The Amino Acid Composition of Hemoglobin. II. Analytical Technics

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The amino acid composition of human hemoglobin has been the subject of sustained interest over the course of many years. Such studies have taken on increasing significance since the classical investigations of Ingram who discovered that only one of the 300 odd amino acids constituting the hemoglobin half molecule was different in Hgb S when contrasted to Hgb A.1,2 With the impetus provided by this fascinating observation, many other types of hemoglobin have come under scrutiny in order to determine the nature and site of the amino acid abnormality resulting from the genetic aberration which exists.

Although the "fingerprinting" technic of Ingram1,3 has been the approach usually employed in these investigations, various groups have utilized other methods of study, including variations in the basic fingerprint technic4-6 and separation of the tryptic peptides by column chromatography.8-9,10 Since our laboratory has been interested in the genetic biochemistry of the human hemoglobins, it seemed worthwhile to review in some detail the technics currently in use by us, particularly as they differ in several significant respects from those employed by Ingram.

**METHODS**

**Hemoglobin preparation.** Hemoglobin solutions were prepared from venous blood collected in any of the several types of anticoagulant by the usual method of washing the erythrocytes with normal saline and lysis with toluene and water as described previously.11 Except when the alkali denaturation procedure12 was to be used to isolate Hgb F, all hemoglobin solutions were converted to the CO compound prior to further manipulation. Crystallization of the hemoglobin was rarely carried out, but fractionation against (NH₄)₂SO₄ solutions was frequently employed as an initial purification step. Cold (4 C.) saturated (NH₄)₂SO₄ was diluted with cold distilled water to concentrations of 62 per cent and 70 per cent. Dialysis was carried out in a cold room using the ratio of at least ten volumes of dialysis fluid to one volume of hemoglobin solution. Following dialysis against two changes of 62 per cent sulfate, the small amount of precipitate was discarded and dialysis continued against 70 per cent sulfate at which time most of the hemoglobin precipitated as an amorphous mass. The hemoglobin precipitate was washed with 70 per cent sulfate, dissolved in water, retreated with carbon monoxide and dialyzed free of sulfate. The purified hemoglobin was then prepared as a powder by lyophilization.

Starch block electrophoresis by the method of Kunkle and co-workers13 was employed to separate hemoglobin mixtures when one or more of the hemoglobin fractions was required for study. As much as 1.5 to 3 ml. of a CO hemoglobin solution could be separated...
on each block of dimensions 25 x 35 x 0.7 cm. The isolated fractions were eluted from the block with distilled water, dialyzed free of buffer, retreated with CO and lyophilized.

Hgb F was purified by the modified alkali denaturation technic previously described. Following denaturation, the fetal hemoglobin was also converted to the CO compound before dialysis and lyophilization.

Globin preparation. Globin was prepared by the method of Anson and Mirsky as previously described. An attempt was made to separate the heme by the methylethylketone method of Teale but, in addition to the more laborious procedure involved, subsequent attempts to separate the material into α and β chains proved fruitless and this approach was abandoned.

Alpha and beta chain separation. Alpha and β chain separations were carried out by a modification of the technic of Wilson and Smith as described in the first publication in this series. In brief, globin separates into its component polypeptide chains in an environment of low pH and ionic strength. Separation is carried out on Amberlite CG-50, Type 2, resin in its acid phase by elution from the column with a urea solution of pH 1.9 and varying in molarity from 2 to 8. By employing an interrupted gradient, almost total separation of the α and β chains of hemoglobin is possible while somewhat less distinct separation of the α and γ chains of Hgb F occurs. The effluent was continuously monitored by a UV detector recording system (280 mÅ) (fig. 1). The fractions containing material of interest were dialyzed repeatedly against distilled water at 4 C. until shown to be free of urea by the diacetyl monoxime method. Lyophilization by freeze drying was then carried out.

