generally 2 to 3 times higher than winter rates at any temperature. The transition from winter to summer rates and back again in this instance was very abrupt, occurring during a 2-week period in late April or early May, and in late September; this corresponded to transitions to and from dormancy. A curve or set of curves of respiration rates on temperature can be used to calculate the annual course of CO_2 exchange of the entire ecosystem, including above- and below-ground plant parts and animals, by applying it to records of temperature. These are often available as means over several years, enabling one to calculate rates of respiration which are averages, as opposed to single or short-term measurements. It is hoped that the technique can be adapted for wide application in the study of terrestrial ecosystems (*9*).

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9. Research carried out under the auspices of the AEC. We thank M. Smith and his colleagues for assistance with various aspects of the work. R. H. Whittaker allowed us to use previously unpublished data from his studies of the Brookhaven forest.

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Multiple Gene Loci for the Monomeric Hemoglobin of the Hagfish (Eptatretus stoutii)

Abstract. *The fact that members of the subclass* Cyclotomata *possess monomeric hemoglobin molecules has been known for some time. Electrophoresis of hemolysates from 12 hagfish* (Eptatretus stoutii) *revealed five hemoglobin phenotypes with four to six distinct zones of hemoglobin. Each zone is believed to represent a monomer containing one heme group on a single polypeptide chain with a molecular weight of approximately 18,000. It is postulated that these monomers are controlled by genes at four loci.*

The present study on the hagfish has been undertaken to estimate the number of gene loci for hemoglobin polypeptides, and to determine if all the hemoglobin molecules exist only in the monomeric form. It has been postulated that the genes for hemoglobin and the gene for myoglobin of vertebrates evolved from a common ancestral gene (*1*). While myoglobin molecules remained monomeric, consisting of a single polypeptide chain attached to one heme group, the hemoglobin molecule of higher vertebrates, from teleost fish to mammals, is a tetramer. The tertiary structure of each subunit of the hemoglobin molecule, however, has been shown to be nearly identical with the single polypeptide myoglobin (*2*). In this respect, previous reports (*3*) which have indicated the monomeric nature of hemoglobin molecules of the hagfish have been viewed with special interest. Previous works, however, have not established whether the hagfish produces more than one kind of hemoglobin polypeptide.

Twelve live specimens of the hagfish (*Eptatretus stoutii*) were used (*4*). All exceeded 30 cm in body length. Seven were gravid females, and two were males, as confirmed by the presence of spermatozoa in testicular squashes. The sex of the remaining three was not readily determinable. Blood samples of 2 to 3 ml were collected in acid citrate dextrose solution directly from a major blood vessel on the upper ventral area. From twice-washed erythrocytes, a freeze-thaw hemolysate of 1 : 2 dilution was prepared in distilled water. Nuclei and stroma were removed by centrifugation at 3000 rev/min for a period of 10 minutes.

The monomeric nature of all the hemoglobin molecules of the hagfish was confirmed by the following experiments. Thin-layer gel filtrate was used to estimate the molecular weight of hemoglobin. A layer of 0.250 mm of superfine Sephadex G-75 was spread on glass plates of 20 by 20 cm. The Sephadex had previously been equilibrated for 3 days with 0.1*M* phosphate buffer, *p*H 7.4. After the plates were spread, they were placed in a chromatographic chamber at an angle of approximately 10° and brought to equilibrium. The hemolysates were run in duplicate for at least 8 hours with a series of three marker proteins of known molecular weight. The marker proteins were monomeric cytochrome 12,000, soya bean trypsin inhibitor 21,500, and lactoperoxidase 80,000. In addition to the hagfish hemolysate, the hemolysates of man as well as the rainbow trout (*Salmo irideus*) were also added. The distance migrated was then plotted against the log of the molecular weight. In order to keep the integrity of the thin-layer plate, a piece of Whatman No. 1 paper was gently smoothed over the surface of the plate. The plate was then sprayed with the benzidine reagent solution in order to localize hemoproteins. After the localization of hemoglobin spots, the paper which was removed from the plate was quickly dried in a warm oven. The location of hemoglobin spots was marked and the position of the other proteins was determined, by using the naphthol blue black solution. A mean molecular weight value of 18,000 was obtained on the hagfish hemoglobin, while both the human and trout hemoglobins gave molecular weights in the range of 60,000. Next, the area containing the hagfish hemoglobin was scraped from the plate. The scraped material, moistened with a small amount of distilled water, was inserted into a slot in the starch-gel plate and subjected to the electrophoretic procedure, together with the original hemolysate. All the bands originally present in the hemolysate were identified in the eluate.

The fact that each monomeric hemoglobin molecule of the hagfish contains one heme group was ascertained by determining the heme/protein ratio.

First, purification of hemoglobin was obtained by a passage down a Sephadex G-50 column. Five milliliters of the hemolysate was passed into a column 2 by 50 cm prepared in 0.05*M* phosphate buffer. The high-molecular-weight proteins contained in the hemolysate were eluted prior to the hemoglobin and discarded. The hemoglobin moved down as a discrete band and was thus obtained free of contaminating material of high and low molecular weight. Direct hemochromogen was prepared by adding 0.5 ml of the purified hemoglobin to 1.5 ml of a 0.066*N* sodium hydroxide solution. One milliliter of pyridine was then added to this solution and mixed. To the optically clear solution thus obtained, a small amount of solid sodium dithionite was added, and after 3 minutes the absorbancy at 557 mμ was determined. The concentration of heme was then calculated; an extinction coefficient of 34 was used for the pyridine hemochromogen of protoheme. Protein was determined by both the Folin and the microbiuret procedures.

