ABNORMAL HUMAN HAEMOGLOBINS

I. THE COMPARISON OF NORMAL HUMAN AND SICKLE-CELL HAEMOGLOBINS BY "FINGERPRINTING"

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NEEL[1] demonstrated in 1949 that sickle-cell anaemia is a disease inherited in a strictly Mendelian manner, and PAULING *et al.*[2], showed in the same year that the blood of sickle-cell patients contains a haemoglobin which differs electrophoretically from normal haemoglobin. Since then, several other abnormal haemoglobins have been discovered. They have been studied intensively by many workers for the sake of both their medical and their genetic interest, because they provide one of the clearest cases where mutations in a Mendelian gene are reflected in changes in the chemical structure of a protein. The medical aspect is important, because several of the abnormal haemoglobins give rise to severe haemolytic anaemias, which thus provide one of the rare instances when a disease is directly attributable to an inherited change in a protein molecule. However, in this series of papers it will be the genetic and biochemical aspect with which we shall be primarily concerned. Like other proteins, haemoglobin is thought to be the first protein product of the gene. The sequence and spacing of the mutations on the gene responsible for its synthesis should therefore be reflected in the sequence and spacing of the changes in amino-acid composition which these mutations produce in the haemoglobin.

The first three papers of this series will contain a comparative study of the amino-acid composition of sickle-cell and normal haemoglobin. The results have already been described in two preliminary notes[5,8]. In this fuller account the experimental work will be described in detail.

Chemical studies on haemoglobins A (normal) and S (sickle-cell) by previous authors[5] may be summarised by saying, first, that whatever difference exists between them must be small, and secondly, that the known charge difference of two to three units per molecule[2] is probably due to haemoglobin S having fewer carboxyl groups[6]. When it is remembered that human haemoglobin is made up of two identical halves[7], this difference is probably one of a single carboxyl group in the half molecule. It was not known whether the two haemoglobins differed in amino acid sequence or on the other hand, in the folding of the polypeptide chains leading to masking of some groups. As a result of the present work we can now say for certain that there is a difference in amino acid composition and that this does involve a carboxyl group, and explain the known electrophoretic behaviour. In fact, the polypeptide chains of the two haemoglobins differ in only one amino acid residue; one of the glutamic acid residues of haemoglobin A had changed to valine in haemoglobin S[5,8]. No other

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changes could be detected. The detailed evidence which led to these conclusions will now be described.

Part I will deal with the comparison of tryptic digests of the two haemoglobins and Part II, the paper published simultaneously[13], with the examination of the trypsin-resistant "cores". Part III will describe the chemical studies on the differing peptides derived from the two proteins.

MATERIALS AND METHODS

The blood samples which reached us had usually been collected in citrate–dextrose solution. The red cells had been sedimented by centrifuging at low speed and washed 4–6 times, sedimenting each time from about 4 volumes of 0.9% NaCl. The samples of normal European blood were worked up in the same manner in our laboratory. Sickle-cell red cells from patients homozygous for the sickle-cell gene, which had already been washed before they arrived, were given a few more washes. The cells were lysed by freezing and thawing; the cell ghosts were removed by centrifugation at 20,000 × *g* for 20 min. The clear solution of haemoglobin—usually 10 ml—was dialysed for 3 days against 3 × 1 l of cold distilled water containing 1 ml of 1 *M* PO_4 of pH 7.2 per l. This dialysis was followed by another high-speed centrifugation; the samples were stored frozen. The early experiments were performed on a sample of sickle-cell haemoglobin kindly supplied in pure solution by Dr. A. C. ALLISON of Oxford.

The purity of these haemoglobin solutions was checked by paper electrophoresis at pH 7.2 in 0.1 *M* PO_4 buffer and at pH 8.6 in 0.06 *M* Veronal buffer. Whatman 3MM was used as a free hanging strip and 1–2 mg of haemoglobin were applied along a 1″ line on the paper. All samples gave clear single bands, though a small amount of slightly faster material was visible in haemoglobin S. This was probably foetal haemoglobin, but the quantities present were not likely to be large enough to interfere with the examination of the trypsin digests of these proteins. Experience has borne this out. Paper electrophoresis of haemoglobin samples at pH 6.4 in the pyridine–acetic acid–water buffer of MICHL[9] showed only traces of 3–4 ninhydrin-positive substances which were the same in both haemoglobin A and S.

