Biochemical Pharmacology, 1965, Vol. 14, pp. 1049–1058. Pergamon Press Ltd., Printed in Great Britain.

# DEMETHYLATION OF ERYTHROMYCINS BY RABBIT TISSUES *IN VITRO*

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(*Received* 4 *December* 1964; *accepted* 23 *March* 1965)

**Abstract**—Erythromycin is rapidly demethylated by a microsomal enzyme of rabbit liver to yield des-N-methyl erythromycin and formaldehyde. Formaldehyde was derived from the N-methyl group of D-desosamine exclusively, none coming from the O-methyl group of L-cladinose. The enzyme is most abundant in liver. Other tissues do not demethylate the antibiotic, with the exception of the adrenal gland which showed some demethylating activity. The distribution of the enzyme and its cofactor requirements indicate that it is similar to enzymes that demethylate other alkylamines. Specificity of the enzyme is rather low, since a number of derivatives of erythromycin can be demethylated. End products from demethylation of erythromycin and 2′-propionyl erythromycin, and their possible intermediates, are discussed.

ERYTHROMYCIN A (Ery. A, Fig. 1), a macrolide antibiotic which is widely used therapeutically, is converted to des-N-methyl Ery. A and carbon dioxide by rats and dogs *in vivo*.[1, 2] However, the nature of the demethylating reaction and the enzyme system involved have not been established.

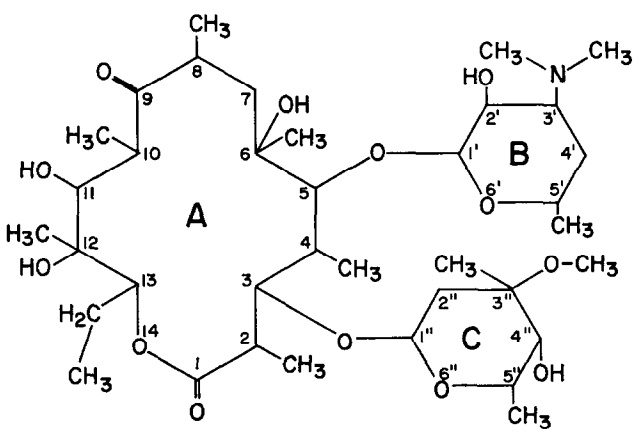


FIG. 1. Chemical structure of erythromycin A. A, erythronolide; B, desosamine; C, cladinose.

Many drugs containing N-methyl or O-methyl groups undergo demethylation by a microsomal enzyme of mammalian tissue,[3] usually most abundant in the liver, to give formaldehyde and demethylated compounds. Since Ery. A contains both N-methyl and O-methyl groups, it is interesting to test whether a similar enzyme is involved in the metabolism of Ery. A in mammalian tissues.

In the present communication we report the demethylation of Ery. A *in vitro* by a microsomal enzyme present in rabbit liver, and some properties of this enzyme. The activity of the enzyme against other substrates related to Ery. A, such as erythromycin B and C, anhydroerythromycin A, and 2′-esters of erythromycin A, is also reported. Evidence will be presented that formaldehyde is formed only from the dimethylamino group of D-desosamine.

## MATERIALS AND METHODS

Erythromycin A, B, and 2′-ethylsuccinyl erythromycin A[4] are products of Abbott Laboratories; 2′-acetyl erythromycin A and 2′-propionyl erythromycin A were prepared by the method of Murphy.[5] Erythromycin C was isolated by countercurrent distribution.[6] Anhydroerythromycin A and erythromycin A N-oxide were prepared as described by Wiley *et al.*[7]

Des-N-methyl erythromycin A and N-methyl-$^{14}$C-erythromycin A were prepared according to the methods of Flynn *et al.*;[8] 2′-propionyl des-N-methyl erythromycin A (m.p. 152°) was prepared by the procedure similar to that used for des-N-methyl erythromycin A but with 2′-propionyl Ery. A as the starting material. $^{14}$C-Erythromycin A (labeled in the aglycone) was prepared from propionate-1-$^{14}$C by a method similar to that of Kaneda *et al.*[9] The purity of all substrates was established by paper chromatography and thin-layer chromatography in the systems described under Chromatography.

