DNA-Cytophotometry and Histology of Lymphatic Organs in Relation to the Skin Homograft Reaction in Hypoxic Mice

RESEARCH REPORT

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Supported by Public Health Service Grant GM-05112 from the National Institute of General Medical Sciences, National Institutes of Health, and in part by NASA Grant NGR-39-009-015

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INTRODUCTION

Hypoxia acclimation is known to be accompanied by a host of adaptive hemodynamic, circulatory and hemopoietic changes which ameliorate localized effects of oxygen lack at the cellular or tissue level. The extensive literature in this area has been reviewed by Korner (1959), VanLiere and Stickney (1963) and Weihe (1964). For example, among the most marked immediate responses to hypoxia are: (a) a hemoconcentration due to a negative water balance (DeAngelo and Anthony, 1967; Stickney and VanLiere, 1953), (b) increased blood volume (Kreider, 1960; Grieshaber and Anthony, 1968), (c) an increase in tissue vascularization (Oster and Anthony, 1967; Anthony and Kreider, 1961; Stickney and VanLiere, 1953), and (d) myeloid hyperplasia and lymphatic involution (Hunt, 1964; Stickney et al., 1964).

It has been generally accepted that functional changes in hemopoietic organs are critical for successful acclimation since increased erythropoiesis rapidly restores the oxygen carrying capacity of blood, whereas the lymphatic responses are somehow related to the immunological or resistance potential of the hypoxic animal. Significantly, there is very scant information on lymphatic alterations associated with hypoxia exposure in contrast to the numerous reports which deal with hypoxia-induced erythropoietic activity.

For example, several workers have dealt with the problem of immunity and resistance to disease at natural or simulated high altitudes, but have failed to either consider what changes are taking place in the lymphatic system or have simply reported average thymic and splenic weight changes without interpreting these in terms of the acclimation process (Altland et al., 1963; Highman and Altland, 1964; Weihe and Hurni, 1964). Also, except for the exploratory work of Trapani (1964), no data are available on the influence of hypoxia on immunological tolerance mechanisms. In short, a review of the available literature makes it readily apparent that the role of the lymphatic system during acclimation has been neglected by workers in the area of altitude physiology. This is understandable since only recently has there been extensive research into the main functions of the lymphatic system and its role in the maintenance of immunological tolerance. Equally important, it is only within the last few years that developments in cytophotometric and transplantation procedures made it feasible to investigate cytophysiological changes in lymphoidal organs.

Thus, one of the aims of the present study was to investigate the relationship between histologic and cytochemical response patterns of the thymus, spleen, and lymph nodes of mice exposed to moderate hypoxia (380 mm Hg). A secondary aim was to determine, by histologic analysis, whether the skin homograft reaction, used as an index of immunologic potential, is affected by hypoxia exposure. Through this combined approach involving microspectrophotometric analysis of Feulgen-DNA profiles in lymphocytes and histologic

analysis of graft rejection patterns between two inbred mouse strains, it was hoped to gain some insight into the role of the lymphatic system during the acclimation process.

GENERAL CONSIDERATIONS

A. Definition of Terms

Hypoxia is a general term that may be applied to all cases of oxygen deficiency. It replaces two previously used terms, anoxemia and anoxia. Anoxemia actually signifies a deficiency of blood oxygen and strictly speaking should be used only in that sense. Anoxia, on the other hand, means "without oxygen," and, for this reason is misleading. Therefore, the best term to describe an "oxygen want" is hypoxia which simply means less than a normal amount of oxygen. Hypoxia can be classified into four major types depending on the mechanisms involved in producing an oxygen deficiency: (a) anoxic hypoxia defined as a lowered oxygen concentration in arterial blood, such as occurs when hemoglobin is not completely saturated; (b) anemic hypoxia which results from a shortage of functional hemoglobin; (c) stagnant hypoxia due to a diminution in local or generalized circulatory rate; and (d) histotoxic hypoxia in which body cells, because of poisoning, are not able to utilize oxygen even though the amount of oxygen in the blood may be quite normal (VanLiere and Stickney, 1963). None of these subclasses exactly describes the condition in animals exposed to a moderate reduction in barometric pressure. This stems largely from the rapidity with which compensatory cardiovascular adjustments

occur in the first week of hypoxia exposure. Thus, mice subjected to reduced oxygen concentrations in inspired air should properly be designated as simply hypoxia-exposed animals.

Tissue grafts may be divided into four classes: (a) autograft, a tissue transplant from one site to another in the same individual; (b) isograft (syngeneic graft), a tissue transplant between animals of the same inbred strain; (c) homograft (allograft), a transplant from one inbred strain to a different inbred strain, both of the same species; and (d) heterograft (xenograft), a transplant of tissue between two species. Thus, the skin grafts employed in this study are properly designated as homografts involving strains C57BL/6J and A/J of the laboratory mouse, Mus musculus.

Acclimatization and acclimation continue to be terms used interchangeably despite attempts to restrict the former to natural environmental stimuli and the latter to laboratory situations. Suggested definitions proposed by Prosser (1961) are as follows: (a) acclimatization refers to compensatory changes which occur in response to a complex of environmental factors as in seasonal or climatic changes; (b) acclimation refers to responses to a single environmental factor, as in controlled experiments. Based on these definitions, the term acclimation is used throughout this study.

B. Altitude and Pressure

The total barometric pressure (mm Hg/sq cm) at a particular altitude is equal to the weight of a square centimeter column of

air extending upward to the limits of our atmosphere. Because air is compressible, the pressure decrease is not uniform. For example, in going from sea level to 10,000 ft the pressure decrease is almost ten times greater than that from 60,000 to 70,000 ft; at 100,000 ft, air pressure is about 10 mm Hg; and at 100 miles it is essentially non existent.

The percentage of various constituents in the gaseous environment remains constant from sea level to high altitude. However, a lowering of barometric pressure causes a reduction in the partial pressures of constituent gases and thus in tensions of these gases in inspired air. Proportions of atmospheric gases in volume percent are: nitrogen, 78.10; oxygen, 20.94; carbon dioxide, 0.03; and other gases, 0.93. The discussion below is limited to a consideration of how altitude hypoxia influences tensions of O_2 and CO_2 in various parts of the respiratory tract. For simplicity the partial pressures are expressed as mm Hg rather than mm Hg/sq cm.

At sea level the air pressure is 760 mm Hg. Since oxygen comprises about 21% of the air, its partial pressure at sea level is 159 mm Hg while that of CO_2 is 0.3 mm Hg. Oxygen tension in tracheal air is lower (ca. 149 mm Hg) than that of atmospheric air due largely to a mixing of inspired air with lung water vapor. The alveolar P_{O_2} undergoes a further reduction to 100 mm Hg due to mixing of inspired air with residual air from which oxygen has been absorbed. Alveolar P_{CO_2} has been shown to be 40 mm Hg due to release of CO_2 from hemoglobin in capillary blood. For all practical purposes, tensions of oxygen and carbon dioxide in lung capillaries

are the same as in alveoli. It should be added that the alveolar P_{H_2O} (47 mm Hg) remains relatively constant at all altitudes, being determined entirely by the deep body temperature.

An altitude of 18,000 ft corresponds to the barometric pressure used in the present study (380 mm Hg) which is half that at sea level. In this case, P_{O_2} of inspired air is reduced to 80 mm Hg and drops below 40 mm Hg in alveoli. Alveolar P_{CO_2} is reduced to 30 mm Hg, a drop that is proportionately less than the drop in oxygen tension. This is because alveolar CO_2 tension is not passively dependent upon barometric pressure but is determined by the balance between respiration and metabolism. It is noteworthy that the highest points on the earth's surface at which people permanently reside are found at altitudes of about 18,000 ft. As expected, arterial oxygen tensions of these people are between 29 and 42 mm Hg. Oxyhemoglobin dissociation curves begin to show their steepest slopes in the range of P_{O_2} just below 40 mm Hg. Consequently, small further increases in altitude will make large differences in saturation. Compensation for this by polycythemia may lead to decreased blood flow, particularly through the brain. In other words, bodily mechanisms of acclimation may not adequately compensate for altitudes which approach 20,000 ft (Bard, 1961). On the basis of above considerations most physiologists use pressures which simulate altitudes of about 18,000 ft in research concerned with mechanisms of hypoxia acclimation.

C. The Homograft Reaction and Antibody Production

With the exception of one or two tissues such as the cornea and cartilage, organ or tissue grafts from one person to another do not usually survive in the host for longer than a week or two. In man, only identical twins are excepted from this generalization and, there are now several cases on record of survival of tissues and organs transplanted between identical twins (Brown, 1937; Converse, 1947; Merril et al., 1956; Murray et al., 1958).

This state of affairs is certainly not limited to man for it has been found in all mammals so far investigated. Failure of homografts is not attributable to inadequacies of grafting procedures, such as temporary loss of blood, since it has been repeatedly shown that autografts made in the same manner as homografts do survive (Billingham, 1959).

In grafting relatively large organs such as kidneys it is, of course, necessary to couple the main blood vessels with those of the host if the grafts are to have any chance of survival. However, with small grafts such as thin sheets or pieces of skin close approximation with living, freshly exposed and vascularized mesenchymal tissues of the host is all that is necessary. Through the activity of fibroblasts the host and graft tissues soon grow together, and revascularization, another facet of the healing process, is accomplished within a day or two as the consequence of end to end unions between some of the small vessels of the host and those of the graft. All homografts enjoy at least a short period

of well being during which they are almost impossible to differentiate from autografts. The duration of this period depends upon the amount of foreign tissue grafted; small homografts usually live longer than larger ones, though this dosage effect is weak. The onset of inflammation and swelling in a skin homograft, accompanied at the microscopic level by an infiltration of mononuclear leucocytes (mainly lymphocyte-like cells) from the host, the eventual cessation of its blood supply and its rapid deterioration to a necrotic scab, are all consequences of the host's immunological reaction (the so-called homograft reaction) against the foreign cellular antigens in the graft.

Because of the ease with which they may be grafted and their subsequent accessibility for observation and removal for histological examination, skin homografts have been studied most extensively.

One of the most fundamental advances in homograft research was Medawar's demonstration that skin homograft incompatibility is not an innate but is an immunological phenomenon. Medawar (1957) states "The homograft reaction has three properties which identify it beyond reasonable doubt as an immunological reaction: (a) the existence of a phenomenon superficially akin to the immunologist's secondary response; (b) the existence of a phenomenon analogous to the passive transference of immunity; and (c) the fact that reactivity against homografts can be prevented from developing by taking advantage of a principle, that of immunologic tolerance, which is known to apply to other and entirely orthodox immunological

systems." It should be noted here that immune responses are of two major types: one results in production of humoral antibodies by plasma cells following antigenic stimulation; the other, dependent on sensitized "cell-bound antibody," takes part in delayed hypersensitivity reactions. The homograft reaction is the second type, and though the mechanisms differ, basic to each is production of antibody, interaction of antibody and antigen, and a tissue response resulting from this interaction.

It has become apparent that the lymphoid cell involved in homograft rejection is the small lymphocyte (Gowans, 1965). External drainage of thoracic duct lymph, in which the lymphocyte population is almost entirely of the small cell variety, depletes lymphoid tissue of lymphocytes and decreases circulating lymphocyte counts in certain animals (Gowans, 1962). As the animal runs out of lymphocytes there is prolonged graft survival (McGregor and Gowans, 1964). Lymphocytes, but not hyperimmune serum from an animal sensitized to a graft are capable of passive transfer of sensitization to an indifferent animal (Brent et al., 1962; Billingham and Brent, 1956). Passively sensitized animals then demonstrate accelerated graft destruction. Recent experiments in vitro have supported the idea that direct contact between activated lymphocytes and target cells may be important in the destruction of homografts (Rosenau and Moon, 1961, 1962). Placing grafts in a millipore chamber (which prevents direct contact with host lymphocytes) permits graft survival (Weaver et al., 1955). Furthermore, Govaerts (1960) and Wilson (1963) have demonstrated that in vitro destruction of donor kidney cells by

lymphocytes from sensitized donor rats with skin homografts is associated with a clustering of lymphocytes around the target cells.

It is generally conceded that alterations in lymphoid elements are a reflection of changes in "immunological tolerance" of organisms. The possibility that lymphoid tissue changes may prove to be adaptive has not been considered. Thus, it is important to consider implications of possible changes in the functional capacities of adrenal and lymphatic organs in animals exposed to hypoxia.

Among the changes known to occur when animals are exposed to environmental stress, including hypoxia or reduced pressure, are: (a) increased production of corticoids by the adreral cortex (initiated by ACTH stimulation), and (b) involution of the thymus (Sayers, 1952; Turner, 1966). Of these changes, those occurring in the thymus and related lymphatic organs are intimately associated with immune responses to antigens. In view of the relationship between the lymphatic system and the antigen-antibody reaction of the organism, the clarification of the nature of the responses of the lymphatic system is a mandatory prelude to the complete understanding of altitude physiology.

A current hypothesis of antibody formation centers upon a functional relationship between the thymus, spleen, and lymph nodes. One of the most direct methods of showing that an organ forms or stores antibodies is to demonstrate the presence of antibodies in the organ after injection of antigen. This was shown some time ago to occur in the spleen and lymph nodes, but no one has yet succeeded

in showing raised antibody titre in the thymus (Coons et al., 1951; Burnet, 1962; Miller et al., 1963).

