Anal. Calcd. for $C_{18}H_{18}NO_8 \cdot ClO_4$: C, 54.61; H, 4.58; Cl, 8.96; 3 OCH_3, 23.52. Found: C, 54.59; H, 4.60; Cl, 8.77; OCH_3, 23.49.

Action of Heat on Galanthine.—A sublimation tube was packed with 12 mg. of galanthine and heated to 280° (3 mm.). A fluorescent oil sublimed along with much unchanged galanthine. The total solid was dissolved in benzene, and the solution was chromatographed on a short column of aluminum oxide. A fluorescent band was eluted easily with benzene and 50% benzene-ethyl acetate. The infrared spectrum of the crude product (2–3 mg.) was nearly identical with that of synthetic II. It exhibited the same action during melting point determination as authentic II[1] and, therefore, was converted to the corresponding 7-phenanthridone by air at 150° and sublimed at 200° (0.25 mm.). The m.p. of the crude product, 265–270° (reported[1] 272–274°), was not depressed by admixture with authentic phenanthridone.[1] The infrared spectrum (KBr) also was identical with that of authentic phenanthridone.[1]

BETHESDA 14, MARYLAND

[CONTRIBUTION FROM THE SOUTHERN UTILIZATION RESEARCH BRANCH, AGRICULTURAL RESEARCH SERVICE, UNITED STATES DEPARTMENT OF AGRICULTURE]

Formation of Copper Complexes During Tyrosinase-catalyzed Oxidations

BY JETT C. ARTHUR, JR., AND T. A. MCLEMORE

RECEIVED DECEMBER 27, 1955

The formation of Cu complexes with products of catechol, hydroquinone, chlorogenic and caffeic acids, during tyrosinase-catalyzed oxidation, was determined, using radioactive Cu^{64} as a tracer and an ion-exchange method. The most stable Cu complexes formed were with *o*-quinone, with their stability decreasing with the introduction of groups on the initial aromatic ring of the compounds investigated. The maximum amount of Cu was complexed with *o*-quinone at the time of its formation. If the polymerization of the *o*-quinone was allowed to proceed prior to the addition of Cu^{++}, a smaller amount of metal was bound.

Introduction

An investigation of the formation of Cu complexes with products of tyrosinase-catalyzed reactions could indicate some differences in the mechanism of oxidation of different substrates, particularly *o*-dihydric and *p*-dihydric phenolic donors. The determination of the formation of Cu complexes using ion-exchange methods,[1,2] radioactive Cu^{64} and catalytic quantities of tyrosinase oxidizing the substrates would offer a new approach to this problem. It is the purpose of this report to present experimental data which indicate formation of Cu complexes with *o*-quinones.

Experimental

The activity of the tyrosinase was measured manometrically (Warburg apparatus) at 25° by determining the rate of oxygen uptake. The flasks, about 17–18 ml., contained two side arms and a center well. The enzyme and $Cu^{64}SO_4$ solutions were usually put in separate side arms. The center well contained a filter paper wick wetted with 0.2 ml. of 10% KOH to absorb CO_2 which might be released. Phosphate buffer (0.3 ml. of 0.5 *M*) and substrate solution (optimum amount) previously determined were placed in the main reservoir of the flask, sufficient Cu-free distilled water being added to bring the total liquid volume to 3 ml.

During the measurements of the rate of oxidation of different substrates by tyrosinase, $Cu^{64}SO_4$ solution was added. After a predetermined time, cation exchange material, 0.2 g. of phosphorylated cotton fabric,[3] was added to remove the copper ions from the solution. The Cu^{64} complexed was determined by drying an aliquot of the solution (which had been extracted with cation-exchange material by shaking the material with the solution for 1 hr.) and measuring its radioactivity in a gas flow counter. The use of this method to determine formation of metal complexes has been outlined by Schubert and Richter.[1,2]

The mushroom tyrosinase used was a commercial preparation. About 0.1 ml. of the preparation as received, about 0.5 mg. of enzyme preparation, was diluted with Cu-free water to 25 ml. One-ml. portions of this diluted preparation, about 20 μg. of enzyme, were used per test.

The Cu^{64} (half-life 12.88 hr.; β-0.57, 0.65; γ-1.34 mev.) was obtained as Cu wire, initial specific activity about 300 mc./g. About 0.64 g. of Cu was dissolved in 5 ml. of concentrated HNO_3, then 3 ml. of concentrated H_2SO_4 were added and the mixture was heated until SO_3 was evolved. The acid $Cu^{64}SO_4$ solution was diluted with Cu-free distilled water to yield a stock solution containing 3–4 μg. of Cu per ml. The other chemicals used were C.P. or reagent grade.

