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Important Variables in Granulocyte Chemiluminescence (40633)

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The first report of granulocyte chemiluminescence (CL) during phagocytosis was by Allen and co-workers (1). The light production correlated with metabolic activation which occurs during phagocytosis. Chemiluminescence has been increasingly used as a means to follow granulocyte metabolic activity associated with microbicidal events (2, 3). It also has been used as a measure of phagocytosis and opsonins (4–6). Techniques of CL assays have varied widely, including differences in temperature, granulocyte isolation procedures, and cell media. This report examines some of these variables in order to achieve more optimal conditions for future CL studies.

Materials and methods. Granulocyte preparations. Blood was obtained from healthy mongrel dogs that had been dewormed, and immunized against hepatitis and distemper. The blood was anticoagulated with either heparin (10 U/ml of blood) or EDTA (0.4 ml of 5% EDTA/16 ml of blood). Red blood cells (RBCs) were sedimented by adding 3 ml of 5% dextran in saline (average MW 255,000) to every 10 ml of anticoagulated blood. After the RBCs had settled out for 30–40 min, the supernatant solution was removed and centrifuged (750 rpm, 66*g* for 10 min) to pellet the white blood cells (WBCs) and residual RBCs. The cell pellet was suspended in 10 ml of cold NH_4Cl RBC lysing solution (NH_4Cl 8.32 g/liter, $NaHCO_3$ 0.84 g/liter, EDTA 43.2 mg/liter) for 10 min at 4° (7). In some experiments the hypotonic lysis procedure of Goldstein *et al.* (8) was used to remove RBCs. RBCs were hypotonically lysed by suspending the cell pellet in 8 ml of 0.9% NaCl, adding 24 ml of distilled water for 20 sec and then adding 8 ml of 3.6% NaCl to return to isotonic conditions. After the RBC lysis procedure the remaining WBCs were washed twice (500 rpm, 30*g* for 10 min) with cell media, resuspended, and counted. Large clumps or aggregates were removed by pouring the WBC suspension through a fine-mesh stainless-steel filter (0.2-mm openings). WBC counts were determined with a Coulter counter (Model Zf), and differential counts were done by microscopic evaluation of Wright-stained smears. On the basis of the WBC count and differential the volume of the cell suspension was adjusted to give the desired granulocyte count.

Chemiluminescence measurement. Granulocyte chemiluminescence was measured with a specifically designed chemiluminescence spectrometer which had temperature control, magnetic mixers, and rubber injection ports to permit the addition of reagents to the reaction vial when positioned in front of the photomultiplier tube (9). The disposable reaction vials were made of polyethylene plastic (Minivials, Rochester Scientific Co., Rochester, N.Y.). During each CL run the samples were maintained at 37° with continuous mixing. Emitted light was measured and recorded as counts per minute (cpm). Most CL experiments were assayed for 10 min after addition of the stimulus. Background counts of unstimulated granulocytes were determined immediately before adding the CL stimulus. The granulocytes (5×10^6) were stimulated in all CL experiments with 2 mg of opsonized zymosan. Zymosan was prepared by suspending the particles in saline, heating in a boiling waterbath for 60 min, and washing three times with saline. Zymosan was opsonized by incubating 2 mg of zymosan with 0.1 ml of autologous serum at 37° for 10 min. All experiments were run in triplicate unless otherwise stated, using granulocytes from the same donor. In most cases the experiments were repeated at least once using granulocytes from different donors. Consistent differences were found.

Chemicals and cell media. All chemicals were of reagent grade. The following cell media were used: Hanks' balanced salt solution (HBSS; pH 7.4; Gibco, Grand Island,

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N.Y.), Earles' balanced salt solution (EBSS; pH 6.8; ISI, Cary, Ill.) Ringers' solution (Baxter, Travenol), medium 199 with Earles' salts (M199; pH 7.4; Gibco), minimal essential medium with Earles' salts (MEM; pH 7.6; Microbiological Associates, Bethesda, Md.), and Hepes medium (pH 7.5; Microbiological Associates). Compositions of the cell media are shown in Table I. None of the media contained phenol red in order to avoid color quenching during the CL assay. ACD formula A was obtained from Aminco, Travenol, and disodium ethylenediamine tetraacetate (EDTA) from Fisher Scientific Company.