The spleen and lymph nodes have in common a profuse plasma cell proliferation during the formation of antibodies (Ehrich et al., 1949; Burnet, 1962). The role of the plasma cell in antibody formation has been elucidated in vitro (Fagraeus, 1948). The thymus has been shown to contain relatively few plasma cells and no increase in these cells has been observed to correspond with that taking place in lymph nodes and spleen during increased antibody formation. Thus, there are no grounds for assuming that either the incorporation of antigen by thymocytes or significant antibody formation takes place in the thymus (Fichtelius, 1960). The major role of the thymus appears to be the formation of lymphocytes which migrate to the spleen and lymph nodes (Diderholm and Fichtelius, 1959; Burnet, 1962; Murray and Murray, 1964; Murray and Woods, 1964). Thymectomy experiments have confirmed that the part played by the thymus in the general process of antibody formation is the production of cells which colonize the spleen and lymph nodes. It is in these sites, and especially the regional lymph nodes in the case of homografts (Billingham, 1959; Barker and Billingham, 1967), where the cells are transformed into plasma cells or graft rejection cells (Ham, 1965).

In summary, one can safely state that even though much has been learned with regard to immune reactions, the involvement of the lymphatic system in these reactions, and the role of the lymphocyte in these processes, our present understanding of transplantation immunity and hypersensitivity reactions is most imperfect. Much is

still to be learned in connection with the route of sensitization, the exact nature of the antigenic stimulus, the transformation of lymphoid cells to graft rejection cells, and the means by which these cells seek out and destroy the graft.

Furthermore, our knowledge of the adaptive changes of lymphatic organs and the effect these changes may have on immune mechanisms, especially at high altitude, are at best sketchy. Those few reports which have appeared (Smith et al., 1961; Altland et al., 1962; Trapani, 1964) have been somewhat contradictory emphasizing the need for further research into the effects of hypoxia on the lymphatic system and the immune response.

D. Restatement of Aims

The general aim of the present study is to investigate possible relationships between hypoxia exposure, the cytological response pattern of lymphatic organs and the skin homograft reaction in two inbred mouse strains, C57BL/6J and A/J. Specific aims which emphasize three related phases of the overall study are as follows:

1. To determine the extent to which functional alterations occur in selected lymphatic organs during hypoxia acclimation using organ weight and histological analyses as indices of functional involvement.
2. To determine the influence of hypoxia exposure on cell proliferation and patterns of DNA

synthesis in lymphatic organs using cytophometric analyses of Feulgen-DNA profiles of the thymus, spleen and lymph nodes.

3. To determine the extent to which lymphatic responses in hypoxia exposed animals are related to the immune response potential using the skin homograft response as an index of immunological tolerance.

MATERIALS AND METHODS

All experiments in this study were conducted according to the principles outlined in the "Guide for Laboratory Animal Facilities and Care" adopted by the Institute of Laboratory Animal Resources, National Academy of Sciences--National Research Council.

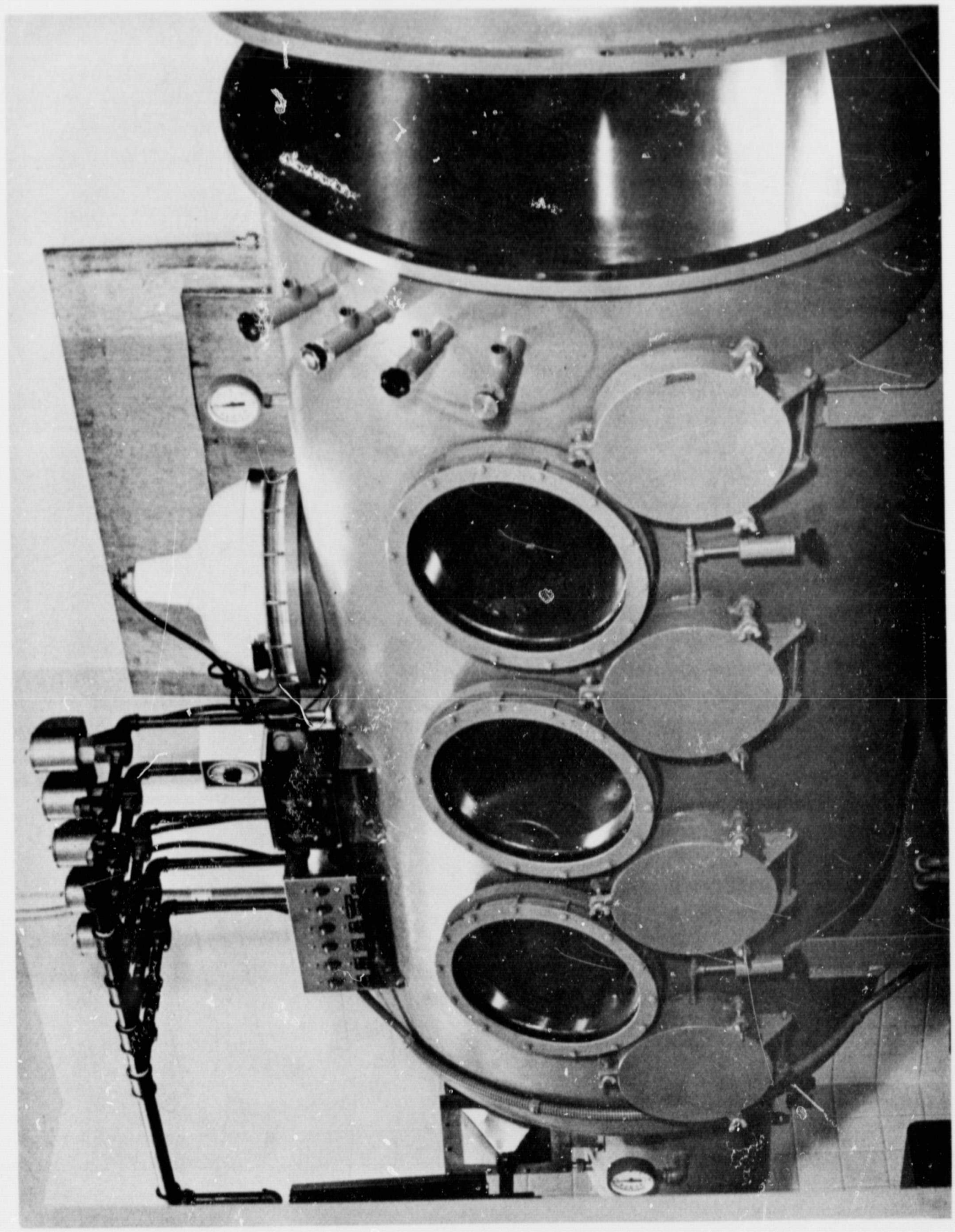
A. Altitude Chamber

The altitude chamber (Blickman Instruments, Weehawken, New Jersey--Figure 1) used was capable of maintaining simulated altitudes up to 25,000 feet above sea level. It had a volume of 42 cubic feet and an air turnover of 15 cubic feet per minute. The chamber had a 12 hour photoperiod and its temperature was 26 ± 1C. During experiments the chamber was maintained at 380 mm Hg which corresponds to a simulated altitude of 18,000 feet above sea level.

B. Animals

A total of 264 adult male mice (strains A/J and C57BL/6J, The Jackson Laboratory, Bar Harbor, Maine) was used in this study. Animals were housed in stainless steel cages (9 3/4 in. x 8 1/4 in.), six mice per cage, a temperature of 26 ± 1C, a 12 hour photoperiod, and given an excess of food and water at all times. Newly received

Figure 1 An outside view of the altitude chamber.



animals were permitted to adapt to laboratory conditions at least one week prior to use in experiments.

Throughout this paper, mice referred to as hypoxic have been exposed to a reduced barometric pressure of 380 mm Hg for one, two, or three weeks depending on the experiment. Animals referred to as "acclimated" have been exposed to reduced pressure (380 mm Hg) for three weeks. Control mice were maintained at the ambient pressure of State College, Pennsylvania (725 mm Hg).

C. Organs and Tissues

At the end of each experimental period, mice were sacrificed and the thymus, spleen and representative brachial and inguinal lymph nodes removed. Organs were weighed on a Roller Smith double hook balance, fixed in buffered 4% formalin for 24 hours and processed using the paraffin technique. All tissues were cut at 8μ and alternate sections stained with Feulgen (Feulgen and Rossenbeck, 1924) or hematoxylin-eosin (Humason, 1967). To minimize staining error, control and experimental sections for any one experimental period were stained simultaneously.

D. Microspectrophotometry

Cytophotometric measurements were made with a single beam microspectrophotometer (Precision Development Corporation, LaPorte, Indiana--Figure 2). Relative amounts of nuclear DNA of thymus, spleen and lymph nodes were determined using the two-wavelength method of microspectrophotometry (Patau, 1952). The two-wavelength

Figure 2 Microspectrophotometer assembly showing:

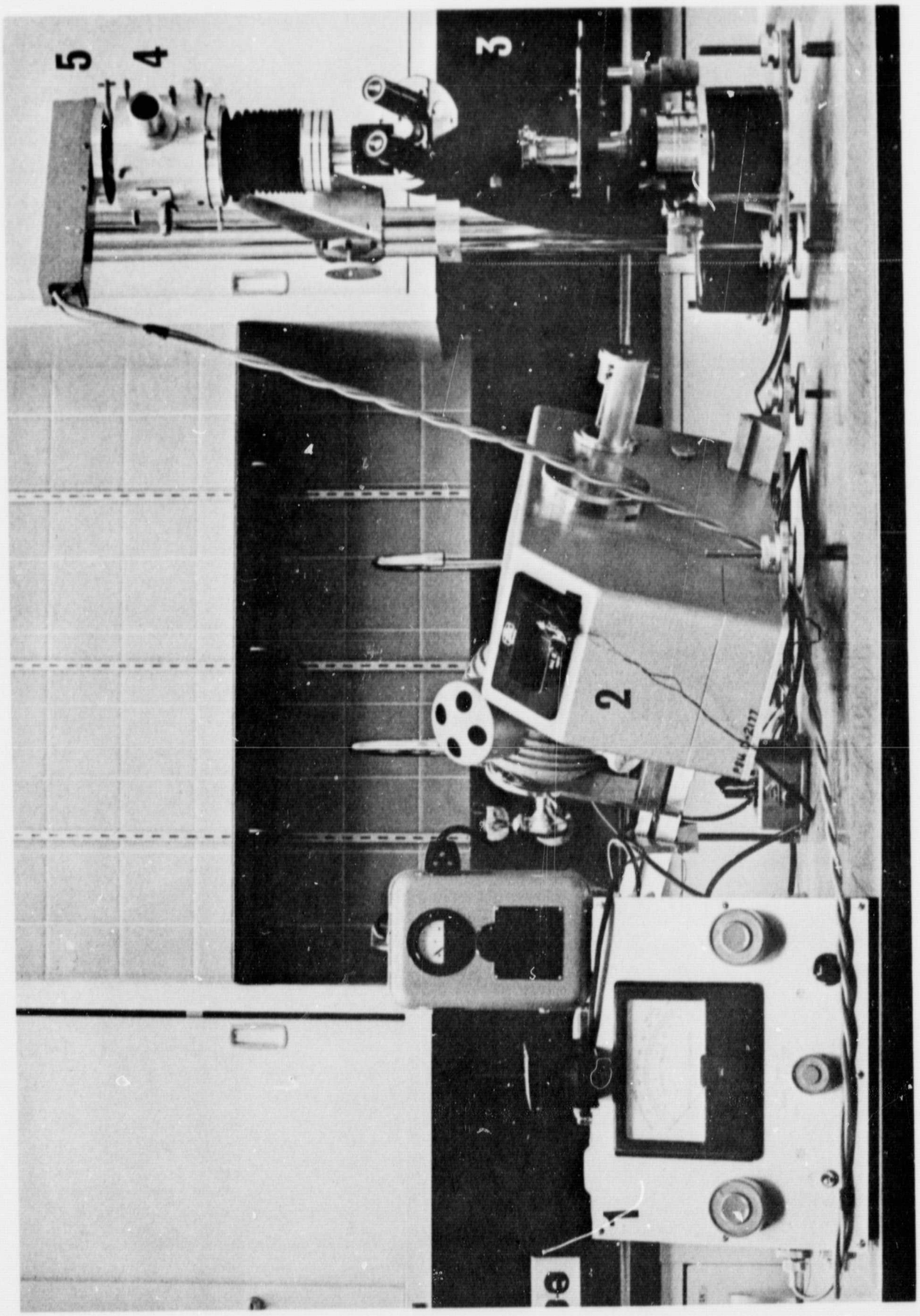
(1) Photovolt power supply and meter

(2) Monochromator and tungsten light source

(3) Microscope with 20X, 0.4 N. A. condenser and 95X objective lens

(4) Photometer head with viewing telescope and fixed apertures

(5) Photodetector (RCA 1P21 photomultiplier).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2

technique is useful when the material being measured is irregular or when it is desired to keep distributional error at a minimum. The procedure for measuring DNA with the two-wavelength method is quite simple. However, since the estimate of absorbing substances depends upon the difference between two low extinctions (or high transmissions), these must be determined with a high degree of accuracy. The illuminated area must be homogeneous, the light as nearly monochromatic as possible, and all chromophores compared must have the same shape of absorption curve (Swift and Rasch, 1956). Since hydrolysis conditions may alter the shape of the Feulgen absorption curve, where Feulgen sections are measured they should all be on the same slide or hydrolyzed and stained at the same time. In practice, a homogeneous area is selected, and two wavelengths are chosen λ_1 and λ_2 such that the extinction (E_1) at one wavelength is half the extinction (E_2) at the other. Thus, $2E_1 = E_2$ where $E_1 = \log I_o/I_s$ at λ_1 and $E_2 = \log I_o/I_s$ at λ_2 and where I_o is the intensity of incident or background light at λ_1 or λ_2 and I_s is the intensity of the incident beam reduced by passing through the specimen. A representative absorption curve for Feulgen stained sections used in this study is shown in Figure 3. After the wavelengths have been properly chosen, inhomogeneous regions may be measured.

