References and Notes

1. D. Bloom, *A.M.A. J. Diseases Children* **88**, 754 (1954).
2. ———, *J. Pediat.*, in press.
3. Experiments conducted in laboratories at the Rockefeller Institute and at Cornell University Medical College.
4. J. German, *Science* **144**, 298 (1964).
5. R. Archibald, M. Erlandson, J. German, in preparation.
6. A. Sawitsky, D. Bloom, J. German, in preparation.
7. B. Lewis, *A.M.A. Arch. Dermatol.* **75**, 772 (1957).

8. This investigation was supported by PHS research grants HD-00635-01 from the National Institute of Child Health and Human Development; FR-102 from the General Clinical Research Center Branch, Division of Facilities and Resources; and 5 T1GM577 from the National Institute of General Medical Sciences. Aided by a grant from The National Foundation.

1 March 1965

Mutagenic Effects of Hydroxylamine in vivo

Abstract. *Hydroxylamine can induce any one of the four types of transition mutations in bacteriophage S13 if the mutagen is added directly to agar plates seeded with phage and bacterial indicators. The phage can also be mutated by treating the host cell with hydroxylamine before infecting it with phage. These effects of hydroxylamine in vivo contrast with the mutagenic effect in vitro, which seems to be exclusively on cytosine.*

Hydroxylamine has a highly specific mutagenic effect on bacteriophage treated in vitro. From a study of the induction of forward and reverse mutations, it has been inferred that in the single-stranded DNA of phage S13, only one of the four bases is altered in mutations induced by treatment of the free phage with hydroxylamine (*1*). By comparing that inference with data on the chemical reactivity of hydroxylamine (*2*, *3*), it was concluded that it is cytosine that is altered, being replaced after replication by thymine. This result is consistent with mutation experiments on double-stranded DNA (*2*, *4*), although in double-stranded DNA the mutagenic effect cannot be restricted to cytosine, but must ultimately involve both members of the guanine-cytosine base pair. In S13, it was concluded that the mutagenic specificity of hydroxylamine for cytosine was at least 500 times greater than for adenine, guanine, or thymine, in sharp contrast with the effects of two other frequently used mutagens, nitrous acid and the alkylating agent, ethyl methanesulfonate, each of which appeared to mutate all four bases (*1*).

In this report we discuss the mutagenic effect of hydroxylamine on S13 when the treatment is in vivo—that is, when the host cell is treated either during or before infection. Under these conditions, hydroxylamine causes the mutation of all four bases almost indiscriminately.

A set of reference mutations that includes all four types of transitions was studied (Table 1). The base change for each mutation was previously inferred from the effects of a variety of chemical mutagens (*1*, *5*), and reasons have been given (*1*) for the belief that each mutation involves only the one particular base change indicated. The mutants have been described (*1*) except *su*HT9, which is a hydroxylamine-induced mutant that is able to infect both *Escherichia coli* C and *Shigella dysenteriae* Y6R, but which can produce progeny only in the *Shigella* strain. It is shown in Table 1 that all the mutations were induced when hydroxylamine was added directly to the overlay agar together with the parental phage and bacterial indicators. The identities of the mutants were verified by picking at least three mutant plaques of each type and replating on hydroxylamine-free plates. Of all the mutations shown in Table 1, only $h_i1 \longrightarrow h+$ can be induced in vitro; therefore it is clear that hydroxylamine is less specific in its mutagenic action in vivo, regardless of what base changes are assumed in Table 1.

The initial concentration of hydroxylamine was $2 \times 10^{-2}M$, but the rate of diffusion into the 35 ml (approximately) of bottom-layer agar is not known. The mutations must have occurred within 1 hour after plating because mutant plaques were already clearly visible after only 4 hours of incubation at 37°C. The effect of adding from 10 to 80 μmole of hydroxylamine was tested on the mutation $h_i1 \longrightarrow h^+$. The optimum amount of hydroxylamine was between 40 and 60 μmole. Above 60 μmole, the bacterial lawn would not develop well.

The plates contained free phage, infected cells, and uninfected cells, and any of the three could have participated in the mutagenic process. It seems that a mutagenic effect can occur without a direct interaction of the hydroxylamine with the phage because we were able to induce phage mutations by infecting cells that had previously been treated with hydroxylamine but which had been separated from it by centrifugation before infection. This is shown in Fig. 1 for an experiment in which the cells were both grown and treated in broth. The hydroxylamine concentration ($2.5 \times 10^{-2}M$) used for the treatment was nearly the same as the initial concentration in the overlay agar in the plate experiments. We do not know whether some hydroxylamine might become bound to the cells and thus not be removed by centrifugation. We also do not know why there was a large decrease in the number of mutants after 30 minutes of the treatment.

