Anal. Calcd. for $C_6H_{10}O_3N_2$: C, 45.56; H, 6.37; N, 17.71. Found: C, 45.24; H, 6.18; N, 17.88.

Ethyl 2,3-Dihydro-5-methyl-2-oxo-4-imidazolecarboxylate.—α-Oximinoacetoacetic ester was reduced to the amino ketone by the method described by Ochiai and Ikuma[13] except that 5% palladium on charcoal catalyst was used in place of the catalyst containing 40% metal described by them. Treatment of the crude amino ketone hydrochloride with potassium cyanate afforded 85% of the theoretical yield of imidazolone. In order to obtain this high yield it was necessary to acidify the original mother liquors to *p*H 4.5 with mineral acid. The micro melting point of the product was 220–221° which agrees with the melting point given by Gabriel and Posner.[14]

5-Methyl-2-imidazolidone.—A mixture of 2.55 g. of ethyl 2,3-dihydro-5-methyl-2-oxo-4-imidazolecarboxylate and 9 cc. of 2 *N* sodium hydroxide was heated at 90–95° for three hours. The resulting solution was then adjusted to *p*H 7 by the addition of hydrochloric acid (vigorous evolution of carbon dioxide) and 45 cc. of 5% sodium bicarbonate was added. The mixture was hydrogenated at 150 atmospheres and 100° for forty-five hours. The solution was filtered and then acidified to congo red by the addition of 50% sulfuric acid. The solution was then concentrated to a volume of 40 cc. under reduced pressure and finally extracted continuously with a mixture of ethyl acetate and ether (85:15 by volume) for several days. The extraction liquid was concentrated to dryness. The residual crude product, m. p. 110–116°, weighed 1.28 g., 86% of the theoretical amount. After one recrystallization from benzene the slightly colored crystals melted at 120–122°. A sample for analysis was obtained by sublimation at 75–80° (1 mm.). The compound then melted at 121–122°.

Anal. Calcd. for $C_4H_8ON_2$: C, 47.98; H, 8.05; N, 27.98. Found: C, 48.22; H, 7.94; N, 27.95.

It was also possible to obtain the cyclic urea in one step by carrying out the saponification, decarboxylation and reduction with excess sodium bicarbonate in the hydrogenation apparatus.

Ethyl 5-Methyl-2-thiol-4-imidazoleacetate.—To a solution containing 2.18 g. of ethyl β-aminolevulinate hydrochloride, prepared from ethyl levulinate[28] as described in the general procedures above, was added 2.1 g. of potassium thiocyanate in a minimum quantity of water. The mixture was heated at 65–70° in an open 50-cc. Erlenmeyer flask for fourteen hours. At the end of this period the volume of liquid was about 15 cc. White plates separated. The mixture was cooled to room temperature and the product (604 mg.) was collected and washed with a small quantity of water. The mother liquor deposited additional material when it was cooled to 5°, but this precipitate was not investigated. The thiolimidazole ester was recrystallized from 95% ethanol and then melted at 217–218°.

Anal. Calcd. for $C_8H_{12}O_3N_2S$: C, 47.97; H, 6.04; S, 16.01. Found: C, 47.73; H, 5.80; S, 16.46.

Summary

The preparation of *homodesthiobiotin*, 5-methyl-2-oxo-4-imidazolidineheptanoic acid, from azelaic acid has been accomplished through the intermediate, ethyl 9-oxodecanoate. 5-Methyl-2-imidazolidone has been prepared and homologs in the dehydrodesthiobiotin series have also been described.

(28) Ruzicka, *Ber.*, **50**, 1362 (1917).

NEW YORK, N. Y. RECEIVED NOVEMBER 20, 1945

[CONTRIBUTION FROM THE DIVISION OF DAIRY RESEARCH LABORATORIES, BUREAU OF DAIRY INDUSTRY, AGRICULTURAL RESEARCH ADMINISTRATION, U. S. DEPARTMENT OF AGRICULTURE]

The Microbiological Synthesis of Riboflavin—A Theory Concerning its Inhibition

BY ABRAHAM LEVITON

In the microbiological synthesis of riboflavin, the presence of traces of iron salts results in striking decreases in yield. This phenomenon is of practical significance; and the definition of the lower limit of concentration at which a significant decrease in yield begins to take place forms the basis of one or more patents[1] which have been issued and put into practice.

