Brit. J. Pharmacol. (1953), 8, 297.

THE THERAPY OF EXPERIMENTAL PSITTACOSIS AND LYMPHOGRANULOMA VENEREUM (INGUINALE)

II. THE ACTIVITY OF QUINOXALINE-1 : 4-DIOXIDE AND SUBSTITUTED AND RELATED COMPOUNDS, WITH A NOTE ON THE MORPHOLOGICAL CHANGES INDUCED IN LYMPHOGRANULOMA VIRUS BY THESE COMPOUNDS AND BY ANTIBIOTICS

BY

E. WESTON HURST, J. K. LANDQUIST, P. MELVIN, J. M. PETERS, N. SENIOR, J. A. SILK, AND G. J. STACEY

From Imperial Chemical Industries Limited, Biological Laboratories and Research Department, Hexagon House, Manchester, 9

(RECEIVED FEBRUARY 4, 1953)

In previous communications (Hurst, 1948; Hurst, Peters, and Melvin, 1950) we have recorded the therapeutic action of Nitroakridin 3582 and compared those of penicillin, chloramphenicol, aureomycin, and terramycin against psittacosis and lymphogranuloma venereum in mice or in developing chick-embryos. For the past eight years we have used these experimental infections (among others) in routine screening-tests of synthetic organic compounds for antiviral properties, and during this time have encountered a number of quite dissimilar chemical types each exhibiting some degree of activity against these particular infections. The most active compounds were quinoxaline-1 : 4-dioxide and various substituted derivatives, the best of which closely approached aureomycin in therapeutic potency. Alergant (1953) has confirmed this action in human lymphogranuloma, though toxic side-reactions rule out the possibility that the compounds will be useful additions to the pharmacopoeia. The quinoxaline oxides also manifest anti-amoebic and antibacterial properties (Jones, Landquist, and Stewart, 1953; McIlwain, 1943; Francis, 1953).

METHODS

The strains of virus used, the general method of working, and the method of computing 40% end-points (not 40% mortalities) in titrations of virus are sufficiently described in our earlier publications. When this work first began aureomycin was unknown, and in the earlier experiments we had no suitable standard of therapeutic activity. When later aureomycin became available in strictly limited quantities, we used it as a standard of reference in all experiments, giving a single dose of 1 mg. to eggs or 2 mg. twice daily to mice. Although when the antibiotic was more freely available it appeared that these were perhaps not quite the optimal doses (see Hurst, Peters, and Melvin, 1950), for the sake of consistency we decided to retain them throughout this investigation. Our experience over more than a hundred experiments, however, strongly suggests that the earliest samples of aureomycin were less effective than those issued somewhat later, so that in fact we did not achieve perfect consistency over the years, though we believe that we have done so for the greater part of the time. The largest amounts administered of other compounds were usually the "maximal tolerated doses" defined as follows: (*a*) for the mouse, as that quantity which, given twice daily (once on Saturday and Sunday) over a period identical with that in the chemotherapeutic test, caused no deaths and allowed the animals to attain a final weight not less than 90% of that of the controls; (*b*) for the egg, as that quantity which killed no embryos within five days of a single injection into the yolk-sac on the seventh day of incubation. At this point it may be noted that on many occasions we found that the maximal tolerated dose did not necessarily give results as favourable as a rather smaller one.

RESULTS

The Action of Quinoxaline-1 : 4-dioxide

Twice-daily oral doses of 0.5 mg. quinoxaline-1 : 4-dioxide per 20 g. greatly reduced mortality in groups of 30 mice infected intraperitoneally with psittacosis or lymphogranuloma virus. Thus in

nine experiments 25 to 30 control mice died as against 0 to 5 treated mice, while in three others the deaths among treated animals numbered 10, 10, and 11; the mean period of survival of mice ultimately dying was very materially lengthened in the treated groups. These results obtained whether treatment began 4 hours before infection with virus or 24 to 72 hours after, and an appreciable effect on mortality followed even when treatment was deferred until some animals of the group were moribund. Daily titrations of virus in the pooled spleens of six treated and six control mice showed this therapeutic effect to be associated with a tenfold to a hundredfold reduction in the growth of virus, though *in vitro* the drug did not inactivate even weak concentrations of virus within a period of 24 hours.

