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Further chromatography of fraction Kc on a cellulose column revealed that this fraction consisted of two components. The one component, having the absorption which was closely similar to that of thymidine within the range between 250 mμ and 290 mμ but much higher than thymidine at shorter wavelengths, showed a deoxyribosidic growth activity after snake-venom digestion. On the other hand, authentic TMP, TDP and TTP were eluted, upon chromatography on Dowex-1 with the formic acid system, at the positions corresponding to those of fraction H, M and O, respectively. It is evident, therefore, that the compound under consideration is not identical with those thymidine nucleotides. It is suggested that the main deoxyribosidic compound in the acid-soluble extract of *L. acidophilus* is a derivative of one of the thymidine nucleotides. The other compound present in fraction Kc appears to be a derivative of uracil ribonucleotide.

EFFECT OF NITROGEN AND SULFUR MUSTARD ON NUCLEIC ACID SYNTHESIS IN *ESCHERICHIA COLI*[*]

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INTRODUCTION

The mustards are prominent members of that group of substances whose biological activities are described as "radiomimetic". On the cellular level, both mustards and radiation inhibit cell division, induce chromosome fragmentation and gene mutation and may ultimately kill the cell. Thus it appears that the nuclear apparatus of the cell is particularly susceptible to the action of these agents and may well be the primary

[*] The opinions expressed in this paper are those of the authors, and do not necessarily represent the official views of any governmental agency.

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site of attack by both mustards and radiation. While the mode of action of these agents need not be the same, studies on the biological effects of the mustards may contribute also to our understanding of radiation injury.

The emergence, in recent years, of strong evidence identifying deoxyribonucleic acid (DNA) as the genetic material of the cell, has directed the attention of investigators in the field of radiobiology to the effects of radiation and radiomimetic drugs on this substance. There appears to be considerable support for the view that the physiological basis of the nucleotoxic action of the mustards (as that of radiation) is to be sought in their interaction with DNA. These substances reduce the viscosity of DNA, perhaps by partial degradation of the molecule[1]. The biological activity of transforming principle is lost upon treatment with nitrogen mustard, even in concentrations which do not affect the viscosity[2]. *In vivo*, inhibition of DNA synthesis after mustard treatment has been observed both in bacteria[3,4] and in mammals[5].

The present study is an attempt to extend our knowledge of the effect of mustards on the metabolism of the nucleic acids.

MATERIALS AND METHODS

Chemicals

Two mustards were selected for investigation:

(a) Sulfur mustard, bis (β-chloroethyl) sulfide, abbreviated HD, was furnished by Dr. M. D. GAON of the Rocky Mountain Arsenal, U. S. Army Chemical Corps, Denver, Colorado.

(b) Nitrogen mustard, methyl bis (β-chloroethyl) amine hydrochloride ("Mustargen") was purchased from Sharp & Dohme, Inc. This material is henceforth referred to as HN2.

Purines and pyrimidines were purchased from Nutritional Biochemicals Corporation, and were recrystallized before use. 6-Methylaminopurine was a gift from Dr. G. HITCHINGS of the Wellcome Research Laboratories, Tuckahoe, N.Y., to whom our thanks are due.

A sample of purified salmon-sperm DNA, obtained from Prof. I. L. CHAIKOFF of the University of California, Berkeley, served as standard for the DNA determinations. For ribonucleic acid (RNA), a sample of commercial yeast RNA was purified as described by VISCHER AND CHARGAFF[6].

Bacterial strains

Escherichia coli, strain B, was obtained from Prof. S. S. COHEN of the University of Pennsylvania School of Medicine, Philadelphia. Strain B/r, a radiation resistant mutant of B first described by WITKIN[7], was obtained from the collection of the Department of Biophysics, University of Colorado School of Medicine. The bacteria were maintained on slants of nutrient agar and were transferred at intervals of four to six weeks.

