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The Comparison of Rotenone and an Environmentally Safe Insecticide (Azadirachtin) on Cultured Cell Respiration

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Abstract

Introduction: Both azadirachtin and rotenone, two botanical pesticides have shown inhibit the growth of cultured insect cells. The inhibitory effect of rotenone on cell growth is through disruption of electron transport chain, but exact mechanism of effect of azadirachtin on growth of cell is unknown. This study was carried out to compare the effect of these phytochemical on oxygen consumption by cultured cells.

Methods: Electron transport and oxygen uptake were measured using the oxygen electrode, which continuously determines the concentration of oxygen in solution. Rotenone was used as a positive control to investigate if azadirachtin had its effect on respiration of the cells. Estimates of oxygen uptake for Sf9 and C6/36 were done at 30 °C and for L929 at 37 °C.

Results: Preliminary results indicated that concentration of 10^{-4} M of azadirachtin had the maximum effect on cell respiration. Also rotenone in concentration of 10^{-8} M reduced the cell respiration by 50%. However using purified mitochondria, the effect of azadirachtin on respiration rate was not significant.

Conclusion: Interestingly in present test, both azadirachtin and rotenone could reduce the total oxygen consumption. Azadirachtin had only a minor effect on the respiration rate in both mammalian and insect cultured cells and there was only a slight difference between them. Due to low sensitivity of insect cells and also presence of same sensitivity in mammalian cells it seems that the effect of azadirachtin on cell respiration is different to that of rotenone. Also the results of this study suggest that while cell growth assessment is not appropriate for all phytochemical pesticides, it is useful for those, such as azadirachtin and rotenone, whose effect is on the essential mechanisms of insect cells in general.

Keywords: *azadirachtin, cell line, respiration, rotenone*

Introduction

The inhibitory effect of azadirachtin and rotenone on the growth of cells reported in previous study (1) showed that both azadirachtin and rotenone reduce the growth of insect cultured cells in a dose and time-dependent manner. Although azadirachtin, even in high concentration, only slightly reduced the growth of mammalian cell line L929, both mammalian and insect cells were quite sensitive to the effect of rotenone. Interference with the respiration process and an effect on cell division are the ways which rotenone affects cultured cells. Because of the effect of rotenone on the respiratory chain, it interferes with energy metabolism, which eventually causes reduction in growth of cultured cells. Also effect of rotenone on polymerization of tubuline causes arrest of cell cycle, which decreases cell growth (2, 3). In higher organisms, energy metabolism depends on respiration, which takes place through oxidation of organic molecules (4). The electron-transferring molecules of the respiratory chain and the enzymes responsible for ATP synthesis are located in and on the inner membrane, while the matrix contains the enzymes of the TCA cycle. A large family of transporters shuttles metabolites such as ATP and citrate across the inner mitochondrial membrane. The enzyme systems primarily

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responsible for the release and subsequent oxidation of reducing equivalents are thus closely related so that the reduced coenzymes formed during catabolism (NADH and FADH) are available as substrates for respiration (5).

A number of toxins (including some pesticides) can affect electron transport in mitochondria. Rotenone is a respiratory enzyme inhibitor, acting between NAD^+ (a coenzyme involved in oxidation and reduction in metabolic pathways) and coenzyme Q or ubiquinone oxidoreductase (complex I) (2, 3). Since this is a respiratory enzyme responsible for carrying electrons in some electron transport chains, the result is failure of the respiratory functions. Azadirachtin, a tetranortripenoid from the Neem Tree (Azadirachtin indica), has been shown to affect insects in many ways. Antifeedancy, repellency, and profound effects on growth, development and reproduction, are the most important pesticidal properties of this phytochemical. Furthermore it has been shown that the compound is cytotoxic to cultured insect cells, and despite significant effects on insects, its differential toxicity on various cell lines, biodegradation and some other aspects have made this compound as one of potentially safe bio-pesticides (6-8). However in spite of much work, it is still unclear why the terpenoid is toxic to insects and innocuous to mammals. In the present study the effect of rotenone on total oxygen consumption of cultured cells has been compared to that of azadirachtin. Then using rat liver mitochondria the effect of these phytochemicals on mitochondrial respiration has been studied.

