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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TABLE I. Effects of Methyl and Propyl Parabens, and EDTA on Growth of Chick Embryo Femora *in vitro.*

| | | | | | |
|---|---|---|---|---|---|
| A. *Effect of methyl paraben* | | | | | |
| Methyl paraben concentration ($\mu$g/ml) | .025 | .25 | 2.5 | 25 | 250 |
| (M) 1.64x | $10^{-7}$ | $10^{-6}$ | $10^{-5}$ | $10^{-4}$ | $10^{-3}$ |
| Dry wt difference ($\mu$g) $\bar{D}$ | 25.6 | 81.5 | 75.3 | 10.4 | 224.6 |
| $\bar{Sd}$ | 19.6 | 21.4 | 18.6 | 22.3 | 31.9 |
| % Change | −2.7 | +9.2 | +7.7 | −1.1 | −19.6 |
| Significance (P) | >.05 | <.01 | <.01 | >.05 | <.01 |
| B. *Effect of propyl paraben* | | | | | |
| Propyl paraben concentration ($\mu$g/ml) | .0025 | .025 | .25 | 2.5 | |
| (M) 1.38x | $10^{-8}$ | $10^{-7}$ | $10^{-6}$ | $10^{-5}$ | |
| Dry wt difference ($\mu$g) $\bar{D}$ | 17.7 | 65.0 | 99.8 | 88.4 | |
| $\bar{Sd}$ | 14.6 | 17.0 | 16.8 | 19.1 | |
| % Change | −1.8 | +6.8 | +11.7 | +8.8 | |
| Significance (P) | >.05 | <.01 | <.01 | <.01 | |
| C. *Effect of methyl and propyl parabens combined* | | | | | |
| Methyl paraben concentration ($\mu$g/ml) | .025 | .25 | 2.5 | 25.0 | |
| Propyl paraben concentration ($\mu$g/ml) | .0025 | .025 | .25 | 2.5 | |
| Dry wt difference ($\mu$g) $\bar{D}$ | 78.5 | 57.7 | 54.5 | 1.4 | |
| $\bar{Sd}$ | 42.3 | 47.9 | 5.8 | 1.0 | |
| % Change | −10.1 | +7.6 | +6.8 | +1.6 | |
| Significance (P) | <.01 | <.01 | <.01 | >.05 | |
| D. *Effect of EDTA* | | | | | |
| EDTA concentration (M) 1.64x | $10^{-7}$ | $10^{-6}$ | $10^{-5}$ | $10^{-4}$ | $10^{-3}$ |
| Dry wt difference ($\mu$g) $\bar{D}$ | 54.9 | 2.8 | 2.2 | 10.0 | 216.6 |
| $\bar{Sd}$ | 17.8 | 20.2 | 21.5 | 25.4 | 15.9 |
| % Change | −7.6 | +0.4 | +0.3 | −1.1 | −23.0 |
| Significance (P) | .01<.05 | >.05 | >.05 | >.05 | <.01 |

control medium, while the other femur is grown in the same medium, to which has been added the compound in question. Generally 12 pairs were grown for each level of paraben. After 2 days in culture, the bones were dried in an oven and weighed on a micro balance. The differences in weights between the control and experimental cultures were averaged and the data were analyzed statistically by the paired variate method using the Student T-test(5).

*Results and discussion.* Table I, A, shows that at 0.25 and 2.5 $\mu$g/ml of methyl paraben there resulted a significant increase in dry weight over the controls (9.2 and 7.7% respectively). The propyl paraben is less water soluble than the methyl ester, and therefore the concentration levels assayed were more restricted. However there was a significant growth stimulation at 0.025, 0.25, and 2.5 $\mu$g/ml of the propyl paraben (Table I, B). When the two compounds were combined in the same ratio occurring in the pharmaceutical preparation originally used, the growth stimulatory effect was not found to be additive (Table I, C). The stimulatory range was also more restricted. An interesting development was the significant growth inhibition resulting in the lowest levels used (10.1% inhibition at 0.025 $\mu$g/ml methyl paraben plus 0.025 $\mu$g/ml propyl paraben).

Whitehouse and Boström have studied the action of salicylate analogues in inhibiting the biosynthesis of mucopolysaccharides(6,7,8,9). However these salicylates were only inhibitory when the ortho phenolic group was present, and Whitehouse found that m- and p-hydroxybenzoates did not inhibit mucopolysaccharide synthesis(10). No mention was made of stimulatory effects. Whitehouse pointed out that the compounds he studied were able to form complexes with metal ions,

and that they were lipophilic in character.

To determine whether the parabens might be complexing with a metal ion, EDTA (disodium ethylenediaminetetraacetate) was added to the medium, in molar concentrations equivalent to the molar concentration used for methyl paraben (Table I, D). No stimulatory effect was noted, although there was an inhibitory effect at the lowest level ($10^{-7}$ M) which may be significant. The second criterion Whitehouse suggested for biological activity of these compounds, a lipophilic character, was not fulfilled by EDTA. Although the para structure of the parabens would appear to exclude the possibility of chelate formation with metal ions, it would not exclude the possibility of other types of coordination complexes. Whitehouse notes that the m- and p-hydroxybenzoates do not form complexes with ferric ion, however this may not be the case with other ions, *e.g.*, cupric ion.

