Acta vet. scand. 1971, *12*, 365—374.

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SERUM ENZYME CHANGES IN LAMBS WITH EXPERIMENTALLY INDUCED ACUTE MUSCULAR DYSTROPHY

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In an earlier paper (*Tollersrud* 1970) the thermostability of lactate dehydrogenase (LDH, EC 1.1.1.27) and its isoenzymes in serum of cattle and sheep have been reported. Based upon the isoenzyme pattern of skeletal muscle in sheep where the cathodic, thermo-labile fractions LDH_4 and LDH_5 dominate, suggestions were made that a so-called relative heat stability test might be a useful aid in differential diagnosis of nutritional muscular dystrophy.

In recent years determination of serum ATP: creatine phosphotransferase or creatine phosphokinase (CPK, EC 2.7.3.2) has been considered to be the most sensitive and specific enzyme test of myopathic conditions.

In the present experiment lambs were made dystrophic to enable the study of serum enzyme changes taking place during the disease.

MATERIAL AND METHODS

Animals and feeding

Five male lambs of the old Norwegian short-tailed breed were taken away from their dams when 10 days old. They were fed by bottle five to six times a day. The diet consisted of skim milk powder suspended in temperated water 1:10, to which was added 5 % cod liver oil. The vitamin E content of the cod liver oil had been extracted with 96 % ethanol and filtered through aluminium oxide. By this procedure the α-tocopherol content was reduced

from 250 µg to 35 µg per g. The cod liver oil was supplemented with 750 i.u. of vitamin A and 75 i.u. of vitamin D per g. The lambs had access to ground barley ad lib.

Five lambs, twins to the experimental animals, were allowed to suck their dams and served as controls. All animals were kept in indoor pens.

Blood was taken at three days' intervals by venepuncture and serum obtained through centrifugation at 3,000 r.p.m. for 15 min.

Enzyme assays

Aspartate aminotransferase (AspAT, EC 2.6.1.1) and alanine aminotransferase (AlAT, EC 2.6.1.2) were determined in serum according to Sigma Technical Bulletin No. 505 (1964). The activity is expressed as Sigma-Frankel (S-F) units.

Determinations of total lactate dehydrogenase and electrophoretic separation of LDH isoenzymes in serum were performed as reported earlier (*Baustad & Tollersrud* 1969) with the modifications described by *Tollersrud* (1970). The isoenzymes are designated LDH_1 to LDH_5, LDH_1 being the fast-moving, anodic fraction.

Creatine phosphokinase (CPK) was determined according to the colorimetric method outlined in Sigma Technical Bulletin No. 520 (1967). The activity is expressed as Sigma units of CPK.

RESULTS

The experimental period lasted for three weeks. After some reluctance the lambs took the food well, and the average daily consumption per animal was 1.250 g. The weight gain during the period, however, was about 5 kg less per experimental lamb compared to the controls.

After three weeks two of the lambs, which had shown the best appetite, suddenly died. The three animals left in this group and two of the control lambs were then killed. All were subjected to post-mortem examinations.

Clinical symptoms of muscular dystrophy were not pronounced. Even in the lambs which died symptoms were absent until shortly before death.

Serum enzyme changes

A rapid increase of all the enzymes investigated occurred in the experimental lambs. Serum values of the two groups at different stages of the experiment are listed in Table 1.

Already after two weeks on the experimental diet all serum enzyme values were considerably elevated. After three weeks the

Table 1. Mean serum levels of aspartate aminotransferase (AspAT), alanine aminotransferase (AlAT), total lactate dehydrogenase (LDH), and creatine phosphokinase (CPK) in the experimental and control group; initially (0) and after one, two, and three weeks.

Enzyme	Weeks			
	0	1	2	3
Exp. group				
AspAT	73.3	71.7	731.3	2280.0
AlAT	17.7	14.0	130.7	280.0
LDH	1418	1098	6983	10967
CPK	2.7	2.0	1862	8550
Contr. group				
AspAT	63.0	60.7	55.0	59.7
AlAT	11.7	12.3	13.3	15.3
LDH	1462	1333	1272	1240
CPK	3.3	2.3	2.7	3.3

levels were further increased and indicated severe pathologic conditions. The highest values obtained in a single lamb, which died on the same day, were: AspAT — 3480, AlAT — 340, LDH — 18050, and CPK — 12900.

