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Inhibition of Coronary Atherosclerosis in the X-Irradiated, Cholesterol-Fed Rat by Chondroitin Sulfate A.* (31635)

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Considerable data are available indicating that administration of chondroitin sulfates, which are widely distributed in connective tissues, cartilage and other tissues(1) have an inhibitory effect on the occurrence of atherosclerosis. As early as 1955, Kurita(2) reported that intravenous injections of chondroitin sulfate C at a level of 5 mg/kg of body weight daily reduced serum cholesterol levels and inhibited atherosclerosis in cholesterol-fed rabbits. Ohdoi(3) observed that sodium chondroitin sulfate inhibited the elevation of serum cholesterol, total lipids and the beta/alpha lipoprotein ratio as well as the formation of atheromatous aortic lesions in cholesterol-fed cockerels when administered orally at a level of 20 mg/kg of body weight per day. Murata(4) found that daily intravenous injections of 5 mg/kg of body weight of a chondroitin polysulfate which was prepared by sulfation of chondroitin sulfate from shark cartilage (chondroitin sulfate C) significantly reduced the serum total lipid and serum cholseterol levels of cholesterol-fed rabbits and had an ameliorating effect on the severity of cholesterol-induced atherosclerosis. More recently Morrison *et al*(5) observed that chondroitin sulfate A (prepared from bovine nasal septa and tracheae) when administered subcutaneously at a level of 20 mg/kg of body weight per day reduced serum total lipids and the incidence and severity of atheromatous aortic lesions in squirrel monkeys (*Saimiri sciurea*) fed a cholesterol- and butter-containing diet. It has been demonstrated the x-irradiation of the thorax (5 weekly exposures of 500 r each) increases the incidence and severity of coronary atherosclerotic lesions in rats fed a cholesterol-containing diet when compared to non-irradiated rats on the same diet or x-irradiated rats on a cholesterol-free diet(6, 7). Similar findings have also been obtained in the rabbit(8). Data are presented here on the effects of a single dose of total body x-irradiation upon the incidence and severity of coronary atherosclerotic lesions in rats fed a cholesterol-containing diet and the response to chondroitin sulfate A administration thereon.

Methods. One hundred and forty-four male rats of the Holtzman strain which had

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been raised from weaning on a natural food stock ration were selected for the present experiment. One group of 24 rats averaging 196 g in body weight (range 178 to 222 g) at the start of the experiment served as non-irradiated controls. The remaining rats averaging 197 g in body weight (range 176 to 226 g) were exposed on the first day of the experiment to a single dose of 600 r total body x-irradiation. The following radiation factors were employed: GE Model Maximar 250; 250 kv; 15 ma; 0.5 mm Cu and 1 mm A_1 filters plus a Cu parabolic filter,† HVL, 2.15 mm Cu; target distance to top of box, 78 cm; and exposure dose rate, 17.92 r per minute (measured in air, at top of box). The animals to be irradiated were placed in a wooden box divided into 14 compartments, 7 cm wide, 16 cm long and 10 cm deep. The partitions and top were made of ⅛-inch cellulose acetate sheeting; and the top, one side and bottom of each compartment were perforated with holes for purposes of ventilation. The container was rotated slowly on an electrically driven turntable to insure equivalent irradiation.

