Determination of the Glycosylated Hemoglobins (Hb A_I) with a New Microcolumn Procedure

Suitability of the Technique for Assessing the Clinical Management of Diabetes Mellitus

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SUMMARY

Fifty-seven children who attended a diabetes camp were divided into five groups on the basis of retrospective evaluation of the management of their diabetes mellitus over the previous three or more months. Shortly after their arrival at camp, fasting blood samples were obtained for the measurement of glucose and the quantitation of Hb A_I and for hemoglobin typing. Samples were obtained from 12 normal (nondiabetic) camp personnel at the same time. The Hb A_I components were measured by the "macrocolumn" procedure of Trivelli et al., by the colorimetric procedure of Flükinger and Winterhalter, and by a new microcolumn procedure employing columns and reagents provided by Isolab, Inc. As might be expected from a number of previous reports by other investigators, there was no significant correlation of the percentage of Hb A_I with single fasting blood sugar values. Hb A_I tended to decrease as the management of diabetes became more adequate according to clinical ratings. Comparison of the Hb A_I (Hb A_{Ia+b+c}) determinations showed an acceptable correlation of percentages obtained by the relatively laborious macrocolumn procedure and the more facile microcolumn procedure, indicating that the latter would be clinically useful. Values of Hb A_{Ic} obtained by the colorimetric procedure, which has been proposed as a procedure suitable for the clinical laboratory, did not correlate well with Hb A_{Ic} values determined by the macrocolumn technique, nor did these show as good an inverse relationship to the ratings of the management of diabetes. DIABETES *27*:931-37, September, 1978.

Several chromatographically separable minor hemoglobins are present in red cell hemolysates of normal adults; these are designated as hemoglobins A_{Ia}, A_{Ib}, A_{Ic}, A_{Id}, and A_{Ie}.[1-4] Hb A_{Ic}, which accounts for the major portion of the minor hemoglobins, has a hexose moiety (1-amino, 1-deoxy-fructose) attached to the amino terminal of the β-chains by virtue of formation of a Schiff base and Amadori rearrangement.[3,5-8] The glycosylation of Hb A appears to be a slow, nonenzymatic, posttranscriptional event taking place over the approximately 120-day life span of the red blood cell.[8-10] Hb A_{Ic} comprises 3 to 6 per cent of total hemoglobin in normal human red cells but is increased, as are Hb A_{Ia+b} albeit with less quantitative effect, in patients with overt diabetes mellitus.[11-14] Within the past several years, a number of investigators have suggested that control of diabetic hyperglycemia could be monitored by measuring Hb A_{Ic} or Hb A_{Ia+b+c}.[15-21]

Two recent editorials have emphasized the need for a procedure that would permit rapid analysis of Hb A_I in clinical laboratories.[22,23] In this paper we describe investigations of a microcolumn chromatographic procedure for the analysis of Hb A_I that has recently become available commercially in kits consisting of prepacked columns with reagents for hemolysis of red

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Accepted for publication April 10, 1978.

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cells and chromatographic development. We have compared the results with those obtained using the macrocolumn procedure of Trivelli et al.[12] Judging from the results of our studies, the micromethod should fulfill the requirement of a rapid, easy, and reliable procedure.

METHODS

Sixty-one children, ages 9 to 15 years, with insulin-dependent diabetes mellitus attended a two-week camp sponsored by the Georgia Diabetes Association in July 1977. Informed consent was obtained from the parents to collect an extra 7 ml. of blood for Hb A_I determination at the time of a routine fasting glucose determination. Each child brought a dossier describing his own physician's evaluation of the control of his diabetes for the previous three to six months. Criteria of the management of diabetes were applied without knowledge of Hb A_I measurements so that a score of 1 to 5 could be assigned to each patient in a manner similar to Gonen et al.,[20] a score of 1 indicating poor management and control and a score of 5 indicating good management. The data used were: blood and urinary glucose records from the three months preceding camp, when available; records from the prior year, when available; an evaluation by the patient's family physician of exceptionally good or poor control; and observations of the acceptance of diabetes management techniques by the patient and family as noted by the family physician and his associates and by the diabetes teaching nurses and staff physicians at the diabetes camp. Table 1 lists 57 children, their management ratings, and the results of the hemoglobin determinations described below. Two children were eliminated from this study because there were not enough data to allow a rating of the management of their diabetes. Two more were omitted because they were found to have a sickle-cell trait upon hemoglobin typing: whether Hb S is glycosylated in diabetics is presently under study..

