Rapid Changes in Chromatographically Determined Haemoglobin A_{1c} Induced by Short-term Changes in Glucose Concentration

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Summary. Chromatographically determined haemoglobin A_{1c} concentration was measured during short-term (1–24 h) changes in glucose concentration in vitro and in vivo. In vitro at 37 °C the HbA_{1c} concentration increased with glucose concentration and time both in normal and diabetic erythrocytes. In normal erythrocytes incubated in 20–100 mmol/l glucose, the increases in the HbA_{1c} concentration were maximal after 4–6 h and then stable for the next 18–20 h. During the first hour, increases in the HbA_{1c} concentration were linear with time and on average $0.034\% \text{ HbA}_{1c} \times \text{h}^{-1} \times \text{mmol/l glucose}^{-1}$. In erythrocytes, after a rapidly produced increase (2 h), HbA_{1c} decreased to preincubation concentrations during a further incubation of the erythrocytes in a glucose-free medium at 37 °C for 4–6 h. The mean rate of linear decrease was $0.017\% \times \text{h}^{-1} \times \text{mmol/l glucose}^{-1}$. After incubation of erythrocytes in 100 mmol/l glucose for 24 h, 1.3% HbA_{1c} remained stable for 6 h in saline. The rapid increase in HbA_{1c} concentration, as determined by chromatography, was not due to stable HbA_{1c} (ketoamine linked glucose) as no increase was found in the HbA_{1c} concentrations determined by the thiobarbiturate method. In juvenile diabetics controlled by an artificial beta-cell, rapid changes of blood glucose concentration (up to 20 mmol/l) resulted in increases in HbA_{1c} concentration of as much as 1.9% within 12 h (mean 1.1%). Rapid in vivo increases in HbA_{1c} concentration were reversible by normalization of the blood glucose concentration. That rapid changes in HbA_{1c} may occur in daily diabetic life was evidenced by differences in HbA_{1c} concentration between blood samples from out-patient diabetics incubated in saline for 16 hours at 4 °C and 37 °C (range of differences 0.2–1.4% HbA_{1c}). The differences correlated to the blood glucose concentration at the time of sampling blood for HbA_{1c} determination. Thus, incubation of blood at a low glucose concentration prior to determination of

the glycosylated haemoglobin concentration may overcome interference from rapidly produced HbA_{1c}.

Key words: Haemoglobin A_{1c}, rapid glycosylation, chromatography, glucose, 2-ketohexose derivatives, artificial pancreas.

Biochemical studies have shown that both the alpha and beta chain of human haemoglobin (Hb) is glycosylated by glucose and glycolytic intermediates in a non-enzymatic process in vitro and in vivo [1, 2, 3].

Since glucose bound in ketoamine-linkage to the N-terminal valine of the haemoglobin beta chain, haemoglobin A_{1c} (HbA_{1c}), is believed to be stable within the erythrocyte, the relative concentration of HbA_{1c} may reflect the integrated blood glucose concentration over a period of time. HbA_{1c} thus has potential for assessment of long-term glycaemic control in diabetics.

A number of studies have shown a statistically significant correlation between diabetic “control” based on conventional parameters and the relative concentration of HbA_{1c} or total fast chromatographing haemoglobins (HbA₁) [4, 5, 6]. So far the magnitude of correlation coefficients reported, especially in insulin-treated diabetics, does not support unambiguous assessment of glycaemic control in the individual diabetic patient by HbA_{1c}.

Precise assessment of glycaemic control in insulin-treated diabetics is a major difficulty in studying the value of HbA_{1c}, but, as previously reported [7, 8], codetermination of unstable, rapidly formed glycosylated haemoglobin may add to the uncertainty in evaluating the clinical value of HbA_{1c}.

The present study deals with further investigation of the rapid glycosylation of haemoglobin.

Methods

For routine measurement of HbA_{1c}, 0.15 ml venous or capillary blood was diluted in 3 volumes of heparin-saline (50 IU/ml – 0.15 mol/l) and stored for a maximum of 48 h at 4 °C before preparation of haemolysates. After 3 washings in 0.15 mol/l saline, lysis of packed red blood cells (RBC) was achieved by adding one volume of distilled water and 0.4 volumes of tetrachloromethane. After centrifugation at 5000 g, clear haemolysates were diluted to 1 mmol/l Hb (16 g/l) with eluting buffer (46 mmol/l sodium phosphate and 10 mmol/l potassium cyanide, pH 6.68) and chromatographed within 2 h. For in vitro studies and for quality control, haemolysates were prepared similarly but in larger volumes.

