methyl iodide. A portion (84.3 g.) of the esters when fractionated yielded 3% methyl caproate, 26° (4 mm.), 26% methyl 2-phenylbuten-3-oiate, 84° (2.7 mm.), 17% of unidentified monomethyl esters, 88° (1.5 mm.), 39% methyl β-benzalpropionate, 103° (1.9 mm.), 4% of an unidentified fraction 110° (1.8 mm.), a 5% methyl ester of a dicarboxylic acid of composition approximately $C_{13}H_{14}O_4$, *ca.* 125° (0.5 mm.), 5% (determined by calculation of saponification equivalents) and 6% of residue. The unidentified monomethyl esters in fraction 3 gave some evidence that suggested *o*-propenylbenzoic acid might be present but the isolation of pure material proved more difficult than the time available for the work permitted.

Activity of the Methylstyrenes in Polymerization of Butadiene.—α-Methylstyrene, 0.25 mole, was metalated by amylsodium prepared from 1 g. atom of sodium, 0.52 mole of amyl chloride and 0.20 mole of isopropyl alcohol in 500 ml. of pentane. This mixture was allowed to stand 9 days before its activity in polymerization of butadiene by bottle polymerization was tested. The conversions were 10, 30, 80 and 100% in about 60 minutes when 10, 20, 40 and 60 ml. of the reagent were used. The corresponding intrinsic viscosities of these four polymers were 0.40, 0.34, 0.38 and 0.39. The corresponding gels were 17, 0, 19 and 17%.

Three similar preparations of β-methylstyrenylsodium reagents were made but the third one gave typical results. The conversions of butadiene to a polymer were 14, 34, 80 and 90% as 10, 20, 40 and 60 ml. of the reagent were used. The corresponding intrinsic viscosities were 1.5, 1.7, 0.9 and 1.0 and the corresponding gels were 26, 32, 28 and 43%.

The effect of the methylstyrenes was tested also on an Alfin catalyst of known activity prepared in the usual way (two separate preparations) from a total of 6 g. atoms of sodium, 3 moles of amyl chloride and 1.2 moles of isopropyl alcohol, and gaseous propylene. The whole was diluted to a volume of 5250 ml. and stored in a well stoppered bottle. This master batch, which should have a concentration of 0.23 milliequivalent of allylsodium per ml. was treated in 250-ml. aliquots with 0, 1.69, 3.38, 6.67 and 13.5 g. of α-methylstyrene, the last quantity being twice the amount needed to react with all of the allylsodium. Each of these five aliquot portions was stirred for one hour at 8000–9000 r.p.m. at 25 ± 5° and then transferred to well stoppered bottles and diluted to 350 ml. with dried *n*-pentane. Tests for the catalytic activity caused by 5 ml. of the reagent on 30 ml. of butadiene in 210 ml. of pentane were made at the end of 7, 29 and 167 days. The control (or no. 1) caused 64, 39 and 41% of polymerization. The corresponding intrinsic viscosities of the polymers were 14, 11 and 11 and the gel contents were 84, 22 and 60. The fifth reagent, that is, the one with the most α-methylstyrene caused a little more polymerization, 66, 61 and 57%. The corresponding intrinsic viscosities were 16, 13 and 12 and the gels were 50, 43 and 47. The intermediate preparations gave conversions and other values within this general range. Actually, out of a total of twelve tests, the controls were exceeded seven times.

The effect of β-methylstyrene was tested in the same way but the catalysts were allowed to stand for 1 and 36 days before each test. The control (or No. 1) showed 45 and 35% conversions. The intrinsic viscosities were 19 in both cases and the gels were 77 and 80%. The fifth sample, which contained the largest amount of β-methylstyrene, caused 57 and 39% polymerizations. The corresponding intrinsic viscosities were 18 and 16 and the gels were 50 and 42%. In every test (eight comparisons) the reagents with β-methylstyrene showed an increase over the corresponding control, and the usual increase was 20 to 30%.

CAMBRIDGE 39, MASS.

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, UNIVERSITY OF WISCONSIN]

The Isolation of Flavin Nucleotides[1]

BY E. DIMANT,[2] D. R. SANADI[3] AND F. M. HUENNEKENS[4]

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Based upon chromatographic methods a procedure is described for the isolation of the coenzyme, flavin-adenine-dinucleotide (FAD), from animal tissues and yeast. The authenticity and purity of the product are established by means of (a) enzymatic assay; (b) absorption spectrum; and (c) paper chromatography in several solvent systems.

