ysis may have interesting consequences when applied to the known kinetic data of hydrolytic reactions.

α-Chymotrypsin did not catalyze oxygen exchange between benzoyl-D-phenylalanine or β-phenylpropionic acid and the solvent. The results of the exchange experiments are given in Table III. The fact that benzoyl-D-phenylalanine does not undergo oxygen exchange in the presence of α-chymotrypsin while the corresponding L-enantiomorph is a substrate, is another example of the stereospecificity requirements of the enzyme. It might be anticipated that β-phenylpropionic acid would participate in an oxygen exchange reaction since the corresponding ester is a substrate of α-chymotrypsin.[20] However, the ester is a poor substrate and the amide is not a substrate at all. Since the oxygen exchange is considerably slower than ester hydrolysis, it is possible that the former is undetectably slow.[23]

TABLE III

OXYGEN EXCHANGE OF β-PHENYLPROPIONIC ACID AND BENZOYL-D-PHENYLALANINE[d]

Acid	Time, hr.	Atoms % O^{18}
β-Phenylpropionic acid[a]	0	1.30
	3.0	1.25
	3.0	1.26[c]
	5.0	1.26
	6.0	1.28[c]
	24.0	1.26
Benzoyl-D-phenylalanine[b]	0	0.817
	4.8	.813
	6.3	.815
	8.6	.812
	10.1	.812

[a] Acid concn. = 1.3×10^{-2} *M*; enzyme concn. = 1.5 mg. N/ml., *p*H 7.8. [b] Acid concn. = 4.2×10^{-2} *M*; enzyme concn. = 2 mg. N/ml., *p*H 7.7. [c] Blank runs in the absence of enzyme. [d] 25.04°.

The Michaelis constants, K_O, and the rate constants, k', found for the oxygen exchange of benzoyl-L-phenylalanine and acetyl-L-tryptophan are of the same order of magnitude as the constants of the hydrolysis of the corresponding amides.[3,21] Vaslow noted the same similarity with respect to acetyl-3,5-dibromotyrosine and its corresponding amide.[7] This similarity suggests that the Michaelis constants of the amides may be regarded as equilibrium constants. This suggestion has also been made by Huang and Niemann[24] from a consideration of the ratios of Michaelis constants and inhibition constants of enantiomorphic pairs, by Shine and Niemann[25] from a consideration of the effect of ionic strength on the kinetic constants and by Bernhard[6] from the consideration mentioned above of the Michaelis and inhibition constants of a poor substrate.

Acknowledgment.—The authors acknowledge valuable discussions with Drs. R. A. Alberty, R. M. Bock and F. Vaslow. The mass spectrometer on A. E. C. Contract At(11-1)-92 was made available through the courtesy of Dr. H. Taube. A generous gift of α-chymotrypsin from Armour and Co. is gratefully acknowledged.

(23) Personal communication from Dr. R. M. Bock indicates that benzoyl-D-phenylalanine methyl ester may be a substrate (although a poor one) of α-chymotrypsin. In this case the failure to observe oxygen exchange with the D-acid would again be due to the extreme slowness of the reaction.

(24) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951).

(25) H. Shine and C. Niemann, *ibid.*, **77**, 4275 (1955).

CHICAGO, ILLINOIS

[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES]

Hygromycin. III. Structure Studies

BY ROBERT L. MANN AND D. O. WOOLF

RECEIVED JUNE 20, 1956

Hygromycin, an antibiotic with a wide antibacterial spectrum, has been degraded to three principal units: 3,4-dihydroxy-α-methylcinnamic acid, an inosamine and 5-keto-6-deoxy-D-arabohexose. The hexose is attached by a glycosidic linkage at the 4-hydroxy position of the 3,4-dihydroxy-α-methylcinnamic acid. The inosamine is linked to the acid as a carboxamide.

Among the metabolic products of the actinomycete *Streptomyces hygroscopicus* (Jensen) Waksman and Henrici is hygromycin,[1,2] which has a relatively broad spectrum of activity against gram-positive and gram-negative bacteria. This paper is concerned with the elucidation of the structure of the antibiotic.

Hygromycin has been isolated as an amorphous substance of the composition $C_{23}H_{29}NO_{12}$,[3] a formula that has been confirmed by analysis of a crystalline 2,4-dinitrophenylhydrazone. Titration of the antibiotic gave a pK_a' of 8.9 in water and 10.7 in 66% dimethylformamide, indicating an acidic group.[4] A positive reaction with the Folin–Ciocalteu reagent and the pK_a' value suggested that the acid function may be phenolic. This was also indicated by the strong absorption of hygromycin in the ultraviolet at 214 and 272 mμ in acid; the intensity and position of the maxima varied with the *p*H of the solution.

Reactivity of the antibiotic with carbonyl reagents, its capacity for reducing Fehling and Benedict solutions and the strong infrared absorption at 5.84 μ indicated the presence of an aldehyde or ketone group. At least one C-methyl was demon-

(1) R. C. Pittenger, R. N. Wolfe, M. M. Hoehn, Phoebe Nelms Marks, W. A. Daily and J. M. McGuire, *Antibiotics and Chemotherapy*, **3**, 1268 (1953).

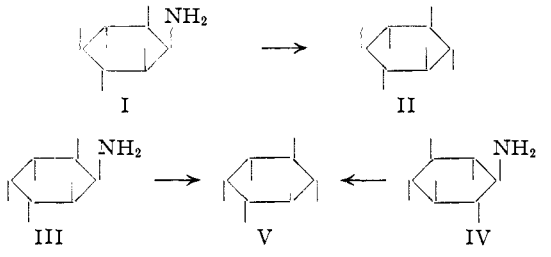
(2) R. L. Mann, R. M. Gale and F. R. Van Abeele, *ibid.*, **3**, 1279 (1953).

(3) The formula for hygromycin was previously reported to be $C_{25}H_{33}NO_{13}$.[2]

(4) T. V. Parke and W. W. Davis, *Anal. Chem.*, **26**, 642 (1954).

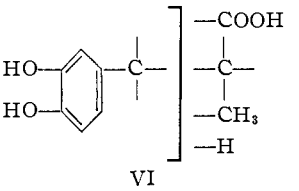
strated by group analysis, but no alkoxyl was detected. Distillation of a 2 *N* sulfuric acid solution of hygromycin resulted in the formation of formaldehyde, which was isolated as the dimedone derivative.