Chromatography was performed on 20 simultaneously run 0.9 × 7 cm glass columns packed with the ion exchange resin Bio-Rex 70, minus 400 mesh (BioRad Laboratories, USA). A constant elution speed of 0.5 ml/min was obtained with a 20 channel Multiflex Roller Pump (O. Dich Instruments, Denmark). Between determinations the resin was reequilibrated with eluting buffer for at least 6 h. Diluted haemolysate, 0.5 ml (8–10 mg Hb), was applied to each column through the pump and fast chromatographing haemoglobins collected in 18 fractions, each of 2.5 ml, directly into disposable cuvettes for a 2074 Calculating Absorptiometer (LKB, Sweden). Thereafter haemoglobin A (HbA) was eluted in one fraction by running phosphate buffer pH 6.42 as described by Trivelli [9] for 30 min followed by eluting buffer for 60 min, giving a total of 45 ml for elution of HbA. Elution of the HbA_{1c} peak in fractions 8–10 was achieved without thermostating the columns, by adjusting the ionic strength of eluting buffer according to the ambient temperature. The ionic strength was decreased by approximately 1%/degree rise in ambient temperature above 18 °C. pH, measured at 37 °C, was kept constant at 6.68 ± 0.01.

For each sample absorbance of the fractions, 18 for glycosylated haemoglobins and 1 for HbA, was read at 417 nm and the relative concentration of the haemoglobin fractions (HbA_{1a+b}, HbA_{1c} and HbA) was calculated from the absorbance values, defining the curve minimum (cut off point) between HbA_{1a+b} and HbA_{1c} peaks by parabolic regression analysis and correcting for baseline deviation after the HbA_{1c} peak.

Purified HbA_{1c} for recovery studies was prepared according to McDonald [10].

Studies of short-term changes in HbA_{1c} concentration were performed in vitro by incubation of 0.15 ml blood with 1.0 ml 0.15 mol/l saline with additions as specified. Changes during incubation were calculated as the differences between aliquots of the

sample incubated with a specified glucose concentration and aliquots incubated in 0.15 mol/l saline for the same time and at the same temperature. In these experiments, all samples from any one subject were chromatographed in the same run.

Blood glucose concentration was determined by a modification of the Trinder glucose oxidase method [11].

The thiobarbiturate reaction (TBA) for 2-keto-hexose derivatives was performed according to Gabbay [12], but haemolysates were dialyzed overnight at 4 °C against 0.15 mol/l saline before determination of TBA-reactive material was performed. 5-hydroxy-methylfurfural (5-HMF) (Merck) was used as standard. The results were corrected for individual blanks before calculation of µmol 5-HMF liberated/mmol Hb.

The least square regression and Wilcoxon's rank sum tests were used for statistical analysis.

Patients

The studies were performed on

1) 6 insulin-treated male diabetics aged 16–47 years (mean 36 years) and with a duration of diabetes of 7–29 years (mean 15 years). Glycaemic control on SC insulin before connection to the artificial pancreas was achieved by 0.71 U kg⁻¹ day⁻¹ (range 0.48–1.16 U kg⁻¹ day⁻¹). Mean blood glucose concentration over 3 days before connection to the artificial pancreas was 11.5 mmol/l (range 8.9–18.0). All had a body-weight within 103–109% of the ideal body-weight [13], and none of the patients showed a C-peptide response to a 1 mg IV glucagon stimulation test. All six patients were treated with an artificial pancreas for 48 h before start of the experiment.

2) 22 diabetics from the out-patient clinic of Steno Memorial Hospital. Based on 24-hour urinary glucose excretion they were divided into two groups: D_I, excreting low amounts of glucose (3–20 g) and group D_{II} excreting high amounts (70–228 g). Eight patients from group D_I were insulin-dependent, 1 patient controlled by diet alone and 1 by diet and a sulfonylurea. The patients' ages ranged from 23 to 70 years (mean 44 years) with diabetes for 3–21 years (mean 10 years). The insulin-treated patients received an average of 0.48 U kg⁻¹ day⁻¹ and had body-weights within 85–115% (mean 103%) of ideal. The 2 non-insulin-dependent diabetics had body-weights of 121 and 142% of the ideal, respectively. In group D_{II} the patients' ages ranged from 19 to 67 years (mean 45 years) with diabetes for 4–40 years (mean 16 years). One patient was controlled by diet plus sulfonylurea and the other 11 by 0.23–2.22 U insulin kg⁻¹ day⁻¹ (mean 0.67). All had body-weights within 85–115% of ideal. One patient in group D_I had elevated serum creatinine (143 µmol/l). All other patients had normal values (50–120 µmol/l). Blood was collected as described above after informed consent was obtained.

