Acta vet. scand. 1969, *10*, 372—381.

From the Department of Animal Husbandry and Genetics,
Veterinary College of Norway, Oslo.

ISOENZYMES OF LACTATE DEHYDROGENASE IN SWINE
STABILITY DURING STORAGE AT DIFFERENT TEMPERATURES AND BY HEAT TREATMENT

By
Börge Baustad and *Sverre Tollersrud*

Several serum enzymes have been shown to occur in multiple molecular forms, which are usually named isoenzymes. Isoenzymes catalyze the same reaction, but can be separated by electrophoresis or by chromatography. One of the most thoroughly investigated enzymes is lactate dehydrogenase (LDH, L-lactate: NAD oxidoreductase, EC 1.1.1.27). This enzyme has been shown to have at least five distinct isoenzymes (*Wieland & Pfleiderer* 1957).

The lactate dehydrogenase molecule is a tetramer built up from four subunits. The monomers are separable into two types (A and B), and a combination of both types in some manner in groups of four yields five different molecular species, A_4B_0, A_3B_1, A_2B_2, A_1B_4, and A_0B_4 (*Markert* 1963a). Heterogeneity of the monomers may result in more than five isoenzymes. Such heterogeneity has been shown to occur in the A as well as in the B monomer in human serum (*Boyer et al.* 1963, *Nance et al.* 1963). In swine, *Hyldgaard-Jensen et al.* (1967) have shown polymorphism of serum LDH isoenzymes.

The isoenzymes of LDH differ in chemical composition as well as in substrate specificities, thermal stabilities, abilities to utilize coenzyme analogues, and susceptibilities to inhibitors. A high degree of immunochemical specificity has also been reported (*Wilkinson* 1965). Recently, *Hyldgaard-Jensen* (1968)

has given a review of the lactate dehydrogenase and its isoenzymes in swine.

The present paper is a study of the stability of different isoenzymes of LDH in swine serum at storage under various conditions and when exposed to heat treatment at different temperatures.

MATERIAL AND METHODS

Animals. The pigs were females and castrated males of the Norwegian Landrace, weighing between 30 and 90 kg. The diets were composed to meet the requirements for protein, minerals, and vitamins. Blood samples were drawn from the anterior vena cava and centrifuged immediately for 10 min. at 3,000 r. p. m.

Enzyme analyses. Total LDH was analysed by the colorimetric method described by *Berger & Broida* (1960). α-hydroxybutyrate dehydrogenase (HBD) was analysed as outlined in Sigma tentative Technical Bulletin No. 495 (1964). Isoenzymes of LDH were determined by using the method described by *van der Helm* (1962), with small modifications.

For electrophoresis an LKB electrophoresis apparatus with equipment for immunoelectrophoresis was used. Eighteen object slides of 76×26 mm were run simultaneously. The slides were covered with an 1 % agar gel (Ionagar No. 2, Oxoid) dissolved in barbital buffer, pH 8.6, ionic strength 0.025. A volume of 10 ml of the agar solution was used for a row of 3 object slides. 10 µl of the serum sample was applied in a groove (1×14 mm) across the slide.

Barbital buffer, pH 8.6, ionic strength 0.1, was used in the electrode vessels. The electrophoresis was run for 90 min., at 250 v and 30—35 mamp.

A substrate solution was mixed from solutions A and B immediately before use.

Solution A: 60 ml substrate agar, consisting of 50 ml 1 % agar in barbital buffer (pH 8.6, ionic strength 0.1), mixed with 10 ml of a 0.05 M sodium cyanide solution. Solution A was melted and kept in a water bath at 50°C.

Solution B: 4.5 ml sodium lactate (0.9 M)
45 mg NAD (β-diphosphopyridine nucleotide, Sigma)
36 mg MTT-tetrazolium (Sigma)
1.2 mg phenazine methosulphate (Sigma)
11 ml distilled water.

Solution B is extremely sensitive to light, but may be kept frozen for some weeks. The solution was filtered before use.

