CHLOROMYCETIN: BIOLOGICAL STUDIES

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Numerous organisms have been examined in our laboratories to determine their antibiotic potentialities. Among those that were selected for more intensive study was a soil actinomycete that had been isolated and found to possess antibacterial activity in Dr. Burkholder's[1] laboratory. On Moyer's sporulation agar, the organism produces a spreading, slightly wrinkled thallus. Aerial mycelium is white until covered with pale tan-gray spores. Oblong to oval spores are formed in unbranched, slightly curved chains on simple or dichotomously branched aerial hyphal tips. Abundant sporulation occurs at room temperature; little or none occurs at 37 C. The organism, which is evidently a *Streptomyces*, will be described more fully elsewhere.

This organism produces an antibiotic substance that is different chemically from any thus far described. The antibiotic has been isolated in crystalline form and has been found to contain both nitrogen and nonionic chlorine. The proposed name for this antibiotic is "chloromycetin" (Ehrlich *et al.*, 1947). Recently Carter, Gottlieb, and Anderson (1948) announced the independent isolation of this substance from culture filtrates of a *Streptomyces* obtained from central Illinois.

This paper describes some antibiotic properties of culture filtrates and crystalline material, microbiological assay methods, the cultural conditions employed, and some toxicity and chemotherapy data for the crystalline chloromycetin.

ANTIBIOTIC ACTIVITY OF THE STREPTOMYCES

Agar cultures. This organism in company with many others was first tested against various bacteria and fungi on agar. The *Streptomyces* was streaked across the center of agar plates and allowed to grow for 4 days at 28 C. Several different agar media were employed. After this incubation period the test bacteria were streaked at right angles to the *Streptomyces* and were incubated for an additional 48 hours at 37 C. Similar plates were cross-streaked with test fungi and incubated for an additional 7 days at 28 C. At the end of these incubation periods the zones of inhibition of the test organism were measured in millimeters. Table 1 gives the results of such tests.

The data in table 1 indicated that the organism produced on several solid media a substance or substances inactive against the yeasts and filamentous fungi tested but active against certain gram-negative, gram-positive, and acid-fast bacteria. It was also noted that although the character of the nitrogenous

[1] Paul R. Burkholder, Osborn Botanical Laboratory, Yale University, New Haven, Connecticut.

TABLE 1

Streak plate data on the Streptomyces

TEST SPECIES	STRAIN	MEDIA‡								
		1	2	3	4	5	6	7	8	9
		Width of zone of inhibition (mm)								
Bacillus mycoides	Waksman	—	—	—	—	—	—	—	—	>30*
Bacillus subtilis	Waksman	—	—	—	—	—	—	—	—	>30*
Escherichia coli	PD 01495	9	17	10	14	1	13	1	12	—
" "	Waksman	—	—	—	—	—	—	—	—	22*
Klebsiella pneumoniae, type A	PD 04544	16	28	21	>30	1	24	0	11	—
Mycobacterium tuberculosis v. *hominis*	ATCC 607	—	—	—	25†	—	—	—	25†	>30*,9†
" "	H37Rv	—	—	>37†	>25†	6†	0†	3†	—	13†
Pseudomonas aeruginosa	PD 01925	0	0	0	0	0	0	0	0	—
Salmonella schottmuelleri	PD 01180	10	14	13	18	2	17	2	10	—
Shigella paradysenteriae (Sonne)	PD 04628	15	23	21	28	2	26	2	22	—
Staphylococcus aureus	N.I.H. 209	10	16	13	22	2	18	0	10	—
" "	Cowles	—	—	—	—	—	—	—	—	>30*
Streptococcus pyogenes	PD 04472	18	25	32	>36	9	23	14	15	—
Various pathogenic yeasts and filamentous fungi		0	0	0	0	0	0	0	0	0*

* Tested by Dr. Paul R. Burkholder, Yale University.

† Tested by Dr. Guy P. Youmans, Elizabeth H. Williston, and Pari Zia-Walrath, Northwestern University Medical School, employing the cross-streak method of Williston, Zia-Walrath, and Youmans (1947).

‡ Composition of media:

COMPONENTS	MEDIA								
	1	2	3	4	5	6	7	8	9
	per cent								
Carbohydrates									
Cerelose	1.0	—	1.0	—	1.0	—	1.0	—	—
Maltose	—	1.0	—	1.0	—	1.0	—	1.0	1.0
Protein digests									
Peptone (Difco)	0.5	0.5	—	—	—	—	—	—	0.5
Casamino acids (Difco)	—	—	—	—	0.5	0.5	0.5	0.5	—
Meat extracts									
Beef extract (Difco)	—	—	—	—	—	—	—	—	0.3
Beef extract (Cudahy)	0.5	0.5	—	—	—	—	—	—	—
Distillers' solubles									
B-Y ferment. solubles (Comm. Solv.)	—	—	—	—	—	—	0.5	0.5	—
Curbay BG (U. S. Indus. Chem.)	—	—	—	—	0.5	0.5	—	—	—
Corn steep liquor (Corn Prod. Ref.)	—	—	1.0	1.0	—	—	—	—	—
Salts									
NaCl	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	—
K_2HPO_4	—	—	0.2	0.2	—	—	—	—	—
Agar	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
	pH								
Adjusted reaction after sterilization (approx.)	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0

material supplied to the organism had little or no effect on antibiotic production, cerelose (commercial glucose) was evidently a less productive carbohydrate than maltose. The organism was next grown in the same media without agar, in order to ascertain whether it would yield antibiotic activity under conditions adaptable to volume production.

Evaluation of Antibacterial Activity

Agar diffusion test. Early in our work on chloromycetin we attempted to follow the activity of culture filtrates (and concentrates) by a paper-disk agar-plate test, employing a strain of *Bacillus subtilis* relatively low in sensitivity to streptomycin (Loo *et al.*, 1945) and expressing activity in terms of equivalent streptomycin base. Later, when a strain of *B. subtilis* (ATCC 6633) more

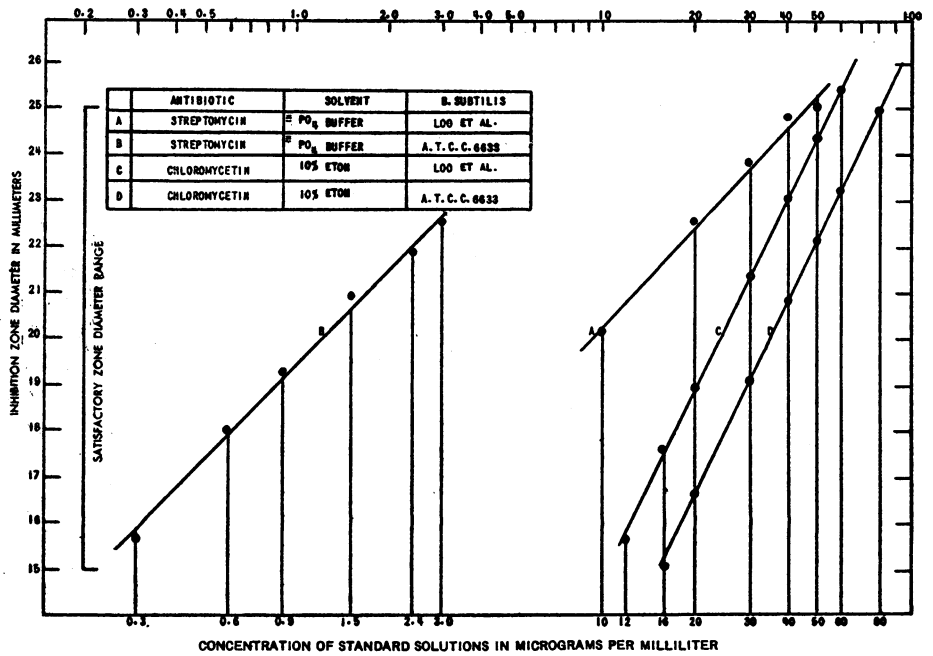


FIG. 1. COMPARISON OF STANDARD CURVES FOR CHLOROMYCETIN AND STREPTOMYCIN

sensitive to streptomycin was employed, it became impossible to follow the activity of low-potency chloromycetin solutions by this method. With both strains, inhibition-zone margins were ill-defined and detracted from the precision of the assay. Upon learning subsequently of Dr. Gottlieb's experience with the assay of his material (Gottlieb *et al.*, 1948), we compared the standard curves shown in figure 1. This comparison showed that the relative sensitivity of these two strains of *B. subtilis* to chloromycetin is the inverse of their relative sensitivity to streptomycin. The shortcomings of attempts to express the antibacterial activity of an unknown substance in terms of a known one are incidentally apparent. A turbidimetric broth dilution test was therefore developed.