Blood samples from non-diabetic subjects were obtained from volunteers of this hospital (n = 40) and from old people from a geriatric unit (n = 18). Some of the latter were taking drugs for cardiovascular atherosclerotic disease including mild to moderate hypertension.

The artificial beta-cell (Biostator, Miles) was employed to control blood glucose as previously described [14].

Results

Methodology

Hb elution curves for a normal (5% HbA_{1c}) and a diabetic sample (12% HbA_{1c}) are shown in Figure 1.

Fig. 1. Elution curves for haemoglobin from a normal (open circles, 5% HbA_{1c}) and a diabetic sample (solid circles, 12% HbA_{1c})

Linearity of HbA_{1c} measurements in the expected range was tested by analysis of mixtures of haemolysates prepared from one normal subject (5% HbA_{1c}) and from one newly diagnosed diabetic (13% HbA_{1c}) (Table 1). Deviation from linearity was less than 2% with a coefficient of correlation of 1.00 (Table 1). Recovery of purified HbA_{1c} chromatographed on 5 routine columns average 98.6% (SEM 1.4%).

Table 1. Linearity of HbA_{1c} measurements assessed by analysis of mixtures of haemolysate from a normal subject and a newly diagnosed diabetic patient

% haemolysate in mixture		% HbA _{1c} measured	% HbA _{1c} expected		Deviation from linearity %
Diabetic	Normal	Mean (n = 4)	SD		
100	0	12.03	0.06		
75	25	10.39	0.18	10.31	0.8
50	50	8.70	0.17	8.60	1.1
25	75	6.99	0.09	6.88	1.6
0	100	5.16	0.12		

Table 2. Stability of glycosylated haemoglobin fractions during storage. Mean values on 6 diabetic samples stored as whole blood, blood diluted in 0.15 mol/l saline and as haemolysates

Storage time, days	Heparinized blood 4 °C		Blood diluted in saline 4 °C		Haemolysate 8 mmol/l Hb -40 °C	
	A _{1a+b}	A _{1c}	A _{1a+b}	A _{1c}	A _{1a+b}	A _{1c}
0	2.19	9.70	2.17	9.78	2.25	9.75
1	2.23	9.70	2.22	9.70		
3	2.18	9.58	2.34	9.32 ^a	2.53 ^a	9.41 ^a
7	2.06	9.34 ^a	2.47 ^a	8.90 ^a	2.63 ^a	9.23 ^a
16			2.51 ^a	8.73 ^a	2.92 ^a	9.15 ^a
34					3.10 ^a	8.88 ^a
62					3.47 ^a	8.90 ^a

^a Difference from values on day 0 significant (2 P < 0.05) by paired non-parametric test

The HbA_{1c} concentration remained stable for at least 3 days in whole blood stored at 4 °C and for 1–2 days in blood diluted with 0.15 mol/l saline and stored at 4 °C. After an initial decrease of 9% during the first month of storage at -40 °C, the HbA_{1c} values in haemolysates were unchanged (Table 2). Accordingly haemolysates stored at -40 °C for more than 1 month were used for assessment of analytical variation. Except in whole blood stored at 4 °C, the HbA_{1a+b} fraction continued to increase during storage. Changes during storage could not be accounted for by oxidation as no changes in glycosylated fractions were found when samples of haemolysate were partially oxidized to haemoglobin by potassium ferric cyanide (haemoglobin-fraction up to 0.28) before chromatography.

Intraassay variation for fresh samples chromatographed on three different days and interassay variation in HbA_{1c} concentration for a normal and a diabetic sample are given in Table 3.

