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Hemoglobin Alc: Structure, Biosynthesis and Clinical Significance In Diabetes Mellitus

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Hemoglobin A_{Ic}:
Structure, Biosynthesis and Clinical Significance
In Diabetes Mellitus

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by

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PREFACE

The work described in this thesis was undertaken in the Laboratory of Medical Biochemistry at The Rockefeller University. I am indebted to my thesis advisor, Dr. Anthony Cerami, for his guidance, insight and willingness to explore new fields of biology. I am equally indebted to Dr. Charles Peterson for showing me the satisfactions and frustrations of clinical medicine and for his expert guidance during the clinical studies.

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ABSTRACT

Diabetes mellitus is a chronic metabolic disorder characterized by abnormally high blood glucose concentrations and a relative or absolute deficiency of insulin. Approximately 5 per cent of the American population are afflicted with this disease, which is a leading cause of morbidity and mortality in the United States today. Diabetes may result in the dysfunction of many different organ systems, yet the biochemical mechanism(s) underlying these dysfunctions is unknown. Even the importance of hyperglycemia in the development of the sequelae of diabetes is unsettled.

One of the metabolic abnormalities known to characterize this disease is an increase in the peripheral blood concentration of hemoglobin A_{Ic}. This minor red cell component comprises 3-5 per cent of the total hemoglobin in non-diabetic humans but up to 15 per cent in diabetics. There is evidence to suggest that it is a glycosylated derivative of hemoglobin A. The aim of these investigations has been to increase our understanding of the significance of increased hemoglobin A_{Ic} concentrations in diabetics.

The studies described here in the diabetic mouse demonstrate increased hemoglobin A_{Ic} to be a marker for the diabetic phenotype regardless of the cause of diabetes. The increase in hemoglobin A_{Ic} concentration occurs 3-4 weeks after the onset of diabetes. Hemoglobin A_{Ic} is made as a post-synthetic modification of hemoglobin A at a constant slow rate throughout the life of the red cell.

In diabetic humans, hemoglobin A_{Ic} concentration correlates with the severity of disease. Changes in the quality of diabetic control are followed, after a 3-4 week delay, by proportionate changes in hemoglobin A_{Ic} concentration. Hemoglobin A_{Ic} concentration reflects the mean blood glucose concentration for the 3-4 weeks prior to the measurement. Thus, infrequent hemoglobin A_{Ic} measurements would be sufficient to assess the quality of long-term diabetic control, a feature unique to

this measurement. The periodic monitoring of hemoglobin A_{Ic} concentration should permit patients to achieve better diabetic control than is currently possible and should make it possible to determine whether hyperglycemia is important in the development of the sequelae of diabetes.

The structure of hemoglobin A_{Ic} is identical to that of hemoglobin A, with the addition of 1-deoxy fructose attached at the amino terminus of the β chains. The increased formation of hemoglobin A_{Ic} in diabetes is an example of the increased glycosylation of a protein (hemoglobin A) occurring in this disease process. The biosynthesis of hemoglobin A_{Ic} provides a conceptual framework that may explain the molecular basis for many of the sequelae of diabetes. Thus, the abnormal or excess glycosylation of other proteins may cause structural or functional changes in those proteins and thereby result in the diverse sequelae known to occur secondary to this disease.

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I. BACKGROUND

Diabetes mellitus is a chronic metabolic disorder characterized by abnormally high blood glucose concentrations and an absolute or relative deficiency of insulin (Marble). It is a syndrome of unknown etiology and variable clinical course, the pathophysiology of which is poorly understood in biochemical terms.

Despite the fact that diabetes can be defined in only the most general of terms and very little is known about the basic disease process, it is a syndrome common to many animal species (Renold) and has afflicted man for thousands of years. The earliest known written record of diabetes in man is in the Ebers papyrus, ca. 1500 B.C. (Marble). In 400 B.C. an Indian, Sushruthra, noted the urine of diabetics to have a sweet taste, but it was not until 1775 (A.D.) that Dobson demonstrated this sweetness to be due to sugar. In 1889 von Mering and Minkowski produced diabetes in dogs by total pancreatectomy. In 1921 Banting and Best extracted from the pancreas a substance with hypoglycemic properties. This discovery revolutionized the treatment of diabetes and drastically altered the clinical course of the disease. Indeed, the administration of exogenous insulin remains the mainstay of therapy in diabetes. The introduction of orally active hypoglycemic agents in 1955 added a measure of convenience to the treatment of many cases of diabetes, although the use of these agents is still controversial.

Clinical diagnosis of diabetes is based upon measurements of blood glucose concentrations, and therefore attempts are frequently made to define the normal limits of glucose metabolism. Yet no standard values have been agreed upon, largely because carbohydrate tolerance is distributed in the population as a continuous function (Williams). This problem has helped produce a large body of conflicting epidemiological data concerning diabetes. No matter which data one subscribes to, however, it is clear diabetes is one of the major public health problems in the United States today.

Estimates of the prevalence of diabetes range from two per cent to five per cent of the American population (4 million to 10 million cases)

(Fajans; Diabetes Source Book). Twenty-five per cent of all diabetes remains undiagnosed, and 400,000 new cases are diagnosed each year. Seventy per cent of diabetes is diagnosed after age 45 and only eight per cent of all cases are diagnosed prior to age 25. The prevalence of diabetes increases with age: whereas only one per cent of Americans between ages 18-24 are diabetic, over twenty per cent of the 65-and-older population have this disease (Diabetes Source Book). Race and sex also bear relation to the prevalence of diabetes: non-white females have twice the rate of this disease as non-white males and white males or females. Interestingly, diabetes is ten times as common in the Pima Indians as in the general United States population, and is virtually non-existent among Eskimos.

Obesity is also a contributory factor to the prevalence of diabetes. In the population 10 per cent above ideal weight, the prevalence of diabetes is 50 per cent increased between ages 20-29 and 400 per cent increased after age 60. Furthermore, the more overweight one is, the greater the risk of becoming diabetic.

The morbidity of diabetes costs the United States 2 billion dollars per year, 65 per cent of which is accounted for by lost earnings. The average diabetic loses 15.4 working days per year, compared to 5.7 days for the general population. Every diabetic has a 67 per cent chance of being hospitalized in a given year, with an average stay of 5.4 days. Non-diabetics, on the other hand, have only a 12 per cent annual chance of being hospitalized, with an average stay of but one day. Eighty-two per cent of all diabetics visit a physician each year, with an annual average of 6.6 visits per patient.

The life expectancy of diabetics (at all ages) is only 67 per cent of that of the general population (Diabetes Source Book). Myocardial infarction is the most common cause of death in diabetics (50 per cent of cases). Clinically significant coronary artery disease is up to 10 times more prevalent in diabetics than in age-matched controls (Goldenberg). Renal failure is the other major source of mortality, accounting for death in nearly 50 per cent of diabetics who suffer from the disease for more

than 20 years (Robbins, 1971). In the pre-insulin era ketoacidosis was the cause of death in 15 per cent of diabetics (Diabetes Source Book); now it accounts for less than one per cent of diabetic mortality (Fajans).

Cases of diabetes diagnosed before the age of 25 years are frequently classified as juvenile-onset, whereas those diagnosed after age 25 are maturity-onset disease. This division is somewhat arbitrary but remains useful because the typical clinical course of juvenile-onset diabetes differs markedly from that of maturity-onset disease (Wintrobe). Juvenile-onset diabetics produce little if any insulin of their own and thus require insulin replacement therapy. Maturity-onset diabetics are capable of producing their own insulin, and treatment with oral hypoglycemic agents (most of which increase endogenous insulin secretion [Goodman]) may be a suitable therapy. Maturity-onset diabetics are frequently obese, and weight reduction tends to alleviate or eliminate their disease.

A genetic predisposition exists toward both forms of diabetes (Tattersall; Nerup), although studies with identical twins (Tattersall) have demonstrated the predisposition toward maturity-onset disease to be greater. Attempts at defining the mode of inheritance have led to conflicting hypotheses and a general lack of agreement (Stanbury).

Acute environmental insults, such as viral infections, are thought to underlie many cases of juvenile-onset diabetes. Epidemiological evidence suggests Coxsackie B4 virus can cause this form of diabetes (Steinke; Craighead). Other viruses, e.g., Mumps, are also known to infect the pancreas.

The onset of maturity-onset diabetes is usually insidious and patients may remain asymptomatic for years. Juvenile-onset diabetes is typically of abrupt onset, with the classical symptoms of polyuria and polydipsia hailing the onset of the disease. Insulin therapy has virtually eliminated ketoacidosis as a major cause of morbidity and mortality in diabetes, but juvenile-onset diabetics remain particularly susceptible to this complication during periods of hypoinsulinization.

The introduction of insulin replacement therapy and the advent of the antibiotic era have greatly extended the lifespan of patients with diabetes, causing a refocusing of attention on the long-term complications, or sequelae, of this disease. Diabetes may result in pathological changes in practically every organ system but those of the large blood vessels, eyes, kidneys, and peripheral nerves now account for the bulk of the morbidity and mortality associated with the disease.

Atherosclerotic heart disease is the leading cause of mortality among diabetics and is as prevalent in pre-menopausal diabetic women as in diabetic men (Fajans). Although the atherosclerotic process appears identical to that seen in non-diabetics, for unknown reasons diabetics suffer from this disease prematurely. Furthermore, whereas the mortality rate immediately following myocardial infarction is 20-28 per cent in non-diabetics, diabetics have a 33-58 per cent mortality rate. The five-year survival of diabetics post-MI is only 17-28 per cent. Clinically significant atherosclerosis is also common in the arteries of diabetics' legs. The Framingham study showed intermittent claudication to be 4.5 times as common in diabetics as in the general population (Fajans).

Diabetic retinopathy is the third leading cause of blindness in the United States, following senile cataracts and glaucoma (Diabetic Source Book; Fajans). Its prevalence appears related to the duration of disease rather than age at diagnosis. In patients who have been diabetic for 24 years the rate of retinopathy is between 42-82 per cent. The average age at onset of blindness from retinopathy is 60 years, versus 72-78 years for cataracts and glaucoma. Diabetic retinopathy is the leading cause of blindness among people 41-60 years of age.

Diabetic retinopathy may be classified as non-proliferative (background) or proliferative. Non-proliferative retinopathy is characterized by microaneurysms, hemmorhages, hard and soft exudates, edema and increased venous tortuosity. Microaneurysms are sac-like capillary dilations (10-200 μ) found most frequently around the edges of areas of non-perfused, obliterated capillaries. Edema is the most common cause of

visual impairment in background retinopathy, and presumably reflects increased capillary permeability. Hard exudates represent areas of extravasated protein and lipid and also reflect increased capillary permeability. Soft exudates are areas of microinfarction.

Proliferative retinopathy accounts for 6 per cent of all cases and is characterized by neovascularization. New thin-walled retinal vessels form, along with connective tissue. Adhesions may occur between this fibrovascular network and the vitreous body; vitreous contraction can then result in vitreous hemmorhaging or retinal detachment - the two major causes of blindness in diabetic retinopathy.

Although the prevalence of senile cataracts is not increased in diabetes, these cataracts do appear to mature faster in the diabetic population. Thus, the frequency of diabetics undergoing cataract extraction is 4-15 times greater than expected (Marble). Diabetes also contributes to glaucoma, the second leading cause of blindness in this country. Primary open angle glaucoma occurs in 4.8 per cent of diabetics but only 1.8 per cent of the general population. Glaucoma secondary to neovascularization of the iris occurs in 10 per cent of all cases of proliferative retinopathy (Fajans).

Renal failure is the leading cause of death in juvenile-onset diabetes and accounts for mortality in nearly 50 per cent of cases of diabetes of 20 or more years duration (Robbins, 1971). The most common lesions are glomerulosclerosis and arteriolosclerosis. Glomerulosclerosis initially develops as a diffuse process characterized by mesangial cell hyperplasia, increased deposition of mesangial matrix, and basement membrane thickening. It is present in at least 90 per cent of patients who have had diabetes for more than 5-10 years, but is not specific for this disease.

Nodular glomerulosclerosis (Kimmelstiel-Wilson lesion) is found in only 10-35 per cent of diabetics but is pathognomnonic for this disease. It is characterized by the presence of ball-like intramesangial deposits in the periphery of the glomerulus. The pathological material contains

mucopolysaccharides, lipids, and collagen, and appears to be chemically identical to the deposits of diffuse glomerulosclerosis. The diabetic glomerulus allows excess amounts of plasma proteins to filter into the urine, presumably because the basement membrane filter does not function properly.

Arteriolosclerosis is found in both the afferent and efferent arterioles to the glomerulus, the latter type being specific for diabetes. The lesion is characterized by hyalin deposits in the arteriolar walls, narrowing of the lumina and basement membrane thickening.

Although neuropathy is an extremely frequent complication of diabetes, its prevalence and resultant morbidity are difficult to estimate. The most typical pattern of involvement is a peripheral symmetrical neuropathy of the lower extremities, affecting sensory more than motor function (Robbins, 1971). Visceral neuropathy may result in bowel and bladder dysfunction and sexual impotence.

Anatomically the lesions are characterized by segmental demyelinization, implying a Schwann cell defect (Thomas). Early lesions may be detected by measuring a decrease in nerve conduction velocity. Occlusive lesions of the vasa nervosa do occur, but are too infrequent to account for the pathology observed.

Diabetes represents one of the most common and serious threats to pregnancy today. Gestational diabetes develops in 7 per cent of pregnancies, and by 6 years post-partum 67 per cent of these cases become permanently diabetic (Fajans). Toxemia occurs in 4 per cent of non-diabetic pregnant women but in 20 per cent of diabetics. Perinatal death occurs in 10-20 per cent of diabetic pregnancies (Fajans; Marble).

The pathological events leading to the organ dysfunctions of diabetes are not understood. However, the observation that capillary basement membranes are thickened in the placenta (Burstein), nerve (Fagerberg), kidney (Farquhar), retina (Bloodworth, 1963), muscle (Benscome) and other tissues of diabetics has led to the hypothesis that it is this thickening that accounts for many of the dysfunctions of diabetes (Bloodworth, 1963).

Routine biopsies of kidneys or retinas for the purpose of assessing basement membrane thickness are not possible, so most studies have utilized skeletal muscle capillaries for this purpose. Williamson has shown that some degree of basement membrane thickening normally occurs with aging, but that compared with those of age-matched controls, the basement membranes of diabetics may be several fold increased in thickness. Basement membrane disease is essentially non-existent in diabetics whose duration of illness is less than 6 months, but is present in 80 per cent of all cases with 20 or more years duration. Capillary basement membrane thickening is segmental and focal in character, perhaps implying that regional factors are important in its development.

The chemical composition of basement membrane in diabetes remains unsettled. Renal glomerular basement membrane from non-diabetics is composed of a collagen-like glycoprotein which characteristically contains 22 per cent glycine, 8 per cent hydroxyproline, 2 per cent hydroxylysine and 7 per cent carbohydrate (Spiro, 1968). Glucosylgalactosylhydroxylysine residues account for 55 per cent of the carbohydrate. Beisswenger has shown that glomerular basement membrane from diabetics is characterized by a decreased content of lysine with an equivalent increase in hydroxylysine and hydroxylysine-linked disaccharide units. He postulates this may render some lysine (or hydroxylysine) residues unavailable for cross-linking and thus result in the increased permeability that characterizes diabetic glomerular basement membrane.

Kefalides and Westburg have been unable to corroborate Spiro's data. They find glomerular basement membrane from diabetics to have a decreased content of cysteine and sialic acid, but normal levels of lysine, hydroxylysine and glucosylgalactosylhydroxylysine. They argue that a decreased ability to form disulfide crosslinks may account for the increased permeability of diabetic glomerular basement membranes. Thus, although it is clear diabetics have more basement membrane than non-diabetics (Klein), it is uncertain what, if any, chemical abnormalities exist in the diabetic basement membrane.

The tissues most commonly affected by the diabetic process (retina, kidney, nerve, blood vessels, lens) are freely permeable to glucose in the absence of insulin (Fajans). Thus, unlike adipose and muscle, these tissues will have elevated intracellular glucose concentrations in the diabetic state. It is conceivable, then, that excess intracellular glucose in some way accounts for the sequelae of diabetes.

Recent investigations suggest the metabolism of glucose via the sorbitol pathway may contribute to certain complications of diabetes, especially cataracts and neuropathy (Gabbay, 1966; 1973). The sorbitol pathway consists of two enzymes, aldose reductase and sorbitol dehydrogenase, which convert glucose first to sorbitol and then to fructose. Since neither of these products is capable of leaving the cell and since fructose is poorly metabolized, hypertonicity and a large osmotic effect result. Furthermore, the sorbitol pathway results in the oxidation of NADPH and the reduction of NAD^+, which may influence other intracellular metabolic processes. The *in vitro* incubation of lens in high glucose or galactose media results in cataract formation, and inhibitors of aldose reductase prevent this process from occurring (Chylack). The concentration of polyol per lens is proportional to the rapidity at which the cataracts develop. Aldose reductase is also present in Schwann cells, and sorbitol and fructose are increased in the sciatic nerve of alloxan diabetic rats (Gabbay, 1973).

Intracellular glucose may suffer other metabolic fates in diabetes. Hyperglycemia results in increased renal UDP-glucose (Spiro, 1976), which may contribute to the increased glycoprotein (Klein) and glycogen (Ritchie) production in diabetic kidneys. UDP-N-acetyl-glucosamine is also synthesized in excess in the hyperglycemic state, and this is reflected by an increased vitreous humor glucosamine content in diabetic rabbits (Walker, 1968) and humans (Walker, 1967). Spiro (1976) hypothesizes that the intracellular metabolism of glucose through insulin-independent pathways in diabetes results in the production of excess or abnormal glycoproteins, or some other metabolic abnormality, which then produces the sequelae of

diabetes. The sequelae would therefore be a direct consequence of excess intracellular glucose and an indirect consequence of hyperglycemia.

This hypothesis has not been subjected to rigorous testing, largely because the methodology has not been available to answer even the most basic of questions, "Does the degree of hyperglycemia correlate with the presence or severity of the sequelae of diabetes?" Many physicians believe good carbohydrate control does not decrease the incidence or severity of the sequelae, and studies addressed to this question have yielded ambiguous results (Knowles). Bloodworth (1973) has shown in dogs that good control of diabetes prevents or retards the onset of cataracts, retinal microaneurysms, and glomerulosclerosis, but it has not been possible to undertake comparable studies in humans. The difficulty in determining the relationship between diabetic control and sequelae hinges on two points: 1. It has not been possible to assess accurately the degree of diabetic control over the years it takes the sequelae to develop. 2. It is difficult to quantify the severity of the major sequelae of the disease (nephropathy, neuropathy, retinopathy).

In order to overcome these problems, I have investigated the synthesis of hemoglobin A_{Ic} as a biochemical model for the development of the lesions of diabetes. In addition, these studies have led to the development of hemoglobin A_{Ic} quantification as a means of assessing long-term diabetic control.

The adult human erythrocyte normally contains several hemoglobin species. Hemoglobin A, the major component, comprises 90-95 per cent of the total adult hemoglobin. Hemoglobins A_{Ic} (3-5 per cent), A_2 (2.5 per cent), $A_{Ia + Ib}$ (1-2 per cent) and F (0.5 per cent) are all minor components of the adult red cell population (Bunn, 1976). Hemoglobin $A_{Ia + Ib}$ consists of at least two species (A_{Ia}, A_{Ib}), but they are rarely separated and are thus quantified as the sum, $A_{Ia + Ib}$. The discovery that the concentrations of hemoglobins A_{Ic} and $A_{Ia + Ib}$ are elevated two-fold in diabetics (Trivelli) has aroused special interest in the structure and synthesis of these molecules.

Hemoglobin A_{Ic} has the same amino acid sequence as hemoglobin A (Holmquist, 1966) but contains, in addition, a sodium borohydride-reducible blocking group at the amino-terminus of the β chain. The blocking group was presumed to form a Schiff base with the amino terminal valine of the β chains. The molecular weight of the blocking group has been reported to be 180 by mass spectroscopy data (Bookchin) but 280 by chemical analysis (Holmquist, 1966). Chemical studies (Holmquist, 1966) failed to reveal the presence of carbohydrate, amino acid, steroid or aromatic groups in hemoglobin A_{Ic}, but mass spectroscopic data (Bookchin) was consistent with a hexose as the blocking group. Recently it has been shown that acid hydrolysis of hemoglobin A_{Ic} liberates 0.3 moles of hexose, consisting of glucose and mannose in a 3:1 ratio, per mole of hemoglobin (Bunn, 1975). This led to the proposal that the blocking group is glucose, and that it undergoes an Amadori rearrangement subsequent to Schiff base formation.

Although these data could not result in a definitive assignment of the structure of hemoglobin A_{Ic}, they did point strongly toward its being a glycosylated form of hemoglobin A. Thus, hemoglobin A_{Ic} would be another glycoprotein synthesized in excess in diabetes. Its synthesis would be an example of the type of process postulated by Spiro to underlie the sequelae of this disease (see p. 9). The excess production of hemoglobin A_{Ic} in diabetes would be a consequence of the fact that erythrocyte glucose uptake is insulin-independent and therefore the intracellular glucose concentration is proportional to the plasma glucose concentration.

Due to the accessibility of blood, studies of the biosynthesis of hemoglobin A_{Ic} could offer a unique way of investigating the biochemical events leading to the sequelae of diabetes, providing both processes do proceed via a common mechanism. It is with this goal in mind that these investigations were undertaken. Hemoglobin A_{Ic} was studied with regard to its biology, clinical significance, and chemistry. The biosynthesis of hemoglobin A_{Ic} in erythrocytes of wild-type and diabetic mice will be described in Chapter 2. Chapter 3 details the relationship between hemoglobin A_{Ic} concentration and the quality of diabetic control in human patients.

