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Synthesis of hemoglobin A_{Ic} in normal and diabetic mice: Potential model of basement membrane thickening

(hyperglycemia/^{59}Fe)

RONALD J. KOENIG AND ANTHONY CERAMI

The Rockefeller University, New York, N.Y. 10021

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ABSTRACT **Adult diabetic mice (C57Bl/KsJ-*db/db*) have increased amounts of a minor hemoglobin in their peripheral blood compared to wild-type (+/+) mice. This increase is analogous to the 2-fold increase of a glycohemoglobin with similar chromatographic mobility (Hb A_{Ic}) seen in the blood of patients with diabetes mellitus. Although the exact chemical nature of human or mouse Hb A_{Ic} is unknown, both contain a sodium-borohydride-reducible linkage on the β chain which is a presumed Schiff base between a sugar moiety and the protein. The *db/db* animals, which have normal amounts of mouse Hb A_{Ic} at weaning, show the increase approximately 4 weeks after the onset of the signs of diabetes. This rise is brought about by an increase in a circulating factor that determines directly or indirectly the synthesis of mouse Hb A_{Ic} as a post-synthetic modification of Hb A. Evidence for this was obtained by showing that the rate of synthesis of the modified Hb is linear for at least the first 50 days of the life of the red cell and that the rate of synthesis is dependent on the environment in which the cells circulate. Thus the rate of mouse Hb A_{Ic} synthesis in +/+ cells is greater when those cells circulate in a *db/db* host than when they circulate in a +/+ host. The nature of the humoral factor is unknown. If glycosylations of basement membrane proteins and hemoglobin proceed via a common mechanism, then the monitoring of Hb A_{Ic} could provide a useful model for studying the early events of basement membrane thickening.**

The introduction of insulin as a treatment for diabetes mellitus by Banting *et al.* (1) drastically altered the clinical history of this disease. The classical signs of hyperglycemia, polyuria and glycosuria, which led to the cachectic state and early death, could be prevented by the judicious use of exogenous insulin. However, despite insulin administration diabetics continue to develop other complications which are now responsible for the significant morbidity and mortality associated with the disease (2). Many of these complications, e.g., retinopathy, neuropathy, nephropathy, are believed to result from a thickening of the basement membrane of capillaries (2). This thickening is thought to arise from an increase in the amount of glycoproteins present following the derangement of carbohydrate metabolism (3). Because of the difficulty in obtaining representative samples of basement membranes, the pathophysiology of the early events of deposition is difficult to analyze (4, 5).

In addition to an increase in basement membrane glycoproteins diabetics have an increased amount of the glycosylated hemoglobin A_{Ic} (Hb A_{Ic}) (6). Patients with diabetes have 6–10% of their total hemoglobin as Hb A_{Ic} (7), while normal individuals range from 3 to 6% of their hemoglobin as Hb A_{Ic} (7–9). The exact nature and linkage of the glycosyl group has not been determined, although evidence points to a hexose attached via a Schiff's base linkage to the beta chain(s) of Hb A (10, 11). Two other minor human hemoglobins of unknown structure, Hb A_{Ia} and Hb A_{Ib}, are also present in elevated concentrations in patients with diabetes (7).

Studies of the glycosylation of hemoglobin would offer a unique way of investigating the early events of basement membrane thickening provided that the glycosylation of both components proceeds via a common mechanism. With this goal in mind we have initiated studies on the formation of Hb A_{Ic} in the diabetic mouse (C57Bl/KsJ-*db/db*). This animal model for human diabetes has, in addition to hyperglycemia and its sequelae (12), evidence for a thickening of basement membrane in the capillaries of the renal glomeruli (13).

In the current paper we report that, in analogy to the human condition, the diabetic mouse has an increased amount of what is presumed to be a glycohemoprotein, Hb A_{Ic}. The mouse Hb A_{Ic} is formed as a postsynthetic modification of Hb A throughout the life of the red cell. The amount of mouse Hb A_{Ic} formed appears to be determined by an unknown circulating factor which reflects the status of carbohydrate metabolism of the animal.

