actually controls the biosynthesis of thyroxine. The result would be a short, but critical, period at the onset of hypoxia exposure during which elaboration of thyroxine and the subsequent utilization of oxygen by peripheral tissues are reduced.

Summary. Exposure of rats to a reduced pressure (380 mm Hg) simulating an altitude of 18,000 feet resulted in a markedly elevated I^{131} uptake in the thyroid, a relative increase in the amount of intrathyroidal I^{131} MIT and a lowered I^{131} DIT. The hypoxia induced alteration of thyroid hormone synthesis was found to be a transient response since the I^{131} MIT/DIT ratio returned to control levels after 60 hours of exposure. A possible mechanism which could account for these thyroidal changes is discussed.

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Toxicity of Malathion and Mercaptosuccinate to Growth of Chick Embryo Cells *in vitro*.* (31023)

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Much evidence indicates that many of the acute toxic effects of malathion (0,0-Dimethyl S-(1,2 dicarbethoxyethyl) phosphorodithioate) and other organophosphorus insecticides are due to their action on the nervous system, and are caused by their inhibition of the enzyme acetylcholinesterase(1,2). The phosphorus group in the insecticide molecule is believed to be responsible for this inhibition(2,3). The possible effects of other groups in the molecule have received little attention. Such effects would be difficult to investigate in the intact animal because the presence of the nervous system complicates studies of the responses of other cells and tissues to organophosphorus insecticides and their breakdown products. Isolated systems such as cell cultures seem appropriate for this kind of investigation. However, until recently(4,5,6) cell cultures have been little used in research on insecticide toxicity to vertebrates, although they have been utilized extensively in other areas of pharmacology such as the

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study of carcinogenic agents(7). The experiments reported here were designed to find out whether malathion is toxic to primary cultures of chick embryo cells and whether compounds having a part of the carbon skeleton of malathion affect cell growth.

Materials and methods. Cells from the pectoral region of 14-day-old chick embryos were grown as fibroblasts in monolayers according to standard culture procedures as described in detail elsewhere(8). The cells were dissociated from the tissue in a solution of a protease, Pronase(9), and concentrations of 100,000 to 200,000 cells/ml were inoculated into plastic petri dishes (Falcon Plastics Inc.) or ½ oz pharmaceutical bottles (Brockway Glass Inc.). The cells that had not become attached to the surface of the culture vessels after 24 hours were removed and the inoculation medium was replaced by test media. The media were changed again 48 hours later. The experiments were usually terminated after a total of 5 or 6 days. The growth medium consisted of 50% Medium 199(10), 43% phosphate buffered saline(8), 5% chicken or fetal calf serum (GIB Inc. or Microbiological Associates) and 2% embryo extract(8). All solutions were sterilized by filtration through 0.45 μ cellulose membrane filters (Millipore Filter Inc.). No antibiotics were used. The gas phase was air and the incubation temperature was 37 $\pm$ 0.5°C.

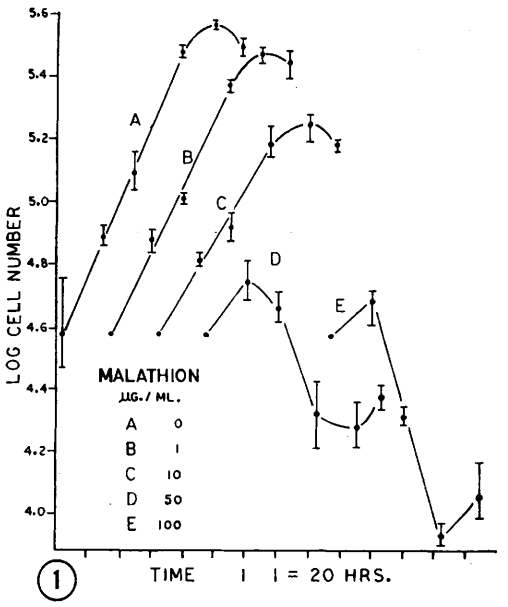
Growth was measured as increase in cell number determined by the Coulter Electronic Cell Counter, Model A(8,11). The cells were removed from the plastic or glass surface by treatment with Pronase and dispersed by repeated pipetting. The means of the counts of at least 3 culture vessels were used to determine the points on the growth curves.

