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MUTAGENICITY AND CLASTOGENICITY POTENTIAL OF GUSATHION A, CARVIL AND LANNATE

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ABSTRACT

A study on the mutagenic and clastogenic potential of three commonly used insecticides in rice farming was conducted. These insecticides are Gusathion A, Carvil and Lannate.

Results with Rec assay suggested DNA damaging capacity of the three insecticides. The Ames test revealed that the three insecticides are not direct acting mutagens. However, Gusathion A at 6, 8 and 10 mg/kg; Carvil at 155.55, 207.67 and 311.50 mg/kg; and Lannate at 4.50, 6.75 mg/kg were found mutagenic after metabolic activation as shown by the results of the host-mediated assay. All insecticides have also been found to fragment the chromatin material of the bone marrow cells of mice. The micronucleus test suggested the clastogenic or chromosome-breaking potential of these insecticides.

Folic acid at 100, 200 and 400 ug/kg reduced the mutagenic and clastogenic potential of Gusathion A. It is therefore an anticlastogen and antimutagen to Gusathion A.

Introduction

The tremendous boost in the use of chemicals especially in agriculture for the control of plant pests and diseases has alarmed the public in the possible health hazards these chemicals pose.

Studies in the persistence of pesticides in the environment and its effect in host organism are voluminous. Only recently has there been studies concentrated on the genotoxicity of pesticides and their residues in animals.

This report deals with the investigation into the genotoxicity of Gusathion A, Carvil and Lannate.

Review of Literature

Gusathion A, Carvil and Lannate are three insecticides in current use in the country's agriculture. Gusathion A is an organophosphate, Carvil a carbamate and Lannate a thiocarbamate.

Gusathion A is more toxic to mammals than its corresponding methyl homolog, Guthion (1). However, more toxicity studies have been focused on Guthion than Gusathion A.

Carvil is a carbamate insecticide which is widely used against rice and mango pests. The active component is a choline esterase (inhibitor (2).

Lannate, a thiocarbamate, has 10% methomyl which is a broad spectrum insecticide and agaricide with some nematocidal properties (3). These make it useful and effective against a variety of pests; a reason for its wide use in agriculture.

The genotoxicity of 228 pesticides and related chemicals were studied (4). About 22% were found to be mutagenic.

Experimental Methods

The Rec assay (5) was used to determine DNA damaging potential. This assay utilizes *Bacillus subrilis* strains H 17 Rec^+ and M 45 Rec^-, courtesy of Tsueno Kada, National Institute of Genetics, Mishima, Japan. The strains were grown overnight in separate test tubes containing 5 ml of B-2 broth at 37°C. On the day of the experiment, each tester strain was radially streaked on the dry surface of the B-2 agar. A paper disk of 16 mm diameter was saturated with 0.5 ml of the test solution and was placed over the starting points of the streaks. The streaked plates with samples were kept at cold storage (4-5°) for 24 hours followed by incubation at 37°C for 20 hours. The length of inhibition zone was then measured starting from the edge of the paper disk. Five trials were done with five plates per trial per concentration of the test solution. Positive and negative controls were also done side by side. Quinoline was used as positive control. Water was the negative control.

The Ames test was used to study mutagenicity potential before metabolic activation (6). *Salmonella typhimurium* strains TA 1535, TA 1537, TA 97 and TA 98 were used. Tester strains were inoculated into separate test tubes containing 5 ml of nutrient broth and were incubated overnight at 37°C. A 0.1 ml culture was transferred into 13 x 100 mm test tube. Into the same tube was pipetted 2 ml of molten top agar and mixed. The mixture was then poured into a hardened bottom agar plate. After the top agar hardened, a sterile paper disk of 8 mm in diameter was saturated with the test solution and was placed in the center of the plate. The plates were incubated for 48 hours at 37°C after which the revertant colonies were counted.

The host-mediated assay (7) was used to study mutagenicity potential after metabolic activation. *Salmonella typhimurium* his G 46 was used as the indicator organism. The test animal was albino mouse of the Swiss Webster strain. Centrifuged stock culture of the indicator organism (2 ml) was injected intraperitoneally into each mouse. The test compounds were administered orally. Three hours later, the mice were sacrificed by cervical dislocation. The abdominal region was sterilized with ethanol and 2 ml of 0.9% saline solution was injected into the peritoneal cavity. About 2 ml of the injected bacteria was removed from the cavity. Plating was done to determine the number of survivors and the number of revertants.

