A STUDY OF THE CLEARANCE RATES OF LACTATE DEHYDROGENASE FROM THE SERUM OF NORMAL CATTLE AND CATTLE AFFLICTED WITH BOVINE LYMPHOCYTOMA

by

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INTRODUCTION

The enzyme lactate dehydrogenase (LDH) (L-lactate: NAD^+ oxidoreductase, EC I.I.I. 27) participates in the anaerobic reduction of pyruvate to lactic acid, with DPNH oxidized to DPN^+. This mechanism functions in those glycolytic situations where the DPN^+ reduced by oxidation of 3-phosphoglyceraldehyde is not reoxidized. These conditions are frequently present in actively metabolizing tissues, especially when intracellular oxygen transport is diminished.

This knowledge, added to the work of Markert and Møller (1959), Plageman, et al. (1960); and Cahn, et al. (1962) dealing with the serum levels of LDH and its isoenzymes in different organs, has made the enzyme LDH a pivotal one in the study of any disease resulting in target organ necrosis or increased anaerobic glycolysis. Neoplastic diseases are an example of the latter, and reports by Wieme (1963), Goldman and Kaplan (1963) and Goldman, et al. (1964) indicate alterations in LDH isoenzymes in these conditions.

If serum levels of total LDH may be altered in neoplastic diseases, the rate of clearance of the enzyme from the plasma may also be affected in neoplastic disease. This observation, first explored in the canine species in 1955 by Wroblewski and La Due, has not been defined in the bovine species. A comparison of the normal bovine LDH blood clearance with the LDH blood clearance of cattle exhibiting signs of bovine lymphocytoma may show significant differences. Further, the observation that the isoenzyme pattern of serum LDH is different in the bovine species as compared to man, sheep, and the dog may reflect transformations in the isoenzyme prior to degradation. Massive doses of particular

fractions of isoenzymes, injected intravenously into adult cattle, both normal and those afflicted with lymphocytoma, may reflect these transformations.

REVIEW OF LITERATURE

Biochemical Properties of Lactate Dehydrogenase

Many proteins with enzymatic activity exist in multiple molecular forms. The functional molecule is a polymer or aggregate of smaller polypeptide chains.

Straub (1940) prepared crystalline lactate dehydrogenase (LDH) from beef heart. The first evidence of polymer constituents of LDH was produced by Meister (1950) who, using moving boundary electrophoresis, divided beef heart LDH into two fractions. No further work was reported on the separation of LDH fractions until 1957 when Sayre and Hill reported the fractionation of serum LDH by salt concentration gradient elution and paper electrophoresis. Vessell and Bearn (1957) utilized paper electrophoresis for the same purpose. Starch-gel electrophoresis was applied to LDH separations by Dewey and Conklin (1960).

The biochemical properties of the molecule were not known by 1960, and techniques applied were modified from electrophoretic methods employed in the separations of other proteins. Appella and Markert (1961) and later Pesce, et al. (1964) described several physical properties of the molecule and its isoenzymes. By binding crystalline guanidine HCl to the enzyme in varying concentrations and measuring the fluorescent intensity at 340 mu, it was established that 2M guanidine HCl ruptured the hydrogen bonds of the secondary structure of the enzyme as measured by decrease in fluorescence. Changes in optical rotationconfirmed this mechanism since the increasing levorotation at higher concentrations, up

to 5M guanidine HCl, characterized the unfolding of the molecule. At 5M concentration of guanidine HCl, the optical rotation was measured at 589 mu with a specific rotation of -30^{o} in buffer to -72^{o} in 5M guanidine HCl. The unfolding caused parallel loss of enzymatic activity. Using equilibrium sedimentation techniques, the molecular weight of the entire molecule was established at 134,000. A homogenous peak was repeatedly demonstrated with a lower sedimentation constant in guanidine HCl. This suggested dissociation into subunits of equal molecular weight. By correcting for the 4% calculated binding of HCl to the protein and extrapolating to zero time, the molecular weight of the polypeptide subunits was established at 34,000 $\pm$ 2,000. This led the authors to suggest four dissociable polypeptide chains from one intact enzyme molecule. The same workers determined nitrogen and carbon terminal residues, total amino acid composition, and patterns of peptides after trypsin digestion. The number of peptides corresponded to about one fourth of the original peptides, plus lysine residues found on total amino acid analysis. This supported evidence that LDH was made up of four similar polypeptide chains.

Cahn, et al. (1962), Markert and Møller (1959), Plageman, et al. (1960) reported the presence of five distinct forms of LDH from tissues. This work did not correspond completely with that of Appella and Markert (1961), and it was the theory of Markert (1963) that united the conflicting data. This theory suggested that a random combination of four polypeptides in pairs produced five electrophoretically distinct moieties. Markert's original description combined two basic subunits with a protein control structure; the theory was simplified to a combination

of subunit pairs. The structures then appeared as follows with band 1, or LDH_1, designating the more anodic fraction and band 5, or LDH_5, designating the more cathodic fraction:

MMMM--designated band 5

MMMH--designated band 4

MMHH--designated band 3

MHHH--designated band 2

HHHH--designated band 1

Markert (1963) supported this theory by preparing pure LDH_1 and pure LDH_5 by separation of crystalline LDH from beef tissues. These were electrophoresed separately and in combination on the same electrophoretic strip. The two pure fractions migrated singly, the LDH_1 being distinctly anodic, while LDH_5 remained near the point of origin. The combination was prepared by mixing equal portions of the pure fractions in 1M NaCl and freezing overnight. The migration of the combination of LDH_1 and LDH_5 generated all five subunits in the expected proportions of 1:4:6:4:1. These subunits, defined as different proteins with similar enzymic activity, were called isoenzymes, as officially recommended by the Standing Committee on Enzymes of the International Union of Biochemistry. The term isozyme was regarded as an acceptable alternative to isoenzyme by Webb (1964).

Fritz and Jacobson (1963;, 1965) added to this theory by results obtained from polyacrylamide gel electrophoresis. After soaking the gel in 0.005 β-merceptoethanol for 13 hours prior to electrophoresis, fifteen separate bands were obtained. Assuming each monomeric unit to contain a molecule of nicotinamide adenine dinucleotide (NAD) and that

the β-mercaptoethanol removed those NAD molecules attached to M subunits, but not those attached to H subunits, the following diagram explained the existence of 5 M_4 bands, 4 M_3H bands, 3 M_2H_2 bands, 2 M_1H_3 bands, and 1 H_4 band:

MN-MN-MN-MN MN-MN-MN-M MN-MN-M-M MN-M-M-M M-M-M-M	LDH_5
MN-MN-MN-HN MN-MN-M-HN MN-M-M-HN M-M-M-HN	LDH_4
MN-MN-HN-HN MN-M-HN-HN M-M-HN-HN	LDH_3
MN-HN-HN-HN M-HN-HN-HN	LDH_2
HN-HN-HN-HN	LDH_1

Koen and Shaw (1965) reported similar findings with starch gel electrophoresis with bridge buffer pH 7.0. Anderson and Weber (1966) demonstrated the reversibility of the hybridization whether partial or complete.

Fondy and Kaplan (1965) found the following differences in the H and M fractions: 1) H contained the following amino acids in higher concentrations than did the M type: histidine, threonine, glutamic acid, isoleucine, leucine, tyrosine; 2) chicken LDH_1 contained 34-38 ninhydrin positive spots; chicken LDH_5 contained 35-38; 3) immunologic reactions were sensitive only to the specific fraction; and 4) temperature stabilities as LDH_1 was stable under conditions that destroyed the LDH_5 form. This aspect of temperature stability was a confirmation of the technique described by Bell (1963). Fondy and Kaplan (1965) noted

the following similarities: 1) molecular weight and shape; 2) identical active sites of sulfhydryl peptide; and 3) LDH subunits of a wide variety of species combined to form hybrids. Fondy, et al. (1965) demonstrated the presence of one active thiol group per subunit and suggested the importance of this group in the catalytic operation of the various LDH tetramers.

Markert (1963) suggested the possibility of genetic influence on the presence of M or H fractions within any tissue. Nance, et al. (1963) and Vessell (1965) supported this theory by describing mutants of the usual isoenzyme pattern. The genetic loci suggested an autosomal condominant transmission.

Physiologic Considerations of Lactate Dehydrogenase

The physiologic function of the isoenzymes of LDH was defined by Cahn, et al. (1962) as follows: activity of skeletal muscle utilizes much energy in a selectively short-time period. This tends toward an aerobiasis, which produces much lactic acid, which in turn is necessary for anaerobic metabolism, since reduced DPN would then not be formed by oxidation of threose phosphate. Hence LDH_5 could be expected to operate in high pyruvate concentrations. Heart musculature does not require sudden utilization of energy, operates in a relative state of aerobiasis, and pyruvate is directed toward oxidation rather than reduction of lactate. Hence, one would expect LDH_1 to operate most efficiently only in low pyruvate concentrations. The authors further observed that LDH_1 was the predominant LDH isoenzyme of the embryonic chick even in breast muscle. In view of earlier observations that

lactic acid was toxic to embryonic chick tissues, this may be a mechanism for keeping lactic acid concentrations low. Also, it is likely that embryonic chick relied on aerobic metabolism. This observation is contrasted with that of the embryonic rat, which contained principally LDH_5 isoenzyme. It appeared that fetal rat tissue was more anaerobic than that of embryonic chick. They further proposed that all four subunits were necessary for enzymatic activity, being held together by hydrogen or hydrophilic bands, and that this was necessary for stability. The importance of the constituents of the molecule to the host cell was the relative number of muscle and heart units present at any one time.

This proposal of Cahn et al. (1962) was examined by several subsequent workers. Fine, et al. (1963) reported on the LDH present in the development and maturation of the various tissues. Much of this was amended by Hinks and Masters (1964) who measured preparations of the isoenzymes in different organs of ruminants throughout the gestation period. Their work indicated that the H isoenzyme was present during early embryonic life. As gestation continued, the LDH isoenzyme of skeletal musculature became more of the M type, while that of the liver and heart remained of the H type. This led to the hypothesis that both genes responsible for the synthesis of the LDH subunits (M & H) were functional in the newly fertilized ova of most avian and mammalian species. Rather than being dictated by parental isozymes, the embryo or fetus may have either or both types, perhaps related to environment. This evidence was supported by Wiggert and Villee (1964), Burch et al. (1963) and by Masters (1964). Vessell and Philip (1963) supported this evidence, and added to it by preparing tissue culture lines of embryonic

and adult human chick and rabbit tissues. They reported a general shift in the LDH zymogram of the definitely anodal ten-day old embryonic chick muscle to a definite cathodic configuration after 16 days of tissue culture. Liver, skin, muscle, and heart tissue of an eight-day old embryonic chick demonstrated a similar change. Hela cells grown in culture for more than ten years were found to be aneuploid, but revealed no alterations in their isoenzyme pattern than other cells found to be euploid. This suggested that changes following initial alterations in early subcultures remained and a stable pattern was established. Bloom et al. (1967) reported similar findings in tissue culture of human leukocytes.

Blatt et al. (1966), Fieldhouse and Masters (1966), and Takso and Hughes (1966) conducted studies on the development of mammalian LDH isoenzyme patterns up to adult LDH constituents. Each reported the emergence of the adult form two to three weeks after birth. The relative ratio of H type to M type of LDH increased in tissues studied with the exception of the liver and skeletal muscle.

All evidence on the developmental aspects of LDH isoenzymes pointed to a gradual shift in the distribution of total LDH activity from a preponderance of anodal bands to a preponderance of cathodic bands. That is, there was a quantitative generic change so that the gene controlling subunit H produced more of subunit H, whether in vitro or in vivo.

The theory of Cahn, et al. (1962) coincided adequately with the work of Vessell and Philip (1963) in respect to the relative state of

aerobiasis controlling the physiologic state of the cell, and the genetic response producing the optimum proportion of M or H subunit.

Dawson, et al. (1964) found that certain muscles, the soleus, for example, exhibited LDH isoenzyme patterns similar to that found in the heart. This may be explained by the fact that these muscles were involved with posture and support, and thus they remained in more or less continuous contraction. Hence an aerobic state would be more likely to be maintained. This work was supported by a report by Dawson and Romanul (1964).

Rosa and Schapira (1964) reported the presence of faint cathodic bands in the young erythrocyte but the older RBC contained none.

The intracellular distribution of LDH isoenzymes was consistent with the aerobic function of the H subunit of LDH. Nuclear and microsomal fractions contained only M subunits while the mitochondrial portion contained M and H subunits of LDH according to Agostoni et al. (1966). The stability of the organ constituents of the LDH subfractions throughout life was reported by Schmukler and Barrows (1967). No changes in LDH subfractions in liver, kidney, skeletal muscle (thigh), heart, and brain were noted between 12 and 24 month old Wistar female rats.

