of Stein, Moore and Bergmann were very similar. The agreements generally are surprisingly good when one considers that the three methods of investigation were very different. In no instance have any corrections been applied. Under these conditions of hydrolysis one may then expect to find about twice as much ala-gly as gly-ala. The significance of this result is difficult to assess. Levy and Slobodian point out that one would expect such a ratio from the sequence -gly-X_1-ala-gly-ala-gly-X_2-[17] because Synge[11] found identical rates of hydrolysis for ala-gly and gly-ala. Although one would, indeed, expect twice as much ala-gly as gly-ala from the peptide ala-gly-ala-gly, on the other hand, this reasoning ignores the influence of the bonds X_1-ala and gly-X_2 on the amounts of ala-gly and gly-ala in a partial hydrolysate of silk fibroin.

If we assume that gly-(ala, gly) of zone S-11 actually is gly-ala-gly, the 22.5 μmoles/250 mg. is not unreasonable as compared with the 62 μmoles/250 mg. of Slobodian and Levy[5] because their time of hydrolysis was only 24 hours instead of 48. If we assume that the ala-(gly, ala) of zone S-9 is ala-gly-ala, then the 8 μmoles is considerably less than the 22.5 μmoles of gly-ala-gly whereas we would predict equal amounts from their sequence. (We assume that any losses are equal.)

The sequence in ala-ala definitely is at variance with the repeating unit and it accounts for about 2% of the peptides which were isolated. Because the sequence in most of the tripeptides is unknown, all can be made to fit the sequence -gly-X_1-ala-gly-ala-gly-X_2- but certain data must be ignored if this is done. Gly-tyr and tyr-gly have been isolated but no tyr-ala. If tyrosine were in X_1 we might expect to find tyr-ala but the isolation of gly-tyr and tyr-gly would appear to limit tyrosine to X_2. Ser-gly may be fitted into the position X_2-gly, but ser-(gly, ala) would have to be ser-ala-gly in order to conform to the sequence in the position X_1-ala-gly because X_2-gly-X_1 is not satisfactory. At least 40% of the serine is in the sequence ser-gly but no ser-ala was observed although, if present, it should have been in zone S-10. Hence, it is more reasonable to conclude that ser-(gly, ala) is ser-gly-ala, which would not fit the above sequence.

It would appear that at the present state of our knowledge one can draw few definite conclusions about the structure of silk fibroin. The preliminary chromatographic experiments have shown that the peptide mixture in this partial hydrolysate of silk fibroin is relatively simple and it is to be hoped that future experiments with less completely hydrolyzed material will yield longer peptides from which it may be possible to deduce more about the structure of silk fibroin.

Acknowledgment.—Certain of the experiments which have been described were carried out by Mr. Setty Porto and Miss Nancy Munger. The investigation was supported in part by a contract with the Quartermaster Corps, U. S. Army.

PASADENA 4, CALIFORNIA

(17) Subscripts have been added in order to distinguish between the "X"s.

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF CHAS. PFIZER & CO., INC., AND THE CONVERSE MEMORIAL LABORATORY OF HARVARD UNIVERSITY]

The Structure of Aureomycin[1]

BY C. R. STEPHENS, L. H. CONOVER, R. PASTERNACK, F. A. HOCHSTEIN, W. T. MORELAND, P. P. REGNA F. J. PILGRIM, K. J. BRUNINGS AND R. B. WOODWARD

RECEIVED FEBRUARY 22, 1954

The antibiotic Aureomycin has been shown to have the structure II.

Aureomycin is a broad spectrum antibiotic which is produced by *Streptomyces aureofaciens*.[2] Comparison of chemical, biological and physical data for Aureomycin and Terramycin[3] suggested at an early date a close structural similarity between the two substances. When the investigations which led to the establishment of the structure I for Terramycin had been completed,[4] it was possible to propose directly the corollary hypothesis that Aureomycin had the structure II. The correctness of this expression was then confirmed by a series of experiments which are described in this communication.[1,5]

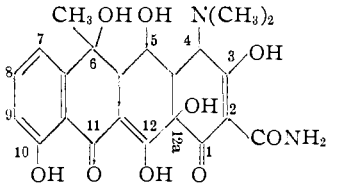
(1) Terramycin XII. The investigations described in this paper were first outlined in part in two preliminary communications: (a) THIS JOURNAL, **74**, 4976 (1952); (b) **75**, 4622 (1953). Terramycin is the registered trade mark of Chas. Pfizer & Co., Inc., for the antibiotic whose generic name is oxytetracycline. Aureomycin is a registered trade mark of Lederle Laboratories Division, American Cyanamid Co., for the antibiotic chlortetracycline.

(2) (a) R. W. Broschard, A. C. Dornbush, S. Gordon, B. L. Hutchings, A. R. Kohler, G. Krupka, S. Kushner, D. V. Lefemine and C. Pidacks, *Science*, **109**, 199 (1949); (b) B. M. Duggar, U. S. Patent 2,482,055 (1949).

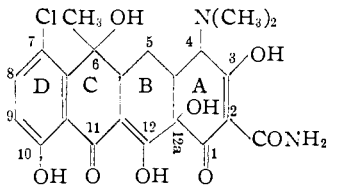
(3) (a) A. C. Finlay, G. L. Hobby, S. Y. P'an, P. P. Regna, J. B. Routien, D. B. Seeley, G. M. Shull, B. A. Sobin, I. A. Solomons, J. W. Vinson and J. H. Kane, *Science*, **111**, 85 (1950); (b) P. P. Regna, I. A. Solomons, K. Murai, A. E. Timreck, K. J. Brunings and W. A. Lazier, THIS JOURNAL, **73**, 4211 (1951).

(4) (a) F. A. Hochstein, C. R. Stephens, L. H. Conover, P. P. Regna, R. Pasternack, K. J. Brunings and R. B. Woodward, *ibid.*, **74**, 3708 (1952); (b) F. A. Hochstein, C. R. Stephens, L. H. Conover P. P. Regna, R. Pasternack, P. N. Gordon, F. J. Pilgrim, K. J. Brunings and R. B. Woodward, *ibid.*, **75**, 5455 (1953).

(5) In a series of preliminary communications another group has also suggested the structure II as one of two possibilities for Aureomycin. (a) C. W. Waller, B. L. Hutchings, C. F. Wolf, R. W. Broschard, A. A. Goldman and J. H. Williams, *ibid.*, **74**, 4978 (1952); (b) C. W. Waller, B. L. Hutchings, A. A. Goldman, C. F. Wolf, R. W. Broschard and J. H. Williams, *ibid.*, **74**, 4979 (1952); (c) B. L. Hutchings, C. W. Waller, R. W. Broschard, C. F. Wolf, P. W. Fryth and J. H. Williams, *ibid.*, **74**, 4980 (1952); (d) C. W. Waller, B. L. Hutchings, C. F. Wolf, A. A. Goldman, R. W. Broschard and J. H. Williams, *ibid.*, **74**, 4981 (1952); (e) C. W. Waller, B. L. Hutchings, R. W. Broschard, A. A. Goldman, W. J. Stein, C. F. Wolf and J. H. Williams, *ibid.*, **74**, 4981 (1952).



