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### Lung Fibrinolytic Activity and Bovine High Mountain Disease\* (32694)

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The etiology of high mountain disease of cattle (brisket disease) is still unknown though the pathological lesions have been studied in detail by many investigators (1). Characteristic is a medial muscular hypertrophy of the small pulmonary arteries and arterioles with a concomitant adventitial proliferation. There is also diffuse interstitial and alveolar septal fibrosis. Speculations on the etiology have centered upon the change in altitude and the lowered oxygen pressure, but these are not sole etiologic factors since other domesticated animals (sheep, goat) do not develop the same disease pattern under identical conditions. An etiologic factor specifically related to cattle, but not to other animal species, has to be sought. Confusing the issue are the large individual variations in sensitivity to the disease (2,3).

Search for some property to discriminate the bovine lung from lungs of other species drew our attention to the presence of a potent inhibitor of fibrinolysis in the bovine lung. The bovine lung inhibitor (pulmin), first reported in 1950 (4,5), was later purified (6) and its effect on fibrinolysis studied in detail (7,8). Its probable identity with the inhibitor of Kallikrein and trypsin isolated from bovine parotid gland (Trasylol), and with the pancreatic trypsin inhibitor of Kunitz has recently been demonstrated (9,10).

Since brisket disease is characterized by

proliferative processes, indicating a response to frequent tissue injury with release locally of tissue thromboplastin and plasminogen activator, it could be anticipated that the presence in certain tissues of a potent anti-fibrinolytic compound would affect the process of repair by delaying resolution of fibrin. Such considerations led to the suggestion that the trypsin inhibitor in bovine lung could be the etiologic factor in brisket disease distinguishing cattle from other animal species (11). To substantiate this concept the fibrinolytic activity (tissue plasminogen activator concentration), trypsin inhibitor content, and thromboplastin concentration in lungs of various breeds of cattle were estimated.

*Materials and Methods.* Lungs were obtained fresh at slaughter houses and from agencies under the U.S. Department of Agriculture. Lungs from Brahman cattle were provided fresh by Dr. J. A. Boehm, then at Meat Inspection Division, Mid-State Meat Packers, Inc., Bartow, Florida. Samples were carefully rinsed under water, dried with absorbent paper, weighed and stored in tightly closed tubes at  $-20^{\circ}\text{C}$  until investigated.

For *plasminogen activator* assays (12) tissue samples were homogenized with 3.0 ml of 2M KSCN per 100 mg tissue followed by slow mechanical stirring for 1 hour and centrifugation. An aliquot of the supernatant was diluted with 7 vol. of water, the pH was adjusted to 1 with N HCl, and the mixture

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was centrifuged after 15 min. The sediment was redissolved in an amount of 1 M KSCN, containing 0.25% gelatin, equal to the volume of the original aliquot; solid  $\text{NaHCO}_3$  was added until neutrality, tested by litmus paper was reached. Of each stock solution serial dilutions were prepared in the 1 M KSCN and each concentration was assayed in triplicate on regular fibrin plates (9 ml) using 0.1% bovine, plasminogen-rich fibrinogen (13). Averages of diameter products were plotted in a double logarithmic diagram, and activities were converted into concentrations of plasminogen activator by interpolation on a reference curve obtained from a standard preparation. Results are given in tissue activator units (Astrup and Albrechtsen units) per gram of fresh tissue. One A. and A. tissue activator unit approximates 0.1 urokinase unit as defined by the Committee on Thrombolytic Agents (advisory to the National Heart Institute) (14). Figure 1 shows representative

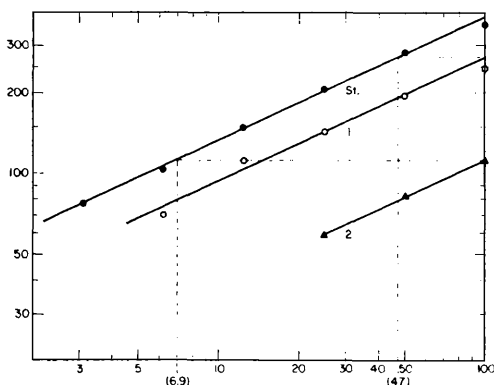


FIG. 1. Assay of plasminogen activator in the lungs of two different breeds of cattle. Abscissa: concentrations of lung extract in per cent of stock solution (logarithmic); Ordinate: fibrinolytic activity as diameter products in  $\text{mm}^2$  of the lysed zones (logarithmic), averages of triplicate.

