The Electrophoretic and Spectroscopic Characterization of Hgb M

By Park S. Gerald

In their investigation of a family showing dominant transmission of a cyanotic condition, Hörlein and Weber in 1948 were able to demonstrate that the cyanosis was due to the presence of a methemoglobin with unusual spectral characteristics.1,2 By interchanging the heme and the globin with similar components prepared from normal blood, they obtained evidence that the anomalous spectral behavior was attributable to an abnormality of the globin. Although this antedated by a year the first report of an abnormal globin (in sickle cell hemoglobin) by Pauling et al.,3 it was not until 1955 that the hemoglobin of Hörlein and Weber was included among the hemoglobinopathies and the designation Hgb M applied.4 In recent years, three additional instances of cyanosis associated with Hgb M have been observed,5-7 and in two6,7 sufficient historical evidence was available to confirm the dominant inheritance pattern observed by Hörlein and Weber.

In the earlier reports,1,2,5 the properties of Hgb M were determined by examination of whole hemolyzates. The greatest deviation in spectral characteristics of "Hgb M" from normal hemoglobin was found in these studies to occur when the acid methemoglobin (methgb) forms were compared. Little or no abnormality in the spectral absorption curve was found for the other derivatives of "Hgb M" that were examined (alkaline methgb, methgb fluoride, methgb cyanide and carbonmonoxy-, oxy-, and reduced hemoglobin).

As noted in a brief initial report,6 both normal hemoglobin and an abnormal hemoglobin with the spectral properties of Hgb M have now been electrophoretically isolated from the hemolyzate of a patient with clinical evidence of Hgb M disease. Subsequent to this publication, it was discovered that the separation of the normal and the abnormal components could be greatly improved by electrophoresis of the hemolyzate after conversion to the methgb form. Examination of the material so isolated has demonstrated that the cyan methgb form of HgbM, as well as the methgb form, deviated markedly from the normal in its spectroscopic behavior. Moreover, evidence has been obtained to indicate that a portion of the heme groups in the methgb form of Hgb M reacts with cyanide essentially normally and a further portion reacts quite abnormally. These and related studies are presented herein and their clinical and genetic implications discussed in detail.

Materials and Methods

All of the investigations reported in this and the previous publication were conducted upon specimens of blood obtained from the same patient. This patient, an adult white...
male, exhibited slight but definite cyanosis which had been present for as long as the patient could remember. He had never experienced any other significant symptoms related to his cardiac, pulmonary or hematologic systems and had always led a normal, active life. As noted previously, this patient's venous blood was chocolate-brown in color. The unusual color was not altered by shaking in the presence of air (and thus could not be due to excess reduced hemoglobin) nor was there a significant increase in methemoglobin as determined by the method of Evelyn and Mallory.

The blood samples, obtained by venipuncture, were collected in ACD or mixed oxalate. The specimens containing oxalate were stored at 4°C. until hemolyzed (a few hours to one day). One specimen preserved in sterile ACD was kept at 4°C. for 5 weeks before hemolyzing, without apparent alteration of the sample. After washing three times with 5 to 10 volumes of buffered normal saline, the red cells were hemolyzed by the addition of 2 volumes of distilled water and 0.4 volumes of toluene. After standing at 4°C. for one hour, the stroma was removed by high speed centrifugation (25,000 x G). The resulting hemolyzate was kept at 4°C. until used; in this state it was stable for 4 to 5 weeks.

The hemolyzate without further treatment is designated the "untreated hemolyzate." The "oxidized hemolyzate" (presumably methgb) was obtained by incubating at room temperature (for 5 to 15 minutes) six volumes of the untreated hemolyzate with one or two volumes of 5 per cent potassium ferricyanide. The hemolyzate was converted to the cyan-methgb form by incubating (for five minutes at room temperature) the oxidized hemolyzate (six volumes of original hemolyzate) with one or two volumes of 2 per cent sodium cyanide (neutralized with 1/4 volume of 10 per cent acetic acid).

The technic of starch block electrophoresis developed by Kunkel was used for all electrophoretic separations. After completion of the electrophoretic run, the buffer was removed as far as possible from the starch block by absorption with filter paper and the several portions of the electrophoretic pattern excised. The starch fragments were transferred to a sintered glass funnel and the pigment eluted by addition of increments of the desired buffer.

