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Effects of Purines on Fluorescent Solutions

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The starting-point of the present investigation was the observation that the fluorescence of an aqueous benzpyrene-caffeine solution disappears on acidification and reappears on neutralization. This led to a systematic examination of the effects on fluorescence of a large number of purines and purine derivatives. Not only the fluorescence of 3:4-benzpyrene, but also that of other hydrocarbons and non-hydrocarbons was studied.

METHODS

Fluorescence measurements were carried out with a direct-reading fluorimeter (Weil-Malherbe, 1944b). Unless otherwise stated, measurements were done in air, since the quenching effect of oxygen is not very marked in aqueous solvents owing to the slight solubility of the gas in water. Moreover, preliminary experiments had shown that the quenching effect of acid aqueous caffeine solutions is the same in air and in pure nitrogen.

RESULTS

The effect of acid caffeine solutions

If H_2SO_4 is added in increasing concentrations to an aqueous caffeine solution of 3:4-benzpyrene, the

fluorescence diminishes progressively and disappears completely at a 0.2N-concentration of acid (Table 1). Other acids have a similar effect, depending on their degree of dissociation. On neutralization the fluorescence is almost completely restored.

Table 1. *Reversible quenching of the fluorescence of 3:4-benzpyrene (c. 1 $\mu\text{g./ml.}$) in 1% aqueous caffeine solution by acids*

Final concentration of acid added	Fluorescence as % of fluorescence in aqueous caffeine solution	
	With acid	After neutralization with N-NaOH
0.001N- H_2SO_4	86.6	—
0.002N- H_2SO_4	66.7	—
0.01N- H_2SO_4	36.1	—
0.02N- H_2SO_4	13.5	94.4
0.2N- H_2SO_4	0	91.3
0.02N HCl	11.6	96.6
2 vol % H_3PO_4 (sp.gr. 1.75)	2.1	92.7
0.4N acetic acid	57.3	96.5
2% citric acid	28.0	98.2

The first question to be decided was, whether the H-ions as such or the caffeine ions formed on

acidification were responsible for the quenching of fluorescence. An experiment in 60% aqueous ethanol showed that neither H_2SO_4 in 1.0N-concentration nor caffeine alone affected the fluorescence of the solution. Quenching was observed, however, when the two components were present together (Table 2). It is clear, therefore, that the caffeine ion is the active quenching agent.

Table 2. *Reversible quenching of the fluorescence of 3:4-benzpyrene (0.8 $\mu\text{g./ml.}$) in 60% aqueous ethanol*

Concentration		As % fluorescence
H_2SO_4 (N)	Caffeine (%)	
0	0	100
1.0	0	100
0	0.3	104
0.04	0.3	56.5*
0.2	0.3	43.0
1.0	0.3	26.6

* After neutralization, 89.5.

Table 3. *Irreversible quenching of the fluorescence of 3:4-benzpyrene (0.8 $\mu\text{g./ml.}$) by high concentrations of HCl in 60% aqueous ethanol. Fluorimetry in nitrogen*

Concentration of HCl (N)	Fluorescence
0	100
0.04	100
0.4	96.5
0.8	86.0
1.2	69.0
1.6	52.4
2.0	40.6
4.0	3.2

If high concentrations of HCl are added to a solution of 3:4-benzpyrene in 60% aqueous ethanol, the fluorescence is quenched even in absence of caffeine (Table 3). This quenching is however irreversible, as the original fluorescence cannot be restored by subsequent neutralization. The effect is not due to H^+ -ions, as H_2SO_4 in similarly high concentrations leaves the fluorescence intact.

Quenching effects of different purines

A number of purines and allied compounds was examined in 60% aqueous ethanol. This solvent was chosen in order to be independent of the variations of solvent power of different compounds as well as of one and the same compound in different ranges of pH. The results are shown in Table 4. Three groups can be distinguished: (1) purine bases, i.e. hypoxanthine, xanthine and *N*-methylxanthines, do not quench in either neutral or alkaline solution, but only in acid solution. The

quenching is completely reversible by neutralization. If caffeine ions are added in the form of caffeine hydrochloride or in the form of caffeine methochloride or methiodide, quenching occurs without addition of acid. The latter substances are more effective than the former, as they are more strongly dissociated. In both cases fluorescence is restored on making alkaline. (2) Uric acid and *N*-methyluric acids have a pronounced quenching effect which is hardly affected by changes of pH. The quenching effect increases with increasing *N*-methylation and is strongest with 1:3:7:9-tetramethyluric acid. Although quenching persists in an alkaline medium, it is of the reversible type, as can easily be shown by extraction of benzpyrene with light petroleum. Tetramethyluric acid is destroyed by brief boiling in alkaline solution, and this is sufficient to restore the initial fluorescence in this particular case. (3) The third group consists of substances other than uric acids with electro-negative substituents, such as 8-methoxytheobromine or -caffeine, or 8-chlorotheobromine or -caffeine. These substances quench, like uric acids, already in absence of H_2SO_4 , but their quenching effect is somewhat intensified in acid solution. They thus occupy an intermediate position between the substances of groups 1 and 2. Adenine and guanine have to be included in this group. Adenosine and guanosine on the other hand behave like the purine bases of group 1.

