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Two New Synthetic Substances Active against Viruses of the Psittacosis–Lymphogranuloma–Trachoma Group

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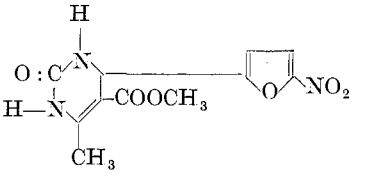
The great majority of substances active chemotherapeutically against viruses of the psittacosis–lymphogranuloma–trachoma group are also well recognized antibacterials. We have, however, mentioned the dissociation of antiviral and antibacterial activity in a spirothiohydantoin (20,065) which is wholly devoid of an effect on bacteria *in vitro* or *in vivo*.[1] The present report concerns two other synthetic compounds, therapeutically active against the largest viruses in the chick-embryo and the mouse, yet which at doses near the maximum tolerated fail to influence murine infections with *Salmonella dublin* or *Streptococcus pyogenes*.[2] The compounds are:

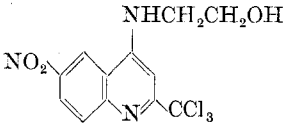
17,025: 5-Methoxycarbonyl-6-methyl-4-(5-nitrofuryl)-2-oxo-1,2,3,4-tetrahydropyrimidine (I)[3]

25,187: 4-β-Hydroxyethylamino-6-nitro-2-trichloromethylquinoline (II)[4]

(I)



(II)



Methods

Eggs. Groups of eight or twelve 7-day-old chick-embryos received virus into the yolk-sac, followed 2½ hours later by a dose of the compound in a dispersing agent or by the dispersing agent alone. Deaths were recorded up to the thirteenth day after infection, when remaining eggs were opened and living embryos were deemed to have survived for one additional day. The mean increased period of survival over that of controls was expressed as a percentage of the similar effect of a standard dose of chlortetracycline within the same experiment. The viruses of trachoma and inclusion blennorrhoea were incubated at 35°[5,6] the others at 38°. The first pair of viruses multiply much better and cause a heavier mortality at the lower temperature.

Mice. Groups of 10 randomized mice weighing 17 g ± 0·5 g for trachoma and infectious blennorrhoea and 19 g ± 0·5 g for the other infections were dosed twice daily (once Saturdays and Sundays) up to the twelfth day. Unless otherwise stated, dosing began 4 to 48 hours after introduction of the virus by one or other route. Often the groups of 10 consisted of 5 males and 5 females. With the ophthalmic viruses more consistent and better infections resulted in the BSVS strain of mice.[7] The mice were observed for 28 days.

Viruses. The virus here designated P is the MOH 154 strain of psittacosis originally obtained through the courtesy of Sir Samuel Bedson. The LGI virus came from another laboratory as a lymphogranuloma virus: it is infective by the intraperitoneal route and is not sulphonamide-susceptible. The JH and Ross strains of lymphogranuloma virus were kindly supplied by Professor C. Barwell; the first is a classical strain long passaged in the yolk-sac, the second a strain recently isolated in the yolk-sac. Professor Barwell also provided the T'ang strain of trachoma virus and Dr. L. H. Collier the Gambian strains of trachoma (G 1 and G 17) and the two strains of inclusion blennorrhoea (Dav and LB 1).

Infection with LGI Virus

Results in the Chick-Embryo

Table I combines the results of three experiments which gave closely similar results. It is clear that with suitable dosage in

the chick-embryo, 17,025 and 25,187 approximate to chlortetracycline in antiviral activity.

Table I. Effect of chlortetracycline, 17,025 and 25,187 on infections of the chick-embryo with LGI virus[a]

Treatment and dose in mg		Survivors/24	Mean period of survival in days	Percentage effect
None		0	5·0	—
Chlortetracycline	1	20	13·5	100
	0·1	0	7·9	34
	0·01	0	5·3	4
	0·001	0	4·9	−1
17,025	5	10	11·2	73
	1	17	12·6	89
	0·1	0	5·7	8
	0·01	0	5·1	1
25,187	0·5	19	13·1	95
	0·1	2	7·1	25
	0·01	0	5·0	0

[a]The table combines the results of 3 experiments which gave closely similar results. We administered a single dose of drug 2½ h after virus into the yolk-sac on the seventh day of incubation. The eggs were opened 13 days later and surviving embryos given a value of 14. The lengthened period of survival resulting from a given treatment is expressed as a percentage of that due to 1 mg chlortetracycline.

