pathological changes in wasting disease must be considered.

Finally, it is suggested that the members of the normal flora, and their ecological relationships, may play an important role in the pathogenesis of other wasting syndromes.

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6 May 1965

Hybridization Experiments: Evidence of Dissociation Equilibrium in Hemerythrin

Abstract. *Partial succinylation of hemerythrin alters its electrophoretic mobility even though it remains an octameric macromolecule. Mixtures of this modified protein and unmodified hemerythrin generate species of intermediate electrophoretic mobility. Such behavior provides strong evidence that the octameric macromolecule is in mobile equilibrium with monomeric subunits.*

Hemerythrin, the iron-containing (in a form other than heme iron), oxygen-carrying protein of sipunculids, interacts with iron-coordinating ligands (*1*) as well as with sulfhydryl-blocking reagents (*2*). These interactions are easily detected since the iron-coordinating ligands alter the visible spectrum of the protein, and the sulfhydryl-blocking reagents cause dissociation of the protein into subunits.

Since the reaction of the –SH groups with organic mercurials or *N*-ethylmaleimide does not change the spectrum of hemerythrin, these groups cannot be in the immediate environment of the iron. Nevertheless, *N*-ethylmaleimide does not react with the –SH groups of hemerythrin in the absence of iron-coordinating ligands whereas the –SH groups become readily accessible in the presence of N_3^- ion (*1*). Thus, an interaction at one locus on the protein affects the reactivity of a second, and we have a clear example of a cooperative interaction.

A molecular interpretation of this cooperative interaction is provided by the assumption that native octameric hemerythrin is always in equilibrium with a very small amount of the monomeric form (Fig. 1). The key role of the –SH groups in preserving the native structure of hemerythrin suggests that these groups take part in some intra- or intermolecular interaction in the octamer. It seems reasonable to assume that these groups are more exposed in separated subunits. Indeed there is experimental evidence (*3*) for the masked character of –SH groups in native hemerythrin and for their availability in 8*M* urea, where dissociation occurs. Since ions such as N_3^- are bound more strongly by monomeric hemerythrin (produced by reaction with *N*-ethylmaleimide) than by octameric (*1*), these ions should shift the dissociation equilibrium (Fig. 1) to the right, increase the concentration of exposed –SH groups and hence accelerate the rate of reaction with sulfhydryl-blocking reagents.

This explanation is not the only one possible, however; a conformational rearrangement (without dissociation) of the octamer might be generated by the binding of ligands, and such a rearrangement could be responsible for the cooperative interaction.

We have searched, therefore, for experiments that might provide evidence in favor of one of these alternative explanations. In particular we have attempted to design an experiment that might detect very small amounts of subunit in equilibrium with octamer, if such an equilibrium does exist (*4*).

Such an experiment became feasible when we found that mildly succinylated hemerythrin remains in the octameric form. Succinylated, octameric hemerythrin was obtained by two methods. In the first, the –SH group of the protein was protected with a mercurial, the resulting monomer was succinylated, and the modified octamer was regenerated by removal of the mercurial. Modified octamer was also obtained by succinylating the native hemerythrin with a small amount of succinic anhydride in relation to the amount of protein. Either procedure

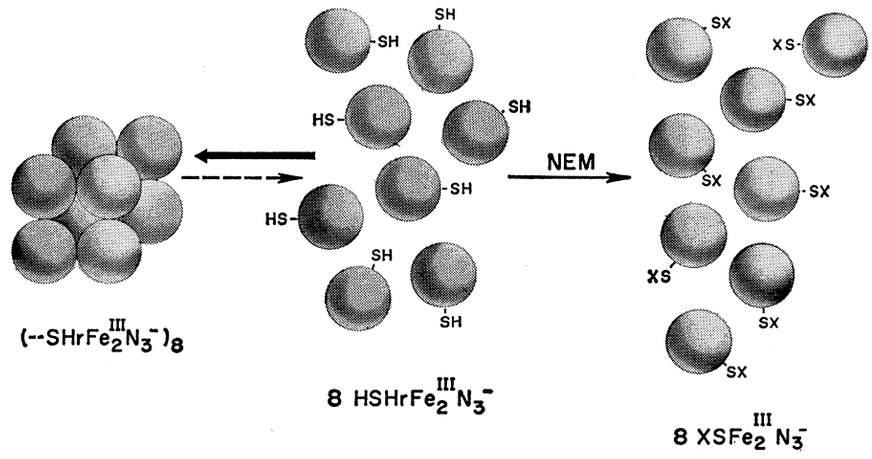

Fig. 1. Representation of equilibrium between intact hemerythrin (as azide complex) and its subunits, showing also the shift to monomers when *N*-ethylmaleimide combines with the sulfhydryl group.

