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GLYCOGENOSIS TYPE III IN THE DOG

By

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ČEH, LOVRO, JENS GABRIEL HAUGE, ROLF SVENKERUD and ANDERS STRANDE: *Glycogenosis type III in the dog.* Acta vet. scand. 1976, *17*, 210—222. — Enzyme and glycogen structure studies have been carried out on tissues of a glycogenotic dog, the clinical and pathological characteristics of which are reported in the accompanying paper. Liver glucose-6-phosphatase, leukocyte and liver acid maltase, and liver and skeletal muscle glycogen phosphorylase all appeared largely unaffected. The activity of the muscle and liver debranching enzyme (amylo-1,6-glucosidase), determined by two independent assay methods, was, however, reduced to between 0 and 7 % of normal activity. Glycogen structure studies with phosphorylase or iodine spectra revealed that the abnormally large amounts of glycogen found in liver and skeletal muscle had abnormally short branches, as would be expected for a deficiency of debranching enzyme. It is thus clear that the dog had suffered from the equivalent of Cori's disease (limit dextrinosis, type III glycogen storage disease). Preliminary data indicate that it may be possible to identify heterozygotes based on a study of the debranching enzyme of leukocytes.

dog; glycogenosis; Cori's disease; enzymes.

Hereditary glycogen storage disease is well known in human medicine, with eight main types clearly distinguished (*Howell* 1972). One would expect to find equivalent metabolic disturbances in animals, and some recent reports indicate that this indeed is the case. *Bardens* (1966) reported cases of glycogenosis in dogs which, on the basis of glucagon tolerance tests, were diagnosed as type I, II and III, the majority being of type II (Pompe's disease). *Mostafa* (1970) presented a case of glycogenosis in the dog where the histological findings suggested a case of type II. The occurrence of type II glycogenosis in cats as well is suggested by the electronmicroscopic study of *Sandström et al.* (1969). *Rafiquzzaman et al.* (1976) in the accompanying report, describe

four cases of glycogenosis in German Shepherd dogs, which on the basis of clinical and pathological findings fail to conform fully to any of the human types.

Because of the many possible types of glycogen storage disease, definite classification is seldom possible without studies of glycogen structure and enzymes from the affected tissues. We report here such studies for one of the German Shepherd dogs described by *Rafiquzzaman et al.*

MATERIAL AND METHODS

Animals

The tissues studied were taken from a German Shepherd dog designated "case 4" in the accompanying paper (*Rafiquzzaman et al.* 1976). Pieces of tissue were rapidly removed, frozen in liquid nitrogen and stored at —70°C. Blood samples were obtained from this dog, from three of the parents of the glycogenotic dogs, as well as from three control dogs.

Preparation of cells, fractions and compounds

a) *Liver fractions for α-1,4-glucosidase assay.* Twenty % homogenates in 0.25 M mannitol were prepared by grinding in a Potter-Elvehjem homogenizer. Debris was removed by centrifuging 10 min. at 2500 r.p.m. The mitochondrial + lysosomal fraction was then obtained by centrifuging 14,000 r.p.m. for 10 min. Microsomes and particle-free supernatant were obtained by centrifuging the supernatant from the last step 50,000 r.p.m. for 60 min. Fractions containing particles were assayed in the presence of 0.1 % Triton X-100.

b) *Liver and muscle samples for enzyme assays and glycogen determination.* Ten % liver homogenates in distilled water were prepared with a Potter-Elvehjem homogenizer, distributed in small portions and stored at —70°C. When preparing a corresponding skeletal muscle homogenate, the tissue was first cut into small pieces, frozen with liquid N_2, and pulverized in a mortar.

c) *Leukocytes* were isolated from heparinized blood by the fibrinogen sedimentation method (*Nitowsky & Grunfeld* 1967). The leukocytes from 5 ml blood were finally suspended in 0.5 ml 0.85 % NaCl and homogenized, either by ultra sonic vibration or using a small Potter-Elvehjem homogenizer. The homogenate was stored at —70°.

d) *Erytrocytes* were obtained by repeated centrifugation and resuspension in 0.5 M-NaCl as described by *Van Hoof* (1967). A 1:1 hemolysate was used for further studies. Erytrocytes may be obtained from the same blood sample as used for leukocyte isolation after removal of the latter.

e) *Glycogen* was isolated from the tissues by heating about 1 g liver or muscle with 5 ml 30 % KOH for 1 hr. in a boiling water bath. The solution was then clarified by centrifugation and the glycogen precipitated by the addition of 6.5 ml 96 % ethanol. The glycogen was further purified essentially as described by *Somogyi* (1957), and finally dried with ethanol and then ether.

f) *Phosphorylase limit dextrin* was prepared from a commercial glycogen preparation by incubating it in a dialysis bag in phosphate buffer at pH 7.4 with crystalline phosphorylase in the presence of 1 mM AMP (*Hers et al.* 1967). The outer solution was changed several times, and the progression of the reaction followed by assaying its glucose concentration. In the end, the reaction was stopped by adding trichloroacetic acid, and the limit dextrin isolated and purified by precipitation with ethanol. Fig. 2 includes an iodine spectrum of this preparation.

