Separation and size determination of human serum lipoproteins by agarose gel filtration

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ABSTRACT A method is described for the separation of the three major classes of human serum lipoproteins by gel filtration on columns of 4 and 6% agarose gel. After calibration of the columns, the elution volumes of the lipoproteins were used to calculate the molecular sizes and molecular weights of these macromolecules. The technique was employed to demonstrate aggregation of low density lipoprotein following partial delipidation, partial proteolysis, or mild heat denaturation. Agarose gel filtration shows promise as a useful method for the isolation, purification, and characterization of lipoproteins.

KEY WORDS gel filtration · serum · lipoproteins · agarose · separation · molecular weight · molecular size · aggregation · delipidation · denaturation · proteolysis

Molecular sieve, or gel filtration, column chromatography has provided a valuable method for the separation and characterization of macromolecules (1–3). With this technique the elution positions are determined solely by the dimensions of a macromolecule, in contrast to adsorption, partition, and ion exchange chromatographies, which depend largely on surface and charge properties. Since separation depends upon the ability of the macromolecule to penetrate to the interior of the gel material, good separations can be achieved only between macromolecules that are smaller in size than the pores of the gel.

The column bed materials most commonly used are gel particles of cross-linked dextran or polyacrylamide. Because these gels have rather small pore sizes, macromolecules with molecular weights greater than 400,000 are totally excluded from the gel interior and cannot be separated from one another. The small pore sizes of these gels have greatly limited their utility for the study of very large particles, such as the serum lipoproteins. Agar and agarose, on the other hand, form gels with much larger pores and have been used successfully to separate macromolecules with molecular weights of many millions, including virus particles (4–6), subcellular particles (7), nucleic acids (8, 9), and macroglobulins (10).

Most of the previous gel filtration studies of serum lipoproteins were done with cross-linked dextran gels. The first serum protein peak from Sephadex G-200 columns was initially reported to contain all the serum lipoproteins (1, 11). The lipoproteins were also found to migrate as a single spot on Sephadex G-200 thin-layer gel filtration (12). Later work indicated that HDL and LDL were eluted from Sephadex G-200 as separate, but overlapping peaks. Thus, LDL and VLDL emerged with the first protein peak, but HDL emerged just ahead of the second peak of serum proteins (13–16). During fractionation of human serum macroglobulins on 3.5% agar gels, Killander, Bengtsson, and Philipson (10) noted the separation of serum lipoproteins into three or four bands. Recently, Werner (17) has reported partial fractionation of serum lipoproteins from normal and hyperlipemic subjects on 2% agarose gels.

In the present studies agarose gel columns have been used to separate the lipoproteins of human serum. The technique was also employed to assess the homogeneity of an LDL preparation, to estimate the molecular diameters and weights of the serum lipoproteins, and to demonstrate aggregation resulting from mild heat treatment, partial proteolysis, or partial delipidation of LDL.

EXPERIMENTAL PROCEDURES

Materials

Agarose was obtained from Seravac Laboratories, Maidenhead, Berks., England. Porous polyethylene

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; SDS, sodium dodecyl sulfate.

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sheets ($^1/_8$ inch thick) were bought from Bel-Art Products. Sodium dextran sulfate 2000 (from dextran with molecular weight of 2×10^6) was obtained from Pharmacia Fine Chemicals Inc., Piscataway, N.J. Bushy stunt virus and tobacco mosaic virus were kindly provided by Dr. Gary Ackers. Bovine serum albumin was purchased from Armour Pharmaceutical Co., Chicago, Ill.

Preparation of Lipoproteins

All lipoprotein fractions were prepared from ACD plasma obtained by plasmapheresis of one fasting, normal subject. The plasma was recalcified and serum was separated from the fibrin clot. The serum was centrifuged at 0°C in the 870 head of a model B-20 International Centrifuge at 26,000 *g* for 30 min to remove chylomicrons. Dextran sulfate was added to a portion of the infranatant serum to precipitate both VLDL and LDL, which were then separated by ultracentrifugation (18, 19). The remaining serum was repeatedly ultracentrifuged to yield HDL (20). Protein concentrations were determined by dry weight and by the colorimetric method of Lowry, Rosebrough, Farr, and Handell (21).

