Runs 59 and 61.—Both runs resembled run 31 except that in 59, 0.007 g. of benzoyl peroxide was added, and in run 69, 0.010 g. of benzoquinone was employed.

Attempted Reduction of Cyclohexene.—A mixture of 25 ml. of 0.13 *M* aqueous potassium hydroxide, 0.800 g. of racemic II and 2 ml. of cyclohexene was heated to 100 ± 2° for 2 hr. in a 150-ml. heavy-walled pressure bottle. The product was isolated as in run 31 except that special care was taken to avoid loss of cyclohexene, and was submitted to gas chromatographic analysis on a 30% pimelonitrile on firebrick column at 108°. No trace of cyclohexane was observed.

Control Runs.—In previous experiments, optically active 2-phenylbutane was found not to racemize at 240° for 24 hr. in a 1 *M* solution of potassium diethylene glycoxide in diethylene glycol.[13b] This experiment controls all runs made in primary alcohols or water as solvents. Other control experiments[3a] were carried out in dioxane at 150° and in *tert*-butyl alcohol at 102° in the presence of potassium *tert*-butoxide without racemization of active III. In dimethyl sulfoxide which was 0.11 *M* in potassium *tert*-butoxide, active 2-phenylbutane racemized completely at 110° for 28 hr.[3a] A control run was conducted under the conditions of run 54. After 90 hr. at 52.5 ± 0.1°, (+)-III was 23% racemized.

[CONTRIBUTION FROM THE CALIFORNIA CORPORATION FOR BIOCHEMICAL RESEARCH, LOS ANGELES, 63, CALIF. AND CONTRIBUTION NO. 1 FROM SYNTEX INSTITUTE FOR MOLECULAR BIOLOGY, PALO ALTO, CALIF.]

The Synthesis of Orotidine-5′ Phosphate

BY J. G. MOFFATT[1]

RECEIVED SEPTEMBER 4, 1962

The reaction of the silver salt of orotidine with methyl iodide gives orotidine methyl ester which is converted to its isopropylidine derivative and phosphorylated with the cyanoethyl phosphate–dicyclohexylcarbodiimide reagent. After removal of protecting groups the resulting orotidine-5′ phosphate is both chemically and enzymatically identical with the natural material. Attempted esterification of the nucleoside by a variety of other methods gave products in which N_3 was also methylated. Several derivatives of these N_3 methylated nucleosides are described and the rates of alkaline hydrolysis of the methyl ester groups in both the N_3-methylated and unmethylated series are described.

During the past ten years the biosynthetic pathways leading to both the purine and pyrimidine ribonucleotides have been clearly defined.[2] Information is also accumulating as to the steps involved in the conversion of ribonucleotides into the corresponding deoxyribonucleotides.[3] The involvement of orotic acid (XI) as a precursor of the pyrimidine ribonucleosides was demonstrated by Hurlbert and Potter[4] and it remained for Lieberman, Kornberg and Simms[5] to clarify this key process. The latter workers[5] showed that in yeast the first intact pyrimidine nucleoside derivative in the biosynthetic pathway was orotidine-5′ phosphate (IV), formed by the condensation of orotic acid (XI) and 5-phosphoribosyl α-1-pyrophosphate (PRPP),[6] and that IV was subsequently decarboxylated to uridine-5′ phosphate by the enzyme orotidylate decarboxylase. The same pathway also obtains in mammalian systems.[7,8]

The nucleoside orotidine (I) was originally isolated from mutants of *Neurospra crassa* which possess relatively low levels of orotidylate decarboxylase.[9] More recently it has been demonstrated in several laboratories[10-12] that this enzyme is inhibited by the nucleotide analog 6-azauridine-5′ phosphate or by large amounts of uridine-5′ phosphate[7] and that accordingly there is a build-up of orotidylic acid and its dephosphorylated derivative orotidine. We have recently had occasion to isolate relatively large amounts of orotidine from the urine of tumor-bearing subjects who have received azauridine therapy. The availability of this pure material has prompted a study of the chemistry of orotidine derivatives and in particular the first synthesis of the key biological intermediate orotidine-5′ phosphate (IV). It is of interest to note that with the exception of its naturally occurring phosphate (IV) no derivatives of orotidine have previously been described.

The most obvious key intermediate in a specific synthesis of orotidine-5′ phosphate (IV) is 2′,3′-O-isopropylidineorotidine methyl ester (III) and our first goal was the preparation of this compound. Since some difficulties were to be expected due to spontaneous hydrolysis of the isopropylidine group in compounds bearing a free carboxyl group (as in VII), it was decided first to prepare orotidine methyl ester (II) and then form the isopropylidine derivative III. The formation of II without complication, however, proved to be quite difficult. This is not unexpected in view of the confusion in the earlier literature regarding the methylation of orotic acid itself.[13] It was not possible, for example, to effect selective methylation of the free carboxyl group using diazomethane since N_3 of the pyrimidine ring was concurrently alkylated.[14] The resulting crystalline N_3-methylorotidine methyl ester (V) was isolated by chromatography on silicic acid. It was shown that the compound was an N-methyl rather than an O-methyl derivative by its stability to acid[14a] and by its hydrolysis with mild alkali to the potentially interesting antimetabolite N_3-methylorotidine (VIII) which was isolated as its crystalline cyclohexylammonium salt. It is interesting to note that some cleavage of the glycosidic bond apparently resulted during the methylation reaction since a small amount of crystalline material that was neutral and periodate negative was isolated from the reaction. This compound had the chromatographic behavior and elemental analysis expected for N_3-methylorotic acid methyl ester (X).

The reaction of crude N_3-methylorotidine methyl ester (V) with acetone containing 2,2-dimethoxypropane

(1) Syntex Institute for Molecular Biology, 3221 Porter Drive, Palo Alto, Calif.

(2) For recent reviews, see J. M. Buchanan and S. C. Hartman, in *Advan. in Enzymol.*, **21**, 199 (1959), and P. Reichard, *ibid.*, **21**, 263 (1959).

(3) See, *e.g.*, P. Reichard, *J. Biol. Chem.*, **236**, 2511 (1961), and preceding papers; S. S. Cohen, H. D. Barner and J. Lichtenstein, *ibid.*, **236**, 1448 (1961).

(4) R. B. Hurlbert and V. R. Potter, *ibid.*, **209**, 1 (1954).

(5) I. Lieberman, A. Kornberg and E. S. Simms, *ibid.*, **215**, 403 (1955).

