[23] C. WEIBULL, *J. Bacteriol.*, 66 (1953) 668.
[24] R. REPASKE, *Biochim. Biophys. Acta*, 22 (1956) 189.
[25] A. G. MARR AND E. H. COTA-ROBLES, *J. Bacteriol.*, 74 (1957) 79.
[26] A. TISSIERES, H. G. HOVENKAMP AND E. C. SLATER, *Biochim. Biophys. Acta*, 25 (1957) 336.
[27] J. W. NEWTON AND G. H. NEWTON, *Arch. Biochem. Biophys.*, 71 (1957) 250.
[28] J. SYKES, Thesis, University of Leeds, 1958.
[29] R. M. BOCK AND W. C. GILLCHRIEST, *Federation Proc.*, (1958) 193.
[30] B. MAGASANIK AND E. CHARGAFF, *Biochim. Biophys. Acta*, 7 (1951) 396.
[31] J. R. G. BRADFIELD, in *Bacterial Anatomy*, Ed. T. C. SPOONER AND B. A. D. STOCKER, C.U.P. England, 1956, p. 296.
[32] A. TISSIERES, *Nature*, 174 (1954) 183.
[33] A. TISSIERES AND E. C. SLATER, *Nature*, 176 (1955) 736.
[34] J. WAGMAN, E. POLLACK AND E. S. WENECK, *Arch. Biochem. Biophys.*, 73 (1958) 161.
[35] D. BILLEN AND E. VOLKIN, *J. Bacteriol.*, 67 (1958) 191.

MECHANISM OF INHIBITION OF D-AMINO ACID OXIDASE

I. INHIBITORY ACTION OF CHLORTETRACYCLINE

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SUMMARY

1. The complex formation of chlortetracycline with flavins was demonstrated both by its quenching action on the fluorescence of flavins and by the shift of the absorption spectrum of flavins by chlortetracycline. The binding site of FAD for complex formation with chlortetracycline is in its riboflavin part.

2. Chlortetracycline had a single inhibitory effect on D-amino acid oxidase which may be attributed to the complex formation of chlortetracycline with FAD, resulting in a competition with the oxidase protein for the FAD. The dissociation constant of the complex was calculated to be $3.9 \cdot 10^{-4}$ *M* at pH 8.3 and 38°.

3. It was demonstrated that chlortetracycline can combine with free FAD but not with FAD which is bound with the oxidase protein. This suggests that the binding site of FAD for the complex formation with the apo-protein is also responsible for the complex formation with chlortetracycline.

INTRODUCTION

YAGI *et al.*[1] found that various phenol derivatives prevent photodecomposition of flavins in aqueous solution. Complex formation of riboflavin with phenol derivatives was demonstrated by the quenching action of these compounds on the fluorescence of riboflavin and by their effect on the absorption spectrum[2,3]. Kinetic studies showed that complex formation also took place between *p*-aminosalicylic acid and the FAD

Abbreviations: FAD, flavinadenine dinucleotide; FMN, flavin monophosphate.

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prosthetic group of D-amino acid oxidase, causing an inhibition of the activity of this enzyme[4].

Another compound which was found to be a strong quencher of the fluorescence of FAD was the antibiotic chlortetracycline. It appeared, therefore, interesting to determine whether this compound inhibited flavoenzymes, especially since it has been reported in clinical studies to cause ariboflavinosis or an ariboflavinosis-like disease[5–7].

This paper deals with the effect of chlortetracycline on D-amino acid oxidase *in vitro*. A preliminary report has appeared[8].