All irradiated rats lost weight during the first week following x-irradiation. During the second week 12 x-irradiated rats died and an additional 9 continued to lose weight. Of the remaining 99 x-irradiated rats most had regained their initial body weight by the 10th day and exhibited an average weight increment of 34 g over their initial weight by the morning of the 15th day after x-irradiation. At this time 84 rats from the x-irradiated group (these were animals with the largest weight increment post x-irradiation) and the 24 non-irradiated rats were selected for the following study. The non-irradiated rats which averaged 286 g in body weight (range 272 to 304 g) were divided into 2 comparable groups of 12 animals each (Groups I and II). The x-irradiated rats which averaged 237 g in body weight (range 222 to 274 g) were divided into 7 comparable groups of 12 animals each (Groups III to IX). Groups I and III were fed a highly purified diet consisting of sucrose, 61%; casein,‡ 24%; cottonseed oil, 10%; salt mixture,§ 5%; and the following vitamins per kg of diet: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; p-aminobenzoic acid, 400 mg; inositol, 800 mg; 2-methyl-1,4 napthoquinone, 5 mg; vit B_{12}, 150 μg; choline chloride, 2 g; vit A, 5000 U.S.P. units; vit D_2, 500 U.S.P. units; and alpha tocopheryl acetate, 100 mg. The vitamins were added in place of an equal amount of sucrose. Groups II, IV, V, VI, VII and VIII were fed a purified diet similar to the above supplemented with 1% cholesterol. Group IX was fed the purified diet + 1% cholesterol + 0.4% chondroitin sulfate A.‖ The cholesterol and chondroitin sulfate A supplements were added in place of an equal amount of sucrose. Rats in Groups V to VIII inclusive received daily subcutaneous injections of the following: Group V, saline solution; Group VI, 2 mg chondroitin sulfate A; Group VII, 4 mg chondroitin sulfate A; and Group VIII, 8 mg chondroitin sulfate A. All injections were ½ ml amounts of either saline solution or chondroitin sulfate A dissolved in saline solution. No injections were administered to rats in the other groups. Animals were placed in metal cages with raised screen bottoms (3 rats per cage) and were provided the above diets and water *ad libitum*. The animals were fed on alternate days and all food not consumed 48 hours after feeding was discarded. Body weights were recorded weekly.

After 12 weeks of feeding, the rats were

† A non-uniform filter which produces a flat isodose surface of x-ray intensity and constructed by the method of Greenfield and Hand(12).

‡ Vitamin-Free Test Casein, General Biochemicals, Chagrin Falls, Ohio.

§ Hubbel *et al*(13) Salt Mixture, obtained from General Biochemicals, Chagrin Falls, Ohio.

‖ Chondroitin sulfate A was prepared from bovine nasal septa and tracheal cartilage obtained from a commercial source and purified by our group. Analysis of this material showed a typical chondroitin sulfate A infra-red spectrophotometric absorption curve. Optical rotation determinations gave values of $[\alpha]_D^{24} = -24°$, and the nitrogen content was 3.3%.

anesthetized with sodium pentobarbital, and blood was withdrawn from the aorta into a heparinized syringe. Livers were excised, blotted to remove excess blood, weighed and stored in a freezer until analyzed. Lipid was extracted from the livers by the method of Thompson *et al*(9), and total and free cholesterol were determined on liver and plasma by a modification of the method of Schoenheimer and Sperry as reported by Nieft and Deuel(10). Total lipids were determined gravimetrically on an aliquot of the liver extract. At necropsy the hearts were fixed in 10% neutral formalin and frozen sections were prepared of the hearts of 6 animals in each group selected at random. Sections were cut at 15 μ in thickness of the entire heart of each of these animals. One hundred and fifty to 200 sections were selected from each heart at random and were stained with Oil-Red-O for the demonstration of lipids and counterstained with hematoxylin. The remaining hearts in each group were prepared for paraffin embedding in the routine manner, sectioned at 7 μ in thickness and stained with Gomori's aldehyde fuchsin.

Results. Body weight. The final body weight of rats in the various groups is indicated in Table I. The average increment in body weight of rats in the x-irradiated series was less than that of rats in the non-irradiated series. No significant differences in body weight were observed between x-irradiated rats in the various groups.

Plasma and liver cholesterol and liver total lipid. Findings indicate that the increment in liver cholesterol and liver total lipid induced by cholesterol feeding was significantly less in x-irradiated rats than in non-irradiated rats. The increment in plasma cholesterol induced by cholesterol feeding was also less in x-irradiated rats than in non-irradiated rats but the differences between the latter groups in respect to plasma cholesterol levels were less marked than were the differences in liver cholesterol and liver total lipid levels. No significant differences in plasma and liver total lipid levels were observed between x-irradiated, cholesterol-fed rats administered chondroitin sulfate A (either by subcutaneous injection or by oral feeding, *i.e.,* Groups VI, VII, VIII

