FETAL HEMOGLOBIN DETERMINATIONS IN THE NEWBORN BY THE SINGER AND ACID ELUTION METHODS

Imran Özalp, M.D.,* Şinasi Özsoylu, M.D., and Burhan Say, M.D.*****

It is an established fact that physical and chemical characteristics of hemoglobin in the normoblasts and erythrocytes during early fetal life differ from those of hemoglobin in the adult. Until the sixteenth week of pregnancy, oxygen in the fetus is carried only through normoblasts containing fetal hemoglobin.[1] Although erythropoiesis in the fetal bone marrow starts during the twentieth week of pregnancy, synthesis of adult hemoglobin (HbA) along with fetal hemoglobin (HbF) begins after the thirty-first week. Cells containing only adult hemoglobin are seen in the fetal circulation at the thirty-seventh and thirty-eighth weeks of pregnancy. Synthesis of fetal cells stops after the fortieth week of intra-uterine life, but the formation of cells containing both fetal and adult hemoglobin continues until the fourth month after birth, and decreases from then on. After the age of two years, fetal Hb composes only two per cent of the total hemoglobin.

Since erythropoiesis takes place mostly in the fetal liver, spleen and bone marrow, some investigators tried to establish a relation between HbF synthesis and erythropoiesis in the liver.[2] Other investigators however noted a decrease in the percentage of fetal hemoglobin in cases of erythroblastosis despite increased erythropoiesis in the liver.[3–6]

Although there may be other explanations of this phenomenon, the most logical one seems to be that, as the fetus grows older, there is a decrease in the production of erythrocytes containing HbF in the bone marrow. The ferroprotoporphyrin ring is the same in both HbF and HbA, the main difference being a gamma chain synthesis in the former and a beta chain synthesis in the latter in the globin molecules.[7] Gamma chain synthesis in the fetus decreases or stops after a certain period, and beta chain, and in small quantities, delta chain synthesis takes place, forming HbA, and in small quantities, Hb A_2.

From Ankara University Hacettepe Faculty of Medicine and Hacettepe Medical Center.

* Pediatrician

** Pediatrician

*** Chief, Hematology Department

These two kinds of hemoglobin are differentiated by their antigenic characteristics, speed in color chromatography,[8] differences in amino acid sequences,[7] solubility, crystallography, and x-ray diffraction. These tests indicate that the molecules of the two types of hemoglobin have different primary and tertiary structures. They can be differentiated quite simply by their resistance to alkali[9] and by electrophoretic mobilities.[10] In electrophoresis, HbF migrates more slowly (pH 8.6 buffer) than HbA. Furthermore, it is known that denaturation of HbF with alkaline substances takes considerably longer than that of HbA. This characteristic makes diagnosis of "swallowed blood syndrome" in newborns quite easy.

The percentage of fetal hemoglobin becomes abnormally high in many hematologic disorders. A marked elevation is seen in patients with beta thalassemia major and with persistent high fetal hemoglobin syndrome.[11,12] It may also be high in patients with certain hemolytic anemias, sickle cell anemia, and in some cases of spherocytosis. It is rarely encountered in hereditary non-spherocytic hemolytic anemia.[13] An increase in fetal hemoglobin is also reported in some cases of leukemia.[14] A rise in HbF in aplastic anemia may serve as an important factor in the differential diagnosis of this disease and in determining the prognosis in cases of acquired aplastic anemia.[15]

In sickle cell anemia, a high ratio of fetal hemoglobin helps to minimize the formation of sickle cells *in vitro* and also alleviates the clinical symptoms of the disease.[16] An increase in the percentage of cells containing fetal hemoglobin following an aplastic crisis seems to support this view.[17]

Chemical tests have proved that fetal hemoglobin in various hemolytic diseases is not structurally different from the fetal hemoglobin in the fetus.[11,18] It follows therefore that in the disorders in which fetal hemoglobin increases, either the transition from HbF to HbA does not occur (beta thalassemia major or persistent high fetal Hb syndrome) or there is a regression to HbF synthesis (acquired aplastic anemia).