I



II

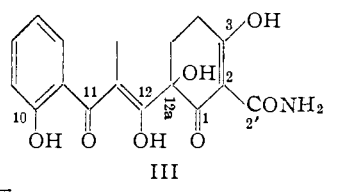
The early available data relevant to a comparison of Aureomycin and Terramycin are collected in Table I.

TABLE I

	Aureomycin	Terramycin
Empirical formula	$C_{22}H_{23}N_2O_8Cl$[a]	$C_{22}H_{24}N_2O_9$[b]
pK_a (hydrochlorides)	3.4, 7.4, 9.2[c]	3.4, 7.6, 9.2[d]
Ultraviolet absorption ($\lambda\lambda_{max}$, $\epsilon\epsilon_{max} \times 10^{-3}$)	267–14.8[e] 375–11.8	267–21.2[d] 357–12.3
Space group (hydrochlorides)[f]	$P2_12_12_1$	$P2_12_12_1$
Unit cell dimensions[f]	11.22 × 12.89 × 15.55 Å.	11.19 × 12.49 × 15.68 Å.
From (drastic) alkaline degradation	$(CH_3)_2NH$, NH_3, 5-chlorosalicylic acid[g]	$(CH_3)_2NH$, NH_3, salicylic acid[g,h]
Antibacterial spectra	similar[i]	

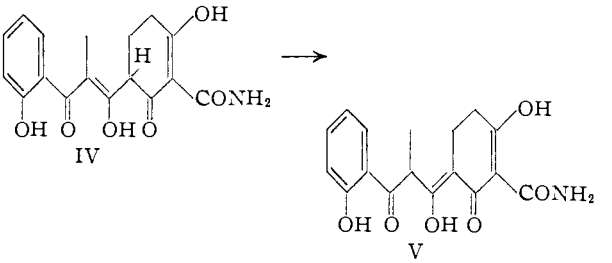
[a] Our data (*cf.* ref. 1 and Experimental part); an earlier erroneous formula, $C_{22}H_{27}N_2O_8Cl$ (B. L. Hutchings, C. W. Waller, S. Gordon, R. W. Broschard, C. F. Wolf, A. A. Goldman and J. H. Williams, THIS JOURNAL, **74**, 3710 (1952)) was later revised (*cf.* footnote 6 of ref. 5*d*). [b] Ref. 4. [c] G. B. Hess, private communication. [d] Ref. 3b. [e] Our data in ethanolic hydrochloric acid (0.01 *N*); see also ref. 2 and D. J. Hiscox, *J. Am. Pharm. Soc.*, **40**, 237 (1951). [f] J. D. Dunitz and J. H. Robertson, THIS JOURNAL, **74**, 1108 (1952); R. Pepinsky and T. Watanabe, *Science*, **115**, 541 (1952). [g] R. Kuhn and K. Dury, *Ber.*, **84**, 563 (1951). [h] R. Pasternack, A. Bavley, R. L. Wagner, F. A. Hochstein, P. P. Regna and K. J. Brunings, THIS JOURNAL, **74**, 1926 (1952). [i] Ref. 3a and T. F. Paine, Jr., H. S. Collins and M. Finland, *J. Bact.*, **56**, 489 (1948).

These facts alone suggested strongly the hypothesis that Aureomycin must be a 7-chloro-x-desoxyterramycin.[6] Further, the studies on Terramycin had demonstrated that the ultraviolet absorption



III

characteristics of the antibiotic, as well as its acidity properties, depended upon the entire system III. Thus, the oxygen atoms at C.10, C.11 and C.12 are responsible for one acid constant, and define the detailed nature of the long wave length absorption of Terramycin, while those at C.1, C.3 and C.2′ provide another acid constant and are associated with ultraviolet absorption in the medium wave length region. The removal of any of these oxygen atoms should be attended by changes in spectrum and acidity more marked than those which differentiate Aureomycin and Terramycin (*cf.* Fig. 1 and Table I). Beyond that, it was known that the removal of the hydroxyl group at C.12a in Terramycin led to very marked changes in properties, associated with a shift in the direction of enolization within the polycarbonyl system IV → V.[4b] Consequently, it could be assumed that the molecule of Aureomycin possessed all seven of the oxygen atoms just dis-

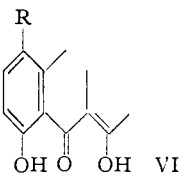


IV V

cussed, and it remained only to place a single hydroxyl group. Since Aureomycin had been shown to give rise to substances exhibiting naphthaleneoid absorption in the ultraviolet on acid treatment,[7] it seemed very likely that this hydroxyl group could be placed at C.6, and that Aureomycin must be formulated as 7-chloro-5-desoxyterramycin (II).

We turn now to a discussion of the new experiments, suggested by our experience with Terramycin, which threw light successively on various portions of the Aureomycin molecule, and confirmed in detail the structure II for the antibiotic.

The attachment of the chlorine atom at C.7 in Aureomycin was very simply deducible from the observation (Table I) that 5-chlorosalicylic acid was produced on alkaline fusion of Aureomycin under conditions which led to salicylic acid in the case of Terramycin. Since the system (VI, R = H) is the major ultraviolet chromophore of the Terramycin molecule, it seemed likely that the di-



VI

rect attachment of the chlorine atom of Aureomycin to the aromatic system should be responsible for the definite, albeit small, differences in the ultraviolet spectra of the two antibiotics. The correctness of this presumption was established when it was found that Aureomycin could be transformed by hydrogenation over palladized charcoal to *deschloro-*

(6) Dunitz and Robertson (THIS JOURNAL, **74**, 1108 (1952)), as well as Pepinsky and Watanabe (*Science*, **115**, 541 (1952)) suggested, on the basis of the X-ray data, that the chlorine atom in Aureomycin took the place of an hydroxyl group in Terramycin. This suggestion is clearly inadmissible in the light of the results obtained on drastic alkaline degradation of the two antibiotics (Table I, *g* and *h*).

(7) D. J. Hiscox, *J. Am. Pharm. Assoc.*, **40**, 237 (1951).

aureomycin, $C_{22}H_{24}N_2O_8$,[1b,8-10] *whose ultraviolet absorption characteristics in acidic and basic media were identical with those of Terramycin (Figs. 1 and 2).*

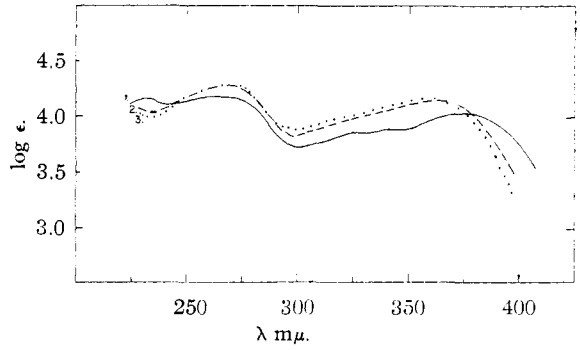


Fig. 1.—Ultraviolet spectra in acidic (0.01 *N*) ethanol: 1, Aureomycin; 2, Deschloroaureomycin; 3, Terramycin.

