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THE REACTION BETWEEN THE SUPEROXIDE ANION RADICAL AND CYTOCHROME *c*

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SUMMARY

1. The superoxide anion radical (O_2^-) reacts with ferricytochrome *c* to form ferrocytochrome c. No intermediate complexes are observable. No reaction could be detected between O_2^- and ferrocytochrome *c*.

2. At 20 °C the rate constant for the reaction at pH 4.7 to 6.7 is $1.4 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ and as the pH increases above 6.7 the rate constant steadily decreases. The dependence on pH is the same for tuna heart and horse heart cytochrome *c*. No reaction could be demonstrated between O_2^- and the form of cytochrome *c* which exists above pH $\approx$ 9.2. The dependence of the rate constant on pH can be explained if cytochrome *c* has pKs of 7.45 and 9.2, and O_2^- reacts with the form present below pH 7.45 with $k = 1.4 \cdot 10^6$ $M^{-1} \cdot s^{-1}$, the form above pH 7.45 with $k = 3.0 \cdot 10^5$ $M^{-1} \cdot s^{-1}$, and the form present above pH 9.2 with $k = 0$.

3. The reaction has an activation energy of 20 kJ mol^{-1} and an enthalpy of activation at 25 °C of 18 kJ mol^{-1} both above and below pH 7.45. It is suggested that O_2^- may reduce cytochrome *c* through a track composed of aromatic amino acids, and that little protein rearrangement is required for the formation of the activated complex.

4. No reduction of ferricytochrome *c* by HO_2 radicals could be demonstrated at pH 1.2–6.2 but at pH 5.3, HO_2 radicals oxidize ferrocytochrome *c* with a rate constant of about $5 \cdot 10^5$–$5 \cdot 10^6$ $M^{-1} \cdot s^{-1}$.

INTRODUCTION

Superoxide anion radicals (O_2^-) are known to reduce cytochrome *c*. Rate constants at pH 8.4, 8.5 and 10.4 have been given as $1.6 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ [1], $1.1 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ [2] and $8 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ [2], respectively, while a recent communication has briefly reported the dependence of the rate constant on pH in the range 6–10.5 [3]. The reaction is interesting in itself, and the rate constant has been used in a determination of the rate constant for the reaction between O_2^- and superoxide dismutase [4]. We now report a study of the reaction kinetics.

The superoxide anion radicals have been generated by pulse radiolysis of oxygen-containing neutral or alkaline solutions of formate (0.1 M) containing cytochrome *c*. In these solutions O_2^- is produced through the following reactions:

$$H_2O \rightsquigarrow e^-_{aq} + H + OH + H_2 + H_2O_2$$
$$e^-_{aq} + O_2 \rightarrow O_2^-$$
$$OH + HCOO^- \rightarrow H_2O + CO_2^-$$
$$H + HCOO^- \rightarrow H_2 + CO_2^-$$
$$CO_2^- + O_2 \rightarrow CO_2 + O_2^-$$
$$H + O_2 \rightarrow HO_2$$
$$HO_2 \overset{pK=4.9}{\rightleftharpoons} O_2^- + H^+$$

The combined yield of the radical species on the right hand side of the first of these equations is taken to be $G = 6.5$ molecules produced per 100 eV of energy absorbed [5]. Doses (50–200 rads, producing $3.4 \cdot 10^{-7}$–$1.4 \cdot 10^{-6}$ M O_2^-) and concentrations of cytochrome *c* (4×10^{-6}–10^{-4} M) were such that the reaction between O_2^- and cytochrome *c* always followed simple first order kinetics (pseudo unimolecular) and that, especially important in the least alkaline solutions, a negligible fraction of O_2^- reacted according to:

$$O_2^- + HO_2 \xrightarrow{+H^+} O_2 + H_2O_2$$

a reaction which proceeds with a rate constant of $8.5 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ [6].

