A review and selection of simple laboratory methods used for the study of glycosaminoglycan excretion and the diagnosis of the mucopolysaccharidoses

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SYNOPSIS The current state of knowledge on the biochemical abnormalities in the mucopolysaccharidoses is reviewed. Methods for the estimation and identification of glycosaminoglycans excreted in the mucopolysaccharidoses are discussed and a selection of simple and reliable methods of value in a small hospital laboratory have been given in detail in an appendix.

The mucopolysaccharidoses are a rare group of inherited disorders of connective tissue metabolism of which the condition most familiar to clinicians is Hurler's syndrome (previously given the unpleasant name 'gargoylism' due to the unusual facial appearance). The clinical features have been reviewed by McKusick and others (McKusick *et al*, 1965; McKusick, 1969, 1972). These disorders have long been known to be associated with excessive mucopolysaccharide excretion (Dorfman and Lorincz, 1957; Meyer *et al*, 1958). The term mucopolysaccharide, although still retained in medical literature, does lead to confusion between polysaccharides containing both hexosamines and hexuronic acids and a variety of mucins and other mucosubstances which do not contain hexuronic acids. Jeanloz (1960) introduced the term glycosaminoglycuronans (GAG) for the acidic polysaccharides only, and the current nomenclature and suitable abbreviations for these compounds are given in table I. Knowledge of the detailed structure of these compounds has been reviewed by Muir (1969) and Dorfman and Matalon (1972) and is summarized in table II. The amino sugar residues may be N-acetylated, N-sulphated or O-sulphated at carbon 4 or carbon 6. In the interests of clarity, the trivial names of the residues will be used throughout the rest of this article, for example, 2-acetamido-2-deoxy- α -D-glucopyranose will be referred to as N-acetylglucosamine.

In 1964 Van Hoof and Hers identified the mucopolysaccharidoses as lysosomal storage disorders by electron microscopy, and since that date it has become increasingly apparent that they are due to genetic defects in the synthesis and activity of

Past Name	Current Name	Abbreviation
Acid mucopolysaccharide	Glycosaminoglycuronan ¹	GAG
Hyaluronic acid	Hyaluronic acid	HA
Chondroitin	Chondroitin	Ch
Chondroitin sulphate A	Chondroitin-4-sulphate	C-4-S
Chondroitin sulphate B	Dermatan sulphate	DS
Chondroitin sulphate C	Chondroitin-6-sulphate	C-6-S
Heparin	Heparin	Hep
Heparitin sulphate	Heparan sulphate	HS
Heparin monosulphate		
Corneal keratosulphate	Keratan sulphate I	KS I
Skeletal keratosulphate	Keratan sulphate II	KS II
Chondroprotein	Proteoglycan	
Protein-polysaccharide complex		

Table I Current nomenclature based on the work of Jeanloz (1960)

¹The term glycosaminoglycan is usually preferred in current literature since glycosaminoglycuronans contain hexuronic acids, and this term would not include the keratan sulphates which contain galactose instead of hexuronic acid residues.

enzymes essential for the degradation of connective tissue GAG. Fratanoti *et al* (1968a) incubated cultured fibroblasts from patients with mucopolysaccharidoses, in the presence of ³⁵S-labelled sulphate, and showed increased accumulation and delayed release of label in abnormal cells compared with normal controls and concluded that this indicated defective degradation of synthesized GAG. In the same year they (1968b) also published the exciting finding that when Hurler and Hunter cells were mixed, there was a normal sulphate uptake as a result of the diffusion of 'corrective factors' from one cell type to the other; these factors were found in the supernatant fluid and have since been isolated from

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GAG	Abbreviation	Amino Sugar	Hexuronic Acid	Sulphate
Hyaluronic acid	HA	Glu N	GUA	—
Chondroitin	Ch	Gal N	GUA	—
Chondroitin-4-sulphate	C ₄ S	Gal N	GUA	O-SO ₄
Chondroitin-6-sulphate	C ₆ S	Gal N	GUA	O-SO ₄
Dermatan sulphate	DS	Gal N	GUA	O-SO ₄
			IDA	
Heparan sulphate	HS	Glu N	GUA	O-SO ₄
			IDA	N-SO ₄
Heparin	Hep	Glu N	GUA	O-SO ₄
			IDA	N-SO ₄
Keratan sulphate	KS	Glu N	None ¹	O-SO ₄

Table II Structure of GAG

Abbreviations: Glu N glucosamine
Gal N galactosamine
GUA glucuronic acid
IDA iduronic acid

¹KS contains galactose instead of hexuronic acid.

The hexosamine residues are N-acetylated except in the case of Hep and HS where some residues are N-sulphated. IDA is the C-5 epimer of GUA.

normal human urine. Their enzymic nature was suggested by Cantz *et al* (1970, 1972) and Bach *et al* (1972).

Recently, a number of specific enzyme defects have been identified and have led to a slight modification of the earlier classifications (McKusick, 1972). The current state of knowledge on enzyme defects is summarized in table III. The importance of these recent enzyme discoveries is threefold:

1. With suitable assay procedures they should enable an unequivocal diagnosis of the different mucopolysaccharidoses to be made.
2. Enzyme assays on cultured amniotic fluid cells will enable early intrauterine diagnosis to be made.
3. Suitable enzyme-replacement therapy may be possible.