Blood Collection and Preparation of Hemolysate

Using Vacutainer tubes, two tubes of blood were collected from each patient, one for hemoglobin analyses and one for a fasting blood sugar determination. Red cell hemolysate was prepared by hemolyzing one volume of washed red cells with an equal volume of distilled water and half volume of CCl_4. Cellular debris was removed by low-speed centrifugation.

Hb A_I Determination with the Microcolumn Procedure

The microcolumns were supplied by Isolab, Inc., Akron, Ohio. The chromatograms were developed according to the procedure proposed by the manufacturer, which is described in the informational publications supplied by this company.

Hb A_{Ia+b} and Hb A_{Ic} Determinations with the Macrocolumn Procedure

The procedure of Trivelli et al.[12] was followed. A 1:2 suspension of Biorex 70 in developer 6 was used to pack a column of 1 × 30 cm. This column was equilibrated by passing it through about 500 ml. of developer 6. After equilibration the length of the column was usually reduced to about 25 cm. Twenty-five to 30 mg. of hemoglobin (0.3 ml. of an 8 to 10 gm. per deciliter hemolysate) was dialyzed overnight against developer 6, which was diluted with an equal volume of water. Some of the buffer above the column was removed, leaving about 0.5 cm. The top of the resin was stirred evenly to a depth of 1 cm. After the dialyzed sample was applied to the column and allowed to flow in, the chromatogram was developed with developer 6. A flow rate of 14 to 15 ml. per minute was maintained, and fractions of 4.5 to 5.0 ml. were collected. After the minor hemoglobins A_{Ia+b} and A_{Ic} were eluted (in about 16 to 20 hours), developer 6 was replaced with a high-molarity phosphate (0.15 M) buffer. The elution of the remaining hemoglobin required an additional 8 to 10 hours. The calculation of the percentages of the minor hemoglobin components were based on the absorbances at 415 nm. in a Beckman spectrophotometer. Figure 1 shows the chromatogram of a normal adult (top panel) and that of a poorly controlled patient with diabetes (bottom panel).

The Chemical Procedure for Hb A_{Ic} Determination

The colorimetric procedure of Flükinger and Winterhalter[24] was employed. One milliliter of 0.3 N oxalic acid was added to 2 ml. of hemolysate (10 gm. per deciliter), and the mixture was incubated for one hour at 100° C. After cooling at room temperature, 1 ml. of a trichloroacetic acid solution (40 gm. per deciliter) was added, and the mixture was shaken and then centrifuged for 10 minutes at 3,000 rpm. Two milliliters of the supernatant was mixed with 0.5 ml. of 0.05 M thiobarbituric acid and incubated for 40 minutes at 40° C. Optical density (O.D.) was read at 443 nm. in a cuvette with a lightpath of 1 cm. The percentages of Hb A_{Ic} were calculated by assuming that 1 per cent Hb A_{Ic} gave an O.D. reading of 0.029.

Other Procedures

The hemoglobin concentration, packed cell volume, and red cell indexes of blood samples were determined in a model S Coulter Counter. Hb F levels

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were determined by the alkali denaturation technique of Betke et al.[25] Starch gel electrophoresis followed the procedure described elsewhere.[26] Blood sugar concentrations were determined by the Beckman automatic glucose analyzer.