The effect of 8-thiouric acids is the same as that of ordinary uric acids. 7:9-Dimethyldeoxyuric acid, on the other hand, appears to belong to the third group. 1:3:7-Trimethyl-4:5-dimethoxydihydrouric acid in which the 4:5-double bond is absent has a slight quenching effect alone, which is still further reduced in acid solution and is abolished in alkaline solution. Its behaviour is thus quite atypical. 8-Hydroxy-9-methyl-8:9-dihydrocaffeine, without a double bond adjacent to C_8 , has no quenching effect.

No quenching effects are observed with deoxycholic acid which, like purines, has solubilizing properties, or with a large number of organic bases unrelated to purines.

Quenching effects in different solvents

Fluorescence quenching by purines is greatest in aqueous solution and decreases in less polar solvents (Table 5). This is not only due to the higher degree of dissociation which might be expected in the case of caffeine and other purine bases in acid solution, as the same differences are observed with tri- or tetramethyluric acids which are not dissociated. Moreover, quenching is lower in formamide than in water; this solvent has the same dielectric constant as, but a lower polarity than, water.

Table 4. *Quenching effect of various purines on the fluorescence of 3:4-benzpyrene (0.8 µg./ml.) in 60 % aqueous ethanol*

Purine	Conc. of purine (%)	Fluorescence (as % of fluorescence in purine-free solvent)		
		No acid	1 N-H ₂ SO ₄	After neutralization
Hypoxanthine	0.2	103	68.6	—
Xanthine	0.5	—	21.8	100*
Theobromine	0.5	100	32.3	94.5
Theophylline	0.5	102	51.1	96.1
Paraxanthine	0.5	100	30.2	—
Caffeine	0.5	104	26.6	93.0
Caffeine hydrochloride	0.5	69.6	—	98.5
Caffeine methiodide	0.5	26.5	35.2	92.3
Caffeine methochloride	0.5	28.7	30.4	94.2
Adenine	0.2	82.5	43.8	—
Adenosine	0.5	102	60.0	—
Adenylic acid	0.1	—	78.4	90.0*
Guanine	0.5	—	25.0	38.3*
Guanosine	0.5	100	40.6	—
Guanylic acid	0.1	85	78.3	—
8-Methoxytheobromine	0.5	—	31.3	64.1*
8-Chlorotheobromine	0.5	21.6	16.1	—
8-Methoxycaffeine	0.5	82.6	68.8	—
8-Ethoxycaffeine	0.5	52.2	33.7	—
8-Chlorocaffeine	0.5	40.0	25.8	—
Uric acid	0.25	—	—	56.8*
1:3-Dimethyluric acid	0.5	—	31.3	36.7*
3:7-Dimethyluric acid	0.5	—	20.6	41.9*
3:9-Dimethyluric acid	0.5	—	—	22.7*
1:3:7-Trimethyluric acid	0.5	25.2	35.0	32.4
1:3:9-Trimethyluric acid	0.5	18.5	17.8	18.5
3:7:9-Trimethyluric acid	0.5	—	16.1	28.1*
1:7:9-Trimethyl-8-thiouric acid	0.4	17.1	17.1	32.5
1:3:7:9-Tetramethyluric acid	0.5	9.4	10.0	8.4
1:3:7:9-Tetramethyl-8-thiouric acid	0.3	21.4	24.2	—
8-Hydroxy-9-methyl-8:9-dihydrocaffeine	0.5	100	100	100
7:9-Dimethyldeoxyuric acid	0.5	57.7	39.8	57.5
1:3:7-Trimethyl-4:5-dimethoxydihydrouric acid	0.5	69.5	83.0	96.0
(Deoxycholic acid	0.5	100	—	100)

* These substances were dissolved in 60 % ethanol containing 0.1–0.2 % NaOH.