The yellow compound, flavin-adenine-dinucleotide (FAD),[5] which functions as the prosthetic group for many oxidative enzymes, was first isolated and characterized by Warburg and Christian.[6,7] Their method consists of extracting the flavins from yeast or animal tissues with hot water and trichloroacetic acid and subsequently purifying the FAD by means of repeated extractions into phenolic solvents, selective precipitation as the silver, lead or mercury complexes, and, finally, recrystallization of the barium complex.

The ultimate preparation of pure FAD by the above method is partially offset by the disadvantages of a low over-all yield and by the contamination with other adenine nucleotides until the final stages of purification.

In an effort to overcome these difficulties a method has been devised which makes use, principally, of the following chromatographic techniques: (1) adsorption chromatography on Florisil which separates the extracted flavins from other nucleotides; and (2) partition chromatography between phenol–butanol and water on Celite, or adsorption chromatography on dicalcium phosphate, which separates the various flavins.

The FAD is obtained in a 40–50% over-all yield and has a dry weight purity of 0.40–0.60; the remaining impurity is innocuous in various enzymatic test systems. The quantitative data on yield, purity, R value, *etc.*, given for the various procedures in the experimental section, are, unless otherwise specified, average values derived from many individual preparations.

(1) A report of this work was presented earlier—D. R. Sanadi and F. M. Huennekens, 117th Meeting of the American Chemical Society, Detroit, Mich., April, 1950, Abstracts, p. 60C.

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(5) The following abbreviations will be used throughout this paper: FAD, flavin-adenine-dinucleotide; FMN, flavin mononucleotide; Rb, riboflavin; CoA, coenzyme A; AMP, adenosine-5-phosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

(6) O. Warburg and W. Christian, *Biochem. Z.*, **298**, 150 (1938).

(7) For an excellent review of the flavins and the flavin nucleotides, see H. Theorell, "Die Alloxazin-Proteide (gelbe Fermente)," in Methoden der Fermentforschung, Vol. III, edited by E. Bamann and K. Myrbäck, Photo Offset Reproduction by Academic Press, Inc., New York, N. Y., 1945, pp. 2361–2384.

In addition to the isolation of flavin nucleotides, the technique of Florisil chromatography has general applicability in effecting a separation of other biologically important nucleotides. By-products such as: CoA, DPN, TPN, AMP, ADP and ATP are obtained as partially purified concentrates by this method.

Experimental

Materials.—All of the organic reagents used in this investigation were of a C.P. grade and were used without further purification. Merck and Co., Inc., C.P. reagent phenol is recommended because of its freedom from colored impurities.

Riboflavin was either the commercial Merck or Eastman product; additional flavins in pure form were generously supplied as follows: flavin mononucleotide (synthetic) by Hoffmann-La Roche, Inc.; L-lyxoflavin (synthetic) by Dr. Karl Folkers of Merck and Co., Inc.; and FAD (isolated by the Warburg and Christian procedure)[6] by the Sigma Chemical Co.

Florisil, a magnesium silicate adsorbent, was obtained as the 30/60 mesh grade from the Floridin Co., Warren, Pa. Hyflo Supercel and Celite (No. 535) were products of the Johns-Manville Co. The Celite was purified by heating with an excess of 5% H_2O_2 on a steam-bath for three hours, whereupon it was filtered, washed with deionized water and dried at 100° for two days.

The unsuitability of several commercial preparations of dicalcium phosphate for our chromatographic purposes led to the following special preparation: Solution A, 150 g. of $CaCl_2 \cdot 2H_2O$ (C.P.) dissolved in 750 ml. of water. Solution B, 240 g. of $K_2HPO_4 \cdot 3H_2O$ dissolved in 1000 ml. of water. After both solutions have been heated to 70–75°, A is added to B with stirring. The precipitate, in the form of a thick sludge, is allowed to stand for 20 minutes and then filtered on a buchner funnel, washed with 1000 ml. of deionized water without allowing the cake to dry and crack during the washing, and dried at 100° for 24 hours. The hard, dry adsorbent is pulverized in a mortar, and the particles between 40 and 100 mesh mixed with an equal weight of Hyflo Supercel. The temperature of precipitation is critical, and if carried out at a lower temperature, a weaker adsorbent, easily crushed by hand, is obtained. Although 24-hour drying is usually satisfactory, the activity of the adsorbent may be increased by prolonged drying.