A ratio of 20 mg of protein per micromole of heme was found, indicating that a single heme prosthetic group was present on each molecule. The number of gene loci for monomeric hemoglobin in the hagfish was estimated by vertical starch-gel electrophoresis at *p*H 8.6, a continuous borate buffer system being used. Electrophoresis was continued for 14 hours at 4°C with a gradient of 4 volt/cm. One slice of the gel plate was then stained with the benzidine reagent, while the other half was stained with the naphthol blue black solution. Five hemoglobin phenotypes were revealed by electrophoresis. These five phenotypes are schematically illustrated in Fig. 1, and three of the five phenotypes are also shown by an actual photograph of a starch-gel plate (Fig. 2).

Six of the twelve hagfish demonstrated the identical four-band pattern. They were interpreted to be the homozygote for the wild-type allele at each of the four gene loci for monomeric hemoglobin. Under the condition employed, the No. 1 band, which moved farthest toward the anode, migrated about 30 mm from the starting line. The No. 2 and No. 3 bands were closely bunched together at positions 22 and 19 mm from the starting line, respectively. The No. 4 band stayed at the starting point, but showed a slight tendency to migrate toward the cathode [Fig. 1A and Fig. 2 (left)]. In one female and one male, the No. 2 band was missing altogether. Instead, a new band, which was slower moving than the No. 3 band, was found. These two fish were judged as homozygous for the slower-moving variant of the No. 2 hemoglobin [Fig. 1B and Fig. 2 (middle)]. The male just mentioned also showed an additional band of hemoglobin at the position 12 mm toward the cathode. Apparently he was also heterozygous for the slower-moving variant of the No. 4 hemoglobin (Fig. 1C). The same heterozygous condition for the No. 4 hemoglobin was also found in one female and one immature fish. The five-band pattern of these two fish is illustrated in Fig. 1D. One female and one immature fish demonstrated six electrophoretically distinct bands of hemoglobin. They were apparently doubly heterozygous. In addition to the four wild-type bands, the presence of the slower-moving variant No. 2 band and the cathodally migrating variant No. 4 band were recognized [Fig. 1E and Fig. 2 (right)].

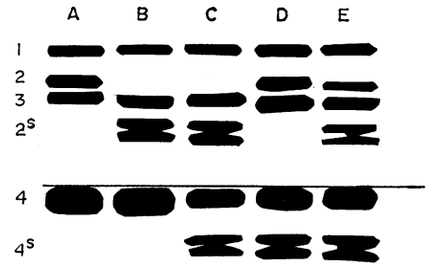

Fig. 1. Five hemoglobin phenotypes of hagfish as revealed by starch-gel electrophoresis are schematically illustrated. Anodal direction is upward. The horizontal line indicates the starting points. The four wild-type hemoglobin bands are numbered: Nos. 1, 2, 3, and 4. No. 2^s and No. 4^s denote the slower-moving variants of No. 2 and No. 4, respectively. Columns: (A) The four wild-type bands only, as shown by 6 of the 12 hagfish. (B) The four-band pattern shown by one female hagfish. A No. 2^s band is present, but the No. 2 band is missing. (C) The five-band pattern shown by one male hagfish, showing a No. 4^s band in addition to Nos. 1, 3, 2^s, and 4. (D) The other five-band pattern shown by one female and one immature fish: Nos. 1, 2, 3, 4, and 4^s bands. (E) The six-band pattern demonstrated by one female and one immature hagfish which were presumed to be doubly heterozygous. In addition to the four wild-type bands, two variant bands, No. 2^s and No. 4^s, are seen.

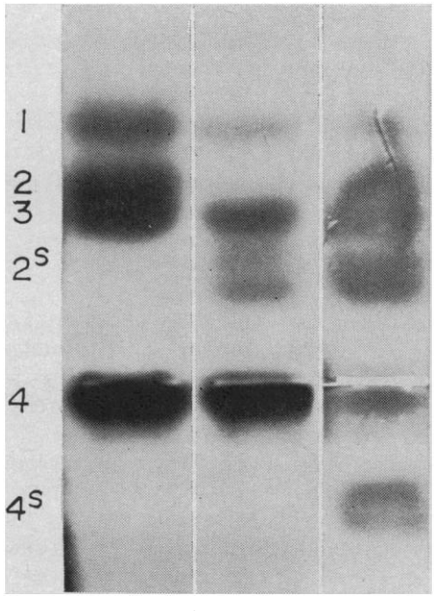

Fig. 2. Starch gel (*p*H 8.6) showing hemoglobin bands of three individual hagfish. The gel plate is stained with naphthol blue black. (Left) An immature hagfish showing the four wild-type bands only. The pattern is shown in Fig. 1A. (Middle) A female hagfish demonstrating the pattern illustrated in Fig. 1B. (Right) A female hagfish which is presumed to be doubly heterozygous. The pattern is illustrated in Fig. 1E.

The above findings were considered to be compatible with the interpretation that there are four independent gene loci for the monomeric hemoglobin in the hagfish. The unlikely possibility that bands No. 1 and No. 3 are produced by a pair of codominant alleles and that all 12 hagfish were heterozygous for those alleles cannot be excluded at this time.

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