LACTIC DEHYDROGENASE PATTERNS IN MURINE VIRUS-INDUCED LEUKEMIA

by

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TABLE OF CONTENTS

	Page
I. INTRODUCTION	1
Statement of the problem	1
Survey of the literature	2
Molecular weight	3
Stability	4
pH	4
Anticoagulants	5
Isozymes	5
LDH agent	12
Properties of the LDH agent	15
Rauscher virus	17
Description of the disease	17
Histological findings	18
II. MATERIALS AND METHODS	20
Animals	20
Experimental groups	20
Virus suspension	21
Inoculation of animals	21
Collection of blood samples	22
Total LDH assay	23
Reagents for assay	25
$NADH_2$ standardization	25
Assay procedure	26
Starch and acrylamide gels	27
Procedure for starch gel electrophoresis	28

TABLE OF CONTENTS (Continued)

	Page
II. MATERIALS AND METHODS (Continued)	
Staining procedure..	30
Reagents used in the staining procedure.............	31
Acrylamide gel..	31
Reagents required for acrylamide procedure...........	32
Procedure for polyacrylamide electrophoresis..........	32
III. RESULTS...	34
Hemoglobin and nucleated cells.........................	34
LDH activity and nucleated cells.......................	34
Hematocrit values and LDH activity....................	37
LDH activity and spleen to body weight ratio..........	37
Liver and spleen weight...................................	37
Isozymes..	41
Hematology..	41
Total LDH activity...	47
Body weights..	60
Death records...	61
IV. DISCUSSION..	62
V. SUMMARY AND CONCLUSIONS...............................	67
VI. REFERENCES..	69

INTRODUCTION

Statement of the problem

In 1962, Rauscher (63) described a virus recovered from Balb/c mice which resulted in a mononuclear infiltrate of the tissues examined, beginning 30-45 days after inoculation. Nucleated erythrocytes were observed in large numbers in the red pulp of the spleen and in the sinusoids of the liver as well as in the peripheral blood smears. Siegel et al.(82) in investigating the pathogenesis of the virus induced leukemia noted the existance of malignant proliferation of the red cell series. Philp et al.(59) described the disease as producing cellular hypoplasia particularly of the erythrocytic series in the spleen, liver, lung and the bone marrow. The mature erythrocyte contains approximately one hundredfold lactic dehydrogenase or LDH activity compared to that of the blood serum levels volume for volume (32).

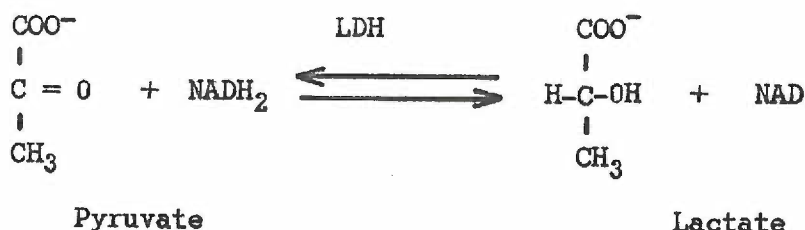
Since this disease produced extensive proliferation and destruction of the erythrocytes, it was felt that the LDH patterns would prove to be a valuable parameter for establishing stages of pathogenesis. To verify a relationship between LDH patterns and the stages of pathogenesis necessitated measuring total serum LDH activity and correlating this activity with gross and microscopic findings. Limited volumes of blood serum available for assay required the development of a microchemical prodecure to determine total enzyme activity. Established electrophoretic procedures had to be modified to elicit data whereby the origin of the enzyme activity could be determined.

Kun et al.(35) investigated the influence of virus multiplication on the metabolism of the host tissue. Chorioallantoic membranes of

embryonated White Leghorn eggs, 12 days old, were infected with a suspension of a variant avian strain of canary pox virus. A marked increase was seen in LDH levels in response to the virus infection. Wenner et al.(97) has shown increased LDH activity in mice that were inoculated with nononcogenic viruses, Newcastle, Vaccinia, Columbia and SK. It was of considerable interest to determine if the Rauscher leukemogenic virus would also alter the level of the glycolytic enzyme LDH.

Survey of the literature

Lactic dehydrogenase is a member of the glycolytic cycle and occurs ubiquitously in animal cells. The enzyme reversibly catalyzes the conversion of pyruvate to lactate, utilizing the oxidizable-reducible coenzyme nicotinamide adenine dinucleotide ($NADH_2$ or NAD):



Pyruvate Lactate

Green (27) cites Meyerhof in 1919 (45) as the first investigator to describe LDH in muscle. Schwert and Winer (80) in their review make reference to Thunberg (89) in 1930 as recognizing an enzyme in tissue which oxidizes lactate and which depends for its activity on a water-soluble cofactor. The utilization of cell-free extracts containing LDH activity was first used in 1932 by Banga et al.(6). Szent-Gyorgyi (88), von Euler (91), Boyland (11) and Green et al.(27) were responsible for much of the early basic work which characterized the enzyme system. In 1940 Straubs (87) prepared from bovine heart a crystallized enzyme which had LDH activity. Kubowitz and Ott (34) crystallized the enzyme from rat skeletal muscle and Jensen rat sarcoma

in 1943. With the advent of improved methods of isolation and purification of LDH more sophisticated work was done to elicit the enzyme's physical and biochemical properties.

Molecular weight

Meister (44) reported that crystalline beef heart LDH prepared as described by Straub (87), and recrystallized four times exhibited molecular weights in the range of 100,000 to 150,000, based on sedimentation constants. Kegeles and Gutter (31) in very exact measurements of hog heart LDH found sedimentation constants of 7.65 Svedberg units at zero protein concentration. Work done by Neilands (48) indicates that two components appear upon electrophoretic separation of bovine heart enzyme, and both have the same sedimentation velocity. He determined that the separate compounds had a molecular weight of 135,000. By fluorometric titration of bovine heart enzyme with $NADH_2$, Velick (90) was able to compute the minimal combining weight of the enzyme as 37,500 g. The molecular weight of the enzyme was estimated as 150,000 $\pm$ 7,500. Molecular weight of pig heart enzyme was estimated from light-scattering measurements as being 127,000 (58). Nisselbaum and Bodansky (51) found that purified human heart LDH upon electrophoretic and ultracentrifugal analysis showed the purified enzyme to be essentially homogeneous, and to have a molecular weight of about 140,000. On the basis of data obtained from velocity ultracentrifugation, approach to equilibrium sedimentation, and diffusion studies, Millar (46) con-concluded that a minomer of beef heart LDH has a molecular weight of 72,000 and it exhibits a concentration-dependent association. He furthermore calculated from amino acid analysis the minimal LDH molecular weight of 72,000. Davisson *et al.* (15) determined from values of the sedimentation constant, diffusion coefficient, and partial specific

volume, the molecular weight of LDH, calculated by the Svedberg equation, was 126,000. The work was with undenatured enzyme.

Stability

LDH is a relatively stable enzyme but certain precautions must be taken to maintain original level of activity. In determination of serum LDH or SLDH, the presence of only slight hemolysis can significantly elevate serum levels in a specimen. Heieh et al. (29) states that clotted blood samples which remained at room temperature for as long as 60 minutes showed an increase in LDH activity by approximately 25 percent over those stored at 4° C. or at room temperature for no more than 30 minutes. Bovine heart enzyme did not lose any activity at 30° C. if held at a pH between 6 and 10 for a period of one hour (28). Human heart LDH was stable for at least 24 hours at values of pH from 5.2 to 9.2 at 4° C. (51). Nisselbaum et al. (51) determined that suspensions of the human heart preparation in 50 percent saturated $(NH_4)_2SO_4$ (pH 5.3) were stable at -10° C. for at least one year. King (32) noted no loss in enzyme activity in specimens, separated from cells, standing at room temperature for 48 hours or at 0° to 4° C. for periods up to three weeks. Erickson and Morales (18) in their experience with serums found that samples could be stored frozen for a period up to a month with negligible loss of LDH.

pH

LDH from rat skeletal muscle retains activity through the pH range of 5.0 to 8.5 (24). Nisselbaum et al. (51) did studies on the influence of pH on human heart LDH. The enzyme was stable for at least 24 hours in 0.1 M sodium phosphate buffer adjusted for pH values between 5.2 to 9.2 at 4° C. When the pH was lowered to 4.0 the enzyme was completely inactivated in 90 minutes. Optimal activity for the heart enzyme appears

to lie between pH 7 to 8 (51). Working with crystalline LDH of beef heart, Winer et al.(100) measured reaction rates at 28.5° C. in tris-tris hydrochloride, 0.2 M buffer over the pH range of 5.6 to 10.5. Results were similar to those of Nisselbaum (51) who utilized human heart LDH. The enzyme attained maximum activity in the pH range of 5.6 to 10.5. The enzyme is stable in 0.05 M phosphate buffer of pH 6.8, at 25° C. for about 24 hours (100). Human serum was measured spectrophotometrically and had optimal activity at pH 7.2 to 7.6 (101).

Anticoagulants

Potassium oxalate and sodium citrate assume the role of LDH inhibitors when used as anticoagulants in the collecting of blood samples. Potassium oxalate produced 75 percent inhibition of serum LDH at concentrations below those normally used in anticoagulation procedures (50). The use of sodium citrate appears also to lower serum LDH activity (32).

Isozymes

Many individual proteins have recently been shown to have molecular heterogeneity. This molecular heterogeneity has proven true with LDH, and occurs not only between different species, but also within the tissue of individual animals. Markert and Møller (41) in 1959 proposed the name isozymes for enzymes which exist in multiple molecular forms within the cell of a single organism. Isozymes may be distinguished from heteroenzymes in that heteroenzymes are proteins of different origin that differ in their physical, chemical and biochemical properties, but have the same biological action, while the isozymes have the same origin and consist of very similar, but distinguishable, proteins (99). Nielands (49) by using Straub's (87) method for crystallizing enzyme from beef heart muscle was able to produce two components when crystal-

line LDH was subjected to electrophoretic separation. The components were designated as "A" and "C". Studies by Wieland and Pfleiderer (98) and by Markert and Møller (41) demonstrated the presence of five electrophoretic forms of LDH. These electrophoretic forms have been shown by means of chemical, immunological and physical tests not to be artifacts of enzyme analysis, but exhibit characteristic patterns of distribution in each individual tissue. Neilands (48) demonstrated that the sedimentration velocity for the two components of ox-heart LDH found electrophoretically were the same and not the product of polymerization of a single molecular type.

There is a preponderance of evidence (2, 12, 16, 19, 30, 99) which now supports the view that LDH is of two types. In adult animals, one type is characteristic of heart tissue and the second type characteristic of skeletal muscle. The two types have been designated as the "H" (heart), and "M" (muscle) forms. Four "H" subunits (H_4) form the pure heart enzyme with the pure muscle enzyme formed by four "M" subunits (M_4) (16). The other three forms of LDH are molecular hybrids of the two parent types (M_3H, M_2H_2, and M_1H_3). Appella et al. (2) stated that the critical test of the subunit hypothesis would involve the construction of the five isozymes from the subunits. Using beef tissue containing all five isozymes, he proceeded to separate the isozymes by electrophoretic and column chromatographic procedures. The two "pure" heart and muscle isozymes were then mixed in equal proportions in 1 M NaCl. Starch gel electrophoresis results revealed a reassociation equilibrium to form the five isozymes in the ratio of 1:4:6:4:1. Thus it appears that quantitative concentration of each hybrid is attributed to the different relative amounts of the parent polypeptides (40). The LDH

isozymes have a molecular weight which is approximately 135,000 and can be dissociated into subunits of 34,000 molecular weight (2). Smithies (83) in 1955 described a technique of zone electrophoresis in starch gel. Starch gel along with a more recent development of a synthetic medium, polyacrylamide gel (65, 66) has found wide use in separating LDH isozymes. The H_4 isozyme has been designated LDH-1 and is the most rapidly migrating (toward the anode) under zone electrophoresis, while M_4 is the slowest migrating and is designated LDH-5. The hybrid intervening bands are LDH-2, 3 and 4. The difference in charge between the isozymes may be due to the fact that LDH-1, the more rapidly migrating protein, contains more carboxylate side-chains or the change may also be due to chemically bound phosphates, sulfate or other polycharged residues (99). It has been determined that there is a pronounced drop in aspartic acid content from LDH-1 through LDH-3, which may alter their rate of migration (99).

It has been proposed that the two types of parent forms of LDH have significantly different physiological roles (12). There is a marked difference in the degree of activity of the two types in high and low concentrations of pyruvate. Heart LDH is maximally active in low concentration of pyruvate, and is strongly inhibited by excess pyruvate. The muscle LDH functions very well under relative high concentrations of pyruvate (16). The significance of the difference in their physiological role may be related to the energy requirements of the individual tissues. Heart muscle demands a steady supply of energy and must resort to complete oxidation of pyruvate and lactate in an aerobic environment. Muscles have sudden requirements for energy, and must function in an anaerobic state. Muscle energy is derived by glycolysis which produces

large amounts of pyruvate with the subsequent production of lactate. It is therefore apparent that the two types have evolved to meet the metabolic needs of the individual cells. Chan *et al.*(12) showed that the two "pure" tetramers (LDH-1 and LDH-5) are immunologically distinct and that the three hybrid isozymes (LDH-2, 3 and 4) are cross reactive with both the parent forms. With distinct immunological properties for the two parent forms it seems reasonable to speculate that they are under the control of separate genes. Numerous reports have now substantiated the two gene assumption (10, 13, 39, 41, 81). It is believed that the isozyme composition of any tissue depends on the relative activity of the two genes at their loci (106). Genetic potential is gained through the processes of cellular differentiation. Each animal's adult tissue has a characteristic pattern of isozymes and this pattern changes during embryonic development until the adult form is reached (41). The patterns and relative concentrations of isozymes are specific for each tissue at each stage of development (41). Markert and Ursprung (42) stated that through the study of isozymes, which are refined expressions of the enzymatic differentiation of cells, one may reveal some insight into basic mechanisms of cellular differentiation. Incubation of fertile chick eggs in 100 percent oxygen results in the suppression of production of the muscle LDH subunits in embryonic skeletal muscle (16). This high level of oxygen encouraged synthesis of the heart LDH subunits in the embryonic heart. When uterine tissue fragments were placed into tissue culture the production of muscle subunits increased (16). The increase in oxygen tension reduced the over-all production of muscle subunits (16). Studies of ontogeny of isozymes patterns in the mouse (42) disclose that the major differentiation of

isozyme patterns occurs during the neonatal and juvenile period of life. Initially mouse embryonic tissue is nearly all LDH-5. Those tissues which will ultimately acquire anodal end of the spectrum activity do so by gradual displacement of the LDH-5 band (42). The same observations are noted in mouse liver tissue. Each organ or tissue matures at its own characteristic rate and, as in the case of kidney tissue, the "adult state" is reached before it is revealed in heart tissue (42). It is of interest to note that all human embryonic tissue appears to be predominantly LDH-1 (19). Closely related rodents as the rat, mouse and hamster have muscle type units in the adult liver, contrasted with the adult liver of the sheep, goat, beef and deer, which have almost completely the heart type units (19). Recent investigations have resulted in evidence that variant forms of LDH do exist (9, 10, 13, 47, 105). A variant of human blood LDH was described by Boyer and Fainer (8). The source of the blood sample was a 25 year-old Nigerian male of the Yoruba tribe. Two samples were drawn over a two month interval. Erythrocytes were washed in 0.9 percent NaCl, hemolyzed in water, extracted with toluene, centrifuged and subjected to vertical starch gel electrophoresis. A normal blood sample was used as a control. Electrophoretic patterns revealed the occurrence of bands LDH-1, 2, 3, and 4 as five, four, three and two components each, respectively. In the normal sample isozyme separation did not reveal molecular subunits. The only explanation that can be given is that a mutant allele exists at the genetic locus and produces a variant component (8).

