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1 : 6 - DI - 4' - CHLOROPHENYLDIGUANIDOHEXANE ("HIBITANE"*). LABORATORY INVESTIGATION OF A NEW ANTIBACTERIAL AGENT OF HIGH POTENCY

BY

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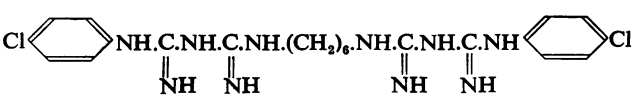
During an investigation of the biological properties of certain polydiguanides it was found that bisdiguanides of the general type:

aryl.NH.C(:NH).NH.C(:NH).NH.$(CH_2)_n$.
NH.C.(:NH).NH.C(:NH).NH.*aryl*‡

exhibited marked antibacterial activity *in vitro* against a wide range of micro-organisms, including *Pseudomonas pyocyanea*. Such bisdiguanides offered considerable scope for structural variation, and many substances of this kind were synthesized and tested (Table I) before it was ultimately decided that one compound, Serial No. 10,040, had the most outstanding bacteriostatic properties.



10,040 (Base)

This compound, in one form or another, has since been the subject of extensive laboratory, clinical, and veterinary study. The present communication is intended to record some of the background to the discovery of 10,040, and to indicate its useful potentialities through a brief account of the initial experimental observations made in these laboratories.

Two salts of the bisdiguanide 10,040, itself a colourless strongly basic substance, m.p. 134° C., were selected for study. One was the diacetate, m.p. 154° C., soluble to approximately 1.9% in distilled water at 20° C., and the second the dihydrochloride, m.p. 257° C., having a corresponding solubility of only 0.06%. The diacetate was clearly the more convenient for the preparation of stock solutions for laboratory work and for that reason has been used almost entirely throughout this research, although repeated tests *in vitro* have shown that the lower solubility of the dihydrochloride does not alter its antibacterial potency.

In addition to the determination of the antibacterial properties of 10,040, a number of other investigations, bearing on the potential applications of the agent, have been undertaken. These have included tests for compatibility with antibiotics such as penicillin; the effect of various possible vehicles known to support bacterial growth, such as milk and serum; the influence of *p*H, size of inoculum, presence of detergent substances; and the action of the drug on bacterial spores. The possible development of resistant organisms has also been studied. Finally, the behaviour in and towards the animal host has been followed by examining the influence of 10,040 on phagocytosis of bacteria by human leucocytes; by feeding experiments in rats extending over several generations; and by its local application to infected artificial wounds in mice.

METHODS

Measurement of Bacteriostatic Action.—Dilutions of the compound were made in Difco brain-heart infusion. Each tube was inoculated with one loopful of a 24 hr. culture of the test bacteria. The results were read after incubation for 24 hr. at 37° C.

Measurement of Bactericidal Action.—For this it was necessary to have available a substance which would neutralize, more or less completely, the action of the drug on the test bacteria in the medium used for subculture. It was ultimately found that egg-yolk (see Table III) contained such a compound, and this was applied as follows:

(*a*) *Preparation of Egg-yolk Solution.*—The yolks of six eggs were separated from the whites and

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‡ Patent protection pending.

emulsified with 600 ml. of Difco brain–heart infusion; 20 g. of kaolin was stirred in and the solution clarified by filtration through paper and then sterilized by Seitz-filtration.

(*b*) *Performance of Test.*—Standard inocula were prepared by growing the bacteria on the surface of 5 ml. of nutrient agar in a 50 ml. conical flask and suspending the growth in water with the aid of a few glass beads, as described by Davies (1951). Five ml. of this suspension of bacteria, diluted as required, was mixed with 5 ml. of the test solution. After the desired period of contact, 1 ml. of the mixture was added to 5 ml. of egg-yolk solution and 1 ml. samples of the inactivated solution plated out to obtain viable counts.