Results. Temperature of cells during isolation. The temperature of granulocytes during CL assays has a marked effect on the amount of light emitted (9). In the present experiment we evaluated the influence of temperature during the isolation of cells on the CL reaction. Figure 1 illustrates the effect of leukocyte isolation temperature on granulocyte CL responses (temperature of CL assay was 37° for all experiments). The best results were obtained with cells kept at 4° during the isolation procedure with approximately twice as much light produced as cells isolated at 37°.

An important correlary finding was that leukocyte aggregation increased with temperature during granulocyte isolation. Cells prepared at 37° had many large aggregates grossly visible, while few were found at 27° and none were visible at 4°. The decreased CL observed from cells isolated at higher temperatures may have been due to increased aggregation at these temperatures. Maintaining cells at 4°, however, did not completely eliminate aggregation in all experiments. This led to the investigation of other techniques designed to further reduce granulocyte aggregation.

Aggregation. In evaluating procedures for their ability to reduce aggregation we have compared cell preparations after incubating separate aliquots at 4 and 37° for 1 hr. Since incubation at 37° encourages the development of aggregation, those techniques which prevented aggregation at that temperature were considered the most useful for CL studies. Aggregation was determined by a decrease in the total WBC count as measured by a Coulter counter. This served as a meas-

TABLE I. COMPOSITION OF CELL MEDIA (g/liter)

	Ringers	HBSS	Earles	Hepes	Medium 199	MEM
$CaCl_2$	0.25	0.14	0.20	0.20	0.20	0.20
KCl	0.42	0.40	0.40	0.40	0.40	0.40
KH_2PO_4	—	0.06	—	—	—	—
$MgCl_2 \cdot 6H_2O$	—	0.10	—	—	—	—
$MgSO_4 \cdot 7H_2O$	—	0.10	0.20	0.20	0.20	0.20
NaCl	9.00	8.00	6.80	6.80	6.80	6.80
$NaHCO_3$	—	0.35	2.20	—	1.25	2.20
$Na_2HPO_4 \cdot 7H_2O$	—	0.09	—	—	—	—
$NaH_2PO_4 \cdot H_2O$	—	—	0.14	0.14	0.12	0.14
$Fe(NO_3)_3 \cdot 9H_2O$	—	—	—	—	7.2×10^{-4}	—
Glucose	—	1.00	1.00	1.00	1.00	1.00
Hepes[a]	—	—	—	238	—	—
Other components (mg/liter)	—	—	—	—	[b]	[c]

[a] *N*-2-Hydroxyethylpiperazine-*N*-2-ethanesulfonic acid.

[b] Adenine sulfate—10.0, adenosinetriphosphate—1.0, adenylic acid—0.2, cholesterol—0.2, deoxyribose—0.5, glutathione—0.05, guanine—0.3, hypoxanthine—0.3, ribose—0.5, sodium acetate—50.0, thymine—0.3, tween 80—20.0, uracil—0.2, xanthine—0.3, DL-α-alanine—50.0, L-arginine—70.0, DL-aspartic acid—60.0, L-cysteine—0.11, L-cystine—20.0, DL-glutamic acid—150.0, L-glutamine—100.0, glycine—50.0, L-histidine—21.88, l-hydroxyproline—10.0, DL-isoleucine—40.0, DL-leucine—120.0, L-lysine—70.0, DL-methionine—30.0, DL-phenylalanine—50.0, L-proline—40.0, DL-serine—50.0, DL-threonine—60.0, DL-tryptophan—20.0, L-tyrosine—40.0, DL-valine—50.0, ascorbic acid—0.05, α-tocopherol phosphate—0.01, D-biotin—0.01, calciferol—0.1, Ca-pantothenate—0.01, choline chloride—0.5, folic acid—0.01, *i*-inositol—0.05, menadione—0.01, niacin—0.025, niacinamide—0.025, *p*-aminobenzoic acid—0.05, pyridoxal—0.025, pyridoxine—0.025, riboflavin—0.01, thiamine—0.01, vitamin A—0.14.