The micronucleus test (8) was done to study the effects of the test substances on the chromatin material of the bone marrow cells. It is a method for determining mutagenic as well as clastogenic potential. In this method, seven to ten-week old Swiss Webster albino mice were used. Two applications of the test system were done. The second application was done 24 hours after the first. The femora were excised. About 0.2 ml fetal calf serum was used to flush the bone marrow from the femora. The suspension was centrifuged at 1,000 rpm for 5 minutes. The pellet was used to prepare a smear on glass slide. The slides were air-dried overnight and were stained the following morning using May-Grunwald solution and Giemsa. The slides were screened for micronucleated polychromatic erythrocytes.

The antimutagenic effect of folic acid was studied using the micronucleus test.

Results and Discussion

The results of the Rec assay for Gusathion A, Carvil and Lannate are shown in Tables 1, 2, and 3. In Table 1, Gusathion A caused inhibition of bacterial growth in both the Rec^+ and Rec^- strains in all concentrations used. The result showed a slightly greater extent of growth inhibition on the Rec^- strain. It is this strain that does not possess the recombination repair mechanism. A similar trend of results was obtained with Carvil (Table 2). Lannate, on the other hand, showed growth inhibition only at 108 and 180 mg/ml concentrations (Table 3). The greater extent of zone of inhibition of the Rec^- strain indicates the capability of the insecticide to damage cellular DNA (1, 2).

Table 1. DNA damaging capacity of Gusathion A as revealed by Rec assay

	*Zone of inhibition (mm)**	
	Rec^+ H 17	*Rec^- M 45*
Control		
Positive**	13.2 ± 0.3	16.6 ± 0.3
Negative ***	0.0 ± 0.0	0.0 ± 0.0
Gusathion A		
16 mg/ml	0.2 ± 0.1	1.0 ± 0.2
80 mg/ml	4.6 ± 2.0	5.1 ± 2.0
160 mg/ml	5.1 ± 0.5	5.9 ± 0.2
240 mg/ml	5.6 ± 0.3	6.6 ± 0.3
400 mg/ml	6.5 ± 0.7	7.6 ± 1.0

*Ayerage of 25 plates.
** Quinoline.
***Distilled water.

Table 2. DNA damaging capacity of Carvil as revealed by Rec assay

	*Zone of inhibition (mm)**	
	Rec^+ H 17	*Rec^- M 45*
Control		
Positive**	13.2 ± 0.3	16.6 ± 0.3
Negative***	0.0 ± 0.0	0.0 ± 0.0
Carvil		
20 mg/ml	7.6 ± 1.4	8.3 ± 0.3
100 mg/ml	8.7 ± 0.3	9.7 ± 0.3
200 mg/ml	9.5 ± 2.0	11.6 ± 2.9
300 mg/ml	9.1 ± 1.4	10.9 ± 1.7
500 mg/ml	10.3 ± 1.2	12.3 ± 2.7

*Average of 25 plates.
**Quinoline.
***Distilled water.

Table 3. DNA damaging capacity of Lannate as revealed by Rec assay

	*Zone of inhibition (mm)**	
	Rec^+ H 17	*Rec^- M 45*
Control		
Positive**	13.2 ± 0.3	16.6 ± 0.3
Negative***	0.0 ± 0.0	0.0 ± 0.0
Lannate		
7.2 mg/ml	0.0 ± 0.0	0.0 ± 0.0
36 mg/ml	0.0 ± 0.0	0.0 ± 0.0
72 mg/ml	0.0 ± 0.0	0.0 ± 0.0
108 mg/ml	0.2 ± 0.2	0.6 ± 0.2
180 mg/ml	0.8 ± 0.2	3.2 ± 0.4

*An average of 25 plates.
**Quinoline.
***Distilled water.

Mutagenicity potential without metabolic activation can be determined by the use of Ames test (6) using *Salmonella typhimurium* mutant strains. Results in Tables 4, 5 and 6 show that Gusathion A, Carvil and Lannate are not mutagenic prior to metabolic activation. These are not direct frameshift or base-pair mutagens.