Nance et al. (47) observed a genetically determined variant of LDH in the erythrocytes of four members of two generations of a Brazilian family. Starch gel electrophoretic pattern displayed bands

LDH-2, and 3 as being double, but not band LDH-1. From their observations they have concluded that the mutant and normal heart subunits are not randomly associated in the erythrocyte of their subjects. It was suggested that this phenomenon appears analogous to the non-random association of human hemoglobin polypeptide chains. The lack of random association between the products of the mutant and normal alleles may be due to technique. LDH in human testes have been investigated with a unique form of LDH being observed (9). When the testicular tissue was homogenized followed by centrifuging the supernatant upon fractionation by vertical starch gel an area of major activity appeared between bands LDH-3 and 4. This area of activity was designated as LDH "band X", and was visible in thirteen out of sixteen patients postpubertal. In prepubertal testicular tissue LDH "band X" was not seen. That the presence of "band X" might be an index of male fertility appears a possibility. Goldberg (25) cleverly derived an experiment which would indicate that one individual cell was capable of producing the five isozymes. Human spermatozoa were selected as they represent a homogeneous population of postmitotic cells. The spermatozoa contained five electrophoretically distinct LDH bands. Thus, there seems to be proof that molecular heterogeneity of enzymes is characteristic of the individual cell and not a reflection of heterogeneity of cell types within a tissue. By treating polyacrylamide gel with 0.1 M β-mercaptoethanol, with subsequent electrophoresis, Fritz and Jacobson (23) were able to produce fifteen LDH bands. They postulated that each monomeric subunit of LDH has one molecule of nicotinamide adenine dinucleotide attached to it and that the β-mercaptoethanol acts to detach NAD from only the muscle LDH form. It was presumed there were partial stages of

detachment with the result that the five tetramers are able to achieve fifteen bands. Boyer et al. (10) found that by treating gels with B mercaptoethanol their Nigerian LDH variants were unaltered. When the cathodic chamber contained 10^{-3} M NAD the variant pattern remained unchanged.

Working with mouse tissue Costello (13) found fifteen sub-bands. They appeared as real entities and are not environmental dependent conditions. Multiple sub-bands have been found in wild mice as well as the laboratory mouse (13). Tissue from deer mice (Peromyscus manicutatus) revealed several extra or "ghost" bands anodal to some major bands (81). It was felt (81) that since the "ghost" bands occur inconstantly, they represent in vitro artifacts. The ionic strength of the buffers used in homogenizing tissue appears to have a significant influence on isozyme patterns (68). As the strength of the buffer is decreased bands LDH-5 and 4 tend to merge in the direction of the anode in starch gel. With variation in buffer pH the phenomenon did not appear (68). A word of caution was given by Zondag (107) on the effect of sample storage temperature on LDH isozyme patterns. Data showed a pronounced loss of activity in the slower moving isozyme band LDH-5 and 4 when concentrated tissue homogenates in barbital buffer at about pH of 7.9, were kept overnight at -20° C. There was no apparent loss in activity if the extracts of homogenation were maintained at -20° C. Recent evidence has shown that LDH from human heart muscle is more heat stable than when the enzyme is derived from other tissue (7, 86, 102). Comparative studies have been made on the heat stabilities of crystalline H_4, M_4 and M_2H_2 (22). H_4 remained stable at 60° C. while M_4 became inactive in two minutes at the same temperature. After a few moments at

60° C., 50 percent of the hybrid M_2H_2 was inactivated, but with further incubation no additional loss of activity was noted. Kinetic values relate that the heart subunits can retain enzyme activity even though the muscle subunits have been denatured (22). It has been reported that heart subunits are specifically inhibited in human blood serum by diluted iodine, oxalic acid, and pyridine-3-sulphonate (62), and also heavy metals particularly zinc (92). Warburton et al. (94) in repeating the inhibitory actions of the above mentioned compounds, found the inhibitory properties against not only heart subunits, but on serum LDH irrespective of its source.

(LDH agent)

Riley et al. (75) in 1960 first described a transmissible agent or factor which elevated mouse plasma LDH levels and was found to be associated with transplanted and spontaneous experimental tumors. The transmissible enzymatic "lesion" demonstrated its presence by an increase in plasma LDH of four to five times that of normal control animals (75). This increase in enzyme activity appeared within 48 hours after inoculation (75). Infected plasma, whole blood, tumor extracts or organ extracts from tumor-bearing or leukemic animals will result in transmitting the factor (75). The presence of the agent can be detected by determination of enzymatic activity, but no preceptible pathological change is found in animals where the agent is consecutively transferred from animal to animal. The inoculum with increased dilution will not remain viable and must be allowed to replicate in passage to be effective (75). Riley and Wroblewski (74) reported that the plasma level of LDH approached normal activity with regressed tumors, but after a period of at least a year levels were still elevated four to sixfold

compared to control animals. Radiation-elicited, chemically induced, and spontaneous primary tumors do not generally exhibit evidence of an LDH agent (70, 77). Riley (70) found a virus-tumor synergism in primary tumors which had also received the LDH agent. He inoculated "virus free" tumor-bearing mice with Ehrlich carcinoma-associated virus. The results were more rapid tumor cell growth and an increased blood LDH activity, in excess of what tumor or virus additives would have contributed alone or together. Riley (70) described the five separate phases of the serial plasma LDH curve found associated with many standard transplanted mouse tumors. Phase one is the period of normal LDH levels post inoculation; phase two occurs between 28 and 72 hours after inoculation and is indicated by an abrupt increase of LDH levels; phase three is a plateau reached where host-virus equilibrium is established with the LDH levels five to tenfold over normal values; phase four indicates the synergistic metabolic reaction between virus and tumor with gradual increase in LDH levels; phase five is a decrease in LDH activity, which normally is an indication of the imminent death of the host.

Notkins (54) reported results similar to those obtained by Riley in the virus tumor synergistic action. His experiments consisted of three groups of mice. Group I received LDH agent; Group II received SS (70429) tumor which was free of a transferrable agent; Group III received tumor implants plus LDH agent. The mice inoculated with LDH agent only had increased blood LDH levels of four to fivefold within 72 hours. Mice bearing tumors only had gradual increases in LDH levels which paralleled increases in tumor size. Mice receiving both tumor and LDH agent had LDH activity jump to 88 times that of normal control mice, and 8 times that of tumor mice alone. This synergistic action

was noted when mice received Moloney virus and the Riley LDH agent although no pathological alterations in the disease patterns could be observed (77). The Moloney leukemia virus is normally contaminated with the LDH agent, but by carrying the leukemia virus in rats, the LDH agent is eliminated without loss of the leukemogenic properties of the Moloney virus (52). Tissue extracts from mice infected with the LDH agent can transmit the factor to a new host (75). When enzyme levels were investigated in numerous tissues of infected mice, no significant difference from the normal mice was noted (45). So it appears that increased plasma LDH level cannot be directly correlated to enzyme levels in body tissue. This has led to speculation that the erythrocyte contributes to increased LDH activity (97). It has been calculated that to increase LDH levels by fourfold, approximately 2.2 percent of the total red blood cells would need to be lysed (4). The red cell contains high levels of glutamic-oxalacetic transaminase and malic dehydrogenase. Therefore, one would anticipate high plasma levels of these two enzymes with animals infected with the LDH agent, if the source of LDH is the erythrocyte. Preliminary results indicate that the two enzymes are not elevated in infected mice (4). Mahy _et al._(38) did find an increase in glutamic pyruvic transaminase which followed closely the plasma LDH rise associated with the LDH agent (38). Hematocrits and the rate of erythropoiesis in normal and infected mice following serial bleeding appeared analogous (5). Notkins _et al._(54) has summarized current theories on the source of LDH increase in LDH agent infected mice: 1) the LDH agent destroys, damages, or alters the permeability of the cells; 2) the LDH agent increases enzyme production by altering the metabolism of the cell; 3) the LDH agent may increase

the activity of the already existing enzyme; 4) LDH agent may impair the rate of clearance of an enzyme from plasma.

Properties of the LDH agent

In freezing and thawing the agent five times a slight drop was recorded in the infective titer in one out of three experiments (56). When the LDH agent was treated with 15 percent ether, the results revealed a complete loss of infectivity in as little as 15 minutes at 4° C. (56). The elevation of temperature completely inactivated the LDH agent. At 4° C. for 24 hours the agent was stable, but partial inactivation occurred at 37° C. for 24 hours. Complete inactivation took place at 80° C. for 30 minutes (56).

Estimation of the size of the LDH agent has been reported by a number of investigators (8, 56, 71, 75, 78). Riley (71) estimated the particle size of the virus elevating plasma LDH activity as being 1 to 3 mμ. Notkins (55), in contradiction, found a particle size of about 60 mμ. Enzyme-elevating virus isolated from a mouse bearing sarcoma 37 was passed through Gradocol membranes (78). Membrane of 83 mμ APD allowed the virus to pass through readily. A membrane of 74 mμ passed only a minute amount of virus through, no passage was indicated with a membrane of 67 mμ. Calculation of the Riley LDH virus diameter from the preceding data gives an approximate diameter of 45 mμ (78). Electron microscopy demonstration of the LDH agent by Balden (8) revealed essentially speroidal particles, 69 mμ x 76 mμ in diameter. Adams et al. (1) has found evidence obtained from centrifuging studies that there are at least two particles or groups of particles present in the infected mice. He reports that, if the plasma sample is drawn 15 to 18 hours after injection of the LDH agent, 99.9

percent of the particles are readily sedimentable. Two weeks post inoculation plasma samples contained 50 percent relatively unsedimentable particles. These observations may account for the two different size particles that Notkins (55) and Riley (71) noted. Preliminary findings suggest that the Riley agent is an RNA virus from studies done by Notkins and Scheele (55). In more recent work Notkins (53) treated intact virus in the absence of plasma with ether and recovered an infectious ribonucleic acid which was inactivated by both RNase and normal plasma, but not by DNase. The presence of an LDH virus has been found in wild mice (Mus musculus) (81). Arison et al. (3) in administration of an arsenical to tumor-bearing animals lowered the plasma LDH. This suggested an arsenic-sensitive agent which might contribute to the LDH increase.

Eperythrozoon coccoides is parasitic to erythrocytes (72) which could lead to elevated LDH levels. Riley (72) inoculated mice with both LDH elevating virus and E. coccoides which produced synergism. The synergism is delineated by quantitative change in several blood elements, host anemia, splenomegaly and a marked increase in plasma LDH (72). In splenectomized animals, latent E. coccoides are activated, or allow a new inoculation to have more virulent destructive effect on host erythrocytes (43). It was found that the time required for removal of the enzyme in the virus infected host is approximately double that required in the uninfected animals (43). Studies reveal the presence of the LDH agent in the saliva and urine of mice at 36 hours post inoculation, but not at five weeks after infection (14). Feces also contain the LDH agent as early as 36 hours and as late as 105 days after infection. The fecal material is infectious even after 96 hours

at room temperature (14). Mice are able to infect themselves with the LDH agent by biting an infected animal and receiving the agent orally via the blood of the infected host (57). It, therefore, appears caution must be taken in the experimental situation to reduce the possibility of infecting control animals.

Rauscher virus

In October 1959, Rauscher began studies on a virus induced leukemia of adult random-bred Swiss mice which had been previously reported by Schoolman *et al.* (79). Rauscher (63) produced one lymphocytic tumor in 508 Balb/c mice eleven weeks after inoculation of a filtrate of the Schoolman-Schuartz Swiss mouse lymphoblastoma. This neoplasm appears to be the source of the virus which subsequently was isolated after a period of six months. An extract of the tumor tissue of the ninth serial whole cell transplant produced lymphocytic leukemia in three mice out of forty-six that were inoculated. From tissue of these three mice, Rauscher made a cell-free extract which, when injected into normal Balb/c, virtually produced 100 percent contraction of the disease.

Description of the disease

The latent period before the spleen is palpable following inoculation to different age groups was found to be approximately equal. But, the death rate decreases as the age of the animal increased at the time of inoculation. Macroscopic findings showed easily palpable spleens within 10 to 15 days after inoculation, and mice began to die between 28 and 37 days. These mice generally had spleens which were as high as 30 to 40 fold increase in weight compared to control mice of the same age. The enlarged spleens are spongy in texture and contained enormous sacs of blood. Hepatomegaly was noted with a two to fourfold increase in

weight at time of death. Slight enlargement of the peripheral lymph nodes was seen while the thymus showed no gross enlargement. Survivors of the early mortality peak associated with splenohepatomegaly developed enlargements of the peripheral lymph nodes and the thymus. Spleens were firm and not as enlarged as the early mortality group. A chronic course was followed by the surviving mice and death generally came between 62 and 90 days.

Histological findings

Microscopic findings of various organs revealed: 1) extreme numbers of nucleated erythrocytes and erythroblastic cells in the red pulp of the spleen; 2) replacement of much of the red pulp by recent hemorrhage or older areas of hemorrhage where organization had taken place; 3) many nucleated red cells are seen in the sinusoids of the liver; 4) accumulations of granulocytes in the medullas of the lymph nodes; 5) in those animals which did not develop lymphocytic leukemia the thymus underwent atrophy with a decrease in thymocytes; 6) in the lymphocytic leukemia mice the thymus, lymph nodes and spleens were infiltrated by large mononuclear basaphilic cells. Lymphocytic leukemia cells and an increase in the number of nucleated red cells were seen in the peripheral blood smears of the leukemic mice. Rauscher and his colleagues tested the efficiency of the several routes of inoculation of the virus. Mice inoculated via the interperitoneal or intravenous route had measurable infection in one-third to one-fourth the time taken by mice inoculated by other routes. Electron microscopy examination of diseased tissue gave no evidence that two viruses were present which would have accounted for the two separate phases of the disease. The virus is very stable and has a high leukemogenic potency when frozen at

-70° C. for five months. Rauscher provided data in which he showed that there was no loss of potency of the virus after incubation with rabbit antiserum prepared against the leukemogenic viruses of Friend, Moloney, and Schoolman-Schwartz (63). In a more recent paper Zeigel and Rauscher (103) studied virus particles associated with a virus induced leukemia in mice. Using a technique of negative staining they were able to show by electron micrographs that 80 percent of the particles showed tail-like projections extending from the "head". The head was approximately 120 mμ in diameter and were angular with a hexagonal shape. Zeigel and Rauscher (104) have published the effects of physiochemical treatments on the morphology and histological activity of the virus as depicted by electron microscopic studies. Immunological studies of the virus have been carried out by Fink and Rauscher (21).

MATERIAL AND METHODS

Animals

All mice were female Balb/c/jax and were purchased from the Jackson Memorial Laboratory, Bar Harbor, Maine. Their mean weight was 15.3 g at the beginning of each experimental procedure, and age ranged from four to five weeks. The animals were divided into groups of five or six, and each individual group was housed in a plastic cage. Food was a standard diet of Purina laboratory chow. Individual animals were numbered by a color code system using various dyes to denote number.

Experimental groups

Data incorporated in this thesis was derived from three separate groups of animals. Group I consisted of 60 mice and was inoculated with the leukemogenic virus and at random six animals were killed at weekly intervals for a period of nine weeks. Blood samples were obtained by cardiac puncture, and spleen and liver weights were determined at the time of sacrifice. Concurrent differentials and hematocrits were also done. Groups II and III of mice consisted of 12 and 10 mice respectively. Five mice of the Group III did not receive the leukemogenic virus and served as control animals. The remaining 17 animals were inoculated with the leukemogenic virus. Blood samples were drawn weekly via the suborbital plexus, starting one day before inoculation. Concurrent nucleated blood cell counts, and differentials were also done. Death records were maintained and, when possible, autopsies were performed. In addition to the information provided by the control animals, normal values have been well established from numerous preceding experiments done in this laboratory.

