No evidence has been found to show that the protease is a mixture of enzymes or that the protease of one plant is distinguishable from another. It has been customary to name a protease after the generic name of the plant in which it is found. This custom, both here and in future work, implies an indefinite extension in the number of proteases. It is therefore recommended that the proteases described in this paper shall be considered identical until evidence to the contrary is adduced, and that they be called leaf proteases, adding where necessary the name of the plant.

## SUMMARY

1. Protease activity is shown by both sap and fibre of the green leaves of the plants examined.

2. This activity is less than one tenth of that shown by the leaves of pineapple.

3. The activation of the enzyme by reducing agents and the pH optimum suggest that the protease is similar to the papain enzymes.

4. It is suggested that the enzyme be referred to as 'leaf protease' until evidence is obtained of the existence of more than one protease in the leaf or of differences between the leaf proteases of different plants.

5. The method of Pope & Stevens (1939) has been modified to allow smaller amounts of amino nitrogen to be estimated in the presence of reducing agents.

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# The Free Amino Groups of Haemoglobins

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Since the original work of Reichert & Brown (1909) on the crystal form of the haemoglobins from a large number of species, many workers have shown them to differ in chemical and physical properties. Among the properties studied were the CO-combining capacity (Douglas, Haldane & Haldane, 1912), oxygen dissociation (Macela & Seliškar, 1925), absorption spectra (Anson, Barcroft, Mirsky & Oinuma, 1925), amino-acid composition (Vickery & White, 1933; Block, 1934), rate of alkali denaturation (von Krüger, 1887) and electrophoretic mobility (Landsteiner, Longsworth & van der Scheer, 1938). Apart from the occasional ability of haemoglobins of closely related species such as the horse and the donkey to form mixed crystals, the property which has brought out marked similarities as well as differences is their immunological behaviour. The most extensive survey was made by Hetkoen & Boor (1931), who showed that, although the haemoglobins were species-specific, cross reactions occurred between the antibodies and antigens of allied species. The extensive work of Landsteiner (1945) and others has suggested a relationship between the antigenic behaviour of a protein and its chemical structure. As an investigation of the free α-amino-groups of a protein brings out some details of the molecular structure (Sanger, 1945), it was of interest to make a comparative study of the terminal residues of several haemoglobins, and to see if any correlation between structure and immunological behaviour could be detected.

## EXPERIMENTAL

### *Preparation of DNP-amino-acids*

The method for the preparation of 2:4-dinitrophenylamino-acids (DNP-amino-acids) using 1:2:4-fluorodinitrobenzene (FDNB) has been previously described (Sanger, 1945).

Details for the purification of the various derivatives are given below.

N-2:4-*Dinitrophenyl*-DL-*isoleucine*. The amorphous product obtained by acidifying the alkaline reaction mixture was filtered off and crystallized from methanol-water; m.p. 166°. (Found: C, 48·1; H, 4·9%. $C_{12}H_{15}O_6N_3$ requires C, 48·6; H, 5·0%.)

N-2:4-*Dinitrophenyl*-DL-*methionine*. On acidification of the reaction mixture an oil separated which was extracted into ether. The ether extract was dried over anhydrous $Na_2SO_4$ and taken to dryness leaving an oil which crystallized overnight. Yield 80% theoretical. The material was recrystallized by dissolving in dry ether and adding an equal volume of light petroleum (b.p. 80–100°). On evaporation of the ether crystalline plates were obtained, m.p. 117°. (Found: C, 41·9; H, 4·2; N, 13·3%. $C_{11}H_{13}O_6N_3S$ requires C, 41·9; H, 4·1; N, 13·3%.)

N-2:4-*Dinitrophenyl*-L-*proline*. On acidification an oil was formed which solidified overnight. Yield 85% theoretical. After recrystallization from ether-light petroleum and acetic acid-water it had m.p. 137°. (Found: C, 46·8; H, 3·9; N, 14·6%. $C_{11}H_{11}O_6N_3$ requires C, 47·0; H, 3·9; N, 14·9%.)

N-2:4-*Dinitrophenyl*-L-*tryptophan*. A gum which was obtained on acidification crystallized readily from aqueous methanol; m.p. 175° (decomp.). (Found: C, 55·5; H, 3·7%. $C_{17}H_{15}O_6N_4$ requires C, 55·0; H, 3·8%.)

