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Evaluation of Bensulide for Mutagenic Properties in Microbial Test System

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The herbicide bensulide was evaluated for its ability to induce point mutations in the T_4 bacteriophage/ *Escherichia coli* B system by measuring the frequency of inducing rII-type mutants of T_4 bacteriophage.

Statistical analyses of the experimental data suggest that bensulide *per se* is not mutagenic in this particular microbial test system.

In a previous article, Andersen *et al.* (1972) reported on the evaluation of 110 herbicides for their ability to induce point mutations in one or more of four different microbial systems. With the exception of inconclusive evidence relating to four herbicides within one test of one of the test systems, the mutation rates of organisms treated with herbicides did not differ significantly from the spontaneous mutation rates.

In this one system, T_4 bacteriophage and *Escherichia coli* B, as the host bacterium, were used to detect chemically induced point mutations caused by selected herbicides. T_4 bacteriophage treated with each of these four herbicides exhibited mutation frequencies of 0.25 to 0.27%. In this particular test the spontaneous mutation rate was 0.11%. These data were subjected to statistical analysis and an increase from 0.11 to 0.25% was shown to be significant at the 5% level for one analysis method and at about the 10% level for a second method of analysis.

One of the herbicides that was associated with these slightly higher mutation rates was bensulide [O,O-diisopropyl phosphorodithioate S-ester with N-(2-mercaptoethyl)benzenesulfonamide]. Because of the serious implications of this finding, an extensive study was undertaken, employing the T_4 bacteriophage/*E. coli* B system to determine if bensulide is indeed mutagenic.

EXPERIMENTAL

Methodology. This test system involved the use of T_4 bacteriophage to detect chemically induced mutations of the rII type (Benzer, 1955) which might be caused by herbicides. The procedure followed was the same as used by Benzer and

Freese (1958) in their study of the induction of mutations with 5-bromouracil. The T_4 bacteriophage and *Escherichia coli* B host are the same as described previously (Andersen *et al.*, 1972). For the purposes of this research program, 240 agar plates were used for each of the experiments to determine the following: spontaneous mutation rate (no test chemical); the mutation rate of a known mutagenic chemical (5-bromouracil, 5-BU); the mutation rate of bensulide; and the mutation rate of domestic table salt (a nonagricultural chemical control). Because of the limited solubility of bensulide in an aqueous medium, it was necessary to use 0.5 ml of acetone to aid in solubilization. In order to determine the effects of acetone on the test system, 240 tubes containing acetone as the test chemical were also evaluated.

Rather than running each of the 240 tube tests on separate days, a series of experiments were performed on different days involving 40 tubes containing no chemical, 40 tubes containing 5-BU, 40 tubes containing acetone, 40 tubes containing bensulide plus acetone, and 40 tubes containing table salt. The same T_4 bacteriophage stock suspension was used throughout all of these experiments.

Source of Herbicides and Other Chemicals. Bensulide was supplied by Stauffer Chemical Company and was the same supply that was used in the earlier study (Andersen *et al.*, 1972). At the initiation of this research program the purity of bensulide was checked and found to be 98–99% pure. 5-Bromouracil and acetone were obtained from P-L Biochemicals, Inc., and Baker and Adamson, Inc., respectively.

Statistical Analyses. The experimental series on rII mutant induction was conducted as a randomized complete block design. Blocks or replications were classified as days. The data were obtained as frequency counts per culture plate. These frequencies consisted of "normal" plaque and rII mu-

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Table I. The Effects of Sodium Chloride, Acetone, Bensulide, and 5-Bromouracil on the Frequency of rII Mutant Plaque Formation on Individual Plates

Additive	rII plaques per plate	Experiment number						
		I	II	III	IV	V	VI	Total
None	0	35	34	28	32	24	29	182
	1	5	6	9	7	15	10	52
	2	0	0	2	1	0	0	3
	3	0	0	0	0	1	1	2
	≥4	0	0	1ᵃ	0	0	0	1
Sodium chloride	0	34	31	28	30	31	28	182
	1	6	9	10	10	9	12	56
	2	0	0	2	0	0	0	2
	3	0	0	0	0	0	0	0
	≥4	0	0	0	0	0	0	0
Acetone	0	34	26	24	27	24	23	158
	1	5	10	11	9	14	14	63
	2	0	4	5	4	2	2	17
	3	1	0	0	0	0	0	1
	≥4	0	0	0	0	0	1	1
Bensulide	0	35	30	30	26	28	29	178
	1	4	9	10	8	9	9	49
	2	1	1	0	3	3	1	9
	3	0	0	0	1	0	1	2
	≥4	0	0	0	2ᵇ	0	0	2
5-Bromouracilᶜ	0	6	5	3	7	2		23
	1	7	7	5	6	7		32
	2	13	6	5	7	5		36
	3	5	7	5	10	9		36
	4	3	4	10	0	7		24
	5	1	4	7	3	4		19
	6	2	3	3	5	4		17
	7	2	1	1	1	1		6
	≥8	1	3ᵈ	1	1	1		7