The normal range for HbA_{1c} concentration was 4.4–6.4% (mean ± 2 SD, n = 58, mean age 54 years, range 20–92 years). For the same normal subjects a statistically significant correlation was found between age and HbA_{1a+b} concentration ($\text{HbA}_{1a+b} = 0.0093 \times \text{years} + 1.4$, $r = 0.64$, $2 P < 0.001$). For the sum of HbA_{1a+b} and HbA_{1c} fractions, HbA₁, the correlation with age was also significant ($\text{HbA}_1 = 0.016 \times \text{years} + 6.4$, $r = 0.43$, $2 P < 0.001$). The HbA_{1c} concentration was not correlated to age ($r = 0.06$). Using samples from normal and diabetic subjects less than 70 years of age (HbA_{1c} concentration between 4.5 and 16%), a significant correlation was found between concentration of HbA_{1a+b} and HbA_{1c} ($\text{HbA}_{1a+b} = 0.14 \times \text{HbA}_{1c} + 1.0$, $r = 0.85$, $n = 79$, $2 P < 0.001$).

In Vitro Experiments

Rapid increases in chromatographically determined HbA_{1c} in red blood cells (RBC) from normal subjects were demonstrated after incubation at 37 °C in glucose-saline (Table 4). At each glucose concentration, the maximum increase in HbA_{1c} concentration was

Table 3. Analytical variation in HbA_{1a+b} and HbA_{1c}. Intraassay variations measured on fresh samples from 2 diabetics and one normal subject on 3 different days. Interassay variations determined on haemolysates prepared from one diabetic and one normal sample and stored at -40 °C

	Intraassay						Interassay	
	HbA ₁		HbA ₁		HbA ₁		HbA ₁	HbA ₁
	a+b	c	a+b	c	a+b	c	c	c
N	18		10		8		34	48
Mean	2.73	10.18	2.53	8.30	1.73	5.15	6.11	10.15
SD	0.071	0.244	0.074	0.081	0.019	0.122	0.251	0.435
CV	2.6	2.2	2.9	1.0	1.1	2.4	4.1	4.3

Table 4. Increase in % HbA_{1c} after incubation of red blood cells from normal subjects in glucose-saline at 37 °C

Glucose concentration mmol/l	Increase in % HbA _{1c}	Incubation time, h				
		1	2	4	6-8	16-24
20-30	Mean		0.57	1.04	1.15	0.87
	SEM		0.07	0.07	0.17	0.10
	n		10	3	10	13
40-50	Mean		1.19	2.01	1.96	2.02
	SEM		0.13	0.27	0.29	0.20
	n		9	5	5	5
80-100	Mean	2.30	3.24	4.08		3.96
	SEM	0.19	0.15	0.15		0.17
	n	6	14	8		17

Table 5. Short-term changes in % HbA_{1c} during incubation of red blood cells from 6 normal subjects in 100 mmol/l glucose-saline and subsequent incubation in saline at 37 °C

Incubation medium Incubation time, min	100 mmol/l glucose-saline				Saline		
	30	60	90	120 ^a	150	180	240 ^a
Increase in % HbA _{1c}							
Mean	1.25	2.60	3.50	3.68	3.11	2.23	1.13
SEM	0.03	0.18	0.21	0.22	0.19	0.14	0.09

^a The glucose concentrations determined in the incubation medium at 120 and 240 min were 81.8 ± 0.4 and 5.1 ± 0.7 mmol/l respectively

reached within 4-8 h, the maxima being proportional to glucose concentration. No significant changes were observed during an additional 12-20 h.

In blood samples from 6 normal subjects incubated at 37 °C with 100 mmol/l glucose-saline, the increase in HbA_{1c} concentration was linear only during the first 60 min (Table 5). Mean increase = $0.034\% \text{ HbA}_{1c} \times \text{h}^{-1} \times \text{mmol/l glucose}^{-1}$ (SEM 0.003). In 2 h the HbA_{1a+b} concentration (not shown) increased by 0.32% (SEM 0.05). After 2 h incubation, the medium was changed to saline without glucose and changes in the HbA_{1c} concentration followed for another 2 h (Table 5). For the individual subjects, the resulting decrease in the HbA_{1c} concentration was linear with time during the 2 h investigated. The mean change was $-0.017\% \text{ HbA}_{1c} \times \text{h}^{-1} \times \text{mmol/l glucose}^{-1}$ (SEM 0.001). During the 2 h at low glucose, a decrease in HbA_{1a+b} concentration of 0.18% (SEM 0.06) was found. Incubation of RBC from normal and diabetic subjects at 3 different glucose concentrations for 2 h showed no difference in response (Table 6).

