Zinc acetate as a precipitant of unstable haemoglobins

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SUMMARY Whereas the addition of four moles of zinc acetate to one of normal human haemoglobin at pH 7.4 results in a clear solution, addition to unstable haemoglobin results in precipitate formation. Within certain limits no false-positive results are obtained with methaemoglobin, and with fetal haemoglobin. With excess zinc, normal haemoglobin also precipitates, but this process is reversible and can be used for a rapid concentration of dilute solutions of normal haemoglobin.

We have recently been searching for a means to precipitate selectively very small amounts of an unstable haemoglobin from a haemolysate as found, for example, in heterozygotes for Hb A and Hb Coventry.1 In the course of this study which is not yet finished it became apparent that zinc acetate may be a useful laboratory tool in a routine test for discovering the common unstable haemoglobins. Within certain limits it does not give false-positive results with methaemoglobin and with fetal haemoglobin. Furthermore it can be used for the reversible precipitation of normal haemoglobin from dilute solutions.

Material and methods

A sample (0.2 ml) of 10 g/dl haemolysate is added to 1:8 ml of a solution containing 0.35 ml of 0.003 M zinc acetate and 1.45 ml of TRIS (trimethamine) buffer 0.1 M, pH 7.4. After mixing, the tubes are incubated for 30 min at 37°C. With unstable haemoglobins an immediate flocculation is seen which has settled as a precipitate by the end of the incubation period. For routine tests this proportion of 3.5 mol zinc:1 mol Hb is preferred because slight precipitates (±) may appear with 0.4 ml zinc acetate and 1.4 ml of buffer, with some normal haemolysates. They can of course be disregarded, but there is no disadvantage in arranging the routine test as described here using 0.35 ml of zinc acetate and 1.45 ml of buffer (3.5 mol zinc:1 mol Hb).

Haemolysates containing various concentrations of Hb F were prepared by mixing 10 g/dl haemolysates from adults and babies. To obtain a 3% methaemoglobin solution, 0.1 ml of K3 Fe(CN)6 (116 mg in 100 ml H2O) was added to 2 ml of 10 g/dl fresh normal haemolysate.

A typical procedure for concentration of dilute haemoglobin solutions would be the following concentration of 10 ml of a 0.5 g/dl haemoglobin solution. On addition of 2 ml TRIS buffer (0.1 M pH 7.4) and 1 ml of 0.003 M zinc acetate a true precipitate appears which after a few minutes at 37°C redissolves. On centrifuging, a compact gel forms and the clear supernatant is decanted. On adding 0.2 ml of water and two drops (about 0.04 ml) of 0.1 M EDTA (ethylenediaminetetraacetate) containing 0.3 μM NaCN the precipitate redissolves on gentle mixing.

Results

ROUTINE TESTS FOR NORMAL HAEMOGLOBINS

The test shows no precipitation of normal adult haemoglobin below a proportion of 4 mol zinc:1 mol Hb. It requires a pH > 7.0 and works equally well at room temperature, 37°C and 50°C. The solutions have to be made up and measured out accurately because above a proportion of 4:1, normal Hb also precipitates, and this may be a handicap in a busy routine laboratory (see Table). The test has the advantage, however, of being negative when up to 20% Hb F are present, and that no precipitate appears with up to 3% methaemoglobin. Potentially therefore this is a suitable test with aged haemolysates. On adding zinc in a proportion of 10:1, normal haemoglobin precipitates almost completely but can then be fully redissolved by the addition of EDTA. Precipitates of unstable...
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Precipitation of haemoglobin by zinc acetate (all volumes in ml)

<table>
<thead>
<tr>
<th>TRIS buffer pH 7-4</th>
<th>Zinc acetate in TRIS buffer (0·1 M)</th>
<th>Water</th>
<th>Haemolysate 10 g Hb/dl</th>
<th>Immediate precipitate</th>
<th>Precipitate after 1 hr at 37°C</th>
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</thead>
<tbody>
<tr>
<td>1·0</td>
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<td>0·9</td>
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<td>0·5</td>
<td>0·2</td>
<td>±</td>
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</tr>
</tbody>
</table>

A parallel experiment with haemolysate from a patient with Hb A + Christchurch showed an immediate precipitate at all concentrations of zinc.

Haemoglobin redissolve only partially. The redissolved normal haemoglobin shows no features of denaturation on checking the Soret band absorption at 415 Å, on scanning the visible spectrum, and on electrofocusng v normal controls.

Routine Tests for Unstable Haemoglobin

The test has been clearly and immediately positive with fresh haemolysates containing Hb E and Hb A plus respectively Hbs E, H, Köln, Christchurch, Hasharon and four not yet identified unstable haemoglobins. In the case of Hb Christchurch the precipitate consisted of abnormal β-chains (Fig. 1) and in the case of Hb Hasharon the abnormal haemoglobin precipitated as a whole—that is, abnormal α-chains and normal β-chains, but no normal Hb A.