MATERIALS AND METHODS

Four-week-old male mice of the genotypes C57Bl/KsJ-+/+, C57Bl/KsJ-+/?, and C57Bl/KsJ-*db/db* were obtained from the Jackson Laboratory, Bar Harbor, Maine. In our laboratory the mice were fed Purina Mouse Chow *ad libitum*. To eliminate possible laboratory variations each procedure was carried out on age-matched pairs (*db/db* and +/+ or +/?) at the same time. Blood was drawn from the retroorbital sinus into heparinized capillary tubes and centrifuged immediately. The plasma was removed, stored at −20° and later analyzed for glucose. The red cells were washed once with 5 volumes of 0.15 M NaCl and lysed by adding 2 volumes of distilled water. The lysate was saturated with carbon monoxide and stored at −20°. Preliminary experiments demonstrated that storage at −20° for 4 days did not significantly alter the amount of mouse Hb A_{Ic}. The amount of hemoglobin was determined by the method of Drabkin (14). The amount of plasma glucose was measured on a 5 μl sample by the glucose oxidase–peroxidase method (Sigma Co., St. Louis, Mo.).

The hemoglobins were fractionated by the column chromatographic method of Trivelli *et al.* (7) on the polymethacrylic acid resin Bio-Rex 70 (Bio-Rad Lab, Richmond, Calif.). The elution profile of the hemoglobins was determined by measuring the absorbance of the fractions at 415

Abbreviations: Hb, hemoglobin; *db*, gene causing diabetes when present in homozygous state; s.a., specific activity.

Table 1. Minor hemoglobins (% of total ± SEM) in wild-type and diabetic mice

Genotype	N	Peak 1 hemoglobins†	Hemoglobin A_{Ic}†
+/? } +/+ }	22	1.09 ± 0.05	1.74 ± 0.06
*db/db**	18	1.73 ± 0.11	4.67 ± 0.23

* >10 weeks of age.
† Difference between *db/db* and +/? significant ($P < 0.001$)

nm. The quantification of the minor hemoglobins was achieved by measuring the area under their peaks with a compensating polar planimeter. Initially, the amount of Hb A_{Ic} was determined by the method of Drabkin (14) to give milligrams of hemoglobin per unit area of peak, and this value was used in subsequent experiments. The amount of Hb A was determined by the method of Drabkin. The quantities of the minor hemoglobins are expressed as a percentage of the total amount of hemoglobins placed on the column.

One hundred microcuries of NaB^3H_4 (7.1 Ci/mmol, Amersham/Seafle, Arlington Heights, Ill.) were mixed with 160 mg of carrier $NaBH_4$ (Fisher Scientific Co., Fair Lawn, N.J.). Hemoglobins A and A_{Ic} were reacted separately with this NaB^3H_4 according to the method of Bookchin and Gallop (11). After extensive dialysis to remove unreacted NaB^3H_4, globin was precipitated in acetone–2% HCl at −20° by the method of Clegg *et al.* (15), and the α and β chains were separated by chromatography on CM-cellulose (CM52, Whatman, Maidstone, Kent, England) according to the method of Clegg *et al.* (16). Radioactivity was determined in a Packard Tri-Carb Liquid Scintillation Counter.

Approximately 2 mCi of $^{59}FeCl_3$ (specific activity 22 mCi/mg; New England Nuclear Co., Boston, Mass.) were added to 1 ml of mouse plasma. The solution was adjusted to pH 6 with 6.25 N NaOH and injected intraperitoneally into a donor mouse; a total of 3 +/+ and 2 *db/db* mice were studied. Three days after the injection of the radioiron, the blood was removed and the specific activity was determined in a Packard gamma counter (4% efficiency). The radio-labeled cells (about 0.20 ml) from each donor were injected via the tail vein into one +/+ and one *db/db* recipient. These 10 recipient mice were then bled at intervals and the specific activities and total cpm of the hemoglobin fractions were determined. Mice used in the $^{59}FeCl_3$ experiments were 110–120 days old at the start of the experiment.

