West *et al*(16) have reported that symptoms of mouse muscular dystrophy were alleviated slightly after treating newborns daily with reserpine, a potent serotonin and catecholamine releasing agent. Exceedingly large concentrations of serotonin, which are directly related to the severity of the myopathy, have been demonstrated in the cerebrospinal fluid of human dystrophic patients(18).

Proportionally larger adrenal and pituitary glands were demonstrated in dystrophic mice as compared to normal animals, and short term treatment with the serotonin antagonist did not significantly affect the weight of these organs (Table I). Enlarged adrenal glands have been reported previously in these animals(4,10), and may be ascribed to the stress of the disease. Serotonin, incidentally, also can cause adrenal enlargement(17).

Administration of serotonin antagonist to mice with hereditary muscular dystrophy did not eliminate the symptoms of dystrophy, but instead, seemed to retard the progressive rate of their development, allowing for longer survival of affected animals. Further studies, possibly on fetal stages, may determine if serotonin is associated with the cause or with one of the many consequences of the myopathy.

*Summary.* Survival time was increased over 90% in mice with hereditary muscular dystrophy treated orally with a serotonin antagonist, methysergide bimaleate. There was a concurrent increase in body weight in treated animals. Short term treatment for 3 weeks with the drug caused a significant enlargement of the ovaries of dystrophic mice, but had no effect on body weight or other endocrine organ weights of normal mice.

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## Mitotic Abnormalities Produced by Juglone in Ehrlich Ascites Tumor Cells.* (32513)

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The cytological and biochemical effects of quinones have been studied in a variety of biological systems(1,2,7,8,9). We now have studied the cytological effects of 16 quinone compounds on Ehrlich ascites tumor cells *in vivo* and have found that one of them, juglone (5-hydroxy-1,4-naphthoquinone), produced

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TABLE I. Influence of Juglone on Mitotic Frequency of Ehrlich Ascites Tumor Cells.

| | Mitotic cells (% of total) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Dose of juglone per mouse (mg) † | | | | | | | | | | | |
| Hour after injection | 0 | | 0.1 | | 0.25 | | 0.4 | | 1 | | 2 | |
| | N* | A* | N | A | N | A | N | A | N | A | N | A |
| 1 | 4.3 | 0 | 3.6 | 0 | 2.2 | .9 | | | 1.3 | 2.9 | 1.3 | 2.5 |
| 3 | 3.7 | 0 | 3.1 | 0 | 1.4 | 1.3 | 1.7 | 2.2 | .3 | 3.8 | 1.0 | 2.8 |
| 6 | 3.5 | 0 | 3.1 | 0 | 1.2 | 1.5 | 1.3 | 2.3 | .3 | 4.5 | .9 | 3.1 |
| 12 | — | — | — | — | — | — | 1.1 | 2.7 | — | — | .8 | 2.4 |
| 24 | 3.9 | 0 | 3.8 | 0 | 2.7 | 1.1 | 1.8 | 1.1 | 4.2 | 1.5 | 1.4 | 1.2 |

* N, normal mitotic figures; A, abnormal mitotic figures.
† Average obtained from 5 to 10 mice at each time interval.

TABLE II. Effect of Juglone (2 mg) on Mitosis of Ehrlich Ascites Tumor Cells.

| | % of mitotic cells* | | | | | |
|---|---|---|---|---|---|---|
| Hr after injection | Control (azalectin) | | | Juglone | | |
| | Prophase | Metaphase | Ana-telophase | Prophase | Metaphase | Ana-telophase |
| 1 | 61.5 | 20.5 | 18.0 | 17.3 | (71.3) † | 11.4 |
| 3 | 40.9 | 33.4 | 25.6 | 16.1 | (83.9) | 0 |
| 6 | 53.7 | 24.5 | 21.7 | 11.9 | (88.1) | 0 |
| 24 | 52.7 | 30.1 | 17.6 | 9.4 | 35.6 (56.0) | 0 |

* Averages obtained in from 5 to 10 mice at each time interval.
† Numbers in parentheses represent abnormal mitoses.

mitotic abnormalities in the tumor cells. The latter substance has long been of interest because it is produced by black walnut trees and is responsible for the wilting and death of plants in a circular area around the trees(6).

