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Numerical Data Concerning the Sensitivity of Anaerobic Bacteria to Oxygen

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Upon comparison of results obtained from growing eight different species of anaerobic bacteria in deep agar under two and three atm of pure oxygen with results of growing them in air under atmospheric pressure, it appears possible that the old terms of "microaerophobic," "very strict anaerobe," and "microaerophilic" can be replaced by precise values corresponding to the size of the inhibition zone produced by oxygen under various pressures when the bacteria are grown in a solid medium. The simultaneous effect of the dilution of the inoculum and the pressure of oxygen used was determined with the help of an electronic computer according to the standard multiple regression method. Two indices have been worked out which express the sensitivity of the species at different oxygen pressures when varying numbers of anaerobic bacteria are used: *X* sp, which is the size in millimeters of the inhibition zone when 1:10 dilutions of the culture are grown under pure oxygen at atmospheric pressure (lp = 0), and *B* sp, which is the result of multiplying by 1.43 the difference in size of inhibition zone when 1:10 dilutions of the culture are grown under atmospheric air (lp = −0.7) and under pure oxygen at 1 atm absolute.

Since Pasteur's discovery in 1861 of the first anaerobic bacteria (9) it has been customary to divide bacteria into three main categories according to their sensitivity to atmospheric oxygen: these are the strictly aerobic bacteria which cannot grow in the absence of oxygen; the facultative bacteria which can thrive either in the absence or in the presence of oxygen; and the so-called anaerobic bacteria which cannot grow in the presence of atmospheric oxygen.

Among the anaerobic bacteria, some species are strict anaerobes, others are called microaerophobic, still others are said to be microaerophilic, etc. These terms, which are used more or less loosely (8), have never been defined precisely, with the exception, perhaps, of Rist's attempt to group the bacteria into seven types: four anaerobic, two facultative, and one aerobic (12). This same classification was found again when Frei studied the respiratory enzymes found in bacteria (7).

With the advent of hyperbaric oxygen as a therapeutic measure for some infections caused by anaerobic bacteria, it was of interest to see if this tool could provide precise measurement of the sensitivity of the anaerobic bacteria to oxygen.

MATERIALS AND METHODS

Preliminary experiments showed that measurements could be more accurate in solid media, although the use of liquid media had already turned up interesting results (3-6). All of the data presented here were obtained with the use of deep agar. In our experiments, 6-ml amounts of Trypticase Soy Agar (BBL) were placed in tubes measuring 8 to 9 mm × 180 mm; this volume gives a height of about 120 mm in the agar column and permits the ready use of decimal dilutions. All of the media were regenerated immediately before use by placing them in an unpressurized autoclave for 20 min, then removing them to a water bath held at 45 C. This procedure results not only in the decolorization of methylene blue (rH = 14), but also (1) of tetrasulfoindigotine (rH = 12.5), nile blue (rH = 9.2), cresyl violet (rH = 8.0), and indigo carmine (rH = 7.4).

Eight different species of anaerobes were studied: one representative each of the gram-negative cocci (*Veillonella* isolated locally with the help of Rogosa's medium), the gram-positive cocci (*Micrococcus niger* obtained from A. Prévot in Paris), the gram-negative nonsporeforming bacilli [*Ristella* (*Bacteroides*) *fragilis* (10)], and five representatives of the gram-positive sporeformers [*Welchia* (*Clostridium*), *Plectridium* (*Clostridium*), and *Clostridium* (10)].

The cultures were stored at room temperature in brain medium sealed under vacuum. When needed,

these cultures were rejuvenated by transferring one drop to the bottom of a Hall tube (reduced design made for us by Canadian Laboratory Supplies, Montreal, Canada) containing 9 ml of freshly regenerated Trypticase Soy Broth (BBL).

These young, fresh broth cultures (overnight at 37 C) were decimally diluted directly into the molten and cooled agar by using Dispo pipettes (Canadian Laboratory Supplies) which were rinsed 10 times for each tube and discarded after every dilution. Triplicate series of media were thus seeded; one set was placed under 30 lb gauge pressure [3 atm absolute (ATA)] of pure oxygen, another set was placed under 15 lb gauge pressure (2 ATA) of pure oxygen, and the third set was left under atmospheric air. After overnight incubation at 37 C, the size of the zone of inhibition was measured in millimeters under a dissecting microscope.