RESULTS

The chromatographic pattern (Fig. 1) of wild-type (+/+) mouse hemoglobins closely parallels that of normal human hemoglobins (7). By convention, the minor human hemoglobins (Hb A_{Ia}, Hb A_{Ib}, Hb A_{Ic}) are named according to their elution sequence on Bio-Rex 70; we have followed this same convention in naming the minor mouse hemoglobins.

Peak 1, which elutes near the void volume, is asymmetric and thus probably contains at least two hemoglobin species. It is likely that these hemoglobins are analogous to the human hemoglobins A_{Ia} and A_{Ib}, which also separate incompletely on Bio-Rex 70 and elute near the void volume. Mouse hemoglobin A_{Ic} elutes at approximately 10 column volumes and separates completely from the other species. Mouse hemoglobin A is eluted with buffer B.

The phenotypically normal littermates (+/?) of diabetic

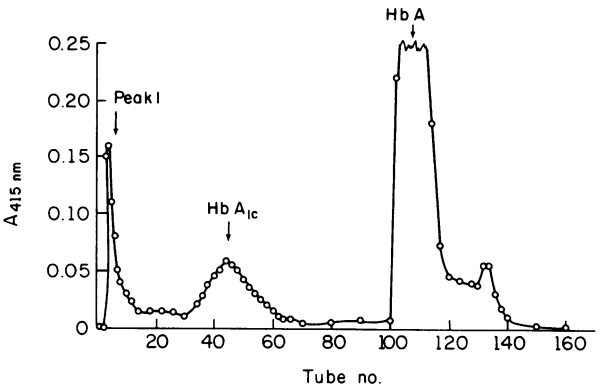

FIG. 1. Elution profile of hemoglobins from C57Bl/KsJ-+/+ mice on Bio-Rex 70. Thirteen milligrams of carbonmonoxy-hemoglobin were eluted with developer 6 (fractions 1–95) and buffer B (fraction 96–160) on a 6 mm × 25 cm column. Fractions of approximately 1.2 ml were collected. The total yield from the column (about 80%) did not vary with mouse genotype (+/+ or *db/db*).

(*db/db*) mice are of either genotype +/+ or +/*db*. The chromatographic pattern of the hemoglobins from +/? mice was qualitatively and quantitatively identical to that of known +/+ mice. Although the hemoglobins of *db/db* mice (>10 weeks of age) chromatograph similarly to those of wild-type mice, the amounts of the minor hemoglobins are elevated, as shown in Table 1. Thus, diabetic mice demonstrate a 1.6-fold increase in peak 1 hemoglobins and a 2.8-fold increase in mouse Hb A_{Ic} as compared to wild-type mice. This compares favorably with the 2-fold increase in Hb A_{Ic} observed in humans with diabetes mellitus (7, 15).

Mice of the genotype C57Bl/KsJ-*db/db* are normoglycemic (150 mg/dl) until approximately 6 weeks of age, at which time their plasma glucose concentrations rise rapidly to reach 500–600 mg/dl. Wild-type (+/?) and *db/db* mice have identical Hb A_{Ic} levels for their first 10 weeks of life, after which the *db/db* mouse displays a rapid increase in the amount of Hb A_{Ic} (Fig. 2). This rise in Hb A_{Ic} concentration is nearly parallel to, but occurs 4 weeks after, the rise in plasma glucose levels (Fig. 3).

Mouse hemoglobins A and A_{Ic} were separately reacted with NaB^3H_4. The average of two experiments shows the β chains of Hb A_{Ic} contain approximately one (0.82) borohydride-reducible linkage per monomer. The α chains of Hb A_{Ic} and both the α and β chains of Hb A had less than 0.04 residues per monomer. Similar results have been reported with human Hb A_{Ic} (11, 17).