*Materials and methods.* A mouse tumor, Ehrlich ascites tumor (hyperdiploid strain), was employed to study the effects of the quinones. The ascites tumor was transmitted serially in Swiss/HaICR mice weighing about 30 g, fed on a stock diet of Purina chow and water. Intraperitoneal injections of freshly prepared suspension of the chemicals were begun on the 7th day after transplantation of the tumor, when the tumor was well established and active proliferation of the tumor cells was taking place in the peritoneal cavity. At various intervals samples of tumor ascites were taken by peritoneal puncture, squashed with acetic dahlia, and examined cytologically. The numerical data were calculated on the basis of approximately 500 interphase and dividing cells for every sample.

Compounds tested in this study were: vitamin $K_1$, vitamin $K_1$ diacetate, vitamin $K_1$ diphosphate, dihydrophytyl vitamin $K_1$, 6 acetylchromanol of vitamin $K_1$, 6 chromanyl phosphate of vitamin $K_1$, $\alpha$-lapachone, $\beta$-lapachone, lapachol, methyl ether, menadione, menadione oxide, monomethyl monophosphate of menadione, monoethyl monophosphate of menadione, menadione sodium bisulfite, lomatiol, and juglone. Compounds were put in suspension in phosphate buffer solution at pH 7.0, usually with azalectin (1 mg/0.1 ml). Azalectin produced good suspensions of the water-insoluble compounds and did not induce any observable effect on tumor cell morphology when administered alone in the above amounts.

*Results. Cytological effects of intraperitoneally administered juglone.* Preliminary experiments showed that juglone induced abnormal mitotic figures in the tumor cells and appeared to prevent cells from entering division. The other 15 compounds were ineffective. Single intraperitoneal injections of juglone then were given to the following numbers of mice at the indicated dose levels: 10, 0.1 mg; 10, 0.25 mg; 10, 0.4 mg; 10, 1 mg; and 20, 2 mg (Table I). Death of the mice often occurred within 3 hours after the injection of doses greater than 2 mg. In Table II are shown average values for mitotic fre-

quencies in tumor cell populations from control mice and from the mice receiving juglone at various periods up to 24 hours after the injection. The values are averages obtained from counts of 500 cells from samples obtained from 5 to 10 mice at each time interval at each dose level. At the 0.1 mg dose there was no observable effect on the cells, but at 0.25 mg abnormalites were evident even in the earliest samples. The degree of abnormality was greatest at the higher doses and the period of maximal occurrence of abnormalities was at 6-12 hours after the injection.

The subsequent description of the sequential cytological effects of juglone is based on observations made on cells from mice receiving a single intraperitoneal dose of 2 mg per animal (Table II). At all times in the controls more of the mitotic cells were in prophase than in metaphase or ana-telophase. However, even within 1 hour after the injection of juglone most of the mitotic cells seen were in metaphase and exhibited abnormalities. An accummulation of abnormal metaphase figures persisted for the period of observation, and after 3 hours no ana-telophase figures were observed. The results suggest that under the conditions of the experiment juglone prevents cells from entering mitosis and also produces abnormalities in metaphase cells so that they cannot proceed further to cell division. Chromosomes in many of the cells in prophase appeared diffuse and sticky (Fig. 1). Disintegration of some tumor cells had occurred at 1 hour, and there was blebbing of the cell surfaces of most interphase cells. The chromosomes in metaphase were short and sticky. Some of the chromosomes appeared clumped and a few were scattered in the cytoplasm, their appearance being similar to that observed in C-mitosis (Fig. 2). Some cells in tripolar division were found. Three to 6 hours after the injection of the 2 mg dose there were many cells containing clumped chromosomes in the cytoplasm (Fig. 3) and many metaphase figures contained chromosomes contracted into balls or masses (Fig. 4). Twenty-four hours after the injection there were fewer tumor cells in the ascites than in the controls and many leukocytes were present in the ascitic fluid.

TABLE III. Mitotic Frequency of Ehrlich Ascites Tumor After Treatment with 8-Hydroxyquinoline (1 mg) and 2,2′-Dipyridyl (2 mg).

| Hr after injection | Control | 8-Hydroxy-quinoline | 2,2′-Dipyridyl |
|---|---|---|---|
| 1 | 3.8* | 3.8 (.3)† | — — |
| 3 | 3.7 | 3.8 (.4) | 3.1 ( .7)† |
| 6 | 3.6 | 2.3 (.1) | 2.6 (1.1) |
| 24 | 3.9 | 6.5 (.1) | 2.7 (1.0) |

* Values represent the average obtained in from 5 to 10 animals.

