OXIDATIVE DECOMPOSITION OF RIBOFLAVIN

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It has been believed that riboflavin is comparatively stable to oxidizing agents such as potassium permanganate ($KMnO_4$), hydrogen peroxide, bromine, nitric acid, etc. But its ribityl side-chain is oxidized with ease by lead tetra acetate ($PbAc_4$) and periodic acid, (HIO_4), and this has been employed in the determination of the structure of this side-chain[1], though sufficient investigations have not yet been made on the oxidation products of the isoalloxazine nucleus. $KMnO_4$ destroys the colored or other fluorescent substances such into colorless or non-fluorescent substances, and in colorimetric or fluorimetric determinations, is frequently applied in removing the other colored or other fluorescent substances. Regarding this destruction of riboflavin by $KMnO_4$, Sakurai *et al.*[2] considered the decrease in riboflavin to be due not directly to the $KMnO_4$ but to adsorption of riboflavin by MnO_2 produced from the potassium permanganate, while recently Fujita[3] also noted this destruction of riboflavin though he reported no details of it.

Our investigations so far have been directed to studies on the various decomposition products of riboflavin with the ultimate aim of clarifying their biochemical significances, but we decided to investigate too the oxidative decomposition of riboflavin with a similar purpose, as well as to obtain information regarding the method of assay of this vitamin. Further, it was believed that the decomposition mechanism of riboflavin by HIO_4 and $PbAc_4$ has something in common with the photochemical reaction and reactive products, and that a study of such will be a subject of interest.

EXPERIMENTS

1) Decomposition of riboflavin by KMO_4

i. Materials

B_2: a) U.S.P. reference standard riboflavin
b) Roche riboflavin, regular type, purity 99.0%
c) Roche riboflavin, regular type, recrystallized 2 times with hot water, purity 99.5%

FMN: a) Roche product, riboflavin content 72.0%
b) Roche product, riboflavin content 71.5%

FAD: Purity 85%

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$KMnO_4$: J.I.S. special grade reagent, used as a 4% solution
HAc: J.I.S. special grade reagent
H_2O_2: 33%, J.I.S. special grade reagent

ii. Apparatus

Beckmann's spectrophotometer Model DU
Klett fluorimeter

iii. Method

1) Riboflavin solution—500 γ/ccm solution prepared by dissolving with heat riboflavin in a mineral free double distilled water prepared with a pyrex apparatus.

2) Oxidation—Into an amber glass test tube with a glass stopper a mixture of 2 ccm each of 4% $KMnO_4$ solution and HAc was placed and after dipping in a thermostat for about 30 minutes, 0.8 ccm of a riboflavin solution kept in the same thermostat, was rapidly added and mixed. After a definite interval of time 0.3 ccm of 33% H_2O_2 was rapidly added and mixed. Water was then added to make the total volume 20 ccm (Method A). b) An amber glass test tube with a glass stopper containing 11 ccm of riboflavin solution (10 γ/ccm) and 1 ccm of HAc, was dipped into a thermostat, and after about 30 minutes, 0.5 ccm of a 4% $KMnO_4$ solution kept at the same temperature was rapidly added and mixed. After a definite interval of time 0.5 ccm of 3% H_2O_2 was added and mixed (Method B).

3) Determination of flavin

In order to avoid adsorption by MnO_2 and non-uniformity of photolysis of riboflavin by the lumiflavin (Lf) fluorescent method, the colorimeteric method employing three points correction was employed. For FAD, the Lf fluorescent method was employed.

iv. Results

1) As the results of paper chromatography indicate, the $KMnO_4$ decomposed riboflavin solution contains complex products, and among them are found substances weakly fluorescent and yellow in color. During the process of $KMnO_4$ decomposition (Fig. 1), and as the absorption spectra of the completely decomposed B_2 solution (Fig. 2) indicate, there is an absorption at around 445 mμ, so that for determination of the riboflavin retention rate during oxidation, direct colorimetry will not indicate clearly the exact value of the remaining riboflavin. Again, when the Lf fluorescent method is employed the alkaline reaction induces a large amount of MnO_2 to precipitate, obstructing thereby the passage of light rays markedly, and renders riboflavin adsorption by MnO_2 difficult. Hence the writer attempted colorimetric quantitative estimations based on the following 3 points correction method. The principle of this is that employed by Morton and Stubbs[4] in the estimation of vitamin A, but which has not been applied so far for riboflavin. The principle is as follows. In Fig. 3, if the absorption curve of a pure substance, curve I, produces an observed curve II due to the presence of impurities, curve III, and when between wave lengths λ_1, λ_2, λ_3, curve II is seen to be approximately a straight line, it is possible to

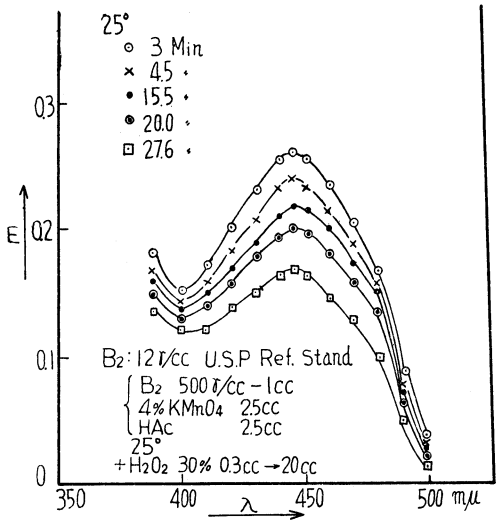


FIG. 1. Absorption spectra of $KMnO_4$ treated B_2 solution.