The substrate solution was poured onto the electrophoresis agar, 10 ml for each three object slides, and the slides were incubated at

37°C in the dark for 1 hr. After fixation in a solution of 2 % acetic acid in 70 v/v % ethanol for 2 hrs., the slides were washed in several baths of distilled water, 24 hrs. in each bath. The gels were dried at 37°C and scanned on a Vitatron densitometer for quantitative determination of the isoenzyme fractions.

Experiment 1

Sera from 16 pigs were stored 1) at room temperature, 22°C, 2) in the refrigerator, 4°C, and 3) in the deep-freezer, — 20°C. Total LDH and HBD activity and LDH isoenzymes were determined in the fresh samples and thereafter every day for 4 days in the sera stored at room temperature and in the refrigerator, and after 32 days in the deep-freezer (*Tollersrud* 1969).

Experiment 2

Serum samples from 10 pigs were divided into four portions. One was held at room temperature and the others were kept in water baths for 30 min. at 50°C, 53°C, and 56°C, respectively. From some of the pigs a fifth portion was heat treated at 65°C in the same manner. Subsequently, total LDH, HBD, and LDH isoenzymes were determined in all samples.

RESULTS AND DISCUSSION

Experiment 1

The results from the isoenzyme determinations are given in Table 1, which shows that great changes in the isoenzyme pattern occurred after storage at different temperatures. As shown

Table 1. The percentage distribution of LDH isoenzymes in pig serum after storing at different temperatures ± standard deviation (s), n = 16.

Storage	LDH_1±s	LDH_2±s	LDH_3±s	LDH_4±s	LDH_5±s
fresh serum	46.4±3.4	31.6±1.9	13.4±2.6	5.6±1.0	3.0±0.8
1 day at 22°	43.1±3.4	29.7±1.9	15.1±1.9	8.6±2.0	3.5±1.4
2 days at 22°	44.8±3.1	25.4±1.9	15.1±2.1	11.0±1.5	3.7±1.3
3 days at 22°	50.8±4.6	24.7±2.2	8.5±2.2	11.3±1.8	4.7±1.5
4 days at 22°	50.9±4.6	24.5±1.7	7.3±2.7	11.8±1.6	5.5±1.7
1 day at 4°	53.7±9.1	18.7±2.3	12.7±2.7	10.9±1.7	4.0±1.1
2 days at 4°	52.6±4.4	13.0±3.0	15.6±2.9	14.3±1.9	4.5±1.6
3 days at 4°	63.3±5.1	6.8±1.7	7.0±2.0	16.0±2.5	6.9±2.2
4 days at 4°	65.5±4.7	7.7±1.6	2.7±1.7	15.6±2.2	8.5±2.8
32 days at —20°	37.3±2.9	27.6±1.8	22.7±1.5	9.2±1.2	3.2±1.2

by *Tollersrud* (1969) great losses were found in total LDH activity in swine serum after storage at 22°C as well as at 4°C. It would therefore be of value to compare the changes in the different isoenzyme fractions at the various storage conditions to the changes in total LDH activity.

As judged from the Michaelis constants (K_m) found by *Hyldgaard-Jensen* (1968) it is obvious that in swine LDH_1 has greater affinity to lactate as substrate than has LDH_5. However, calculations of the different isoenzyme fractions from total LDH activity may give a good indication of the changes taking place in the isoenzymes during storage at different temperatures, as it must be assumed that the substrate affinity is independent of the storage of the serum samples.

In Table 2 the calculated activity of the different fractions as well as of total lactate dehydrogenase and α-hydroxybutyrate dehydrogenase activity are shown.

Table 2. Total LDH and HBD, and calculated LDH activity in isoenzyme fractions at different storage conditions.