ᵃ This frequency count was 14 rII plaques. ᵇ This frequency count included 6 and 12 rII plaques. ᶜ This set of experiments was performed independently from the randomized complete block design involving control, NaCl, acetone, and bensulide. ᵈ This frequency count included 15 rII plaques.

Table II. The Effects of Sodium Chloride, Acetone, Bensulide, and 5-Bromouracil on the Spontaneous Mutation Induction Frequency of rII Mutants of T_4 Bacteriophage

Additive	Concentration		Experiment number					
			I	II	III	IV	V	VI
None	...	No. of plaques	3636	3970	5500	4673	5756	5398
		No. of rII plaques	5	6	13	9	18	13
		Mutation freq., %	0.137	0.151	0.236ᵃ	0.192	0.313	0.241
Sodium chloride	1 mg	No. of plaques	3563	4228	5964	4213	3921	4372
		No. of rII plaques	6	9	14	10	9	12
		Mutation freq., %	0.168	0.213	0.235	0.237	0.230	0.274
Acetone	50 μl	No. of plaques	4210	4956	5470	5742	3976	4146
		No. of rII plaques	8	18	21	17	18	22
		Mutation freq., %	0.190	0.363	0.384	0.296	0.453	0.531
Bensulide	10 μl	No. of plaques	3084	3611	3682	4566	3019	3381
		No. of rII plaques	6	11	10	23	15	14
		Mutation freq., %	0.195	0.305	0.272	0.504	0.497	0.414
5-Bromouracil	1 mg	No. of plaques	2525ᵇ	4849ᵇ	4013ᵇ	4028ᵇ	3913ᵇ	
		No. of rII plaques	99	119	139	112	131	
		Mutation freq., %	3.921	2.454ᵃ	3.464	2.780	3.348	

ᵃ In calculation of these frequencies, a single outlier value has been deleted. Outlier values are defined as those frequencies of rII plaque counts which are separated from their nearest neighboring count frequency by three or more null classes. The contribution to the total number of plaques yielded by plates containing these outlier values has also been deleted from the total plaque population. ᵇ These values were obtained in a separate experimental series and are not incorporated into the randomized complete block design as presented here.

tant plaque counts. Each treatment was represented by 240 culture plates subdivided into 40 plates per replication.

The data obtained were subjected to distribution analysis using the χ-square test for Poisson distributed variables. Analysis of variance was also performed with Tukey's test for nonadditivity being applied in order to determine the need for data transform. Comparison of treatment means was accomplished using a sequential variant of the Q method (Snedecor and Cochran, 1967).

RESULTS

The data of all of the experiments are summarized in Tables I and II, with Table II being a condensation of the results presented in Table I.

The data in Table I are the actual frequency counts of the rII mutant plaques with respect to block and treatment. It should be noted that the frequency data obtained for the 5-bromouracil treatment was obtained in a separate experimental series and is not incorporated into the original randomized complete block design (RCB). The χ-square test for Poisson distributed variables was applied independently to each treatment and showed consistency between replications at the 5% level. The exception to this, however, occurred with the 5-bromouracil treated material. In this separate series it was noted that not only was the distribution not that of a Poisson variable, but the mean of the treatment was approximately ten times larger than the pooled treatment means of the RCB experiment.