METHODS

Unless otherwise stated, the cytochrome *c* used here was horse heart, 90–100 %, from Koch-Light. Spectrophotometric measurements with and without added sodium dithionite showed that these samples consisted 98 % of cytochrome *c*, 93 % of which was in the oxidized form. Ferrocytochrome *c* was prepared by dissolving 0.1 g ferricytochrome *c* in 10 ml water, adding excess sodium dithionite and passing the solution through a Sephadex G25 column. The fraction containing the cytochrome *c* was tested spectrophotometrically to determine the concentrations of the reduced and oxidized forms, and to check for the absence of dithionite. Tuna cytochrome *c* was 95–100 %, obtained from Sigma, 90 % being in the oxidized form. Other reagents were of Analar grade except for sodium formate which was pure grade from Fluka. Water was distilled from alkaline permanganate. Buffers used (10^{-2} M) were for pH 5–8, Na_2HPO_4/KH_2PO_4 and for pH 8–10, H_3BO_3/NaOH. For pH 3–5, sodium formate/formic acid was used, the total formate concentration being 0.1 M. Extremes of acidity and alkalinity were achieved using appropriate concentrations of $HClO_4$ or NaOH. All solutions were saturated with oxygen (Air Products) at ambient temperature and pressure.

Pulse radiolysis experiments were conducted using the Paterson Laboratories linear accelerator facility [7, 8]. A single Bausch and Lomb monochromator was normally used, with bandwidths 5 nm, but a double monochromator was used to obtain the results presented in Fig. 1, bandwidths being 2.5–5 nm. For these results the monochromator was calibrated using both narrow-band interference filters and reduced cytochrome *c*. Cells had optical path length of 0.4, 2.5 or 5.0 cm.

RESULTS

At pH 8.6 the production of O_2^- in the presence of excess cytochrome *c* led to a first-order increase in absorption at 550 nm, the rate of which was directly proportional to the cytochrome *c* concentration in the range $3.85 \cdot 10^{-6}$–$1.2 \cdot 10^{-5}$ M. The second order rate constant for the reaction at 20 °C was $2.2 \pm 0.4 \cdot 10^5$ $M^{-1} \cdot s^{-1}$. Fig. 1 shows the increase or decrease in absorption on completion of the reaction, i.e. after a few seconds. The figure also shows the change which would be expected for conversion of ferricytochrome *c* to ferrocytochrome *c* with $G = 6.5$, based on published absorption spectra [9]. The correspondence between the changes shows that the sole product of the reaction is ferrocytochrome *c*, in 90–100 % yield. Similar results were obtained at pH 6.75.

The possibility of further reaction of O_2^- with ferrocytochrome *c* was examined by delivering enough pulses to ferricytochrome *c* solutions ($3.85 \cdot 10^{-6}$ and $6.8 \cdot 10^{-6}$ M, pH 8.6) to reduce all of the cytochrome *c*, and then looking for any further effect produced by delivery of one extra pulse. No change was seen at 550 nm or 450 nm over a period of 20 s, indicating that O_2^- does not react with ferrocytochrome *c*. An additional test was made by delivering 100 rads ($7 \cdot 10^{-7}$ M O_2^-) to a $5 \cdot 10^{-6}$ M ferrocytochrome *c* solution which contained $2 \cdot 10^{-7}$ M ferricytochrome *c* as an impurity (pH 8.4). The absorption at 550 nm was found to increase rather than decrease and the absorption at 450 nm to decrease rather than increase. This is attributable to reaction of O_2^- with the ferricytochrome *c* impurity, and strengthens the conclusion that O_2^- does not react with cytochrome *c* in its reduced form.

The effect of pH on the reduction of cytochrome *c* was examined using cytochrome *c* solutions of concentration $3.85 \cdot 10^{-6}$–$9.8 \cdot 10^{-5}$ M. Doses were in the range 50–150 rads ($3.4 \cdot 10^{-7}$–$1 \cdot 10^{-6}$ M O_2^-). For each pH, the concentrations of cytochrome *c* and the doses were chosen so as to avoid the duration of the reaction being unduly long as well as to minimise errors due to the reaction between O_2^- and

