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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, $6 \times 10^{-8}M$

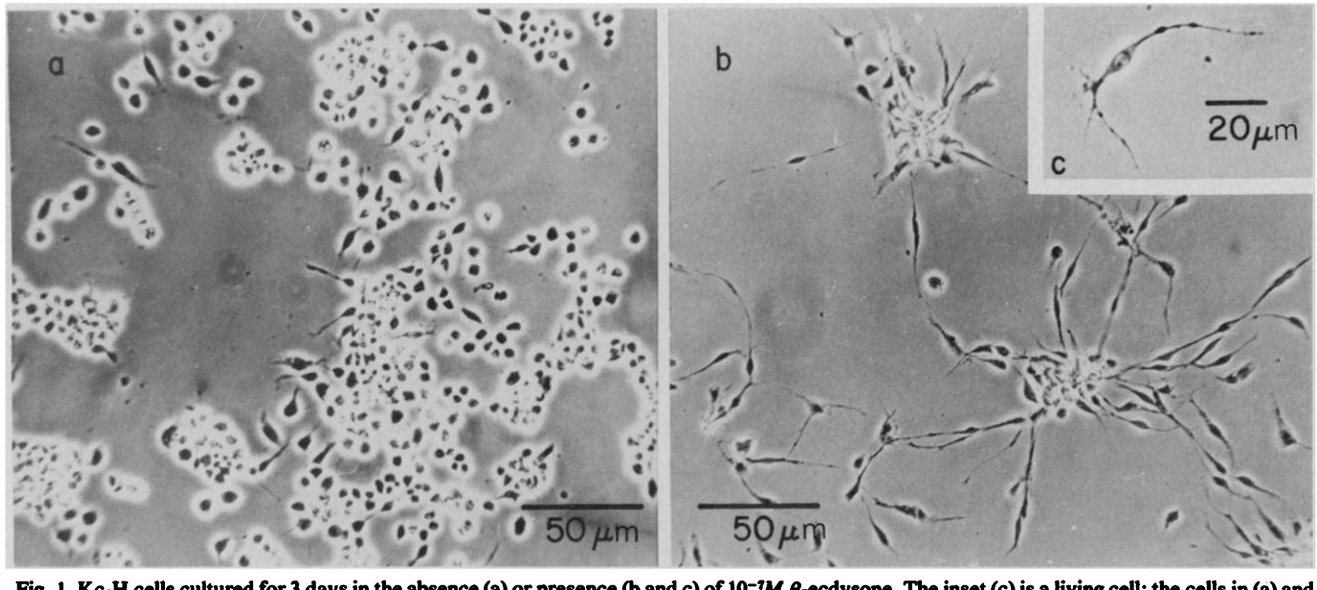

Fig. 1. Kc-H cells cultured for 3 days in the absence (a) or presence (b and c) of $10^{-7}M$ β-ecdysone. The inset (c) is a living cell; the cells in (a) and (b) were fixed in glutaraldehyde, dehydrated, and mounted by standard techniques (photography in phase contrast optics).

BW284c51 (Burroughs Wellcome), or $3 \times 10^{-5}M$ iso-Ompa (*N,N'*-diisopropyl pyrophosphorodiamide anhydride) (*8*).

The two enzyme preparations were also compared by polyacrylamide gel electrophoresis. Figure 3 shows a 7.5 percent gel stained for AChE activity. The enzymatic activity from Kc-H cells was less disperse than that from whole flies and was coincident with a minor band in the fly extract. The mobilities of the cell band and of the most intense band of activity in the fly extract were 16 and 22 mm, respectively. The corresponding mobilities on a 4.5 percent gel were 49 mm and 68 mm (gel not shown). The ratio of the mobility at 7.5 percent to that at 4.5 percent was 0.32 for both bands, good evidence that the molecular weights of the enzymes are very similar (*9*).