(6) A. Kornberg, I. Lieberman and E. S. Simms, *ibid.*, **215**, 389 (1955).

(7) D. G. R. Blair, J. E. Stone and V. R. Potter, *ibid.*, **235**, 2379 (1960).

(8) W. A. Creasey and R. E. Handschumacher, *ibid.*, **236**, 2058 (1961).

(9) A. M. Michelson, W. Drell and H. K. Mitchell, *Proc. Natl. Acad. Sci. U.S.*, **37**, 396 (1951).

(10) R. E. Handschumacher, *J. Biol. Chem.*, **235**, 2917 (1960).

(11) V. Habermann, *Biochem. Biophys. Acta*, **43**, 137 (1960).

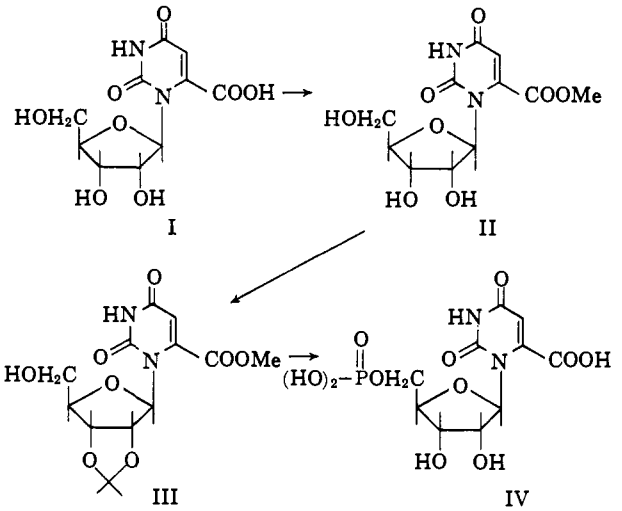
(12) R. E. Handschumacher, *Nature*, **182**, 1090 (1958).

(13) For a clarification of this problem, see J. J. Fox, N. Yung and I. Wempen, *Biochem. Biophys. Acta*, **28**, 295 (1957).

(14) Numerous examples of alkylations at N_3 of pyrimidine nucleosides by diazomethane are known, *e.g.*, (a) H. T. Miles, *J. Am. Chem. Soc.*, **79**, 2565 (1957); (b) W. Szer and D. Shugar, *Acta Biochem. Polon.*, **7**, 491 (1960). Since the present work was completed it has been found that Dr. R. E. Handshumacher (personal communication) has obtained crystalline II by the use of limiting amounts of diazomethane. A sample of the resulting material kindly provided by Dr. Handschumacher was chromatographically identical with the non-crystalline product obtained from the silver salt of orotidine and methyl iodide as described later.

and dried Dowex 50 (H^+) resin rapidly gave N_3-methyl-2′,3′-O-isopropylidineorotidine methyl ester (VI) which was isolated in crystalline form by chromatography on neutral alumina. During chromatography, however, considerable hydrolysis of the methyl ester resulted since elution of the column with water removed almost a 40% yield of N_3-methyl-2′,3′-O-isopropylidineorotidine (VII) which was isolated as its crystalline cyclohexylammonium salt.[15]

The methylation of orotidine by methanolic hydrogen chloride was accompanied by methanolysis of the glycosidic bond and gave orotic acid methyl ester (IV)[13,16] and methyl riboside in high yield. It was thought that, in view of the highly selective esterification of nucleotides by methanol and dicyclohexylcarbodiimide,[17] a similar procedure applied to orotidine would give exclusively II. Treatment of an anhydrous methanol solution of free acid orotidine with dicyclohexylcarbodiimide, however, gave both the desired product II and its N_3-methylated derivative V together with traces of other compounds. The reaction was accordingly not studied further.



A relatively successful preparation of II was achieved through the heterogeneous reaction of the silver salt of orotidine with methyl iodide in methanol. This is similar to the successful syntheses of orotic acid methyl ester previously described by Biscaro and Belloni[18] and by Bachstez[19] which were recently clarified by Fox, *et al.*[13]

Stirring of an aqueous solution of free acid orotidine (which, incidentally, has not previously been described in the crystalline form; see Experimental) with 0.5 molar equivalent of finely divided silver oxide resulted in a quite rapid rise of the *p*H from an initial value of 1 to roughly 7.5 leaving a clear solution. Storage of this solution overnight, however, resulted in the separation of a voluminous gray precipitate and a drop of the *p*H to 3.5. The addition of a further 0.5 mole of silver oxide then caused the *p*H to rise to 7.5 once again, leaving the insoluble precipitate. This is interpreted as meaning that the soluble silver salt of the carboxylate ion disproportionates to give a water-insoluble disilver salt in which the second silver ion is associated with the enolic oxygen at C_4 of the pyrimidine ring. Frequently, the soluble silver salt is never obtained and the gray precipitate forms immediately. Also in this case a full molar proportion of silver oxide is required to bring the *p*H to neutrality. Some support for this idea comes from observations on the relative ease of enolization of the C_4-keto group in orotidine derivatives. Thus while uridine has a very low mobility on paper electrophoresis at *p*H 7.6 (0.05 *M* ammonium bicarbonate) and N_3-methylorotidine methyl ester (V), which is unable to enolize, has no mobility, orotidine methyl ester (II) is found to have the mobility expected of a compound bearing a single negative charge. These results would lead one to suspect that the pK_2 of orotidine[13] is lower than that of uridine. In point of fact, potentiometric titration of crystalline free acid orotidine showed pK's of <3 (carboxyl group) and 8.8,[20] while uridine is normally given a value of 9.2.

Treatment of the crude dried silver salt of orotidine as described above with methyl iodide gave an excellent yield of crude orotidine methyl ester (II) which contained only trace amounts of the corresponding N_3-methyl ester V. This material was not obtained in a crystalline form[14] but was directly treated in anhydrous acetone with dried Dowex 50 (H^+) resin.[21] A small amount of 2,2-dimethoxypropane was also added as a water scavenger in order to draw the equilibrium toward the isopropylidine compound.[22] The resulting 2′,3′-O-isopropylidine orotidine methyl ester (III) was obtained directly in 62% yield (from orotidine) by crystallization from the crude reaction mixture. The actual yield was higher than this but the presence of minor side products prevented the crystallization of a second crop. Chromatography of the mother liquors on silicic acid did permit the isolation of some further product.

