# MUCOPOLYSACCHARIDE EXCRETION IN DWARF CATTLE AND IN PATIENTS WITH HURLER'S DISEASE

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ABSTRACT

MUCOPOLYSACCHARIDE EXCRETION IN DWARF CATTLE AND IN PATIENTS WITH HURLER'S DISEASE

by Jary S. Mayes

Several heritable disorders of mucopolysaccharide metabolism have been reported of which Hurler's syndrome in humans has been best characterized. In patients with Hurler's disease, excessive amounts of chondroitin sulfate B and heparitin sulfate are known to be excreted in the urine and to accumulate in the tissues. The disorders are probably not confined to humans but may appear in other mammals, and several reports have appeared concerning unusual mucopolysaccharide excretion in cattle. Using chondroitinase to quantitatively measure chondroitin sulfates, hexuronic acid tests to measure total mucopolysaccharides and the carbozole and naphthoresorcinol reactions to estimate the amount of chondroitin sulfate B, the excretion of mucopolysaccharides has been compared in several types of dwarf and normal cattle. The amounts and types of mucopolysaccharides appeared to be similar in normal and dwarf cattle tested in the present study. A significant decrease in the excretion of mucopolysaccharides relative to creatinine was evident with advancing age.

In order to verify the methods the recovery of mucopolysaccharides from urine was studied. Both chondroitin sulfate A and chondroitin sulfate B when added to the urine of cattle could be recovered in good yields. In addition urine samples were obtained from Hurler's patients and the mucopolysaccharides isolated and determined as in the

animal specimens. The Hurler's patients showed the expected abnormal excretion of mucopolysaccharides and different variants excreted either heparitin sulfate or chondrotin sulfate B and heparitin sulfate in excessive amounts.

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By

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To Faye

# VITA

Jary S. Mayes was born on October 19, 1938 at Walters, Oklahoma. He graduated from Walters High School in 1956. After attending Cameron Jr. College he transferred to Oklahoma State University and received the B. S. degree in Agriculture in 1960 from the Department of Agricultural Chemistry. His graduate studies were pursued at Michigan State University in the Department of Biochemistry to complete the requirements for the degree of Master of Science. The graduate studies will be continued at the same institution with the aid of a National Institutes of Health Predoctoral Fellowship. He is married and has two sons.

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# INTRODUCTION

Several hereditary disorders have been reported in which there are abnormal metabolism and excretion of mucopolysaccharides. Of these Hurler's syndrome (gargoylism, lipochondrodystrophy, dysostosis multiplex) is the only severe mucopolysaccharide disorder that has been conclusively documented. There are two hereditary forms of Hurler's syndrome, one an autosomal recessive and the other a sex-linked recessive. In both genotypes mucopolysaccharides are excreted in the urine and accumulate in the tissues, although there has to date been no indication of a qualitative difference between the two genotypes in the excretion pattern or tissue deposits of mucopolysaccharides. Although it does appear to be an inborn error in the metabolism of mucopolysaccharides, the relationship between the two hereditary forms and the cause of the defect is largely unknown.

More recently mucopolysaccharide excretion in the urine and accumulation in the tissues has been suggested in snorter dwarf cattle (brachycephalic dwarf, short headed dwarf) and the metabolic defect has been compared to the human form of dwarfism, Hurler's syndrome, but in some cases tests have failed to discriminate between the dwarf animal and the normal animal in mucopolysaccharide metabolism. With the conflicting results it is still questionable whether the snorter dwarf syndrome in cattle and Hurler's syndrome in humans are manifestations of the same metabolic defect.

A big problem in the metabolic studies is the lack of quantitative methods for measuring individual naturally occurring variants of the mucopolysaccharides. The isolation, characterization, separation and assessment of purity of the complex polymers are all difficult tasks. Most of the methods available are time consuming, require large amounts of the mucopolysaccharides, or lack specificity and subject to interference by other substances.

Since the metabolic defect in snorter dwarf cattle had been reported to be analogous to that of the human form of dwarfism, Hurler's syndrome, it appeared profitable to study the defect in cattle where tissue samples could be more readily obtained. Preliminary experiments indicated that the metabolism of mucopolysaccharides was similar in the dwarf and control animals. Therefore a survey of a large number of various types of dwarf cattle for abnormal excretion of mucopolysaccharides was conducted.

A spectrophotometric method for the quantitative measurement of the chondroitin sulfates was developed employing the enzyme, chondroitinase. Using this method for chondroitin sulfates, uronic acid tests for total mucopolysaccharides, and the carbazole and naphthoresorcinol uronic acid color reactions for the estimation of chondroitin sulfate B the excretion of mucopolysaccharides in the urine of dwarf cattle and Hurler's syndrome was studied.

# LITERATURE REVIEW

## Chemistry of the Mucopolysaccharides

The mucopolysaccharides are linear polymers of high molecular weight. They usually consist of a disaccharide repeating unit of a hexosamine and a hexuronic acid moiety joined by a glycosidic bond and usually sulfate is part of the molecule. Currently eight distinct connective tissue acid mucopolysaccharides are known and Table I gives a survey of the compounds and their components. Some recent reviews discuss their chemistry and distribution in tissues (1,2,3,4).

Only two of the mucopolysaccharides found in connective tissue are non-sulfated, e.g. hyaluronic acid and chondroitin. Hyaluronic acid is widely distributed and has been extensively studied being the first mucopolysaccharide for which a complete structure was well established. It has alternating β-1,3-D-glucopyranosyluronic acid and β-1,4-N-acetyl-D-glucopyranosylamine units. Chondroitin is less common in nature, but has been obtained by the removal of sulfate from the chondroitin sulfates. It has the same structure as hyaluronic acid except that it contains galactosamine instead of glucosamine.

Among the sulfated mucopolysaccharides, three types of chondroitin sulfates, A, B, and C, are recognized as distinctive isomers and chondroitin sulfate-D has been isolated from shark cartilage, but its characterization is less firmly established. The repeating units of chondroitin sulfate-A are β-1,3-D-glucopyranosyluronic acid and β-1,4-N-acetyl-D-galactopyranosylamine-4-sulfate. Chondroitin sulfate-B differs from A in that the D-glucopyranosyluronic acid moiety is replaced

Table I. Constituents of Acid Mucopolysaccharides

| Name | Hexosamine | Uronic Acid | Sulfate |
|---|---|---|---|
| Hyaluronic acid | Acetylglucosamine | Glucuronic acid | - |
| Chondroitin | Acetylgalactosamine | Glucuronic acid | - |
| Chondroitin sulfate A | Acetylgalactosamine | Glucuronic acid | + |
| Chondroitin sulfate B | Acetylgalactosamine | Iduronic acid | + |
| Chondroitin sulfate C | Acetylgalactosamine | Glucuronic acid | + |
| Heparitin sulfate | Acetylglucosamine | Glucuronic acid | + |
| Heparin | Glucosamine | Glucuronic acid | + |
| Keratosulfate | Acetylglucosamine | * | + |

*Contains galactose as the other sugar moiety.

by its C-5 epimer, L-idopyranosyluronic acid. Chondroitin sulfate-C differs from A in that it is the 6-O-sulfate instead of the 4-O-sulfate. Chondroitin sulfate-D appears to differ from C in that it has a higher sulfur content.

The other sulfated mucopolysaccharides of connective tissue are heparin, heparitin sulfate, and keratosulfate. The characteristics of these compounds are not as firmly established as the chondroitin sulfates or hyaluronic acid. Heparin contains glucosamine, glucuronic acid, and sulfate and has 1-6 and 1-4 linkages. It contains both O-sulfate and N-sulfate. Heparitin sulfate is probably related to heparin but appears to have less sulfate and a larger proportion of 1-4 linkages. It is a by-product of heparin preparation and is present in organs and urine of Hurler's syndrome. Keratosulfate is a polysaccharide that contains equal proportions of N-acetylgluosamine, galactose, and sulfate. Little is known about the characteristics of this compound.

Metabolism of Mucopolysaccharides

The general outline of the biosynthesis of the mucopolysaccharides has been fairly well established with the use of radioactive isotopes and the discovery of the uridine nucleotide co-enzymes (review see 5). However, the mechanism and extent of removal from tissues is still quite obscure. The half life of hyaluronic acid, 2 to 4 days, and chondroitin sulfate, 7 to 16 days, has been calculated from isotopic studies of rabbit skin (6) and rat cartilage (7). However, this rate of turnover changes considerable with age (8).

Mucopolysaccharides are excreted in the urine in normal individuals; the daily excretion in both men and women being about 8 to 15 mg. (9),

but this is also dependent upon the age of the individual (10,11). The mucopolysaccharide excreted in normal individuals appears to be mainly chondroitin sulfate-A (12). Kaplan and Meyer (13) have studied the fate of injected chondroitin sulfates A, B, and C and heparitin sulfate in man and dog. After injection of chondroitin sulfate A and C there was a rapid disappearance from the blood but no significant amount was demonstrable in the urine. On injection of these mucopolysaccharides in heavier doses into a dog a small amount was recovered in the urine, but the blood level remained high for several hours. The fate of the unrecovered polysaccharides is unknown and why normal individuals excrete almost exclusively chondroitin sulfate-A cannot be explained at this time. After injection of chondroitin sulfate-B and heparitin sulfate they disappeared from the blood stream and a large per cent could be recovered unchanged in the urine. On long continued injection of chondroitin sulfate-B, a polysaccharide was excreted with the properties of heparitin sulfate. Therefore, the different variants of Hurler's syndrome may involve just one mucopolysaccharide and some interrelationship may exist between chondroitin sulfate-B and heparitin sulfate.

The removal of mucopolysaccharides from tissues may be accomplished both by extensive degradation and by excretion of the unchanged polymers.

## Heritable Disorders of Mucopolysaccharide Metabolism

Heritable disorders in which altered mucopolysaccharide metabolism has been implicated in humans are Hurler's syndrome (14), Multiple exostoses (15), artho-osteo-onychodysplasia (15), Morquio-Ullrich's

disease (16,17), and Marfan syndrome (18). Hurler's syndrome has been carefully studied and is the best understood of the heritable disorders of mucopolysaccharide metabolism. Brante (14) was the first to show that the disorder was a mucopolysaccharidosis. By histochemical methods and by isolation procedures he showed the presence in the tissues and urine of large quantities of a sulfated mucopolysaccharide which he believed to be chondroitin sulfate. Dorfman and Lorincz (19, 20) and Meyer and his associates (21,22) demonstrated that two sulfated mucopolysaccharides, chondroitin sulfate B and heparitin sulfate, are excreted in the urine and present in the tissues in abnormal amounts in these individuals. In general there appears to be three patterns of mucopolysaccharide accumulation and excretion involving either chondroitin sulfate B, heparitin sulfate, or both of these mucopolysaccharides in varying proportions.