St: reference curve of standard preparation from pig heart, containing 8.5 A. and A. units per ml. 1: Brahman cattle (*Bos indicus*). 2: white short horn steer (*Bos taurus*). Curve 1 represents an assay of 273 mg of tissue treated with 8.2 ml of 2 M KSCN. The stock solution (100%) yields the same activity as 47% of the standard stock solution which contains 8.5 units per ml. Hence, the concentration of plasminogen activator in the assay is  $(8.5 \times 47)/100$  units per ml. The total amount in the 8.2 ml from 273 mg tissue then

estimations of plasminogen activator concentration in the lungs of two different breeds of cattle.

The *trypsin inhibitor* was determined in an aliquot of the 2 M KSCN extract described above by preparing serial dilutions (in 2 M KSCN) and mixing 1 ml of each with 1 ml of a solution containing 10  $\mu\text{g}$  of crystalline trypsin (Novo Laboratories, Copenhagen) per ml of saline-barbital buffer (pH 7.75, 0.05 M barbital, 0.1 M NaCl). The activity of each dilution was estimated in triplicate on fibrin plates. Averages of diameter products were plotted against relative concentrations on normal graph paper (Fig. 2A). Interpolation on a curve produced from serial dilutions of a trypsin solution (5  $\mu\text{g}/\text{ml}$ ) gave the concentration of free trypsin in the mixture. Plotting for each dilution the estimated inhibition in per cent against the concentration of tissue extract yielded a linear curve from which the concentration of inhibitor could be read in terms of  $\mu\text{g}$  trypsin neutralized as shown in Fig. 2B.

*Tissue thromboplastin* suspensions were prepared in a Potter-Elvehjem homogenizer using per 100 mg tissue 0.9 ml of saline-barbital buffer containing 0.25% gelatin, followed by freezing overnight at  $-20^\circ\text{C}$ , thawing and rehomogenizing. The assay system consisted of 0.2 ml of human, platelet-poor, citrated plasma, 0.1 ml of saline-barbital buffer, 0.1 ml of thromboplastin solution and 0.2 ml of 0.03 M  $\text{CaCl}_2$ . Calculations were performed as described before (15). With human plasma the arbitrary unit, defined as the amount needed to produce coagulation in 60 sec under the experimental setup, differed from that previously defined on the basis of rabbit plasma. Human plasma is less sensitive to tissue thromboplastin. Comparisons (to be published elsewhere) have shown that an unit of tissue thromboplastin assayed on human plasma, is about twice the rabbit plasma unit, though qualitative differences also exist making a direct conversion of activities meas-

gives  $(8.5 \times 47 \times 8.2 \times 100)/(100 \times 273) = 120$  A. and A. units per gram of tissue. Curve 2 was obtained from 522 mg tissue in 16 ml. It corresponds to 6.9% of the standard and equals 18 A. and A. units per gram of tissue.

