Flavine Adenine Dinucleotide

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Principle

The method[1,2] (cf.[3]) described here for the determination of flavine adenine dinucleotide (FAD) depends on the specific reactivation of the apoenzyme of D-amino acid oxidase from pig kidney by this coenzyme. With low FAD concentrations (up to 25 μg./ml.) the oxygen uptake, measured manometrically, is proportional to the amount of FAD. Comparison of the reactivation with a standard solution of FAD is necessary, since the Michaelis constant of the enzyme for FAD varies with different preparations and temperatures. The apparent dissociation constant for FAD varies between 0.13 and 0.33 μM., *i.e.* over a range of approximately two and a half-fold (refer to[4,5]). Other reasons for the use of an FAD standard are given below.

Reagents

1. Sodium pyrophosphate, $Na_4P_2O_7$ or $Na_4P_2O_7 \cdot 10\ H_2O$
2. DL-Alanine
3. Sulphuric acid, 1 N
4. Sodium dihydrogen phosphate, $NaH_2PO_4 \cdot H_2O$
5. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 12\ H_2O$
6. Potassium hydroxide, 20% (w/v)
7. Flavine adenine dinucleotide, FAD
   free acid; commercial preparation, see p. 1012.
8. Apoenzyme of D-amino acid oxidase
   from pig kidney; preparation, see Appendix, p. 598.

Preparation of Solutions

I. Pyrophosphate buffer (0.1 M; pH 8.5):
Dissolve 5.32 g. $Na_4P_2O_7$ or 8.92 g. $Na_4P_2O_7 \cdot 10\ H_2O$ in 150 ml. distilled water, adjust to pH 8.5 with *ca.* 4 ml. 1 N H_2SO_4 and dilute with distilled water to 200 ml.

II. DL-Alanine (1.0 M):
Dissolve 0.891 g. DL-alanine in distilled water and make up to 10 ml.

III. Phosphate buffer (10^{-2} M; pH 7.0):
a) Dissolve 7.164 g. $Na_2HPO_4 \cdot 12\ H_2O$ in distilled water and make up to 1000 ml.
b) Dissolve 2.760 g. $NaH_2PO_4 \cdot H_2O$ in distilled water and make up to 1000 ml.
Mix 61 ml. solution a) with 39 ml. solution b) and dilute to 200 ml.

IV. Flavine adenine dinucleotide, FAD:
a) Stock solution (*ca.* 1.5×10^{-3} M):
Dissolve 6 mg. pure FAD (dried *in vacuo* at 50 to 60°C over P_2O_5) in 5 ml. phosphate buffer (solution III).

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1) *O. Warburg* and *W. Christian*, Biochem. Z. *298*, 150 [1938].
2) *F. B. Straub*, Biochem. J. *33*, 787 [1939].
3) *F. M. Huennekens* and *S. P. Felton* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 950.
4) *K. Burton* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955 Vol. II, p. 199.
5) *E. Diamant, D. R. Sanadi* and *F. M. Huennekens*, J. Amer. chem. Soc. *74*, 5440 [1952].

b) Dilute solution (*ca.* 3×10^{-6} M):
Just before use dilute the stock solution 500-fold with distilled water.

After diluting the stock solution determine the exact FAD content spectrophotometrically. Pure FAD has an extinction coefficient at 450 mμ of 11.3 cm.²/mmole (pH 7.0). A solution containing exactly 6 mg. FAD/5 ml. is 1.5275×10^{-3} M and after 50-fold dilution*) has an optical density at 450 mμ of 0.341 (pH 7.0 and 1 cm. light path).

For pure FAD preparations the ratio of the optical densities at 260 and 450 mμ (pH 7) is exactly 3.25. The purity can also be checked by paper chromatography[3]. FAD can be freed from FMN, riboflavin and several nucleotides by electrophoresis on Whatman No. 1 paper and subsequent elution of the fluorescent material[6]. Excessive exposure to ultraviolet light is to be avoided. FAD is sensitive to acids, bases and light.

V. Apoenzyme of D-amino acid oxidase:
Use the solution prepared according to p. 598.

Stability of the solutions

The phosphate and pyrophosphate buffer keep for weeks if no growth of micro-organisms occurs. The FAD and apoenzyme solution keep for several weeks at −15° C.

