MODE OF ACTION OF CHLORAMPHENICOL

IV. Failure of Selected Natural Metabolites to Reverse Antibiotic Action

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Chloramphenicol has been shown to exert a strong inhibitory action on microbial protein synthesis (Gale and Folkes, 1953; Maxwell and Nickel, 1954; Wisseman *et al.*, 1954). Hence, the hypotheses that the antibiotic may act simply as the antagonist of one amino acid or another are especially attractive (Mentzer *et al.*, 1950; Woolley, 1950; Bergmann and Sicher, 1952; Molho and Molho-Lacroix, 1952). Nevertheless, examination of the evidence in support of this proposed mechanism of action reveals that it is derived from experiments in which reversal of growth inhibition by the respective substances was generally minimal and was demonstrable only under special circumstances. Moreover, results of this kind are not limited to selected amino acids; indeed, a wide variety of other substances has also been found to yield comparable degrees of reversal of growth inhibition (Smith, 1952; Swenseid *et al.*, 1952; Foster and Pittillo, 1953; Roblin, 1954; Hitchings and Elion, 1955). Although it is conceivable that a complex relationship of antagonists exists here, as in the case of the sulfonamides (Work and Work, 1948), it is also possible that these minimal "reversal effects" may be due to indirect factors which influence bacterial growth in some other manner and whose relation to chloramphenicol inhibition is more non-specific in nature than is that usually included in the concept of antimetabolites as discussed by Work and Work (1948), Martin (1951) and Woolley (1952).

The structural relation of chloramphenicol to the aromatic amino acids phenylalanine, tryosine and tryptophan in particular, bears reconsideration in discussing this problem. When the formula is written in the conventional two-dimensional projection (see A, figure 1), there is a suggestion of a resemblance to the aromatic amino acids or to D-serine. However, the x-ray diffraction studies of Dunitz (1952) on crystalline chloramphenicol and bromamphenicol indicate that in this physical state, a second six membered ring may be formed by a hydrogen bond in the propanediol moiety, giving the molecule a much different appearance (see B, figure 1). It is, of course, possible that this ring does not actually exist in solution or under the conditions of antibiotic action, but compounds in which a similar but more stable ring structure has been substituted actually have been synthesized and possess antibiotic activity (U. S. Patent, 1952; Taylor, 1953). Formula C in figure 1 illustrates one of these compounds. An extensive discussion of the structural and configurational factors which contribute to the antibiotic activity of chloramphenicol is published elsewhere (Hahn *et al.*, 1956). Furthermore, the theoretical relation of phenylalanine to chloramphenicol requires the assumption of four distinct substitutions (Woolley, 1950). This multiplicity of alterations might change the properties of the resulting compound to such an extent that it could no longer function as an antimetabolite of phenylalanine. In any case, the structural basis for relating chloramphenicol to the aromatic amino acids in an antimetabolite relationship would appear less well-founded than formerly supposed.

The present report is an outgrowth of studies which have been continued intermittently since our first reported failure to detect reversal of growth inhibition by a variety of natural substances (Wisseman *et al.*, 1954). The results are presented at this time because of their bearing on hypotheses concerning the mechanism by which protein synthesis is inhibited by chloramphenicol. In the course of these studies there have been many variations in conditions of testing, including different strains of organisms, different media and small as well as large inocula. The particular results selected for inclusion in this report were obtained under conditions designed to eliminate in so far as possible the influence of non-specific factors. In our experience

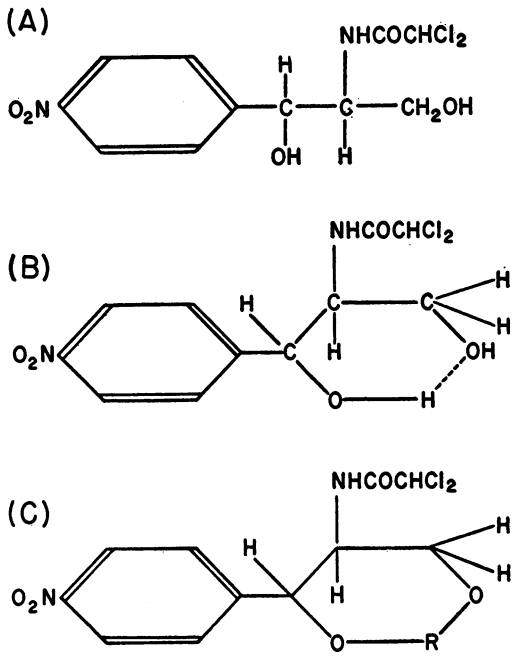

Figure 1. Structure of chloramphenicol and one of its cyclic derivatives.

all substances tested have uniformly failed to reverse specifically the growth inhibiting property of chloramphenicol.

