CCLI. SPECTROPHOTOMETRY OF PROTEINS.

I. ABSORPTION SPECTRA OF TYROSINE, TRYPTOPHAN AND THEIR MIXTURES.

II. ESTIMATION OF TYROSINE AND TRYPTOPHAN IN PROTEINS.

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I. ABSORPTION SPECTRA OF TYROSINE, TRYPTOPHAN AND THEIR MIXTURES.

MEASUREMENTS of the absorption spectra of the aromatic amino-acids have been frequently reported in the literature but the results are not in good agreement. For purposes evident in Part II it was required to know the absorption curves of tyrosine and tryptophan with accuracy. Their absorption curves were therefore redetermined. The method of Vierordt for estimating spectrophotometrically the concentration of the components of a mixture was also investigated in its application to mixtures of tyrosine and tryptophan.

Method.

Absorption measurements were made throughout on a Hilger Medium Quartz spectrograph E 316 (the dispersion of which is 18.6 cm. from 200 to 400 $m\mu$) with Spekker photometer, using a condensed spark between tungsten steel electrodes as light source. The spectrograph was fitted by the makers with a wave-length scale which was checked at intervals with the mercury arc. The maximum error of the scale between 230.2 and 333.9 $m\mu$ was found to be + and - 0.2 $m\mu$. When exposing a plate on the spectrophotometer the wave-length scale was printed at the top and the bottom of the plate. The matching was made by eye as follows. The plate to be matched was placed gelatin face downwards on a piece of glass with a fine cursor line engraved on it at right angles to the spectral strips. Owing to the small play which must be allowed for the plate in the plate holder the spectra on two plates are not always similarly orientated with respect to the edge of the plate. An adjustor was therefore made by which the plate could be rotated slightly and the cursor line made to cut the wave-length scales at identical readings. The spectra were then viewed through a low-power microscope, the plate being moved so that the point at which each pair of spectra appeared to match lay on the cursor line. The wave-length at which the cursor line cut the scales was then read off. This is a more laborious method than the usual one of placing an ink mark below the match point but was adopted in order that several sets of matchings could be made on the same plate without bias.

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Since it is well known that ions of acids and bases often exhibit marked differences in absorption as compared with the unionized forms, the absorption spectra were measured at values of pH removed at least 2 units from the pK values of the amino-acids. The values for the dissociation constants of tyrosine are well established, those of Neuberger [1934] have been taken; for tryptophan those of Schmidt *et al.* [1929].

	COOH pK	$-\text{NH}_2$ pK	$-\text{OH}$ pK
Tyrosine	2.35	9.16	10.41
Tryptophan	2.38	9.39	—

The imino-nitrogen of tryptophan has extremely weak acidic and basic properties but the dissociation constant is outside the range of useful measurement.

Accordingly measurements of absorption were made on solutions in water, in $N/10$ alkali and in $N/10$ HCl.

Amino-acids. Tyrosine was obtained from three sources, (a) isolated from silk according to Morrow [1927]; (b) a gift from Mr Philpot; (c) a commercial sample of Hoffmann La Roche. These all gave nitrogen values by Kjeldahl estimation between 7.72 and 7.73% (theoretical 7.73). Their absorption curves were

Fig. 1. I. Tryptophan in $N/10$ HCl. II. Tryptophan in $N/10$ NaOH. III. Tyrosine in $N/10$ HCl. IV. Tyrosine in $N/10$ NaOH.

Table I. *Molecular extinction coefficients of tyrosine and tryptophan.*

Wave-length		In $N/10$ NaOH $\epsilon \times 10^{-3}$.											
mμ	...	260	265	270	275	280	285	290	295	300	305	310	315
Tyrosine	1.75	1.14	1.08	1.24	1.54	1.96	2.24	2.26	1.89	1.14	0.471	0.182	
Tryptophan	3.48	4.33	4.90	5.30	5.43	4.57	4.13	2.17	1.14	0.500	0.221	0.141	
Wave-length		In $N/10$ HCl $\epsilon \times 10^{-3}$.											
mμ	...	260	265	270	275	280	285	290	295	300	305	310	315
Tyrosine	0.56	1.00	1.21	1.24	1.23	0.55	0.16	0.07	0.02	—	—	—	—
Tryptophan	4.07	4.84	5.33	5.47	5.43	4.33	3.67	1.33	0.50	0.13	—	—	—

