[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

The Photochemistry of Riboflavin and Related Compounds

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The photolysis of riboflavin and of a number of related flavins in acid solution is a case of general acid and base catalysis. The proton involved in the catalysis has been identified in the case of 9-(2'-hydroxyethyl)-isoalloxazine. In the photolysis of this compound, the side chain produces acetaldehyde, formaldehyde and an acid, probably formic. A mechanism is proposed to account for these results.

The fact that riboflavin is photosensitive was first observed by Warburg and Christian.² They found that if riboflavin (I) is irradiated in alkaline solution, it is converted into lumiflavin (II). A short time later, Karrer and co-workers³ showed that irradiation in neutral or acid solution yields a different product, which they named lumichrome (III). taken up was the effect of the photolysis on the hydroxylated side chain. If Karrer's hypothesis is correct, then it should be possible to find a carbonyl-containing side chain product in the photolyzed solution. However, the compounds to be expected from the side chain of riboflavin itself are rare substances which have been little studied, and their isolation, identification and estimation



These reactions have been investigated by Kuhn and co-workers,^{4,5} by Koschara,⁶ by Theorell,⁷ by Brdička,⁸ and especially by Karrer and coworkers.^{9–13} Karrer concluded that the first step in the photolysis of riboflavin and related compounds in neutral or acid solution is an oxidation to carbonyl of the 2'-hydroxyl group of the side chain. The carbonyl compound then splits, giving lumichrome or an analogous compound.

The present study is confined to the photolysis in neutral or acid solution. One of the points

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 - (12) P. Karrer, T. Köbner and F. Zehender, ibid., 19, 261 (1936)
 - (13) P. Karrer and R. Naef, ibid., 19, 1029 (1936)

identification and estimation might prove very difficult. For this reason, the simpler flavin, 9-(2'-hydroxyethyl)isoalloxazine (IV) was used. This substance shows the same photoreactions as riboflavin, and the photolysis products to be expected from its side chain are simple compounds, whose properties have been extensively studied.

Four chief possibilities must be taken into account: (1) oxidation of the side chain hydroxyl group followed by a reductive cleavage of the N-C bond, forming acetaldehyde; (2) oxidation of the hydroxyl group followed by a hydrolytic

cleavage, forming glycolic aldehyde; (3) reductive cleavage without oxidation of the hydroxyl group, forming ethyl alcohol; (4) hydrolytic cleavage without oxidation of the hydroxyl group, forming ethylene glycol.



Experimental

9-(2'-Hydroxy-ethyl)-isoalloxazine was prepared by the method of Karrer, Schlitter, Pfaehler and Benz.¹⁰ To effect the photolysis, 80 mg. was dissolved in a liter of water and exposed to sunlight in a Pyrex flask. This was done out of doors, at temperatures that varied from 8 to 31°. The photolysis was allowed to proceed until the yellow fluorescence of the solution had fallen to less than 1% of its original value. This required 3 to 4 hours. Toward the end of the photolysis, in most cases, and in all cases after standing a few hours, pale yellow crystals gradually deposited. These have been shown by Karrer to be alloxazine (V). The crystals were filtered off, and in some cases, the total yield of alloxazine, both crystals and the portion remaining in solution, was determined by a method given below. The filtrate was used to detect and determine the side chain photolysis products.

Detection of Acetaldehyde .- The presence of acetaldehyde in the photolyzed solution was immediately evident from its odor, which can be detected in a concentration as low as two parts per million: 190 ml. was distilled off 2 liters of photolyzed solution, with the delivery tube dipping into 10 ml. of ice-cooled water. The distillate was again distilled, 30 ml. being collected. This was diluted to 100 ml. and 20 ml. distilled off. This third distillate was diluted to 100 ml. and 20 ml. distilled off. The purpose of these repeated distillations was to obtain acetaldehyde free of formaldehyde (see below). To the final distillate was added 33 ml. of a 0.4% aqueous solution of dimedon (methone). The crystals which deposited in the course of three days melted at 142.1° after recrystallizing once by dissolving in a small amount of alcohol and diluting with water. A known sample of the dimedon derivative of acetaldehyde melted at 142.0°. The mixed melting point was unchanged.

