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Evolutionary trends in the hemoglobins of murine animals

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Abstract. The evolutionary origin of murine line based on a phylogenetic tree made on sequence data of ∝-and β-hemoglobin chains, followed by the diversity spectrum of hemoglobin genes in two wild species of murine rodents: *Rattus rattus rufescens* (house rat) and *Bandicota indica* (bandicoot rat) has been reported. Each house rat contains six hemoglobin types involving two ∝-and three β-chains, which suggests a probable gene duplication at the ∝ chain locus and a gene triplication at the β-chain locus. Each bandicoot rat contains one ∝-and two β-chains suggesting a probable gene duplication at the β-chain locus. Peptide pattern analysis of the polypeptide chains of these murine hemoglobins further indicates that intraspecies differences among duplicated chains of the same kind are less than interspecies differences among corresponding ∝- and β-chains.

Keywords. Murine rodents; phylogenetic relationship; hemoglobin gene diversity; gene reiteration.

Introduction

Rodents presumably arose from the insectivore placental stem (Romer, 1966). From this base, primitive rodents have evolved along many lines of adaptive radiation giving rise to the rat-mouse stock, the most successful of all living mammals. The hemoglobins of these murine animals likewise show a spectrum of wide diversity. For instance, the laboratory mouse and rat show multiple hemoglobin forms which contain several ∝- and non-∝ -polypeptide chains (Ranney and Glueckshon-Waelsch, 1955, Gluecksohn-Waelsch *et al.,* 1957; Russel and Gerald, 1958; Popp, 1969; Travnicek *et al.,* 1971; Gilman, 1972; Garrick *et al,* 1975). Clearly, the rat-mouse stock is an excellent experimental source for studying the evolutionary trends of hemoglobins. However, any attempt that tries to study the extent of diversity of the hemoglobin genes within any mammalian species, must take into consideration the evolutionary relationships of the hemoglobin genes in that species with those in other mammalian groups. This is necessary since hemoglobin gene diversities appear not only at the level of the species but also at that of broader categories.

In the present report, we have first located the origin of the murine line of descent by constructing a phylogenetic tree based on the combined sequences of ∝ and β-chains of the hemoglobins of representative animals species belonging to different mammalian orders. Next, we have structurally characterised the multiple hemoglobin forms of two wild species of murine animals, namely *Rattus rattus rufescens* (house rat) and *Bandicota indica* (bandicoot). Finally, we have made a comparative assessment of the hemoglobin gene diversity in *Rodentia* and those in other mammalian orders.

Materials and methods

The phylogenetic tree for the combined ∝-and β-chain sequences of hemoglobins was constructed by using parsimony methods (Barnabas *et al.,* 1972; Moore *et al,* 1973; Barnabas *et al.,* 1978). The two murine species under study were trapped in and around Ahmednagar (19°5′ N; 74°45′ E). Blood samples of individual animals were collected by cardiac puncture using EDTA or sodium citrate as anticoagulants. Hemoglobin typing was carried out by starch gel electrophoresis using Tris-EDTA-boric acid buffer, pH 8.1, as the gel buffer and boric acid-NaOH buffer, pH 9.0, as the electrode vessel buffer (Huisman, 1963). Hemoglobin variants were isolated by means of both carboxymethyl (CM)-cellulose chromatography (Huisman *et al.,* 1958) and diethylaminoethyl (DEAE)-Sephadex A-50 chromatography (Huisman and Dozy, 1965). Globins were prepared according to the method of Anson and Mirsky (1930). The separation of polypeptide chains was achieved by using the urea-polyacrylamide gel electrophoretic method of Moss and Ingram (1968). Polypeptide chains were isolated following the method of Clegg *et al.,* (1966) with necessary modifications as reported earlier (Pratap *et al.,* 1978). The polypeptide chains were identified as ∝-or β-chains by locating the respective characteristic peptides on fingerprints. The globins were aminoethylated prior to mapping the peptides (Jones, 1964). Using the fingerprint of ∝-and β-chains of mouse hemoglobin as standard, the tryptophan containing ∝-Tp-3 and methioninei containing ∝-Tp-5 peptides were identified on the fingerprints by appropriate colour tests.The tryptophan containing β-Tp-2 and β-Tp-4 peptides were also similarly identified.

