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STUDIES ON THE BIOCHEMISTRY OF HEART VALVES

I. ON THE BIOSYNTHESIS OF MUCOPOLYSACCHARIDES IN BOVINE HEART VALVES

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SUMMARY

Sections of individual heart valves obtained from cattle of various ages were incubated *in vitro* in a medium containing radioactive inorganic sulphate ([^{35}S]S_I). Incorporation of [^{35}S]S_I into sulphated mucopolysaccharides of the valves increased linearly with time at least until 6 h of incubation. The [^{35}S]S_I incorporation (expressed as radioactivity per mg of dry tissue or as radioactivity per μg of sulphated mucopolysaccharides) was higher in the aortic valves than in the pulmonary valves and also higher in mitral, aortic and pulmonary valves of young animals than in those of old animals. Similar differences with respect to [^{35}S]S_I were noticed between different parts of individual mitral valves.

The inhibition of [^{35}S]S_I incorporation into sulphated mucopolysaccharides of the heart valves by some antiphlogistic drugs was also studied. A marked inhibition was obtained with sodium salicylate, chloroquine and phenylbutazone, while hydrocortisone showed no significant inhibition of this reaction.

The physiological and clinical implication of these findings are discussed.

INTRODUCTION

There is little information concerning the biochemistry of heart valves. The presence in these tissues of small amounts of acid mucopolysaccharides, the content of which amounts to only 0.5–1.0 % of dry tissue, was reported by DEISS AND LEON[1] and by MEYER *et al.*[2]. According to these authors this fraction consists mainly of chondroitin sulphate B (also known as β-heparin or derman sulphate) and chondroitin sulphate C with hyaluronic acid as a minor component.

Autoradiographic studies in rabbits following the injection of sodium [^{35}S]sulphate (see refs. 3 and 4) have revealed a considerably higher incorporation of ^{35}S into the heart valves than into any other tissues of the heart. These findings suggest that

Abbreviation: MPS, mucopolysaccharide sulphates; S_I, inorganic sulphate.

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the sulphated mucopolysaccharides in heart valves are being continuously synthesised *in vivo*.

This paper reports studies of the incorporation of [^{35}S]S_I *in vitro* into heart-valves mucopolysaccharides and the effect of aging and of anti-inflammatory drugs thereon.

EXPERIMENTAL

Principle

Slices of heart valves from cattle were incubated with sodium [^{35}S]sulphate in a Krebs–Ringer phosphate solution. The ^{35}S incorporation into mucopolysaccharide sulphates was studied under various conditions.

Preparation and incubation of slices

Hearts from cattle of different ages were collected at the slaughter house immediately after slaughter, opened in both ventricles, filled with and embedded in ice and transported to the laboratory. The individual cusps of the aortic, pulmonary and mitral valves were dissected out and placed in ice-cold Krebs–Ringer phosphate solution.

"Slices" were prepared from aortic and pulmonary valves by punching out the cusps with a cork borer, 0.5 cm in diameter. During this operation the slices were kept moist by continuous addition of cold modified Krebs–Ringer phosphate solution (see below). The slices were then transferred to 25-ml erlenmeyer flasks for incubation. In some experiments slices prepared from the cusps of one individual valve of one single heart were used, but usually slices from the same type of valve from 2 or 3 hearts were pooled and incubated together as indicated in the tables. Sections of mitral valves were cut freehand from 2-year-old cattle hearts.

All incubations were carried out with continuous shaking at 37° in an atmosphere of oxygen in 10 ml of a modified Krebs–Ringer phosphate solution (with 0.11 M $MgCl_2$ instead of 0.15 M $MgSO_4$) containing 0.1 mC of carrier-free sodium [^{35}S]sulphate (see footnote*). Incubations were conducted for varying periods as indicated in the tables. At the end of the incubation period the reaction was stopped by adding sodium monoiodoacetate to a final concentration of $5 \cdot 10^{-3}$ M. The slices were then thoroughly washed with isotonic saline, finally rapidly washed with water and defatted with acetone for 36 h. The dry weight was then determined.

