that these two fragments were adjacent and were connected through a simple peptide linkage involving the carboxyl group of tyrosine and the amino group of isoleucine, and the structure for oxytocin was postulated in accordance with this view.[18] However, inasmuch as such a degradation had not, so far as was known, been previously encountered in the chemistry of polypeptides and proteins, it was desirable to apply the degradative procedure to the synthetic compound. The early experiments[19] in this Laboratory on the cleavage of oxytocin itself with bromine water had given low yields of the fragments. Rather than apply the reaction to performic acid-oxidized synthetic material, the bromine water cleavage directly on oxytocin was reinvestigated. It has been found that cleavage in good yield can be obtained at $-10°$ with oxytocin under the conditions used in the case of the performic acid-oxidized oxytocin. A comparison of this reaction with the synthetic and natural compounds was made. Both give rise on treatment with bromine water followed by chromatography on paper in phenol to the two ninhydrin-positive spots, R_f 0.3 and 0.85, which have been identified as β-sulfoalanyldibromotyrosine and a sulfonic acid heptapeptide, respectively.[13,19]

The comparisons of the biological activities of the synthetic and natural oxytocin including the rat uterus contraction *in vitro*, the induction of labor in the human, the avian vasodepressor activity and the milk-ejecting activity in the human have been presented and discussed in the first section of this paper.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES]

Erythromycin. I. Properties and Degradation Studies[1]

BY EDWIN H. FLYNN, MAX V. SIGAL, JR., PAUL F. WILEY AND KOERT GERZON

RECEIVED JANUARY 16, 1954

The physical and chemical properties of erythromycin, a new and useful antibiotic, have been studied. Degradative studies have led to partial structures for erythromycin and some of its derivatives and degradation products.

Erythromycin[2] is a therapeutically useful, wide-range antibiotic produced by a strain of *Streptomyces erythreus*.[3] A brief description of some of its properties has been given.[3] It is a crystalline, colorless compound which is slightly soluble in water but dissolves easily in most of the common organic solvents. Crystals are obtained readily from aqueous acetone, aqueous alcohol or chloroform. Other solvents have been employed but those mentioned are the most useful. Crystals can be obtained from aqueous solution, but in this solvent solubility varies inversely with temperature in the ordinary temperature range. As might be expected from this temperature effect, the resulting crystals are hydrated. The hydrated crystals lose water on drying at 56° under reduced pressure. When dried in this manner, the crystals are hygroscopic and if exposed to moisture, regain the weight which was lost on drying. When erythromycin is allowed to crystallize from aqueous acetone, unstable solvated crystals are obtained which lose crystallinity on drying over phosphorus pentoxide. When crystallized from either solvent (see Experimental) the compound melts at 135–140°, and, if a slow rate of heating is continued, it partially resolidifies. It then melts at 190–193°. Solvated crystals are also obtained from other organic solvents. A detailed report on the crystallography of erythromycin is being published.[4]

Erythromycin is a base exhibiting a pK'_a of 8.6 when titrated in 66% dimethylformamide. It dissolves readily in dilute aqueous acids and forms crystalline salts with mineral acids. It is optically active with a specific rotation of $-73.5°$ in methanol (corrected to an anhydrous basis). The compound absorbs weakly in the ultraviolet; the single broad band has a maximum at about 278 mμ, ε 27. Its infrared absorption (Fig. 1) in chloroform solution is characterized by intense bands in the 8.5–10.0 μ region. These bands are probably due to the presence of C–O–C groupings. In the 5.5–6.0 μ region two maxima are observed. The first, at 5.78 μ, is comparable in intensity and position to the carbonyl band observed for a normal ester or six-membered lactone. The second band is at 5.91 μ and has an intensity which is about half that of the 5.78 μ band. A maximum at 2.84 μ indicates the presence of hydroxyl groupings in the molecule.

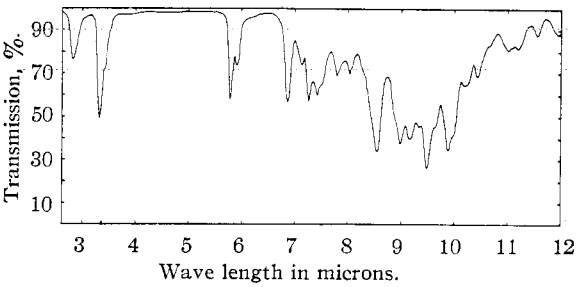


Fig. 1.—Infrared spectrum of erythromycin: 5.5% in $CHCl_3$, cell path, 0.093 mm.

The molecular weight of erythromycin was estimated by two independent methods. The free base, two salts and two other derivatives were subjected to X-ray crystallographic analysis.[5] Five independent molecular weight values were calculated. The average value derived for the molecular weight of the free base was 736. Maximum deviation from the average was 6 units. This method has a possible maximum error of ±2%, although it is felt that the results are more precise

(1) Presented before the Division of Organic Chemistry at the National Meeting of the American Chemical Society at Chicago, Illinois, September 6–11, 1953. Subsequent to the presentation of this paper, an article appeared which described some experiments similar to those recorded here. See R. B. Hasbrouck and F. C. Garven, *Antibiotics and Chemotherapy*, **3**, 1040 (1953).

(2) The Eli Lilly and Company trade-mark for the antibiotic erythromycin is "Ilotycin."

(3) J. M. McGuire, R. L. Bunch, R. C. Andersen, H. E. Boaz, E. H. Flynn, H. M. Powell and J. W. Smith, *Antibiotics and Chemotherapy*, **2**, 281 (1952).

(4) S. F. Kern and H. A. Rose, in preparation.

(5) A detailed report of the method used will be published by H. A. Rose and S. F. Kern.

than this. Table I gives the values obtained for the compounds used.

TABLE I

Compound	Mol. wt. of compound	Derived mol. wt. of free base
Erythromycin dihydrate	773	737
Erythromycin oxalate	863	737
Erythromycin hydroiodide dihydrate	902	738
Erythromycin N-oxide[a]	758	742
O,N-Dicarbethoxydes-N-methylerythromycin[a]	861	730

[a] See text for description of the chemistry of this compound.

The apparent molecular weight of erythromycin also was determined by titration, using a technique which is precise to $\pm 0.10\%$. The value obtained was 738.1. These values, together with analytical data given in the Experimental section, indicate the molecular formula to be $C_{37}H_{67-69}NO_{13}$ (mol. wt., 733.9–735.9). This composition is also supported by degradation studies.

Functional group analyses on erythromycin indicate the presence of one methoxyl, two N-methyl, and eight or more (18%) C-methyl groups. No hydrogen uptake was observed when an ethanol solution of erythromycin was treated with platinum oxide and hydrogen. In glacial acetic acid solution, however, one mole of hydrogen was absorbed per mole of erythromycin. Acetylation of the antibiotic with acetic anhydride, using pyridine as catalyst, yielded an amorphous product having two acetyl groups per molecule. Weak hydroxyl absorption was still detectable in the infrared spectrum of this product, suggesting the presence of tertiary hydroxyl. Active hydrogen determinations indicated three replaceable hydrogens in erythromycin.

Degradation Studies

Mild acid hydrolysis of erythromycin (I) yielded two compounds. One of these is a basic, crystalline solid melting at 207–208° and has the probable composition $C_{29}H_{49}NO_8$. We propose to name this compound *erythralosamine* (II). The other product is a high boiling liquid for which we propose the name *cladinose* (III). It is a neutral substance and has the molecular formula $C_8H_{16}O_4$.

Hydrolysis of erythromycin with 25% hydrogen bromide in acetic acid yielded dimethylamine. Hydrolysis with 6 *N* hydrochloric acid resulted in extensive decomposition but a crystalline, basic compound was isolated as the hydrochloride and proved to have the composition $C_8H_{17}NO_3 \cdot HCl$. This substance, which appears to be a dimethyl aminodeoxy aldose, has been named *desosamine* (VI). Propionaldehyde was also isolated from the hydrolysis mixture as the 2,4-dinitrophenylhydrazone.

When erythromycin was dissolved in dry methanol containing 1% hydrogen chloride, two products were formed. The first of these was erythralosamine ($C_{29}H_{49}NO_8$) which has been mentioned. The second was a liquid which has the composition $C_9H_{18}O_4$; it has been designated as methyl cladinoside (IV).

Early work on erythromycin had shown that biological activity disappeared rapidly when the antibiotic was placed in solution at *p*H 10 or higher. Accordingly, alkaline degradation was investigated to determine the nature of these changes. It was found that treatment with 0.01 *N* sodium hydroxide for 24 hours at room temperature produced a new compound which behaved like a zwitterion. This substance contained an acidic group which had a pK'_a of 4.3 in water. The basic function had a pK'_a of 9.1. The infrared absorption curve no longer had the 5.78 μ band present in erythromycin, but there was a new absorption maximum at 6.3 μ. A carbonyl band at 6.0 μ also was present. The ultraviolet absorption was characterized by a single maximum at 235 mμ, $\epsilon = 6000$, indicating formation of the grouping $>C{=}C{-}\overset{\overset{O}{\|}}{C}-$. Treatment with diazomethane gave a basic compound which had a carbonyl band at 5.76 μ.

Reaction of erythromycin with 0.005 *N* sodium hydroxide formed a product VIII with similar properties, *i.e.*, a zwitterionic compound which differed in composition from erythromycin by the addition of water to the molecule. However, its ultraviolet absorption spectrum differed from that of the product obtained from 0.01 *N* sodium hydroxide. It appears, therefore, that two or more reactions occur in dilute sodium hydroxide. It is felt that these results indicate the presence of a lactone grouping in erythromycin. A possible alternative grouping consistent with these reactions and with the changes in infrared absorption, is a 2,2-disubstituted-1,3-diketone.

