mouse Embryos. *Journal of Embryology and Experimental Morphology,* **55**: 33-51.

Pollack, R.D., and Fallon, J.F. 1974. Autoradiographic Analysis of Macromolecular Synthesis in Prospectively Necrotic Cells of the Chick Limb Bud. I. Protein Synthesis. *Experimental Cell Research* **84**: 9-14.

Pollack, R.D., and Fallon, J.F. 1976. Autoradiographic Analysis of Macromolecular Synthesis in Prospectively Necrotic Cells of the Chick Limb Bud. II. Nucleic Acids. *Experimental Cell Research,* **100**: 15-22.

Potten, C.S. 1985. Cell Death (Apoptosis) in Hair Follicles and Consequent Changes in the Width of Hairs after Irradiation of Growing Follicles. *International Journal of Radiation Biology,* **48(3)**: 349-360.

Primmett, D.R.N., Norris, W.E., Carlson, G.J., Keynes, R.J., and Stern, C.D. 1989. Periodic Segmental Anomalies Induced by Heat Shock in the Chick Embryo are Associated with the Cell Cycle. *Development,* **105**: 119-130.

Raff, M.C. 1992. Social Controls on Cell Survival and Cell Death. *Nature,* **356**: 397-400.

Rennie, P.S., Bruchovsky, N., Buttyan, R., Benson, M., and Cheng, H. 1988. Gene Expression During the Early Phases of Regression of the Androgen-dependent Shionogi Mouse Mammary Carcinoma. *Cancer Research,* **48**: 6309-6312.

Ritter, E.J., Scott, W.J., and Wilson, J.G. 1973. Relationship of Temporal Patterns of Cell Death and Development to Malformations in the Rat Limb. Possible Mechanisms of Teratogenesis with Inhibitors of DNA Synthesis. *Teratology,* **7**: 219-226.

Rizzino, A., Rizzino, H., and Sato, G. 1979. Defined Media and the Determination of Nutritional and Hormonal Requirements

of Mammalian Cells in Culture. *Nutrition Reviews,* **37(12)**: 369-378.

Robaye, B., Mosselmans, R., Fiers, W., Dumont, J.E., and Galand, P. 1991. Tumor Necrosis Factor Induces Apoptosis (Programmed Cell Death) in Normal Endothelial Cells *in Vitro. American Journal of Pathology,* **138(2)**: 447-453.

Rogers, A.W. 1973. *Techniques of Autoradiography.* 2nd Ed. Elsevier Scientific Publishing Co., Amsterdam.

Rotello, R.J., Lieberman, R.C., Purchio, A.F., and Gerschenson, L.E. 1991. Coordinated Regulation of Apoptosis and Cell Proliferation by Transforming Growth Factor $\beta 1$ in Cultured Uterine Epithelial Cells. *Proceedings of the National Academy of Science, USA,* **88**: 3412-3415.

Rouleau, M., Leger, J., and Tenniswood, M. 1990. Ductal Heterogeneity of Cytokeratins, Gene Expression, and Cell Death in the Rat Ventral Prostate. *Molecular Endocrinology,* **4(12)**: 2003-2013.

Sambhara, S.R., and Miller, R.G. 1991. Programmed Cell Death of T Cells Signaled by the T Cell Receptor and the α_3 Domain of Class I MHC. *Science,* **252**: 1424-1427.

Sanderson, C.J., and Glauert, A.M. 1979. The Mechanism of T-cell Mediated Cytotoxicity. VI. T-cell Projections and their Role in Target Cell Killing. *Immunology,* **36**: 119-129.

Sandritter, W., and Riede, U.N. 1975. Morphology of Liver Cell Necrosis. Pp. 1-14 in *Pathogenesis and Mechanisms of Liver Cell Necrosis,* Ed. Keppler, D. MTP Press Ltd., Lancaster.

Sarraf, C.E., and Bowen, I.D. 1986. Kinetic Studies on a Murine Sarcoma and an Analysis of Apoptosis. *British Journal of Cancer,* **54**: 989-998.

