Reports

Hemoglobin M

A methemoglobin possessing a unique spectral absorption curve was found by Hörlein and Weber (*1*) in a family exhibiting a cyanotic condition in four generations. By interchanging the heme and the globin with the corresponding components of normal methemoglobin, the abnormality was found to reside in the globin fraction. On the basis of this description, the condition has been included among the abnormal hemoglobin syndromes, and the designation *hemoglobin M* has been applied to this protein (*2*).

A family of German extraction has now been discovered in New England possessing a similar "cyanotic" condition in at least four generations, both males and females being affected. This preliminary report concerns the findings in an extensive study of the hemoglobin from one of these affected individuals (*3*).

Venous blood from this individual had a chocolate-brown color, which was unaffected by shaking in the presence of air. Since methemoglobin as determined by the method of Evelyn and Malloy (*4*) was not increased, the presence of an abnormal pigment such as that described by Hörlein and Weber was suspected. When a modification of the starch electrophoretic technique of Kunkel (*5*) was used, the fresh hemolyzate was found to migrate to the cathode at *p*H 7.2 (sodium phosphate buffer containing 0.029 mole of Na_2HPO_4 and 0.010 mole of Na_2HPO_4 per liter) as a diffuse band. After prolonged electrophoresis, the diffuse band was seen to consist of three regions distinguishable by their color. The leading (cathodic) and the trailing margins of the hemoglobin band (hereafter identified as zone A and zone C, respectively) were of an oxyhemoglobin hue, while the area between these zones was brownish in color (zone B) (*6*). When hemoglobin from a normal individual was subjected to electrophoresis under these conditions, only a single band (paralleling zone A) was seen. Zone A was therefore presumed to be normal adult oxyhemoglobin.

Zones B and C were too narrow to permit isolation of a sufficient quantity of pigment from the individual zone without contamination from the adjoining regions. Zone C was therefore removed in its entirety, along with a small amount of zone B; the eluate from this is designated as "the eluate from zone C." Zone A was sufficiently broad to permit isolation of its pigment without contamination, and its eluate was used throughout as a control, representing the reactions of normal adult oxyhemoglobin. The spectral characteristics of the hemoglobin derivatives prepared from this eluate did not differ significantly from the published data for normal hemoglobin (*7*). Spectrophotometric studies were made of the eluates of the various regions, and as is discussed in the following paragraphs, evidence was obtained indicating that the zone-C eluate contained both an abnormal oxyhemoglobin and an abnormal methemoglobin.

The pigments of the two regions were recovered by eluting with sodium phosphate buffer of *p*H 6.5. The spectral absorption curve of the zone-A eluate exhibited maxima at 540 and 570 mμ (*8*). These maxima were indistinguishable in their location from those of normal oxyhemoglobin, as was evidenced by comparison with untreated hemolyzates of normal bloods.

The spectral absorption curve of the zone-C eluate exhibited maxima at 495, 530, and 570 mμ and, in addition, a slight inflection at 600 mμ. The spectral characteristics at 495 and 600 mμ were thought to be the result of the contaminating pigment of zone B. The maxima at 530 and 570 mμ indicate the presence of an oxyhemoglobin, in agreement with the visual impression of an oxyhemoglobin hue in zone C. The discrepancy between 530 and 540 mμ for the respective maxima for zones A and C is of doubtful significance, in view of the limitations of the spectrophotometer used and the fact that the zone-C eluate contains a mixture of pigments.

Further verification of the presence of an oxyhemoglobin in zone C was obtained by the addition of an oxidizing agent (potassium ferricyanide) to the eluate. After such treatment, the peaks at 530 and 570 mμ were obliterated (Fig. 1, zone C), indicating their origin from an oxidizable pigment such as an oxyhemoglobin. The curve so obtained closely resembles that published by Hörlein and Weber (*1*) for their abnormal methemoglobin.

