lesions indicate that the monkey was able to discriminate 1.4 minutes of visual angle (approximately 20/30 vision) with a target brightness of 220 lux. Data collected after binocular destruction of the foveas indicate loss in acuity such that 9.0 minutes (20/180) was now the smallest visual angle correctly discriminated at the same brightness (Table 1).

When the eyelids of one eye were sewed closed, the monkey showed no signs of discomfort and began work as soon as the effects of the anesthetic disappeared. Performance on these monocular tests did not differ significantly from that when either eye could be used. Thus, in the macaque monkey, foveal lesions appear to produce decrements in visual acuity similar to those in man (*3*). The generality of these findings, of course, awaits further study with a larger number of subjects.

M. YARCZOWER
Department of Psychology, University of Maryland, College Park
M. L. WOLBARSHT
U.S. Naval Medical Research Institute, Bethesda, Maryland
W. D. GALLOWAY
Department of Psychology, University of Maryland
K. E. FLIGSTEN
U.S. Naval Medical Research Institute
R. MALCOLM
Department of Psychology, University of Maryland

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10. We thank J. R. Hayes for the histological examination and for help in all phases.
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2 March 1966

Sea Cucumber Sibling Species: Polypeptide Chain Types and Oxygen Equilibrium of Hemoglobin

Abstract. *The hemoglobin of the "thin" sibling species of* Thyonella gemmata *(phylum: Echinodermata; class: Holothuria) has three electrophoretically distinct polypeptide chains. In "stout" sibling species of* T. gemmata *there are only two chain types. These results account for the greater number of multiple hemoglobins in "thins" than in "stouts," as well as for differences in the amounts of some of the multiple hemoglobins when comparisons are made of hemolyzates of erythrocytes from the water vascular system and from the main body cavity of the "thin" but not the "stout" sibling species.*

Survey of a population of common intertidal sea cucumbers (holothurians) for hemoglobin variants resulted in the observation that what had been previously considered a single species, *Thyonella gemmata* (Pourtalès), was in fact two distinct populations consistently separable on the basis of elec-