The unambiguous determination of the structure of hemoglobin A_{Ic}'s blocking group is described in Chapter 4. Finally, Chapter 5 details the potential significance of this work and future uses of hemoglobin A_{Ic} measurements.

II. BIOSYNTHESIS OF MOUSE HEMOGLOBIN A_{Ic}

A. Introduction

Many aspects of human diabetes can be investigated with animal models. In the mouse several mutations are known to result in a diabetic syndrome. Diabetes may also be induced in wild-type animals by the administration of specific β-cell toxins. Both genetic- and chemically-induced diabetes mellitus in the mouse appear to mimic the human condition.

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 (Hummel, 1966). 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 polydipsia. 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 (Like). The failure to observe advanced lesions is presumably due to the fact that these animals die at a relatively early age.

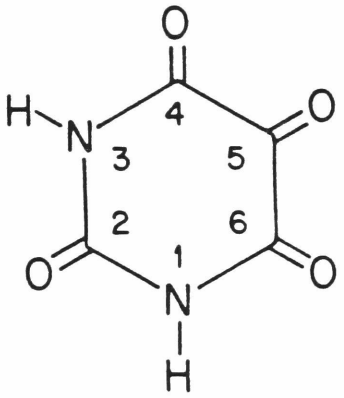
The obese gene (ob) is also inherited in an autosomal-recessive pattern. When present in the homozygous state on the C57BL/KsJ background, the ob/ob genotype results in pathology very similar to that observed in C57BL/KsJ-db/db mice (Coleman). Genetic studies have shown that these mutations are not alleles (Hummel, 1972) and are located on different linkage groups (Green).

In contrast to the above cases, when either the db or ob gene is present in the homozygous state on the C57BL/6J background, only transient diabetes results (Coleman; Hummel, 1972). These mice are hyperglycemic for a few months until pancreatic β-cell hyperplasia and hyperinsulinemia eventually return the animals to a nearly normoglycemic state. These mice are hyperphagic and obese throughout their lives, which are of normal duration.

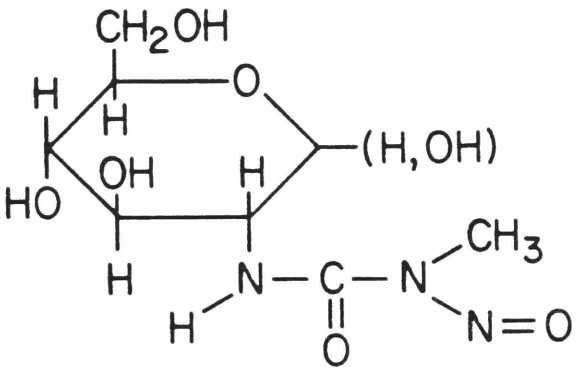
Two drugs commonly used to induce diabetes in rodents are streptozotocin and alloxan. The structures of these compounds are shown in Fig. 1. The intracellular reduction of alloxan at C5 yields dialuric acid, which in the presence of molecular oxygen is rapidly reconverted to alloxan, plus hydrogen peroxide (Deamer). Alloxan and dialuric acid thus cycle back and forth to generate large amounts of hydrogen peroxide, which accounts for the cytotoxicity of the drug either directly or through the formation of free radicals (Heikkila, 1974; 1976).

Streptozotocin is 1-methyl, 1-nitroso, 3-glucosamine-urea. The toxicity of this compound is apparently due to the nitrosourea portion since 1-methyl, 1-nitrosourea itself results in similar β-cell toxicity, albeit at a higher dosage (Anderson; Gunnarsson). The glucosamine portion of the molecule allows specific uptake by the β cells and thus accounts for the relative specificity of streptozotocin. The toxicity of nitrosoureas in general, and of streptozotocin in particular, is not well understood, although decreased levels of nicotinamide adenine dinucleotide (NAD) may play a role. In mice treated with streptozotocin or 1-methyl, 1-nitrosourea a dose dependent decrease in β cell NAD content occurs and cell killing clearly correlates with the degree of this decrease. Furthermore, the administration of nicotinamide protects against the diabetogenic action of streptozotocin in mice.

The administration of streptozotocin or alloxan to wild-type mice (Rerup) results in a triphasic response of blood glucose concentration: a state of acute hyperglycemia is followed by several hours of hypoglycemia until finally chronic hyperglycemia (diabetes) ensues. The acute



Alloxan



Streptozotocin

Fig. 1

hyperglycemic stage occurs 45 minutes after injection of alloxan and 2 hours after streptozotocin, and is related at least in part to hepatic glycogenolysis. Degranulation of the β cells, hyperinsulinemia and hypoglycemia occur 7 hours post-alloxan administration and 10 hours post-streptozotocin. Chronic diabetes secondary to β cell necrosis is established by 24 hours. The severity of diabetes, as assessed by fasting blood sugar, varies greatly between individual mice, but the diabetes is always sensitive to exogenous insulin. The major difference between alloxan- and streptozotocin-induced diabetes is that the former appears to be spontaneously reversible with time (Bunnag). That is, alloxan diabetic mice have been noted to replenish their β cell population gradually over a course of three to six months and return to the normoglycemic state.

Mice with chemically-induced diabetes are never obese and frequently weigh less than age-matched controls. The mice may be ketotic and develop a progressive glomerulosclerosis characterized by mesangial thickening and the deposition of immunoglobulins and complement.

B. Materials and Methods

Animals. Four-week-old male C57BL/KsJ and C57BL/6J mice, as well as diabetic (db/db) and obese (ob/ob) mutants of each strain, were obtained from the Jackson Laboratory, Bar Harbor, Maine. Six-week-old female $B6D2F_1/J$ mice were obtained from the same source. Mice were fed Purina mouse chow ad libitum.

Hemoglobin isolation and quantification. Blood was withdrawn from the retro-orbital sinus of nonfasted animals in midafternoon, placed in heparinized tubes, and centrifuged. The plasma was separated from the cells immediately, stored at -20°C, and later analyzed for glucose. The red blood cells were washed in five volumes of 0.9 per cent sodium chloride, lysed in two volumes of distilled water, and saturated with carbon monoxide (Matheson Gas Products, East Rutherford, New Jersey). Hemoglobin concentration was determined by the method of Drabkin.

Hemoglobins (A_{Ia}, A_{Ib}, A_{Ic}, A) were separated by column chromatography on the methacrylic acid polymer Bio Rex 70 (Bio Rad Laboratories, Richmond, California). Approximately 13 mg of carbonmonoxy hemoglobin, 50-100 mg/ml, were applied to a 6 mm x 25 cm column of the cation-exchange resin. Hemoglobins A_{Ia}, A_{Ib}, and A_{Ic} were eluted with developer 5A (0.055 M sodium phosphate, 0.01 potassium cyanide, pH 6.8). Hemoglobin A was eluted with buffer B (0.25 M sodium phosphate, 0.01 M potassium cyanide, pH 6.8). The rate of elution was 10 ml/hr, with fractions of approximately 1.2 ml being collected. The elution profile of the hemoglobins was determined by measuring the absorbance of the fractions at 415 nm. Hemoglobin components were quantified either by the method of Drabkin or by measuring the areas under the peaks with a compensating planimeter. The quantities of the minor hemoglobins are expressed as a percentage of the total amount of hemoglobin applied to the column.

Glucose quantification. Plasma glucose concentrations were determined on 5-µl samples by a glucose oxidase-peroxidase method (Sigma Chemical Company, St. Louis, Missouri).

$NaBH_4$-reactivity. One hundred microcuries of NaB^3H_4 (7.1 Ci/mmol, Amersham/Searle, Arlington Heights, Illinois) were mixed with 160 mg of carrier $NaBH_4$ (Fisher Scientific Company, Fair Lawn, New Jersey). Hemoglobins A and A_{Ic} were reacted separately with this NaB^3H_4 according to the method of Bookchin. After extensive dialysis to remove unreacted NaB^3H_4, globin was precipitated in acetone-2% HCl at -20°C by the method of Clegg (1966), and the α and β chains were separated by chromatography on CM-cellulose (CM52, Whatman, Maidstone, Kent, England) according to the method of Clegg (1968).

Forty milligrams of the B^3H_4-reduced globin were applied in 4 ml of starting buffer (0.005 M sodium phosphate, 0.05 M β-mercaptoethanol, 8 M urea, pH 6.8) to a 0.9 cm x 15 cm column of CM cellulose. The column was developed with a linear gradient of sodium phosphate, using 150 ml of starting buffer and 150 ml of limit buffer (0.04 M sodium phosphate, 0.05 M β-mercaptoethanol, 8 M urea, pH 6.8). Fractions of 1.2 ml were collected at a flow rate of 20 ml/hour. The absorbance of the effluent was monitored

at 280 nm. Radioactivity of the fractions was determined in a Packard Tri-Carb liquid scintillation counter. The fractions containing the β chains were pooled, dialyzed extensively against distilled water, and lyophilized.

Tryptic peptide map of mouse hemoglobin. Two milligrams (0.13 μmol) of β chains isolated from NaB^3H_4-reduced hemoglobins A_{Ic} and A were separately dissolved in 1 ml of 1 M Tris, 6 M guanidine buffer, pH 8.0. A 50 molar excess (6.5 μmol) of dithiothreitol was added, and the mixture was incubated for 4 hours at 37°C. The mixture was cooled to room temperature and 0.5 ml of Tris-guanidine buffer was added, along with 14.3 μmol of iodoacetamide. The mixture was incubated in the dark for 20 minutes, dialyzed extensively in the dark against distilled water, and lyophilized.

The samples were then dissolved in 2 ml of 1% NH_4HCO_3 and 0.1 mg of trypsin (180 μ/mg, Worthington Biochemical Corporation, Freehold, New Jersey) was added. The reaction mixtures were incubated 4 hours at 37°C, lyophilized, redissolved in 0.5 ml distilled water, and relyophilized.

The tryptic digests were then dissolved in 0.2 ml of distilled water. Approximately 0.5 mg of each was spotted onto Whatman 3 MM paper and subjected to electrophoresis at pH 4.7, 3000 V, for 50 minutes according to the procedure of Clegg (1966). This was followed by descending chromatography in butanol:acetic acid:water:pyridine (15:3:12:10).

The maps were dried and sprayed with ninhydrin (Brenner). Approximately 5 per cent of each spot was oxidized in a Packard Sample Oxidizer and counted for tritium. The remainder of the sole ^3H-containing spot in the $\beta^{A_{Ic}}$ map and the corresponding spot in the β^A map were eluted with 1:1 pyridine:water and lyophilized to dryness. The samples were digested in 6 N HCl for 16 hours at 105°C in vacuo and analyzed on a Beckman 121 M amino acid analyzer.

Hemoglobin A_{Ic} biosynthesis. Approximately 2 mCi of $^{59}FeCl_3$ (specific activity 22 mCi/mg; New England Nuclear Company, Boston, Massachusetts) 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 wild-type and 2 db/db C57BL/KsJ 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 (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.

Chemically-induced diabetes. Diabetes was chemically induced in eight-week-old wild-type C57BL/KsJ, C57BL/6J, and B6D2F$_1$/J mice with intraperitoneal injections of either alloxan (Schwartz-Mann, Orangeburg, New York) or streptozotocin (Calbiochem, La Jolla, California, and a gift from Dr. W. E. Dulin, Upjohn Company, Kalamazoo, Michigan). Both drugs were dissolved immediately before use in 0.9 per cent NaCl adjusted to pH 4.5 with citric acid. Diabetogenic doses of alloxan (300 mg/kg) and streptozotocin (170 mg/kg) were used. Control animals were injected with either 0.9 per cent NaCl adjusted to pH 4.5 with citric acid or subdiabetogenic doses of alloxan (40 mg/kg) or streptozotocin (80 mg/kg).

C. Results

The chromatographic pattern (Fig. 2) of C57BL/KsJ wild-type (+/+) mouse hemoglobins closely parallels that of normal human hemoglobins (Huisman). By convention, the minor human hemoglobins (hemoglobin A_{Ia}, hemoglobin A_{Ib}, hemoglobin 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.

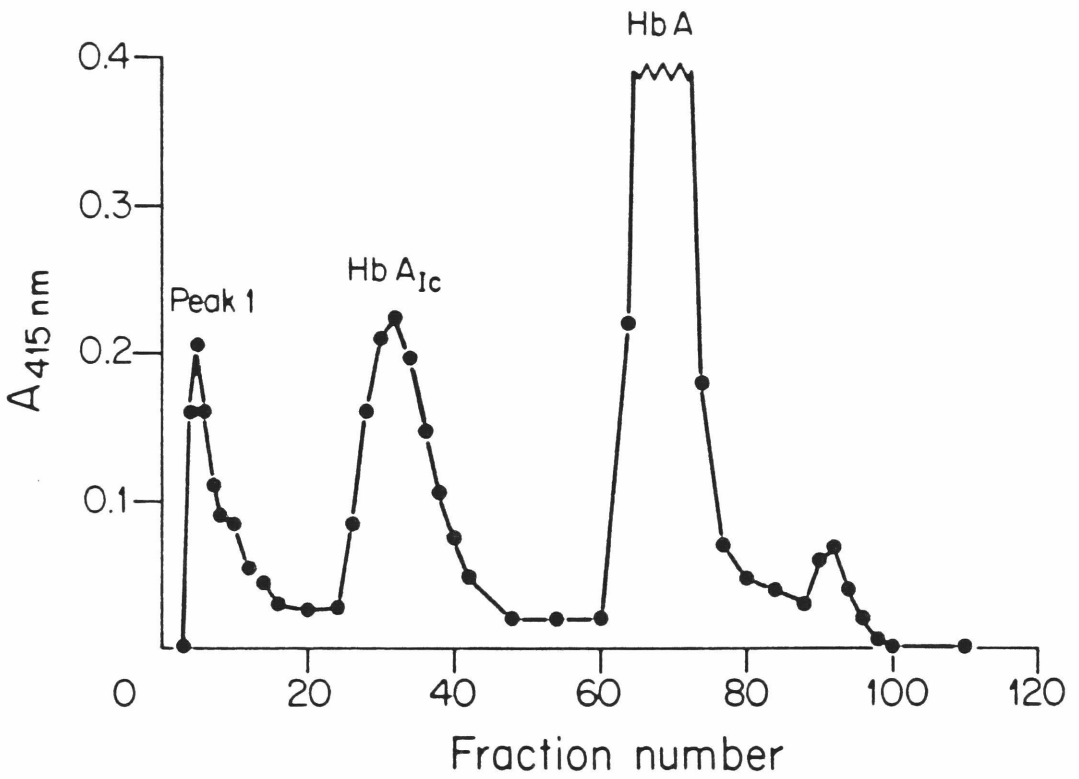


Fig. 2. Elution profile of hemoglobin from C57BL/KsJ +/+ mice on Bio Rex 70. Thirteen milligrams of carbonmonoxy-hemoglobin were eluted with developer 5A (fractions 1-55) and buffer B (fractions 56-120) on a 6 mm x 25 cm column. Fractions of approximately 1.2 ml were collected at a flow rate of 10 ml/hour.

The structure of mouse hemoglobin A_{Ic} was investigated further. Mouse hemoglobins A_{Ic} and A were reacted with NaB^3H_4, the heme was extracted in acetone - 2% HCl, and the α and β chains were separated by ion exchange chromatography. The elution profile for globin from hemoglobin A_{Ic} is shown in Fig. 3. It was found that there is approximately one BH_4-reducible linkage per mouse β^{AIc} chain (Table I). The α chains from mouse hemoglobin A_{Ic} do not react significantly with $NaBH_4$, nor does hemoglobin A. Similar results have been obtained with human hemoglobins A_{Ic} and A (Table I).

TABLE I

Moles of B^3H_4-reducible linkages per mole of hemoglobin monomer for mouse and human hemoglobins A_{Ic} and A

	Mouse Hemoglobins		Human Hb's*	
	A_{Ic}	A	A_{Ic}	A
α chains	0.04	0.03	0.12	0.12
β chains	0.82	0.04	0.92	0.12

* (Bookchin; Rahbar)

The borotritiide-reduced β chains from mouse hemoglobins A_{Ic} and A were digested with trypsin. The tryptic peptides were separated by a 2-dimensional analysis using electrophoresis at pH 4.7 followed by descending chromatography in butanol:acetic acid:water:pyridine.

The peptide maps for β^{AIc} and β^{A} chains are shown in Fig. 4. The spot with the arrow is the only ^3H-containing peptide in the β^{AIc}-map. It contains 2×10^4 cpm, or 90% of the radioactivity initially spotted onto the paper. The ^3H-containing peptide is in a slightly different position compared to the corresponding peptide in the β^{A} map. Amino acid analysis shows these spots to be the NH_2-terminal octapeptides of the β^{A} and β^{AIc} chains (Table II). The low recovery of valine in the β^{AIc} map is

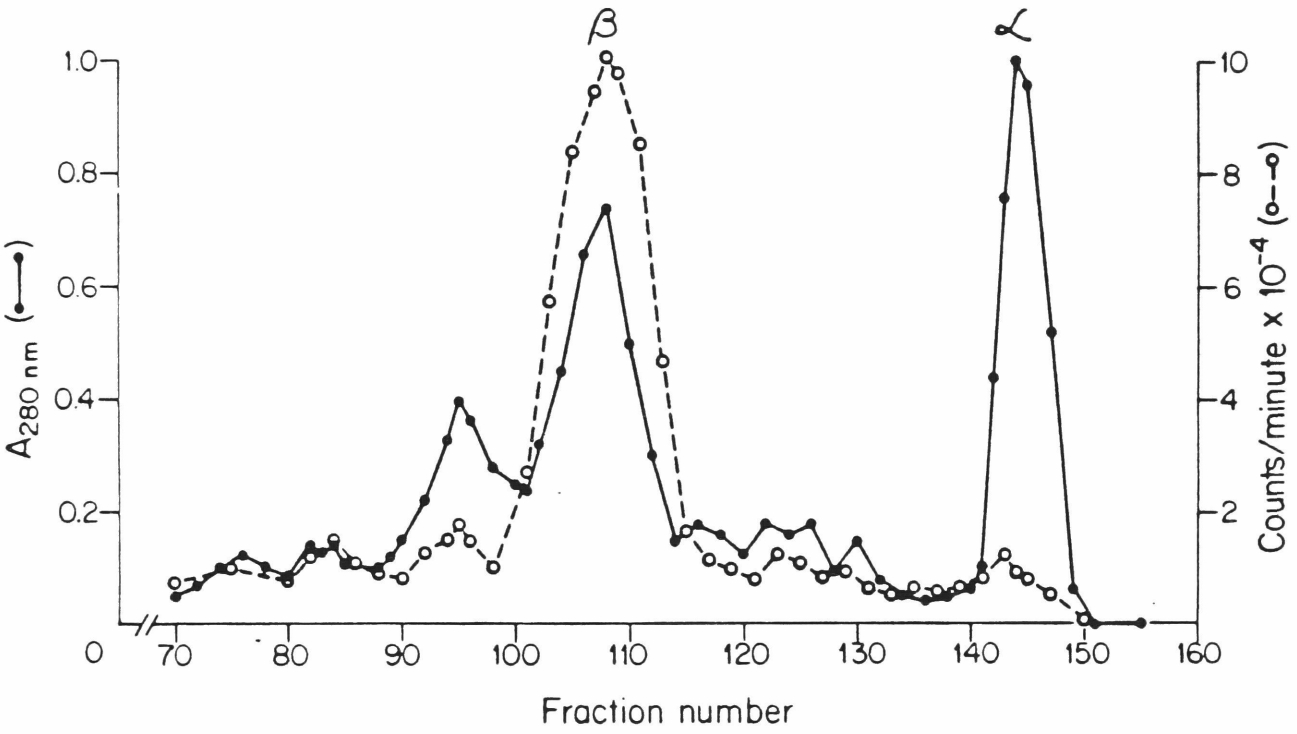


Fig. 3. Separation of α and β chains of NaB^3H_4-reduced mouse hemoglobin A_{Ic} by ion exchange chromatography on CM-cellulose. Forty milligrams of globin (prepared by extraction of heme into acetone-2% HCl) were applied to a 0.9 cm x 15 cm column of CM-cellulose and eluted with a sodium phosphate buffer gradient (0.005 M → 0.04 M) containing 8 M urea and 0.05 M β-mercaptoethanol, pH 6.8. Fractions of 1.2 ml were collected and monitored both for absorbance at 280 nm (●—●) and radioactivity (o---o).

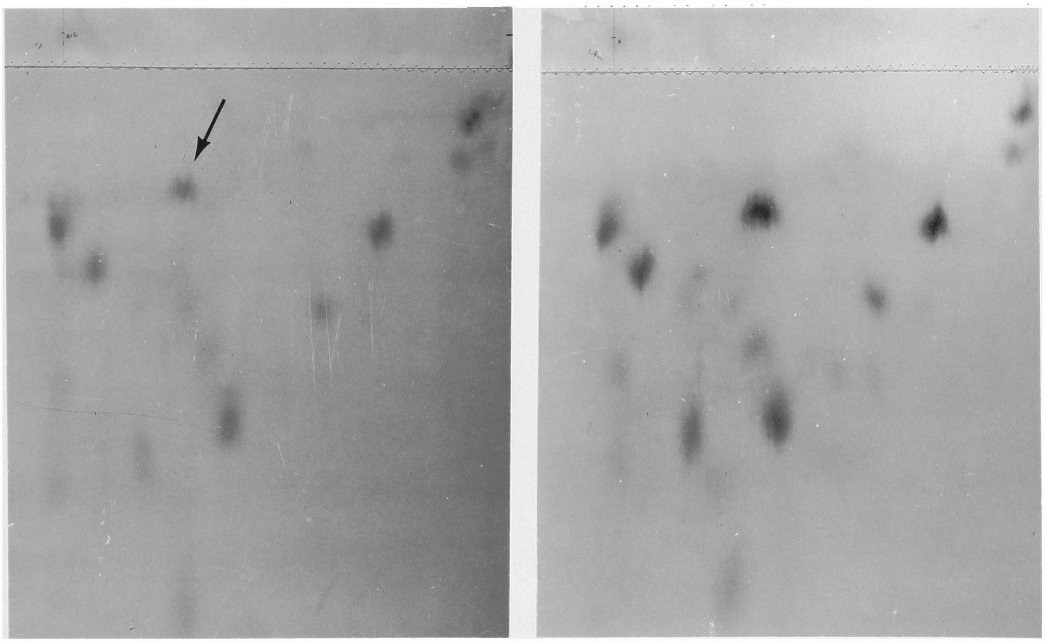


Fig. 4. Peptide maps of NaB^3H_4-reduced mouse β^{AIc} (left) and β^{A} (right) chains after digestion with trypsin. The tryptic peptides were separated by electrophoresis at pH 4.7 followed by descending chromatography in butanol:acetic acid:water:pyridine 15:3:12:10. The maps were stained with ninhydrin. The sole ^3H-containing peptide in the β^{AIc} map (arrow) has the amino acid composition of the amino-terminal octapeptide.