Two *db/db* and three +/+ mice were injected with $^{59}FeCl_3$ and bled 3 days later. At the time of bleeding the specific activity (s.a.) (cpm/mg) of mouse Hb A_{Ic} was only

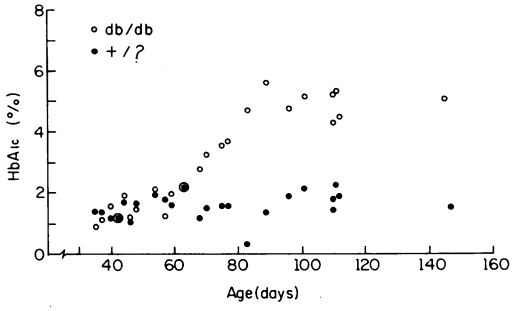

FIG. 2. Relationship between the percentage of peripheral blood hemoglobin as Hb A_{Ic} and age, in mice of +/? (●) and *db/db* (○) genotypes. The percentage of Hb A_{Ic} was quantitated as described in *Materials and Methods*.

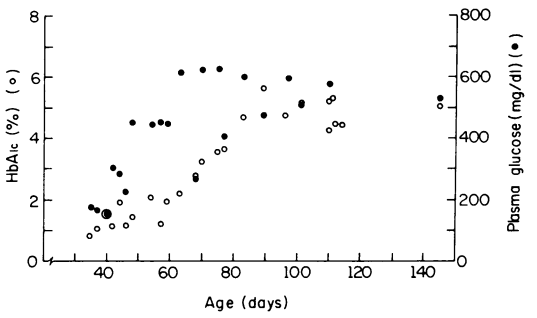

FIG. 3. Relationship of both percentage of total hemoglobin as Hb A_{Ic} (○) and plasma glucose (●) to age in *db/db* mice.

0.33 ± 0.05 that of Hb A. The specific activity for each mouse Hb A_{Ic} peak was constant throughout that peak, denoting molecular homogeneity. There was no significant difference in the ratio (s.a. Hb A_{Ic}/s.a. Hb A) between the *db/db* and +/+ mice. The radio-labeled blood from each of these five mice was injected into one +/+ and one *db/db* mouse. At intervals these 10 recipient animals were bled and the specific activities and total counts per minute (cpm) of the various hemoglobins were determined.

It is important to point out that the ^{59}Fe of the donor's red cells is located only within newly formed reticulocytes (about 2% of red cell mass). Under ideal circumstances these radio-labeled cells would remain in the circulation approximately 50 days (18). Since the amount of radio-labeled blood transferred from donor to recipient (about 0.2 ml) equals approximately 10% of one animal's blood volume, only 0.2% of the circulating red cells in any recipient mouse contain ^{59}Fe. Furthermore, unlike the unlabeled red cells in each recipient, these labeled cells are all approximately the same age. While the average age of the labeled cells is constantly increasing, that of the animal's unlabeled cells remains constant. Thus, any age-dependent change in mouse Hb A_{Ic} concentration will not be reflected in the percent of total hemoglobin as Hb A_{Ic} (since 99.8% of the red cells are of constant average age), but will be reflected in both the total cpm in the mouse Hb A_{Ic} fraction and the specific activity (cpm/mg) of that fraction.

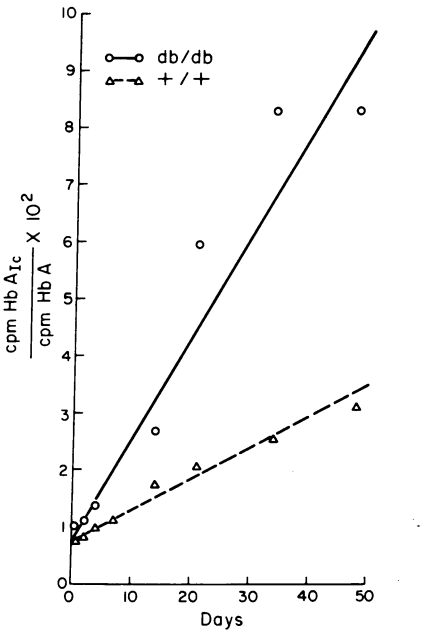

FIG. 5. The ratio of total cpm of Hb A_{Ic} to that of Hb A as a function of time in +/+ (△) and *db/db* (○) mice which received radio-labeled blood from a +/+ donor. This figure is a replotting of Figs. 4A and B to reflect net synthesis of Hb A_{Ic}.