Results

The effect of substrate on tyrosinase oxidation at *p*H 6.7 and 25° with $Cu^{64}SO_4$ equivalent to 3.84 μg. of Cu^{64} added at zero time is shown in Fig. 1. The tyrosinase preparation has *o*-dihydric phenolase activity; however, hydroquinone can be oxidized by the enzyme on addition of a trace of catechol as a mediator. Using about 20 μg. of enzyme per test, the data indicate that initially the mechanism of oxidation of catechol and hydroquinone containing a trace of catechol approximates zero order.

Oxidation was also determined at *p*H 5.6 and 7.8 at 25°. Very little deviation from the data shown in Fig. 1 for *p*H 6.7 was noted.

The effect of *p*H and substrate on the amount of Cu ion complexed is shown in Table I. $Cu^{64}SO_4$, equivalent to 3.84 μg. of Cu, was added to the enzyme–substrate solution at zero time. After 60 minutes, cation-exchange cotton fabric was added. An aliquot of the extracted solution was analyzed for radioactive Cu^{64}, and, after appropriate corrections for decay time, the μg. of Cu associated with

TABLE I

EFFECT OF *p*H AND SUBSTRATE ON Cu^{++} COMPLEXED[a]

Substrate	Cu^{++} complexed, μg. *p*H 5.6	*p*H 6.7	*p*H 7.8
Hydroquinone + trace catechol	0.04	0.19	0.50
Catechol	.77	1.01	.90
Chlorogenic acid	.22	0.08	.38
Caffeic acid	.40	.23	.23
Protocatechuic acid	.00	..	.00
Hydroquinone	.06	.00	.00

[a] Age of diluted enzyme, 24 hr., estimated Cu content of tyrosinase used per test, 0.04 μg.; values reported are in excess of controls.

(1) J. Schubert, *J. Phys. Colloid Chem.*, **52**, 340 (1948).

(2) J. Schubert and J. W. Richter, *ibid.*, **52**, 350 (1948); THIS JOURNAL, **70**, 4259 (1948).

(3) J. D. Guthrie, *Ind. Eng. Chem.*, **44**, 2187 (1952).

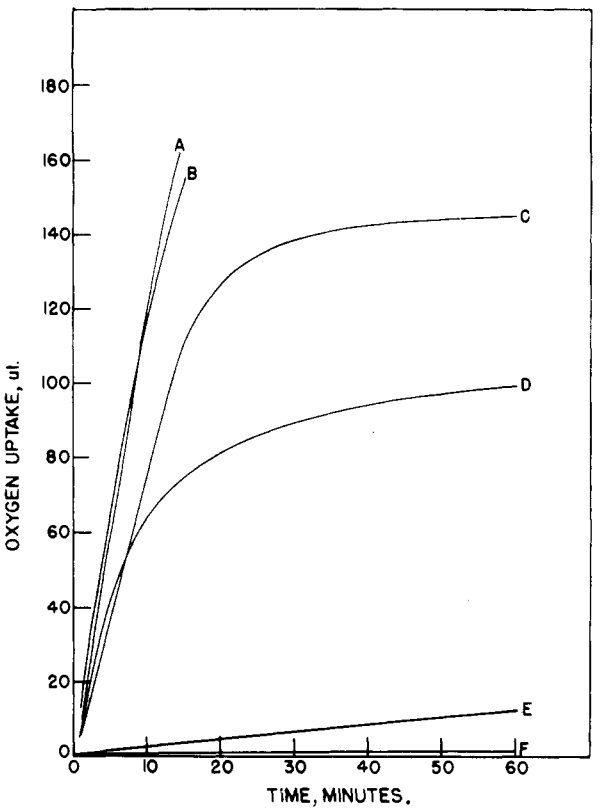


Fig. 1.—Effect of substrate on tyrosinase oxidation at *p*H 6.7, 25°: A, hydroquinone 3 mg., catechol 0.4 mg.; B, catechol 20 mg.; C, chlorogenic acid 3 mg.; D, caffeic acid 3 mg.; E, protocatechuic acid 3 mg.; F, hydroquinone 3 mg.

the enzyme, substrate and/or oxidation product was calculated. Controls, consisting of buffered solution of the chemicals and of buffered solution containing the enzyme in absence of substrates, were also run. Residual radioactivity in the extracted solutions in these cases indicated less than 0.05 p.p.m. of Cu^{++}, equivalent to about 0.15 μg. of Cu in the solution.

