JOURNAL OF BACTERIOLOGY, May 1967, p. 1671-1676

Vol. 93, No. 5
Printed in U.S.A.

Measurement of Binding of Chloramphenicol by Intact Cells

CHARLES HURWITZ AND CATHERINE B. BRAUN

Basic Science Research Laboratory, Veterans Administration Hospital, and Department of Microbiology, Albany Medical College, Albany, New York 12208

Received for publication 28 January 1967

The binding of chloramphenicol to intracellular components of intact cells was measured by procedures based on a silicone-wash technique. The number of stereospecifically bound molecules of chloramphenicol increased with external concentration to a saturation value equal to the number of ribosomes per cell. Chloramphenicol is therefore believed to be attached stereospecifically by a weak bond, most probably to a single site on the 50*S* ribosome. This bond was found to be temperature-dependent and appeared to be responsible for inhibition of protein synthesis.

Chloramphenicol (CM) is predominantly bacteriostatic for most bacteria, and is so weakly held that ribosomal-bound CM is displaced on centrifugation of ribosomes through a sucrose gradient (10). Its inhibitory action in protein synthesis can be readily reversed by washing by centrifugation. Vasquez (8, 9), Wolfe and Hahn (11), and Das et al. (3) measured binding of CM to ribosomes and to intact cells by sedimenting the particles (cells or ribosomes) from suspension and correcting for nonspecifically bound or entrained CM. Calculations of the bound CM by this procedure have necessitated large corrections for nonspecifically entrained CM and have therefore introduced considerable uncertainty in the values obtained.

We have described a method for determining intracellular concentrations of freely diffusible ions in bacteria. The method depends on centrifuging bacteria from an aqueous suspension through a more dense layer of immiscible solvent (a mixture of silicones), which is also less dense than the bacterial cells (6). Since such a procedure seemed applicable for the measurement of intracellular concentrations of solutes held by weak bonds, we have used it to measure the effect of external concentrations of CM on the CM bound to specific sites in intact cells.

The purpose of this report is to describe the results obtained with this technique, which enabled us to obtain accurate values for the intracellular binding of CM; to show that CM is attached stereospecifically by a weak bond, most probably to a single site on a ribosome; to show that this weakly bound CM is responsible for inhibition of protein synthesis; and to show that this weak bond is stable to cold (0 to 4 C) washes.

MATERIALS AND METHODS

Organism and media. Escherichia coli ML 35 cells growing exponentially in nutrient broth or in half-strength minimal medium (4) at 37 C were used for these studies.

Labeling with tritium. The cells, at about 3×10^7 per ml, were exposed to ^{3}H-thymidine and were further grown for about two generations. The cells were then washed free of ^{3}H-thymidine and reincubated in fresh medium until cell growth reached about 3×10^8 per ml, when ^{14}C-CM was added at the indicated concentrations.

Isolation of cells from medium. The cells were isolated from aqueous suspension by layering 4-ml samples at indicated intervals on top of a 2-ml layer of a cold (0 to 4 C) silicone mixture in cellulose nitrate tubes, as described elsewhere (6). The tubes were centrifuged for 30 min at 15,000 $\times$ *g* in an HS-2 swinging bucket rotor in a Sorvall (model RC2) centrifuge, and the cells were isolated by decantation of the supernatant fluid and excision of the curved bottom of the tube.

Measurement of radioactivity. Radioactivity was extracted from the cells as indicated. ^{3}H and ^{14}C disintegrations in 0.5-ml samples of the extracts were measured in a Packard Tri-Carb liquid scintillation counter to a counting error of less than 2%. Bray's solvent (1), modified to contain 13% bis(2-methoxyethyl)ether, was used. The counting efficiency was 35% for ^{14}C and 11% for ^{3}H in the presence of 5% trichloroacetic acid, which exerts a strong quenching effect.

Determination of numbers of cells. Viable counts were made in quadruplicate on nutrient agar. Direct counts were made in duplicate with a Petroff-Hausser chamber. Turbidity measurements were made with a

Coleman (model 7) photonephelometer, one nephelos unit being equivalent to 10^6 cells in exponential growth phase in nutrient broth. Determination of the number of ribosomes per cell was made according to the procedure of Goldstein et al. (5).

