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# Kinetics of Fast Haemoglobin in Diabetic Rats

B. Vialettes[1], L. Vovan[2], M. C. Simon[1], V. Lassmann[1], E. Altomare[1], and Ph. Vague[1]

[1]Department of Internal Medicine, Hôpital Universitaire Michel Levy and [2]Haemoglobinopathy and Paediatric Haematology Laboratory, Hopital Universitaire La Timone, Marseille, France

**Summary.** This study was designed to examine the appearance and disappearance kinetics of glycosylated haemoglobin during abrupt changes of blood glucose in the rat. The concentration of the fast haemoglobin component, which has similar chromatographic and electrophoretic profiles to human haemoglobin $A_1$, was measured after the induction of diabetes by streptozotocin and its cure by syngeneic intraportal islet transplantation. Fast haemoglobin was increased in 12 diabetic rats compared with 22 controls (15.8 ± 0.8 versus 8.2 ± 0.3%, mean ± SEM). In a group with mild diabetes ($n$ = 8, blood glucose < 22 mmol/l), fast haemoglobin rose to 13.7 ± 1.0% by week 8. In a group with severe diabetes ($n$ = 4, blood glucose > 22 mmol/l), fast haemoglobin rose more quickly (in 3 weeks) to a higher level (18.2 ± 3.3%) and changed little thereafter. This suggests a saturable system in which the rate of increase and final value depend upon the degree of hyperglycaemia. After islet transplantation, fast haemoglobin returned to normal in 4 weeks ($n$ = 5, 17.6 ± 1.4 to 9.4 ± 0.9%). This delay is shorter than expected from the red cell lifespan (around 60 days), suggesting that haemoglobin glycosylation may be partly reversible. These results suggest that in unstable diabetes the interpretation of haemoglobin $A_1$ levels is not as simple as was supposed previously.

**Key words:** Glycosylated haemoglobin, streptozotocin diabetes, islet transplantation, rat.

Increase of haemoglobin $A_{1c}$ ($HbA_{1c}$) in diabetic patients was first described by Rahbar et al. [21] and later linked to hyperglycaemia by Trivelli et al. [27]. There is convincing evidence that glycosylation of HbA to form $HbA_{1c}$ is secondary to hyperglycaemia and occurs during the entire erythrocyte lifespan [2, 5, 6, 24]. Accordingly, $HbA_1$ ($HbA_{1(a+b+c)}$) determinations have been proposed as a means of evaluating blood glucose control over a prolonged period of time [7, 9, 16, 19]. Unfortunately, despite clinical studies [3, 4, 28], the kinetics of $HbA_1$ level during abrupt changes of diabetic control remain unknown.

In an attempt to elucidate this point, an experimental rat model was designed to achieve successively the rapid induction of hyperglycaemia, stable sustained diabetes, and rapid normalization of plasma glucose. We have studied a fast minor haemoglobin component in rats which has similar electrophoretic and chromatographic profiles to human $HbA_1$. The animals were rendered diabetic by streptozotocin and subsequently cured by intraportal islet transplantation.

## Material and Methods

The animals used were female inbred Lewis rats (CNRS Centre de Sélection et d'Elevage d'Animaux de Laboratoire, Orléans) weighing 170–210 g at the beginning of experiment.

Diabetes was induced by IV injection of 65 mg/kg streptozotocin (Sigma) in 0.005 mol/l citrate buffer (pH 4.5). Eighteen rats were injected. No death occurred. Four animals did not become diabetic. Fourteen became diabetic and of them 12 were followed in this study. Severe diabetes was defined as a mean plasma glucose above 22 mmol/l ($n$ = 4) during follow-up in any animal. Mild diabetes was taken as a mean plasma glucose between 11 and 22 mmol/l ($n$ = 8).

Islets of Langerhans were isolated from rats as described by Lacy and Kostianowsky [17]. The donor pancreases were distended in vivo by Hanks' balanced salt solution (pH 7.4), removed, minced and digested with 15 mg of collagenase (type IV, Worthington Biochemicals) for every two pancreases for 5–9 min with hand shaking in a waterbath at 37 °C. After two washes in Hank's solution, the islets were counted and hand-picked under a dissecting microscope. For transplantation, 500–1 000 islets were slowly embolized into the liver by infusion into a mesenteric vein tributary of the portal vein under ether anaesthesia.

