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DIFFERENCES IN EFFECTS OF ROTENONE ON INSECT CELL LINES[1]

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Growth of insect cells in vitro was inhibited by addition of rotenone to culture media. The rate of inhibition did not vary in cell lines originating from the same tissue of the same species. However, there were great differences between cell lines derived from different species or different tissues. Among the tested cell lines, the ones from *Mamestra brassicae* ovaries were most sensitive and the ones from *M. brassicae* hemocytes were most insensitive to rotenone. Respiratory rate did not vary in cell lines from the same species regardless of the original tissues. However, it varied considerably between cell lines from different species. The fluctuation in rates of respiratory inhibition by rotenone showed a tendency similar to that in the rates of growth inhibition.

Introduction

Vast numbers of insects are killed every year as bioassay materials, especially for developing new insecticides. Rearing of these insects is usually laborious, time consuming and costly. Cultured insect cells have been considered a good alternative bioassay material, and the effects of insecticides on cultured insect cells have been studied.[1-3] In these studies it has become clear that cultured insect cells are rather insensitive to most insecticides which are neurotoxins, compared to whole body insects. On the contrary, cultured cells were found to be very sensitive to respiratory inhibitors, such as rotenone. However, these studies have been made on limited insect cell lines. We have been examining the effects of various insecticides on various insect cells cultured in vitro, and report herein the effects of rotenone on cell growth and cell respiration which varied according to the species and tissues from which the cell lines were derived.

Materials and methods

Cells :

Cell lines used were NIAS - MB - 25 and NIAS - MB - 32 derived from pupal ovaries of the cabbage armyworm, *Mamestra brassicae*, NIAS - MaBr - 92 and NIAS - MaBr - 93 from larval hemocytes of *M. brassicae*, SES - MaBr - 1 and SES - MaBr - 4 from larval fat bodies of *M. brassicae*, and NIAS - PX - 64 from pupal ovaries of the swallowtail butterfly, *Papilio xuthus*. All cell lines had been maintained in Mitsuhashi and Maramorosch's medium[4] with additional 3 % fetal bovine serum at 25 ℃. This medium was used throughout the experiments.

Insecticides :

Rotenone (99 %, Aldrich Chemical Company, Inc., Milwaukee) was dissolved in ethyl alcohol at the concentration of 3×10^{-3} M. From the solution a ten - fold dilution was made with ethyl alcohol. The diluted rotenone solution and the culture medium were mixed at the ratio of 1 : 300 so as to give required final concentration of rotenone in test media. All the test media, therefore, contained ethyl alcohol at the concentration of 0.33 % and the rotenone was evenly dispersed in the test media.

Test cultures :

Cells were harvested from stock cultures and counted with a hemocytometer. The cell suspension was spun down at 1,500 r.p.m. for 3 min. The cell pellet was dispersed in fresh

1. This study was supported by a grant from the Research Group Japan for Alternatives to Animal Testing.

culture medium so as to provide a cell density of 2×10^6 cells per ml. Three ml of this cell suspension was dispensed to each culture bottle (MB - 30, 30 x 60 x 30, Tsukuba Flat, Co. Ltd., Nagareyama, Chiba). To these cell suspensions, 10 μl of alcoholic rotenone solution was added by means of a microsyringe. This resulted in a fine rotenone emulsion. The culture bottles were capped with silicone rubber stoppers and maintained at 25 ℃ for 6 days. As controls, the cultures given 10 μl ethyl alcohol alone were prepared. At least three replicates were made for each rotenone concentration.

Cell enumeration :

During the experimental cultures, living cells were counted at an interval of 24 hr or 48 hr. Living and dead cells were distinguished by their different morphology. The counting was done directly on the cultured cells by projecting a square grid into the field of the microscope. The cells were counted at 10 places selected randomly for each bottle. The density of cells were calculated from the recorded numbers of cells, the area of the bottom of the culture bottles and the amount of media.

