[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

The Chemistry of Antimycin A. VI. Separation and Properties of Antimycin A Subcomponents[1,2]

BY WEN-CHIH LIU AND F. M. STRONG

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Antimycin A is a mixture of at least four active components which have been separated and shown to resemble each other very closely although differing somewhat in molecular weight. Component A_3 has several times the antibiotic potency of A_1, but both are equally effective as inhibitors of the electron transport system of mammalian tissue. Blastmycin and virosin are mixtures of certain antimycin components.

Although antimycin A[3] was isolated[4] in apparently homogeneous form in 1949, later work revealed that the crystalline antibiotic was in fact a complex of several active components.[5,6] Lockwood, *et al.*,[6] demonstrated by paper chromatography that a sample recrystallized to constant melting point (148–149.5°) contained at least 4 different active substances and designated these as antimycins A_1, A_2, A_3 and A_4, respectively, in the order of increasing R_f values.

Efforts to separate the individual components by chromatographic and other methods on a scale sufficient for chemical characterization met with little success.[5,7] Recently, however, larger supplies of antimycin have become available[8,9] and it has now been possible to effect separation of gram quantities of the antimycin A complex by countercurrent distribution. The components were thus individually obtained as chromatographically homogeneous crystalline solids, except for antimycin A_4, the least abundant component, which although apparently homogeneous could not be induced to crystallize.

The present report describes the separation, analysis and properties of the individual antimycin A components. Two other antibiotics, blastmycin[10] and virosin[11] were also examined and found to be identical with certain combinations of antimycin A components.

Experimental

Equipment.—The countercurrent distribution apparatus used was a 200 chamber robot-driven instrument.[12] Twenty ml. of solvent mixture was used in each chamber, *i.e.*, 10 ml. of each liquid phase.

Materials.—Antimycin A-35 (m.p. 139–140°) was prepared by Tener, *et al.*,[13] and antimycin A-102 (m.p. 148–149.5°) by Lockwood *et al.*[6] The antimycin A sample from Hoffman–LaRoche was a preparation of Burger, Teitel and Grundberg.[14] The antimycin A preparations used in this work for all countercurrent distribution studies were obtained from Y. Harada, Kyowa Fermentation Industry Co., Tokyo, Japan, and melted at 138–139°. Blastmycin (m.p. 166–167°) was prepared by Watanabe, *et al.*,[10] and virosin (m.p. 132–134°) by Sakagami, *et al.*,[11] both of Tokyo University, Japan.[15]

Assays of antibiotic activity were carried out as previously described.[4,6] Samples for elemental analysis were dried over P_2O_5 at 70–80° and 0.1 mm. for 12–16 hours.

Solvent Systems for Countercurrent Distribution.—The solvent system methanol:water:carbon tetrachloride:Skellysolve B[16] (87:13:80:20 by volume) was prepared by shaking the pure solvents together for 30 min. or more at room temperature and separating the phases. The partition coefficient[17] of the antimycin complex in this system was 1.04, the solubility in the upper phase being approximately 80 mg./ml.[18]

The above solvent system was selected after a systematic study of 11 different solvent mixtures. Each contained 3 or 4 of the following: water, methanol, ethanol, acetone, benzene, carbon tetrachloride, Skellysolve B. The desired solvents were equilibrated in such proportions as to give two phases of approximately equal volume. Distribution coefficients ranging from 0.10 to 2.60 were observed.[7] In addition to the system finally used, another consisting of ethanol:water:benzene:Skellysolve B (85:15:105:95 by volume) also gave a coefficient very close to one. However, a 200 transfer distribution carried out in this system showed no appreciable separation of the antimycin A components, but gave a distribution curve so nearly symmetrical that it could have been regarded as good evidence that the test sample was a single homogeneous substance.

Countercurrent Distribution and Isolation of Individual Components.—The distribution curve shown in Fig. 1 resulted when 3 g. of antimycin A complex was subjected to 800 transfers. Recycling was carried out after the initial 200 transfers in order to complete the distribution. Data for plotting the curve in Fig. 1 were obtained by determining the total dry weight (combined upper and lower phases) in every seventh tube of the series.