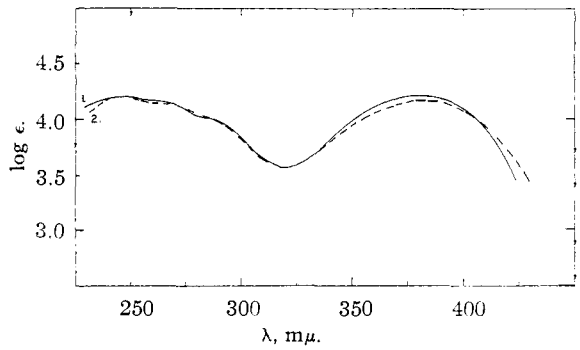
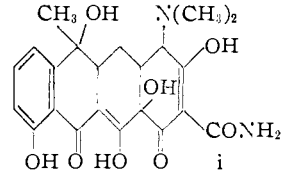


Fig. 2.—Ultraviolet spectra in methanolic sodium hydroxide (0.01 *N*): 1, deschloroaureomycin; 2, Terramycin.

(8) J. H. Boothe, J. Morton, II, J. P. Petisi, R. G. Wilkinson and J. H. Williams, THIS JOURNAL, **75**, 4621 (1953).

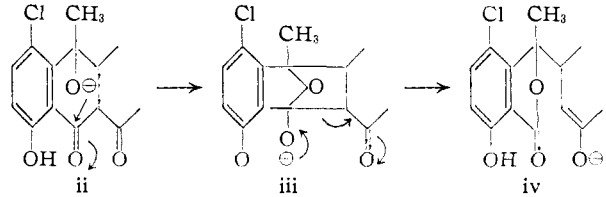
(9) It will be noted that the structure i of deschloroaureomycin is common to both Terramycin (I) and Aureomycin (II). We have earlier assigned the name "tetracycline" to i (*cf.* reference 1a), thus



i

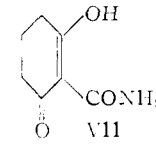
forming a basis for applying the generic names "oxytetracycline" to Terramycin and "chlortetracycline" to Aureomycin. Tetracyn is the registered trade mark of J. B. Roerig & Co., Division of Chas. Pfizer & Co., Inc., for the antibiotic, tetracycline. The antibacterial activity of i has been reported elsewhere (*cf.* reference 1b).

(10) It is of considerable interest that removal of the chlorine atom from Aureomycin effects a marked stabilization of the molecule toward destruction in alkaline media. Thus, whereas Aureomycin at room temperature shows a half-life in aqueous bicarbonate solution of less than three hours, deschloroaureomycin (i) under similar conditions shows a half-life of considerably over 20 hours. These relationships may be a consequence of a neighboring group effect, whereby a repulsion between the chlorine atom and substituents in the *peri*-position (C.6 in structure II) strongly facilitates an internal cleavage reaction, *i.e.*, ii → iii → iv.



ii iii iv

Since the system VII makes a definite contribution to the absorption of Terramycin, in the median wave length region, these observations suggest that VII also is incorporated in the Aureomycin molecule.

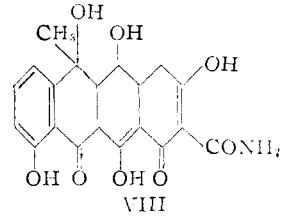


VII

The nitrogen atoms in Aureomycin were shown to be components of a carboxamido and a basic dimethylamino group. Thus, alkali treatment of the antibiotic liberated one mole each of ammonia and dimethylamine. The basic nature of the dimethylamino function became apparent (*vide infra*) when a number of non-basic C_{20} degradation products lacking this function were isolated. The carboxamido function was identified by the observation that *p*-toluenesulfonyl chloride in pyridine smoothly converts Aureomycin to **aureomycinonitrile,** $C_{21}H_{21}NO_7Cl(CN)$, whose infrared spectrum exhibits a characteristic nitrile band at 4.6 μ.

Comparison of the behavior of Aureomycin and Terramycin on reduction with zinc and acetic acid was of the greatest value in demonstrating conclusively the similarities in the structures of the two antibiotics. Like Terramycin, Aureomycin was found to be reduced under mild conditions to a **desdimethylamino compound,** $C_{20}H_{18}NO_8Cl$, whose absorption properties closely resembled those of the parent antibiotic. Thus, it was clear that the dimethylamino group of Aureomycin, like that of Terramycin: (a) must be α to a carbonyl group, to permit activation for removal by reduction, (b) could not be directly attached to a chromophoric system, and (c) could not be placed in such a way as to block alternative enolization within the polycarbonyl system (*vide infra*). These limitations require that the dimethylamino group be attached at C.4 (*cf.* II).

Vigorous treatment of Aureomycin with zinc and acetic acid, or further reduction of desdimethylaminoaureomycin with the same reagents, gave **desoxydesdimethylaminoaureomycin,** $C_{20}H_{18}NO_7Cl$, whose acidity constants and ultraviolet spectrum (Fig. 3) are very similar to those of the Terramycin analog (VIII)[4] but quite different from those of Aureomycin. Thus, as in the case of Terramycin, removal of the oxygen atom is attended by marked



VIII

changes in the direction of enolization of the polycarbonyl system. The close similarity of the phenomena in the two cases leaves no doubt that Aureomycin possesses an hydroxyl group at C.12a (*cf.* II) which is removed by vigorous zinc reduction.

When desoxydesdimethylaminoaureomycin was

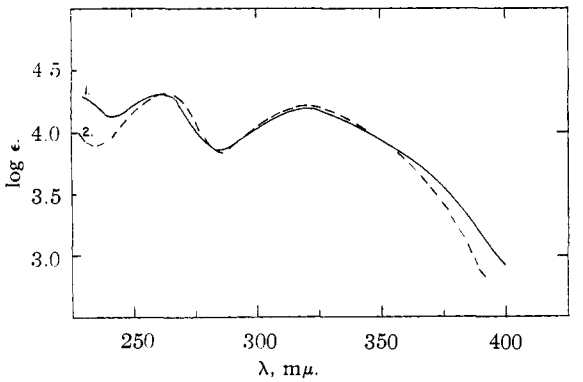
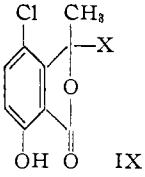


Fig. 3.—Ultraviolet spectra in acidic (0.01 *N*) methanol: 1, desoxydesdimethylaminoaureomycin; 2, desoxydesdimethylaminoterramycin (VIII).

