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*A COMPARISON OF ANIMAL HEMOGLOBINS BY TRYPTIC PEPTIDE PATTERN ANALYSIS**

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The complete amino acid sequences (primary structure) of hemoglobins can, in principle, be determined by methods currently available. Although detailed studies of the primary structure of human and horse hemoglobins are in progress in several laboratories,[1] the methods are so laborious that complete sequences have not yet been established. Important questions in the realm of genetics and evolution require the immediate examination of the structure, primary and other, of

many different hemoglobins. The application of methods that are quicker, though less informative and reliable, than the techniques required for complete sequence determination is therefore in order as a provisional means of securing useful information. Such a method is the analysis of peptide patterns obtained by combined paper electrophoresis and chromatography of tryptic hydrolysates of denatured hemoglobin.[2] Of particular interest are comparisons between hemoglobin components present in (*a*) organisms of one animal species at a given time in development, (*b*) organisms of one species at different stages of development, and (*c*) organisms of different species. The present paper is concerned exclusively with the last type of comparison. In order to scan the range of variation of hemoglobin structure throughout evolution, hemoglobins from a number of animals both closely and distantly related to man have been selected and compared as to tryptic peptide patterns with human hemoglobin A. Whole hemoglobin preparations from adult animals have been studied throughout. The problem of individual heterogeneity will be treated elsewhere.

Materials and Methods.—Ape bloods[3a] were anticoagulated and transported in Alsever's solution. The apes studied included two lowland gorillas (*Gorilla gorilla*), two chimpanzees (*Pan troglodytes*), and three orangutans (*Pongo pygmaeus*). Erythrocytes from Rhesus monkeys (*Macaca mulatta*) were obtained from clotted blood.[3b] Heparinized porcine and bovine bloods were secured during bleedings at Los Angeles slaughter houses. Heparinized bloods were obtained from the marine lungfish, *Pimelometopon pulcher* (sheepshead), and from the cartilaginous fish *Cephaloscyllium uter* (swell shark).[3c] Blood was also obtained from a live specimen of *Lepidosiren paradoxa* (Dipneust, South American fresh water lungfish).[3c, d] Contamination of the latter blood by tissue fluid was unavoidable. Blood of the Pacific hagfish, *Polistotrema stouti*, was also examined.[4] Coelomic cells (hemoglobin cells) were obtained from *Urechis caupo* (Echiurid marine "worm")[5] collected at low tide from mud flats near the Kerckhoff Marine Laboratory at Corona del Mar, California.[3e]

In general, the hemoglobin preparations examined were from single individuals. However, in the case of *Urechis* single as well as pooled samples of coelomic cells were used without apparent differences. Only a pooled sample of blood from twelve hagfish was examined. All of the animals studied were judged to be adults either from their size or their known age. The youngest apes were one orangutan 2 years old and one gorilla 2 years old. Peptide patterns of their hemoglobins were indistinguishable from those of older individuals of the same species. The *Lepidosiren*, 14 inches long, was at a minimum 8 months of age, and might be considered a subadult.

The erythrocytes were washed four times with cold 0.9% NaCl with the exception of *Pimelometopon* and *Cephaloscyllium* cells, which were washed with 1.2% NaCl.[6] The red cells from hagfish were washed with chilled 3% NaCl and the coelomic cells of *Urechis* with 3.2% NaCl. Washing with 0.9% NaCl led to considerable hemolysis in the case of the fresh water fish *Dipneust*.

The washed cells were hemolyzed in a standard fashion with distilled water and toluene[7] with the exception of *Urechis*, where ether was used instead of toluene. The washed red cells of hagfish were stored for one day at 4°C before lysing. A major portion of the hemoglobin obtained was insoluble possibly due to acidifica-

tion during storage. The peptide pattern shown below was obtained from the remaining soluble fraction. After lysis, each sample was centrifuged at high speeds in order to remove solid debris and the toluene phase. The resulting hemoglobin solutions were saturated with carbon monoxide and dialyzed for a week against distilled water saturated with carbon monoxide (three changes of water with the ratio of hemoglobin solution to water of the order of 1:100). This prolonged dialysis was employed to permit the flocculation of non-hemoglobin proteins.[6] A crystal of thymol was added to one *Urechis* preparation in order to eliminate the possibility of the formation of peptides by bacterial contamination. No differences were found in the peptide patterns obtained with and without thymol.