In order to render haemoglobin susceptible to trypsin attack, it first had to be denatured. A 2% haemoglobin solution was brought to pH 8.0 with dilute NaOH and heated in a narrow test tube in a water bath at 90° for 4 min. The sample was then immediately cooled. A fine light brown suspension was obtained; this showed little tendency to settle and trypsin attacked it readily.

The trypsin used for these digestions was the 2 × crystallised, salt-free protein obtained from Worthington Biochemicals, Freehold, New Jersey, U.S.A. It was used without further purification. The first trypsin digestions were carried out in dilute phosphate buffer under the following conditions: Haemoglobin concentration 2% or 4% in 4.0 ml; trypsin to haemoglobin ratio, by weight, 1:50; 0.05 *M* Na_2HPO_4–HCl buffer pH 8.1; 38°. Two drops of toluene were added to prevent contamination. The incubation of the suspension lasted for 43–48 h, with occasional mixing. As the haemoglobin had been denatured in the presence of the buffer, the precipitate was much coarser and settled easily, unlike the preparation described in the previous paragraph. As the digestion proceeded, a lot of the precipitate disappeared, but a dark brown residue remained undissolved, presumably containing all the haeme groups. The supernatant had little colour. Surprisingly this method of digestion gave reproducible peptide mixtures. It was used for the early "fingerprints"[5].

Since then improved methods of trypsin digestion have been developed and all later digests were carried out in a type of "pH-stat". 4-ml samples of a 2.2% haemoglobin solution, denatured by heating in salt-free solution at pH 8, were stirred continuously at 38° in an atmosphere of nitrogen; trypsin 0.10 ml of a 0.5% solution in 0.001 *M* HCl was added at zero time. The vessel contained a glass electrode and an agar–KCl bridge to a calomel electrode. The splitting of peptide bonds liberated hydrogen ions; 0.5 *N* NaOH was added manually by means of an Agla micrometer burette to keep the pH between 7.95 and 8.00 using an EIL direct reading pH meter. It is well known that when alkali consumed is plotted against time the slope of this curve never flattens completely. In preliminary experiments, the end point was determined by adding further aliquots of trypsin solution and observing that after a certain time—about 90 min—there was no additional alkali uptake (Fig. 1). After the digestion, the pH was adjusted to 6.5 with 1 *N* HCl and the precipitate removed by centrifugation. The supernatant was stored frozen; it was used for fingerprinting and for the preparation of various peptides. Thus the improved procedure reduced digestion time from about 40 h to less than 2. The purification and chymotryptic digestion of the trypsin-resistant "core" is described in part II of this series.

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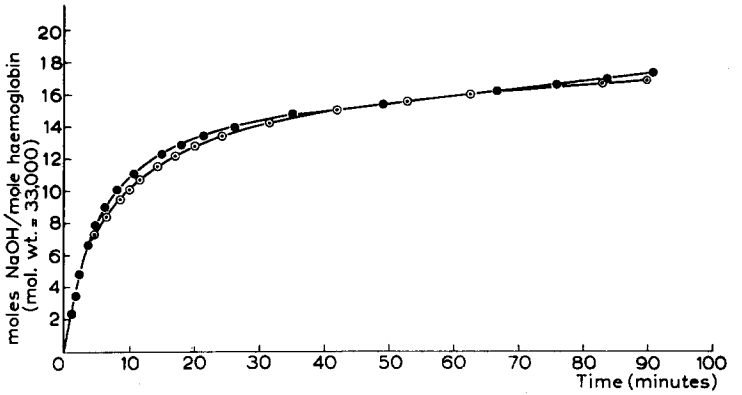

Fig. 1. Course of the trypsin digestion of haemoglobin A and S. ⊙—⊙ Haemoglobin A; ●—● Haemoglobin S.

