Estimation of Bloodstain Age by Rapid Determinations of Oxyhemoglobin by Use of Oxygen Electrode and Total Hemoglobin

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Bloodstain age could be estimated from the ratio of oxyhemoglobin/total hemoglobin (fractional oxyhemoglobin) in the bloodstain by this present method, if the temperature at which the bloodstain had been kept was known.

The oxyhemoglobin was determined with an oxygen electrode immersed in water in which the oxygen had been depleted, and the total hemoglobin was determined by conventional colorimetry (cyanomethemoglobin method). Ages of prepared bloodstain samples (within 24 h after bleeding) were estimated by this present method, which requires only 20 μl of bloodstain and only 5 min for the whole analysis.

Key words bloodstain age; oxygen electrode; fractional oxyhemoglobin; cyanomethemoglobin method

In a criminal investigation, it is very important to estimate bloodstain ages. Formerly, the age of a bloodstain was estimated by macrographic color changes of the stain after exposure to ultraviolet rays,[1] by transfer of chloride from the bloodstain to some bloodstain-support (paper and cloth)[2] or by solubility of the bloodstain in water, glycerol, acid, or alkaline.[3] Since these methods seemed to be affected by the subjective interpretation of the investigator, several methods using novel analytical equipment were recently developed for exact determination of bloodstain age. For instance, the degree of freshness of a bloodstain was proved by detection of hemin in red blood cells by thin-layer chromatography.[4] The spectrum of the electron spin resonance (ESR) of methemoglobin (MetHb) in bloodstains was measured to estimate their age.[5] Decrements of activities of several enzymes in bloodstains were also determined to estimate the age.[6]

Moreover, it was reported that bloodstain ages could be obtained from the decrease in oxyhemoglobin (HbO_2) and the increase in MetHb by determining the ratio of absorbance at two wavelengths,[7] or by measuring the absorbance spectrum in the visible region[8] or the reflective spectrum of hemoglobin (Hb).[9] These methods, however, seem to be inadequate to make a precise determination of bloodstain age, because the difference between the absorbance spectrum of the sample and that of the control is very small and because the color of the sample itself interferes with the reflective spectrum. Furthermore, these methods are tedious or require the use of expensive instruments (for instance, ESR).

In the field of forensic science, Van Kampen's method[10] (colorimetric determination of MetHb) has been used as a standard method for estimation of bloodstain age. However, the method requires a large amount of sample (200 μl). Furthermore, the absorbance of cyanomethemoglobin (CNMetHb) at 630 nm is too small to give the total Hb concentration.

In preliminary experiments, we clearly demonstrated that HbO_2 in a bloodstain decreased and was oxidized to MetHb almost stoichiometrically with the lapse of the time after bleeding and with elevation of the temperature. So, we conjectured that bloodstain age could be estimated by measurement of the ratio or percentage of HbO_2/total Hb (fractional HbO_2[11]) in the bloodstain, if the temperature at which the bloodstain had been kept was known.

MATERIALS AND METHODS

Reagents Ferrous ethylenediamine sulfate (FES, $[(CH_2NH_2)_2 \cdot FeSO_4 \cdot 4H_2O]$) was purchased from Dojin Chemical Laboratories, Kumamoto, Japan; and 131 mmol/l of FES solution was prepared as an external standard of oxygen by dissolving 0.25 g of this reagent in 5 ml of water containing 50 μl of 1 N H_2SO_4. Iron(II) ammonium sulfate hexahydrate, *i.e.*, ferrous ammonium sulfate (FAS, $[FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O]$; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was also available as a substitute for FES at the same molar concentration.

All other reagents, of analytical grade, were purchased from Wako Pure Chemical Industries, Ltd., Osaka.

Preparations of Bloodstain Samples Blood samples were taken from healthy adults and supplemented with anticoagulant EDTA (final concentration, 5 mmol/l), which is a calcium chelator frequently used in forensic science investigations. In our preliminary experiments, we demonstrated that analytical data for HbO_2 in blood and bloodstain samples with EDTA added were in fair agreement with those for HbO_2 in the same heparinized blood and bloodstains. So, EDTA was used in our experiments.