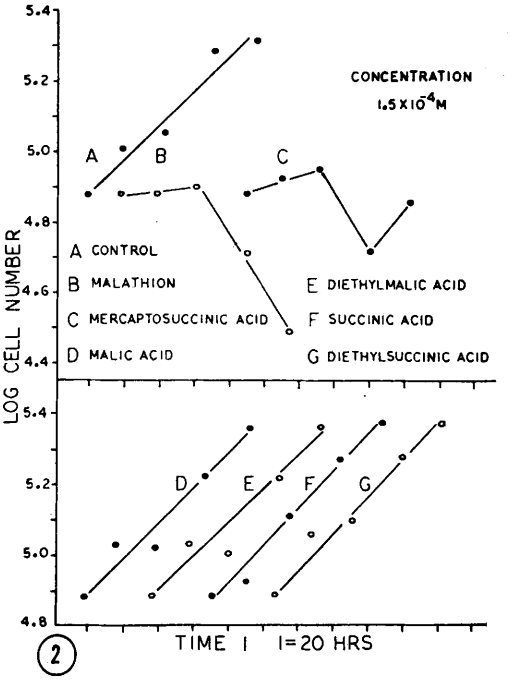
Two malathion samples were used: one of 95% purity was obtained from the Agricultural Toxicology Dept., Davis, and another of 99+% purity was obtained from American Cyanamid Co. Both samples yielded the same experimental results. Malathion was dispersed by adding it to the complete basal medium and stirring the solution for 2 to 3 hours at 37°C. No more than 10 mg/100 ml of malathion was added to the medium in the experiments reported here. This concentration was slightly less than the solubility of malathion in H_2O(12).

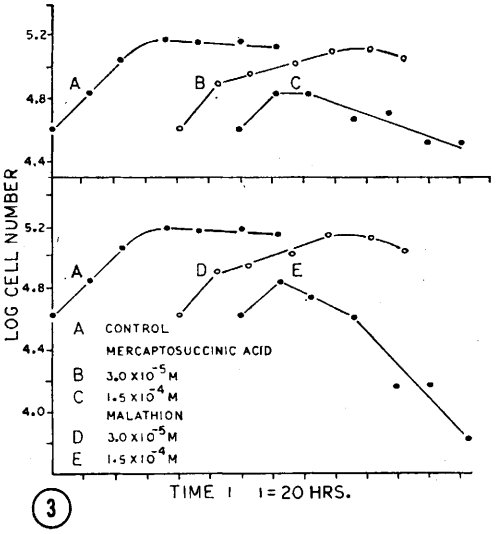
Results. The first series of experiments studied the effect of malathion concentration on growth of the cells. The results of a typical experiment are shown in Fig. 1. For convenience, the growth curves are displayed along the horizontal axis of the graph. Concentrations of malathion above 3.0×10^{-6} M (1 μg/ml) progressively inhibited the growth rate and the peak populations of the cells. Malathion was strongly toxic to the cells above 3.0×10^{-5} M (10 μg/ml), causing a net decrease in cell number from that of the original inoculum. This decrease was not immediate; in every experiment with media containing 1.5×10^{-4} M (50 μg/ml) and 3.0×10^{-4} M (100 μg/ml) concentrations of malathion the cell number either remained unchanged or increased for at least 24 hours after addition of the pesticide and then rapidly declined.

Staining *in situ* with Oil-Red-O and Janus Green B revealed that cells attached to the surface of the vessels in the presence of malathion differed little in their morphology from those grown on the control medium.