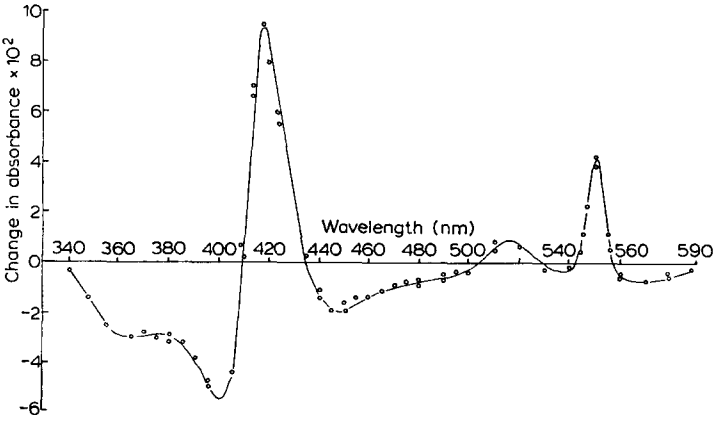

Fig. 1. Change in absorbance after reaction of O_2^- with horse-heart ferricytochrome *c*. The solutions contained $3.85 \cdot 10^{-6}$–$1.2 \cdot 10^{-5}$ M cytochrome *c* and 0.1 M sodium formate. They were buffered to pH 8.6, and saturated with oxygen. The change is that produced by the action of $1.35 \cdot 10^{-6}$ M O_2^- in a cell of optical path length 1.6 cm. The points are experimental. The line is calculated from reference 9 assuming $G = 6.5$.

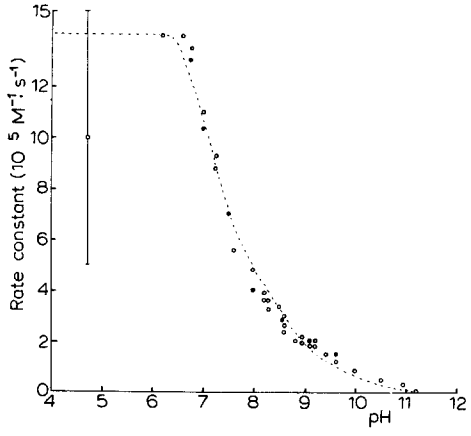

Fig. 2. Effect of pH on the rate constant for the reaction of O_2^- with ferricytochrome *c* at 20 °C. The solutions contained $3.85 \cdot 10^{-6}$–$9.8 \cdot 10^{-5}$ M cytyochrome *c* and 0.1 M sodium formate. They were saturated with oxygen. ○, horse heart cytochrome *c*. ●, tuna heart cytochrome *c*. The point at pH 4.7 with the large error is estimated (see discussion). The dotted line is theoretical, based on pKs of 7.45 and 9.2 with rate constants of $1.4 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ for the form below pH 7.45, $3.0 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ for the form between pH 7.45 and 9.2, and zero for the alkaline form.

HO_2. Results for 18–22 °C are shown in Fig. 2. At pH 8.0 the same rate constant was found in the phosphate buffer as in the borate buffer. The reaction of O_2^- with tuna heart cytochrome *c* was also investigated as a function of pH, using tuna heart cytochrome *c* concentrations of between 10^{-5} and 10^{-4} M. Results are incorporated in Fig. 2.

Earlier work has shown that the reduction of mildly alkaline cytochrome *c* by hydrated electrons or CO_2^- [2, 10] or dithionite [11] gives rise to an unstable form of ferrocytochrome *c* which changes in a first order manner into the normal form within about 0.5 s. To see if reduction in the present system proceeds through a similar intermediate, pulses were given to cytochrome *c* solutions ($5.7 \cdot 10^{-5}$ and $1.25 \cdot 10^{-4}$ M, pH 9.2) at 12.7° and 10.8 °C, respectively. From the earlier work [2, 10] any production of the unstable reduced form would be expected to be followed by a marked decrease in absorbance at about 410–420 nm with a half life of about 0.15–0.2 s. Examination of absorption charges showed the expected reduction of the

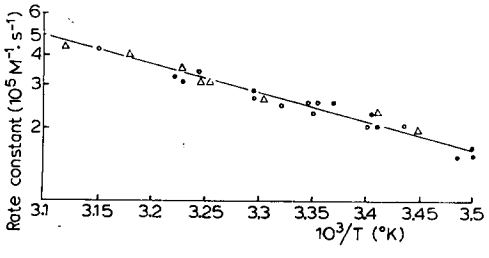

Fig. 3. Temperature dependence of the rate constant for the reactions between O_2^- and horse-heart cytochrome *c* at pH 8.6, ○, $3.85 \cdot 10^{-6}$ M ferricytochrome *c*. △, $6.8 \cdot 10^{-6}$ M ferricytochrome *c*. ●, $1.2 \cdot 10^{-5}$ M ferricytochrome *c*.

cytochrome *c*, but the absorbance at 410–420 nm did not decrease afterwards, showing that the unstable reduced form had not been produced.