Turbidimetric test. Before the pure substance was obtained, a method of following antibacterial activity was employed in which the end point was considered to be that dilution of the antibiotic culture filtrate or concentrate that inhibited the growth of an actively growing test organism in broth by 50 per cent (Joslyn and Galbraith, 1947).

Turbidimetric assay of chloromycetin. Methods for isolation of chloromycetin from culture liquids and certain physical and chemical properties of the crystalline substance have already been reported (Bartz, 1948). With the pure compound available,[2] it became possible to express the antibacterial activity of solutions of the antibiotic (culture liquids, body fluids, etc.) gravimetrically in terms of a crystalline reference standard. A daily standard curve is drawn by plotting the percentage of growth of *Shigella paradysenteriae* (Sonne) on a linear scale against broth dilution of the standard on a logarithmic scale. Samples for assay, in the form of clear but not necessarily sterile solutions, are diluted on the basis of estimated potency and their assay potency is calculated from the standard curve. A more detailed description of the turbidimetric method of assay is in preparation.

Broth Culture Filtrates

Antibacterial activity. The organism was first grown in media 1 to 8, without agar, in 100-ml quantities in 500-ml Erlenmeyer flasks on a rotary shaking machine. After an appropriate growth period the culture liquid was withdrawn, filtered to remove the organism, and tested. The data on a typical early experiment are given in table 2. The antibiotic titer was relatively low. The greatest dilutions of shaken culture filtrate giving 50 per cent inhibition of the test species were not determined in most cases because of the labor involved. Yet the data demonstrated that antibiotic activity was present, that the sensitivity of five test species was in approximately the same order as in the agar plate test, and that antibiotic titer was consistently greater in the maltose media. The results of a later experiment using medium 8 are given in table 3. Here the antibiotic titer was considerably higher, permitting calculation of the dilution for the 50 per cent inhibition end point. Since *Shigella paradysenteriae* (Sonne) proved to be consistently the most sensitive of these five test species, it was decided to employ this culture as the assay organism.

Antiviral and antirickettsial activity. When a Seitz filtrate of an early low-potency shaken flask culture in Waksman's streptomycin medium was tested by methods described below, no significant effect was observed against the viruses of St. Louis encephalitis or of type A influenza in mice, but indications of activity against *Rickettsia prowazeki* in chick embryos were obtained. These results are presented below in the section on chemotherapy of experimental typhus (figure 2).

PRODUCTIVITY OF SUBMERGED CULTURE MEDIA

Equipment and methods. Chloromycetin has been produced in aerated, submerged culture in several types of containers. Erlenmeyer flasks on rotary

[2] Early lots were prepared by Quentin R. Bartz; later lots, by Clark E. Cottrell.

shaking machines and laboratory fermenters were employed for exploratory media studies, for the furnishing of material for the development of extraction

TABLE 2

Turbidimetric data on culture filtrates of the Streptomyces in shaken flasks

TEST SPECIES	DILUTION	SHAKEN CULTURE MEDIA							
		1	2	3	4	5	6	7	8
		percentage of inhibition							
Escherichia coli	1:10	0	85	89	100	0	73	0	81
	20	—	71	72	100	—	42	—	60
Klebsiella pneumoniae type A	1:10	0	100	93	—	0	90	12	87
	20	—	88	83	100	—	70	14	81
	25	—	—	—	—	—	43	—	—
	40	—	—	55	—	—	—	—	42
	45	—	50	—	—	—	—	—	—
	125	—	—	—	51	—	—	—	—
Salmonella schottmuelleri	1:10	0	87	90	—	0	73	0	84
	20	—	77	77	100	—	50	—	63
Shigella paradysenteriae (Sonne)	1:10	0	92	100	—	0	89	0	94
	20	—	86	85	100	—	73	—	81
Staphylococcus aureus	1:10	0	100	71	90	0	58	0	63
	20	—	85	46	80	—	28	—	32
Streptococcus viridans									
" PD 04171	1:100	—	—	—	41*	—	—	—	—
" PD 04150	1:100	—	—	—	55*	—	—	—	—
" PD 04365	1:100	—	—	—	41*	—	—	—	—
" PD 04409	1:100	—	—	—	50*	—	—	—	—

* These values are based upon a filtrate that caused 50 per cent inhibition of *Klebsiella pneumoniae* when diluted 1:200.

TABLE 3

Turbidimetric data on a shaken culture filtrate of the Streptomyces using medium 8

TEST SPECIES	DILUTIONS					CALC. DILUTION FOR 50% INHIB.
	1:100	1:200	1:250	1:300	1:400	
	percentage of inhibition					
Escherichia coli	84	60	49	36	21	1:250
Klebsiella pneumoniae A	87	69	54	44	29	270
Salmonella schottmuelleri	86	62	56	47	36	285
Shigella paradysenteriae (Sonne)	100	84	78	67	57	>400
Staphylococcus aureus	43	17	15	<10	<10	<100

and purification procedures, and for initial chemical work on the properties and structure of crystalline chloromycetin. Larger amounts of culture liquid to

furnish material for further chemical work and for pharmacological studies were produced in horizontal and vertical fermenters. Either spore suspensions from agar culture or vegetative liquid cultures were used as inoculum. The temperature in all equipment was maintained between 23 and 27 C.

Culture media. Studies on media composition have been made largely on the shaking machines.

Reference has been made above to the superiority of maltose to glucose in various media. Table 4, portraying a typical experiment, shows that, whereas

TABLE 4

Effect of carbohydrates on biosynthesis of chloromycetin in shaken flasks

BASE MEDIUM: CASAMINO ACIDS 0.5%, B-Y FERMENTATION SOLUBLES 0.5%, SODIUM CHLORIDE 0.5%, PLUS		MEAN POTENCIES OF QUADRUPLICATE FLASKS AFTER 5 DAYS
carbohydrate	*per cent*	*μg/ml*
Glucose	1.0	<19
Lactose	1.0	<19
Maltose	1.0	24
Glycerol	1.0	47

TABLE 5

Effect of various proteins and protein hydrolyzates on biosynthesis of chloromycetin in shaken flasks

BASE MEDIUM: GLYCEROL 1.0%, B-Y FERMENTATION SOLUBLES (CSC) 0.5%, SODIUM CHLORIDE 0.5%, PLUS		MEAN POTENCIES OF QUADRUPLICATE FLASKS AFTER			
		3 days	4 days	5 days	6 days
protein	%	*μg/ml*			
Soya					
Soybean oil meal (Staley)	0.5	59	60	55	66
Alpha protein (Glidden)	0.5	69	72	74	81
Protein hydrolyzate (Publicker)	0.5	76	89	84	86
Milk					
Labco casein (Borden)	0.5	54	83	92	87
N-Z amine B (Sheffield)	0.5	23	78	82	84
Meat					
Hog stomach residue (P., D. & Co.)	0.5	96	118	105	116
Tryptone (Difco)	0.5	136	169	155	164
Peptone (Difco)	0.5	103	106	115	110

results with lactose were of the same order as those with glucose, glycerol was even more effective than maltose in effecting biosynthesis of chloromycetin.

A variety of animal and vegetable proteins and protein hydrolyzates, including milk, corn, soya, wheat, cotton seed, and meat products, were tested for their effectiveness in glycerol media. The meat products were found superior, in most cases, to the others: Difco peptone, Difco tryptone, and a hog stomach residue[3] produced the best yields. The latter is a waste product and was most

[3] A concentrated and dried residue from ground hog stomachs extracted with saline for the production of "ventriculin" (Parke, Davis and Company).

often used because of its availability. Table 5 depicts an experiment in which various soya, milk, and meat proteins and hydrolyzates were compared. It will be noted that not only were the activities produced by the meat products considerably higher, but these high activities were reached in a shorter period of time than the highest activities produced by the soya or milk products.