However, at the time of writing, enzyme assays are not widely used for diagnosis because suitable substrates are not readily available (with the exception of p-nitrophenyl- α -D-glucosamine for type III B), and substrate preparation and the culture of tissue cells are difficult and time-consuming. Urine

analysis for excessive and abnormal GAG excretion is, therefore, likely to remain important for some time to come as the initial diagnostic test.

An extensive literature has developed in recent years on methods used for detection and evaluation of abnormal GAG excretion. Most have been developed in research laboratories and require special apparatus and expertise not usually available in clinical chemistry departments. As a result many laboratories are reluctant to enter this field owing to a belief that the methodology required is too elaborate. It is the purpose of this article to discuss a number of simple procedures which are reliable and adequate for diagnostic purposes and to recommend those which the author has found to be most useful. The latter are described in detail in the appendix.

The Sample

The diagnosis of mucopolysaccharidoses is most likely to be required in early childhood when 24-hour specimens of urine are difficult to collect. The problem of incomplete collections may be overcome

Mucopolysaccharidosis	Type ¹	Enzyme Defect	References
Hurler	IH	α L-iduronidase	Matalon and Dorfman (1972)
Scheie	IS (formerly V)		
Hunter	II	L-iduronosulphate sulphatase	Bach <i>et al</i> (1973); Sjöberg <i>et al</i> (1973)
Sanfilippo	IIIA	Heparan sulphate sulphamidase	Kresse (1973)
Sanfilippo	IIIB	α N acetylglucosaminidase	O'Brien (1972); von Figura <i>et al</i> (1973)
Morquio	IV	N-acetyl hexosamine-4-sulphate sulphatase	Matalon <i>et al</i> (1974)
Maroteaux Lamy	VI	Arylsulphatase B	Stumpf <i>et al</i> (1973)
β glucuronidase ² deficiency	VII	β glucuronidase	Sly <i>et al</i> (1973); Beaudet <i>et al</i> (1972)

Table III Current views on the enzyme defect in the mucopolysaccharidoses

¹McKusick (1972).

²Only two patients have been described with this disorder. In one, urinary GAG were normal (Sly *et al*, 1973) and in the other DS excretion was elevated (Beaudet *et al*, 1972).

by relating GAG excretion to creatinine excretion, and this may be done on random samples of urine collected from outpatients. Although creatinine excretion varies during a 24-hour period and the GAG/creatinine ratio is increased at night (Di Ferrante and Lipscomb, 1970), the excretion of both substances remains fairly constant during waking hours (say 0900-1800 hours) and the GAG/creatinine ratios in samples obtained during this period are similar to those obtained on complete 24-hour urine collections (Pennock *et al*, 1973).

Infected samples should not be used, but some preservatives may interfere with the analyses, eg, thymol interferes with some screening tests and toluene lowers the efficiency of precipitation methods (Di Ferrante, 1967). Their use is best avoided if possible, and the author prefers samples to be frozen solid as soon as possible after collection, and kept frozen during transport to the laboratory and while awaiting analysis. However, freezing is not essential, and adequate diagnostic information can be gained from the analysis of samples which have been sent through the post provided the sample is not infected. For postal delivery, the author requests three or more random samples of urine collected at different times; all non-infected samples can be pooled, and this usually gives enough material for a full qualitative analysis if necessary (see below).

Some urine samples may be very concentrated, which may lead to inadequate isolation of GAG and hence error in quantitative analyses (Di Ferrante, 1967). The sample should be diluted with distilled water to lower the specific gravity to below 1.020 before any tests, including estimation of creatinine, are done. Very dilute samples of low specific gravity and creatinine content should be discarded since small errors in the estimation of the latter may give false positive or false negative results.

Screening Tests

The main objectives in selecting any chemical screening test should be that the test (1) should never give a false negative result, (2) is easy and quick to perform, and (3) gives a minimum number of false positive results which would require further analysis. Although a large number of screening tests have been devised, the majority fail on at least one of these criteria, mainly because they take no account of urine concentration. If an identical volume of urine is used in each case, a concentrated urine from a normal individual may give a false positive result while a dilute urine from a patient with a mucopolysaccharidosis will give a false negative result.

Screening tests fall into two main categories, paper spot tests based on the interaction of urinary GAG

with a suitable cationic dye, and precipitation tests based on the interaction of GAG with acidified albumin, quaternary ammonium compounds, or alcian blue.

Spot Tests

A number of paper spot tests have been described in which a fixed volume of urine is applied to filter paper, dried, and stained with a solution of suitable dye. The dyes used have included toluidine blue (Berry and Spinanger, 1960), alcian blue (Carson and Neill, 1962; Procopis *et al*, 1968), and azur A (Berman *et al*, 1971). None of these tests takes urine concentration or age-related changes in GAG excretion into account. Carter *et al* (1968) found 31.5% false negative results using toluidine blue to test 54 urine samples from patients with Hurler's syndrome, and Procopis *et al* (1968) found a false negative result using alcian blue. Allowing for the urine concentration by spotting a urine volume containing a fixed amount of creatinine does not eliminate false negative results. The method using azur A (Berman *et al*, 1971) was based on a study of only 17 samples. This test does not take urine concentration into account, and the recommended procedure seems unlikely to be an improvement on the other screening tests.

In view of the high incidence of false negative results, paper spot tests should not be used as screening tests for the mucopolysaccharidoses.