MATERIALS AND METHODS

Test organisms and media. Escherichia coli strain B, obtained from Dr. Mark Adams, was fully adapted to growth in the synthetic test medium by serial transfer prior to its use in the experiments. In like manner, a phenylalanine-requiring mutant of *E. coli* strain B/r (strain C-2), furnished by Dr. Joseph Gots, was adapted to phenylalanine-fortified synthetic medium. The basic test medium employed in most experiments with the *E. coli* strains was that of Fisher and Armstrong (1947) with glucose substituted for glycerol. However, in some experiments indicated in the text below, the synthetic medium of Hook *et al.* (1946) was employed.

Leuconostoc citrovorum ATCC 8081 was maintained on micro-inoculum agar (Difco) while actual reversal studies were carried out in citrovorum broth (Difco).

Test substances. Stock solutions of the various amino acids and other substances were prepared in distilled water in a concentration 10 times that desired in the test media. Citrovorum factor was donated by Dr. T. H. Jukes of Lederle Laboratories in the form of the calcium salt. Chloramphenicol, a gift of Parke, Davis and Co., was dissolved in distilled water to give a stock solution containing 1000 μg/ml. All solutions were sterilized by passage through a Seitz filter.

General procedure for reversal experiments with strains of E. coli. The following general procedure was employed for all the experiments with strains of *E. coli*, except as indicated in the exploratory studies. Ten ml of a 16 hr culture grown in the appropriate synthetic medium was transferred to a 1-L flask containing 500 ml of fresh medium. This culture was incubated in a water bath at 34 C and growth was followed turbidimetrically at 420 mμ until it attained the early logarithmic phase. (The number of cells present in an early logarithmic phase culture did not influence the minimal inhibitory concentration of chloramphenicol; preliminary experiments in which *E. coli* was tested in the conventional serial dilution type of sensitivity test indicated that the minimal inhibitory concentration of chloramphenicol after incubation for 18 hr at 37 C was constant, regardless of the size of inoculum which was varied between 5×10^1 organisms per ml and 5×10^6 organisms per ml.) Then, 8.0 ml aliquots were quickly distributed to sterile cuvettes which contained 1.0 ml of test substance and 1.0 ml of chloramphenicol, each 10 times the desired final concentration. Each series of tubes contained a constant amount of test substance but the final chloramphenicol concentration was varied from 0.039 to 10 μg/ml in 2-fold increments of concentration. A control tube containing all additions except chloramphenicol was included in each series. A similar series of tubes containing chloramphenicol in which distilled water was substituted for the metabolite to be tested for reversing action was prepared for comparison and was run simultaneously with every series containing test substances. The cuvettes were then incubated at 34 C in a water bath equipped with forced circulation. Optical densities of each cuvette were read in a Coleman Model 14 spectrophotometer at a wave length of 420 mμ at 30-min intervals over a 3-hr period or until the maximum stationary growth phase was attained.

Procedure for reversal studies with L. citrovorum. Two flasks of citrovorum factor medium (Difco Manual—9th edition) were inoculated with washed (3 times) *L. citrovorum* strain 8081.

TABLE 1

Failure of L-phenylalanine or L-tryptophan to reverse inhibition of growth of Escherichia coli strain B by a sub-bacteriostatic concentration of chloramphenicol

	Chloramphenicol	L-phenylalanine (growth rate)		L-tryptophan (growth rate)	
	μg/ml	0 μg/ml	2000 μg/ml	0 μg/ml	1000 μg/ml
Control	0	0.294	0.340	0.270	0.315
Chloramphenicol	1.0	0.183	0.220	0.171	0.206
Relative growth rate*		62	65	63	65

* Relative growth rate = $\frac{\text{growth rate with chloramphenicol}}{\text{growth rate of control}} \times 100.$

The medium in one flask contained 0.3 mμg calcium citrovorum per ml, the minimal amount yielding optimal growth under the conditions of the test, while the medium in the other flask contained 30 mμg calcium citrovorum per ml. The cells from each flask were collected by centrifugation, washed three times and resuspended in citrovorum factor broth. Two series of cuvettes, one containing a final concentration of 0.3 and the other containing 30 mμg calcium citrovorum per ml, were inoculated with a sufficient amount of washed cell suspension to give an initial optical density of 0.150 at a wavelength of 620 mμ in the Coleman instrument. Each series contained a chloramphenicol-free control and a range of chloramphenicol concentrations as described in the preceding section. The tubes were incubated at 34 C and the turbidity was measured at 30 min intervals over a period of 5 hr.