RESULTS AND DISCUSSION

Figure 2 presents the correlation between the Hb A_I values determined by the microchromatographic procedure and those measured by conventional Biorex

TABLE 1

Summary of clinical characteristics and hemoglobin determinations of 57 diabetic children

Name	Age (yr.)	Duration of disease	Fasting blood sugar (mg./dl.)	Hb A_{Ia+b} Macro. (%)	Hb A_{Ic} Macro. (%)	Hb A_I Macro. (%)	Hb A_I Micro. (%)	Hb A_{Ic} Colorimet. (%)	FAD (%)	Hb (gm./dl.)
Management Rating: 1										
K.M.	10	1 yr.	188	3.3	10.2	13.6	11.0	11.6	0.3	14.2
R.C.	10	14 mo.	95	3.7	13.3	16.9	13.5	13.3	1.5	15.4
M.C.	9	2 yr.	229	3.8	12.8	16.6	10.8	—	3.8	14.3
G.A.	12	5½ yr.	340	3.4	10.4	13.8	12.6	16.9	0.9	16.5
R.C.	12	3–5 yr.	281	1.6	8.2	9.8	10.7	6.2	1.2	15.9
L.S.	12	2 yr.	189	2.4	6.8	9.1	7.6	6.3	0.6	14.2
B.S.	11	6 yr.	454	4.0	12.5	16.5	13.4	13.4	1.1	12.9
N.C.	12	3 yr.	95	2.6	9.1	11.7	10.0	13.4	1.3	13.2
A.C.	12	10 yr.	56	4.5	11.7	16.2	16.7	—	0.7	15.0
D.M.	15	5 yr.	120	3.7	11.1	14.8	14.6	10.4	0.8	17.9
G.H.	14	4 yr.	143	4.1	15.0	19.2	14.7	—	0.8	16.0
G.H.	12	10 yr.	96	4.5	10.0	14.5	17.4	—	2.5	13.5
D.K.	13	11 mo.	298	5.2	14.8	20.1	12.6	13.8	1.2	14.5
V.D.	15	6 mo.	183	2.7	9.4	12.0	11.7	—	0.8	14.0
T.A.	15	7 mo.	455	3.2	9.7	12.9	11.9	7.5	1.2	14.2
Mean			215	3.5	11.0	14.5	12.6	11.3	1.2	
±S.D.			127	0.9	2.3	3.2	2.6	3.6	0.9	
Management Rating: 2										
S.L.	10	6 yr.	284	2.8	7.7	10.5	9.1	6.6	0.8	15.4
R.J.	10	4 yr.	317	4.6	7.3	11.4	10.7	10.5	1.1	16.6
J.McG.	10	7 yr.	260	4.5	14.2	18.7	13.6	5.8	0.7	15.6
M.M.	9	2½ yr.	132	4.08	.2	17.3	15.1	10.3	0.7	14.3
C.W.	11	3 yr.	394	3.2	10.7	14.0	12.2	14.6	0.6	13.8
P.T.	11	1 yr.	203	5.6	9.9	15.4	9.7	—	1.6	14.5
K.C.	14	2 yr.	87	4.2	14.6	18.8	14.1	6.8	0.4	16.6
M.B.	14	4 yr.	140	2.6	8.4	11.0	12.3	—	0.2	16.9
T.R.	16	7 yr.	—	3.9	12.3	16.2	12.9	5.7	1.1	15.3
Mean			227	3.9	10.9	14.8	12.2	8.6	0.8	
±S.D.			105	1.0	2.8	3.3	2.0	3.3	0.4	
Management Rating: 3										
J.G.	10	7 yr.	248	2.5	7.2	9.7	9.5	5.4	0.8	14.2
B.W.	10	1 yr.	177	2.9	9.3	12.2	9.5	9.7	0.9	15.7
T.P.	10	6 yr.	293	3.0	6.6	9.7	10.7	5.7	1.3	15.6
T.R.	10	1 mo.	244	2.5	8.3	10.8	10.4	4.8	0.8	14.4
S.P.	9	2½ yr.	172	3.1	7.6	10.7	9.9	13.1	0.5	15.8
D.H.	9	1 yr.	94	5.6	7.4	13.0	8.4	13.4	0.3	14.5
B.C.	11	6 yr.	59	2.2	3.1	5.3	7.8	9.4	0.5	14.3
P.P.	11	1½ yr.	231	3.3	10.0	13.3	11.7	13.9	0.8	14.6
T.Y.	15	9 yr.	219	2.6	8.0	10.6	11.0	—	1.1	16.0
K.B.	15	4 yr.	81	3.3	11.8	15.0	14.7	10.5	0.9	16.3
T.A.	13	1 yr.	75	2.4	7.6	10.0	8.4	—	1.4	14.9
S.McL.	13	1 yr.	84	3.0	12.4	15.5	10.1	13.5	1.2	15.4
C.M.	13	8½ yr.	271	3.4	9.7	13.1	11.3	—	0.4	14.6
W.H.	12	4 yr.	167	1.9	6.0	7.9	10.1	6.5	1.0	15.3
J.H.	13½	10 mo.	156	4.5	11.5	16.0	12.1	12.5	1.1	15.3
L.McC.	15	9 mo.	61	2.5	6.4	8.9	7.9	10.6	1.3	11.4
Mean			165	3.0	8.3	11.4	10.3	9.9	0.9	
±S.D.			80	0.9	2.4	2.9	1.8	3.4	0.3	