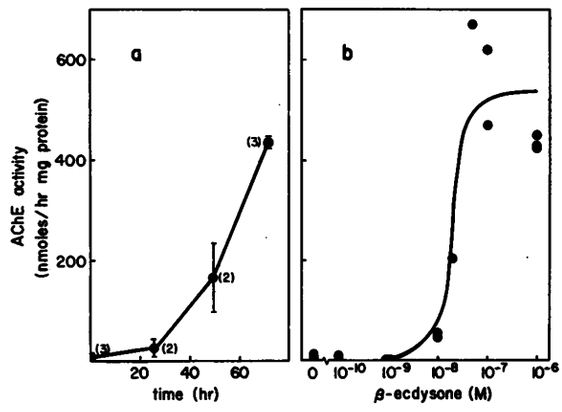

Fig. 2. (a) AChE specific activity after different periods of culture in $10^{-6}M$ β-ecdysone. The points are mean values and the bars are standard deviations for the number of independent extractions given in parentheses. (b) AChE specific activity after 71 hours of treatment with β-ecdysone at different concentrations. Each point represents a separate extraction. Cells were maintained in exponential growth (1×10^6 to 10×10^6 cell/ml) in suspension in petri dishes. They were treated with hormone in ethanol or with ethanol alone and harvested at the appropriate time. They were washed with saline (*16*), then homogenized in a Dounce homogenizer in 0.1*M* phosphate buffer, *p*H 7.5, containing 0.5*M* NaCl, 0.25 m*M* EDTA, and 0.5 percent Triton X-100. After incubation for 20 minutes in ice, extracts were centrifuged for 10 minutes at 20,000*g*; supernatants were frozen in liquid nitrogen and then stored at −90°C for assay later. AChE was assayed by a modification of the method of Ellman *et al.* (*17*) under conditions based on those of Sanes and Hildebrand (*18*). Reaction mixtures contained 0.1*M* phosphate buffer, *p*H 8.0, 0.5 m*M* AtCh, 0.3 m*M* dithiobisnitrobenzoic acid (DTNB), and 50 μl of extract in a final volume of 1.47 ml. Blank tubes contained $2.6 \times 10^{-4}M$ eserine sulfate. Reactions were stopped after 1 hour at 25°C by the addition of eserine, and the absorbancy at 412 nm (A_{412}) was measured. Protein was determined by the procedure of Lowry *et al.* (*19*), with standards containing an appropriate concentration of Triton X-100. Assaying AChE by these procedures, we obtain values for whole fly extracts comparable to those reported (*13–15*).