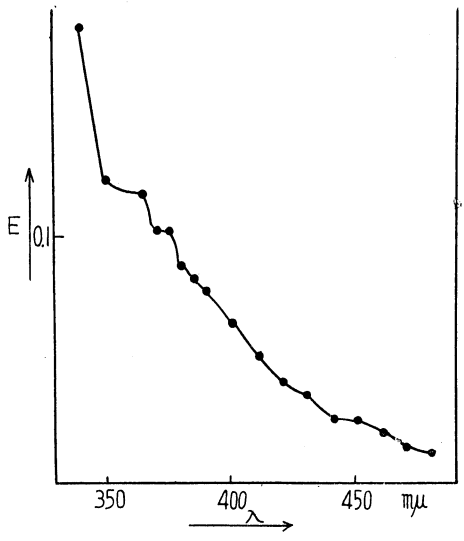


FIG. 2. Absorption spectrum of $KMnO_4$ treated B_2 solution.

compute from the observed absorbancy, E obs, the real absorbancy E corr, according to the following equation,

$$\frac{E\lambda_1 \text{ obs.} - ML - x}{E\lambda_3 \text{ obs.} - x} = k \tag{1}$$

$E\lambda_1$ obs. $- Ml - x = E\lambda$,

$$\text{corr.} = kE\lambda_1 \text{ obs.} - k\left\{\frac{kE\lambda_3 \text{ obs.} - E\lambda_1 \text{ obs.} + \dfrac{(\lambda_3 - \lambda_1)(E\lambda_2 \text{ obs.} - E\lambda_3 \text{ obs.})}{\lambda_3 - \lambda_2}}{k-1}\right\}$$

Based on the absorption spectra (Table 1) of pure riboflavin (U.S.P. Reference Standard) and employing equations (1) and (2), equation (3) for E 446 was obtained as follows:

$\lambda_1 = 446$ mμ, $\lambda_2 = 418$ mμ, $\lambda_3 = 474$ mμ

$E\lambda_1/E\lambda_2 = E\lambda_1/E\lambda_3 = 1.387$

Correction formula being:

$$E_{446}\,\text{corr} = 3.58\,E_{446}\,\text{obs.} - 1.79\,(E_{418}\,\text{obs.} + E_{474}\,\text{obs.}) \tag{3}$$

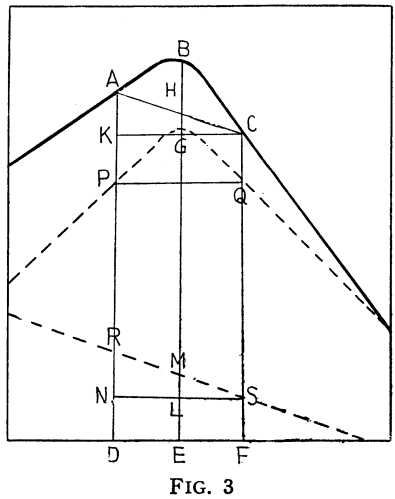


FIG. 3

(2) Decomposition of riboflavin by $KMnO_4$

An aqueous solution of riboflavin (500

TABLE 1. Absorption Spectra of B_2 Solution

λ_i (mμ)	E_{λ_i}	Ratio E_{λ_i}/E_{446}	λ_i (mμ)	E_{λ_i}	Ratio E_{λ_i}/E_{446}	λ_i (mμ)	E_{λ_i}	Ratio E_{λ_i}/E_{446}	λ_i (mμ)	E_{λ_i}	Ratio E_{λ_i}/E_{446}
410	0.406	0.6198	426	0.541	0.8259	446	0.652	1.0000	466	0.552	0.8427
411	0.417	0.6366	428	0.559	0.8534	447	0.651	0.9984	468	0.534	0.8153
412	0.421	0.6427	430	0.575	0.8779	448	0.647	0.9878	470	0.515	0.7863
414	0.438	0.6687	432	0.595	0.9084	450	0.644	0.9832	471	0.506	0.7761
416	0.456	0.6994	434	0.608	0.9282	452	0.634	0.9710	472	0.498	0.7638
417	0.463	0.7101	436	0.620	0.9466	454	0.628	0.9558	473	0.485	0.7438
418	0.471	0.7224	438	0.632	0.9649	456	0.618	0.9435	474	0.469	0.7193
419	0.480	0.7362	440	0.640	0.9771	458	0.610	0.9313	475	0.458	0.7024
420	0.493	0.7561	442	0.646	0.9862	460	0.597	0.9114	476	0.443	0.6763
422	0.506	0.7725	444	0.651	0.9984	462	0.587	0.8962	478	0.414	0.6321
424	0.520	0.7939	445	0.651	0.9984	464	0.570	0.8702	480	0.380	0.5801

γ/ccm) was oxidized by $KMnO_4$ according to method A, and by the above mentioned 3 points correction method, the retention per cent of riboflavin was obtained, and is shown in Table 2 and Fig. 4.