Adult organ LDH constituents tend to follow a consistent pattern, as indicated by Pesce et al. (1967). Lowenthal (1964) reported that peripheral nerve extracts contained principally LDH_5. Katz and Kalow (1965) found intracellular LDH patterns to be stable for some time after death. Those samples collected at autopsy from heart, liver, and skeletal muscle were essentially the same as those from fresh surgical specimens of the same organs. Ressler et al. (1965) reported a sixth

band in the rat kidney. Its biochemical properties resemble that of the sixth band found by other workers in bovine testes. Hinks and Masters (1966) reported the predominant LDH isoenzyme from various organs of the cat. They were: 1) spleen, LDH_3; lung, LDH_3; erythrocytes, LDH_1; brain, LDH_1; kidney, LDH_2; skeletal muscle, LDH_5; heart, LDH_1; pancreas, LDH_3; and liver, LDH_5. The serum contained a predominance of the LDH_1 isoenzyme of LDH. Walter and Selby (1966) and Starkweather et al. (1966a) reported finds on erythrocytes. Predominant LDH bands obtained from LDH assay of the erythrocytes of the calf were LDH_4 and LDH_3; of the dog, LDH_4 and LDH_5; of the lamb, LDH_3; of the pig, LDH_4 and LDH_3; of the horse, LDH_2 and LDH_1; and of the rabbit, LDH_1.

If anaerobic or aerobic metabolism has an influence on genetic production of LDH subunits, one could hypothesize on the effect of hormones, particularly the sex hormones, the effect of catecholamines, and the effect of physical training and exercise. Goodfriend and Kaplan (1964) and Kaplan (1965) reported that the M subunit was found in smaller proportions in the immature than the mature uterus, and the injection of estradiol caused a marked increase in the M subunit in the immature uterus. Testosterone led to an active synthesis in the M subunit of the seminal vesicles in the immature rat.

Garbus et al. (1964) and Gollnick et al. (1967) studied the effect of exercise and training in rats. Garbus et al. (1964) administered epinephrine in oil and dibenamine to some of their experimental rats. Halonen and Konthinen (1962) measured the serum LDH in military trainees during exercise. Results of these studies were: 1) training in rats produced a significant increase in LDH activity of the heart

muscles, but skeletal muscle LDH activity decreased. Exercise did not produce any change in LDH activity in either heart or skeletal muscle; 2) the administration of epinephrine in oil to rats caused an elevation of serum LDH in both exercised trained rats and in exercised untrained rats. Conversely, dibenamine, which ameliorated changes by epinephrine or anoxia, did not decrease the elevation of serum LDH in untrained rats subjected to prolonged exercise; and 3) in man, increased serum LDH activity was noted in trained subjects during exercise of only one hour in duration, while the subjects (military trainees) did not have an increase in serum LDH over an extended period of marked exercise. Conclusions drawn from the effects of physiologic alteration on tissue and serum levels of LDH and LDH isoenzymes pointed towards an increase in certain tissues after hormonal or training influences, and that cell permeability, as well as cellular death, may have released LDH from the intracellular substance.

The presence of LDH in serum has been known since the report by Wroblewski and LaDue (1955) and later by Hill (1956). Lactate dehydrogenase activity in the dog was not listed, but interpretation of pre-experimental levels suggested that levels of 500 to 1,100 units per ml. of serum were typical. Normal serum levels in the sheep were not reported precisely, but fractionation data suggested 1500 units per ml. of serum (Hinks and Masters, 1965).

Wroblewski and La Due (1955) recognized that the serum level of LDH may be a reflection of disease or health. After injecting 19,200 units of LDH per kilogram of body weight intravenously into a dog, the authors recorded marked elevations in the serum LDH levels for one hour

following injection. This was the first attempt to measure the serum elimination, or clearance, of LDH from an experimental animal. This observation led the authors to produce an experimental myocardial infarction in a dog with subsequent observations on the serum LDH levels. The serum LDH alterations were significant, and this led to investigations by other workers and on other diseases.

Boyd (1967) determined the rates of disappearance of intravenously injected LDH isoenzymes from the plasma of sheep. The findings suggested two phases of clearance of the M_4 and H_4 isoenzymes, and these phases fit a biphasic exponential curve. Hence two phases of disappearance could be characterized. He observed a half-life of two and eight hours for the injected M_4 isoenzymes and a half-life of 2.4 and 48.0 hours for the injected H_4 isoenzyme. By creating mathematical model systems, the author concluded that isoenzyme M_4 must have been released at a rate 7.5 or 15.0 times greater than isoenzyme H_4, depending on the model system adopted. Hence the more rapid disappearance rate of isoenzyme M_4 accounted for its relatively low activity in the plasma of this species.

Assay Techniques of Lactate Dehydrogenase

The diagnostic potential of LDH isoenzymes necessitated development of techniques suitable for routine laboratory use. The method of serum LDH assay first described was by Wroblewski and La Due (1955). The mechanism of the test was concerned with the reduction of LDH in the presence of reduced nicotinamide adenosine diphosphonucleotide (NAD^+, DPNH) of pyruvic acid, but adjusted for the fact that alpha-keto and gamma-diketo acids may also have been reducing agents in the system.

The test described was a measurement of the rate of the first order kinetic reaction of LDH. A 20 minute incubation time at 24 - 27 C removed all exogenous reducing agents from the system. By measuring spectrophotometric optical density at 340 mu for 3 to 5 minutes at intervals of 10, 15, or 30 seconds, the rate of decrease of optical density representing the rate of oxidation of NAD^+ was considered a measure of LDH in the serum. The activity was expressed as units/ml. of serum per minute. One unit then equalled a decrease in optical density of 0.001 minute ml. at 24 - 27 C. This was altered in 1958 by Berger and Broida (1958) who specified a reaction temperature of 25 C.

Isoenzyme separation utilizing electrophoresis was first described by Neilands (1952). Smithies (1955) described zone electrophoresis in starch gels. The technique proved adaptable to LDH methods, as described by Vessell and Bearn (1957), Markert and Møller (1959) and Wroblewski and Gregory (1961). Agar electrophoresis was first described by Wieme (1959). The use of cellulose acetate was first described by Barnett (1962). Resseler, et al. (1963) observed several factors involving LDH isoenzyme electrophoretic migration. First, in increasingly dilute buffers, the more basic isoenzymes, band five of LDH especially, tended to migrate cathodically. This was likely to be in association with anodic compounds in the buffer. Secondly, the isoenzymes registered an increase in interaction as the concentration of agar was increased. Thirdly, incubation with ribonuclease prior to electrophoresis had an increasingly detrimental effect on the isoenzyme as the buffer became more dilute and fourthly, the authors observed a lesser degree of isoenzyme interaction with Difco special agar, Noble, than with Difco* purified agar.

*Difco Laboratories, Detroit, Michigan.

Agar gel had the advantage in LDH separation over starch and acrylamide gel in that its separation was based solely on electrophoretic factors rather than partial separation due to molecular sieve effects. Wieme (1964) established several requisites for high-resolving zone electrophoresis utilizing agar gel. Firstly, zone separation was considered optimal if fractions as narrow as possible were separated over a distance as large as practical. Likewise, a small amount of fluid sample, 1.4 to 2.0 ml. was suggested. Secondly, the thickness of the agar slab should be thin; 1.6 mm. was optimal. Thirdly, the ionicity of the buffer should be 0.03 to 0.06. The author reported that lower values caused an excessive conductivity, and that higher values created cooling problems. Fourthly, an adequate coolant was necessary. Fifthly, electro-osmotic flow should be regulated perhaps by agar blocks.

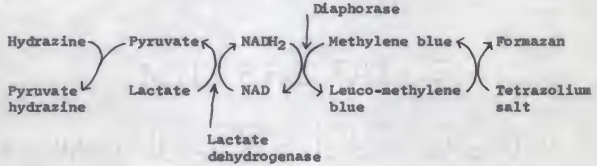
Wieme (1964) also described scanning and recording equipment of the electrophoresed material. A slow scanning speed with a narrow optical slit was suggested.

Mull and Starkweather (1965) modified Wieme's technique by using a scanning attachment consisting of a monocular microscope with a mechanical stage to which a 4 r.p.m. synchronous motor was attached. A 500 mu filter was placed in the light path and a front photocell from a Spinco model RA analytrol* was incorporated in a box over the microscope eyepiece. The 43X objective was used with a substage illuminator. Leads connected the photocell to a Spinco model RA analytrol. The authors reported increased sensitivity of the recording equipment with this technique.

*Beckman Instruments, Inc., Fullerton, Calif.

Starkweather, et al. (1966a) utilizing only slight modifications of Wieme's (1964) technique made a number of observations: 1) changes of enzyme activity did not effect the mobility of LDH_5 on Difco agar; 2) isoenzyme patterns varied with homogenizing media of varying strength. LDH_5 lost 25 to 100% of its activity in 0.25 M sucrose compared to 0.15 M buffers; 3) the practice of preparing an application medium by cutting a slit in the gel was undesirable because, after electrophoresis, reactants reached isoenzymes close to the slit immediately, resulting in erroneously high values for that fraction.

The detection of isoenzymes in electrophoretic media have been described as part of a total isoenzyme technique, but some individual consideration of the reagents involved was necessary. The reaction scheme described by Markert and Møller (1959) was as follows:



This reaction was based on the observation by Kun and Abhood (1949) that LDH could undergo reduction to a distinctly colored substance, formazan. In 1962, Van der Helm used methylphenazonium methosulfate (PMS) which catalyzed the reaction between $NADH_2$ and the tetrazolium salt, eliminating diaphorase and methylene blue. Likewise, cyanide was found to be a better pyruvate acceptor than hydrazine. Van der Helm (1962) suggested an isoenzyme development medium containing nitroblue tetrazolium (1 mg/ml).

Incubation was suggested for one hour at 37 C. Raabo (1963) described a technique for visualization of LDH on paper electrophoresis which employed 2 (p-iodide phenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT). The color developed was violet tinted, and it was adaptable to elution and photometry techniques.

Starkweather et al. (1966a) recommended the use of tetranitro blue tetrazolium (TNBT). The reasons were: 1) TNBT did not require "pyruvate traps" or anerobic conditions; 2) TNBT did not vary in color on the same gel, precluding accurate comparisons at the same wavelength.

A problem encountered by the authors in the use of TNBT was the apparent reduction of NAD by LDH isoenzymes without added lactate. Since the reaction required PMS and NAD, nonspecific reduction of the ditetrazolium by protein sulfhydryl groups or lipids was eliminated as a causative factor. The reaction also occurred when diaphorase was substituted as the immediate electron carrier. Lactate bound to the enzyme was not implicated as reaction mechanisms of LDH required the addition of NAD to the enzyme followed by lactate--the reaction may have been related to the "nothing dehydrogenases" referred to in cellular localization of dehydrogenases.

Homolka et al. (1966) described the use of 2-p-nitrophenyl-5-phenyl-3-($3_1 3^1$--dimethoxy-4-diphenylil) tetrazolium chloride (TBMT) in the visualization of LDH isoenzymes. A considerable increase in sensitivity was noted. Wright et al. (1966) utilized para nitro blue tetrazolium with consistent results.

The stability of LDH isoenzymes in blood serum was important in any technique utilizing the enzyme. Kreutzer and Fennis (1964) reported a considerable difference in cold stability at temperatures below zero.

Fraction 4 was least stable, followed by fraction 5, then 3, then 2 and fraction 1 was very stable at low temperatures. Because of these characteristics, it was suggested that sera in which LDH activity and LDH isoenzymes were studied should not be frozen, and that storage at room temperatures was better than storage at refrigeration temperatures. Chilson et al. (1965) reported the effect of one or more freezes on LDH isoenzyme activity. Using an equal mixture of M_4 and H_4, they found that one freezing (placing in freezer at -20 C until completely frozen) and one thawing (room temperature, 25 C for 20-30 minutes) resulted in considerable hybridization of all except M_3H_1 (band 4). A second freeze-and-thaw resulted in nearly complete equilibration of all fractions.

Diagnostic Aspects of LDH

The diagnostic significance of LDH followed the observation that cardiac tissue enzymes were released into the blood following myocardial infarction. Wroblewski and La Due (1955) reported a two-fold increase in serum LDH following myocardial infarction in man. Wroblewski (1957) reported two- to ten-fold elevation of serum LDH_1 24 - 48 hours following infarction. A grave prognosis was suggested with a ten-fold increase. A quantitative relationship between serum LDH levels and rapidity of neoplastic growth was observed. Serous effusions in diseases involving non-neoplastic cells had less LDH activity than serum LDH of the same patient, while serous fluids bathing cells had more LDH activity than serum LDH of the same patient. The author also observed three-fold elevations in serum LDH of patients with diagnosed chronic myelogenous leukemia.