The total amount of absorbing material in the measured area (A), regardless of its distribution, is given by $M = KAL_1C$. Here K is a constant ($K = 1/E_1$) where E_1 is the extinction at λ_1, and may be disregarded for relative determinations. From the two

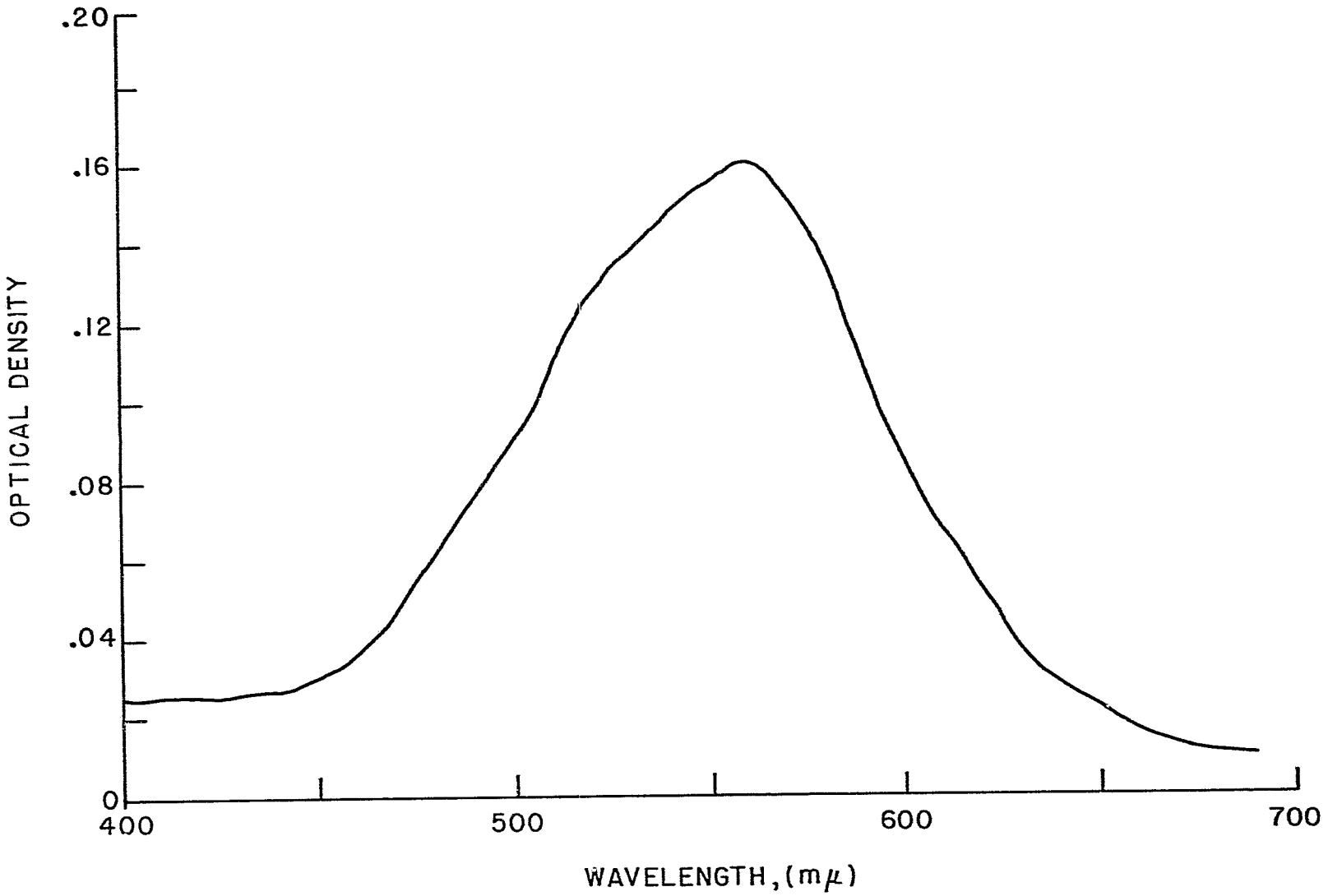


Figure 3 Typical absorption curve for Feulgen stained tissue.

transmissions, T_1 and T_2, obtained from each area, L_1 and L_2 are computed, such that $L_1 = (1 - T_1)$ and $L_2 = (1 - T_2)$. The ratio between these values, Q, is obtained--$(Q = L_2/L_1)$, and the corresponding value for C which is a correction factor is found. Since the extinction ratio at the two wavelengths is known as 2:1, Q permits an elimination of the influence of the unoccupied portion of the measuring area. The function with Q as its parameter is tabulated in the literature (Patau, 1952), and the correction factor, C, is listed for corresponding values of Q. The value of M is a close approximation of the total number of absorbing molecules in the measured area. A similar value would be obtained were the molecules homogeneously distributed throughout area A and measured at λ_2 using the plug technique (Patau, 1952). The two wavelength method has highest accuracy when the wavelengths chosen do not give excessively high or low transmissions so that the values of L_2/L_1 fall somewhere above 1.5. Also, where the object is surrounded by unstained regions, it should be included in as small a measured area as possible, since random errors due to noise become increasingly large as the ratio of unabsorbing to absorbing material increases.

E. Skin Grafting Technique

Skin transplants were made between A/J and C57BL/6J adult male mice. The following combinations of donor and host were used: (a) acclimated donor to acclimated host, (b) unacclimated donor to unacclimated host, (c) acclimated donor to unacclimated host, and (d) unacclimated donor to acclimated host.

After several exploratory operations using a variety of different grafting techniques, a modification of the technique devised by Gottfried and Padnos (1959) was finally adopted.

There are two main factors which may interfere with the technical success of a graft. One deals with the graft, the other with the bed. It is advantageous that the graft size equals the size of the bed so that the cut edges remain in approximation until the graft is firmly adherent. The union of a skin graft and its bed should heal by first intention, i.e., by direct union of immediately exposed raw surfaces. Techniques in which the size of the bed is not constant are subject to criticism simply because there is a disparity between the graft and the bed.

During the grafting operation mice were anesthetized with intraperitoneal injections of Nembutal (5 mg/cc in 20% propylene glycol and 10% ethyl alcohol). The dosage used was 0.1 cc/10 gms body weight. Anesthetized animals were placed on an operating board so that all extremities were held down by elastic bands. An area of the dorsum of each animal was shaved and swabbed with ether. A three-inch strip of surgical tape, No. 1530 (Minnesota Mining and Manufacturing Company), was applied firmly to the back of the animal. The tape, still applied to the skin, was pinched at the midline and a circular full thickness graft 1.27 cm in diameter produced with a Weck Stainless Steel Skin Graft Punch (Weck Instrument Company, Brooklyn, New York). The graft was immediately placed in a sterile petri dish containing Hank's buffered salt solution. This procedure was repeated for another animal and the grafts exchanged between the

two. Thus each animal served as both a donor and a recipient. A four-inch strip of surgical tape No. 1530 was then spread over the tape already on the dorsum of the animal, covering and adhering to the tape affixed to each graft, thereby holding each graft in place. The tape was then drawn tightly together girding the animal just proximal to the lower extremities. Gauze with a small amount of neomycin ointment was placed over the grafted area and another four-inch strip of surgical tape placed over the gauze to hold it in place. The grafting procedure is shown in Figures 4 and 5.

Postoperatively, mice were maintained in individual cages. The bandages were removed after five days. At this time through the proliferative activity of fibroblasts, the cut edge of the graft appeared healed and there was anastomosis of blood vessels between the graft and host. The animals were then observed daily to determine the fate of the graft.

F. Design of Experiments

1. Effect of Hypoxia on the Lymphatic System of Mice

A total of 72 adult male mice (36 A/J and 36 C57BL/6J) of the same age and weight (25 ± 3 g) was used in this series of experiments. Experimental mice were maintained at a pressure of 380 mm Hg for one, two, or three weeks. Hematocrit determinations were made on alternate days by bleeding from the orbital sinuses. That is, on day one all mice were bled from the right orbital sinus and on day three all mice were bled from the left orbital sinus. This

Figure 4 Procedure for making skin homotransplants:

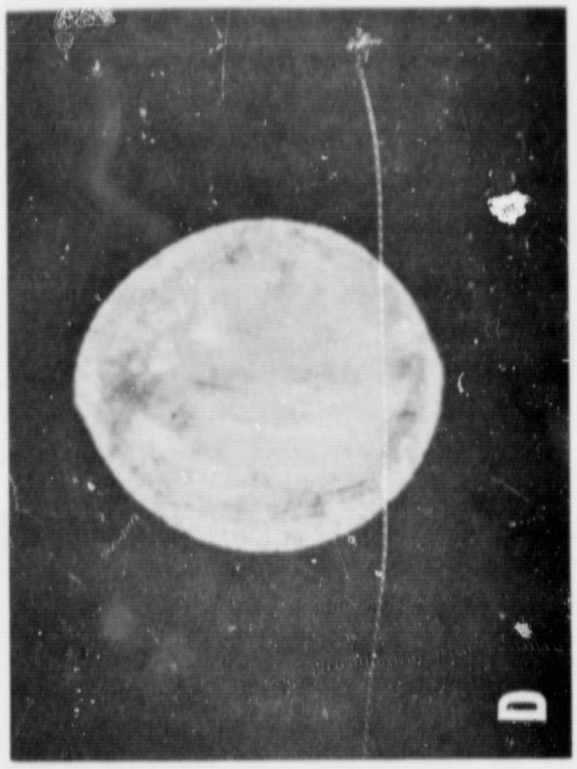
A. Method used in restraining the mouse during the grafting operation and the appearance of the prepared area for the homografts

B. Three-inch strip of surgical tape placed over the shaved area

C. Cutting of circular graft with Weck Skin Graft Punch

D. Appearance of circular graft cut with the punch.


B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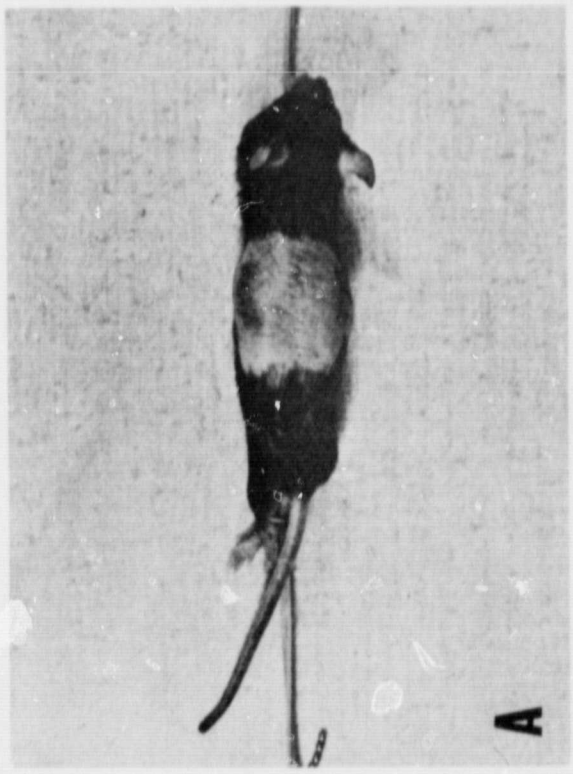
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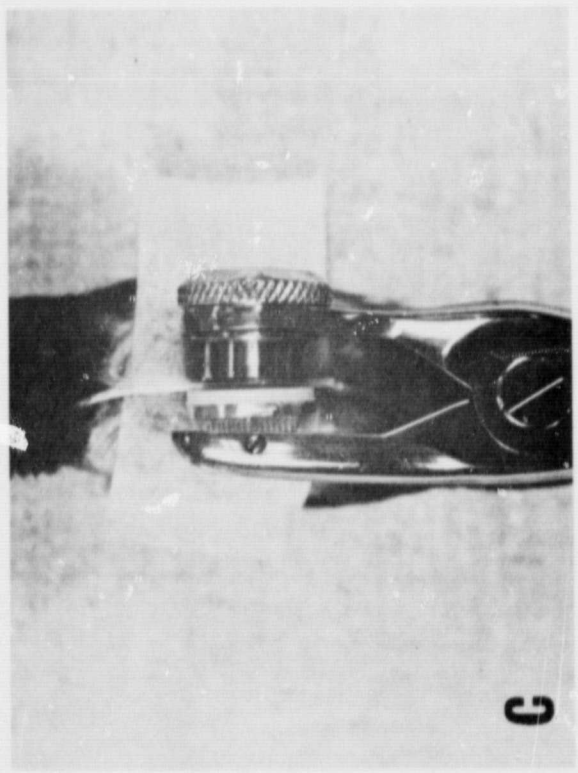
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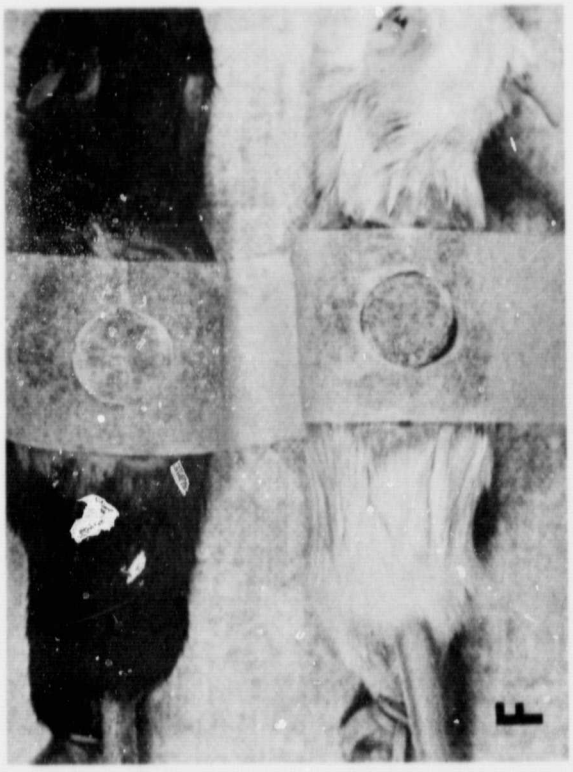
Figure 5 Procedure for making skin homotransplants (continued):