The velocity of oxidation of riboflavin by $KMnO_4$ according to method B is shown in Fig. 5.

TABLE 2. Decomposition of B_2 by Method A

Temperature	Time(t) (min.)	Retension % of B_2 (C_t) (%)	k (hr^{-1})
0°C	5	98.4	2.80×10^{-3}
	10.5	97.9	2.02
	12	94.9	4.35
	15	95.2	3.28
	20	94.0	3.09
	25	95.8	1.71
	30	92.1	2.74
Average			2.86×10^{-3}
25°C	2.83	82.1	6.96×10^{-2}
	4.33	73.8	7.00
	8.15	58.6	6.55
	10	53.3	6.28
	13	42.0	6.66
	15.15	29.0	8.15
	30.0	11.7	7.15
Average			6.96×10^{-2}
40°C	1.0	61.1	0.493
	2.17	45.6	0.561
	4.0	20.6	0.395
	5.0	9.6	0.471
Average			0.480

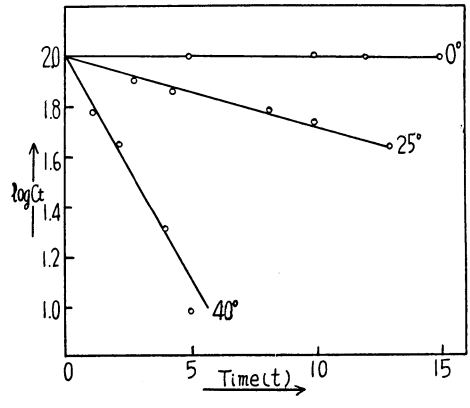


FIG. 4. Oxidative decomposition of B_2 on method A: relationship of log C_t vrs. t.

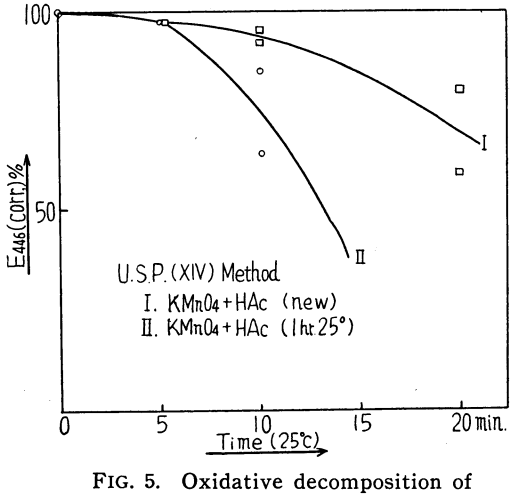


FIG. 5. Oxidative decomposition of B_2 by methode B.

(3) *Relation of temperature to action of $KMnO_4$*

Riboflavin decomposition of method A can be said to be a first order reaction.

As may be seen from Fig. 4 $\log C_t$ and reaction time are in a linear relationship, and k (first order) can be said to be a constant from the following formula:

$$k = \frac{2.303}{t} \log \frac{C_0}{C_t}$$

Where k is a rate constant, C_0 and C_t initial concentration and concentration after t hours of reaction of riboflavin respectively, and t the reaction time.

Arrhenius' equation, is as follower (Fig. 6):

$$\log k = 15.14 - \frac{4734}{T},$$

and activating energy 21.654 cals.

From the above it will be clear that the effective temperature of $KMnO_4$ on riboflavin is great. For example, when we observe the relation of the period of half decay of riboflavin to temperature, at 40° C it is 86 secs., at 25° C 10–30 minutes and at 0° C 4 hours.

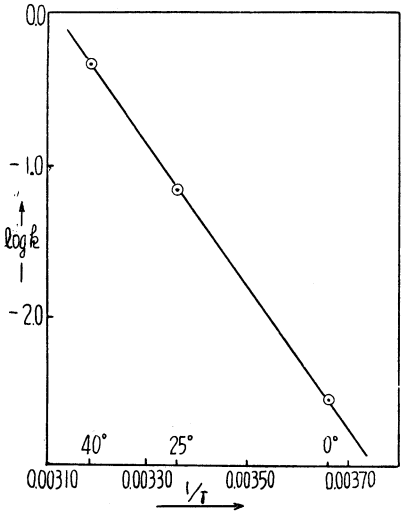


FIG. 6. Arrhenius temperature dependence of rate constant.

(4) *Effects of acids on the action of $KMnO_4$*

When the concentration and type of acid employed in method A are made to vary, the decomposition rates of riboflavin change as in Table 3.

TABLE 3. Effect of Acids on the Action of $KMnO_4$

Acids added (Method A)	Retention % of B_2 after 10 min. at 25° C (%)
10% HAc	70.5
100% HAc	89.6
10% H_2SO_4	14.6

(5) *Destructive action of $KMnO_4$ for various substances*

Comparative studies were made of the action of $KMnO_4$ for flavin and other substances attached to flavin during the process of method A, when it was found that FMN and lumiflavin were attacked to the same degree while FAD was destroyed much more rapidly. B_2-keto acid, pteroic acid, oxoviolet and 2-amino-4-hydroxy-6-methylpteridine were extremely more rapidly decomposed than flavin (Table 4).