Tryptic digestion. Preparations of hemoglobin, globin or the individual peptide chains were subjected to identical procedures for obtaining the tryptic peptides. Inasmuch as trypsin is incapable of exerting its digestive action on native proteins, denaturation must be carried out as a preliminary step. Both heat and alkali denaturation procedures have

*Gilson Medical Electronics Company, Middleton, Wis.
been employed with comparable results. Since the former is simpler, we have confined our activities to this procedure except in special instances. From 50 to 100 mg. of the protein are dissolved or suspended in water at a concentration of 10 mg. per ml. of water. The material is heated at 96 to 99 C. for 4 to 6 minutes with shaking, cooled to room temperature under tap water and one half volume of 0.6 M \((\text{NH}_4)_2\text{CO}_3\) added to give a final solution of 0.2 M carbonate. Trypsin (Worthington, 2x crystallized, salt free) prepared as a 2 mg. per ml. solution in \(10^{-3}\) N HCl, is added in sufficient quantity to yield a protein to enzyme ratio of 50:1. The pH of the reaction mixture is adjusted to 8.2 to 8.4 with 3N HCl and a drop of 0.1 per cent phenol red (in 20 per cent EtOH) added as an inert indicator for use in subsequent steps. Digestion is permitted to proceed for three hours at 32 C., with occasional shaking of the reactants. Dissolution of the precipitate takes place in most instances within a period of 30 minutes although some preparations may go into solution completely within a matter of minutes. Enzymatic digestion is probably complete in 90 minutes or less at this temperature. We have carried out studies which indicate no advantage to longer periods of digestion (up to 24 hours) or higher temperatures (up to 38 C.). Comparable "fingerprints" have resulted provided a minimum period of two hours is permitted for digestion at any temperature between 24 and 37 C. No attempt was made to follow the liberation of peptide bonds as a result of tryptic digestion by determining the quantity of sodium hydroxide utilized under conditions of a pH stat experiment. Such studies have been carried out by Ingram who found a period of 90 minutes at room temperature sufficient for the liberation of essentially all the sites at which the enzyme acts. Following digestion, the mixture is freeze dried to remove the volatile \((\text{NH}_4)_2\text{CO}_3\) buffer and to concentrate the resulting peptides.

"Fingerprinting" techniques. On the basis of the known starting weight of the hemoglobin or globin and an assumed tryptic digestion of 80 per cent of the molecule (approximately 20 per cent of the hemoglobin or globin remains immune to the action of trypsin and constitutes the "resistant core")\(^{19}\), sufficient de-ionized water is added to yield a concentration of 1 mg. of tryptic peptides per 30 to 50 lambda of solution. Adequate mixing must be carefully carried out to ensure maximal solution of the peptides. The insoluble core is removed by centrifugation and the clear yellow (phenol red) supernatant is utilized for chromatographic purposes.

From 40 to 60 \(\lambda\) (approximately 1 to 1\(\frac{1}{2}\) mg.) of solution is applied to one corner of an 18 \(\frac{3}{4}\) by 22 \(\frac{1}{4}\) inch sheet of Whatman No. 3 MM filter paper in 10 \(\lambda\) aliquots. The spots are dried by a stream of cold air between applications to keep the area of the spot in the range of 1 to 1\(\frac{1}{2}\) cm. in diameter. Descending chromatography in n-butanol:acetic acid:H\(_2\)O (4:1:5) is carried out in standard chromatography cabinets for 22 hours. Fresh solvent is prepared daily by mixing the ingredients in a separatory funnel, discarding the aqueous phase after a suitable period of settling (about two hours) and filtering the solvent free of any droplets of water which may remain. The spotted peptide papers are permitted to equilibrate from three to five hours in the cabinets in an atmosphere saturated with the solvent itself or its expendable aqueous phase. Following descending chromatography, the papers are dried in an oven at 90 C. for approximately 30 minutes.

Electrophoresis. Electrophoresis is carried out in large plastic tanks as described by Katz, Dreyer, and Anfinsen\(^{20}\) (fig. 2). These tanks contain unequal volumes of buffer on each side of the center baffle, while the remainder is filled with an inert organic solvent, Varsol,\(^*\) into which is immersed a large stainless steel cooling coil. Nichrome-platinum electrodes dip into the respective buffer areas. The dried papers are moistened with the electrophoretic buffer (pyridine, 10 ml.; glacial acetic acid 100 ml.; H\(_2\)O, 2890 ml.; pH 3.7) in such a fashion that the peptides are washed into a sharp line by the advancing buffer fronts. A convenient method is pictured in figures 3 and 4. A small quantity of buffer fills the shallow pan into which is placed a plastic rack containing three movable glass rods. The paper is draped over the center rod so that the original point of the peptide application and the phenol red indicator spot are directly over this rod. The remaining two rods hold the

