PHAGOCIDAL ACTION OF OXIDIZED SPERMINE AND ITS ANALOGUES.

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Received May 22, 1967

Spermine (Ia), a naturally occurring polyamine, is oxidized by beef plasma amine oxidase (Hirsh, 1953; Tabor, et al., 1954; Yamada, et al., 1962). The oxidation product, oxidized spermine, is known to be toxic to certain bacteria (Tabor, et al., 1961) and mammalian cells in culture (Bachrach, et al., 1967), and to inactivate the coliphages of T-uneven series (Bachrach, et al., 1963). These findings have been interpreted in terms of the formation of a biological inactive complex between oxidized spermine and nucleic acids (Bachrach, et al., 1965). The oxidation product has been established to be N,N'-bis-(2-formylethyl)-butan-1,4-diamine (IIa) after the reduction with $NaBH_4$ to the corresponding alcohol (IIId) (Tabor, et al., 1964). However, because of the difficulty of isolating the formyldiamine (IIa) in a pure form, any direct evidence that the formyldiamine (IIa) is responsible for the toxic effects has not yet been provided.

The present communication deals with re-investigations of the phagocidal action of oxidized spermine with a large variety of bacteriophages. The synthesis of the formyldiamine (IIa) as well as its homologous compounds and evaluations of their phagocidal activities is also presented.

Oxidized spermine was prepared at 30° in a Warburg apparatus. The reaction mixture consisted of 30 µmoles of spermine, 250-500 units of the crystalline plasma amine oxidase (Yamada, et al., 1962), 300 units of catalase and 150 µmoles of potassium phosphate buffer, pH 6.0, in a final volume of 3.0 ml. The reaction was usually completed within 3 hours, with the consumption of the theoretical amount of oxygen. The analogues of oxidized spermine were prepared from N,N'-bis-(3-aminopropyl)-ethan-1,2- (Ib) and N,N'-bis-(3-aminopropyl)-hexan-1,6-diamine (Ic) (Israel, et al., 1964) by similar enzymatic oxidation.

No attempt was successful in the synthesis of the formyldiamine (IIa) by reduction of N,N'-bis-(2-cyanoethyl)- (IIIa), N,N'-bis-(2-carboxyethyl)- (IIIb) or N,N'-bis-(2-carbomethoxyetnyl)-butan-1,4-diamine (IIIc) or by oxidation of N,N'-bis-(3-hydroxypropyl)-butan-1,4-diamine (IIId) (Tabor, et al., 1964; Israel, et al., 1964). The chemical synthesis of the formyldiamine (IIa) was performed through the following steps. 3,3-Diethoxypropylamine (b.p. 78°/17mm) was condensed with succinyl chloride in dry benzene containing an equivalent amount of triethylamine. The condensation proceeded without any difficulties to give N,N'-bis-(3,3-diethoxypropyl)-succinamide (IVa) (m.p. 84-85°). The succinamide (IVa) was reduced with $LiAlH_4$ in tetrahydrofuran to afford N,N'-bis-(3,3-diethoxypropyl)-butan-1,4-diamine (Va) as a yellow oil. The crude diamine (Va) was introduced to a crystalline salt as its nitrate (m.p. 144-145°), (Anal. found: C, 45.46; H, 8.93; N, 12.10: calcd. for $C_{18}H_{42}O_2N_4$: C, 45.55; H, 8.92; N, 11.81%). The formyldiamine (IIa) was obtained as its oxalate (m.p. 235-245° (decomp.)) by treatment of the bis-diethoxypropyl diamine (Va) with excess of oxalic acid in aqueous solution overnight and by subsequent recrystallization from aqueous ethanol. According to similar procedures, N,N'-bis-(4,4-diethoxybutyl)-butan-1,4-diamine (Vb) was prepared from 4,4-diethoxybutylamine (b.p. 104-105°/30mm) via the corresponding succinamide (IVb) (m.p. 101-102°).

Table I. Effect of oxidized spermine on the viability of various bacteriophages.