For preparation of bloodstain samples, 200-μl aliquots of the blood were put into shallow wells (diameter, 10 mm) of ceramic plates, and the plates were separately kept at 5, 14, 24, and 37 °C in a dark place. For the determination of HbO_2 and CNMetHb, the dried bloodstains were dissolved in 400—600 μl of saline.

Use of Oxygen Electrode[12] for Analyses of HbO_2 and Deoxyhemoglobin (DeoxyHb) in Bloodstains For measurements of HbO_2 and deoxyHb, a polarographic oxygen analyzer (Central Kagaku, Co., Ltd., Tokyo, Japan) was used (Fig. 1). A galvanic-type oxygen electrode may also be suitable. A cylindrical water-jacketed glass cell (1.0 ml

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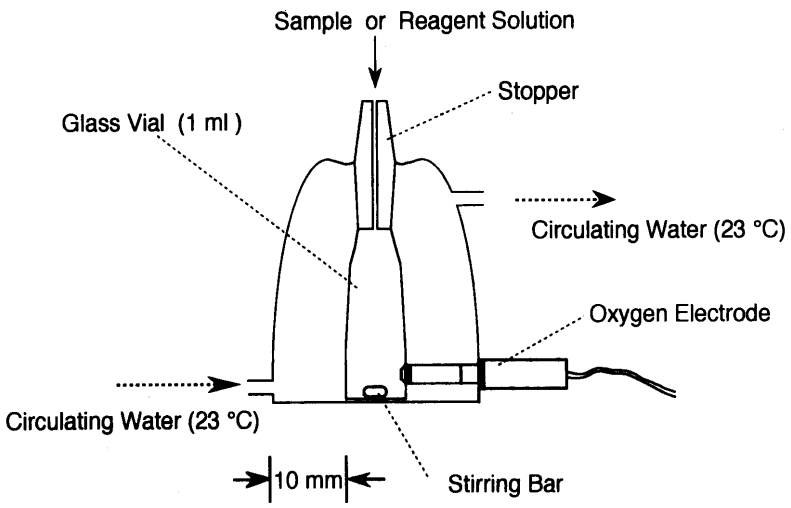

Fig. 1. Glass Cell-Attached Oxygen Electrode for Determination of HbO_2 and DeoxyHb in Blood

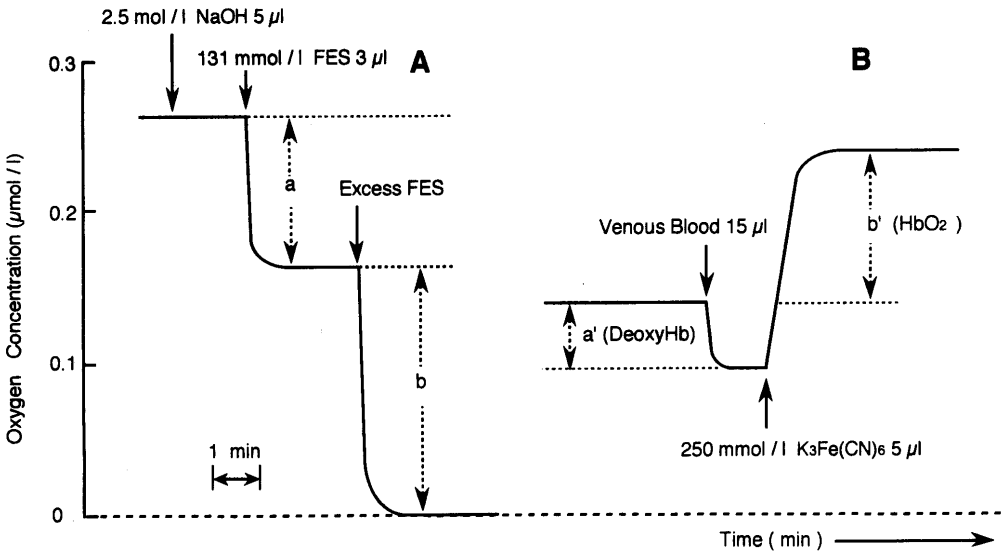

Fig. 2. Determination of Dissolved Oxygen in Air-Saturated Pure Water with FES and Oxygen Electrode (A) and that of HbO_2 and DeoxyHb in Venous Blood with the Oxygen Electrode and Oxygen-Depleted Water of Which Oxygen Was Determined with FES (B)

in volume, with temperature maintained at 23 °C) was placed on a magnetic stirrer, and microsyringes (10 or 25 μl in volume capacity) were used for additions of reagent and sample into the cell.