The control lambs maintained normal values during the experiment. A decrease of total lactate dehydrogenase has previously been observed in adolescent lambs.

Of special interest was the lactate dehydrogenase isoenzyme distribution in serum of the experimental lambs. Results of the experimental and control groups at the initial stage and after three weeks are shown in Table 2.

Table 2. Mean percentage distribution of LDH isoenzymes in serum of experimental and control lambs, initially and at the end of the experiment.

Group	Initial					Final				
	LDH_1	LDH_2	LDH_3	LDH_4	LDH_5	LDH_1	LDH_2	LDH_3	LDH_4	LDH_5
Exp.	31.1	17.1	33.8	17.2	0.8	36.3	13.1	33.3	16.3	1.0
Contr.	31.1	15.8	32.8	18.5	1.8	33.4	14.1	37.0	14.6	0.9

Contrary to expectations, no proportional increase of the cathodic LDH isoenzymes could be observed in serum of the experimental lambs. Repeated analyses during the research

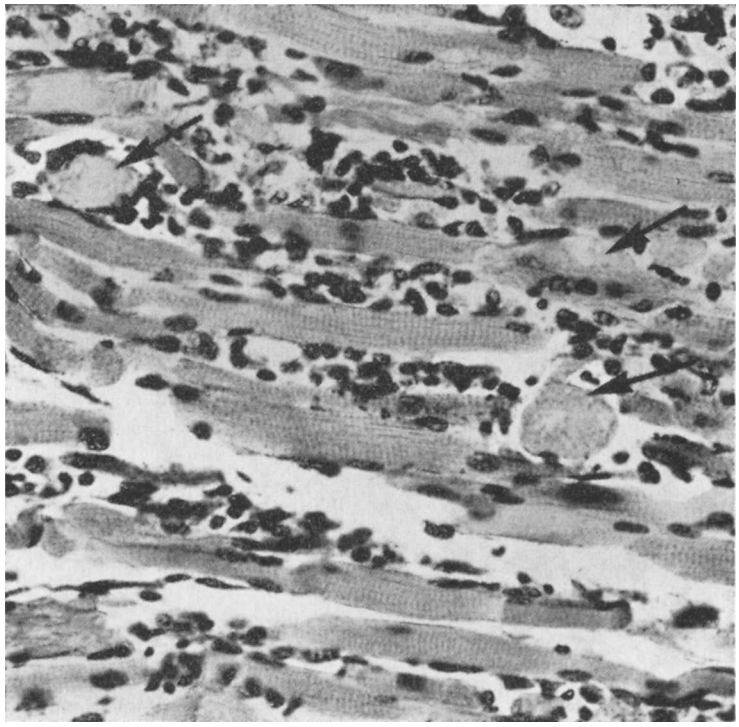

Figure 1. M. supraspinatus. Hyaline swelling with fragmentation (arrows) and reactive inflammation. H & E, × 360.