Xanthine oxidase was isolated from cream by the method of Corran, *et al.*[8]

The split D-amino acid oxidase was prepared from pig kidney by a slight modification of the methods of Krebs[9] and Warburg and Christian.[6]

Methods.—Absorption spectra were determined in the Beckman spectrophotometer, model DU. All determinations were carried out in quartz cuvettes of 1 cm. thickness upon solutions at *p*H 7.0 unless otherwise specified.

Concentrations of flavin solutions were determined from light absorption measurements at 450 mμ. From the usual equation $\log I_0/I = \epsilon cd$, the molal extinction coefficient (ϵ) at this wave length is assumed to be 11.3×10^3 cm.²/mole for FAD,[6] (*cf.* Discussion) (below *p*H 3, $\epsilon = 9.83 \times 10^3$).

Assuming the following molecular weights: FAD ($C_{27}H_{33}N_9O_{15}P_2$, 785.6), FMN ($C_{17}H_{21}N_4O_9P$, 456.4) and Rb ($C_{17}H_{20}N_4O_6$, 376.4), it follows that solutions containing 10 μg./ml. of each of these compounds will exhibit, respectively, $\log (I_0/I)$ values of 0.144, 0.249 and 0.301. Purity of a sample, on a dry weight basis, was obtained by use of the above considerations.

Ascending paper chromatograms with the indicated solvent systems (*cf.* Table II) were run on 40 × 40 cm. sheets of Whatman No. 1 paper, the flavins being detected by means of their yellow-green fluorescence when observed under ultraviolet light from a "Mineralite" lamp.[10] The details of these, and other, solvent systems and the *RF* values for various other flavins are given elsewhere.[11]

(8) H. S. Corran, J. G. Dewan, A. H. Gordon and D. E. Green, *Biochem. J.*, **33**, 1694 (1940).

(9) H. A. Krebs, *ibid.*, **29**, 1620 (1935).

(10) C. E. Carter, THIS JOURNAL, **72**, 1466 (1950).

(11) F. M. Huennekens, D. R. Sanadi, A. I. Schepartz and E. Dimant, *ibid.*, to be submitted.

Preparations. A. Preparation of FAD. 1. Extraction of Flavins from the Tissue.—Fresh pig liver is packed in ice and transported immediately to the laboratory. A 1-kg. portion is cut into small pieces, divested of connective tissue, and extracted in the following manner with trichloroacetic acid: 150 g. of the tissue and 450 ml. of cold deionized (or distilled) water are ground in a Waring Blendor for one minute. 70 ml. of 50% (1:1 by weight) trichloroacetic acid is added and the blendorization continued for 1/2 minute. The deproteinized mixture is centrifuged in the cold for 5 minutes at 2300 × *g*. The opalescent, yellow supernatant is removed by decantation and filtered through coarse, fluted paper to remove fat and debris. The instability of flavins in acid solution and in the presence of light (*cf.* Part C) makes it imperative that the extraction from the tissue and subsequent Florisil chromatography be carried out as rapidly as possible and with a minimum exposure to strong light.

2. Chromatography on Florisil.—A glass chromatographic tube 3.5 × 30 cm. is filled with Florisil to a height of about 20 cm., and topped with a plug of glass wool. The adsorbent is not tamped, since this greatly reduces the rate of flow. The column is prepared in advance and stored in the cold room (*ca.* 5°). Water is passed through the column just prior to use followed by the filtered trichloroacetic acid extract (*ca.* 2600 ml.). The flavins remain adsorbed at the top of the column. A constant-level separatory funnel is useful for increasing the rate of flow and avoiding frequent addition to the column. After the extract has passed through, the column is washed, in turn, with 2 l. of 2% acetic acid, 2 l. of deionized water and finally with 2 l. of 0.5% pyridine. It should be noted that during the water wash a very *pale* yellow color (not a flavin) moves down the column and appears in the effluent (*cf.* Part D). The intense yellow flavin band, however, diffuses slowly down the column under the influence of water and 0.5% pyridine, and the pyridine wash should be terminated if the band reaches the bottom of the column.