The incubation mixture contained 0.1 ml of the indicated bacteriophage suspension, 0.1 ml of oxidized spermine (0.6 µmoles) and 0.8 ml of the dilution buffer, pH 7.4. The incubation was carried out at 30° for 90 minutes. The number of phage (plaque forming unit, PFU) was determined by the agar-layer method (Adams, 1959).

Bacteriophage	Host	PFU/ml Concentration (mM) 0	0.6
T_2	Escherichia coli	$2\ 10^9$	$3\ 10^8$
T_3	"	$9\ 10^5$	$<10^2$
T_4	"	$1\ 10^9$	$9\ 10^8$
T_5	"	$2\ 10^8$	$<10^2$
T_6	"	$4\ 10^8$	$3\ 10^8$
T_7	"	$6\ 10^8$	$6\ 10^3$
λ	"	$4\ 10^7$	$<10^2$
ϕ80c	"	$2\ 10^8$	$<10^2$
MS2	"	$4\ 10^9$	$<10^2$
ϕX174	"	$2\ 10^7$	$1\ 10^7$
P22	Salmonella typhimurium	$5\ 10^8$	$3\ 10^7$
ϵ^{15}	Salmonella anatum	$4\ 10^8$	$2\ 10^4$
ϵ^{34}	"	$1\ 10^7$	$4\ 10^5$
M2	Bacillus subtilis	$5\ 10^8$	$2\ 10^7$
SP10	"	$4\ 10^3$	$3\ 10^3$
A1	"	$2\ 10^5$	$<10^2$
F4	Brevibacterium lactofermentum	$2\ 10^8$	$<10^2$
P465	"	$6\ 10^8$	$<10^2$
Ap85III	"	$8\ 10^7$	$<10^2$
I128T	Pseudomonas glycinea	$3\ 10^5$	$<10^2$
I2418	Xanthomonas phaseoli	$9\ 10^8$	$1\ 10^3$
PK66	Streptomyces griseus	$6\ 10^8$	$<10^2$

The bis-diethoxybutyl diamine (Vb) was crystallized in a state of its phosphate (m.p. 148-150°, Anal. found: C, 41.46; H, 9.09; calcd. for $C_{20}H_{44}O_4N_2$-$2H_3PO_4$: C, 41.95; H, 8.80%).

The phagocidal action of oxidized spermine on various bacteriophages is summarized in Table I. It was shown that coliphages of the T-uneven series were markedly inactivated and that the T-even series were inhibited only slightly. Similar findings on the coliphages have been reported by Bachrach et al. (1963). Other phages; MS2, ϕ80C, λ (Escherichia coli); ϵ^{15} (Salmonella anatum); P465, P468, Ap85, F4 (Brevibacterium lactofermentum); I128T (Pseudomonas glycinea); I2418 (Xanthomonas phaseoli); A1 (Bacillus subtilis) and PK66 (Streptomyces gliseus) were also inactivated. The inactivation of phages by oxidized spermine proceeded in a linear function of both incubation time and concentration of oxidized spermine. It is conceivable that the inactivation is due to the interaction between oxidized spermine and phagal nucleic acids.

The phagocidal actions of chemically synthesized formyldiamine (IIa) and other structurally homologous compounds on coliphages of T_2 and T_5 are summarized in Table II. The formyldiamine (IIa) inactivated T_5 at almost the same rate as that of oxidized spermine. Essentially the same activity was observed with oxidized polyamines as well as the acid hydrolyzed bis-acetal diamines (Va) and (Vb). However, the cyanoethyl- (IIIa), carboxyethyl- (IIIb), carbomethoxyethyl- (IIIc) and hydroxypropyl-diamine (IIId) were inert for both phages of T_2 and T_5. In a separate experiment, it was shown that other aldehydes, such as butyraldehyde, γ-aminobutyraldehyde, phenylacetaldehyde, imidazoleacetaldehyde and indoleacetaldehyde did not inactivate T_5 phage even at much higher concentration (6 mM).

