STUDIES ON THE CLOT STABILIZING ENZYME IN AORTA OF RABBITS UNDER NORMAL CONDITIONS AND AFTER CHOLESTEROL FEEDING

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In the pathogenesis of atherosclerosis, the fibrin clot plays an initiating role as Rokitansky pointed out more than one hundred years ago (Rokitansky, 1852). More recently, Duguid restated this concept, namely that the growing fibrous tissue plays the most important role in atherosclerosis subsequent to thrombus formation (Duguid, 1948, 1959). Page (1959) suggested that the laying down of fibrin on the endothelium damaged by the haemodynamic effect of blood flow is the first step in atherogenesis. Eventually endothelial cells slowly overgrow the fibrin strands and the overgrown tissue finally provides a cushion into which later lipid may be deposited in the form of lipid drops inside the foam cells (Geer, et al., 1961; Robertson et al., 1962).

According to Astrup (1959), a well functioning dynamic balance between fibrin formation and fibrinolysis represents the optimal condition for the integrity of intima. But the possibility of a disturbance of this balance exists in case of intima lesion since intima contains fairly large amounts of thromboplastin (Perlick, 1960). Traces of thrombin are enough to produce a fibrin network and to aggregate the platelets (Haslam, 1964; Born and Cross, 1964; Cross, 1964; Tromzer and Baumgartner, 1967).

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All of these together appear to be protecting factors of the intima against the trauma of blood flow (Szalontai, 1963, 1968; Haslam, 1964; Apitz, 1942; Zucker and Boreli, 1962; Hugues, 1953; Irsigler, 1966). But these events slowly lead to a thickening of the intima, the primary change of the atherosclerotic plaque (Constantinides, 1965). The presence of fibrinogen in the wall of blood vessels has been noticed (Gitlin, 1959) and recently the School of Page found an increase of fibrinogen in atherosclerotic aortas (Shainoff et al., 1968).

It is now firmly established that in the transformation of fibrinogen to fibrin two enzymes are involved: the well-known proteolytic enzyme, thrombin, and the more recently described fibrin stabilizing enzyme (Laki and Lorand, 1948) which, in the presence of thrombin and calcium ions, brings about the formation of an urea insoluble fibrin clot. The clot stabilizing enzyme appears to be a transamidase which catalyzes a cross-linking reaction between amino groups and certain glutamine or asparagine residues in adjacent fibrin monomers. The cross-linking enzyme transforms the loose fibrin clot to a solid gel (Loewy, 1968).

Since the clot stabilizing enzyme is involved in wound healing (Gerendas, 1968; Laki, 1968) by providing the cross-bonded clot, we thought it important to explore whether such an enzyme is also involved in atherosclerosis. In this paper, we report a study concerned with the quantitation of this enzyme in the wall of the aorta of normal rabbits and those made sclerotic by feeding a cholesterol-rich diet.

Materials and Methods

Animals. In these experiments we used adult male albino rabbits (New Zealand strain) of body weight 1500-2500 g. The rabbits were supplied by the Animal Production Branch at the National Institutes of Health. The normal rabbits were sacrificed and examined immediately after obtaining them. Six cholesterol treated rabbits were kept in cages and were supplied with water and with a cholesterol-rich diet

produced by Nutritional Biochemical Corporation, Cleveland, Ohio. The diet contained: alphacel, 60 g.; butter fat (salt free), 400 g.; cholesterol, 53 g.; choline dihydrogen citrate, 4.0 g.; salt mixture W, 40 g.; sucrose, 203 g.; sodium cholate, 20 g.; vitamin diet fortification mixture, 20 g.; vitamin free casein, 200 g. The daily food consumption of an animal was about 30 g. The cholesterol feeding lasted for 10 weeks.

Tissue Preparation. Ten minutes before sacrificing, the animals were given intravenous injection of heparin (200 units, 0.1 ml solution). When the animals were bled out after carotis cutting, the heart, aorta, and the liver were removed. The aortas of cholesterol treated rabbits were divided into two parts. The proximal piece of about 2.0 cm long exhibiting the cholesterol induced plaques was prepared separately from the distal part appearing normal. The tissue pieces were soaked in cold Tris buffer (pH 8.0). Then about 0.200 g. of each tissue specimen were weighed and added to Tris buffer solution (pH 8.0) in the weight ratio of 1 to 9. The tissues to be tested were then homogenized at 1500 RPM for 3 minutes in the cold by using a glass homogenizer of Greiner Scientific Corporation. The aorta samples were homogenized for 15 min. strictly under cold conditions. Based on the weight of the aorta pieces, it was calculated that the homogenate contained about 10 mg protein per ml. Actual Kjeldahl nitrogen determinations on three homogenates (samples of which were washed repeatedly with TCA)[1] gave 15.5, 17.5, 16.0 mg as the protein content of those homogenates.