Detection of Formaldehyde .--- In agreement with Brdička's observations on riboflavin,⁸ formaldehyde was found among the photolysis products. For its detection a liter of photolyzed solution was distilled, collecting 200 ml. This contained all the acetaldehyde and part of the formaldehyde. The distillate was redistilled, 40 ml. being dis-tilled off. The residue contained formaldehyde free of acetaldehyde. The presence of formaldehyde in this solution was demonstrated by the phenylhydrazine-ferricyanide test.¹⁴ For final identification, 25 ml. of a 0.4% aqueous dimedon solution was added to the formaldehyde-containing solution. After several days, the crystals were filtered off. They melted at 191.5° after one recrystallization from an alcohol-water mixture. A known sample of the dimedon derivative of formaldehyde also melted at 191.5°. The mixed melting point was unchanged.

The detection of ethyl alcohol was attempted by combin-ing the procedures of Bacon,¹⁵ Kostytschew¹⁶ and Argenson.¹⁷ No alcohol could be detected. A test for ethylene glycol was made by evaporating a liter of photolyzed solution in was induce of orapidating a induct of photosystem solution in M yacuto to 25 ml., destroying formaldehyde by heating for 10 minutes at 100° with a mixture of 5 ml. of 3% hydrogen peroxide and 5 ml. of 4 M sodium hydroxide, acidifying, removing excess peroxide by addition of permanganate, and testing for ethylene glycol by the periodic acid method of Feigl.¹⁸ No ethylene glycol was found.

A test for glycolic aldehyde was made which depends on the fact that, like ethylene glycol, it is oxidized to formaldehyde by treatment with periodic acid. Since formaldehyde is already present in the photolyzed solution, and since no feasible way of removing it without affecting the glycolic aldehyde could be found, it was necessary to determine formaldehyde before and after oxidation, and show that if an appreciable amount of glycolic aldehyde were present, the concentration of formaldehyde would have been detectably increased.

A liter of photolyzed solution was evaporated in vacuo to 25 ml., at a bath temperature not exceeding 35° Another liter of photolyzed solution to which 0.5 mg. of glycolic

 (17) G. Argenson, Bull. soc. chim. France, [3] 27, 1000 (1902).
(18) F. Feigl, "Spot Tests," Nordemann Publishing Co., Inc., New York, N. Y., 1939, 2nd ed., p. 272.

aldehyde had been added as a control was treated in a similar manner. One portion of each of these solutions, acidified with dilute sulfuric acid, was oxidized for one-half hour at room temperature with 0.1% periodic acid. The latter added to determine the formaldehyde. To other portions of the photolyzed solutions, fuchsin reagent was added without oxidizing. The color intensities produced were measured with a spectrophotometer. The oxidized portion of the solution to which no glycolic aldehyde had been added showed a loss in color compared with the unoxidized por-tion owing to partial destruction of formaldehyde. The tion, owing to partial destruction of formaldehyde. oxidized portion of the solution to which glycolic aldehyde had been added showed an increase in color compared with the unoxidized portion, owing to conversion of glycolic aldehyde to formaldehyde. Thus, there must be less than 0.5 mg. of glycolic aldehyde, if any, in the photolyzed solution. Determination of Formaldehyde, Acetaldehyde and

Alloxazine.—Formaldehyde was determined by the phenyl-hydrazine-ferricyanide method.¹⁰ Acetaldehyde was determined by concentrating the photolyzed solution tenfold by distillation and applying the bisulfite method of Ripper.20 A small amount of formaldehyde accompanies the acetaldehyde into the distillate and was determined separately by the phenylhydrazine-ferricyanide method.

Alloxazine was determined in the precipitated crystals and in the supernatant solution. The precipitate was dissolved in dilute ammonia, the pH adjusted to 10.2, and the yellow fluorescence compared in a fluorometer with alloxazine solutions of known concentration. The alloxazine used as a standard was prepared by the method of Kühling.²¹ The alloxazine in the supernatant solution was determined by adjusting to pH 10.2 and measuring the fluorescent intensity excited by 436 mµ light, making a small correction for a fluorescence that remained on acidifying to pH 4.6.

