Review Letter

# CHALONES
## Specific Endogenous Mitotic Inhibitors

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## 1. Introduction

In the mid-1950's, Paul Weiss [1] applied the then very current concept of cybernetics to a major biological problem; namely, the control of mitosis in cells *in vivo*. Thus, Weiss and Kavanau [2] proposed that a "negative feedback inhibitor" pre-existed in, or on, the surface of cells which specifically inhibited the mitosis of those cells until they were either displaced or destroyed. In the absence of these specific negative feedback inhibitors of mitosis, the cell would enter into the mitotic cycle*. Bullough and Laurence [3, 4], applying this concept to their own work on the control of epidermis, suggested that, since extracts of various epidermal tissues could inhibit the mitosis of epidermal cells *in vivo* and *in vitro* without apparently inhibiting the mitosis of other cell types in the skin, this negative feedback inhibitor could be called a "chalone," from the Greek word meaning to "brake" or slow down [4, 5]. These workers have proposed that there exists in a wide variety of cells, specific and endogenous mitotic inhibitors which, by a negative feedback mechanism, control the mitotic activity of these cells [4, 5]. Their primary biological characteristics are, i) total cell specificity, ii) lack of species-specificity, and iii) reversibility. A number of workers have attempted to establish that the wide variety of cells are under chalone control, and the major systems under chalone control to be proposed to date are described in table 1.

Table 1
Characteristics of various putative chalones.

| Chalone | References | Chalone assay | Mol. wt. of chalone (in daltons) |
|---|---|---|---|
| Epidermis | 5, 10, 34 | $G_1$, $G_2$ | $G_1$: 30,000–40,000<br>$G_2$: > 100,000 |
| Liver | 35, 36 | $G_1$ | < 2000 |
| Melanocyte | 30, 31 | $G_1$ | ca. 4000 |
| Fibroblast | 37 | $G_1$ | 30,000–50,000 |
| Kidney | 38, 39 | $G_2$ | – |
| Granulocyte | 40, 41, 42 | $G_1$ | < 5000 |
| Lymphocyte | 43, 44, 45 | $G_1$, $G_2$ | $G_1$: 30,000–50,000<br>$G_2$: – |

We will review only four chalone systems: epidermis, melanocyte, fibroblast, and lymphocyte. The evidence for the existence of these four chalones is probably both largest in scope and most convincing in quality.

* The reproductive cycle of cell division has been divided into five phases. The newly formed daughter cells are said to be in $G_0$ if they are permanently or temporarily withdrawn from the division sequence; if they remain in this sequence or re-enter it they first go through a period termed $G_1$ during which they make protein and RNA but no DNA; then an abrupt burst of DNA synthesis occurs and the cells are said to be in S phase. After this time, while macromolecular synthesis decreases, the cells, having twice as much DNA as normal, are said to be in $G_2$. Finally the cells go into metaphase and divide (M phase) and the resulting daughter cells are once again in $G_0$ until they re-enter the the cycle through $G_1$.

## 2. Chalone systems

### 2.1. *Epidermal chalone*

The existence of a chalone in the epidermis was suggested by the study of epidermal wound healing by Bullough and Laurence in 1960 [3]. The epidermis and superficial dermis were removed from one side of the ears of mice, then the number of mitoses was counted in the intact epidermis across from the wound. An increased mitotic rate was found opposite from the *centre* of the wound. If a stimulator had been released from the injured cells, then more mitoses should have been found opposite the edges of the wound. But if the increase in number of mitoses was due to the release from an inhibition normally present, then the highest mitotic activity would be found opposite from the centre of the wound, as they found, suggesting the presence of a mitotic inhibitor in the epidermis. Finegold [6] extended these experiments to show that the replacement of the removed tissue with a graft of intact epidermis suppressed the proliferation opposite the wound, further supporting the existence of an epidermal chalone.