Assays. Fibrin cross-linking transamidase activity was assayed as described previously by Tyler and Laki (1966) with the incorporation of labeled glycine ethyl ester (GEE)[2] into casein. Labeled GEE (11.6 mg μc/mM) was diluted with cold GEE (58.0 mg) Tris buffer solution (5.0 ml, 0.1 molar, pH 7.8) to give a stock solution containing 1 μc/1 μmole/10

[1] Trichlor acetic acid

[2] Glycine ethyl ester

µl of 0.1 molar Tris buffer (pH 8.0). (0.5 micromoles of this solution gave 500,000 c.p.m.).

Casein (Hammersten) was dissolved in 0.2 M Tris-HCl buffer (pH 7.9) and dialyzed against Tris buffer. The casein solution was adjusted to give a 3% solution with buffer and was stored frozen until used (casein stock solution). Assays were carried out routinely in the presence of 10 mM $CaCl_2$ and 5mM GSH.[3]

Incubation mixture. 0.4 ml of casein stock solution, 0.4 ml of Tris buffer (pH 8.0), 0.05 ml of GSH solution (5 mM pH 8.0) and 0.1 ml of 0.1 M $CaCl_2$ solution and 0.1 ml tissue homogenate were pipetted into a test tube and 0.05 ml of labeled GEE stock solution was added. One-tenth ml samples of incubation mixture were removed and added to 5.0 ml of TCA solution (7.5%) at start and after 45 and 90 minutes incubation on water bath of 37°C.

Washing. The centrifuge tubes with the samples in TCA solution were allowed to stand on ice for about 20 min., then again for 20 min. at room temperature. Afterwards the precipitates were centrifuged down at 1500 RPM for 5 min. and the supernatant was sucked off. The sedimented precipitates were resuspended in 5.0 ml TCA. This washing procedure was repeated three times and to the final precipitate 0.5 ml, 1.0 N NaOH was added.

Counting. Aliquots (0.45 ml) of these solutions were pipetted into counting vials containing 10.0 ml scintillation fluid (Bray, 1960) and the radioactivity was counted in a Nuclear-Chicago liquid scintillation counter (Mark I).

The counts were corrected for casein blank samples; then the results were expressed as counts per minute per 1.0 ml of reaction mixture. The tables give the values obtained after 90 min. incubation.

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[3] Glutathione

Experimental and Results

The liver homogenates exhibited the greatest transamidase activity. (See Table I). The counts per minute vary from 15,000 to 138,000 in the normal rabbits (column 2) and from 62,000 to 168,000 in the cholesterol treated rabbits (column 1). Significant difference was not found between these two groups.

TABLE I

FIBRIN CROSS-LINKING TRANSAMIDASE ACTIVITY IN LIVER

COLUMN 1		COLUMN 2	
CHOLESTEROL TREATED RABBITS		NORMAL RABBITS	
NO.	COUNTS PER MIN.	NO.	COUNTS PER MIN.
III	62,150	I	14,000
IV	69,200	II	37,800
V	96,500	III	34,180
VI	168,000	IV	56,570
		V	15,000
		VI	65,000
		VII	77,000
		IX	138,000

Legend to Table I

Fibrin cross-linking transamidase activity in liver. Column 1 presents the counts per min. incorporated into casein during 90 min. incubation by the liver of rabbits treated with cholesterol. Column 2 presents incorporation catalyzed by liver of normal rabbits. If we assume 100 mg casein to represent one micromole, then 56,530 counts incorporated into 10 mg casein correspond to about half a micromole of GEE incorporated. The Roman numerals in the tables are the identification of the rabbits.

TABLE II

FIBRIN CROSS-LINKING TRANSAMIDASE ACTIVITY IN AORTA

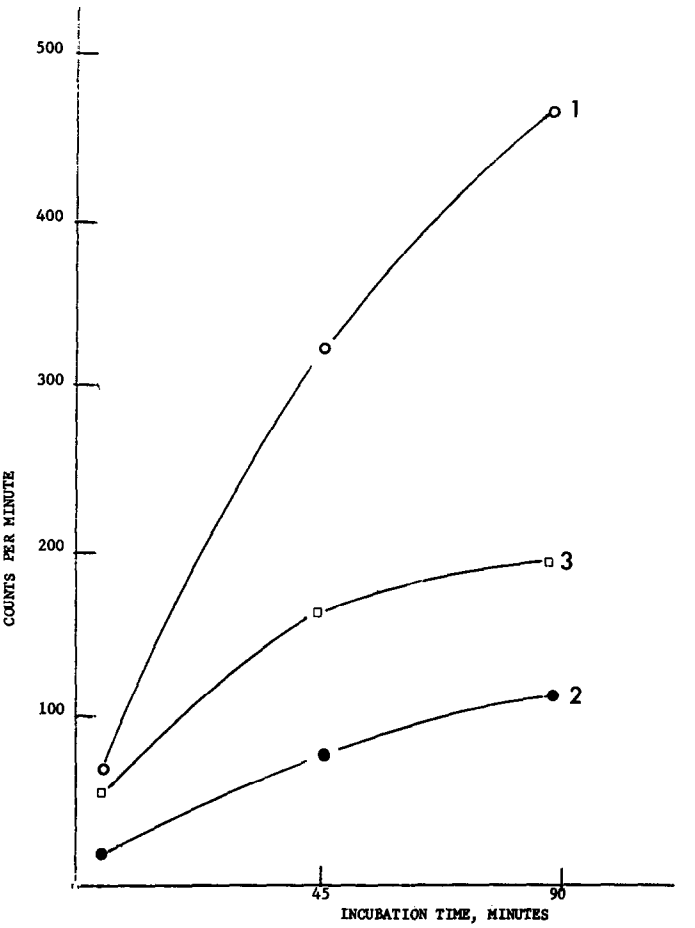
COLUMN 1*		COLUMN 2	
CHOLESTEROL TREATED RABBITS		NORMAL RABBITS	
NO.	COUNTS PER MIN.	NO.	COUNTS PER MIN.
III	2460	I_1	1000
IV	4770	I_2	1600
VI	3410	III_1	1100
		III_2	810
		V	1630
		VII	820