Preparation of Agarose Columns

Dry agarose was added to distilled water in amounts sufficient to give gels of the desired percentage. The mixtures were heated in an autoclave for 15 min at 15 psi. After removal from the autoclave, the solution was allowed to gel at room temperature. The block of gel was sliced into large pieces with a spatula and then reduced to small fragments with a Waring Blendor. Only one-third to one-half of the blendor volume was filled with gel so that sufficient excess of water was available to keep the particles well suspended during the cutting. Cutting periods of 60–90 sec were employed. After each cutting period, the suspended gel particles were washed with a strong stream of tap water through a 40-mesh sieve and collected on a 60-mesh sieve. Particles larger than 40-mesh were returned to the blendor for another cutting. Three or four cutting cycles reduced most of the gel to particles small enough to pass through the 40-mesh sieve. Gel fragments smaller than 40- and larger than 60-mesh were washed into a vacuum flask, which was evacuated with a water pump aspirator to remove trapped air from the gel particles.

The columns were packed in glass tubing (122×1.3 cm). The agarose gel was supported on a porous polyethylene disc, cut to size with a number 8 cork borer, that was held in place with a size 0 rubber stopper. A 16 gauge stainless steel needle, with hub removed, was passed through the rubber stopper so that the flat end of the needle was flush with the inner surface of the stopper. Polyethylene tubing was attached to the needle and the column effluent was directed into a flow-through cell of a model 139 Hitachi Perkin-Elmer spectrophotometer and then into a fraction collector. Absorption was monitored at 280 mμ. This type of column has the advantage of negligible dead-space volume for possible mixing of proteins after elution.

Columns were established by pouring a suspension of the gel particles into the top of the glass tube and allowing excess water to drain from the bottom of the tube. In order to obtain an evenly packed column we continually added fresh agarose to maintain a layer of suspended gel particles above the lengthening column of packed gel. When the level of packed gel reached the desired height (110 cm), a buffer reservoir was attached and the column was equilibrated with the buffer. The eluting fluid in all experiments consisted of 0.4 M NaCl solution containing 0.1 M potassium phosphate buffer, pH 7.0, and 0.1 g/liter of the sodium salt of EDTA. Toluene (1 drop/liter) added to the buffer prevented bacterial growth in the column.

Although a disc of filter paper is frequently placed on top of gels to prevent a disturbance of the column surface when samples are added, no disc was used on these columns because lipoproteins tend to adsorb to filter paper. The protein sample, in a volume of 0.5–1.0 ml, could easily be applied without disturbing the surface of the gel. All columns were run at room temperature. Initial flow rates varied from 10 to 20 ml/hr. After a column had been used five or six times the flow rate fell to about 3 ml/hr. The initial flow rate was regained when the gel was removed and the column was refilled. Attempts were made to study proteins solubilized in SDS, but addition of SDS to the eluting buffer, even in the absence of protein, caused such a great reduction in flow rate that these experiments were abandoned.

Calculation of Molecular Sizes and Molecular Weights

A number of different equations have been proposed for the calculation of the size and molecular weight of a macromolecule from data obtained by gel filtration (22–26). The use of such equations requires the definition of several column parameters. These include: V_e, the elution volume required to reach the peak concentration of the solute; V_0, the void volume of the column, which corresponds to the elution volume of a protein so large that it is totally excluded from the gel pores; V_t, the total volume of the gel bed, which equals the elution volume of a molecule so small that it is distributed throughout the interior of the gel; and V_i, the volume of the interior stationary solvent phase, which can be determined from the relationship $V_i = V_t - V_0$.

In these studies the molecular sizes of the lipoproteins were calculated by the method of Ackers and Steere (26). This first involves the determination of two column calibration constants, using two particles with known mo-

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lecular radii whose elution volumes are intermediate between V_0 and V_t. From the elution volume of these particles with radii a_1 and a_2, corresponding sieve coefficients, σ_1 and σ_2, are calculated from the equation $V_e = V_0 + \sigma V_t$. Using the relationship $\sigma = 1 - \text{erfc}(x)$, values for x_1 and x_2, corresponding to σ_1 and σ_2, are obtained from error function tables. The calibration constants are then determined from the equations $b_0 = (a_1 - a_2)/(x_1 - x_2)$ and $a_0 = {}^1/_2[a_1 + a_2 - b_0(x_1 + x_2)]$. Once these calibration constants have been determined, the Stokes radius, a, of an unknown particle can be calculated. The σ of the unknown is calculated from its elution volume, and the corresponding x is determined from error function tables. The Stokes radius is then given by the formula $a = a_0 + b_0x$.