(*continued*)

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TABLE 1 (*continued*)

				Management Rating: 4						
C.J.	10	1 yr.	101	3.2	9.1	12.4	10.9	5.7	2.0	14.9
J.W.	10	3 yr.	47	2.9	8.5	11.4	9.9	10.7	0.9	14.9
T.N.	9	3 yr.	97	3.4	12.2	15.6	11.1	12.3	1.5	15.0
D.L.	9	6 yr.	90	4.0	9.3	12.0	8.9	8.4	0.8	15.4
S.O.	9	4 yr.	86	2.7	6.2	8.8	8.3	10.4	1.0	15.4
K.C.	9	2 yr.	68	3.0	6.1	9.1	7.2	8.2	0.7	14.6
F.G.	9	4 yr.	84	2.1	7.0	9.2	7.6	6.9	0.8	14.3
K.R.	11	2 yr.	70	3.0	8.6	11.6	10.1	9.7	1.3	15.8
M.S.	12	3 mo.	199	3.8	8.7	11.9	10.5	17.7	0.7	14.4
S.K.	12	10 yr.	80	3.0	8.4	11.4	9.8	—	1.1	15.7
K.S.	13	2 yr.	155	3.7	12.3	16.0	16.0	12.8	0.8	15.9
Mean			98	3.2	8.8	11.8	10.0	10.3	1.1	
±S.D.			43	0.5	2.0	2.4	2.4	3.4	0.4	
				Management Rating: 5						
D.P.	11	6 mo.	111	2.5	6.8	9.2	9.4	4.2	1.7	14.7
J.B.	11	4 yr.	216	3.0	8.6	11.6	7.5	7.3	0.1	14.6
C.B.	9	6 mo.	130	3.5	8.9	12.4	6.9	4.4	0.9	15.1
M.C.	11	4 mo.	71	—	—	—	6.6	5.7	0.8	14.9
H.A.	12	4 mo.	106	3.4	8.6	12.0	7.1	13.2	0.0	16.3
W.D.	13	3 yr.	89	2.7	5.2	7.9	8.3	—	1.5	14.6
Mean			121	3.0	7.6	10.6	7.6	6.9	1.0	
±S.D.			51	0.4	1.6	2.0	1.1	3.7	0.7	
				Normal Controls (12 cases)						
Mean				2.2	4.3	6.5	5.9	7.2	1.2	
±S.D.				0.5	1.0	1.5	1.1	3.1	0.4	