\*Standard Oil Company of New Jersey.
Fig. 2.—Electrophoretic tanks utilized for high voltage electrophoresis. Visible are the stainless steel cooling coils, unequal layers of buffer, the overlying layer of Varsol and the plastic frames holding the peptide papers.

paper in the solution. As the buffer fronts advance along a sharp line, the peptides are washed into a narrow zone which facilitates subsequent separation. The moistened paper is blotted lightly and draped over a plastic rack so as to permit the ends of the paper to make contact with the buffer while the chromatographed line of peptides is kept out of the buffer (fig. 3). Electrophoresis is carried out at 2000 volts and approximately 90 milliamps per paper for 60 minutes in an "uphill" fashion in which the shallow buffer section

Fig. 3.—Schematic diagram of method of “washing” chromatographed peptides into a sharp line.
Fig. 4.—Method of “washing” peptides into a sharp line in actual use.

serves as anode, the deep buffer section, as cathode. Following electrophoresis, the papers are dried in an oven at approximately 90°C for 30 minutes.

Reverse phase procedure. At times, it has been advantageous to run the electrophoretic separation first, followed by descending chromatography. We have found that several of the trypic peptides of hemoglobin are more cleanly separated by the reverse phase procedure, although the separations obtained for the majority of the peptides are inferior to those found in the procedure described above. Technical details are identical with those already described except for the change in order of manipulation.

Peptide elution. Glass distilled, constant boiling HCl (essentially 6 N) is used to elute the peptides identified on the “fingerprints”. The peptide papers are lightly stained with 0.025 per cent ninhydrin in absolute ethanol (see staining technics below), the areas of interest removed from the paper in such a manner as to have one end of the peptide “cut out” pointed and the other end squared off. Elution is carried out in a plastic chamber containing an inclined chromatography trough partially filled with the 6 N HCl (fig. 6). The cut out peptides are carefully handled with teflon covered tweezers, rinsed lightly in reagent acetone to remove excess ninhydrin and carefully placed between two acid cleaned glass microscope slides cut in half, the squared off end being caught between the slides which act as a siphon for the HCl. Ten by 75 mm. test tubes are set beneath the freely hanging pointed tips so that the paper makes contact with the tube at a single point inside the rim. Elution of the peptide occurs within 15 to 20 minutes when carried out in the covered chamber, approximately four drops being sufficient to remove the entire peptide content of the paper. The elution tubes are sealed in a high temperature flame and acid hydrolysis carried out for 20 hours in an oven at 108°C.

Amino acid analysis. The contents of the opened hydrolysis tubes are evaporated to dryness over sodium hydroxide in vacuo, diluted with a small drop of triple distilled water and, after thorough mixing, applied in their entirety to sheets of Whatman No. 1 filter paper measuring 18¼ inches by 22½ inches. Two samples may be applied to one sheet
Fig. 5.—Plastic frames for holding papers during high voltage electrophoresis.

of paper by proper orientation about a center line. Extreme care is taken to confine the material being placed in the paper to an area no greater than five mm. in diameter by applying one to two λ at a time and drying the spots between applications by heated air. Descending chromatography is carried out in standard cabinets using the same butanol, acetic acid, water solvent system described above, except that the solvent need not be made up the very same day. Equilibration is permitted to take place for about one hour, following which the solvent is added and descending chromatography along the short dimension of the paper carried out for 18 hours. The papers are air dried or heated to 90 C. for 30 minutes, cut along the center line and subjected to ascending chromatography in covered jars. The solvent system consists of 80 per cent pyridine: 20 per cent water and chromatography is carried out for three hours at room temperature. The air dried papers may then be stained for amino acid identification.

Staining technics.21 A. Ninhydrin stain for peptides: 0.5 per cent ninhydrin in absolute ethanol. Moderately stable at room temperature, this preparation is usually used as a dip. The wet papers are heated to 90 C. for 10 minutes for full color development.