NN′-*bis*(2:4-*Dinitrophenyl*)-L-*lysine*. The amorphous precipitate obtained on acidification was crystallized from formic acid with some difficulty and was recrystallized from methanol-water; m.p. 146°. (Found: C, 45·2; H, 3·73; N, 17·8%. $C_{18}H_{18}O_{10}N_6$ requires C, 45·1; H, 3·77; N, 17·6%.)

N-2:4-*Dinitrophenyl*-DL-*aspartic acid*. This compound crystallized on acidification and was recrystallized from water; m.p. 196° (decomp.). (Found: C, 40·2; H, 3·0; N, 13·9%. $C_{10}H_9O_8N_3$ requires C, 40·2; H, 3·0; N, 14·0%.)

N-2:4-*Dinitrophenyl*-DL-*serine*. The oil which separated on acidification crystallized on standing and was recrystallized from methanol; m.p. 199°. (Found: C, 39·5; H, 3·2; N, 15·4%. $C_9H_9O_7N_3$ requires C, 39·8; H, 3·3; N, 15·5%.)

N-2:4-*Dinitrophenyl*-DL-*threonine*. Crystallized on acidification and recrystallized from aqueous methanol; m.p. 152°. (Found: C, 41·7; H, 3·8%. $C_{10}H_{11}O_7N_3$ requires C, 42·1; H, 3·8%.)

NN′-*bis*(2:4-*Dinitrophenyl*)-L-*cystine*. On acidification an amorphous precipitate separated. It was crystallized from ethylene glycol monoethyl ether-water and recrystallized from acetic acid-water. Yield 80% theoretical; m.p. 109°. (Found: C, 38·2; H, 3·1; S, 11·2%. $C_{18}H_{16}O_{12}N_6S_2$ requires C, 37·8; H, 2·8; S, 11·2%.)

$N^1$-2:4-*Dinitrophenyl*-L-*arginine*. After removal of the ethanol from the reaction mixture, the residue was treated with water. *DNP-arginine* was insoluble and was filtered off and washed with ethanol and ether. It was recrystallized by solution in dilute HCl and neutralization with ammonia; yield 70%, m.p. 252° (decomp.). For analysis it was crystallized from conc. HCl as the hydrochloride; m.p. 168°. (Found: C, 37·7; H, 4·4; N, 22·5%. $C_{12}H_{16}O_6N_6$.HCl requires C, 38·3; H, 4·5; N, 22·6%.)

The DNP-derivatives of glutamic acid and hydroxyproline have not yet been obtained in crystalline form.

$N^5$-2:4-*Dinitrophenyl*-L-*lysine*. A simpler preparation of this compound similar to that described for DNP-ornithine (Sanger, 1946) has now been achieved. L-Lysine (0·5 g.) was dissolved in 10 ml. water and $CuCO_3$ added slowly to the boiling solution. The excess $CuCO_3$ was filtered from the dark blue solution and washed with 2–3 ml. water. Excess $NaHCO_3$ was added followed by a solution of 1·5 g. DNFB in 20 ml. ethanol and the mixture shaken for 2 hr. at room temperature. The yellow-green precipitate was filtered off and washed with water, ethanol and ether. It was then suspended in 5 ml. water and sufficient N-HCl added to produce a clear solution. This solution was cooled in ice, $H_2S$ bubbled through for 2 min., a trace of charcoal added and the mixture filtered immediately. The filtrate was rapidly taken to dryness *in vacuo*, crystallized from water and recrystallized from 20% HCl; yield 0·45 g.

### *Chromatographic separation of DNP-derivatives*

The methods of separation and identification of the DNP-derivatives are essentially the same as those previously described (Sanger, 1945). It should be emphasized again that the $R_F$ values recorded in that paper apply only to a certain batch of silica gel (cf. Tristram, 1946). The absolute, and to a lesser extent the relative, $R_F$ values vary considerably with different batches of gel. It appears that the DNP-amino-acids are partly held in the aqueous phase by adsorption on the silica and that the fractionation is not entirely the result of liquid-liquid partition. In general an adsorbent gel, giving low $R_F$ values, produces the maximum resolution for any given solvent, though increased 'tailing' of the bands sometimes prevents clean separation. Table 1 shows a scheme of fractionation which has been found applicable to most batches of gel used in this laboratory.