Saunders, J.W. 1966. Death in Embryonic Systems. *Science*, **154**: 604-612.

Saunders, J.W., Jr., Cairns, J.M., and Gasseling, M.T. 1957. The Role of the Apical Ridge of Ectoderm in the Differentiation of the Morphological Structure and Inductive Specificity of Limb Parts in the Chick. *Journal of Morphology*, **101**: 57-88.

Saunders, J.W., Jr., Gasseling, M.T., and Saunders, L.C. 1962. Cellular death in Morphogenesis of the Avian Wing. *Developmental Biology*, **5**: 147-178.

Savage, N.W., Kruger, B.J., and Adkins, K.F. 1985. The Effects of Fractionated Megavoltage X-irradiation on the Rat Submandibular Gland: an Assesment by Electron Microscopy. *Australian Dental Journal*, **30(3)**: 188-193.

Schoenwolf, G.C. 1981. Morphogenetic Processes Involved in the Remodeling of the Tail Region of the Chick Embryo. *Anatomy and Embryology*, **162**: 183-197.

Schulte-Hermann, R., Timmermann, I., Barthel, G., and Bursch, W. 1990. DNA Synthesis, Apoptosis, and Phenotypic Expression as Determinants of Growth of Altered Foci in Rat Liver During Phenobarbital Promotion. *Cancer Research*, **50**: 5127-5135.

Schwartz, L.M., Kosz, L., and Kay, B.K. 1990a. Gene Activation is Required for Developmentally Programmed Cell Death. *Proceedings of the National Acadamy of Sciences, USA*, **87**: 6594-6598.

Schwartz, L.M., Myer, A., Kosz, L., Engelstein, M., and Maier, C. 1990b. Activation of Polyubiquitin Gene Expression During Developmentally Programmed Cell Death. *Neuron*, **5**: 411-419.

Schwartzman, R.A., and Cidlowski, J.A. 1991. Internucleosomal Deoxyribonucleic Acid Clevage Activity in Apoptotic Thymocytes: Detection and Endocrine Regulation. *Endocrinology*, **128(2)**: 1190-1197.

Schweichel, J.U., and Merker, H.J. 1973. The Morphology of Various Types of Cell Death in Prenatal Tissues. *Teratology*, **7**: 253-266.

Searle,J., Kerr, J.F.R., and Bishop, C.J. 1982. Necrosis and Apoptosis: Distinct Modes of Cell Death with Fundamentally Different Significance. *Pathology Annual*, **17 (2)**: 229-259.

Searle, J., Lawson, T.A., Abbott, P.J., Harmon, B., and Kerr, J.F.R. 1975. An Electron-microscope Study of the Mode of Cell Death Induced By Cancer-chemotherapeutic Agents in Populations of Proliferating Normal and Neoplastic Cells. *The Journal of Pathology*, **116(3)**: 129-138.

Sentman, C.L., Shutter, J.R., Hockenbery, D., Kanagawa, O., Korsmeyer, S.J. 1991. *bcl*-2 Inhibits Multiple Forms of Apoptosis but Not Negative Selection in Thymocytes. *Cell*, **67**: 879-888.

Servomaa, K., and Rytomaa, T. 1990. UV Light and Ionizing Radiations Cause Programmed Death of Rat Chloroleukaemia Cells by Inducing Retropositions of a Mobile DNA Element (*L1Rn*). *International Journal of Radiation Biology*, **57(2)**: 331-343.

Seto, S., Carrera, C.J., Kubota, M., Wasson, D.B., and Carson, D.A. 1985. Mechanism of Deoxyadenosine and 2-Chlorordeoxyadenosine Toxicity to Nondividing Human Lymphocytes. *Journal of Clinical Investigation*, **75**: 377-383.

Shackney, S.E. 1973. A Cytokinetic Model for Heterogeneous Mammalian Cell Populations I. Cell Growth and Cell Death. *Journal of Theoretical Biology,* **38**: 305-333.

Sheldrake, A.R. 1974. The Ageing, Growth and Death of Cells. *Nature,* **250**: 381-385.

