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Effect of Connective Tissue and Collagen on Platelet Lactate Production: Role of Acid Mucopolysaccharides* (33318)

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Glycolysis, a major source of energy for platelets (1, 2), is responsible to some extent for serotonin uptake (3), potassium transport (4), and clot retraction (5, 6). Other investigators have already studied the effect on glycolysis of substances such as thrombin (5, 7–9), epinephrine (8), and ADP (8) which may be involved in the formation of an *in vivo* platelet plug. The first stage in the formation of a thrombus or a hemostatic plug is probably the interaction of platelets with the collagen of connective tissue (10, 11). The present report describes the different effects of connective tissue and collagen on the rate of formation of lactate, the end product of glycolysis, and establishes that platelet glycolysis is inhibited by the mucopolysaccharides of connective tissue.

Materials and Methods. Concentrated platelet suspension. Blood was collected from normal human volunteers in polyethylene centrifuge bottles containing one-quarter vol of acid-citrate-dextrose (ACD) (12) anticoagulant. It was centrifuged at 100 *g* for 20 min. Platelet-rich plasma (PRP) was collected in 50-ml polycarbonate tubes and spun at 1465 *g* for 15 min. All centrifugations were carried out at 4° to minimize metabolic activity. The platelet-poor plasma (PPP) was removed, and the pH was adjusted to 6.7 at 18° with either 0.1 *N* HCl or 0.1 *N* NaOH. The sedimented platelets were resuspended in a small volume of PPP. The volume of packed platelets was measured in a microhematocrit tube; it comprised 5–7.2% of the suspension, equivalent to 0.75 to 1.1 $\times$ 10^{10} platelets/ml suspension respectively.

Imidazole buffered saline (IMB). One part of 0.2 *M* imidazole buffer, pH 7.2, was diluted with 10 parts of isotonic saline.

Connective tissue suspension. Human connective tissue (CT) was obtained from regions of radical mastectomy specimens with no gross evidence of tumor. It was stored frozen, and prepared as described by Spaet and Zucker (13) except that the particles were washed twice in IMB instead of four times. Each milliliter of suspension was de-

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rived from 2 g CT. Earlier work (14) indicated that the final suspension prepared from 67 mg CT contained only 0.5 mg dry weight exclusive of salt, largely because much of the tissue is discarded during preparation of the final suspension. We thus estimated that our final suspension contained 15 mg dry weight/ml, of which 50% was estimated to be collagen. The suspension was maintained at 4° and used within 10 days, after which its platelet-aggregating ability diminished rapidly. Prior to use, the CT particles were sedimented at 48,000 *g* for 30 min at 4°. The sediment was resuspended in IMB—one-third of the volume of the original suspension.

Collagen suspension. A collagen suspension was prepared by grinding 100 mg of collagen[3] in 10 ml of IMB in a homogenizer[4] — at approximately 1000 RPM for 2 hours at 4°. It was left overnight at the same temperature and then further homogenized until the particles were very fine.

Collagenase. One-tenth milliliter of an aqueous 0.1% solution of highly purified collagenase from *Clostridium histolyticum*[3] was added to 2.5 ml of collagen or 1.1 ml of thrice-concentrated CT, estimated to contain 25 mg collagen. Five μl of 1 *M* $CaCl_2$ was added to each of the suspensions and the volume brought to 5 ml with Tris buffer (0.06 *M* hydroxy-methyl amino-methane and 0.09 *M* NaCl, pH adjusted to 7.5 with 1 *N* HCl). The suspension was combined in a ratio of 100:1 with an antibiotic solution consisting of 20 mg penicillin sodium,[5] 10 mg streptomycin sulfate,[6] 0.01 ml chloromycetin sodium succinate[7] in 5 ml Tris buffer and was incubated at 37° for 18 hours. It was then centrifuged at 48,000 *g* for 30 min at 4°, and the sedimented particles were resuspended in the original volume of IMB.

Hyaluronidase. One mg (150 USP units) hyaluronidase[8] was incubated with 0.75 ml of suspension for 1 hr at 37°. The suspension was centrifuged at 48,000 *g* for 30 min and the sediment resuspended in 0.25 ml of IMB.

Lactic acid levels. Lowry's method (15) as adapted by Karpatkin (8) was used to determine the lactic acid levels of platelet suspensions. The method depends on the reduction of acetyl-DPN in the presence of lactic dehydrogenase.