All the fractions under both the A_1 and A_3 peaks of two similar runs were then pooled, the solvents removed and the solid residue crystallized from ethyl acetate:Skellysolve B. In this manner, there was obtained 2.3 g. of a mixture of antimycin A_1 and A_3 which contained much less of the other components than the original material. An 800 transfer redistribution of this sample gave the result shown in Fig. 2,

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(2) Presented in part before the Division of Agricultural and Food Chemistry, 133rd National Meeting, American Chemical Society, San Francisco, April, 1958, abstracts p. 8a. Based on the Ph.D. Dissertation of When-chih Liu, 1958.

(3) F. M. Strong, "Topics in Microbial Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1958, pp. 1–43.

(4) B. R. Dunshee, C. Leben, G. W. Keitt and F. M. Strong, THIS JOURNAL, **71**, 2436 (1949).

(5) H. G. Schneider, G. M. Tener and F. M. Strong, *Arch. Biochem. Biophys.*, **37**, 147 (1951).

(6) J. L. Lockwood, C. Leben and G. W. Keitt, *Phytopathology*, **44**, 438 (1954).

(7) Wen-chih Liu, Ph.D. Thesis, University of Wisconsin, 1958.

(8) K. Nakayama, F. Okamoto and Y. Harada, *J. Antibiotics (Japan)*, **A9**, 63 (1956).

(9) Y. Harada, private communication.

(10) K. Watanabe, T. Tanaka, K. Fukuhara, N. Miyairi, H. Yonehara and H. Umezawa, *J. Antibiotics (Japan)*, **A10**, 39 (1957).

(11) Y. Sakagami, S. Takeuchi, H. Yonehara, H. Sakai and M. Takashima, *ibid.*, **9**, 1 (1956).

(12) Manufactured by H. O. Post, Scientific Instrument Company, Maspeth, N. Y. Permission to use this equipment in the School of Pharmacy, and the advice and assistance of Professor H. Mitchner in operating it are gratefully acknowledged.

(13) G. M. Tener, F. M. Bumpus, B. R. Dunshee and F. M. Strong, THIS JOURNAL, **75**, 1100 (1953).

(14) J. Burger, private communication.

(15) We wish to thank Dr. H. Yonehara for supplying samples of these two antibiotics.

(16) Purified petroleum ether, b.p. 60–71°, Skelly Oil Co.

(17) Defined as the concentration in the upper phase divided by that in the lower case.

(18) The solubility of antimycin in carbon tetrachloride has recently been observed to be at least 300 mg. per ml.; much higher than previously supposed (J. P. Dickie, unpublished experiments).

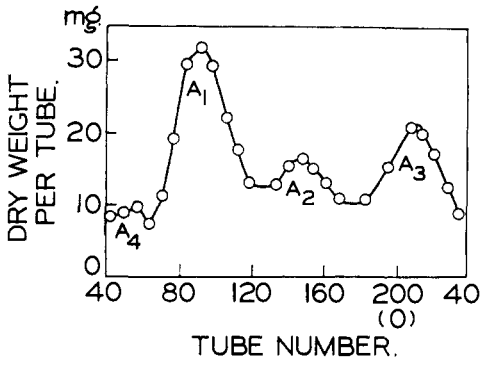

Fig. 1.—Countercurrent distribution of antimycin A complex (200 tubes, 800 transfers); weight of sample 3 g. (in 3 tubes).

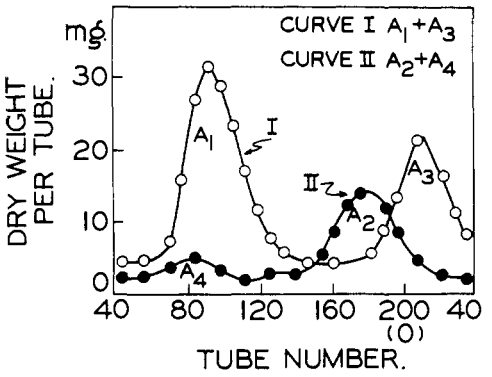

Fig. 2.—Countercurrent redistribution of antimycin A_1 + A_3 (curve I) and A_2 + A_4 (curve II) (200 tubes, 800 transfers). Weight of samples A_1 + A_3 2.3 g. (in 4 tubes). A_2 + A_4 1.0 g. (in 1 tube).

curve I. From the total estimated dry weight (approximately 780 mg.) in tubes 85–115 which were shown by paper chromatography to contain only antimycin A_1, a yield of 350 mg. of pure A_1 was obtained. This was achieved by concentration of the pooled fractions under reduced pressure and recrystallization of the residue from ethyl acetate: Skellysolve B to a constant m.p. of 149–150°, $[\alpha]^{26}D$ +76° (1% in chloroform).