Storage conditions	Total LDH	HBD	LDH_1	LDH_2	LDH_3	LDH_4	LDH_5
fresh serum	991	408	460	313	133	55	30
1 day at 22°	886	286	382	263	134	76	31
2 days at 22°	811	236	363	206	122	89	30
3 days at 22°	761	236	386	188	65	86	36
4 days at 22°	767	251	390	188	56	91	42
1 day at 4°	736	221	395	138	93	80	29
2 days at 4°	643	182	338	84	100	92	29
3 days at 4°	620	183	392	42	43	99	43
4 days at 4°	553	184	362	43	15	86	47
32 days at —20°	973	361	363	269	221	90	31

The isoenzyme distribution found in normal serum (Table 1) is in good agreement with the results of *Hyldgaard-Jensen & Jensen* (1967). Small differences, however, were found, especially in LDH_5. This may be explained by the fact that we used serum obtained from clotted blood where the thrombocytes will partly be destroyed. This may cause a leakage of the enzymes from the thrombocytes to serum and cause a change in the isoenzyme pattern.

From Table 2 it is seen that a strong correlation existed between total LDH and HBD (correlation coefficient 0.91). It is

further seen that the greatest loss of enzyme activity occurred in the intermediate isoenzymes LDH_2 and LDH_3. This loss was most clearly demonstrated after storage in the refrigerator (4°C), where LDH_2 had lost more than the half of its activity after only 24 hrs. A further decrease was seen after 2 days, and after 3 days only 13 % of the original activity of this isoenzyme was present. LDH_3 also lost much of its activity through storage at 4°C, and only 11 % of its initial activity was present after 4 days. With storage at room temperature, too, the decrease in activity was considerable, since LDH_2 lost about 40 % and LDH_3 50 % of the initial activity during 4 days.

The cathodic isoenzymes LDH_4 and LDH_5 showed a small increase in activity during storage. No explanation for this can be given at present. Analytical discrepancies must be taken into consideration, since total lactate dehydrogenase and isoenzymes were determined by two different procedures. *Feissli et al.* (1966), however, also reported an increase in the activity of some serum enzymes after storage.

In LDH_1 a decrease of about 20 % was seen at storage in the refrigerator as well as at room temperature after 24 hrs., whereupon the activity seemed fairly stable.

Storage in the deep-freezer caused small changes in total lactate dehydrogenase activity, but the isoenzyme pattern indicated an increase in LDH_3. This may perhaps be explained by the findings of *Markert* (1963b). By freezing and thawing of a mixture of the pure isoenzymes LDH_1 and LDH_5, he found that the intermediate forms LDH_2, LDH_3, and LDH_4 were produced by dissociation and recombination from the original isoenzymes.

In swine plasma *Hyldgaard-Jensen* (1966) found the LDH activity to be quite stable for 4 days when kept at room temperature. With this not unimportant exception our results from the determinations of enzyme activity in the LDH fractions at different storage conditions are in good agreement with the results of *Hyldgaard-Jensen* (1968).

Kreutzer & Fennis (1964) found no loss of lactate dehydrogenase activity in human cord blood during the first 10 days when serum was stored at room temperature. At 4°C and at —10°C a rapid loss of activity was seen in the isoenzymes LDH_4 and LDH_5. LDH_4 in particular lost much of its activity when exposed to the cold, and no activity was found after 2 days at —10°C.

Human liver is especially rich in the cathodic fractions LDH_4 and LDH_5. Swine liver, on the other hand, exhibits great activity of the intermediate forms, LDH_2 and LDH_3 (*Hyldgaard-Jensen & Jensen*). A possible explanation of the obvious difference in stability at storage of the different isoenzymes in swine and human serum may be that the enzymes originating from liver tissue are especially sensitive to storage at 4°C.

Experiment 2

The results from the isoenzyme determinations are given in Table 3.

Table 3. The percentage distribution of LDH isoenzymes in Experiment 2. n = 10.

Heat treatment	$LDH_1 \pm s$	$LDH_2 \pm s$	$LDH_3 \pm s$	$LDH_4 \pm s$	$LDH_5 \pm s$
normal serum	38.2± 4.7	29.3±2.8	23.2±2.9	7.2±2.9	2.1±1.7
50°C for 30 min.	44.8± 6.0	28.1±3.3	18.9±5.1	6.4±3.2	1.8±1.5
53°C for 30 min.	62.6±11.5	24.6±3.3	8.2±5.7	2.9±2.0	1.7±1.4
56°C for 30 min.	90.6± 3.4	9.1±3.7	0.3±0.6	0	0

As in Experiment 1 the total enzyme activity in the different isoenzyme fractions was calculated. In Table 4 total LDH and HBD activity and calculated activity of the five isoenzymes are given.