Results and discussion

In recent years, the idea that sequences of proteins could be used to decipher phylogenetic relationships in animal species has gained fairly good acceptance. Hemoglobin, the principal protein of vertebrate blood has in particular been extensively studied in this context. Consequently, the sequences of ∝ and non-∝-chains of vertebrate hemoglobins have not only been correlated with genetics (Ingram, 1957; Baglioni, 1963) but their variation in different species have been used to derive gene species phylogeny as well (Fitch and Margoliash, 1967; Barnabas *et al.,* 1971; Goodman *et al.,* 1972; Goodman *et al.,* 1974). We have constructed a phylogenetic tree for the hemoglobins of representative animal species of six eutherian orders along with those of a protherian and metatherian (figure 1). From this figure, it is evident that the hemoglobins of eutherian mammals separate after the divergence of those of echidna (Prototherian) and kangaroo (Metatherian). In the eutherian line of descent, the hemoglobins of rodent *(Mus musculus* and *Rattus norvegicus)* separate prior to those of ungulates *(Equus caballus* and *Bos taurus)* and a primate *(Homo sapiens),* but after the divergence of those of a lagomorph *(Oryctolagus cuniculus)* and a carnivore *(Canis familiris).* This is consistent with the presumed origin of rodents (Romer, 1966). It is noteworthy that rat and mouse which belong to the same subfamily *Murinae* show considerable nucleotide replacements (17 for mouse and 29 for rat) in their hemoglobins since the time of their immediate common ancestor (figure 1). This also confirms earlier immunological and DNA hybridisation data (Laird *et al.,* 1959; Sarich, 1972). Furthermore, Morrison and co-workers (1977) suggest that two cricetine genera: *Peromyscus* and *Calomys* stand closer in their hemoglobin make-up to *mouse* than to rat.

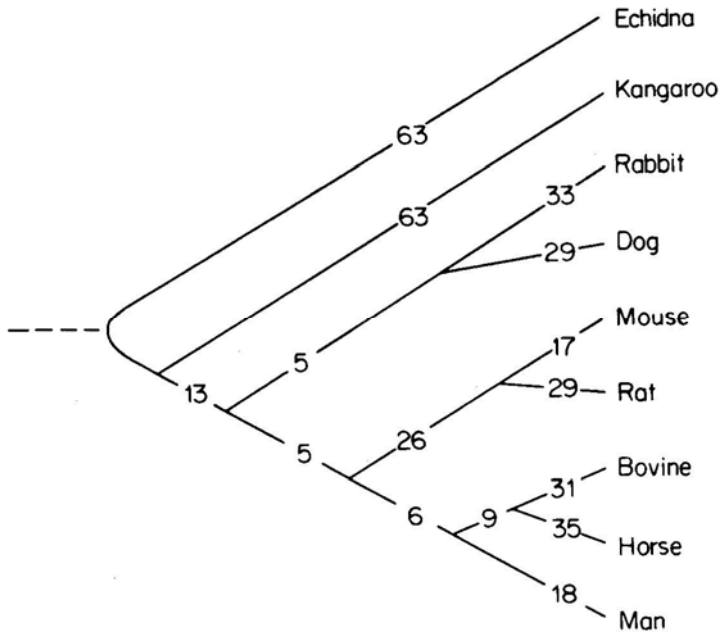

Figure 1. An evolutionary tree of combined hemoglobin ∝ -β-chain sequences. Man *(Homo sapiens)* (Braunitzer *et al.,* 1961), horse *(Equus caballus)* (Smith, 1964; Kilmartin and Clegg, 1967), bovine *(Bos taurus)* (Schroeder *et al.,* 1967), rat *(Rattus norvegicus)* (Chus *et al.,* 1975; Garrick *et al,* 1978), mouse *(Mus musculus)* (Popp, 1967; Popp, 1972), dog *(Canis familiaris)* (Jones *et al.,* 1971; Dresler *et al.,* 1974), rabbit *(Oryctolagus cuniculus)* (von Ehrenstein, 1966; Best *et al.,* 1969), kangaroos *(Macropus giganteus)* (Beard and Thompson, 1971; Air and Thompson, 1972), and echidna *(Tachyglossus aculeatus)* (Whittaker *et al.,* 1972; Whittaker *et al.,* 1973). Numbers represent nucleotide replacements between ancestor and descendant sequences.