Following the washing with 0.5% pyridine, the flavins are eluted from the column with approximately 500 ml. of 5% pyridine. The yellow flavin solution is adjusted to *p*H 8, extracted four times with 3 volumes of chloroform (the chloroform being discarded), filtered through a water-wetted paper, and finally warmed under reduced pressure (water aspirator). If the flavin has been washed adequately on the column, the *p*H of the 5% pyridine eluate will be 7 or higher, the *R* value 5 or less, and no emulsions will result when the solution is extracted with chloroform. Prior to the next step in the preparation, the flavin solution may be stored in the frozen state or lyophilized to yield 0.190 g. of a fluffy, light yellow powder having a dry weight purity of 0.10 and an *R* value (ratio of light absorption at 260 and 450 mμ; *cf.* Discussion) of 4.0–7.0. The *R* value for the original trichloracetic acid extract is *ca.* 120.

3. Partition Chromatography between Phenol–butanol and Water.—The various flavins are next separated from each other by means of partition chromatography. The column is prepared on the previous day in the following manner: 1800 g. of phenol and 800 ml. of *n*-butanol are mixed by warming on a steam-bath and then cooled to room temperature. 120 g. of washed Celite is added, followed by 40 ml. of water from a buret or pipet. During the latter two additions the solution is stirred vigorously. The solution is next stirred intermittently for about one hour and then allowed to stand for about three hours. As much as possible of the clear supernatant is decanted and saved as the *developing solvent.* The residual slurry is used to prepare a column *ca.* 14 cm. in height in a tube *ca.* 3.5 × 19 cm. For this size column no more than 10 mg. of total flavin should be chromatographed; hence, the sample from Part 2 (above) should be chromatographed on two separate columns. The desired rate of flow (1–3 ml./min.) may be achieved by pressure from a constant-level separatory funnel or, preferably, by compressed gas (N_2 or CO_2, but not O_2). After preparation, the column is stored by leaving several cm. of fluid over the adsorbent bed and sealing each end of the tube with a cork stopper.

Prior to the partition chromatography, the flavin solution from the Florisil column is treated with ammonium sulfate, as described below; or, if the solution had been lyophilized previously, the powder is extracted with successive 10-ml. portions of water by centrifugation in a clinical centrifuge. Three or four extractions suffice to remove most of the color

leaving a pale yellow, gelatinous residue of colloidal Florisil, which is discarded. The combined extracts are diluted to approximately 200 ml., treated with one-half the weight (100 g.) of ammonium sulfate and extracted successively with 10, 5 and 5 ml. of the developing solvent. The combined, dark-orange phenolic extracts are centrifuged for 10 minutes at top speed in a clinical centrifuge, and the upper layer, carefully excluding the interface region, drawn off with a pipet. The column is allowed to drain to incipient dryness and the flavin solution in the developing solvent added dropwise with a pipet—care being taken not to disturb the adsorbent bed. Under gravity or tank pressure the flavin solution is passed into the column, again to the point of incipient dryness. Five ml. of the developing solvent is added and the column again brought to near-dryness. When the flavin zone has been drawn completely into the column the developing solvent may be added in quantity. Since the flavins are added to the column in a phenol–butanol solution which is saturated with respect to water, equilibrium is not immediately re-established with the bulk of the solvent on the column, which is less than saturated with water. This is reflected by the behavior of the flavins on the column: Initially, owing to the high concentration of water, the combined flavins move rapidly down the column and may appear to be resolving into individual bands. Upon reaching a point about one-third the distance down the column, however, the diffuse yellow band is virtually stopped as the true equilibrium under the defined solvent system is established. At this point, a brown-yellow band separates from the combined zone and, after its slow passage down the remainder of the column, is collected in the effluent (*ca.* 30–60 ml.). This flavin, FAD-X, will be described in a later publication.[11] It may be transferred to water and worked up by the same procedures which will be described below for FAD.