[c] L-Arginine—126.4, L-cystine—24.0, L-glutamine—292.0, L-histidine—41.9, L-isoleucine—52.5, L-leucine—52.4, l-lysine—73.1, l-methionine—14.9, L-phenylalanine—33.0, L-threonine—47.6, L-tryptophan—10.2, L-tyrosine—36.2, L-valine—46.8, D-calcium pantothenate—1.0, choline chloride—1.0, folic acid—1.0, *i*-inositol—2.0, nicotinamide—1.0, pyridoxal—1.0, riboflavin—0.1, thiamine—1.0.

ure of aggregation since aggregated cells are counted as single cells in the Coulter counter. The WBC counts were closely observed to be sure that large aggregates did not occlude the counting chamber and give incorrect counts. Table II shows the effect of Ca^{2+}- and Mg^{2+}-free HBSS when compared with HBSS containing these ions. Also shown are the results obtained when the Ca^{2+}- and Mg^{2+}-free HBSS contained chelating agents (ACD formula A 1.5 ml/100 ml of Ca^{2+}-Mg^{2+}-free HBSS or EDTA 0.125% in Ca^{2+}-Mg^{2+}-free HBSS). Marked aggregation occurred at 37° in HBSS containing Ca^{2+} and Mg^{2+}. There was a slight reduction in aggregation when Ca^{2+}- and Mg^{2+}-free HBSS was used, however, both chelating agents caused a dramatic reduction in aggregation. Gross and microscopic examination of the samples and measurements of light scattering (OD 550 nm) correlated well with the Coulter counter results (data not shown). When heparin (10 U/ml) was used as an anticoagulant, and the same amount was added to HBSS in the isolation and preparation of the granulocytes, the level of aggregation was greater than that found when granulocytes were prepared with Ca^{2+}- and Mg^{2+}-free HBSS (data not shown).

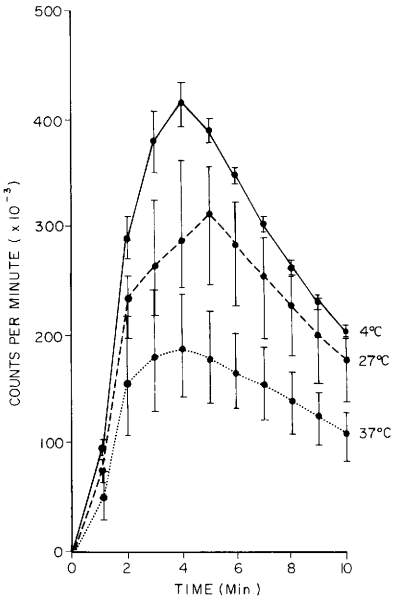

FIG. 1. Granulocytes were isolated and maintained at 4, 27, or 37° until 5 min before the CL assay when they were placed in a 37° water bath. The CL assay was run at 37° with continuous mixing. Opsonized zymosan was used as the CL stimulus. The lines indicate the average of triplicate determinations, and the brackets show the ranges.

Another factor contributing to aggregation was the technique of RBC lysis. In CL assays it is necessary to remove the RBCs because of their light quenching ability (9). Two techniques commonly used are hypotonic lysis (8) and ammonium chloride lysis (7). Table III summarizes the amount of aggregation associated with these procedures. The ammonium chloride method had a degree of aggregation which was similar to hypotonic lysis performed once. When hypotonic lysis was performed twice, however, there was a marked increase in the degree of aggregation. A similar increase in aggregation might have been seen if ammonium chloride lysis had been performed twice; but, in our experience it is rarely necessary to repeat ammonium chloride lysis in order to remove all the RBCs while it is frequently necessary to perform hypotonic lysis twice.

The force of centrifugation was another cause of aggregation. The centrifugation forces listed in the materials and methods section were the minimal values that would

TABLE II. EFFECT OF CALCIUM AND MAGNESIUM IONS ON GRANULOCYTE AGGREGATION[a]

	White blood cell counts per mm^3 using Coulter counter			
	Incubated 1 hr at 4°		Incubated 1 hr at 37°	
Cell media	Average	Range	Average	Range
HBSS	11,129	(11,055–11,194)	6,650	(6,570–6,697)
Ca^{2+}-Mg^{2+} Free HBSS	12,879	(12,822–12,956)	7,860	(7,667–8,195)
Ca^{2+}-Mg^{2+} Free HBSS + ACD	13,037	(12,905–13,263)	10,420	(10,339–10,546)
Ca^{2+}-Mg^{2+} Free HBSS + EDTA	11,105	(11,038–11,201)	10,953	(10,786–11,074)

[a] In this experiment the cell media listed were used for washing the cells following RBC lysis and in the final cell suspension used during incubation. Cells were maintained at 4° during the isolation procedure prior to the final incubations indicated above. All samples were equal aliquots of a single WBC preparation taken through the point of RBC lysis. The values shown are the average and range of triplicate WBC counts using a Coulter counter.

