Inhomogeneities in Alkali-Resistant Hemoglobin. Demonstration of Zone Electrophoretic Differences Using a Cationic Detergent Electrolyte

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ATTEMPTS TO demonstrate differences between alkali-resistant hemoglobin of the new-born and of patients with severe thalassemia have met with varying degrees of success. Electrophoretic differences of carbonmonoxyhemoglobin from cord blood and patients with thalassemia have been revealed.[1] It was pointed out, however, that in a free boundary system where the pH of the medium was less than the isoelectric points of the proteins involved heterogeneity could result from carbonmonoxide oxidation or low pH.[2] Immunologic differences have been shown.[3,4] Interference by non-pigmented protein components of hemolysates was a possibility in antigen-antibody reactions.[5] Allen, Schroeder and Balog[6] demonstrated by crystallization and chromatography that fetal hemoglobin (Hgb F) was indeed inhomogeneous. Four distinct components of the crystallized Hgb F fraction were demonstrated. Unfortunately no similar study of thalassemia hemoglobin was undertaken.

As far as could be determined, zone electrophoretic methods have failed to reveal alkali-resistant hemoglobin inhomogeneities. One of the prime contributing factors could be that strongly cationic proteins such oxyhemoglobin, cytochrome c, crystalline DNase and trypsin resist electrophoretic resolution because of adsorption on negatively charged solid media.[7] Reversal of the adsorptive property of filter paper supports has been accomplished by impregnating the paper with a strong cationic detergent.[8] The danger of such procedures, however, lies in the possibility of producing undesirable protein-detergent interactions.[9]

In this report we have extended the principle of modifying the charge on solid supporting media to include starch gel.[10] Recognizing inherent dangers of protein-detergent interaction while at the same time attempting to devise a simple method for demonstrating differences in alkali-resistant hemoglobin, we have used a trimethyl substituted cationic active detergent instead of the more common dimethylbenzyl substituted agent. Jacox[9] has shown little protein-detergent interaction with trimethyl substituted cationic detergents.

MATERIALS AND METHODS

The starch gel electrophoretic methods of Smithies[10] were used throughout the experiments. The choice of solid supporting medium was made solely on the basis of its high hold up volume and excellent physical properties afforded in handling.

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A non-continuous buffer system was used. The gel mixing solution consisted of 0.07 M Tris-maleate buffer, pH 8.7. The bridge solution was 0.3 M boric acid adjusted to pH 8.4 with sodium hydroxide.

Trimethyloctadecyl ammonium chloride (TMOD),* a cationic detergent was added to the gel mixing solution at an optimum concentration of 20 mg. per 100 ml.

Starch gels containing 13 Gm. of potato starch† in 100 ml. of TMOD gel mixing solution were formed in plastic trays 17 x 2.0 x 0.7 cm. Usually six of these trays were cemented on a glass plate of appropriate dimension and the whole unit with hemoglobin samples in place was stood vertically contacting by means of gauze wicks electrode vessels filled with borate solution. A platinum electrode system was used. Electrophoresis was in the cold (4°C) against gravity for 16 hours at a potential gradient of 7 volts per cm. (15 to 20 M.A.).

Red blood cell hemolysates were prepared by the method of Jonxis and Huisman.[11] All samples, in the oxyhemoglobin form, were adjusted to uniform concentration by addition of distilled water until an optical density of 0.7 was reached at 575 mμ on a Beckman DU spectrophotometer. The hemoglobin samples were then placed on filter paper strips (Whatman #3, 1.0 x 0.6 cm.) and carefully edged into the gels on an unvaried line of origin.

Alkali-denaturation tests were performed according to the method of Singer, Chernoff and Singer.[12]

All cases of thalassemia studied in this report were detected in Egypt.[13] The clinical, laboratroy and radiographic documentation of these cases was the subject of another report.[14]

Results

Selective Action of TMOD

Five starch gels were formed in which the concentration of TMOD was varied systematically from 0 to 20 mg. per 100 ml. of gel mixing solution. Hemoglobin from a patient with severe thalassemia (32 per cent alkali-resistant hemoglobin) was placed in each of the gels. A photograph of the unstained gels immediately after electrophoresis is shown in figure 1. No detectable effect was noted until the concentration of TMOD reached 8 mg. per 100 ml. (fig. 1*A*). The major hemoglobin zone (Hgb A and the alkali-resistant fraction of thalassemia, Th^T) migrated as a single zone at the lower concentrations (fig. 1*B*). When TMOD was at a level of 12 mg. per 100 ml. first signs of cleavage were noted. (fig. 1*C*). Resolution of the two zones was complete at a TMOD concentration of 16 mg. per 100 ml. (fig 1*D*). An additional zone evolved just off the line of origin when TMOD was at 20 mg. per 100 ml. Since the new zone Th^T was distinct at a concentration of 20 mg. per 100 ml., it was considered that TMOD at this level in the system was optimum.