Growth experiments

Unless otherwise stated, growth experiments were conducted as follows: Bacteria were taken from the slants, inoculated into 20 ml of the glucose-salts medium of COHEN AND ARBOGAST[8], and the cultures were incubated at 37° overnight. They were then diluted with an appropriate volume of the medium and incubated at 37° with vigorous aeration. Growth was followed by measurement of the optical density at 600 mμ in a Beckmann DU spectrophotometer and by viable cell count. The generation time was 60–65 min. When the bacteria, growing logarithmically, had undergone at least one division and had attained an optical density of about 0.3 (corresponding to $4–5 \cdot 10^8$ cells/ml), they were harvested by centrifugation, washed once with the medium lacking glucose and resuspended in the same. The desired quantity of HD (dissolved in a trace of ethanol) or of HN2 (aqueous solution) was then added*; the mixture was incubated for 15 min and the cells were again collected by centrifugation. They were then resuspended in pre-warmed glucose-salts medium and aerated at 37° as before**. Samples were withdrawn at frequent intervals for chemical analysis and for determination of the viable cell count. Whenever possible, control and experimental cultures were run concurrently.

* Concentrations of mustard throughout this paper refer to the concentration of mustard in the cell suspension.

** This point corresponds to 0 minutes in Fig. 1–6.

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Analytical methods

Viable cell counts were performed by plating on nutrient agar supplemented with 0.5% sodium chloride. The bacteria were incorporated into soft agar before plating.

For the determination of nucleic acids, 25 ml aliquots of the bacterial suspension (optical density 0.06 or higher) were chilled and centrifuged. The cells were then extracted with 0.25 *N* perchloric acid for 20 min in the cold to isolate the acid-soluble fraction (when this fraction was not needed, the cell suspension was precipitated directly with 0.25 *N* perchloric acid). Nucleic acids were then extracted from the cell residue by heating it with two successive 5-ml portions of 0.5 *N* perchloric acid at 95° for 10 min. The pooled extracts were brought to volume. DNA was determined by BURTON's method[9] and RNA by that of CERIOTTI[10]. The ultraviolet absorption of the extracts at 260 mμ agreed very well with that of artificial mixtures of nucleic acids in the proportions indicated by the analysis.

In order to determine deoxyribose and ribose in the acid-soluble fraction, growth experiments had to be conducted on a larger scale. Samples of 400 ml (optical density about 0.5) were centrifuged. The cells were washed with saline and extracted in the cold with two portions of 0.25 *N* perchloric acid, to give a final volume of 5 ml. Under these conditions about 70% of the acid-soluble fraction was recovered. Ribose and deoxyribose were determined as before; hydrolysis of the extract proved unnecessary.

Determination of the base composition of DNA

Bacteria were grown on a large scale, either on the surface of agar or in liquid medium. DNA was isolated either by a modification of the method of ZAMENHOF *et al.*[11] or by that of SMITH AND WYATT[12]. In either case, RNA was removed by alkaline digestion. The RNA content of our DNA preparations did not exceed 4% as determined by analysis for uracil on an ion-exchange column[13].

Adenine, guanine, thymine, and cytosine were determined after digestion with perchloric acid[14]. They were separated by paper chromatography, eluted and estimated by means of their ultra-violet absorption[15]. 6-Methylaminopurine was determined as described by DUNN AND SMITH[16]. Phosphorus was determined after digestion of the DNA[17] by the method of ALLEN[18]. Some of the methods applied in this phase of the study have been described in greater detail elsewhere[19].

RESULTS

1. *Growth and viability of* E. coli *after mustard treatment*

Survival curves for *E. coli* B and B/r after treatment with mustards are presented in Fig. 1. These were obtained by treating the bacteria with mustard in the logarithmic phase of growth as described in the experimental section, and plating them onto nutrient agar 15–20 min after addition of the mustard. It will be noted that survival of B/r was much higher than that of B, in agreement with the previous work of BRYSON[20].