Action of 10,040 on Bacterial Spores.—Several strains of sporing bacilli were isolated freshly from garden soil. They all resisted the action of boiling water for 1 hr. Pure cultures of the bacilli were grown for 4 days on agar, by which time nearly all the cells had spored. Growth was washed off the agar with sterile distilled water and the suspension heated at 80–90° C. for 15 min. The heated suspension was then adjusted to contain 10,000,000 spores per ml. as determined by haemocytometer counts. These spores were suspended in 1% and 0.1% solutions of 10,040 to give final concentrations of 1,000,000 spores per ml. After varying intervals of time samples were inactivated with egg-yolk and tested for the presence of viable spores.

Treatment of Artificial Wounds in Mice.—The technique of Gordon *et al.* (1947) was used. An ellipse of skin was cut from the back of a mouse anaesthetized with ether, and 2 drops of a 24 hr. culture of Group A streptococci (Kruger) dropped on to the exposed fascia, which was then lightly scarified. Fifteen minutes later 2 drops of the test solution were dropped into the wound and the skin closed with a single Michel clip.

Therapeutic Activity in Mice.—Groups of 10 mice were infected intraperitoneally with 0.2 ml. of a 10^{-4} dilution of a blood broth culture of *Streptococcus pyogenes*. The mice were treated with 0.2 mg. per 20 g. of 10,040 diacetate intraperitoneally, treatment being given just before, and at various periods after, infection.

RESULTS

Bacteriostatic Action.—Table I provides a selection of the several hundred substances prepared during the course of this work, and illustrates the connexion between chemical structure and bacteriostatic effect. Activity in each compound is related to 10,040 as standard. The data for the first three compounds (12,483, 10,040, and 11,383) showed the influence of the length of the polymethylene chain and, with other results not recorded here, illustrated the superior effect provided by hexamethylene. Other compounds were made using different kinds of conjunctive groupings—for example, aromatic and mixed aromatic-aliphatic systems such as those in 11,384 and 11,385—but in general these were much less active. It was also found that the solubility in water, of bisdiguanides varied in this manner, was often greatly diminished. Many variations were made in the type of group or groups substituted in the terminal benzene rings. The relative activities of a few of these preparations are shown in Table I, selecting for convenience of comparison those based on the hexamethylene conjunctive system. Omission of the chlorine atoms (10,387), or the introduction of a second halogen into each benzene ring (11,386), reduced activity, while the depressant effect of the acidic hydroxyl and carboxyl groups, in 11,108 and 10,691 respectively, was

TABLE I

COMPARATIVE BACTERIOSTATIC ACTIVITY OF A NUMBER OF AGENTS RELATED TO 10,040

Compounds of type: R.NH.C.NH.C.NH(X)NH.C.NH.C.NH.R
‖ ‖ ‖ ‖
NH NH NH NH

Code No.	Terminal Group R	Central Unit X	Comparative Bacteriostatic Effect		
			Bact. coli	*Staph. aureus*	*Ps. pyocyanea*
12,483	4-Chlorophenyl	Trimethylene	0·3	1	0·3
10,040	,,	Hexamethylene	1	1	1
11,383	,,	Decamethylene	0·3	0·3–1	<0·01
11,385	,,	(4 : 4′)-Diphenylmethane	1	0·3	0·3
11,384	,,	(1 : 4)-Phenylene	0·1	0·1	0·03
10,387	Phenyl	Hexamethylene	0·3–1	0·3–1	0·1
11,386	3 : 4-Dichlorophenyl	,,	0·3	0·3	1
11,108	4-Hydroxyphenyl	,,	0·03	0·01	<0·01
10,689	4-Methoxyphenyl	,,	0·3	0·1	0·01
10,691	4-Carboxyphenyl	,,	<0·01	<0·01	<0·01
9,381	RNH. = Et_2N	,,	0·1	0·3	<0·01
14,575	RNH. = 4-ClC_6H_4NMe-	,,	0·3	1	0·1
10,030	RNH.C. = hydrogen ‖ NH	,,	<0·01	<0·01	<0·01
11,717	RNH.C. = 4-chlorophenyl ‖ NH	,,	0·03	0·03	0·03

especially marked. The need for the terminal aromatic nucleus was illustrated by the antibacterial results obtained with the bisdiguanide 9,381, while the comparative uselessness of the guanidine system—in contrast to that of the diguanide—was shown with compounds 10,030 and 11,717.