The spectral changes occurring when normal oxyhemoglobin is oxidized to methemoglobin are illustrated by the response of the eluate of zone A (Fig. 1). The oxyhemoglobin peaks at 540 and 570 mμ are absent, and the peaks due to the presence of methemoglobin (500 and 630 mμ) are apparent. The failure of a peak to appear at 630 mμ after oxidation of the oxyhemoglobin from zone C indicates that its methemoglobin differs from this derivative of normal hemoglobin in its spectral characteristic. This oxyhemoglobin, which differs both electrophoretically and spectroscopically (as the methemoglobin derivative) from normal adult hemoglobin, has been named *hemoglobin M* in accordance with the application of this term (*2*) to the clinically and chemically similar cases of Hörlein and Weber (*1*).

The pigment of zone B which contaminates the eluate of zone C can be shown to be a methemoglobin, as follows. Addition of sodium cyanide to the zone-C eluate results in a decrease in optical density at 500 mμ and a considerable increase in optical density at

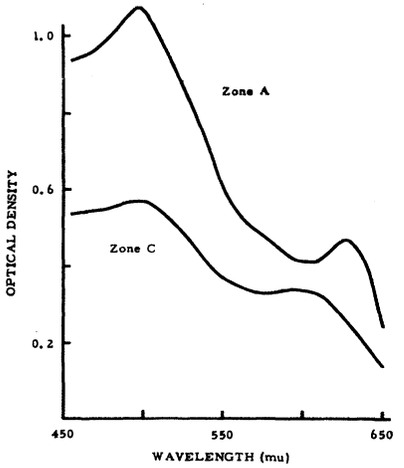

Fig. 1. Spectral absorption curves for the oxidized eluates from different regions of the hemoglobin electrophoretic pattern. Concentration of pigments: zone-A eluate, 0.9×10^{-4} mole/lit; zone-C eluate, not determined.

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540 mμ. These are the results expected if a cyanmethemoglobin is formed (by the reaction of cyanide with methemoglobin), with spectral properties resembling the normal compound. If this solution is now oxidized with potassium ferricyanide and again treated with sodium cyanide, a curve with a single maximum, in the visible region, at 535 mμ results. (When the zone-A eluate was similarly treated, the maximum was found to be at 545 mμ. In addition, the maximum for the zone-C pigment was broader than that for zone A. As was noted in the case of the oxyhemoglobin spectra, such differences cannot be considered significant at the present time.) From this it is concluded that all of the pigment in the oxidized eluate of zone C is reactive with cyanide and, hence, presumably is methemoglobin. On the basis of these findings, it may be stated that the original eluate from zone C is a mixture of methemoglobin and oxyhemoglobin.

We have as yet no direct proof that the pigment of zone B is formed from the oxyhemoglobin of zone C, since we have not succeeded in separating them sufficiently to study their interconversion in detail. The relatively greater cathodic migration of the zone-B pigment suggests this relationship however. If the two pigments differ only in the valence of the iron of the heme group (+2 for the oxyhemoglobin and +3 for the methemoglobin), then the methemoglobin would be expected to migrate cathodically slightly in advance, since it would have the greater positive charge. If we assume that these two pigments are so related, the spectral curve of the oxidized eluate of zone C (Fig. 1) is that of pure methemoglobin M. The absence of a maximum at 630 mμ explains the failure to detect methemoglobin by the method of Evelyn and Malloy (*4*), since this test is dependent on the disappearance of this maximum after chemical treatment. The finding of a hemoglobin derivative with spectral characteristics in the visible region differing from those of the normal compound is unique, since the location of maxima is nearly identical, even when hemoglobin derivatives from different vertebrate species are compared (*7*).

In summary, a second family with methemoglobinemia of the Hörlein and Weber type has been discovered. Both normal oxyhemoglobin and an abnormal oxyhemoglobin, designated *hemoglobin M*, have been identified in the hemolyzate from one affected individual. An abnormal methemoglobin differing in its visible spectral characteristics from normal methemoglobin is also present. It is tentatively concluded that the abnormal oxyhemoglobin is the precursor of the abnormal methemoglobin, and in consequence the term *methemoglobin M* has been applied to the latter pigment. From the available family history, it is expected that hemoglobin M will show the dominant inheritance pattern usually found with the abnormal hemoglobins. The individual described herein then represents the hemoglobin-M trait.