E. Appearance of animal after circular graft is removed

F. Exchange of grafts between A/J and C57BL/6J mice, notice close approximation of graft and bed

G. Protection of grafted area with neomycin ointment and covered with surgical tape

H. Appearance of animals after complete dressing of grafted area.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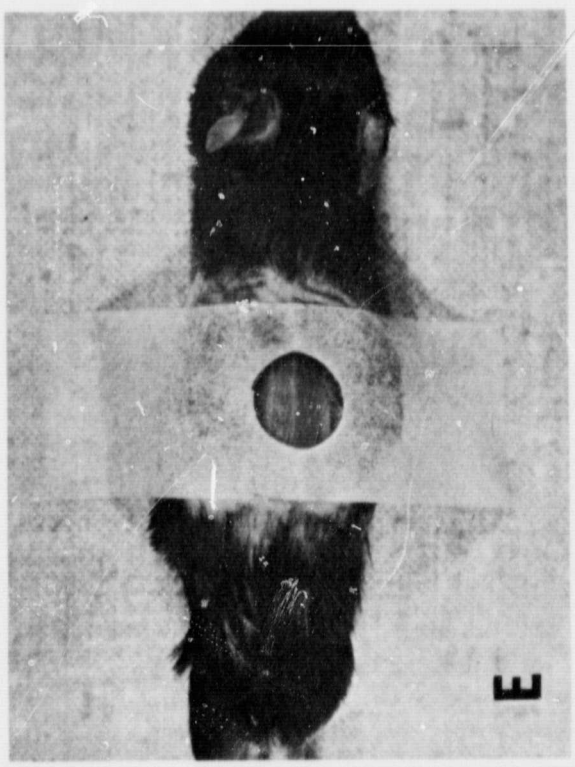
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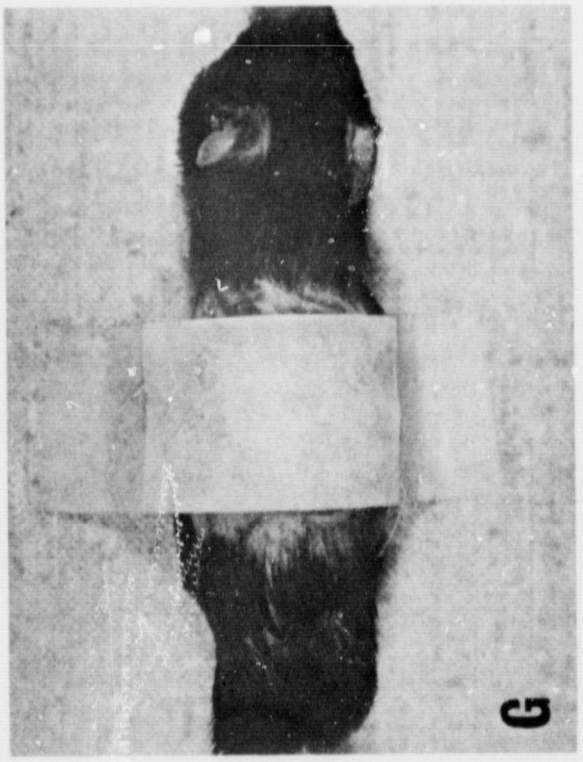
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schedule was followed for the extent of each experimental period. A modified method described by Pansky et al. (1961) was used to obtain blood for hematocrit determinations. A heparinized glass capillary tube (75 mm x 1.2 mm I.D.) was inserted into the eye at the anterior angle formed by the lids and was gently pushed downward into the orbital sinus. The tube was sealed by heat and centrifuged for four minutes in an International micro-capillary centrifuge, Model MB, at 11,500 rpm (13,000 X G) The hematocrit value was then determined from a Critocap micro hematocrit tube reader.

Changes in organs were assessed by changes in weight and by comparison of hematoxylin-eosin stained sections of experimental and control tissues. Relative amounts of nuclear DNA of thymus, spleen, and lymph nodes, used to assess differences at the cellular level, were determined using the two-wavelength method of micro-spectrophotometry and checked using the plug technique. In practice, a minimum of 100 nuclear measures per organ were made for each of the groups of mice used. Thus, the photometric data presented are based on a combined total of more than 4800 nuclear measurements of DNA content.

2. Homografts

A total of 192 adult male mice (96 A/J and 96 C57BL/6J) was used in the homograft experiments. The first series of experiments involving 144 mice was performed to determine the survival time of homografts between acclimated and unacclimated mice. The grafting schedule for this series is shown in Table 1 and is schematically represented in Figure 6.

TABLE 1

SCHEDULE FOR SKIN HOMOGRAFTS BETWEEN A/J AND C57BL/6J MICE

	Donor	Host	n
I.	Donor and Host Acclimated[a]		
	A/J	C57BL/6J	24
	C57BL/6J	A/J	24
II.	Donor and Host Maintained at Ambient Pressure[b]		
	A/J	C57BL/6J	24
	C57BL/6J	A/J	24
III.	Acclimated Donor and Unacclimated Host		
	A/J	C57BL/6J	12
	C57BL/6J	A/J	12
IV.	Unacclimated Donor and Acclimated Host		
	A/J	C57BL/6J	12
	C57BL/6J	A/J	12

[a]Animals maintained at reduced pressure (380 mm Hg) for three weeks prior to making reciprocal grafts.

[b]Animals maintained at ambient pressure of State College, Pennsylvania (725 mm Hg) prior to making reciprocal grafts.

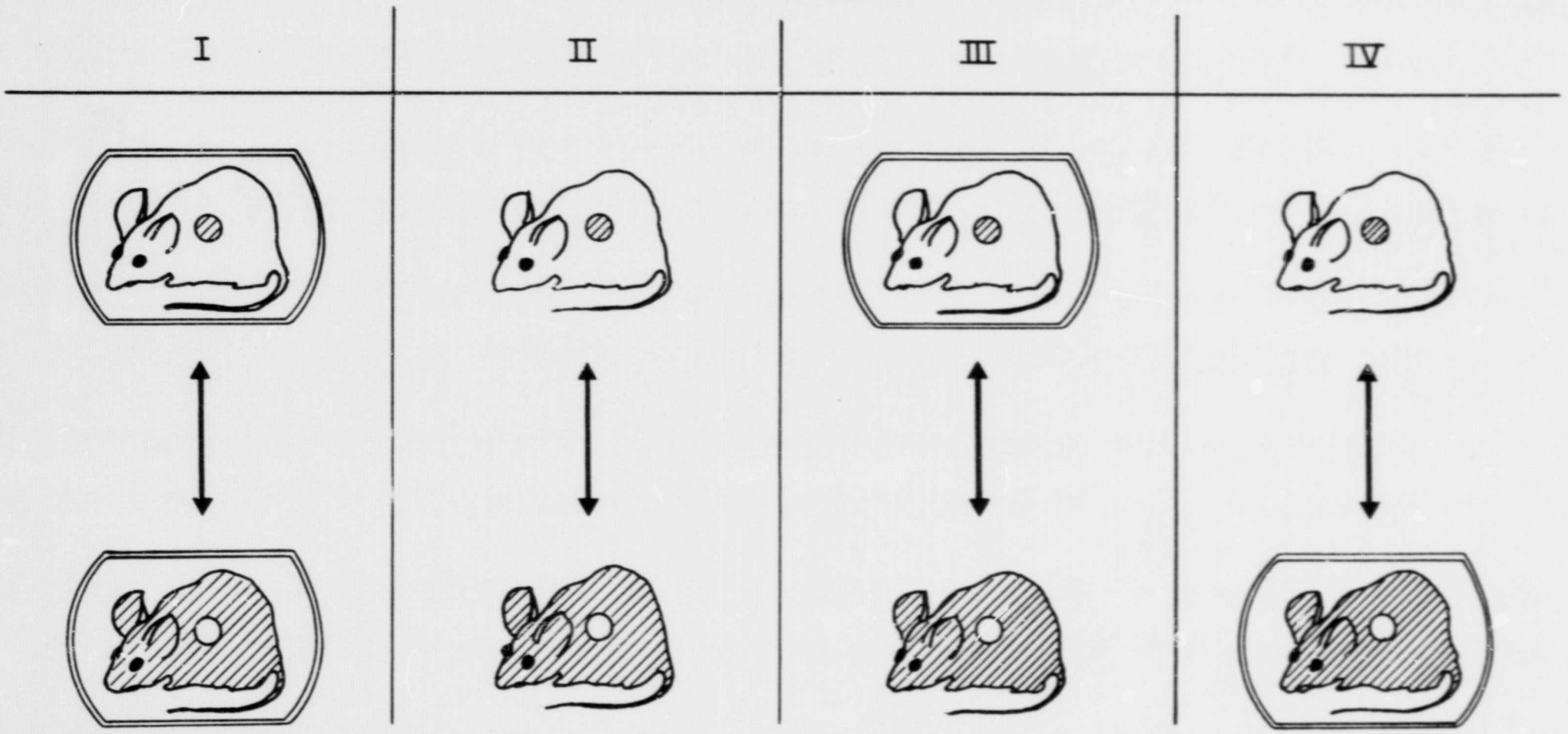

Figure 6 Skin homograft schedule for homografts between A/J and C57BL/6J mice. Animals in chambers represent mice maintained at reduced pressure (380 mm Hg) for three weeks prior to grafting. Animals not enclosed represent mice maintained at ambient pressure (725 mm Hg) prior to grafting.

Histological analysis was used in a second series of experiments using 48 mice to determine if the homograft reaction in unacclimated mice differed from that in acclimated mice. In these experiments homografts were exchanged between 12 acclimated A/J and 12 acclimated C57BL/6J mice, and between 12 unacclimated A/J and 12 unacclimated C57BL/6J mice. On each succeeding day following grafting, up to the day of sloughing the graft, animals from each group were sacrificed. The graft with a small area of the host's skin was removed, placed in 4% formalin, and processed in paraffin. Sections were cut at 6μ and stained with hematoxylin-eosin for subsequent histological analysis.

RESULTS

The major findings of this study are presented in four major subdivisions:

A. Response of Lymphatic Organs to Hypoxia,

B. Hematocrit Changes,

C. Cytophotometry of Lymphatic Organs Exposed to Hypoxia, and

D. Homograft Reaction in Acclimated and Unacclimated Mice.

A. Response of Lymphatic Organs to Hypoxia

1. Organ Weight Changes

Thymus weights of both A/J and C57BL/6J animals after one, two, or three weeks of exposure were significantly lower than controls (Figures 7 and 8). A/J mice exhibited the greatest loss of weight after one week and then began to recover, but after three weeks the thymus weight was still significantly lower than that of controls. The thymus weight change of C57BL/6J mice, however, was greatest after three weeks of exposure to 380 mm Hg. In these animals there was a steady decline in thymus weight during the three week experimental period.

