Quenching Effect of Electrolytes on the Fluorescence Intensity of Riboflavin and Thiochrome

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After the discovery of riboflavin, and its identification as vitamin B_2, assay methods based on the measurement of its fluorescence intensity were described (v. Euler & Adler, 1934; Cohen, 1934; Josephy, 1934). Similar methods of assay were developed for vitamin B_1 after Jansen (1936) had shown that thiochrome, the fluorescent oxidation product of thiamin, could be used as the basis for the fluorimetric estimation of this vitamin. These methods have been developed by numerous workers for use in the determination of the vitamin content of foodstuffs, tissues and body fluids. In all such assays the fluorescent intensity of riboflavin is measured in aqueous or butanol solution; thiamin is extracted, oxidized to thiochrome and measured fluorimetrically in *iso*butanol solution. The methods used for both vitamins (cf. papers quoted by Najjar, 1941, and the Report of the Vitamin B_1 Sub-Committee (Appendix A), Medical Research Council, 1943), do not prevent the appearance in the purified extract of a number of substances present in the original material, particularly since a considerable amount of water is soluble in butanol. Such substances might significantly influence the fluorescence intensity of riboflavin or thiochrome, and thus interfere with the accuracy of the assay. An investigation has therefore been made on the effect of a number of substances on the fluorescence intensity of the two fluorescent pigments. The quenching effect of oxygen, which was stressed by Weil-Malherbe & Weiss (1942), was not taken into account because all measurements were made in equilibrium with air and the standards used were equally affected.

METHODS

(*a*) *Preparation of riboflavin and thiochrome solutions*

Riboflavin. Solutions of riboflavin in water containing 100, 50, 20 and 2 mg./l. were diluted with equal amounts of 0·2 N solutions of various inorganic and organic compounds,* so that the riboflavin concentration of the solution to be measured was 50, 25, 10 and 1 mg./l., and the salt concentration 0·1 N. With Na_2HPO_4, NaCl, $FeCl_3$ and $FeSO_4$, solutions of other concentrations were also examined. To study the effect of pH on the fluorescence intensity, solutions of riboflavin in water containing 25 mg./l. were diluted with equal amounts of buffer solutions (Sörensen's borate and phosphate buffers; Michaelis's acetate and tartrate buffers) to obtain pH values between 11·2 and 2·4. The pH of each solution was measured in a glass-electrode pH meter. Only those salts which did not quench the fluorescence in the neutral region were used as buffers.

Thiochrome. Thiochrome was prepared from thiamin according to the procedure of Barger, Bergel & Todd (1935). The effect of the presence of quenching substances, and of changes in pH on the fluorescence of thiochrome dissolved in water or in non-fluorescent *iso*butanol was examined. The aqueous solutions were prepared by dilution of a solution of thiochrome containing 20 mg./l. with equal amounts of 0·2 N salt solutions. To find the effect on *iso*butanol solution two series of preparations were made. In one series *iso*butanol solutions of thiochrome of 5 and 2·5 mg./l. were saturated with aqueous 0·2 N salt solutions. The saturation point of *iso*butanol with water and salt solutions at room temperature was found to be approximately 10%. Therefore 9 parts of the *iso*butanol solutions were thoroughly shaken with 1 part of the aqueous salt solutions and kept at room temperature for about 18 hr. The fluorescence intensity of this solution was then compared with that of a dry *iso*butanol solution, and with that of one shaken with 10% of distilled water. In the other series conditions were chosen which correspond to those of some of the assay methods in use. Two ml. of an aqueous solution containing 10 mg./l. of thiochrome and a 0·1 N concentration of the salt were shaken with 5 ml. *iso*butanol for 3 min. The *iso*butanol layer was removed, dried with anhydrous Na_2SO_4, and its fluorescence intensity compared with extracts prepared in the same way from a thiochrome solution in distilled water.

The effect of changes in pH on the fluorescence of thiochrome in aqueous and *iso*butanol solutions was also investigated. The aqueous solutions were prepared, by methods similar to those used for riboflavin, from a thiochrome solution of 20 mg./l. The *iso*butanol solution was saturated with the corresponding aqueous buffer solution.

