THE FLUOROMETRIC MEASUREMENT OF THE NUCLEOTIDES OF RIBOFLAVIN AND THEIR CONCENTRATION IN TISSUES

By OTTO A. BESSEY, OLIVER H. LOWRY, AND RUTH H. LOVE

(From The Public Health Research Institute of The City of New York, Inc., New York, the Department of Biological Chemistry, College of Medicine, University of Illinois, Chicago, and the Department of Pharmacology, Washington University School of Medicine, St. Louis)

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Warburg and Christian (1), in describing the preparation of flavin-adenine dinucleotide (FAD), mentioned that whenever dinucleotide solutions developed a greenish fluorescence they were found to have lost their coenzyme activity. On further investigation of this phenomenon it has been found that there occurs a 10-fold increase in fluorescence when FAD is split to riboflavin phosphate (flavin mononucleotide) or to free riboflavin. This change in fluorescence has proved to be a practical means of measuring FAD. Since flavin mononucleotide (FMN) can be distinguished from riboflavin on the basis of its distribution coefficient between benzyl alcohol and aqueous solutions (2), ready analytical means are available for measuring separately FAD, FMN, and riboflavin, the three forms of riboflavin encountered in biological material.

A description is given below for the fluorometric measurement of these three forms of riboflavin. The measurements are considered to be much simpler than the enzymatic procedures which were the available methods hitherto. Data are given for the fluorescent behavior of FAD, FMN, and riboflavin, the stability of FAD, and the concentration of each of these three forms of riboflavin in normal and riboflavin-deficient tissues of the rat.

Measurement of Riboflavin, FMN, and FAD in Tissues

Extraction of Flavin Compounds—The fresh tissue sample is ground in a mortar or blended (in a Waring blendor) with 25 to 50 times its volume of ice water. The cold suspension, or an aliquot, is mixed immediately with an equal volume of ice-cold 20 per cent trichloroacetic acid (final concentration 10 per cent). After 15 minutes, the sample is centrifuged and an aliquot of the extract is neutralized at once with one-fourth its volume of 4 M K_2HPO_4. Until neutralized, the sample is kept as cold as possible to prevent hydrolysis of FAD (Fig. 1). A second aliquot of the suspension is stored in the dark at 38° overnight, or at room temperature for 2 days, to produce complete hydrolysis of FAD to FMN. After

hydrolysis, this sample is neutralized in exactly the same manner as the initial sample. Considerable care is taken with both samples after neutralization to prevent undue exposure to light, since both riboflavin and FMN are much more sensitive to destruction by light in the presence of this high salt concentration than they are in dilute salt solutions. The high tissue dilution (50- to 100-fold) is necessary to effect complete flavin extraction. A lower dilution (10-fold) results in only 80 to 90 per cent recovery of the flavins.

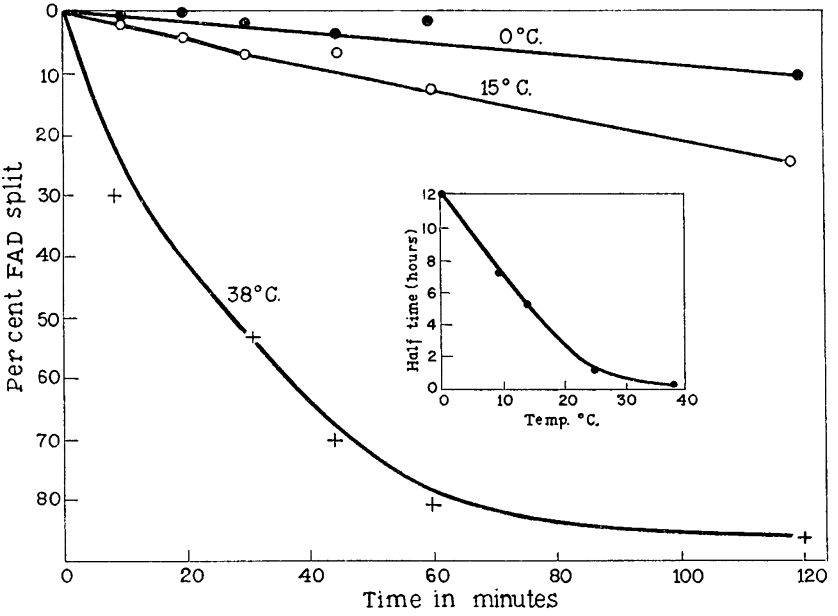

FIG. 1. Rate of splitting of FAD by 10 per cent trichloroacetic acid at different temperatures.

Measurement of FAD—The fluorescence of the two neutralized extracts is determined in the usual manner (3). An aliquot of suitable volume is measured into a fluorometer cuvette and readings are made: (*a*) initially $= F_1$; (*b*), after the addition of a standard amount of riboflavin approximately equal to that present, $= F_2$; and (*c*), after reduction with 1 per cent of the volume of 10 per cent sodium hydrosulfite in 5 per cent $NaHCO_3$, $= F_3$.

The second and third readings are corrected for the dilution of the sample with standard and reducing agents to give F_2' and F_3'. The apparent riboflavin in the aliquot is calculated as follows: Apparent riboflavin = standard riboflavin $\times (F_1 - F_3')/(F_2' - F_1)$.