RESULTS

Ten experiments were performed with each species of anaerobe concerned; the results are compiled in Table 1 as arithmetical means of the readings for all 10 experiments.

It can be seen that *W. perfringens* (*C. perfringens*), one of the most easily cultivated anaerobes (10), is in fact not very sensitive to oxygen in comparison with the other species studied. This may explain the frequency of isolation of this species in comparison with other less-known anaerobes. At atmospheric pressure, the zone of inhibition measured about 5 mm, whereas at 15 and 30 lb pressure of pure oxygen, they were, respectively, 13 and 15 mm. There is a sort of leveling effect between 15 and 30 lb of oxygen.

The anaerobic species which appeared next in sensitivity to oxygen was *C. histolyticum*, another rather easily grown anaerobe. The average zones measured 6, 15, and 17 mm, respectively. Here again, a leveling effect is noted at the higher pressures.

Similarly, the first anaerobic species ever discovered, *C. butyricum*, is not very sensitive to oxygen: 8, 16, and 18 mm. The tapering off effect is again noticeable at 15 and 30 lb pressure of pure oxygen.

C. sporogenes is the last of the series of not very sensitive anaerobes. The zones of inhibition measured, respectively, 9, 19, and 22 mm.

With *P.* (*Clostridium*) *tetani* (10), the zones of inhibition due to oxygen reach 10 and 20 mm. As is well known, this species is much more difficult to isolate and to grow than the previous species, indicating that zones of inhibition of these sizes mark a boundary in the sensitivity of anaerobes to oxygen.

The nonsporeforming anaerobes show a still

TABLE 1. *Size of inhibition zone in millimeters of anaerobic bacteria by oxygen at different pressures*

Organism	Dilution	Atmospheric pressure	15 lb of O_2	30 lb of O_2
Welchia perfringens	10^{-1}	3.2	9.2	11.6
	10^{-2}	4.2	11.4	13.4
	10^{-3}	4.6	12.6	15.0
	10^{-4}	5.4	14.2	16.6
	10^{-5}	5.6	16.6	18.4
	10^{-6}	6.0	16.8	18.8
	Total	29.0	80.8	93.8
	Avg	5	13	15
Clostridium histolyticum	10^{-1}	5.3	13.3	13.6
	10^{-2}	5.6	15.3	15.6
	10^{-3}	8.0	15.6	17.0
	10^{-4}	5.0	16.1	17.8
	10^{-5}	7.2	16.6	18.0
	10^{-6}	8.1	17.6	19.3
	Total	39.2	94.5	101.3
	Avg	6	15	17
C. butyricum	10^{-1}	6.0	13.0	15.0
	10^{-2}	7.0	15.8	17.0
	10^{-3}	11.0	14.5	17.2
	10^{-4}	8.4	19.8	18.7
	10^{-5}	9.0	16.7	21.4
	10^{-6}	8.6	21.5	25.0
	Total	50.0	101.3	109.3
	Avg	8	16	18
C. sporogenes	10^{-1}	6.3	17.0	18.3
	10^{-2}	8.3	17.8	20.0
	10^{-3}	8.1	20.0	24.5
	10^{-4}	10.8	20.1	22.1
	10^{-5}	10.6	21.1	21.8
	10^{-6}	10.0	20.5	24.9
	Total	54.1	116.5	131.6
	Avg	9	19	22
Plectridium tetani	10^{-1}	8.6	21.3	20.8
	10^{-2}	10.0	23.3	23.0
	10^{-3}	10.2	24.1	25.3
	10^{-4}	11.5	25.0	25.2
	10^{-5}	13.3	26.0	26.0
	10^{-6}	13.0	25.6	25.6
	Total	66.6	125.3	125.9
	Avg	11	21	21
Veillonella spp.	10^{-1}	7.4	17.4	20.4
	10^{-2}	8.8	21.8	25.4
	10^{-3}	9.2	24.6	25.6
	10^{-4}	12.2	24.7	32.0
	10^{-5}	9.7	25.3	27.5
	10^{-6}	14.2	25.6	27.7
	Total	61.5	139.4	178.6
	Avg	10	23	29