Results in the Mouse

The results of chemotherapeutic experiments in mice can only partly be evaluated on the basis of figures for survival. One drug may be sufficiently active not only to allow survival of all treated animals, but also so to suppress signs of disease, so that the treated animals at no stage manifest symptoms either of infection or of toxicity. This state of affairs always obtains with chlortetracycline at a dose of 1 mg twice daily by mouth to animals infected intraperitoneally, and frequently at a dose of 0·1 mg. Another drug, on the other hand, may permit all treated animals to survive, but during the period of dosing the animals suffer from obvious illness, either infectious or toxic; the degree of activity of such a substance can hardly be rated as high as that of chlortetracycline. With remedies having still less effect one may see: (*a*) partial

sparing of mortality with an increased mean period of survival of animals ultimately dying; or (*b*) an increased mean period of survival with no reduction in mortality. In either event, some or all of the deaths may occur well after the arbitrarily determined period of dosing has expired. Finally, the efficacy of a drug in sterilizing the infection, and the ease or not with which drug-resistance may be induced, are further points of importance with the psittacosis–lymphogranuloma group of organisms.

Table II presents the results of oral dosing of mice infected intraperitoneally with LGI virus. When treatment begins 4 h after infection, 17,025 and 25,187 both show considerable antiviral activity—on a weight-for-weight basis possibly rather less

Table II. Effect of orally-administered chlortetracycline, 17,025 and 25,187 on infections in the mouse with LGI virus[a]

Treatment and dose in mg		B.i.d. from 4 h after virus		B.i.d. from 5 days after virus	
		deaths/10	symptoms	deaths/10	symptoms
None		9 (6·7)	++	9 (7·2)	++
Chlortetracycline	1	0	0	—	–
	0·1	0	0	2 (7·0)	+
	0·01	3 (8·0)	+	7 (8·7)	++
17,025	10	0	0	—	–
	5	0	0	3 (6·3)	+
	1	0	±	1 (12·0)	+
	0·2	6 (8·0)	+	6 (5·9)	++
25,187	10	0	±(T)	—	–
	5	0	0	4 (6·0)	+
	1	0	±	5 (9·2)	++
	0·2	5 (10·2)	++	10 (6·4)	++

[a] Infecting dilution of virus $10^{-5.5}$ intraperitoneally. The figures in parentheses are the mean periods of survival of animals which died. (T) indicates that the symptoms probably arose from toxicity. Dosing ceased on the twelfth day after virus. Mice were observed for 28 days.

than one-tenth that of chlortetracycline. When treatment is delayed for 5 days, by which time, as will be apparent later, virus in the spleens and doubtless elsewhere has reached a titre of about 10^{-6}, no remedy is successful in curing all animals subjected to this

very severe experience; under these circumstances, the activity of 17,025 approaches that of chlortetracycline more nearly than does that of 25,187. On the sixth day of this experiment the first deaths occurred, yet after the third dose of chlortetracycline or 17,025 clinical improvement was clearly evident in mice which were not actually moribund when treatment began. Improvement followed rather more tardily with 25,187.

Experiments which will not be described in detail showed that broadly similar results follow intraperitoneal dosing with 17,025 and 25,187, beginning either 4 or 48 h after intraperitoneal infection. Moreover, oral dosing beginning 4 h after infection is capable of countering up to 10,000 times the dose of virus used to infect the mice in Table II; twice-daily amounts of 5 mg of either 17,025 or 25,187 completely controlled intraperitoneal infections with a dilution of $10^{-1.5}$ LGI virus. On the other hand, curtailment of the periods of oral dosing to 8 and 5 days (beginning 4 h after virus) leads to less satisfactory results with chlortetracycline and with the two synthetic substances.

