A NEW METHOD FOR PREPARING FLAVIN-ADENINE-DINUCLEOTIDE

by

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Flavin-adenine-dinucleotide* was first isolated and characterized by WARBURG AND CHRISTIAN[1,2]. Subsequently this method has been modified by other workers in order to simplify the procedure and obtain a better yield and a purer product[3,4,5,6,7,8,9,10].

The preparations of FAD obtained up to now were identified and tested for purity by means of enzymic assay, absorption spectrum and sometimes[8,9] by paper chromatography.

WHITBY succeeded in obtaining products containing 90% FAD by chromatography on powdered cellulose. A very accurate determination of the molecular extinction coefficient of FAD has been done by the same author[9].

Considering his own results and those obtained by preceding workers as well, WHITBY states that: "It is probably true to say that pure FAD has not yet been prepared".

Recently CHRISTIE *et al.*[11] succeeded in obtaining pure FAD by synthesis, but this achievement is very difficult to reproduce.

In the present paper we described a new and simple procedure for preparing FAD from baker's yeast, making use of both ion-exchange resins and column electrophoresis. The product obtained, and tested by enzymatic assay, absorption spectrum, paper chromatography and electrophoresis, was found to be pure.

EXPERIMENTAL

Ion-exchange chromatography. Carboxylic acid exchanger (Amberlite IRC-50, 50–80 mesh) in the hydrogen form, packed in a 5 × 70 cm column was used.

Column electrophoresis. The column electrophoresis procedure as described by FLODIN AND PORATH[12] was followed. A 50 × 3 cm column was packed with cellulose powder previously washed with the same buffer solution (acetate buffer pH 5.10; $\mu = 0.05$) used for the electrophoresis run. The electrophoresis was run in a dark ice-cold room applying a 30 mA current for 14 hours. At the completion of the run, the column was disconnected from the electrode vessels, fitted on a fraction collector and the elution carried out with the same acetate buffer. A flow rate of 40 ml per hour was used and fractions of 8 ml collected.

Paper chromatography. Ascending paper chromatography was used and the solvents suggested by DIMANT *et al.*[8] were adopted.

Paper electrophoresis. The procedure for the separation of riboflavine and its coenzymes, elsewhere described[13], was followed.

Coenzymic activity. The activity of FAD as a coenzyme for D-amino-acid oxidase was determined by the method of WARBURG AND CHRISTIAN[1]. The apoenzyme, however, was prepared from pig kidneys, according to BESSEY *et al.*[4]. A commercial sample (Sigma Co.) 60% purity was used as reference.

* FAD = Flavin-adenine-dinucleotide.
FMN = Flavin-mononucleotide or riboflavin-phosphate.
R = Riboflavine.

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Spectrophotometric measurements. Absorption spectra and the extinction coefficients were determined in a DU Beckman spectrophotometer. Measurements were made in 0.05 *M* phosphate buffer at pH 7.0. The FAD molecular extinction coefficient (ε) at 450 mμ was assumed to be $11.3 \cdot 10^3$ l mole^{-1} cm^{-1}.

Extraction of FAD (A). Samples of different Italian baker's yeasts were first analyzed for their FAD content by manometric method in order to choose the yeast richest in this coenzyme. "Titano" baker's yeast was choosed as the richest, its FAD content being 11 mg/kg. For the extraction of FAD from the yeast the method of WARBURG with minor modifications was followed (see also WHITBY)[9]. Four kg of yeast were crumbled and added to 6,000 ml of water maintained at 80°; the suspension was kept at this temperature for 10 minutes after the addition of the last portion of yeast and then rapidly cooled to room temperature by means of a cooled coil.

After centrifuging at 2,500 r.p.m. for 20 minutes solid ammonium sulphate (3460 g = = $^2/_3$ saturation) was added to the supernatant fluid and the solution extracted three times with 820 ml of phenol.

