[CONTRIBUTION FROM THE DEPARTMENTS OF BIOCHEMISTRY AND CHEMISTRY, UNIVERSITY OF WISCONSIN, MADISON 6, WISC.]

The Chemistry of Antimycin A. X. Structure of the Antimycins[1]

BY E. E. VAN TAMELEN, J. P. DICKIE, M. E. LOOMANS, R. S. DEWEY AND F. M. STRONG

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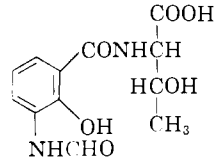
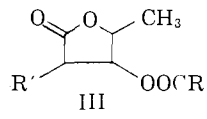
Chemical and physical evidence are combined in establishing the constitution of the antimycins (XVII), the *streptomyces* antibiotic group noted for its effect in inhibiting electron transport mechanisms in aerobic systems. Specific structures are proposed for antimycin A_1 and antimycin A_3, two components of the antibiotic complex.

The *streptomyces* antibiotic complex, antimycin (I), was discovered at the University of Wisconsin in the 1940's,[2] and has since been isolated by several other groups.[3] This family of antibiotics assumed considerable importance when it was found to be remarkably effective in inhibiting the hydrogen transport systems of aerobic organisms, and consequently useful in the study of certain enzyme mechanisms.[4] Previous chemical investigations, which were carried out first on a mixture of the closely related components[5] and more recently on pure materials,[6] revealed the general chemical nature of the constitutive degradation products, although the structure of the intact antibiotic remained uncertain.[7] We present herein a generalized formula for the antimycins based upon findings secured in these laboratories which represent the totality of the pertinent chemical information available up to the present time.

Separation studies have shown antimycin A to be a mixture of at least four active components, designated as antimycins A_1, A_2, A_3, and A_4.[2c,8,9] Characterization of A_1 and A_3 (blastmycin[8]) demonstrated the molecular formulas $C_{28}H_{40}N_2O_9$ and $C_{26}H_{36}N_2O_9$, respectively, and showed moreover that the two materials possessed very similar structures. Mild hydrolysis with base resulted in formation of N-formylantimycic acid (blastmycic acid), II,[6,10,11] and antimycin lactone, III ($R = -CH_2CH(CH_3)_2$, $R' = n\text{-}C_6H_{13}$) from antimycin A_1[6,12]; while, in addition to the same acid II, the lactone III ($R = -CH_2CH(CH_3)_2$, $R' = n\text{-}C_4H_9$) was generated from antimycin A_3.[10]



II



III

In a formal sense, the structure of the antibiotic itself is then derivable from the component units II and III, with loss of one molecule of water. A great variety of combinations is possible, and the derivation of the structure may be looked upon as a problem in selecting the correct formal reconstruction. This selection was accomplished by operating in a stepwise fashion: the various part structures and functions making up the total antibiotic system were regarded as separate subproblems, and accordingly the presence of each was established independently, for the most part, of the others. Since most of the structures which can be written could accommodate the observed basic cleavage of the antimycins to II and III, this behavior, or any other degradation which might involve initial conversion by base to II,[13] was not regarded as compelling evidence for any particular set of structures; but, rather, independent chemical and physical data bearing on the matter were sought. Because of the indisputable structural similarity between antimycin A_1 and A_3,[8] results obtained through study of one or the other antibiotic were brought to bear without reservation on the structural problem as a whole.

The evidence pertaining to the aromatic portion of the antimycin molecule will be discussed first. Color tests suggested early in the course of investigations that antimycin possessed an enolic, presumably phenolic, hydroxyl group. This view was supported by the observation that treatment of the antibiotic with diazomethane afforded a monomethyl derivative Ia, which did not exhibit the tests characteristic of enols. This fragmentary evidence was taken to indicate the presence of

(1) This contribution corresponds to our preliminary Communication to the Editor, part IX of the series "The Chemistry of Antimycin A," *J. Am. Chem. Soc.*, **82**, 1513 (1960). Parts I to VIII correspond to ref. 2b, 5, 11, 12, 14a, 8, 14b and 6 of the present paper, respectively. This work was supported in part by grants G-3527 from the National Science Foundation, and E-585 from the National Institutes of Health, U. S. Public Health Service.

(2) (a) C. Leben and G. W. Keitt, *Phytopath.*, **38**, 899 (1948); (b) B. R. Dunshee, C. Leben, G. W. Keitt and F. M. Strong, *J. Am. Chem. Soc.*, **71**, 2436 (1949); (c) J. L. Lockwood, C. Leben and G. W. Keitt, *Phytopath.*, **44**, 438 (1954).

(3) (a) J. Berger, Hoffmann–LaRoche, Inc., Nutley, N. J., personal communication, 1949; (b) K. Nakayama, F. Okamoto and Y. Harada, *J. Antibiotics (Japan), Ser. A*, **9**, 63 (1956); (c) K. Watanabe, T. Tanaka, K. Kukuhara, N. Miyaira, H. Yonehara and H. Umezawa, *ibid.*, **10**, 39 (1957); (d) Y. Sakagami, S. Takeuchi, H. Yonehara, H. Sakai and M. Takashima, *ibid.*, **9**, 1 (1956); (e) H. G. Schneider, Parke, Davis and Co., Detroit, Mich., personal communication, 1959; (f) A. D. Argoudelis, The Upjohn Co., Kalamazoo, Mich., personal communication, 1960.

(4) (a) K. Ahmad, H. G. Schneider and F. M. Strong, *Arch. Biochem.*, **28**, 281 (1950); (b) V. R. Potter and A. E. Reif, *J. Biol. Chem.*, **194**, 287 (1952); (c) A. L. Tappell, *Biochem. Pharmacol.*, **3**, 289 (1960).

(5) G. M. Tener, F. M. Bumpus, B. R. Dunshee and F. M. Strong, *J. Am. Chem. Soc.*, **75**, 1100 (1953).

(6) W. Liu, E. E. van Tamelen and F. M. Strong, *ibid.*, **82**, 1652 (1960).

(7) F. M. Strong, "Topics in Microbial Chemistry," Squibb Lectures on Chemistry of Microbial Products (1956), John Wiley and Sons, Inc., New York, N. Y., 1958, p. 32.

(8) W. Liu and F. M. Strong, *J. Am. Chem. Soc.*, **81**, 4387 (1959).

(9) Y. Harada, K. Uzu and M. Asai, *J. Antibiotics (Japan), Ser. A*, **11**, 32 (1958).

(10) H. Yonehara and S. Takeuchi, *ibid.*, **11**, 122, 254 (1958).

(11) G. M. Tener, E. E. van Tamelen and F. M. Strong, *J. Am. Chem. Soc.*, **75**, 3623 (1953).