Incubation procedure. One-half–milliliter aliquots of concentrated platelet suspension were placed in siliconized glass tubes. Five tubes were used for each additive, providing samples to be tested at 0, 15, 30, 45, and 60 min. After preincubating the samples at 37° for at least 10 min, 5 μl of IMB, CT, hyaluronidase-treated CT, collagenase-treated CT, collagen, or collagenase-treated collagen was added to each set of five tubes. In some experiments, an additional set of tubes was prepared that contained both CT suspension and 5 μl $MgCl_2$ (0.04 *M*). The tubes were shaken for 90 sec; the end of this period was considered zero time. If marked aggregation was not observed when expected, an additional 5 or 10 μl of aggregating agent was added. The reaction was stopped by adding 0.05 ml of cold (4°) 5.5 *N* perchloric acid at the established intervals; the tubes were immediately agitated[9] and then centrifuged for 25 min at 4080 *g*. The supernatant fluids were neutralized with a very small volume of concentrated KOH and spun at 48,000 *g* for 30 min. The clear supernatant fluid was frozen, and lactic acid levels were subsequently determined in aliquots.

Results. Large platelet aggregates formed when platelets were shaken with collagen, CT alone, CT treated with hyaluronidase, or CT with added magnesium, although as previously reported (14), there was no aggregation with collagen or CT treated with collagenase.

Platelets had already produced some lactate during the preparation and preincubation of the concentrates. To calculate this lactate produced prior to 0 time, it was necessary to correct for the lactate in normal plasma in which the platelets were suspended. The lac-

[3] Worthington Biochemical Corp., Freehold, New Jersey.

[4] Krebs Electric Manufacturing Co., New York, New York.

[5] The Upjohn Co., Kalamazoo, Michigan.

[6] E. R. Squibb & Sons, New York, New York.

[7] Parke, Davis & Co., Detroit, Michigan.

[8] Nutritional Biochemicals Corp., Cleveland, Ohio.

[9] Vortex Jr. Mixer, Scientific Industries Inc., Queens Village, New York.

TABLE I. Lactate Concentration (μmoles/ml Platelets) at 0 Time after Aggregation.

	Experiment no.					
	1	2	3	4	5	6
IMB	31.9	34.1	33.2	33.5	29.2	20.9
Collagen	—	—	38.6	40.7	41.4	48.6
Collagen + collagenase	—	—	33.6	—	30.1	22.5
CT	37.2	31.4	33.3	49.2	42.6	—
CT + hyaluronidase	37.2	33.2	—	29.9	—	—
CT + Mg^{2+}	36.5	34.1	—	—	—	—
CT + collagenase	—	—	34.9	—	46.4	—

tate concentration of the supernatant fluid obtained by perchlorate precipitation of the control platelet suspensions at 0 time averaged 2.68 μmoles/ml in six experiments. After subtracting the "normal" plasma lactate concentration of 1.1 μmoles/ml (16, 17), and correcting for reagent dilution and the platelet volume in the suspension, it was determined that the average amount of lactate produced was 30.3 μmoles/ml of packed platelets.

Table I shows the lactate levels present at 0 time in the tubes with various additives. Values higher than those in the controls represent lactate produced within 90 sec after aggregation (when it occurred). Collagen caused a burst of lactate production, whereas collagenase-treated collagen did not. With untreated CT, results were variable.

The duration of lactate production after 0 time varied with different additives (Figs. 1 and 2; Table II). In the control suspension and with collagenase-treated collagen, production had decreased markedly or ceased within 45 min. With CT and collagenase-treated CT, production had ceased within 15 min. With collagen, hyaluronidase-treated CT, and CT with added magnesium, production continued in a linear fashion for 60 min. The hourly rate of lactate production in the control samples was 37.9 (S.E. $\pm$ 1.7) μmoles/ml of packed platelets. It was not affected by the additives except that the increment noted at 15 min in four of the five CT experiments was considerably greater than with any other additive. (The one experiment that failed to show the greater increment appears in Fig. 1.) Since there was no subsequent lactate production, this finding is indicated in Table II as an elevated hourly rate. Much of the burst of activity presumably occurred shortly after CT was added.

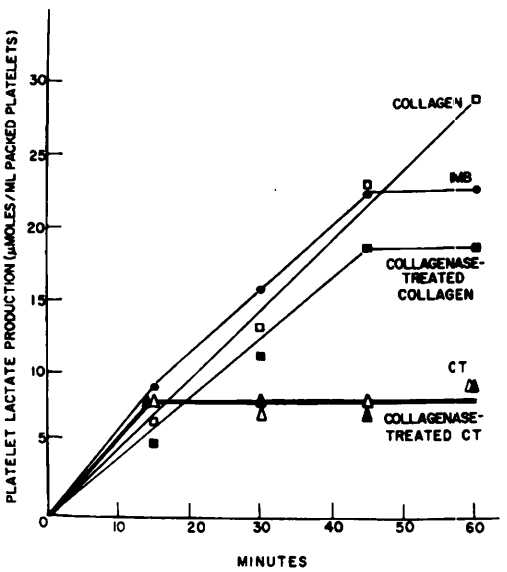

FIG. 1. Representative experiment showing lactate production after the addition of connective tissue and collagen alone and after treatment with collagenase.