Phosphorylation of this blocked intermediate (III) was readily accomplished by the elegant method of Tener[23] using 2-cyanoethyl phosphate and dicyclohexylcarbodiimide in anhydrous pyridine. After removal of the cyanoethyl and methyl esters and destruction of excess cyanoethyl phosphate with 1 *N* lithium hydroxide at 100° for one hour, the isopropylidine group was hydrolyzed by heating the free acid solution at 100° for thirty minutes. The resulting material contained 95% orotidine-5′ phosphate, 4% free orotidine and 1% of what appears to be an orotidine diphosphate. The desired product IV was readily purified from these minor impurities by ion exchange chromatography. The final yield of pure product was 50% and it is uncertain at what point the losses occurred since at several stages during the reaction the desired products were virtually the only ultraviolet-absorbing compounds present as determined by paper chromatography. A similar situation has been observed during several other phosphorylations with this reagent.[23]

The orotidine-5′ phosphate obtained as above was homogeneous under a variety of paper chromatographic and electrophoretic conditions and gave excellent elemental analyses. It was periodate positive, thus confirming its structure as a 5′-phosphate. Interestingly enough, it was completely resistant to the action of the 5′-nucleotidase present in crude *Crotalus adaman-*

(15) No doubt the yield of VI could be greatly improved by using silicic acid rather than alumina for the chromatography.

(16) G. D. Daves, F. Baiocchi, R. K. Robins and C. C. Cheng, *J. Org. Chem.*, **26**, 2755 (1961).

(17) M. Smith, J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6204 (1958).

(18) G. Biscaro and E. Belloni, *Ann. Soc. Chim. Milano*, **11**, 18, 71 (1905).

(19) M. Bachstez, *Chem. Ber.*, **63**, 1000 (1930); **64**, 2683 (1931).

(20) The kind assistance of Dr. B. H. J. Hofstee of the Palo Alto Medical Research Foundation in obtaining these data is gratefully acknowledged.

(21) The use of the ion exchange resin as the acid catalyst greatly facilitated the work-up of the reaction in view of the marked lability of the methyl ester toward alkaline hydrolysis. This method has been used successfully in this Laboratory for the preparation of several other isopropylidine nucleosides (see, *e.g.*, J. P. Verheyden and J. G. Moffatt, in preparation).

(22) Dimethoxypropane itself has recently been used for the preparation of isopropylidine compounds. See, *e.g.*, C. H. Robinson, L. E. Finckenor, R. Tiveria and E. P. Oliveto, *J. Org. Chem.*, **26**, 2863 (1961); M. Tanabe and B. Bigley, *J. Am. Chem. Soc.*, **83**, 757 (1961); A. Hampton, *ibid.*, **83**, 3640 (1961).

(23) G. M. Tener, *ibid.*, **83**, 159 (1961). Thanks are extended to Dr. Tener for many discussions on the use of this method prior to publication.

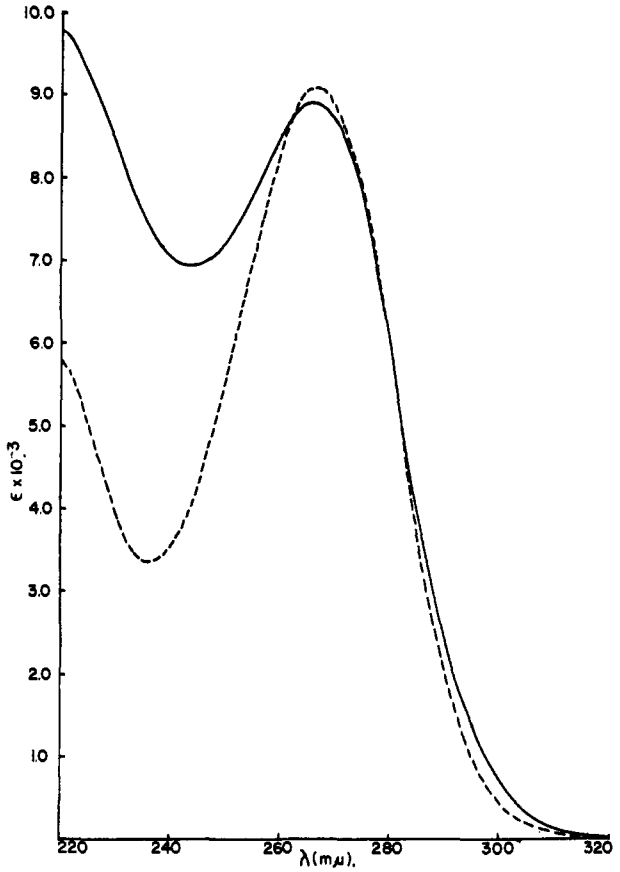


Fig. 1.—Ultraviolet spectra in methanol of: - - - - orotidine; ———, N_3-methylorotidine.

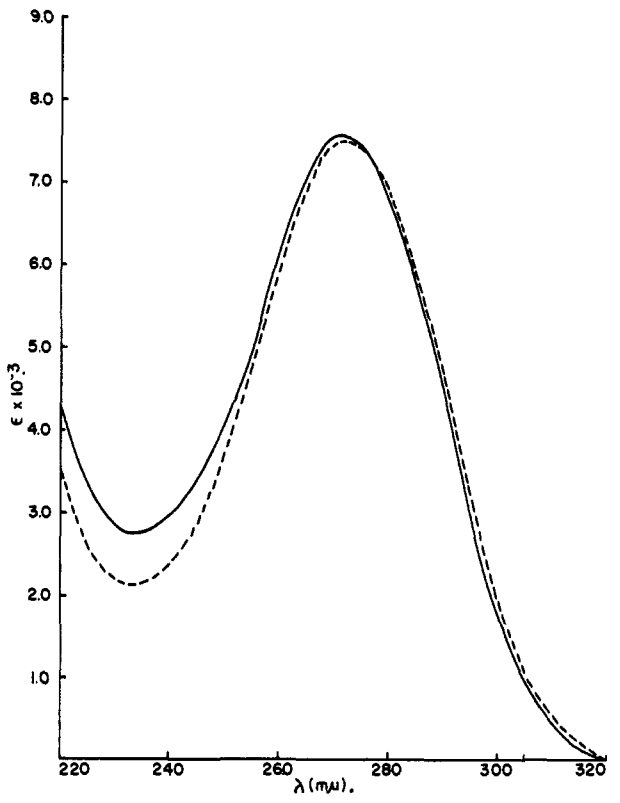


Fig. 2.—Ultraviolet spectra in methanol of: - - - -, 2′,3′-O-isopropylidineorotidine methyl ester; ———, N_3-methylorotidine methyl ester.