The selection of two different methods for obtaining blood serum

samples was taken to allow: 1) the comparison of LDH activity to spleen and liver weights in the group in which six random mice were killed weekly; 2) serial bleeding provided information pertaining to LDH activity in one individual animal over the course of the disease process.

Virus suspension

Suspension of spleen extracts of virus originally obtained from Dr. Rauscher was maintained by serial passage in Balb/c mice in our laboratory. The virus suspension used as the inoculum was prepared in the following manner: infected mice were maintained until such time as they showed evidence by palpation of gross splenomegaly. The mice were then killed by cervical dislocation and the spleen excised and weighed under sterile procedure. Pooled spleen hemogenates were prepared in an all-glass homogenizer, using Hank's biological balance salt solution with antibiotics added, as the diluent. The homogenate suspension was transferred to a centrifuge tube and centrifuged in an International PR-2 centrifuge at 2500 rpm, and 4° C for a period of twenty minutes. The supernate was drawn off and centrifuged a second time. Diluent was added in proportions to produce a 40 percent spleen extract suspension. The result of the second centrifugation was a cell debris free virus suspension. The 40 percent virus suspension was stored at -70° C. in sealed glass ampules until time of inoculation.

Inoculation of animals

The sixty mice in the Group I received virus suspension from the second passage with the remaining seventeen animals receiving virus suspension from the fifth passage. The mice were inoculated intraperitoneally in the lower right abdominal quadrant with 0.2 ml of a 15 percent virus suspension.

Collection of blood samples

Two methods were employed in obtaining blood samples as previously stated: 1) ether was administered to those animals from which blood samples were drawn by cardiac puncture. While the animal was anesthetized, an incision was made starting at the xiphisternum and extending upward to the sternal notch. The palpitating heart was exposed, and snipped open with a pair of scissors. At the moment the heart was incised, eight to ten micro non-heparinized capillary tubes were thrust into the heart and allowed to fill with blood by capillary action. 2) Mice followed serially were bled weekly by drawing samples from the ophthalmic venous plexus (69). This method could be repeated any number of times, and only rarely was there induced tissue damage or infection. The animal was laid on its side with the head held firmly between the thumb and middle finger. A micro non-heparinized capillary tube was gently placed at the lower or inner corner of the eye and pushed into the ophthalmic venous plexus which lines the back of the orbit. The reservoir formed by the orbital cavity filled with blood as the result of rupturing of the venous capillaries. The tube was filled to within one cm of the exposed end by capillary action. An average of three tubes were drawn per animal. Differential slides were made by placing a drop of blood from the capillary tube on a glass slide and smearing. Although anesthetic is not absolutely necessary, it was found that a larger number of animals could be handled per unit of time if an anesthetic were administered prior to bleeding. The procedure followed for processing the samples was identical for both methods after the whole blood had been collected. The capillary tubes were allowed to clot for a period of fifteen minutes, and then the open ends of the tubes were sealed with

the aid of an oxygen-gas flame.

A Safeguard centrifuge (Clay Adams Inc., New York) equipped with a swinging bucket head was used to centrifuge the capillary tubes. The tubes underwent centrifugation at approximately 1,000 x G for a period of fifteen minutes at room temperature. Hematocrits were obtained at this stage by measuring the volume percent of the packed red cells. The tubes were snapped in two at the serum-erythrocyte interface. The packed cells were discarded and the serum was transferred to a clean capillary tube by placing the new clean and old tubes end-to-end. The two tubes were then held vertically and by gravity, and capillary action, the serum was transferred. Any serum that showed evidence of hemolysis was discarded. The serum was centered in the new tube and immediately both ends were sealed by an oxygen-gas flame. Individual tubes were identified by a code system. The tubes were marked by using a colored felt pencil. The group number was denoted by a corresponding number of dots on the left end of the tube when the tube was held in a horizontal position. The individual animal number was indicated by a single dot, which was located the same number of centimeters from the left end of the tube as the animal number it represented. One complete week of samples was placed in a single glass tube and stored at -10° C. until used for assay.

<u>Total LDH assay</u>

Current assay methods for the determination of LDH in general are based on the change of the optical density of the oxidizable-reducible coenzyme $NADH_2$ and NAD (nicotinamide adenine dinucleotide) as measured at 340 mμ, and the colorimetric measurement of the intensely colored "hydrozone", formed when pyruvic acid reacts with 2, 4-Dinitrophenylhydrazine, in the wave length range of 400 to 555 mu. While investi-

gating various methods for measuring serum LDH it was found that no single method was entirely suitable for our use. It was desirous to obtain information derived from a number of different analytical procedures performed on a single serum sample. Under these conditions the amount of serum which may be allotted to each individual assay became a critical factor. Since repeated serial bleedings could introduce an experimental anemia with subsequent erythropoiesis, we thought it feasible to keep the quantity of the sample to a minimum. To achieve this micro-assay, a modified Lowry micro-chemical procedure was adapted to measure total enzyme activity (37). In this procedure, use is made of the principle that the reduced form of the coenzyme nicotinamide adenine dinucleotide or $NADH_2$ has native fluorescence when excited by a source of ultraviolet light, while the oxidized form, NAD, exhibits negligible native fluorescence. We were able to measure LDH activity in the presence of pyruvate.

The spectrophotometric assay measuring the oxidation of $NADH_2$ in the presence of pyruvate, and the colorimetric method both require a pre-incubation period prior to taking of optical density readings. This step is not necessary with the fluorometric method. The spectrophotometric and colorimetric methods require concentrations of the coenzyme far in excess of that essential for fluorometric analysis. Thus, a substantial reduction in the cost per assay is realized when working with large number of samples. Lowry (37) measured LDH activity in a single nerve cell body (less than 0.01 µg of tissue, dry weight) which exemplifies the extreme sensitivity of fluorometry when applied to enzymology. He (36) states that the use of fluorometry has not been "exploited as much as it deserves, particularly for micro-chemical

application...It is inherently much more sensitive than colorimetry, since the emitted fluorescence is directly related to the substance measured and the illumination...Fluorometry also may be used over a much wider concentration range than colorimetry...Thus colorimetric readings are ordinarily restricted to the range of 5 to 90 percent transmission, a twenty fivefold concentration range, but fluorometric measurements may be satisfactory over a 1000 to 10,000-fold range."

Reagents for assay

1) Tris buffer (pH 7.4 and pH 8.7, 0.1 M). The tris buffers are prepared from Tris (hydroxymethyl) aminomethane (Sigma Chemical Company, Trizma base, reagent grade) using glass distilled water. The pH is adjusted with 0.1 N HCl. Trizma base is utilized for it is low in fluorescent impurities. 2) Nicotinamide adenine dinucleotide ($NADH_2$) solution. Pipette 2 ml of tris buffer, pH 8.7 into a vial containing 2 mg of $NADH_2$ (Sigma Chemical Company, Grade III, approximately 96 percent purity). 3) Bovine serum albumin (10 percent) solution. In a 25 ml volumetric flask place 2.5 g bovine serum albumin and dilute to 25 ml with glass distilled water. This solution is stable for many months under refrigeration. 4) Nicotinamide (9.8×10^{-1} M) solution. In a 10 ml volumetric flask place 1.2 g nicotinamide and dilute to 10 ml with glass distilled water. This solution is stable under refrigeration. 5) Sodium pyruvate (4.58×10^{-3} M) solution. To a 50 ml volumetric flask dissolve 2.52 mg of sodium pyruvate (Sigma Chemical Company, type II) with glass distilled water to make 50 ml. This solution is freshly prepared for each series of sample runs.

$NADH_2$ standardization

The $NADH_2$ is received from the Sigma Chemical Company in a

deliquescent state. To prepare for use 2 ml of tris buffer, pH 8.7 is added to the vial containing 2 mg of $NADH_2$. To obtain the exact concentration of $NADH_2$ in the solution, spectrophotometric measurements were taken. To one of two matched 2.5 ml capacity quartz cuvettes 150.9 microliters of $NADH_2$ solution was added followed by 2.0 ml of tris buffer, pH 8.74, 0.1 M. To the second cuvette which is used as the standard blank, 2.0 ml of tris buffer, pH 8.74, 0.1 M is added. Readings were made on a Beckman D. U. spectrophotometer. The spectrophotometer was first standardized with the blank cuvette at a wave length of 340 mμ, followed by taking the initial reading of the sample cuvette. To both cuvettes 402.4 microliters of 5 N HCl was then added. The spectrophotometer was restandardized and a second reading was taken of the sample cuvette and recorded. The second reading was subtracted from the initial reading with the resulting value indicating the drop in optical density of the $NADH_2$ solution due to the enzymatic denaturing by the HCl. A value of 627 was used as the extinction coefficient (33). The following equation was used to evaluate the molar concentration of the $NADH_2$ in the sample being tested.

$$\frac{\Delta \text{ O.D.}}{\epsilon} \times 0.1 \times \frac{\text{total volume in sample cuvette}}{\text{volume of } NADH_2 \text{ added to cuvette}}$$

Δ O.D. = change in optical density

ϵ = extinction coefficient

Assay procedure

All glassware was rinsed with glass distilled water to reduce contamination by fluorescent impurities. The assay reagents were premixed with 1 ml of reagent mixture needed for each sample run. To a flask of suitable size for the number of anticipated sample runs, the following reagents are pipetted: 1) 5 ml of tris buffer, pH 7.4; 2)

21.7 microliters of nicotinamide solution. The reagent mixture flask was covered with aluminum foil to reduce the decomposition of the $NADH_2$ as the result of exposure to light. The flask was placed in ice for the duration of the sample runs.

Using volumetric pipettes the following reagents were added to a fluorometric sample tube with sample tubes matched according to inherent fluorescence: 1) 1 ml of reagent mixture; 2) 8.6 microliters of sodium pyruvate solution. The final substrate concentration in the sample tube was 5.4×10^{-6} mM of $NADH_2$, and 3.94×10^{-5} mM of sodium pyruvate. The sample tube mixture was mixed thoroughly by a mechanical vibrator. The sample tube was wiped clean, placed in the fluorometer and standardized by setting the galvanometer (0 to 100 scale) to a reading of 100. This setting indicated 100 percent fluorescence for the total concentration of $NADH_2$ in the sample tube. The run was started by the addition of 1 or 2 microliters of blood serum, then immediately the sample tube was vibrated, wiped clean, and placed in the fluorometer. Readings were taken at set time intervals of thirty seconds for a period of two minutes. A drop of one unit on the galvanometer scale represented oxidation of one percent of the total sample $NADH_2$ to NAD.

Measurements were made in a Farrand fluorometer (Farrand Optical Company, New York). Primary filter used with the fluorimeter was #5840 Corning while the secondary filters were Corning #4308 (next to incoming light source), and Corning #3387 (positioned away from incoming light source).

Starch and acrylamide gels

Both starch gel and acrylamide gels were used to elicit LDH isozyme patterns. Characteristics which distinguished gel electrophoresis from

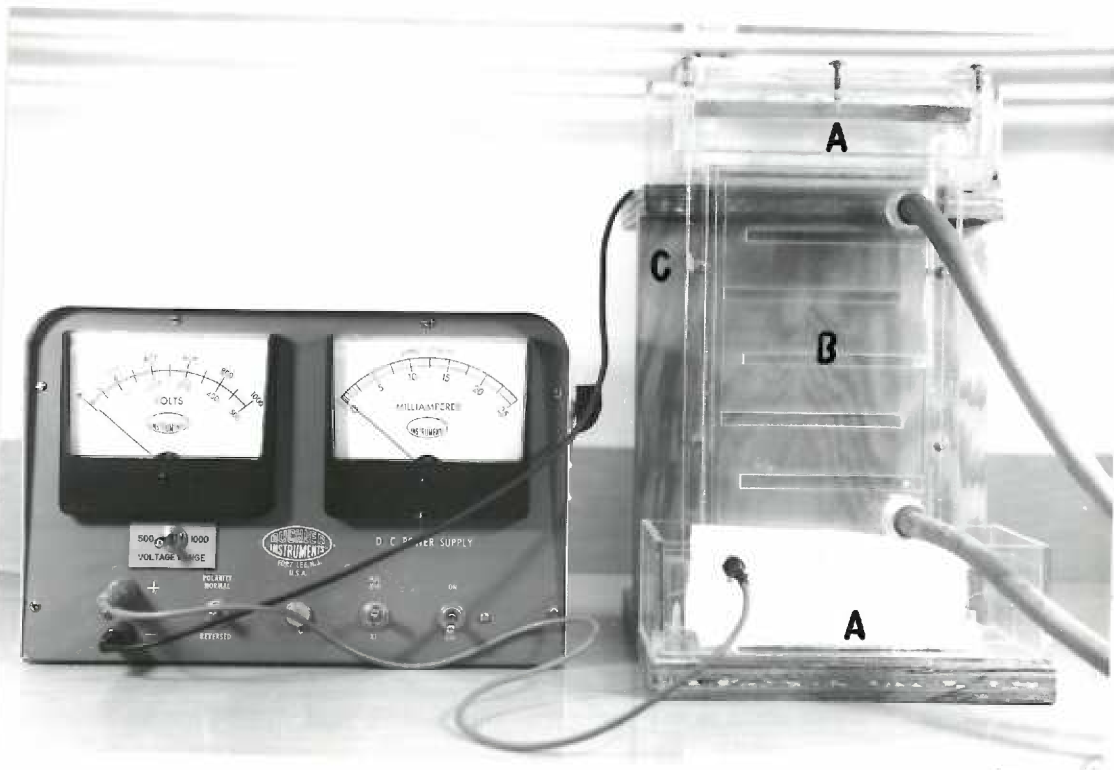
other techniques made it especially useful in the analysis of protein. Raymond (64) consideres gel electrophoresis as having a "sieve" effect. In filter paper electrophoresis, a gaseous film is present around the supporting material; with the gel system the migrating ions must pass through the gel matrix (85). Poulik and Smithies (61) showed the comparisons of the mobilities of proteins of known size in starch gel and on filter paper. Results indicated that starch gel hinders migration of larger proteins more than smaller proteins. Smithies (85) postulated that the molecular chains of the gel are not a static network, and thermal motion caused the distance between neighboring chains to constantly vary. Raymond *et al.* (67) cautions that "methods of preparation, such as the degree of hydrolysis of the starch or variation in the synthetic starting materials of acrylamide gel, can significantly effect the gel structure and therefore also the electrophoresis patterns produced in the gel".

A modification of the apparatus designed by Smithies (84) was constructed in our laboratory by this writer. This apparatus (Figure 1) consisted of acrylic plastic water cooled mold with removable end plates, a pressure plate, two baffled electrode chambers with platinum electrodes, and a supporting stand with shelves for the electrode vessels. Direct current electrical power source was provided by a Beckman Model RD-2 Duostat power supply.

Procedure for starch gel electrophoresis

43.2 g of hydrolyzed starch, purchased from Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada, were added to a standard one liter, round-bottom, Pyrex flask containing 400 ml of pH 8.6 buffer. The buffer was prepared by the addition of

(Figure 1). Pictured is the vertical starch gel apparatus constructed in this laboratory and used to elicit isozyme patterns. Seen are the two baffled electrode chambers with platinum electrodes (A), the water cooled mold with removable end plates (B), and the supporting stand with shelves for the two electrode chambers (C).