Both 17,025 and 25,187 are but sparingly soluble in water. When injected intramuscularly these compounds persist locally for at least 4 days, the latest time at which observation was made. Histological examination reveals no sign of appreciable local irritation. It seemed, therefore, that the viral infection might be treated adequately by infrequent doses given intramuscularly or intraperitoneally. Table III shows that this is in fact the case; two intraperitoneal doses completely suppress symptoms and prevent deaths, while a single intraperitoneal dose of 17,025 and two intramuscular doses of either drug are nearly as effective in saving mortality and in almost abolishing signs of infection.*

Many antiviral remedies are less efficient against cerebral infections than against those established elsewhere. The intracerebral route of introduction is, of course, a highly artificial one, and many drugs do not reach effective concentrations in the brain. Unfortunately, however, several viruses of the group under consideration have been transmitted to mice of the age used in this

* As already stated these experiments were carried out with the compound dispersed in an aqueous medium. We have since found that if suspended in arachis oil the compound is even more effective and a single intramuscular dose given 4 h after intraperitoneal inoculation completely abolishes all signs of disease.

Table III. Effect of infrequent parenteral dosing with 17,025 and 25,187 on infections in the mouse with LGI virus[a]

Treatment and dose in mg		Intraperitoneal dosing				Intramuscular dosing 4 h and 7 days after virus	
		4 h after virus		4 h and 7 days after virus			
		deaths/10	symptoms	deaths/10	symptoms	deaths/20	symptoms
None		9 (6·8)	++	9 (7·2)	++	19 (7·5)	++
17,025	10	0	±	0	0	1 (15·0)	±
25,187	5	2 (8·0)	+	0	0	1 (26·0)	±

[a] Infecting dilution of virus $10^{-5.5}$ intraperitoneally. The figures in parentheses are the mean periods of survival of animals which died.

Table IV. Comparison of the efficacy of chlortetracycline, 17,025 and 25,187 in the therapy of intraperitoneal and intracerebral infections with LGI virus in the mouse[a]

Treatment and dose in mg			Virus given intraperitoneally		Virus given intracerebrally	
			deaths/10	symptoms	deaths/10	symptoms
None			9 (6·8)	++	10 (8·2)	++
Chlortetracycline	1,	orally b.i.d.	0	0	1 (9·0)	±
	0·1,	orally b.i.d.	0	±	7 (8·9)	++
17,025	10,	i.p. once	0	±	4 (8·8)	±
	10,	orally b.i.d.	0	0	8 (10·9)	+
	2,	orally b.i.d.	0	0	10 (7·1)	++
25,187	5,	i.p. once	2 (8·0)	±	2 (6·5)	±
	5,	orally b.i.d.	0	0	0	±
	2,	orally b.i.d.	0	0	10 (9·5)	+

[a] Infecting dilution of virus $10^{-5.5}$. Drugs administered from 4 h after virus. Other details as in previous tables.

work only by intracerebral injection,[8,9] so that it is a necessary expedient for comparing the relative chemotherapeutic susceptibilities of these viruses in the mouse. Table IV illustrates the difficulty attending therapy of a cerebral as opposed to an intraperitoneal infection. Despite this, an effect is evident with all three therapeutic agents; with 17,025 the single intraperitoneal dose seemed more effective than repeated oral doses, which were more satisfactory with 25,187 and with chlortetracycline.

We made two attempts to demonstrate a synergistic action of chlortetracycline paired with 17,025, 25,187 or 2,3-dimethyl-1,4-quinoxaline oxide,[10] or of the synthetic substances paired with each other. Suboptimal doses of each substance were administered singly to groups of mice, while other groups received half the dose of each of two compounds in combination. Clinical assessment furnished no indication of a striking advantage accruing from combining two remedies.

Daily titrations of the pooled spleens of 6 mice infected intraperitoneally and treated with one or other substance afford clear proof of their antiviral properties (Table V). Neither of the synthetic substances, at near maximum tolerated doses, suppress viral growth to the same degree as a large dose of chlortetracycline; 17,025 is more efficient than 25,187 in this respect, but even so the latter usually reduces the titre of 2 log units or more. All treatments abolished mortality in parallel groups of mice kept under observation.