The host, *E. coli* C, used in this experiment is normally nonpermissive for growth of the parent phage, *su*HT9. Two other mutations, $h^+ \longrightarrow h_i1$ and hH43$\longrightarrow h^+$, were also tested for inducibility by prior treatment of *E. coli* C with hydroxylamine, and in these

Table 1. Induction of the four types of transition mutations by addition of hydroxylamine (HA) directly to the agar plates. The plates (*1*) were incubated at 37°C within 10 minutes after the overlay agar containing phage and indicator bacteria were added. Hydroxylamine hydrochloride (Fisher) was made up as a 1*M* solution in 0.2*M* sodium phosphate buffer, *p*H 6.0, and was stored frozen at −10°C. Similar stored stocks were used in previous in vitro experiments (*1*). Mixed bacterial indicators had to be used for the host-range mutants (*1*). The *p*H of the plates was measured with pHydrion papers (Micro Essential Laboratory). With *E. coli* C in the overlay agar, the *p*H changed from 6.3 at the time the plates were seeded, to approximately 6.0 after 6 hours of incubation at 37°C, and to approximately 5.5 after overnight growth.

Mutation			Amount of parental phage plated	No. of mutant phage plaques appearing on a plate	
Phage		Base change*		No HA	50 μmole HA†
Parent	Mutant				
h^+	$\longrightarrow h_i1$	G $\longrightarrow$ A	4×10^7	20	450
h_i1	$\longrightarrow h_i1$	A $\longrightarrow$ G	1×10^8	8	100
h_i1	$\longrightarrow h^+$	C $\longrightarrow$ T	1×10^8	8	90
hH43	$\longrightarrow h^+$	T $\longrightarrow$ C	1×10^8	1	110
hH76	$\longrightarrow h^+$	T $\longrightarrow$ C	1×10^8	2	80
*su*HT9	$\longrightarrow su^+$	T $\longrightarrow$ C	1×10^8	6	≧300
*su*HT9	$\longrightarrow su^+$	T $\longrightarrow$ C	2×10^7	1	100

* The abbreviations are: G, guanine; A, adenine; C, cytosine; T, thymine. † Added to 2.5 ml of overlay agar.

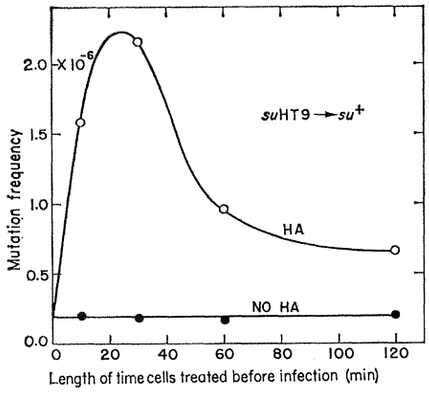

Fig. 1. Mutation frequency of *su*HT9 ⟶ *su*⁺ relative to the number of input phage as a function of the length of time the host cells were treated with hydroxylamine (HA) before infection. *Escherichia coli* C was grown to 1×10^8/ml in tryptone broth (13 g of Bacto tryptone per liter of H_2O), *p*H 6.4. To 20 ml of the bacterial suspension was added 0.5 ml of 1*M* HA at *p*H 6.0. This was shaken at 37°C. At the indicated times, 5.0 ml was removed and centrifuged, the pellet then being resuspended in 5.0 ml tryptone broth, centrifuged again and resuspended to give a cell concentration between 5 and 10×10^8/ml in 1.0 ml tryptone broth-salt (13 g of Bacto tryptone plus 7 g of NaCl per liter of H_2O) containing $1 \times 10^{-2}M$ $CaCl_2$. To 0.2 ml of this bacterial suspension was added 0.05 ml of phage at 1×10^9/ml. Ten minutes at 37°C was allowed for adsorption, and then the entire phage and cell mixture was plated with indicator bacteria for phage assay. A control culture of cells was handled in the same way but with no HA added. Before treatment with HA, approximately 100 percent of the bacteria observable in the microscope could form colonies, but after 120 minutes of treatment, approximately 50 percent could not.

cases the host normally permits growth of the parent phage types. Both mutations were induced.

A similar effect could be obtained in an inorganic buffer. Cells grown in broth were treated with hydroxylamine in 0.1*M* sodium phosphate buffer, *p*H 6.8, containing $10^{-3}M$ $MgSO_4$. The one mutation studied, *su*HT9⟶*su*⁺, was induced. At 10 minutes the effect was about half as great as the value shown in Fig. 1 for broth. But no mutagenic effect was observed at 30 and 60 minutes. The reason could well be the extremely rapid inactivation of cells treated in the buffer; the survival decreased roughly exponentially, reaching 10^{-2} at 60 minutes.

When cells were treated with hydroxylamine, centrifuged, and then aerated in tryptone broth at 37°C, the mutagenic effect of the hydroxylamine was reduced, but even after 80 minutes of aeration there was still a threefold increase in the mutation *su*HT9⟶*su*⁺ compared with control cells not treated with hydroxylamine.