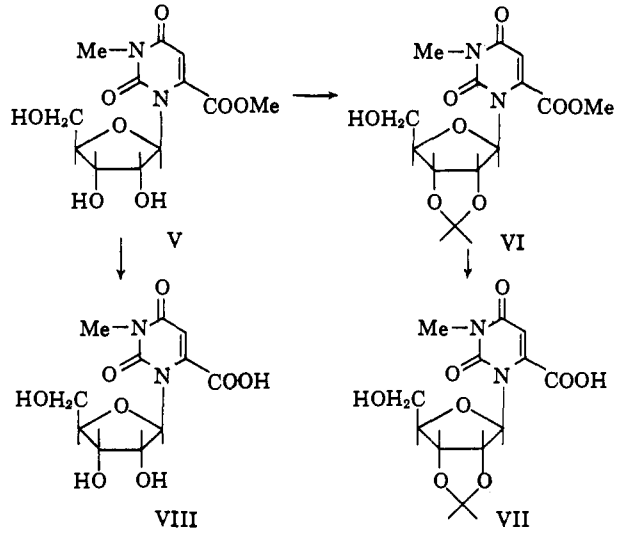
teus venom. It did not, however, inhibit the dephosphorylation of an equivalent amount of cytidine-5′ phosphate. This same behavior has been previously observed by Lieberman, *et al.*,[5] using bull semen 5′-nucleotidase. A recent study by Mizumo, *et al.*,[24] has also demonstrated that certain unusually substituted pyrimidine-5′ nucleotides (*e.g.*, 5-dimethylaminouridine-5′ phosphate and 4-deoxyuridine-5′ phosphate) are also resistant to the 5′-nucleotidase present in *Trimeresurus flavoviridis* (Japanese Habu) venom.

In view of the method of preparation and the properties of the final product, there can be no doubt that the above described an unequivocal synthesis of orotidine-5′ phosphate.

The final test of purity came as the result of an enzymatic assay of the synthetic material using the orotidylate decarboxylase system. This assay, which was very kindly performed by Dr. R. E. Handschumacher of Yale University, showed synthetic IV to be completely active in his system. It was, in fact, slightly more active than a purified preparation of the natural compound.

The phosphorylation of III was also attempted using dibenzyl phosphorochloridate as the phosphorylating agent. Phosphorylation proceeded well, but during hydrogenolysis of the benzyl groups using a commercial 10% palladium-on-carbon catalyst at room temperature the pyrimidine ring was also reduced giving, presumably 5,6-dihydroörotidine-5′ phosphate (XII) as the final product.

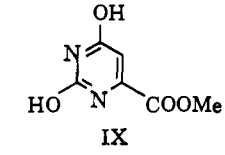
A small amount of the desired orotidine-5′ phosphate was also present after reduction, but the two products were not separated. In view of the successful phosphorylation using the 2-cyanoethyl phosphate method, no effort was made to determine whether or not the reduction of the pyrimidine ring could be avoided using other types of palladium catalysts. A similar reduction of the cytidine ring frequently accompanies hydrogenolysis of benzyl groups.



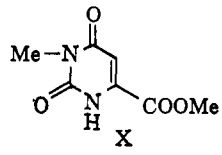
The ultraviolet spectra in methanol of orotidine compounds and their N_3-methyl analogs are grossly similar, although the shapes of the absorption curves vary slightly (Fig. 1–2). Attempts to differentiate spectrally between the two series of compounds at higher *p*H (Fig. 3) where the N_3-methyl derivatives are incapable of enolization led to the observation of a pronounced difference in hydrolysis rates of the methyl esters. These rates were readily determined by following the release of the free carboxyl group spectrally at

(24) Y. Mizuno, M. Ikehara, T. Veda, A. Nomura, E. Ohtsuka, F. Ishikawa and Y. Kanai, *Chem. and Pharm. Bull.*, **9**, 338 (1961).

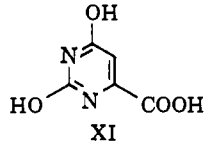
266 mμ. Good first-order kinetic plots were obtained with all compounds under comparable conditions (0.25 *N* methanolic sodium hydroxide at 23-25°). In general, the derivatives of N_3-methylorotidine methyl ester were found to be hydrolyzed much more rapidly (k = 0.018 sec.$^{-1}$ for V and 0.039 sec.$^{-1}$ for VI) than the corresponding compounds with a free N_3 (k = 3.3 × 10^{-4} sec.$^{-1}$ for III and 1.1 × 10^{-3} for II). The greater stability of the non-N-methylated compounds can doubtless be explained by the ready enolization of their C_4-carbonyl groups, the presence of the resulting anion then repressing nucleophilic attack at the carboxyl carbonyl. In the N_3-methyl compounds such enolization is, of course, impossible.



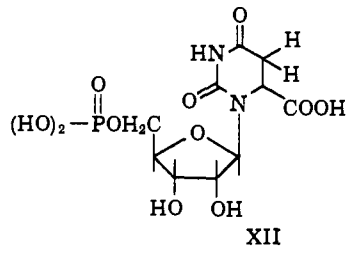
IX



X



XI



XII

In order to complete the characterization of the various compounds as fully as possible, several substances have been prepared purely as chromatographic standards and without isolation. For example, 2′,3′-isopropylidineorotidine was prepared from the corresponding, characterized, methyl ester by alkaline hydrolysis. This provided a chromatographically pure reference compound which was not subsequently isolated and purified. The chromatographic behavior of all compounds related to this work is compiled in Table I.