With species closely related to man the hemoglobin concentration in the final preparation could be assayed approximately by the use of spectrophotometric constants established for human hemoglobins. In other species the quantities of material to be used on peptide patterns were estimated roughly from the consumption of sodium hydroxide during tryptic digestion.

Hydrolysis with trypsin of heat-denatured hemoglobin preparations was performed at constant pH using a Radiometer automatic titrator as a pH-stat. The hydrolyses were carried out at 40°C at pH 8.0 in the presence of Ca ion (0.01 *M*) with an enzyme to hemoglobin ratio of between 1 and 2% by weight (Worthington twice crystallized trypsin). Ninety minutes was allowed for the hydrolysis except in the case of the apes, where the digestion was stopped after sixty minutes.

Those tryptic peptides which are soluble at pH 6.5 were analyzed by a combination of electrophoresis and chromatography on paper. These peptide patterns were obtained essentially as specified by Ingram[2] except that the voltage rate was 900–1,000 V for a duration of $3^1/_2$ hours. In general, a human control sample was run as a pair mate with every animal hemoglobin in order to have a reference pattern for each unknown. Even with such parallel determinations, the spreading occurring during electrophoresis was not always identical. The polarity of the electrophoretic field is marked on each pattern and the point of application of the sample is designated by a cross (+). The peptide spots resulting from tryptic hydrolysis of human hemoglobin have been assigned arbitrary numbers by Ingram.[2] This convention has been employed in the present study. The neutral band region is comprised of peptide spots 1 through 7. These peptides do not migrate to any extent in the electric field, but do move some from the point of application due to fluid movement. Phenylalanine and lysine have been added as reference spots at least on one pattern in the case of every species. Chromatography was ascending in direction and the peptides were detected mainly by their reaction with ninhydrin. The Sakaguchi[8] test for arginine and the cinnamaldehyde[9] reaction for trypophan were also employed. Patterns from each species were obtained at least in triplicate.

Results.—Figure 1 is a peptide pattern of human hemoglobin A as currently obtained in our laboratories. A diagram indicating the peptide spot numbering system is also included.

Figure 2 shows peptide patterns from three different apes (gorilla, chimpanzee, and orangutan) and one monkey (Rhesus). The gorilla, chimpanzee, and human patterns are almost identical in appearance. In the case of the gorilla peptide patterns, spot numbers 12 and 24 both appear to be double. In humans the peptides that make up these two spots are from the beta chain. No difference was

