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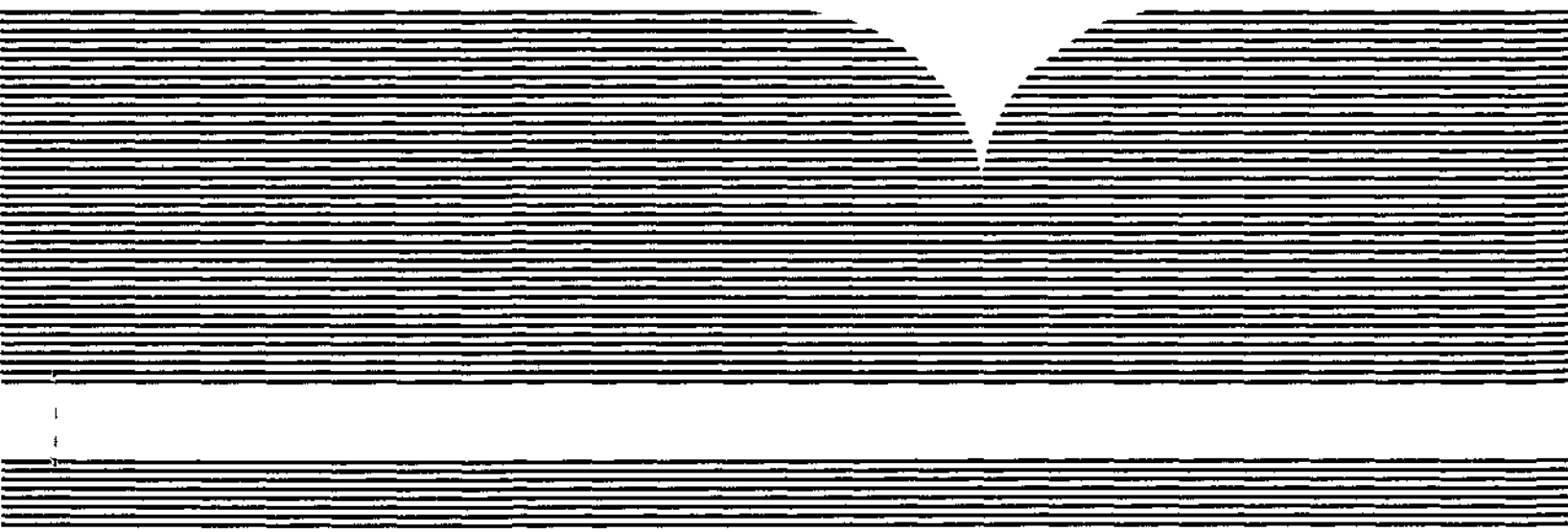
Evaluation of Carcinogenic, Teratogenic, and Mutagenic Activities of Selected Pesticides and Industrial Chemicals. Volume III: Mutagenic Study

Bionetics Research Laboratories
Bethesda, Maryland

August 1968



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Initial Planning - Dr. A. J. Pallotta and Dr. E. Ross Hart, BRL

16. Abstracts Carbamates and structurally related compounds were studied for their effects on growth, morphology, mutation rates, and the DNA of B. subtilis (168i). The bacteria were inoculated into Penassay broth containing various concentrations of each compound. Visible turbidity was recorded after incubation at 37°C for 18 hrs. Turbidimetric readings were obtained after inoculation and incubation with aeration at 37°C for 6 hrs. Insoluble compounds could not be studied by this technique. Of those studied, growth inhibition was greatest by substitution of chlorine, branched chains, and/or hydroxy radicals in the amino portion of ethyl-N-hydroxy carbamate. A marked decrease in inhibition resulted when the hydroxy moiety was attached to the ethyl portion. Morphological changes were phenotypic rather than genetic and unrelated to amino acid inbalance, to interference of thymidine synthesis, or to purine or purine (cont.)

17. Key Words and Document Analysis. 17a. Descriptors

Bioassay	Growth
Mutagens	Morphology
Pesticides	Carcinogens
Carbamates	Environments
Thymidines	Mutations
Purines	
Bacillus subtilis	
Salmonella typhimurium	
Colorimetric tests	

17b. Identifiers/Open-Ended Terms

Teratogens	Mutagenicity
Mutagenesis	Teratogenicity
Chemical structure	Pesticide Residue
Deoxyribonucleic acid inactivation	Mutations

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16. Abstracts (continued)

nucleotide metabolism. Thus, carbamates affect many different metabolic sites in these biological systems. No carbamate proved mutagenic to the indole locus of B. subtilis; however, B-chloroethyl-N-hydroxy carbamate appears slightly mutagenic (3.6X) at the histidine locus of B. subtilis Sb 25. DNA inactivation capabilities of some compounds were negative at their "safe" levels. While N-OH carbamate was not mutagenic, it was able to inactivate DNA. This may be due to conversion to ethyl carbamate, which is unable to inactivate DNA.

EVALUATION OF THE CARCINOGENIC, TERATOGENIC AND MUTAGENIC ACTIVITY OF SELECTED PESTICIDES & INDUSTRIAL CHEMICALS

VOLUME III
EVALUATION OF THE MUTAGENIC ACTIVITY OF SELECTED PESTICIDES AND INDUSTRIAL CHEMICALS IN BACTERIA

SUBMITTED UNDER
CONTRACTS PH43-64-57 and PH43-67-735
WITH THE
NATIONAL CANCER INSTITUTE

BIONETICS RESEARCH LABORATORIES, INC.
7300 Pearl Street
Bethesda, Maryland 20014

VOLUME III

TABLE OF CONTENTS

LISTING OF TABLES/FIGURES

INTRODUCTION

There is strong scientific evidence that mutagenic events are an important part of the etiology of cancer. Bacteria are an ideal system in which to study mutagenic effects because of their defined genetic systems and their ease of handling. The purpose of this part of the program was to investigate the ability of pesticides and related compounds to effect genetic and/or physiologic changes in bacteria.