Table 4. Mutagenicity potential without metabolic activation of Gusathion A using *Salmonella typhimurium* tester strains

	*Average number of revertants per plate**			
	TA 1535	*TA 1537*	*TA 97*	*TA 98*
Control				
Positive**	TNTC***	TNTC	TNTC	TNTC
Negative****	6.2 ± 2.6	4.1 ± 0.4	121 ± 2	15.4 ± 3.8
Gusathion A				
16 mg/ml	3.2 ± 1.0	4.4 ± 1.5	113 ± 2	15.1 ± 3.9
80 mg/ml	3.6 ± 1.0	5.4 ± 2.2	114 ± 0	15.4 ± 4.4
160 mg/ml	7.3 ± 1.2	6.9 ± 1.8	117 ± 3	16.6 ± 3.7
240 mg/ml	8.1 ± 1.9	2.3 ± 0.6	119 ± 3	17.6 ± 3.7
400 mg/ml	8.7 ± 3.2	2.6 ± 0.5	122 ± 2	20.1 ± 3.8

*Average of 15 plates.
**Ethylmethane sulfonate for TA 1535.
Amino acridine for TA 1537 and TA 97.
Malathion for TA 98.
***TNTC – too numerous to count.
****Distilled water.

Table 5. Mutagenicity potential without metabolic activation of Carvil using *Salmonella typhimurium* tester strains

	*Average number of revertants per plate**			
	TA 1535	*TA 1537*	*TA 97*	*TA 98*
Control				
Positive**	TNTC***	TNTC	TNTC	TNTC
Negative****	6.3 ± 0.3	4.1 ± 0.4	78 ± 5	12.9 ± 0.2
Carvil				
20 mg/ml	6.0 ± 0.3	1.1 ± 0.5	60 ± 6	9.7 ± 0.9
100 mg/ml	6.1 ± 0.2	0.8 ± 0.2	53 ± 4	10.7 ± 0.3
200 mg/ml	8.9 ± 0.5	0.8 ± 0.2	56 ± 6	13.3 ± 0.3
300 mg/ml	10.9 ± 1.7	4.7 ± 0.6	57 ± 4	13.8 ± 1.4
500 mg/ml	7.1 ± 1.0	3.0 ± 0.3	48 ± 4	11.1 ± 0.2

*Average of 15 plates.
**Ethylmethane sulfonate for TA 1535.
Amino acridine for TA 1537 and TA 97.
Malathion for TA 98.
***TNTC – too numerous to count.
****Distilled water.

Table 6. Mutagenicity potential without metabolic activation of Lannage using *Salmonella typhimurium* tester strains

	*Average number of revertants per plate**			
	TA 1535	*TA 1537*	*TA 97*	*TA 98*
Control				
Positive**	TNTC***	TNTC	TNTC	TNTC
Negative****	6.1 ± 0.5	2.4 ± 0.4	84 ± 7	8.7 ± 0.6
Lannate				
36 mg/ml	8.4 ± 1.4	4.8 ± 0.8	52 ± 6	3.8 ± 0.7
72 mg/ml	8.9 ± 1.0	4.0 ± 0.3	67 ± 7	3.8 ± 0.8
108 mg/ml	9.5 ± 0.7	3.8 ± 0.2	67 ± 3	5.1 ± 0.3
180 mg/ml	12.4 ± 1.1	2.6 ± 0.4	68 ± 8	8.4 ± 0.5

*Average of 15 plates.
**Ethylmethane sulfonate for TA 1535.
Amino acridine for TA 1537 and TA 97.
Malathion for TA 98.
***TNTC – too numerous to count.
****Distilled water.

Tables 7, 8, and 9 show that Lannate, Carvil and Gusathion A are genotoxic after metabolic activation. Evaluation of the results of the host-mediated assay using analysis of variance revealed statistically significant increases in mutation frequencies in all insecticides used. Results of Duncan's Multiple Range test revealed statistically significant difference between the control and all treatments used and also in between treatments for Gusathion A and Carvil treated mice at 99% confidence level. Lannate gave significant results only at 4.5 and 6.75 mg/kg concentrations.