Precipitation Tests

The interaction of GAG with acidified bovine albumin to form a flocculent precipitate has also been used as a screening test (Dorfman, 1958; Denny and Dutton, 1962). This test also does not take urine concentration into account, and false negative results have been found in three patients with Hurler's syndrome and three patients with Sanfilippo syndrome using the test in its original form (Pennock *et al*, 1973). Even when refined by quantitative measurement of the turbidity (Steiness, 1961) false negative results have also been found in 9.2% of samples from patients with Hurler's syndrome (Carter *et al*, 1968) and in patients with Sanfilippo syndrome (Lewis *et al*, 1974).

The observation that GAG will react with quaternary ammonium compounds to give a precipitate (Scott, 1960) has also been used as a screening test. Renuart (1966) added a buffered solution of cetyltrimethyl ammonium bromide (CTAB) to urine and observed a flocculent precipitate after 30 minutes in samples with high GAG content. In the author's experience this test is often difficult to interpret since

normal urines frequently give a peculiar precipitate and a true flocculent precipitate is so rarely seen that it is difficult to explain to an inexperienced observer what to look for.

A semiquantitative test in which cetylpyridinium chloride (CPC) is added to urine and the turbidity recorded was described by Manley and Hawksworth (1966). They found that timing was critical since aggregation of particles occurred after four minutes. They also found that results changed with age throughout childhood, but again this test does not take urine concentration into account.

Precipitation of GAG with CPC or CTAB is affected by ionic strength and pH (Scott, 1960) as well as by concentration and, if these factors are not taken into account, urine samples may give false positive or false negative results. The effects of ionic strength and pH can be overcome by buffering the CPC solution, and the use of citrate buffer at pH 4.8 (Pennock, 1969) also overcomes the critical time dependence of the CPC test as described by Manley and Hawksworth (1966). The inclusion of standards enables a quantitative result to be obtained. It is essential that allowance is made for urine concentration effects by relating the results to the creatinine content of the sample, and that it is appreciated that GAG excretion changes with age throughout childhood so that the correct normal range can be used (Pennock, 1969). The CPC citrate test has the following advantages over other screening tests:

1. The effects of differences in ionic strength and pH of the sample are overcome.
2. The precipitate is stabilized so that timing of the reaction is less critical.
3. It is quantitative and thus lends itself to quality control.
4. By relating results to creatinine, account is taken of urine concentration.
5. It may be applied to random urine samples.
6. The test has not been known to give false negative results even in patients with Sanfilippo syndrome when other screening tests have failed (Pennock, 1973; B. Turner—personal communication).
7. The incidence of false positive results is acceptable; 5% in the first year of life and 2.6% in older children were found in one study of 3000 children (Pennock *et al*, 1973) and 3.6% in another study of 1000 children (Valdivieso *et al*, 1973).

It is for these reasons that the CPC citrate test is given in more detail as the screening method of choice in the appendix.

Quantitative Estimation of Urinary GAG

Quantitative estimation is based on measurement of

hexuronic acid residues which all the GAG contain, except keratan sulphate. Separation of GAG from interfering substances has been achieved in a number of ways. Hexuronic acid may be measured on the dialysed sample directly (Segni *et al*, 1964; Rosenfeld, 1971) or after a further purification step, such as fractionation on ECTEOLA cellulose columns, which enables sulphated and non-sulphated GAG to be measured separately (Wessler, 1967; Blackham and Raine, 1970), or by precipitation with CTAB or CPC (Thompson and Castor, 1966; Teller *et al*, 1962) or aminoacridine (Bitter *et al*, 1970; Dean *et al*, 1971). CPC and CTAB are poor precipitants of GAG in the absence of salts, and it is preferable to use the method described by Di Ferrante and Rich (1956) in which these agents are added to untreated urine. CPC is the best of the three precipitants in the presence of urinary salts (Di Ferrante, 1967) although glycoproteins tend to be co-precipitated. The latter problem is easily overcome as a higher concentration of precipitant than recommended by Di Ferrante (1967) leaves glycoproteins in solution (Goldberg and Cotlier, 1972; Pennock, 1973). Aminoacridine has not been widely used because, in addition to being less efficient than CPC, the method is more time consuming.

Column chromatography of either dialysed or CPC precipitated samples has held an important place in research into GAG excretion patterns but is time consuming and results in a large number of fractions for further analysis. However, the addition of urine directly to an ECTEOLA cellulose column and elution of GAG with a single solvent gives the same results as CPC precipitation (Di Ferrante, 1967).

The amount of hexuronic acid containing material present after dialysis varies with the duration of dialysis, the number of changes of distilled water, and pore size of the membrane, and considerable losses of GAG may occur (Rosenfeld, 1971). This applies particularly to samples from patients with Hurler's syndrome in which up to 50% of the GAG may be dialysable compared with 11% in samples from normal subjects (Constantopoulos, 1968); similar losses have been found in Scheie's syndrome even when Visking dialysis tubing was heated to reduce the pore size (Pennock, 1973).

However, despite losses, the hexuronic acid content of a dialysed sample is usually, but not always, greater than that recovered by CPC precipitation of untreated urine. The CPC precipitable hexuronic acid was found by Rosenfeld (1971) to be 25.55% of non-dialysable hexuronic acid in normal adults and 70% in children, and a similar range of 11–65% in adults but a greater range of 71–156% in children was found by Pennock *et al* (1975), ie, in samples from

some normal children a large portion of CPC precipitable material will be lost on dialysis. This has also been observed in some patients with mucopolysaccharidoses (see above). It is not known whether the high results in some dialysed samples are due to interfering chromogens, as suggested by Manley *et al* (1968), or to the presence of GAG which is not CPC precipitable, as suggested by Rosenfeld (1971).