Acid and Alkali Degradations.—The fact that the nitrogen in hygromycin was not titrable, coupled with strong infrared absorption bands at 6.22 μ and at 6.62 μ, suggested the possibility of an amide structure. This consideration received support when a crystalline amine hydrochloride having the empirical formula $C_6H_{14}NO_5Cl$ with a pK_a' of 7.6 was isolated from an acid hydrolysate of hygromycin. This amine and its hexaacetyl and N-acetyl derivatives showed no optical activity. It did not reduce Fehling or Benedict solution. In addition, no formaldehyde could be detected on oxidation with periodate.[5] The evidence, thus, was indicative of a cyclic structure. That the compound was an inosamine seemed a possibility; and, indeed, on deamination with nitrous acid myo-inositol (V) was formed. Posternak[6] has shown that deamination of both myo-inosamine-2 (I) and scyllo-inosamine (IV) takes place with Walden inversion leading to the formation of scyllo-inositol (II) and myo-inositol, respectively. Assuming that a Walden inversion occurred in the present study, the formation of myo-inositol would imply that scyllo-inosamine or neo-inosamine (III) occurs in the antibiotic. Only these two of the eight possible optically inactive inosamines could yield myo-inositol by this mechanism.



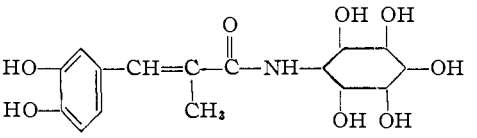
The infrared spectrum and the X-ray diffraction pattern of the inosamine differed from those of an authentic sample of scyllo-inosamine.[7] Therefore, the compound was tentatively identified as the heretofore unknown neo-inosamine-2.

Aside from the inosamine just described, acid degradation led to a tarry material from which no definable compounds were isolated. On the other hand, alkaline hydrolysis destroyed the inosamine but released an acidic substance, $C_{10}H_{10}O_4$, that contained at least one C-methyl group. This compound possessed two titrable acid groups; one with pK_a' 8.9 in water (11.4 in 66% dimethylformamide) similar to that of the antibiotic, the other with pK_a' 4.5 in water (7.6 in 66% dimethylformamide) attributed to a carboxyl function. A phenolic carboxylic acid was suspected on the basis of the pK_a' values and a positive ferric chloride test. The presumption was confirmed when, after fusion of the acid with alkali, 3,4-dihydroxybenzoic acid was isolated. Partial formula VI could now be postulated for the C_{10} acid. The compound was identified as 3,4-dihydroxy-α-methylcinnamic acid by comparison with a synthetic specimen.



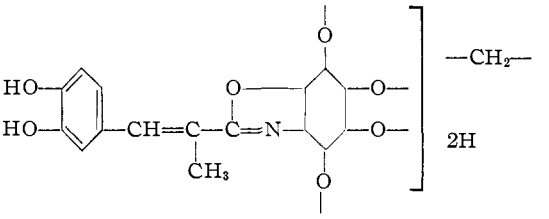
VI

Having obtained an amine and a carboxylic acid as degradation products, the hypothesis of an amide structure in hygromycin seemed even more tenable. Thus, an attempt was made to obtain the postulated inosamide of 3,4-dihydroxy-α-methylcinnamic acid ($C_{16}H_{21}NO_8$) (VII) from the antibiotic by treatment with dilute hydrochloric acid at room temperature for several days. However, the



VII

only compound which could be isolated readily from the reaction mixture was a yellow crystalline substance obtained as a hydrochloride and having the formula $C_{17}H_{20}NO_7Cl$. The free base which was generated by treatment with an anion exchange resin (Amberlite IR-45) analyzed for $C_{17}H_{19}NO_7$. This formula contained *one carbon more* and *one mole of water less* than that of the simple amide (VII). The yellow hydrochloride was degraded to the inosamine (III) and 3,4-dihydroxy-α-methylcinnamic acid. Electrometric titration of the yellow compound indicated two titrable groups with pK_a' values of 9.4 and 4.0 in 33% dimethylformamide. In 66% dimethylformamide the pK_a' values were 10.7 and 4.0. This indicated that one of the groups was a weak acid and the other was a weak base. Since hygromycin has only a phenolic group, the basic function was assumed to be associated with the inosamine nitrogen and must have been derived from a rearrangement involving the amide linkage. The presence of the weak basic group, the lack of one mole of water in the formula as compared to the formula for the expected inosamide, the infrared spectrum (see Experimental) and a consideration of the acidic nature of the degradation reaction justified the hypothesis that the compound was the C_{17}-oxazoline (VIII) formed as an intermediate during an N → O acyl shift.



VIII

A most significant feature of structure VIII in comparison with the C_{16}-amide (VII) was the pres-

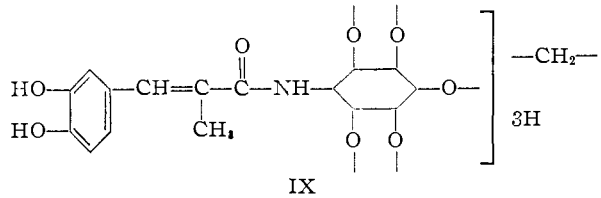
(5) Richard E. Reeves, THIS JOURNAL, **63**, 1476 (1941).
(6) Theodore Posternak, *Helv. Chim. Acta*, **33**, 1597 (1950).
(7) We wish to thank Dr. Henry A. Lardy, Univ. of Wisconsin, for supplying us with a sample of scyllo-inosamine.

ence of the additional carbon atom. Distillation of an acid solution of the C_{17} compound resulted in the formation of formaldehyde. This observation is similar to that noted earlier with hygromycin and is consistent with the result to be expected with a dioxymethylene group. Since the phenolic function was titrable (pK_a' 9.4 in 33% dimethylformamide) the methylene must link two oxygens of the inosamine moiety.