Measurement of oxygen consumption :

For oxygen consumption experiments, the *M. brassicae* cell lines (NIAS - MB - 32, NIAS - MaBr - 92 and SES - MaBr - 4) and a *P. xuthus* cell line (NIAX - PX - 64) were used. The harvested cells from stock cultures were spun down at 1,500 r.p.m. for 3 min and the cells were dispersed in fresh culture media so as to give a cell density of 2×10^6 cells per ml. Respiration of cells was measured with a Clark type oxygen electrode (Rank Brothers, Ltd., Cambridge). Two ml of cell suspension was introduced into a culture chamber of the device. Surface of the cell suspension was covered with a drop of liquid paraffin to disturb the contact of the cell suspension with air. Measurements were made for 45 min from 15 min after the setting up the test cultures. When the inhibitory effects of rotenone were examined, 6 μl of alcoholic rotenone solution was added to the cell suspension at the time the culture was set up.

Topical application of rotenone to whole body insects :

M. brassicae larvae were reared on an artificial diet[5], and *P. xuthus* larvae on fresh leaves of citrus plants. The third instar larvae were used as test animals. Two μl of rotenone solution in acetone at various concentrations was applied to the dorsal cuticle of thorax or abdomen of the test animal. Then the deaths of the test animals were recorded daily.

Evaluation of the inhibitory effects by rotenone :

For cellular growth inhibition, median inhibitory concentration (IC_{50}) was calculated by means of least square method from the growth inhibitory rates against the controls. For cell respiration, 25 % inhibitory concentration (IC_{25}) was calculated, because respiratory inhibition did not exceed over 50 %. The calculation was made in the same manner as for IC_{50} for growth inhibition. From the results of insecticidal tests, the median lethal dose (LD_{50}) was calculated by means of probit analysis[6] on Abbott's compensated[7] mortality of test animals.

Results

Effects of rotenone on cell growth :

Growth inhibition was not observed in the control culture which contained ethyl alcohol at the concentration of 0.33 %. It was, therefore, assumed that ethyl alcohol, the solvent of rotenone, had no effects on cell growth in the experimental cultures. Figs. 1 A, 1 C, 2 A, 2 C, 3 A, 3 C, and 4 A show the growth curves of cells in rotenone - containing media. These results, clearly show that the sensitivity to rotenone was different in cell lines originating from different tissues or species. The critical concentration of rotenone was further examined for each cell line by setting up three additional concentrations between the highest concentration which permitted cell growth and the lowest one which decreased the cell population (Figs. 1 B, 1 D, 2 B, 2 D, 3 B, 3 D, and 4 B). Based on the above results, IC_{50}s were calculated (Table 1). Cell lines from the same tissue of the same species had similar sensitivity to rotenone, but cell lines derived from different tissues even of the same species had different sensitivities : it varied also between cells

derived from the same tissue of a different species.