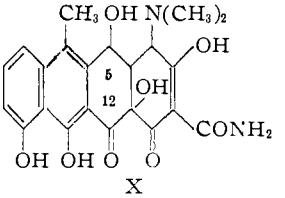
treated with alkali, it was converted smoothly to **isodesoxydesdimethylaminoaureomycin,** $C_{20}H_{18}NO_7Cl$, which clearly contains the hydroxyphthalide system (IX), since its infrared spectrum possesses a characteristic new band at 5.72 μ, and it gives 4-chloro-7-hydroxy-3-methylphthalide (**IX**, X = H) on pyrolysis. These changes, which find precise analogs in the Terramycin series,[4b] clearly



IX

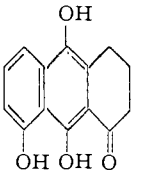
indicate the presence in Aureomycin of hydroxyl and methyl groups at C.6.

The first intimation that Aureomycin resembled Terramycin in possessing an hydroxylated six-membered ring C (*cf.* II) was obtained through the observation that treatment of Aureomycin with acidic reagents resulted in the formation[7] of reaction-mixtures with ultraviolet absorption similar to that of anhydroterramycin (X).[4b] This view was consolidated through the isolation from



X

Aureomycin, deschloroaureomycin, and desdimethylaminoaureomycin, after treatment with acids, of a series of monoanhydro compounds, **anhydroaureomycin,** $C_{22}H_{21}N_2O_7Cl$,[11,12] **deschloroanhydroaureomycin,** $C_{22}H_{22}N_2O_7$,[11] and **desdimethylaminoanhydroaureomycin,** $C_{20}H_{16}NO_7Cl$. The remarkable resemblance of the ultraviolet spectra (*cf.* Fig. 4) of these substances, in particular that of deschloroanhydroaureomycin, to those of anhydroterramycin (X) and the synthetic model XI,[4b] leaves no doubt that the anhydro compounds contain a 2-acyl-1,8-dihydroxynaphthalene system.



XI

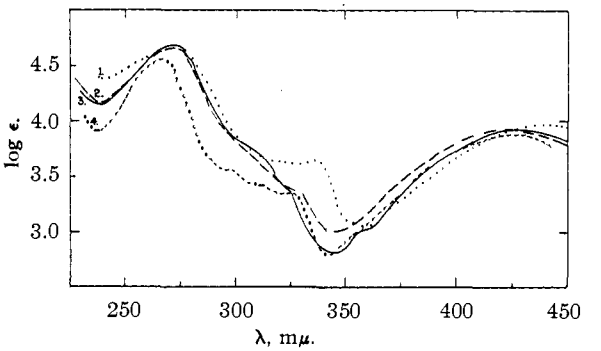
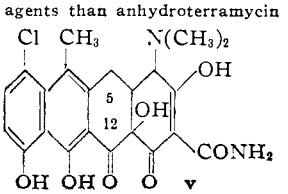


Fig. 4.—Ultraviolet spectra in acidic (0.01 *N*) methanol: 1, anhydroaureomycin (v); 2, anhydroterramycin (X); 3, deschloroanhydroaureomycin; 4, 3,4-dihydro-8,9,10-trihydroxy-1(2)-anthracenone (XI).

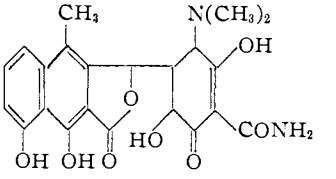
Conclusive evidence of the close similarity of the A/B ring systems of Aureomycin and Terramycin is provided by the reductive experiments described

(11) These derivatives were independently reported in a preliminary communication by Waller, *et al.* (ref. 5e).

(12) Anhydroaureomycin (v) was found to be much more stable toward hydrolytic agents than anhydroterramycin (X). The latter is rapidly converted into a mixture of the isomeric α- and β-apoterramycins (vi) (*cf.* ref. 4), by the action of acids or bases in dilute aqueous media, while anhydroaureomycin is relatively stable under the same conditions. This striking difference is explicable only through the

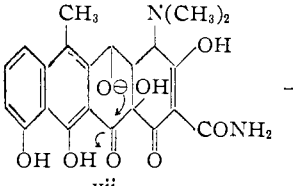


v

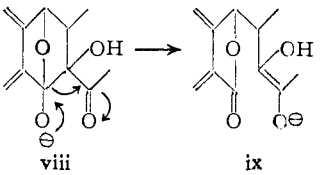


vi

assumption that the ready cleavage of anhydroterramycin is a consequence of internal attack by the C.5 hydroxyl group upon the carbonyl function at C.12 (vii → viii → ix). Since anhydroaureomycin lacks the hydroxyl group at C.5, no parallel change is possible.

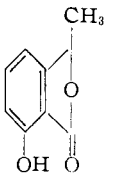


vii

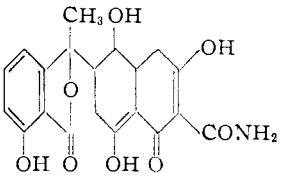


viii ix

above, taken with the following considerations. When the ultraviolet absorption spectrum of 7-hydroxy-3-methylphthalide (XII)[13] is subtracted from that of isodesoxydesdimethylaminoterramycin[4b] (XIII), a difference curve is obtained which

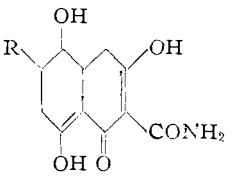


XII



XIII

represents the absorption attributable to the unique chromophoric system represented in XIV. *The*



XIV

identity (Fig. 5) of the curve obtained by subtracting the spectrum of 4-chloro-7-hydroxy-3-methylphthalide (IX, X = H) from that of isodesoxydesdimethylaminoaureomycin demonstrates that the chromophoric system of XIV is also present in the Aureomycin analog.

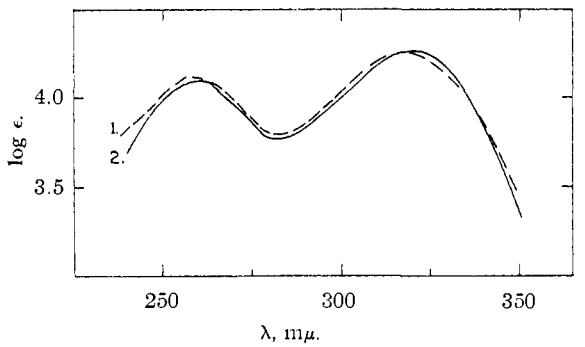
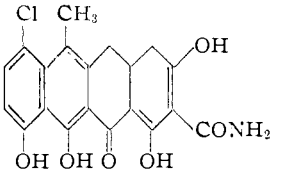
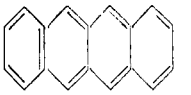


Fig. 5.—Ultraviolet spectrum derived for the chromophoric system XIV: 1, difference curve obtained by subtracting the ultraviolet absorption of 7-hydroxy-3-methylphthalide from that of isodesoxydesdimethylaminoterramycin (XIII); 2, difference curve obtained by subtracting the ultraviolet absorption of 4-chloro-7-hydroxy-3-methylphthalide from that of isodesoxydesdimethylaminoaureomycin.

The hydronaphthacene skeleton common to Terramycin and Aureomycin was also confirmed in the latter case. Desoxydesdimethylaminoaureomycin loses *one* molecule of water on treatment with acids, giving a red insoluble *anhydro* compound, $C_{20}H_{16}NO_6Cl$,[14] formulable as XV, or a tautomer. When this substance was distilled from zinc dust, naphthacene (XVI) was obtained.



