THE THERMAL DESTRUCTION OF RIBOFLAVIN

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Summary

The thermal destruction of riboflavin in buffer solutions from pH 0.5 to 12.0 has been studied by means of fluorimetric analysis and the application of the first-order reaction and Arrhenius equations. The buffer solutions used were a modified universal buffer (phosphate, borate, acetate), McIlvaine's citric acid-phosphate buffer, veronal-HCl, and glycine-NaOH.

Riboflavin in concentrations of 5 μg/ml is completely stable for 1 hr from pH 1.3 to 6.5 at 80°C, from pH 1.7 to 5.5 at 100°C, and from pH 2.0 to 5.0 at 120°.

Destruction at the higher pH's follows the first-order reaction and the Arrhenius equation relating rate of destruction to temperature. There is also a linear relation between the logarithm of the concentration of riboflavin and k, the rate constant.

At pH values below 7, the relation between log k and pH is roughly linear, but over the whole range studied kinetically (pH 5.0 to 9.5), and especially above pH 7.5, there is clear evidence of the varying catalytic effect of different buffer anions on the rate of destruction.

It has been shown by paper chromatography that the products of the thermal hydrolysis of riboflavin are ammonia, urea, 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxalinecarboxylic acid, and an unidentified compound giving a weak mauve fluorescence in ultraviolet light.

Destruction of riboflavin at the very low pH values is a reversible reduction rather than a hydrolysis, as most of the fluorescence lost can be regenerated by aeration. Chromatography of these solutions yielded only riboflavin.

I. Introduction

Since the days when 'water soluble B' was first separated into heat-labile B_1 and heat-stable B_2, riboflavin has been considered to be stable to heat, at least under the conditions to which foodstuffs are exposed.

It is known that riboflavin is not stable in alkaline solutions (Chick and Roscoe 1930; Kuhn and Moruzzi 1934), and its alkaline hydrolysis has been described (Surrey and Nachod 1951). Loy, Haggerty, and Combs (1951) have recently recorded the loss of riboflavin on heating for 1 hr at 121-3°C at pH 0-13. They showed that, under these conditions, there is a rapid destruction below pH 1 and above pH 5.4.

This paper presents a kinetic study of the thermal destruction of riboflavin, records the catalytic effect of various buffer salts, temperature, and riboflavin concentration, and describes the products of the reaction. While it was being

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completed, a report of the work of Shimizu (1950) has appeared. Unfortunately, the original reference is not available in this country, but it would appear that similar results were obtained at much lower temperatures. The different effects of various acids and buffer salts on riboflavin destruction in solutions of increasing pH were noted, and destruction was found to be greater at low concentrations.

The chromatography of riboflavin and its photodegradation products has been described by Hais and Pecakova (1949). Good separation of the various compounds, riboflavin, lumiflavin, and lumichrome, as well as two unidentified spots, was obtained using butanol-acetic acid-water (3:4:7). These authors studied only the products of the irradiation of riboflavin.

According to Surrey and Nachod (1951), the most likely products of the thermal destruction of riboflavin at high pH values are urea and 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxalinecarboxylic acid (referred to hereafter as 'the quinoxaline compound') which they found on hydrolysis with NaOH. Accordingly, it was decided to find out whether paper chromatography could be used to detect these compounds. This work is described in the second part of this paper.

II. Experimental

(*a*) *Reagents*

(i) *Buffer Solutions.*—The following solutions were prepared from A.R. reagents and glass-distilled water:

A mixed acid solution 0.04M with respect to phosphoric, boric, and acetic acids.
Citric acid, 0.1M.
Boric acid, 0.2M.
Disodium hydrogen phosphate, 0.2M.
Glycine, 0.1M.
Sodium acetate, 1M.
Sodium borate, 0.05M.
Sodium diethyl barbiturate (veronal, B.P.), 0.1M.
Sodium hydroxide, 0.2M.
Hydrochloric acid.

Analysis with the dithizone method has shown heavy metals to be present in the test solutions in quantities less than 0.5 p.p.m. (total) and usually of the order of 0.1 p.p.m.

(ii) *Riboflavin Stock Solution.*—Riboflavin (25 mg) was dissolved in glass-distilled water, acidified with a few drops of glacial acetic acid, and made up to 500 ml; 1 ml of this solution contained 50 μg of riboflavin.