In aqueous solutions, FAD is only 9 per cent as fluorescent as riboflavin

(see below). However, under the conditions of these measurements (high salt concentration) FAD (calculated as riboflavin) has a fluorescence equal to 15 per cent of riboflavin, whereas FMN (calculated as riboflavin) and riboflavin are equal in fluorescence. Therefore, if the apparent riboflavin of the initial sample is R_i and the apparent riboflavin of the hydrolyzed sample is R_t, FAD = $(R_t - R_i)/0.85$. The balance of the flavin consists of FMN plus free riboflavin. Total riboflavin (R_t) = FAD + non-FAD riboflavin (FMN + free riboflavin).

Measurement of Free Riboflavin—Since, as is shown below, there is ordinarily very little free riboflavin in tissues, *i.e.* non-FAD flavin = FMN, the above measurements will suffice for most purposes. If it is, however, desirable to distinguish between FMN and riboflavin, the procedure is as follows: An aliquot of the initial (non-hydrolyzed) neutralized sample

TABLE I

Partition Coefficients for Riboflavin and Derivatives between Benzyl Alcohol and Various Aqueous Solutions

Ratios of benzyl alcohol to aqueous layer (25–28°).

Aqueous layer	Riboflavin	FMN	FAD
0.008 M phosphate buffer, pH 6.8	3.3	0.010	0.004
4 volumes 10% CCl_3COOH + 1 volume 4 M K_2HPO_4 (final pH = 6.6)	4.1	0.032	0.020
1.5 M $(NH_4)_2SO_4$ in 2.5% CCl_3COOH and 0.45 M K_2HPO_4 (final pH 6.6)	24.0	0.082	0.111

is thoroughly shaken with an equal volume of water-saturated, redistilled, C.P. benzyl alcohol, in a glass-stoppered centrifuge tube. An aliquot of the benzyl alcohol layer is diluted in a fluorometer cuvette with 2 or more volumes of 45 per cent ethyl alcohol, which is 0.05 M in both sodium acetate and acetic acid. The apparent riboflavin in this solution is measured in this sample exactly as before (R_{Bz}). The partition coefficients for riboflavin, FMN, and FAD between benzyl alcohol and 10 per cent trichloroacetic acid, neutralized as described, are 4.1, 0.032, and 0.020 respectively (Table I). Since FAD has a fluorescence approximately 60 per cent as large as free riboflavin, when present in a mixture of benzyl alcohol and 45 per cent alcohol, the apparent riboflavin in the benzyl alcohol extract (R_{Bz}) = 4.1/5.1 free riboflavin + 0.032/1.032 FMN + 0.02/1.02 × 0.6 FAD. By letting R non-FAD = FMN + free riboflavin, and solving, free riboflavin = 1.30 R_{Bz} − 0.040 R non-FAD − 0.015 FAD.

The concentration of free riboflavin is so low in normal tissues that a special fluorometer is required to measure the fluorescence (4). In addition, it is desirable to avoid the fluorescence of even redistilled benzyl

alcohol by driving the extracted riboflavin back into an aqueous solution. This is accomplished by shaking 1 volume of the benzyl alcohol extract in a glass-stoppered vessel with 15 volumes of toluene and 1 volume of water which is 0.05 M in both sodium acetate and acetic acid. All of the flavins extracted by the benzyl alcohol are quantitatively driven into the aqueous layer; hence, the calculation is made as described above, except that the FAD correction term is changed from -0.015 to -0.003 because of the lesser fluorescence of FAD in water. The benzyl alcohol and toluene are prepared before use by shaking with large volumes of water to remove possible traces of water-soluble fluorescent materials.

The above analytical procedures would appear to be applicable, with appropriate modifications, to materials other than animal tissues.[1]

Properties of Flavin Nucleotides

FAD—The fluorescence of the FAD, used as originally prepared,[2] increased about 6-fold on hydrolysis. It was thought possible that even the small initial fluorescence might be due to contamination with free riboflavin or FMN. A sample of FAD was accordingly submitted to a counter-current extraction process (6) (with individual glass tubes) between 2 M ammonium sulfate in 0.02 N NH_4OH and water-saturated benzyl alcohol. After a total of eighteen plates, the maximum FAD was found in the fourth tube. The initial fluorescence values, expressed as the per cent of the fluorescence after hydrolysis in the third, fourth, and fifth tubes, were 9.2, 9.2, and 8.9 respectively. It, therefore, seems strongly indicated that pure FAD in water at a neutral pH is about 9 per cent as fluorescent as free riboflavin. It is, of course, barely possible that part of this fluorescence is still due to the presence of an impurity.

It is rather unexpected that FAD should be less fluorescent than riboflavin or FMN, since it seems likely that the FMN and adenylic acid moieties are joined through their respective phosphate groups, *i.e.* at a

[1] Other means of hydrolyzing FAD may be more expedient in certain cases. Complete hydrolysis may be effected by heating for 10 minutes at 100° in 5 or 10 per cent trichloroacetic acid or in 0.1 N HCl. The light must of course be kept very dim while the sample is hot to avoid destruction of riboflavin. An alternative method of hydrolysis is to add FAD-splitting enzyme from potato to the neutralized sample (Lowry, O. H., Bessey, O. A., and Love, R. H., in preparation, and Kornberg (5)). In this case the concentration of trichloroacetate should be kept as low as possible to minimize inhibition of the enzyme. With low substrate concentration the velocity of reaction increases with increasing acidity to a pH of about 4. An advantage of the enzymatic splitting is that a single sample may be used to measure both FAD and total riboflavin.