(6) *Chromatography of substances obtained by decomposition of various flavines by $KMnO_4$*

100 mg each of riboflavin, FMN, Lf and Lumichrome (Lm) were dissolved

TABLE 4. Decomposition of Several Substances by $KMnO_4$

Substance	Retension %	
	25° C 10 min.	25° C 30 min.
B_2	82.5*** 85.7*	49.4*** 49.4*
FMN	89.0*** 89.8*	52.2*** 57.1*
FAD	69.0***	27.9***
Lf	88.6**	—
B_2-keto acid	2.0**	—
Oxo-violet	4.8**	—
Pteroic acid	0.0**	—
2-amino-4-hydroxy-6-methyl-pteridine	0.0**	—

* 3 points correction method.
** Direct fluorometry.
*** Lumiflavin fluorometry.

or suspended in 3 ccm of water, and after addition of 80 mg of concentrated sulfuric acid and 200 mg of $KMnO_4$, were heated over a water bath for 30 minutes. The decomposition solutions thus obtained were filtered and the filtrates were subjected to chromatography. The following results were obtained.

a. Paper chromatography (Toyo filter paper ♯51, solvent—HAc : BuOH : H_2O, 1 : 4 : 5, room temperature, "descending" method) (Fig. 7).

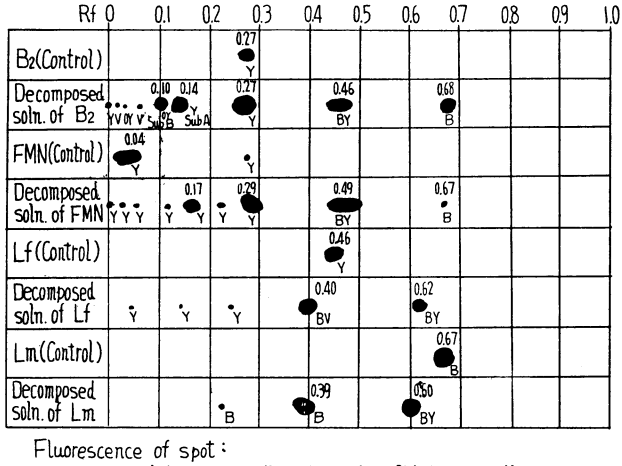


FIG. 7. Paper chromatogram of decomposition product of various flavins by $KMnO_4$ (HAc : BuOH : H_2O).

b. Paper chromatography (Toyo filter paper #51, solvent—$Na_2HPO_4 \cdot 12\,H_2O$-5%, aqueous solution, room temperature, "ascending" method) (Fig. 8).

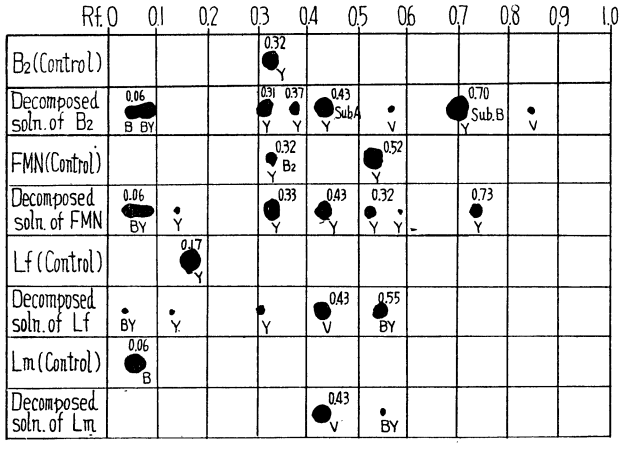


FIG. 8. Paper chromatogram of decomposition product of various flavins by $KMnO_4$ (Phosphate buffer).

c. Paper ionephoresis (Toyo filter paper #51, Theorell's buffer of pH 7.0, time 2 hours) (Fig. 9).

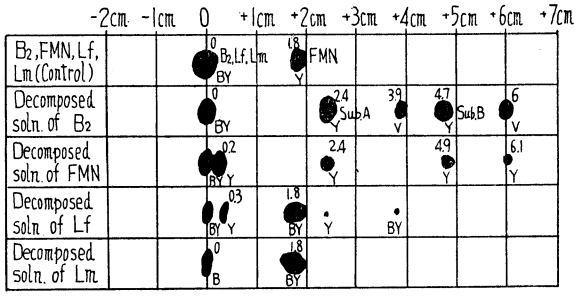


FIG. 9. Paper ionophoresis of decomposition products of various flavins by $KMnO_4$.

From chromatograms of B_2 decomposition solutions developed with a 5% Na_2HPO_4 solution, the substance with Rf 0.43 was termed Sub. A, and that with Rf 0.70 Sub. B. The spots of Sub. A and B were extracted with N-NaOH and developed with HAc : BuOH : H_2O as solvent when Rfs of 0.14 and 0.10 were respectively obtained. When ionophoresis with Theorell's buffer was conducted there were noted migrations of 2.4 cm and 4.9 cm respectively to the anode. From the above experiments the fluorescent substances obtained by $KMnO_4$ treatment of various flavines will be as follows.

