2. Ribose from each nucleotide has been degraded and the distribution of isotope has been determined.

3. The distribution of isotope suggests that oxidation and decarboxylation of glucose 6-phosphate is largely responsible for synthesis of ribose.

4. Other reactions that contribute to ribose synthesis probably include those catalysed by transaldolase, transketolase, aldolase and triose phosphate isomerase.

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The Time Course of the Recombination of Human Adult and Canine Haemoglobins

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The composition of the new haemoglobin species formed in a wide variety of recombination experiments by using either acid or alkaline conditions to bring about dissociation (Itano, Singer & Robinson, 1959; Vinograd, Hutchinson & Schroeder, 1959; Gammack, Huehns, Lehmann & Shooter, 1961) suggests that they have arisen by a simple exchange of α_2 - and β_2 -sub-units between the two parent haemoglobins as indicated by the equation:

Moreover, the formation, in the recombination of carbonmonoxyhaemoglobin S and methaemoglobin C (Singer & Itano, 1959) of species which behave electrophoretically as though both α -chain haem groups are in the carbonmonoxy-form and both β -chain haem groups in the met-form, and vice versa, but not of species where one α -chain (or one β -chain) carried carbonmonoxy-haem and the

other the ferrihaem group, appears to provide direct evidence in favour of this mechanism. There are, however, a number of experiments that are difficult to explain on this basis; these have been summarized by Antonini, Wyman, Bucci, Fronticelli & Rossi-Fanelli (1962).

One of them is the finding that, although the initial dissociation of each haemoglobin into half-molecules occurs rapidly, it is necessary with certain mixtures of haemoglobins, e.g. normal human adult and canine haemoglobins (Robinson & Itano, 1960) and abnormal human haemoglobins and haemoglobin A₂ (Huehns & Shooter, 1961), to keep them together at either acid or alkaline pH for a long time before maximum exchange of sub-units occurs. It could be argued that the rate of exchange is here dependent on the occurrence of a slow conformational change in one or both of the α_2 - and β_2 -sub-units of normal adult or canine

haemoglobins before they could combine in complementary pairs to produce hybrid species. However, Vinograd & Hutchinson (1960) found that little exchange of sub-units occurred when haemoglobins A and S, which had been separately kept at alkaline pH for a long period, were mixed and held at this pH for only a very short period before being rapidly neutralized. These authors suggested that the delay in exchange could be explained if it occurred with the single α - and β -chains rather than α_2 - and β_2 -dimers. They envisaged that small numbers of α - and β -chains could arise from a slow dissociation of the symmetric $\alpha\beta$ -sub-units postulated to be the main products of the initial dissociation of the ($\alpha_2\beta_2$) haemoglobin molecule. Such a mechanism has a time-dependent step in the dissociation of the $\alpha\beta$ -sub-units into single chains but also requires a high degree of specificity in the recombination of the single chains, such that only like α -chains (or β -chains) combine to form dimer sub-units, to the final exclusion of tetramer hybrids of the type $\alpha^x\alpha^y\beta_2^x$ etc.

The effects of prolonged incubation of the haemoglobin sub-units under conditions which bring about dissociation have now been reinvestigated at acid pH with the normal adult haemoglobin-canine haemoglobin system, where the rate of formation of hybrid haemoglobin is sufficiently slow to allow it to be followed in detail.

METHODS

Preparation of haemolysates. Fresh blood was obtained from a normal adult and an adult dog. The red cells from approx. 5 ml. of blood were washed five times with iso-osmotic NaCl solution, lysed by shaking with 1 vol. of water and 0.3 vol. of toluene and the clear haemoglobin solutions collected after centrifugation. The haemoglobin in the canine haemolysate and in the normal adult haemolysate after dilution with an equal volume of iso-osmotic NaCl was converted into the cyanide form of methaemoglobin by the addition, for each 100 mg. of haemoglobin in the haemolysate, of 0.02 ml. of a solution containing 0.75 g. of potassium ferriocyanide and 0.15 g. of KCN in 10 ml. of sodium phosphate buffer, pH 6.8 and *I* 0.10. The haemoglobin solutions were subsequently dialysed against two separate 1 l. volumes of a buffer containing 0.01M- NaH_2PO_4 made 0.01M with respect to KCN and adjusted at room temperature to pH 7.0 with conc. H_3PO_4 (phosphate-KCN buffer, pH 7.0). The concentrations of the two haemoglobin solutions were determined from the extinctions at 540 μm , by using the $E_{1\text{cm}}^{1\%}$ value of 6.84 (based on the millimolar extinction value of 11.0; Zijlstra & van Kampen, 1960) for both normal adult and canine haemoglobins.