"Fingerprinting"

The separation of the peptides obtained by tryptic digestion was achieved by a two-dimensional combination of paper electrophoresis and chromatography. Paper electrophoresis was carried out on Whatman No. 3MM paper which had been dipped into MICHL's volatile buffer[9]: pyridine–glacial acetic acid–water, pH 6.4, in the proportions 10: 0.4: 90 by volume. AnalaR grade chemicals were used without further purification. Excess liquid was removed carefully by blotting firmly between two sheets of fresh blotting paper. The moist No. 3MM paper was immediately placed onto a horizontal piece of $^1/_4$" polished plate glass which rested on the two buffer vessels, each 3" high. The sample of peptides—0.10 ml of the original solution which had been evaporated and dissolved in 0.020 ml of water—was applied with a construction pipette at the point indicated in Fig. 2. Similarly, a second peptide mixture could be applied to the other paper. At once, a second sheet of plate glass was placed on top of the first one, thus enclosing the moist filter paper as a sandwich. Fig. 2 shows a plan of the arrangement and in particular the way the paper is cut.

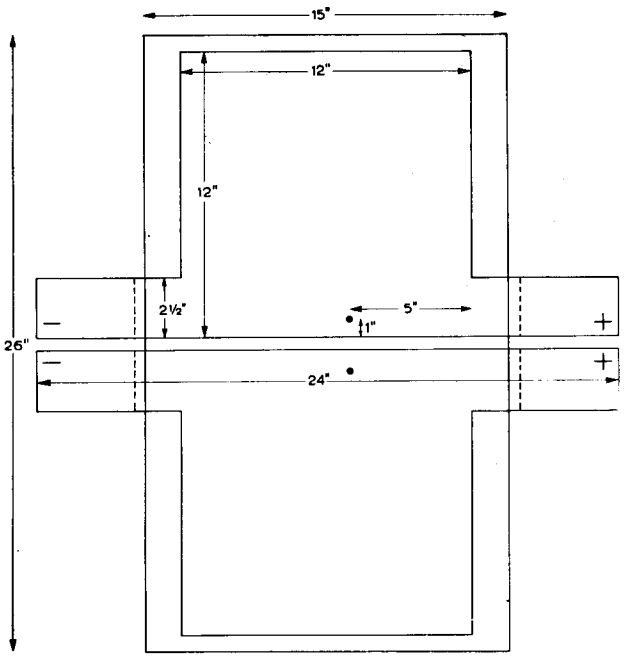

Fig. 2. Dimensions of filter paper used for fingerprinting.

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The tags dip into buffer vessels (not shown). This arrangement allows two separations to be carried out under identical conditions; it is now generally used to compare peptide mixtures from normal haemoglobins with those from the abnormal proteins. Great care is taken to handle pairs of papers in the same way. Preferably they are cut from a single sheet of paper, 24″ × 24″, with the machine direction of the paper always pointing in the same direction.

After the sample had been applied, 15 min were allowed to elapse to give the buffer time to diffuse into the sample, even though the latter was already at pH 6.5. A potential of 19 V/cm was applied, and as the current rose, this fell to about 14 V/cm after 150 min. The glass sandwich got comfortably warm during the electrophoresis. The tags were trimmed off the papers and the buffer was removed by drying at room temperature in a current of air for not less than two hours and preferably longer. The dry papers, now 12″ × 12″, were hung for two hours in the air space above the butanol–acetic acid chromatographic solvent. This was made up from AnalaR grade chemicals in the proportions *n*-butanol–glacial acetic acid–water, 3:1:1 by volume; it was freshly prepared each Saturday for use in the next week beginning on Monday.

Ascending chromatography was carried out overnight, usually for 15 h. The papers were again dried in a current of air at room temperature. The peptide spots were revealed by dipping the papers into 0.2% ninhydrin in acetone and allowing development to take place at room temperature or in a warm place. This usually took 24 h to reach maximum intensity. Since it was difficult to preserve these "finger prints" even with the copper spray[10], reflex prints were taken on document paper or colour transparencies were made.