The replacement of B-Y fermentation solubles (CSC) by molasses has recently been found possible. Yeast products, beef extract, distillers solubles, and corn steep solids resulted in lowered activity. A comparison of several of these materials is made in table 6. The quantities used had been found in preliminary experiments to be the optimum concentrations in the base medium.

TABLE 6

Effect of various supplementary materials on biosynthesis of chloromycetin in shaken flasks

BASE MEDIUM: GLYCEROL 1.0%, HOG STOMACH RESIDUE 0.5%, SODIUM CHLORIDE 0.5%, PLUS		MEAN POTENCIES OF QUADRUPLICATE FLASKS AFTER			
		3 days	4 days	5 days	6 days
supplementary material	%	*μg/ml*			
Corn steep solids (Corn Prod. Ref.)	0.8	25	98	88	83
Brewers' yeast type 2019 (Standard Brands)	0.7	<25	35	33	34
Distillers' solubles (Brown and Forman)	1.0	<25	<25	<25	30
B-Y fermentation solubles (CSC)	0.5	114	119	120	113
Molasses (Brer Rabbit green label)	1.0	142	137	118	137

TABLE 7

Course of chloromycetin potency and pH of culture liquid

AGE	POTENCY	REACTION
hours	*μg/ml*	*pH*
0	—	6.70
65	68	6.10
89	82	5.25
97	78	5.79
113	78	5.89

It would appear that there are as yet unidentified substances in both molasses and B-Y fermentation solubles, which is the residue from a molasses fermentation process for the production of industrial alcohol, that are capable of stimulating chloromycetin production by the organism.

During the course of the elaboration of chloromycetin by the organism, the pH of the culture liquid tends to drop during the early part of the incubation period. It has frequently been observed that the concentration of chloromycetin in the culture liquid is greatest when the pH has fallen to its lowest point or shortly after it has begun to rise again. Table 7 shows the course of the pH and the increase in amount of chloromycetin in a typical experiment.

ANTIBIOTIC ACTIVITY OF CRYSTALLINE CHLOROMYCETIN

In Vitro Activity

The results of *in vitro* tests of antibiotic activity of crystalline chloromycetin, already reported in part (Ehrlich *et al.*, 1947), are summarized in table 8.

Antibacterial activity. Although methods of testing and recording bacteriostatic activity necessarily varied for different species, the results in the first section of table 8 show that chloromycetin is considerably more active in broth culture against several gram-negative species than against the gram-positive and acid-fast species tested. When compared with streptomycin (table 8, notes p and q), chloromycetin is thus approximately one-tenth as active against streptomycin-sensitive strains of *Mycobacterium tuberculosis*, 1 to 2 times as active against *Bacillus mycoides* and *Staphylococcus aureus*, and 2 to 16 times as active against the gram-negative organisms compared. When compared with penicillin (table 8, notes o and p), chloromycetin is 7 to 36 times as active against the gram-negative species tested, approximately one-fiftieth as active against the 209 strain of *Staphylococcus aureus*, and remarkably active against a Schuhardt tick strain of *Borrelia recurrentis* in a 2-hour test in which 100 I.U. per ml of penicillin are inactive.

The data on *M. tuberculosis* show that chloromycetin maintains its activity sufficiently at 37 C in the presence of beef plasma to prevent growth. They show also that a substrain of *M. tuberculosis* highly resistant to streptomycin is not more resistant to chloromycetin than the streptomycin-sensitive parent strain.

Antifungal activity. Neither 200 μg of chloromycetin per ml of broth nor 12,500 μg per ml of agar—highest concentrations tested—inhibited the growth of the pathogenic yeasts and filamentous fungi tested.

Antiprotozoal activity. When *Pelomyxa carolinensis*, a large multinucleate rhizopod, and *Tetrahymena geleii*, a small ciliated protozoan, were placed into chloromycetin-saturated buffered culture solution and into chloromycetin-saturated 2 per cent proteose-peptone solution, respectively, the appearance and observable activities of these two free-living species remained unchanged over a 48-hour period. Similarly, when *Trichomonas foetus* was placed in a nearly saturated solution of chloromycetin in 0.7 per cent saline, the organisms were not killed in 7 hours, as compared with 1.5 hours' death time in an equal concentration of calcium penicillin solution. Likewise when *Endamoeba histolytica* was placed in diphasic egg-Locke medium containing graded dilutions of a boiled Locke overlay solution of chloromycetin, the numbers of motile amebae after incubation at 37 C for 48 hours decreased only to an extent attributable to inhibition of the associated mixed bacterial flora.

Chemotherapy

A number of tests on experimentally infected animals have been initiated. Certain of these tests are being extended, but the following descriptions are indicative of the activity of chloromycetin in infected animals.

TABLE 8

Antibiotic activity of chloromycetin in vitro

TEST SPECIES AND STRAIN	CULTURE MEDIUM	METHOD OF TESTING	END POINT OBSERVED	CONCENTRATION OF CHLOROMYCETIN REQUIRED
				μg/ml
Bacteria				
Bacillus mycoides (PD 04595)	Difco brain heart infusion (pH 7.4)	Broth dilution	50% of growth (turbidimetrically) of control after 4–5 hours at 37 C	0.50[p]
Borrelia recurrentis[a] (Schuhardt)	2.5% Rat serum + buffer (pH 7.0)	Buffer dilution	Death within 2 hours at 37 C	2.50[o]
Borrelia recurrentis[a] (Schuhardt)	2.5% Rat serum + buffer (pH 7.0)	" "	50% motile after 2 hours at 37 C	0.00625
Brucella abortus (Huddleson 1335)	Difco tryptose (pH 6.9)	Agar dilution	No growth (by unaided eye) after 24 hours at 37 C	2.00
Brucella melitensis[b] (recent reisolate from guinea pig)	Difco tryptose (fortified)[c]	Broth dilution	No growth (by unaided eye) after several days at 37 C	0.50[d]
Brucella suis[b] (moderate guinea pig virulence)	Difco tryptose (fortified)[c]	" "	No growth (by unaided eye) after several days at 37 C	0.50[d]
Eberthella typhosa (N.I.H. "Hopkins")	Difco brain heart infusion (pH 7.4)	" "	50% of growth (turbidimetrically) of control after 3–4 hours at 37 C	0.25[p]
Escherichia coli (PD 01495)	Difco brain heart infusion (pH 7.4)	" "	50% of growth (turbidimetrically) of control after 3–4 hours at 37 C	0.33[p]
Hemophilus pertussis, phase I,[r] high mouse virulence (PD 04692)	Sauer's (rabbit) blood	Agar dilution	No growth (by unaided eye) after 72 hours at 35 C	0.2
Klebsiella pneumoniae, type A (PD 04544)	Difco brain heart infusion (pH 7.4)	" "	50% of growth (turbidimetrically) of control after 3–4 hours at 37 C	0.33[p]
Mycobacterium tuberculosis v. *hominis*[e] (H37Rv)	Youmans' synthetic[g] (pH 7.0)	" "	No growth (by unaided eye) after 2 weeks at 37 C	12.5[q]
Mycobacterium tuberculosis v. *hominis*[e] (H37RvR)[f]	Youmans' synthetic[g] (pH 7.0)	" "	No growth (by unaided eye) after 2 weeks at 37 C	12.5
Mycobacterium tuberculosis v. *hominis*[e] (H37Rv)	Youmans' synthetic[g] + 10% beef plasma	" "	No growth (by unaided eye) after 2 weeks at 37 C	12.5
Mycobacterium tuberculosis v. *hominis*[e] (H37RvR)[f]	Youmans' synthetic[g] + 10% beef plasma	" "	No growth (by unaided eye) after 2 weeks at 37 C	12.5
Pasteurella tularensis[h] (Schu, and Church)	Synder *et al.*, peptone[i]	" "	No growth (turbidimetrically and by plate counts) after 96 hours	0.4–10[d]

