Origin of Lactate Dehydrogenase in Fluid of Cantharidin Produced Blisters.* (32275)

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Lactate dehydrogenase (LDH) in man occurs in at least 5 electrophoretically distinct forms, the relative amounts of each isozyme present being characteristic of each tissue. It is thus possible to investigate the origin of this enzyme in a transudate or exudate. This technic has been used to determine the origin of LDH in cerebrospinal fluid(1,2) and synovial fluid(3,4). In this paper, the technic has been used to determine the origin of LDH in cantharidin-produced blister fluid.

Materials. Cantharidin was obtained from Inland Alkaloid Inc., Tipton, Ind.; Siliclad from Clay-Adams Inc., New York; Plasmagel from Laboratoire Roger Bellon, Neuilly, Seine, France; nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), NADH, NAD, and sodium pyruvate from Sigma Chemical Co., lithium lactate from Nutritional Biochemicals Corp.; trypsin in Hanks' BSS from Robbin Laboratories, Chapel Hill, N. C.; and 7 × 100 mm tubes from Johnsen and Jorgensen Ltd., London, England.

Methods. Blister fluid. Cantharidin (100 μg) in acetone (100 μl) was applied to each of 4 areas 2 cm in diameter on the upper lateral aspect of the arms of male volunteers. A wet occlusive dressing was applied under a vaccination shield. The 4 blisters were aspirated successively at 7, 12, 24, and 48 hours. The fluid was stored at 4°C until all the specimens had been collected. Before use, the specimens were centrifuged at 3,000 rpm (r. max. = 14.5 cm) for 20 minutes to remove the clot(5) and cells.

Epidermis and dermis. A Castroviejo keratotome with a setting of 0.3 mm was used without anaesthesia to remove about 1 cm^2 skin from an area near a blister. After rinsing away blood in 0.9% NaCl, the skin slice was incubated with 0.25% trypsin in Hanks' BSS for 30 minutes at 37°C. After this time, the dermis was easily separated from the epidermis.

Fractionation of blood. Leucocytes and platelets were separated from blood using a method based on that of Amos and Peacocke (6). All glassware was washed with chromic acid and siliconized with Siliclad. Freshly drawn blood (10 ml) was added to 5% EDTA Na_2 (1 ml). To this was added 3% Plasmagel (3 ml). The contents of the tube were mixed by inverting and bubbles were removed with a Pasteur pipette. After standing 20 minutes, the pink plasma, containing most of the leucocytes and platelets and many erythrocytes, was transferred to a 10 × 75 mm tube and centrifuged at 1,000 rpm (r. max. = 14.5 cm) in swing-out head for 4 minutes. The sediment and supernate were then treated separately as follows.

The supernate was transferred to a 10 × 75 mm tube and centrifuged at 3,000 rpm for 20 minutes to yield plasma and platelets. The sediment was resuspended in 0.1 ml platelet-free plasma, transferred to a 6 × 50 mm tube, and allowed to stand for 10 minutes. The leucocyte-rich supernatant was diluted with an equal volume of buffered EDTA saline (Na_2HPO_4 1.3 g, Na_2EDTA 1.5 g, NaCl 8.5 g in 1 liter) and allowed to stand for 5 minutes to remove more erythrocytes. A portion of this supernatant (0.3 ml) was then layered on 1.6 ml platelet-free plasma in a 7 × 100 mm tube. The leucocytes were then centrifuged down through the plasma at 2,000 rpm for 2 1/2 minutes leaving the platelets in suspension and yielding a sediment consisting of leucocytes and a few erythrocytes. The erythrocytes were removed by suspending the sediment in 1.5 ml buffered ammonium chloride (2% tris adjusted to pH 7.65 with HCl, 1 vol: 0.83% NH_4Cl, 9 vol) incubated at 37°C for 5 minutes and cen-

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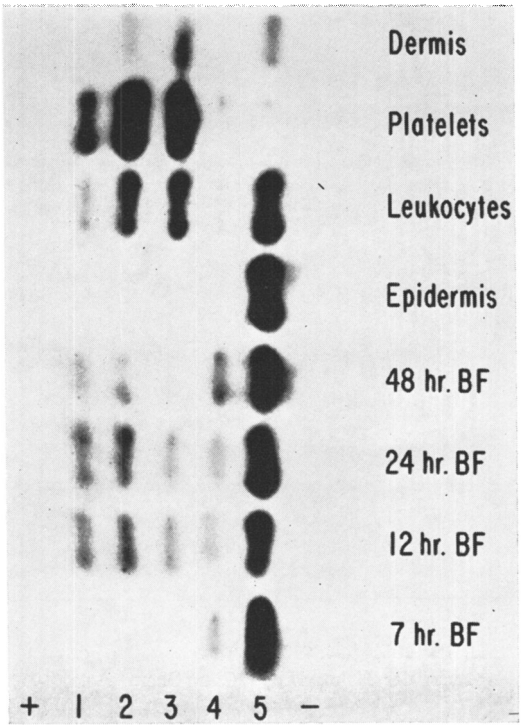

FIG. 1. LDH isozyme pattern of dermis, platelets, leukocytes, epidermis and 7, 12, 24, and 48 hr blister fluid (BF).

trifuged at 500 rpm for 4 minutes to yield a white residue of leucocytes. The platelets obtained from the first centrifugation were freed from erythrocytes similarly. Platelet-free plasma was concentrated approximately 10 times by ultrafiltration.

