vitro; to identify and study differences between abnormal and normal cells in vitro; to study any changes that may occur in cell surface components during development; and, potentially, to sort specific cell types by means of a fluorescent activated cell sorter, a technique now used with lymphocytes (*12*). Surface labeling of the proteins of the cells, by standard membrane probes, can now be used to determine the nature of the antigens responsible for the specific antiserum produced.

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Dibutyryl Cyclic AMP Mimics Ovariectomy: Nuclear Protein Phosphorylation in Mammary Tumor Regression

Abstract. *Growth of mammary carcinoma induced by 7,12-dimethylbenz(a)anthracene is arrested by either ovariectomy or treatment with* $N^6,O^{2'}$*-dibutyryl cyclic adenosine 3',5'-monophosphate (dibutyryl cyclic AMP). When this occurs, a new nonhistone protein species becomes the predominant endogenous substrate of cyclic AMP-dependent protein kinase in the tumor nuclei. Phosphorylation of this regression-associated protein ceases when resumption of tumor growth is induced by either the injection of 17β-estradiol or cessation of dibutyryl cyclic AMP treatment. Thus phosphorylation of regression-associated protein may play a role in the regression of hormone-dependent mammary tumors.*

Studies from several laboratories have suggested that most mammalian cytoplasmic protein kinases are stimulated by adenosine 3',5'-monophosphate (cyclic AMP) and preferentially phosphorylate basic proteins, such as histones, in vitro (*1*). In contrast to the cytoplasmic enzymes, protein kinases from nuclear fractions have generally been found to be unresponsive to cyclic AMP and to prefer acidic proteins, such as casein and phosvitin, as substrates in vitro (*2*).

A mechanism that would allow cytoplasmic protein kinase to influence nuclear events has been implicated in several tissues. Redistribution of protein kinase from the cytoplasm to the nuclear fraction has been shown to occur in rat liver after stimulation with glucagon or dibutyryl cyclic AMP (*3*), in rat uterus with hormonal stimulation of adenylate cyclase (*4*), and in calf ovary in response to chorionic gonadotropins (*5*). This translocation of cytoplasmic protein kinase into the nucleus was also found in tumors regressing after dibutyryl cyclic AMP treatment (*6*). Thus a correlation exists between growth arrest in vivo and nuclear accumulation of protein kinase.

The growth of mammary carcinoma induced by 7,12-dimethylbenz(*a*)anthracene (DMBA) is arrested by either treatment with dibutyryl cyclic AMP (*7*) or by ovariectomy (*8*). Furthermore, soon after the initiation of either treatment, microsomal isozyme activity of glucose-6-phosphate dehydrogenase disappears; and the activity and amount of acid ribonuclease doubles in the neoplastic tissues (*7, 9*). Growth arrest of hormone-dependent mammary tumors induced by either deprivation of estrogen (ovariectomy) or treatment with dibutyryl cyclic AMP, may be mediated through a common mechanism that could involve phosphorylation of nuclear proteins. Therefore, we investigated the role of protein kinase–dependent phosphorylation of nuclear proteins in the growth control of a hormone-dependent DMBA-induced mammary carcinoma in Sprague-Dawley random-bred female rats.

Tumor nuclei were incubated with [γ-^{33}P]ATP to phosphorylate the nuclear proteins via intrinsic protein kinase. These nuclear proteins were then treated with 1 percent sodium dodecyl sulfate (SDS) and 1 percent mercaptoethanol and subjected to electrophoresis. As shown in Fig. 1, five major groups of protein bands were apparent with Coomassie blue staining. Similar patterns of protein bands were observed for unincubated nuclei and nuclei incubated with adenosine triphosphate (ATP) in the presence or absence of cyclic AMP. Protein species IV was found to be the major endogenous substrate for nuclei-associated protein kinase in the growing tumor (Fig. 1A), whereas in nuclei of the growth-arrested tumor (Fig. 1B) and regressing tumor (Fig. 1C) the radioactivity peaks associated with protein species IV decreased by about 40 percent, and new radioactivity peaks coincident with protein species I appeared. Injection of 17β-estradiol into the host for 5 days (5 μg/day; 200-g rat; subcutaneously) or cessation of dibutyryl cyclic AMP treatment for 5 days produced regrowth of the tumors and reversed the pattern of nuclear protein phosphorylation to that of the growing tumor. The phosphorylation of protein species I was not observed in a few DMBA tumors that grow autonomously despite ovariectomy.

