tent of saturated fat. Thus it is possible that these or other yet unidentified dietary factors present in both regimes are responsible for the thrombosis.

Our model provides an opportunity to determine whether the thrombotic tendency is the result of altered hemostatic mechanisms and therefore is a possible progenitor of the atherosclerotic lesion itself, or whether this tendency is simply secondary to a particularly virulent form of atherosclerosis induced by these dietary factors. Apart from the fulminant nature of the disease that occurred under the two dietary regimes, the observed pathologic events appear to be similar to those that occur in man. The availability of an animal model in which it is possible to study these important questions may represent a key discovery that will allow final clarification of the role of hemostasis in the genesis, progression, morbidity, and mortality of atherosclerosis.

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8. Dogs were given free access to food. The basic dog chow was a mixture of 60 percent growth diet and 40 percent meat-bone meal (Riviana Foods, Topeka). To the diet of the euthyroid dogs were added equal amounts of beef tallow (Litvak Meat Packing Co., Denver) and lard (Loveland Packing Co., Loveland) to a final concentration of 22 percent fat by weight in the diet. The hypothyroid dogs received the same basic chow supplemented with a mixture of tallow and lard (total fat in diet, 22 percent) and 0.75 percent taurocholic acid. The cholesterol in the diet was varied from 1 to 5 percent by weight in an attempt to regulate the concentration of cholesterol in the plasma to between 750 and 1500 mg per 100 ml. The propylthiouracil was fed at a level of 1.5 mg per kilogram of body weight per day but was increased to 15 mg per kilogram per day after 28 weeks. Propylthiouracil, in addition to surgical thyroidectomy, was necessary to suppress the function of aberrant thyroid tissue, which occurred frequently in the dogs. The supplement was prepared by solubilizing the tallow and lard at 70°C and adding the cholesterol, taurocholic acid, and propylthiouracil slowly and with stirring.
9. We thank A. Groome and S. Lewis for technical assistance. A portion of the work described was performed under NHLI contract (NO1-HI-42903) with Colorado State University.

26 January 1976; revised 25 March 1976

Chloramine Mutagenesis in *Bacillus subtilis*

Abstract. *Chloramine (which occurs widely as a by-product of sanitary chlorination of water supplies) is shown to be a weak mutagen, when reversion of* trpC *to* trp$^+$ *in* Bacillus subtilis *is used as an assay. Some DNA-repair mutants appear to be more sensitive to chloramine, suggesting the involvement of DNA targets in bactericide. The influence of plating media on survival of cells treated with chloramine suggests a bacterial repair system acting upon potentially lethal lesions induced by chloramine.*

Chloramine (NH_2Cl), the reaction product of chlorine and ammonia in aqueous solution, is widely used directly for, or is generated as a by-product of, the disinfection of public water supplies and swimming pools. Nevertheless, little is known of the biological mechanism of action of chlorine or of possible genetic effects. Our own study (*1*) has shown that chloramine reacted with *Bacillus subtilis* DNA in vivo and in vitro; however, Boyle (*2*) did not obtain auxotrophs by treating *Escherichia coli* cells with chloramine; and a preliminary experiment with bacteriophage lambda by Hayatsu (*3*) did not show mutagenicity of hypochlorite. On the other hand, while this manuscript was in preparation, Wlodkowski and Rosenkranz (*4*) reported that sodium hypochlorite was a weak base-substitution mutagen in *Salmonella typhimurium*. We now report the mutagenicity of chloramine, using reversion of *trp*C to *trp*$^+$ in *B. subtilis* as an assay.

The strains used in this study were derivatives of indole-requiring *B. subtilis* strain 168 (*5*). As an index of the involvement of DNA targets in bactericide by chloramine, the sensitivity of different *B. subtilis* strains carrying various DNA-repair mutations was examined. Cell concentrations of each mutant and of its control for chloramine treatment were adjusted to about the same. In addition, the mixed cultures of strains 168 + SB879 and strains BD170 + BD194 were treated with chloramine and their survivors were sorted out by their respective nutritional markers. The surviving fractions of a representative test for each mutant as a function of chloramine doses are shown in Fig. 1. While *uvr* (SB879) and *rec*B (BD191) showed no evident increases in sensitivity, *rec*3 (BD193), *rec*A (BD194), and *pol*A5 (SB1060) were consistently

