OXIDATION OF ASCORBIC ACID BY TERRAMYCIN *

by

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While studying the effect of antibiotics on the cytochrome oxidase of beef heart[1], an unusually high endogenous oxygen uptake was observed in controls in which cytochrome C, terramycin and ascorbic acid were incubated together in the absence of the enzyme. This enhanced oxygen consumption was subsequently established to be due to the oxidation of ascorbic acid by terramycin to the dehydro form. In view of the importance of ascorbic acid in the maintenance of oxidation reduction potentials of biological systems[2], it was considered of interest to study in some detail the factors involved in this interesting catalytic property of the antibiotic.

EXPERIMENTAL

Antibiotics and other chemicals

The following crystalline antibiotic obtained through the courtesy of their respective manufacturers were used in this study. Penicillin G (Indian Penicillin Committee), Dihydrostreptomycin sulphate (Glaxo Laboratories), Aureomycin hydrochloride (Lederle Laboratories), Chloramphenicol (Parke Davis & Company), Neomycin sulphate (Upjohn & Company) and Terramycin hydrochloride (Charles Pfizer & Company).

Ascorbic acid and other chemicals employed were of reagent grade purchased from British Drug Houses Ltd., Charles Pfizer or Nutritional Bio-chemicals.

Manometry

Conventional Warburg techniques were adopted using flasks with centre cups and single side arms.

Analytical method

Ascorbic acid was estimated according to ROE AND OESTERLING[3].

Spot tests for copper in the drugs used were carried out with dithiozone reagent[4] (10 mg in 100 ml chloroform).

Antibiotic assay

Terramycin was tested by the standard cup assay method[5] with *S. aureus* (Oxford) and *E. coli* as test organisms.

Fluorimetry

The fluorescence of terramycin was measured in a Lumetron Fluorimeter 402 E using the filter accessories for riboflavin.

* An abstract of this paper was presented at a symposium on "Antibacterial substances from soil, plants and other sources" held under the auspices of the Pharmaceuticals and Drugs Committee, Council of Scientific and Industrial Research, at Bombay on the 26th March 1953.

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RESULTS

Action of six crystalline antibiotics on ascorbic acid

2 mg ascorbic acid neutralised to pH 7 were tipped in from the side arm of the flask to a mixture of 1 ml *M*/15 phosphate buffer pH 7.0 and 1 ml of antibiotic solution (1 mg/ml). Manometric readings were taken over a period of one hour at 10 minute intervals. Oxygen consumed by the various reacting systems in 60 minutes is given in Table I.

TABLE I

OXYGEN CONSUMPTION OF ASCORBIC ACID IN PRESENCE OF 6 ANTIBIOTICS

Antibiotic added	*Oxygen consumed microliters*	*% activation + or inhibition —*
Water (Control)	25.0	—
Aureomycin hydrochloride	24.2	— 3.2
Chloramphenicol	25.7	+ 2.8
Neomycin sulphate	21.5	— 14.0
Dihydrostreptomycin sulphate	30.0	+ 20.0
Penicillin G	18.6	— 25.6
Terramycin hydrochloride	168.4	+ 573.6

All the antibiotics were found to affect the oxidation one way or the other. Aureomycin, neomycin and penicillin inhibited the oxidation, the maximum effect being shown by penicillin G. Dihydrostreptomycin and terramycin accelerated the rate of oxidation. The catalytic action of terramycin was most pronounced, subsequent work was, therefore, confined to this antibiotic.

Specificity of the reaction with respect to substrate

In order to find out whether terramycin exerted a similar action on other reducing agents and chemicals of biological interest following substances were tested:

Glucose, gluconic acid, lactic acid, pyruvic acid, sorbitol, sorbose, quercitol, glutathione, cysteine hydrochloride, sodium bisulphite, sodium thiosulphate, phenylenediamine, sodium nitrite, adrenalin, cholesterol and vegetable lecithin (all these substances in 1 mg/ml adjusted to pH 7.0); a mixture of ten B vitamins (cf. Table II), a mixture of twelve amino acids (cf. Table II), a mixture of five purine bases (adenine, allantoin, guanine, hypoxanthine, xanthine and uric acid—0.1 ml supplying 20 μ each) and three pyrimidines (cytosine, uracil and thymine—0.1 ml supplying 33 μ each), menadione (1 mg/ml in 50% alcohol), dicumarol (1 mg/ml in 50% alcohol) and adenylic acid (1 mg/ml).

None of these substances was found to take up any oxygen in the presence of terramycin under the conditions of the experiment, indicating that the action of the antibiotic was specific for ascorbic acid.