A
C
B
VOLTS
MILLIAMPERES
INSTRUMENTS
A

1) 8.06 g of tris; 2) 0.8 g of EDTA; 3) 0.6 g of boric acid, and brought up to 400 ml with distilled water (20). Continual mixing by swirling was carried out as the suspension was heated over a naked flame. With increased temperature the suspension turned semi-solid. Heating was continued until the semi-solid mass became a viscous liquid. Degassing was then conducted by applying a negative pressure with a vacuum aspirator until the contents of the flask boiled vigorously, and the liquid was then poured into the mold with the end plates in place. The gel was allowed to cool to room temperature. A razor blade was used to cut a slit across the full width of the gel, a distance of one-third its length, from the cathode end. Eight serum samples were inserted in the slit by placing each sample on individual four thickness (4 mm x 4 mm) Whatman No. 1 filter paper wicks. The wicks were placed an equal distance from each other. At this stage, a sheet of household plastic wrap was placed over the exposed surface of the gel, and then the pressure plate was applied. The end plates of the mold were removed and the mold stood upright in the lower anodal buffer vessel on cut strips of Whatman No. 1 filter paper. Electrical connection with the upper cathodal vessel is made by a flannel wick and were held by refastening the end plate over the wick. The electrode vessels were then filled with electrode buffer, which consisted of: 1) 30 ml of 0.2 M citric acid; 2) 240 ml of 0.2 M Na_2HPO_4; and 3) 1230 ml of distilled water. The resulting pH is 7.0 (20). Separation was conducted with continuous flow of cold tap water through the water jacket for a period of up to sixteen hours, at 190 volts.

Staining procedure

Detection of LDH activity following electrophoresis was accomplished

by staining the gel with a nitro-blue tetrazolium-substrate solution. LDH was indicated in the gel by discrete purple spots, whose size and color intensity were proportional to enzyme activity. The staining technique employed was a modified Dewey and Conklin (17) procedure (20). The staining reaction appeared to take place in the following manner: when LDH catalyzed lactate to pyruvate in the presence of NAD, the NAD was reduced to $NADH_2$. At this stage, phenazine methosulfate transferred electrons from the reduced coenzyme to nitro blue tetrazolium with the subsequent conversion to formazan which precipitates as a purple stain.

Reagents used in the staining procedure

1) Tris buffer, pH 8.0, 0.1 M, 26.5 ml; 2) Lithium lactate, 2.0 M, 1.5 ml; 3) Phenazine methosulfate (PMS), 5 mg per ml, 0.12 ml; 4) Nitro blue tetrazolium (NBT), 10 mg per ml, 1.0 ml; 5) NAD, 30 mg per ml, 0.6 ml (20).

Acrylamide gel

Acrylamide gel has been utilized recently in studies of isozyme sub-bands (23). The acrylamide gel was found to have some distinct advantages when compared to starch gel in the ease of preparation and handling. Time required to prepare starch gel for electrophoresis is two to three hours, while acrylamide gels are ready to accept samples in less than one hour. Acrylamide gel is able to stand mechanical trauma following removal from the electrophoretic cell which would fragment the starch gel slab. Duration of acrylamide gel runs an average of three to three and one-half hours, while a comparable run with starch gel requires as long as sixteen hours. It was for these reasons, as well as the anticipation that the acrylamide procedure would produce results

which would substantiate data obtained from starch gel, that the second procedure was enlisted.

Reagents required for acrylamide procedure

1) Electrolytic tris buffer, pH 9.2. The buffer was prepared by the addition of 30.0 g of trishydroxy-methylamino methane; 3.0 g of EDTA; 1.14 g of boric acid. The volume was then brought up to three liters with distilled water. 2) The acrylamide gel was prepared by the addition of 6.5 g of acrylamide (Cyanogum 41, American Cyanamid Company, Wayne, New Jersey); 0.26 ml of TEMED (N, N, N^1, N^1 tetramethyl ethylenediamine); 0.26 g of ammonium per sulfate; 127 ml of tris buffer (same as electrolytic buffer).

Procedure for polyacrylamide electrophoresis

The vertical electrophoresis cell used (E-C Apparatus Corp., Philadelphia, Pa.) consisted of two cooling pressure plates which formed the gel mold and a lower and upper electrode compartment. Slots were premolded in the gel slab to accept samples in liquid form; samples were applied beneath the surface of the buffer by means of a micro-capillary pipette. The gel was formed by mixing all reagents except the catalyzing agent ammonium per-sulfate which was rapidly dissolved in the Cyanogum immediately prior to pouring in the electrophoresis cell which was in the horizontal position. Tap water was run continuously through the cooling jackets during the gel forming operation and electrophoresis run. After a period of approximately fifteen minutes the acrylamide had gelled, and the slot former was removed. The cell was placed in the vertical position, and the electrode buffer was poured into the two electrode chambers. A pump was attached to circulate the buffer from the lower chamber to the upper chamber where it completed

the cycle by flowing back to the lower chamber via a buffer overflow tube. Power was applied to the system to equilibrate the gel. At this time the samples were applied to the gel slots. A power setting of 270 volts at 90 ma was maintained for three hours. The gel was removed from the cell, and stained, utilizing the same reagents and procedure as that for starch gel. The isozyme separation results were quantitatively revealed by densitometer readings (Hromoscan densitometer, Joyce, Loebl and Company, Ltd., Gaeshead, England).

Micropipettes used in the total enzyme fluorometric assay procedure were purchased from Microchemical Specialties Company, Berkeley, California. Volumes employed in enzyme determinations were based on pipette calibrations provided by the vendor. In attempting to duplicate the assay procedure it is not necessary to use identical pipettes as those employed in this thesis. For critical measurements pipettes should have a variance no larger than $\pm$ 5 percent. It would be important for reproducibility that the final reagent concentrations in the sample run have a variance no larger than $\pm$ 5 percent.

RESULTS

Hemoglobin and nucleated cells

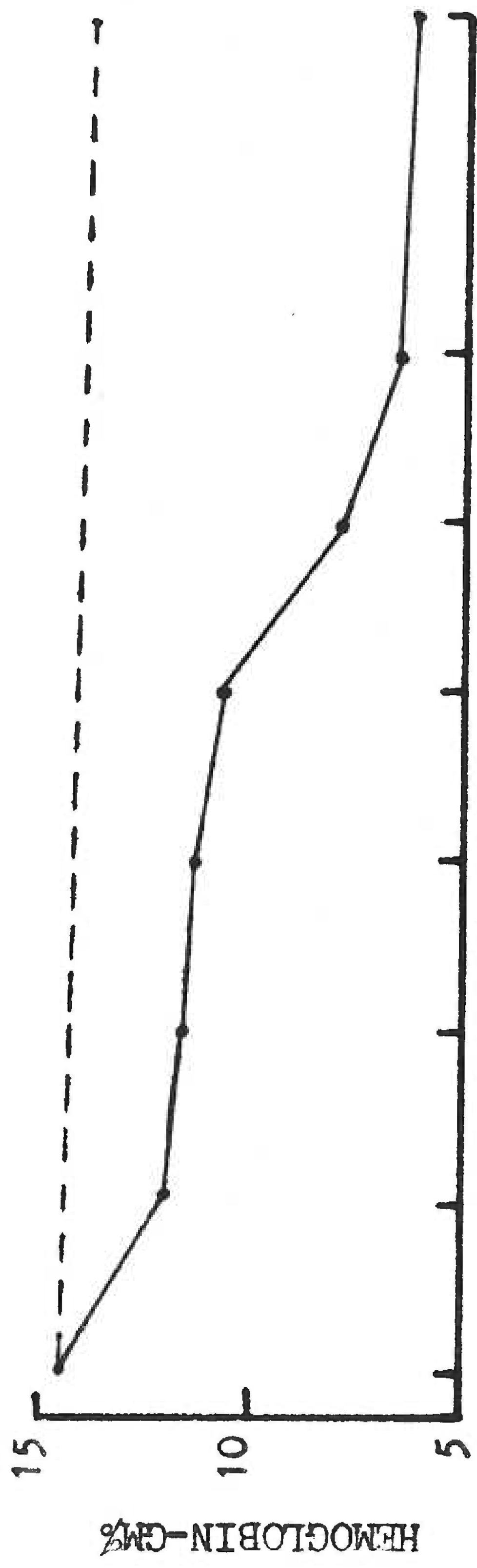
In the infected animals there was a marked decrease in hemoglobin values with time (Figure 2). This anemia could be observed grossly near the terminal stages of the disease. Blood drawn during orbital bleeding had a pale red hue, with a readily seen drop in normal blood viscosity. This anemia may be due to: 1) a deficiency in the production of red blood cells; or 2) increased loss or destruction of red cells. Evidence from bone marrow smears support the latter proposition as the probable cause for the anemia. The bone marrow is markedly hyperplastic with this hypercellularity predominantly due to increased erythropoesis associated with increased numbers of prorubricytes, and maturing erythrocytes. In this same group (Figure 3) of animals nucleated cell counts were determined by a Coulter electronic cell counter. Results show a progressive increase in total nucleated cells with time in the infected animals. If results from Figures 2 and 3 are compared an inverse relationship between hemoglobin and total nucleated cells is observed. The drop in total nucleated cell counts between day 42 and day 56 is apparently due to data derived from animals that were more resistant to the leukemogenic virus, thus enabling them to survive to day 42.

LDH activity and nucleated cells

In Figure 4 LDH activity of one representative animal is compared to normal LDH activity values of the control group. There is a progressive increase in serum LDH activity with duration of the infection. Activity is expressed in micromoles of $NADH_2$ converted to NAD per ml of blood serum per minute. The LDH activity in the infected animal rose by a factor of 10.7 during the course of seven weeks. It was

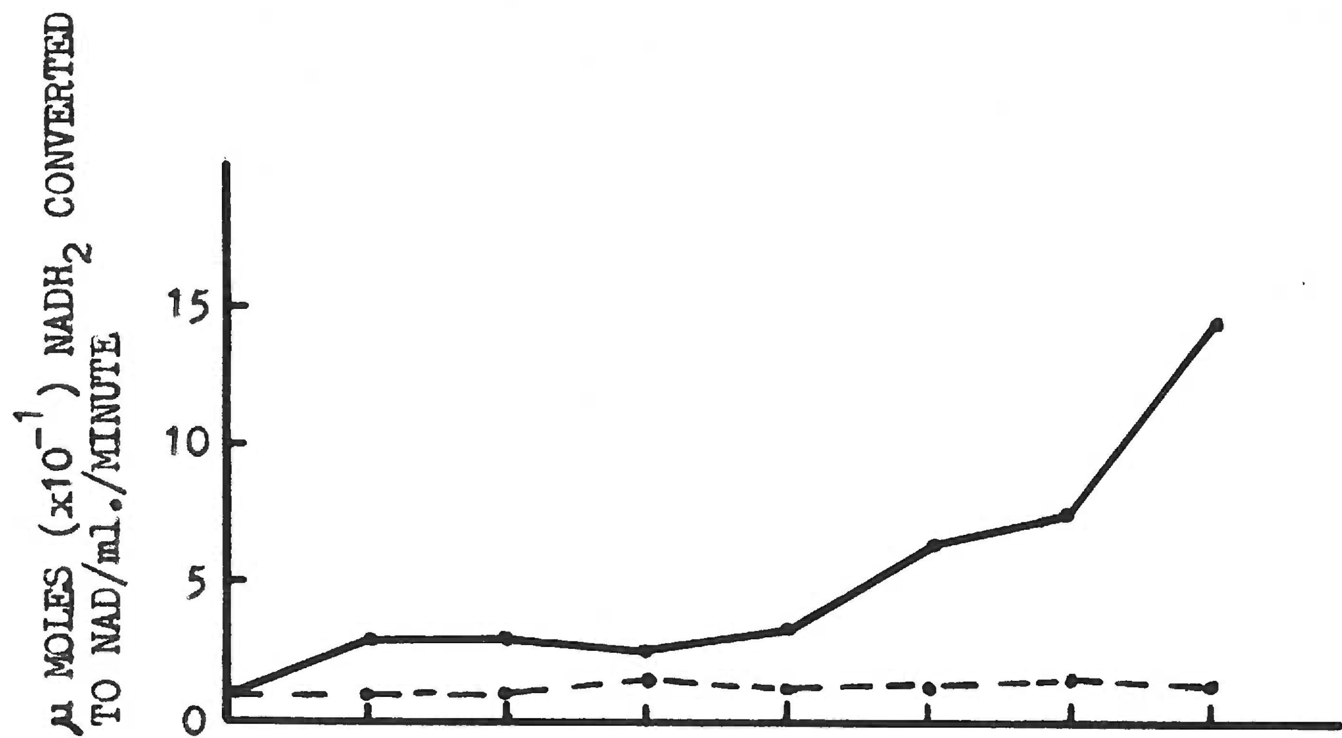
(Figure 2). Infected and control animals hemoglobins were assayed by the cyanmethemoglobin method in a photometer. A marked decrease in hemoglobin is seen with time following inoculation. Each point represents a mean value of eight mice. The solid line indicates the infected mice, with the broken line representing normal values. This data was derived from a previous experiment done in this laboratory (82).

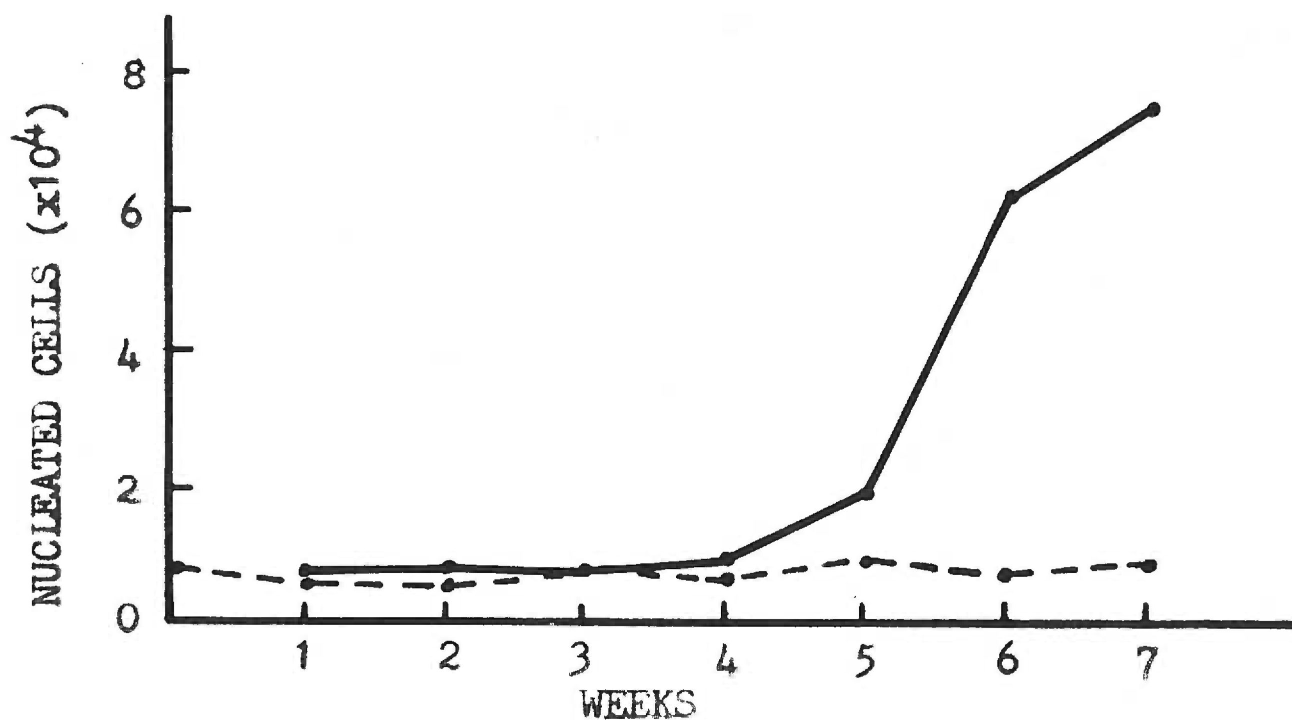
(Figure 3). Total nucleated cells were determined by a Coulter electronic cell counter. An abrupt increase in nucleated cells is seen the fourth week following inoculation. Each point represents a mean value of eight mice. The solid line indicates the infected mice, with the broken line representing normal values. This data was derived from a previous experiment done in this laboratory (82).


