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REVIEW

Cell death: questions for histochemists concerning the causes of the various cytological changes

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Summary

The question of cell death is accessible to study by histochemists and many questions remain to be resolved. From a physiological point of view, the most important are the causal relationships. (1) At what phase in cell death is the synthesis of RNA disrupted and at what phase is the rate of degradation of RNA increased? (2) Does the disruption of synthesis result from a direct genetic command, or does it result indirectly from gradual deterioration of energy resources or optimal ionic conditions? (3) What properties, presumably of the substrate organelles, marks them for specific absorption into autophagic vacuoles? (4) What proteases and other hydrolases operate currently undetected in the cytoplasm? How are they controlled and regulated? (5) Why does the physiologically dying cell shrink and appear more dense? To what extent is a cell in this state able to regulate any metabolic parameter? The advent of newer, more sensitive and quantitative techniques, and greater attention to the controls and causes as opposed to the phenomena, should help to resolve these questions.

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Introduction

For the physiologist, histochemistry provides answers unattainable from *in vivo* studies or biochemistry. In the study of cell death, for instance, cytologists and histochemists have defined the autophagic vacuole and indicated its specificity (Locke & Collins, 1965; Beaulaton, 1967; Locke & Sykes, 1975; Beaulaton & Lockshin, 1977); recognized the phenomenon of apoptosis (Kerr, 1973) and helped us to understand the order of shutdown of synthetic events (Pollak & Fallon, 1976; Fallon *et al.*, 1980). Physiology, however, is a science of causes and controls, and the very precision of the histochemist's results raises new questions and reveals how much further we must carry our analyses. Happily, several new techniques are now coming on line, most notably the use of fluorescent substrates and computer-assisted monitoring and quantification of results, and it is to be hoped that these techniques will lead to a higher level of understanding. It will be gratifying, if ironic, to find that this precise science of the study of fixed tissues will ultimately explain the mechanisms of control in the living tissue.

We now know that many types of cells die under physiological control (Lockshin, 1980, 1981; Wyllie, 1981; Hinchliffe, 1981; Levi-Montalcini & Aloe, 1981; Munck & Crabtree, 1981; Bowen, 1981) and that the destruction of their cytoplasm is carried out in autophagic vacuoles, the relative temporal and quantitative importance of these latter depending on tissue, species and situation. Often in developmental situations impending cell death is presaged by the cell's dropping out of the mitotic cycle (Fallon *et al.*, 1980). We do not understand the intimate causes of these events, and here our histochemical techniques are yet lacking. The problems can be illustrated by the fate of the intersegmental muscles of silkmoths and Sphingid moths, and are summarized in roughly the chronological order in which they appear. The intersegmental muscles are destroyed during the first two days after the moth emerges from its pupal shell; the synchronous death of the cells, as defined by pycnosis and collapse of the resting potential, occurs 15–18 h after emergence (Lockshin & Beaulaton, 1979).

How does synthesis and breakdown of nucleic acids relate to cell death?

The death of the cell seems to result from a physiological demand with which the cell cannot cope (Bidlack & Lockshin, 1976; Finn & Lockshin, 1980). It is not clear whether the demand arises from intracellular or extracellular sources; nor is it clear whether the demand–here hypothesized to be extertion in an hypoxic state–is unusual or the response is inadequate. In many other systems, most notably the silk glands of *Bombyx mori*, there appears to be an early failure of the synthesis of messenger and perhaps other RNAs, and a rapid autophagic destruction of much of the RNA (Matsuura *et al.*, 1968; Okabe *et al.*, 1975; Fournier, 1979). Thus arises the first question: Does the failure of synthesis of RNA derive from a direct genic command, or does it result from a changed cytoplasmic and nuclear milieu? In neither case do we have means of resolving these questions by histochemical techniques. We obviously need far more knowledge of the

histones and nucleic acids of involuting tissues. The synchronous involution of metamorphosis and the isolation and cloning of specific genes will ultimately provide enough material for the analysis of specific genes and gene products, but the histochemist can explain the milieu of the nucleus in which these events occur. Electron probe analysis can contribute a knowledge of the ionic milieu, and we await interpretations of the localization and activity of specific genes during this period.

