The Enzymic Oxidation and Assay of Adenine

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Booth (1938) showed that adenine is oxidized by xanthine oxidase from milk with the uptake of 1 molecule of oxygen per molecule of adenine oxidized, and that no ammonia is given off. Recently Bendich, Brown, Philips & Thiersch (1950) observed that after oral or intraperitoneal administration of large amounts of adenine to rats, crystals of 2:8-dihydroxyadenine were deposited in the kidneys; they therefore suggested that adenine is oxidized by xanthine oxidase to 2:8-dihydroxyadenine.

The aim of the present work was to characterize the enzymic oxidation product of adenine and to describe a sensitive assay method for this compound.

EXPERIMENTAL

Materials

Adenine (Hoffman-La Roche) was recrystallized as adenine hydrochloride semihydrate.

Xanthine oxidase was prepared from cream according to the method of Ball (1939) with a few modifications (Kalckar, Kjeldgaard & Klenow, 1950*a*).

Characterization of the enzymic oxidation product of adenine

A concentrated solution of adenine (about 10 mg./ml.) in phosphate buffer was incubated under aerobic conditions with catalase and xanthine oxidase (about 5 mg. of protein/ml.); a precipitate gradually formed. The incubation mixture was left at room temperature overnight and the precipitate then washed with water and dissolved in a small amount of 2N-hydrochloric acid. This solution was treated with charcoal and the enzymic reaction product reprecipitated by neutralization with concentrated ammonia. The reprecipitation was repeated three times and the final product was washed and dried over phosphorus pentoxide in vacuum at 100° for 2 hr. This procedure is similar to that used by Bendich *et al.* (1950) for the isolation of 2:8-dihydroxyadenine from rat kidneys. The sample analysed as the semihydrate of 2:8-dihydroxyadenine. (Found: C, 34·8; H, 3·4; N, 40·0. Calc. for $C_5H_5O_2N_5 . \frac{1}{2}H_2O$: C, 34·1; H, 3·4; N, 39·8 %.) After reprecipitation, washing once more, and drying at 140° for 2 hr. the analysis was N, 39·9 %.

The extinction curve of the sample (see Fig. 1) was measured in the Beckman spectrophotometer together with that of the 2:8-dihydroxyadenine isolated from rat kidney (kindly furnished by Dr G. B. Brown, Sloan-Kettering Institute). The measurements were performed with 2 μg./ml. in 0·067 M-phosphate buffer pH 7·0.

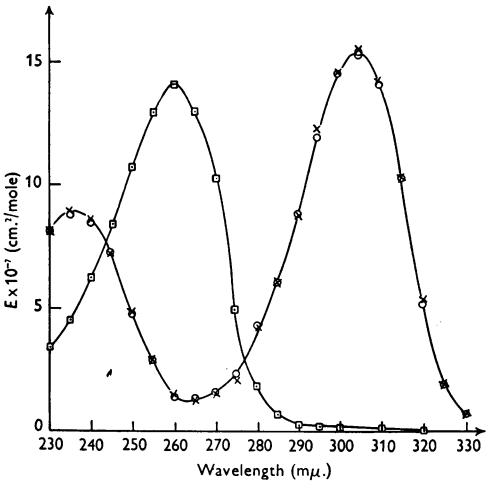

Fig. 1. Molar extinction curves of adenine (⊡), of the enzymic oxidation product (×) and of 2:8-dihydroxyadenine (○). M/15-Phosphate buffer, pH 7·0.

Addition of crystalline uricase to the oxidation product of adenine as well as to 2:8-dihydroxyadenine did not cause any changes in the absorption at $\lambda = 305$ mμ.

Since apparently the solubility, composition, and the molar absorption curves of the enzymic oxidation product of adenine are the same as those of 2:8-dihydroxyadenine it is reasonable to assume that this compound is formed by the oxidation of adenine by milk xanthine oxidase preparations. In a private communication Dr G. Hitchings has informed us that he also has isolated 2:8-dihydroxyadenine as the oxidation product of adenine after incubation with xanthine oxidase.

Estimation of small amounts of adenine

The assay method is based on enzymic differential spectrophotometry as developed by Kalckar (1947).