For studies of the effect of salicylate on [^{35}S]S_I incorporation into the heart valves, two slices were punched out from adjacent areas of any one valve and kept separated. One batch of slices so obtained was incubated with [^{35}S]S_I alone in the medium as described. The second batch of slices was incubated with [^{35}S]S_I and potassium salicylate (10^{-2} M). The described procedure for stopping the reaction and washing and defatting the slices was then applied.

Isolation of MPS

Slices were defatted with acetone, denatured by heating in a water bath at 100° for 15 min and digested with activated papain at 65° for 12–16 h in 1.0 M NaCl containing 0.1 M phosphate buffer (pH 6.5), containing 1.0 M NaCl and 0.005 M each

* Obtained from the Radiochemical Centre, Amersham (Great Britain).

of cysteine, HCl and of EDTA, following the procedure described by SCOTT[5]. If undigested material was still present, more papain was added and the digestion continued. The residue remaining after papain digestion was very small, accounting for only 3–5 % of the original dry weight of the slices; this residue contained very little ^{35}S.

The pH of the digestion mixture was then adjusted to 1.5 with 0.5 N HCl and the precipitate removed by centrifugation. The residue was washed with distilled water and the supernatants were collected. A small aliquot (usually 10 μl) of the supernatant was then taken for radioactivity measurement.

The MPS were precipitated from the digest with Rivanol, following the procedure of WHITEHOUSE AND BOSTRÖM[6]. This method quantitatively precipitates very small amounts of MPS but hyaluronate and inorganic sulphate are not precipitated. In artificial mixtures of either sodium sulphate or hyaluronate with chondroitin sulphate over 95 % of the chondroitin [^{35}S]sulphate (30–200 μg) was precipitated, whilst 100 % of the inorganic sulphate and hyaluronate remained in the supernatant.

After the samples had been precipitated with Rivanol and centrifuged, an aliquot (50 μl) was taken from the supernatant for measurements of the radioactivity (inorganic sulphate). The radioactivity of the MPS fraction was calculated by subtracting the radioactivity of the inorganic sulphate fraction from the total radioactivity in the papain digest.

Radioactivity measurements

Aliquots (10 or 50 μl) were plated on frosted aluminium plates, dried at 80° for 15 min and counted with a Geiger–Müller tube with a mica end-window (1.9 mg/cm^2).

Quantitative analyses for MPS

The MPS content of papain digests was determined turbidimetrically with Rivanol in 0.5 M ammonium formate[6], after removing the bulk of the nucleic acids by precipitation with dilute hydrochloric acid. Fur further analyses, acetone-dried samples of both semilunar and mitral valves from new-born calves and from 6–10 year old cattle were chopped finely and defatted in chloroform–methanol (1:1, v/v). After drying at 100° for 3 h, these tissues were digested with twice-crystallized papain (British Drug Houses) added in the proportions of 2 mg per 100 mg dry tissue. After 18 h incubation at 65°, additional papain (1 mg/100 mg of dry tissue) was added and the digestion allowed to continue for a further 24 h. The digests were clarified by centrifugation and the very small precipitate was discarded. Trichloroacetic acid was added at 3° to give a final concentration of 5 % (w/v). Precipitated nucleic acids and proteins were removed by centrifugation at 3°. Polysaccharides were precipitated at 3° overnight from the supernatants by addition of 3 vol. of ethanol, pre-saturated with sodium acetate. The precipitated polysaccharides were washed free of trichloroacetic acid with ethanol and redissolved in water. Mucopolysaccharide sulphates were then precipitated with Rivanol in dilute hydrochloric acid as previously described. These precipitates were hydrolysed with 4 N hydrochloric acid for 8 h at 100° to liberate amino sugars. After hydrolysis, HCl was removed from the hydrolysate by repeated evaporation under reduced pressure using a rotary evaporator. The residues were dissolved in water. Aliquots of these solutions were submitted to paper chromatography following the method of FISCHER AND NEBEL[7] in parallel with standard glucos-

amine and galactosamine. The chromatograms were stained with alkaline silver nitrate[8]. Other aliquots of the above solutions were passed through columns of Dowex-50 X8 (H^+ form, 200–400 mesh) prepared as described by BOAS[9]. After washing with 20 ml of water, the hexosamine was eluted with 20 ml of 2 N HCl. The eluates were collected and dried in desiccators over solid sodium hydroxide pellets. The residues were dissolved in water and the hexosamine determination was carried out on aliquots of these solutions by the method of BOAS[9] as modified by BOLOGNANI *et al.*[10].