The copper content of tyrosinase is about 0.2%, and for 20 μg. of enzyme only about 0.04 μg. of Cu is present.

It was observed that in the case of catechol substrate, the largest amount of Cu was complexed. For the *o*-dihydric phenols of chlorogenic, caffeic and protocatechuic acids, the addition of other groups to the aromatic ring affected the apparent activity of the enzyme and the amount of Cu complexed.

In the case of hydroquinone, a *p*-dihydric phenol, the amount of Cu complexed was very low. Hydroquinone containing a trace of catechol was a very active substrate (see Fig. 1); the amount of Cu complexed was significant.

The effect of denaturation of the diluted tyrosinase by aging and the time of addition of $Cu^{64}SO_4$ on the amount of the Cu complexed is shown in Table II. The decrease in O_2 uptake in the first minute is a measure of the denaturation of the enzyme. It is observed that the amount of Cu complexed (measurements at the end of 60 minutes) decreased with denaturation of the enzyme.

TABLE II

EFFECT OF *p*H, DENATURATION OF TYROSINASE AND TIME OF ADDITION OF Cu^{64} ON Cu^{++} COMPLEXED[a]

Age of diluted enzyme	O_2 Uptake at 1 min., μl.		Cu^{++} Complexed, μg.	
	*p*H 5.6	*p*H 6.7	*p*H 5.6	*p*H 6.7
	Cu^{64} added at 0.0 hr.			
Fresh	23	34	1.88	2.45
24 hr.	10	10	0.77	1.01
	Cu^{64} added at 0.5 hr.			
Fresh	17	18	1.03	0.23

[a] Substrate catechol; values reported are in excess of controls.

When $Cu^{64}SO_4$ was added after 0.5 hr. of oxidation, the amount of Cu complexed was less than when the Cu was added at zero time. It is also seen that the effect of the presence of Cu^{++} at zero time was to increase the oxidation.

Discussion

The determination of the Cu^{++} complexed is based primarily on the fact that the quantity of a cation bound to a definite amount of cation exchanger at equilibrium is proportional to the concentration of free ions in the solution.[1,2] If measurements are made in the absence and presence of complexing agents, then at equilibrium the total metal species in solution, free metal ions and complexed metal, is proportional to metal on the cation exchanger.[4] Therefore, under given experimental conditions, an increase in the amount of Cu remaining in the solution is directly proportional to the formation of Cu complexes.

The data indicate that the most stable Cu complex formed, in the cases investigated, were with *o*-quinone. *o*-Quinones of chlorogenic and caffeic acids also formed Cu complexes; however, the formation constants were apparently less than for *o*-quinone. Protocatechuic acid and hydroquinone were poor substrates for this tyrosinase preparation.

Although hydroquinone, containing a trace of catechol, is a good substrate for this tyrosinase (see Fig. 1), the Cu complex formed is less stable than the Cu–*o*-quinone complex and is also more dependent on *p*H. The mechanism of the oxidation of hydroquinone, using catechol as a mediator, has been proposed as (1) the catalyzed oxidation of catechol, (2) the chemical reaction of *o*-quinone and hydroquinone to yield catechol and hydroxyhydroquinone and then (3) catalyzed oxidation of the former to *o*-quinone and of the latter to hydroxy-*o*-quinone.[5]

The data in Table I would indicate that Cu complexes of the oxidation products of catechol and hydroquinone have significantly different formation constants. If the proposed mechanism of oxidation of hydroquinone is accepted, it would appear that the presence of the OH group of hydroxy-*o*-quinone is a determining factor in the formation constants of its Cu complexes. Another possibility is that hy-

(4) A. E. Martell and M. Calvin, "Chemistry of the Metal Chelate Compounds," Prentice-Hall, Inc., New York, N. Y., 1952.

(5) J. M. Nelson and C. R. Dawson, *Advances in Enzymol.*, **4**, 99 (1944).

droquinone is oxidized to *p*-quinone. Then the differences in Cu, not extracted from the solution by the cation-exchange materials, would indicate a higher formation constant for Cu–*o*-quinone than for Cu–*p*-quinone.

The apparent effect of denaturation of the tyrosinase on the Cu^{++} complexed, as shown in Table II, is probably related to the amount of *o*-quinone formed rather than change in the enzyme. The apparent effect of time of addition of $Cu^{64}SO_4$ to the oxidizing solution on the amount of Cu^{++} complexed is probably due to the polymerization of the *o*-quinone and the decrease in active binding sites. That is, if the Cu^{++} was present at the time of formation of *o*-quinone, a greater amount was complexed than if the *o*-quinone was allowed to begin polymerization prior to addition of the $Cu^{64}SO_4$.