After calibration of the zero point of the analyzer with 400 mmol/l of sodium sulfite solution, determination of the oxygen concentration in water was performed with FES reagent[12b] under alkalin conditions. For instance, as shown in Fig. 2A, 3 μl of 131 mmol/l FES corresponding to 0.0983 μmol (3.141 μg) of oxygen was added, because 1 mol of FES consumes 1/4 mol of oxygen under alkaline conditions as shown in the following Eqs. 1, 2:

$$(CH_2NH_2)_2 \cdot FeSO_4 + 2NaOH \rightarrow Fe(OH)_2 + (CH_2NH_2)_2 + Na_2SO_4 \quad (1)$$

$$4Fe(OH)_2 + O_2 + 2H_2O \rightarrow 4Fe(OH)_3 \quad (2)$$

As shown in Fig. 2A, total oxygen (μmol/l) dissolved in the water was calculated by the following Eq. 3:

$$\text{Total } O_2\ (\mu\text{mol/l}) = 0.0983 \times (a+b)/a \quad (3)$$

We confirmed that FAS could be replaced with FES; however, in this case, the value 0.0956 should be used instead of 0.0983 in Eq. 3.

Then, 15 μl of blood was added to the cell filled with water in which the concentration of oxygen had been reduced to about 0.14 μmol/l by bubbling with N_2. DeoxyHb in the sample bound to the oxygen remaining in the medium, and the consumption of oxygen was recorded immediately (Fig. 2B, a'). Successive addition of potassium ferricyanide (250 mmol/l, 5 μl) oxidized the HbO_2 to MetHb, and all oxygen in the HbO_2 was released into the medium (Fig. 2B, b'). Moles of oxygen measured by this method precisely reflect one-fourth mol of HbO_2 in the sample blood. Dried bloodstains, corresponding 200 μl of blood, were dissolved in 400—600 μl of saline, and HbO_2 in the dissolved bloodstain solution (15 μl) was assayed by use of the oxygen electrode (Fig. 3). No deoxyHb was detected, because deoxyHb in the bloodstain bound oxygen in the air to form HbO_2 during drying.

The precision of determination of HbO_2 by the oxygen detector was on the order of 1.5% CV, and a highly significant linear correlation was found between our

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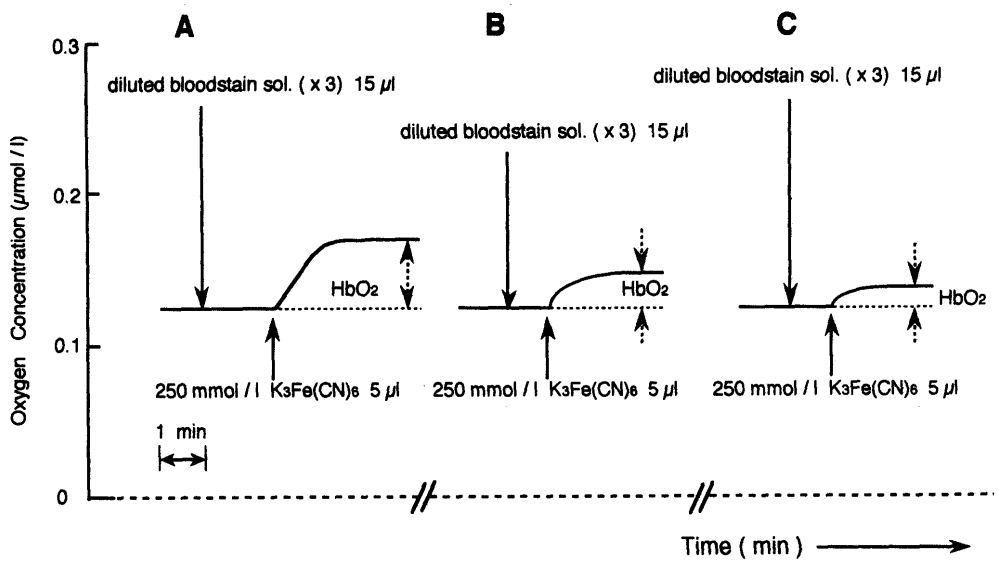