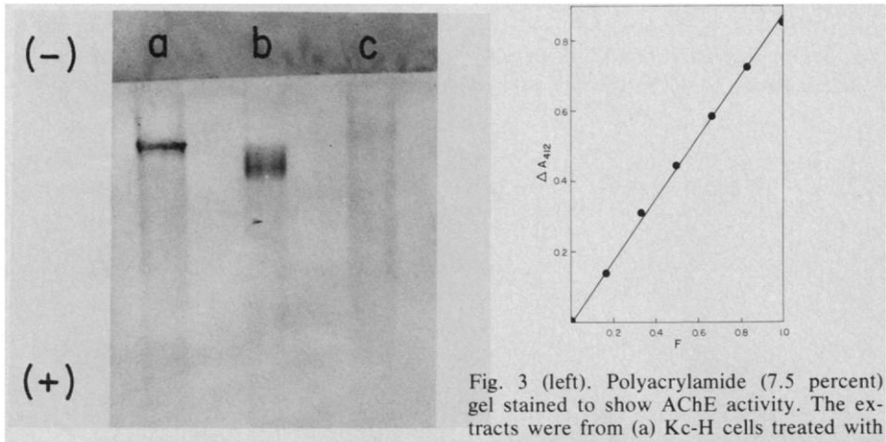

Fig. 3 (left). Polyacrylamide (7.5 percent) gel stained to show AChE activity. The extracts were from (a) Kc-H cells treated with β-ecdysone (73 hours, $5 \times 10^{-8}M$); (b) whole adult Oregon-R flies; and (c) control Kc-H cells. Extracts were prepared as described in the legend to Fig. 2. The channels received 25-μl samples containing total AChE activity and protein as follows: (a) 10 nmole/hour, 15 μg; (b) 23 nmole/hour, ~ 0.8 μg; (c) 0.2 nmole/hour, 18 μg. The gel was subjected to the conditions described by Laemmli (*20*) except that sodium dodecyl sulfate was omitted; but 0.5 percent (by volume) Triton X-100 was present throughout. When the moving front had reached nearly to the bottom of the gel, electrophoresis was ended, and the gel was stained for AChE activity (*10*). The top half of the gel is shown; no bands were visible in the bottom half. The bands visible in (a) and (b) were absent when gels were stained in the presence of $10^{-5}M$ eserine sulfate. Fig. 4 (right). AChE activity in mixtures of extracts. Two extracts were mixed in varying proportions by volume. One was a control cell extract (the specific activity was about 3 nmole/hour per milligram), the other was an extract of cells treated with β-ecdysone ($10^{-7}M$; 71 hours; the specific activity was 640 nmole/hour per milligram); *F* is the fraction of the latter in each mixture; Δ_{412} is the difference between A_{412} of the experimental and eserine-blank tubes; $\Delta_{412} = 1.0$ would correspond to a specific activity of about 735 nmole/hour per milligram in this experiment.

The mixing experiment (Fig. 4) shows that control cell extracts do not inhibit the activity of AChE in extracts of hormone-treated cells. It seems likely, but remains to be proved, that the increase in AChE activity is due to de novo synthesis of AChE.

Histochemistry of AChE (*10*) shows that very few control cells have detectable eserine-sensitive AChE. By contrast, cells treated with β-ecdysone exhibit fairly uniform staining, with virtually no unstained cells (*11*). The extent of cell-to-cell variation in AChE has not yet been determined quantitatively. It is possible that the effect of β-ecdysone is not to induce AChE at the cellular level but rather to cause a relative stimulation in the growth of an AChE-constitutive subpopulation. Thus β-ecdysone might cause selection rather than enzyme induction. This hypothesis can be rejected because no subpopulation with the requisite enzymatic and growth properties can be demonstrated by histochemistry or by analysis of time-lapse films (*12*).

Our experiments demonstrate that physiologically reasonable concentrations of β-ecdysone induce AChE activity in Kc-H cells. That this activity is AChE and not a minor cholinesterase is suggested by the following considerations. It is known that the largest part, if not all, of the AChE in adult flies is due to a closely related group of isozymes, all probably coded at least in part by the *Ace* gene (*13*). Kc-H cell AChE is indistinguishable from this major *Drosophila* AChE in all of the enzymatic properties we have tested as well as in size. The differences in electrophoretic behavior between Kc-H cell AChE and the isozymes of whole Oregon-R flies are, in any case, no greater than those among the isozymes themselves. Thus it is simplest to suppose that the Kc-H cell AChE is that determined by the *Ace* gene. Our hypothesis, most strongly stated, is that β-ecdysone induces synthesis of the *Ace* gene product in Kc-H cells.

Our results, taken in conjunction with Courgeon's, suggest that Kc-H cells may be precursors of neurons or glial elements. This idea is supported by histochemical evidence that in *Drosophila* AChE is restricted to the nervous system (*13*). Moreover, Dewhurst *et al.* (*14*)

have found that AChE and choline acetyltransferase (an exclusively neural enzyme in vertebrates) are present in constant ratio in whole adult *Drosophila*, in their brains, and in their thoracic ganglia; this is another indication that AChE in *Drosophila* is essentially restricted to the nervous system. Still, whether Kc-H cells are actually differentiating along neural lines must remain an open question since the highest AChE specific activity we have observed in these cells is only 1 percent of that in a *Drosophila* brain (*13–15*) and it is not known whether AChE at such low specific activities is a good marker for nervous tissue in *Drosophila*. Only by examining additional tissue-specific markers will it be possible to say whether Kc-H cells are neural elements or, indeed, whether they are faithful to any normal differentiative pathway. Nonetheless, the induction at physiological hormone concentrations of readily measurable amounts of a well-defined biochemical activity greatly enhances the utility of Kc-H cells for the study of ecdysone's action.