Table 5. *Quenching of the fluorescence of 3:4-benzpyrene (0.8 µg./ml.) by purines in different solvents. Fluorimetry in nitrogen*

Purine	Conc. of purine (%)	Solvent	Fluorescence (as % of fluorescence without purine)
1:3:7-Trimethyluric acid	0.25	Chloroform	67.6
		Dioxane	63.4
		Formamide	43.2
		Ethanol	33.4
1:3:7:9-Tetramethyluric acid	0.4	Benzene	53.0
		Dioxane	52.5
		Formamide	29.5
	0.5	Ethanol	16.2
		80 % aqu. ethanol	14.5
Caffeine hydrochloride	0.5	60 % aqu. ethanol	13.2
		40 % aqu. ethanol	3.4
		2 % aqu. caffeine sol.	2.9
		60 % aqu. ethanol	69.6
		Water	14.3*

* As percentage of fluorescence in 0.5 % aqueous caffeine solution.

Effect of purine concentration on fluorescence quenching

The quencher has to be present in vast excess over the fluorescent substance. To achieve a quenching of 50 % in 60 % ethanol a 150,000-fold excess of tetramethyluric acid over benzpyrene is required (Table 6). In the case of caffeine, where the active agent is the caffeine ion only, increase of

Table 6. *Quenching of the fluorescence of 3:4-benzpyrene (0.8 µg./ml.) by different concentrations of 1:3:7:9-tetramethyluric acid (T.M.U.) in 60 % aqueous ethanol*

Conc. of T.M.U. (%)	Ratio mol. T.M.U./mol. benzpyrene	Fluorescence (as % of fluorescence without T.M.U.)
0.0001	1.41×10^5	100
0.001	1.41×10^4	98.3
0.01	1.41×10^3	94.8
0.1	1.41×10^2	55.4
1.0	1.41×10^1	5.0

caffeine concentration without simultaneous increase of acid concentration has no effect; on the contrary, it somewhat diminishes the quenching owing to the buffering action of caffeine (Table 7).

Table 7. *Effect of concentration of caffeine and of acid on the quenching of the fluorescence of 3:4-benzopyrene (c. 1 $\mu\text{g./ml.}$) in water*

Caffeine concentration (%)	Fluorescence (as % of fluorescence before acidification)	
	0.04N-H ₂ SO ₄	0.002N-H ₂ SO ₄
0.158	6.3	62.3
0.55	10.6	66.5
3.0	12.9	73.9

Nature of the quenching effects

As the quenching of fluorescence observed might have been due merely to a 'filter effect', i.e. an absorption of primary or secondary radiation by the quenching substance, this possibility had to be excluded. It was *a priori* very improbable, as purines do not absorb light of wave-lengths above 300 m μ . But it was ruled out by direct experiment with the aid of a special vessel previously described (Weil-Malherbe, 1944a). This vessel consists of an inner tube and an outer jacket; if there is a filter effect, a solution of the quenching substance in the jacket causes an inhibition of fluorescence equal to that observed if the inhibitor is added directly to the fluorescent solution. If the quenching is due to a reversible photochemical reaction between the fluorescent and the inhibitor substances, the fluorescence of the solution in the inner tube is not dimmed by the solution of the inhibitor in the jacket. Purines in the outer jacket did not affect the fluorescence of a solution of benzopyrene in the inner tube and the effects described are therefore not due to a filter effect.

Effects on the fluorescence of other hydrocarbons

The fluorescence of a number of hydrocarbons in aqueous caffeine solution was found to be quenched in varying degree on addition of acid and the quenching was reversible on neutralization (Table 8). On the whole, those hydrocarbons which show the highest fluorescence intensity also show the highest degree of quenching. Only pyrene deviates from this correlation in combining a moderate fluorescence intensity with a very high degree of quenching. Naphthacene and rubrene which are insoluble in aqueous caffeine solution were tested in 60% ethanol (Table 9). The observations with rubrene were very similar to those made with other hydrocarbons; in the case of naphthacene however caffeine alone caused a considerable increase of fluorescence which on addition of acid was only reduced to the level observed without caffeine.

Table 8. *Reversible quenching of the fluorescence of various hydrocarbons in 1-3% aqueous caffeine solution by 0.04N-H₂SO₄. The saturated caffeine solutions of hydrocarbons were used undiluted or diluted to the required fluorescence intensity with 1% caffeine solution*

	Fluorescence (as % of fluorescence before acidification)	
	With 0.04N-H ₂ SO ₄	After neutralization
Phenanthrene	73.8	90.0
Anthracene	30.8	88.5
9:10-Dimethylanthracene	12.9	88
Pyrene	4.2	93.2
Chrysene	54.6	100
1:2-Benzanthracene	39.5	89.5
9:10-Dimethyl-1:2-benzanthracene	27.5	86
20-Methylcholanthrene	10.7	88.8
1:2:5:6-Dibenzanthracene	33.4	95
Coronene	37.5	89