Table 4. Total LDH, HBD, and calculated LDH activity in isoenzyme fractions at different heat treatments.

Heat treatment	Total LDH	HBD	LDH_1	LDH_2	LDH_3	LDH_4	LDH_5
normal serum	1221	382	466	358	281	88	27
50°C for 30 min.	1083	331	485	304	205	69	19
53°C for 30 min.	847	257	530	208	70	24	14
56°C for 30 min.	616	187	558	56	2	0	0

On exposure to 65°C for 30 min. no activity was found in LDH or HBD.

A close correlation was found between total LDH and HBD activity in this experiment too (correlation coefficient r = 0.98), and the regression line went through zero. The correlation

was better between total LDH and HBD than between any combination of isoenzymes and HBD. This seems to indicate that, with the analytical methods used, determination of lactate dehydrogenase and α-hydroxybutyrate dehydrogenase is an expression of the same thing. This is in contrast to what is found in human serum, where LDH_1 and LDH_2 exhibit much greater HBD activity than LDH_4 and LDH_5 (*Wilkinson* 1965).

In Experiment 2 the total LDH activity and especially the activity of LDH_3 were found to be higher than in Experiment 1. The pigs, however, proved to be normal at necropsy.

From Tables 3 and 4 is seen that inactivation of isoenzymes occurred at different temperatures of heat treatment. At 50°C LDH_2 had already lost 15 %, and LDH_3, LDH_4, and LDH_5 about 25 % of their original activity. At 53°C LDH_2 had lost 40 %, and LDH_3, LDH_4, and LDH_5 more than ⅔ of their activity. At 56°C LDH_2 retained only about 15 % of its original activity and LDH_3, LDH_4, and LDH_5 had lost all the lactate dehydrogenase activity. LDH_1, on the other hand, showed no loss of activity even at 56°C, but all activity had disappeared at 65°C.

Hyldgaard-Jensen (1968) has studied heat inactivation of the pure isoenzymes LDH_1 and LDH_5 from swine, and his results are in good agreement with what we found in serum.

Lactate dehydrogenase in human serum seems to show greater heat stability than we found in swine serum. *Wróblewski & Gregory* (1961) found by heating samples of serum, to which $NADH_2$ had been added, to 57°C or 65°C that the cathodic fractions, principally LDH_5, were destroyed at 57°C, whereas the anodic fraction, LDH_1, was stable even by heating to 65°C. The intermediate isoenzymes, LDH_2, LDH_3, and LDH_4 were inactivated between 57°C and 65°C. A "heat stable test" based on these findings has been employed in the routine clinical diagnosis, since heat treatment is a more convenient procedure than the more laborious electrophoresis procedure (*Strandjord et al.* 1962).

In swine serum, it seems to be possible to distinguish between LDH_1 and the other isoenzymes by heating the sample to 57°C for 30 min. These findings are based on normal sera, and nothing is known of the results in serum with elevated enzyme activity.

REFERENCES

Berger, L. & D. Broida: The colorimetric determination of lactic dehydrogenase. Sigma Techn. Bull. No. 500, 1960.

Boyer, S. H., D. C. Fainer & E. J. Watson-Williams: Lactate dehydrogenase variant from human blood: Evidence for molecular subunits. Science 1963, *141,* 642—643.

Feissli, S., G. Forster, G. Laudahn, E. Schmidt & F. W. Schmidt: Normal-Werte und Alterung von Hauptketten-Enzymen im Serum. (Normal values and changes of main chain enzymes in serum). Klin. Wschr. 1966, *44,* 390—396.

Helm, H. J. van der: A simplified method of demonstrating lactic dehydrogenase isoenzymes in serum. Clin. chim. Acta 1962, *7,* 124—128.

Hyldgaard-Jensen, J.: Mælkesyredehydrogenase hos svin. I. Mælkesyredehydrogenase (LDH) i blodplasma. (Lactic dehydrogenase (LDH) in pig plasma). Kgl. Vet.- og Landbohøjsk., Inst. Sterilitetsforskn. Aarsberetn. 1966, 117—125. København.