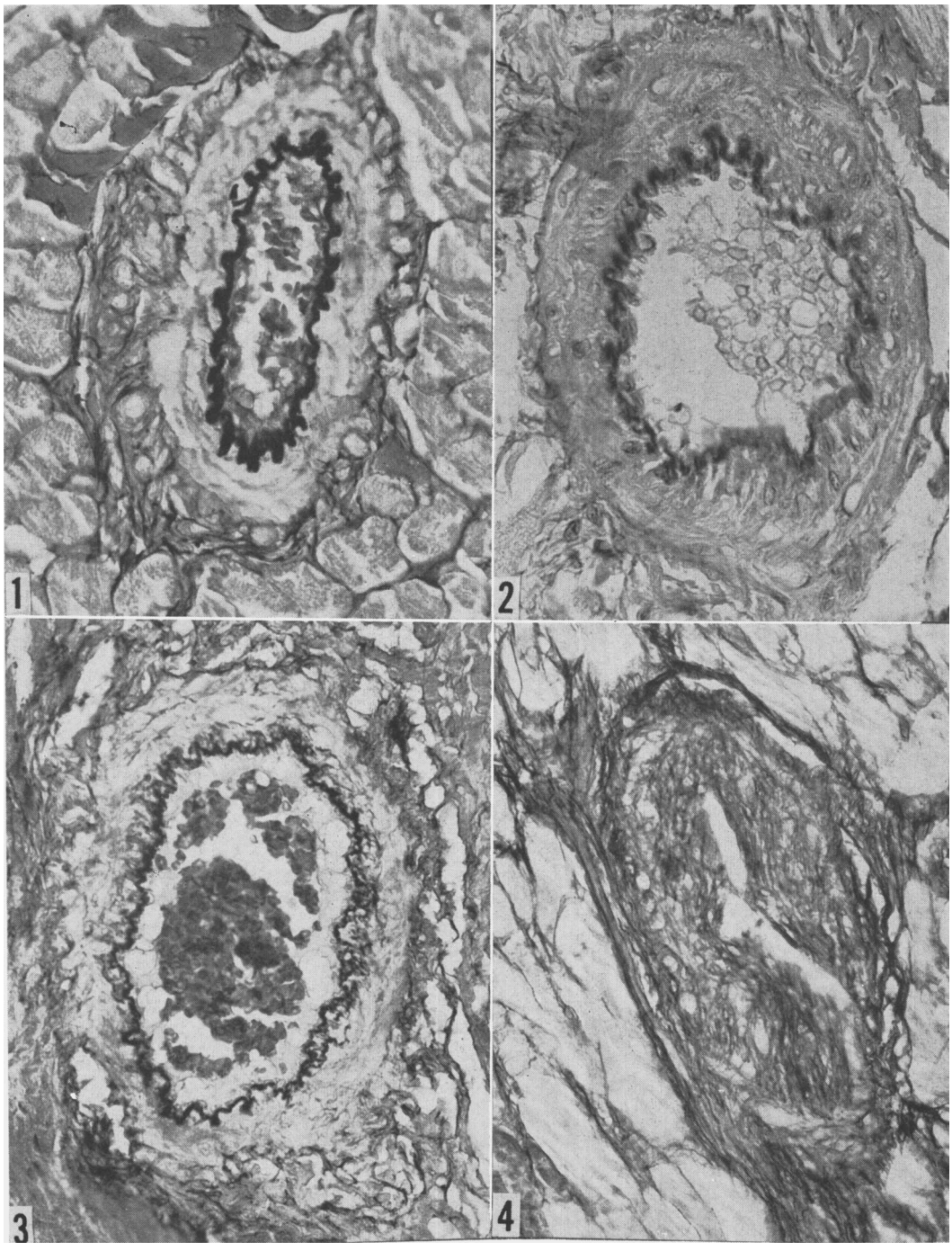
TABLE I. Effects of X-Irradiation on Plasma and Liver Cholesterol and Liver Total Lipid in Cholesterol-Fed Rats (12 Animals per Group).*