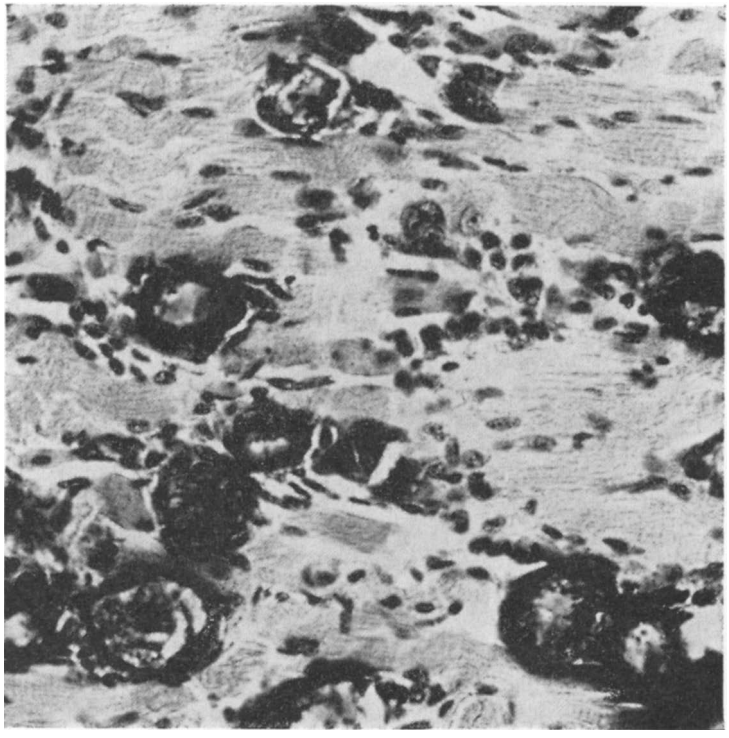

Figure 2. M. ext. dig. long. Calcification of degenerated fibres. H & E, × 360.

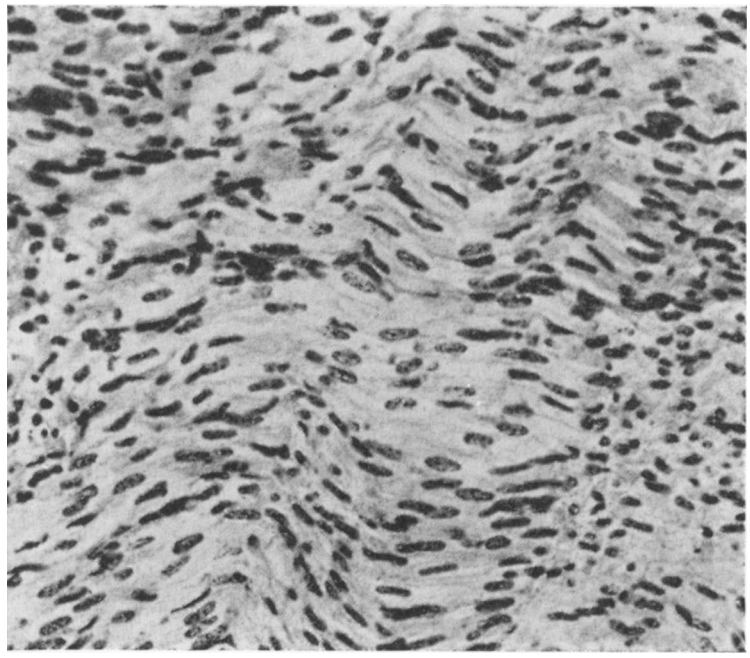

Figure 3. Myocardium. Loss of cross and longitudinal striation and granular degeneration of the sarcoplasm. Proliferation of sarcolemmal nuclei. H & E, × 360.

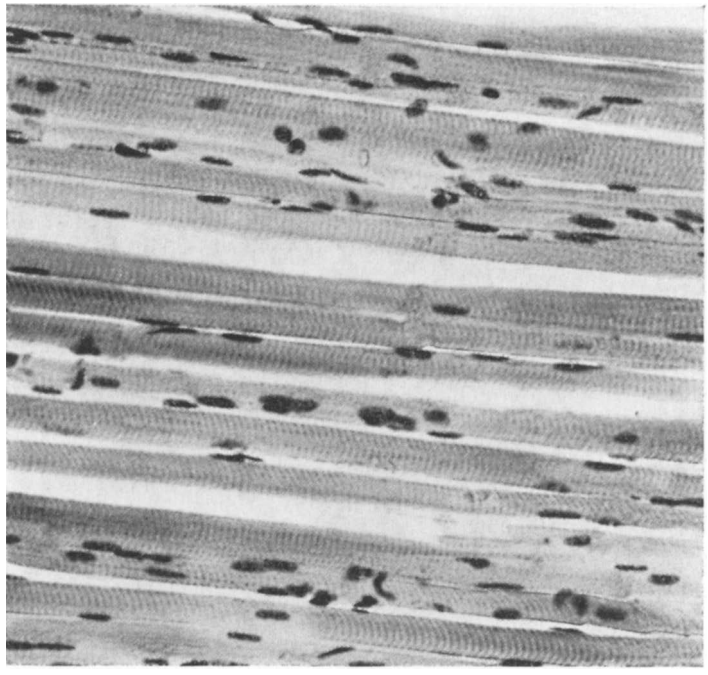

Figure 4. Skeletal muscle of control lamb. H & E, × 360.

period gave similar results. The small changes which had occurred in the isoenzyme distribution from the initial to the final stage of the experiment are conformable with a normal trend towards an adult isoenzyme pattern (*Tollersrud* 1970).