B. Ninhydrin stain for elution of peptides: 0.025 per cent ninhydrin in absolute ethanol. We have applied this stain mainly as a spray. The sprayed papers are heated to 90 C. for 10 minutes for full color development.

C. Ninhydrin-collidine stain for peptides: 0.5 Gm. ninhydrin dissolved in 300 ml. absolute EtOH to which are added 40 ml. collidine and 100 ml. glacial acetic acid. This solution is stable for up to two weeks if kept under refrigeration but deteriorates rapidly at room temperature. The papers are dipped in this solution and dried at 90 C. for 10 minutes. The chief advantage of this stain lies in the fact that a greater variety of colors appear in the peptide spots permitting in some instances easier identification than is possible with stain A.

D. Ninhydrin-collidine stain for amino acids: The solution consists of 0.3 Gm. ninhydrin dissolved in 300 ml. absolute EtOH, 100 ml. glacial acetic acid and 40 ml. of collidine. At
with stain C above, this preparation is stable for up to two weeks in the cold but deteriorates rapidly at room temperature. The amino acid papers are liberally sprayed and dried at 80 to 90°C for 10 minutes.

E. Pauly's stain for histidine and tyrosine: The following solutions are prepared and are stable indefinitely at room temperature. I—5 per cent NaNO₃ (aqueous). II—0.9 Gm. sulfanilic acid dissolved in 9 ml. concentrated HCl and made up to 100 ml. with H₂O. III—5 per cent Na₂CO₃. A freshly prepared mixture of I (50 ml.) and II (10 ml.) is applied as a spray. While the paper is still slightly damp, spray with solution III. An orange color develops almost immediately, reaching full intensity within 10 minutes, in the presence of histidine and tyrosine. This stain may be used as an overstain after B or D.

F. Pauly's stain for histidine: (Tyrosine is less well stained by the following procedure). Solution I—0.8 Gm. sulfanilic acid dissolved in 8 ml. concentrated HCl made up to 100 ml. with H₂O. Solution II—0.69 per cent NaNO₃ in water. Solution III—10 per cent aqueous Na₂CO₃. Mix equal volumes of solution I and II and apply as a fairly heavy spray. When the paper is nearly dry, spray with solution III. The orange color due to histidine appears almost immediately reaching full intensity within 10 minutes. The light orange hue of tyrosine fades quickly. Stain E may also be used as an overstain after either B or D.

G. α Nitroso β naphthol stain for tyrosine and tryptophane²²,²³: Solution I—0.1 per cent α nitroso β naphthol in acetone. Solution II—10 per cent HNO₃ in acetone. Dip
the paper in solution I and dry at room temperature. The paper is then dipped in solution II and heated for three minutes at 80 to 90°C. Care should be taken to make the dip as smooth as possible. Tyrosine is recognized by the resulting pink color against a pale green background while tryptophane causes the appearance of a dirty yellow to brown color. The resulting colors are stable for only a few hours. May be used as an overstain after B but cannot be used in the presence of phenol or collidine.

H. Sakaguchi’s stain for arginine: Solution I—0.1 per cent diacetyl in water. Solution II—20 per cent KOH in water. Solution III—2.5 per cent α naphthol in absolute EtOH. Mix equal volumes of I, II and III in the order given. The paper is dipped into the solution with a smooth even motion. The pink color of arginine appears within a few minutes reaching its peak intensity within 5 to 10 minutes. The color fades within a few hours. Sakaguchi’s stain may be used as an overstain after B or I. When used as an overstain after I, care should be taken that the paper be completely dry before proceeding with the Sakaguchi stain.

I. Ehrlich’s stain for tryptophane: 1 Gm. Ehrlich’s reagent (p-dimethylaminobenzaldehyde) dissolved in 90 ml. acetone to which are added 10 ml. concentrated HCl. The chromatographic papers may either be sprayed with or dipped in this solution. Must be used immediately after preparation. The blue color of tryptophane appears within five minutes and reaches its maximum intensity within ten minutes. May be used as an overstain after B.