For some time this Laboratory has been engaged in a study of the factors involved in the microbiological synthesis of riboflavin. In one series of experiments it was found that in media containing iron salts in critical concentrations, added riboflavin was destroyed. This result suggested that the action of iron was at least to some extent destructive rather than inhibitory. The explanation of the destructive action seemed to lie in the possibility that one or more riboflavin-destroying compounds were formed during the fermentation. The view that hydrogen peroxide in the presence of ferrous ion would bring about the decomposition of riboflavin suggested itself, in spite of the fact that riboflavin in the presence of pure hydrogen peroxide is quite stable.

This paper is concerned with experiments which serve to support this view; and the results of both chemical and microbiological studies are presented. The chemical studies describing the influence of ferrous salts (ferric salts are ineffective) on the decomposition of riboflavin by hydrogen peroxide were designed to demonstrate the existence of a parallelism between the influence on one hand of ferrous ion concentration on the rate of decomposition of riboflavin by hydrogen peroxide and the influence on the other hand of ferrous ion concentration on the yield of microbiologically synthesized riboflavin. The chemical studies were designed furthermore to demonstrate the antagonistic effect of catalase and reducing agents on the action of hydrogen peroxide; and corresponding microbiological studies were designed to show that the effect of catalase and reducing agents on the yield of riboflavin and on the rate of fermentation could reasonably be ascribed to a mechanism involving hydrogen peroxide.

(1) Meade, Pollard and Rogers, U. S. Patent 2,239,680 (1942).

Experimental

Chemical Studies.—Rate studies were carried out on solutions in stoppered, light-shielded bottles held at 37.0°. These solutions were, for the most part, made 26.5 × 10^{-6} M in riboflavin, 0.035 M in hydrogen peroxide and in most instances, 0.01 N in H_2SO_4. They were also made to contain the ferrous ion in concentrations varying from 0.18 to 1.8 milligram-atoms per liter. The riboflavin employed was a Merck synthetic product which had been purified by the recovery of crystals resulting from dilution of a solution of riboflavin in 46% nitric acid. Merck superhydrol was used as a source of hydrogen peroxide. The ferrous ion was added in the form of washed crystals of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ of reagent grade. Tests with potassium thiocyanate indicated the absence of Fe^{+++} Each solution was prepared to contain at first all of its constituents with the exception of Fe^{++}. After thermal equilibrium at the temperature of the thermostat had been established, a solution of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was added in appropriate quantities, and zero time was taken as the time at which the ferrous salt was added. As soon as possible thereafter, a 10 ml. sample was withdrawn for analysis and introduced into a large test-tube containing 0.35 ml. of N sodium hydroxide and 1.65 ml. of a solution containing 5% sodium hydrosulfite and 5% sodium bicarbonate. The sodium hydroxide neutralized the sulfuric acid and the hydrosulfite prevented further decomposition of riboflavin, and reduced it to its leuco form, from which it could be regenerated by vigorous agitation. The regenerated riboflavin was determined colorimetrically by means of a photoelectric colorimeter employing Corning filter No. 511, and a 6–8 volts, 3 c. p. incandescent lamp. Standard curves relating transmission to riboflavin were prepared to correspond to each variation in Fe^{++}. Samples were withdrawn periodically and subjected to the same treatment and analysis. The rate of decomposition of hydrogen peroxide was followed by iodimetric analysis.

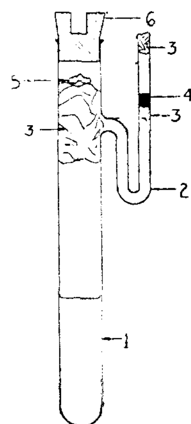

Fig. 1.—Anaerobic culture tube: 1, 22 × 175 mm. test tube; 2, trap; 3, cotton plugs; 4, one-way mercury valve; 5, pellet of solid carbon dioxide to sweep out air from tube through mercury valve; 6, rubber stopper, semisolid with recessed top.