Materials and Methods

Cell lines: Sf9 cell line, derived from ovarian cells of *Spodopteraa frugiperda,* and L929 culture, derived from mouse fibroblast cells, were obtained from stocks in the Division of Biochemistry, IBLS, University of Glasgow. C6/36 cells derived from the mosquito *Aedes albopictus* was the kind gift of Dr. A. Bridgen, Division of Virology, IBLS, University of Glasgow.

Chemicals: The pure azadirachtin was produced in the University of Glasgow. The other chemicals (including rotenone, PMS, and MTT, NADH, KCl, Sodium dithionite, malic acid, glutamic acid, Succinate, ADP, TMPD, and catalase were purchased from Sigma (Sigma-Aldrich).

Measurement of oxygen consumption:

The water-jacketed electrode was maintained at a constant temperature by means of water circulating from a thermostatically controlled water-bath. Estimates of oxygen uptake for Sf9 and C6/36 were done at 30 °C and for L929 at 37 °C. Throughout the reported work, the volume of PBS, in which the cells were suspended, was 3 ml. The signal from the electrode was fed into a chart recorder with a 10 mV maximum range. The concentration of the NADH solution was measured accurately by spectrophotometer at 340 nm based on an extinction coefficient of $6.3 \times 10^3\ M^{-1}.cm^{-1}$.

Using mitochondria in the respiration assay

Isolation buffer:

Isolation buffer contained 0.225 M mannitol, 0.07 M sucrose, 0.4 mM EGTA and 2 mM MOPS (pH 7.2).

Preparation of mitochondria from rat liver:

After killing the animal, a medial incision was made from groin to sternum of a rat, and 100 ml of ice-cold 0.9% NaCl was poured into peritoneal cavity. Then by cutting it off at the base the liver was removed and dropped into a second beaker of ice-cold saline solution. Before homogenizing, the liver was washed three times in ice-cold isolation buffer. Then tissues were chopped with a pair of scissors and transferred to a 50 ml glass homogenizing tube in 20 ml isolation buffer. Homogenization was done using a loose-fitting pestle, afterwards was repeated with a tight-fitting pestle to ensure complete breakage of cells. Homogenized cells (including washing) were transferred to a 50 ml centrifuge tubes and after making up the tube, the homogenate with isolation buffer was

centrifuged at 2000 rpm for 10 min at 4 °C. To bring down the mitochondria pellet the supernatant, carefully poured into a clean centrifuge tube and without filling the tube, was centrifuged at 11000 rpm for 10 min to collect the mitochondria. The supernatant was discarded and a Pasteur pipette used to remove the last bit of liquid. A glass rod was used to gently resuspend the remaining pellet. The paste was transferred to an eppendorf tube and air spaces removed by pipetting with a yellow micro pipettor and stored at 4°C.

Effect of azadirachtin on oxygen Consumption of cultured cells.

Acute effect:

Insect and mammalian cells grown to confluence were harvested, and washed with PBS after centrifugation at 800 rpm for 5 min, and finally resuspended in 3ml PBS at a concentration of $5\text{-}10\times10^5$ cells.ml^{-1}. The electrode was then closed and the rate of oxygen consumption followed over a period of 15 min before adding azadirachtin or rotenone to the suspension of cells.

Various concentrations of the two possible inhibitors of respiration were made up in DMSO, before being added to the respiring suspension in the oxygen electrode, in a total of 100 ml to give final concentrations from 10^{-7}-10^{-4} M for azadirachtin and 10^{-11}-10^{-6} M for rotenone (the highest concentrations were dictated by the solubility of compounds). The final concentration of DMSO was 1%, shown in controls to reduce oxygen consumption by 14 ± 4%. The respiration was followed for a 30 min after the addition of the azadirachtin or rotenone. The rate of respiration after the addition of compounds was compared to that before, with each cell suspension acting as its own control.