Glycogen structure determination

Glycogen structure variation was displayed by recording the absorption spectrum in the presence of iodine and saturated calcium chloride (*Krisman* 1962). The length of the glycogen branches were more directly measured as the proportion of glucose residues liberated by phosphorylase. The method described by *Hers* (1964) was used, except that we found it necessary to add AMP.

Chemical analyses

Glycogen was determined directly in muscle and liver homogenate by the anthrone method (*Seifter et al.* 1950), after heating 50 µl of the 10 % homogenate with 250 µl 20 % KOH to 100°C for 30 min. The samples were diluted to 5 ml, centrifuged, and 1 ml supernatant used with 2 ml anthrone reagent. When determining glycogen in hemolysates, the proteins were first precipitated with trichloroacetic acid, as suggested by *Sidbury et al.* (1961), and glycogen measured in the dialyzed supernatant.

Hemoglobin was determined spectrophotometrically as cyanohemoglobin according to *Van Kampen & Zijlstra* (1961). Protein was determined according to *Lowry et al.* (1951), glucose by the glucose oxidase method (*Hugget & Nixon* 1957), and inorganic phosphate by the method of *Gomorri* (1942).

Enzyme assays

a) *α-1,4-glucosidase* was assayed by measuring the amount of glucose being liberated from maltose, essentially according to *Hers*. The reaoction was stopped by heating the mixture for 2 min. at 100°C and the denatured proteins removed by centrifugation. Glucose was determined in the supernatant, and correction, when necessary, made for the glucose content of the tissue preparation.

b) *Amylo-1,6-glucosidase* was most directly assayed by measuring the liberation of glucose from a phosphorylase limit dextrin (*Hers*). This assay measures both activities of the debranching enzyme: the (1,4 → 1,4) transglycosylation as well as the hydrolysis of the 1,6-linkage. The reverse reaction is the basis for a very sensitive and specific assay, measuring the incorporation of radioactive glucose in glycogen. The procedure used by *Hers* was adapted for our purpose. After repeated precipitation of the radioactive glycogen, it was dissolved in water and determined according to *Noll* (1969) in a scintillation liquid using Triton X-100 as the hydrophilic component. Care must be taken to avoid alkaline conditions due to KOH adsorbed to the glycogen, as this may lead to chemiluminescence. We prevented this by adding 50 µl of glacial acetic acid to each counting vial.

c) *Phosphorylase and glucose-6-phosphatase* assays were performed according to *Hers*. Both assays measure the amount of inorganic phosphate liberated in a given time.

d) *Glutamate-pyruvate transaminase* was determined by the method of *Reitman & Frankel* (1957).

Enzymes and chemicals

Glycogen phosphorylase a and rabbit liver glycogen (type III) were obtained from Sigma Chemical Company, glucose oxidase reagent (Glox Novum) from Kabi A/S, D-glucose [^{14}C(U)] from New England Nuclear Corporation.

RESULTS

Glycogen accumulation

The observation made by histochemical methods in the accompanying paper (*Rafiquzzaman et al.* 1976) that liver and skeletal muscle contain larger than normal amounts of glycogen, was confirmed by chemical analyses of the tissues (Table 1).

While the protein to glycogen ratio was 5.5 for the control liver, the ratio in the sick dog was 0.7. This congestion with glycogen led to rupture of liver paranchymal cells, as evidenced by a serum GPT-value of 302 mU/ml, compared to 8.7 for the control.

Table 1. Glycogen content.

Dog	Amount of glycogen (g/100 g wet tissue)	
	liver	skeletal muscle
sick	14.5	6.5
normal	4.1	1.0

Studies of acid maltase

Lack of the lysosomal enzyme α-1,4-glucosidase (acid maltase) is one of the most commonly encountered human glycogen storage diseases (type II). The few reports that exist for animals suggest that the same might be the case here. The glycogen observed in the material of Rafiquzzaman furthermore had a rather generalized occurrence. We thus first suspected a lack of acid maltase in these dogs.

