Growth and Respiration of Monolayer Cell Cultures of Chick Embryo Heart and Skeletal Muscle: Action of Malathion and Malaoxon* (33481)

B. W. WILSON AND H. O. STINNETT
(Introduced by F. H. Kratzer)
Department of Poultry Husbandry, University of California, Davis, California, 95616

The organophosphorus agent, malathion [*O,O*-dimethyl *S*-(1,(1,2-2dicarbethoxyethyl) phosphorodithioate], is toxic to the growth of chick embryo cells (13) and established mammalian cell lines (4). It has been shown (11) to inhibit the respiration of chick embryo skeletal muscle homogenates and mitochondria; in contrast, malaoxon [*O, O*-dimethyl *S*-(1, 2-dicarbethoxyethyl) phosphorodiolate], a product of malathion metabolism (1, 7) with strong anticholinesterase activity, does not. The experiments reported here were designed to find out whether these results would obtain with intact avian cells grown *in vitro*. Two cell types, chick embryo heart and pectoral muscle were studied using a respiration chamber constructed specifically to measure the oxygen consumption of monolayers of growing cells.

Materials and Methods. Tissues from 11-day embryos of a commercial egg-laying chicken line (Donsing Breeding Farm and Hatchery, Rio Linda, California) were dissociated by treatment with the protease Pronase (California Biochemicals) and inoculated into 15 × 60-mm plastic petri dishes (Falcon Plastics Inc.) containing 40-mm diameter acid-cleaned cover slips. A 5-ml portion of growth medium consisting of 50% medium 199 (Grand Island Biologicals, Inc.), 43% phosphate-buffered saline (12), 5% fetal calf serum (Grand Island Biologicals, Inc.) and 2% embryo extract was added to each dish. All medium components were sterilized by filtration through 22-μ cellulose membrane filters (Millipore Corp.) according to the method recommended by Cahn (2). No antibiotics were used. Incubation temperature was 38°; initial pH was 7.5; and the gas phase was air. Cultures were fed fresh media every other day during exponential growth starting 1 day after their inoculation. Malathion and malaoxon (99 + % pure primary standards donated by the American Cyanamid Co.) were dissolved in the medium by stirring for several hours at 38°. These insecticide-containing media were added to the cultures 1 day after their inoculation. Cell number was determined by treating the cultures with 10 mg/100 ml of Pronase solutions (12, 13) and counting the resulting suspensions with an electronic cell counter (Coulter Electronics Inc.). Protein was determined by the method of Lowry *et al.* (6) as modified by Oyama and Eagle (8).

The respiration chamber is depicted in Fig. 1. Respiration of a monolayer of cells was determined with it in the following manner: A coverslip was transferred from its plastic culture dish to the 20 × 60-mm glass petri dish that served as the bottom of the chamber. A 1-mm thick neoprene ring was placed in the dish and the Delrin plastic top was firmly clamped in place. The device was lowered into a water bath maintained at 38.00 ± 0.05° and prewarmed, aerated medium was carefully added to the chamber. Chamber volume was 3.0 ml. The solution in the chamber was stirred at 60 rpm and the oxygen tension of the solution was measured for periods up to 60 min. The oxygen sensor was a polyethylene-membrane covered platinum, silver–silver chloride polarographic electrode (Beckman Instruments Inc.) connected to a model 160 physiological gas analyzer (Beckman Instruments Inc.) and a strip chart recorder (Leeds and Northrup, Inc.). The device was calibrated with N_2 gas and aerated culture medium before and after each run. Coverslips with more than 200,000

* Supported by Agricultural Experiment Station Project H-2221 and USPHS Grant, ES-00202.

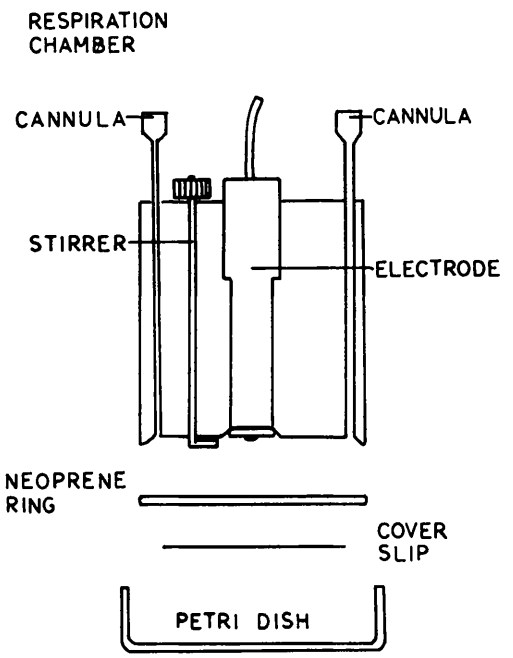

FIG. 1. Respiration chamber for monolayer cultures. Delrin plastic top, glass petri dish bottom.

cells yielded satisfactory rates of respiration. At the end of each run, the cells were removed from the coverslip and counted. Comparison of these counts with those from coverslips that were not placed in the respiration chamber showed that few cells were lost during the process.