The optimal period of medication was 7 to 12 days; dosing for only 4 days gave inferior results, while no advantage accrued from prolonging treatment to 18 days. Twice-daily dosing was more effective than was exhibition of larger doses once daily or once every two days. Doses of 0.1 mg./20 g. were much less successful, as were usually the larger doses of 1 and 2 mg./20 g. Treatment was successful against even massive infecting doses of virus; indeed on several occasions we noted the curious phenomenon that, when treatment started 4 hours before infection with virus, it was relatively more effective against large than against smaller doses of virus. Table I illustrates this

TABLE I

EFFECT OF DOSE OF VIRUS ON THE OUTCOME OF TREATMENT OF PSITTACOSIS WITH QUINOXALINE-1:4-DIOXIDE

Virus was injected intraperitoneally four hours after the first dose of drug. Drug was given orally in doses of 0.5 mg./20 g. twice daily for 12 days. The animals were observed for 46 days. The figures in parentheses are the mean periods of survival of fatal cases in days

Infecting Dilution of Virus	Mortalities in Groups of 30 Mice			
	Experiment 1 (KLG Virus)		Experiment 2 (Ps Virus)	
	Treated	Untreated	Treated	Untreated
10^{-8}	2 (17·0)	3 (19·3)	8 (21·6)	9 (24·3)
10^{-7}	3 (15·0)	5 (10·8)	16 (23·1)	22 (10·1)
10^{-6}	3 (14·6)	7 (10·4)	16 (27·6)	23 (9·6)
10^{-4}	3 (17·6)	24 (8·8)	15 (29·2)	25 (8·8)
10^{-2}	8 (21·3)	25 (8·2)	12 (21·8)	28 (4·3)

phenomenon with psittacosis virus. It is probably explained in terms of the greater antigenic stimulus provided by the larger dose of virus during the period when its growth was largely suppressed by the drug; the smallest doses of virus evoked no immunity, and, as virus was not completely eradicated by therapy (see below), in the absence of immunity it still led to death of the more susceptible animals after dosing ceased.

Passage at about the 45th day of the spleens of mice recovered from one or other infection showed that 166 of 193 (86%) carried virus. In a long-term experiment in which 45 of 82 treated survivors from an initial group of 100 infected with psittacosis were killed at intervals between the 45th and 268th days, virus was still present in about three-quarters of the animals (74%). The 37 mice surviving to the 277th day were reinoculated with virus. Twenty-nine (78%) remained well, while the remainder developed psittacosis and died as did each of six previously uninfected controls. The close correspondence between the number of mice proving immune to reinoculation and that of animals carrying virus in the spleen is unlikely to be coincidental. The observations suggest that many clinically recovered mice carry virus for the rest of their lives. Nevertheless, 24 fresh mice introduced into the cages containing these mice, and left there from the 34th to the 277th day, developed no sign of disease; at the end of the period of contact they were not immune. The mice carrying virus were not therefore contagious for others. Meyer and Eddie (1951) have recorded analogous behaviour in human psittacosis.

Combined therapy of infected mice with quinoxaline-1:4-dioxide and aureomycin or procaine-penicillin usually gave results inferior to those with one drug alone.

A single dose of 0.25 mg. quinoxaline-1:4-dioxide given into the yolk-sac of developing eggs two hours after infection with the virus of lymphogranuloma venereum prolonged the mean period of survival of the embryos by about 50%. Doses of 0.1 and 0.5 mg. were rather less effective.

Mice infected intramuscularly with the virus of herpes febrilis or louping-ill, intracerebrally with mouse-adapted human poliomyelitis, or intranasally with influenza A received no benefit from medication with quinoxaline-1:4-dioxide.

Although thus possessing very marked activity against the largest viruses, quinoxaline-1:4-dioxide appeared to be therapeutically inferior to aureomycin. This opinion was based not so much on the figures for mortality as on the better condition of the aureomycin-treated mice during the active stages of the infection, and on their greater mean weight at the end of the experimental period. In both infected and non-infected animals the quinoxaline compound caused some retardation of growth, and an appreciable difference in weight from undosed controls still existed six and a half months after treatment ended. Among the substituted quinoxaline dioxides we have found compounds apparently free from this disadvantage and

at the same time possessing rather greater therapeutic activity than quinoxaline dioxide itself.

Examination of Substituted Quinoxaline-1:4-dioxide and Related Compounds

We have examined 93 compounds (Landquist and Stacey, 1952; Landquist, 1951; Landquist, 1952) for activity against lymphogranuloma in the chick-embryo, and 46 of these for activity against this disease and often psittacosis in the mouse. Groups of 18 eggs received a single dose of compound into the yolk-sac two hours after injection of virus. Groups of 30 mice received a large intraperitoneal dose of virus, and 48 to 72 hours were allowed to elapse before treatment started, usually by oral dosing twice daily for 12 days. Table II summarizes the results obtained with 10 of these compounds as typical of the observations made in the larger series. The results are expressed as percentages of the effect produced by a standard dose of aureomycin in the same experiment. Thus, with mice, if only 2 untreated controls survived while 28 of the aureomycin-treated animals did so, representing a saving of 26 lives, and in a quinoxaline-treated group 15 survived representing a saving of 13 lives, the percentage effect was held to be 50.