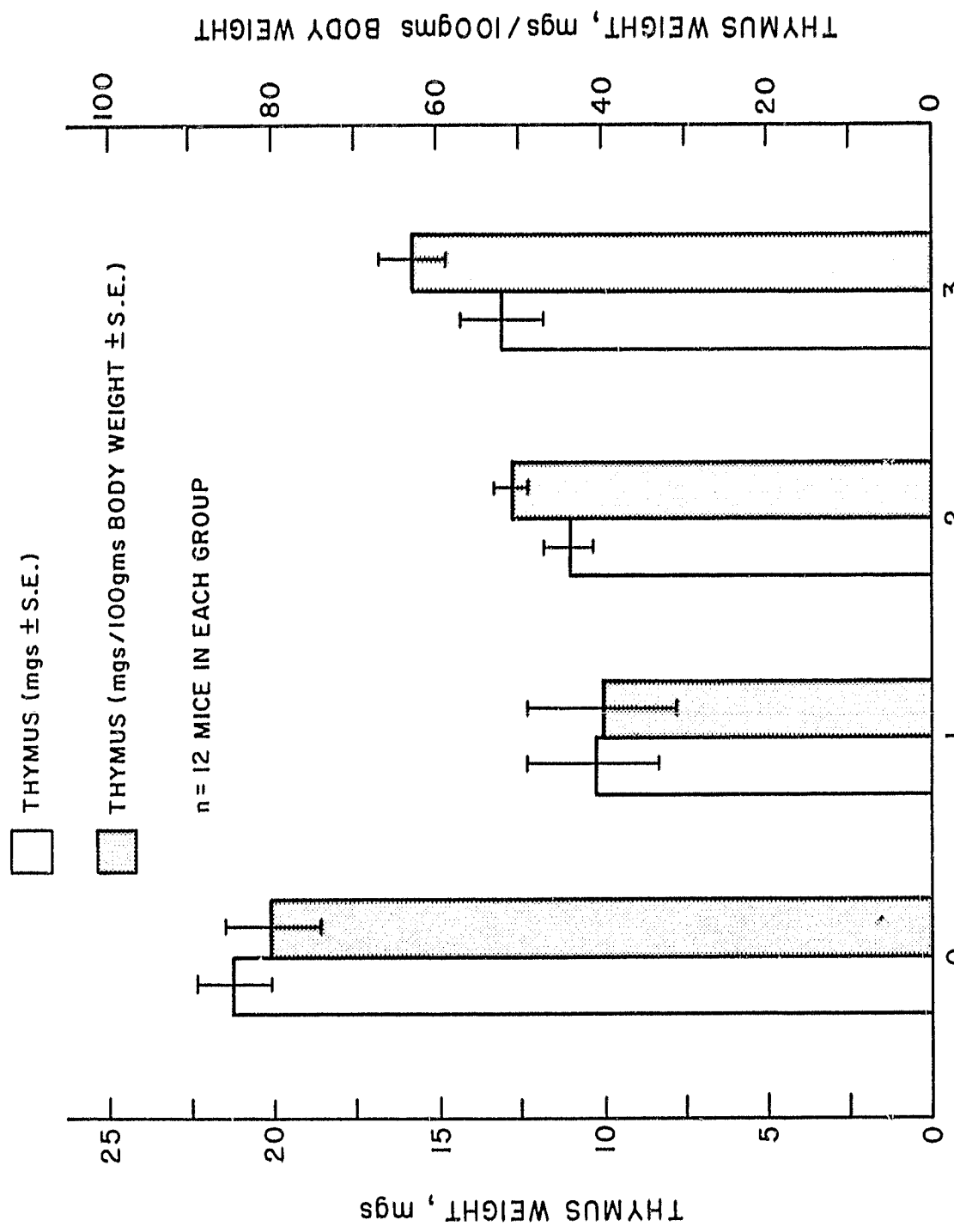

Figure 7 Average thymus weight of A/J mice exposed to reduced barometric pressure (380 mm Hg).

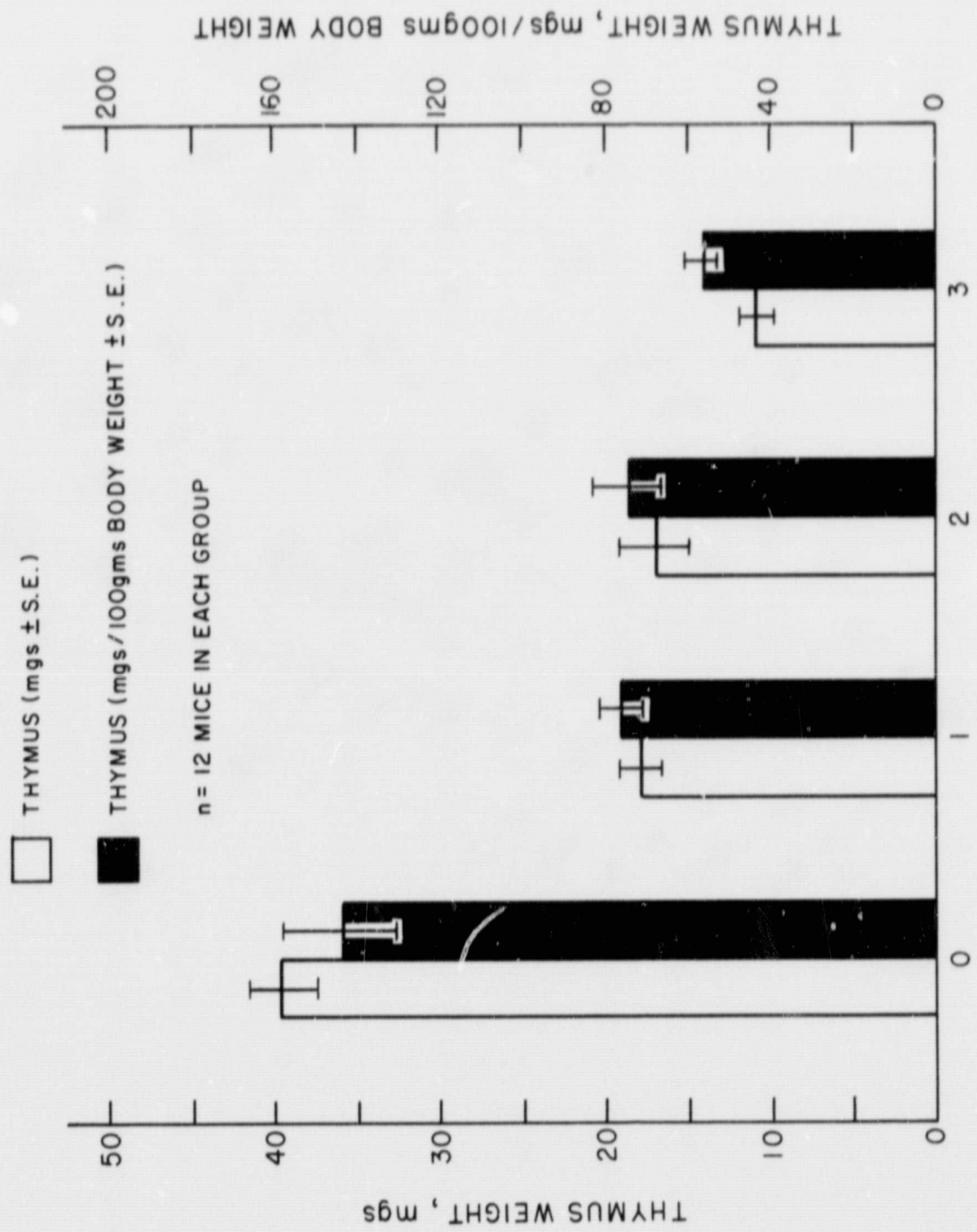

Figure 8 Average thymus weight of C57BL/6J mice exposed to reduced barometric pressure (380 mm Hg).

The spleen weight of C57BL/6J mice after one, two, or three weeks of hypoxia exposure was significantly higher than that of controls (Figure 9). A/J strain mice, however, were more variable and exhibited a significant increase in weight, relative to controls, after two weeks of exposure, but not after one or three weeks exposure to reduced pressure (Figure 10).

As with spleen weight, brachial and inguinal lymph node weights of A/J strain mice were variable over the three week experimental period (Figures 11 and 12). After one week of exposure of A/J mice to reduced pressure, there was a significant decrease in the weight of brachial lymph nodes, but not inguinal nodes. After two weeks of exposure neither brachial nor inguinal nodes were significantly different from controls. However, after three weeks values for both brachial and inguinal nodes were significantly lower than controls.

C57BL/6J strain mice exhibited a steady decline in both brachial and inguinal lymph node weight during the three week experimental period (Figures 13 and 14). No significant weight decrease occurred in brachial nodes after one week, but there was a significant decrease in weight of these lymph nodes after two and three weeks exposure. Inguinal nodes, on the other hand, exhibited a significantly lowered weight, relative to controls, after one, two, or three weeks of exposure to reduced pressure.

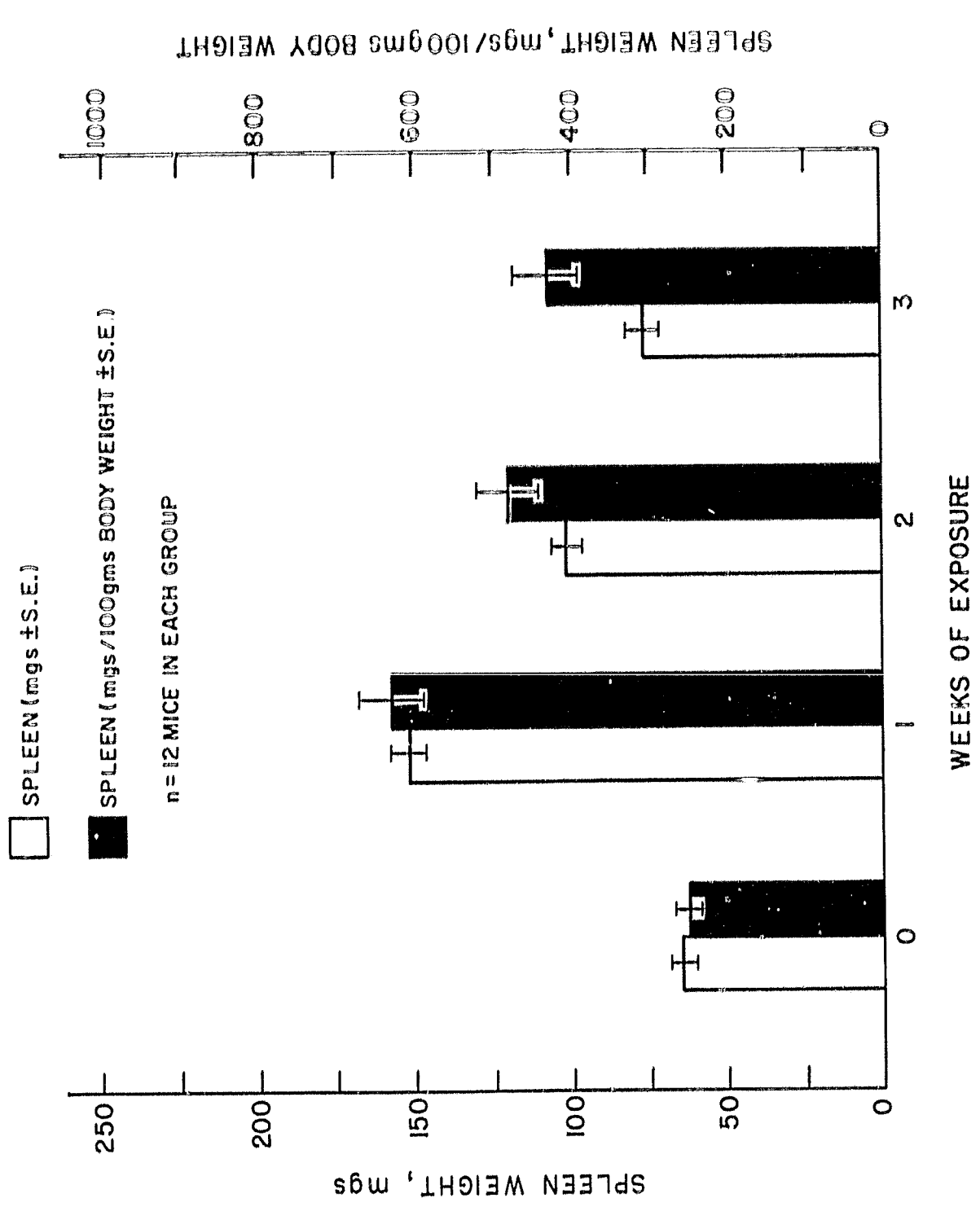

Figure 9 Average spleen weight of C57BL/6J mice exposed to reduced barometric pressure (380 mm Hg).