TABLE 8—*Continued*

TEST SPECIES AND STRAIN	CULTURE MEDIUM	METHOD OF TESTING	END POINT OBSERVED	CONCENTRATION OF CHLOROMYCETIN REQUIRED
				μg/ml
Bacteria—*Continued*				
Proteus vulgaris (PD 04736)	Difco brain heart infusion (pH 7.4)	Agar dilution	50% of growth (turbidimetrically) of control after 3–4 hours at 37 C	0.33
Salmonella schottmuelleri (PD 01180)	Difco brain heart infusion (pH 7.4)	" "	50% of growth (turbidimetrically) of control after 3–4 hours at 37 C	0.33[p]
Shigella paradysenteriae (Sonne) (PD 04628)	Difco brain heart infusion (pH 7.4)	Broth dilution	50% of growth (turbidimetrically) of control after 3–4 hours at 37 C	0.20[p]
Staphylococcus aureus (N.I.H. 209)	Difco brain heart infusion (pH 7.4)	" "	50% of growth (turbidimetrically) of control after 3–4 hours at 37 C	1.00[p]
Streptococcus pyogenes (PD 04472)	Difco brain heart infusion (pH 7.4)	" "	50% of growth (turbidimetrically) of control after 5–6 hours at 37 C	0.63
Yeasts and filamentous fungi[j]			Results obtained with highest concentration tested	
				mg/ml
Candida albicans (PD 04600)	Sabouraud's glucose + neopeptone (pH 5.8)	Broth dilution	No inhibition after 24 hours at 37 C	0.20
Candida albicans (PD 04600)	Sabouraud's glucose + neopeptone (pH 5.8)	Agar diffusion	No inhibition after 24 hours at 37 C	12.5
Cryptococcus neoformans (N.I.H. 3713)	Sabouraud's glucose + neopeptone (pH 5.8)	Broth dilution	No inhibition after 24 hours at 37 C	0.20
Cryptococcus neoformans (N.I.H. 3713)	Sabouraud's glucose + neopeptone (pH 5.8)	Agar diffusion	No inhibition after 24 hours at 37 C	12.5
Microsporum audouini (N.I.H. 239)	Sabouraud's glucose + neopeptone (pH 5.8)	Broth dilution	No inhibition after 10 days at 28 C	0.20
Microsporum canis (N.I.H. 237)	Sabouraud's glucose + neopeptone (pH 5.8)	" "	No inhibition after 10 days at 28 C	0.20
Trichophyton interdigitale (N.I.H. 640)	Sabouraud's glucose + neopeptone (pH 5.8)	" "	No inhibition after 10 days at 28 C	0.20
Trichophyton interdigitale (N.I.H. 640)	Sabouraud's glucose + neopeptone (pH 5.8)	Agar diffusion	No inhibition after 6 days at room temperature	12.5
Protozoa			Results obtained with highest concentration tested	
				mg/ml
Endamoeba histolytica[k] with mixed bacterial flora (U. of Chicago)	Egg—Locke diphasic (pH 7.9)	Broth dilution in Locke overlay	No significant decrease in number of motile amebae after 48 hours at 37 C	1.0
Pelomyxa carolinensis[l]	Pace and Kimura buffer[m] (pH 6.8–7.0)	Sat. soln. in buffer	No change in appearance, locomotion, etc., during 48 hours at 25 C	2.5

TABLE 8—*Concluded*

TEST SPECIES AND STRAIN	CULTURE MEDIUM	METHOD OF TESTING	END POINT OBSERVED	CONCENTRATION OF CHLOROMYCETIN REQUIRED
Protozoa—*Continued*			Results obtained with highest concentration tested	
				mg/ml
Tetrahymena geleii[l]	2.0% proteose peptone, Difco (pH 6.8)	Sat. soln. in broth	No change in appearance, locomotion, etc., during 48 hours at 25 C	2.5
Trichomonas foetus[n] (B. B. Morgan	0.7% sodium chloride	Saline dilution	No deaths during 7 hours at 37 C	2.0

[a] Tested under the direction of Paul E. Thompson by Donald L. Bush at the Research Laboratories of Parke, Davis and Company, Detroit.

[b] Tested by E. H. Kelly and A. N. Gorelick at Camp Detrick, Frederick, Maryland.

[c] Fortified with 1 per cent glucose, 0.01 mg per cent thiamine hydrochloride, and 1 mg per cent ferrous sulfate. Organisms were added in 5 per cent volume of a solution containing 0.1 per cent tryptose and 0.5 per cent sodium chloride.

[d] These values are tentative and may be modified as the result of repetition.

[e] Tested by Guy P. Youmans at the Department of Bacteriology, Northwestern University Medical School, Chicago.

[f] Streptomycin-resistant substrain of H37Rv (Williston and Youmans, 1947).

[g] Youmans and Karlson, 1947.

[h] Tested by H. T. Eigelsbach and I. W. Gibby at Camp Detrick, Frederick, Maryland.

[i] Two per cent Difco peptone, 1 per cent sodium chloride, and 0.1 per cent glucose; adjusted to pH 7.0 before sterilization (Snyder *et al.*, 1946).

[j] Tested under the direction of Arthur B. Hillegas by Bessie D. Moore at the Research Laboratories of Parke, Davis and Company, Detroit.

[k] Tested under the direction of Paul E. Thompson by Betty Lou Lilligren at the Research Laboratories of Parke, Davis and Company, Detroit.

[l] Tested under the direction of Donald M. Pace by David Russell at the Department of Physiology, University of Nebraska, Lincoln.

[m] Pace and Kimura, 1946.

[n] Tested by Thomas F. Reutner at the Research Laboratories of Parke, Davis and Company, Detroit.

[o] Although penicillin was effective against *Borrelia recurrentis* after prolonged contact, 100 I.U. per ml had no effect in 2 hours (Schuhardt).

[p] Concentrations required for comparable effect:

ORGANISM	PENICILLIN		CHLOROMYCETIN	RATIO
	I.U./ml	*μg/ml*	*μg/ml*	*P/C*
E. coli	14	8.4	0.33	25
K. pneumoniae	4	2.4	0.33	7
S. schottmuelleri	4	2.4	0.33	7
S. paradysenteriae (Sonne)	12	7.2	0.20	36
S. aureus	0.03	0.018	1.00	0.018

ORGANISM	IN DIFCO PENASSAY BROTH		
	STREPTOMYCIN BASE	CHLOROMYCETIN	RATIO
	μg/ml	*μg/ml*	*S/C*
B. mycoides	0.66	0.63	1.05
E. typhosa	1.66	0.36	4.61
E. coli	2.50	0.50	5.00
K. pneumoniae	0.55	0.25	2.20
P. vulgaris	8.00	0.50	16.0
S. schottmuelleri	1.66	0.50	3.32
S. paradysenteriae (Sonne)	1.00	0.33	3.00
S. aureus	1.66	1.00	1.66

[q] Most virulent strains of the tubercle bacillus are completely inhibited under comparable conditions by less than 2.0 μg streptomycin base per ml (Youmans and Karlson, 1947).

[r] Tested by R. W. Sarber at the Research Laboratories of the Parke, Davis and Company, Detroit.

Experimental avian malaria.[4] Starting 6 hours before inoculation with the 12A strain of *Plasmodium lophurae* by the intravenous injection of parasitized blood, groups of three 100-g ducklings were treated twice daily by the intraperitoneal route with chloromycetin in 50 per cent propylene glycol until the untreated controls reached their parasitemia peaks on the fifth day. Maximum tolerated doses (200 mg per kg per day) were without antimalarial effect, as determined by comparison of the parasitemia in the treated birds with that in the untreated controls.

Experimental rabbit syphilis. Rabbits infected with the Nichols strain of *Treponema pallidum* and receiving chloromycetin intramuscularly at the rate of 25 mg per kg per day in two divided doses for 8 days showed no change in lesions or disappearance of spirochetes. Although daily dosages of 50 and 100 mg per kg cleared the lesions of spirochetes, the effect proved to be temporary.