MATERIALS

The following preparations were used: chlortetracycline (hydrochloride), a product of Lederle Ltd.; riboflavin, chemically synthesized and crystallized preparation; FMN, of lederle Ltd.; riboflavin, chemically synthesized and crystallized preparation; FMN, sodium salt, prepared from riboflavin with phosphoryl chloride; FAD, obtained from *Eremothecium ashbyii* by the method of YAGI *et al.*[9]. The purity of this FAD sample was more than 92% as determined by fluorimetry and spectrophotometry; it was free from other flavins, nucleic acids, and metals. D-Amino acid oxidase was prepared by the method of NEGELEIN AND BRÖMEL[10]. From 10 hog kidneys, 90 g of acetone-dried powder were obtained, and FAD was removed by acidification at 0.2 saturation of $(NH_4)_2SO_4$. After repeating the salting-out procedure, a colourless precipitate was obtained and dissolved in 0.1 M pyrophosphate buffer (pH 8.3). From this solution, a white powder containing 600 μg of the oxidase protein was obtained by lyophilization.

In each experiment, an enzyme solution containing 15 μg enzyme protein/ml was used. When the substrate, DL-alanine, was mixed with 1.0 ml of the enzyme solution with excess FAD at 38°, the O_2 uptake was 132 μl/30 min. There was no O_2 uptake in the absence of FAD.

METHODS

pH-Fluorescence curve of chlortetracycline

Chlortetracycline was dissolved in the appropriate buffer to give a concentration of $2 \cdot 10^{-5}$ M. Citrate buffer ($5 \cdot 10^{-2}$ M) was used for experiments below pH 4, phosphate buffer ($5 \cdot 10^{-2}$ M) for those from pH 4 to 7, and monoethanolamine buffer ($5 \cdot 10^{-2}$ M) for those above pH 7. The total fluorescence energy at different pH's was measured at 20° by a fluorimeter designed by YAGI *et al.*[11].

Quenching action of chlortetracycline on flavins

As chlortetracycline has no fluorescence below pH 7 (Fig. 1), the quenching action of chlortetracycline on flavins was examined at pH 6.0, and at 20°.

Assuming that the complex of chlortetracycline with flavin has no fluorescence and that the reaction is bimolecular, the dissociation constant K of the complex will be presented by the following formula:

$$K = \frac{(f - x)\,(c - x)}{x}, \qquad (1)$$

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where f is the molar concentration of flavin, c is that of chlortetracycline, and x is that of a complex. In the actual measurement, f and $(f - x)$ correspond to the intensities of fluorescence in the absence and presence of chlortetracycline, respectively.

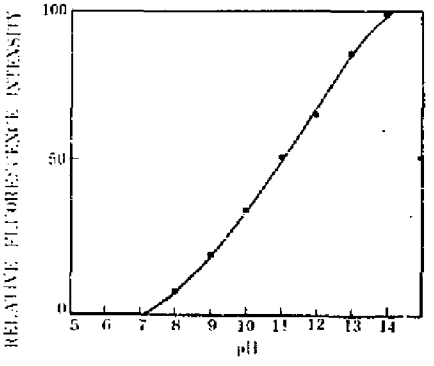

Fig. 1. pH-Fluorescence curve of chlortetracycline. Chlortetracycline was dissolved in 0.05 M citrate, phosphate, or monoethanolamine buffer to a final concentration of $2 \cdot 10^{-5}$ M.

Measurement of the enzyme activity

Enzyme activity was measured by the conventional Warburg method at pH 8.3, 38°. The gas phase was air and the center well contained 0.2 ml of 20 % NaOH. The reactants were mixed after the temperature equilibrium had been reached. The pressure changes were followed for 60 min at intervals of 5 min.

Kinetic analysis

The dissociation constant of FAD with the oxidase protein (K_f) was calculated to be $1.1 \cdot 10^{-7}$ M by using Michaelis–Menten's formula.

Let:

(e) = total concentration of the oxidase protein
(f) = total concentration of FAD
(i) = total concentration of the inhibitor which competes with the protein for the FAD
(x) = concentration of the FAD–protein complex
(y) = concentration of the inhibitor–FAD complex
K_{if} = dissociation constant of the inhibitor–FAD complex
v = reaction velocity in the presence of the inhibitor
V = maximum velocity in the presence of a large excess of the protein.