Many of the commonly used herbicides and pesticides are carbamates and the major emphasis of study by these investigations was on compounds of this class. Ethyl carbamate (urethane) produces a variety of effects in many biological systems and has close homologues which show different spectra of activity, depending upon the phenomena investigated. Higher homologues are more effective as mitotic poisons for sea urchin eggs[1], more toxic to mice[2], but less effective as leucopenic[2] and carcinogenic[3,4] agents. With respect to bacteria, the carbamates can be toxic whereas some act as weak mutagenic agents[5]. Of particular interest is the report that ethyl-N-hydroxy carbamate, previously reported to be a carcinogen for skin or lung of mice[3], is a metabolite of ethyl carbamate and may be the active form of this compound that produces a carcinogenic action in rats, rabbits and man[6].

In these studies, carbamates and compounds structurally related to them were studied for their effects on growth, through visual and colorimetric sensitivity tests, morphology, mutation rates by standard analysis, revised standard analysis, plate mutation analysis and reconstruction analysis, and on DNA of bacteria. Table I lists these compounds studied.

The specific studies are discussed in the following sections.

TABLE I

COMPOUNDS STUDIED FOR MUTAGENESIS

SOURCE	CODE #	COMPOUND NAME
Outside	032	2,4-D Isooctyl Ester
	033	Methyl carbamate
	034	Ethyl carbamate
	035	n-Propyl carbamate
	036	iso-Propyl carbamate
	037	n-Butyl carbamate
	038	Allyl carbamate
	039	B-Chloroethyl carbamate
	040	Ethyl-N-acetyl carbamate
	041	Ethyl-N-hydroxy carbamate
	042	iso-Butyl carbamate
	043	sec-Butyl carbamate
	044	Methallyl carbamate
	045	Phenyl carbamate
	046	Benzyl carbamate
	047	Sevin
	048	IPC
	049	Sodium diethyl dithiocarbamate
	051	Zineb
	053	Diuron
	058	Thiram
	059	Monuron
	062	Ferbam
	063	2,4-D
	069	Captax
	071	Phenylisothiocyanate
	078	Ethylene imine
	079	Nabam
	149	Zectran
	150	CIPC
	152	Isolan
	154	B-Hydroxyethyl hydrazine
	157	Ethylene urea
	194	Acetohydroxamic acid
	227	Methoxy ethyl carbamate
	228	Methoxyethyl dimethylol carbamate
		Urea
		Trypan blue

TABLE I (CONT.)

COMPOUNDS STUDIED FOR MUTAGENESIS

SOURCE	COMPOUND NAME
Synthesized (BRL)	Amyl carbamate
	Hexyl carbamate
	B-Hyroxy ethyl carbamate
	3-Chloroethyl-N-hydroxy carbamate
	Propyl-N-hydroxy carbamate
	3-Chloropropyl-N-hydroxy carbamate
	Butyl-N-hydroxy carbamate
	Ethyl-N-cyanoacetyl carbamate
	Ethyl-N-(B-hydroxyethyl) carbamate
	Ethyl-N-methoxy carbamate
	Naphthyl-N-methyl carbamate
	Ethyl-N-(O-carbethoxy) carbamate
	Ethyl-N-methyl-N-hydroxy carbamate
	Butyl-N-methyl-N-hydroxy carbamate
	Amyl-N-methyl-N-hydroxy carbamate
	Diethyl bicarbamate
	Pyramate
	Dimethyl ethyl carbamate
	N-Methyl-N-hydroxy butyl carbamate
	N-Methyl-N-hydroxy amyl carbamate
	B-Chloroethyl-N-hydroxy carbamate
	Ethyl-N-(O-carbethoxy) carbamate
	N-Methyl-N-hydroxy methyl carbamate
	N-Methyl-N-hydroxy-B-chloroethyl carbamate
	N-Methyl-N-hydroxy propyl carbamate
	N-Methyl-N-hydroxy-3-chloropropyl carbamate
	N-Methyl-N-hydroxy allyl carbamate
	N-Methyl-N-hydroxy butyl carbamate
	N-Methyl-N-hydroxy aryl carbamate
	N-Methyl-N-hydroxy hexyl carbamate
	N-methyl-N-hydroxy phenyl carbamate
	N-methyl-N-hydroxy benzyl carbamate
	N-(B-methoxyethyl) acetamide
	N-Acetyl ethanolamine
	Hydroxylamine HCl
	Methoxyamine HCl
	N-(B-hydroxyethyl) formamide
	N-Hydroxyethyl hydrazine
	Acetyl urea
	Hydroxy urea
	3-Bis-(2-Hydroxyethyl) urea
	Propylene imine

TABLE I (CONT.)

COMPOUNDS STUDIED FOR MUTAGENESIS

B-hydroxy ethylene imine
Ethyl-N-carbamoyl aziridine
n-Propyl-N-carbamoyl aziridine
n-Butyl-N-carbamoyl aziridine
N-(Propionyl) aziridine
N-(Butyryl) aziridine

GROWTH STUDIES

Sensitivity Test - Visual

The degree of growth inhibition for Bacillus subtilis, strain 168$\bar{I}$ (indole requiring), was scored by determining the level of various carbamates and structurally related compounds that caused complete inhibition of growth. Most of these studies were made on a strain of B. subtilis obtained from Dr. S. Zamenhof (University of California). The bacteria (10^3-10^4 cells/ml, obtained from six-hour growth cultures) were inoculated into Penassay broth (Difco) containing various concentrations of selected compounds. Visible turbidities were recorded in each case after incubation at 37°C for 18 hours. The results are shown in Table II.

Using ethyl carbamate as a reference, it appears that an increase in growth inhibition is obtained when the ethyl radical is replaced by a larger radical having a branched chain, or when an hydroxy group is added to the carboamino portion of the molecule. Growth inhibition was further increased by the addition of a chlorine atom to the carboethoxy portion of the ethyl-N-hydroxy molecule. Indeed, the simpler compounds that bear a structual similarity to the amino portion of ethyl-N-hydroxy carbamate were found to be extremely inhibitory to growth. On the other hand the addition of a hydroxy moiety to the same portion in the ethyl carbamate molecule caused a marked decrease in inhibition, emphasizing the importance of the location of the hydroxy radical.

Because several compounds presented solubility problems, the solvents dimethyl sulfoxide and propylene glycol were tried; but they were found not suitable for study with B. subtilis. Dimethyl sulfoxide is extremely toxic to these bacteria and propylene glycol induces the formation of long chains of cells.