The main $KMnO_4$ oxidative decomposition products of riboflavin are Sub. A and B, Sub. A being produced in greater amounts than B. Besides these,

small amounts of Lm and numerous undefined substances are recognized. By $KMnO_4$ oxidation of FMN, Sub. A and B as well as small amounts of Lm will also be demonstrated.

Whether these are due to production from the riboflavin present in FMN or directly from FMN is not clear. Further a strong spot corresponding to the Rf of riboflavin was confirmed. With Lf and Lm there were recognized mainly 2 types of violet-blue spot only, and production of Sub. A and B was not recognized.

The results of paper ionophoresis suggested that Sub. A and B are acid substances.

(7) *Nature of Sub. A*

In the riboflavin decomposition substances there was noted a large production of Sub. A, and hence studies were made to determine the nature of this substance. In the decomposition solution there were noted numerous decomposition substances besides unreacting riboflavin, so that isolation of Sub. A was rendered difficult. Hence, chromatograms with phosphate buffer were prepared, and the spot of Sub. A was isolated, and from this Sub. A was extracted with alkali and its nature determined.

Characteristics to light—In a neutral to acid medium there was seen photolysis with production of Lm and Lf as end products. In an alkaline medium there was seen no decomposition. The substance was insoluble in chloroform (an acetic acid solution of Sub. A could not be extracted with chloroform). There was noted migration to the anode with paper ionophoresis. The substance

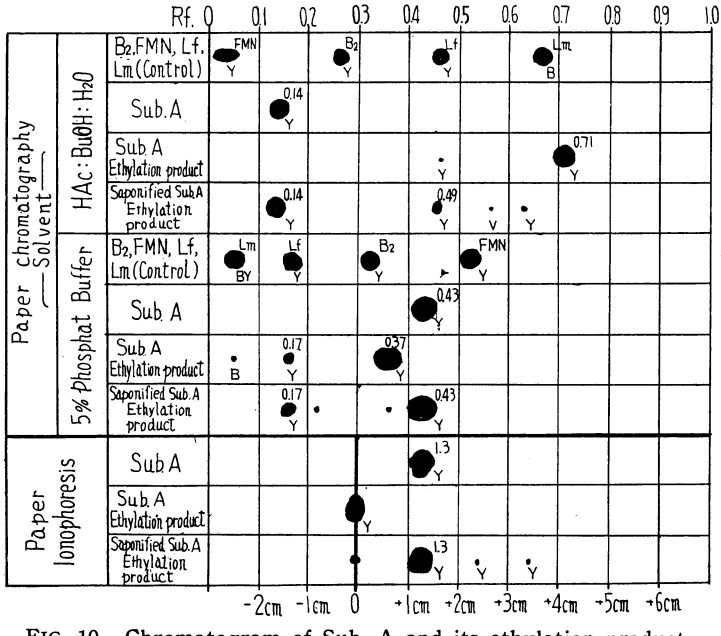


FIG. 10. Chromatogram of Sub. A and its ethylation product.

reacted with the alcohols such as methanol and ethanol, to form esters. The Sub. A spot on the chromatogram was cut into pieces and after suspending them in absolute ethanol was next saturated by passage through dessicated hydrogen chloride gas. After 2 days the spot of Sub. A disappears, and a fresh yellow colored spot believed to be that of the ethyl ester of Sub. A is formed. This is soluble in chloroform. This substances is also soluble in dilute alkali, and by leaving at room temperature again becomes capable of producing the spot of Sub. A (Fig. 10).

From the above it was surmised that Sub. A is an acid substance possessing the isoallaxazine nucleus and COOH. Its nature to light resembles that of B_2-9 acetic acid.

(8) *Chemical constitution of Sub. A*

As isolation of Sub. A was difficult, indirect experiments to determine the chemical constitution of this substance were conducted. The results of PPC undertaken with the $KMnO_4$ decomposed solution of riboflavin and photolytic solution obtained by treating riboflavin with NaOH in the presence of H_2O_2 according to Sakurai's method[5], are shown in Fig. 11. The Rf of Sub. A was found to resemble closely that of B_2-9 acetic acid.

Next, the ethyl ester of this substance was prepared according to the steps indicated in Table 5, namely.

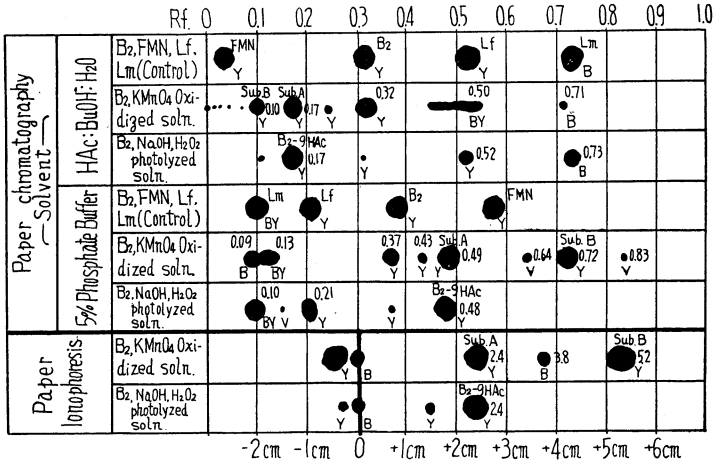


FIG. 11. Paper chromatogram: relationship between Sub. A and B_2 9-acetic acide.