NEW ORLEANS, LOUISIANA

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

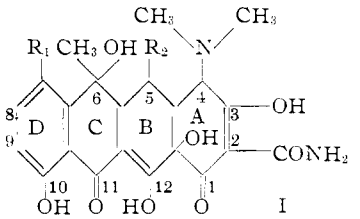
Acidity Constants of the Tetracycline Antibiotics

BY C. R. STEPHENS, K. MURAI, K. J. BRUNINGS AND R. B. WOODWARD

RECEIVED FEBRUARY 20, 1956

The three observed dissociation constants of the antibiotics oxytetracycline, chlorotetracycline and tetracycline have been assigned to specific acidic groupings. In each case, the first dissociation is due to the tricarbonyl system in ring A, the second to the dimethylammonium function[1] and the third to the phenolic β-diketone system (C.10, C.11, C.12). The findings reveal that in neutral solution these antibiotics exist largely as zwitterions.

The antibiotics oxytetracycline, tetracycline and chlorotetracycline may be illustrated by the general structural formula I.[2] It will be noted that the molecule I contains several acid groupings of a

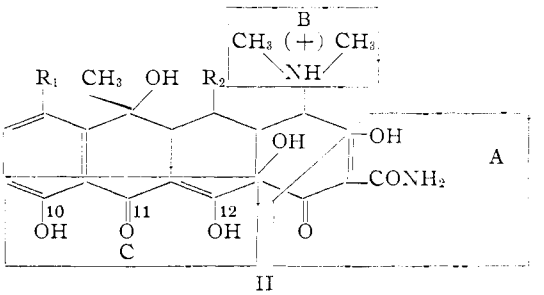


I

Ia, Oxytetracycline
$R_1 = H$, $R_2 = OH$
Ib, Chlorotetracycline
$R_1 = Cl$, $R_2 = H$
Ic, Tetracycline
$R_1 = R_2 = H$

rather unusual type. This communication is concerned with the assignment of the observed acidity constants of each of the three antibiotics to the specific functions responsible for the dissociation. From an examination of Table I, it is apparent that the three antibiotics are closely similar in acidity properties. This, of course, is consistent with their structural relationship. Thus, if we assign the pK_a's in any one of the antibiotics we will assume a similar relationship to apply in the other two.

The general formula I, written as an acid salt II, contains three distinct acid groups—the tricarbonylmethane system A, the ammonium cation B, and the



II

phenolic diketone system C.[3] The problem is thus to associate the 3, 7 and 9 pK_a values with the correct system (A, B or C).

TABLE I

pK_a VALUES[a] (OF HYDROCHLORIDES) IN AQUEOUS SOLUTION AT 25°

Oxytetracycline (Ia)	3.27	7.32	9.11
Chlortetracycline (Ib)	3.30	7.44	9.27
Tetracycline (Ic)	3.30	7.68	9.69

[a] These are carefully corrected thermodynamic pK_a's. They differ very slightly from previously reported values; *cf.* reference 2 and A. Albert, *Nature*, **172**, 201 (1953).

Our earliest data pertinent to the assignment of dissociation constants was derived through etherification studies on Terramycin.[2c] With diazomethane, a 10% yield of a dimethyl ether[2a] ($C_{24}H_{28}N_2O_9$) was obtained in addition to a larger amount of water-soluble, unstable, amorphous material. We have been able to prove that dimethyloxytetracycline contains one methoxyl at C.12 and another at either C.1 or C.3 (*cf.* I) in an otherwise unaltered molecule. Thus, the spectral properties and composition[2a] of the ether indicate that its basic Terramycin skeleton is still intact. Hydrolysis with

(1) ADDED IN PROOF.—Our more recent studies have placed the above assignments for pK_{a1} and pK_{a2} in serious jeopardy; see ref. 11.

(2) (a) 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, THIS JOURNAL, **75**, 5455 (1953); (b) 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, *ibid.*, **76**, 3568 (1954); (c) Terramycin is the registered trade-mark of Chas. Pfizer & Co., Inc., for the antibiotic oxytetracycline. Aureomycin is the registered trade-mark of the Lederle Laboratories Division of American Cyanamid Co. for chlorotetracycline. Tetracyn is the registered trade-mark of Chas. Pfizer & Co., Inc., for tetracycline; Achromycin is the trade-mark of American Cyanamid Co. for this antibiotic.

(3) In the case of system C we will make no effort to speculate as to which hydroxyl function (*i.e.*, the C.10 or C.12 —OH, etc.) actually loses its proton in this strongly chelated grouping of atoms.