The insoluble compounds were also tested in solid media but the conditions were found to be inadequate for study.

A commercial "Sevin Sprayable" preparation (Union Carbide and Chemical Corporation) was tested in agar as was its vehicle. The

TABLE II

Sensitivity of *B. subtilis* (168 $\bar{1}$) to Selected Compounds

Selected Compounds	Percent Compound Required For Complete Inhibition of Growth
Hydroxylamine hydrochloride	0.0035
β-Hydroxy ethyl hydrazine	0.0205
Methoxyamine	0.085
N-methylhydroxylamine	<0.1
Amyl carbamate	>0.1*
2-Chloroethyl-N-hydroxy carbamate	0.13
n-Propyl-N-hydroxy carbamate	0.20
n-Butyl-N-hydroxy carbamate	0.20
Phenyl carbamate	0.25
Amyl carbamate	0.30
Zectran	>0.5*
Sevin	>0.5*
iso-Butyl carbamate	0.80
sec-Butyl carbamate	0.85
n-Butyl carbamate	0.85
IPC	>1.0*
CIPC	>1.0*
Methallyl carbamate	1.5
Acetylurea	>1.5*
n-Propyl carbamate	1.8
Ethyl-N-hydroxy carbamate	2.1
iso-Propyl carbamate	2.5
β-Chloroethyl carbamate	2.5
Ethyl-N-methyl-N-hydroxy carbamate	2.5
Allyl carbamate	2.9
Ethyl-N-methoxy carbamate	4.5
Ethyl carbamate	4.9

TABLE II (cont'd.)

Selected Compounds	Percent Compound Required For Complete Inhibition of Growth
N- (β-Hydroxyethyl)ethyl carbamate	6.2
N-Acetyl-ethyl carbamate	7.0
Methyl carbamate	8.0
N-(2-Methoxyethyl) acetamide	16.8
β-Hydroxyethyl carbamate	19.2
N-Acetyl ethanolamine	19.5
N-(2-Hydroxyethyl) formamide	22.0

* Solubility problems prohibited testing at higher concentrations.

inhibition that was noted with the preparation was matched by the inhibition induced by the vehicle alone.

Therefore, because of problems of solubility, no conclusive results could be obtained with some compounds in this biological system.

Sensitivity Test - Colorimetric

Because of the subjectivity of the visual sensitivity method, described above, a revision in procedure was attempted which might be expected to give more meaningful results. A colorimetric method which had been used by others to study bacterial sensitivity to drugs [7,8] seemed appropriate to this study. By the use of a Bausch and Lomb "Spectronic 20" photoelectric colorimeter, turbidimetric readings obtained at 630mμ were plotted against total cell counts of B. subtilis 168I . The standard calibration curve was plotted as a straight line showing that it is possible to relate optical density to such cell populations. Tubes containing various concentrations of compounds were inoculated, incubated with aeration at 37°C, and turbidity measurements were read photometrically at six hours. The values obtained, expressed as percentage of control growth, were plotted on a probit scale against the logarithm of compound concentration. From the graph obtained, one can compare and quantitate the degree of bacterial growth inhibition induced by various compounds. In addition, the slope of the straight line may be obtained[9] by use of the following formula:

$$\frac{IC\ 16/IC\ 50 + IC\ 50/IC\ 84}{2}$$

The slopes may then be compared. Differences in slope are believed to indicate differences in the mechanism of action of the compound and similar slopes may (or may not) reflect similar mechanisms of action. Table III lists the compounds studied in this manner, along with data obtained from the graphs.

While the colorimetric study has not been carried through for all compounds studied for sensitivity by the visual method, it shows that this system may be used as a means of comparing compound toxicities, as a practical guide for determining concentration levels for standard mutation analyses, and as a monitor of modes of action by comparison of slope measurements. The inhibition data are comparable

but more refined than those obtained by the visual sensitivity method.

It may be seen in Table III that the slope measurements obtained for ethyl carbamate and many of its derivatives, with the exception of ethyl-N-hydroxy carbamate, are quite similar (i.e., 1.44-1.63); however, the slopes obtained for other carbamates and the carbamate pesticides are not. The slopes obtained with urea type compounds were all similar and comparable in range to the ethyl carbamate compounds. While similar slopes do not necessarily mean similar modes of action for the compounds on B. subtilis 168 $\bar{i}$, a change in the mode of action may have occurred, as suggested by Treffers[7]. As was seen in our visual sensitivity tests, the addition of a hydroxy moiety to the amino portion of the basic molecule caused greater inhibition of growth.

TABLE III

Sensitivity of *B. subtilis* 168ī to Selected Compounds

Selected Compounds	IC50*	Slope
B-Hydroxyethyl hydrazine	0.0137	1.04
Hydroxylamine	0.0037	1.04
Methoxyamine	0.0070	1.19
B-Hydroxyethyl carbamate	9.6	1.31
3 bis-(2-hydroxy ethyl urea)	6.6	1.36
Ethyl carbamate	2.09	1.40
Dimethylol ethyl carbamate	0.01	1.44
Hydroxy urea	0.03	1.47
Ethylene urea	3.8	1.55
Methoxy ethyl carbamate	5.8	1.55
B-chloroethyl-N-hydroxy carbamate	0.05	1.63
Urea	4.5	1.67
Maleic hydrazide	0.49	2.10
n-Butyl-N-hydroxy carbamate	0.0610	2.16
Isobutyl carbamate	0.43	2.20
Zectran	0.012	2.24
n-Propyl-N-hydroxy carbamate	0.092	2.46
Ethyl-N-hydroxy carbamate	0.16	2.48
Acetohydroxamic acid	0.490	3.53
CIPC	0.005	3.59
Sevin	0.07	3.89

*Concentration that permits 50 percent of control growth.