Fetal hemoglobin is resistant to both alkali and acids. The resistance to alkali can be demonstrated by chemical methods, such as Singer's method, and resistance to acid can be determined by the acid elution method, which differentiates those erythrocytes carrying HbF.[19]

In using this technique care must be taken to spread the specimen in such a way that the erythrocytes will not lie on top of each other. The pH of the buffer solution should be kept between 3.2 and 3.4, and the specimen should be stained within 60 minutes. An increase of up to 100 per cent of HbF-containing cells could falsely be produced if the smears are not

stained within this period of time.[20] Next, thinly spread areas are detected under low power microscope (16 objective and 10x ocular). Selecting one such area, 500 cells are counted under immersion in order to determine the percentage of cells containing fetal hemoglobin. Erythrocytes containing HbA appear dim and shadowy, whereas the cells containing HbF retain their normal shape and are stained bright pink (Figure 1).

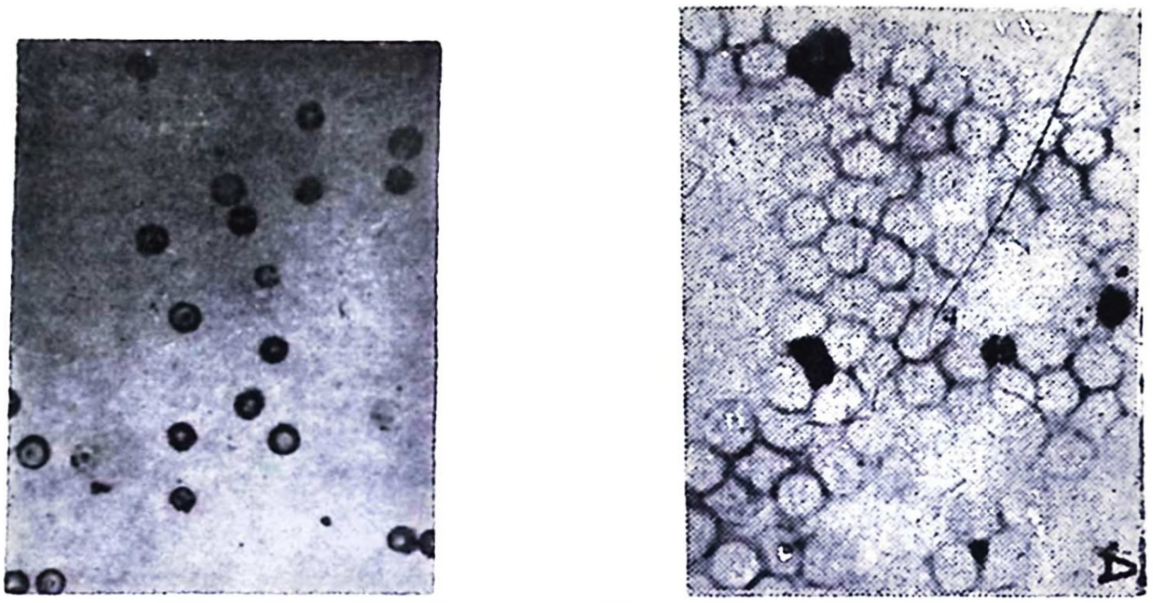
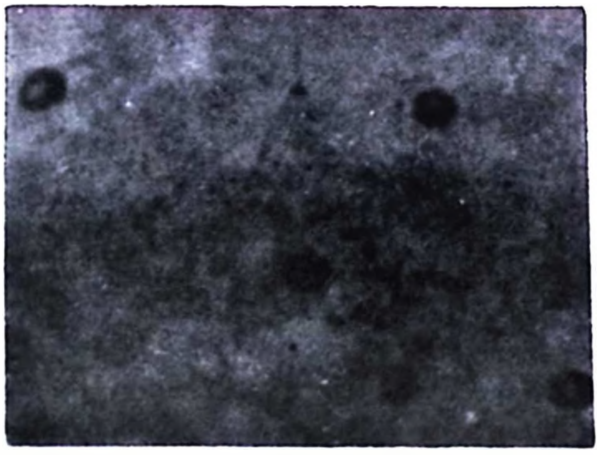


Figure 1. Peripheral smears stained for fetal hemoglobin.
A. From a newborn. B. From a normal adult.



