

BIOCHEMICAL ALTERATIONS IN THE BLOOD, MILK AND TISSUES OF SHEEP MAMMARY GLAND AFTER EXPERIMENTAL MYCOPLASMAL MASTITIS

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To study the biochemical changes in milk, blood plasma and udder tissues, during unilateral mycoplasmal mastitis, 7 lactating sheep were inoculated through the right teat canal with 2 ml of Mycoplasma mycoides sub sp. mycoides (Mmm) Large-colony type (LC) culture containing 10^5 colony forming units per ml. The left udder halves of all the sheep served as controls. One animal was killed at every third day interval up to 21 days post-inoculation. All the sheep developed clinical mastitis within 24 h after inoculation of Mycoplasma, which persisted till the end of the experiment. The total protein, total cholesterol, total phospholipids and free fatty acid contents of mastitic milk and mammary secretions showed progressive increase, whereas total lipids and glycerides decreased substantially. Such results indicate that Mycoplasma use lipolytic enzymes to degrade lipids to meet the needs of energy and for the biosynthesis of membrane lipid bilayer during their multiplication.

Quantitative estimations and histoenzymatic studies revealed increased activities of aspartate aminotransferase (EC 2.6.1.1), alanine aminotransferase (EC 2.6.1.2), acid phosphatase (EC 3.1.3.2), alkaline phosphatase (EC 3.1.3.1), malate dehydrogenase (EC 1.1.1.38), lactic dehydrogenase (EC 1.1.1.27), adenosine triphosphatase (EC 3.6.1.3), and amylase (EC 3.2.1.1) in blood plasma, milk and udder tissues which was partially due to increased total leukocyte counts and the Mycoplasma organisms.

Total protein, total cholesterol, phospholipids, free fatty acids, glycerides, enzymes, M. mycoides.

It has been reported (Prasad et al. 1985; Mistri et al. 1988) that Mycoplasma infection in goat udder produces spontaneous agalactia with marked reduction in the size of secretory mammary tissue and without any systemic reaction. Mycoplasma mycoides sub sp. mycoides (Mmm) Large-colony type (LC) has been reported to cause various diseases exclusively in goats (D a M a s s a et al. 1983) and infection of sheep with this Mycoplasma appears to be rare, especially mastitis in sheep due to Mmm LC has not been reported. The present work was therefore undertaken to study alterations in milk, blood plasma and udder composition and the activities of their enzymes during experimentally produced mycoplasmal mastitis in sheep and with a view to assess the pathogenicity of Mmm LC to lactating sheep udder.

Materials and Methods

Seven lactating sheep aged 2.5 to 3 years were observed for 7 days preexperimentally. All were found healthy and free of sub-clinical mastitis. The total leucocyte count (TLC) of milk varied from 0.25 to 0.35 x 10⁶ ml⁻¹. The milk was free from infectious agents. Two ml of 48h culture of Mmm LC at third passage level, containing 10⁵ colony forming units (CFU).ml⁻¹ was inoculated through the right teat canal into the right half of udder of all the 7 sheep and their left halves were inoculated with 2 ml of sterile mycoplasma broth to serve as control. Any abnormalities in udder, temperature, systemic disturbances and mastitis (assessed by California mastitis test and modified Whiteside test) were recorded. Samples of milk/mammary secretions were obtained from all the sheep before inoculation and at every third day interval up to twentyone days after infection (DAI). These were cultured on mycoplasma medium (B a n e r j e e et al. 1979), blood agar and Sabouraud's Dextrose agar. Total leucocyte (TLC) of milk/mammary secretions and haematological value of sheep were also recorded. The pooled milk/mammary secretions were analysed for total lipids (F o l c h et al. 1957), total phospholipids (A m e s 1966), total cholesterol (Z l a t k i s and Z a k 1969), total free fatty acids (L o w r y and T i n s l e y 1976), total glycerides (by difference) and total proteins (G o r n a l l et al. 1949). Estimations of various enzymes in milk and udder tissues was done by following the methods described by W o o t t o n (1964). Blood plasma samples were analysed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), acid phosphatase (ACP), alkaline phosphatase (ALP), lactic dehydrogenase (LDH), malate dehydrogenase (MDH), adenosine triphosphatase (ATPase), amylase and creatinine (W o o t t o n 1964).