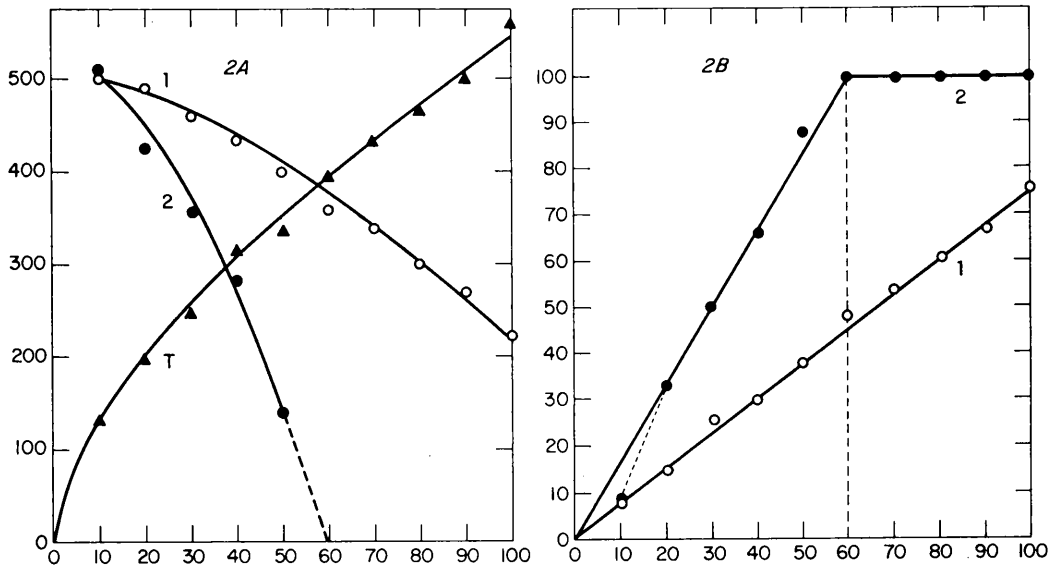


FIG. 2. Trypsin binding by extracts of lungs from 1) calf and 2) Hereford steer. A. Abscissa: Concentrations of lung extracts (1, 2) in per cent of stock solution, mixed with equal volumes of trypsin stock solution; T: Trypsin dilution curve. Concentrations in per cent of stock solution (10  $\mu$ g per ml) mixed with an equal volume of saline and further diluted; Ordinate: Fibrinolytic activity as diameter products in mm<sup>2</sup> of the lysed zones, averages of triplicate. B. Abscissa: Concentration of lung extract in per cent of stock solution; Ordinate: Percentage of trypsin inhibited as read from Figure 2A. Curve 1. The stock solution, neutralizing 76% of the trypsin in the trypsin stock solution, was prepared from 260 mg calf lung tissue in 7.8 ml 2 M KSCN. Hence, one gram tissue neutralizes  $(10 \times 76 \times 7.8 \times 1000)/(100 \times 260) = 230 \mu$ g trypsin. Curve 2. Similarly Curve 2 shows that 60% of the stock solution, prepared from 560 mg adult lung in 17 ml 2 M KSCN, completely neutralizes the trypsin solution. Hence, one gram tissue birds  $(10 \times 100 \times 17 \times 1000)/(60 \times 560) = 506 \mu$ g trypsin.

ured in the two systems questionable. The reproducibility, in particular the constancy of the slope of the linear curves obtained in a double logarithmic graph, was controlled with Simplastin (a calcium-free preparation, Warner-Chilcott Research Institute, courtesy of Dr. George Phillips).

**Results.** Every value in Table I was obtained from dilution curves prepared from triplicates (fibrin plate assays) or duplicates (thromboplastin assays). Data are given as units per gram of fresh tissue, and in the thromboplastin assay also as clotting times produced by the stock solutions.

Table I shows that there are large individual variations. Such individual differences have been observed in all of our assays of tissue fibrinolytic activity, in man as well as in animal species (16). The determinations of trypsin inhibitor confirm previous ob-

servations of high concentrations in the adult bovine lung with lower average concentrations in the calf lung. The reason for the higher average concentration in the unselected group is unknown but might be genetically determined.

The concentration of plasminogen activator is very low in the unselected group and in the other breeds of common cattle (*Bos taurus*). Calf lung showed a marked average activity but still in the lower range. Previously (6,17) plasminogen activator was never observed in lung from adult cattle and only occasionally in calf lungs. However, the fibrin plate method is now more sensitive to low concentrations of activator. Also, the animals studied previously were cows of a Danish red breed raised as dairy cattle. The finding of a higher average concentration of activator in the lungs of the Brahman cattle is significant since it separates

