OXIDATION OF KINETIN BY MAMMALIAN XANTHINE OXIDASE*

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The oxidation of adenine by xanthine oxidase (XO) has been studied by KLEENOW[1], and the pathway of this reaction has been established recently by WYNGAARDEN AND DUNN[2] as follows:

Adenine (I) $\longrightarrow$ 8-hydroxyadenine (II) $\longrightarrow$ 2,8-dihydroxyadenine (III)

We have been studying for some time the enzymic reactions of kinetin (6-α-furfurylaminopurine), in the hope of shedding light on the biological role of this growth factor. The behavior of kinetin towards XO, although in principle similar to that of adenine, differs characteristically from its mother substance in several respects. Our observations are reported in the present paper.

MATERIALS AND METHODS

Kinetin was a gift from Dr. A. DEUTSCH, California Foundation for Biochemical Research, Los Angeles.

A highly purified specimen of milk xanthine oxidase was obtained from Prof. F. BERGEL and Dr. R. C. BRAY, Chester Beatty Institute of Cancer Research, London, England. This preparation, when diluted 1:800, produced 1 γ/ml of uric acid per min, with xanthine ($6.5 \cdot 10^{-5}$ M) as substrate.

Catalase was a commercial product of Worthington Biochemical Corporation, and contained 5,000 units/ml.

Enzyme experiments

Incubation was carried out—unless stated otherwise—with a mixture of XO (1:150) and catalase (1:500) at pH 8.0, in the presence of 10^{-3} M phosphate buffer. The progress of the reaction was observed in a Beckman ultraviolet spectrophotometer at a temperature of 28°, which was maintained by the use of the Beckman thermospacer.

Paper chromatography was carried out according to the method of DIKSTEIN, BERGMANN AND CHAIMOVITZ[3], using an acid solvent (85 ml of 95% ethanol, 5 ml of acetic acid, and 10 ml water) for development.

RESULTS

Spectral changes during the enzymic oxidation of kinetin

When kinetin, $1.5 \cdot 10^{-5}$ M, was incubated with a mixture of XO (1:150) and catalase (1:500), the absorption spectrum changed in a characteristic way (Fig. 1). During the first hour, instead of the original absorption maximum at 268 mμ, two new peaks appeared at 276 and 304 mμ, respectively. When the reaction was per-

* This investigation was supported by a research grant of the Hadassah Medical Organisation. The results reported form part of a Ph. D. thesis of H. KWIETNY, to be submitted to the Faculty of Science, The Hebrew University, Jerusalem.

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mitted to continue until no further optical changes occurred, only the higher peak remained. Fig. 1 shows that kinetin and its ultimate oxidation product possess an isosbestic point at 281 mμ. Therefore, any change of the optical density (O.D.) at this wavelength must be ascribed to the transient appearance of an intermediate substance, responsible for the absorption maximum at 276 mμ. Indeed, it was found that during enzymic oxidation of kinetin, the O.D. at 281 mμ increased rapidly, then remained at a constant level for about 1 h and finally declined, until the original value was reached again (see Fig. 2). With adenine as substrate, similar observations could be made by following the change in optical density at 277 mμ (the isosbestic point of adenine and its oxidation product III).

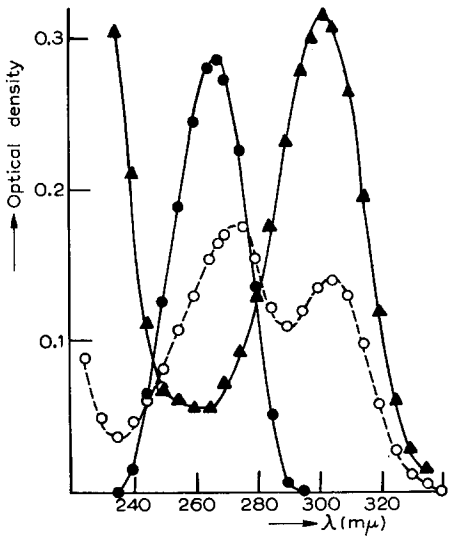

Fig. 1.

Fig. 1. Changes of absorption spectrum during the oxidation of kinetin by xanthine oxidase (1:150). ●—● Zero time: Spectrum of kinetin, $1.5 \cdot 10^{-5}$ *M* (λ_{max} 268 mμ). O—O After 45 min: Two new peaks have appeared, at 276 and 304 mμ, respectively. ▲—▲ After 16 h: Only the peak at 304 mμ has remained and has increased in height.

Fig. 2. Spectroscopic evidence for the appearance of an intermediate in the enzymic oxidation of adenine and kinetin. The changes in optical density at the isosbestic point (1) of adenine and 2,8-dihydroxyadenine (277 mμ), and (2) of kinetin and its final oxidation product (281 mμ) were measured continuously during the first hours of the action of xanthine oxidase and plotted as function of time. Enzymes: XO, 1:150; catalase, 1:500. Substrates: adenine, $4.9 \cdot 10^{-5}$ *M*; kinetin, $4.7 \cdot 10^{-5}$ *M*. Left ordinate: adenine; right ordinate: kinetin.