Rosenfeld (1971) has shown the presence of at least three GAG types and suggested the possibility of a fourth. GAG may be: (1) high molecular weight, non-dialysable but CPC precipitable; (2) low molecular weight, ultrafilterable, and thus lost on dialysis but CPC precipitable; (3) very low molecular weight, ultrafilterable and lost on dialysis, and not CPC precipitable; (4) low molecular weight and attached to proteins which are neither dialysable nor precipitable. Di Ferrante *et al* (1972) have confirmed that low molecular weight GAG exist which are not CPC precipitable and which will pass through a Visking dialysis membrane even after reduction of pore size with heat treatment. However, the actual concentration of these low molecular weight GAG in patients with mucopolysaccharidoses is the same as in normal children so that the estimation of this fraction is of little diagnostic value, although further research into their nature could be enlightening. It is important to emphasize that precipitation of GAG with CPC from untreated urine gives the same results as isolation by ECTEOLA cellulose chromatography. The former is the simpler of the two methods and is therefore described in the appendix. Because the proportion of CPC precipitable GAG which is lost on dialysis varies among normal individuals and patients with mucopolysaccharidoses one must reiterate the comment made by Di Ferrante (1967), 'One cannot be too emphatic in discouraging dialysis of urine prior to precipitation'.

Once a suitable GAG preparation has been isolated the estimation of hexuronic acid presents little problem and it may be measured by the carbazole-sulphuric acid method (Dische, 1947) or the borate modification of that method (Bitter and Muir, 1962). The latter gives almost equal colour yield for glucuronic and iduronic acid and may be carried out as a single step reaction in the modified manner described in the appendix.

Whiteman (1973a, b) has described a quantitative test in which GAG are precipitated with alcian blue and the dye is ultimately eluted from the precipitate and measured colorimetrically. This test may offer advantages over CPC precipitation in that the colour yield for equal amounts of different GAG is almost identical and keratan sulphate is precipitated. Results by this method tend to be slightly higher than those obtained by measuring CPC precipitable

hexuronic acid, and this may be due to other unknown reactants but there is a reasonable correlation between the two methods (Whiteman, 1973b). Full details of this test as an alternative to the CPC citrate test are not included in the appendix since it has been used in only a few laboratories and its true value has not yet been fully assessed. The precipitate is often very finely dispersed and often difficult to harvest without centrifugation at least at 2000 RCF for 15 minutes, facilities for which may not be available in many small clinical laboratories. It may prove to be of special value in the study of Morquio's disease if it proves more reliable than CPC for the isolation of keratan sulphate (*vide infra*).

Identification of Type of GAG excreted

The major GAG excreted by normal individuals is chondroitin-6-sulphate (Di Ferrante, 1969) whereas heparan sulphate, dermatan sulphate, and keratan sulphate are excreted in excess in patients with mucopolysaccharidoses (see interpretation of GAG excretion pattern below and table IV).

Patient	Major GAG Excreted
Normal	Chondroitin-6-sulphate
Type IH	Dermatan sulphate and heparan sulphate in ratio 3:1
Type IS	Dermatan sulphate ¹
Type II	Heparan sulphate
Type III	Heparan sulphate with or without dermatan sulphate
Type IV	Keratan sulphate
	Chondroitin-6-sulphate
Type VI	Dermatan sulphate

Table IV Glycosaminoglycan excretion patterns

¹Pattern may be equal amounts of both GAG or, more often, difficult to distinguish from type I (see text).

Identification of GAG excretion pattern requires simple methods for separating and assessing the relative proportions of heparan, dermatan, and keratan sulphates. Most investigations have used column chromatography but separations are often imperfect (Kaplan, 1969). Full identification requires hydrolysis of the appropriate fractions and structural identification by defining the principal hexosamines (see table II) by further column chromatography which is not only time-consuming but often affords inadequate separation. These methods are quite unsuited to the small hospital laboratory.

Electrophoretic separation may be done in barbitone buffer (Manley and Hawksworth, 1966). Although this method does not separate dermatan

sulphate (DS) from the chondroitin sulphates (CS) nor heparan sulphate (HS) from keratan sulphate, it is a useful method since galactosamine containing GAG and glucosamine containing GAG migrate as two separate bands. Other separations have been described which are based on GAG structure and include electrophoresis in barium acetate (Wessler, 1968) in which keratan sulphate separates poorly from chondroitin sulphates, and electrophoresis in cupric acetate (Hata and Nagai, 1972) and zinc sulphate (Hardingham and Phelps, 1970), both of which fail to separate heparan and keratan sulphates, and the poorly defined separations of other GAG are more difficult to interpret than those achieved in barbitone or barium acetate (Pennock, 1973). Barium acetate gives good separation of DS and HS (Lewis *et al*, 1974), and this method has been selected and described in the appendix because of its speed and simplicity.

Thin layer chromatography of GAG has been used by Wusteman *et al* (1966) who found difficulty in separating dermatan sulphate, and by Teller and Ziemann (1969) who were unable to separate keratan sulphate from other GAG. The best method described so far is that based on the relative solubility of different GAG, as their calcium salts, in alcohol using a sequence of solvents which affords a very rapid and efficient separation of all GAG (Lippiello and Mankin, 1971; Humbel and Chamois, 1972). The method is included in the appendix with comments on interpretation of patterns of GAG excretion. This is the only method which clearly separates keratan sulphate from other GAG and is thus invaluable in the diagnosis of Morquio's disease.