Experimental mouse septicemias. Exploratory experiments, using small numbers of mice infected intraperitoneally with lethal doses of virulent *Klebsiella pneumoniae* (type A), *Shigella paradysenteriae* (Flexner), *Shigella paradysenteriae* (Sonne), *Diplococcus pneumoniae* (type I), *Streptococcus hemolyticus*, and *Streptococcus viridans* and treated subcutaneously with chloromycetin in 20 per cent propylene glycol, streptomycin sulfate, or penicillin G, showed that chloromycetin was qualitatively similar to streptomycin but quantitatively inferior in protective action.

Experimental tuberculosis: Mouse chemotherapy. Subcutaneous and oral chemotherapeutic tests have been undertaken by Dr. Youmans,[5] who will report in a separate publication.

Tuberculostatic blood levels: Guinea pigs.[6] In order to ascertain by a short method whether or not tuberculostatic blood levels of chloromycetin are readily attainable, Drs. Feldman and Karlson performed an "*in vivo, in vitro*" test with 700-g guinea pigs. Each of two animals received subcutaneously 15 ml of an 0.85 per cent sodium chloride solution containing 2.5 mg chloromycetin per ml every 2 hours for 3 injections, so that each animal received a total of approximately 160 mg chloromycetin per kg of body weight; they were bled 1 hour after the last injection. Each of two animals received by intubation 10 ml of a suspension containing 13.5 mg chloromycetin per ml hourly for 5 doses or a total of 964 mg per kg each; these animals were bled 1 hour after the last injection. The specimens of blood from the treated animals and two specimens from normal guinea pigs were placed in a refrigerator overnight. The serum from each animal was collected from the clot about 16 hours after bleeding and mixed with an equal volume of Proskauer and Beck liquid medium. This mixture was dispensed in 3-ml amounts into test tubes and inoculated with 0.1 mg tubercle bacilli H37Rv. After 10 days of incubation at 37 C it was found that the growth

[4] Tested by Paul E. Thompson, the Research Laboratories of Parke, Davis and Company, Detroit 32, Michigan.

[5] Guy P. Youmans, Northwestern University Medical School, Chicago 11, Illinois.

[6] Determined by William H. Feldman and Alfred G. Karlson, The Mayo Foundation, University of Minnesota, Rochester, Minnesota.

in tubes containing serum from treated animals was grossly equal to that in the control tubes. In view of the previously demonstrated stability of chloromycetin under these *in vitro* conditions, it is concluded that tuberculostatic blood levels were not present at the time when the treated animals were bled.

Experimental virus infections. Chloromycetin has given negative results against type A influenza virus in eggs and mice and against St. Louis encephalitis virus and fixed rabies virus in mice. Some indication of protection was obtained against Newcastle disease in young chickens, but tests against the causal virus in embryonated eggs were negative.[7] We have done no work with any member of the psittacosis group of viruses, but Smadel and Jackson (1947) have reported positive results.

Experimental epidemic typhus in chick embryos. Smadel and Jackson (1947) have already reported that chloromycetin has considerable chemotherapeutic activity, under experimental conditions, against several rickettsial agents. Their investigations are continuing and will be reported elsewhere.

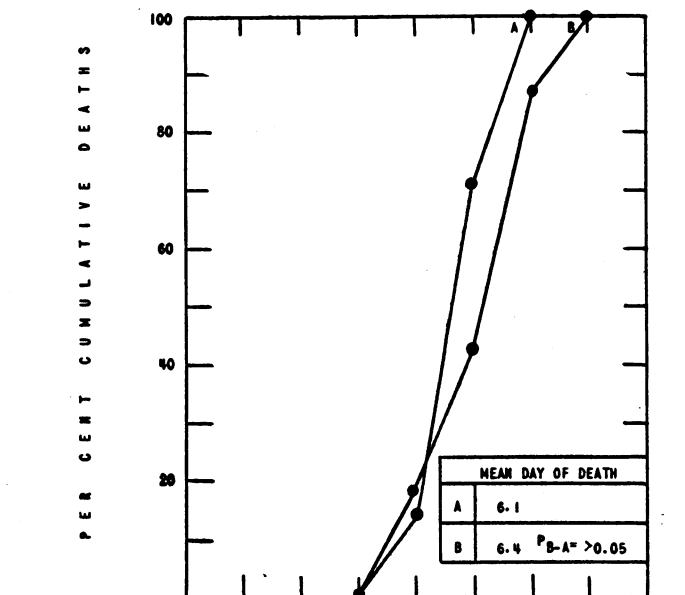
The following studies were made early in the development of this drug in connection with the antiviral and antirickettsial screening program in progress in this laboratory.

Methods. The Breinl strain of *Rickettsia prowazeki*, used throughout the study, has been maintained by yolk-sac passage in 6-day embryonated eggs. The seed for chemotherapeutic tests consists of pools of bacteriologically sterile rickettsia-rich yolk sacs ground as a 10 per cent suspension in buffered milk and stored in sealed ampoules at −70 C. Prior to use a representative ampoule is thawed and the suspension titrated in 6-day embryonated eggs to determine the dilution required to kill approximately 50 per cent of the embryos upon the administration of 0.5 ml into the yolk sac by the sixth day postinfection.

For the tests, the yolk sacs of groups of 20 to 30 fertile eggs previously incubated for 6 days at 37.5 C are injected with 0.5 ml per egg of the properly diluted seed material through a small hole in the shell over the air space. The hole is sealed with collodion and incubation is continued at 35 C for the remainder of the test. Seventy-two hours after inoculation the eggs are candled, the dead and weak embryos are discarded, and treatment is begun. Unless otherwise stated, all treatment was withheld until after this 72-hour incubation period to allow the infection to become established. Treatment consists of administration of 0.5 ml of the test material into the yolk sac daily for 1 to 6 days. In each experiment, a control group of infected eggs injected with 0.5-ml doses of sterile saline solution according to the same schedule is included. All eggs are candled daily, those containing dead embryos opened and yolk-sac smears made and examined for the presence of rickettsiae by a modification of Macchiavello's technique. Embryos dying before the fourth day postinfection are not considered in the test and those surviving 14 days postinfection are sacrificed and examined for rickettsiae. Those of the latter are considered dead if rickettsiae are demonstrated, and survivors if smears are negative.

[7] Tests conducted by Herman M. Salk of Parke, Davis and Company.

Each group of test embryos is compared with the corresponding control group by calculating the harmonic mean death time[8] for each group and the percentage dying after the fifth day with demonstrable rickettsiae in yolk-sac smears. With this strain of *R. prowazeki* and the dose used it is extremely difficult to demonstrate free rickettsiae in untreated embryos dying before the fifth day and, for this reason, only those embryos dying after the fifth day are included in the calculation of the percentage of positives.



DAYS POSTINFECTION		2	3	4	5	6	7	8	Total
A SALINE CONTROLS	Dose ml	.5	.5	.5	.5	.5	.5	-	3.0
	No. Dead	-	-	-	3	12	6	-	21
	No. Pos.	-	-	-	2	12	6	-	20
B BROTH FILTRATE	Dose ml	.5	.5	.5	.5	.5	.5	-	3.0
	No. Dead	-	-	-	4	6	10	3	23
	No. Pos.	-	-	-	4	6	10	3	23

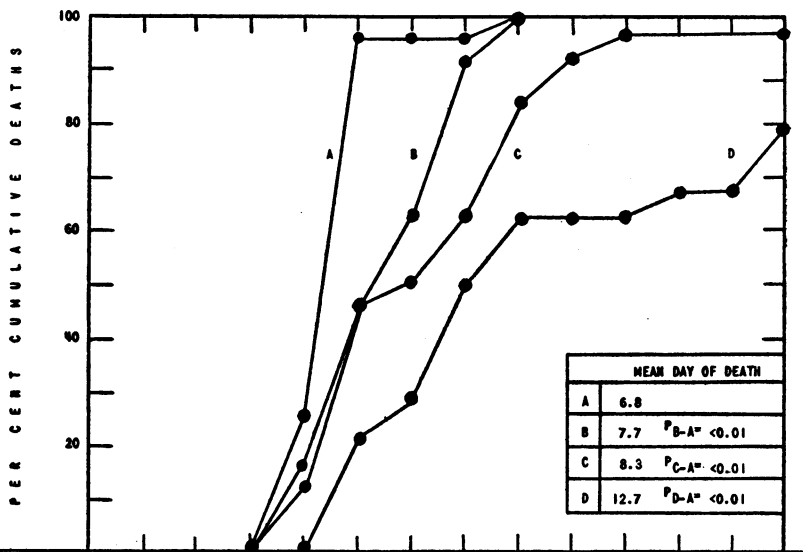
FIG. 2. EFFECT OF AN EARLY SHAKEN-CULTURE FILTRATE OF THE STREPTOMYCES ON R. PROWAZEKI INFECTION IN CHICK EMBRYOS