Table 1. *Extinction coefficients of adenine and 2:8-dihydroxyadenine*

Compound	Maxima (mμ.)	$E \times 10^{-7}$ (cm.²/g.mol.)	pH	Reference
Adenine	260	1·42	7·0	Gulland & Holiday (1936)
	260	1·41	7·0	Klenow (present paper)
2:8-Dihydroxyadenine	235	0·889	6·8	Bendich *et al.* (1950)
	235	0·880	7·0	Klenow (present paper)
	305	1·54	6·8	Bendich *et al.* (1950)
	305	1·55	7·0	Klenow (present paper)
	Minimum (mμ.)			
2:8-Dihydroxyadenine	263	0·11	7·0	Klenow (present paper)

In Table 1 a list of the absorption maxima and minima and the molecular extinction coefficients of adenine and of 2:8-dihydroxyadenine are given. The maximal differences in absorption and the isobestic points of the two substances at pH 7·0 as derived from Fig. 1 are given in Table 2. The spectral changes at $\lambda = 305$ mμ. found in an experiment starting with different amounts of adenine incubated with xanthine oxidase show (see Table 3) that the method allows estimation down to about 0·3 μg. adenine/ml. with an accuracy of about 10 %.

Table 2. *Density changes at various wavelengths on the oxidation of adenine to 2:8-dihydroxyadenine by milk xanthine oxidase at* pH 7·0

(The values are derived from Fig. 1.)

Wavelength (mμ.)	$\Delta E \times 10^{-7}$ (cm.²/g.mol.)	$\Delta \log I_0/I$ per μg. adenine per ml. $\frac{\Delta E}{1{\cdot}35 \times 10^8}$
243	0	0
260	−1·26	−0·093
277	0	0
305	+1·55	+0·115

Table 3. *Determination of adenine*

(Buffer, M/15-phosphate, pH 7·0; enzyme, xanthine oxidase, 0·5 mg. of protein/ml. mixture.)

Concentration of adenine (μg./ml.)	$\Delta \log I_0/I$ at $\lambda = 305$ mμ.	
	Observed	Calculated from Table 2
0·0163	+0·070	+0·073
0·125	+0·146	+0·144
0·187	+0·197	+0·215

Routine determinations should be carried out at $\lambda = 305$ mμ., not only because this gives the greatest sensitivity, but also because the optical density of most proteins and of other compounds usually present in biological filtrates is low at this wavelength.

The extinction of 2:8-dihydroxyadenine at the maximum at $\lambda = 305$ mμ. changes with the pH of the solution, whereas adenine has no detectable absorption at any pH at this wavelength. Since this causes a considerable difference of the ΔE (change in extinction) per unit of adenine oxidized at different pH, as appears from Table 4, the pH of the assay solution should be well controlled. Since 2:8-dihydroxyadenine is a very insoluble substance (2 μg./ml. of water (Bendich *et al.* 1950)), the buffered solutions for the assay should not contain more than about 2 μg. adenine/ml., as a higher concentration may result in a precipitation of the reaction product.

Table 4. *Molar extinction coefficient of 2:8-dihydroxyadenine at $\lambda = 305$ mμ. at different* pH *values*

Buffer	pH	$E \times 10^{-7}$ (cm.²/mole)	$\Delta \log I_0/I$ per μg. adenine per ml. $\frac{E}{1{\cdot}35 \times 10^8}$
M/15-Phosphate	6·4	1·48	0·110
	7·0	1·55	0·115
	7·4	1·55	0·115
	7·7	1·62	0·120
M/10-Pyrophosphate	7·9	1·69	0·125
	8·4	1·76	0·130
	9·0	1·80	0·133

As mentioned later, the reaction taking place under the conditions usually used for analysis is mainly a first-order reaction. The end point for the reaction, therefore, is not well defined unless rather large amounts of xanthine oxidase are used (for an enzyme preparation with the activity mentioned below about 0·5 mg. of protein/μg. of adenine should be used).