(12) E. E. van Tamelen, F. M. Strong and U. C. Quarck, *ibid.*, **81**, 750 (1959).

(13) For example, it was found that selective lithium aluminum hydride reduction of antimycin in ether gave, as one of several products, the antimycin lactone (III). This reaction course strongly suggests that full hydride reduction of antimycin proceeds by way of III, and thus conclusions regarding the structure of the antibiotic based on such reductions are equivocal.

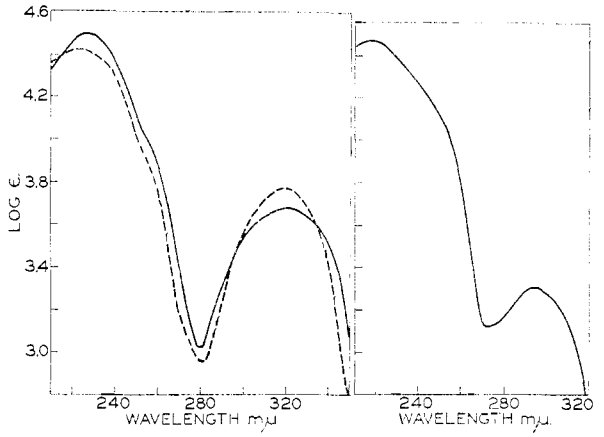
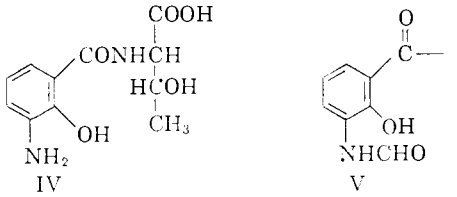


Fig. 1A (Left graph).—Ultraviolet spectra of antimycin A_1(———) and N-formylantimycic acid (--------). Fig. 1B (Right graph).—Ultraviolet spectrum of N-formylantimycic acid methyl ether methyl ester and of antimycin A_3 methyl ether. The two curves coincide.

a phenolic unit corresponding to that in antimycic or blastmycic acid, and the further results presented below confirmed in detail this surmise.

Earlier alkaline degradations carried out in these laboratories resulted in the formation of formic acid and antimycic acid (N-(3-aminosalicylyl)-L-threonine) (IV),[5,7,11] which was studied thoroughly and later synthesized.[14] The observation that the antimycins do not possess basic properties



suggested at an early stage that in the parent system the formic acid unit is bound by means of an amide linkage to the amino group of the 3-aminosalicylic acid residue (V). This assignment was confirmed in several independent ways. In the first place, the subsequent discovery that even milder basic conditions bring about formation of N-formylantimycic acid (blastmycic acid)[6,10] constitutes direct evidence for the original site of attachment of the formic acid fragment. In keeping with this assignment, antimycin A_1 is converted by hot alcoholic hydrochloric acid to deformylantimycin A_1 hydrochloride, m.p. 190–191°, which regenerates the parent material on treatment with formic acid. Finally, ultraviolet spectral comparisons between N-formylantimycic acid, the intact antibiotic, and derivatives of both provide strong confirmation of the above arguments. In the first place, the ultraviolet spectra of antimycin A_1 and N-formylantimycic acid are nearly identical (Fig. 1A). Also, the exact correspondence between the ultraviolet spectra of N-formylantimycic acid methyl ether methyl ester (IIa) and the methyl ether (Ia) of antimycin A_3 (Fig. 1B)

(14) (a) F. S. Okumura, M. Masumura, T. Horie and F. M. Strong, *J. Am. Chem. Soc.*, **81**, 3753 (1959); (b) F. S. Okumura, M. Masumura and T. Horie, *ibid.*, **81**, 5215 (1959).

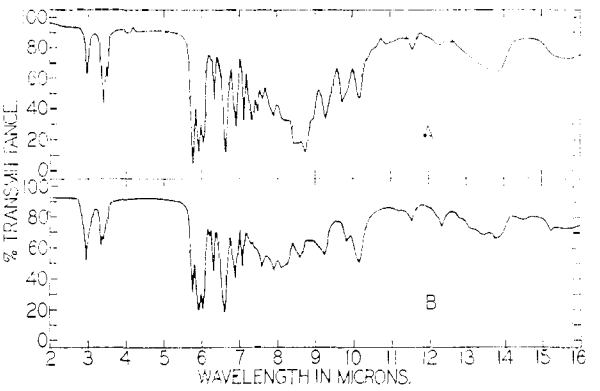
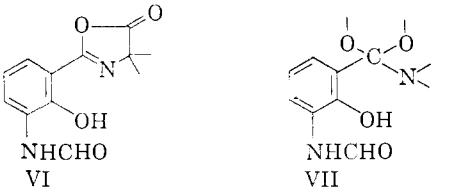


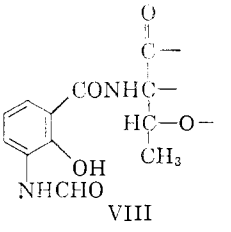
Fig. 2.—Infrared spectra of antimycin A_3 methyl ether (A), and of N-formylantimycic acid methyl ether methyl ester (B). Both spectra were determined in chloroform solution.

suggests identity of the aromatic systems in every detail, and furthermore substantiates the earlier conclusion that methylation of the antibiotic involves reaction of the phenolic hydroxyl group. The hypsochromic shift observed in going to the phenolic methyl ether (compare Fig. 1A and 1B) is again in keeping with the structural change involved. These spectral data require the N-formyl-3-aminosalicylyl chromophore V as a constituent portion of the antibiotic, and render such



possibilities as an azlactone (VI)[15] or an orthoester system (VII) unlikely.

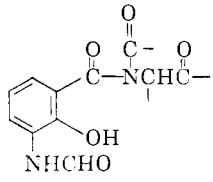
Study of infrared spectra of appropriate substances made it possible to expand the assigned sequence of linkages present in antimycin from V to VIII, a part structure directly implied, but not required, by the corresponding nature of the N-formylantimycic acid formed on degradation. N-Formylantimycic acid methyl ether methyl ester



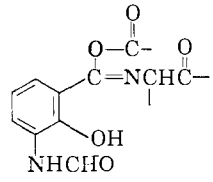
(IIa) again serves as a useful model of established structure, the infrared spectrum of which is reproduced in Fig. 2. The following assignments, which are based in part on considerations presented later, may be made: 5.74 μ (ester carbonyl), 5.89 μ

(15) Hippuric acid azlactone, chosen as a convenient model compound, was prepared as previously described (*C. A.*, **44**, 1096*d* (1950); M. M. Shemyakin, *et al.*, *Zhur. Obshchei Khim. J. Gen. Chem.*, **19**, 769 (1949)), and was found to have a peak in its ultraviolet spectrum at 241 mμ (log ε 4.14).

