Meeting Report

CHALONES *

A report on the International Chalone Conference held at Brook Lodge, Augusta, Michigan, during 5–7 June, 1972

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1. Introduction

It has long been known that the rate of cell division in adult tissues is well controlled, and is at a level characteristic for the cell type concerned. Dramatic changes in the incidence of mitosis can however occur in response to certain circumstances, especially the increase which follows removal of part of the existing cell population, such as during the healing of a skin wound or the regeneration which follows partial hepatectomy. Theories have at various times been put forward to account for this phenomenon; during the last decade experimental attention has been attracted by the 'chalone' concept, that is, that cells produce mitotic inhibitors which specifically repress division in their own type. It is postulated that when some of the cells are taken away, their chalone production will be lost so that the general level will fall and mitosis will be permitted until enough cells are again present to sustain an inhibitory concentration.

The chalone concept was originally developed by *W. Bullough* (Birbeck College, London) and *O.H. Iversen* (Rikshopitalet, Oslo) and their collaborators to explain the local growth regulation system of the epidermis. In this tissue a cellular layer of constant thickness is maintained, with cell proliferation balanced to a standard rate of loss but able to adjust to perturbations caused by sudden cell removal, damage or death. When some of the epidermis of a mouse ear is removed, high mitotic activity follows on the opposite but undamaged side of the ear, and is most marked directly across from the wound. This observation, together with the finding of mitotic inhibitors in tissue extracts, argued that mitosis was not being stimulated by the secretion of a wound hormone, but rather being released from blockage by the absence of a local inhibitor. To denote the proposed messenger in these and similar effects, *Bullough* resurrected from the early endocrinological literature the term 'chalone', from χαλάω, to make slack, in contrast to 'hormone', from ορμάω, to arouse. Towards the end of the Brook Lodge meeting, the definition and general proporties of the term 'chalone' were discussed, and general agreement was reached that chalones were inhibitors affecting the cell cycle before mitosis, they were tissue-specific and present in the tissue of action, but need not be species-specific. Some other apparently common properties have been described, but need be no part of a definition at this stage.

2. Epidermal chalone(s)

A substantial session centered on studies on the epidermis, as the original and the most comprehensive studies have been preformed on the tissue. *Edna Lau-*

* The proceedings of the meeting will be edited by B. Forscher and published as a supplement to the Journal of the National Cancer Institute in early 1973.

Abbreviations. S denotes the phase of the cell cycle when DNA is synthesized, G1 the post-mitotic period which preceeds S, and G2 the period between S and the onset of M, or mitosis.

rence (Birkbeck College, London) reviewed the development from early work on epidermal mitosis to the formulation of the chalone concept and the current experimental approach. Work has progressed from the original finding that skin extracts could suppress mitosis *in vitro*, especially when the tissue was also treated with adrenalin. It has by now been shown that the active factor is specific for epidermis, is present in a wide range of skins (as well as in the urine) and is remarkably species non-specific. The antimitotic effect is reversible, and seems to block during the G2 phase of the cell cycle, but may also act during G1. Purification has demonstrated a glycoprotein which is inactivated by high pH and trypsin, but not pepsin. In a following annotation, *O.H. Iversen* described an *in vivo* system more dramatic than the mouse ear for demonstration of tissue effects on epidermal mitosis. Fruit bats were taken, and the epidermis removed from one side of the wing by repeated stripping with adhesive tape. After 4 days, there was a marked epidermal hyperlasia on the other side of the wing, presumably because much of the adjacent chalone source had been removed. This effect was abolished by thorough application of skin extracts to the wound.

J. Voorhees (Ann Arbor) discussed the differentiation sequence epidermal cells. In psoriasis, the process is accelerated. The levels of cyclic AMP were followed in normal and diseased tissue in relation to division and differentiation, and it was suggested that lowered cyclic AMP levels in psoriasis allowed the greater mitotic rate in this condition, although there were no obvious genetic defects in the relevant enzymes. *Edna Laurence*, in her second contribution, stressed certain biological problems in working with epidermal chalones. Care must be taken that circadian rhythms, or the level of activity of the experimental animals, do not influence the results. 'Skin' is a term which encompasses a range of related tissues; there are major local differences in division rate, the ability to undergo transplantation modulation, and other parameters.The potentiating effects of adrenalin and hydrocortisone on chalone effects vary with the type of epidermis, thereby requiring the use of adrenalectomized animals for many studies. *Laurence* herself has come to regard chalones not as mere mitotic inhibitors, but also as promoters of specific pathways of differentiation. If this is so, the particular features of seperate subtypes of tissue will influence the demonstration of any chalone or antichalone effect.