Other portions of the Rivanol precipitates were redissolved in 2 M potassium acetate with warming in a water bath at 60°. Small aliquots (0.2 ml) of the solutions were diluted to 1 ml with water and heated for 40 min in a boiling-water bath with 2 ml of an orcinol reagent. This reagent contained 0.2 % (w/v) recrystallized orcinol in 11 N hydrochloric acid containing 60 mg $CuCl_2 \cdot 2H_2O$ per litre of reagent. The absorbancy was read at 660 mμ and compared with a glucurone standard. Chondroitin sulphate B and chondroitin sulphate C gave equal colour yields by this procedure.

RESULTS

The present study shows that inorganic sulphate was readily incorporated *in vitro* into the MPS fraction of different types of heart valves. This process seems to occur at different rates in the different types of valves and even at different rates within different parts of the mitral valves.

Incorporation of [^{35}S]S_i into the MPS was completely inhibited either by boiling the slices before incubation or by incubating slices with ^{35}S in the presence of monoiodoacetate (10^{-3} M). In both aortic and pulmonary valves, ^{35}S incorporation increased proportionally with the incubation time, at least until the 6th hour (Fig. 1).

Table I shows that ^{35}S incorporation occurred more rapidly in the aortic valves than in the pulmonary valves. The average rate of ^{35}S incorporation into aortic valves from 24 hearts of new-born calves was 26200 ± 3700 counts/min/mg dry wt. per hour of incubation. The average obtained with pulmonary valves from 17 calf hearts was 9300 ± 1700 counts/min/mg dry wt. per hour of incubation, which is only 35 % of the figure for the aortic valves.

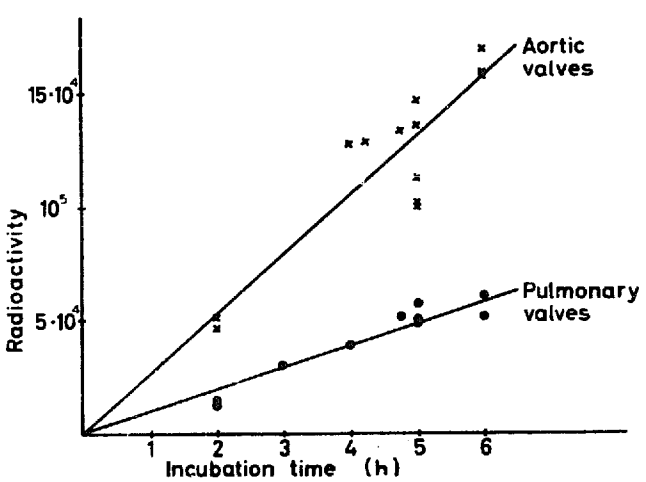

Fig. 1. [^{35}S]Sulphate incorporation *in vitro* into the mucopolysaccharides of aortic and pulmonary heart valves of 1–8 day old calves as a function of time of incubation. Radioactivity expressed as counts/min/mg dry weight of semilunar valves.

TABLE I

COMPARISON OF INCORPORATION OF [^{35}S]S_1 INTO MUCOPOLYSACCHARIDES BY AORTIC AND PULMONARY HEART VALVES FROM 1–8-DAY-OLD CALVES