The epidermal chalone was extracted by Bullough and Laurence [5] from the epidermis of male Strong CBA mice and reduced the mitotic rate of ear epidermis when injected subcutaneously or when added to pieces of mouse ear in organ culture. Epinephrine was necessary for epidermal chalone action *in vitro*, which led to the suggestion that a chalone–epinephrine complex could be the active mitotic inhibitor. The active substance was water soluble, non-dialyzable, precipitable by 80% ethanol, and heat-labile [7]. Epidermal chalone was relatively tissue-specific: aqueous extracts of kidney, liver, lung, brain, rectum, hair bulbs, and hypodermis did not affect the mitotic rate of ear epidermis *in vitro*; addition of the epidermal extract to cultures did not inhibit mitosis in growing hair follicles or rectal crypts, but clearly reduced the mitotic rate in cornea and oesophagus, which are closely related to epidermis embryologically. Extracts of epidermis from male or female CBA or WLL mice, rats, guinea pigs, or rabbits all contained epidermal chalone activity; thus, chalone activity was not dependent on the sex, strain, or species of animal tested. This work has been confirmed and extended by Iversen, Elgjo, and their co-workers in Oslo [8–10] and by Marrs and Voorhees [11, 12].

A major effort was put into fractionation and purification of epidermal chalone from pig skin, leading to the suggestion that the epidermal chalone was a protein or glycoprotein with a molecular weight of 30,000 to 40,000 daltons. Although the material was purified perhaps 2000 times, the high level of hydroxyproline in the most purified material indicates probable serious contamination with collagen fragments from the dermis [12a].

Until 1968, all experiments on epidermal chalone utilized a mitosis assay; although it had become clear that a physiological growth regulator should act in the $G_1$ phase of the cell cycle [13], prior to DNA synthesis, rather than in $G_2$ or in mitosis. Most cells in a tissue are at rest in $G_1$ (or $G_0$), from which state they may differentiate or may be induced to prepare for DNA replication by synthesizing RNA and protein molecules necessary for initiating and proceeding through S phase. The addition of epidermal chalone to organ cultures of rat skin did not affect DNA synthesis within 1–3 hr [14]. This lack of immediate effect of [$^3$H]thymidine incorporation into epidermal DNA was confirmed *in vivo* [15]. However, DNA synthesis was clearly inhibited 9–13 hr after intraperitoneal injection of epidermal chalone, suggesting that the chalone was acting at a point in $G_1$ several hours prior to the beginning of S phase [15]. DNA synthesis in organ cultures of hamster cheek pouch was inhibited 16–20 hr after the addition of an aqueous extract of cheek pouch epidermal cells [16]. Epinephrine was not required for this inhibition. In an autoradiographic study, it was found that [$^3$H]thymidine labeling index of mouse forestomach cells was clearly lowered 11 hr after injection of epidermal chalone [17].

Following these preliminary finding indicating the presence in the epidermis of an inhibitor acting in $G_1$, evidence for the $G_1$ epidermal chalone has accumulated. Treatment of mouse skin with actinomycin D 24 hr before chalone extraction resulted in a complete loss of the $G_2$ chalone activity extractable from mouse skin, with no loss in the $G_1$ activity [18]. Epidermal basal cells were separated from differentiated cells and extracts of each cell type were made to determine their chalone activity. Basal cells were found to contain primarily $G_2$ chalone activity [19],

while the differentiating cells contained most of the $G_1$ chalone activity [20]. Marks [21] injected mice with pig skin extracts and found an inhibition of DNA synthesis after a lag of about 5 hr, with a peak inhibition between 10 and 20 hr after chalone injection. In contrast to the $G_2$ chalone, activity of the $G_1$ chalone was not affected by boiling at neutral pH or by excessive digestion with trypsin or pronase. The $G_1$ chalone has been purified about 50.000-fold, but the active fraction, which contains protein, carbohydrate, and RNA, may still be heterogeneous.

Regarding the tissue specificity of the $G_1$ epidermal chalone, it has been found that the skin extract did not inhibit DNA synthesis in lung, liver, kidney, or spleen, while extracts of lung, liver, or kidney did not affect DNA synthesis in skin [21]. Extracts of liver [15, 17] or connective tissue [16] did not affect epithelial DNA synthesis.

Thus, it is clear that the material which has been referred to as the "epidermal chalone" is not a single substance. Both the $G_2$ inhibitor and the $G_1$ inhibitor are apparently tissue-specific in their action, a necessary characteristic of a chalone. Which of these substances is more likely to be the physiological growth regulator for epidermis – the epidermal chalone? Since most cells rest in and differentiate from the $G_1$ phase, we submit that the chalone acting in $G_1$ is primarily responsible for epidermal growth control. The $G_2$ inhibitor is more likely to be involved in stress situations requiring an immediate stoppage of mitosis (the $G_1$ inhibitor would require several hours to effect a decrease in the number of epidermal mitoses). While we feel that factors acting in $G_1$ should properly be designated "chalones" for clarity in the literature, chalones should be referred to as "$G_1$ chalone", "$G_2$ chalone", "maturation chalone", etc., depending on the assay used for estimating chalone activity.