In summary, the simple tests required include:

1. A reliable screening test for which the CPC citrate test is recommended.
2. Isolation of GAG and estimation as hexuronic acid.
3. Isolation of GAG and identification of excretion pattern by thin layer chromatography and/or electrophoresis.

In most urines, these methods enable a diagnosis to be made but rarely equivocal results may be obtained, and in these cases a more elaborate procedure is necessary such as measuring the proportion of hyaluronidase resistant GAG (Pennock *et al*, 1973), estimation and identification of hexosamine content by gas liquid chromatography (Murphy *et al*, 1974), specific estimation of dermatan sulphate (Di Ferrante *et al*, 1971), estimation of amino sulphate as a measure of heparan sulphate (Lagunoff *et al*, 1967) or measurement of keratan sulphate as galactose (Robertson and Harvey, 1972). All of these methods are rarely required and are not included in the appendix.

Interpretation of Results and Diagnostic Difficulties

False positive CPC citrate screening tests may be found in some patients on certain drugs such as barbiturates, phenytoin, and penicillins but the mechanism is not understood (Pennock *et al*, 1970). Patients with renal disease and mucoproteinuria will also give false positive results, since mucoproteins precipitate with CPC, but CPC precipitable hexuronic acid is usually within normal limits.

Any patient with excessive connective tissue destruction may give a positive CPC citrate screening test and an increased excretion of CPC precipitable hexuronic acid. Notable examples include rickets (Valdivieso *et al*, 1973), malabsorption syndromes with gross osteomalacia, malignant disorders with extensive secondary deposits (including leukaemia) and patients with disseminated lupus erythematosus (Di Ferrante *et al*, 1957); some patients with rheumatoid arthritis (Di Ferrante, 1957; Thompson and Castor, 1966) and some with Marfan's syndrome (Berenson and Dalferes, 1965; Bitter *et al*, 1970) as well as a few other rare disorders (Brimacombe and Stacey, 1964). Qualitative examination of excreted GAG shows that chondroitin sulphates predominate and hence the excretion pattern is similar to normal excretion and easily differentiated from the pattern in the mucopolysaccharidoses.

Patients with one of the mucopolysaccharidoses excrete excessive amounts of heparan, dermatan or keratan sulphates, and the excretion patterns are shown in table IV. The excretion pattern in Hurler's, Scheie's, and Maroteaux-Lamy syndromes is consistent and easy to interpret. In a detailed study of excretion patterns, Kaplan (1969) found roughly equal amounts of heparan sulphate in patients with Hunter's syndrome. Others have failed to confirm this, finding that the pattern in Hunter's syndrome more frequently resembles the pattern in Hurler's syndrome (Maroteaux, 1970; Pennock, 1973). Therefore, these two conditions cannot always be distinguished by the simple methods described. This does not detract from the value of the simple methods since distinction is also difficult to make using more sophisticated techniques, and furthermore the problem is not usually great since distinction between the two syndromes may be made on clinical grounds. Similar comments apply to Scheie's syndrome (MPS Type IS) where the enzyme defect and excretion pattern are identical with those of Hurler's syndrome (Wiesmann and Neufeld, 1970) but the clinical features are distinctly different (McKusick, 1972).

In Sanfilippo syndrome, in which there are two different enzyme defects, there appear to be two patterns of heparan sulphate excretion which cannot

be distinguished by electrophoresis (Lewis *et al*, 1974). However, by thin layer chromatography one type shows a heparan sulphate fraction which migrates in the same position as heparan sulphate from normal urine while the other type shows a heparan sulphate fraction which behaves as if it were chondroitin-4-sulphate. However, the pattern is still distinct from normal and gives no problem in interpretation if electrophoresis in barium acetate is also done when any doubt exists.

The diagnosis of Morquio's disease, although it is clearly a mucopolysaccharidosis with abnormal keratan sulphate metabolism (Robins *et al*, 1963), should be regarded as a separate problem. This is mainly because keratan sulphate excretion may sometimes be normal, especially in adult life (Maroteaux and Lamy, 1967; Linker *et al*, 1970; Jenkins *et al*, 1973). When keratan sulphate excretion is increased the CPC citrate screening test is usually positive and usually there is also an increase in CPC precipitable hexuronic acid since there is a parallel increase in chondroitin sulphate excretion (Pennock, 1973). Although it has been disputed that keratan sulphate is precipitated by CPC, it is now clear that although precipitation is difficult from aqueous solutions it is readily achieved in urine (Di Ferrante, 1967). Indeed the first demonstration of increased keratan sulphate excretion in Morquio's disease by Pedrini *et al* (1962) was achieved using quaternary ammonium precipitants. Furthermore, it seems likely that most of the keratan sulphate is actually excreted as a complex with chondroitin sulphate similar to the form in which it exists in cartilage although probably of reduced molecular size. Since it is only soluble in excess CPC when it is totally released from its hybrid chondroitin sulphate complex (Goldberg, 1973; Kaplan *et al*, 1968), most urinary keratan sulphate would precipitate with the chondroitin sulphate fraction. Recently the excretion of keratan sulphate throughout childhood has been studied by Robertson and Harvey (1972), using ion-exchange chromatography followed by GAG precipitation and estimation of keratan sulphate as galactose. This is a potentially useful method for the study of Morquio's disease and other spondyloepiphyseal dysplasias. This disease clearly requires further study, and its separation from the other mucopolysaccharidoses in this sense appears justified.