### *Stability of DNP-amino-acids to acid hydrolysis*

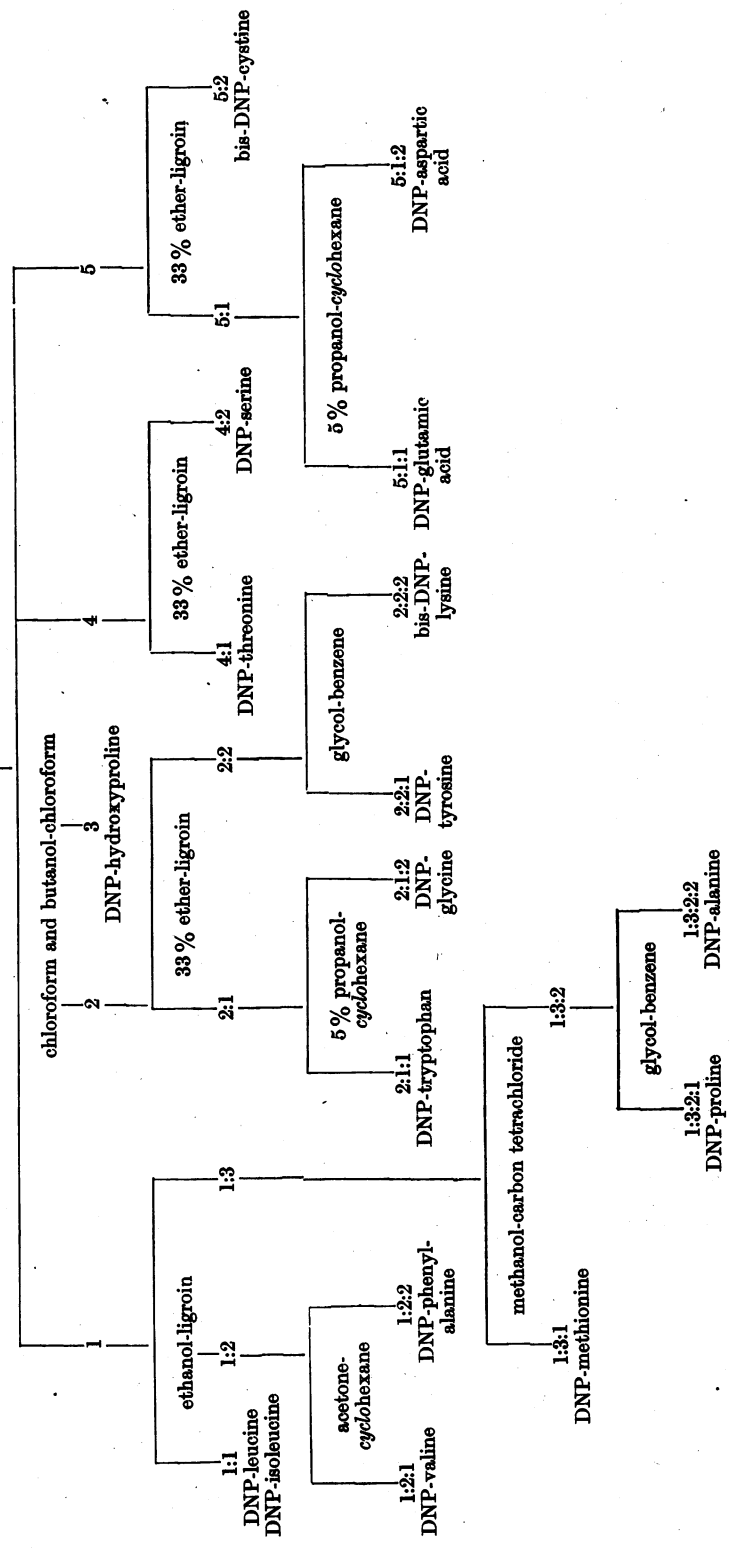
*Hydrolysis in boiling* 5·7 N-HCl. In order to ascertain whether any terminal amino-acids might be overlooked due to the instability of their DNP-derivatives during hydrolysis, a survey was made of the rates of breakdown of all the DNP-derivatives.