The bacteriostatic activity of 10,040 against a wider range of organisms is shown in Table II. Gram-negative bacteria were sensitive to its action, but, on the whole, they were rather less sensitive than Gram-positive species.

TABLE II

BACTERIOSTATIC ACTION OF 10,040 ON VARIOUS BACTERIA IN HEART-BRAIN INFUSION

Organism	Minimal Concn. Inhibiting Growth at 37° C. in 24 hr.
Bacillus subtilis (1)	1 : 1,000,000
Streptococcus lactis (1)	1 : 2,000,000
Streptococcus pyogenes (4)	1 : 500,000–1 : 1,000,000
Corynebacterium diphtheriae (1)	1 : 500,000
Streptococcus pneumoniae (1)	1 : 500,000–1 : 1,000,000
Staphylococcus aureus (20)	1 : 500,000–1 : 2,000,000
Proteus vulgaris (1)	1 : 200,000
Salmonella pullorum (1)	1 : 300,000
Vibrio cholerae (1)	1 : 200,000
Streptococcus faecalis (1)	1 : 200,000
Salmonella dublin (1)	1 : 100,000
Salmonella typhi-murium (1)	1 : 100,000
Aerobacter aerogenes (1)	1 : 100,000
Bacterium coli (1)	1 : 100,000
Pseudomonas pyocyanea (10)	1 : 50,000–1 : 100,000

The figures in brackets indicate the number of strains tested.

In most bacteriostatic tests the transition from no growth to full growth was sharp. Occasionally, when the test organisms were staphylococci, a number of the tubes, which at first sight appeared clear, were found to contain a tiny white speck of growth at the bottom. When the supernatant broth was removed by centrifugation, and replaced by fresh broth not containing 10,040, the bacteria divided further until they produced full normal turbidity. These trace growths appeared at random throughout the series. Their appearance bore no relation to the concentration of 10,040 over a fairly wide range. Further experiments on these survivors are now in progress.

Neutralization.—The powerful inhibition of the bacteriostatic activity of 10,040 by broth containing egg-yolk, and the ultimate use of this phenomenon to distinguish between bacteriostatic and bactericidal effects, has already been commented upon. The earliest attempts to achieve this end concerned the use of simple anions such as sulphate, which might, for example, have caused deactivation of the agent by precipitation of the very sparingly soluble disulphate ; but no demonstrable difference in growth was observed. Many

TABLE III

THE EFFECT OF VARIOUS SUBSTANCES ON THE BACTERIOSTATIC ACTIVITY OF 10,040 ON *STAPH. AUREUS B.E.*

Substance Added to Heart-Brain Infusion	Minimal Inhibitory Concn. of 10,040
None	1 : 500,000
1% yeast nucleic acid	1 : 500,000
1% suramin	1 : 200,000
0·1% thioglycollic acid	1 : 500,000
10% full cream evaporated milk	1 : 100,000
10% rabbit serum	1 : 200,000
1% Lubrol W	1 : 200,000
1% Tween 80	1 : 500,000
0·1% egg lecithin	1 : 500,000
Egg-yolk (1 yolk per 100 ml.)	1 : 4,000

other substances examined for the same purpose (Table III) also permitted the retention of a considerable degree of bacteriostatic activity. These included serum, nucleic acid, milk, suramin (chosen as a polyanionic agent), and Lubrol W (which neutralizes quaternary ammonium compounds). It will be noted that lecithin was without action.

Bactericidal Action.—The bactericidal action of 10,040 was unusual. A very high proportion of the inoculum was killed within a short time even at high dilutions. Much higher concentrations were required to produce a 100% kill.

TABLE IV

BACTERICIDAL ACTION: EFFECT OF VARYING THE CONCENTRATION OF 10,040 ON THE NUMBER OF SURVIVING BACTERIA. *STAPH. AUREUS B.E.*: *p*H 7.0, 20° C.