Recently Laurence and Randers-Hansen have found that the $G_2$ chalone acts as well in adrenalectomized mice as in intact mice [22], casting doubt on the importance of epinephrine in $G_2$ chalone action *in vivo*. These authors have concluded that "... the epidermal chalone may not act directly with adrenalin to reduce the mitotic rate." An alteration in the hypothesis of $G_2$ chalone action is necessary. The existence of a $G_2$ chalone antagonist (a mitotic stimulant perhaps produced by the dermis), the action of which may be blocked by epinephrine, has been postulated [23, 24].

If chalones are the physiological growth regulators, then their synthesis, or the sensitivity of a tissue to their action, may be altered during carcinogenesis and in tumours. The skin carcinogen 7,12-dimethylbenz [a]-anthracene binds to the $G_2$ chalone-containing 80% ethanol precipitate from mouse skin to a much greater extent than to the inactive 55% or 70% ethanol precipitates [25]. This suggestive result will remain inconclusive until relatively pure chalones can be prepared. After purification is accomplished, then it will be possible to determine whether carcinogens interact directly with $G_1$ or $G_2$ chalones, or whether the rate of chalone synthesis is altered during carcinogenesis.

Three transplantable epidermal carcinomas (rabbit Vx2, Hewitt mouse carcinoma, Chernozemski hamster carcinoma) have been studied with respect to their chalone content and whether they respond to the epidermal chalone prepared from normal skin. Extracts with $G_2$ chalone activity have been prepared from all three tumours [26–29]. In addition, all three tumours responded with a reduced mitotic rate to the normal epidermal chalone. Only the Chernozemski tumour has been tested to determine whether it retains the response to inhibition in $G_1$ after chalone injection. A 70–84% depression of DNA synthesis was found 4–8 hr after chalone injection, with no effect at 2 hr [28]. Thus, transplantable epidermal tumours appear to retain the ability to synthesize at least the $G_2$ chalone, although perhaps in reduced amounts, and still respond to normal chalone by inhibition of mitosis and DNA synthesis. Attempts to induce regression of epidermal tumours by repeated chalone injections have been unsuccessful.

### 2.2. *Melanocyte chalone*

A $G_2$ chalone control mechanism for melanocytes, the pigment-producing cells of the skin, was first suggested by Bullough and Laurence [30]. An aqueous extract of pig skin, which contained epidermal chalone activity, was found to inhibit the mitotic rate of Harding Passey melanomas in mice. When the extract was added to the culture medium of pieces of hamster amelanotic melanomas, the mitotic rate was inhibited, but only in the presence of epinephrine and hydrocortisone. Both *in vivo* and *in vitro*, a more highly purified preparation of epidermal chalone was not active against the melanomas.

This work has recently been extended by Dewey [31], who has shown that extracts of Harding Passey melanoma inhibit the growth of the Harding Passey melanoma grown in cell culture, but do not affect the growth of Chinese hamster lung cells, transformed liver cells, or normal human fibroblasts. The active inhibitor appears to differ from that studied by Bullough and Laurence [30], since it acts *in vitro* in the absence of added epinephrine and hydrocortisone. Melanocyte chalone activity is destroyed by heating to $>50^{\circ}$, or by treatment with trypsin, chymotrypsin, or neuraminidase. The material was dialyzable, with a molecular weight of about 2000 daltons, as indicated by Sephadex chromatography. Thus, the melanocyte chalone appears to be a small glycoprotein or glycopeptide.

The pig skin extract used by Bullough and Laurence was tested for its effect on mice bearing Harding Passey melanomas and Syrian hamsters bearing amelanotic melanomas [32]. The tumours dramatically regressed and ulcerated in all treated animals. Since purified epidermal chalone was not active against the melanomas, the tumour regressions were thought to be the result of melanocyte chalone action. Subsequently, however, it was discovered that the activity was not present in all pig skin extracts, and that active preparations were inactivated by sterile filtration, suggesting that the activity was the result of bacterial contamination [33]. Mohr has recently concluded that [33], "... the striking oncolytic activity of some of our pig skin fractions is caused by contaminating Clostridium spores and not by possible chalone components."