Hemoglobin gene iteration in house rat and bandicoot

Alkaline starch gel electrophoresis of 1025 blood samples of house rat revealed four hemoglobin components in each referred to as C_1,C_2, C_3 and C_4 in the decreasing order of their anodic mobilities (figure 2). The relative proportions of each component varied considerably in different samples. For practical purposes, we tentatively characterised phenotypes of the house rat into two broad categories, P_1 and P_2. In P_l, C_1 concentration was very low (2 to 4% of the total) whereas in P_2, C_1 concentration varied from 7 to 20%. The bandicoot on the other hand showed 2 Hemoglobin components in the ratio 40:60 in each of the 140 samples examined on starch gels.

The hemoglobins from representative samples from each species were subjected to chromatography on CM-cellulose as well as on DEAE-Sephadex A-50 columns. By subjecting each sample to both a cation and an anion exchanger, it was possible to get the component hemoglobins in a pure state. Figure 3 shows the chromatographic profile on DEAE -Sephadex A-50 columns.

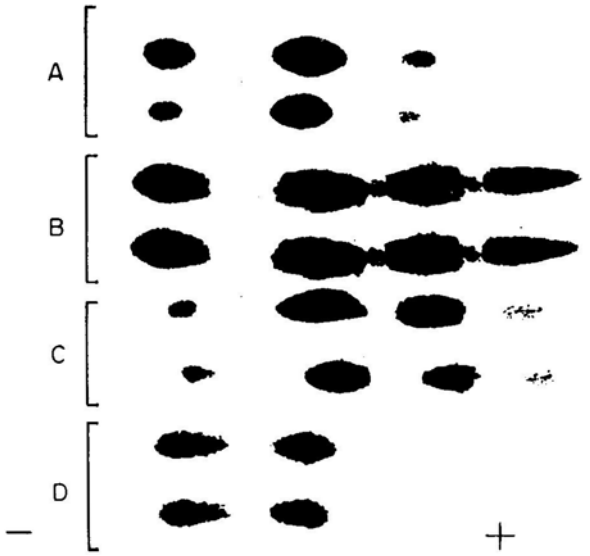

Figure 2. Starch gel electrophoretic pattern of murine hemoglobins. A. house rat (P_1); B. house rat (P_2); C. house rat (P_2); D. bandicoot
Buffer Gel: Tris-EDTA-Boric acid, pH 8.1 Trough: Boric acid–NaOH, pH 9.0

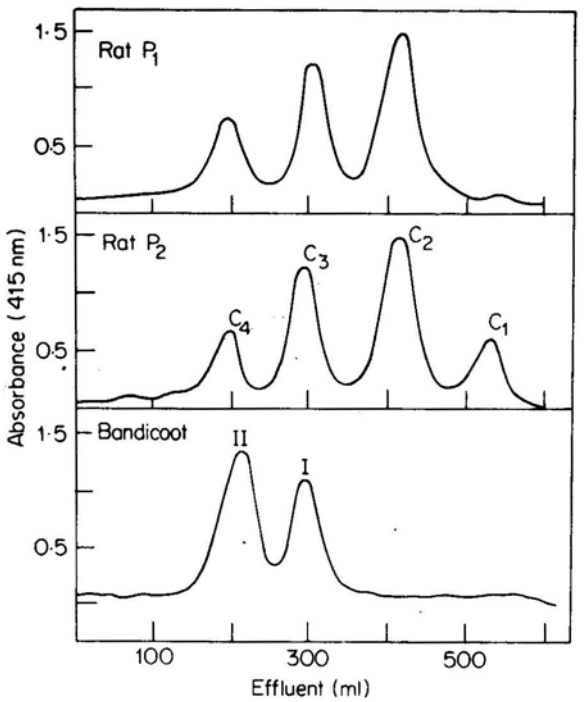

Figure 3. DEAE-Sephadex A-50 chromatographic separation of hemoglobins of house rat (P_1 and P_2) and bandicoot. A pH gradient of Tris-HCl buffers (0.05M) was used.