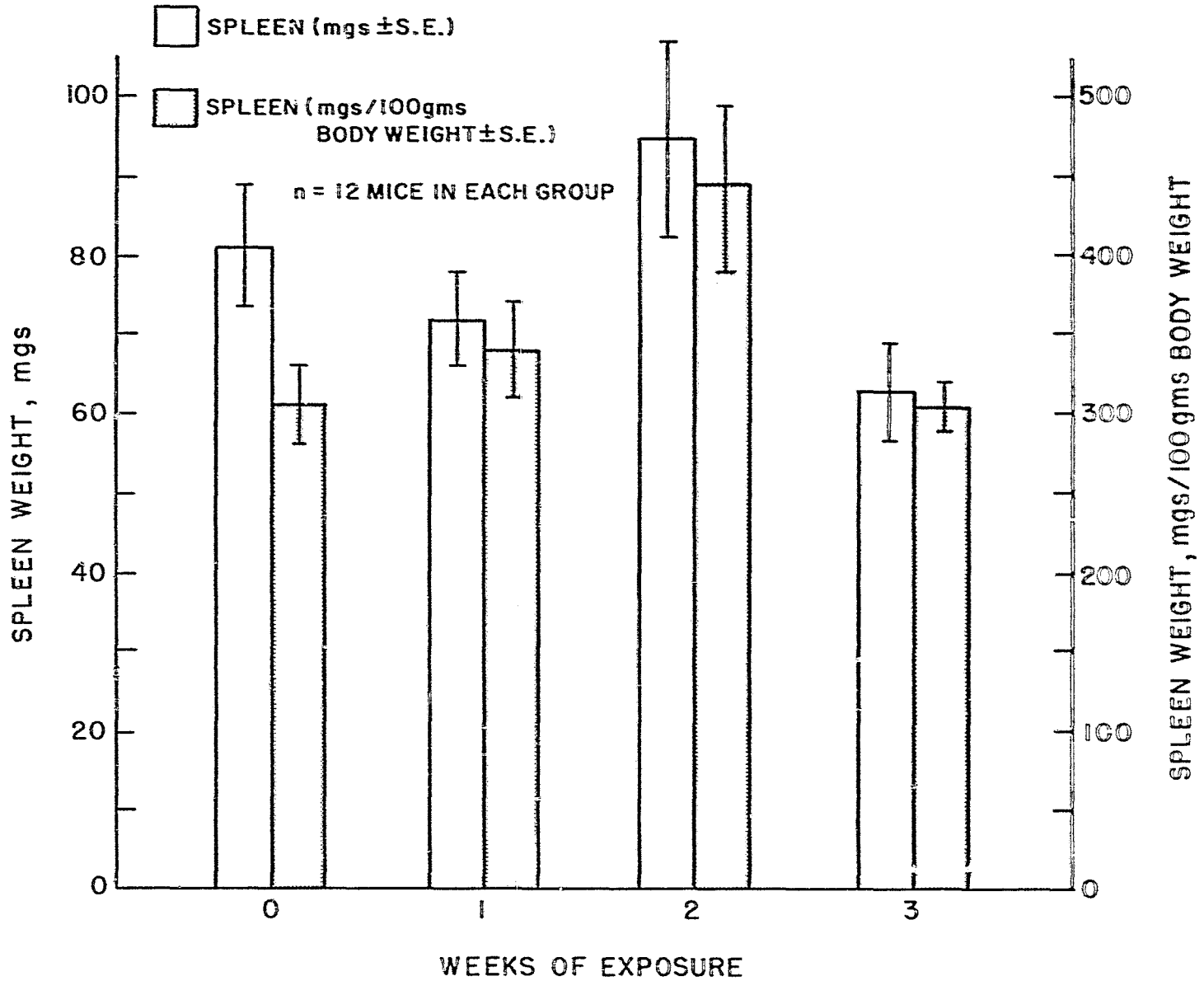

Figure 10 Average spleen weight of A/J mice exposed to reduced barometric pressure (380 mm Hg)

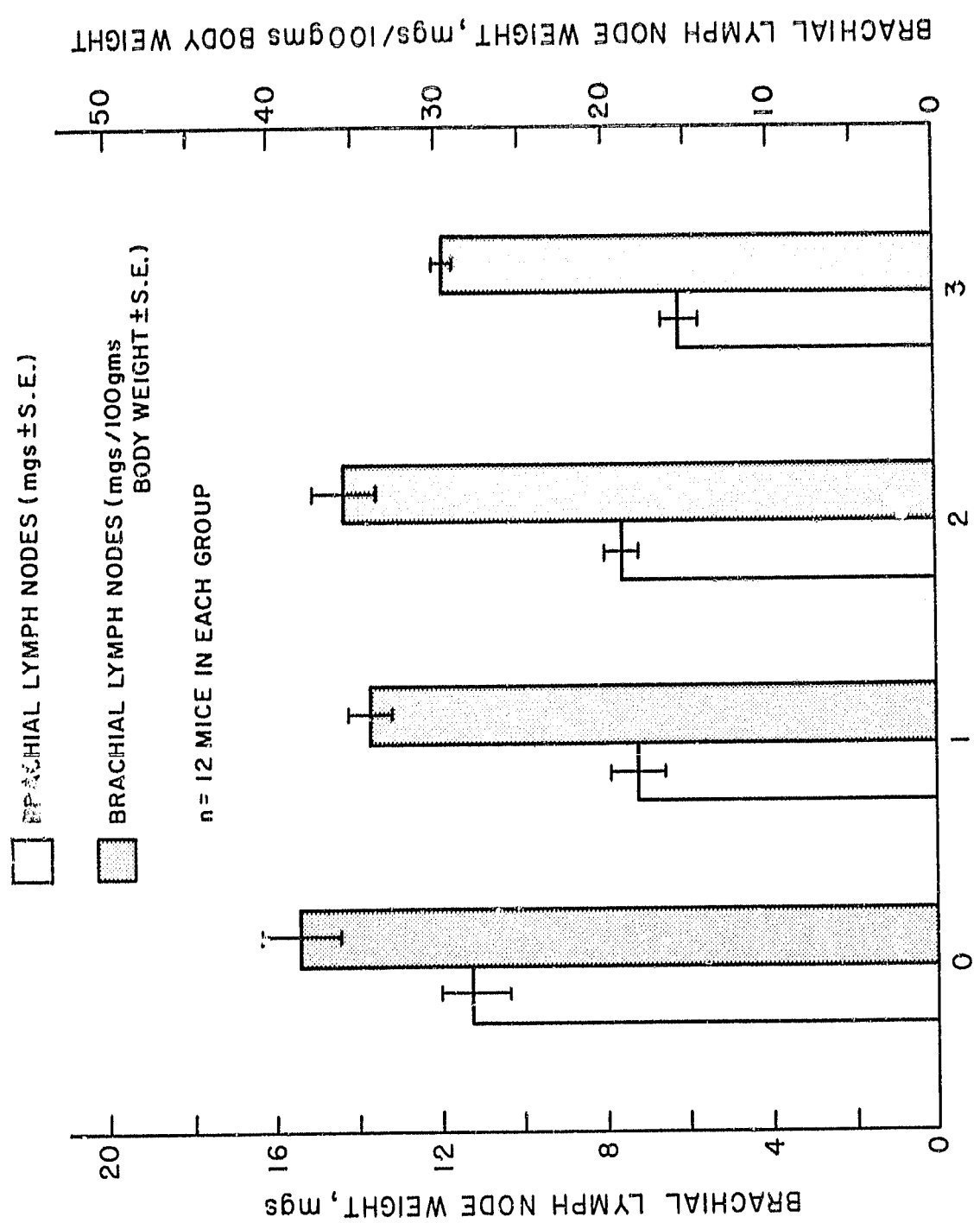

Figure 11 Average brachial lymph node weight of A/J mice exposed to reduced barometric pressure (380 mm Hg).

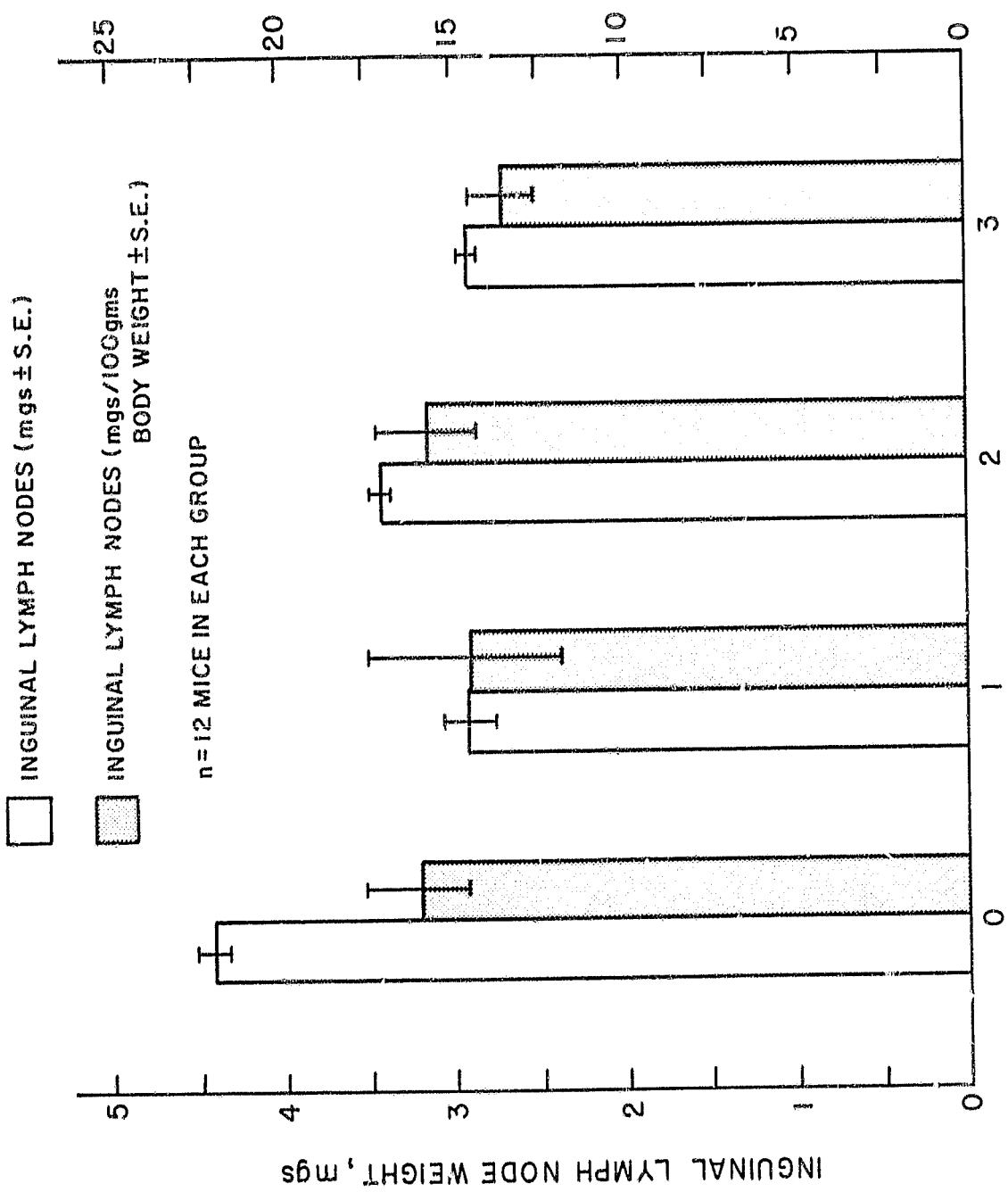

Figure 12 Average inguinal lymph node weight of A/J mice exposed to reduced barometric pressure (380 mm Hg).

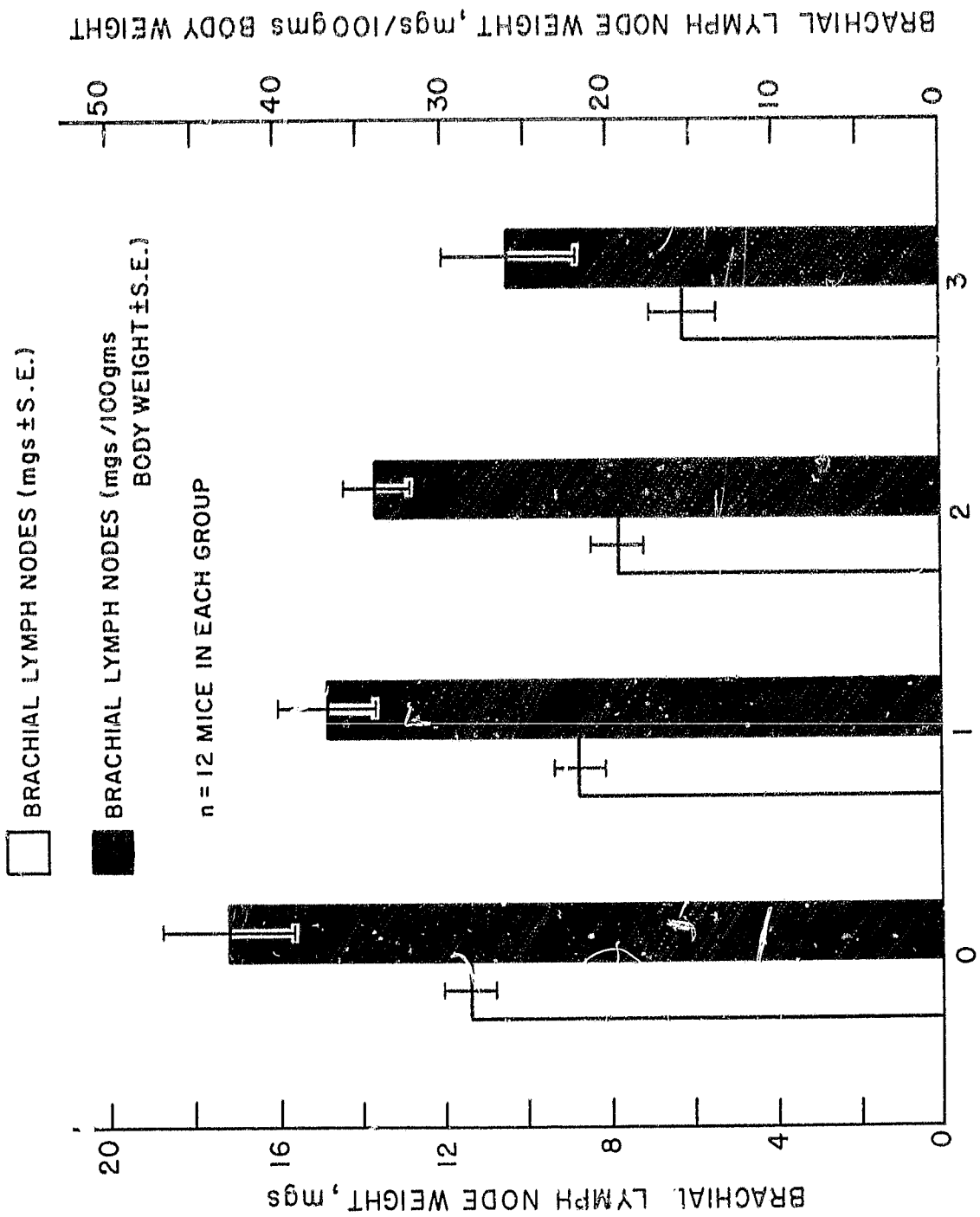

Figure 13 Average brachial lymph node weight of C57BL/6J mice exposed to reduced barometric pressure (380 mm Hg).