Three determinations of the alloxazine produced in the photolysis of 9-(2'-hydroxyethyl)-isoalloxazine, initial concentration 80 mg. per liter, at pH 5.5 by sunlight showed yields of 86, 88 and 89%, average 88%. It was found that for every mole of alloxazine, there were produced, on the average, 0.20 mole of acetaldehyde and 0.58 mole of formaldehyde, a total yield of side chain products of 0.78 mole per mole of alloxazine. Thus, if it is arbitrarily assumed that a given side chain can produce either one acetaldehyde or one formaldehyde, approximately 22% of the side chains remain unaccounted for. The results show, moreover, that under these conditions, the chief product is not acetaldehyde but formaldehyde. The distribution of side chain products between formaldehyde and acetaldehyde was found to depend markedly on the initial concentration of the flavin. At an initial concentration of 16 mg. per liter, 0.69 mole of acetaldehyde and 0.15 mole of formaldehyde were produced. Photolysis with monochromatic light of 436 mµ wave length instead of with sunlight also favored the formation of acetaldehyde.

The pH of the solution fell about 1.5 units on photolysis. The drop is not due to the formation of alloxazine, whose acidic properties are very feeble. It is possible that the un-accounted portion of the side chain forms an acid, most likely formic.

The Photolysis of Riboflavin and Other Flavins as Cases of General Acid and Base Catalysis.-It was observed that riboflavin photolyzes more rapidly in buffered solutions than in unbuffered solutions of the same pH. Investigation showed that the photolysis of riboflavin, in neutral or acid solution, is a case of general acid and base catalysis. The same is true of the other flavins investigated.

Experimental

Relative photolysis rates were measured by irradiating a very dilute solution of the flavin and determining the rate at which the fluorescence diminished. Since conditions varied somewhat from day to day, one system was chosen as

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- (21) O. Kühling, Ber., 24, 2363 (1891).

^{(14) &}quot;Methods of Analysis of the Assoc. Official Agr. Chemists," Washington, D. C., 6th ed., 1945, p. 530.

⁽¹⁵⁾ R. F. Bacon, U. S. Dept. Agric., Bur. Chem. Circular #74 (1911).

⁽¹⁶⁾ S. Kostytschew, Z. physiol. Chem., 79, 359 (1912).

⁽¹⁹⁾ F. D. Snell and C. T. Snell, "Colorimetric Methods of Analysis," D. Van Nostrand Co., Inc., New York, N. Y., 2nd ed., 1939, Vol. 2, p. 59.

a standard of reference. This system consisted of riboflavin (obtained from Merck and Company, Inc., and recrystallized once from water), concentration 0.605 mg./liter, in a solution 0.05 M with respect to both sodium acetate and acetic acid, at 25° . This system showed a first order rate constant of about 0.1 reciprocal minute under the conditions used. It was arbitrarily assigned a value of exactly 0.1. At least one determination of the rate constant of this reference solution was made with each series of determinations, and all rate constants were referred to the value of 0.1 for the standard solution.

The solution was placed in a glass cell of depth 0.5 cm., immersed in a bath at $25 \pm 0.1^{\circ}$. The light source was an AH4 mercury arc, from which wave length 436 m μ was isolated by means of glass filters. Photolysis was allowed to proceed until about 50% of the fluorescence had disappeared. The residual fluorescence was then compared with that of the unphotolyzed solution in a fluorimeter. A small correction was made for the fluorescence of the photolysis products. The initial concentration of each flavin was chosen so that a 0.5 cm. layer transmitted 98% at 436 m μ . The reaction velocity was found to be first order with respect to flavin concentration in every case. Using this method, the effect of three acid-base pairs on the rate of photolysis of riboflavin, initial concentration 0.605 mg./l., was determined, with results given in Fig. 1.



Fig. 1.—Effect of buffer mixtures on photolysis rate of riboflavin: (1) acetic acid-sodium acetate buffer, ratio of acetic acid to sodium acetate 0.5, pH 4.9; (2) ratio of acetic acid to sodium acetate 1, pH 4.6; (3) ratio of acetic acid to sodium acetate 2, pH 4.3; (4) pyridinium hydrochloride-pyridine buffer, ratio of pyridinium hydrochloride to pyridine 0.5, pH 5.4; (5) ratio of pyridinium hydrochloride to pyridine 1, pH 5.1; (6) formic acid-sodium formate buffer, ratio of formie acid to sodium formate 1, pH 3.5; (7) ratio of formic acid to sodium formate 2, pH 3.3.