The resistance of Hgb A and A_2 to the action of TMOD at the concentrations used confirmed the selectivity of the cationic agent for alkali-resistant hemoglobin.‡

Comparison of Zones Th^T and Hgb F in the TMOD Modified System

Normal adult hemoglobin, hemoglobin of a patient with thalassemia and cord blood hemoglobin were submitted concomitantly to electrophoresis in

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‡The author has recently learned that concentrations up to 40 mg. per 100 ml. have been used without deleterious effect on Hgb A and A_2.

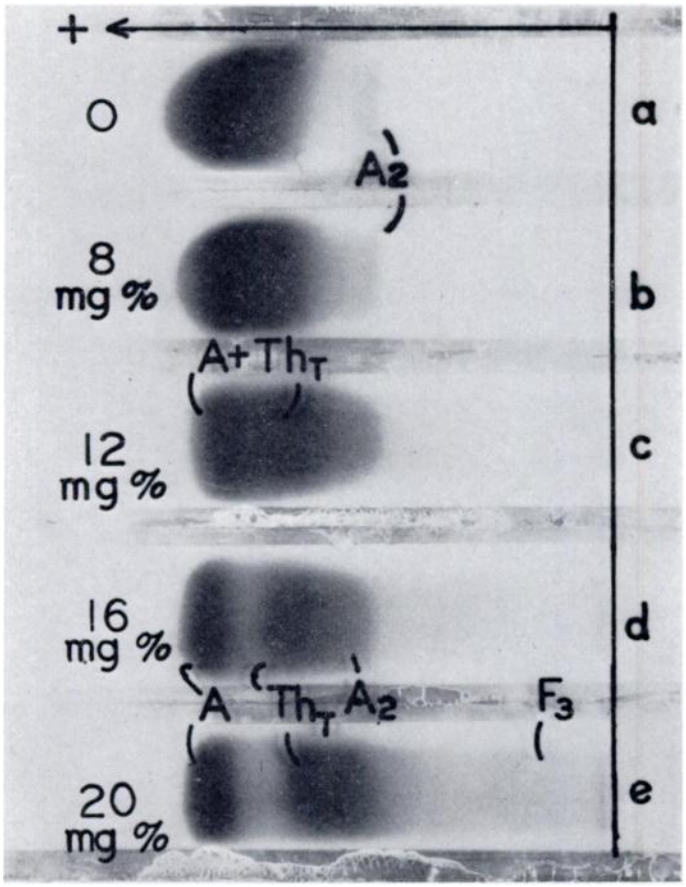

Fig. 1.—Photograph of unstained starch gels immediately after electrophoresis. The effect of varying concentration of cationic detergent, trimethyloctadecyl ammonium chloride (TMOD) on the migration of alkali-resistant hemoglobin of thalassemia is shown. A selective loss of negative charges on Th^T is inferred.

six gels, three of which contained TMOD and three without the cationic agent. A photograph of the unstained gels following the electrophoretic run is shown in figure 2. The odd numbered gels were TMOD-modified. Gels 1 and 2 contained normal adult hemoglobin 3 and 4 thalassemia hemoglobin and 5 and 6 cord blood hemoglobin. The absence of effect of TMOD on normal adult hemoglobin was marked (gels 1 and 2). Resolution of zone Th^T was clearly noted in the hemoglobin of thalassemia (gel 3). In gel 4 which contained thalassemia hemoglobin but no TMOD, zone Th^T was not resolved and the migration pattern was similar to that of normal adult hemoglobin. In gel 5 the effect of TMOD on cord blood hemoglobin was observed. Four distinct pigmented zones were resolved. The single zone migration characteristic of cord blood hemoglobin without the influence of TMOD is shown in gel 6.

