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LDH ASSESSMENT OF DIFFERENTIAL EARLY STRESS IN RATS

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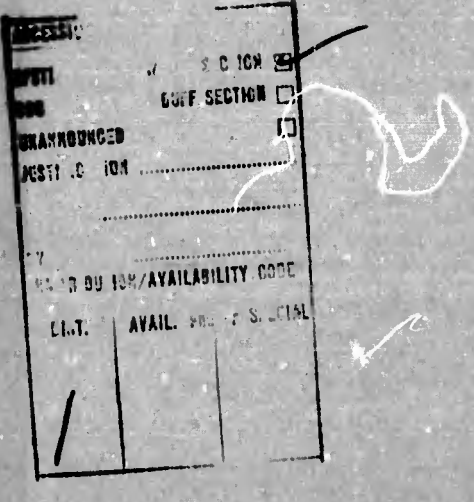
The animals used in this study were handled in accordance with the "Guide for Laboratory Animal Facilities and Care" as promulgated by the National Academy of Sciences - National Research Council.



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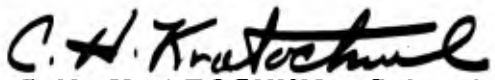
FOREWORD

This experimentation, which began on 1 November 1966 and was completed on 31 July 1967, was performed by the Primate Neuro-Sciences Laboratory at Florida Presbyterian College, St. Petersburg, Florida. The research was conducted under contract F29600-67-C-0011, Project 7906, with the 6571st Aeromedical Research Laboratory, Holloman AFB, New Mexico, in coordination with the Office of Aerospace Research. The research was conducted under the monitorship of Lt Colonel Herbert H. Reynolds, whose advice and support of this study is gratefully acknowledged.

The assistance of the following persons is gratefully acknowledged: Marjorie I. Lathrop, typing of manuscript; Bert Alberts and Steve Leeper, autopsies; and Sylvia Hargan, biochemical analyses.

This technical report has been reviewed and approved for publication.



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ABSTRACT

A study was performed to determine the effects of differential early stress (immobilization) upon the LDH response in rats. Ninety-six hooded rats (48 of each sex) were used as subjects. Three groups of subjects were stressed at ages 6-8, 24-26, and 38-40 days. These three groups were also stressed again at 120 days of age. Three more groups of animals were stressed at identical ages during early development, but not stressed later in life. One group was not stressed early but stressed later in life, and the last group was not stressed at all. The results of the LDH analyses showed that the earlier stress is induced in the rat, the greater is the protective effect of this initial stress, when the animal is exposed again to a stress in adulthood. Specifically, this protective effect was found to occur in the cardiac tissues, and to a smaller extent in hepatic tissues and-or skeletal muscles. No sex differences with regard to LDH-isoenzyme response to stress at different periods of development were found; but age-specific differences between males and females, thought to be due to the female hormonal system, were found for LDH-1 and to some extent for LDH-4 and LDH-5.

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INTRODUCTION

Early Studies

The theory that there are certain critical periods in the development of organisms is not a new one. In experimenting with the effects of various inorganic chemicals upon the development of fish embryos, Stockard (Ref. 1) found that almost any chemical would produce the same effect, provided that it was applied at the proper time during development. These early studies and those of Child (Ref. 2) established that the specificity of effects at particular periods is due to the fact that rapidly growing tissue in an embryo are most sensitive to any change in conditions. Another early investigator, McGraw (Ref. 3), discovered the existence of critical periods for optimal learning of motor skills in the human infant. More recently, investigators have attempted to study the critical period by manipulating S at a certain period of development, and then comparing adult performance on a behavioral task or physiological measure to Ss manipulated at some other period of growth.

Behavioral Studies

Most data support the finding that early shock or handling causes superior performance in avoidance conditioning, when Ss are compared to non-manipulated controls (Levine, Ref. 4; Stanley and Monkman, Ref. 5; Baron, Brookshire and Littman, Ref. 6; Denenberg and Bell, Ref. 7; and Brookshire, Littman and Stewart, Ref. 8). There is disagreement, however, as to whether the age at prior treatment is a significant factor influencing later avoidance learning. Levine (Ref. 4) found that early handled Ss (1 to 20 days) were superior in avoidance learning to late handled Ss (50 to 70 days), while Brookshire, et al, (Ref. 8) failed to find any differences between early shocked and late shocked Ss.

Henderson (Ref. 9) has stated that a simple early-late distinction is misleading in that a curvilinear trend exists, and Ss which were treated at 30 days were affected the most. This age (30 days) has often been suspected of being a critical period for le[illegible]ng (Scott, Ref. 10). Bell and Denenberg (Ref. 11) administered sh[illegible] mice on either 2 and 3, 8 and 9,

or 15 and 16 days of age. At 60 days significant differences were found between groups with regard to activity measures, open-field performance, and avoidance learning scores. This finding substantiated results found in a previous study by Denenberg and Karas (Ref. 12).

There have been many other behavioral findings related to critical periods. Meyers (Ref. 13) found significant differences in adult exploratory behavior related to certain periods of infantile stimulation. Hale (Ref. 14) found differences in survival time to terminal schedules of reinforcement caused by manipulation at certain periods. Battig (Ref. 15) found differences in the acquisition and maintenance of conditioned responses to be dependent on particular periods of growth. Hess (Ref. 16) has found that the frequency of fear responses elicited by the potential imprinting object in chicks starts to increase at the age of 13 to 16 hours, which could account for the decrease in frequency of successful imprinting beyond the age of 15 to 16 hours, thus suggesting a definitely critical period. Forgays and Read (Ref. 17) exposed rats to a complex environment for 21 days at different periods of growth, and found that Ss exposed immediately after weaning were superior in a series of testing situations. Denenberg (Ref. 18) states that where findings are consistent with the critical period hypothesis, further research has shown that the critical period is a complex function of amount of infantile stimulation.

Physiological Studies

There have been significantly fewer studies of critical periods utilizing a physiological or biochemical measure. In a pioneering study measuring biochemical response to a stress situation, Bevan (Ref. 19) stated that "it is conceivable that the stability of a metabolic process at the time of exposure to continued stress may be an important factor in determining what modifications might occur", and this stability is attained at a certain period of development of the organism. Beach and Jaynes (Ref. 20) found that visual deprivation may prevent the development of a visual response during an early critical period, when it would have been learned readily.

More recently, Levine (Ref. 21) found that rats manipulated in infancy show a significant elevation of corticosteroids as early as 15 seconds after shock, whereas non-manipulated controls do not. Schaefer, Weingarten, and

Towne (Ref. 22) found that rats which have been handled or which have been subjected to lowered temperature without handling during the first week of life show significant reduction of adrenal ascorbic acid when stressed in adulthood, while controls do not.

Ader and Friedman (Ref. 23) exposed rats to manipulation during the first, second and third week of life, or throughout pre-weaning, and found significant differences in responses to inoculation with a suspension of Walker 256 carcinosarcoma. Among other things, they felt that these responses were influenced by the period of life during which manipulation occurred. In another study, Ader (Ref. 24) immobilized one group of rat Ss just as it approached its period of maximum activity, and another group was restrained during the inactive phase of their cycle. Half of the group immobilized during the first period developed ulcers, whereas no animals of the group stressed during their inactive phase developed this condition. This fact suggests a recurring critical period. Denenberg and Haltmeyer (Ref. 25) found differences in rat corticosterone response to shock stress, as a result of handling at different periods prior to weaning.

In a classic study Levine and Mullins (Ref. 26) proposed that the presence of hormones (sex, thyroid, or adrenal) during critical periods of development exerts a direct action on the central nervous system, producing profound and permanent changes in the subsequent psychophysiological processes of the organism.

It was the purpose of this investigation to determine whether or not there are critical periods in the development of the rat with regard to adult physiological response to stress. More specifically, the author wished to determine if stress induced at different periods of early development would produce differences in adult organ response to a stressor. If this would be the case, then specific relationships between a particular period of early stress, and a particular physiological response could be examined.

It is well documented that an extended period of exposure to a stressor produces a series of adaptive responses within the organism that will eventually lead to the exhaustion of the organ(s), primarily the heart, gastrointestinal tract, and adreno-cortical system, involved in this adaptive role. (Selye, Ref. 27). During the course of this syndrome of organ response (General Adaptation Syndrome), deterioration or damage of the organs involved occurs prior to the final exhaustive breakdown, and loss of function.

The author utilized this damage as an indication of differential physiological response, and measured this damage, both location and degree, using clinical procedures for the determination of plasma Lactic Dehydrogenase (LDH). A detailed text explaining the theory and applications of this procedure will be found in the next section.

Isoenzymes (of Lactic Dehydrogenase)

A serum component which catalyzes the transfer of hydrogen from reduced diphosphopyridine nucleotide (DPN, formerly NAD) to pyruvic acid was determined to be an enzyme and was named lactic dehydrogenase, or LDH. All of the organs of the body contain LDH. That LDH consists in multiple molecular forms has been demonstrated by electrophoresis in a variety of supports and numerous buffer media (Markert and Moller, Ref. 28; Kaplan and Ciotti, Ref. 29; Markert, Ref. 30; Fritz and Jacobson, Ref. 31; Boyer and Fainer, Ref. 32; Blanco and Zinkham, Ref. 33; Nance, et al, Ref. 34; and Preston, et al, Ref. 35). These studies have produced convincing evidence for the existence of at least five molecular forms of LDH. Each form appears to be one of five possible tetrameric combinations of two basic monomers or subunits: LDH-1, or H, since it occurs predominantly in the heart; and LDH-5, or M, since it occurs predominantly in the skeletal muscle and liver. The five isoenzymes might be illustrated as follows:

LDH-1	LDH-2	LDH-3	LDH-4	LDH-5
HHHH	HHHM	HHMM	HMMM	MMMM

Increasing evidence for the genetic control of these monomers has been found (Nance, et al, Ref. 34).

The ratio of these five LDH isoenzymes to one another varies with each organ, so that each has a particular isoenzyme-percent-pattern (for instance, the heart contains mostly LDH-1, with some LDH-2, and very little of LDH-3, 4, or 5; whereas the liver contains mostly LDH-5, with some 4, and very little of the other three isoenzymes).