Hauss and Leppelman (1958) studied several enzymes, including glutamic-oxalacetic transaminase (GOT), LDH, aldolase (ALD), tributyrinase (TRI), and cholinesterase (CHE) in serum in an effort to define the problem of whether the change in serum enzyme activities in myocardial infarction represented a specific observation or whether it was nonspecific and represented a general occurrence in acute diseases characterized by the "acute syndrome", including high temperature, leukocytic increase, changes in hemogram, increse in blood sedimentation rate, blood sugar and urea nitrogen and variations in serum protein. Their findings demonstrated that myocardial infarction was followed by an increase in GOT, LDH, and ALD, as well as a decrease of serum TRI and CHE. Serum enzymes in cases of surgery, infectious diseases and pulmonary embolism reacted in the same manner as in myocardial infarction. They felt that quantitative differences may have been linked with the severity of the condition, but that increases per se were non-specific, as they occurred in a variety of acute diseases. Wroblewski (1958) and Hill (1958) reported an elevation of serum LDH in myocardial infarction, as well as an elevation in various neoplasms.

An earlier paper by Vessell and Bearn (1957) reported a technique that was to become widely accepted because it increased the specificity of LDH as a diagnostic enzyme. By electrophoresis of the enzyme, the authors reported three separate peaks of LDH, one pattern of which appeared to be specific for myocardial infarction and another for leukemia. Vessell and Bearn (1957) refined the technique and established the presence of five isoenzyme fractions with further evidence on the findings reported initially in 1957.

The observation regarding the differences in heat stability of the H_4 fraction and the M_4 fraction of LDH led Wroblewski and Gregory (1961) and later Strandjord et al. (1962) to describe a technique for quantitation of these techniques. The M_4, or heat labile fraction, was destroyed by incubation at 57 C for 30 minutes, while the H_4, or heat stable fraction, remained after incubation at 65 C for 30 minutes.

Van der Helm (1962) described a simplified method for electrophoretic separation of LDH isoenzymes. The report indicated the diagnostic specificity of isoenzymes of LDH in myocardial infarction and hepatic cirrhosis. This information was followed by several publications on LDH isoenzymes in disease states. Wieme (1963) suggested three main patterns of increased levels of LDH isoenzymes: 1) hepatic necrosis M_4 (band 5); 2) malignancy, M_1H_3 (band 2); and 3) myocardial infarct, H_4 (band 1). The author also noted an increase in H_4 (band 1) in muscular dystrophy, thus supporting earlier reports, and an increase in M_4 (band 5) in prostatic carcinoma in relapse. Goldman and Kaplan (1963) reported results of LDH isoenzyme assays on numerous neoplasms. They noted a shift toward a predominance of the M fractions on "solid" tumors, and a shift toward the H fractions on the more malignant ones. Pure cell populations were not utilized. Richterich and Burgur (1963) reported an absolute and relative increase in M fractions in 7 malignant human neoplasms. Wermeskerken and Kammeraat (1963) reported an increase in the H fractions in LDH isoenzyme electrophoretic separations in neoplasms in man, and Cenciotti and Mariotti (1964) reported an increase in the M_2H_2 LDH isoenzyme in leukogenic myeloma and Erlich's carcinoma.

Goldman et al. (1964) found a definite and consistent shift to the M fractions of LDH in tissue extractions obtained from neoplastic tissues, as well as an increase in total LDH values from the same tissues. The authors found no correlation between the degree of shift to the anaerobic types of LDH isoenzyme and the histologic grading of the neoplasms. These findings were compatible with the expected increase in glycolytic activity of malignant neoplasms.

Freeman and Opher (1965) demonstrated the sensitivity of LDH isoenzyme interpretation in patients evidencing vague symptoms of myocardial infarction. Whereas 93 per cent of a group of 48 patients with established diagnoses of myocardial infarction possessed a serum H_4 band of more than 30% of the total LDH activity, 73% of a group of 48 patients with only vague symptoms of myocardial infarction exhibited similar H_4 activity. A control group had no H_4 activity over 30%. Similar findings were reported by Taylor and Preston (1965), Holzer and Binzus (1966), Bohn (1966) and Schneider and Lehmann (1966).

Brody (1965), Paulson et al. (1966) and Pearson and Kar (1966) suggested the possibility of diagnosing myopathies with accuracy equal to that of diagnosis of myocardial infarction. Their work also emphasized the effects of variations in adult levels of the isoenzymes on diagnostic accuracy and of the effect of concommitant lesions in other organs in certain myopathies such as neuromuscular disease in lambs.

Recent work defining the LDH isoenzyme diagnostic possibilities in neoplasms include a report by Poznanska-Linde et al. (1966) which indicated that tissue neoplasms, including those of lymphoid tissue had a shift in LDH isoenzyme pattern to the M_4, M_3H_1 and M_2H_2 fractions.

Ng and Gregory (1966) reported work indicating that substances inhibiting LDH activity were capable of significantly inhibiting aerobic glycolysis in certain standard tumor cell lines, and not in others. *In vitro* studies with similar constituents produced inhibition of tumor cell reproduction. Non-malignant cells were not affected.

Some disagreement in the nature of normal and neoplastic lymphocytes and granulocytes was evident. Starkweather *et al.* (1965) and Dioguardi *et al.* (1966) agreed with Rabinourtz and Dietz (1967) in stating that normal granulocytes possessed considerable M_4 LDH isoenzyme activity, and that neoplastic granulocytes tended towards predominance of M_2H_2. However, the former paper disagreed with the latter as to the normal lymphocytic LDH isoenzyme configuration, with the former paper stating that lymphocytes contained the M_4 configuration, while the latter stated that a high total H_4 LDH isoenzyme was present in normal lymphocytes. Both agreed that a shift toward the M_2H_2 configuration occurred in leukemic cells.

Spector, *et al.* (1966) reported a predominance of M_2H_2 LDH isoenzymes in leukemic cells of bone marrow, with a higher total LDH in bone marrow than in corresponding serum total LDH assays.

MATERIALS AND METHODS

Experimental Animals

The normal animals utilized for controls were 30 - 32 months old Jersey cattle; seven were steers and one was a nulliparous female. Prior to experimentation, the animals were held in a dry lot for 8 months and fed a maintenance ration of alfalfa hay and a corn-oats concentrate supplement. These conditions were maintained throughout the experiment. The cattle designated as affected with bovine lymphocytoma were adult Holstein-Fresian cows varying in age from 2 to 7 years. They were held in dry lot for a minimum of 12 months prior to experimentation and fed a ration of alfalfa hay with corn-oats supplement. All of these animals originated from a southeastern Kansas dairy herd exhibiting an unusually high herd incidence of bovine lymphocytoma. The original herd consisted of 35 mature Holstein cows plus replacement heifers. No cattle had been introduced into this herd since 1949. Artificial insemination had been utilized exclusively since 1959. Most of the herd were daughters and granddaughters of animals purchased at a dispersal sale.

Since 1952, 1 to 3 cows had been removed annually as a result of a condition diagnosed as "malignant lymphoma". Most of these animals were slaughtered with a similar diagnosis reported upon post-mortem inspection. Between 1964 and 1966, the animals selected for this study were purchased from the owner and placed under observation. The criteria for selection was: 1) elevated peripheral lymphocyte count, established as leukemic by Bendixen (1959), and 2) clinical evidence of lymphocytoma as indicated by clinical diagnosis.

The animals selected on the basis of being leukemic were as follows with criterion:

Animal	Ave. lymphocyte/cu. mm. during past 18 months	Age in years	Diagnosis of leukemia, based on Bendixen (1959)
2	105,000	6	Positive
3	12,000	5	Positive
4	38,000	5	Positive
8	11,000	3	Positive
10	5,900	6	Suspect
11	39,000	NA	Positive

Cow 9 was selected because of the following findings: upon rectal palpation, several irregularly diffuse abdominal masses anterior to the right ileum were detected that had neither the shape nor the consistency of normal lymph nodes. No encapsulation was evident and the size was 3.8 x 2.5 x 2.5 cm. The right kidney lacked the usual lobulations. These findings were taken as suggestive of bovine lymphocytoma due to the absence of any criteria incriminating any other disease condition.

Five control animals received injections of crude heart muscle extract (H), and 7 animals received injections of crude skeletal muscle extract (M). No pairing of experiments nor of animals was attempted. The only restriction upon selection was a two-week waiting period between injection of animals that were to participate in 2 experiments. Six of the 7 test animals were used for paired experiments, that is, they received one crude extract, and following at least 2 week's rest, received the remaining crude extract. However, the death of one animal from other causes necessitated the introduction of a seventh unpaired

animal into the research project. No animal of any group received more than one H injection and one M injection.

Preparation of Crude Extract of Lactate Dehydrogenase Monomers

Bovine heart muscle was obtained from local meat retailers or from the veterinary necropsy room at Kansas State University from adult cattle without gross lesions of cardiac or skeletal muscular disease. All bovine skeletal muscle was obtained from the necropsy room. Extract preparation techniques were the same for both types of muscle.

The extraction procedure was as follows: 100 gm. of muscle tissue, trimmed free of fat and large vasular tissue was minced with scissors to pieces about 0.5 cm. diameter. The fragments were washed in cold tap water by flushing through a Buchner funnel until the effluate was nearly colorless, indicating a removal of blood from the minced tissue. The negative pressure created by the Buchner funnel left the minced tissue relatively dry. The minced tissue was placed in a three-speed food blender, 100 ml. of 0.1 M monobasic-dibasic potassium phosphate buffer, pH 7.5*, cooled to 6 C, was added, and the tissue blended at high speed for 3 minutes and at the low speed for 2 minutes. The mixture was poured into centrifuge tubes and centrifuged at 28,000 g for 20 minutes. The supernatant was withdrawn and quickly refrigerated. The precipitate was discarded.

The supernatant (designated as crude extract) was passed through a Seitz** filter apparatus, equipped with a medium bacterial filter, pressure no greater than 20 p.s.i., and maintained under sterile

* Hereafter referred to as phosphate buffer, pH 7.5.
**Republic Seitz Filter Corporation, Newark, New Jersey.

conditions thereafter. The crude extract was cultured and established as bacteria-free, and the continued sterility was determined by periodic culturing on blood agar.

The total LDH activity of the crude extract was determined by the following method, modified from the technique of Wroblewski and La Due (1955): 2.8 ml. of 0.1 M monobasic dibasic potassium phosphate buffer, pH 7.5; containing 0.2 mg. of β-Dihydro-Nicotinamide adenine Dinucleotide* ($NADH + H^+$) was prepared, to which 0.1 ml. of serum or serum diluent in phosphate buffer was added. This mixture was incubated at 25 C for 20 minutes to allow any exogenous oxidizing agent to react with the $NADH + H^+$ after establishing a zero O.D. with a water blank. One-tenth ml. of 0.02 M pyruvate was added, mixed and immediately placed in a spectrophotometer, 340 mu, utilizing cells of 1 cm. light-path. A Hitachi-Coleman model 101** spectrophotometer was used initially, and a Gilford model 240† spectrophotometer was used during later phases of this investigation. Optical density was read at least four times in the first 3 minutes following addition of pyruvate. Since 1 Sigma unit of LDH is the amount of enzyme necessary to reduce the optical density of 1 ml. serum .001 units per minute at 25 C with a wavelength of 340 mu, the LDH activity was calculated directly. One Sigma unit was 2.08 International units (I.U.). The dosage of crude extract was determined from the LDH activity ml.

* Sigma Chemical Company, St. Louis, Missouri.
**Coleman Instrument Company, Maywood, Illinois.
† Gilford Instruments, Oberlin, Ohio.

Whole and clotted blood samples were collected prior to injection of the crude extract. The site of injection was scrubbed with an alcohol pleget. Indwelling catheters (Venocath-14)* were fixed in place in both external jugular veins prior to injection. California bleeding needles, 16 ga, were used alternatively to the indwelling catheters. The LDH activity of the crude extract, was established at 9,700 I.U./kg. in the control animals, and 26,700 I.U./kg. in the test animals. The calculated quantity of crude extract was injected intravenously into the right jugular vein, with an injection time span of 2 minutes.

Post-injection blood samples were drawn from the left external jugular via the indwelling catheter or, alternatively, via a California bleeding needle.

Collection time intervals for the post-injection samples were irregular in the control animals but were collected at regular intervals from the test animals. Six minutes after completion of the injection of crude extract, a blood sample was collected. Sampling was continued at the following intervals: 0.25, 0.50, 1.0, 1.5, 2.0**, 3.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10,00**, 12.0, 15.0, 20.0**, 30.0, 40.0, 50.0**, 60.0, 70.0, and 80.0** hours. Asterisks (**) represent collection of whole blood samples.

Laboratory data obtained from all whole blood samples, including pre-injection samples, were:

1. Packed cell volume (PCV), capillary tube method, recorded as cellular percentage of blood volume.

*Abbot Pharmaceutical Company, Chicago, Ill.

2. Hemoglobin (Hb), oxyhemoglobin method, utilizing an American Optical* hemoglobin meter, recorded as gm. hemoglobin/100 ml. blood.
3. Total leukocyte count (WBC), recorded as cells/cmm., performed with the aid of a Coulter** Model A electronic particle counter.
4. Differential leukocyte count, recorded as per cent of the total leukocyte estimation, prepared by staining with a Wright's Leishman stain.
5. Blood urea nitrogen, recorded as mg./100 ml., performed with the Harnstoff† Urease technique.