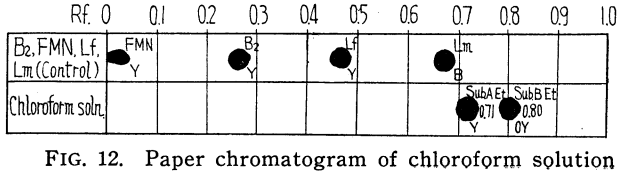


FIG. 12. Paper chromatogram of chloroform solution (Solvent—HAc : BuOH : H_2O).

TABLE 5. Ethylation of Sub. A

$KMnO_4$ decomposed solution of riboflavin (using 2 g of riboflavin)

↓

Precipitate — filtrate (dark red in color)

↓

extracted with $CHCl_3$ after acidifying with HAc

↓

aqueous solution — $CHCl_3$ solution (Im)

↓

dried under reduced pressure (below 40° C)

↓

residue

↓ suspended in 20 ccm of absolute alcohol, saturated with dried HCl gas, left for 2 full days

reacting solution

↓ 20 ccm of water added, acidified to weak acid with sodium acetate, extracted with $CHCl_3$

$CHCl_3$ solution* — aqueous solution (riboflavin, others)

↓ elution, dehydrated, evaporated, dried

residie

↓ recrystallized with glacial acetic acid (warm)

yellow needle crystals (Sub. A ethylation product)

After much investigations it became clear that Rf 0.80 (OY) was the ethyl ester of Sub. B. Hence, Sub. B was believed to be an acid substance containing COOH.

In the crude state the ethyl ester of Sub. A contains as impurities the ester of Sub. B (Fig. 12), but this could be removed by recrystallization with glacial acetic acid.

Nature of the ethyl ester of Sub. A

With the B_2-9 HAc ethyl ester synthesized by Sakurai[5] as control, the chromatogram of the ethyl ester of Sub. A turned out to be as follows, and the Rfs of the two were found to correspond when developed with two types of solvents (Fig. 13).

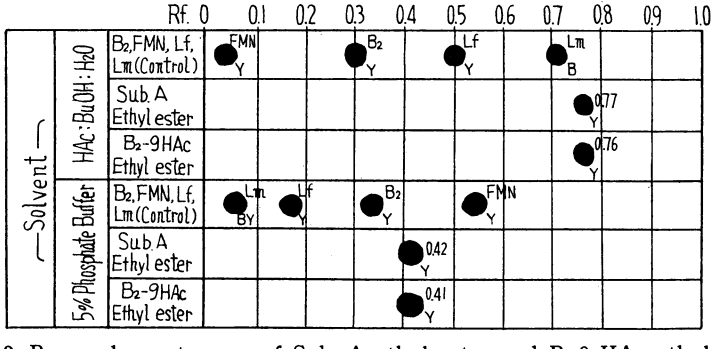


FIG. 13. Paper chromatogram of Sub. A ethyl ester and B_2-9 HAc ethyl ester.

The maximal absorption spectra were found to be at 268, 350 and 448 mμ, and the minimal at 300 and 388 mμ, (Fig. 14). They were therefore found to correspond with that of Sakurai's B_2-9 acetic acid ethyl ester (ethyl-6, 7-dimethyl flavin-9-acetic ester).

The substance was insoluble in water, soluble in $CHCl_3$, glacial acetic acid, and hot alcohol. In glacial acetic acid it was stable and heat produced no decomposition. It was easily soluble in N-NaOH, rapidly yielding Sub. A. Melting point was 288–290° C (decomposition point), while Sakurai's B_2-9 acetic acid ethyl ester indicated a decomposition point of 288° C.

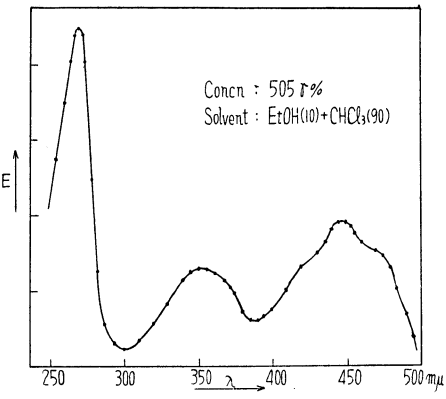


FIG. 14. Absorption spectrum of Sub. A ethyl ester.

Ultimate analysis:

Experimental value: C 57.81, H 5.17, N 16.22.

Calculated (as ethyl-6, 7-dimethyl flavin-9-acetic ester $C_{16}H_{160}O_4N_4$). C 58.54, H 4.8, N 17.07.

From the above results it became clear that Sub. A ethyl ester closely resembles B_2-9 acetic acid ethyl ester. Hence the B_2 oxidative decomposition product, Sub. A, is believed to be 6, 7-dimethyl-isoalloxazine-9-acetic acid.

