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Short Communication

Problems Connected with in Vivo Labelling of Embryonic Glycosaminoglycans with $Na_2{}^{35}SO_4$ in Teratological Studies *

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Summary. The time course in which radioactivity is found in maternal serum after a single injection of $Na_2{}^{35}SO_4$ may be drastically changed under the influence of drugs. This must influence the rate of incorporation of ^{35}S labelled precursors into the GAG fractions of embryonic tissue. Therefore it is not possible to draw any conclusions from in vivo labelling experiments unless at least the change in the radioactivity of the maternal serum has been taken into consideration.

A method is presented which allows an evaluation of an effect of drugs on GAG also in such cases in which the availability of the isotope from the maternal organism is changed: instead of measuring the rate of incorporation into the total GAG, a subfractionation of GAG according to their anionic behaviour is performed. In a routine assay three fractions of GAG are obtained after fractionation on ECTEOLA-cellulose. The ratio of incorporation of radioactively labelled precursors into the different subfractions is taken as indication of an effect of a drug on GAG metabolism. As an example the effect of vitamin A and Na-salicylate is analysed.

Key words: Glycosaminoglycans — ^{35}S Labelling — Embryonic Metabolism — Pharmacological Studies.

Pharmacological or teratological studies on the metabolism of sulfated glycosaminoglycans often are performed by measuring the incorporation of $Na_2{}^{35}SO_4$ into glycosaminoglycans (GAG). In most such studies the ^{35}S incorporation rate found in the GAG after a single injection of $Na_2{}^{35}SO_4$ is measured and the difference between control and treated animals is taken as an indication of an effect of the drug on the metabolism of GAG (Perumal *et al.*, 1965; Kochhar *et al.*, 1968; Robertson, 1968; Nanda, 1970).

A prerequisite for conclusions to be drawn from such data is that the concentration of the radioactively labelled precursor has the same kinetics in the controls as well as in the treated animals.

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We have measured the kinetics of $Na_2{}^{35}SO_4$ in the serum of rats treated with some drugs or hormones. In this paper data on the change in maternal serum concentration and in the rate of labelling of embryonic GAG are given. A method which helps to evaluate data on GAG metabolism independent of different serum activities is presented.

Methods

Animals. Female rats, strain Wistar S.W. 69, 190 ± 20 g were mated for 2 h. The following 24 h were called day 0 pregnancy. Drugs or hormones were given at times as indicated. 12 h prior to sacrifice the animals received an intravenous injection of 1 mCi/kg $Na_2{}^{35}SO_4$. Radioactivity in the maternal serum was measured in 0.05 ml samples using a Packard-Tricarb 3380. The embryos were removed from the mother animals at 4° C and dried in acetone. 30 mg dry weight of the acetone powder was hydrolysed with 1 ml 0.1 N NaOH for 12 h. After neutralisation with 0.1 N HCl a proteolysis was performed with 2 mg papain (Merck 7144), 3 mg EDTA-Na in 1 ml 0.1 M phosphate buffer, pH 6.4 and 0.005 M cystein for 24 h at 65° C. The 10,000 × g supernatant was used for chromatography with ECTEOLA-cellulose.

Column Chromatography with ECTEOLA-Cellulose. 0.1 g ECTEOLA-cellulose per column was transformed into the chloride form with 6 N HCl (30 min) and the columns packed, compressed with air and extensively washed with 0.02 N HCl. Preparation of the columns and details on the fractionation procedure have been published previously (Schimmelpfennig *et al.*, 1971). GAG were stepwise eluted from the column with 0.4—0.8—1.5 M guanidinium chloride. The three fractions were dialysed and lyophilised. Uronic acids were determined in each of the fractions with the carbazole reaction (Bitter and Muir, 1962). ^{35}S was measured with a Packard-Scintillation-Counter in a toluol-triton-X-100 mixture. Electrophoretic analysis showed that three fractions with different anionic behaviour had been eluted: Fraction I predominantly contained hyaluronic acid; it therefore showed the lowest ^{35}S incorporation rate. Fraction II and III represented the sulfated GAG, the third one showing the highest ^{35}S incorporation.