Experimental results. The first indications of the antirickettsial activity of chloromycetin were obtained from tests on early crude culture filtrates.[9] The results of the first test, with a filtrate, are given in figure 2. Since at that time methods of evaluating activity had not been well developed, the concentration of chloromycetin used is unknown and was undoubtedly very low. A total of 3.0 ml of the crude broth filtered through a no. 10 Mandler filter candle

[8] The reciprocal of the mean of the reciprocals or $1/n(1/x_1 + 1/x_2 + 1/x_3 \ldots + 1/x_n) = 1/\bar{X}$. This mean is preferable to the arithmetic mean death time since it allows inclusion of survivors inasmuch as $1/\infty = 0$.

[9] This work was done under the direction of A. H. Killinger, then of Parke, Davis and Company.

was administered to each egg in 6 daily doses of 0.5 ml each, starting in this case on the second day postinfection. The resulting 0.3-day delay in mean death time is just below the level of statistical significance;[10] however, when considered in conjunction with subsequent work and contrasted with the results on other substances tested during that period, these results probably cannot be attributed to chance alone.



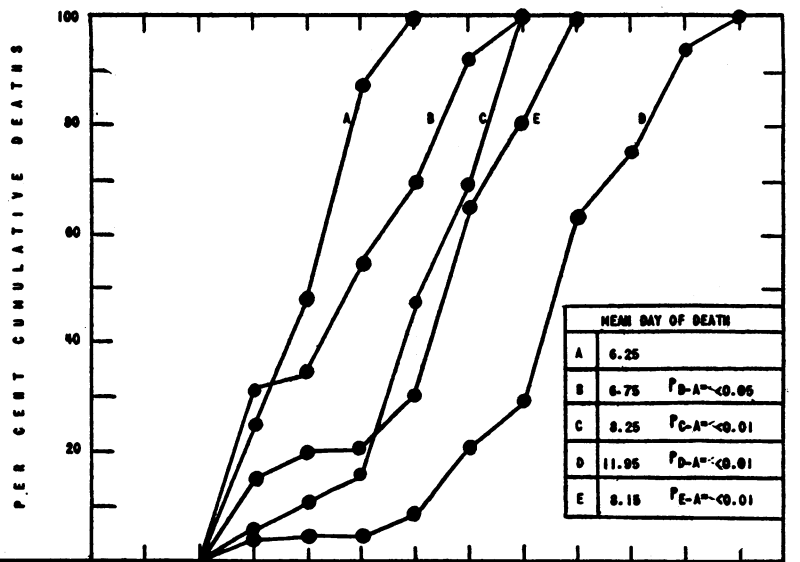
DAYS POSTINFECTION		3	4	5	6	7	8	9	10	11	12	13	14	Surv.	Total
A SALINE CONTROLS	Dose ml	.5	.5	.5	.5	-	-	-	-	-	-	-	-	-	2.0
	No. Dead	-	-	0	6	17	0	0	1	-	-	-	-	0	24
	No. Pos.	-	-	-	6	17	-	-	1	-	-	-	-	-	24
B CHLORO-MYCETIN 167γ	Dose γ	½2	½2	½2	½2	-	-	-	-	-	-	-	-	-	167
	No. Dead	-	-	0	2	8	4	7	2	-	-	-	-	0	24
	No. Pos.	-	-	-	1	2	0	0	0	-	-	-	-	-	3
C CHLORO-MYCETIN 333γ	Dose γ	83	83	83	83	-	-	-	-	-	-	-	-	-	333
	No. Dead	-	-	0	4	7	1	3	5	2	1	0	0	1	24
	No. Pos.	-	-	-	0	0	0	0	0	0	1	-	-	0	1
D CHLORO-MYCETIN 667γ	Dose γ	167	167	167	167	-	-	-	-	-	-	-	-	-	667
	No. Dead	-	-	0	0	5	2	5	1	0	0	1	0	8	24
	No. Pos.	-	-	-	-	0	0	0	0	-	-	0	-	3	3

FIG. 3. EFFECT OF INCREASING DOSES OF CHLOROMYCETIN ON EXPERIMENTAL INFECTION OF CHICK EMBRYOS WITH R. PROWAZEKI

When crystalline chloromycetin was obtained, the question of antirickettsial activity was reopened. In the first experiment, treatments were made with a Seitz EK filtrate of an aqueous solution containing 1 mg per 3 ml, and with 1:2 and 1:4 dilutions of this solution in sterile physiological saline. Starting on the third day postinfection, each concentration was administered in 4 daily 0.5-ml doses for totals of 167, 333, and 667 μg per egg, respectively (figure 3; B, C, and

[10] Statistical analysis was made by "Student's" method for measurement data. P in the figures refers to probability that delay in death time could be due to chance alone.

D). Even the smallest dosage resulted in a statistically significant prolongation of mean death time and in marked decrease in demonstrable rickettsiae in the smears. As the total dose was increased, the mean death time was correspondingly prolonged; with the largest dose, 667 μg per egg, 8 embryos or 33.5 per cent survived the 14 days' incubation period. When the survivors were sacri-



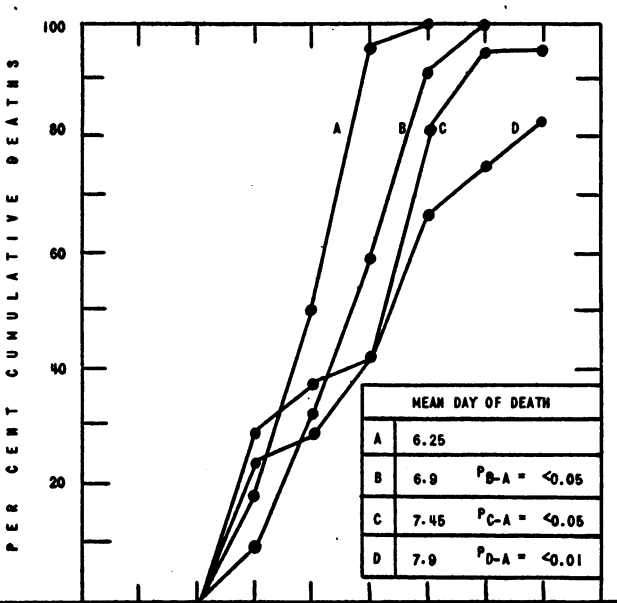
DAYS POST INFECTION		3	4	5	6	7	8	9	10	11	12	13	14	Total
A	Dose ml	.5	.5	-	-	-	-	-	-	-	-	-	-	1.0
SALINE	No. Dead	-	-	11	10	17	6	-	-	-	-	-	-	44
CONTROLS	No. Pos.	-	-	4	10	17	6	-	-	-	-	-	-	37
B	Dose μg	17	-	-	-	-	-	-	-	-	-	-	-	17
CHLOROMYCETIN	No. Dead	-	-	8	1	5	4	6	2	-	-	-	-	26
17 μg	No. Pos.	-	-	1	0	5	4	6	2	-	-	-	-	18
C	Dose μg	167	-	-	-	-	-	-	-	-	-	-	-	167
CHLOROMYCETIN	No. Dead	-	-	1	1	1	6	4	6	-	-	-	-	19
167 μg	No. Pos.	-	-	0	0	0	2	3	6	-	-	-	-	11
D	Dose μg	167	167	-	-	-	-	-	-	-	-	-	-	334
CHLOROMYCETIN	No. Dead	-	-	1	0	0	1	3	2	8	3	5	1	24
334 μg	No. Pos.	-	-	0	-	-	0	2	2	2	1	5	1	13
E	Dose μg	167 μg one hour before inoculation of Rickettsiae												167
CHLOROMYCETIN	No. Dead	-	-	3	1	0	2	7	3	4	-	-	-	20
167 μg PRE-INFECTION	No. Pos.	-	-	0	0	-	1	7	3	4	-	-	-	15

FIG. 4. EFFECT OF AMOUNT AND TIME OF STARTING TREATMENT WITH CHLOROMYCETIN ON EXPERIMENTAL INFECTION OF CHICK EMBRYOS WITH R. PROWAZEKI

ficed, however, occasional rickettsiae were found in yolk-sac smears from 3 of them. The occurrence of rickettsiae 3 to 5 days after the termination of treatment, associated with a sharp increase in number of deaths, has also taken place in other experiments. Since the drug cannot be excreted from the egg, it must be assumed either that inactivation occurs *in ovo* or that the embryo concentrates

the drug in certain tissues or fluids at the expense of its concentration in the yolk sac.