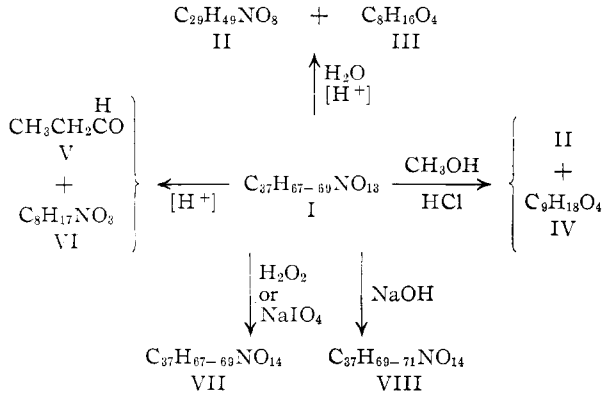
Heating erythromycin with 1 *N* sodium hydroxide yielded propionaldehyde which was isolated as the 2,4-dinitrophenylhydrazone. No dimethylamine and no volatile acid could be detected in the reaction mixture.

The antibiotic has been oxidized with sodium metaperiodate in dilute aqueous ethanol and in aqueous dioxane solution. Two moles of oxidant was consumed, the first in less than five minutes and the second after an additional 24 hours. Very slow additional uptake continued. Such a reaction mixture yielded erythromycin N-oxide (VII) as one of the products. This derivative was also obtained by oxidation of erythromycin with dilute hydrogen peroxide. Its formulation as an N-oxide is based on the facts that one oxygen atom was introduced into the molecule and that the product showed a large decrease in basicity. Its pK'_a was 6.0 when titrated in 80% dimethylformamide. Periodate oxidation of erythromycin did not appear to be a simple reaction, however, since evidence was obtained which indicated the presence of other reaction products in the reaction mixture. This conclusion is supported by the fact that erythromycin N-oxide reacted with periodate, one mole being consumed in less than 20 hours. Oxidation of erythromycin in a buffered solution (aqueous sodium acetate-acetic acid and dioxane) followed a different course than is indicated above. Periodate uptake was slower, one mole being used in about nine hours. Uptake continued but at a decreased rate until nearly two moles was used in five days.

Erythromycin reacts readily with acid chlorides to form crystalline monoacyl derivatives. The preparation of a number of such compounds has been described.[6] In every case, the monoacyl erythromycin showed a decrease in basicity from the value of the parent compound. Typical of the acyl derivatives prepared were the benzoate, propionate, allyl carbonate, propargyl carbonate and ethyl carbonate. The pK'_a values of the acyl derivatives fell in the range 6.4–6.8 as compared to 8.6 for erythromycin when titrated under the same conditions, *i.e.*, in 66% dimethylformamide solution. It is believed that the decrease in basicity after acylation indicates that the esterified hydroxyl is on a carbon adjacent to the one bearing the dimethylamino group XV.

A second compound, formed as a by-product in the acylation with ethyl chloroformate, was isolated as a crystalline solid melting at 218–220°. This substance was neutral and analyzed satisfactorily for an erythromycin bis-(ethyl carbonate) less one methyl group. Its structure is indicated by the fact that hydrolysis with hydrogen bromide in acetic acid yielded methylamine instead of dimethylamine, which had been obtained from erythromycin under similar conditions. The compound, therefore, must be O,N-dicarbethoxydes-N-methylerythromycin (XVI). Elimination of an N-methyl group has been reported by reaction of a tertiary amine with ethyl chloroformate.[7]

Hydrolysis of erythromycin ethyl carbonate occurred very readily. A high yield of erythromycin was obtained when the ethyl carbonate ester was dissolved in 40% methanol and allowed to stand at room temperature for two days.



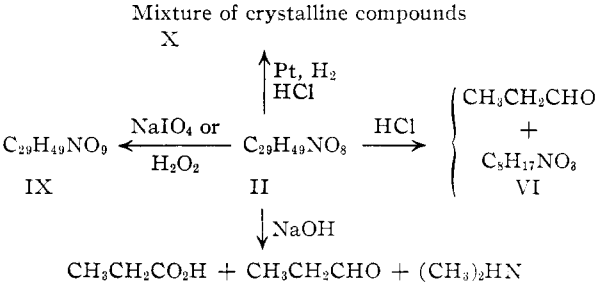
Erythralosamine, $C_{29}H_{49}NO_8$

Erythralosamine, described above as a product resulting from mild acid hydrolysis of erythromycin, contained no methoxyl group. The C-methyl content measured by the Kuhn–Roth procedure was 16%, indicating at least six C-methyl groups per molecule. An active hydrogen determination with lithium aluminum hydride gave variable results. Two, or possibly three, active hydrogens were indicated. Erythralosamine N-oxide (IX) was formed by treatment with dilute hydrogen peroxide. Erythralosamine was also oxidized by periodate, consuming one mole of oxidant and forming the N-oxide. This N-oxide gave a pK'_a of 5.70 when titrated in 66% dimethylformamide but in water the pK'_a was 4.35. This change in dissociation of a base with change in solvent has not been experienced previously in these laboratories although acidic groups are known to undergo such a shift.[8]

Erythralosamine absorbed one mole of hydrogen when dissolved in acetic acid with platinum as catalyst. Hydrogenation of a dilute aqueous hydrochloric acid solution resulted in the uptake of 1.8 moles of hydrogen. From this reaction a mixture is obtained which is incompletely characterized as yet.

When erythralosamine was hydrolyzed with 6 *N* hydrochloric acid, propionaldehyde and desosamine were formed. Hydrolysis with 1 *N* sodium hydroxide in aqueous methanol liberated propionic acid, propionaldehyde and dimethylamine. Erythralosamine was relatively stable to base, however, compared to erythromycin. It was recovered unchanged after standing for 27 hours at 25° in 8% ethanolic potassium hydroxide solution. This stability points to a change in the lactone grouping which may be present in erythromycin. The presence of a carboxyl type carbonyl in erythralosamine is indicated, however, by infrared absorption at 5.78 μ and the lack of ultraviolet absorption. Erythralosamine was recovered unchanged after treatment with anhydrous ammonia in methanol at 100° or with anhydrous hydrazine in ethanol at reflux temperature.

The following chart summarizes the principal reactions discussed above.



Desosamine, $C_8H_{17}NO_3$

Desosamine, isolated after drastic acid hydrolysis of erythromycin, was crystallized as the hydrochloride. Its formulation as $C_8H_{17}NO_3 \cdot HCl$ is compatible with elemental analyses and a molecular weight of 214, estimated by electrometric titration. The calculated molecular weight is 211.7. The free base, m.p. 83–83.5°, was isolated by dissolving the hydrochloride in water, adjusting to *p*H 10, and extracting with ether. The compound had a pK'_a of 8.6. It exhibited no ultraviolet absorption. Its infrared absorption indicated that hydroxyl and –C–O– groups were present. No carbonyl was apparent. Group analyses suggested one C-methyl and two N-methyl groups.

Desosamine reduced Fehling solution and gave a positive iodoform reaction. It dissolved in concentrated sulfuric acid to give a colorless solution. Acetylation of the hydrochloride with acetic anhydride and pyridine furnished the diacetate hydro-

(6) H. Murphy, *Antibiotics and Chemotherapy*, in press.

(7) F. Bayer and Co., German Patent 255,942 (1911).

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

chloride. Desosamine was oxidized readily by periodate but the uptake varied, depending on experimental conditions. In dilute solution at a *p*H of 4.5–5.5, one mole was used in less than five minutes. In more concentrated, basic solution three moles was used in 28 hours. In concentrated, weakly acidic solution, uptake of one mole occurred rapidly but additional periodate was consumed after several hours. The results indicate the presence of two hydroxyl groups on adjacent carbon atoms.

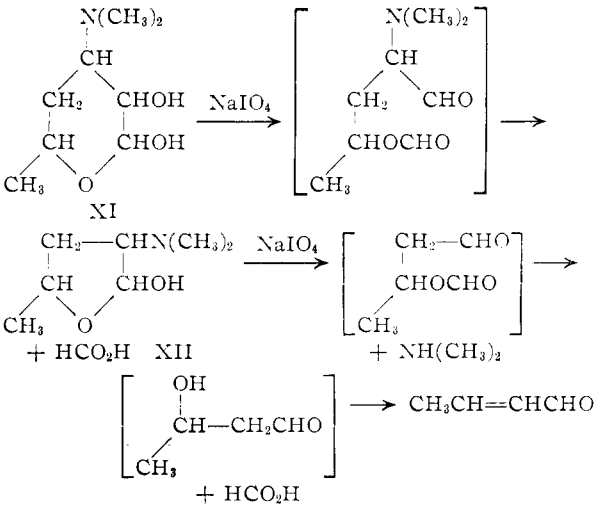
Treatment of desosamine with methanol and hydrogen chloride led to the formation of methyl desosaminide. This product did not reduce Fehling solution. Methyl desosaminide did not give dimethylamine when hydrolyzed with base, while desosamine did. This is in keeping with the expected differences between a glycoside and an amino aldose. The analysis was satisfactory for the methyl glycoside. Ethyl desosaminide and *n*-butyl desosaminide were isolated in small amounts from acid hydrolysates of erythralosamine when ethanol or *n*-butanol was used during isolation of desoamine.

The foregoing results indicate the presence of a hemiacetal grouping in the molecule, a dimethylamino function, and a C-methyl group. The quantitative periodate studies suggest that the two hydroxyl groups are on adjacent carbon atoms. The high yield of acetic acid obtained in the Kuhn–Roth determination suggests that the C-methyl is on a carbon bearing an oxygen function. This conclusion is supported by the fact that the reaction mixture resulting from periodate oxidation of desosamine gave a positive iodoform test with extreme ease.

The variability in periodate uptake exhibited by desosamine, depending on experimental conditions, suggests that the dimethylamino group may be involved. It has been reported that tertiary amines are not oxidized by periodate at an appreciable rate.[9] However, experiments in this Laboratory have shown that diethylaminoethanol and 3-diethylaminopropanol-1 are readily oxidized. The former consumed 0.9 mole of periodate in 51 hours, and the latter, one mole in 45 hours.