[2] The FAD used was prepared from yeast by the method of Warburg and Christian (1) to a purity of about 60 per cent, judging from its riboflavin content. The absorption spectrum (peaks at 452, 375, and 265 mμ) was in agreement with this purity.

point quite remote from the fluorescent isoalloxazine nucleus. If the phosphate groups are the point of union, the limited fluorescence of FAD would indicate a second linkage between the isoalloxazine nucleus and some group of the adenylic acid.

Evidence that a loose linkage, possibly electrovalent, may exist is furnished by the behavior of FAD at different pH values and in different solvents. As the pH of an aqueous solution of FAD is decreased, the fluorescence increases reversibly to a maximum at pH 2.9 and then decreases in parallel with riboflavin itself. The fluorescence *relative* to

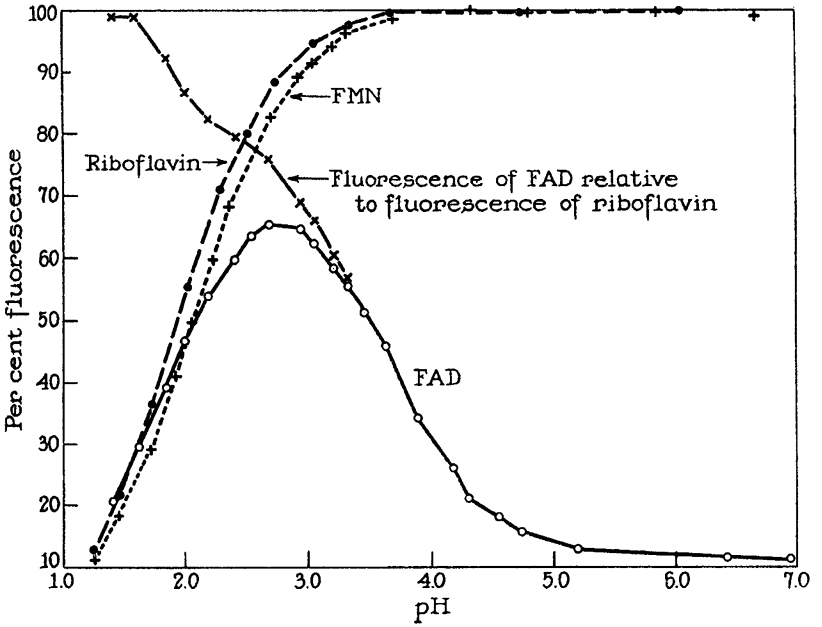

FIG. 2. Influence of pH on fluorescence of riboflavin, flavin mononucleotide, and flavin-adenine dinucleotide (FAD).

riboflavin increases steadily until the two compounds are equally fluorescent at about pH 1.5 (Fig. 2). This behavior would be compatible with a salt linkage between a positively charged group on one-half of the molecule and a negatively charged group with a pK_a of about 3.5 on the other part. The data fit this interpretation quantitatively only in the less acid part of the pH range (Fig. 2). The behavior of FAD in organic solvents is consistent with the above interpretation (Table II). Various combinations of alcohol and benzyl alcohol increase the fluorescence of FAD in the neutral range to more than 50 per cent of that of riboflavin. It is well known that lowering the dielectric constant of the medium decreases

the dissociation constant of those acids which become negatively charged on ionization (7). (The pH effect on the fluorescence, of course, does not prove the presence of an electrovalent bond, since secondary bonding of other sorts might be similarly affected.[3])

Weil-Malherbe (8) has reported that various purines are able to quench the fluorescence of a number of compounds, including riboflavin. Accordingly, the fluorescence of riboflavin and its derivatives was measured in the presence of adenosine and yeast or muscle adenylic acid. Quenching was, in fact, observed (Table III). Adenine (not shown) had a similar effect. The quenching could be reversed by lowering the pH, and this pH effect was quantitatively equal to that found for FAD (Fig. 2). It seems likely, therefore, that adenylic acid is responsible for the inhibition

TABLE II

Fluorescence of Flavin-Adenine Dinucleotide in Various Solvents

The fluorescence is expressed as per cent of fluorescence of free riboflavin in the same solvent. Ph, 0.01 M phosphate buffer, pH 6.8; Et, ethyl alcohol; Ac, 0.1 M acetate buffer, pH 4.6; and Bz, benzyl alcohol.

Solvent	Fluorescence	Solvent	Fluorescence
4 M $(NH_4)_2SO_4$	6		
100% Ph	9	55% Ac, 45% Et	40
85% " 15% Et	22	43% " 45% " 12% Bz	66
75% " 25% "	31	33% " 45% " 22% "	70
55% " 45% "	42	43% Ph, 45% " 12% "	52
5% " 95% "	48	33% " 45% " 22% "	64

of fluorescence in FAD. Although the absolute concentration of adenylic acid is far too low in a dilute FAD solution to explain the quenching, the local "concentration" of adenylic acid in the neighborhood of the riboflavin portion of the FAD molecule would quite probably be high enough to produce the phenomenon. It may be of interest to note that the presence of a high muscle adenylic acid concentration did not permit FMN to substitute for FAD as a coenzyme for D-amino acid oxidase.