XV



XVI

The observations reported here thus provide detailed information about the nature and arrangement of all the functional groups of Aureomycin, and require that the structure II be assigned to the antibiotic.

Experimental

Melting points were taken in Pyrex capillary tubes using a calibrated thermometer. Most of the compounds described herein showed decomposition points which varied considerably with the rate of heating. Specific rotations, unless otherwise specified, were all taken at a concentration of 1%. The acid constants reported are apparent values determined with a glass electrode instrument in 50% aqueous dimethylformamide solution, unless a different solvent is specified. Infrared spectra were determined on a Baird double beam recording spectrophotometer equipped with a sodium chloride prism. Ultraviolet absorption spectra were usually measured either in 95% ethanol or in absolute methanol; acid and alkali, when present, were at a concentration of 0.01 *N*. As was noted with Terramycin, Aureomycin and certain of its degradation products form unusually stable solvates with a wide range of solvents.

Aureomycin (II).—One gram of Aureomycin hydrochloride was dissolved rapidly in 20 ml. of hot water, cooled rapidly to 40°, treated with 0.1 ml. of 2 *N* hydrochloric acid, then chilled in an ice-bath. The product so obtained was recrystallized twice more in a similar manner to give 0.3 g. of yellow crystals which showed physical constants (m.p. 234–236° dec., $[\alpha]^{25}D$ −235° (water)) in good agreement with previously reported[2] values. For analysis this material was dried to constant weight at 0.1 mm. and 100°.

Anal. Calcd. for $C_{22}H_{23}N_2O_8Cl\cdot HCl$: C, 51.27; H, 4.69; N, 5.43; Cl, 13.76. Found: C, 51.24; H, 4.66; N, 5.40; Cl, 13.80.

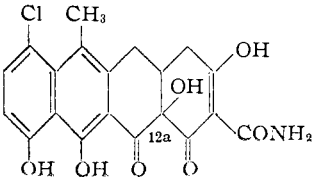
Titration of Aureomycin hydrochloride in aqueous solution shows pK_a's 3.4, 7.4 and 9.2.

The low solubility of Aureomycin hydrochloride in water made the following procedure necessary for a clean conversion of the commercial acid salt to the base: Aureomycin hydrochloride (51.5 g., 0.1 mole) was slurried in dimethylformamide (250 ml.) and stirred mechanically while one equivalent (8.4 g.) of sodium bicarbonate in aqueous solution (175 ml.) was gradually added. A clear solution was obtained which rapidly deposited amphoteric Aureomycin in good yield. Material obtained in this way was freed of water of hydration by azeotropic distillation in toluene solution. The crystalline anhydrous Aureomycin which separated on cooling was recrystallized from reagent grade benzene and dried to constant weight *in vacuo* at 100° over paraffin wax. The pure anhydrous antibiotic decomposes at 172–174°. The Zerewitinoff determination showed 7.4 equivalents of active hydrogen per mole (calcd. 7.0).

Anal. Calcd. for $C_{22}H_{23}N_2O_8Cl$: C, 55.17; H, 4.84; N,

(13) F. A. Hochstein and R. Pasternack, THIS JOURNAL, **74**, 3905 (1952).

(14) The absorption properties of this compound furnish an additional example of the role of the blocking hydroxyl group at C.12a in isolating two otherwise conjugated chromophoric systems. Thus, whereas desdimethylaminoanhydroaureomycin (x) and anhydroaureomycin (v) show identical ultraviolet spectra which closely resemble the absorption of the 2-acyl-1,8-dihydroxynaphthalene model (XI), compound XV exhibits absorption of a much more complicated nature.



x

5.85; Cl, 7.40. Found: C, 55.10; H, 4.90; N, 5.72; Cl, 7.27.

When anhydrous Aureomycin is recrystallized from methanol, it separates as yellow plates, m.p. 172–174° dec., containing methanol of crystallization which cannot be removed by analytical drying. A sample of this solvate showed the following analysis:

Anal. Found: C, 54.06; H, 5.33; N, 5.60; OCH_3, 3.12.

The carbon, hydrogen and nitrogen analytical values above are in agreement with the originally reported[2] analyses for Aureomycin; it may be suggested that solvation of this type led to the previously mentioned (*cf.* ref. *a*, Table I) incorrect formula assignment.

Deschloroaureomycin (Tetracycline) (i).—A solution of 9.6 g. (0.02 mole) of anhydrous Aureomycin in 50 ml. of dry dioxane was treated with 100 ml. of absolute methanol and 1 g. of 5% palladium-on-charcoal catalyst. The mixture was shaken at 3 atmospheres pressure in a Parr hydrogenation apparatus. After 18 hours, one equivalent of hydrogen had been absorbed and reaction had ceased. The catalyst was removed and the cold solution was concentrated to dryness *in vacuo*. The resulting crude hydrochloride was dissolved in 100 ml. of distilled water, adjusted to *p*H 5 with dilute ammonium hydroxide and extracted continuously with ether for 24 hours. Crystalline product separated from both the ether and the aqueous phases. There was obtained a total of 3.8 g. of fairly pure product, $[\alpha]^{25}D$ −224° (methanol). A sample prepared for analysis by recrystallization from toluene and drying exhaustively at 100° *in vacuo* showed $[\alpha]^{26}D$ −239 (methanol), m.p. 170–175° dec.

Anal. Calcd. for $C_{22}H_{24}N_2O_8$: C, 59.45; H, 5.44; N, 6.30. Found: C, 59.35; H, 5.41; N, 6.14.

Titration showed pK_a's 8.3 and 10.2, neut. equiv. 454 (calcd. mol. wt. 444). The ultraviolet absorption properties of this compound are identical with those of Terramycin (*cf.* Figs. 1 and 2).

Comparative Stabilities of Deschloroaureomycin (i) and Aureomycin (II).—A qualitative test for distinguishing between deschloroaureomycin and Aureomycin was developed which takes advantage of the observed marked difference in their stabilities in neutral or alkaline solutions. Samples of the two substances were placed in 100-ml. volumetric flasks, filled to the mark with 5% aqueous sodium bicarbonate, and maintained at room temperature. At appropriate time intervals, comparable samples were pipetted out, quenched by neutralizing with dilute hydrochloric acid, diluted to a standard volume and subjected to the Terramycin bioassay against *Klebsiella pneumoniae*.[15] Decomposition (as measured by the decrease in biological activity) followed pseudo first-order kinetics in both cases. For each of several samples followed in this manner the time for one-half decomposition of Aureomycin was 1–3 hours while that for deschloroaureomycin was 24–30 hours.

The results from a typical run are shown in Table II.

TABLE II

BIOLOGICAL POTENCY OF SOLUTION (TERRAMYCIN UNITS/ML.)