Molecular weights were determined from the relationship $MW = 4\pi NR^3/3\bar{V} \times 1.46$, where N = Avogadro's number, $\bar{V}$ = partial specific volume of the lipoprotein, R = Stokes radius of the lipoprotein, and the factor 1.46 is a correction for hydration of the macromolecule, based on an assumed value of 0.35 g of water per g of anhydrous protein (26, 27). The partial specific volumes of the lipoproteins were calculated from the average density of each lipoprotein.

RESULTS

Elution Pattern of LDL

Fig. 1 illustrates a typical elution pattern of LDL from a 6% agarose gel column. The lipoprotein began to emerge from the column after 80 ml, the peak was reached at 90 ml, and the protein was completely eluted after 110 ml of buffer. The elution volume of LDL varied by less than ±1 ml when the same preparation of LDL was repeatedly applied to the same agarose column, and by less than ±2.5 ml when it was applied to other 6% agarose columns of the same size. The sharp symmetrical elution peak suggests that LDL is not very heterogeneous in size. No protein peaks were eluted either before or after the LDL peak, which indicates that this preparation of LDL was not contaminated with significant amounts of either larger or smaller proteins.

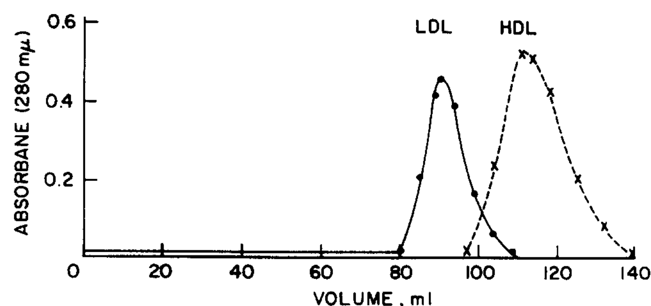

FIG. 1. Elution patterns of LDL and HDL applied separately to a 6% agarose gel column. 8 mg of each lipoprotein was applied in a volume of 0.5 ml.

TABLE 1 REPORTED MOLECULAR PARAMETERS AND ELUTION VOLUME OF PROTEINS FROM A COLUMN OF 6% AGAROSE GEL

Protein	Molecular Size	Molecular Weight	Elution Volume
	A		*ml*
Tobacco mosaic virus (28)	3000 × 152	39 × 10^6	66.6
VLDL (27)	300–700	5–90 × 10^6	76
Bushy stunt virus (28)	309*	8.9 × 10^6	84.5
LDL (27)	350 × 150	3 × 10^6	90
HDL (27)	80–100	1–4 × 10^5	112.5
Bovine serum albumin (29)	72*	6.8 × 10^4	120.5

Numbers in parentheses are reference numbers.
* Stokes diameters for assumed spherical particles.

Molecular Sizes and Weights of VLDL, LDL, and HDL

Table 1 lists the molecular sizes and weights reported for the proteins used to calibrate the agarose gel columns, along with their elution volumes from a typical column. Tobacco mosaic virus was eluted after 65 ml, which represents the void volume of the column. Potassium iodide emerged after 155 ml, the total fluid volume of the column. Bushy stunt virus and serum albumin were chosen as standards because of their well-studied physical properties. Their elution patterns are shown in Fig. 2. We applied substances of grossly differing sizes in order to test the behavior of the column throughout the size range of the lipoproteins. The correlation between the size and elution volume of these macromolecules demonstrates that the 6% agarose gel column was in fact acting as a molecular sieve.