TABLE II

Relative amino acid compositions of the radioactive peptide from mouse $\beta^{A}Ic$ chains and the corresponding β^{A} peptide, and the amino acid composition of the NH_2-terminal octapeptide of β^{A} chains ($\beta^{A}T1$)

	Peptides from maps		
	$(^{3}H\text{-})\beta^{A}Ic$	β^{A}	$\beta^{A}T1$*
Lysine	1.1	1.0	1.0
Histidine	1.1	0.9	1.0
Aspartic acid	0.9	1.0	1.0
Threonine	0.9	0.9	1.0
Glutamic acid	1.1	1.0	1.0
Alanine	0.9	1.1	1.0
Valine	0.4	0.9	1.0
Leucine	1.0	1.0	1.0

* (Rifkin)

presumably due to the formation of a borotritiide-reduced modified valine that is only incompletely hydrolyzed in 6 N HCl. Previous studies with human β^{A} and $\beta^{A}Ic$ chains have shown their NH_2-terminal octapeptides to be located in a similar position in tryptic digest maps with incomplete recovery of the NH_2-terminal valine of the $B^{3}H_4$-reduced $\beta^{A}Ic$ chains (Bookchin; Rahbar).

Having demonstrated that mouse hemoglobin A_{Ic} is structurally analogous to human hemoglobin A_{Ic}, we then determined whether diabetic mice have more hemoglobin A_{Ic} than wild-type 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 III. Thus, diabetic mice demonstrate a 1.6-fold increase in peak 1 hemoglobins and a 2.8-fold increase in mouse hemoglobin A_{Ic} as compared to wild-type mice.

TABLE III

Minor hemoglobins (% of total ± SEM)
in wild-type and diabetic C57BL/KsJ 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 ($P < 0.001$)

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 (Hummel, 1966). Wild-type (+/+) and db/db mice have identical hemoglobin A_{Ic} levels for their first 10 weeks of life, after which the db/db mouse displays a rapid increase in the amount of hemoglobin A_{Ic} (Fig. 5). This rise in hemoglobin A_{Ic} concentration is nearly parallel to, but occurs 3 to 4 weeks after, the rise in plasma glucose levels (Fig. 6).

The 3 to 4 week time delay between the onset of hyperglycemia and increase in hemoglobin A_{Ic} concentration is a consequence of the mechanism of hemoglobin A_{Ic} biosynthesis.

The biosynthesis of mouse hemoglobin A_{Ic} was investigated using the following experimental design. Wild-type mice were injected with ^{59}Fe. Three days were allowed for incorporation of radiolabel into reticulocyte hemoglobin. These animals were bled and 0.2 ml of the blood of each donor animal was injected intravenously into one wild-type and one db/db recipient. The recipients were then bled at intervals and the specific activities of hemoglobins A_{Ic} and A were determined.

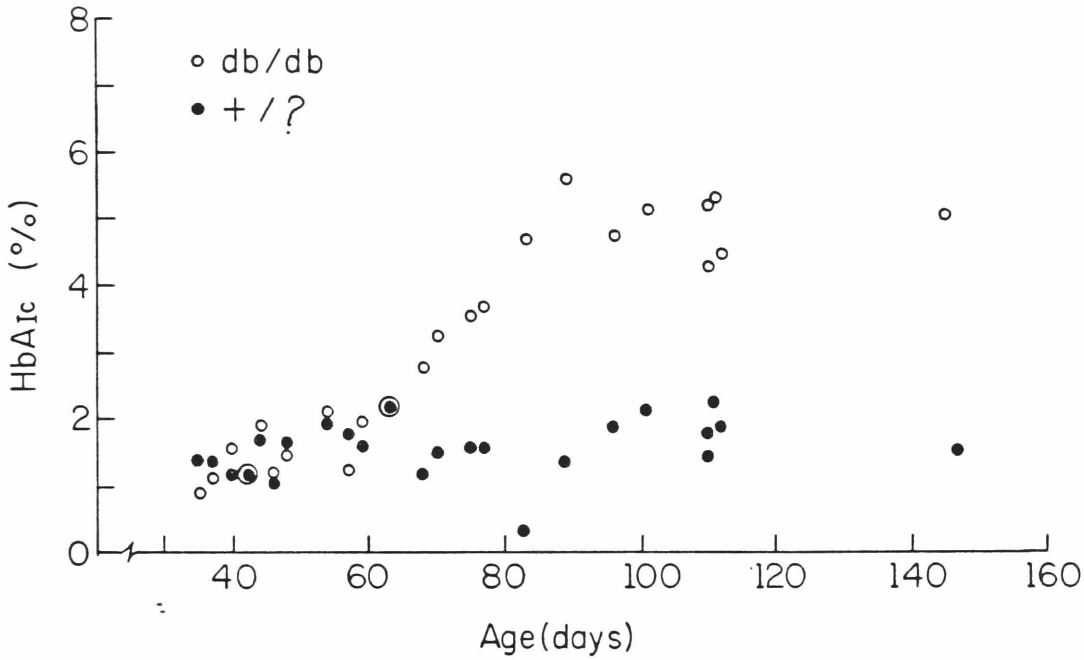


Fig. 5. Relationship between the percentage of peripheral blood hemoglobin as hemoglobin A_{Ic} and age in C57BL/KsJ - +/? (•) and db/db (o) mice.

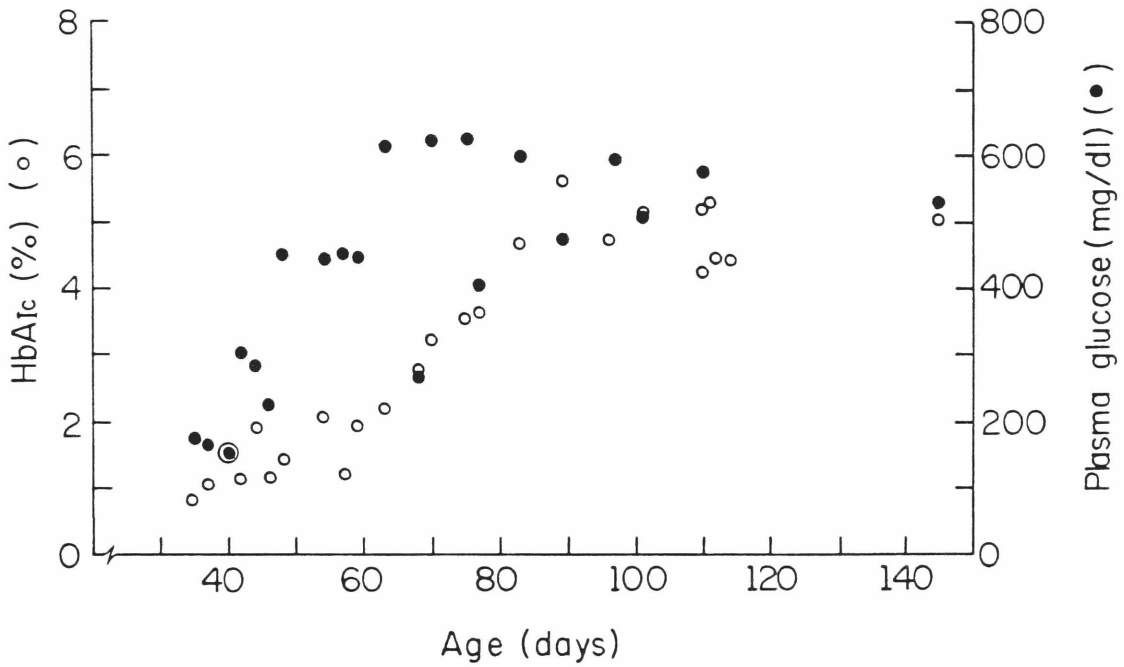


Fig. 6. Relationship of both percentage of total hemoglobin as hemoglobin A_{Ic} (o) and plasma glucose (•) to age in C57BL/KsJ - db/db mice.

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 (Russell). Since the amount of radio-labeled blood transferred from donor to recipient (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 hemoglobin A_{Ic} concentration will not be reflected in the per cent of total hemoglobin as hemoglobin 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 hemoglobin A_{Ic} fraction and the specific activity (cpm/mg) of that fraction.

When the 5 mice which received i.p. $^{59}FeCl_3$ were bled, the specific activity of hemoglobin A_{Ic} was found to be only 0.33 ± .05 that of hemoglobin A (Table IV). If hemoglobins A and A_{Ic} were synthesized at the same time in the life of an erythrocyte, they would be expected to have identical specific activities. (Because hemoglobin A is present in a 25-50-fold excess over hemoglobin A_{Ic}, the gross counts/minute of the two hemoglobins should not be equal.)

TABLE IV

Specific activities of hemoglobins A_{Ic} and A in the five donor mice three days after injection of $^{59}FeCl_3$

	Genotype	s.a. Hb A_{Ic}	s.a. Hb A	$\frac{A_{Ic}}{A}$
1	+/+	39,280	108,000	0.364
2	+/+	17,781	35,560	0.500
3	+/+	11,033	45,971	0.240
4	db/db	15,427	51,420	0.300
5	db/db	27,327	120,000	0.228

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 hemoglobins A_{Ic} and A were determined.

Figs. 7A and 7B 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 hemoglobin A remained constant or rose slightly over the first 4 to 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 hemoglobin A, which occurs after 4 to 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 hemoglobin A_{Ic} in all recipients continued to rise or remained constant for the first 2 to 3 weeks and then declined at a slow rate.

Since hemoglobin A and hemoglobin A_{Ic} are presumably equally distributed within the radio-labeled cells, it is possible to correct for premature loss of radio-labeled cells at each time point by dividing the total cpm of mouse hemoglobin A_{Ic} by that of hemoglobin A, which would remain constant if the radiolytic process were not shortening cell survival. The data presented in Fig. 8 are a replotting of the information contained in Figs. 7A and 7B using this technique. From this figure it is seen that the amount of mouse hemoglobin 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 hemoglobin A_{Ic} production in any recipient mouse can be estimated by comparing the ratio (cpm hemoglobin A_{Ic}/cpm hemoglobin A) at the time of cell transfer to that ratio after 47 days. These data for all mice studied are shown in Table V and summarized in Table VI. When labeled blood from any genotype donor is placed in diabetic recipients, the rate of mouse hemoglobin A_{Ic} synthesis in the labeled cells is 2.7 times that rate

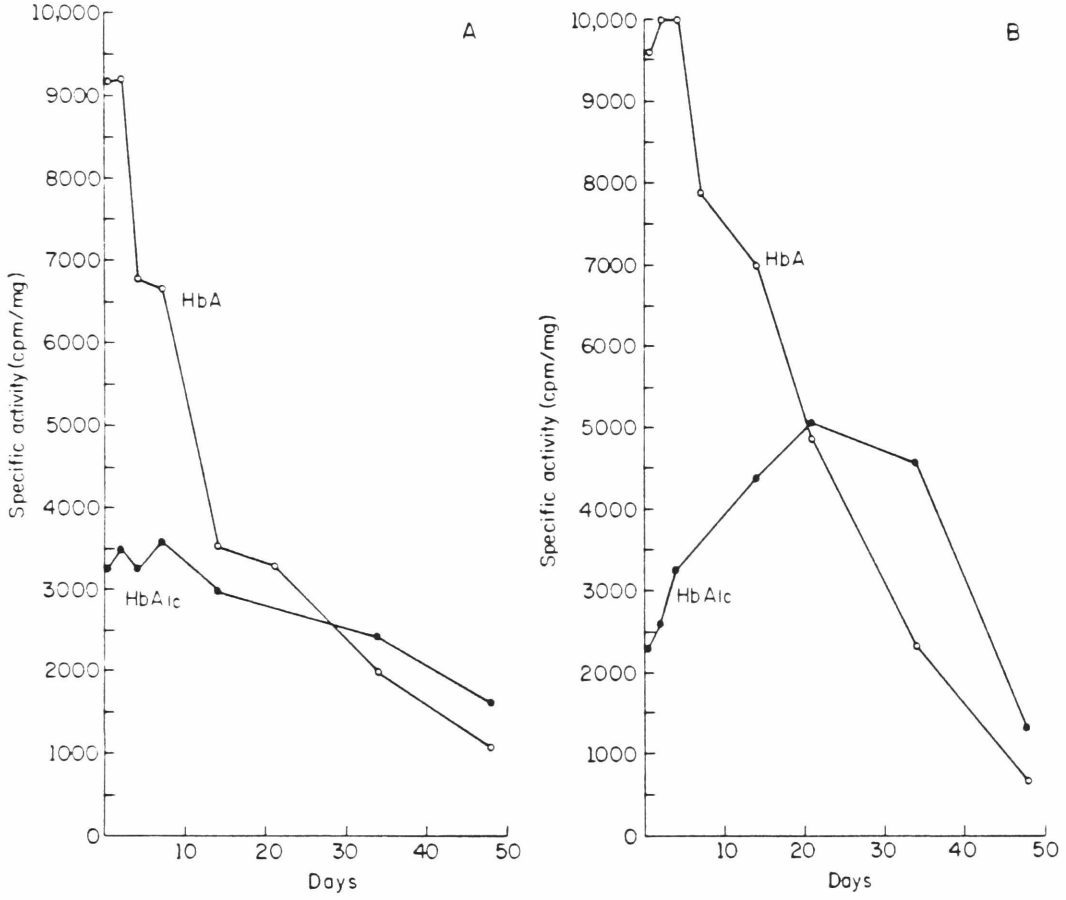


Fig. 7. Specific activities of hemoglobins A_{Ic} and A as a function of time in C57BL/KsJ wild-type (A) and diabetic (B) mice. Wild-type (+/+) mice were injected intraperitoneally with 2 mCi of $^{59}FeCl_3$. Three days later approximately 0.2 ml of radio-labeled blood from each +/+ mouse was injected intravenously into one +/+ (A) and one db/db (B) mouse. These recipient mice were bled at intervals and the specific activities of their hemoglobin A and hemoglobin A_{Ic} fractions were determined. Time zero represents the time of the transfer of radio-labeled blood.

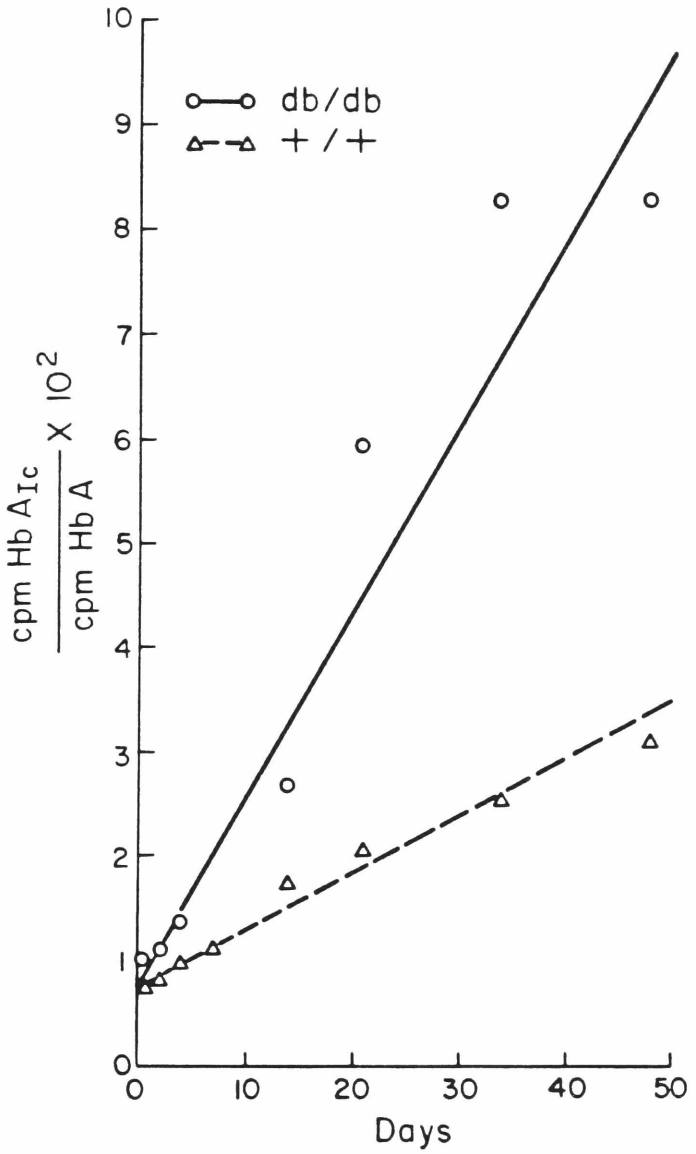


Fig. 8. The ratio of total cpm of hemoglobin A_{Ic} to that of hemoglobin A as a function of time in +/+ (Δ) and db/db (o) mice which received radio-labeled blood from a +/+ donor. This figure is a replotting of Figs. 7A and B to reflect net synthesis of hemoglobin A_{Ic}.

TABLE V

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).

seen when the cells are transferred to wild-type recipients (Table VI; $P < 0.005$). This agrees well with the 2.8-fold increase in hemoglobin A_{Ic} levels of diabetic mice, as compared to wild type. This production of hemoglobin A_{Ic} must be the result of post-synthetic modification of already existing hemoglobin A, since de novo protein synthesis is not possible. 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 hemoglobin A_{Ic} achieved can be correlated with the genotype and carbohydrate status of the recipient mouse and not with the genotype of the donor.

In fact, it has been found that increased hemoglobin A_{Ic} concentration is a marker for the diabetic phenotype regardless of the cause of diabetes or genetic composition of the mouse. Thus, C57BL/KsJ mice that are homozygous for the obese gene (ob/ob) are diabetic and have a two-fold increase in their hemoglobin A_{Ic} concentration (3.53%, Table VII). However, when C57BL/6J mice are homozygous for either the db or ob mutation, only a transient diabetes results. C57BL/6J-ob/ob mice that are nondiabetic (and

TABLE VI

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

Donor genotype	Number of Donors	Recipient genotype	Number of recipients	Hb A_{Ic}* / Hb A	Δ db/db Recipient / Δ +/+ recipient
db/db	2	+/+	2	3.34 ± .08	2.40
		db/db	2	8.03 ± .64	
+/+	3	+/+	3	2.53 ± .06	3.05
		db/db	3	7.72 ± .65	
All	5	+/+	5	2.86 ± .20	2.74
		db/db	5	7.84 ± .42	

* The column heading Hb A_{Ic}/Hb A represents (cpm Hb A_{Ic}/cpm Hb A) x 10^2, Mean ± S.E.M.

hyperinsulinemic) have normal levels of hemoglobin A_{Ic} (1.86%, Table VII). Diabetic C57BL/6J-db/db mice have elevated hemoglobin A_{Ic} concentrations (4.50%, Table VII), but when hyperinsulinemia returns these mice to near normoglycemia, their hemoglobin A_{Ic} values also approach nondiabetic levels (Table VIII).

Furthermore, wild-type mice with chemically-induced diabetes have increased hemoglobin A_{Ic} concentrations (3.30%, Table IX). In this experiment, diabetes was induced in 6 mice by the administration of alloxan, and in 7 mice by the administration of streptozotocin. Both of these drugs show selective toxicity toward the β cells of the islets of Langerhans.

TABLE VII

Minor hemoglobin components and carbohydrate status of C57BL/KsJ-+/+, ob/ob, and db/db, as well as C57BL/6J-+/+, ob/ob, and db/db mice*

Strain	Mutation	N	Hb $A_{Ia + Ib}$ (% ± S.E.M.)	Hb A_{Ic} (% ± S.E.M.)	Plasma glucose (mg/dl ± S.E.M.)
C57BL/KsJ	+/+	28	1.15 ± .05	1.79 ± .04	151 ± 6
	db/db	24	1.73 ± .11$^{\xi}$	4.37 ± .17$^{\infty}$	551 ± 24$^{\infty}$
	ob/ob	6	1.55 ± .16**	3.53 ± .28$^{\xi}$	472 ± 51$^{\xi}$
C57BL/6J	+/+	16	1.35 ± .06	1.90 ± .11	156 ± 6
	db/db †	6	1.51 ± .17**	4.50 ± .21$^{\infty}$	468 ± 55$^{\xi}$
	ob/ob ‡	10	2.05 ± .29**	1.86 ± .18**	153 ± 35**

* Eighty days of age and greater except as noted.

† 80-170 days of age.

‡ 353-503 days of age.

$^{\xi}$ Difference statistically signficant, P <0.01, compared with appropriate wild-type controls.

$^{\infty}$ Difference statistically significant, P <0.001, compared with appropriate wild-type controls.