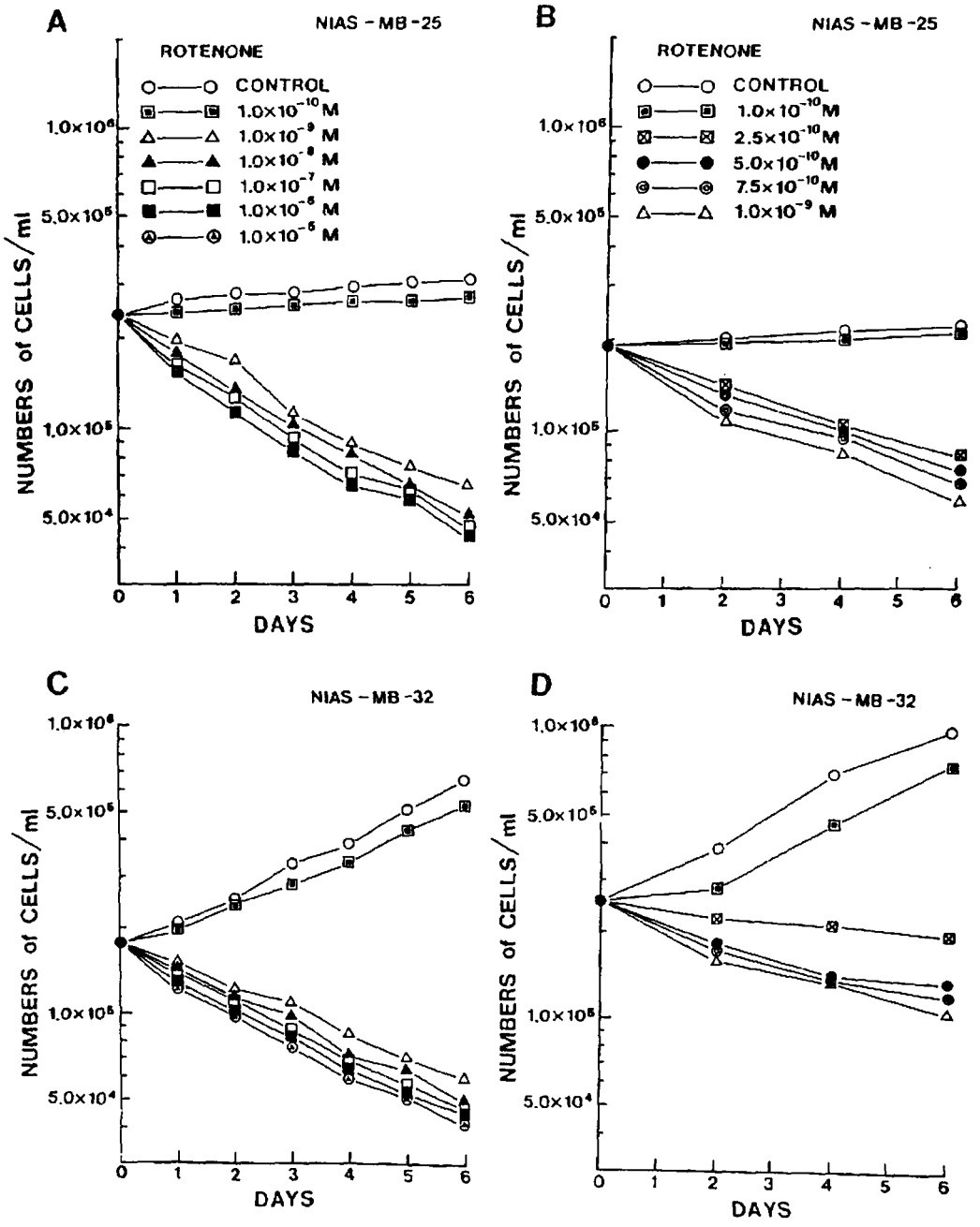

Fig. 1. Growth inhibition by rotenone at various concentrations on the cell lines derived from *M. brassicae* pupal ovaries. A and B, NIAS - MB - 25 cell line ; C and D, NIAS - MB - 32 cell lines. Symbols in C and D are the same as in A and B respectively.