D-Desosamine, L-cladinose, and methyl cladinoside were prepared by the method of Flynn *et al.*[10]

Nicotinamide-adenine dinucleotide phosphate (NADP) was purchased from Sigma Chemical Co.

### *Tissue preparation*

Male Dutch belted rabbits, weighing 2–3 kg, were killed by injecting air through ear veins. The liver or other tissues were immediately removed and placed in ice-cold 1·15% KCl solution. Tissues were homogenized at 0°–3° in 2 volumes of Krebs-Ringer phosphate buffer, pH 7·4, with a Potter–Elvehjem-type homogenizer. The homogenates were spun in a refrigerated centrifuge at 9,000 *g* for 10 min to remove unbroken cells, nuclei, and mitochondria. The supernatant (9s) which contains microsomes and soluble enzymes was used for most of the experiments.

### *Measurement of enzyme activity*

Enzymatic demethylation of erythromycins was measured by determining the amount of formaldehyde formed. A typical reaction mixture was prepared as follows. To a 25-ml Erlenmeyer flask was added 1·0 ml of a mixture containing the following in μmoles: nicotinamide 50, $MgCl_2$ 30, NADP 0·3, and neutralized semicarbazide 50; 3·0 ml of 5 mM erythromycin in 0·2 M sodium phosphate buffer, pH 8·0; and 2·0 ml of supernatant (9s). The final pH of the mixture was 7·8. The mixture was incubated in air in a Dubnoff metabolic shaking water bath for 1 hr at 37°. At the end of the incubation period, the reaction was stopped by addition of 2·0 ml of 20% $ZnSO_4$ and then 2·0 ml of saturated $Ba(OH)_2$. After centrifugation, 5·0 ml of supernatant fluid was taken to determine formaldehyde by the modified Nash method.[11]

*Estimation of radioactive formaldehyde*

N-Methyl-$^{14}$C-erythromycin A, 15 $\mu$moles, was incubated with supernatant (9s) as described above. At the end of the reaction period, an equal volume of 10% trichloroacetic acid was added. An aliquot of 8·0 ml was distilled over; 5·0 ml was used for formaldehyde determination by the Nash reagent and 1·0 ml in duplicate for determination of radioactivity in a Tri-Carb automatic liquid scintillation spectrometer (model 314E, Packard Instrument Co.). The composition of the scintillation solution was Dioxane:anisole:1,2-dimethoxyethane (6:1:1), 1·5% 2,5-diphenyl oxazole, and 0·6% 1,4-*bis*-2(5-phenyloxazolyl) benzene. The counting efficiency was about 61%.

*Chromatography*

Eaton–Dikeman no. 613 paper strips (0·8 × 30 cm), after samples were applied, were developed in an ascending direction for 3 hr at 28°. Two solvent systems were employed: (A) methylisobutylketone:butylethylketone:methylethylketone (1:3:3); (B) 25 g $NH_4Cl$, 50 ml *p*-dioxane dissolved in 1 liter of water, pH 5·5. The location of antibiotics was determined by placing the paper strips on an agar plate seeded with *Bacillus subtilis* and incubating at 28° for 15 hr. Radioactive erythromycins were also detected by radioautography.