Because hydroxylamine is such a highly specific mutagen in vitro, it might at first seem surprising that it should be so unspecific in vivo. But the mutagenic effects in vivo are actually not unreasonable considering the widespread effects that HA and its derivatives have on *E. coli*. At $10^{-3}M$ concentrations, hydroxylamine is known to seriously affect many distinct cellular processes, immediately stopping DNA, RNA, and protein synthesis (*6*); and inhibitory effects have been observed even at $10^{-4}M$ (*7*). It should not be surprising if such damages were to impair the fidelity of the replication process and thus increase the frequency of many types of mutations.

It is also conceivable that hydroxylamine can react with cytosine to produce an analogue for both cytosine and thymine that is mutagenic. Furthermore, mutagens might be produced by the reactions of hydroxylamine with other substances besides cytosine. For example, a large class of hydroxamates could be formed (*8*), particularly with the aid of intracellular enzymes; and some of the hydroxamates would be expected to react with bases other than cytosine (*9*). Perhaps in that way each base could be chemically modified. But actually, the formation of just one base analogue could be enough to explain the induction of all the mutations observed (*5*). In any case, it is clear that for the in vivo mutagenic effects there are many conceivable explanations that require further study.

The S13 experiments suggest possible limitations of hydroxylamine as a specific cellular mutagen. Hydroxylamine appears to be highly specific for just cytosine in vitro, but cellular mutations are usually induced in vivo. It is possible to mutate some cells by treatment in vitro of transforming DNA or transducing viruses, in which cases we would expect only cytosine to be affected. But if cellular mutations were produced by hydroxylamine treatment of the cells directly, then the in vivo results for phage S13 should alert us to the possibility of a mutagenic effect on any one of the four bases. Other mutagens might also behave quite differently in vivo than they do in vitro.

IRWIN TESSMAN
HIROMI ISHIWA
SANTOSH KUMAR
Department of Biological Sciences, Purdue University, Lafayette, Indiana

References and Notes

1. I. Tessman, R. Poddar, S. Kumar, *J. Mol. Biol.* **9**, 352 (1964).
2. E. Freese, E. Bautz-Freese, E. Bautz, *ibid.* **3**, 133 (1961); E. Freese, E. Bautz, E. B. Freese, *Proc. Nat. Acad. Sci. U.S.* **47**, 845 (1961).
3. H. Schuster, *J. Mol. Biol.* **3**, 447 (1961); D. M. Brown and P. Schell, *ibid.*, p. 709; D. W. Verwoerd, W. Zillig, H. Kohlhage, *Z. Physiol. Chemie* **332**, 184 (1963).
4. S. P. Champe and S. Benzer, *Proc. Nat. Acad. Sci. U.S.* **48**, 532 (1962).
5. B. Howard and I. Tessman, *J. Mol. Biol.* **9**, 364 (1964).
6. H. S. Rosenkranz and A. J. Bendich, *Biochim. Biophys. Acta* **87**, 40 (1964).
7. S. Béguin and A. Kepes, *Compt. Rend.* **258**, 2427, 2690 (1964).
8. W. P. Jencks, M. Caplow, M. Gilchrist, R. G. Kallen, *Biochemistry* **2**, 1313 (1963).
9. N. K. Kochetkov, E. I. Budowsky, R. P. Shibaeva, *Biochim. Biophys. Acta* **87**, 515 (1964).
10. This research was supported, in part, by NSF research grant GB-2748.

1 February 1965

Thin-Layer Chromatography of Plant Pigments on Mannitol or Sucrose

Abstract. *Fat-soluble tobacco leaf pigments were separated by chromatography on thin layers of mannitol and sucrose. Resolution of pigments, simplicity, and rapidity of preparation and development offer advantages over previous methods.*

Successful separation of plant pigments has long been obtained by column chromatography on adsorbents such as powdered sugar or powdered cellulose (*1*). Thin-layer chromatography on sucrose (powdered confectioners' sugar containing 3 percent cornstarch) was recently described (*2*). A method with mannitol as the adsorbent has been developed in this laboratory.

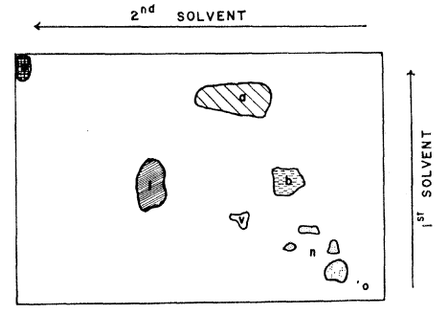

Fig. 1. Diagrammatic figure showing the two-dimensional separation of chloroplast pigments from a young tobacco leaf obtained on a mannitol thin-layer plate. *1st solvent*, 2 percent (by volume) methanol in petroleum ether (b.p. 30° to 60°C); *2nd solvent*, 5 percent (by volume) acetone in petroleum naphtha (b.p. 60° to 70°C); *a*, chlorophyll *a*; *b*, chlorophyll *b*; *c*, carotenes; *l*, lutein; *v*, violaxanthin; *n*, other xanthophyll pigments; *o*, origin.