The response of the cells to several of the carbon fragments of malathion was next investigated. The compounds tested were mercaptosuccinate, malate, diethylmalate, succinate and diethylsuccinate. Results such as those shown in Fig. 2 indicated that mercaptosuccinate but not malate, succinate or their diethylesters was toxic to chick embryo cells at a concentration of 1.5×10^{-4} M. Fig. 3 illustrates results of an experiment comparing the effects of malathion and mercaptosuccinate on the cells at 2 concentrations, 3.0×10^{-5} M and 1.5×10^{-4} M. The results of this and several other experiments indicated that the growth inhibition due to mercaptosuccinate was not evident until 24 hours after the compound was added to the cells and that mercaptosuccinate was acutely toxic to the cells at 3.0×10^{-5} M, but not at 1.5×10^{-6} M. Although mercaptosuccinate severely inhibited cell growth at a concentration of 1.5×10^{-4} M (Fig. 2 & 3), a rapid decrease in the number of cells in the flasks did not occur.







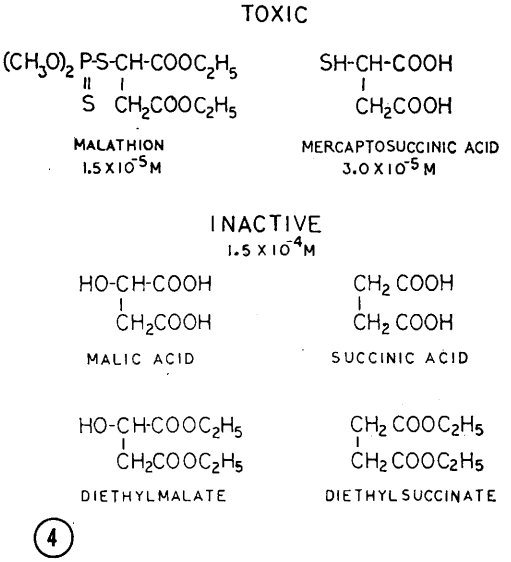


FIG. 1. Effect of malathion on growth of chick embryo cells. Cells from 14-day pectoral muscle; incubated in chicken serum, Medium 199, PO_4 buffer, embryo extract basal medium. I indicates range.

FIG. 2. Effect of 1.5×10^{-4} M malathion, mercaptosuccinate, malate, diethylmalate, succinate and diethylsuccinate on growth of chick embryo cells. Cells from 14-day pectoral muscle; incubated in fetal bovine serum, Medium 199, PO_4 buffer, embryo extract basal medium.

FIG. 3. Growth of chick embryo cells on 3.0×10^{-6} M and 1.5×10^{-4} M malathion and mercaptosuccinate. Cells from 14-day pectoral muscle; basal medium contained fetal bovine serum, embryo extract, Medium 199, and PO_4 buffered saline.

FIG. 4. Structural relationships between malathion and mercaptosuccinate, malate, succinate and their diethylesters.

Discussion. The data obtained in this investigation indicate that malathion is acutely toxic to the growth of primary cultures of chick embryo fibroblasts *in vitro* above concentrations of 3×10^{-6} M (1.0 μg/ml). Concentrations of 1.5×10^{-4} M and 3.0×10^{-4} M (50 and 100 μg/ml) invariably caused a severe decrease in the number of cells in the cultures. Concentrations of 3×10^{-5} M (10 μg/ml) usually inhibited the rate of increase of cell number and the peak populations reached by the cells.

It is plausible to interpret the data as suggesting that the rate of death of the cells increased in direct proportion to the malathion concentration. However, other factors may also be involved. It is possible that malathion, particularly at low concentrations, affected the generation time of the cells, *i.e.*, the time spent by the cells between divisions. Furthermore, it should be noted that only cells attached to the surface of the growth vessels were counted; the viability of cells that may have floated up into the medium in response to the pesticide was not investigated.

There have been few studies on the toxicity of insecticides to vertebrate cells cultured *in vitro*. Gabliks and Friedman(4,5,6) recently investigated the acute and chronic effects of several organophosphorus, organochlorine and dinitrophenol insecticides to Chang human liver, HeLa human carcinoma and mouse L-929 established cell lines. They found that 13 to 15 μg/ml of malathion caused a 50% inhibition of the rate of protein synthesis of the human cell cultures. The minimal toxic dosage of malation was 0.1 μg/ml(5). Comparing the results of these studies with the data reported here, it would seem that malathion may be less acutely toxic to primary cultures of chick embryo cells than it is to established mammalian cell lines.