(*b*) *Method*

Riboflavin was assayed fluorimetrically with a Coleman Model 12B Photofluorimeter according to the method of Scott *et al.* (1946), but without the acid-acetone extraction, which was not necessary with aqueous solutions.

To confirm that the fluorescent quinoxaline compound was unlikely to interfere with the fluorimetric determination of riboflavin, a solution of the former of the same concentration as the riboflavin solutions used (5 μg/ml) was examined in the fluorimeter. The solution scarcely fluoresced in unfiltered ultraviolet light, and with the same filters as are used for riboflavin estimations there was no galvanometer deflection.

TABLE 1

LOSS OF RIBOFLAVIN FROM SOLUTION AT LOW pH

Reagent	pH	Percentage Riboflavin Recovered			
		After Time at 100°C		After 1 Hr Aeration	After 1 Hr 100°C, Then 1 Hr Aeration
		0 Min	60 Min		
Conc. H_2SO_4, 10 ml	0·73	96·2	98·8	98·8	—
Conc. H_2SO_4, 15 ml	0·30	88·5	90·6	98·5	100·0
75% H_3PO_4, 10 ml	1·38	94·2	94·2	98·8	—
75% H_3PO_4, 15 ml	0·78	91·1	90·0	98·5	100·0
Conc. HCl, 15 ml	0·75	88·7	89·7	95·3	98·2
Conc. HCl, 17·5 ml	0·60	83·5	83·5	95·0	100·0
1M NaAc:1M HCl buffer	1·02	93·5	91·2	98·6	98·5
1M NaAc:1M HCl, buffer	1·07	91·8	92·1	97·2	100·0
McIlvaine buffer	7·14	92·0	56·5	—	58·2
McIlvaine buffer	8·23	92·0	17·7	—	20·8

(c) *Procedure*

(i) *Riboflavin Recovery Tests*

pH 0-2.0.—Ten ml of riboflavin stock solution were diluted with water, the pH adjusted to the desired value with HCl, and the volume then made up to 100 ml.

pH 2.0-8.3.—Ten ml of riboflavin stock solution and 20 ml of McIlvaine's phosphate-citric acid buffer solution (Britten 1932) at the desired pH were mixed together and diluted to 100 ml.

pH values above 8.5.—Ten ml of riboflavin stock solution were diluted with water, the pH adjusted to the desired value with NaOH, and the volume then made up to 100 ml.

Each of these solutions contained 5 μg of riboflavin per ml. They were all treated similarly.

Twenty ml of the solution were sealed into 50-ml glass ampoules for tests at 120 and 80°C. These were wrapped in tin foil to protect the solution from

light, and held in an oil bath at 120-3 and 79-82°C respectively. After 1 hr, the ampoules were cooled, opened, and riboflavin determined. For tests at 100°, the solution as prepared was boiled under reflux in a flask protected from the light. After 1 hr the cooled sample was assayed.

The pH of all test solutions was determined before and after heating. The values given are "mean" readings and all fell within a range of ± 0.1 pH units. All pH values reported were determined with the glass electrode.

(ii) *Experiments at Low pH*

One hundred ml of various solutions as shown in Table 1 were made up to contain 5 μg of riboflavin and the volume of acid or concentration of buffer shown in the table. pH and riboflavin concentration were determined immediately, and the sample divided into two parts. The first part was aerated for 1 hr at room temperature. The second was boiled for 1 hr, assayed, and in most cases then aerated for 1 hr at room temperature and again assayed.

(iii) *Determinations of Rate Constants*

Universal buffer solution.—Ten ml of riboflavin stock solution and 50 ml of the mixed acids buffer solution were mixed together and adjusted to the desired pH with 0.2M NaOH. The volume was then made up to 100 ml with glass-distilled water and the pH checked.

Phosphate-citric acid buffer solution (McIlvaine's).—Ten ml of riboflavin stock solution, x ml of 0.2M Na_2HPO_4, and $20 - x$ ml of 0.1M citric acid were mixed together in a 100-ml volumetric flask. The value of x depended on the pH desired. The solution was made up to volume with distilled water and the pH checked.

Sodium diethyl barbiturate-hydrochloric acid buffer solution.—These solutions were prepared as described above (see (ii)) using x ml of 0.1M sodium diethyl barbiturate solution and $10 - x$ ml of 0.1M HCl.

Glycine-sodium hydroxide buffer solution.—These solutions were prepared as described above, using x ml of 0.1M glycine and $10 - x$ ml of 0.1M NaOH.