Group		Body wt at sacrifice, g	Plasma cholesterol, mg/100 ml Free	Plasma cholesterol, mg/100 ml Total	Liver total lipid, mg/g	Liver cholesterol, mg/g Free	Liver cholesterol, mg/g Total
	Non-irradiated series						
I	Basal diet	479	28.5 ± 2.0	114.3 ± 4.9	42.2 ± 1.9	1.8 ± .1	2.6 ± .3
II	Basal diet + 1% cholesterol	493	35.9 ± 2.1	180.4 ± 7.2	204.5 ± 13.7	5.6 ± .3	77.4 ± 4.9
	X-irradiated series						
III	Basal diet	383	22.2 ± 1.8	103.7 ± 7.5	44.4 ± 1.7	1.8 ± .2	2.9 ± .1
IV	Basal diet + 1% cholesterol	392	28.8 ± 2.6	147.8 ± 9.0	119.6 ± 9.4	4.0 ± .4	42.2 ± 2.4
V	*Idem* (inj with saline solution)	384	22.8 ± 1.1	131.5 ± 4.0	130.0 ± 12.2	4.6 ± .2	29.2 ± 2.4
VI	" (inj with 2 mg CSA)†	387	24.8 ± 2.8	120.9 ± 7.2	130.2 ± 9.1	4.7 ± .1	36.1 ± 2.1
VII	" (inj with 4 mg CSA)	388	21.3 ± 2.6	124.7 ± 6.4	129.8 ± 12.1	4.0 ± .6	24.5 ± 1.5
VIII	" (inj with 8 mg CSA)	380	27.9 ± 1.9	134.2 ± 6.9	144.1 ± 5.6	4.9 ± .4	36.3 ± 2.8
IX	" + 0.4% CSA (oral)	385	29.2 ± 3.5	136.6 ± 7.8	130.8 ± 6.5	4.5 ± .1	39.2 ± 3.6

* Including standard error of mean.
† Chondroitin sulfate A.

FIG. 1. Section of coronary artery from a non-irradiated rat fed a cholesterol-free diet (Group I). The internal elastic membrane is intact and abuts against the endothelial layer. Gomori's aldehyde fuchsin. × 500.

FIG. 2. Section of coronary artery from a non-irradiated rat fed a cholesterol-containing diet (Group II). Internal elastic membrane is fragmented and there is a slight thickening of the



intima. Note change in polarity of the smooth muscle cell nuclei from a circular position to one perpendicular to endothelial surface. Gomori's aldehyde fuchsin. × 500.

FIG. 3. Section of coronary artery from an x-irradiated rat fed a cholesterol-free diet (Group III). Note splitting of internal elastic membrane. Gomori's aldehyde fuchsin. × 500.

FIG. 4. Section of coronary artery from an x-irradiated rat fed a cholesterol-containing diet (Group IV). Note extreme narrowing of the lumen of the vessel due to extensive proliferation of cellular and fibrillar components of the intima. Gomori's aldehyde fuchsin. × 500.