(*b*) *Measurement of fluorescence*

The fluorescence intensity, excited by the 366 mμ line of a mercury lamp, was measured with a photoelectric fluorimeter designed by the authors (Ellinger & Holden, 1944), in which 2 mm. containers for the solutions were used. Most determinations were made by an electrical balancing method and some by a direct deflexion method (Ellinger & Holden, 1944). Quenching was determined on the basis of a comparison of the observed fluorescence intensity of the experimental solution with that expected from the known concentration of fluorescent material. The extent of quenching was expressed as a percentage of the fluorescence intensity of the pigments in solvents containing no quenching substances. The absorption of the 366 mμ line was measured for the riboflavin-salt mixtures with the same instrument.

* With uric acid, the solution was saturated.

RESULTS

The results of the quenching effect of electrolytes and organic substances are given in Tables 1–3.

Table 1. *Quenching effect of various inorganic and organic compounds on the fluorescence intensity of aqueous riboflavin solutions of various concentrations*

(I_0 = fluorescence of unquenched solution. I = fluorescence of quenched solution.)

Quenching substance	100 ($I_0 - I$)/I_0 determined by Direct deflexion method for solutions of riboflavin containing (mg./l.) 50	10	1	Electrical balancing method 10	25
NaF	None	None	None	None	—
NaCl	—	7·4	7·0	6·5	7·4
NaBr	65·9	65·8	65·5	68·2	—
NaI	85·8	85·4	87·5	85·5	—
NaCN	88·2	85·2	—	84·1	—
NaCNS	71·2	73·1	69·0	73·7	—
$NaNO_2$	83·6	83·9	—	84·6	—
$NaNO_3$	5·8	5·6	6·5	7·4	—
$NaHCO_3$	None	None	None	None	—
Na_2SO_3	45·2	44·7	43·7	42·0	—
Na_2SO_4	None	None	None	None	—
Na_2HPO_4	12·6	13·4	14·1	9·0	—
Sodium acetate	None	None	None	None	—
Sodium citrate	None	None	None	None	—
Sodium tartrate	None	None	None	None	—
Sodium lactate	None	None	None	None	—
Urea	None	None	None	None	—
Uric acid	None	None	None	None	—
KCl	—	—	—	—	6·9
NH_4Cl	—	—	—	—	6·9
$MgCl_2$	—	—	—	—	6·9
$CaCl_2$	—	—	—	—	6·9
$FeSO_4$	—	—	—	—	44·4
$FeCl_3$	—	—	—	—	92·3

Table 2. *Quenching effect of various salt concentrations on the fluorescence intensity of riboflavin*

(Quenching is calculated from $(I_0 - I)/I_0 \times 100$: I_0 = fluorescence of unquenched solution; I = fluorescence of quenched solution.)

Quenching substance	Quenching with solutions containing quenching substances in final concentration (normality) 0·20	0·10	0·05	0·02	0·01	0·005
NaCl	23·1	6·9	—	2·4	1·0	—
Na_2HPO_4	—	13·1	—	5·9	3·0	—
$FeCl_3$	—	92·3	83·3	57·2	—	25·9
$FeSO_4$	—	44·4	31·6	—	—	5·9

Table 3 shows that distilled water has a quenching effect on thiochrome in dry *iso*butanol. The values of quenching obtained for the salts allow for the quenching effect of the water dissolved in the *iso*butanol. In column 5 of Table 3 the quenching values are based on an average yield of 82 % for the extraction of thiochrome from distilled water by *iso*butanol under the conditions of these experiments. The values for the percentage of quenching in this column are related to this reduced value. The shaking of the *iso*butanol with aqueous thiochrome solutions containing $MgCl_2$ or $CaCl_2$ resulted in the formation of an emulsion which was broken slowly. The values given in the last column of Table 3 were obtained after these solutions had stood for 18 hr. If measurements were carried out without complete separation of the layers, quenching up to 30 % was observed.

The measurement of the absorption of light of wave-length 366 mμ by the various salt solutions showed that absorption was increased by about 60 % in the presence of $NaNO_2$ and by a very small extent in that of NaI. All the other substances did not affect the absorption.