Shi, Y., Bissonette, R.P., Parfrey, N., Szalay, M., Kubo, R.T., and Green, D.R. 1991. *In Vivo* Administration of Monoclonal Antibodies to the CD3 T Cell Receptor Complex Induces Cell Death (Apoptosis) in Immature Thymocytes. *The Journal of Immunology,* **146(10)**: 3340-3346.

Sinex, F.M. 1977. The Molecular Genetics of Aging. Pp. 37-62 in *Handbook of the Biology of Aging.* Ed. Finch, C., and Hayflick, L. Van Nostrand Reinhold Co., New York.

Snow, M.H.L. 1987. Cell Death in Embryonic Development. Pp.202-228 in *Perspectives on Mammalian Cell Death.* Ed. Potten, C.S., Oxford University Press.

Sorenson, C.M., Barry, M.A., and Eastman, A. 1990. Analysis of Events Associated With Cell Cycle Arrest at G_2 Phase and Cell Death Induced By Cisplatin. *Articles,* **82(9)**: 749-755.

Sorenson, C.M., and Eastman, A. 1988. Influence of *cis*-Diamminedichloroplatinum(II) on DNA Synthesis and Cell Cycle Progression in Excision Repair Proficient and Deficient Chinese Hamster Ovary Cells. *Cancer Research,* **48**: 6703-6707.

Stanners, C.P., and Till, J.E. 1960. DNS Synthesis in Individual L-strain Mouse Cells. *Biochimica Biophysica Acta,* **37**: 406-419.

Strasser, A., Harris, A.W., Cory, S. 1991. *bcl*-2 Transgene Inhibits T Cell Death and Perturbs Thymic Self-Censorship. *Cell*, **67**: 889-899.

Steel, G.G. 1968. Cell Loss from Experimental Tumours. *Cell and Tissue Kinetics,* **1**: 193-207.

Steel, G.G. 1977. *Growth Kinetics of Tumours.* Clarendon Press, Oxford.

Stephens, L.C., King, G.K., Peters, L.J., Ang, K.K., Schultheiss, T.E., and Jardine, J.H. 1986. Acute and Late Radiation Injury in Rhesus Monkey Parotid Glands: Evidence of Interphase Cell Death. *American Journal of Pathology,* **124**: 469-478.

Strauss, B.S. 1977. Molecular Biology of the Response of Cells to Radiation and to Radiomimetic Chemicals. *Cancer,* **40**: 471-480.

Sulik, K.K., Cook, C.S., and Webster, W.S. 1988. Teratogens and Craniofacial Malformations: Relationships to Cell Death. *Development,* **103**: 213-232.

Summerbell, D. 1974a. A Quantitive Analysis of the Effect of Excision of the AER from the Chick Limb-bud. *Journal of Embryology and Experimental Morphology,* **32(3)**: 651-660.

Summerbell, D. 1974b. Interaction Between the Proximo-distal and Antero-posterior Co-ordinates of Positional Value During the Specification of Positional Information in the Early Development of the Chick Limb-Bud. *Journal of Embryology and Experimental Morphology,* **32(1)**: 227-237.

Tanaka, S., and Kato, Y. 1983a. Epigenesis in Developing Avian Scales I. Qualitative and Quantitative Characterization of Finite

Cell Populations. *The Journal of Experimental Zoology,* **225**: 257-269.

Tanaka, S., and Kato, Y. 1983b. Epigenesis in Developing Avian Scales II. Cell Proliferation in Relation to Morphogenesis and Differentiation in the Epidermis. *Journal of Experimental Zoology,* **225**: 271-283.

Tata, J.R. 1966. Requirement for RNA and Protein Synthesis for Induced Regression of the Tadpole Tail in Organ Culture. *Developmental Biology,* **13**: 77-94.

Tartaglia, L.A., Weber, R.F., Figari, I.S., Reynolds, C., Palladino, M.A., Jr., and Goeddel, D.V. 1991. The Two Different Receptors for Tumor Necrosis Factor Mediate Distinct Cellular Responses. *Proceedings of the National Academy of Sciences, USA,* **88**: 9292-9296.