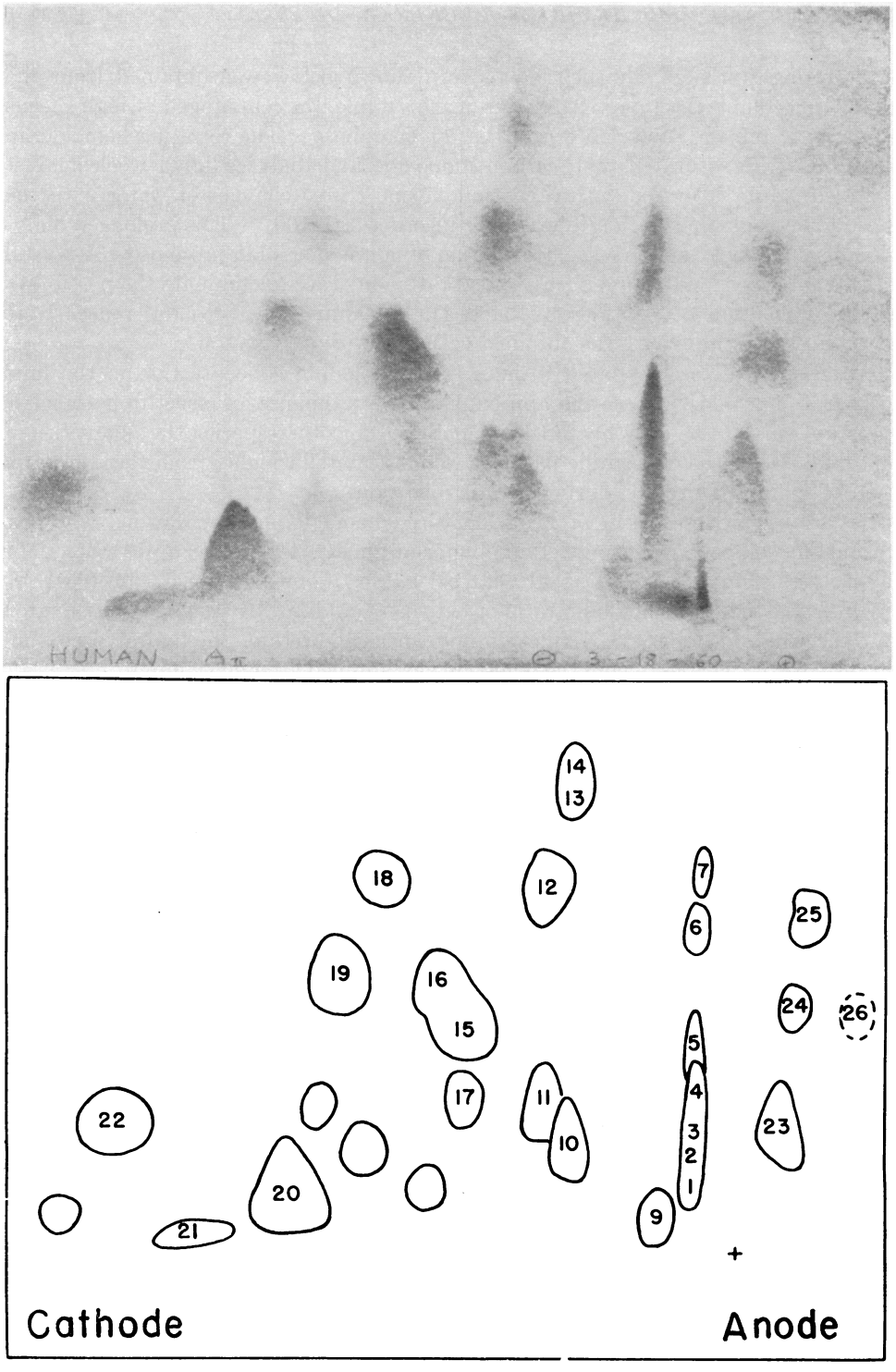

FIG. 1.—A tryptic peptide pattern of adult human hemoglobin and its schematic representation. The spots are numbered according to the convention introduced by Ingram.[2] The site of spot 26 is shown on the diagram, although not seen on the pattern represented. The locations of four hitherto undescribed spots usually found on patterns obtained in our laboratories are indicated.

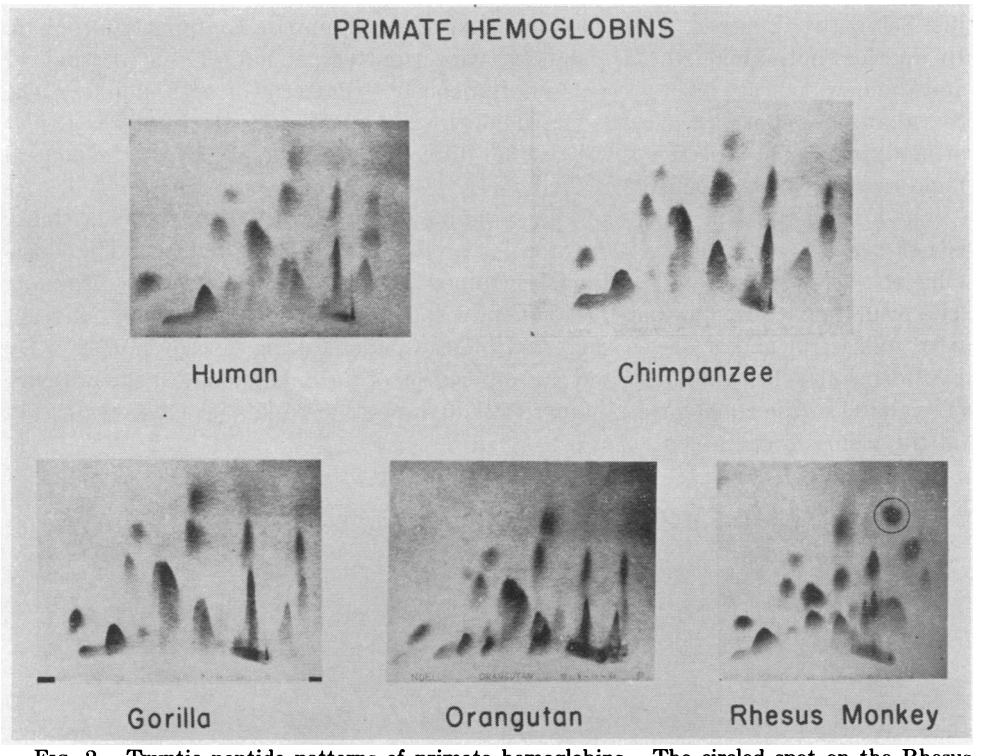

FIG. 2.—Tryptic peptide patterns of primate hemoglobins. The circled spot on the Rhesus monkey pattern represents phenylalanine added two and a half inches to the anodal side of the point of application of the peptide mixture.