(-NHCHO), 6.01 μ (ArCONH-), 6.31 μ (Ar-H) and 6.60 μ (ArCONH-). Except for the relative intensity of the ester carbonyl absorption, the carbonyl region of this infrared spectrum is nearly superimposable on that of antimycin A_3 methyl ether (Fig. 2). This similarity disposes of such modifications as the imide IXa or the related O-acyl enol IXb, and supports the formulation VIII put forth.



IXa



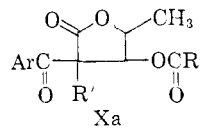
IXb

It was originally assumed that antimycin contained no free carboxylic acid function. Conversion of various antimycins to monomethylated products regarded (see above) as phenolic methyl ethers also intimated that a center more acidic than the original phenolic function was absent. However, contrary to the indications stated, the nuclear magnetic resonance (n.m.r.) spectrum of antimycin A_1 or A_3 displayed a signal (−380 c.p.s.)[16] in the region characteristic of carboxylic acid protons.

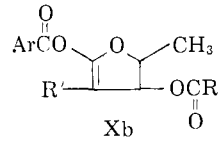
It should be borne in mind that the extreme lability of the molecule to base would prevent unequivocal determination of the neutralization equivalent and it was indeed found[17] that by slight variation of conditions one, two or three equivalents of base could be used to titrate antimycin components. The methylated derivatives did not consume any base on attempted titration with sodium hydroxide.

Spectrophotometric determination of the pK_a value of antimycin by the method of Bendich[18] gave a value of 5.1. Although the shape of the curve obtained indicated that there probably was only one acidic function present in the molecule, the extreme lability of the compound in base and the need to establish the presence or absence of a free carboxyl with certainty prompted further investigation of this point.

It may be stated first of all that the formation of antimycin lactone (III) during the degradation of antimycin by aqueous alkali can under no circumstances be interpreted in terms of a structure wherein the lactone carbonyl group of III existed originally as a free carboxyl group. At the same time, the absence of carbonyl absorption below 5.73 μ in the infrared spectrum of antimycin demanded that a γ-lactone system not be present in the parent natural product. Modified five-membered lactones, in particular Xa or Xb, also should, if present, be responsible for absorption at considerably lower wave lengths than observed (refer

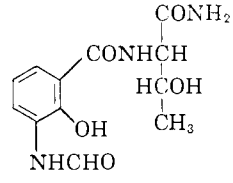


Xa



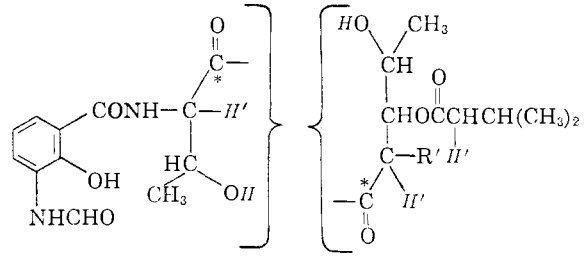
Xb

also to the later discussion of O-acyl enols).[19] Therefore, it may be concluded that the potential carboxyl group of the lactone system in III is present in antimycin in a masked state, and not incorporated into a five-membered ring. Turning to the N-formylantimycic acid moiety, it was reasoned that, should the carboxyl function be bound in the antibiotic, attack by base other than hydroxide ion should lead to formation of, not II, but a carboxylic acid derivative of same. Ammonia, chosen as a suitable and convenient base, converted antimycin A_1 to N-formylantimycyl amide (XI). Consequently it was concluded that a free carboxyl group does not appear in the antimycins.



XI

At this stage, the partial structure of the antibiotic may be represented as XII, wherein N-formylantimycic acid and the hypothetical hy-



XII

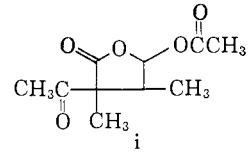
droxy acid corresponding to antimycin lactone are connected in some fashion, less two molecules of water. More explicitly, the possible variants are those which arise by attachment of the asterisked carboxyl groups to carbon or oxygen atoms bearing active hydrogen (italicized), in addition to those which derive by way of bond formation through simple enol linkages, the latter possibilities being indicated by the symbol H'. Finally, the array of structures requiring attention is compounded by those cases featuring hemiketal ester (*e.g.*,

(16) The n.m.r. spectra were run in deuterated chloroform using Varian Associates instruments. The spectra of antimycin A_1 and A_3 were determined by Varian Associates, Palo Alto, Calif., and the spectrum of blastmycin diacetate by the University of Wisconsin. Signals are reported at 60 megacycles, relative to benzene = 0.

(17) L. A. Ehrhart, M.S. thesis, University of Wisconsin, 1960.

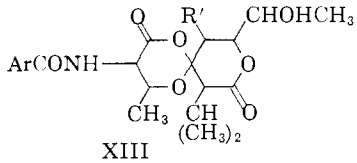
(18) A. Bendich, in E. Chargaff and J. N. Davidson, "The Nucleic Acids," Vol. I, Academic Press, Inc., New York, N. Y., 1955, p. 166.

(19) α-Acetyl-γ-butyrolactone possesses a lactone band at 5.64 μ, the same position as that in γ-butyrolactone itself (determined in these laboratories); and the antibiotic acetomycin (i) absorbs as low as

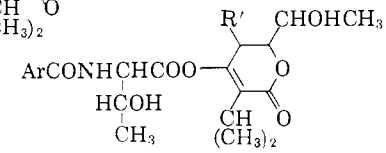


i

5.57 μ (W. Keller-Schierlein, M. L. Mihailovic and V. Prelog, *Helv. Chim. Acta*, **41**, 220 (1958)).

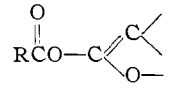


XIII

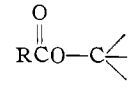


XIV

XIII) or 1,3-dicarbonyl enol ester (*e.g.*, XIV) systems. Although certain of the structures included in the above treatment cannot accommodate the behavior of the antimycins in base as well as others, it was deemed unwise, again, to eliminate possibilities on this basis alone; therefore other, more reliable evidence was utilized in the structural development. Attention is drawn first to esters of enols or hemiketals, which group may be excluded on the basis of the following considerations. Such structures are regarded as incompatible with the observed resistance of the antibiotic to drastic treatment with acid: hot alcoholic hydrochloric acid removes the formyl group, but leaves the remainder of the molecule untouched. Second, the close correspondence in the ultraviolet of the antimycins with N-formylantimycic acid (Fig. 1A) does not allow for the presence of a second point of unsaturation (such as in XIV) which would be expected to absorb to any reasonable degree in this region of the spectrum. Finally, a sufficiently large number of cases[20] has been recorded to justify the generalization that O-acyl derivatives of ester enols (XV) possess carbonyl absorption at about 0.1 μ shorter wave length in the infrared than the



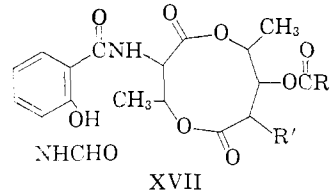
XV



XVI

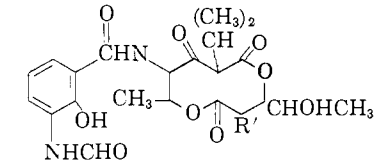
corresponding O-acyl derivatives (XVI) of simple alcohols. Consequently, should the structure XV form part of the antimycin molecule, a carbonyl peak at about 5.63 μ would be observed, contrary to fact.