RESULTS

Reversal studies with E. coli strain B. Exploratory experiments were designed to study the effect of L-phenylalanine and L-tryptophan on the growth retarding action on *E. coli* strain B of a chloramphenicol concentration well below that required to inhibit growth completely. The low antibiotic concentration was employed to increase the chances of detecting minor reversing actions. Hook's (1946) synthetic medium was employed here. A 16-hr culture of the organism grown in this medium was washed by centrifugation and resuspended in sufficient fresh medium to give an optical density in the Beckman model DU spectrophotometer of approximately 0.030 in a 1-cm cell at 420 mμ. This diluted suspension was divided into four 100-ml aliquots; additions were then made to give cultures of the following composition: (1) basal medium alone, (2) basal medium containing 1.0 μg chloramphenicol/ml, (3) basal medium with L-phenylalanine (2000 μg/ml) or L-tryptophan (1000 μg/ml), and (4) basal medium containing chloramphenicol (1.0 μg/ml) and L-phenylalanine (2000 μg/ml) or L-tryptophan (1000 μg/ml). The cultures were placed in a 34 C waterbath and after a 10–15 min equilibration period, were found to have resumed the logarithmic phase of growth. Samples were removed from each culture at 10-min intervals over a period of 1.5–2.0 hr for optical density measurements. The slope of the straight line which resulted from plotting log optical density against time was used as a measure of the growth rate. The average of the results of three separate experiments each with L-phenylalanine and L-tryptophan are recorded in table 1.

The concentration of chloramphenicol employed in these experiments permitted the test organism to grow in the basal medium at a rate 62–63 per cent of that of the uninhibited culture in the same medium. The addition of phenylalanine or tryptophan to the basal medium regularly caused the growth rate of *E. coli* strain B to increase up to 15 or 20 per cent over that observed in the basal medium alone. However, despite the stimulatory action of the amino acids on growth, the relative magnitude of the chloramphenicol effect on growth remained unaltered; that is, 1.0 μg/ml of chloramphenicol in the amino acid-fortified medium still reduced the rate of growth of the test organism to 65 per cent of that of the control culture, which in this instance was a drug-free culture in the fortified medium. This value does not differ significantly from the one obtained in the unfortified basal medium. Had a specific reversal of chloramphenicol action occurred in the fortified medium, the

relative growth rate in the presence of 1.0 μg/ml of the antibiotic in this medium should have been significantly higher. Since this is not the case, these experiments give no indication of any specific reversing action that could be ascribed to antagonism of an antimetabolite.

More extensive studies were then performed with these same amino acids, as well as with a number of other natural metabolites. The method employed here was more sensitive and reproducible[1] than that employed in the exploratory studies described above. It was based on a determination of the 50 per cent effective dose (ED_{50}), that is, the amount of chloramphenicol required to reduce the *rate* of growth of a logarithmic phase culture of the test organism to 50 per cent of the rate of the corresponding antibiotic-free control. This value was determined simultaneously in every instance both in the presence and in the absence of the compound being tested for reversing action. The method was broadly patterned after those described by Kohn and Harris (1941) and by Joslyn and Galbraith (1950); special modifications, however, were introduced to cope with factors, other than reversal of antimetabolic action, which have been suggested by several investigators as potentially capable of influencing this type of experiment. These modifications were: (1) elimination or minimization of the lag phase which may introduce serious variations into the results, (2) minimization of the effects due to bacterial degradation of the antibiotic (Smith and Worrel, 1950) by reducing the time of exposure of the drug to the microorganisms, (3) permitting only a few cell divisions to occur during the experiment so as to give little opportunity for overgrowth of resistant variants or mutants (Foster and Pittillo, 1953), (4) compensation for nonspecific effects of added nutrients on either the rate or the maximal final levels of growth attained.