Spectroscopic studies were made of these eluates after clarification by high speed centrifugation (25,000 x G). The Beckman model DU spectrophotometer with photomultiplier attachment, calibrated against the 446 mμ mercury line, was used throughout. Quantitative estimation of Hgb A was based upon an extinction coefficient at 500 mμ of 9.04 for its methgb form (extinction/millimole of Fe L. for 1 cm. light path, at pH 6.5) calculated from data obtained with crystallized horse hemoglobin.

The following abbreviations will be used throughout: hemoglobin = hgb; optical density = O.D. In general the designation "Hgb A" will be applied to the sum of the spectroscopically normal pigments; i.e., to the nonabnormal hgb. It should be realized that when so used it includes the alkali-resistant fraction as well as the three components distinguishable in the starch electrophoretic pattern at pH 8.6."

Results

Spectroscopy of the whole hemolyzate: Immediately after clarification by high speed centrifugation, the spectral absorption curve of the untreated hemolyzate in M/15 sodium phosphate buffer (pH 6.5) was obtained. Only the normal maxima for oxyhgb (at 542 and 577 mμ) were present in the visible portion of the spectrum; there was no peak or inflection at 600 or at 630 mμ.

The acid methgb form of the whole hemolyzate was prepared by adding 0.05 ml. of 5 per cent potassium ferricyanide to 3 ml. of a dilute solution of the untreated hemolyzate in M/15 sodium phosphate buffer (pH 6.5). The spectral curve of this acid methgb solution is given in figure 1. This spectral curve does not differ dramatically from that of Hgb A (see fig. 4, dotted curve) contrary to the findings noted in previous investigations. On close inspection however it is apparent that the minimum at 600 mμ in
figure 1 is less prominent than that in figure 4. This was critically evaluated by determination of the ratio of the O.D. at 500 m\(\mu\) to that at 600 m\(\mu\). The ratio for the patient's hemolyzate was found to be 2.56 while that obtained with an hemolyzate of normal blood was 3.22. This discrepancy corroborates the visual impression, and indicates that a spectroscopically abnormal pigment is present.

*Electrophoresis at alkaline pH:* At pH 8.6 (barbital buffer, ionic strength 0.05) the electrophoretic pattern of the untreated hemolyzate did not differ from the normal\(^{11,12}\) in the mobility of the components nor in the concentration of the A\(_2\) component\(^*\) (visual estimation). At times however an inhomogeneity in the color of the A\(_1\) spot\(^*\) was detected, a bluish tinge being evident at its leading (anodic) margin.

Electrophoresis of the methgb form (prepared by oxidation with potassium ferricyanide) at this same pH was more successful in demonstrating an abnormality. A double spot was now evidently present in place of the single major spot observed with untreated hemolyzate. The two components were

\(^*\)The three components of the starch block electrophoretic pattern at pH 8.6 are: (1) the major component, known as Hgb A\(_1\), comparable to that recognized with paper and Tselius electrophoresis; (2) a “slow” minor component, designated Hgb A\(_2\), having an electrophoretic mobility indistinguishable from Hgb E; and (3) a “fast” minor component presenting as a tongue-like extension from the major component in the anodic direction.\(^{13}\)
present in comparable concentration, as judged by visual estimation of color intensity. The slower of the two components was of the normal methgb hue while the faster exhibited the peculiar grayish-blue tint that is characteristic of the methgb form of Hgb M. The faster mobility of Hgb M at this pH is consistent with the impression gained from the observation of color inhomogeneity in the alkaline electrophoretic pattern of the untreated hemolyzate. Although splitting of the major component was quite obvious, the separation of the two spots was inadequate for the isolation of the two pigments in pure form.

After conversion to cyanmethgb, the electrophoretic pattern of the hemolyzate reverted to that seen with the untreated material. The double spot seen with the methgb preparation was no longer present! This is a major discrepancy, since cyanide should have a comparable effect upon both kinds of hgb, and the relative mobility of the cyanmethgb forms should not differ from that seen with the methgb forms. The explanation for this was found to lie in the unusual reactivity of methgb M with cyanide, as is subsequently detailed.

