Biochemical Pharmacology, Vol. 19, pp. 391–399. Pergamon Press. 1970. Printed in Great Britain

BIOCHEMICAL BASIS FOR THE SELECTIVE TOXICITY OF ERYTHROMYCIN

JAMES C.-H. MAO, MARY PUTTERMAN and RONALD G. WIEGAND

Department of Pharmacology, Abbott Laboratories,
North Chicago, Ill. 60064, U.S.A.

(*Received* 3 *March* 1969; *accepted* 30 *May* 1969)

Abstract—Erythromycin inhibits bacterial protein synthesis in either intact cells or cell-free systems, but it does not inhibit protein synthesis of rat liver or rabbit reticulocytes. The mechanism for the selective toxicity was investigated. Results show that erythromycin binds to *S. aureus* ribosomes in a stoichiometric manner, but no binding of erythromycin to rat liver or rabbit reticulocyte ribosomes can be detected. It is proposed that differences between the ribosomes of bacteria and mammals, i.e. size and composition, account for the difference in binding ability and that the difference in binding ability accounts for the selectivity of erythromycin.

ALTHOUGH erythromycin is known to inhibit bacterial protein synthesis in intact cells[1] and in cell-free systems,[2, 3] its effect on mammalian protein synthesis has not been studied extensively. Clinical data show no recognizable effect of erythromycin on the protein synthesis of mammalian whole cells.[4, 5] No systematic study on the effect of erythromycin on mammalian protein synthesis has been reported. Only one publication, which mainly dealt with chloramphenicol, briefly showed that erythromycin could not inhibit the endogenous protein synthesis in a cell-free preparation from rabbit reticulocytes, but could inhibit protein synthesis induced by added m-RNA.[6] Recently, it has been reported that erythromycin binds to bacterial ribosomes in a stoichiometric manner.[7, 8] Evidence indicates that binding of erythromycin to ribosomes is a prerequisite for the inhibition of protein synthesis.[9, 10]

In this study the effect of erythromycin on bacterial (*Staphylococcus aureus*) and mammalian (rat liver and rabbit reticulocytes) protein synthesis was compared in cell-free preparations under various conditions. In addition, the ability of various ribosomes to bind erythromycin was determined as a secondary method to test the effect of erythromycin on mammalian protein synthesis.

Evidence is presented that under various experimental conditions erythromycin neither inhibits mammalian protein synthesis nor does it bind to mammalian ribosomes. It is therefore concluded that the ability of erythromycin to selectively inhibit bacterial but not mammalian protein synthesis resides in the structural difference between bacterial and mammalian ribosomes.

MATERIALS AND METHODS

Materials. *N*-^{14}C-methyl-erythromycin was synthesized by reductive methylation of des-*N*-methyl-erythromycin[11] and purified by counter-current distribution. The product was chromatographically pure on silica gel thin-layer plates in three solvent

systems,[12] had a specific activity of 15 c/mole, and had antibacterial activity of 730 units per μmole (theoretical value: 734 units per μmole). Uniformly ^{14}C-labeled phenylalanine (375 c/mole), lysine (240 c/mole) and leucine (252 c/mole) were purchased from New England Nuclear Corp. Triphosphates of adenosine, cytidine, guanosine and uridine (ATP, CTP, GTP, UTP respectively), phosphoenolpyruvate, pyruvate kinase, poly-U, poly-A and dithiothreitol were obtained from Calbiochem. Medium A contained: Tris-HCl, 0·01 M, pH 7·8; KCl, 0·06 M; magnesium acetate, 0·005 M; and dithiothreitol, 1 mM. Medium B contained: Tris-HCl, 0·01 M, pH 7·8; NH_4Cl, 0·05 M; magnesium acetate, 0·016 M; and dithiothreitol, 1 mM.

