A HEMOGLOBIN FRACTION (Hb Pb) migrating more rapidly than hemoglobin A during electrophoresis at pH 8.6 has been described in children with lead poisoning. The component disappeared after recovery. The abnormality was not detected in all patients with lead poisoning. It could not be produced in acute experiments in which animals were treated with lead acetate, nor could it be detected in adults with acute hemolytic anemia due to lead ingestion. Small amounts of a hemoglobin with similar electrophoretic properties, hemoglobin A3 (Hb A3), are found in hemolysates from normal individuals. This minor component is thought to be formed in vivo during aging of red cells and in vitro after prolonged storage of blood at 0–4 °C. Hemoglobin A3 has been shown to consist of several chromatographically distinct species, which differ from the major normal component in their β chains.

A hemoglobin similar to Hb A3 has been described in patients receiving tolbutamide. It is not known whether hemoglobin A3 is related to the tolbutamide hemoglobin (Hb Tol). Rapidly migrating hemoglobins can be produced in vitro by attachment of metal ions (CrO₄²⁻) or by formation of mixed disulfides with oxidized glutathione (GSSG) or cystine. These ligands appear to attach to the β chain, but the fast hemoglobins they produce probably differ from hemoglobin A3 formed in vivo.

In the present study, the clinical characteristics of lead-poisoned patients in whom Hb Pb appeared have been delineated. Electrophoretic and chromatographic characteristics of Hb Pb have been compared with those of Hb A3 and also with those of other fast hemoglobins produced in vivo or in vitro. An attempt has been made to determine whether the altered electrophoretic mobility of Hb Pb can be ascribed to attachment of some ligand. Hemoglobins A3 and Pb have been subjected to trypsin hydrolysis, and the results of these studies have been compared.

METHODS

Blood was collected using Na₂EDTA (0.6 mg./ml. blood), ammonium and potassium oxalate (1.2 and 0.8 mg./ml. blood) or heparin (0.2 mg./ml. blood) as anticoagulant.

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This investigation was supported by graduate training grant number TI-AM-5260 and research grant number HE-02799 from the National Institutes of Health.


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Hematologic studies were performed by standard methods in the clinical laboratories of participating hospitals. Blood lead was determined by the Division of Chemistry, Bureau of Laboratories of the Baltimore City Health Department, using a wet digestion dithizone method. Urine coproporphyrin was measured by the semiquantitative method of Benson and Chisolm.

**Globin Characterization**

Hemolysates were prepared from washed red cells with distilled water and carbon tetrachloride. Buffers were made with distilled, but not deionized, water. The phosphate-agar chromatographic technic of Naiman and Gerald was slightly modified to permit use of larger samples. Globin was prepared with cold acid acetone and was subjected to starch gel electrophoresis in a 6M urea-barbital buffer. Paper and vertical starch gel electrophoresis and citrate agar electrochromatography were performed by previously described technics. Peptide mapping of trypsin-digested hemoglobin was performed by the method of Ingarn as modified by Baglioni. Alpha and beta chains were separated by carboxymethyl cellulose chromatography in 8M urea containing mercaptoethanol.

**Elution from Agar**

Hemoglobin was eluted from phosphate agar by imbedding strips of agar in a starch block prepared with phosphate buffer, pH 6.5, ionic strength 0.10, and applying 8 volts/cm. until most of the hemoglobin had migrated into the starch.

**Lead Staining**

To assess the sensitivity of lead staining procedures, serial twofold dilutions of a 0.01 M solution of lead acetate were prepared at pH 7.0. and immediately subjected to electrophoresis in starch gel at pH 8.6 for 3 hours.

Portions of the gel were immersed in aqueous solutions of 0.2 per cent dipotassium rhodizionate, 3 per cent potassium dichromate or hydrogen sulfide. After 24 hours, staining had occurred only with the H₂S solution. At a lead acetate concentration of 1.25 × 10⁻² M, a discrete spot appeared. Lower concentrations could not be detected; higher concentrations produced a broad smear along the path of migration. Starch gels containing hemoglobin fractions were then stained for 24 hours with H₂S solution.