Anal. Calcd. for $C_{28}H_{40}O_9N_2$: C, 61.30; H, 7.35; N, 511; mol. wt., 548.62. Found: C, 61.33, 61.30; H, 7.29, 7.34; N, 5.05, 5.37; mol. wt. (Rast), 527; neut. equiv. (in ethanol), 502.

Similarly, from tubes 195–34 (Fig. 2, curve I), which contained an estimated total weight of approximately 660 mg. of antimycin A_3, 350 mg. of pure A_3, m.p. 170.5–171.5°, $[\alpha]^{26}D$ +64.3° (1% in chloroform) was obtained.

Anal. Calcd. for $C_{26}H_{36}O_9N_2$: C, 59.99; H, 6.97; N, 5.38; mol. wt., 520.56. Found: C, 59.67; H, 7.06; N, 5.21, 5.43; mol. wt., (Rast) 513; neut. equiv. (in ethanol), 485.

An exactly analogous procedure was followed for isolation of components A_2 and A_4. The fractions under the appropriate peaks in the first distribution (Fig. 1) and in a second similar run were pooled, evaporated and crystallized to give 0.98 g. of a mixture of A_2 and A_4 relatively free of the other components. An 800 transfer redistribution of this preparation gave the result shown in Fig. 2, curve II. When the fractions from tubes 150–5 under peak A_2, containing a total dry weight of approximately 550 mg., were combined, evaporated and the residue crystallized from ethyl acetate: Skellysolve B, well-formed elongated crystals appeared. After two recrystallizations from the same solvent mixture, the m.p. was constant at 147–148°, and the product (110 mg.) was designated antimycin A_{2a}. Mixed with A_1, the m.p. was 143–149°.

Anal. Calcd. for $C_{26}H_{36}O_9N_2$: C, 59.99; H, 6.97; N, 5.38; mol. wt., 520.56. Found (on separate preparations): C, 60.30, 61.01; H, 6.66, 7.00; N, 5.57, 5.59; mol. wt. (Rast), 478; neut. equiv. (in ethanol), 481; ash, 3.40, nil.

When the mother liquor from A_{2a} was concentrated, a cotton-like mass of crystals formed, which after recrystallization reached a constant m.p. of 168°. About 30 mg. of this product, designated antimycin A_{2b}, was obtained. Analyses were variable but were close enough to those of A_{2a}, to suggest that the two fractions were isomeric. More extensive purification was not attempted.

Attempts to crystallize antimycin A_4 from tubes 75–105 of the redistribution (Fig. 2, curve II) were unsuccessful.

Paper Chromatography.—A solvent system consisting of water:ethanol:acetone (7:2:1 by volume) was used throughout this work for examining various preparations of the antimycin A complex, for detecting components in the countercurrent distribution fractions and for verifying the purity of isolated individual components. The antimycin components were well resolved in this system, except for A_4 which traveled only slightly faster than A_3 and in the bioautography sometimes was overshadowed by the more abundant and biologically more active A_3. A few paper chromatograms also were developed with the system water:absolute ethanol:glacial acetic acid (62:33:5 by volume) used by Lockwood, *et al.*[6] Since the R_f values found increased in the order A_1, A_{2a}, A_{2b}, A_3, A_4, the designations of the individual components as used in the present paper correspond to those of Lockwood, *et al.*[6]

Eaton-Dikeman No. 613 filter paper strips of 1/2 inch width were cut into 45 cm. lengths and holes were punched 1 inch from each end with a paper punch. Each strip was spotted with approximately 0.1–1.0 μg. of the sample[19] at a point 2 inches from one end, and glass rods were inserted through the holes so as to maintain the strips in a position parallel to each other and about 1.5 inches apart. The entire assembly was then suspended with the sample spots downward in a sealed cylindrical chromatographic jar (61 cm. high × 29 cm. in diameter), the atmosphere of which had been previously saturated with the developing solvent for at least 1 hr. After equilibration for 4 additional hours the assembly of paper strips was lowered carefully into the solvent, keeping the line of the sample spots parallel to the surface of the solvent. Development was allowed to proceed by upward flow of the solvent until the solvent front had traveled approximately 39–41 cm. (21–24 hr.). The temperature in the chromatographic room was 24–26° At the termination of the development, the strips were removed from the jar and dried in a previously steamed inoculation room in order to minimize microbial contamination.