Structure XI has been proposed for desosamine, based on studies analogous to those reported here.[10] When desosamine was oxidized with two moles of periodate, crotonaldehyde was isolated from the reaction mixture as its 2,4-dinitrophenylhydrazone. Formic acid and dimethylamine also were formed.[10] Crotonaldehyde formation agrees with data obtained here. The formation of crotonaldehyde probably occurs as indicated in the reaction scheme, *via* formylacetaldol and acetaldol. When desosamine was treated with one mole of periodate a product was isolated readily, simply by adjusting the reaction mixture to *p*H 10 and extracting with ether. This oxidation product formed a crystalline hydrochloride and appears to be the 2-dimethylaminopentose (XII) resulting through the loss of the aldehyde carbon from desosamine.

A "novel amino sugar," $C_8H_{17}NO_3$, has been reported as a degradation product of picromycin.[11] Although no properties were given, it is interesting to speculate on its possible identity with desosamine in view of certain chemical similarities between erythromycin and picromycin. Furthermore, the antibacterial spectra of these two antibiotics are similar.



Cladinose, $C_8H_{16}O_4$

Cladinose, $C_8H_{16}O_4$, has been mentioned as the second product formed by mild acid hydrolysis of erythromycin. The presence of one methoxyl group was shown by a Zeisel determination. Absorption in the infrared at 2.81 and 2.91 μ indicated the existence of two hydroxyl groups in the molecule. A low absorption was noted in the carbonyl region. Cladinose was soluble in water, alcohol, ether, benzene, chloroform and carbon tetrachloride. The presence of a reducing group was shown by positive Fehling and Tollens tests. It underwent an iodoform reaction but was not oxidized by periodate. Treatment with concentrated hydrochloric acid gave a dark green solid. Titration with iodine by the Willstätter–Schudel procedure[12] gave an uptake of slightly more than one mole of iodine in less than two minutes. Cladinose reacted with acetic anhydride to yield a low-melting diacetate which was easily purified by distillation. It also reacted with methanol containing dry hydrogen chloride to yield methyl cladinoside, the same compound obtained by methanolysis of erythromycin.

Methyl cladinoside was a mobile liquid which boiled at a lower temperature than did cladinose. It, too, was optically active and was soluble in water and most organic solvents. Analytical data indicated the molecular formula $C_9H_{18}O_4$, the presence of two C-methyl groups and two methoxyl groups. The infrared absorption spectrum had a band at 2.81 μ, indicating the presence of hydroxyl in the molecule. Fehling and Tollens tests were negative. No iodoform reaction occurred. Periodate had no effect on methyl cladinoside. The compound formed two isomeric mono-(3,5-dinitrobenzoates). These were separated by chromatog-

(9) R. Adams, "Organic Reactions," Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1944, p. 343.

(10) R. K. Clark, *Antibiotics and Chemotherapy*, **3**, 663 (1953).

(11) H. Brockman, *Angew. Chem.*, **65**, 257 (1953).

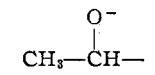
(12) R. Willstätter and G. Schudel, *Ber.*, **51**, 780 (1918).

raphy and presumably differ in configuration at carbon 1. This would indicate, in conjunction with data which follows, that the methyl cladinoside formed by reaction with methanol is a mixture of the α- and β-methyl glycosides. Aqueous 1.2 *N* hydrochloric acid hydrolyzed methyl cladinoside to yield cladinose.

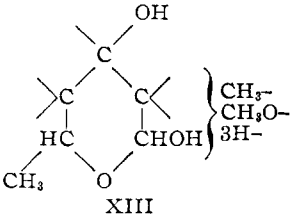
Cladinose reacted with ethyl mercaptan and dry hydrogen chloride, forming ethyl thiocladinoside, analogous to the reaction with methanol.

Positive reduction tests with Fehling and Tollens reagents indicated the presence of an aldehyde or potential aldehyde group in cladinose. Since very little carbonyl absorption was apparent in the infrared spectrum, a hemiacetal structure is indicated. The ready formation of methyl cladinoside and its conversion back to cladinose by acid hydrolysis is in keeping with this conclusion. The very rapid oxidation observed in the Willstätter–Schudel titration can be explained by a hemiacetal structure.

The formation by cladinose of a diacetyl derivative and the presence of two absorption bands of equal intensity in the hydroxyl region of its infrared spectrum provides adequate evidence for the existence of two hydroxyl groups. Methyl cladinoside contains one hydroxyl group since it formed a mono-(3,5-dinitrobenzoate) which showed no hydroxyl absorption in its infrared spectrum. The positive iodoform reaction with cladinose indicates the presence of the grouping

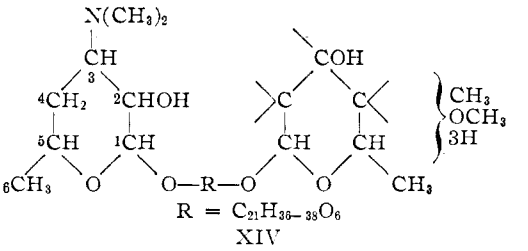


The oxygen on the carbon alpha to the methyl group must be in a ring since methyl cladinoside did not give a positive iodoform test. The two hydroxyl groups in cladinose are not situated on adjacent carbon atoms since no periodate uptake occurred. Those carbon atoms bonded to the ring oxygen probably are not adjacent to a carbon bearing a hydroxyl group because of the lack of reaction with periodate. The properties of cladinose are consistent, therefore, with a six-carbon chain and a pyranose ring structure. Structures containing a five-membered ring are not excluded by these data but they are thought to be less likely. The following partial structure XIII may be written from these data if the presence of a six-membered ring is assumed



XIII

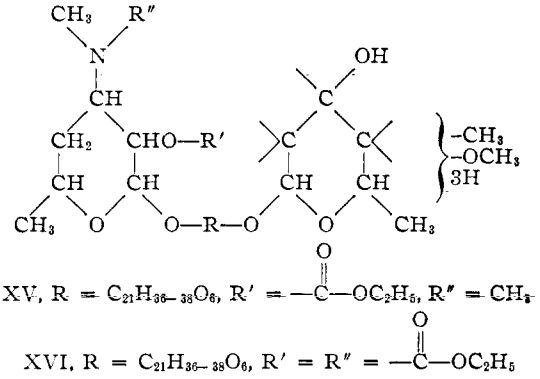
With structure XI for desosamine and the partial structure XIII for cladinose, a partial structure for erythromycin may be postulated as XIV. The glycosidic linkage to desosamine is indicated by the monoacyl derivatives described earlier, which make attachment unlikely through the single free hydroxyl group on carbon 2 in desosamine. The glycosidic linkage to cladinose is indicated by the



XIV

formation of methyl cladinoside on methanolysis of erythromycin and both glycosidic linkages are substantiated by the fact that erythromycin is not reducing in nature. That the desosamine moiety is not removed by methanolysis may be explained by analogy with the known stability of glycosides of 2-amino sugars and probably also 3-amino sugars.[13]

Erythromycin ethyl carbonate may be formulated as XV and O,N-dicarbethoxydes-N-methylerythromycin as XVI.



XV, R = $C_{21}H_{36-38}O_6$, R′ = $-C(=O)-OC_2H_5$, R″ = CH_3

XVI, R = $C_{21}H_{36-38}O_6$, R′ = R″ = $-C(=O)-OC_2H_5$

Acknowledgment.—The authors are grateful to Messrs. W. L. Brown, H. L. Hunter and G. M. Maciak for microanalyses; to Drs. H. Boaz, S. F. Kern and H. A. Rose, and Messrs. J. Forbes and D. O. Woolf, Jr., for physical chemical data; and to Drs. R. G. Jones and E. R. Shepard for helpful advice.

Experimental[14]

Crystallization of Erythromycin. (a) From Chloroform.—Two kilograms of erythromycin was dissolved in sufficient chloroform to give a solution volume of 20 liters. It was necessary to warm the suspension to 40° to effect complete solution. After filtration, the solution was kept at −15° for two days. The crystals were removed by filtration and dried in an oven at 80° for 16 hours. The yield was 1760 g. of nearly colorless product with a m.p. of 135–140°. Recrystallization in this manner served to remove much of the color found in crude preparations of erythromycin. (b) **From Aqueous Acetone.**—Erythromycin crystallized readily from water–acetone mixtures when it was first dissolved in acetone (5 ml./g.), filtered, and an equal volume of water added to the filtrate. Such crystals gave a satisfactory X-ray diffraction pattern before drying but when dried the X-ray pattern lost all but the strongest lines. Similar behavior was encountered when water–ethyl alcohol mixtures were used, except that crystallization proceeded more

(13) A. B. Foster and M. Stacey, "Advances in Carbohydrate Chemistry," Vol. VII, Academic Press, Inc., New York, N. Y., 1952, pp. 271, 275.

(14) Melting points were determined on a Kofler micro melting point apparatus unless indicated otherwise. Infrared spectra were obtained with a Beckman IR-2T spectrometer. Ultraviolet measurements were made with a Cary recording spectrometer. We wish to acknowledge the technical assistance of Miss Ione Thompson, Miss Ollidene Weaver and Dr. H. Latham Breunig in carrying out part of the experimental work.

slowly. (c) **From Water.**—Erythromycin (6.1 g.) was suspended in one liter of distilled water and the suspension was stirred at −15° until the mixture was partially frozen (four hours in a chill room). Nearly all of the solid had dissolved. The cold solution was filtered and the filtrate was warmed slowly to 55°. The crystals were removed and dried for 2.5 hours at 8 mm. Four and six-tenths grams was recovered, m.p. 135–140°, resolidifying and melting at 190–193°.