Results and Discussion

1. Effect of Some Drugs and Hormones on the Elimination of Radioactivity from the Serum

In Fig. 1 data are compiled which have been obtained using a number of drugs which are of interest in teratological studies. In this experimental set-up ^{35}S radioactivity in serum has been measured 12 h after a single injection of 1 mCi/kg $Na_2{}^{35}SO_4$. The concentration of ^{35}S radioactivity in the serum is changed significantly by some of these drugs when compared with controls. While some of the drugs are able to retard the elimination of the isotope from the maternal serum (vitamin A, tolbutamide, and even rape-seed oil which often is used as a vehicle for the oral application of lipid-soluble drugs), other substances accelerate the elimination of the isotope from the serum (Na-salicylate, triiodothyronine).

These data indicate that in many studies it cannot be expected that the incorporation of ^{35}S into GAG is the same in controls and in treated

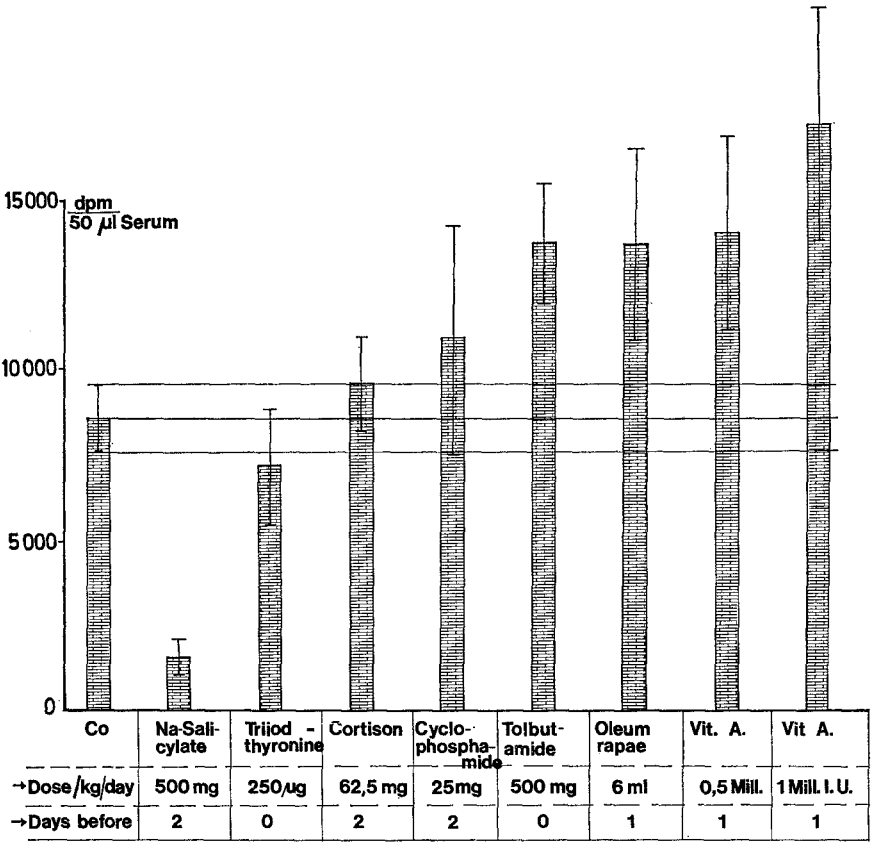

Fig. 1. ^{35}S activity in serum 12 h after injection of 1 mCi/kg $Na_2{}^{35}SO_4$. The animals received the drug every 12 h over a period as indicated. The last dose was given 30 min (= day 0) before i.v. injection of the ^{35}S-activity. 12 h later the serum sample was obtained. Application route: oral intubation of Na-salicylate (Merck), vitamin A-palmitate (Merck), triiodothyronine (Schuchardt), tolbutamide (Rastinon®), oleum rapae. Intraperitoneally: cortisone (Merck), cyclophosphamide (Endoxan®). One column represents values obtained from 5 animals

experimental animals. Under the influence of drugs the ^{35}S incorporation gives no information on the degree of biosynthesis or the extent of sulfatation of GAG.