A standard solution was made of DNP-amino-acid in 5·7 N-HCl. A suitable portion was taken, some globin was added in case the rate of breakdown was influenced by the presence of hydrolytic products of protein, and the mixture boiled under reflux for the required time. The hydrolysate was extracted as previously described and the extract passed through appropriate columns in order to separate any breakdown products. The DNP-amino-acid was collected and the breakdown estimated by colorimetric comparison with the original solution. The results obtained are shown in Table 2.

It is clear that, with the exception of proline (Consden, Gordon, Martin & Synge, 1946), all amino-acids with a free amino or imino group could be detected using this method of hydrolysis. It was found, however, that during hydrolysis, some DNP-amino-acids gave rise to coloured breakdown products which were liable to cause confusion in the chromatographic separation. DNP-methionine and DNP-tyrosine both produced a coloured 'artefact' band stationary on a chloroform column. DNP-cystine yields a decomposition product which hardly moves on a 3% butanol-chloroform column. DNP-tryptophan on hydrolysis gave

Table 1. *Scheme of fractionation of ether-soluble DNP-amino-acids*

(Numbering refers to fractions and subfractions.)



a coloured product which remained in the aqueous solution during ether extraction and moved fast on a 66% -methyl ethyl ketone-ether column. Their behaviour on other columns distinguishes these breakdown products from the DNP-amino-acids. No other DNP-amino-acids give rise to 'artefact' bands with $R_F$ values less than 1·0 on a chloroform column, though some of them give a band moving fast which is probably 2:4-dinitroaniline.

Table 2. *Approximate breakdown of DNP-amino-acids on acid hydrolysis*

| Amino-acid derivative | Hydrolysis in boiling 5·7 N-HCl: Time of hydrolysis (hr.) | Hydrolysis in boiling 5·7 N-HCl: Amount unchanged (%) | Hydrolysis for 16 hr. in 12 N-HCl at 105° (minimal figures). Amount unchanged (%) |
|---|---|---|---|
| DNP-alanine | 12 | 80 | 75 |
| DNP-arginine | 12 | 90 | 75 |
| DNP-aspartic acid | 24 | 60 | 75 |
| bis-DNP-cystine | 12 | 25 | 0 |
| DNP-glutamic acid | 12 | 75 | 75 |
| DNP-glycine | 8 | 40 | 50 |
| DNP-hydroxyproline | 4 | 40 | 50 |
| DNP-isoleucine | 12 | 80 | 75 |
| DNP-leucine | 12 | 80 | 75 |
| bis-DNP-lysine | 8 | 95 | 75 |
| ε-DNP-lysine | 12 | 95 | 75 |
| DNP-methionine | 12 | 75 | 75 |
| DNP-phenylalanine | 12 | 70 | 50 |
| DNP-proline | 2 | 10 | 50 |
| DNP-serine | 12 | 90 | 75 |
| DNP-threonine | 24 | 90 | 75 |
| DNP-tryptophan | 12 | 90 | 0 |
| DNP-tyrosine | 12 | 75 | 50 |
| DNP-valine | 12 | 80 | 75 |

*Hydrolysis in approx.* 12 N-HCl *at* 100°. Many conditions of hydrolysis were investigated in order to find a method suitable for the estimation of DNP-proline. It was found that if DNP-proline and globin were heated in a sealed glass tube with 12 N-HCl at 105° for 16 hr., less than 50% decomposition was produced. The stability of DNP-hydroxyproline at the higher acidity was greater than in boiling 5·7 N-HCl, but with a number of other DNP-amino-acids (e.g. tryptophan and cystine) the decomposition was greatly increased. Earlier observations had suggested that proline might be a terminal amino-acid in salmine, and hence this protein was used to test the efficiency of the method of hydrolysis. Using 12 N-HCl in a sealed tube we were able to confirm that proline is in fact the only terminal amino-acid of salmine. This was expected from the inability of salmine to yield nitrogen in the Van Slyke apparatus. The great lability of DNP-proline had not been observed during the earlier experiments carried out on insulin (Sanger, 1945) and gramicidin S (Sanger, 1946). Using the above method of hydrolysis we have been able to confirm that in neither is proline a terminal residue.

### *Reaction of FDNB with valine*

In order to test whether the reaction with valine is complete in 2 hr., two samples of DL-valine were allowed to react with FDNB for 2 and 16 hr. respectively, the DNP-valine being estimated after purification on a chloroform column. The results obtained are shown in Table 3.