Procedure

Experimental material

Solutions containing free FAD can be analysed directly. Protein-bound FAD can be liberated in the manometer vessel by heat denaturation (place the vessel in a boiling water bath for 5 min.; after cooling, add the other reagents to the protein coagulum[3]). Strongly bound FAD can be liberated by heat denaturation followed by proteolysis (cf.[7]).

Since biological material must be diluted considerably to obtain a final FAD concentration of 10^{-7} to 10^{-6} M, interference from other compounds present in the sample can usually be ignored. Much higher concentrations (10^{-4} to 10^{-3} M) of partial structural analogues, such as FMN, AMP, ATP, DPN, free riboflavin, *etc.*, can cause appreciable competitive or non-competitive inhibition[8]. In doubtful cases, inhibition can be ruled out by the use of internal standards. If purification of the sample is necessary[3,6], the use of internal standards corrects for any losses.

Manometric measurements

Warburg manometers: manometer vessels with centre well and side arm; gas phase: air; temperature: 37° C. The following vessels are required: 1—2 experimental vessels, 3—4 standards, 1 control (without FAD) and 1 thermobarometer.
Prepare the vessels as follows:

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*) For the spectrophotometric measurements it may be necessary to dilute the solution with phosphate buffer (solution III) instead of with distilled water.

6) *O. Walaas* and *E. Walaas*, Acta chem. scand. *10*, 118 [1956].
7) *H. Kondo*, *H. C. Friedmann* and *B. Vennesland*, J. biol. Chemistry *235*, 1533 [1960].
8) *E. Walaas* and *O. Walaas*, Acta chem. scand. *10*, 122 [1956].

		Experimental and Standard	Control	Thermo-barometer
Main compartment	buffer (soln. I)	1.0 ml.	1.0 ml.	—
	apoenzyme (soln. V)	0.3 ml.	0.3 ml.	—
	sample or FAD standard solution (IVb)	0.6 ml.	—	—
	distilled water	—	0.6 ml.	2.1 ml.
Side arm	alanine soln. (II)	0.1 ml.	0.1 ml.	—
Centre well	20% KOH + filter paper	0.1 ml.	0.1 ml.	—

Equilibrate at 37°C, tip the contents of the side arms into the main compartments and close the manometer taps. Start a stopwatch and read the manometers at 5 to 10 min. intervals. The ideal range of oxygen consumption is between 10 and 40 µl. per 10 min.

Calculations

The oxygen consumption, ΔO_2/min. or ΔO_2/10 min., of the experimental and standard vessels is obtained from the manometer readings (mm. manometer fluid) after correction for the thermobarometer and control (refer to p. 40). The values for the successive 10 min. intervals should agree within ±5% and are averaged[3].

For the standards plot

$$\frac{1}{O_2/\text{min.}} \text{ or } \frac{1}{O_2/10\,\text{min.}} \text{ against } \frac{1}{\text{FAD content}}.$$

Obtain from this standard curve the amount of FAD corresponding to the oxygen consumption of the experimental vessels. The molecular weight of FAD is 785.6. For low concentrations of FAD (refer to "Principle") there is a linear relationship between the oxygen uptake and the amount of FAD, and therefore a reciprocal plot is not necessary.

Appendix

Preparation of the D-amino acid oxidase apoenzyme*)

Extract an acetone-dried powder of pig kidney at room temperature with distilled water and centrifuge. The supernatant should contain *ca.* 10 mg. protein/ml. For the following steps work at 0°C. To 9.8 ml. of the supernatant add 3.4 ml. saturated ammonium sulphate solution. Slowly add, with stirring, 5.6 ml. 0.1 N H_2SO_4 and then centrifuge. Discard the supernatant, wash the precipitate with 4.9 ml. saturated ammonium sulphate solution, centrifuge again and discard the supernatant. Suspend the precipitate in 3.5 ml. 0.1 M Na phosphate buffer (pH 7.2), centrifuge and discard the precipitate. Use the supernatant undiluted or store at −15°C.

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*) The method described here[9] is similar to that of *Warburg* and *Christian*[1], but H_2SO_4 is used instead of HCl. A method employing acetic acid has been described by *Huennekens* and *Felton*[3]. For the effect of various anions, such as chloride and sulphate, on the rate constants for the dissociation and association of the FMN-containing old yellow enzyme refer to[10].

9) *J. Koukol*, Ph. D. Thesis, University of Chicago, Department of Biochemistry, 1959.

10) *H. Theorell* and *A. P. Nygaard*, Acta chem. scand. *8*, 1649 [1954].