TABLE 1. $R_F$ VALUES, RELATIVE ANTIBIOTIC ACTIVITIES AND PARTITION COEFFICIENTS OF ERYTHROMYCINS

| Compounds | $R_F$, Paper chromatography A | B | $R_F$, Thin-layer chromatography A | B | C | Relative antibiotic* activity (U/$\mu$mole) | $k$† |
|---|---|---|---|---|---|---|---|
| Ery. A | 0·27 | 0·72 | 0·60 | 0·42 | 0·30 | 726 | 0·57 |
| Ery. B | 0·30 | 0·66 | 0·60 | 0·42 | 0·30 | 558 | 1·38 |
| Ery. C | 0·20 | 0·87 | 0·56 | 0·47 | 0·30 | 147 | 0·19 |
| Des-N-methyl Ery. A | 0·35 | 0·81 | 0·20 | 0·22 | 0·36 | 56 | 0·12 |
| Anhydroery. A | | | 0·64 | 0·50 | 0·50 | 0 | 1·05 |
| Ery. A N-oxide | | 0·63 | | | 0·50 | 5 | 0·18 |
| 2′-Acetyl Ery. A | 0·82 | 0·67 | | | | 677 | >20 |
| 2′-Propionyl Ery. A | 0·67 | 0·49 | | | | 676 | >20 |
| 2′-Ethylsuccinyl Ery. A | 0·68 | 0·45 | | | | 650 | >20 |
| 2′-Propionyl des-N methyl Ery. A | 0·70 | 0·81 | 0·86 | 0·90 | 0·90 | 25 | 9·0 |

* Antibiotic activity was determined by zone-diameter with *B. subtilis* as test organism.

† The partition coefficient $k$ is as determined by countercurrent distribution in the solvent system 0·1 M phosphate buffer, pH 6·5, acetone and methylisobutylketone.[19]

Thin-layer chromatography was performed on silica gel G (E. Merckag, Darmstadt, Germany). Three solvent systems were used: (A) diethylcarbinol:ethylacetate: dimethylformamide (1:7:2); (B) carbon tetrachloride:ethanol:dimethylformamide (7:2:1); and (C) chloroform:methanol:dimethylformamide (2:7·5:0·5). Spots were detected by spraying with arsenomolybdate reagent[12] and heating on a hot plate until the blue color appeared.

The $R_F$ values, relative antibiotic activities, and lipid solubilities of compounds used in these experiments are listed in Table 1.

## RESULTS

*Tissue distribution of the demethylating enzyme*

Erythromycin A, 15 μmoles, was incubated with 2·0 ml of homogenized liver, lung, kidney, brain, heart, muscle, spleen, whole blood, and adrenal gland of the rabbit under the conditions described under Methods. After 1 hr, formaldehyde formed with each homogenate was determined. Liver homogenates gave large amounts of formaldehyde (1·6 μmoles), and adrenal gland homogenates gave significant amounts of formaldehyde (0·32 μmole); the other tissues were unable to demethylate Ery. A.

*Subcellular distribution of the demethylating enzyme*

Rabbit liver homogenates were centrifuged at 9,000 *g* for 10 min to precipitate nuclei and mitochondria. The supernatant was centrifuged again at 140,000 *g* for 1 hr to precipitate microsomes. Each fraction was examined for its ability to demethylate Ery. A. Little activity was found in any individual fraction. However, when microsomes and soluble fraction were recombined, almost full enzyme activity was restored. Combining the soluble fraction with the mixture of nuclei and mitochondria resulted in very low activity, probably resulting from contamination with microsomes. NADP is the obligate cofactor for the enzyme reaction. When NADP was omitted, very little formaldehyde was formed with the supernatant (9s) of liver. These observations indicate that the erythromycin demethylation system is similar to other drug-demethylating systems[13–15] in requiring components present in both the microsome and soluble fraction.