What activates lysosomes and determines their specificity?

During the early phases of involution, the function of lysosomes is evident but the control is not. Two features are particularly striking: first, at any given time, lysosomes may be observed to contain only one type of organelle (Locke & Sykes, 1975; Beaulaton & Lockshin, 1977; Fig. 1). Second, in some insects, a pronounced but not lethal regression which accompanies larval molts manifests non-lysosomal destruction of organelles (Locke & Sykes, 1975; Matsuura & Tashiro, 1976) while at metamorphosis the same organelles may be swept away by waves of autophagic destruction. Non-lysosomal destruction is also seen in the intersegmental muscle: during the early phases of lysis, myofilaments are lost from intact, contractile cells, and none are seen in autophagic vacuoles. Flagrant development of autophagic vacuoles is rare in muscles (Lockshin *et al.*, 1980). In insects (Colon, 1980) and tadpoles (Chin, 1980) the accumulation of intermediate degradation products of myosin indicates that proteolysis occurs in several steps.

How much non-lysosomal digestion takes place?

These phenomena raise several questions: What non-lysosomal proteinases operate within the cytoplasm? How are they activated and controlled? Do lysosomal enzymes escape from the lysosome, and can they function in the cytoplasm? In tadpole tail, as in mammalian muscle, calcium-activated protease requires unphysiologically high levels of calcium, but cyclic adenosine monophosphate lowers the requirement ten-fold (Chin, 1980). Perhaps other co-factors also play a role. The use of the extraordinarily sensitive fluorescent dye substrates, coupled to appropriate insoluble salts (Smith & van Frank, 1975; Dorsey, 1980) will help resolve these questions, as will the isolation of some of the enzymes and the preparation of antibodies to them (Barrett, 1977). Although the recent use of *p*-nitrophenyl phosphate as substrate has helped to re-open the question of the distribution of lysosomal phosphatases (Bowen & Ryder, 1976; Bowen, 1981), ultimately the localization of the enzymes will be best defined by antibodies. Enzymes may also be found under appropriate circumstances by the use of marker substrates or inhibitors to which they bind; such techniques have been used for the purification of enzymes (Chua & Bushuk, 1969; Smith & Turk, 1974) and some histochemical procedures.

From a logical standpoint, it would seem more reasonable to relate the specificity with

which organelles are scavenged to a flaw in the organelle rather than specificity of the autophagic membrane. Recent evidence has related phagocytosis of foreign particles to the surface chemistry of the particle, particularly the presence of specific glyoproteins or carbohydrate moieties (Böhmer *et al.*, 1979) or as influenced by cyclic compounds (Kielan & Cohn, 1980; Oates & Touster, 1980). It is theoretically possible that a damaged organelle also develops an alteration of surface chemistry, perhaps in a protective component of high turnover rate, so that, the damaged organelle not being able to replace the component, its membrane automatically manifests a signal for destruction. The histochemistry of surface proteins is a field urgently in need of more specific techniques. Scanning electron microscopy has revealed many subtle alterations of the surfaces of cells as they change activity; we need to know more about their chemistry.

Why do dying cells shrink?

