The Effects of Insect Hormones on the Multiplication Rates of Cultured Insect Cells *in Vitro*

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The effects were examined of some insect hormones on the multiplication rates of cells in cultures obtained from *Antheraea eucalypti* pupal ovarioles. 20-hydroxyecdysone and ponasterone A showed stimulating effects on cell multiplication at the concentration of 0.1 μg per ml. Proteinic brain hormone, and farnesol had no stimulating effect on the cell growth. At the concentration of 10.0 μg per ml, 20-hydroxyecdysone, ponasterone A and farnesol impaired the cell growth markedly if no protein was present in the culture media, but this deleterious effect was moderated in each instance by the addition of bovine plasma albumin to the culture medium. Combinations of 20-hydroxyecdysone and farnesol at various ratios caused no stimulation of the cell multiplication.

INTRODUCTION

Several insect cell lines have been established recently. Some of them are maintained in culture media containing insect haemolymph, and others in media containing vertebrate sera, as it has been found that insect haemolymph or vertebrate sera are essential for the growth of insect cells *in vitro* and without them, the cells cannot multiply. The growth of insects is controlled by hormones, and the tissues are thought to be exposed to these hormones, therefore, it was speculated that insect hormones might stimulate the growth of insect cells *in vitro*. Furthermore, it is known that substances which mimic the action of some insect hormones are distributed in various tissues of vertebrates, and therefore, it was possible that the beneficial effect of vertebrate sera on the growth of insect cells could be due to these substances. The present study was made to investigate the effects of insect hormones on the multiplication of insect cells *in vitro*.

MATERIALS AND METHODS

Cell Lines and Media : The cell line established from pupal ovarioles of *Antheraea eucalypti* SCOTT (GRACE, 1962) was used throughout the experiments. The stock cultures of the cells had been maintained for 8 years in GRACE's culture medium (G. M. A.) at 28°—30°C by changing the medium every 6 days. The culture medium (G. M. A.) consisted of 21 amino acids, 3 sugars, 4 organic acids, 10 vitamins, 6 inorganic salts, 2 antibiotics, bovine plasma albumin (fraction V), and

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heat-treated haemolymph from *Antheraea pernyi* (GRACE and BRZOSTOWSKI, 1966). In addition to G. M. A., media without insect haemolymph (G. M. B.) or without bovine plasma albumin and insect haemolymph (G. M. O.) were prepared, leaving the other constituents unchanged.

Insect Hormones : 20-hydroxyecdysone and ponasterone A were used as representatives of moulting hormones (kindly supplied by Dr. D. H. S. HORN of CSIRO, Melbourne, Australia). Proteinic brain hormone isolated from the pupal brain of *Bombyx mori* (KOBAYASHI and YAMAZAKI, 1966) was made available by courtesy of Dr. M. KOBAYASHI, Sericultural Experiment Station, Tokyo, Japan. Farnesol was used as juvenile hormone although it was fully realized that it is not the corpus allatum hormone. All the hormones were kept in the refrigerator at 4°C until used. The moulting hormones were dissolved in ethyl alcohol at the concentration of 1.0 mg per ml, and the brain hormone in distilled water at the same concentration. Ten-fold dilutions were made with culture media from the stock solutions immediately before they were tested. For farnesol, 10.0 mg was mixed with 1.0 ml of ethyl alcohol, and then added to culture medium to make 10.0 ml. This resulted in a very fine emulsion. Test solutions were prepared by ten-fold dilutions of the emulsion with culture medium. The emulsion of farnesol was prepared just before use, because farnesol separated if the emulsion was kept for long.

Determination of Cell Growth : The cells were cultivated in 2 inch diameter petri dishes containing 3.0 ml of the medium containing the test substances. The cultivation was carried out at 25°C for 4 or 6 days without a change of the medium. The cells were counted every day or every 2 days. The method for the enumeration of cells was described previously (MITSUHASHI et al., 1970).

RESULTS

20-Hydroxyecdysone : When 20-hydroxyecdysone was added to G. M. A., the growth rates of the cells were either stimulated or inhibited, depending upon the concentrations of the hormone in the medium (Fig. 1). The effects of the hormone were not recognizable at any concentration tested on the first day after the cultures were set up. On the second day, the number of the cells in 0.1 µg per ml of the hormone was markedly increased (significant at 5 per cent level), while those in other concentrations of the hormone were about the same as that in the control. On the third day, the stimulative effect on the growth rate in 0.1 µg per ml of the hormone became prominent (significant at 0.5 per cent level). A similar stimulative effect was observed in 1.0 µg per ml of the hormone (significant at 1.0 per cent level). There was no significant difference in the numbers of cells between the control and 0.01 µg per ml of the hormone. A slightly inhibitory effect on the growth rate was observed in 10.0 µg per ml of the hormone (significant at 5 per cent level). On the fourth day, the stimulating effect in 0.1 µg per ml of the hormone was still maintained (significant at 0.5 per cent level). The difference in the cell numbers between the control and 1.0 µg per ml of the hormone became less prominent (significant at 5 per cent level), while the hormone at the concentration of 0.01 µg per ml showed the more prominent stimulative effect (significant at 1.0 per cent level). The inhibitory effect in 10 µg per ml of the hormone was reduced (the difference from the control was no longer significant).