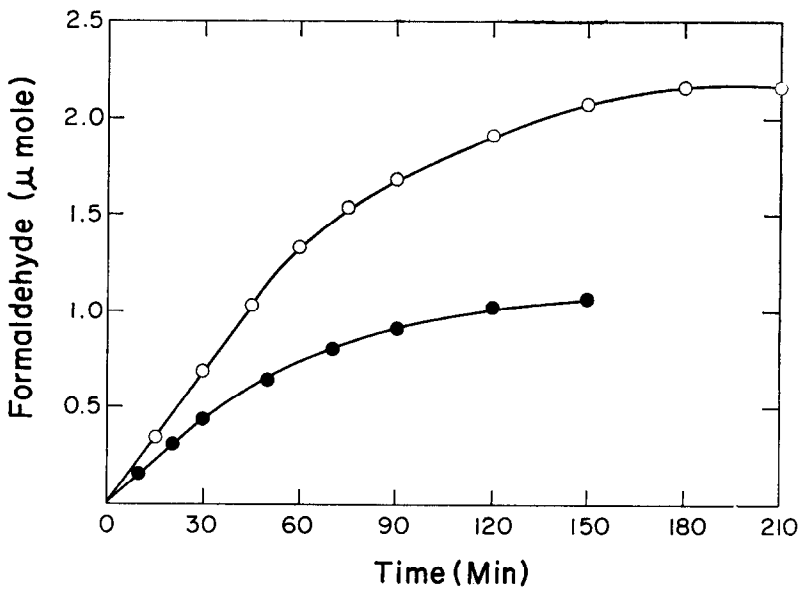


FIG. 2. Rates of N-demethylation of erythromycin A (○), and 2′-propionyl erythromycin A (●) by the rabbit liver enzyme.

*Rate of demethylation*

The rates of enzymatic demethylation of Ery. A (15 μmoles per assay) and 2′-propionyl Ery. A (3 μmoles per assay) by the rabbit liver supernatant (9s) are shown in Fig. 2. The reaction was linear for 1 hr and 0·5 hr respectively. After 2·5 hr no further

demethylation occurred. Axelrod[13] reported a similar time curve for the enzymatic demethylation of ephedrine.

The relationship between substrate concentration and formaldehyde release is shown in Fig. 3. Maximal activity was observed at substrate levels of 15 $\mu$moles per

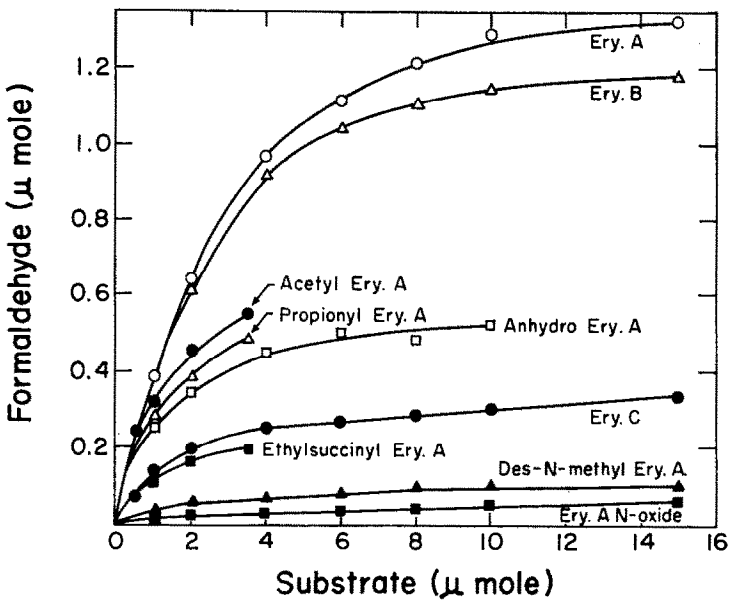


FIG. 3. Substrate specificity of the N-demethylating enzyme. Substrates at several concentration were incubated with rabbit liver enzyme for 1 hr at 37°. Amounts of formaldehyde formed were determined as described in Methods.

assay or above. Substrate levels up to 60 $\mu$moles showed no inhibition of enzyme activity.