Preparation of cell-free protein-synthesizing systems. The protein-synthesizing extracts of rabbit reticulocytes and rat liver were prepared by the procedure of Weinstein *et al.*,[13] and the protein-synthesizing extract from *S. aureus* was prepared by the procedure of Mao.[14]

Assay for amino acid incorporation. The standard reaction mixture, for rat liver or rabbit reticulocytes extracts, in a total volume of 0·5 ml, consisted of 10 mM Tris-HCl (pH 7·8), 60 mM KCl, 5 mM magnesium acetate, 0·1 mM dithiothreitol, 1 mM ATP, 0·05 mM GTP, 5 mM phosphoenolpyruvate, 0·02 mg of pyruvate kinase, 0·25 μc of ^{14}C-amino acid, 0·06 mM of each of the remaining amino acids and 0·1 ml cell fraction from either rat liver or reticulocytes. In the study of phenylalanine or lysine incorporation, 50 μg of poly-U or 100 μg of poly-A were added respectively. The standard reaction mixture for *S. aureus* extract was described previously.[14] Protein synthesis was measured by the incorporation of radioactive amino acid into hot trichloroacetic acid-precipitable material. Polylysine was precipitated by a tungstate–trichloroacetic acid mixture.[15] The precipitate was collected and washed on a membrane filter. Radioactivity was measured by scintillation counting.

Assay for binding of ^{14}C-erythromycin to ribosomes. Binding of ^{14}C-erythromycin to ribosomes was determined by three methods.

(1) Sucrose density gradient centrifugation. Ten A^{260} units of ribosomes from rat liver, reticulocytes or *S. aureus* were incubated with 14 mμmoles of ^{14}C-erythromycin at 35° for 10 min in buffer A or B. The mixtures were cooled and layered on top of a 12-ml linear sucrose gradient (3–15%). Samples were centrifuged at 40,000 rpm for 3 hr in an SW-41 rotor (Spinco). Fractions were collected by a density gradient fractionator equipped with a u.v. analyzer. The radioactivity of the fractions was determined by the liquid scintillation method.

(2) Nitrocellulose membrane filtration. The binding reaction was performed in 0·1 ml containing 0·14 mμmoles ^{14}C-erythromycin and 5 A^{260} units of ribosomes in medium A or B. After a 30-min incubation at 37° the reaction mixtures were diluted with 3 ml of cold medium, filtered through nitrocellulose membranes and washed with three 3-ml portions of cold medium. The radioactivity remaining on the filter was determined by the liquid scintillation method.

(3) Ultrafiltration. Six ml of medium A or B containing 150 A^{260} units of ribosomes and 8·2 mμmoles of ^{14}C-erythromycin were filtered through cellulose dialysis tubing under 15 psi of pressure at room temperature. After about 2-ml aliquots had been filtered, the radioactivity of the filtrate and the sample remaining in the tubing were analyzed. The difference between them was taken as the erythromycin bound to ribosomes.

RESULTS

Effect of erythromycin on protein synthesis in rat liver, rabbit reticulocyte and S. aureus *cell-free extracts.* The effect of erythromycin on the incorporation of ^{14}C-leucine directed by endogenous m-RNA, ^{14}C-lysine directed by poly-A and ^{14}C-phenylalanine directed by poly-U was compared in cell-free extracts of liver, reticulocyte and *S. aureus*. Concentrations of 1μM to 5 mM erythromycin were used. The incorporation of leucine (Fig. 1), phenylalanine (Fig. 2) and lysine (Fig. 3) into polypeptide was inhibited by erythromycin when the *S. aureus* extract was used. However, the protein-synthesizing extracts from either rat liver or rabbit reticulocytes were not inhibited by erythromycin. In fact, usually erythromycin stimulated protein synthesis in the mammalian cell-free extracts.

