The methylnitrosoguanidine, recrystallized from alcohol at 50°, melted with decomposition at 95°. Analysis for nitrogen: found 54.19, 53.90%; calcd. for $C_2H_6ON_4$, 54.90%.

The reduction of methylnitroguanidine with zinc dust and aqueous ammonium hydroxide was carried out in the same way as with zinc dust and ammonium chloride except that it was slower, ten hours being required for its completion. The red filtrate from the reduction mixture deposited white flocculent material as long as it had any odor of ammonia. After all of the ammonia had disappeared, the liquid was filtered again, and the filtrate, now apparently free from zinc, on evaporation gave crystals of methylnitrosoguanidine or was used directly for the preparation of the silver salt which came down as a voluminous white precipitate when silver nitrate was added; yield of silver salt 39.6%. Analysis of silver salt of methylnitrosoguanidine: found Ag 51.74, 51.90%; calcd. for $C_2H_5ON_4$, Ag 51.19%.

N-*n*-Butyl-, and N-benzyl,N′-nitrosoguanidine, prepared by reduction of the nitro compounds with zinc dust and aqueous ammonium hydroxide, were procured as orange-red oils, rather unstable and very readily soluble in water. The zinc-free filtrates from the reduction mixtures were used directly for the preparation of the silver salts, yields, respectively, 30.9 and 32.9%. Analysis of silver salt of butylnitrosoguanidine: found Ag 42.76, 42.91%; calcd. for $C_5H_{11}ON_4$, Ag 42.96%. Analysis of silver salt of benzylnitrosoguanidine: found Ag 36.21, 36.42%; calcd. for $C_8H_{10}ON_4$, Ag 36.28%.

N,N′-Di- and trialkylguanidines, isolated as the picrates, were prepared by the interaction of the corresponding amines with the zinc-free red filtrates from the reduction of methyl-, *n*-butyl-, and benzylnitroguanidines by means of zinc dust and aqueous ammonium hydroxide. The procedure was the same as has been described for the reaction of nitrosoguanidine with aliphatic amines. N-Methyl,N′-*n*-butylguanidine prepared from the reaction of *n*-butylamine with methylnitrosoguanidine, yield 13.6%, was found, by a mixed melting point determination on the picrates, to be identical with that prepared from the reaction of methylamine with *n*-butylnitrosoguanidine, yield 22.4%.

Summary

Nitrosoguanidine reacts in aqueous solution with ammonia to produce guanidine, and with primary and secondary aliphatic amines to produce the corresponding alkylguanidines.

Nitrosoguanidine does not react with aniline or methylaniline in boiling aqueous solution but yields dicyandiamide quantitatively.

N-Methyl,N-*n*-butyl, and N-benzyl,N′-nitrosoguanidines have been prepared, the methyl compound as a crystalline solid, the others as orange-red oils.

N-Alkyl,N′-nitrosoguanidines react with primary and secondary aliphatic amines to produce di-(or tri-) alkylguanidines in which the substituent groups are attached to two different nitrogen atoms. Seven new N,N′-dialkylguanidines and one new N,N,N′-trialkylguanidine have been prepared by this reaction.

Nitrosoguanidine and the N-alkyl,N′-nitrosoguanidines dearrange in aqueous solution to produce cyanamide and the alkylcyanamides, respectively. The other product of the dearrangement is evidently nitrosoamide which accounts for the nitrogen which is evolved in these experiments.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGY OF PRINCETON UNIVERSITY]

Chemical Studies on Bioluminescence. IV. Salt Effects on the Total Light Emitted by a Chemiluminescent Reaction[1]

BY RUBERT S. ANDERSON

An aqueous solution containing cypridina luciferin and the enzyme, cypridina luciferase, materials extracted from a small crustacean, emits light in the presence of oxygen.[2] The total light emitted during the reaction of a given volume of luciferin solution can be changed by various means,[3] and particularly it can be increased by the addition of sodium chloride to the reaction mixture. Other chemiluminescent reactions have not been studied in this way but salt effects on fluorescence are well known.[4] Since the radiation from fluorescent solutions is in general decreased by salts, if they have an effect at all, a more detailed study of the effects of sodium chloride and a series of other salts on the luminescent reaction of luciferin and luciferase has been made.

Experimental Procedure

The procedure and solutions have been described recently.[5] In addition the luciferase solutions were dia-

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[1] These results were presented at the Chapel Hill meeting of the American Chemical Society, April, 1937.

[2] E. Newton Harvey, "Nature of Animal Light," 1920, p. 103.

[3] Rubert S. Anderson, *J. Cell. Comp. Physiol.*, **3**, 45 (1933).