The separation of the yellow phenol phase from the emulsion was accomplished by centrifuging the mixture at 3,000 r.p.m. for 45 minutes. 1800 ml of clear yellow phenolic solution were recovered. An equal volume of ether was added to the phenolic solution and the mixture extracted 10 times with 50 ml of water. The aqueous extract was washed with 90 ml of ether and the residual ether was then boiled off under reduced pressure at 20° C. The solution was then acidified to Congo-red using about 45 ml of *N* nitric acid and the crude Ag salt of FAD was precipitated with 12 ml of 30% silver nitrate. The precipitate was washed 2 times with 10 ml of water, resuspended in 50 ml of water and dissolved by adding excess of saturated KCl; a few drops of 2 *N* HNO_3 were then added to precipitate the AgCl, and the suspension centrifuged. The precipitate was washed 3 times with a little water (10 ml) and the washings combined with the supernatant (final solution = 100 ml). Ammonium sulphate was added (1 g/2 ml solution) and the FAD extracted twice with 10 ml *p*-cresol; this solution was washed three times with 50 ml of the following mixture: water 90 ml; 2 *N* H_2SO_4 10 ml; ammonium sulphate 50 g and FAD re-extracted with 30 ml of water (10 ml each time).

Analysis of the extract by paper electrophoresis and by paper chromatography showed a large yellow fluorescent spot corresponding to FAD, a second smaller spot corresponding to FMN and four spots with blue fluorescence due to unidentified compounds. The purity of this extract was determined by estimating the content of FAD spectrophotometically (see Table I).

TABLE I

YIELDS OF FAD AND RATIOS OF LIGHT ABSORPTION AT 260 AND 450 mμ AT DIFFERENT STAGES OF PREPARATION

Stage	*Crude FAD expressed as equivalent of pure FAD mg/kg of yeast*	$R = \frac{260\ m\mu}{450\ m\mu}$
A	11.5	16.2
B	7.1	10.2
C	2.25	3.42

For the explanation of these Stages see text.

TABLE II

ACTIVITY OF FAD AS COENZYME OF AMINO-ACID-OXIDASE DETERMINED ACCORDING TO WARBURG AND CHRISTIAN PROCEDURE[1-2]

Time (minutes)	*Oxygen uptake (mm³)*	
	FAD (our prep.)	*FAD (Sigma)*
15	42.3	25.2
30	79.2	48.4

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Ion-exchange chromatography (B). The solution of crude FAD was concentrated *in vacuo* to about 20 ml and applied to the top of the column containing activated Amberlite IRC-50. 8 ml fractions were collected and the extinction coefficients determined at 260 and 450 mμ for the yellow fluorescent fractions and at 260 mμ for the others. During the washing with water (sorption step) a yellow fluorescent fraction appears immediately after the dead volume and three other peaks follow this. A fifth peak is obtained when the column is eluted with 1/50 *N* HCl (see Fig. 1).

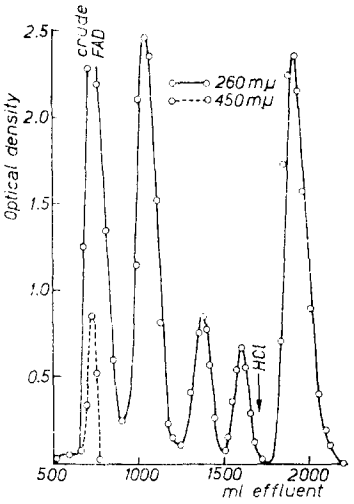

Fig. 1. Ion-exchange (Amberlite IRC-50) separation of crude FAD from other unidentified substances from yeast.

The yellow fluorescent fractions, when examined by paper chromatography and electrophoresis, revealed the presence of FAD and FMN in the same proportions as in stage (A); no other spot was, however, detected in the U.V. light. The purity of FAD in this solution is higher than in stage (A) (see Table I).

Column electrophoresis (C). The FAD solution obtained in the preceding stage was concentrated to about 6 ml under reduced pressure at 25° C. Two ml of this solution, corresponding to 6.3 mg of dried residue were submitted to electrophoresis on cellulose powder column. The current was applied after the flavine mixture had been allowed to flow down the column for about 10 cm. The top of the column was connected to the cathode, the bottom to the anode; a current of 30 mA for 14 hours was then applied. Both FAD and FMN migrate to the anode, the former faster than the latter, so that they can be well separated.