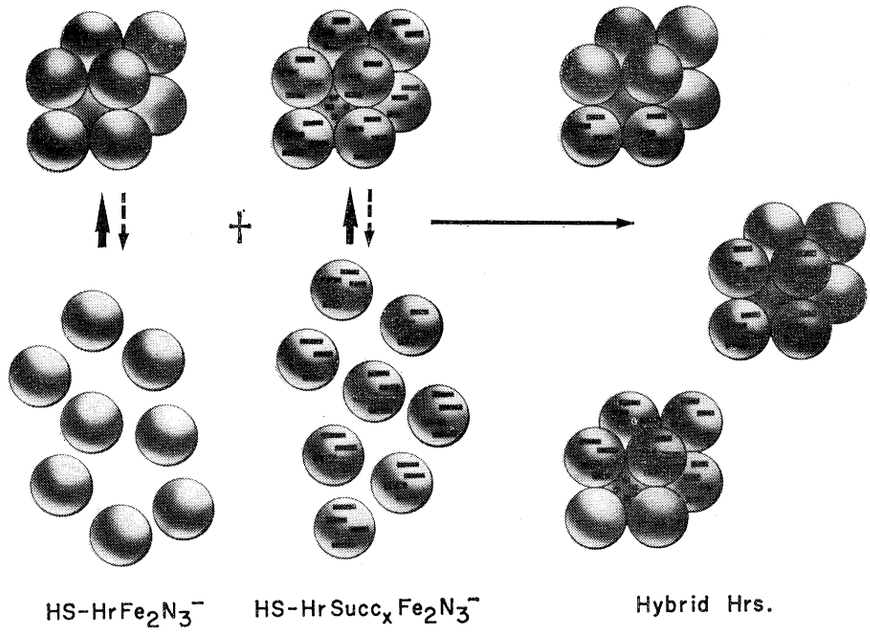

Fig. 2. Schematic representation of hybridization experiment with hemerythrin and succinylated hemerythrin.

yields a marked hemerythrin with a substantially larger negative charge than that of the native unmodified protein. The extra charge does not interfere with the aggregation process, and does provide an aggregate with a much greater electrophoretic mobility than that of the native protein.

Using the succinylated octamer we performed an experiment (Fig. 2) to test for the presence of a very small amount of subunit in equilibrium with octamer.

If native hemerythrin is in equilibrium with a small amount of its subunit and succinylated hemerythrin is in equilibrium with a small amount of its monomer, a mixture of the two octameric proteins should generate hybrid octamers with various proportions of the two types of subunit. These hybrids will have different charges and hence mobilities different from either of the parent octamers. They should be easily detectable by electrophoresis experiments in a starch-gel matrix.

A sample of hemerythrin with its –SH group blocked by salyrganic acid was succinylated (*6*) by addition of 20 mg of succinic anhydride per 100 mg of protein. Excess reagent, salts generated in the reaction, and any subunits were removed by chromatography on Sephadex G25, which simultaneously transferred the (octameric) protein into the desired buffer, usually tris-(hydroxymethyl)-aminomethane at about *p*H 8. A sample of this solution, adsorbed on a small piece of filter paper, was placed in starch-gel prepared in a tris-citrate buffer of *p*H 8.8 (*7*). Electrophoresis was then carried out (*8*) for a period of 18 hours at 330 volts (18 volt/cm) and with a 10-ma current.

The position of succinylated octameric hemerythrin is shown in column 1 of Fig. 3. A corresponding solution of native hemerythrin subjected to the same electric field in the same gel moved very little, as is demonstrated in column 3. A third solution was prepared containing a 1:1 mixture of native and succinylated hemerythrins. This mixture was allowed to stand for 24 hours at 4°C. It was then also placed on the starch gel and subjected to the same electric field. The pattern obtained is shown in column 2 of Fig. 3. Very little density is found at the position of either original component hemerythrin. On the other hand, species of all intermediate electrophoretic mobilities are strongly visible. Since succinylation produces a statistical distribution of hemerythrins of modified charge, and labeled and unlabeled monomers are randomly combined, this result is exactly what one would expect from the hypothesis of octamer-monomer equilibrium, but would not be expected if the protein does not dissociate reversibly.