The effect of variation in pH upon the fluorescence intensity of thiochrome and riboflavin is given in Fig. 1.

DISCUSSION

From Table 1 it is evident that a considerable quenching effect on riboflavin fluorescence is observed only with a number of anions and with iron salts (ferrous and ferric), and that the quenching effect does not depend on the concentration of riboflavin. The observations parallel those of Eisenbrand (1934) on the quenching effect of various salts on the fluorescence intensity of quinine sulphate; he found that cations have little or no effect, while, of the anions, the effect of the halogens is important and increases with increasing atomic number. Of the anions the halides, cyanide, thiocyanide, sulphite and nitrite have a considerable effect on the fluorescence intensity of riboflavin. That of nitrite is mainly due to increased absorption of the primary beam. The effect of the chlorides of Na, K, NH_4, Mg and Ca is due to the anion. The considerable effect of ferrous and ferric ions may be explained as due to a reversible oxidation-reduction process, as observed by Weiss (1938) for the quenching of the fluorescence of certain dyes by iron ions. The quenching effect of phosphate and bicarbonate, and in some measure of cyanide, is due to the alkalinity of the solutions containing these anions. Neutral phosphates do not quench. Table 2 shows that the quenching effect is a function of the concentration of the quenching substance.

The quenching effect of electrolytes on thiochrome fluorescence is more complex. In aqueous solution considerable quenching occurs only with halides, nitrite, ferricyanide, lactate, NH_4 and iron salts. In the halide series the fluorides and iodides have a much higher quenching effect than chlorides and bromides. The effect of the nitrite ion is due to its

Table 3. *Quenching effect of inorganic and organic compounds on the fluorescence intensity of thiochrome solutions*

(Quenching is calculated from $(I_0 - I)/I_0 \times 100$, where I_0 = fluorescence of unquenched solution, and I = fluorescence of quenched solution.)

Quenching substance	Quenching observed in: Aqueous solution containing 0·1 N quenching substance and 10 mg./l. of thiochrome	*Iso*butanol solution saturated with 0·2 N solution of quenching substance. Concentration of thiochrome (mg./l.) 5	*Iso*butanol solution saturated with 0·2 N solution of quenching substance. Concentration of thiochrome (mg./l.) 2·5	*Iso*butanol extract of an aqueous solution of thiochrome containing 0·1 N quenching substance and 10 mg./l. of thiochrome
H_2O	—	4·7	5·0	—
NaF	28·5	22·7	21·7	11·5
NaCl	5·0	16·0	17·5	10·1
NaBr	4·0	22·2	23·0	7·4
NaI	20·5	39·0	39·8	24·2
NaCN	None	9·5	8·8	None
NaCNS	None	—	7·1	6·0
$NaNO_2$	57·0	25·7	26·9	8·8
$NaNO_3$	None	12·3	10·1	7·5
Na_2CO_3	None	—	—	None
Na_2SO_3	None	5·2	5·1	None
Na_2SO_4	None	12·3	11·3	None
Na_2HPO_4	None	9·1	8·4	None
Sodium acetate	None	8·7	7·7	None
Sodium citrate	None	—	6·8	None
Sodium tartrate	None	6·6	7·4	None
Sodium lactate	43·9	—	7·1	1·2
Urea	None	9·1	8·7	5·8
Uric acid	None	8·5	8·0	8·3
$K_3Fe(CN)_6$	100	—	19·2	25·4
$K_3Fe(CN)_6$ (0·02 N)	100	—	—	17·9
KCl	8·7	18·7	18·3	10·1
NH_4Cl	23·6	18·4	18·0	6·5
$MgCl_2$	6·4	20·6	21·4	0·3
$CaCl_2$	6·4	24·0	22·3	1·6
$FeCl_3$	88·4 (0·04 N)	81·8 (0·04 N)	80·0	33·2
$FeCl_3$	100·0	88·9	—	96·9

NOTE. Bracketed values refer to concentrations of salt used in these instances.

