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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

ON THE LUMINESCENCE OF LUMINOLS VII: THE EFFECT
OF FOREIGN MATTER ADDITION ON THE
LUMINOL REACTION

Following is a translation of an article by
K. Weber et al., Medical Faculty of the Uni-
versity of Zagreb, Croatia, Yugoslavia, in
the German-language, published in Croatica
Chemica Acta 28 (1956), pages 25-32.

An investigation was conducted to deter-
mine the effect of foreign matter additions on
the chemoluminescence of Luminol, using vari-
ous complex ferruginous compounds as catalysts.
Foreign additions show a cancelling effect on
luminescence or an increase in maximal luminos-
ity. It was possible to distinguish between a
truly inhibiting effect, complex formation, ef-
fect of solvent and electrolytic effect, accord-
ing to Broensted's theory.

The chemoluminescent reaction of Luminol takes place
in alkaline solution containing hydrogen peroxide. Complex
ferro(III)hydrochlorides or other heavy-metal compounds show
a positive catalytic effect on this reaction, which moreover,
may be strongly influenced by foreign additions. The latter,
although not taking part in the basic reaction itself, may
change the reaction speed or direction. A decrease in the
brightness of luminescence, caused by foreign additions, has
been frequently observed [1] [numbers in brackets refer to
similarly numbered items in Bibliography at end] and inter-
preted as a consequence of the restraint or inhibition of the
Luminol reaction. Thus far, no detailed test results have
been published on this phenomenon (in particular on the use
of ferruginous hydrochlorides as catalysts), although they
may certainly be of interest in view of the general import-
ance of the Luminol reaction as a model reaction for peroxi-
dizing effects. The following presents the results of an

investigation of the effects of various foreign additions on the chemoluminescence of Luminol in the presence of different ferro(III)hydrochloride catalysts.

Test methods and working conditions were identical with those used in previous work. In all cases the relative intensity values of luminescence were measured in their dependence on the reaction time, with foreign additions absent or present in various concentrations. Particular attention was devoted to the "maximal intensity" of luminescence, whose increase or decrease, as caused by addition of foreign matter, reflects the latter's affectiveness. The "half-value concentration" of inhibition (cancellation of luminescence) denotes that molar concentration of the inhibiting agent at which maximal intensity of luminescence is reduced to half its value. The term "light sum" denotes the total light energy emitted by the reaction compound over the duration of luminescence. In addition to the foreign matter added, all reaction compounds contained Luminol (3-aminophthalhydrazide) in a concentration of 1.76×10^{-2} mol/l and the respective catalyst: 2×10^{-6} mol/l heminchloride or $1 \times 10^{-3}\%$ hemoglobin /See Note/, 2×10^{-4} mol/l salicyl-aldehyde-ethylen-diimin-Ferro(III)chloride (denoted as SK), 4×10^{-3} mol/l potassium-ferro(III)cyanide or $1 \times 10^{-2}\%$ Ferritin. The reaction volume in all tests was 50 ml. Relative intensity values of luminescence were measured photoelectrically, at room temperature, using the same selenium element and mirror galvanometer (sensitivity 10^{-9} Amp/sec). G denotes the deflection of the test apparatus galvanometer. This deflection is proportional to the intensity of luminescence.

(/Note:7 The preparation used in this test is being marketed as "lamellar hemoglobin". However, since it obviously contains trivalent iron it is to be denoted as hemoglobin or methemoglobin.)

Test Results

Predominantly cancelling (inhibiting) effects were noted in the examination of the effect of a large number of foreign additions on the chemoluminescence of Luminol, in the presence of the aforementioned ferruginous compounds. A number of the foreign additions caused an increase in maximal brightness of luminescence when used in low concentration but showed strong inhibiting effects when their concentration was substantially increased. In single cases, in particular in the presence of Potassium-ferro(III)cyanide, only an increase in maximal brightness could be noted as an effect of the admixture of certain foreign agents. In accordance with the

effects noted for foreign additions on the maximal intensity of luminescence we may, in the case of cancellation, differentiate between inhibiting agents, admixtures forming compounds and such acting as solvents, whereas the exclusive increase in luminescence intensity may be interpreted (primarily) as an electrolytic effect. This interpretation also takes into consideration the assumed reaction mechanism of these foreign additions. A rigid grouping of these additions with respect to their effects on luminescence, however, is not possible because the various individual compounds may show several different effects; the effect obtained may even be determined by the concentration of such a compound.