70 macrochromatography. Results of samples from 60 persons, most of whom had diabetes mellitus, are included. The minor hemoglobins Hb A_{Ia}, Hb A_{Ib}, and Hb A_{Ic} are eluted together on the microcolumns; however, since Hb A_{Ia+b} and Hb A_{Ic} are separated from each other on the macrocolumns, the per cent Hb A_I is the sum of the percentages of these two separate zones. The correlation between the Hb A_I values obtained by these two methods is reasonable

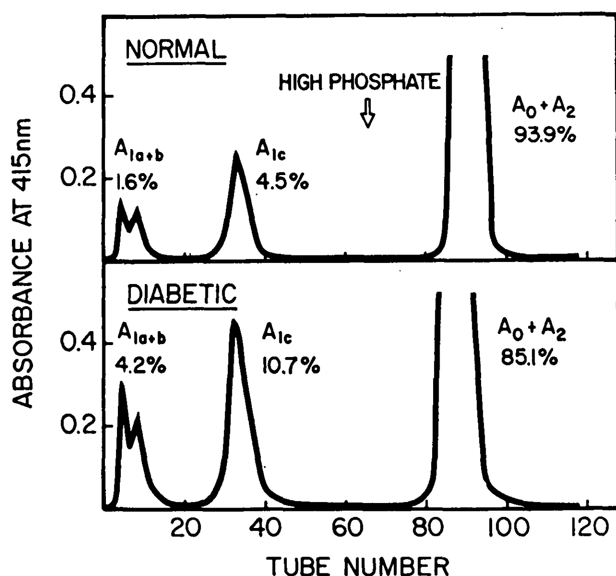

FIG. 1. Chromatographic separation on columns (1 × 25 cm.) of Biorex 70 of the hemoglobins in a hemolysate from a normal control and a diabetic patient.

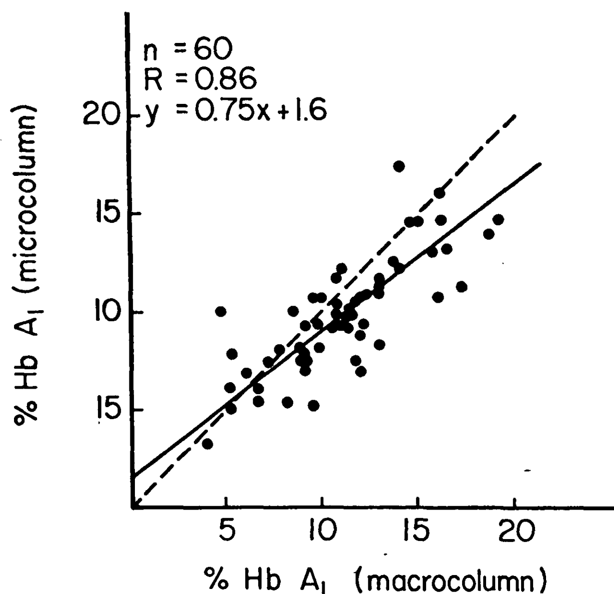

FIG. 2. The correlation of the Hb A_I values determined by microchromatography (Isolab columns) and the conventional Biorex 70 macrochromatography.[12] The solid line is the least-square regression line and the broken line is the theoretic line correlation.