† Abnormal mitoses in parentheses.

*Is the action of juglone attributable to chelation?* The possibility was considered that juglone, a good chelating agent, was exerting its action by chelating an essential cationic constituent in the tumor cells. Three relevant experiments were performed. In the first, the action of juglone was compared with that of isomolar amounts of 8-hydroxyquinoline and 2,2′-dipyridyl. In the second, juglone (2 mg/0.1 ml) was premixed with an isomolar amount of $CaCl_2$ (1.1 mg/0.1 ml) or $MgCl_2$ (2 mg/0.1 ml) and then administered to the animals. In the third, juglone was injected first and $CaCl_2$ or $MgCl_2$ in isomolar amounts were administered immediately thereafter.

As shown in Table III, 8-hydroxyquinoline (1 mg/mouse) and 2,2′-dipyridyl (2 mg/mouse) induced some abnormal mitotic figures in the ascites tumor. After injection of either compound, chromosomes in some mitotic cells scattered in the cytoplasm and clumped together (Fig. 5). There were some cells with 3-group metaphase (Fig. 6). Throughout the whole experimental period sticky chromosomes were never observed. The frequency of abnormal mitotic cells was not high. Twenty-four hours after the injection the tumor cells appeared to be morphologically normal and the mitotic frequency of tumor cells in animals treated with 8-hydroxyquinoline was higher than that of the controls. The mitotic abnormalities produced by the latter two compounds were not as widespread or prolonged as those seen after juglone.

Many abnormal mitotic figures were observed after injection of juglone with $CaCl_2$ or $MgCl_2$. Neither premixture with $Ca^{++}$ or $Mg^{++}$ or administration of these ions subsequent to the injection of juglone diminished the effectiveness of juglone.

The above results do not rule out the possibility that the effects of juglone may be attributable to chelation with metallic cations. They suggest that if the activity of juglone is exerted through chelation, it would have to be with a substance (or substances) with