Another phenomenon of interest is the shrinkage of the cell. Traumatically injured cells swell and lyse, as one might expect from the hypothesis of an accumulation of acid intermediates of anaerobic glycolysis, but physiological death is characterized by shrinkage necrosis or apoptosis, in which the cell condenses (Wyllie, 1981). The mechanism of this shrinkage is not understood. Although some of the apparent density at light and electron levels may be an artifact owing to increased exposure of binding sites for stains at acid pH, there appears to be a very real contraction of cell size (Lockshin, 1980, 1981; Trump *et al.*, 1981; Fig. 2). Many authors consider the cell to be capable of exerting some control at this time, in which case one might hypothesize contraction of the cytoskeleton or precipitation at acid isoelectric points of major proteins. Otherwise a passive loss of water would imply a previous hypotonic state, which is difficult to explain. Increasing acidity would permit exodus of K^+ from the cell and, although several assumptions would be necessary relating to the movement of anions, it would be possible to derive the loss of water from this process. In insect muscle, this phase does not develop until the cell depolarizes (Lockshin & Beaulaton, 1979). Other authors

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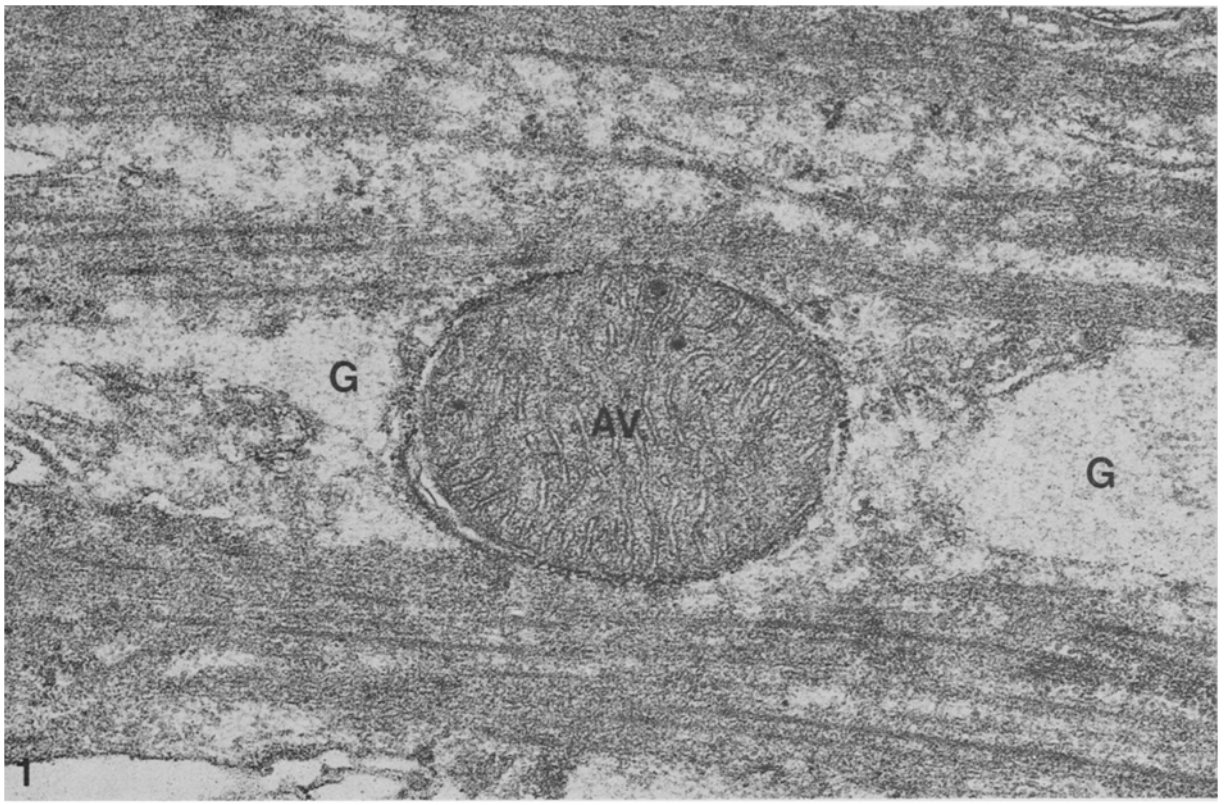
Figs. 1 and 2. Electron micrographs of intersegmental muscles from the silkmoth *Antheraea polyphemus* fixed according to the procedure of Peracchia & Mittler (1972).