Test for the presence of copper and other metallic impurities in terramycin

In view of the fact that traces of heavy metals, especially copper[6] can bring about catalytic oxidation of ascorbic acid the antibiotic was tested for the presence of such impurities. This is important because terramycin is known to form complexes with heavy metals and this property is actually utilised in the commercial extraction of the antibiotic from culture broths[7]. However, the antibiotic failed to give a positive

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test with dithiozone even after digestion with sulphuric acid, whereas copper salts up to 0.16 μg concentration responded to this test.

The catalytic activity of the antibiotic was found to be thermostable and not affect either its antibacterial activity or fluorescence in the ultraviolet region.

The pH optimum of the reactions was found to be around 8 and under optimal conditions the reaction was complete within two hours. The catalytic activity of the antibiotic was demonstrable at concentrations as low as 1 μg.

Comparative mechanism of oxidation of ascorbic acid by copper and terramycin

In order to study the mechanism of oxidation of ascorbic acid, the effect of several reagents known to inhibit the oxidation by copper was tried on the catalytic action mediated by terramycin. The results obtained have been summarised in Table II where the percentage inhibition on the endogenous, copper-catalysed and terramycin-catalysed oxidations of ascorbic acid has been given. Out of the nineteen substances tried only 8-hydroxyquinoline and penicillin G exerted an inhibitory action on the oxidation catalysed by terramycin. Sodium cyanide, glutathione and cysteine completely inhibited both the auto- and copper-catalysed oxidation of ascorbic acid. All the other reagents excepting dicumarol, menadione and allantoin, had antioxidant action on copper catalysis.

TABLE II

EFFECT OF CERTAIN SUBSTANCES ON OXIDATION OF ASCORBIC ACID

	Reagent	*Effective Concentration in reaction mixture*	*% inhibition on*		
			Endogenous	*Copper oxidation*	*Terramycin oxidation*
1.	Sodium cyanide	0.03 *M*	100	100	0
2.	Boric acid	0.003 *M*	0	0	0
3.	Glutathione	0.2 mg	100	100	0
4.	Cysteine-HCl	0.2 mg	95	100	0
5.	Yeast nucleic acid	1.0 mg^{-3}	40	100	0
6.	8-Hydroxyquinoline	$3 \cdot 10$ *M*	16	80	30
7.	Mixture of amino acids	*	85	70	0
8.	Mixture of B vitamins	**	50	33	0
9.	Yeast adenylic acid	1.0 mg	100	30	0
10.	Adenine sulphate	0.1 mg	100	84	0
11.	Guanine dihydrochloride	0.1 mg	100	87	0
12.	Hypoxanthine	0.1 mg	95	82	0
13.	Allantoin	0.1 mg	3.0	3.0	0
14.	Uric acid	0.1 mg	65	80	0
15.	Xanthine	0.1 mg	94	92	0
16.	Mixture of pyrimidines	***	82	86	0
17.	Menadione	1 mg	0	0	0
18.	Dicumarol	1 mg	0	0	0
19.	Penicillin G	1 mg	72	0	30
20.	Urea	0.1 *M*	0	0	0

* Amino acids (Alanine, aspartic acid, arginine, cystine, glycine, glutamic acid, lysine, proline, phenylalanine, valine, threonine, and serine 231 μg each).

** B. vitamins (thiamin, riboflavin, niacinamide, cal. pantothenate, pyridoxal, inositol, *p*-aminobenzoic acid, biotin, folic acid and B_{12}, 4 μg each).

*** Pyrimidines (cytosine, thymine and uracil 33 μg each).

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DISCUSSION

From the foregoing experimental evidence it is apparent that the oxidation of ascorbic acid by terramycin is an inherent property of the antibiotic itself. The possibility of any metallic impurities being involved in this function is ruled out because 1. the antibiotic, even in large concentrations, fails to give positive colour reaction which was found to be sensitive for concentrations of copper as low as 0.16 μg; 2. the addition of substances like cyanide, glutathione and purine bases does not affect the action of the antibiotic on ascorbic acid whereas its oxidation by copper is completely prevented by these agents, and 3. kinetics of the oxidation of ascorbic acid by the metal and the antibiotic are different.

Manifestation of this oxidizing property by the antibiotic even in traces would indicate that the reaction is catalytic in nature. Since the antibiotic retains its characteristic antibacterial and fluorescent properties after catalysing the oxidation, it does not seem to be chemically involved. Attempts made to elucidate the exact mechanism of catalysis by the use of inhibitors have not been very fruitful. None of the inhibitors tried excepting penicillin G and 8-hydroxyquinoline had any anti-oxidant action on ascorbic acid in presence of the antibiotic. Even these two reagents gave only partial protection to the vitamin.