To further characterize the regression-associated phosphorylation of protein species I, differential extractions of nuclear proteins were used after incubation of the nuclei with [γ-^{33}P]ATP. The protein bands and phosphorylation patterns of basic proteins isolated from the nuclei of growing and regressing tumors are shown in Fig. 2, A and B, respectively. Three major classes of protein bands were designated BI, BII, and BIII, with BIII being the fastest band migrating toward the anode. In nuclei from the growing tumor, basic protein species BIII were found to be the major substrates for nuclei-associated protein kinase, and the radioactive peak was coincident with the major radioactive peak shown in Fig. 1A (Fig. 2A). In nuclei from the regressing tumor, two prominent radioactive peaks

were found; one was associated with BIII as in the nuclei from the growing tumor and the other coincided with BI protein bands (Fig. 1B). Band BI was apparently composed of four protein components, designated a, b, c, and d, respectively, with d being the fastest migrating protein species. The peak of radioactivity associated with BI protein species in regressing tumor coincided with the BIc protein band which was not apparent in the nuclei from the growing tumor. BIc protein had an apparent molecular weight of 76,000 in the SDS–acrylamide gel electrophoresis (data not shown). The electrophoresis of purified histone components showed that histone H1 comigrated with the BII protein band, whereas histone H3, H2A, H4, and H2B (Sigma) migrated in close sequence with the BIII band (data not shown). It should be noted that lysine-rich proteins migrate much more slowly in SDS-electrophoresis as shown with histone H1. This suggests that the estimated apparent molecular weight of 76,000 for BIc may not be valid if BIc protein is a lysine-rich basic protein. Until more evidence is obtained about the molecular properties and functional significance of BIc protein, we propose to refer to BIc protein as the "regression-associated protein" (RAP).

Isolation of histone H1 (*10*) from the nuclei incubated with [γ-^{33}P]ATP showed no radioactivity associated with the protein peak. The small peaks of radioactivity associated with acidic proteins that were isolated (*23*) from the incubated nuclei (with [γ-^{33}P]ATP) of both growing and regressing tumors were similar.

When the phosphorylated nuclear proteins from both growing and regressing tumors were treated with hydroxylamine prior to electrophoresis, no loss of incorporated ^{33}P was observed. All radioactivity incorporated was released, however, when the nuclei were treated with 1*N* NaOH at 95°C for 5 minutes. These observations indicated that the phos-