We may denote as ideal inhibitors such substances which will show an inhibiting effect only on the incipient reaction of luminescence, e.g. acting as a negative catalyst on the reaction speed, or deactivate the carbonyl type Luminol molecules which are in a stimulated state, the carbonyl type Luminol molecules which are in a stimulated state, capable of light emission. In the first case the inhibitors will cause a deceleration of the stimulation, causing a smaller number of stimulated and light emitting molecules to be formed per time unit, while in the second case they will make possible a conversion without emission (radiation) of stimulation energy into other forms of energy. Both effects will decrease the initial intensity of luminescence. In the first case, i.e. in the inhibiting of the stimulating reaction, a prolongation of the duration of luminescence with no change or hardly any change in the "light sum" value. The maximal brightness of luminescence (G_m) generally decreases in these cases, in accordance with the general inhibitor equation

$$G_0/G_m = 1 + \beta \cdot c \quad (1)$$

where c = inhibitor concentration,

G_0 = brightness without addition of inhibitor and

β = the inhibitor constant.

The half-value concentration (\bar{c}) obtained by means of graphical interpolation will then be equal to the reciprocal inhibitor constant ($1/\beta$).

Figure 1 shows decay curves for the luminescence of Luminol as a typical example of such inhibitor effects, using hemoglobin as a catalyst and hydrochinon as inhibitor. (G is the relative intensity and t the reaction time).

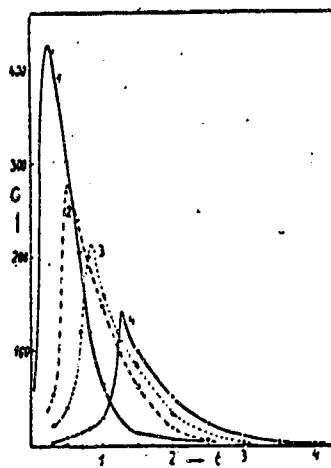


Figure 1. Luminol + hemoglobin 1, without hydrochinon 2, 1.4×10^{-3} mol/l 3, 2.0×10^{-3} mol/l and 4, 3.0×10^{-3} mol/l hydrochinon.

The illustration shows clearly that the maximal brightness, as well as the "light sum" (i.e. the integral of the decay curves) show diminishing values for increasing inhibitor concentration. A second example shows the effect of aniline on the same luminescence by means of the numerical values in Table I. Good conformity with Equation (1) may be noted in this case.

Table I

Luminol + H_2O_2 + Hemoglobin

Aniline (mol/l $\times 10^3$)	—	0,2	1	2	6	12	20	41	123
Maximal brightness G	220	210	160	124	71	45	31	14	3,9
Inhibitor constant β	—	235	374	387	346	324	304	359	446

Mean value: $\beta = 347$

$\bar{c} = 2,5 \cdot 10^{-3}$
 $1/\beta = 2,88 \cdot 10^{-3}$

Potassium cyanide is a well known inhibitor of biocatalytic chemical reactions and it is generally assumed that

its effect is caused by formation of a complex compound with the biocatalyst. Depending on the type of catalyst used, this substance has a varying effect on the Luminol reaction. When using potassium-ferro(III)cyanide as catalyst, the adding of KCN merely leads to an increase of luminescence brightness with a simultaneous decrease in luminescence duration, i.e. the light sum will show a relatively constant value. The speed of chemoluminescence is obviously increased by an electrolytic effect, since it has been noted that other hydrochloride compounds will, in principle, show an identical effect on this reaction. The saline concentrations which will increase the maximal intensity of luminescence to double its value are (in mol/l):

KCN 0.39; KCNS 0.35; KCl 0.27; KI > 0.50

For the purpose of a closer investigation of this electrolytic effect the logarithm of the measured maximal luminescence intensity ($\log G_m$) as a function of the square root of the ion concentration of the solution ($\sqrt{\mu}$) was entered in a graph. The resulting curves are shown in Figure 2.