Growth stops very rapidly when concentrations of chloramphenicol down to the bacteriostatic level are introduced into an actively growing culture of *E. coli* (Bozeman *et al.*, 1954; Wisseman *et al.*, 1954). Over a broad range of chloramphenicol concentrations below the minimum bacteriostatic level, growth proceeds more slowly than in the antibiotic-free control but it is still exponential in character over most of the observation period so that the logarithm of the optical density plotted against time forms a straight line; its slope bears an inverse relation to chloramphenicol concentration. The slope (K), which is an expression of the growth rate, was calculated by the equation

$$K = \frac{\log D_2 - \log D_1}{T_2 - T_1} \qquad (1)$$

in which D_1 and D_2 are the optical densities at the beginning and end of the straight segment of the growth curve and T_1 and T_2 are corresponding times in hours. Then, in order to compensate for any general stimulation or retardation of growth caused by the addition of the metabolite being tested, the *relative growth rate* at each chloramphenicol concentration in a given test was expressed as per cent of the growth rate exhibited by the *corresponding* antibiotic-free control culture, i. e., the one which contained all additions except the antibiotic. This was calculated by the equation.

$$\text{Relative growth rate} = \frac{K_{CM}}{K_{\text{control}}} \times 100 \qquad (2)$$

in which K_{CM} is the slope of the growth curve for any given chloramphenicol concentration and K_{control} is the slope of the growth curve of the control. When the relative growth rates of the partially inhibited cultures are plotted against the logarithm of the corresponding concentrations of chloramphenicol, a nearly symmetrical sigmoid curve is obtained (figure 2) which has the general form of dose-response curves given by many biological systems (Bliss, 1950). The central portion of the curve approaches linearity and, hence, the 50 per cent growth inhibition level of chloramphenicol is readily determined graphically.

Phenylalanine, tryptophan, aspartic acid, glutamic acid, glycine and *p*-aminobenzoic acid were tested for their capacity to reverse the growth inhibition effect of chloramphenicol on *E. coli* strain B. The results obtained, in each

[1] The competitive antagonism which exists between *p*-aminobenzoic acid and sulfadiazine was readily demonstrated by this method in preliminary studies. Furthermore, it would be anticipated that minimal degrees of reversal, of the kind expected in the case of non-competitive antagonism, would be detectable at the lower end of the dose-response curve in the form of divergence of the curve obtained in the fortified medium from that obtained in the basal medium.

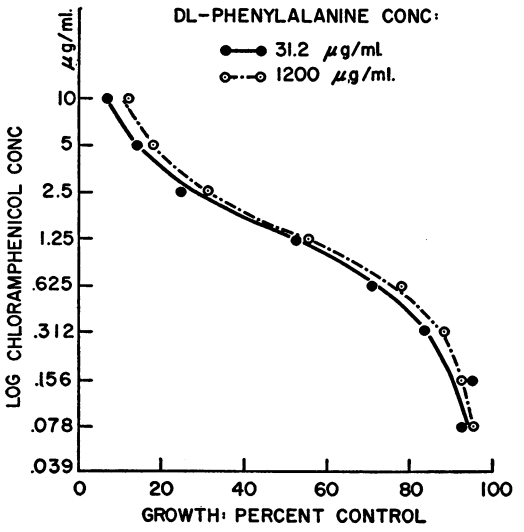

Figure 2. Dose-response curves of cultures of *Escherichia coli* strain C-2 to chloramphenicol in the presence of phenylalanine.

instance the average of three or more separate experiments, are recorded in table 2 and are expressed as the ratio

$$\frac{ED_{50}\ (CM) \text{ with metabolite}}{ED_{50}\ (CM) \text{ without metabolite}} \qquad (3)$$

Thus, the ratio obtained in a test with a substance having no reversing effect would be 1.0; the ratio for a substance which had a reversing action would be significantly greater than 1.0. It is clear from the table, in which all the ratios are near 1.0, that none of the substances caused any significant reversal of growth inhibition.