All solutions were transferred to a 250-ml flask connected to a reflux condenser by means of a ground-glass joint, and heated within a small enclosed boiling water-bath fitted with a lid, so that no light fell on the solution. Samples were withdrawn at regular intervals, cooled, and 5-ml aliquots taken for assay.

(*d*) *Paper Chromatography*

Paper chromatography was used to investigate solutions of riboflavin which had been boiled under various conditions. All chromatograms were developed by ascending flow in closed tanks covered with brown paper to exclude light. Developing solutions were butanol-acetic acid-water (4:1:5) and butanol-pyridine-water (3:2:5). Whatman No. 3 MM chromatography paper was used throughout and the chromatograms examined in daylight and in ultraviolet light.

III. Results and Discussion

(*a*) *Recovery at Different Temperatures*

The first step was to repeat the work of Loy, Haggerty, and Combs (1951) in which solutions of riboflavin at various pH's and in various buffer solutions were held at 120°C. Temperatures of 100 and 80°C were also explored. As already noted, the concentration of riboflavin was only 5 μg/ml, not 50. The former is closer to potencies normally found in foods. Results are shown in Figure 1.

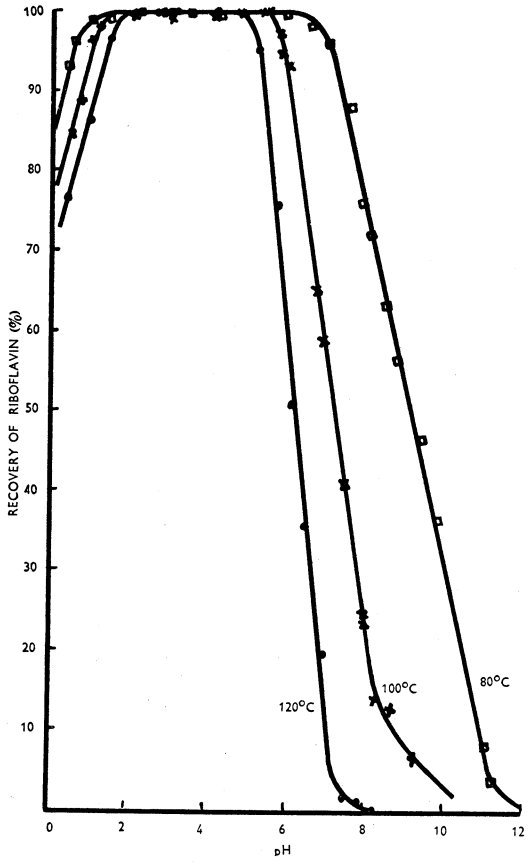

Fig. 1.—Recovery of riboflavin from solution (5 μg/ml) after heating at various pH for 1 hr.

It can be seen that the results obtained at 121-3°C are very close to those obtained by Loy, Haggerty, and Combs although the 'plateau' is rather narrower, the range of complete stability being from pH 2.0 to 5.0 (1.0-5.4 for Loy, Haggerty, and Combs). This is probably due to the much lower riboflavin concentration. The point of complete destruction (pH 8) is almost identical with that shown by the earlier workers.

As may be expected, the range of complete stability is lengthened by lowering the temperature. At 100°C it is 1.7-5.5 and at 80° 1.3-6.5.

It was then asked whether the loss of riboflavin at very low pH was a true thermal destruction, or whether it was in fact due to the high hydrogen ion concentration. Preliminary tests suggested the latter, as a loss obtained on making up the test solutions was not increased by subsequent boiling.

The series of experiments recorded in Table 1 was then carried out. It is seen that at low pH there is incomplete recovery of riboflavin from cold unheated solutions, and that subsequent boiling for 1 hr does not increase the apparent loss. On bubbling air through the solutions, either before or after heating, the loss can be almost completely recovered. This is in marked contrast to the behaviour of the solutions at alkaline pH, in which large losses occur and subsequent aeration has no effect. It would appear that the losses of riboflavin at very low pH are due to a reversible reduction very different from the irreversible hydrolysis which occurs at the higher pH.