Rapidly formed HbA_{1c} was not completely dissociated by a 2 h incubation of RBC in glucose-free

Table 6. Increase in % HbA_{1c} after 2 h incubation at 37 °C in normal and diabetic red blood cells (RBC)

RBC	n	Increase in % HbA _{1c}	Glucose concentration, mmol/l		
			20	40	80
Normal	5	Mean	0.44	1.01	2.32
		SD	0.13	0.15	0.32
Diabetic	5	Mean	0.53	1.01	2.01
		SD	0.10	0.08	0.25

Table 7. Stable HbA_{1c} produced in red blood cells from normal subjects after 2 h and 24 h incubation in 100 mmol/l glucose-saline at 37 °C and subsequent dissociation of unstable HbA_{1c} by 6 h incubation in saline

% HbA _{1c}	Before incubation	2 h in 100 mmol/l glucose	+ 6 h in saline	Before incubation	24 h in 100 mmol/l glucose	+ 6 h in saline
Mean	5.53	8.73	5.71 ^a	5.30	9.09	6.59 ^b
SEM	0.08	0.22	0.10	0.23	0.32	0.21
n	7			6		

^a $2P > 0.05$, ^b $2P < 0.001$ for difference from preincubation value

medium (Table 5). In other experiments it was found that the disappearance of rapidly formed HbA_{1c} continued for 4-6 h at 37 °C in saline. Thereafter the HbA_{1c} concentration was stable for at least 24 h. Using a 6 h incubation in saline at 37 °C to dissociate unstable HbA_{1c}, we investigated whether stable HbA_{1c} was produced in RBC from normal subjects by incubation in 100 mmol/l glucose-saline for 2 and 24 h at 37 °C. After 24 h a statistically significant amount of non-dissociable HbA_{1c} (mean 1.3%) was produced (Table 7).

From Table 2 it is seen that HbA_{1c} in diabetic RBC diluted in 0.15 mol/l saline is stable for at least 24 h. Blood samples from 10 diabetics (mean % HbA_{1c} 9.8, range 7.5-13.8) and from 10 normal subjects (mean % HbA_{1c} 5.2, range 4.4-5.7) were diluted in 0.15 mol/l saline and incubated both at 4 ° and 37 °C for 24 h. Mean difference in the HbA_{1c} concentration, % HbA_{1c} at 4 °C - % HbA_{1c} at 37 °C, for diabetic samples was 0.85% HbA_{1c} (range 0.3-1.6) and for normal samples 0.15% HbA_{1c} (range 0.03-0.3). By non-parametric testing, the decrease in HbA_{1c} in diabetic samples was significantly different from that of normal samples.

In another 18 freshly drawn diabetic samples it was tested whether decreases in HbA_{1c} concentration, like decreases in samples after rapid in vitro induced increases in the HbA_{1c} concentration, were maximal after a 6 h incubation period in 0.15 mol/l saline at 37 °C. The mean value for HbA_{1c} concentration after a 6 h incubation in saline was 9.34% (SEM 0.57) and still 9.34% (SEM 0.56) after a further 18-hour incubation.

To test whether a decrease in HbA_{1c} concentration during incubation of RBC in 0.15 mol/l saline at 37 °C was dependent on glycaemic control during the previous 24 h, blood samples from diabetic outpatients excreting low amounts of glucose in a 24 h urine collection, Group D_I (n = 10, mean excretion 10 g, range 3–20, mean HbA_{1c} concentration 9.2%) and high amounts of glucose, Group D_{II} (n = 12, mean excretion 105 g, range 70–228, mean HbA_{1c} concentration 10.7%) were incubated in 0.15 mol/l saline at 4 °C and 37 °C for 16 h. Samples from non-diabetics (n = 10, mean HbA_{1c} concentration 5.2%) were treated identically. The mean differences between the HbA_{1c} concentration in samples incubated at 4 °C and 37 °C were not significant for Groups D_I and D_{II} (mean decrease 0.61 and 0.68%, ranges 0.2–1.2 and 0.1–1.4%), but in both cases higher than the decrease in Group N (0.21% HbA_{1c}) (2 P < 0.01 in both cases). In the combined D_I and D_{II} groups a significant correlation between the actual concentration of glucose in blood at time of sampling and the decrease in HbA_{1c} concentration during incubation at 37 °C was calculated ($\bar{r} = 0.54$, 2 P < 0.01).

