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Hydra Reaggregation: A Rapid Assay to Predict Teratogenic Hazards Induced by Environmental Toxicity

Arthur J. Kudla

Fitzgerald Public Schools Warren, Michigan

ABSTRACT

Before drugs, chemicals, and food additives are safety certified, they need to be tested in lengthy and expensive experiments. Such evaluation, however, does not keep up with the rapid development and production of new compounds. The purpose of this study, therefore, was to design, develop, and validate a new rapid assay to simulate embryogenesis and to determine the teratogenic potential of chemicals.

After testing many organisms, adult *Hydra attenuata* were used to evaluate the toxicity of several substances, including salicylic acid, lithium chloride, 2,4,5-trichlorophenoxyacetic acid, and Agent Orange. Each chemical's lowest toxic adult and "embryo" dosages were determined, through a series of 28 tests, to within one-tenth of a log concentration. Unique methods of tissue culturing of "embryo" hydra were developed to dissociate *Hydra attenuata* into their component cells and to form pellets which were ejected into reaggregation media containing specific concentrations of the test substances. While studying, at six time intervals, the growth reactions of adult and "embryo" hydra exposed to the chemicals, numerous controls were maintained.

Through an analysis of the results, validation by comparison with published rodent studies, and statistical verification, it may be noted that the *Hydra attenuata* assay is a viable screening technique which can predict the magnitude of a chemical's toxicity to developing organisms. Since many compounds cannot be adequately tested by traditional methods before being distributed within the environment, this new assay will identify those which mandate further testing before dissemination.

Introduction

The teratogenic potential or developmental mutagenicity of drugs, chemicals, and food additives is routinely tested in lengthy and expensive experiments which call for administration of a test substance to pregnant rodents during the period of major organogenesis. Military and industrial facilities are introducing new chemical compounds at such an increased rate, however, that the absence of rapid and inexpensive means for detecting teratogenic hazards is a major obstacle in society's efforts to make safety evaluations of chemicals.

The purpose of this study, therefore, was to design, develop, and validate a rapid assay

which would simulate embryogenesis and determine the teratogenic potential of chemicals. Hydra, the most primitive organism with complex structures, were chosen as the experimental animals because they exhibit, during regeneration, many of the phenomena required of a zygote in becoming an embryo and then a fetus. Some of the developmental cell processes exhibited by the regenerating hydra are: changes in cell size and shape,[1] spatial orientation,[2] cell migration,[3] intercellular matrix formation,[4] cell division and differentiation,[5] and organ field[6] and tissue[7] formation.

It was hypothesized that this new hydra assay could be used as a screening technique to identify chemicals potentially dangerous to an organism's developmental processes. Such a procedure would prioritize new substances according to their toxicity and their need for further study by more elaborate means.

Procedure

Three cultures of adult *Hydra sp.* were continually maintained within separate, aerated tanks regulated at a constant temperature of 21°C, using a method[8] which included daily feeding with iodine treated brine shrimp and complete tank draining 5 hours after feeding. Each tank contained hydra media (pH 7) which consisted of 0.147 g $CaCl_2$, 0.115 g TES, and 0.004 g EDTA dissolved in 1 liter distilled water.

Because extensive attempts using *Hydra littoralis* were unsuccessful, adult *Hydra attenuata*[9] were used to evaluate the toxicity of lithium chloride, salicylic acid, acetaminophen, lead dioxide, carbon tetrachloride, formaldehyde, 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and Agent Orange (50% 2,4-D and 50% 2,4,5-T). Each chemical's lowest toxic dose was identified and verified to within one-tenth of a log concentration through a series of twenty-eight tests which were divided into four experiments. Experiment I determined the lowest toxic whole-log concentration by exposing hydra to solutions ranging from 0.0 mg/1 to 1000 mg/1. Experiment II confirmed the lowest toxic whole-log concentration. Experiment III divided the lowest toxic whole-log concentration into tenths of a log and Experiment IV confirmed the lowest toxic one-tenth of a log concentration. In each of the tests, 3 adult hydra were placed in 9 ml of hydra media containing 150 mg/1 of Amikacin sulfate (an anti-bacterial agent), and the appropriate concentration of the substance being evaluated. Every 24 hours, the hydra were placed in a new solution with the identical chemical composition of the solution used within the first trials. Amikacin sulfate was used for all testing of adult and "embryo" hydra, as well as with all of the controls.