**Studies of Metal and Sulfide Binding**

Hemolysates containing the fast hemoglobin from a patient with lead poisoning, to which 0.03 M cysteine, 2-mercaptoethanol or reduced glutathione had been added, were incubated for 18 hours at 37 C. and pH 7.3. The hemolysates were dialyzed for 24 hours against Drabkins solution (1 Gm. NaHCO₃, 0.2 Gm. K₃Fe(CN)₆, 0.05 Gm. KCN/L. distilled water) and then subjected to electrophoresis. Normal hemolysates were incubated with GSSG (15 mg./ml.) for 3 hours at 37 C., dialyzed against Drabkin’s solution, and then subjected to electrophoresis.

Solutions of 0.01 M sodium chromate, lead acetate and chromous chloride were prepared. Immediately after these solutions were adjusted to pH 6–8, portions were added to equal volumes of a normal hemolysate and incubated for 3 hours at 37 C. The hemolysates were then dialyzed for 48 hours against several changes of Drabkin’s solution, precipitated hemoglobin was removed by centrifugation and the supernatant hemoglobin solution was subjected to electrophoresis.

*The gas evolved from one “Aitch-tu-ess” tube (Will Corporation) was bubbled through 1 liter of distilled water.*
FAST HEMOGLOBIN IN LEAD POISONING

Fig. 1.—Comparison of fast hemoglobin with normal hemoglobin by electrophoresis on paper and starch gel at pH 8.6: † = origin; A\textsubscript{1} = major normal hemoglobin component; A\textsubscript{2} = hemoglobin A\textsubscript{2}; Fast = hemoglobins A\textsubscript{3} and Pb; CA = carboxylic anhydrase. The cathode is to the left.

Results

Clinical Studies

Records of the 82 patients with lead poisoning seen at the Johns Hopkins Hospital within the past 5 years were reviewed. The original paper strips from patients studied by hemoglobin electrophoresis were examined and clinical and hematologic data were tabulated. In 6 of the 21 paper electrophoretic patterns, Hb Pb appeared in front of the major hemoglobin component. This finding had been overlooked in only one instance.

Starch gel electrophoresis was performed on fresh hemolysates from 24 new patients with lead poisoning in the University of Maryland, Baltimore City, and Johns Hopkins Hospitals. In 10 of 24 hemolysates, Hb Pb could be detected in front of the major hemoglobin component. The appearance of Hb Pb after paper and starch gel electrophoresis is illustrated in Figure 1. In 13,000 electrophoretic analyses performed in our laboratory before the present study, fast hemoglobins of this appearance had been detected only in lead poisoning. In contrast to the results of electrophoresis at pH 8.6, Hb Pb could not be demonstrated by agar electrophoretography at pH 6.0 or starch gel electrophoresis at pH 7.0.

The diagnosis of lead poisoning was based upon multiple abnormal blood lead levels in 35 patients; in the remainder the diagnosis rested upon a single abnormal determination and a convincing clinical picture. The 3 adults in the combined series of 45 patients had normal electrophoretic patterns, and they were not anemic. The children ranged in age from 1 to 6 years; the majority were 2–3 years old. Hb Pb occurred more commonly in children below the age
Table 1.—Comparison of Patients with Lead Poisoning, with and without Fast Hemoglobin

<table>
<thead>
<tr>
<th></th>
<th>Fast Hemoglobin Present</th>
<th>Fast Hemoglobin Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic</td>
<td>5/16 (31%)</td>
<td>10/29 (34%)</td>
</tr>
<tr>
<td>Coproporphyrinuria (3–4+)</td>
<td>8/13 (62%)</td>
<td>6/9 (67%)</td>
</tr>
<tr>
<td>Reticulocytosis (&gt;2%)</td>
<td>6/9 (67%)</td>
<td>8/12 (67%)</td>
</tr>
<tr>
<td>Red cell stippling (moderate-marked)</td>
<td>0/5 (0%)</td>
<td>3/16 (19%)</td>
</tr>
<tr>
<td>Increased fetal hemoglobin (2.2–8.1%)</td>
<td>8/9 (89%)</td>
<td>1/5 (20%)</td>
</tr>
</tbody>
</table>