Another possible explanation relates to the effects of the salicylate analogues on glucose metabolism. Sturman and Smith found that p-hydroxybenzoic acid and a series of related compounds inhibited glucose utilization by the human erythrocyte(11). Both glycolysis and the pentose phosphate pathway was affected. The salicylate analogues *inhibited* glucose utilization from $5 \times 10^{-3}$ M to $20 \times 10^{-3}$ M which is in the concentration range we find growth inhibition. These authors do not mention if lower concentrations of the salicylates stimulate glucose utilization. One would expect that an increase in growth rate would be correlated with an increase in glucose utilization rather than an inhibition.

A third approach to the mechanism of action of the parabens relates to the observation of Miller and Smith that rat liver lysosomes were stabilized by acetylsalicylic acid at concentrations of $10^{-3}$ to $10^{-5}$ M(12). We obtained growth stimulation at $10^{-5}$ M with both parabens, however the methyl ester was active at $10^{-6}$ M while the propyl ester was active at $10^{-6}$ and $10^{-7}$ M. This may reflect the relatively greater lipid solubility of these esters at neutral pH as compared to acetylsalicylic acid.

In other studies we have observed that there is a period of "adaptation" of the femora to *in vitro* conditions. During this period, which may last from 6 to 12 hours, there is a lag period during which little growth occurs. This is followed by a rapid increase in dry weight. It is not unreasonable to conclude that there is a labilization of lysosomes during the initial part of the adaptation period, after which the lysosomal integrity is restored. There are a variety of factors present during the early culture period which might labilize lysosomes. These include rapid changes in pH, oxygen tension and temperature, and the trauma involved in the removal of the femur from the chick. An attractive hypothesis would be that the parabens stabilize lysosomes, and therefore prevent a partial autolysis during the early period of the culture. This stabilization would result in a more rapid adaptation to culture, and consequently a greater final weight.

While the mechanism of action of the parabens is unknown, the significance of these observations is considerable. Anomalous results have been reported by others when pharmaceutical preparations of hydrocortisone were utilized(13). In this Department, Caster, Garner and Luckey noted an increase in the size of lymph nodes when mice were injected with saline containing the parabens(14). This had actually been done as a control for another experiment, and for convenience, hospital saline was used. It appears that most hospital saline solutions contain the parabens, as do pharmaceutical preparations of many drugs. The possible biological effects of the parabens should not be ignored.

*Summary*. When 10-day-old chick embryo femora were cultured for 2 days in medium containing methyl or propyl paraben, there resulted an increase in dry weight as compared with femora cultured in the control medium. The methyl paraben concentrations found to stimulate growth were $10^{-5}$ and $10^{-6}$ M. Propyl paraben stimulated at $10^{-5}$ to $10^{-7}$ M. The effects of the two were not additive. It is suggested that these compounds may stabilize lysosomes.

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## Effect of Inhibitors of Protein Synthesis on Pyrogen Production by Granulocytes.* (32515)

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Polymorphonuclear leukocytes from inflammatory exudates of rabbits release a pyrogen when incubated in saline(1-3). This leukocytic pyrogen (LP) is a protein(4-6) which may play an important role in the pathogenesis of fever in rabbits.

There is little specific information available concerning the process of pyrogen formation by leukocytes. Fessler and co-workers(7) and Kaiser and Wood(3) found that mechanical disruption of granulocytes did not release significant amounts of LP and that subcellular fragments of leukocytes were not pyrogenic. On the other hand, Herion and his colleagues showed that intact granulocyte lysosomes or their lysates induced fever when injected intravenously into rabbits, suggesting that there may be a relationship between some constituent of lysosomes and leukocytic pyrogen(8). These studies permit the hypothesis that, during incubation, granulocytes either synthesize pyrogen *de novo* or release it from an inactive precursor. Gander and Goodale favored the precursor theory because puromycin failed to abolish the production of pyrogen by granulocytes *in vitro*(9). The experiments to follow were undertaken to assess further the effect of inhibitors of protein synthesis on production of pyrogen.

*Materials and methods.* Rabbits weighing 2 to 3 kg were used in all experiments. Glassware was sterilized and rendered pyrogen-free by heating at 170°C for 2½ hours. Commercially available sterile, nonpyrogenic distilled water was used to prepare all solutions.

Leukocytes, 80 to 90% of which were granulocytes, were obtained by procedures outlined by others(5). Leukocyte-saline suspensions containing $3.5 \times 10^7$ cells/ml were incubated for 20 hours at 37°C unless otherwise specified, and the cells were then removed by centrifugation. Extracts were passed through a bacterial filter, frozen and stored at 0°C until used.

The procedures employed for assaying the pyrogenicity of leukocyte extracts by intravenous injection into rabbits were essentially the same as those previously described (10). Febrile responses were quantitated by measurement of the fever index ($F.I._{120}$), the area in cm² under a 2-hour fever curve (10 cm on the ordinate equaled 1°C and 4 cm on the abscissa represented 60 minutes).

Rabbits used for test purposes were obtained in groups which were treated as units and kept intact as long as they were suitable for pyrogen assays. Only results obtained within a given group of test animals were used to compare the pyrogenicity of various

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