MORPHOLOGY OF *B. SUBTILIS* 168ī:

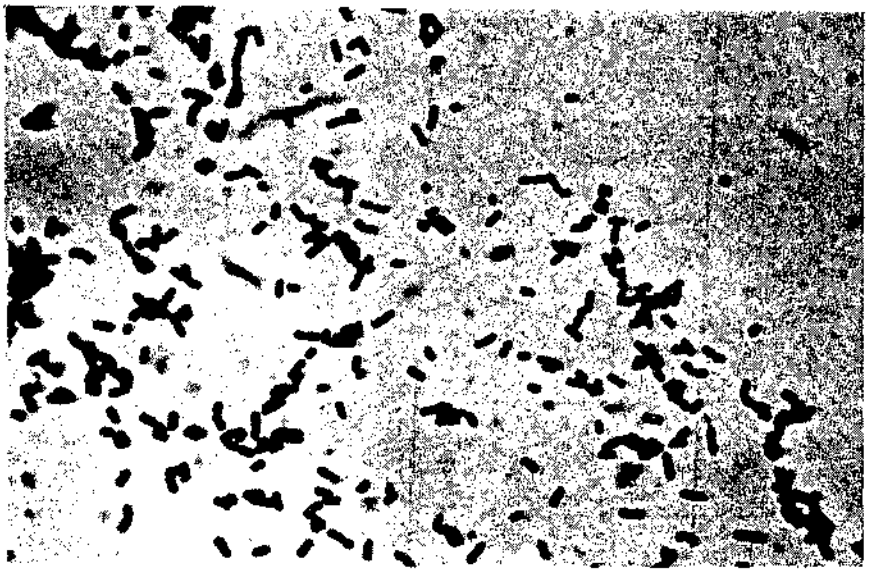
The growth inhibition induced by carbamate compounds was accompanied by a change in cell morphology. The bacteria form extended chains of cells when grown in the presence of high carbamate concentrations; they continue to divide but do not separate (Figure 1). This change is not genetic but phenotypic. When transferred to control medium, the chains break and give rise to normal cell units. Under control conditions, *B. subtilis* formed chains during the early exponential phase of growth which usually broke down well before the stationary phase. Chains varied in length; in extreme cases, chains containing up to 600 cells were seen. Attempts were made to reverse the pattern by the addition of various supplements to ethyl carbamate-containing media, but all were unsuccessful. The addition of Lankford's glucose-phosphate complex (GP factor) which has been reported to initiate early cell division in various *Bacillus* strains[10] had no effect neither did the addition of 2,6-diaminopurine, contrary to the results obtained with *E. coli* strains inhibited by ethyl carbamate[11]. This may indicate that various strains of bacteria respond differently to the presence of carbamates, and may be a further reflection of the fact that carbamates are able to affect many different metabolic sites in biological systems.

In our brief study with a thymine requiring strain of *E. coli*, ethyl carbamate and ethyl N-hydroxy carbamate induced elongated forms characteristic of thymine starvation, but thymidine offset this pattern. Supplements of thymine, thymidine and thymidine-monophosphate were ineffective in diminishing the extent of chain formation in *B. subtilis*. Further, the phenotypic effect of carbamates on *B. subtilis* seems unrelated to amino acid imbalance, to interference of thymidine synthesis or to interference of purine or purine nucleotide metabolism.

The inability of thymine to counteract chain formation is comparable to its inability to reverse the carbamate effect on mitosis in sea urchin eggs[1].

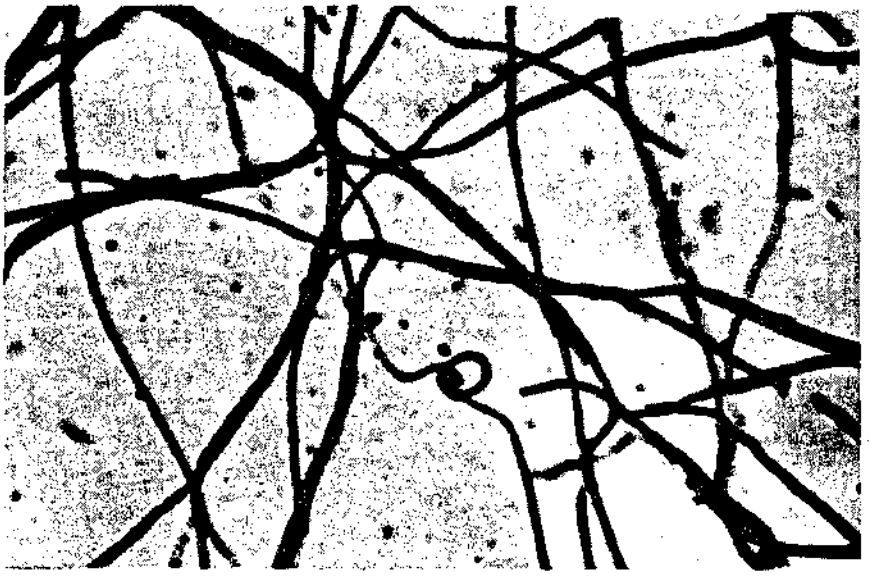
FIGURE 1

B. Subtilis Control (Penassay Broth)



B. Subtilis Experimental

(Penassay Broth + 4.5% Ethyl Carbamate)



MUTATION STUDIES

Back Mutation - Standard Analysis

The mutagenic effect of compounds on bacteria was studied by determining the back mutation rate of amino acid-requiring strains to independence of amino acid supplement. Six control flasks and six experimental flasks, each inoculated with a colony of the bacteria (obtained from an 18 hour incubated Penassay agar plate), were incubated and aerated for 18 hours at 37° C. The concentrations of compounds chosen for the experiments, in most cases, inhibited growth and induced a tendency for long chain formation. After the incubation period, the cells in each independent culture were harvested by centrifugation, washed twice in 0.85% NaCl and resuspended in 3-5 ml of saline. Mutant counts were determined on Spizizen minimal agar[12] and total counts on Pennassay agar. The plates were examined after 48-72 hours incubation at 37°C. for the presence of reverse mutants. From the median number of mutants in these independent cultures and the median number of cells plated, the mutation rate for each condition was calculated using the Lea and Coulson formulae and tables[13]. The results obtained with *B. subtilis* 168ī are listed in Table IV.

Of all the carbamates tested, only ethyl N-hydroxy carbamate appeared mutagenic. It was noted, however, that the compound had a marked effect on viability of the cultures and further scrutiny of the data led to the discovery that the apparent mutagenicity was erroneous. In our study, no carbamate was mutagenic at the indole locus of *B. subtilis*.