Of 122 consecutive bloods seen at the Abnormal Haemoglobin Reference Centre, Old Addenbrooke’s Hospital, Cambridge, 42 were normal, 45 came from β-thalassaemia heterozygotes (4 of them δβ thalassaemia) and 40 contained haemoglobin variants. In the first two groups very fine precipitates (±) were occasionally seen with a proportion of 4 mol zinc:1 mol Hb, 6 of the 42 normal, and 7 of the 45 β-thalassaemia heterozygotes, but none with a proportion of 3·5:1. Of the 40 haemoglobin variant samples nine gave positive results, both at zinc:Hb proportions of 4:1 and 3·5:1. They were derived as follows: 4 Hb H disease, 1 Hb Hasharon, 1 homozygous Hb E, 1 Hbs A and E, and two contained unstable haemoglobins not yet identified. Negative results were obtained with 23 heterozygotes for Hb A and respectively Hbs D Punjab (1), J Baltimore (1), G Philadelphia (1), O-Arab (2), M

Fig. 1 “Fingerprint” of zinc precipitate from haemolysate of heterozygote for Hb Christchurch (β71 Phe→Ser). The precipitate consists predominantly of the abnormal β-chain. The abnormal trypsic peptide β8-9 is indicated. The area where the normal β-chain peptide would be expected is ringed.
Iwate (1), C(4), S(9), Lepore (4) and doubly abnormal heterozygotes for Hbs O-Arab and Lepore (2), S and C (2), as well as 3 from homozygous sickle-cell anaemia and one with a double Hb A₂. These specimens (with two exceptions) gave negative results with zinc:Hb proportion of 4:1. Slightly positive results were obtained with Hbs O-Arab plus Lepore, but the results were negative with a zinc:Hb proportion of 3:5:1.

CONCENTRATION OF HAEMOGLOBIN SOLUTIONS

One problem for laboratories handling haemoglobin is the time and expense involved in the concentration of dilute solutions such as column eluates; see Fig. 2 as an example. Provided that the pH is alkaline and sequestrants are not present, the addition of a tenfold molar ratio zinc:Hb gives virtually complete precipitation of haemoglobin. When phosphates are present some zinc phosphate will be formed and this may complicate, though not devalue, the procedure. The supernatant can be removed and the precipitate then dissolved in a small volume of water with EDTA allowing at least 20-fold concentration. The resulting solution behaves as normal haemoglobin both as regards its spectrum and on isoelectric focusing. Precipitated unstable haemoglobins, however, substantially do not redissolve.

**Fig. 2** Concentration of haemoglobin solution (pH 7-4) with zinc acetate. Left: 10 ml 0-1% haemolsyate (0-05 M TRIS buffer, pH 7-4); middle: 0·3 ml of 0·1 M zinc acetate were added; the resulting precipitate was centrifuged down; right: The supernatant was removed and 0·1 M EDTA were added followed by 5 ml water to redissolve the haemoglobin which is now of double strength.

**Discussion**

Zinc is used for the selective precipitation of some human proteins in Cohn's "Method 10." The amounts used differ and the required pH is acid rather than alkali as in our test.² The binding sites of zinc in haemoglobin are not known though Oelshlegel and colleagues³ have proposed subunit contact areas as likely sites. These areas are involved in the conformational changes of oxy- to deoxyhaemoglobin and earlier work has demonstrated that this step is affected by zinc.⁴ In the whale myoglobin monomer, a single zinc binds at the corner between the G and H helices to the imidazole ring of the external histidine residue GH1.⁵ In the human haemoglobin α-chain this position is occupied by leucine and in the β, γ and δ-chains by phenylalanine. However the penultimate residue of the G helix is, in all chains, one of histidine. Mutations of this external histidine residue result in only a mild abnormality if any.

Our interest in zinc as a precipitant for unstable haemoglobins stems from the observation that oxidation to methaemoglobin invariably preceded the selective heat precipitation of Hb Köln.⁶ In a study on the inhibition of the enzymic conversion of glucose-1-phosphate to hexose-6-phosphate by oxidising agents it was noted that zinc at low concentrations behaved like the oxidants.⁷ We therefore used zinc to precipitate, the unstable haemoglobin Hammersmith thus separating this electrophoretically "neutral" mutant from Hb A.

As mentioned in the introduction, we returned to this technique in the hope that it might help with the routine discovery of small amounts of Hb Coventry and similar variants which might occur much more frequently than is recognised at present. This has not been achieved as yet, but the zinc precipitation test may be a useful adjunct to present laboratory tests. The isopropanol precipitation test⁸ tends to give false-positive results when excess amounts of Hb F and methaemoglobin are present, and it is here that the zinc acetate precipitation test may find a place. At present a most useful application of zinc acetate seems the rapid concentration of dilute normal haemoglobin solutions.

We thank Miss Laurene Robb and Miss Gwen Surreys for help with testing haemoglobin samples in Cambridge.

References


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