TABLE I

Compound	R_f in Solvent I	R_f in Solvent II
Orotidine	0.51	0.13
Orotidine methyl ester	.67	.42
2′,3′-O-Isopropylidineorotidine methyl ester	.80	.79
2′,3′-O-Isopropylidineorotidine	.73	.44
Orotidine-5′ phosphate	.41	.10
N_3-Methylorotidine	.65	.25
N_3-Methylorotidine methyl ester	.74	.60
N_3-Methyl-2′,3′-O-isopropylidineorotidine methyl ester	.84	.86
N_3-Methyl-2′,3′-O-isopropylidineorotidine	.81	.69
Orotic acid	.53	.26
Orotic acid methyl ester	.65	.59
N_3-Methylorotic acid methyl ester	.75	.76

Experimental[25]

General Methods.—Paper chromatography by the descending technique was done on Schleicher and Schuell 589 orange ribbon paper using the following systems: solvent I, ethyl alcohol–0.5 *M* ammonium acetate buffer, *p*H 3.8 (5:2); solvent II, *n*-butyl alcohol–acetic acid–water (5:2:3); solvent III, isobutyric acid–1 *N* NH_4OH–0.1 *M* tetrasodium ethylenediamine tetraacetic acid (60:100:1.6). Paper electrophoresis was done on the same paper impregnated with 0.05 *M* lithium acetate buffer, *p*H 5.0, or in some cases with 0.05 *M* ammonium bicarbonate, *p*H 7.5, or 0.05 *M* ammonium acetate buffer, *p*H 3.5; R_f's of all compounds described are given in Table I. All compounds were run simultaneously so that relative mobilities should be reproducible. Phosphorus-containing spots were located on paper chromatograms using the Hanes and Isherwood spray[26] followed by ultraviolet irradiation.[27] Phosphorus analysis was done by the method of King.[28] Ultraviolet spectra were recorded on a Cary model 15 spectrophotometer.

(25) Melting points are uncorrected and elemental analyses were done by Midwest Microlab, Inc., Indianapolis, Ind.

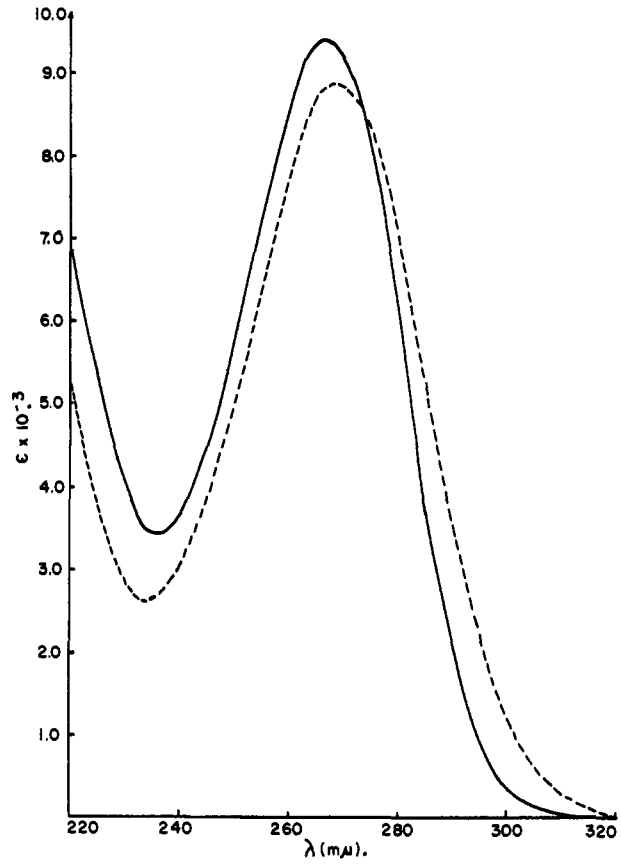


Fig. 3.—Ultraviolet spectra in 0.1 *N* methanolic sodium hydroxide of: ———, orotidine; – – – –, N_3-methylorotidine.

Orotidine Free Acid (I).—Crystalline cyclohexylammonium orotidine[29] (1 g.) was dissolved in water and passed through a column containing 5 ml. of Dowex 50 (H^+) resin. The acidic solution was evaporated to dryness, dissolved in hot methanol, and brought to turbidity with benzene. Free acid orotidine (0.55 g., 75%) separated as fine needles which slowly turned brown near 200° but failed to melt at 400°, probably indicating polymerization; λ_{max} (MeOH) 268 mμ, ϵ_{max} 8,900 (Fig. 1); λ_{max} (0.1 *N* HCl) 267 mμ, ϵ_{max} 9570; λ_{max} (0.1 *N* methanolic NaOH) 265 mμ, ϵ_{max} 8,960 (Fig. 3).

Anal. Calcd. for $C_{10}H_{12}N_2O_8$: C, 41.67; H, 4.19; N, 9.72. Found: C, 41.85; H, 4.22; N, 10.08.

Orotidine Methyl Ester (II).—An aqueous solution of the crystalline cyclohexylammonium salt of orotidine (11.6 g., 30 mmoles) was passed through a column containing 50 ml. of Dowex 50 (H^+) resin and the resin was then washed with water until the effluent was neutral (total volume 200 ml.). Finely powdered silver oxide (3.5 g., 15 mmoles) was added to the aqueous solution and the mixture was stirred to break up solid lumps. During this procedure, a gray solid separated and the *p*H rose to 3.5–4 and then remained unchanged.[30] A further 3.5 g. of silver oxide was added and the stirring continued until the *p*H rose to 7.5. The entire heterogeneous mixture was evaporated to dryness under reduced pressure and rendered anhydrous by three evaporations of a suspension of the residue in methyl alcohol–benzene (1:1). The final residue was dried on an oil pump, finely pulverized, and further dried *in vacuo* over phosphorus pentoxide. The resulting gray powder was suspended in a mixture of methyl alcohol (100 ml.) and methyl iodide (100 ml.) and vigorously shaken overnight in the dark. During this time

(26) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

(27) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).

(28) E. J. King, *Biochem. J.*, **26**, 292 (1932).

(29) Available from the California Corporation for Biochemical Research.

(30) In one experiment, a clear aqueous solution of *p*H 7.5 was quickly obtained using 0.5 equivalent of silver oxide. On storage overnight, however, the *p*H dropped to 4.0 and a heavy gray precipitate separated. See text.

the gray precipitate acquired a yellowish color. The mixture was filtered and the solids washed thoroughly with methyl alcohol. The combined filtrates were evaporated to dryness, dissolved in a small volume of methyl alcohol, and filtered through Celite to remove some finely divided yellow solids. Evaporation to dryness and evacuation then left crude orotidine methyl ester (6.0 g.) as a hygroscopic white froth. The insoluble salt mixture from the above process was dried *in vacuo*, finely pulverized, and retreated as above with methyl alcohol (50 ml.) and methyl iodide (50 ml.) to give a further 3.1. g. of the crude methyl ester. These materials could not be obtained crystalline and were shown by paper chromatography and electrophoresis to contain a very small amount of N-methylorotidine methyl ester. They were, however, directly used in the next step.