The rate constant for the reduction of cytochrome *c* was found to depend on temperature. The logarithmic dependence of the rate constant on the inverse of the absolute temperature is shown for cytochrome *c* solutions ($3.85 \cdot 10^{-6}$–$1.2 \cdot 10^{-5}$ M) at pH 8.6 in Fig. 3. The activation energy, E_a, calculated from Fig. 3 is 19.9 kJ · mol^{-1}. The enthalpy of activation at 25 °C, $\Delta H^{\ddagger} = E_a - RT$, is 17.4 kJ · mol^{-1}. The entropy of activation at 25 °C, $\Delta S^{\ddagger}$, was calculated using the expression:

$$k = (\boldsymbol{k}T/h) \exp(-\Delta H^{\ddagger}/RT) \exp(\Delta S^{\ddagger}/R)$$

where k is the rate constant at temperature T, $\boldsymbol{k}$ is Boltzman's constant and h is Planck's constant, and found using $k = 2.4 \cdot 10^{5}$ $M^{-1} \cdot s^{-1}$ at 25 °C to be −83.5 J $deg^{-1} \cdot mol^{-1}$.

The temperature dependence of the rate of the reaction was also investigated at pH 6.75. The Arrhenius plot was again linear, corresponding to an activation energy of 21.2 kJ mol^{-1}, an enthalpy of activation of 25 °C of 18.7 kJ · mol^{-1} and an entropy of activation at 25 °C of −64.2 J · deg^{-1} · mol^{-1}. The activation energy and enthalpy of activation are not significantly different from the values at pH 8.6, and mean values of 20 kJ · mol^{-1} and 18 kJ · mol^{-1}, respectively, may be adopted. The entropy of activation however appears to be significantly lower at the lower pH.

The reaction of the hydrodioxyl radical, HO_2, with ferricytochrome *c* was examined by giving pulses of approximately 50, 100 and 200 rads ($3.4 \cdot 10^{-7}$–$1.4 \cdot 10^{-6}$ M HO_2) to cytochrome *c* solutions (10^{-5} M) at pH 1.2, 2.0 and 3.1. For none of the solutions was any change in absorption seen at 415, 445 or 550 nm up to a period of 20 s. This indicates that if HO_2 radicals do reduce ferricytochrome *c*, the rate constant for the reaction under these conditions must be less than $3 \cdot 10^{3}$ $M^{-1} \cdot s^{-1}$. The reaction was also investigated at pH 4.7. A solution containing $8 \cdot 10^{-6}$ M cytochrome *c* was given a dose of 45 rads (combined concentrations of O_2^- and $HO_2 = 3.1 \cdot 10^{-7}$ M). The absorbance at 550 nm was found to increase by about $6 \cdot 10^{-3}$. This small increase can be quantitatively attributed to the reaction of O_2^- with cytochrome *c* occurring together with reaction between O_2^- and HO_2, and is consistent with HO_2 radicals not reacting with cytochrome *c*.

Attempts were also made to investigate the reaction of HO_2 with ferrocytochrome *c*. Experiments could not be done below pH 4 because of the rapidity of the reaction of oxygen with ferrocytochrome *c*. However, in a typical experiment at pH 5.3 and 19.4 °C, it was found that when a pulse of 150 rads ($1 \cdot 10^{-6}$ M O_2^-) was delivered to a solution containing approximately $1.4 \cdot 10^{-5}$ M ferrocytochrome *c* together with about $2 \cdot 10^{-6}$ M ferricytochrome *c* impurity there was a decrease in absorbance at 550 nm which could be attributed to oxidation of ferrocytochrome *c* by HO_2. The conditions did not permit the rate constant to be determined accurately but it could be estimated to lie in the approximate range $5 \cdot 10^{5}$–$5 \cdot 10^{6}$ $M^{-1} \cdot s^{-1}$.