One-dimensional paper electrophoresis on 3MM paper in the above volatile pH 6.4 buffer was frequently used for examining peptide or amino acid mixtures and for isolating peptides. The arrangement was a glass sandwich similar to the fingerprinting apparatus but measuring only 12″ × 6″. Another volatile buffer[9] at pH 3.6: pyridine–glacial acetic acid–water, 1:10:90 by volume, was useful for one-dimensional separations. For these, the bottom member of the glass sandwich was replaced by a copper plate $^1/_4$″ thick, cooled by copper coils carrying tap water. A thin layer of silicone greases and another of thick polythene film separated the copper plate from the paper. Up to 35 V/cm could be used without noticeable rise in the temperature of the upper glass plate. When the isolation of a peptide was required, the papers were usually first washed by descending irrigation with the pH 6.4 buffer, followed by soaking in water and drying. Such papers were developed by spraying with 0.025% ninhydrin in butanol and gentle heating. Alternatively guide strips were used. The peptides were eluted with water or 20% aqueous acetic acid. Descending chromatography on No. 1 paper *n*-butanol–glacial acetic acid–water, 4:1:5, was used for separating peptide mixtures, especially those which are uncharged at pH 6.4. Amino acids were identified by the two-dimensional system of REDFIELD[11] using Whatman No. 1 paper, 20 × 20 cm. A multiframe holding 12 papers could be processed in about 7 h. After steaming for 10 min and dipping into 0.2% ninhydrin in acetone, the papers were developed at room temperature. Good separations were obtained when the solvents were made up each day and were kept at the fairly constant temperature of a basement room. Occasionally amino acids were separated by ascending chromatography on No. 1 paper butanol–acetic acid–water solvent, 3:1:1, overnight.

RESULTS AND DISCUSSIONS

Comparison of haemoglobin A and S tryptic digests

The most striking feature of the fingerprints of haemoglobin A and S (Fig. 3) is the faithful reproduction of the intricate and highly characteristic pattern of peptide spots in both proteins. All except one; this is the No. 4 peptide which appears amongst the uncharged peptides of a haemoglobin A fingerprint, but is positively charged in haemoglobin S and appears in a new position nearer to the cathode. Four questions arose: is such a fingerprint characteristic of all individuals of a certain genotype; is it a true representation of all the peptides in a trypsin digest; are all the peptides, which occupy similar positions really identical in structure, and finally what is the difference between the haemoglobin A and S No. 4 peptides?

The haemoglobins of five different normal white persons and of five different negro sickle-cell patients have been examined by the method of trypsin digestion followed by separation of the peptides by the "fingerprinting" technique. No differences in behaviour were found within either group. The comparison with normal negro haemoglobin has yet to be done, but it is known that electrophoretically it is like