The mode of identification of subunits of component hemoglobins as either ∝- or -β involved isolation of the polypeptide chains by urea-CM-cellulose chromatography

The mode of identification of subunits of components hemoglobins as either ∝ or β involved isolation of the polypeptide chains by urea-CM-cellulose chromatography and subsequent location of typical tryptic peptides in the fingerprints of the amino-ethylated globins. Figure 4 presents the urea-CM-cellulose chromatographic profile

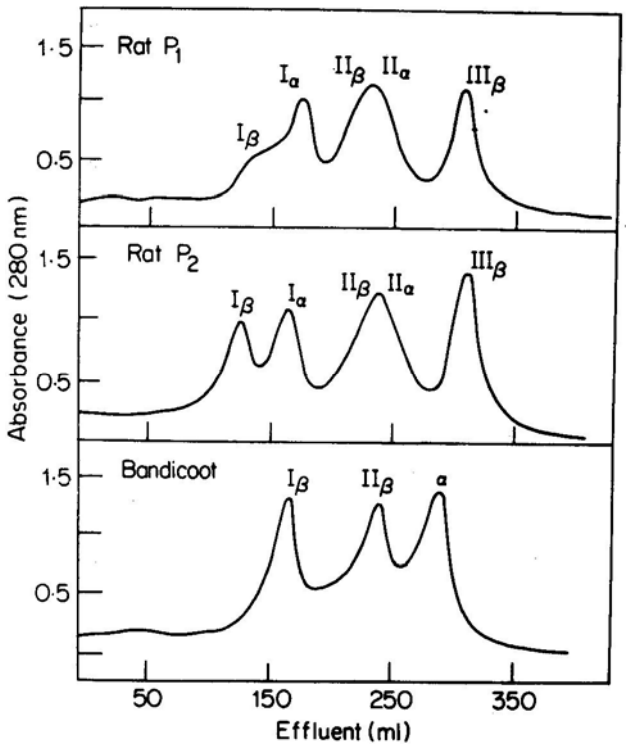

Figure 4. Chromatographic separation of polypeptide chains of globins of house rat (P_1 and P_2) and bandicoot, by urea-CM-cellulose chromatography.

and figure 5 presents the relative mobilities of different polypeptide chains on urea-polyacrylamide gel electrophoresis. Since the extreme components on chromatographs or electrophoregrams are likely to be least contaminated, we first analyzed these components. Thus C_1, from P_2, C_2 from P_1 and C_4 from either P_1 or P_2 from

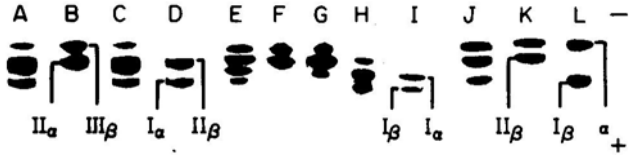

Figure 5. Separation of polypeptide chains of globins in urea-polyacrylamide gel electrophoresis. A. house rat (P_1) total globin; B. house rat (P_1) C_4 globin; C. house rat (P_1) C_3 globin; D. hause rat (P_1) C_2 globin; E. house rat (P_2) total globin; F. house rat (P_2) C_4 globin; G. house rat (P_2) C_3 globin; H. house rat (P_2) C_2 globin; I. house rat (P_2) C_1 globin; J. bandicoot total globin; K. bandicoot Hb-II globin; L. bandicoot Hb-I globin.