Of the remaining individual structures which are permitted by the collective expression XII, one is a cyclic diester (XVII); several must be formulated as mono-(*sec*)-hydroxymonoketones (*e.g.*, XVIII); and the rest constitute a group each member of which has two ketonic and two secondary alcoholic functions (*e.g.*, XIX). In an endeavor to place the antibiotic in one of these categories, experiments designed to reveal the

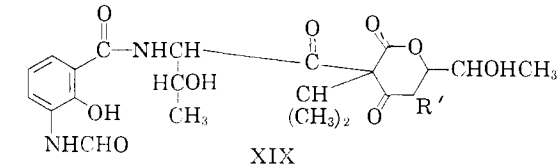


XVII

(20) H. H. Wasserman and P. S. Wharton, *J. Am. Chem. Soc.*, **82**, 661 (1960).



XVIII



XIX

number of such functions were carried out. Almost without exception, however, these attempts, which are outlined below, proved negative and therefore forced the conclusion that antimycin must possess the unique dilactone structure XVII.

Efforts to secure ketone carbonyl derivatives from antimycin were uniformly unsuccessful, and even under relatively drastic catalytic reduction conditions, *viz.*, platinum in acetic acid in the presence of hydrochloric acid, no hydrogen was consumed. Under conditions which normally suffice for conversion of a ketone to the corresponding dithioketal (or a hemiketal to the monothioketal), antimycin was modified only to the extent that the N-formyl group was lost. On treatment with pyridine–acetic anhydride, antimycin O-methyl ether (Ia) was recovered totally. The same derivative, on prolonged exposure to chromic acid in acetone–sulfuric acid, was unattacked, again intimating that no oxidizable alcoholic grouping is present. Such experiments, although individually perhaps only suggestive, on the whole require that antimycin can have no structure other than XVII. Since, on the basis of previous work, the structures of the antimycin lactones derived from antimycins A_1 and A_3 are known,[6,10] the exact formula XVII may be written for antimycin A_1 (R = $-CH_2CH(CH_3)_2$, R′ = n-C_6H_{13}) and antimycin A_3 (R = $-CH_2CH(CH_3)_2$, R′ = n-C_4H_9).[21]

It was anticipated that acetylation attempts on antimycin would shed light on the structure by providing a measure of the number of alcoholic hydroxyl groups present, and indeed results of this type had been recorded in the recent literature by Yonehara and Takeuchi.[10] These workers reported that antimycin A_3, on treatment with acetic anhydride–pyridine, formed a *diacetate*, which on saponification liberated 3.78 moles of volatile acid. On the assumption that only phenolic and alcoholic hydroxyl groups would be esterified under these conditions, this result would suggest that antimycin possesses a structure other than that (XVII) proposed herein. Our inspection of the experimental account of the Japanese investigators revealed that the analytical results did not permit differentiation between a monoacetate and a di-

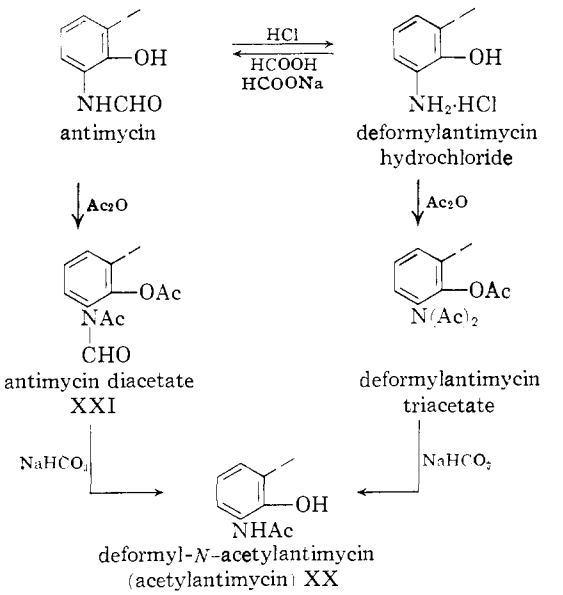
(21) The dilactone formulation was also proposed for blastmycin (antimycin A_3) by A. J. Birch, D. W. Cameron, R. W. Richards and Y. Harada, *Proc. Chem. Soc.*, 22 (1960). However, the evidence cited was insufficient to allow rigorous exclusion of other plausible structures, and in particular completely failed to explain the reported formation of a *diacetate* of blastmycin.[10]

acetate formulation, and consequently the matter was reinvestigated in our laboratories.

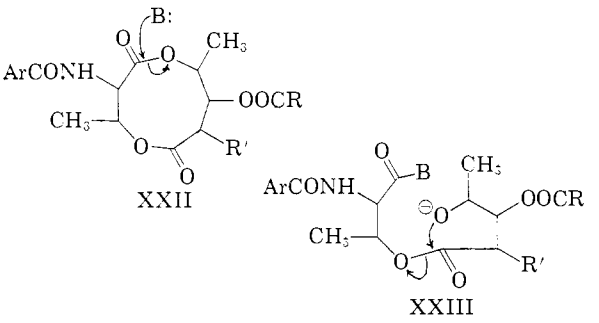
Crystalline acetates of antimycin A_1 and A_3 and of the antimycin complex were obtained without difficulty, and gas chromatographic studies on their basic and acidic hydrolysates were undertaken. Saponification of the derivatives in alcoholic base for 2.5 hours followed by methylation of the liberated acids and chromatography of the esters showed that there were two moles of methyl acetate per mole of methyl isovalerate present in the hydrolysate. When the hydrolysis was carried out in *acid*, the gas chromatogram also showed approximately two moles of methyl acetate per mole of methyl isovalerate.

Our finding, which was in agreement with the results of C-methyl and n.m.r. determinations on the antimycin acetate, verified the assumption of the Japanese school that the derivative was indeed a diacetate, but at the same time constituted an obstacle to acceptance of the dilactone structure for the antibiotic. One of the acetyl groups had become attached to the phenolic oxygen atom of the antimycin, as indicated by negative ferric chloride test and by the disappearance of the phenolic hydroxyl resonance peak in the n.m.r. spectrum. Surprisingly enough, the second acetyl group was bound to the aromatic ring nitrogen, as indicated by the following studies.