** Difference statistically not significant, P> 0.05, compared with appropriate wild-type controls.

TABLE VIII

Relationship between period of transient diabetes and Hb A_{Ic} levels in C57BL/6J-db/db mice

N	Age (days)	Hb A_{Ic} (% ± S.E.M.)	Plasma glucose (mg/dl ± S.E.M.)
6	81-170	4.50 ± .21	468 ± 55
6	171-270	2.55 ± .20*	242 ± 23**

* Difference statistically significant, P <.01, compared with 81-170-day-old group.

** Difference statistically significant, P <.05, compared with 81-170-day-old group.

TABLE IX

Minor hemoglobin and glucose concentrations in mice with chemically-induced diabetes

Phenotype	N	Hb $A_{Ia + Ib}$ (% ± S.E.M.)	Hb A_{Ic} (% ± S.E.M.)	Plasma glucose (mg/dl ± S.E.M.)
Normal	13	1.04 ± .10	1.53 ± .13	151 ± 7
Diabetic				
Alloxan	6	1.49 ± .21	3.38 ± .46	342 ± 38
Streptozotocin	7	1.49 ± .16	3.22 ± .29	400 ± 30
All	13	1.49 ± .13*	3.30 ± .25**	373 ± 24**

* Difference not statistically significant, P >.05, compared with normals.

** Difference statistically significant, P <.001, compared with normals.

D. Discussion

Chromatographic analysis on Bio-Rex 70 of mouse hemoglobins (C57BL/KsJ +/+ and C57BL/6J +/+) revealed several minor components, one of which had similar chromatographic behavior to human hemoglobin A_{Ic} and comprised 1.5-2% of the total mouse hemoglobin. Although the chemical structure of mouse hemoglobin A_{Ic} is undetermined, it is probably analogous to human hemoglobin A_{Ic} (i.e., a glycosylated hemoglobin) since both hemoglobins contain a $NaBH_4$-reducible linkage at the amino terminus of the β chains and have similar chromatographic behavior.

Hemoglobin A_{Ic} concentration is increased two- to three-fold in all mice with genetic- or chemically-induced diabetes, but is not increased in non-diabetic db/db or ob/ob mice. Erythrocytes from diabetic db/db mice, when infused into wild-type mice, synthesize normal levels of hemoglobin A_{Ic}. Wild-type erythrocytes circulating in a diabetic host synthesize hemoglobin A_{Ic} at diabetic (two- to three-fold increased) rates. Thus, the increased hemoglobin A_{Ic} concentration of diabetes is not the result of a genetic defect of the animal or red cell, nor is it the direct result of obesity, but rather, it is a consequence of, and a marker for, the diabetic phenotype.

The red cell is an ideal cell type in which to study the time of protein modification, since there is no protein synthesis in the mature erythrocyte. Therefore, the appearance of new hemoglobins in the peripheral blood must come about by a postsynthetic modification of existing hemoglobins. A comparison of the specific activities of hemoglobin A_{Ic} and hemoglobin A of the reticulocytes from both db/db and +/+ animals revealed a lower specific activity in the mouse hemoglobin A_{Ic} fraction. This reflects either a greater percentage of the total mouse hemoglobin A_{Ic} than the total hemoglobin A being synthesized in the pre-reticulocyte state, or the synthesis of hemoglobin A_{Ic} from hemoglobin 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 hemoglobin A_{Ic} present whether they circulated in a +/+

or db/db recipient. In fact, a linear increase in the ratio (cpm hemoglobin A_{Ic}/cpm hemoglobin A) was observed for 50 days, denoting a constant rate of synthesis.

The rate of synthesis of mouse hemoglobin 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 hemoglobin A_{Ic} from hemoglobin A. The onset of diabetes causes an increase in this factor(s) which promotes more rapid hemoglobin A_{Ic} synthesis.

The 3 to 4 week time delay between the onset of hyperglycemia in the C57BL/KsJ db/db mice and the increase in hemoglobin A_{Ic} (Fig. 6) can now be explained. The ^{59}Fe data show hemoglobin A_{Ic} to be synthesized at a very slow rate: only 0.04% of hemoglobin A is modified per day in wild-type mice, and 0.1% per day in diabetic mice. Thus, hyperglycemia must be sustained for several weeks before enough new hemoglobin A_{Ic} is synthesized to see an increase in the total mouse hemoglobin A_{Ic}. The slow conversion of hemoglobin A to hemoglobin A_{Ic} in the mouse red cells has since been confirmed in human erythrocytes by Bunn (1976). This slow rate of synthesis will turn out to be a major factor in the clinical utility of hemoglobin A_{Ic} measurements.

The continual linear synthesis of hemoglobin 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 hemoglobin A_{Ic} seen in peripheral blood is brought about primarily by a balance of new synthesis of hemoglobin A_{Ic} and the replacement of old cells laden with hemoglobin A_{Ic} by new cells with less hemoglobin A_{Ic}. If the nature of the modification of basement membrane proteins is similar to that of hemoglobin A_{Ic}, then a slow turnover of basement membrane proteins could easily lead to a substantial buildup of glycosyl groups in diabetes. In addition, the increase 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.

III. CLINICAL SIGNIFICANCE OF HEMOGLOBIN A_{Ic}

A. Introduction

Studies in the diabetic mouse demonstrated that hemoglobin A is glycosylated to form hemoglobin A_{Ic} at a constant, slow rate throughout the life of the red cell. Elevated hemoglobin A_{Ic} concentrations appear to reflect the diabetic phenotype regardless of the underlying cause of diabetes. The monitoring of hemoglobin A_{Ic} concentrations might therefore be a potentially useful method of diagnosing diabetes in humans or of assessing the quality of control in a given patient.

The relationship between hemoglobin A_{Ic} concentration and the clinical syndrome of diabetes was investigated to answer the following questions. 1) Does the degree of the elevation of hemoglobin A_{Ic} concentration correlate with the severity of disease? 2) Will changes in the quality of diabetic control be reflected by proportionate changes in hemoglobin A_{Ic} concentration? These basic questions were answered in studies of 32 diabetic out-patients and 5 diabetic in-patients at The Rockefeller University Hospital.

B. Materials and Methods

Hemoglobin isolation and quantification. The minor hemoglobins $A_{Ia + I b}$ and A_{Ic} were isolated and quantified from heparinized or EDTA-treated venous blood as described in Chapter 2, except that the ion exchange columns were developed with developer 6 (0.055 M sodium phosphate, 0.01 M KCN, pH 6.75).

Out-patient study. A total of 32 diabetic out-patients were studied to determine the relationship between fasting blood sugar, response to an oral glucose tolerance test, and hemoglobin A_{Ic} concentration. The patients ranged in age from 16 to 82 years. Fifteen were maintained on insulin therapy, 9 on oral hypoglycemic agents, and 8 on diet alone. Eleven patients were females; 21 were males.

Fasting blood sugars were measured on at least two occasions in the 32 patients. Oral glucose tolerance tests were performed on 22 patients by giving a 75-gm load of glucose after an overnight fast.

Glucose concentrations were determined by an AutoAnalyzer (Hoffman). The response to the glucose challenge was determined by measuring the area under the curve by planimetry of a three-hour glucose tolerance test. These studies were approved by the Clinical Review Boards of The Rockefeller University Hospital and Washington University School of Medicine, St. Louis, Missouri.

In-patient study. Five diabetic in-patients were studied at The Rockefeller University Hospital to determine whether changes in the quality of control of a patient's diabetes result in proportionate changes in that patient's hemoglobin A_{Ic} concentration. The case histories are summarized as follows:

Case 1, a 57-year-old black man with an eight-year history of diabetes mellitus, was maintained on 36 units of insulin per day, and manifested a mild peripheral neuropathy (by electromyography), with occasional numbness of the digits.

Case 2, a 38-year-old white woman with a 28-year history of diabetes, was maintained on 35 to 40 units of insulin per day. She had mild peripheral neuropathy and gangrene of the distal phalanx of the right great toe.

Case 3, a 38-year-old white woman with a 33-year history of diabetes, was maintained on 15 to 20 units of insulin per day. In 1970 she underwent hypophysectomy for retinitis proliferans with frequent vitreous hemorrhages, which had not been controlled by photocoagulation. She had mild peripheral neuropathy. Her retinopathy has been stable for four years.

Case 4, a 61-year-old white woman with a 14-year history of diabetes, had been treated with phenformin before hospitalization, when she received 33 units of insulin per day. She weighted 78 kg on admission and lost 10 kg during hospitalization through a diet and exercise program. She had retinal microaneurysms and exudates and peripheral neuropathy.

Case 5, a 64-year-old black woman with a 30-year history of diabetes, had received insulin in the past, but had not taken insulin or oral

hypoglycemic agents for the two months before admission. She had extensive background retinopathy, peripheral vascular disease, peripheral neuropathy and mild proteinuria.

All patients were under poor diabetic control at the time of induction into the study. Blood sugar concentrations were brought to more optimal levels within approximately one to two months after hospital admission. This improvement was accomplished by the careful regulation of diet, exercise, and the administration of insulin. Patients were hospitalized on a metabolic ward for the duration of this study (3 to 6 months per patient).

Several methods were used to assess the regulation of each patient's blood sugar concentration. Urinary sugar was measured semi-quantitatively (on a scale of 0 to 4+) four times per day by standard methods (Clinitest). A daily average of 1+ is equivalent to approximately 8 grams of urinary glucose/day. These measurements were summed every seven days to obtain a mean weekly index of the degree of glycosuria. If a patient's urine frequently gave a negative test for sugar by this technique, 24-hour urine collections were made three times per week, and the total sugar content of these specimens was determined quantitatively (Hoffman). Fasting blood sugar was measured once per week by an Auto-Analyzer (Hoffman). To assess each patient's response to meals, blood sugar was measured just before and one hour after each meal for one day both before and during optimal control of blood sugar levels. These six blood sugar concentrations were then summed, and this value, called the glucose brackets, was taken as an estimate of each patient's response to dietary intake since the diet remained constant during each test. These studies were approved by the Clinical Review Board of The Rockefeller University Hospital.

C. Results

Glucose tolerance tests were performed on 22 diabetic patients. In this group, hemoglobin A_{Ic} levels varied from 4.49 per cent to 11.20 per cent. The peak glucose response to the glucose tolerance test ranged from 184 mg/dl to 880 mg/dl. The correlation coefficient between these

two variables is highly significant ($r = 0.82$, $P < 0.001$) (Fig. 9). The results are unaltered by utilizing the areas under the glucose tolerance curves ($r = 0.76$, $P < 0.001$) instead of the peak values. A significant but less impressive correlation was also found between fasting blood sugar and hemoglobin A_{Ic} levels in the 32 diabetics studied ($r = 0.62$, $P < 0.001$) (Fig. 10). Because fasting blood sugar concentration and response to a glucose tolerance test are interdependent variables that correlate well with each other ($r = 0.77$, $P < 0.001$), their respective correlations with hemoglobin A_{Ic} concentration might simply reflect their interdependence. Partial correlation coefficients (Hays), which take into account the interdependence of response to a glucose tolerance test and fasting blood sugar, revealed that hemoglobin A_{Ic} concentration and response to a glucose tolerance test are indeed correlated (partial $r = 0.61$, $P < 0.005$); whereas the correlation between fasting blood sugar and hemoglobin A_{Ic} concentrations loses its significance (partial $r = 0.22$, $P > 0.05$).

These data demonstrate that hemoglobin A_{Ic} concentration correlates with the severity of diabetes, at least as measured by response to an oral glucose tolerance test.

Five diabetic in-patients were studied to determine whether changes in diabetic control result in proportionate changes in hemoglobin A_{Ic} concentration. The patients were poorly controlled at the time of induction into the study, but were brought to a state of improved diabetic control during hospitalization (Table X).

The mean fasting blood sugar for the group was 343 mg per deciliter (range of 280 to 450) at the start of the study and decreased to a mean of 84 mg per deciliter (range of 70 to 100) at the point of optimum control. The ability to respond to dietary carbohydrate as evidenced by summing of the blood sugar concentrations measured before and after breakfast, lunch and dinner (glucose brackets) was also improved. This summed value decreased by an average of 51 percent (1865 to 914 mg per deciliter). The amount of sugar excreted in the urine was also reduced after control. Semiquantitative estimates of urinary sugar performed four times per day revealed a decrease of an average of about 90 per cent (mean of 89 to 10)

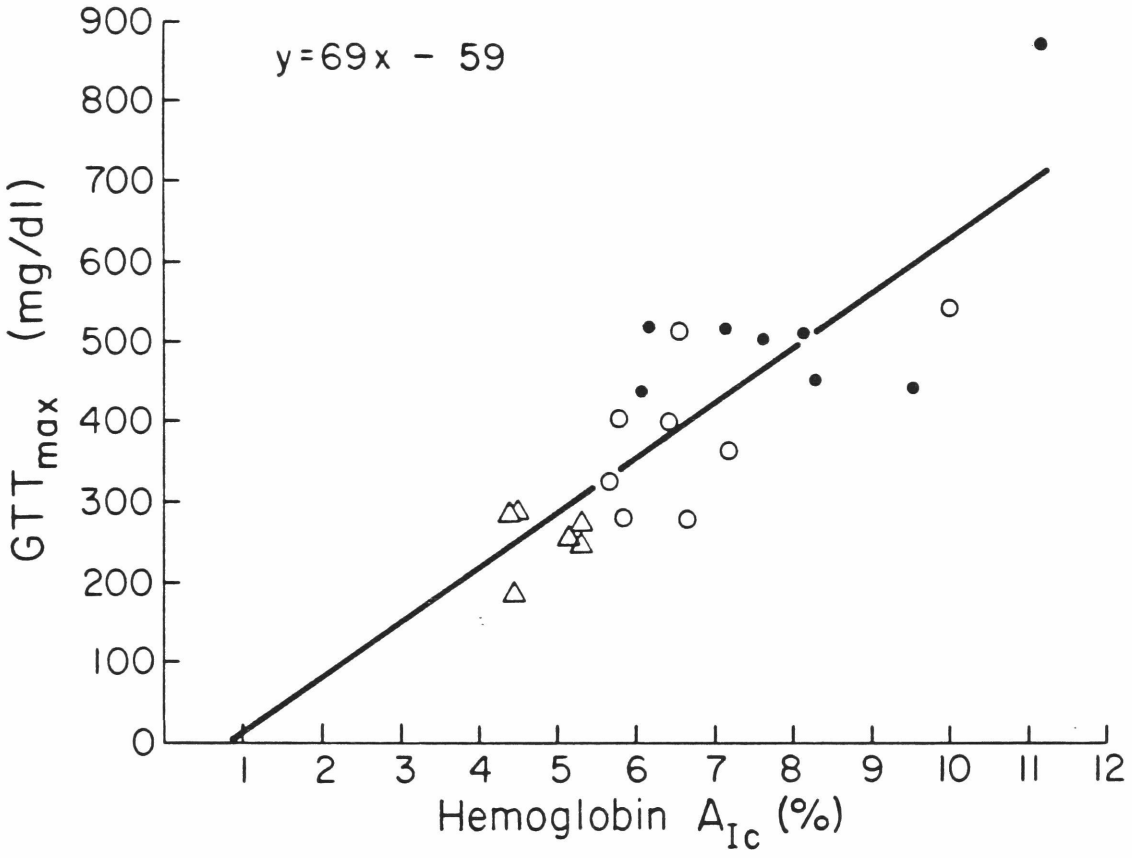


Fig. 9. Correlation of hemoglobin A_{Ic} concentration and maximal response to an oral glucose tolerance test in 22 diabetic patients (• patients treated with insulin, o patients treated with oral hypoglycemic agents, Δ patients treated with diet alone).

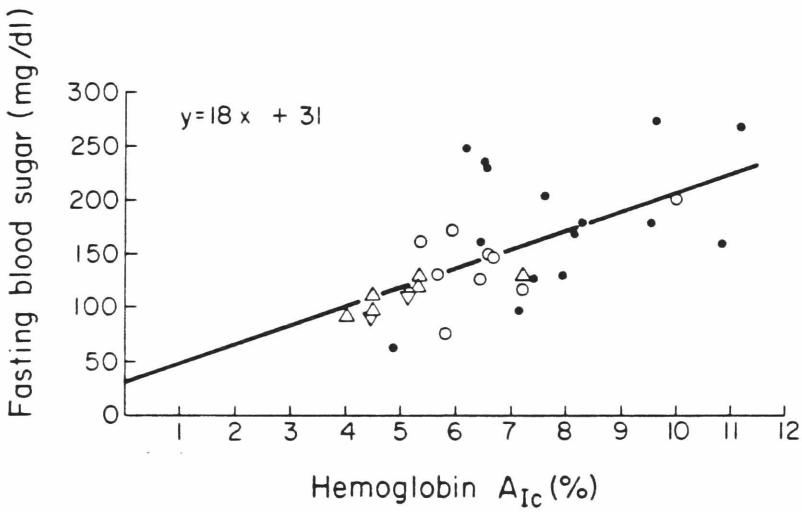


Fig. 10. Correlation between hemoglobin A_{Ic} concentration and fasting blood sugar in 32 diabetic patients (● patients treated with insulin, o patients treated with oral hypoglycemic agents, Δ patients treated with diet alone).

TABLE X

Changes in minor hemoglobin and glucose measurements with control of diabetes

Case No.	Fasting Blood Sugar	Weekly Urinary Sugar*	Glucose Brackets †	Hemoglobin A_{Ic}	Hemoglobin $A_{Ia + Ib}$
	mg/dl		mg/dl	%	%
1:					
Precontrol	390	85	1060	10.1	3.5
Control	77	0	718	5.8	2.3
2:					
Precontrol	280	52	1613	6.8	2.5
Control	100	14	1063	4.2	1.9
3:					
Precontrol	450	112	2716	12.1	4.8
Control	70	9	653	5.4	2.1
4:					
Precontrol	312	96	1978	10.0	3.2
Control	97	0	918	5.8	2.1
5:					
Precontrol	282	100	1959	10.2	3.6
Control	75	28	1218	7.6	3.1
Mean ± S.E.M.					
Precontrol	343 ± 33	89 ± 10	1865 ± 270	9.8 ± .8	3.5 ± .4
Control	84 ± 6	10 ± 5	914 ± 105	5.8 ± .5	2.3 ± .2
Normal	70 - 100	0	500 - 800	3 - 5	2

* Semiquantitative (0 to 4+) urinary sugar concentration was determined four times per day, and values for consecutive 7 day periods summed to determine weekly urinary sugar.

† Sum of blood sugar concentrations measured just before and 1 hour after breakfast, lunch and dinner.

when the values 0 to 4+ were summed over consecutive seven-day periods. Quantitative 24-hour urinary sugar determinations performed three times per week on several of the patients revealed a similar decrease in urinary sugar (mean of 33 to 5 g per day).

The concentration of hemoglobin A_{Ic} also showed a decrease after the improvement of control in all patients, from a mean of 9.8 per cent at the onset to 5.8 per cent at the point of optimum control. Because the absolute amounts of hemoglobin $A_{Ia + Ib}$ are small, slight errors in quantification could obscure changes in the concentrations of these hemoglobins. Despite this problem, all patients showed a decrease in hemoglobin $A_{Ia + I b}$ levels during control (Table X).

Case 3 responded most dramatically to therapy, decreasing her glucose brackets by 76 per cent (2716 to 653 mg per deciliter) and hemoglobin A_{Ic} by 55 per cent (12.1 to 5.4 per cent). She was also the most hyperglycemic patient (fasting blood sugar of 450 mg per deciliter) on admission to the hospital. Case 2 had the mildest glycosuria on admission and also the lowest hemoglobin A_{Ic} concentration (6.8 per cent); the modest drop to 4.2 per cent is all that would be expected since that value is well within the limits seen in nondiabetic populations (Trivelli). Of the methods used in this study to assess blood sugar concentration, the glucose brackets probably best reflect mean daily blood sugar levels. The glucose brackets before and during optimum control of diabetes correlate well with the respective patient's hemoglobin A_{Ic} concentrations at those times (Fig. 11), demonstrating that improvements in diabetes control result in proportionate decreases in hemoglobin A_{Ic} concentration.

The temporal relationship between the decrease in hemoglobin A_{Ic} concentration and improvement in carbohydrate metabolism, as judged by the weekly summation of semiquantitative urinary sugar determinations, is illustrated for one patient (Case 3) in Fig. 12. It can be seen that the decrease in urinary sugar precedes the change in hemoglobin A_{Ic} concentration by approximately three to four weeks. The change in the concentration of hemoglobin $A_{Ia + Ib}$, although smaller in absolute amount, occurs at the same time as the decrease in hemoglobin A_{Ic} levels (Fig. 13).