observed between two gorillas, one male and one female. Spots 12 and 24 also seem to be double in the peptide patterns of chimpanzee. In addition, extra material may be present in the 15–16 region. In humans at least four major peptides are present in this region.[1] Two chimpanzees, both females, were found to have identical patterns. Of course further differences between these two types of apes and humans may be discovered upon the analysis of the individual peptides or the study of the protein residue which remains insoluble after hydrolysis with trypsin.

The difference from human patterns is somewhat greater for orangutan peptide patterns than for the patterns of the two apes just mentioned. In two orangutans (one male and one female) spots 12 and 24 are double. The third orangutan (female), whose pattern is illustrated in Figure 2, was observed to have single 12 and 24 spots. Further differences between the patterns of the individual orangutans are not apparent; however, each differs from human patterns by an apparent increase in peptides in the 15–16 region. The orangutans also differ from humans and the other two apes studied by the appearance of two new spots, one anodal to spot 20 and the other cathodal and below spot 10. The latter does not appear to be the same as spot 9 of human. All of the spots containing arginine and tryptophan are the same as in human.

Spot 12 is absent in the peptide patterns of Rhesus monkey hemoglobin. A new spot, possibly representing a modification of the peptide of spot 12, is present

just below the 12 region. The new spot, like 12 from human, contains tryptophan. In another individual Rhesus monkey, whose pattern is not presented, spot 12 and the new spot are both present simultaneously. Patterns of both monkeys also reveal another spot on the cathodal side of the 2, 3, and 4 region. Spot 7 is absent or modified (almost fused with 6). Other differences may be present in the neutral band region, which is poorly resolved.

The human spot 26, which contains arginine and is derived from the beta chain, is not always seen because of poor color production with ninhydrin. The existence of this spot can, however, be ascertained by the Sakaguchi test for arginine. The primate patterns presented do not show spot 26, either because it did not react with ninhydrin or because it migrated off the paper during electrophoresis. By undimensional electrophoresis and the application of the Sakaguchi test the presence of arginine in the zone corresponding to human peptide 26 has been ascertained in all the primates examined.

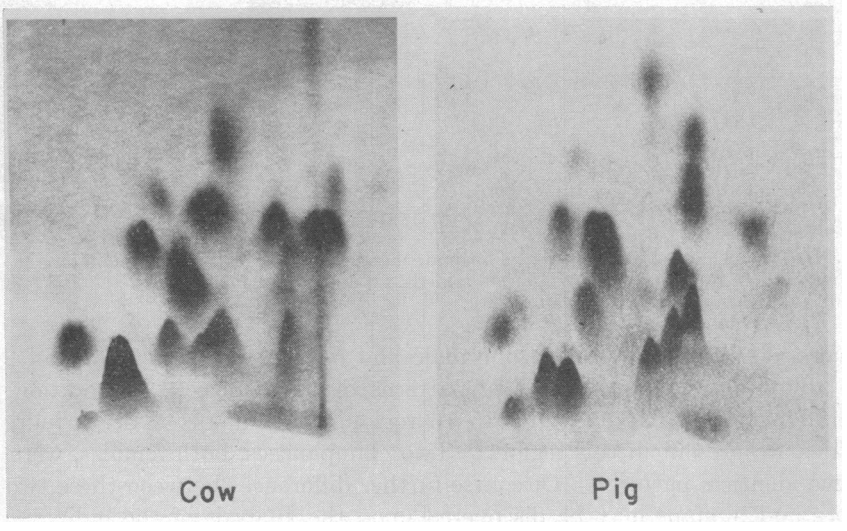

FIG. 3.—Tryptic peptide patterns of bovine and porcine hemoglobins.

Tryptic peptide patterns of bovine and porcine hemoglobins are presented in Figure 3. Cow and pig belong to the same order. In many respects these patterns appear similar to human patterns. However, the differences are numerous and striking.