Microbiological Studies.—The basal medium used in the microbiological experiments contained per liter: KH_2PO_4, 0.5 g.; $MgSO_4 \cdot H_2O$, 0.5 g.; $CaCl_2 \cdot 2H_2O$, 0.3 g.; $(NH_4)_2SO_4$, 2.0 g.; asparagine, 2.0 g.; starch, 30 g. on dry basis; biotin, 1 γ; p-aminobenzoic acid, 50 γ; yeast dialysate, 10 ml.; trace elements in p. p. m. B, 0.01; Mn, 0.01; Zn, 0.07; Mo, 0.01. The medium contained in addition 125 ml. per liter of either $M/5$ sodium pyruvate or sodium butyrate buffer pH 6.0. All chemicals employed were of the highest quality. The butyric acid was a Kahlbaum product purified by distillation. Yeast dialysate was prepared according to Weizman and Rosenfeld.[2] It contained 7.18 g. of solids per liter, and furnished to the medium supplementary factors which were required to obtain a normal rate of fermentation, that is, a rate comparable to that obtained with corn-mash media.[2] The butyric acid, sodium butyrate solution or the sodium pyruvate solution was also required to effect a normal rate of fermentation. Without its use, the rate curve was characterized by two lag periods, one a short period occurring at the outset, and the other a prolonged period occurring when the fermentation was approximately 25% complete. In this respect sodium butyrate and sodium pyruvate are only two of a large number of salts of organic acids which act in the same way to produce a smoother and more rapid fermentation. More important than their function to normalize the rate of fermentation, however, is their contribution to the microbiological synthesis of riboflavin. Without these salts, no significant quantity of riboflavin is synthesized. The subject of the influence of organic acids on the yield and synthesis of riboflavin will be presented in a subsequent paper.

The preparation of media consisted in the dispensing of 12.5 ml. of basal media (1.2 times normal concentration) in anaerobic culture tubes, adding the heat-stable substances under investigation and enough water to bring the volume to 15 ml., sterilizing at 120° for fifteen minutes and cooling to room temperature. Heat-labile substances were sterilized by filtration through a Seitz filter and added to the main portion of the medium after inoculation. A 2% inoculum of active cells in 5% corn mash was employed This inoculum was derived by a series of transfers from a heat-activated spore culture in corn mash. Activation was obtained by heat treatment for one minute at 100°. The organism *Clostridium acetobutylicum*, Weizmann strain no. 4259, was obtained from the American Type Culture Collection. Transfer to the synthetic media was made after approximately 25% by weight of the available gas had been evolved. The physiological state of the organism influences riboflavin yield, and consequently the age of the culture at the time of transfer is a factor which requires control. The rate of fermentation was followed by measurement of the weight of the evolved gases. The anaerobic tube employed was quite adaptable for this purpose. The design of this tube is shown in Fig. 1.

The catalase employed was a twice-recrystallized product obtained from beef liver by the method of Dounce.[3] Sterile solutions in phosphate buffer, pH 7.0, were prepared by filtration, and, based on the Kat. f. value (26,000) of the catalase, a sterile solution containing one microgram per ml. was prepared.

Solutions of the reducing agents KI and $NaHSO_2$ were prepared just prior to use and were sterilized by filtration.

Riboflavin was determined fluorimetrically on an aliquot which had been brought to a pH of 6.8–7.0, and to which approximately 0.01 g. of calcium chloride had been added. Upon heating at 100° for five minutes with light excluded, cooling to room temperature, diluting and centrifuging, a clear solution was obtained which could be used for fluorimetric analysis.

A variety of analyses was run to yield complete carbon balances. In all samples under investigation a normal fermentation was obtained with respect to the yield of volatile solvents. The yield of acetone and ethyl alcohol showed significant increases relative to that of butyl alcohol as the iron content of the medium was increased.

Results

Table I contains the results of microbiological experiments in which the destructive action of iron on riboflavin is illustrated. Comparison of media containing less than and more than the critical concentration of iron shows that iron in more than the critical concentration contributes to the destruction of practically all of the added riboflavin. Under the conditions of this experiment, the addition of catalase, potassium iodide and $NaHSO_2$ exerts a pronounced stabilizing action on the riboflavin added to the medium. In the medium containing less than the critical concentration of iron, the recovery of added riboflavin is assumed to be complete inasmuch as the total quantity of riboflavin recovered exceeds appreciably the quantity added, and since no attempt was made to distinguish between added and microbiologically synthesized riboflavin. In media containing more than the critical concen-

(2) Weizman and Rosenfeld, *Biochem. J.*, **31**, 628 (1937).