The lysosomal acid maltase has been studied for several human tissues, including leukocytes. Study of the leukocyte enzyme has obvious advantages. We therefore established the leukocyte isolation and maltase assay procedure for dogs, in anticipation of further patients with suspected glycogen disease, and also for the purpose of determining levels in heterozygote relatives.

When the dog designated "case 4" in the accompanying paper, was brought to the clinic, investigation of its leukocyte maltase activity at pH 4 gave a value of 0.39 μmol glucose liberated per mg protein per hr., while 0.30 and 0.36 was recorded for two control dogs. The leukocyte level thus appeared normal. Occasionally this occurs in humans with manifest disease (*Stei-*

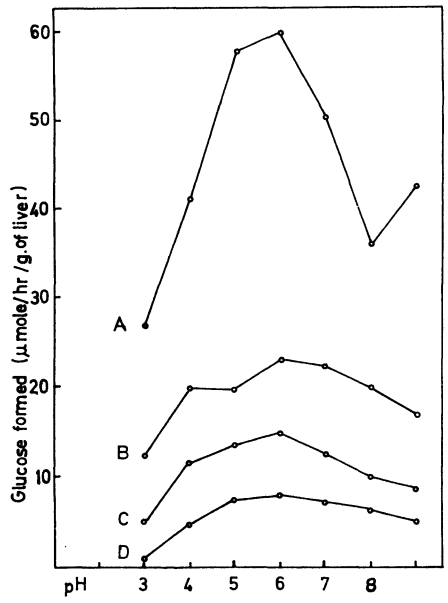

Figure 1. α-1,4-glucosidase activity in liver fractions as a function of pH. A, homogenate; B, particle free supernatant; C, microsomes; D, mitochondria plus lysosomes.

nitz & Rutenberg 1967), while muscle or liver biopsies show lack of the enzyme. When the dog suddenly died, pieces of liver and muscle were therefore rapidly removed, frozen i liquid N_2, and stored at —70° for later enzyme studies.

Studies of α-1,4-glucosidase in the tissue homogenates and its subfractions showed activities per mg protein at pH 4 which were 50—80 % of those of the same tissue of a control dog. The results were not easily interpreted because control livers, even when unfrozen, did not give a mitochondrial plus lysosomal fraction with an acid pH optimum for maltase activity. A maltase activity with optimum at pH 6 dominated in all fractions (Fig. 1). Further study of the maltase enzymes in the dog is necessary before safe judgements on the state of its lysosomal maltase can be made.

Glycogen structure

The acid maltase studies did not give a clear answer to whether the dog had suffered from type II glycogen storage disease or not. The lack of evidence of membranes surrounding the glycogen deposits even in rapidly fixed tissue specimens on the other hand (*Rafiquzzaman et al.*), suggested that the disease might not be of the lysosomal type. Attention was thus turned to the other types and biochemical diagnosis of these. Type III and IV are characterized by abnormally short and long branches

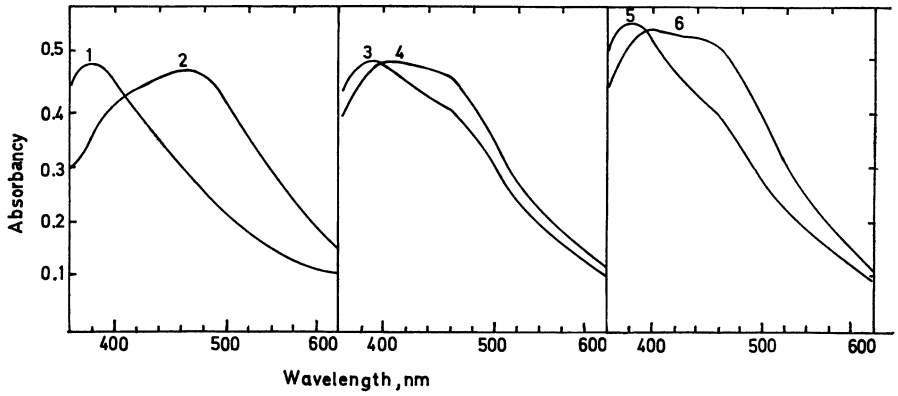

Figure 2. Glycogen-I_2 absorption spectra. The curves were obtained with the following preparations: 1, phosphorylase limit dextrin; 2, rabbit liver glycogen; 3 and 4, liver glycogen from the glycogenotic and normal dog, respectively; 5 and 6, skeletal muscle glycogen from the glycogenotic and normal dog, respectively.

in the glycogen molecules respectively. These differences from the normal structure of glycogen are revealed in the absorption spectrum for the polysaccharide-iodine complex. Phosphorylase limit dextrin (glycogen with branches maximally shortened) thus had a maximum at 380 nm, and normal liver glycogen a maximum at 465 nm in our hands (Fig. 2), while glycogen preparations with longer branches are known to have maxima at wave-lengths above 500 nm. The figure further shows curves for liver and muscle isolated from the diseased dog, as well as from a control dog. The increased proportion in the affected dog of glycogen molecules with a structure related to limit dextrin is seen. The control dog has evidently been in a physiological state before it was killed, however, in which its liver glycogen branches in particular were partly degraded.