Since total lactate dehydrogenase showed a heavy increase in the experimental lambs, the activity of all LDH isoenzyme fractions were, when calculated, found elevated.

Post-mortem examinations

Necropsies were performed at the Department of Pathology. On gross examination of the experimental lambs, pronounced discoloured, greyish streaks were seen, especially in the extensor, but also in the flexor muscle groups of all extremities. The same degenerated appearance was also distinct in the intercostal and diaphragmatic muscles. The heart muscle, in particular the right ventricle, showed discoloured spots.

Histological examination of the skeletal muscles revealed a high-grade hyaline degeneration, with calcification, reactive inflammation, and increments of connective tissue. In the cardiac muscle an extensive diffuse granular degeneration of the fibres was seen, but no hyaline degeneration or calcification. One lamb showed histological kidney changes indicating an acute nephrosis. The capsular spaces were dilated and contained a protein-rich fluid; the glomeruli were contracted and hypercellular with degenerative changes in the proximal tubular epithelium.

In the control lambs no pathologic changes were observed.

DISCUSSION

Acute muscular dystrophy can be induced in various animal species by feeding of a ration deficient in α-tocopherol. Supplements of unsaturated fatty acids have been shown by many authors to accelerate and aggravate the myopathic changes (*Naf-stad & Tollersrud* 1970).

In the present experiment pronounced hyaline degenerations of skeletal muscles and myocardial changes were demonstrated. *Culik et al.* (1951) have reported that heart lesions are generally found in the right ventricle of dystrophic lambs while very few are observed on the left side.

Liver changes were not present in the diseased lambs, whereas kidney lesions indicating an acute nephrosis were observed.

Serum elevations of aspartate aminotransferase, alanine aminotransferase, and total lactate dehydrogenase are regularly obtained in cases of heart and skeletal muscle injuries. Creatine phosphokinase proved in this experiment to be the most sensitive indicator of myopathic damages.

Normal serum CPK levels of sheep have not been available from the literature. With the present method, normal values of lambs seem to be 0—10 Sigma units of CPK. In 24 healthy lambs three and a half months old the author found 4.1±3.6 units and in 10 adult ewes 6.9±4.6 units.

A more than 1000-fold increase was thus recorded in serum CPK in one of the experimental lambs. *Park & Pennington* (1966) observed a maximum rise of about 400 times the normal levels in serum from dystrophic humans and mice, and a 6-fold increase of LDH.

Creatine phosphokinase is released from heart as well as from skeletal muscle, and a differentiation between lesions in these organs can hardly be made by means of a total CPK assay of serum. Electrophoretic fractionation into three CPK isoenzymes has been made in extracts of human and rat tissues (*Van der Veen & Willebrands* 1966). In myocardial infarction two, and in muscular dystrophy one or two of the isoenzymes could be detected in serum.

In the present experiment the dramatic rise of serum CPK obviously occurred at a relatively late stage of the disease.

Electrophoretic separation of LDH isoenzymes in serum of dystrophic lambs was expected to reflect a high proportion of the cathodic isoenzymes abundantly represented in skeletal muscle. Such findings have been reported by *Boyd* (1964) and *Paulson et al.* (1966). As seen from Table 2, this could not be observed.

In an attempt to explain the results it must be emphasized that *Boyd* (1967) using C^{14}-labelled LDH_5 intravenously injected into sheep, found the disappearance rate of LDH_5 from the blood to be about seven times faster than that of LDH_1. *Hyldgaard-Jensen et al.* (1969) report corresponding elimination rates of LDH isoenzymes from pig blood, the half time of LDH_5 being six to seven hours.

The fact that the cathodically migrating isoenzymes are removed very quickly from the circulating blood may explain the small activity of LDH_5 regularly found in normal serum, and

might also contribute to the present findings in serum of dystrophic lambs.

A second important aspect is that metabolic changes in diseased organs might cause a shift in the subunit constellation of the isoenzymes.