Expt. No.	Incubation time (h)	Aortic valves				Pulmonary valves			
		Number of hearts	Dry weight (mg)	Radioactivity in MPS (counts/min/mg dry wt.)	Radioactivity in MPS (counts/min/mg dry wt. per hour of incubation)	Number of hearts	Dry weight (mg)	Radioactivity in MPS (counts/min/mg dry wt.)	Radioactivity in MPS (counts/min/mg dry wt. per hour of incubation)
1	2	5	38.5	46 174	23 087	3	29.6	14 414	7 207
	2	5	40.1	50 872	25 436	3	32.0	11 806	5 903
2	6	3	16.6	159 906	26 651	3	11.9	60 858	10 143
	6	3	11.3	158 184	26 364	—	—	—	—
	5	1	7.0	101 860	20 372	—	—	—	—
	5	1	7.0	136 425	27 285	1	7.0	50 000	10 000
3	5	1	8.1	112 400	22 480	1	7.6	48 350	9 670
	4.66	1	6.7	134 021	28 760	1	7.6	51 050	10 955
	4.25	1	7.5	129 493	30 469	—	—	—	—
	3	—	—	—	—	1	11.6	29 925	9 975
	4	1	9.1	128 352	29 422	1	12.1	38 512	9 628
4	5	1	7.1	147 110	28 400	1	8.4	57 070	11 414
	6	1	7.3	170 400	20 166	1	8.7	51 516	8 586
5	5	3	14.4	100 833	20 166	3	12.1	48 839	9 767
				Average:	26 229				9 348
					± 3 735				± 1 714

Table II shows that in both aortic and pulmonary valves the ^{35}S incorporation decreases with increasing age of the animals. The principal decline in metabolic activity appears to take place between birth and the age of the second group of calves studied. These were about 10–12 weeks old. This aging effect was more pronounced with aortic valves than with the pulmonary valves.

A further comparison was made between [^{35}S]S_1 incorporation into MPS by the semilunar valves and the mitral valves. Since it was not possible to apply the "punching-out" technique to obtain slices of mitral or tricuspidal valves, these were cut

TABLE II

^{35}S INCORPORATION INTO SULPHATED MUCOPOLYSACCHARIDES OF AORTIC AND PULMONARY HEART VALVES OF CATTLE OF VARIOUS AGES

Group	Age	Number of hearts	Aortic valves		Pulmonary valves	
			Radioactivity (counts/min/mg dry wt. of tissue per hour of incubation)	Decrease (%)	Radioactivity (counts/min/mg dry wt. of tissue per hour of incubation)	Decrease (%)
I	1–8 days	4	21 095	—	10 437	—
II	10–12 weeks	4	12 778	40	5 362 (2 hearts)	(49)
III	1–1.5 year	4	9 687	54	7 424	29
IV	8–10 years	4	8 978	57.5	6 784	35

freehand and the sections then incubated with [^{35}S]S_I. For purposes of strict comparison, semilunar valves were also cut freehand, usually into three sections, and similarly incubated with [^{35}S]S_I. It was found that when the nine sections of a semilunar valve (aortic or pulmonary) so obtained from one animal were separately incubated with [^{35}S]S_I, the incorporation of ^{35}S into at least 5 of these sections was comparable to within 15%. Such "harmonious" values are quoted in Table III. To correct for any large difference in the MPS content of semilunar and mitral valves, all values for ^{35}S incorporation were calculated as counts/min/mg dry wt. of tissue as in the previous experiments and also as the specific activity of the [^{35}S]MPS after determining the MPS content of the tissue digests by a turbidimetric method[6].

Table III (A) shows the relative activities of aortic, pulmonary and mitral valve sections, each incubated separately. All valves represent harmonious values between [^{35}S]MPS content of incubated individual slices from one animal donor. Tricuspidal valves manifested approximately equal biosynthetic activity to mitral valves, both being substantially less active than the semilunar valves. Table III (B) shows the distribution of biosynthetic activity within different portions of the mitral valvular tissue. Incubations with the chordae tendineae were conducted with pooled bundles of this particular tissue; all other incubations were with an individual tissue section. These comparative experiments were carried out with valves from animals approx. 3 years old.

TABLE III

COMPARISON OF BIOSYNTHETIC ACTIVITY IN AORTIC, PULMONARY AND MITRAL VALVES

Tissues from 2-year-old animals were cut freehand and sections (20–40 mg dry wt.) incubated separately for 4 h with [^{35}S]S_I (0.5 μC/ml medium). All values represent harmonious values for separate incubations with individual sections of valves from one animal donor.