HEMOGLOBIN-GM%
15
10
5
NUCLEATED CELLS (x10⁴)
12
8
4
0
0
7
14
21
28
35
42
56
DAYS

(Figure 4). In Group III, animals were individually followed at weekly intervals, and there was an increase in serum LDH activity with duration of the infection. A representative infected animal is contrasted with the mean values of the control group. The LDH activity in the infected animal rose by a factor of 10.7 during the course of seven weeks. The solid line indicates the infected animal, with the broken line representing normal values.

(Figure 5). The nucleated blood cell counts of the infected animal that was depicted in Figure 4 is compared with normal values. A close parallel appears to exist between LDH levels and total nucleated cell counts. The solid line indicates the infected animal, with the broken line representing normal values.


μ MOLES ($x10^{-1}$) $NADH_2$ CONVERTED TO NAD/ml./MINUTE
15
10
5
0


NUCLEATED CELLS ($x10^4$)
8
6
4
2
0
1
2
3
4
5
6
7
WEEKS

found that there was a wide range of variation between individual animals in respect to LDH activity at any given stage of the infection. In Figure 5 the mean nucleated cell counts of the control group is compared to the nucleated cell count of the same representative animal as depicted in Figure 4. The nucleated cell count took a sharp rise after the fourth week of the infection. It can be seen that a close parallel exists between LDH levels (Figure 4) and total nucleated cell counts (Figure 5).

Hematocrit values and LDH activity

At the time of sacrifice of group I, the percent of packed red blood cells was determined. It was observed that when total LDH levels were increased above normal values, in general a drop in the hematocrit could be seen (Figure 6). Statistical evaluation of the hematocrits compared to levels of serum LDH showed a correlation coefficient of 0.94 with a probability of chance of less than 0.001.

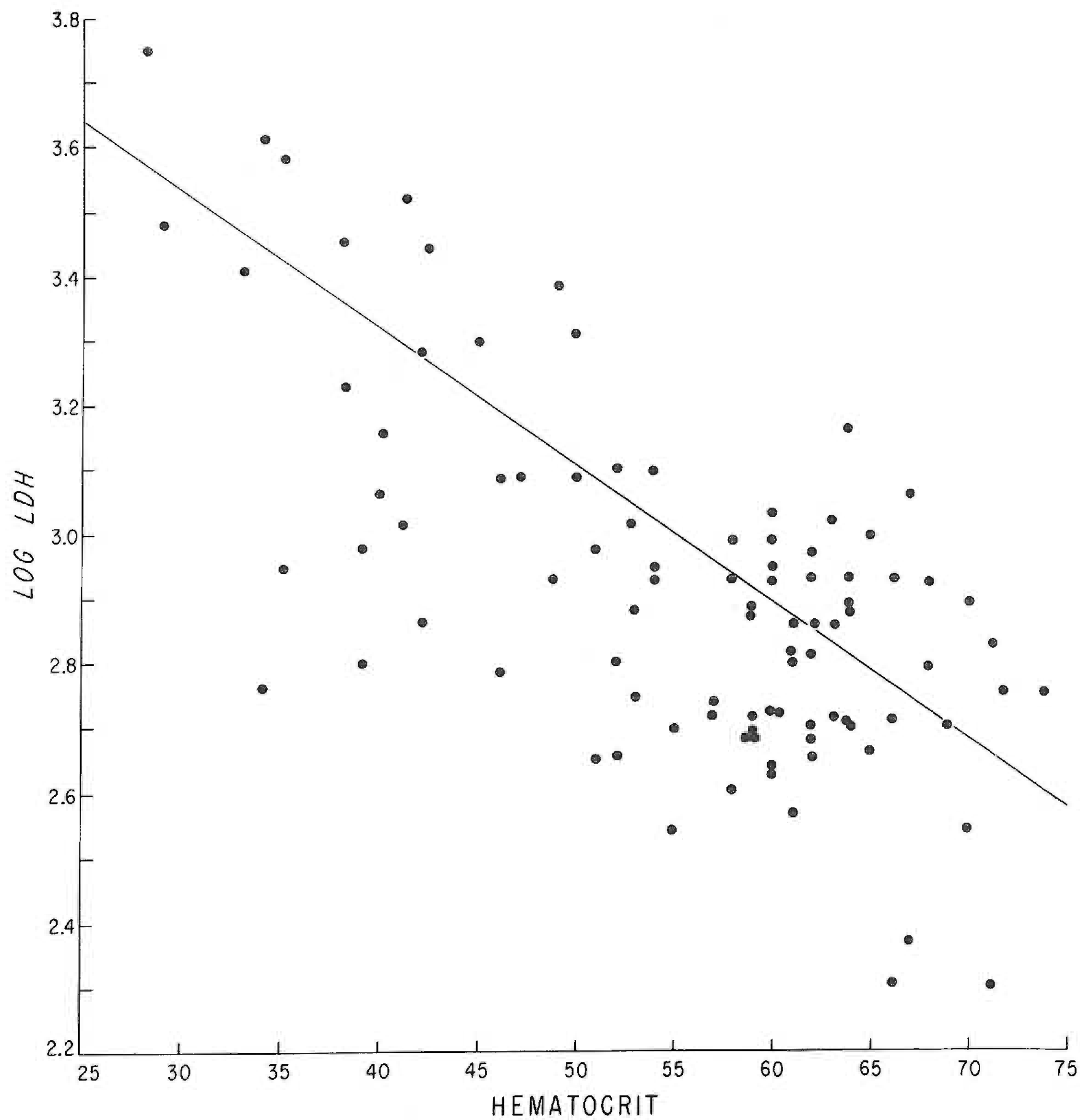
LDH activity and spleen to body weight ratio

When the normal spleen weight to body weight ratio was determined, results gave a range of 4.3×10^{-3} to 6.5×10^{-3}, with a mean value of 5.5×10^{-3}. This ratio was found to be as high as 208×10^{-3} in the infected mice (Table 1). If LDH activity is plotted against these ratios (Figure 7), increased LDH activity is seen to be coterminous with increased spleen weights. Statistical evaluation of the spleen to body weight ratio and absolute values of LDH revealed a correlation coefficient of 0.79 with a probability of chance of less than 0.001.

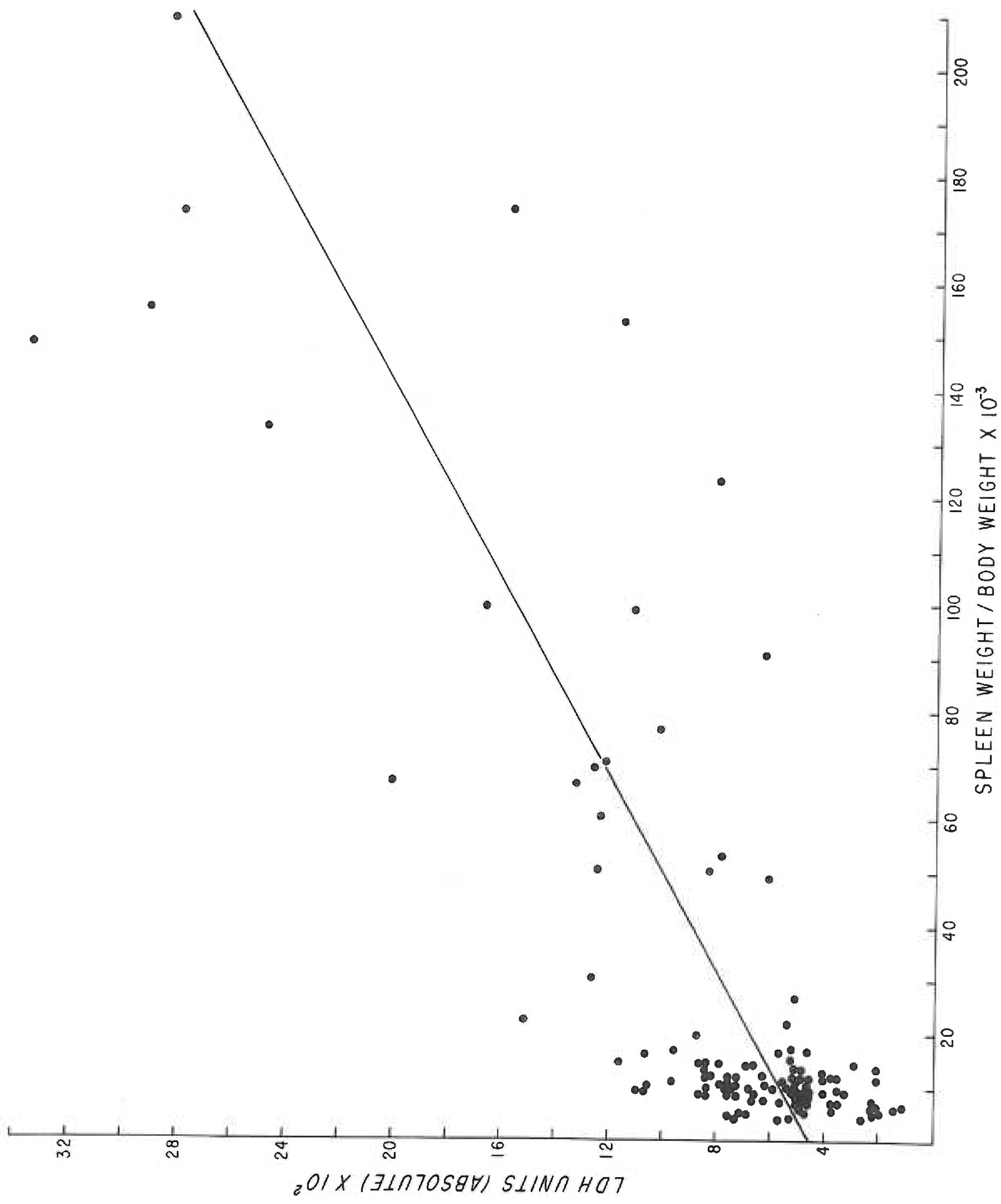
Liver and spleen weight

Livers at autopsy were enlarged in the infected animals and often showed evidence of necrosis by being pale in color and soft in consistency. It is of interest to note, (Figure 8), the direct parallel

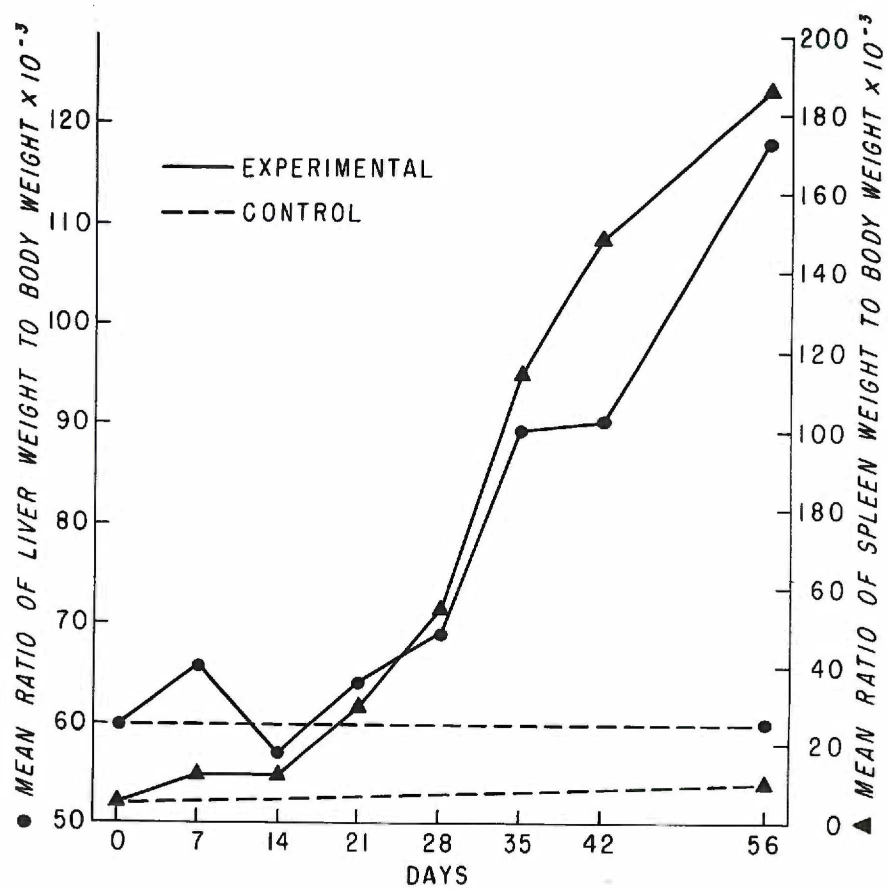
(Figure 6). Percent of packed red blood cells or hematocrit was measured at the time of orbital bleeding. The log of LDH activity is compared to the hematocrit in those mice killed at weekly intervals following inoculation. As the LDH activity increased the volume percent of packed red blood cells diminished.


3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
LOG LDH
25
30
35
40
45
50
55
60
65
70
75
HEMATOCRIT

(Figure 7). Spleen and body weights were recorded at time of sacrifice. When normal spleen weight to body weight ratio was determined, results gave a mean value of 5.5×10^{-3}. This ratio was found to be as high as 208×10^{-3} in the infected mice. If LDH activity is plotted against these ratios, increased LDH activities are seen to be coterminous with increased spleen weights.


LDH UNITS (ABSOLUTE) X 10²
SPLEEN WEIGHT / BODY WEIGHT X 10⁻³
4
8
12
16
20
24
28
32
20
40
60
80
100
120
140
160
180
200

(Figure 8). Hepatosplenomegaly was present in the infected mice. The direct parallel which exists between increased spleen and liver weights during the course of the disease is graphically illustrated. Livers often showed evidence of necrosis grossly by being pale in color and soft in consistency.



● MEAN RATIO OF LIVER WEIGHT TO BODY WEIGHT x 10⁻³
120
110
100
90
80
70
60
50
EXPERIMENTAL
CONTROL
0
7
14
21
28
35
42
56
DAYS
200
180
160
140
120
100
80
60
40
20
0
▲ MEAN RATIO OF SPLEEN WEIGHT TO BODY WEIGHT x 10⁻³

which exists between increased spleen and liver weights during the course of the disease.