To test "embryo" hydra, hundreds of adult *Hydra attenuata* were bathed in iodine to reduce the potential of bacterial contamination, rinsed, and kept in a glass jar, separate from the main culture, for 3 days. These adult hydra were then placed into 3 ml of 70 mosmol reaggregation media[10] (pH 7) which consisted of 0.29 g KCl, 0.97 g $CaCl_2$, 0.16 g $MgSO_4$, 1.94 g Na citrate, 0.73 g Na pyruvate, 3.00 g TES, and 0.10 g phenol red dissolved in 1 liter distilled water. 150 mg/1 of Amikacin sulfate was added. After 30 minutes, the hydra were removed from the reaggregation media and were dissociated into their component cells and tissue fragments by repeated pipetting. After this mixture of cells was centrifuged to form a cell mass, the supernatant was removed and the mass was resuspended. This suspension was drawn into ID 0.58 mm polyethylene tubing which was then sealed with wax. The tubing was centrifuged to manipulate the hydra cells into a long, thin pellet. This pellet of cells was slowly ejected into sterilized, covered glass dishes containing 9 ml of 70 mosmol reaggregation media, Amikacin sulfate, and specific concentrations of the substance being tested. Because the pellets were formed in a high molarity reaggregation media which was not suitable for adult hydra, the molarity was reduced to 35 mosmol and 17.5 mosmol at 4 and 18 hours, respectively. At 26 hours the pellets were transferred to hydra media, which was replaced with fresh media at 42 and 66 hours. The pH and the concentration of both the Amikacin sulfate and the substance being tested

remained constant through all the media changes.

During all experimentation, both the adults and ''embryos'' were incubated at 21°C and were observed at 4, 18, 26, 42, 66, and 90 hours of chemical exposure. Any abnormalities, as compared to the controls, were noted.

Results

Figure I displays the reaction of ''embryo'' *Hydra attenuata* to 2,4,5-T in Experiment I. Figure II is a summary of the identification of the lowest toxic concentration of 2,4,5-T for ''embryo'' *Hydra attenuata*.

After identifying the lowest toxic dose of a substance for both adult (A) *Hydra attenuata* and ''embryo'' (E) *Hydra attenuata*, a ratio of the adult concentration to the ''embryo'' concentration (A/E ratio) was calculated. Table I displays the lowest toxic concentrations of the test substances on adult and ''embryo'' Hydra, as well as the ratios calculated using these two figures. A small A/E ratio indicates that the substance disrupts