Fig. 1. Longitudinal section of a muscle fibre 7 h after emergence showing an autophagic vacuole (AV) with a mitochondrion entirely sequestered. The figure illustrates the osmiophilic aspect of isolation membranes and the beginning of disorganization in the contractile material. Note the electron-lucent glycogen (G) in the interfibrillar sarcoplasm. This configuration is the most common form of autophagic vacuole between 7 and 10 h.

Figs. 2. Low magnification photograph illustrating the contraction of fibre size 22 h after emergence. At this phase of degeneration the contractile material has entirely disappeared and the residual sarcoplasm contains numerous autophagic vacuoles (AV) sequestering ribosomes. Note the sarcoplasmic delamination (SD). Such a cell would also have a pycnotic nucleus. Similar condensed cells are seen in many forms of physiological or programmed cell death.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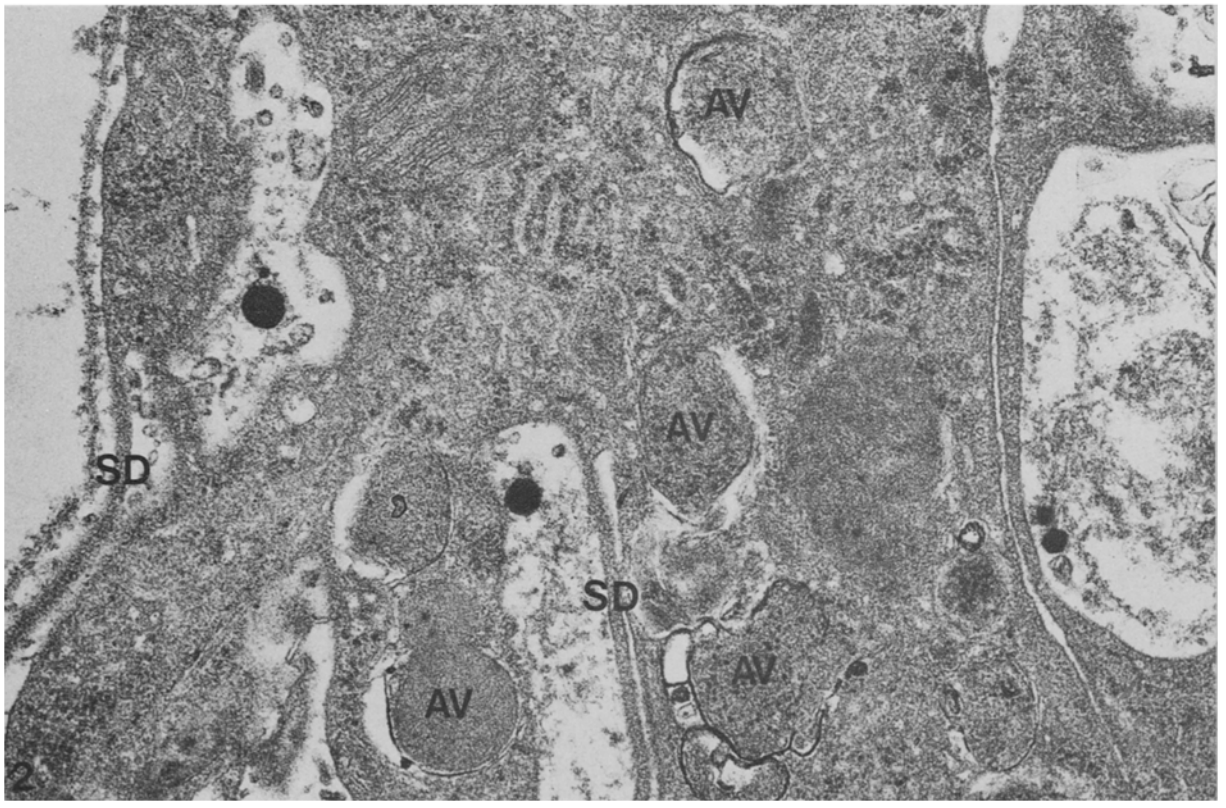
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feel that pycnosis occurs in cells which have lesions of sufficient size that tracers such as ethidium bromide (Bank & Mazur, 1972) or lanthanum (Humbert, 1978) can penetrate. Such lesions are envisaged as being smaller than that required to swamp the cell immediately, but slightly larger than that with which the cell can cope. Thus, over a period ranging from hours to days, the cell gradually loses the battle, and finally succumbs (Bank & Mazur, 1972; Humbert, 1978; Ross *et al.*, 1981). Since this argument is theoretically quite different from that forming the basis of the concept of apoptosis, it would be very useful for more histochemists to examine pycnosis as the starting point of a query, rather than a manifestation of an event which has occurred.