These disorders may not be confined to the human species but may also appear in other mammals and Lorincz (15,23) and Koger et al (24) have recently reported the excretion and accumulation of mucopolysaccharides in the tissues of snorter dwarf cattle. After isolation and fractionation Lorincz (15) identified the mucopolysaccharide as chondroitin sulfate B. Therefore, according to Lorincz (15) the snorter dwarf cattle syndrome appears to be analogous in abnormal mucopolysaccharide metabolism to the human form of dwarfism, Hurler's syndrome. In contrast Tyler et al (25) found mainly chondroitin sulfate A in the urine of two snorter dwarf calves. They could find no evidence of chondroitin sulfate B or heparitin sulfate.

Methods Used in Detecting Mucopolysaccharides and Their Application to Dwarf Cattle and Hurler's Syndrome

One of the problems in studying the different disorders of mucopolysaccharide metabolism has been methods to differentiate between the various mucopolysaccharides. One method is to isolate, separate, and identify the individual mucopolysaccharides. This is laborious, requires a large sample and is not suitable for a rapid screening test. Another method is to chemically measure one of the components, but this lacks specificity and many substances cause interference. A test for uronic acids has been extensively used to estimate mucopolysaccharides. Teller et al (11) have used the carbazole test for uronic acids to study the urinary excretion of mucopolysaccharides in normal children and patients with Hurler's disease. The normal range of excretion was determined and the effect of age was reported. Excretion of all Hurler's patients, when expressed in terms of mucopolysaccharides per gram of creatinine were above normal range. Teller et al (26) have also developed a method for detecting mucopolysaccharides employing the carbazole to naphthoresorcinol uronic acid color reactions (C/N ratio). Most mucopolysaccharides give a high C/N ratio while chondroitin sulfate B gives a low C/N ratio. Using this method they have been able to identify the heterozygous carriers of Hurler's syndrome. McIlwain and Eveleth (27) have used the uronic acid reactions to study the excretion of mucopolysaccharides in snorter dwarf cattle. The mucopolysaccharides isolated from urine of snorter dwarf cattle showed a low C/N ratio which indicates chondroitin sulfate-B and a high C/Creatinine ratio which indicates a higher excretion of mucopolysaccharides. Other workers have analyzed some urine specimens

from snorter dwarf cattle using the C/N and C/Creatinine ratios and were unable to discriminate through testing urine which animals were affected and which were not affected.

Dorfman (28) has developed a turbidity method based on the fact that mucopolysaccharides form an insoluble complex with bovine serum albumin. Although lacking specificity this method has been used as a rapid screening test. Lorincz (29) and Steiness (30) observed that all the patients with the confirmed Hurler's disorder gave positive values by the turbidity method. Lorincz (15) also used this method in his studies with dwarf cattle and all were reported to have abnormally elevated turbidity values. The turbidity test, though lacking specificity, is a very rapid method and thus may be a valuable diagnostic screening test for Hurler's syndrome and other mucopolysaccharide disorders. Campbell and Fried (31) have used this method, together with the increase in reducing activity after hydrolysis, periodate oxidation, and the uronic acid carbazole test, to study the excretion of mucopolysaccharides in the sex-linked form of the Hurler's syndrome. In general the various methods yielded parallel data which indicated that the clinically affected individuals excrete significantly increased quantities of mucopolysaccharides. The known carriers and most of the siblings were in or near the normal range.

Berry (32) has developed a procedure for detecting chondroitin sulfate in urine dried on filter paper. She employed the dye, toluidine blue, which gives a purplish color when urine specimens contain large amount of chondroitin sulfate while negative specimens are light blue. In surveying approximately 700 urine samples from mentally retarded children for various metabolic disorders she (33) found one patient

that was positive for chondroitin sulfate. Carson and Neill (34) in surveying 2,081 mentally retarded individuals for inborn errors of metabolism found three cases of Hurler's syndrome by using a specific staining reaction on urine dried on paper. Since screening programs are time consuming and the finding of metabolic disorders is rare, the simple paper spot tests could serve as a preliminary screening procedure.

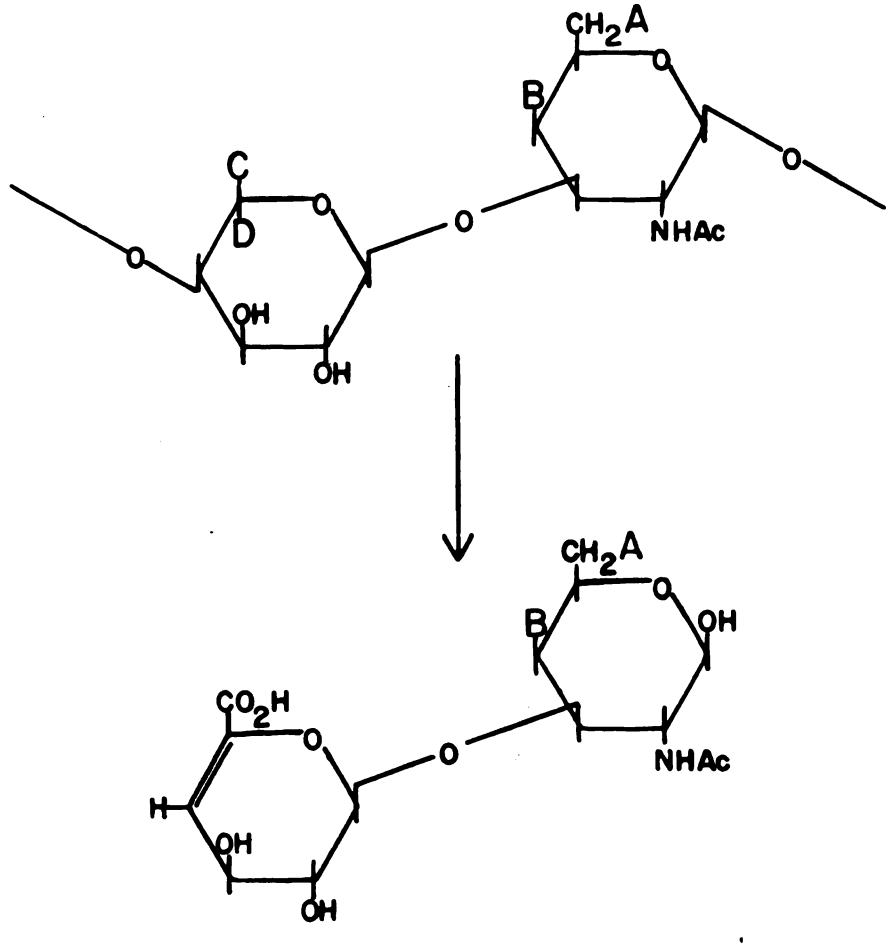
By using various stains Mittwoch (35) has suggested that abnormal inclusions are present in the lymphocytes of Hurler's syndrome. He further suggested that the inclusions are mucopolysaccharides and could provide a simple diagnostic test. More recently Mittwoch (36) has reported that the heterozygous carriers of the disorder have an unusually large number of segments on the nuclei of the polymorphonuclear neurophils, but there was considerable overlap with the controls and other disorders are known to cause an increase in nuclear segmentation. It might nevertheless prove of value in detecting the heterozygous carriers in the families of patients with Hurler's syndrome and differentiate between the sex-linked and the autosomal forms of inheritance.

## The Enzymic Degradation of the Chondroitin Sulfates

Chondroitinase is the enzyme that catalyzes the breakdown of the chondroitin sulfates to an unsaturated disaccharide as shown in Fig. 1. An unusual feature of this bacterial mucopolysaccharidase is the introduction of $\Delta 4,5$ unsaturation in the uronic acid at the site of cleavage. This unsaturated disaccharide has a strong absorption in the ultra violet and the equilibrium of the reaction is in the direction of

Figure 1. Reaction of Chondroitinase.

The abbreviation used is ChS for chondroitin sulfate.




| | A | B | C | D |
|---|---|---|---|---|
| ChS-A | OH | $OSO_3H$ | $CO_2H$ | H |
| ChS-B | OH | $OSO_3H$ | H | $CO_2H$ |
| ChS-C | $OSO_3H$ | OH | $CO_2H$ | H |

complete degradation; therefore, it would be of interest if a quantitative method for measuring the chondroitin sulfates could be developed employing chondroitinase.

Chondroitinase has been obtained from Flavobacterium heparinum (37) and Proteus vulgaris (38). The enzyme has been purified over 700 fold from Proteus vulgaris and some of its properties studied (39,40). It was free of chondrosulfatase activity and produced a single detectable product. The purified enzyme degraded the chondroitin sulfates and hyaluronic acid, but did not degrade heparin or blood group substances. Chondroitin sulfate was degraded much faster than hyaluronic acid and the pH optimum for the degradation of chondroitin sulfate was about 8.0 while for hyaluronic acid it was 6.7 to 6.8. Heparin was inhibitory as were several heavy metals. Both cations and anions have a marked effect upon the activity of the enzyme. Cations appear to effect the activity by influencing the physical configuration of the substrate molecule while the anions effects have been interpreted as enzyme activation.

# MATERIALS AND METHODS

## Reagents and Substrates

Crude chondroitin sulfate and heparin were obtained from General Biochemical Inc. Chondroitin sulfate A was purified from the crude chondroitin sulfate by fractionation with cetyl pyridinium chloride and by column chromatography on Dowex-1-X2-Cl according to the method of Schiller *et al* (41). It was further purified by alcohol fractionation as the calcium salt according to the method of Meyer *et al* (4). Chondroitin sulfate B was a generous gift of Dr. Karl Meyer and it was also isolated in large quantity from pig skin by the method of Meyer *et al* (4), then further purified by cetyl pyridinium chloride fractionation and alcohol fractionation as the calcium salt. Chondroitin sulfate C was a generous gift of Dr. C. W. Castor. Hyaluronic acid and hyaluronidase were obtained from the Worthington Biochemical Co. Heparitin sulfate was a gift from the Upjohn Co. Sugars were obtained from Phansteihl Chemical Co. as reagent grade chemicals. *Proteus vulgaris* type NCTC 4636 was purchased from the American Type Culture Collection.

## Qualitative Measurements

Descending chromatography was carried out on Whatman No. 1 filter paper for the identification of the end product of the enzyme reaction and for the preparative chromatography of the compound. The chromatograms were developed in butanol-acetic acid-water (50:12:25 v/v) for 48 hours (42). The unsaturated disaccharide was detected by ultraviolet

light (Mineralight SL 2537) and by ammonia silver nitrate according to the method of Trevelyan et al (43).