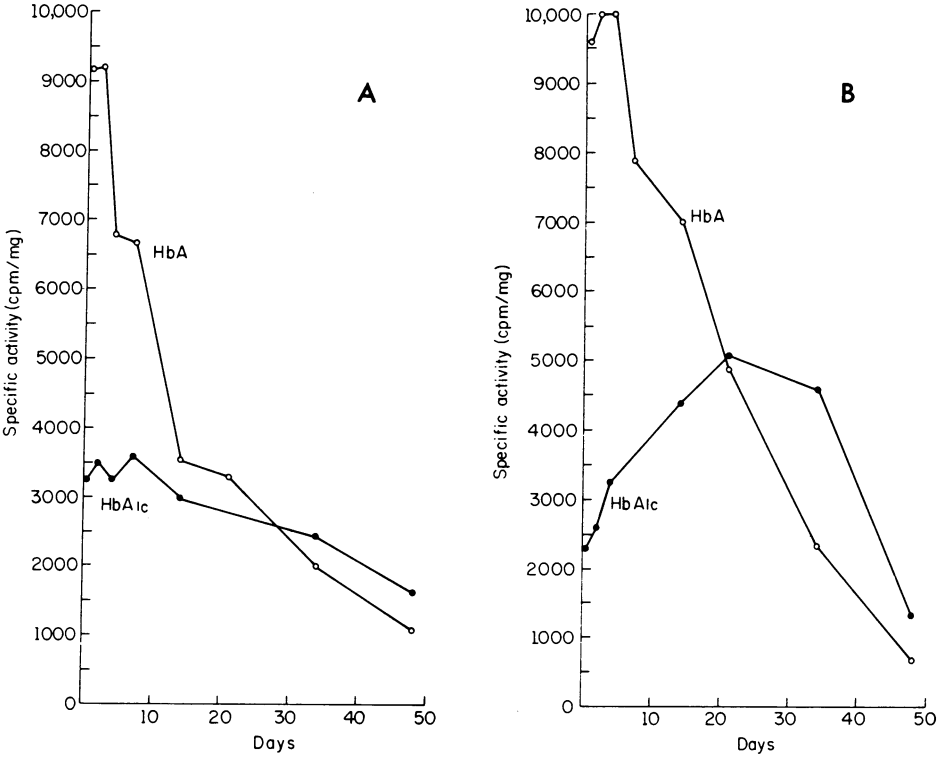

FIG. 4. Specific activities of Hb A_{Ic} and Hb A as a function of time in wild-type (A) and diabetic (B) mice. Wild-type (+/+) mice were injected intraperitoneally with 2 mCi of $^{59}FeCl_3$. Three days later approximately 0.2 ml of radio-labeled blood from each +/+ mouse was injected intravenously into one +/+ (A) and one *db/db* (B) mouse. These recipient mice were bled at intervals and the specific activities of their Hb A and Hb A_{Ic} fractions were determined. Time zero represents the time of the transfer of radio-labeled blood.

Table 2. Hemoglobin A_{Ic} synthesis in mice receiving radio-labeled red cells

Pair	Donor genotype	Recipient genotype	$\left(\frac{A_{Ic}}{A}\right) \times 10^2$ day 0	$\left(\frac{A_{Ic}}{A}\right) \times 10^2$ day 47	$\Delta\left(\frac{A_{Ic}}{A}\right) \times 10^2$
1	+/+	+/+	0.75	3.40	2.65
		db/db	0.77	9.10	8.33
2	+/+	+/+	1.03	3.46	2.43
		db/db	0.86	9.27	8.41
3	+/+	+/+	0.66	3.18	2.52
		db/db	0.60	7.02	6.42
4	*db/db*	+/+	1.15	4.41	3.26
		db/db	1.34	8.74	7.40
5	*db/db*	+/+	1.11	4.53	3.42
		db/db	1.03	9.70	8.67

A_{Ic}/A in the column headings represents (cpm Hb A_{Ic}/cpm Hb A).