Serum glutamic oxalacetic-transaminase (GOT), (Dade††), determinations were performed on the serum of the clotted blood samples collected at the time intervals of the whole blood collections, including the pre-injection sampling. Urine was collected for analysis prior to and once during experimentation.

Total LDH and LDH Isoenzyme Electrophoretic Techniques

All clotted blood samples were held at ambient temperature for 4 to 6 hours. The serum was harvested and immediately refrigerated until assayed.

Serum samples were assayed for total LDH activity within 36 hours following collection. Dilution of one part serum to five parts phosphate buffer was made prior to assay with the Hitachi-Coleman spectrophotometer

* American Optical Company, Instrument Division, Buffalo, New York.
**Coulter Electronics Company, Hileaha, Fla.
† Fisher Chemical Company, Chicago, Ill.
††Dade Reagents, Inc., Miami, Fla.

while a one in eight serum dilution was carried out prior to assay with the Gilford spectrophotometer. The assay technique was similar to that for the crude extract.

The LDH isoenzyme technique was as follows:

A 2 cm. strip of unemulsified 35 mm. P.40-B film leader* was marked from left to right at the 5, 8, 9, 12, 13, and 16 cm. lengths. Sixty mg. of Agarose**, mixed and dissolved in 10 ml. of warm phosphate buffer, was evenly layered over the strip. As the agar began to solidify, strips of plastic microscope slide cover slip†, 18 x 3 mm., were placed on edge at a right angle to the length of the film leader at the 5, 9, and 13 cm. marks. After several minutes at ambient temperatures the microscope slide cover slip strips were removed from the gel. The film strips were then placed in a Gelman†† horizontal electrophoresis chamber containing approximately 200 ml. of phosphate buffer, pH 7.5. Eight microliters (ul.) of undiluted serum were placed in the gel slits left from the cover slips, using a Hamilton syringe‡. The electrophoretic chamber was covered and placed in a plastic box 60 x 30 x 8 cm. Crushed ice at -10 C was packed around the electrophoretic chamber. This maintained an internal chamber temperature of approximately 4 C at all times. Electrophoresis was carried out with a Gelman power supply set at -4 v. per cm. per strip. Because of the high electrical resistance of the gel, only three strips per electrophoretic chamber were utilized.

* Du Pont de Nemours & Company, Wilmington, Del.
**Fisher Scientific Company, Pittsburg, Penn.
† Scientific Products Company, Evanston, Ill.
††Gelman Instrument Company, Ann Arbor, Mich.
‡ Hamilton Instruments, Inc., Whittier, Calif.

After 5-1/2 hours of electrophoretic migration, the strips were removed and an overlay composed of the following was applied evenly over the surface:

1. 18.0 mg. Agarose dissolved in 3 ml. water at 56 C.
2. 01.8 mg. Nitro-blue tetrazolium*
3. 03.7 mg. nicotinamide adenine dinucleotide (NAD, diphosphopryridine nucleotide, DPN).*
4. 0.09 mg. Phenazine methosulfate in 0.09 ml. water.*
5. 3.00 ml. of a solution containing 48 mg. sodium cyanide, 16 mg. magnesium chloride and 544 mg. lithium lactate dissolved in 80 ml. of phosphate buffer.

Immediately after the overlay solidified, the entire preparation was placed in a humid, dark environment at 37 C for 30 minutes. The strips were then placed in a pan containing a minimum of 250 ml. of 5% acetic acid per strip for 12 to 36 hours. The strips were then placed in a warm, forced air current until the gel was completely dehydrated.

Densitometric scanning of the stained, fixed, and dried LDH isoenzyme bands was performed with the aid of a Beckman Model RB Analytrol** with a Gelman Scan-A-Tron conversion kit, including a B-2 balancing cam, a slit width of 1/2 mm. and an interference filter of 550 mu. Recording paper was a commercial grade number 12-188 graph paper†, 10 mm./cm..

* Sigma Chemical Company, St. Louis, Missouri.
**Beckman Instruments, Inc., Fullerton, California.
† National Blank Book Company, Holyoke, Mass.

Following the generally accepted European system of nomenclature of LDH isoenzyme bands, the most anodic or fast-moving band was designated as band one or LDH_1. The remaining bands, each equidistant from adjacent mates, were numbered progressively in order of descending migration rate, with the most cathodic band, or slow-moving band being designated as band five or LDH_5. Bands two, three, and four, being hybrids, were grouped together as the intermediate bands, or $LDH_{2,3,4}$. Methods utilized in this experiment gave rise to straight, blue-staining bands, band five remained at or just anodal to the point of origin. Since increased resistance caused a voltage drop, the most cathodic sample in the three-sample strip migrated less total distance than the anodic sample. As a result, identification of individual isoenzyme bands within a sample was accomplished by proportionating migration distance relative to LDH_1 rather than quantitation by migration distance.

Analysis of LDH Data

Isoenzyme quantitation was determined by calculating the percentage of total decrease in light transmittance of the specifically stained isoenzyme bands created by each specific isoenzyme band with the aid of the integrator tracings recorded by the Analytrol. Gaussian distribution curves were sketched for each component initially, but this technique was dropped in favor of delineating of components by drawing a straight line downward from the lowest point between each peak of the recording trace after evidence accumulated that similar accuracies existed for both techniques.

Absolute levels of individual LDH isoenzyme components was determined by multiplying the percentage value of the LDH isoenzyme com-

ponents by the total LDH value. This data was punched into computer cards with an IBM Model 029* keypunch machine. Data was sorted into four groups:

1. Crude heart muscle extract injectate in control cattle; designated as H-control.
2. Crude heart muscle extract injectate in lymphocytoma or leukemic cattle; designated as H-test.
3. Crude skeletal muscle extract injectate in control cattle designated as M-control.
4. Crude skeletal muscle extract injectate in lymphocytoma or leukemic cattle; designated M-test.

In each of these animal groups, 9 treatment groups were established. These were:

1. Total LDH
2. LDH_1
3. $LDH_{2,3,4}$
4. LDH_5

pre-test sample (t_o)

5. Time of sample collection (t_{time}), or (t_t)

6. Total LDH
7. LDH_1
8. $LDH_{2,3,4}$
9. LDH_5

post-injection sample collection at t_t

New dependent variables were generated from these nine. From the H-test and H-control groups, these were:

*International Business Machines, Inc., White Plains, N. Y.

10. $\frac{\text{Total LDH}_{t_o}}{\text{Total LDH}_{t_t}}$ designated variable 10

11. $\frac{LDH_{1,t_o}}{LDH_{1,t_t}}$ designated variable 11

12. $\frac{LDH_{1,t_o}}{LDH_{5,t_t}}$ designated variable 12

13. $\frac{LDH_{1,t_o}}{LDH_{2,3,4,t_t}}$ designated variable 13

14. $\frac{LDH_{1,t_o}}{LDH_{1,2,3,4,t_t}}$ designated variable 14

15. $\frac{LDH_{1,t_o}}{LDH_{2,3,4,5,t_t}}$ designated variable 15

16. $\frac{LDH_{2,3,4,t_o}}{LDH_{t_t}}$ designated variable 16

17. $\frac{LDH_{2,3,4,t_o}}{LDH_{2,3,4,t_t}}$ designated variable 17

18. $\frac{LDH_{2,3,4,t_o}}{LDH_{5,t_t}}$ designated variable 18

19. $\frac{\text{Total LDH}_{t_o}}{LDH_{1,t_t}}$ designated variable 19

20. Repeat of dependent variables 10 through 18 listed above, with the quantity t_t changed to $(t+t^2)_t$ designated variable 20.

New dependent variables generated from the M-test and M-control were:

21. $\frac{\text{Total LDH},t_o}{\text{Total LDH},t_t}$ designated variable 21

22. $\frac{LDH_1,t_o}{LDH_1,t_o}$ designated variable 22

23. $\frac{LDH_{1,2,3,4},t_o}{LDH_{1,2,3,4},t_t}$ designated variable 23

24. $\frac{\text{Total LDH},t_t}{LDH_5,t_t}$ designated variable 24

25. $\frac{LDH_{2,3,4},t_o}{LDH_{2,3,4},t_t}$ designated variable 25

26. Repeat of dependent variables 21 th ough 25 of the group listed above, with the quantity t_t changed to $(t + t^2)_t$, designated variable 26.

27. $\log_e \frac{\text{total } LDH_1,t_o}{\text{total } LDH_1,t_t}$ designated variable 27

28. $\log_e \frac{LDH_1,t_o}{LDH_1,t_t}$ designated variable 28

29. $\log_e \frac{LDH_{1,2,3,4},t_o}{LDH_{1,2,3,4},t_t}$ designated variable 29

30. $\log_e \frac{\text{total } LDH_t}{LDH_5,t_t}$ designated variable 30

This data was subjected to a simple least squares regression analysis by an IBM 36-50 computer.

The half-life of total LDH was determined mathematically for H-test and H-control groups as well as M-test and M-control groups.

Analysis of Parameter Data

Data of the PCV, BUN, and GOT were arithmetically analyzed, with means ($\bar{X}$) and unbiased estimations of standard deviations (s) being the determinants. Total leukocyte and absolute neutrophils/cmm. were grouped into test and control animal groups, and high and low means were determined for each. Changes in the absolute lymphocytes/cmm. were also evaluated.

RESULTS

Experimental Parameter Data

Most animals resisted restraint and injection procedures. Consequently, increases in pulse, respiration, and body temperature were of no significance. Animals in 4 of the 24 experiments had a moderate increase in respiration that could not be attributed solely to resistance to restraint. One cow aborted a late mid-term fetus ten days after injection. Failure to detect and remove a second fetus from the uterus of this animal resulted in death from bacterial metritis 12 days after abortion.

There were no significant changes in the PCV, Hb, and BUN in any group (see Figs. 1 and 2). GOT was elevated significantly in all groups (Fig. 3). The total leukocyte count and number of segmented neutrophils decreased significantly during the experiment (Table 1).

Table 1. Mean High and Mean Low Neutrophils and Total Leukocytes/cmm. of Experimental Animals During the Inclusive Experimental Time

Test Groups	High absolute neutrophils/ cmm.	Low absolute neutrophils/ cmm.	High absolute leukocytes/ cmm.	Low absolute leukocytes/ cmm.
Normal cattle	3,095 ± 1,230	625 ± 1,690	10,569 ± 3,560	5,092 ± 1,730
Lymphocytoma	5,528 ± 2,590	1,246 ± 1,120	32,300 ± 11,200	23,905 ± 10,450

One animal in the control group had a drop in the number of lymphocytes/cmm. from a pre-injection sampling of 5,190 to 1,320 at 11.0 hours after injection. Seven animals of the control group had a decrease from

a mean of 5,740 lymphocytes/cmm. prior to injection to a mean of 2,255 lymphocytes/cmm. 5.0 - 11.0 hours post-injection. In this group of 7 animals, the absolute lymphocytes/cmm. rose to 6,470 by 48 - 72 hours after injection. Three animals in the test group registered a decrease in lymphocytes/cmm. from an average of 7,670 pre-injection to 3,030 at 2.0 - 20.0 hours post-injection. Each of the 3 test animals had a lymphocyte count at least equal to the pre-injection sample by 80.0 hours post-injection. When averages of greatest differences in absolute lymphocytes/cmm., regardless of time, were calculated for both control and test groups, no significant differences existed between the average high lymphocyte count and the average low lymphocyte count, given 90% confidence interval levels.

Urine analysis revealed no significant alterations during any portion of this study.

Serum Lactate Dehydrogenase Data

Animals in all groups responded with elevated serum LDH levels during some phase of these experiments. In general, the isoenzyme electrophoretic patterns of the H-test and H-control groups appeared as in Fig. 4, and the M-test and M-control groups appeared as in Fig. 5. The confidence interval between pre-injection levels of the total LDH and LDH_1 test animals and control animals showed differences at the 90% confidence interval but not at 95%.

Table 2. Pre-injection Activity of Lactate Dehydrogenase and Lactate Dehydrogenase Isoenzymes in International Units in Normal Adult Cattle and Adult Cattle Afflicted with Lymphocytoma

Group	Total LDH	LDH_1	$LDH_{2,3,4}$	LDH_5
Normal cattle	1,474 ± 191	595 ± 98.5	831 ± 123	38.5 ± 26.4
Lymphocytoma	886 ± 234	405 ± 139	461 ± 147	20.0 ± 4.3

Analysis of data from the animals which received the crude heart muscle extract indicated:

1. The reestablishment of normal total LDH levels did not occur during the H-test period for either test or control groups, but the rate of decrease of LDH was greater in the H-test group (Fig. 7).
2. The total quantity of LDH_1 was markedly elevated initially and did not return to pre-injection levels in the H-control groups, whereas a less marked initial elevation was observed in the H test group, and the pre-injection level was reached by 40 hour's post-injection time (Figs. 7 and 8).
3. $LDH_{2,3,4}$ was elevated initially and did not return to pre-injection levels during the course of the experiment. The H-control group was elevated more than H-test at any given time during the experiment (Figs. 9, 10, and 11).
4. LDH_5 was not significantly elevated during the experiment (Fig. 11).