A more direct chemical determination of glycogen branch length is afforded by determining the proportion of glucose residues which may be liberated by phosphorylase action. These values are recorded in Table 2 along with the iodine spectral ratios, A_{460}/A_{390}.

Table 2. Glycogen structure.

Source of glycogen	Phosphorolysis (%)	A_{460}/A_{390}
rabbit liver	31.5	1.20
limit dextrin	0	0.63
normal dog, liver	16.7	0.92
sick dog, liver	11.7	0.81
normal dog, muscle	19.8	0.97
sick dog, muscle	4.3	0.70

Amylo-1,6-glucosidase activity

The glycogen structure study suggested that the dog had suffered from a deficiency in the debranching enzyme, amylo-1,6-glucosidase. A confirmation of this was sought by direct assay of the enzyme in tissue homogenates. The enzyme was assayed in two ways: 1) by release of glucose from phosphorylase limit dextrin, 2) by attachment of radioactive glucose to glycogen, thus measuring the reverse of the debranching process. With both assays very low levels of activity were found, in liver as well as in skeletal muscle (Table 3). It is clear from this that

Table 3. Activity of debranching enzyme.

Tissue	Incorporation of ^{14}C-glucose (units*/g tissue)	Glucose liberation from limit dextrin** (μmol/min./g tissue)
normal dog, liver	414	0.57
sick dog, liver	22	0.04
normal dog, muscle	395	0.26
sick dog, muscle	9	0

* The unit of activity is defined as the amount of enzyme incorporating 0.1 % of the radioactivity added in 1 hr.
** Total glucose production, without correction for general glucosidase activity.

the enzyme affected was indeed amylo-1,6-glucosidase, that the dog had suffered from the equivalent of Cori's disease, also called limit dextrinosis or type III glycogen storage disease.

Other tests of glycogen metabolic activity

A simultaneous loss of two different enzymes in glycogen metabolism would be extremely rare. One would thus expect to find other enzymes in the tissues of the diseased dog intact. This was found to be the case for glucose-6-phosphatase and muscle and liver glycogen phosphorylase, the enzymes missing in types I, V and VI of glycogen storage disease respectively (Table 4). Assays for the remaining three potentially affected enzymes were not developed.

The weak blood sugar response upon injection of adrenalin in the dog (Fig. 3) is in agreement with the lesion being present in the debranching enzyme. If the dog had been thoroughly fasted, there should have been no response. The glycogen structure study showed, however, that the branches, although short, were not degraded to the extent found in phosphorylase limit dextrin.

Table 4. Glycogen phosphorylase and glucose-6-phosphatase.

Tissue	Liberation of inorganic phosphate (μmol/min./g tissue)	
	phosphorylase	phosphatase
normal dog, liver	25	2.6
sick dog, liver	28	2.1
normal dog, muscle	34	
sick dog, muscle	37	

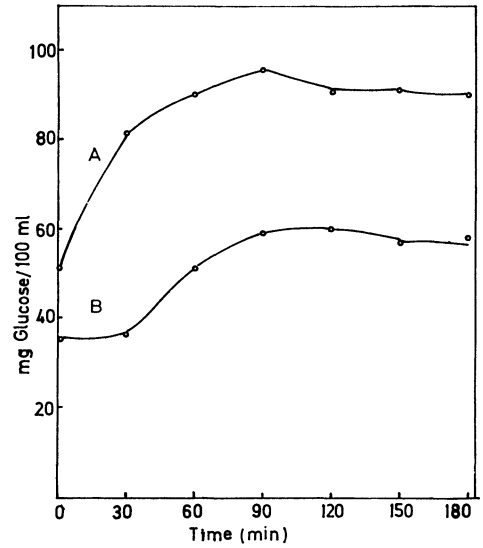

Figure 3. Blood glucose response after injection of adrenalin. 1 ml 1 % adrenalin was injected intramuscularly. A, normal dog; B, glycogenotic dog.