One final problem is worthy of mention. It is often difficult to distinguish high abnormal GAG excretion from normal results in the neonatal period since GAG excretion in relation to creatinine is high at this time of life even in normal individuals (Pennock *et al*, 1971). A request for examination of urinary GAG in the first year of life should, therefore, be 2*

completed by qualitative examination of the excretion pattern since the latter is usually abnormal from birth in the mucopolysaccharidoses even when total GAG excretion as hexuronic acid is within normal limits, and well before radiological changes are demonstrable (Pennock, 1973).

In conclusion, the methods outlined in the appendix are adequate for the diagnosis of the various mucopolysaccharidoses provided note is taken of the difficulties which may be encountered and which have been discussed in the preceding paragraphs. The methods are simple and require the minimum of apparatus and technical skill and should, therefore, be easily performed in any hospital or laboratory. They will remain of value in the investigation of the mucopolysaccharidoses until such time as suitable enzyme substrates are made available commercially. More specialized or new methods are required for the study of keratan sulphate excretion and the identification of patients with Morquio's disease.

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APPENDIX

Details of methods suitable for a simple study of excess glycosaminoglycan (GAG) excretion for the diagnosis of mucopolysaccharidoses

A. COLLECTION AND PREPARATION OF THE SAMPLE

Early morning specimens should not be used for screening tests, and infected samples collected at any time are unsuitable for analysis. Either single specimens passed between 0900 and 1800 hours or complete 24-hour specimens should be collected without preservative and stored frozen until analysed. (Freezing is not absolutely essential provided the sample does not become secondarily infected.)

The specific gravity of the sample should be measured and adjusted to less than 1.020 with distilled water if necessary. The sample should be centrifuged and the supernatant used for the estimation of creatinine and for the following tests.

B. SCREENING TEST

(CPC-citrate turbidity: Pennock, 1969)

Principle

Under controlled conditions of pH and electrolyte concentration cetylpyridinium chloride (CPC) reacts with GAGs to form an insoluble precipitate. In the presence of a citrate buffer at pH 4.8 this precipitate

is sufficiently stabilized and dispersed to measure the absorbance at 680 nm in a spectrophotometer or filter colorimeter or its turbidity may be measured using a nephelometer.

Reagents

1. Citrate buffer pH 4.8 (0.1 M). Dissolve 9.68 g of citric acid (AR) and 15.88 g of trisodium citrate (AR) in 1 litre of deionized water. Adjust the pH to 4.8 using a pH meter (little adjustment is usually required as this pH is close to the pK_2 of citric acid).
2. CPC reagent. Dissolve 1 g of cetylpyridinium chloride in 1 litre of the above buffer. Store at room temperature—shelf life at least one year.
3. Standards. Prepare standard aqueous solutions of chondroitin sulphate (CS) (Calbiochem Ltd) to contain 50 and 100 mg per litre. Store at 4°C.

Method

1. Centrifuge the sample and use the supernatant for the test. Allow standards and samples to reach room temperature (20–25°) before testing. (This is extremely important as CPC comes out of solution at low temperature and will do so if added to a cold sample, thus giving a falsely high turbidity.)
2. Mix 1 ml of sample with 1 ml of citrate buffer as a blank.
3. Mix 1 ml of sample with 1 ml of CPC reagent for the test.
4. Mix 1 ml of standard with 1 ml of CPC reagent.
5. Allow to stand at room temperature for at least 30 minutes, remix, and read absorbance of blanks, tests, and standards at 680 nm. (Lower wave lengths, but not less than 450 nm, or filter instruments may be used.) Absorbance (OD) is linear up to a concentration of 100 mg/l.

Calculation and Normal Range

$$\frac{\text{OD Test} - \text{OD Blank}}{\text{OD Standard}} \times \frac{\text{Concentration of standard (mg/l)}}{10} = \text{CPC units per litre}$$

One CPC unit is equivalent to the absorbance given by a solution containing 10 mg CS per litre. The results are not expressed directly in milligrams since different GAGs give slightly different turbidity readings at the same solution concentrations. The results are finally expressed as CPC units per gram of creatinine or per mole of creatinine. The normal range at different ages throughout childhood is given in table I. The upper limit in the first year of life is an arbitrary one chosen to ensure detection of abnormal samples but which gives a slightly

Age	CPC units/g creatinine	CPC units/mMole creatinine
0-6 months	300 ^a	34 ^a
6 months-1 year	280	32
1-2 years	262	30
2-3 years	244	28
3-4 years	228	26
4-5 years	213	24
5-6 years	198	22
6-7 years	185	21
7-8 years	173	20
8-9 years	161	18
9-10 years	150	17
10-11 years	140	16
11-12 years	131	15
12-13 years	122	14
13-14 years	114	13
14-18 years	100	9
Adults	50	5

Table I Upper limit of normal range for CPC citrate screening test (figures given to nearest whole number)

^aResults higher than this may be found in normal infants in the first 2 weeks of life.

higher percentage of false positive results at this age than is usually found later in life (see text).

C. QUANTITATIVE ESTIMATION OF GAG BY MEASUREMENT OF HEXURONIC ACID CONTENT

Principle

All the glycosaminoglycans, except keratan sulphate, contain hexuronic acids as part of the molecule. This will react with carbazole in the presence of concentrated sulphuric acid to give a magenta coloured product. The GAGs must first be isolated from other hexuronic acid-containing metabolites, and this is simply achieved by using CPC as precipitant.