Extraction of enzymes. Epidermis and dermis were ground with tris buffer (0.05 M, pH 7.5/25°C) in a glass tissue grinder (0.1 ml buffer per 5 mg wet tissue). Insoluble material was removed by centrifuging at 3,000 rpm (r. max. × 14.5 cm) for 30 minutes. Platelets and leucocytes from 10 ml blood were suspended in 0.5 ml tris buffer (0.05 M, pH 7.5/25°C) and subjected to 4 freeze-thaw cycles using solid CO_2-acetone and water at 25°C. Insoluble material was removed as before.

Determination of LDH activity. The activity of LDH in plasma, blister fluids, and tissue extracts was measured by recording the rate of decrease of optical density at 340 mμ of a solution containing in 3 ml, 300 μM phosphate buffer pH 7.0, 1.0 μM sodium pyruvate, 0.35 μM NADH and 20 μl tissue extracts.

Electrophoresis. Separations were performed on cellulose acetate using a Beckman microelectrophoresis apparatus. Approximately the same amount of LDH activity was applied to each station. This was done by diluting samples of high specific activity with tris buffer (0.05 M, pH 7.5/25°C) or by making up to 4 applications of low activity samples. Electrophoresis was performed in barbiturate buffer (pH 8.6, I = 0.075) at 250 volts for 30 minutes.

Visualization of LDH activity on cellulose acetate strips. LDH activity was visualized with nitro blue tetrazolium using the technic described by Preston, Briere, and Batsakis (8).

Results. Fig. 1 shows the pattern of LDH isozymes found in blister fluid, epidermis, dermis, leucocytes, and platelets. Similar patterns were obtained in all the 6 subjects studied. When lactate was omitted from the incubation medium, no color appeared confirming that the bands did indeed indicate the presence of LDH.

Discussion. LDH in epidermis has been shown by us to occur almost exclusively in the form of LDH_5. When larger amounts of activity were applied to the strip, isozymes 4, 3, and 2 were also seen as reported by Weber and Pfleiderer(9); LDH_1 was not detected (10).

What little LDH activity there is in the upper dermis resides mainly in LDH_5 and LDH_3 with some LDH_4. This does not agree with the findings of Weber and Pfleiderer(9) who found most of the dermal activity in LDH_5 with some LDH_4 and less LDH_3.

Platelets were shown to contain predominantly LDH_3 and LDH_2 as reported by Vesell(11). The main LDH isozymes found in leucocytes were LDH_5, LDH_3, and LDH_2 in general agreement with Vesell *et al* (4). Plasma contained LDH_1, LDH_2, and LDH_3 with traces of LDH_4, and LDH_5 as reported by many workers(12,13,14). Erythrocytes have been shown by Cohen *et al*(15) to contain LDH_1, LDH_2, and some LDH_3.

Blister fluid collected 7 hours after application of cantharidin contained LDH_5 with

some LDH_4. This excludes plasma, dermis, platelets, leucocytes, and erythrocytes as the source of the enzyme. At 12 hours, appreciable amounts of LDH_1, LDH_2 and LDH_3 are present in blister fluid, which could be derived from leucocytes or platelets. This is consistent with the observations of Smith *et al* (16) who found few leucocytes present in blister fluid 7 hours after the application of cantharidin, but found many cells 5 hours later.

Summary. Lactate dehydrogenase (LDH) isozyme patterns in the blister fluid of cantharidin-produced blisters, epidermis, dermis, leucocytes, platelets, and serum have been studied. Blister fluid collected 7 hours after cantharidin application contained mainly LDH_5 with some LDH_4. LDH_2 and LDH_3 also appeared in blister fluid collected 12 hours or longer after application of cantharidin. This finding is consistent with the hypothesis that LDH in cantharidin-produced blisters is derived initially from epidermis and is later augmented from leucocytes or platelets.

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Pregnanolone: A Highly Potent, Naturally Occurring Hypnotic-Anesthetic Agent. (32276)

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The first findings of Selye in 1940(1) on the hypnotic effect of certain hormonal steroids have since been substantially expanded. A group of water soluble ester salts of 5 beta-pregnane derivatives with hypnotic effects and lacking hormonal activities were described 10 years ago(2). One of these compounds, hydroxydione, the sodium hemisuccinate salt of 21-hydroxy pregnanedione has been introduced as an intravenous anesthetic agent. Some side effects, however, limited its therapeutic usefulness. Recent efforts to obtain suitable and more potent water soluble anesthetic steroids did not meet with much success(3,4).

Of the hormonal steroids studied by Selye (1) progesterone showed considerable potency. Since it was subsequently shown that some of the naturally occurring metabolic products of this hormone are even more potent hypnotics (2) a neuropharmacologic exploration comparing progesterone with these hormonally inactive but hypnotically potent metabolites has been made(5). It was found that a) the free

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