Source of ^{14}C-CM. ^{14}C-CM was obtained from several sources. CM labeled in the *N*-dichloroacetyl moiety was obtained from New England Nuclear Corp., Boston, Mass., and from Nuclear Research Chemical Corp., Orlando, Fla. CM labeled in the 3-propanediol carbon was obtained from Nuclear-Chicago Corp., Des Plaines, Ill. Nonradioactive CM was a gift from Parke, Davis & Co., Detroit, Mich.

The purity of a number of the samples was improved by paper chromatography (100 parts of butanol plus 14 parts of 2.5% acetic acid) and by column chromatography (water elution from Sephadex G-10).

In this publication, CM is used to mean the active D(−)-*threo* isomer of chloramphenicol, D(−)-*threo*-2,2-dichloro-*N*-[β-hydroxy-α-(hydroxymethyl)-*p*-nitrophenethyl]acetamide. The inactive L(+)-*erythro* isomer, obtained from Parke, Davis & Co., is labeled as such when used. Analogues of CM, designated as WIN, were obtained through the courtesy of Sydney Archer from Sterling Winthrop Research Institute, Rensselaer, N. Y.

RESULTS

Exponentially growing cells containing tritium-labeled deoxyribonucleic acid were exposed to ^{14}C-CM at different concentrations. At each concentration, a duplicate sample was exposed to the same concentration of ^{14}C-CM to which was added a 100-fold concentration of ^{12}C-CM. Samples were removed, chilled in crushed ice, and washed by centrifugation through a silicone layer; the cellular pellet was then extracted with hot 5% trichloroacetic acid for 20 min. Tritium and ^{14}C were then measured.

Figure 1 shows the results obtained when such cells were exposed to 1 and 3 μg (per ml) of ^{14}C-CM, plus or minus ^{12}C-CM, for various lengths of time. The values are corrected for recovery of cells, measured as percentage recovery of tritium (6). Recoveries averaged between 85 and 95% when a Sorvall HS-2 swinging-bucket rotor was used, and about 10% lower when a fixed 40°-angle rotor (Sorvall SE-12) was used. The values are also corrected for the growth which occurs during the exposure to CM. This growth was determined both by nephelometry and by direct counts.

At both 1 and 3 μg of ^{14}C-CM per ml, a 100-fold dilution with unlabeled CM markedly reduced the amount of ^{14}C recovered from the cells, the amount of reduction being greater at 3 μg/ml than at 1 μg/ml. Since the reduction in label resulting from the dilution effect of unlabeled CM must be a measure of sites for which

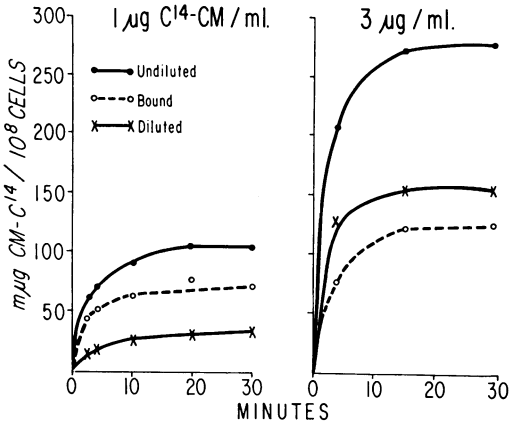

FIG. 1. *Effect of isotopic dilution on binding of ^{14}C-CM by intact cells. Escherichia coli ML 35 cells, growing exponentially at 3 × 10^8 cells per ml in nutrient broth, were exposed to ^{14}C-CM ± ^{12}C-CM as indicated. ^{12}C-CM, when added, was present in 100 times the concentration of ^{14}C-CM. Cells from 4-ml samples were isolated from the aqueous medium by centrifugation through silicone (6). The ^{14}C recovered in the cells is expressed as ^{14}C-CM, calculated from the specific activity of 2.2 × 10^4 counts per min per μg of CM.* (●) *Cells exposed to undiluted ^{14}C-CM;* (×) *diluted ^{14}C-CM;* (○) *specifically bound CM, calculated as the difference between the two determined values.*

both ^{14}C-CM and ^{12}C-CM compete, the difference between the two obtained values (i.e., with and without added ^{12}C-CM) is assumed to measure specific binding of CM. This isotopic dilution technique is based on the same considerations as described by Wolfe and Hahn (11) and Das et al. (3).