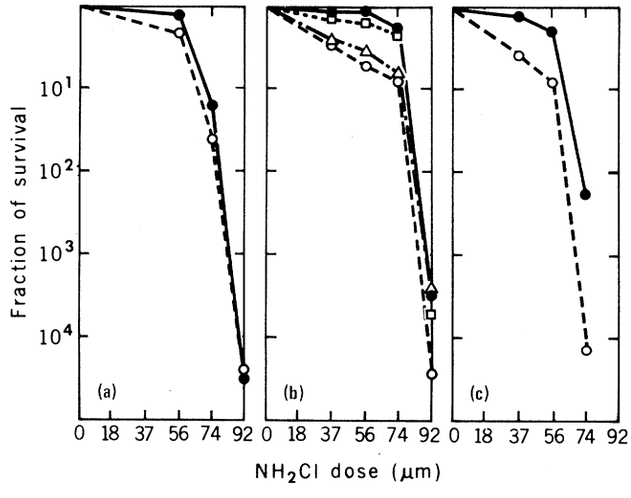

Fig. 1. Chloramine sensitivity of different *B. subtilis* strains. (a) ●, 168 (*uvr*$^+$, *trp*C); ○, SB879 (*uvr*, *trp*C, *his*B); (b) ●, BD170 (*rec*$^+$, *trp*C, *threo5*); □, BD191 (*rec*B, *trp*C, *threo5*); △, BD193 (*rec*3, *trp*C, *threo5*); ○, BD194 (*rec*A, *trp*C); (c) ●, SB1058 (*pol*$^+$, *phe*A, *trp*C, *his*B); ○, SB1060 (*pol*A5, *phe*A, *trp*C, *his*B). For the chloramine treatment, NaOCl was diluted in 0.05*M* phosphate buffer, *p*H 7, to different concentrations. Chloramine (NH_2Cl) was formed by incubating eight parts of NaOCl and one part of 0.1*M* NH_4Cl at 37°C for 1 hour. The prepared early stationary phase cells (*1*) were diluted ten times into chloramine solution and treated for 30 minutes at 37°C. The reaction was stopped by the addition of one volume of 0.02*M* sodium thiosulfate. Viable cell counts were scored by averaging the colonies of two plates (amino acid media, see Table 1) after incubation at 37°C for 2 days. The minimum number of colonies per plate was 161 ± 8 percent.

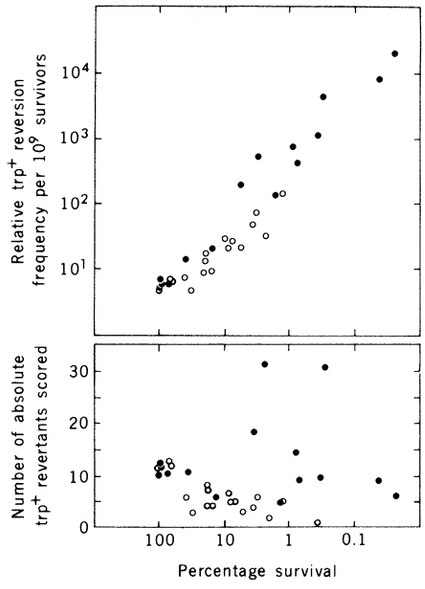

Fig. 2. Relative trp^+ reversion frequencies and absolute trp^+ revertants plotted against the percentage of survival after chloramine treatment (●) and ultraviolet irradiation (○). The revertants and survival were scored on CH and CH+trp media, respectively. Cell counts on CH+trp medium were 2.26×10^9 to 2.61×10^9 for controls.

more sensitive than their respective controls. Another test of the *pol*A5 mutant in a different strain, SB1059 (*pol*A5, *phe*A, *trp*C), demonstrated the same chloramine sensitivity as SB1060 did. [This finding is consistent with that obtained by Rosenkranz (*6*) which was also reported in the course of these experiments.] Our data suggested that at least *pol*A5$^+$, and possibly *rec*A$^+$ and *rec*3$^+$, are involved in the repair of chloramine-induced damage in DNA.

Strain 168 (*trp*C) was used to test the influence of plating media on survival after chloramine treatment and the effect of chloramine on mutation. Ultraviolet irradiation was employed to give comparative results. In ultraviolet-irradiated cells, the survival on nutrient agar was much lower than that observed on all the other media (Table 1). Minimal medium, however, showed the lowest survival after chloramine treatment. Since chloramine readily reacted with amino acids and proteins (*2*), the supplementation of nutrients after chloramine treatment seemed to be essential in the recovery of the cells. Among the nutrient-supplemented media, however, amino acid supplements (AA, CH-trp) afforded more efficient repair than nutrient agar for both ultraviolet-irradiated and chloramine-treated cells. The tests for the prototrophs (SB19 and SB850, trp^+) gave similar results. This phenomenon was similar to that observed in ultraviolet irradiation of *E. coli* (*7*), and it was suggested that the higher survival on synthetic medium than on complex medium resulted from delaying the growth of the irradiated cells until repair of ultraviolet damage occurred. The differential recoveries of chloramine-treated cells also suggested a bacterial repair system acting upon potentially lethal lesions, similar to well-known findings with ultraviolet irradiation.