(3) Dounce, *J. Biol. Chem.*, **143**, 499 (1942).

TABLE I

INFLUENCE OF CATALASE AND REDUCING AGENTS ON RECOVERY OF ADDED RIBOFLAVIN IN MEDIA CONTAINING MORE THAN THE CRITICAL CONCENTRATION OF IRON

Medium	Total Fe in medium, mg. atoms per liter	Catalase added, mg. per liter	Millimoles per liter KI added	Millimoles per liter $NaHSO_2$ added	Micromoles per liter Riboflavin added	Total riboflavin recovered	Added riboflavin recovered	Added riboflavin recovered, %
Basal	0.20	...	...	...	..	18.6	..	...
	.20	...	...	...	13.3	26.5	13.3	100
	.38	...	...	...	..	0.9	..	...
	.38	...	...	...	13.3	1.0	0.1	0.8
	.38	0.1	...	...	13.3	9.6	8.7	66.0
	.38	...	0.1	...	13.3	10.0	9.1	69.0
	.38	...	...	0.2	13.3	10.0	9.1	69.0

tration, recovery values were calculated on the assumption that the yield of microbiologically synthesized riboflavin was 0.9 micromole per liter, the yield obtained in the absence of catalase, potassium iodide and $NaHSO_2$.

Table II contains the results of microbiological experiments in which the concentration of iron is varied from 0.20 to 0.36 mg. atom per liter. The results are given for two sets of experiments, one of which shows the influence of iron on the yield of riboflavin, and on rate of fermentation, and the other of which shows the influence of iron in corresponding media containing per liter 0.07 mg. of crystalline catalase. Of interest is the sharp decrease in yield at approximately the same concentration of iron in both sets of experiments. Of particular interest is the influence of catalase in markedly increasing the yield of riboflavin approximately 250% in media containing up to 0.30 mg. atom of iron per liter.

TABLE II

SHOWING INFLUENCE OF CATALASE ON THE MICROBIOLOGICAL SYNTHESIS OF RIBOFLAVIN IN MEDIA CONTAINING VARYING QUANTITIES OF IRON. BASAL MEDIA EMPLOYED CONTAINING SODIUM PYRUVATE BUFFER

Total Fe in medium,[a] mg. atoms per liter	Catalase added, mg. per liter	Riboflavin synthesized, micromoles per liter	Maximum rate of fermentation, g. gas per liter per hour
0.200	..	18.8	0.19
.218	..	18.8	.19
.254	..	11.7	.30
.290	..	6.1	.60
.344	..	1.6	.70
.200	0.07	40.0	.50
.218	.07	43.9	.70
.254	.07	35.0	1.00
.290	.07	27.9	1.00
.344	.07	1.3	1.00

[a] *p*H of medium 6.0. Temp. of incubation −37°.

The maximum rates of fermentation are also given. According to Weizmann and Rosenfeld, the maximum rate of fermentation is independent of the amount of bacteria in the inoculum or of any other influence on the initial growth period. The rate values were obtained from the plot of the weight of evolved gases as a function of the time of fermentation. Comparison of these values with the values usually associated with a normal maize mash fermentation (0.6–0.7 g./liter/hr.) shows that even in the presence of only insignificant quantities of particulate matter, and of suboptimal concentrations of iron, catalase activates the fermentation to an extent considerably beyond the activity of a normal maize fermentation.

Table III illustrates how catalase, potassium iodide and $NaHSO_2$ influence the yield of riboflavin in media containing iron at or near its critical concentration. Although inhibitory to the fermentation at high concentrations, $NaHSO_2$ at lower concentrations neutralizes in part at least the destructive action of iron. Catalase is much less effective in this respect, and potassium iodide actually contributes to the inhibition. Values are given for the maximum rate of fermentation and these indicate a common function of the reducing agents and catalase in increasing the rate. A concentration of sodium hydrosulfite of approximately one millimole per liter is inhibitory, and maximum stimulation is obtainable below 0.3 millimole per liter.