C. From a person with thalassemia minor.

It should be emphasized that these two methods do not yield the same results. The alkali denaturation method determines chemically the amount of HbF in the hemoglobin solution. The purpose of the acid elution method is to count the number of cells containing HbF.

From the genetic point of view, it would be expected that fetal hemoglobin would be distributed equally in all the erythrocytes, but the acid elution method demonstrates that this is not the case. A similar phenomenon is encountered in patients with heterozygote glucose 6 phosphate dehydrogen-

ase deficiency, where the activity of this enzyme in red blood cells differs from one cell to another.[21]

Although the amount of HbF shows no increase in beta thalassemia minor when measured by Singer's method, the acid elution method indicates an increase in the number of cells containing HbF and is quite useful in diagnosing the disease.

The acid elution method is also useful in diagnosing materno-fetal transfusions, since it readily demonstrates the presence of an increased number of erythrocytes containing HbA in the circulation of the newborn. The technique is also useful in cases of feto-maternal hemorrhage, which is one of the causes of anemia in the newborn period. This method, together with other serological tests, is of great diagnostic value.[22]

Comparison of the two methods has been made by various authors in a variety of diseases. Kiasoglou and his colleagues determined the amount of HbF in the blood of 82 persons, of whom 19 were healthy and 63 were suffering from various blood disorders.[20] They were able to demonstrate that in patients with high HbF levels, there was also a proportional increase in the number of fetal hemoglobin-containing red blood cells. They also indicated that the staining technique gives somewhat higher percentages than Singer's method.

We have also compared the two methods, using cord blood and capillary samples from 98 newborns. Cord blood was used for HbF determination by Singer's method, and peripheral smears were used for the acid elution technique. The staining was done not later than one hour after the samples were obtained. The results of this study are given in Figures 2 and 3. It can be seen clearly that the number of cells containing fetal hemoglobin is significantly higher when calculated by the acid elution method as compared to Singer's method. Although it was not possible to obtain complete correlation between these two determinations, as a whole the results are comparable. This study confirms the results obtained by Zipursky and his colleagues.[4]

Fetal hemoglobin determinations by the two methods are compared in Figure 4. Here, the specimens were obtained from ten normal newborns and ten infants with neonatal hyperbilirubinemia. Both methods indicated a higher level of HbF in the jaundiced infants when compared to the normal ones. Blood samples from newborns with hyperbilirubinemia were obtained before exchange transfusions were given. Hemoglobin levels in these infants were between 15 and 17 Gm per cent, and there was no evidence of erythroblastosis. Since it has been reported that there is a relative decrease in

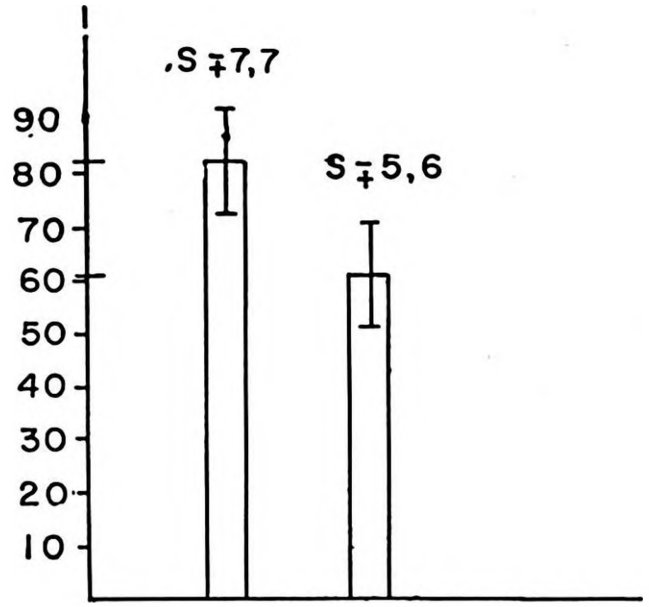


Figure 2. Fetal hemoglobin determinations of 98 newborns.
On the left by the acid elution technique.
On the right by the Singer method.