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References and Notes

1. H. Hörlein and G. Weber, *Deut. med. Wochschr.* 73, 476 (1948).
2. K. Singer, *Am. J. Med.* 18, 633 (1955).
3. We are indebted to G. N. French for referring this patient to us.
4. K. A. Evelyn and H. T. Malloy, *J. Biol. Chem.* 126, 655 (1938).
5. H. G. Kunkel and G. Wallenius, *Science* 122, 288 (1955).
6. We are unable to illustrate this, since the distinction between the colored zones is not adequately reproduced in black and white.
7. R. Lemberg and J. W. Legge, *Hematin Compounds and Bile Pigments* (Interscience, New York, 1949).
8. All spectrophotometric measurements were made with a Bausch and Lomb "Spectronic 20."

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3 June 1957

Nonspecific Inhibition of Vaccinia Hemagglutinin

Knowledge concerning the occurrence of nonspecific (nonantibody) inhibitors of viral hemagglutinins in body fluids is essentially limited to influenza virus. The present report (*1*) concerns the hemagglutinin of vaccinia virus. The hemagglutinin inhibitor to be described was found in the plasma of the Lettré, hyperdiploid line of the Ehrlich ascites carcinoma of the mouse. The procedure used to measure the inhibitor was the same as that described in a previous paper (*2*), except that calcium chloride was omitted from the diluent. Five agglutinating doses of the IHD-E strain of virus (*3*) were employed in the hemagglutination inhibition tests. The method of inoculating mice has been described previously (*2*).

Variation of inhibitor concentration with the age of the tumor is shown in Table 1. The concentration is highest in the older tumors. That the inhibitor is not antibody is apparent from two facts: (i) it is not present in the serum of tumor-bearing mice; and (ii) it is inactivated by heating at 60°C for 30 minutes. Of additional interest is the observation that it has no neutralizing action on the infectivity of vaccinia virus.

A sample of the ascites tumor was disintegrated by shaking with glass beads (*4*). The supernatant of this preparation, following centrifugation, did not show an increase in titer over that of the cell-free fluid from an untreated control specimen. This was equally true of both the 5- and 13-day tumors. Disintegrated preparations made from washed cells, which had been resuspended in 0.85-percent NaCl, yielded no inhibitor at all.

When cell-free tumor fluid was mixed with an equal volume of packed, agglutinable fowl cells, and specimens were held at room temperature and at 4°C for 1 hour, the inhibitor was not adsorbed from the fluid in either case. A 1-percent suspension of fowl cells was prepared in tumor fluid, and samples were held at room temperature and at 4°C for 1 hour. The cells were subsequently washed with 0.85-percent NaCl and used to determine the hemagglutination titer of a suspension of vaccinia virus. The titers were the same as those of control suspensions that had not been in contact with tumor fluid. The evidence suggests that the inhibitor acts against the hemagglutinin, and not against the fowl cells.

Inhibition was also observed with other strains of vaccinia virus. In comparative tests in which five agglutinating doses of the IHD-E, Nelson, and Levaditi strains were employed, inhibition occurred with all three, and to the same titer.

Upon the dialysis of cell-free tumor fluid against distilled water at 4°C for 24 hours, the inhibitor was not removed. However, it was found only in the precipitate in the dialyzing bag. When the volume in the bag was adjusted to what it had been prior to dialysis, it was found that the precipitate represented 7 percent of the original volume. A sample of the thoroughly resuspended contents of the dialyzing bag was brought back to solution by adding NaCl. The titer of this sample was the same as that of the

Table 1. Vaccinia hemagglutinin inhibitor concentration in tumors of different ages. Titers were determined individually on the cell-free tumor fluids obtained from each of three mice which were sacrificed daily.

Age of tumor (day)	Titer		
	Mouse No. 1	Mouse No. 2	Mouse No. 3
3	< 10	10	< 10
4	20	20	20
5	20	40	10
6	40	40	80
7	80	80	80
8	80	20	40
9	40	40	40
10	160	160	80
11	40	80	40
12	160	160	160
13	320	160	80
14	160	160	160