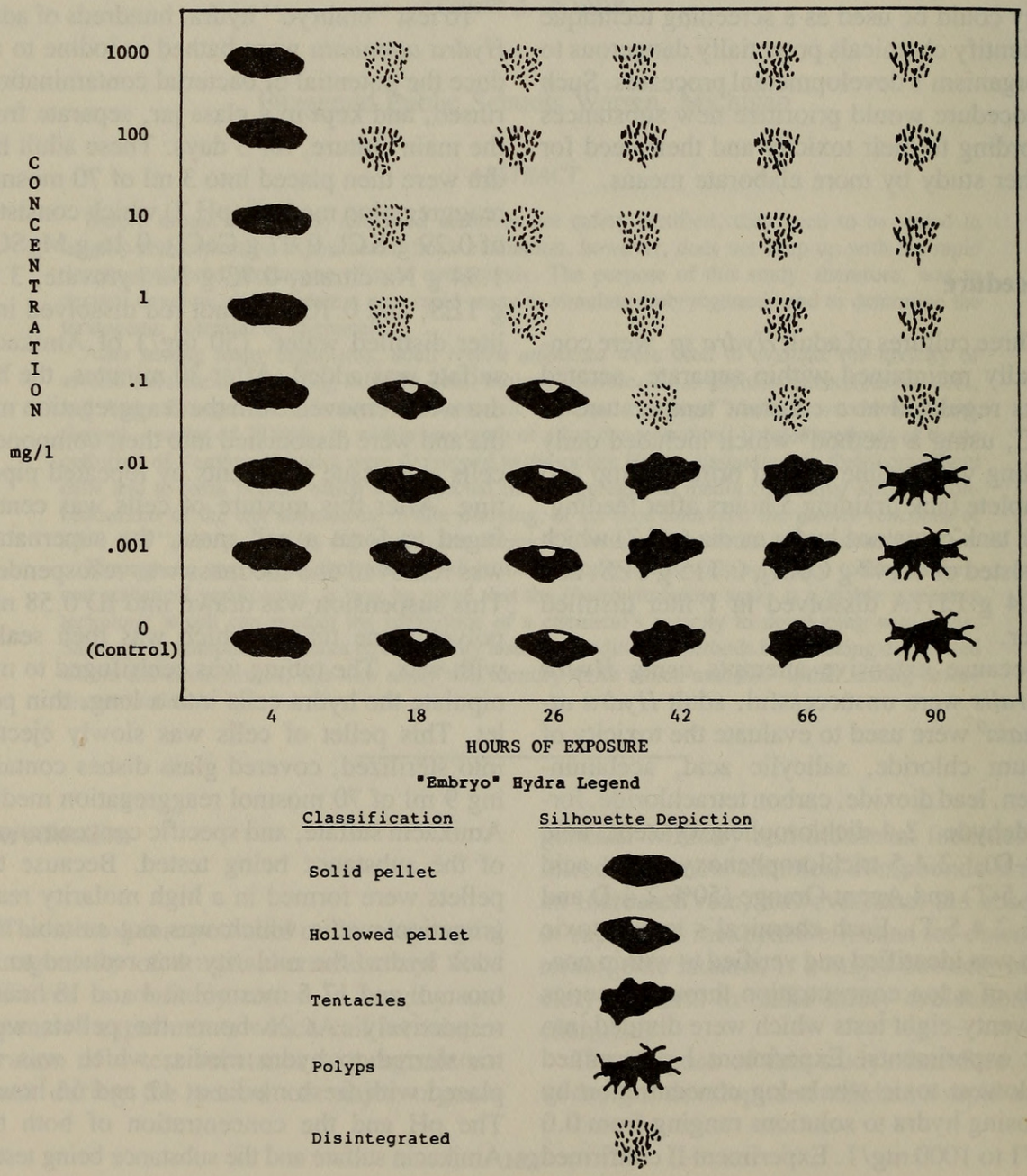

Fig. 1.

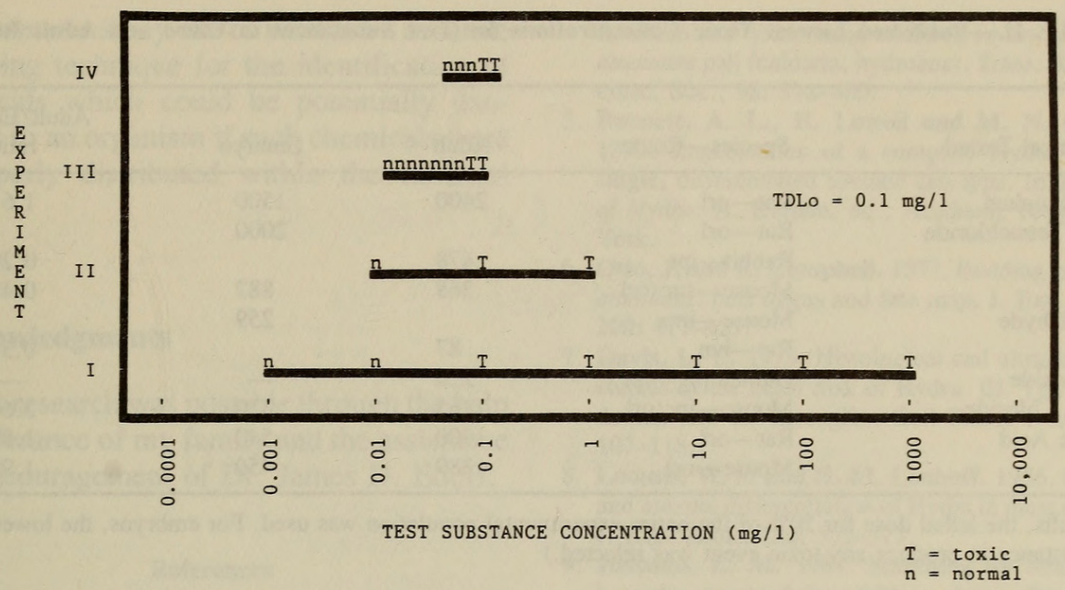

Fig. 2. Summary of experimental identification of lowest toxic concentration of 2,4,5-T for "Embryo" *Hydra attenuata*

development only at or near the concentration also toxic to the adult (a developmentally non-hazardous substance). A large A/E ratio indicates that the substance disrupts developmental events at a small fraction of the exposure toxic to adults (a teratogenic hazard). A rank order of a group of substances, beginning with the lowest A/E ratio and finishing with the highest A/E ratio, results in a substance list showing increasing teratogenic potential.