Electrophoresis at neutral pH: Although the isoelectric point of normal hemoglobin is near pH 7.0\textsuperscript{13} there is still significant cathodic migration of the protein at pH 7.0 to 7.2 due to the considerable electroosmotic flow in the starch block. Under these conditions untreated hemolyzates from normal adults show only a single electrophoretic band. The untreated hemolyzate from our patient showed a diffuse band in which three zones could be distinguished on the basis of color.\textsuperscript{6} The leading (cathodic) band, which possessed the electrophoretic mobility of normal hgb, was colored the normal oxyhgb hue and exhibited the spectroscopic behavior expected of normal hgb both before and after treatment of the eluted pigment with ferricyanide and subsequently cyanide.\textsuperscript{6} The middle zone was brownish in color, it could not be eluted without excessive contamination with the two adjacent zones. The third (anodic) zone—previously designated zone C\textsuperscript{a}—was also of a normal oxyhemoglobin hue. The prominence of this last zone seemed to vary from specimen to specimen and to decrease with the age of the individual specimen. When eluted separately with pH 6.5 buffer, the pigment of this third zone showed a complicated spectral curve (fig. 2, solid line) with maxima at 407, 490–495, 532–540, and 577 m\textmu; and an inflection at 600 m\textmu.. After treatment with potassium ferricyanide, the region from 450 to 650 m\textmu; was re-examined and was found to contain only two peaks, at 495 and 600 m\textmu.. The spectral curve after oxidation (fig. 2, dotted curve) resembles very closely that given by Hörlein and Weber\textsuperscript{1,2} and by Kiese et al.\textsuperscript{5} for the methgb derivative of "Hgb M."

The middle, brownish zone of the three-zoned pattern was interpreted as being the methgb form, and the trailing red zone as the oxyhgb form of Hgb M.\textsuperscript{6} The electrophoretic pattern was thought to be complicated because of the presence of three pigments with only slightly different mobilities. Conversion of the methgb and the oxyhgb forms to a single species was expected to simplify the electrophoretic pattern and facilitate separation into...
Fig. 2.—Ordinate, optical density; abscissa, wave length (\( \text{m}_{\Lambda} \)). Spectral curve of eluate of electrophoretically separated "oxyhgb M" (zone C' of the electrophoretic pattern at neutral pH) before (solid line) and after (dotted line) oxidation with potassium ferri-cyanide. The break in the curve indicates a change of phototube. Concentration of hgb 8.4 x 10^{-3} \text{mM/L.}; in M/15, pH 6.5 sodium phosphate buffer.

pure components. Accordingly the hemolyzate was converted to the methgb form by oxidation and then electrophoresed at neutral pH. The excellent separation depicted in figure 3 was now obtained. The spectroscopy of the material so separated is described subsequently.

It was expected that a similar two-banded pattern would result upon conversion of the methgb present in the original hemolyzate to cyanmethgb, since the latter complex has essentially the same electrophoretic mobility as oxyhgb. Surprisingly, this proved not to be the case. Addition of cyanide to the original hemolyzate did not alter the pattern from that obtained before treatment. Moreover, attempted conversion of all the hgb present to cyanmethgb, by oxidation and subsequent treatment with cyanide, only altered the pattern in that with such specimens the anodic oxyhgb-hued zone was never visible. Neither procedure eliminated the brownish, "middle" zone. This anomalous reactivity with cyanide corresponds to that described in the electrophoretic studies at alkaline pH.

Spectroscopy of the eluates of the methgb electrophoretic pattern: Optimum separation of the hemolyzate into normal and abnormal components was achieved by electrophoresis of the methgb preparation at pH 7.0 to 7.1. Although at lower pH's a greater distance of migration and hence greater separation could be expected, this advantage was offset by more rapid denaturation. The pH range adopted (pH 7.0 to 7.1) permitted satisfactory separation with minimal denaturation. In every instance the more rapidly moving pig-
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Fig. 3.—Starch block electrophoretic pattern of oxydized hemolyzates. Upper pattern, normal adult. Lower pattern, Hgb M disease (present patient). Electrophoresis in sodium phosphate buffer (pH 7.08, ionic strength 0.1).

ment (fig. 3) exhibited normal spectroscopic behavior and has been used as a control to illustrate the reactions of the normal (Hgb A) derivative. The more slowly moving pigment has been similarly constant in displaying abnormal spectral characteristics and has been designated the Hgb M derivative.