When *E. coli* B, treated with a concentration of HD or HN2 sufficient to inactivate 90% or more of the cells, were resuspended in the growth medium, growth* began immediately. The turbidity increased exponentially for some 3 to 4 h, after which growth slowed down. The *rate* of turbidity increase depended on the concentration of mustard used, diminishing as the dose of mustard was increased. Typical turbidity-doubling times after treatment with various concentrations of HD and HN2 are shown in Table I. It must be emphasized that the bulk of the bacteria in these and subsequent experiments were unable to give rise to colonies. The only viable bacteria in the system were the few which had survived the mustard treatment. These multiplied at the normal rate (generation time 60–65 min) but were never sufficiently numerous to contribute significantly to the optical density or to the chemical determinations.

* Growth is here defined as an increase in the turbidity of the suspension at 600 mμ. This is considered to be a measure of the increase of cell protoplasm, whether or not accompanied by cell division.

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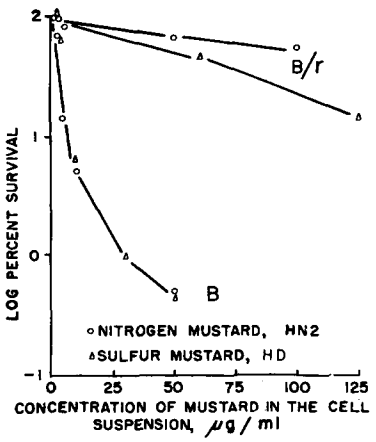

Fig. 1. Effect of nitrogen- and sulfur mustard on viability of *E. coli* B and B/r.

TABLE I

APPROXIMATE TURBIDITY-DOUBLING TIMES OF *E. Coli* B AND B/r AFTER TREATMENT WITH MUSTARD COMPOUNDS

The bacteria were harvested during the logarithmic phase of growth, suspended in buffer and treated with mustard. They were then collected by centrifugation and resuspended in growth medium

Mustard	*Concentration, µg/ml*	*Turbidity-doubling time, (minutes)*	
		E. coli B	*E. coli B/r*
HD	0	60	60
	85	80	85
	110	95–100	100
HN_2	0	60	60
	50	60	—
	250	75–80	75
	500	85–90	85

Microscopic examination of the cultures after several hours revealed that the majority of the organisms, having lost the ability to divide, had grown into long filaments. Cultures of B/r treated with mustards showed the same reduction of the growth rate observed with B. However, filament formation was much less pronounced than with B and the number of viable cells was much higher.

2. *Nucleic acid content of* E. coli *B and B/r*

It has been reported[21] that the nucleic acid content of some strains of B/r is higher than that of B. We were unable to confirm this report with our strains. In both the logarithmic and stationary phases of growth, the nucleic acid content of B and B/r were the same (Table II).

TABLE II

NUCLEIC ACID CONTENT PER CELL OF *E. Coli* B AND B/r

Strain	*DNA, µg/cell*		*RNA, µg/cell*	
	Logarithmic phase	*Stationary phase (aerated for 24 h)*	*Logarithmic phase*	*Stationary phase (aerated for 24 h)*
B	$16–18 \cdot 10^{-9}$	$24–26 \cdot 10^{-9}$	$100–120 \cdot 10^{-9}$	$65–75 \cdot 10^{-9}$
B/r	$16–18 \cdot 10^{-9}$	$22–27 \cdot 10^{-9}$	$100–120 \cdot 10^{-9}$	$50–65 \cdot 10^{-9}$

We also compared the base compositions of DNA samples isolated from the two strains. In agreement with previous reports[22], the molar ratios of adenine to thymine, guanine to cytosine, purines to pyrimidines and 6-amino groups to 6-keto groups were all close to one. The molar ratio of thymine plus adenine to cytosine plus guanine was selected as a measure of the ratio of the two base pairs. Statistical analysis of the results shown in Table III revealed no significant difference (at the 1 % level) between the DNA's of the two strains.