Dilution of ^{14}C-CM with smaller increments of ^{12}C-CM (i.e., ^{14}C-CM to ^{12}C-CM ratios of 1:1, 1:2, 1:4, etc.) reduced the specifically bound CM proportionately, providing the ^{14}C-CM concentration was greater than 3 μg/ml. At values lower than 3 μg/ml (Fig. 2), saturation of the binding sites was not achieved in the undiluted sample. Consequently, addition of ^{12}C-CM at low ratios to ^{14}C-CM resulted in skewed values.

For example, if external ^{14}C-CM is sufficient for half saturation of specifically bound CM, dilution with an equal amount of ^{12}C-CM would almost double the amount of bound CM, but at 50% of the original specific activity. Consequently, the amount of bound ^{14}C-CM recovered would be about the same with and without added ^{12}C-CM. If ^{12}C-CM is instead added at a 100:1 ratio, the amount of specifically bound CM would be increased to twice the amount, but at a specific activity of 1:100 that of the undiluted ^{14}C-CM. Under the latter condition, the amount of specifi-

cally bound ^{14}C-CM recovered would be reduced by a factor of 50. At higher external ^{14}C-CM concentrations, addition of ^{12}C-CM at 100:1 would approach the predictable reduction factor of 100. At all external concentrations of ^{14}C-CM, addition of ^{12}C-CM at 100:1 concentration was sufficient to reduce residual ^{14}C-CM to minimal values, indicating that the remaining bound ^{14}C-CM was reduced to undetectable levels. Above 5 μg of CM per ml, the total amount of CM recovered per cell was proportional to the external concentration, ruling out the possibility that the internal concentration was not in linear equilibrium with the external concentration.

As ^{14}C-CM concentration increases, the amount of specifically bound CM becomes smaller compared to the two determined values from which it is calculated by difference. Consequently, the error of determination of the difference value becomes disproportionately large as external concentration increases, setting an upper limit of 3 to 5 μg (per ml) of ^{14}C-CM for the accurate measurement of specifically bound CM by this dilution method.

The finding that more CM was specifically bound at 3 μg/ml than at 1 μg/ml indicated an effect of concentration on the amount bound. In the following experiments, the cells were exposed to concentrations of ^{14}C-CM varying from 0.25 to 5 μg/ml, all plus or minus a 100-fold dilution with ^{12}C-CM. The amount of specifically bound CM (the difference between plus or minus ^{12}C-CM) after 15 min of exposure is plotted as a function of external concentration of ^{14}C-CM. The values in Fig. 2 are plotted as molecules per cell. It can be seen that the amount of specifically bound CM increases to a maximum of about 26,000 molecules per cell at an external concentration of about 5 μg/ml.

The source of ^{14}C-CM, or the position of the label, had no effect on the amount of specifically bound chloramphenicol, although the amount of residual ^{14}C after dilution with ^{12}C-CM was higher with some samples than with others. The amount of residual label in some samples was markedly reduced by prior purification by column and paper chromatography of the ^{14}C-CM. The compounds received from Nuclear Chicago Corp. and from New England Nuclear Corp. could not be further purified by our procedures.

Experiments in which the L(+)-*erythro* isomer was substituted for ^{12}C-CM indicated that the dilution technique measures stereospecifically bound CM, since no dilution effect was obtained with the inactive isomer. Similar effects were obtained with stereoisomers of analogues of CM, as described below.

It is possible that the lack of effect of the L(+)-*erythro* isomer on synthesis of protein and on binding of the active D(−)-*threo* isomer may be due to lack of permeability of cells to the former, rather than to difference in binding affinity to the ribosome. The possibility that the cells are permeable to only the D(−)-*threo* configuration would, however, seem to be unlikely, since (i) the L(+)-*erythro* isomer had no effect on binding of CM by isolated ribosomes (10, 11); (ii) similar effects were obtained with stereo isomers of analogues of CM (see below); and (iii), in the latter case, partial effects were obtained in intact cells, indicating that the cells were permeable to both stereoisomers. The unavailability of the radioactive L(+)-*erythro* isomer prevented a direct experimental determination of whether the cells were equally permeable to both stereoisomers.

The following experiments suggest that the stereospecific binding of CM is an integral part of the mechanism by which the drug inhibits protein synthesis. In Table 1, the effectiveness of several analogues in reducing the stereospecific binding of CM is compared with the relative ability of each drug in blocking induced synthesis of β-galactosidase. WIN 5094-2, a racemic mixture in which the nitro group of CM is replaced by methyl sulfide, is 68% as effective as CM in reducing the stereospecific binding, and is about 50% as effective in inhibiting protein synthesis. WIN 10446, a racemic mixture of the methylsulfinyl derivative, is almost devoid of activity in either category. WIN 5063, a methylsulfonyl derivative, has intermediate values in both categories.