When tissues are damaged so as to alter the permeability of cell walls, release of organ specific LDH isoenzymes into the bloodstream results. This

release raises the Total LDH level of the serum, a photometric measure of number of units of LDH present, given in units (one unit will reduce 4.8 x 10^{-4} moles of pyruvate per minute at 25° C). Also this release alters the LDH isoenzyme pattern, determined by an electrophoretic technique, measured in percentages of the serum, elevating those isoenzymes that are most specific to that particular organ. Thus with heart damage, we would expect to find an elevation of serum LDH-1, the predominant isoenzyme found in the heart; conversely, with liver damage an elevation of serum LDH-5 would be expected.

Therefore, if we know the normal or control pattern of isoenzymes in the serum or plasma of a particular species, we can determine whether or not organ damage is present within the organism by comparing the individual's LDH isoenzyme pattern with the normal pattern for the species under question. By carefully determining in which isoenzymes elevations occur, and comparing these elevations with organ isoenzyme patterns (found in the literature as a result of excising an organ, homogenating it, and running the electrophoresis technique on a sample of this homogenation; see Table II), we can determine the source of the damage.

Thus by examining the serum LDH isoenzyme pattern we can determine in which particular organ or organs damage has occurred; and by examining the Total LDH value of the serum sample, we can determine the total amount of damage, by comparing the individual's total with the total normal value of the species. Utilizing the following formula we can determine the amount of LDH attributable to each particular isoenzyme in units:

UNITS OF LDH/isoenzyme =
MEAN TOTAL SERUM LDH/group X MEAN PERCENT
OF SERUM ISOENZYME/group

We have thus pinpointed the site of serum isoenzyme elevation; now, with the above formula, it is possible to determine the amount of the elevation, in units of LDH, of the specific isoenzyme elevated.

This paper will utilize both the mean percentages/group of each isoenzyme present in the serum samples for a group to determine in which organs, if any, damage has occurred; and the amount of LDH/isoenzyme

to determine the extent of the damage. Both measures are necessary for an accurate general assessment of the internal state of the organism.

METHOD

Subjects

Forty-eight male and 48 female hooded rats were used as Ss. All animals were born and raised in our laboratory from pure strain mothers.

Apparatus

A durable nylon hardware cloth was used for the restraining procedure and fashioned into a bag by the use of staples. The size of the bag varied with the age of the animals stressed, so that it would fit firmly around S's body, and thus uniformly restrict movements for all subject-groups. A box, made of plywood, with 30 individual compartments, each 6 x 12 x 12 inches, was used to hold the bags containing the animals during periods of stress, so that visual and tactile contact between Ss during stress was prohibited. The box was raised 6 inches off the table level by means of four legs. A stopwatch was used to time placement, and removal of Ss upon completion of the stress period.

Procedure

All Ss were housed with their respective litters from birth, with a social cage assigned to each litter. These cages were 18 x 18 x 9 inches in size, and placed in a controlled environment apart from the auditory and visual variables of the laboratory. Ss were kept on a 12 hours on/12 hours off light schedule, so that endogenously and exogenously controlled mechanisms would operate by the same normal conditions for all Ss. Only one person was allowed in the room to maintain Ss, and this same person fed Ss Purina Laboratory Chow and water at the same time each day.

Ss were divided into eight major groups:

Group	Initial Stress	Final Stress (120 Days)
1	6-8 Days	Yes
2	24-26 Days	Yes
3	38-40 Days	Yes
4	6-8 Days	No
5	24-26 Days	No
6	38-40 Days	No
7	No	Yes
8	No	No

Initially two litters were assigned to each condition. At 23 days all litter groups were weaned, and at age 45 days, the two litters were sexed, combined, and excess animals removed, so that each condition had 12 Ss assigned to it: 6 males and 6 females. These three particular early periods were chosen because they seemed to conform to the following important periods of growth: early maternal period--pre-weaning (6-8 days); loss of mother immediately following weaning (24-26 days); and the onset of puberty (38-40 days). Handling during sexing was for a period of no more than 5 to 10 seconds, and Ss were handled at no other time during development, except, of course, during S's particular stress period.

The following procedure was utilized for the stressing of Ss:

A. Initial Stress

1. The animals were removed from the housing cage and immediately placed in a restraining bag.

2. E timed himself using a stopwatch, so that the time between removal from the home cage and final placement within the bag was identical for all Ss. This time period was exactly 3 minutes for all Ss.

3. The exact time of placement into the holding compartment was noted, and after a period of 3 hours Ss were removed in the same temporal succession in which they were placed, so that each S was exposed to exactly 3 hours of immobilization within the holding compartment.

4. Removal was timed with a stopwatch, so that exactly 3 minutes elapsed between removal from the holding apparatus and return to the home cage.

B. Final Stress (120 Days)

1. All procedures as stated in part A (above) were followed, except that Ss remained in the restraining bag for a period of 18 hours. (Ader, Ref. 36).

2. Final stress occurred when Ss were 120 days of age. Immediately upon removal from final stress, Ss were anesthetized using ether, and 2.5 cc of blood was obtained by cardiac puncture (Garbus, Highman and Altland, Ref. 37). Heparin was utilized so that plasma could be obtained from the blood, as hemolysis had to be avoided (hemolyzed serum contains spuriously high concentrations of LDH). Blood drawing was also performed in the same temporal order in which Ss were withdrawn from the stress, so that blood was drawn at the same time following stress for all Ss.

Following blood drawing, bloods were centrifuged at 3,000 rpm for 10 minutes, and the separated plasma was placed in aliquots. Plasma was kept at room temperature at all times, as LDH-5 is particularly unstable under conditions of cold, and all clinical procedures were performed within 48 hours. Plasma Total LDH analysis was performed according to the technique of Cabaud and Wroblewski (Ref. 38). (See Appendix I) LDH isoenzymes were determined by a modification (Hargan and Nay, Ref. 39, unpublished) of the Preston, et al, technique (Ref. 35). (See Appendix II)

Ss that were not stressed at any time during their development (Group 8) remained unhandled in their home cages throughout the periods of stress, except for sexing at 45 days of age.

RESULTS

The results of a three-dimensional analysis of variance (Lindquist, Ref. 40) appear in Table I. The remainder of the results appear in Table II and Figures 1-9. (Raw data appear in Appendix III.)

DISCUSSION

From Table 1 it is apparent that highly significant differences in units of LDH existed among the A factors (time of initial stress) for each isoenzyme except LDH-5. Significant differences were found also among the B factors (late stress vs. no late stress) in all isoenzymes except LDH-3. We can also conclude that in general no apparent differences between males and females exist, as only one of the C factors (LDH-2) showed significant differences (at the . 05 level).

The findings for Total LDH essentially reinforce the findings for the isoenzymes: there are significant differences between the A factors, the B factors, but not the C factors.

The only interactions in which significant differences appeared were the A X B interaction (time of initial stress; late or no late stress) for LDH-1, 4, and 5, and Total units; and the B X C interaction late or no late stress; sex) for LDH-2 and 3 only. The highly significant B X C interaction of LDH-3 in particular, is a provocative finding. The individual B and C factors for LDH-3 did not reach significance, indicating a very specific interactive effect that bears future investigation.

GROUP DIFFERENCES

Groups 1, 2, 3, and 7 were considered the experimental groups, as they determined the effects of early differential, or no early stress, on adult response to a stressor, which was the goal of this paper. Groups 4, 5, and 6

TABLE I

RESULTS OF THREE-DIMENSIONAL ANALYSES OF VARIANCE

FACTORS	Total LDH	LDH-1	LDH-2	LDH-3	LDH-4	LDH-5
A^a	5.39**	9.78***	6.34***	7.95***	5.15(.005)	1.21 N/S
B^b	65.88***	33.79***	5.77(.025)	2.38 N/S	7.87**	27.22***
C^c	2.00 N/S	2.37 N/S	5.13*	1.14 N/S	1.00 N/S	1.25 N/S
A X B	8.88***	15.92***	1.12 N/S	1.00 N/S	10.79(.005)	4.05**
A X C	1.00 N/S	1.04 N/S	1.00 N/S	1.00 N/S	1.14 N/S	1.30 N/S
B X C	2.38 N/S	1.00 N/S	6.26(.025)	15.25***	1.00 N/S	3.70 N/S
A X B X C	1.00 N/S	3.29 N/S	1.00 N/S	1.00 N/S	1.00 N/S	1.00 N/S

* = F value at the .05 level

** = F value at the .01 level

*** = F value at the .001 level

A^a = Time of initial stress

B^b = Late or no late stress

C^c = Male or female

(differential early stress, but no late stress) served as control groups, so that differences among the experimental groups due to the effects of differential early treatment on adult stress responses could be shown, and not those due to the time of initial stress alone. Group 8 (never stressed) served as an over-all control group, providing normal values of LDH for Ss.

Experimental Group Differences (Early Stress and Late Stress)

In Figure 1 we see the zymograms, based on the percent relationships of the five isoenzymes of both male and female experimental groups. From these two zymograms it may be concluded that by far the greatest percentage elevations from the control or normal values (Group 8) occurred in LDH-1 (as much as 26.7 percent in Group 7). A comparison of these zymograms to those established in the literature for particular organs (see Table II) indicates that this almost singular rise of LDH-1 is indicative of cardiac damage (the alteration or breakdown of cellular permeability through which enzymes are released into the bloodstream are here defined as tissue damage).