The experiment was continued for 21 days. One animal was killed at every 3rd day interval i.e. up to twentyone days after infection. Udders and their lymph nodes were examined grossly and microscopically. For microscopic examination 5 to 6 μ thick paraffin sections were stained with haematoxylin and eosin. Cryostat sections of the udder tissues were used for histochemical and histoenzymic studies (B a r k a and A n d e r s o n 1963).

Results

Body temperature was increased transitorily from third to sixth DAI without affecting the appetite. The infected

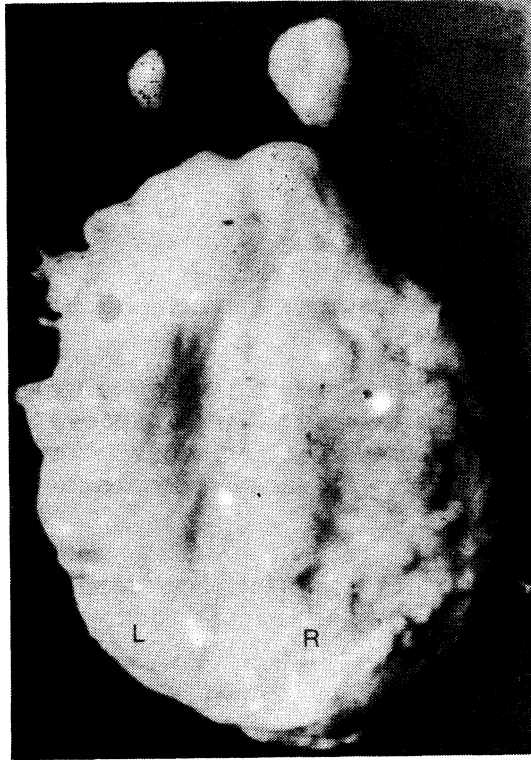


Fig. 1. Increase in the size of right udder half (R) and its corresponding lymph node 21 days after inoculation with M. mycoides sub sp. mycoides Large-colony type.

udder halves of all the 7 sheep were inflamed, hot, tender and painful at second DAI and remained enlarged (Fig. 1) throughout the experiment. Between day 3 and 21 post infection there was a marked decrease in the volume of milk/mammary secretions from the right infected halves and the secretions were yellowish, thick, turbid with shreds of blood and on standing, the proteins settled down as aggregates leaving clear whey-like fluid. On the other hand, there was only slight change in colour, consistency and amount of milk drawn from moninfected left udder halves. Mastitis in the right infected halves was severe (Fig. 2) from 2nd DAI to 21st DAI.

The total leucocyte counts of milk from infected halves increased from the basal value of $0.30 \times 10^6 \text{ ml}^{-1}$ to $2.14 \times$

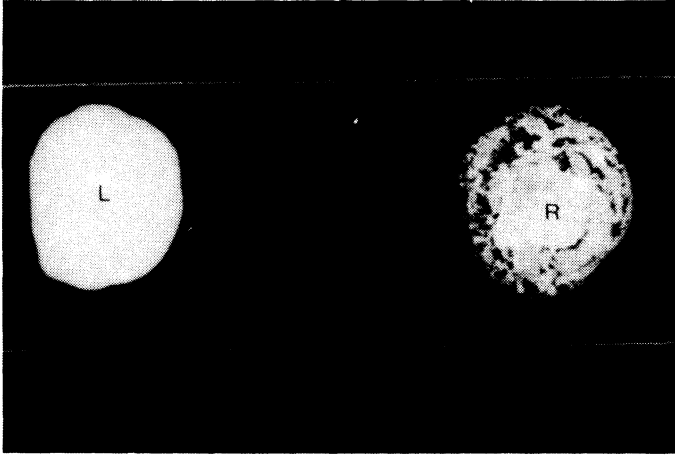


Fig. 2. Right(R)-Milk from right infected udder half showing severe positive reaction with modified Whiteside test. Left(L)-Milk from left control udder half of the same animal was negative for mastitis test.

$\times 10^6 .\text{ml}^{-1}$ on 3rd DAI, $17.4 \times 10^6 .\text{ml}^{-1}$ on 6th DAI and $27.8 \times 10^6 .\text{ml}^{-1}$ on the 9th DAI.