Quantitative Measurements

Uronic acid determinations were performed by the carbazole method of Dische (44) with added borate (45) as modified by Bitter and Ewins (46) and by the naphthoresorcinol method of Pelzer and Staib (47). N-acetylamino sugars were determined by the method of Reissig et al (48). Sulfate was determined by the method of Dodgson and Spencer (49) as modified by Cifonelli and Dorfman (50) to determine the benzidine sulfate directly in a Beckman spectrophotometer at 250 mμ. Creatinine was determined by the method of Folin and Wu (51) and protein was measured by the method of Lowry (52). Chondroitinase assays were performed on a Beckman DU spectrophotometer equipped with a Gilford changer and recording attachment (53,54). The chondroitinase activity was measured according to the method of Nakada et al (55).

Urine Samples

Urine samples were collected from dwarf cattle at the University of California, Davis through the courtesy of Dr. J. Meyer and Dr. P. W. Gregory and from normal cattle at Michigan State University through the courtesy of Dr. W. T. MaGee and at University of California, Davis through the courtesy of Dr. F. D. Carroll. Also, blood and tissue samples were obtained from a snorter dwarf calf through the courtesy of Dr. E. J. Turman, Oklahoma State University. Urine samples from Hurler's patients and controls were obtained from Dr. S. J. Sanfilippo of the University of Minnesota and from the Mt. Pleasant Home and

Training School. The samples from out of state were frozen and shipped by air express frozen in dry ice.

Mucopolysaccharides were isolated by a modification of the method of Diferrante and Rich (9). A thirty ml sample was dialyzed against distilled water for 2-3 hours. After dialysis five ml of cetyl trimethylammonium bromide (CTAB) was added and the mixture was held overnight. The resulting precipitate was centrifuged at 2,300 R.P.M. and washed twice with 30 ml of 95% ethanol saturated with sodium chloride. The precipitate was dissolved in water and analyzed.

## EXPERIMENTAL PROCEDURES AND RESULTS

### The Enzymic Measurement of the Chondroitin Sulfates

a. Induction of Chondroitinase: Proteus vulgaris was grown on 1.0% peptone, 0.1% sodium chloride, and 0.02% mucopolysaccharide (for induction of the enzyme) for 48 hours. The bacteria were harvested, washed twice with distilled water, and ruptured by sonic oscillation. The sonic extract was centrifuged at 30,000 x g for 30 minutes at 4° C and the supernatant liquid was assayed for activity. The results from the various inducers are shown in Table II. The chondroitinase formed was not specific for the inducer but resulted from the presence of chondroitin sulfate A, B or hyaluronic acid. This pattern of induction could be expected because of the similarity of structures.

b. Purification of Chondroitinase: Proteus vulgaris was grown in a quantity of 64 liters using 0.1% crude chondroitin sulfate as inducer. The cells were harvested by continuous flow centrifugation and after washing, the wet cells were suspended in an equal volume of water and ruptured by sonic oscillation. The sonic extract was centrifuged at 20,000 x g to remove cellular debris. Ammonium sulfate was added to 60% saturation to the supernatant while stirring at 4° C. The precipitate which contained the activity was collected by centrifugation and dissolved in 0.01 M phosphate buffer pH 6.5. The pH was adjusted to 5 with 1.0 N HCl and the precipitate that formed was centrifuged, washed with 0.1 M phosphate buffer pH 5, and discarded. The wash and supernatant were combined and 2% protamine sulfate was added to a 0.2%

Table II. Activity of chondroitinase from different inductions on various substrates.

| | Substrates | | | | | | |
|---|---|---|---|---|---|---|---|
| Inducer | Chs | Chs A | Chs B | Chs C | H | HS | HA |
| Glucose | 4.6 | 4.6 | 3.4 | 2.3 | 0 | 0 | 0 |
| Chs | 50.9 | 63.2 | 42.1 | 40.0 | 0 | 0 | 0 |
| Chs A | 96.0 | 105.1 | 64.0 | 61.7 | 0 | 0 | 0 |
| Chs B | 76.4 | 88.0 | 56.5 | 51.5 | 0 | 0 | 0 |
| H | 5.0 | 5.0 | 4.0 | 3.5 | 0 | 0 | 0 |
| HS | 6.6 | 7.1 | 5.2 | 4.2 | 0 | 0 | 0 |
| HA | 30.9 | 41.8 | 29.1 | 26.8 | 0 | 0 | 0 |

The abbreviations are: Chs, chondroitin sulfate; H, heparin; HS, heparitin sulfate; and HA, hyaluronic acid. The activity is given as specific activity. The assay system consisted of 0.005 ml of the crude extract, 0.1 ml (100 ugm) of substrate (optimum concentration) and 0.2 M Tris buffer pH 8.0 to a total volume of 0.5 ml.

volume. The small amount of precipitate was removed by centrifugation and discarded. The supernatant was dialyzed overnight against distilled water. The precipitate that formed upon dialysis was also removed by centrifugation. Ammonium sulfate was again added to 55% saturation to precipitate the enzyme. The precipitate was collected by centrifugation, drained and dissolved in water.

Calcium phosphate gel was added to the supernatant to a gel to protein ratio of 0.6 without adsorption of the activity. After 15 minutes it was centrifuged and the gel discarded. Calcium phosphate gel was added to the supernatant to a ratio of gel to protein of 1.5 and the activity was adsorbed. The solution was centrifuged and the supernatant discarded. The gel was washed by suspending it in 0.02 M phosphate buffer for 20 minutes and collected by centrifugation. The activity was eluted with 2 M sodium acetate pH 7.6 by extracting for 12 hours in the cold. After removal of the gel by centrifugation the supernatant which contained the activity was dialyzed against distilled water.

A DEAE cellulose column (4x25 cm.) was equilibrated overnight with 0.005 M potassium phosphate buffer pH 8.0 and the dialyzed enzyme was placed on the column. Chondroitinase was eluted with 0.005 M potassium phosphate buffer pH 8.0, the eluate lyophilized to approximately 70 ml, dialyzed against distilled water for 4 hours and again lyophilized to approximately 10 ml. This solution was placed on a DEAE cellulose column that had been equilibrated overnight with 0.005 M glycylglycine buffer pH 6.0. The enzyme was then eluted with the same buffer, concentrated by precipitation with ammonium sulfate, collected by

centrifugation and finally suspended in 1 ml of 60% saturated ammonium sulfate. This was stored at -15° C and appeared to be stable under these conditions. There was an overall purification of 200 fold with a yield of about 12% (Table III).

c. Activity of the Purified Chondroitinase: As seen in Fig. 2 the increase in optical density per unit time is proportional to the amount of enzyme added. The order of activity with the various substrates is chondroitin sulfate A > B > C. This relative activity was seen throughout purification. At pH 8.0 the enzyme was specific for the three chondroitin sulfates and showed no activity with heparin, heparitin sulfate, or hyaluronic acid. At pH 6.0 a trace of activity was observed with hyaluronic acid.

d. Standard Curve for Substrate Concentration: Since the equilibrium of chondroitinase is far in the direction of complete breakdown, it is possible to quantitatively measure the amount of chondroitin sulfate present by an end point assay. As seen in Fig. 3 the change in absorbancy is proportional to the amount of substrate. The reaction contained a large excess of enzyme in order to obtain a sharp end point. As would be expected on a dry weight basis the sodium salt of chondroitin sulfate A yielded somewhat higher absorbing values than the calcium salt. The sample of chondroitin sulfate B from Dr. Meyer was a trace higher than the calcium salt of chondroitin sulfate A and the sample of chondroitin sulfate C from Dr. Castor was somewhat lower. The later sample probably contains a small quantityof hyaluronic acid. The sodium salt of chondroitin sulfate A served for a reference to calculate chondroitin sulfate in the urine specimens.

Table III. Purification of chondroitinase.

| | Units/ml | ml | Total units | Protein mg./ml | Spec. Act. |
|---|---|---|---|---|---|
| Crude | 10,760 | 555 | $5.92 \times 10^6$ | 66.0 | 163 |
| Protamine sulfate (After dialysis) | 2,770 | 935 | $2.59 \times 10^6$ | 8.7 | 318 |
| 55% $NH_2SO_4$ | 5,040 | 345 | $1.74 \times 10^6$ | 13.7 | 368 |
| $Ca_3(PO_4)_2$ Gel | 7,710 | 200 | $1.54 \times 10^6$ | 2.59 | 2,980 |
| DEAE Cellulose I | 3,350 | 350 | $1.16 \times 10^6$ | 0.17 | 19,500 |
| DEAE Cellulose II | 7,000 | 112 | $.7 \times 10^6$ | 0.214 | 32,700 |

Assay system consisted of 0.005 or 0.01 ml of enzyme, 0.1 ml (100μgm) of chondroitin sulfate and 0.2 M Tris buffer pH 8.0 to a total volume of 0.5 ml. A unit of activity is defined as that amount of enzyme necessaryfor an O. D. change of 0.01 per minute.

Figure 2. Activity of the purified chondroitinase.

Circles, chondroitin sulfate A; triangles, B; squares, C. Each reaction cuvette contained 100 μgm (0.1 ml) of mucopolysaccharide, the enzyme, and 0.2 M tris buffer pH 8.0 to a total volume of 0.5 ml.