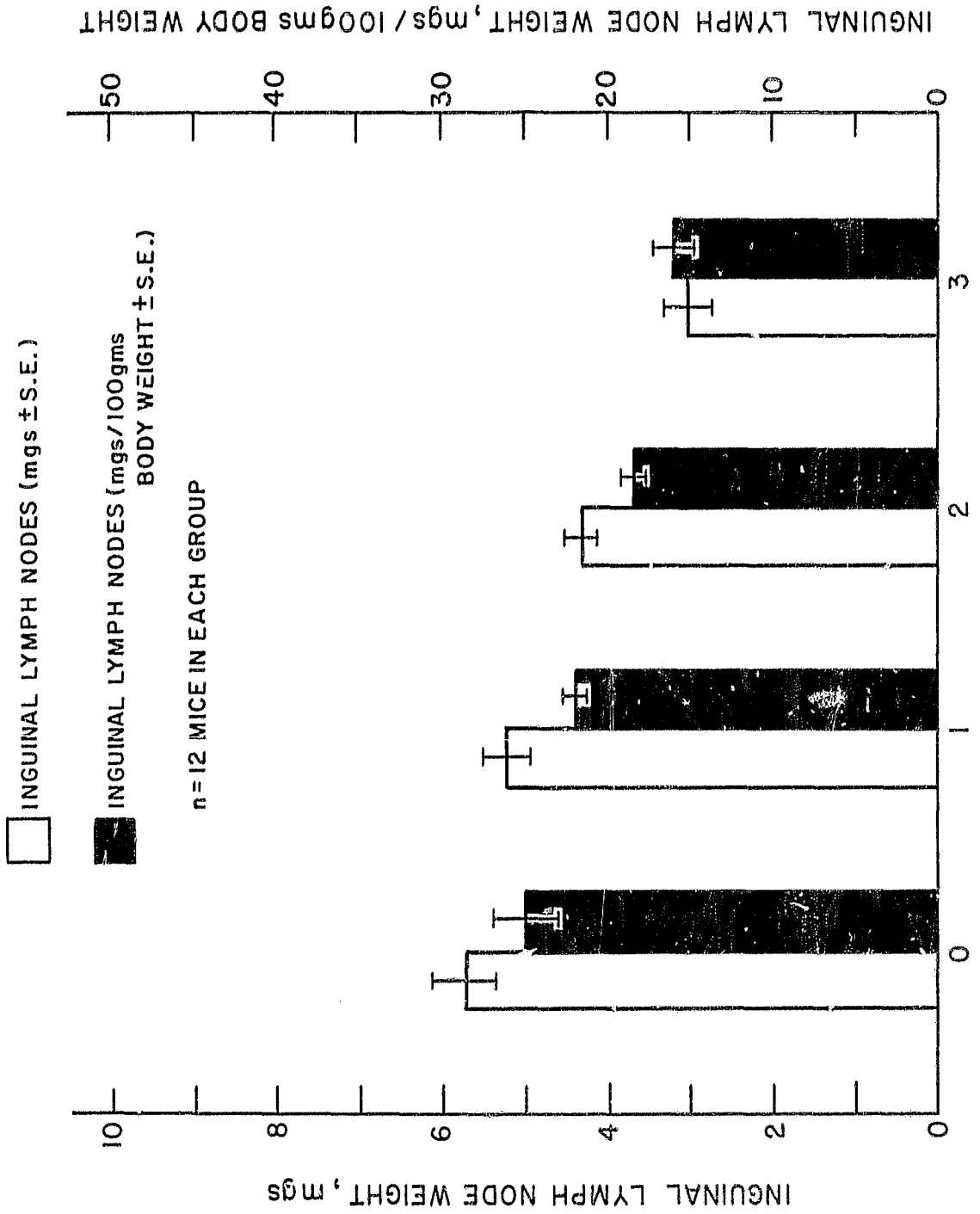

Figure 14 Average inguinal lymph node weight of C57BL/6J mice exposed to reduced barometric pressure (380 mm Hg).

2. Histology of Lymphatic Organs

Although weight changes in lymphatic organs demonstrated some degree of inter- and intrastrain variability, changes at the tissue level, as assessed by histological examination of hematoxylin-eosin stained paraffin sections of lymphatic organs of exposed animals were equivalent for both strains of mice used. Thus, the following description of histological changes refers to both A/J and C57BL/6J mice.

Histological examination of the thymus after one, two, and three weeks of exposure confirmed that there was a marked hypoplasia of thymic lymphocytes in all three exposure groups. Because of its greater mass, involution of cortical elements accounted for most of the weight loss of the thymus gland. However, medullary elements also proved to be reduced and not well demarcated in hypoxic mice. It was further observed that the cortex of one week exposed mice had fewer mature lymphocytes with considerably more cytoplasm or reticular matrix separating individual nuclei than corresponding controls. On the other hand, in two and three week exposed animals, nuclei of small lymphocytes were closely packed giving the cortex a densely stained appearance. In no instance were there any signs of lympholysis, pyknosis or other indications of tissue degeneration.

Microscopic examination of splenic tissue after one week of exposure revealed a reduction in the extent of white pulp comprising nodules, but not in the number of primary nodules per unit area. Furthermore, during this period, more small lymphocytes were found in the venous sinuses, relative to controls. In spleens from two

and three week exposed mice primary nodules were equivalent in size to those of controls, but were much more densely packed with lymphocytes.

Lymph nodes of one week exposed animals also exhibited a depletion of mature lymphocytes and an increase in the number of larger blast cell types, relative to controls. As with the spleen and thymus, nodal tissue of animals exposed for two to three weeks of hypoxia again presented a microscopic picture of very densely packed lymphocytes.

B. Hematocrit Changes

The average hematocrit, used as an index of hemopoiesis, was measured in A/J and C57BL/6J strains over a 21 day period of altitude exposure. The major findings, based on approximately 70 determinations for each strain of mice are as follows. First, the greatest increase in hematocrit occurred during the first four days of exposure, i.e., after four days both strains had increased by approximately 10%. Second, the shape of the hematocrit curve was nearly identical in both strains (Figures 15 and 16). The two strains differed, however, with respect to the maximum hematocrit attained over a three week period. A/J mice exhibited a hematocrit value of 61% after three weeks of exposure, while the hematocrit for C57BL/6J mice during the same period was 76%. Third, the rate of increase was greatest during the first two weeks. For example, A/J mice attained 99% of their response during this period, and,

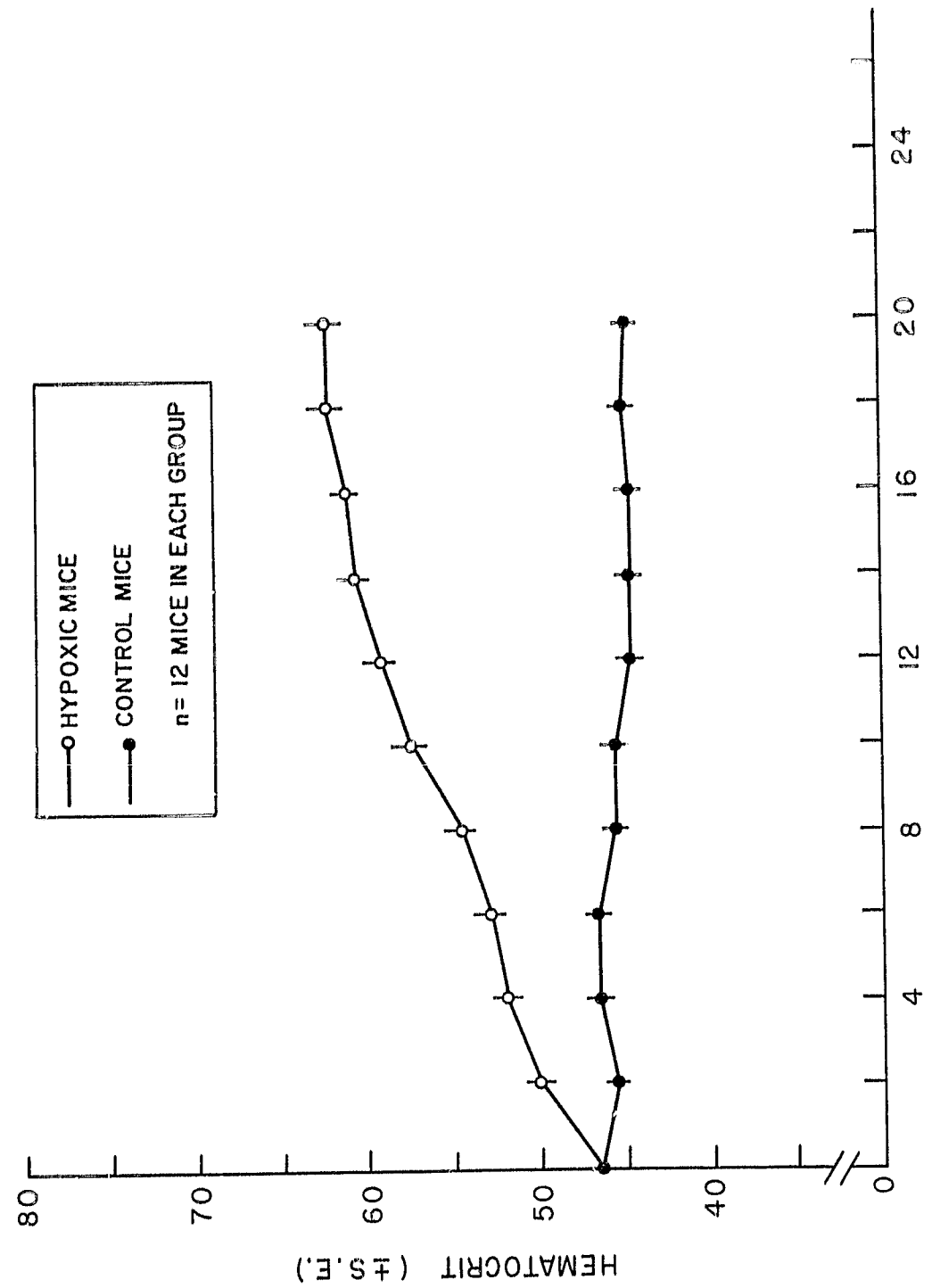

Figure 15 Mean hematocrit response in A/J mice exposed to reduced barometric pressure (380 mm Hg).

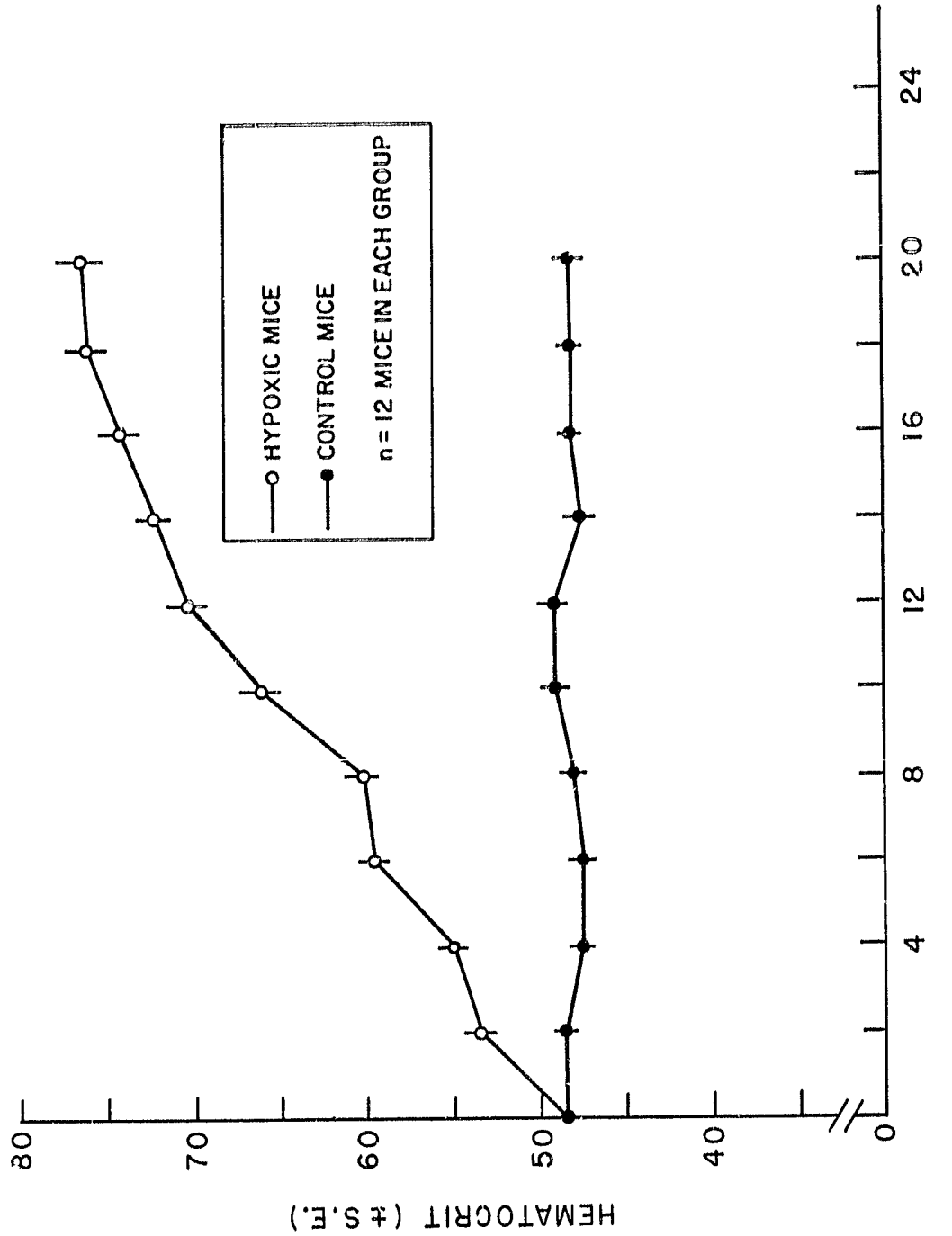

Figure 16 Mean hematocrit response in C57BL/6J mice exposed to reduced barometric pressure (380 mm Hg).

correspondingly, 95% of the total increase in C57BL/6J mice occurred during the first two weeks.