After elution of FAD-X is complete, the top of the column, to a depth of 2–3 cm., is removed as a slurry by means of a pipet. This mixture, which contains the small amount of FMN present in the original flavins, may be discarded. The developing solvent is now changed by adding one part of water to each 30 parts of original solvent, and the chromatography continued with the water-enriched mixture. The FAD moves slowly down the column as a discrete yellow zone and is collected in the effluent (*ca.* 100–300 ml.). In a large separatory funnel the effluent is mixed with 3 volumes of chloroform and extracted with 25 ml. of water. The organic layer, in turn, is re-extracted with two or more additional 25-ml. portions of water until it is colorless, and the combined water fraction containing the flavins is extracted three times with 200-ml. portions of chloroform, the chloroform being discarded subsequently. The flavin solution is freed from traces of chloroform by the procedure described previously under Part 2, and lyophilized to dryness. The product (original flavin from the Florisil column divided, chromatographed on two separate Celite columns, and the FAD fractions recombined) is an orange powder weighing 38 mg., having a dry weight purity of 0.30 and an R value of 3.8–4.0. The recovery of FAD is 55–70% in this step.

4. **Adsorption Chromatography on Dicalcium Phosphate.**—Adsorption chromatography on dicalcium phosphate offers an alternate method which can be used in place of partition chromatography for separating the flavins. The dry adsorbent is carefully packed in a glass chromatographic tube (1.3 × 15 cm.) to a height of about 10 cm. The capacity of this column is about 20 mg. of flavin. The adsorbent should be added (under gentle suction from a water aspirator) in about 10 separate portions, each one being evenly tamped.

Before adding the flavins, water is allowed to pass through the column and it is observed that the rate of travel through the adsorbent itself appears to differ from that between the adsorbent and the glass wall. The water aspirator is adjusted so that by visual observation these two solvent fronts nearly coincide.

The mixed flavin sample, previously lyophilized after the Florisil chromatography, is extracted by centrifugation with successive 3-ml. portions of water until the extracts are essentially colorless. The combined extracts (*ca.* 10–15 ml.) are added with a pipet to the top of the column, which has been brought to the point of incipient dryness. After the flavins have been completely brought into the column, water is added and the chromatography continued. The flavins immediately separate into two yellow zones: the second remaining at the top of the column (FAD-X), while the first migrates rapidly downward and is collected in test-tubes in 10-ml. portions. When this first band has been completely eluted from the column, the developing solvent is changed to 25% pyridine which causes slow migration downward and elution of the top band. It has been observed that more active batches of dicalcium phosphate (prepared by prolonged heating at higher temperatures, *i.e.*, 3 days at 150°) will separate the mixed flavins into three bands: FMN, FAD and FAD-X. This advantage is offset, however, by the fact that the FAD must now be eluted with 5–10% pyridine while the FAD-X can be eluted only with concentrated pyridine which causes extensive breakdown into its mononucleotide.

The fractions of the first band are assayed by means of paper chromatography in either the phosphate buffer or the butanol–acetic acid systems. Tubes 1 and 2 usually contain exclusively FMN, 3 and 4 are mixtures of FMN and FAD, while the bulk of flavin, FAD, is found in the remaining tubes. It should be noted that if the column has not been tamped properly, the mixture of FMN and FAD will extend over many tubes, hence decreasing the yield of pure FAD. The FAD fractions are pooled and lyophilized to dryness yielding a light yellow powder weighing 115 g. and having a dry weight purity of 0.10 (contaminated with dicalcium phosphate) and an R value of 4.0–4.5. The recovery is 75% for this step.

5. **Further Purification of FAD.**—The impurities remaining after the chromatographic steps appear to have been derived both from the original tissue and from the columns. Further purification (up to 0.50–0.60) may be achieved by repetitive extraction into phenol, but this advantage is partially offset by the relatively high losses. The dry material (10–20 mg.) is dissolved in 50 ml. of water and ammonium sulfate (5 g. per 10 ml. of solution) added. Following the procedure for the phenolic extraction in Part 3, the solution is extracted three times with 2-ml. portions of a mixture of 100 g. of phenol and 50 ml. of benzyl alcohol. The combined phenol extract is dried over freshly ignited sodium sulfate, decanted, filtered through a paper wetted with phenol–benzyl alcohol, and the sodium sulfate washed with two 5-ml. portions of previously dried, phenol–benzyl alcohol mixture. The flavins are transferred from the phenolic solution to water in the manner described previously (*cf.* Part 3) and lyophilized to dryness.

