FLAVINS AS LABELS IN IMMUNOASSAYS

I. Design and Synthesis of Flavin Labels

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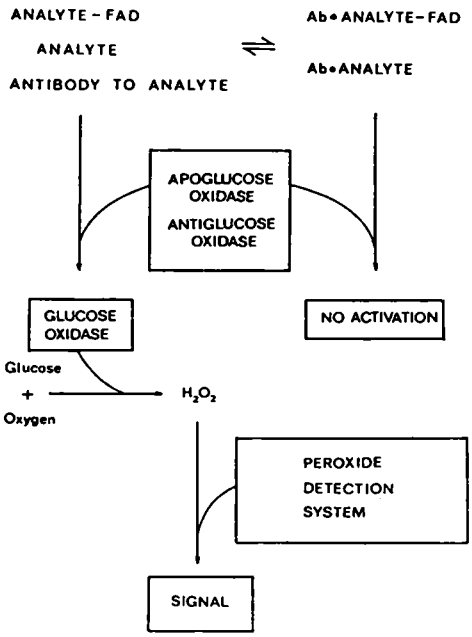
The Apoenzyme Reactivation Immunoassay System (ARIS) is a competitive binding assay in which FAD is used as a label.[1] The concept of the assay is shown below in Figure 1.

FIG. 1 CONCEPT OF THE APOENZYME REACTIVATED IMMUNOASSAY SYSTEM



Flavins and Flavoproteins

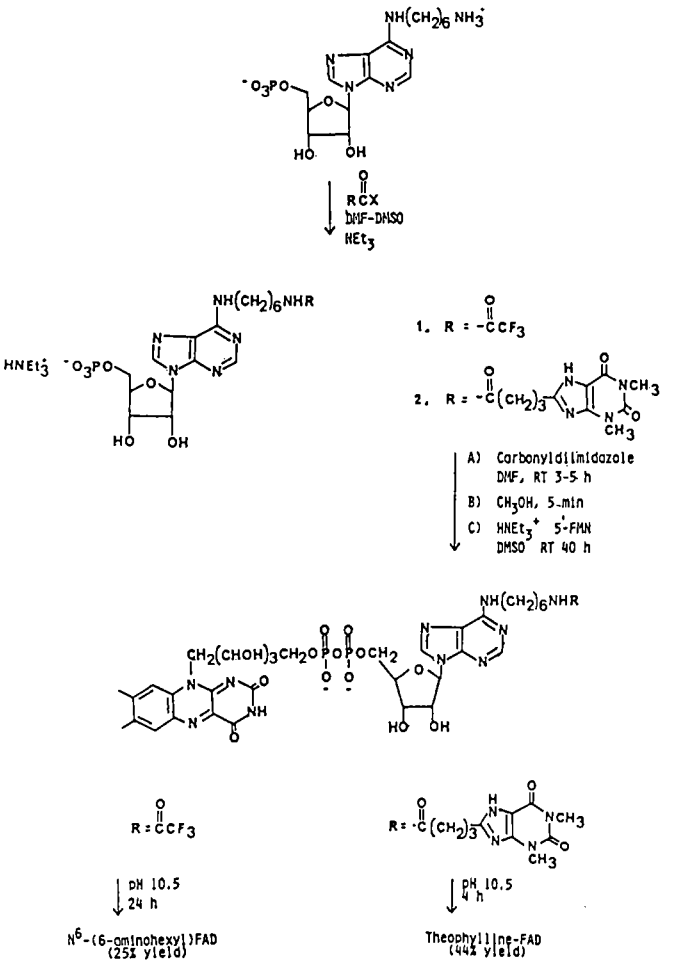
The FAD label is detected by the measurement of the glucose oxidase activity generated when excess apoglucose oxidase is added to the assay mixture. The assay requires no separation because the free FAD label can be detected in the presence of antibody-bound analyte-FAD conjugate, which does not activate apoenzyme. This homogeneous assay has high sensitivity because of the intrinsic amplification from the conversion of FAD labels into active centers of glucose oxidase. The hydrogen peroxide thus generated in the assay can be detected by a variety of peroxidase catalyzed chromogenic reactions.