TABLE IV

THE EFFECT OF CARBAMATES ON THE MUTATION RATE OF B. SUBTILIS 168$\bar{1}$

Carbamate	%	Mutation Rate*		Factor of Increase Over Control
		Control	Experimental	
Methyl	5.0	11.4	20.0	1.75
	6.0	3.9	1.9	0.49
Ethyl	2.5	3.5	2.2	0.63
	3.0	27.6	5.3	0.19
Allyl	2.0	5.0	8.0	1.60
n-Propyl	1.0	8.0	7.0	0.88
	1.5	11.4	4.5	0.39
Isobutyl	0.6	7.0	10.0	1.43
Amyl	0.1	6.2	6.8	1.10
	0.2	7.8	10.1	1.30
Formaldehyde-Condensation Products				
Dimethylolethyl	0.015	13.0	11.0	0.9
Methoxy ethyl	9.0	34.0	23.0	0.7
	9.5	34.0	27.0	0.8

*Number of mutations per bacterium per generation x 10^{-9}

TABLE IV
(cont.)

THE EFFECT OF CARBAMATES ON THE MUTATION RATE OF B. SUBTILIS 168ī

Carbamate (OH Substituted)	%	Mutation Rate*		Factor of Increase Over Control
		Control	Experimental	
Ethyl-N-hydroxy	0.025	28.0	30.0	1.07
	0.10	6.4	24.0	3.75
	0.150	9.5	230.0	24.00
	0.250	6.7	500.0	75.00
	1.0	8.0	2060.0	250.00
β-Hydroxy-ethyl	10.0	13.5	21.3	1.6
	10.5	14.1	45.3	3.2
n-Propyl-N-hydroxy	0.026	12.0	13.0	1.09
	0.10	12.3	27.4	2.42
	0.125	4.7	4.5	0.96
	0.150	4.7	8.6	1.83
n-Butyl-N-hydroxy	0.075	7.2	8.8	1.22
	0.105	7.2	4.1	0.57
2-Chloroethyl-N-hydroxy	0.05	17.3	3.4	0.19
	0.10	7.1	10.5	1.48
	0.125	6.4	17.6	2.75
	0.15	6.4	14.1	2.21

*Number of mutations per bacterium per generation x 10^{-9}

TABLE IV
(cont.)

THE EFFECT OF CARBAMATES ON THE MUTATION RATE OF *B. SUBTILIS* 168I

Pesticide	%	Mutation Rate*		Factor of Increase Over Control
		Control	Experimental	
IPC	0.01	8.0	9.0	1.1
	0.025	8.0	36.0	4.5
	0.2	4.5	7.0	1.55
	0.5	7.4	17.0	2.30
	0.66	5.75	48.9	8.5
	0.77	6.2	27.6	4.5
	0.88	7.6	21.8	2.87
	1.0	5.4	6.1	1.13
CIPC	0.006	17.0	39.0	2.3
	0.007	17.0	11.0	0.65
	0.66	9.3	20.3	2.18
	0.75	1.8	2.4	1.33
	0.86	3.3	2.8	0.86
	0.88	7.55	23.3	3.1
	0.89	5.4	6.5	1.2
	1.0	5.5	8.8	1.6
Sevin	0.04	10.0	10.0	1.0
	0.07	10.0	13.0	1.3
	0.15	6.3	3.6	0.57
	0.20	7.8	10.9	1.4
Zectran	0.01	12.0	35.0	3.0
	0.60	11.0	24.0	2.18
	0.80	11.0	19.7	1.79

*Number of mutations per bacterium per generation x 10^{-9}

Revision of Standard Mutation Analysis

In the standard mutation analysis the mutation rate was calculated on the basis of the number of cells plated initially on the synthetic medium. Further study showed that calculations should be based on the number of cells present after 24 hours incubation. It had been previously assumed from experiments with control levels of plating (10^8 cells/plate) that if additional divisions took place on the synthetic agar plate, they would be few (2-3) and the same for all plates. Attempts were made to try to match the number of plated cells of the controls with those of the experimental, by taking into account the viability and degree of inhibition characteristic of the compound studied. This was not always possible. It was believed, however, that dilution of the control culture to the level of the experimental would render a population too low for mutant appearance and estimation of control mutation rates.

Further experiments were performed to determine whether mutant expression would be altered by background populations of different titers. This was done by plating a known number of mutants (200; approximately 20 x the number usually scored in a mutation experiment) on synthetic agar plates containing 4×10^8; 5×10^7; 3×10^6; 5×10^5; 1×10^4 parent cells. It was found that the number of parent cells plated did have a slight effect on the time required for mutant expression. Those with the highest background appeared last; those with the lowest appeared first. In most cases all appeared within the 48-hour period used for scoring mutant numbers. The plates containing the highest titer of parent cells showed 1/4 less mutants than the others, but this concentration of parent cells was rarely used in an experiment. The experimental conditions, therefore, allowed for true mutant expression.

The next item checked, to see if there was a sizeable change in parent cell density, was the number of cells present after 24 hours' incubation on synthetic agar. The unexpected discovery was that the parent cells are able to undergo 1-12 divisions on the plate after

24 hours' incubation, depending on the number of cells plated. Since the mutants are not scored until after 48 hours of incubation, the number of cells present on the plate after 24 hours' incubation should be used to calculate the mutation rate/bacterium/generation. This information was obtained by plating various concentrations of control cells on synthetic and Penassay agar and incubating for 24 hours at 37° C. The initial viable counts obtained from the Penassay plates were recorded and compared with viable counts on Penassay-agar obtained by "washing" the surface of 24-hour old synthetic plates with 10 ml of saline to remove all the cells. This procedure also allowed for the estimation of the number of cell divisions that had taken place. Knowing the mean number of mutants scored for each level, it was possible to compare the mutation rates one would obtain by comparison of the initial median number of bacteria plated with those obtained on the basis of the median "wash off" number of bacteria present after 24 hours. It can be seen in Table V that the "wash off" values are valid, whereas those based on initial cell counts are not.