Reagents

The following reagents are used for isolation of GAG (Di Ferrante, 1967) and estimation of hexuronic acid (Bitter and Muir, 1962).

1. CPC reagent. Dissolve 100 g of cetylpyridinium chloride in 1 litre of deionized water. Store at room temperature. The CPC is only poorly soluble and solution can be achieved by gentle agitation in a warm water bath at approximately 40°C. Excessive heating should not be used since the CPC will be degraded.
2. Potassium acetate in ethanol. Dissolve 10 g of potassium acetate in 1 litre of absolute ethanol.
3. Borate-sulphuric acid reagent. Dissolve 9.5 g of sodium tetraborate decahydrate (AR) in 1 litre concentrated sulphuric acid (AR). Store at 4°C—shelf life in excess of one year.
4. Carbazole reagent. Dissolve 1.25 g carbazole in

1 litre of 95% ethanol (AR). (In practice it is best to prepare a smaller volume and store in a dark bottle as the shelf life does not exceed three months.)

5. Hexuronic acid standards. Dissolve glucuronolactone (previously dried in a vacuum desiccator over phosphorus pentoxide for three days) in a solution containing 30 g of benzoic acid per litre, to give final concentrations of 100, 80, 60, 40, 20 mg/l or prepare a stock solution containing 1.7613 g/l (10 mM) and dilute to give standards containing 500, 400, 300, 200, 100 μ M/litre of benzoic acid solution.

Method

(a) Isolation of GAGs

1. Place 5 ml of filtered sample prepared as described in section A in a 15 $\times$ 90 mm disposable polystyrene tube (Metal Box Co Ltd) and add 0.2 ml of CPC reagent. Mix well and stand overnight at 4°C.

2. Centrifuge for 15 minutes in a standard bench centrifuge at 3 000 rev/min. Carefully remove supernatant with a Pasteur pipette and discard.

3. Add 5 ml of potassium acetate in ethanol to the precipitate, mix well, and stand overnight at 4°C. Centrifuge again at 3000 rev/min for 15 minutes, discard supernatant, and invert the tube on filter paper to drain. (The precipitate should be sufficiently well packed not to become dislodged.)

4. Add 1 ml of deionized water and mix well to dissolve the precipitate. (If the screening test result was very high add 5 ml of water.) If the precipitate does not dissolve immediately, leave at room temperature for 1 hour with frequent mixing. Centrifuge, if necessary, to remove insoluble material and use supernatant for hexuronic acid assay.

(b) Hexuronic Acid Assay

1. Place 0.2 ml of the final sample in a 15 $\times$ 125 mm Pyrex tube.

2. Add 0.2 ml of carbazole reagent.

3. Add 2.0 ml of cold borate-sulphuric acid reagent.

4. Stopper the tubes. Mix well (at least 15 seconds on a vortex mixer). The mixing is extremely important. If narrower bore tubes are used, adequate mixing is difficult owing to the viscosity of the sulphuric acid.

5. Set up water (blank) and standards in the same way.

6. Transfer to a vigorously boiling water bath for 7 minutes (the time is not critical between 7 and 10 minutes). Deionized water should be used in the water bath since chlorine in tap water may affect the reaction and give a green product.

7. Remove from water bath, cool to room temperature, and read the absorbance in a 1 cm light path cuvette at 530 nm. (The colour is stable for several hours if stored in the dark.)

8. Calculate the concentration per litre from the

Age	Hexuronic Acid	
	mg/g creatinine	mMoles/mole creatinine
0-3 weeks	Rising from 40-85	Rising from 23.3-49.5
3 weeks-6 months	Falling to 38.4	Falling to 22.4
6 months-1 year	38.4	22.4
1-2 years	35.6	20.7
2-3 years	27.2	16.0
3-4 years	26.9	15.7
4-5 years	25.7	15.0
5-6 years	20.7	12.0
6-7 years	16.5	9.6
7-9 years	13.6	7.9
9-10 years	11.3	6.6
10-11 years	10.3	6.0
11-19 years	9.1	5.3
Adults	6.2	3.6

Table II Upper limit of normal range for GAG hexuronic acid in relation to creatinine at different ages (Pennock et al, 1971 and 1972)

standard curve. Note that this value is five times the original urine concentration if 1 ml of water was added at step 4 of the isolation procedure and is equal to the original urine concentration if 5 ml was added. Results are expressed in relation to the creatinine content of the urine sample, and the normal range is given in table II.

D. IDENTIFICATION OF GAG EXCRETION PATTERN

1. Isolation of GAG

Isolation of GAG is achieved by the method described in section C using 100 ml of urine (SG adjusted to less than 1.020) and increasing the CPC reagent volume to 4 ml. The larger volumes involved may present difficulties in centrifugation if only a standard bench centrifuge is available. In the author's laboratory the precipitation is usually done in 25 ml aliquots of urine, adding 1 ml of CPC reagent, in universal glass specimen collection containers. After CPC precipitation overnight at 4°C the harvested precipitate is mixed with 2-3 ml of potassium acetate in ethanol, and the precipitates are pooled from all bottles and transferred to a single bottle. A further aliquot of alcohol reagent is added to wash final traces from each bottle, and the washings are added to the final bottle. The final volume is made up to approximately 25 ml with more alcohol reagent and left overnight at 4°C. The precipitate is harvested by centrifugation and washed once with a few millilitres of diethyl ether, dried, and taken up in deionized water. Sufficient water should be added to give a final GAG concentration in the region of 2.5-5 mM per litre in terms of hexuronic acid. This volume is calculated from the known concentration of CPC precipitable hexuronic acid

in the original urine. The final solution is centrifuged and the supernatant is used for electrophoresis and chromatography.