DISCUSSION

From the redox potentials for O_2^- ($E^0(O_2/O_2^-) = -0.33$ v, $E'^0(O_2^-, H^+/H_2O_2) = 0.94$) [12] and cytochrome *c* ($E^0(Fe^{III}$ cyt *c*/Fe^{II} cyt *c* = 0.26 v) [13] it is evident that on thermodynamic considerations alone, O_2^- could either oxidize

or reduce cytochrome *c*. The results in the present paper indicate that under the conditions employed here the reaction of O_2^- with cytochrome *c* is a simple reduction. No evidence has been seen for the formation of any intermediate complexes during the course of the reaction.

Experimental values are given here for the rate constant of the reaction at pH 11.2–6.2 (Fig. 2). The experiments done at pH 4.7 can now be analysed to provide one further value: if HO_2 radicals do not react with ferricytochrome *c*, and if the reaction of HO_2 with ferrocytochrome *c* impurity can be neglected, the only reactions to be considered in this experiment are the reaction between O_2^- and cytochrome *c*, rate constant *k*, and the reaction between O_2^- and HO_2, rate constant $8.5 \cdot 10^7\ M^{-1} \cdot s^{-1}$. It can be shown that the concentration of ferrocytochrome *c* produced under the conditions is given by:–

$$\frac{2.3k\,K[Fe^{III}]}{8.5 \cdot 10^7[H^+]} \log\left(1 + \frac{8.5 \cdot 10^7\,[H^+][O_2^-]}{kK[Fe^{III}]}\right)$$

where *K* is the dissociation constant of the HO_2 radical and $[Fe^{III}]$ and $[O_2^-]$ are the initial concentrations of ferricytochrome *c* and O_2^-, respectively. The concentration of ferrocytochrome *c* which corresponds to an increase in absorbance of $6 \cdot 10^{-3}$ is $6.8 \cdot 10^{-8}$ M, from which the rate constant *k* is $1 \cdot 10^6\ M^{-1} \cdot s^{-1}$. From the various errors in the calculation this value may be considered accurate to $\pm 0.5 \cdot 10^6\ M^{-1} \cdot s^{-1}$. Since the value is not significantly different from the experimental values determined at pH 6.2–6.7 it can be concluded that the rate constant for the reaction between O_2^- and cytochrome *c* is essentially independent of pH from pH 6.7 to 4.7.

The rate constants reported here supersede those obtained in previous pulse radiolysis experiments [2] but are still in reasonable agreement with the single value obtained independently [1]. They are in agreement with the limited number of values obtained recently for a narrower pH range [3]. It should be noted that since cytochrome *c* is positively charged the rate constants would be expected to increase with decreasing ionic strength, so that in unbuffered solution in the absence of salt, the rate constant would be expected to be greater than given here. The rate constants, together with the other features of the reaction make it likely that the reduction of the ferric ion proceeds through an outer sphere (remote) pathway rather than an inner sphere (adjacent one).

In seeking an explanation for the shape of Fig. 2. It may be noted that from the results at pH 8.0 the rate constants appear to be independent of the nature of the buffer, suggesting that ion binding to cytochrome *c* is unimportant. Also the variation in ionic strength in the various solutions is much too small to play any part in the explanation. Any reaction of cytochrome *c* with hydrogen peroxide (such as molecular hydrogen peroxide produced by the radiation pulse) would be much too slow to play any part [14]. Now above pH $\approx$ 9 cytochrome *c* exists in a form in which the ε amino group of Lysine 79 appears to replace the sulphur of Methionine 80 in the inner sphere of the ferric iron. Reduction of this form by powerful reducing agents gives rise in the first instance to an unstable form of ferrocytochrome *c* which has different spectroscopic properties from the form which is stable in neutral solution [2, 10, 11, 15]. The unstable form does not appear when cytochrome *c* is reduced in the present system at pH 9.2, which shows that either O_2^- reduces the alkaline form of cytochrome *c*