Expt.	*Valve*	*Specific activity of [^{35}S]MPS (counts/min/μg)*	*[^{35}S]MPS/mg dry wt. (counts/min/mg)*
A	Aortic	60, 53, 57	810, 820, 860
	Pulmonary	63, 65, 72	640, 690, 630
	Mitral (without chordae tendineae)	25, 28	300, 260
B	Mitral		
	whole-valve sections	33, 31	220, 240
	basal portion only	65, 52	610, 550
	distal portion only	14, 13	260, 300
	chordae tendineae	23, 20	120, 110

Table IV shows that salicylate inhibits [^{35}S]S_I incorporation into aortic and pulmonary valves. Salicylate inhibition was greater in the younger animals than in older animals, and was greater in the aortic valves than in the pulmonary valves.

In the experiments illustrated in Table V the inhibition of [^{35}S]MPS formation by four different types of antiphlogistic drugs (hydrocortisone, sodium salicylate, chloroquine and phenylbutazone) was studied and compared. As indicated in the table, sodium salicylate, chloroquine and phenylbutazone had a marked inhibitory effect on MPS formation in all three types of valves investigated, while no significant inhibition was obtained with hydrocortisone.

Table VI shows the MPS content (expressed both as aminosugar and uronic acid) of the valves from new-born calves and those from elderly cattle (6–10 years). Valves from old animals contain less MPS per mg of dry tissue than those from new-born calves. Aortic valves contained at least 20 % more MPS than the pulmonary valves regardless of age and in the younger age groups this difference was even larger (30 %). In both valves of both ages, the paper chromatography of the hydrolysates of sulphated MPS fractions revealed the presence of only one type of amino sugar, *i.e.*, galactosamine. This finding shows that the sulphated MPS fractions are constituted by chondroitin sulphates.

TABLE IV

EFFECT OF SALICYLATE ON THE [^{35}S]SULPHATE INCORPORATION *in vitro* INTO THE MUCOPOLYSACCHARIDES OF PULMONARY AND AORTIC HEART VALVES IN YOUNG AND OLD CATTLE

Group	Age	Number of hearts	Aortic valves			Pulmonary valves		
			Radioactivity (counts/min/mg dry wt. per hour of incubation)		Inhibition (%)	Radioactivity (counts/min/mg dry wt. per hour of incubation)		Inhibition (%)
			No salicylate	Salicylate		No salicylate	Salicylate	
I	1–8 days	3	17 705	7 451	58	9 768	6 058	38
		3	20 166	6 904	66	8 733	5 029	42.5
		3	26 364	12 777	52	10 143	5 895	42
II	6–8 years	3	9 000	4 316	52	8 116	5 064	37.6
		3	14 324	9 618	33	10 546	6 682	36.6

TABLE V

EFFECT OF SOME ANTI-INFLAMMATORY DRUGS ON THE METABOLISM OF AORTIC, PULMONARY AND MITRAL VALVES

[^{35}S]MPS after drug incubations computed per mg dry wt. semilunar valves and per μg MPS from mitral valves and expressed as percentage of [^{35}S]MPS in drug-free controls. Valves from 2-year-old animals cut in segments and incubated for 5 h.

Drug	Concentration ($M \times 10^{-3}$)	[^{35}S]MPS in valves (per cent controls)		
		Aortic (%)	Pulmonary (%)	Mitral (%)
None	—	100	100	100
Hydrocortisone	0.25	106	104	85
Sodium salicylate	5	58	55	61
Chloroquine diphosphate	1	63	42	
Phenylbutazone	0.9	61	73	43

DISCUSSION

Studies on the synthesis of sulphated MPS in connective tissues have established the close relationship between the introduction of the sulphate group into the molecule and the synthesis of the polysaccharide skeleton, as reviewed elsewhere[11]. Thus it may be assumed that the incorporation of [^{35}S]S_I into heart-valve MPS actually represents

synthesis of sulphated mucopolysaccharides in the bovine-heart valves. As this study has shown, the rate of synthesis is dependent upon many factors, notably age.

Biosynthetic activity declined with increasing age of the animals from which the heart valves were taken. Thus the difference in metabolic rate between the youngest and the oldest age group amounted to 57 % for the aortic valves and 35 % for the pulmonary valves. The difference in the MPS content between young aortic and old aortic valves, young pulmonary and old pulmonary valves was of the same order of magnitude (Table VI).