TABLE II

SOME TYPICAL ORGAN-SPECIFIC LDH ISOENZYME PATTERNS IN THE RAT

HEART	Significant amounts of LDH-1, small amount of LDH-2 (Garbus, et al, Ref. 37) (Fine, et al, Ref. 41)
KIDNEY	Significant amounts of both LDH-1 and LDH-2 (Garbus, et al, Ref. 37)
LIVER	Significant amount of LDH-5, slight elevation of LDH-4 (Wiggert and Villee, Ref. 42)
SKELETAL MUSCLE	Significant amounts of both LDH-4 and LDH-3 (Komatsu and Michaelis, Ref. 43)
BRAIN	Elevations of all five isoenzymes (Komatsu and Michaelis, Ref. 43)

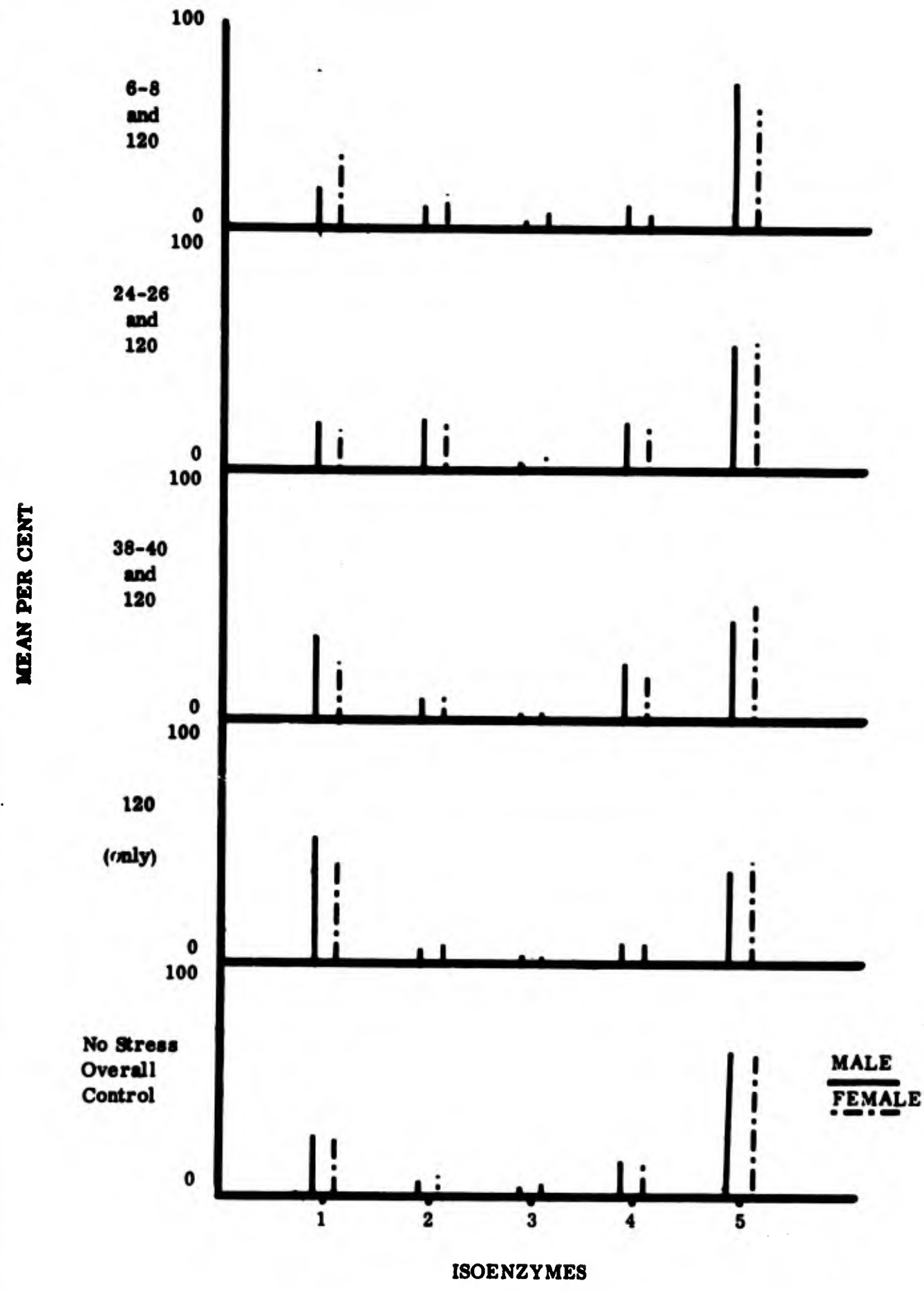

Figure 1. Mean Zymograms for Experimental Groups 1, 2, 3, and 7; and Over-all Control Group 8.

The amount of cardiac damage as indicated by units of LDH for each of the male and female experimental groups is shown in Figure 2. LDH-1 unit release increases with time of initial stress, when Ss are stressed at 120 days, with the largest amount of unit release occurring with stress at 120 days only. More specifically, LDH-1 for male Ss shows a gradual increase from Ss stressed on days 6-8 and 120 days to those stressed at 24-26 days and 120 days. Ss stressed at 38-40 days and 120 days have significantly more LDH-1 unit release, and Ss stressed at 120 days (only) exhibited the highest amount of unit release. In terms of units, Ss stressed at 120 days (only) showed an increase over the earliest stress group, 6-8 days and 120 days, of over 450 units--a truly significant difference in amount of cardiac damage.

For female Ss, the same increase with age is indicated, but with one outstanding difference: LDH units released with stress at 6-8 days and 120 days are significantly higher than those of the male group stressed at the same times. At 24-26 days and 120 days, both males and females show essentially the same unit release; but at 38-40 days and 120 days and at 120 days (only) the females exhibit less cardiac damage as indicated by less unit release, than similarly stressed male Ss. These sex differences, not shown in the analysis of variance due to their specificity, could be due to the onset of the protective mechanisms inherent in the female's hormonal systems, which are not significantly active until puberty (which more nearly approximates our 38-40 day stress period).

Thus, the primary source of LDH unit release, indicating tissue damage by the criterion cited above, appears to be the cardiac tissues, as seen in Figure 1 and Table II. Also, an increase in amount of this damage with time of initial stress is indicated, as seen in Table I and Figure 2, with age-specific differences between male and female groups (Fig. 2).

The zymogram (see Fig. 1) based on absolute percent values has limitations in that it will indicate primarily the predominant source of damage, since smaller amounts of organ damage do not significantly alter the percent pattern. In this flaw lies the value of unit comparisons, such as are made in Figure 1, which indicate the amount of unit release. Figures 3, 4, 5 and 6 show unit variations among the experimental groups for LDH isoenzymes 2, 3, 4 and 5, respectively. No apparent differences or trends are indicated in Figures 3 and 4 for LDH-2 and LDH-3. Significant variations among groups

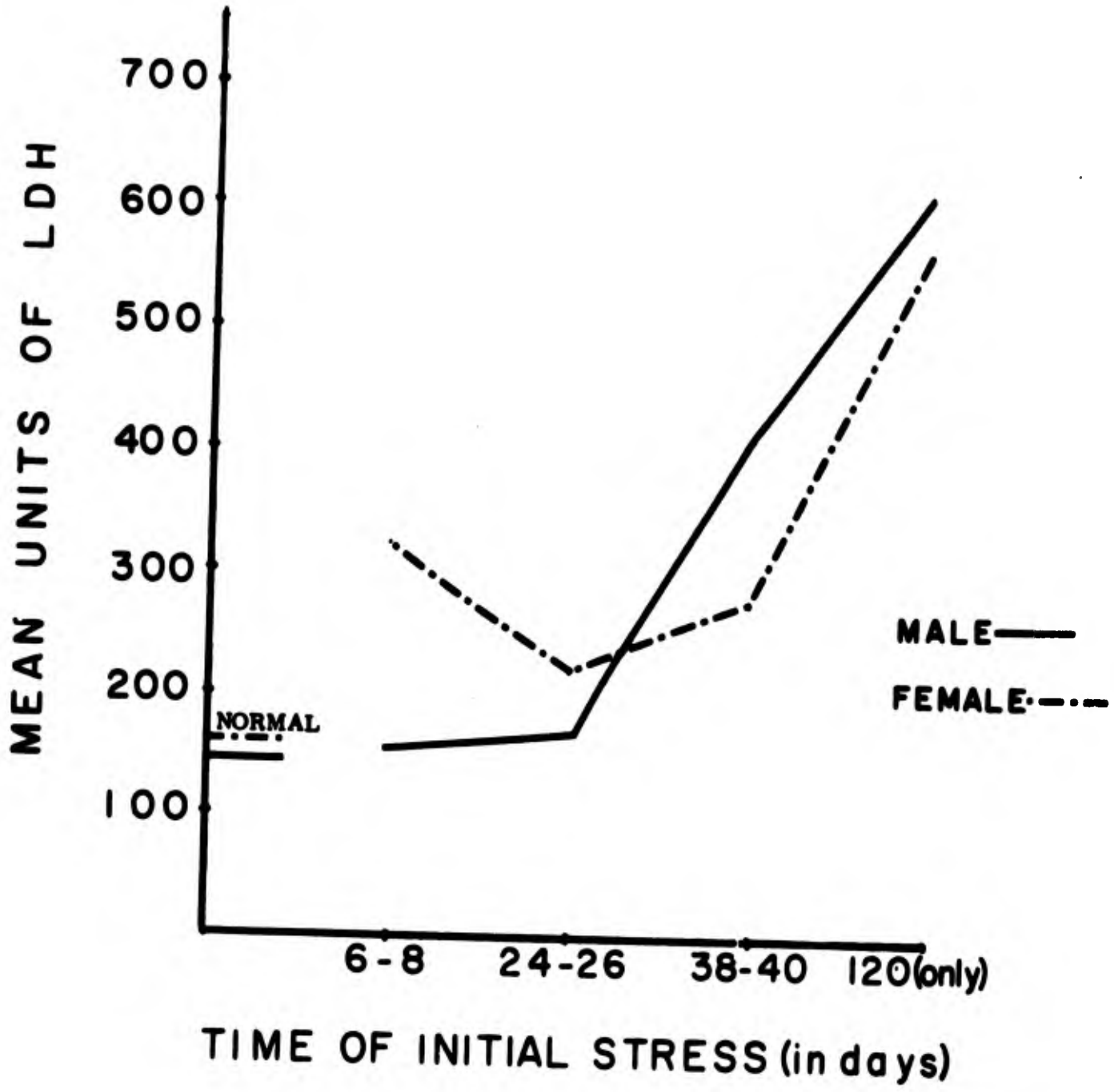

Figure 2. Mean Units of LDH-1 Release for Experimental Groups.