Also included in Table 1 are representative values reported for the size and molecular weight of each class of human serum lipoproteins, and their elution volumes from a 6% agarose gel column. These elution volumes, along with the calibration data, were used in the method

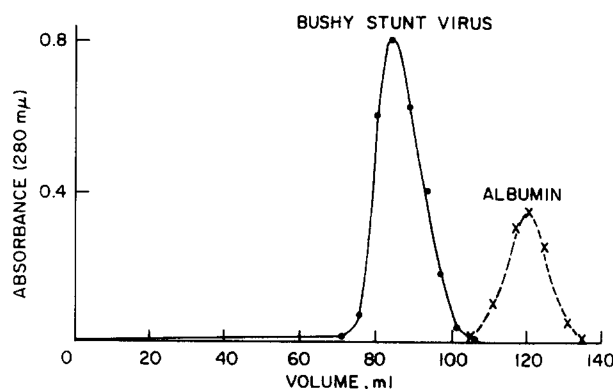

FIG. 2. Elution patterns of bushy stunt virus and bovine serum albumin applied separately to a 6% agarose gel column. 8 mg of bushy stunt virus and 4 mg of bovine serum albumin were applied, each in a volume of 0.5 ml.

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TABLE 2 Size and Molecular Weights of Human Serum Lipoproteins Calculated from Elution Volumes from Agarose Gel Columns

Lipoprotein	Molecular Size*	Mol Wt $\times 10^{-6}$†
	A	
VLDL	414 (380–440)	15
LDL	258 (246–258)	3.5
HDL	113 (113–117)	0.365

* All values are Stokes diameters, assuming a spherical particle. The initial values listed were calculated from the elution volumes of Table 1. Indicated in parentheses are the ranges of values calculated from separate experiments using one 4% and two 6% agarose gel columns.

† Calculated from the mean values of the Stokes radius.

of Ackers and Steere (26) to calculate the molecular size of each lipoprotein. The results are shown in Table 2. The calculated molecular size of each lipoprotein is presented in terms of the Stokes diameter of a spherical molecule of equivalent size. Such equivalent Stokes diameters provide a good estimate of the dimensions of LDL and VLDL, which are almost spherical particles, but are not as useful for more asymmetrical particles such as HDL.

Although gel filtration data have been used to calculate the molecular weights of proteins, the elution volume of a macromolecule correlates better with its Stokes radius than with its molecular weight (23, 30, 31). Only when the tested macromolecules and the calibrating proteins have similar partial specific volumes and frictional ratios can accurate molecular weights be obtained from elution volumes alone. Since both the partial specific volumes and frictional ratios of the lipoproteins are quite different from those of the standards, the molecular weights of the lipoproteins cannot be directly calculated from the empirical equations alluded to earlier. An alternative method for estimating molecular weights employs the partial specific volumes of the lipoproteins along with the Stokes radius determined from the gel filtration experiments (26). The molecular weights estimated in this manner tend to be higher than the true anhydrous weight of the particle, especially for asymmetrical particles such as HDL. The mean molecular weights calculated are listed in Table 2.

The sizes of lipoproteins determined by gel filtration (Table 2) agree reasonably well with those found by other methods (Table 1). The wide range of values calculated for the size of VLDL is not surprising since this lipoprotein emerged as a broad band which made it difficult to determine its elution volume accurately. The size of HDL obtained by gel filtration is greater than the average values reported for the whole HDL class (27), but it is similar to values calculated for the larger HDL_2 subfraction (32). Although the molecular weights estimated for HDL and VLDL fall within the range of values reported for these lipoproteins, the molecular weight of 3,500,000 determined for LDL is higher than most reported values.

Thus, gel filtration provides a simple and reasonably accurate method for determining the molecular size of lipoproteins that does not require the use of costly equipment and highly specialized techniques. Although this method allows an approximate determination of the molecular weights of lipoproteins, these values can be determined accurately only when gel filtration is used in conjunction with a second physical method.