The previously mentioned failure to obtain a new compound on attempted acetylation of the methyl ether Ia suggested that the second acetyl group was not attached to the lactone part of the molecule. Final confirmation of the position of the second acetyl function derived from studies on deformylantimycin. Acetylation of this substance gave a product which was shown by gas chromatography as a *triacetate*. Both the triacetate and diacetate gave the identical *monoacetate* on mild hydrolysis with sodium bicarbonate. This compound had regained the free phenolic hydroxyl function, showed the infrared band at 5.90 μ characteristic of the Ar–N-acyl group, was recovered unchanged after treatment with acid under conditions normally utilized for converting antimycin to deformylantimycin, and had recovered the full enzyme inhibitory activity of antimycin which had been lost when the formyl group was removed. For these reasons and because of its formation from both the di- and triacetates, the monoacetate is formulated as shown in formula XX, *i.e.*, as the "acetyl analog" of antimycin. Consequently, antimycin diacetate must be XXI, and relationships between the various acetates of antimycin are represented in the diagram.



One of the noteworthy features of antimycin chemistry is the facile hydrolytic cleavage brought about by dilute sodium hydroxide at room temperature. In terms of the developed structure XVII, this fragmentation must be promoted by predominant attack at the lactone carbonyl of the potential N-formylantimycic acid unit (XXII). The ethereal oxygen released is then favorably disposed for attack on the second lactone function (XXIII), leading to formation of the antimycin lactone; and it is possible that the two processes are concerted, with the result that the activation energy for the over-all reaction would be lower than that for a simple ester hydrolysis.



In regard to the infrared spectrum of antimycin, the bands at 5.73, 5.90, 6.09, 6.20(weak) and 6.56 μ are consistent with the established structure XVII. The lactone carbonyl groups and the ester group of the side chain are responsible for the composite absorption at 5.73 μ. Deformylantimycin displays absorption in the 5.7–7.0 μ region very similar to that of antimycin itself, with the exception that the 5.90 μ band is missing; consequently this peak must be due to the N-formyl group of the natural product. The remaining bands are ascribable to the secondary amide function (6.09, 6.56 μ) and to benzenoid ring absorption. The shift of the N-formyl carbonyl absorption in antimycin O-methyl ether to a somewhat shorter wave length (5.88) can be explained readily in terms of decreased hydrogen bonding in the ether. It should be emphasized that, since all of the carbonyl bands of antimycin can be accounted for by referral to the proposed structure, possible formulas for the antibiotic containing a ketone function are not supported by the infrared spectral behavior, in agreement with the results already presented. This point becomes especially clear in the spectrum of deformylantimycin, wherein the normal ketone carbonyl region (5.75–5.85 μ) is devoid of absorption. It should be recognized, however, that

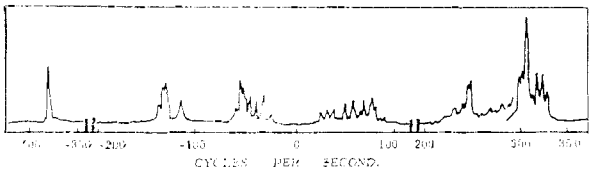


Fig. 3.—Nuclear magnetic resonance spectrum of antimycin A_1. Above +290 c.p.s. the sensitivity of the instrument was reduced by one-half.

this evidence by itself is not compelling, in that a structure in which a ketone function is present, but masked as a hemiketal, is not excluded.

Although the nuclear magnetic resonance behavior of antimycin and its derivatives was not of great value in the structural elucidation, interpretation of the spectra in light of the known constitution is of interest. The signal which appears at lowest field (−380 c.p.s., Fig. 3) is very probably a consequence of the hydrogen of the phenolic hydroxyl group, since (i) this proton is the most acidic in the molecule, and (ii) in the acetylated antibiotic this low field peak is missing. The variety of resonances found in the region −130 to +90 c.p.s. are associated with the hydrogens present in the amide and formyl units, on the aromatic ring, and on carbon atoms bearing acyloxy groupings; the remaining peaks at higher field are due to the methine, methylene and methyl groups of the lactone portion of the molecule. The peak areas confirm the separation into these two broad categories.

The assignment of the O,N-diacetate structure XXI to antimycin diacetate is corroborated by its n.m.r. spectrum, which is, in its over-all pattern, similar to that of the parent material, except for the following differences. Two new, sharp singlets are found at 244 and 257 c.p.s., the proper positions for the methyl group hydrogens of O-acetyl groups. The appearance of two such peaks, and the area under the curves corresponding to six hydrogens, indicate that the substance is in fact a diacetate. In addition to the disappearance of the low field phenolic hydroxyl peak, a new band appears (−189 c.p.s.), apparently produced by the formyl hydrogen in the newly formed imide grouping. Because of the negative substituents in the imide function, the resonance due to the hydrogen should come in at lower fields, as observed.

A comparison of derivatives of blastmycin and antimycin A_3 further confirms the earlier conclusion[8] that blastmycin consists mainly of the A_3 component. The infrared spectra of the methylated derivatives are extremely similar and the spectra of the acetylated compounds are essentially superimposable. Although the melting points of the blastmycin derivatives are always a few degrees lower than the corresponding A_3 derivatives, admixture of the compounds causes no melting point depression. The probable presence of a small amount of antimycin A_4 in blastmycin[8] could well account for these lower melting points.

Studies on the ability of the antimycin derivatives to inhibit electron transport have shown that deformyl-N-acetylantimycin (XX) is as active as the natural material and that none of the other derivatives bears comparable activity.[4c]

Experimental[22]

Blastmycin O-Methyl Ether.—To 100 mg. (0.19 mmole) of blastmycin (Kyowa Fermentation Co., Tokyo, Japan), m.p. 170.5–172.5°, was added 15 ml. of ether. The solution was cooled in an ice–salt mixture and an excess of ethereal diazomethane solution was added. After 5 minutes in the ice–salt mixture the solution was allowed to remain for 20 minutes at room temperature before the solvent was removed by heating in a 50° water-bath under a stream of dry air. The colorless oil which remained after the removal of the solvent was crystallized from ethyl acetate–Skelly solve C to yield 76 mg. (0.14 mmole, 74%) of white crystals. Recrystallization from the same solvent pair gave an analytical sample which melted at 110–111°. The compound gave a negative ferric chloride test. The ultraviolet spectrum (Fig. 1B) showed λ_{max} 294 mμ (log ε 3.30), 225 mμ (log ε 4.47). The infrared spectrum is reproduced in Fig. 2.