Erythromycin Molecular Formula.—Data supporting the formula $C_{37}H_{67}NO_{13}$ for erythromycin have been cited in the Discussion. In the course of this work numerous elemental analyses have been made on erythromycin and its derivatives. Early work, which led to the postulation of a C_{34-36} formula[3] and which later seemed to favor a C_{39} formula, has been supplanted by the evidence described in the Discussion. The change is a result of more experience in purifying the compounds involved and of a better understanding of the properties of erythromycin and its derivatives. Analytical values supporting the C_{37} formula are given below. These data are the most reliable in our opinion and were obtained specifically to attempt to establish the molecular formula with some exactitude. (a) **Erythromycin.**—The analytical sample was prepared by recrystallizing commercial erythromycin twice from chloroform and twice from water as described above. The erythromycin used had maximum microbiological activity before these procedures were applied to it. The sample was pig-dried for 2 hours at 56° and 0.1 mm. before being analyzed in quadruplicate.

Anal. Calcd. for $C_{37}H_{67}NO_{13}$: C, 60.58; H, 9.20; N, 1.91; N-CH_3 (2), 4.03; C-CH_3 (9), 18.4; OCH_3 (1), 4.2; wt. loss, 4.68 ($2H_2O$). Found: C, 60.48 ± 0.20; H, 9.25 ± 0.09; N, 1.82; N-CH_3, 4.6; C-CH_3, 17.3, 18.3; O-CH_3, 4.3; wt. loss, 4.27 ± 0.11.

The above values are illustrative. Other samples have been analyzed with comparable results. (b) **Erythromycin N-Oxide.**—Erythromycin N-oxide was isolated after oxidation of erythromycin with periodate. It was recrystallized from chloroform–ether twice and from methanol–ether once. It was pig-dried at 100° and 0.1 mm. for two hours before being analyzed.

Anal. Calcd. for $C_{37}H_{67}NO_{14}$: C, 59.26; H, 9.01. Found: C, 59.15, 59.22; H, 8.94, 9.01.

Other preparations gave values comparable to those cited.

The C_{37} formula is also indicated by the fact that a C_{29} compound (erythralosamine) and a C_8 compound (cladinose) result from mild acid hydrolysis. No small fragments were detectable in addition to these two substances. A definite decision regarding the number of hydrogens in the erythromycin molecule cannot be made on the basis of available data.

Acetylation of Erythromycin.—Erythromycin (1.0 g.) was dissolved in 10 ml. of pyridine and 2.0 ml. of acetic anhydride was added. The reaction mixture was kept at room temperature overnight, and then it was poured onto cracked ice. The turbid solution was extracted three times with 50-ml. portions of chloroform. The combined chloroform extract was washed with saturated aqueous sodium bicarbonate solution, dried with anhydrous magnesium sulfate, and the chloroform removed under reduced pressure. The product was dissolved in ether and petroleum ether was added. The solution was concentrated to dryness, giving a white, finely divided powder.

Anal. Calcd. for $C_{37}H_{67}NO_{13}\cdot 2CH_3CO$-: CH_3CO-, 10.5. Found: CH_3CO-, 11.0, 10.25.

Hydrogenation of Erythromycin.—Erythromycin (35 mg.) was reduced in the presence of platinum catalyst with glacial acetic acid as the solvent. One mole equivalent of hydrogen was absorbed. The glacial acetic acid was removed under reduced pressure, leaving a white, amorphous solid. When submitted for assay this product had about 2.5% of the microbiological activity of erythromycin.

Attempted reduction of erythromycin in the presence of platinum catalyst with ethanol as the solvent resulted in no hydrogen uptake.

Erythromycin N-Oxide.—Erythromycin (20 g.) was dissolved in 1200 ml. of 60% methanol containing 3% hydrogen peroxide. The resulting solution was allowed to stand at room temperature for 48 hours and then was concentrated under reduced pressure to remove methanol. The remaining aqueous suspension was extracted with one 400-ml. portion and three 200-ml. portions of chloroform. The chloroform extract was concentrated to dryness under reduced pressure. The residue was redissolved in 300 ml. of chloroform; the addition of 500 ml. of petroleum ether (Skellysolve B) caused an immediate precipitation of a white solid. This product weighed 19.0 g. and when recrystallized from methanol–ether, melted at 218–222°; after another recrystallization the melting point was 220–223°.

Anal. Calcd. for $C_{37}H_{67}NO_{14}$: C, 59.26; H, 9.01; N, 1.87. Found: C, 59.44; H, 9.02; N, 1.93.

O,N-Dicarbethoxydes-N-methylerythromycin.[15]—Erythromycin was allowed to react with ethyl chloroformate as described by Murphy.[6] O,N-Dicarbethoxydes-N-methylerythromycin was relatively insoluble in acetone while erythromycin ethyl carbonate was very soluble. Thus the two compounds were separated readily by dissolving the crude reaction product in acetone. The insoluble dicarbethoxy derivative was recrystallized from ethanol–water mixtures. The purified product melted at 218–220°. The less pure preparations melted at 200–210°.

Formation of the dicarbethoxy compound was minimized when one mole equivalent of ethyl chloroformate was used in the reaction. Increasing the molar ratio of ethyl chloroformate to erythromycin increased the amount of O,N-dicarbethoxydes-N-methylerythromycin formed. With one mole equivalent, the yield was about 5%; with two mole equivalents, the yield increased to 25%; and when the molar ratio was increased to five, a 70% yield of the dicarbethoxy derivative was obtained based on the erythromycin present.

Hydrolysis of Erythromycin Ethyl Carbonate.—Erythromycin ethyl carbonate (10.0 g.) was dissolved in 1 l. of methanol, then 1 l. of water was added. The solution was allowed to stand at room temperature for 48 hours. It was concentrated under reduced pressure to about one-half of the original volume. The water solution which remained was extracted three times with 200-ml. portions of benzene. The benzene extract was concentrated to dryness under reduced pressure, leaving 8.0 g. of white solid. This was dissolved in 60 ml. of acetone and 75 ml. of water was added to the warm solution. The crystals which formed over a 48-hour period were removed and identified as erythromycin. The yield was 5.65 g. (64%) having full microbiological activity.

Methylamine from O,N-Dicarbethoxydes-N-methylerythromycin.—O,N-Dicarbethoxydes-N-methylerythromycin (3.0 g.) was dissolved in a mixture of 22.5 ml. of 48% hydrobromic acid and 22.5 ml. of glacial acetic acid. The solution was heated under reflux for 16 hours, and then concentrated to dryness under reduced pressure. Water was added and removed under reduced pressure to ensure complete removal of excess hydrobromic acid. The residue was dissolved in chloroform and extracted continuously with water for 24 hours. The aqueous extract was concentrated, leaving a partially crystalline residue. The product was recrystallized by dissolving in ethyl alcohol and adding ether. The resulting crystals melted at 245–250°. After an additional recrystallization, the product was shown to be methylamine hydrobromide by comparison of the X-ray patterns obtained from it and from an authentic sample.

Methanolysis of Erythromycin.—Erythromycin (40 g.) was dissolved in two liters of methanol containing 1% hydrogen chloride. The reaction mixture was allowed to stand for 24 hours and then was poured into 600 ml. of water containing 46 g. of sodium bicarbonate. The resulting suspension was concentrated under reduced pressure to remove methanol and the remaining aqueous suspension was then extracted with chloroform (300 ml., followed by two 150-ml. portions). The chloroform extract was washed with 100 ml. of 2% aqueous sodium carbonate, with 200 ml. of water, and then concentrated to dryness under reduced pressure. The residue was dissolved in 200 ml. of benzene. When 800 ml. of petroleum ether (Skellysolve B) was added a crystalline product precipitated which weighed 16.5 g. and melted at 205–207°, with prior sublimation. After further recrystallization the melting point was 207–208°. The pK'_a in 66% dimethylformamide was 8.5.

Anal. Calcd. for $C_{29}H_{49}NO_8$: C, 64.53; H, 9.17; N, 2.59; C-CH_3 (6), 16.7; mol. wt., 539.7. Found: C, 64.38,

(15) This compound was isolated and supplied in quantity by Dr. H. Higgins. He also studied reaction conditions favoring its formation.

64.50, 64.97; H, 9.15, 9.19, 9.45; N, 2.58, 2.59, 2.80; O-CH_3, 0.0; C-CH_3, 16.9; mol. wt., 549 (electrometric titration).

Hydrolysis of Erythromycin with 0.01 *N* Sodium Hydroxide.—Erythromycin (4.0 g., 5.45 m*M*) was suspended in 1.0 l. of 0.010 *N* sodium hydroxide and the mixture was shaken vigorously with a mechanical shaker for 24 hours. All of the solid had dissolved after this time. The colorless solution was extracted once with 100 ml. of chloroform, then three times with 50-ml. quantities of chloroform. This procedure removed no material, showing that the erythromycin had reacted completely. Extractions at *p*H 6.4 and 2.9 also failed to remove any material. The solution was adjusted to *p*H 7.0 and concentrated to dryness under reduced pressure. The residue was triturated with three 25-ml. portions of ethyl alcohol. The alcohol solution was evaporated to dryness under reduced pressure and the residue was dissolved in chloroform. Filtration removed a small amount of inorganic contaminant, leaving 3.80 g. of chloroform soluble product. This material showed the presence of two titratable groups, the original basic group and a new acidic group, indicating it to be a zwitterion.

Reaction of Alkaline Hydrolysis Product with Diazomethane.—The zwitterion described above (1.0 g.) was dissolved in 10 ml. of methylene chloride and the solution was cooled to 0°. An excess of diazomethane in methylene chloride was added. Nitrogen was evolved immediately. The solution was taken to dryness under reduced pressure leaving a colorless product. This material showed an ester carbonyl absorption at 5.76 μ in the infrared, in contrast to the 0.01 *N* sodium hydroxide hydrolysis product which lacked this band but had a 6.3 μ band characteristic of a carboxyl group.