TABLE II

SUMMARY OF THE THERAPEUTIC ACTIVITY AGAINST PSITTACOSIS AND LYMPHOGRANULOMA OF 10 SUBSTITUTED QUINOXALINE-1:4-DIOXIDES

The entries in the Table (except in the last column) represent the therapeutic effects of the compounds expressed as percentages of that produced by aureomycin in the same experiment. The exact method of calculation is described in the text. The standard dose of aureomycin in eggs was 1 mg., in mice 2 mg. orally twice daily for 12 days. Figures in parentheses are means. The entries in the last column are the percentages of surviving mice carrying virus in the spleen at about the 40th day. R = quinoxaline-1:4-dioxide

Compound	Dose in mg.	LGI Virus in Chick-embryo, % Effect	LGI Virus in Mouse, % Effect	Ps Virus in Mouse, % Effect	% Surviving Mice Carrying Virus*
7331 2-Methyl—R	1·5	67	—	—	—
	0·5	99	45, 40 (43)	11	—
	0·1	—	5	0	—
8183 6-Methyl—R	5	—	104 88 (96)	50, 100 (75)	—
	1·5	49, 50 (50)	100	100, 96 (98)	92
	0·5	68, 59 (64)	50	24, 40 (32)	—
	0·2	73, 25 (49)	—	—	—
7218 2:3-Dimethyl—R	5	53, 34, 42 (43)	84, 125 (105)	54	38
	1·5	—	117, 95, 91, 125, 111, 104, 100, 125 (109)	24	67
	1	48, 64, 61 (58)	—	—	—
	0·5	86, 69, 122, 109, 83, 72, 108, 86, 66 (89)	81, 105, 121, 100 (102)	40	100
	0·2	37	37, 43 (40)	—	—
	0·1	—	4	—	—
9630 2-Methyl, 3-amyl—R ..	10	41, 39 (40)	30	—	—
	5	30	—	—	—
	2	2	25	—	—
8218 6-Chloro, 2:3-dimethyl—R	5	—	96, 88 (92)	46, 48 (47)	—
	1·5	—	113, 115 (114)	16	—
	0·5	44, 51 (48)	104	36	—
	0·2	33, 63, 44 (47)	—	—	—
	0·1	24	—	—	—
9677 6-Bromo, 2:3-dimethyl—R..	5	—	83, 105 (94)	100	64
	2	43	—	—	—
	1	30, 47 (39)	—	—	—
	0·5	88	—	—	—
8811 5-Methoxy—R	1	—	92, 113 (103)	93, 92 (93)	87
	0·5	34, 38 (36)	—	—	—
	0·2	46, 59, 53 (53)	113	80	—
	0·1	57	25	16	—
	0·05	33, 39 (36)	—	—	—
9265 5-Methoxy, 2:3-dimethyl—R	5	5, 5 (5)	—	—	—
	2	—	−16, −4 (−12)	50, 24 (37)	—
	0·5	3	−7	−12	—
9275 6-Methoxy, 2:3-dimethyl—R	5	37, 27 (32)	121	100	65
	1·5	—	67	52	—
	1	0, 5 (3)	—	—	—
9592 6-Acetamido—R	5	—	110	—	—
	2	41, 40, 68 (50)	85	88	—
	1	55	—	—	—
	0·5	51, 47 (49)	11	36	—

* Virus carriers among mice treated with aureomycin = 65%.

With developing eggs a similar calculation was based upon the mean period of survival of the embryos in days after infection ; embryos surviving until the eggs were opened 13 days after infection (20th day of incubation) were deemed to have survived for one additional day.

From the data summarized in Table II and the full series of observations we reached the following conclusions:

(i) The standard doses of aureomycin were not quite the best which could have been chosen. Larger doses gave slightly superior results, but were not possible in the earlier stages of the work, when great economy was needed in the use of the antibiotic. As explained previously, for the sake of consistency we adhered to these doses when the antibiotic became more freely available.

(ii) Of the quinoxaline oxides the largest doses used were near the maximum tolerated as previously defined. The relation between the tolerated dose for mice and that for chick-embryos varied. Often, weight for weight, mice tolerated larger doses, and of course in mice these doses were repeated. The reverse obtained with 7331, 9265, and some other compounds. The best therapeutic results did not necessarily follow administration of the largest amount tolerated.