Anal. Calcd. for $C_{27}H_{38}N_2O_9$: C, 60.66; H, 7.17; OCH_3, 5.80. Found: C, 60.60; H, 7.03; OCH_3, 5.75.

Antimycin A_1 O-Methyl Ether.—Antimycin A_1,[23] m.p. 149–150°, was treated with diazomethane as described for the preparation of the blastmycin counterpart. The solid residue which remained after removal of the solvent was recrystallized twice from ethyl acetate–Skellysolve B to yield 14 mg. (0.025 mmole, 68%) of the analytical sample, m.p. 93–95°. The ultraviolet and infrared spectra showed the same major bands as the blastmycin derivative. This material also gave a negative ferric chloride test.

Anal. Calcd. for $C_{29}H_{42}N_2O_9$: N, 4.98; OCH_3, 5.51. Found: N, 5.14; OCH_3, 5.57.

Antimycin A_3 O-Methyl Ether.—Ethereal diazomethane (20 ml.) which had just been washed with cold water was added slowly to 100 mg. (0.19 mmole) of antimycin A_3, m.p. 177–178.5°. The solid material remaining after removal of the solvent was recrystallized from ether–Skellysolve B to yield 80 mg. (0.15 mmole, 79%) of material which melted at 88–90°. A sample of blastmycin O-methyl ether, m.p. 110–111°, prepared as described above, was recrystallized from ether–Skellysolve B, and by seeding with a few crystals of A_3 O-methyl ether, material could be obtained which melted at 86–87°. The melting point on admixture of this sample of blastmycin O-methyl ether with the A_3 derivative was 86–87°. The infrared spectrum of the antimycin A_3 O-methyl ether was extremely similar to, although not quite superimposable on, the spectrum of the blastmycin counterpart. The ultraviolet spectrum of A_3 O-methyl ether was identical to that of the blastmycin derivative.

N-Formylantimycic Acid Methyl Ether, Methyl Ester.—A 50-mg. sample of N-formylantimycic (blastmycic) acid, m.p. 141.5–142.5° (obtained by basic degradation of the antimycin complex[6]), was dissolved in methanol and cooled in an ice-salt-bath. Excess ethereal diazomethane was added. The yellow solution was allowed to remain in the ice-bath for 5 min., and then at room temperature for 20 min. The very light yellow oil remaining after removal of the solvent gave a negative ferric chloride test. The ultraviolet spectrum was identical with that of antimycin A_3 O-methyl ether (Fig. 1B). The infrared spectrum (Fig. 2) was virtually superimposable, in the 5–7 μ region, on that of the antimycin A_3 O-methyl ether.

N-Formylantimycyl Amide.—The antimycin A complex (100 mg., 0.18 mmole, m.p. 141–142.5°) was dissolved in 10 ml. of anhydrous methanol. The solution was cooled in an ice-bath and anhydrous ammonia (which had been passed through a soda-lime tube) was bubbled into the solution for 15 minutes. The mixture was allowed to stand in ice for

(22) All melting points were determined on a Kofler type hot-stage melting point block (Monoscope model IV, Michigan Scientific Co., Ann Arbor, Mich.) and are uncorrected. Ethanol and ether when used in this discussion will mean 95% ethanol and diethyl ether, respectively. Infrared spectra were determined on a Beckman model IR-5 double beam recording spectrophotometer. All ultraviolet spectra were run in ethanol on a Cary recording spectrophotometer, model 11. Unless otherwise indicated, the analyses were performed at the Hoffman Micro-analytical Laboratories at Wheatridge, Colo.

(23) All antimycin subcomponents were separated as described by Liu and Strong, ref. 8.

another 30 minutes and then at room temperature for 90 min. before the solvent was removed under a stream of nitrogen in a 30° water-bath. Extraction of the residue with chloroform left 30 mg. of insoluble material as a white powder. After two recrystallizations from propanol–Skellysolve B there remained 21 mg. (0.075 mmole, 40%) of white crystals, m.p. 186–189°. Three more recrystallizations from anhydrous ethanol gave the analytical sample, which exhibited a melting point of 189.5–191°; λ_{max}^{KBr} 5.88 μ (N-formylcarbonyl), and 6.06μ (NH-acyl); λ_{max} 318 mμ (log ϵ 3.76), 228 mμ (shoulder) (log ϵ 4.41), 223 mμ (log ϵ 4.43). The material gave a dark blue color with ferric chloride. When a sample of N-formylantimycic acid was substituted for antimycin in the above procedure only starting material could be isolated. A mixture of N-formylantimycic acid, m.p. 139–140°, and the amide isolated from the above reaction, m.p. 189.5–191°, exhibited a melting point of 170–180°.

Anal. Calcd. for $C_{12}H_{15}N_3O_5$: C, 51.24; H, 5.38; N, 14.94. Found: C, 51.55; H, 5.43; N, 15.07.

Antimycin A_3 diacetate (XXI) was prepared as described by Yonehara and Takeuchi[10] for the blastmycin analog. From 50 mg. (0.096 mmole) of antimycin A_3, m.p. 177–178.5°, 40 mg. (0.066 mmole) of the analytical sample, m.p. 152-153°, was obtained. The melting point of a mixture of A_3 acetate with the analogous blastmycin derivative, m.p. 148–150°, prepared in an identical fashion, was 149–151°. The infrared spectra of the two acetates were identical.

Anal. Calcd. for $C_{30}H_{40}N_2O_{11}$: C, 59.58; H, 6.67; N, 4.63. Found: C, 60.01; H, 7.10; N, 4.75; C-methyl detn. on the acetate and antimycin A_3, 4.43 and 2.43, resp.

Deformylantimycin A_1 Hydrochloride.[24]—To 60 mg. (0.11 mmole) of antimycin A_1 was added 2 ml. of ethanol and 1 ml. of concentrated hydrochloric acid. The mixture was boiled for 5 min. and allowed to cool. The material which crystallized was separated from the mother liquor by filtration and washed with cold water. One recrystallization from ethanol–hydrochloric acid yielded 55 mg. (0.099 mmole, 90%) of the analytical sample, m.p. 190–191° dec.

Anal. Calcd. for $C_{27}H_{41}ClN_2O_8$: C, 58.20; H, 7.42; Cl, 6.37. Found: C, 58.63; H, 7.33; Cl, 5.86.

Other Deformylantimycin Hydrochlorides.—Deformylblastmycin hydrochloride, m.p. 170–172°, deformylantimycin A_3 hydrochloride, m.p. 171–172°, and the deformylantimycin complex hydrochloride, m.p. 178–180°, were prepared using the same procedure described for the A_1 counterpart. The melting point on admixture of the hydrochlorides obtained from blastmycin and antimycin A_3 was 170–172°. Both hydrochlorides showed some softening at 145° and rapid decomposition at their melting temperature.