Analysis of data from animals receiving the crude skeletal muscle extract indicated:

1. The initial elevation of total LDH following injection of the extract diminished to pre-injection levels by 57 hours in the M-test group, but the initial elevation of the M-control group did not return to pre-injection levels of LDH, during the test period. The rate of decrease of total LDH in the M-test group was more rapid than the rate of decrease of the total LDH of the M-control group (Fig. 12).
2. LDH_1 was elevated initially in both M-test and M-control groups, returning to normal 45-50 hours after injection. The M-test group was elevated more initially than the M-control group (Fig. 13).
3. $LDH_{2,3,4}$ was elevated initially and returned to normal between 50 and 55 hours in the M-test group, while the M-control group was less elevated initially but continued to be elevated during the 48 hour experimental period (Figs. 14 and 16).
4. The ratio of total LDH to LDH_5 did not return to pre-injection levels during the 48 hour experiment of the M-control group or the 80-hour experiment of the M-test group (Fig. 15).

The period of time required for clearance one-half of the total LDH from the experimental animals in this study may be defined as the half-time of the total LDH, designated by the mathematical term $T_{\frac{1}{2}}$. This term can be derived from any portion of the clearance measurement period, provided the rate of clearance is constant relative to time, as defined by the term $Y_{ij} = \alpha + \beta t + \epsilon_{ij}$. Observation of Figs. 7 and 12 indicate this relationship exists for both test and control groups during H and M experiments.

Calculations resulted in the following $T_{\frac{1}{2}}$ values:

H-test	145 hr.
H-control	188 hr.
M-test	81 hr.
M-control	133 hr.

DISCUSSION

The uniformity from group to group of experimental animals was desirable as adult cattle on maintenance rations were utilized, but undesirable in that breed and sex homogeneity was not maintained. Also, the separate experiments were conducted during three seasons of the year. The significance of these factors is not known.

Control serum was not used in this experiment for the following reasons: 1) a commercial stable bovine serum for LDH assay was not available. The alternative of an in-house control was rejected because of the instability of the enzyme in fresh serum and the impracticality of consistent dessication techniques for this material; 2) due to differences in kinetic constants between human and bovine LDH, standard human enzyme control serum was rejected. This species difference was due to differences in isoenzyme components. These differences may be overcome by adding an excess of NADH + H^+, a technique which was not possible with all equipment used during the project. Human enzyme control serum assayed in our laboratory had less day-to-day reproducibility than did fresh bovine sera; 3) each sample tended to be its own control since all exogenous oxidizing substances were allowed to oxidize the NADH + H^+ in solution before assay. Hence the rate of decrease of O.D. after addition of pure 0.02 M pyruvate measured the first order enzyme-substrate reaction of LDH.

The isoenzyme electrophoresis of bovine LDH presented unique problems. The rate of migration was considerably slower than for man or other animals as performed in our laboratory. Heat dispersion, drying of the gel, and electrostatic crystalization of the buffer ions

were factors affecting the technique. Despite attempts to move LDH_5 from the artifact-creating application well, a potential source of experimental error remained with LDH_5 (Fig. 6).

Preparation of the Crude Extracts

As is consistent in several species, the heart extract did not contain only LDH_1. Lesser amounts of $LDH_{2,3,4}$ were present in the crude heart extracts. This was not true of the crude skeletal muscle extracts, which contained almost all LDH_5 (Fig. 6).

Evaluation of data was based on ratios of total LDH or LDH isoenzyme fractions. This permitted accurate evaluation of the changes within the isoenzyme constituents of the enzyme. Attempts were made to fit data to both straight and curvilinear regression analysis: the straight line regression analysis was chosen for interpretation. Criterion for acceptance was the r test, which would not be accepted below 0.2, as was a 90% confidence interval utilizing a "t" distribution. The r test is a measure of dispersion of data about a mean at a given time. The "t" distribution is an indication of the correlation of a given regression line to a straight line. Only a small proportion of the analyzed data was acceptable. This was due to the difficulty of characterizing data relationships rather than being due to the lack of agreement of the data. Of the H-test and H-control groups, those accepted were variables designated: 10, 11, 13, 14, and 15. Likewise, of the M-test and M-control groups, variables 21, 22, 23, 24, and 25 were accepted.

Evaluation of Experimental Parameter Data

Increase in respiration of animals in 4 of the 24 experiments may have been a physical disclosure of a less obvious fright than other animals in other experiments. An alternative may have been respiratory acidosis resulting from the potassium present in the potassium phosphate buffer used as a vehicle for the enzyme preparation. This alternative was unlikely, as an elevation of only 1% would be noted, considering 60.0 ml. of blood per kg. and 4-5 mEq. of potassium/100 ml. of blood per animal and 100 ml. of 0.1 M potassium phosphate buffer being administered.

The lack of change in PCV, Hb, and BUN indicated an absence of dehydration, erythrocytic destruction, or alteration of systemic or renal urea metabolism and excretion. These parameters would probably have been altered in disease resulting from toxic foreign substance, but normal findings suggest an absence of disease.

The moderate elevation of GOT was considered to be due to the injection of large amounts of that enzyme in the crude extract and a blockage of the reticulo-endothelial (R-E) system in a manner similar totthat described by Fleisher and Wakim (1963) and Wakim and Fleisher (1963). If the latter mechanism was present, some interference with LDH clearance may have been possible.

Any changes in the total leukocyte and differential leukocyte estimations were of lesser importance because of the existing neoplastic disease in the test animals. However, the control animals exhibited an absolute neutropenia and an absolute leukopenia during the experiments (Table No. 1). The mean high and mean low WBC of the test animals was

a reflection of the leukemic condition of most of the group, but the mean low neutropenia suggested a reaction similar to the control group. Two possible mechanisms may have contributed to this observation: 1) a microcytic response to subcellular particles injected, and 2) migration of the neutrophils to the extravascular spaces in response to the stress of the experiment, including the R-E overload created by the injected material.

The drop in lymphocytes/cmm. in individual control animals and individual test animals was most probably a response to stress. These individuals apparently responded more markedly than others, because the decrease in lymphocytes per cu. mm. of the entire group was not significant at the 90% confidence interval level.

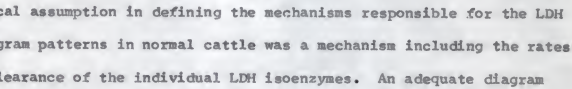
Evaluation of Lactate Dehydrogenase Data

Isoenzymes were released as tetramers rather than as individual monomers because an individual monomer release would result in a ratio approaching the theoretical values of 1:4:6:4:1 in all mammals if equal units of H and M were available. In cattle, the ratio was approximately 1:2:6:12:16, which was skewed toward LDH_1 and LDH_2. Either LDH_1 and LDH_2 were indicative of tissue destruction, such as erythrocytes, or the rate of removal of LDH_5, LDH_4, and LDH_3 was more rapid. The erythrocyte, because of rapid destruction, may be responsible for the prevalence of LDH_1 and LDH_2 in the serum if it can be assumed that tissue destruction was the primary mechanism determining the LDH isoenzyme pattern in the serum.

If the genetic linked correlation between H or M monomers and aerobiasis or anaerobiasis of organ metabolism was assumed, and organ metabolism in mammals was similar, tissue destruction was unlikely to

be the sole mechanism in determining serum LDH isoenzyme patterns. Two reasons supporting this statement are: 1) the normal equine serum LDH zymogram contained LDH_3 predominantly, while erythrocyte LDH was predominantly LDH_1 and LDH_2. Organ constituents were consistent with other mammalian organs; and 2) the normal canine serum LDH zymogram, which contained nearly all LDH_5, while erythrocytes and other organs were consistent with those of other mammals (Coffman, 1968)*. The most logical assumption in defining the mechanisms responsible for the LDH zymogram patterns in normal cattle was a mechanism including the rates of clearance of the individual LDH isoenzymes. An adequate diagram depicting these relationships was taken from Cawley (1968)**.

LACTIC ACID DEHYDROGENASE



A formula derived from this diagram, also from Cawley (1968)** was:

$$\text{"serum level} \cong \frac{\text{rate of release}}{\text{rate of removal}} + \text{bound fraction"}$$

It was difficult to account for the preferential concentration of LDH_1 and LDH_2 in the serum of normal adult cattle if one assumed that the rate of clearance of all five LDH isoenzymes was identical. The

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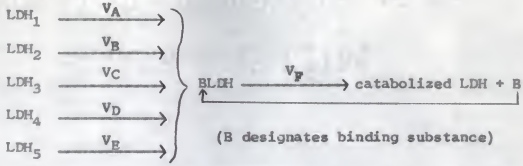
presence of individual LDH isoenzymes in the serum based on their independent rate of clearance was more likely to be an important factor in explaining the bovine serum LDH profile. This hypothesis correlated adequately with the observation that various mammalian organ LDH constituents were similar with few exceptions, but their normal serum LDH isoenzyme constituents were dissimilar.

An analogy in other protein moieties of the mammalian body was the immunoglobulins γ G, γ A, γ M, and γ D. Barth, et al. (1964) reported similar rates of synthesis, but different rates of catabolism of these compounds. Their difference in rates of catabolism were then responsible for their different serum levels.

If different rates of catabolism were responsible for normal serum levels of LDH, one must assume an equilibrium existed normally rather than a steady state. This avoided the unlikely conclusion of predicting an ever increasing serum concentration of the less rapidly catabolized LDH isoenzymes.

This does not prevent external factors from altering the equilibrium and a steady state may well be present between the old and the new equilibria. An example of this is the effect of the Riley virus, which causes permanent increase in serum LDH in man due to R-E blockage (Notkins, 1965). Obviously, a new equilibrium was established after an infection due to Riley virus, but one could not assume that a steady state in existence during the interval would be identical to the new equilibrium.

A multiple reaction scheme depicting a clearance mechanism, given equal amounts of isoenzymes, is as follows:



If V designates reaction velocity, whether at steady state or at equilibrium, and $V_5 > V_4 > V_3 > V_2 > V_1$, each by a factor of two, an equilibrium ratio of 1:2:4:8:16 of the LDH isoenzymes would be expected. This would be true if the availability binding substance (B) were greater than or equal to the sum of the reaction velocities.

If there were no surplus of B, any excess of any LDH isoenzyme added to the system would shift the equilibrium away from LDH_1 and LDH_1 would appear in excess. If massive amounts of one LDH isoenzyme were added in a system containing B in concentration greater than the sum of the reaction velocity, the rate of effect on other LDH isoenzyme concentrations would vary if a gradient of velocities existed.

If a marked gradient existed, one would expect a rapid shift to more rapid velocities and a rapid reverse shift if massive amounts of LDH_1 were introduced. Likewise, in a marked gradient system, if LDH_5 were introduced, a less rapid shift would occur because of the velocity of V_E, but the reverse shift would be less rapid.

If only a small gradient existed, regardless of total velocities ($V_A + V_B + V_C + V_D + V_E$), the rate of shift of equilibria to other LDH isoenzymes would be more nearly equal from either the VA $\longrightarrow$ ($V_B + V_C + V_D + V_E$) direction, or the $V_E \longrightarrow$ ($V_D + V_C + V_B + V_A$) direction.

The kinetics of the LDH $\xrightarrow{V_{xc}}$ BLDH reaction was dependent upon the BLDH $\xrightarrow{V_F}$ Degraded LDH + B reaction because the rate of V_F will

determine the availability of B, and this can be a limiting factor, regardless of the velocity of $V_{A\text{—}E}$. The LDH data produced by this project tended to fit this clearance scheme with several alterations. An arbitrary assumption that excessive amounts of B are available was made prior to evaluation, since more than one fraction was elevated after either injection. Whereas the clearance scheme depicted a consistent gradient, the experimental data indicated that intermediate fractions tended to be removed more slowly when heart muscle extract was injected, as depicted by Figs. 7 and 8. With injection of skeletal muscle extract $LDH_{2,3,4}$ and LDH_1 were removed prior to removal of LDH_5 as depicted in Figs. 15 and 16. The application point artifact undoubtedly was a factor (Starkweather, 1966b) in the graphic representation of the clearance of LDH_5. However, if a gradient existed, the rate of shift described would result in velocity rank of $V_E > V_A > V_{B,C,D}$. The difference between $V_E > V_A$ cannot be definitely established, but it is undoubtedly significant, because an elevation of LDH_1 was noted when skeletal muscle extract was injected (Fig. 13), but no elevation of LDH_5 was noted when heart muscle extract was injected (Fig. 11). Similarly, the difference between V_E and $V_{B,C,D}$ is greater than the difference between V_E and V_A because, after skeletal muscle extract injection, LDH_1 returned to normal before $LDH_{2,3,4}$ (Figs. 13, 14, and 16). An artifact existing in the evaluation of LDH_5 was likely in view of the observation that, after skeletal muscle extract injection, LDH_1 was normal after 45-50 hours, $LDH_{2,3,4}$ was normal after 30 hours (in the test animals) and the total LDH returned to normal in 55-60 hours (Figs. 12, 13, and 15).