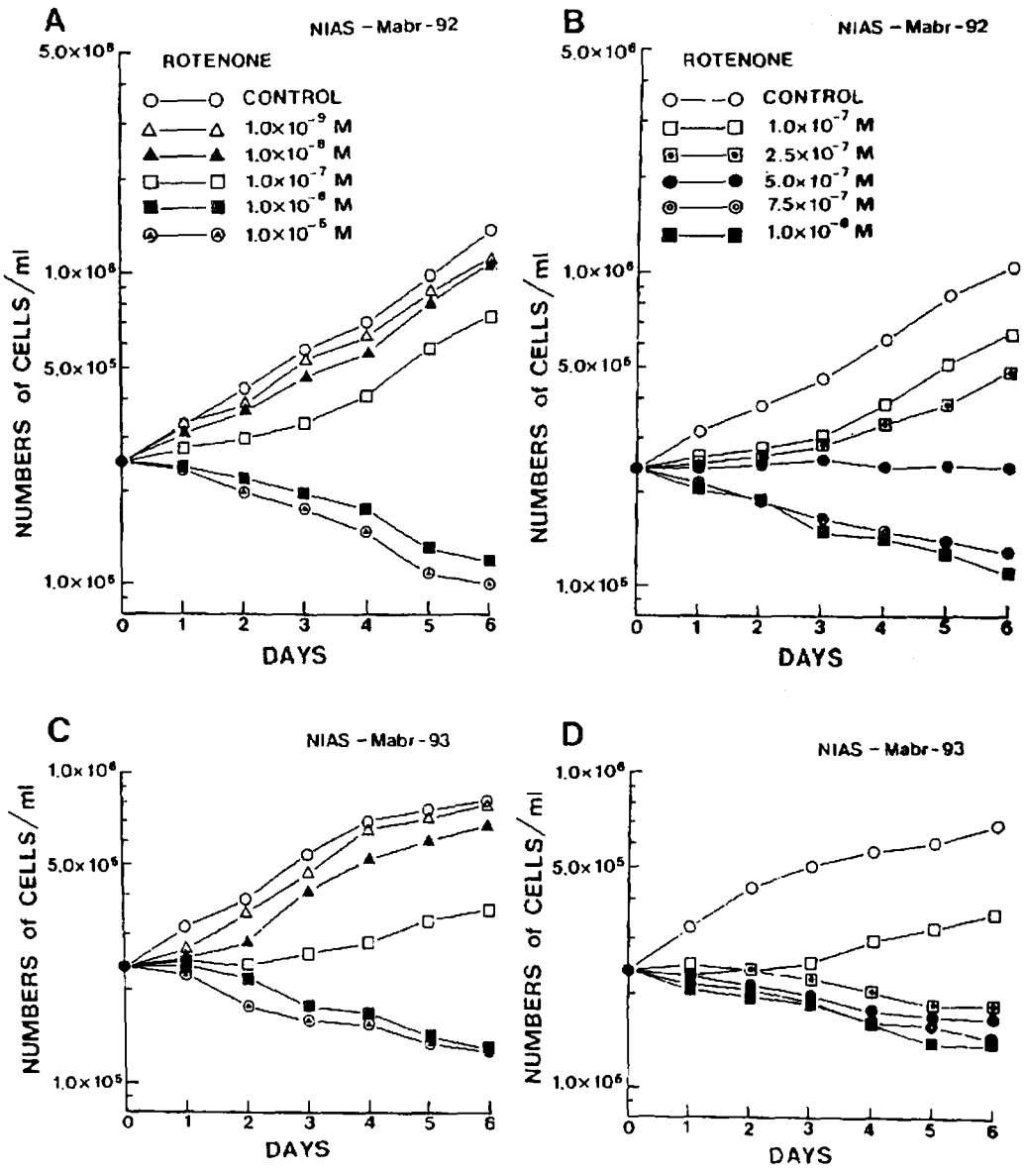

Fig. 2. Growth inhibition by rotenone at various concentrations on the cell lines derived from *M. brassicae* larval hemocytes. A and B, NIAS - MaBr - 92 cell line ; C and D, NIAS - MaBr - 93 cell line. Symbols in C and D are the same as in A and B respectively.