TMOD Effect on Cord Blood Hemoglobin

The disclosure of four distinct zones in cord blood hemoglobin as shown in figure 2, gel 5; figure 3, gel 1; figure 4, gels *a* and *b* occurred consistently in a

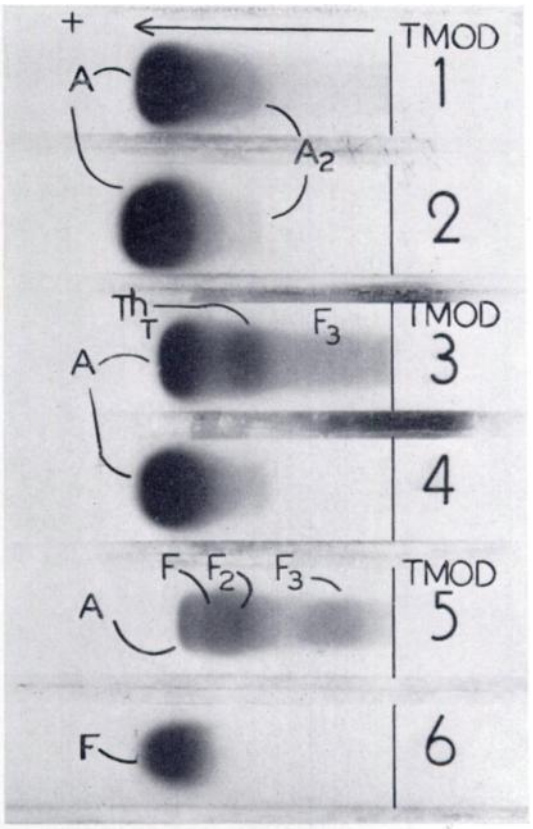

Fig. 2.—Photograph of an unstained series of starch gels immediately after removal from the electrophoresis experiment. The comparative effect of the addition of cationic detergent (trimethyloctadecyl ammonium chloride, 20 mg. per 100 ml.) is shown. Gels 1 and 2 contain normal adult hemoglobin. Note slight effect of TMOD (gel 1). Gels 3 and 4 contain thalassemia hemoglobin. Note the development of zone Th^T in the TMOD treated gel. Gels 5 and 6 contain cord blood hemoglobin. Note the development of four distinct zones in the TMOD treated gel.

series of 16 TMOD modified electrophoretic procedures. One of the striking features was the separation of Hgb A and Hgb F. Although known to be present in cord blood,[6] Hgb A has never been divulged by electrophoretic means. Two new zones, Hgb F_2 and F_3 were revealed. This finding was in good agreement with the chromatographic results obtained by Allen, et al.[6]

Comparison of Migration Rates of Alkali-resistant Hemoglobin of the New-Born and of Patients with Thalassemia

A single starch gel of the dimension 17 x 12 x 0.7 was formed. Hemoglobin samples of cord blood, of patients with thalassemia and Hgb E trait and of normal adults were submitted to TMOD modified electrophoresis simultaneously. Figure 3 shows a photograph of the stained gel slab (amido-black 10-B) in which the various hemoglobin patterns are displayed. Inhomogeneity of zones Hgb F (pattern 1, fig. 3) and Th^T (patterns 2 and 3, fig. 3) was based on their