Fig. 3. Determination by Oxygen Electrode of HbO_2 in Bloodstain Kept at 24 °C

Bloodstains (each 200 μl) stood for 1 h (A), 8 h (B), and 96 h (C) at 24 °C were dissolved in and diluted to 600 μl with saline, and HbO_2 in 15 μl of each diluted bloodstain solution was assayed by the oxygen electrode.

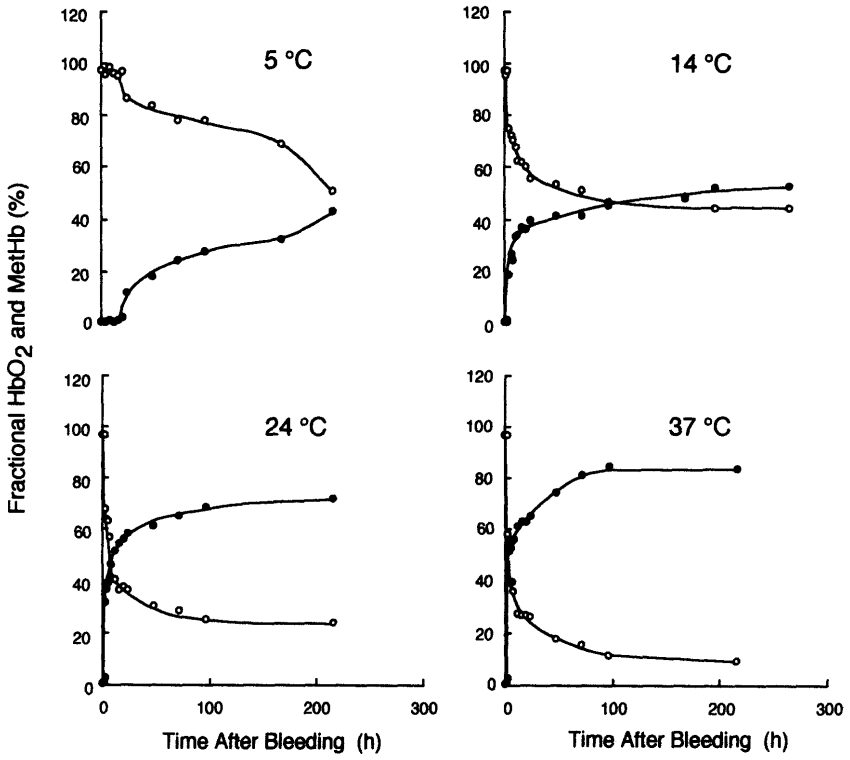

Fig. 4. Changes in Fractional HbO_2 and MetHb in Bloodstains (200 μl) at 5, 14, 24, and 37 °C

Fractional HbO_2 (○) and fractional MetHb (●) to total Hb were determined by the present method.

method and the Van Slyke method[13] ($r=0.996$; $n=18$).[12b]

Colorimetric Determinations of Total Hb and MetHb in Bloodstains The amounts of total Hb and MetHb in bloodstain samples were assayed by the CNMetHb method (Hemoglobin Test Wako, Wako Pure Chemical Industries Ltd., Osaka) and Van Kampen's method,[10] respectively.

RESULTS

Oxidation of HbO_2 to MetHb in Bloodstains Kept at 5—37 °C It was already reported that HbO_2 in bloodstain becomes oxidized to MetHb upon storage.[9b] To confirm this, we measured the decrease in HbO_2 and the increase in MetHb during storage of bloodstains at 5, 14, 24, and 37 °C. As shown in Fig. 4, clear contrasts of the decrease in HbO_2 with the increase in MetHb in bloodstains were demonstrated; and the reaction rate increased with a rise in temperature.