Discussion. In our studies, lactate production in the control tubes containing a concentrated suspension of platelets in plasma at a pH of 6.7 and IMB (i.e., no aggregation) proceeded linearly for 45 min and then decreased markedly or ceased entirely. Karpatkin (8), using washed platelets suspended in an artificial medium containing glucose, observed a rate of 37.0 μmoles/ml/hr (S.E. $\pm$ 4.5) that is similar to ours. Lactate production was linear for 60 min and continued at a diminishing rate for 2 more hr. Other investi-

TABLE II. Effect of Collagen and Connective Tissue on Platelet Aggregation and Lactate Production.

Agents used	No. expt.	Degree of aggregation	Duration of linearity (min)	Average rate of lactate production[a] (μmoles/ml packed platelets/hr) $\pm$ SE
IMB	6	0	45	37.9 $\pm$ 1.7
Collagen	4	++++	>60	39.7 $\pm$ 4.3
Collagen + collagenase	3	0	45	35.1 $\pm$ 5.1
CT	5	++++	15	53.6 $\pm$ 3.4
CT + hyaluronidase	3	++++	60	44.8 $\pm$ 3.7
CT + Mg^{2+}	2	++++	60	43.1 $\pm$ 4.4
CT + collagenase	2	0	15	42.4 $\pm$ 5.8

[a] Values for rate of lactate production over linear portion were obtained by extrapolation.

gators observed higher values for washed platelets (1, 18).

Although collagen, CT and hyaluronidase-treated CT caused the formation of huge platelet clumps, their effects on lactate production differed. In the presence of collagen and hyaluronidase-treated CT, production continued linearly for slightly longer than in the control system and at essentially the same rate. In contrast, CT arrested lactate production within 15 min in all five experiments. Collagenase-treated CT, which failed to induce aggregation (14), also arrested lactate production. These findings suggest that the inhibition noted with untreated CT could be attributed to mucopolysaccharide rather than to the collagen or to aggregation itself. Connective tissue contains up to 20% of mucopolysaccharide polyelectrolytes whose main component is hyaluronic acid (19). Since this acid has high affinity for divalent cations (19) and might reduce their concentration significantly, added magnesium was evaluated. It was found that lactate production was no longer arrested; production occurred at about the same rate as with the controls or hyaluronidase-treated CT and, as with the latter, continued for 60 min. Magnesium deprivation may thus explain the early arrest of lactate production caused by CT. The limited concentration of magnesium ions in the heavily citrated plasma may also account for the arrest of lactate production at 45 min in the control samples. When aggregation occurs in the absence of mucopolysaccharide (as with hyaluronidase-treated CT, or with collagen), magnesium may leak from the platelets, as do ADP and serotonin (13, 20), permitting lactate production to continue for at least 60 min.

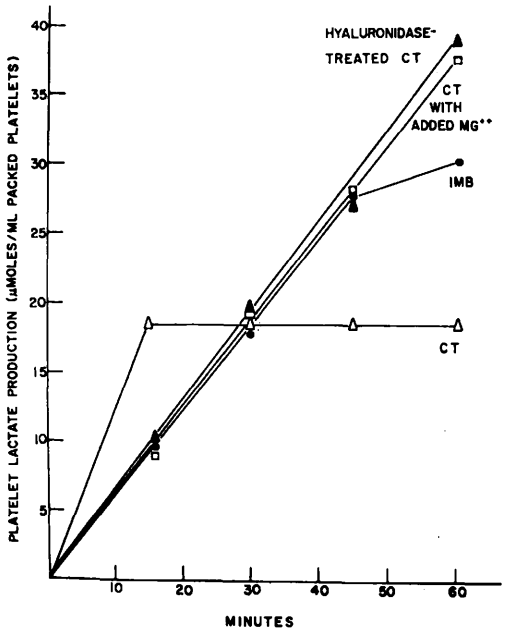

FIG. 2. Representative experiment showing the inhibitory effect of connective tissue on lactate production and abolition of this effect by hyaluronidase treatment or the addition of magnesium.