Mercaptanolysis.—The proposed amide structure now accounted for all of the hygromycin molecule with the exception of six carbons and four oxygens. The high ratio of oxygen to carbon suggested that hygromycin, like many other antibiotics produced by actinomycetes, contained a sugar residue. Hence, mercaptanolysis under mild conditions was undertaken to remove the sugar and permit the isolation of the amide.

By carrying out the mercaptanolysis at 5° in ethyl mercaptan saturated with dry hydrogen chloride, a compound was obtained that analyzed for $C_{16}H_{21}NO_8$. The product was degraded to the inosamine and 3,4-dihydroxy-α-methylcinnamic acid. It had one titrable group, pK_a' 9.5 in 33% dimethylformamide (10.8 in 66% dimethylformamide) and gave an infrared spectrum (see Experimental) consistent with the C_{16}-amide structure VII.

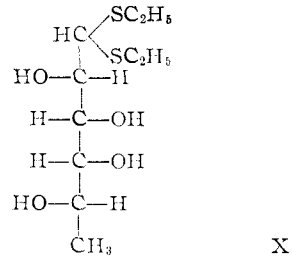
The amide with the methylene group intact, however, was not detected among the mercaptanolysis products of hygromycin. Furthermore, no sugar derivative was obtained. If any such derivative had been formed, it should have contained the aldehyde or ketone function known to be present in hygromycin and might well have been unstable. To avoid such stability difficulties during mercaptanolysis, hygromycin was treated with sodium borohydride to reduce the carbonyl group. After mercaptanolysis of reduced hygromycin with ethyl mercaptan and 6 *N* hydrochloric acid at 5°, two crystalline compounds were isolated. One analyzed for $C_{17}H_{21}NO_8$ and had a pK_a' of 9.5 in 33% dimethylformamide (10.8 in 66% dimethylformamide). It gave an infrared spectrum essentially identical in the 6–6.7 μ region with that of the C_{16}-amide. Degradation yielded formaldehyde, the inosamine, and 3,4-dihydroxy-α-methylcinnamic acid. This is clearly the C_{17}-amide IX.



IX

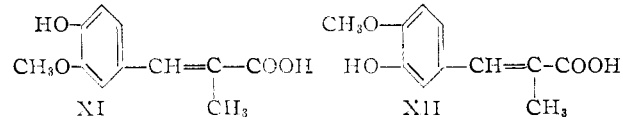
The conversion of IX to the C_{17}-oxazoline hydrochloride by treatment with acid and the reverse reaction observed in the presence of alkali substantiated the amide constitution of IX and clarified the relation between this amide and the C_{17}-oxazoline hydrochloride.

The second product obtained by mercaptanolysis of reduced hygromycin had the molecular formula $C_{10}H_{22}S_2O_4$. It was identical with the diethylmercaptal of L-fucose (X). Thus, degradation products accounting for the entire molecular formula of hygromycin had been isolated.



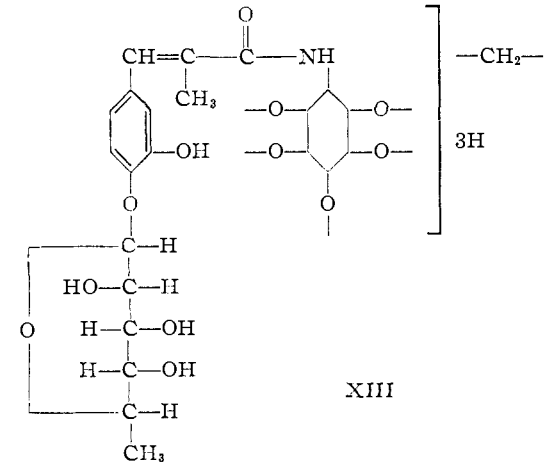
X

Studies were initiated to determine the position of the linkage between the amide and the sugar. All degradation products which contained the free 3,4-diphenol structure were readily oxidized in air when in solution at a slightly alkaline *p*H. Hygromycin, however, showed no such tendency toward oxidation. Apparently one of the phenolic hydroxyls in the intact antibiotic is not free and is probably the point at which the sugar is attached. The free phenolic group of hygromycin was methylated with diazomethane and the methoxyl derivative was hydrolyzed with alkali under the conditions which originally produced 3,4-dihydroxy-α-methylcinnamic acid. The phenolic carboxylic acid that was obtained had the formula $C_{11}H_{12}O_4$ and must necessarily be one of the two isomers, 4-hydroxy-3-methoxy-α-methylcinnamic acid (XI) or 3-hydroxy-4-methoxy-α-methylcinnamic acid (XII).



XI XII

Comparison with a synthetic preparation showed that the degradation product was 4-hydroxy-3-methoxy-α-methylcinnamic acid (XI). This definitely established the position of the glycosidic linkage at the 4-hydroxyl and made possible formulation of the partial structure XIII for sodium borohydride-reduced hygromycin.[8]

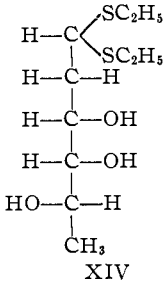


XIII

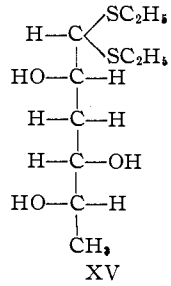
Aside from the position of the dioxymethylene group, the only other uncertain structural feature is the location of the free carbonyl group on the sugar.

(8) The structure of the sugar residue is arbitrarily drawn in pyranose form. No experimental evidence exists for this structure.