TABLE III. EFFECT OF RBC LYSING PROCEDURE ON GRANULOCYTE AGGREGATION[a]

	White blood cell counts per mm^3 using Coulter counter			
	Incubated 1 hr at 4°		Incubated 1 hr at 37°	
Lysing procedure	Average	Range	Average	Range
NH_4Cl Lysis*	12,199	(12,060–12,342)	10,060	(10,039–10,102)
Hypotonic lysis**				
Performed once	11,646	(11,486–11,807)	9,251	(9,162–9,300)
Performed twice	11,695	(11,574–11,758)	4,573	(4,497–4,622)

[a] All cells were prepared using Ca^{2+}-Mg^{2+}-free HBSS + EDTA 0.125%. Cells were maintained at 4° during the isolation procedure prior to the final incubations indicated above. The average of three determinations is shown.

* Weening *et al.* (7).

** Goldstein *et al.* (8).

completely sediment the granulocytes in a 10-min period. Centrifugation at higher speeds caused an increase in gross and microscopic aggregates that could not be broken up by vigorous vortex mixing.

In an attempt to further reduce cell aggregation, heated and unheated 10% autologous serum was used with HBSS. Neither of these preparations had any effect in reducing aggregation (data not shown).

Calcium and magnesium requirements for optimal chemiluminescence. Granulocytes require Ca^{2+} and Mg^{2+} for optimal light production. Since it was shown above that cells prepared in the absence of these ions were less likely to aggregate, we wanted to determine whether adding these ions immediately prior to a CL assay would allow the cells to produce normal levels of light. Figure 2 shows the CL responses of zymosan-stimulated granulocytes when the cells were suspended in either standard HBSS or in Ca^{2+}- and Mg^{2+}-free HBSS. Very little light was detected in the absence of Ca^{2+} and Mg^{2+}. The results of experiments in which granulocytes were prepared in Ca^{2+}- and Mg^{2+}-free HBSS containing EDTA 0.125% (3.4×10^{-3} *M*) are also shown in Fig. 2. In these experiments excess Ca^{2+} and Mg^{2+} were added immediately before initiating the CL run in order to bind EDTA and permit free Ca^{2+} and Mg^{2+} for granulocytes. The lowest final concentration of Ca^{2+} and Mg^{2+} was obtained by adding: $CaCl_2$, 0.28 g/liter; $MgSO_4 \cdot 7H_2O$, 0.24 g/liter; and $MgCl_2 \cdot 6H_2O$, 0.24 g/liter ($Ca^{2+} = 2.5 \times 10^{-3}$ *M*, $Mg^{2+} = 2.2 \times 10^{-3}$ *M*). The other three concentrations tested were 1.5, 2, and 3 times this level. All four concentrations gave similar levels of CL which exceeded the CL of cells prepared in standard HBSS (Fig. 2).

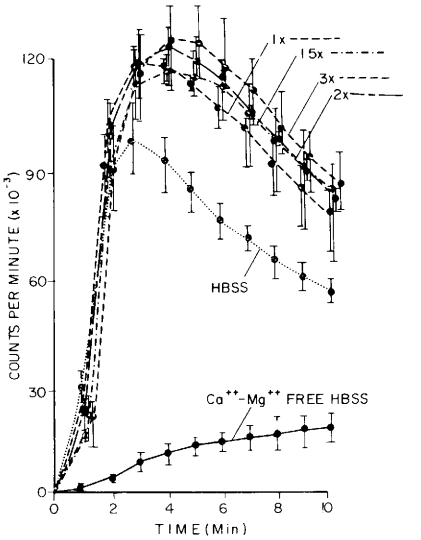

FIG. 2. The CL response of zymosan-stimulated granulocytes in various cell media is shown. The bottom two lines show the difference in CL responses of granulocytes in HBSS and Ca^{2+}- and Mg^{2+}-free HBSS. Granulocytes used in the four top lines were isolated in Ca^{2+}- and Mg^{2+}-free HBSS containing EDTA (0.125%; 3.4×10^{-3} *M*). In the experiment shown by the line marked 1X, Ca^{2+} and Mg^{2+} were added (see text) just prior to the CL assay. In the other experiments 1.5, 2, or 3 times the amount of Ca^{2+} and Mg^{2+} as used in the 1X experiment were added prior to the CL assay. The assay was performed at 37° with continuous mixing. Each line is the average of three observations and the brackets show the range.