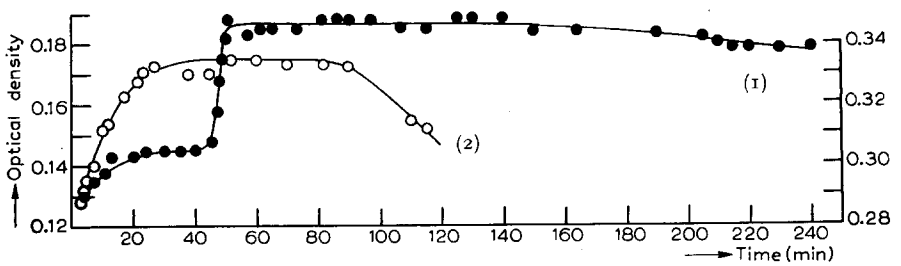

Fig. 2.

It is, therefore, difficult to understand why WYNGAARDEN AND DUNN[2] did not observe the transient increase of O.D. at 277 mμ. Possibly, these authors worked with such high enzyme concentrations that their reaction mixture entered into the prolonged steady state, shown in Fig. 2 for adenine, as early as the initial phase of the experiment. However, the over-all change in the absorption spectrum of adenine does not reveal the intermediate II, *i. e.* in contrast to kinetin no secondary peak between 260 and 305 mμ can be distinguished.

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Comparison of the value for λ_{max} of the compounds I–III with the analogous series of kinetin derivatives (Table I) indicates that in both cases the same oxidation pathway is followed. Since 2-hydroxyadenine—the other potential intermediate in adenine oxidation—exhibits a maximum at 287 mμ, it can be reasonably expected that 2-hydroxykinetin should possess a λ_{max} above the value of 287 mμ.

Characterisation of the oxidation product of kinetin

The absorption maxima of III and of the end-product of kinetin oxidation are so close that the two substances may be suspected to be actually identical, *i.e.* it might be supposed that XO removes the furfuryl side-chain from the 6-amino group. If this should prove to be the case, kinetin could serve as precursor of purine metabolites, which are usually derived from adenine, and this may represent one of the important biological functions of the new growth factor. In order to test this theory, both oxidation products were submitted to paper chromatography. Each chromatogram was cut into 20 strips, and each strip was extracted with 8 ml of 10^{-2} *M* phosphate buffer, pH 8.0. The extracts were then analysed spectrophotometrically. This procedure established that the R_F values of the oxidation products of adenine and kinetin were totally different (see Table I).

TABLE I

PHYSICAL CHARACTERISTICS OF THE ENZYMIC OXIDATION PRODUCTS OF ADENINE AND KINETIN

Substance	λ_{max} (mμ)	R_F*
A. Adenine (I)	260	0.4
8-Hydroxyadenine (II)	272.5	
2-Hydroxyadenine (= isoguanine)	287	
2,8-Dihydroxyadenine (III)	305	0.28
B. Kinetin	268	0.45
8-Hydroxykinetin (IV)	276	
2,8-Dihydroxykinetin (V)	304	0.66

* Solvent: 85 vol. of 95 % ethanol; 5 vol. of glacial acetic acid; 10 vol. of water. R_F values were determined in a descending chromatogram, solvent front 35–40 cm.

Conclusive evidence that the oxidation product of kinetin still contained the furan nucleus, was obtained by the use of DISCHE's reagent[4,5]. It has been reported[6], that kinetin gives in this test a characteristic red-violet colour, with λ_{max} at about 490 mμ. The same was found to be true for the final oxidation product of kinetin after it had been purified by paper chromatography. These experiments establish beyond doubt that kinetin—under the influence of XO—is transformed first into 8-hydroxykinetin (IV) and finally into the 2,8-dihydroxy derivative (V).

Relative rate of oxidation of adenin and kinetin

Since the intermediate (IV) has not yet been isolated in the pure state, its absorption spectrum and especially its molar extinction are still unknown. Therefore, the rate of conversion of kinetin into its 8-hydroxy derivative and of the latter into the end-product cannot yet be determined. We have evaluated approximately the rate of appearance of the end-product from the increase in O.D. at 304 mμ. During

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the first 30 minutes of an experiment, in which both adenine and kinetin, $1.5 \cdot 10^{-5}$ *M*, were incubated with XO (1:300), 31% of (V), but only 23% of (III) were formed. Likewise, the curves in Fig. 2 indicate, that the kinetin intermediate (IV) is formed and consumed faster than (II). Although no definite conclusions can as yet be drawn on the relative rate of the second reaction step, it appears probable that 8-hydroxykinetin is also oxidized at a higher rate than 8-hydroxyadenine.

DISCUSSION

The above results show that kinetin is easily attacked by xanthine and is converted into the 2,8-dihydroxy derivative, in analogy with the observations of WYNGAARDEN AND DUNN[2] on adenine. The oxidation of kinetin proceeds considerably faster than that of adenine, and this fact may be connected with the biological activity of kinetin. It appears also possible that kinetin may react similarly to adenine in other enzymic systems. A search for kinetin metabolites in living systems is thus called for. If such metabolites can be found, they may shed light on the mechanism of action of kinetin, which so far has remained obscure.

ACKNOWLEDGEMENTS

The authors wish to thank Prof. F. BERGEL and Dr. R. C. BRAY of the Chester Beatty Institute of Cancer Research, London, for a generous gift of highly purified milk xanthine oxidase. They are also obliged to Dr. A. DEUTSCH of California Foundation for Biochemical Research for the supply of kinetin.

SUMMARY

Kinetin is oxidized by mammalian xanthine oxidase to 2,8-dihydroxykinetin, 8-hydroxykinetin being the intermediate. The furfuryl side-chain is not removed during these reactions. The enzymic rate of kinetin exceeds that of adenine.

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Received November 5th, 1957