Hydrolysis of Erythromycin with 0.005 *N* Sodium Hydroxide.—A mixture of 1.0 g. of erythromycin and 300 ml. of 0.005 *N* sodium hydroxide was refluxed for 1.5 hours. The hot solution was filtered, and 14 ml. of 0.1 *N* hydrochloric acid was added to the filtrate which was then cooled and adjusted to *p*H 7.0 with 0.01 *N* hydrochloric acid. The solution was evaporated to dryness under reduced pressure at room temperature. The residue was extracted with four 5-ml. portions of hot, dry acetone. The extracts were filtered hot, combined and evaporated to dryness. This procedure was repeated using two 5-ml. portions of hot, dry acetone except that the combined extracts were filtered through a layer of Celite. The amorphous residue weighed 0.39 g. and melted at about 120–130°. Titration in water showed a pK'_a of 4.1 and 9.12, apparent molecular weight, 750. The infrared absorption spectrum showed strong absorption at 2.9, 5.8 and 6.3–6.4 μ, with a shoulder at 6.0 μ. The ultraviolet spectrum showed a peak at 229 mμ, ϵ 3,650 and a shoulder at 263 mμ, ϵ 1,110.

Anal. Calcd. for $C_{37}H_{69}NO_{14}$: C, 59.04; H, 9.24; N, 1.86. Found: C, 59.39; H, 9.54; N, 1.92.

Dimethylamine from Erythromycin.—Four grams of erythromycin and 6.0 g. of selenium were thoroughly mixed. The flask containing the mixture was connected to a distillation apparatus and placed in a metal-bath previously heated to 320°. After considerable evolution of gas, a liquid was distilled which boiled at 87–98°. The distillate was diluted with 10 ml. of water, and the water layer was removed. A saturated solution (2.5 ml.) of picric acid in alcohol was added to the water layer. The resulting solution was allowed to stand at room temperature until about two-thirds had evaporated, then the remainder was refrigerated. This solution deposited 43 mg. of yellow crystals, m.p. 149–153°. Two recrystallizations from alcohol gave a product melting at 156°; the melting point was undepressed by admixture with dimethylamine picrate. An X-ray diffraction pattern also indicated identity with dimethylamine picrate.

Anal. Calcd. for $C_8H_{10}N_4O_7$: N, 20.43. Found: N, 20.14.

Propionaldehyde from Acid Hydrolysis of Erythromycin.—Erythromycin (12.4 g.) was dissolved in 500 ml. of 10% sulfuric acid. The solution was then distilled, keeping the volume in the distilling flask above 360 ml. The distillate (600 ml.) was mixed with 2,4-dinitrophenylhydrazine reagent (1 g. of 2,4-dinitrophenylhydrazine dissolved in 300 ml. of 6 *N* hydrochloric acid, then diluted to 1 l.) until no further precipitate formed. The precipitated 2,4-dinitrophenylhydrazone weighed 0.75 g. An additional 0.24 g. was obtained by repeating the procedure, for a total yield of 25%. The product was identified as a mixture of the two forms of propionaldehyde 2,4-dinitrophenylhydrazone.[16]

Propionaldehyde from Alkaline Hydrolysis of Erythromycin.—A mixture of 10 g. of erythromycin and 500 ml. of 1.0 *N* sodium hydroxide solution was refluxed for two hours. The reaction mixture was distilled until 250 ml. of distillate had been collected. One hundred and fifty milliliters of 2,4-dinitrophenylhydrazine reagent[17] was added to the distillate. A yellow solid (179 mg.) was formed. The solid was dissolved in 40 ml. of benzene–Skellysolve B (30:70 mixture) and chromatographed on 6.0 g. of silica. The same solvent was used for elution. The effluent was collected in 15-ml. fractions after colored eluate began to appear.

Fractions 7–10 inclusive were combined, concentrated to dryness, and recrystallized twice from alcohol to give a product melting at 140–142°. This material was identified as the 2,4-dinitrophenylhydrazone of propionaldehyde by X-ray diffraction pattern and by mixed melting point.

The material from fractions 3, 4 and 5 was combined and recrystallized twice from alcohol. The product melted at 142–144°. Admixture of this material with the other 2,4-dinitrophenylhydrazone or the 2,4-dinitrophenylhydrazone of propionaldehyde caused no lowering of melting points. Comparison of the X-ray diffraction pattern of this compound with that of a second form[16] of propionaldehyde 2,4-dinitrophenylhydrazone indicated that they were identical.

Anal. Calcd. for $C_9H_{10}N_4O_4$: N, 23.53. Found: N, 23.23.

Desosamine from Erythromycin.—Erythromycin (200 g.) was dissolved in one l. of ethyl alcohol. The solution was added to 3.2 l. of 6 *N* hydrochloric acid and heated at reflux temperature for 4 hours. The dark reaction mixture was cooled in an ice-bath, then the liquid was decanted from a dark insoluble material which had formed during hydrolysis. The solution was extracted with five 250-ml. portions of chloroform. The aqueous layer was concentrated under reduced pressure to about one-half volume, to remove ethyl alcohol. 1-Butanol (500 ml.) was added, followed by 200 ml. of water. Two phases formed. The lower layer was extracted with 1-butanol (500-ml. portions) four times. More water was added during the extraction to maintain constant volume. The combined butanol extracts were washed once with 250 ml. of water and this wash was added to the aqueous layer. The aqueous layer was concentrated to dryness under reduced pressure. The viscous oil which remained was dissolved in 230 ml. of 85% ethyl alcohol. Ether (220 ml.) was added until turbidity appeared. After standing, the crystalline desosamine hydrochloride was removed (26.7 g., m.p. 182–183°). Additional desosamine hydrochloride was obtained by adding ether to the filtrate. The total yield of pure desosamine hydrochloride, m.p. 183–184°, was 26 g., 49% of theory.

Anal. Calcd. for $C_8H_{17}NO_3 \cdot HCl$: C, 45.40; H, 8.59; N, 6.63; Cl, 16.78; C-CH_3 (1), 7.1. Found: C, 45.41; H, 8.53; N, 6.44; Cl, 16.42; C-CH_3, 7.96.

Desosamine hydrochloride has also been isolated as a crystalline hydrate, melting at about 130°, then resolidifying and melting at the higher temperature. It could be converted to the anhydrous form by crystallization from an ethyl alcohol and ether mixture.

Isolation of *n*-Butyl and Ethyl Desosaminides from Acid Hydrolysates of Erythromycin.—Erythromycin (200 g.) was dissolved in a mixture of 3 liters of 6 *N* hydrochloric acid and 1 liter of water. The mixture was boiled under reflux for 16 hours. It was cooled to room temperature, the dark supernatant was decanted from insoluble material, then extracted with four successive 500-ml. portions of chloroform. The aqueous layer was concentrated to a volume of about 500 ml. and extracted four times with 250-ml. portions of *n*-butanol; additional quantities of water were added occasionally to ensure a two-layer system. The aqueous fraction was retained (A) for work up as described below. The combined butanol extracts were concentrated under reduced pressure. The residue was taken up in 250

(16) G. L. Clark, W. I. Kaye and T. D. Parke, *Ind. Eng. Chem., Anal. Ed.*, **18**, 310 (1946).

(17) R. L. Shriner and R. C. Fuson, "The Systematic Identification of Organic Compounds," 2nd Ed., John Wiley and Sons, Inc., New York, N. Y., 1940, p. 65.

ml. of *n*-butanol and the mixture was saturated at room temperature with dry hydrogen chloride gas. The reaction mixture was boiled under reflux for 30 hours. The solvent and excess hydrogen chloride were removed under reduced pressure. The residue was dissolved in 50 ml. of water, the solution was filtered, then adjusted to a *p*H of about 11 with 20% sodium hydroxide solution. Extraction with chloroform and distillation of the chloroform extract gave 5.6 g. of butyl desosaminide, b.p. 90° (0.1 mm.) which crystallized upon standing at room temperature. Recrystallization from petroleum ether (60–64°) gave white needles melting at 50–51°.

Anal. Calcd. for $C_{12}H_{25}NO_3$: C, 62.34; H, 10.86; N, 6.06. Found: C, 62.53; H, 10.71; N, 5.81.

The aqueous layer (A) remaining after the initial butanol extraction described above, was utilized for the isolation of desosamine hydrochloride (20 g.) by the procedure described earlier. The mother liquor from its isolation was then concentrated to dryness under reduced pressure. The residue was dissolved in 150 ml. of absolute ethanol, then the solution was saturated with dry hydrogen chloride gas and heated under reflux for 18 hours. Ethyl desosaminide was isolated from the resulting reaction mixture as described above for the *n*-butyl analog. Distillation gave about 10 g. of a pale yellow liquid, b.p. 65–67° (0.2 mm.).

Anal. Calcd. for $C_{10}H_{19}NO_3$: N, 6.89. Found: N, 7.05.

Periodate Oxidation of Erythromycin.—Erythromycin (1.25 g., 1.7 m*M*) was dissolved in 50 ml. of pure dioxane. The solution was diluted with 100 ml. of water, then a solution of 1.06 g. (4.98 m*M*) of sodium metaperiodate in 90 ml. of water was added. The mixture was diluted to exactly 250 ml. with water. Titrations were done with 0.010 *M* sodium arsenite as described under "Periodate Oxidation of Desosamine in the Presence of Sodium Bicarbonate," using 1-ml. aliquots of the reaction mixture.