Cell media and additives. A variety of cell media (compositions shown in Table I) were examined for their influence on granulocyte CL. Many of these media have been used by other investigators in CL studies. HBSS gave

the best CL levels while EBSS was next best (Table IV). All of the other media, however, gave markedly reduced levels of CL. In order to clarify the reasons for these differences some of the components present in these cell media were individually examined.

The effects of various medium components on granulocyte CL are summarized in Table V. All of the proteins added to HBSS inhibited the CL responses of zymosan-stimulated cells. In some cases, such as the reactions containing 2% ovalbumin, almost no CL was detected. The suppressive effect of the proteins did not appear to be due to light quenching since most of the solutions were colorless or only faintly yellow. Of the two amino acids tested, tryptophan caused a significant suppression of CL while tyrosine had no effect. Glucose enhanced the CL response of cells to opsonized zymosan. CL reactions in HBSS (normally containing 1 g of glucose/liter) were greater than reactions in HBSS with the glucose omitted. The CL response was further augmented when the glucose level was increased to 2 g/liter. Granulocytes in HBSS with 4 g of glucose/liter had similar levels of CL as cells in HBSS with 2 g of glucose/liter. Ascorbic acid and ferrous and ferric salts all had suppressive effects on the CL of zymosan-stimulated granulocytes.

Discussion. One of the greatest limitations in the use of granulocyte CL as a research technique or a clinical assay is its variability. In one of the first experiments in evaluating the factors contributing to this variability, it was found that the temperature at which the cells were isolated significantly influenced the amount of observed CL. Cells prepared at 4° gave much better CL than those prepared at 37° when exposed to opsonized zymosan at 37°. Since increased granulocyte isolation temperature correlated with increased cell aggregation, it appeared to be a likely cause of reduced CL. Aggregated granulocytes would have difficulty phagocytizing a particulate stimulus such as zymosan. Even soluble stimulators of granulocyte CL might be hindered by aggregation. Aggregation could also lead to inhomogeneity of samples and consequently greater variability.

Using the observed potentiation of aggre-

TABLE IV. THE INFLUENCE OF CELL MEDIA ON THE CHEMILUMINESCENCE OF GRANULOCYTES STIMULATED WITH OPSONIZED ZYMOSAN

Cell media	Chemiluminescence[a]	
	Average (%)	Range (%)
HBSS	100	89–111
EBSS	60	54–65
M199	49	46–53
Hepes	2	1–2
Ringers	1	0–1
MEM	<1	0–1

[a] This table includes data from experiments performed on different days, but in each experiment HBSS was compared with other cell media. For the purpose of comparison CL (total counts per 10-min run minus background values) was expressed as percentage of HBSS. The average CL value for HBSS was arbitrarily considered to be 100%. The range of duplicate determinations is shown.

TABLE V. THE INFLUENCE OF ADDITIVES ON THE CHEMILUMINESCENCE OF GRANULOCYTES STIMULATED WITH OPSONIZED ZYMOSAN

Cell media	Chemiluminescence[a]	
	Average (%)	Range (%)
HBSS—No additives	100	89–117
HBSS + additives		
Protein		
Autologous serum 2% (v/v)	76	72–79
Autologous serum 10% (v/v)	51	47–54
Bovine serum albumin 2% (w/v)	29	15–38
Fetal calf serum 2% (v/v)	59	59–60
Fetal calf serum 10% (v/v)	39	38–40
Gelatin 3% (w/v)	45	16–78
Ovalbumin 2% (w/v)	2	2–2
Amino Acids		
Tryptophan 3×10^{-4} *M*	73	64–82
Tyrosine 5×10^{-4} *M*	110	104–117
Glucose (g/liter)		
None	84	80–86
1[b]	100	89–117
2	124	122–125
4	123	117–129
Ascorbic acid 1×10^{-4} *M*	61	58–64
$FeSO_4$ 3.7×10^{-5} *M*	52	46–58
$FeCl_3$ 3.7×10^{-5} *M*	54	41–61

[a] This table includes data from experiments performed on different days, but in each experiment HBSS with and without additives were compared. For the purpose of comparison CL (total counts per 10-min run minus background values) was expressed as percentage of HBSS without additives. The average CL value for HBSS—no additives—was arbitrarily considered to be 100%. The range of triplicate determinations is shown.