Blood (500 µl) was collected into heparinized capillary tubes from a tail vein of non-fasting normal or diabetic rats and was microfuged (Eppendorf microfuge) for 2 min. A 20 µl aliquot of plasma was kept frozen at −20 °C for subsequent glucose assay (glucose oxidase method, peroxidase-glucose oxidase diagnostica, Roche). The remaining plasma was discarded and the cells were washed three times in 0.154 mol/l saline. Erythrocytes were haemolysed by the addition of two volumes of distilled water and two

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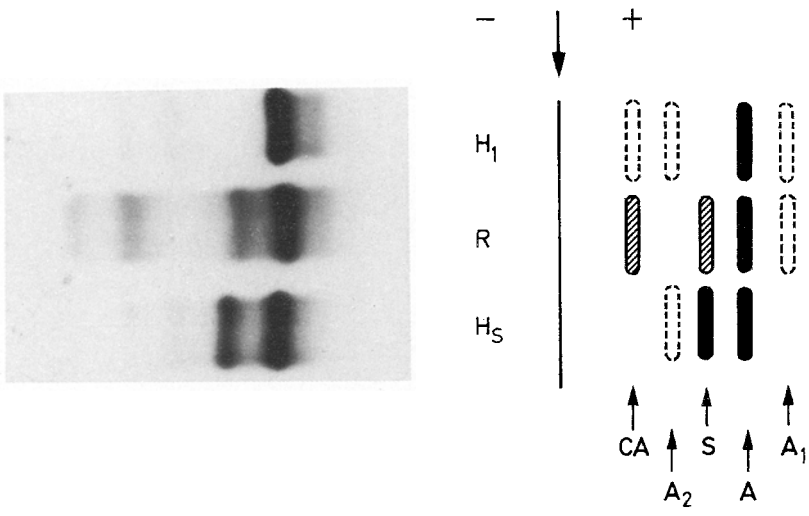


**Fig. 1.** Electrophoretic profiles of normal man ($H_1$) diabetic rat (*R*) and sickle-cell disease ($H_S$) haemolysates. (*CA:* carbonic anhydrase; $A_2$: $HbA_2$; *S:* HbS; *A:* HbA; $A_1$: $HbA_1$ and fast Hb)

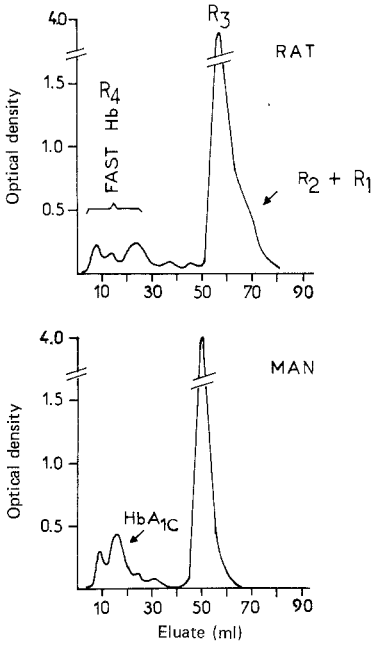


**Fig. 2.** Chromatographic profile on cation exchange resin of human and rat haemolysates (Symbols are described in materials and methods)

drops of toluene. After vortex mixing the blood was kept for one night at 4 °C. The solution was then spun at 10000 rev./min for 1 min.

Electrophoretic isolation of haemoglobin fractions was done on cellulose acetate (cellogel 2.5 × 17 cm band) in Tris-EDTA-borate buffer (pH 9.0) under 200 volts for 2 h. Bands were stained after migration by amido-Schwartz colouration. Fractions were then located, cut out and dissolved in acetic acid 80% (v/v). Optical density (OD) was read at 625 nm. Four main fractions could be isolated from haemolysate by this procedure (Fig. 1). The major component, R3, migrated as human HbA and represented approximately 50% of total Hb. R2 migrated coincidently with human HbS of sicklecell disease and R1 as carbonic anhydrase. R2 and R1 represented about 45% of whole Hb. Fast Hb (R4) migrated as human glycosylated Hb and its level was determinated by the following calculation:

$$\text{Fast Hb} = \frac{\text{OD Hb R4}}{\text{OD (Hb R1} + \text{R2} + \text{R3} + \text{R4)}} \times 100$$

Reproducibility of fast Hb assay was tested on duplicated electrophoresis of ten rat haemolysates. Coefficient of intra-assay variation was 2.2%.