After electrophoretic separation, the central portion of each band was eluted in M/15 sodium phosphate buffer (pH 6.5). After standing overnight at 8 C., the eluates were diluted with further amounts of the same buffer and clarified by high-speed centrifugation (25,000 x G). The spectroscopic characteristics for the visible region of the resulting eluates are given in figure 4. In addition, both pigments showed a maximum in the Soret band region at 405-406 mμ.

The cyanmethgb form of each pigment was then prepared by the addition of a small amount of neutralized sodium cyanide solution (final concentration of cyanide, 1 x 10⁻³ M/L.). The spectral curves for the resulting compounds differed considerably in the visible region (fig. 5). The Soret band region for Hgb M was also anomalous in that a double peak (406 and 418 mμ) appeared. That this difference was in part the consequence of an abnormal reactivity (failure to react) of some of the heme groups in the Hgb M preparation was demonstrated by the further changes occurring when a large excess of cyanide ions were added (final concentration of cyanide, 31 x 10⁻³ M/L.). In contrast to this behavior, no change in the Hgb A eluate was produced by a similar large excess of cyanide. Figure 6 illustrates the actual O.D. changes resulting
from these reactions with cyanide. The O.D. increments (and decrements) resulting from the addition of a minimal amount of cyanide to the two different methgb solutions are qualitatively similar (curves a and b of fig. 6). Note that the locations of the maxima and minima are identical and the interceptions of the zero line (the isobestic points) are nearly superimposed. A very different curve is obtained in the case of Hgb M when the cyanide concentration is increased from $1 \times 10^{-3}$ to $31 \times 10^{-3}$M/L. (fig. 6, curve c). Note that the locations of the maxima and minima differ significantly from curves a and b of figure 6 and in particular that the isobestic points show no correspondence to the aforementioned curves. This indicates that the difficultly reacting hemes of the Hgb M preparation are largely, if not totally, responsible for the abnormal spectral properties of the methgb form.

Spectral constants for the methgb form of Hgb M. The molecular extinction coefficients for derivatives of Hgb M were not determined. This failure is the consequence of the difficulty of determining the iron content of such dilute solutions as were available. The alternative methods of standardization, based on color reactions of the heme group, were not utilized. In the absence of suitable standardization, only the ratios of O.D. for the various maxima are available: O.D.406/O.D.500=12.4; O.D.500/O.D.600=1.60.

Determination of the Hgb M content in the hemolysate. Quantitation of the Hgb M fraction depends upon knowledge of the molecular extinction coeffici-
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Fig. 5.—Ordinate, optical density; abscissa, wavelength (μ). Spectral curves obtained after addition of small amounts of neutralized sodium cyanide (final concentration of cyanide, 1 x 10⁻³ M/L.) to the solutions of figure 4. Solid line, Hgb M derivative; dotted line, Hgb A derivative.

ents for Hgb M and Hgb A. As noted previously, such coefficients are not available for Hgb M. This difficulty may nonetheless be circumvented by assuming the data of Kiese et al.² to be applicable in part to pure Hgb M. The whole hemolysate from their patient evidently possessed a large amount of Hgb M, inasmuch as the methgb form of the whole hemolysate resembled in its spectral curve that obtained with electrophoretically isolated Hgb M (fig. 5). Kiese et al.² showed that the molecular extinction coefficients of their patient’s hemolysate in the methgb form (pH 6.5) was nearly identical at 500 mμ with the value for a normal hemolysate, standardization being based upon iron content. Accordingly, we have assumed that the extinction coefficient at 500 mμ (pH 6.5) of the methgb form of Hgb A and Hgb M at pH 6.5 are equal. From this and the observed ratio of the O.D. for the methgb derivative at 500 mμ to that at 600 mμ (1.60 for Mgb M and 3.22 for whole normal hemolysate) the following equation can be derived:

\[ \% \text{Hgb M} = \left( \frac{3.17 - 0.98}{R} \right) \times 100; \]