Table 3. *Reaction of FDNB with valine*

| Time of reaction (hr.) | Yield (mg. α-amino N) |
|---|---|
| 2 | 0·22 |
| 16 | 0·24 |
| Calculated | 0·225 |

The discrepancy is close to the experimental error and is not considered significant. In order to ensure that the time necessary for the reaction was not increased when the amino-acid was in combination in a protein, the effect of increasing the time of reaction of FDNB with haemoglobin from 2 to 24 hr. was investigated. Again the quantitative results showed no significant difference.

### *Preparation of the DNP-globins*

Horse haemoglobin was prepared by the method of Adair & Adair (1934). A similar method was used for goat and donkey haemoglobins, but crystallization was induced by dialysis against ammonium sulphate solution of suitable concentration. The cow, sheep and human haemoglobins were prepared by the method of Boor & Hetkoen (1930), human foetal haemoglobin from the blood of a 30-week foetus by the method of Haurowitz (1935), and horse myoglobin by the method of Theorell (1932).

The DNP-globins were prepared as described for DNP-insulin (Sanger, 1945), but two types of preparation were made. In the first the haem was removed (Anson & Mirsky, 1934) before the reaction with FDNB, and in the second afterwards. This was done in order to determine if the coupling of the haem with the globin blocked any of the free amino groups. The amount of globin in the air-dried DNP-globins was calculated from the amide content as previously described. The values are shown in Table 4.

Table 4. *Globin content of air-dried DNP-globins*

| DNP-globin | Globin (%) |
|---|---|
| Cow | 72 |
| Donkey | 75 |
| Goat | 75 |
| Horse | 76 |
| Human adult | 77 |
| Human foetal | 80 |
| Sheep | 70 |
| Horse myoglobin | 70 |

### *Measurement of the DNP-amino-acids*

The DNP-amino-acids were measured colorimetrically with a photoelectric absorptiometer after solution in N-HCl or 1% (w/v) $NaHCO_3$. Contrary to a previous statement it was found that the colorimetric curves obtained with the DNP-derivatives of the L- and DL-amino-acids studied were identical. The losses of DNP-valine and DNP-methionine during hydrolysis in the presence of globin are shown in Table 5.

Table 5. *Breakdown of DNP-amino-acids on acid hydrolysis*

| Method of hydrolysis | DNP-amino-acid | Time of hydrolysis (hr.) | Amount unchanged (%) |
|---|---|---|---|
| Boiling 5·7 N-HCl | DNP-valine | 16 | 75 |
| | | 24 | 64 |
| | DNP-methionine | 8 | 85 |
| | | 24 | 46 |
| 12 N-HCl at 105° | DNP-valine | 16 | 90 |
| | | 40 | 75 |

As the factor which is used to correct for breakdown of DNP-amino-acids during hydrolysis is probably the principal source of error, the time of hydrolysis was always reduced until peptides were detected. In the globins about 10% of DNP-valine was still in peptide form after 16 hr. hydrolysis with 5·7 N-HCl whilst only a trace of a methionyl peptide was detected after 8 hr. hydrolysis. DNP-valyl peptides could just be detected after 40 hr. hydrolysis in 12 N-HCl at 100°. It is interesting to note that the intensity of the colour of DNP-valine and DNP-glycine is considerably reduced when they are in peptide form. Hence, peptides, if present, were separated on the columns, hydrolyzed further and estimated separately.

## RESULTS

### *Identification of the terminal residues in haemoglobins*

In human adult, human foetal, horse and donkey haemoglobins only one terminal amino-acid was found. By passing the DNP-derivative obtained from the hydrolysate through a number of different columns and comparing the $R_F$ values with that of synthetic DNP-amino-acids, the terminal residue was found to be valine. This was confirmed by showing that the DNP-derivative could be separated on the appropriate column from all other DNP-amino-acids except DNP-valine. In a similar manner it was demonstrated that cow, sheep and goat haemoglobins contain two terminal residues which were identified as valine and methionine. Glycine was found to be the only terminal amino-acid in horse myoglobin.