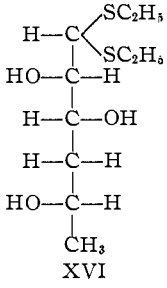
This point was clarified in the following manner. Hygromycin, treated with ethyl mercaptan and 1 *N* hydrochloric acid, was not degraded but yielded a product that contained sulfur and that could be converted to the antibiotic by treatment with mercuric chloride. The mercaptan-treated product no longer reacted with carbonyl reagents. It gave an infrared spectrum that differed significantly from that of hygromycin by the lack of absorption at 5.84 μ. The product appeared to be hygromycin mercaptol. Desulfurization with Raney nickel led to a product designated as dihydrodeoxyhygromycin in which the ketone group of hygromycin was reduced to methylene. Mercaptanolysis of this reduced compound (dihydrodeoxyhygromycin) gave a mercaptal with the formula $C_{10}H_{22}S_2O_3$. It follows that this mercaptal must be XIV, XV, XVI or XVII and that the location of the methylene



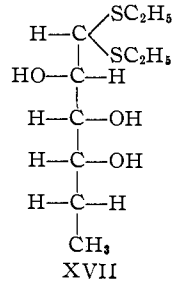
XIV



XV

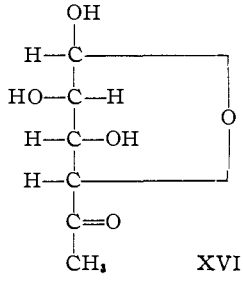


XVI



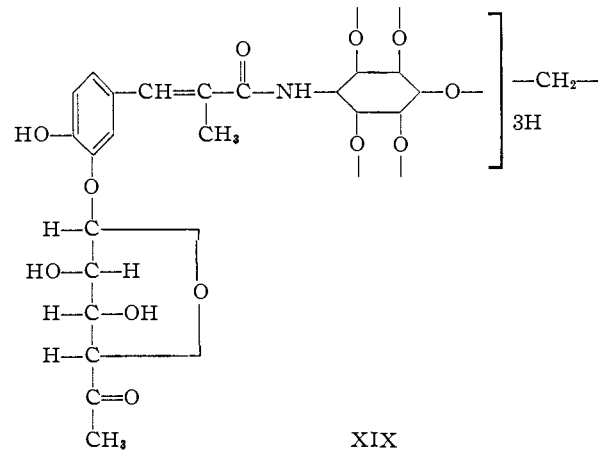
XVII

group establishes the position of the original carbonyl group in hygromycin. When the mercaptal was oxidized with sodium metaperiodate, six moles of periodate was consumed, and propionaldehyde was obtained in 70% yield. Thus, the mercaptal must have the structure XVII, since XIV, XV and XVI will not yield propionaldehyde on periodate oxidation. The sugar in hygromycin was thus shown to be 5-keto-6-deoxy-arabohexose (XVIII).



XVIII

Based on the evidence in this paper, the structure of hygromycin is XIX. The only remaining uncertainties are the location of the methylene group in the inosamine fragment and the configuration of the glycosidic linkage.



XIX

Acknowledgments.—The authors are grateful to Drs. H. A. Rose, H. Boaz and Mr. J. Forbes for physicochemical data; to Messrs. W. L. Brown, H. L. Hunter, G. M. Maciak and Miss Gloria Beckmann for microanalyses; to Mr. E. H. Presti for technical assistance; and to Drs. G. B. Kline, F. R. Van Abeele and C. W. Pettinga for helpful advice.

Experimental[9]

Hygromycin (XIX).—Hygromycin was isolated and purified by a previously described procedure.[2] The material used for structure studies showed only one microbiologically active spot on papergrams and followed a theoretical distribution curve in a 300-transfer countercurrent distribution utilizing a solvent system composed of amyl alcohol, water and acetic acid in the ratio 24:24:1. The ultraviolet absorption spectrum in dilute hydrochloric acid was characterized by major maxima at 272 mμ ($E^{1\%}_{1\,cm.}$ 280) and at 214 mμ ($E^{1\%}_{1\,cm.}$ 372). In dilute alkali the maxima occurred at 254 mμ ($E^{1\%}_{1\,cm.}$ 350), 286 mμ ($E^{1\%}_{1\,cm.}$ 194) and 323 mμ ($E^{1\%}_{1\,cm.}$ 116). The infrared spectrum showed strong absorption bands at 3.0, 5.84, 6.08, 6.22 and 6.62 μ.

Anal. Calcd. for $C_{23}H_{29}NO_{12}$: C, 54.01; H, 5.71; N, 2.74; O, 37.54; C-methyl (one), 2.94. Found: C, 53.85; H, 6.15; N, 2.78; O, 37.53; C-methyl, 2.56.

Hygromycin 2,4-Dinitrophenylhydrazone.—One gram of hygromycin in 5 ml. of water was added with stirring to 200 ml. of a solution containing 30 ml. of concentrated hydrochloric acid and 800 mg. of 2,4-dinitrophenylhydrazine. The solution was cooled to 5°. After one hour, the precipitate that formed was removed by centrifugation and was washed twice with 10-ml. portions of cold water. The dried amorphous 2,4-dinitrophenylhydrazone weighed 1260 mg. The product was crystallized 3 times from water; m.p. 154–156°.

Anal. Calcd. for $C_{29}H_{33}N_5O_{15}$: C, 50.36; H, 4.81; N, 10.13. Found: C, 50.33; H, 5.00; N, 9.95.

Recovery of Formaldehyde from Hygromycin.—Hygromycin (300 mg.) was dissolved in 20 ml. of 2 *N* sulfuric acid and the solution was distilled for 2 hr. into a flask containing 300 mg. of dimedone in 75 ml. of water. Periodically water was added to the distillation flask to maintain the volume. The distillate was allowed to stand overnight at room temperature. The dimedone derivative of formaldehyde was removed by filtration, washed with water and dried. The yield was 56 mg. Identification was made by comparison of the X-ray diffraction pattern with that of an authentic sample.

Inosamine Hydrochloride.—Hygromycin (52 g.) was dissolved in 1 liter of 6 *N* hydrochloric acid and the solution was heated under reflux for 17 hr. The hydrolysate was

(9) Melting points were determined on a Kofler micro melting point apparatus unless otherwise indicated. Infrared spectra were obtained as mulls in mineral oil with a Beckman IR-2T spectrophotometer or a Baird double-beam recording spectrophotometer. Ultraviolet measurements were made with a Cary recording spectrophotometer.

cooled and filtered, and the filtrate was extracted 5 times with 1-liter portions of ether. The aqueous phase was concentrated *in vacuo* to 500 ml. and 2 liters of ethanol was added. A crystalline precipitate of inosamine hydrochloride was obtained, and this was recrystallized twice from water by the addition of ethanol. The yield was 17.3 g. The melting point determined on the Kofler hot stage was 114–115°, but when determined in a capillary was 215–216° (uncorrected). Titration in water showed a pK_a' of 7.6. The compound was optically inactive.