Reversal studies with phenylalanine employing a phenylalanine-requiring mutant of E. coli. Extensive studies with phenylalanine were performed using a phenylalanine-requiring mutant of *E. coli* as test organism since experience has shown that demonstration of antagonism may prove unsuccessful except with an organism dependent upon an extrinsic source of the metabolite under consideration (Woolley, 1952). The effect of chloramphenicol on the growth of this mutant was tested over a range of antibiotic concentrations which extended from one whose effect was barely detectable to one which caused almost complete inhibition of growth; two concentrations of DL-phenylalanine were employed, i. e., (1) 31.2 μg/ml which was 5 times the minimal level that gave optimal growth under the conditions of the tests and (2) 1200 μg/ml which was 192 times the minimal optimal concentration. The dose-response curves for each series are presented in figure 2. The $ED_{50}(CM)$ was 1.22 ± 0.09 μg/ml for the lower phenylalanine level and 1.24 ± 0.11 μg/ml for the higher concentration, giving a ratio of 1.01. The difference between the two values is small and statistical analysis showed that it is well within the limits of accuracy of the method. The possibility remained that a noncompetitive antagonism not apparent at the $ED_{50}(CM)$ level might be demonstrable at chloramphenicol concentrations well below the $ED_{50}(CM)$ level. However, from inspection of the dose-response curves it is seen that, even at the lowest antibiotic levels giving perceptible growth retardation, the two curves are almost identical. This impression is substantiated by a probit analysis of the data which indicated that the differences were not significant and that the two curves were essentially superimposable (95 per cent confidence limits).

Reversal studies with citrovorum factor and Leuconostoc citrovorum. Three-tenths mμg of citrovorum factor per ml were sufficient for optimal growth of this test organism. The $ED_{50}(CM)$ for this organism was the same regardless of whether the medium contained this minimal concentration of citrovorum factor or 100 times as much, i. e., 30 mμg per ml.

TABLE 2

Failure of selected metabolites to reverse inhibition of growth of Escherichia coli strain B by chloramphenicol

Test Compound	Concentration	Reversal Ratio*
	μg/ml	
L-Aspartic acid	100	0.95
L-Glutamic acid	100	1.09
Glycine	100	1.04
p-Aminobenzoic acid	10	1.11
DL-Phenylalanine	100	1.10
DL-Phenylalanine	1000	1.04
L-Tryptophan	100	1.07

* Reversal ratio = $\frac{ED_{50}\ (CM) \text{ with test compound}}{ED_{50}\ (CM) \text{ without test compound}}$

DISCUSSION

The failure of selected amino acids to reverse the action of chloramphenicol under the de-

scribed experimental conditions is in discord with the idea that the drug acts as an antimetabolite of an amino acid. Despite the negative nature of the findings, they bear significantly on the theories concerning the mode of action of chloramphenicol.

Particular effort was made to employ test conditions which were heavily weighted in favor of obtaining reversal effects. For example, the widest possible range of chloramphenicol concentrations employed should have permitted the detection even of minimal antagonistic effects of a noncompetitive nature when the proper amino acids were supplied in sufficiently high concentrations. Furthermore, the present experiments were carried out with bacteria in the logarithmic phase of growth, i. e., under cultural conditions almost identical with those under which the action of chloramphenicol on bacteria protein synthesis has been demonstrated (Wisseman *et al.*, 1954).

While it may be inferred that antagonism of amino acids is not the mechanism by which chloramphenicol inhibits protein formation, the possibility is not excluded that the drug might be a biological antagonist of some other essential substance, for example, a catalyst which perhaps is involved in protein synthesis. Until such action is actually demonstrated, however, the possibility must also be considered that the mechanism of action of chloramphenicol is fundamentally different and not based on antimetabolism of structural analogues.

SUMMARY

The presence of phenylalanine, tryptophan, glycine, aspartic acid and *p*-aminobenzoic acid in logarithmic growth phase cultures of *Escherichia coli* strain B failed to reverse the inhibitory effect of chloramphenicol under a variety of experimental conditions. Similarly, no reversal was demonstrable in systems which employed a phenylalanine-requiring mutant of *E. coli* or in *Leuconostoc citrovorum* which requires citrovorum factor for growth. Observations which lead to the belief that chloramphenicol does not act as a simple amino acid antagonist are discussed.

REFERENCES

BERGMANN, E. D. AND SICHER, S. 1952 Mode of action of chloramphenicol. Nature, **170**, 931–932.

BLISS, C. I. 1950 The design of biological assays. Ann. N. Y. Acad. Sci., **52**, 817–888.

BOZEMAN, F. M., WISSEMAN, C. L., JR., HOPPS, H. E., AND DANAUSKAS, J. X. 1954 Action of chloramphenicol on T-1 bacteriophage. I. Inhibition of intracellular multiplication. J. Bacteriol., **67**, 530–536.

DUNITZ, J. D. 1952 The crystal structure of chloramphenicol and bromamphenicol. J. Am. Chem. Soc., **74**, 995–999.

FISHER, K. C. AND ARMSTRONG, F. H. 1947 The effects of sulfathiazole and of propyl carbamate on the rate of oxygen consumption and growth in *Escherichia coli*. J. Gen. Physiol., **30**, 263–278.

