

URINARY EXCRETION OF ACIDIC GLYCOSAMINOGLYCANS IN CONGENITALLY MALFORMED CHILDREN

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The embryonic midline fusion, which fails in spina bifida and in hare lip and cleft palate, may be regarded as analogous to the normal process of wound healing. Since it has been reported that the strength of healing wounds is associated with their content of acidic glycosaminoglycans (AGAG), it seemed worth while measuring the urinary output of AGAG in these and other congenital malformations. The study was extended to the mothers of cases because of the known familial distribution of certain malformations.

Materials and methods: Urine samples from 57 congenitally malformed infants and children and their mothers were provided through out-patient clinics in Newcastle-upon-Tyne and London. Cetylpyridinium chloride was a gift of F.W. Berk Ltd.; Alcian Blue 8GX was purchased from George T. Gurr Ltd. (Lot No. 19742) and cellulose acetate membranes from Millipore Corporation, because these gave the least background staining. Standard AGAG were given by Professor J.E. Scott or purchased as follows: chondroitinases were purchased from Seikagaku Kogyo Co. Ltd., Tokyo, Hyaluronidase from Koch-Light, papain from Sigma and all other chemicals from B.D.H.

A portion of urine was diluted to give a conductance equal to 50 mmol/l sodium chloride, subjected to papain digestion (1) and the AGAG isolated as the potassium salts (2), dried *in vacuo* and dissolved in water. A volume equivalent to 1 ml diluted urine was frozen at -20°C and dried in the frozen state before being taken up in 14 μl buffer (80 g/l sodium chloride, 1.35 g/l sodium acetate, 2.31 ml/l glacial acetic acid). A second aliquot was treated similarly but with buffer solution containing 100 IU (200 μg) hyaluronidase: both samples were incubated in a moist chamber at 37°C for 3 h before use. Where chondroitinase studies were done, an aliquot dried as above was taken up in 14 μl Tris buffer (57 mmol/l, pH 8.0) containing chondroitinase ABC (0.06 units) and incubated as above. The hyaluronidase digestion was done as routine and was supplemented by chondroitinase digestion when this seemed likely to give additional information.

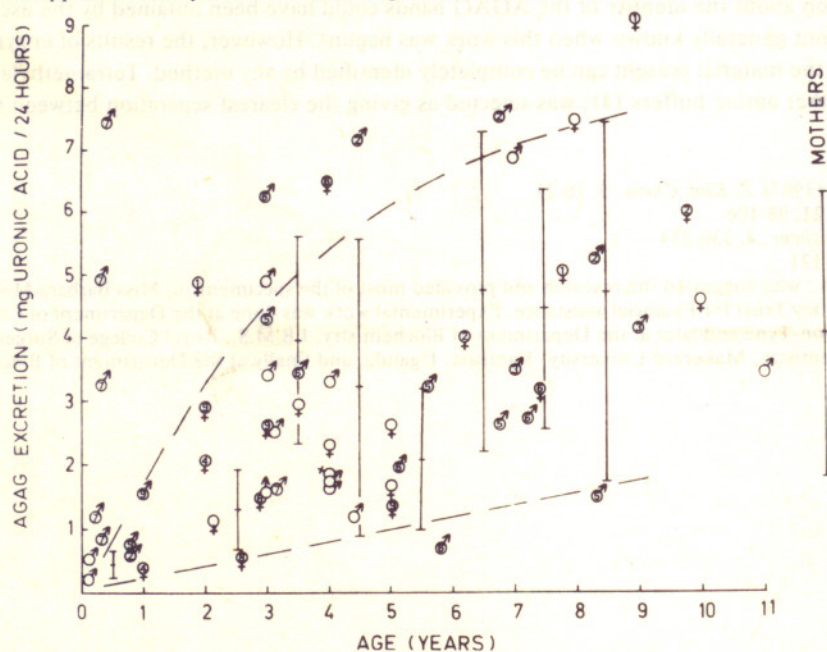


Figure 1: Total AGAG (as mg glucuronic acid / 24 h) in 57 congenitally malformed children. Each child is represented by a symbol showing its sex and diagnosis as follows: - 1 = syndactyly; 2 = absence of parts of limb(s); 3 = limb abnormality (unspecified); 4 = presence of extra tissue in limb(s); 5 ring constriction of a limb; 6 = heart defect (Fallot's Tetralogy, septal defect, isolated pulmonary stenosis, or unspecified); 7 = hare lip or cleft palate (combined in all cases studied); 8 = spina bifida; 9 = diagnosis not recorded; no number = normal. Vertical bars and cross lines represent means \pm SD computed by age group and excluding cases of hare lip and cleft palate. Dotted lines are smooth curves showing the approximate relationship of mean \pm SD to age. The mean \pm SD for the mothers is shown on the right.