identical within the limits of error of the method. Three samples of tryptophan were used, (a) a gift from Mr B. C. G. Knight, (b) a gift from Dr K. G. Stern of a sample of Fraenkel and Landau, (c) a preparation of Hoffmann la Roche. The absorption curve of tryptophan seems to be very sensitive to the presence of impurities. The two commercial samples required recrystallization twice from dilute alcohol before their absorption curves agreed with the sample of Mr Knight. Further purification yielded no change in the absorption spectrum. It is probable that the impurities in the commercial samples were decomposition products of the tryptophan as they were both some years old. These finally gave colour values according to Folin and Ciocalteau [1927] between 88.4 and 88.9% by weight of that given by tyrosine (theoretical 88.7).

The absorption curves of the amino-acids are given in Fig. 1. The actual values for tyrosine and tryptophan are summarized in Table I.

Determination of tyrosine and tryptophan in mixtures of the two.

It is possible to estimate the concentrations of two components of a mixture knowing the absorption curves of standard solutions of each component and the absorption curve of the mixture provided that they do not interact. The method is due to Vierordt [quoted by Twyman & Allsopp, 1934].

If E_a and E_b be the extinction coefficients of the mixture at wave-lengths a and b ,

ϵ_{1a} and ϵ_{2a} be the molecular extinction coefficients of components 1 and 2 respectively at λ_a ,

ϵ_{1b} and ϵ_{2b} be the molecular extinction coefficients of components 1 and 2 respectively at λ_b ,

M_1 and M_2 be the molar concentrations of components 1 and 2 respectively in the mixture,

then it can be shown that

$$M_1 = \frac{E_a \epsilon_{2b} - E_b \epsilon_{2a}}{\epsilon_{1a} \epsilon_{2b} - \epsilon_{2a} \epsilon_{1b}}, \quad \dots\dots(1)$$

$$M_2 = \frac{E_b \epsilon_{1a} - E_a \epsilon_{1b}}{\epsilon_{1a} \epsilon_{2b} - \epsilon_{2a} \epsilon_{1b}}. \quad \dots\dots(2)$$

Given a solution of a mixture of, say, tyrosine and tryptophan it is therefore necessary to measure the extinction coefficients of this mixture at two wave-lengths and to know the extinction coefficients of solutions containing known weights of tyrosine and tryptophan. From considerations discussed below it has been found that the most suitable wave-lengths are 280 and 305 $m\mu$ to be measured on solutions of tyrosine and tryptophan in alkaline solutions whose pH is > 12.0.

Substituting in equations (1) and (2) the values given in Table I there result

$$M_{\text{tyrosine}} = (1.0 E_{305} - 0.092 E_{280}) \times 10^{-3},$$

$$M_{\text{tryptophan}} = (0.21 E_{280} - 0.288 E_{305}) \times 10^{-3}.$$

A test on a series of mixtures of tyrosine and tryptophan is reported in Table II.

Errors of the spectrographic method and of the analysis of mixtures.

The accuracy of the photographic method of spectrophotometry as here employed depends on the reproducibility of blackening of neighbouring small areas of a photographic plate as a result of equal exposures. This has been shown to be 5% of the light intensity under favourable conditions [cf. Weigert, 1927].

This corresponds to an absolute error in E of 0.02. Von Halban & Eisenband [1927] put the error higher at 0.04 E . Besides this error there is that of matching. To assess this, 32 matchings were made at each of two points on a typical plate chosen at random. Some time was allowed to elapse between matchings to avoid eye strain or any possibility of remembering the spectral structure of those regions. Matchings were made alternately with the plate one way up and then inverted. It was found that there was a systematic difference between matchings made with the two orientations of the plate, the magnitude of which was very different with different observers, being large with the inexperienced and becoming almost negligible with practice. The eye appears to give preference of blackening to the lower of the pair of spectra in view. It is for this reason always advisable to match a plate in both ways. It was found that for the 32 matchings the standard deviation (σ) at 281.7 m μ was 0.288 m μ and at 301.6 m μ was 0.259 m μ which in each case corresponded to a standard deviation in extinction (σE) of 0.005 E . Taking an arbitrary value of $3 \times \sigma E$ as the maximum error then this error of matching is $\pm 0.015 E$. It may therefore be conservatively stated that the error of the visual match point method of spectrography is less than $\pm 0.055 E$ for a single matching. By ensuring that the E measured is as high as possible the relative error is reduced. With the Spekker photometer densities up to 2.0 can be measured and it has been the practice in this work to arrange the concentration and thickness of the absorbing layer so that the density at the desired wave-length shall be greater than 1.0. It was more usually > 1.5 and in the cases of the measurements given in Table I was always so. The values there given are the average of 6 matchings on each of 4 plates, i.e. 24 matchings for each point, and have a standard error of 0.003 E .