Consequently, the results of experiments using pig skin extracts may not be valid. However, Bullough and Laurence obtained active extracts from melanomas as well as from pig skin, and their extracts displayed the necessary tissue specificity [30], while Mohr's Clostridium-containing preparations were active against a wide spectrum of transplanted tumours. Results showing that melanomas contain and respond to aqueous extracts, along with Dewey's isolation and partial characterization of active material from the Harding Passey melanoma, must be considered as the best evidence supporting the existence of melanocyte chalone.

### 2.3. *Fibroblast chalone*

The fibroblast represents another cell type which suggests chalone control mechanisms. That is, normally fibroblasts do not divide until the tissue is disturbed, as in injury. At this time, the defect is repaired in part by the proliferation of fibroblasts into the injured area. Sometime after the onset of this fibroblast proliferation, the cell population becomes quite dense and the fibroblasts cease to proliferate. At this particular point, it starts to make glycosaminoglycans and eventually collagen to repair the defect.

In culture, the fibroblast represents a cell type which demonstrates "contact inhibition". That is, initially after seeding the cultures with various types of fibroblasts, there is a short lag which is followed in turn by a logarithmic growth phase. Eventually, the logarithmic growth phase reaches the point of crowding the culture so that many cells are obviously closely in contact with each other. At this point the kinetics of culture growth reach a stationary phase; that is, the cells cease to divide. This contact inhibition of mitotic activity can be easily overcome by either increasing the concentration of serum or by scratching a hole into the layers of cells (usually a monolayer) which cover the bottom of the culture vessel. Thus, an initially very rapid burst of cell proliferation is followed by an almost complete inhibition of mitotic activity of these cells when they have become crowded and are in contact. It is believed that this *in vitro* contact inhibition might be a model for the *in vivo* phenomenon of the termination of the initial proliferation of fibroblasts during wound healing.

We have found that extracts of either the medium from cultured diploid human fibroblasts or of the cells themselves possess a trypsin-labile and thermolabile material with a molecular weight between 30,000 and 50,000 daltons which will inhibit significantly the uptake of [$^3$H]thymidine by other diploid human fibroblasts during normal growth *in vitro* [37]. These extracts of either fibroblasts or their used medium did not influence the proliferation rate of various types of lymphocytes or other cultured cells, thus suggesting some specificity in mitotic inhibition.

It is possible that as these fibroblasts proliferate *in vitro* and approach and make contact one with the other, they begin to share their chalone and at some critical concentration of shared chalone, mitotic

activity essentially ceases. Thus, the elaboration of a chalone by diploid human fibroblasts in culture suggests a possible explanation for a phenomenon of contact-inhibition so characteristic of these cell types *in vitro*.

It has been known for perhaps half a century that serum is an extremely helpful component in culture medium. Recently [50] we have shown that diploid human fibroblasts in culture *stringently* require serum in order to divide. We have isolated and purified this serum-derived mitogen for diploid human fibroblasts and have found it to be a macromolecule weighing approximately 116,000 daltons, made up of two identical dimers. Each dimer contains two free —SH groups and two sialic acid components. Neuraminidase will destroy the activity of this serum-derived mitogen. Each dimer also contains at least one and possibly two disulfide pairs. The thermostable serum macromolecule can be destroyed by trypsin. This macromolecular species of sialoprotein is found in all species of mammalian sera that we have investigated to date (human, cow, horse, dog, pig, and rat). This sialoprotein which serves as the critical mitogen for diploid human fibroblasts can substitute completely for serum over at least 15 generations of the lifetime of these cells *in vitro*.

Preliminary data suggests that an interesting relationship exists between the fibroblast chalone and the serum mitogen. Namely, that as one increases the concentration of the serum mitogen *in vitro* for a given amount of chalone, the activity of the chalone is decreased; conversely, given concentrations of chalone have a much larger anti-mitotic effect in the presence of less serum mitogen. The relationship between serum mitogen and chalone concentration in terms of the proliferation of diploid human fibroblasts *in vitro* is straightforwardly one of an apparent competition for either the same or similar sites on the surface of the cell. Neither the serum mitogen nor the chalone, when administered one to the other, shows a strong interaction; that is, the chalone weighing less than 50,000 and the serum mitogen weighing over 100,000 can easily be separated by molecular sieving and, quantitatively, after mixing purified serum mitogen and crude fibroblast chalone. Our proposal is that the serum mitogen serves to displace preexisting chalone from the surface of the fibroblast, thus permitting the cell to enter the mitotic phase of its cell cycle. It would also explain in part the rapid proliferation of resting fibroblasts into a wound (since this is associated with the insuspation of serum into the area), and the termination of cell proliferation which occurs kinetically about the same time that the biological continuity of the microcirculation is restored to normal and the cells are no longer bathed with the serum-derived mitogen. Thus, we are of the opinion that the serum mitogen functions as an "anti-chalone" which, in quantitative relationship with the chalone concentration of the fibroblast, effectively controls the proliferation of this cell *in vitro* and *in vivo*.