K. Elgio (Oslo) discussed the action of epidermal chalones on the cell cycle. An epidermal extract gave substantial reversible blocking in G1 and G2, but none at all on the S phase. However, if an extract was made of only the keratinizing cells, a factor was demonstrated which did inhibit only the onset of DNA synthesis. This emphasized the difficulties of analysing a tissue situation in which a number of cellular activities were in progress, and where there seemed to be at least two chalone factors produced. In a subsequent annotation, which attracted much interest in later discussions, Dame *Honor Fell* (Cambridge) spoke of her studies on the differentiation of embryonic chicken skin. In culture, the administration of excess vitamin A causes an otherwise keratinizing epithelium instead to become of the mucus-producing type with continued mitosis. Since vitamin A labilizes lysosomal membranes and liberates the enclosed enzymes, she suggested that lysosomal enzymes could be responsible for the gradation of chalones and the ensuing release of mitosis.

F. Marks (Heidelberg) described his thorough and continuing attempt to purify a chalone. He chose the G1 inhibitor of pig skin, which can be assayed by its effect on the incorporation of labelled thymidine into the DNA of epidermal cells *in vitro*. An aqueous extraction was followed by alcohol fractionation, giving a product which stayed in the phenol phase of a water–phenol extraction. The activity behaved as if it were in a heterogeneous fraction with a molecular weight of about 200,000, yet survived a number of drastic treatments such as boiling at pH 6 or digestion with a number of enzymes. After pronase digestion, the molecular weight dropped to 10,000–20,000, but without loss of biological potency. Marks proposed a scheme by which the chalone would normally be sequestered in a layer outside the plasma membrane proper until freed by the death or displacement of adjacent cells; this model would be compatible with the behaviour of the chalone fraction during purification.

S. Rothberg (Virginia) has used embryonic chicken skin in organ culture to study mitotic control mechanisms. There is initially much mitosis in embryonic epidermis, but after about 13 days of incubation the rate falls. Extracts of older skin from this or other sources will inhibit mitosis in younger skin, espe-

cially if adrenalin is added, but attempts to purify the factor concerned have proven difficult, with some activity appearing in many fractions, if the inhibition of thymidine incorporation into DNA is used as the assay technique.

W. Bullough (London) discussed how chalones could act as controlling agents in the biology of the epidermis. In many, but not all, mammals, changes in the thickness of the skin are accompanied by changes in the mitotic rate. If the rate increases somewhat, hyperplasia ensues; if further, carcinoma. The mitotic index depends firstly on the speed at which divisions follow each other and secondly on the proportion of the basal cell population which will divide. There are several possible sites for controlling mechanisms to operate here: on the mitotic population itself, on the recruitment of cells to the mitotic population, and on the non-mitotic differentiation pathway. *Bullough* considers that cells may respond to changes in the chalone concentration rather than to the absolute level.

3. Chalones of haemopoietic tissue

L. Lajtha (Christie Hospital, Manchester) reviewed the differentiation of blood cells in the small rodent, and the possible sites at which chalones could affect proliferation or maturation of erythrocytes, lymphocytes and granulocytes. The difficulties of looking for cell-specific mitotic inhibitors in a tissue as complex as bone marrow were emphasized, and pleas made for colony assay methods for a convenient but conclusive demonstration of such effects, and for incubation times long enough to reveal G1 as well as S phase inhibitors. *J.C. Houck* (Children's Hospital, Washington) described the isolation from lymphoid tissue of a substance which inhibits DNA synthesis in lymphocytes without being merely cytotoxic. Comparable extracts from other tissues show little such effect, while the lymphoid factor is not inhibitory to other cell types. If precautions are taken to remove cathepsins and oxidizing lipids, the inhibitor can be partially purified and turns out to have a molecular weight in the range 30,000–50,000 daltons and to be trypsin-sensitive. It seems that at least some lines of leukaemic lymphocytes can make a little chalone, and can respond to it to some extent, but are deficient in their ability to bind it. Inhibition of immune responses by partially purified lymphoid extracts have been demonstrated by *E. Garcia-Giralt* (Villejuif), *A. Chung* (Georgetown University) and *Nicole Kiger* (Villejuif), and some reduction in the growth of lymphoid organs noted after repeated administration of these preparations.