20-HYDROXYECDYSONE IN G. M. A.

Fig. 1. Growth of the cells in G. M. A. containing 20-hydroxyecdysone.

20-HYDROXYECDYSONE IN G. M. O.

Fig. 2. Growth of the cells in G. M. O. containing 20-hydroxyecdysone.

As G. M. A. is a medium which supports vigorous cell growth, the effect of the addition of growth stimulating substances could be difficult to detect. For this reason the experiment was repeated using G. M. O. instead of G. M. A. It is clear in Fig. 2, that G. M. O. maintained the population density of the cells for a short period, but did not permit the continuous growth of the cells. When 20-hydroxyecdysone was added to this medium the growth stimulating effect at $0.1 \mu\text{g}$ per ml disappeared, and the deleterious effect at $10.0 \mu\text{g}$ per ml became marked (Fig. 2). This result suggests that 20-hydroxyecdysone has a stimulating effect only in the presence of serum or other protein. In vertebrates, it has been said that sterol is present in the serum, conjugated with protein (AVIGAN, 1959). Therefore, the effect of 20-hydroxyecdysone was tested in medium containing bovine plasma albumin (G. M. B.). In G. M. B. without hormone the cells survived for a short period but did not multiply continuously. No stimulative effect of the hormone was observed, but the inhibitory effect of the hormone at $10.0 \mu\text{g}$ per ml was somewhat moderated compared with the growth rate in G. M. O.

Ponasterone A : Ponasterone A showed similar effects to 20-hydroxyecdysone on the cell growth. On the first day of the cultivation no difference in the growth rate of the cells was observed between the control and the groups treated with the hormone. On the second day, however, stimulative effects of the hormone were observed in $0.01 \mu\text{g}$ per ml and $0.1 \mu\text{g}$ per ml of the hormone (significant at 5.0 per cent and 1.0 per cent levels respectively). After the third day the stimulative effects were not significant. An inhibitory effect of the hormone was observed at $1.0 \mu\text{g}$ per ml and $10.0 \mu\text{g}$ per ml of the hormone (significant at 1.0 per cent level) on the second day and was maintained throughout the cultivation period. As in the case of 20-hydroxyecdysone, the toxic effect at $10.0 \mu\text{g}$ per ml was greatly emphasized in G. M. O., and was moderated in G. M. B.

Brain Hormone : Brain hormone did not affect the cell growth at all when it was tested in G. M. A. or in G. M. O.

Farnesol : Farnesol did not show any effect on cell growth when it was incorporated into G. M. A., but a marked decrease of the cell population was observed at $10.0 \mu\text{g}$ per ml when tested in G. M. O. As in the case of the moulting hormones, this toxicity was moderated when farnesol was incorporated into G. M. B.

Interaction of Moulting Hormone and Juvenile Hormone : The development of intact insects is controlled by the balance of moulting hormone and juvenile hormone. It was interesting, therefore, to see whether or not the presence of both hormones at various ratios could effect the cell growth *in vitro*. For this purpose, 8 different media were prepared;

- (1) G. M. O. containing $0.1 \mu\text{g}$ 20-hydroxyecdysone and $0.1 \mu\text{g}$ farnesol per ml;
- (2) G. M. O. containing $0.1 \mu\text{g}$ 20-hydroxyecdysone and $0.001 \mu\text{g}$ farnesol per ml;
- (3) G. M. O. containing $0.001 \mu\text{g}$ 20-hydroxyecdysone and $0.1 \mu\text{g}$ farnesol per ml;
- (4) G. M. O. containing $0.001 \mu\text{g}$ 20-hydroxyecdysone and $0.001 \mu\text{g}$ farnesol per ml;
- (5) G. M. A. containing $0.1 \mu\text{g}$ 20-hydroxyecdysone and $0.1 \mu\text{g}$ farnesol per ml;
- (6) G. M. A. containing $0.1 \mu\text{g}$ 20-hydroxyecdysone and $0.001 \mu\text{g}$ farnesol per ml;
- (7) G. M. A. containing $0.001 \mu\text{g}$ 20-hydroxyecdysone and $0.1 \mu\text{g}$ farnesol per ml;
- (8) G. M. A. containing $0.001 \mu\text{g}$ 20-hydroxyecdysone and $0.001 \mu\text{g}$ farnesol per ml.