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TABLE III

BASE COMPOSITION OF DNA FROM *E. coli* B, B/r AND HD-TREATED B

Bacterial strain	*Growth medium*	*Proportion in moles of bases per 4 gram-atoms of phosphorus corrected for 100 % recovery*					*Percent actual recovery of phosphorus*	*Ratio of base pairs:*
		Thymine	*Cytosine*	*Adenine*	*Guanine*	*6-Methyl-amino-purine*		*Thymine + Adenine / Cytosine + Guanine*
B	Nutrient	0.92	1.01	0.95	1.10	0.02	100	0.89
	Nutrient	0.92	1.06	0.94	1.08	0.02	108	0.87
	Glucose–salts	0.91	1.08	0.94	1.05	0.02	88	0.87
	Glucose–salts	0.91	1.06	0.95	1.06	0.02	88	0.88
B, Treated with 85 μg/ml HD	Glucose–salts	0.91	1.09	0.92	1.08	0.02	113	0.85
	Glucose–salts	0.95	1.02	0.96	1.09	0.02	88	0.90
B/r	Nutrient	1.00	1.00	0.98	1.00	0.02	100	0.99
	Nutrient	0.94	1.00	0.97	1.09	0.02	90	0.92
	Glucose–salts	0.89	1.04	1.01	1.04	—	86	0.91
	Glucose–salts	0.97	1.06	0.93	1.02	0.02	104	0.91
	Glucose–salts	0.95	1.06	0.93	1.04	0.02	108	0.90

3. *Effect of mustard on nucleic acid synthesis*

Under the conditions of logarithmic growth used in the present study, the rates of DNA and RNA synthesis equaled that of turbidity increase (growth) (Fig. 2, A). Treatment of *E. coli* B with a concentration of HD or HN2 (*e.g.*, 10 μg/ml) just sufficient to reduce the viable count by 90 % had no detectable effect on the synthesis of these cell constituents. However, with higher concentrations of mustard a specific, albeit transient, inhibition of DNA synthesis was observed (Fig. 2, B and C).

It will be noted that after treatment of *E. coli* B with 85 μg/ml HD (viable count less than 0.5 % of untreated controls) the growth rate was but slightly reduced. The synthesis of RNA paralleled turbidity as in the untreated cells, but DNA synthesis was inhibited for about 20 min. DNA synthesis then resumed and its rate returned to normal (Fig. 2, B).

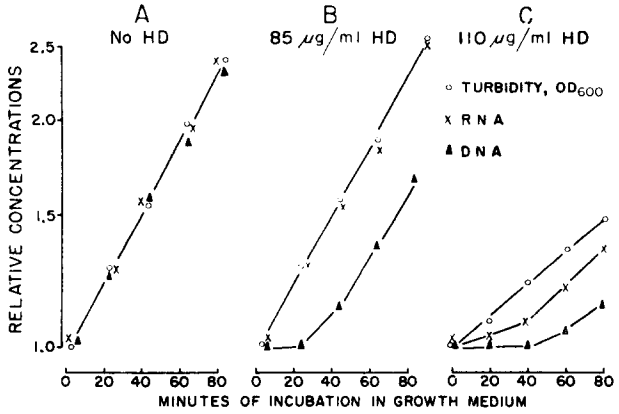

Fig. 2. Effect of sulfur mustard on growth and nucleic acid synthesis in *E. coli* B. The bacteria were treated with HD as described in the text and resuspended in growth medium at 0 minutes. Ordinates $= \frac{\text{concentration of cells and nucleic acids at time} = t}{\text{concentration of cells and nucleic acids at time} = 0}$ plotted on a logarithmic scale.

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If the bacteria were treated with still higher concentrations of HD, DNA synthesis was inhibited for a longer period of time. Thus, 110 μg/ml HD inhibited DNA synthesis for about 40 min (Fig. 2, C). Some inhibition of RNA synthesis was generally observed after treatment with concentrations of HD greater than 100 μg/ml, after which RNA synthesis again paralleled the increase of turbidity.

Transient inhibition of DNA synthesis was also demonstrated after treatment with HN2, although the concentration required was considerably higher (Fig. 3).