The diabetic rat and human haemolysates were also submitted to ion exchange chromatography on a Bio-Rex column (Bio-Rex 70, Pharmacia) as described by Trivelli et al. [27] (Fig. 2) and isoelectric focussing as described by Beccaria et al. [1]. Both techniques isolated a fast rat Hb fraction which had a migration profile similar to human $HbA_1$. We assume that this fast fraction separated by electrophoresis is an analogue of human $HbA_1$.

Student's t-test and Pearson's coefficient of correlation were used for statistical analysis. Results are presented as mean ± SEM.

## Results

### *Fast Haemoglobin in Normal and Diabetic Rats*

In 22 normal rats mean non-fasting plasma glucose level was 5.9 ± 0.2 mmol/l and fast Hb 8.2 ± 0.3%. In contrast in 12 rats in whom diabetes had been established 8 weeks previously, the mean plasma glucose was 21.7 ± 0.8 mmol/l and fast Hb 15.8 ± 0.8%. These two populations were statistically different for both parameters ($p < 0.001$).

### *Correlation Between Plasma Glucose and Fast Haemoglobin Level*

Sixty blood samples were collected from 12 diabetic animals regardless of the date of streptozotocin injection (Fig. 3). A correlation was found between the plasma glucose and fast Hb level ($r = 0.30, p < 0.05$). When samples collected before, 20 days and 50–60 days after streptozotocin injection were considered separately, a statistically significant correlation was found only for the last group ($r = 0.58, p < 0.05$; Fig. 4).

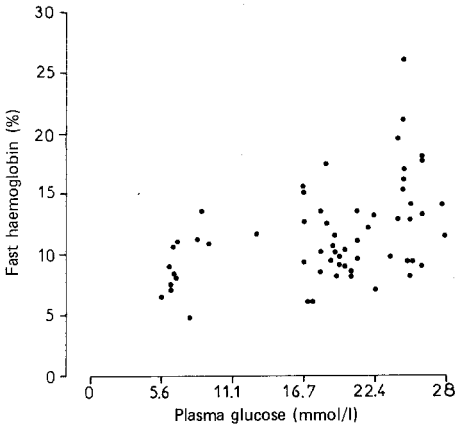


**Fig. 3.** Fast haemoglobin levels expressed as a function of simultaneous plasma glucose measurement in 60 blood samples regardless of the time of streptozotocin injection

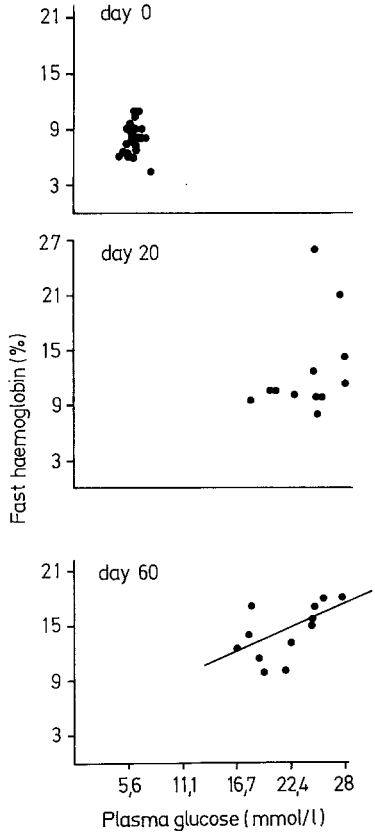


**Fig. 4.** Fast haemoglobin levels expressed as a function of plasma glucose before, 20 and 60 days after streptozotocin injection

### *Kinetics of Fast Haemoglobin Changes After Induction of Diabetes by Streptozotocin*

Two groups of diabetic rats were isolated according to severity of hyperglycaemia (Fig. 5): group 1 with severe diabetes and group 2 with mild diabetes. In the first group stable hyperglycaemia was established by day 5 following the streptozotocin injection (mean