Precipitating FAD at pH 2 as the lead or silver complex and recovering the flavin by treatment with H_2S[6] does not lead to any significant increase in purity. Furthermore, even under mild conditions there is always some breakdown of FAD to FMN.

The relatively high solubility of the barium salt of FAD in water (2.5 mg./ml. at 25°)[6] does not permit purification by recrystallization unless large quantities are available. However, FAD is conveniently precipitated from solution at pH 8.7 by adding barium acetate and 5 volumes of cold acetone. When decomposed with an excess of sodium sulfate the recovery of FAD is quantitative with no breakdown to FMN. Precipitation by acetone in the absence of barium leads to extensive breakdown of FAD.

B. **Use of Other Tissues.**—Both heart and kidney may also be used as a source of FAD although the yields are somewhat lower than in the case of liver. Tissues may be stored in the frozen state prior to use without decreasing the yield of FAD.

Bakers' yeast (Red Star) may also serve as the starting material, although in this case the usual procedure for extraction of the flavin is modified as follows[12]: 3 kg. of yeast is crumbled and added to 6000 cc. of water kept at 70–80°; the solution is kept at this temperature for 10 minutes after the last of the yeast has been added and then rapidly cooled to room temperature by the addition of ice or by means of a cooling coil. The solution is centrifuged for 20 minutes at 2300 × g, and the supernatant is filtered through a pad of Celite 1 cm. in depth. Solid trichloracetic acid is added to the filtrate to make a final concentration of 5%, the solution centrifuged for 10 minutes at 2300 × g, and the supernatant processed thereafter in the same manner as the acid extract from liver.

The yield of FAD from various animal tissues and from

(12) This method was devised by Dr. A. I. Schepartz of this Laboratory.

bakers' yeast is shown in Table I. It should be noted that these figures are values after the Florisil chromatography and represent only one extraction from the tissue.[13] The dry weight purity of the flavins at this stage is *ca.* 0.10 in all cases except for yeast which is *ca.* 0.03 (the R value is likewise high in the latter case, *i.e.*, 10–20).

TABLE I

YIELDS OF FAD FROM VARIOUS SOURCES

Source	Flavin (as FAD), mg./kg. of wet tissue
Pig liver	19.0
Pig kidney	12.6
Pig heart	7.5
Rabbit liver	11.0
Pigeon breast muscle	9.4
Bakers' yeast	18.5

C. Stability of Flavin Nucleotides.—The instability of FAD below pH 3 and above pH 10, at elevated temperatures and in the presence of light has been confirmed using paper chromatography (phosphate buffer system). Unless the paper chromatograms are prepared and run in near darkness, a spot of lumichrome[14] (RF = 0.05 in the phosphate system) will always be observed, even with pure samples.

Although in the first stage of purification FAD is chromatographed on a Florisil column without breakdown, more purified samples undergo extensive decomposition to FMN if rechromatographed under the same conditions. This anomaly, noted also by Bessey, *et al.*,[15] would appear to be due either to a protective action of impurities in the crude state or subtle differences in experimental conditions during the first chromatography.

D. Nature of the Various Effluents from the Florisil Column.—Since many nucleotides, other than the flavins, are extracted from the tissues by trichloroacetic acid, it is of interest to ascertain their location in the various eluates from the Florisil column. The trichloroacetic acid and acetic acid effluents from the column contain no nucleotides according to the criterion of paper chromatography.

Adenine-containing nucleotides are found in high concentrations in both the water and 0.5% pyridine effluents, and may be detected by their characteristic dark blue "quenching" spots on paper chromatograms using 5% Na_2HPO_4 as the developing solvent.[10] If the water wash of the column is increased to 3 l., essentially all of the CoA is found in the pale, straw-yellow effluent.[16] As yet no attempt has been made to isolate the above nucleotides, but the ability of the Florisil column to effect a preliminary separation of these compounds from a crude extract suggests the possibility that this method might be used to prepare "concentrates."

After the flavins have been eluted with 5% pyridine, a pale yellow band is often observed at the top of the Florisil column. Eluted with 25% pyridine, this material is identified as FAD, but no explanation can be offered for this apparent anomaly where a small fraction (*ca.* 2–5%) of the total flavin is tightly bound to the top of the Florisil column.