where R is the ratio of O.D. at 500 mμ to O.D. at 600 mμ for the methgb form of the hemolysate under study. The methgb is prepared by adding 0.010 ml.
Fig. 6.—Ordinate, optical density; abscissa, wavelength (mµ). The change in optical density produced by the addition of neutralized sodium cyanide to the solutions of figure 4. (a) Interrupted line, change produced by addition of cyanide (1 x 10^{-3} M/L.) to Hgb A derivative. (b) Solid line, change produced by addition of cyanide (1 x 10^{-3} M/L.) to Hgb M derivative. (c) Dotted line, further change (actual values multiplied by 5) produced in this Hgb M solution by increasing cyanide concentration from 1 x 10^{-3} M/L. to 31 x 10^{-3} M/L. No significant change occurred in the Hgb A solution when the cyanide concentration was similarly increased.

of 5 per cent potassium ferricyanide to 3.0 ml. of a dilute solution of the hemolyzate in M/15, pH 6.5 sodium phosphate buffer.

Direct estimation of the Hgb M may also be achieved by electrophoresis of the hemolyzate (methgb form), quantitative elution of each band in pH 6.5 buffer, and determination of the O.D. at 500 mµ after dilution to equal volumes.

Both of these methods were applied to the hemolyzate of the patient. The spectroscopic method gave a Hgb M content of 26 per cent; the electrophoretic technic, 30 per cent.

**Determination of the alkali-resistant fraction.** The alkali-resistant fraction was determined by the technic of Singer et al.\textsuperscript{14} The total Hgb concentration of the hemolyzate was estimated by measurement of the O.D. at 500 mµ of the methgb at pH 6.5, as discussed before. A value of 1.4 per cent was obtained for the alkali-resistant fraction.

**Discussion**

“Hgb M disease” is a syndrome characterized by hereditarily transmitted cyanosis in the absence of increased amounts of reduced hgb or “normal”
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methgb. The oxidized hgb of a patient with clinical evidence of “Hgb M disease” is separable into two major fractions (fig. 3), one normal in its electrophoretic and spectroscopic behavior and the other abnormal. The unusual spectral curve of the abnormal fraction resembles very closely that given for acid methgb prepared from whole hemolyzates in the previous investigations of Hgb M disease.\(^1\)\(^2\)\(^3\) On the basis of this similarity we have applied the designation Hgb M, originally proposed by Singer\(^4\) for the hereditary hemoglobinopathy of Hörlein and Weber, to the abnormal hemoglobin fraction of the present patient.\(^6\)

The first investigators interchanged the heme and the globin of their patient's hgb with similar components prepared from normal hgb.\(^1\)\(^2\) The combination of “normal” heme with “abnormal” globin resulted in a compound with spectral properties comparable to the original hemolyzate from their patient. The compound obtained by the reciprocal interchange on the other hand showed a normal spectral curve. From this they concluded that the spectral abnormality was attributable to the globin portion of the molecule. Similar results were obtained by Kiese et al.\(^5\) These findings are assumed to apply to the present case, although duplication of this experiment was precluded by the lower concentration of the abnormal fraction. Localization of the abnormality to the globin is consistent with current opinion regarding the biochemical defect in the abnormal hemoglobins.

As judged by the presence of cyanosis, the transmission of Hgb M\(^1\)\(^6\)\(^7\) conforms to that of a dominant characteristic. The present demonstration of both Hgb A and Hgb M in a member of such a pedigree (cyanosis was said to be present in two of the present patient’s three children as well as in his sister, father and paternal grandfather) is in accordance with the postulation of a heterozygous state. In its transmission as a dominant defect and in the presence of both Hgb M and Hgb A in a probable heterozygote, Hgb M follows the general principles established for the more frequent hemoglobinopathies. The spectroscopic behavior of Hgb M, despite its uniqueness, does not necessarily indicate that Hgb M deserves a special category apart from the hemoglobinopathies of common experience.

