NATURE AND MECHANISM OF INDUCTION OF MUTATIONS

Annual Progress Report

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The main results during the past year have been an increased understanding of the pathway by which the azide metabolite is synthesized *in vivo*, the development of a technique for producing an abundant amount of the metabolite *in vitro* and in furthering the understanding of the effect of azide and its metabolite on chromosomes through analyses of sister chromatid exchanges. Some additional information has been obtained on the chemical nature of the metabolite-- specially possible differences of those from barley and from bacteria. Since the final budget was considerably less than requested, research on the chemical nature and identification of the metabolite had to be curtailed.

Considerable progress was made on understanding the nature of azide-induced mutations through continued mapping of mutant alleles at the waxy pollen locus and characterization of the gene products and karyotype analysis of these mutant alleles. Most of this progress had only minimal support from DOE.

Most of the results obtained are contained in the attached reprints and manuscripts and thus the narrative of this report will be considerably reduced. Preliminary results of certain specific experiments are described in the proposal.

A. Metabolite Studies

1. Characterization and identification of the mutagenic metabolite

Crystallization of the bacterial metabolite from methanol-carbon tetrachloride systems was successful and mass spectroscopic data were obtained for the crystallized metabolite. However, the data were difficult to interpret due to the fact that the metabolite is unstable at high temperatures.

Preliminary results suggested that the barley and the bacterial metabolite may be identical. The solubility of both metabolites is similar in polar and organic solvents. Both are highly mutagenic in base substitution strains of *Salmonella*, but neither reverts the frameshift mutants. Recent results indicated that the bacterial metabolite exhibits a positive charge in aqueous solutions. Thus it adheres strongly to Dowex-50 cation exchange column. However, the barley metabolite lacks this property. Therefore, more chemical and physical data should be obtained to account for the similarity between the metabolites if indeed it exists.

The isolation and purification procedures for the *Salmonella* metabolite are described in the attached manuscripts (Owais et al., 1979; Owais et al., submitted to Mutation Research).

2. Metabolite synthesis

As indicated above, considerable effort during the past year has been placed on the pathway by which the metabolite is synthesized *in vivo*. In summary, our results suggest an involvement of L-cysteine biosynthetic enzymes in the activation of azide to the mutagenic metabolite. Apparently, O-acetyl-L-serine, the precursor of L-cysteine, may act *in vivo* as an intermediate in the metabolite biosynthesis. This is

described in the attached manuscript by Owais et al. (Mutation Research, in press).

A major breakthrough has been the *in vitro* conversion of azide to the metabolite using a bacterial cell-free system. In this conversion O-acetyl-L-serine is essential. This was conducted in *S. typhimurium* TA1530 strain and the results are shown in Table 1. This *in vitro* process will enable production of sufficient quantities of the metabolite for purification, chemical characterization, etc.

3. Interaction of metabolite with the DNA

In order to understand the interaction of the azide metabolite with DNA it is necessary to label the metabolite. Tentative data indicate that ^{14}C [U] serine label is incorporated into the metabolite. *Salmonella typhimurium* was grown in the presence of 5×10^{-4} M sodium azide and about 25 μCi ^{14}C L-serine in a 100 ml solution of minimal salts. The supernatant containing the azide metabolite was passed through a Dowex-50W ion exchange chromatography column and was eluted with 0.75 N HCl. The mutagenic fractions were evaporated and applied to a second Dowex-50W column and eluted with 0.1 N-0.5 N HCl gradient. The azide metabolite came off the column at 0.4 N HCl and was then applied to a high pressure liquid chromatograph (HPLC). Fractions were collected every 15 seconds and assayed for both mutagenicity and radioactivity. As shown in Figure 1, the two peaks show good correlation. Also, the specific activity in counts/revertants was measured throughout the purification. This specific activity started to level out as greater purity was obtained. It seems that serine is probably a precursor to the azide metabolite.

Thin layer chromatography (TLC) will be used in the final purification process. Preliminary experiments show chloroform:methanol:t-butylamine (62:28:10) gives the ideal separation, giving an R_f value of about 0.4 for the metabolite.

4. Azide metabolite experiments in barley

a. The effect of bacterial azide metabolite in barley

An important question in our research has been whether the metabolite from *Salmonella* can induce mutations in barley. A very extensive experiment was conducted during the past year which is described in the attached article from the Barley Genetics Newsletter (Hodgdon and Nilan, in press). It is concluded that the bacterial azide metabolite is not mutagenic in barley.

b. Relation between azide and metabolite concentrations and mutation frequencies

A sodium azide concentration experiment with aerated and non-aerated azide treatment was conducted on 'Himalaya' barley as described in the 1979 grant proposal (see attached Abstract, Hodgdon and Nilan, 1980). Samples of treated seeds were assayed

for metabolite level following the method of Owais et al. (1978). Other samples of the same treated seeds were planted for mutation data. These data were taken for all concentrations of azide treatment. Azide metabolite data were determined from aerated azide treatments for the concentrations of 10^{-5} M, 10^{-4} M, 2×10^{-4} M, 5×10^{-4} M, and 10^{-3} M. Metabolite levels for all non-aerated azide treatments up to 2×10^{-3} M were assayed. The mutation data are summarized in Table 2. Mean azide metabolite levels for the various treatments are shown in Table 3.

Azide treatments at the concentration of 10^{-5} M produced no significant mutations, while those of 2×10^{-5} M induced significant mutation frequencies above controls. Non-aerated azide treatments gave significantly lower mutation rates than aerated in the concentration range of 2×10^{-5} M to 5×10^{-4} M. To attain the same mutational effect, about twice the azide concentration was necessary for non-aerated treatment as with aerated treatment. Comparisons between azide concentration with aerated treatment and the next highest concentration non-aerated generally showed no significant difference. These results are probably explained by greater azide uptake under aeration.