Thereafter these values decreased gradually ($18.8 \times 10^6 .\text{ml}^{-1}$, $13.1 \times 10^6 .\text{ml}^{-1}$, $8.7 \times 10^6 .\text{ml}^{-1}$ and $2 \times 10^6 .\text{ml}^{-1}$ on 12th, 15th, 18th and 21st DAI, respectively) but remained much higher than the basal values. Leucocyte counts of milk from noninfected left halves were unaffected. The total leucocyte counts of blood increased from the basal value of $6.4 \times 10^3 .\text{cmm}^{-1}$ to $12 \times 10^3 .\text{cmm}^{-1}$ on 15th DAI. There was a gradual decrease of haemoglobin content also.

Mmm LC was re-isolated from mammary secretions of all the right infected udder halves up till 18th DAI, but not from the milk of noninfected udder halves. No bacteria or fungal agent was detected in the milk of any sheep during the experiment.

Microscopically, the infected udder halves of sheep killed on 3rd and 6th DAI showed acute diffuse purulent mastitis characterised by marked infiltration of neutrophils in the lumina of acini (Fig. 3) and vacuolar degeneration of epithelial cells lining the acini. The animals killed on 9th, 12th, 15th, 18th and 21st DAI showed chronic interstitial mastitis. Atrophied acini, proliferated fibrous connective

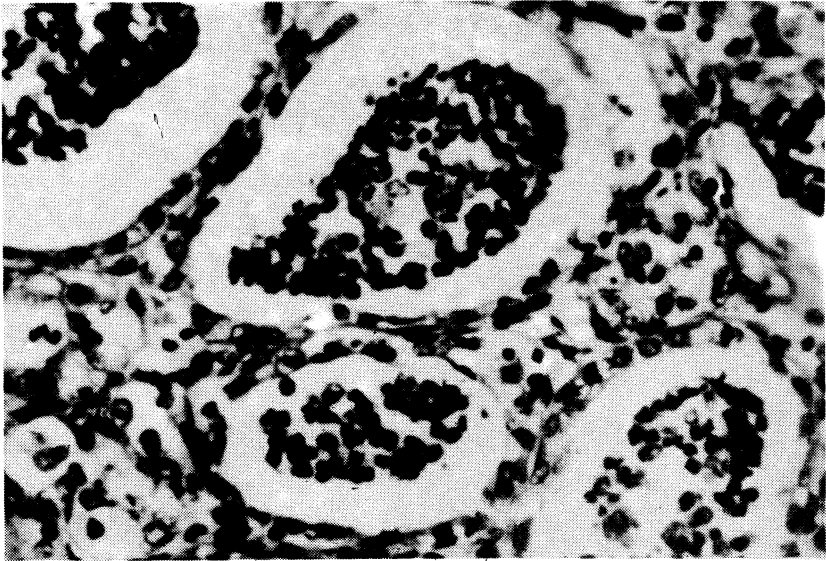


Fig. 3. Section of right infected udder half of sheep killed on 3rd day after infection showing acute diffuse purulent mastitis characterised by marked infiltration of neutrophils in the lumina of acini. H. E. X 300.

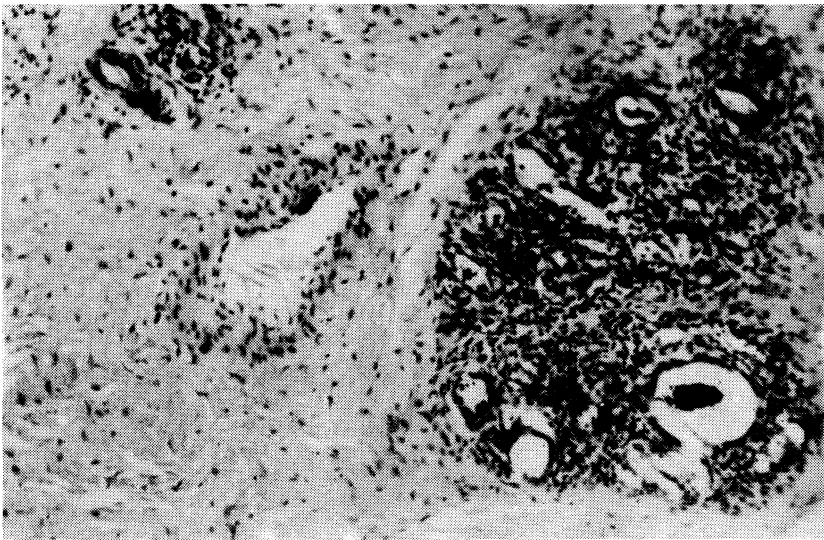


Fig. 4. Section of right udder half of sheep killed on 21st day after infection showing chronic mastitis with intense fibrosis replacing the glandular parenchyma. H. E. X 70.