The essentially normal nature of the O.D. changes following reaction of the methgb (of Hgb M) with minimal amounts of cyanide (fig. 6, curve c) attests to the presence of normally-reactive heme groups in the molecule. The further reaction of the molecule with a large excess of cyanide (fig. 6, curve c) on the other hand indicates that abnormally reactive heme groups are also present.\(^8\) As noted, the methgb-colored band characteristic of Hgb M persists in the electrophoretic pattern (at neutral pH) after addition of minimal amounts of cyanide to either the original or the oxidized hemolyzate. This persistence is independent evidence of abnormal reactivity of some of the heme groups.

That both classes of heme groups occur together in the molecule and are equally present

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\(^*\)This hypothesis was developed conjointly by the author and Dr. Philip George (University of Pennsylvania) during a survey of the Soret band characteristics of methgb complexes of Hgb M prepared from material isolated from the patient described in the present report.\(^9\)
in all Hgb M molecules was demonstrated by spectroscopic study following electrophoresis of the hemolyzate in the cyanmethgb form at neutral pH. The Hgb M band, as noted, still possessed its methgb hue in contrast to the normal cyanmethgb color of the Hgb A band. The Hgb M band was divided, perpendicular to the direction of migration, into two equal parts. Spectroscopy of the eluates from the two portions showed them to be identical, a circumstance possible only if the number of reacted and unreacted heme groups was constant from molecule to molecule. This last follows from the alteration in the charge of the heme group consequent to its reaction with cyanide (Fe$^{3+} \rightarrow$ FeCN$^{-2}$). Inhomogeneity among the Hgb M molecules in their reactivity with cyanide would thus be associated with inhomogeneity in electrical charge, which in turn would be reflected in their distribution in the electrophoretic band.

The existence of normally reactive heme groups in the Hgb M molecule, in addition to the abnormally reactive hemes, can be logically predicted if the biochemical abnormality of Hgb M is analogous to that found for Hgb S and Hgb C. In the case of Hgb S, Ingram has shown that it differs from Hgb A in only a single amino acid. Specifically, in Hgb A the sequence -glu,glu,lys- occurs while in Hgb S it is -val,glu,lys-. Similarly Hgb C shows an alteration of one amino acid residue, possessing the sequence -lys,glu,lys-. If a comparable and very localized abnormality exists in Hgb M, then the alteration would be expected to be in proximity to some heme groups, whose reactivity (and spectroscopic properties) it would influence, and simultaneously far from others. The latter groups could exhibit the normal reactivity seen experimentally.

The observation of an abnormal spectral curve for the cyanmethgb is in variance with the findings of previous workers. They were unable to detect any significant spectroscopic difference between the various derivatives of normal hgb and their "Hgb M" preparations, except when the methgb forms were compared. This is perhaps attributable to their use of whole hemolyzates, which could be expected to contain significant amounts of Hgb A. The degree of this latter contamination can be estimated from the spectroscopic data given by Kiese et al. When the equation given previously is used, the amount of Hgb A in their whole hemolyzate is calculated to be 15 per cent. Considering the similarity of the spectral curve published by Hörlein and Weber to that of Kiese et al., the former group must also have been dealing with an hemolyzate containing a comparable concentration of Hgb M.

The considerably lower concentration of the abnormal pigment in the patient presently reported is in itself a significant distinction. With the exception of Hgb J the uncomplicated abnormal hgb traits (heterozygotes) have consistently shown an electrophoretic pattern in which Hgb A comprised the major portion of the pigment. If the three unrelated individuals studied to date do indeed represent the same disease, then Hgb M is additionally unusual in occurring in widely varying amounts.

Although estimation of the total concentration of Hgb M is possible, determination of the proportion of the hgb in the oxidized (methgb) state would now seem to be more complicated than has been suspected. It is reasonable

*The symbols glu, lys, and val stand for amino acid residues of glutamic acid, lysine, and valine, respectively.
to assume that only the abnormally reactive hemes cannot be reduced to the ferrous state by the erythrocyte enzyme systems normally responsible for this. Certainly the methgb reducing power of red cells in Hgb M patients has not been impaired.\textsuperscript{5} Determination of the methgb concentration by reaction with cyanide would hence be complicated not only by the abnormal spectroscopic properties but also by the abnormal reactivity of the oxidized heme groups. The failure to detect methgb by the Evelyn and Mallory technic\textsuperscript{8} in the present patient\textsuperscript{6} and in another independently studied case\textsuperscript{7} is consistent with this.