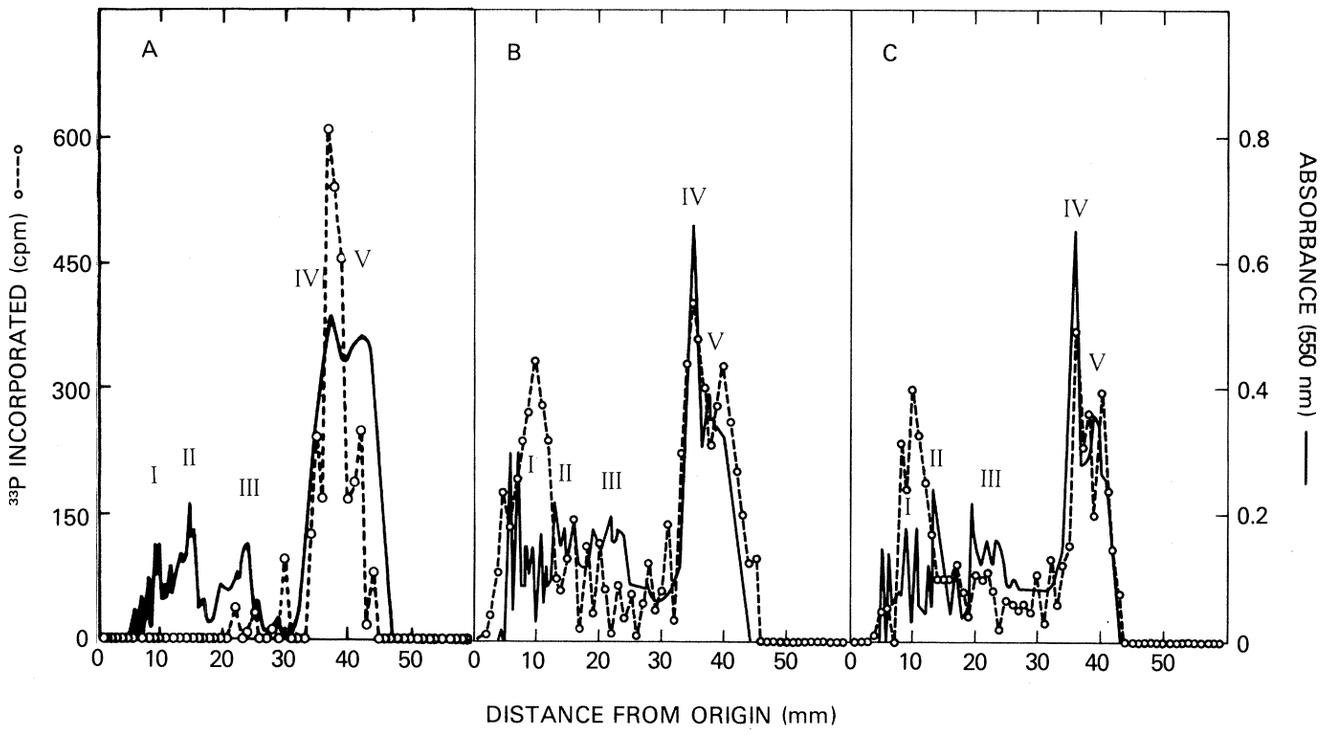

Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of phosphorylated nuclear proteins from growing, regressing, and growth-arrested DMBA tumors. (A) Growing tumors; (B) growth-arrested tumors, 3 days after dibutyryl cyclic AMP treatment (*7*); (C) regressing tumors (regressed by 20 percent of the original size) 3 days after ovariectomy. Tumors (0.2 g) were homogenized immediately after removal in a Teflon-glass homogenizer with five volumes of buffer (0.25*M* sucrose, 2 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 10 m*M* KCl, 20 m*M* tris-HCl, *p*H 7.5). The homogenates were filtered through three layers of gauze and centrifuged at 755*g* for 10 minutes. The pellets were resuspended in an original volume of buffer A containing 0.2 percent (v/v) Triton X-100, then slurred in a Vortex and centrifuged at 755*g* for 10 minutes. The resulting pellets were resuspended in an original volume of buffer A, slurred in a Vortex, and centrifuged at 755*g* for 10 minutes. The final nuclear pellets (consisting of mostly morphologically intact nuclei with minimal cytoplasmic contamination, as seen by phase contrast microscopic examination) were added to a reaction mixture (0.3 ml) containing 100 m*M* potassium phosphate buffer, *p*H 7.5; 1 m*M* theophylline; 20 m*M* magnesium acetate; 1 μ*M* cyclic AMP; and 12.6 μ*M* [γ-^{33}P]ATP (19.2 c/mmole; ICN Pharmaceuticals, Inc., Irvine, Calif.); [γ-^{33}P]ATP was used since ^{33}P has a half-life two times longer than ^{32}P and emits a radiation energy similar to that of ^{14}C). The mixtures were incubated at 30°C for 15 minutes, immediately cooled to 4°C, and centrifuged; supernatants were discarded. To the nuclear pellets were added 0.3 ml of 10 m*M* potassium phosphate buffer, *p*H 7.5, containing 1 percent SDS, and 1 percent β-mercaptoethanol; the mixture was incubated at 37°C for 4 hours. The dissolved samples were then dialyzed at room temperature for 3 hours against 500 volumes of 10 m*M* sodium phosphate buffer, *p*H 7.0, containing 0.1 percent SDS and 0.1 percent β-mercaptoethanol, and the polypeptide chains were resolved by electrophoresis in 0.1 percent SDS–10 percent polyacrylamide gels by the method of Weber and Osborn (*19*). Samples containing 40 μg of protein were subjected to electrophoresis. Proteins were stained with Coomassie blue (*19*) and destained with 7.5 percent acetic acid in an Ames quick gel destainer (Canalco). The protein tracings were done in a Gilford model 240 spectrophotometer at 550 nm (*20*). For the determination of ^{33}P incorporation into nuclear proteins, gels were sliced into 1 mm slices and incubated with 1 ml of 30 percent H_2O_2 at 85°C for 6 hours in capped scintillation vials; 10 ml of Insta gel (Packard) was then added, and the radioactivity measured by a Beckman LS-355 liquid scintillation spectrometer with a window setting of 400 to 700. The maximum phosphorylation of nuclear proteins was reached within 15 minutes after incubation. Recovery of radioactivity after the electrophoresis was 60 percent in all gels. The data represent one of seven similar experiments. Protein concentrations were determined as described (*21*). Protein concentrations of nuclei isolated from per gram of growing and regressing tumors were similar. The studies on physiopathology or regressing DMBA tumors showed that regression is an endocellular event, and is an energy-dependent process that appears to involve new synthesis of RNA and proteins (*22*).

phorylated proteins contained phosphoester bonds rather than acylphosphate moieties (*11*).