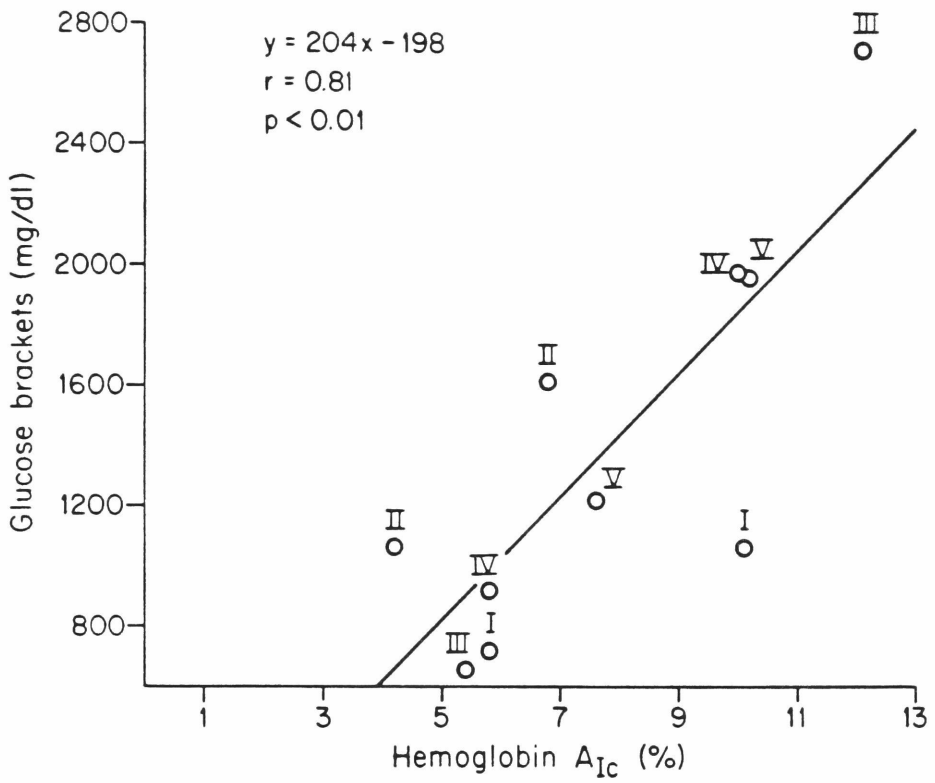


Fig. 11. Correlation between hemoglobin A_{Ic} concentration and glucose brackets before and during carbohydrate control in five diabetic patients. The glucose brackets represent the sum of blood sugar levels measured just before and one hour after breakfast, lunch and dinner, and the Roman numerals the five cases.

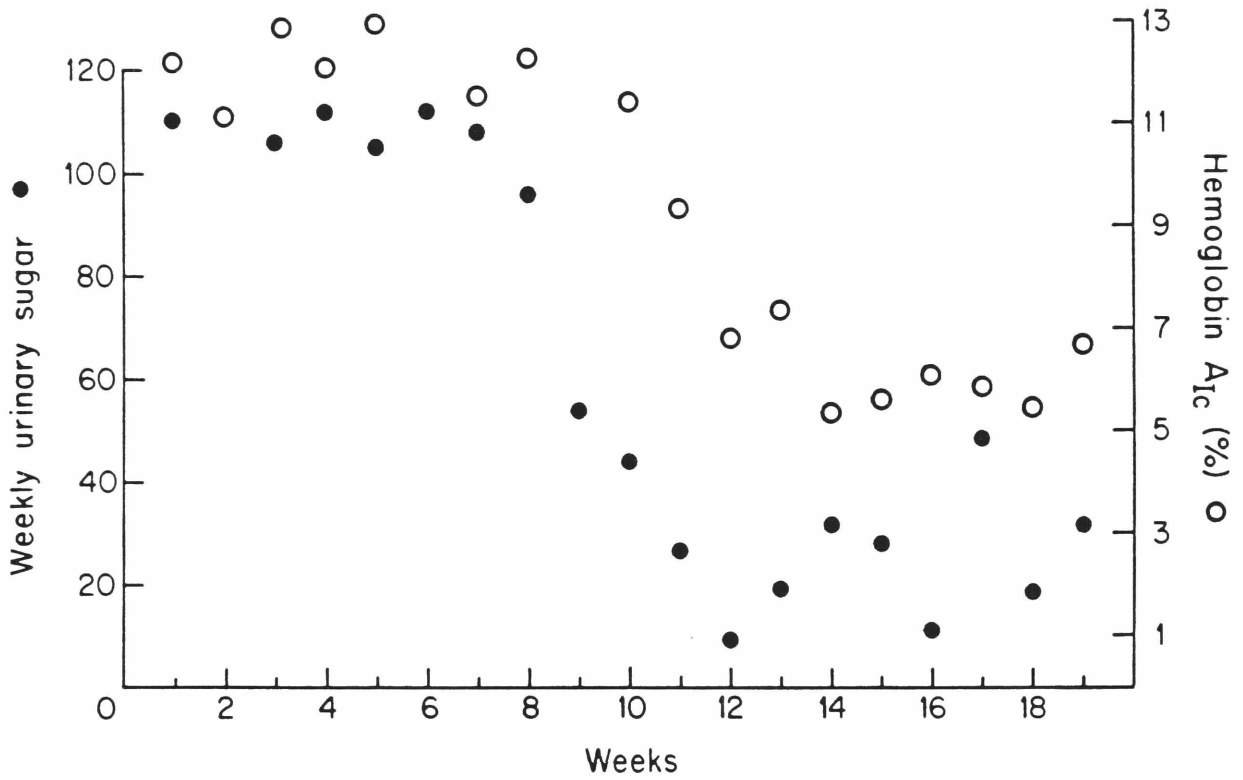


Fig. 12. Temporal relationship between weekly urinary sugar (●) and hemoglobin A_{Ic} (o) levels in Case 3. Semiquantitative (0 to 4+) estimates of urinary sugar levels, performed four times per day, were summed over consecutive seven-day periods to obtain weekly urinary sugar values.

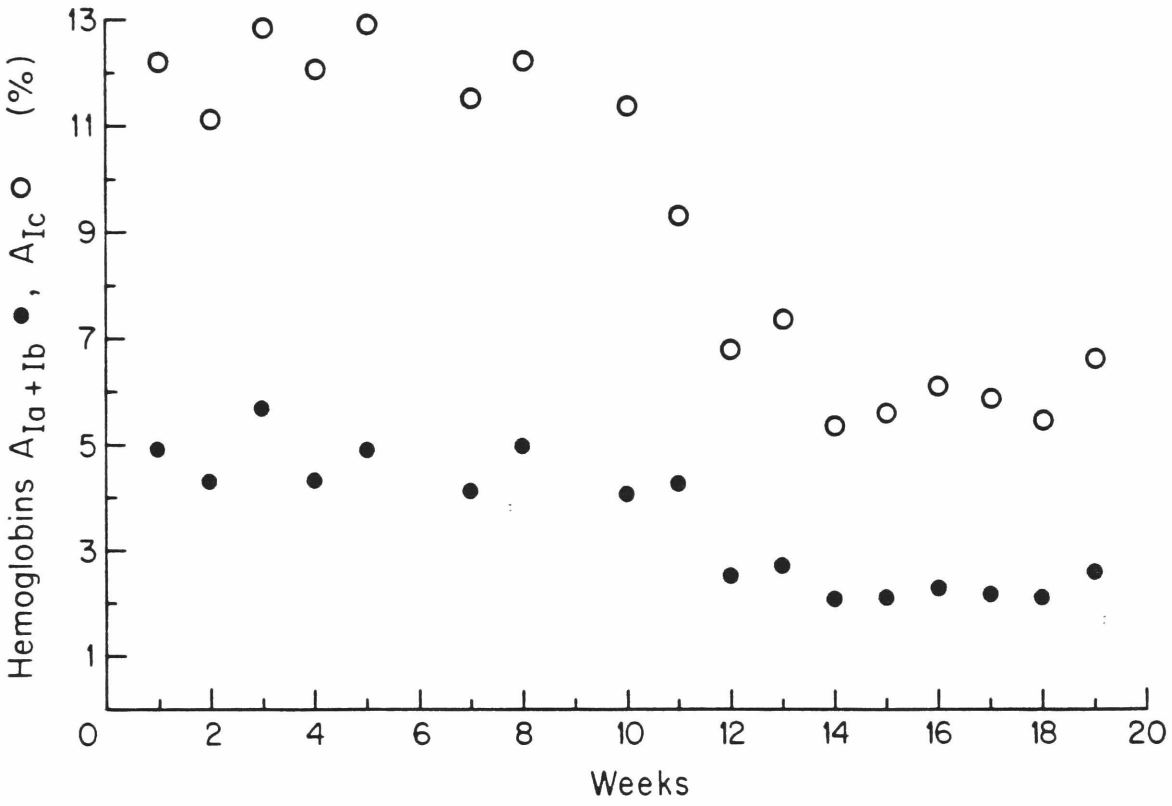


Fig. 13. Temporal relationship between changes in the levels of hemoglobins $A_{Ia + Ib}$ (●) and A_{Ic} (o) in Case 3.

The time lag between changes in glucosuria and minor hemoglobin concentrations is reminiscent of that seen in the db/db mouse between the onset of hyperglycemia and the rise in hemoglobin A_{Ic} concentration. It results from the fact that hemoglobin A_{Ic} is synthesized at a very slow rate, so that a change in rate of synthesis must be maintained for many days before it is reflected by a change in total hemoglobin A_{Ic} concentration.

D. Discussion

The measurements of the concentrations of hemoglobins $A_{Ia + Ib}$ and hemoglobin A_{Ic} represent a means of assessing the presence of diabetes and monitoring the degree of diabetic control. These minor hemoglobins serve as indicator molecules that reflect the mean blood sugar concentration for the previous weeks, not just at the time of measurement. In other words, hemoglobin A_{Ic} concentration is not influenced by short-term fluctuations in blood glucose concentration. Rather, hemoglobin A_{Ic} reflects the patient's mean blood glucose concentration for the three to four weeks prior to the measurement. Thus, short-term fluctuations in diet, exercise, emotional status, etc., that greatly influence fasting blood sugar will not affect hemoglobin A_{Ic}. Hemoglobin A_{Ic} could be measured at infrequent intervals in non-fasted patients to determine whether the patient is well controlled, a feature unique to this measurement. Currently, clinical assessment of diabetic control is based on fasting blood sugar and urinary sugar because these are easy parameters to assess - but they also are not very reproducible from day to day. Most diabetics are rarely in a position to have these parameters checked frequently enough for them to know if they really are well controlled.

The ability of hemoglobin A_{Ic} to indicate the carbohydrate status of the patient over the previous weeks imparts important potential clinical utility to hemoglobin A_{Ic} quantification. For instance, this measurement could be used to diagnose diabetes mellitus. At the present time there are estimated to be more than a million undiagnosed cases of diabetes in the United States (Fajans). Glucose tolerance tests are too time

consuming and expensive to identify these cases. Fasting blood sugar determinations are not reliable for this purpose. A rapid method for determining hemoglobin A_{Ic} concentrations would be useful in screening large populations for diabetes.

Hemoglobin A_{Ic} will be useful in assessing the quality of diabetic control and in determining whether carbohydrate control influences the onset or progression of the sequelae of this disease. This question remains unsettled largely because it has not been possible to document the degree of patients' diabetic control on an out-patient basis over the years it takes for the sequelae to develop. Periodic monitoring of hemoglobin A_{Ic} will circumvent this problem and allow this important question to be answered.

Hemoglobin A_{Ic} should also be useful in determining relative efficacies of various therapies, such as insulin versus oral hypoglycemic agents - or the value of new drugs in the treatment of diabetes.

IV. STRUCTURE OF HEMOGLOBIN A_{Ic}

A. Introduction

Hemoglobin A_{Ic} has been shown to be an indicator molecule whose concentration reflects the patient's mean blood glucose concentration for the 3 to 4 weeks prior to the measurement. This results from the fact that hemoglobin A_{Ic} is synthesized at a slow rate, so that changes in the rate of synthesis must be maintained for several weeks before the total blood concentration of hemoglobin A_{Ic} is significantly altered. The potential value of hemoglobin A_{Ic} in the assessment of long-term diabetic control is established, but the mechanism of hemoglobin A_{Ic} synthesis and its possible relevance to the glycosylation of other proteins in diabetes could be understood only after unambiguous assignment of the structure of this hemoglobin.

Previous work (Holmquist, 1966) has indicated that the amino acid sequences of hemoglobins A and A_{Ic} are identical. The only detectable difference is a low molecular weight sodium borohydride reducible moiety, presumably a Schiff base, at the NH_2-terminus of the β chain of hemoglobin A_{Ic}. Subsequent work has pointed to a carbohydrate as the modifying group on the $\beta^{A_{Ic}}$ chains (Bookchin). Recently, Bunn (1975) has proposed that glucose is the carbohydrate and that it undergoes an Amadori rearrangement following Schiff base formation. This proposal was based on the observation that acid hydrolysis of hemoglobin A_{Ic} liberated 0.3 moles of hexose, consisting of glucose and mannose in a 3:1 ratio, per mole of hemoglobin. In addition to the low recovery of hexose, another unknown component was liberated under these conditions. These and other ambiguities in the experimental design and data precluded a definitive assignment of the structure of hemoglobin A_{Ic}.

We investigated the structure of the amino-terminal blocking group in the following way. Hemoglobin A_{Ic} was isolated free of the other minor hemoglobins and reduced with NaB^3H_4 to stabilize the molecule and radiolabel the blocking group. The ^3H-containing β chains were isolated and a series of mild enzymatic digestions led to the purification of the amino terminal modified dipeptide, (^3H)-R-valylhistidine. Comparison by thin layer chromatography, gas liquid chromatography, and proton magnetic resonance spectroscopy of R-Val-His with synthetic standards permitted the unambiguous assignment of the structure of the blocking group.

B. Materials and Methods

Reagents. All chemicals and solvents were reagent grade. Trypsin, papain, protease VI and Sil-A (silylating reagent) were obtained from Sigma Chemical Co., St. Louis, Missouri. The ion exchange resins Bio-Rex 70, AG 50W-X2, AG 50W-X8 and AG 1-X8 were purchased from Bio Rad Laboratories, Richmond, California; and cellulose phosphates P1 and P11 from Whatman, Inc., Clifton, New Jersey. Sephadex G-10 was obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey. Silica gel GF thin layer chromatography plates were purchased from Analtech, Inc., Newark, Delaware. L-valyl-L-histidine was purchased from Calbiochem, Los Angeles, California, NaB^3H_4 and ^{14}C-valine from Amersham/Searle, Arlington Heights, Illinois, and 100%-d deuterium oxide, 99.5% pyridine d-5, 99.5% acetic acid d-4, and sodium-3-trimethylsilylpropionate-2,2,3,3-d-4 (TSP) from Wilmad Glass Company, Buena, New Jersey.

Isolation of hemoglobin A_{Ic}. Blood was drawn from normal human volunteers into heparinized blasks. The red cells were separated from the plasma by centrifugation, washed twice with two volumes of 0.14 N NaCl, lysed by the addition of two volumes of distilled water, and the hemoglobin solution saturated with carbon monoxide. The carboxyhemoglobin solution was dialyzed against Developer 6 (0.05 M sodium phosphate, 0.01 M KCN, pH 6.75) saturated with CO. Fifty to 75 grams of hemoglobin (50-75 mg/ml) were loaded onto a 15 x 50 cm column of Bio-Rex 70 previously equilibrated with Developer 6. Hemoglobins $A_{Ia + Ib}$ and A_{Ic} were eluted with Developer 6 at a rate of 3 liters per hour; 1.5 liter fractions were collected. The absorbance of the eluent at 415 nm was monitored to determine the elution profile. Two to three grams of hemoglobin A_{Ic} were isolated. The pH of the pooled hemoglobin A_{Ic} fractions (7 liters) was lowered to 6.4 with H_3PO_4, and the solution was applied to a 5 x 70 cm column of cellulose phosphate P1, previously equilibrated with 0.05 M sodium phosphate buffer pH 6.4 at 4°C. Hemoglobin A_{Ic} adsorbed to the resin while contaminating hemoglobin $A_{Ia + Ib}$ did not. The column was then brought to room temperature and the hemoglobin A_{Ic} eluted with 0.25 M sodium phosphate, 0.01 M

KCN pH 7 buffer. This procedure resulted in further purification and a ten-fold concentration of the hemoglobin A_{Ic}.

Isolation of glycodipeptides from hemoglobin A_{Ic}. The hemoglobin A_{Ic} solution (2 g in 130 ml) was dialyzed against 0.1 M sodium phosphate buffer pH 7 and subsequently reacted with a 200 molar excess of NaB^3H_4 (1.4×10^7 dpm/μmol) at room temperature for 1 hour. After removing the unreacted sodium borohydride by dialysis, the heme was extracted in acetone - 2% HCl at -20°C and the α and β chains were separated by ion exchange chromatography on carboxymethyl cellulose as described in Chapter 2, except that the procedure was scaled up to 600 mg batches of globin on 5 cm x 20 cm columns of the cation exchange resin. The columns were developed with 8-liter (total volume) gradients of sodium phosphate buffer and 16 ml fractions were collected at a flow rate of 180 ml/hour.

The absorbance of the eluent was monitored at 280 nm and the radioactivity of the fractions was determined in a Packard Tri-Carb liquid scintillation counter. The fractions containing the α and β chains were pooled separately. The (^{3}H-) β chains were dialyzed extensively against distilled water and lyophilized.

The (^{3}H-) β chains were dissolved in 1% NH_4HCO_3 to a final concentration of 1 mg/ml and digested for 4 hours at 37°C with trypsin (50 μg/mg β chain). The digest was lyophilized, dissolved in distilled water, and relyophilized.

Partial separation of the tryptic peptides was achieved by ion exchange chromatography on the sulfonated polystyrene resin AG 50W-X2 (Schroeder). Six micromoles (90 mg) of digested β chains were dissolved in 2 ml of distilled water, the pH was adjusted to 2 with 6 N HCl, and the sample was applied to a 0.9 cm x 110 cm column of the cation exchange resin. The column was developed with a non-linear pH gradient using 350 ml of starting buffer (pyridine acetate, 0.2 M pyridine, pH 3.1) and 700 ml of limit buffer (pyridine acetate, 0.2 M pyridine, pH 5.0). Fractions of 2 ml were collected at a flow rate of 15 ml/hour. The fractions comprising the ^{3}H-containing peak were pooled and lyophilized. This procedure was repeated three times.

The (^{3}H-) NH_2-terminal octapeptide ($\beta^{A}Ic$T1) was further purified (in 3 batches) by ion exchange chromatography on cellulose phosphate P11 (Holmquist, 1964). The samples were dissolved in 2 ml of distilled water, the pH was lowered to 3 with 3 N HCl and the samples were applied to 0.9 cm x 100 cm columns of resin. The columns were developed at 15 ml/hour with a non-linear gradient using 200 ml of starting buffer (pyridine acetate, 0.125 M pyridine, pH 4.55) and 400 ml of limit buffer (pyridine acetate, 0.52 M pyridine, pH 5.5). Fractions of 2 ml were collected and monitored for radioactivity. The fractions containing tritium were pooled and lyophilized. Purity of the NH_2-terminal octapeptide ($\beta^{A}Ic$T1) was assessed by hydrolysis of a portion in 6 N HCl at 105°C in vacuo for 16 hours followed by amino acid analysis (Beckman 119C amino acid analyzer).

The peptide $\beta^{A}Ic$T1 was dissolved (1 mM) in a solution of 0.05 M NaCN, 0.002 M EDTA and 0.1 M ammonium acetate buffer pH 5.5 and digested for 16 hours at 39°C by the addition of papain (45 units/μmol peptide). The NH_2-terminal glycodipeptide (glycovalylhistidine) was isolated by ion exchange chromatography on a 0.8 x 46 cm column of AG 50W-X8 according to the procedure of Holmquist (1966), except that the glycopeptide was eluted at room temperature using a linear gradient (250 ml total volume) of pyridine acetate pH 5.28, where the pyridine concentration increased from 0.175 M to 0.290 M. . The purity of the glycodipeptide was determined by hydrolysis in 6 N HCl at 105°C in vacuo for 16 hours followed by amino acid analysis. Digestion of the glycovalylhistidine with protease VI was not successful although under similiar conditions complete digestion of valylhistidine was observed.

Preparation of adducts of valine and valylhistidine with hexoses. Adducts of various hexoses (D-glucose, D-mannose and D-galactose) with L-valine were synthesized according to the method of Dixon. Seven millimoles of ^{14}C-valine (specific activity 1.4 μCi/mmol) and 9 millimoles of hexose were stirred for 3 days at room temperature in 150 ml of pyridine:acetic acid, 1:1. The solutions were lyophilized and the residues dissolved in 180 ml of 1 M morpholine. A 25-molar excess of sodium borohydride was added to each reaction mixture and mixtures stirred for 3 hours at room temperature. The progress of the reduction was monitored by thin layer

chromatography on silica gel GF in methyl ethyl ketone:isobutanol:water: diethylamine (80:80:40:9). The compounds were visualized by spraying the plate with 0.5% $KMnO_4$ in 1 M NaOH. The R_f value of the unreduced adducts was 0.35 and those of the reduced materials are listed in Table XII. The reaction mixtures were lyophilized, dissolved in 10 ml of 0.5 M morpholine and applied to 2.5 x 50 cm columns of Sephadex G 10. The reduced adducts were eluted with 0.5 M morpholine and thus separated from the bulk of the salts and unreacted reagents. Fractions containing the glycitol valines (reduced adducts) were lyophilized and the oily residues were dissolved in 150 ml of distilled water. The solutions were made basic (pH 11-12) by the addition of piperidine and then applied to 4 x 50 cm columns of AG 1-X8 previously equilibrated with morpholine acetate buffer pH 8.6 (0.25 M morpholine). The adducts were eluted with this buffer in approximately 5 column volumes and lyophilized. The residual morpholine acetate was removed by repeated evaporations from pyridine on a rotary evaporator until constant weight was achieved. The products were recrystallized from ethanol:water (2:1) with an overall yield of 30%.

Glucose (275 µmol) was reacted with L-valyl-L-histidine (180 µmol) in 2 ml of pyridine:acetic acid (1:1) according to Dixon. The reaction mixture was lyophilized and the residue dissolved in 1.5 ml of 1 M morpholine containing 6.9 mmol of NaB^3H_4 (2×10^5 dpm/µmol). After 6 hours at room temperature the material was lyophilized and the unreacted $NaBH_4$ was quenched with 2 M acetic acid. The material was dried by rotary evaporation, dissolved in 7 ml of 1 M acetic acid and the pH adjusted to 2 with 6 N HCl. The sample was applied to a 0.9 x 100 cm column of AG 50W-X8. The column was developed with pyridine acetate buffers C (pH 4.21, 0.057 M pyridine) and D (pH 5.28, 0.11 M pyridine) according to Holmquist (1966), followed by a linear gradient (400 ml total volume) of pyridine acetate buffer pH 5.28, 0.175 M to 0.260 M pyridine. Fractions of 2.7 ml were collected at a flow rate of 10 ml per hour.