RESULTS AND DISCUSSION

Though similar to the technical approach used by Ingram and co-workers, as well as by others, the methods described in this communication differ in at least two major respects from those previously reported. By performing the chromatographic separation first, a system of somewhat higher resolving power precedes the system of slightly lower resolving power, electrophoresis. Sharper, cleaner peptide separations have, as a result, been achieved. Furthermore, the methods adopted in this study employ a more acidic pH for electrophoresis which leads to more complete separation of the peptides and eliminates the heavy bulk of the so called neutral peptides seen at pH 6.5.

Many of the technical details, problems and pitfalls have already been touched upon in the previous section and need not be repeated here. It goes without saying that compounds of the highest degree of purity possible must be employed in such studies for the results to be meaningful. That some denaturation may occur in the process of hemoglobin purification need not be a source of concern inasmuch as denaturation is a necessary step for tryptic digestion, as well as for the action of certain other enzymes. Of the many technics of denaturation available, we have preferred to use heat rather than alkali or urea. The use of globin rather than hemoglobin has been found to be advantageous since with the latter the associated heme occasionally causes less clear-cut separations. However, either preparation is quite satisfactory. The use of individual alpha, beta, or gamma chain preparations is highly desirable since overlapping peptides are almost eliminated and less confusing patterns result. Nevertheless, it is important to keep in mind that the separation of globin into its component chains may induce some changes in the peptide structure. This conclusion is based on observations to be reported in which the chromatographic positions of several of the tryptic peptides of hemoglobin are found to be altered somewhat in fingerprints of the individual alpha and beta chains.
Although many enzymes are available for digestion of the hemoglobin molecule, none has the specificity and usefulness of trypsin. Stable in acid medium, it has its maximum activity in the pH range 8 to 8.5. Salt free, crystallized preparations must be used and the reagent should be checked for possible chymotryptic activity which may lead to bonds other than arginyl and lysyl being broken. The material utilized in these studies was found to be virtually free of such contamination and did not have to be purified further. Other enzymes which find ready applicability in these studies are chymotrypsin (for the resistant core) and pepsin (for less specific cleavage). Leucine aminopeptidase, carboxypeptidase, and other enzymes serve useful roles in both N-terminal and COOH-terminal group studies.

Scrupulous attention to details forms one of the mainstays of successful peptide fingerprinting. Thus, papers must be carefully handled, chromatographic “hardware” must be rigorously cleaned, solvent systems must be fresh, and equilibration must be complete. Somewhat greater latitude is permissible with the amino acid analyses, but here too, details are unavoidably important. Through the judicious use of specific stains, much information relative to the composition of the peptides can be obtained. Nevertheless, amino acid analysis is essential for definitive studies on the resulting peptides. Although two dimensional amino acid chromatography yields at best only semiquantitative information and may be somewhat inaccurate in terms of detecting all the amino acids comprising a given peptide, this method still serves as an initial approach to the problem. More elegant quantitative paper chromatographic studies, quantitative column chromatographic procedures and sequence studies, using methods such as Sanger’s FDNB* technique or the Edman degradation are obviously required for more definitive information. The results reported in this and the several papers in preparation must be regarded primarily, therefore, as an initial approach to the problem of the structural differences of these proteins.

A great deal of information has been gathered concerning the primary, secondary and tertiary structure of hemoglobin. Suffice it to say that the classic studies of Perutz, Kendrew, Moore and Stein, Schroeder, Braunitzer, Itano, Ingram and many others have brought our state of knowledge of the chemistry of the human hemoglobins to an advanced stage. It seems reasonably certain that hemoglobin, composed of four heme groups attached to the globin moiety, consists primarily of four coiled polypeptide chains. The protein mass consists of identical half molecules, each with approximately 300 amino acids, symmetrically oriented about a central axis. Under conditions of moderate acid and alkaline pH, dissociation of the molecule takes place, presumably in an asymmetric fashion. It has been shown that two dissimilar peptide chains, designated alpha and beta, exist in the hemoglobin molecule and that two members of each variety are present. These four polypeptide chains are oriented about two different axes of symmetry and may be readily separated by taking advantage of the phenomena referred to above. End