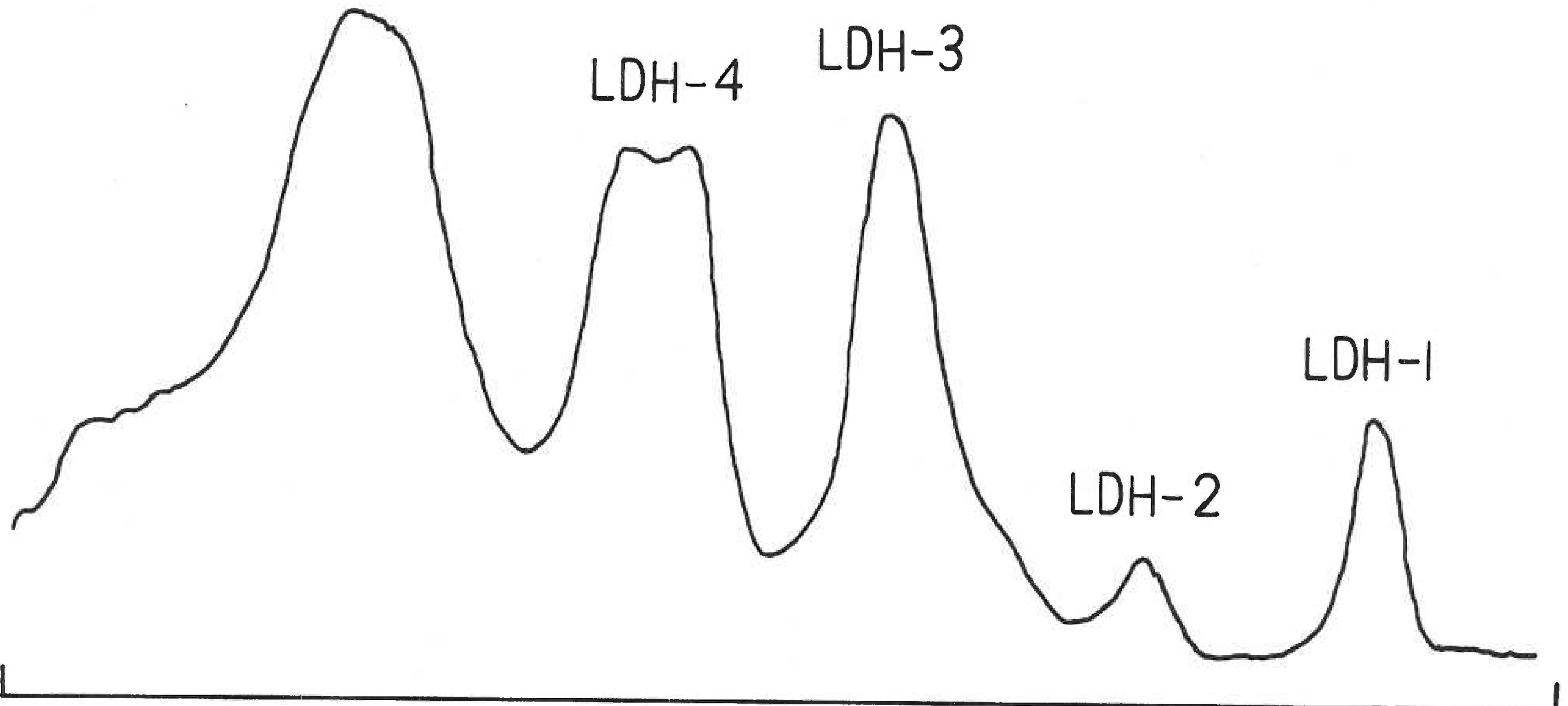
Isozymes

Isozyme studies were undertaken to determine the relative concentrations that the individual isozyme made toward the total increase in LDH activity in the disease state. In Figure 9 is an example of the normal mouse isozyme pattern as produced by acrylamide gel electrophoresis and evaluated by means of a densitometer. LDH bands 5 contributes the largest proportion of the total enzyme activity with the other isozymes LDH-4, LDH-3, LDH-1, and LDH-2 (listed in descending order of activity) completing the total serum activity. There is a definite change in the LDH pattern in the infected animal. As seen in Figure 10, the infected animal maintains the five isozyme bands, but LDH bands 5, 4 and 3 increase in relative activity to LDH bands 1 and 2. It therefore appears that LDH bands 5, 4 and 3 are quantitatively responsible for the major increase in the total LDH activity. By serially bleeding individual mice, the isozyme pattern may be followed during the course of the infection. In Figure 11 a graphic reproduction from starch gel is seen representing isozyme results of a serial bleeding, from the first week following inoculation through the eighth week of the infection. Starting with the fourth week, the major increase in total LDH activity is seen to be contributed by LDH bands 5, 4 and 3. During the eighth week a drop in LDH activity of LDH bands 5, 4 and 3 is evident. It is of interest to note that a drop in nucleated cells is often seen during this same stage of the infection (Figure 3).

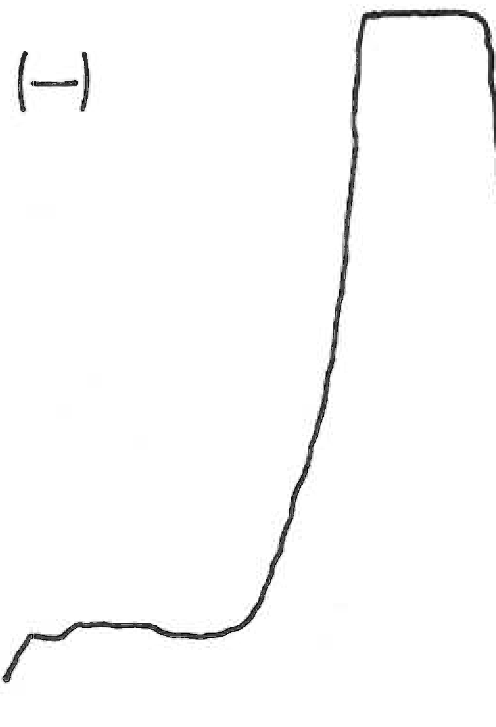
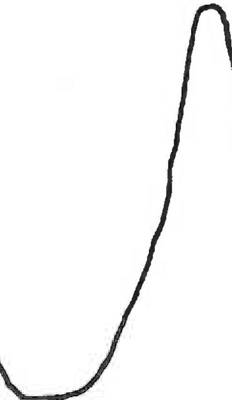
Hematology

Blood smears were taken at time of orbital bleeding. Examples of

(Figure 9). This figure illustrates the distribution of isozymes in the normal mouse. Five bands are seen: the pure muscle, or LDH-5 migrating towards the cathode, and the pure heart muscle isozyme of LDH-1 migrating toward the anode with the three hybrid forms located between the parent forms. The area under each individual curve denotes relative quantitative amounts.

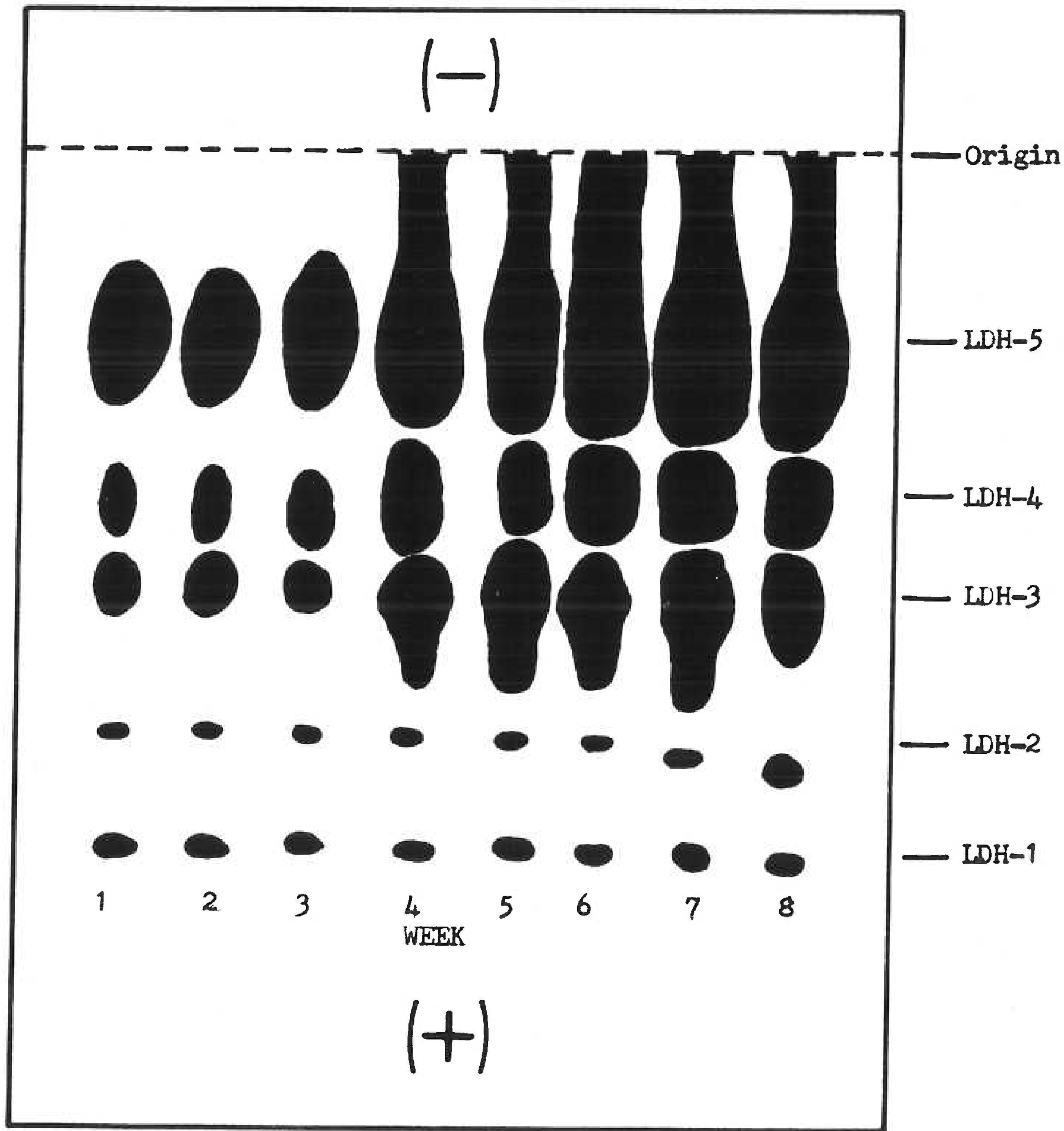

(−)
(+)
LDH-5
LDH-4
LDH-3
LDH-2
LDH-1
42

(Figure 10). In the infected mouse LDH bands 5, 4 and 3 are seen to be quantitatively responsible for the major increase in total LDH activity when contrasted with the normal mouse as seen in Figure 9. The rate of migration of individual isozymes appears not to be altered by the pathogenic process.

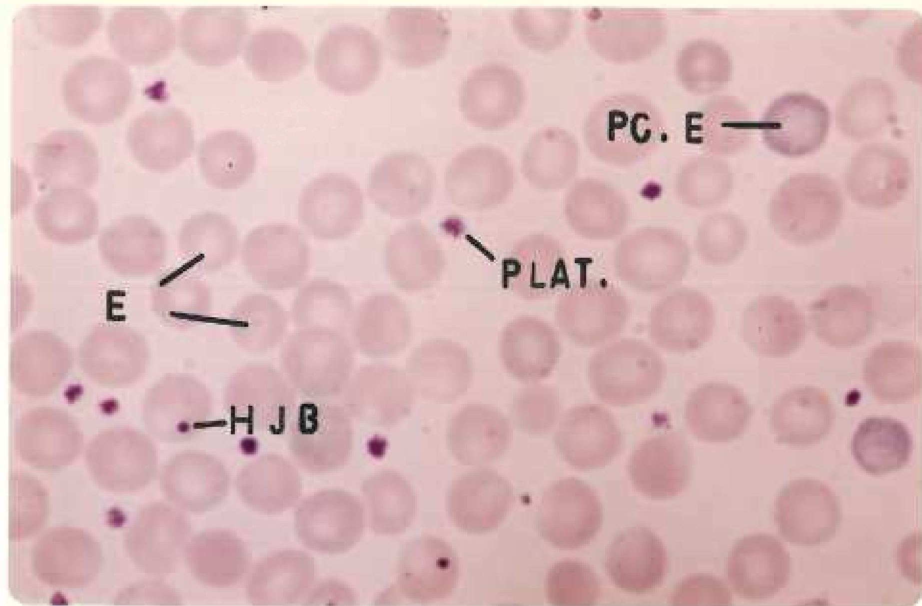
(−)
LDH-5
LDH-4
LDH-3
LDH-2
LDH-1
(+)

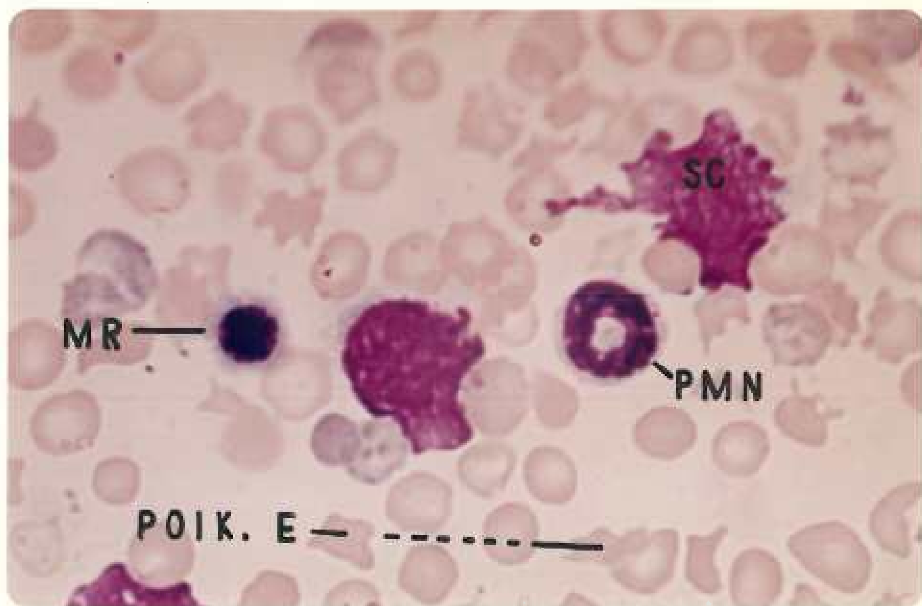
(Figure 11). In this figure a graphic reproduction from starch gel is seen representing the results from electrophoresis of eight weekly serial blood serum samples. Commencing with the fourth week after inoculation, a conspicuous increase in the activity of LDH bands 5, 4 and 3 is seen, while LDH bands 1 and 2 remain normal. Electrophoresis was carried out for a period of sixteen hours at 6 volts per running cm of gel or a total of 190 volts.


(−)
Origin
LDH-5
LDH-4
LDH-3
LDH-2
LDH-1
1
2
3
4
5
6
7
8
WEEK
(+)

(Figure 12). Normal buff colored mature erythrocytes (E); polychromatophilic erythrocyte (Pc. E); Howell-Jolly body (HJB); and normal platelets dispersed between these cells (Plat).

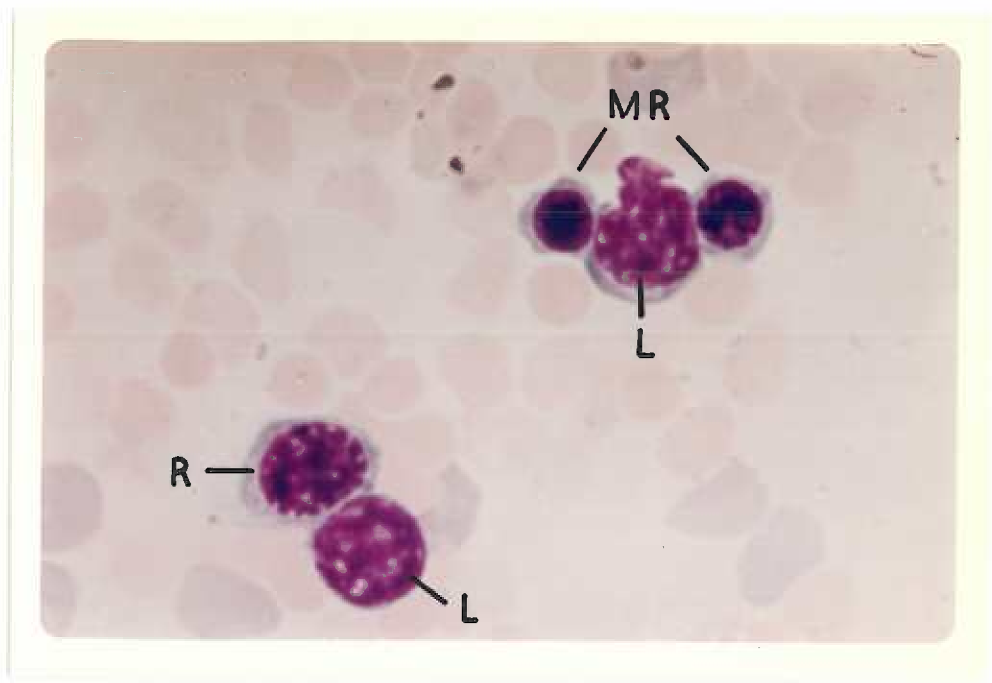
(Figure 13). A basophilic metarubricyte (MR); two smudge cells (SC); a ring form poly-morphonuclear neutraphil (PMn); erythrocytes with polychromatophilia and poikilocytosis (Po.K.E.).