It is also noteworthy that WIN 5094-2, the D(+) isomer of 5094, is 10 times as effective as the L(−) isomer (5094-3) in reducing the stereospecific binding of CM, whereas the racemic mixture has an intermediate value. This finding, which is in agreement with the lack of effect of the L(+)-*erythro* isomer of CM, further supports the conclusion that the dilution technique measures stereospecifically bound CM.

Vasquez (10) also found that WIN 5094 competes with chloramphenicol for bacterial binding sites. In his studies, the WIN derivative was somewhat less effective than in our experiments, but it should be noted that we used twice the ratio of WIN 5094 to ^{14}C-CM that he used. The ability of a number of other analogues to reduce the sterospecific binding of CM was also determined. In each instance, the ability of the analogue to reduce the stereospecific binding of CM agreed with the relative effectiveness of the drug in preventing growth, as previously reported

TABLE 1. *Comparison of degree of competition for chloramphenicol binding sites with degree of inhibition of protein synthesis*[a]

Competitor for binding of ^{14}C-CM	Stereoisomer	Reduction of binding of ^{14}C-CM	Inhibition of protein synthesis at 2 μg/ml
		%	%
Chloramphenicol	D(−)-*threo* (nitro, dichloro-*N*-acetyl)	100	91
WIN 5094-2	D(+)-*threo* (methylthio)	89	
WIN 5094	(±)-*threo* (methylthio)	67	43
WIN 5094-3	L(−)-*threo* (methylthio)	9	
WIN 5063	(±)-*threo* (methylsulfonyl)	37	28
WIN 10446	D(+)-*threo* (methylsulfinyl)	0	5
WIN 5243	(±)-*threo* (methylsulfonyl, *N*-acetyl)	6	
WIN 8535	D(+)-*threo* (allysulfonyl)	17	

[a] The binding studies were performed by measuring the effect of reduction of the binding at 5 μg (per ml) of ^{14}C-CM in the presence of 100 μg (per ml) of the indicated analogue. The reduction in binding by 100 μg (per ml) of nonradioactive chloramphenicol was taken as 100%. The effect on protein synthesis was measured as the degree of inhibition of induced synthesis of β-galactosidase by *Escherichia coli* B.

(2, 7). Thus, both the methylmercaptophenyl and methylsulfonyl derivatives are effective growth inhibitors, and both are effective in reducing binding of CM. Replacement of the dichloracetyl with acetyl (WIN 5243), and lengthening the chain attached to the sulfone (WIN 8,535), decreases the antibiotic effectiveness of the analogues and also decreases their ability to reduce the stereospecific binding of CM. In all instances studied, the degree of inhibition of protein synthesis is directly related to the degree of reduction of the binding of ^{14}C-CM.

That CM is bound by weak forces is shown by the finding that ribosome-bound CM is displaced on centrifugation through a sucrose gradient (11). We have also observed such an effect and were somewhat puzzled that bound CM could be measured by the centrifugation procedures used by Vasquez (8), Wolfe and Hahn (11), and Das et al. (3).

The key to this paradox became apparent when we noted that the stereospecifically bound CM is stable to washing by cold neutral solution (a procedure common to all the above-mentioned procedures) and that CM appeared to be bound to specific sites by weak forces which are temperature-dependent. Support for this concept came from the following sequential washing experiment which indicated that, although a considerable amount of entrained ^{14}C-CM was removed by washing with ice-cold water, only the cold water-insoluble fraction showed a specific dilution effect with ^{12}C-CM.

After isolation of cells from media by the silicone method, the cells were further washed according to the following sequence: (i) the cells were washed once by centrifugation from 1 ml of the ice-cold distilled water, 0.5 ml of the supernatant fluid being removed for counting; (ii) 0.5 ml of ice-cold 10% trichloroacetic acid was added, the cells were again centrifuged, and 0.5 ml of this supernatant liquid was removed for counting; (iii) 0.5 ml of 5% trichloroacetic acid was added, and the cells were heated in a boiling-water bath for 30 min before centrifugation and counting of the last supernatant fluid. An equivalent amount of acid was added to the water extract before counting so that the degree of quenching due to the acid was the same in all samples counted. The radioactivity in each of the two acid extracts was corrected for residual counts in the 0.5 ml retained from the previous wash.