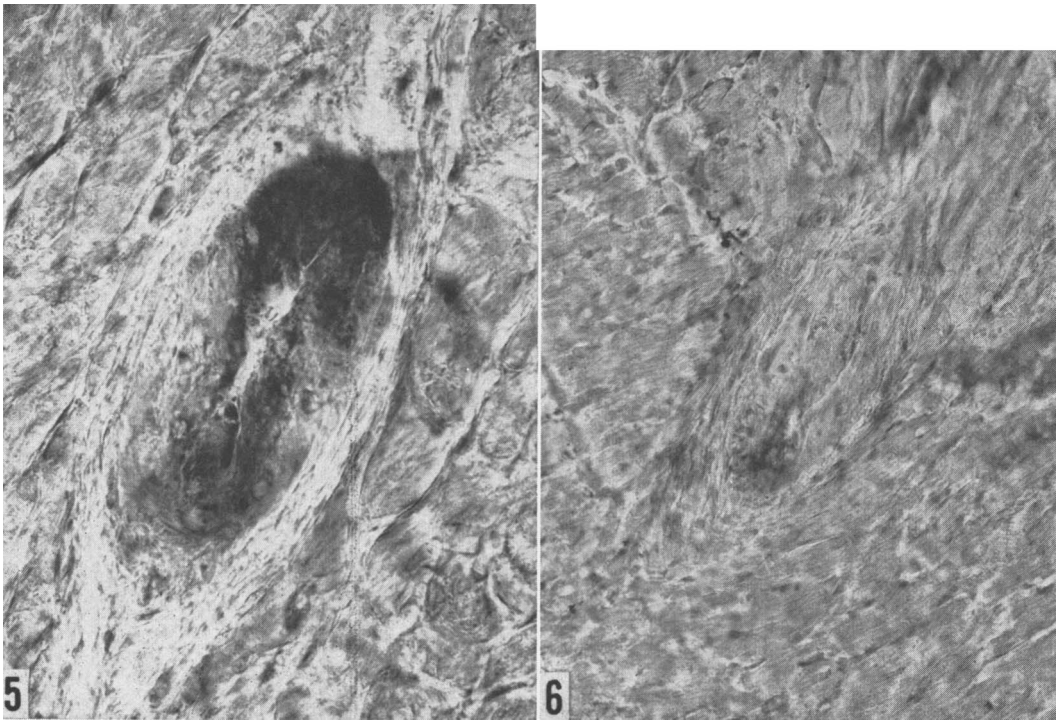


FIG. 5. Frozen section of coronary artery from an x-irradiated rat fed a cholesterol-containing diet (Group IV). The section was stained with Oil-Red-O for the demonstration of lipids. Note lipid infiltration of intima and media. × 400.

FIG. 6. Frozen section of coronary artery from an x-irradiated rat fed a cholesterol-containing diet supplemented with 0.4% chondroitin sulfate A (Group IX). The section was stained with Oil-Red-O for demonstration of lipids. Note that there is only slight deposition of lipid in the vessel wall. Compare with preceding figure. × 400.

and IX) and x-irradiated, cholesterol-fed rats not administered chondroitin sulfate A (Groups IV and V). Results are summarized in Table I.

Microscopic appearance of coronoray arteries. In non-irradiated rats fed the cholesterol-free diet (Group I) the internal elastic membrane of the coronary arteries was continuous, narrow and sharply defined with the endothelial layer directly adjacent to the elastic membrane (Fig. 1). Non-irradiated rats fed the cholesterol-containing diet (Group II) exhibited fragmentation of the internal elastic membrane, a slight thickening of the intima and reorientation of the smooth muscle cells of the media (Fig. 2). Whereas in Group I the smooth muscle cells were circularly arranged, in Group II many of them were radially arranged, indicating what Thomas *et al*(11) refer to as a "pre-atheroma phase of atherosclerosis." X-irradiated rats fed the cholesterol-free diet (Group III) exhibited a splitting and reduplication of the internal elastic membrane but no intimal thickening (Fig. 3). No lipid deposits were observed in the coronary arteries of any of the rats in the above groups. In contrast to the above, x-irradiated rats fed the cholesterol-containing diet (Group IV) exhibited extensive atherosclerotic lesions characterized by fragmentation of the internal elastic membrane and extreme intimal proliferation to the extent that the lumen was reduced to a mere slit (Fig. 4). The intimal proliferation consisted of an increased number of cells, collagenous elements and elastic fiber hyperplasia. Frozen sections of the coronary arteries of this group stained with Oil-Red-O exhibited a heavy infiltration of lipid deposits throughout the various layers (Fig. 5). These lipid deposits were observed in from 50% to 60% of all frozen sections examined in this

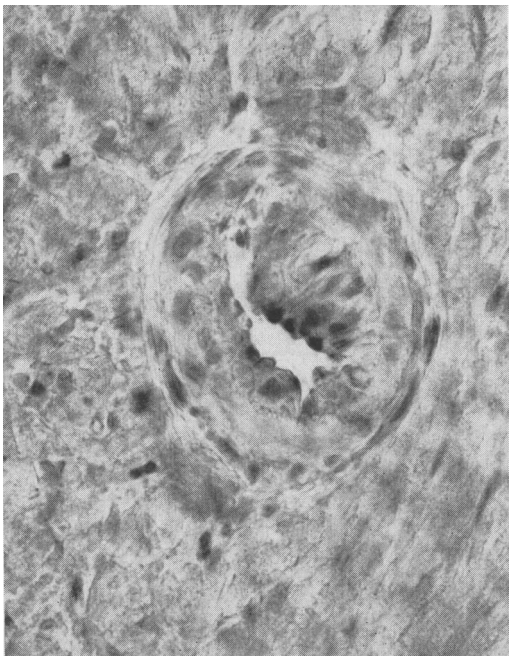


FIG. 7. Section of coronary artery from an x-irradiated rat fed a cholesterol-containing diet supplemented with 0.4% chondroitin sulfate A (Group IX). Intima is markedly thickened as a result of both cellular and fibrillar proliferation. Note that smooth muscle cells nuclei are radiating into the intima. Hematoxylin. × 400.