Each isoenzyme is evidently a tetramer of subunits of two main types, H (heart, LDH_1) and M (muscle, LDH_5). Besides the genetic control in synthesis of H and M subunits, in vitro investigations have shown that changes in the oxygen tension will cause a shift in the subunit proportions. An increase of oxygen tension is followed by an enlarged intracellular synthesis of H subunits, whereas a fall induces an increment of M subunits (*Dawson et al.* 1964).

In liver diseases in man, a change of LDH subunits in plasma towards the M-form (LDH_5) is very often observed without a simultaneously increased activity of total LDH (*Maier & Hölzer* 1969). These authors conclude that the shift in plasma isoenzyme distribution is due to an altered hepatic metabolism rather than to an overflow of normally occurring liver isoenzymes.

In serum of human patients suffering from primary muscular dystrophy the total LDH level is raised. In human skeletal muscle it is LDH_5 that dominates; one thus expects a serum pattern as seen in acute liver necrosis. However, it was demonstrated by *Wieme & Herpol* (1962) that the LDH pattern of diseased muscle changes towards isoenzymes of greater electrophoretic mobility, so that LDH_5 may become the weakest fraction. This phenomenon was interpreted as a dedifferentiation of the muscle cell.

Since no increase of the cathodic, thermolabile isoenzymes was found in serum of dystrophic animals, the previous suggestion of a relative heat stability test as a diagnostic aid in this disease seems to be without validity. Further, if our technique has been inadequate at this point, a thermal LDH test would be complicated by the fact that myocardial lesions, theoretically releasing thermostable isoenzymes, frequently seem to occur simultaneously with skeletal muscle degenerations.

ACKNOWLEDGEMENTS

I wish to thank colleagues in the Department of Pathology, Veterinary College of Norway, for their kind and valuable assistance: Mrs. Inger Nafstad and Mr. N. Koppang for histological examinations and Mr. J. Teige jr. for supplying extracted cod liver oil.

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SUMMARY

An experiment was performed to study some serum enzyme changes taking place in artificially fed lambs made dystrophic on a skim milk ration supplemented with α-tocopherolextracted cod liver oil. In the course of two to three weeks the lambs showed highly increased serum values of aspartate- and alanine aminotransferase (AspAT = GOT and AlAT = GPT), total lactate dehydrogenase (LDH), and creatine phosphokinase (CPK). The latter enzyme proved to be the most sensitive test of myopathies, with serum values above 1000 times the normal level. A high-grade hyaline skeletal muscle degeneration was confirmed by histology. Myocardial changes were also present.

Contrary to expectations, electrophoretic separation of LDH isoenzymes in the serum of the dystrophic lambs did not reflect any increase of the prevalent muscular cathodic thermolabile fraction LDH_5. The use of a so-called LDH heat stability test as an aid in clinical diagnostic work, as previously suggested, therefore does not seem to be valid in this disease. The results are discussed.

SAMMENDRAG

Forandringer i serumenzymer hos lam med eksperimentelt fremkalt muskeldystrofi.

Et forsøk ble utført for å undersøke noen av de enzymforandringer som finner sted i serum hos lam med eksperimentelt fremkalt muskeldystrofi. Dietten besto av skummet melk og α-tokoferolekstrahert tran. I løpet av to—tre uker viste lammene en sterk stigning i serumnivået av aspartat- og alaninaminotransferase (AspAT = GOT og AlAT = GPT), total laktatdehydrogenase (LDH) og kreatinfosfokinase (CPK). Det siste enzymet viste seg å være den mest sensitive indikator på myopatiske tilstander med serumverdier over 1000 ganger det normale nivå.

En sterkt uttalt hyalin degenerasjon av skjelettmuskulaturen ble bekreftet ved histologisk undersøkelse, samtidig som forandringer i myocardiet ble påvist.

I motsetning til hva en hadde ventet, viste elektroforetisk separasjon av LDH isoenzymene i serum av dystrofiske lam ingen økning av den katodiske termolabile fraksjon LDH_5, som er sterkt representert i skjelettmuskulatur. En såkalt relativ varmestabilitetstest av LDH som et diagnostisk hjelpemiddel ved muskeldystrofi hos lam, ser derfor ikke ut til å være relevant, slik som antydet i en tidligere artikkel. Mulige forklaringer er diskutert.

(Received August 14, 1970).