The manner in which errors in measurement of the extinction coefficients of a mixture and in the values for the molecular extinction coefficients of the components are transmitted to the values obtained for the concentrations of the components in the mixture has been calculated by Mr J. St L. Philpot (private communication) to whom I am grateful for the following solution. It can be shown that in equations (1) and (2), the variances of M_1 , i.e. ($\sigma^2 M_1$), and of M_2 , i.e. ($\sigma^2 M_2$), are determined as follows:

$$\frac{\sigma^2 M_1}{M_1^2} = \frac{\left[2 \left(\frac{M_2}{M_1} \right)^2 + \alpha^2 + \beta^2 \right] (\sigma^2 E + \sigma_\epsilon^2) + 2 \frac{M_2}{M_1} (\alpha + \beta) \sigma^2 E}{(\alpha - \beta)^2}, \quad \dots\dots(3)$$

$$\frac{\sigma^2 M_2}{M_2^2} = \frac{\left[2 \left(\frac{M_1}{M_2} \right)^2 + \xi^2 + \eta^2 \right] (\sigma^2 E + \sigma_\epsilon^2) + 2 \frac{M_1}{M_2} (\xi + \eta) \sigma^2 E}{(\xi - \eta)^2}, \quad \dots\dots(4)$$

where

$$\alpha = \frac{\epsilon_{1a}}{\epsilon_{2a}} = \frac{1}{\xi}, \quad \beta = \frac{\epsilon_{1b}}{\epsilon_{2b}} = \frac{1}{\eta},$$

and

$$\sigma_{Ea \text{ or } b}^2 = \sigma_E^2 \quad \text{and} \quad \sigma_{\epsilon_{1a}, \epsilon_{1b}, \epsilon_{2a}, \epsilon_{2b}}^2 = \sigma_E^2.$$

It follows that errors in M_1 and M_2 are more dependent on errors in E than on errors in ϵ . Further, in order that the errors in M_1 and M_2 shall be small it is required that $(\alpha - \beta)^2$ and $(\xi - \eta)^2$ shall be large. This is fulfilled in the case where the wave-lengths a and b are chosen on opposite sides of that wave-length at which the absorption curves of the two components cross one another. It will follow that if α is > 1 then ξ and β will be < 1 and $\eta > 1$. The curves of tyrosine and tryptophan in acid solution do not cross at any point but the curves of equimolar solutions in alkali cross at 294.5 m μ . The two wave-lengths 280 m μ and 305 m μ have been chosen to give the maximum reversal in the value of ϵ_1/ϵ_2 consistent with accurate measurement.

From Table I

$$\begin{aligned}\alpha &= 0.284, & \beta &= 2.31, \\ \xi &= 3.53, & \eta &= 0.432.\end{aligned}$$

As an example of the application of this to the analysis of mixtures the case of a mixture of tyrosine and tryptophan in the molar ratio 3 : 1 is taken.

Substituting for α , β , ξ and η in equations 3 and 4 we get

$$\frac{\sigma^2 M_{\text{tyrosine}}}{M^2_{\text{tyrosine}}} = 1.8\sigma_E^2 + 1.37\sigma_\epsilon^2,$$

and

$$\frac{\sigma^2 M_{\text{tryptophan}}}{M^2_{\text{tryptophan}}} = 5.8\sigma_E^2 + 3.21\sigma_\epsilon^2.$$

σ_ϵ has been estimated as 0.003 and σ_E as 0.012. Inserting these values

$$\frac{\sigma M_{\text{tyrosine}}}{M_{\text{tyrosine}}} = 0.014 \quad \frac{\sigma M_{\text{tryptophan}}}{M_{\text{tryptophan}}} = 0.026$$

giving an error of about $\pm 4\%$ of tyrosine and $\pm 8\%$ of the tryptophan concentrations.