The over-all reaction may be written as follows:

$$\underset{(e)}{E} + \underset{(f-x-y)}{F} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{(x)}{EF}$$

and

$$\underset{(f-x-y)}{F} + \underset{(i)}{I} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \underset{(y)}{FI}$$

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The amount of (x) is

$$x = \frac{fe}{K_f(1 + i/K_{if}) + e} \quad (2)$$

The maximum velocity, V, will be obtained when FAD is completely bound in the form of the protein–FAD complex.

Rearranging Eqn. (2) yields:

$$\frac{1}{v} = \frac{K_f(1 + i/K_{if})}{Ve} + \frac{1}{V} \quad (3)$$

V was found to be 143 μl O_2/30 min in the presence of $1.6 \cdot 10^{-7}$ M FAD and $3 \cdot 10^{-5}$ M of the oxidase protein. The molecular weight of the oxidase protein was calculated to be $1 \cdot 10^5$ from its sedimentation coefficient measured by the Spinco type E ultracentrifuge. The concentration of the oxidase protein in the enzyme solution was estimated by its extinction coefficient at 280 mμ using KALCKER's formula[12]. K_{if} can be evaluated from the slope of the line obtained by plotting $1/v$ against $1/e$.

If v_0 and v are the reaction velocities in the absence and presence of chlortetracycline, respectively,

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\frac{i}{K_{if}}. \quad (4)$$

Eqn. (4) yields a straight line with the intercept 1 and the slope $(1 - v_0/V)/K_{if}$ by plotting v_0/v against i. From the slope of the straight line thus obtained, K_{if} can also be calculated.

If chlortetracycline inhibits with respect to FAD in more than one way, plots of v_0/v give a curve which is concave to i axis. For example, in the case of *p*-aminosalicylic acid[1] which inhibits the oxidase activity by both complex formation and competition with FAD, v_0/v can be shown to be

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\left(\frac{K_{if} + K_{ip} + i}{K_{if}K_{ip}}\right)i, \quad (5)$$

where K_{ip} is the dissociation constant of the complex of the inhibitor with the oxidase protein in competition with FAD. In this way, it can be determined whether chlortetracycline inhibits the oxidase by single or plural inhibitory mechanism.

RESULTS

Quenching action of chlortetracycline on flavins

Riboflavin. A straight line was obtained when $(f - x)$ was plotted against $x/(e - x)$ (Fig. 2). The dissociation constant K was calculated from the slope of this line to be $6.6 \cdot 10^{-7}$ M.

FMN. The same quenching action was observed as in the case of riboflavin under the same condition, K also being $6.6 \cdot 10^{-7}$ M.

FAD. By the same method, the dissociation constant of chlortetracycline-FAD complex was calculated from Fig. 2 to be $1.1 \cdot 10^{-6}$ M.

Effect of chlortetracycline on the absorption spectrum of flavins

Fig. 3 shows that chlortetracycline shifted the absorption of FAD to the longer wavelength region. A similar result was obtained with riboflavin and FMN.

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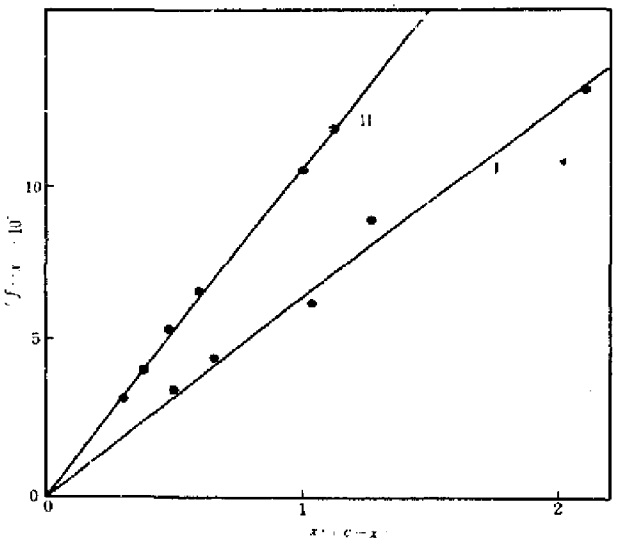