TABLE I. Assays on Fibrin Plates and of Thromboplastin.\*

Sample (no.)	Plasminogen activator		Thromboplastin		Sample (no., letter, name)	Plasminogen activator		Thromboplastin	
	(units/gm)	(units/gm)	(units/gm)	t (sec)		(units/gm)	(units/gm)	(units/gm)	t (sec)
Steer (un- selected)									
1	20	—	640	37	Short horn a) (steer)	4	245	1100	32
2	—	—	460	40.5	b)	4	275	—	—
3	73	660	720	36	c)	22 (10)	160 (227)	—	—
4	36	2250	150	54	Hereford a) (steer)	4	600	2150	27
5	34	2160	180	52	b)	10	645	—	—
6	—	—	190	51	c)	16 (10)	645 (630)	—	—
7	68	1485	530	39	Hereford a) (steer)	10	—	—	—
8	36	950	640	37	b)	14	850	1650	29
9	34	190	180	52	c)	16 (13)	—	—	—
10	59	1950	700	36.5	Angus a) (steer)	4	240	1550	29.5
11	54	—	800	35	b)	37	400	—	—
12	13	122	800	35	c)	20 (20)	475 (388)	—	—
13	77	53	530	39	Short horn a) (steer)	22	400	1000	33
14	42	—	1200	31.5	b)	4	765	—	—
Average	46	1450	550	41.1	c)	14 (13)	765 (645)	—	—
Calf (un- selected)									
1	34	276	1450	30	Short horn (beef)	20	600	1450	30
2	113	330	640	37	Jersey (steer)	27	600	2000	27.5
3	45	228	800	35	Average	16	562	1560	29.7
4	65	99	640	37	Brahman				
5	117	329	640	37	1	56	1500	530	39
6	77	450	2000	27.5	2	120	600	800	35
7	255	75	900	34	3	151	660	400	42
8	23	752	1200	31.5	4	134	174	280	46
Average	91	317	1030	33.6	5	296	243	1000	33
					Average	151	608	600	39

\* Plasminogen activator is recorded in A. and A. units per gram of fresh tissue. The inhibitor is recorded in units indicating the amount of trypsin bound per gram of fresh tissue. Thromboplastin is recorded in units assayed on human plasma per gram of fresh tissue, and also as the clotting time produced by the undiluted stock solution. Letters a), b), and c) indicate samples obtained from various sections of the lung of the same animal, in which case averages are given in parentheses.

this breed from other breeds of cattle, and brings it into the medium range of activities recorded among animal tissues.

The wide variation in tissue thromboplastin content prevents any serious considerations except to notice that the Brahman breed is in the lower activity group. Cattle are among the species with the highest thromboplastin content in their lungs (15).

*Discussion.* Plasminogen activator is chiefly related to the endothelial cells of veins and capillaries. In tissue repair the sprouting capillaries are particularly rich in activator (18). It is believed that plasminogen activator assists in removing fibrin laid down in a normal process of tissue repair thereby decreasing immigration of fibroblasts and limiting the formation of reparative connective tissue (16). The lesions in high mountain disease suggest that tissue injury followed by cellular proliferation and tissue repair is a salient part of the pathological process. If the concentration of inhibitor is increased locally delay in removal of fibrin deposits could be anticipated, followed by additional fibroblastic migration with formation of connective tissue leading to local fibrosis. The observed wide individual variations in inhibitor concentration could possibly explain the known variations in individual susceptibility to the disease. It would be interesting to know whether animals succumbing to brisket disease have a higher concentration of inhibitor in their lungs than those which are not affected.

The Brahman breed was included in this study in order to obtain data from a breed of cattle belonging to a species (*Bos indicus*) different from that which is most common in temperate climates (*Bos taurus*) (19). It is significant that there is on the average a considerably higher plasminogen activator concentration in the lungs of the Brahman cattle than in the *Bos taurus* breeds, even including the calves of the latter. Breeds of *Bos indicus* are reported to be more resistant to many diseases than breeds of *Bos taurus*. Our data suggest that their resistance could also be greater to mountain disease since fibrin de-

posits in their lungs might redissolve more quickly.

*Summary.* The concentrations of plasminogen activator, trypsin inhibitor, and tissue thromboplastin in lungs of different breeds of cattle have been determined. Brahman cattle (*Bos indicus*) has a higher average concentration of plasminogen activator than breeds of normal cattle (*Bos taurus*). There are large individual variations in concentrations of trypsin inhibitor. It is suggested that these variations could explain differences in susceptibility to high mountain disease.

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