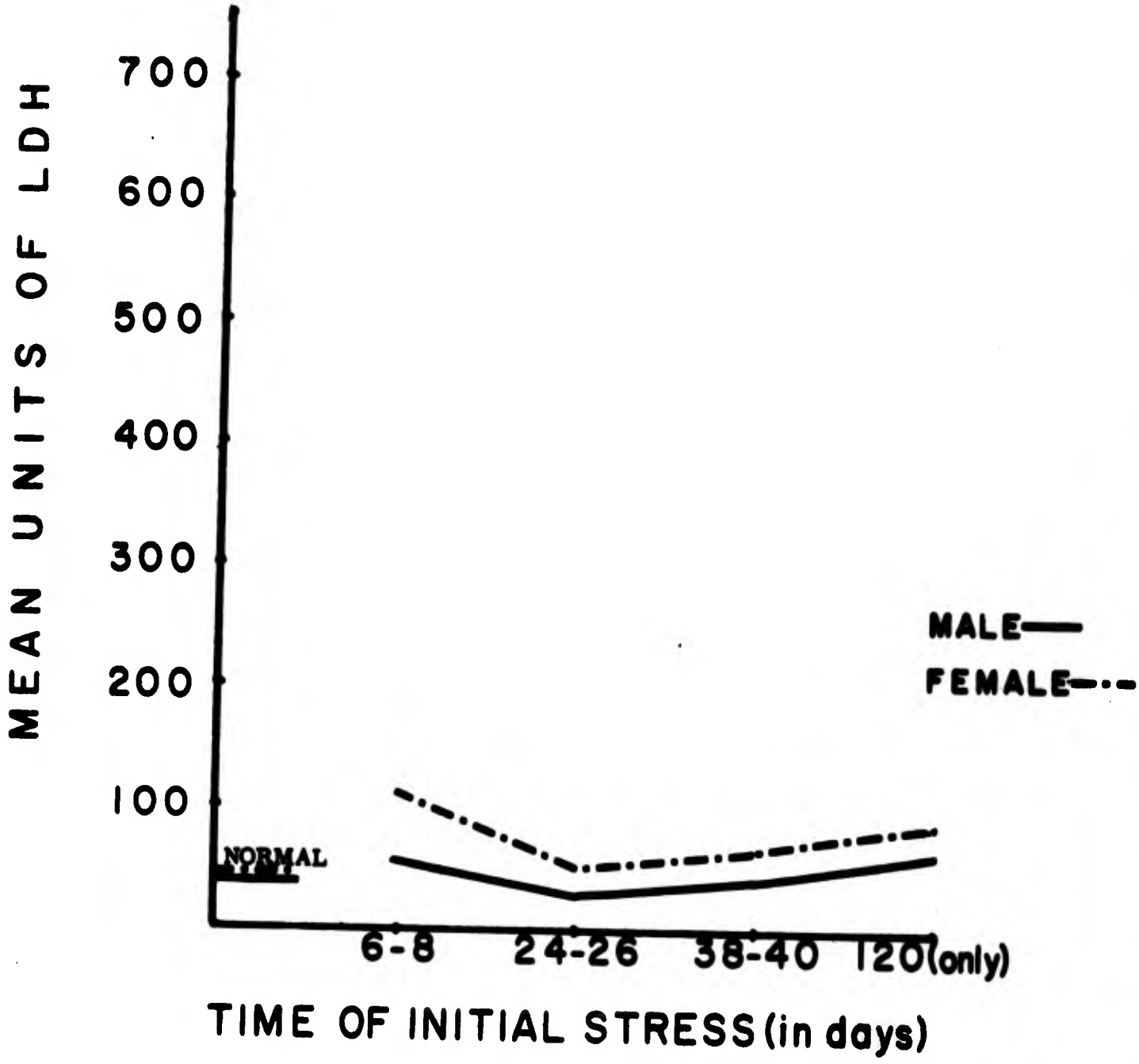

Figure 3. Mean Units of LDH-2 Release for Experimental Groups.

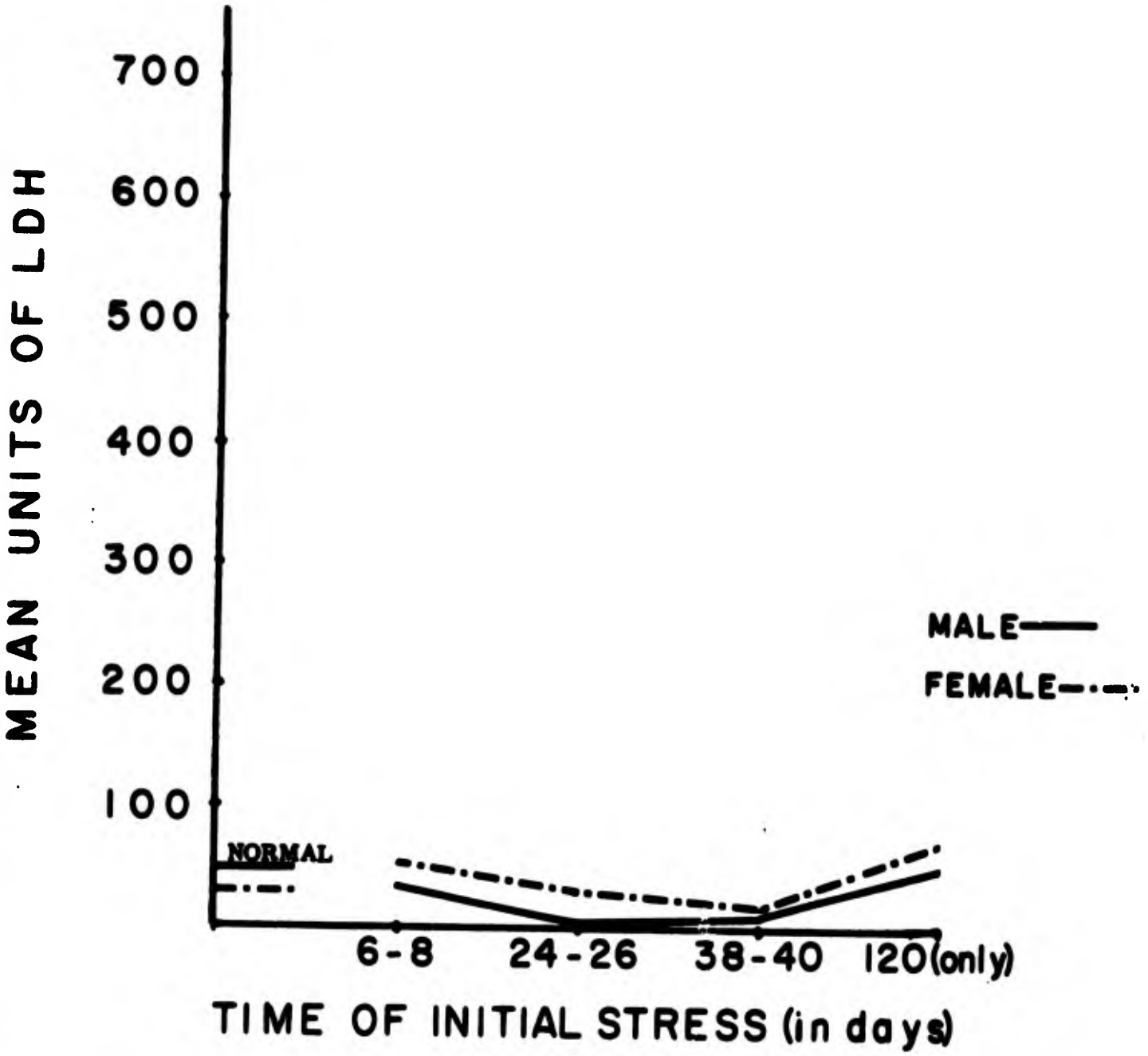

Figure 4. Mean Units of LDH 3 Release for Experimental Groups.

with regard to unit release are apparent for LDH-4 and LDH-5, as seen in Figures 5 and 6.

Figure 5 shows a steady increase in LDH-4 unit release with time of initial stress of 275 units, when male groups are compared: stress 6-8 days and 120 days to stress at 38-40 days and 120 days. For males, with stress at 120 days (only), however, a highly significant decline was apparent when compared to the 38-40 day group (258 units). This same type of relationship seems to hold true for females, except that unit release for the 38-40 days and 120 days stress group is not quite as high for the females. It is apparent, then, that for LDH-4, very early stress at 6-8 days and 120 days; and stress at 120 days (only) tended to yield a protective effect, with unit release near normal values (as indicated by the horizontal lines). Stress at 24-26 days and 38-40 days appeared to elicit much greater amounts of LDH unit release, with the latter yielding the highest amount.

Figure 6 presents a comparison of groups with regard to LDH-5 unit release. Here, as with LDH-4, it is apparent that stress at 38-40 days and 120 days elicits more LDH unit release than any other group for both males and females, with groups: 6-8 day and 120 day stress; and 120 day stress (only), exhibiting values just slightly higher than normal values (as indicated by the horizontal lines). With regard to the 24-26 day and 120 day stress groups, females exhibit more LDH unit release (approx. 150 units) than do males. A look at Table 2 indicates that elevations of LDH-4 and LDH-5 could be indicative of liver and/or skeletal muscle damage, although the amounts of this type of damage are not nearly as great for experimental groups, as is the cardiac damage mentioned previously.

In general, it may be stated that, for male and female Ss, heart and hepatic (or skeletal muscle) damage tends to increase with time of initial stress, when Ss are stressed in adulthood. With regard to adult stress (120 days) only, there appears to be a significant difference in organ response, with a significant amount of cardiac specific damage, and very little or no hepatic (or skeletal muscle) damage.