Table III. The Analysis of Variance of Raw and
Transformed Data for rII Mutation Frequency

Source of variation	Degrees of freedom	Sum of squares	F Ratio
The analysis of variance of the raw percentage data			
Total	23	0.3109	
Treatments	3	0.1323	9.76[a]
Replicates	5	0.1108	4.90[b]
Error	15	0.0678	
Nonadditivity	1	0.0187	4.32[b]
Remainder	14	0.0491	
The analysis of variance of the transformed percentage data $Y = \chi^{-1/2}$			
Total	23	3.1697	
Treatments	3	1.3138	15.79[a]
Replicates	5	1.4399	10.38[a]
Error	15	0.4160	
Nonadditivity	1	0.0086	<1 N.S.[c]
Remainder	14	0.4074	

[a] F Ratio significant at the 1% level. [b] F Ratio significant at the 5% level. [c] N.S., F Ratio is not significant at 5% level or below.

An analysis of variance was performed on the data shown in Table II. The 5-bromouracil treated material was not used in this analysis. It should also be noted that three outlier values were deleted from this analysis. Outlier values are defined in this instance as those frequencies of rII mutant plaque counts which are separated from their nearest neighboring count frequency class by three or more null classes. The contribution to the total number of plaques yielded by plates containing these outlier values was deleted from their respective plaque population. The analysis of variance for the raw percentage data is shown in Table III. An additional degree of freedom was split from the error variance estimate in order to help decide if a transformation was necessary and to suggest a suitable transformation to be used. This application of Tukey's test for nonadditivity showed that a significant nonadditive effect of treatment *vs.* replicates did exist at the 5% level. Further application of this technique suggested a transform value quite close to $\chi^{-1/2}$. This transform value would suggest that the variance of the treatment would be proportional to the cube of the expected treatment mean (Brownlee, 1965).

Analysis of the transformed raw percentages is shown also in Table III. No significant nonadditivity between treatments and replications is demonstrated. A significant difference between treatments is observed at the 1% level. Comparison of the error variance of the RCB experiment with the variance of the separate 5-bromouracil experimental data subjected to the $\chi^{-1/2}$ transform using the two-tailed F test yields no basis for the rejection of the null hypothesis of equality of variance. It is obvious that the 5-bromouracil treated material has a much greater mutation frequency than any of the treatment samples in the RCB experimental series.

Comparison of the treatment means by a sequential variant of the Q method was conducted. The data show no significant difference between the control and sodium chloride treatments. There was also no difference between the bensulide and acetone treatments. However, the bensulide and acetone treatments were significantly greater in rII mutant induction than the control and sodium chloride treatment groups at the 5% level.

DISCUSSION

The herbicide bensulide has been examined in the T_4 bacteriophage/*Escherichia coli* B test system to determine if it is mutagenic. In a previous study, Andersen and coworkers (1972) were unable to detect any conclusive evidence of point mutations induced by any of the 110 herbicides evaluated, with the exception of several herbicides within one test, including bensulide, that produced mutation frequencies that were slightly in excess of the control. The results of the present study have also produced inconclusive evidence for the mutagenicity of bensulide.

When all the evidence in this present series of experiments (Tables I–III) is evaluated and examined in-depth it reveals that the mutation frequencies obtained with acetone and bensulide were slightly higher than the control. Although it cannot unequivocally be stated that bensulide is not mutagenic, it should be noted that acetone alone produced mutation frequencies in excess of the control. It should also be stated that these data demonstrate that bensulide does not significantly increase the mutation frequency over that induced by the acetone diluent system. This observation with acetone strongly presents a need for further research.

We hasten to add that failure to demonstrate mutagenicity in this one specialized system does not prove that the compound is genetically safe. However, this test system has been generally recommended as a first step in the evaluation of chemicals for mutagenic properties and has the advantage of being a rapid and quantitative assessment of a compound. It should also be mentioned that this system is designed to detect mutagens causing base substitutions, deletions or additions, or grosser alterations, but does not detect mutations involving DNA transformation or genetic alterations caused by chromosome breaks or other chromosome changes which are restricted to diploid cells.

In presenting the results of the statistical analyses, outlier values were deleted. We define these outlier values as an unusually high number of rII mutant plaques clustered in one area on one plate, with the remaining plates in the test containing either no mutant plaques or an extremely low number of mutants. One possible explanation of the outlying values, which are a common occurrence in microbial mutation studies, is probably a clonal distribution of mutants in a growing culture. On one plate in Experiment No. 4, with bensulide, we counted 14 rII mutants, while the remaining 39 plates contained one mutant plaque or no mutant plaques. A similar observation was noted on one control plate containing no test chemicals in Experiment No. 3 where 12 rII mutant plaques were observed and the remaining 39 showed either zero or one mutant plaque per plate.

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