### *Estimation of the terminal residues in haemoglobins*

The results obtained are listed in Table 6. With the exception of the human foetal haemoglobin the figures for the numbers of the free amino groups

Table 6. *The numbers of free amino groups of haemoglobins.*

| Globin | Conditions of hydrolysis | No. of free α-amino groups/mol. of mol. wt. 64,000: Valine | No. of free α-amino groups/mol. of mol. wt. 64,000: Methionine | No. of free lysine ε-$NH_2$ groups/mol. wt. of 64,000 |
|---|---|---|---|---|
| Horse | 24 hr. boiling 5·7 N-HCl | 6·3, 6·1, 5·8 | Nil | 42, 41, 40 |
| | 16 hr. boiling 5·7 N-HCl | 5·9 | — | — |
| | 40 hr. 100° 12 N-HCl | 6·0 | — | — |
| | Mean | 6·0 | Nil | 41 |
| Donkey | 16 hr. boiling 5·7 N-HCl | 5·6, 6·0 | Nil | 40, 42 |
| | Mean | 5·8 | Nil | 41 |
| Cow | 24 hr. boiling 5·7 N-HCl | 2·2, 2·0 | 1·9, 2·3 | 46, 48 |
| | Mean | 2·1 | 2·1 | 47 |
| Goat | 16 hr. boiling 5·7 N-HCl | 2·4 | 1·8 | 47, 49 |
| | 8 hr. boiling 5·7 N-HCl | 2·3, 2·0 | 1·7, 1·8 | — |
| | Mean | 2·3 | 1·8 | 48 |
| Sheep | 24 hr. boiling 5·7 N-HCl | 2·1, 2·1, 2·0 | 2·0, 1·9, 1·9 | 46, 48, 48 |
| | 8 hr. boiling 5·7 N-HCl | 2·1 | 2·2 | — |
| | 40 hr. 100° 12 N-HCl | 2·3 | — | — |
| | Mean | 2·1 | 2·0 | 47 |
| Human adult | 24 hr. boiling 5·7 N-HCl | 5·1, 4·8, 5·3 | Nil | 42, 43, 45 |
| | 16 hr. boiling 5·7 N-HCl | 4·8 | — | — |
| | 40 hr. 100° 12 N-HCl | 5·2 | — | — |
| | Mean | 5·1 | — | 43 |
| Human foetal | 24 hr. boiling 5·7 N-HCl | 2·5, 2·6, 2·7 | Nil | 47, 45, 50 |
| | Mean | 2·6 | — | 47 |
| | | Glycine/mol. wt. 17,000 | | Lysine/mol. wt. 17,000 |
| Horse myoglobin | 4 hr. boiling 5·7 N-HCl | 1·2, 1·2, 1·3 | | 17, 21, 24 |
| | 2 hr. boiling 5·7 N-HCl | 0·9, 1·1 | | 20 |
| | 8 hr. 100° 12 N-HCl | 0·9 | | — |
| | Mean | 1·1 | | 20 |

Table 7. *Summary of results*

| Haemoglobin | Assumed mol. wt. | Terminal residues | Free lysine ε-amino groups |
|---|---|---|---|
| Horse | 66,000 | 6 valine | 41 |
| Donkey | 66,000 | 6 valine | 41 |
| Human adult | 66,000 | 5 valine | 43 |
| Cow | 66,000 | 2 valine, 2 methionine | 47 |
| Sheep | 66,000 | 2 valine, 2 methionine | 47 |
| Goat | 66,000 | 2 valine, 2 methionine | 48 |
| Human foetal | 66,000 | 2·6 valine | 47 |
| Horse myoglobin | 17,000 | 1 glycine | 20 |

have been approximated to the nearest whole number and are summarized in Table 7.

No accurate figure is yet available for the molecular weight of human foetal haemoglobin. The sedimentation constant (Andersch, Wilson & Menten, 1944) suggests a molecular weight about half that of the adult haemoglobin, but as the diffusion constant was not measured the true value could not be calculated. The results obtained would suggest one terminal residue/molecule of mol.wt. 25,000 or two/molecule of mol.wt. 50,000.

The accuracy of the figures obtained is of particular importance in assessing the significance of the difference between the six terminal valyl residues found in horse haemoglobin and the five found in human haemoglobin. When a breakdown correction of 25% is used there must be doubt as to the importance of a difference of only 15–20%; however, the consistency of the figures obtained under a variety of conditions of hydrolysis and the absence of any overlapping justifies, it is felt, the conclusion that there is a structural difference between the two proteins.