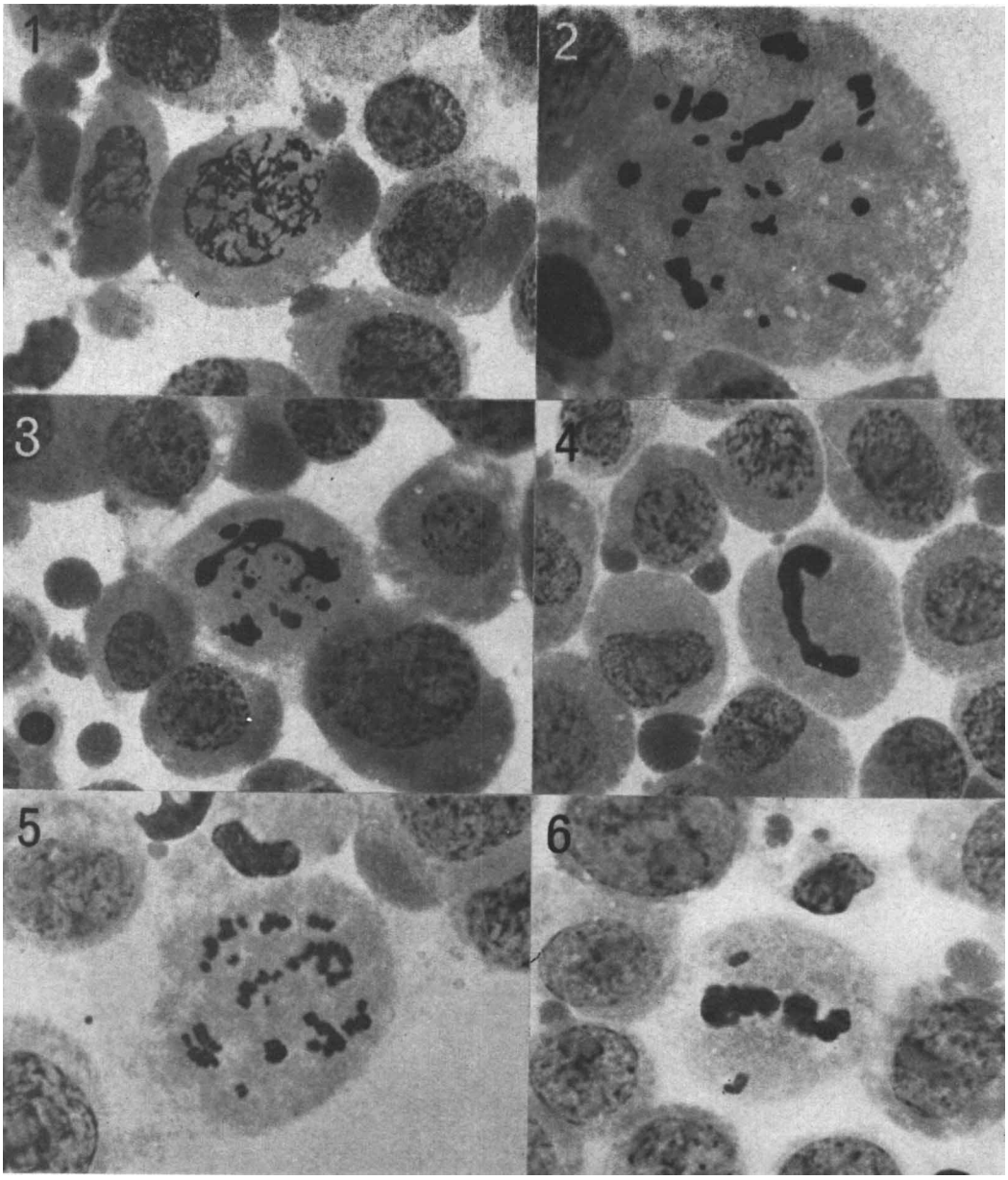


FIG. 1-4. Photomicrographs of Ehrlich ascites tumor. Squash preparation stained with acetic dahlia. × 1000.

FIG. 1. Diffuse and sticky chromosomes in prophase after injection of juglone.

FIG. 2. A cell with clumped chromosomes scattered in cytoplasm after injection of juglone.

FIG. 3. A cell with clumped chromosomes scattered in cytoplasm after injection of juglone.

FIG. 4. A cell with chromatin mass after injection of juglone.

FIG. 5. A cell with clumped chromosomes scattered in cytoplasm after injection of 8-hydroxyquinoline.

FIG. 6. A cell with three-group metaphase after injection of 8-hydroxyquinoline.

TABLE IV. Effects of Orally or Intraperitoneally Administered Juglone on Weights of Tumor-Bearing Animals and on Mitotic Frequency of Tumor Cells.

| Days after treatment | Oral route* | | | | |
|---|---|---|---|---|---|
| | Control | | | | |
| | Mitotic frequency (%) | Body wt (g) | Mitotic frequency (%) | Body wt (g) | Body wt after intraperitoneal injection† |
| 0 | 4.2‡ | 34.5‡ ± 2.67 | 4.3‡ | 37.2‡ ± 1.8 | 35.0‡ ± 3.84 |
| 1 | 4.0 | 34.3 ± 4.16 | 3.8 | 35.6 ± 4.89 | 31.2 ± 4.84 |
| 2 | 4.9 | 37.4 ± 4.81 | 4.8 | 37.2 ± 5.60 | §27.0 ± 5.13 |
| 3 | 4.8 | ‖40.3 ± 4.98 | 3.5 | 36.5 ± 5.67 | §29.0 ± 3.93 |
| 4 | 4.3 | ‖41.9 ± 4.69 | 4.3 | §34.2 ± 3.96 | §29.7 ± 4.81 |
| 5 | 3.3 | ‖42.9 ± 4.38 | 3.2 | §33.2 ± 2.57 | 30.2 ± 5.53 |
| 6 | 3.4 | ‖43.3 ± 3.52 | 1.2 | §30.4 ± 2.31 | —— |

* Daily administration.
† Single injection.
‡ Values represent the average obtained from 5 animals.
§ Values significantly lower than body wt of 6-days tumor-bearing animals. $P < 0.05$ or less.
‖ Values significantly higher than body wt of 6-days tumor-bearing animals. $P < 0.05$ or less.

higher affinity for juglone than shown by $Ca^{++}$ and $Mg^{++}$.