The data illustrated in Fig. 2 & 3 show that mercaptosuccinate was toxic to chick embryo cells *in vitro* at concentrations above 3.0×10^{-5} M, results similar to that found for malathion, and that succinic acid, malic acid and their diethylesters were not toxic to the cells at concentrations as high as 1.5×10^{-4} M. The structural relationships between these molecules and malathion are shown in Fig. 4. The non-phosphorus containing breakdown products of malathion have not been intensively studied, nor are the metabolic products of malathion known for cells grown *in vitro*. Regardless, the results suggest the need for further study of the effects of the non-phosphorus-containing parts of organophosphate insecticides on vertebrate cells, both *in vivo* and *in vitro*.

It is doubtful, as pointed out by Gabliks and Friedman(4) that organophosphorus insecticides are toxic to cells in culture because of their inhibitory action on acetylcholinesterase. However, cultured cells contain other esterases which could be important to cell survival. Moreover, Freedland and McFarland(13) showed that malathion and several other insecticides inhibited the activity of purified beef liver glutamate dehydrogenase. It is interesting to note that parathion, an organophosphorus insecticide, had no appreciable effect on the activity of the enzyme.

The mechanism by which mercaptosuccinate inhibits cell growth is not known. Considering its structure, mercaptosuccinate may act as an inhibitor of cell respiration, or it may function as a competitor in sulfhydryl reactions.

The lag between time of addition of malathion and mercaptosuccinate and decrease in the rate of growth of the cells merits investigation. It may signify that both compounds are taken up relatively slowly by the cells so that it is some hours before the substances reach toxic intracellular levels. These two compounds may even be selectively toxic to cells that are in a particular stage of their division cycle. These and other possible causes for the phenomenon are under investigation.

Summary. The growth of primary cultures of chick embryo cells was examined in the presence of the organophosphorus insecticide, malathion, and several of its molecular fragments, mercaptosuccinate, malate, succinate and their diethylesters. The results showed that malathion was toxic to the growth of the cells in concentrations as low as 3×10^{-6} M, mercaptosuccinate inhibited cell growth in concentrations as low as 3×10^{-5} M, and that malate, succinate and their

diethylesters had no effect on the growth of the cells in concentrations as high as 1.5×10^{-4} M. The inhibitions of cell growth obtained with malathion and mercaptosuccinate were first evident 24 hours after the compounds were added to the cultures. The implications of these results with respect to the actions of insecticides on cells and tissues are discussed.

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Attempt at Immunization by Oral Feeding of Live Rhinoviruses in Enteric-Coated Capsules. (31024)

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The extreme antigenic diversity(1) of rhinoviruses renders it important to devise simplified means for inducing immunity if reasonable control of infections caused by these agents is ever to be achieved. Present evidence indicates that rhinoviruses do not ordinarily infect the gut and the virus does not appear in the feces(2). The rapid inactivation of rhinoviruses at pH 3 to 5(3,4) suggests that in natural infections, the agents are destroyed during passage through the stomach. The possibility remains that infection of the intestines might be achieved if the acidity of the stomach could be bypassed. Accordingly, live preparations of rhinovirus types 32 and 44 were prepared in enteric-coated capsules and fed to human volunteers. The fate of the capsules and the serologic findings in human subjects fed the virus are described here.

Materials and methods. Vaccines and placebo. Rhinovirus strains 1955M (type 32) (1) and 1863H (type 44)(1) were isolated from throat washings from cases of common cold (upper respiratory illness) in university students and were propagated in serial passage in human diploid cell strain WI-38(5) which was monitored for cell alterations according to the proposed Minimum Requirements(6). Both viruses used to prepare the capsules were in fourth passage in cell culture. The WI-38 strain of diploid cells used to cultivate the viruses was in passages 18 to 29. Preparation of the vaccine viruses and tests for identity, freedom from extraneous agents, sterility and safety, whenever applicable, were consistent with contemporary regulations of the Division of Biologics Standards, National Institutes of Health, U. S. Public Health Service. To prepare vaccine,