None of the remedies at present available for treatment of diseases caused by the large viruses sterilizes the infection with certainty, unless possibly when given at overwhelming doses. Examination of the spleens of individual mice 30–35 days after infection, i.e. a minimum of 18 days after dosing with compound ceased, showed that with moderate dosage affording good clinical protection the majority of mice still harbour infection (Table VI). We have already commented upon the frequent reappearance of virus, when dosing ceases, in animals treated with chlortetracycline,[11] in spite of the data obtained from growth-curves which suggest complete elimination of virus (Table V). On the other hand, sufficiently large doses of 17,025 and 25,187 come near to eradicating virus. The spleens were tested for the presence of virus by passage intraperitoneally in mice and into the yolk-sac

Table V. Growth-curves of LGI virus in mice treated with chlortetracycline, 17,025 or 25,187[a]

Treatment		Days after infection				
		1	2	3	4	7
None		3·3	4·5	5·7	6·2	7·0
Chlortetracycline	1 mg orally b.i.d.	N.V.	N.V.	1·5	0·6	N.V.
17,025	5 mg orally b.i.d.	0·6	0·8	1·9	1·5	2·1
25,187	5 mg orally b.i.d.	1·9	2·1	3·0	2·5	2·4

[a] Infecting dilution of virus $10^{-4.5}$ intraperitoneally. Dosing began 4 h later. The pooled spleens of 6 mice were titrated at the times stated. The figures are the negative logarithms, to the base 10, of suspensions giving a 50 per cent endpoint as defined elsewhere.[11]

N.V. = No virus detected.

Mortality in parallel groups of 10 mice: No treatment 10 (6-8)
Chlortetracycline 0
17,025 0
25,187 0

Table VI. Persistence of LGI virus in the spleens of treated mice

	Treatment	Number examined	Number positive	% positive
Chlortetracycline	1 mg orally b.i.d. from 4 h after infection	40	22	55
	0·1 mg ,, ,,	68	61	90
17,025	10 mg orally b.i.d. from 4 h after infection	30	1	3
	5 mg ,, ,,	52	26	50
	2·1 mg ,, ,,	44	41	93
	10 mg i.p. 4 h and 7 days after infection	10	0	0
25,187	10 mg orally b.i.d. from 4 h after infection	29	4	14
	5 mg ,, ,,	58	27	47
	2·1 mg ,, ,,	35	21	60
	5 mg i.p. 4 h and 7 days after infection	6	1	17

of chick-embryos. The two hosts gave similar results, except that if only small amounts of virus were present all the eggs commonly succumbed, whereas in groups of 3 mice only 1 or 2 might manifest definite signs of disease. Thus the developing chick-embryo possesses some advantage over the mouse for the detection of residual virus; it is, however, necessary to culture or to examine microscopically one or more yolk-sacs from each group, to exclude deaths due to possible contamination with a different pathogen.

A serious disadvantage of the quinoxaline oxides as antiviral agents was the readiness with which drug-resistance developed.[10] Indeed, these compounds are in current use in at least two laboratories as experimental tools for the study of the phenomenon.[12, 13] Our studies seem to show that with 17,025 and 25,187 drug-resistance is much less prone to appear. Groups of mice received virus intraperitoneally. Some remained untreated and others were given twice-daily doses of compound in an amount insufficient to prevent deaths. On the day of the first death in a group, the remainder of the mice of that group were sacrificed and the pooled spleens passed immediately to fresh mice, which in turn received treatment forthwith. Doses of each compound were initially 0·2 mg and they rose progressively during the experiment to 0·5 mg. The virus was thus continuously in contact with the drug during the experimental period of 115 days. After 28 passages each pooled virus—untreated, 17,025-treated and 25,187-treated—was tested in dilutions of 10^{-3} and 10^{-5} intraperitoneally against three dose-levels of each compound. No virus was more or less resistant to therapy than any other.

Comparative Observations with Other Viruses of the Group and with Other Chemotherapeutic Agents

Results in the Chick-Embryo

If groups of infected embryos are treated with single doses of drug diminishing by tenfold or fivefold steps, at some point there comes a sharp break in the efficacy of the treatment (Table I). At one level of dosage, a level depending on the particular chemotherapeutic agent and the virus under study, the increased mean

period of survival of the eggs expressed as a percentage of that obtained with a standard dose of chlortetracycline is frequently well above 50 per cent, whereas at the next level of dosage it is well below. The results are usually consistent, so that confidence can be placed in the average of three to five replicate tests. The larger of the two doses may be considered as the 'minimally effective dose (M.E.D.)'.

