Increased Activity of Lysosomal Enzymes in Experimental Atherosclerosis, and the Effect of Cortisone* (33766)

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The activity of beta-glucuronidase is increased in atherosclerotic human arteries (1–3). Noting that beta-glucuronidase is a key marker enzyme in evaluating lysosomal enzyme activity, we decided to investigate other lysosomal enzymes in human atherosclerotic vessels. It was found that in addition to beta-glucuronidase, the lysosomal enzymes, acid phosphatase, cathepsin and aryl sulfatase showed increased activity in human atherosclerotic aortas (4). The present report describes the activities of these four enzymes in the aortas of rabbits in which atherosclerosis has been induced by adding cholesterol to the diet.

The effect of cortisone added to the diet has been observed. Cortisone tends to increase the level of serum cholesterol in the cholesterol-fed animals; nevertheless, this compound greatly decreases the intensity of atherosclerosis (5, 6). It is of interest that cortisone exerts a strong effect on lysosomal membranes (7). We have therefore investigated the effect of cortisone on the activity of the lysosomal enzymes in the cholesterol-fed rabbits.

Materials and Methods. Male, white New Zealand rabbits, weighing 2.0 to 2.5 kg were fed the diets *ad libitum* for a period of 100 days. The control group of 5 rabbits received standard rabbit chow. Another 10 animals were fed the same chow to which was added 1% cholesterol acetate. Of these, five survived the 100-day period. A third group of 8 rabbits received the cholesterol-containing diet to which was added cortisone acetate to give a concentration of 0.005%. Of the third group, 5 rabbits were alive at the end of the experiment. The animals were sacrificed instantly by mechanical means at 100 days. The aortas were dissected out and scored grossly with a hand lens for atherosclerotic involvement by two observers working independently. The arch, the thoracic and abdominal portions of the aorta were graded separately on a scale of 1 to 4, and the total score was recorded: 1+ represents fatty streaks and small plaques covering up to 10% of the area; 2+ indicates 10–25%; 3+, 25–50%; 4+ from 50% to total involvement.

Representative samples of each portion of the aorta were taken for histologic examination, and the remainder were employed for the enzyme assays. The histological examinations were performed by Dr. M. A. Farooki, Professor of Pathology, Khyber Medical College, Peshawar University, who at the time of these experiments was Visiting Pathologist at the Philadelphia General Hospital.

For the determination of enzyme activities, the aortas were carefully stripped of adventitia. The intima–media residuals were then homogenized by hand in a cold 0.25 *M* sucrose solution with a glass tissue grinder. The homogenate was centrifuged at 2520*g* for 10 min to remove debris. The supernatant was employed for the estimation of the enzyme activities. The enzymic tests were all carried out at 37°. Activities are expressed in units/gram of tissue; 1 unit refers to the decomposition of 1 μg or 1 μmole of substrate/hr under the conditions of assay. For beta-glucuronidase and aryl sulfatase the results are in micrograms, and for acid phosphatase and cathepsin in micromoles.

Beta-glucuronidase activity was determined by the method of Fishman *et al.* (8) as modified by Plaice (9). Incubation was carried out for 6 hr in 0.2 *M* acetate buffer, pH 4.5, containing phenolphthalein glucuronide to give final concentration of 0.006 *M*. For *cathepsin* the incubation medium con-

* Aided by a grant from The William H. Donner Foundation.

tained in a total volume of 1 ml, 0.00026 *M* denatured hemoglobin and 0.17 *M* acetate buffer (pH 3.6) in addition to the enzyme preparation. The reaction was stopped by 2 vol of 5% TCA. The mixture was filtered in the cold. The product of reaction was measured on the filtrate by the method of Lowry *et al* (10). The modified method of Valentine and Beck (11) was used for the determination of *acid phosphatase* activity. Assay mixture contained 1.8 ml, 0.052 *M* Na-β-glycerophosphate in 0.05 *M* acetate buffer (pH 5.0) and the enzyme preparation. The liberated inorganic phosphate was determined by the method of Chen *et al.* (12). *Aryl sulfatase B* activity was determined by the method of Roy (13); 0.06 *M* nitrocatechol sulfate was used as the substrate in a volume of 1 ml containing 0.05 *M* acetate buffer (pH 5.0). The end product was determined by alkaline quinol reagent. In the present studies the addition of phosphotungstic acid was omitted. The enzyme activities are reported on the basis of wet weight of the aortas. It has been our experience and that of other laboratories that nothing is gained by referring the activities to a DNA basis. Serum cholesterol was determined on blood drawn from the rabbits the day before they were sacrificed at the end of the experimental periods. The method of Zlatkis *et al.* (14) was employed. All the animals gained weight satisfactorily during the 100-day period of the experiment.