TABLE 1—*Continued*

Organism	Dilution	Atmospheric pressure	15 lb of O_2	30 lb of O_2
Micrococcus niger	10^{-1}	11.8	18.6	13.6
	10^{-2}	13.2	20.6	25.8
	10^{-3}	14.6	21.4	31.0
	10^{-4}	15.0	21.8	25.8
	10^{-5}	16.8	22.7	25.2
	10^{-6}	15.7	27.0	27.0
	Total	87.1	132.1	147.8
	Avg	16	22	24
Ristella fragilis	10^{-1}	11.6	24.5	24.8
	10^{-2}	12.8	27.8	26.6
	10^{-3}	14.1	27.6	28.6
	10^{-4}	14.5	29.8	28.1
	10^{-5}	13.8	30.1	29.3
	10^{-6}	16.0	35.9	29.1
	Total	82.8	175.7	166.5
	Avg	13	29	28

greater sensitivity to oxygen, a fact which might explain the failures to isolate these bacteria as regularly as some of the more hardy species. For example, a species of *Veillonella* isolated locally gave 10, 23, and 29 mm; similarly, *Micrococcus niger* gave 16, 22, and 24 mm. Finally, *Ristella fragilis* (Prévot) gave the widest zones, namely, 13, 29, and 28 mm. Thus, it would appear that in this particular case the specific epithet is well chosen.

The results seem to suggest that the size of the zones of inhibition is directly proportional to the logarithm of the partial pressure of oxygen used (lp) as well as to the logarithm of the dilution of the bacterial suspension (ld). The values of lp corresponding to the data are: −0.699 (which is the log of 0.2, or partial pressure of oxygen in atmospheric air); 0.301 (log of 2.0 ATA); and 0.477 (log of 3.0 ATA). The values of ld corresponding to the data are 1, 2, 3, 4, 5, 6, which are the logarithms of dilutions by 10, 100, 1,000, 10,000, 100,000, and 1,000,000. The simultaneous effect of factors lp and ld for each species was therefore examined by means of a double regression formula based on the method of the least squares (11):

$$Y = a + b_1 \text{ lp} + b_2 \text{ ld} \qquad (1)$$

The values obtained for parameters a, b_1, and b_2 are contained in Table 2. The different partial regression coefficients were all significantly different from zero ($P < 0.01$). The double regression planes for the eight species of anaerobes studied are illustrated in Fig. 1–8 according to Finney's method (2). The ordinate corresponds to the size of the inhibition zone, whereas the abscissa shows the sum of lp and ld for the combinations considered for these two "independent variables." The theoretical values of Y form a parallelogram containing a series of smaller parallelograms limited by two series of straight lines respectively parallel to two adjacent sides of the large figure. In each diagram, the six lines with the steeper slope represent simple logarithmic regressions of the inhibition of growth as a function of partial oxygen pressure for a given dilution. In a complementary fashion, the four lines with the weaker slope represent the simple logarithmic regressions of the inhibition of growth as a function of dilution when the partial oxygen pressure is equal to 0.2, 1.0, 2.0, and 3.0 atm, the value for 1.0 ATA being an interpolation.

These equations permit a quantitative comparison of the sensitivity of the eight species of anaerobes to oxygen. We propose to use as a main index the value obtained when a 1:10 dilution of the culture is grown under 1 atm of pure oxygen (lp = 0). This specific index of anaerobiosis, which we propose to call "X sp,"

TABLE 2. *Double regressions of growth inhibition as a function of the pressure of oxygen and of initial dilution of the culture in eight anaerobic species*[a]

Species	a	b_1	b_2	R
Welchia perfringens	6.85	9.00 ± 0.45	1.21 ± 0.14	0.984
Clostridium histolyticum	10.21	8.94 ± 0.41	0.74 ± 0.12	0.986
C. butyricum	10.13	8.92 ± 0.83	1.25 ± 0.25	0.950
C. sporogenes	13.45	10.78 ± 0.55	0.87 ± 0.17	0.982
Veillonella spp.	14.93	13.49 ± 0.92	1.34 ± 0.28	0.970
Micrococcus niger	15.51	8.28 ± 0.14	1.34 ± 0.42	0.870
Plectridium tetani	16.37	11.87 ± 0.53	0.91 ± 0.16	0.986
Ristella fragilis	19.25	13.10 ± 1.02	1.15 ± 0.31	0.961

[a] $Y = a + b_1\text{lp} + b_2\text{ld}$; Y = inhibition zone (in mm), lp = logarithm of partial pressure of oxygen (in atmospheres), ld = logarithm of dilution, R = correlation coefficient for the double regression, ± = standard deviations of partial regression coefficients b_1 and b_2.