2',3'-O-Isopropylidineorotidine Methyl Ester (III).—Crude orotidine methyl ester (9.1 g.) was dried *in vacuo* over phosphorus pentoxide and suspended in dry acetone (100 ml.) containing 2,2-dimethoxypropane (5 ml.) and dried Dowex 50 (H^+) (2.0 g.). After being shaken at room temperature for 45 minutes the starting material had completely dissolved and the reaction was continued for a total of 3 hours. The slightly brown solution was quickly filtered and the resin washed with dry acetone. Evaporation of the filtrates *in vacuo* left the crude product as a pale tan froth which was dissolved in hot acetone (50 ml.) and treated with charcoal (0.5 g.). After filtration, hot cyclohexane was added to turbidity and the product allowed to crystallize, giving 7.6 g. of white needles of m.p. 156–160°. One further recrystallization raised the m.p. to 161–163° with a recovery of 6.3 g. (yield 62% from orotidine). Paper chromatography and electrophoresis of this product showed a trace contamination by isopropylidineorotidine, but the material was quite suitable for the next step. An analytical sample crystallized from isopropyl alcohol melted at 163–164° and was chromatographically homogeneous; λ_{max} (MeOH) 272 mμ, ϵ_{max} 7980.

Anal. Calcd. for $C_{14}H_{18}N_2O_8$: C, 49.10; H, 5.30; N, 8.18. Found: C, 49.45; H, 5.47; N, 8.04.

In an earlier experiment, the crude product (3.2 g.), following reaction of the silver salt with methyl iodide, was chromatographed on 75 g. of silicic acid. Elution with dichloromethane–ether (1:1) gave 300 mg. of an oil that partially crystallized from isopropyl alcohol giving slightly impure orotic acid methyl ester (IX) (m.p. 235–240°) which was identified chromatographically and spectrally (see later). Elution with dichloromethane–ether (1:3) gave 2.1 g. of amorphous, almost pure 2',3'-O-isopropylidineorotidine methyl ester which was crystallized with good recovery from acetone–cyclohexane (m.p. 163–164°) and was chromatographically pure.

Orotidine-5' Phosphate (IV).—Barium 2-cyanoethyl phosphate dihydrate (12 g., 36 mmoles) was suspended in 100 ml. of water containing 50 ml. of Dowex 50 (pyridinium) resin and stirred until the initially insoluble salt had dissolved. The entire mixture was then added to the top of a column containing a further 20 ml. of the same resin and the resin was washed with water (200 ml.). The effluent was evaporated to dryness *in vacuo* and the residue rendered anhydrous by three evaporations with anhydrous pyridine[31] (150 ml. each). The final residue was dissolved in dry pyridine (150 ml.), 2',3'-O-isopropylidineorotidine methyl ester (6.1 g., 18 mmoles) and dicyclohexylcarbodiimide (14.9 g., 72 mmoles) were added, and the mixture was shaken in the dark for 2 days. Water (10 ml.) was added and after 1 hour a further 100 ml. of water was added and the insoluble dicyclohexylurea was removed by filtration. The filtrate was evaporated to a sirup and residual pyridine was removed by evaporation with water (100 ml.). The residue was partitioned between water (150 ml.) and ether (150 ml.), and the water layer re-extracted with ether. After removal of residual ether *in vacuo*, lithium hydroxide monohydrate (6.3 g., 150 mmoles) was added and the solution was heated in a boiling water-bath for 1 hour. After cooling in an ice-bath, the precipitated lithium phosphate was removed and washed with 0.01 *N* lithium hydroxide. The filtrate was adjusted to *p*H 7 by portionwise addition of Dowex 50 (H^+), filtered, and concentrated to 50 ml. It was then passed through a column containing 150 ml. of Dowex 50 (H^+) and the resin washed with water until the effluent was neutral. The acidic solution (250 ml.) was heated in a boiling water-bath for 30 minutes, cooled, and neutralized to *p*H 8 with ammonium hydroxide. Chromatography in solvent III showed the presence of small amounts of free orotidine (R_f 0.17, 4%) and what is apparently an orotidine diphosphate (R_f 0.037, 1%) as well as the major product orotidine-5' phosphate (R_f 0.07).

This material (11.7 mmoles by spectra) was passed onto a column (3 × 40 cm.) of Dowex 2 (Cl^-) resin and washed with water. Elution with 0.12 *M* lithium chloride in 0.003 *N* hydrochloric acid removed 0.5 mmole of orotidine. Subsequent elution with 0.20 *M* lithium chloride in 0.003 *N* hydrochloric acid gave 11.0 mmoles of chromatographically pure orotidine-5' phosphate. The pooled peak was adjusted to *p*H 7.5 with lithium hydroxide and evaporated to a volume of 50 ml. Calcium chloride (3 g., 20 mmoles) was then added followed by methyl alcohol (500 ml.) and acetone (1 liter). The precipitate was collected and washed with methyl alcohol–acetone until the washings were free of chloride ion. It was then washed with ether and dried *in vacuo* giving 4.6 g. (50%) of the pure hydrated calcium salt of orotidine-5' phosphate.

Anal. Calcd. for $C_{10}H_{10}N_2O_{11}PCa_{1.5}\cdot 5H_2O$: P, 6.00; N, 5.44. Found: P, 5.94; N, 5.32.

An aqueous suspension of this calcium salt was stirred with 15 ml. of Dowex 50 (Na^+) resin until it dissolved and then was passed through a further 15 ml. of the same resin. After washing the resin with water, the effluent was evaporated to dryness and the residue triturated with acetone giving the dry, white, water-soluble trisodium salt of orotidine-5' phosphate (4.4 g.) which was chromatographically and electrophoretically completely pure: λ_{max} (0.1 *N* HCl) 267 mμ, ϵ_{max} 9430.

Anal. Calcd. for $C_{10}H_{10}N_2O_{11}PNa_3\cdot 3H_2O$: C, 24.60; H, 3.30; N, 5.74; P, 6.34. Found: C, 24.20; H, 3.38; N, 5.74; P, 6.34.