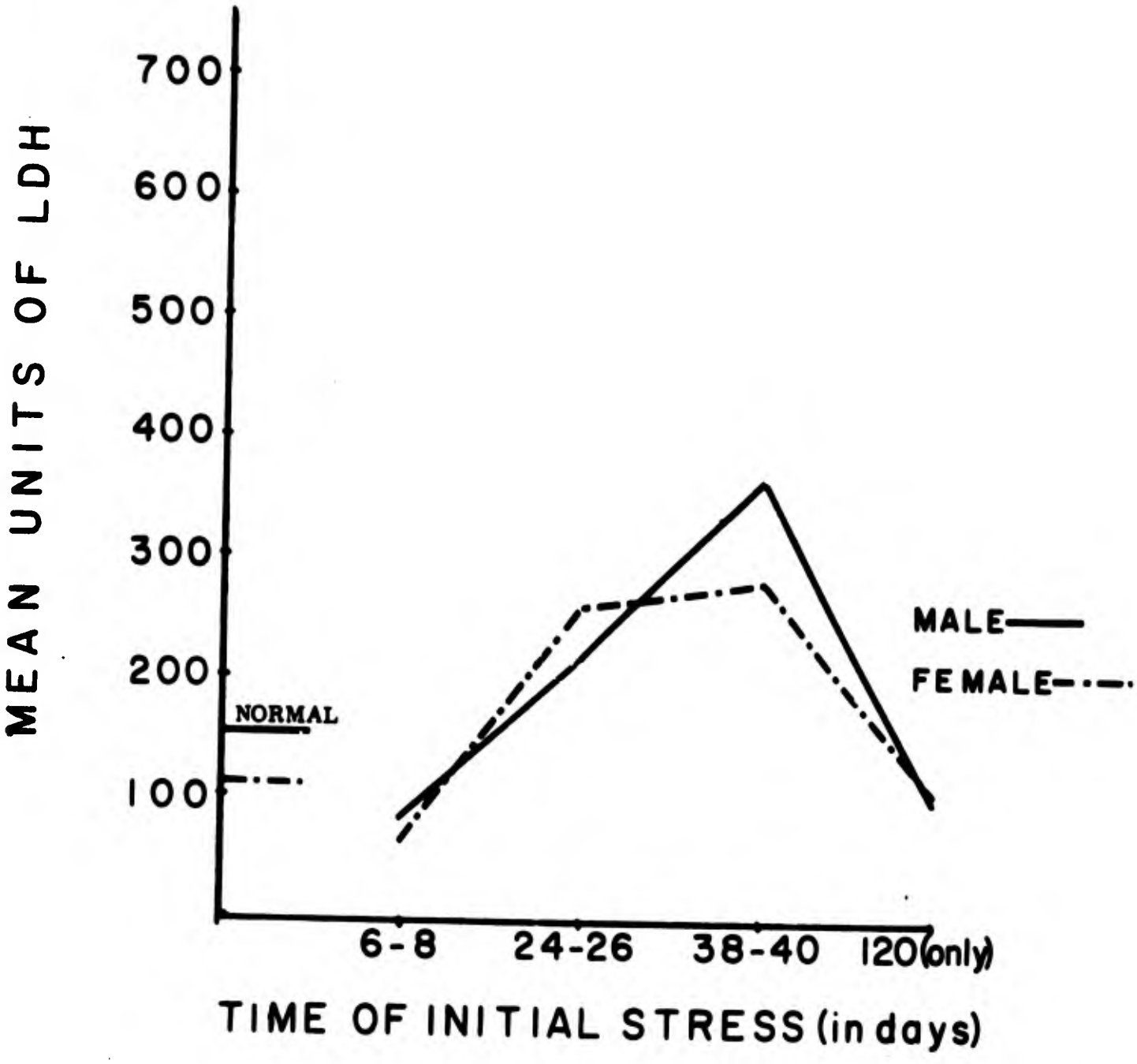

Figure 5. Mean Units of LDH-4 Release for Experimental Groups.

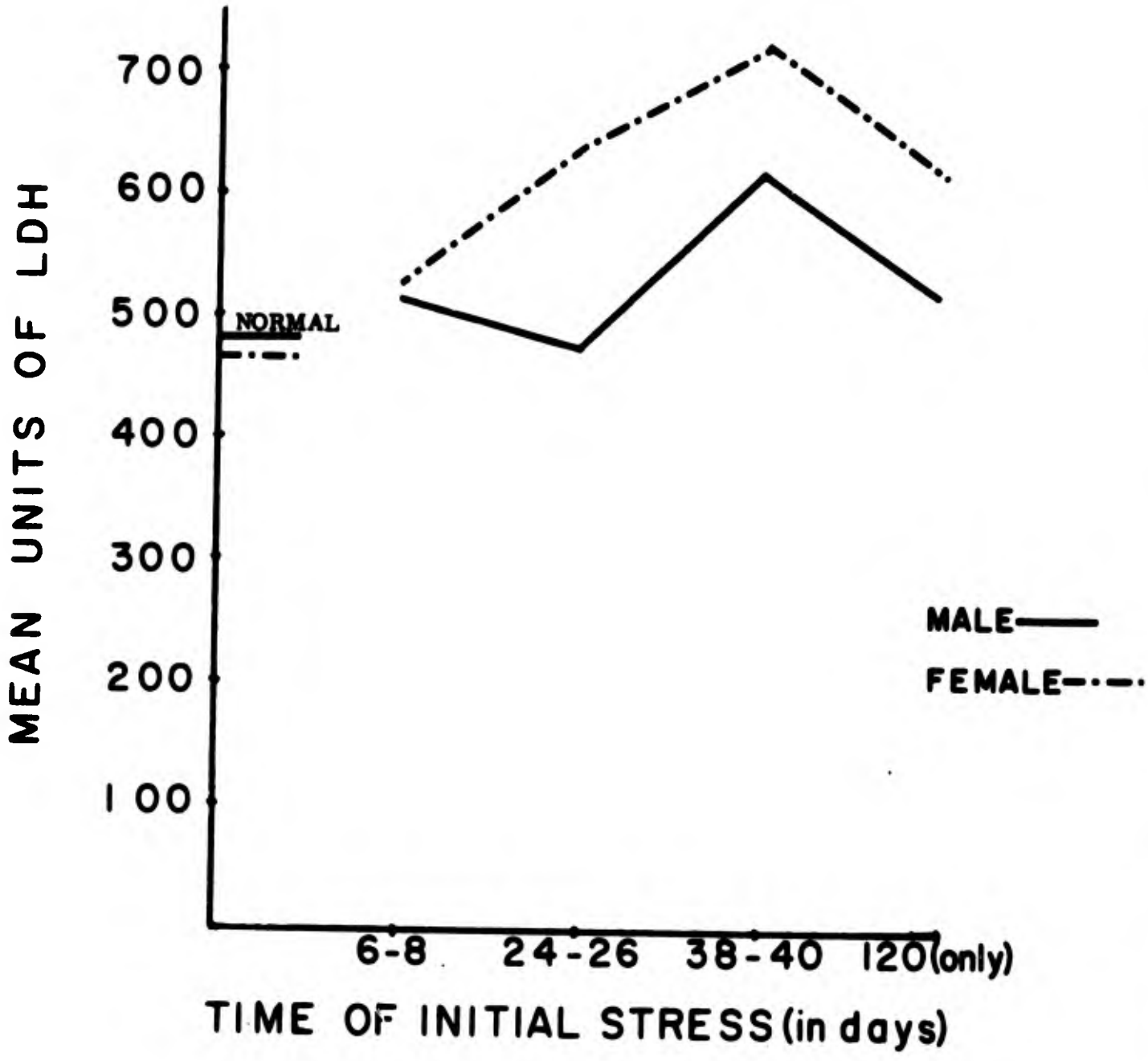

Figure 6. Mean Units of LDH-5 Release for Experimental Groups.

Control Group Differences (Early Stress With no Late Stress)

Figures 7, 8, and 9 show the LDH unit pattern for experimental groups 1, 2, and 3, respectively, as compared to their particular control groups (no late stress). With increase in time of initial stress, more differences between the experimental and control groups are apparent, with experimental values being greater in all cases for the 38-40 day experimental groups as compared to their controls. This increase in differentiation, mostly indicated by an increase in unit release among experimental groups, probably reflects the higher amounts of cardiac and hepatic and/or skeletal muscle damage seen in Figures 2, 5, and 6. As would be expected, (from Figs. 7-9), most of the increase in unit release is exhibited by LDH-1, LDH-4, and LDH-5, a fact which reinforces the above explanation.

These findings support the work of Selye (Ref. 44), who found that prior immobilization, as well as a number of other systemic and drug stressors, seemed to have a "protective effect" when Ss were exposed to a technique which caused topical tissue injury. The findings of Garbus, et al. (Ref. 37) are also borne out by the data. These investigators found that prior exercise stress tends to protect the rat from the damage of prolonged exercise, as indicated by the serum isoenzymes of LDH; whereas non-exercised controls showed a significant serum LDH elevation when exposed to the stress.

The data presented here seem to indicate this same type of "protective effect" of prior stress with regard to tissue damage as a result of a later stressor. Importantly, however, these data link this phenomenon with that of the "critical periods" findings. Not only do the data show the protective effects of early stress in general, but they seem to depend on a particular period of development of the organism during which stress is induced. Specifically, these findings indicate that, for the rat, the earlier this prior treatment, the less damage to cardiac and possibly hepatic (and/or skeletal muscle) tissues occurred when the organism was exposed to a stressor in adulthood. Also apparent age-specific differences with regard to these protective effects have been indicated.

What mechanism is operating within the organism to alter cell permeability, thus releasing these isoenzymes into the bloodstream? The theory that tissue hypoxia, known to occur after prolonged exercise, results in serum LDH elevations has been supported by Highman and Altland (Ref. 45). It is