Attempted Acetylation of Antimycin A_3 O-Methyl Ether.—To 20 mg. of antimycin A_3 O-methyl ether, m.p. 87–89°, was added 1 ml. of acetic anhydride and 2 drops of pyridine. The solution was allowed to stand 14 hours at room temperature in the dark. At the end of this time water was added and a light yellow oil separated. The aqueous layer was decanted from the mixture and the oil washed with water and crystallized from ethanol–water. The material isolated (16 mg.) exhibited a melting point of 88–90° and had an infrared spectrum superimposable on that of the starting material. Admixture of the isolated sample with the starting material gave material melting at 87–89°.

Formylation of Deformylantimycin A_3 Hydrochloride.—To 25 mg. (0.047 mmole) of deformylantimycin A_3 hydrochloride was added 48.6 mg. (0.072 mmole) of sodium formate and 0.4 ml. of 88% formic acid. The mixture was heated at 50° for 1 hour and allowed to stand at room temperature for an additional 13.5 hours. On addition of 2 ml. of water, white crystals formed which were separated from the mother liquor by filtration, washed with cold water, and dried. Recrystallization from ethyl acetate–Skellysolve B yielded 14.8 mg. (0.029 mmole, 62%) of antimycin A_3, m.p. 173–175°. The melting point on admixture of this compound with authentic antimycin A_3, m.p. 177–178°, was 174–177°. The infrared spectrum of the isolated material was superimposable on the spectrum of authentic antimycin A_3. The reformylated material also was as effective in inhibiting succinoxidase as the natural product (Table I).

TABLE I

ACTIVITY OF ANTIMYCIN A_3 AND DERIVATIVES IN ELECTRON TRANSPORT INHIBITION

Compound tested[a]	Inhibition, %
Antimycin A_3	95
Antimycin A_3 O-methyl ether	0
Antimycin A_3 diacetate	0
Deformylantimycin A_3 hydrochloride	10
Deformylantimycin A_3 triacetate	10
Antimycin A_3 (reformylated deformylantimycin A_3 hydrochloride)	95
Deformyl-N-acetylantimycin A_3 (XX)	85

[a] 0.15 μg./flask containing a total volume of 3 ml.

Attempted Reaction of Antimycin A Complex with Benzyl Thiol.—To 46 mg. of antimycin A complex in 1 ml. of dichloromethane was added 4 drops of benzyl thiol, and dry hydrogen chloride gas was passed slowly through the solution for 0.5 hour while the mixture was cooled in an ice-bath. After 2 hours at 0° the reaction mixture was concentrated under reduced pressure and the crystals which formed were removed by filtration. The crystalline material had an infrared spectrum identical with that of the starting material. The product was treated further with 6 drops of benzyl thiol and hydrogen chloride gas as described above and allowed to stand for 12 hours at 0°. Concentration of the solution followed by extraction of the excess thiol with petroleum ether gave 37 mg. of crystalline material. This material had an infrared spectrum identical with the starting material and mixture of isolated material with starting material caused no depression of melting point.

Attempted Reaction of Antimycin A Complex with 1,2-Ethylene Dithiol.—A 30-mg. sample of antimycin A in 0.5 ml. of dichloromethane was treated with 4 drops of 1,2-ethylene dithiol, *ca.* 50 mg. of freshly fused zinc chloride was added, and anhydrous hydrogen chloride gas was bubbled through the reaction mixture for 15 min. at room temperature. After 12 hours at room temperature the reaction mixture was concentrated under reduced pressure (15 mm.) and extracted with petroleum ether. The oil which was isolated had an infrared spectrum which indicated that it was deformylated antimycin (absence of carbonyl absorption at 5.8–5.9μ). The oil formed a crystalline hydrochloride (5 mg.) which had an infrared spectrum identical with that of the deformylantimycin complex hydrochloride.

Attempted Oxidation of Antimycin A Complex O-Methyl Ether.—The O-methyl ether of the antimycin complex was prepared as described for the A_3 derivative to yield material melting at 107–108.5°. A 46-mg. sample of antimycin A complex O-methyl ether was dissolved in 1.5 ml. of acetone and treated with 0.03 ml. of a solution of 0.5 g. of sodium chromate, 0.5 ml. of sulfuric acid and water to 2 ml. After 2 hours at −10° the reaction mixture was diluted with water and sodium bicarbonate was added until the *p*H was *ca.* 6. Sodium bisulfite was added to destroy the remaining chromate, and the mixture was extracted with ether. The material, 30 mg., isolated from the ether extract had an infrared spectrum identical with that of the starting material, and mixture of the two caused no depression of melting point.

Deformylantimycin A_3 Triacetate.—Antimycin A_3 (84 mg., 0.16 mmole) was deformylated as previously described. The hydrochloride was washed with water and shaken with 5 ml. of 10% sodium bicarbonate and 4 ml. of ethyl acetate. The layers were separated and the aqueous layer washed twice with 2-ml. portions of ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate, and the solvent was removed at room temperature under a stream of dry nitrogen. To the yellow oil remaining after the evaporation of the solvent was added 3 ml. of acetic anhydride and 6 drops of pyridine. The mixture was heated at 60° for 10 min. and then allowed to stand for 17 hours at room temperature. About 8 ml. of water was added, and the mixture was stirred in an ice-bath until crystals formed. The solid material was removed by filtra-

(24) This substance was first prepared by Dr. W. Liu in this laboratory.

tion and washed with water. Two recrystallizations from ethanol–water yielded 56 mg. (0.090 mmole, 56%) of the analytical sample, m.p. 142–144°.

Anal. Calcd. for $C_{31}H_{42}N_2O_{11}$: C, 60.17; H, 6.84; N, 4.53. Found: C, 60.43; H, 6.77; N, 4.51.

Hydrogenation Studies on Antimycin A_1.—These experiments were done using the method and apparatus described by Clauson-Kaas, *et al.*[25]

An attempt was made to hydrogenate a solution of 9.9 mg. of antimycin A_1 in 5 ml. of acetic acid (which had been distilled from potassium permanganate) over 6 mg. of 5% palladium-on-carbon at room temperature and atmospheric pressure. There was no uptake of hydrogen in 15 hours. Substitution of platinum oxide for palladium-on-carbon and addition of hydrochloric acid in the above experiment also gave no hydrogen uptake.[26]

Basic Degradation of Antimycin A Complex Diacetate.—To 20 mg. of acetylated antimycin complex, m.p. 126–129°, was added 1 ml. of 10% aqueous potassium hydroxide and enough ethanol (*ca.* 5 drops) to dissolve about one-tenth of the solid material. After heating 5 min. in a warm water-bath the solution was extracted with Skellysolve A. After removal of the solvent from the extract, the infrared spectrum of the remaining material was superimposable on that of the antimycin lactone, isolated by identical treatment of the unacetylated complex.