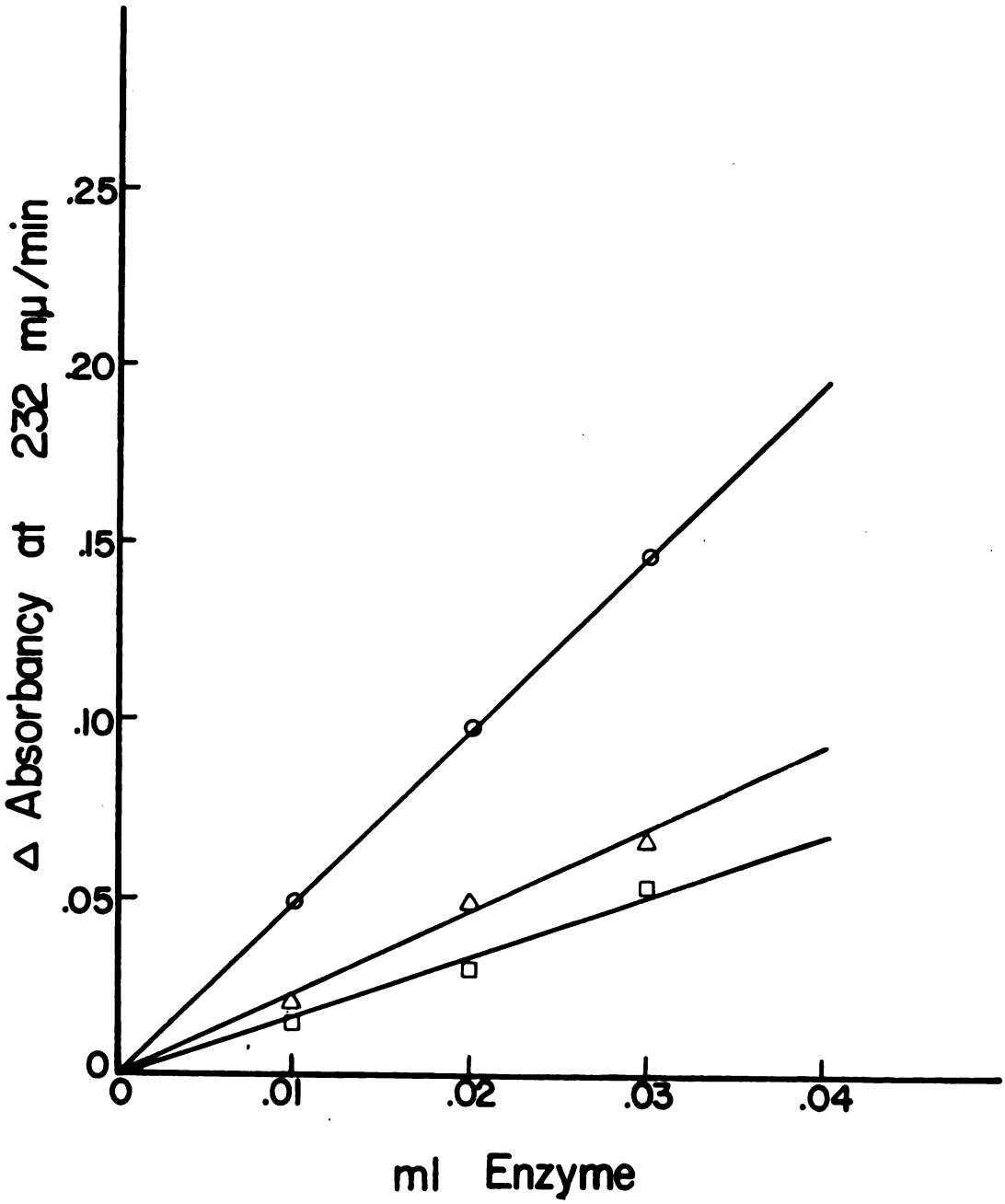
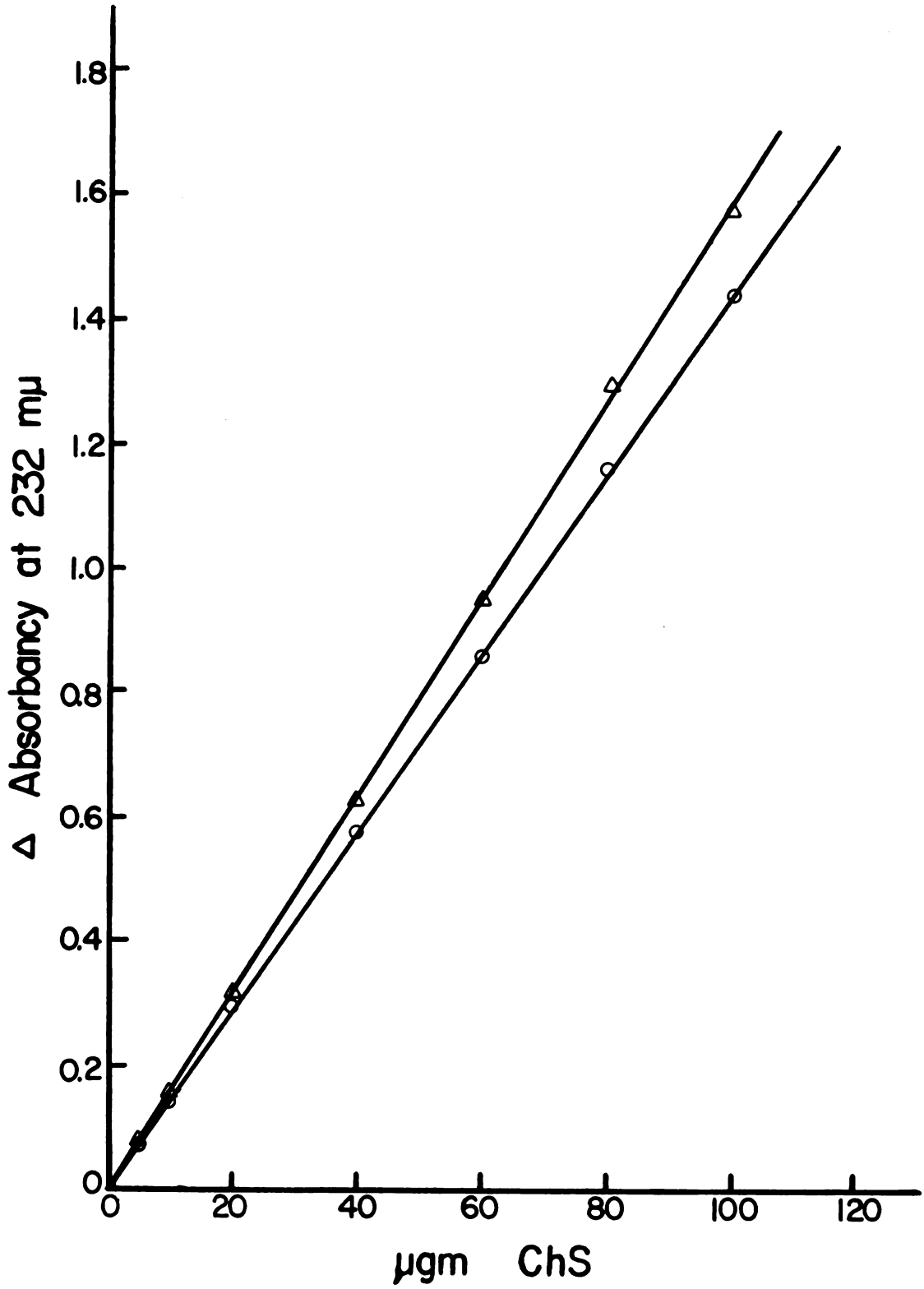

Δ Absorbancy at 232 mμ/min
.25
.20
.15
.10
.05
0
0
.01
.02
.03
.04
ml Enzyme

Figure 3. Standard Curve for substrate concentration.

Circles represent the calcium salt of chondroitin sulfate A while the triangles represent the sodium salt. Each reaction cuvette contained 0.025 ml of a one to a hundred dilution of the enzyme, the mucopolysaccharide, and 0.2 M tris buffer pH 8 to a total volume of 3.5 ml.

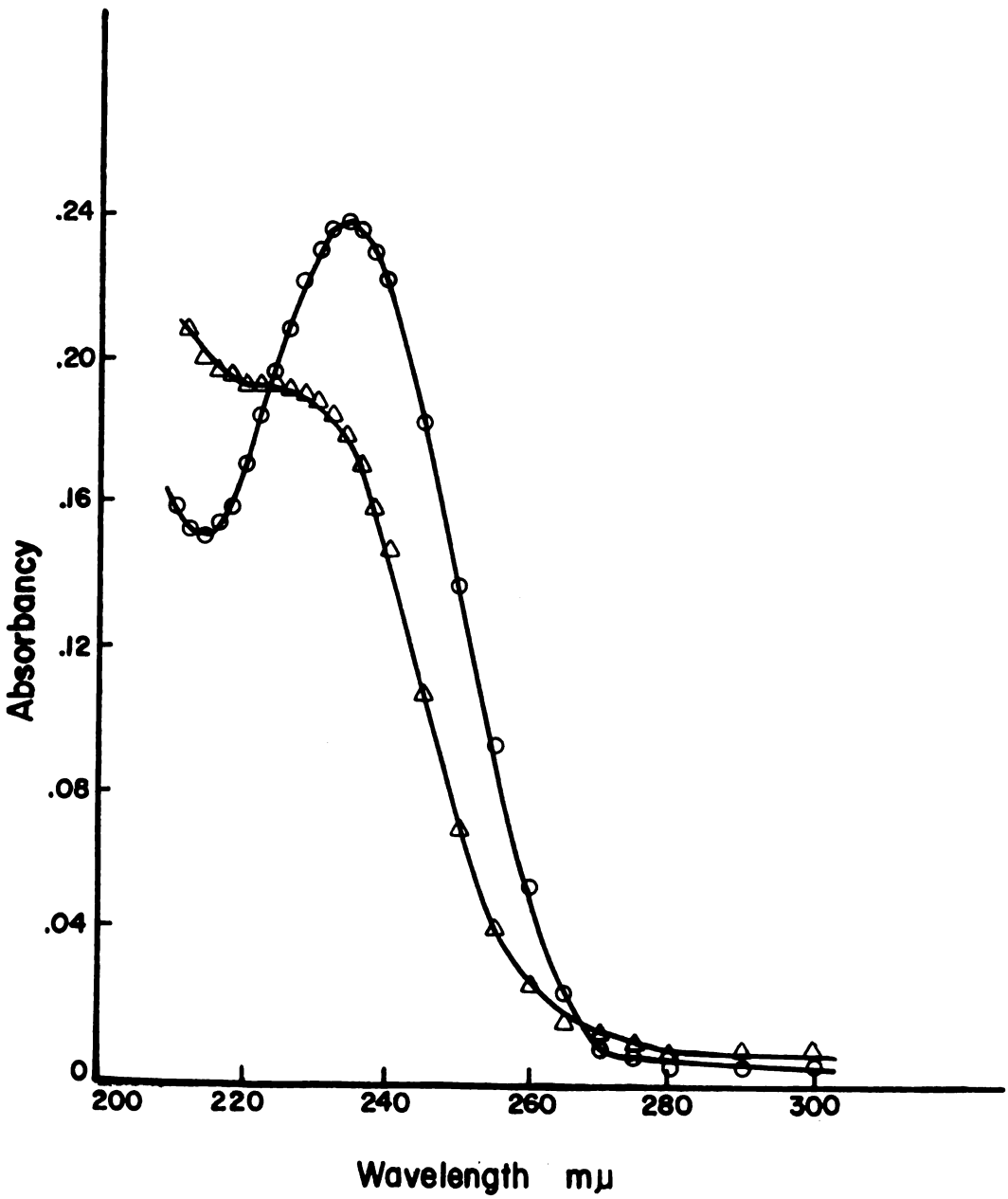

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Δ Absorbancy at 232 mμ
0
20
40
60
80
100
120
μgm ChS

e. End Product of the Chondroitinase Reaction: Chondroitinase (0.01 ml of a one to ten dilution) was incubated with 5 mg. each of the calcium salt and sodium salt of chondroitin sulfate A, for 1/2 and 2 hours respectively in 0.49 ml of 0.02 M tris buffer pH 8.0. After the reaction had been incubated at 37° C for the given amount of time, the tubes were placed in a boiling water bath for 2 minutes. After cooling the reaction mixtures were streaked on Whatman #1 chromatography paper. All the chromatograms showed two bands upon exposure to ultraviolet light; one much more dense than the other. When a strip of the chromatogram was developed with silver nitrate, the sample from the sodium salt of chondroitin sulfate A showed only one spot, which corresponded to the dark ultraviolet band. The light ultraviolet band was probably due to the enzyme. The sample from the calcium salt of chondroitin sulfate A had several spots when developed with silver nitrate. This difference in effect with the calcium salt is unclear at the present time and will be further explored.