The phosphorylation of nuclear proteins measured in our study is dependent on the activity of protein kinase in the nucleus. The activity of protein kinase in the nuclei of growing and regressing tumors was, therefore, measured (*6*) with the use of exogenous substrates of histones and casein. Protein kinase from growing tumor nuclei exhibited an activity of casein phosphorylation threefold higher than that of histone phosphorylation (data not shown). Dibutyryl cyclic AMP treatment or ovariectomy resulted in a change in the protein substrate specificity of nuclear protein kinase. In the regressing tumor nuclei, the histone kinase activity increased threefold, whereas the casein kinase activity decreased to 30 percent of that in the growing tumor nuclei (data not shown). Cyclic AMP had no effect on the activity of kinase from either growing or regressing tumor nuclei, whereas protein kinase inhibitor protein prepared from rat skeletal muscle (*12*) inhibited the kinase from growing and regressing tumor nuclei by 10 and 80 percent, respectively (data not shown). These results suggest that the differential phosphorylation of nuclear proteins in growing and regressing tumors, as shown in Figs. 1 and 2, is probably due to the presence of different protein kinases in the respective tumor nuclei.

That phosphorylation of RAP is indeed a nuclear event rather than being due to a cytoplasmic contaminant, is suggested by the experiments with highly purified nuclei (*13*); results were similar to those shown in Figs. 1 and 2. Moreover, phosphorylation of RAP is not associated with incipient cell death in general, since treatment with the cytotoxic chemotherapeutic agent, cyclophosphamide (10 mg/day; 200-g rat; subcutaneously) (*14*) did arrest the growth of DMBA tumors but had no effect on the phosphorylation of RAP (data not shown).

Our study presents the first evidence, so far as is known, of a differential endogenous phosphorylation of nuclear proteins during growth and regression of hormone-dependent mammary carcinoma. The phosphorylation of RAP via nuclear protein kinase is stimulated by either dibutyryl cyclic AMP treatment or ovariectomy and is depressed by the cessation of dibutyryl cyclic AMP treatment or readministration of 17β-estradiol. The results support our finding (*15*) that a steroid hormone and cyclic AMP may counteract each other at the nuclear level in the growth control of a hormone-dependent mammary tumor. The counteraction between steroid hormones and cyclic AMP in the endogenous phosphorylation of a specific cytosol protein, designated SCARP, has also been demonstrated in several target tissues (*16*).