Time (hours)	1.0	3.1	4.2	18
Sample (ml.)	0.65	0.50	0.49	0.24
Blank (ml.)	1.65	1.65	1.64	1.67
Moles IO_4^-/mole of erythromycin	1.47	1.69	1.70	2.11

Erythromycin N-oxide was isolated as described below and identified as one of the periodate oxidation products. Erythromycin (10.0 g., 13.6 m*M*) was dissolved in 450 ml. of ethyl alcohol. A solution of 3.2 g. (15 m*M*) of sodium metaperiodate in 500 ml. of water was added. After 45 minutes a qualitative test showed that all of the periodate had disappeared from the reaction. The solution was concentrated under reduced pressure until the alcohol was removed. A white precipitate formed. The mixture was extracted four times with 100-ml. portions of chloroform. The chloroform extract was washed with 100 ml. of water, dried with anhydrous magnesium sulfate, and then concentrated to dryness under reduced pressure. An amorphous residue (9.95 g.) remained. It was dissolved in 25 ml. of acetone. A crystalline product deposited, 3.25 g., m.p. 216–219°. After recrystallization from methanol–ether, 2.17 g. was obtained, melting at 228–229°. It was shown to be erythromycin N-oxide by comparison with an authentic sample prepared by oxidation of erythromycin with hydrogen peroxide.

Periodate Oxidation of Erythralosamine.—Erythralosamine (0.198 g., 0.367 m*M*) was dissolved in 250 ml. of a 20% aqueous dioxane solution of 0.008 *M* sodium metaperiodate. Five-ml. aliquots were removed at intervals and the remaining periodate was determined by titration with 0.01 *M* arsenite in the usual manner. The consumption of periodate was essentially complete in about 20 hours; 0.97 mole equivalent of periodate was consumed.

Erythralosamine N-Oxide. (a) **By Sodium Metaperiodate Oxidation.**—Erythralosamine (1.0 g., 1.85 m*M*) was dissolved in 625 ml. of a 20% aqueous dioxane solution containing 2.14 g. of sodium metaperiodate and the resulting solution was allowed to stand for 60 hours at room temperature. The solution was then concentrated under reduced pressure to remove dioxane and the aqueous residue was extracted with chloroform. The chloroform extractable material weighed 1.04 g. and when crystallized from a mixture of chloroform–petroleum ether (Skellysolve B, 1:3), yielded 0.87 g., m.p. 183.5–187°. After two recrystallizations the melting point was 186–193°. When titrated in aqueous solution, the product had a pK'_a of 4.3.

Anal. Calcd. for $C_{29}H_{49}NO_9$: C, 62.5; H, 8.88; N, 2.52; mol. wt., 555.7. Found: C, 62.12; H, 8.92; N, 2.69; mol. wt., 570 (electrometric titration).

(b) **By Hydrogen Peroxide Oxidation.**—Erythralosamine (1.0 g., 1.85 m*M*) was dissolved in 100 ml. of a solution containing 3% hydrogen peroxide in 50% methanol. The solution was allowed to stand for 48 hours at room temperature. The reaction mixture was concentrated under reduced pressure and then was extracted with chloroform. The chloroform extractable material weighed 1.1 g. After two recrystallizations from chloroform–petroleum ether, 0.68 g. of product was obtained which melted at 188–192°. The identity of this compound with that obtained from the reaction of erythralosamine and sodium metaperiodate was established by comparing respective infrared spectra and X-ray diffraction patterns.

Attempted Reaction of Erythralosamine and Ammonia.—Liquid ammonia (25 ml.) was dissolved in 50 ml. of methanol at 0° and this solution was added to a solution of erythralosamine (5 g.) in 25 ml. of methanol. The mixture was heated in a sealed vessel at 95–100° for 18 hours. After removal of the solvent and excess ammonia, the residue was crystallized by trituration with petroleum ether (60–64°). Recrystallization of this material (5 g.) from benzene gave unchanged erythralosamine (m.p. 207–208°). A mixed melting point with starting material showed no depression and the infrared spectrum was identical with that of authentic erythralosamine.

Attempted Reaction of Erythralosamine and Hydrazine.—One ml. of anhydrous hydrazine was added to a solution of erythralosamine (2 g.) in 5 ml. of absolute ethanol. The mixture was heated under reflux for 18 hours. It was protected from the air by a drying tube filled with soda lime. After removal of solvent under reduced pressure the residue solidified, yielding 1.8 g. of unchanged starting material. When recrystallized from benzene it melted at 207–208°. A mixed melting point with authentic erythralosamine showed no depression.

Acid Hydrolysis of Erythralosamine.—Erythralosamine (2.0 g.) in 150 ml. of aqueous hydrochloric acid (4 *N*) was heated to boiling and 50 ml. of the solution was distilled. The distillate was treated with 100 ml. of 2,4-dinitrophenylhydrazine reagent and a precipitate was obtained. This precipitate (120 mg.) on crystallization from absolute ethanol gave 70 mg. of product (m.p. 134–150°) which was identified as propionaldehyde 2,4-dinitrophenylhydrazone by its X-ray diffraction pattern.

Alkaline Hydrolysis of Erythralosamine. (a).—Erythralosamine (2.0 g.) was dissolved in 100 ml. of 1 *N* aqueous–methanolic (1:1) sodium hydroxide and the solution was refluxed for 16 hours. Volatile base evolved during the heating period and was absorbed in a trap containing 75 ml. of 2% hydrochloric acid. The reaction mixture was then concentrated to 50 ml. by distillation and the distillate was collected in the acid trap. The hydrochloric acid solution was evaporated to dryness and the residue dissolved in ethyl alcohol. Addition of ether caused a white solid to precipitate. It had a melting point of 172–173° and formed a picrate which had a melting point of 158–160°. Dimethylamine hydrochloride melts at 170° and the picrate melts at 158°.

(b).—After hydrolysis of erythralosamine (2.0 g.) as described in (a), the reaction mixture was steam distilled, keeping the volume constant, until 300 ml. of distillate had been collected. The distillate was treated with 1300 ml. of 2,4-dinitrophenylhydrazine reagent; 0.73 g. of 2,4-dinitrophenylhydrazone derivative was obtained which was identical with an authentic sample of propionaldehyde 2,4-dinitrophenylhydrazone. The yield corresponds to 0.83 mole equivalent of propionaldehyde.

The aqueous reaction mixture remaining after removal of the propionaldehyde by steam distillation, was acidified with 1 *N* sulfuric acid. The solution was subjected to steam distillation, keeping the volume in the distilling flask constant. The distillate (275 ml.) was titrated with 0.1 *N* sodium hydroxide. The steam-volatile acid corresponded to 0.99 mole equivalent. The solution was concentrated to a small volume (*ca.* 2 ml.) and an equal volume of ethyl alcohol was added. *p*-Bromophenacyl bromide (0.3 g.) was added and the mixture was refluxed for one hour. A solid product was

obtained which had a melting point of 55–56°. Recrystallization gave material with a melting point of 59–60°. An authentic sample of *p*-bromophenacyl propionate gave an infrared spectrum and X-ray pattern which were identical, respectively, with the infrared spectrum and X-ray pattern obtained from the unknown.

Desosamine Hydrochloride from Acid Hydrolysis of Butyl Desosaminide.—Butyl desosaminide (10 g.) was dissolved in 250 ml. of 6 *N* hydrochloric acid and the mixture was heated under reflux for 16 hours. The cooled solution was extracted with ether, then concentrated to dryness under reduced pressure. Desosamine hydrochloride (4.5 g.) was isolated by crystallization from ethanol and ether as described earlier. Recrystallization from 95% ethanol gave material melting at 183–184°; a mixed melting point with previously obtained material showed no depression. X-Ray patterns of the present material and previous samples were indistinguishable.

Methyl Desosaminide.—Desosamine hydrochloride (5 g.) was dissolved in 150 ml. of methanol and the solution was saturated at ice-bath temperature with dry hydrogen chloride. The mixture was heated under reflux for 48 hours. The solvent and excess hydrogen chloride were removed under reduced pressure, then the residue was dissolved in 10 ml. of cold water. The *p*H of the solution was adjusted to about 11 with the aid of cold 20% sodium hydroxide solution. The methyl desosaminide was extracted with three successive 25-ml. portions of chloroform. The combined chloroform layers were washed twice with water, then dried with anhydrous sodium sulfate. Distillation of the residue after removal of the solvent gave 2.5 g. of a colorless liquid (55% of the theoretical), b.p. 61–62° (0.3 mm.), $n^{25}D$ 1.4600.

Anal. Calcd. for $C_9H_{19}NO_3$: N, 7.42; CH_3O–, 16.10. Found: N, 7.16; CH_3O–, 15.89.

Reaction of Desosamine with Base.—Desosamine hydrochloride (235 mg., 1.1 m*M*) was dissolved in 25 ml. of 1 *N* sodium hydroxide in 50% methanol. The mixture was heated for 16 hours under reflux. Escaping gas was collected in a flask containing 20 ml. of 0.1 *N* hydrochloric acid. Back titration of the acid required 9.29 ml. of 0.1 *N* sodium hydroxide solution, indicating the evolution of 1.05 m*M* of volatile base or 95% of theory based on dimethylamine.

Reaction of Ethyl Desosaminide with Base.—Treatment of ethyl desosaminide under conditions identical to those described above for desosamine produced less than 3% of the theoretical amount of volatile amine.

Diacetyl Desosamine Hydrochloride.—Desosamine hydrochloride (0.20 g.) was suspended in 5 ml. of pyridine and one ml. of acetic anhydride was added. The mixture was heated to 70° on the steam-bath, causing the solid to dissolve. After 2 hours at room temperature the solution was concentrated to dryness under reduced pressure. The residue was recrystallized by dissolving in 10 ml. of chloroform and adding 25 ml. of ether. Crystals (0.22 g.) were deposited, m.p. 180–185° dec. Recrystallization from chloroform–ether and ethylene dichloride–ether gave 0.130 g., m.p. 194–195° dec.

Anal. Calcd. for $C_8H_{17}NO_3 \cdot HCl \cdot 2(CH_3CO-)$: C, 48.8; H, 7.48; Cl, 12.0; CH_3CO–, 28.4. Found: C, 49.39; H, 7.53; Cl, 11.54; CH_3CO–, 23.7.