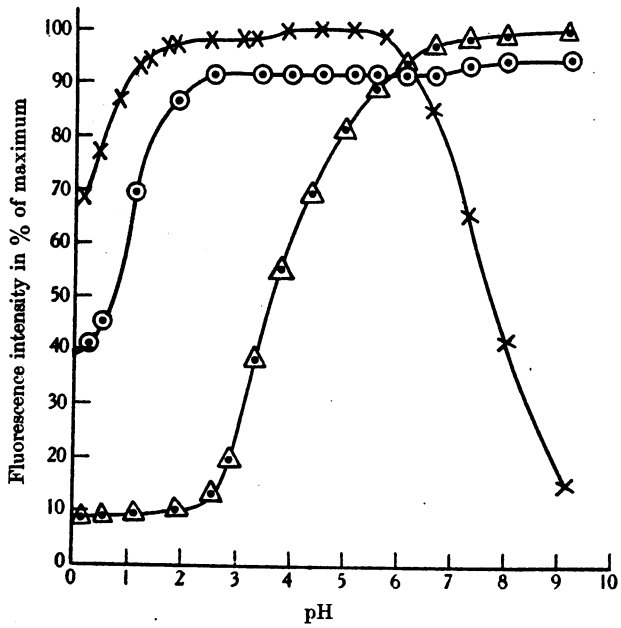

Fig. 1. Effect of pH on fluorescence intensity of riboflavin in aqueous solution ×——×; and of thiochrome in aqueous solution △——△ and in *iso*butanol ⊙——⊙.

absorption of the exciting light. The high quenching effect of lactate is remarkable, and requires further investigation for its explanation.

The quenching effect in *iso*butanol solutions is influenced by the absolute and relative solubilities of the quenching compounds in water and *iso*butanol. In the saturated *iso*butanol solution it is higher for most substances than in aqueous solution. For a number of substances which do not quench in aqueous solution, slight quenching occurs in *iso*butanol saturated with water, but for a number of substances, particularly chloride, bromide and iodide, the quenching in *iso*butanol is considerably greater than in aqueous solution. The effect of potassium chloride is probably due to the chloride ion; the slightly greater one of Mg and Ca may be partly due to the cation. The quenching effect seen in the *iso*butanol extracts of the aqueous solutions is governed by the partition coefficient of the quenching substance between water and *iso*butanol.

Apart from the quenching effect of the halide and the slight effect of some others, the considerable quenching by the ferric cation and the ferricyanide anion is remarkable. Potassium ferricyanide in a concentration of 0·02N has a quenching effect of 18%. Since this substance is used in the preparation of thiochrome for the assay of vitamin B_1 the concentration of this salt in the final extract may be of great influence.

The curve of the dependence of the fluorescence intensity of riboflavin on the pH shows a maximum between pH 5·9 and 7·7; between 3·8 and 5·9 the intensity is about 97% of the maximum. At pH values below 3·8 and above 7·7 there is a rapid fall in the fluorescence intensity. The curve corresponds to that published by Kuhn & Moruzzi (1934).

With thiochrome in aqueous buffer solutions the maximum fluorescence intensity is reached at the alkaline end of the range examined (pH 11). The intensity decreases slowly at pH 8·8, then more rapidly at pH 7·5 and falls steeply at pH 5. This is in accordance with the findings of Kuhn & Vetter (1935). Below this pH the blue fluorescence has completely disappeared and there is only a small amount of green fluorescence. In *iso*butanol solutions saturated with buffer solutions the effect is less marked. There is very little alteration between pH 11 and 4·5, but below 4·5 the fluorescence intensity falls steeply.

SUMMARY

The influence of changes in pH of the solution, and of the presence of a number of inorganic and a number of organic compounds on the fluorescence intensity of riboflavin and thiochrome, has been investigated.

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A Method for the Estimation of Nicotinamide Methochloride in Urine

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Najjar & Wood (1940), Najjar & Holt (1941 *a*, *b*) and Najjar, Stein, Holt & Kabler (1941) described a fluorescent pigment 'F_2' in the urine of normal dogs and of man, the elimination of which increased after ingestion of nicotinamide and related compounds, but which was absent from the urine of pellagrins. It was suggested by Coulson, Ellinger & Platt (1942) that information about the metabolism of these compounds could be expected from an investigation of the urinary elimination of F_2, and indeed a preliminary method of assay was developed for this specific purpose before the chemical