T. Rytömaa (Helsinki) has studied the effects of a low molecular weight endogenous factor on granulocyte production in perfusion chambers. This factor met the requirements of a chalone in that it was cell type but not species specific, it inhibited DNA synthesis but was not cytotoxic, and its effects were short-lived and reversible. *W.R. Paukovits* (Vienna) has studied the purification and chemical nature of this substance. There were clearly considerable difficulties in the isolation of sufficient material, and much remains to be done, but results so far indicate a peptide of 20 to 30 residues, all hydrophilic, with many acidic but no sulphur amino acids. Its biological activity appears to be confined to immature members of the granulocytic series.

4. Miscellaneous chalones

Throughout the history of cell culture, serum has been known to promote division in the *in vitro* fibroblast and the closer the cell is to its original karyotype, the more it seems to require a serum factor. *J.C. Houck* discussed both factors which promote fibroblast growth and factors which limit it. That which allows diploid fibroblast proliferation in serum-free medium is extracted from serum, has a molecular weight of 116,000–120,000, is composed of 2 subunits and possesses substantial sulphydryl and aromatic amino acids and 2 moles of sialic acid. The chalone factor, that which depresses fibroblast growth, is endogenous and is a potent inhibitor of DNA synthesis and mitosis, and has a molecular weight of 30,000–50,000. This superficial similarity to the lymphocytic chalone does not extend further, however, as it is without effect on lymphocyte transformation.

Liver regeneration after partial hepatectomy is a standard experimental system for the investigation of growth regulation. *W. Verly* (Montreal) has partially purified from liver a low molecular weight soluble factor, perhaps a cyclic peptide, which depresses thymidine uptake by liver but not other organs. *M.P. Stack-Dunne* (Mill Hill) stressed the complexity of the evidence for factors controlling liver growth, and the

possible plurality of agents affecting the various events in the cell cycle.

With both lung and kidney, unilateral excision leads to compensatory hyperplasia. *D.P. Chopra* (Temple University, Philadelphia) described how extracts of these organs depressed division in their type tissue, especially if stress hormones were added. If antiserum to tissue extract was added to the culture medium for the relevant organ, mitosis was promoted, perhaps because an endogenous chalone was neutralized.

Feedback control of division may be apparent even with malignant cells, in that aspiration of an ascites tumour can promote a burst of division, which again slows down as cells reach a characteristic density. As *P. Bichel* (Aarhus) described, extracts of ascites tumours can inhibit the proliferation of cells which would otherwise exhibit exponential growth. At least 2 factors seem to be concerned: a low molecular weight (<10,000) agent which blocks in G2, and a larger (10,000–50,000) factor which inhibits G1. Another small factor was reported by *D.L. Dewey* (Mount Vernon Hospital, Middlesex) which inhibited the multiplication of the Harding–Passey melanoma in culture; this possible melanocyte chalone was smaller than 10,000 daltons and was sensitive to attack by trypsin, chymotrypsin and neuraminidase.

In the final sessions, some speakers described aspects of cellular control mechanisms which might be related to chalone studies. *M. Smulson* (Washington) has investigated nuclear poly-ADP ribosylation and its possible significance during the cell cycle. A nuclear polymerase, using NAD as a substrate, covalently links ADP to chromatin and polymerises on further units with the freeing of nicotinamide. ADPR polymerase activity was highest in M and G1, and could be restricted by end-product inhibition with nicotinamide, as well as treatment with actinomycin D or cordycepin. DNA was required for the polymerization; but when polymerization was complete the product inhibited DNA synthesis, and from this latter observation speculations can be made about the role of poly-ADPR in the cell cycle and its relationship to chalone action.