There was no neutral salt effect. Adding potassium chloride to give a concentration of $0.05\ M$ to a solution already

0.05~M with both sodium acetate and acetic acid had no effect on the rate constant. One determination of the effect of temperature was made, using a riboflavin solution, concentration $0.605~{\rm mg./l.}$, in 0.05~M sodium acetate plus 0.05~M acetic acid. The rate constant was 19% greater at 35° than at 25°.

It is seen from Fig. 1 that the rate constant is a linear function of the concentration of catalyst in each case. The lines representing the acetate and formate buffers extrapolate nearly to the same point. Thus it may be said that at least within the pH range 3.3 to 4.9, the pH of the solution has little or no effect on the rate. The two pyridine buffers extrapolate to a different point as compared with the acetate and formate systems.

The effect of buffers on the rate constant can be expressed by the relation $k = k_0 + k_A[A] + k_B[B]$. Here k_0 is the uncatalyzed (or solvent-catalyzed) rate constant, k_A is the catalytic constant for the acid A, and k_B for the base B. k_0 will have the same value for the acetate and formate buffers, but a different value for the pyridine buffers. The value of k_0 for the acetate and formate buffers is 0.018, for the pyridine buffers, it is 0.007. The former is much the closer of the two to the value of 0.015 found in pure water.

The catalytic constants, k_A and k_B , were evaluated from the slopes of the lines in Fig. 1. They are given in Table I. The most striking feature of this table is the low catalytic power of formic acid as compared with the other two. This is surprising, in view of the fact that formic acid is the strongest of the three. The cause of this effect is obscure, but may be connected with the fact that the still stronger acid, hydrogen ion, shows no catalytic activity (see Table II).

TABLE	I
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CATALYTIC	CONSTANTS	OF	Some	Acids	AND	BASES	IN	THE
	PHOTOLYSIS	S OF	RIBO	FLAVIN	AT 2	5°		

Species	Acid strength	$k_{\rm A}$, min. $^{-1}$ × 1./mole
	A. Acids	
Acetic	$1.8 \times 10^{-5^{a}}$	1.09
Formic	$2.1 imes10^{-4}$	0.02
Pyridinium	$7.4 imes10^{-6}$	0.70
Species	Base strength	$k_{\rm B}, \min_{n=1}^{n-1} \times 1./mole$
	B. Bases	
Acetate	$5.6 imes10^4$	0.53
Formate	4.8×10^{3}	.28
Pyridine	$1.2 imes10^5$. 26

^a The values of the acid strength (K_a) are from R. P. Bell, "Acid-Base Catalysis," Clarendon Press, Oxford, 1941, p. 47. Base strength = $1/K_a$.

Table II shows the effect of acids alone. This table shows the interesting fact that hydrogen ion, which one might expect to be the most effective of the acid catalysts, shows no catalytic effect. In fact, it interferes with other catalysts. Thus, 0.1 M acetic acid shows a definite catalytic effect, which is suppressed by the presence of 0.01 Mhydrochloric acid. Evidently, lowering the pHalters the riboflavin molecule in such a way as to make it resistant to photolysis. The effect of pHis not due to a lowering of the extinction coefficient, since this is the same in 0.01 M hydrochloric acid as in water.

TABLE II

EFFECT OF ACIDS ON THE RATE OF PHOTOLYSIS OF RIBO-

PLAVIN		
Acid	⊅H	k, min1
None	5.3	0.0147
Acetic, 0.1 M	2.80	.0288
HCI, 0.1 M	1.98	. 0143
HCl, 0.01 M + acetic, 0.1 M	1.98	.0153