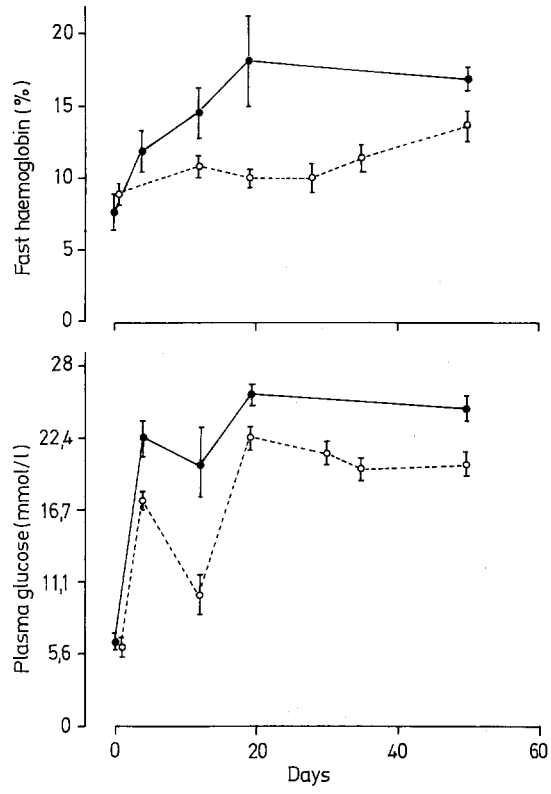


**Fig. 5.** Changes in fast haemoglobin levels and plasma glucose concentrations after streptozotocin injection (day 0) in mild (○----○, $n = 8$) and severe (●——●, $n = 4$) diabetes (mean ± SEM)

25.9 ± 0.8 mmol/l). In the second group the rise in plasma glucose concentration was less regular and led to a lower level (20 ± 2.2 mmol/l).

The mean plasma glucose level was significantly different in the two groups ($p < 0.005$). Fast Hb changes followed those of plasma glucose with different kinetics in each group. In group 1, fast Hb level increased rapidly towards a maximum (18.2 ± 3.3%) at 20 days and was no higher (16.8 ± 0.7%) at 50 days. In contrast, in group 2 the increase was more progressive and led to a lower value (13.7 ± 1%) 60 days after streptozotocin injection. There was a significant difference between the two groups ($p < 0.05$) in fast Hb levels at 2 months.

### *Changes of Fast Haemoglobin After Islet Transplantation*

Five diabetic rats with high plasma glucose and fast Hb levels sustained for a least 2 weeks (24.7 ± 1.4 mmol/l and 17.6 ± 1.4%, respectively) were transplanted intraportally with islets of Langerhans isolated from adult syngeneic donors. Patterns of plasma glucose and fast Hb changes are represented in Figure 6. Plasma glucose dropped rapidly by the day after transplantation and became normal between weeks 2 and 3. Fast Hb levels diminished more slow-

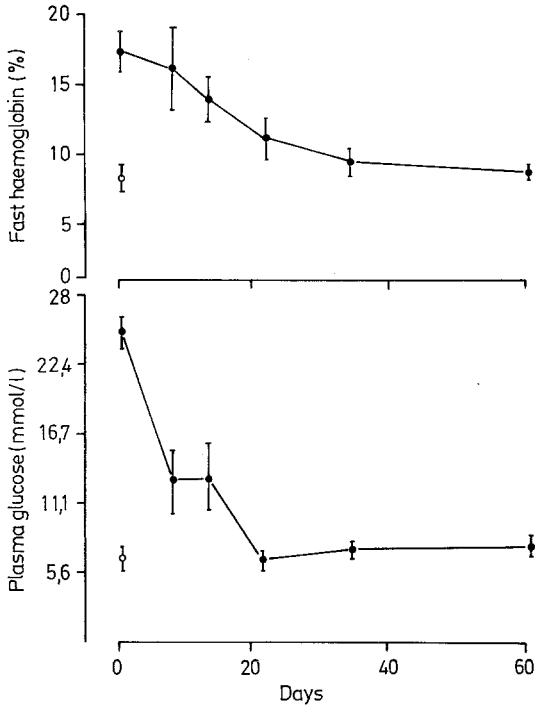


**Fig. 6.** Changes in fast haemoglobin levels and plasma glucose concentrations after syngeneic islet transplantation (day 0) in five diabetic rats (●——●). The open circles indicate values in a series of normal rats (mean ± SEM)

ly, became comparable with non-diabetic values about 5 weeks after transplantation and thereafter remained stable throughout the observation period of 60 days.