Table 2.—Comparison of Patients with Lead Poisoning, with and without Fast Hemoglobin

<table>
<thead>
<tr>
<th></th>
<th>Fast Hemoglobin Present</th>
<th>Fast Hemoglobin Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>Number</td>
<td></td>
</tr>
<tr>
<td>Blood lead mg./100 Gm. blood</td>
<td>16</td>
<td>29</td>
</tr>
<tr>
<td>Hematocrit %</td>
<td>16</td>
<td>28</td>
</tr>
<tr>
<td>Hemoglobin concentration Gm./100 cc.</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin concentration %</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Mean ± 2SD</td>
<td>Mean ± 2SD</td>
</tr>
<tr>
<td></td>
<td>10 ± 42</td>
<td>13 ± 1.30</td>
</tr>
<tr>
<td></td>
<td>28 ± 9.2</td>
<td>34.4 ± 10.6</td>
</tr>
<tr>
<td></td>
<td>6.9 ± 2.6</td>
<td>9.9 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>25.1 ± 3.5</td>
<td>29.5 ± 5.31</td>
</tr>
</tbody>
</table>

of 3 (48 per cent) than in those from 3–6 years of age (29 per cent). All children were paint and plaster eaters. The duration of pica was generally unknown, but was usually thought to have been present for many months. Older siblings of 3 patients with Hb Pb also were paint eaters, but Hb Pb could not be detected in these siblings or in the patients' mothers. Some patients were under treatment with chelating agents at the time blood was obtained for electrophoresis.

The presence of Hb Pb was not related to presence of symptoms on admission, degree of coproporphyrinuria, reticulocyte count, or amount of red cell stippling (Table 1). Proportion of fetal hemoglobin was measured in 14 children. The 9 whose blood contained Hb Pb were 1½–3 years old; the 5 children with normal hemoglobin were 3–5 years old. Elevated levels of fetal hemoglobin (2.2–8.1 per cent) were more common in the patients with Hb Pb. Presence of Hb Pb was not related to blood lead level (p > 0.4) (Table 2). The majority of the lead in blood is bound to red cells. If blood lead levels were adjusted to compensate for variations in hematocrit values between patients, presence of Hb Pb still was not related to blood lead level. Patients with and without Hb Pb differed significantly in hematocrit value (p < 0.01), hemoglobin concentration (p < 0.01), and mean corpuscular hemoglobin concentration (p < 0.001) (Table 2). When the same comparison was made after exclusion of patients older than 3½ years, the statistical significance of these differences was unchanged. Uncomplicated hypochromic anemia was not associated with a fast hemoglobin, for Hb Pb could not be detected in a control group of 50 young children with severe iron deficiency anemia.
FAST HEMOGLOBIN IN LEAD POISONING

Hemolysates from 55 patients receiving tolbutamide were studied by starch gel electrophoresis. Hb Tol was detected in 5 hemolysates. The 5 patients were not anemic. They had taken 0.5–1.5 Gm. tolbutamide daily for periods of 3 months to 2 years. When studied on starch gel and agar, hemolysates containing Hb Tol appeared identical to those containing Hb Pb.

Studies of Metal and Sulfide Binding

Blood obtained from a single affected donor was mixed with heparin and with anticoagulants known to bind both lead and calcium. No difference could be detected in the amount of fast hemoglobin present. Hemolysates from this donor were incubated with mercaptoethanol and with reduced glutathione. When compared with control samples, no difference could be detected in the amount of fast hemoglobin present. Incubation of a normal hemolysate with sodium chromate produced a fast hemoglobin component which appeared similar to Hb Pb after starch gel electrophoresis but migrated as a heavy band in the approximate position of fetal hemoglobin during agar electrophoresis (Fig. 2). Incubation with lead or chromous ions under the same conditions did not produce any alterations in electrophoretic mobility.