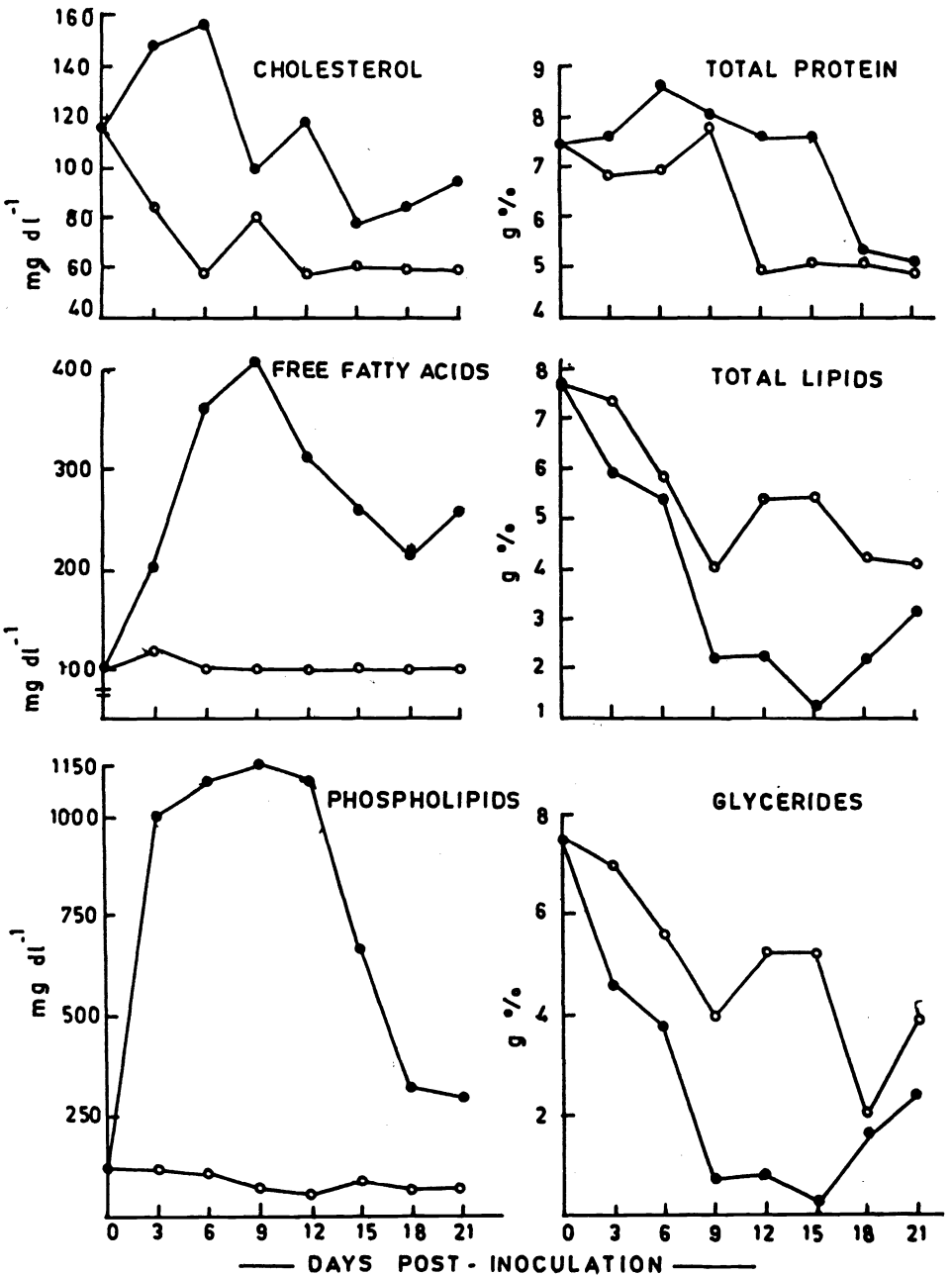


Fig. 5. Changes in the composition of cholesterol, free fatty acids, phospholipids, total protein, total lipids and glycerides in mammary secretions from control O—O and inoculated ●—● udder halves of sheep.

tissue (Fig. 4), thickening of interlobular septa, chronic galactophoritis with squamous metaplasia of the lining epithelium and marked infiltration of lymphocytes were observed. The mammary lymph nodes of infected udder halves showed reactive lymphadenitis. There was no pathological change in the non-infected udder halves and their lymph nodes.