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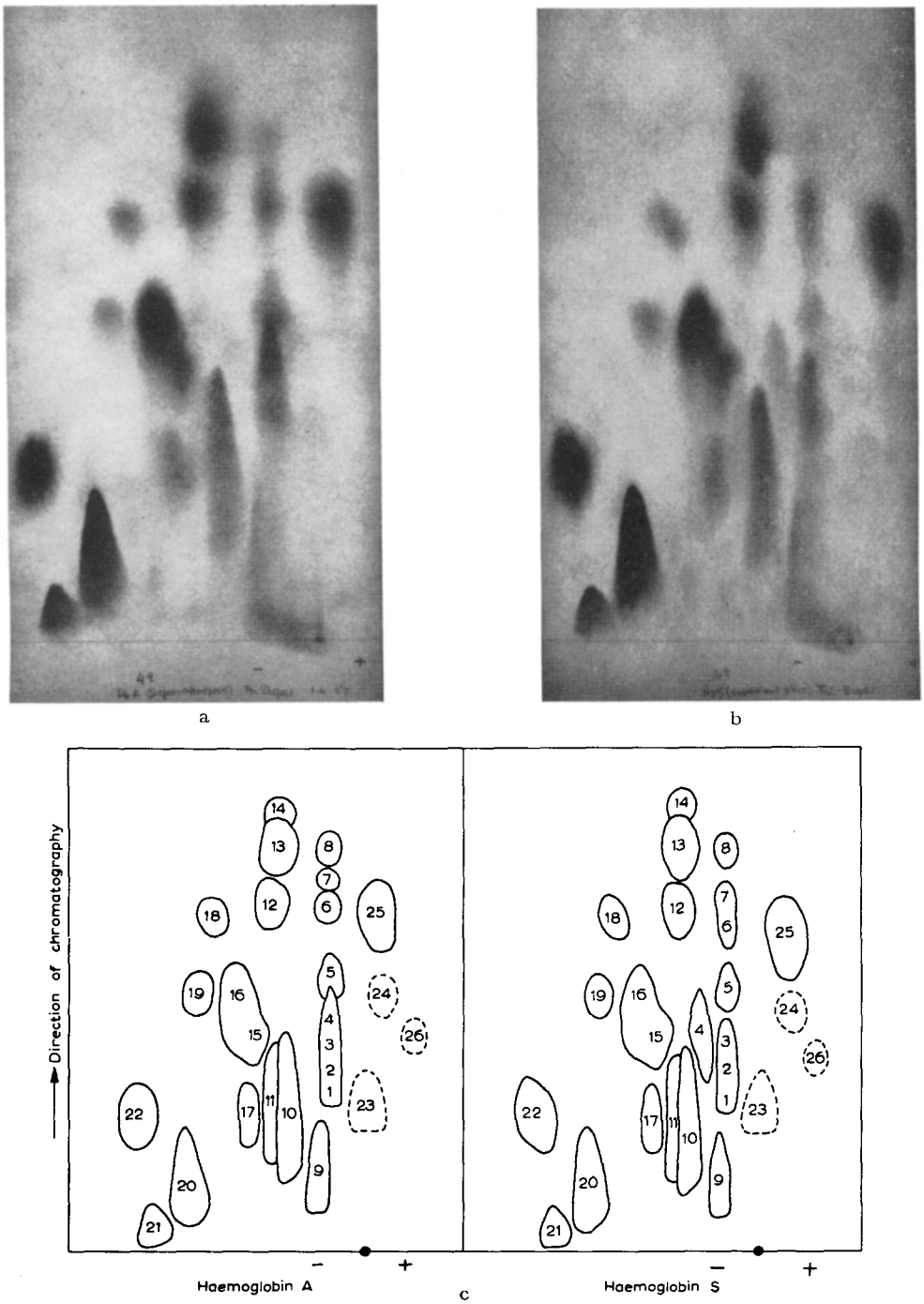

Fig. 3. Fingerprints of trypsin digests of haemoglobin A and S. a, Haemoglobin A; b, Haemoglobin S; c, Tracings, showing the system for numbering peptide pairs. Dotted lines indicate peptides which only became visible after heating the chromatogram.

normal European haemoglobin A. The fingerprint contains altogether some 26 peptides, though, as the numbering in the figure shows, not all of them are separated. Their further separation which will be discussed below, has provided good evidence that the tracing in Fig. 3 shows all the peptides present in the tryptic digest with the exception of the trypsin-resistant core which is discussed in Part II. As judged by the intensity of their ninhydrin colours and those of their constituent amino acids, the majority of peptides are present in good yield. Earlier "fingerprints"[5] had been prepared from 43-h trypsin digests in buffered solutions, as compared with the 90-min incubation periods now used and the reduction in the proportion of trypsin to a quarter of the old value. The earlier fingerprints showed thirty peptide spots which may have corresponded to some thirty-two or -three peptides. The extra peptides had probably arisen through traces of chymotrypsin which were present in the trypsin and which got a chance to act because the period of digestion was too long and the enzyme concentration too high. The present fingerprints show 18–19 lysine-containing and 6 arginine-containing peptides. The trypsin-resistant core probably contains another 3 lysine peptides, but no arginine. These figures compare very well with the latest data given by STEIN *et al.*[12] for normal haemoglobins, namely about 21 lysines and 6 arginines per half molecule of haemoglobin.