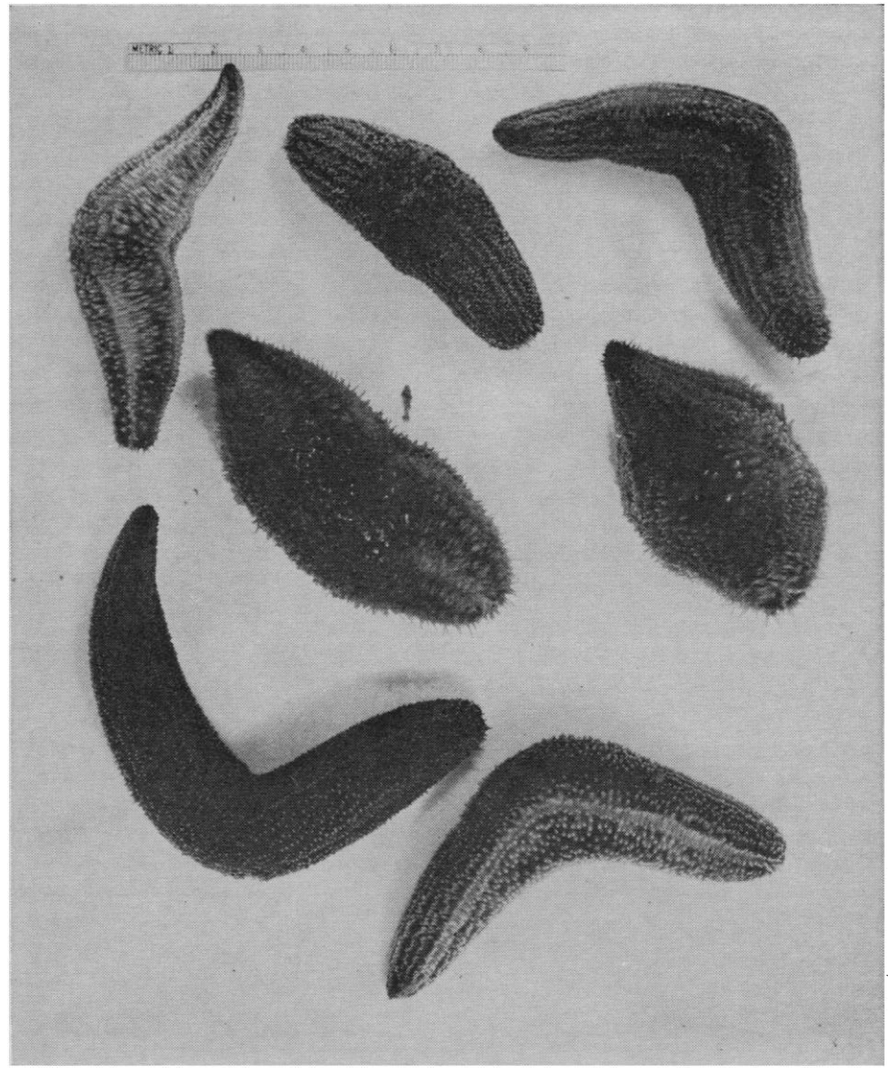

Fig. 1. The sibling species of *Thyonella gemmata*. Five "thins" are at the bottom and top of the picture; two "stouts" are in the middle. The animals have been removed from sea water for 20 minutes; during that time the "thins" retract their tube feet almost completely, whereas the "stouts" do not. This gives the two "stouts" a slightly bushier appearance. "Stouts" are on the whole somewhat fatter than "thins", but the plastic shape of sea cucumbers makes this character unreliable. Other convenient behavioral differences are given in (*1*). "Stouts" tend to have bushier oral tentacles than "thins" and this characteristic is useful when the tentacles are exposed—at night.

trophoresis of hemoglobin, esterases, and proteins extractable in solutions of low ionic strength (*1*). These sibling species are designated "thins" and "stouts" (Fig. 1). Previously (*1*) two unusual properties of the hemoglobins were not explained: (i) Hemoglobin from all "thins" is readily resolved by electrophoresis into at least eight distinct hemoglobin components, whereas hemoglobin from "stouts" resolves into only five components (*2*). (ii) These particular sea cucumber species have erythrocytes in two different body-fluid compartments, the perivisceral coelom and water-vascular system. Comparison of hemoglobin from coelomic erythrocytes and water-vascular erythrocytes of "stouts" indicated no differences, whereas comparison of hemoglobin from the two body-fluid compartments in "thins" showed that the relative proportions of some of the multiple hemoglobins were different, although no qualitative difference, such as occurs in certain worms, was found (*3, 4*). New studies on these sea cucumber hemoglobins provide the explanation for these unusual facts.

Collection of sea cucumbers, identification of sibling species by behavioral characteristics and by hemoglobin electrophoresis, and preparation of uncontaminated coelomic and water vascular hemoglobin samples have been described (*1*). Smithies' vertical starch-gel electrophoresis was used to separate the multiple hemoglobins in *p*H 8 to 8.5 buffers (*5*), and to separate the polypeptide chain types of globin prepared (by the acid-acetone method) from each of the separated multiple hemoglobins, the gels being *p*H 2 or with 8*M* urea at *p*H 8 (*6*). The hemoglobins were crystallized prior to many of the electrophoretic and oxygen-binding experiments (*7*). Oxygen equilibria were evaluated spectrophotometrically with the crystalline hemoglobins, having been dialyzed against common phosphate buffers (*8*).