The total protein, total cholesterol, total phospholipids and free fatty acid content of mastitic mammary secretions, from infected udder halves increased markedly after infection and remained higher than those in the milk from left control halves throughout the experiment (Fig. 5). The total lipids and glycerides content were decreased substantially (Fig. 5). Esterification of cholesterol was higher in mastitic milk/mammary secretions from infected udder halves as compared to the control left halves.

In blood plasma, gradual increase in the levels of ALT, AST, ALP, ACP, LDH, MDH, ATPase and amylase was observed without any appreciable change in the levels of creatinine (Fig. 6). In milk, increased activities of AST, ALT, ALP, ACP, and ATPase were observed from both infected and control udder halves throughout the experiment but the increase was more marked in the right infected halves. There was no significant change in the amylase levels in milk either from the right or left udder halves (Fig. 7a).

The AST, ALT, ACP, ALP, LDH and ATPase increased gradually in the tissues of both udder halves after inoculation of Mycoplasma but increase in these enzymes was more marked (about 2 times) in the right infected halves than those in left halves (Fig. 7b). Histoenzymic studies also revealed increase in the activities of ALP, ACP, LDH and succinic dehydrogenase (SDH) in the infected right udder halves.

Discussion

This study clearly indicated that Mycoplasma mycoides sub sp. mycoides (Mmm) Large-colony type (LC) was highly pathogenic to sheep udder and caused mastitis by damaging the udder tissue. These effects have been reported to be due to binding of Mycoplasma to secretory cells leading to their death by peroxide, nucleases and toxic metabolites