group. The coronary arteries of x-irradiated, cholesterol-fed rats administered saline solution (Group V) and subcutaneous injections of chondroitin sulfate A (Groups VI, VII and VIII) were comparable to those of Group IV in appearance. X-irradiated, cholesterol-fed rats administered chondroitin sulfate A orally (Group IX) however, exhibited a striking reduction in both the incidence and extent (Fig. 6) of lipid deposition in the coronary arteries although the degree of intimal proliferation (Fig. 7) and fragmentation of the internal elastic membrane was comparable to that of x-irradiated, cholesterol-fed rats in Group IV. Lipid deposition was observed in less than 10% of the sections examined of the hearts of rats in Group IX; and in contrast to the marked deposition observed in the coronary arteries of rats in Groups IV-VIII inclusive, when it did occur in the hearts of rats in Group IX it was present in only minimal amounts (Fig. 6). Data on the incidence and degree of lipid deposition in the coronary arteries of rats in the various groups are summarized in Table II.

Discussion. In this study lipid-containing atherosclerotic coronary lesions were produced in rats exposed to a single dose of 600 r total body x-irradiation and subsequently fed a cholesterol-containing diet. Non-irradiated rats fed a similar diet did not develop such lesions nor did x-irradiated rats fed a cholesterol-free diet. The incidence and extent of lipid deposition in the coronary arteries of x-irradiated, cholesterol-fed rats was significantly reduced in animals administered chondroitin sulfate A orally at a level of 0.4% of the diet. Subcutaneous daily injections of chondroitin sulfate A at levels of 2 mg, 4 mg, or 8 mg per day per rat, however, were

TABLE II. Effects of X-Irradiation on Incidence and Degree of Lipid Deposition in Coronary Arteries of X-Irradiated, Cholesterol-Fed Rats.

Group		No. of hearts studied	No. of hearts showing lipid deposition in coronary arteries	% of sections per heart showing lipid deposition in coronary arteries	Degree of lipid deposition
	Non-irradiated series				
I	Basal diet	6	0	0	None
II	Basal diet + 1% cholesterol	6	0	0	"
	X-irradiated series				
III	Basal diet	6	0	0	"
IV	Basal diet + 1% cholesterol	6	6	50–60	Medium to severe
V	*Idem* (inj with saline solution)	6	6	50–60	*Idem*
VI	" (inj with 2 mg CSA*)	6	6	50–60	"
VII	" (inj with 4 mg CSA)	6	6	50–60	"
VIII	" (inj with 8 mg CSA)	6	6	50–60	"
IX	" + .4% CSA	6	4	0–10	None to slight

* Chondroitin sulfate A.

without significant effect. Although oral administration of chondroitin sulfate A had an ameliorative effect on the incidence and extent of lipid deposition in the coronary arteries of x-irradiated, cholesterol-fed rats, it did not prevent the occurrence of intimal proliferation and fragmentation of the internal elastic membrane in the coronary arteries of these animals. The ineffectiveness of subcutaneously administered chondroitin sulfate A in the present experiment is in contrast to its activity in reducing the incidence and severity of atheromatous aortic lesions in squirrel monkeys fed a cholesterol- and butter-containing diet(5). Further studies are indicated to determine to what extent metabolic differences between the two species may have contributed to the diverse results. The reason why chondroitin sulfate A was active in reducing lipid deposition in the coronary arteries of x-irradiated, cholesterol-fed rats when administered orally but not subcutaneously is not readily apparent. Since the amount of chondroitin sulfate A ingested daily by rats was greater than that administered by subcutaneous injection, it is possible that the diverse results obtained between rats administered this material orally and parenterally were due to differences in amount. Blood levels of chondroitin sulfate A might also have been elevated for longer periods per day when it was provided in the diet (which was ingested intermittently over a 24-hour period) than when it was administered once daily by subcutaneous injection. It has not been established, however, that chondroitin sulfate A is absorbed from the intestinal tract of the rat; and the possibility that the activity of orally administered chondroitin sulfate A under conditions of the present experiment was due to some effect that may have occurred in the intestinal tract and that did not occur following parenteral administration has not been excluded.