Evidence to support the term $V_A > V_{B,C,D}$ is provided in Fig. 8, which indicates a return of LDH_1 to normal by 40 hours (in test animals) following heart muscle extract injection, while Figs. 9 and 10 indicate that $LDH_{2,3,4}$ did not return to normal during the sampling time. Thus, the higher level of $LDH_{2,3,4}$ probably accounts for the fact that the total LDH did not return to normal during the sampling time following injection of heart muscle extract (Fig. 7).

The comparison of total LDH values supported the term $V_B > V_A$, as a comparison of Figs. 7 and 12 indicated that there was a more rapid reestablishment of preinjection level following the injection of crude skeletal muscle extract than following injection of crude heart muscle extract.

Overall comparison of injections of crude heart muscle as extract and crude skeletal muscle extract of the animals tested was explained best by a reaction diagram in which each isoenzyme was bound to a binding substance with a specific reaction velocity or rate constant. A limited excess of binding substance was assumed to be available. If an equal affinity for the binding substance was present for each isoenzyme, the effect of introduction of massive amounts of exogenous heart muscle extract would have shown a skewing toward the LDH_1 fractions, while introduction of massive amounts of exogenous skeletal muscle extract would have shown a skewing to the LDH_5 fractions of equal intensity. This was not observed experimentally. The specific velocity constants were most likely involved, and the equation $V_B > V_A > V_{B,C,D}$ fit all data except the clearance time of LDH_5 after skeletal muscle extract injection, which may have been due to an artifact of the serum application slit.

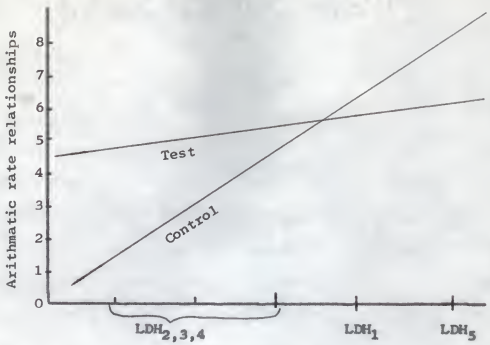
The comparison of test and control animals pointed to general similarities with the following exceptions: 1) regardless of the isoenzyme comparison, all significant responses following H injection in the test animals had a less initial elevation and the return to pre-injection levels generally paralleled the results observed in the control animals; 2) regardless of the isoenzyme comparison, all significant responses in the test animals following M extract injection had a greater initial elevation with a rate of return to normal that was faster than that of the control group; and 3) following M injection, the $LDH_{2,3,4}$ of the control animals continued to be increased during the sampling time, as shown in Figs. 14 and 16.

Reference is again made to the equation written by Cawley (1968):

$$\text{"serum level} \simeq \frac{\text{rate of production}}{\text{rate of removal}} + \text{bound fraction"}$$

A more rapid rate of removal of the enzyme is the most probable explanation for lesser initial amounts of enzyme following H injection in the test animals. This conclusion is not incompatible with the observation that generally parallel rates were maintained when the steady state concept is accepted. Conversely, the M injection was more rapidly removed initially by the control animals than by the test animals.

These observations suggested a cross-over in rates of removal. A graphic example follows:



The M-test and M-control differences in the $LDH_{2,3,4}$ clearance may have been due to differences in gradients of V_E and $V_{B,C,D}$ between test and control animals. The differences in gradients are depicted graphically by differences in slope of the graphic example above. If a marked gradient existed at a steady state, a saturated LDH_5 binding system would exhibit a preferential affinity for the binding agent to the extent that the $LDH_{2,3,4}$ would continue to increase until the concentration of the exogenous material began to return to near normal. Conversely, if gradients between V_E and $V_{B,C,D}$ were not great, the two velocities would have approached saturation quickly and a steady state would produce a slow, but positive rate of return to normal by all constituent LDH isoenzyme levels. Figures 14 and 16 indicated that the data fit the mechanisms suggested.

The estimations of $T_{\frac{1}{2}}$ of total LDH were combined to form ratios of the H and M groups. The ratio of H-test to H-control was 1:1.265, and the ratio of M-test to M-control was 1:1.65. This indicated the greater ability of the test animals to clear LDH from the circulating blood, and it indicated the LDH_5 isoenzymes were cleared more quickly than the

LDH_1 isoenzymes. This coincides with the degradation scheme proposed.

The proposed mechanisms describing rate of removal of LDH from bovine plasma supported the data derived from the experiment only for the time span defined--that is, 0.3 to 48 - 80 hours. Hence the more rapid blocking mechanism involving removal of LDH was assumed to have reached significant proportions by 0.3 hour. The experimental design did not permit accurate differentiation between a physical mixing phenomenon in the circulating blood of the experimental subjects and any blocking mechanism involving a binding substance. Also, the experimental design was not intended to differentiate between exogenous isoenzyme and endogenous isoenzyme at any sampling.

The type of statistical analysis must be considered in any attempt to fit the data to a complex model. Trend lines may not have defined biological mechanisms when reaction kinetics were involved but one can predict reproducibility about a trend line. This is why all ratios appeared as straight lines. Even though reaction kinetics did not react in a straight line manner from start to finish, the data presented fit a straight line better than two mathematical curvilinear models, namely $(t + t^2)_t$ and $(Ln)_t$. Also, several of the ratios of $\frac{LDH_x\ t_o}{LDH_x\ t_t}$ returned to normal and continued to rise during the sampling time. This may have represented a rebound effect, but it was more likely to have represented an inability of few samples at later sampling times to skew the trend line established by many early collected samples with no significant interference with "t" or r values.

This work tended to agree with that of Freedland et al. (1963) which stated that "there were no differences in plasma LAD activity and

kinetic values between normal cattle and cattle with persistent lymphocytosis"*. The report does not state the confidence intervals utilized, but the work presented here agreed with the report of Freedland if 95% confidence intervals were utilized in both analyses.

*LAD was defined as Lactic acid dehydrogenase earlier in the report.

SUMMARY

A comparison of the clearances of LDH from the serum of normal and leukemic cattle following IV injection of crude heart and crude muscle extracts suggested differences other than direct degradation mechanisms of individual constituent LDH isoenzymes. Exogenous and endogenous LDH isoenzymes were measured collectively. The time span of this study apparently did not include a reestablishment of equilibrium of the resting LDH clearance mechanism in the experimental animals. A hypothesis of the interaction of LDH isoenzymes and clearance mechanisms that fits the data collected is proposed.

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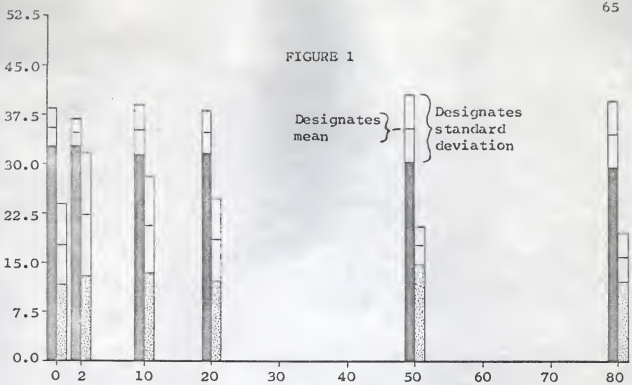
APPENDIX

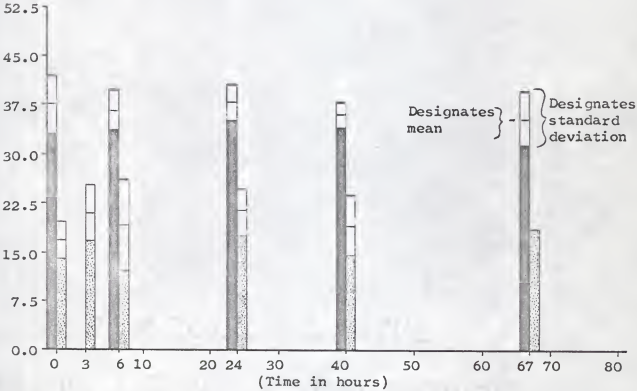
FIGURE 1



Packed cell volume and Gms. Hemoglobin/100 ml. after injection of crude skeletal muscle extract in test animals

Packed cell volume in per cent cell volume

Hemoglobin in Gms./100 Ml.

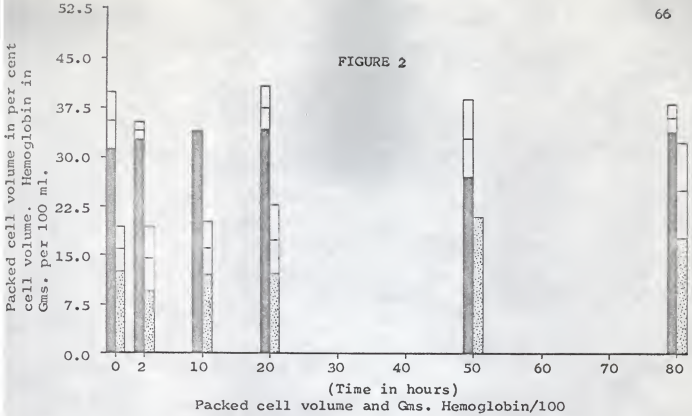


Packed cell volume and Gms. Hemoglobin/100 ml. after injection of crude skeletal muscle extract in control animals

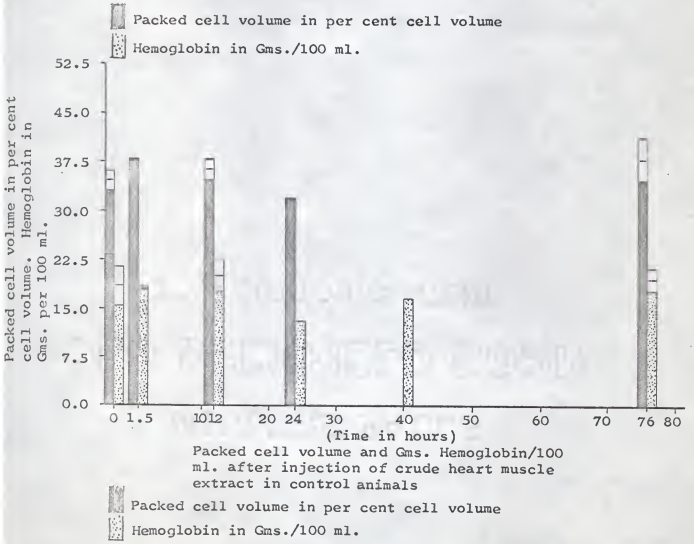
Packed cell volume in per cent cell volume

Hemoglobin in Gms./100 ml.

FIGURE 2



Packed cell volume and Gms. Hemoglobin/100 ml. after injection of crude heart muscle extract in test animals

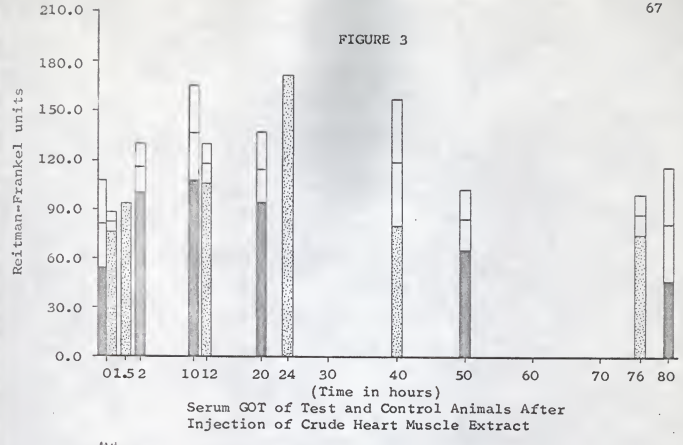


Packed cell volume and Gms. Hemoglobin/100 ml. after injection of crude heart muscle extract in control animals

Packed cell volume in per cent cell volume

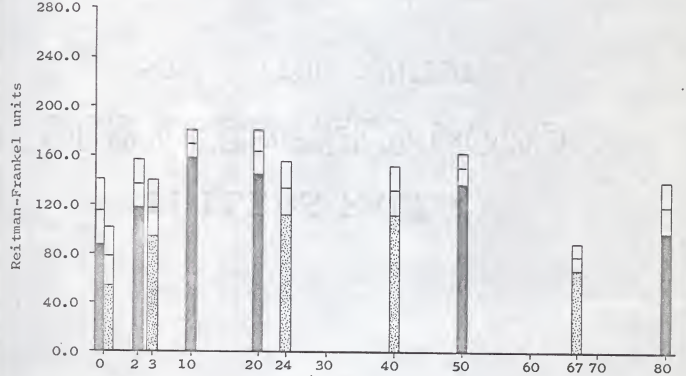
Hemoglobin in Gms./100 ml.