Chronic effect of azadirachtin on consumption of oxygen by Sf9 cells.

In order to investigate the long-term effect of azadirachtin on respiration of insect cells, azadirachtin dissolved in DMSO was added to culture of Sf9 cells in final concentration of 10^{-8} M. After appropriate time of incubation (30min to 15 h) cells were harvested and resuspended in PBS in the absence of azadirachtin and oxygen consumption was measured as indicated above. The controls contained only DMSO. Samples were taken to estimate oxygen uptake at 30 min intervals.

Effect of serum on activity of azadirachtin and rotenone.

Since albumin in serum can bind unspecifically to many compounds such as pesticides, (9, 10) it was important to investigate the possibility of an interaction of serum albumin with phytochemicals which might reduce the effect of the phytochemicalson cell respiration. To achieve this, concentrations of 10% and 25% of FBS were used in cell suspensions containing and respiration was monitored.

Results

The effect of foetal bovine serum on inhibition of cell respiration by azadirachtin or rotenone

Preliminary results indicated that concentration of 10^{-4} M of azadirachtin had the maximum effect on cell respiration. Also rotenone in concentration of 10^{-8} M reduced the cell respiration by 50%. However it was shown that bovine serum in concentration of 10% and 25% could effectively reduce the acute inhibitory property of rotenone and azadirachtin in Sf9 cells (Table 1).

Table 1: The effect of Foetal Bovine Serum on activity of azadirachtin and rotenone on respiration of Sf9 cell line

Phytochemical	Concentration of F.B.S	Reduction in consumption of oxygen (% of control)
Azadirachtin	0%	16±3.5
	10%	12±1.8
	25%	9.3±0.7
Rotenone	0%	51.8±4
	10%	40.5±4
	25%	29.9±2.44

The effect of azadirachtin on oxygen consumption of cell lines.

The basal respiratory rates of Sf9, C6/36 and L929 cells were shown to be $8.3 \pm 0.53 \times 10^{-9}$, $1.75 \pm 0.19 \times 10^{-9}$, and $27 \pm 0.22 \times 10^{-9}$ m O_2/cell/min respectively.

Over the range of concentrations used, azadirachtin showed only a small inhibitory effect on the total cellular oxygen consumption of either insect or mammalian cells. At the highest feasible concentration, 10^{-4}M, this was 16 ±3.5%, 10.25±1.2% and, 10±2.1%, for Sf9, C6/36 and L929 respectively which in all case it was statistically significant ($P<0.01$), (Figs: 1-3).

The chronic effect of azadirachtin on total cellular oxygen consumption of Sf9 cells.

When Sf9 cells were incubated with azadirachtin at a concentration of 10^{-8} M, there was no effect on oxygen consumption over 5h incubation. After 5h there was slight difference and after 15h it was 3-5% that was not statistically significant.

Effect of rotenone on acute respiration of cell lines.

In contrast to these results with azadirachtin, the results with rotenone showed that it was effective as an inhibitor of respiration at much lower concentrations and to a much greater extent. At the highest concentration (10^{-6} M) it was 78±7.9%, 82.5±3.5% and 61±7.5% for Sf9, C6/36 and L929 respectively. Rotenone even in lower concentration, 10^{-11} M could effectively reduce the respiration between 10- 15% in cell lines (Figs: 4-6).

Effect of azadirachtin on mitochondrial respiration.

Rotenone at a concentration of 10^{-6} M reduced the respiration rate of rat liver mitochondria up to 90%. In contrast, azadirachtin even in concentrations as high as 10^{-4} M did not affect respiration in those mitochondria (data has not been presented).

