Table III. Effect of various drugs and sulfhydryl reagents on cyclic 3', 5'-nucleotide phosphodiesterase of various tissues

Compound	Concentration (mM)	Enzyme activity (% control)						
		Guinea-pig heart	Beef heart	Rat heart low K_m	Human platelet low K_m	Human platelet high K_m	Rat platelet	Rat brain
Mersalyl	0.2	41	24	38	54	30	—	84
	2.0	28	11	—	—	5	31	—
Meralluride	0.2	56	42	54	64	46	—	81
	2.0	33	9	—	—	3	67	—
Ethacrynic acid	2.0	65	14	33	17	22	45	12
Phenylmercuric acetate	0.2	10	30	47	47	35	—	56
	2.0	4	22	—	—	—	—	—
Methylmercuric chloride	0.2	46	54	66	76	62	—	94
	2.0	24	33	—	—	7	28	—
Mercuric chloride	0.2	32	37	37	26	23	—	87
	2.0	8	8	—	—	2	15	—
p-chloromercuribenzoic acid	0.2	48	56	56	69	41	—	100
	2.0	35	21	—	—	—	—	—
N-ethylmaleimide	2.0	95	74	—	—	—	—	—
Iodoacetamide	2.0	100	—	—	—	—	—	—
Iodoacetic acid	2.0	95	—	—	—	—	—	—
Theophylline	2.0	75	53	—	—	36	92	—

Incubation conditions were as previously described. The amount of the substrate [H³] c-AMP used was as follows: a) 35 nmoles for the guinea-pig heart, rat brain and rat platelet preparations. b) 4.38 nomles for the beef heart preparation (equal to the reported K_m value[27]). c) 0.38 nmoles for the rat heart preparation (equal to the K_m value of 3.0×10^{-6} M as determined in this laboratory). d) 60 nmoles and 4.13 nmoles for the human platelet enzyme (equal to K_m values of 4×10^{-6} M and 3.3×10^{-5} M respectively as determined in this laboratory).

enzyme is of physiological importance. Since sulfhydryl reagents have been shown to inhibit the basal but not the ACTH or NaF stimulated activity of adrenocortical adenyl cyclase[17], drugs used in this study may be expected to elevate in vivo c-AMP levels in the normal catecholamine stimulated hearts by inhibiting c-AMP phosphodiesterase.

Platelet aggregation can be prevented by elevated intracellular levels of c-AMP[18]; therefore the present observation that the organomercurial diuretics inhibit platelet c-AMP phosphodiesterase may account for the inhibition of platelet aggregation in vitro[19].

Résumé. Différent composés organo-mercuriels ainsi que l'acide éthacrinique inhibent les phosphodiestérases de l'AMP cyclique, du cœur de cobaye, de bœuf et de rat, des plaquettes de rat et de l'homme ainsi que du cerveau de rat.

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Effect of Age on Distribution of Acidic Glycosaminoglycans in Normal Human Urine: Special Reference to Proportion of Chondroitin Sulfate A to the C-Isomer

The qualitative and quantitative analyses of urinary acidic glycosaminoglycans (AGAG) in relation to connective tissue disorders are the focus of interest and study. There is no general agreement, however, regarding the proportional constituents of AGAG in normal human urine, though previous studies have indicated that the major components of urinary AGAG are chondroitin sulfates A and/or C[1-3]. Age-dependent changes of the excreted urinary AGAG in normal human have been reported with respect to the comparative changes of the AGAG resistant to testicular hyaluronidase[4]: dermatan sulfate (chondroitin sulfate B) or heparan sulfates[5]. In these circumstances, we have studied the effects of age on a possible change of urinary AGAG in normal subjects, chondroitin sulfates in particular, at the disaccharide subunits by an enzymatic assay with chondroitinases[6,7].

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Materials and methods. Urinary AGAG were freshly obtained from 24-h specimens of normal subjects ranging in age from 3 to 80 years in both sexes and were prepared by the method reported in previous papers[6,7]. The urinary specimens were adjusted to pH 5.0, precipitated with cetylpyridinium chloride (15 ml of 5% solution/l of urine) by standing for 24 h at 4 °C and collected by centrifugation. Following sequential washing with ethanol and water, the specimens were digested with pronase-P (20 mg/l of urine) in 1/15 *M* phosphate buffer (pH, 7.8) for 72 h. After cold trichloroacetic acid was added to a final concentration of 10%, the supernatants were dialyzed against running tap water and concentrated. Crude AGAG were then precipitated by addition of 4 vol. of ethanol and kept at 4 °C overnight. The urinary AGAG were applied to Dowex 1-X2 columns (Cl form, 200–400 mesh) to separate glycopeptides which were eluted with 0.25 *M* NaCl. The AGAG eluted at the 3.0 *M* NaCl were dialyzed, concentrated and subjected to the following enzymatic assay for chondroitin sulfate isomers with chondroitinase-ABC[8].