Thraves, P.J., and Smulson, M.E. 1982. Acceptors for the Poly ADP-ribosylation Modification of Chromatin Structure are Altered by Carcinogen-induced DNA Damage. *Carcinogenesis,* **3(10)**: 1143-1148.

Tobias, C.A., Blakely, E.A., Ngo, F.Q.H., and Yang, T.C.H. 1980. The Repair-misrepair Model of Cell Survival. Pp.195-230 in *Radiation Biology in Cancer Research,* Ed. Meyn, R.E., and Withers, H.R. Raven Press, New York.

Tomei, L.D., Kanter, P., and Wenner, C.E. 1988. Inhibition of Radiation-Induced Apoptosis *In Vitro* by Tumor Promoters. *Biochemical and Biophysical Research Communications,* **155(1)**: 324-331.

Tomei, L.D., Shapiro, J.P., and Cope, F.O. 1993. Apoptosis in C3h/10T$^{1/2}$ Mouse Embryonic cells: Evidence for Internucleosomal DNA Modification in the Absence of Double-

strand Cleavage. *Proceeding of the National Acadamy of Sciences, USA.* **90**:853-857.

Tone, S., Tanaka, S., and Kato, Y. 1983. The Inhibitory Effect of 5-Bromodeoxyuridine on the Programmed Cell Death in the Chick Limb. *Development, Growth and Differentiation,* **25(4)**: 381-391.

Tone, S., Tanaka, S., and Kato, Y. 1988. The Cell Cycle and Cell Population Kinetics in the Programmed Cell Death in the Limb-buds of Normal and 5-Bromodeoxyuridine-treated Chick Embryos. *Development, Growth and Differentiation,* **30(3)**: 261-270.

Truman, J.W. 1984. Cell Death in Invertebrate Nervous Systems. *Annual Review of Neuroscience,* **7**: 171-188.

Trump, B.F., Goldblatt, P.J., and Stowell, R.E. 1965. Studies on Necrosis of Mouse Liver *in Vitro*: Ultrastructural Alterations in the Mitochondria of Hepatic Parenchymal Cells. *Laboratory Investigation,* **14(4)**: 343-371.

Trump, B.F., Berezesky, I.K., and Osornio-Vargus, A.R. 1981. Cell Death and the Disease Process. The Role of Calcium. Pp. 209-242 in *Cell Death in Biology and Pathology.* Ed. Bowen, I.D., and Lockshin, R.A. Chapman and Hall Ltd., London.

Umansky, S.R. 1982. The Genetic Program of Cell Death. Hypothesis and Some Applications: Transformation, Carcinogenesis, Ageing. *Journal of Theoretical Biology,* **97**: 591-602.

van der Starre-van der Molen, L.G., and Otten, L. 1974. Embryogenesis of *Calliphora erythrocephala* Meigen IV. Cell Death in the Central Nervous System During Late Embryogenesis. *Cell Tissue Research,* **151**: 219-228.

Virchow, R. 1853. *Cellular Pathology as Based Upon Physiological and Pathological Histology.* Translated by F. Chance, 1860. Churchill, London.

Vukmanovic, S., and Zamoyska, R. 1991. Anti-CD3-Induced Cell Death in T Cell Hybridomas: Mitochondrial Failure and DNA Fragmentation are Distinct Events. *European Journal of Immunology*, **21**: 419-424.

Wadewitz, A.G., and Lockshin, R.A. 1988. Programmed Cell Death: Dying Cells Synthesize a Co-Ordinated, Unique Set of Proteins in Two Different Episodes of Cell Death. *Federation of European Biochemical Societies*, **241**: 19-23.

Walker, N.I., Harmon, B.V., Gobe, G.C., and Kerr, J.F.R. 1988. Patterns of Cell Death. *Meth. Achiev. Experimental Pathology,* **13**: 18-54.