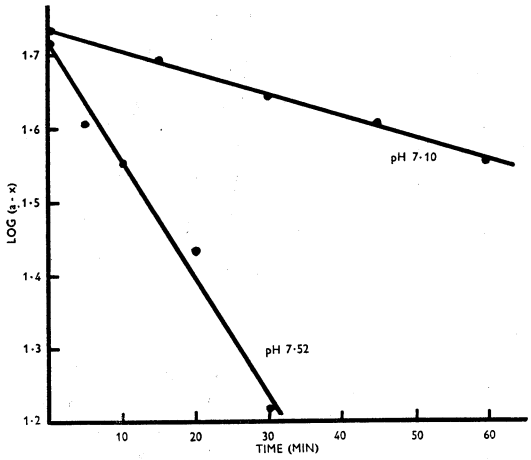

Fig. 2.—First-order reaction curves for riboflavin destruction at 100°C.

(*b*) *Effect of Buffer Anions*

Generally speaking, the results of this work have been interpreted in terms of simple reaction kinetics. The galvanometer reading for any given sample is proportional to the riboflavin in solution, and hence is equivalent to the term $(a-x)$ in the first-order reaction equation

$$k = \frac{1}{t} \ln \frac{a}{a-x} ,$$

where k is the rate constant, a the initial concentration of the substance studied, and x the amount of substance destroyed in t min.

That the thermal destruction of riboflavin is a first-order reaction is shown by the linearity of the curves in Figure 2 in which are plotted log $(a-x)$ against

t for riboflavin destruction in, for example, phosphate-citric acid buffer solutions at pH 7.10 and 7.52. The temperature in the examples given was 100°C. Surrey and Nachod (1951) in their discussion of the alkaline hydrolysis of riboflavin showed from optical density figures that the degradation of a 5 per cent. solution in 0.25N NaOH at 25°C was a first-order reaction for which $k = 2.7 \times 10^{-4}$ min^{-1}.

From the gradients of these curves k, the rate constant, can be calculated, and this has been done for all buffer solutions studied. Log k has been plotted against pH and the curves shown in Figure 3 obtained.

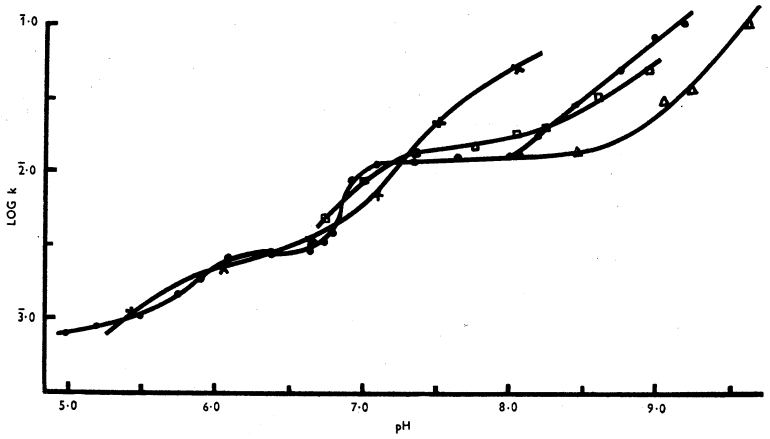

Fig. 3.—Log k/pH curves for the destruction of riboflavin in buffer solutions at 100°. ●, Modified universal buffer; ×, McIlvaine's citric acid-phosphate buffer; □, veronal-HCl buffer; △, glycine-NaOH buffer.

The first buffer studied was the phosphate-citric acid (McIlvaine's) buffer. The shape of the curve strikingly resembles a similar curve obtained with aneurin in the same buffer solution, i.e. a sigmoid curve with points of inflexion corresponding with the points of inflexion in the titration curve of H_3PO_4 (Farrer 1945). With riboflavin this curve is displaced towards the higher pH values but nevertheless it would appear that the considerations of the effect of ionic environment on the rate of destruction of aneurin (Farrer 1945) apply also to riboflavin.

The pH/log k curve for the modified universal buffer solution shows the same effect and reinforces the suggestion that the destruction of riboflavin as well as that of aneurin proceeds at different rates under the influence of different anions. It is noteworthy that below pH 7, if pH alone and not buffer ions also were considered, the curve obtained would be almost linear. It is above neutral point that big differences appear. The modified universal buffer solution contains boric acid, which has been shown to form with riboflavin (Frost 1942) a complex which is stable on the alkaline side of 7 but is unstable on the acid side. This appeared to provide a likely explanation for the very different behaviour of riboflavin in the two buffer solutions at pH's above 7. However, of repeated attempts to demonstrate the presence of a complex by

using paper chromatography, none succeeded. Frost (1942) has already failed to show that the complex had any effect on freezing point depression, electrometric titration, precipitation with heavy metals, specific rotation, and transmission curves.