## Discussion

Although extrapolation of results from animal experiments to man requires caution, some similarities exist between our animal model and human diabetes. Rat fast Hb seems to be analogous to human $HbA_1$ on both chromatographic and electrophoretic study. The increase in fast Hb induced by hyperglycaemia is an additional argument in favour of similarities in structure and glycosylation. The lifespan of rat erythrocytes is not established but results of isotopic studies range between 45 and 70 days with an average of 60 days [8, 22]. Diabetes induced by beta-cytotoxic drugs in rats displays the same metabolic and structural features observed in human diabetes, including insulin deficiency [11, 22], hyperglucagonaemia [12], hypertriglyceridaemia [23] and glomerulopathy [18].

Koenig et al. [14, 15] have studied other animal models from which an analogue of human $HbA_{1c}$ has been isolated, These include C57Bl/Ksj mice, among which bearers of the *db/db* gene are spontaneously diabetic, and mice rendered diabetic by the administration of the B cell-cytotoxic drugs, alloxan and streptozotocin. Studies by these authors in chemically induced diabetes suggested that the $HbA_{1c}$ levels, although increased in the diabetic population, did not correlate with either the degree of hyperglycaemia or the duration of diabetes. In contrast, our results show that fast Hb changes in the diabetic rat depend on both the plasma glucose level and the duration of hyperglycaemia, at least in the early stages. Hyperglycaemia induced by streptozotocin injection caused a progressive rise in fast Hb levels over the succeeding weeks. The degree of hyperglycaemia seemed to act more upon the speed of formation of fast Hb than upon its final level. A maximum value of fast Hb was obtained by week 3 in the group with severe diabetes, whereas in the group with mild diabetes the fast Hb level was still rising up to 8 weeks after streptozotocin. In *db/db* mice studied by Koenig et al. [15], $HbA_1$ rose in parallel with the plasma glucose from 11 to 40 mmol/l with a delay of 4 weeks.

The rapidity of fast Hb formation and the sustained high values after the first month in our group 1 rats and in mice [15] do not agree with the kinetics deduced from the persistence of a hypothetical glycosylation throughout the entire erythrocyte lifespan. These results suggest a saturation of glycosylation beyond a certain value. This saturation level could be reached sooner or later depending on the degree of hyperglycaemia. This phenomenon could explain the lack of difference in $HbA_1$ level between severely and mild diabetic mice found by Koenig et al. [15]. Saturable glycosylation of HbA has also been suspected in man by Graf et al. [10]. Clinical studies by Dunn et al. [4] and Vague et al. [28] have shown that in insulin treated diabetic patients, $HbA_1$ correlated well with the last month's glycosuria but less well with those of the two preceding months.

The second part of this work confirmed the ability of syngeneic islet transplantation to reverse the metabolic consequences of chemically induced diabetes [13, 20, 25, 29, 30]. Islet transplantation also prevents the development of specific glomerular lesions of diabetes in the rat [18]. This study shows that rapid normalization of the $HbA_1$ analogues is another feature of this treatment. The rate of change of fast Hb was again more rapid than expected. The short delay of 5 weeks necessary to achieve normalization of fast Hb does not agree with the hypothetical very slow reversibility of haemoglobin glycosylation described by Fluckiger and Winterhalter in human eythrocytes in vitro [6]. A simple progressive disappearance of erythrocytes previously exposed to hyperglycaemia would take longer to normalize $HbA_1$. Clinical studies are in agreement with the results presented here. In cases of remission of juvenile diabetes, Ditzel and Kjaergaard [3] and Vague et al. [28] reported that normalization of

$HbA_1$ occurred by the first month following the return of plasma glucose to normal values. There are two possible explanations for these findings. First the decay curve of glycosylated haemoglobin following normalization of blood glucose would be expected to be sigmoidal since the earliest cells to disappear are the oldest (and hence more glycosylated) ones and the last to go the least glycosylated. The other explanation is that glycosylation of haemoglobin may at least in part be reversible. Svendsen et al. [26] have demonstrated with human $HbA_{1c}$ isolated by chromatography that rapid changes in vivo or in vitro are not related to the stable $HbA_{1c}$ (ketoamine linked glucose), but to reversible adduct of glucose to haemoglobin. Variations of this adduct may, at least in part, account for the rapid decrease in glycosylated haemoglobin found after glucose normalization in both animals and man.

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Dr. Bernard Vialettes
Service de Medecine Interne
Hôpital Universitaire Michel Levy
84a Rue de Lodi
13281 Marseille, France