TABLE III

SHOWING INFLUENCE OF CATALASE AND REDUCING AGENTS ON YIELD OF RIBOFLAVIN IN MEDIA CONTAINING IRON AT OR NEAR CRITICAL CONCENTRATIONS. BASAL MEDIUM EMPLOYED CONTAINING SODIUM BUTYRATE BUFFER

Total Fe in medium,[a] mg. atoms per liter	Catalase added, mg. per liter	Millimoles per liter KI added	Millimoles per liter $NaHSO_2$ added	Riboflavin synthesized, micromoles per liter	Maximum rate of fermentation, g. gas per liter per hour
0.20	..	..	...	19.0	..
.33	..	..	...	2.1	0.22
.33	..	..	0.057	5.3	.60
.33	..	..	0.285	4.8	.22
.33	..	..	1.14	..	No action
.33	..	0.03	...	1.3	.75
.33	..	.15	...	1.3	.60
.33	..	.60	...	1.1	.40
.33	0.07	..	...	2.6	.40

[a] *p*H of medium, 6.0. Temp. of incubation, 37°.

The results obtained in chemical experiments are shown in Fig. 2. The rates of decomposition of riboflavin and of hydrogen peroxide are plotted

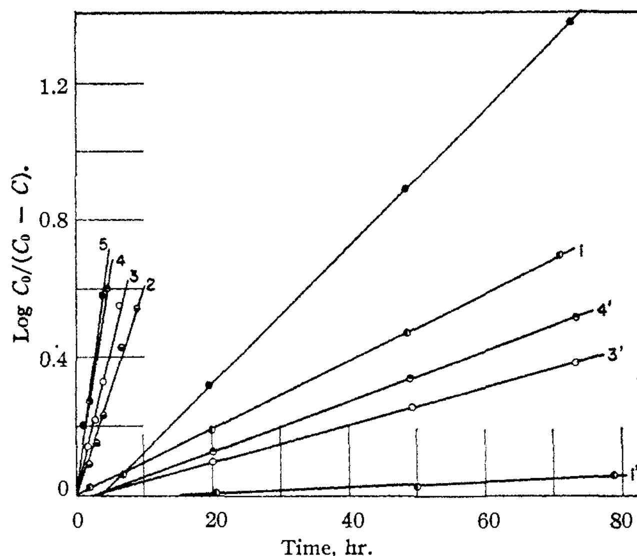

Fig. 2.—The rate of decomposition of riboflavin and hydrogen peroxide in the presence of varying concentrations of the ferrous ion. Primed numbers refer to rate curves for peroxide decomposition. Unprimed numbers refer to rate curves for riboflavin decomposition: 1, 1′ 0.18 mg. atom per liter Fe^{++}; 2, 0.36 mg. atom per liter; 3, 3′, 0.54 mg. atom per liter; 4, 4′, 0.90 mg. atom per liter; 5, 5′, 1.8 mg. atoms per liter.

as functions of ferrous ion concentration. In making these plots it was assumed that no instantaneous decomposition reactions were involved, and that changes in light absorption were due uniquely to changes in riboflavin concentration. These assumptions, it is believed, do not influence the main body of conclusions reached in this paper, although from the standpoint of reaction kinetics, a subject outside the scope of this paper, the assumptions require careful examination.

Appropriate control runs (data not given) indicate that riboflavin is quite stable in solutions containing peroxide but no ferrous ion, and in solutions containing ferrous ion but no peroxide.

Of interest in the rate studies is the sharp increase in the rate of decomposition occurring between 0.18 and 0.54 mg. atom per liter of ferrous ion. Thus in this region the rate constant changes from 2.3 to 18.9% per hour. Of further interest is the crowding together of the rate curves for solutions containing 0.9 and 1.8 mg. atoms per liter of ferrous ion. The rate process is essentially that for a unimolecular reaction. Rate constants wherever they are stated are obtained from the slopes of the lines (shown in Fig. 2) multiplied by the logarithmic conversion factor, 2.3. The simultaneous catalytic decomposition of hydrogen peroxide is characterized by a lag phase followed by a phase in which the rate process is unimolecular. The fractional rate of decomposition of hydrogen peroxide is quite small compared to the corresponding rate for the decomposition of riboflavin.