Cellulose acetate strips, 14.5×2.5 cm, were soaked in buffer (182 ml tetramethylammonium hydroxide solution, 25% w/v, as supplied, plus 818 ml 2 mol/l formic acid), arranged horizontally in a suitable chamber and current passed at 3 mA per strip for 15 min. Two samples (1 μl) were applied to each strip 4.5 cm from the cathode end, with a standards mixture between them. Current was then passed as before for 2 h. Strips were stained in fresh 0.1% (w/v) Alcian Blue in 3% (v/v) acetic acid, differentiated by soaking in water for 1 h, with two changes, and 3% acetic acid for 1 h (one change). Total AGAG was measured in extracts by the method of Bitter and Muir (3).

Results: Figure 1 shows total AGAG excretion in all 57 children and their mothers. The means \pm SD for each age group, excluding cases of hare lip and cleft palate, agree well with values in the literature for normals. The AGAG values for very young baby boys with hare lip and cleft palate were very high.

In tetramethylammonium/formate buffer the order of migration of standard AGAG was as follows: - heparin (125), chondroitin sulphate (CS) (100), heparan sulphate (93), dermatan sulphate (DS) (82) and hyaluronate (HA) (64) - the figure in brackets representing observed migration distance relative to chondroitin sulphate. This migration velocity sequence differs from what is observed in barium or calcium buffers. It was expected that separation of this extent, combined with the differential effect of enzyme treatment, would allow clear identification of the AGAG extracted from urine.

Figure 2 shows electrophoretic patterns from five selected urines: migration distance of standard AGAG, and enzyme

pre-treatment used, are also shown on the figure. In practice, the commonest pattern was a single weak band spanning the region of standard CS and DS and only partially sensitive to either hyaluronidase or chondroitinase ABC (figure 2a), whereas standard substances added to the urine extracts were undetectable after only 30 min of appropriate enzyme digestion. This pattern may be regarded as "normal". Less common pattern elements occurring in addition to this main band, singly or in combination, were: (i) material migrating faster than CS but hyaluronidase-sensitive (figure 2b); (ii) a weak band coincident with or slower than HA but enzyme-resistant (figures 2c and d); (iii) a heavy band in this same region which might be sensitive to hyaluronidase (figure 2b) or resistant to both enzymes (figure 2e); (iv) enzyme treatment producing an apparent shift in migration of the main band (figures 2a, c and d). Such variant patterns occurred apparently at random in samples from patients, their mothers, and normal controls, without any relationship to clinical condition.

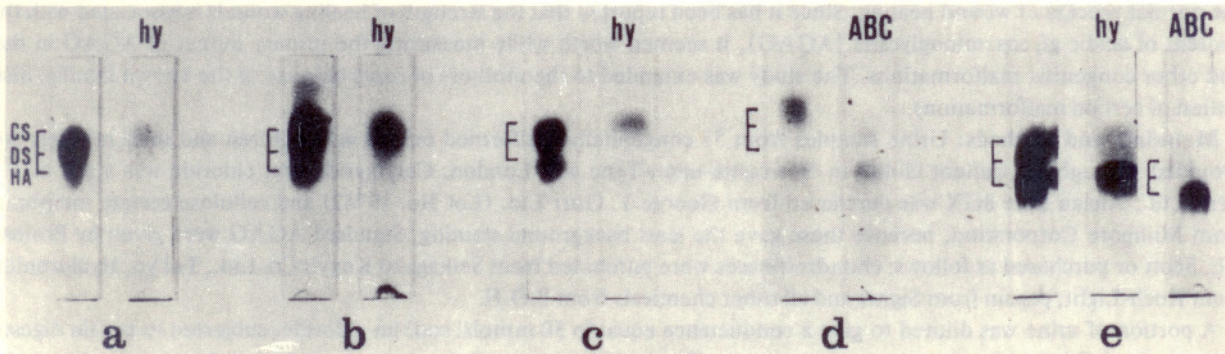


Figure 2: Electrophoretic patterns from five selected urines. hy = hyaluronidase, ABC = chondroitinase ABC. For explanation see text.

Discussion: There seems little doubt that the excretion of AGAG in very young baby boys with hare lip and cleft palate is extraordinarily high, on average. This may not persist and proper comparison requires reliable collections from age-matched controls, which are naturally difficult to obtain. Results with other congenital abnormalities were negative but it will be noted that, by chance, no cases were in the same age range. It would apparently be worth extending the study in these conditions also into the first few weeks of life.

It may be argued that more information about the identity of the AGAG bands could have been obtained by the use of barium or calcium buffers (which were not generally known when this work was begun). However, the results of enzyme treatment make it seem unlikely that all the material present can be completely identified by any method. Tetramethylammonium, though behaving rather like other amine buffers (4), was selected as giving the clearest separation between the standard AGAG tested.

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