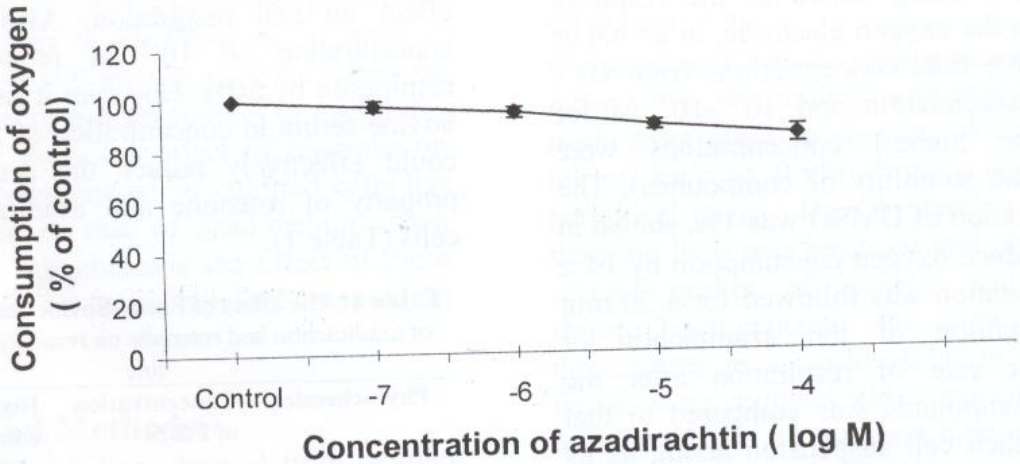

Fig. 1: The effect of azadirachtin on consumption of oxygen by Sf9 cells

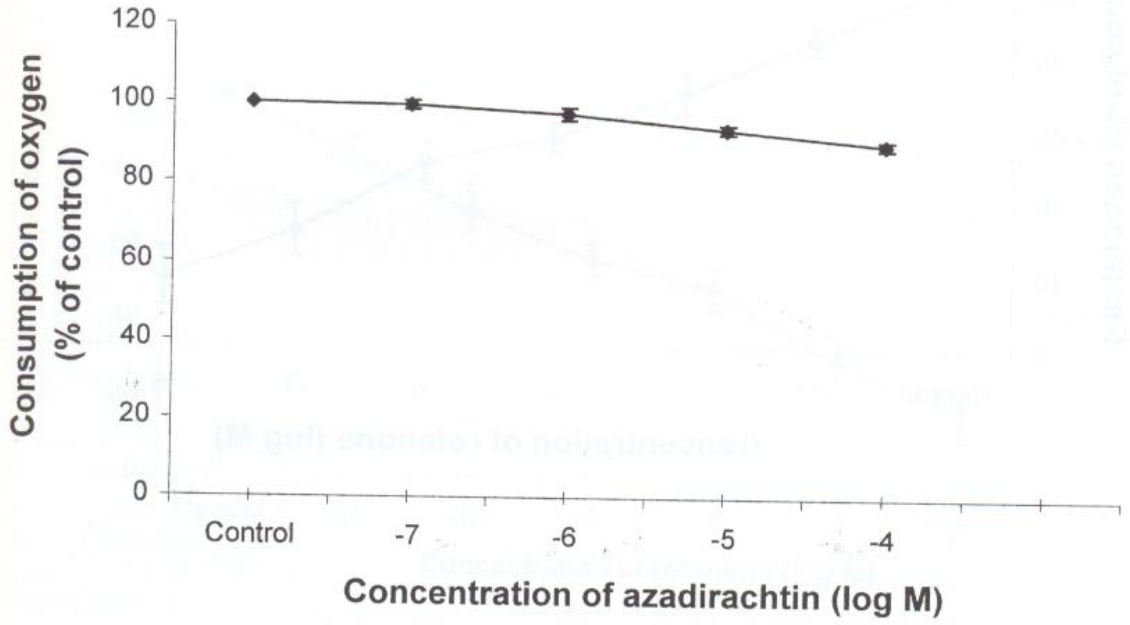

Fig. 2: The effect of azadirachtin on consumption of oxygen by C6/36 cells

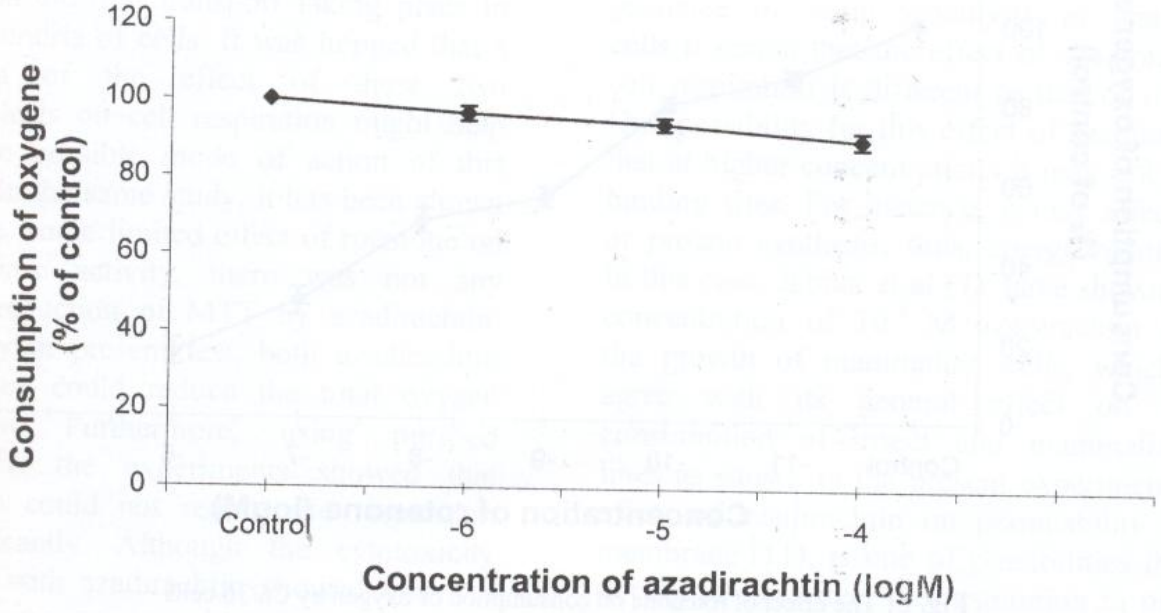

Fig. 3: The effect of azadirachtin on consumption of oxygen by L 929 cells

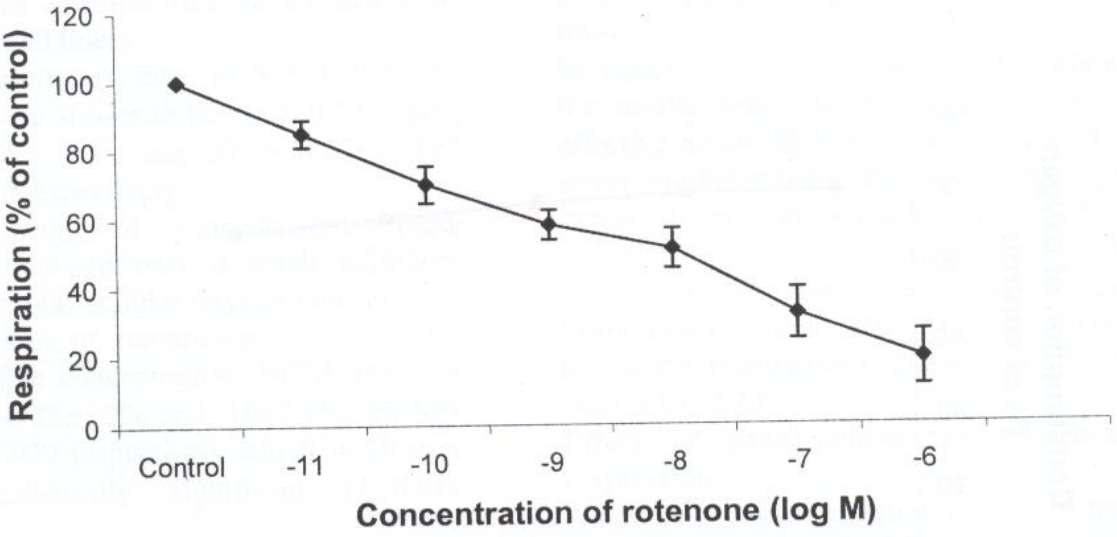

Fig. 4: The effect of rotenone on consumption of oxygen by Sf9 cells

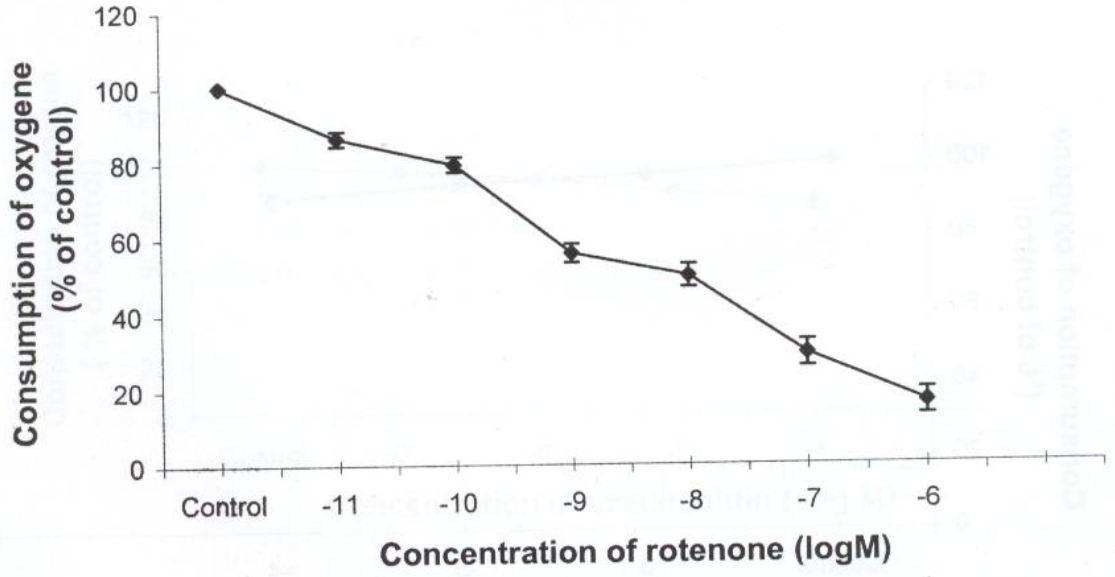

Fig. 5: The effect of rotenone on consumption of oxygen by C6/36 cells

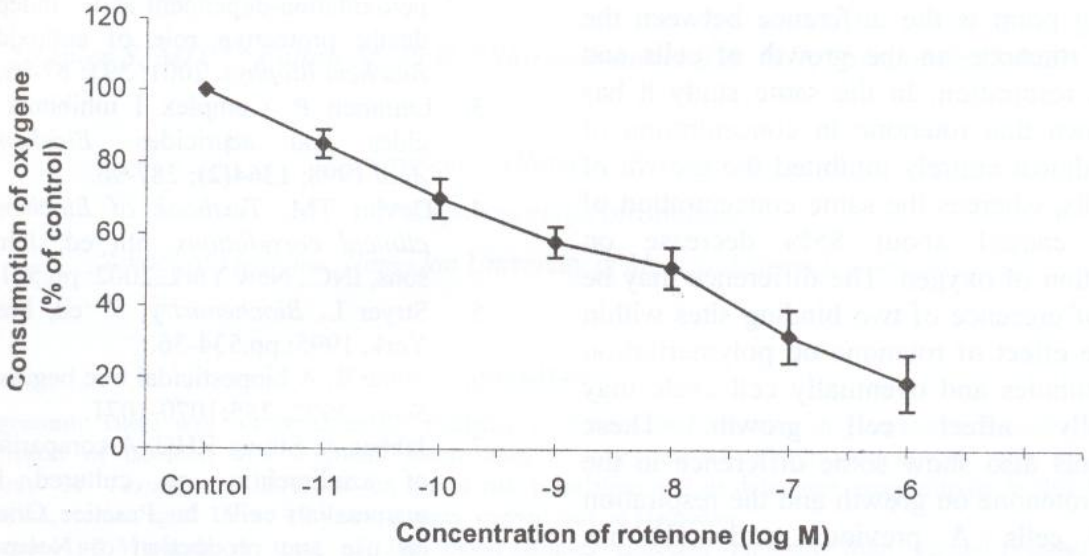

Fig. 6: The effect of rotenone on consumption of oxygen by L929 cells

Discussion

In previous study (1) it was shown that both rotenone and azadiarachtin significantly reduced the growth of cell lines. The effect of rotenone on electron transport taking place in the mitochondria of cells. It was hopped that a comparison of the effect of these two phytochemicals on cell respiration might help to find the possible mode of action of this terpenoid. In the same study, it has been