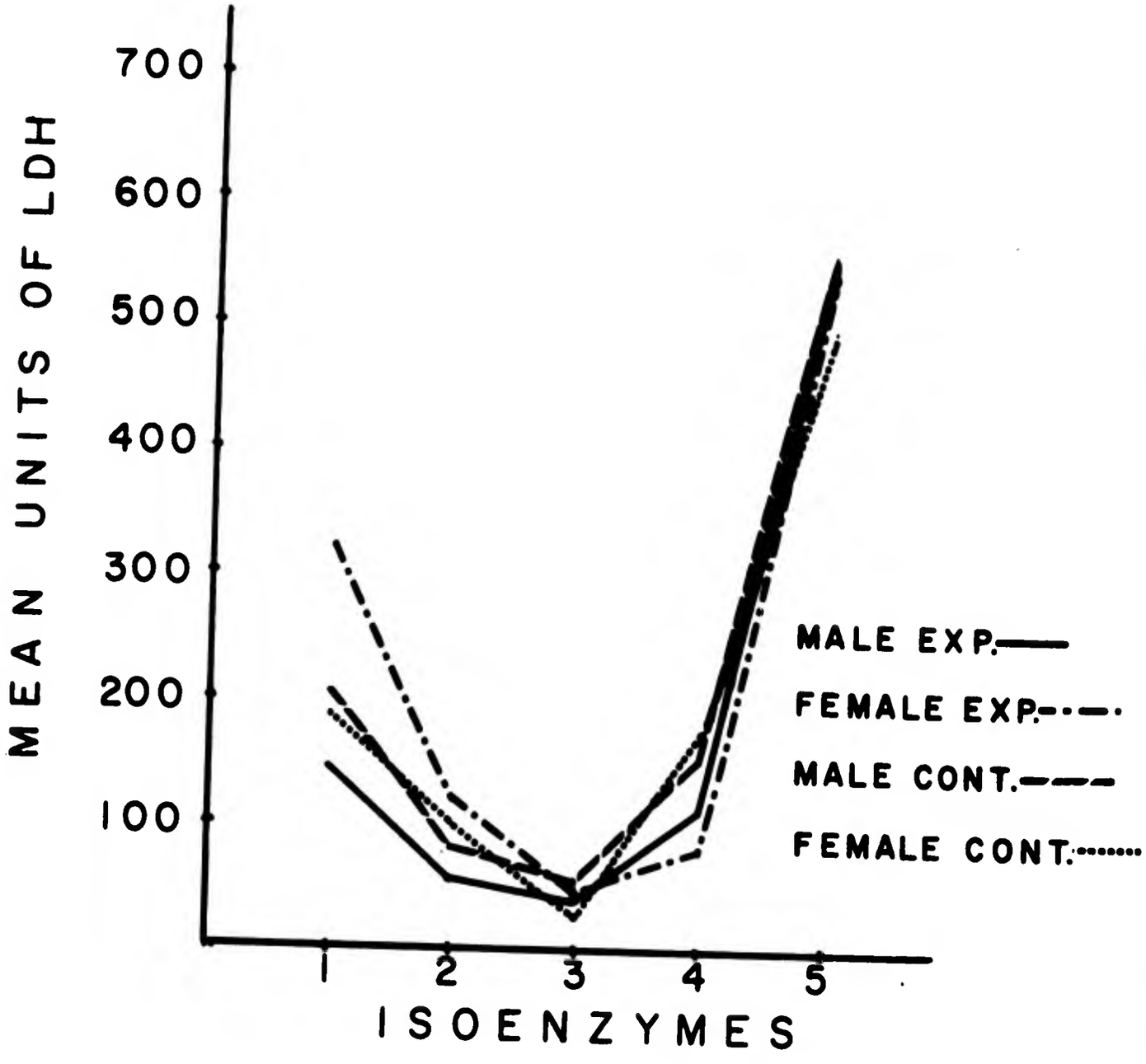

Figure 7. Comparison of LDH Isoenzyme Unit Pattern Between Experimental and Control Groups: Groups 1 and 4.

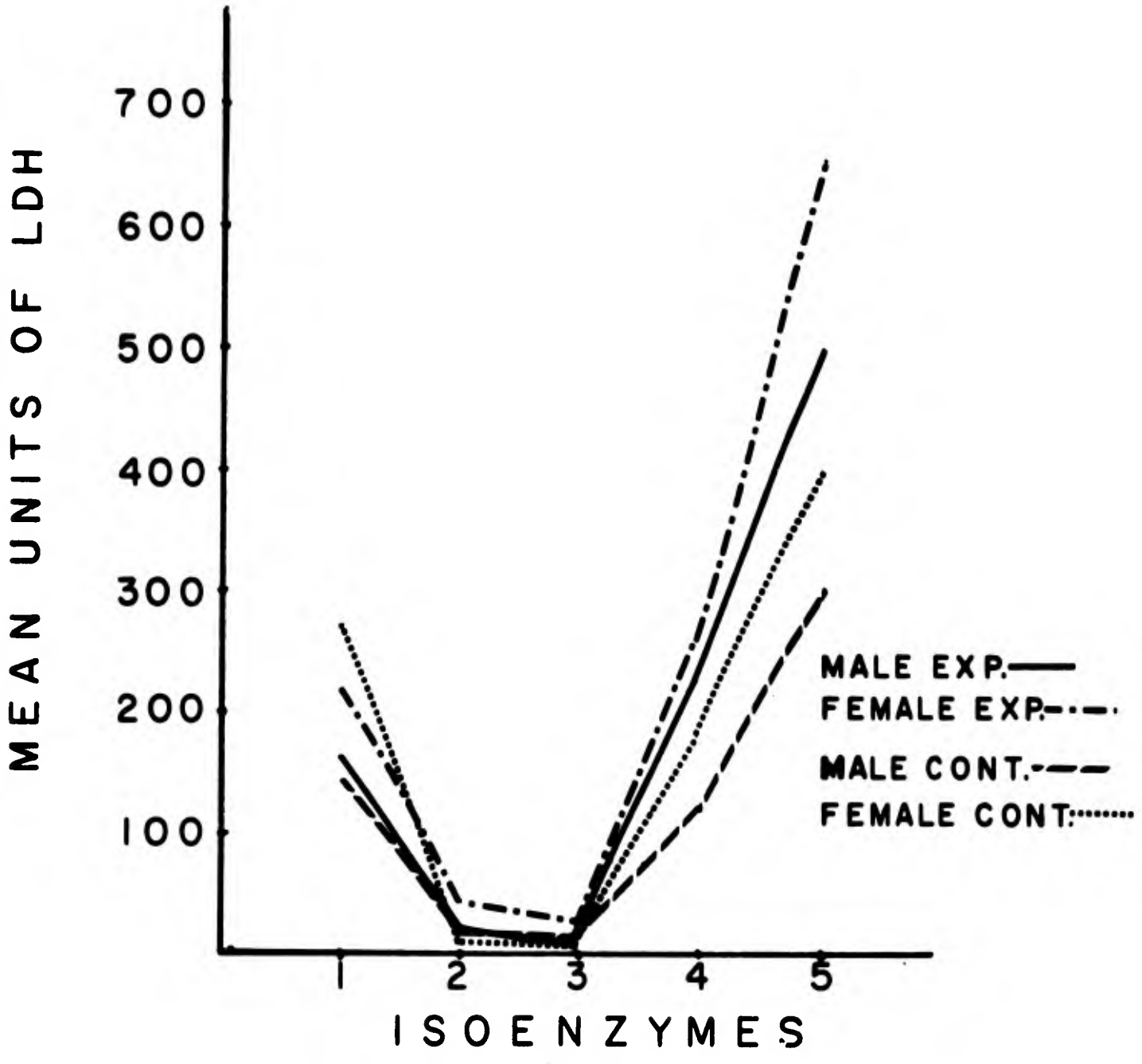

Figure 8. Comparison of LDH Isoenzyme Unit Pattern Between Experimental and Control Groups: Groups 2 and 5.

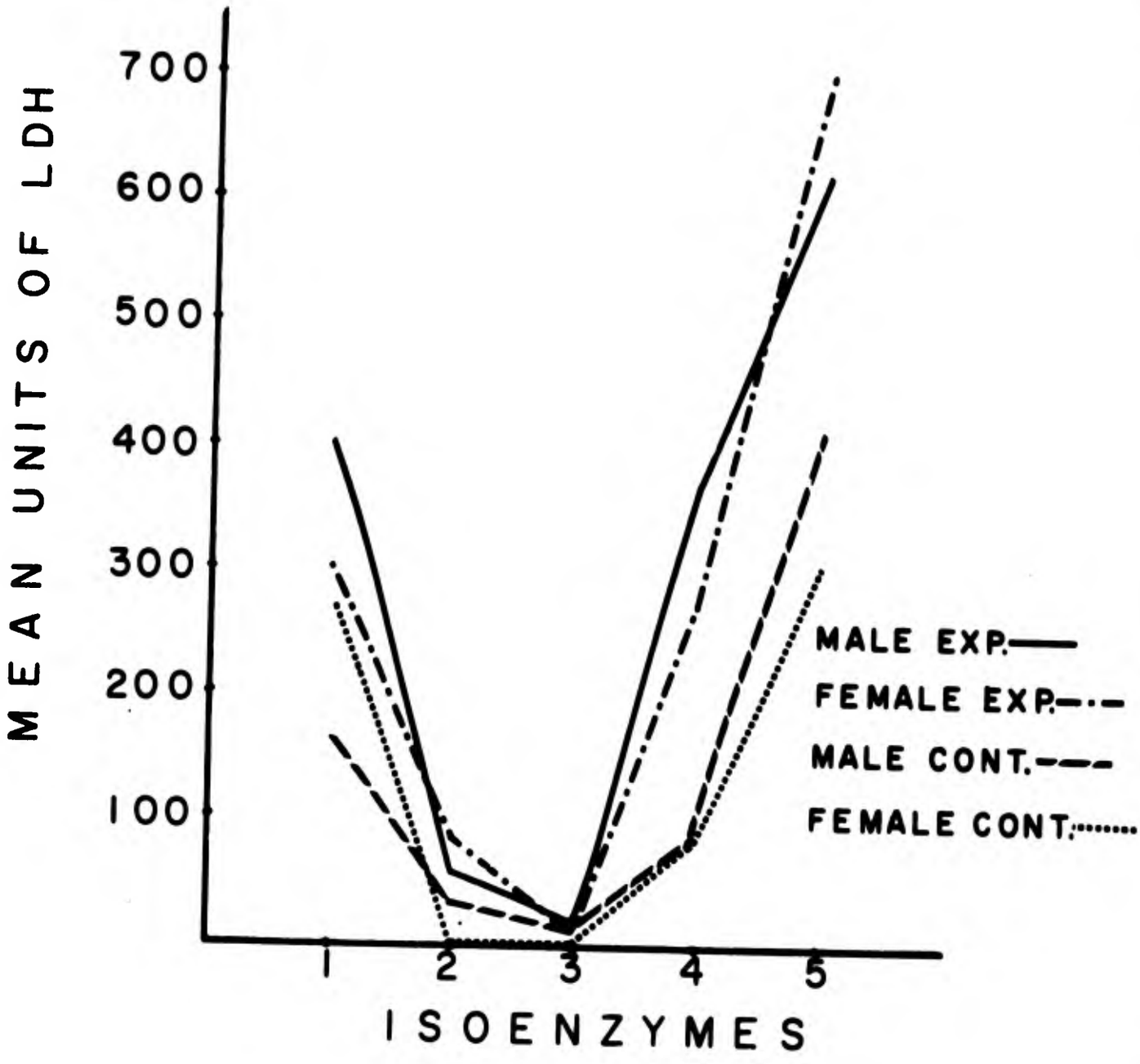

Figure 9. Comparison of LDH Isoenzyme Unit Pattern Between Experimental and Control Groups: Groups 3 and 6.

conceivable that under conditions of immobilization, in which the organism must physically struggle for freedom of movement, tissue hypoxia of the cardiac tissues and/or skeletal muscles could occur, due to increased oxygen requirements. Release of catecholamines from the adrenal medulla has been shown to alter cellular permeability, and thus allows a release of enzymes (Highman, et al, Ref. 46). Possibly the increase in LDH unit release with time of initial stress is a reflection of increased adrenal involvement acting on organ tissues.