[b] HBSS + glucose 1 g/liter is the same as HBSS—no additives.

gation at 37°, we examined various techniques to reduce aggregation. Other authors (10, 11) have noted that divalent cation chelators were helpful in reducing granulocyte aggregation. The present study has shown that the addition of EDTA and ACD to Ca^{2+}- and Mg^{2+}-free media caused a significant reduction in aggregation at 37° compared to Ca^{2+}- and Mg^{2+}-free media without chelators. It was possible to obtain high CL responses from cells in these media by adding excess Ca^{2+} and Mg^{2+} to the cells immediately prior to the CL assay. The responses were, in fact, better than those observed from cells prepared with standard HBSS. This improvement was probably due to reduced cell aggregation.

Physical trauma such as that produced by centrifugation and resuspension of cell pellets contributes to granulocyte aggregation. Reducing centrifugation speed and gentle mixing to resuspend cell pellets appeared to be beneficial in reducing granulocyte aggregation. The technique of RBC lysis is another important cause of aggregation. The ammonium chloride method caused less aggregation than the hypotonic lysis of RBCs under conditions which permitted complete lysis of the RBCs. By careful attention to all of the factors that prevent aggregation granulocyte CL should be greater and more reproducible.

Reports of CL studies show a confusing array of cell media ranging from buffered salt solutions containing no glucose, Ca^{2+}, or Mg^{2+} (4) to buffered salts containing proteins, glucose, Ca^{2+}, Mg^{2+}, and complex mixtures of amino acids, vitamins, and other additives (12–14). The present study demonstrates that the more complex media containing multiple amino acids and vitamins gave much lower levels of CL than the buffered salts with glucose, Ca^{2+}, and Mg^{2+}. It was found that proteins in general, tryptophan, ascorbic acid, and ferrous and ferric salts all had a significant suppressive effect on granulocyte CL. Some of these observations appear to contradict the results of Nelson *et al.* (12) who reported augmented CL in the presence of 3% gelatin and 3×10^{-4} *M* tryptophan. They suggested that these substances were serving as substrates for the reactive oxygen intermediates produced by the activated granulocytes. The systems are not identical, however, since these investigators used human granulocytes in a CL assay performed at ambient temperature.

Since glucose is an energy source for CL, it is possible that added glucose might augment the supply from cellular glycogen stores and thereby cause increased CL. This idea was supported by these studies. Although CL was observed without added glucose, the maximum response occurred when the glucose concentration was approximately 2 g/liter. Another, but less likely, possibility was that oxygen intermediates were reacting directly with glucose to produce a chemiluminescent reaction.

This study demonstrates that optimal granulocyte CL occurs when cells are in a buffered salt solution with glucose, Ca^{2+}, and Mg^{2+} without proteins, amino acids, vitamins, and iron salts. Minimizing the physical forces acting on the cells, reducing the temperature of cell isolation to 4°, and the use of Ca^{2+}-Mg^{2+}-free media with added divalent cation chelators are helpful in reducing aggregation and improving CL. These observations may be useful in improving the design of future CL studies.

Summary. A CL system consisting of canine granulocytes stimulated with opsonized zymosan was used to examine factors which lead to variability in light production. Variability of CL response was associated with granulocyte aggregation. Granulocyte aggregation was reduced by: (i) reducing centrifugation and mixing forces used to prepare cell suspensions, (ii) preparing cell suspensions at 4° in Ca^{2+}- and Mg^{2+}-free cell media containing divalant cation chelating agents, and (iii) lysing red blood cells by the ammonium chloride technique.

The type of cell media used during the CL run was another important variable. Solutions containing complex mixtures of amino acids and vitamins gave markedly reduced levels of CL. Tryptophan, ascorbic acid, iron salts, and all proteins tested caused significant suppression of CL. Glucose, Ca^{2+}, and Mg^{2+} in the final reaction mixture were necessary for maximal CL response. The optimal cell media for CL, therefore, was buffered salt solution containing glucose, Ca^{2+}, and Mg^{2+}, but not amino acids, vitamins, proteins, or iron salts.

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