[4] W. West, R. H. Müller and E. Jette, *Proc. Roy. Soc.* (London), **A121**, 294, 299 (1928).

[5] Rubert S. Anderson, *J. Cell. Comp. Physiol.*, **8**, 261 (1936).

lyzed so that the control runs would be free of salts.[6] Since the activity of the luciferase often decreased during dialysis, the amount used for a reaction was adjusted so that the reaction would be practically complete under standard conditions in one and one-half or two minutes.

Formation of oxidized luciferin[5] in the high sodium chloride concentrations was largely avoided either by starting the reaction within a few seconds after adding the luciferin to the sodium chloride solution or by having present about 5×10^{-5} *M* toluhydroquinone. The former will be referred to as the special technique.

Sodium chloride, potassium thiocyanate and potassium iodide decreased the velocity of the reaction. This was a limiting factor on the range of concentrations that could be studied with a single luciferase concentration, particularly in mixtures of these salts.

Although consecutive duplicate measurements of total light agreed to within 2 or 3%, uncertainties of the photocell and properties of the luciferin, such as the difficulty of getting complete reactions in high salt concentrations,[3,5] made this agreement somewhat illusory. For this reason the data have been used only to illustrate general relations on the basis of large differences.

Results

Table I shows that even 0.001 *M* sodium chloride produced a substantial increase in the total light emitted, while 0.5 *M* sodium chloride increased it to three and a half times the control value. The shift toward a more acid *p*H caused by the addition of sodium chloride was not a primary factor in the increase.

TABLE I

THE TOTAL LIGHT EMITTED FROM REACTION MIXTURES CONTAINING VARIOUS CONCENTRATIONS OF SODIUM CHLORIDE

Sodium chloride, moles per liter	Light emitted, arbitrary units 1	2	3
0.0	[1.0]	[1.0]	[1.0]
.00048	1.08		
.00095	1.14	1.3	
.0019	1.34		
.0048	1.8		
.0095	2.1	2.2	2.2
.019	2.4		
.048	2.8		
.095	3.1	3.0	3.2
.48		3.4	3.4

	Temp., °C.	*p*H	Technique
1	25–26	7.0–7.1	0.0003 *M* 1:1 phosphate buffer + sodium hydroxide to constant *p*H
2	21–22		0.00167 *M* 1:1 phosphate buffer, "special technique"
3	21–22		0.00167 *M* 1:1 phosphate buffer + 10^{-5} *M* toluhydroquinone

This luminescent reaction when produced by the organism takes place in sea water. The yield of light when sea water that had been adjusted to *p*H 8.0 to 8.3 was used as the reaction medium was about five times as great as from 0.00167 *M* phosphate buffer within the same *p*H range.

TABLE II

THE TOTAL LIGHT EMITTED FROM REACTION MIXTURES CONTAINING A FIXED CONCENTRATION OF VARIOUS SALTS. ALL OF THE SOLUTIONS ALSO CONTAINED 6 (ACID) : 4, 0.00033 *M* PHOSPHATE BUFFER

0.0095 *M* salt	Light emitted, arbitrary units
NaCl	2.3[a]
KCl	2.2
NaBr	1.9
KBr	2.0
KF	1.4
KNO_3	1.3
K_2OX	1.2
K_2SO_4	1.1
No salt	[1.0]
KCNS	0.12
KI	.08

[a] The average deviation of 11 experiments from 21–27° was ±0.05.

Table II shows the highly specific character of the salt effects and that the negative ions of these salts, as in fluorescence, exerted the major part of the influence. Otherwise the results seemed to show little relationship to those obtained for quenching the fluorescence of quinine bisulfate and other compounds in aqueous solutions.[4] In those cases all of the salts given either were without effect or quenched the fluorescence, although the order of ions was not completely constant. In this luminescent reaction only two salts decreased the total light emitted while all of the others increased it. However, the four ions which usually had the greatest quenching effect on fluorescence, iodide, thiocyanate, bromide and chloride, also had the largest effect on the total light emitted by the luciferin reaction. Unless this is a coincidence, it suggests a relationship between the positive and negative effects on light emission.