The reversible increase in fluorescence of FAD at pH 2 to 3 could be put to analytical use, but it has been found more convenient, in general, to hydrolyze FAD before measurement, as described above.

Fig. 1 records the rate of hydrolysis of FAD at different temperatures

[3] Dr. C. W. Sondern of the White Laboratories, Inc., Newark, New Jersey, kindly furnished us with a synthetic compound which is believed to be diriboflavin diphosphate with a pyrophosphate linkage, as in FAD. This compound showed a 50 per cent increase in fluorescence after mild hydrolysis, which suggests a similar secondary linkage.

in 10 per cent trichloroacetic acid (pH 0.67). These data are useful for the preparation of tissue extracts by serving as a guide to permissible times of exposure of the samples to acid, if significant splitting of FAD is to be avoided. They also indicate the necessary time required at higher temperatures for complete hydrolysis. At 90°, in 0.075 N HCl, FAD is 50 per cent hydrolyzed in 2.2 minutes. Abraham (9) measured the liberation of adenylic acid under nearly comparable conditions and observed a similar rate of hydrolysis. FAD is hydrolyzed approximately 10 times faster than diphosphopyridine nucleotide under the same conditions (10). The instability of FAD is further indicated by the fact that adsorption on Florisil at a neutral pH followed by elution with organic solvents results in nearly complete conversion to FMN.

TABLE III

Quenching of Fluorescence of Riboflavin and Its Derivatives by Adenosine and Adenylic Acid

The values are reported as per cent of fluorescence in 0.008 M phosphate buffer, pH 6.8.

Medium	Riboflavin	FMN	FAD
Adenylic acid,* 0.03 M, pH 6.8	38	49	66
" " 0.15 " " 6.8	20	14	36
Adenosine, 0.04 " " 6.8	28	28	45
" 0.08 " " 6.8	12†	17	31

* Yeast adenylic acid; muscle adenylic acid also quenched riboflavin fluorescence.
† This rose to 25 per cent at pH 3.5 and 44 per cent at pH 2.9.

The rate of increase of fluorescence of a trichloroacetic acid extract of rat liver held at 38° was found to be the same as for a similar solution of purified FAD from yeast (Fig. 3). This is strong evidence for the validity of the proposed method for FAD. Since both the liver extract and purified FAD increased in fluorescence according to a single monomolecular curve (Fig. 3), it would appear that a single molecular species is concerned in both instances.

One further property of FAD which is of some interest is its relative insensitivity to light. Under comparable conditions of exposure to ultraviolet light of wave-length 365 mμ, riboflavin and FMN are destroyed 20 times faster than FAD.

FMN—On mild hydrolysis of FAD, the fluorescence increases to a maximum with the liberation of little or no free riboflavin, as judged by the failure of benzyl alcohol to extract more than small amounts of fluorescent material. The fluorescent compound liberated is presumably FMN. Abraham (9) has shown that the product of mild acid hydrolysis is active as a

coenzyme for the "old yellow enzyme" and is, therefore, probably FMN. It will also be shown below that the hydrolytic product has the same partition coefficient as the "FMN" of tissues. More drastic hydrolysis (autoclaving in 0.1 N HCl for 15 minutes at 15 pounds pressure) induces little further change. Kuhn and Rudy (11) also noted that FMN is but slowly hydrolyzed by acid. Prolonged autoclaving with higher acid concentration results in only partial hydrolysis of FMN with accompanying destruction of part of the riboflavin. Treatment with acid phosphatase (clarase) liberates free riboflavin with no change in fluorescence. Thus, FMN has

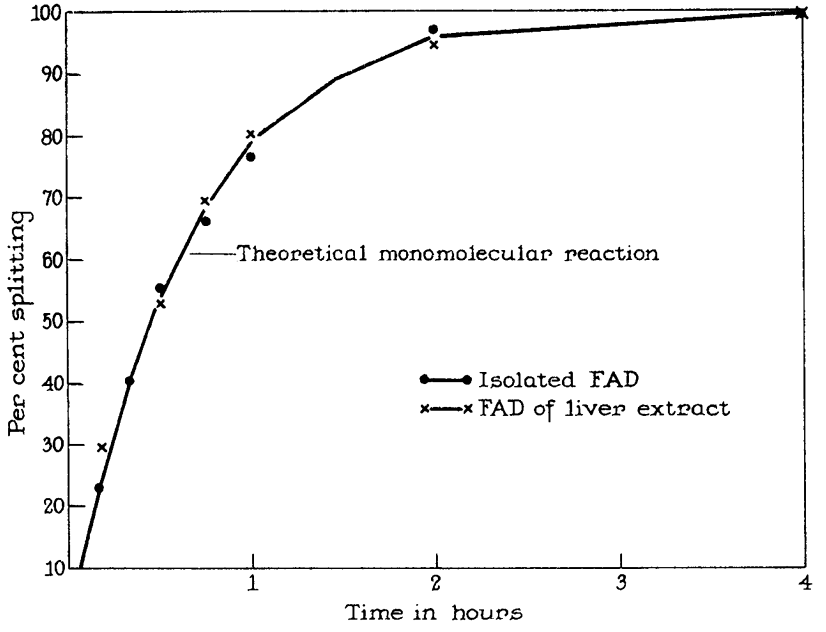

FIG. 3. Splitting of isolated FAD and FAD present in an extract of rat liver by 10 per cent trichloroacetic acid at 38°.