(iii) When activity was expressed in terms of that of aureomycin under identical conditions, few compounds were as effective against lymphogranuloma in the egg as against the same disease in the mouse ; in this respect 7331, 9630, and a few other compounds were exceptional. Allowing for the considerable margin of experimental error inherent in these observations, fair agreement usually existed between the relative order of activity of various quinoxaline oxides in eggs and in mice. Activity against psittacosis in the mouse usually corresponded with that against lymphogranuloma in the egg rather than lymphogranuloma in the mouse. However, 8183, 8811, 9275, 9592, and 9677 were highly active against both diseases in the mouse ; 9265 was exceptional in showing some activity against psittacosis in the mouse and none against lymphogranuloma in either mouse or egg, while 7331 showed good activity against lymphogranuloma in the egg, moderate activity against lymphogranuloma in the mouse, and negligible activity against psittacosis in the mouse.

(iv) Against lymphogranuloma in the mouse, the best compounds exhibited activity roughly comparable with that of aureomycin ; as with the antibiotic, activity was evident whatever the route of inoculation of virus—intraperitoneal, intranasal, or intracerebral. While a large amount (10 mg.) of aureomycin (or an associated diluent in the capsules—cf. Cabasso, Moore, and Cox, 1952) inactivated 10 LD50 psittacosis virus *in vitro* in less than 2 hours at room temperature, a similar dose of 7218 did not do so within 6 hours. Yet, as seen from Table III, 7218 was highly active in restraining or suppressing growth of virus in the mouse. Despite this marked effect during the period of medication, virus often reappeared when treatment ceased (cf. Hurst, Peters, and Melvin, 1950), and as seen from Table II many animals carried virus in the spleen at about 40 days after infection ; the proportion so doing appeared to diminish with increased dosage of the drug. The apparent absence of virus during therapy was probably not due to the masking effect of drug or a metabolite, since serum or splenic suspensions from treated uninfected animals had no power to inactivate even small quantities of virus when the two were mixed and injected into fresh mice.

(v) Against equine encephalomyelitis, herpes febrilis, and ectromelia in mice several substituted quinoxaline dioxides had no action. Furthermore, Dr. M. G. P. Stoker very kindly tested 7218 against

TABLE III

PSITTACOSIS VIRUS: DAILY TITRES IN THE SPLEENS OF MICE TREATED OR UNTREATED WITH 7218 OR AUREOMYCIN

Virus was inoculated intraperitoneally. At the times stated the pooled spleens of 6 mice were titrated and the dilution of virus giving 40% end-point in the passage-mice was derived by the method described elsewhere (Hurst, Peters, and Melvin, 1950). The $\log_{10}$ dilutions are given below as positive quantities. N.V. = no virus detected

Infecting Dilution of Virus	Treatment	Treatment Begun	Days After Infection				
			1	2	3	4	7
10^{-5}	None	—	2·5	4·1	5·9	5·7	5·2
	7218 5 mg./20 g. orally twice daily	4 hours before virus	N.V.	N.V.	N.V.	N.V.	N.V.
10^{-3}	None	—	2·7	6·5	6·8	7·0	4·9
	7218 5 mg./20 g. as above ..	4 hours after virus	1·5	N.V.	N.V.	N.V.	N.V.
	7218 1 mg./20 ,, ,, ,, ..	,, ,, ,, ,,	2·3	5·5	4·0	3·2	1·1
10^{-4}	None	—	3·1	5·0	6·0	6·9	6·5
	7218 5 mg./20 g. as above ..	4 hours after virus	0·1	N.V.	1·0	N.V.	N.V.
	Aureomycin 2 mg./20 g. as above	,, ,, ,, ,,	N.V.	N.V.	1·5	0·2	N.V.

R. burneti infection in the chick-embryo and found that it showed no useful activity, while we ourselves could demonstrate no activity against *Toxoplasma* in mice.

In view of the foregoing results it was disappointing that Alergant (1953), while confirming the therapeutic activity in man, found it inseparable from unpleasant toxic side-reactions. At various times and in various places† quinoxaline-1:4 dioxide itself, 7218, or 8218 have been tried clinically in man and have always shown these toxic effects when given systemically in adequate therapeutic doses (see also Jones, Landquist, and Stewart, 1953). Of the toxic effects, dermatitis has not been observed in animals, but tonic spasms of the abdominal muscles, hind limbs and tail, and to a less extent of the back and neck, may be caused in mice by proportionately very much larger doses than are responsible for symptoms in man. Although the toxicity of different derivatives varies materially, so also does therapeutic activity, and in experiments on mice we have never met with any indication that the therapeutic ratio of a particular compound was more favourable than that of the best in Table II.