Time, hr.	Aureomycin	Deschloro-aureomycin
0	555	390
1.5	265	375
3.0	120	360
4.5	52	365
9	7	320
24	0	225

Identification of Dimethylamine and Ammonia from Aureomycin Hydrolysis.[16]—One gram (1.94 mmoles) of Aureomycin hydrochloride was boiled for 24 hours in 20% sodium hydroxide. The volatile amines were quantitatively collected and determined by the procedure described[17] for Terramycin. There was obtained 1.78 mmoles (0.92 equivalent) of ammonia and 1.81 mmoles (0.93 equivalent) of dimethylamine.

Aureomycinonitrile.—A slurry of 2.0 g. of amphoteric Aureomycin in 6 ml. of pyridine was stirred in a water-bath and treated with 3.0 g. of *p*-toluenesulfonyl chloride.[18] The solid dissolved rapidly, and the temperature rose to 60° in 45 seconds, then began to fall. The solution was rapidly cooled in an ice-bath to 33° over 1.25 minutes. After a total reaction time of only 2 minutes, the mixture was diluted with 50 ml. of water, and the crystalline precipitate which separated was dissolved in 15 ml. of dimethylformamide and treated with 40 ml. of acetone. Pure aureomycinonitrile separated rapidly as pale yellow plates, wt. 0.6 g. (31% yield). A sodium fusion on this product indicated the total absence of sulfur. For analysis the compound was recrystallized twice from dimethylformamide–acetone and dried at 100° and 0.05 mm. for five hours.

Anal. Calcd. for $C_{22}H_{21}N_2O_7Cl$: C, 57.33; H, 4.59; N, 6.08; Cl, 7.69. Found: C, 56.99; H, 4.58; N, 6.12; Cl, 7.46.

Like the nitriles from Terramycin, aureomycinonitrile shows no significant basic properties, *i.e.*, it does not form a hydrochloride. The compound does not show a definite melting point but sinters at around 252° without liquefying. It shows $[\alpha]^{25}D$ −324° (dimethylformamide). Titration shows pK_a's 7.1, 9.5; neut. equiv. 460 (calcd. mol. wt. 461). The ultraviolet absorption spectrum has peaks at λ_{max} 232 mμ, log ε 4.23; λ_{max} 277 mμ, log ε 4.11 and λ_{max} 374 mμ, log ε 4.02. The infrared absorption curve (in mull) shows a characteristic 4.6 μ absorption band due to the nitrile function.

Desdimethylaminoaureomycin.—A slurry of 10.0 g. of Aureomycin hydrochloride, 2.5 g. of sodium acetate and 20.0 g. of zinc dust (Mallinckrodt Analytical Reagent—from a freshly opened bottle) in 100 ml. of glacial acetic acid was stirred under nitrogen at 30–32° for six hours. The unreacted zinc dust was removed by filtration,[19] and the filtrate was freeze dried. A yellow residue (17.0 g.) containing the product in the form of a zinc complex was obtained. This solid was slurried in 85 ml. of methanol and treated with 6 ml. of concentrated hydrochloric acid. An initially clear solution was obtained which on being scratched rapidly deposited a heavy crystalline mass. After about 30 minutes at room temperature the product was isolated and washed with methanol. There was obtained 3.7 g. (41% yield) of light yellow plates, m.p. 156–159° with effervescence. These crystals contained one mole of methanol of crystallization; efforts to prepare a crystalline methanol-free product were not successful. For analysis, the product was recrystallized from dimethylformamide–methanol and dried at 100° and 0.5 mm. The analytical sample melted with methanol evolution at 160–161°.

Anal. Calcd. for $C_{20}H_{18}NO_8Cl \cdot CH_3OH$: C, 53.91; H, 4.72; N, 2.99; Cl, 7.57; OCH_3, 6.62. Found: C, 54.08; H, 4.95; N, 3.12; Cl, 7.58; OCH_3, 6.24.

Titration showed pK_a's of 7.4, 8.9 and a neutral equivalent of 482 (calcd. mol. wt. 468).

Desdimethylaminoaureomycin shows $[\alpha]^{25}D$ −302° (dimethylformamide). Its ultraviolet absorption spectrum has the same peak positions as Aureomycin (*cf.* Table I).

When a solution of 2 g. of desdimethylaminoaureomycin in 80 ml. of 2% sodium hydroxide was kept under nitrogen at room temperature overnight, then acidified, *isodesdimethylaminoaureomycin* ($C_{20}H_{18}NO_8Cl$) was obtained as colorless microcrystals (1.4 g., 70% yield). Recrystallization from dioxane–water and then from benzene gave colorless needles which showed $[\alpha]^{25}D$ −132° (dimethylformamide) and decomposed at about 255°.

Anal. Calcd. for $C_{20}H_{18}NO_8Cl$: C, 55.12; H, 4.16; N, 3.21. Found: C, 55.46; H, 4.29; N, 3.10.

Isodesdimethylaminoaureomycin has infrared absorption peaks in the carbonyl region at 5.71 and 5.81 μ (in mull); upon pyrolysis it furnishes 4-chloro-7-hydroxy-3-methylphthalide.[20] The ultraviolet absorption spectrum of iso-

(15) R. C. Kersey, *J. Am. Pharm. Assoc.*, **39**, 252 (1950).

(16) We are indebted to Dr. R. L. Wagner for this determination.

(17) R. Pasternack, A. Bavley, R. L. Wagner, F. A. Hochstein, P. P. Regna and K. J. Brunings, THIS JOURNAL, **74**, 1926 (1952).

(18) The conversion of amides to nitriles by the action of acid halides in pyridine has precedent, *cf.* ref. 4b; Q. E. Thompson, THIS JOURNAL, **73**, 5841 (1951) and J. Mitchell, Jr., and C. E. Ashby, *ibid.*, **67**, 161 (1945).

(19) *Caution:* the zinc became pyrophoric when dry.

(20) The pyrolysis behavior and infrared absorption of isodesdi-

desdimethylaminoaureomycin is substantially identical with that of isoaureomycin.[5d] Titration of the compound showed pK_a's 7.6 and 8.5, neutral equivalent 440 (calcd. mol. wt. 436).

Desoxydesdimethylaminoaureomycin.—The same quantities of reactants (10 g. of Aureomycin hyrochloride, 2.5 g. of sodium acetate, 20 g. of zinc dust and 100 ml. of acetic acid) and conditions were employed as were used with desdimethylaminoaureomycin, with the exception that the reaction time was extended to 72 hours. The bright yellow residue which remained after freeze drying the reaction solution was slurried in 60 ml. of cold methanol, then treated with 10 ml. of concentrated hydrochloric acid. The resulting solution was quickly poured into 200 ml. of water. The precipitated solid was taken up in ether, and the ethereal solution was evaporated to yield a yellow, amorphous powder. This weighed 5.5 g. and consisted largely of the desired product—as was apparent from its infrared and ultraviolet absorption spectra. Efforts to crystallize this substance were not successful, although it could be converted in good yield into crystalline degradation products.

A sample was prepared for analysis by reprecipitation from ether with light petroleum. Analysis of this amorphous material suggested that a small amount of the deschloro analog was present as a side product.