Discussion

The authenticity and purity of the FAD have been established by the following criteria: (a) enzymatic assay in the D-amino acid oxidase system, (b) paper chromatography in various solvents and (c) absorption spectrum.

(a) In the D-amino acid oxidase system,[6,9] the FAD is fully active at each stage of preparation, the impurities being innocuous.[17]

(b) A second criterion of purity is provided by the method of paper chromatography using several different solvent systems (*cf.* Table II). The phosphate buffer system is simple and extremely useful, for it achieves a clean separation in the case of riboflavin, FMN and FAD; the only disadvantages are the diffuse nature of the spots and the slight breakdown of the various flavins to lumichrome (RF = 0.05). The formation of this degradation product may be minimized by preparing and running the chromatogram in the absence of light. A second system, composed of butanol-acetic acid–water,[18] has the disadvantage of relatively low RF values for the phosphorylated compounds, but the advantage of small, discrete spots which permit of the assignment of accurate RF values. The third solvent system, phenol–butanol–water, effects the separation of FAD and FAD-X.

TABLE II

RF VALUES OF THE FLAVINS BY ASCENDING PAPER CHROMATOGRAPHY

Solvent Systems: 1, 5% Na_2HPO_4 in water[10]; 2, 4/1/5 *n*-butanol/acetic acid/water (top phase)[18]; 3, 160 g. phenol/30 ml. *n*-butanol/100 ml. water (lower phase).

Flavin	Solvent system 1	2	3
FAD	0.35	0.05	0.23
FAD-X	.35	.05	.47
FMN	.48	.13	.17
Rb	.30	.30	.80

Using these systems, the purity of the FAD preparation may be verified at each step. No spot is detected at the expected positions of riboflavin and only occasionally a faint spot at the position of FMN. This test for the presence of breakdown products of FAD is rendered even more delicate because of the 7-fold greater molal fluorescence of riboflavin and FMN as compared to FAD.[14] After separation of the flavins on Celite or dicalcium phosphate, the three components FAD, FAD-X and FMN, each exhibit single fluorescent spots in each of the solvent systems.

(c) The absorption spectrum of FAD (purity 0.5) is shown in Fig. 1. It is apparent that the ratio, R, of light absorption at 260 and 450 mμ, may be used as a criterion of purity for a flavin nucleotide. Contaminants, such as other adenine-containing nucleotides or traces of organic solvents (*i.e.*, phenol and pyridine), will absorb at 260 mμ but not at 450 mμ, and the R value will be increased over that of the pure flavin. It is of some importance, therefore, to establish the correct R value for pure FAD.

The commercial availability and known molecular weights of pure Rb and FMN make it possible to determine the absolute values of the molecular extinction coefficients, ϵ, for these substances. Since the preparation of pure FAD is not as readily accomplished, the R value, being independent of the dry-weight purity, may be used, nevertheless, as a measure of the purity relative to other nucleo-

(13) Under the conditions described in Part 1, a single extraction of the tissue with trichloroacetic acid liberates 80–85% of the total flavin.

(14) P. Karrer and T. Korber, *Helv. Chim. Acta*, **18**, 266 (1935).

(15) O. Bessey, O. H. Lowry and R. H. Love, *J. Biol. Chem.*, **180**, 755 (1949).

(16) F. M. Huennekens, H. R. Mahler and E. Dimant, unpublished observations.

(17) We are indebted to Dr. Henry R. Mahler for assaying our preparation in this system. Using Protein E of Negelein and Brömel (*Biochem. Z.*, **300**, 225 (1939)) from pig kidney as a source of the enzyme, a value of the Michaelis constant, K_m, equal to 3.3×10^{-7} M was obtained. This may be compared to the value 2.5×10^{-7} M reported by Warburg and Christian[6,7] for their FAD preparation tested with the corresponding sheep kidney enzyme.

(18) J. L. Crammer, *Nature*, **161**, 349 (1948).