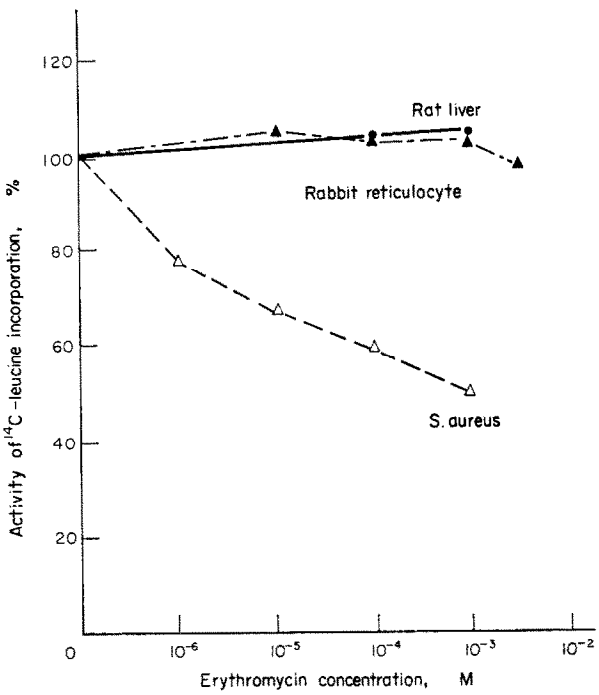

FIG. 1. Effect of erythromycin on the incorporation of ^{14}C-leucine directed by endogenous mRNA in cell-free extracts. The reaction mixtures were incubated at 37° for 30 min. Without addition of erythromycin, the total incorporation of ^{14}C-leucine in the cell-free extracts of rat liver, rabbit reticulocytes and *S. aureus* was 1650, 3700 and 9800 cpm per mg of protein respectively. The zero time samples were less than 100 cpm.

The time course of the incorporation of leucine in rat liver extracts in the presence and absence of erythromycin was also tested. Figure 4 shows that the rate of synthesis was not changed significantly by the presence of 2 mM erythromycin.

Effect of erythromycin on rat liver protein synthesis at various concentrations of Mg^{2+}. The 5 mM Mg^{2+} used in rat liver cell-free protein synthesis probably is not the physiological concentration. Because of the extreme importance of the Mg^{2+} concentration in protein synthesis, the effect of erythromycin on rat liver protein synthesis

at various concentrations of Mg^{2+} was examined. Figure 5 shows a normal Mg^{2+}-dependent protein synthesis curve with an optimal Mg^{2+} concentration of 6 mM. Erythromycin did not cause any inhibition of protein synthesis in liver extracts at Mg^{2+} concentrations ranging from 3 to 18 mM.

Effect of other macrolides on rat liver protein synthesis. Other macrolides such as niddamycin, oleandomycin, spiramycin, carbomycin and tylosin were also tested in rat liver extracts. The results are presented in Table 1. The concentration of macrolides used in this experiment is more than 1000-fold higher than the therapeutic concentration, yet there is no inhibitory effect on the rat liver protein synthesis. Also included in the table for the purpose of comparison is tetracycline, a known inhibitor of