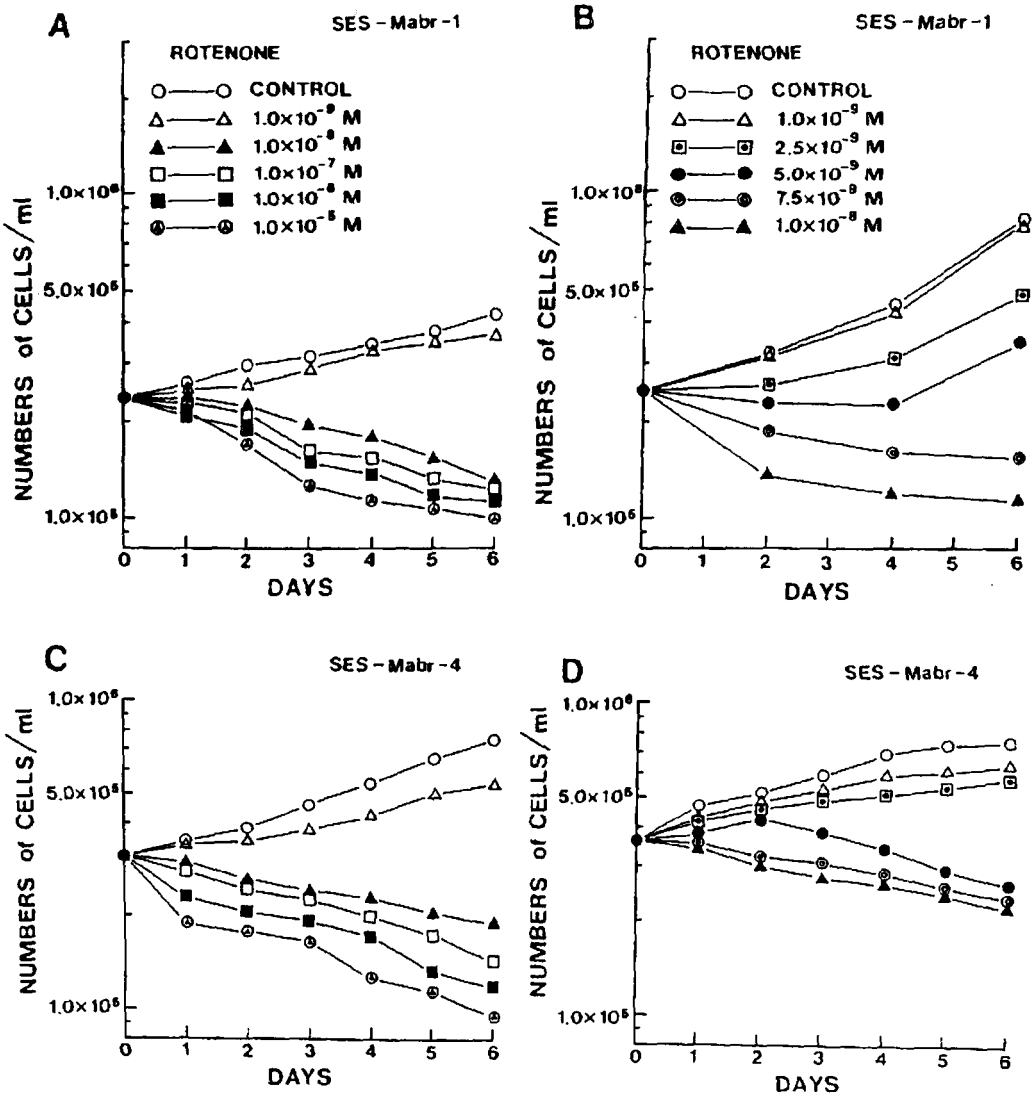

Fig. 3. Growth inhibition by rotenone at various concentrations on the cell lines derived from *B. brassicae* larval fat bodies. A and B, SES-MaBr-1 cell line; C and D, SES-MaBr-4 cell line. Symbols in C and D are the same as in A and B respectively.