As an example may be quoted the findings of an actual experiment. Two solutions were made up in $N/10$ NaOH: (a) tyrosine $0.785 \times 10^{-3} M$, (b) tryptophan $0.33 \times 10^{-3} M$. These were mixed in the proportions $a : b = 3 : 1$, $1 : 1$ and $1 : 3$ and the absorption curves of the five solutions measured. Table II shows found and calculated concentrations of tyrosine and tryptophan expressed in terms of $10^{-3} M$. The maximum error in any of the values obtained is $+5.0\%$ for tyrosine in mixture $a/b = 1$. This compares favourably with chemical estimation of tyrosine and tryptophan in mixtures.

Table II.

Solution		$a/b = 3 : 1$	$a/b = 1 : 1$	$a/b = 1 : 3$	b	a
Tyrosine:	Found	0.59	0.412	0.195	—	0.793
	Calc.	0.589	0.392	0.196	—	0.785
	% error	+0.2	+5.0	-0.5	—	+0.1
Tryptophan:	Found	0.080	0.162	0.248	0.340	—
	Calc.	0.083	0.166	0.248	0.330	—
	% error	+3.6	+2.4	0.0	+0.3	—

Concentrations are given in molarity $\times 10^{-3}$.

II. ESTIMATION OF TYROSINE AND TRYPTOPHAN IN PROTEINS.

Dhr [1909] suggested that the selective character of the absorption spectrum of proteins was due to the contained aromatic amino-acids. Stenstrm & Reinhard [1925] showed that a solution containing tyrosine and tryptophan in concentrations calculated from the values of Abderhalden and Kiyotaki for blood serum proteins gave an absorption spectrum very similar to that of serum. They also showed that when a simple solution of tyrosine was made alkaline the absorption curve was shifted towards longer wave-lengths and that the same occurred, though to a lesser extent, on making serum alkaline. Smith [1929] and Coulter *et al.* [1936] have also pointed out the dependence of the selective absorption of

proteins on the contained aromatic amino-acids. No quantitative measurements of this dependence are to be found in the literature.

Since it was desired in this laboratory to estimate tyrosine and tryptophan in small quantities (5 mg.) of protein, the spectrophotometric method was investigated as a means to this end. Such application can only be successful (a) if the absorption spectra of the aromatic amino-acids are not altered by combination in the protein, and (b) if it is possible to select a region of the spectrum where only tyrosine and tryptophan absorb. It is obvious that if a region can be found where only one of them absorbs, then that one may be estimated directly by comparison with a standard solution. Inspection of the absorption curves of amino-acids recorded in the literature [Guthmann *et al.* 1931; Abderhalden *et al.* 1927; 1928; 1929; Ward, 1923] shows that a region can be found ($>280\text{m}\mu$) where tyrosine and tryptophan are the only absorbing amino-acids. Further, there is evidence to show that a chromophoric group is only affected through a system of conjugated double bonds, and that combination of an absorbing molecule with a much larger but transparent molecule is possible without altering the absorption [Holiday, 1930; Arnold & Kistiakowsky, 1932; Gulland *et al.* 1934]. It is therefore feasible that the absorption of tyrosine and tryptophan should not be altered by peptide linking in a protein molecule, since there is no conjugated double bond system linking the chromophoric group (aromatic nucleus) with the site of substitution ($-\text{NH}_2$ or COOH groups). Abderhalden & Haas [1927] have shown that the absorption spectrum of glycyltyrosine is identical with that of tyrosine. If this condition be fulfilled in a protein, then its absorption spectrum will be identical with that of a simple mixture of its contained amino-acids, and

(1) hydrolysis of protein should not lead to any change of absorption;

(2) application of the method of spectrophotometric analysis of mixtures detailed in the previous section should give values of concentrations of tyrosine and tryptophan agreeing with those obtained by chemical determination.

Below are reported results obtained by the chemical and spectrophotometric methods. From considerations discussed in Part I it was found that alkaline solutions of proteins ($N/10$ NaOH) were the most suitable for the measurements. An additional advantage is the fact that most proteins are quite soluble in $N/10$ NaOH.