Concn. of 10,040	No. of Bacteria per ml. Surviving		
	0 min.	5 min.	10 min.
1 : 20,000	2×10^8	200	70
1 : 30,000	2×10^8	4,000	200
1 : 40,000	2×10^8	600	5
1 : 50,000	2×10^8	11,000	0
1 : 60,000	2×10^8	2,500	40
1 : 70,000	2×10^8	40,000	150
1 : 80,000	2×10^8	7,000	120
1 : 90,000	2×10^8	5,000	800
1 : 100,000	2×10^8	23,000	2,000
1 : 200,000	2×10^8	150,000	600

Table IV illustrates this phenomenon. A concentration of 1 : 200,000 of 10,040 killed more than 99.9% of the bacteria present in 5 min., but 1 : 20,000 did not kill completely in 10 min. Reduction in the size of inoculum, with any given period of contact between 10,040 and the bacteria still resulted in the appearance of substantially the same number of survivors (Table V). The first hypothesis advanced to account for the survivors was that they represented a few cells which were naturally resistant to the action of 10,040. When, however, the survivors were re-grown and re-tested, the same phenomenon appeared. The bactericidal action was influenced by the *p*H of

TABLE V

BACTERICIDAL ACTION: EFFECT OF INOCULUM-SIZE ON THE NUMBER OF SURVIVING BACTERIA. *STAPH. AUREUS B.E.*: *p*H 7.0, 20° C.; 1: 20,000 OF 10,040

No. of Bacteria per ml. Added	No. of Bacteria per ml. Surviving After		
	5 min.	15 min.	60 min.
6×10^8	1,300	40	4
6×10^6	1,200	150	15
6×10^3	70	14	8

TABLE VI

BACTERICIDAL ACTION: INFLUENCE OF *p*H ON THE NUMBER OF SURVIVING BACTERIA. *STAPH. AUREUS B.E.*: 20° C.; 1: 20,000 OF 10,040

*p*H of Solution	No. of Bacteria per ml. Surviving After		
	0 min.	5 min.	15 min.
5·25	4,000,000	>1,000,000	>1,000,000
6·22	4,000,000	2,300,000	50,000
7·08	4,000,000	90,000	5,000
7·97	4,000,000	17,000	900

NOTE: The 10,040 was dissolved in M/15 phosphate buffer; portions of the solution were adjusted to the desired *p*H using a glass electrode.

the solution ; the activity increased with increasing alkalinity (Table VI).

Compatibility with Antibiotics.—10,040 is compatible with penicillin, streptomycin, chloramphenicol, oxytetracycline, and aureomycin. With penicillin, for example, bacteriostatic assays were made against *Staphylococcus aureus A.B.* and *A.C.*, using six serial dilutions of penicillin ranging from 0 to 0.25 u./ml., divided into four groups each containing concentrations of 10,040 of 0, 0.1, 0.5, and 1.0 μg./ml., respectively. In no case was there any indication that the action of the one agent was influenced by the presence of the other.

The Toxicity of 10,040.—The acute toxicity of 10,040 given by various routes to mice is shown in Table VII. The mice received one dose of the compound and were observed for a period of 10 days.

The compound appeared to have a very low toxicity when given orally to rats over long periods.

TABLE VII

THE ACUTE TOXICITY OF 10,040 IN MICE

(Albino mice weighing 16–22 g. given one dose of the compound and observed for ten days. The numbers of mice used are given in parentheses.)

Salt of 10,040	Route of Administration	Weighted Mean LD50 (mg./kg.) ± S.E.
Dihydrochloride	Subcutaneously in 0·5 ml.	>5,000 (40)
Diacetate ..	,, ,, 0·5 ,,	325±201 (20)
,, ..	Intraperitoneally in 0·1 ,,	38±3·8 (95)
,, ..	Intravenously in 0·1 ml.	25±2·0 (90)
,, ..	Orally in 0·5 ml.	2,000±580 (100)

Two groups of rats, each of 12 females and 2 males, were selected. The control group was given water to drink and the other group received a 1 in 2,000 solution of 10,040 diacetate as its only source of water. Litters were obtained from all the females. Two litters were produced from these animals of the second generation, the dosing with 10,040 being maintained throughout. The original parents had been drinking 10,040 for one year when the experiment ended.

The rats were weighed weekly throughout the experiment. Every three months a total and differential blood-cell count was made and the blood haemoglobin was estimated. At the end of the experiment the rats were killed and the tissues examined histologically. In every respect the treated animals were similar to the controls.