Walker, P.R., Smith, C, Youdale, T., Leblanc, J., Whitfield, J.F., and Sikorska, M. 1991. Topoisomerase II-reactive Chemotherapeutic Drugs Induce Apoptosis in Thymocytes. *Cancer Research*, **5**: 1078-1085.

Waring, P., Kos, F.J., and Mullbacher, A. 1991. Apoptosis or Programmed Cell Death. *Medicinal Research Reviews*, **11(2)**: 219-236.

Watanabe, I., and Okada, S. 1967a. Effects of Temperature on Growth Rate of Cultured Mammalian Cells (L5178Y). *Journal of Cell Biology,* **32**: 309-323.

Watanabe, I., and Okada, S. 1967b. Reproductive Death of Irradiated Cultured Mammalian Cells and its Relation to Mitosis. *Nature,* **216**: 380-381.

Waymouth, C. 1974. To Disaggregate or Not to Disaggregate. Injury and Permanent Cell Disaggregation, Transient or Permanent? *In Vitro,* **10(1 & 2)**: 97-111.

Webster, D.A., and Gross, J. 1970. Studies of Possible Mechanisms of Programmed Cell Death in the Chick Embryo. *Developmental Biology,* **22**: 157-184.

Weibel, E.R., Kistler, G.S., and Scherle, W.F. 1966. Practical Stereological Methods for Morphometric Cytology. *Journal of Cell Biology,* **30**: 23-28.

Weiss, S.A., Lester, T.L., Kalter, S.S., and Heberling, R.L. 1980. Chemically Defined Serum-free Media for the Cultivation of Primary Cells and Their Susceptibility to Viruses. *In Vitro,* **16(7)**: 616-628.

Whittaker, M., and Patel, R. 1990. Calcium and Cell Cycle Control. *Development,* **108**: 525-542.

Williams, R.J.P. 1990. The Mineral Elements in Homeostasis and Morphogenesis. *Biochemical Society Transactions*, **18**: 689-705.

Williams, G.T., Smith, C.A., Spooncer, E., Dexter, T.M., and Taylor, D.R. 1990. Haemopoietic Colony Stimulating Factors Promote Cell Survival by Suppressing Apoptosis. *Nature,* **343**: 76-79.

Wilson, D.J., and Hichliffe, J.R. 1985. Experimental Analysis of the Role of the ZPA in the Development of the Wing Buds of Wingless (*ws*) Mutant Embryos. *Journal of Embryology and Experimental Morphology,* **85**: 271-283.

Wise, L.D., and Scott, W.J. Jr. 1982. Incorporation of 5-Bromo-2'-deoxyuridine into Mesenchymal Limb-bud Cells Destined to

Die: Relationship to Polydactyly Induction in Rats. *Journal of Embryology and Experimental Morphology,* **72**: 125-141.

Wolman, M. 1985. Diferent Modes of Cell Death and Related Phenomena: A Proposal for a Meaningful Terminology in Relation to Tests of Lethality. *Journal of Theoretical Biology,*.**114**: 343-350.

Wright, N.A. 1981. The Tissue Kinetics of Cell Loss. Pp.171-209 in *Cell Death in Biology and Pathology*. Ed. Bowen, I.D., and Lockshin, R.A. Chapman and Hall, London.

Wright, N.A., and Allison, M. 1984. The Tissue Kinetics of Cell Death and Cell Loss. Pp. 211-222 in *Cell Aging and Cell Death,* Ed. Davies, I., and Sigee, C.D. Cambridge University Press, Cambridge.

Wyllie, A.H. 1980. Glucocorticoid-Induced Thymocyte Apoptosis is Associated with Endogenous Endonuclease Activation. *Nature*, **284**: 555-556.

Wyllie, A.H. 1981. Cell Death: a New Classification Separating Apoptosis from Necrosis. Pp.9-34 in *Cell Death in Biology and Pathology*. Ed. Bowen, I.D., and Lockshin, R.A. Chapman and Hall, New York.

Wyllie, A.H. 1985. The Biology of Cell Death in Tumours. *Anticancer Research,* **5**: 131-136.

Wyllie, A.H. 1986. What is Apoptosis? *Histopathology*, **10**: 995-998.