Desosamine from Desosamine Hydrochloride.—Desosamine hydrochloride (0.20 g.) was dissolved in 30 ml. of water. The *p*H of the solution was adjusted to 9.8 with 0.1 *N* sodium hydroxide. The solution was extracted continuously with chloroform for 24 hours. The chloroform extract was dried with anhydrous magnesium sulfate, then concentrated to dryness under reduced pressure. The light colored oil which remained was triturated with ether, causing white crystals to form. The product melted at 82–84° and weighed 0.070 g. Recrystallization from ether–petroleum ether gave desosamine free base melting at 83–83.5°.

Anal. Calcd. for $C_8H_{17}NO_3$: C, 54.83; H, 9.78. Found: C, 54.69; H, 9.84.

Periodate Oxidation of Desosamine. (a) With One Mole of Periodate.—Desosamine hydrochloride (1.0 g., 4.68 m*M*) was dissolved in 50 ml. of water and 1.0 g. (4.67 m*M*) of sodium metaperiodate was added. The solution was allowed to stand for 15 minutes, then the *p*H was adjusted to 10.3 and the solution was extracted continuously with ether for 4 hours. The ether extract was evaporated to dryness under reduced pressure and the residue (0.44 g.) was redissolved in 5 ml. of 0.5 *N* hydrochloric acid. This was evaporated under reduced pressure and the product was crystallized from ethyl alcohol–ether. Two recrystallizations gave 150 mg., m.p. 166–168° with sublimation.

Anal. Calcd. for $C_7H_{15}NO_2 \cdot HCl$: C, 46.28; H, 8.87; N, 7.71. Found: C, 45.99; H, 8.94; N, 7.62.

(b) **Quantitative Oxidation.**—Desosamine hydrochloride (0.200 g., 0.94 m*M*) was dissolved in 75 ml. of water containing 2.0 g. (9.3 m*M*) of sodium metaperiodate. The solution was diluted to 100 ml. Aliquots (5.0 ml.) were titrated at intervals by adding 5.00 ml. of 0.100 *M* standard sodium arsenite solution, then titrating the arsenite which remained with 0.05 *M* iodine. The arsenite destroyed is a measure of periodate remaining in the aliquot of reaction mixture.

Time (hours)	0.3	1.6	19.2	26.4	46
Blank (ml. I_2)	0.83	0.83	0.84	0.84	0.83
Sample (ml. I_2)	2.09	2.45	2.41	2.48	2.51
Moles IO_4^-/mole compound	1.34	1.72	1.68	1.79	1.78

Oxidations were also run using the titration method described under "Periodate Oxidation of Desosamine in the Presence of Sodium Bicarbonate." In this case, however, variable results were obtained since free iodine is formed in the reaction mixture in the presence of sodium bicarbonate. The reaction products give a facile iodoform test, so the iodine which is normally titrated by this method reacts rapidly with the products of the reaction, resulting in apparent excessive periodate consumption.

Periodate Oxidation of Desosamine in the Presence of Sodium Bicarbonate.—Desosamine hydrochloride (161.2 mg., 0.765 m*M*) was dissolved in 30 ml. of sodium metaperiodate solution (2.247 g./100 ml.) to which 1 g. of sodium bicarbonate had been added. The reaction mixture was diluted to 100 ml. The initial *p*H was 7.8. After oxidation was complete (45 hours) the *p*H was 7.6. At regular intervals during the course of the reaction, 1-ml. aliquots were removed and titrated to measure unreacted periodate. This was done by diluting the aliquot with 5 ml. of water, adding an excess of aqueous potassium iodide solution, and titrating the iodine formed with standard sodium arsenite (0.0101 *M*). One mole of sodium arsenite is equivalent to one mole of sodium metaperiodate.

PERIODATE CONSUMED BY DESOSAMINE

Time, hr.	0.03	0.08	0.50	4.0	21	28	45
Blank, ml.	..	..	2.91	2.87	2.94	2.94	.
Sample, ml.	2.00	1.64	1.58	1.27	0.76	0.69	0.58
Moles IO_4^-/mole of desosamine	1.22	1.66	1.80	2.17	2.83	2.92	3.08

Periodate Oxidation Products of Desosamine.—Desosamine hydrochloride (1.0 g., 4.7 m*M*) was dissolved in 50 ml. of water and 2.0 g. (9.4 m*M*) of sodium metaperiodate was added. The solution was extracted continuously with ether. The ether reservoir contained 800 ml. of 2,4-dinitrophenylhydrazine reagent (800 mg. of 2,4-dinitrophenylhydrazine dissolved in 800 ml. of 2 *N* hydrochloric acid). The reservoir was kept at 45–50° which was adequate to maintain ether flow through the liquid–liquid extractor. A precipitate appeared in the reservoir after about three hours. After extracting for 48 hours the ether was distilled out of the reservoir and the remaining aqueous suspension was extracted with three 75-ml. portions of chloroform. The chloroform extract was washed once with 50 ml. of water, dried with anhydrous magnesium sulfate, and concentrated to dryness under reduced pressure. The bright red crystalline residue weighed 0.77 g. and melted somewhat indefinitely at 165–175° with some prior softening. The solid was dissolved in 50 ml. of benzene and chromatographed on 50 g. of silica gel which had been packed into a column as a slurry in 1:1 benzene–petroleum ether. The column was developed with benzene. The first dark band to move off the column was collected after 325 ml. of benzene had passed through. About 600 ml. of benzene was required to elute it completely. When the benzene was removed, 0.365 g. of bright red crystals remained, m.p. 195–

197°. The infrared spectrum and X-ray pattern obtained from this material were identical with the spectrum and pattern obtained from an authentic sample of crotonaldehyde 2,4-dinitrophenylhydrazone.

Periodate Oxidation of Methyl Desosaminide.—Methyl desosaminide (123.9 mg., 0.655 m*M*) was dissolved in 100 ml. of a sodium metaperiodate solution (1.2076 g./250 ml.). The initial *p*H of the reaction mixture was 7.5. At periodic intervals, 1-ml. aliquots were titrated in the manner described above, except that sodium bicarbonate was added to the aliquot before adding potassium iodide solution. When periodate consumption had ceased (22 hours) the *p*H of the reaction mixture was 6.0.

Time, hr.	0.08	0.16	0.5	1	4	7	22
Blank, ml.	2.15	2.14	..	2.14	..	2.15	2.18
Sample, ml.	2.15	2.12	2.04	1.95	1.71	1.65	1.58
Moles IO_4^-/mole of compound	0	0.03	0.15	0.29	0.64	0.79	0.90

Periodate Oxidation of Diethylaminoethanol and 3-Diethylaminopropanol-1.—A 0.01 *M* aqueous solution of diethylaminoethanol containing 0.02 *M* sodium metaperiodate was allowed to stand at room temperature. Aliquots (10 ml.) were removed at intervals and titrated with 0.01 *N* sodium arsenite in the usual manner. The periodate consumption after 22 hours was 0.75 mole equivalent and after 51 hours was 0.90 mole equivalent.

When a 0.0108 *M* aqueous solution of 3-diethylaminopropanol-1 containing 0.02 *M* sodium metaperiodate, was allowed to stand at room temperature, 0.59 mole equivalent of periodate was consumed in 17 hours and 0.99 mole equivalent was consumed in 45 hours.

Cladinose.—Forty grams of erythromycin was dissolved in 2 l. of 0.75 *N* hydrochloric acid and the solution was allowed to stand at room temperature for 20 hours. The acid solution was poured into a solution of 128 g. of sodium bicarbonate in 4 l. of water. A little solid sodium bicarbonate was added to ensure alkalinity; this was followed by extraction with four 600-ml. portions of chloroform. Evaporation of the combined chloroform extracts and crystallization of the residue from benzene–petroleum ether (60–70°) gave 23.6 g. of erythralosamine, m.p. 200–203°. The erythralosamine was shown to be identical with the product from methanolysis of erythromycin by comparison of the X-ray diffraction patterns.

The aqueous solution after chloroform extraction was evaporated to dryness under reduced pressure at room temperature. The residue was extracted with four 300-ml. portions of boiling benzene, filtering each portion of extract. The benzene was removed by evaporation under reduced pressure, leaving a white gummy residue which was dissolved in 60 ml. of water. The aqueous solution was extracted with 24 ml. of chloroform and again evaporated to dryness under reduced pressure, leaving a light brown sirup. Distillation of the sirup gave 3.5 g. of colorless liquid boiling at a bath temperature of 120–132° at 0.25 mm., $[\alpha]^{25}D$ −23.1° (*c* 2.6, water).

The infrared spectrum of this product had peaks at 2.81 and 2.91 μ of almost equal intensity, with weak absorption in the carbonyl region. The ultraviolet spectrum showed only end absorption. Cladinose gave positive Fehling and Tollens tests and a positive iodoform reaction.

Anal. Calcd. for $C_8H_{16}O_4$(1 CH_3O): C, 54.54; H, 9.09; CH_3O, 17.6. Found: C, 54.41; H, 9.30; CH_3O, 17.04.

Methyl Cladinoside.—The filtrate remaining from the crystallization of erythralosamine from methanolysis of a 100-g. quantity of erythromycin, was concentrated under reduced pressure. The residue was dissolved in 1 l. of ether, and the ether solution was extracted with two 400-ml. portions of 1% hydrochloric acid. The ether solution was dried over magnesium sulfate, filtered and concentrated on the steam-bath. Distillation of the residue gave 7.0 g. of colorless liquid boiling at 54–56° at 0.3 mm. This was dissolved in 350 ml. of 0.1 *N* sodium hydroxide solution. The basic solution was extracted with 50 ml. of ether by shaking in a separatory funnel followed by continuous extraction with ether for 40 hours. The second ether extract was evaporated to dryness *in vacuo* at room temperature. The colorless residue was distilled to give 4.9 g. of methyl cladinoside boiling at 51–53° at 0.2 mm., $n^{25}D$ 1.4508, d^{30}_4 1.080, $[\alpha]^{30}D$ −6.9°, (*c* 3, water).