shown that despite some limited effect of rotenone on dehydrogenase activity, there was not any effect on reduction of MTT by azadirachtin. Interestingly in present test, both azadirachtin and rotenone could reduce the total oxygen consumption. Furthermore, using purified mitochondria, the experiments showed that azadirachtin could not reduce the respiration rate significantly. Although the cytotoxicity experiment with azadirachtin showed that Sf9 and C6/36 were more sensitive than L929 cells, estimation of total oxygen consumption of cells demonstrated that azadirachtin had only a minor effect on the respiration rate in both mammalian and insect cultured cells and there was only a slight difference between them. Due to low sensitivity of insect cells and also presence of same sensitivity in mammalian cells it seems that the effect of azadirachtin on cell respiration is different to that of rotenone. One possibility for this effect of azadirachtin is that at higher concentrations it may affect other binding sites. For instance, it may affect DNA or protein synthesis, thus, energy requirement. In this case, Jabbar et al (7) have shown that in concentration of 10^{-3} M azadirachtin inhibits the growth of mammalian cells, which is in agree with its general effect on oxygen consumption of insect and mammalian cell lines as shown in the present experiments. The effect of azadirachtin on permeability of cell membrane (11), is one of possibilities that this terpenoid can affect the respiration in different cell lines. Other possibility is that azadirachtin affect cells in a special sector of cell cycle (e.g. M or G2/M phase) as only part of cells are in this phase at the time of measurement of the

respiration rate, even a 100% inhibition of respiration would only cause about 33% reduction in overall respiration. The other interesting point is the difference between the effect of rotenone on the growth of cells and effect on respiration. In the same study it has been shown that rotenone in concentration of 10^{-6} M, almost entirely inhibited the growth of insect cells, whereas the same concentration of rotenone caused about 85% decrease on consumption of oxygen. The difference may be a result of presence of two binding sites within cells. The effect of rotenone on polymerisation of microtubules and eventually cell cycle may additionally affect cell growth. These experiments also show some difference in the effect of rotenone on growth and the respiration of L929 cells. A previous study (1) has demonstrated that in a concentration of 10^{-6} M, rotenone inhibits the growth to about 39% of controls, whereas the same concentration of rotenone causes about 80% reduction in oxygen consumption of these cells. One possible explanation of the difference between the effect on growth and respiration could be the degradation or metabolism of rotenone during the experiment. In this case Bowman et al (12) have revealed that the half- life of rotenone in the presence of animal chow is between 7 and 8 days. The difference in the temperature of the incubators of insect and mammalian cells may affect this situation. A favourite possibility is that this difference is related to anaerobic metabolism in L929 cell line.

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