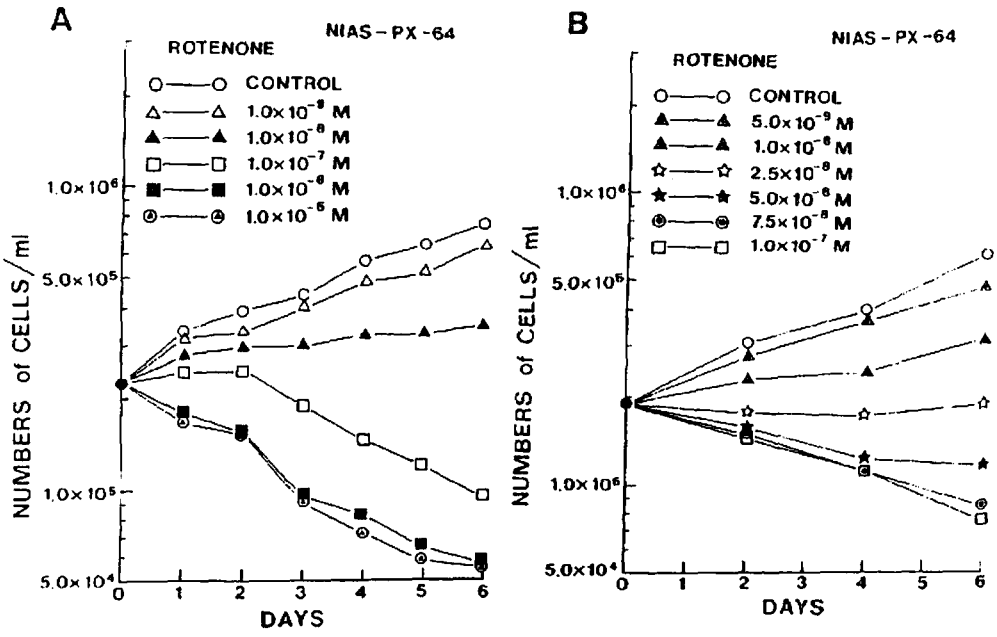

Fig. 4. Growth inhibition by rotenone at various concentrations on the cell line, NIAS - PX - 64, derived from *P. xuthus* pupal ovaries.

Table 1. Effects of rotenone on cell growth and respiration

Cell lines	Growth inhibition IC_{50} ($\times 10^{-10}$ M)	Respiratory inhibition IC_{25} ($\times 10^{-10}$ M)
NIAS - MB - 25	3.2	–
NIAS - MB - 32	1.7	2.6
SES - MaBr - 1	36	–
SES - MaBr - 4	42	12
NIAS - MaBr - 92	1800	230
NIAS - MaBr - 93	740	–
NIAS - PX - 64	140	130

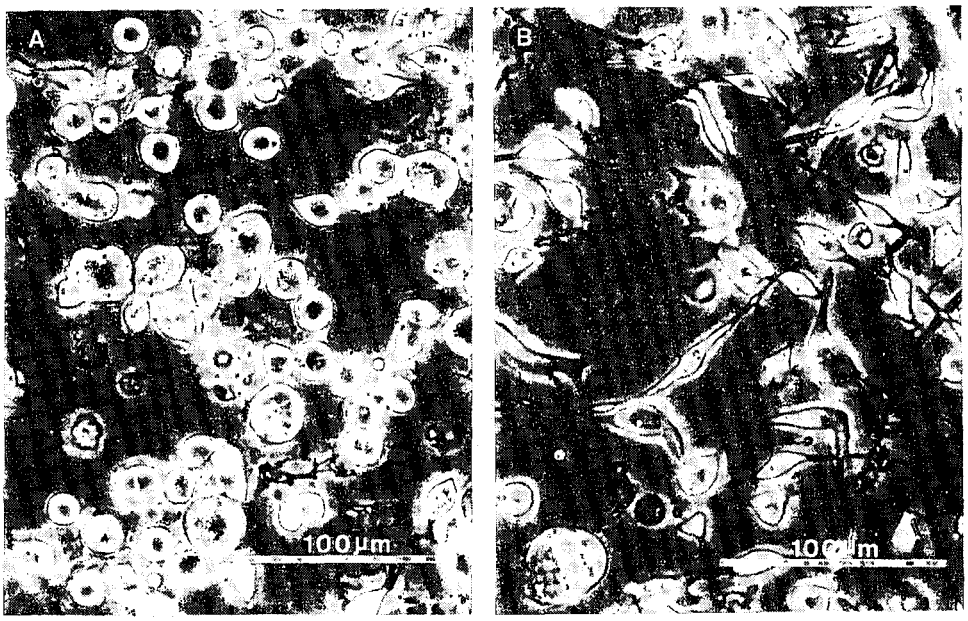

Fig. 5. Elongation of cells treated by rotenone at sublethal concentrations. A. non-treated *M. brassicae* hemocyte cell line (NIAS-MaBr-93); B. the same cell line as in A treated with rotenone at the concentration of 1.0×10^{-7} M.