When B_2 is subjected to oxidative decomposition with $KMnO_4$ many complex decomposition products are formed, but of these that showing fluorescence consists mainly of Sub. A, followed by Sub. B, Sub. A was examined by chromatography when it was found to resemble closely B_2-9 acetic acid. Next its ethyl ester was synthesized and various investigations were made, and it was found to coincide with ethyl-6, 7-dimethyl flavin-9-acetic ester, and from this it was surmised that Sub. A is 6, 7-dimethyl-9-acetic acid.

The chemical consitution of Sub. B remained undefined, but as the result of paper chromatography it was found to be more hydrophyllic than Sub. A, and from the fact that its ethyl ester can be derived it was surmised that it is a COOH compound with the OH of the ribityl remaining.

2. 2. Decomposition of flavin with lead tetra acetate ($Pb(Ac)_4$)

The reacting solution was examined by PPC (BuOH : HAc : H_2O 4 : 1 : 5) and Toyo filter paper #51. As may be seen from Fig. 15, under mild conditions the decomposition products of $Pb(Ac)_4$ produced besides Lm, a yellow fluorescent substance with Rf 0.52 and a blue-violet fluorescent substance with Rf 0.9. Under rather powerful conditions there was produced besides Lm a blue-violet fluorescent substance with Rf 0.9. From FMN there were produced two substances of unknown nature of Rfs 0.1 (Y) and 0.77 (Y), and no Lm was demonstrated. B_2 tetra acetate comes to produce a weak fluorescence at Rf 0.3, but it does not seem to undergo decomposition. Lf also seems to under little decomposition, but a yellow spot is produced at Rf 0.2. B_2-keto acid is oxidized

with case and changes into substances with Rf 0.88 (V) and Rf 0.97 (V?).

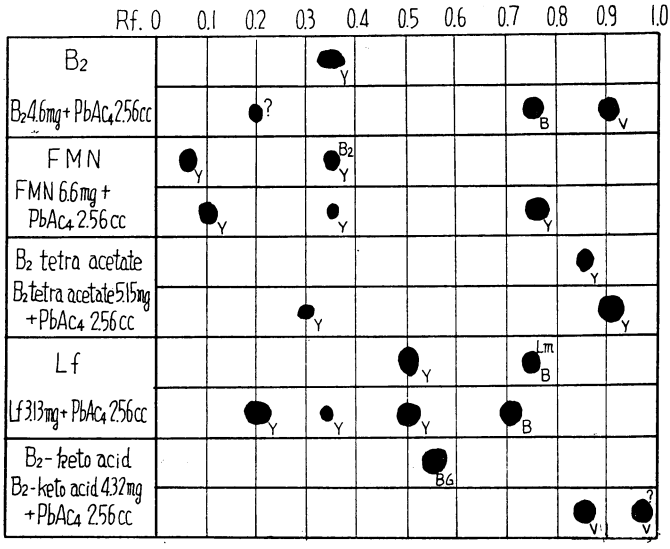


FIG. 15. Paper chromatogram of decomposition products of various flavine by $Pb(Ac)_4$.

2. 3. Riboflavin decomposition with periodic acid (HIO_4)

2. 3. i. Method of oxidation with HIO_4

1) Karrer's method[6)7)]—To 20 mg of B_2 were added 7 ccm of water, 0.4 ccm of N/10 H_2SO_4, and 1.2 ccm of N/10 HIO_4, and the reaction was allowed to proceed at room temperature for 30 minutes.

2) Wickström's method[8)]—To about 50 mg of B_2 was added 20 ccm of M/20 HIO_4, and after thorough shaking was allowed to react at room temperature for 30 minutes.

2. 3. ii. PPC of HIO_4 oxidized products ($PuOH : HAc : H_2O$ 4 : 5 : 1; Toyo # 51)

(1) Karrer's method

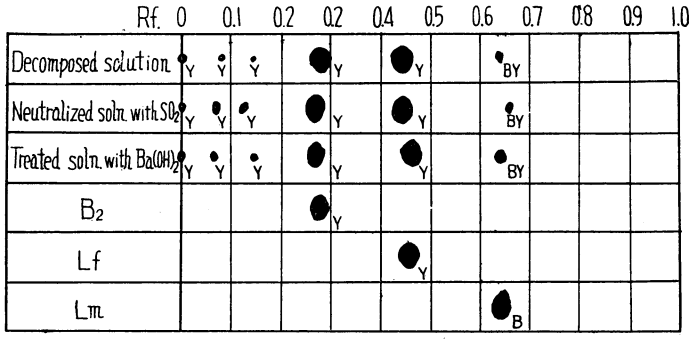


FIG. 16. Paper chromatogram of decomposition product of B_2 by HIO_4 (Karrer's method).

By this method B_2 was not completely decomposed and some remained.

The solutions examined consisted of 1) the entire decomposed solution as itself, 2) one in which SO_2 gas was passed into the reacting solution and the HIO_4 was made to decompose, 3) where $Ba(OH)_2$ was passed into the reacting solution and HIO_4 and iodic acid were removed. The chromatograms are indicated in Fig. 16.