*Optimal pH of the demethylating system*

The demethylating activity of rabbit livers was measured at various pH values in sodium phosphate buffer at a final concentration of 0·1 M. Two substrates, Ery. A and 2′-propionyl Ery. A, were tested. The maximal demethylation of Ery. A occurred at pH 7·8 (Fig. 4), while optimal demethylation of 2′-propionyl Ery. A was seen at pH 7·4. At pH 6·0, 2′-propionyl Ery. A was not demethylated, but Ery. A still showed 30% of maximal activity. Determination of rates of hydrolysis of 2′-propionyl Ery. A in 0·1 M sodium phosphate buffer at pH 6·5 and 7·4 have shown that 2′-propionyl Ery. A is hydrolyzed more slowly in the buffer with the lower pH. This fact combined with a greater resistance of the ester to demethylation would explain in part the differences in pH-activity curves for the demethylation of Ery. A and 2′-propionyl Ery. A. The reason for the drop in efficiency of demethylation of 2′-propionyl Ery. A at higher pH values is not clear.

*Substrate specificity*

The specificity of the demethylating enzyme present in rabbit liver was examined by measuring formaldehyde liberated from a series of erythromycins and derivates of Ery. A at several concentrations. The results are shown in Fig. 3.

Erythromycin B gave the same amount of formaldehyde as Ery. A at low concentrations, but gave slightly lower amounts at high concentrations. Apparently the

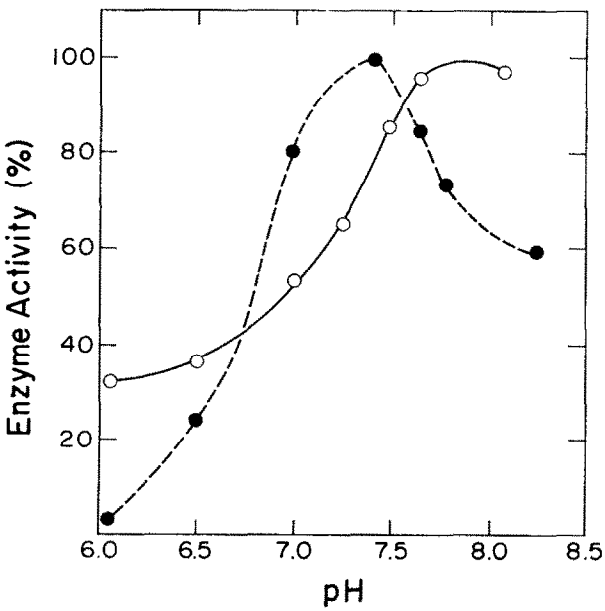


FIG. 4. pH-Activity curve for N-demethylating enzyme. Activity of the enzyme was measured in 0·1 M sodium phosphate buffer, with erythromycin A as the substrate (○), and with propionyl erythromycin A as the substrate (●).

hydroxyl group at C-12 exerts only a slight effect on demethylation. On the other hand, Ery. C which does not have an O-methyl group on the neutral sugar gave a very low rate of demethyltaion. At first glance, these results indicate that at least part of formaldehyde is derived from the O-methyl group in Ery A. However, experiments with N-methyl-$^{14}$C-Ery. A showed that the formaldehyde comes exclusively from the dimethyl amino group of desosamine, suggesting that the function of the O-methyl group is to increase the affinity of substrate for the enzyme. Des-N-methyl Ery. A can be further demethylated to di-des-methyl Ery. A. However, the rate was extremely low. Anhydroery. A, a spiroketal obtained from erythromycin A with oxygen bridges between C-6 and C-9, and C-11 and C-9 (see Fig. 1) gave less formaldehyde than Ery. A. Demethylation of Ery. A N-oxide was negligible.

The demethylation of three alkyl esters of Ery. A, 2′-acetyl, 2′-propionyl, and 2′-ethylsuccinyl, was compared. It was found that the rate of demethylation diminished as the size of the attached group increased. However, these esters are not very stable in solution under the conditions used for demethylation. Thus, part of formaldehyde comes from Ery. A formed by hydrolysis of the ester bond during the incubation period. Therefore, the true rate of formaldehyde formation from erythromycin esters

should be less than the apparent rate shown in Fig. 3. Nevertheless, 2′-propionyl Ery. A itself was demethylated, since des-N-methyl propionyl Ery. A was found as an end product in a later experiment. When desosamine and cladinose were used as substrates, no formaldehyde was detected, probably because the demethylating enzyme is encased in a lipophilic surrounding. Therefore, the active site of the enzyme was inaccessible to substrates with low lipid solubility.[15, 16] Another interesting observation is that when methyl cladinoside, which also has very low lipid solubility, was used as the substrate, large amounts of formaldehyde were detected. This formaldehyde appears to be formed from the glycosidic methyl group, suggesting that metabolism of erythromycin in liver is not limited to demethylation but that a glycosidase which can hydrolyze the glycosidic bound between erythronolide and cladinose may also be present.