Detection of disease and heterozygotes with erytrocytes and leukocytes

It would be valuable for the study of the distribution of the gene for type III glycogen storage disease if samples of blood would give sufficient information. Studies of Cori's disease in humans suggest three blood assay parameters as being of interest: 1) Erytrocyte glycogen content, 2) Erytrocyte amylo-1,6-glucosidase, and 3) Leukocyte amylo-1,6-glucosidase. *Van Hoof* (1967) thus found mean erytrocyte enzyme activities of 4.3 % of normal for a group of type III patients, while the glycogen content was about ten fold increased. Heterozygotes, on the average, had intermediate values, with some individual overlapping, however. Similar observations were made by *Chayoth et al.* (1967), who in addition studied the enzyme activity in leukocytes. The separation of heterozygotes from normals was found to be more complete using this assay, but individual overlapping could still be observed.

Blood cell values for some normal dogs and some close relatives of the glycogenotic dogs are given in Table 5. It is evident that erytrocyte glycogen content as well as erytrocyte 1,6-glu-

Table 5. Blood cell glycogen content and debranching enzyme activity.

Dog	Erytrocyte glycogen (μg/g hemoglobin)	Erytrocyte 1,6-glucosidase (units/g hemoglob.)	Leukocyte 1,6-glucosidase (units/g protein)
case 1, 2 father	144	20	
case 4, father	54	22	106
case 4, mother	56	19	100
normal 1	104	22	228
normal 2	38	26	176
normal 3	37	13	180

cosidase activity display considerable variability within the groups, making it impossible on this basis to detect heterozygotes. The leukocyte enzyme activity does, however, look promising in this respect.

DISCUSSION

The studies of glycogen structure and enzyme activities presented leave no doubt that the dog whose tissues we have studied suffered from a lack of the glycogen debranching enzyme. The occurrence of type III glycogenosis has thus definitely been established also for animals. Based on a comprehensive set of biochemical data, *Van Hoof & Hers* (1967) could further divide their type III patients into subgroups, corresponding to different mutational changes in this complex enzyme. The present case fits best the characteristics of subgroup III A: There are very low activities of limit dextrin hydrolysis and ^{14}C-glucose incorporation in both muscle and liver, together with heavy accumulation of glycogen in both tissues.

The accumulation of glycogen in the liver in this disease is particularly dramatic, the liver containing 50 % more glycogen than protein, while the normal glycogen to protein ratio is around 1:5. Destruction of parenchymal cells as evidenced by high GPT-values are thus not surprising. The mild reduction in α-1,4-glucosidase activities per mg protein in various liver fractions are probably to be regarded as secondary effects of this heavy glycogen deposition.

The distribution of glycogen is not so widespread in human tissues as found in the dog (*Rafiquzzaman et al.* 1976). The

disease is for humans in addition often less severe in adulthood. Transaminase levels and liver size return to normal at puberty (*Howell* 1972). It would be interesting to find out whether a similar adaption may take place in dogs, what the mechanism is of this adaption, and whether the difference in tissue distribution is parallelled by differences in enzyme contents.

The disease is for humans readily detected by assay on erytrocytes and leukocytes. It would be valuable if the same were the case for dogs, and there is no reason to believe that they are different in this respect. It was unfortunately not possible to carry out a test of this assumption in the present study.

It would also be valuable if blood tests could detect heterozygote individuals. This would make it easier to establish firmly the genetics of the disease, and to devise procedures to limit the spreading of the affected gene. The data obtained from three parents of glycogenotic dogs and three controls indicate that enzyme analyses on leukocytes give the best separation between normals and heterozygotes, as found for humans. A larger material must, however, be analyzed in order to ascertain if and how often there may be individual overlaps using the leukocyte assay.

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SAMMENDRAG

Glykogenose type III hos hund.

Enzym- og glykogenstudier er utført med vev fra en glykogenotisk hund, hvis kliniske og patologiske karakteristika er rapportert av *Rafiquzzaman et al.* (1976). Glukose-6-fosfatase fra lever, sur maltase fra leukocytter og lever, og glykogenfosforylase fra lever og skjelettmuskel var ikke nevneverdig berørt. Aktiviteten for avgrenings-

enzymet (amylo-1,6-glukosidase) i muskel og lever, bestemt med to uavhengige metoder, var imidlertid redusert til mellom 0 og 7 % av normal aktivitet.

Glykogenstrukturundersøkelser med fosforylase eller jodspektra viste at de unormalt store glykogenmengder i lever og muskel hadde unormalt korte grener, slik en skulle vente ved et lavt nivå av avgreningsenzymet. Det er således klart at hunden led av en sykdom tilsvarende Cori's sykdom ("limit dextrinosis", glykogenavleiringssykdom type III).

Preliminære data antyder at det skulle være mulig å identifisere heterozygoter, basert på undersøkelse av avgreningsenzymet i leukocytter.

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