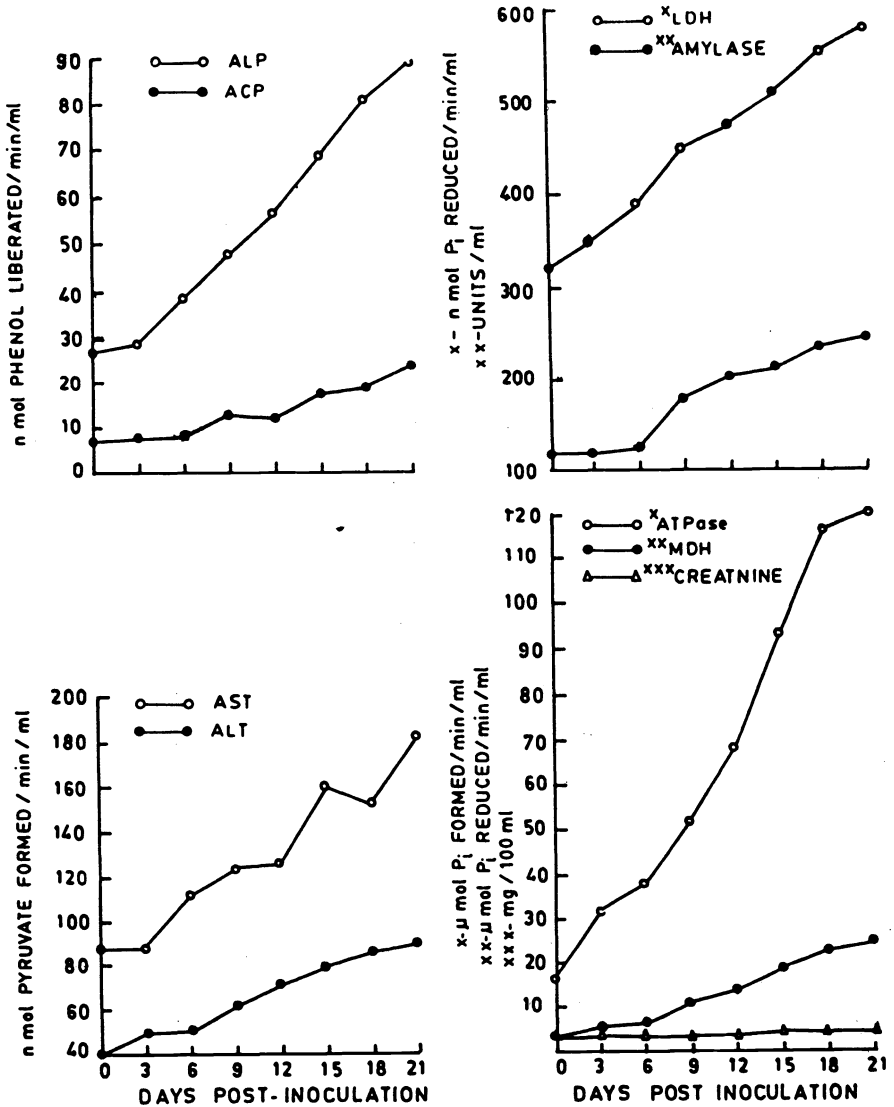


Fig. 6. Changes in the levels of creatinine and enzymes in blood of sheep administered *Mycoplasma* in the right half of the udder.

(Cherry and Taylor Robinson 1970).

The re-isolation of Mmm LC from milk/mammary secretions even eighteen DAI confirmed that the mastitis was of mycoplasmal origin. The increased TLC in milk and other gross and microscopic changes in udder of sheep observed in this study were similar to those described in contagious agalactia caused by *M. agalactiae* (Heidrich and

Renk 1967; Bar-Moshe and Rapaport 1978; Cottew 1979; Barton and Cottew 1980), and in experimental mastitis in sheep caused by M. canadense (Ball and Mackie 1986), animal ureaplasmas (Ball and Mackie 1985 ab), M. ovi-pneumoniae (Jones 1985), in goats inoculated with M. putrefaciens (Adler et al. 1980), M. agalactiae sub sp. bovis (Ojo and Ikede 1976), M. bovi-genitalium (Pal et al. 1983), M. arginini (Prasad et al. 1985), M. mycoides sub sp. capri (Misri et al. 1988) and in cows inoculated with M. bovis (Bennet and Jasper 1978; Meszáros et al. 1986).

The lipid changes indicated increased cellular content of mycoplasma, as well as leucocytes and mycoplasmal lipase activity in the milk. Cryostat sections stained with Oil-Red-O, also showed less neutral lipid in the infected right udder halves. These biochemical and histochemical changes are similar to those reported by Misri et al. (1988) in caprine experimental mastitis due to M. mycoides sub sp. capri. The nonspecific lipase showing optimum activity in alkaline pH range has been observed in some species of Mycoplasma (Smith 1979). The increase in cholesterol content indicated hydrolysis of cholesterol esters by Mycoplasma for incorporation of cholesterol into its own membranes. The presence of sterol esterase has also been demonstrated in M. arthritidis, M. gallinarum and A. laidlawii (Smith 1979). Mycoplasmas are known to require preformed cholesterol (Smith 1979), therefore they may depend upon cholesterol present in mammary secretions for incorporation into their own membranes during their multiplication. The increase in total phospholipids and protein content of mastitic milk/mammary secretions may be due to higher content of membranous material either from Mycoplasma and/or inflammatory cells. The latter might also be due to albuminous secretions induced by Mycoplasma infection (Schalm 1977).

Although an effect of bacterial mastitis on composition of mammary secretions of sheep has been reported (Agarwal and Narayana 1976; Mandal et al. 1977 and Mandal and Raheja 1985) yet such information with regard to Mycoplasma-induced mastitis in sheep is not available. An increase in free fatty acids (Randolph and Erwin, 1974; Mandal et al., 1977), cholesterol (Mandal and Raheja