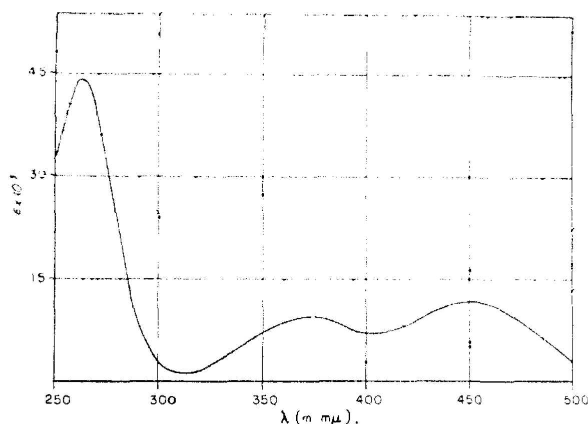

Fig. 1.—Absorption spectrum of FAD.

tides. The ϵ values in Fig. 1 are calculated on the basis that $\epsilon = 11.3 \times 10^3$ at 450 mμ.[6]

From Fig. 1, R is determined to be 3.8 and no sample of FAD was encountered in this investigation where R was lower, unless breakdown to FMN had occurred. From the data of Warburg and Christian,[6] however, R is calculated as 3.3 and it is possible that our best preparations (purity 0.50) still are contaminated by relatively large amounts of material which absorb at 260 mμ. In support of the higher value, however, are the findings of several investigators who have reported spectral data on FAD obtained by deproteinizing purified flavoproteins containing this nucleotide as the prosthetic group, and subsequent purification of the flavin. Thus, Corran, *et al.*,[8] and Gordon, *et al.*,[19] found R values[20] of 3.7 for FAD liberated from milk xanthine oxidase and liver aldehyde oxidase, while from the data of Singer and Kearney[21] it may be calculated that R = 4.0 in the case of the pure L-amino acid oxidase from snake venom. Recently we have been informed by Dr. Henry R. Mahler (private communication) that his preparation of highly purified DPN-cytochrome *c* reductase from beef heart contains a flavin prosthetic group, which, when resolved from the apoenzyme, has an R value of 3.9. Contrary to the above findings, however, Ball[22] has obtained a value of 3.3 for FAD released from xanthine oxidase. In principle this method is capable of giving accurate values for FAD, since purification of the enzyme should eliminate all contaminant nucleotides and leave only the bound prosthetic group. The possible existence of a second prosthetic group in the case of xanthine oxidase and aldehyde oxidase renders the data in these cases less reliable but the L-amino acid oxidase data would seem to be unequivocal since FAD is the only known prosthetic group of this enzyme. Furthermore, it is unlikely that remnants of native protein could survive the purification procedures in sufficient amount to interfere with the R determination.

Finally, the R value of FAD may be calculated as 3.6[23] on the assumption that the light absorption in the dinucleotide is additive for the isoalloxazine moieties. Warburg and Christian[6] considered that their lower experimental value could be explained on the grounds that the absorptions were not additive and this position is apparently strengthened by the finding[14] that at *p*H 7.0 the fluorescence of FAD is only 0.14 of that for FMN or Rb, but that as the *p*H is lowered, the fluorescence of FAD rises until at *p*H 2 it is equivalent to that of the mononucleotide. A possible interpretation of these fluorescence data, suggested by Bessey, *et al.*,[15] is that in FAD the adenine acts as an "internal quencher" of the isoalloxazine nucleus, and that at low *p*H values, this interaction is prevented, perhaps owing to a change in configuration. While this explanation is probably correct, it does not follow from any theoretical considerations that such an interaction should affect the *absorption* of light by the individual absorbing structures.

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(19) A. H. Gordon, D. E. Green and V. Subrahmanyan, *Biochem. J.*, **34**, 764 (1939).

(20) In the two cases cited, the R value is based upon readings at 265 mμ rather than 260 mμ. As seen in Fig. 1, however, the change in absorption at 260 and 265 mμ is only 2–3%.

(21) T. P. Singer and E. B. Kearney, *Arch. Biochem.*, **27**, 348 (1950).

(22) E. G. Ball, *J. Biol. Chem.*, **128**, 51 (1939).

(23) Dr. G. S. Whitby has informed us that pure riboflavin has $\epsilon = 12.2 \times 10^3$ at 450 mμ, in agreement with Singer and Kearney,[21] and $\epsilon = 28.0 \times 10^3$ at 260 mμ. Assuming the same values for FMN and from the known values (H. Kalckar, *ibid.*, **167**, 445 (1949)) of $\epsilon = 15.9 \times 10^3$ for AMP, the calculated R value for FAD, assuming additivity, would be $(28.0 + 15.9)/12.2 = 3.6$.