*Fluoro 2:5 dinitrobenzene.
group analysis has shown the beta chain probably to have peptide No. 14 at its N-terminal end\textsuperscript{1,30} with the N-terminal sequence val−his−leu.\textsuperscript{1,35} For the α chain, the N-terminal sequence is val−leu.\textsuperscript{34,35} Hgb A consists of two α and two β chains and may be referred to as a α\_β or α\textsubscript{2}β\textsubscript{2}. Hgb F shares the same α chains but has polypeptide chains significantly different from β, which have been designated γ\textsubscript{4,30,37-39}. Hence, its formula may be expressed as α\textsubscript{2}γ\textsubscript{2}. It has recently been reported by Stretton and Ingram that Hgb A\_2 has yet a different second set of peptide chains, designated δ and its formula presumably is α\textsubscript{2}δ\textsubscript{2}.\textsuperscript{40} Abnormalities of the various hemoglobins have been found in either α or β chains and can be conveniently designated by superscript letters as follows: Hgb S = α\_β\_S; Hgb I = α\_β\_I; Hgb C = α\_β\_C; Hgb H = β\_I, etc.

Initial studies in our laboratory have been directed at a number of types of hemoglobin including A, S, F, C, D, E, and R (Durham No. 1\textsuperscript{41,42}). Although in some respects these studies are incomplete, it was felt worthwhile to describe some of these patterns to serve as reference points for studies to be reported shortly as well as for comparative purposes with findings in other laboratories.

**Hgb A** (fig. 7): Some of the difficulties encountered in fingerprinting studies

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\*The system of peptide numbering referred to in this paper differs from that of Ingram and co-workers inasmuch as different methods of separation have been utilized and the fingerprint patterns are not entirely comparable. Peptide No. 14 is identical with peptide No. 4 of Ingram. Peptide 26 in the present study is also No. 26 in Ingram's work. Unfortunately, not all peptides identified in our respective laboratories have yet been correlated.

\textsuperscript{1}The nomenclature for the amino acids utilized in this paper is consistent with that adopted by most workers in this field.
Table 1.—Peptides Comprising the α and β Chains of Human Hemoglobin A

<table>
<thead>
<tr>
<th>Peptide No.</th>
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Table 2.—Peptides Reacting with Specific Stains for Amino Acids

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*See “Methods” for identification of stain.
†Peptides not included fail to react with any of these stains.

are well illustrated in the findings pictured in the accompanying schematic drawing. Overlapping peptides, particularly 11 and 12, 15 and 16, and 17, 18 and 21 make interpretation difficult. Those peptides which have in subsequent studies been found in the α or β chains are listed in table 1. Finally, several peptides, all found in the β chain, are still indefinite and are listed in par-
entheses. It is very probable that the apparent presence of peptides 29, 30 and 31 in the $ chain is due to some cross contamination with the $ chain, inasmuch as their location corresponds to those of peptide 17, 18 and 21 of the $ chain, while their intensity on the fingerprints is considerably less than that of definite members of the $ chain. Peptide 28 has been inconsistent in appearance.
Table 3.—Amino Acid Sequence in Peptides No. 14 and 26

<table>
<thead>
<tr>
<th>Peptide No. 14*</th>
<th>Peptide No. 26*</th>
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<tr>
<td><strong>Hgb A</strong></td>
<td>Val - his - leu - thr - pro - glu - glu - lys</td>
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<tr>
<td><strong>Hgb S</strong></td>
<td>Val - his - leu - thr - pro - val - glu - lys</td>
</tr>
<tr>
<td><strong>Hgb C</strong></td>
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<td><strong>C&lt;sub&gt;2&lt;/sub&gt;</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Hgb A</strong></td>
<td>Val - asp - val - asp - glu - val - gly - gly - glu - ala - leu - gly - arg</td>
</tr>
<tr>
<td><strong>Hgb E</strong></td>
<td>Val - asp - val - (asp - glu - val - gly) - gly - lys † E&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>ala - leu - gly - arg</td>
</tr>
<tr>
<td><strong>E&lt;sub&gt;2&lt;/sub&gt;</strong></td>
<td></td>
</tr>
</tbody>
</table>

† Additional sites at which trypsin breaks the respective peptides.
*From data presented in the literature (5, 43–45).

and requires further study. Peptides reacting to specific stains are listed in table 2.