When the barbiturate buffer was used, it was found that the curve obtained was very close to that of the universal buffer. This too discounted the suggestion that the shape of the latter was due to a boron-riboflavin complex.

Further point is given to the buffer effect by the results obtained with the glycine-NaOH buffer (pH range 8.2-10.0) It can be seen that it extends still further the range of pH maximum (of about 1.5 pH units) over which it is possible to obtain the same rate of destruction. These curves show clearly the effect of different anions at given pH values, especially above pH 7.

Calculations and some preliminary experiments suggest that the variations of ionic strengths in the concentrations of the buffer solutions employed will not be sufficient to influence the general conclusions to be drawn from Figure 3. The salt effect has not yet been studied.

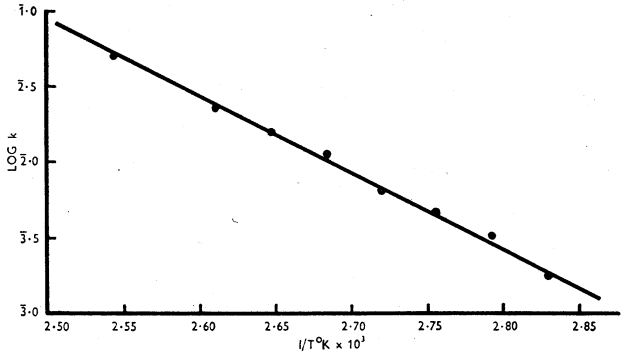

Fig. 4.—Arrhenius equation curve for riboflavin destruction in citric acid-phosphate buffer solutions at pH 7.27 ± 0.03.

(*c*) *Effect of Temperature*

Rate constant is related to temperature by the Arrhenius equation, which may be written as

$$\ln k = I - {}^{E}/_{RT},$$

where k is the rate constant, T the temperature (°K), and I, E, and R are constants. The curve log $k/{}^{1}/_{T}$ should thus be linear.

The thermal destruction of riboflavin was tested against this equation by determining k at eight temperatures from 80°C to 120°C in phosphate-citrate buffer solutions at pH 7.27. The curve obtained by plotting log k against ${}^{1}/_{T}$ is shown in Figure 4. It is apparent that the Arrhenius equation is followed very closely.

(*d*) *Effect of Concentration of Riboflavin*

To facilitate the chromatography of solutions of riboflavin heated in the presence of various buffers a concentration of 500 μg of riboflavin per ml of solution was used. It was noticed that spots of riboflavin persisted long after

the time when, according to the thermal destruction curves for riboflavin solutions of 5 μg/ml, all the riboflavin should have been destroyed.

It seemed then that the rate of destruction of riboflavin depends also on its concentration in solution. This is not a new observation as Daglish, Baxter, and Wokes reported it in 1948 and Shimizu (1950) had also referred to it, but no attempt seems to have been made to express quantitatively what must be a simple relationship.

Accordingly the rate constant k was determined for riboflavin destruction at 100°C in universal buffer solutions at pH 7.70 ± 0.03 at concentrations of 5, 50, and 500 μg riboflavin per ml.

The results obtained were:

Riboflavin Concn.	k (min^{-1})
5 μg/ml	0.0155
50 μg/ml	0.0120
500 μg/ml	0.00823

If log concentration is plotted against k, a straight line results (Fig. 5). It has already been shown (Farrer 1948) that there is a relationship between rate constant and concentration of aneurin which underlines what is already clearly recognized, that the thermal breakdown of these substances is not a true first-order reaction.