The synthetically prepared analyte-FAD conjugate is a key component in the assay. Its structure must be designed to ensure the following certain essential characteristics. First, the FAD moiety must retain its function as a prosthetic group for glucose oxidase. The analyte portion of the conjugate must bind to the analyte-specific antibody such that the FAD activity of the label is quenched. Free analyte must compete effectively with the conjugate for the antibody binding sites. Finally, a general synthetic strategy should be devised to obtain a wide variety of stable, highly purified FAD conjugates.

FAD derivatives for ARIS are prepared from N^6-(6-aminohexyl) AMP according to the synthetic scheme outlined in Figure 2. N^6-(6-aminohexyl) FAD has found wide application in our laboratory for the labeling of numerous drug derivatives, proteins, and hormones of biological interest. Its synthesis begins with the protection of 6-aminohexyl AMP with ethyl trifluoroacetate to give the crystalline N-TFA derivative in 76% yield. This compound is then converted into its FAD derivative by carbonyldiimidazole activation and coupling with triethylammonium 5'-FMN in 1:1 DMF-DMSO. Optimum coupling reaction conditions were found to be with 1.5 molar equivalents of 5'-FMN at 0.1 molar concentration at 30° for 20 h.

The crude conjugate is purified by silicic acid chromatography. The N-trifluoroacetamide group is then removed by treatment at pH 10.5 for 24 h. 6-Aminohexyl FAD is finally isolated in 25% yield by either Bio-Gel P-2 chromatography or semi-preparative reverse phase HPLC.

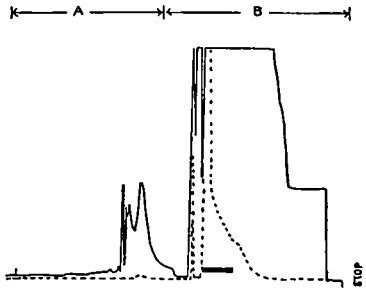
FIG. 2 SYNTHESIS OF FAD DERIVATIVES



The synthesis of haptenic-FAD conjugates is illustrated in Figure 2 by the method for the preparation of theophylline-FAD. The lactam of 8-(3-carboxypropyl)theophylline is reacted with N^6-(6-aminohexyl) AMP, and is then converted to the FAD conjugate by the carbonyldiimidazole activation method. The crude conjugate and its 2',3'-ribosyl carbonate derivative[2] are isolated by silicic acid chromatography and then hydrolyzed by alkali. Theophylline-FAD is then isolated in 44% yield by either Bio-Gel P-2 or reverse phase chromatography.

A general semi-preparative procedure has been developed in our laboratories for the isolation of 10-300 μmolar quantities of FAD conjugates from crude reaction mixtures.[3] This rapid method, replacing the previous chromatography protocol, is illustrated below for the purification of theophylline-FAD.

FIG. 3 PURIFICATION OF THEOPHYLLINE-FAD BY SEMIPREPARATIVE HPLC



Column: Whatman ODS-3, MM-9
Flow Rate: 4.0 mL/min
Mobile Phase: A = H_2O
B = 85:15 (v/v)
PPB, 0.02M, pH 5.5: CH_3CN
Detector: Solid line 290 nm
Dashed line 510 nm

Product was eluted from the column in the area designated by the solid bar (—).

REFERENCES

1. D.L. Morris, et. al., Anal. Chem. 53, 658 (1981).

2. M. Malda, A.P. Patel and A. Hampton, Nucleic Acids Res. 4, 2843 (1977).

3. K.F. Yip and J.P. Albarella, Fourth International Symposium on the HPLC of Proteins, Peptides, and Polyncleotides, Dec. 10-12, 1984, Baltimore, MD.