Anal. Calcd. for $C_{20}H_{18}NO_7Cl$: C, 57.21; H, 4.31; N, 3.33; Cl, 8.46. Found: C, 57.81; H, 4.92; N, 3.02; Cl, 7.42.

Titration of the substance showed pK_a's of 7.1 and ≃11, neutral equivalent 423 (calcd. mol. wt. 420). The infrared and ultraviolet (*cf.* Fig. 3) absorption spectra of desoxydesdimethylaminoaureomycin are strikingly similar to those of the Terramycin analog VIII.[4b]

When desdimethylaminoaureomycin was subjected to the above conditions, an identical product resulted.

Isodesoxydesdimethylaminoaureomycin (xii).—A solution of 2.0 g. of amorphous desoxydesdimethylaminoaureomycin in 50 ml. of 0.5 *N* alcoholic potassium hydroxide was stirred under nitrogen at room temperature for four hours. The solution was then poured into 200 ml. of water and acidified with concentrated hydrochloric acid. An oil separated which soon crystallized on standing. There was obtained 1.5 g. (75% yield) of a tan crystalline product. Recrystallization from aqueous acetone, then from ethanol, gave colorless needles decomposing at 208–210° when placed in a bath at room temperature and heated rapidly to 185°, then at 3° per minute; when the substance is placed in a bath preheated to 185° it decomposes immediately.

Anal. Calcd. for $C_{20}H_{18}NO_7Cl$: C, 57.21; H, 4.31; N, 3.33; Cl, 8.46. Found: C, 57.42; H, 4.58; N, 3.34; Cl, 8.00.

The compound shows pK_a's 7.5 and 8.6, neut. equiv. 414 (calcd. mol. wt. 420). The ultraviolet absorption curve (which is very similar to that of isodesoxydesdimethylaminoterramycin (XIII)[4b]) shows λ_{max} 240 mμ, log ε 4.15; λ_{max} 257, log ε 4.15; λ_{max} 317 mμ, log ε 4.26 in acidic methanol. The infrared spectrum in chloroform shows a single sharp band (5.72 μ) in the carbonyl region.

Pyrolysis of Isodesoxydesdimethylaminoaureomycin.—Approximately 50 mg. of isodesoxydesdimethylaminoaureomycin was placed in a Pyrex test-tube, covered lightly with glass wool and warmed over a soft flame under reduced pressure (100 mm.). Decomposition occurred, accompanied by distillation of an oil onto the sides of the test-tube. This distillate was washed out with ether. Removal of the ether left a crystalline residue. The products from five such pyrolyses were combined and resublimed twice to give 20 mg. of slightly impure 4-chloro-7-hydroxy-3-methylphthalide, m.p. 110–112°,[21] pK_a 8.0 (50% ethanol), neut. equiv. 197 (calcd. mol. wt. 198).

Anal. Calcd. for $C_9H_7O_3Cl$: C, 54.42; H, 3.55. Found: C, 55.25; H, 3.99.

The ultraviolet absorption spectrum of 4-chloro-7-hydroxy-3-methylphthalide shows λ_{max} 235 mμ, log ε 3.80; λ_{max} 310 mμ, log ε 3.53 (acidic methanol).

Application of the above pyrolysis procedure to isodesdimethylaminoaureomycin (xi) also furnished 4-chloro-7-hydroxy-3-methylphthalide in comparable yield.

Desdimethylaminoanhydroaureomycin (x).—One and one-half grams of pure desdimethylaminoaureomycin in 60 ml. of 1:1 dioxane–methanol was treated with 60 ml. of saturated methanolic hydrogen chloride solution and left overnight in the refrigerator. The long orange needles of pure desdimethylaminoanhydroaureomycin which separated were dried to constant weight at 100° *in vacuo*.

Anal. Calcd. for $C_{20}H_{16}NO_7Cl$: C, 57.49; H, 3.86; N, 3.35; Cl, 8.49; mol. wt., 418. Found: C, 57.13; H, 4.04; N, 3.17; Cl, 8.70; mol. wt. (titration), 421.

Desdimethylaminoanhydroaureomycin shows $[\alpha]^{25}D$ −229° (dimethylformamide). The compound decomposes without liquefaction at about 240°. Its ultraviolet absorption spectrum is superimposable upon that of anhydroaureomycin (*cf.* Fig. 4).

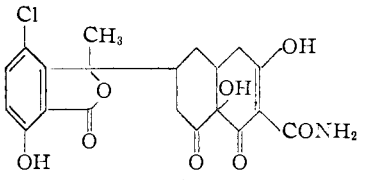
Anhydroaureomycin (v).—A solution of 0.5 g. of Aureomycin hydrochloride in 20 ml. of saturated methanolic hydrogen chloride was kept at 5° for 2 days. An excess of anhydrous ether was then added. The resulting deep orange amorphous precipitate was isolated, dissolved in water and adjusted to *p*H 4.0. The precipitate which formed was dried by azeotropic distillation with benzene, then recrystallized twice from benzene. Pure anhydroaureomycin was obtained as orange yellow needles, m.p. 210° dec. when placed in a bath at 200° and heated at a rate of 2° per minute. The decomposition point[22] varied considerably with the rate of heating.

Anal. Calcd. for $C_{22}H_{21}N_2O_7Cl$: C, 57.33; H, 4.59; N, 6.08. Found: C, 57.50; H, 4.53; N, 5.92.

The ultraviolet absorption spectrum of anhydroaureomycin shows a close similarity to the spectra of anhydroterramycin and deschloroanhydroaureomycin (*cf.* Fig. 4). Anhydroaureomycin, in sharp contrast with anhydroterramycin, is quite stable in both acidic and alkaline solutions. In one experiment a considerable portion of the material was recovered unchanged after warming in 1% alkali on the steam-bath for one hour. (Anhydroterramycin is almost immediately isomerized[4b,12] to the *apo*terramycin compounds by 0.01 *N* alcoholic alkali at room temperature).

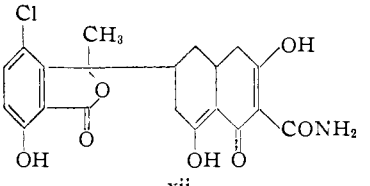
Deschloroanhydroaureomycin.—Five grams of crude deschloroaureomycin was dissolved in a mixture of 10 ml. of methanol and 40 ml. of concentrated hydrochloric acid and

methylaminoaureomycin permits the assignment of structure xi to this substance. The interesting observation that this compound

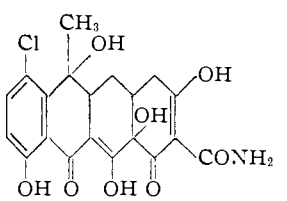


xi

(unlike isodesoxydesdimethylaminoaureomycin (xii) or desdimethylaminoaureomycin xiii) exhibits a strong 5.81 μ infrared peak (due to



xii



xiii

the isolated ketone group) serves to demonstrate further the presence of the C.12a hydroxyl group in Aureomycin (II).

(21) S. Kushner, *et al.*, THIS JOURNAL, **75**, 1097 (1953) report a melting point of 114–115.5° for a synthetic sample of this compound.