Methyl cladinoside showed only end absorption in the ultraviolet. The infrared spectrum showed a narrow band at 2.81 μ and no absorption in the carbonyl region. The Fehling and Tollens tests were negative on methyl cladinoside, as was the iodoform reaction.

Anal. Calcd. for $C_9H_{18}O_4$ (2 CH_3O, 2 CH_3C and 1 active H): C, 56.84; H, 9.54; CH_3O, 32.6; CH_3C, 15.8; active H, 1; mol. wt., 190. Found: C, 56.99, 56.68, 56.62; H, 9.76, 9.58, 9.62; CH_3O, 31.7; CH_3C, 14.4; active H, 1.02; mol. wt., 201.5.

Conversion of Methyl Cladinoside to Cladinose.—Two hundred milligrams of methyl cladinoside was dissolved in 10 ml. of 0.6 *N* hydrochloric acid. This solution was allowed to stand at room temperature for 24 hours. The solution was neutralized with 1.0 g. of silver oxide. The resulting mixture was filtered and the solid was washed thoroughly with water. The washings were combined with the filtrate, and the entire solution was evaporated to dryness under reduced pressure at room temperature. The residue was dissolved in 1:1 chloroform–benzene, and the solution was filtered. Evaporation left a viscous oil weighing 100 mg., which gave a positive Fehling test and a positive iodoform reaction. The infrared spectrum of this material indicated that it was identical with cladinose.

Conversion of Cladinose to Methyl Cladinoside.—One gram of cladinose was dissolved in 100 ml. of anhydrous methanol containing 1% of hydrogen chloride, and the solution was allowed to stand at room temperature for 24 hours. It was poured into a solution of 3 g. of sodium bicarbonate in 100 ml. of water. The methanol was removed by evaporation under reduced pressure. The aqueous residue was extracted with three 20-ml. portions of chloroform. The combined chloroform extracts were evaporated until the chloroform was removed. The residue was distilled twice to give a colorless liquid, b.p. 53–55° at 0.2 mm., $n^{25}D$ 1.4501. The infrared spectrum was identical with that of methyl cladinoside.

Diacetylcladinose.—Five hundred and sixty milligrams (3.3 m*M*) of cladinose was dissolved in 12 ml. of dry pyridine. Acetic anhydride (1.1 ml., 10.0 m*M*) was added, and the solution was heated on the steam-bath for two hours. Twelve milliliters of water was added, and then solid sodium bicarbonate until reaction no longer occurred. Enough water was put in to give a clear solution, and the solution was evaporated to dryness under reduced pressure. The residue was dissolved in 50 ml. of water, and the solution was extracted with three 10-ml. portions of ether. The ether extracts were combined, concentrated, and the residue was distilled. The product distilled at a bath temperature of 135–145° under a pressure of 0.1 mm. to give a colorless liquid which solidified on standing.

Anal. Calcd. for $C_{12}H_{20}O_6$ (2 CH_3CO): C, 55.37; H, 7.74; CH_3CO, 33.07. Found: C, 55.25; H, 8.18; CH_3CO, 32.58.

Willstätter–Schudel Titration of Cladinose.—This was done by the usual Willstätter–Schudel procedure.[12] The sample was dissolved in 10 ml. of water. Approximately 0.1 *N* iodine solution and 0.1 *N* sodium hydroxide solution were added. The solution was then acidified with 0.5 *N* sulfuric acid and titrated with 0.1 *N* sodium thiosulfate solution in less than two minutes.

Run	Sample wt., g.	Ml. I_2 0.116 *N*	Ml. NaOH 0.1 *N*	Ml. $Na_2S_2O_3$ 0.1 *N*	Cladinose, m*M*	I_2 uptake, m*M*	Ratio
1	0.0416	8.56	10	3.00	0.236	0.330	1.40
2	.0609	12.20	21	4.05	.345	.461	1.33

3,5-Dinitrobenzoate of Methyl Cladinoside.—Three hundred milligrams of methyl cladinoside and 600 mg. of 3,5-dinitrobenzoyl chloride were dissolved in 6 ml. of dry pyridine. The solution was heated on the steam-bath for one-half hour and then diluted with 18 ml. of water. Overnight refrigeration of the mixture gave 200 mg. of a white crystalline product melting at 134–140°. An infrared spectrum of the mixture showed no hydroxyl absorption. This material was chromatographed on acid-washed alumina using benzene as solvent and eluant. Fractions 2–7 of the eluate were evaporated to dryness and combined. Six recrystallizations from ethanol gave a product melting at 195–196°.

Anal. Calcd. for $C_{16}H_{20}N_2O_9$: C, 50.00; H, 5.22; N, 7.29. Found: C, 50.11; H, 5.49; N, 7.43.

Fractions 9–20 were evaporated to dryness, combined and recrystallized three times from ethanol–water. The final melting point was 159–161°.

Anal. Calcd. for $C_{16}H_{20}N_2O_9$: C, 50.00; H, 5.22; N, 7.29. Found: C, 49.88; H, 5.24; N, 7.27.

Ethyl Thiocladinoside.—Three grams (0.017 mole) of cladinose was dissolved in 60 ml. of dry ethyl mercaptan containing 1.5 g. of dry hydrogen chloride. After the solution had stood at room temperature for one day, it was concentrated under reduced pressure at room temperature. The residue was dissolved in 60 ml. of chloroform, and the solution was washed with 15 ml. of 5% sodium hydroxide followed by 15 ml. of water. The solvent was again removed under reduced pressure and the residue was distilled. The yield of colorless liquid boiling at a bath temperature of 110–120° at 0.3 mm., was 2.8 g. (74%), $n^{25}D$ 1.4901.

Anal. Calcd. for $C_{10}H_{20}O_3S$: C, 54.52; H, 9.15; S, 14.55. Found: C, 54.67; H, 9.50; S, 14.83.

INDIANAPOLIS, INDIANA

[CONTRIBUTION FROM THE UNIVERSITY LABORATORY OF PHYSICAL CHEMISTRY RELATED TO MEDICINE AND PUBLIC HEALTH, HARVARD UNIVERSITY]

Dimerization of Serum Mercaptalbumin in Presence of Mercurials. II. Studies with a Bifunctional Organic Mercurial[1a,b,c]

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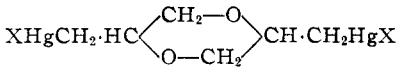
The dimerization of human serum mercaptalbumin (ASH) has been studied with a bifunctional organic mercurial (XHgRHgX) which serves to link two albumin molecules together through the sulfhydryl groups to form a dimer (ASHgRHgSA). When the reagents are mixed in the proportion 0.5 mole mercurial per mole albumin, equilibrium lies almost completely on the side of dimer formation. The dimer dissociates in the presence of an excess of the mercurial or of added cyanide. The equilibrium attained in the latter reaction has provided a method of estimating the equilibrium constant for dimer formation, $(ASHgRHgSA)/(ASHgRHg^+)(As^-)$ as being near $10^{18.2}$ at *p*H 4.75, in acetate buffer at ionic strength 0.05, and 25°. The corresponding equilibrium constant for the mercury dimer (ASHgSA) previously studied is $10^{13.5}$, which is less by a factor of 50,000. The two albumin molecules must approach about 10 Å. closer in ASHgSA than in ASHgRHgSA, and the steric repulsions involved are probably primarily responsible for the difference between the two equilibrium constants. The rate of dimer formation is faster by two to three thousand-fold for ASHgRHgSA formation than for ASHgSA formation. However, both reactions are affected by *p*H to about the same extent. The bimolecular velocity constant for dimer formation with the bifunctional organic mercurial falls from approximately 69,000 l. $mole^{-1}$ $min.^{-1}$ at *p*H 5 and 25° to 4100 at *p*H 6. The temperature coefficient of the velocity constant is large, the energy of activation being of the order of 20 kcal./mole both at *p*H 5.5 and *p*H 6. The standard entropy of activation is large and positive. Possible explanations for this unusual entropy value are discussed.

Equilibrium and kinetic studies on the dimerization of mercaptalbumin in the presence of mercuric salts have already been reported in detail.[2] In the present paper, a briefer report is presented of studies on dimerization with a bifunctional organic mercurial



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X may be chloride, acetate or nitrate; the acetate was the salt most commonly used in our experiments. When X is nitrate, the resulting compound has been termed by Chatt,[3] 3,6-bis-(nitratomercurimethyl)-dioxane. However, in what follows, we shall refer to it either as XHgRHgX or as "the bifunctional mercurial." A preliminary report on the interaction of this mercurial with mercaptalbumin already has been given.[4] The reactions involving formation and dissociation of the albumin dimer may be formulated by equations which exactly correspond with those previously employed[2] to describe the reaction with mercuric salts.

$$ASH + XHgRHgX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ASHgRHgX + H^+ + X^- \quad (1)$$

$$ASHgRHgX + ASH \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ASHgRHgSA + H^+ + X^- \quad (2)$$

$$ASHgRHgSA + XHgRHgX \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} 2ASHgRHgX \quad (3)$$

No quantitative evidence is yet available concerning the velocity of reaction 1 but it appears to be extremely rapid, so that reaction 2, here as in our earlier studies, appears to be the rate-controlling step. The velocity constant of this reaction, however, is far greater for dimerization with the organic mercurial than with simple mercuric salts. The temperature coefficient of both reactions is large. The significance of the energy and entropy of activation is considered below in the discussion.

Materials and Methods

Materials.—The albumin preparations were the same as those used in the study[2] of the formation and dissociation of the mercury dimer (ASHgSA). In the earlier experiments preparations of the class previously denoted as type A were employed. The later studies of velocity constants at *p*H 5.5 and *p*H 6 at different temperatures were carried out with a preparation previously denoted as of type C.

The organic mercurial XHgRHgX was prepared in the form of 3,6-bis-(acetatomercurimethyl)-dioxane, essentially

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(3) J. Chatt, *Chem. Revs.*, **48**, 7 (1951).

(4) R. Straessle, THIS JOURNAL, **73**, 504 (1951).