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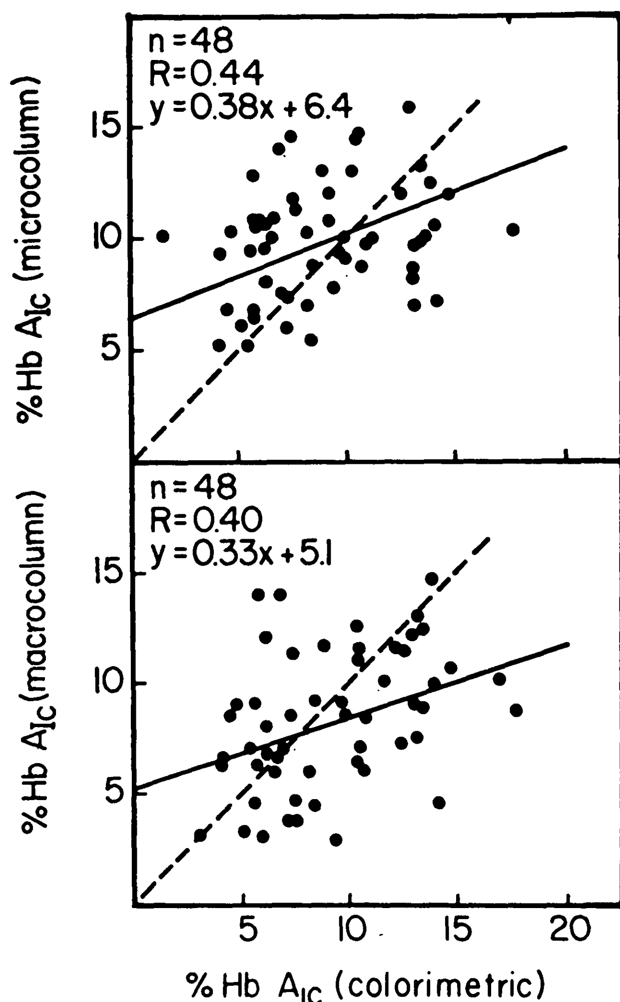

FIG. 3. The correlation of the concentrations of Hb A_{Ic} determined by the chemical procedure of Flükinger and Winterhalter,[24] with the percentages of Hb A_I obtained by microchromatography and those of Hb A_{Ic} by macrochromatography.

with an R-value of 0.86. The microcolumn Hb A_I values tend to be slightly lower at all levels, as shown in table 1 and as indicated by a slope of the least-square regression line of 0.75 (figure 2). Rather surprising is the observation that neither the microcolumn Hb A_I values nor the macrocolumn Hb A_{Ic} values correlated well with the Hb A_{Ic} values obtained by the colorimetric procedure (figure 3). Inspection of table 1 shows, moreover, that the standard deviations of the Hb A_{Ic} values of each rating group of children were greater for the colorimetric procedure than for the macrocolumn method. The standard deviations of Hb A_I values for the macrocolumn and microcolumn procedures were, on the other hand, quite similar.

The reproducibility of the microchromatographic procedure was evaluated by repeated analyses of three blood samples. Each blood sample, namely one from a normal control and two from diabetic patients, was analyzed 10 times. The data given in table 2 indicate an excellent reproducibility.

TABLE 2

Hb A_I determined by microchromatography

Case	n*	Hb A_I %	S.D.	Range
Patient I	10	10.4	0.4	10.0–11.2
Patient II	10	18.2	0.3	17.9–18.5
Normal subject	10	5.6	0.2	5.3– 6.1

*Number of analyses.

Figure 4 presents the various Hb A_I values of the diabetic children as a function of the fasting blood sugar concentrations determined simultaneously. That no correlation is evident is not surprising, inasmuch as our patients were relatively unstable juvenile diabetics. Paulsen[13,16] and Lanoe et al.[21] have reported the same finding, but they suggested that glycosuria over the previous three to four months did correlate with high Hb A_I and A_{Ic} levels. In more stable, maturity-onset diabetics, correlations with fasting blood sugar rates have been reported (for example, see reference 20).

Rather significant information was obtained when the Hb A_I and Hb A_{Ic} values from the diabetic children were divided among the five different groups, established by clinical and laboratory tests. Figure 5 shows that Hb A_I concentrations are decreased in the