We shall not present detailed results in respect of the other compounds examined, mostly against lymphogranuloma only but sometimes against psittacosis in addition. A classified list follows, indicating the substituent group in each together with the degree of their activity in eggs (E) or mice (M). Compounds showing 80–120% of the activity of aureomycin were considered to be highly active (++), 25–80% as moderately active (+), below 25% as slightly active or inactive (0). The compounds marked with asterisks were examined on two to four occasions and with these the classification is beyond doubt. Because of experimental error it is possible that repeated examination of some of the others might have caused them to be moved into a neighbouring class; it is certain, however, that a slightly active compound would not have appeared as highly active and vice versa.

Monosubstituted Quinoxaline-1 : 4-dioxides

10,856	2-Ethyl	E++,M+*
11,861	2-Ethoxymethyl	E+
14,026	2-Acetoxymethyl	E++
8333	6-Chloro	E+,M++*
9553	6-Bromo	E+*
10,309	6-Amino	E++,M++*
8358	6-Methoxy	E++,M+
10,203	6-Ethoxy	E+,M+
12,563	6-*n*-Butoxy	E+
12,371	6-β-Methoxyethoxy	E++*

Disubstituted Quinoxaline-1 : 4-dioxides

9804	2-Methyl-3-ethyl	E+,M++*
9915	2:3-Diethyl	EO,M++*
10,323	2-Methyl-3-*n*-propyl	EO,M++*
10,931	2-Ethyl-3-*n*-propyl	EO,M++*
9833	2:3-Di-*n*-propyl	EO,MO*
8175	2:3-Diphenyl	E+,MO*
12,248	2-Methyl-3-carbethoxy	E+
11,160	2-Methyl-3-β-ethoxyethyl	E+,M++
12,922	2:3-Di(hydroxymethyl)	E+
12,879	2:3-Di(iodomethyl)	EO
12,911	2:3-Bis(dimethylamino-methyl)	EO
12,880	2:3-Di(anilinomethyl)	EO
12,634	2:3-Di(piperidino-methyl)	EO
13,079	2:3-Di(β-piperidino-ethyl)	EO
12,818	2:3-Di(bromomethyl hexamine compound)	E+
12,963	2:3-Di(trimethylammonium methyl bromide)	EO
12,964	2:3-Di(pyridinium methyl bromide)	EO
9947	6:7-Dimethyl	E++,M++*
9270	6:7-Dichloro	E+*
10,389	6-Chloro-7-methyl	E++,M+*
10,791	6-Bromo-7-methyl	E++,M+*

Substituted 2 : 3-dimethylquinoxaline-1 : 4-dioxides

8219	6-Methyl	EO,M++*
8986	6-Nitro	EO,MO
11,306	6-Trifluoromethyl	E+,M++*
9378	6-Cyano	E+*
10,685	6-Carbethoxy	E+
10,628	6-Carbamyl	E+
10,879	6-γ-Piperidinopropyl-carbamyl	E+
11,070	6-Methylsulphonyl	E++*
11,736	6-Ethylsulphonyl	E+*
12,373	6-(2′-Methoxyethyl)-sulphonyl	E+
11,971	6-Carboxymethyl-sulphonyl	E+
10,226	6-Amino	E+,MO
9586	6-Acetamido	EO,MO
10,821	6-*p*-Toluenesulphonamido	EO

† We are greatly indebted to our colleague Dr. G. I. Robertson, at present in Nairobi, and to Drs. A. R. McKelvie (Nairobi), R. Lewthwaite (Colonial Office), A. H. Lowther (Kuala Lumpur), F. Maxwell Lyons (Giza), and A. O. Ross (Liverpool) for help with one or other aspect of the clinical trials.

11,031	6-*p*-Aminobenzene-sulphonamido	EO
10,328	6-(2′-Amino-1′:6′-dimethyl-pyrimidinium-4′-amino)iodide	E+*
11,788	5-Hydroxy	EO
10,513	6-Hydroxy	EO
10,234	6-Ethoxy	EO,MO*
12,564	6-*n*-Butoxy	EO
12,253	6-(2′-Hydroxyethoxy)	EO*
12,370	6-(2′-Methoxyethoxy)	EO
12,250	6-(2′-Acetoxyethoxy)	EO
9920	6:7-Dimethyl	E+,MO
10,548	6-Chloro-7-methyl	E++,M++*
11,147	6-Bromo-7-methyl	E+,M+
9629	6:7-Dimethoxy	EO,MO

Phenazine Di-N-oxide and Related Compounds

2781	Phenazine di-N-oxide	EO,MO
11,975	2-Piperidinophenazine di-N-oxide	EO
10,000	1:2:3:4-Tetrahydrophenazine di-N-oxide	E+,M++*
10,501	6-Methyl-1:2:3:4-tetrahydrophenazine-di-N-oxide	EO,M+
10,819	6-Chloro-1:2:3:4-tetrahydrophenazine-di-N-oxide	E+,M++
10,965	6-Methoxy-1:2:3:4-tetrahydrophenazine-di-N-oxide	E+,MO
12,833	2:3-Pentamethylenequinoxaline-1:4-dioxide	EO