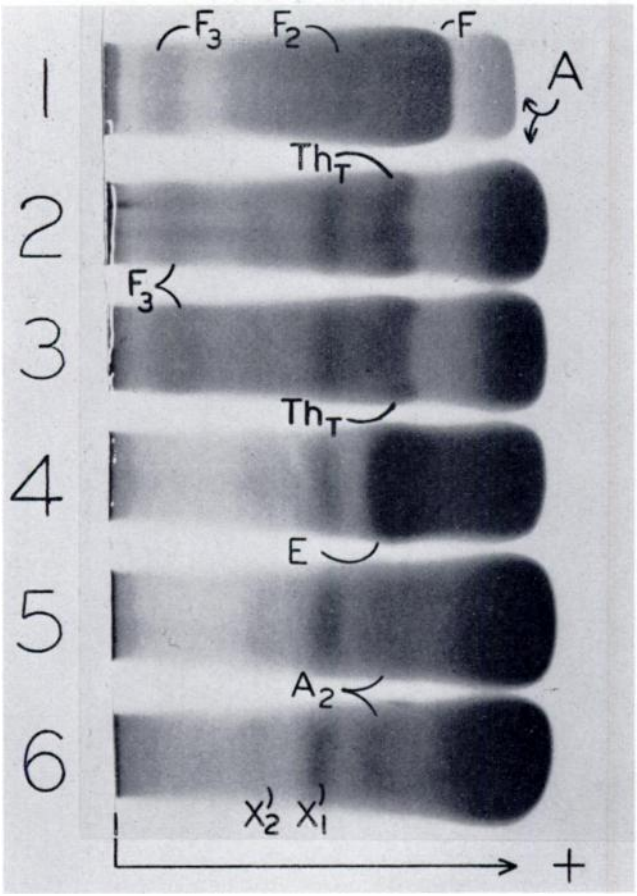

Fig. 3.—Photograph of amido-black 10-B stained starch gel slab after electrophoresis of hemoglobins in a TMOD (trimethyloctadecyl ammonium chloride) modified medium. Difference in rates of migration of hemoglobin of the new-born (gel 1, zone F) and of patients with thalassemia (gels 2 and 3, zones Th^T) is shown. Note separation of Hgb A and F in gel 1. Note resolved components of cord blood hemoglobin (gel 1, zones F_2 and F_3). The distinctive pattern of thalassemia hemoglobin as compared to normal adult and Hgb AE patterns is noted. (gels 4, 5 and 6) Zones x_1 and x_2 are non-pigmented components of hemolysates.

difference in mobility rates. The faster migration of Hgb F was confirmed by comparing patterns of 12 cases of thalassemia with those of randomly selected cord blood hemoglobins. The result of one such experiment is shown in figure 4. The cord blood hemoglobins (gels *a* and *b*, fig. 4) varied in alkali-resistant hemoglobin concentration, 76 and 92 percent respectively. The thalassemia hemoglobins contained 73 per cent alkali-resistant hemoglobin (gel *c*, fig. 4) and 24 per cent alkali-resistant hemoglobin (gel *d*, fig. 4).

The TMOD modified electrophoresis procedure was successful in identifying cases of thalassemia where the level of alkali-resistant hemoglobin was as low as 12 per cent. The occurrence of zone Th^T in the electrophoretic pattern of thalassemia hemoglobin was the cardinal element of identification.

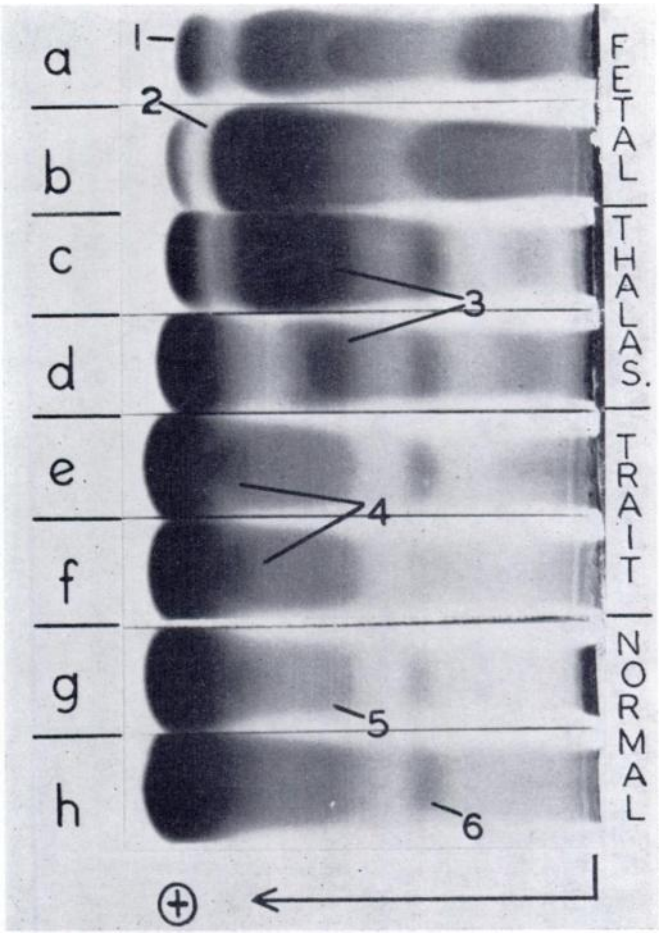

Fig. 4.—Photograph of amido-black 10-B stained starch gels after electrophoresis of hemoglobins in a TMOD (trimethyloctadecyl ammonium chloride) modified medium. Zone no. 1 indicates Hgb A which has been separated from components of cord blood hemoglobin (zone 2 indicates Hgb F). Zone 3 shows the component (Th^T) which is found consistently in hemoglobin of patients with severe thalassemia. Zone 4 shows a post Hgb A components present in hemoglobin from known thalassemia gene carriers. Zone 5 is Hgb A_2 and zone 6 is a non-pigmented minor component.

It was noted, however, that zone Th^T and normal Hgb A_2 migrated approximately the same distance. The relationship of the two components in the present system of electrophoresis was not studied, nonetheless it was almost certain that they were not homogeneous. Upon close inspection of the patterns shown in figure 3, one could distinguish Hgb A_2 in apposition and on the cathode side of zone Th^T.

