Thus an aldoheptose travelling between glucosamine and maltose in butanol-pyridine-water could be none other than D-glycero-D-galacto-heptose (or its optical enantiomorph L-glycero-L-galacto-heptose) since the missing heptoses could all be confidently expected to travel faster than this. Similarly, D-glycero-D-talo-heptose or its optical enantiomorph could be checked between different pairs of common sugars in each of the four solvent systems. By these methods a probable identification or a small number of alternatives could be deduced for any aldoheptose sugar.

Whereas the aldohexoses almost always occur in Nature in the D- form, the two aldoheptoses known thus far from natural sources are one D- and one L-. The determination of optical form can only be made on the isolated sugars or their derivatives.

SUMMARY

1. The behaviours of 12 of the 16 D-aldoheptose sugars have been recorded for four different solvent systems on paper chromatograms.

2. The positions of a number of common sugars have been determined in relation to the heptoses so that an idea of the identity of an unknown heptose may be gained without reference to authentic samples.

3. The order of separation of the eight D-aldohexoses and their homomorphous D-aldoheptoses are in tolerably good agreement.

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Heterogeneity of Normal Adult Human Haemoglobin

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Studies with three different techniques have indicated that normal adult human haemoglobin is heterogeneous. These techniques are: solubility in variable solvents (Roche, Derrien & Roques, 1953*a, b*; Roche, Derrien, Reynaud, Laurent & Roques, 1954; Roche & Derrien, 1953); electrophoresis in starch slabs (Kunkel & Wallenius, 1955); and chromatography on columns of ion-exchange resins (Huisman & Prins, 1955; Morrison & Cook, 1955).

In the variable-solvent test a system is set up containing a constant amount of protein at a

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constant pH and varying concentrations of salts. The extent of precipitation of the protein or proteins depends upon the salt concentration in the way described by the Cohn equation, $\ln S = \beta - K(\Gamma/2)$, where S is the solubility in g./l., K is the salting-out constant, $\Gamma/2$ is the ionic strength of the precipitating salt and β is the extrapolated intercept of the linear portion of the curve on the ordinate ($\log S$) axis (Cohn, 1925). Using phosphate buffer, pH 6.5, as the precipitating salt, Roche and his colleagues (Roche *et al.* 1953*a, b*, 1954) described six breaks in the salting-out curve of normal adult carboxy-haemoglobin, and concluded that it contains three adult and three small foetal components. This interpretation has been criticized by Itano (1956), on the grounds that changes in the solid phase, which are

reflected in discontinuities in the salting-out curve, might arise from alterations in the degree of aggregation of haemoglobin as a result of interaction with small molecules or ions in the solvent. This objection is partially overcome by the fractionation of three components in horse carboxyhaemoglobin (Roche *et al.* 1954). However, Ogston & Tombs (1956) have described a simple way of recognizing discontinuities due to changes in the solid phase and of distinguishing these from discontinuities due to the presence of more than one solute component. If two or more different species of protein are present in the solution, the position of the breaks will vary with the concentration of protein in solution at the start of the experiment. On the other hand, if there is a single species of solute giving different solid forms in different parts of the curve, the position of the breaks, representing transition points, will be invariant. A further complication is introduced when the solid is precipitated in a macrocrystalline form, as opposed to a microcrystalline or amorphous form; Smithies (1954) describes anomalous breaks in the precipitation curve of lactoglobulin which were the result of crystallization.

In this paper the results of experiments on the solubility of normal adult human carboxyhaemoglobin in ammonium sulphate at pH 6.8 are reported.

MATERIAL AND METHODS OF INVESTIGATION

Haemoglobin specimens were obtained from three normal adult subjects, two male and one female, as follows. About 10 ml. of blood was collected into 0.2 M-citrate buffer, pH 7.4, with Na^+ as the only cation and 200 mg. of glucose/100 ml. The erythrocytes were washed three times in a large volume of 0.85% NaCl. After the final packing of the cells, an equal volume of distilled water was added, and the mixture was frozen twice to lyse all the cells. Carbon monoxide was bubbled through the haemolysate, which was then dialysed for 24 hr. at 4° against several changes of distilled water saturated with CO. The haemolysate was centrifuged at 5000 *g* for $\frac{1}{2}$ hr. to sediment the cell stroma. The clear supernatant was decanted and the concentration of carboxyhaemoglobin measured in a Unicam SP. 600 spectrophotometer at 539 *mμ*. The aqueous solution of carboxyhaemoglobin was diluted 1 to 20, 30 and 40 (v/v) with *m*-phosphate buffer, pH 6.8 (prepared by mixing *m*-NaOH with *m*- KH_2PO_4). Then 1 ml. samples of the mixtures were pipetted into 6 ml. centrifuge tubes, followed by 4 ml. of $(\text{NH}_4)_2\text{SO}_4$ solution to give the final concentration required. All solutions had been saturated with CO. The tubes were sealed with silicone-treated rubber bungs, and, after being mixed by inversion, were allowed to stand at room temperature ($18 \pm 3^\circ$) for 24 hr. The tubes were centrifuged at 5000 *g* for 20 min. to sediment the precipitate. The protein concentration in the supernatant was determined by its absorption at 539 *mμ*.