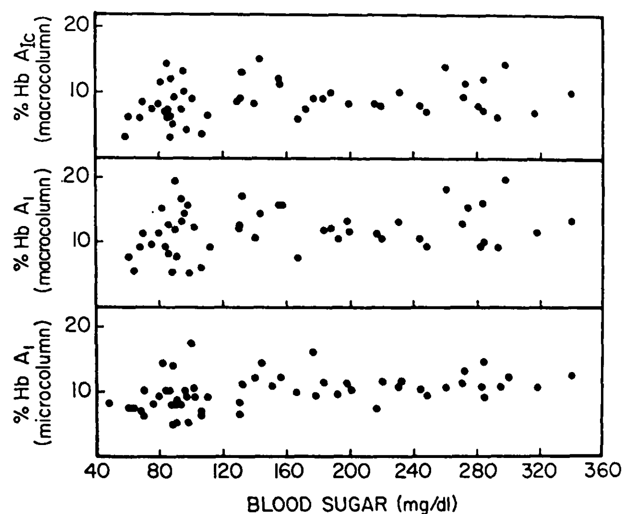

FIG. 4. The relationship of fasting blood sugar concentrations and the percentages of Hb A_I or Hb A_{Ic}.

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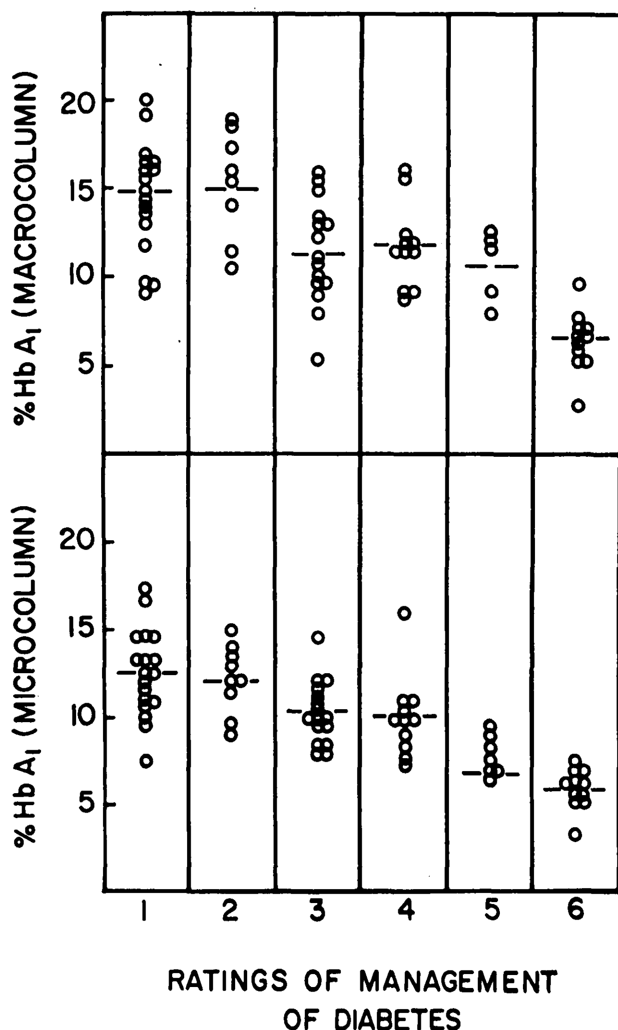

FIG. 5. The levels of Hb A_I in patients with juvenile diabetes who had varying degrees of management of their diabetes. Results obtained with the macro- and microcolumns are similar. All patients had normal hemoglobin types and had levels of Hb F (FAD) less than 1.5 per cent. For further details, see text.

patient groups showing the better management ratings. Means and standard deviations are given in table 1. Statistical analyses have not been carried out because no a priori assumption of the statistical distribution of the management-rating groups can be made. There is considerable overlap in the Hb A_I or the Hb A_{Ic} (figure 6) values between the different groups, except for the values of group 1 (poor control) and those of the normal controls. The correlation of Hb A_I and A_{Ic} levels (figure 6) with the different management groups was similar for both column chromatographic procedures. Correlation between patient management groups and Hb A_{Ic} concentrations determined by the colorimetric procedure, however, was decidedly poorer.[24]

Our findings establish an acceptable correlation between the Hb A_I values obtained by a microchromatographic procedure (the Isolab microcolumns) and those obtained by a more conventional, macrochromatographic technique. Thus, for routine clinical purposes the microcolumn procedure can replace the more time-consuming macrocolumn procedure; moreover, from our observations of diabetic children, the determinations of total Hb A_I rather than Hb A_{Ic} values are adequate for this purpose. The fact that