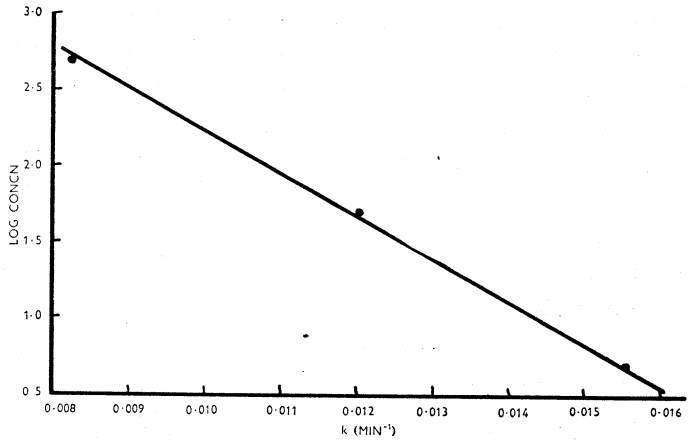

Fig. 5.—Relation between concentrat on of solution (μg/ml) and reaction velocity for the thermal destruction of riboflavin at 100°C in universal buffer solutions at pH 7.70 ± 0.03.

(e) *Thermal Degradation Products of Riboflavin*

The quinoxaline compound was prepared by the method described by Surrey and Nachod (1951), and the free acid and sodium salt chromatographed using butanol-acetic acid-water (4:1:5). Each gave a yellow spot which, when examined under ultraviolet light, emitted a characteristic aquamarine fluorescence. The R_F values of free acid and sodium salt were the same, 0.41.

To confirm that the quinoxaline compound could be detected in alkaline hydrolysates containing unchanged riboflavin, 1 g of riboflavin was dissolved

in 250 ml of 0.2N NaOH, and heated at 100°C. Samples withdrawn at 10-min intervals were chromatographed for 4 hr at 20°C using butanol-acetic acid-water (4:1:5). At 0 min, the intense yellow riboflavin spot was obtained, together with an unidentified pale yellow spot seen in ultraviolet light only at the point of application of the solution. The latter has previously been reported by Hais and Pecakova (1949) and attributed by them to an adsorbate on the paper. In all samples taken at 100°C, exposure to ultraviolet light showed:

(i) The pale yellow spot, R_F value 0—an adsorbate on the paper;

(ii) A trace of an elongated bluish white spot, R_F value approximately 0.70, identified as lumichrome;

(iii) A spot consisting of an aquamarine fluorescence with a mauve upper edge suggesting a composite spot of two compounds. The R_F value was 0.49.

The best separation of the composite spot was obtained by developing the chromatogram with butanol-pyridine-water (3:2:5) and this solvent was used in all subsequent work unless otherwise stated. Comparative chromatograms run side by side then showed that the aquamarine spot in these mixtures was the quinoxaline spot, and that the mauve spot was due to a so far unidentified compound present apparently in very small amounts, and detectable also in the mother liquors remaining from the preparation of the quinoxaline compound according to Surrey and Nachod's directions. This substance is possibly a further breakdown product of the quinoxaline compound.

After 10 min at 80°C a similar solution, 1 g riboflavin in 250 ml 0.2N NaOH, showed only riboflavin and the adsorbate. Samples taken at 20, 30, and 40 min showed:

(i) The adsorbate,

(ii) Riboflavin,

(iii) The quinoxaline compound,

(iv) The unidentified mauve spot, and

(v) A trace of lumichrome.

Chromatograms were then prepared, as described above, from the solutions of riboflavin in various buffers used for the kinetic studies. These solutions contained originally 5 μg riboflavin per ml and proved to be too dilute to give detectable spots after hydrolysis.

More concentrated solutions (500 μg/ml) were therefore used for chromatographic studies. These follow the same kinetic pattern as the more dilute riboflavin solutions although the rate of reaction is slow, as has already been described.

Figure 6 is a typical chromatogram. It refers to a solution of riboflavin (500 μg/ml) at pH 8.12 in universal buffer solution which was heated at 100°C away from the light. Samples were withdrawn at 20-min intervals from 0 to 180 min, cooled, and spotted in duplicate. After development with butanol-pyridine-

water (3:2:5) and drying, the chromatogram was examined in ultraviolet light and the spots observed were:

A, bluish white, elongated spot av. R_F 0.71. Lumichrome.

B, mauve av. R_F 0.63. Unidentified.

C, intense yellow av. R_F 0.53. Riboflavin.

D, aquamarine av. R_F 0.48. Quinoxaline compound.

In ordinary light, both *C* (riboflavin) and *D* (quinoxaline compound) are yellow.

Because of its low sensitivity (50 μg/ml was the smallest concentration which could be detected), the quinoxaline compound was not observed in this chromatogram until after 40 min hydrolysis. At this stage 55 per cent. of the riboflavin remained. After the appearance of the quinoxaline compound the riboflavin spots were clearly less intense and decreased in size until, at 180 min, the spot was quite small and only faintly yellow. The mauve spot appears at 80 min and the lumichrome spot is just discernible at the beginning of the hydrolysis.