Anal. Calcd. for $C_6H_{14}NO_5Cl$: C, 33.42; H, 6.54; N, 6.49; Cl, 16.44. Found: C, 33.53; H, 6.72; N, 6.50; Cl, 16.54.

Inosamine hydrochloride was converted to the free base III by adjusting an aqueous solution to *p*H 10.0 and adding ethanol. The resulting crystals were recrystallized twice from water by the addition of ethanol to give a product melting at 234–236°.

Anal. Calcd. for $C_6H_{13}NO_5$: C, 40.22; H, 7.31; N, 7.82. Found: C, 39.92; H, 7.58; N, 7.50.

Deamination of the Inosamine.—The deamination procedure followed was essentially that of Posternak.[8] One hundred mg. of the inosamine hydrochloride was dissolved in a mixture of 1.5 ml. of water and 0.2 ml. of glacial acetic acid. The solution was cooled to 0°, and 250 mg. of barium nitrite in 1.25 ml. of water was added. The reaction mixture was held at 5° for 16 hr. Barium was removed by precipitation with 5 *N* sulfuric acid followed by filtration. The filtrate was concentrated to 1 ml. in a stream of nitrogen, and on standing overnight at 5° crystallization occurred. The product weighed 15 mg. and was identified as myo-inositol by X-ray diffraction. A mixture melting point with an authentic sample was not depressed.

Hexaacetyl Inosamine.—Inosamine hydrochloride (1 g.) was dissolved in 10 ml. of anhyd. pyridine and 5 ml. of acetic anhydride. The mixture was heated under reflux for 10 minutes, cooled and poured into 60 ml. of ice-water. The resulting precipitate was removed by filtration and was recrystallized 3 times from ethanol–water; yield 580 mg., m.p. 277–279°. The compound was optically inactive.

Anal. Calcd. for $C_{18}H_{25}NO_{11}$: C, 50.11; H, 5.84; N, 3.25. Found: C, 50.40; H, 5.88; N, 2.98.

N-Acetylinosamine.—Hexaacetylinosamine (130 mg.) was dissolved in 4 ml. of methanol saturated with ammonia and was allowed to stand at 5° overnight. The crystals that separated were filtered, washed with methanol and recrystallized twice from water–methanol; yield 54 mg., m.p. 238–242°. The compound was optically inactive.

Anal. Calcd. for $C_8H_{15}NO_6$: C, 43.43; H, 6.84; N, 6.33. Found: C, 43.56; H, 6.76; N, 6.16.

3,4-Dihydroxy-α-methylcinnamic Acid.—A solution of hygromycin (4 g.) in 250 ml. of 10% sodium hydroxide in the presence of 15 g. of zinc was heated under reflux in a nitrogen atmosphere for 16 hr. The hydrolysate was acidified to *p*H 2.0 with concentrated sulfuric acid and was extracted twice with 200-ml. portions of ether. The combined ether solutions were extracted twice with 100-ml. portions of 5% sodium bicarbonate solution. The alkaline solution was acidified to *p*H 2.0 with 5 *N* sulfuric acid, and the ether extraction was repeated. The ether solution was dried over sodium sulfate. Benzene was added, and on slowly removing the ether *in vacuo* a crystalline product precipitated which weighed 1.4 g. and melted at 145–154°. A recrystallization from benzene followed by one from water raised the melting point to 160–162°. The X-ray diffraction pattern and the infrared spectrum of this compound were identical with those of the synthetic 3,4-dihydroxy-α-methylcinnamic acid described below.

Anal. Calcd. for $C_{10}H_{10}O_4$: C, 61.85; H, 5.19; C-methyl (one), 7.74. Found: C, 62.14; H, 5.29; C-methyl, 6.44.

Synthesis of 3,4-Dihydroxy-α-methylcinnamic Acid.—Piperonal (4.5 g., 0.03 mole), sodium propionate (3.27 g., 0.034 mole) and propionic anhydride (5 ml., 0.04 mole) were heated 2 hr. in an oil-bath at 155°. Fifty ml. of water was added to the brown reaction mixture. The resulting solution was adjusted to *p*H 8.0 with sodium hydroxide and was extracted with three 50-ml. portions of ether to remove unreacted aldehyde. The extracted solution was acidified to *p*H 1.5 with concentrated sulfuric acid and was again extracted with three 50-ml. portions of ether to remove the reaction product. The ether solution was extracted twice with 25-ml. portions of 0.5 *N* sodium hydroxide. On acidification of the alkaline extract, piperonyl methacrylic acid precipitated. It was removed by filtration, washed with water and dried. The yield was 1.85 g. of crude material that was not purified further.

Piperonyl methacrylic acid (618 mg., 0.003 mole), phosphorus pentachloride (1.87 g., 0.009 mole) and 1.5 ml. of phosphorus oxychloride were heated together on a steam-bath for 10 minutes, followed by heating under gentle reflux for 20 minutes. The reaction mixture was concentrated *in vacuo* until all volatile materials were removed, and 25 ml. of cold water was added to the residue. The aqueous solution was allowed to stand at 5° for 2 hr. and then was heated under reflux for 1.5 hr. The boiled solution was treated with charcoal, filtered and concentrated *in vacuo* to 3 ml., at which point crystallization took place. The product was recrystallized twice from water; yield 75 mg., m.p. 160–162°. Two titrable groups were found, pK_a' 8.9 and 4.5 in water.

Anal. Calcd. for $C_{10}H_{10}O_4$: C, 61.85; H, 5.19. Found: C, 62.03; H, 5.30.

Fusion of 3,4-Dihydroxy-α-methylcinnamic Acid with Alkali.—A mixture of 0.7 g. of potassium hydroxide and 140 mg. of 3,4-dihydroxy-α-methylcinnamic acid in a nickel crucible was heated slowly in a metal-bath to 240°. After the further addition of 0.7 g. of potassium hydroxide, the mixture was heated to 300° and held at this temperature for 10 minutes. The melt was cooled, dissolved in 30 ml. of ice and water and acidified to *p*H 1.5 with 6 *N* sulfuric acid. The acid solution was extracted with three 25-ml. portions of ether. The ether extract was dried over calcium chloride and was concentrated to dryness on a steam-bath. The residue was crystallized from benzene and was recrystallized twice from ethylene chloride, to yield 20 mg. of 3,4-dihydroxybenzoic acid. Identification was made by comparison of the X-ray diffraction pattern with that of an authentic sample and by the lack of depression of a mixture melting point.