The product from the sodium salt was further studied. The dark ultraviolet bands were sectioned and eluted with water to a total volume of 1 ml. The absorption spectra of the product is shown in Fig. 4 with 0.01 ml of the eluate in a total volume of 0.5 ml. In 0.1 M KCl-HCl buffer pH 1.8 a spectrum with a minimum at 214 mμ and a maximum of 234 mμ was observed. In 0.02 M tris buffer pH 7.9 no distinct peak was observed but a break in the curve was found at about 220 mμ. At pH 1.8 the spectrum is similar to that reported by Nakada et al (55).. However, at pH 7.9 the spectrum is different in that they found no break in the curve at lower wave lengths. This difference is unexplained.

Figure 4. Ultra violet spectra of the unsaturated disaccharide resulting from chondroitinase reaction with chondroitin Sulfate A.

The spectrum is represented by circles at pH 1.8 and triangles at pH 7.9.


.24
.20
.16
.12
.08
.04
0
Absorbancy
200
220
240
260
280
300
Wavelength mμ

The linear relationship between absorbancy and concentration of the end product at four wave lengths is shown in Fig. 5. In these cases absorbancy was measured at pH 8.0 because it is the optimum pH for chondroitinase and thus the reaction can be followed directly. These data demonstrate that it is possible to measure the reaction at various wave lengths and when interfering materials are present which absorb at 232 mμ a higher wave length could be chosen. Also, the sensitivity of the reaction can be regulated by approximately selecting the wave length of light for measuring the absorption. In this report all reactions were conducted at 232 mμ for maximum sensitivity.

To further characterize the chondroitinase end product from the sodium salt of chondroitin sulfate A, chemical analyses were performed. The ratio of uronic acid to N-acetyl-galactoamine to sulfate was 1:1.01:1.24.

Excretion of Mucopolysaccharides in Dwarf and Normal Cattle

A total of 58 urine specimens were analyzed. The samples were divided into three groups: brachycephalic dwarfs, dwarf controls, and normal controls. The dwarf controls include dolichocephalic, compressed, and unclassified dwarfs and dexters. The normal controls were from beef cattle herds. For a review on the various types of dwarfs see review by Julian et al (56). Table IV summarizes the data resulting from the various analytical procedures applied to the urine of the cattle. The C/N ratio showed considerable variation. The variation appeared to be more a function of the amount of mucopolysaccharide measured and not the type present in the urine. If the mucopolysaccharide

Figure 5. Absorption of the unsaturated disaccharide at various wave lengths.

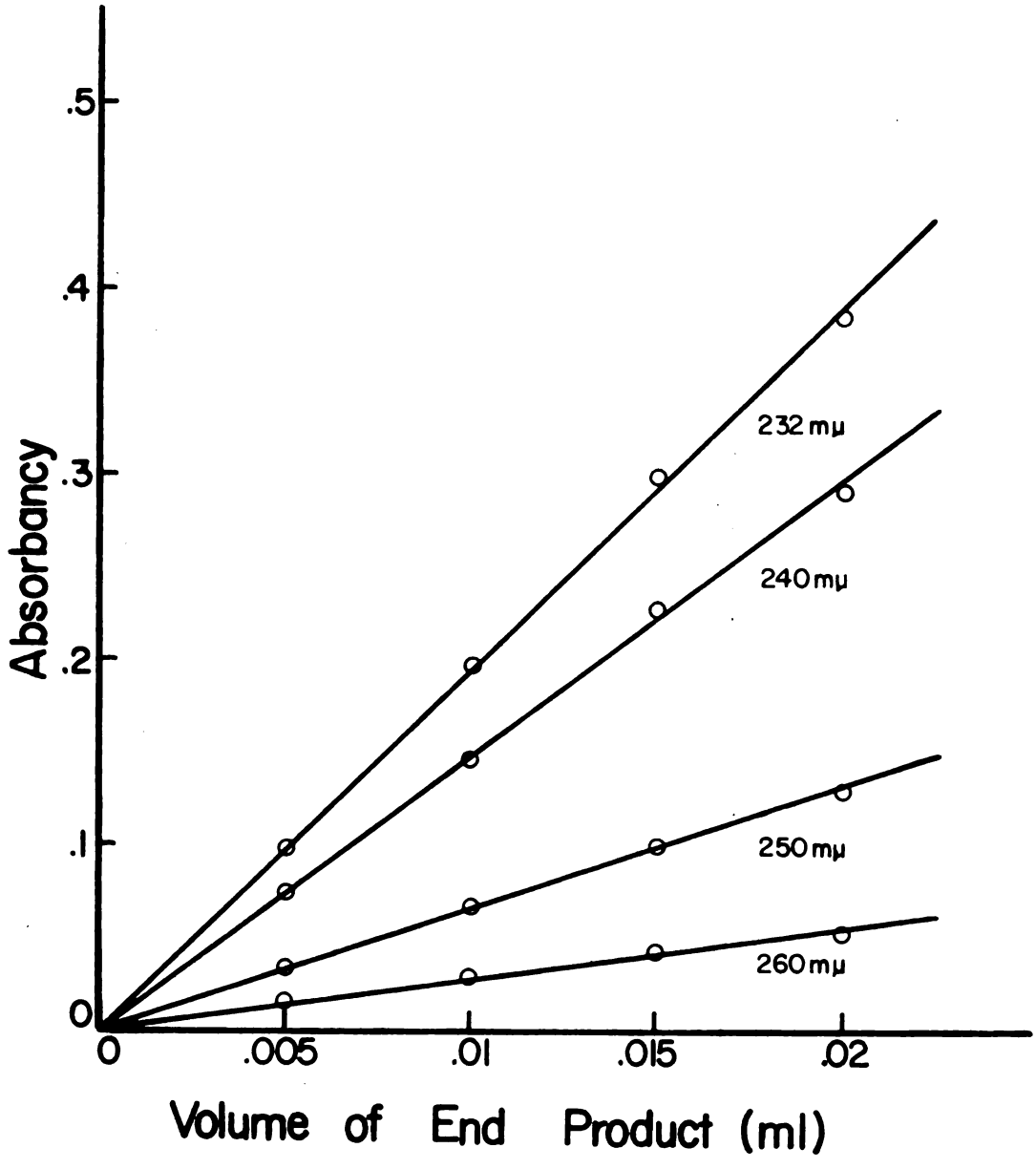

Absorbancy
.5
.4
.3
.2
.1
0
232 mμ
240 mμ
250 mμ
260 mμ
0
.005
.01
.015
.02
Volume of End Product (ml)

Table IV. Excretion of mucopolysaccharides in dwarf and normal cattle.

| Age[1] | Sex | C/N[2] | Cr/ml[3] | ChS/ml[4] | Chs/Cr | MPS/ml[5] | MPS/Cr. |
|---|---|---|---|---|---|---|---|
| a. Brachycephalic Dwarfs | | | | | | | |
| 51 | F | 6.7 | 0.920 | 8.8 | 9.6 | 14.2 | 15.5 |
| 39 | M | 6.3 | 0.385 | 2.9 | 7.5 | 5.0 | 14.1 |
| 48 | M | 7.4 | 0.640 | 7.6 | 11.9 | 10.0 | 15.6 |
| 35 | F | 7.2 | 0.335 | 4.8 | 14.3 | 9.2 | 27.4 |
| 20 | F | 7.6 | 1.440 | 30.2 | 21.0 | 37.8 | 26.2 |
| 21 | F | 7.3 | 0.420 | 9.6 | 22.9 | 13.6 | 32.4 |
| 17 | F | 7.9 | 1.030 | 24.5 | 23.8 | 29.8 | 29.0 |
| 11 | F | 7.6 | 1.100 | 33.7 | 30.6 | 41.1 | 37.3 |
| 22 | F | 7.8 | 0.295 | 2.5 | 8.5 | 5.3 | 18.0 |
| 6 | M | 9.5 | 1.190 | 42.7 | 35.9 | 53.3 | 44.8 |
| 6 | M | 9.3 | 0.320 | 14.8 | 46.2 | 19.0 | 59.5 |
| 6 | F | 12.1 | 1.980 | 89.3 | 45.1 | 103.3 | 52.1 |
| 4 | M | 9.3 | 0.440 | 27.6 | 62.7 | 30.4 | 69.2 |
| 2 | M | 8.9 | 0.255 | 18.9 | 74.1 | 25.4 | 99.6 |
| 2 | M | 9.4 | 0.280 | 18.0 | 64.3 | 24.5 | 87.5 |
| 9 | M | 7.2 | 0.295 | 6.1 | 20.7 | 9.8 | 33.0 |
| 10 | F | 7.3 | 1.180 | 34.4 | 29.2 | 39.6 | 33.6 |
| 13 | F | 7.4 | 0.695 | 32.0 | 46.0 | 35.3 | 50.8 |
| 15 | F | 7.8 | 1.180 | 32.0 | 27.1 | 35.8 | 30.3 |
| $\bar{X}$ | | 8.1 | 0.760 | 23.2 | 31.6 | 28.5 | 40.8 |
| b. Dwarf Controls | | | | | | | |
| 67 | M | 4.3 | 0.860 | 3.9 | 4.5 | 7.1 | 8.5 |
| 50 | M | 6.4 | 2.940 | 30.3 | 10.3 | 51.1 | 17.4 |
| 40 | F | 5.0 | 0.300 | 2.3 | 7.7 | 3.8 | 12.8 |
| 64 | M | 4.9 | 2.070 | 9.9 | 4.8 | 13.9 | 6.7 |
| 27 | F | 7.2 | 0.560 | 7.1 | 12.7 | 11.1 | 19.9 |
| 26 | F | 7.8 | 1.020 | 15.3 | 15.0 | 20.8 | 20.4 |
| 18 | F | 7.5 | 0.820 | 20.0 | 24.4 | 25.8 | 31.5 |
| 16 | M | 7.9 | 0.440 | 6.3 | 14.3 | 8.8 | 19.9 |
| 23 | F | 6.8 | 0.995 | 22.1 | 22.0 | 29.3 | 29.5 |
| 21 | F | 4.4 | 0.193 | 1.9 | 9.8 | 3.8 | 19.7 |
| 11 | M | 8.7 | 1.310 | 44.3 | 33.8 | 52.3 | 39.9 |
| 16 | F | 8.1 | 1.110 | 21.3 | 19.2 | 30.4 | 27.4 |
| 18 | F | 6.2 | 0.675 | 8.5 | 12.6 | 12.3 | 18.3 |
| 21 | F | 6.3 | 0.620 | 10.4 | 16.8 | 15.6 | 25.2 |
| 16 | F | 7.6 | 1.150 | 13.5 | 11.7 | 18.4 | 16.0 |
| 9 | M | 6.4 | 0.945 | 27.1 | 28.7 | 31.3 | 33.1 |
| 9 | F | 7.1 | 0.975 | 28.7 | 29.4 | 38.2 | 39.2 |
| 9 | F | 8.7 | 0.610 | 17.9 | 29.3 | 23.4 | 38.4 |
| 3/4 | M | 7.4 | 0.310 | 40.3 | 130.0 | 43.8 | 141.4 |
| $\bar{X}$ | | 6.8 | 0.940 | 17.4 | 23.0 | 23.2 | 29.7 |