To validate this new rapid assay using *Hydra attenuata,* the A/E ratio for each of the substances evaluated in this research was compared to results of published rodent studies. Table II lists the lowest toxic concentrations, obtained from the *Registry of Toxic Effects of Chemical Substances,*[11] for both adult and embryo rodents, and the ratios calculated using these dosages.

Limitations

Though all experimentation was carefully controlled, the following limitations affected analysis of results:

1. The technique described has been found to be viable with only a specific hydra species.
2. The development of all experimental pellets must be verified and control pellets at all times because of the general tendency for some pellets not to be viable due to incorrect dissociation processes.
3. This hydra assay is capable of evaluating the toxicity of any compound except those containing copper since copper interferes with protein synthesis in hydra.

Table I.—Lowest Toxic Concentrations for Test Substances on Adult and "Embryo" Hydra (mg/l).

Chemical Tested	Adult	"Embryo"	Adult/"Embryo" ratio
Acetaminophen	100.0	—	—
Agent Orange	0.08	0.01	8.0
Carbon Tetrachloride	0.50	—	—
2,4-D	4.0	—	—
Formaldehyde	0.003	—	—
Lead Dioxide	1.0	—	—
Lithium Chloride	50.0	50.0	1.0
Salicylic Acid	7.0	400.0	0.018
2,4,5-T	0.80	0.10	0.86

TABLE II.—Published Lowest Toxic Concentrations for Test Substances in Utero and Adult Rodents (mg/kg).

Chemical Tested	Species—Route	Adult	Embryo	Adult/Embryo ratio
Acetaminophen	Rat—orl	2400	1500	1.6
Carbon Tetrachloride	Rat—orl		2000	
	Rabbit—ipr	478		0.24
2,4-D	Mouse—suc/orl	368	882	0.42
Formaldehyde	Mouse—ims		259	
	Rat—ivn	87		0.34
Lead dioxide	Guinea pig—ipr	220	—	—
Lithium Chloride	Mouse—ipr/orl	1165	320	3.64
Salicylic Acid	Rat—orl	1000	540	1.85
2,4,5-T	Mouse—orl	389	450	1.95

(For adults, the lethal dose for 50% of the entire experimental population was used. For embryos, the lowest dose of a substance to produce any toxic event was selected.)

4. Though cautious comparison was made with published rodent toxicity statistics, not all comparisons could be validated by published studies employing the same organism or the same method of chemical exposure.

Analysis

In comparison to published results of in utero and adult rodent toxicity studies, the following general characteristics of the *Hydra attenuata* assay have been confirmed and statistically verified by the Spearman's Correlation Coefficient, with an alpha level equal to 0.05:

1. Concentrations of chemicals toxic to adult hydra are predictive to the magnitude of toxicity for adult rodents but are not predictive of the magnitude of toxicity for rodent embryos.
2. Concentrations of chemicals toxic to "embryo" hydra are predictive of the magnitude of toxicity for rodent embryos but are not predictive of the magnitude of toxicity for adult rodents.
3. A ratio (adult/"embryo") of hydra toxic chemical concentrations is predictive of a ratio (adult/embryo) of rodent toxic chemical concentrations.
4. Concentrations of chemicals toxic to adult hydra are not predictive of the magnitude of toxicity for "embryo" hydra nor are adult rodents predictive of the magnitude of toxicity for embryo rodents.

Conclusion

Through the previous analysis of the results and verification with the Spearman Correlation Coefficient, it may be noted that the *Hydra attenuata* assay, which was designed, developed, and validated through this research, is a viable screening technique for the rapid identification of chemicals which could be potentially dangerous to developing organisms.

Calculation of the adult/"embryo" hydra ratio by this system quickly and accurately reflects conclusions possible for complex animal studies since it predicts the magnitude of a chemical's toxicity to developing organisms. The great majority of substances are no more hazardous to embryos than to adults, but the *Hydra attenuata* assay quantitatively identifies the chemicals with the greatest possibility of disrupting developmental processes. This allows for rapid separation of developmentally nonhazardous substances from potential teratogenic hazards, which would mandate further testing before dissemination.

The extensive experimentation and the careful use of controls within this project allow acceptance of the hypothesis that the *Hy-*

dra attenuata assay can be used as an accurate screening technique for the identification of chemicals which could be potentially dangerous to an organism if such chemicals were improperly distributed within the environment.

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