FIGURE 3



Serum GOT of Test and Control Animals After Injection of Crude Heart Muscle Extract

H - Control Group
H - Test Group



Serum GOT of test and control animals after injection of crude skeletal muscle extract

M - Control Group
M - Test Group

EXPLANATION OF FIGURE 4

Photograph depicting in sequence, from top to bottom, three typical densitometric scans of serum samples collected during an H injection experiment. The stained and dehydrated electrophoretic strips were attached for qualitative comparison. The pre-injection sample (top sequence) is representative of normal bovine LDH isoenzyme constituents. Following injection of H extract, a relative change is seen in the intermediate bands (middle sequence). These changes decrease during the experiment as seen in the bottom sequence. X2.40:1.0.

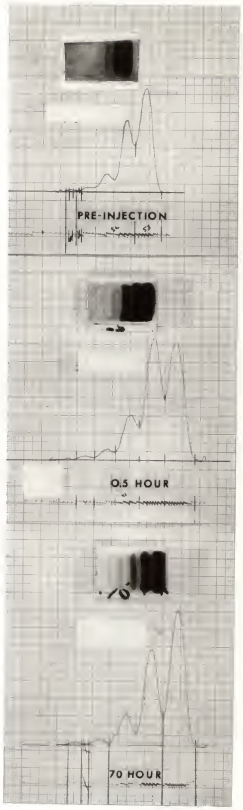


FIGURE 4

Pre, 0.5, and 70.0 hour zymograms
of typical H injections

EXPLANATION OF FIGURE 5

Photograph depicting in sequence, from top to bottom, three typical densitometric scans of serum samples collected during a M injection experiment. The stained and dehydrated electrophoretic strips were attached for qualitative comparison. The pre-injection sample (top sequence) is representative of normal bovine LDH isoenzyme constituents. Following injection of M extract, a significant amount of LDH_5 and LDH_4 appear as seen in middle sequence and these decrease during the experiment as seen in the bottom sequence. X2.50:1.0.

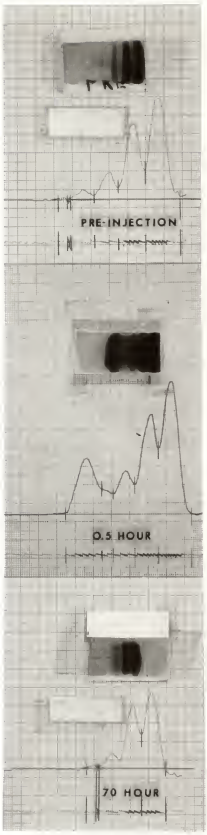


FIGURE 5

Pre, 0.5, and 70.0 hour zymograms
of typical M injections

EXPLANATION OF FIGURE 6

Top photograph is a densitometric scan of a cardiac muscle extract. The stained and dehydrated electrophoretic strip was attached for qualitative comparison. LDH_2 and LDH_3 are present in lesser proportions than LDH_1. X2.08:1.0.

Bottom photograph is a densitometric scan of a skeletal muscle extract. Both the tracing and the dehydrated zymogram suggest a predominance of LDH_5. Arrow defines an artifact created by the serum application well. X2.08:1.0.

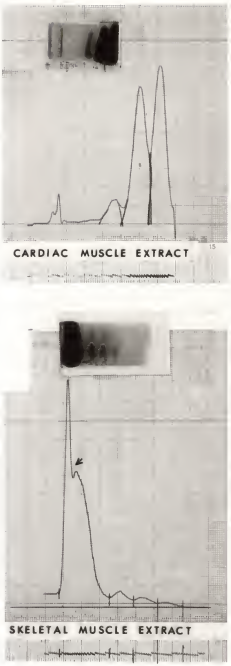
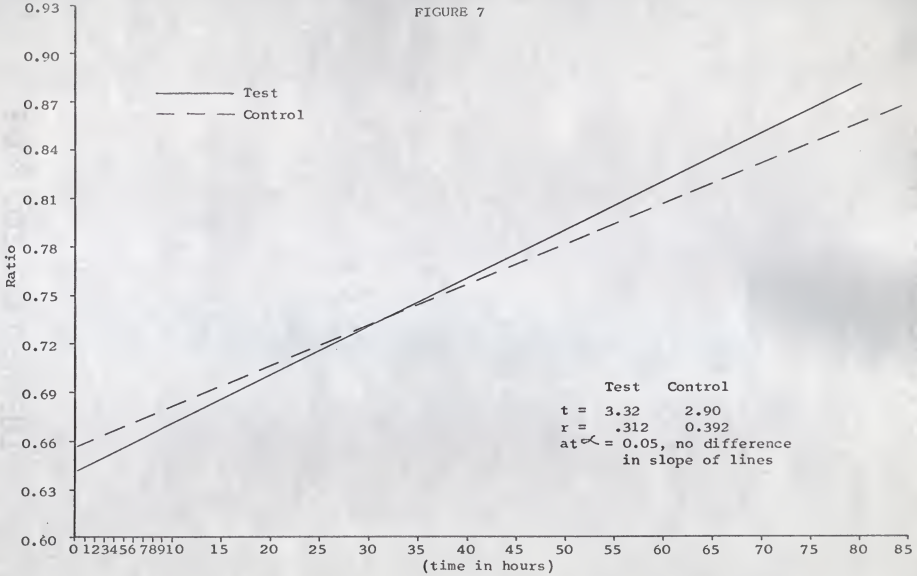


FIGURE 6

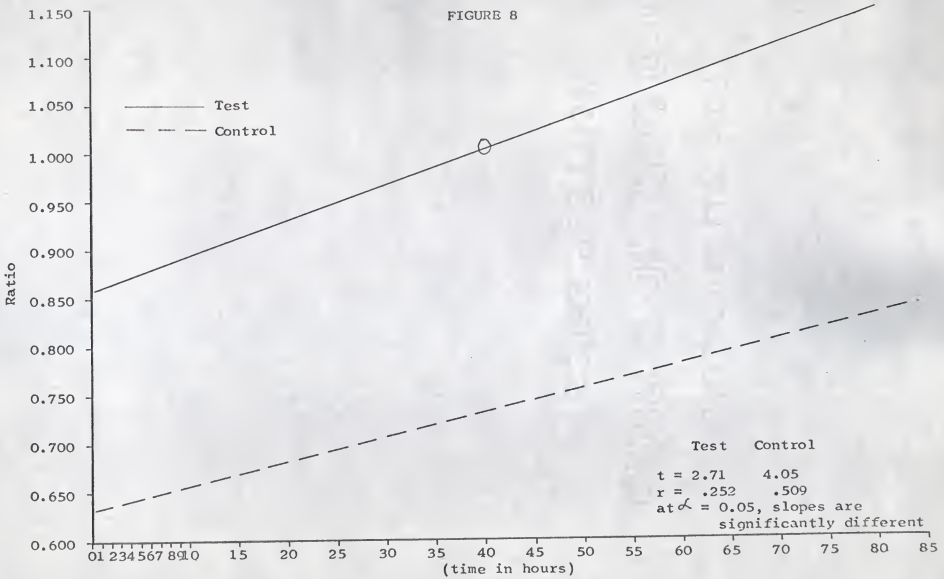
Typical cardiac and skeletal muscle extract zymograms

FIGURE 7



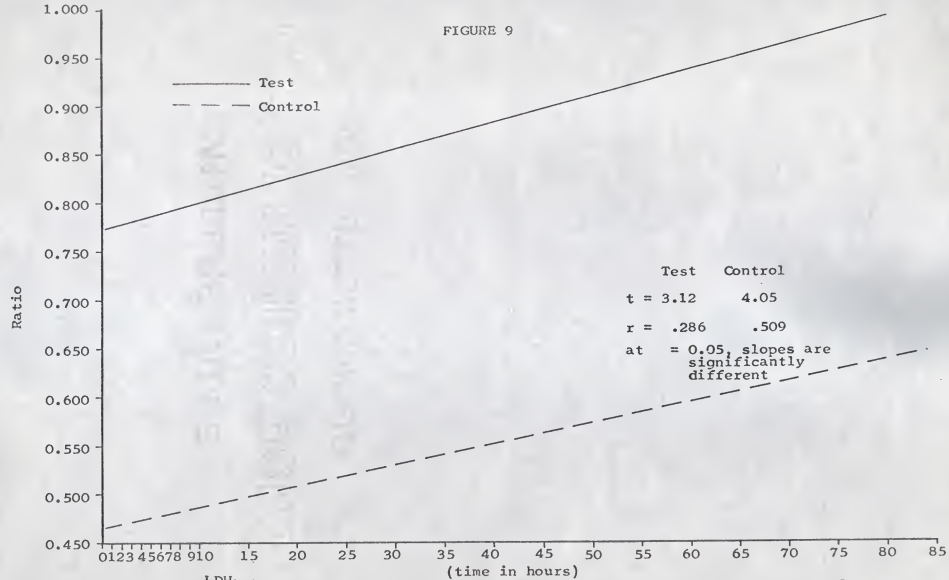
Ratio of $\frac{\text{Total LDH}_{t_o}}{\text{Total LDH}_{t_t}}$ of test and control animals after injection of crude heart muscle extract, with 1.0 being the resting ratio.

FIGURE 8

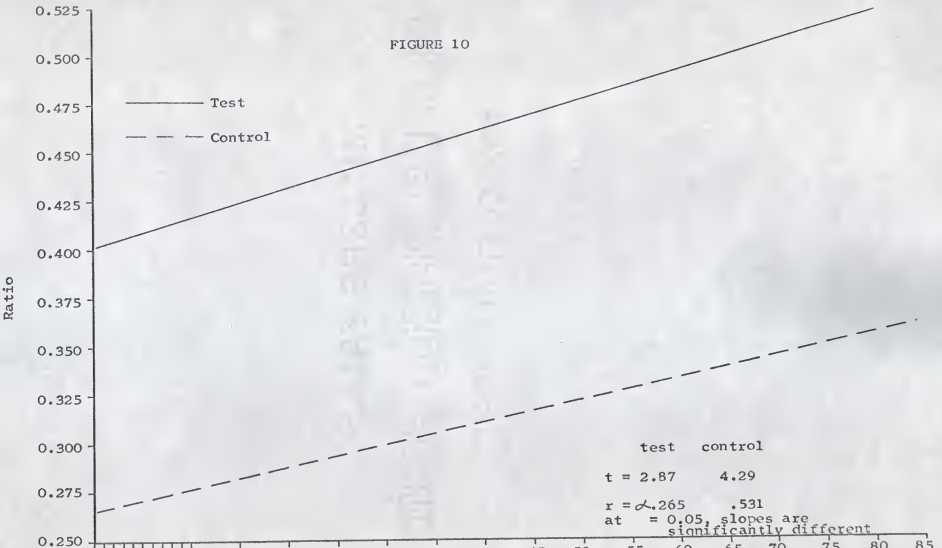


Ratio of $\frac{LDH_1, t_o}{LDH_1, t_t}$ of test and control animals after injection of crude heart muscle extract, with 1.0 being the resting ratio.

FIGURE 9

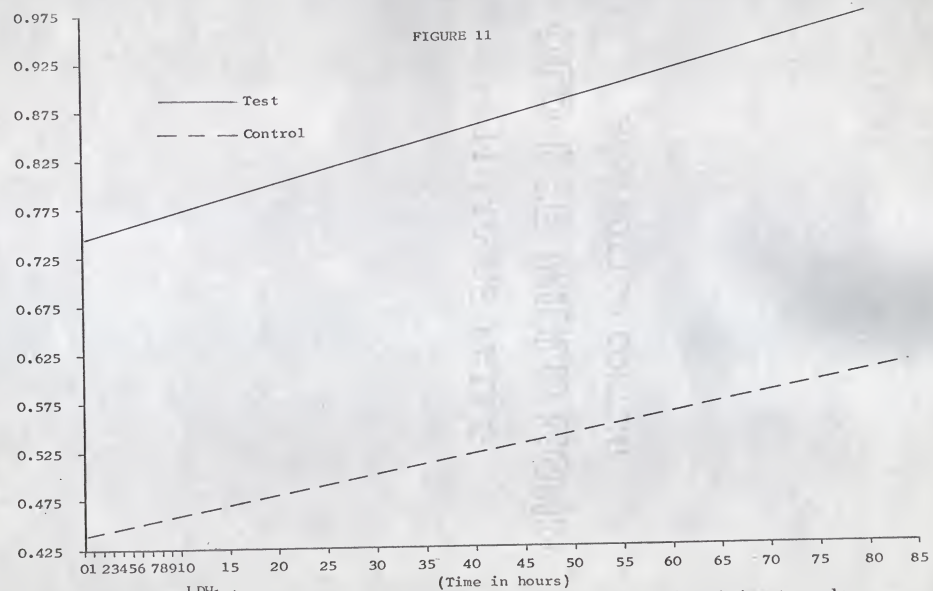


Ratio of $\frac{LDH_{1},t_{o}}{LDH_{2,3,4},t_{t}}$ of test and control animals after injection of crude heart muscle extract, with 1.3 the pre-test ratio of test animals, and 0.69 the pre-test ratio of control animals.