A series of correlation coefficients was calculated between the chlorophyll mutation data, both spike and seedling, and the metabolite level data for the azide concentration experiments. T-statistics were calculated for each correlation coefficient.

The best correlations between azide metabolite level and chlorophyll mutation rate, as shown in Table 4, were between the azide concentrations of 10^{-5} M and 5×10^{-4} M. All correlations using data from non-aerated azide treatment up to 2×10^{-3} M were high (probability .01). The correlations between aerated treatments and azide metabolite from 10^{-5} M to 10^{-3} M were not as good due to fewer data points. However, they were still fairly high (probability .05). These data add further evidence for the conversion *in vivo* of azide to a mutagenic metabolite.

B. Sister Chromatid Exchange Studies

The results of the analyses of sister chromatid exchanges in mammalian cells are described in a manuscript by Arenaz and Nilan (submitted to Mutation Research) and in abstracts from the 11th Annual Meeting of the Environmental Mutagen Society (Arenaz and Nilan, 1980a) and the 49th Annual Meeting of the Genetics Society of America (Arenaz and Nilan, 1980b). In summary, it is apparent that neither azide nor the bacterial metabolite has any influence on sister chromatid exchange in human lymphocytes and Chinese hamster cells.

C. Nature of Mutations

Most of the data on understanding the nature of mutations through the analysis of azide-induced mutant alleles at the waxy locus is described in several manuscripts (Hodgdon et al., in press; Nilan et al., 1979;

Nilan et al., in press; Rosichan et al., submitted). In addition, the development of an Ames-type tester system utilizing mutant alleles at the waxy barley locus is also described in these manuscripts. Much of this research is now supported by the National Institute of Environmental Health Sciences and very little of this research was conducted by Department of Energy funds--as mandated following the last review of this contract.

D. Mutagenic Effect of Sodium Azide in Rice

The most recent data on the very high mutagenicity of azide in rice is described in the attached manuscript by Awan et al. (in press).

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Figure 1.

TABLE 1. *IN VITRO* ACTIVATION OF AZIDE BY BACTERIAL CELL HOMOGENATES

Exponentially growing *S. typhimurium* TA1530 cells were homogenized in phosphate buffer pH 7.4, centrifuged, the supernatants then treated with azide and/or O-acetyl-serine. The treated extracts were incubated (37°C) for 10 min., acidified to get rid of free azide and then tested for mutagenicity.

Crude Extract (5 ml)	Azide (N ₃ ⁻) (mM)	O-acetyl-serine (mM)	<i>His</i> ⁺ TA1530 revertants
heated (100°C)	2	2	4
heated (100°C)	-	-	5
native (37°C)	2	-	5
native (37°C)	-	2	15
native (37°C)	2	2	TMTIC*

*Too many to count.

TABLE 2. EFFECT OF AERATION AND NON-AERATION ON AZIDE MUTAGENICITY IN

HORDEUM VULGARE

Conc. of Azide	Aeration	Spikes Scored	# Seedlings Scored	Seedlings/ Spike	% Spike Mut.	% Seedling Mut.
10^{-5} M	+	1304	48,537	37.22	0.37	4.77
10^{-5} M	-	1386	51,234	36.97	0.35	3.81
2×10^{-5} M	+	1216	44,476	36.60	0.60	6.89
2×10^{-5} M	-	1354	52,312	38.64	0.31	3.29
5×10^{-5} M	+	1277	42,172	33.03	1.80	22.54
5×10^{-5} M	-	1328	47,748	35.95	0.04	6.03
10^{-4} M	+	1230	32,168	26.15	3.03	33.09
10^{-4} M	-	1351	46,994	34.78	0.89	15.04
2×10^{-4} M	+	1300	32,165	23.81	3.70	39.42
2×10^{-4} M	-	1341	41,715	31.11	2.24	22.94
5×10^{-4} M	+	1258	27,062	21.51	5.66	43.51
5×10^{-4} M	-	1213	32,005	26.36	3.49	33.35
10^{-3} M	+	1257	29,155	23.19	4.88	40.81
10^{-3} M	-	1285	31,489	24.51	4.06	37.44
2×10^{-3} M	+	1206	28,227	23.41	4.83	38.89
2×10^{-3} M	-	1196	26,146	21.86	4.83	40.61
5×10^{-3} M	-	1100	24,304	22.09	4.86	41.92
Control		947	36,370	38.00	0.28	2.30

TABLE 3. METABOLITE PRODUCTION IN AZIDE-TREATED BARLEY EMBRYOS

Azide Concentration	Aeration	Azide Metabolite level (rev/.1 ml)
10^{-5} M	+	53
10^{-5} M	-	9
2×10^{-5} M	-	3
5×10^{-5} M	-	58
10^{-4} M	+	124
10^{-4} M	-	81
2×10^{-4} M	+	198
2×10^{-4} M	-	91
5×10^{-4} M	+	223
5×10^{-4} M	-	163
10^{-3} M	+	300
10^{-3} M	-	131
2×10^{-3} M	-	151

TABLE 4. CORRELATIONS BETWEEN AZIDE METABOLITE LEVEL AND
CHLOROPHYLL MUTATION FREQUENCIES

Experiment	Correlation Coefficient	T (P > .05)
Azide aerated		
Spike mutation rate	.95	.1 to 0.5
Azide aerated		
Seedling mutation rate	.918	.05
Azide non-aerated		
Spike mutation rate	.95	.01
Azide non-aerated		
Seedling mutation rate	.91	.02