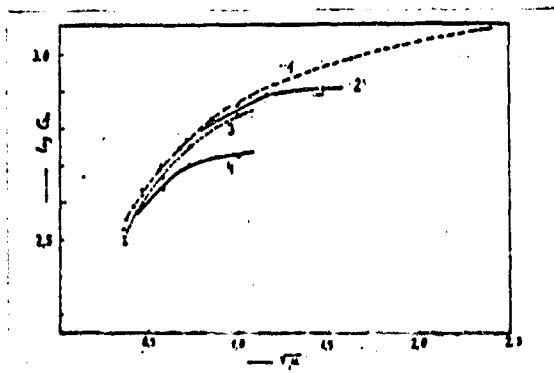
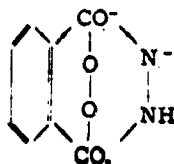


Figure 2. Luminescence intensity as a function of ion concentration. Electrolytic additions: 1. KCN 2. KCNS 3. KCl 4. KI; Catalyst: Potassium-ferro(III)cyanide

Evidently, a linear relationship can only be obtained in the case of low ion concentrations. This fact may be traced to two causes: The ion concentrations used in these tests are very high which means that true linearity may not

even be expected in the case of a true primary electrolytic effect and, secondly, an inhibitor effect may be superimposed on the electrolytic effect. This inhibitor effect will cause a deceleration of the reaction speed with increasing saline concentration and thus results in the type of curves shown in Figure 2. The assumption of an inhibitor effect being superimposed over the electrolytic effect proper is further strengthened by the fact that deviation from the linear pattern is most pronounced in the KI-curve, since generally a higher inhibiting effect is also observed for this substance than for the other salines used. The electrolytic effect is positive, i.e. the reaction speed is increased by admixture of salines, which fact is understandable from the point of Broensted's theory [2] since the critical complex of the reaction is formed by the following anions:



According to the number of charges shown for the ions listed the electrolytic effect should be much more pronounced and the curves shown in Figure 2 should have a much steeper slope. We are led to assume that this behavior (which is in conflict with the theory) is also caused by the simultaneously present inhibitor effect.

In the presence of the other catalysts potassium cyanide shows a cancelling effect on the luminescence. The respective half-value concentrations have been compiled in Table II. It should be noted, however, that in the case of reaction catalysis by means of hemoglobin or ferritin only cancellation may be observed, whereas small cyanide concentrations will cause an increase in luminescence intensity, in the case of the other catalyses.

In the complex SK and chlorohemin molecules, only four of the available six coordination points of iron are filled and we may, therefore, assume that occupation of the fifth position by cyanide increases the catalytic effect of these substances. This would be the case for low KCN concentration, while a retardation of catalysis may ensue when even the sixth coordination point and would be filled, i.e. in case of

high cyanide concentration. Only one coordination place of the iron atom is unoccupied, however, in the hemoglobin and ferritin molecules and, when occupying this one free space, cyanide will show an inhibiting effect, even in low concentration.

Table II

Luminol + H₂O₂ + KCN

Catalyst	\bar{c}	Remarks
Chloride-hemin SX	4.6 x 10 ⁻² 5.1 x 10 ⁻²	In case of low cyanide concentration maximal brightness is increased
Hemoglobin Ferritin	3.4 x 10 ⁻² 1.2 x 10 ⁻²	

The effect of cyanide thus consists in its embedding in the complex molecules of the ferruginous catalyst and the same assumption seems to explain the inhibiting properties of the foreign additions whose half-value concentrations have been compiled in Table III. The chemical composition of these substances corresponds to an effect of this nature. Sulfides as well as organic nitrogen bases are generally capable of forming complex compounds and the trilons are compounds which in chemical practice are used for the removal of traces of copper, since they are capable of forming complex combinations with heavy metals ⁴⁷. Trilon A is the sodium salt of nitrilo-acetic acid and Trilon B is the sodium saline of ethylen-bis-imino-di-acetic acid.

Of the substances generally known for their inhibiting effect on chemical reactions we have examined potassium iodide, thiocyanide, phenol (carbolic acid), hydrochinon and aniline with regard to their effect on the chemoluminescence of Luminol. A cancellation of luminescence was observed in all case. This effect is, however, not very intensive, in particular in the case of the inorganic salines. Half-value concentrations for cancellation are relatively high for these salines (see Table IV) and the general cancellation equation (1) is hardly satisfied.

Table III

Half-value concentrations of complex forming substances

Inhibitor	a) Katalysator				
	Na ₂ S	Pyridin	Nicotin	Trilon A	Trilon B
b) Chlorhämmin	—	2,5 vol %	0,30 Mol/l	—	—
SK	0,49 Mol/l	0,26 vol %	—	0,6 %	8,6 %

Legend: a) Catalyst; b) Chlorohemin.