Although much of the radioactivity was removed by the cold-water wash, no significant difference in amount of cold water-soluble ^{14}C was found between the two samples exposed to the same amount of ^{14}C-CM but differing by a 100-fold addition of ^{12}C-CM to one of them (Table 2).

Only the cold water-insoluble ^{14}C (cold trichloroacetic acid-soluble extract in Table 2) was decreased by the dilution with ^{12}C-CM. No significant amount of radioactivity was found in the hot acid extract. Cells washed at 37 C contained much less residual ^{14}C-CM (cold acid-extractable) than those washed with ice-cold water.

Experiments of this type were performed over a range of external CM concentrations, and the data (Fig. 2) show that the determinations of stereospecifically bound CM by either the dilution method or by the cold-water wash procedure are equally valid at CM concentrations below 5 μg/ml. At values above 5 μg/ml, the cold-water

TABLE 2. *Extraction of entrained chloramphenicol by sequential treatment with cold water, cold trichloroacetic acid, and hot trichloroacetic acid*[a]

Treatment[b]	Extract 1 (cold water)	Extract 2 (cold acid)	Extract 3 (hot acid)
^{14}C-CM	70.9[c]	27.35	−0.2
^{14}C-CM + ^{12}C-CM	78.3	0.25	1.8

[a] Samples (4 ml) of the cell suspension were centrifuged through a silicone layer to remove all but entrained ^{14}C-CM. The sedimented cells were then extracted in the order indicated. The counts per minute in each of the two acid extracts were corrected for residual counts in the 0.5 ml of supernatant fluid remaining from the previous wash. The total cell counts were 1.3×10^9 for the 5 μg/ml exposure and 1.16×10^9 for the 505 μg/ml exposure, reflecting the small amount of growth occurring during the 20-min exposure at 5 μg/ml. The specific activity of the ^{14}C-CM was 2.2×10^4 counts per min per μg.

[b] ^{14}C-CM was added at a level of 5 μg/ml; ^{12}C-CM at 500 μg/ml.

[c] Results expressed as counts per minute per 10^8 cells.

wash procedure yields more valid results, since it does not depend on the difference between two determined values.

By use of the procedure of Goldstein et al. (5), the number of ribosomes per cell in the ML 35 strain growing exponentially in nutrient broth was estimated to be 26,000 ± 2,000. From this, it appears likely that each ribosome has a single, specific site to which CM can bind. The value of one specific binding site per ribosome is in agreement with the proposals of Wolfe and Hahn (11) and Das et al. (3), derived from results with isolated ribosomes. At external concentrations of less than 5 μg/ml, presumably the percentage of ribosomes having no bound CM increases.

One further type of experiment was performed to test the hypothesis that each ribosome has a single binding site for CM. If the hypothesis is valid and the number of ribosomes per cell is varied, the number of molecules of bound CM at saturation should also vary proportionally. The growth rate of *E. coli* in minimal medium is about one-half that in nutrient broth, and the number of ribosomes per cell is likewise smaller. Our estimation of the number of ribosomes per cell of our ML 35 strain grown in minimal medium turned out to be 13,000, about 50% of the number found when grown in nutrient broth. The amount of cold water-insoluble CM at saturation was found to be 54% of that bound to cells in nutrient broth.

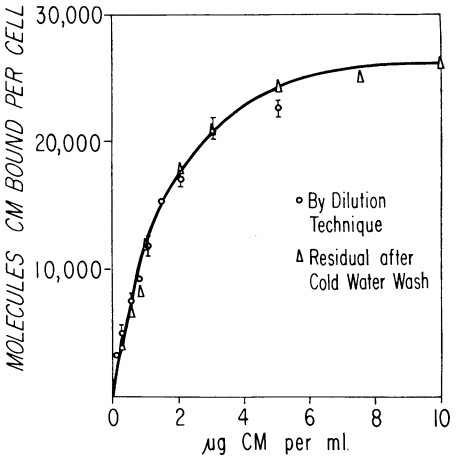

FIG. 2. *Effect of external concentration of CM on the amount of stereospecifically bound CM per cell. Specific activity of the ^{14}C-CM was 2.2×10^4 counts per min per μg.*

DISCUSSION

Since only the cold water-insoluble fraction showed a specific dilution effect with ^{12}C-CM, it is concluded that only this fraction measures stereospecifically bound CM. This conclusion is supported by the good agreement between the values of stereospecifically bound CM obtained by the two methods used. That this fraction also measures the CM bound to sites resulting in inhibition of protein synthesis is indicated by the agreement between the degree of inhibition of protein synthesis and the degree of competition for binding sites by analogues and isomers of CM.