Hgb C (fig. 8): As reported by Ingram, peptide No. 14 (Ingram’s No. 4), presumably the N-terminal peptide of the β chain, is replaced by two fragments C<sub>1</sub> and C<sub>2</sub> due to the introduction of a lysine residue in place of glutamic acid, permitting trypsin to split this area of the chain at an additional locus (table 3). Specific stains are listed in table 2.

Hgb E (fig. 9): The only discernible change relates to peptide No. 26 which has been replaced by two fragments, E<sub>1</sub> and E<sub>2</sub>, because of the introduction of lysine in the place of glutamic acid present in the same position of peptide 26 of Hgb A<sup>44</sup> (tables 2 and 3).

Hgb S (fig. 10): No additional fragments are found in Hgb S, but a shift of peptide No. 14 has occurred, due, as Ingram<sup>2,36</sup> and Hill and Schwartz<sup>2</sup> have described, to the replacement of one of the glutamic acid residues by valine in this portion of the β chain (table 3).

In order to clarify the differences in the peptide patterns of Hgbs A, S, C and E, fingerprints of the isolated β chain of each are summarized in figure 11.

Results of studies with other hemoglobins are as yet incomplete but the amino acid abnormality of Hgb D (from the patient with homozygous Hgb D disease previously reported<sup>46</sup>) appears to be in peptide No. 22; of Hgb R (Durham No. 1) in peptide No. 14 and of a sample presumed to be Hgb Stanleyville I (47, 48) in the α chain in either peptide 17 or 18. This latter observation is of some interest in that Hgb Dα (49, 50) has also been reported to have its abnormality in peptide 18 (No. 23 of Ingram). It is tempting to consider that Dα and Stanleyville I are, in fact, one and the same hemoglobin. Preliminary studies of Hgb F have been reported in an earlier publication.<sup>29</sup> However, changes in our technics have necessitated certain revisions in our previous data. In addition to the adoption of a uniform numbering system for the tryptic peptides, we have noted minor errors in the amino acid composition previously reported and these findings will be the subject of a report soon to be completed.
A detailed description of our current methods of “fingerprinting” and of amino acid analysis of the hemoglobins has been presented. They differ in several respects from comparable technics used elsewhere. Preliminary results of analyses of several human hemoglobins are briefly described.

SUMMARY IN INTERLINGUA

Es presentate un description detaliate de nostre currente methodos “characterologic” e technicas de analyse amino-acidic del hemoglobinias. Illos dif-
fere in plure respectos ab le comparabile procedimentos usate in altere laboratorios. Es describite brevemente le resultatos preliminari del analyse de plure hemoglobinas human.

REFERENCES


21. Block, R. J., Durrum, E. L. and Zweig,
AMINO ACID COMPOSITION OF HEMOGLOBIN, ANALYTICAL TECHNIQUES


23. Ingram, V. M.: Personal communication.


45. Ingram, V. M.: Abnormal Human Hem-


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A method is described for the production of experimental thrombi in rats by the direct application of 10 per cent formalin in 60 per cent methyl alcohol to the jugular vein. This method produced thrombi in about 65 per cent of animals within 24 hours. Both “depo heparin” and “Treburon” reduced the incidence of thrombosis when injected subcutaneously 5 hours after operation. The incidence of thrombosis fell with increasing dosage of these drugs, to a minimum of about 40 per cent when 20 to 40 mg./100 Gm. was given. Many deaths from hemorrhage occurred with the higher dosages used. There was a significant negative correlation between the incidence of thrombosis and the prothrombin time of rats receiving dicooumarol or “warfarin,” but the dosage of these drugs was poorly correlated with both prothrombin time and the incidence of thrombosis.—R. M. H.


Rabbit bone marrow suspended in Krebs Ringer phosphate was incubated at 37 C. in open Erlenmeyer flasks with glycine 2-C¹⁴ for 4 hours and the rate of glycine incorporation into heme and into globin was determined. The ratio between the rate of incorporation into heme and into glycine was found to be almost constant for all immature red cells although the biosynthetic activity of reticulocytes was much less than that of nucleated red cells. The addition of cobalt changed this constant ratio by inhibiting heme synthesis without changing the incorporation into glycine. The effects of substrate concentration, method of preparing the bone marrow suspension, temperature and time of incubation were examined.—A. J. E.
The Amino Acid Composition of Hemoglobin. II. Analytical Technics

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