Table 9. *Fluorescence of naphthacene and rubrene (both approx. 1 $\mu\text{g./ml.}$) in 60% aqueous ethanol*

	Substance added	Fluorescence (as % of fluorescence in pure solvent)
Naphthacene	1.0N-H ₂ SO ₄	100
	1.2% caffeine	142
	1.0N-H ₂ SO ₄ + 1.2% caffeine	104
Rubrene	1.0N-H ₂ SO ₄	100
	1.2% caffeine	115
	1.2% caffeine + 1.0N-H ₂ SO ₄	55.2

Effects on the fluorescence of non-hydrocarbons

Table 10 contains the results of experiments with some organic bases which are either water-insoluble or non-fluorescent except in acid solution. The effect of caffeine alone could not be investigated here, but only that of caffeine in acid solution, i.e. of caffeine ions. Strong quenching was observed in every case.

Table 10. *Quenching of the fluorescence of various organic bases by caffeine in acid solution. (Solutions made up in 0.1N-H₂SO₄ and contained approx. 2 $\mu\text{g./ml.}$)*

	Conc. of caffeine (%)	Fluorescence (as % of fluorescence in 0.1N-H ₂ SO ₄ without added caffeine)
Quinine	2.4	19.6
Acridine	1.8	2.1
Benzylidene quinaldine	1.8	17.0
3-Aminoquinaldine	1.8	9.1

Some other substances were tested which are both soluble and fluorescent in pure water. Here it was possible to study the effect of acid alone, of caffeine or tetramethyluric acid alone and of

caffeine in acid solution. The fluorescence of all these substances was more or less decreased in acid solution (Table 11). With the exception of eosin,

Table 11. *Effects of purines on the fluorescence of various substances in aqueous solution containing approx. 2 μ g./ml.*

	Fluorescence (as % of fluorescence in aqueous solution)			
	With 0.03 N- H_2SO_4	With 1.8 % caffeine	With 1.8 % caffeine + 0.03 N- H_2SO_4	With 1.2 % T.M.U.*
Thiochrome	13.7	258	10.9	17.1
Riboflavin	54.7	13.0	7.1	13.0
Acriflavine	70.6	74.0	52.8	0.6
Mepacrine	85.5	142	60.0	4.7
hydrochloride				
Fluorescein	49.1	62.0	10.2	—
sodium				
Eosin	19.2	182	35.6	3.4

* T.M.U. = 1:3:7:9-tetramethyluric acid.

caffeine in acid solution depressed the fluorescence still further. Tetramethyluric acid alone had a very strong quenching effect in every case, often stronger than that of caffeine in acid solution. The effects of caffeine alone are very interesting. In some cases it has a considerable, or, in the case of riboflavin, even strong quenching action, while it causes a large increase of fluorescence in other cases, notably with thiochrome where the fluorescence is more than doubled. The result obtained with mepacrine (atabrine) confirms the finding of Auerbach & Eckert (1944) who utilized this property of caffeine for analytical purposes.

DISCUSSION

When the fluorescence effects of the purines were first observed, it was natural to assume a connexion with their solubilizing properties. If a correlation between the two effects could be established, the fluorescence quenching might be interpreted as the consequence of the same type of interaction which is responsible for the solubilization of hydrocarbons and, in some cases, for the formation of molecular compounds (Weil-Malherbe, 1946). At first sight and on superficial consideration such a correlation does not seem to exist. In the series of purine bases quenching is observed only if the substances are present in the form of cations. Yet, at any rate as far as caffeine methiodide or methochloride is concerned, these cations have a reduced solubilizing power. No quenching is observed with purine bases either in the undissociated form in which they have a good solubilizing action or in anionic form in which they have practically none. Uric acids, on the other hand, have a strong quenching effect in undissociated form in which they are potent

solubilizers, but about the same quenching effect in anionic form in which they are almost inactive as solubilizers. On the other hand, just as an increase in the number of *N*-methyl groups causes an increase of solvent power, so it leads to an intensification of quenching, and the importance of the 4:5-double bond and the double bond adjacent to C_8 for both phenomena is shown by the fact that the absence of either double bond causes the reduction or disappearance of quenching as well as solvent power.