* One of the six animals put on high cholesterol diet died before the experiment was completed. Another lost hair and did not develop sclerotic plaques.

Legend to Table II

Fibrin cross-linking transamidase activity in aorta. Column 1 presents the values in aorta (proximal portion, atherosclerotic) of rabbits treated with cholesterol. Column 2 contains the values in aorta of normal rabbits. Numbers 1 and 2 in column 2 refer to proximal and distal portions of the aorta.

The results of the experiments carried out with aortas are seen in Table II and Figure 1. Column 2 gives the counts obtained on normal aortas and Column 1 the counts on the atherosclerotic aortas. The transamidase activity of the diseased aorta exhibits a threefold increase. In spite of the small number of animals used, this difference seems real since,



Legend to Figure 1

Fibrin clot stabilizing enzyme activity in aorta of normal and cholesterol-fed rabbits

Curve 1 illustrates the enzyme activity in the sclerotic portion of aorta after cholesterol feeding.

Curve 2 represents the behavior of the enzyme activity in the aorta of untreated rabbits.

Curve 3 demonstrates the enzyme activity of the distal, healthy portion of the aorta of cholesterol-fed animals.

In order to compare the data with these in the tables, the counts per min. should be multiplied by ten.

TABLE III

FIBRIN CROSS-LINKING TRANSAMIDASE ACTIVITY
IN AORTA OF RABBITS FED A HIGH CHOLESTEROL DIET

COLUMN 1		COLUMN 2	
ATHEROSCLEROTIC, PROXIMAL PORTION		NOT SCLEROTIC, DISTAL PORTION	
NO.	COUNTS PER MIN.	NO.	COUNTS PER MIN.
III	2460	III	1660
IV	4770	IV_1	1520
		IV_2	1960
VI	3410	VI_1	1560
		VI_2	870

Legend to Table III

Fibrin cross-linking transamidase activity in aorta of rabbits fed with rich cholesterol diet. Column 1 shows the values in the proximal portion (atherosclerotic) of aorta. Column 2 presents the values in distal portion (not sclerotic). Numbers 1 and 2 refer to two separate segments of the distal portion of the aorta.

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when the values of the sclerotic portions of the same aorta are compared to those of the healthy distal portions, the same difference appears. (Table III). This conclusion is strengthened by the observation that the two portions of the aorta of normal animals do not have different activities.

Discussion.

The experiments presented in this paper demonstrated that in atherosclerosis the transamidase (clot stabilizing enzyme) activity of the sclerotic portion of the aorta increases considerably as compared to the normal portion of the same aorta. In normal rabbits the transamidase

activity of the distal and the proximal portions of the aorta are similar. It is interesting to notice that this increase is about the same (threefold) as the increase in fibrinogen content of the wall of the affected aorta (Shainoff et al., 1968). This accumulation of fibrinogen and the clot stabilizing enzyme may be an indication of a need for the stabilized clot in the diseased regions.

Here we may be confronted with a situation similar to wound healing in which the clot stabilizing enzyme has an important role (Gerendas, 1968; Laki, 1968). In atherosclerosis also, tissue regeneration takes place; thus, an increase of the clot stabilizing enzyme may be expected.

Since not only blood plasma but also tissues contain clot stabilizing enzymes (Laki, 1968; Laki et al., 1966; Laki and Yancey, 1968) we cannot at present decide whether we are dealing with the tissue or with the plasma enzyme. The low activity of the enzyme in the wall of normal aorta as compared to liver may be taken as an indication that it comes from the plasma. On the other hand, if we take into consideration that the increase in enzyme content takes place only in the sclerotic plaques which represent only a small portion of the wall material, the amount of this enzyme becomes comparable to what we find in the liver. A true tissue transamidase in the uterus muscle of rabbit increases several fold during pregnancy (Alving and Laki, 1966).

The question naturally arises whether the augmented activity of the fibrin stabilizing enzyme is advantageous for the vessel's wall. It is reasonable to assume that the stabilized clot contributes to the strengthening of the damaged wall. Yet the subsequent tissue proliferation narrows the lumen of the arteries and thus hinders blood flow. It appears then that beyond a certain point the increased activity of the enzyme may not be advantageous. A specific inhibitor of this enzyme may be expected to be useful in the control of this disease.

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