During the above experiments, we noticed that cells derived from *M. brassicae* hemocytes and fat bodies changed their shapes in the media containing a sublethal dose of rotenone. These cells were originally spherical in shape. However, elongated cells became predominant after the rotenone treatment (Fig. 5). The elongated cells continued to multiply even in the media containing rotenone.

Effects of rotenone on cell respiration :

Respiration rate of cells was examined in the rotenone-free media. The oxygen consumption per min per cell did not differ among cell lines derived from different tissues of *M. brassicae*, while that of *P. xuthus* cells differed markedly from that of the *M. brassicae* cells (Fig. 6).

Addition of rotenone to the culture media decreased the oxygen consumption in all cell lines, but the inhibitory rate did not exceed over 50 %. The rates of respiratory inhibition by rotenone were varied among cell lines (Fig. 7). From these results IC_{25} was calculated for each cell line (Table 1).

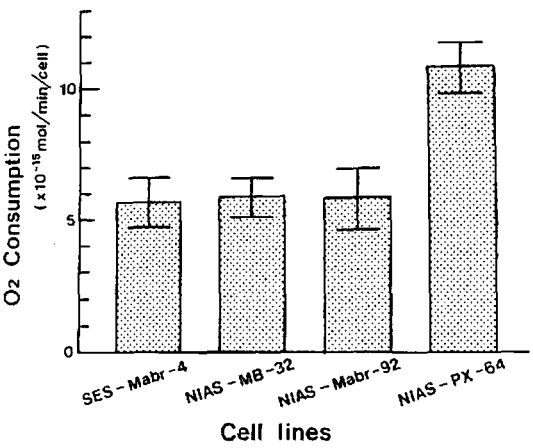

Fig. 6. Respiratory activity of several insect cell lines. For origins of the cell lines, refer to materials and methods in the text.