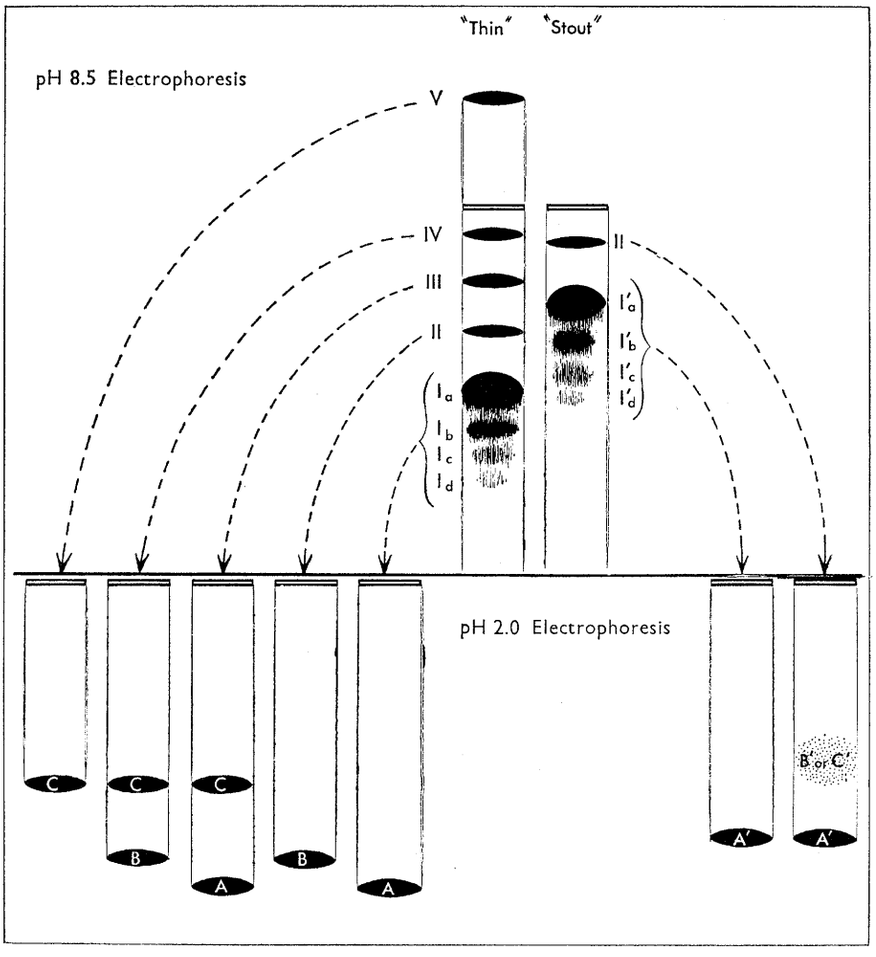

Fig. 2. Representation of the starch gel electrophoresis of the multiple hemoglobins of "thin" and "stout" sibling species of *T. gemmata*, followed by electrophoresis in formate-HCl (*p*H 2) for separation of the globins into polypeptide chains. In the upper part of the diagram the cathode is at the top and the anode at the bottom; in the "second dimension" of separation, the anode is at the top and the cathode at the bottom.

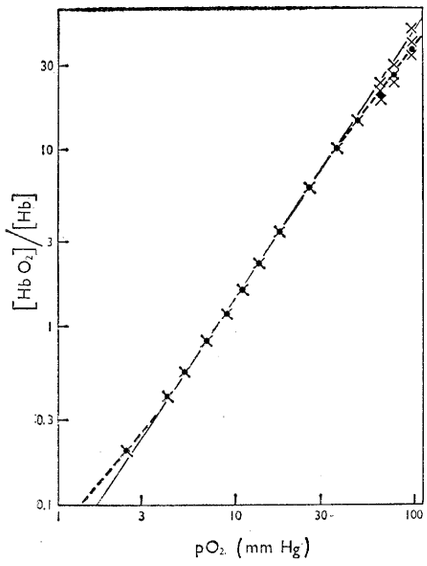

Fig. 3. Oxygen equilibrium curves for crystallized and dialyzed hemoglobins of three different individual "thins", all designated **X**, and one "stout", represented ●. Two percent hemoglobin solution in potassium phosphate buffer; ionic strength 0.6; *p*H 7.5; 25°C.