Table 7. Mutagenicity potential after metabolic activation of Gusathion A as revealed by the host-mediated assay

	*Mutation frequency**
Control**	10.6 ± 0.2^{a}
Gusathion A	
6 mg/kg	2.06 ± 0.1^{b}
8 mg/kg	2.24 ± 0.1^{b}
10 mg/kg	4.23 ± 0.2^{c}

*Average of 5 plates.
**Distilled water.
[a]Means with different letter notation indicates significant difference at $P<0.01$.

Table 8. Mutagenicity potential after metabolic activation of Carvil as revealed by the host-mediated assay

	*Mutation frequency**
Control**	1.06 ± 0.2[a]
Carvil	
155.55 mg/kg	2.04 ± 0.1[b]
207.67 mg/kg	2.97 ± 0.4[c]
311.50 mg/kg	4.72 ± 0.6[d]

*Average of 5 plates.
**Distilled water.
[a]Means with different letter notation indicates a significant difference at P<0.01.

Table 9. Mutagenicity potential after metabolic activation of Lannate as revealed by the host-mediated assay

	*Mutation frequency**
Control**	1.06 ± 0.2[a]
Lannate	
2.25 mg/kg	1.39 ± 0.1[a]
4.50 mg/kg	2.74 ± 0.9[b]
6.75 mg/kg	3.00 ± 0.4[b]

*Average of 5 plates.
**Distilled water.
[a]Means with different letter notation indicates significant difference at P<0.01.

In a metabolic pathway of Gusathion A that has been proposed (9), formaldehyde is formed. Formaldehyde has been shown to be reactive with adenine to form bis adenosine (10). This reactivity may induce mutation. A similar explanation for the mutagenicity of Carvil after metabolic activation is possible. Carvil is also metabolized to give formaldehyde (9). Lannate may give thioformaldehyde after metabolism. This interacts with DNA like formaldehyde.

To further validate the results in *in vitro* studies and the host-mediated assay, another *in vivo* test, the micronucleus test was carried out in order to evaluate the chromosome breaking potential of the insecticides. The end point of this test is observed at the chromosomal level of the bone marrow cells.

Tables 10, 11 and 12 give the summary of the dose-effect relation of Gusathin A, Carvil and Lannate, respectively, for micronuclei formation. For Gusathion A, 6 mg/kg concentration does not differ statistically with the control at 99 per-

Table 10. Induction of micronuclei formation by Gusathion A in bone marrow cells of mice

	*Number of micronucleated polychromatic erythrocytes per thousand**
Control**	0.89 ± 0.19[a]
Gusathion A	
6 mg/kg	1.78 ± 0.19[a]
8 mg/kg	3.78 ± 0.69[b]
10 mg/kg	6.78 ± 0.77[c]

*Average of 15 slides from 5 mice.
**Distilled water
[a]Means with different letter notation indicates significant difference at P<0.01.

Table 11. Induction of micronuclei formation by Carvil in bone marrow cells of mice

	*Number of micronucleated polychromatic erythrocytes per thousand**
Control**	0.89 ± 0.19[a]
Carvil	
155.55 mg/kg	2.22 ± 0.39[b]
207.67 mg/kg	3.33 ± 0.67[c]
311.50 mg/kg	3.89 ± 0.19[c]

*Average of 15 slides from 5 mice.
**Distilled water
[a]Means with different letter notation indicates significant difference at P<0.01.

Table 12. Induction of micronuclei formation by Lannate in bone marrow cells of mice

	*Number of micronucleated polychromatic erythrocytes per thousand**
Control**	0.89 ± 0.19[a]
Lannate	
2.25 mg/kg	1.89 ± 0.84[b]
4.50 mg/kg	2.22 ± 0.51[b]
6.75 mg/kg	2.44 ± 0.51[b]

*Average of 15 slides from 5 mice.
**Distilled water
[a]Means with different letter notation indicates significant difference at P<0.01.

cent confidence level. However, higher concentrations (8 and 10 mg/kg) gave statistically significant results. In Carvil, all concentrations used were statistically different from the control but inter-dosage correlation showed that 207.67 mg/kg and 155.55 mg/kg were not statistically different at 99 percent confidence level. Lannate, which showed significant increase in micronuclei formation at 95 percent confidence level, gave statistically insignificant differences between dose-treatments. The observed significant increase in micronuclei formation induced by the insecticides, which strongly supports the results of the host mediated assay, clearly showed that the insecticides used were genotoxic upon metabolism.