FOSTER, S. W. AND PITTILLO, R. F. 1953 Reversal by complex natural materials of growth inhibition caused by antibiotics. J. Bacteriol., **65**, 361–367.

GALE, E. F. AND FOLKES, J. P. 1953 The assimilation of amino acids by bacteria. 19. The inhibition of phenylalanine incorporation in *Staphylococcus aureus* by chloramphenicol and *p*-chlorophenylalanine. Biochem. J. (London), **55**, 730–735.

HAHN, F. E., HAYES, J. E., WISSEMAN, C. L., JR., HOPPS, H. E. AND SMADEL, J. E. 1956 Mode of action of chloramphenicol. VI. Relation between structure and activity in the chloramphenicol series. Antibiotics and Chemotherapy, **6**, 531–543.

HITCHINGS, G. H. AND ELION, G. B. 1955 The *Lactobacillus casei* screening test. Investigation of diverse systems for cancer chemotherapy screening. Cancer Research, Supplement No. 3, 66–68.

HOOK, A. E., BEARD, D., TAYLOR, A. R., SHARP, D. G., AND BEARD, J. W. 1946 Isolation and characterization of the T_2 bacteriophage of *Escherichia coli*. J. Biol. Chem., **165**, 241–258.

JOSLYN, D. A. AND GALBRAITH, M. 1950 A turbidimetric method for the assay of antibiotics. J. Bacteriol., **59**, 711–716.

KOHN, H. I. AND HARRIS, J. S. 1941 On the mode of action of the sulfonamides. I. Action on *Escherichia coli*. J. Pharmacol. and Exptl. Therap., **73**, 343–382.

MARTIN, G. J. 1951 *Biological antagonism*. The Blakiston Co., Philadelphia.

MAXWELL, R. E. AND NICKEL, V. S. 1954 The antibacterial activity of the isomers of chloramphenicol. Antibiotics and Chemotherapy, **4**, 289–295.

MENTZER, C., MEUNIER, P., AND MOLHO-LACROIX, L. 1950 Faits de synergie et d'antagonism

entre la chloromycétine et divers amino acides vis-à-vis des cultures d'*E. coli*. Compt. rend. soc. biol., **230**, 241–243.

MOLHO, D. AND MOLHO-LACROIX, L. 1952 Étude comparée de l'antagonisme entre quelques dérivés de la phénylalanine et la chloromycétine, la B_2-thiénylalanine et la β-phénylsérine. Bull. Soc. Chim. Biol., **34**, 99–107.

ROBLIN, R. O., JR. 1954 Metabolite antagonists. Ann. Rev. Biochem., **23**, 501–526.

SMITH, G. N. AND WORREL, C. S. 1950 The decomposition of chloromycetin (chloramphenicol) by microorganisms. Arch. Biochem. **28**, 232–241.

SMITH, G. N. 1952 The influence of chloromycetin decomposition products on the growth of *Escherichia coli* and their effects on reversing the growth inhibitory action of the antibiotic. Arch. Biochem. and Biophys., **40**, 314–322.

SWENSEID, M. E., WRIGHT, P. D., AND BETHELL, F. H. 1952 A growth factor for *L. citrovorum* synthesized by hemopoietic tissue reversing aminopterin and chloromycetin inhibition. Proc. Soc. Exptl. Biol. Med., **80**, 689–690.

TAYLOR, E. P. 1953 A tasteless derivative of chloramphenicol. J. Pharm. and Pharmacol., **5**, 254–256.

U. S. Patent Number 2,587,641. Nov. 4, 1952 (Ring derivatives of chloramphenicol).

WISSEMAN, C. L., JR., SMADEL, J. E., HAHN, F. E., AND HOPPS, H. E. 1954 Mode of action of chloramphenicol. I. Action of chloramphenicol on assimilation of ammonia and on synthesis of proteins and nucleic acids in *Escherichia coli*. J. Bacteriol., **67**, 662–673.

WOOLLEY, D. W. 1950 A study of non-competitive antagonism with chloromycetin and related analogues of phenylalanine. J. Biol. Chem., **185**, 293–305.

WOOLLEY, D. W. 1952 *A study of antimetabolites*. John Wiley and Sons, Inc., New York.

WORK, T. S. AND WORK, E. 1948 *The basis of chemotherapy*. Interscience Publishers, Inc., New York.