TABLE I. Growth and Respiration of Chick Embryo Cells.[a]

Culture type	Generation time (hr)	Protein (μg/10^6 cells)	Respiration (mμmoles of O_2/hr/10^6 cells)
Pectoral muscle	47	265	178
	52	215	168
	58	264	133
Grand mean	52	248	160
Heart	30	271	219
	41	287	272
	48	261	176
	43	406	191
Grand mean	41	306	215

[a] Samples grown 2 to 6 days *in vitro*.

TABLE II. Effect of Malathion and Malaoxon on Chick Embryo Cell Respiration.

Cell type	(mμmoles of O_2/hr/10^6 cells) No inhibitor	Malathion 3×10^{-4} *M*	Malaoxon 3×10^{-4} *M*
Pectoral muscle[b]	163 ± 46[a] (33)[d]	43 ± 25 (13)	168 ± 51 (13)
Heart[c]	215 ± 84 (42)	110 ± 94 (24)	211 ± 83 (13)

[a] Standard deviation.
[b] Average of 3 experiments.
[c] Average of 4 experiments.
[d] No. of values.

Results. The growth and respiration of four heart and three pectoral muscle cultures are shown in Table I. The initial number of cells attached to the coverslips ranged from 150,000 to 250,000. Examination of the cultures with a phase contrast microscope showed that pectoral muscle cultures exhibited extensive myotube formation; multinucleated cells became apparent after 48 hr and increased in size and number until day 5 or 6 of incubation. Heart cell cultures consisted of mononucleated cells. Pectoral muscle cultures had a mean generation time of 52 hr, a mean protein content of 248 μg/10^6 cells and a mean respiration rate during day 2 through 6 of incubation of 160 mμmoles of O_2/hr/10^6 cells. Heart cultures exhibited a mean generation time of 41 hr, a mean protein content of 306 μg/10^6 cells and a mean respiration rate of 215 mμmoles of O_2/hr/10^6 cells.

The effects of malathion and malaoxon on the respiration of pectoral muscle and heart cell cultures grown in inhibitor-free medium are shown in Table II. Malathion (3×10^{-4} *M*) but not malaoxon, inhibited the respiration of both cell types. Malathion-containing medium reduced the respiration of pectoral muscle cells to 26% of normal and the respiration of heart cells to 51% of normal. Several experiments indicated that this inhibition of respiration brought about by short-term incubation of the cells with malathion could be reversed by placing the cells into inhibitor-free medium. However, the cells did not

TABLE III. Effect of Malathion and Malaoxon on the Growth of Embryo Pectoral Muscle Cultures.

Condition	Nf/No[a]	(%)[b]
0 inhibitor	5.86	100
Malathion (M)		
3×10^{-4}	0.26	4.4
6×10^{-5}	1.55	26.4
3×10^{-5}	3.55	60.5
Malaoxon (M)		
3×10^{-4}	1.31	22.4
6×10^{-5}	3.09	52.6
3×10^{-5}	4.55	72.7

[a] Nf/No; relative growth of the cells, Nf = cell number at 120 hr, No = cell number at 24 hr.

[b] 100 × relative growth of inhibitor-treated cells /relative growth of control (0 inhibitor) cells.

always return to their normal respiration level. Several studies of the effect of inhibitor concentration on the respiration of heart cells indicated that 3×10^{-5} M malathion and below did not inhibit respiration over the short periods of time involved in the study.

Several studies indicated that 3×10^{-4} M malathion and malaoxon were both strongly toxic to the growth of pectoral and heart muscle cultures. Table III contains data obtained from an experiment with pectoral muscle cells that illustrates the toxicity of malathion and malaoxon to this cell type. The data represent the ratio of the cell number present at 120 hr of incubation to the cell number present at 24 hr of incubation, the time when the organophosphorus agents were added to the cultures. The results indicate that both malathion and malaoxon inhibited growth and that malathion was more toxic than malaoxon. Microscopic examination revealed that none of the cultures except those grown in inhibitor-free medium formed myotubes.

The results of three experiments were combined to construct the graph shown in Fig. 2 representing the toxic actions of malathion and malaoxon on the growth of heart cell cultures. The values are the ratios of cell numbers at 120 hr compared to those at 24 hr of incubation expressed as percentage of the growth of inhibitor-free cell cultures. The data indicate that concentrations of inhibitors above 10^{-5} M reduced growth and that, as was true for pectoral muscle cultures, malathion was more toxic than malaoxon. Using these data it can be calculated that the LD_{50} for malathion was 5.6×10^{-5} M and the LD_{50} for malaoxon was $8.2 \times 10^{-5}M$.

