

Preparation of Flavin Adenine Dinucleotide by Paper Electrophoresis

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Satisfactory separation of flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and riboflavin has been carried out by paper electrophoresis. By the same method FAD is separated from adenine nucleotides and pyridine nucleotides. Paper electrophoresis has been employed in the final fractionation of FAD from bakers' yeast. The FAD obtained is pure, judging from absorption measurement and enzymatic testing in the D-amino acid oxidase system. The fluorescence of FAD was 20 % of that of pure FMN.

The preparation of pure FAD* from bakers' yeast was originally carried out by Warburg and Christian¹. Whitby^{2,3} and Siliprandi and Bianchi⁴ have recently given a summary of the modifications which have been employed later in order to produce a product with a higher yield. The main problem is to separate FAD from other nucleotides in the latter stages of the fractionation. A complete purification has not been achieved by paper chromatography⁵, adsorption chromatography on alumina or powder cellulose². A completely pure FAD was prepared by ion exchange chromatography followed by column electrophoresis⁴. As previously mentioned⁶, we have prepared pure FAD by using paper electrophoresis in the final purification of FAD extracted from bakers' yeast.

EXPERIMENTAL

Paper electrophoresis. An apparatus similar to that described by Foster⁷ has been used. In most experiments Whatman No. 1 filter paper was employed. Three strips of filter paper, 30 cm × 8 cm, were used simultaneously. The material to be separated was applied to the paper by means of an Agla micrometer syringe (Burroughs Wellcome Ltd.) and a volume of 10 μ l was applied 5 cm from the end of the paper strip and dried in a stream of air. Repeated applications could be carried out. The strips were wetted with buffer and placed on the glass plate and connected with vessels each containing 400 ml

* Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; A-5-P, adenosine-5-phosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

buffer. Each vessel was connected with another by means of a siphon filled with buffer. Carbon electrodes connected to a source of direct current were placed in the latter vessels. The voltage could be regulated by means of a transformer which permitted the use of 200–750 V. Usually 600 volt was employed which gave a potential gradient of 20 V per cm and a current of 5–10 mA, and the experiment was run for 4–5 hours. Cooling was achieved by the circulation of tap water through glass tubes attached to the bottom side of the lower glass plate. This prevented condensation on the upper glass plate covering the apparatus.

After each run the paper was dried in a stream of air. Riboflavin and its derivatives were detected under the fluorescent lamp. Other nucleotides were detected by photography in ultraviolet light⁸. Elution and quantitative estimation of the different products⁹ were also carried out. Pieces of paper, 1 × 2 cm, were cut from the starting line and successively in the longitudinal direction of the paper, and each piece eluted with 4 ml of distilled water for 30 min. The spectra of the eluates in the ultra-violet, and near ultra-violet region, were determined using a Beckman spectrophotometer model DU. For the quantitative determination the absorptions at 260 m μ , 375 m μ and 450 m μ were used. By this method 90 % of the material was eluted from the paper. For analytical separation 20–50 μ g FAD was applied to each spot. However, as much as 0.5 mg could be purified at the time in this apparatus.

For the preparative purification of larger amount of FAD an apparatus for continuous electrophoresis on paper was employed¹⁰. Isolation of dry FAD was achieved by freeze-drying the collected fractions.

In the experiments designed to test the possible separation of FAD from other nucleotides, the following materials from "Sigma" Chemical Company were used: A-5-P, ADP, ATP, DPN, TPN, FMN (synthetical product), riboflavin. FAD was prepared from bakers' yeast.

RESULTS

As shown in Fig. 1 complete separation of FAD, FMN and riboflavin was achieved at a pH near neutral both in phosphate buffer and ammonium acetate buffer. Furthermore it appears that the method permits the separation of FAD from DPN, TPN, A-5-P, ADP and ATP. The migration of the two latter compounds was so rapid that they moved off the paper in the time employed.

FAD was prepared from bakers' yeast as described by Warburg and Christian¹. The main problem in obtaining pure FAD consists of the removal of other nucleotides which contaminate the preparation at a later stage in the fractionation.

Separation by paper electrophoresis was attempted after the stage in which the silver-salt of FAD was decomposed. As shown in Fig. 2 two products with absorption maximum at 260 m μ were found in addition to FAD. FMN was not present. The first peak corresponded to DPN, its presence was verified enzymatically by the alcohol dehydrogenase assay. The fastest moving substance corresponded to the position of TPN and A-5-P. Enzymatic assay with glucose-6-phosphate dehydrogenase gave, however, no evidence for the presence of TPN. FAD was somewhat contaminated by the other components as the ratio R between the light absorption at 260 m μ and 450 m μ was 5.6 calculated from the spectrum. At this stage in the fractionation, however, further purification of FAD was achieved by two-dimensional electrophoresis, initially at pH 3.4 and finally at pH 6.8. It was however more advantageous to continue the fractionation of crude FAD until the extraction with cresol. Electrophoresis at this stage showed the amount of contaminating ultraviolet absorbing