It is apparent from these results that unbound and otherwise entrained CM can be removed by washing with ice-cold water or medium without significantly altering the amount of stereospecifically bound CM. We shall show elsewhere that this technique can be used to determine accurately the stereospecifically bound CM, enabling measurements to be made of the effect of temperature on rates of its release. These studies (*in preparation*) confirm the indication obtained from the experiments described in the present report that CM is held on specific sites by weak, temperature-dependent bonds.

The significance of the ^{14}C remaining in the cells after dilution with 100:1 ^{12}C-CM is difficult to assess. When the accessible intracellular water space is determined from the residual ^{14}C (6), the volume calculates to be about two times too large. This discrepancy indicates that CM may be held in solution against a concentration gradient, or that a labeled impurity is present in the ^{14}C-

CM. Repeated repurifications of our ^{14}C-CM by column and paper chromatography failed to do away with the residual ^{14}C, although considerable reduction was obtained with certain samples. No evidence could be found that label from ^{14}C-CM was incorporated into protein or ribonucleic acid.

Vasquez (9) reported that CM is bound specifically on the 50*S* ribosomal subunit. The finding that a close parallel exists between the number of ribosomes per cell and the number of bound CM molecules indicates that each 50*S* subunit has only one stereospecific site for binding the antibiotic, and that a single CM molecule bound at this site inactivates the 70*S* ribosome.

Inhibition of protein synthesis by CM therefore appears to result from a weak interaction between a small molecule (molecular weight, 323) and a large molecule (molecular weight, 3.1×10^6) in such a way as to inactivate the latter. Weak bonds between small and large molecules play many important roles in biological phenomena, such as enzyme-substrate interactions and allosteric or coenzyme-monitored interactions involving catalytic proteins. The use of temperature sensitivity as a means of isolating and studying such interactions may prove to be applicable to many such weak-bonded interactions. Detailed studies of the temperature sensitivity of the weak bond between CM and ribosomes in intact cells will be published elsewhere.

ACKNOWLEDGMENT

This investigation was supported by AEC contract (30-1)3236.

LITERATURE CITED

1. BRAY, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. **1**:279–285.
2. CUTLER, R. A., R. J. STENGER, AND C. M. SUTER. 1952. New antibacterial agents. I. J. Am. Chem. Soc. **74**:5475–5481.
3. DAS, H. K., A. GOLDSTEIN, AND L. C. KANNER. 1966. Inhibition by chloramphenicol of the growth of nascent protein chains in *Escherichia coli*. Mol. Pharmacol. **2**:158–170.
4. DAVIS, B. D., AND E. S. MINGIOLI. 1950. Mutants of Escherichia coli requiring methionine or vitamin B_{12}. J. Bacteriol. **60**:17–28.
5. GOLDSTEIN, A., D. B. GOLDSTEIN, AND L. I. LOWNEY. 1964. Protein synthesis at 0°C in *Escherichia coli*. J. Mol. Biol. **9**:213–235.
6. HURWITZ, C., C. B. BRAUN, AND R. A. PEABODY. 1965. Washing bacteria by centrifugation through a water-immiscible layer of silicones. J. Bacteriol. **90**:1692–1695.
7. SUTER, C. M., S. SCHALIT, AND R. A. CUTLER. 1953. New antibacterial agents. II. J. Am. Chem. Soc. **75**:4330–4333.
8. VASQUEZ, D. 1963. Antibiotics which affect protein synthesis; the uptake of ^{14}C-chloramphenicol by bacteria. Biochem. Biophys. Res. Commun. **12**:409–413.
9. VASQUEZ, D. 1964. The binding of chloramphenicol by ribosomes from *Bacillus megaterium*. Biochem. Biophys. Res. Commun. **15**:464–468.
10. VASQUEZ, D., 1966. Mode of action of chloramphenicol and related antibiotics, p. 169–191. *In* B. A. Newton and P. E. Reynolds [ed.], Biochemical studies of antimicrobial drugs. University Press, Cambridge.
11. WOLFE, A. D., AND F. E. HAHN. 1965. Mode of action of chloramphenicol. IX. Effects of chloramphenicol upon a ribosomal amino acid polymerization system and its binding to bacterial ribosome. Biochim. Biophys. Acta **95**: 146–155.