the same fluorescence as riboflavin. There is, however, a slight difference in the effect of acid on the fluorescence of FMN and of free riboflavin (Fig. 2). The phosphorylated compound loses its fluorescence at a slightly higher pH than does riboflavin. Thus, at pH 2 riboflavin is 18 per cent more fluorescent than FMN. The loss of fluorescence of both compounds fits a titration curve almost perfectly. Assuming, therefore, that this change represents the conversion of a single group to an acid, this group has a pK_a of 1.97 in riboflavin and a pK_a of 2.17 in FMN. (Kuhn and Moruzzi (12) estimated a pK_a of about 1.7 for riboflavin from the effect of pH on its fluorescence.)

Partition Coefficients of Flavins—Emmerie (2) used the difference between the benzyl alcohol-water partition coefficients of riboflavin and FMN to separate these two compounds. The differences in the distribution of the flavin compounds between aqueous solutions and benzyl alcohol are useful for both analytical and characterization purposes (Table I). FMN is more readily extracted than FAD from dilute salt solutions, whereas the reverse is true from strong ammonium sulfate solutions.

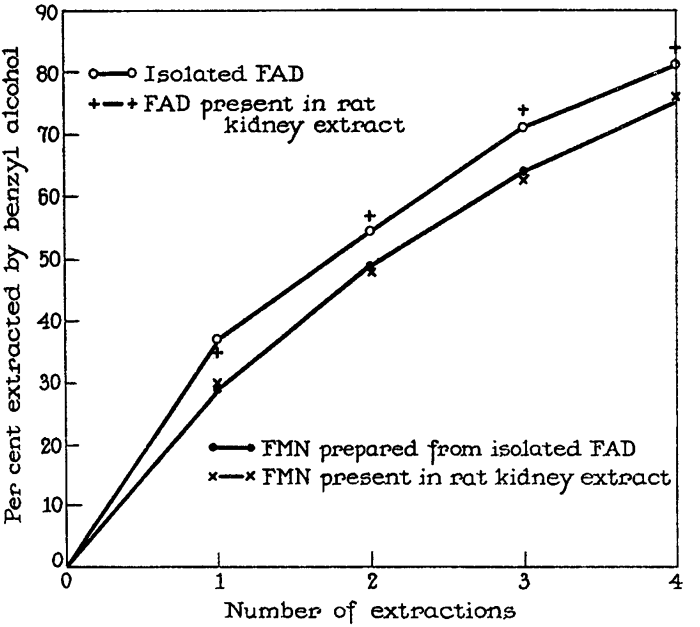

FIG. 4. Extraction of FMN and FAD by benzyl alcohol from extracts of rat kidney. A 10 per cent trichloroacetic acid extract of rat kidney was neutralized with K_2HPO_4, made 1.5 M in $(NH_4)_2SO_4$, and extracted four times with 5 volumes of benzyl alcohol.

A neutralized trichloroacetic acid extract of rat kidney was brought to a concentration of 1.5 M in ammonium sulfate and extracted successively with 5-fold volumes of benzyl alcohol, and the FMN and FAD left behind were measured by the proposed method. The extraction curves (Fig. 4) concur within experimental error with similar curves found for purified yeast FAD and for FMN obtained by mild hydrolysis from this yeast FAD. These data indicate the identity of the rat kidney flavins with the purified compounds and suggest that no significant amounts of riboflavin derivatives other than these two exist in rat kidney. These data may also be regarded as further proof for the validity of the proposed analytical procedure.

Stability of FAD in Tissues and Tissue Extracts—In measuring the FAD in tissues there are two possibilities of loss other than incomplete extraction, *viz.* enzymatic splitting of FAD prior to extraction and acid splitting during extraction. There exists an enzyme or enzymes in at least kidney and liver capable of splitting FAD (13). This enzymatic effect is apparently less marked in the intact tissue than in tissue mince (Table IV). The data indicate that even kidney mince, which is quite active in this respect, may safely be allowed to stand for 30 minutes at 10°. There is a greater danger of hydrolysis after the addition of 10 per cent trichloroacetic acid (Table IV; Fig. 1). To prevent serious loss it appears that the acid extracts should be neutralized within 60 minutes at 0°, 30 minutes at 10°, or 15 minutes at 15°.

Comparison of Coenzymatic and Fluorometric Assay for FAD—FAD was measured in a number of tissues by its function as a coenzyme for D-amino

TABLE IV

Stability of FAD in Rat Kidney and Kidney Extracts

	Temperature	FAD remaining		
	°C.	*15 min. per cent of initial*	*30 min. per cent of initial*	*60 min. per cent of initial*
Intact kidney	38		99	
Kidney blended with water	10	100	102	95
" " " "	38	66	59	40
" 1:50 extract in 10% CCl_3COOH	15	93	87	84

acid oxidase. The apoenzyme was prepared from pig kidneys by essentially the method described by Warburg and Christian (1).