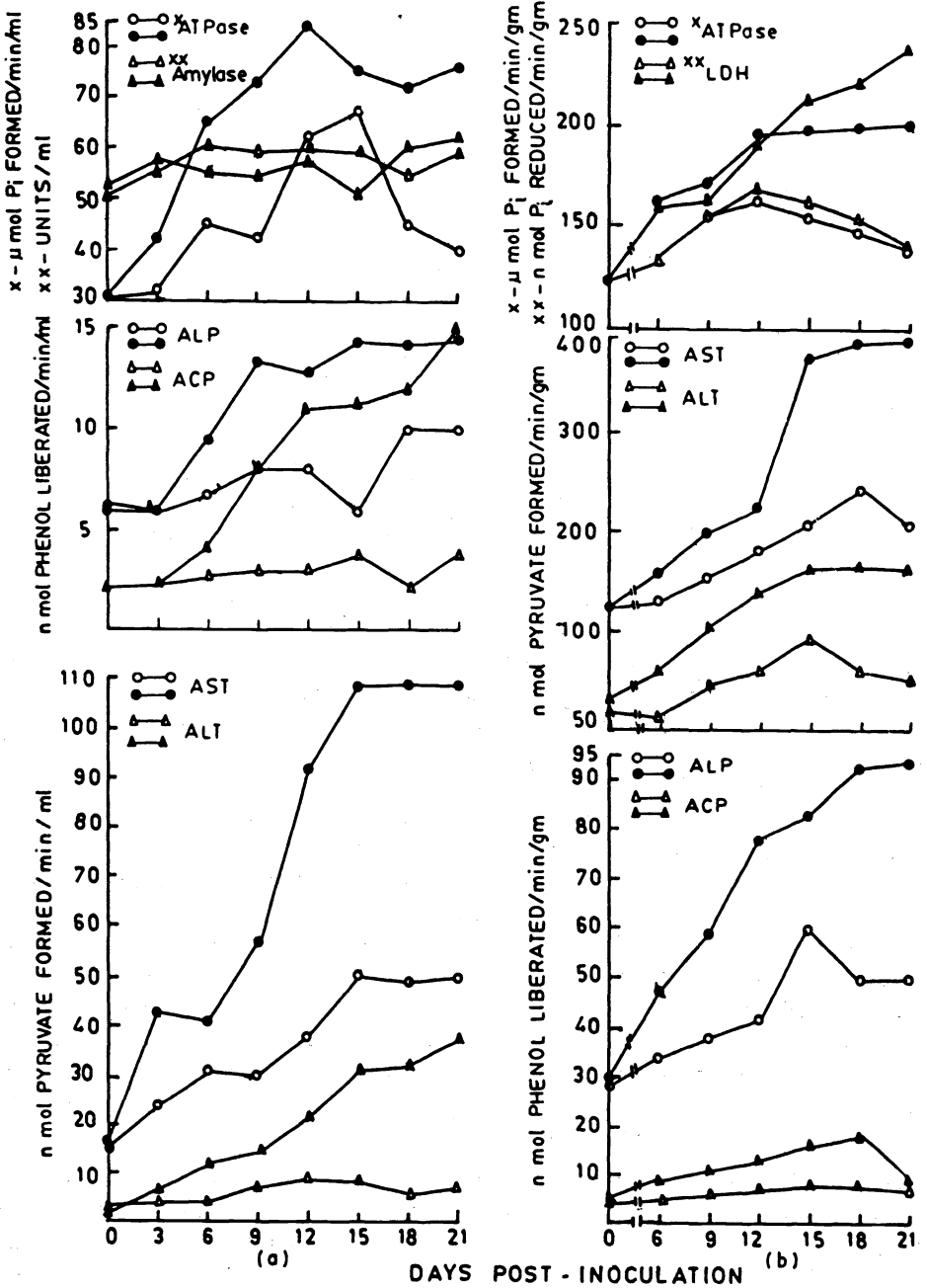


Fig. 7 Changes in the levels of various enzymes in mammary secretions (a) and tissues (b) from control (O—O, Δ — Δ) and inoculated (●—●, \blacktriangle — \blacktriangle) udder halves of sheep.

1985), phospholipids (A g a r w a l and N a r a y n a n 1976) and decrease in total lipids/glycerides (M a n d a l and A h u j a 1985) has been reported after bacterial infection. Such reports and findings of M i s r i et al. (1988) in experimental mastitis in goats due to M. mycoides sub sp. capri, and the results of the present study indicate that mastitis due to bacteria or Mycoplasma generally cause hydrolysis of milk glycerides and catabolise the released fatty acids for their anabolic needs.

Increase in the levels of ALT and AST in plasma and an appreciable increase in the levels of ACP, in udder tissues, blood plasma and milk have been reported to indicate acute or chronic tissue damage (W i l s o n 1970). Increased activity of ALP enzyme in udder might be due to marked aggregation of neutrophils (H o r n et al. 1964; J a i n 1968) and due to damage to udder tissues (K a n e k o 1980). Increased activities of LDH and SDH in infected udders indicate increased glycolysis (L e h n i n g e r 1982). B o g i n et al. (1976) also reported increased activity of LDH in bovine udder tissue during acute or chronic mastitis. The increase in amylase in blood plasma, and ATPase levels in plasma and udder tissue might have increased to meet the increased demand for supply of energy.