### 2.4. *Lymphocyte chalone*

Moorhead was the first to apply the chalone concept to the control of the proliferation of lymphocytes [43]. He showed that extracts of pig lymph node would inhibit significantly the blast transformation of human lymphocytes in culture when stimulated with lectins such as phytohemagglutinin (PHA). This inhibition of blast transformation (determined morphologically) was also associated with a significant inhibition of the uptake by these cells of tritiated thymidine culture. This inhibition of lymphocyte transformation when stimulated by PHA was paralleled by Moorhead's further finding that lymph node extracts from pig would also inhibit the spontaneous transformation and eventual mitosis of leukemic lymphocytes derived from tumour patients *in vivo* [44]. These observations were confirmed by Garcia-Giralt et al. [45] and by ourselves [46, 47]. We also indicated that extracts of not only lymph nodes but also spleen and thymus from cow, pig, and rat were all capable of inhibiting the transformation of PHA-stimulated lymphocytes *in vitro*. Further, we were able to establish that extracts of non-lymphoid tissues had no effect upon the transformation rate of human lymphocytes in culture and that these lymphoid extracts could be purified with the "chalone" activity concentrated in a molecular weight range between 30,000 and 50,000 daltons. This purification process also removed the various cytotoxic components, particularly complement and IgM, from the aqueous extracts of the lymphoid tissue, and these extracts could then be demonstrated to be without effect upon the proliferation rates of diploid human fibroblasts, HeLa

cells, or colon carcinoma cells *in vitro*. Thus we have been able to demonstrate a correspondence between the mitotic inhibition activity of lymphoid tissue vis a vis stimulated lymphocytes and Bullough and Laurence's definition of a chalone.

Obviously the material which is capable of inhibiting lymphocyte transformation may find utility as an immunosuppressive agent, and both Garcia-Giralt et al. [48] and Kiger et al. [49] have published recently on the application of lymphocyte chalone concentrate to the inhibition of graft rejection *in vivo*.

The properties of the lymphocyte chalone are relatively unknown, save in that the molecular weight of the reactive species must be between 30,000 and 50,000 daltons. Further, the material is trypsin-labile and it must contain very large amounts of carbohydrate, since a portion of this chalone activity can be recovered through cold trichloracetic or perchloric acids. Thus, it is most probably a glycoprotein. Preliminary evidence suggests its isoelectric point is someplace between pH 4 and 5.

A comparison of the response of tumour cells (lymphocytic leukaemia and lymphomas) to the chalone with that of normal diploid human lymphocytes suggests some important differences between these two cell types: namely, the tumour cells, while responding to the addition of chalone in the medium *in vitro*, require two to four times as much chalone to demonstrate an equivalent percent inhibition as do diploid cells. This increased requirement for chalone concentration seems to be associated with evidence that we have presented elsewhere that tumour lymphocytes will not bind chalone nearly as well as will diploid human lymphocytes. Our evidence suggests that the leukaemic lymphocyte or lymphoma cell will produce a small amount of chalone but, because of this failure of binding, cannot build a sufficiently large concentration on the surface of the cell to preclude the unremitting mitotic activity characteristic of these types of cells in culture.

Finally, the lymphocyte chalone does not appear to be enormously cytotoxic, since the cells which are inhibited with respect to tritiated thymidine uptake are not inhibited with respect to their rate of uptake of labeled amino acids into protein.

## 3. Discussion and critique

The primary criticism that has been offered over the last ten years, during which the chalone proposal has been increasingly noted and studied, relates firstly to the issue of cytotoxicity and secondly to the issue of how does one determine mitosis experimentally.