TABLE V

INFLUENCE OF DENSITY OF PARENT CELLS
ON CALCULATION OF MUTATION RATE

Cell Number			Mutation Rate*	
Initial	After 24 Hrs.	Divisions	Based on Initial No.	Based on No. After 24 Hrs.
9.6×10^8	3.8×10^9	2	5	1.2
9.6×10^7	1.5×10^9	4	20	1.2
9.6×10^6	2.4×10^9	8	159	0.7
9.6×10^5	1.9×10^9	11	2146	1.1

* Number of mutations/bacterium per generation 10^9

Where viability is good and growth in the experimental and control cultures is comparable, the mutation rates obtained may be compared for the "Factor of Increase Over Control" value as used in Table IV. However, where there is a difference in the number of cells plated initially (due to poor viability or poor growth in experimental flasks) it is possible that the cell numbers, while not comparable at initial plating, are comparable after 24 hours. Thus, if the mutation rate is calculated on the basis of the initial cell populations and the number of mutants is scored after 48 hours, the "Factor of Increase Over Control" value would be misleading and give an indication of an increase or a "mutagenic effect". In light of these findings, ethyl N-hydroxy carbamate and ß-chloroethyl-N-hydroxy carbamate were re-examined and the results are presented in Table VI. It may be seen that these carbamates are not mutagenic at the trytophane or indole locus of B. subtilis; however, ß-chloroethyl-N-hydroxy carbamate appears slightly mutagenic (3.6X) at the histidine locus of B. subtilis Sb 25*.

*Strain requires indole and histidine for growth.

TABLE VI

Comparison of Experimental Mutation Rate Analyses
of *B. subtilis*

Exp.	Carbamate	Conc. %	Strain	Locus*	Initial No. Bacteria	Mutation Rate**	No. Bacteria After 24 Hrs.	Mutation Rate**
A	Control	----	168$\bar{i}$	i	9.0×10^8	4.0	4.8×10^9	0.75
	Ethyl-N-hydroxy	0.25	168$\bar{i}$	i	4.6×10^8	6.6	3.1×10^9	1.02
B	Control	----	168$\bar{i}$	i	9.0×10^8	6.3	7.6×10^9	0.75
	Ethyl-N-hydroxy	1.0	168$\bar{i}$	i	5.4×10^7	46.3	4.4×10^9	0.57
C	Control	----	Sb25	i	1.2×10^9	2.6	6.5×10^9	0.91
	Ethyl-N-hydroxy	0.25	Sb25	i	9.2×10^8	3.6	4.2×10^9	0.78
	Control	----	Sb25	h	1.2×10^9	28.2	5.5×10^9	5.95
	Ethyl-N-hydroxy	0.25	Sb25	h	9.2×10^8	44.9	4.9×10^9	8.40
D	Control	----	Sb25	i	6.5×10^8	6.4	5.0×10^9	0.84
	Ethyl-N-hydroxy	1.0	Sb25	i	1.4×10^6	150.0	2.3×10^9	0.91
	Control	----	Sb25	h	6.5×10^8	45.6	4.4×10^9	6.75
		1.0	Sb25	h	1.4×10^6	4507.0	2.6×10^9	2.47
E	Control	----	Sb25	i	1.7×10^9	3.6	1.0×10^9	0.6
	β-chloroethyl-N-hydroxy	0.24	Sb25	i	4.8×10^8	7.7	3.8×10^9	1.0
	Control	----	Sb25	h	1.7×10^9	19.8	4.6×10^9	7.3
		0.24	Sb25	h	4.8×10^8	240.0	4.4×10^9	26.2

*i = indole requiring, h = histidine requiring

**Per bacterium per generation x 10^9

In light of the results obtained with B. subtilis, some additional assays were carried out with a strain of Salmonella typhimurium (C527), showing a positive response on the plate assay. This was done to determine whether a positive response on the plate assay was meaningful and if ethyl N-hydroxy carbamate's mutagenicity for S. typhimurium could be quantitated by use of the revised standard mutation analysis. It was found that by growing S. typhimurium in the presence of 0.2% ethyl N-hydroxy carbamate, the mutation rate was increased ten-fold. To further check whether or not the compounds ethyl N-hydroxy carbamate and B-chloroethyl N-hydroxy carbamate were mutagenic, they were subjected to reconstruction experiments, as described below in the section devoted to these studies.

Plate Mutation Analysis

A relatively quick and simple method was used for the screening of a large number of compounds for mutagenic properties. The system, suggested by Dr. Bruce Ames of the National Institute of Allergy and Metabolic Diseases, consists essentially of placing test compounds on a "lawn" of nutritionally deficient bacteria and observing for the appearance of back mutants. Several histidine-requiring strains of S. typhimurium of known genetic defect[14] were used. It was hoped that differential responses to test compounds would give a clue to their mode of action.

The procedure is as follows: The strains are grown on Penassay plates. A single colony is inoculated into Spizizen minimal medium containing casein hydrolysate, glucose and histidine, incubated for four hours with aeration at 37° C. One tenth of a milliliter of this culture is added to 2.0 ml of soft agar containing yeast, glucose and histidine, being held at 45-50°C in a water bath. The 2.0 ml cell suspension is poured on Spizizen Minimal Agar plates containing yeast and glucose. After the overlay hardens, the test compound (crystals or liquid) is dropped in the center of the plate.

Control plates are left untreated. All plates are incubated at 37°C for 48 hours or more. A compound is considered "mutagenic" when large histidine independent colonies are concentrated around or just beyond the zone of inhibition caused by the compound. This is the arrangement of back mutant colonies observed after treatment with reference compounds of known strong mutagenic activity. Colonies randomly scattered on the "lawn" are considered to be spontaneous mutations, the number of which is fairly constant for a particular strain.

Table VII describes the type of histidine requiring Salmonella typhimurium strains used in the screening tests and their reaction to the known mutagenic compounds, B-propiolactone and diethyl sulfate.

TABLE VII

RESPONSE OF STRAINS TO MUTAGENIC COMPOUNDS*

Strain** Type**	C207 Frame Shift	C117 Ochre	D130 Mis-sense	C151 Ochre	C527 Amber	G46 Ochre
Diethyl sulfate	-	++	+++	+	+++	++++
B-Propiolactone	-	+++	+++	-	+++	++

*Back mutants/plate: - = 0, ± = <50 questionable, + = <50
++ = >50<400, +++ = >400<2000, ++++ = >2000

**Dr. Ames' nomenclature and classification.

The response of the strains to any test compound may then be compared to the response obtained with these reference compounds, and information can be derived with respect to its type of mutagenic action. For example, should a test compound induce mutation of strain 207, its effect would be different from those of the two reference compounds since these are not mutagenic for that strain.