Collagen and CT resulted in aggregation and in a marked initial burst of lactate production whereas collagenase-treated collagen did not cause either. Although it is tempting to attribute the early increase in lactate pro-

duction to aggregation, it did not occur with CT treated with hyaluronidase or added magnesium, both of which induced platelet aggregation.

Corn (7) observed that connective tissue did not affect the amount of lactate produced in 45 min by washed platelets although it caused aggregation. We obtained similar results on unwashed platelets with low concentrations of connective tissue.

Bettex-Galland and Lüscher (5) noted an increase in glycolysis up to 10 min after the addition of thrombin. Production then slowed and ceased completely within 30 min. Others who reported that thrombin increases lactate production (7, 8) measured lactate only at 45 or 60 min, so whether increased production occurs only early, or throughout the study period, cannot be determined. ADP is the final mediator of other aggregating agents and does not itself affect lactate production (8). Thus, the burst of lactate production caused by other agents is presumably not the result of aggregation *per se* but rather of the processes leading to ADP release from the platelets. Taken together, the data suggest that thrombin (5), epinephrine (8), CT, and collagen cause a burst of lactate production without a sustained increase.

Summary. Although connective tissue and collagen induced the formation of huge platelet clumps, they had different effects on lactate production. With collagen, platelet lactate production was linear for 60 min, as compared with 45 min for control platelet suspensions. The rate of lactate production increased during actual aggregation and was similar to that of the control thereafter. Collagenase-treated collagen behaved like the control samples. In contrast to collagen, CT caused an arrest of lactate production within 15 min. Prior to this time, more lactate was produced than in the controls. Collagenase-treated CT also arrested lactate production within 15 min but caused no aggregation. After the addition of hyaluronidase-treated CT or CT with added Mg^{2+}, however, aggregation occurred, and lactate production proceeded linearly for 60 min, similar to the rate for control or collagen-treated platelets. These findings appear to show that the arrest of lactate production by CT was due to the binding of divalent cations by acid mucopolysaccharides.

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Bagassois III. Isolation of Thermophilic and Mesophilic Actinomycetes and Fungi from Moldy Bagasse* (33319)

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Dried sugar cane fiber from which the liquid sucrose content has been extracted is known as bagasse. This material is commonly baled and allowed to remain exposed in the field for months before being processed into wall board and paper products. During storage it is subject to high environmental temperature and humidity and serves as a growth medium for myriads of soil fungi and bacteria. Approximately 5×10^8 fungal spores/g of dry weight have been estimated in the exposed specimens (1).

Some industrial workers who crush and grind the dry, raw material develop a characteristic respiratory illness called bagassosis that resembles other forms of "extrinsic allergic alveolitis" (2) or "hypersensitivity pneumonitis" (3). These include farmer's lung, sequoaosis, maple bark stripper's, mushroom picker's, thatched roof, pigeon breeder's, and pituitary snuff inhaler's diseases among others (4–12). Individuals with bagassosis demonstrate precipitating antibody against extracts of exposed moldy sugar cane fiber, suggesting that certain microorganisms growing in bagasse at high temperatures are involved in the etiology and pathogenesis of the disorder.

This report describes the most common thermophilic species isolated from bagasse samples obtained in Louisiana, Puerto Rico, and India.

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Materials and Methods. Organisms were isolated by grinding dry bagasse fiber in a Waring blendor for 10 min followed by exposure of sterile gelatin-coated Whatman No. 1 filter paper strips in the blendor flask atmosphere at 5, 10, 15, and 20-min intervals. Strips were immediately transferred by means of a sterile forceps into Petri dishes containing several media.

The media used included half-strength nutrient agar, yeast extract agar, V_8 agar, potato agar, and peptone iron agar. Petri dishes were incubated at 45°, and 0.5 mg of actidione/ml was added to inhibit fungal growth. Half-strength nutrient agar and yeast extract agar generally yielded the highest numbers of thermophilic microorganisms; peptone iron agar was used only for the melanin test.

Amino acid and sugar compostion of selected actinomycete whole-cell hydrolysate preparations were determined by descending paper chromatography on Whatman No. 1 filter paper by a modification of the technique of Becker, Lechevalier, and Lechevalier (13). Organisms from which cell-wall preparations were made were harvested from yeast extract or half-strength nutrient broth after incubation periods varying between 3 and 5 days. Broth was centrifuged at 1500 rpm then washed directly with sterile distilled water and ground for 15–20 min under sterile conditions using a Kontes glass grinder in an ice bath. Ground cells were immediately lyophilized. Hydrolysis and chromatography