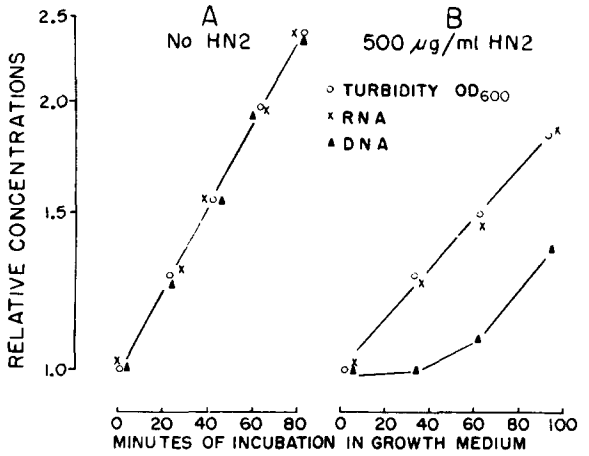

Fig. 3. Effect of nitrogen mustard on growth and nucleic acid synthesis in *E. coli* B. Experimental technique and plot as in Fig. 2.

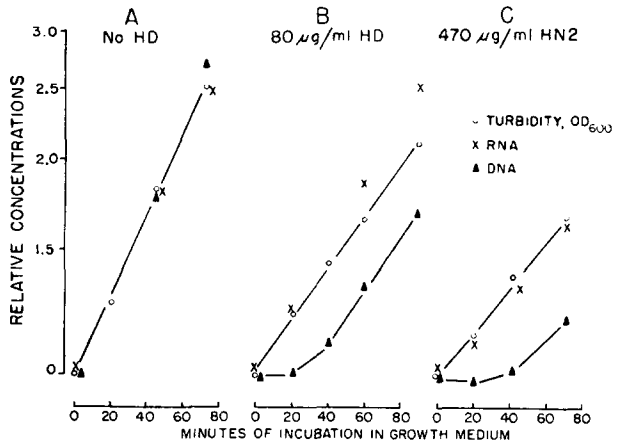

Fig. 4. Effect of sulfur- and nitrogen mustards on *E. coli* B/r. Experimental technique and plot as in Fig. 2.

Of great interest is the observation that a given concentration of HD or HN2 induced about the same inhibition of DNA synthesis in B/r as in B. Thus, in Fig. 4, B, 80 μg/ml HD inhibited DNA synthesis in B/r for 20 min, which is the same result as shown for B in Fig. 2, B.

It seemed of interest to characterize more fully the DNA that is synthesized

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after treatment with HD. To this end DNA was isolated as described above from *E. coli* B which had been treated with 85 μg/ml HD and which had then been permitted to grow for 3–4 h. About 80 % of the DNA in this system had been synthesized after the mustard treatment. The material was readily isolated in good yield in fibrous form and gave a viscous solution when dissolved in saline[11]. The base composition, determined after removal of RNA by alkaline digestion, is shown in Table III and appears the same as that of DNA from untreated *E. coli* B.

4. *Accumulation of deoxyribose in the acid-soluble fraction after mustard treatment*

The observation that DNA synthesis is inhibited after mustard treatment prompted us to search for effects on the intracellular nucleotide pool. In experiments, conducted as described in the experimental section, it proved feasible to measure approximately the deoxyribose and ribose content of the acid-soluble fraction of *E. coli*. Deoxyribose and ribose, both free and purine-bound, would be found in this fraction.

In untreated, growing *E. coli* B the acid-soluble deoxyribose and ribose contents paralleled turbidity. By contrast, in HD-treated cells the intracellular deoxyribose level rose rapidly (much of the increase took place prior to the start of incubation), reaching a peak after about 15 min of incubation, and then dropped. The ribose content was not increased over that of untreated cells and parallelled growth (Fig. 5). Treatment of the cells with HN2 also raised the level of intracellular deoxyribose without affecting that of ribose. However, with this mustard the deoxyribose level rose steadily throughout the period of incubation (Fig. 5).