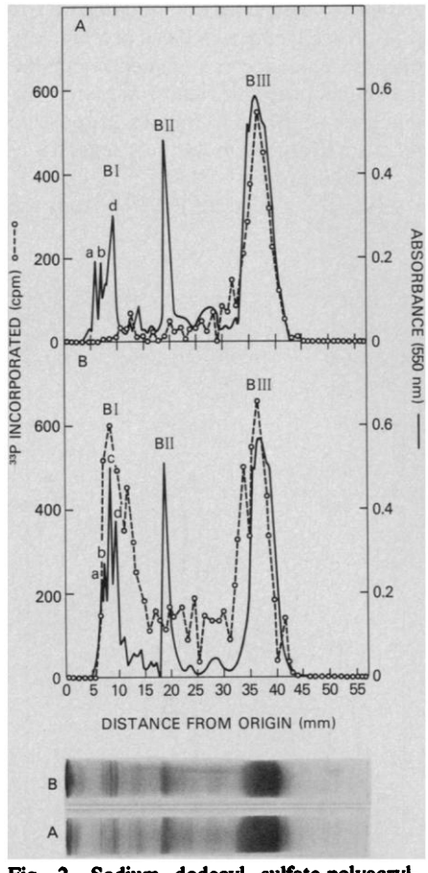

Fig. 2. Sodium dodecyl sulfate-polyacrylamide electrophoresis of nuclear basic proteins from growing and regressing DMBA tumors. (A) Growing tumors; (B) regressing tumors (regressed by 20 percent of the original sizes) 3 days after ovariectomy. Coomassie blue stainings of basic nuclear proteins from growing and regressing tumors are shown as gel pictures A and B, respectively. Nuclei were isolated and incubated with [γ-^{32}P]ATP as described in the legend to Fig. 1. Basic proteins were isolated from the incubated nuclei (*23*). The nuclear pellets (from a 0.2-g tumor) were suspended in five volumes (1 ml) of 0.2*N* HCl, and sonicated for 30 seconds at 4°C (Heat System–Ultrasonics, Inc., sonifier cell disrupter model W-350, in pulse mode, output setting of 3, 50 percent duty cycle, with the microtip). The samples were centrifuged at 17,300*g* for 10 minutes. Six volumes of acetone were added to the supernatants, and samples were cooled in ice for 30 minutes. The mixtures were then centrifuged at 17,300*g* for 10 minutes, and the pellets consisted of the basic proteins, which were then stored at −80°C until used. The basic proteins were treated in the manner described in Fig. 1 for the nuclear pellet, and the polypeptide chains were resolved by electrophoresis (as in Fig. 1). Recoveries of radioactivity after electrophoresis were 90 percent in all gels. The data represent one of three similar experiments.

Translocation of cytoplasmic cyclic AMP binding and protein kinase activities was found in the regressing tumor after ovariectomy (*15*) or dibutyryl cyclic AMP treatment (*6*). This finding, together with other results (*16, 17*), suggests that the phosphorylated RAP may be the regulatory subunit of cyclic AMP–dependent protein kinase which is derived from the holoenzyme by the mechanism of autophosphorylation as described for purified protein kinase (*18*). It should be emphasized, however, that steroid hormones and cyclic AMP can also affect the phosphorylation of protein bands other than RAP and that, if nuclear proteins had been phosphorylated fully in vivo prior to the isolation of the nuclei, then further phosphorylation in vitro could not be demonstrated. The role of RAP in the regression process of hormone-dependent mammary tumors remains to be clarified.

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Induction of Acetylcholinesterase Activity by β-Ecdysone in a *Drosophila* Cell Line

Abstract. *When cells of the* Drosophila *Kc-H line are treated with ≥ 10^{-8} molar β-ecdysone, they extend long processes and acquire acetylcholinesterase activity. Thus, this permanent line, derived originally from embryo cultures, may be composed of cells having some neural or glial characteristics.*

The Kc cell line, derived originally from cultures of *Drosophila melanogaster* embryos, responds morphologically to treatment with physiological doses of the insect molting hormone, β-ecdysone (ecdysterone) (*1*, *2*). We have been studying this phenomenon using a strain of cells derived from the Kc line. Here we report that in this strain, henceforth designated Kc-H cells, β-ecdysone causes not only a striking morphological transformation but also the appearance of acetylcholinesterase (AChE) (E.C. 3.1.1.7.) activity.

Kc-H cells were separated from the parent line about 2 years ago. The two strains differ in karyotype in that the Kc-H strain is virtually exclusively XO and haplo-IV, while the Kc strain is heterogeneous but contains many XX, haplo-IV cells (*1*, *3*). Furthermore, the morphological response to β-ecdysone is more extreme in Kc-H cells than in the parent line. Thus, during exposure to β-ecdysone at concentrations of $10^{-8}M$ or greater, Kc-H cells extend long thin processes. After 3 days, the average overall length of the cells has increased more than fivefold, and many of the processes are more than 40 μm long (Fig. 1). Other aspects of the response to β-ecdysone by Kc-H cells are similar to the Kc response as described by Courgeon (*2*): the cells cease dividing, aggregate, and eventually die.

The appearance of cells like those in Fig. 1, b and c, suggested to us that Kc-H cells might have some neural properties. Therefore we tested for the presence of AChE, a neural marker that is readily assayed. Cells were grown in suspension in medium D-22, including 10 percent fetal calf serum (*1*). Our assay for AChE is described in the legend to Fig. 2. Operationally, the AChE activity is taken to be equal to the net rate of eserine-sensitive hydrolysis of acetylthiocholine (AtCh)—that is, to the overall rate of hydrolysis minus any component not eliminated by $2.6 \times 10^{-6}M$ eserine sulfate. We found that the residual, eserine-insensitive component was not inhibited by eserine sulfate at concentrations as high as $6 \times 10^{-4}M$.