TABLE 3. *Numerical indices suggested for the characterization of the sensitivity to oxygen of eight anaerobic species of bacteria*[a]

Species	*X* sp	*B* sp
Group 1		
Welchia perfringens	8.06	9.18 (*B* sp = 9.00 ± 0.45)
Clostridium histolyticum	10.95	9.18 (*B* sp = 8.94 ± 0.41)
C. butyricum	11.38	9.18 (*B* sp = 8.92 ± 0.83)
C. sporogenes	14.32	9.18 (*B* sp = 10.78 ± 0.55)
Micrococcus niger	16.85	9.18 (*B* sp = 8.28 ± 0.14)
Group 2		
Veillonella spp.	16.27	12.82 (*B* sp = 13.49 ± 0.92)
Plectridium tetani	17.28	12.82 (*B* sp = 11.87 ± 0.53)
Ristella fragilis	20.40	12.82 (*B* sp = 13.10 ± 1.02)

[a] *X* sp = inhibition zone (in mm) under 1 ATA of pure oxygen when a 1:10 dilution of the culture is used (lp = 0; ld = 1; *X* sp = $a + b_2$). *B* sp = increase in the size of the inhibition zone when the partial pressure of oxygen is multiplied by 10 (*B* sp = b_1). General equation representing the ensemble of the 144 results from Table 1: $Y = -1.37 + 1.019X$ sp + *B* sp lp + 1.1 ld ($R = 0.905$) (*B* sp = 9.18, 12.82).

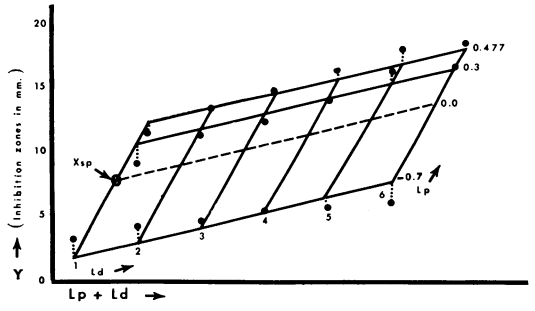

FIG. 1. *Double regression planes for Welchia perfringens.*

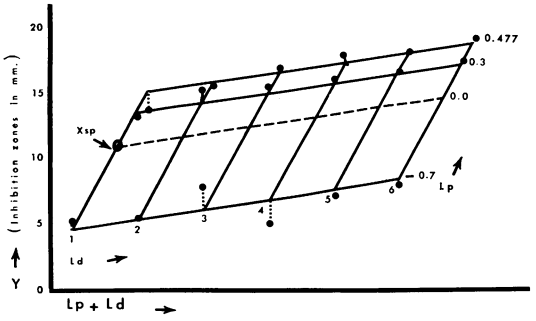

FIG. 2. *Double regression planes for Clostridium histolyticum.*

corresponds to the value of $a + b_2$ in equation 1. In Fig. 1–8, it is represented by the point of intersection (circle) of the straight lines coresponding to lp = 0 and ld = 1. With the use of this criterion, the following classification is obtained: *W. perfringens*, 8.06; *C. histolyticum*, 10.95; *C. butyricum*, 11.38; *C. sporogenes*, 14.32; *Veillonella* spp., 16.27; *M. niger*, 16.85; *P. tetani*, 17.28; and *R. fragilis*, 20.40.