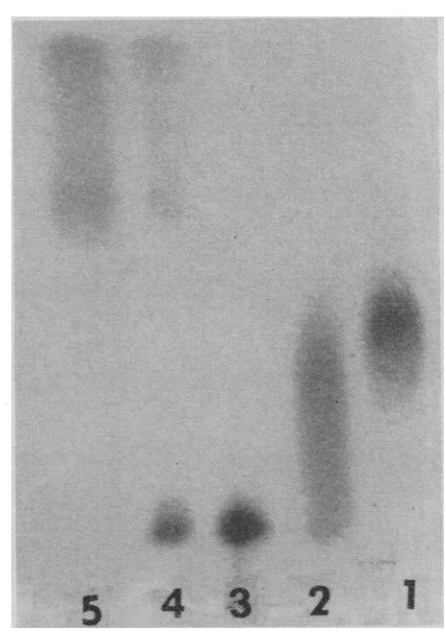

Fig. 3 Starch-gel electrophoresis of mixtures of hemerythrins: Pattern 1, succinylated hemerythrin octamer; pattern 2, mixture of 1 and 3; pattern 3, native hemerythrin octamer; pattern 4, mixture of 3 and 5; pattern 5, heavily succinylated hemerythrin (azide complex) irreversibly in monomeric state.

A control experiment was carried out as follows. A sample of heavily succinylated hemerythrin was prepared which disaggregated into monomers and could not reassociate. It was stabilized against denaturation by addition of 0.001*M* azide ion. This preparation had a very high mobility in starch gel (column 5, Fig. 3), and incidentally showed evidence of two components. A mixture of this material with native hemerythrin was also prepared, allowed to stand for 24 hours at 4°C, and placed on starch gel. As column 4 shows, the original components are present, unmodified in mobility. Furthermore there is no density in the intermediate range of mobilities. Thus the octamers contain no succinylated subunits.

This method of detecting subunits in equilibrium with native protein should be quite general in applicability. Essentially all proteins contain lysyl residues and thus should react with succinic anhydride to produce macromolecules with altered electrophoretic mobility. If hybridization occurs between unmodified and succinylated aggregates, as can readily be tested either by starch-gel or free-boundary electrophoresis, then strong evidence has been provided that a mobile equilibrium exists between aggregated protein and its subunits.

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29 June 1965

Synaptic Connections of the Centrifugal Fibers in the Pigeon Retina

Abstract. *The centrifugal fibers in the pigeon retina end in the inner nuclear layer and form two kinds of terminals, convergent and divergent. In the inner nuclear layer the fibers synapse with amacrine and displaced ganglion cells. Because of their great number and their even distribution these fibers appear to constitute a system for the localized centrifugal control of the retinal functions.*

There is abundant and conclusive anatomic evidence demonstrating the existence in vertebrates of centrifugal fibers which come from the brain to end in the retina (*1*, *2*, *3*, *4*). However, their mode of connection and their functional role are not as yet well known. According to Cajal (*1*) and Dogiel (*2*), who first studied them, the centrifugal fibers end in the retina in the inner aspect of the layer of bipolar cells (sublayer of the amacrine cells, according to Cajal), forming synapses only with the association amacrines and ordinary amacrines. Physiological studies (*5*) in various vertebrates confirm the existence of centrifugal fibers.

We have made a careful examination of pigeon retinas vitally stained with methylene blue (*3*). Our observations indicate that the centrifugal fibers enter the retina at the optic papilla, follow a path amidst the optic axons, then cross obliquely the inner plexiform layer, and bend a few microns before reaching the inner nuclear layer, where they follow, parallel to it, a course of variable length and direction, and end in its innermost aspect. Although the course of each centrifugal fiber seems erratic, the fiber endings are evenly distributed over the entire expanse of the retina; the centrifugal fiber which enters a particular retinal segment ends in it. This relation is in agreement with the findings of McGill (*6*) with respect to the projection on the retina of the nuclei from which the centrifugal fibers originated.