*Source of formaldehyde*

The microsomal fraction of mammalian liver will not only catalyze N-demethylations but also O-demethylations.[17, 18] Since Ery. A contains both N-methyl and O-methyl groups it was interesting to ascertain whether formaldehyde is released by demethylating N-methyl or O-methyl groups or both. Fifteen μmoles of N-methyl-$^{14}$C-Ery. A, with a specific activity of 720,000 cpm per μmole, were incubated with rabbit liver supernatant (9s) for 1 hr. The formaldehyde and radioactivity released were determined as described in Methods. If formaldehyde comes from N-methyl groups exclusively, the specific activity of the formaldehyde (cpm/μmole) should be half that of Ery, A, since only one N-methyl group in Ery. A. is labeled. If some of the formaldehyde comes from the O-methyl group, the specific activity of formaldehyde will be less than half that of Ery. A, dependent on the amount of formaldehyde contributed by the O-methyl group. The results of the test are shown

TABLE 2. SOURCE OF FORMALDEHYDE

| μmole HCHO/ml | Theoretical cpm if HCHO is derived from N-methyl only | Found (cpm) |
|---|---|---|
| 0·1338 | 48,000 | 49,000 |
| 0·1365 | 59,000 | 51,000 |

in Table 2. The results from runs 1 and 2 both strongly indicate that formaldehyde came exclusively from N-methyl groups.

*End products of demethylation*

The end products from Ery. A and 2′-propionyl Ery. A were determined. Two flasks, each containing 12 ml of 5 mM $^{14}$C-Ery. A (labeled in the lactone, specific activity 31,700 cpm/μmole), 4·0 ml of cofactor mixture, and 8·0 ml enzyme solution, were incubated at 37°. Four flasks, each containing 18·0 ml of 1 mM $^{14}$C-2′-propionyl Ery. A (labeled in the aglycone, specific activity 1,380 cpm/μ mole), 6·0 ml of cofactor mixture, and 12·0 ml of enzyme solution, were incubated at 37°. After 1 hr, the contents of flasks containing the same substrate were combined and filtered. The residues were extracted five times with chloroform. The filtrate and extracts of each

substrate were combined and evaporated to dryness under vacuum at 40°. The residue was dissolved in 20 ml of the upper phase of countercurrent solvent, and components were separated by counter current distribution in the system described by Pettinga *et al.*,[19] with 120 transfers. The control for each substrate, treated in the same manner except that the enzyme solution was added at the end of the reaction period, was used for comparison. Each tube was evaporated under vacuum at 40° and finally dissolved in 10 ml water. One-ml aliquots were used for radioactive determinations. The results are shown in Figs. 5 and 6.