A microbioautographic technique, essentially that of Lockwood, *et al.*,[6] was used to detect the antibiotic on the paper strips. Results are shown in Figs. 3 and 4.

Results and Discussion

The evidence presented above demonstrates that at least four active substances were separated from a particular antimycin A prepration and that three of them were obtained in substantially pure form. The proportions of the individual components in the original sample, as roughly estimated from the countercurrent distribution results, are listed in Table I together with their relative biological potencies against *Glomerella cingulata*. The major component from the standpoint of weight was A_1 whereas the most active component was A_3. The latter made up only 20% of the weight but accounted for some 60% of the total activity. It was about 5 times as active as A_1.

Components A_{2a} and A_{2b} were intermediate in biological activity and together accounted for about one-fifth of the original weight. Whether these were distinctly different substances or per-

(19) The amount of sample applied to the strips had to be carefully controlled because the separation achieved was very sensitive to overloading of the chromatogram. It was found that after a little practice the proper amount of sample could be conveniently estimated by observing the strips under ultraviolet light and noting the intensity of the fluorescence produced by the antimycin present on the paper.

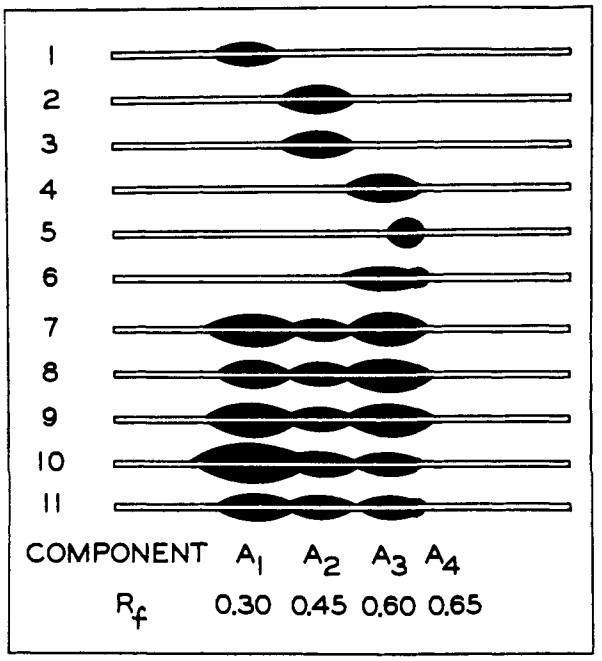

Fig. 3.—Schematic diagram showing paper chromatograms of antimycin A complex and separated subcomponents. Solvent system water:ethanol:acetone (7:2:1). Temperature 24–26°, time of development 21–24 hours, R_f values averages of seven runs. Samples: 1, component A_1; 2, component A_{2a}; 3, component A_{2b}; 4, component A_3; 5, component A_4; 6, blastmycin; 7, antimycin A-35; 8, antimycin A-102; 9, antimycin A complex (Harada); 10, antimycin A complex (Hoffman–LaRoche); 11, virosin.

haps polymorphic forms of the same substance was not certain, but they were distinct from both A_1 and A_3. The minor component A_4 was definitely active, but since it could not be obtained in pure form, no estimate of its activity is included in Table I. It probably contributed no more than 5% to the total activity of the original sample.

TABLE I

WEIGHT AND ANTIBIOTIC POTENCY OF SEPARATED COMPONENTS OF ANTIMYCIN A COMPLEX[a]

Component	% of total wt.	Potency[b] units/μg.	% of total activity[c]
Complex	100	0.9	100
A_1	40	0.5	20
A_{2a}	20	1.1	20
A_{2b}		0.8	
A_3	20	2.6	60
A_4	10	..	..