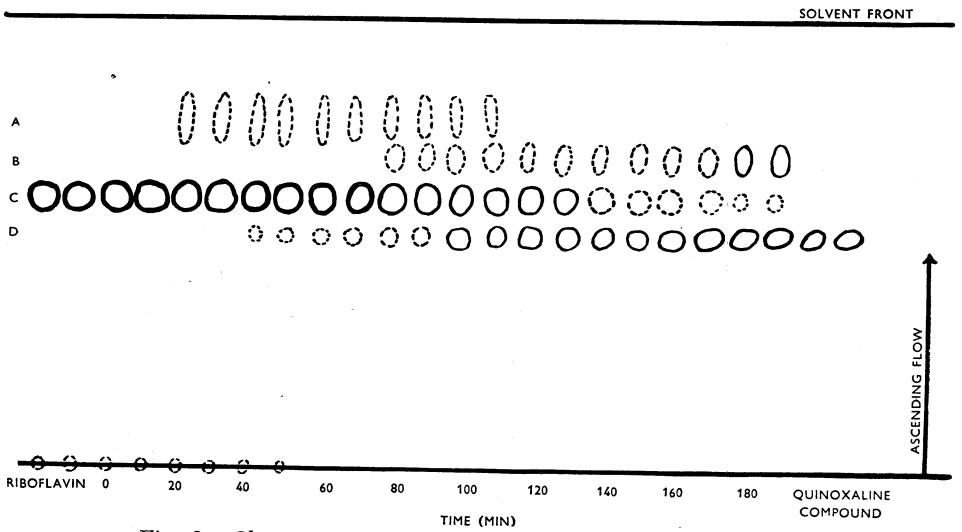

Fig. 6.—Chromatograms for hydrolysis of riboflavin at pH 8.12.

According to Surrey and Nachod (1951) urea too is found in alkaline hydrolysates of riboflavin. One would expect the degradation of urea to free ammonia under alkaline conditions, and this was detected with litmus paper when 1 g of riboflavin was heated for 30 min in 250 ml of 0.2N NaOH. A chromatogram of this solution was developed with the butanol-pyridine-water (3:2:5) solvent, and, when dry, sprayed with *p*-dimethyl-aminobenzaldehyde which gives a yellow spot with urea. Such a spot was found and compared with a urea chromatogram. The R_F value was 0.49.

Examination in ultraviolet light showed that the urea spot on the test chromatogram coincided with that of the quinoxaline compound, which is also yellow in ordinary light. Separation of the two was obtained on chromato-

grams developed with butanol-acetic acid-water (4:1:5). Thus, for example, urea and ammonia, as well as the quinoxaline compound, were obtained from a riboflavin solution (500 μg/ml) boiled for 3 hr at pH 7.80 in a McIlvaine citric acid-phosphate solution. None of them was obtained in a similar solution boiled at pH 6.13 for 4 hr in which no destruction of riboflavin would be expected because of the high concentration of riboflavin used.

These chromatograms are typical of all studied, and it is clear that the thermal degradation of riboflavin always followed the mechanism outlined by Surrey and Nachod (1951).

Shimizu (1951), in a second report only recently available in Australia, has also used paper chromatography for the study of the photodecomposition of riboflavin. He confirmed the occurrence of riboflavin and lumichrome, but noted that "the photodecomposition at higher temperatures in alkaline media seemed to belong to a completely different type of decomposition from those under other conditions." It would appear from our work that the decomposition under these conditions follows the course already described above, i.e. due to an alkaline hydrolysis, and is in no way the same as in irradiated solutions.

There still remained the question of the destruction at very low pH values, which seemed, at least in the greater part, to be very similar to the formation of leucoflavin.

Repeated chromatograms using butanol-pyridine-water (3:2:5) have shown only riboflavin in the solutions. Attempts have been made by using inert atmospheres to demonstrate the presence of leucoflavin, and even to chromatograph, for comparative purposes, chemically prepared solutions of leucoflavin. In every test only riboflavin was found. There is enough evidence, however, to permit us to say that the small destruction at very low pH is quite different in every way from that found at the higher pH values and from that already well described in the literature which occurs in irradiated solutions.

IV. Acknowledgment

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