To what extent are histochemical results quantifiable?

Finally, although the advent of newer substrates is gradually making the point moot, it would be helpful if more histochemists allied themselves more closely to physical chemists. It is perturbing, for instance, to find lead salt techniques so poorly understood. Most techniques for identifying acid phosphatase, modifications of the Miller–Palade technique, involve addition of lead nitrate to a solution of sodium β-glycerophosphate, whereupon the mixture is heated and filtered. The precipitate removed is lead phosphate, formed from the degradation of the organic phosphate during storage of the stock solution. If sodium fluoride is added to the incubation medium rather than to the tissue section separately, a further precipitate of lead fluoride may form. Assuming no loss of lead at the first step and calculating from solubility products, a presumed control would in fact contain as little as one-third of the lead that the experimental solution contains. Although admittedly under the conditions of preparation the experimenter must be working with a solution near the saturation point of lead phosphate, the variability in the actual concentration of lead would change the concentration of phosphate necessary to precipitate the lead by a factor equal to $[Pb^{2+}]^{3/2}$, or 6–7 times as much phosphate would be necessary. In the absence of fluoride, free contaminant phosphate ranging in concentration from 1 μM to 1 mM would leave residual lead at concentrations of 250–25 M respectively, and these levels of lead would require 1 and 20 μM phosphate for precipitation. It would be much more satisfying to work with well-understood conditions and concentrations.

These several reflections represent some of the questions perceived by a physiologist and a cytochemist. Undoubtedly others will be on the minds of other scientists, and undoubtedly the naivety of some of these questions reflect our ignorance of the field. Even so, in this period of a surfeit of information, perhaps these remarks will serve to maintain the dialogue.

References

BANK, H. & MAZUR, P. (1972) Relation between ultrastructure and viability of frozen-thawed chinese hamster tissue-culture cells. *Exp. Cell Res.* **71**, 441–54.

BARRETT, A. J. (1977) Cathepsin D and other carboxyl proteinases. In *Proteinases in Mammalian Cells and Tissues* (edited by BARRETT, A. J.), pp. 209–248. Amsterdam, New York: North-Holland.

BEAULATON, J. (1967) Localisation d'activités lytiques dans la glande prothoracique du ver à soie du chêne (*Anthéraea pernyi* Guer.). II. Les vacuoles autolytiques (cytolysomes). *J. Microscopie* **6**, 349–70.

BEAULATON, J. & LOCKSHIN, R. A. (1977) Ultrastructural study of the normal degeneration of the intersegmental muscles of *Antheraea polyphemus* and *Manduca sexta* (Insecta, Lepidoptera) with particular reference to cellular autophagy. *J. Morphol.* **154**, 39–58.

BIDLACK, J. M. & LOCKSHIN, R. A. (1976) Evolution of LDH isozymes during programmed cell death. *Comp. Biochem. Physiol.* **55B**, 161–6.

BÖHMER, F. D., KIRSCHKE, H., BOHLEY, P., & SCHÖN, R. (1979) Membrane sialoglycoprotein from human erythrocytes activates lysosomal proteinases. *Acta biol. med. germ.* **38**, 1521–6.