Discussion. The results of this study of two cell types grown *in vitro* demonstrate that an acute dose of malathion, 3×10^{-4} M, inhibits cell respiration and that incubation of the cells in 3×10^{-4} M malaoxon does not. Wilson and Fry (11) found that malathion, but not malaoxon, inhibited the respiration of pectoral muscle homogenates and mitochondria and that one of the effects of malathion was to block the oxidation of α-ketoglutarate. Thus, evidence has now been obtained on three levels of organization that malathion inhibits the energy metabolism of chick embryo cells and that the conversion of its P→S group to the P→O group of malaoxon results in a disappearance of the inhibition. The experiments presented here also suggest that malathion is more inhibitory to the respiration of pectoral than it is to heart muscle cultures. However, neither of the two cell cultures used in the study consisted of a single cell type as would obtain if they had been grown from clones, and the possibility that malathion does not affect all of the cells in a culture in the same way must be considered when interpreting the data.

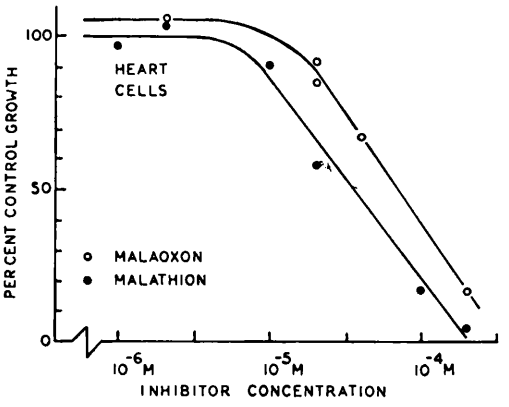

FIG. 2. Toxicity of malathion and malaoxon to the growth of chick embryo heart cells. Values represent the ratio of cell numbers at 120 and 24 hr of incubation expressed as percentage of growth in inhibitor-free medium.

Although the results of this and previous studies (11) establish that malathion inhibits the respiration of chick embryo cells, it is still unclear as to what role this inhibition plays in its toxicity to cell growth *in vitro*. The conversion of malathion to potent anticholinesterase agents such as malaoxon (1) is widely accepted as the major cause of its toxicity to insects and vertebrates (1, 7). However, several lines of evidence suggest that this conversion is not the major cause of the toxicity of malathion to embryo cell growth. The results of this study show that malaoxon, on an equimolar basis, is less toxic to the growth of heart and pectoral muscle cultures than is malathion. Moreover, recent experiments (10) indicate that malaoxon strongly inhibits the activity of cell cholinesterases at concentrations where it does not appreciably affect cell growth and that cells grown in the presence of toxic levels of malathion have higher cholinesterase activities than those grown in less toxic levels of malaoxon. Thus, the possibility exists that inhibition of cell energy metabolism, and other as yet unexplored effects of malathion on cell metabolism may be important factors in its toxicity to cell growth. However, malathion did not inhibit respiration at concentrations where it inhibited growth. Studies of the respiration of cells grown in the presence of various concentrations of malathion and malaoxon are in progress.

Few studies have been carried out on the respiration of metazoan cells while they are attached to the surfaces upon which they have been growing. Harris (5) studied the respiration of rat connective tissue cells using a growth chamber containing a platinum electrode and a KCl–agar bridge. The oxygen uptake values obtained ranged from 810 to 870 mμmoles/hr/10^6 cells for three experiments. Most studies concerning the respiration of cultured cells have been carried out with manometric techniques using cells that have been removed from the surfaces upon which they have been growing, and placed into concentrated suspensions. The results of many such studies have been reviewed by Paul (9). The respiration rates obtained, both for mammalian and avian cells, were often somewhat higher than those presented here. For example, when the data are recalculated in terms of cell number, Cristofalo and Kritchevsky (3), using warburg manometry, found that the mean respiration rate of 1–5 $\times$ 10^7 cells/ml of a human diploid cell strain WI-38 was 1290 mμMoles of O_2/hr/10^6 cells, 5–10 times the respiration of the avian cells used in this study. Attempts to determine the respiration of avian cells that had been scraped from or enzymatically removed from the surfaces upon which they were attached damaged their capacity to respire. Considering the fact that the situation in the polarographic respiration chamber closely approximates that found during growth of the cells, and the ease with which the media surrounding the cells can be changed to test the effects of inhibitors and nutrients, chambers such as the one used in this study should prove useful in investigations into the energy metabolism of growing cells.