In bovine and porcine hemoglobin, sequence val-leu has been found to be N-terminal in one of the polypeptide chains.[10] Both hemoglobins therefore contain alpha chains, according to the terminology introduced by Rhinesmith, Schroeder, and Martin.[11] However, the cow alpha chain is different in several respects from the human alpha chain. Although the chains have not yet been separated, the peptide patterns indicate that the alpha chain peptides 5, 10, and 17 are absent from bovine patterns, while the alpha chain peptides 12, 13, 18 and an alpha component of spot 16, detected by a positive Sakaguchi test, are present.[1,12] Although the second chain by definition is not a beta chain,[10, 11] the human beta chain spots

12 and 19 are seen clearly. The human pure beta spots 2, 4, 14 (as ascertained by a negative reaction with cinnamaldehyde) and 25 are absent.

The porcine alpha chain may be somewhat more similar to the human alpha chain since the alpha spots 10, 13, and 18 are seen, and the presence of the alpha spots 11, 17, and 23 is suspected. The presence of an alpha-component in the composite spot 15 is likely because of a positive cinnamaldehyde test, and the presence of an alpha-component in the composite spot 16 is indicated by a positive Sakaguchi test. The alpha spot 5 is absent. Among the beta spots, 14 (cinnamaldehyde positive), 26 (Sakaguchi positive), 4, 19, 24, and possibly 25 are present; while 12 is absent. Like bovine hemoglobin, porcine hemoglobin does not contain beta chains; however, there are a number of tryptic peptides similar to those from the human beta chain observed.

The composite human spots 13–14, 15–16, 20 and 21 as well as the lysine spot 22 (arising from both α and β chains[1]) are seen in pig as well as in cow. Thus the

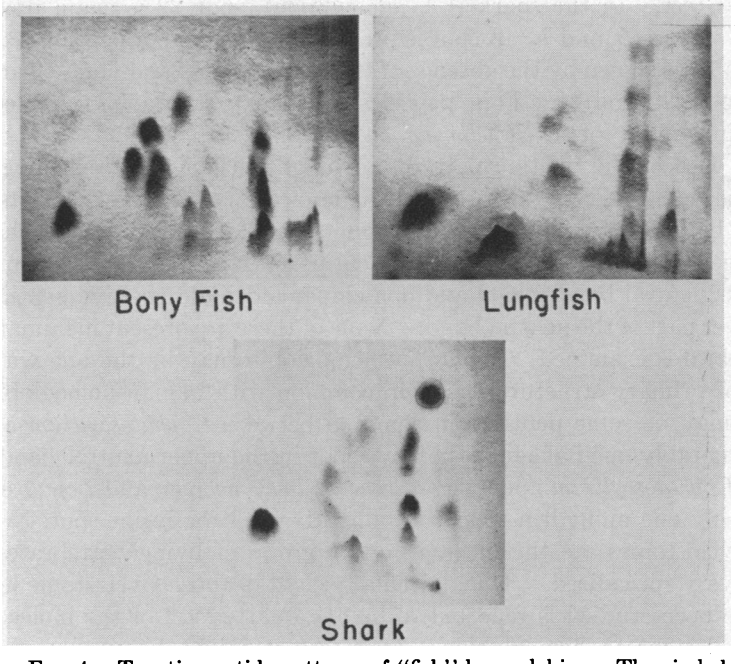

FIG. 4.—Tryptic peptide patterns of "fish" hemoglobins. The circled spot on the shark pattern represents phenylalanine added two and a half inches to the anodal side of the point of application of the peptide mixture.

apparent similarities between bovine and human patterns are in many respects the same as those noted between porcine and human; however, the differences are not the same. From three independent pattern studies of the same bovine sample it appears that many of the new spots are slightly on the anodal side of the neutral band region, whereas in porcine patterns most of the new spots are on the cathodal side. Arginine is present in at least two of the new porcine peptides, one neutral and one acidic.

Spot 12, as stated, and as confirmed by cinnamaldehyde tests, is absent from pig, but present in the cow. The decapeptide[1] that accounts for this spot in humans may have undergone independent alteration (mutation?) at different times. In pig, a novel tryptophan peptide is found in the upper neutral region. It may correspond to an altered peptide No. 12.