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Acid-Base Catalysis in the Photolysis of 9-(2'-Hydroxyethyl)-isoalloxazine and Related Compounds.-Like riboflavin, the photolysis of 9-(2'-hydroxyethyl)-isoalloxazine was found to show general acid and base catalysis, as shown in Fig. 2, where the rate constants in sodium acetate-acetic acid buffers are plotted. Since acid and base catalysis involve the transfer of one or more protons, those hydrogen atoms in 9-(2'-hydroxyethyl)-isoalloxazine which seemed most likely to be involved in the reaction were replaced by other groups, and the effect on the photolysis rate determined. First, the labile hydrogen at position 3 in the ring was replaced by methyl, giving 3-methyl-9-(2'-hydroxyethyl)-isoalloxazine. However, as Fig. 2 and Table III show, this compound behaves much like the parent compound. On the other hand, replacing the hydrogen of the hydroxyl group in the side chain by acetyl, producing 9-(2'-acetylethyl)-isoalloxazine produces a striking effect (Tables III, IV). Acid and base catalysis all but cease, and the rate of photolysis becomes very slow. This is in agreement with Karrer's observation on acetylated riboflavin.9 It is clear, therefore, that the acidbase catalysis in the case of 9-(2'-hydroxyethyl)isoalloxazine involves the removal of the hydrogen atom of the side chain hydroxyl group. The same is undoubtedly true of riboflavin, although here there are several hydroxyl groups, and it has not yet been established whether several or only one participate.

TABLE III

CATALYTIC CONSTANTS FOR ACETIC ACID AND ACETATE ION OF 9-(2'-HYDROXYETHYL)-ISOALLOXAZINE AND RELATED COMPOUNDS

	Catalytic constant, min.			
Compound	Acetic acid	Acetate ion		
9-(2'-Hydroxyethyl)-isoalloxazine	3.98	1.12		
3-Methyl-9-(2'-hydroxyethyl)-				
isoalloxazine	4.98	0.82		
9-(2'-Hydroxy-2'-methyl-n-				
propyl)-isoalloxazine	8.14	.87		
9-(2'-Acetylethyl)-isoalloxazine	0.02	.00		

TABLE IV

PHOTOLYSIS RATES OF FLAVINS IN WATER,	<i>p</i> H 5.3
9-(2'-Hydroxyethyl)-isoalloxazine	0.0456
3-Methyl-9-(2'-hydroxyethyl)-isoalloxazine	.0379
9-(2'-Methyl-2'-hydroxy-n-propyl)-	
isoalloxazine	.0071
9-(2'-Acetylethyl)-isoalloxazine	.0021

It has been mentioned that Karrer concluded that the photolysis of the flavins begins with the oxidation of the 2'-hydroxyl group to a carbonyl. To test this conclusion, he synthesized the compound 9-(2'-hydroxy-2'-methyl-n-propyl)-isoalloxazine (VI), which is incapable of forming a carbonyl compound. He found that this compound still photolyzed, although much more slowly than 9-(2'-hydroxyethyl)-isoalloxazine.

To determine whether this compound shows acidbase catalysis, it was synthesized by the method of Karrer¹¹ and tested, with results given in Fig. 2 and Tables III and IV. In agreement with the observations of Karrer, a very low rate of photolysis



moles/l.

Fig. 2.—Effect of acetic acid-sodium acetate buffers on photolysis rate of three isoalloxazines: (1) 9-(2'-hydroxyethyl)-isoalloxazine, ratio of acetic acid to sodium acetate 1, pH 4.6; (2) 9-(2'-hydroxyethyl)-isoalloxazine, ratio of acetic acid to sodium acetate 0.5, pH 4.9; (3) 3-methyl-9-(2'-hydroxyethyl)-isoalloxazine, ratio of acetic acid to sodium acetate 1; (4) 3-methyl-9-(2'-hydroxyethyl)-isoalloxazine, ratio of acetic acid to sodium acetate 0.5; (5) 9-(2'-methyl-2'-hydroxy-*n*-propyl)-isoalloxazine, ratio of acetic acid to sodium acetate 1; (6) 9-(2'-methyl-2'-hydroxy*n*-propyl)-isoalloxazine, ratio of acetic acid to sodium acetate 0.5.

in the absence of catalysts was found. However, the compound shows a very strong acid catalysis. It also shows basic catalysis of about the same order as $9 \cdot (2'-hydroxyethyl)$ -isoalloxazine.