To investigate the possible effect of minimal amounts of chloromycetin and of preinfection and postinfection administration of the drug, the study shown in



DAYS POSTINFECTION		3	4	5	6	7	8	9	10	Surv.	Total
A SALINE CONTROLS	Dose ml	.5	.5	.5	.5	.5	-	-	-	-	2.5
	No. Dead	-	-	4	7	10	1	-	-	0	22
	No. Pos.	-	-	2	7	10	1	-	-	-	20
B STREPTOMYCIN BASE	Dose mg	1.32	1.32	1.32	1.32	1.32	-	-	-	-	6.6
	No. Dead	-	-	2	5	6	7	2	-	0	22
	No. Pos.	-	-	0	4	5	7	2	-	-	18
C METHYLENE BLUE	Dose mg	.2	.2	.2	.2	.2	-	-	-	-	1.0
	No. Dead	-	-	5	1	3	8	3	0	1	21
	No. Pos.	-	-	4	0	0	6	3	-	0	13
D P-AMINO-BENZOIC ACID	Dose mg	2.5	2.5	2.5	2.5	2.5	-	-	-	-	12.5
	No. Dead	-	-	7	2	1	6	2	2	4	24
	No. Pos.	-	-	0	0	0	0	0	0	0	0

FIG. 5. EFFECT OF MASSIVE DOSES OF STREPTOMYCIN, METHYLENE BLUE, AND PARA-AMINOBENZOIC ACID ON R. PROWAZEKI INFECTION IN THE CHICK EMBRYO

figure 4 was performed. It can be seen that as little as 17 μg (B) of chloromycetin administered in one dose on the third day postinfection caused a definite prolongation of life and a change in the shape of the cumulative death curve. The larger doses, 167 μg (C) and 334 μg (D), the latter given in two doses, caused increasingly greater prolongations of life. Little difference was demonstrated

in the effectiveness of a 167-μg dose, whether given 1 hour preinfection (E) or 3 days postinfection (C).

For comparison with chloromycetin the results of treatment with high concentrations of three drugs that have been reported active against experimental infections with rickettsiae (Greiff *et al.*, 1944; Hamilton *et al.*, 1945; Kikuth and Schilling, 1944; Morgan *et al.*, 1947; Smadel, Jackson, and Gauld, 1947) are given in figure 5. This study was made at about the same time and in the same manner as the foregoing experiments and should be comparable in all respects. Relatively large amounts of a low-potency streptomycin hydrochloride, of methylene blue, and of *para*-aminobenzoic acid were given over a 5-day period to three groups of infected embryos (figure 5; B, C, and D). From a comparison of these and similar data, chloromycetin, gram for gram, appears to be decidedly more effective against *R. prowazeki* than any other agent tested under these experimental conditions in chick embryos.

PHARMACOLOGY OF CHLOROMYCETIN IN ANIMALS

Toxicity for Small Animals

When administered intravenously to 20-g white mice in propylene glycol, the maximum tolerated dose of chloromycetin was 200 mg per kg; the LD_{50} was approximately 245 mg per kg. Administered orally as an acacia suspension, 1 g per kg produced depression in some animals, with recovery in less than 24 hours; tremors and prostration occurred after 1.25 g per kg, followed by recovery. When 100 mg per kg per day were administered subcutaneously in 20 per cent propylene glycol in two divided daily doses, only slight depression of weight gain was noted in 15 days. Divided subcutaneous doses of 200 mg per kg per day were tolerated at least 11 days; doses above 400 mg per kg per day produced ataxia, weight loss, and death in a few days. Local ulceration occurred at the site of repeated subcutaneous injection. When chloromycetin was administered to 20-g mice for 14 days in a ground diet, normal weight gain occurred at 0.25 per cent concentration (360 mg drug per kg per day); on 1 per cent drug diet (1,290 mg per kg per day) there were no deaths but the mice lost an average of 15 per cent in body weight during the treatment period. Rabbits tolerated 100 mg per kg per day in two daily subcutaneous injections in 20 per cent propylene glycol for at least 8 days.

Dogs

Toxicity. Two dogs received acute intravenous doses of chloromycetin as an 8 per cent solution in 75 per cent propylene glycol, injected at the rate of approximately 3.5 ml per minute. The doses employed were 50 and 100 mg per kg. No symptoms were noted other than a transient rise in body temperature (0.5 and 1.1 F) within an hour after injection and a return to normal in several hours.

The effects of intravenous chloromycetin in pure propylene glycol (100 mg of drug per ml of solution) upon blood pressure were tested in four nembutalized

dogs.[11] A dose of 12.5 mg per kg injected at the rate of 100 mg of drug per minute was without effect. Single doses of 25, 50, and 100 mg per kg at the same injection rate resulted in declines in blood pressure of 15, 40, and 60 per cent, followed by recovery in 8, 10, and 20 minutes, respectively. A single dose of 150 mg per kg injected at the rate of 450 mg per minute caused sudden death as a result of fall in blood pressure and respiratory failure. One dog survived two 100-mg per kg doses one half-hour apart, but not a third such injection.

On intramuscular injection of a 1.0-g dose of chloromycetin suspended in 3 ml peanut oil into the thighs of three dogs, mild swelling occurred at the injection site in two animals. On autopsy 7 days postinjection, a large thick-walled cyst containing a gelatinous fibrotic mass was found in each injection area. There was little or no cellular infiltration at the periphery of the cyst. When single doses of 150 to 300 mg of chloromycetin in 2 ml of 70 per cent propylene glycol were injected intramuscularly in dogs, considerable pain was evidenced at the time of injection, but no swelling was noted 24 hours later at the injection site, nor was tissue injury evident at autopsy 7 days later.

Four 7- to 14-kg dogs were given chloromycetin twice daily, 5 days a week, for 38 doses during a 24-day experiment. Three of the animals received the antibiotic twice daily as an intramuscular dose of 0.5 g in colloidal solution in 2 ml of 62 per cent propylene glycol (72 to 88 mg per kg per day). One dog received 0.5 g of the drug twice daily in gelatin capsules (143 mg per kg per day).

The three animals receiving chloromycetin by injection gained slightly in body weight (0.25 to 1.0 kg) during the treatment period. A series of injection sites were used in rotation such that each site was reinjected every fourth day. Induration occurred at the sites, and on autopsy the muscle area appeared pale, indurated, and necrotic. Following injections, there was a slight (1 to 2 F) transient rise in body temperature, accompanied by an increase in pulse rate of 15 to 40 beats per minute. Anemia developed in varying degrees in these three animals. In the most severe case, the initial red cell count and hemoglobin percentage (5.3×10^6 per cu mm, 85 per cent) fell to a minimum by the tenth day (2.3×10^6 per cu mm, 42 per cent) and rose to 3.7×10^6 per cu mm and 60 per cent, respectively, by the end of the experiment. The anemia in another animal developed gradually during the treatment period, and the final values were approximately those of the first animal. In the mildest case, the initial values (5.8×10^6 per cu mm, 91 per cent) fell to 4.3×10^6 per cu mm and 85 per cent by the tenth day and returned to final values of 5.2×10^6 per cu mm and 85 per cent.

The one dog receiving chloromycetin by the oral route lost 0.3 kg in body weight during the 24-day treatment period. There were no changes in body temperature or pulse rate associated with drug intake, nor did alteration of the red cell count or hemoglobin value occur.