With Karrer's method a large amount of non-reacting riboflavin remains. The main products are Lf, a small amount of Lm, and small amounts of three yellow colored fluorescent substances with Rfs 0(?), 0.06–0.08 and 0.11–0.14. The last is frequently not found at all.

(2) Wickstrom's method

The results are shown in Fig. 17. In this case oxidation is complete, and the main substances are Lm and If. Besides these, small amounts of three unknown substances with Rfs of 0.0, 0.03 and 0.06–0.07 were demonstrated.

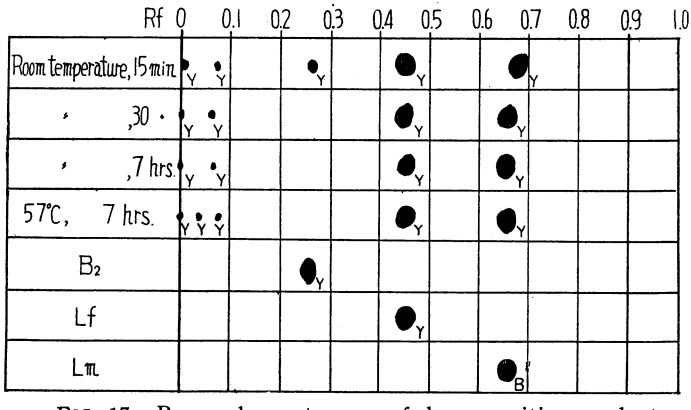


FIG. 17. Paper chromatogram of decomposition product of B_2 by HIO_4 (Wickström's method).

The substance with Rfs 0.64–0.69 is a yellow fluorescent substance but its Rf coincides with that of Lm. When the reacting solution is left for long at room temperature or at 57° C, the yellow fluorescent substance on the chromatogram gradually changes into blue yellow to blue in color, but the Rf value does not change. This substance is believed to be the same as isoallaxazine reported previously.

Karrer's method is a comparatively milder one than Wickström's method. Under mild conditions the main substance is Lf, and there is little production of isoallaxazine and Lm. Contrary to this, under intense conditions it is interesting that the main products Lf and isoallaxazine are produced in almost equal proportions. During the process of riboflavin oxidation by HIO_4 Wickström considered that B_2-9-acetoaldehyde is produced as an intermediate substance, but in our experiments such a substance could not be demonstrated. The substance with an Rf of 0.06–0.08 could not be ascertained regarding its nature. It appeared under all conditions in small amounts. It was found at 57° C, and

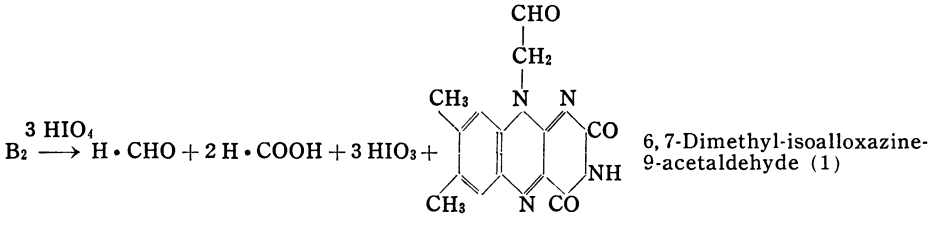
remained stable even when kept for long periods at this temperature, so that it can be said to be a fairly stable substance, but not 9-acetoaldehyde.

DISCUSSIONS

Investigations were made of riboflavin oxidative products with fluorescence as the main clue, and it was found that as main product the cleavaged product of the ribityl side chain is demonstrable. Thus with $KMnO_4$ 6, 7-dimethyl-isoallaxazine, and with $PbAc_4$ and HIO_4 Lf and Lm are produced. That these riboflavin light decomposition products are also produced by oxidizing agents is a matter of deep interest. $KMnO_4$ which has hitherto been employed in the quantitative estimation of riboflavin is a substance that destroys the flavines fairly powerfully, and special caution has to be paid to the fact that it acts more powerfully on FAD than on riboflavin and FMN. Sub. A one of the main fluorescent substances resulting from the oxidation by $KMnO_4$, was considered to be 6, 7-dimethyl-isoallaxazine-9 acetic acid.

But according to the researches of Hais[9)10)], the heterocyclic portion of B_2 is weakly resistant to H_2O_2 and $KMnO_4$, while from our experiments too. that the decomposition products have lost markedly the light absorption peculiar to flavine, it would seem that substances besides Sub. A with the flavine nucleus cleavaged are produced in large amounts. But as these substances possessed no fluorescence they could not be demonstrated in the present series of experiments.

According to Wickström[8)] HIO_4 does not act on the heterocyclic part of riboflavin but partakes in the following reaction:



6, 7-Dimethyl-isoalloxazine-9-acetaldehyde (1)

In this reaction Lf and Lm were demonstrated and confirmed by PPC, but (1) remained within the range of conjecture. In our experiments (1) could not be demonstrated, and doubt remains regarding its existence. At any rate the actions of HIO_4 and $Pb(Ac)_4$ on riboflavin were limited practically to the ribityl side chain, while $KMnO_4$ acts on not only the side chain but also on the isoallaxazine nucleus.

And in this respect deserves attention as being markedly different.

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