extremely slowly, or it does reduce it at all (like ascorbate [16]). It may therefore be supposed that O_2^- reduces the neutral and alkaline forms of cytochrome *c* at widely different rates. This hypothesis is not however in itself sufficient to explain the shape of Fig. 2, since it would predict that the rate constant for the reduction of cytochrome *c* should be independent of pH below about pH 8. The failure to detect any reaction between HO_2 and ferricytochrome *c* is consistent with HO_2 radicals playing no part in the reaction so it is suggested that the shape of the curve below pH 8 is due to the existence in that pH range of at least two forms of cytochrome *c* with which O_2^- reacts at different rates. At least squares analysis on this basis shows that a good fit to the experimental results of Fig. 2 is obtained if there is a pK at 7.45 ± 0.5 as well as at 9.2 ± 0.2 and that the rate constants for reactions with the three forms of cytochrome *c* are $1.4 \pm 0.15 \cdot 10^6\ M^{-1} \cdot s^{-1}$ for the form which predominates below pH 7.45, $3.0 \pm 0.4 \cdot 10^5\ M^{-1} \cdot s^{-1}$ for the form between pH 7.45 and 9.2, and zero for the form which predominates above pH 9.2. A line drawn on this basis is shown in Fig. 2.

The unreactivity of O_2^- towards the alkaline form of cytochrome *c* is in line with electrode potentials [13], with the decreased reactivity of many other reducing agents towards this form, and with the unreactivity of O_2^- towards ferriprotoporphyrin IX dimers [17]. The marked change in reactivity at pH 7.45 ± 0.5 resembles the change at pH 7.0 ± 0.4 observed in recent experiments using other anionic reductants [18]. The similarity between results with cytochrome *c* from horse heart and tuna heart demands that any explanations of this pK must apply to both cytochromes *c*. It could be that the O_2^- reacts at the exposed edge of the haem and this would be consistent with the results from the activation energy experiments at pH 8.6 and pH 6.75 which indicate that little rearrangement of the structure of the cytochrome is required for the electron transfer process to take place. However, this mechanism cannot readily accommodate a dramatic change in rate constant at pH 7.45, particularly as there exists a cluster of localized positively charged groups around the haem edge [19]. While various other explanations of the pK can be considered it is possible to propose a hypothesis based on an examination of the amino acid sequences in cytochrome *c* from various sources [19] which shows that residues 47–53 and 63–83 are reasonably invariant. In horse-heart cytochrome *c* these two blocks of residues border a distinct channel into the cytochrome interior, with some of the side chains (for example, tyrosine 67 and 74 and tryptophan 59) being along the channel [19]. It is suggested that O_2^- may transfer its electron to the haem group through this track. Protonation of some amino acid at pH values below 7.45 enhances the electron transfer either because O_2^- reacts more rapidly with the protonated group or because protonation opens up the way through the track. The pK cannot be assigned to any particular amino acid because the pK of many of the amino acid residues in the cytochromes must be different from those of free amino acids or amino acids in peptides because of the existence of hydrophobic regions and hydrogen bonding. Although this mechanism may apply to our results and perhaps some other systems too, it is not proposed that it necessarily applies to all reductions of all types of cytochrome *c*.

The smallness of the energy and enthalpy of activation at both pH 8.6 and 6.75 are consistent with the proposed mechanism. The entropies of activation are less easy to interpret as they may be controlled by a variety of interrelated factors, in particular the slight contraction of the surface area as a consequence of reduction [20],

the loss of bound water molecules and anions [13] and the resulting change of the charge of the molecule.

The rate constant for the reaction of HO_2 radicals with ferrocytochrome *c* ($5 \cdot 10^5$–$5 \cdot 10^6$ $M^{-1} \cdot s^{-1}$) is of the same order of magnitude as that for the reaction of HO_2 with hydrated ferrous ions (10^6 $M^{-1} \cdot s^{-1}$ [21]) and for the reaction of O_2^- with the two more acid forms of cytochrome *c* studied in this paper. It seems likely that, as in the other cases, the mechanism is an outer sphere (remote) one, and that since in ferrocytochrome *c* the haem group is even more buried than in ferricytochrome *c* [20], the electron transfer may again proceed through a route through the protein.

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