### 3.1. *Cytotoxicity*

Extracts of many tissues which have not been purified extensively contain, among other things, complement and IgM; the first portion of the complement chain together with the IgM will fix to the surface of a number of cells in such a fashion as to activate the interdigitated proenzymes which constitute the complement chain, leading to the activation of a number of proteases and most particularly enzymes similar to phospholipase-C ($C'_{6-8}$). In this fashion, a cell may be enzymatically injured to the point of being grossly dead, as judged from morphology or, more subtly and yet still profoundly, altered by these cytotoxic components of blood-rich tissues. Further, these tissue extracts contain extensive amounts of lysosomal acid hydrolases, and many of the lysosomal cathepsins are also cytolytic. Thus was born "Houck's Law" which states that i) "Dead cells do not divide," and ii) "Dying cells divide damn slowly." This profound philosophical statement indicates one of the major experimental hazards, then, to accepting incredulously the observation that tissue extracts will cause a given cell species to cease dividing *in vitro*.

We have studied the metabolic and biochemical parameters of cell death by subjecting diploid human fibroblasts in culture to radiation. These cells, which are programmed to die at some particular finite time, have a kinetics of death which can be observed in terms of increasing failure firstly to incorporate labeled thymidine into DNA, and secondly an increasing failure to incorporate either uridine or phenylalanine into RNA or proteins, respectively. After the cells have "died" to an extent in which no further incorporation of precursors to macromolecular synthesis can be demonstrated, these cells still retained labeled chromium and only after a few more hours would start to release both the chromium and lysosomal and cytoplasmic enzymes into the medium. Eventually, in a period of some 8 more hours the cell no longer possessed the

ability to exclude vital dye and finally its morphology itself began to depart significantly from normal. Even in cells which were no longer excluding vital dye, they were still metabolically consuming glucose, releasing $CO_2$, and synthesizing ATP. Thus, the rate of cell death depends on what parameters are being measured of what particular organelle; mitochondrial activities obviously will endure considerably longer than those involving synthesis on the endoplasmic reticulum. It thus is almost as difficult to pinpoint the precise moment of death of a cell as it is to determine the precise moment of clinical death in a patient.

Thus, if a chalone extract contains sufficient amounts of cytotoxic materials to injure the cell so that it will at some future date "die" by whatever morphological or other criteria may be at hand, it is quite possible that while still apparently in clinical health, the cell has lost the ability to incorporate thymidine into DNA. This inhibition proceeds, then, not because of specific inhibition of mitosis but, rather, a generalized kinetic phenomenon of cytotoxicity. The end point of the exclusion of vital dyes by cells is a late manifestation of the cells' earlier injury.

Perhaps the most effective way of demonstrating the health of cells and hence the "chalone activity" is specific to mitosis rather than relating to Houck's Law is to demonstrate that these cells, while not incorporating thymidine, can still incorporate amino acid precursors. An even more subtle and sophisticated characteristic of the chalone is that it not only inhibits mitosis but it also stimulates differentiated cell function. Thus, for example, we find that fibroblast chalone, when added to diploid human fibroblasts in culture, will inhibit their incorporation of [$^3$H]thymidine considerably while at the same time the cell is still capable, when exposed to ascorbic acid of synthesizing hydroxyproline from radioactive proline. The bulk of the *in vitro* studies relating to chalones has not been carefully controlled with respect to the cytotoxicity of the crude tissue extracts under study. However, the *in vivo* studies demonstrating the existence of chalone are not nearly as compromised by this problem of cytotoxicity as are the *in vitro* studies.

### 3.2. *Errors in determination*

The most rapid technique for determining the entrance of cells into the mitotic phase of their cycle is by studying the incorporation rates of [$^3$H]thymidine into macromolecular material. There are a number of criticisms which are quite important to the interpretation of the apparent chalone inhibition of this process. For example, the extracts of many lymphoid tissues, as well as of liver, contain a sufficient amount of thymidine kinase to alter effectively a very large amount of the thymidine label added to the biological system under study. Thus, the apparent inhibition of thymidine uptake by chalone might not be due to the inhibition of mitosis in the cell but rather to the enzymatic catabolism of the thymidine itself. Further, many tissue extracts which have not been subjected to dialysis are known to contain "cold" nucleotides which are capable of competitively diluting the pool size of the added labeled thymidine. In this case, then, mitotic inhibition is really a demonstration of pool size dilution rather than true chalone activity. Again, *in vivo* demonstrations of chalone activity would probably not be subject to the particular criticism nor would those experimental techniques for studying mitosis which would involve the actual counting of either the number of cells or, alternatively, the proportion of those cells which were in metaphase.

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