Effect on Phagocytosis.—A 1:10,000 solution of 10,040 in normal saline did not affect the phagocytosis of staphylococci by human leucocytes when tested by the method of Davies (1951).

Action on Bacterial Spores and on Mycobacterium tuberculosis.—No spores were recovered from a 1% solution of 10,040 after 2 hr. at room temperature, but a 0.1% solution had caused no reduction in numbers after 24 hr. at room temperature.

10,040 inhibited the growth of *M. tuberculosis* at 1 in 1,000,000 in the medium of Dubos and Middlebrook (1947). It was inactive at 1 in 1,000 on Löwenstein's medium (which contains egg-yolk).

Failure of Bacteria to Develop Resistance to 10,040.—Many attempts have been made to demonstrate bacterial resistance to 10,040, but without success. Serial transfers were made in a variety of media with several strains, both of *Staph. aureus* and *Ps. pyocyanea.* No increase in resistance was noted.

The Action of 10,040 in the Treatment of Artificial Wounds in Mice.—Table VIII shows that 10,040 exerted a marked action on artificial wounds in mice infected with pathogenic streptococci. Fourteen out of 15 mice treated with 1%

TABLE VIII

THE EFFECT OF 10,040 AND PROFLAVINE APPLIED TO ARTIFICIAL WOUNDS INFECTED WITH STREPTOCOCCI

Application	No. of Mice	Mean Survival Time in Hr. (max. 120 hr.)	No. of Survivors at 5 Days
1% 10,040 ..	15	117	14
1% proflavine ..	15	60	3
Controls	15	35	0

10,040 survived for five days. All 15 control mice died with a mean survival time of 35 hr. Proflavine (1%) exerted very little effect under the same conditions.

Therapeutic Activity in Mice.—Table IX shows that 10,040 exerted a slight but definite therapeutic effect against a streptococcal infection in mice when both drug and infection were given intraperitoneally. Treatment given 1, 6, 24, and 30 hr. after infection was no more effective than a single treatment given 1 hr. after infection. Thus 10,040 does not appear to influence those organisms which survive contact with it in the peritoneal cavity and subsequently invade the body. No therapeutic activity was demonstrable when 10,040 was given by the oral or subcutaneous routes to mice infected intraperitoneally.

TABLE IX

THE EFFECT OF 10,040 ON THE SURVIVAL TIME OF MICE INFECTED WITH STREPTOCOCCI

Interval between Dosing and Infection	Expt. No.	No. of Deaths on Day 1	2	3	4	5	Total Deaths	Mean Survival Time* (Days)
Dosed 1 hr. before	256	1	9				15/20	3·5
	258		4		1			
„ 1 min. „ ..	256		6	4			20/20	2·3
	258		9	1				
„ 1 „ after ..	256		7	1	1	1	16/20	3·5
	258		6					
„ 1 hr. „ ..	256		8	1		1	19/20	2·6
	258		7	2				
„ 2 „ „ ..	256	1	7	2			19/20	2·3
	258	1	8					
„ 4 „ „ ..	256	5	5				20/20	1·8
	258		9		1			
„ 1, 6, 24, and 30 hr. after	256		8	2			19/20	2·7
	258		5	3	1			
Untreated controls	256	8	2				20/20	1·1
	258	10						

* Mice dying on Day 1 allotted a survival time of 1 day; on Day 2 of 2 days, and so on. No mice died on the 6th or the 7th day; all those alive at 7 days were allotted a survival time of 8 days.

SUMMARY

1. A bisdiguanide, 10,040, "Hibitane," 1:6-di-4'-chlorophenyldiguanidohexane, was selected from an extensive series of related compounds as the one possessing the highest degree of antibacterial activity.

2. Its antibacterial action is exerted against a wide range of vegetative bacteria both Gram-positive and Gram-negative.

3. The compound is feebly active against bacterial spores.

4. Activity is maintained in the presence of body-fluids but is antagonized by a substance contained in egg-yolk.

5. Attempts to demonstrate development of drug-resistance were unsuccessful.

6. 10,040 has a very low oral toxicity for laboratory animals and does not interfere with phagocytosis by human leucocytes.

7. It has no true systemic antibacterial activity in mice but is highly active in artificial wounds infected with haemolytic streptococci.

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