The catalytic activity of terramycin reported in these studies has been shown to be highly specific for ascorbic acid. If the antibiotic were to function as an oxidation-reduction catalyst, formation of hydrogen peroxide as a secondary reaction product would be indicated. Since hydrogen peroxide even in low concentrations is toxic to the cell, it is conceivable that the catalytic property of terramycin reported in this study may be related to its mode of action.

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SUMMARY

1. Six crystalline antibiotics, penicillin G, dihydrostreptomycin sulphate, aureomycin hydrochloride, chloramphenicol, neomycin sulphate and terramycin hydrochloride were tested for their oxidation on ascorbic acid. Only terramycin was found to catalyse the reaction.

2. Terramycin has been shown to be free from traces of metals. The catalytic activity is thermostable. The antibiotic activity and fluorescent property of terramycin are not altered by its catalysis of ascorbic acid oxidation.

3. Terramycin oxidizes ascorbic to dehydroascorbic acid without liberating any carbon dioxide. pH optimum of the reaction is around 8. MICHAELIS' constant of the reaction is 0.0103 M whereas the catalyst substrate dissociation constant is $5 \cdot 10^{-6}$.

4. Sodium cyanide, boric acid, glutathione, cysteine, yeast nucleic acid, purines, pyrimidines, amino acids, vitamins of B complex, menadione and dicumarol do not inhibit the oxidation of ascorbic acid by terramycin, whereas 8-hydroxyquinoline and penicillin G exert 30% inhibition.

5. Significance of the present observations on the mode of action of terramycin is briefly discussed.

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RÉSUMÉ

1. Les auteurs ont étudié l'action de six antibiotiques cristallisés (Penicilline G, sulfate de dihydrostreptomycine, chlorhydrate d'auréomycine, chloramphénicol, sulfate de néomycine et chlorhydrate de terramycine) sur l'oxydation de l'acide ascorbique. Seule la terramycine catalyse la réaction.

2. Les échantillons de terramycine ne renfermaient pas de traces métalliques. Leur activité catalique est thermostable. Leur activité antibiotique et leur fluorescence ne sont pas modifiées au cours de la catalyse de l'oxydation de l'acide ascorbique.

3. La terramycine oxyde l'acide ascorbique en acide déhydroascorbique sans libération d'anhydride carbonique. Le pH optimum de la réaction est d'environ 8. La constante de MICHAELIS est de 0.0103 *M*; la constante de dissociation du complexe catalyseur-substrat est de $5 \cdot 10^{-6}$.

4. La cyanure de sodium, l'acide borique, le glutathion, la cystéine, l'acide nucléique de la levure, les purines, les pyrimidines, les aminoacides, les vitamines du groupe B, la ménadione et le dicoumarol n'inhibent pas l'oxydation de l'acide ascorbique par la terramycine; au contraire, la 8-hydroxy-quinoline et la pénicilline G produisent une inhibition de 30%.

5. La signification des observations précédentes pour le mode d'action de la terramycine est brièvement discutée.

ZUSAMMENFASSUNG

1. Die Wirkung von 6 kristallisierten Antibiotika, Penicillin G, Dihydrostreptomycinsulfat, Aureomycinchlorhydrat, Chloramphenicol, Neomycinsulfat and Terramycinchlorhydrat bei der Oxydation von Ascorbinsäure wurde geprüft. Es wurde gefunden, dass nur Terramycin die Reaktion katalysiert.

2. Es wurde gezeigt, dass Terramycin frei von Metallspuren ist. Die katalytische Aktivität ist thermostabil. Die antibiotische Aktivität und die Fluoreszenzeigenschaft des Terramycins werden bei der Katalyse der Ascorbinsäureoxydation nicht verändert.

3. Terramycin oxydiert Ascorbinsäure zu Dehydroascorbinsäure ohne Freisetzung von Kohlendioxyd. Das pH-Optimum der Reaktion liegt bei 8. Die MICHAELISkonstante der Reaktion beträgt 0.0103 *M*, die Katalysator-Substrat-Dissozziationskonstante $5 \cdot 10^{-6}$.

4. Natriumcyanid, Borsäure, Glutathion, Cystein, Hefenucleinsäure, Purine, Pyrimidine, Aminosäuren, Vitamine des B-Komplexes Menadion und Dicumarol hemmen die Oxydation von Ascorbinsäure mit Terramycin nicht, dagegen üben 8-Oxyquinolin and Penicillin G eine Hemmung van 30% aus.

5. Die Bedeutung der vorliegenden Beobachtungen auf die Art der Wirkung des Terramycins wird kurz besprochen.

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