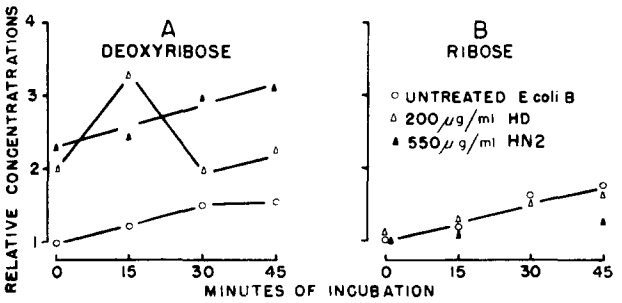

Fig. 5. Effect of nitrogen- and sulfur mustards on the acid-soluble deoxyribose and ribose content of *E. coli* B. *E. coli* B was treated with mustard as described in the text and resuspended in growth medium at 0 minutes. The ordinates are corrected to the same cell density at 0 minutes.

5. *Excretion of ultraviolet-absorbing material into the medium after mustard treatment*

In addition to the transient accumulation of deoxyribose inside HD-treated *E. coli* B we also noted a rapid release into the medium of acid-soluble, ultraviolet-absorbing material from such cells. The ultraviolet absorption spectrum of the excreted material showed a strong peak about 260 mμ, suggesting that it consisted of compounds related to the nucleic acids. The initial rate of accumulation of this substance in the medium increased with increasing concentrations of HD (Fig. 6). Excretion of ultraviolet-absorbing material from HD-treated *E. coli* B also occurred if the cells were suspended in buffer, but at a reduced rate.

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By contrast, treatment of *E. coli* B with HN2 did not induce an increased excretion of ultraviolet-absorbing material over that of untreated cells (Fig. 6), even when the dose of HN2 was sufficient to inhibit DNA synthesis for 50 min. The behavior of *E. coli* B/r with respect to excretion of ultraviolet-absorbing material paralleled that of B.

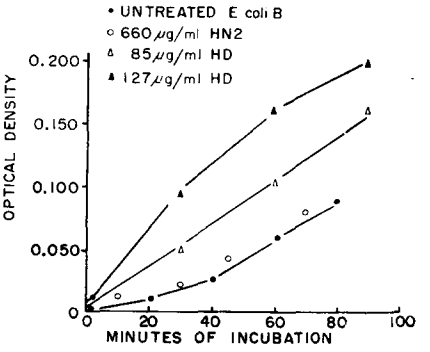

Fig. 6. Excretion of ultraviolet-absorbing materials by mustard-treated *E. coli* B. *E. coli* B was treated with mustard as decribed in the text and resuspended in growth medium at o minutes. The ordinate represents optical density of the growth medium at 260 mμ, using the medium at o minutes as blank. All the curves are corrected to the same initial cell density.

DISCUSSION

There appears to be general agreement that nitrogen- and sulfur mustards block cell division in bacteria and lower the viable count. However, as is also the case with both ultraviolet and ionizing radiations, bacteria inactivated by mustards may continue to enlarge giving rise to long filaments[3, 4, 20, 23, 24, 25]. Our observations are in full agreement with these previous reports. It is of interest that filament formation in the mustard-resistant strain, B/r, is much less pronounced than in B. A similar effect has been reported with respect to induction of filamentous growth in B and B/r by ultraviolet light[7] and by furacin[26].

The literature dealing with the effect of mustards on nucleic acid metabolism of *E. coli* presents a more confusing picture. HERRIOTT[3] and PARDEE[4] found that *E. coli* inactivated with HD are unable to synthesize DNA. On the other hand, KATCHMAN *et al.*[25] reported normal amounts of DNA in filamentous *E. coli* resulting from treatment of bacteria with low doses of the mustard triethylene melamine. Under our experimental conditions we found that both HD and HN2 induce a transient inhibition of DNA synthesis, the duration of which depends on the concentration of mustard. Ultimately DNA synthesis resumed and its rate returned to normal. In agreement with previous work[3, 4, 21] the synthesis of RNA and of protein (*i.e.*, growth) continued even while DNA synthesis is blocked.