Fig. 2. Quenching action of chlortetracycline on fluorescence of flavins. Line I was obtained from the measurements of solutions containing $2 \cdot 10^{-6}$ *M* riboflavin and graduated concentrations of chlortetracycline between $1 \cdot 10^{-6}$ *M* and $5 \cdot 10^{-6}$ *M*. Line II was obtained from measurements of the solutions containing $1.5 \cdot 10^{-6}$ *M* of FAD and the same graduated concentrations of chlortetracycline. f, c and x represent the molar concentrations of flavin, chlortetracycline and their complex, respectively. The values of f and $(f - x)$ correspond to the intensities of fluorescence of flavin in the absence and presence of chlortetracycline, and the values of x were calculated from these values.

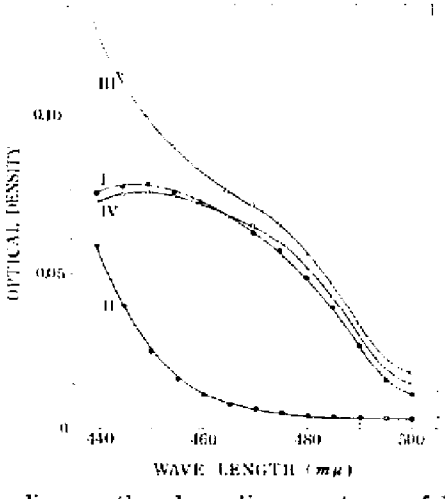

Fig. 3. Effect of chlortetracycline on the absorption spectrum of FAD. I: FAD ($6.85 \cdot 10^{-6}$ *M*) in 0.1 *M* phosphate buffer (pH 5.5); II: Chlortetracycline ($2.06 \cdot 10^{-3}$ *M*) in the same buffer; III: FAD ($6.85 \cdot 10^{-6}$ *M*) + chlortetracycline ($2.06 \cdot 10^{-3}$ *M*) in the same buffer; IV: Difference spectrum, III — II.

Effect of the oxidase protein on the fluorescence and absorption spectrum of chlortetracycline

The addition of the oxidase protein ($4 \cdot 10^{-6}$ *M*) did not quench the fluorescence of chlortetracycline ($1 \cdot 10^{-5}$ *M*) irradiated in 0.1 *M* pyrophosphate buffer (pH 8.3)

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by 365-mμ light. The presence of the oxidase protein did not shift the absorption spectrum of chlortetracycline under these conditions.

Inhibitory action of chlortetracycline on the oxidase activity

In the measurement of enzyme activity, chlortetracycline and FAD were placed in the side bulb of a manometer flask. After temperature equilibrium had been reached, the contents of the side bulb were transferred to the main chamber containing the oxidase protein and a large excess of DL-alanine. A marked inhibitory effect of chlortetracycline was observed in the initial 10 min, but the inhibition subsequently decreased, reaching an equilibrium after 30 min, as shown by curve 1 in Fig. 4. The values of v were measured during 30 to 60 min after the contents of the side bulb had been mixed with those of the main flask. Using Eqn. (3), the values of $1/v$ were plotted against $1/e$ in the absence and presence of chlortetracycline, as shown in Fig. 5.