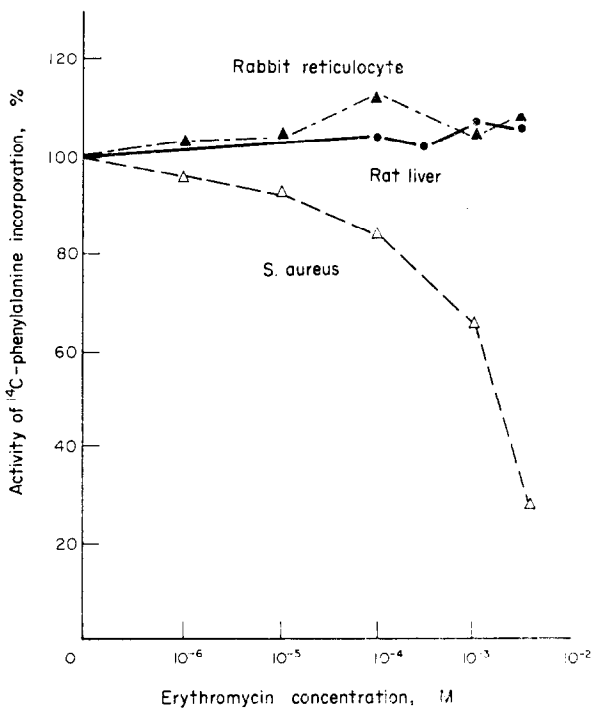

FIG. 2. Effect of erythromycin on the incorporation of ^{14}C-phenylalamine directed by poly-u in cell-free extracts. The reaction mixtures were incubated at 37° for 30 min. Without addition of erythromycin, the total incorporation of ^{14}C-phenylalanine in the cell-free extracts of rat liver, rabbit reticlocytes and *S. aureus* was 5300, 9600 and 78,000 cpm per mg of protein, and samples without poly-U were 480, 430 and 1900 cpm per mg of protein respectively.

mammalian protein synthesis.[16] Its inhibitory effect on rat liver protein synthesis was confirmed.

Binding of erythromycin to ribosomes. The binding of erythromycin to ribosomes isolated from *S. aureus*, rat liver and rabbit reticulocytes was determined by three methods. Figure 6 shows the results of the sucrose gradient centrifugation analysis of ^{14}C-erythromycin binding to ribosomes. It clearly demonstrates that erythromycin binds to *S. aureus* ribosomes, since the radioactivity curve coincides with the absorbance curve of *S. aureus* ribosomes. From absorbance readings and radioactivity data, it was

TABLE 1. EFFECT OF OTHER MACROLIDES ON THE RAT LIVER PROTEIN SYNTHESIS

		Concn	Incorporated cpm	Incorporated % activity	^{14}C-phenylalanine incorporated cpm	^{14}C-phenylalanine incorporated % activity
	Control		1650	100	5915	100
Macrolides	+ Erythromycin	1 mM	1563	95	5918	100
	+ Oleandomycin	1	1634	99	5678	96
	+ Tylosin	1	1720	104	5560	94
	+ Spiramycin	1	1533	93	6255	105
	+ Carbomycin	0·1	1675	102	5738	97
	+ Niddamycin	0·1	1582	96	6066	103
	+ Chalcomycin	1	1602	97	5702	96
	+ Lankamycin	1	1632	99	5737	97
	+ Tetracycline	1	671	41	197	3

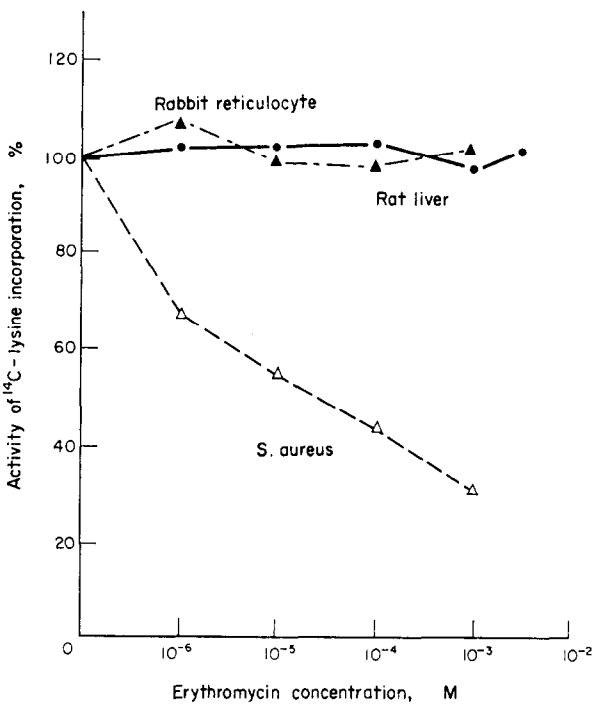

FIG. 3. Effect of erythromycin on the incorporation of ^{14}C-lysine directed by poly-A in cell-free extracts. The reaction mixtures were incubated at 37° for 30 min. Without addition of erythromycin the total incorporation of ^{14}C-lysine in the cell-free extracts of rat liver, rabbit reticulocytes and *S. aureus* was 1600, 2700 and 22,000 cpm per mg of protein, and samples without poly-A were 560, 480 and 1200 cpm per mg of protein respectively.