Not shown in the figure, but pertinent to the subject, are the results obtained in the presence of potassium iodide, and catalase. Thus the value of the unimolecular rate constant for the decomposition of riboflavin by hydrogen peroxide in an unacidified solution containing 0.36 mg. atom per liter of ferrous ion is 11.7% per hour. The corresponding value for the decomposition in a solution containing 0.167 millimole per liter of potassium iodide is less than 0.2% per hour. The respective values for the rate constants governing the decomposition of hydrogen peroxide are 0.62 and 2.7 per cent. per hour. Thus potassium iodide accelerates the rate of decomposition of hydrogen peroxide, and at the same time stabilizes riboflavin against peroxide. Catalase in a concentration of 0.07 mg. per liter behaves in the same manner as potassium iodide in stabilizing riboflavin against peroxide. The concentration of catalase employed was sufficient to reduce the concentration of peroxides approximately 80% in fifteen minutes. Although the stabilizing action of catalase can be explained on the basis of relative reaction rates, it is doubtful whether the action of potassium iodide can be explained on a similar basis. The function of potassium iodide goes further than its function in reducing the concentration of peroxide as a comparison of the relative reaction rates indicates.

Discussion

The peroxidase-like effect of heavy metal systems is well known. With regard, however, to the participation of heavy metals in anaerobic process, "our knowledge," to quote Oppenheimer and Stern,[4] "has so far not penetrated beyond a few isolated observations and details."

The synthesis of abnormally large quantities of riboflavin by the organism *Clostridium acetobutylicum* is in itself suggestive of the possibility that hydrogen peroxide is a product of fermentation. Riboflavin combined with adenine and phosphoric acid forms the prosthetic group of enzyme systems which are capable of producing hydrogen peroxide. Bertho and Glück[5] in a series of papers showed that catalase-free facultative anaerobic lactic acid bacteria underwent a forced respiration in the presence of oxygen to yield hydrogen peroxide. Later Warburg and Christian[6] proved that in Bertho's experiments the presence of a yellow flavin-containing enzyme (Warburg's yellow ferment) was responsible for the entire respiration.

Although in the *Clostridium acetobutylicum* fermentation it has not been possible to demonstrate the presence of hydrogen peroxide, the cause for this failure can be attributed, as a matter of speculation, to the decomposition of hydrogen

(4) Oppenheimer and Stern, "Biological Oxidations," Verlag W. Junk, Amsterdam, Holland, 1939, p. 71.

(5) Bertho and Glück, *Ann.*, **494**, 159 (1932)

(6) Warburg and Christian, *Biochem. Z.*, **260**, 499 (1933).

peroxide in the course of the fermentation. Regarding the possibility of the formation of hydrogen peroxide, it is necessary to assume either the presence of traces of oxygen, or the existence of hydrogen acceptors other than molecular oxygen which will react with reduced flavin-containing enzyme systems to yield hydrogen peroxide.

The data presented in this paper are difficult to interpret on any other basis. The occurrence of an abrupt change in values in the same concentration range of ferrous ion in the curves (see Fig. 2) showing (1) the change in the velocity constant of the decomposition of riboflavin by hydrogen peroxide, and (2) the change in the quantity of microbiologically synthesized riboflavin (see Table II) can hardly be fortuitous. The action of 0.07 mg. per liter of catalase in increasing the yield of microbiologically synthesized riboflavin approximately 250% can only be interpreted safely on the basis of its enzyme activity. This coupled with the observation that in the presence of catalase, riboflavin is not attacked by the combination hydrogen peroxide and ferrous ion, appears to be strong evidence in support of the view that hydrogen peroxide is the active agent responsible for the microbiological destruction of riboflavin. It is interesting to note that the yield of riboflavin is not increased by catalase once the critical concentration of iron is exceeded. This can be interpreted to mean that the synthesis and destruction of riboflavin is largely an intracellular process, in which the added catalase functions extracellularly. Thus if the riboflavin is destroyed before it can diffuse through the cell wall, the presence of catalase in the medium is without influence. On the other hand, if the concentration of the ferrous ion is low enough, riboflavin and hydrogen peroxide can accumulate, and will diffuse through the cell wall; and under these conditions it is reasonable to expect that catalase will inhibit the relatively slow decomposition of riboflavin. Supporting this conclusion are the experiments (see Table III) with catalase and with reducing agents in which the medium was adjusted to contain ferrous ion near its critical concentration. The significantly greater yield of riboflavin in the media containing sodium hydrosulfite compared with the yields in the control medium and in that containing catalase, indicates a function of the reducing agent not shared by catalase, a function which may reasonably be ascribed to its diffusibility and ability to penetrate into the interior of the cell.