Dissociation and recombination experiments. Samples of the two haemolysates were diluted to a haemoglobin concentration of 2.5% with a buffer containing 0.1M-sodium acetate made 0.01M with respect to KCN and adjusted at room temperature to pH 4.7 (acetate-KCN buffer, pH 4.7). Immediately equal volumes (usually

0.1 ml.) of each solution were mixed and dialysed at 4° with stirring against 1 l. of the acetate-KCN buffer, pH 4.7, for various times. At the end of the given period the dialysis sac was removed, rinsed with distilled water and dialysed at 4° again with stirring against 1 l. of the phosphate-KCN buffer, pH 7.0, for 24 hr. Samples of the original 2.5% solution of the two haemoglobins were also dialysed separately at 4° for 24 hr. against the acetate-KCN buffer, pH 4.7. Mixtures of equal volumes of these two acid-dialysed solutions were then made and dialysed as above, first against the acetate-KCN buffer, pH 4.7, for various periods before being neutralized by the dialysis against the phosphate-KCN buffer, pH 7.0, for 24 hr.

A control mixture of normal adult and canine haemoglobins was made as follows. Samples of the two haemolysates were diluted to a haemoglobin concentration of 2.5% with the phosphate-KCN buffer, pH 7.0, and 0.1 ml. of each of these solutions mixed and dialysed for 48 hr. against the same buffer. Similar experiments were performed with solutions of 1.0% haemoglobin concentration. No haemoglobin was precipitated during any of the above procedures and the recovery of haemoglobin was complete in each experiment.

Electrophoretic analysis. The haemoglobin mixtures were analysed in starch gel by using the discontinuous tris-citrate-borate buffer system (Poulik, 1957) as described in detail by Gammack, Huehns, Shooter & Gerald (1960). Thin (1 mm.) slices from the centre of the gel were stained with Amido Black 12B in glycerol-water-acetic acid (5:4:1, by vol.), and differentiated exhaustively in the same acid-glycerol solvent mixture (Gratzer & Beaven, 1960). The transparent gels were evaluated by transmission densitometry, followed by planimetric analysis of the densitometer records (G. H. Beaven & B. L. Stevens, unpublished work).

RESULTS

The dissociation and recombination of normal adult and canine haemoglobins produces two new hybrid species:

which are resolved from each other and the parent haemoglobins by electrophoresis at pH 8.5 (Fig. 1b). Since only the more rapidly migrating hybrid species is formed when canine haemoglobin is dissociated and recombined with haemoglobin H, i.e. β_4^A , its composition is $\alpha_2^{\text{Can}}\beta_2^A$ and that of the slower hybrid species is $\alpha_2^A\beta_2^{\text{Can}}$ (Huehns, Shooter & Beaven, 1962).

The amounts of the two hybrid species in the dissociated and recombined mixture increased with time of exposure to acid pH, reaching a maximum value after about 24 hr. (Fig. 2). These values have been corrected for the small quantities (1–2% of the total protein in the mixture) of haemoglobin A_3 and non-haem protein present in the original haemolysates. Haemoglobin A_3 migrates at approximately the same rate as the species $\alpha_2^{\text{Can}}\beta_2^A$, and one band of the non-haem protein moves with the species $\alpha_2^A\beta_2^{\text{Can}}$. The amounts of these two extraneous species were determined from the analysis of

Fig. 1. Dissociation and recombination of normal adult and canine haemoglobins. (a) Mixture of normal adult and canine haemoglobins dialysed against phosphate-KCN buffer, pH 7.0, for 48 hr. (b) Mixture as in (a) dialysed against acetate-KCN buffer, pH 4.7, for 24 hr. and then against phosphate-KCN buffer, pH 7.0, for 24 hr. (c) Normal adult and canine haemoglobins separately incubated at pH 4.7 for 24 hr., mixed and immediately dialysed against phosphate-KCN buffer, pH 7.0, for 24 hr. The mixtures were made equal with volumes of 1% normal adult and 1% canine haemoglobin. Analysis was in starch gel, with the tris-citrate-borate buffer system. The migration time was 2 hr. The picture is a direct photograph of *o*-dianisidine-stained gel.

Fig. 2. The approach to equilibrium in the dissociation and recombination of normal adult and canine haemoglobins. Mixtures of equal volumes of 1% normal adult and 1% canine haemoglobin solutions were dialysed against acetate-KCN buffer, pH 4.7, for various times and then against phosphate-KCN buffer, pH 7.0, for 24 hr. Analysis was in starch gel, with the tris-citrate-borate buffer system. ●, Haemoglobin A₂; ×, canine haemoglobin; □, 'fast' hybrid, $\alpha_2^{\text{Can}}\beta_2^{\text{A}}$; ○, 'slow' hybrid, $\alpha_2^{\text{A}}\beta_2^{\text{Can}}$.

the control mixture of the normal adult and canine haemoglobins and it was assumed that they were present in these quantities in the acid-treated mixtures.