Resolution of the multiple hemoglobins by electrophoresis at *p*H 8 to 8.5, followed by resolution of the polypeptide chains from the separated globins by electrophoresis at *p*H 2, is shown diagrammatically in Fig. 2. The electrophoretic pattern is not modified by the presence of 1 percent mercaptoethanol and, thus, no disulfide (–S–S–) bonds are involved in the quaternary structure of these sea cucumber hemoglobins. Electrophoresis of the dissociated globins indicates clearly that the "thin" sibling species has three different polypeptide chain types and the "stout" species has only two. The mobilities of these sea cucumber hemoglobin chain types are similar to human α- and β-chains, which were run as "controls." In both "thin" and "stout" species the most anodal hemoglobins have only one polypeptide chain type—like abnormal human Hb H. That the sea cucumber hemoglobins I_a, I_b, I_c, and I_d have the same polypeptide chain type is also suggested by the interconvertibility of this series; that is, reaction of the hemoglobin with iodoacetamide results in an increase of I_a at the expense of the other three components, whereas reaction with iodoacetate shifts the equilib-

rium in favor of I_b. The other hemoglobin components are composed of either a single chain type (II is B-chain only, V is C-chain only) or are composed of equal amounts of two different chains. Type III is AC, IV is BC. Under favorable conditions of electrophoretic resolution a small component cathodal to I_a is obtained and in acid gels this has the chain types A and B in equal amounts (*9*).

In contrast to the "thin" sibling species, "stouts" have only two chain types, the major one being most likely equivalent to the A-chain of "thins." Thus, a simple explanation is provided for the greater heterogeneity of "thin" sibling species hemoglobin: there are more polypeptide chain types.

The possibility that the difference in chain types might have some physiological advantage is contradicted by studies on oxygen binding, for there is not even a slight difference in oxygen equilibria when "thin" and "stout" hemoglobins are compared (Fig. 3). Both hemoglobins have identical oxygen affinities, low heme-heme interactions ($n = 1.4$), and no Bohr effect, at least over the range of *p*H's used (6.5 to 8.0). These allosteric properties are similar to those observed in other studies on sea cucumber hemoglobin oxygen equilibria (*4, 10*).

In that studies on the multiple hemoglobins of the related sea cucumber *Thyone* (now *Sclerodactyla*) *briareus* indicate three different polypeptide chain types, each with an electrophoretic mobility similar to A-, B-, and C-chains of the "thin" sibling species, the most plausible explanation of the results is the loss of expression of one of the three hemoglobin genes in the course of evolution of "thin" and "stout" *Thyonella gemmata* from a common ancestor. This loss might be due to actual gene deletion, to mutation of some hypothetical control gene to the "off" position, or to mutation to the point the protein product is no longer recognizable as hemoglobin. No unusual nonhemoglobin erythrocyte protein occurs in "stouts" when they are compared with "thins" in starch-gel electrophoresis of erythrocyte extracts.

The subtle difference in electrophoretic pattern between water-vascular and coelomic hemoglobin preparations of "thin" *T. gemmata* is shown in Fig. 4. Consistently (30 specimens) the water-vascular erythrocytes have a higher concentration of hemoglobin component II, which is composed only of B-chains, whereas the coelomic erythrocytes have a higher concentration of hemoglobin component III, which is composed of A- and C-chains. Thus, although A-chains are produced in greatest amounts in both erythrocyte locations, there are more B- than C-chains in the water-vascular erythrocytes and more C- than B-chains in the perivisceral coelom erythrocytes. The reason that "stouts" do not show this relative difference in amounts of certain hemoglobins in the water-vascular and coelomic body-fluid compartments is clear: "Stouts" have only one kind of minor chain type and, thus, cannot modulate the ratio of B- to C-chains. The extreme situation is seen in the related *Thyone briareus*: only the water vascular system has erythrocytes, there being no hemoglobin in the perivisceral coelom.