Chondroitinase-ABC was prepared from *Proteus vulgaris* and purified through DEAE-cellulose chromatography[9]. The enzymatic activity was examined against standard chondroitin sulfates[10], when the enzyme was used for analytical procedure.

Adequate amounts of urinary AGAG (approximately 200–300 μg) were completely digested with chondroitinase-ABC (1 unit) at 37 °C for 120 min[11]. The resulting unsaturated disaccharides were applied on a filter paper (Whatman No. 1) and the desalting paper chromatography was made in *n*-butanol-ethanol-water (52:32:16, in vol.)[11]. Descending paper chromatographic separation of the unsaturated disaccharides was then carried out in 1-butyric acid – 0.5 *M* ammonia (5:3, in vol.) for 48 h at room temperature[6,7,11]. The separated unsaturated disaccharides visualized under a Mineralight at 232 nm were individually cut out and eluted with distilled water. The qualitative measurement of the eluted unsatured disaccharides was individually performed by the borate carbazole reaction[12]. The preliminary examination revealed that the values could be estimated with not more than 7% error. In this procedure, the AGAG resistant to chondroitin ase-ABC remained intact at the origin on the paper chromatography.

Results and discussion. The amounts of AGAG in 24-h urinary specimens were distributed between 2.3 mg and 5.2 mg and there was no trend in age-dependent changes. The undigested urinary AGAG accounted for 9% to 18% of the AGAG and their amounts tended to increase with advancing age. The Figure illustrates the analytical results of urinary chondroitin sulfate isomers in normal subjects at the unsaturated disaccharide unit, where total amounts of chondroitin sulfates were expressed as 100%. The unsaturated 4-sulfated disaccharides, which were the degraded products of chondroitin sulfate A and dermatan sulfate, consisted of more than half parts of total unsaturated disacharides in childhood. But it decreased significantly with advancing age. Dermatan sulfate exists in quite small amounts in normal human urine[2,3,6] so that the values obtained as unsaturated 4-sulfated disaccharides were designated as chondroitin sulfate A.

Contrariwise, chondroitin sulfate C (measured as unsaturated 6-sulfated disaccharide) was composed of 30% to 40% of total chondroitin sulfates in childhood and it tended to increase with advancing age. Accordingly, the proportion of the chondroitin sulfate A to the C-isomer decreased with the advance of age, as shown in the Figure: the ratio was higher than 1.0 in children whereas it was lower than 1.0 in adults after adolescence. It is noteworthy that the proportional changes of these major urinary AGAG occur at adolescence.

[8] Chondroitinase-ABC degrades chondroitin sulfates A and B to the unsaturated 4-sulfated disaccharides and chondroitin sulfate C to the unsaturated 6-sulfated disaccharides; the detailed information appeared in ref. [6,7,9].

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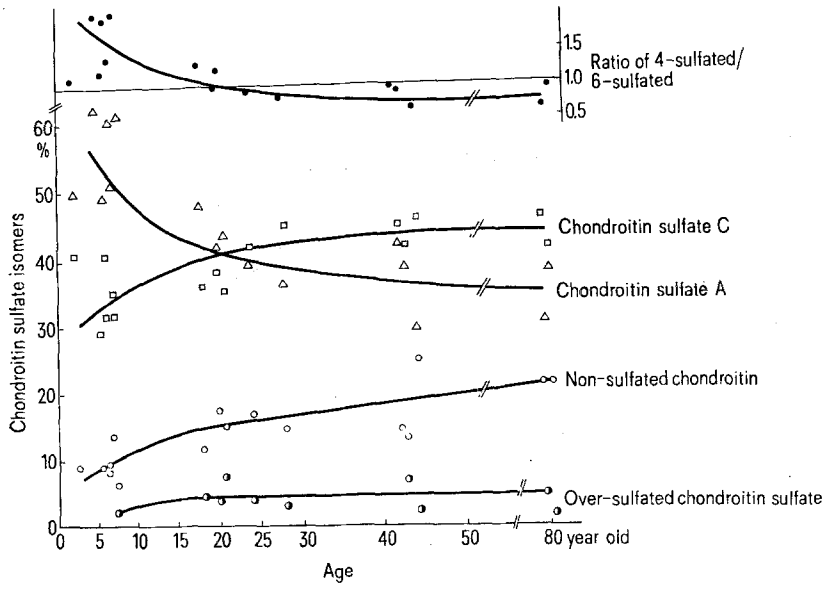

Age-dependent changes of chondroitin sulfate isomers in normal human urine at the disaccharide subunits. Note decrease of chondroitin sulfate A and increase of chondroitin sulfate C with the advance of age. Chondroitin also increases with advancing age.