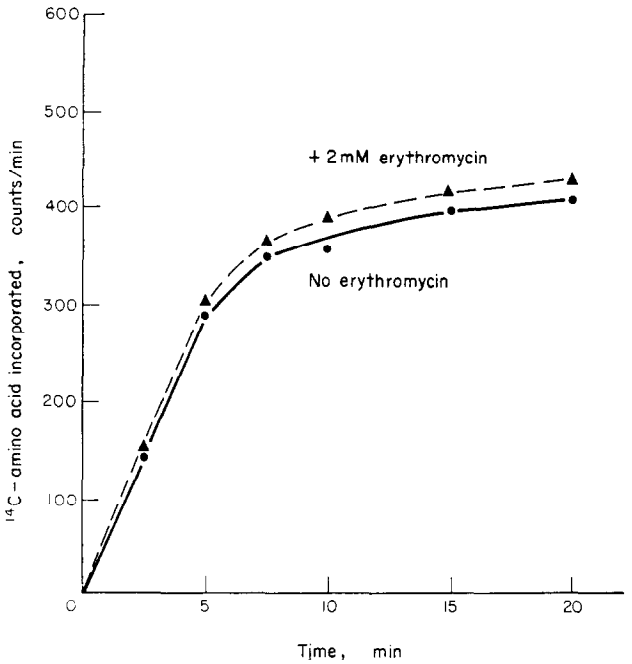

FIG. 4. Time course of amino acid incorporation in the rat liver extract with or without erythromycin. The reaction was run in the standard conditions but the volume was increased to 1·5 ml which contained 4 mg of protein. At the given time intervals, 0·2-ml aliquots were withdrawn and amino acid incorporation was determined as described in Methods.

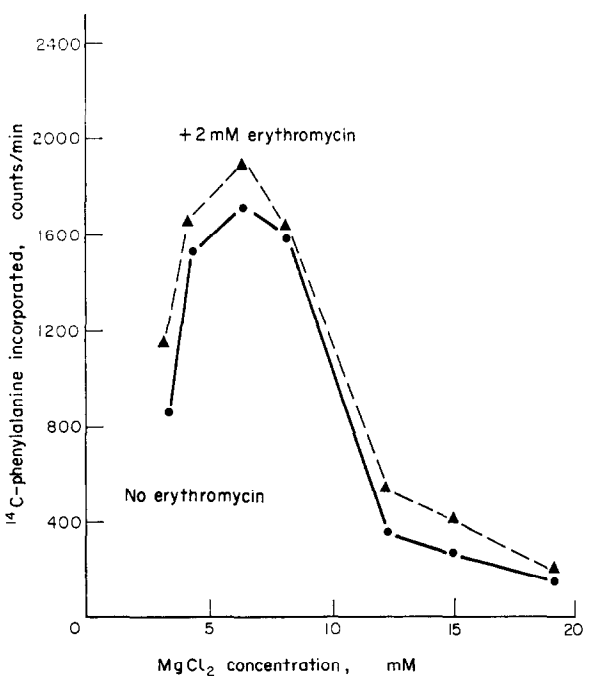

FIG. 5. Effect of erythromycin on rat liver protein synthesis at various concentrations of magnesium ion.

calculated that each 70S ribosome binds approximately one molecule of erythromycin. The 100S ribosome, which is a dimer of 70S ribosomes, binds about two molecules of erythromycin. But no erythromycin was associated with liver or reticulocyte ribosomes.