Separation of Serum Lipoproteins

The elution volumes in Table 1 indicate that the major classes of serum lipoproteins were fairly well separated on a single agarose gel column. Fig. 1 depicts the elution patterns obtained when LDL and HDL were applied separately to the same column. There was some overlap between the elution peaks of the two lipoproteins, but the use of a longer column or a gel with smaller pore size should allow complete separation. As illustrated in Fig. 3, the separation between LDL and VLDL was not as good as that obtained between LDL and HDL. VLDL began to emerge with the void volume of the column, but there was considerable overlap between the VLDL and LDL peaks. The broad elution peak of VLDL is largely a reflection of its great heterogeneity in size, but could also indicate some contamination with LDL. The breadth of the VLDL elution peak suggests that gel filtration might also prove useful for the separation of this lipoprotein into subfractions of more homogeneous size. Such subfractionation has been previously achieved by ultracentrifugation (33, 34). The present method, however, would provide separation according to particle size alone, rather than as a function of both density and particle size.

Demonstration of Aggregation of LDL

Agarose gel columns were used to document physical changes in LDL after gentle heat treatment, partial digestion by proteolytic enzymes, and partial delipidation. Fig. 1 demonstrates that native LDL emerged as a single symmetrical peak with an elution maximum of 90 ml. This lipoprotein was then heated at 65°C for 5 min, a procedure that produced no visible turbidity, though changes in spectral properties were not assessed. Fig. 4 shows the elution pattern observed when heat-treated LDL was placed on the same agarose gel column. The major elution peak occurred at 72 ml, and there was a smaller peak with the same elution volume as native LDL. The faster-moving peak presumably consists of lipoprotein molecules aggregated by the heat treatment.

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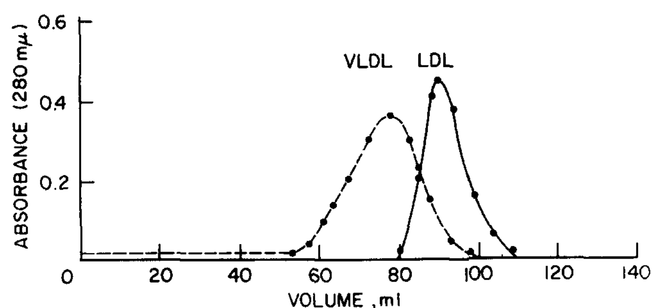

FIG. 3. Elution patterns of VLDL and LDL applied separately to a 6% agarose gel column. 8 mg of LDL and 15 mg of VLDL were applied, each in a volume of 0.5 ml.

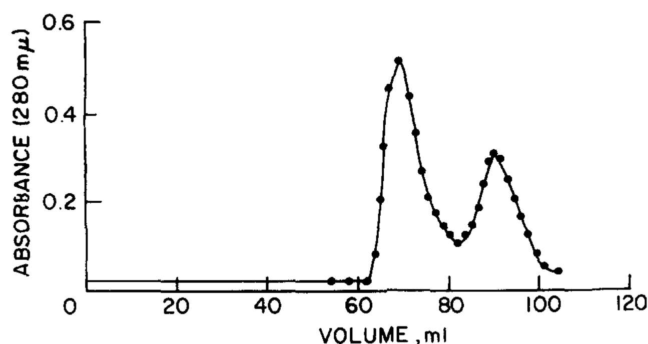

FIG. 4. Elution pattern of heat-denatured LDL applied to a 6% agarose gel column. 12 mg of lipoprotein was applied in a volume of 0.75 ml.

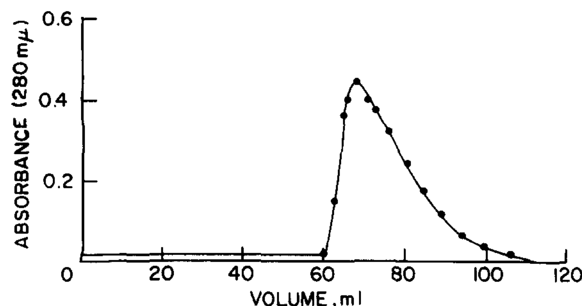

FIG. 5. Elution pattern of pronase-digested LDL applied to a 6% agarose column. 16 mg of partially digested lipoprotein was applied in a volume of 1.0 ml.

LDL was also digested for 8 hr with pronase, a bacterial proteolytic enzyme, which released about 25% of the peptide during this period (35). The digested lipoprotein did not appear turbid. On a 6% agarose column the main elution peak (Fig. 5) was at 68 ml and tailed to 105 ml. Similar aggregation was noted following the release of 14% of LDL peptide during a 24 hr digestion with trypsin.