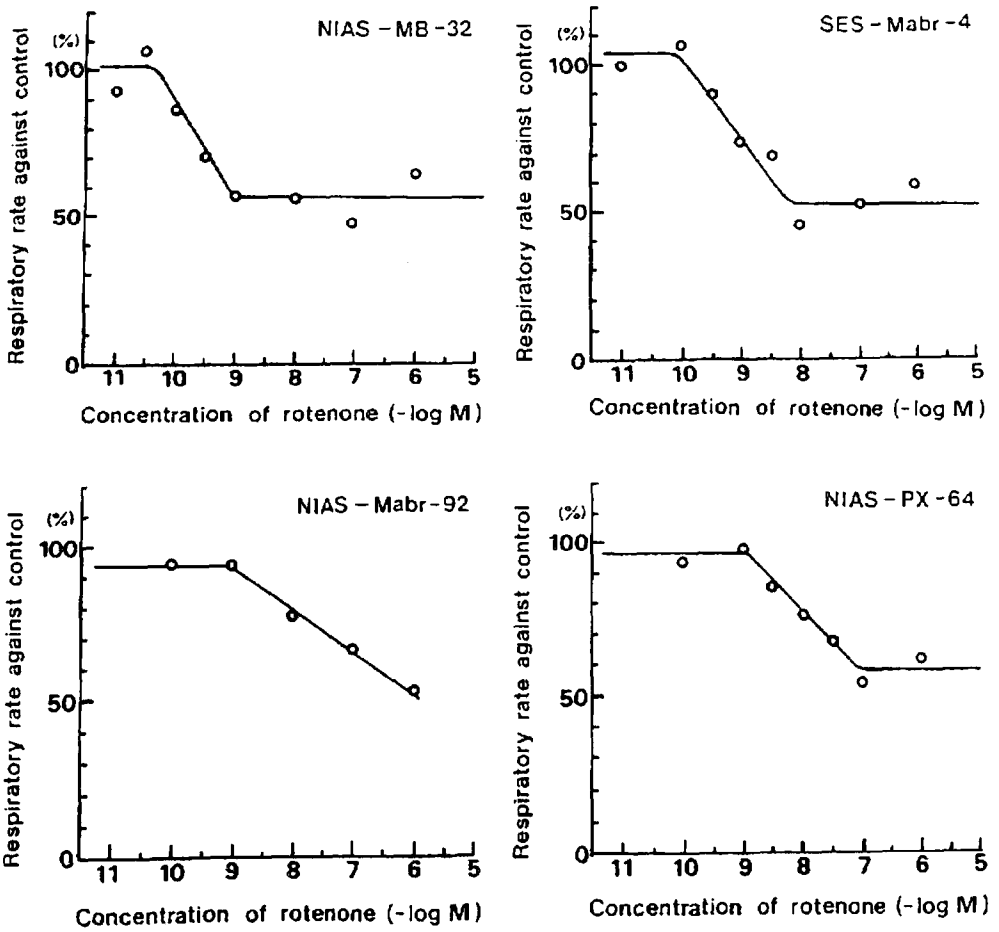

Fig. 7. Inhibition of cellular respiration by rotenone at various concentration. For origins of the cell lines, refer to materials and methods in the text.

Insecticidal activity of rotenone :

Table 2 shows the results of the tests on *M. brassicae* larvae. *P. xuthus* larvae were not killed at all by rotenone at the concentration tested. From the data in Table 2, LD_{50} was calculated as 5 mg per g.

Discussion

In general, rotenone decreased markedly the growth rates of all the cell lines. However, the rates of the decrease were considerably different in cell lines derived from different species or tissues.The most sensitive cell lines, *M. brassicae* ovarial cells, were about one thousand times more sensitive than the *M. brassicae* hemocyte cell lines. Between cell lines derived from the same tissue, ovaries, of different species, differences in sensitivity to rotenone were also observed. On the other hand, no difference in the sensitivity was observed between cell lines derived from the same tissue of the same species. The intraspecific differences due to the original tissue were much greater than the interspecific difference of the same tissues.

Mitsuhashi et al.[1] and Yoshida et al.[2] have studied the effects of various insecticides on the growth of insect cell lines. For the cell lines derived from the emperor gum moth, *Antheraea*

Table 2. Mortality of *Mamestra brassicae* larvae at 144 hr after rotenone application[1].

Dose of rotenone (μg/larva)	Numbers of surviving larvae[2]	Percentage of survivors	Compensated mortality[3] (%)
0	9.8 ± 0.5	98	–
5	9.5 ± 0.6	95	2.5
6	8.8 ± 1.3	88	10.6
10.8	8.0 ± 1.4	80	17.5
18	6.8 ± 0.5	68	30.6
30	6.8 ± 2.2	68	31.4
50	4.0 ± 1.6	40	59.5

1) The 3rd instar larvae (average body weight was 9 mg) were used

2) Average + S. D. of 4 replicates (for each replicate, 10 larvae were used).

3) Compensated according to Abbott's equation.[7]

eucalypti, pupal ovaries and the yellow fever mosquito, *Aedes aegypti*, embryos, order of magnitude in IC_{50} of rotenone was 10^{-3} M.[1] The IC_{50} for a cell line from the Japanese cellar mosquito, *Culex molestus*, adult ovaries was 1.95×10^{-6} M.[2] These values fall into the range of the IC_{50} values obtained in the present study.

Rotenone is known to block NAD and coenzyme Q in the respiratory chain[8]. Since such a basic pathway is considered to be present in all kinds of cells, differences found among cell lines from different tissues are puzzling. Respiration rates of all the cell lines from the same species showed similar respiratory activity regardless of the tissues from which cell lines derived. Therefore, differences in sensitivity to rotenone may be due to other factors than cell line specific respiratory activity. It may be due to differences in permeability of rotenone or in metabolic activity against rotenone. Experiments along this line are now under way.

Respiratory inhibition caused by rotenone differed with the cell line to which it was applied. These differences seem to be parallel to the differences in growth inhibition. Furthermore, cells from the insects sensitive to rotenone may be said to be sensitive also, and their respiration seems to be more inhibited by rotenone.

References

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