Experimental.

The source of the proteins. The serum proteins were prepared from horse serum, using the method of Groh & Weltner [1934], in which the serum is diluted 10 to 20 times before precipitation with ammonium sulphate. Besides giving sharp fractionation the samples of euglobulin and pseudoglobulin are remarkably free from pigment, as compared with those obtained from undiluted serum. The euglobulin is that fraction which separates out on dialysing total globulin and which redissolves in 0.9% saline. The pseudoglobulin is the distilled water-soluble fraction precipitated from serum diluted 10 times with distilled water by 40% saturation with ammonium sulphate. The albumin is the further fraction obtained by complete saturation with ammonium sulphate. The fractionation of the total globulin was repeated 3 times. All samples were dialysed 7–10 days against distilled water, until the dialysate had been negative 2 days to Nessler reagent. One serum N. IV had been extracted with ether-alcohol according to Hardy & Gardner [1910] as modified by Hewitt [1927]. Except for N. III and N. IV the serum proteins had not been brought to dryness. Concentrations were estimated by N determination by the micro-Kjeldahl method of Parnas & Wagner,

using the factor 6.45 to convert to protein. The gliadin was prepared by the method of Dill & Alsberg [1925] and the caseinogen according to the method of Van Slyke & Baker [1918], with the further extraction of the caseinogen with 60% alcohol. This extraction removed an appreciable amount of protein and it is to be noted that the tryptophan content of the extracted protein is considerably lower than before extraction. The two samples of zein were a gift of Dr A. Neuberger. The sample of crystalline insulin examined was a product of Messrs Burroughs and Wellcome. The gelatin was an ash free sample of Eastman Kodak, Ltd.

The concentration of tyrosine and tryptophan in various proteins determined by the spectrophotometric and chemical methods. Absorption curves of proteins were determined on simple watery solutions (when soluble) and on solutions in *N*/10 NaOH. In the case of the serum proteins the concentration was determined by estimation of total N; in other cases weighed quantities of the dried sample were dissolved to give concentrations varying between 0.1 and 0.2%. In order to reduce errors the value of the density measured was always made > 1.0, and in

Table III.

Protein	Tyrosine		Tryptophan		
	Spect.	Chem.	Spect.	Chem.	
Horse serum euglobulin:					
N. E. G. IV	7.7	6.9	2.3	2.1	Dried preparation
IV	7.5	7.2	2.3	2.2	Dried preparation, second separation
V	6.0	—	2.8	—	—
VI	6.8	—	2.9	—	—
Horse serum pseudoglobulin:					
N. P. G. I	6.25	—	3.2	—	—
II	6.4	—	2.8	—	—
III	5.6	6.9	2.8	2.7	Dried preparation
		7.2			
IV	5.8	5.7	2.6	2.1	Dried preparation
V	5.12	—	2.6	—	—
VI	5.3	—	2.7	—	—
Horse serum albumin:					
1A	(6.3)	5.5	(0.82)	0.94	—
	7.46		0.6		
2A	(6.45)	6.23	(0.79)	0.63	—
	8.3		0.65		
Caseinogen:					
A & B	6.95	6.2	1.02	1.1	—
		6.4		1.0	
		5.7		0.8	
	7.5	—	1.26	—	—
V. S. & B.	6.7	—	1.04	—	Acc. to Van Slyke & Baker
V. S. & B. E.	6.45	6.55*	0.85	1.4*	Acc. to Van Slyke & Baker but extracted with ether and 60% alcohol
Gelatin	0.46 (0.26)	—	0.05	—	—
Gliadin	(3.4)	3.1	(0.65)	0.84	—
		3.4*		1.14*	
	4.3	—	0.66	—	—
Zein	(6.3)	5.9*	—	0.17*	—
	10.0				
Insulin	12.7	12.0†	0.0	—	—

* Given by Folin & Ciocalteu [1927].

† Given by Jensen & Wintersteiner [1932].

most cases was >1.5 . Plates were matched as described in Part I, and the extinction coefficients (E) of the alkaline protein solutions at $280.0\text{ m}\mu$ and $305.0\text{ m}\mu$ determined. Substituting these values in the equations previously given, values for molar concentration of tyrosine and tryptophan in the solution examined were obtained from which could be calculated the percentage by weight of the free amino-acids in the protein.