Analyses. All synthetic and natural glycopeptides were analyzed by thin layer chromatography on Silica gel GF using the solvent systems propanol:water (85:15) and methyl ethyl ketone:isobutanol:water:diethylamine (80:80:40:9). The plates were developed twice and sprayed with either

0.5% $KMnO_4$ in 1 N NaOH, Pauly reagent (Brenner), or ninhydrin (Brenner). Gas liquid chromatography was performed on the trimethylsilyl derivatives (Sweeley) of both the glycovalines and glycovalylhistidine methyl esters (formed in methanolic - 1.5 N HCl). Analysis was performed using a 4 mm x 6 foot OV-17 column (Supelco Company, Bellefonte, Pennsylvania) in a Packard 417 gas chromatograph. The gas flow rates were: carrier nitrogen, 45 ml/min; hydrogen flow to detector, 30 ml/min; air flow to detector, 230 ml/min. For analysis of the glycitol valine derivatives the instrument was programmed from an initial temperature of 175°C with a temperature increase of 0.1°/minute. The glycovalylhistidine derivatives were analyzed from an initial temperature of 145°C with a temperature increase of 0.1°/minute.

All compounds were analyzed at 220 MHz by proton magnetic resonance spectroscopy employing a Varian HR/NTC-220 proton magnetic resonance spectrometer having pulse Fourier transform and decoupling facilities. Two-hundred-fifty-six acquisitions were taken for the naturally-derived glycodipeptides using a 90° pulse. Solvent for the glycovalines was deuterium oxide; for the glycovalylhistidines deuterated pyridine acetate (12 mM pyridine, pD 6.04) was used. Chemical shifts of the glycitol valine derivatives are relative to TSP. Acetate was used as internal reference for the glycitolvalylhistidines. The chemical shift of acetate (1.92 ppm, in the pD range employed) was determined using TSP as an internal standard and the chemical shifts of the glycitol dipeptides are related to TSP in this manner.

C. Results

Isolation of glycodipeptides from hemoglobin A_{Ic}. The elution profile of hemoglobins $A_{Ia + Ib}$ and A_{Ic} isolated from the 15 x 50 cm column of Bio Rex 70 is shown in Fig. 14. Liters 8-15 were pooled and concentrated by chromatography on cellulose phosphate as described in Materials and Methods. A portion of this concentrated hemoglobin A_{Ic} solution was rechromatographed on Bio Rex 70 and demonstrated to be free of hemoglobin $A_{Ia + Ib}$ (Fig. 15). The small amount of hemoglobin A present is presumably

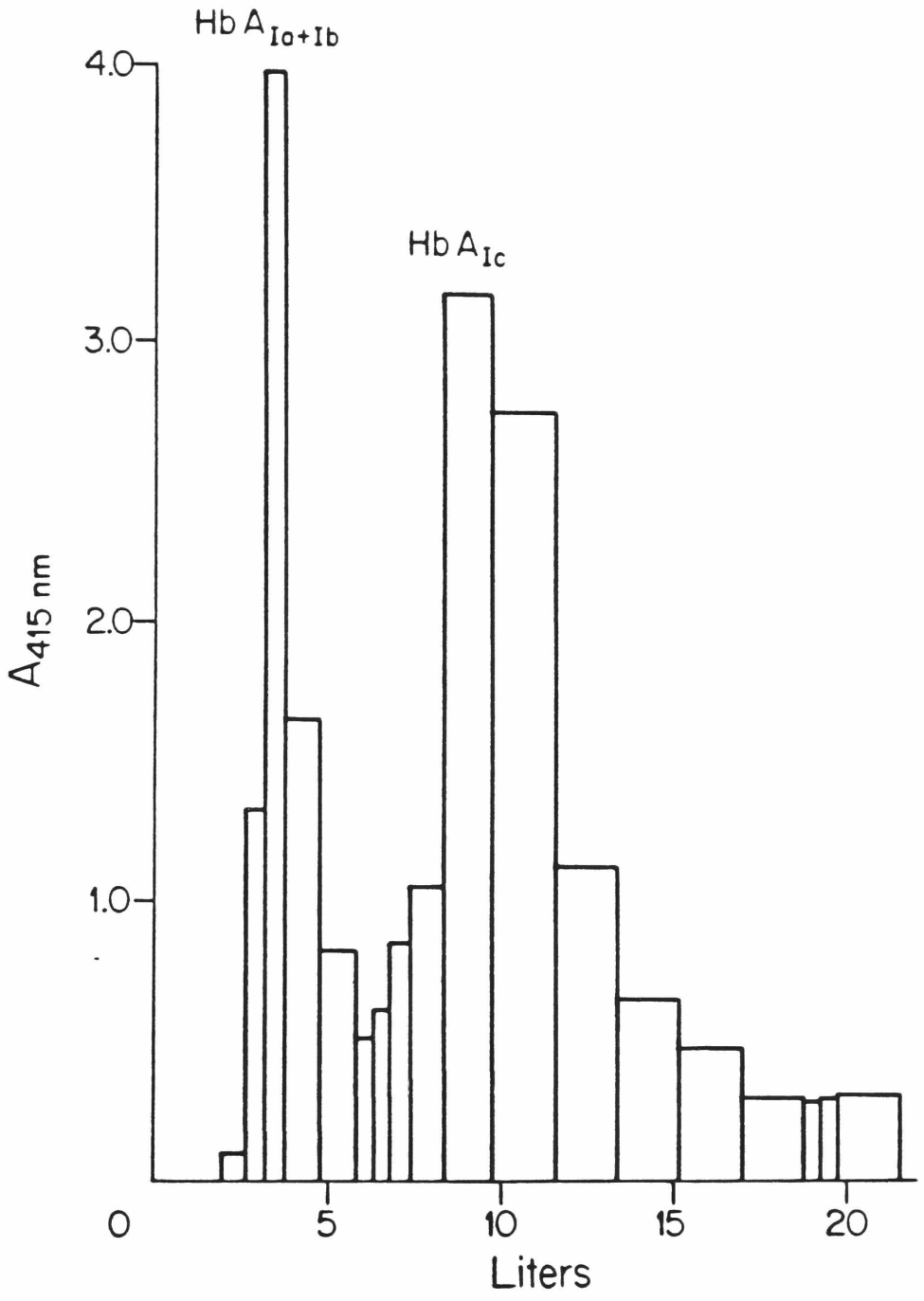


Fig. 14. Elution profile of human hemoglobins $A_{Ia + Ib}$ and A_{Ic} following ion exchange chromatography on Bio Rex 70. Seventy-five grams of carbonmonoxy hemoglobin in 1 liter of developer 6 were applied to a 15 cm x 50 cm column of Bio Rex 70 and eluted with developer 6 at a rate of 3 liters per hour. Hemoglobin $A_{Ia + Ib}$ was eluted in liters 2-6 and hemoglobin A_{Ic} in liters 8-15.

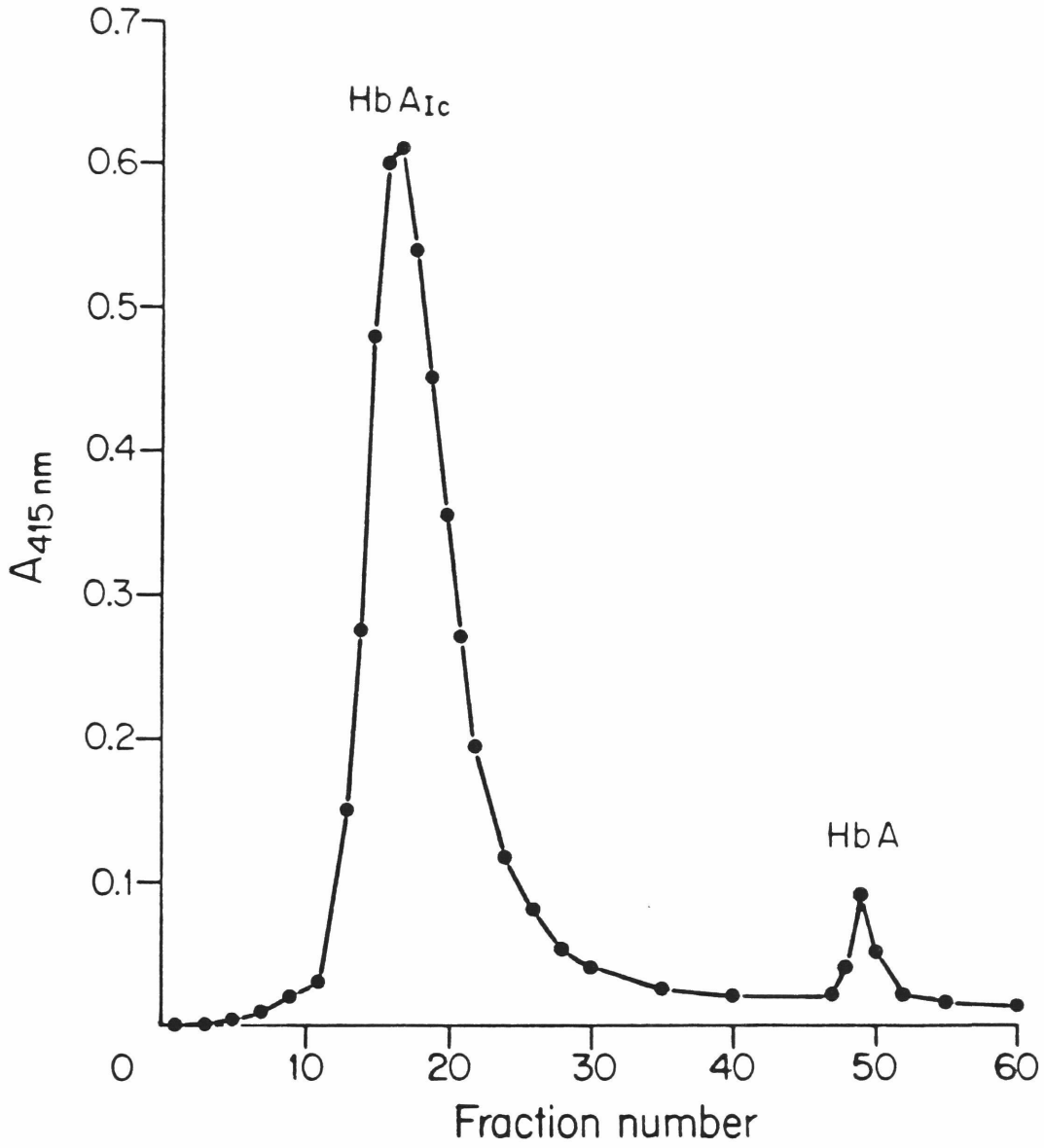


Fig. 15. Elution profile of purified human hemoglobin A_{Ic} following Bio Rex 70 chromatography. Hemoglobin A_{Ic} was isolated and purified in gram quantities as described in Materials and Methods. One milligram of this hemoglobin A_{Ic} preparation was applied to a 6 mm x 25 cm column of Bio Rex 70 and eluted with developer 6 (fractions 1-44) and buffer B (fractions 45-60). Fractions of 1.2 ml were collected at a flow rate of 10 ml/hour.

due to loss of the glycosyl group of hemoglobin A_{Ic} (hemoglobin A is not glycosylated).

The hemoglobin A_{Ic} was reduced with NaB^3H_4 and the β^{AIc} chains were isolated by chromatography on CM-cellulose as shown in Fig. 16. The ^3H-β^{AIc} chains were then digested with trypsin, and the ^3H-containing, amino terminal tryptic peptide was partially purified by ion exchange chromatography on AG 50W-X2, as shown in Fig. 17. Fractions 159-188 were pooled, lyophilized and rechromatographed on cellulose phosphate P11 (Fig. 18). Fractions 95-105 of this chromatogram were pooled and lyophilized. A portion of the peak was digested in 6 N HCl at 105°C for 16 hours in vacuo and its amino acid composition was determined (Table XI). The amino acid composition of the ^3H-containing peptide agrees well with the known composition of the amino-terminal octapeptide of β chains from hemoglobin A (β^AT1), except that a valine peak was not observed in the analysis. Instead, a tritium containing peak which was ninhydrin negative eluted prior to the position of aspartic acid and presumably was a substituted valine which is stable to acid hydrolysis. Thus, the tryptic peptide isolated is shown to be the purified NH_2-terminal modified octapeptide of β^{AIc} chains (β^{AIc}T1).

TABLE XI

Amino acid composition of the ^3H-containing tryptic peptide isolated from β^{AIc} chains and the known amino acid composition of the NH_2-terminal octapeptide of β^A (and β^{AIc}) chains (β^AT1)

	Relative moles	
Amino acid	Peptide isolated	β^AT1*
Valine	0.0	1.0
Histidine	0.9	1.0
Leucine	1.0	1.0
Threonine	1.0	1.0
Proline	1.1	1.0
Glutamic acid	2.1	2.0
Lysine	1.0	1.0

* (Holmquist, 1966)

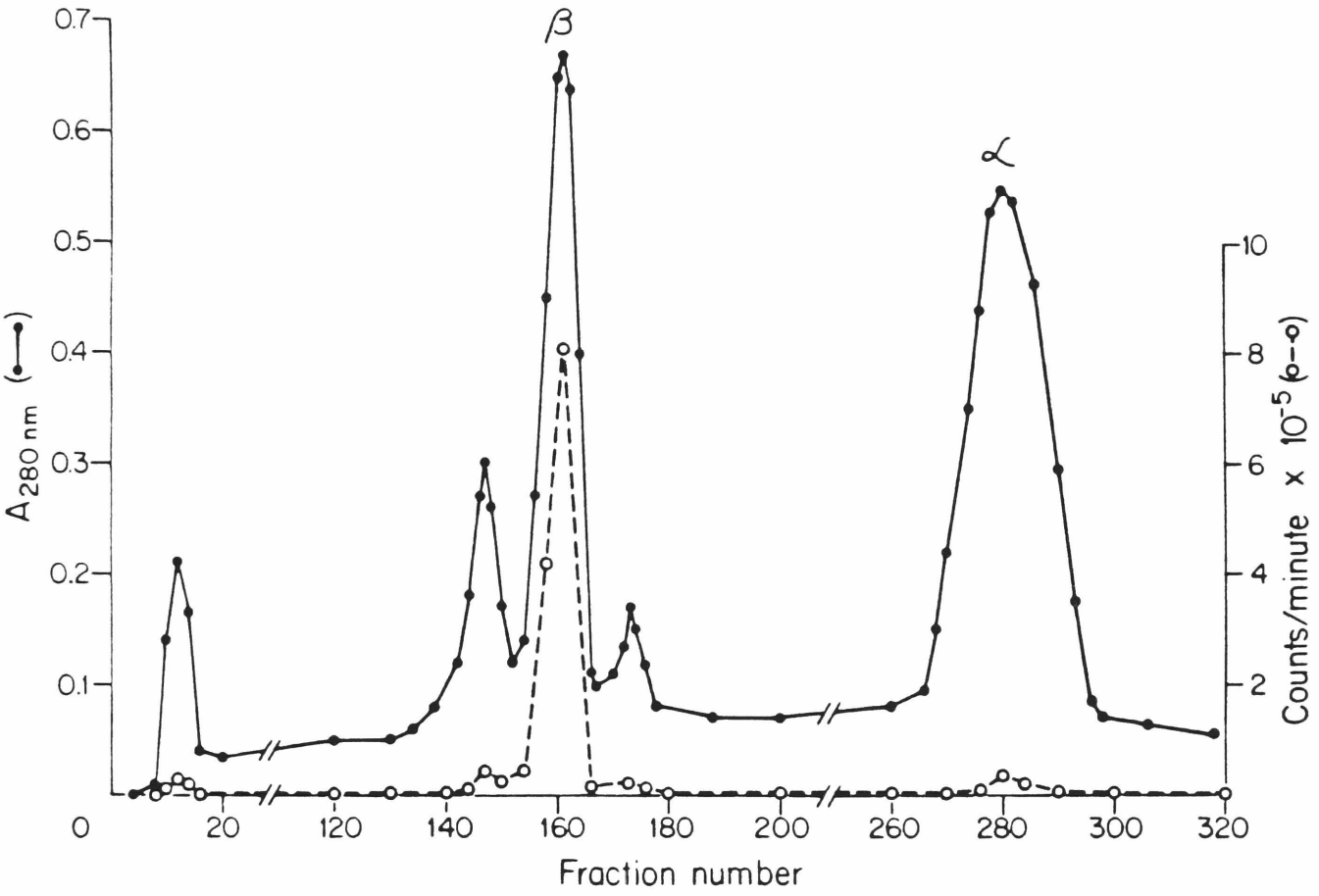


Fig. 16. Separation of α and β chains of NaB^3H_4-reduced human hemoglobin A_{Ic} by ion exchange chromatography on CM-cellulose. Six hundred milligrams of globin (prepared by extraction of heme into acetone-2% HCl) were applied to a 5 cm x 20 cm column of CM-cellulose and eluted with a sodium phosphate buffer gradient (0.005 M → 0.04 M) containing 8 M urea and 0.05 M β-mercaptoethanol, pH 6.8. Fractions of 16 ml were collected at a flow rate of 180 ml/hour and monitored both for absorbance at 280 nm (●—●) and radioactivity (o--o).

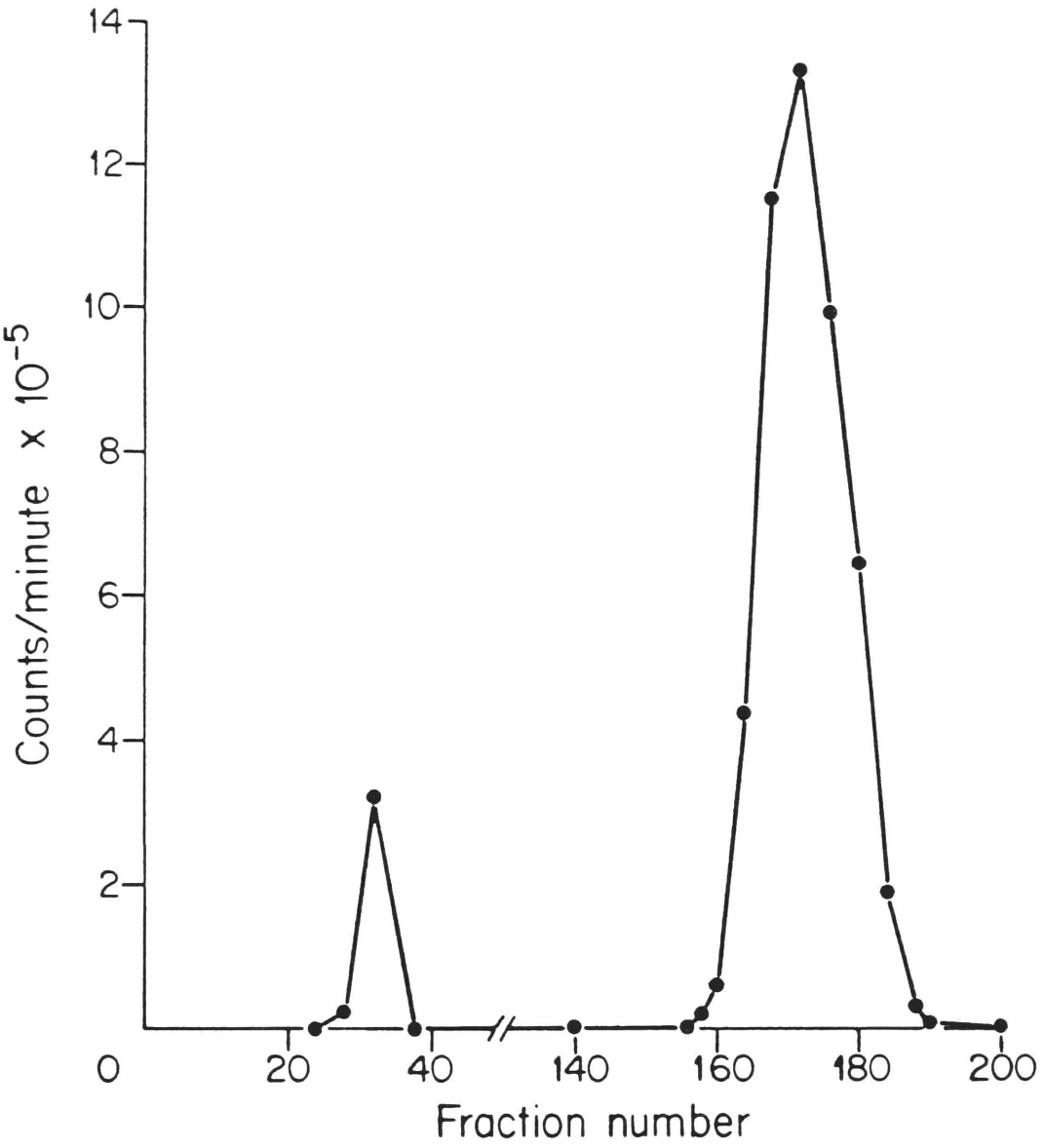


Fig. 17. Partial purification of the NH_2-terminal glyco-octapeptide of NaB^3H_4-reduced β^AIc chains (β^AIcT1) by ion exchange chromatography on AG 50W-X2. Reduced β^AIc chains were digested with trypsin. Ninety milligrams of the tryptic digest were applied to a 0.9 cm x 110 cm column of AG 50W-X2 and eluted with a pyridine acetate pH gradient (pH 3.1 → pH 5.0, 0.2 M pyridine). Fractions of 2 ml were collected at a flow rate of 15 ml/hour and monitored for radioactivity. Fractions 159-188 were pooled and further purified.