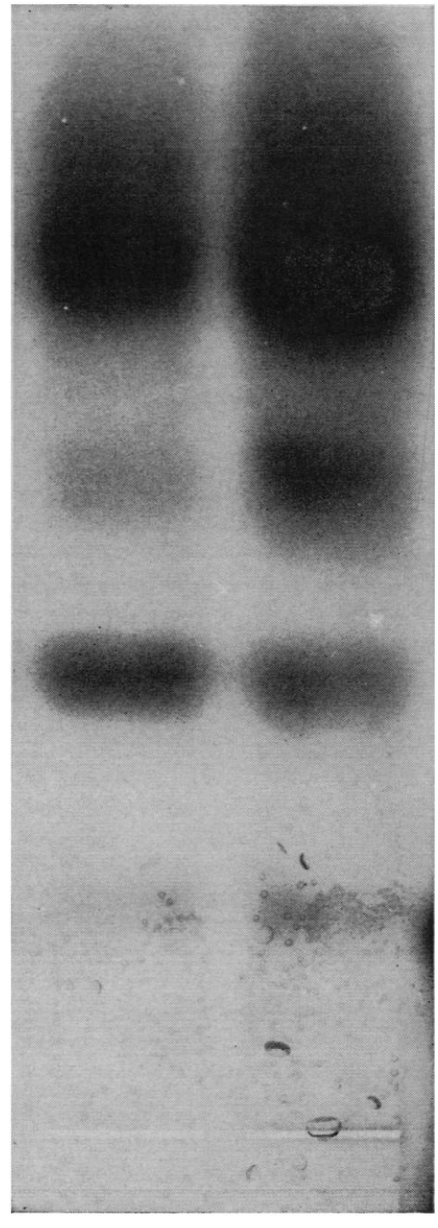

Fig. 4. Gel electrophoresis in *p*H 8.4 borate buffer of hemoglobin from the perivisceral coelom (left) and water vascular system (right) of the same individual "thin" sibling species of *Thyonella gemmata*. The anode is at the top, the cathode at the bottom of the picture. Note the greater concentration of hemoglobin in component II, containing B-chains, in the water vascular sample and the greater concentration of hemoglobin in component III, containing A- and C-chains, in the perivisceral coelom sample. The gel has been stained for hemoglobin with *o*-dianisidine and hydrogen peroxide; the bubbles are from the decomposition of hydrogen peroxide by the very active erythrocyte catalase in this species. The cathodal hemoglobin, component V, is not shown.

The relative differences in the amounts of B- and C-chains in coelomic and water-vascular erythrocytes of "thins" is reminiscent of lactate dehydrogenase isozyme differentiation; only rarely does a given organ or cell type have completely H or M types of LDH polypeptide chain; usually a mixture of five isozymes occurs, the proportions varying during the course of development and, possibly, in reflection of the relative significance of aerobic to anaerobic pathways (*11*). Except for the sea cucumber, tissue and developmental specificity of hemoglobin have appeared to be absolute; that is, muscle hemoglobin (myoglobin) does not occur in red blood cells. Thus, the sea cucumber example of relative, rather than absolute, difference in tissue distribution of polypeptide-chain types makes hemoglobin differentiation more comparable to that for lactate dehydrogenase.

CLYDE MANWELL
Department of Biological Science, Florida State University, Tallahassee

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7. *Thyonella gemmata* hemoglobin was crystallized by the following method, which has also been used successfully on the sea cucumbers *Thyone briareus, Cucumaria elongata, C. miniata*, and *Molpadia intermedia*. Most of the hemoglobin is precipitated between 35 and 45 percent saturation with ammonium sulfate at 0°C, at *p*H 7.0 to 7.5. The precipitate is dissolved in approximately 10 volumes of cold potassium phosphate buffer (ionic strength, 0.6; *p*H 7.5), and the hemoglobin solution is dialyzed against distilled water at 0°C. Within from 12 hours to, at the most, 3 days good-sized crystals are obtained. Oxyhemoglobin, CO-hemoglobin and methemoglobin can be crystallized. In my studies the hemoglobins were kept under CO except where destined for oxygen equilibrium measurements. The hemoglobin heterogeneity is not altered by crystallization.
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9. Molecular-weight data might also be explained by the interconvertibility of hemoglobin components I_a, I_b, I_c and I_d in terms of successive dissociation of a tetramer, although the data are still equivocal. An experiment on a preparation of hemoglobin from the "thin" sibling species was performed by Dr. C. D. Trader, Dept. of Chemistry, Florida State University. Only a single sedimenting component appears in "thin" hemoglobin examined in the analytical ultracentrifuge; the molecular weight estimated from the corrected sedimentation constant is 41,000. This is too high for the expected molecular weight of the hemoglobin dimer (32,000 to 34,000) and too low for that of the tetramer (66,000 to 68,000). The unusual molecular weight may result from either (i) a polypeptide chain size different from that of the 16,000 to 17,000 unit typical of all vertebrate and some invertebrate hemoglobins, or (ii) a sufficiently rapid dissociation and association of subunits so that a single boundary is observed in sedimentation equilibrium. Whatever the explanation for the anomalous molecular weight, it does not detract from the significance of the studies on polypeptide-chain type, although it means that at present it is not possible to tell if the multiple hemoglobins are dimers (A_2, AB, AC, etc.) or tetramers (A_4, A_2B_2, A_2C_2, etc.), or some mixture.
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Stimulation of the Proliferation of Cortical Neurons by Prenatal Treatment with Growth Hormone