The results are given in Table III, together with the results of chemical analysis. The chemical estimations were made exactly as described by Folin & Ciocalteu [1927] except that 0.1 g. quantities were taken for the analysis. The estimations were not very satisfactory on such small quantities as shown by the divergence of duplicated results.

DISCUSSION OF RESULTS.

Except in the case of N.P.G. III the values for tyrosine are all higher in varying degree than those obtained by chemical determination. Where the discrepancy is large, as in the samples of albumin, gliadin and zein, it can be attributed to the interference of pigment. These samples were all considerably coloured. It is to be noted that this interference does not disturb the tryptophan values to the same extent. The reason for this lies in the form of the equations for the derivation of the concentration of tyrosine and tryptophan:

$$\begin{aligned} M_{\text{tyrosine}} &= 1.0 E_{305} - 0.092 E_{280}; \\ M_{\text{tryptophan}} &= 0.21 E_{280} - 0.288 E_{305}. \end{aligned}$$

If $IE_{305.0}$ and $IE_{280.0}$ be the extinction coefficients at the two wave-lengths due to interfering substances, then the error in molar concentrations (IM) will be:

$$\begin{aligned} IM_{\text{tyrosine}} &= 1.0 IE_{305} - 0.092 IE_{280}; \\ IM_{\text{tryptophan}} &= 0.21 IE_{280} - 0.288 IE_{305}. \end{aligned}$$

In the case of most proteins examined the pigment impurities show "general" absorption gradually increasing with decrease in wave-length, so that IE_{280} is only slightly greater than IE_{305} . The difference is such that $IM_{\text{tryptophan}}$ is very small, whereas IM_{tyrosine} is approximately $0.9 IE_{305}$. To correct for this it has been found possible to estimate IE_{305} on solutions of proteins soluble in water or dilute acids. In neutral solution or acid solution a mixture of tyrosine and tryptophan shows no absorption beyond $310.0\text{ m}\mu$. The absorption of a protein solution at this wave-length has therefore been taken as an estimate of IE_{305} . It has been found that solutions of pigment-free proteins which show little light scattering give a very small value for IE_{305} (limits 0.02 – 0.06). With the proteins gliadin, zein and serum albumin IE_{305} may be as much as 0.4 . Tyrosine values corrected in this manner are given in the table in brackets. These corrected values are not so reliable as those obtained from pigment-free protein solutions.

A final check of the values obtained can be made by comparing the summation curve of tyrosine and tryptophan in the concentrations found with the absorption curve of the protein. Such a summation curve should approximate closely to that of the protein at wave-lengths longer than $275\text{ m}\mu$; at shorter wave-lengths than this the protein curve will exceed the summation curve owing to the absorption of other amino-acids. It should also be noted that at no wave-length should the extinction coefficient of the summation curve exceed that of the protein curve by more than the experimental error, i.e. $0.05 E$. If this should be

found to occur the presence of interfering substances may be suspected, for which it may or may not be possible to introduce a correction. No difficulty was experienced in fitting the curves, except where there was much pigment. A small correction was required in the curves of most serum proteins for the general absorption due to scattering of light. Such solutions generally show visible opalescence in concentrated solution (3-5%) but none in the concentrations here used (0.1-0.2%). There is, however, even with the clearest dilute solutions, some scattering of ultraviolet light which must be corrected for.

From the results reported in this paper it is concluded that by the spectrographic method it is possible to arrive at a good estimate of the tyrosine and tryptophan concentrations in a protein. This method has the advantages over the chemical methods of requiring only about 5 mg. of protein, of being much less laborious and subject to small risk of failure in contrast to the case where alkaline hydrolysis must precede estimation.

SUMMARY.

1. The absorption curves of tyrosine and tryptophan have been measured between 260 and 305 μ in acid, neutral and alkaline solutions.
2. The spectrophotometric method of estimating their concentrations in mixtures of the two is described and the errors of the method discussed.
3. The estimation of tyrosine and tryptophan in proteins by the spectrophotometric method is described.
4. The results are compared with those derived from chemical analysis.
5. The errors of the method and a means of correcting for pigment impurities are discussed.

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