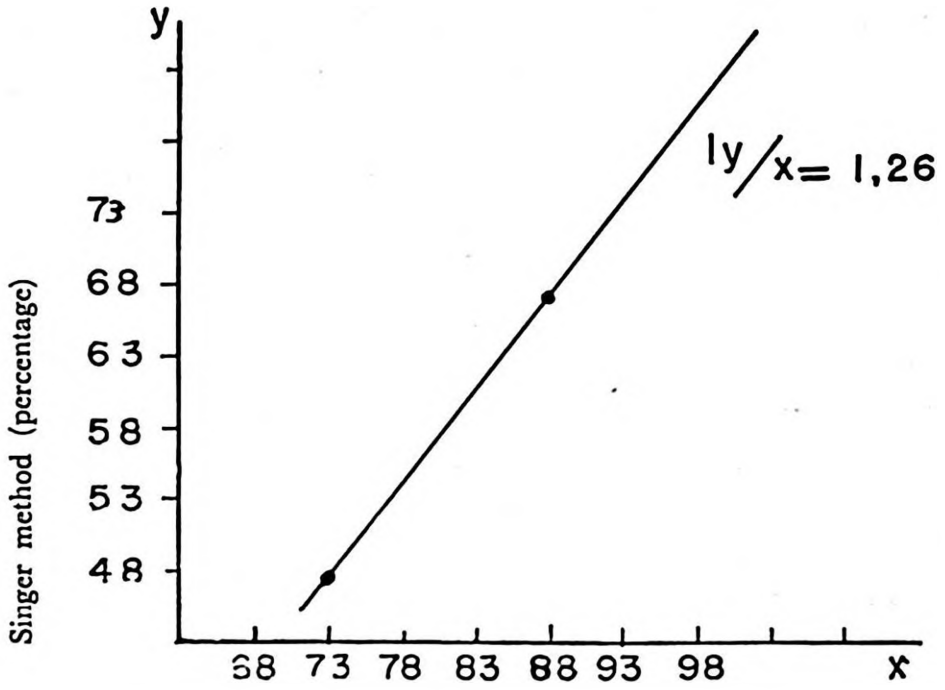


Figure 3. The comparison of fetal hemoglobin determinations using both the Singer and acid elution methods.

the amount of HbF in cases of erythroblastosis fetalis,[3,5,6] it seems logical to investigate the relations between the low HbF and neonatal hyperbilirubinemia of unknown etiology.