Abstract. *Subcutaneous or intravenous injections daily of purified bovine pituitary growth hormone into pregnant rats from the 7th till the 20th day of pregnancy (total dose 36 mg) resulted in offspring with unchanged body weight but with significant increases in brain weight, brain DNA content, cortical cell density, and ratio of neurons to glia.*

This report deals with the problem of whether the final number of cortical neurons can be experimentally increased for the eventual purpose of studying a possible correlation between such an increase and behavior.

Conceivably, such an increase in number could be achieved by stimulating mitoses during the period in which neurons are still capable of dividing (proliferation period). In mice and rats this period ceases around birth (*1*, *2*). Conceivably such stimulation will be more effective for cells with a short proliferation period (neurons) than for cells for which this period extends longer throughout life. Pituitary growth hormone was chosen as the proper stimulant since this hormone is known to stimulate protein synthesis and to improve nitrogen retention. Nitrogen retention may also result in stimulation of synthesis of other essential nitrogenous constituents of the cell, such as nucleic acids.

These concepts were supported by experimental evidence. Previous studies (*3*, *4*) have demonstrated that subcutaneous injections of bovine pituitary growth hormone into tadpoles (*3*) or pregnant rats (*4*) resulted in a statistically significant increase in brain weight and in number of brain cells (of tadpoles or of rat offspring, respectively). These experiments had certain inadequacies which, at that time, could not be rectified. One was the possible side effects of the impurities in the growth hormone preparations that were then available; another was that the finding of an increase in total number of cells, which also included glia, did not prove that the number of neurons themselves has increased. Still another was that

Table 1. Offspring of rats injected subcutaneously with bovine pituitary growth hormone from the 7th till the 20th day of pregnancy.

Newborn				20-day-old	
Weight (g)		Brain wt/ body wt	Total DNA per brain (μg)	Cortical cell density (10^6 cells)	Neuron-glia index†
Brain	Body				
Control rats					
0.156 ± .006*	6.25 ± .47	0.0247 ± .0019	438 ± 37	1.9 ± .05	1.87 ± .25
Experimental rats					
0.182 ± .024	6.4 ± .71	0.0285 ± .0017	495 ± 32	3.1 ± .15	3.2 ± .32
Increase (% of control)					
17	2.4	15.4	13	63	71
Probability					
.004	.60	< .001	< .001	< .001	< .001

* Standard deviation. † Estimates on 35 (5625 μ^2) grids per slide (2nd and 3rd layer), 21 slides (*11*, *12*).

Table 2. Offspring (newborn) of rats injected intravenously with bovine pituitary growth hormone from the 7th till the 20th day of pregnancy.

Weight		Brain wt/body wt	Total DNA per brain (μg)
Brain (g)	Body (g)		
Control			
0.152 ± .008*	5.98 ± .44	0.0248 ± .0017	426 ± 37
Experimental			
0.197 ± .018	6.06 ± .62	0.0329 ± .0011	530 ± 19
Increase (% of control)			
30	1	33	24
Probability			
< .001	.66	< .001	< .001

* Standard deviation.