C_{17}-Oxazoline Hydrochloride (VIII) from Hygromycin.—Hygromycin (5 g.) was dissolved in 250 ml. of 4 *N* hydrochloric acid. The solution was placed in a liquid–liquid extractor and was extracted continuously with ether for 6 days. It had been observed earlier that ether extraction would prevent much of the darkening of the acid reaction mixture. The extracted aqueous phase was concentrated *in vacuo* to 10 ml. and was allowed to stand at 5° overnight. The yellow crystals that separated were removed by filtration and were recrystallized twice from ethanol to which was added an equal volume of 6 *N* hydrochloric acid. The product was finally washed with a small amount of cold water; yield 480 mg., m.p. 221–226°. The infrared spectrum showed a strong band at 6.30 μ which appeared to indicate a C=N, especially in view of the high degree of conjugation in the molecule due to the 3,4-dihydroxy-α-methylcinnamic acid moiety. A band of medium intensity at 6.63 μ was considered to be too weak for an amide II band.

Anal. Calcd. for $C_{17}H_{20}NO_7Cl$: C, 52.92; H, 5.23; N, 3.63; Cl, 9.19. Found: C, 52.87; H, 5.28; N, 3.46; Cl, 9.26.

Conversion of the C_{17}-Oxazoline Hydrochloride to the Free Base.—C_{17}-Oxazoline hydrochloride (120 mg.) was treated with 50 ml. of anion-exchange resin (Amberlite IR-45). There was immediate formation of a flocculent crystalline precipitate which was separated from the resin by decantation. The product was recrystallized from dimethylformamide–water; yield 45 mg., m.p. 248–249°.

Anal. Calcd. for $C_{17}H_{19}NO_7$: C, 58.45; H, 5.48; N, 4.01. Found: C, 58.92; H, 6.05; N, 3.73.

Inosamine Hydrochloride from the C_{17}-Oxazoline Hydrochloride.—A solution of the C_{17}-oxazoline hydrochloride (50 mg.) in 10 ml. of 6 *N* hydrochloric acid was heated under reflux for 4 hr. The solution was treated with decolorizing carbon and filtered. After addition of 20 ml. of ethanol to the filtrate a crystalline precipitate formed. Recrystallization from water–ethanol yielded 15 mg. of product which was found by comparison of X-ray diffraction patterns to be identical with the inosamine hydrochloride obtained directly from hygromycin.

3,4-Dihydroxy-α-methylcinnamic Acid from the C_{17}-Oxazoline Hydrochloride.—A solution of C_{17}-oxazoline

hydrochloride in 10 ml. of 10% sodium hydroxide in the presence of 0.3 g. of zinc was heated under reflux for 4 hr. The hydrolysate was acidified to *p*H 1.5 with concentrated sulfuric acid and was extracted with two 15-ml. portions of ether. The ether extract was dried over sodium sulfate. Five ml. of benzene was added and the ether was removed *in vacuo*. The crystals that formed were recrystallized from water to yield 20 mg. of product. Identity with 3,4-dihydroxy-α-methylcinnamic acid was proved by X-ray diffraction.

Recovery of Formaldehyde from the C_{17}-Oxazoline Hydrochloride.—Twenty mg. of the dimedone derivative of formaldehyde was obtained when an acid solution of 50 mg. of C_{17}-oxazoline hydrochloride was distilled by the same procedure as described for hygromycin. Identification was made by X-ray diffraction.

C_{16}-Amide (VII) from Hygromycin.—Hygromycin (2 g.) was suspended in 40 ml. of ethyl mercaptan and was cooled to 5°. The mixture was saturated with dry hydrogen chloride and was allowed to stand at 5° overnight. The brown, partly crystalline solid phase which formed during the reaction was dissolved in hot water and was allowed to crystallize. Two additional recrystallizations from water yielded 978 mg. of product melting at 251–252°.

Anal. Calcd. for $C_{16}H_{21}NO_8$: C, 54.08; H, 5.96; N, 3.94. Found: C, 54.18; H, 6.21; N, 3.92.

In the infrared spectrum the bands at 6.13 μ (M), 6.24 μ (S) and 6.52 μ (S) were given the assignments C=C, amide I and amide II, respectively; pK_a' 9.50 in 33% dimethylformamide (10.8 in 66% dimethylformamide).

Inosamine and 3,4-dihydroxy-α-methylcinnamic acid were recovered in 85 and 60% yields, respectively, from the C_{16}-amide by the degradative procedures described for the C_{17}-oxazoline hydrochloride.

Reduction of Hygromycin with Sodium Borohydride.—Hygromycin (100 g.) was dissolved in 800 ml. of methanol, and a solution of 3.34 g. of sodium borohydride in 50 ml. of methanol was added slowly with stirring. The reaction mixture stood at room temperature for 1.5 hr., after which it was acidified to *p*H 6.5 with 6 *N* hydrochloric acid. The solution was filtered and concentrated to dryness *in vacuo*. The residue was dissolved in methanol and again was concentrated to dryness. This was repeated twice more. The final dried powder weighed 106 g. The gain in weight over the starting material was due probably to the presence of inorganic salts formed during the reaction and decomposition of excess sodium borohydride.

For subsequent reactions the product as obtained above was found to be satisfactory. For characterization, 5 g. of the reduced hygromycin was purified by a 60-transfer countercurrent distribution in a solvent system composed of butanol, water and acetic acid in the ratio 24:24:1. The pure amorphous product gave an infrared spectrum which showed no carbonyl absorption in the 5.8 μ region but otherwise did not differ appreciably from that of hygromycin. The microbiological activity of the reduced compound was about half that of hygromycin.

Anal. Calcd. for $C_{23}H_{31}NO_{12}$: C, 53.79; H, 6.09; N, 2.73. Found: C, 54.38; H, 6.33; N, 2.78.