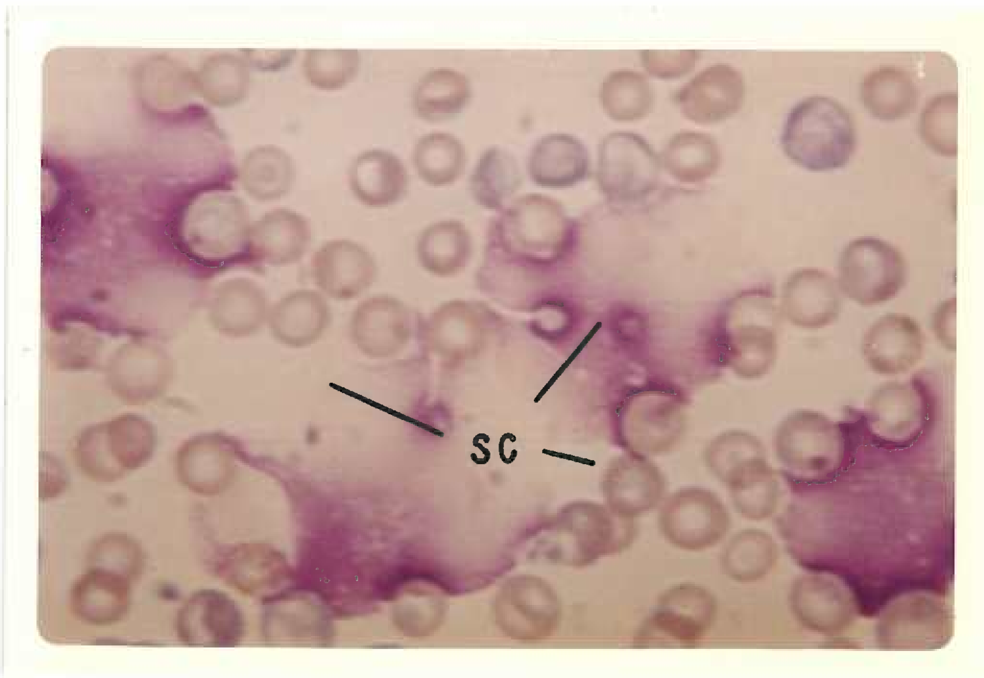

PC. E
PLAT.
E
HJB


SC
MR
PMN
POIK. E

(Figure 14). A rubricyte (R) and two metarubricytes; one mature lymphocyte with damaged nucleus, and a band lymphocytic nucleus.

(Figure 15). Four large smudge cells (SC) found in large numbers in the advanced stages of the disease.


MR
L
R
L

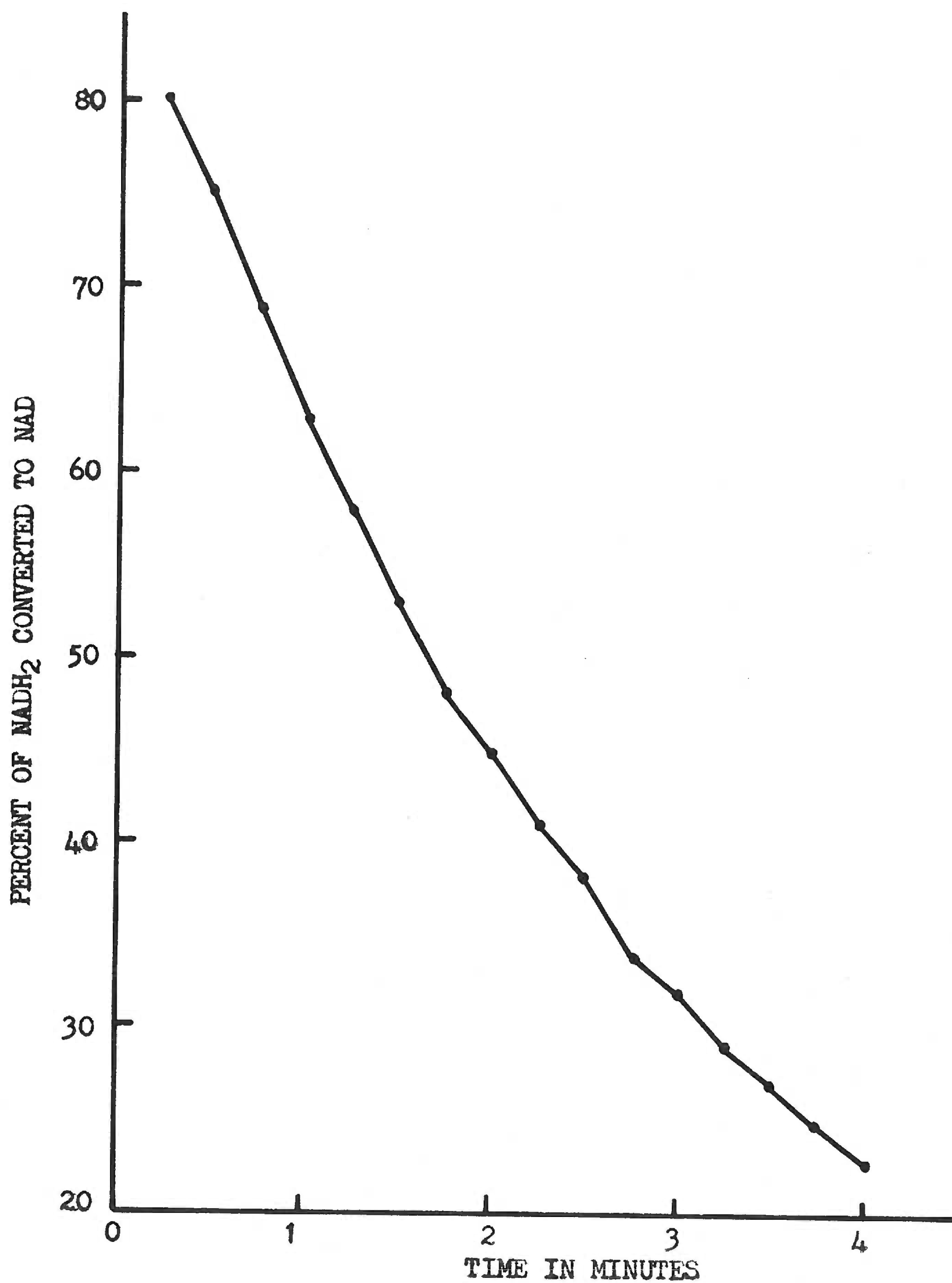

SC

blood cells seen in the infected mouse are shown in Figures 12 to 16. Normal values established in this laboratory from 26 female Balb/c mice are in percent: lymphocytes 67.42; granulocytes 30.57; monocytes 0.345; eosinophils 1.65; and smudge cells (per one hundred nucleated cells) 8. Results of serial differential cell counts from a normal mouse are shown in Table 5. In the healthy mouse only mature forms of the blood elements are normally seen in the peripheral blood smears. In contrast the infected animal presents a picture of early morphological stages of the red blood cell, specifically the rubricyte and the metarubricyte (Table 6). In the last stages of the infection the prorubricyte appears (Table 6). Other pathological findings of the mature red cell series are: polychromatophilia and anisocytosis. In Table 6 the presence of these cell forms are indicated as being in the range of slight to marked in the last five weeks of the experiment. Howell-Jolly bodies are also shown to be present in the infected animals. In Table 6 the animal's smudge cell count went from 1 to 231, per one hundred nucleated cells counted, during the first weeks of the infection. LDH activity is arranged in Table 6 by weekly intervals to show the close, direct relationship between increased LDH activity and increased proliferation of pathological red cells.

Total LDH activity

All total LDH activity is expressed in micromoles of $NADH_2$ converted to NAD per ml of blood serum per minute in the presence of pyruvate. This unit of activity was selected because the procedure makes use of the loss of native fluorescence of the coenzyme substrate, and not of change in optical density. The so-called "international unit" requires an optical density reading to compute. A graphic example

(Figure 16). This graph depicts a representative sample run by the modified Lowry method in which $NADH_2$ is converted to NAD in the presence of pyruvate. The rate of the reaction is plotted against time in minutes. The substrate concentration was adjusted to produce a linear reaction for a period of one minute or more at all sample enzyme activity levels.



80
70
60
50
40
30
20
0
1
2
3
4
PERCENT OF $NADH_2$ CONVERTED TO NAD
TIME IN MINUTES

TABLE 1

COMPOSITE EXPERIMENTAL DATA FROM GROUP I
(All Weights in Grams)

	Animal Number	Liver Weight	Spleen Weight	Body Weight	Spleen/Body Weight Ratio ($x10^{-3}$)	Hematocrit	Micromoles ($x10^{-2}$) $NADH_2$ Converted to NAD/ml/minute
Week Zero	1	0.81	0.08	15	5.33		30.6
	2	0.86	0.11	17	6.47		54.6
	3	0.92	0.10	18	5.55		68.6
	4	0.77	0.18	15	5.33		30.6
	5	0.72	0.06	14	4.29		38.3
	6	0.79	0.04	15	6.0		34.7
Week One	1	0.98	0.15	18	8.33		33.7
	2	0.78	0.08	16	5.00		99.3
	3	1.10	0.22	19	11.50		29.8
	4	0.73	0.14	15	9.33	70	52.0
	5	0.96	0.13	18	7.22	55	50.5
	6	0.97	0.11	17	6.47	67	34.7
Week Two	1	0.77	0.17	15	11.34	57	78.0
	2	0.60	0.16	12	13.3	66	30.0
	3	0.73	0.20	16	12.5	60	60.7
	4	0.72	0.17	16	10.6	58	70.0
	5	0.70	0.31	14	22.14	64	77.3
	6	0.73	----	14	---	71	31.2

TABLE 1 (Continued)

	Animal Number	Liver Weight	Spleen Weight	Body Weight	Spleen/Body Weight Ratio ($x10^{-3}$)	Hematocrit	Micromoles ($x10^{-2}$) $NADH_2$ Converted to NAD/ml/minute
Week Three	1	1.07	1.63	18	90.60	34	85.9
	2	1.53	1.94	20	99.50	39	169.1
	3	0.84	0.26	19	13.70		43.3
	4	1.35	2.34	19	123.20	42	131.7
	5	1.01	1.37	18	76.10	51	158.9
	6	0.86	0.78	18	48.80	46	96.2
Week Four	1	0.73	0.18	15	12.00		73.7
	2	1.07	0.48	18	26.70		87.5
	3	0.82	0.22	19	11.50		80.0
	4	1.14	1.05	20	52.50		123.3
	5	0.80	0.21	17	12.40		60.1
	6	0.87	0.28	17	16.40		78.8
Week Five	1	1.23	0.32	21	15.20	60	81.6
	2	1.09	0.21	20	10.50	59	113.7
	3	2.05	3.60	23	156.50	40	187.1
	4	1.93	3.83	22	174.10	41	255.2
	5	0.93	0.13	18	7.20	62	74.4
	6	1.04	0.13	18	7.20	58	77.3

TABLE 1 (Continued)

	Animal Number	Liver Weight	Spleen Weight	Body Weight	Spleen/Body Weight Ratio (x10^{-3})	Hematocrit	Micromoles (x10^{-4}) $NADH_2$ Converted to NAD/ml/minute
Week Six	1	1.00	0.15	20	7.50	62	104.3
	2	2.00	4.58	22	208.20	35	465.6
	3	0.90	0.15	20	7.50	61	103.1
	4	0.93	0.15	19	7.90	61	103.3
	5	0.87	0.19	19	10.0	61	120.3
	6	1.01	0.15	19	7.90	55	77.7
Week Seven	1	1.00	0.15	22	6.80	62	76.6
	2	1.14	0.15	23	6.81	61	57.8
	3	1.24	0.19	24	7.92	59	78.0
	4	1.06	0.16	22	7.27	57	88.5
	5	1.08	0.21	21	10.00	64	81.6
	6	1.10	0.27	21	12.86	59	80.0
Week Eight	1	1.03	0.25	23	10.87	61.5	84.5
	2	1.05	1.00	20	50.00	47	217.9
	3	1.34	1.15	23	50.00	60	148.8
	4	0.94	0.16	20	8.00	64	136.4
	5	0.93	0.17	20	8.50	62	149.8
	6	0.87	0.22	18	12.22	68	108.9

TABLE 1 (Continued)

	Animal Number	Liver Weight	Spleen Weight	Body Weight	Spleen/Body Weight Ratio ($x10^{-3}$)	Hematocrit	Micromoles ($x10^{-4}$) $NADH_2$ Converted to NAD/ml/minute
Week Nine	1	1.19	0.24	24	10.00	65	173.4
	2	1.02	0.17	21	8.10	66	142.7
	3	0.95	0.16	21	7.62	63	122.5
	4	1.10	0.26	21	12.38	63	127.1
	5	1.15	0.37	23	16.09	53	181.6
	6	1.00	0.22	21	10.48	54	145.3

TABLE 2

TOTAL SERUM LDH ACTIVITY GROUP II (INFECTED)
Micromoles ($x10^{-2}$) $NADH_2$ Converted to NAD/ml/minute

Animal Number	Week 0	1	2	3	4	5	6	7	8	11
1	13.5	27.0	54.0							
2	13.5	18.9	10.8	43.2						
3	10.8	18.9	18.9	27.0	32.4	24.3	26.6	78.3	78.3	
4	8.1	35.1	21.6	67.5	86.1	89.1				
5	10.8	24.3	35.1	35.1	27.9					
6	21.6	21.6	24.3	21.6	29.7	45.9	43.2	72.9	199.8	210.6
7	13.5	27.0	27.0	24.3	32.4	64.8	72.9	140.4		
8	--	13.5	8.1	--	37.8					
9	24.3	29.7	32.4	62.1	64.8	97.2	72.9	70.2	151.2	83.7
10	16.2	10.8	37.8	29.7	24.3	10.8	21.6	35.1	48.6	40.5
11	10.8	29.7	32.4	48.6	83.7					
12	8.1	21.6	32.4	64.8	72.9	62.1	75.6			

TABLE 3

TOTAL SERUM LDH ACTIVITY GROUP III
Micromoles ($x10^{-2}$) $NADH_2$ Converted to NAD/ml/minute

	Animal Number	Week 0	1	2	3	4	5	6	7	8
Controls	1	8.1	8.1	13.5	10.8	10.8	8.1			
	2	8.1	---	8.1	13.5	13.5	8.1	13.5	8.1	
	3	10.8	8.1	---	5.4	8.1	8.1	8.1	10.8	8.1
	4	---	10.8	8.1	10.8	5.4	2.7	8.1	8.1	10.8
	5	10.8	8.1	5.4	---	5.4	8.1	16.2	5.4	8.1
	Mean									
Infected	1	8.1	16.2	16.2	37.8					
	2	8.1	13.5	16.2	54.0					
	3	5.4	18.9	21.6						
	4	---	18.9	16.2	48.6	48.6	145.8			
	5	8.1	21.6	13.5	37.8					
	Mean									