The pattern of growth and of nucleic acid synthesis presented here bears a striking resemblance to that reported by KANAZIR AND ERRERA[27], who demonstrated transient inhibition of DNA synthesis in *E. coli* subjected to ultraviolet irradiation. Surprisingly, a given dose of ultraviolet light[27] or of mustard inhibits DNA synthesis to the same extent in both B and B/r. Transient inhibition of DNA synthesis has also been observed after X-irradiation of *E. coli* under special conditions[28]. KANAZIR AND ERRERA, in their investigation of nucleic acid metabolism of ultraviolet-irradiated *E. coli*, demonstrated an accumulation of deoxyribonucleotides inside the irradiated cells and have presented evidence for the view that these represent precursors of DNA accumulating behind the block[27, 29, 30]. In the experiments reported here, accumulation of

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acid-soluble, deoxyribose-containing compounds was observed in *E. coli* B treated with quite high concentrations of HD or HN2. However, pending more detailed investigation, it is not possible to state whether these represent precursors of DNA or acid-soluble degradation products. In this connection, we wish to point out an apparent difference between the actions of HD and HN2: in HD-treated *E. coli*, the intracellular, acid-soluble deoxyribose level rises to a peak, followed by a drop. Concomitantly, material with an ultraviolet-absorption peak at 260 mμ appears in the growth medium. By contrast, the intracellular deoxyribose content of *E. coli* treated with HN2 rises steadily and no ultraviolet-absorbing material is excreted into the medium. While these observations must be interpreted with great caution, they suggest that HD (but not HN2) may act upon the cell wall to cause leakage of cell constituents. It is of interest that leakage of nucleic acid derivatives from *E. coli* has also been observed after irradiation with X-ray and ultraviolet light[31].

It remains to consider the possible relationship between the cytotoxic and mutagenic actions of the mustards and the effects of these substances upon nucleic acid metabolism. COHEN AND BARNER[32] have suggested that the lethal action of the mustards on bacteria might be a consequence of "unbalanced growth" resulting from an inhibition of DNA synthesis while cytoplasmic growth continues. The present results do not lend support to this proposal, at least in its original form. Thus it was found possible to inactivate *E. coli* B with concentrations of mustard which have no detectable effect on DNA synthesis. Moreover, inhibition of DNA synthesis was found to be transient, showing that the inactivated bacteria are still capable of extensive DNA synthesis. Finally, DNA synthesis in B/r is as sensitive to inhibition by mustards as is that of B, but survival is much higher.

On the other hand, our results should not be construed as disproving the view that the lethal action of the mustards is due to their interaction with nucleic acids. The degree of survival of *E. coli* after mustard treatment appears to depend upon subsequent treatment*, which renders it difficult to correlate inactivation (measured on solid, nutrient medium after 18 h) with the inhibition of DNA synthesis (measured immediately in liquid, glucose–salts medium). Further, while the DNA synthesized by *E. coli* inactivated by HD grossly resembles that from normal cells, there is no evidence that it is genetically competent. Synthesis of an incomplete DNA, for instance, might not permit continued cell division.

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SUMMARY

1. Treatment of *Escherichia coli* with nitrogen- or sulfur mustard resulted in a cessation of cell division and a marked reduction of the viable count. Strain B/r was much more resistant to

* For instance, survival is higher on glucose–salts agar than on nutrient agar.

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mustards than was B. Bacteria inactivated with mustard could still grow for several hours, giving rise to filaments.

2. The nucleic acid content, per cell, of *E. coli* B and B/r was found to be the same. Samples of DNA isolated from these two strains were found to have the same base composition.

3. Nitrogen- and sulfur mustards induced a transient inhibition of DNA synthesis in both *E. coli* B and B/r. The duration of the inhibition depended on the concentration of mustard. Ultimately, DNA synthesis resumed and the rate of DNA synthesis of the filaments returned to normal.

4. Growth (increase of turbidity) and RNA synthesis continued while DNA synthesis was blocked.

5. Deoxyribose accumulated in the acid-soluble fraction of *E. coli* treated with either sulfur- or nitrogen mustard.

6. *E. coli* B and B/r, treated with sulfur mustard, excreted into the growth medium acid-soluble material, whose absorption spectrum showed a peak at 260 mμ. Bacteria treated with nitrogen mustard did not release such substances.

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