Hyldgaard-Jensen, J.: Mælkesyredehydrogenase hos svin. En oversigt. (Lactic Dehydrogenase in Pigs. A survey). Kgl. Vet.- og Landbohøjsk., Inst. Sterilitetsforskn. Aarsberetn. 1968, 243—284. København.

Hyldgaard-Jensen, J. & S. E. Jensen: Mælkesyredehydrogenase (LDH) hos svin. II. LDH-isoenzymer i blodplasma og væv. (Lactic Dehydrogenase (LDH) in Pigs. II. LDH-isoenzymes in blood plasma and tissues). Kgl. Vet.- og Landbohøjsk., Inst. Sterilitetsforskn. Aarsberetn. 1967, 109—120. København.

Hyldgaard-Jensen, J., M. Valenta & J. Moustgaard: Mælkesyredehydrogenase (LDH) hos svin. III. Polymorfi af LDH-isoenzymer hos svin. (Polymorphism of LDH-isoenzymes in pigs). Kgl. Vet.- og Landbohøjsk., Inst. Sterilitetsforskn. Aarsberetn. 1967, 121—134. København.

Kreutzer, H. H. & W. H. S. Fennis: Lactic dehydrogenase isoenzymes in blood serum after storage at different temperatures. Clin. chim. Acta 1964, *9,* 64—68.

Markert, C. L.: Lactate dehydrogenase isoenzymes: Dissociation and recombination of subunits. Science 1963a, *140,* 1329—1330.

Markert, C. L.: Epigenetic control of specific protein synthesis in differentiating cells. *In* Cytodifferential and Macromolecular Synthesis, ed. M. Locke, Acad. Press, New York 1963b.

Nance, W. E., A. Claflin & O. Smithies: Lactic dehydrogenase: Genetic control in man. Science 1963, *142,* 1075—1077.

Strandjord, P. E., K. J. Clayson & E. F. Freier: Heat stable lactate dehydrogenase in the diagnosis of myocardial infarction. J. Amer. med. Ass. 1962, *182,* 1099—1102.

Tollersrud, S.: Stability of some serum enzymes in sheep, cattle, and swine during storage at different temperatures. Acta vet. scand. 1969, *10,* 359—371.

Wieland, T. & G. Pfleiderer: Nachweis der Heterogenität von Milchsäuredehydrogenasen verschiedenen Ursprung durch Trägerelektrophorese. (Demonstration of heterogeneity in lactate dehydrogenases by electrophoresis). Biochem. Z. 1957, *329*, 112—116.

Wilkinson, J. H.: Isoenzymes. E. & F. N. Spon Ltd., London 1965.

Wróblewski, F. & K. F. Gregory: Lactic dehydrogenase isozymes and their distribution in normal tissues and plasma and in disease states. Ann. N.Y. Acad. Sci. 1961, *94*, 912—932.

SUMMARY

The isoenzymes of lactate dehydrogenase (LDH) in serum from normal pigs were studied after separation by agar gel electrophoresis with subsequent staining with a tetrazolium salt.

Experiment 1. The stability of isoenzymes was investigated for 5 successive days after storage at room temperature (22°C), in the refrigerator (4°C), and once after storage for 32 days in the deep-freezer (—20°C). Greatest loss of activity was seen after storage in the refrigerator, where LDH_2 and LDH_3 lost most of its activity after 5 days. In LDH_4 and LDH_5 no loss had occurred at this time. Also at room temperature great losses were seen in LDH_2 and LDH_3. After storage in the deep-freezer an increase in LDH_3 activity was recorded.

Experiment 2. Serum samples were kept in water baths for 30 min. at 50, 53, and 56°C. A simultaneous and increasing loss in activity of LDH_3, LDH_4, and LDH_5 was seen from 50° to 56°C. At 56° no activity was left in LDH_3, LDH_4, or LDH_5, and only about 15 % of the original activity was present in LDH_2. LDH_1 showed no loss at 56°, but all activity was lost at 65°.