Diquinoxalyl Tetra-N-oxides

11,078	1:2-Di-(6′-quinoxalyloxy)ethane tetroxide	E+,MO*
10,367	1:3-Di(6′-quinoxalyloxy)propane tetroxide	EO
11,074	1:5-Di-(6′-quinoxalyloxy)pentane tetroxide	E+*
10,368	1:3-Di-(2′:3′-dimethylquinoxalyl-6′-oxy)propane tetroxide	EO
11,073	1:5-Di-(2′:3′-dimethylquinoxalyl-6′-oxy)pentane tetroxide	EO
11,734	1:2-Di-(2′:3′-dimethylquinoxalyl-6′-sulphonyl)-ethane tetroxide	EO
10,514	2:3:2′:3′-Tetramethyl-6:6′-diquinoxalylsulphone tetroxide	E+,M+

Miscellaneous Compounds

8271	Quinoxaline-l-oxide	E+,MO*
9989	2-Methyl-3-acetylquinoxaline-1-oxide	EO
11,954	2:3-Dimethylquinoxaline-6-sulphonic acid mono-N-oxide	EO
9591	1-Methylquinoxal-2-one-4-oxide	EO
9502	1-Methyl-3-hydroxyquinoxal-2-one-4-oxide	EO
10,565	1-Methylbenz-1:2:3-triazole-3-oxide	EO,MO
12,514	2:3-Dimethyl-l-methoxyquinoxalinium-4-oxide *p*-toluenesulphonate	EO
13,489	1-Methylquinoxalinium-4-oxide iodide	EO
8379	2:3-Diphenylpyrazine-1-oxide	E+,MO
8378	2:3-Diphenylpyrazine-1:4-dioxide	EO,MO
11,232	2:5-Diphenyl-3:6-dimethylpyrazine-1:4-dioxide	EO

These results warrant few conclusions regarding the relation between chemical structure and biological activity. However, in view of the importance of the $-NO_2$ group in determining antiviral activity in some acridine derivatives (Eaton, van Allen, and Wiener, 1947), it is worth drawing attention to the inactivity of 6-nitro-2:3-dimethylquinoxaline-1:4-dioxide (8986) when a number of closely related compounds in which the $-NO_2$ group was replaced by Cl, Br, CF_3, etc., showed considerable activity.

Miscellaneous Observations

Combined Treatment with Vitamin K and a Substituted Quinoxaline Dioxide.—It may be recalled that McIlwain (1943) prepared quinoxaline oxides as potential vitamin K antagonists, although he did not establish the fact that such antagonism exists. When we treated mice infected with lymphogranuloma with both 7218 and maximal tolerated doses of "Synkavit" (Messrs. Roche Products, Limited) or other preparations of synthetic vitamin K, the quinoxaline dioxide still exerted its usual therapeutic effect. Given alone, the vitamin did not itself affect mortality.

Morphological Changes in the Virus of Lymphogranuloma Treated with Antibiotics or a Substituted Quinoxaline Dioxide.—We examined

histologically the yolk-sacs of over 90 embryos sacrificed at various times after treatment with single doses of crystalline penicillin G (sodium salt) ranging from 250 to 4,000 units, of aureomycin from 0.025 to 2 mg., or of 7218 from 0.2 to 1.5 mg. The tissues were fixed in Zenker with acetic acid and sectioned at 2 μ; in staining we obtained much better results with Giemsa than by Noble's method (Yanamura and Meyer, 1941), which differentiated less clearly the virus from normal constituents of the yolk.

Rake and Jones (1942) described in detail the development of lymphogranuloma virus in the yolk-sac, and it would be superfluous here to duplicate their admirable account. Suffice it to say that, with the doses of virus used in our experiments, infected cells containing virus particles of 0.5 to 2 μ in diameter ("initial bodies") were readily detected at 16 hours when the first observation was made. Thereafter elementary bodies appeared and, together with a proportion of the larger forms, increased rapidly in number as the colonies of virus enlarged greatly. More and more cells became infected, until at 72 hours practically every cell lining the yolk-sac showed larger or smaller colonies. Soon afterwards signs of impending disintegration were evident, and abundant elementary bodies and colonies of virus escaping from ruptured cells lay free in the yolk. A typical smallish colony of virus is depicted in Fig. 1.