Finally, LDL was partially delipidated by cold ether extraction according to Avigan (36). This procedure resulted in the removal of essentially all of the neutral lipids. The partially delipidated lipoprotein was readily soluble in isotonic saline, but the resulting solution was slightly turbid. Since neutral lipids account for about half the weight of LDL, the modified lipoprotein should be eluted after native LDL. Instead, the partially delipidated LDL emerged as a single peak with its maximum at 75 ml, which indicates that this treatment also leads to aggregation.

DISCUSSION

Agar, which is widely employed as a supporting medium for electrophoresis, contains a large number of charged groups which may result in adsorption of proteins to the gel. Such adsorption to agar gels has been observed specifically for serum lipoproteins (37, 38). Agarose, one of the two major components of agar, is an uncharged linear galactose polymer (39). Thus, nonspecific adsorption of protein and ion-exchange effects are less likely to interfere with the simple relationship between elution volume and molecular size (40). Although we used a high ionic strength buffer in these studies to minimize adsorption of lipoproteins to the agarose gels, this precaution was probably unnecessary. In several experiments HDL, LDL, and VLDL were applied separately to small 6% agarose columns that were developed with 0.15 M NaCl. Protein determinations on the starting lipoproteins and on pooled eluates from each column indicated that each lipoprotein was completely recovered (21).

The advantages of using carefully prepared agarose are obvious since, in impure agarose, a large number of sulfate and other charged groups may persist from the starting agar. The method of preparing agarose gel particles used in these studies results in granules of irregular shape. Several techniques have been described for preparing spherical agarose gel granules (6, 41). Such gel particles give more homogeneous packing of columns, and, consequently, narrower zones of elution than irregularly-shaped granules. Several commercial sources now sell agarose of high purity and have also made available spherical agarose gel granules with agarose concentrations varying from 2 to 10%.

The widespread use of gel filtration in biochemical studies attests to the utility of this technique, and the results reported in this paper clearly indicate that agarose gel chromatography extends many of these advantages to the study of lipoproteins. Gel filtration can be employed as either a preparative or an analytical tool. Complete separation of lipoprotein classes was not a major objective of these studies. However, by proper choice of gel pore size and column dimensions, agarose gel chromatography should allow the isolation of a narrow size range of serum lipoproteins. This method may also prove useful for subfractionation of these major components.

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The technique may be as gentle as, or perhaps more gentle than, preparative ultracentrifugation and should not alter the structure of the isolated lipoproteins.

As an analytical technique, gel filtration can be used to evaluate homogeneity, to estimate molecular size and weight, and to assess changes in these properties following various modifications of lipoproteins. This test of homogeneity is particularly useful since it depends almost exclusively on the size of the lipoprotein. In contrast, ultracentrifugation is influenced by size, shape, and density, while electrophoresis is largely dependent on charge properties. Agarose gel filtration provides an additional method for determining the molecular size and weight of lipoproteins, especially when combined with a second physical method.

Aggregation of LDL was demonstrated after gentle heat treatment, partial proteolysis, and partial delipidation. It is noteworthy that little or no turbidity was evident in the treated samples. Such changes in the physical properties of modified lipoproteins must be taken into consideration when attempts are made to interpret the effects of modification. For example, earlier studies showed that lipids were more readily extracted from partially digested LDL than from native LDL (42). These results were interpreted to indicate that the peptide portion of the intact lipoprotein prevented access of solvent to the lipid portion of the molecule. An alternative interpretation is that partial digestion of LDL leads to aggregation which in turn makes the lipids of LDL more susceptible to extraction. The increased extractability of lipids from heat-denatured LDL has been clearly demonstrated in this laboratory (unpublished experiments).

Although these studies were all carried out with soluble lipoproteins, agarose column chromatography should also serve as a useful tool for purification and characterization of solubilized structural lipoproteins. In this regard, it was disappointing to find that the addition of SDS to the elution buffer caused an intolerable reduction in the flow rate of the column. However, other detergents, as yet untested, may prove effective in maintaining lipoproteins in solution without disturbing the flow rate.

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