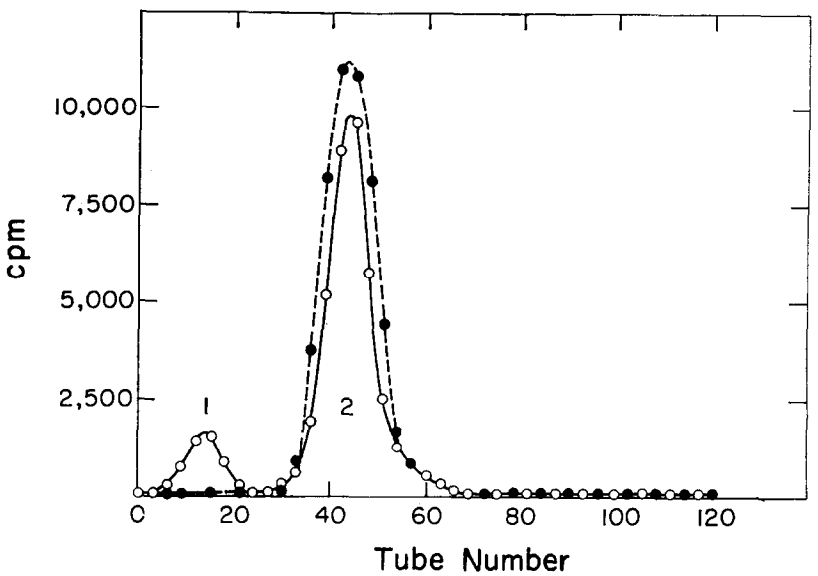


FIG. 5. Countercurrent distribution of the products of the demethylation of erythromycin A. $^{14}$C-erythromycin A was incubated with rabbit liver enzyme at 37° for 1 hr (○—○), and without rabbit liver enzyme at 37° for 1 hr (●—●).

Peak 1 of Fig. 5 is des-N-methyl Ery. A, identified by paper and thin-layer chromatography. Peak 2 is unreacted Ery. A. Apparently des-N-methyl Ery. A is the major product of the demethylation of Ery. A in liver. As shown in Fig. 3, des-N-methyl Ery. A can be further demethylated to di-des-N-methyl Ery. A. Since the rate of demethylation of des-N-methyl Ery. A is very low, it cannot be detected among the end products from the demethylation of Ery. A.

Metabolic products from 2′-propionyl Ery. A are more complex. Peak 1 of Fig. 6 is des-N-methyl Ery. A. Peak 2 is Ery. A produced from 2′-propionyl Ery. A during incubation. Since the sample incubated without enzyme contained more Ery. A than the sample with enzyme, it would appear that part of des-N-methyl Ery. A was formed from Ery. A. Peak 3 is unreacted 2′-propionyl Ery. A. The shoulder of peak 3 (tubes 100–112) was identified as 2′-propionyl des-N-methyl Ery. A, by comparison with an authentic sample of 2′-propionyl des-N-methyl Ery. A prepared as described under Methods with countercurrent distribution and a number of chromatographic systems.

## DISCUSSION

When administered at doses recommended for clinical use, the level of Ery. A in the liver is usually below 0·2 μmole/g.[20] At this concentration about 45% of Ery. A is demethylated within 1 hr by rabbit liver. Therefore, the N-demethylating system in liver is extremely active, and N-demethylation is a major metabolic pathway resulting in the deactivation of erythromycin.

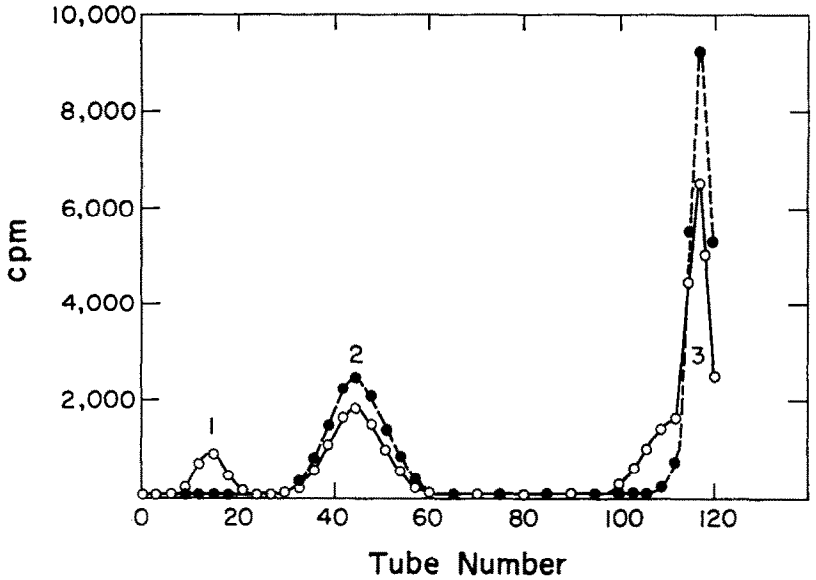


FIG. 6. Countercurrent distribution of the products of the demethylation of 2′-propionyl erythromycin. 2′-Propionyl-$^{14}$C-erythromycin A was incubated with rabbit liver enzyme at 37° for 1 hr (○—○), and without rabbit liver enzyme at 37° for 1 hr (●—●).