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12. In order to account for a 20-fold increase in AChE specific activity during the first 2 days of ecdysone treatment, the relative frequency of AChE-constitutive cells would have to increase 20-fold. Given the concurrent increase in the total population of 1.8-fold and the absence of cell death during this period, a 36-fold increase in the number of constitutive cells would be required. This cohort would have a mean division time of about 9 hours and, if it were sufficiently large, we should detect it in films. We have collected the pedigrees of more than 200 cells during β-ecdysone treatment. No intermitotic time less than 12 hours has been observed. From the absence of the rapidly dividing cells in our sample, we conclude that these cells, if present, represent ≤ 2.5 percent of the population initially. This being so, they should represent ≤ 50 percent of the population at 48 hours, and this heterogeneity would be detectable histochemically. The smaller the cohort of constitutive cells, the higher must be the activity of each cell and the more readily detectable in histochemical tests. We do not detect heterogeneity of nearly the magnitude required by the hypothesis.
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Proton-Induced X-ray Emission Analysis of Single Human Hair Roots

Abstract. *Collimated beams of 3.75 million electron volt protons were used to examine a 2-millimeter length of the root end of human hair; the concentrations of some hair root elements were correlated with the results of standard clinical assays of blood samples. The technique should be useful for the analysis of micro amounts of biological tissue.*

With the rapid development and application of proton-induced x-ray emission (PIXE) analysis, considerable interest has been generated in measurement of the elemental content of biological samples. Horowitz and Grodzins (*1*) presented an interesting example of PIXE analysis of elements along the length of a hair strand. Cookson and Pilling (*2*) reported elemental distributions determined by scanning a proton microbeam across the diameter of a single hair. Lazar (*3*) commented that many difficulties are encountered in obtaining valid correlations between the elemental content of a hair strand and medical factors, primarily be-

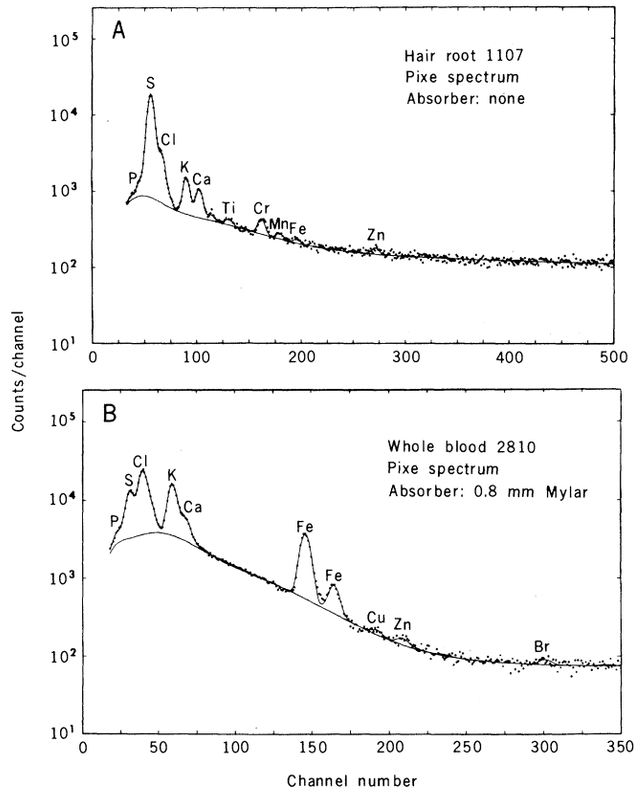

Fig. 1. Proton-induced x-ray emission spectra of (A) hair root and (B) whole blood samples. Values for Fe, Cu, Zn, and Br in 2 μl of blood are 642, 8.0, 11.0, and 7.2 ng, respectively. The blood spectrum is for the only child in whom copper was detected in both blood and hair root.