Experiments with potassium iodide, although not as convincing, are not inconsistent with the views advanced. The results given in Table I show that riboflavin added to a medium containing iron in excess of its critical concentration is largely stabilized by potassium iodide. Thus, toward preformed riboflavin, potassium iodide acts as a stabilizer in the same manner as catalase and sodium hydrosulfite, a result in harmony with the results of the chemical experiments on the stabilizing influence of potassium iodide. However, potassium iodide in the presence of ferrous ion near its critical concentration brings about a decrease rather than an increase in the yield of microbiologically synthesized riboflavin. This phenomenon can best be explained on the basis of the specific inhibitory action of potassium iodide in the presence of ferrous ion, on the synthesis of riboflavin. Tests indicate that the iodide ion is transformed during the fermentation into organic iodine. This transformation quite likely proceeds through a mechanism involving the oxidation by peroxide of the iodide ion to iodine which then reacts in part with the intermediates involved in the synthesis of riboflavin.

A feature in common possessed by catalase, sodium hydrosulfite and potassium iodide is their effect on the rate of fermentation. The maximum rate may be increased more than 180% due to the presence of catalase, more than 280% due to the presence of sodium hydrosulfite, and more than 340% due to the presence of potassium iodide. These increases are not necessarily maximal. The reducing agents also exert a specific retarding effect in addition to their accelerating action, with the first mentioned action predominating at relatively high concentrations. The results nevertheless are suggestive of a common mechanism involving possibly a mutual capacity to accelerate the destruction of the inhibitory agent—hydrogen peroxide.

Although it is not the purpose of the paper to discuss reaction kinetics, and as a matter of fact the data are far too incomplete to permit it, there are interesting qualitative aspects of the data which at this time can only be indicated.

(1) The proximity of the curves showing the rate of decomposition of riboflavin at concentrations of the ferrous ion equal to 0.90 and 1.8 milligram atoms per liter, would seem to indicate that the rate determining reaction involves a complex compound of riboflavin and the ferrous ion.

(2) The inactivity of the ferric ion compared with the ferrous ion may be taken to mean that any complex involving riboflavin and the ferric ion is highly dissociable. The existence of stable ferrous ion complexes with organic reagents (*o*-phenanthroline, α,α'-dipyridyl and dithizone) to the practical exclusion of similar complexes with the ferric ion are analogous phenomena.

(3) The mechanism involving the reaction with hydrogen peroxide is not clear, but the data are not inconsistent with the views advanced by Michaelis and Barron[7] to explain the oxidation of cysteine in the presence of the ferrous ion, through the formation of the intermediate, ferrous cysteine. The primary reaction on this basis would involve the formation of a ferric riboflavin complex which would decompose instantaneously to yield oxidation products of riboflavin and the

(7) Michaelis and Barron, *J. Biol. Chem.*, **81**, 29 (1929).

ferrous ion. This reaction it must be assumed would be much more rapid than the dissociation of the complex into the ferric ion and riboflavin.

(4) The catalytic decomposition of hydrogen peroxide proceeds simultaneously with the reaction involving hydrogen peroxide and riboflavin. The existence of a lag period may mean that the ferrous ion reacts preferentially with riboflavin, and that consequently the initiation of the decomposition of peroxide depends upon the availability of the ferrous ion. This type of mechanism would indicate that the decomposition of riboflavin proceeds independently of the catalytic decomposition of peroxide or of intermediate products such as ferrates, the formation of which have been demonstrated to attend peroxide decomposition in the presence of iron. This conclusion is supported further by the experiments with catalase and potassium iodide. In these experiments the formation of intermediates leading to an acceleration of the decomposition of peroxides does not lead to a corresponding acceleration of the decomposition of riboflavin but rather to a sharp retardation.