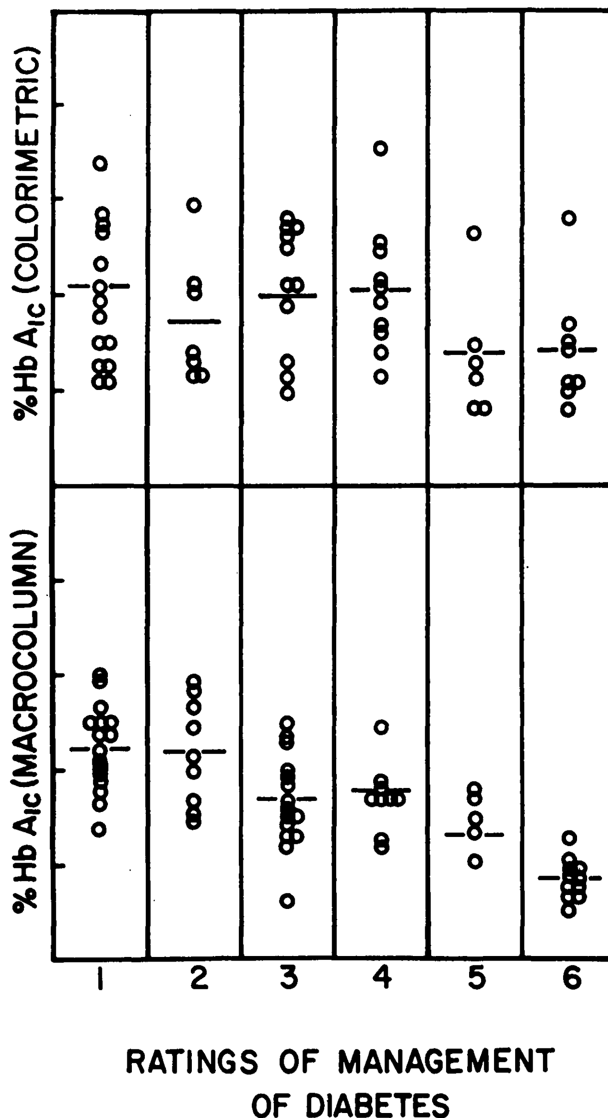

FIG. 6. Concentrations of Hb A_{Ic} in juvenile diabetics. Comparison of the macrocolumn procedure of Trivelli et al.[12] and the colorimetric procedure of Flükinger and Winterhalter.[24] See text for further details.

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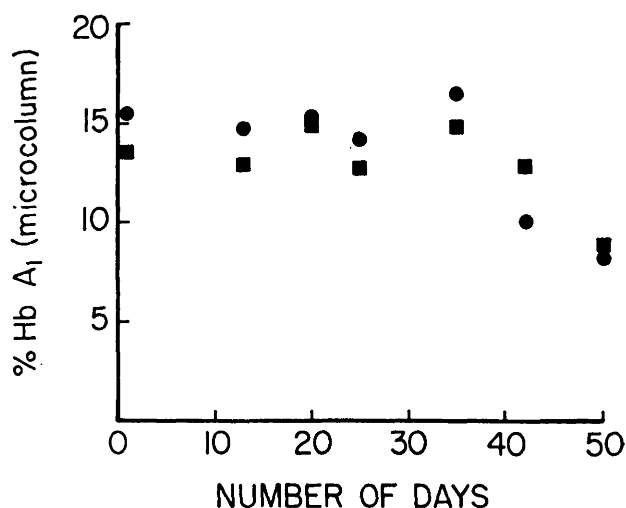

FIG. 7. The effect of storage of blood samples at 4° C. on the levels of Hb A_I determined by microchromatography.

blood stored for one month at 4° C. gives fairly constant values of Hb A_I (figure 7) is encouraging and suggests that no significant amount of Hb A_I is formed under these circumstances.

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