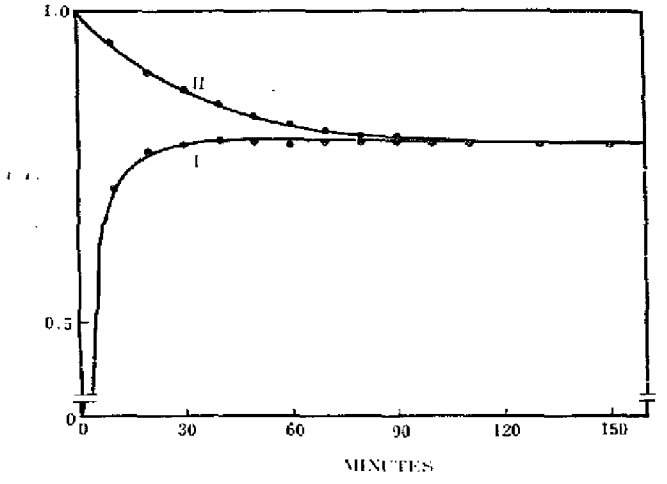

Fig. 4. Change of inhibition of D-amino acid oxidase by chlortetracycline during incubation. In the case of curve I, manometer flask contained 15 μg oxidase protein and $6.25 \cdot 10^{-2}$ M of DL-alanine in the main chamber. After temperature equilibrium had been reached, a mixture of $1.6 \cdot 10^{-7}$ M (final concn.) of FAD and $2.5 \cdot 10^{-4}$ M (final concn.) of chlortetracycline was added into the main chamber from the side bulb. In the case of II, the main chamber contained 15 μg of oxidase protein, $1.6 \cdot 10^{-7}$ M of FAD, and $6.25 \cdot 10^{-2}$ M of DL-alanine. After temperature equilibrium was reached, chlortetracycline was added from the side bulb to the final concn. of $2.5 \cdot 10^{-4}$ M. Each plot shows the value of v/v_0 obtained for each 10 min interval of the incubation.

The results show that chlortetracycline inhibits the oxidase activity by competing with the oxidase protein for FAD. The values of v_0/v plotted against the concentration of chlortetracycline fell on a straight line, as shown in Fig. 6, which indicates according to Eqn. (4) that chlortetracycline inhibits the oxidase by a single mechanism.

When the oxidase protein, DL-alanine, and chlortetracycline were incubated in the main chamber for 10 min and then FAD was added from the side bulb, the inhibition also decreased and reached an equilibrium after 30 min, similarly to curve I in Fig. 4. On the other hand, when the oxidase protein, DL-alanine, and FAD were incubated in the main chamber for 10 min and then chlortetracycline was added from the side bulb, the degree of inhibition slowly increased (see curve II, Fig. 4). After 120 min, the inhibition also reached an equilibrium at the same level as that

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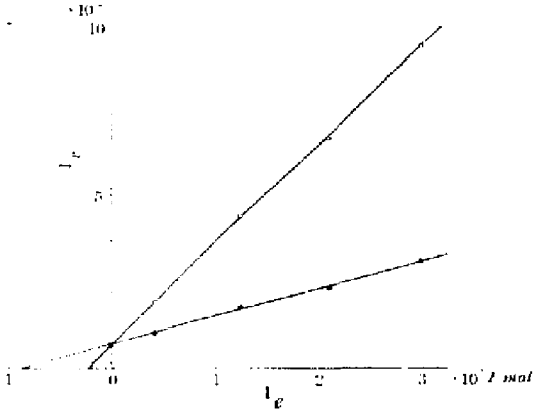

Fig. 5. Inhibitory action of chlortetracycline on D-amino acid oxidase. Each cup contained the oxidase protein as indicated and 0.25 *M* (final concentration) of DL-alanine in the main chamber. After a temperature equilibrium had been reached, $1.6 \cdot 10^{-7}$ *M* (final concentration) of FAD with or without $1.2 \cdot 10^{-3}$ *M* of chlortetracycline was added to the main chamber from the side bulb.

observed when chlortetracycline and FAD were added together to the mixture of the protein and DL-alanine. Values of v_0/v at 120 min of incubation were on a straight line, which agreed with that shown in Fig. 6.

DISCUSSION

The quenching of the fluorescence of flavins by chlortetracycline and the shift of the absorption spectrum of the former by the addition of chlortetracycline suggests the formation of a complex in aqueous solution between the antibiotic and the isoalloxazine ring of FAD. Fluorimetric and spectrophotometric measurements gave no evidence of complex formation between chlortetracycline and the oxidase protein.