Against all but overwhelming infecting doses, chlortetracycline and oleandomycin have an M.E.D. of 0·1 mg for the JH and Ross strains of lymphogranuloma, and for the three trachoma and the two inclusion blennorrhoea viruses (Table VII). As pointed out elsewhere,[1] in the chick-embryo tetracycline, oxytetracycline and erythromycin are consistently more active than is chlortetracycline; they have an M.E.D. of 0·01 mg against all these viruses, as does also sodium penicillin. For chloramphenicol, carbomycin and 17,025 the M.E.D. is 1 mg. Compound 25,187 has an M.E.D. of 0·1 mg for the strains of lymphogranuloma and trachoma, but both strains of inclusion blennorrhoea require about five times this amount to control an equal number of infective units. Sulphadiazine is often more active than is sulphadimidine, by a factor of rather less than ten, but with these substances differences appear in the response of the different strains of a single virus. Actually, inter-strain differences are demonstrable also with penicillin, though they are less marked than with the sulphonamides.

The other two viruses included in this investigation (P and LGI) contrasted sharply with the foregoing ones. In infections with these viruses, the M.E.D. of the various antibiotics and synthetic compounds were commonly five to ten times greater than with the viruses considered in the preceding paragraph (see also chlortetracycline and 25,187 in Table I). To penicillin and the sulphonamides, however, the P and LGI viruses were still more resistant; the M.E.D. for the former was in excess of 1 mg and no therapeutic effect whatsoever was apparent with doses of the sulphonamides of 10 mg. Only two remedies possessed equal activity against these infections, namely 17,025 and oleandomycin which controlled them at doses of 1 mg and 0·1 mg respectively, as was the case with trachoma, etc.

Table VII. Minimally effective doses of antiviral remedies in mg for viruses of the psittacosis–lymphogranuloma–trachoma group in the chick-embryo

Drug	Psittacosis	Lymphogranuloma		Trachoma			Inclusion blennorrhoea	
	P	JH	Ross	T'ang	G1	G17	Dav	LB1
Chlortetracycline	1	0·1	0·1	0·1	0·1	0·1	0-1	0·1
Tetracycline	0·1	0-01	0·01	0·01	0·01	0·01	0·01	0-01
Erythromycin	0·1	0·01	0·01	0·01	0·01	0·01	0·01	0·01
Oleandomycin	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1
Chloramphenicol	1	1	1	1	1	1	1	1
Carbomycin	>1	1	1	1	1	1	1	1
Penicillin–sodium salt	>1	0·01	0·01	0·01	0·01	0·01	0-01	0·01
17,025	1	1	1	1	1	1	1	1
25,187	0-5	0·1	0·1	0·1	0-1	0-1	0·5	0·5
Sulphadiazine	>10	0·1	0·01	0·1	0·1	0·01	0-1	0-01
Sulphadimidine	>10	0-1	0·1	1	0·1	0·1	0·1	0·1

Results in the Mouse

A similar procedure may be adopted in assessing the M.E.D. in mice. Unfortunately, with the viruses of present interest, a number of difficulties arise. In the first place, only some of these have been adapted to mice, and then only by the intracerebral route[8, 9] which as shown above militates severely against efficient therapy by many chemotherapeutic agents. Secondly, none of the newly-adapted viruses kill more than a small minority of the mice, which after a period of symptomatic abnormality gradually recover. With these viruses, therefore, the effect of therapy has to be judged on the presence or absence of signs of disease, a distinction which is a fairly sharp one at about 7–12 days after infection. Although in psittacosis it is far easier to produce a definite effect on mortality than it is to suppress symptomatology completely, to preserve uniformity in this study the same criterion has been adopted for psittacosis virus, while to estimate how far differences in therapeutic efficiency are due to the introduction of virus by different routes, with this virus data have been obtained for both intraperitoneal and intracerebral inoculation. Table VIII summarizes the observations with doses of the various remedies diminished by tenfold or fivefold steps. Oral dosing began 48 h after virus and was continued twice daily to the twelfth day after infection, except with procaine-penicillin which was administered subcutaneously every third day. The dose of psittacosis virus was an approximate LD_{100}.