A constant-solvent phase diagram, of the type devised by Northrop & Kunitz (1930), was also constructed. An appropriate final concentration of $(\text{NH}_4)_2\text{SO}_4$ was selected

as the solvent by inspection of the variable-solvent curve. The protein concentration was varied by carefully pipetting different amounts of the buffered haemoglobin solution used in the variable-solvent test into a series of tubes. The volume was made up to 1 ml. with buffer and 4 ml. of $(\text{NH}_4)_2\text{SO}_4$ was added, to give the same final concentration of buffer and salts in each tube. After standing at room temperature for 24 hr. the concentration of protein which remained in solution was determined as before.

RESULTS

The variable-solvent salting-out curve of normal adult human carboxyhaemoglobin at pH 6.8 in ammonium sulphate solution is shown in Fig. 1. In the curve three breaks occur, the positions of which are all dependent on the concentration of haemoglobin originally in solution. Similar results were obtained with samples from three subjects. The absorption spectra of the carboxyhaemoglobin remaining in solution and in the redissolved precipitates were indistinguishable.

Fig. 1. Precipitation curves of normal adult human carboxyhaemoglobin in aqueous ammonium sulphate, pH 6.8, 18°. Discontinuities in the curves are shown by arrows A, B, C.

Fig. 2. Variable-solute phase diagram in aqueous 4.36M ammonium sulphate, pH 6.8, 18°. Discontinuities in the plot occur at the points indicated by arrows A, B, C.

The constant-solvent phase diagram is shown in Fig. 2. Three breaks are seen, as expected of a three-component system. Precipitation was visible at haemoglobin concentrations above the first break. The precipitate was examined microscopically in some experiments at a magnification of 600 and did not appear to be crystalline.

DISCUSSION

Our results are similar to the ammonium sulphate solubility curves obtained with horse carboxy-haemoglobin by Roche *et al.* (1954). Three breaks in the precipitation curve have been found, the position of all being dependent upon the concentration of protein originally in solution. There was no evidence of crystallization, and it is concluded that normal adult human carboxyhaemoglobin comprises three molecular species.

Kunkel & Wallenius (1955) submitted normal adult human carboxyhaemoglobin to electrophoresis in starch slabs and obtained three components. The eluted components all had the same absorption spectra as the original pigment, and when they were submitted to electrophoresis separately they retained the mobility shown in the first experiment. It is therefore unlikely that these components represent denaturation products, or different forms of a single chemical substance. Morrison & Cook (1955) were also able to obtain three components from normal adult oxyhaemoglobin by chromatography on a column of Amberlite IRC-50 ion-exchange resin. The spectra of the components were found to be normal, from which it was concluded that no significant denaturation had taken place.

The discontinuities in the precipitation curve at high concentrations of phosphate reported by Roche *et al.* (1953*a, b*) and attributed by them to the presence of three small foetal haemoglobin components in the haemoglobin of normal adults,

were not observed in our experiments. It was not possible in our tests to obtain very high salt concentrations without modifying the conditions, so that we have not excluded the possibility that other breaks occur in this region. However, other results, including accurate measurements of the rate of denaturation of haemoglobin in alkali (Beaven, Ellis & White, 1956), suggest that the proportion of foetal haemoglobin in the blood of normal adults is less than 1% of the total, if any is present at all.

SUMMARY

The results of variable-solvent and variable-solute tests indicate that normal adult human carboxy-haemoglobin contains three distinct molecular species, thus confirming the heterogeneity observed by Kunkel & Wallenius by electrophoresis and by Morrison & Cook by chromatography on ion-exchange resin columns.

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Determination of Creatine in Biological Fluids

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The colour reaction between diacetyl and creatine in the presence of α -naphthol can be used for the determination of creatine (Barritt, 1936). A number of inhibitory substances occur in urine, plasma and tissue extracts which interfere with the formation of

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the coloured product (Eggleton, Elsdon & Gough, 1943). It was shown by Ennor & Stocken (1948) that sulphhydryl compounds could produce marked inhibition of the diacetyl reaction and that the interference could be prevented by the addition of *p*-chloromercuribenzoic acid. On applying their method to urine, Ennor & Stocken (1953) found that