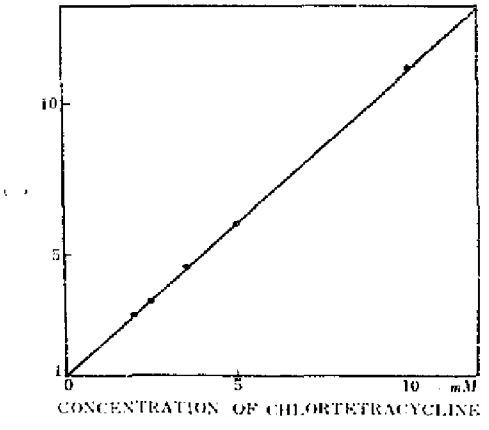

Fig. 6. Inhibitory action of chlortetracycline on D-amino acid oxidase. Each cup contained 15 μg oxidase protein and $6.25 \cdot 10^{-2}$ *M* (final concn.) of DL-alanine in the main chamber. After a temperature equilibrium had been reached, a mixture of $1.6 \cdot 10^{-7}$ *M* (final concn.) of FAD and graduated concentrations of chlortetracycline was added to the main chamber from the side bulb. v was 143 μl/30 min and v_0 was 87.5 μl/30 min.

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Kinetic studies showed that chlortetracycline inhibits D-amino acid oxidase by competing with the oxidase protein for FAD, and that the inhibition is consistent with a single mechanism. The inhibition can be attributed to the complex formation of chlortetracycline with FAD.

Since it was also demonstrated that chlortetracycline did not inhibit the initial activity of the oxidase and a long period was required to reach a constant inhibition when FAD was previously bound with the oxidase protein, it can be concluded that chlortetracycline can combine with free FAD but not with bound FAD.

These observations suggest that the binding site of FAD involved in complex formation with the oxidase protein may be responsible for its complex formation with chlortetracycline.

The dissociation constant of the complex between chlortetracycline and FAD was calculated from the slope of the straight lines in Figs. 5 and 6 to be $3.9 \cdot 10^{-4} M$. The difference between the value of K_{if} obtained by fluorimetry and that obtained by enzymic analysis may be attributed to the variations in pH and temperature.

In the field of clinical research, it has been reported by MEIKLEJOHN *et al.*[5], HARRIS[6], MERLISS *et al.*[7] and others that the ariboflavinosis or an ariboflavinosis-like disease was caused by chlortetracycline as one of its side effects. This may possibly be explained by the direct action of chlortetracycline on FAD *in vivo*, which will result in the inhibition of flavin enzymes.

REFERENCES

1 K. YAGI AND I. ISHIBASHI, *Vitamins (Kyoto)*, 7 (1954) 935.
2 K. YAGI AND Y. MATSUOKA, *Vitamins (Kyoto)*, 7 (1954) 874.
3 K. YAGI AND Y. MATSUOKA, *Biochem. Z.*, 328 (1956) 38.
4 K. YAGI, J. OKUDA, T. OZAWA AND K. OKADA, *Biochem. Z.*, 238 (1957) 492.
5 G. MEIKLEJOHN AND R. I. SHRAGG, *J. Am. Med. Assoc.*, 140 (1949) 391.
6 H. J. HARRIS, *J. Am. Med. Assoc.*, 142 (1950) 161.
7 R. R. MERLISS AND B. HOFFMAN, *New Engl. J. Med.*, 245 (1951) 328.
8 K. YAGI, J. OKUDA, T. OZAWA AND K. OKADA, *Science*, 124 (1956) 273.
9 K. YAGI, Y. MATSUOKA, S. KUYAMA AND M. TADA, *J. Biochem. (Tokyo)*, 43 (1956) 93.
10 E. NEGELEIN AND D. BRÖMEL, *Biochem. Z.*, 300 (1939) 225.
11 K. YAGI AND T. ARAKAWA, *Vitamins (Kyoto)*, 6 (1953) 523.
12 H. KALCKER, *J. Biol. Chem.*, 167 (1947) 461.