It is known that ribosomes cannot pass through a nitro cellulose filter.[17] Thus any erythromycin which forms a complex with the ribosome should be retained on the

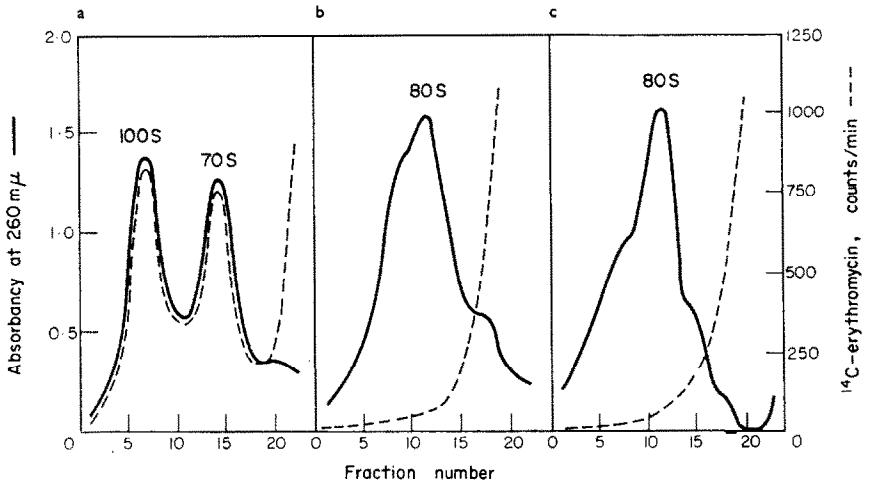

FIG. 6. Sucrose gradient centrifugation analysis of binding of ^{14}C-erythromycin to *S. aureus* (a), rat liver (b) and rabbit reticulocyte (c) ribosomes.

TABLE 2. BINDING OF ^{14}C-ERYTHROMYCIN TO VARIOUS RIBOSOMES

		Ultrafiltration	
	^{14}C-erythromycin retained on the nitrocellulose filter	^{14}C-erythromycin ratio $\frac{in}{out}$	
— Ribosomes	495 cpm	$\frac{in}{out}$ $\frac{4050 \text{ cpm}}{3550}$	1·14
S. aureus ribosomes	3194	$\frac{in}{out}$ $\frac{5470}{2130}$	2·57
Rat liver ribosomes	451	$\frac{in}{out}$ $\frac{4020}{3580}$	1·12
Rabbit reticulocyte ribosomes	405	$\frac{in}{out}$ $\frac{3880}{3730}$	1·04

filter. Results of studies using this method are shown in the second column of Table 2. *S. aureus* ribosomes retained a significant amount of radioactivity on the filter but rat liver and reticulocyte ribosomes failed to retain radioactivity, showing the failure of these mammalian ribosomes to bind erythromycin.

In the above two methods, the initial equilibrium binding conditions are not maintained throughout the procedures, and loosely bound erythromycin might be lost

during centrifugation or filtration. The third method, ultrafiltration, is under equilibrium conditions and should be able to detect loosely bound erythromycin. The results are shown in the third column of Table 2. Again, only *S. aureus* ribosomes can retain radioactivity. This is indicated by the much higher radioactivity inside the dialysis tubing than in the filtrate. The slight accumulation of radioactivity by rat liver and reticulocyte ribosomes is not significant because the sample without ribosomes also accumulated a slight amount of erythromycin.

DISCUSSION

The endogenous protein synthesis and poly-A or poly-U stimulated incorporation of lysine and phenylalanine were not inhibited by erythromycin when the protein synthesis system was from rat liver or rabbit reticulocytes. Neither the extent nor the rate of incorporation was affected by erythromycin.