Present findings indicate that exposure to total body x-irradiation significantly reduced the increment in liver cholesterol and liver total lipid induced by cholesterol feeding in the rat.¶ The deposition of lipids in the coronary arteries of x-irradiated, cholesterol-fed rats occurred despite the fact that these animals had lower liver and plasma cholesterol and lower liver total lipid levels than did non-irradiated, cholesterol-fed rats whose coronary arteries were free of lipid deposition. No significant differences in plasma and liver cholesterol and liver total lipids were observed between x-irradiated, cholesterol-fed rats administered chondroitin sulfate A (either by subcutaneous injection or by oral feeding) and x-irradiated, cholesterol-fed rats not administered chondroitin sulfate A. It would appear that the ameliorative effect of orally administered chondroitin sulfate A on lipid deposition in the coronary arteries of x-irradiated, cholesterol-fed rats was independent of any effect of this supplement on plasma and liver cholesterol and liver total lipid level.

Summary. Lipid-containing atherosclerotic coronary lesions were produced in rats exposed to a single dose of 600 r total body x-irradiation and subsequently fed a cholesterol-containing diet. Non-irradiated rats fed a similar diet did not develop such lesions nor did x-irradiated rats fed a cholesterol-free diet. The incidence and extent of lipid deposition in the coronary arteries of x-irradiated, cholesterol-fed rats was significantly reduced by the oral administration of chondroitin sulfate A at a 0.4% level in the diet. Exposure to total body x-irradiation significantly reduced the increment in liver cholesterol and liver total lipid and to a lesser extent plasma cholesterol levels induced by cholesterol feeding in the rat. No significant differences in plasma and liver cholesterol and liver cholesterol and liver total lipid levels were observed between x-irradiated, cholesterol-fed rats administered chondroitin sulfate A and x-ir-

¶ Food consumption was not determined for rats in the various groups. Since the average weight increment of x-irradiated rats was significantly less than that of non-irradiated rats, it is possible that the lower liver cholesterol and liver total lipid levels of x-irradiated, cholesterol-fed rats were due, at least in part, to a lower food consumption with an accompanying reduction in the amount of cholesterol ingested. It should be noted, however, that approximately half of the weight differences between x-irradiated and non-irradiated rats were due to differences in growth in the 2-week period following x-irradiation and prior to the time that cholesterol feeding was initiated.

radiated, cholesterol-fed rats not administered this supplement.

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Molecular Weight of Human Renin.* (31636)

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Whitaker first noted a linear correlation between the logarithm of the molecular weight of a protein and the ratio of its elution volume to the column void volume using G-75 and G-100 Sephadex(1). This work was extended by several investigators using G-75, G-100 and/or G-200 Sephadex to determine the molecular weights of proteins ranging from 13,000 to 225,000(2,3).

As renin has not been obtained in pure form, classical molecular weight (m.w.) determinations have not been possible. However, with the introduction of gel-filtration it proved possible to determine the molecular weight of a protein in a crude preparation (3). Gel-filtration has already been applied to *hog* renin, which had an elution volume between that of ^{125}I labelled human albumin (m.w. 69,000) and pepsin (m.w. 35,000) and was estimated as having a molecular weight between 42,000 and 49,000(4). The present study applied the method of Whitaker(1) toward the determination of the molecular weight of *human* renin.

Methods. Proteins of known molecular weight. Thyroglobulin: porcine, water soluble, lot 1923-60. Catalase: beef liver, slightly soluble, lot 45B-0440. Gamma-globulin: bovine, Cohn fraction II, lot 15B-2920. Serum albumin: human, grade III, lot 65B-1630. Ovalbumin: grade V, lot A102B-250. Pepsin: 2× crystallized, lot 15B-1370. All proteins used were obtained from the Sigma Chemical Co., St. Louis, Mo.

Human renin. The dog unit (DU) of renin is defined as that quantity of renin required to raise the mean femoral blood pressure of an unanesthetized dog by 30 mm Hg(5). Renin, of a specific activity of 0.5 DU/mg protein was prepared from fresh-frozen human kidneys by the method of Dexter, Haynes and Bridges(6), followed by lyophilization and gel filtration on G-75 Sephadex. Final preparations were lyophilized and stored un-

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