house rat hemoglobins were initially analyzed. The C_1 from P_2 on urea-CM-cellulose chromatography separated into two fractions which on fingerprinting could be identified as an ∝- chain and a β-chain. These chains were designated as $^{I}\alpha$ and $^{I}\beta$ chains respectively. In naming the polypeptide chains, preceeding superscripts have been used to denote more than one copy of the gene according to the nomenclature proposed by Huisman and Schroeder (1970). The C_2 from P_1 on similar treatment showed two major fractions which on structural analysis could be identified at $^{I}\alpha$ and $^{II}\beta$ chains respectively. Similarly C_4 from either P_1 or P_2 separated into two major fractions which could respectively be identified as $^{II}\alpha$ and $^{III}\beta$ chains. The relative mobilities of these polypeptide chains of urea-polyacrylamide gel at alkaline pH are shown in figure 5. Clearly $^{II}\alpha$ and $^{II}\beta$ chains have more or less the same anodic mobility, $^{I}\beta$ having highest and $^{III}\beta$ the lowest. From these observations, it is evident that C_1 represents $^{I}\alpha_2\ ^{I}\beta_2$. Similarly C_2 from P_1 has $^{I}\alpha_2\ ^{II}\beta_2$ as the major component; and C_4 represents $^{II}\alpha_2\ ^{III}\beta_2$. Based on the elctrophoretic and chromatographic mobilities of different plypeptide chains, we assumed that C_2 from P_2 would contain two molecular form viz $^{I}\alpha_2\ ^{II}\beta_2$ and $^{II}\alpha_2\ ^{I}\beta_2$. Similarly C_2 from either P_1 or P_2 was assumed to contain $^{II}\alpha_2\ ^{II}\beta_2$ and $^{I}\alpha_2\ ^{III}\beta_2$. The structural analysis of these component hemoglobins confirmed our assumption. The components C_2 from P_2 showed four polypeptide chains namely $^{I}\alpha$, $^{II}\alpha$, $^{I}\beta$ and $^{II}\beta$. Similarly C_3 showed four chains namely $^{I}\alpha$, $^{II}\alpha$, $^{II}\beta$ and $^{III}\beta$ (figure 5). On the basis of these findings, we believe that there are six molecular forms of hemoglobins in the wild population of *Rattus rattus rufescens.* These molecular forms are:

$$^{I}\alpha_2\ ^{I}\beta_2,\ ^{I}\alpha_2\ ^{II}\beta_2,\ ^{I}\alpha_2\ ^{III}\beta_2,\ ^{II}\alpha_2\ ^{I}\beta_2,\ \ ^{II}\alpha_2\ ^{II}b_2 \text{ and } ^{II}\alpha_2\ ^{III}\beta_2.$$

The two components of the bandicoot were isolated through DEAE-Sephadex A-50 (figure 3) and subjected to urea-CM-cellulose chromatography (figure 4)-and urea-polyacrylamide gel electrophoresis (figure 5). The isolated chains were mapped for their tryptic peptides. The two components were found to contain a common ∝ -chain but differeing β-chains designated as Iβ and IIβ. The molecular forms in the bandicoot are $\alpha_2\ ^{I}\beta$, and $\alpha_2\ ^{II}\beta_2$.

Mice and rats show both non-allelic and allelic hemoglobin variants. For instance, several alleles at the hemoglobin loci including the ones in which 'breeding unit alleles' contain two DNA sequences are known in the inbred strains of mice (Hilse and Popp, 1968). Starting with the observation that hemoglobins of mice showed a 'single' and a 'diffuse' band (Ranney and Gluecksohn-Waelsch, 1955) on paper electrophoresis, Gluecksohn-Waelsch and co-workers (1957) have subsequently shown that these two traits are controlled by allelic genes. Three hemoglobin alleles have been identified at the β-chain loci of the inbred strains of mice (Ranney and Gluecksohn-Waelsch, 1955; Ranney *et al*.,1960; Popp, 1962; Gilman 1972). Two of them Hbb^{d} and Hbb^{P} which give rise to the 'diffuse' trait contain a major (β^{d} major and β^{P} major respectively) and a minor hemoglobin component, while the third (Hbb^{s}) has a single