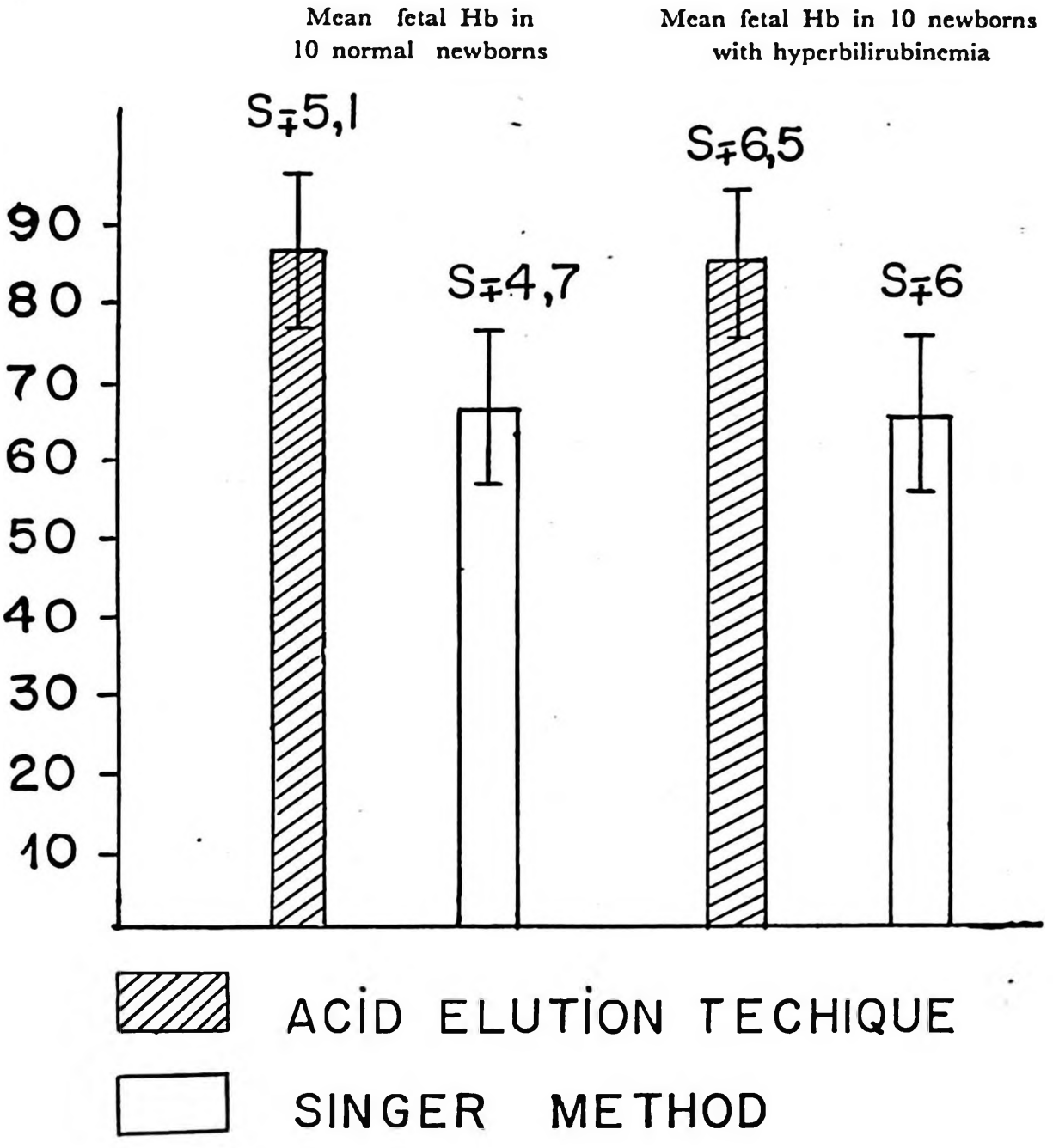


Figure 4. Comparison of the mean fetal hemoglobin determination in newborns with and without hyperbilirubinemia.

The study presented here has led to the following conclusions concerning the acid elution method:

1. It is a relatively simple test that can be carried out in even a modest hospital laboratory.
2. It is of value in diagnosing cases of beta thalassemia minor because it demonstrates clearly the abnormally high number of erythrocytes containing fetal hemoglobin.

3. It is also useful in the diagnosis of persistent high fetal hemoglobin syndrome, since this syndrome is characterized by almost equal distribution of fetal hemoglobin in the red cells.
4. It confirms the theory that both HbA and HbF are produced by the same cells.
5. It has a definite diagnostic value in cases of feto-maternal and materno-fetal hemorrhages.

Summary

In this paper the literature in this subject is reviewed, and the results of a comparative study of the two methods of fetal Hb determination are presented. It is concluded that the two methods give comparable results, although the acid elution method has certain practical advantages over Singer's method.