Some properties of the enzymic reaction

The catalytic activity of xanthine oxidase towards adenine is not very high. For the preparation used the initial rate measured as mentioned later corresponds to the oxidation of about 6×10^{-7} g.mol. of adenine/mg. protein/hr. (for hypoxanthine measured under the same conditions according to the method of Kalckar (1947) the figure is about 2×10^{-5} g.mol./mg. protein/hr.). In an assay method the reaction should go to completion at least within

1–2 hr. and a fairly large amount of enzyme, therefore, has to be used. In this case, however, the enzyme protein apparently is present in such a high concentration that, under the experimental conditions, it is no longer saturated with substrate, since the reaction appears to be mainly of the first order (see Fig. 2), whereas most other reactions by xanthine oxidase appear to be mainly of the zero order.

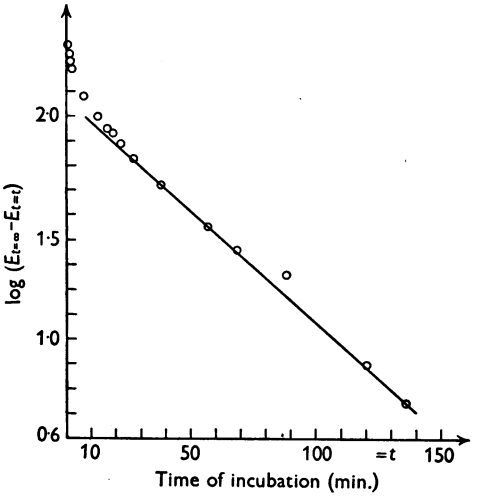

Fig. 2. Enzymic oxidation of adenine. M/15-Phosphate buffer containing about 1·9 μg. adenine and about 0·5 mg. protein/ml.

For the determination of the pH optimum of the reaction it is not sufficient to compare the initial ΔE per minute at different pH values, since the ΔE per minute is dependent not only upon the reaction rate but also upon the total ΔE at a given pH. The pH optimum for the reaction is found, therefore, by comparison of $\frac{\text{initial } \Delta E/\text{minute}}{\text{total } \Delta E}$ at different pH values. The pH optimum was found to be located at about pH 7.

As appears from Fig. 3 the reaction was temporarily inhibited by the presence of 2-amino-4-hydroxy-6-formylpteridine. Thus, the initial reaction rate was only about 25 % of the original in the presence of about 2×10^{-10} g.mol. inhibitor/ml. when the reaction mixture contained about 2×10^{-8} g.mol. adenine/ml. The fact that the inhibition is only temporary is probably due to the enzymic oxidation of the inhibitor (Klenow, 1951). Adenine itself, however, appears to inhibit the activity of xanthine oxidase towards hypoxanthine. Thus, using the method of Kalckar (1947), it was found that in the presence of 7×10^{-8} g.mol. adenine/ml. 0·2 M-phosphate buffer, pH 7·0, containing xanthine oxidase (0·05 mg. protein/ml.) and 7×10^{-8} g.mol. of hypoxanthine/ml. the oxidation rate of hypoxanthine was reduced to about 40 % of the original.

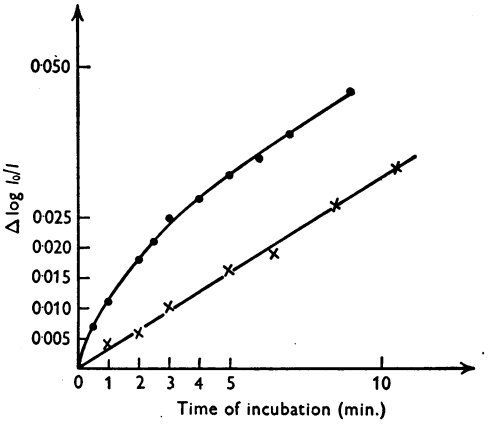

Fig. 3. Inhibition of the enzymic oxidation of adenine by 2-amino-4-hydroxy-6-formylpteridine. ●, 2×10^{-8} Mole of adenine/ml. of M/15-phosphate buffer pH 7·0; ×, the same with 2×10^{-10} mole of inhibitor added/ml. of mixture. Xanthine oxidase (about 0·2 mg. of protein/ml. of mixture) added at zero time.