In order to study the chemical nature of rapidly produced HbA_{1c}, blood samples from 3 normal and 4 diabetic subjects were incubated in 0.15 mol/l saline or saline plus 50 or 100 mmol/l glucose for 2 h at 37 °C. Haemolysates from each incubate were dialyzed overnight at 4 °C against 0.15 mol/l saline and the next day subjected to chromatographical determination of glycosylated haemoglobins and to estimation of 5-HMF liberated during boiling with oxalic acid according to Gabbay [13].

From Table 8 it will be noted that the mean increase in chromatographically determined HbA_{1c} concentration of 25 and 50% respectively did not result in any increase in μmol 5-HMF/mmol Hb. For samples incubated in 0.15 mol/l saline a significant correlation between HbA_{1c} concentration and μmol 5-HMF/mmol Hb was calculated (5-HMF = $2.31 \times \text{HbA}_1 + 1.35$, $r = 0.99$, 2 P < 0.001).

Clinical Studies

The results from a diabetic patient studied after feedback control in an artificial pancreas are shown in Figure 2. Feed-back control with the Biostator for up

Table 8. Changes in % HbA_{1c}, and 5-hydroxymethylfurfuraldehyde (5-HMF) liberated by the thiobarbiturate reaction, after incubation of normal and diabetic red blood cells in 0, 50 and 100 mmol/l glucose-saline at 37 °C

Subject	% HbA _{1c}			μmol HMF/mmol Hb		
	Glucose concentra-			Glucose concentra-		
	0	50	100	0	50	100
Normal no. 1	5.5	7.4	9.5	19.1	16.8	17.9
Normal no. 2	5.2	7.1	8.8	16.7	16.7	17.1
Normal no. 3	4.3	7.2	8.5	16.4	17.0	18.1
Diabetic no. 1	10.5	11.9	13.9	32.2	32.4	33.6
Diabetic no. 2	6.2	7.9	10.2	20.5	20.9	22.5
Diabetic no. 3	10.0	11.5	13.4	31.5	32.5	33.3
Diabetic no. 4	8.3	9.9	11.5	26.7	24.7	24.3
Mean	7.2	9.0	10.8	23.3	23.0	23.8

Fig. 2. Short-term changes in HbA_{1c} in a diabetic patient during a period with elevated blood glucose concentration following a 48-hour control period with near-normal blood glucose (broken line glucose, horizontal part representing mean blood glucose during the past 24 hours) obtained by feedback control with an artificial pancreas (solid circles HbA_{1c})

to 48 h before the start of the experiment resulted in near normal glucose concentrations in all 4 diabetics investigated (mean blood glucose concentration during 24 h before experiment 6.3 mmol/l, range 5.1–7.8). Rapid changes in blood glucose concentration accomplished by withdrawal of insulin and IV infusion of glucose produced an increase in HbA_{1c} concentration in all 4 patients (mean increase 1.1%, range 0.7–1.9%) within 6–11 h after increase in blood glucose concentration of 8–19 mmol/l above the mean over the previous 24 h.

Another 2 diabetics had subcutaneous insulin withdrawn 24 h before connection to the Biostator,

resulting in blood glucose concentrations of 28 and 18 mmol/l when feed-back control was started. In these 2 patients, a decrease of 1.8 and 0.8% HbA_{1c} respectively was measured within 6 h of normalisation of blood glucose.

Discussion

Several pitfalls in the chromatographic determination of glycosylated haemoglobins have been described, including instability of glycosylated haemoglobins, column temperature, presence of lipaemia or abnormal haemoglobins, and variation in erythrocyte life-span. Our results suggest that the rapid formation of an unstable glycosylated haemoglobin fraction should be added to the list.

The chromatography method used in this study is essentially that described by Trivelli [9], using however an ion exchange resin of low particle size, a slightly lower pH in the eluting buffer, complete re-equilibration of resin between runs, and adjustment of the ionic strength of the eluting buffer according to ambient temperature. In this way a reproducible separation of HbA_{1a+b} and HbA_{1c} is obtained over 90 min and using 45 ml of eluant.