Discussion

The photolysis of the flavins is a complicated reaction or set of reactions. The mechanism which is presented here is admittedly incomplete, but it is hoped it will prove suggestive of further experiments. It is presented for 9-(2'-hydroxyethyl)-isoalloxazine, but may be extended by analogy to riboflavin itself. In brief, the absorption of a photon produces an activated molecule. With the help of the acids and bases present in the solution, the proton is removed from the side chain hydroxyl group, and a proton is taken on by the ring nitrogen atom to which the side chain is attached. The proton transfer is not necessarily in the above order. The bond between the nitrogen atom and the first carbon of the side chain now breaks, the electron pair remaining with the nitrogen—that is, the proton displaces the side chain from the ring. The side chain now rearranges to form acetaldehyde and the body of the molecule tautomerizes, forming alloxazine.

The proposed mechanism is symbolized below. A^- represents a typical base, HA a typical acid.

photolysis, which requires the presence of a proton at position 9.

The presence of formaldehyde may be due to the mechanism proposed by Brdička⁸ for the anaerobic photolysis, involving the transfer of a hydrogen atom from the 2'-carbon atom to one of the ring nitrogens. The photolysis of the flavins is a



According to this view, the conversion of the side chain hydroxyl to a carbonyl is simply one of the results of the photo-reaction and not, as Karrer believed, the prime cause of the split. Karrer's hypothesis would lead us to expect that 9-(2'-methyl - 2' - hydroxy - n - propyl) - isoalloxazine, in which the formation of a carbonyl would require the rupture of a carbon-carbon bond, would photolyze only very slowly or not at all. Actually it has been shown that when properly catalyzed, it photolyzes faster than any of the other flavins studied.

In the schematic representation of the photolysis given above, the removal of the proton from the side chain has been shown as preceding the addition of the proton to the ring nitrogen. However, the reserve process is equally likely. Presumably, the presence of a proton on the ring nitrogen facilitates the subsequent removal of the proton from the OH group. Thus, either the removal or addition may be a rate-determining step, so that the reaction shows both acid and base catalysis. The photolysis nearly stops when the OH hydrogen is replaced because the formation of the ion is an essential part of the driving force of the reaction.

The failure of hydrogen ion itself to show catalytic activity may be connected with the amphoteric nature of the flavins. Both azine nitrogens are basic. The assumption is made that the nitrogen at position 10 is much more strongly basic than that at position 9. At low pH values, protons attach themselves predominantly to position 10, producing a positive charge which makes it difficult for the nitrogen at position 9 to acquire a proton of its own. This diminishes the likelihood of complex reaction and it is distinctly possible that more than one mechanism is involved. Or, it may be due to a breakdown of energy-rich acetaldehyde.

Preparation of Compounds

3-Methyl-9-(hydroxyethyl)-isoalloxazine.—Methylalloxan was prepared from theobromine by the method of Biltz²²; 1.5 g. of N-hydroxyethyl-o-nitraniline, prepared by the method of Karrer¹⁰ was reduced to the diamine by catalytic hydrogenation. The theoretical amount of hydrochloric acid was added, then an aqueous solution of 1.0 g. of methyl alloxan monohydrate. The solution was boiled a few minutes, then cooled overnight in a refrigerator. Yellow crystals formed, which were recrystallized from boiling 5% acetic acid with the addition of a little animal charcoal. After washing with water, then with alcohol and ether, and drying to constant weight, the compound melted at 275–277° dec. cor. Elementary analysis for C, H and N showed good agreement with theory.

9-(2'-Acetylethyl)-isoalloxazine.—The procedure of Kuhn²³ for the acetylation of riboflavin was adapted. 0.2 g. of 9-(2'-hydroxyethyl)-isoalloxazine was dissolved in 5 ml. of boiling pyridine. After cooling, 50 g. of acetic anhydride was added. The solution was allowed to stand several hours, then boiled several minutes and cooled. The solution was made acid to brom phenol blue by addition of hydrochloric acid, and extracted five times with methylene chloride. The combined methylene chloride solutions were washed once with dilute hydrochloric acid, then with water. After drying over sodium sulfate, the solvent was evaporated off. The yellow residue was recrystallized three times from boiling water and dried to constant weight. The product melted at 240° d. cor. Analysis for C, H and N showed good agreement with theory.

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⁽²²⁾ H. Biltz, Ber., 45, 3674 (1912).

⁽²³⁾ R. Kuhn and T. Wagner-Jauregg, ibid., 66, 1577 (1933)