Wyllie, A.H. 1987. Apoptosis: Cell Death Under Homeostatic Control. *Mechanisms and Models in Toxicology, Archives of Toxicology, Supplement* **11**: 3-10.

Wyllie, A.H., Duvall, E., and Blow, J.J. 1984a. Intracellular Mechanisms in Cell Death in Normal and Pathological Tissues.

Pp. 269-294 in *Cell Ageing and Cell Death*, Ed. Davies, I. and Sigee, D.C. Cambridge University Press.

Wyllie, A.H., Kerr, J.F.R., and Currie, A.R. 1980. Cell Death: The Significance of Apoptosis. *International Review of Cytology*, **68**: 251-306.

Wyllie, A.H., Morris, R.G., Smith, A.L., and Dunlop, D. 1984b. Chromatin Cleavage in Apoptosis: Association with Condensed Chromatin Morphology and Dependence on Macromolecular Synthesis. *Journal of Pathology*,**142**: 67-77.

Xeros, N. 1962. Deoxyriboside Control and Synchronization of Mitosis. *Nature,* **194**: 682-683.

Yuan, J., and Horvitz, H.R. 1990. The *Caenorhabditis elegans* Genes *ced*-3 and *ced*-4 Act Cell Autonomously to Cause Programmed Cell Death. *Developmental Biology,* **138**: 33-41.

Zelenka, P.S. 1990. Proto-oncogenes in Cell Differentiation. *BioEssays,* **12(1)**: 22-26.

ACKNOWLEDGMENTS

No research project is possible without the help and understanding of a great number of people, in my case more than can be easily named. I would like to express my gratitude to the staff (academic and technical) and students of the SBS, for putting up with me for so long.

Special mention is due to several people, but most notably to Dave Burton, my supervisor, to Bill Jordan for help and advice, particularly with autoradiography, to John Miller for help and equipment for cell culturing, and to Alan Hovard for his histological know-how.

Also, particular thanks are due to Ann van der Veen, for typing my references (not without protest!), and more importantly for motivating me to finish this thesis.

ABBREVIATIONS USED IN THIS THESIS:

@	ln(2/Tc)
Aa	Tissue area at time a
Ab	Tissue area at time b
AER	Apical ectodermal ridge
BrDU	5-Bromo-2-Deoxyuridine
d	The histological half-life of apoptotic cells
Da	Cell density at time a
Db	Cell density at time b
DI	The death index (Nac/N)
Dp	The proportion of newly born cells that die
G1	The gap between mitosis and DNA synthesis
G2	The gap between DNA synthesis and mitosis
Ip	The proportion of cells in the cell cycle
Ir	The increase in the cell immigration rate
K	The thickness of an histological section
Kb	The cell birth rate
K_G	The actual increase in cell number (Growth rate)
$K_{Gt(b-a)}$	The growth rate from time a to b
K_L	The rate of cell loss
Kla	The cell loss rate due to apoptosis
Kp	The cell production rate
LI	The labelling index (Ns/N)
MI	The mitotic index (Nm/N)
N	The total cell number in the population
Nac	The number of apoptotic cells
Nc	The number of cells in the cell cycle
N_{G1}	The number of cells in G1
N_{G2}	The number of cells in G2
Nm	The number of cells in mitosis
Ns	The number of cells in S-phase
P	Proliferating cells
PCD	Programmed cell death
Q	Quiescent, non-proliferating or resting cells
ra	The average radius of apoptotic nuclei
rm	The average radius of mitotic nuclei
Ta	The histological duration of apoptosis
Tc	The duration of the cell cycle
Td	The actual doubling time of the population
T_{G1}	The duration of G1
T_{G2}	The duration of G2
Tm	The duration of mitosis
TNF	Tumour necrosis factor
Tpd	The potential doubling time of the population
TRPM-2	Testosterone-repressed prostate-messenger-2
Ts	The duration of S-phase
VBL	The line of ruffle formation and PCD in the VBW
VBW	Ventral body wall, between neck and yolk stalk.