## DISCUSSION

The significance of the results will be discussed, first, as to the light they can throw on the molecular structure of the haemoglobins, and secondly, as to the correlation that can be found between the structural pattern and the immunological specificity of the molecule.

*Structure.* The number of free α-amino groups gives the number of open peptide chains present in the protein molecule. Possibly there also exist in the molecule cyclic chains or chains branching off the ω-carboxyl groups of glutamic or aspartic acid. Such chains would contain no free α-amino groups. The presence of cyclic chains has been demonstrated in gramicidin S (Sanger, 1946) and tyrocidine (Christensen, 1945) and thus may occur in proteins. However, in the absence of further evidence, the simplified assumption will be made in the following discussion that the number of open peptide chains is equal to the total number of chains in the molecule. Even if this is not true, it is likely that the number of open chains has a similar significance in relation to structure and immunological specificity.

The marked structural difference between the human adult and foetal haemoglobins was to be expected in view of the other differences that have been observed, e.g. rate of alkali denaturation (Brinkman & Tonxis, 1935), immunological specificity (Darrow, Nowakovsky & Austin, 1940), electrophoretic mobility and sedimentation rate (Andersch *et al.* 1944). It would seem clear that the difference in the two molecules arises from dissimilarity of chemical structure, and not from a physical association or dissociation of the same chemical units.

It is also apparent from lysine content, terminal amino-acid residue, molecular weight and other properties that myoglobin is chemically quite distinct from haemoglobin of the same species.

Of the normal haemoglobins, those from the cow, sheep and goat have four chains/molecule, two with a terminal valyl residue and two with a terminal methionyl residue. Roche, Michel & Schiller (1944) claim to have isolated the phenyl hydantoin of phenylalanine from a hydrolysate of sheep globin which had been treated with phenyl *iso*cyanate, but we could find no evidence of a terminal phenylalanyl residue. The human haemoglobin has five chains, each with a terminal valyl residue, and the horse and donkey haemoglobins six chains with terminal valyl residues. Boyes-Watson & Perutz (1943), from an X-ray study of a single crystal of horse haemoglobin, deduced that the molecule probably consisted 'of four equal and parallel layers of scattering matter'. This need not conflict with our results as the lengths of the six chains are not known, and it is possible that they could be grouped or folded in such a manner as to give four such equal layers.

The results given in Table 6 were obtained on preparations of DNP-globin from which the haem had been removed either before or after the reaction with FDNB. No difference was found in the numbers of the terminal amino-acids, thus proving that the haem is not linked to the globin through an α-amino group. The accuracy of the method is such that a difference of one or two in the number of free lysine ε-amino groups could not be detected with certainty. The possibility of the linkage of the haem through this group must therefore remain.

The lysine contents of three of the haemoglobins used have been determined by Dr Macpherson in this laboratory and are given in Table 8. Comparison of the number of free $\epsilon$-amino groups as measured by this method and the lysine contents shows good agreement. It is therefore probable that all these groups are free in these proteins, and that no branching or cross linking of the peptide chains occurs through them.

Table 8. *Lysine contents of haemoglobins*

| Haemoglobin | Mol. wt. | Number of lysine residues/mol. | Number of free lysine $\epsilon$-amino groups/mol. |
|---|---|---|---|
| Horse | 66,000 | 39 | 40 |
| Sheep | 66,000 | 45 | 47 |
| Horse myoglobin | 17,000 | 19 | 20 |

*Relation of molecular structure to immunological specificity.* An extensive study of the immunological behaviour of the haemoglobins has been made by Hetkoen & Schulhof (1923), Hetkoen & Boor (1931) and Heidelberger & Landsteiner (1923). The results show that in their serological properties the haemoglobins are similar to the other pure proteins that have been examined, and the conclusions may be summarized as follows: (1) Proteins having the same function in genealogically related animals cross-react with each other. (2) The serological differences between such proteins increase with the distance apart in the zoological scale, but some similarity persists even in proteins from distantly related animals. (3) The specificity of a protein is altered by certain types of denaturation, but some species specificity remains.