The dramatic nature of the presenting complaint (cyanosis) in this condition renders it probable that further examples will be found in the near future now that detection of lesser amounts of Hgb M is possible. All examples of familial cyanosis in the absence of increased amounts of reduced hgb or methemoglobinemia (by conventional tests) should be examined for the presence of Hgb M.

**Summary**

The hemoglobin (hgb) from a patient with Hgb M disease was resolved into two components by starch block electrophoresis (at pH 7.0-7.2) of the oxidized hemolysate. One component was identified electrophoretically and spectroscopically as Hgb A, and the other as Hgb M. Methods for the determination of the relative concentration of Hgb M were given. In the patient reported, Hgb M was found to comprise approximately 30 per cent of the total hgb.

Spectroscopic studies of electrophoretically isolated Hgb M demonstrated that both the methgb and the cyanmethgb form were abnormal in their spectral curves. The reactions of the methgb form with low and high concentrations of cyanide were found to differ. The nature of the spectral changes were such as to indicate that some of the heme groups of the methgb form react abnormally and others apparently normally. The electrophoretic behavior of the patient's hemolysate after treatment with various combinations of cyanide and ferricyanide was consistent with this hypothesis. The differing reactivity of the heme groups was explained in the light of the biochemical genetics of the abnormal hemoglobins.

**Summario in Interlingua**

Le hemoglobina de un patiente con morbo a hemoglobina M esseva resolvite in duo componentes per medio de electrophorese a bloco de amylo (a pH 7,0 a 7,2) post oxydation del hemolysato. Un componente esseva identificate electrophoreticamente e spectroscopicamente como hemoglobina A, le altere como hemoglobina M. Es presentate methodos pro le determinacion del concentration relative de hemoglobina M. In le presente caso il esseva trovate que hemoglobina M representava circa 30 pro cento del hemoglobina total.

Sudios spectroscopic del electrophoreticamente isolate hemoglobina M demonstrava que tanto le forma methemoglobininic como etiam le forma cyanmethemoglobininic esseva anormal in lor curvas spectral. Esseva constata.te
que le forma methemoglobinicom reageva differentemente con basse e alte concentrazioni de cyanuro. Le natura del alterationes spectral suggesteva que certes del gruppos hemic in le forma methemoglobinicom reageva anormalmente durante que aleres pareva reager normalmente. Le comportamento electrophoretic del hemolysato del patience post tractamento con varie combinationes de cyanuro e ferricyanuro esseva compatibile con ille hypothese. Le non-uniforme reactivitate del gruppos hemic esseva explicate in le lumine del genetica biochimic del hemoglobinas anormal.

ADDENDUM

Because of their pertinence to the foregoing presentation, recent experimental findings which will be detailed in another publication (P. George and P. S. Gerald, manuscript in preparation) warrant summarization at the present time. (1) The blood of the cyanotic sister of the patient described in the preceding article has now been examined and an abnormal hemoglobin identical in its spectroscopic and electrophoretic properties to that described in the main presentation has been isolated. (2) A second family residing in Canada and similarly affected with a dominantly transmitted cyanosis (Canad. M. A. J. 62:348, 1950) has been studied by the same technics. (We are indebted to Dr. D. M. Baltzan for the opportunity to examine the blood from 3 cyanotic members of this family.) Again, both a normal and an abnormal hemoglobin were demonstrable in each blood specimen. The isolated abnormal methemoglobin, while exhibiting maxima at the same location as the pigment obtained from the New England family, nonetheless was significantly different in the intensities of these maxima. The difference between these two pigments was more dramatically demonstrated by the essentially normal reactivity of the Canadian specimen with cyanide.

As a temporary expedient we have adopted the following terminology: the Hgb M described in the main text will be called “Boston type” (symbolized by Hgb M1), while that recently discovered in the Canadian family is identified as “Saskatoon type” (Hgb M5).

Since the spectrochemical findings reported by the German workers cited in the main text more nearly resemble Hgb M5 than Hgb M1, the methods used for quantitating the amount of Hgb M5 must be set aside until the extinction coefficients of the methemoglobin derivative are independently determined.

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PARK S. GERALD

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