Mycoplasma mycoides sub sp. mycoides Large-colony type, although thought to be infective exclusively in goats, was found to be highly pathogenic to lactating sheep udder. It produced severe mastitis, with extensive damage to secretory tissue of udder which led to agalactia.

Biochemické změny v krevní plazmě, mléce a ve tkáních
mléčné žlázy ovcí po experimentální infekci
Mycoplasma mycoides

Biochemické změny v krevní plazmě, mléce a tkáních mléčné žlázy byly sledovány u 7 laktujících ovcí s unilaterální mastitidou, vyvolanou experimentálně aplikací 2 ml kultury Mycoplasma mycoides subsp. mycoides (Mmm) typu velkých kolonií (LC) s obsahem 10^5 buněk schopných reprodukce (CFU) v 1 ml. Kultura M. mycoides byla vpravena do strukového kanálku pravé poloviny mléčné žlázy. Její levá polovina sloužila jako kontrolní. Každý 3. den po inokulaci

až do 21. dne bylo utraceno 1 zvíře. U všech ovcí se vyvinula klinická mastitida do 24 h po inokulaci a přetrvávala do konce poku su. Koncentrace celkových bílkovin, celkového cholesterolu, fosfolipidů a volných mastných kyselin v mléce (resp. sekretu mléčné žlázy) progresivně stoupala, zatímco koncentrace celkových lipidů a glyceridů výrazně poklesla. Tyto výsledky ukazují, že M. mycoides využívá lipolytické enzymy k degradaci lipidů nutných ke krytí energetických potřeb a k syntéze lipidové membránové vrstvy během multiplikace zárodků.

Kvantitativní stanovení a histochemické studie prokázaly vzestup aktivity aspartát-aminotransferázy (EC 2.6.1.1), alanin-aminotransferázy (EC 2.6.1.2), kyselý fosfatázy (EC 3.1.3.2), alkalické fosfatázy (EC 3.1.3.1), malátdehydrogenázy (EC 1.1.1.38), laktátdehydrogenázy (EC 1.1.1.27), adenosintrifosfatázy (EC 3.6.1.3) a amylázy (EC 3.2.1.1) v krevní plazmě, mléce a tkáních mléčné žlázy zřejmě v důsledku vzestupu celkového počtu leukocytů a zárodků M. mycoides.

Биохимические изменения в кровяной плазме, молоке
и тканях молочной железы овец
после экспериментальной инфекции
Mycoplasma mycoides

Наблюдения за биохимическими изменениями в кровяной плазме, молоке и тканях молочной железы проводились у 7 лактирующих овец с односторонним маститом, экспериментально вызванным применением 2 мл культуры Mycoplasma mycoides subsp. mycoides (Mmm) типа крупных колоний (LC) с содержанием 10^5 способных к воспроизведению клеток (CFU) в 1 мл. Культуру M. mycoides вводили в канал соска правой половины молочной железы. Ее левая половина была контрольной. Каждые 3 суток после инокуляции до 21 суток было умерщвлено одно животное. У всех овец развился клинический мастит до 24 часов после инокуляции и длился до конца эксперимента. Концентрация общих белков, общего холестерина, фосфолипидов и несвязанных жирных кислот в молоке (или секрете молочной железы) прогрессивно увеличивалась, между тем как концентрация общих липидов и глицеридов существенно понижалась. Полученные

данные свидетельствуют о том, что M. mycoides использует липолитические ферменты для деградации липидов, необходимых для покрытия расхода энергии и синтеза липидного мембранного слоя в процессе мультпликации зародышей.

Количественные определения и гистохимические исследования выявили повышение аспарат-аминотрансферазы (ЕС 2.6.1.1, аланин-аминотрансферазы (ЕС 2.6.1.2), кислой фосфатазы (ЕС 3.1.3.1), малатдегидрогеназы (ЕС 1.1.1.38), лактатдегидрогеназы (ЕС 1.1.1.27), аденозинтрифосфатазы (ЕС 3.6.1.3) и амилазы (ЕС 3.2.1.1) в кровяной плазме, молоке и тканях молочной железы, видимо, в результате увеличения общей численности лейкоцитов и зародышей M. mycoides.

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