Table IV. cont.

| Age[1] | Sex | C/N[2] | Cr/ml[3] | ChS/ml[4] | Chs/Cr | MPS/ml[5] | MPS/Cr |
|---|---|---|---|---|---|---|---|
| c. Normal Controls | | | | | | | |
| 6 | M | 8.2 | 0.290 | 14.7 | 50.7 | 19.0 | 65.7 |
| 5 | M | 8.0 | 0.285 | 12.7 | 44.6 | 14.7 | 51.6 |
| 80 | M | 3.3 | 0.480 | 1.2 | 2.4 | 2.7 | 5.6 |
| 68 | M | 4.1 | 0.560 | 4.3 | 7.7 | 7.7 | 13.7 |
| 44 | M | 5.9 | 2.960 | 30.3 | 10.2 | 37.0 | 12.5 |
| 44 | M | 7.7 | 3.000 | 50.0 | 16.7 | 65.2 | 21.7 |
| 21 | M | 8.2 | 2.110 | 54.0 | 25.6 | 72.1 | 34.1 |
| 21 | M | 5.0 | 1.740 | 52.9 | 30.4 | 50.3 | 28.9 |
| 21 | M | 5.5 | 1.650 | 46.8 | 28.2 | 51.0 | 30.9 |
| 21 | M | 6.8 | 1.230 | 24.4 | 19.8 | 33.2 | 27.0 |
| 20 | M | 7.2 | 1.700 | 29.9 | 17.6 | 34.5 | 20.3 |
| 20 | M | 3.6 | 0.180 | 1.5 | 8.3 | 2.4 | 11.9 |
| 9 | M | 9.0 | 0.170 | 5.2 | 30.4 | 7.0 | 40.7 |
| 9 | M | 8.2 | 1.900 | 63.3 | 33.3 | 71.1 | 37.4 |
| 9 | M | 8.0 | 0.575 | 18.9 | 32.8 | 22.8 | 39.8 |
| 8 | M | 6.5 | 2.160 | 78.3 | 36.2 | 85.9 | 39.8 |
| 4 | M | 10.7 | 1.460 | 86.7 | 59.4 | 99.5 | 68.2 |
| 5 | M | 8.1 | 0.405 | 17.1 | 42.2 | 17.0 | 42.0 |
| 2 | M | 6.4 | 0.235 | 17.1 | 72.8 | 19.7 | 83.8 |
| 6 | M | 8.4 | 0.410 | 17.9 | 43.7 | 21.4 | 52.3 |
| $\bar{X}$ | | 6.9 | 1.175 | 31.4 | 30.6 | 36.7 | 36.4 |

[1]Age is in months.

[2]C/N is the carbazole hexuronic acid/naphthoresorcinol hexuronic acid ratio.

[3]Creatinine/ml (Cr/ml) is given as mg./ml.

[4]Chondroitin sulfate/ml (ChS/ml) is expressed as µgm/ml and is based upon the purified sample of the sodium salt of chondroitin sulfate A.

[5]Total mucopolysaccharides/ml (MPS/ml) is expressed as µgm/ml and is based upon the purified sample of the sodium salt of chondroitin sulfate A as standard in the carbazole huxuronic acid reaction.

concentration was very low, a low C/N ratio usually resulted. This variation in the C/N ratio could probably have been reduced if a more representative amount of mucopolysaccharide had been measured. The C/N ratio for the various mucopolysaccharides are given in Table V. When chondroitin sulfate B was present, the ratios were very low. Low ratios were also observed when chondroitin sulfate B was added and recovered from urine. Therefore, if chondroitin sulfate B was present in the urine of the dwarf cattle a low C/N ratio should have been obtained, but it did not serve to distinguish the brachycephalic dwarfs from other dwarfs or controls and in no case was there a higher than normal value with the naphthoresorcinol reaction.

There was also considerable variation in the amount of creatinine, chondroitin sulfates, and total mucopolysaccharide as seen in Fig. 6. The mean ($\bar{X}$) and standard deviation were calculated, but the standard deviation was very high and is not given because all the variables could not be taken into account. The mean is given in Tables IV and VII only for comparison and it does not suggest representative samples. When the ratio of chondroitin sulfates (Fig. 7) or total mucopolysaccharide (Fig. 8) to creatinine were plotted against age, a significant decrease was evident with advancing age. In all cases the brachycephalic dwarfs fell within the same range as other dwarfs and controls and no abnormal qualitative or quantitative excretion of mucopolysaccharide was observed. Chondroitin sulfates accounted for most of the mucopolysaccharides excreted in the urine, although small amount of other uronic acid positive material appeared to be present. From the naphthoresorcinol reaction and by analogy to findings of others the chondroitin sulfate excreted in all animals was probably chondroitin sulfate A and not chondroitin sulfate B.

Table V. C/N ratio for the various mucopolysaccharides.

| Mucopolysaccharide | C/N ratio |
|---|---|
| Chondroitin sulfate A | 13.6 |
| Chondroitin sulfate B | 0.78 |
| Chondroitin sulfate C | 12.25 |
| Hyaluronic acid | 10.3 |
| Heparitin sulfate | 10.0 |
| Heparin | 21.0 |
| 25% ChS A and 75% ChS B | 1.11 |
| 50% ChS A and 50% ChS B | 1.75 |
| 75% ChS A and 25% ChS B | 3.34 |

Figure 6. Scattergram of analytical values for creatinine (Cr), chondroitin sulfates (ChS), and total mucopolysaccharides (MPS) in the urine of dwarf and normal cattle.

■, brachycephalic dwarfs; Δ, dwarf controls; O, normal controls.

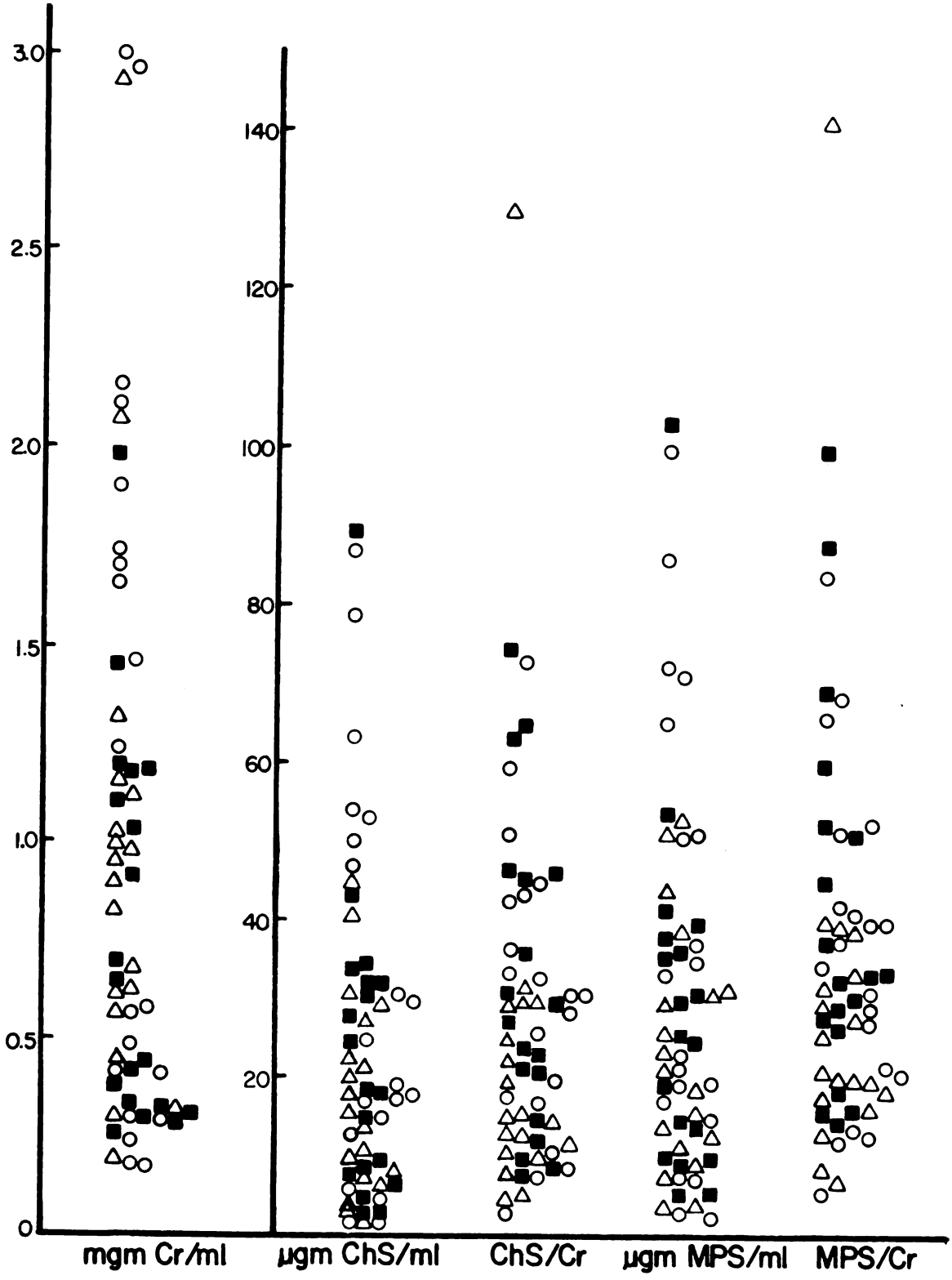

3.0
2.5
2.0
1.5
1.0
0.5
0
140
120
100
80
60
40
20
mgm Cr/ml
µgm ChS/ml
ChS/Cr
µgm MPS/ml
MPS/Cr

Figure 7. The amount of chondroitin sulfates (ChS) relative to creatinine in urine of dwarf and normal cattle of various ages.