Extracts of cultures not exposed to β-ecdysone showed little AChE activity. When cells were exposed to $10^{-6}M$ β-ecdysone, the specific activity of AChE began to increase after 1 day; after 3 days it had increased at least 50-fold (Fig. 2a). Meanwhile the eserine-insensitivie activity was unchanged.

Induction is detectable at $10^{-8}M$ and maximal at $10^{-6}M$ β-ecdysone (Fig. 2b). Thus, with respect to its dependence on hormone concentration, AChE induction is similar to other effects of β-ecdysone in culture (*2*, *4*, *5*). Concentrations of β-ecdysone equal to or greater than these do occur in pupariating and pupating *Drosophila* (*5*, *6*).

The AChE present in ecdysone-treated Kc-H cells has low K_m's (Michaelis constant) for AtCh (0.13 mM) and for butyrylthiocholine (< 0.26 mM). The latter substrate is hydrolyzed about half as rapidly as AtCh, each being tested at optimal substrate concentration. These values are similar to those for AChE activity in whole fly extracts (*7*). We have directly compared the enzyme from hormone-treated Kc-H cells with that present in extracts of whole Oregon-R flies in terms of their sensitivities to three AChE inhibitors. The two preparations were indistinguishable, both enzymes being 50 percent inhibited by $1 \times 10^{-8}M$ eserine sulfate, $6 \times 10^{-8}M$

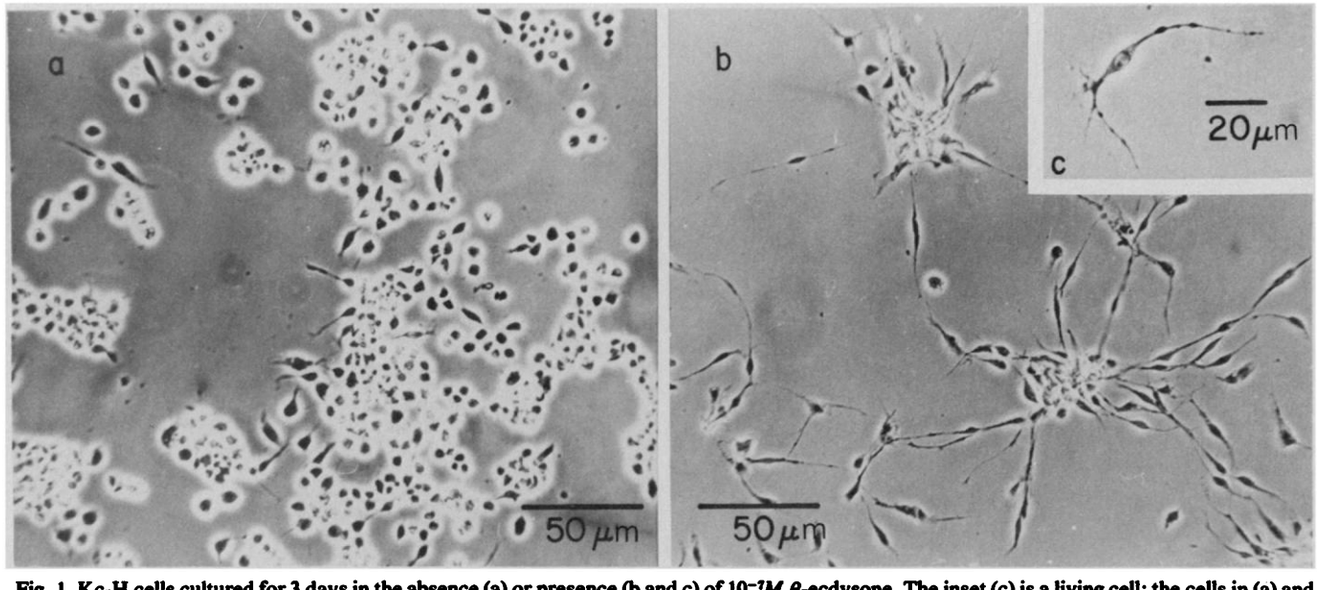

Fig. 1. Kc-H cells cultured for 3 days in the absence (a) or presence (b and c) of $10^{-7}M$ β-ecdysone. The inset (c) is a living cell; the cells in (a) and (b) were fixed in glutaraldehyde, dehydrated, and mounted by standard techniques (photography in phase contrast optics).