The assay was conducted in 0.05 N pyrophosphate buffer at pH 8.3, with 3 mg. of DL-alanine per ml. as substrate and fluorometrically assayed yeast FAD of 60 per cent purity as a standard. The concentration of FAD required for half activity was found to be 0.155 γ per ml. as compared to 0.196 γ per ml. reported by Warburg and Christian (1) for enzyme from sheep kidney. The tissues were blended in ice water and then heated at 100° for 5 minutes to liberate the FAD. Aliquots of the supernatant fluids after centrifuging were assayed for their FAD content by the enzymatic procedure and the values found were compared with the values obtained fluorometrically on the same extracts (Table V). The data obtained by the two procedures are seen to concur within experimental limits. Similar comparative analyses were made on a sample of FAD which was heated for different lengths of time in 0.075 N HCl at 90°. The decrease in FAD as measured fluorometrically was found to be accompanied by a correspond-

ing fall in coenzymatic activity, thus further demonstrating the relationship between potential fluorescence and FAD activity.

TABLE V

Comparison of Values for FAD Obtained by Fluorometric and Enzymatic Methods

The values are based on wet tissue weight.

	Fluorometric		As coenzyme*
	Free riboflavin plus FMN	FAD (as riboflavin)	FAD (as riboflavin)
	γ per gm.	*γ per gm.*	*γ per gm.*
Liver 1	7.0	28.3	28.5
" 2	5.7	36.8	34.9
Kidney 1	21.8	19.2	21.8
" 2	17.0	16.4	13.8
Heart 1	1.5	18.1	17.6
" 2	2.4	21.6	18.8
Average		23.4	22.6

* For D-amino acid oxidase.

TABLE VI

Recovery of Flavins Added to Minced Liver

Substance	Initial	Addition	Found	Calculated	Recovery
	γ per gm.	*γ per gm.*	*γ per gm.*	*γ per gm.*	*per cent*
Riboflavin	0.6	10.3	10.9	10.9	100
"	0.6	20.3	20.0	20.6	97
Average					98
FMN	6.0	14.7	18.1	20.7	82
"	6.0	14.7	19.3	20.7	91
"	7.9	15.0	25.1	22.9	115
"	7.9	15.0	25.4	22.9	117
"	13.5	31.4	43.9	44.9	97
Average					100

Substance	Initial	Addition	Found	Calculated	Recovery
	γ per gm.	*γ per gm.*	*γ per gm.*	*γ per gm.*	*per cent*
FAD	31.6	38.8	67.8	70.4	93
"	31.6	38.8	66.2	70.4	91
"	31.6	38.8	72.0	70.4	104
"	33.3	29.6	59.7	62.9	89
"	33.3	29.6	55.0	62.9	73*
"	29.9	39.0	69.2	68.9	101
"	29.9	39.0	64.5	68.9	89
Average					95
Riboflavin	0.6	9.9	12.6	10.5	121
FMN	4.9	14.6	20.7	19.5	108
FAD	29.6	73.5	109.0	103.1	108
Total	35.1	98.0	142.3	133.1	108

* Omitted from the average.

Recovery of Flavins Added to Tissues—Riboflavin, FMN, and FAD were added to the cold minced tissues before extraction with trichloroacetic acid. The recovery of the separate flavins averaged 95 to 100 per cent (Table

VI). Recovery was, however, less complete (85 per cent) if the final dilution was only 1:10 instead of 1:100.

TABLE VII

Three Riboflavin Fractions of Five Tissues of Control and Riboflavin-Deficient Rats

The values are calculated as riboflavin on the basis of wet tissue weight.

	Control					Riboflavin-deficient						
	Total riboflavin	FAD	FMN	Free riboflavin	FAD	Total riboflavin	FAD	FMN	Free riboflavin	FAD		FMN
	γ per gm.	γ per gm.	γ per gm.	γ per cent	per cent of total	γ per gm.	γ per gm.	γ per gm.	γ per gm.	per cent of total	per cent of average control	per cent of average control
Liver	39.8	32.3	6.6	0.90	81	11.8	10.9	0.7	0.24	92	36	11
	40.4	34.0	5.8	0.62	84	13.3	12.0	1.1	0.24	90	39	18
	31.4	26.6	4.8*		71	9.7	8.8	0.5	0.36	91	29	8
	33.4	24.2	9.2		72							
	40.5	35.2	5.3		87							
	37.1†	**30.5**	**6.2**	**0.76**	**79**	**11.6**	**10.6**	**0.8**	**0.28**	**91**	**35**	**12**
Kidney	38.2	23.2	14.0	1.02	61	16.2	12.6	3.3	0.28	78	53	28
	41.4	26.2	14.4	0.82	63	15.2	12.9	2.1	0.19	85	55	18
	40.5	32.4	7.2	0.92	80	18.4	16.9	1.1	0.38	92	72	9
	27.2	18.1	9.1*		66							
	26.5	18.3	8.2		69							
	34.8	**23.6**	**11.9**	**0.92**	**68**	**16.6**	**14.1**	**2.2**	**0.28**	**85**	**60**	**18**
Heart	21.6	19.5	1.9	0.19	90	7.8	6.8	1.0	0.07	86	44	50
	21.6	19.5	2.0	0.11	90	8.6	7.7	0.9	0.07	89	50	45
	21.5	17.7	3.8*		82	9.3	8.0	1.2	0.05	86	52	60
	24.4	20.6	3.8		84							
	19.3	13.8	5.5		72							
	21.7	**18.2**	**2.0**	**0.15**	**84**	**8.6**	**7.5**	**1.0**	**0.06**	**87**	**49**	**51**
Brain	2.95	2.19	0.62	0.14	74	2.04	1.56	0.40	0.08	76	63	58
	3.25	2.37	0.76	0.12	73	2.24	1.73	0.44	0.07	77	70	64
	3.47	2.62	0.74	0.11	75	1.89	1.46	0.37	0.06	77	59	54
	3.69	2.69			73							
	3.34	**2.47**	**0.71**	**0.12**	**74**	**2.06**	**1.58**	**0.40**	**0.07**	**77**	**64**	**58**
Skeletal muscle	3.56	3.13	0.43	0.00	88	0.86	0.74	0.15	−0.02	85	22	38
	3.48	3.10	0.33	0.05	89	1.24	1.08	0.09	0.07	87	32	23
	4.47	3.99	0.44	0.04	89	0.78	0.64	0.05	0.08	83	19	13
	3.84	**3.41**	**0.40**	**0.04**	**89**	**0.96**	**0.82**	**0.10**	**0.04**	**85**	**24**	**25**