N_3-Methylorotidine Methyl Ester (V).—Cyclohexylammonium orotidine (1.0 g., 2.5 mmoles) was converted to the free acid on a column of Dowex 50 (H^+) resin (10 ml.) and evaporated to dryness. After a further evaporation with methyl alcohol and evacuation, the free acid was obtained as a white froth which was dissolved in methyl alcohol (25 ml.) and treated with an ethereal solution of diazomethane until a pale yellow color persisted. The solution was then evaporated to dryness leaving a hygroscopic white froth which did not readily crystallize. This was dissolved in warm dichloromethane and applied to a column of 30 g. of silicic acid. Elution with ether gave a small amount (30 mg.) of crystalline material that was recrystallized from ethanol and had m.p. 215–216°. It was neutral, periodate negative, and gave the analysis and chromatographic behavior expected for N_3-methylorotic acid methyl ester (X); λ_{max} (MeOH) 286 mμ, ϵ_{max} 6020.

Anal. Calcd. for $C_7H_8N_2O_4$: C, 45.63; H, 4.38; N, 15.21. Found: C, 46.10; H, 4.93; N, 15.47.

The main component was removed using ether–methyl alcohol (4:1). Crystallization of this material from isopropyl alcohol gave 520 mg. of N_3-methylorotidine methyl ester (V) as stout colorless crystals, m.p. 135–137°; λ_{max} (MeOH) 271 mμ, ϵ_{max} 7620.

Anal. Calcd. for $C_{12}H_{16}N_2O_8$: C, 45.57; H, 5.10; N, 8.86. Found: C, 45.82; H, 5.39; N, 8.94.

N_3-Methylorotidine (VIII).—N_3-Methylorotidine methyl ester (V) (75 mg.) was dissolved in 0.25 *N* methanolic sodium hydroxide (2 ml.) and kept at room temperature for 1 hour. The entire mixture was then passed through 3 ml. of Dowex 50 (H^+) resin which was then washed with water. Cyclohexylamine was added until the solution was alkaline and the solvent was removed *in vacuo*. The white residue was crystallized from ethanol–ether giving 85 mg. of cyclohexylammonium N_3-methylorotidine as clusters of tiny needles. After recrystallization from ethanol to remove a trace of a chromatographically slower moving material the product melted with decomposition and charring between 180 and 190° depending upon the rate of heating; λ_{max} (MeOH) 266 mμ, ϵ_{max} 9400; λ_{max} (0.1 *N* methanolic NaOH) 266 mμ, ϵ_{max} 9,110.

Anal. Calcd. for $C_{17}H_{27}N_3O_8$: C, 50.88; H, 6.78; N, 10.46. Found: C, 50.88; H, 6.99; N, 10.31.

Attempted Methylation of Orotidine with Methanolic Hydrogen Chloride.—Cyclohexylammonium orotidine (387 mg., 1 mmole) was converted to the free acid with Dowex 50 (H^+) and evaporated to dryness. The free acid was dried by several evaporations with anhydrous methyl alcohol and then *in vacuo* over phosphorus pentoxide. The resulting brittle froth was dissolved in 20 ml. of freshly prepared 20% methanolic hydrogen chloride and left at room temperature. After standing overnight a white crystalline material had separated (140 mg., 82%) which after recrystallization from methanol had m.p. 244–245° and λ_{max} (MeOH) 286 mμ, λ_{max} (*p*H 9.75) 317.5 mμ. Fox[13] reports m.p. 243–245°, λ_{max} (*p*H 4) 285 mμ, λ_{max} (*p*H 9.75) 317.5 mμ for orotic acid methyl ester. The filtrate, after removal of the crystalline material, contained a single strong non-reducing (aniline phthalate spray), periodate positive[32] spot with the same R_f as methyl ribofuranoside[33] in several solvents.

N_3-Methyl-2',3'-O-isopropylidineorotidine and N_3-Methyl-2',3'-O-isopropylidineorotidine Methyl Ester.—Cyclohexylammonium orotidine (2.0 g., 5 mmoles) was converted to the free acid by passage of its aqueous solution through Dowex 50 (H^+) resin (10 ml.) and evaporated to dryness. The residue was dried by

(31) Distilled and dried over calcium hydride.

(32) M. Viscontini, D. Hoch and P. Karrer, *Helv. Chim. Acta*, **38**, 642 (1955).

(33) P. A. Levene, A. L. Raymond and R. T. Dillon, *J. Biol. Chem.*, **95**, 699 (1932).

evaporation with methanol and evacuation giving free acid orotidine (1.5 g.) as a white froth. This was dissolved in methanol (25 ml.) and treated with an ethereal solution of diazomethane as above. After evaporation to dryness and high-vacuum evacuation, the crude N_3-methylorotodine methyl ester (1.57 g.) was dissolved in dry acetone (18 ml.) containing 2,2-dimethoxypropane (2 ml.) and dried Dowex 50 (H^+) resin (1 g.). After 3 hours, the pale brown solution was filtered and the filtrate evaporated to dryness leaving 1.73 g. of crude N_3-methyl-2′,3′-O-isopropylidineorotidine methyl ester as a tan colored froth which smelled strongly of acetone polymers. This material was dissolved in dichloromethane and applied to a column containing 50 g. of neutral alumina (activity 1). Brown, oily acetone polymers were removed with dichloromethane, while elution with ether–methanol (1:1) gave fairly pure N_3-methyl-2′,3′-O-isopropylidineorotidine methyl ester (0.6 g.) as an oil which crystallized from benzene–petroleum ether giving chromatographically homogeneous white needles of m.p. 110–111°[15]; λ_{max} (MeOH) 272 mμ, ϵ_{max} 7060; λ_{min} (MeOH) 232 mμ.

Anal. Calcd. for $C_{15}H_{20}N_2O_8$: C, 50.60; H, 5.66; N, 7.86. Found: C, 50.88; H, 5.92; N, 7.90.

Nothing was eluted from the column with methanol, but water removed 0.7 g. of an oil which was electrophoretically shown to have a free carboxyl group. The *p*H of the eluate was, however, neutral. This material was dissolved in water and rapidly passed through a cold column containing Dowex 50 (H^+) resin (5 ml.) into an excess of aqueous cyclohexylamine. The eluate was evaporated to dryness leaving a crystalline residue which was recrystallized from isopropyl alcohol giving 600 mg. of chromatographically pure cyclohexylammonium N_3-methyl-2′,3′-O-isopropylidineorotidine, m.p. 210–211°; λ_{max} (MeOH) 266 mμ, ϵ_{max} 9320; λ_{max} (0.1 *N* NaOH–MeOH) 265 mμ, ϵ_{max} 9100.

Anal. Calcd. for $C_{17}H_{30}N_3O_8$: C, 54.60; H, 7.08; N, 9.52. Found: C, 54.11; H, 7.33; N, 9.52.