[a] Preparation from Y. Harada, Kyowa Fermentation Industry Co., Tokyo, Japan. [b] Antimycin A-35 used a standard and assigned a potency of 1 unit per μg. [c] Precision of bioassay estimated to be approximately ±5–10%.

Following the completion of our separation studies, Harada, *et al.*,[20] reported a similar separation of the antimycin A complex by countercurrent distribution. Antimycins A_1 and A_3 were obtained in crystalline form and were reported to melt at 147–148° and 167–168°, respectively. The physical and chemical properties of A_1 and A_3

(20) Y. Harada, K. Uzu and M. Asai, *J. Antibiotics (Japan)*, **A11**, 32 (1958).

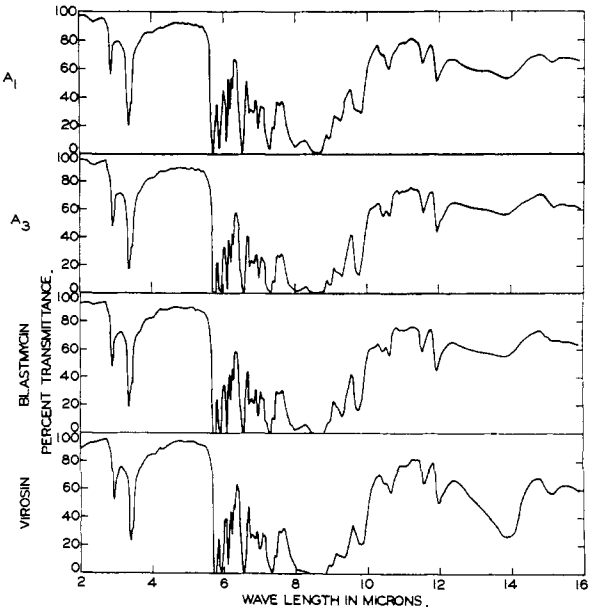

Fig. 4.—Comparison of infrared spectra of antimycin A_1, A_3, blastmycin and virosin (in chloroform).

reported by these authors corresponded well with those found in the present study. With *Torula utilis* as the test organism they reported A_3 to be 13 times as active as A_1.

In view of the marked differences in the antibiotic potencies of the two chief components, it was of interest to compare their inhibitory power at the enzymatic level. When tested in the enzymatic oxidation system of rat kidney mitrochondria as described by DeLuca, *et al.*,[21] with glutamate as substrate, the results summarized in Table II were obtained.[22] In this test A_1, A_3, and the entire

TABLE II

RELATIVE EFFECTIVENESS OF ANTIMYCIN A COMPONENTS IN ELECTRON TRANSPORT INHIBITION

Inhibitor		
Component	Amount per flask,[a] μg.	Oxygen uptake, QO_2 (N)[b]
Control	..	583
A complex	0.25	0
	.125	514
A_1	.25	0
	.125	506
	.05	566
A_3	.25	0
	.125	523
	.05	567

[a] Total volume of 3.0 ml. per flask. [b] Microliters of oxygen per mg. mitochondrial nitrogen per hour.

complex were all equally effective. The differences in antibiotic potency must therefore be caused by factors which come into play before the inhibitor actually reaches the susceptible enzymatic site.

The behavior of the isolated components on paper strip chromatograms is compared with that of the original complex and with blastmycin in

(21) H. F. DeLuca, F. C. Gran, H. Steenbock and S. Reiser, *J. Biol. Chem.*, **228**, 469 (1957).

(22) The assistance of Dr. Hector De Luca in carrying out these enzyme inhibition tests is gratefully acknowledged.

Fig. 3. This is a schematic representation based on average R_f values appearing in 7 different chromatographic runs. It is evident that the various antimycin A components were obtained in chromatographically homogeneous form. Further, from these results blastmycin appeared to consist mainly of antimycin A_3 with minor contamination by the A_4 component.

Paper chromatograms of the various antimycin complex preparations are also given in Fig. 3. It is apparent that the four antimycin preparations all contained at least the A_1, A_2 and A_3 components, although in somewhat varying proportions. Virosin was similar in composition but also contained a significant proportion of antimycin A_4. The other preparations may also have contained small amounts of A_4 as it certainly was present in the Harada sample but yet was not detected by the chromatogram.