TABLE 4

NUMBER OF NUCLEATED CELLS PER CMM OF BLOOD IN THE INFECTED ANIMAL
GROUP II

Animal Number	Week 0	1	2	3	4	5	6	7	8	11
1	---	8,100	6,500							
2	8,950	8,050	8,000	10,700						
3	---	5,500	5,700	8,600	9,100	5,800	10,900	7,000	62,000	
4	8,750	6,750	4,550	23,000	44,600	66,200				
5	8,700	5,100	7,400	11,600	48,000					
6	---	6,350	7,350	9,350	7,150	7,050	15,350	90,000	128,800	109,400
7	---	7,150	5,600	7,100	6,250	18,650	60,200	74,200		
8	10,350	7,400	7,050	---	8,550					
9	6,650	6,800	6,700	25,800	---	46,600	110,200	59,000	93,600	55,600
10	10,200	8,900	7,550	4,200	78,650	4,650	9,800	44,400	5,500	4,900
11	10,450	7,400	7,200	11,650	8,700					
12	---	7,250	4,050	10,700	29,551	66,400	105,400			

LEGEND FOR TABLE 5 AND 6

L. L.	-	Large Lymphocyte
S. L.	-	Small Lymphocyte
At. Ly.	-	Atypical Lymphocyte
V.	-	Virus cell
Pro. L.	-	Pro Lymphocyte
Total L.	-	Total Lymphocytes
S. C.	-	Smudge Cell
P. C.	-	Polychromatophilin
A.	-	Aniscocytosis
P.	-	Poikilocytosis
H. J. B.	-	Howell Jolly Bodies
Bs. Stp.	-	Basophilic Stippling
M. R.	-	Metarubricyte
R.	-	Rubricyte
P. R.	-	Prorubricytes
Hypo. C.	-	Hypochromia
Pmn.	-	Polymorphonuclear Leukocytes
M.	-	Monocyte
E.	-	Eosinophile
S.	-	Slight
M.	-	Moderate
MK	-	Marked
X	-	Present

TABLE 5

HEMATOLOGY AND LDH ANALYSIS
FOR NORMAL MICE - GROUP III

	Week				
Cell Type (%)	1	5	6	7	8
L. L.	11	9	21	11	9
S. L.	62	73	61	81	74
At. Ly.	2		2		2
V.					
Pro. L.					
T. L.	75	82	84	92	85
S. C.	10	9	6	4	13
P. C.					
A.					
P.					
H. J. B.					
Bs. Stp.					
M. R.					
R.					
P. R.					
Hypo. C.					
Pmn.	22	17	16	6	14
M.				2	
E.		1	2		1
LDH Activity	8.1	8.1	8.1	10.8	8.1

TABLE 6

HEMATOLOGY AND LDH ANALYSIS
FOR INFECTED MICE - GROUP III

Cell Type (%)	Week 0	1	2	3	4	5	6	7	8
L. L.	31	10	31	7	2	9	4	1	8
S. L.	38	58	36	37	38	38	6	12	13
At. Ly.			2	2	8	6	4		5
V.					1	2	4	5	2
Pro. L.									
T. L.	69	68	69	46	49	53	38	18	26
S. C.	1	1	5	33	118	231	173	113	76
P. C.				S	MK	MK	MK	MK	MK
A.					MK	M	MK	MK	MK
P.					MK	M	MK	MK	MK
H. J. B.					X	X	X	X	X
Bs. Stp.					X	X	X	X	X
M. R.				21	27	18	28	58	57
R.						5	4	15	2
P. R.								4	1
Hypo. C.							MK	MK	MK
Pmn.	20	28	29	29	20	25	27	11	30
M.	5				1	2	1	1	1
E.	6	4	2	1	3	1	1		1
LDH Activity	8.1	16.2	16.2	37.8	37.8	97.2	156.6	135.0	91.8

TABLE 7

MEAN BODY WEIGHT'S IN GRAMS - GROUP II (INFECTED)

Week									
0	1	2	3	4	5	6	7	8	11
14.6	16.5	17.6	16.7	17.0	16.5	17.7	19.7	22.2	22.0

TABLE 8

MEAN BODY WEIGHT'S IN GRAMS - GROUP III

	Week								
	0	1	2	3	4	5	6	7	8
Infected	15.3	15.6	17.2	18.1	18.5	19.4	20.7	19.9	21.5
Control	15.5	12.4	17.8	18.7	18.5	17.6	21.0	23.0	21.0

TABLE 9

DEATH RECORD - GROUP II (INFECTED)

	Week									
	0	1	2	3	4	5	6	7	8	11
Number of Living Animals	12	12	12	12	9	8	6	5	4	3

TABLE 10

DEATH RECORD - GROUP III (INFECTED)

	Week								
	0	1	2	3	4	5	6	7	8
Number of Living Animals	5	5	5	4	2	2	1	1	0

TABLE 11

DEATH RECORD - GROUP III (CONTROL)

	Week								
	0	1	2	3	4	5	6	7	8
Number of Living Animals	5	5	5	5	5	5	4	4	4

of a single fluorometric run is seen in Figure 16. The ordinate axis represents reaction time in minutes. Substrate concentrations were adjusted to produce a linear reaction for a period of one minute or more at all sample enzyme activity levels. Inspection of the absolute number of micromoles of $NADH_2$ converted to NAD in Group I (Table 1) appears larger than comparable values in Groups II and III (Tables 2 and 3). This discrepancy appears because the pyruvate concentration was changed in the reaction mixture for the assays in Groups II and III. This situation does not alter the value of the results, for emphasis is placed on relative changes rather than absolute values. LDH activity increased progressively with time in all three groups of animals. Individual animals showed a wide range of variation in LDH activity at any given time interval, but the difference in LDH activity can be directly correlated to the differences in the degree of severity of the infection. A direct parallel has been shown to exist between increased enzyme activity and increases in: 1) spleen weight (Figure 7); 2) number of nucleated cells (Tables 1 and 4), (Figures 3 and 5); 3) liver weight (Figure 8); 4) red blood cell precursors (Table 6); 5) disintegrated cells in peripheral blood smears (Table 6). An inverse relationship exists between enzyme activity in: 1) hematocrits (Figure 6); and 2) degree of anemia (Figure 2).

Body weights

Records of body weights were maintained throughout the experiments (Tables 1, 7 and 8). No significant statistical difference could be established between body weight of the infected and the control animals, but it is apparent that in the infected animals as the liver and spleen weights increased there was also a proportional loss of body mass in

other organs.

Death records

Deaths occurred most frequently at the time of orbital bleeding as a result of the physical trauma involved with the procedure (Tables 9, 10 and 11). As the degree of severity of the infection increased, the animals were less able to sustain blood loss and appeared to succumb as a result of shock.

DISCUSSION

In 1964, Siegel et al. (82) while investigating the pathogenesis of the virus induced disease, was able to demonstrate reticulocytosis, anemia, and increase in nucleated cell count. Beginning at the third week, blood smears demonstrated progressive changes consisting of polychromatophilia, anisocytosis and Howell-Jolly bodies of the mature erythrocytes, and increase in the total nucleated cells. Siegel interpreted the changes he described as being due to malignant proliferation of the red cell series, or erythroleukemia which is remarkably similar to the erythroleukemia of de Guglielmo's disease seen in man. Data derived from this present study confirms the early work done by Siegel et al. (82). Robbins (76) states that di Guglielmo's disease appears as a neoplastic proliferation of erythroblasts in the marrow and flooding of the peripheral blood with these same immature cells. In this condition there is usually an anemia. The immature erythroblasts in di Guglielmo's disease do not mature and do not form increased numbers of metarubricytes and red cells as is the case with polycythemia (76). The mouse erythrocyte originates from the mesenchymal or stem cell of the bone marrow, and undergoes a progressive morphological differentiation until the adult form is reached. These steps in order of increasing maturity are: undifferentiated stem, rubriblast, prorubricyte, rubricyte, metarubricyte, and entry into the circulating blood as an adult erythrocyte.

Differential cell counts demonstrate the presence of the early morphological stages of the erythropoietic cell series, the prorubricyte, rubricyte and metarubricyte. When bone marrow smears were examined (59), cellular hyperplasia was observed particularly in the erythrocytic series.

Other pathological findings were hepatosplenomegaly, increased number of nucleated blood cells, anemia, decrease in hematocrit values and the presence of numerous smudge cells. Although the initial site of the infection is unknown, evidence strongly suggests the reticuloendothelial system. Assuming the virus is able to invade this tissue successfully, it would undergo viral multiplication in all susceptible cells. These susceptible cells would ultimately lyse and release virus particles into the blood. A subsequent viremia would ensue. The presence of a viremia has been demonstrated in this laboratory by injecting blood serum from infected mice into healthy mice. The inoculated mice developed erythrocytic leukemia in approximately four to five weeks. This intense viremia may cause numerous virus particles to attach to the erythrocytes where the enzymatic groups of the virus particle would destroy the specific mucopolysaccharides on the surface of the erythrocyte. This enzymatic insult would, in all probability, lead to lysis of the erythrocyte, releasing the cytoplasmic content into the blood plasma. It is well known that the mature erythrocyte contains approximately one hundredfold LDH activity compared to that of the blood serum level, volume for volume (32). In the event that the erythrocyte is not lysed in the blood stream, an alternate condition may exist to allow cytoplasmic release of LDH. The spleen and/or liver may recognize these virus coated erythrocytes as being abnormal and remove them from the circulating blood. While in the confinement of the spleen and/or liver, degeneration of the erythrocyte would take place releasing LDH. This mechanism would account for the marked anemia with the succeeding erythropoiesis response seen in the infected animals. Splenomegaly would be produced by the collection of abnormal erythrocytes and the

increased cellular hyperplasia.

It has been established by Rauscher (63) and Siegel et al. (82) that a definite malignant condition exists in the infected animals. Warburg (93) postulated that viruses produced their effects by destroying the ability of normal tissue to take up oxygen and oxidize the respiratory substrate, glucose to carbon dioxide. He believes that most of these cells die, but a few survive by resorting to a primitive type of energy release, glycolysis. According to Warburg, these surviving cells are permanently damaged in that they cannot revert to oxidative catabolic processes. Numerous investigators since Warburg's report have found that in general all enzymes of the glycolytic series are present in amounts that would permit normal functioning. There seems little doubt that anaerbic glycolysis is high in tumor cells and that aerobic glycolysis is characteristic of malignant neoplasms (26). Isozyme results in this study show a consistant shift towards the muscle type LDH. This shift would be anticipated with malignant tissue derives increasing amounts of energy via the glycolytical cycle. The muscle type LDH reaches maximal activity under these conditions (16).

Since LDH is an ubiquitous enzyme, serum elevation might logically be anticipated in any process resulting in significant cellular damage of necrosis regardless of the etiology. Histological sections of liver and spleen tissue has revealed numerous areas of tissue necrosis. These areas of necrotic tissue unquestionably contribute to the total LDH serum level.

Notkins et al (54), Riley et al (73) and Bailey et al (5) have recently reported that elevated LDH levels in the blood plasma are due to an impaired rate of enzyme clearance. Data derived from mice bled

serially show the conspicuous increase in the activity of LDH bands 5, 4 and 3, while LDH bands 1 and 2 remain normal during the course of the infection (Figure 11). An obvious question is why do LDH bands 1 and 2 remain at normal levels of activity if the mechanism for enzyme clearance is altered? A plausible explanation might be that the impaired rate of clearance predominantly affects the muscle type of LDH.

Riley (70, 72, 74, 75) and others have shown the presence of an LDH elevating agent which is thought to be viral in nature. The agent has been found associated with transplanted and spontaneous experimental tumors. The plasma LDH is elevated through five separate phases in mice in response to growing or regressing tumors in association with the LDH agents (74, 75). Riley (74) reports that when normal mice are inoculated with the LDH agent alone there is a slight drop in the hematocrit, but no gross pathologic lesion is seen. A five to tenfold increase in plasma LDH usually appears within 48 hours following inoculation and remains abnormally high for the life of the animal. While the presence of the LDH agent has not been ruled out thusfar, we do not obtain the five to tenfold increase in the enzyme activity within the first week of the infection. Further, in animals that show natural resistance to the leukemogenic virus, there is also apparently the same resistance to the LDH agent, for we have not incurred elevated LDH levels in animals independent of the pathogenic process produced by the leukemogenic virus.

Recent evidence has been presented that indicates the red blood cells are not the source of additional LDH in plasma following infection of mice with LDH agent (5). Hematocrit levels were slightly lower in infected mice, but erythropoiesis itself was not directly affected by

the LDH agent (5). In contrast there is a marked drop in hematocrit levels of as high as 30 to 40 percent in the leukemogenic inoculated animals. In the leukemic mice, a definite increase in the rate of erythropoiesis is seen in both bone marrow smears and spleen tissue studies. The skeletal muscle has the greatest amount of LDH enzyme followed in order of tissue LDH concentration by the liver and spleen. Bailey *et al*. (4) found that in none of the numerous tissues or cells he tested were the LDH levels in mice infected with the LDH factor, significantly higher than in the normal animal.

It has been determined that enzyme levels of five times normal could be provided by as little as 0.5 percent of the total liver tissue, or by 2.2 percent of total red blood cells (4). Wenner *et al* (97) in studying serum LDH levels of mice inoculated with oncogenic and non-oncogenic viruses drew the conclusion that erythrocyte destruction is a likely source of elevated LDH activity. Microscopic examination of the enlarged spleen shows focal hemorrhage, necrosis and some collection of hemosiderin in macrophages. This evidence strongly suggests erythrocyte destruction and resultant splenic congestion as the possible source of serum LDH. It seems reasonable to postulate that the source of the increased LDH is derived from the erythropoietic system (96).

While the mechanisms involved in the production of increased LDH levels in the blood serum have yet to be established, it would appear that the determination of serum LDH patterns may provide a valuable parameter for studying the pathogenesis of virus-induced leukemias.

SUMMARY AND CONCLUSIONS

The patterns of lactic dehydrogenase (LDH) production in Balb/c mice infected with murine leukemogenic (Rauscher) virus were followed for approximately 11 weeks. Data was derived from three separate groups of animals. The first group (Group I), consisting of 60 mice, was inoculated with the leukemogenic virus and at random six animals were killed at weekly intervals for a period of nine weeks. Blood samples were obtained by cardiac puncture and spleen and liver weights determined at time of sacrifice. Concurrent differentials and hematocrits were also done. The second and third groups of mice consisted of 12 and 10 mice respectively. Five of the third group did not receive the virus and served as control animals. The remaining 17 animals were inoculated with virus. Blood samples were drawn weekly via the suborbital plexus, starting one day before inoculation. Concurrent nucleated blood cell counts and differentials were also done. Death records were maintained and when possible autopsies were performed. LDH blood serum assays were carried out by a modified Lowry microchemical procedure, making possible measurement of total enzyme activity in one or two microliters of blood serum. Isozyme studies were carried out on parallel samples by vertical polyacrylamide and starch gels to determine the relative concentration that the individual isozyme made toward the total increase in LDH activity in the disease state. Vertical gels of starch and polyacrylamide were stained with a nitro-blue tetrazolium-substrate solution to detect LDH activity following electrophoresis.

These studies with a virus induced mouse leukemia demonstrate that the level of LDH is an indicative feature of the stage of patho-

genesis. A direct parallel has been shown to exist between enzyme activity, the number of nucleated cells, weights of the spleen and liver, number of red blood cell precursors, and disintegrated cells found in the peripheral blood smears with an inverse relationship in hematocrits. Isozyme patterns indicate a relative increase in the amounts of bands LDH-5, 4 and 3, while bands LDH-2, and 1 continues normal. It seems reasonable to postulate that the source of the increased LDH is derived from the erythropoietic system.

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