2. Incorporation of Radioactivity into the GAG of Embryonic Tissue

As may be seen from the results given in Table 1 all the experimental conditions which alter the kinetics of the radioactivity in the serum are also bound to change the rate of incorporation of ^{35}S into embryonic GAG. Although this result is not very surprising, the fact that a changed rate of

Table 1. *The influence of several drugs on the ^{35}S incorporation (^{35}Si) in GAG fractions and the distribution of ^{35}S labelled GAG on the fractions ($R^{35}Si$)*

	$\frac{\text{dpm}}{\mu\text{g U. A.}}$ = Incorporation-equivalent (^{35}Si)			Ratio of ^{35}Si-values of fraction III over I and III over II	
	Fraction I	Fraction II	Fraction III	$R^{35}Si\ \frac{III}{I}$	$R^{35}Si\ \frac{III}{II}$
Embryo day 13					
Controls	53.8 ± 11.9	551 ± 63	1666 ± 189	30.9 ± 3.8	3.0 ± 0.61
Rape-seed oil	139 ± 37.6	1130 ± 360	3360 ± 769	24.2 ± 3.5	2.97 ± 0.52
Na-Salicylate	96 ± 12.7	449 ± 36	747 ± 71	7.8 ± 2.2 ($p < 0.001$)	1.66 ± 0.17 ($p < 0.01$)
Embryo day 15					
Controls	80 ± 10	1294 ± 147	2004 ± 487	24.8 ± 2.1	1.57 ± 0.13
Vitamin A	129 ± 19	1425 ± 353	1948 ± 573	14.9 ± 3.2 ($p < 0.001$)	1.36 ± 0.1 ($p < 0.1$)

Pregnant rats received 500 mg/kg and day of Na-salicylate over a period of 2 days (oral intubation). An other group was given 6 ml/kg rape-seed oil for 1 day. The last dose was applied on day 13 + 0 h. 30 min later the animals got 1 mCi/kg $Na_2{}^{35}SO_4$ i.v. The embryos were taken 8 h later.—1 mill I.U./kg and day of vitamin A was given every 12 h over a period of 2 days. 30 min later the animals received the ^{35}S-activity. The embryos were taken 14 h later.

Rape-seed oil influences the ^{35}S incorporation equivalent $\left(\frac{\text{dpm}}{\mu\text{g U. A.}}\right)$, not however the ratio of incorporation equivalents (R^{35}Si-values) of different fractions. Na-salicylate and vitamin A influence this ratio (R^{35}Si-values) significantly, indicating an altered distribution of ^{35}S-labelled GAG among the fractions.

incorporation may reflect a different availability of the radioactivity from the maternal organism apparently has not been taken into consideration by many previous investigators. Our data show that a change in the rate of ^{35}S incorporation into GAG of a given tissue may not be taken a priori as an indication of an effect of the drug on GAG metabolism.

In order to get some indication of a possible impairment of GAG metabolism in embryonic tissue of drugs, we have subfractionated sulfated GAG according to their anionic behaviour. The anionic character apparently represents the most important functional property of these macromolecules (Mathews, 1968). The method used and the classification of GAG species into groups of different anionic behaviour gives us the advantage of studying the distribution of radioactive label on the different GAG fractions rather than measuring the overall incorporation rate. We feel that the ratio of incorporation of ^{35}S into different GAG fractions

offers a reasonable measure of possible pathological changes occuring in GAG metabolism. Data given in Table 1 indicate that a substance—e.g. rape-seed oil—which alters the kinetics of ^{35}S in maternal serum does not necessarily change the ratio of incorporation of radioactivity into the different GAG fractions. Although the mode of interference of this oil is not understood at the moment its effect apparently is restricted to the kinetics of ^{35}S in the maternal organism. Thus the effect obtained closely resembles that seen after an injection of a larger dose of $Na_2{}^{35}SO_4$ into the mother animals. Apparently the specific activity of the precursor is changed for all GAG fractions in the same way. The ratio of ^{35}S incorporation into different GAG fractions may therefore be taken under normal conditions as being rather independent of the precursor supply. It is also seen from the data compiled in Table 1 that after Na-salicylate or vitamin A given to experimental animals a quite different situation occurs: Under the influence of these drugs the ratio of the incorporation of ^{35}S into the different GAG fractions changes drastically. This suggests that these substances—besides an effect on the kinetics of the isotope in maternal serum—may change the GAG metabolism within the embryo in a typical way or may lead to a dissociation of the different precursor pools within the embryo (Schimmelpfennig *et al.*, 1972).

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