The three fish from which peptide patterns have been obtained (Fig. 4) belong to entirely different groups which modern systematicians do not unite under the heading of a single class. None of the three patterns is similar to the patterns of human hemoglobin. In the bony fish (*Pimelometopon*), spot 22 is present as are spots 18 and 19, which in humans are dipeptides.[1] Possibly spots 24 and 25 and some material in the regions of 4 and 15 are common to human and *Pimelometopon*. The remaining spots appear to be different from human. Three spots containing tryptophan are seen with no analogy in human hemoglobin. One other spot at the base of the neutral band resembles a similarly located spot on pig patterns in that it gives a blue rather than purple color with ninhydrin and a positive Sakaguchi test. In the shark (*Cephaloscyllium*), spot 22 is again present as are probably spots 4, 5, and 7. A spot is present in the region of peptide 12, but differs from spot 12 as shown by the absence of tryptophan. Spots 18 and 19 are absent as well as spots 20 and 21. Four peptides containing tryptophan are seen with no analogy in human or in *Pimelometopon*.

In the case of the peptide patterns of lungfish (*Lepidosiren*) hemoglobin, spot 22, (lysine, identified by position and shade of color) is present. Spots 19 and 23 may also be present. The absence of spots 20 and 21 appears to be characteristic of all three types of fish. Three Sakaguchi-positive peptides are seen in the middle and upper neutral band region and one cinnamaldehyde positive peptide adjacent to the lower part of the neutral band. None of these are present in human. Among the vertebrates examined, *Lepidosiren* hemoglobin may be the one with the least number of primary structural traits in common with human hemoglobin.

There may be some peptides in common between *Cephaloscyllium* and *Pimelometopon*, notably one Sakaguchi-positive spot in the upper neutral band and some between *Cephaloscyllium* and *Lepidosiren*. *Pimelometopon* and *Lepidosiren* appear to have only one ninhydrin spot in common beyond the lysine spot, No. 22.

The Cyclostomes are the most primitive group of living vertebrates, although they are very specialized. The molecular weight of those Cyclostome hemoglobins that have been studied is reported to be one quarter[13, 14] of the molecular weight of other vertebrate hemoglobins. Therefore it might be presumed that these Cyclostome hemoglobins are composed of single polypeptide chains. Because direct estimates of the molecular weight of *Polistotrema* hemoglobin are not available, an ultracentrifuge study was made.[15] The soluble fraction of our *Polistotrema* hemoglobin (see *Materials and Methods*) appeared to sediment as a single component with an $s_{20,w}$ of 1.9. This value, in the case of hemoglobin from the related *Petromyzon*, has been interpreted by others[14] to correspond to a molecular weight of approximately 23.600. However, the peptide pattern of *Polistotrema* hemoglobin shown in Figure 5 may not represent a single kind of polypeptide chain because two major and possibly some minor hemoglobin components were shown to be present by starch gel electrophoresis.[16]

It is not possible to make definite statements about the similarities between

Polistotrema and other patterns in the region of the neutral band because of incomplete resolution of the *Polistotrema* spots. Except possibly for the neutral band region and the lysine spot, the *Polistotrema* peptide pattern has no feature in common with human, lungfish, or shark patterns. One or two spots in the bony fish pattern appear similar to spots in the *Polistotrema* pattern.

In order to test the range of variation in hemoglobins in general, a peptide pattern of a single invertebrate, the Echiurid "worm" *Urechis* was studied (Fig. 6). In this group the hemoglobins are intracellular, as in the vertebrates. The heavily

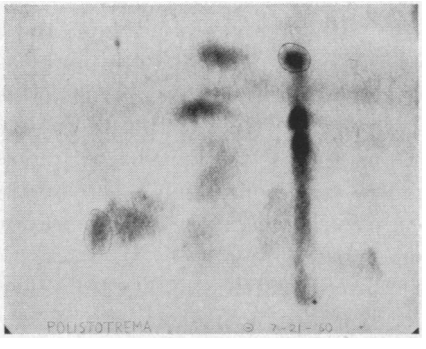

FIG. 5.—Tryptic peptide pattern of hagfish hemoglobin. The circled spots represent phenylalanine and lysine respectively added at the point of application of the peptide mixture.