The results of two typical experiments on the effect of chloramine and ultraviolet irradiation on the yield of *trp*C to trp^+ reversion are shown in Table 1. In ultraviolet-irradiated cells plated on different media and in chloramine-treated cells plated on minimal medium, there was enhancement of mutation frequencies; however, the absolute number of revertants was reduced owing to the superimposed lethality. The use of supplemented media for the chloramine-treated cells, in contrast, yielded small but consistent increases of total numbers of trp^+ revertants (and implies marked enhancement of trp^+ reversion rates). This result resembled that observed in *S. typhimurium* and *E. coli* (*8*): that the yield of trp^- to trp^+ reversion induced by ultraviolet irradiation was profoundly enhanced by certain conditions of incubation after treatment.

The *trp*C marker of *B. subtilis* is a stable locus with a low spontaneous reversion frequency of 2 to 9 per 10^9 cells. Absolute increase of revertants was observed only at survivals between 0.1 and 10 percent. However, chloramine kill was difficult to control because of the presence of other variables (such as cell concentration, amino acid or protein residues in cell samples, and the cleanliness of glassware) and the same dose of chloramine did not always give reproducible kill and reversion. These small increases in absolute numbers of trp^+ revertants reinforced the evidence for mutagenesis by chloramine, as judged by the relative increases among the survivors. The combined results of several experiments were analyzed statistically (Fig. 2). Comparing the regression coefficiencies (relative reversion frequencies and percentage of survivals) of the two killing agents, chloramine ($b = -43.62$) had a significantly ($d = 3.99$) higher effect, for

Table 1. Effects of different media on survival and on *trp*C to trp^+ reversion after treatment with chloramine or ultraviolet irradiation. The media used were as follows: NA, nutrient agar (Difco); MM, Spizizen minimal medium + 0.5 percent sucrose + 1.75 percent agar; MM+trp, MM + tryptophan (25 μg/ml); CH, MM + 4 mg of casein hydrolyzate per milliliter (Nutritional Biochemicals); CH+trp, CH + tryptophan (25 μg/ml); AA−trp, MM plus all the amino acids (25 μg/ml) except tryptophan; AA, MM plus all the amino acids (25 μg/ml). Viable cells were counted as described in Fig. 1. The revertants were counted after 4 days of incubation at 37°C.

Treatment	Survival (%)				trp^+ reversion*		
	NA	MM+trp	CH+trp	AA	MM	CH	AA−trp
NH_2Cl							
None	95.11	95.74	100†	100.53	11 (5.1)	8 (3.5)	6 (2.6)
18 μ*M*	78.19	70.29	98.94	90.43	10 (5.3)	9 (4.0)	11 (5.4)
37 μ*M*	39.88	28.09	56.25	64.19	4 (6.3)	14 (11.1)	11 (7.6)
56 μ*M*	0.69	0.25	2.47	13.67	2 (315)	32 (574)	24 (78)
74 μ*M*	0.0037	0.0013	0.042	0.27	0 (–)	8 (8547)	6 (983)
Ultraviolet							
None	101.15	100.38	100†	98.08	10 (5.1)	12 (4.6)	13 (4.7)
1 min	23.53	71.33	65.89	65.87	8 (3.9)	13 (7.7)	12 (7.6)
3 min	4.70	17.59	15.63	20.65	4 (8.7)	4 (9.8)	9 (15.2)
6 min	0.30	2.76	2.10	5.13	3 (41.6)	2 (36.4)	3 (22.3)
9 min	0.0068	0.38	0.38	1.00	1 (–)	1 (–)	2 (762)

*Upper numbers are actual numbers of revertants scored; lower numbers in parentheses are relative frequencies per 10^9 survivors. For ultraviolet treatment the prepared cells were suspended in phosphate buffer (3.5 ml) and held at 37°C for 30 minutes; they were then treated with ultraviolet (5 erg sec^{-1} mm^{-2}) in plastic petri dishes (5 cm in diameter). †Cell counts are 2.26×10^9 for NH_2Cl treatment and 2.61×10^9 for ultraviolet irradiation.

a given kill, than ultraviolet did ($b = -0.66$), in enhancing the trp^+ reversion. The chloramine sensitivity of 12 trp^+ revertants was also studied, and they were as sensitive to chloramine as the *trp*C cells.

The effect of chloramine on the yield of *trp*C to trp^+ of other different strains carrying various mutation pertinent to DNA repair was also studied on CH medium. The preliminary experiments showed that the trp^+ yields of *pol*A5 (SB1060), *rec*3 (BD193), *rec*A (BD194), and *uvr* (SB879) were not significantly different from the yields of $polA5^+$ rec^+ control strains (168, BD170) when treated with chloramine. Strain *rec*B (BD191), however, had a comparatively lower reversion frequency whether the cells were chlorinated or not. No increase of absolute number of revertants in chloramine-treated cells was seen. Similar phenomena have been reported also in studying the mutability of recombination-deficient and ultraviolet-sensitive strains of *E. coli* toward ultraviolet light (*9*).