Hemoglobin Characterization

Hemolysates containing Hb Pb were subjected to starch block electrophoresis; the fast hemoglobin was eluted and concentrated about 100-fold. Hb A₃
from a fresh normal hemolysate was prepared in a similar fashion. The purified hemoglobins A₃ and Pb were subjected to electrophoresis on starch gel which was then stained with H₂S solution. A lead sulfide precipitate could not be detected in any sample.

Both A₃ and Pb hemoglobins separated into 2 bands during agar electrochromatography (Fig. 3). Elution of fast and slow bands from agar and repeated electrochromatography yielded only one band from each eluate. Both “fast” and “slow” eluates migrated more rapidly than hemoglobin A on starch gel in the position of Hb A₃.

Hemoglobin Pb and hemoglobin A₃ were separated into heme and globin fractions. The globin of both samples was subjected to electrophoresis in 6M urea-barbital, and the globin from Hb A₃ was subjected to chromatography in 8 M urea-mercaptoethanol-phosphate. On starch gel, the alpha chains migrated as discrete bands, but the beta chains migrated with diffuse, anodal tails (Fig. 4). Fingerprints of hemoglobins Pb and A₃ did not differ significantly from those of hemoglobin A. During chromatographic separation, in 8 M urea, the beta chain of Hb A₃ was eluted in exactly the same position as the beta chain of Hb A.

**DISCUSSION**

A fast hemoglobin (Hb Pb) was found in about 40 per cent of preschool children with elevated blood lead levels, but not in lead-poisoned older siblings or adults. Hb Pb occurred more often when hypochromic anemia was present, it could not be detected in children with marked hypochromic anemia but no evidence of lead poisoning. Failure of previous investigators to produce
Fig. 4.—Comparison of globins prepared from purified Hb A₃ and Hb Pb with normal globin and globin from a patient with sickle cell anemia, by starch gel electrophoresis in 6 M urea. The cathode is to the left. The β chains of Hb Pb and Hb A₃ have diffuse anodal trails.

A fast hemoglobin in animals by administration of lead may have been due to species differences, to the use of adult animals, to the acute nature of the experiments, or possibly to the absence of iron deficiency.

A fast hemoglobin could be detected in only 9 per cent of adults receiving tolbutamide. Hb Tol appeared similar to both Hb Pb and Hb A₃ after electrophoresis. In contrast to Hb Pb, it was found in patients with no hematologic abnormalities. Its presence could not be related to dosage of tolbutamide or duration of therapy. Its relationship, if any, to Hb Pb remains unclear.

Altered electrophoretic mobility of Hb Pb could not be attributed to binding
of glutathione since Hb Pb differed electrophoretically and chemically from hemoglobin-glutathione complex. Altered electrophoretic mobility also did not appear to be due to binding to lead to hemoglobin. Direct chemical analysis of Hb Pb for heavy metals was impractical, for lead-free reagents had not been used in its preparation, but starch gels containing Hb Pb were stained with H2S. The most dilute lead solution which could be detected after electrophoresis in starch gel contained lead ions in a concentration of about 10^{-2} M, suggesting that there was less than a 10^{-2} M concentration of lead in the semipurified hemolysates which were subjected to electrophoresis. Lead cation did not bind to hemoglobin in vitro, although chromate anion behaved as a ligand under the same conditions. Reagents known to combine with lead ions by chelation or binding to sulfhydryl groups did not alter the electrophoretic mobility of Hb Pb.

Hemoglobin Pb appeared identical to normal hemoglobin A3 in its electrophoretic and chromatographic behavior. Globin prepared from Hb Pb and Hb A3 appeared to have abnormal β chains after electrophoresis in 6M urea. In contrast, the β chain of Hb A3 appeared identical to that of Hb A after chromatography in 8 M urea, and fingerprints of both Hb Pb and Hb A3 did not differ significantly from that of hemoglobin A.