Summary. The respiration of chick embryo heart and pectoral muscle cell cultures was determined using a polarographic respiration chamber designed specifically to measure the oxygen consumption of monolayers of cells. Heart cultures had a mean respiration rate of 215 $\pm$ 84 mμmoles O_2/hr/10^6 cells; pectoral muscle cultures had a mean respiration rate of 163 $\pm$ 46 mμmoles O_2/hr/10^6 cells. Malathion 3 $\times$ 10^{-4} *M* reduced the respiration of pectoral muscle cultures to 26% of normal and the respiration of heart cultures to 50% of normal. Malaoxon 3 $\times$ 10^{-4} *M* had no effect upon the respiration of either cell type. Growth studies indicated that malathion was more toxic than malaoxon to the growth of both cell types.

The authors gratefully acknowledge the skillful assistance of Mr. D. M. Fry and the technical aid of Miss Elizabeth Jones.

1. Aldridge, W. N., Biochem. J. **54**, 442 (1953).

2. Cahn, R. D., Science **155**, 195 (1967).

3. Cristofalo, V. J. and Kritchevsky, D., J. Cell Physiol. **67**, 125 (1966).

4. Gabliks, J. and Friedman, L., Proc. Soc. Exptl. Biol. Med. **120**, 163 (1965).

5. Harris, H., Brit. J. Exptl. Pathol. **37**, 512 (1956).

6. Lowry, A., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

7. O'Brien, R. D., J. Econ. Extomol. **50**, 159 (1967).

8. Oyama, V. I. and Eagle, H., Proc. Soc. Exptl. Biol. Med. **91**, 305 (1956).

9. Paul, J., *in* "Cells and Tissues in Culture" (E. N. Willmer, ed.), Vol. 1, p. 239. Academic Press, New York (1965).

10. Wilson, B. W., Stinnett, H. O., Hooker, W. P., and Asmundson, R. V., J. Cell Biol. **39** 143a (1968).

11. Wilson, B. W. and Fry, D. M., submitted for publication.

12. Wilson, B. W., Peterson, D. W., Stinnett, H. O., Nelson, T. K., and Hamilton, W. H., Proc. Soc. Exptl. Biol. Med. **121**, 954 (1966).

13. Wilson, B. W. and Walker, N. E., Proc. Soc. Exptl. Biol. Med. **121**, 1260 (1966).

Received July 22, 1968. P.S.E.B.M., 1969, Vol. 130.

The Influence of Vasoactive Amines on Interferon Production in Mice* (33482)

MARCUS M. JENSEN (Introduced by A. F. Rasmussen, Jr.)

Department of Medical Microbiology and Immunology, UCLA School of Medicine, Los Angeles, California 90024

As part of a study to better understand the influences of environmental factors on resistance to infectious diseases, we have demonstrated that various stressors alter susceptibility to viral infections (1, 2). In some cases the changes in susceptibility, as well as changes in host–defense mechanisms, were transient and were not associated with the increased adrenocortical secretions induced by stress (3, 4). Because vasoactive amines are also secreted in response to environmental stimuli and in turn induce transient physiologic responses, we are investigating the possible mediating role of these amines in the stress-induced suppression of host–defense mechanisms. A previous report (5) described the transitory impairment of interferon production in mice treated with serotonin. This paper describes primarily the influence of epinephrine on interferon production in mice, with comparative data on the influence of norepinephrine and of histamine.

Materials and Methods. Seven to 8-week-old Swiss-Webster female mice were injected i.p. with either epinephrine hydrocholoride (Parke, Davis & Co.), norepinephrine (Levophed, Winthrop Labs.) or histamine phosphate (Eli Lilly & Co.) in 0.25 ml of saline. Controls received 0.25 ml of saline. At intervals from 1 hr before to 24 hr after the drug injection, mice were stimulated to produce interferon by 0.2-ml i.v. injections of either 5.6×10^7 plaque forming units (pfu) of Newcastle disease virus (NDV), or of 100 μg of *Escherichia coli* lipopolysaccharide endotoxin (Difco). Blood was collected 4 hr after NDV and 2 hr after endotoxin injections; sera from 2 or 3 mice were pooled.

Interferon assays employed secondary mouse embryo tissue cultures in 30-ml plastic flasks which were treated for 3 hr at 37° with serum dilutions, then challenged with about 100 pfu of vesicular stomatitis virus (VSV). An overlay of Tris buffer with 0.6% agar and 5% agamma globulin calf serum was added after 1-hr viral adsorption at 37°. Four flasks were used per dilution and plaques were counted after about 40-hr incubation. Results were expressed as the percentage of plaque reduction from virus control flasks, either at a single predetermined interferon dilution or, when necessary, as the dilution inducing 50% pfu reduction. Levels of significance were determined by the Student *t* test.

Experiments and Results. Epinephrine. Figure 1 shows the influence of different

* Supported by USPHS Grant AI-05995 from the National Institutes of Allergy and Infectious Diseases, and USPHS Mental Health Training Grant 5 T1-MH-6415.