2. Electrophoresis (based on Wessler (1968))

Electrophoresis is done on any type of cellulose acetate membrane. (In the author's laboratory 5×18 cm strips of Oxoid cellulose acetate are used. Other workers prefer Sephrapore III which gives slightly better separation. The running times given below apply to Oxoid cellulose acetate and may need to be shortened for other types of cellulose membrane.) The membrane is soaked in the electrolyte solution for at least 20 minutes before use. It should be floated on the surface to allow electrolyte solution to soak up through the membrane before full submersion, since immediate submersion will result in uneven wetting of membrane and ultimate poor separations. The membrane is then blotted between Whatman No. 1 filter paper to remove excess electrolyte and then placed across a Shandon electrophoresis tank with a 10 cm bridge gap. Conducting wicks are made of Whatman No. 1 filter paper. Current should be applied to the membrane for about 10 minutes, before application and separation of the sample, in order to obtain an even distribution of electrolyte and water through the membrane.

The sample is applied with a $20 \mu\text{l}$ pipette, using a ruler as a guiding edge, and approximately $10 \mu\text{l}$ is applied as an even streak approximately 2 cm in length and 3 cm from the anode. A marker solution containing 2 mg of chondroitin sulphate and 2 mg of heparin per ml should be applied to a separate strip.

Electrophoresis is done in 0.1 M barium acetate at 2 mA/cm constant current for three hours. The strips are stained as described below.

Staining of Separated GAG

Electrophoretic strips are stained for 30 minutes in 1% alcian blue in 2.5% acetic acid. The strips are floated onto the stain to allow complete uptake of dye before full submersion. The strips are then washed in running tap water until a clear white background is achieved. They may be inspected visually or dried between Whatman No. 1 filter paper overnight, cleared in Whitmor transparency oil (Shandon Southern Ltd) and scanning using a scanning densitometer.

Electrophoresis in barium acetate gives separation with the following order of decreasing mobility—CS, KS, DS, HS, Hep. KS separates poorly from CS, HS separates poorly from DS, and HA migrates with DS. Some samples of heparin contain an HS fraction but neither Hep nor HA are prominent fractions in normal urine. This electrophoretic

method is not perfect but, taken in conjunction with thin layer chromatography, sufficient data are obtained for reliable interpretation.

3. Thin Layer Chromatography (TLC) (Humbel and Chamoles, 1972)

This method is based on the differences in solubility of the calcium salts of GAG in different concentrations of alcohol. The thin layer plate is chromatographed through six separate solutions of decreasing alcohol concentration. Cellulose plates 0.1 mm thick, 20×20 cm (Polygram CEL 300 Camlab, Cambridge), are marked with a soft pencil at 2 cm distances from the bottom of the plate. Approximately $10 \mu\text{l}$ of GAG solution is applied on the first line in a thin streak, 1-2 cm in width, and dried. The plate is then placed in the first solvent and this is allowed to ascend to the second line. The plate is then removed, excess solvent being quickly removed by touching the bottom of the plate on Whatman No. 1 filter paper, and transferred to the second tank containing solvent 2, which is then allowed to ascend to the third line. This sequence is repeated until all six solvents have been used. If insufficient tanks are available, two may be used, and the tank not in use at any one time can be cleaned out and the next solvent added. Fifty millilitres solvent is adequate for each tank and the solvent concentrations are as follows:

1. 25 g calcium acetate per litre of 70% v/v ethanol in 0.5 M acetic acid
2. 50 g calcium acetate per litre of 50% v/v ethanol in 0.5 M acetic acid
3. 50 g calcium acetate per litre of 40% v/v ethanol in 0.5 M acetic acid
4. 50 g calcium acetate per litre of 30% v/v ethanol in 0.5 M acetic acid
5. 50 g calcium acetate per litre of 20% v/v ethanol in 0.5 M acetic acid
6. 50 g calcium acetate per litre of 10% v/v ethanol in 0.5 M acetic acid

The first solution has a slightly higher concentration of alcohol than that used by Humbel and Chamoles (1972) and affords a better separation of KS. The solvents must be prepared fresh for each separation.

After the chromatography is complete the plate is dried and stained for 10 minutes in 1% alcian blue 8GX in 70% ethanol-acetic acid mixture (95/5/v/v). The plate is washed with 10% acetic acid to remove as much excess dye as possible.

If the individual solvent fronts are numbered 1 to 6 from the origin, the migration position of different GAG in relation to these fronts is as follows: DS(1-2), HS(2-3), C_4S (4), C_6S (4-5), and KS(5-6).

Interpretation is easier than with electrophoresis.

Normal urines contain predominantly chondroitin-6-sulphate with trace amounts of other GAG. DS and HS are well separated from each other and from other GAGs, and their relative proportions may be used to establish a diagnosis in line with the results shown in table IV of the main text. The method is excellent for detection of KS in patients with Morquio's disease. HS in some patients with Sanfilippo syndrome migrates a little further than HS in patients with Hurler's syndrome. This is clearly the best simple method for qualitative GAG identification described up to the present time.

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