The results presented in this paper give direct evidences that the formyldiamine (IIa) is responsible for the phagocidal action of oxidized spermine, and that both groups of the terminal -CHO and the internal $-NH-(CH_2)_n-NH-$ are essential for the action. It is not clear, however, how the formyldiamines react with the phagal nucleic acids to form the

Table II. Effect of the formyldiamine and its homologues on the viability of coliphages T_2 and T_5.

Conditions of the incubation were the same as described in Table I, except that 0.5 µmoles of the formyldiamine or its homologues were used.

	Compound*	PFU/ml T_2	PFU/ml T_5
1.	None	$2\ 10^8$	$2\ 10^8$
2.	IIa	$7\ 10^6$	$<10^2$
3.	Va	$1\ 10^8$	$8\ 10^7$
4.	Acid-hydrolyzed Va**	$8\ 10^6$	$<10^2$
5.	IIIa	$2\ 10^8$	$2\ 10^8$
6.	IIIb	$2\ 10^8$	$2\ 10^8$
7.	IIIc	$2\ 10^8$	$2\ 10^8$
8.	IIId	$2\ 10^8$	$2\ 10^8$
9.	Vb	$1\ 10^8$	$2\ 10^8$
10.	Acid-hydrolyzed Vb**	$8\ 10^6$	$<10^2$
11.	Ib	$2\ 10^8$	$2\ 10^8$
12.	Oxidized Ib	$3\ 10^7$	$<10^2$
13.	Ia	$2\ 10^8$	$2\ 10^8$
14.	Oxidized Ia	$5\ 10^6$	$<10^2$
15.	Ic	$2\ 10^8$	$2\ 10^8$
16.	Oxidized Ic	$5\ 10^6$	$<10^2$

*The compounds used were as follows:

I. $H_2N(CH_2)_3NH(CH_2)_nNH(CH_2)_3NH_2$;
a, n=4; b, n=2; c, n=6

II. $OHC(CH_2)_nNH(CH_2)_4NH(CH_2)_nCHO$;
a, n=2; b, n=3

III. $R(CH_2)_2NH(CH_2)_4NH(CH_2)_2R$;
a, R=CN; b, R=COOH; c, R=$COOCH_3$; d, R=CH_2OH

IV. $(C_2H_5O)_2CH(CH_2)_nNHCOCH_2CH_2CONH(CH_2)_nCH(OC_2H_5)_2$;
a, n=2; b, n=3

V. $(C_2H_5O)_2CH(CH_2)_nNH(CH_2)_4NH(CH_2)_nCH(OC_2H_5)_2$;
a, n=2; b, n=3

**The acid hydrolysis was carried out with 0.5 N H_2SO_4, at 30° for 3 hours.

biologically inactive complex, and why coliphages of T-uneven series MS2, φ80C and λ are much more susceptible to the action of the formyldiamine (IIa) than T-even series and φX174. Futher works on the action mechanisms of the formyldiamines with the phagal nucleic acids are in progress in our laboratories.

References

Adams, M. H., Bacteriophages, Interscience, New York, 1959, p. 443.
Bachrach, U., Tabor, C. W. and Tabor, H., Biochim. Biophys. Acta, 78, 768 (1963).
Bachrach, U. and Leibovici, J., Biochem. Biophys. Research Commun., 19, 357 (1965).
Bachrach, U., Abzug, S. and Berkierkunst, A., Biochim. Biophys. Acta, 134, 174 (1967).
Hirsh, J. G., J. Exptl. Med., 97, 345 (1953).
Israel, M., Rosenfield, J. S. and Modest. E. J., J. Med. Chem., 7, 710 (1964).
Tabor, C. W., Tabor, H. and Rosenthal, S. M., J. Biol. Chem., 208, 645 (1954).
Tabor, H., Tabor, C. W. and Rosenthal, S. M., Ann. Rev. Biochem., 30, 579 (1961).
Tabor, C. W., Tabor, H. and Bachrach, U., J. Biol. Chem., 239, 2194 (1964).
Yamada, H. and Yasunobu, K. T., J. Biol. Chem., 237, 1511 (1962).