Data obtained from reactions in mixed salt solutions, Fig. 1, relate the mechanism of the positive effect of sodium chloride and the negative effects of potassium iodide and thiocyanate more closely. (A few experiments with sodium iodide and sodium thiocyanate gave approximately the same results.) The total light emitted at each sodium chloride concentration without added quenching salt is taken as one. If the positive

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(6) A previous qualitative estimate of the sodium chloride effect in the presence of 0.067 *M* phosphate buffer[3] was much too low because the great effect of small concentrations of sodium chloride present in all of the extracts and the difficulties of getting complete reactions in high salt concentrations were not realized.

and negative effects were additive, as would be expected if they were unrelated, the percentage decrease on adding quenching agent would be the same at different sodium chloride concentrations. This evidently is not the case as the relative amount of quenching is greatly decreased by high concentrations of sodium chloride. For instance in the absence of added sodium chloride 0.01 *M* potassium thiocyanate reduced the light emitted by 85–90% but only about 15% in the presence of half molar sodium chloride. As one consequence of this opposed effect, added sodium chloride produced an increase in the light emitted which was much more striking in the presence of added quenching agent than in its absence. In the case just mentioned, the addition of half molar sodium chloride increased the total light perhaps twenty fold instead of to the usual three and a half fold. It will be of interest to see if other conditions which decrease the emitted light, such as increased temperature or increased *p*H, can be counteracted by sodium chloride.

It seems reasonable to assume that the quenching of luminescence by potassium thiocyanate or iodide occurs through the same general mechanism as is believed to be true for the quenching of fluorescence by these same salts,[4] that is, by the deactivation of activated molecules, before radiation has time to take place, with or without a chemical reaction.[7] Since sodium chloride decreased this quenching of luciferin luminescence, it apparently in some way prevented the inactivation of the activated molecules. That this opposed action did not result simply from an increase in the number of activated molecules at a given instant was shown by increasing their concentration through an increased luciferase or luciferin concentration. Most of the potassium iodide experiments in Fig. 1 were repeated with the concentration of luciferase increased by 400% without affecting the results given, by as much as 10% in any case. An increase of the luciferin concentration in two cases by 300% also was without a marked effect on the influence of the quenching salt.

One explanation of the increased light emission in solutions of sodium chloride is that a change occurred in the activated molecule which made it less likely to lose its energy in collisions with the solvent or with added potassium iodide or thiocyanate. Although chemical combination of the activated molecule with the salt or negative ion is one possible mechanism for this, little suggests it so far. Nor were similar observations found in the literature of fluorescence in solutions. However, there may be more than a superficial similarity between these observations and the behavior of mercury vapor in stepwise radiation and in the sensitized fluorescence of thallium vapor.[8, 9] In these processes, small pressures of added nitrogen may increase greatly the intensity of the emitted radiation of certain frequencies.[10] Whether or not the analogy is significant, it seems worth while to look for salt effects similar to these on the luciferin reaction, in other luminescent reactions and in reactions giving more commonly occurring activated molecules than the luminescent ones. This is being done.

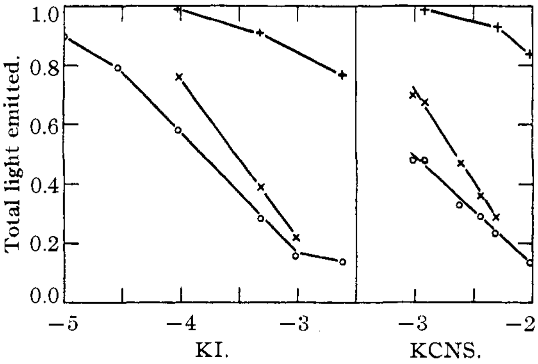

Fig. 1.—○, 0.0 *M* NaCl; ×, 0.0095 NaCl; +, 0.48 NaCl. Most of the above reaction mixtures contained *M*/600 1:1 phosphate buffer and 5×10^{-5} *M* toluhydroquinone. Temp., 22 ± 1°.

Summary

The total light emitted by a fixed quantity of cypridina luciferin increased regularly with increased concentrations of sodium chloride in the reaction mixture. Contrary to results in fluorescence, six out of eight potassium salts which were studied, increased the total light emitted but to smaller extents than the chloride. Iodide and thiocyanate decreased the light emitted. The negative effects of these two salts were partially or completely eliminated by the presence of sodium chloride.

PRINCETON, N. J. RECEIVED JULY 23, 1937

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[7] Erich Schneider, *Z. physik. Chem.*, **B28**, 311 (1935).

[8] K. Donat, *Z. Physik*, **29**, 345 (1924).

[9] S. Loria, *Phys. Rev.*, **26**, 573 (1925).

[10] The mechanism of the increase is supposed to be the production of mercury atoms in a metastable state through the loss of a small fraction of the excess energy during collisions with nitrogen. In this metastable state the mercury atom loses the remainder of its excess energy much less readily by radiation or collisions in general. Thus more atoms retain it until a second quantum is absorbed, in stepwise radiation, or until collision with a thallium atom in the sensitized fluorescence.