Differentiation of the TMOD modified thalassemia pattern from that of Hgb AE presented some difficulty. However, the clear separation of Hgb A and Th^T (fig. 3, patterns 2 and 3) marked the main point of differentiation. As shown in figure 3, pattern 4, the area between Hgb A and E was filled with trailing material.

Two minor non-pigmented components were always present in stained gels in which thalassemia or adult hemoglobin samples were inserted. These components (fig. 3, pattern 6, zones x_1 and x_2) have been mentioned by others.[15] Their migration rates were not altered by the action of TMOD.

In our studies it was difficult to distinguish the patterns of normal adult from thalassemia hemoglobin of gene carriers (trait). However, upon staining, a definite post Hgb A zone appeared (fig. 4, gels *e* and *f*, no. 4). The origin of this zone was undetermined. In any event it had no apparent relationship to Hgb A_2 and did not appear in the TMOD modified patterns of normal adult hemoglobin (fig. 4, gels *g* and *h*).

Special Studies

In order to determine if the new zones of cord blood and thalassemia hemoglobin were products of denaturation and/or protein-detergent interaction, samples of the two alkali-resistant pigments were mixed in tubes with levels of TMOD and buffers equal to those encountered in routine electrophoresis. Untreated controls were prepared at the same time. These reactions were allowed to stand for 24 hours in the cold (4°C). All samples were then submitted to electrophoresis in starch gels without TMOD in the medium. No variation in migration patterns of the treated and untreated samples occurred. These results indicated freedom from denaturation, or, at least, if denaturation did occur, the degree was such that reversal in non-TMOD medium came about easily.

The cationic active electrolyte alone in aqueous solution (5.8×10^{-4} M TMOD) had a specific conductance of approximately 2×10^{-3}. This low conductance verified the purity of the agent.

Neither pH nor conductivity of the gel mixing solution was significantly altered by TMOD at a level of 20 mg. per 100 ml. The same results were observed in conductance tests of molten gel and after solidification.

Electroosmosis in the TMOD modified gel was tested by subjecting the non-charged particle dextran to electrophoresis. The effect of TMOD was nil.

Discussion

The advantages of the present zone electrophoretic method for demonstrating differences in alkali-resistant hemoglobins are: (1) elimination of salt and buffer anomalies by use of solid supporting medium,[10] (2) use of oxyhemoglobin samples thus precluding the possibility of developing intermediate oxidation products, (3) electrophoresis in a medium at a pH above the isoelectric points of the involved proteins, (4) demonstration of selective ionizing agent specific for alkali-resistant hemoglobin, and (5) demonstration of new components of cord blood and thalassemia hemoglobin without the use of dyes after electrophoresis.

Even though there were indications that denaturation was not a factor in the production of new hemoglobin zones in the present electrophoretic system, it must be kept in mind that cationic detergents are, under certain conditions, excellent protein fractionating agents.[9] The use of trimethyl substituted long chain cationic active detergents in the present experiment minimizes this protein-detergent interaction possibility. For the present, however, the most

attractive line of speculation about the mode of action of TMOD would be one of selective cation binding on the alkali-resistant molecule. It is probable that carboxyl and sulfhydryl groups of the alkali-resistant hemoglobin are highly reactive with the ammonium cationic charges of TMOD under the conditions of electrophoresis employed. Thus selective changes in net charge are evolved.

Notwithstanding, the experimental data presented in this report are insufficient to reveal what must surely be exquisite interactions in a three phase colloidal electrophoretic system (starch gel, protein and cationic colloidal electrolyte). Any attempt to define the exact mode of action would be an oversimplification.

Summary

Trimethyloctadecyl ammonium chloride, a cationic active detergent electrolyte, when added to starch gels before electrophoresis, caused selected alteration in the migration rates of alkali-resistant hemoglobin. Other red blood cell pigments were not similarly affected. Electrophoretic inhomogeneity of cord blood and thalassemia hemoglobin was noted. New components of cord blood hemoglobin were revealed by the method. A new component in the hemoglobin of cases of severe thalassemia was demonstrated.

Summario in Interlingua

Chloruro de ammonium trimethyloctadecylic (un electrolyto detergente cationicamente active), quando addite a gels de amylo ante le electrophorese, causava un selegite alteration in le rapiditate migratori de alkali-resistente hemoglobina. Altere pigmentos erythrocytic non esseva afficite in ille maniera. Inhomogeneitate electrophoretic de sanguine de cordon e de hemoglobina thalassemic esseva notate. Nove components del hemoglobina de sanguine de cordon esseva revelate per le methodo. Un nove componente in le hemoglobina de thalassemia sever esseva demonstrate.

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