BOWEN, I. D. (1981) Techniques for demonstrating cell death. In *Cell Death in Biology and Pathology* (edited by BOWEN, I. D. and LOCKSHIN, R. A.), pp. 379–444. London: Chapman and Hall (in press).

BOWEN, I. D. & RYDER, T. A. (1976) Use of the *p*-nitrophenyl phosphate method for the demonstration of acid phosphatase during starvation and cell autolysis in the planarian *Polycelis tenuis* Iijima. *Histochem. J.* **8**, 319–29.

CHIN, W. (1980) *A study of the proteolytic enzyme system involved in the tail resorption of* Xenopus *tadpoles.* M.S. thesis, St. John's University, Jamaica, New York.

CHUA, G. K. & BUSHUK, W. (1969) Purification of wheat proteases by affinity chromatography on hemoglobin-sepharose column. *Biochem. biophys. Res. Commun.* **37**, 545–50.

COLON, A. D. (1980) *Proteolysis and degradation of myosin during the involution (programmed cell death) of the intersegmental muscles of* Manduca sexta. Ph.D. thesis, St. John's University, Jamaica, New York.

DORSEY, A. M. (1980) *The role of lysosomal proteinases in the programmed cell death of intersegmental muscles in* Manduca sexta. Ph.D. thesis, St. John's University, Jamaica, New York.

FALLON, J. F., SIMANDL, B. K., & BOUTIN, E. L. (1980) Histochemistry of the posterior necrotic zone of the chick wing bud. *Abst. VIth Int. Histochem. Cytochem. Cong. 1980*, pp. 115–6.

FINN, A. F., JR & LOCKSHIN, R. A. (1980) Activation by anoxia of latent lactic acid dehydrogenase isozymes in insect intersegmental muscles (*Manduca sexta*, Sphingidae) *Comp. Biochem. Physiol.* **68C**, 1–7.

FOURNIER, A. (1979) Quantitative data on the *Bombyx mori* L. silkworm: a review. *Biochimie* **61**, 283–320.

HINCHLIFFE, J. R. (1981) Cell death in embryogenesis. In *Cell Death in Biology and Pathology* (edited by BOWEN, I. D. and LOCKSHIN, R. A.), pp. 35–77. London: Chapman and Hall (in press).

HUMBERT, W. (1978) Intracellular and intramitochondrial binding of lanthanum in dark degenerating midgut cells of a collembolan (insect). *Histochemistry* **59**, 117–28.

KERR, J. F. R. (1973) Some lysosome functions in liver cells reacting to sublethal injury. *Lysosomes in Biology and Pathology*, Vol. 3 (edited by DINGLE, J. T.), pp. 365–394. Amsterdam: North-Holland.

KIELAN, M. C. & COHN, Z. A. (1980) Phagosome-lysosome function. Characterization of intracellular membrane fusion in mouse macrophages. *J. Cell Biol.* **85**, 754–65.

LEVI-MONTALCINI, R. & ALOE, L. (1981) Mechanism(s) of action of nerve growth factor in intact and lethally-injured sympathetic nerve cells in neonatal rodents. In *Cell Death in Biology and Pathology* (edited by BOWEN, I. D. and LOCKSHIN, R. A.), pp. 295–328. London: Chapman and Hall (in press).

LOCKE, M. & COLLINS, J. V. (1965) The structure and formation of protein granules in the fat body of an insect. *J. Cell Biol.* **26**, 857–84.

LOCKE, M. & SYKES, A. K. (1975) The role of the Golgi complex in the isolation and digestion of organelles. *Tissue and Cell* **7**, 143–58.

LOCKSHIN, R. A. (1980) Aging, cell death and lysosomes. In *Handbook Series in Aging, Section D: Biological Sciences, Vol. II. Cell Biology* (edited by CRISOFALO, V. J.), Boca Raton, Florida: CRC Press, Inc. (in press).