The C_{17}-Amide (IX) from Sodium Borohydride-reduced Hygromycin.—A mixture of 10 g. of sodium borohydride-reduced hygromycin, 10 ml. of 6 *N* hydrochloric acid and 20 ml. of ethyl mercaptan was allowed to stand at 5° for 2 days. The aqueous phase was separated. After dilution with 2 volumes of water and standing at 5° overnight, a crystalline precipitate of the C_{17}-amide separated. This amide did not separate from a strongly acid solution. The product was recrystallized 3 times from water, yield 3.5 g., m.p. 108–112°.

Anal. Calcd. for $C_{17}H_{21}NO$: C, 55.58; H, 5.76; N, 3.81. Found: C, 55.08; H, 5.84; N, 3.58.

This compound gave an infrared spectrum which differed but little from that of the C_{16}-amide, showing the characteristic amide I and amide II bands at 6.29 and 6.57 μ, respectively. Formaldehyde, the inosamine and 3,4-dihydroxy-α-methylcinnamic acid were recovered in yields of 31, 68 and 30%, respectively, from the C_{17}-amide by the procedures described above for the C_{17}-oxazoline hydrochloride. Identification of each of the compounds was made by X-ray diffraction.

Interconversion of the C_{17}-Amide and the C_{17}-Oxazoline Hydrochloride.—A solution of 100 mg. of C_{17}-amide in 100 ml. of 4 *N* hydrochloric acid was allowed to stand at room temperature for 4 days. The solution was concentrated *in vacuo* to 10 ml. and kept at 5° for 4 days. Yellow crystals (10 mg.) slowly separated. The product was identified as the C_{17}-oxazoline hydrochloride by X-ray diffraction.

Fifty mg. of C_{17}-oxazoline hydrochloride was dissolved in 10 ml. of water, and the solution was adjusted to *p*H 10.5 while maintaining the mixture in a nitrogen atmosphere. The solution was heated, still under nitrogen, for 2.5 hr. on a steam-bath, cooled and neutralized with 2 *N* sulfuric acid. An amorphous precipitate was removed by centrifugation and discarded. The solution was concentrated to a volume of 2 ml. and cooled. A partially crystalline product was obtained, and it was recrystallized twice from water to yield 14 mg. of the C_{17}-amide. It was identified by its X-ray diffraction pattern.

L-Fucose Diethylmercaptal (X) from Sodium Borohydride-reduced Hygromycin.—A mixture of sodium borohydride-reduced hygromycin (13.5 g.), 28 ml. of 6 *N* hydrochloric acid and 25 ml. of ethyl mercaptan was allowed to stand with occasional shaking at room temperature for 2 hr. During this time fine, white needle-like crystals separated. The ethyl mercaptan was decanted, and the crystals were removed from the aqueous phase by filtration. Two recrystallizations from water yielded 2.4 g. of product, m.p. 161–162°.

Anal. Calcd. for $C_{10}H_{22}S_2O_4$: C, 44.41; H, 8.20; S, 23.71; O, 23.67. Found: C, 44.23; H, 8.15; S, 23.59; O, 23.85.

The compound was identified as L-fucose diethylmercaptal by comparison of its X-ray diffraction pattern with that of an authentic sample. Mixture melting point was not depressed.

The aqueous phase of the mercaptanolysis was diluted with two volumes of water, and on standing 2.8 g. of the C_{17}-amide crystallized. It was identified by its X-ray diffraction pattern.

Methylation of Hygromycin with Diazomethane.—An excess of diazomethane in 130 ml. of methylene chloride was added to 100 ml. of cold methanol containing 5 g. of hygromycin. The solution was allowed to warm slowly to room temperature and then to stand, unstoppered, overnight. The reaction mixture was concentrated *in vacuo* to a light tan amorphous powder. The methylation apparently was complete because the product no longer contained a titrable phenolic group.

4-Hydroxy-3-methoxy-α-methylcinnamic Acid (XI) from Methylated Hygromycin.—A solution of 1 g. of methylated hygromycin in 50 ml. of 10% sodium hydroxide in the presence of 3 g. of zinc was heated under reflux for 2.5 hr. The hydrolysate was acidified to *p*H 1.5 with concentrated sulfuric acid and was extracted twice with 25-ml. portions of ether. The ether solution was concentrated to dryness *in vacuo* and the residue was dissolved in methanol. On adding water to the methanol solution, crystallization occurred. The product was recrystallized twice from methanol-water; yield 300 mg., m.p. 167–168°.

Anal. Calcd. for $C_{11}H_{12}O_4$: C, 63.45; H, 5.81. Found: C, 63.57; H, 5.75.

Identity with the 4-hydroxy-3-methoxy-α-methylcinnamic acid described below was determined on the basis of similar X-ray diffraction patterns and a mixture melting point which was not depressed.

Synthesis of 4-Hydroxy-3-methoxy-α-methylcinnamic Acid.—A mixture of 9.6 g. (0.1 mole) of fused and powdered sodium propionate, 13.7 g. (0.09 mole) of vanillin and 15.5 ml. (0.12 mole) of propionic anhydride was heated for 3 hr. at 135° in an oil-bath. The reaction mixture was cooled and poured into 50 ml. of water. The aqueous solution was extracted with two 50-ml. portions of ether, and the ether in turn was extracted twice with 10-ml. portions of 2 *N* sodium hydroxide. On acidifying the alkaline solution with 5 *N* sulfuric acid a crystalline precipitate was obtained. The product was recrystallized twice from methanol–water; yield 2 g., m.p. 166–167°. On titration two acidic groups were found, pK_a' 10.4 and 5.58 in 33% dimethylformamide (12.0 and 7.33 in 66% dimethylformamide).

Anal. Calcd. for $C_{11}H_{12}O_4$: C, 63.45; H, 5.81. Found: C, 63.48; H, 5.91.

Hygromycin Mercaptol.—A mixture of 10 g. of hygromycin, 15 ml. of 1 *N* hydrochloric acid and 20 ml. of ethyl mercaptan was shaken at room temperature for 2.5 hr. During this period three phases appeared. The oily bottom layer was separated, washed thoroughly with water and dissolved in ethanol. On concentrating the alcohol solution to dryness 7 g. of a white amorphous powder was obtained. The material had a pK_a' of 11.2 in 66% dimethylformamide, and its infrared spectrum was very similar to that of hygromycin, with the exception that the carbonyl band at 5.84 μ was no longer present.