The substrate specificity of the N-demethylating enzyme is low, since erythromycin A, B, and C and a number of derivatives of Ery. A are demethylated. Structural changes on the lactone ring of erythromycin exert a relatively slight effect on rates of demethylation (Ery. B and anhydroery. A). The effect of esterification at the 2′-position on demethylation is dependent on the size of the ester group. In all cases the rate of demethylation of 2′-esters is lower than that of Ery. A. On the other hand, changes on the amine group (N-oxide and des-N-methyl Ery. A) or the O-methyl group (Ery. C) reduce the rate of demethylation substantially.

The demethylating enzyme is a particulate enzyme. Thus, the specificity is complicated by permeability of the substrate to the active site of the enzyme. McMahon[15] reported that the rate of demethylation shows a positive correlation with lipid solubility. However, our experiments show that esters of Ery. A, which are much more lipid soluble than Ery. A, gave much lower rates of demethylation (see Table 1 and Fig. 3). Apparently, the rate of demethylation is influenced less by lipid solubility than by esterification in the 2′-position of desosamine. Wiegand[21] has observed that the apparent first-order disappearance rate calculated from serum levels of 2′-propionyl Ery. A in human subjects is about half that of Ery. A, a result consistent with the slower demethylation of the ester.

Des-N-methyl Ery. A appears as a product of the demethylation of 2′-propionyl Ery. A, raising a question whether the ester must be hydrolyzed before demethylation.

There is little doubt that des-N-methyl Ery. A is derived in part from Ery. A formed by hydrolysis of 2′-propionyl Ery. A since, under the condition of demethylation (pH 7·8 and 37°), 2′-propionyl Ery. A is hydrolyzed to Ery. A to the extent of about 30% in 1 hr (Fig. 6). The conclusion that 2′-propionyl Ery. A is also a substrate for the demethylating enzyme is supported by the detection of a metabolite identified as 2′-propionyl des-N-methyl Ery. A. Hydrolysis of 2′-propionyl des-N-methyl Ery. A constitutes a second source of des-N-methyl Ery. A in the reaction mixture.

It has been postulated that either the N-hydroxymethyl compound or N-oxide[22, 23] is the intermediate for oxidative N-demethylation. Recently, Ziegler and Pettit[24] identified the N-oxide of N,N-dimethyl aniline by paper chromatography when N,N-dimethyl aniline was used as the substrate. Brodie *et al.*[3] found that the N-oxide of dimethyl aniline could be demethylated by liver microsomes but at a much slower rate than N,N-dimethyl aniline. They speculated that because the N-oxide is highly polar it may have difficulty in penetrating to the active site of the enzyme. The lipid solubility of Ery. A N-oxide is comparable to Ery. C and higher than des-N-methyl Ery. A, yet the rate of demethylation is lower than both Ery. C and des-N-methyl Ery. A. Therefore, the slow rate of demethylation of Ery. A N-oxide cannot be due simply to lipid solubility and must be attributed to low specificity. It is doubtful that Ery. A N-oxide is an intermediate in the demethylation of Ery. A.

*Acknowledgement*—We are indebted to Dr. P. P. Hung for the isolation of Ery. C, Dr .P. H. Jones for synthesizing Ery. A N-oxide, and Mr. A. Alter for the preparation of N-methyl-$C^{14}$-erythromycin A. In addition, we wish to express our appreciation to Mr. B. Wates and Mr. L. F. Graham, Jr., for their technical assistance.

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