LOCKSHIN, R. A. (1981) Cell death in metamorphosis. In *Cell Death in Biology and Pathology* (edited by BOWEN, I. D. and LOCKSHIN, R. A.), pp. 79–122. London: Chapman and Hall (in press).

LOCKSHIN, R. A. & BEAULATON, J. (1979) Programmed cell death. Cytological studies of dying muscle fibers of known physiological parameters. *Tiss. Cell* **11**, 803–9.

LOCKSHIN, R. A., COLON, A. D. & DORSEY, A. M. (1980) Control of muscle proteolysis in insects. *Fed. Proc.* **39**, 48–52.

MATSUURA, S., MORIMOTO, T., NAGATA, S. & TASHIRO, Y. (1968) Studies on the posterior silk gland of the silkworm, *Bombyx mori.* II. Cytolytic processes in posterior silk gland cells during metamorphosis from larva to pupa. *J. Cell Biol.* **38**, 589–603.

MATSUURA, S. & TASHIRO, Y. (1976) Ultrastructural changes in the posterior silk gland cells in the early larval instars of the silkworm, *Bombyx mori. J. Insect Physiol.* **22**, 969–79.

MUNCK, A. & CRABTREE, G. R. (1981) Glucocorticoid-induced lymphocyte death. In *Cell Death in Biology and Pathology* (edited by BOWEN, I. D. and LOCKSHIN, R. A.), pp. 329–360. London: Chapman and Hall (in press).

OATES, P. J. & TOUSTER, O. (1980) *In vitro* fusion of *Acanthamoeba* phagolysosomes. IV. Evidence that cyclic nucleotides and vacuole sub-populations respectively control the rate and extent of vacuole fusion in *Acanthamoeba* homogenates. *J. Cell Biol.* **85**, 804–10.

OKABE, K., KOYANAGI, R. & KOGA, K. (1975) RNA in the degenerating silk gland of *Bombyx mori. J. Insect Physiol.* **21**, 1305–9.

PERACCHIA, C. & MITTLER, B. S. (1972) Fixation by means of glutaraldehyde-hydrogen peroxide reaction products. *J. Cell Biol.* **53**, 234–8.

POLLAK, R. D. & FALLON, J. F. (1976) Autoradiographic analysis of macromolecular synthesis in prospectively necrotic cells of the chick limb bud. II. Nucleic acids. *Expl Cell Res.* **100**, 15–22.

ROSS, M. W., YAMAMOTO, R. S. & GRANGER, G. A. (1981) The role of the LT system in lymphocyte-induced cell destruction *in vitro.* In *Cell Death in Biology and Pathology* (edited by BOWEN, I. D. and LOCKSHIN, R. A.), pp. 361–378. London: Chapman and Hall (in press).

SMITH, R. E. & VAN FRANK, R. M. (1975) The use of amino acid derivatives of 4-methoxy β-naphthylamine for the assay and subcellular localization of tissue proteinases. In *Lysosomes in Biology and Pathology*, Vol. IV, (edited by DINGLE, J. T. and DEAN, R. T.), pp. 194–249. Amsterdam: North-Holland.

SMITH, R. & TURK, V. (1974) Cathepsin D: Rapid isolation by affinity chromatography on haemoglobin–agarose resin. *Eur. J. Biochem.* **48**, 245–54.

TRUMP, B. F., BEREZESKY, I. K. & OSORNIO-VARGAS, A. R. (1981) Cell death and the disease process. In *Cell Death in Biology and Pathology* (edited by BOWEN, I. D. and LOCKSHIN, R. A.), pp. 209–242. London: Chapman and Hall (in press).

WYLLIE, A. (1981) Cell death: A new classification separating apoptosis from necrosis. In *Cell Death in Biology and Pathology* (edited by BOWEN, I. D. and LOCKSHIN, R. A.), pp. 1–34. London: Chapman and Hall (in press).