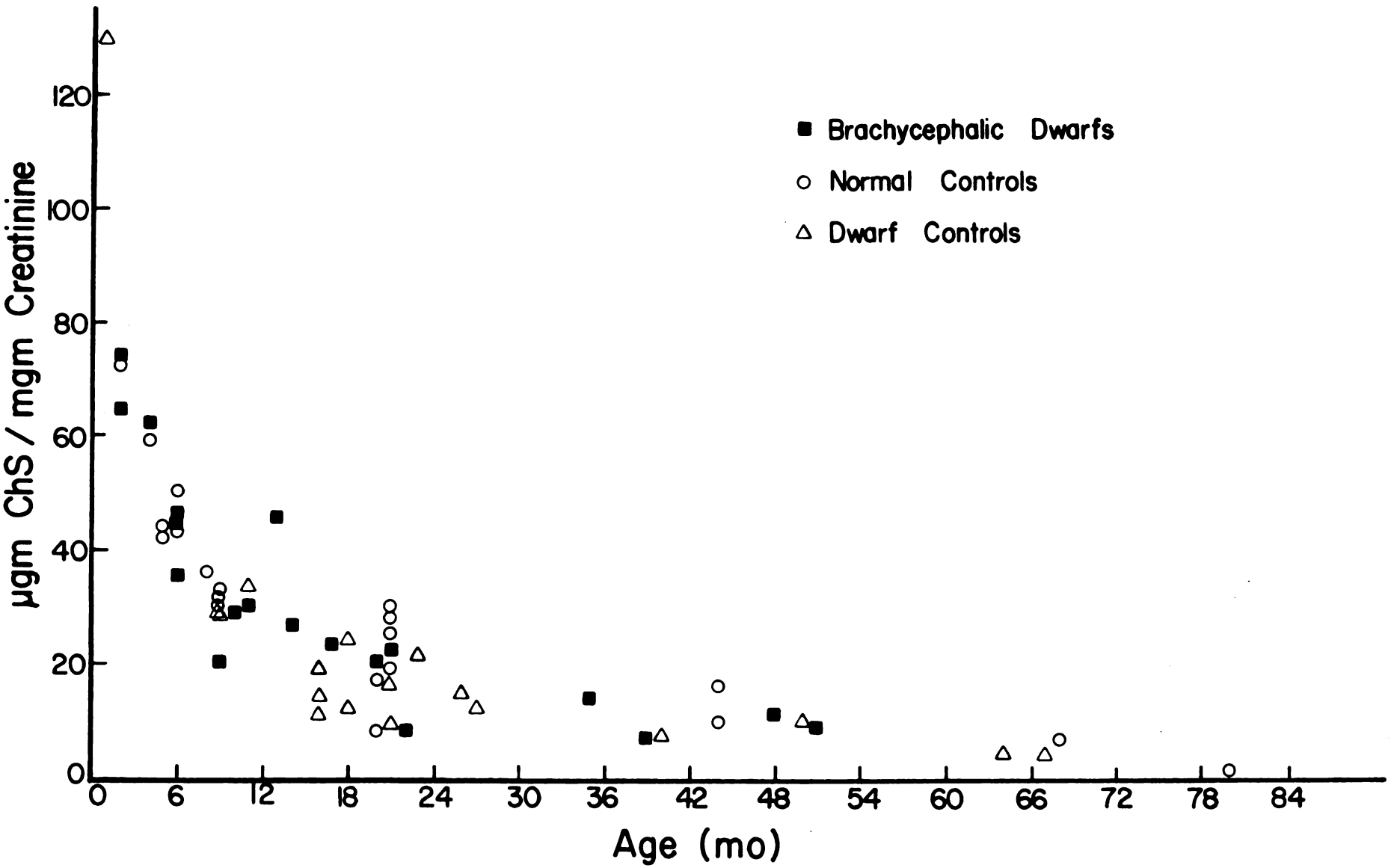
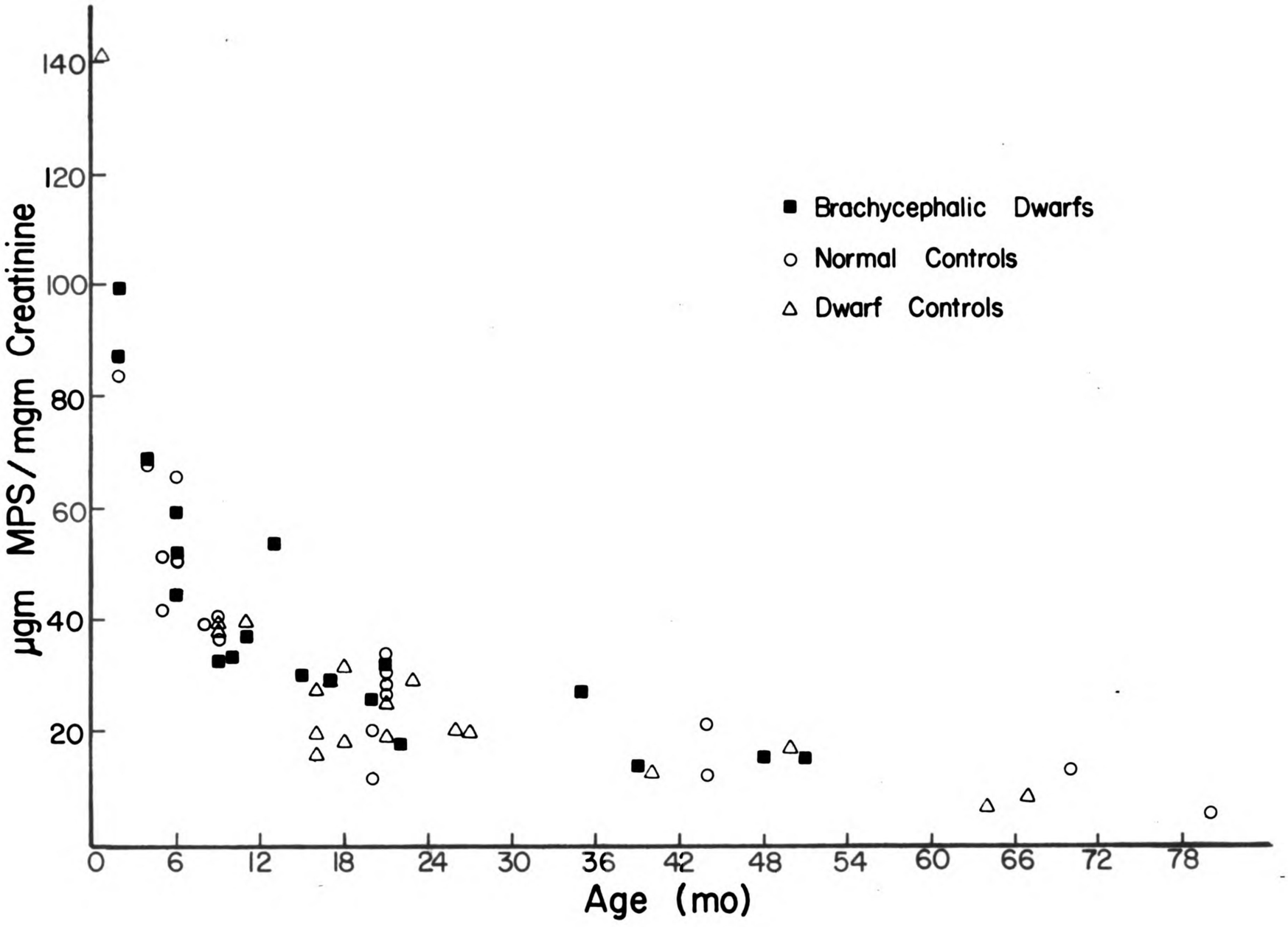

µgm ChS / mgm Creatinine
120
100
80
60
40
20
0
0
6
12
18
24
30
36
42
48
54
60
66
72
78
84
Age (mo)
Brachycephalic Dwarfs
Normal Controls
Dwarf Controls

Figure 8. The total quantity of mucopolysaccharide (MPS) relative to creatinine in the urine of dwarf and normal cattle of various ages.


140
120
100
80
60
40
20
µgm MPS/mgm Creatinine
0
6
12
18
24
30
36
42
48
54
60
66
72
78
Age (mo)
Brachycephalic Dwarfs
Normal Controls
Dwarf Controls

Recovery of Mucopolysaccharides from Urine.

Chondroitin sulfates A and B wee added to urine and the sample treated as previously. As seen in Table VI, the recovery values are satisfactory. There was no significant difference in the recovery of chondroitin sulfate A or B and thus, if chondroitin sulfate B were present in the samples it was precipitated. The C/N ratio was not greatly altered by the addition of chondroitin sulfate A but was significantly decreased with the addition of chondroitin sulfate B. Recovery of chondroitin sulfate A was attempted in distilled water but a precipitate did not form with CTAB. It was later learned that the addition of an electrolyte and albumin was necessary for precipitation.

Excretion of Mucopolysaccharides in Patients with Hurler's Disease and in Normal Humans.

The excretion of mucopolysaccharides in patients with Hurler's syndrome and normal humans is shown in Table VII, but the bar graph (Fig. 9) more clearly depicts the data. Total mucopolysaccharides by the carbazole uronic acid test were higher in all Hurler's patients, however, the chondroitin sulfates per mg. of creatinine in two of the Hurler's patients fell within the normal range. Further the C/N ratio was very low in all the Hurler's except in the two patients that excreted normal quantities of the chondroitin sulfates. This would indicate that these two patients are probably variants that excrete predominantly heparitin sulfate while the other patients excreted both heparitin sulfate and chondroitin sulfate B. Since mucopolysaccharide excretion is known to be variable with age in humans (10,11), it would be important to consider, but to date data pertaining to age of the patients have not been made available.

Table VI. Recovery of mucopolysaccharides from urine

| MPS Added | Quantity Added | Total ChS | Urine ChS | Difference | % Recovered | C/N |
|---|---|---|---|---|---|---|
| - | 0 | - | 488 | - | - | 9.0 |
| ChS A | 91 | 576 | 488 | 88 | 96.7 | 9.2 |
| ChS A | 455 | 920 | 488 | 432 | 94.9 | 9.8 |
| ChS B | 96 | 588 | 488 | 100 | 104.2 | 4.8 |
| ChS B | 480 | 972 | 488 | 484 | 100.8 | 2.1 |
| - | 0 | - | 173 | - | - | - |
| ChS A | 100 | 269 | 173 | 96 | 96.0 | - |
| ChS A | 500 | 690 | 173 | 517 | 103.4 | - |

Table VII. Excretion of mucopolysaccharides in patients with Hurler's disease and normal humans.