Figs. 4A and B display the results of analysis of the specific activities of the hemoglobins A and A_{Ic} from representative +/+ and *db/db* animals injected with radio-labeled blood from a +/+ donor. In nine of the 10 recipient animals the specific activity of Hb A remained constant or rose slightly over the first 4–7 days. This increase is presumably due to further hemoglobin A synthesis by reticulocytes containing ^{59}Fe. The observed decline in the specific activity of Hb A, which occurs after 4–7 days, is believed due to the destruction of the highly radio-labeled cells which are damaged by radioactive decay, as well as injuries incurred during the *ex vivo* handling of the cells. In contrast, the specific activity of mouse Hb A_{Ic} in all recipients continued to rise or remained constant for the first 2–3 weeks and then declined at a slow rate.

Since Hb A and Hb A_{Ic} are presumably equally distributed within the radio-labeled cells, it is possible to correct for premature loss of radio-labeled cells at eact time point by dividing the total cpm of mouse Hb A_{Ic} by that of Hb A, which would remain constant if the radiolytic process were not shortening cell survival. The data presented in Fig. 5 are a replotting of the information contained in Figs. 4A and B using this technique. From this figure it is seen that the amount of mouse Hb A_{Ic} increases in an approximately linear fashion for the first 50 days of the life of the red cell. This implies a constant rate of synthesis.

The rate of Hb A_{Ic} production in any recipient mouse can be estimated by comparing the ratio (cpm Hb A_{Ic}/cpm Hb A) at the time of cell transfer to that ratio after 47 days. These data for all mice studied are shown in Table 2 and summarized in Table 3. When labeled blood from any genotype donor is placed in diabetic recipients, the rate of mouse Hb A_{Ic} synthesis in the labeled cells is 2.7 times the rate seen when the cells are transferred to wild-type recipients (Table 3; $P < 0.005$.) This agrees well with the 2.8-fold increase in Hb A_{Ic} levels of diabetic mice, as compared to wild type. Hemoglobin A_{Ic} synthesis occurs approximately 2.7 times faster in diabetic than wild-type mice, even if the red cells in the diabetic originated in a wild-type mouse. Thus, the rate of synthesis and eventual level of Hb A_{Ic} achieved can be correlated with the genotype and carbohydrate status of the recipient mouse and not with the genotype of the donor.

DISCUSSION

The gene *db* in the house mouse provides an experimental animal model for the human disease diabetes mellitus. The gene *db* is an autosomal recessive mutation which in the homozygous state on the C57Bl/KsJ background results in many of the signs of diabetes mellitus (11). The molecular defect is unknown. Early in life the homozygote *db/db* animals are observed to have a marked tendency for hyperphagia and frank obesity. It is not uncommon to see animals weighing twice as much as normal mice. During this period of rapid weight gain *db/db* mice are normoglycemic and hyperinsulinemic. At approximately 5 weeks of life there is an apparent failure of the pancreas to keep up with the hypersecretion of insulin necessary to maintain normoglycemia. As a result these animals quickly develop many of the signs of diabetes mellitus: hyperglycemia, glycosuria, polyuria, and polydypsia. These signs continue unabated until approximately 6 months of age when for unknown reasons the animals begin to lose weight and eventually succumb. Although these animals do not develop the pathological lesions of the kidney and retina that are hallmarks of the human condition, they do appear to develop with time a thickening of the basement membrane of capillaries in the kidneys (13). The failure to observe advanced lesions is presumably due to the fact that these animals die at a relatively early age. Humans develop these complications many years after the onset of the disease, even when exogenous insulin is used to maintain normoglycemia as closely as possible.

Analysis of basement membrane material from diabetic

Table 3. Mean values for hemoglobin A_{Ic} synthesis according to mouse genotype

Donor genotype	No. of donors	Recipient genotype	No. of recipients	$\Delta\left(\frac{\text{cpm Hb } A_{Ic}}{\text{cpm Hb A}}\right) \times 10^2$ (Mean ± SEM)	$\frac{\Delta\ db/db \text{ Recipient}}{\Delta\ +/+ \text{ Recipient}}$
db/db	2	+/+	2	3.34 ± 0.08	2.40
		db/db	2	8.03 ± 0.64	
+/+	3	+/+	3	2.53 ± 0.06	3.05
		db/db	3	7.72 ± 0.65	
All	5	+/+	5	2.86 ± 0.20	2.74
		db/db	5	7.84 ± 0.42	

humans has revealed an increase in the amount of glycoproteins (3); however, such analysis has not made it possible to ascertain whether this increase is a result of post-synthetic modification of existing proteins or an induced synthesis of new glycoproteins. To gain insight into the mechanism of increased glycosylation in diabetes we have initiated studies in the diabetic mouse. The strategy of the experiments reported here was to study the time of synthesis of the easily accessible and quantitated minor hemoglobins of the red cell.