Microbiologically active hygromycin was regenerated from the mercaptol. One gram of the mercaptol was dissolved in 20 ml. of ethanol. To the solution was added 900 mg. of mercuric chloride in 10 ml. of ethanol. The mixture was warmed on a steam-bath for 15 minutes and then was allowed to stand 1 hr. at room temperature. The insoluble ethyl mercaptide was removed by filtration, and the filtrate was saturated with hydrogen sulfide to remove excess mercuric chloride. Immediately after filtration the solution was neutralized with anion-exchange resin (Amberlite IR-45). The solution was concentrated *in vacuo* to a white powder having a pK_a' of 8.9 and ultraviolet and infrared spectra identical with those of hygromycin.

Preparation of Dihydrodeoxyhygromycin and its Degradation to 5,6-Dideoxy-D-arabohexose.—A mixture of 20 g. of hygromycin mercaptol and 250 g. of Raney nickel in 400 ml. of ethanol was refluxed with stirring for 4 hr. The suspension was filtered, and the Raney nickel was washed twice with 100-ml. portions of ethanol. The filtrate and washings were combined and concentrated *in vacuo* to dryness. Nine grams of amorphous dihydrodeoxyhygromycin was obtained. There was no absorption at 5.84 μ in the infrared, otherwise the spectrum was similar to that of hygromycin.

A mixture of 8 g. of dihydrodeoxyhygromycin, 15 ml. of 6 *N* hydrochloric acid and 25 ml. of ethyl mercaptan was shaken for 3.5 hr. The ethyl mercaptan phase was separated and concentrated to dryness *in vacuo*. The oily, partly crystalline residue was dissolved in hot water and was allowed to crystallize. Two recrystallizations from water yielded 1.3 g. of 5,6-dideoxy-D-arabohexose, m.p. 108–109°.

Anal. Calcd. for $C_{10}H_{22}S_2O_8$: C, 47.21; H, 8.72; S, 25.20. Found: C, 47.40; H, 8.68; S, 24.98.

Periodate Oxidation Product of 5,6-Dideoxy-D-arabohexose.—One hundred mg. of 5,6-dideoxy-D-arabohexose was dissolved in 50 ml. of water, and 1 g. of sodium metaperiodate was added. The solution was allowed to stand at room temperature for 1.5 hr. at which time the maximum consumption of 6 moles of periodate had been attained. The reaction mixture was distilled into a flask containing 50 mg. of 2,4-dinitrophenylhydrazine in 50 ml. of 2 *N* hydrochloric acid. The crystalline 2,4-dinitrophenylhydrazone which formed was recrystallized from 95% ethanol; yield 68 mg. (72%). The compound was shown to be identical with an authentic sample of propionaldehyde-2,4-dinitrophenylhydrazone by comparison of their X-ray diffraction patterns.

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[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY[1]]

The Viscosity and Opacity of Heated β-Lactoglobulin Solutions: The Effect of Salts, and Oxidizing and Reducing Reagents

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The viscosity of heated β-lactoglobulin solutions is *p*H-dependent. When solutions are salt-free the increases in viscosity on heating in the *p*H range 6.2 to 7.5 are relatively slight with a maximum at *p*H 6.8. In general, the viscosities are increased by salts with the greatest increases at the low *p*H values. There are, however, specific salt effects; sodium phosphate and citrate prevent the aggregation and concomitant opacity in the low *p*H region and bring about regular increases in viscosity. Sodium chloride, on the other hand, increases the viscosity but does not prevent opacity at low *p*H values, and there is a viscosity maximum at *p*H 6.7 with 0.025 *M* sodium chloride. In general, however, increases in viscosity and opacity are parallel. The increases in viscosity in the presence of salts are greatest with high concentrations of protein indicating a high degree of cross-reaction. The more viscous solutions also show structural viscosity. Treatment of β-lactoglobulin with iodine leads to some increase in the viscosity and clearing of the solutions which is consistent with sulfhydryl, disulfide participation in formation of opaque gels. Very large increases in viscosity are obtained in the presence of excess sulfhydryl reagents. The probable explanation of this increase is the opening of loops formed by disulfide bridges with consequent elongation of the molecule.

Protein solutions denatured by heat or urea become highly viscous or gel, a property which is strongly *p*H-dependent[2,3] with maximal gelling near the isoelectric point. The diminished gelling tendency at more remote *p*H values is viewed as a consequence of the increased electrostatic repulsion, and thereby decreased interaction, between the like-charged molecules.[2,3] In view of this interpretation of the *p*H dependence, the presence of salts would be expected to increase the viscosity by reduction of the electrostatic repulsion. The present studies with β-lactoglobulin and chiefly with the salts sodium phosphate and chloride, revealed in general the expected increase in viscosity and aggregation with increase in salt concentration. There were, however, exceptions to this generalization, apparent at low salt concentrations and low *p*H values, which indicated that there were also specific salt effects probably due to binding of the salt ions to the protein. The effects of treating β-lactoglobulin with iodine, and mercaptoethanol and other reducing agents before heating, were also studied because of the participation of sulfhydryl and disulfide groups in gel formation.[2,3]

Materials

β-Lactoglobulin.—This protein was prepared from raw milk by the method of Palmer[4] and recrystallized once. It was electrophoretically homogeneous at *p*H 8.6. This material was dried from the frozen state and stored as described previously.[5]

β-Lactoglobulin, Iodine-treated.—Ten milliliters 3.6% β-lactoglobulin in water at *p*H 7.5 was mixed with 1.0 ml.

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(2) (a) E. V. Jensen, V. D. Hospelhorn, D. F. Tapley and C. Huggins, *J. Biol. Chem.*, **185**, 411 (1950); (b) V. D. Hospelhorn and E. V. Jensen, THIS JOURNAL, **76**, 2830 (1954).

(3) H. K. Frensdorff, M. T. Watson and W. Kauzmann, *ibid.*, **75**, 5157 (1953).

(4) A. H. Palmer, *J. Biol. Chem.*, **104**, 359 (1934).

(5) C. A. Zittle and E. S. DellaMonica, *J. Dairy Sci.*, **39**, 514 (1956).