Centrifugal fibers can be divided into two types according to their mode of terminal branching.

1) In the convergent type, the centrifugal fiber ends in several branches of different lengths and thicknesses. These, after following separate paths that make loops sometimes hundreds of microns wide, converge to form a synaptic nest on a single cell body (Figs. 1 and 2). The synaptic nests may give off small branches which make additional loops that end either on the same nest or on some neighboring cell. Sometimes these collateral loops, or others given off earlier, contribute to synaptic nests formed by other converging terminals. Convergent fibers make synapses with very few cells, usually not more than three or four, and frequently with only one. They penetrate the inner nuclear layer and form synaptic nests as deep as the second or third cell row, but some of their slender branches may penetrate deeper. However, most of their synapses are confined to the innermost quarter or fifth of this layer, where the amacrines lie.

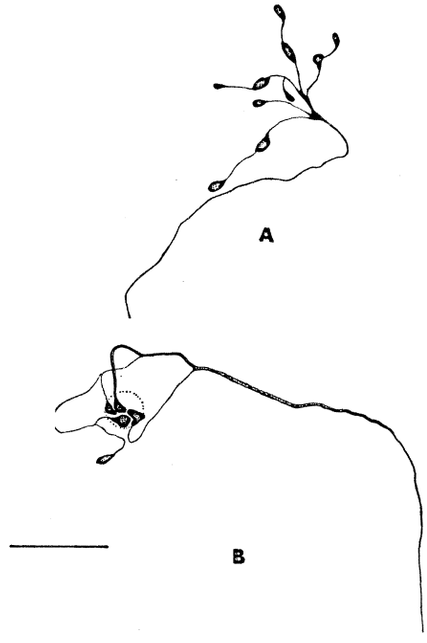

Fig. 1. Camera lucida drawings of retinas mounted flat, vitreal side uppermost. Oil immersion objective of the microscope was used. (*A*) Divergent terminal; (*B*) convergent terminal; the dotted circle represents the outline of a cell body, 7 μ in diameter, on which the terminal impinges. Scale, 20 μ.

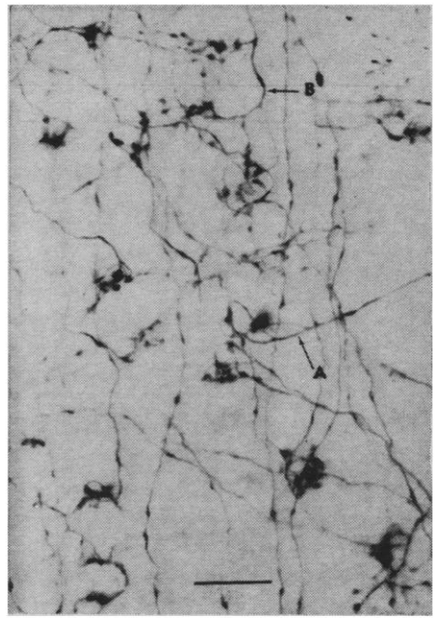

Fig. 2. Centrifugal terminals in a retina stained with methylene blue as seen from the vitreal side: Arrows: (*A*) convergent terminal, (*B*) divergent terminal. Scale, 40 μ.

2) In the divergent type, the centrifugal fiber ends in several fanlike branches which make successive synapses with different cells and sometimes at several separate places on the same cell (Figs. 1 and 2). In general, they penetrate less deeply into the inner nuclear layer than the convergent type does, and usually they are confined to the boundary area between this layer and the inner plexiform layer.

Centrifugal fibers synapse on at least three types of cells. Of these, (i) displaced ganglion cells have their perikaryon (9 to 15 μ in diameter) amidst the amacrines of the inner nuclear layer at the boundary with the inner plexiform layer. A single expansion which enters the inner plexiform layer, where it divides into several thick dendrites, sprouts from the cell body. The dendrites give off many secondary and tertiary branches which form a single dendritic stratum 300 to 600 μ wide and 8 to 10 μ below the inner nuclear layer. The axon issues from one of the main dendrites, crosses the internal plexiform layer, and enters the layer of optic fibers, leaving the retina with these fibers. At the origin of their main dendrites the displaced ganglion cells receive at least one synaptic bouton from one centrif-