* Calculated value.

† Averages.

Concentration of Individual Flavins in Normal and Deficient Tissues—The concentration of riboflavin, FMN, and FAD was measured in five major tissues of normal and riboflavin-deficient rats. The deficient rats

had been maintained for several months on purified diets containing about 5 γ of riboflavin per day. The control animals were young adults on a Purina dog chow diet. The FAD was found to represent the largest frac-

TABLE VIII

FAD and Non-FAD Riboflavin Fractions of Lung, Thymus, Submaxillary Gland, and Adrenals of Control and Riboflavin-Deficient Rats

The values are calculated as riboflavin on the basis of wet tissue weight.

	Control					Riboflavin-deficient						
	Rat No.	Total riboflavin	FAD	FMN + free riboflavin	FAD	Rat No.	Total riboflavin	FAD	FMN + free riboflavin	FAD		FMN + free riboflavin
		γ per gm.	γ per gm.	γ per gm.	per cent of total		γ per gm.	γ per gm.	γ per gm.	per cent of total	per cent of average control	per cent of average control
Lung	1	3.76	3.10	0.66	82	5	3.26	2.52	0.74	77	69	118
	2	4.22	3.66	0.56	87	6	4.24	3.76	0.48	89	103	76
	3	4.80	4.29	0.51	89	7	5.12	4.57	0.55	89	126	88
	4	4.29	3.49	0.80	81	8	3.41	3.05	0.36	89	84	57
		4.27*	**3.64**	**0.63**	**85**		**4.01**	**3.48**	**0.53**	**86**	**96**	**84**
Thymus	1	2.76	2.12	0.64	77	5	1.86	1.41	0.45	76	70	78
	2	2.47	2.11	0.36	85	6	1.60	1.37	0.23	86	68	40
	3	2.47	1.87	0.60	76	7	2.01	1.56	0.45	78	78	78
	4	2.67	1.95	0.72	73	8	2.41	1.68	0.73	70	84	126
		2.59	**2.01**	**0.58**	**78**		**1.97**	**1.51**	**0.46**	**78**	**75**	**79**
Submaxillary gland	1	6.30	5.12	1.18	81	5	4.65	3.12	1.53	67	67	137
	2	5.59	4.85	0.74	87	6	6.75	4.65	2.10	69	100	187
	3	4.94	3.67	1.27	74	7	4.98	3.93	1.05	79	85	94
	4	6.24	4.93	1.31	79	8	4.52	3.30	1.22	73	71	109
		5.77	**4.65**	**1.12**	**80**		**5.22**	**3.74**	**1.48**	**72**	**80**	**132**
Adrenals	1	19.9	17.6	2.3	88	5	19.3	15.1	4.2	78	83	105
	2	20.1	16.2	3.9	81	6	15.8	15.9	−0.1	101	87	
	3	25.0	20.6	4.4	82	8	17.3					
	4	24.2	18.9	5.3	78		**17.5**	**15.4**		**90**	**85**	
		22.3	**18.3**	**4.0**	**82**							
Liver	1	38.6	36.7	1.9	95	5	13.7	11.7	2.0	85	37	69
	2	34.5	32.7	1.6	95	6	18.9	16.8	2.1	89	53	72
	3	31.1	25.8	5.3	83	7	15.3	13.1	2.2	86	42	76
		34.7	**31.8**	**2.9**	**92**		**16.0**	**13.9**	**2.1**	**87**	**44**	**72**

* Averages.

tion in all of the tissues (Table VII). The percentage in the normal tissues varied from nearly 90 per cent of the total in skeletal muscle to less than 70 per cent in kidney. The balance was found to be chiefly FMN. The relatively large quantity of FMN in kidney is of interest and is quite surely

not an artifact, since preparation of extracts with the greatest haste, after killing the animal, did not decrease the percentage of FMN found. The free riboflavin was observed to be consistently low, too low in several of the tissues for accurate measurement by the technique available. The kidney had the highest absolute amount (0.7 γ per gm.) and the brain the highest relative concentration (3 per cent of the total).