Oxidation of HbO_2 to MetHb in Bloodstains Kept at 5—37 °C and the Estimation of Bloodstain Age Figure 5A and B shows a typical example of fractional HbO_2 (%) in bloodstains kept for 0—20 h and for 0—216 h. DeoxyHb, if present in a fresh blood sample, would be

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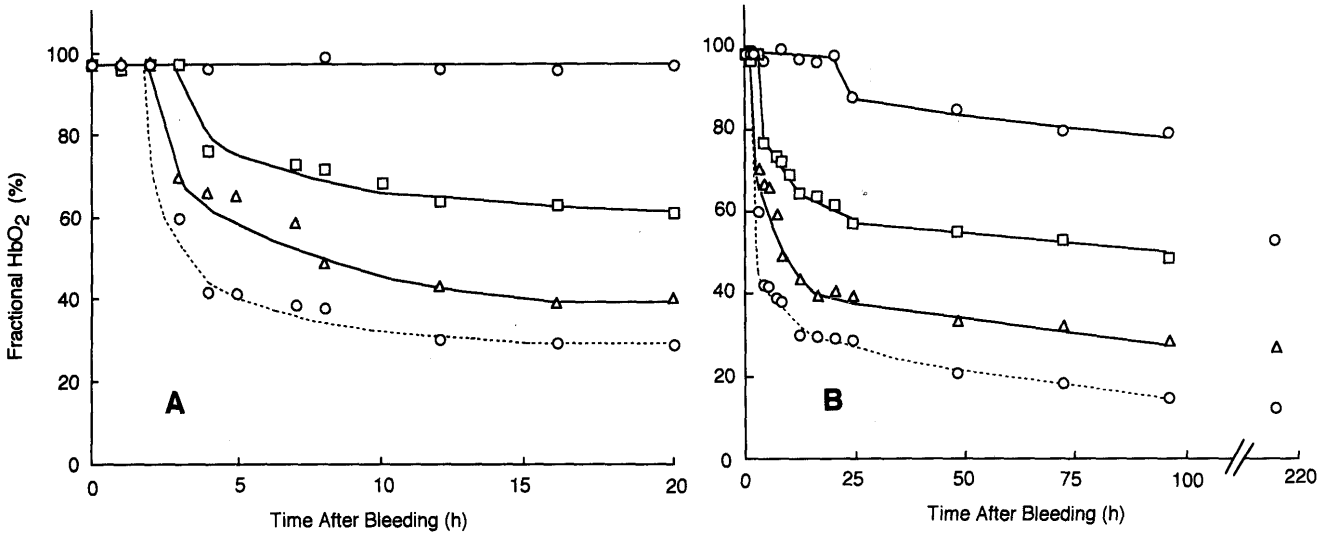

Fig. 5. Changes in Fractional HbO_2 in Bloodstains (200 μl) Stored at 5, 14, 24, and 37 °C for Short (A) and Long (B) Periods
5 °C (—○—), 14 °C (—□—), 24 °C (—△—), 37 °C (---○---).