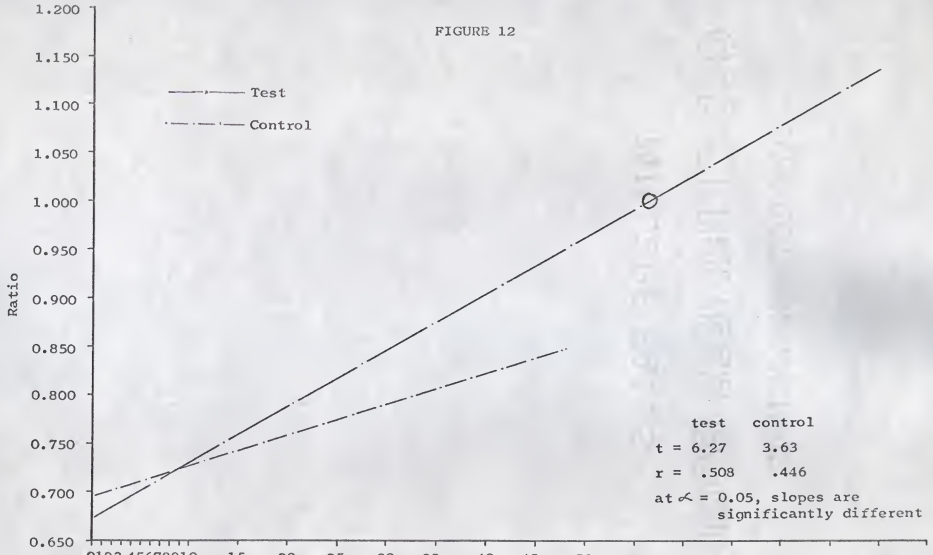
Ratio of $\frac{LDH_1,t_o}{LDH_1,2,3,4,t_t}$ of test and control animals after injection of crude heart muscle extract, with 0.565 the pre-test ratio of test animals and 0.406 the pre-test ratio of control animals.

FIGURE 11



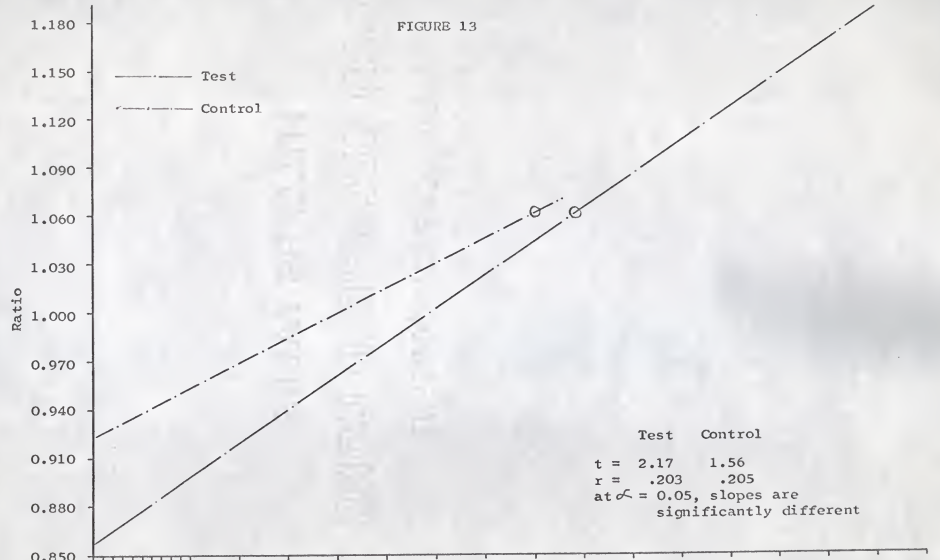
Ratio of $\frac{LDH_{1},t_{o}}{LDH_{2,3,4,5},t_{t}}$ of test and control animals after injection of crude heart muscle extract, with 1.27 the pre-test ratio of test animals and 0.65 the pre-test ratio of control animals.

FIGURE 12



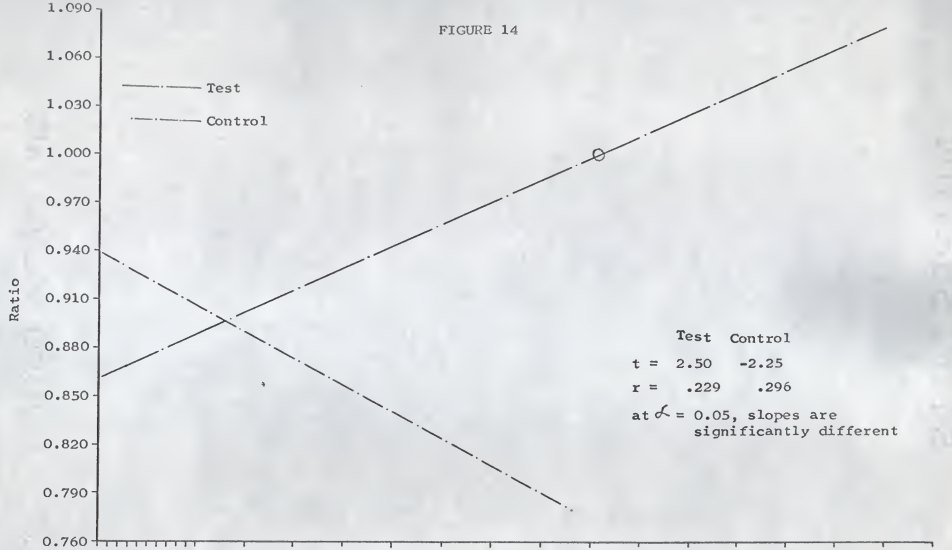
Ratio of $\frac{\text{Total LDH}_{t_o}}{\text{Total LDH}_{t_t}}$ of test and control animals after injection of crude skeletal muscle extract, with 1.0 being the resting ratio.

FIGURE 13



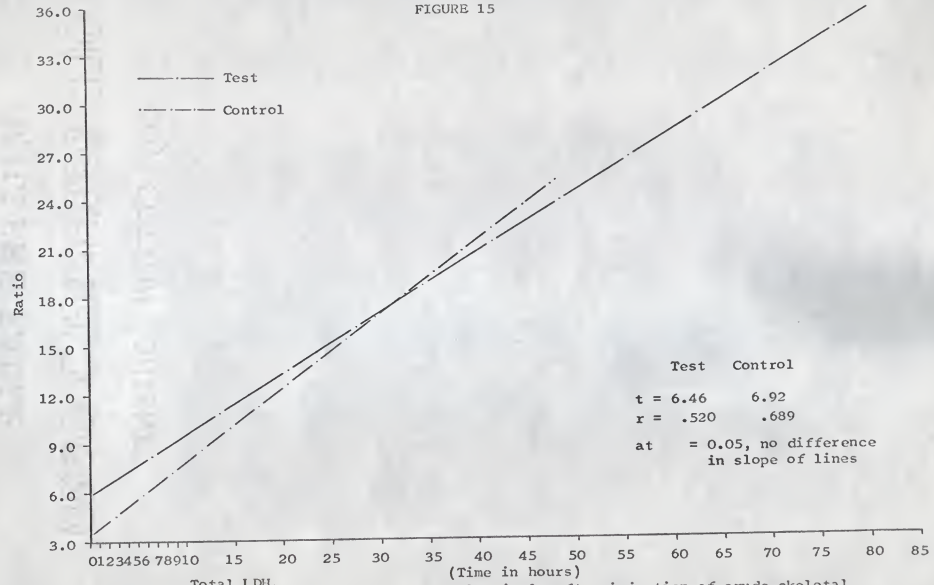
Ratio of $\frac{LDH_1, t_o}{LDH_1, t_t}$ of test and control animals after injection of crude skeletal muscle extract, with 1.0 being the resting ratio.

FIGURE 14



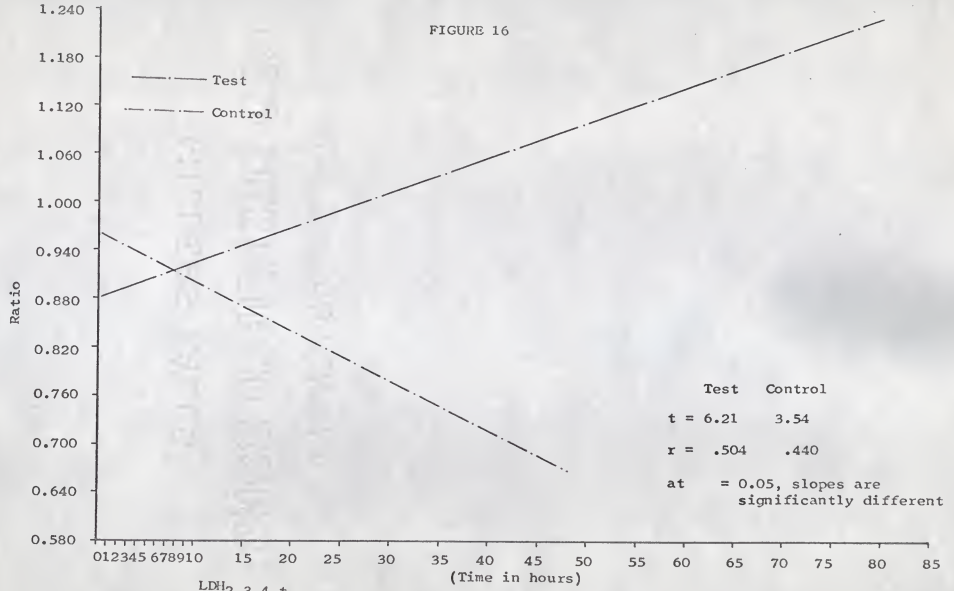
Ratio of $\frac{LDH_{1,2,3,4,t_o}}{LDH_{1,2,3,4,t_t}}$ of test and control animals after injection of crude skeletal muscle extract, with 1.0 being the resting ratio.

FIGURE 15



Ratio of $\frac{\text{Total LDH}_{t_t}}{\text{LDH}_{5,t_t}}$ of test and control animals after injection of crude skeletal muscle extract, with 53.0 the pre-test ratio of test animals, and 37.0 the pre-test ratio of control animals.

FIGURE 16



Ratio of $\frac{LDH_{2,3,4,t_o}}{LDH_{2,3,4,t_t}}$ of test and control animals after injection of crude skeletal muscle extract, with 1.0 being the resting ratio.

A STUDY OF THE CLEARANCE RATES OF LACTATE DEHYDROGENASE FROM THE SERUM OF NORMAL CATTLE AND CATTLE AFFLICTED WITH BOVINE LYMPHOCYTOMA

by

DAVID ROGER HELLAND

D.V.M., Iowa State University, 1964

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Pathology, Parasitology,
and Public Health

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1968

An experiment was conducted to evaluate the clearance of serum LDH and serum LDH isoenzymes from normal cattle and cattle with bovine lymphocytoma. Crude extracts were prepared from fresh bovine heart muscle and fresh bovine skeletal muscle. The animals were divided into four groups. Six adult cattle afflicted with lymphocytoma received 26,700 I.U./kg. of crude skeletal muscle extract intravenously. Six animals with lymphocytoma received 26,700 I.U./kg., IV, of crude heart muscle extract. Seven normal adult cattle received 9,700 I.U./kg., IV, of crude skeletal muscle extract. Five normal adult cattle received 9,700 I.U./kg., IV, of crude heart muscle extract.

Pre-injection levels of total serum LDH were significantly different between the lymphocytoma group and the normal gropp at the $\alpha = 0.10$ level, but not at the $\alpha = 0.05$ level. Blood samples were removed at frequent intervals initially, and less frequently later, during an 80 hour sampling period. Whole blood samples were collected throughout the sampling period for evaluation of hemogram and blood urea nitrogen.

Total LDH activity was determined by spectrophotometric measurement of ultraviolet absorption of NAD^+ at 340 mu, and LDH isoenzyme determinations were made by agar-gel electrophoresis and quantitation of the migrated stain specific bands. Statistical evaluation was performed with the use of an IBM 36-50 computer.

Evaluation of the hemograms and blood urea nitrogen data indicated no change in the general well-being of the animals during experimentation, except for depletion of neutrophils during the 80-hour experimental period. An increase of serum glutamic-oxalacetic transaminase occurred during the experimentation.

Evaluation of the LDH data indicated a significant difference in the rate of clearance of LDH isoenzymes between normal animals and those afflicted with bovine lymphocytoma. These differences could be correlated only after establishing an LDH degradation scheme allowing for depression or reinforcement of isoenzyme degradations depending upon the proportion of the various isoenzymes present in the plasma.