The morphological appearances in embryos treated with penicillin closely resembled those described by Weiss (1950) in eggs infected with the viruses of feline and murine pneumonitis. Such differences as were noted were probably due to the different experimental conditions; Weiss applied treatment 3 days after inoculation of virus, which thus had appreciable time in which to develop normally, while we injected drug only 2 hours after infection. However, since our work was completed before the publication of Weiss's paper and we have not had the opportunity of repeating it under other conditions, we cannot exclude the possibility of slight variations in the response to antibiotics of the different larger viruses. With single doses of 250 or 1,000 units, the appearance of virus in cells lining the yolk-sac was not appreciably retarded, and only slightly after 4,000 units. During much of the period of development virus existed exclusively in the form of large plaques ranging from 2 μ to as much as 6 μ in diameter, with a preponderance of the larger sizes; these usually stained a deep purplish blue with Giemsa, but sometimes a paler blue, and a few showed a vacuolated structure. In some instances a group of intensely coloured plaques seemed to be embedded in a paler blue matrix. The size of these plaques was not influenced by the dose of antibiotic within the range used. With the larger doses of penicillin infection of new cells occurred less readily, so that at 4 days less than 10 to 20% were visibly affected and advanced infection was retarded until the 5th or 7th day, when perhaps the effect of the antibiotic was wearing off. Although now the yolk-sac was visibly disintegrating, most of the virus still existed as large plaques, though a few of the masses had divided into elementary bodies or forms rather larger than these. Some of the plaques free at this time in the yolk stained very poorly and were possibly degenerate. Fig. 2 illustrates the state of affairs at 96 hours.

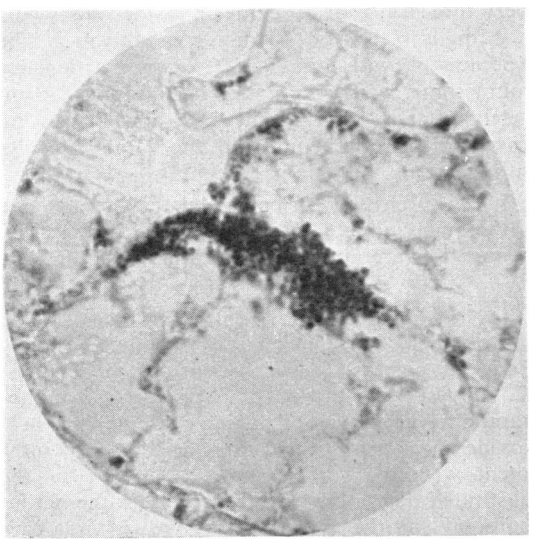

FIG. 1.—Infection of the yolk-sac with the virus of lymphogranuloma; a smallish colony of virus at 48 hours. Giemsa's stain. Magnification, ×1,000 diameters.

The picture with aureomycin was quite different. A dose of 0.025 mg. only slightly retarded the progress of viral growth and the ultimate dissolution of the yolk-sac; the morphological appearances were identical with those in untreated embryos. Larger doses (0.1 and 0.5 mg.) markedly delayed the appearance of recognizable virus, and moderately heavy infections were not present until the 5th to 7th day. In some cells, particularly at intermediate stages, virus tended to occur exclusively in plaques rather larger than normal, while in other cells at this stage and in nearly all later on the normal appearances were seen. Development of large forms was much less pronounced than portrayed by Gogolak and Weiss (1950) in feline

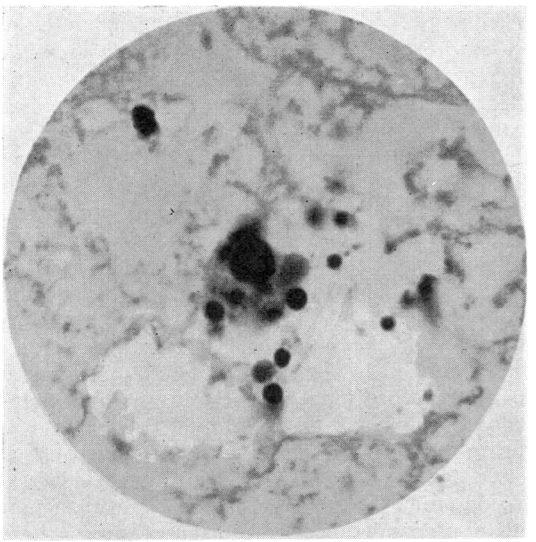

FIG. 2.—Effect of treatment with 1,000 units of penicillin; a virus colony 96 hours after infection. The largest plaque is nearly 5 μ in diameter. Some plaques stain less deeply than others, and around some of the larger group there are suggestions of a still paler matrix which do not appear to be wholly due to an optical effect. Staining and magnification as in Fig. 1.

pneumonitis virus treated with aureomycin under rather different conditions. The gigantic forms seen with penicillin were absent. In a few yolk-sacs treated with the still larger dose of 2 mg. no virus was detected histologically on the 7th or 10th days, though from approximately one-third of the chickens hatched from eggs so treated virus could be recovered by passage in animals.