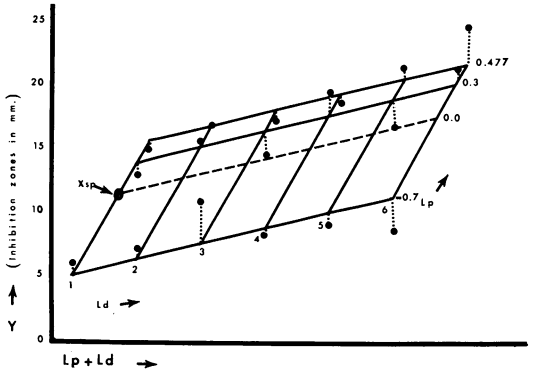

FIG. 3. *Double regression planes for Clostridium butyricum.*

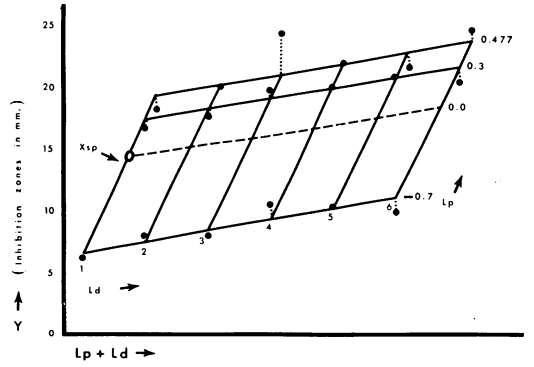

FIG. 4. *Double regression planes for Clostridium sporogenes.*

But a more rigorous examination of the values in Fig. 1–8, especially the analysis of covariance between the corresponding equations, allows a comparison of the slopes of b_1 and b_2. We come

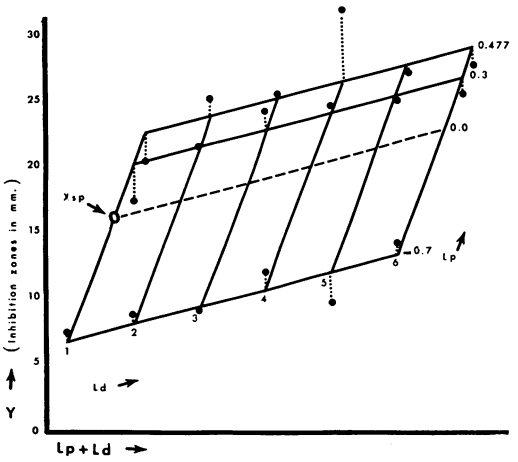

FIG. 5. *Double regression planes for Veillonella spp.*

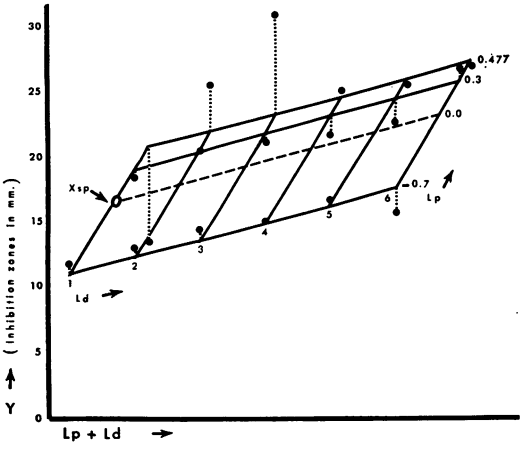

FIG. 6. *Double regression planes for Micrococcus niger.*

to the conclusion, first, that there is no significant difference between the b_2 coefficients applicable to the logarithms of the dilutions used, the common value of b_2 being 1.1, and, second, that the eight regressions cannot be considered parallel to each other in so far as the effect of lp is considered. We therefore would like to consider a second specific index, "*B* sp," which corresponds to the values of b_1 given in Table 2. Biologically speaking, "*B* sp" translates the increase in the size of the zone of inhibitions when the partial pressure of oxygen is increased ten times.

The entire 144 observations reported in Table 1 may be summarized by means of the following equation:

$$Y = -1.37 + 1.019\,X\,\text{sp} + B\,\text{sp lp} + 1.1\,\text{ld} \quad (2)$$

This equation has been established from a simple regression of the following type:

$$(Y - B\,\text{sp lp} - 1.1\,\text{ld}) = a + b\,X\,\text{sp} \quad (3)$$

on the result of which an algebraic transposition was effected to isolate Y anew and replace the terms B sp lp 1.1 ld in the right-hand member of the equation. Factor X sp being a specific index, the partial regression coefficient applying to it should theoretically be equal to 1; the value of 1.019 which was obtained therefore constitutes a correction for the slight error introduced by