The content of nonsulfated chondroitin sulfate isomer increased with the advance of age. It should be of interest to know that the amount of the nonsulfated isomer is quite the reversed of that of chondroitin sulfate A. This may be interpreted by our previous finding that chondroitin sulfate A and chondroitin coexist independently or form dependently undersulfated chondroitin sulfate A in normal urinary AGAG[7]. There was no significant change in oversulfated chondroitin sulfate with advancing age.

These age-dependent changes of urinary AGAG in normal subjects would reflect the effect of age on the distribution of AGAG in body connective tissue. For example, KAPLAN and MEYER[13], and MATHEWS and GLAGOV[14] reported that in human cartilage chondroitin sulfate A decreased significantly with advancing age, whereas the C-isomer decreased moderately. Consequently, the ratio of the A-isomer to the C-isomer in the human cartilage decreased with the advance of age. Thus, the age-related changes of urinary AGAG, chondroitin sulfate isomers in particular, should be an important parameter with respect to age-dependent function of AGAG in body connective tissue[15].

Résumé. Le sulfate A de chondroitine est un acide glycosaminoglycanes principal chez les enfants normaux. Mais cette substance diminue avec l'age, tandis que le sulfate C de chondroïtine a une tendance à augmenter. Il en résulte une diminution, de l'action du sulfate A sur le sulfate C. La prépondérence du sulfate C de chondroïtine se manifeste après l'adolescence. Il augmente continuellement au cours de la vie.

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Changes in Density of Organelles from *Neurospora*

In the course of a study of the biochemical characteristics of the 'glyoxysome-like' particles (GLPs) isolated from *Neurospora crassa*[1], we observed that their density was affected by the growth conditions and by the isolation procedure. If overlooked, these factors might produce a cross-contamination of the organelles, leading to erroneous conclusions regarding their true enzyme content. On the other hand, such alterations might provide a valuable tool for improving the separation of the organelles and gaining information on the binding of enzymes on the particles. The purpose of this report is to assess the effect of: 1. the carbon source provided in the growth medium, 2. the tonicity of the medium in which the particles are suspended, on the mean densities of both mitochondria and GLPs.

Material and methods. Neurospora crassa (wild type, Lindegren +) is grown at 30 °C in a shaken (200 strokes/min) liquid medium[2]. Derepression of the glyoxylate cycle enzymes is achieved either by transfer to a similar basal medium containing 40 m*M* acetate as the only carbon source, or by growing the mold in a complete medium supplemented with acetate (110 m*M*) and with a decreased sucrose concentration (14 m*M*). Homogeneization is performed as described elsewhere[1], in a complex medium made 0.4 *M* in sucrose. Crude particulate pellets are obtained by 3 successive centrifugations at $500 \times g$ (10 min), $3{,}000 \times g$ (30 min) and $10{,}000 \times g$ (45 min). Even though the bulk of the mitochondria sediments at $3{,}000 \times g$, the pellet of the last centrifugation (P 3–10 K) was chosen for this study, after it was shown that the mitochondria sedimenting in both pellets exhibit the same density pattern. The P 3–10 K pellet is suspended in various concentrations of sucrose, 1 m*M* in EDTA. The suspension (10–12 mg protein) is either layered on the surface of a 32–60% (W/W) linear sucrose gradient (1 m*M* in EDTA), or layered within the gradient at a point corresponding to its own density. The isopycnic centrifugation is carried out at $100{,}000 \times g$ for 8 to 16 h in a Spinco SW 27 rotor. The gradient is collected in 1 ml fractions and enzyme activities are measured by established procedures. As a rule, isocitrate lyase (IL) and NAD isocitrate dehydrogenase (NAD IDH) were used as marker enzymes for GLPs and mitochondria respectively.

Results and discussion. The first set of experiments (Table) shows that there is a correlation between the level of IL derepression and the average density of the

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Table I. Correlation between the derepression of isocitrate lyase (IL) and the mean densities of particles isolated from *Neurospora crassa*

Growth conditions	IL activity	Density of:	
		Mitochondria	GLPs
58 m*M* sucrose	0.70	1.182	1.205
Transfer to 40 m*M* acetate, 7 h	5.00	1.184	1.210
Transfer to 40 m*M* acetate, 24 h	10.2	1.194	1.219
Mixture 14 m*M* sucrose – 110 m*M* acetate	12.0	1.205	1.215

IL activity in μmoles of L (+)-isocitrate cleaved per h and per mg protein; densities in $g \times cm^{-3}$ at 20 °C