In riboflavin deficiency all three fractions decreased. In liver and kidney the greatest fall was found for FMN. In heart, brain, and skeletal muscle, in contrast, both FAD and FMN decreased to about the same relative extent. It is perhaps unwise to speak too definitely about the FMN

TABLE IX

FAD and Non-FAD Riboflavin Fractions of Miscellaneous Tissues (Single Values)

The values are calculated as riboflavin on the basis of wet tissue weight.

Tissue	Control				Riboflavin-deficient rat					
	Total riboflavin	FAD	FMN + free riboflavin	FAD	Total riboflavin	FAD	FMN + free riboflavin	FAD	FAD	FMN + free riboflavin
	γ per gm.	γ per gm.	γ per gm.	per cent of total	γ per gm.	γ per gm.	γ per gm.	per cent of total	per cent of control	per cent of control
Gastric mucosa	10.9	9.3	1.6	86	5.27	4.67	0.60	89	50	38
Pancreas	8.14	6.82	1.32	84	5.67	5.28	0.39	93	77	30
Ovary	7.52	5.95	1.57	79	6.11	5.15	0.96	83	87	61
Thyroid	4.56	2.90	1.66	64	3.38	2.43	0.95	72	84	57
Spleen	3.90	3.38	0.52	87	2.63	2.35	0.28	89	69	54
Pituitary	3.68	2.62	1.06	68	2.51	1.20	1.31	48	46	128
Testes	3.37	2.45	0.92	73						
Lymph node	2.86	2.18	0.68	76	1.83	1.46	0.37	80	67	54
Uterus	2.34	1.82	0.52	78	1.99	1.81	0.18	91	99	34
Bladder	2.31	1.59	0.72	69	1.36	1.12	0.24	82	70	33
Cornea	1.12	1.00	0.12	89	0.85	0.66	0.18	78	66	150
Skin	1.00	0.76	0.24	76	0.61	0.55	0.07	89	73	68

concentration from these few data, since the values have not been found to be very consistent. Contrast, for example, the FMN data for liver found in Table VIII with those in Table VII. Unknown age or environmental factors may influence the concentration of this flavin.

Except for brain, the above five tissues are characterized by a marked drop in total riboflavin concentration in riboflavin deficiency. Not all tissues are so severely affected. Lung, thymus, submaxillary, and adrenal glands are presented as typical of tissues or organs in which deficiency produces only minor changes (Table VIII). FAD comprised about 80 per cent of the total riboflavin in these tissues and deficiency was without dramatic effect on this percentage.

Single values were obtained purely for orientation purposes on twelve other normal and deficient rat tissues (Table IX). No striking percentage of FAD or change in deficiency was noted. Until these data are obtained with more animals, they must be interpreted with caution.

SUMMARY

1. A convenient fluorometric procedure is described which permits separate measurement of riboflavin, riboflavin monophosphate, and riboflavin dinucleotide. The measurement of the dinucleotide is based on the fact that it has much less fluorescence than riboflavin, and that it can be readily converted into riboflavin monophosphate which exhibits the same fluorescence as riboflavin. Riboflavin is distinguished from its two derivatives by its very much greater partition coefficient between benzyl alcohol and water.

2. Data are presented concerning some of the properties of riboflavin dinucleotide, particularly its rate of hydrolysis in acid and the influence of pH and solvent on its fluorescence.

3. Evidence is given that riboflavin monophosphate and dinucleotide account for practically all of the riboflavin of rat kidney.

4. The riboflavin dinucleotide content of liver, kidney, and heart was measured both fluorometrically and enzymatically with concurring results.

5. The data are given for the concentration of the three riboflavin fractions in five major rat tissues, both normal and riboflavin-deficient, and for the total riboflavin and dinucleotide concentrations in sixteen others. Free riboflavin was found to be present in quantitatively insignificant amounts. The dinucleotide accounted in general for 70 to 90 per cent of the total riboflavin.

BIBLIOGRAPHY

1. Warburg, O., and Christian, W., *Biochem. Z.*, **298,** 150 (1938).
2. Emmerie, A., *Rec. trav. chim. Pays-Bas*, **58,** 290 (1939).
3. Burch, H. B., Bessey, O. A., and Lowry, O. H., *J. Biol. Chem.*, **175,** 457 (1948).
4. Lowry, O. H., *J. Biol. Chem.*, **173,** 677 (1948).
5. Kornberg, A., *J. Biol. Chem.*, **174,** 1051 (1948).
6. Craig, L. C., *J. Biol. Chem.*, **155,** 519 (1944).
7. Linderstrøm-Lang, K., *Compt.-rend. trav. Lab. Carlsberg*, **17,** No. 4 (1927).
8. Weil-Malherbe, H., *Biochem. J.*, **40,** 363 (1946).
9. Abraham, E. P., *Biochem. J.*, **33,** 543 (1939).
10. Warburg, O., and Christian, W., *Biochem. Z.*, **274,** 112 (1934).
11. Kuhn, R., and Rudy, H., *Z. physiol. Chem.*, **239,** 47 (1936).
12. Kuhn, R., and Moruzzi, G., *Ber. chem. Ges.*, **67,** 888 (1934).
13. Ochoa, S., and Rossiter, R. J., *Biochem. J.*, **33,** 2008 (1939).