Appearances in embryos treated with 7218 closely resembled those in the preceding group. With a dose of 0.2 mg. dissolution of the yolk-sac was retarded by a day or two, with larger doses proportionately longer. Virus occurred mainly in the normal forms, and giant forms were not encountered with the doses of compound used.

It would seem, therefore, that on the basis of morphological changes induced in the virus penicillin differs considerably in its action from aureomycin and the quinoxaline oxides.

Attempts to Engender Drug-resistance in the Virus.—The development of drug-resistance to a new chemotherapeutic agent is commonly observed only after the agent has been for some time in clinical use. In the earliest stages of assessing the relative merits of quinoxaline-1:4-dioxide and the antibiotics, we sought to ascertain the ease or otherwise with which such resistance might be engendered. With this object we infected mice and began treatment within a few hours. After a suitable period, determined largely by information concerning the titre of virus at various times in treated animals, we removed the spleens, passed them as a pool to fresh mice, and again started treatment within a few hours. The virus was thus exposed almost continuously to the drug for as long as the experiments lasted. At each passage a few mice were left untreated to demonstrate that virus still persisted in spite of prolonged treatment. Finally, we passed the stock virus in parallel with that exposed to drug to show that repeated transfer had not altered its properties.

Many such experiments were started with various doses of quinoxaline-1:4-dioxide, 7218, procaine-penicillin, or aureomycin. Some ended in failure because after several transfers in the presence of antibiotic the virus under examination failed to appear in the passage-mice, others because it became contaminated with ectromelia. The facts elicited were as follows:

(i) Both psittacosis and lymphogranuloma viruses developed partial resistance in 3 passages and absolute resistance in 4 passages at 14-day intervals to quinoxaline-1:4-dioxide given orally twice daily in a dose of 0.5 mg. At the 4th passage all (30) mice infected with "treated" virus died in spite of therapy, whereas those infected with passaged stock virus responded in the usual manner.

(ii) Similarly psittacosis virus developed considerable resistance to 7218 after 4 to 5 serial passages within 56 to 66 days.

(iii) There was no evidence of resistance to procaine-penicillin (initial dosage 1,000 units once every three days) or aureomycin (initial doses 0.05 to 1 mg.) in 117 or 42 days respectively. The experiments with the latter were particularly troublesome, as virus tended to disappear completely after a few passages; James, Price, and Kneeland (1951) encountered the same difficulty with the virus of feline pneumonitis.

(iv) Virus partially or completely resistant to one of the quinoxaline dioxides responded in the usual manner to aureomycin or procaine-penicillin.

SUMMARY AND CONCLUSIONS

A number of distinct classes of synthetic organic chemicals possess some degree of activity against the largest viruses of the psittacosis-lymphogranuloma group; of these quinoxaline-1:4-dioxide and its substituted derivatives are the most potent we have yet encountered. Some of the substituted derivatives are preferable to the parent compound on the ground of higher antiviral activity coupled with lower toxicity; the best equal aureomycin in activity against lymphogranuloma

venereum in the mouse, but relatively few are as effective against this disease in the chick-embryo or against psittacosis in the mouse. Like aureomycin and unlike penicillin, they are effective whatever the route of inoculation of virus—intraperitoneal, intranasal, or intracerebral. Therapeutic activity has also been noted in man, but in this host toxic side-reactions preclude the use of these compounds.

The compounds do not inactivate virus *in vitro*, but greatly restrict its growth in the mouse even to the point of apparent complete suppression. Nevertheless, as with the antibiotics, after treatment ceases many mice remain carriers of virus indefinitely (more than 277 days), without being contagious for normal animals. Therapeutic activity is not abolished by simultaneous exhibition of synthetic vitamin K. After several serial passages in the presence of the drug the viruses under consideration become drug-resistant.

The therapeutic activity of penicillin is accompanied by considerable morphological changes in the virus, which for much of its period of development continues to grow into large plaques (up to 6 μ in diameter) without subdivision into elementary bodies. Similar giant forms are not seen with the quinoxaline dioxides or aureomycin ; although at one stage plaques rather larger than normal develop, they ultimately divide into elementary bodies.

The quinoxaline dioxides do not influence infections with the viruses of herpes febrilis, ectromelia, mouse-adapted poliomyelitis, influenza A, equine encephalomyelitis, or louping-ill. One of them (7218) was equally devoid of useful activity against *R. burneti* and *Toxoplasma*.

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