Infrared spectra of various preparations as shown in Fig. 4 reveal extremely close similarity both of the individual components A_1 and A_3 to each other and to blastmycin and virosin. Antimycin A_2 and the antimycin complex also gave essentially the same infrared spectrum. The differences are too slight to be of much aid in establishing differences in the chemical structure. Evidently, the components differ from each other only in the alkyl side chains of the neutral portion of the molecule.[3] Such a relationship is also suggested by the molecular formulas of A_1, A_2 and A_3 which differ among themselves only by the equivalent of one to three methylene groups. The ultraviolet spectra of the individual components also were essentially identical.

The infrared tracing of blastmycin offers further evidence of its very close similarity with antimycin; furthermore, its melting point was close to that of antimycin A_3 and the mixed melting point showed no depression. These data taken together with the above paper chromatographic results establish quite conclusively that the major component of blastmycin is identical with antimycin A_3 and indicate that the minor component very probably is antimycin A_4.

All three of the isolated antimycin A components showed neutral equivalents appreciably lower than theory for their most probable molecular formulas. This discrepancy may be attributable to partial cleavage of the alkali-sensitive bond of the antimycin A structure.[13] The investigation of these and other aspects of the chemistry of antimycin A is being continued.

MADISON, WISCONSIN

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Small-ring Compounds. XXIII. The Nature of the Intermediates in Carbonium Ion-type Interconversion Reactions of Cyclopropylcarbinyl, Cyclobutyl and Allylcarbinyl Derivatives[1a]

BY ROBERT H. MAZUR, WILLIAM N. WHITE,[1b] DOROTHY A. SEMENOW, C. C. LEE, MARC S. SILVER[1c] AND JOHN D. ROBERTS[1d]

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Investigation of the extent of isotope-position rearrangement in carbonium ion-type reactions of ^{14}C-labeled cyclopropylcarbinyl derivatives has revealed that the three methylene groups of the starting material achieve a striking degree of equivalence between reactants and products. These results, taken in conjunction with the abnormally large solvolytic reactivities of cyclopropylcarbinyl and cyclobutyl halides and sulfonate esters, can best be accounted for by assuming rapid but not instantaneous equilibration of three isomeric non-classical unsymmetrical "bicyclobutonium" ion intermediates.

Considerable interest attends the question of how best to formulate the intermediate or intermediates involved in carbonium ion-type interconversion reactions of cyclopropylcarbinyl, cyclobutyl and allylcarbinyl derivatives.[2] The abnormally large solvolytic reactivities of cyclopropylcarbinyl and cyclobutyl halides[2] and sulfonate esters[3-5] are characteristic of reactions for which we believe that *non-classical* cationic intermediates have been well established.[6] Detailed information as to the structures of the intermediates was sought in the present research by measurement of the extent of isotope-position rearrangement in the reactions of cyclopropylcarbinylamine-α-^{14}C with nitrous acid[7] and cyclopropylcarbinol-α-^{14}C with Lucas reagent.

Synthetic and Degradative Methods

Cyclopropylcarbinylamine-α-^{14}C was obtained by lithium aluminum hydride reduction of the amide from cyclopropanecarboxylic-1-^{14}C acid. This acid was prepared by carbonation of the Gri-

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(2) J. D. Roberts and R. H. Mazur, THIS JOURNAL, **73**, 2509 (1951).

(3) J. D. Roberts and V. C. Chambers, *ibid.*, **73**, 5034 (1951).

(4) C. G. Bergstrom and S. Siegel, *ibid.*, **74**, 145 (1952).

(5) R. G. Pearson and S. H. Langer, *ibid.*, **75**, 1065 (1953).

(6) As leading references see (a) S. Winstein, B. K. Morse, E. Grunwald, H. W. Jones, J. Corse, D. Trifan and H. Marshall, *ibid.*, **74**, 1127 (1952); (b) J. D. Roberts, C. C. Lee and W. H. Saunders, Jr., *ibid.*, **76**, 4501 (1954).

(7) For a preliminary communication concerning this work, see J. D. Roberts and R. H. Mazur, *ibid.*, **73**, 3542 (1951).