This separation is shown in Fig. 2 which was obtained by measuring the extinction coefficients, at 260 and 450 mμ, of the fractions recovered by eluting the column. The identification of the compounds was carried out by paper chromatography and electrophoresis.

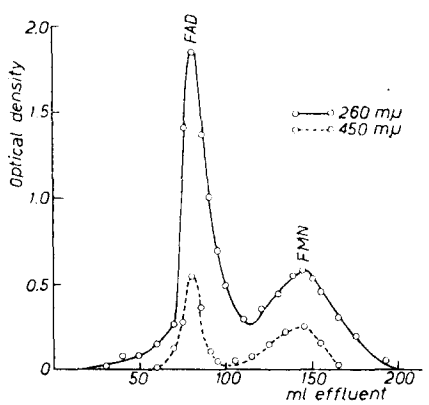

Fig. 2. Separation of pure FAD by cellulose powder electrophoresis (acetate buffer 5.1 pH, $\mu = 0.05$, 30 mA for 14 hours).

Determination of purity. In order to free the FAD from the acetate buffer present in the solution recovered from the electrophoresis column, the solution was rechromatographed on the carboxylic resin column. FAD is "retarded" in respect to the acetate ions and it appears in the aqueous effluent as pure component. The water solution of FAD was evaporated under reduced pressure at 30° and lyophylized.

Samples of FAD (12 μg) were chromatographed on paper by using the various solvents systems described by DIMANT[8]. No other spot could be detected apart from that corresponding to FAD. Other samples of FAD were tested by paper electrophoresis and no other spot besides that corresponding to FAD was obtained (see Fig. 3).

The activity of our preparation of FAD as coenzyme of D-amino-acid oxidase was

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found to be 163% as compared with commercial sample (60% purity) (see Table II).

A solution of FAD in phosphate buffer (10 μg) showed ln (I^0/I) values of 0.143 at 450 mμ; taking $\varepsilon = 11.3 \cdot 10^{-3}$ ml^{-1} mole^{-1} and the molecular weight of FAD = 785.6, the purity was calculated to be 99,30%. The absorption spectrum of FAD is shown in Fig. 4.

The ratio, R, of light absorption at 260 and 450 mμ calculated from this spectrum is 3.275.

Yield. 8.9 mg of FAD were isolated from 4 kg baker's yeast. This yield is somewhat lower than that obtained by former workers; it may be, however, improved if the described procedure is employed to this end.

Discussion. The present procedure for preparing FAD is essentially the same as that of WARBURG in its initial extraction stage. The subsequent stages involve a separation by means of a carboxylic resin and a further separation by

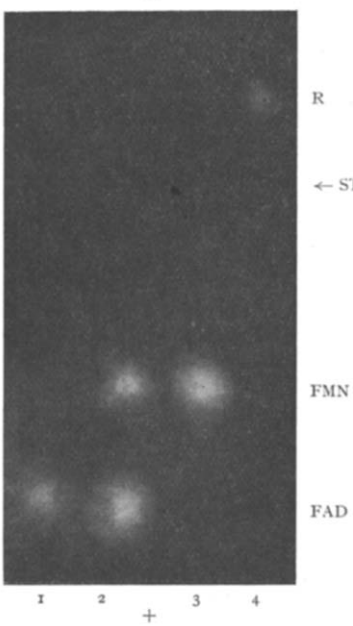

Fig. 3. Separation of flavine compounds (R; FMN; FAD) by paper electrophoresis (acetate buffer pH 5.1, μ = 0.05, 3.5 mA during 7 hours). 1 = pure FAD obtained with the present method. 2 = commercial sample of FAD 60% purity (Sigma Co.). 3 = pure FMN obtained by synthesis (VISCONTINI *et al.*[14]). 4 = riboflavine (commercial sample).