A close correlation was found between total lactate dehydrogenase and α-hydroxybutyrate dehydrogenase activity in both experiments.

ZUSAMMENFASSUNG

Isoenzyme der Laktatdehydrogenase bei Schwein. Stabilität während der Lagerung bei verschiedenen Temperaturen und bei Wärmebehandlung.

Die fünf Isoenzyme der Laktatdehydrogenase (LDH) wurden untersucht in Schweineserum mit Hilfe von Agarelektrophorese und Färbung mit einem Tetrazoliumsalz.

In Versuch 1 wurde die Stabilität der Isoenzyme durch Lagerung von Serum bei verschiedenen Temperaturen untersucht. Der grösste Verlust erfolgte bei Lagerung im Kühlschrank (4°C), wo der grösste Teil von LDH_2 und LDH_3 nach dem Verlauf von vier Tagen verloren gegangen war. Bei Zimmertemperatur (22°C) waren die Verluste ebenfalls erheblich, und auch hier galt dieses besonders LDH_2 und LDH_3. In der Anode-Fraktion, LDH_1, war der Verlust verglichen mit der ursprünglichen Aktivität etwa 20 %. In den Katode-Fraktionen,

LDH_4 und LDH_5, wurde nach viertägiger Lagerung kein Verlust festgestellt. Nach Lagerung im Gefrierschrank (—20°C) über 32 Tage wurde eine Steigerung in der LDH_3-Menge festgestellt.

In Versuch 2 wurde die Wärmestabilität der verschiedenen Isoenzyme untersucht. Schon bei einer Erwärmung auf 50°C über 30 Minuten betrug der Rückgang der Enzymaktivität etwa 15 % bei LDH_2 und etwa 25 % bei LDH_3, LDH_4 und LDH_5. Bei 56° war alle Aktivität in LDH_3, LDH_4 und LDH_5 verschwunden, während 15 % der ursprünglichen Aktivität noch in LDH_2 zugegen war. In LDH_1 fand bei einer Erwärmung auf 56° kein Verlust statt, bei 65° war jedoch alle Aktivität verschwunden.

In beiden Versuchen wurde eine sehr gute Übereinstimmung zwischen Laktatdehydrogenase und α-Hydroxybutyratdehydrogenase festgestellt.

SAMMENDRAG

Isoenzymer av laktatdehydrogenase hos svin. Stabilitet under lagring ved ulike temperaturer og ved varmebehandling.

De fem isoenzymene av laktatdehydrogenase (LDH) ble undersökt i svineserum ved hjelp av agargelelektroforese og farging med et tetrazoliumsalt.

I Forsök 1 ble stabiliteten av isoenzymene undersökt ved lagring av serum ved forskjellige temperaturer. Störst tap fant sted ved lagring i kjöleskap (4°C), der storparten av LDH_2 og LDH_3 var tapt etter 4 dögn. Ved romtemperatur (22°C) var også tapene betydelige, også her först og fremst av LDH_2 og LDH_3. I den anodiske fraksjon, LDH_1, fant en et tap på ca. 20 % sammenliknet med den opprinnelige aktivitet. I de katodiske fraksjonene LDH_4 og LDH_5 ble det ikke påvist tap ved lagring i fire dögn. Etter lagring i fryseboks (—20°C) i 32 dögn ble det funnet en ökning i LDH_3.

I Forsök 2 undersökte en varmestabiliteten hos de ulike isoenzymene. Allerede ved opvarmning til 50°C i 30 minutter var det en nedgang i enzymaktivitet på ca. 15 % i LDH_2 og ca. 25 % i LDH_3, LDH_4 og LDH_5. Ved 56° var all aktivitet i LDH_3, LDH_4 og LDH_5 borte, mens ca. 15 % av den opprinnelige aktiviteten fortsatt var til stede i LDH_2. I LDH_1 fant en ikke noe tap ved oppvarming til 56°, men all aktivitet var tapt ved 65°.

I begge forsökene ble det funnet en meget god korrelasjon mellom laktatdehydrogenase og α-hydroxybutyratdehydrogenase.

(Received March 7, 1969).