(β^s) component (Ranney *et al.,* 1960; Morton, 1962; Gilman, 1972). It has been shown that the β^d major and β^d minor chains differ extensively from each other as well as from the β^s chain (Gilman, 1972). Likewise, the β^p major and β^p minor chains also show large differences between them (Gilman, 1972). A β-gene duplication has been suggested to explain the presence of major and minor bands in the diffuse phenotype of mice (Hutton *et al.,* 1962; Gilman, 1972). Also, the presence of a recombinant gene as the product of an unequal cross-over between genes coding for β^d major and β^d minor has been suggested to account for a single hemoglobin type in a Thai mouse *Mus caroli* (Gilman, 1972). Similarly, there are atleast 5 alleles at the ∝ -chain locusin mice (Popp, 1969). Thus two strains of mice C57BL/Cum and NB/RI have single but differing ∝-chains (Popp, 1969) whereas the BALB/c strain of mouse has two ∝-chains which are due to gene duplication at the ∝ -chain locus in this strain (Hilse and Popp, 1968). Simiarly each of the two species CBA/Cum and C3H/Cum have differing sets of two ∝-chains (Popp, 1969). The hemoglobins in the laboratory rat are equally diverse. Earlier electrophoretic studies on the hemoglobins of the laboratory rat *(Rattus norvegicus)* showed several components in unequal proportions (Stein *et al.,* 1971). It is now known that there are at least six hemoglobin components in the laboratory rat, some in small amounts (Garrick and Charlton, 1969; Garrick *et al.,* 1970; Garrick *et al.,* 1974; Garrick *et al.,* 1975; Garrick *et al.,* 1978). The earlier observation of Travnicek *et al.,* (1971) that the laboratory rat contains two alpha and three beta chains has been recently confirmed by Garrick *et al.,* (1975; 1978) who have characterised these chains and sequenced some of them. The latter authors further suggest that gene duplication at the ∝-chain locus and a gene triplication at the β-chain locus are responsible for the multiplicity of hemoglobins in *Rattus norvegicus.* Our results with *Rattus rattus rufescens* likewise shows that each hemoglobin sample contains 2 types of ∝-and 3 type of β-chains suggesting a probable gene duplication at the ∝-chain locus and a gene triplication at β-chain locus. *Bandicota indica,* on the other hand, probably has a gene duplication at the β-chain locus since each of the 140 hemoglobin samples showed one ∝-chain and two β-chains.

From the foregoing consideration, it is clear that murine hemoglobins provide examples of gene iteration at the hemoglobin loci. Instances of clusters of genes producing sets of closely related globin polypeptide chains are not uncommon in eutherian mammals. In fact, in five of the mammalian orders represented in figure 1 namely *Primates, Perissodactyla, Artiodactyla, Rodentia,* and *Carnivora* gene duplications at the hemoglobin loci have been established.

The first gene cluster to be demonstrated was a pair of linked genes coding for the β-and δ-chains of humans (Ceppellini, 1959). the evolutionary origin of this duplication has now been traced to the anthropoid stem (Goodman *et al.,* 1974). However, the origin of genes coding for the embryonic zeta chains of humans and the typical eutherian ∝-chains has been traced to an ancient duplication which occurred in the basal eutherians (Barbnabas *et al.,* 1978). This observation is further supported by the fact that, in addition to that in man (Capp, 1970), similar zeta globins have also been found in mice and rabbits (Steinheider *et al.,* 1972; Melderis *et al.,* 1974). The more recent gene duplications in the primate line of descent are those at the respective ∝-(Hollan *et al.,* 1972) and γ-chain (Huisman *et al.,* 1972) gene loci of humans. The Perissodactyl branch also shows a recent gene duplication at the ∝-

chain locus in that the two ∝ chains of horse differ by a single amino acid substitution at the 60th residue position (Kilmartin and Clegg, 1967). Similarly, duplication at the ∝-chain loci of dog (Dresler *et al.,* 1974) may also be of recent evolutionary origin. The Artiodactyl branch, on the other hand, shows a series of duplications during its evolutionary history. Among these, the duplications in water buffalo- $I\propto$ -$II\propto$ (Balani and Barnabas, 1965), bison—$I\propto$ -$II\propto$ (Huisman, 1974), deer- $I\propto$-$I\propto$ (Harris *et al.,* 1972), goat— $I\propto$ -$II\propto$ (Huisman *et al.,* 1980; John and Barnabas, 1975) and barbary sheep—$I\propto$-$I\propto$ (Wilson *et al.,* 1970), appear to be relatively more than those in bovid—$^{A}\beta$ $^{F}\beta$ (Huisman, 1974) and caprine $^{A}\beta$-$^{C}\beta$ (Huisman *et al.,* 1968; Wilson *et al.,* 1970; John and Barnabas, 1978).

Our results on the peptide pattern analysis of the polypeptide chains of murine hemoglobins suggest that the two ∝ -chains of the house rat are closer to one another than either of them to the ∝ -chain of the bandicoot. Simiarly, the three β-chains of the house rat resembled each other. The β-chains of the bandicoot likewise, are closer to one another than they are to the β-chains of the house rat. These results collectively suggest that hemoglobin gene diversities in each of the two species appeared independently. These may therefore be of recent origin.

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