The normal range published for HbA_{1c} concentration is clearly dependent upon the method used, but the range found in the present study is within those found using chromatographic methods [15, 16]. As [17] we found a correlation between HbA₁ and age, but in contrast to [18] not for HbA_{1c} in normals aged 20–92 years. This may indicate that HbA_{1c} concentration estimated by our method is unable to detect the decrease in glucose tolerance with age, or that the lifetime of RBC decreases with age. The correlation between HbA_{1a+b} concentration and age has not to our knowledge been reported before. The significance of this finding is obscure. Like other investigators [6, 16] we found a correlation between concentration of HbA_{1a+b} and HbA_{1c} in a combined group of normal and diabetic subjects. As reported by Dunn [19] the HbA_{1a+b} fraction was unstable during storage. The initial decrease in HbA_{1c} during storage of diabetic haemolysates may be the same process but at a slower rate than that occurring during incubation of RBC at 37 °C in low glucose medium (Table 4, 6).

The chemical nature of stable HbA_{1c}, a ketoamine-linkage between glucose and the N-terminal valine of the beta haemoglobin chain, has convincingly been clarified [20, 21], but the Schiff base being proposed as the initial step in the synthesis of HbA_{1c} from HbA and glucose has so far not been identified. Hence its stability and chromatographic behaviour in the systems used for determination of

glycosylated haemoglobins is unknown. Rapid in vitro synthesis of HbA_{1c} in intact erythrocytes or in haemolysates has previously been described [15]. Spicer [22], determining HbA_{1c} by isoelectric focusing, reported parallel increases in HbA_{1c} in normal and diabetic RBC after incubation with glucose at 37 °C for 6–20 h. Flückiger [23] found the increase to be the same after 8 and 18 h incubation. Bunn [24] reported that subsequent dialysis at 37 °C in glucose-free medium reduced the amount of HbA_{1c} formed during incubation of carboxyhaemoglobin with 25 mmol/l glucose, and he concluded that part of glucose in rapidly formed HbA_{1c} is bound in an unstable linkage.

Rapid in vivo changes in HbA₁ concentration have been noted less frequently. Karamanos [25] and Bolli [26] found significant changes in diabetics within 3 days. Leslie [27] reported a mean decrease of 2.5% HbA₁ in 1 week. The present study is to our knowledge the first report of significant changes taking place within hours.

Furthermore, our in vitro experiments show that HbA_{1c} may be increased significantly within a few hours when RBC are incubated in a glucose-containing medium. We also found that the peak increase in HbA_{1c} concentration is reached within 4–6 h at 37 °C (Table 4) and that the increase is reversible by incubation in a glucose-free medium for 4–6 h at 37 °C. The rate of increase and maximal increase were correlated to glucose concentrations in the incubation medium. The HbA_{1c} increase was not different in RBC from normal and diabetic subjects. Elevation of glucose concentration for 24 h, but not 2 h, led to a significant increase in non-dissociable HbA_{1c}.

Previously we reported [8] that rapid increase in HbA_{1c} concentration resulted in a slight change in its chromatographic behaviour. Since only glucose bound in ketoamine linkage is determined in the thiobarbiturate reaction [12], we conclude from the results in Table 8 that no significant amounts of ketoamine-HbA_{1c} are formed by short-term increases in HbA_{1c}, and we have found (unpublished observations) that the HbA_{1c} band on isoelectric focusing may be separated into 2 when rapidly produced HbA_{1c} is present. It is therefore possible that the substance measured after rapidly induced increases in the HbA_{1c} concentration might be the intermediate Schiff base precursor of stable ketoamine-HbA_{1c}.

The rapid changes in HbA_{1c} measured in diabetics after treatment with an artificial pancreas show *directly* formation and dissociation of the unstable HbA_{1c} compound in vivo. The findings in out-patient diabetics of a blood glucose-dependent decrease in HbA_{1c} concentration due to incubation of RBC in a low-glucose medium at 37 °C *indirectly* suggests that rapid changes in HbA_{1c} concentration do occur in

“normal” diabetic life. In diabetic patients we measured as much as 1.9% unstable HbA_{1c} representing up to 15–30% of the usual concentration of HbA_{1c} seen in our diabetic patients (6–12% HbA_{1c}). This clearly represents a problem in the use of HbA_{1c} concentration as an indicator of long-term glycaemic control.

Our results also indicate however that this pitfall may be abolished by incubation of RBC in low-glucose medium for at least 4 h at 37 °C before chromatographic determination.

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