None of the four dogs receiving parenteral or oral doses showed significant changes in total white cell or differential counts, blood nonprotein nitrogen,

[11] By Graham Chen, the Research Laboratories of Parke, Davis and Company.

blood sugar, or bromsulfalein liver function tests, nor were alterations in behavior referable to drug toxicity noted. The urine of all animals remained consistently free of albumin and reducing sugar, and the urinary pH and specific gravity were within normal limits.

Absorption and excretion. Chloromycetin serum and urine concentrations were determined in each dog on the eighth and twenty-second days of treatment; specimens were taken just prior to the first dose of the particular day (18 hours after the last dose), 2 hours after the first and second doses of the same

TABLE 9

Absorption and excretion of single doses of chloromycetin in the dog

TIME	CONCENTRATION OF CHLOROMYCETIN IN BLOOD AND URINE								
	Intravenous route[a] 19 mg/kg			Intramuscular route[b] 101 mg/kg			Oral route[c] 86 mg/kg		
	Serum	Urine[d]	Urine	Serum	Urine[d]	Urine	Serum	Urine[d]	Urine
hours	*μg/ml*	*μg/ml*	*μg/specimen*	*μg/ml*	*μg/ml*	*μg/specimen*	*μg/ml*	*μg/ml*	*μg/specimen*
0	<6	2-3	—	<1	<1	—	<6	0	—
¼	17	—	—	—	—	—	—	—	—
½	13	—	—	<3	—	—	—	—	—
1	10	—	—	5	—	—	<6	—	—
2	7	644	10,300	7	114	5,590	8	283	1,980
4	<6	426	3,620	5	157	6,440	25	1,360	8,160
6	<6	190	1,900	3	198	5,740	25	2,540	13,950
7	—	—	—	3	184	1,360	—	—	—
8	<6	70	700	—	—	—	20	2,080	16,620
24	<6	9	970	<1	75	16,500	<6	121	17,550
30	—	—	—	—	47	1,360	—	—	—
48	—	—	—	—	8	1,890	—	—	—
Total urinary excretion, μg			17,490			38,880			58,260
Percentage of dose excreted in urine			8.7			3.9			7.3

[a] A dose of 200 mg drug as a 4 per cent solution in 60 per cent propylene glycol was administered to a 10.5-kg dog.

[b] A 1-gram dose of drug was given suspended in 3 ml peanut oil to a 9.9-kg dog.

[c] A dose of 800 mg in gelatin capsules was given to a 9.3-kg dog.

[d] Urine samples represented the total collection from the time of the previous specimen.

day, and just prior to the first dose of the following day (18 hours after the last dose and 16 hours after the last specimen). There was no indication of slow accumulation of the drug in the body. Intramuscular injections resulted in 2-hour serum levels ranging from less than 1 to 29 μg per ml; the majority of samples (10 out of 12) fell in the range of 2 to 6 μg per ml. The 18-hour levels after injection were usually (11 samples out of 12) in the range of from less than 1 to 2 μg per ml. Urine concentrations of 36 to 406 μg per ml were noted. In

the one orally treated animal, 2-hour serum levels ranged from 6 to 19 μg per ml. Eighteen-hour serum levels were usually less than 2 μg per ml. Urine concentrations were comparable to those obtained on parenteral administration.

Preliminary observations were made on the absorption and excretion of chloromycetin in dogs after a single dose of drug by the oral (1 animal), intravenous (1 animal), and intramuscular (3 animals) routes. Typical data are summarized in table 9. The antibiotic appears to be fairly rapidly excreted or inactivated. The comparable urinary percentage of recoveries of chloromycetin after oral and parenteral administration and the similar drug serum levels during chronic oral and intramuscular administration indicate that a high percentage of the drug is absorbed when given orally.

BINDING BY SERUM ALBUMIN

The binding of crystalline chloromycetin by serum albumin was measured[12] by a modification of the method of van Dyke *et al.* (1945) using 3 per cent crystalline bovine serum albumin. Chloromycetin was bound to the extent of 45 per cent, which is intermediate between the values of 38 and 70 per cent found for sulfadiazine and sulfathiazole, respectively, at corresponding equilibrium concentrations. However, in contrast to these agents, the binding of chloromycetin is relatively little affected by variations in drug concentration. Since the degree of adsorption varies markedly with the amount of protein present, it is estimated that the binding by albumin at the blood-stream concentration would approach 60 per cent.

DISCUSSION

The work on chloromycetin is still at an early stage, and many pertinent questions await answers. Its antibiotic spectrum *in vitro* is being expanded, and, as indications of activity appear, *in vivo* studies are being undertaken. Since antibacterial *in vitro* studies have already indicated that chloromycetin possesses activity against *Borrelia recurrentis* and several gram-negative pathogens, animal trials with these organisms are proceeding. Of the potentialities of chloromycetin presented in this paper and in that of Smadel and Jackson (1947), the most outstanding is its effectiveness in rickettsial infections of chick embryos and mice. This substance, in small doses, administered either orally or parenterally, afforded greater protection against all the rickettsiae studied than has any othe r agent yet tested. Furthermore, even the largest doses produced no toxic symptoms in the control animals.

Intravenously in mice and intramuscularly in dogs chloromycetin appears to be well tolerated in single doses of approximately 0.1 g per kg of body weight. Somewhat larger doses are tolerated orally, although further studies are necessary to define the upper limits of tolerance. Anemia develops in dogs on chronic parenteral administration, but it has not yet been noted after oral administration; further studies are in progress to clarify this point. The greater part of a

[12] By John M. Vandenbelt, the Research Laboratories of Parke, Davis and Company.

single dose is excreted or presumably destroyed in 6 to 8 hours. Less than 10 per cent of the dose appears in the urine, indicating extensive inactivation by the body and perhaps excretion by other routes. With repeated doses it appears that serum levels of 2 to 6 μg per ml may be maintained. Binding by serum albumin appears to be sufficiently low to permit satisfactory diffusion into body fluids.

Chloromycetin has not been evaluated in man, and little is known of its action on healthy and diseased animals. Yet the information thus far available suggests certain limitations and advantages that might attend its employment in human medicine. The limited water solubility and the irritant properties of suspensions of the drug on intramuscular injection in dogs appear to contraindicate this route of administration for systemic infections in man; the oral route would appear to be feasible, in view of the apparently high degree of gastrointestinal absorption and favorable tolerance by this route in animals. Should the toxicity of the antibiotic in man prove to be no greater than the data on laboratory animals would indicate, its demonstrated antibiotic activity suggests the possibility of usefulness against certain of the spirochetes, several gram-negative species, and notably the rickettsiae.

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SUMMARY

An unidentified *Streptomyces* has been isolated that produces an antibiotic substance called "chloromycetin." Antibiotic screening tests on solid and in liquid media are described. The organism produces chloromycetin in submerged aerated culture in several types of media, those containing meat proteins, glycerol, and supplementary material such as molasses giving the best yields. A turbidimetric assay method, in which 50 per cent inhibition of growth is the end point, has been developed.

Crystalline chloromycetin has been found *in vitro* to be inactive against yeasts and filamentous fungi, inactive against protozoa, moderately active against gram-positive bacteria and *Mycobacterium tuberculosis*, and active against gram-

negative bacteria and *Borrelia recurrentis*. The crystalline antibiotic, *in vivo*, has afforded no protection against avian malaria in ducks, syphilis in rabbits, pneumococcus and streptococcus infections in mice, or against type A influenza, St. Louis encephalitis, and fixed rabies virus infections in mice or in eggs, but moderate protection against *Klebsiella* and *Shigella* infections in mice, and remarkable protection against *Rickettsia prowazeki* in chick embryos.

The toxicity of the antibiotic for laboratory animals is of the order of that of streptomycin. Although chloromycetin is only slightly soluble in water, it may be administered parenterally in propylene glycol solutions; oral administration is also possible, since it appears to be well absorbed from the gastrointestinal tract of the dog. After single doses in the dog, only small amounts of antibacterial substances are excreted in the urine, indicating inactivation in the body and perhaps excretion by other routes.

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