It was particularly necessary to examine the poorly resolved "backbone" of neutral peptides. They were prepared by elution of the neutral band after one-dimensional paper electrophoresis at pH 6.4; this fraction was then examined by descending paper chromatography in butanol–acetic acid–water, 4:1:5, and revealed a number of peptides which were identical in both proteins except for peptide No. 4[5]. A better way of examining peptides 1–4 was by paper electrophoresis at pH 3.6 where they separated well. One can therefore be reasonably certain now that the fingerprint shows all the trypsin peptides.

Duplicate fingerprints of haemoglobin A and S when carefully prepared, are sometimes so good that individual peptide spots are superimposable. This is an indication, but no more, that any peptide from haemoglobin A will have the same structure as the corresponding S peptide. Individual peptides from the two haemoglobins have been compared on the same paper, by extended paper chromatography with butanol–acetic acid or by paper electrophoresis at pH 3.6. In all cases they have formed identical patterns and their ninhydrin colours relative to other peptides have been closely similar. In addition, analogous peptides have been eluted, purified further if necessary, hydrolysed completely and examined qualitatively for their amino acid contents. This procedure does not detect tryptophan or cysteine; neither does it distinguish between, say, 3 or 4 amino acids of the same kind in one peptide, though it would detect a difference between one and two. Careful comparison of pairs of these amino acid chromatograms has failed to show any difference between analogous peptides, except for the two No. 4 peptides. Here the loss of one of two glutamic acids was obvious, as was the gain of the extra valine in the haemoglobin S peptide. With the above reservations in mind, it is possible to say that probably all trypsin peptides, except No. 4, have similar chemical structures. Similar arguments hold for all peptides obtained by the chymotryptic digestion of the "core".

The determination of the detailed chemical structure of the No. 4 peptides from haemoglobins A and S will be described in Part III. The results described here and by HUNT AND INGRAM[13], lead one to conclude with reasonable confidence that most of

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the amino acid sequences of the two haemoglobins are very similar and perhaps identical, and that the only difference lies in the No. 4 peptide. It does not follow that there are not additional differences in the folding of the polypeptide chains, but they must be small, since they are not detectable in the X-ray diffraction patterns from single crystals of these proteins[7]. The genetic implications of these findings have already been discussed[5,8].

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SUMMARY

Samples of human haemoglobin from normal adults and from sickle-cell anaemic patients have been heat denatured and then digested with trypsin. The resultant peptide mixtures were separated in two dimensions by paper electrophoresis and paper chromatography. Such "fingerprints" show that the two proteins differ in only one peptide.

REFERENCES

1 J. V. NEEL, *Science*, 110 (1949) 64.
2 L. PAULING, H. A. ITANO, S. J. SINGER AND I. C. WELLS, *Science*, 110 (1949) 543.
3 H. A. ITANO, *Ann. Rev. Biochem.*, 25 (1956) 331.
4 W. W. ZUELZER, J. V. NEEL AND A. R. ROBINSON, in *Progress in Hematology*, Vol. I, Grune & Stratton, New York, 1956.
5 V. M. INGRAM, *Nature*, 178 (1956) 792.
6 I. H. SCHEINBERG, R. S. HARRIS AND J. L. SPITZER, *Proc. Natl. Acad. Sci. U.S.*, 40 (1954) 777.
7 M. F. PERUTZ, A. M. LIQUORI AND F. ERICH, *Nature*, 167 (1951) 929.
8 V. M. INGRAM, *Nature*, 180 (1957) 326.
9 H. MICHL, *Monatsh. Chem.*, 82 (1951) 489.
10 E. KAWERAU, *Biochem. J.*, 48 (1951) 281.
11 R. R. REDFIELD, *Biochim. Biophys. Acta*, 10 (1953) 344.
12 W. H. STEIN, H. G. KUNKEL, D. COLE, D. M. SPACKMAN, S. MOORE, *Biochim. Biophys. Acta*, 24 (1957) 640.
13 J. A. HUNT AND V. M. INGRAM, *Biochim. Biophys. Acta*, 28 (1958) 546.

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