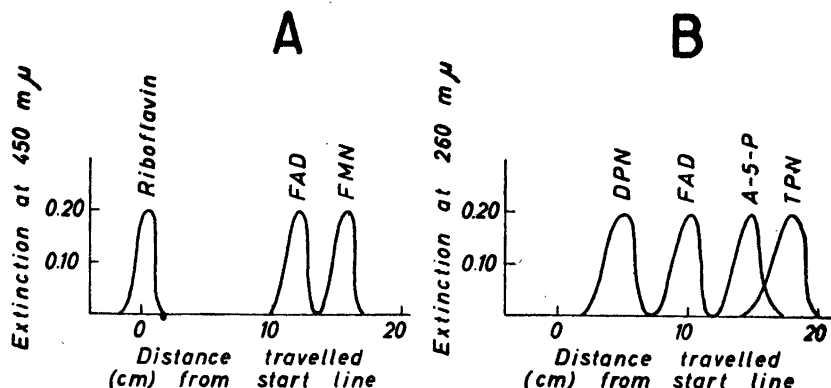


Fig. 1. A: Separation of riboflavin, FAD and FMN by paper electrophoresis. Ammonium acetate pH: 6.8. 5 hours, 600 V, 6 mA. B: Separation of FAD, DPN, TPN, A-5-P by paper electrophoresis. Ammonium acetate pH: 6.7. 4½ hours, 600 V, 10 mA.

material to be considerably reduced. Complete purification of FAD was achieved (Fig. 2) in ammonium acetate between pH 6—7, the best results obtained at pH 6.7.

DISCUSSION

As mentioned elsewhere⁶ the activity of the D-amino acid oxidase is inhibited by nucleotides. Further, anions strongly dissociate the coenzyme-protein complex in the "old yellow enzyme"¹² and D-amino acid oxidase⁶. Thus in studies on flavin enzymes it is of importance to work with pure coenzymes. The electrophoretic separation of FAD obtained after cresol extraction, in ammonium acetate which is easily removed afterwards by evaporation, solves this problem. The great loss which accompanies the crystallization is avoided, and the method is of value as long as synthetic FAD¹³ is not available for

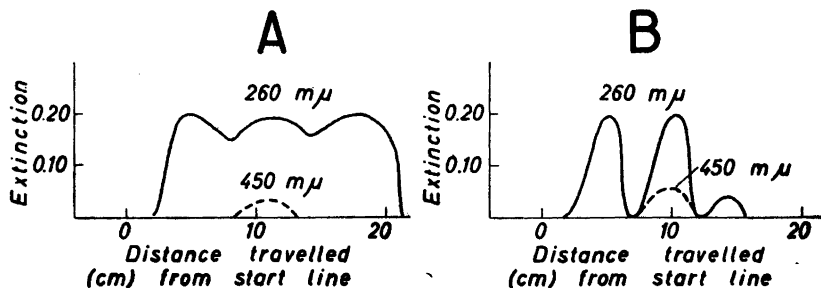


Fig. 2. Purification of FAD extracted from bakers' yeast by paper electrophoresis. A: After decomposition of the silver salt of FAD. Ammonium acetate pH: 6.4, 6 hours, 600 V, 12 mA. B: After extraction of FAD with cresol. Ammonium acetate pH: 6.7. 4½ hours, 600 V, 15 mA.

ordinary work. Electrophoresis on paper for the purpose of analytical work also gives better separation of flavin derivatives than by paper chromatography¹⁴.

The FAD prepared was not contaminated with ultraviolet absorbing material. Thus $R = 260 \text{ m}\mu/450 \text{ m}\mu$ was for several samples at pH 7.0 between 3.30 and 3.40. R values previously quoted are 3.38¹, 3.35² and 3.275⁴. Enzymatic testing of FAD in the D-amino-acid oxidase system with a highly purified apoenzyme¹⁵ gave $K_M = 1.2 \times 10^{-7} \text{ M}$ at pH 8.3 and 38° C. This is in accordance with Warburg and Christian¹ who under the same experimental conditions found $K_M = 1.4 \times 10^{-7} \text{ M}$.

By reduction of FAD with $\text{Na}_2\text{S}_2\text{O}_4$ the absorption at 450 m μ is reduced by 90 %. The fluorescence of FAD was 20 % of that of FMN purified by paper electrophoresis. 90 % quenching of the fluorescence of FAD occurred when $\text{Na}_2\text{S}_2\text{O}_4$ was added. Previous figures quoted for the fluorescence of FAD as compared to FMN are 20 %¹¹ and 9 %⁷. In the latter work FAD was not completely pure and the result can be explained by the well known quenching effect of nucleotides on the fluorescence of riboflavin and riboflavin derivatives^{18,16}.

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