Although a quantitative correlation between the degree of fluorescence quenching and the degree of solubilizing power does not exist, all the substances with quenching action have some solubilizing activity, even though it may be relatively small, as in the case of the urates. As has been discussed in the preceding paper (Weil-Malherbe, 1946), the solubilizing effect depends on an interaction between purine and hydrocarbon. If the forces between the two compounds are strong, the mean lifetime of the complex will be relatively great and the stationary concentration of the hydrocarbon in solution relatively high. On the other hand, a low concentration of the hydrocarbon complex in solution indicates weak van der Waals forces and a relatively short lifetime of the product of combination. But even in this case its lifetime is likely to exceed that of the excited state of a hydrocarbon (10^{-8} sec.), because otherwise solubilization would be immeasurably small.

The fluorescence effects can now be explained on the basis of the theory proposed by Weiss (1943) by considering the fluorescent properties of the relatively stable complex purine-fluorescent hydrocarbon. A combination of a fluorescent molecule with another compound does not necessarily involve quenching. The complex may have the same fluorescence as the fluorescent component, as in the case of the benzpyrene-caffeine combination, or it may even have an enhanced fluorescence, as in the case of the complexes between caffeine and thiochrome, mepacrine or eosin. Whether or not quenching occurs depends on the strength of interaction between electronic vibrations and molecular vibrations (Weiss, 1943). Such an interaction can be intensified when the 'mobility' of the electrons is reduced, e.g. under the influence of an electric field set up by an electric charge or a strong polar group. This is the most probable explanation of the fact that caffeine ions have a quenching action, while caffeine in the undissociated state has not.

In discussing the quenching effect of urates which have only a very small solvent action, another factor must be considered. The fluorescent molecule is far more polarizable in the excited than in the ordinary state. Although the polarizing forces of a urate may be too weak to react appreciably with a hydrocarbon in the ordinary state, they

can be sufficiently strong to react with the small number of the more polarizable excited molecules in solution. These excited molecules are present in extremely small concentration, but they alone are responsible for fluorescence.

We thus arrive at the conclusion that, despite apparent discrepancies, the quenching effects of purines are due to the same intermolecular forces as are responsible for their solubilizing effect and the formation of molecular compounds.

SUMMARY

1. The fluorescence of polycyclic aromatic hydrocarbons in aqueous caffeine solution is quenched by acidification. The quenching is reversible on neutralization. The active quenching agent is the caffeine ion, not the H-ion.

2. Other purine bases, such as hypoxanthine, xanthine and *N*-methylxanthines, have an effect similar to that of caffeine. Uric acids quench even in pure water or aqueous ethanol, and their quenching effect is not appreciably affected by changes in the pH of the medium. A third group of purines, comprising compounds with electro-negative substituents other than uric acids, and including adenine and guanine, occupy an inter-

mediate position in that they quench without acidification, but their quenching effect is somewhat intensified in acid solution. Substances without the 4:5-double bond or the double bond adjacent to C₈ do not quench.

3. The quenching effects are strongest in water and decrease in less polar solvents.

4. The quenching is due to a reversible photochemical reaction and not to absorption of either primary or secondary radiation.

5. The quenching effects were also observed with non-hydrocarbons, such as quinine sulphate, acridine sulphate, quinaldine sulphates, thiochrome, riboflavin, fluorescein. Neutral caffeine solutions sometimes exert a quenching effect, e.g. on riboflavin, and sometimes they cause an intensification of fluorescence, e.g. with thiochrome, mepacrine or eosin.

6. The results are discussed, and it is concluded that, despite apparent discrepancies, the quenching effects of purines are due to the same intermolecular forces as are responsible for the solubilizing effect and the formation of molecular compounds.

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The Influence of Biotin upon the Relative Lipotropic Effects of Choline and Inositol

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It has been reported recently from this department (Beveridge & Lucas, 1945) that choline was more effective than inositol in decreasing the cholesteryl esters in the livers of rats which had been fed on a diet low in the lipotropic factors. This incidental finding was difficult to reconcile with the repeated claims of McHenry and his collaborators (Gavin & McHenry, 1941*a, b*; Gavin, Patterson & McHenry, 1943) that 'inositol is effective under conditions unfavourable for the action of choline particularly when large amounts of cholesterol are present in the liver' (McHenry & Patterson, 1944, p. 159). A more extensive comparison of the relative lipotropic effects of choline and inositol was therefore under-

taken and the results of a number of studies in which biotin was fed are reported in this paper. No evidence has been secured to support McHenry's contentions (*a*) that biotin produces a fatty liver characterized by a high content of cholesterol, or (*b*) that the fatty liver observed when biotin is fed is 'definitely resistant to the action of choline' (McHenry & Patterson, 1944, p. 136). Recently MacFarland & McHenry (1945) have modified the original statement from their laboratory and state that the fatty liver produced by administering biotin is partially responsive to choline. They now report that both choline and inositol exert an effect upon the cholesterol content of the liver. While our