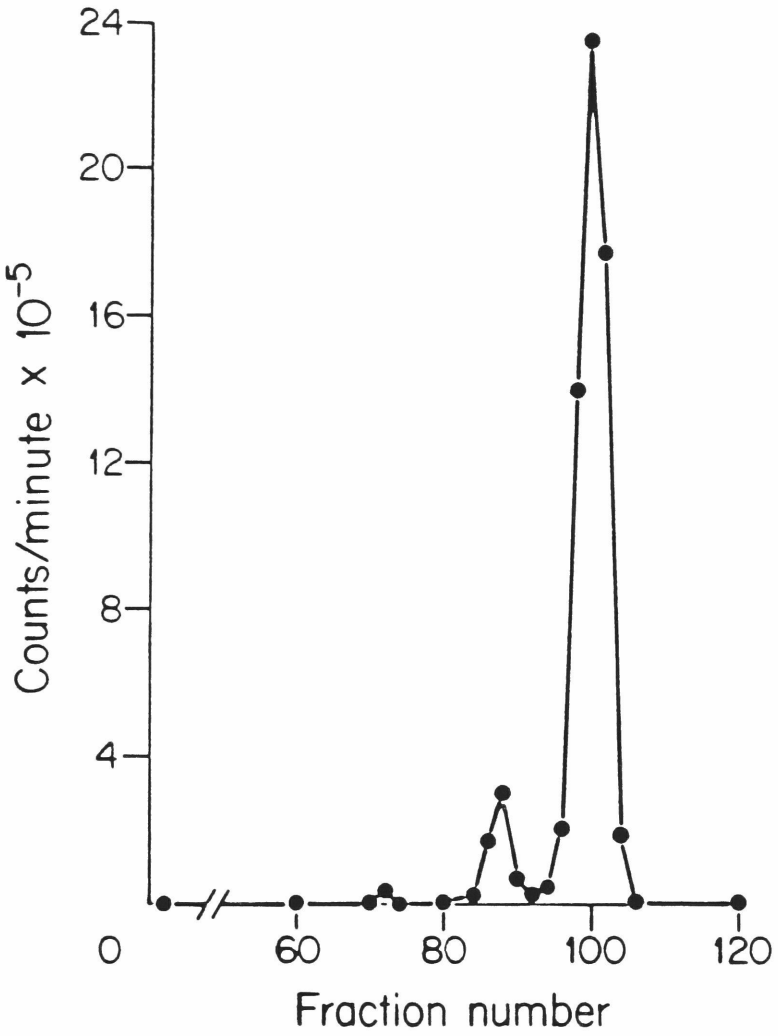


Fig. 18. Further purification of reduced $\beta^{A}Ic$T1 by ion exchange chromatography on cellulose phosphate P11. The partially purified peptide, obtained by chromatography on AG 50W-X2, was applied to a 0.9 cm x 100 cm column of cellulose phosphate P11. The column was developed at 15 ml/hour with a pyridine acetate gradient (0.125 M pyridine, pH 4.55 → 0.52 M pyridine, pH 5.50). Fractions of 2 ml were collected. Fractions 95-105 were pooled and analyzed for purity of reduced $\beta^{A}Ic$T1 as described in Materials and Methods.

Purified $\beta^{A_{Ic}}T1$ was digested with papain and subjected to chromatography on AG 50W-X8. The elution profile of this chromatogram is shown in Fig. 19. Two radioactive peaks, in approximately a 2:1 ratio, were resolved. Amino acid analysis of acid hydrolyzed portions of both of these peaks gave histidine as the only amino acid, in agreement with the structure glycovalylhistidine for both naturally derived products (R_1-Val-His and R_2-Val-His). Four micromoles of R_1-Val-His (fractions 161-168) and two micromoles of R_2-Val-His (fractions 172-178) were isolated from a total of 2 grams of hemoglobin A_{Ic} (62.5 μmol β chains), for an overall yield of 10%.

Preparation of adducts of valine and valylhistidine with hexoses. Glucose, mannose and galactose were each reacted with ^{14}C-valine in pyridine:acetic acid (1:1) and reduced with $NaBH_4$. Each reaction mixture was applied to a Sephadex G 10 column (Fig. 20) to separate the reaction products from salts and unreacted starting materials. Fractions containing the (^{14}C-) reduced adducts were pooled, lyophilized, and chromatographed on AG 1-X8 (Fig. 21). The major radioactive peak of each column was lyophilized and the reduced adducts were crystallized from ethanol:water (2:1). The products were analyzed by thin layer chromatography (TLC), proton magnetic resonance (pmr) spectroscopy, and gas chromatography (GC) of their trimethylsilyl (TMS) derivatives. Each hexose resulted in the formation of 2 reduced adducts with valine, as shown by the TLC and GC data in Table XII. The major product (75%) of the glucose and valine reaction is identical to the major product of the mannose and valine reaction by TLC and GC. The minor products of these two reactions are also identical. The two products of the galactose and valine reaction are clearly different from those of the glucose and mannose reactions.

The pmr spectra confirmed this data. The pmr spectrum of the major product obtained from the reaction of glucose with valine and the assignment of chemical shifts is shown in Fig. 22. Irradiation of the H_β multiplet of valine at 2.26 ppm caused the doublet at 3.55-3.57 ppm to merge into a singlet whereas none of the sugar resonances were affected, thereby establishing the chemical shift of the proton attached to the valine α

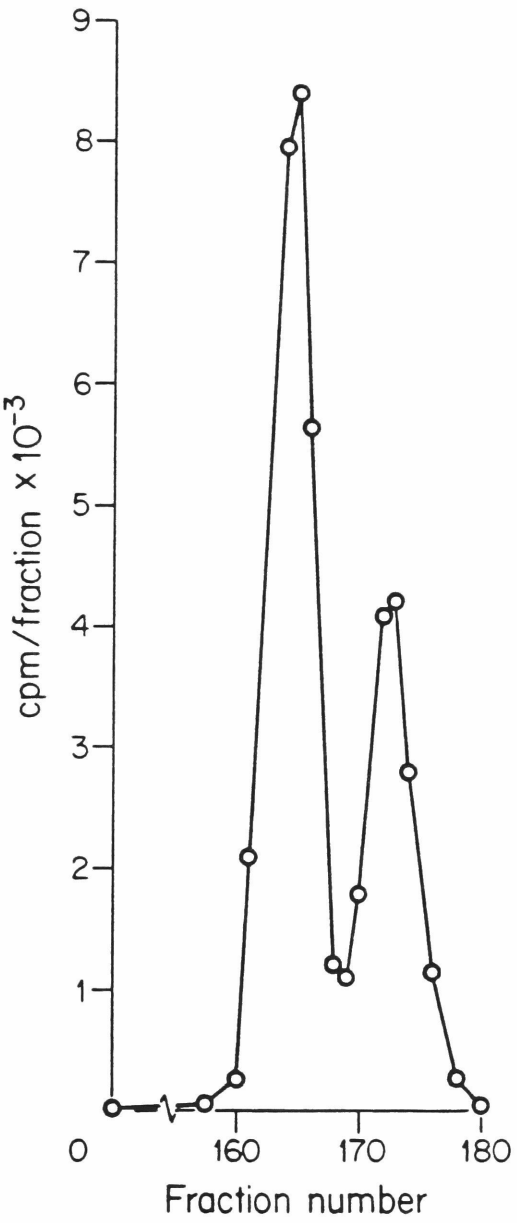


Fig. 19. Elution profile of papain-digested reduced $\beta^{AIc}T1$ on AG 50W-X8. The papain-digested material was applied to a 0.8 cm x 46 cm column of AG 50W-X8. The radioactive NH_2-terminal glycopeptide was eluted with a pyridine acetate gradient (0.175 M pyridine → 0.290 M pyridine, pH 5.28). Two such glycopeptides were recovered (R_1-Val-His, fractions 161-168 and R_2-Val-His, fractions 172-178) and analyzed for purity as described in Materials and Methods.

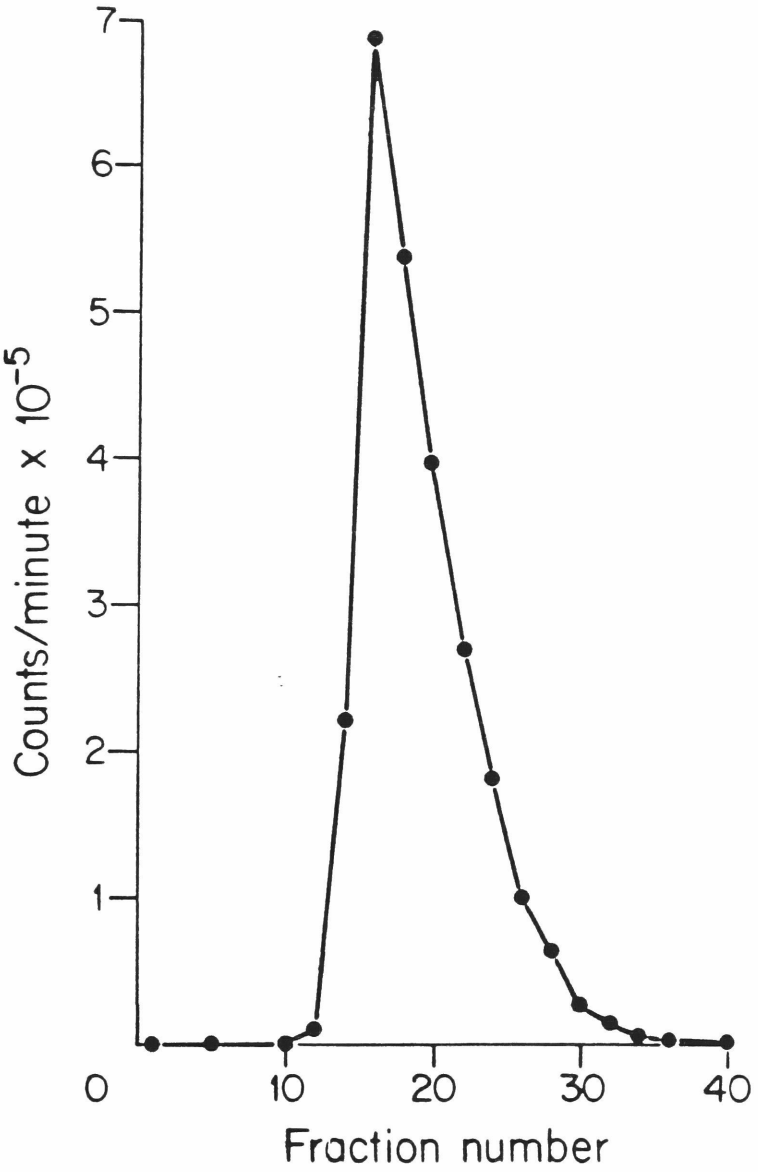


Fig. 20. Partial purification of $NaBH_4$-reduced adduct of glucose and ^{14}C-valine by gel filtration on Sephadex G10. The reaction mixture (See Materials and Methods for details) was applied to a 2.5 cm x 50 cm column of Sephadex G10 and eluted with 0.5 M morpholine. Fractions of 5 ml were collected at a flow rate of 40 ml/hour. Fractions 13-25 were pooled and the reduced adduct purified further.

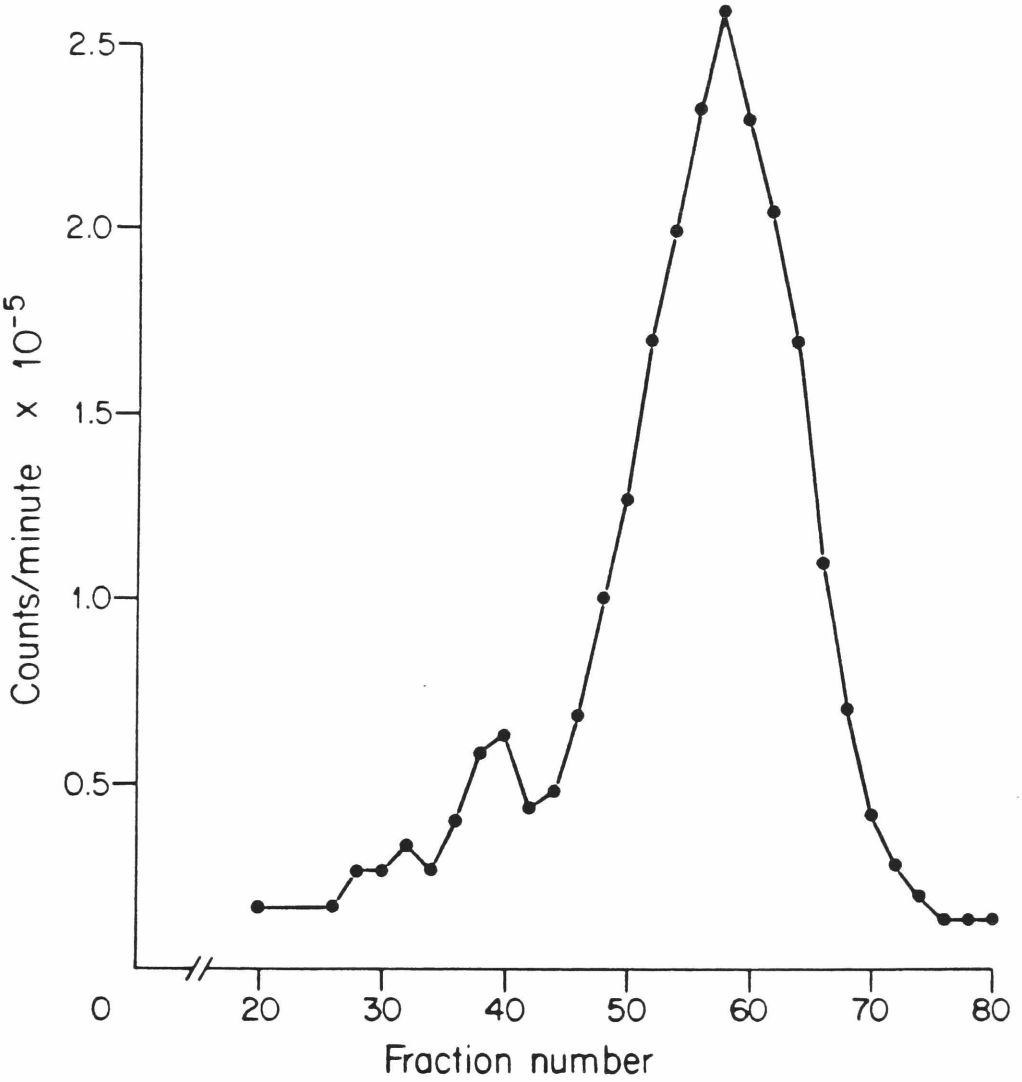


Fig. 21. Elution profile of the $NaBH_4$-reduced adduct of glucose and ^{14}C-valine on AG 1-X8. The peak obtained by Sephadex gel filtration was applied to a 4 cm x 50 cm column of AG 1-X8 and eluted with 0.25 M morpholine acetate buffer, pH 8.6. Fractions of 9 ml were collected at a flow rate of 50 ml/hour. Fractions 47-70 were pooled and lyophilized, and the reduced adduct recrystallized from ethanol:water (2:1). The peak centered at fraction 40 is valine.

TABLE XII

R_f values of synthetic and natural glycopeptides and gas chromatographic data of their trimethylsilyl derivatives

Reactants	Products	R_f System 1*	R_f System 2†	GC Retention Time (min)**
Glucose + valine	major	0.20	0.29	33.5
	minor	0.20	0.36	31.6
Mannose + valine	major	0.20	0.29	33.5
	minor	0.20	0.36	31.6
Galactose + valine	major	0.24	0.32	36.2
	minor	0.24	0.40	33.9
Glucose + Val-His	peak 1 ††	0.09	0.23	----
	peak 2 ††	0.24	0.21	23.0
	peak 3 ††	0.21	0.17	24.1
----------	R_1-Val-His ††∞	0.24	0.21	23.0
	R_2-Val-His ††∞	0.21	0.17	24.1

* methyl ethyl ketone: iso-butanol: water: diethylamine (80:80:40:9); two developments

† propanol: water (85:15); two developments

** See methods for program conditions.

†† Gas chromatography was performed on TMS derivatives of the methyl esters of these compounds.

∞ Naturally-derived products isolated from hemoglobin A_{Ic}.

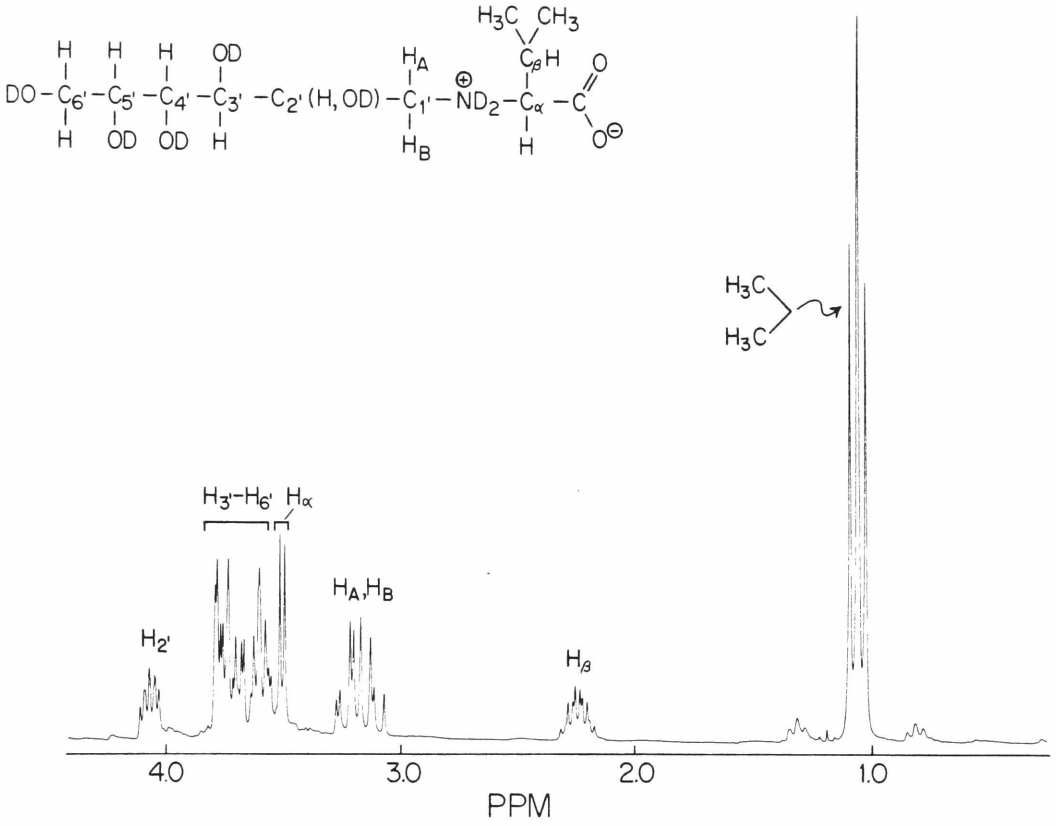


Fig. 22. 220 MHz pmr spectrum of the major product obtained from the reaction of glucose with valine, followed by $NaBH_4$ reduction. The spectrum is recorded in D_2O using TSP as an internal standard. Assignment of chemical shifts is based upon pmr spectra of valine and glucitol and spin decoupling studies described in Results.

carbon (H_α). Similarly, irradiation of the multiplet at 4.1 ppm ($H_{2'}$) converted the two proton octet (AB portion of ABX system) centered at 3.22 ppm into a quartet, establishing the chemical shifts of the H_A, H_B and H_2, protons. The major product (75%) obtained from the reaction of mannose and valine had the identical pmr spectrum (not shown). The minor products of both the glucose and mannose reactions were not isolated free of the major product, but both mixtures had identical pmr spectra. The pmr spectra of the two galactitol valine adducts differed significantly from each other and from the glucitol valine (and mannitol valine) spectra. These data are consistent with initial formation of a hexose-valine Schiff base or aldosylamine linkage followed by an Amadori rearrangement (Gottschalk) prior to borohydride reduction (Fig. 23). The Amadori rearrangement product of the glucose (and mannose) reaction, prior to borohydride reduction, is 1-deoxy, 1-(N-valyl) fructose.

Glucose was reacted with valylhistidine, reduced with NaB^3H_4 and applied to an AG 50W-X8 column. The elution profile is shown in Fig. 24. Peak 1 (fractions 110-119) was eluted with buffer D of Holmquist (1966). Peaks 2 (fractions 258-267) and 3 (fractions 275-281) were subsequently eluted with the pyridine acetate gradient. Analysis by thin layer chromatography and gas liquid chromatography indicated glycitolvalylhistidine peaks 2 and 3 were identical to the naturally derived materials, R_1-Val-His and R_2-Val-His respectively (Table XII). Proton magnetic resonance spectroscopy of these derivatives confirmed this assignment. The pmr spectra of the naturally derived R_1-Val-His and R_2-Val-His are compared in Fig. 25. Assignment of resonances is based on the chemical shift data of valylhistidine and glycitol valine (see above). Small but distinct differences in the splitting patterns of the sugar protons and the two AB portions of the spectra were observed. Upon expansion of these regions, these differences became clearer (Fig. 26). Comparison of the expanded spectra of the naturally derived R_1-Val-His and R_2-Val-His to those of the synthetically derived glycitol-Val-His peaks 2 and 3 respectively, shows them to be identical (Fig. 26).