REFERENCES

1. Fraser, I.D., and Raper, A.B.: Observations on the change from foetal to adult erythropoiesis, Arch. Dis. Childh. **37**: 289, 1962.
2. Thomas, D.B., and Yoffey, J.M.: Human foetal haematopoiesis II. Hepatic haematopoiesis in the human foetus, Brit. J. Haemat. **10**: 193, 1964.
3. Brody, S., and Engstrom, L.: Foetal and adult haemoglobin in newborn infants with erythroblastosis foetalis, Acta Paediat. (Upps) **49**: 868, 1960.
4. Zipursky, A., Neelands, P., Pollock, J., Chown, B., and Israels, L.G.: Fetal Hb synthesis in some hemoglobinopathies, Lancet **2**: 154, 1961.
5. Jonxis, J.H.P.: Foetal haemoglobin and erythroblastosis, Nature **161**: 850, 1948.
6. Oppé, T.E., and Fraser, I.D.: Foetal haemoglobin in haemolytic disease of the newborn, Arch. Dis. Childh. **36**: 507, 1961.
7. Hunt, J.A.: Identity of the chains of adult and foetal human haemoglobins, Nature **183**: 1373, 1959.
8. White, J.C., and Beaven, G.H.: Foetal haemoglobin, Brit. M. Bull. **15**: 33, 1959.
9. Singer, K., Chernoff, A.I., and Singer, L.: Studies on abnormal hemoglobins; their demonstration in sickle cell anemia and other hematologic disorders by means of alkali denaturation, Blood **6**: 413, 1951.
10. Halbrecht, I., and Klibanski, C.: Identification of new normal embryonic haemoglobins, Nature **183**: 327, 1959.
11. Thompson, R.B., Mitchener, J.W., and Huisman, T.H.: Studies on the fetal hemoglobin in the persistent high Hb-F anomaly, Blood **18**: 267, 1961.
12. Olivia, J., and Myerson, R.M.: Hereditary persistance of fetal hemoglobin, Amer. J. Med. Sci. **241**: 215, 1961.
13. Mitchener, J.W., Thompson, R.B., and Huisman, T.H.: Foetal haemoglobin synthesis in some haemoglobinopathies, Lancet **1**: 1169, 1961.
14. Boguslawska-Jawarska, J., and Dobryszycka, I.W.: Hemoglobinopathies in leukemias in children, Pol. Tyg. Lek. **16**: 281, 1961.

15. Shahidi, N.T., Gerald, P.S., and Diamond, L.K.: Alkali-resistant hemoglobin in aplastic anemia of both acquired and congenital types, New Engl. J. Med. 266: 117, 1962.
16. Jackson, J.F., Odom, J.L., and Bell, W.N.: Amelioration of sickle cell disease by persistent fetal hemoglobin, J.A.M.A. 177: 867, 1961.
17. MacIver, J.E., and Parker-Williams, E.J.: The aplastic crisis in sickle cell anemia, Lancet 1: 1086, 1961.
18. Sturgeon, P., Schroeder, W.A., Jones, R.T., and Bergren, W.R.: The relation of alkali—resistant haemoglobin in thalassemia and abnormal haemoglobin syndromes to foetal—haemoglobin, Brit. J. Haemat. 9: 438, 1963.
19. Kleihauer, E., Braun, H., and Netke, K.: Demonstration von fetalem hemoglobin in der erythrocyten eines blutausstrichs, Klin. Wchrschr. 35: 637, 1957.
20. Kiasoglou, K.A., Wolman, I.J., and Garrison, M. Jr.: Fetal hemoglobin-containing erythrocytes I. Counts of cells stained by the acid elution method compared with alkali denaturation measurements, Blood 21: 553, 1963.
21. Beutler, E., and Bahida, M.C.: The separation of glucose-6-phosphate-dehydrogenase-deficient erythrocytes from the blood of heterozygotes for glucose-6-phosphate-dehydrogenase deficiency, Lancet 1: 189, 1964.
22. Zipursky, A., Hull, A., White, F. D., and Israels, L.G.: Foetal erythrocytes in the maternal circulation, Lancet 1: 451, 1959.