It is important that possible mechanisms for this "protective effect" of early stress be discussed. It may well be that this "protection" is due to a regeneration of previously damaged tissue from more resistant cellular elements. This theory is discussed by Highman and Altland (Ref. 47), who found evidence that necrotic portions of muscle fibers were often replaced by regeneration from less injured portions of the fiber. It is possible that some type of selective regeneration occurs to damaged tissues of an organ, making that organ, in effect, more durable to the effects of a later stressor.

Selye (Ref. 44) found that catecholamines, injected at a prior time, offer almost complete protection against necrosis induced by prolonged interruption of the circulation; while Highman, et al, (Ref. 46) have found that catecholamines alter cell permeability. While apparently opposing each other, these two findings could offer a basis for the selective regeneration proposed above. Upon initial catecholamine injection, or exposure to a rigorous systemic stressor (in which catecholamines would be introduced in significant amounts into the blood), this alteration of cell permeability probably does occur; but a selective regeneration of cellular components, much like that spoken of by Highman and Altland (Ref. 47) could follow. Importantly, cells not initially damaged could be structurally or chemically less susceptible to the effects of this catecholamine-induced breakdown; these less susceptible cells then would selectively regenerate over a period of time. In the case of non-regenerative tissues, a selective growth would then take place.

In place of, or in conjunction with such a "selection" theory, an immunity to catecholamine release could be built up on the receptor cell level, much like the immunity established against certain drugs and antibiotics, so that the organ would respond, up to a certain point, in a less violent fashion with each stress repetition. With an increased inhibition of organ response, less damage would occur.

It is possible also that the initial stress period causes increased vascularization of tissues, so that the particular organ or muscle can operate more efficiently under the adult stress conditions. If damage does occur from extended periods of maximum organ or muscle involvement, (Selye, Ref. 27), then probably efficiency would be negatively correlated with damage from usage to a certain extent.

How can this theory be fitted to the findings presented here that the amount of stress protection due to an early stressor depends on the age in which stress is induced? Hypothetically, it is possible that developing tissues are more susceptible to this selective cell response, so that the earlier the initial stress-induced catecholamine release, the sooner this selectivity takes place in the cells of the organ involved, and thus becomes set at the period of maximum growth for that organ. The later this initial release, the more cellular composition is set, and the more resistant to this selective regeneration it becomes.

In addition to the hypotheses discussed above, a protective mechanism based on the adrenal-activating amount of ACTH released into the bloodstream should be discussed. Shapiro, et al, (Ref. 48) have postulated that control of adrenal cortical function in adult organisms is partially accomplished by means of a homeostatic feedback mechanism or hormonostat. In such a system, the concentration of corticosteroids in the blood is monitored in the central nervous system and compared with a controlling set point. If the concentration of circulating steroids is higher than this set point, ACTH secretion and, consequently, adrenal output diminish. If the concentration is below this set point, then ACTH is released and more steroids are produced. Based on this theory, Levine and Mullins (Ref. 26) have formulated an hypothesis to explain the process whereby handling in infancy can permanently affect the adrenal system so that the adult animal can respond more appropriately. Basically, these investigators feel that early handling, by causing variation in the concentration of adrenal steroids in the infant animal, modifies the set point during a critical time in development so that it can vary in a graded manner in the adult, with several possible values between the maximum and the minimum. In the non-handled, newborn rat there is less variation in adrenal steroid concentration during the critical period, and thus the set point develops fewer possible values. The adrenals then tend to operate in an "all or none" fashion when any degree of stress is induced, as no gradient of response has been set for this homeostatic feedback mechanism at its critical period of

development. With regard to the findings of the present study it is possible that the protective effects of the early stress on cardiac and other organ response to a stressor are a result of "more appropriate" ACTH, and thus adrenal, involvement. This theory could also account for the great amount of organ damage, probably due to increased adrenal release acting on organ involvement, when Ss were exposed to stress at 120 days only. In this case, the adrenals could have been functioning in an inappropriately rigorous fashion, which would be due to a lack of an established gradient of ACTH response resulting from previous early stress.

Also the present findings that the earlier the period of early stress, the less the amount of adult stress damage, seems to agree with this theory. At these earlier periods adrenal release would be acting on the central nervous system in its maximum, or possibly "critical" period of post-natal adjustment to the environment and development, and alterations in response mechanisms could more easily be made and set in, than at a later period.

In attempting to account for the mechanism by which early stress at a certain period causes a particular protective effect under conditions of adult stress, we find ourselves theorizing, at best. It is possible that not one, but a series of mechanisms are operating in some complex fashion to cause this effect. One thing is evident, however, and that is the need for more detailed research on the biochemical and physiological changes that take place within the organism during the early periods of maximum growth in which we find evidence for the "critical period". Unless we know what changes are taking place, and thus are aware of the state of the systems on which we impose a variable, we are making only an observation; important enough, but at best only a reflection of an unknown quantity. The determination of these age-specific processes upon which much of the adjustment of the individual to his environment are made, will certainly give man some insights into the unseparable relationship between his internal processes and his behavior... and possibly a more adequate and beneficial means of interrelating the two.

CONCLUSIONS

This study showed that the earlier stress is induced in the rat, the greater is the protective effect of this initial stress, when the animal is exposed to a stressor in adulthood. Specifically, this protective effect was found to occur in the cardiac tissues, and to a less extent in hepatic tissue and/or skeletal muscle.

In general, no sex differences with regard to isoenzyme response to stress at different periods of development were found; but age-specific differences between males and females, thought to be due to the females' hormonal system, were found for LDH-1, and to a lesser extent for LDH-4 and LDH-5.

Significant differences were also found between groups stressed only during their early development, and groups stressed at the same times and stressed in adulthood, with significant interactions between time of early stress and the variable of late or no late stress. An increase in differentiation between experimental and control groups was found to occur with an increase in time of initial stress. This differentiation, seen as increases of LDH-1, LDH-2, and LDH-5, was thought to be the result of increased tissue damage. Also stress in adulthood, with no prior early stress, was found to elicit an organ-specific LDH unit release.

Theories of cellular selectivity and ACTH-controlling mechanisms were discussed in order to explain the protective effects of early stress.

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APPENDIX I

TOTAL LACTIC DEHYDROGENASE

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REAGENTS

1. Pyruvate Substrate - Sigma Stock No. 500L-1

   Stable indefinitely when refrigerated even after several years of storage.

2. Color Reagent - Sigma Stock No. 505-2

   Standardized 2.4 dinitrophenyl hydrazine. Stable indefinitely when refrigerated.

3. B-KPNH preweighed (1.0 Mg/vial) - Sigma Stock No. 340-101

   Dry, stable form. Stable at room temperature when dry and dark.

4. 0.40 N NaOH -

   16 gm NaOH ---- 1 liter. Standardize accurately. Keep free of CO_2. Store in small polyethylene bottles to avoid frequent exposure to atmosphere.

TEST PROCEDURE

1. Prepare 1:6 dilution of serum by diluting 1 part of serum with 5 parts of water.

2. Accurately pipette 1.0 ml substrate (Sigma 500 L-1) into bottom of a B-DPNH vial (Sigma 340-101). Place in water bath at 37^{O} C for 5 minutes to equilibrate.

3. Add 0.10 ml of diluted serum. Shake gently; note time, replace in the water bath. (If perform more than one determination, stagger timing 1/2 - 1 minute apart.)

4. Exactly 30 minutes after adding sample, remove vial from water bath. Add 1.0 ml color reagent (Sigma 505-2) to each vial. Shake well by swirling (stops reaction and starts color development). Leave at room temperature ($25^{o}C \pm 5^{o}C$).

5. Twenty minutes after adding color reagent, add 10 ml 0.40 N NaOH to each vial. Cap and mix well by inversion. Transfer to cuvettes.

6. Wait at least 5 minutes after adding NaOH (but no more than 30 minutes) and read OD (or T percent) vs. H_2O as blank at wave length (540 millimicrons) used in preparation of calibration curve.

7. Determination LDH value from calibration curve.

APPENDIX II

LACTIC DEHYDROGENASE ISOENZYMES BY ELECTROPHORETIC SEPARATION ON CELLULOSE ACETATE

Lactic Dehydrogenase Isoenzymes by Electrophoretic Separation on Cellulose Acetate

(* May be performed simultaneously with Serum Proteins using same Buffer and equipment.)

REAGENTS

A. Stock Solutions: (*Sigma Chemicals)

1. Phosphate Buffer: 0.1 m, pH 7.5 Store at 4°C.

2. Substrate: Sodium lactate, 1M; dilute 60 per cent syrup (5 M) 4:1 with 0.1 M Phos. Buffer, pH 7.5.

3. -Diphosphopyridine nucleotide (B -DPN, B -NAD) Stored at -10°C dessicated in 10 mg. aliquots.

4. Phenazine methosulfate, 1 mg/ml in dist. H_2O and store at 4°C. Caution--highly unstable--make up this reagent in subdued light and store in dark container. Probably stable for 1-2 weeks, although it is advisable to make up 5 ml immediately before use, and discard.

5. Nitroblue Tetrazolium, 1 mg/ml in dist. H_2O and store at 4°C. Also light sensitive--make up in subdued light and store in dark container. Stable for approx. 1 month.

6. Fixing Solution: 50 per cent methanol, 40 per cent dist. H_2O, and 10 per cent glacial acetic acid. It is advisable to make up 200 ml. immediately before use, and discard.

7. Electrophoresis Buffer: Barbital-barbituric acid, pH 8.6-8.8, ionic strength = 0.05.

B. Working Solution: Prepare fresh daily from above, avoiding exposure to light. Add reagents in the following order to aliquot of -DPN.