As reported above the diabetic mouse has an increased amount of hemoglobin A_{Ic} compared to wild type (+/+). This increase occurs approximately 4 weeks after the onset of the signs of diabetes and is sustained for many months (Fig. 2). Although the chemical structure of mouse Hb A_{Ic} is undetermined, it is probably analogous to human Hb A_{Ic} (i.e., a glycosylated hemoglobin) since both hemoglobins contain one sodium-borohydride-reducible linkage per β chain, are increased in concentration in diabetes, and have similar chromatographic behavior.

The red cell is an ideal cell type to study the time of protein modification, since there is no protein synthesis in the mature erythrocyte. Therefore, in the peripheral blood the appearance of new hemoglobins must come about by a post-synthetic modification of existing hemoglobins. A comparison of the specific activities of Hb A_{Ic} and Hb A of the reticulocytes from both *db/db* and +/+ animals revealed a lower specific activity in the mouse Hb A_{Ic} fraction. This reflects either a greater percentage of the total mouse Hb A_{Ic} than the total Hb A being synthesized in the pre-reticulocyte state, or the synthesis of Hb A_{Ic} from Hb A in the post-reticulocyte stage. The latter explanation was found to be the case, since these radio-labeled cells continued to show an increase in the amount of mouse Hb A_{Ic} present whether they circulated in a +/+ or *db/db* recipient. In fact, a linear increase in the ratio (cpm Hb A_{Ic}/cpm Hb A) was observed for 50 days, denoting a constant rate of synthesis.

The rate of synthesis of mouse Hb A_{Ic} was related to the environment in which the radio-labeled cells were circulating; a faster rate was observed when the same donor cells (+/+ or *db/db*) were placed in a *db/db* recipient than in a +/+ recipient. Evidently there is in the circulation a factor(s) which is normally involved in the formation of mouse Hb A_{Ic} from Hb A. The onset of diabetes causes an increase in this factor(s) which promotes more rapid Hb A_{Ic} synthesis. It is not possible to say whether this factor reacts directly with Hb A to form Hb A_{Ic} or possibly proceeds via Hb A_{Ia} or Hb A_{Ib} as intermediates. In addition, it is not possible to ascertain whether this reaction is irreversible or is an equilibrium process which has not attained equilibrium. The identification of the group that is attached to mouse hemoglobin A_{Ic} should facilitate the understanding of the nature of the plasma factor and should suggest new experiments to determine whether an enzyme(s) catalyzes the reaction.

The continual linear synthesis of Hb A_{Ic} observed for 50 days of the life of the red cell points to a slow, nearly irreversible reaction. The plateau value of mouse Hb A_{Ic} seen in peripheral blood is brought about primarily by a balance of new synthesis of Hb A_{Ic} and the replacement of old cells laden with Hb A_{Ic} by new cells with less Hb A_{Ic}. If the nature of the modification of basement membrane proteins is similar to that of Hb A_{Ic} then a slow turnover of basement membrane proteins could easily lead to a substantial build-up of glycosyl groups in diabetes. In addition the increased glycosylation could alter the turnover of basement membrane proteins as well as promote the adherence of other proteins and the eventual thickening of the basement membrane.

It is hoped that further studies will determine whether there is glycosylation of hemoglobin to yield Hb A_{Ic} and whether there is a common mechanism for this process and the glycosylation of basement membrane proteins. If that should prove to be the case, the monitoring of Hb A_{Ic} levels might provide a mechanism for screening in the *db/db* mouse for new pharmacological agents to prevent basement membrane thickening, as well as a means for evaluating current forms of therapy in patients with diabetes.

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