[CONTRIBUTION FROM THE LABORATORY OF ORGANIC CHEMISTRY, UNIVERSITY OF ATHENS, GREECE]

Transformation of Serine to Cysteine. β-Elimination Reactions in Serine Derivatives[1,2]

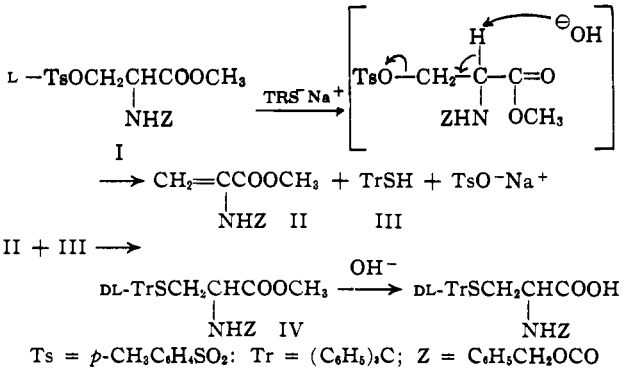
BY IPHIGENIA PHOTAKI[3]

RECEIVED NOVEMBER 16, 1962

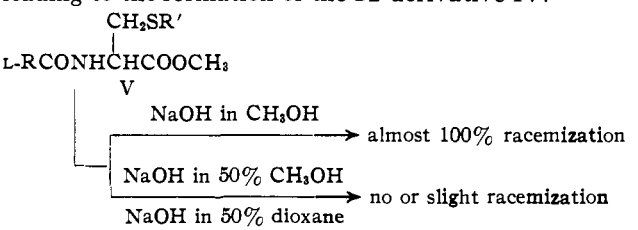
O-Tosylated and O-diphenylphosphorylated serine derivatives undergo a β-elimination reaction on being treated with 0.1 *N* alkali or diethylamine in non-polar solvents, resulting in the formation of dehydroalanine derivatives. N-Carbobenzoxy-O-tosyl-L-serine methyl ester is converted to N-carbobenzoxy-S-trityl-DL-cysteine by reaction with tritylthiocarbinol sodium salt, followed by saponification. It has been observed that N-acyl-S-alkyl-L-cysteine esters are extensively racemized by saponification with alcoholic alkali, but little or no racemization is produced by alkali in 50% aqueous methanol or aqueous dioxane.

Introduction

The synthesis of unsymmetrical cystine peptides containing two or more –S–S– bridges is in progress in this Laboratory. For this purpose, cysteine residues bearing different S-protecting groups which may be removed selectively have been introduced.[4] We thought that this problem might also be solved by distributing L-serine residues in a peptide chain and converting these to cysteine residues. Serine has been transformed to cysteine through formation of phenylthiazolines[5] or phenyloxazolines,[6] but these conversions are not suitable for our purposes. An alternative method of conversion has been realized in the case of N-carbobenzoxy-O-tosyl-L-serine methyl ester (I). As has been stated in a preliminary report,[1a] this ester reacts very quickly with the sodium salt of tritylthiocarbinol to form the corresponding S-trityl-N-carbobenzoxycysteine methyl ester (IV). This route has the advantage that the S-trityl group can be cleaved very smoothly even at 0° either by means of 0.2 *N* HBr in acetic acid or with an equivalent amount of silver nitrate–pyridine.[4] In the meantime, however, it has been found that complete racemization occurs during the replacement reaction. As saponification of the reaction product either by methanolic alkali or by alkali in 50% aqueous methanol or aqueous dioxane leads to fully racemized acid, it is concluded that the loss of the optical activity occurs prior to the saponification, during the replacement of the O-tosyl group with the trityl-thio group.[7] This conclusion is based on the fact that N-carbobenzoxy-S-trityl-L-cysteine methyl ester (Vc) and in general N-acyl-S-alkyl-L-cysteine esters are extensively racemized by saponification with alcoholic alkali, but little or no racemization is produced by alkali dissolved in aqueous methanol or aqueous dioxane. Apparently in the above replacement reaction, a β-elimination is first brought about by the action of the alkaline sodium mercaptide on compound I and this is followed by the addition of tritylthiocarbinol to the dehydroalanine derivative II,[8] leading to the formation of the DL-derivative IV.



Ts = *p*-$CH_3C_6H_4SO_2$: Tr = $(C_6H_5)_3C$; Z = $C_6H_5CH_2OCO$



Va, R = C_6H_5, R′ = $(C_6H_5)_2CH$
Vb, R = $C_6H_5CH_2O$, R′ = $C_6H_5CH_2$
Vc, R = $C_6H_5CH_2O$, R′ = $(C_6H_5)_3C$

(1) (a) A summary of a part of this paper was presented at the 3rd European Peptide Symposium, Basle, Switzerland, September, 1960; L. Zervas and I. Photaki, *Chimia*, **14**, 375 (1960). (b) A summary of this paper was presented at the 5th European Peptide Symposium, Oxford, England, September, 1962.

(2) This investigation was supported by the Royal Hellenic Research Foundation, to which I am greatly indebted.

(3) Present address: Department of Biochemistry, Cornell University Medical College, New York, 21, N. Y.

(4) L. Zervas and I. Photaki, *J. Am. Chem. Soc.*, **84**, 3887 (1962); L. Zervas, I. Photaki and N. Ghelis, *ibid.*, in press.

(5) D. F. Elliot, *Nature*, **162**, 658 (1948).

(6) E. Fry, *J. Org. Chem.*, **15**, 438 (1950).

(7) For examples of exchange of O-tosyl groups to mercapto groups *cf.* H. Gilman and N. J. Beaber, *J. Am. Chem. Soc.*, **47**, 1449 (1925); A. L. Raymond, *J. Biol. Chem.*, **107**, 85 (1934); C. King, R. Dodson and L. Subluskey, *J. Am. Chem. Soc.*, **70**, 1176 (1948); J. H. Chapman and L. N. Owen, *J. Chem. Soc.*, 579 (1950); A. M. Michelson, *ibid.*, 979 (1962); N. F. Blau and C. G. Stuckwish, *J. Org. Chem.*, **25**, 1611 (1960); R. Ireland and J. A. Marshall, *ibid.*, **27**, 1615 (1962).

(8) For addition reactions of mercaptans to dehydroalanine derivatives *cf.* B. H. Nicolet, *Science*, **81**, 181 (1935).