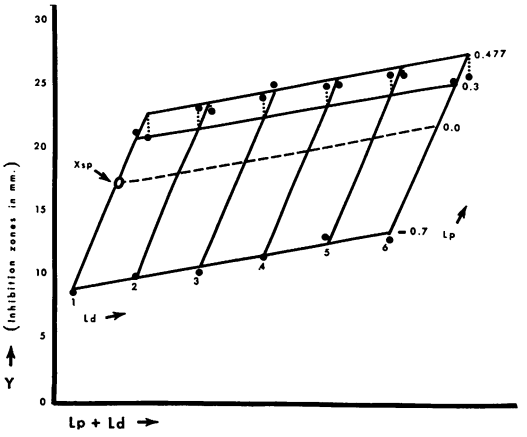

FIG. 7. *Double regression planes for Plectridium tetani.*

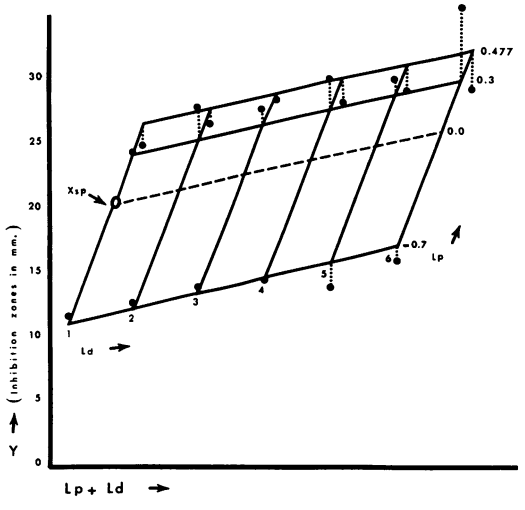

FIG. 8. *Double regression planes for Ristella fragilis.*

considering a single common value for coefficient ld.

Whereas the double regression lines mentioned in Table 2 can easily be represented as parallelograms, which constitute in fact a simplified view of a tridimensional graph, graphic models of equation 2 comprise a five-dimensional graph. In a subsequent article, a solution is proposed towards such graphical representation, obtained by an extension of Finney's method modified with the help of a few devices which permit a direct visualization of abstract multidimensional spaces (Roy et al., Can. J. Microbiol., *in press*).

Simplified procedure for further determinations. Since it seems highly desirable that the indices X sp and B sp be determined as soon as possible for the various species and strains of anaerobic bacteria already known, the authors would suggest a simplification of the procedure, both experimental and mathematical.

It is believed the one valid conclusion of the results reported on the eight species studied is that the zone of inhibition is proportional both to the logarithm of oxygen pressure (or partial oxygen pressure) and to the logarithm of the dilution of the culture. With this in mind, the X sp index can be determined directly as the average inhibition zone on cultures previously diluted 1:10 and incubated under pure oxygen pressure (lp = 0). To determine the B sp index, which will permit a prediction of the inhibition zone under other oxygen pressures, it may be sufficient to perform only one additional series of experiments at an oxygen pressure different from 1 ATA. The simplest procedure would be to use atmospheric air under normal pressure (lp = 0.7). If this suggestion is adopted and the average inhibition zone under atmospheric air is called X_2, then a valid estimation of B sp corresponds to:

$$B\,\text{sp} = (X\,\text{sp} - X_2)/0.7 \qquad (4)$$

or $B\,\text{sp} = 1.43\,(X\,\text{sp} - X_2)$.

DISCUSSION

The use of oxygen under pressure to study the inhibition of growth in different species of anaerobic bacteria under various dilutions would seem to indicate the possibility of attributing precise values to loosely used and ill-defined terms concerning the sensitivity of the anaerobes to oxygen. The use of known and diminishing numbers of bacteria in the inocula brings out evidence that the anaerobes do not follow a simple and regular pattern of sensitivity to oxygen. Nevertheless, such a determination can be made more readily in solid media than in liquid media, but once again it is necessary to use varying numbers in the inoculum as well as different oxygen pressures in order to obtain definite data on this fundamental property of the anaerobic bacteria. Notwithstanding the possible use of hyperbaric oxygen for treatment of diseases produced either in man or in animals by these bacteria, these studies throw some light on the main characteristic of these peculiar bacteria.

ACKNOWLEDGMENTS

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