Results. In the animals on the 1% cholesterol diet there was very significant gross atherosclerosis in the aortas of each rabbit. Dr. Farooki's histologic examinations confirmed the gross diagnosis of typical cholesterol-induced atherosclerotic lesions. Also, in no instance did he observe infiltration of leukocytes; this is important because leukocytes contain relatively high concentrations of lysosomal enzymes.

In each instance where gross atherosclerosis was found, there was observed a distinct elevation of the level of the four lysosomal hydrolases. When one averages these small numbers, the increase in beta-glucuronidase approximates fifteenfold; the cathepsin and aryl sulfatase roughly five to six times; and the acid phosphatase about a five-fold increase.

As shown in Table I the increases in beta-glucuronidase activity appear to follow the severity of the lesions. There is a suggestion of this relationship with the aryl sulfatase, but not with cathepsin or acid phosphatase.

When cortisone was added to the 1% cholesterol diet there was a marked inhibition of the atherosclerosis. Our results agree with those observed by others (5, 6). As shown in Table I, the activities of each of the four lysosomal enzymes remained at the same levels as observed for those for the control rabbits.

Discussion. The marked increase in activity of the four enzymes in the animals given 1% cholesterol-containing diet suggest a lysosomal effect in the affected aortas. Even though the numbers of animals that survived the 100-day period were small, all the values for the four enzymes stand clearly higher than any for the control group. There is no overlapping of enzyme activities between the experimental and control groups. The p values are all $<.001$.

Usually a concomitant increase of activity of the two marker enzymes, beta-glucuronidase and acid phosphatase, is taken as presumptive evidence of increased activity of lysosomes in a tissue (7). Here, in addition to these two enzymes, we found increased activities of cathepsin and aryl sulfatase.

The inhibition of the experimental atherosclerosis by cortisone is in agreement with the results of others (5, 6). The cortisone effect is extremely interesting because this compound tends to increase the serum cholesterol levels over that produced by feeding 1% cholesterol alone. Thus the atherogenic stress is augmented.

Our results show that the animals fed the cholesterol diet with added cortisone did not have increased activities of any of the lysosomal enzymes. This would indicate that the marked increases of activities of these enzymes in the group fed only the 1% cholesterol did not occur as a response to the

TABLE I. Activities of Lysosomal Enzymes in Rabbit Aorta with and without Atherosclerosis.

	Dietary supplement	Gross atherosclerotic score	Serum cholesterol (mg/100 ml)	Beta-glucuronidase (units/g tissue)[b]	Acid phosphatase (units/g tissue)[c]	Cathepsin (units/g tissue)[c]	Arylsulfatase (units/g tissue)[b]
I	Controls	0	104	92	25	17	295
		0	80	113	61	14	505
		0	134	83	38	19	360
		0	79	41	22	20	369
		0	65	86	30	15	328
			92 ± 27.2[a,d]	83 ± 26	35 ± 16	17 ± 2.5	371 ± 80
II	Cholesterol (1%)	9	2000	2220	182	103	2760
		8	3935	1220	155	164	2060
		6	3150	754	168	128	1020
		6	1770	564	208	83	760
		8	1600	1334	182	95	3610
			2491 ± 1009	1218 ± 644	179 ± 20	115 ± 32	2042 ± 1190
III	Cholesterol (1%) + cortisone (0.005%)	0	2430	135	40	11	270
		0	2400	68	19	10	395
		0	6765	131	29	15	470
		0	5720	85	32	11	482
		0	—	148	34	21	390
			4329 ± 2251	113 ± 35	31 ± 7.7	14 ± 4.6	401 ± 85

[a] Mean ± SD.

[b] For β-glucuronidase and arylsulfatase the units are in micrograms per hour.

[c] For acid phosphatase and cathepsin the units are in micromoles per hour.

[d] The mean values for the four lysosomal enzymes in groups I and II differ significantly ($p < 0.001$). The mean values for these enzymes in groups I and III do not show significant differences.

elevated serum cholesterol *per se*. Rather, it suggests that the atherosclerotic process in the aorta is the stimulus for the increase in lysosomal activity.

It is not possible to discern from these experiments the relationship between increased enzyme activities and the appearance of the atherosclerotic lesions. A time sequence study of this relationship will be necessary, and might hopefully lead to an enzymatic assay method for the presence of atherosclerotic changes in the aortas of rabbits before anatomical changes become evident.

Summary. Four hydrolytic enzymes present in lysosomes, beta-glucuronidase, acid phosphatase, cathepsin, and aryl sulfatase were studied in the aortas of rabbits with cholesterol-induced atherosclerosis. Each of the enzymes showed a marked increase in activity. It was found in agreement with others that addition of cortisone to the atherogenic diet inhibits the atherosclerosis despite very high levels of serum cholesterol. In the aortas of the cortisone-treated rabbits, the four lysosomal enzymes showed no increases in activity.

We thank Dr. Johannes Ipsen, Professor of Medical Statistics, for arranging the statistical presentation in Table I.

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Received Nov. 20, 1968. P.S.E.B.M., 1969, Vol. 130.