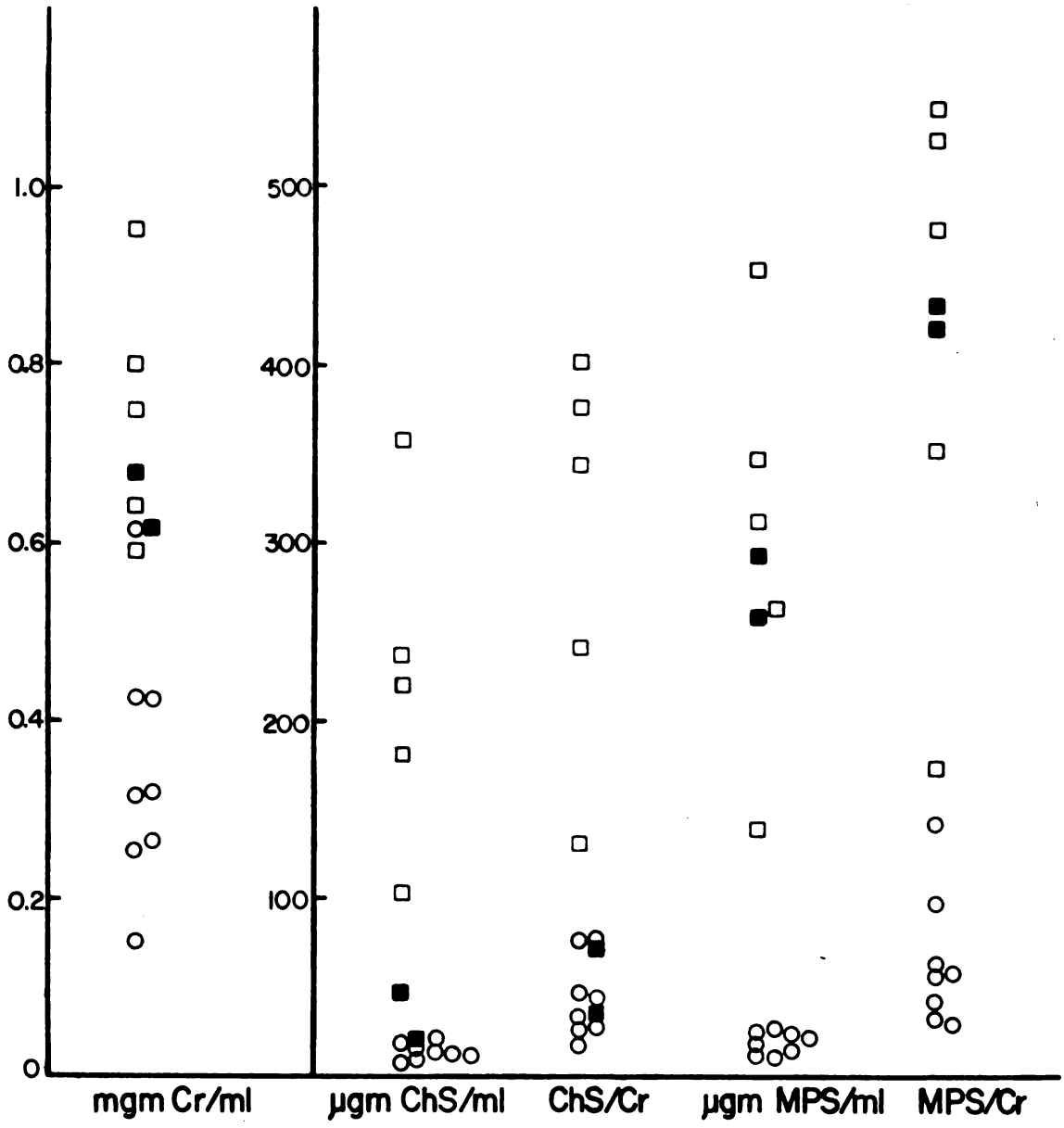
| Name | Type[1] | C/N | Cr/ml | ChS/ml | ChS/Cr | MPS/ml | MPS/Cr |
|---|---|---|---|---|---|---|---|
| CK | C | 6.7 | 0.255 | 19.9 | 78.0 | 25.1 | 98.4 |
| KE | C | 8.6 | 0.425 | 20.9 | 49.2 | 26.9 | 63.3 |
| LG | C | 5.4 | 0.265 | 7.2 | 27.2 | 11.3 | 42.6 |
| DR | C | 12.9 | 0.315 | 14.1 | 44.8 | 18.6 | 59.0 |
| MH | C | 7.3 | 0.425 | 12.1 | 28.5 | 24.5 | 57.6 |
| BBA | C | 5.0 | 0.150 | 11.9 | 79.3 | 21.4 | 142.7 |
| GB | C | 8.0 | 0.320 | 9.7 | 30.3 | 10.9 | 34.1 |
| SA | C | 10.8 | 0.615 | 11.3 | 18.4 | 14.1 | 22.9 |
| $\bar{X}$ | | 8.1 | 0.346 | 13.4 | 44.5 | 19.1 | 65.1 |
| CS | H* | 8.0 | 0.615 | 21.5 | 35.0 | 258.8 | 420.8 |
| KB | H* | 11.9 | 0.675 | 48.0 | 71.1 | 292.8 | 433.8 |
| $\bar{X}$ | | 9.95 | 0.645 | 34.7 | 53.0 | 275.8 | 427.3 |
| TG | H | 1.5 | 0.750 | 180.0 | 240.0 | 263.4 | 351.2 |
| TT | H | 1.7 | 0.640 | 220.0 | 343.8 | 348.0 | 543.8 |
| TS | H | 1.4 | 0.950 | 358.7 | 377.6 | 453.0 | 476.8 |
| DG | H | 1.5 | 0.590 | 237.3 | 402.2 | 310.8 | 526.8 |
| DT | H | 1.5 | 0.800 | 104.0 | 130.0 | 138.6 | 173.3 |
| $\bar{X}$ | | 1.5 | 0.746 | 220.0 | 298.7 | 302.8 | 414.4 |

[1]Types are controls (C), Hurler's (H*) that excrete predominantly heparitin sulfate, and Hurler's (H) that excrete both heparitin sulfate and chondroitin sulfate B.

The other abbreviations are the same as in Table IV.

Figure 9. Scattergram of the excretion values for creatinine (Cr), chondroitin sulfate (ChS), and total mucopolysaccharide (MPS) in urine of Hurler's patients and appropriate controls.

■, Hurler's syndrome that excrete predominantly heparitin sulfate; □, Hurler's syndrome that excrete both heparitin sulfate and chondroitin sulfate B; O, controls.


1.0
0.8
0.6
0.4
0.2
0
500
400
300
200
100
mgm Cr/ml
µgm ChS/ml
ChS/Cr
µgm MPS/ml
MPS/Cr

# DISCUSSION

In attempting to develop a method to differentiate between the various mucopolysaccharides it was hoped that a specific chondroitinase could be induced with purified mucopolysaccharides, but the chondroitinase induced reacted with each of the three chondroitin sulfates whether induced on purified chondroitin sulfate A, B or hyaluronic acid. This non-specific induction has also been shown in Proteus vulgaris (39) and Flavobacterium (37). The enzyme was purified over 200 fold and the order of activity with the chondroitin sulfate was A > B > C throughout purification. These results would indicate that there was one enzyme and it could breakdown all three chondroitin sulfates.

The enzymatic breakdown of the chondroitin sulfates was proportional to the amount of substrate and thus could readily serve as a base for a quantitative measurement. With the rates being different for the three chondroitin sulfates it could possibly serve to differentiate between A, B and C. However, this would require relatively pure samples and no interference from other mucopolysaccharides. Since usually one of the chondroitin sulfates is present in a given tissue, this method could serve to study the metabolism of the chondroitin sulfates. Chondroitin sulfate A, B and C should give the same or similar change in absorbancy since A and B give the same end product and the end product of C differs only in the position of the sulfate group. It is difficult to obtain an absolute value for the change in absorbancy and in this report the sodium salt of a highly purified

sample of chondroitin sulfate A served as a reference to calculate the concentration of chondroitin sulfate.

The purified enzyme appeared to produce a single product with the sodium salt of chondroitin sulfate A with a structure which can be tentatively formulated as an N-acetylchondrosin sulfate that is unsaturated α, β to the carboxyl group of the uronic acid moiety, but formal proof for the structure is lacking.

In attempting to isolate mucopolysaccharides from liver and blood of dwarf and control animals preliminary experiments indicated no difference in the amount or type of mucopolysaccharide. In surveying a large number of various types of dwarf and control cattle no abnormal excretion of mucopolysaccharides was detected whether measuring chondroitin sulfates, uronic acid positive materials, or estimating chondroitin sulfate B by the C/N ratio. The chondroitin sulfates accounted for most of the mucopolysaccharides excreted in the urine and this was in all probability chondroitin sulfate A judging from the C/N ratio. By the same token only a very small amount of chondroitin sulfate B could have been present in the urine of the dwarf animals. These findings are in agreement with the brief note of Tyler et al (25).

Since both chondroitin sulfate A and chondroitin sulfate B when added to the urine of cattle could be recovered in good yields, it would indicate that the methods were adequate. In addition urine samples were obtained from Hurler's patients and the mucopolysaccharides were isolated and determined as in the animal specimens. The Hurler's patients showed the expected abnormal excretion of mucopolysaccharides. The different variants could be identified by the methods employed,

some excreting predominantly heparitin sulfate and others excreting varying amounts of chondroitin sulfate B and heparitin sulfate.

Thus, with these experiments to give confidence to the methods it would appear that the dwarf cattle tested did not excrete abnormal amounts or unusual types of mucopolysaccharides. The marked change of mucopolysaccharide excretion with age especially during the first few months emphasizes the need to consider this factor when making comparisons. Whether the recent conflicting reports in the literature may be due to inadequate concern for age of the animal is difficult to assess since this is not always given. Alternatively different mutants may actually exist, some excreting abnormal amounts of mucopolysaccharides while others are normal in this respect.

Although several theories have been proposed to explain the facts (for a review see Meyer and Hoffman (57)), the metabolic defect in Huler's syndrome is unknown. A recent theory proposed by Dorfman (58) suggests that the disorder involves a change in the protein of an essential mucopolysaccharide-protein complex preventing the polysaccharide from attaching to the protein. These mucopolysaccharides accumulate in the tissues and are excreted in the urine. It is difficult to explain the different variants, but it appears that this may be understood as the pathways of metabolism of the mucopolysaccharides are delineated.

## SUMMARY

The qualitative and quantitative excretion of mucopolysaccharides was measured in dwarf and normal cattle. Chondroitin sulfates were converted to the unsaturated disaccharides by the enzyme chondroitinase and estimated spectrophotometrically. Total mucopolysaccharides were estimated by the carbazole uronic acid test. The amount of chondroitin sulfate B was estimated by the carbazole and naphthoresorcinol reactions. The methods were validated by adding mucopolysaccharides to urine and measuring their recovery and by testing urine samples of known Hurler's patients. The Hurler's patients showed the expected abnormal excretion of mucopolysaccharides. Furthermore chondroitin sulfate A and chondroitin sulfate B could both be quantitatively recovered from cattle urine. By all qualitative and quantitative tests the mucopolysaccharide excretion was similar in dwarf and normal animals.

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