*Effects of oral administration of juglone on accumulation of ascitic fluid.* Intraperitoneal administration of juglone produced mitotic abnormalities, decreased abdominal distension and reduced the body weight of treated animals (Table IV). But the life span of animals given a single dose of juglone was not prolonged. Recently, Rao *et al*(11) tested lapachol, (2-hydroxy-3-(3-methyl-2-butenyl)-1,4 naphthoquinone), against several experimental mouse and rat tumors(10). It was found that the compound was active by the intraperitoneal, intramuscular, subcutaneous and oral routes. By the oral route the compound had a much higher therapeutic index than by the other routes of administration. In the present experiment effects of juglone by the oral route of administration were observed.

A 2 mg dose of juglone suspended in 1 ml of water with the aid of azalectin was administered to tumor-bearing animals by the oral route at 6 days after transplantation of the tumor. The oral administration of 2 mg of juglone was not as toxic as the administration of the same dose by the intraperitoneal route. Mice received the compound once daily by stomach tube until the animals became moribund. The body weights of the animals and mitotic frequencies of the tumors are shown in Table IV. Juglone given by the oral route did not produce abnormal mitotic cells in the ascites and did not appear to suppress mitoses of peritoneally contained tumor cells. However, the body weights of the treated animals stopped increasing after the second administration. At the same time, the abdomens of the treated animals stopped expanding. However, the life spans of the treated animals were not prolonged over those of the controls.

*Discussion.* Most compounds tested in this study that showed no effect on Ehrlich ascites tumor have been studied in other systems and were found to be variously effective in one or another system. Menadione produced a reduction in mitotic rate in the embryonic chick. Vitamin $K_1$ caused both chromosomal and spindle abnormalities(1). A number of the compounds used here showed some influence on oxidative phosphorylation in a bacterial system(2). The functions of oxidative phosphorylation in mitosis have been investigated by several investigators and their results have been reviewed by Mazia(7). It was stated that cells could be prevented from entering division by disturbing oxidative phosphorylation, and in some cases, chromosomal and spindle abnormalities were induced. In the present experiment cells were prevented from entering division. The overall mitotic frequencies of treated tumor cell population were not significantly different from the control. Nevertheless, chromosomes in prophase from juglone-treated tumors appeared diffuse and sticky and accumulation of abnormal metaphase figures occurred. This indicates that when juglone was administered, cells already in division, as well as those which entered

division while the drug was still active, showed the typical cytological effects described. Cells in various stages of interphase also may be affected by juglone, but the effects may not be cytologically detectable until the cells enter division.

For a long time it has been known that potato, tomato, alfalfa and other crop plants growing in a circular area about black walnut and butternut trees became wilted and subsequently died. In all cases the root systems of affected plants were in immediate contact with the tree roots. During the investigation of the cause of wilting and death of plants, juglone was extracted from black walnut. It was suggested that the toxic effects were probably attributable to juglone contained in black walnut and butternut(3,5,6, 11). Although juglone was found to alter the respiratory rate of erythrocytes(4) the mechanism of action was not elucidated.

*Summary.* The cytological effects of 16 quinone compounds were tested on Ehrlich ascites tumor cells *in vivo*. Of these only juglone (5-hydroxy-1,4-naphthoquinone) produced mitotic abnormalities. An accumulation of abnormal metaphase figures occurred and the cells in metaphase were prevented from entering ana-telophase. Chromosomes in prophase cells appeared diffuse and sticky. Juglone administration, either intraperitoneal or oral, caused decreases in the amount of ascitic fluid at a time that marked increases were taking place in control animals.

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## Stimulation of the Growth of Organ Cultures by Methyl and Propyl Parabens.* (32514)

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Some years ago we were studying the effects of hydrocortisone hemisuccinate on organ cultures of embryonic chick bones. We noted that certain levels appeared to stimulate the growth of these cultures, (as measured by dry weight), which was contrary to previous reports(1,2,3). We found that this growth stimulation was difficult to reproduce. When pure hydrocortisone hemisuccinate was substituted for the pharmaceutical preparation we previously used (Solu-Cortef, Upjohn) only inhibitory effects were obtained. The pharmaceutical preparation, in addition to the steroid, contained methyl paraben and propyl paraben as preservatives. These are the methyl and propyl esters of p-hydroxybenzoic acid. When the effects of these compounds upon growth were studied, we found definite stimulatory effects.

*Material and methods.* The femora were dissected from 10-day White Leghorn chick embryos. They were cultured in medium MB 752/1a containing 25% embryo extract, using the roller tube system described by Teaford and White(4). Paired cultures were used, in which the femur from one leg is grown in the

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