The observations with psittacosis emphasize the unsatisfactory nature of the intracerebral test in revealing therapeutic agents likely to be useful against less artificial infections. They also demonstrate the resistance of this virus to therapy with penicillin and the sulphonamides. The LGI virus similarly treated is also resistant to the sulphonamides, but is at least ten times more susceptible to penicillin. Under the conditions of dosing in these experiments, no advantage accrued from using the more persistent sulphonamides, sulphadimethoxypyrimidine, sulphamethoxypyridazine, sulphadimethoxine or 6-methoxy-4-sulphanilamidopyrimidine, in place of sulphadiazine or sulphadimidine. The results also confirm the usual greater activity of sulphadiazine against those viruses which are susceptible to its action, the

Table VIII. Minimally effective doses of antiviral remedies in mg twice-daily for viruses of the psittacosis–lymphogranuloma–trachoma group in mice

Drug	Psittacosis		Lymphogranuloma		Trachoma T'ang i.c.	Inclusion blennorrhoea LB1 i.c.
	P i.p.[a]	P i.c.	JH i.c.	Ross i.c.		
Chlortetracycline	0·1	1[b]	0·1	0·1	0·1	0·1
Tetracycline	1	1[b]	1	1	1	1
Erythromycin	>1	5[b]	>1	1	>1	1
Oleandomycin	>1	10[b]	>1	>1	>1	>1
Chloramphenicol	10	10	2	10	2	2
Carbomycin	>10	>10	10	>10	10	10
Procaine–penicillin	10	10	1	0·1	0·1	1
17,025	5	>5	>5	5	>5	>5
25,187	5	5[b]	1	5	1	5
Sulphadiazine	>5	>5	0·01	0·01	0·1	0·1
Sulphadimidine	>5	>5	0·1	0·1	0·1	1

[a] These figures are based not on mortality but on a complete suppression of signs of disease between the seventh and twelfth days. They are thus in excess of the minimal dose needed to produce a definite effect on mortality from psittacosis introduced by the intraperitoneal route.[1]

[b] Although animals receiving these doses remained free from symptoms during the period of therapy, many relapsed and died 5–15 days after therapy was discontinued.

superiority of chlortetracycline over tetracycline or erythromycin in the mouse,[1] and the favourable response of lymphogranuloma and the ophthalmic infections to therapy with penicillin. Compound 17,025 does not show great activity in these tests, but we have already shown that against intracerebral infections it is more effective when given by a single intraperitoneal injection.

Observations on Toxicity of 17,025

Of the two new compounds with antiviral properties, 17,025 has the lower toxicity; indeed it has been difficult to demonstrate ill-effects of its administration. For single oral doses in mice the LD_{50} is in excess of 5000 mg/kg, and for intraperitoneal injection in excess of 2500 mg/kg. Repeated oral or intraperitoneal dosing of male and female rats produced no abnormalities in the organs attributable to the action of the drug. The schedules of dosing were:

500 mg/kg orally once daily, 5 days weekly, for 20 days
1170 mg/kg orally twice daily for one week
233 mg/kg intraperitoneally twice daily for one week

Sub-acute toxicity tests in mice and guinea-pigs indicate a similarly low toxicity.

Male and female dogs given 100 mg/dog intramuscularly three times weekly for four weeks showed no abnormalities attributable to the drug.

Intramuscular injection into rats, with subsequent examination at intervals of the site of inoculation, revealed no excessive local reaction.

Twice-daily application to the conjunctiva of rabbits for three weeks provoked no sign of irritation. The compound was applied in a water-miscible cream, which is a more effective means of bringing the compound into contact with the conjunctiva than is an ointment.

Comment

The two new synthetic compounds here shown to be therapeutically active against viruses of the psittacosis–lymphogranuloma–trachoma group are but sparingly soluble. One, 17,025,

has very low toxicity, and is both effective and non-irritant when given by the intramuscular route. Although weight for weight it is rather less than one-tenth as active as chlortetracycline, its clinical use might perhaps be envisaged when it is desired to control an infection by infrequent parenteral therapy, rather than by prolonged topical application or by frequent oral dosing.

Summary. 5-Methoxycarbonyl-6-methyl-4-(5-nitrofuryl)-2-oxo-1,2,3,4-tetrahydropyrimidine and 4-β-hydroxyethylamino-6-nitro-2-trichloromethylquinoline are therapeutically active against viruses of the psittacosis–lymphogranuloma–trachoma group. The former is of very low toxicity.

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