B.R. Rabin (University College, London) described how carcinogens can disrupt cellular controls by interfering with ribosome–endoplasmic reticulum interaction. Steroids are involved in this binding, but can be displaced by substances such as aflatoxin. The effects of this displacement can be monitored *in vitro* by following rearrangease activity, and its consequences observed *in vivo* in the loss of export protein production and the development of tumours. A more abstract view of control mechanisms was taken by *J. Bard* (Edinburgh), whose heuristic model of chalone action illustrated sharply how much is unknown. How stable is a chalone? How well does it equilibrate between blood and tissue, how much its action attributable to effects on division rather than cell loss, and do antichalones complicate the story? As *Bard* pointed out, theoretical discussions focus attention on such problems, rather than solve them outright.

5. Some general points and conclusions

An initial difficulty in dealing with reports of chalone effects is the apparent finding of various inhibitory substances which affect different stages of the cell cycle. However, it might well be that there is no *a priori* reason to predict that a chalone must act on any particular stage in the cell cycle; in some situations a block might exist at G1, in others, circumstances might favour a halt in S or G2. *G. Mueller* (Madison) reviewed the multiplicity of events during G1 and S, and gave his general scheme by which events at the cell surface could influence nuclear events such as the initiation of DNA synthesis. In this model, the plasma membrane would be able to take up certain molecules, possibly including chalones, from the intercellular environment and in response release others which would influence the replication or expression of its own intracellular genetic material at many stages of the cycle. There is a methodological corollary to this line of argument about the complexity of cellular events, though, in that those who follow thymidine incorporation rather than mitosis may miss the effect of G2 inhibitors, or even of G1 inhibitors if they employ a short assay time and there is a longer delay before the onset of cell division.

Another difficulty lies in the complexity of tissues. Especially when stem cells are studied, it may not be easy to recognize the types which can respond to an extract of an adult tissue, while other cell types may well fail to respond and mask the effects. It is in situations like this that the agar colony techniques advocated by *Lajtha* may be invaluable.

Lack of knowledge of the basic biology of an organ may well hinder studies. In their work on the skin, *Bullough* and *Lawrence* have been forced to reinvestigate the division and differentiation sequence of epidermal cells and have found previously unknown site and species differences. In the case of the hypertrophy of one lung after the excision of the other, *J.D. Simnett* (Newcastle) has come up with some further difficulties. In the rat, cells in the contralateral lung are stimulated to division and differentiation, although some of the histology is unexpected in that new alveoli are produced from bronchial tissue. In organ culture, crude extracts of lung will inhibit the onset of M from G2, while extracts of other tissues are without effect, but in the intact animal, the physiology of lung hyperplasia can be shown to be more than a mere question of chemical messengers. Here excision of one lung fails to lead to growth of the other, if the cavity had been filled with a moulded sponge. On the other hand, simple collapse of a lung promotes mitosis in the other. *Simnett* also drew attention to a physiological parameter which affects whole animal experimentation, that is, the changes in vascularity which occur, and which can alter the clearance rates and local concentrations of substances.

Especial interest was shown in the attempts to purify chalones by *Marks* and by *Paukovits*. A major difficulty met by all investigators in this field is in the complexity of the assay methods available. To start with a crude extract, with many biologically active molecules present, and to monitor each fraction for an effect demonstrable only by following the division of one cell type is no mean task. Not surprisingly, only small amounts have yet been purified, but many scientists will await with great interest the preparation of enough material to elucidate fully the chemical nature and physiological significance of these substances. So far, the chalones from various sources seem to be of two chemical types: glycoproteins with a molecular weight of 30,000–50,000 and peptides of a tenth or less of that size..

If chalones control growth, can they be used for cancer therapy? Some promising results have been reported, especially on rat chloroleukaemia by *Rytőmaa* and his collaborators. There seems to be no reason why a chalone should destroy a tumour, as *Iversen* has pointed out, but *Rytőmaa* and others feel that the check imposed by the administration of a growth regulatory substance may be enough to allow other defensive mechanisms the chance to reject a neoplasm.

Formidable difficulties are encountered by the experimentalist in any attempt at definitive demonstration and characterization of a chalone as the major growth-regulating factor for any given tissue. Rigorous criteria need to be met in the experimental design, the biological effects may be complicated, and the laboratory work may be difficult, tedious and by no means certain to yield clear-cut conclusions. Yet an increasing number of investigators are being directly stimulated by the chalone concept and even more will await their results with interest.