(22) C. W. Waller, *et al.*, report a decomposition point of 220–235° for this substance; *cf.* reference 5e.

heated on a steam-bath for 30 minutes. The resulting hot solution was neutralized to *p*H 5.5 with dilute aqueous sodium hydroxide, cooled and extracted continuously with ether for three days. During this time considerable material crystallized from the ether solution.

The ether was cooled and filtered, and the resulting crystalline material was recrystallized from benzene–hexane to give 1.0 g. (22% yield based on crude starting material) of deschloroanhydroaureomycin, m.p. 215–220° dec., $[\alpha]^{25}D$ +25°[23] (cellosolve). This compound shows ultraviolet absorption properties substantially identical with those of anhydroterramycin (*cf.* Fig. 4).

Anal. Calcd. for $C_{22}H_{22}N_2O_7$: C, 61.96; H, 5.20; N, 6.57. Found: C, 62.01; H, 5.18; N, 6.45.

Desoxydesdimethylaminoanhydroaureomycin (XV or a Tautomer).—A solution of 3.6 g. of amorphous desoxydesdimethylaminoaureomycin in 100 ml. of methanol was treated with 100 ml. of saturated methanolic hydrogen chloride solution. A rapid reaction occurred during which an orange-red powder separated. After 30 minutes at room temperature, the solid was collected and washed with methanol. There was obtained 2.9 g. of amorphous product. This was boiled in 40 ml. of dioxane, filtered hot and washed with dioxane. Two grams (57% yield) of crystalline product of good quality was obtained. Recrystallization from boiling nitrobenzene gave orange-red needles which decomposed without liquefying at about 240–250° and showed a specific rotation at 25° (0.1% in dimethylformamide) of +955°. This compound shows ultraviolet absorption peaks at 275, 338, 391 and 450 mμ in glacial acetic acid solution.

Anal. Calcd. for $C_{20}H_{16}NO_6Cl$: C, 59.78; H, 4.02; N, 3.49; Cl, 8.83. Found: C, 60.13; H, 4.14; N, 3.57; Cl, 8.90.

Naphthacene from Desoxydesdimethylaminoanhydroaureomycin.—Seventy-five milligrams of desoxydesdimethylaminoanhydroaureomycin was mixed in a mortar with 5 g. of purified[24] zinc dust, packed between asbestos plugs in an 8 × 300 mm. Pyrex tube, flushed with hydrogen, and heated to a dull red heat in a slow stream of hydrogen. The small amount of orange-red distillate which collected in the cooler parts of the tube was sublimed twice at 180° (0.05 mm.) to yield 0.5 mg. of pure naphthacene—identified by its very characteristic ultraviolet absorption peaks at 280.5, 295, 355, 375, 395, 418, 444 and 475 mμ in benzene solution. An additional 0.5–1.0 mg. of less pure naphthacene was also recovered from the reaction.

Acknowledgments.—We should like to express our gratitude to Messrs. G. B. Hess, T. J. Toolan and Dr. J. R. Means for the analytical data and for physical measurements recorded in this paper. We are also indebted to Mr. E. J. Bianco for certain preparative work, and to Dr. D. B. Seeley and associates for the microbiological assays.

(23) This product was also prepared, in a different manner, by C. W. Waller and co-workers who report m.p. 225–226° dec. and $[\alpha]^{25}D$ +24° (cellosolve); *cf.* reference 5e.

(24) The purification procedure is described in the Experimental section of reference 4b.

BROOKLYN 6, N. Y.
CAMBRIDGE, MASS.

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

The Stability of Coenzyme A[1]

BY D. A. BUYSKE, R. E. HANDSCHUMACHER,[2] E. D. SCHILLING AND F. M. STRONG

RECEIVED FEBRUARY 1, 1954

The stability of a high purity preparation of coenzyme A has been followed by use of the transacetylase assay system. It was found that the transacetylase method measured only intact CoA, while the sulfanilamide method was active for fragments of the CoA molecule. Acetone powders and high purity CoA preparations in the free acid form were found to be stable for several years when stored under dry conditions, at room temperature. Drying of high purity powders *in vacuo* produced marked decreases in activity. Aqueous solutions subjected to autoclave temperatures showed considerable destruction. Alkaline solutions of CoA were found to be unstable, while acidic solutions were much more stable and even showed an increase in activity under certain conditions of time, temperature and *p*H.

The great interest in coenzyme A (CoA) and its wide current research use has prompted the present study of its stability under a variety of commonly encountered conditions. Most of the work was carried out on a single highly purified preparation, although a few observations were made on a crude concentrate.

Experimental

Materials.—Crude concentrates containing 2–4% of CoA (acetone powder stage) were prepared from brewers' yeast as previously described.[3] To obtain CoA of the highest purity, the previous procedure[3] was modified by including a reduction step after the second charcoal adsorption and before the copper–glutathione precipitation. The pyridine-free eluate from the second charcoal column was made 0.5 *N* in sulfuric acid and forced by air pressure through a Jones reductor consisting of a 1 × 15 cm. column of freshly amalgamated, granulated zinc (20 mesh). The effluent was kept at 0–5° until all the solution had passed through (about 30 minutes). The solution was passed through a Dowex-50 column, then warmed to 40°, immediately precipitated in the usual manner and carried through to the lyophilized powder as previously described.[3]

The freshly purified material (approximately 95% CoA) was dissolved in water at a concentration of 2.5 mg. per ml. and 0.2-ml. aliquots were dispersed into a series of 10-ml. ampules. The solutions were lyophilized, and the ampules filled with nitrogen, sealed, and stored at −5° in the dark until needed for stability trials. No detectable change in activity occurred under these conditions during the two months required to complete the stability studies.

Analytical Methods.—CoA activity was determined by the transacetylase method,[4] using transacetylase from *E. coli*. Occasional samples were also checked by the sulfanilamide method.[5] Pantothenic acid (PA) was determined microbiologically after enzymatic liberation,[6] and ribose by Drury's method.[7] Adenine was estimated spectrophotometrically after hydrolysis in *N* HCl at 100° for one hour. The molar extinction value of 13,300 for free adenine in acid solution at the maximum wave length of 264 mμ was used for the calculations. The Beckman model DU spectrophotometer was used for all measurements in the ultraviolet.

(1) Presented in part at the 124th Meeting of the American Chemical Society, Chicago, Ill., September, 1953. Supported in part by grants from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

(2) National Science Foundation Predoctoral Fellow, 1952–1953.

(3) H. Beinert, R. W. Von Korff, D. E. Green, D. A. Buyske, R. E. Handschumacher, H. Higgins and F. M. Strong, *J. Biol. Chem.*, **200**, 385 (1953), and preceding papers.

(4) E. R. Stadtman, G. D. Novelli and F. Lipmann, *ibid.*, **191**, 365 (1951).

(5) N. O. Kaplan and F. Lipmann, *ibid.*, **174**, 37 (1948).

(6) J. B. Neilands and F. M. Strong, *Arch. Biochem.*, **19**, 287 (1948).

(7) H. F. Drury, *ibid.*, **19**, 455 (1948).