1. 1 ml of 1M Sodium lactate
2. 1 ml of 0.1 M Phos. Buffer
3. 3 ml of Nitroblue Tetrazolium
4. 0.3 ml of Phenazine Methosulfate

Invert the mixture several times, then place in shallow dish for use.

PROCEDURE

A. Electrophoresis

1. Soak strips in Buffer, and upon removal, blot strips between two absor-sheets.

2. Apply a 10-12 sample of serum (or plasma)--this should be 3 applications on the same place on the strip-- 1.5-2 cm on the cathode side of the center of the strip.

3. Place in chamber and electrophorese for 90 min. with a current of 1-1.5 ms. per strip (about 200-220 volts).

B. Staining

1. While electrophoresis is underway, prepare a clean glass plate, marked off to 8 separations with a wax crayon, and place it in a large chamber containing one large sheet of moist blotter paper.

2. Prepare fresh working solution immediately before expected use, and place in a shallow dish.

3. On completion of electrophoresis, remove the acetate strips one at a time and soak completely in the working solution. Then blot the wet strips between 2 pieces of absorbant blotting paper, and place them sample application side down on the glass plate. Cover the chamber

with cellophane or saran wrap and place chamber in incubator at 37°C for 30 min-2 hrs. (It was found that the anodal fractions are developed more completely with the longer development time.)

4. After development, remove strips and place them in fixative for 10 minutes. Then place strips on glass plate (siliconized) with the ends of each strip secured under glass slides, and allow to dry at room temp.

C. Quantitation

The test strip may be quantitated after drying by scanning the opaque strip (550 mu filter) or by cutting out each fraction and dissolving each in 2 ml acetone, reading OD at 400 mu.

Total LDH must be determined before actual amount of each isoenzyme may be calculated. However, percentages of total may be obtained for each fraction without Total LDH.

RESULTS

5 fractions are found: Fast (lab. and glob. regions) are cardiac; Slow (slowest glob.) are hepatic. Avoid hemolyzed sera--show elevated fast fractions.

APPENDIX III

TOTAL LDH UNITS

TOTAL LDH UNITS

	6-8	24-26	38-40	NO STRESS
LATE STRESS	790	985	1370	1375
	745	540	950	1270
	800	685	1490	1460
	960	1265	1890	1225
	800	820	1070	955
	915	980	1800	1475
NO LATE STRESS	970	335	570	865
	650	460	640	940
	860	800	950	835
	1140	590	590	850
	1200	722	560	895
	1250	332	740	625

MALES

	6-8	24-26	38-40	NO STRESS
LATE STRESS	1180	940	1970	895
	1190	945	1180	2200
	1420	1340	1230	1380
	890	1200	920	1230
	830	1100	1520	1275
	975	1500	1465	1810
NO LATE STRESS	1212	640	515	870
	430	665	440	705
	518	600	860	562
	618	680	320	830
	1320	650	830	1000
	1360	660	890	850

FEMALES

LDH-1 (Units)

MALES

	6-8	24-26	38-40	NO STRESS
LATE STRESS	154.8	180.3	332.9	622.9
	146.8	83.7	294.5	415.3
	192.0	202.1	365.1	865.8
	208.3	182.2	483.8	655.9
	116.0	209.1	452.6	442.2
	92.4	104.9	482.4	618.0
NO LATE STRESS	90.2	133.6	157.3	224.0
	199.6	102.9	145.3	82.7
	169.4	131.7	137.8	80.2
	190.4	158.3	113.3	216.8
	270.0	170.4	161.3	193.3
	366.3	132.8	225.7	76.9

FEMALES

	6-8	24-26	38-40	NO STRESS
LATE STRESS	433.1	127.8	411.7	479.7
	298.7	189.9	310.3	668.8
	271.2	241.2	205.4	505.0
	271.5	292.8	227.2	343.2
	278.1	147.4	205.2	559.7
	445.6	226.5	358.9	832.6
NO LATE STRESS	276.4	130.6	210.0	244.8
	83.4	158.9	212.5	140.1
	111.9	89.4	321.6	173.4
	69.8	129.9	164.5	134.3
	410.5	93.6	302.9	135.3
	183.6	149.8	403.2	164.0

LDH-2 (Units)

	6-8	24-26	38-40	NO STRESS	
	35.6	0.0	6.9	19.2	
	73.8	25.9	16.2	38.1	
LATE	33.6	26.7	4.5	54.0	
STRESS	61.4	53.1	47.3	67.5	
	36.0	0.0	20.3	30.6	
	17.4	27.4	88.2	64.9	
					MALES
	0.0	10.0	0.0	64.0	
	70.9	12.0	0.0	10.3	
NO LATE	46.4	27.6	29.5	14.2	
STRESS	0.0	24.1	0.0	73.9	
	159.6	21.9	0.0	60.0	
	136.3	8.6	88.8	10.6	

	6-8	24-26	38-40	NO STRESS	
	99.1	16.9	106.4	73.4	
	44.0	60.5	97.9	108.0	
LATE	113.6	42.8	6.2	96.4	
STRESS	93.5	63.6	151.8	33.2	
	77.2	26.4	18.2	136.4	
	189.2	46.5	79.1	74.2	
					FEMALES
	0.0	9.0	0.0	91.8	
	29.7	8.6	0.0	26.1	
NO LATE	18.1	18.6	0.0	45.8	
STRESS	69.8	10.9	0.0	25.9	
	302.3	0.0	0.0	27.4	
	53.0	44.9	0.0	37.0	

LDH-3 (Units)

	6-8	24-26	38-40	NO STRESS
LATE STRESS	19.8	0.0	0.0	0.0
	39.5	17.8	0.0	0.0
	17.6	10.3	22.4	20.4
	67.2	0.0	43.5	45.0
	29.6	0.0	10.7	26.7
	17.4	25.5	18.0	10.3
NO LATE STRESS	0.0	18.8	0.0	142.4
	39.0	10.7	17.9	4.7
	44.7	15.7	39.9	0.0
	0.0	8.7	0.0	45.9
	117.6	22.4	0.0	58.2
	67.5	11.8	40.7	0.0

MALES

	6-8	24-26	38-40	NO STRESS
LATE STRESS	35.4	38.5	25.6	79.7
	13.1	41.6	22.4	99.0
	95.2	38.9	0.0	20.6
	72.9	0.0	62.6	22.1
	0.0	30.8	13.7	90.5
	79.0	39.0	38.1	45.2
NO LATE STRESS	0.0	0.0	0.0	28.1
	4.3	11.3	0.0	19.1
	33.2	6.6	0.0	61.3
	0.0	0.0	0.0	9.6
	122.8	0.0	0.0	15.8
	32.6	16.5	0.0	27.0

FEMALES

LDH-4 (Units)

	6-8	24-26	38-40	NO STRESS	
	16.6	303.4	408.2	247.8	
	96.1	109.1	218.5	137.5	
LATE	129.6	141.1	439.6	129.5	
STRESS	30.7	432.6	483.8	30.7	
	77.6	89.4	112.4	22.5	
	161.9	204.8	504.0	46.8	
					MALES
	169.8	110.3	50.7	128.0	
	65.0	150.4	79.4	177.7	
NO LATE	119.5	89.2	225.2	146.9	
STRESS	297.5	30.8	109.2	125.8	
	133.2	38.8	48.2	159.3	
	76.3	198.6	34.8	127.5	

	6-8	24-26	38-40	NO STRESS	
	28.3	181.5	457.1	34.9	
	220.2	164.4	213.6	341.0	
LATE	80.9	353.8	496.9	62.0	
STRESS	22.3	267.6	138.9	143.9	
	6.6	290.4	107.9	14.0	
	18.5	265.5	156.7	114.0	
					FEMALES
	246.0	122.9	38.7	98.6	
	27.1	138.3	40.9	67.0	
NO LATE	75.6	160.2	141.0	112.1	
STRESS	131.6	90.4	24.0	110.7	
	128.0	146.9	139.4	130.3	
	349.5	363.0	164.6	131.0	

LDH-5 (Units)

	6-8	24-26	38-40	NO STRESS	
	563.2	501.3	622.0	595.4	
	388.8	303.5	420.8	687.1	
LATE	427.2	304.8	658.4	489.1	
STRESS	592.4	597.1	831.6	334.1	
	540.8	521.5	474.0	408.7	
	625.9	617.4	707.4	534.0	
					MALES
	710.0	362.4	362.0	406.6	
	275.5	157.7	397.4	664.6	
NO LATE	480.0	132.7	517.6	593.7	
STRESS	652.1	176.2	367.5	387.6	
	519.6	444.8	350.0	424.2	
	603.6	325.1	350.0	410.0	

	6-8	24-26	38-40	NO STRESS	
	584.1	575.3	969.2	227.3	
	614.0	488.6	535.8	983.2	
LATE	859.1	663.3	521.5	695.0	
STRESS	429.8	576.0	339.5	687.6	
	468.1	605.0	1175.0	474.4	
	242.7	922.5	832.2	744.0	
					FEMALES
	689.6	377.5	266.3	617.7	
	285.5	349.7	186.6	312.4	
NO LATE	279.2	325.2	397.4	281.5	
STRESS	346.8	448.8	131.5	521.2	
	356.4	409.5	387.7	641.0	
	741.3	363.0	322.2	386.7	

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13. ABSTRACT

A study was performed to determine the effects of differential early stress (immobilization) upon the LDH response in rats. Ninety-six hooded rats (48 of each sex) were used as subjects. Three groups of subjects were stressed at ages 6-8, 24-26, and 38-40 days. These three groups were also stressed again at 120 days of age. Three more groups of animals were stressed at identical ages during early development, but not stressed later in life. One group was not stressed early but stressed later in life, and the last group was not stressed at all. The results of the LDH analyses showed that the earlier stress is induced in the rat, the greater is the protective effect of this initial stress, when the animal is exposed again to a stress in adulthood. Specifically, this protective effect was found to occur in the cardiac tissues, and to a smaller extent in hepatic tissues and-or skeletal muscles. No sex differences with regard to LDH-isoenzyme response to stress at different periods of development were found; but age-specific differences between males and females, thought to be due to the female hormonal system, were found for LDH-1 and to some extent for LDH-4 and LDH-5.

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