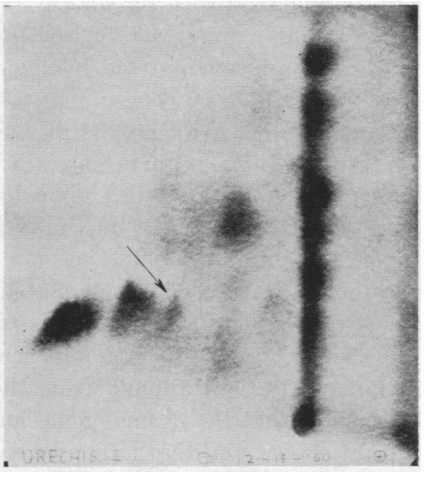

FIG. 6.—Tryptic peptide pattern of hemoglobin from an Echiurid "worm." Most of the spots in the neutral band, as well as the one indicated by the arrow, are not tryptic peptides (see text).

loaded neutral band region obtained has been shown to contain many amino acids[17] that are not the result of tryptic hydrolysis. The spots on the cathodal side of the neutral band, with the exception of the one indicated by the arrow, were found to be from *Urechis* hemoglobin. The latter conclusion was obtained by comparing, peptide patterns of the total undigested hemoglobin preparation with the undigested supernatant obtained after heat denaturation and centrifugation of the precipitate. The only spot identified with any certainty is spot 22. All other *Urechis* spots appeared to be without a match in human.

Discussion.—Peptide patterns as obtained by the present technique do not yield unequivocal information for several reasons: (*a*) spots found in identical locations may not represent identical peptides, (*b*) single spots may contain more than one peptide, (*c*) a single peptide, through partial secondary alteration or because of incomplete splitting into two peptides, may give rise to more than one spot, and (*d*) a significant part of the hemoglobin molecule (about one third in human A, S, and F[18]) is not broken down by trypsin sufficiently to become soluble, and accordingly is not represented on tryptic peptide patterns.

Although, in view of (*d*), all findings must be substantiated by the examination of the undigested core, observations (*a*), (*b*), and (*c*) do not seriously impair the conclusion that Primate and human hemoglobins are very similar, especially

gorilla, chimpanzee and human. There is no doubt that the identification of individual spots needs to be based on data more specific than position, shape, and (sometimes) color shade of the ninhydrin-developed spots, or even reaction with reagents specific for certain amino acid residues. However, when two complex peptide patterns look similar as a whole the probability that most of the spots actually represent identical or highly similar sequences becomes high.

That chimpanzee should be extremely close to man while orangutan and Rhesus monkey are somewhat more distant when examined by hemoglobin peptide patterns is in accord with serological data.[19] On the other hand, while human hemoglobin strongly crossreacts with Rhesus monkey hemoglobin, it has been observed not to do so with porcine hemoglobin and only exceptionally crossreacts with cattle hemoglobin.[20] The similarity between human and Primate hemoglobins suggests that these hemoglobins probably have not been modified extensively since the times of their common ancestor. It is possible that the genes for the α and β chains of a normal adult human hemoglobin are more stable than the mutated genes formed from them by simple mutations, and that the mutated genes often undergo back-mutation to the more stable normal genes. In addition to natural selection, the thermodynamic stability of the genes themselves may be an important factor in determining the distribution of alleles in a population. Our results indicate that stable hemoglobin genes had been developed before the separation of humans and anthropoids from their common stock.

The idea that the normal genes have greater thermodynamic stability than their mutant alleles, and that in consequence the mutant alleles have a greater mutation rate than the normal genes, provides an explanation of the fact that some of the abnormal hemoglobins have their abnormalities at the same site.[21] The alleles corresponding to one site may represent one mutant from the normal gene and other alleles formed by second mutations from this mutant, and involving its unstable site.

Human adult hemoglobin peptide patterns actually differ more from human fetal patterns than from adult gorilla and chimpanzee patterns. In view of the theory of recapitulation it would not appear paradoxical that an adult organism should be in a sense a more distant "relative" of its own embryo than of other closely related adult organisms. Adaptative factors in response to the environment may play an equally important role in establishing the differences. In the only animal examined (sheep) fetal hemoglobin differs from adult hemoglobin in molecular shape.[22]

As one gets further away from the group of Primates, the amount of primary structure that is shared with human hemoglobin decreases. Different primary structures may be compatible with rather constant tertiary structures.[23] The limits to this statement remain to be investigated.

The observation that pig and human on one hand and steer and human on the other show to a large extent apparent identity of the same peptides but diverging differences for the others may again indicate the existence in the hemoglobin molecule of zones more prone to mutation than others, or of zones where the occurring mutations are more liable to be preserved by natural selection. Part of the Primate pattern may have evolved relatively early in mammalian history, if not before, while another part may have varied more frequently throughout the groups.