No effect on the cell growth was obtained in any of the media if G. M. O. was used as solvent. If the hormones were dissolved in G. M. A., the growth rate of the cells increased slightly only in medium 5, but this increase was not significant (not significant at 5.0 per cent level). Since, in the previous experiment, an increase of the cell growth was obtained in G. M. A. containing 20-hydroxyecdysone at 0.1 μg per ml, it may be considered that the effect of 20-hydroxyecdysone was affected by the presence of farnesol.

Cholesterol: Insects cannot synthesize cholesterol, and it is an essential component of the diet for most insects. Furthermore, cholesterol itself was identified as a hormone produced in the neurosecretory cells of the brain of *B. mori* (KOBAYASHI et al., 1962). G. M. O. does not contain sterol. Therefore, the effect of cholesterol on the growth of the cells was examined with G. M. O. Cholesterol showed no stimulative effect on the cell growth, while cholesterol at 100.0 μg per ml decreased the cell population. This toxic effect of cholesterol at high concentration is similar to that of the moulting hormones and of farnesol, although the concentration of cholesterol used was higher. To see whether the toxic effect of cholesterol at high concentration was moderated in the presence of protein in the medium or not, the experiment was repeated with G. M. B. Unlike the results of moulting hormones and farnesol, the toxic effect of cholesterol was not moderated in G. M. B., and no stimulative effect on the cell growth was obtained.

DISCUSSION

In the early stages of insect tissue cultures, the effects of insect hormones had been investigated by cultivating organs with certain endocrine organs. This type of study has been continued up to date by substituting the endocrine organs with pure insect hormones (MICIARELLI, 1967; OBERLANDER and FALCO, 1967; BURDETTE et al., 1968; AGUI et al., 1969a, b; KURODA, 1969; LAVERDURE, 1969; OBERLANDER, 1969; PIHAN, 1969; WILLIAMS and KAMBYSELLIS, 1969; YAGI et al., 1969; MARKS and LEOPOLD, 1970). The effects of insect hormones in insect cell cultures have been studied to a lesser extent.

GRACE (1958) showed that the migration of cells from the explanted ovarioles of *B. mori* as well as the number of mitoses in the migrated cells were increased markedly when the extract of endocrine organs, which were either ring glands of *Lucilia cuprina* or prothoracic glands of *Periplaneta americana*, were incorporated into the culture medium. Recently MARKS et al. (1967) has reported that 0.1 μg per ml of 20-hydroxyecdysone stimulated the migration of cells from cockroach, *Leucophaea maderae*, embryonic legs. This stimulative effect seems to be enhanced with the concentration of the hormone up to 5 μg per ml. On the other hand the same authors stated that in the mono-layer cell culture from the dissociated whole embryos, 5 μg per ml of the hormone arrested mitoses and slowed down the movement of the cells. On the contrary, the cells from various tissues of tobacco horn worm, *Manduca sexta*, have been reported to become very active by the incorporation of 2.5-25 μg per ml of 20-hydroxyecdysone (JUDY, 1969). It is interesting that GRACE's *A. eucalypti* cells did not respond to the same amount of the hormone (JUDY, 1969).

In the present study, a stimulative effect was obtained only from the moulting

hormones, but the effect was not marked and was not present in the absence of insect haemolymph. It may, therefore, be concluded that the insect hormones are not growth promoting factors for insect cells cultivated *in vitro*. The hormones, however, may control the growth of organized tissues. If so, a stimulative effect of hormones may be obtained in some organ cultures, however, the hormone may not act directly on the cells.

Farnesol did not show a stimulative effect on the cell growth in the present study. It has been reported that dodecyl-methyl-ether or phytol, which is known to have juvenile hormone-like activity, showed slight stimulative effect on the outgrowth from the eye-antennal discs of *Drosophila melanogaster* (KURODA and MINATO, 1967).

Cholesterol did not improve the cell growth at all. Cholesterol has been reported to give no effect on the outgrowth from the eye-antennal discs of *D. melanogaster* also (KURODA and MINATO, 1967). Since the cells most likely do not synthesize cholesterol, it seems to be non-essential for their growth. But the sterol is essential for the growth of lepidopterous insects. Sterol may be used for the production of hormones or other substances and may not be needed for the growth of individual cells even *in vivo*.

Growth promoting substance(s) for the insect cells should be present in the haemolymph of *A. pernyi* and it is hoped that chemical analyses of the components of the haemolymph will lead to the finding of the growth factor(s). Once a growth promoting substance(s) is found, it may be easy to formulate a chemically defined medium for the cells, and this could facilitate a large number of biochemical studies on the cultivated insect cells.

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