Most of the compounds tested produced negative results. Those that produced a mutagenic effect are listed in Table VIII. The carbamate compounds that were "active" produced a weak effect compared with the classical mutagens. They should be further tested in standard mutation analyses to check their apparent weak mutagenicity. However, ethylene imine and the related aziridines produced a very strong effect - in some cases, more pronounced than those of the classical mutagens. The chemosterilant Tepa showed some weak mutagenic activity which might have been due to its degradation to ethylene imine[15]. Ethylene imine and carbamates are known alkylating agents and the strains that reacted were those that can be mutated by the classic mutagenic and alkylating agent, diethyl sulfate. The reaction to hydroxy urea, although weak for strain 207, is of interest since this strain (a frame-shift mutant) requires a different mutagenic action than the others. The defects in all strains but 207 are believed to reside in single nucleotide alteration. However, the defect in strain 207 is believed to be due to a deletion, a larger DNA defect, and it previously had responded only to classical acridine half-mustards[16].

An attempt was made to use B. subtilis histidine mutants in this type of assay, and it was proved feasible with classical mutagens, but not all of the test compounds were completed. The compounds tested were ethyl-N-hydroxy carbamate, B-chloroethyl-N-hydroxy carbamate, ethylene imine, and propylene imine. The

strains showed a negative response to the carbamates, but a strong positive response to ethylene imine and propylene imine. The negative response is comparable to the inability of these carbamate compounds to produce a true mutagenic effect when tested in our other assays.

TABLE VIII

Response of Strains to Selected Compounds

Selected Compounds	Strains* C207 Frame Shift	C117 Ochre	D130 Mis-Sense	C120 Mis-Sense	C151 Ochre	C527 Amber	C496 Mis-Sense	G46 Ochre
Diethyl sulfate	-	++	+++	-	+	+++	+++	++++
B-Propiolactone	-	+++	+++	-	--	+++	+++	++
Ethyl-N-hydroxy carbamate	-	±	+	-	--	+	-	+
B-chloroethyl-N-hydroxy carbamate	-	-	±	-	--	±	-	+
Ethyl-N-(O-carbethoxy) carbamate	-	-	-	-	--	±	-	+
Hydroxy urea	±	+	-	-	--	±	-	-
B-Hydroxy ethyl hydrazine	-	-	+	-	--	-	±	+
Ethylene imine	-	++	+++	+	++	+++	+++	++++
Propylene imine	-	+++	++	+	+	++	++	++++
B-Hydroxy ethyl imine	-	+++	++	-	+	++	++	+++
Ethyl-N-carbamoyl aziridine	-	+	+++	-	-	++	+++	++++
n-Propyl-N-carbamoyl aziridine	-	+	+++	-	-	++	+++	+++
n-Butyl-N-carbamoyl aziridine	-	+	+++	-	-	++	+++	++++
n-(butyryl) aziridine	-	-	++	-	-	+	+	++
Tepa	-	-	+	-	-	-	-	++

\- = No colonies above spontaneous background number.
± = <50 colonies; arrangement questionable.
\+ = <50 colonies; arrangement comparable to reference compounds.
++ = >50 <400 colonies; arrangement comparable to reference compounds.
+++ = >400 <2,000 colonies; arrangement comparable to reference compounds.
++++ = >2,000 colonies; arrangement comparable to reference compounds.

*Dr. Ames' nomenclature and classification.

RECONSTRUCTION EXPERIMENTS

Assays were conducted to determine whether or not any suspected mutagenic compound exerted a selective pressure in mutation analyses. Known mixtures of the parent strain and the back mutant (obtained from a back mutation rate analysis) were added to control flasks and to flasks containing the compound under investigation. The cultures were incubated for 18 hours with aeration at 37°C. Serial dilutions were then made and plated on Penassay agar plates and Spizizen minimal agar plates to determine the proportion of mutant and parent cells found under control and experimental conditions. Subsequent to our final analysis of back mutation data, β-chloroethyl-N-hydroxy carbamate (for the histidine locus of *B. subtilis* Sb 25) and ethyl N-hydroxy carbamate (for the histidine locus of *S. typhimurium* C527)appeared to be mutagenic agents. In both cases, however, reconstruction experiments showed that the apparent mutagenicity was due to selection of the back mutant rather than induction of it. Both compounds gave the back mutant an advantage over the parent strain which resulted in higher proportions of back mutants under experimental conditions than under control conditions.

TRANSFORMATION EXPERIMENTS

Transformation studies were initiated to further elucidate whether or not some compounds have an effect on the biological integrity of DNA. That is, can the compound act directly on DNA (inactive DNA *in vitro*)? An efficient transformation system was set up using strains of *B. subtilis*[12,16].

The crude DNA was prepared essentially as described by Ephrate-Elizur, *et al.*[17]. To obtain a more stable DNA, the preparation was further purified by extraction with a 5:1 $CHCL_3$:iso-amyl alcohol mixture for 1/2 hour using a wrist shaker. After centrifugation, the top layer (containing the DNA) was removed and DNA fibers were precipitated with 95% alcohol; collected and washed with 75% alcohol. The excess alcohol was drained and the fibers were resuspended in 10% NaCl and stored at 4°C.

Freese's method for studying the inactivation of DNA[18] was modified and used to determine whether or not suspect compounds could inactivate DNA. The DNA (380 γ/ml in 2M NaCl) was diluted to 10 γ/ml in ice cold reaction mixture (0.02M NaH_2PO_4 adjusted to pH 6.2 with 1N Na OH). The control was further diluted to 2γ in reaction mixture. For the experimental samples, the DNA was diluted to 2 γ/ml in reaction mixture containing the compound under test. The 0.25 ml amounts of all samples were placed in screw cap vials and heated for various time periods at 75°C. After heating, the samples were iced immediately and tested for their efficiency of transformation.