The two hybrid species appeared in equal quantities at any given time during the approach

Fig. 3. Rate of haemoglobin formation from the original and the acid-treated normal adult and canine haemoglobins. The mixture was made of equal volumes of 2.5% haemoglobin solutions. Species $\alpha_2^{\text{Can}}\beta_2^{\text{A}}$: 'fast' hybrid from parent haemoglobins (○) and from acid-treated parent haemoglobins (●). Species $\alpha_2^{\text{A}}\beta_2^{\text{Can}}$: 'slow' hybrid from parent haemoglobins (□) and from acid-treated parent haemoglobins (■).

to the final equilibrium (Fig. 2). As the proportion of the hybrid species increased those of the parent haemoglobins fell, again by equal amounts, until half of each parent haemoglobin remained. The final equilibrium mixture contained approximately equal quantities of the four species.

The analysis of the mixture of normal adult and canine haemoglobins that had been separately exposed to acid pH for 24 hr. before being mixed and dialysed against the phosphate-cyanide buffer, pH 7.0, showed only the bands of the parent species (Fig. 1c). Moreover, the rate at which hybrid species appeared when the acid-treated parent haemoglobins were subsequently kept together at acid pH was the same as the rate of hybrid formation from the untreated parent haemoglobins (Fig. 3).

DISCUSSION

That the relatively slow rate of hybrid haemoglobin formation in the normal adult haemoglobin-canine haemoglobin system is not due to a slow rate of dissociation of canine haemoglobin into dimers is shown by the failure to observe hybrid species in the initial mixture of the acid-treated parent haemoglobins. A mixture of the parent haemoglobins kept at acid pH for the same length of time as the acid pretreatment of the separate haemoglobins showed complete exchange of sub-units. Similarly, the slow rate cannot be due to any slow change in conformation of the sub-units at acid pH. Since such changes would be complete after 24 hr. in acid solution the initial mixture of the acid-treated normal adult and canine haemoglobins should show complete exchange of sub-units without any further exposure to

acid whereas it shows none. Further, the finding that the rates of hybrid formation are the same whether from the original or the acid-treated parent haemoglobins suggests that the haemoglobin sub-units are not altered by prolonged exposure to acid pH. The similar results of Vinograd & Hutchinson (1960) indicate that this is also probably true at alkaline pH. It would appear therefore that the delay in the exchange of sub-units in this system is related in some way to the actual mechanism of the dissociation of the haemoglobin molecule. The scheme proposed by Vinograd & Hutchinson (1960) allows for a time-dependent step in the exchange of sub-units, provided that there is a high degree of specificity in the assembly of the individual chains. An experiment which demonstrates that the assembly of the haemoglobin molecule from its sub-units is a highly specific process has been reported by Antonini *et al.* (1962). These authors found that in the reconstitution of haemoglobin from haem and a mixture of human adult and horse globins, under conditions where both globins were largely in single-chain form, only human adult, i.e. $\alpha_2^A \beta_2^A$, and horse, i.e. $\alpha_2^{Eq} \beta_2^{Eq}$, haemoglobins were formed. No hybrid haemoglobin species of the type $\alpha^A \beta^{Eq} \beta_2^A$ or $\alpha_2^A \beta^A \beta^{Eq}$ appeared.

The experiments at the higher total haemoglobin concentration (Fig. 3) showed a decreased rate of hybrid formation compared with those at the lower concentration (Fig. 2). Since increasing the total protein concentration will decrease the fraction present as single α - and β -chains, it follows that this result is also compatible with the idea that it is the availability of single chains that determines the rate of exchange of sub-units and consequently the rate of formation of hybrid haemoglobins. Finally, the composition of the equilibrium mixture at the lower total haemoglobin concentration, i.e. 25% of each of the four species, suggests that there is no difference in the association constants for the formation of either the $\alpha\beta$ -sub-units or the $\alpha_2\beta_2$ -tetramer of both the parent and the hybrid species.

SUMMARY

1. The proportions of the hybrid haemoglobin species $\alpha_2^A \beta_2^{Can}$ and $\alpha_2^{Can} \beta_2^A$ formed in the dissociation and recombination of normal adult and canine

haemoglobins increase with time of exposure to acid pH up to 24 hr. at 4°. The equilibrium mixture attained after this time contains equal proportions of the two hybrid species and of the two parent haemoglobins.

2. No hybrids are rapidly formed if the two parent haemoglobins are separately exposed to acid pH for 24 hr. before being mixed. The subsequent rate of hybrid formation from the mixed acid-pretreated parent haemoglobins on further exposure to acid pH is the same as from the original parent haemoglobins.

3. These results suggest that the relatively slow rate of hybrid formation in this system is not due either to the slow dissociation of the parent haemoglobins into dimers or to a time-dependent conformational change in one or more of the sub-units. They are consistent with the mechanism proposed by Vinograd & Hutchinson (1960), which involves a symmetric dissociation followed by exchange at the single-chain level.

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