TABLE VI

MPS CONTENT OF INDIVIDUAL HEART VALVES FROM NEW-BORN CALVES AND 8–10-YEAR-OLD CATTLE

MPS have been measured as aminosugar and uronic acid (orcinol) content of MPS fraction per 100 mg dried, defatted valves.

Valves	*MPS Aminosugar (μg)*	*MPS Uronic acid (μg)*	*Decrease in the MPS (as aminosugar) content of old valves compared with the young ones (%)*
Young aortic	960	900	
pulmonary	637	585	
mitral	890	790	
Old aortic	552	494	43
pulmonary	457	450	28
mitral	428	472	51

These findings might indicate that the observed decrease in the rate of [^{35}S]MPS formation with increasing age reflects changes in the composition of the valves. Actual decrease in the turnover rate of MPS can, however, not be excluded. Changes with age of both these types have been observed in other mesenchymal tissues[9–16].

There was a considerable difference between the metabolic activities of the aortic and pulmonary valves particularly within the calf group. In calf valves, the difference in MPS content between the aortic and pulmonary valves was also noteworthy. The biological significance of such a difference between the aortic and pulmonary valves is not clear. The pressure difference between the pulmonary and systemic circulation might require modifications in chemical composition of these two types of heart valves. The higher pressure in the aorta may cause more wear and tear, necessitating higher concentrations and more rapid replacement by biosynthesis of the various constituents of the aortic valves. Such differences in composition and metabolism between the various types of heart valves might also be correlated with the well-known differential response of the various valves to various pathogenic factors.

In agreement with other studies on the metabolism of connective tissues[6,16,17], the synthesis of sulphated mucopolysaccharides both in the semilunar and mitral valves is inhibited by salicylate. This observation would explain one beneficial property of salicylates for the management of rheumatic fever, namely, suppressing growth of scar tissue elicited by inflammation of the endocardium. Some other anti-inflammatory drugs—phenylbutazone and chloroquine, but not hydrocortisone—also depress mucopolysaccharide biosynthesis in the heart valves, paralleling the action of these drugs upon mucopolysaccharide biosynthesis in cornea and cartilage[16].

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REFERENCES

1 W. P. DEISS AND A. S. LEON, *J. Biol. Chem.*, 215 (1955) 685.
2 K. MEYER, E. DAVIDSON, A. LINKER AND PH. HOFFMAN, *Biochim. Biophys. Acta*, 21 (1956) 506.
3 H. BOSTRÖM AND E. ODEBLAD, *Anat. Record*, 115 (1953) 505.
4 W. M. COPENHAVER, *Anat. Record*, 131 (1958) 669.
5 J. E. SCOTT, *Methods Biochem. Anal.*, 8 (1960) 145.
6 M. W. WHITEHOUSE AND H. BOSTRÖM, *Biochem. Pharmacol.*, 7 (1961) 135.
7 F. G. FISCHER AND H. J. NEBEL, *Z. Physiol. Chem.*, 302 (1955) 10.
8 W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 166 (1950) 444.
9 N. F. BOAS, *J. Biol. Chem.*, 204 (1953) 553.
10 L. BOLOGNANI, G. COPPI AND V. ZAMBOTTI, *Boll. Soc. Ital. Biol. Sper.*, 34 (1958) 1950.
11 H. BOSTRÖM, *Polysaccharides in Biology, Trans. of the 4th Conf.*, G. F. Springer, The Josiah Macy, Jr. Foundation, New York, 1959, p. 211.
12 R. KUHN AND H. J. LEPPELMAN, *Ann. Chem.*, 611 (1958) 254.
13 D. KAPLAN AND K. MEYER, *Nature*, 183 (1959) 1267.
14 M. DYRBYE, *J. Gerontol.*, 14 (1959) 32.
15 W. H. HAUSS, F. JUNGE-HÜLSING AND W. SCHULZE, *Z. Alternsforsch.*, 14 (1960) 259.
16 M. W. WHITEHOUSE AND H. BOSTRÖM, *Biochem. Pharmacol.*, 11 (1962) 1175.
17 H. BOSTRÖM AND B. MÅNSSON, *J. Pharm. Pharmacol.*, 7 (1955) 185.