It is clear from the foregoing that the practice in analytical procedures for riboflavin involving the use of peroxides in high concentrations as a reagent for the destruction of interfering pigments is open to criticism. The use of this reagent is justified only in the absence of traces of the ferrous ion and perhaps of other metallic ions.

Summary

Riboflavin which in pure solutions is exceedingly stable to the action of hydrogen peroxide is decomposed rapidly by dilute solutions of this reagent in the presence of traces of the ferrous ion.

The rate of decomposition increases abruptly between 0.18 and 0.36 milligram atom of the ferrous ion per liter.

In the microbiological synthesis of riboflavin by the organism *Clostridium acetobutylicum* a drastic reduction in the yield of riboflavin occurs precisely in this concentration range of the ferrous ion. Added riboflavin is also destroyed in this range, and this suggests that the action of the iron is in part at least destructive rather than inhibitory.

The view that the destruction of riboflavin by the organism *Clostridium acetobutylicum* operates through a peroxide mechanism is supported by experiments in which significant increases in yield of riboflavin are obtained by the use of sodium hydrosulfite and traces of crystalline catalase.

Iodide ion stabilizes riboflavin against the action of hydrogen peroxide *in vitro* and *in vivo* but is inhibitory to the microbiological synthesis of riboflavin. This is explained on the basis of the independent inhibitory action of iodine ion operating through a mechanism in which iodine formed by the action of hydrogen peroxide reacts in the presence of the ferrous ion with the precursors of riboflavin.

Simultaneous with its action on riboflavin, hydrogen peroxide undergoes a thermal decomposition which is catalysed by the ferrous ion. This decomposition is characterized by a lag period during which the greater portion of riboflavin is destroyed.

Ferrous and not ferric ion activates the decomposition of riboflavin.

The use of hydrogen peroxide to destroy interfering pigments in analytical procedures for the determination of riboflavin is justified only in the absence of traces of the ferrous ion and perhaps of other metallic ions.

WASHINGTON, D. C. RECEIVED JANUARY 9, 1946

[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, NORTHWESTERN UNIVERSITY DENTAL SCHOOL]

Pressor Amines Containing Nuclear Chlorine and Fluorine[1,2]

BY L. S. FOSDICK, O. FANCHER[3] AND K. F. URBACH[4]

A great number of pressor amines have been synthesized and investigated in the past; however, compounds of this type containing nuclear halogen have received comparatively little attention.

Zeynek[5] reported the preparation of 3,5-dichloro- and 3,5-dibromotyramine. Glynn and Lynell[6] prepared 1-(3,4-dichlorophenyl)-2-aminoethanol for which they reported both decreased activity and toxicity as compared to epinephrine. Edkins and Lynell[7] also synthesized several derivatives of ω-aminoacetophenone containing nuclear bromine and chlorine. Other compounds of this nature previously described include 3-fluorophenethylamine,[8] 3-fluoro-4-hydroxyphenethylamine,[8] *p*-fluorophenethylamine and its N-methyl analog[9] and the *p*-chloro analog of propadrine.[10] Hansen[11] prepared 3-fluoro- and 3-chloro-4-hydroxy-ω-methylaminoacetophenone. He, as well as Edkins and Lynell, was unable to reduce the

(1) This paper represents part of the thesis material submitted by Mr. Fancher and Mr. Urbach at Northwestern University Graduate School.

(2) This work was done under a grant from the Abbott Foundation.

(3) Present address: G. D. Searle and Co., Chicago, Illinois.

(4) Present address: Pharmacology Dept., Northwestern University Medical School.

(5) Zeynek, *Z. biol. Chem.*, **114**, 275 (1921).

(6) Glynn and Lynell, *Quart. J. Pharmacol.*, **5**, 480 (1932).

(7) Edkins and Lynell, *ibid.*, **9**, 75 (1936).

(8) Schiemann and Winkelmüller, *J. prakt. Chem.*, **135**, 101 (1932).

(9) Suter and Weston, THIS JOURNAL, **63**, 602 (1941).

(10) Hartung, Munch and Crossley, *ibid.*, **57**, 1091 (1935).

(11) Hansen, *ibid.*, **59**, 280 (1937).