The system was found to be feasible for the testing of compounds that appear mutagenic *in vivo*. In addition, we determined, for each compound, the level that had no effect on the competency (ability to accept DNA) of the cells to which the heated DNA was exposed. This was tested by adding specific molar concentrations of the heated compound at the time when untreated DNA was added to the cells for a transformation assay. Comparing the test values

with control values (untreated DNA plus cells), we considered those levels that had no effect on the system as "safe." Further tests determined whether such a level of compound had any effect when heated with DNA prior to exposure to the cells. If the inactivation of transformation was still evident, we then assumed that the compound inactivated DNA. Table IX lists the "safe" levels of the compounds tested. The results indicate that some compounds are not capable of inactivating DNA at their "safe" levels. The data do not distinguish the relative DNA inactivation capabilities of individual compounds.

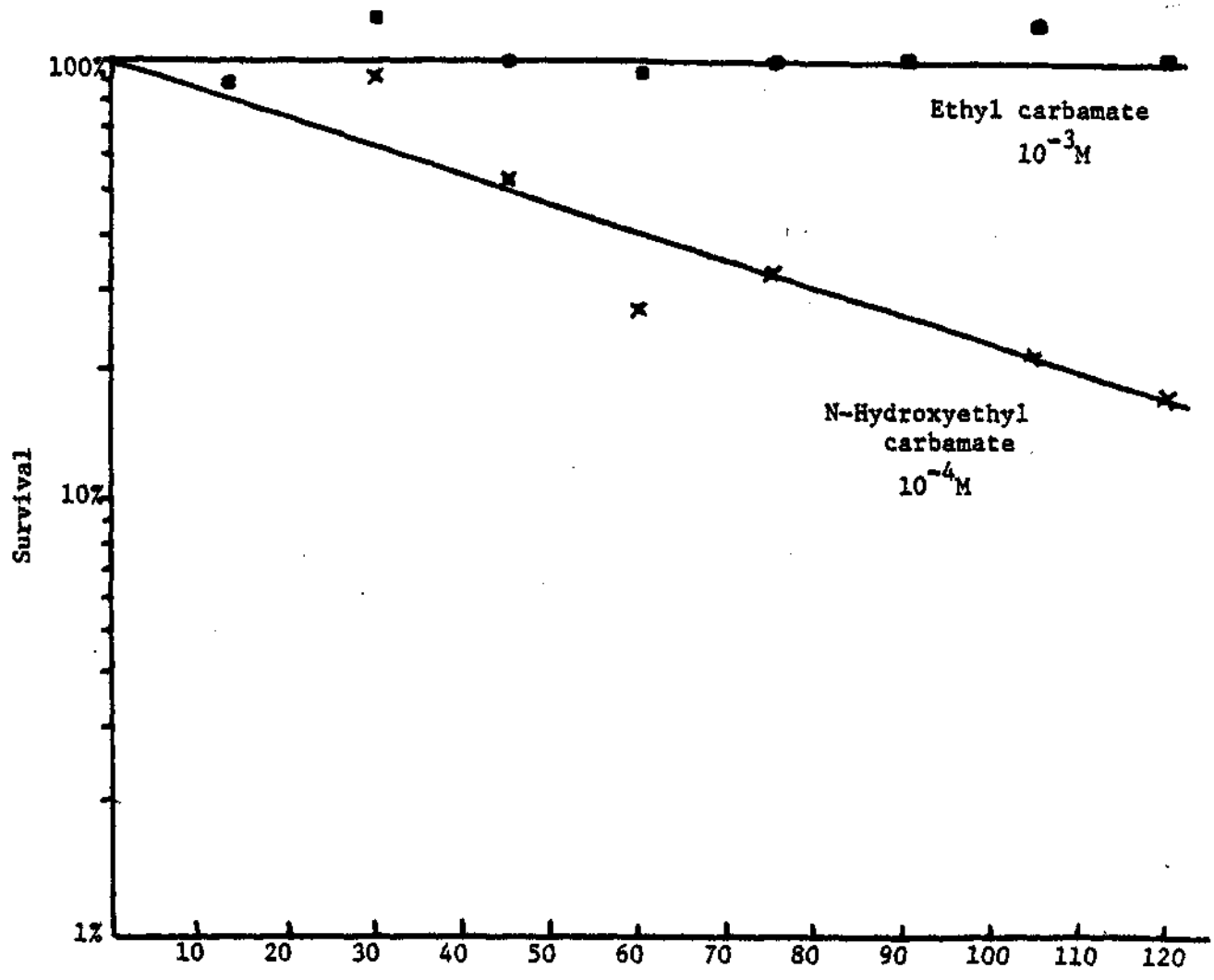
The compounds for which inactivation information was completed are ethyl carbamate and ethyl N-hydroxy carbamate; the results are presented in Figure 2. Survival refers to the percent of transformants obtained with treated DNA as compared to control DNA (heated alone and heated compound added at "zero" time). The pattern of inactivation was essentially that noted by Freese[18] -- no inactivation with ethyl carbamate (10^{-3}M), whereas ethyl-N-hydroxy carbamate (10^{-4}M) caused a great deal of inactivation. It appears that ethyl N-hydroxy carbamate, although not mutagenic for our strain of B. subtilis, is able to inactivate DNA. The negative results obtained in our mutation analysis may be due to the inability of the compound to react with DNA in the in vivo system or, as suggested by Mirvish[19], to metabolic conditions within the cell which may convert it to ethyl carbamate, which is unable to inactivate DNA.

TABLE IX

EFFECTS OF SELECTED COMPOUNDS ON THE TRANSFORMATION SYSTEM OF B. SUBTILIS

Selected Compounds	(M/L) Safe Level	Ability to Inactivate DNA
β-Chloroethyl-N-hydroxy carbamate	10^{-4}	+
Ethyl-N-(O-carbethoxy) carbamate	10^{-4}	+
Butyl N-hydroxy carbamate	10^{-3}	+
Ethyl N-hydroxy carbamate	10^{-3}	+
Propyl N-hydroxy carbamate	10^{-2}	+
Ethyl carbamate	10^{-2}	+
Acetohydroxamic acid	10^{-1}	+
β-Hydroxy ethyl carbamate	10^{-1}	-
Ethylene urea	10^{-1}	-
Methyl carbamate	10^{-2}	-
Propyl carbamate	$5X10^{-2}$	-

Figure 2. The Effects of Carbamates on Transforming DNA



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