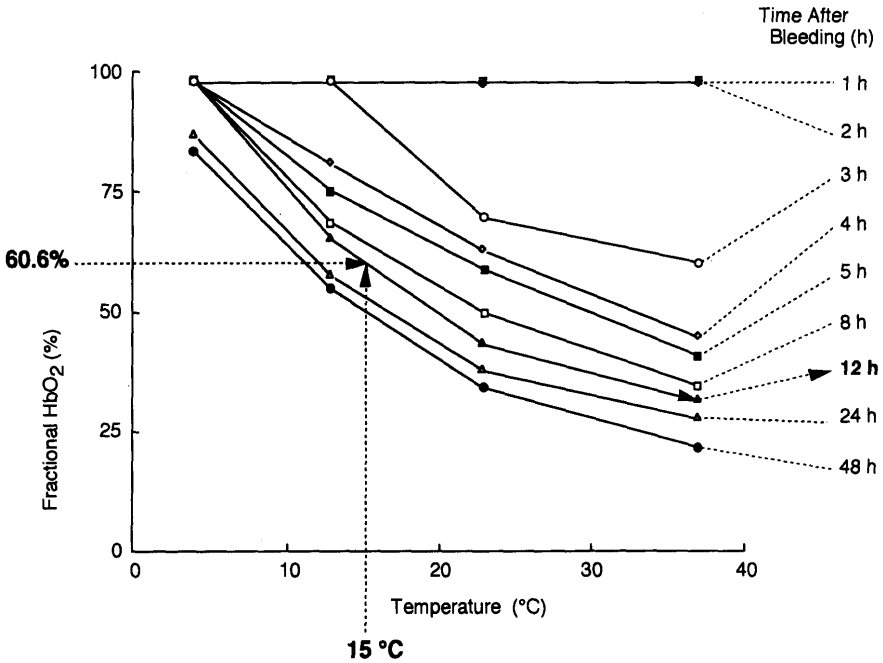

Fig. 6. Standard Curves for the Estimation of Bloodstain Age by Determination of Fractional HbO_2 in the Sample

converted to HbO_2 immediately after the sample was exposed to air in the well of a ceramic plate, and the HbO_2 thus formed as well as the endogenous HbO_2 (previously present) in the blood would be oxidized to MetHb. As shown in Fig. 5, oxidation of HbO_2 to MetHb in bloodstains kept at 5, 14, 24, and 37 °C began at 20, 3, 2, and 2 h, respectively, after the fresh blood had been placed into the well. This indicates that the conversion of HbO_2 to MetHb in bloodstains is more rapid at higher temperatures. As shown in Fig. 5A, no decrease in HbO_2 was observed at 37 °C during the first 2 h after bleeding, the bloodstain had completely dried.

Figure 6 shows the plots of fractional HbO_2 (%) against temperature for the specimens of various ages. The age can thus be estimated from these curves. For example, if the sample had been maintained at 15 °C and the fractional HbO_2 was 60.6%, the age would be estimated to be 12 h.

DISCUSSION

For determination of HbO_2 in blood, Morioka[14] reported the use of an oxygen electrode method employing water saturated with carbon monoxide. Morioka's electrode method required less than 200 μl of whole blood, and the analytical time was 3 min. On the contrary, the present oxygen electrode method for HbO_2 requires only 15 μl of bloodstain (blood) without carbon monoxide, and 2—3 min for the whole analysis of HbO_2 and deoxyHb in the bloodstain (blood). The total Hb content in the bloodstain is determined by a common colorimetric technique (CNMetHb method) in 2—3 min.

In this oxygen electrode analysis, even in fresh blood, we found that the sum of HbO_2 and deoxyHb corresponded to 97—98% of the total Hb determined by the colorimetry (see Figs. 4—6). The remaining 2—3% of Hb may be due to the trace amount of Hb that has no

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ability to bind oxygen, such as carboxyhemoglobin (carboxyHb).

It seems that the thin-layer chromatography[4] for proof of freshness of bloodstain is affected by impurities in the samples and by properties of bloodstain-supports containing benzidine reaction-positive substances (heavy metal ions). These factors may not interfere with the bloodstain age estimation by the present method.

For application of the present method to criminal investigation, it will be necessary to study the effects of sun exposure and humidity during standing of a bloodstain on the fractional HbO_2 determined by the present method, for, in most cases, these environmental conditions of bloodstain would not be constant during the standing period.

If a bloodstain (blood) contains carboxyHb, which is occasionally found in cases of carbon monoxide poisoning or fire victims, the carboxyHb can be easily determined by another oxygen electrode method[15] using erythrocytes saturated with oxygen.

It is a well-known fact that enzymatic reduction of MetHb to deoxyHb with NADH and nonezymatic reduction with ascorbic acid, *etc.* take palce in blood, and thus the similar mechanism probably occurs in wet bloodstains before drying. In our experiments (Fig. 5), 2, 2, 3, and 20 h were necessary to dry completely each 200 μl of bloodstains kept at 37, 24, 14, and 5 °C, respectively, in a dark place. After drying, the rapid oxidation of HbO_2 to MetHb was observed at higher temperature. So, we conjecture that the oxidation of HbO_2 to MetHb owing to the attack of atmospheric oxygen on the HbO_2 occurred in the dried bloodstain.

We are now investigating the oxidation of HbO_2 to MetHb in bloodstain in the changeable environmental conditions (changes of temperature, wind, light, *etc.*) and will report the results elsewhere in the future.

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