Our results are in parallel with the first conclusion; the haemoglobins from the horse and donkey, which cross react strongly, both have the same terminal residues and number of open chains. Those from the cow, sheep and goat fall into another group, both by end-group assay and immunological behaviour, whereas the human adult and foetal haemoglobins are distinct from each other and from the first two groups in both respects. With regard to the second conclusion it is interesting to note that all the haemoglobins examined have at least one chain with a terminal valyl residue, but myoglobin, shown by Hetkoen (1928) to be immunologically distinct from haemoglobin in the dog, has one chain with a terminal glycyl residue. It should be noted that its molecular weight and amino-acid composition are also markedly different.

Landsteiner's study of azo proteins originated the idea of small determinant groups which he designated haptenes. According to this concept certain polar groups in the native protein molecule would function as haptenes and the specificity of the molecule would be the result of their net action. The effect of denaturation by heat, dilute acid or dilute alkali (Macpherson & Heidelberger, 1945), contrary to denaturation by urea or guanidine (Erickson & Neurath, 1943), produces a drastic alteration in the specificity of the protein. This presumably implies that the configuration or folding of the peptide chains within the molecule plays an important part in determining the specificity. This apparently conflicting evidence can best be reconciled by assuming that the haptene-functioning parts of the molecule are influenced by the configuration of the molecule. The determinant groups may arise by the folding of the chain to give a specific array of polar side-chain residues which in the unfolded chain are distantly spaced. Such an array would be dependent on the configuration of the molecule. The disordering of the polar groups, as might occur in certain types of denaturation, would disrupt the specificity-determining parts of the molecule and would lead to a marked change in immunological behaviour. In such a completely denatured protein, where the configuration is believed to be random, the specificity would then depend only on the relative numbers of the different amino-acids and their order along the peptide chain. The haemoglobins of allied species have been shown to have a similar number of open peptide chains and probably have a similar amino-acid composition. It would thus be expected that denaturation would increase the cross reactions of the haemoglobins from related species whilst the distinction between haemoglobins with different numbers of chains/molecule would be maintained. Evidence on this point could not be found in the literature and it is hoped to investigate this further.

Our results suggest a relationship between the terminal residues and immunological specificity. That the free amino groups are part of the determinant groups seems unlikely from the work with toxins and toxoids (Pappenheimer, 1938), and it seems probable that the number of chains in the molecule plays a more predominant role in the immunological behaviour, and this suggests the importance of gross structure in addition to amino-acid composition in determining the specificity of a protein. Our results bring out chemical similarities as well as differences between the haemoglobins, a point not previously shown. The immunological differences that exist between closely allied species can no doubt be accounted for by slight variations in configuration. The results are therefore in agreement with the suggestion as to the basis of the specificity of native proteins outlined above, but much more evidence will be required before more definite conclusions can be drawn.

## SUMMARY

1. Several DNP-amino-acids have been prepared and characterized.

2. Several modifications of the method of measuring the free amino groups of proteins with 1:2:4-fluorodinitrobenzene are described.

3. The method has been applied to several haemoglobins. Horse and donkey haemoglobins contain six terminal valyl residues/molecule, adult human haemoglobin five terminal valyl residues, and cow, sheep and goat haemoglobins two terminal valyl and two terminal methionyl residues. If a molecular weight of 66,000 is assumed, human foetal haemoglobin contains 2·6 terminal valyl residues. Horse myoglobin has one terminal glycyl residue/molecule of molecular weight 17,000. The $\epsilon$-amino groups of the lysine residues are free in the above proteins.

4. The significance of these results in relation to the structure of the haemoglobins and the correlation between structure and immunological specificity of proteins is discussed.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# Metabolism of Acetoacetate in Animal Tissues. 2

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The first paper on this subject (Krebs & Eggleston, 1945) was concerned with the anaerobic reactions of acetoacetate. It was shown that in isolated sheep heart two rapid reactions of acetoacetate occur:

acetoacetate + L-malate
= L-$\beta$-hydroxybutyrate + oxaloacetate,
acetoacetate + $\alpha$-ketoglutarate
= L-$\beta$-hydroxybutyrate + succinate + $CO_2$.

These two reactions account for the *anaerobic* metabolism of acetoacetate in sheep heart muscle: all the acetoacetate removed was recovered, within the limits of error, as $\beta$-hydroxybutyrate. The present paper deals with the *aerobic* reactions of acetoacetate. Whilst this work was in progress some of its problems were solved by Buchanan, Sakami, Gurin & Wilson (1945) with the help of isotopic carbon. These workers showed beyond doubt that the carbon