The trp^+ revertants of 168 have been studied for the transforming activity of the linkage group ($aroB^+$ $trpC^+$ $hisB^+$). Of 75 chloramine-induced revertants, 24 percent showed reduced trp^+ transforming activity compared to wild type trp^+. Spontaneous reversions showed about 8 percent such transforming-defective types. These may represent more complex genetic changes than base substitution.

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Increased Sarcoma Virus RNA in Cells Transformed by Leukemia Viruses: Model for Leukemogenesis

Abstract. *A morphologically flat revertant of mink cells nonproductively infected with Moloney sarcoma virus exhibited contact inhibition and lacked detectable sarcoma virus RNA. Superinfection by usually nontransforming type C mammalian leukemia-causing viruses induced transformation and increased sarcoma virus RNA. The results suggest a model for leukemogenesis in animals by increasing, during replication of usually nontransforming leukemia viruses, the levels of RNA from potentially oncogenic cell or integrated virus transforming genes.*

Mammalian type C RNA tumor viruses exist in two functional categories.

1) There are viruses that, when injected into animals, cause sarcoma or leukemia within a few weeks (*1*). These viruses are able to enter and transform fibroblasts and other cells in cell culture. The viruses that can transform fibroblasts have been well characterized and are defective in their ability to replicate progeny virions in the absence of replicating nontransforming helper viruses (*2*). There is evidence that these replication-defective but transforming viruses directly code for a function required for the maintenance of transformation (*3*).

2) There are helper-type viruses that replicate progeny without causing transformation in cell cultures and are capable of helping transforming viruses in replication of their progeny (*2, 4*). While some of these viruses (for example, the feline-primate viruses RD-114, CCC, and M-7) are not associated with disease, others are known to cause leukemias (*5*). These leukemias, as exemplified by the thymic leukemia of AKR mice, are unlike the leukemias caused by the transforming RNA tumor viruses and occur only after a long latent period (*6*). Since replicating viruses cause no transformation in vitro, the manner by which they induce transformation of hematopoietic cells in vivo, that is, leukemia and lymphoma, is obscure.

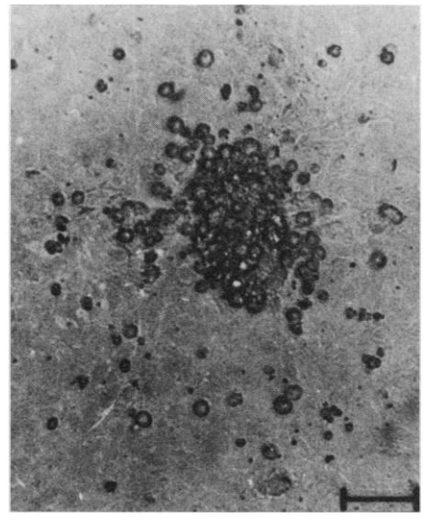

Fig. 1. A focus induced on S+L− mink cells by a single RD-114 replicating helper virus (*8*). The RD-114 superinfected S+L− mink cells continue to divide after confluency and form the focus of transformed cells seen above. The surrounding nonsuperinfected S+L− mink cells maintain contact inhibition and do not divide after the cell monolayer reaches 100 percent confluency. The bar represents 100 μm.

We have recently isolated mink lung cells which, although infected by replication-defective Moloney murine sarcoma virus (S+L−) strain MSV, were not like other murine and nonmurine cells infected by MSV and had phenotypically reverted to a flat morphology exhibiting contact inhibition similar to uninfected mink lung cells (*7*). Superinfection of these MSV infected cells (termed S+L− mink cells) by a variety of replicating but usually nontransforming type C viruses resulted in small foci (Fig. 1) of transformed cells on the contact inhibited monolayer; and this focus induction was useful as a quantitative assay for infectious replicating type C viruses (*8*).

Since this system involved transformation by usually nontransforming type C viruses, we investigated further the mechanism by which S+L− mink cells were caused to undergo transformation by the replicating mammalian type C viruses. We recently were able to prepare a complementary DNA (cDNA) probe that represents the sarcoma specific sequences of Moloney sarcoma virus (*7, 9*). The endogenous reverse transcriptase reaction from MSV obtained by rescue from S+L− dog cells (*7*), superinfected with RD-114 helper virus, was used to synthesize [^{3}H]deoxycytidine-labeled DNA. The DNA transcripts were recycled against RD-114 RNA and Moloney MuLV RNA to leave only MSV specific cDNA. Using this probe, we examined the levels of sarcoma virus specific RNA in S+L− mink cells before and after superinfection with various mammalian type C viruses. In addition, we examined mouse cells derived from 3T3 mouse cells and infected by the same MSV (*10*).