Shown in Table 13 is the summary of the effect of three doses of folic acid on Gusathion-induced micronuclei formation. Results show that folic acid reduced the number of micronucleated polychromatic erythrocytes induced by Gusathion A.

The observed reduction can be a consequence of the interaction of the coenzyme from folic acid with formaldehyde. Folic acid is readily transformed to tetrahydrofolate which can trap formaldehyde as a one carbon fragment which could further reduce the effective concentration of formaldehyde which will interact with adenine of DNA. Folic acid, can therefore reduce the mutagenic and clastogenic effect of Gusathion A. It is an antimutagen and an anticlastogen.

Table 13. Effect of folic acid on micronuclei formation as induced by Gusathion A

Gusathion A (Concentration)	*and Folic Acid*	*No. of micronucleated polychromatic erythrocytes per thousand**
10 mg/kg	0 ug/kg	6.78
10 mg/kg	100 ug/kg	0.77
10 mg/kg	200 ug/kg	0.67
10 mg/kg	400 ug/kg	0.44

*An average of 15 slides from 5 mice.

Conclusion

In vitro studies using the Rec assay and the Ames test revealed that Gusathion A, Carvil and Lannate possess DNA damaging potential but are not mutagenic before metabolic activation.

However, all three insecticides exhibited mutagenic effects after metabolic activation. The results of the host-mediated assay inferred the formation of mutagenic metabolite after metabolic activation.

In addition, the insecticides affect the chromatin material of the bone marrow cells of mice. The three insecticides induced the formation of micro-

nucleated polychromatic erythrocytes. The insecticides are mutagenic and clastogenic.

The chromosome damaging effect of Gusathion A was greatly reduced by folic acid which was administered right after the administration of Gusathion A.

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Alumanda M. de la Rosa, Discussant

The two principal reasons for our concern on environmental mutagenesis are 1) an increase in mutation rate in human germ cells which may cause an increase in genetic diseases for future generations, and 2) an increase in mutation rate in somatic cells which may cause an increase in cancer incidence in the present generation. From this perspective, I consider the research study of Dr. Sylianco and coworkers very relevant and important. Looking from quite a different perspective, I believe their work has shown also that short-term and sensitive mutagenicity assays are within the resources of developing countries which enable them to participate in the worldwide endeavour of detecting environmental mutagens.

The present work succeeded in detecting mutagenic activities in formulations of the insecticides gusathion A, carvil and lannate. In addition to detecting the presence of mutagens, it may also be important to identify the ultimate metabolic products of the abovementioned promutagens. Such information will provide an insight into the mechanism of action of the mutagen, and subsequently, into preventive measures to reduce human exposure towards the mutagen.

Another interesting aspect of the research work is to study the exposed population, in this particular case, the agricultural workers. It involves a more complex situation because the agricultural worker is studied within his environment where mutagens other than the insecticides being studied may be at play. Such a study entails the interaction of three areas of discipline, namely, environmental chemistry, biochemistry and genetic toxicology.

Thank you.

Angelita G. Reyes, Discussant

The results of this study point very strongly to the mutagenicity and potential clastogenicity of the 3 insecticides – gusathion A, carvil and lannate. Four different tests prove this beyond doubt. In addition, it has been pointed out that a vitamin, folic acid, is antimutagenic to gusathion A.

The importance of such a study cannot be over emphasized if we think of the Philippines as a developing country having as one of its priorities the intensification of food production. Definitely, one important factor that cannot be overlooked is the use of insecticides or pesticides in agriculture to boost food production. However, these chemicals do harm to the population and to the environment, more specifically to the farmers and fisherman who are gradually exposed to them as they go about their daily chores.

Our problem, therefore, is how to produce more food without jeopardizing the health of the people and without damaging the environment.

While there may be available data on these harmful chemicals, they would remain merely as that, as facts if proper dissemination of this kind of information is

not carried out. National policies on agricultural programs should be formulated with the help of scientists so that studies like these could benefit our people.

If we, as scientists, are here to raise the quality of human life, then let us start by helping educate our fellowmen. Let us make them appreciate the relevance of our experiments and its results. Let us make them understand and let us both *act here* and *now*.