Procedure for the assay of adenine

The solution containing adenine is buffered with phosphate buffer and the final solution should contain 1–2 μg. adenine/ml. and 0·1–0·2 g.mol. phosphate/ml. The pH of the solution is measured and should be within the range of 7·0–7·5. This solution (3 ml.) is pipetted into a Beckman cuvette. To another cuvette 3·0 ml. phosphate buffer pH 7·0–7·5 and 30 μl. of the xanthine oxidase preparation (containing about 50 mg. protein/ml.) are added. The density at $\lambda = 305$ mμ. of the two solutions is measured; the sum of the densities is called E_{initial}. The reaction is started by addition of 30 μl. of xanthine oxidase to the cuvette containing the adenine solution and is followed at $\lambda = 305$ mμ. until the density does not change for about half an hour; this density is called E_{final}. Then

$$\Delta \log I_0/I = \Delta E = E_{\text{final}} - E_{\text{initial}}$$

and the concentration of adenine is calculated from Table 4.

DISCUSSION

From the experiments mentioned above it is not possible to state if the enzyme oxidizing adenine is identical with the xanthine oxidase from milk. Adenine, however, is probably adsorbed to xanthine oxidase since it turns out to be an inhibitor of this enzyme. In addition the enzymic oxidation of adenine is, like xanthopterin and some aldehydes, inhibited by 2-amino-4-hydroxy-6-formylpteridine (Kalckar, Kjeldgaard & Klenow, 1950*b*). It therefore seems probable that the enzyme oxidizing

adenine is at least of the same type as the enzymes (or enzyme) that oxidize the above-mentioned group of substrates.

SUMMARY

1. The enzymic oxidation product of adenine by milk xanthine oxidase has been identified as 2:8-dihydroxyadenine.

2. An enzymic differential spectrophotometric method for the determination of adenine is described.

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Studies Involving Enzymic Phosphorylation

1. THE HEXOKINASE ACTIVITY OF RAT TISSUES

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Colowick & Kalckar (1941, 1943) have demonstrated that the enzyme hexokinase catalyses the transfer of the terminal phosphate group of adenosinetriphosphate (ATP) to glucose or fructose, yielding the corresponding hexose-6-phosphate and adenosinediphosphate (ADP). Mannose (Berger, Slein, Colowick & Cori, 1946) and glucosamine (Harpur & Quastel, 1949) have also been found to serve as substrates. Mg^{++} is required as a cofactor in these phosphorylations.

The literature concerning the hexokinase activity of animal tissues is extremely fragmentary. Values for homogenates of rat brain and liver have been reported by Utter (1950) and by Vestling, Mylroie, Irish & Grant (1950), respectively. The published figures for rat kidney (Stadie & Haugaard, 1949) and skeletal muscle (Colowick, Cori & Slein, 1947; Stadie & Haugaard, 1949), however, refer to extracts only and were obtained in attempts to discover whether or not the hexokinase activity of animal tissues could be influenced by the *in vitro* addition of hormonal extracts; the lack of agreement in this latter field could conceivably have been due to variations in the experimental conditions adopted by the different workers. Partly in view of this possibility and partly because of the intrinsic value of such information, it seemed desirable to establish the optimal conditions for determining the hexokinase activity of animal tissues and to measure quantitatively the maximal glucose-phosphorylating capacity of each tissue.

In the work to be described, rat-tissue homogenates have been studied and the requirements for maximal activity have been found to be remarkably similar in all tissues. Brain was found to exhibit the greatest hexokinase activity while liver was least active. Other tissues varied over a fourfold intermediate range of activity.

A secondary aim of the present work was to ascertain whether any obvious correlation existed between the hexokinase activity of a tissue and its known function in the economy of the whole animal. As a result of the many enzyme assays performed, no close correspondence could be found for kidney or intestine, in spite of the fact that the phosphorylation of carbohydrate is generally regarded as occupying a special place in the metabolism of these tissues.

EXPERIMENTAL

Materials

Adenosinetriphosphate. ATP was prepared from rabbit muscle as the dibarium salt ($C_{10}H_{12}O_{13}N_5P_3Ba_2.4H_2O$) according to the procedure of Dounce, Rothstein, Beyer, Meier & Freer (1948). Analysis of freshly prepared material: total P, 10·4% (calc. 10·9%); P hydrolysable by N-H_2SO_4 in 10 min. at 100°, 7·3% (calc. 7·3%); hydrolysable P as fraction of total P, 0·70 (calc. 0·67); total N (Kjeldahl),