Weisberger *et al.*[6] reported briefly that erythromycin at a concentration of 0·01 mM completely inhibited the mRNA-induced protein synthesis but had no effect on the endogenous protein synthesis. This result would imply that erythromycin interferes with the attachment of mRNA to ribosomes. However, since then, more has been learned about the mechanism of action of erythromycin[2, 3] which makes this observation difficult to understand. First, the erythromycin binding site is on the 50S subunit of ribosomes[7, 18] and the mRNA binding site is on the 30S subunit of ribosomes.[19] It is not likely that erythromycin can extend its influence to the 30S subunit. Second, the direct evidence from sucrose gradient centrifugation analysis showed that erythromycin does not prevent the binding of ^{3}H-poly-U and ^{3}H-poly-A to ribosomes.[2] Third, the massive clinical data accumulated after more than a decade show no side effect related to the interference with mammalian protein synthesis.[5]

Binding of erythromycin to bacterial ribosomes has been established with many species of microorganisms.[8, 18, 20] Corcoran *et al.* have shown that erythromycin must bind to ribosomes in order to inhibit protein synthesis.[9, 10] In this paper, three methods were used to test the ability of rat liver ribosomes and rabbit reticulocyte ribosomes to bind erythromycin. In no case was there detectable binding of erythromycin.

It is well known that ribosomes of bacterial origins are of the 70S type, containing about 63% RNA and 37% protein. Mammalian ribosomes are of the 80S type, containing equal amounts of RNA and protein. It is therefore concluded that the ability of erythromycin to selectively inhibit bacterial but not mammalian protein synthesis is due to differences in structure of mammalian and bacterial ribosomes. These variations in the ribosomal structure account for the differences in binding ability and, furthermore, the difference in binding ability accounts for the selective toxicity of erythromycin.

REFERENCES

1. T. D. BROCK and M. L. BROCK, *Biochim. biophys. Acta* **33**, 274 (1959).
2. J. C.-H. MAO and R. G. WIEGAND, *Biochim. biophys. Acta* **157**, 404 (1968).
3. S. B. TAUBMAN, A. G. SO, F. E. YOUNG, G. W. DAVIE and J. W. CORCORAN, *Antimicrob. Agents Chemother.* 395 (1964).
4. W. E. HERRELL, *J. Am. med. Ass.* **168**, 1875 (1958).
5. W. J. MARTIN and W. E. WELLMAN, *Postgrad. Med.* **42**, 397 (1967).
6. A. S. WEISBERGER, S. WOLFE and S. ARMENTROUT, *J. exp. Med.* **120**, 161 (1964).
7. J. C.-H. MAO, *Biochem. Pharmac.* **16**, 2441 (1967).

8. N. L. OLEINICK and J. W. CORCORAN, *Fedn Proc.* **26**, 285 (1967).
9. J. M. WILHELM, N. L. OLEINICK and J. W. CORCORAN, *Antimicrob. Agents Chemother.* 236 (1967).
10. J. M. WILHELM and J. W. CORCORAN, *Biochemistry, N.Y.* **6**, 2578 (1967).
11. E. H. FLYNN, H. W. MURPHY and R. E. MCMAHON, *J. Am. chem. Soc.* **77**, 3104 (1955).
12. J. C.-H. MAO and P. L. TARDREW, *Biochem. Pharmac.* **14**, 1049 (1965).
13. I. B. WEINSTEIN, M. OCHOA, JR. and S. M. FRIEDMAN, *Biochemistry, N.Y.* **5**, 3332 (1966).
14. J. C.-H. MAO, *J. Bact.* **94**, 80 (1967).
15. R. S. GARDNER, A. J. WAHBA, C. BASILIO, R. S. MILLER, P. LENGYEL and J. F. SPEYER, *Proc. Natn Acad. Sci. U.S.A.* **48**, 2087 (1962).
16. T. J. FRANKLIN, *Biochem. J.* **90**, 624 (1964).
17. M. NIRENBERG and P. LEDER, *Science, N.Y.* **145**, 1339 (1964).
18. S. B. TAUBMAN, N. R. JONES, F. G. YOUNG and J. W. CORCORAN, *Biochim. biophys. Acta* **123**, 438 (1966).
19. A. KAJI and H. KAJI, *Biochim. biophys. Acta* **87**, 519 (1964).
20. K. TANAKA, H. TERAOKA, T. NAGIRA and M. TAMAKI, *Biochim. biophys. Acta* **123**, 435 (1966).