These findings are consistent with at least 3 hypothetical alterations of the β chains. (1) Small, negatively charged ligands could be present, attached to the β chain by bonds broken during tryptic hydrolysis. Such ligands might not stain with ninhydrin or they might have been lost during fingerprinting due to high electrophoretic or chromatographic mobility. (2) Ligands could be attached to the trypsin resistant “cores” of the β chains of hemoglobin A3 and Pb, and therefore not appear on our fingerprints. The β chain of Hb A3, prepared by chromatography in 8 M urea-mercaptoethanol, did not differ from the β chain of Hb A, suggesting that if a ligand had been present, the bond was broken during chromatography. (3) The tertiary structure of the β chains of hemoglobins Pb and A3 may be different from that of hemoglobin A1. A change of this type could either expose previously “buried” negatively charged portions of the molecule to electrophoretic solvents, or alter protein-solvent interactions in such a manner as to increase electrophoretic mobility.28

Regardless of the type of molecular alteration responsible for increased electrophoretic mobility of hemoglobins A3 and Pb, the evidence presented suggests that the fast moving hemoglobin of lead poisoning is very similar, if not identical, to hemoglobin A3. Little hemoglobin A3 is present in red cells newly released into the circulation, but its proportion increases during the life of the cell. The metabolic processes responsible for these alterations and the nature of the changes produced are unknown. If hemoglobin Pb does represent hemoglobin A3 in increased proportion, its presence may reflect premature senescence of red cells in lead poisoning.

SUMMARY

1. An electrophoretically fast hemoglobin was found in approximately 40 per cent of preschool children with elevated blood lead levels.
2. Fast hemoglobin was found more often in lead-poisoned patients with hypochromic anemia than in patients with normochromic red cells.

3. Fast hemoglobin differed from hemoglobins produced in vitro by incubation with chromate or oxidized glutathione. It had electrophoretic properties similar to that found in a few patients receiving tolbutamide.

4. Fast hemoglobin could not be differentiated from normal hemoglobin A\(_3\) by any technic utilized.

5. Both lead and A\(_3\) hemoglobins were heterogeneous molecular species.

6. The mechanisms leading to the production of hemoglobin A\(_3\) and lead hemoglobin remain unknown.

**Summario in Interlingua**

1. Un electrophoreticamente rapide hemoglobina esseva trovate in approximativemente 40 pro cento de un gruppo de juveniles de etate prescolari con elevate nivellos sanguineo de plumbo.

2. Hemoglobina rapide esseva trovate plus frequentemente in patients invenenate per plumbo quando illes habeva anemia hypochromic que quando lor erythrocytos esseva normochromic.

3. Hemoglobina rapide differeva ab hemoglobinas producite in vitro per incubation con chromato o glutathiona oxydate. Su proprietates electrophoretic esseva simile a illos trovate in un micre numero de patientes sub tractamento con tolbutamida.

4. Hemoglobina rapide non poteva esser differentiate ab normal hemoglobina A\(_3\) per ulla del technicas essayate.

5. Tanto le hemoglobina a plumbo como le hemoglobina A\(_3\) esseva heterogenee species molecular.

6. Le mechanisms ducente al production de hemoglobina A\(_3\) e de hemoglobina a plumbo remane incognosce.

**ACKNOWLEDGMENTS**

We wish to express our thanks to Dr. Phillip Jensen of the University Hospital Pediatric Clinics for referral of patients with lead poisoning, to Mr. Paul Goldstein of the Johns Hopkins Hospital Pharmacy for referral of patients receiving tolbutamide, to Dr. Emanuel Kaplan of the Bureau of Laboratories, Baltimore City Health Department, for blood lead determinations, and to Dr. J. Julian Chisoihn, Jr., of Baltimore City Hospitals for referral of patients and review of the manuscript.

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Fast Hemoglobin in Lead Poisoning

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