Attempted Lithium Aluminum Hydride Reduction of the Antimycin A Complex.—To 20 mg. of lithium aluminum hydride in 20 ml. of anhydrous ether was added 100 mg. of the antimycin complex dissolved in 15 ml. of dry ether. The mixture was stirred at room temperature for 1.75 hours and then at reflux for 2 hours. A saturated aqueous solution of ammonium chloride was added slowly to decompose excess lithium aluminum hydride, and the reaction mixture acidified with hydrochloric acid. The ether layer was separated, evaporated to dryness, and the residue taken up in butanol. A chloroform extract of the material (40 mg.) remaining after evaporation of the butanol had an infrared spectrum which showed strong bands at 5.63μ (5-membered lactone carbonyl) and 5.78μ (normal ester carbonyl) and in all respects was identical with that of antimycin lactone isolated from basic degradation of the antimycin complex.

Deformyl-N-acetylantimycin A_3 (XX).—To 39 mg. (0.065 mmole) of antimycin A_3 diacetate dissolved in 2 ml. of methanol was added 6 ml. of a solution prepared by dissolving 0.40 g. of anhydrous sodium bicarbonate in 40 ml. of water and 60 ml. of methanol. The mixture was warmed to 45° and held at that temperature for 15 min. The reaction was stopped by addition of 10% HCl to *p*H 2, and the mixture diluted with 5 ml. of water and cooled. A white solid material which precipitated was washed with water. One recrystallization from a dioxane–water solvent pair yielded 23.4 mg. (0.044 mmole, 68%) of product, m.p. 152–154°. This material gave a dark blue color with ferric chloride. When treated with hot alcoholic HCl for 10 min., it was recovered unchanged.

Anal. Calcd. for $C_{27}H_{38}N_2O_9$: C, 60.66; H, 7.17; N, 5.24. Found: C, 60.72; H, 6.83; N, 5.49.

Application of this procedure to deformylantimycin A_3 triacetate gave a mixture from which the same monoacetylated compound could be obtained in pure form by chromatographic separation on a silicic acid column. The infrared spectrum was superimposable on that of the material obtained from the diacetate. On admixture of the two compounds no melting point depression was observed.

Spectrophotometric determination of the acidic dissociation constant was carried out as described by Bendich.[18] A stock solution was prepared by dissolving 7.9 mg. of the antimycin A complex, m.p. 141–143°, in ethanol and diluting to 50 ml. with the same solvent. Four-ml. portions of the stock solution were pipetted into each of a series of nine 10-ml. volumetric flasks and diluted to volume with appropriate buffers which were prepared by the method of Clark and Lubs.[27] The *p*H values of the final solutions were determined with a glass electrode and were found to range from 4.7 to 9.7. Solvent blanks were prepared by adding 4 ml. of ethanol to a 10-ml. volumetric flask and diluting to volume with the buffer solution. Ultraviolet spectra were determined at each of the *p*H values in 2-cm. quartz cells. The absorbancy at 345 mμ was read for each of the curves and was plotted *vs.* the *p*H of the solution from which it was obtained. The inflection point of the *p*H *vs.* absorbancy curve was found at *p*H 6.1. The addition of 4 ml. of ethanol to 6 ml. of buffer of approximately *p*H 5 caused the apparent *p*H to increase about 1 unit. Therefore, the pK_a value of antimycin in water was estimated to be about 5.1.

Gas Chromatographic Studies.—To a 50-mg. sample of the antimycin complex diacetate, or of the deformylantimycin complex triacetate (prepared as described for the A_3 analog, m.p. 132–132.5°, with softening at 129°), was added 6 ml. of 30% sulfuric acid and the mixture was refluxed for 2 hours. It then was diluted with 75 ml. of water, distilled at atmospheric pressure until *ca.* 50 ml. of distillate had been collected, and the distillate was made basic and evaporated to dryness in a rotary evaporator at 40°. The residue was acidified to *p*H 2 with concentrated sulfuric acid while the flask was cooled in an ice-bath, and the mixture then extracted 15–20 times with 5-ml. portions of ether. The ether solution of the acids was concentrated to a volume of 0.5 ml. and the sample esterified by passing diazomethane vapors through the solution until a permanent yellow color remained. The mixture was sampled immediately for gas chromatographic studies without removing any of the solvent.

The methyl esters were chromatographed on a 2-meter column packed with polyethylene glycol on Chromosorb in a ratio of 20 parts of liquid to 80 parts of solid[28] with helium as the carrier gas. The gas flow rate was 12.5 ml. per min. and the temperature of the column was 136°. Experiments with known mixtures of formic, acetic and isovaleric acids had verified the reliability of the methylation and extraction procedures and had shown that the above method could be used to determine the molar ratio of methyl acetate to methyl isovalerate present.

After chromatography of the esters the peaks corresponding to the methyl acetate and methyl isovalerate were cut from the chart, weighed and the molar ratio of methyl acetate to methyl isovalerate determined.[29] Calculations showed 1.99 and 2.95 moles of acetate were obtained per mole of isovalerate from antimycin diacetate and from deformylantimycin triacetate, respectively. Samples of antimycin diacetate were also saponified in 10% potassium hydroxide for 2.5 hours, and the acids isolated, methylated and chromatographed as described above. In this experiment 1.72 moles of acetate was obtained per mole of isovalerate. A previous experiment in which the reflux time was 1 hour gave a 1:1 ratio of methyl acetate to methyl isovalerate, presumably because of incomplete saponification.

Inhibition of Electron Transport.—Inhibition experiments were carried out with the system described by Lardy, *et al.*,[30] in which succinate serves as the substrate. The compounds were tested in an ethanol–water solution. A control of ethanol–water in the same proportions as used for the test solutions caused no inhibition of the enzyme system. The results are given in Table I as % inhibition compared to a sample containing no inhibitor.[31]

(25) N. Clauson-Kaas and F. Limborg, *Acta Chem. Scand.*, **1**, 884 (1947).

(26) We wish to thank V. Haarstad for carrying out these hydrogenation experiments, and also for assistance in making n.m.r. measurements.

(27) N. Lange, "Handbook of Chemistry," 7th ed., Handbook Publishers, Inc., Sandusky, Ohio, 1949, p. 1127.

(28) Column "Kx," Perkin-Elmer Corp., Norwalk, Conn.

(29) A. I. M. Keulemans, "Gas Chromatography," Reinhold Publishing Corp., New York, N. Y., 1957, p. 33.

(30) H. A. Lardy and H. Wellman, *J. Biol. Chem.*, **195**, 215 (1952).

(31) We wish to thank H. A. Lardy and Mrs. Diane Johnson for carrying out these measurements.