Table IV

Half-value concentration of inhibition in mol/l

Inhibitor	a) Katalysator				
	KJ	KCNS	Hydrochinon	Phenol	Anilin
b) Chlorhämmin	0,49	4,5	$3,0 \cdot 10^{-3}$	$1,5 \cdot 10^{-4}$	$2,0 \cdot 10^{-3}$
SK	0,47	4,4	$2,3 \cdot 10^{-4}$	$4,4 \cdot 10^{-4}$	$9,0 \cdot 10^{-3}$
c) Hämitglobin	0,66	3,7	$1,9 \cdot 10^{-3}$	$3,0 \cdot 10^{-3}$	$5,5 \cdot 10^{-3}$
Ferritin	0,34	—	—	$2,5 \cdot 10^{-3}$	$4,4 \cdot 10^{-4}$
K ₃ [Fe(CN) ₆]	—	—	$1,1 \cdot 10^{-3}$	$2,2 \cdot 10^{-3}$	$9,0 \cdot 10^{-3}$

Legend: a) Catalyst; b) Chlorohemin; c) Hemiglobin.

We are, therefore, led to assume that the inorganic salines do not act as true inhibitors on the luminescence of Luminol (even when using chlorohemin, SK, hemiglobin or ferritin as catalysts) but in those cases also show a primarily electrolytic effect. This effect, however, causes a deceleration in the speed of reaction, which is to be expected in accordance with the theory: the stimulating reaction for luminescence apparently proceeds via an unstable interim complex formed by the cations of the catalysts enumerated above with the anions of the Luminol peroxyde. The factor $z_A \times z_B$ in the equation by Broensted:

$$\log k = \log k_0 + z_A z_B \sqrt{\mu} \quad (2)$$

consequently has a negative sign and the saline effect shows as an inhibition of the reaction. The logarithm of the maximal brightness as a function of ion concentration in these cases yields a fairly good linear relationship. The slope of the straight line corresponds to a value of $z_A \times z_B = 1$, for catalyses of SK and ferritin, respectively, while definitely lower values were obtained when using the other catalyses. This result indicates a very complicated reaction mechanism for the respective catalyses.

The organic inhibitors tested show approximately the same effect within their effect on the chemoluminescence of luminol as they show under thermal or photochemical reactions. Since varying half-time inhibition values were obtained when using different catalyses (see Table IV), we may assume that these foreign admixtures act predominantly on the stimulating catalytic reaction, rather than on the stimulated molecules of the Luminol.

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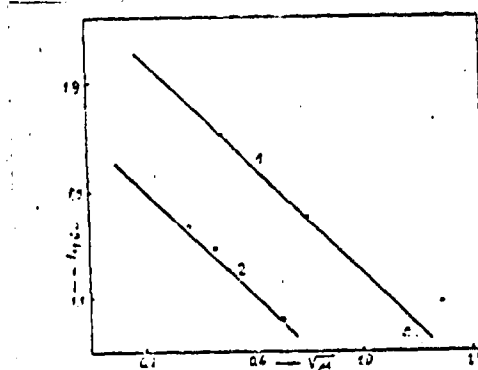


Figure 3. Inhibition by electrolytic effect.
Catalysts: 1. SK 2. Ferritin.

No generally applicable rules or interrelationships between catalytic and inhibiting effects may be derived or deduced from the results of these tests. There is no fixed sequence for the effectiveness of the inhibitors, which should be the same for the majority of the catalyses. It rather appears as if individual properties inherent in the substances of either category were of importance. The general inhibition formula (1) is satisfied by the inhibiting qualities of quite a number of the organic substances listed (compare Table I).

Finally, the effect of organic solvents was examined on the brightness of chemoluminescence. All solvents tested showed a cancelling effect, partly, however, only in very high concentrations. The half-value concentrations for cancellation have been compiled in Table V, in vol %. Simultaneously with the maximal intensity the light sum of luminescence is reduced by the solvents listed. Despite the fact that glycerin causes a slight prolongation of the luminescence duration, the light sum is always less than in the absence of glycerin. The effect of this particular substance may partly be due to a change in viscosity of the solution. True changes in viscosity, however, without other influences, cause a much more marked change in luminescence duration. This effect will be treated in a separate publication.

Table V
Influence of solvents

a) Lösungsmittel	b) Katalysator	c in Vol%	
		c) Chlorhämmin	SK
Aceton		8,6	5,1
Methanol		45,7	41,9
Äthanol		26,4	43,2
Glycerin		—	4,4

Legend: a) Solvent; b) Catalyst; c) Chlorohemin.

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