Sections of both the alpha and beta chains, or of chains taking their place, appear to be involved. So far there is no evidence that one of the chains remains more stable through evolution than the other.

Firm conclusions about the partial stability of chains are premature before the sequence of each of the tryptic peptides that are common to different hemoglobins is determined. On the basis of a chance distribution of amino acids in the hemoglobin molecule, it is evident that the smaller the peptide, the greater the chance of finding it in different species. The presence of spot 22 seems to be the only trait shared by all hemoglobins. Because 22 is pure lysine, this only means that in all of the hemoglobins examined there is, in at least one place, a lysine next to another lysine or an arginine. In human hemoglobin a lys-lys sequence has been found to occur in both the alpha and beta chains.[1] The frequently seen spots 18 and 19 are dipeptides.[1] Spots 20 and 21 are a tetrapeptide and a pentapeptide, respectively, which are closely related.[1] It is true that some of the spots that may be common to pig, steer, and human (spots 10, 11, 24, and 25) contain much higher peptides.

However, the significance of the similarities observed between pig, steer, and human hemoglobins is increased by the observation that many of these similarities are not shared by the three fish and none of them (except the lysine spot) by the Cyclostome and the *Urechis* "worm." The three "fish" patterns differ among themselves considerably more than the mammalian patterns examined. Although shark and bony fish as well as shark and lungfish hemoglobins may share a small number of tryptic peptides, bony fish patterns appear about as dissimilar from lungfish as from human patterns. The findings are consistent with the view that these "fish" belong to widely divergent evolutionary lines.

Unless a greater constancy were found in the undigested core than in the rest of the molecule, no large tryptic peptide would be constant throughout the vertebrate series. Thus, most of the hemoglobin molecule, at least in the portion solubilized by trypsin, has been subject to successful mutation at some time during vertebrate evolution. Successful mutations in each tryptic peptide region have probably occurred more than once considering the differences between groups of fish and between Cyclostomes and fish. This observation, to be sure, does not exclude the existence of preferential sites of (successful) mutation. But such sites, if they exist, must be distributed throughout the molecule and not be concentrated in any one part of it, in contradistinction to what has so far been observed with insulin.[24]

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† On leave from Centre National de la Recherche Scientifique, Paris.

‡ Contribution No. 2618.

[1] Groups investigating the amino acid sequence of human hemoglobin include Dr. G. Braunitzer and co-workers, Drs. R. J. Hill and W. Koingsberg, Drs. V. M. Ingram and J. A. Hunt, and Dr. W. A. Schroeder and co-workers. Information concerning the tryptic peptides is from unpublished sequence studies of W. A. Schroeder and co-workers.

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[3] Various hemoglobin specimens have been supplied for this research. The authors wish to acknowledge the very valuable help in this respect of (*a*) Dr. W. P. Henschele, Head Veterinarian, San Diego Zoo; (*b*) Dr Renato Dulbecco, Division of Biology, California Institute of Tech-

nology; (c) Dr. K. S. Norris, Curator of Marineland of the Pacific, Palos Verdes, California; (d) Dr. W. H. Hildemann, Department of Zoology, University of California at Los Angeles, who drew the lungfish blood; and (e) Dr. A. Tyler, Division of Biology, California Institute of Technology.

[4] Specimens of the Pacific hagfish were caught and bled by Dr. David Jensen of the Oceanography Institute, La Jolla, California. The special methods employed were devised by him.

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*THE KINETICS OF DOUBLE HELIX FORMATION FROM POLYRIBOADENYLIC ACID AND POLYRIBOURIDYLIC ACID**

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Synthetic polyadenylic acid (poly A) and polyuridylic acid (poly U), which are largely in random coil conformation in solution at neutral pH, have been shown to react to form a double-stranded helical complex, poly (A + U), which has a structure similar to that of naturally occurring deoxyribonucleic acid (DNA).[1–4] The reaction is accompanied by a decrease in the optical density at 259 mμ. Under most circumstances the reaction is too rapid for kinetic study by conventional spectrophotometric means, but it can be readily followed by the stopped-flow technique.[5, 6] We report here the results of measurements made by this technique at various polymer concentrations, ionic strengths, and temperatures.

The polymer samples employed were kindly supplied by Dr. A. Rich, who determined the mean sedimentation coefficients to be 6.25 for the poly A and 4.52