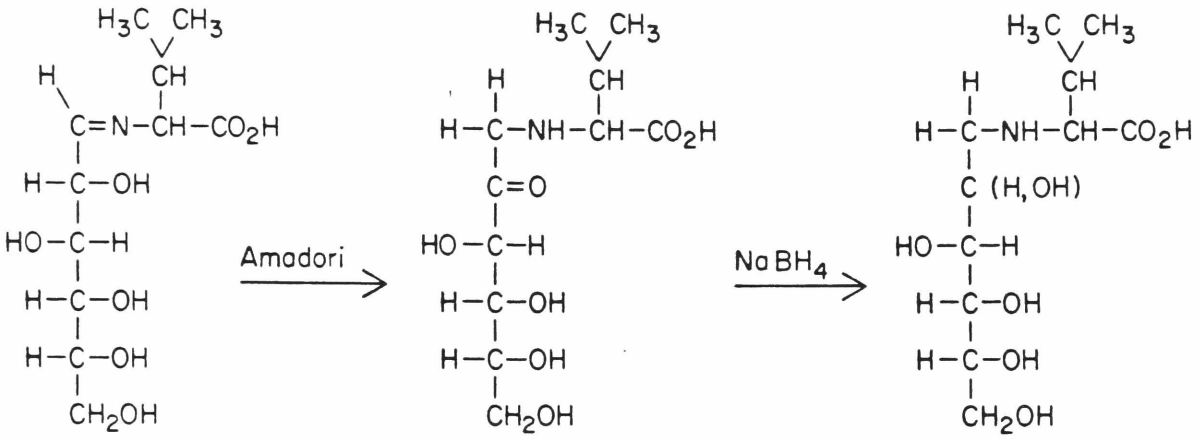


Fig. 23. The Schiff base adduct between valine and glucose undergoes an Amadori rearrangement to form 1-deoxy, 1-(N-valyl) fructose. Borohydride reduction of this compound gives a mixture of the $C_{2'}$ isomeric glycitol valines.

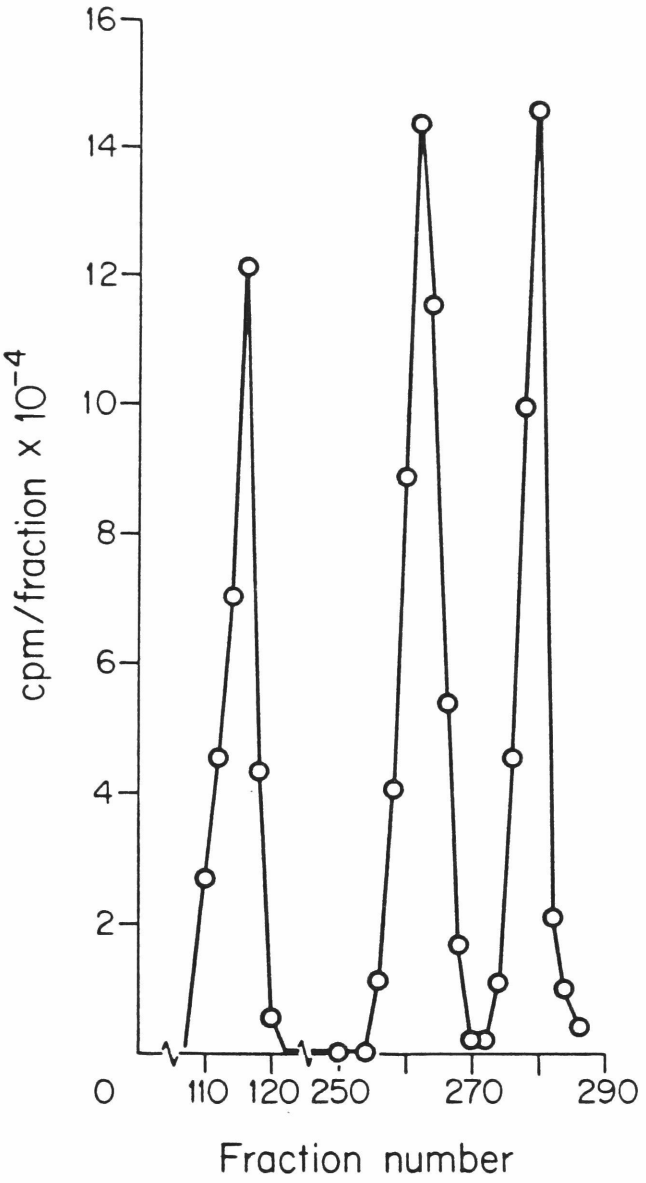


Fig. 24. AG 50W-X8 chromatography elution profile of the NaB^3H_4-reduced adduct formed from the reaction of glucose with valylhistidine. Glucose (275 µmol) was reacted with Val-His (180 µmol) and the resulting mixture was then reduced with NaB^3H_4 (2 x 10^5 dpm/µmol) as described in Materials and Methods. The reaction mixture was dissolved in 7 ml of 1 N acetic acid, and loaded onto a 0.9 x 100 cm column. The materials were eluted as described in Materials and Methods. Fractions of 2.7 ml were collected at a flow rate of 10 ml/hour.

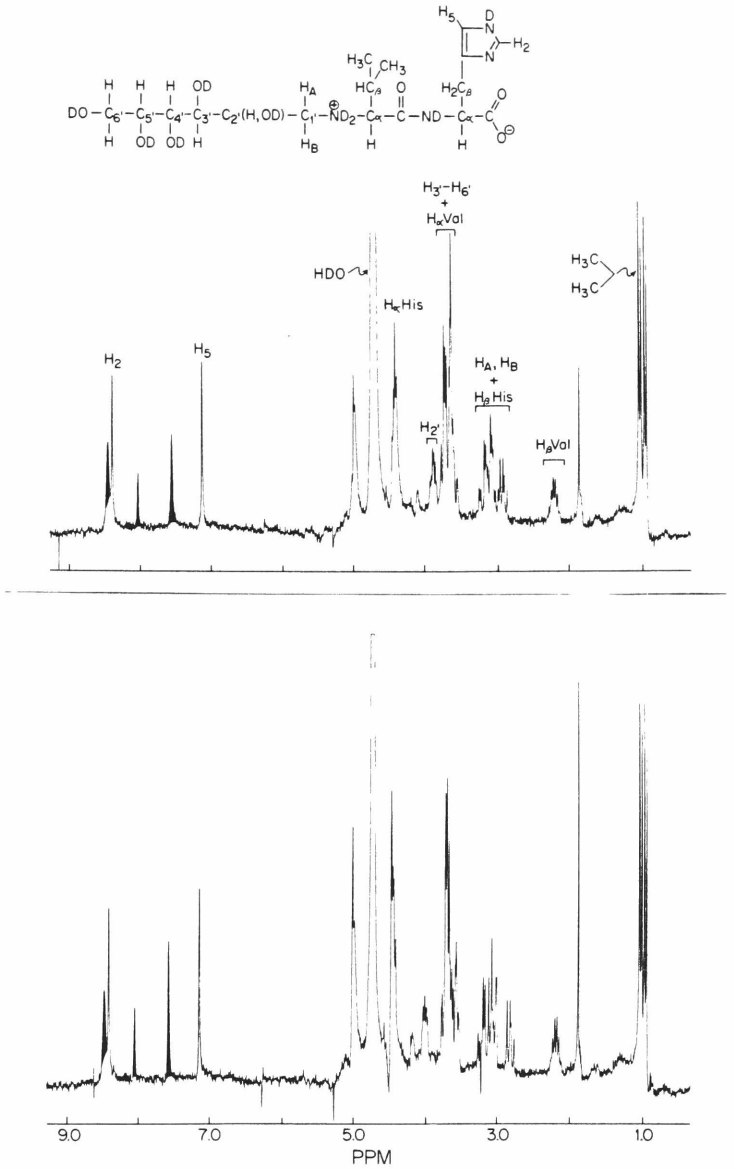


Fig. 25. 220 MHz pmr spectra of the naturally derived R_1-Val-His (upper) and R_2-Val-His (lower). Spectra were recorded in deuterated pyridine acetate buffer, pD = 6.04. Concentrations were about 1 mM each with 256 accumulations taken. The shaded peaks correspond to pyridine and the peak at 1.92 ppm corresponds to acetate, used as an internal reference. Spinning side bands of HDO are evident in both spectra. Assignment of resonances is based upon the pmr spectra of valylhistidine and glycitol valine (Fig. 22).

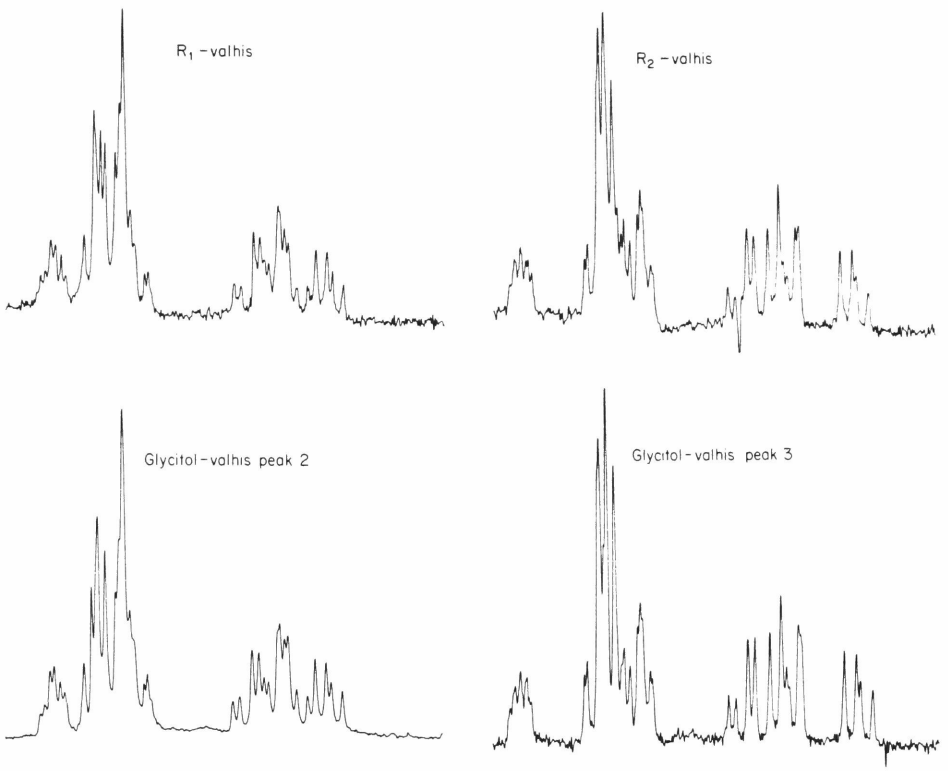


Fig. 26. Comparison of the expanded 220 MHz pmr spectra of the naturally derived and synthetic glycodipeptides (starting in the upper left and going clockwise: R_1-Val-His, R_2-Val-His, glycitol-Val-His peak 3 and glycitol-Val-His peak 2). The region expanded is from 2.5 to 4.1 ppm.

In analogy to the glucose-valine and mannose valine adducts, the two borohydride-reduced synthetic glucose plus valylhistidine adducts must be glucitol-Val-His and mannitol-Val-His. The compound from which they derive, prior to borohydride reduction, is 1-deoxy, I-(N-valylhistidinyl) fructose, which results from an Amadori rearrangement of the glucose adduct. Thus, by TLC, GC and pmr spectroscopy, we have shown the NH_2-terminus of $\beta^{A_{Ic}}$ chains to be 1-deoxy, 1-(N-valyl) fructose.

The adduct of glucose plus valylhistidine found in peak 1 gave a pmr spectrum qualitatively similar to the above derivatives; the major difference being that by integration the substance contained 2 moles of hexose per mole of valylhistidine. The material is tentatively assigned as containing two hexoses attached at the NH_2 group of valine (Gottschalk).

D. Discussion

Holmquist (1966) has shown the structure of hemoglobin A_{Ic} to be identical to that of hemoglobin A except for a low molecular weight borohydride reducible moiety on the NH_2-terminal valine of the $\beta^{A_{Ic}}$ chain. It has been proposed (Bunn, 1975) that a glucose molecule is attached to the valine via a Schiff base which subsequently undergoes an Amadori rearrangement, but a definitive assignment of the structure of the glycosyl group of hemoglobin A_{Ic} has not been made.

In this study hemoglobin A_{Ic} was isolated free of hemoglobins A_{Ia} and A_{Ib} (which is also glycosylated [Stevens]) by cellulose phosphate ion exchange chromatography. The hemoglobin A_{Ic} was then reduced with sodium borohydride - ^3H and the α and β chains separated. The β chains were digested with trypsin, and the NH_2-terminal glyco-octapeptide isolated was then further digested with papain. Two chromatographically distinct NH_2-terminal glycovalylhistidine peptides (R_1-Val-His and R_2-Val-His) were then isolated. The mild conditions used in the isolation of R_1-Val-His and R_2-Val-His assure that the R groups were not modified (except for borohydride reduction) prior to identification. Purity of the NH_2-terminal glycopeptides isolated was assessed after each enzymatic digestion. The NH_2-terminal glyco-octapeptide was shown by amino acid analysis to be

derived solely from hemoglobin (Table XI). Similarly, the amino acid sequence of the glycodipeptides isolated was valylhistidine - the sequence at the NH_2-terminus of hemoglobin β chains. Thus, non-heme proteins that are known to contaminate hemoglobin A_{Ic} isolated by ion exchange chromatography on Bio-Rex 70 (Bunn, 1976) could not have been the source of R_1-Val-His and R_2-Val-His.

The structures of the naturally derived, pure R_1-Val-His and R_2-Val-His were compared with model compounds. Adducts of glucose, mannose and galactose with valine were synthesized, reduced with $NaBH_4$ and purified. Each hexose resulted in the formation of two reduced adducts. The fact that two reduced adducts were obtained and that the glucose and mannose adducts were identical is consistent with intial information of a hexose valine Schiff base or aldosylamine linkage which undergoes an Amadori rearrangement (Gottschalk) prior to reduction. The Amadori rearrangement product formed from the adducts of both glucose and mannose with valine is 1-deoxy, 1-(N-valyl) fructose. Subsequent reduction with $NaBH_4$ is not stereospecific and therefore generates 2 C_2, isomeric products (Fig. 23). In a similar fashion the reaction of glucose with valylhistidine was found to yield, after borohydride reduction, both glucitol and mannitol valylhistidines. These products were separated by ion exchange chromatography and shown to be identical by gas liquid chromatography, thin layer chromatography (Table XII), and pmr spectroscopy (Fig. 26) to R_1-Val-His and R_2-Val-His obtained from hemoglobin A_{Ic}. Thus we conclude that hemoglobin A_{Ic} has, as the NH_2-terminus of the β chain, 1-deoxy, 1-(N-valyl) fructose.

Assignment of the configuration at C_2, of R_1-Val-His and R_2-Val-His has not been attempted although it would be of general interest. However, in situ or <u>in vivo</u>, the material exists in a keto or hemiacetal form and only subsequent to borohydride reduction is the asymmetric C_2, center generated. Of greater interest would be the configuration of the carbohydrate in situ, i.e., an open chain or furanose or pyranose ring.

V. CONCLUSION

The aim of these investigations has been to increase our understanding of the significance of increased hemoglobin A_{Ic} concentrations in diabetes mellitus. Hemoglobin A_{Ic} is a glycosylated hemoprotein that comprises 3-5 per cent of the total hemoglobin in non-diabetic humans and 1.5-2 per cent in wild-type mice. Its concentration is elevated approximately two-fold in diabetic humans and mice.

The studies in the mouse reported here have demonstrated increased hemoglobin A_{Ic} to be a marker for the diabetic phenotype regardless of the cause of diabetes (genetic defect, environmental insult). Hyperglycemia precedes by 3 to 4 weeks the increase in hemoglobin A_{Ic}. Hemoglobin A_{Ic} is made as a post-synthetic modification of hemoglobin A at a constant slow rate throughout the life of a red cell.

In diabetic humans hemoglobin A_{Ic} concentration correlates with the severity of disease, as assessed by response to an oral glucose tolerance test. Changes in quality of diabetic control are followed, after a 3 to 4 week delay, by proportionate changes in hemoglobin A_{Ic} concentration. Hemoglobin A_{Ic} concentration appears to best reflect the mean blood glucose concentration for the 3 to 4 weeks prior to the measurement. This is a result of the slow rate of hemoglobin A_{Ic} synthesis, so that changes in rate of synthesis must be maintained for many days before the total hemoglobin A_{Ic} concentration is significantly altered. Thus, infrequent hemoglobin A_{Ic} measurements are uniquely capable of assessing the quality of long-term diabetic control.

The structure of hemoglobin A_{Ic} is identical to that of hemoglobin A, with the addition of 1-deoxy fructose attached at the amino terminus of the β chains. The origin of this unusual hexose is apparently glucose, which forms a Schiff base adduct with the amino-terminal valine and subsequently undergoes an Amadori rearrangement to yield the 1-deoxy fructose product. Stevens has shown that, in vitro, hemoglobin A and glucose do not readily form an adduct. However, glucose-6-phosphate does react with the amino terminus of the β chains of hemoglobin A to form an adduct which has the same pI as hemoglobin A_{Ib}. It is conceivable that, in vivo,

hemoglobin A_{Ib} is then dephosphorylated to yield hemoglobin A_{Ic}. Apparently, the phosphate group is necessary to orient and stabilize the glucose moiety in the DPG pocket of hemoglobin to allow adduct formation.

These data point to a dual role for hemoglobin A_{Ic} measurement in the management of diabetic patients and research into the basic disease process. The stability of hemoglobin A_{Ic} concentration to short-term fluctuations in blood glucose levels makes its quantification an excellent means of assessing the presence of diabetes and of monitoring the degree of long-term diabetic control. Blood sugar determinations are not sufficiently reproducible to allow accurate assessment of control and are not sensitive enough to diagnose many cases of diabetes. Glucose tolerance tests are too time-consuming and expensive to be of general use in this regard. A rapid assay for hemoglobin A_{Ic} would be useful in screening large populations for diabetes mellitus and would permit the evaluation of diabetic control on an outpatient basis in a more objective and accurate fashion than is now possible. In addition, serial hemoglobin A_{Ic} measurements on an out-patient basis should make it possible to readdress the long debated question of whether control of glucose metabolism influences the development of sequelae.

The pathogenesis of the sequelae of diabetes remains one of the major unsolved mysteries of this disease. Perhaps the increased glycosylation of hemoglobin is a reflection of a mechanism that also accounts for the clinical complications of diabetes. That is, diabetes might be a disease of generalized abnormal protein glycosylation. Just as hemoglobin can be glycosylated in excess in diabetes to form increased hemoglobin A_{Ic}, other proteins might be similarly modified by this disease process. The abnormal glycosylation of these proteins might result in altered enzymatic activity, solubility, etc., and thus lead to the sequelae of diabetes. (The glycosylation of hemoglobin does result in functional changes, e.g., an increase in oxygen affinity [Bunn, 1970]).

This hypothesis leads to an interesting prediction. Even if the patient is hyperglycemic, a cell that <u>requires</u> insulin for glucose entry will not have an elevated intracellular glucose concentration in the

diabetic state. Only cells that are freely permeable to glucose in the absence of insulin will contain excess glucose, and it is therefore in these cells that we would expect increased protein glycosylation and consequent functional impairment.

As Spiro (1976) was first to point out, the tissues that are most commonly affected by diabetes - lens, nerve, glomerulus, blood cells and aorta, for instance - are insulin-independent for glucose uptake. It will be necessary to investigate the structure and carbohydrate content of proteins in these tissues in poorly controlled and well controlled diabetics to determine whether abnormal protein glycosylation can indeed account for the sequelae of this disease.

If abnormal protein glycosylation is a general phenomenon in diabetes, the chemistry of the modification will probably vary with the particular protein and tissue involved.

The postsynthetic modification of the β chain of hemoglobin A is highly specific and is probably contingent upon the relatively low pK of the amino terminus of this protein (Garner) as well as the peculiar affinity of organic phosphates (e.g., glucose-6-phosphate) for the DPG pocket of hemoglobin. Other proteins may form glycosylated Schiff base adducts at amino groups which similarly possesss the right combination of pK_a and steric factors. Amino terminal residues and basic amino acid side chains would both be candidates for such a process.

The Schiff base-Amadori rearrangement class of adduct discussed above probably would form non-enzymatically. However, it is also possible that enzymatic protein glycosylations would increase in the hyperglycemic state. Studies with genetic and chemically-induced diabetic mice (Spiro, 1971; Khalifa; Velasco) have demonstrated increased activities of renal cortical lysyl hydroxylase and glucosyl transferase, two enzymes important in the glycosylation of basement membrane collagen. Although controversial, Beisswenger has found diabetic glomerular basement membrane to have an increased percentage glucosylgalactosyl-hydroxylysine residues.

The deposition of glycogen has been observed in the renal tubules of uncontrolled human diabetics (Ritchie) and alloxan diabetic rats (Robbins, 1950). In the latter the severity of the process reflects the degree of hyperglycemia and can be reversed by lowering the blood glucose level. An elevated glucosamine content of vitreous humor glycoproteins has been found in diabetic rabbits (Walker, 1968) and this defect also corrects toward normal with control.

These glycosylation reactions are all clearly a function of blood (and intracellular) glucose concentration. While the chemical reactions themselves are all probably irreversible, the half life of the particular protein and tissue involved would determine the reversibility of the consequent lesion. The relatively rapid turnover of blood cells accounts for the reversibility of diabetic sequelae in these cells (Peterson). Basement membrane glycoproteins turn over at an exceedingly slow rate (Spiro, 1976) and therefore a substantial buildup of glycosyl groups in this matrix would take longer to occur but would also be more difficult to reverse. In all cases, normalization of blood glucose levels would be necessary for the prevention and/or regression of the lesions of diabetes.

Thus, studies of the glycosylation of hemoglobin might provide a unifying model for the development of the sequelae of diabetes as well as provide a clinical tool for monitoring diabetic control. Hopefully, clinical measurement of hemoglobin A_{Ic} will allow the patient to achieve better diabetic control and thus decrease the incidence and severity of the sequelae.

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