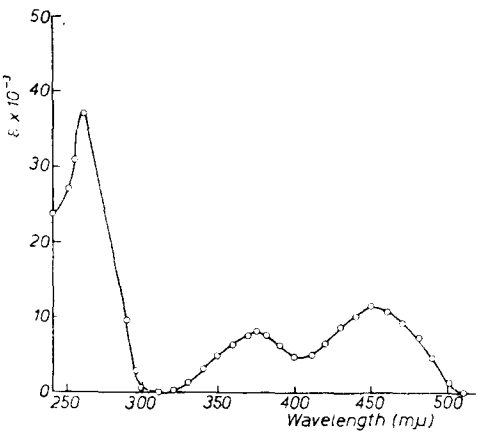

Fig. 4. Absorption spectrum of FAD determined in 0.1 M phosphate buffer, pH 7.0.

column electrophoresis. Attempts to improve the yield and to shorten the procedure are under study. Four criteria were applied in order to test the purity of FAD obtained by this procedure: paper chromatography, paper electrophoresis, manometric measurement of the activity as coenzyme of D-amino-acid oxidase, molecular extinction coefficient.

By chromatographic and electrophoretic analysis FAD appeared to be quite pure. The purity test by paper electrophoresis seems to be more accurate than that by paper chromatography. A crude preparation of FAD, obtained after stage (B) on paper chromatography gave only one spot; however, when it was tested by paper electrophoresis it was resolved into three spots.

Previous authors stress the importance of the ratio, R, between light absorption at 260 mμ and that at 450 mμ as a criterion of purity for flavin dinucleotide. DIMANT, WHITBY and BALL[8,9,10,15] report R values corresponding respectively to 3.8, 3.25 and 3.3 for their preparations of FAD. By assuming that the light absorption at 450 mμ in the dinucleotide is additive for the *iso*alloxazine moieties, DIMANT calculated the R

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value of FAD as 3.6. It will be possible to state the exact value for FAD, however, only when an absolutely pure sample of this compound is available. If it is contaminated by adenine derivatives which absorb at 260 mμ a higher R value will result, and if contaminated by FMN they will be lower. Our preparation of FAD which was absolutely free of contaminating adenine derivatives or FMN, exhibited an R value of 3.275.

From the results obtained by measuring the activity of FAD as D-amino-acid oxidase coenzyme, our preparation was found to be 98% pure assuming that the purity of the commercial sample used for reference was 60%.

Elementary analysis of FAD was not taken into consideration since it is of no value in ascertaining whether the molecule of FAD is intact: in fact both FAD and an equimolecular mixture of FMN + AMP (the breakdown products of FAD) give the same analytical figures.

Our criteria for testing the purity of FAD were essentially the same as those adopted by previous authors, except for the paper electrophoresis; however, if our results are compared with theirs, it seems that we have obtained the purest preparation so far produced.

ACKNOWLEDGEMENT

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SUMMARY

Chromatography on carboxylic resin, followed by electrophoresis on a column packed with cellulose powder, has been used to prepare pure FAD.

The purity of the product has been tested spectrophotometrically, by paper chromatography and electrophoresis and by enzymic activity as coenzyme of D-amino-acid oxidase.

RÉSUMÉ

La chromatographie sur résine carboxylique, suivie d'une électrophorèse sur colonne de cellulose en poudre, a été employé pour préparer du FAD pur.

La pureté du produit a été testée par spectrophotométrie, par chromatographie et électrophorèse sur papier et par l'activité comme coenzyme de la D-aminoacide oxydase.

ZUSAMMENFASSUNG

Chromatographie auf Resin mit Carboxylgruppen, gefolgt von einer Elektrophorese auf einer mit Zellulosepuder bepackten Säule wurde zur Darstellung von reinem FAD benutzt.

Die Reinheit des Produktes wurde getestet spektrophotometrisch durch, Papierchromatographie und Elektrophorese, wie durch Enzymaktivität als Coenzym von D-Aminosäureoxidase.

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