C. Cytophotometry of Lymphatic Organs Exposed to Hypoxia

Results of DNA measurements by the two-wavelength method on lymphatic organs of exposed and control A/J and C57BL/6J animals are shown in Figures 17 and 18. In all cases and with both strains, after one week of hypoxia exposure, there was a shift to the right in the DNA profile of lymphatic cells, indicating an increase in the amount of DNA present in exposed organs as compared with controls. In contrast, DNA profiles of two to three week exposed mice were comparable to controls. Significantly, the shift in the DNA profile was similar in thymic, splenic and nodal lymphocytes and in both strains of mice, indicating that all three organs were responding in concert. Similarly, restoration of control profiles occurred in all three lymphatic organs by week two of exposure.

D. Homograft Reaction in Acclimated and Unacclimated Mice

1. Survival Time of Skin Homografts

Survival time of skin homografts was determined by visual inspection of the transplants after dressings had been removed. The end point for rejection was chosen to be the time at which the graft changed from healthy viable skin to a necrotic scab. It was felt that this point was a better indication of survival time than sloughing of the graft since the scab-like grafts, even though they

Figure 17 Relative amounts of DNA, as determined by two-wavelength method, of A/J mice exposed to reduced barometric pressure (380 mm Hg) for one, two, or three weeks.

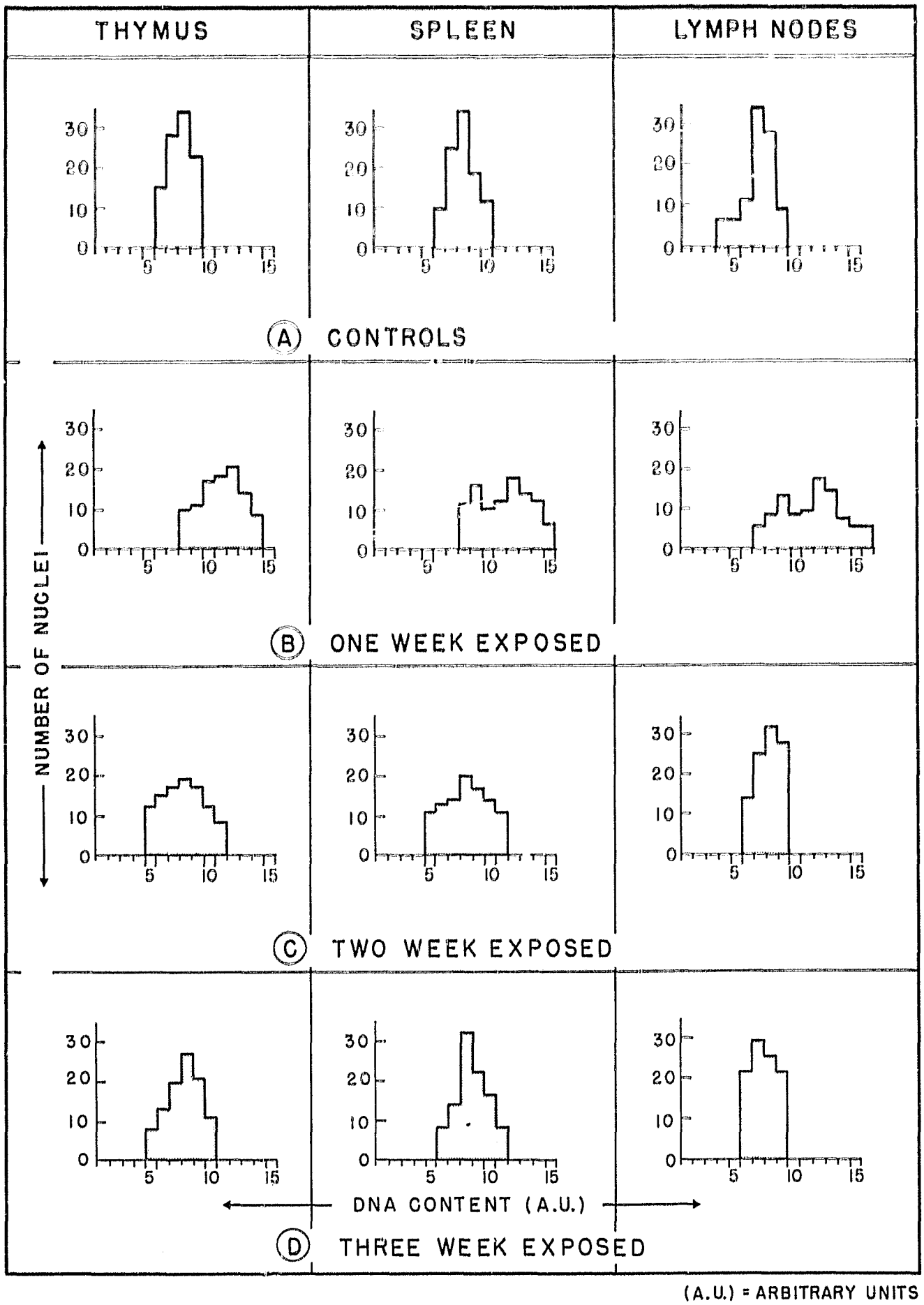

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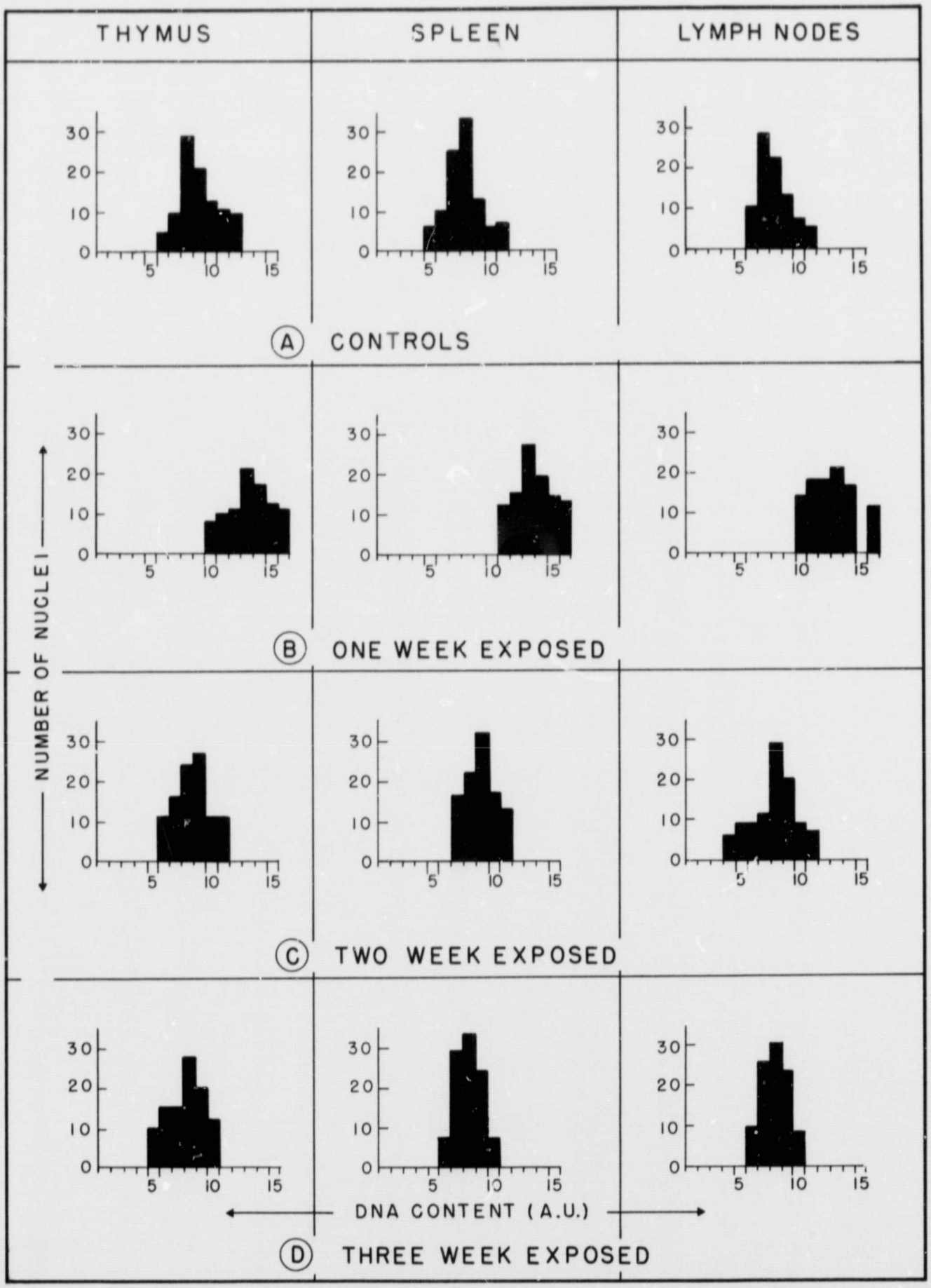
Figure 18 Relative amounts of DNA, as determined by two-wavelength method, of C57BL/67 mice exposed to reduced barometric pressure (380 mm Hg) for one, two, or three weeks.



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were dead tissue, remained on the animals for variable periods of time after their formation.

In all cases, regardless of whether A/J or C57BL/6J was host or donor, grafts between acclimated animals had a mean survival time of about 15 days which was significantly longer than the approximately nine-day survival time for transplants between unacclimated (control) mice.

When skin from an unacclimated donor was transplanted to an acclimated host, survival time was increased from 9 to about 12 days. Also, when skin from an acclimated donor was transplanted to an unacclimated host, survival time of the graft increased from 9 to about 12 days. These results are summarized in Table 2.

2. Gross Observations and Histology of Homografts

Homograft rejection can be divided into three stages: (a) the period of granulation, (b) the period of retraction, and (c) sloughing. The granulation period is marked by anastomoses of blood vessels of the host and the graft. At this time the transplant has a pink, healthy appearance. The onset of the period of retraction is characterized by invasion of the graft with mononuclear leucocytes; this is associated with breakdown of the vascular supply to the transplant. During this period the graft bed retracts and the graft itself becomes necrotic. The terminal stage of rejection is characterized by a lifting or sloughing of the scab-like graft leaving a small scar in its place.

TABLE 2

SURVIVAL TIME OF SKIN HOMOGRAFTS BETWEEN A/J AND C57BL/6J MICE

	Donor	Host	n	Mean Survival Time of Skin Graft in Days ± S.E.
I.	Donor and Host Acclimated[a]			
	A/J	C57BL/6J	24	15.1 ± 0.2
	C57BL/6J	A/J	24	15.6 ± 0.4
II.	Donor and Host Maintained at Ambient Pressure[b]			
	A/J	C57BL/6J	24	9.3 ± 0.2
	C57BL/6J	A/J	24	9.2 ± 0.3
III.	Acclimated Donor and Unacclimated Host			
	A/J	C57BL/6J	12	11.9 ± 0.3
	C57BL/6J	A/J	12	12.2 ± 0.4
IV.	Unacclimated Donor and Acclimated Host			
	A/J	C57BL/6J	12	12.5 ± 0.4
	C57BL/6J	A/J	12	12.1 ± 0.3

[a]Animals maintained at reduced pressure (380 mm Hg) 21 days prior to grafting.

[b]Animals maintained at ambient pressure of State College, Pennsylvania (725 mm Hg) prior to grafting.

Groups I, III, and IV are significantly higher than the control group (II) at the 0.01 level using t-test.

Daily inspection of the grafts showed that the three stages of rejection were identical in appearance in both acclimated and control mice. However, retraction and scab formation commenced on about day 15 if skin transplants were between two acclimated mice and on day 12 if either the donor or recipient were acclimated prior to grafting. In contrast, if both host and donor were unacclimated, retraction and subsequent scabbing of the graft occurred about nine days after grafting. Thus, an extension of the granulation period occurs in hypoxic mice.

Microscopic analyses revealed that the granulation period of homografts was completed within a week after grafting in unacclimated mice as evidenced by infiltration of the graft site with mononuclear leucocytes. On the other hand, few or no monocytes were evidenced in grafts between acclimated animals until 10 to 12 days after grafting (Figure 19). In brief, histological data of skin homografts confirmed gross observations that the increased survival of transplants in hypoxic animals was due to an increase in the length of the granulation period.

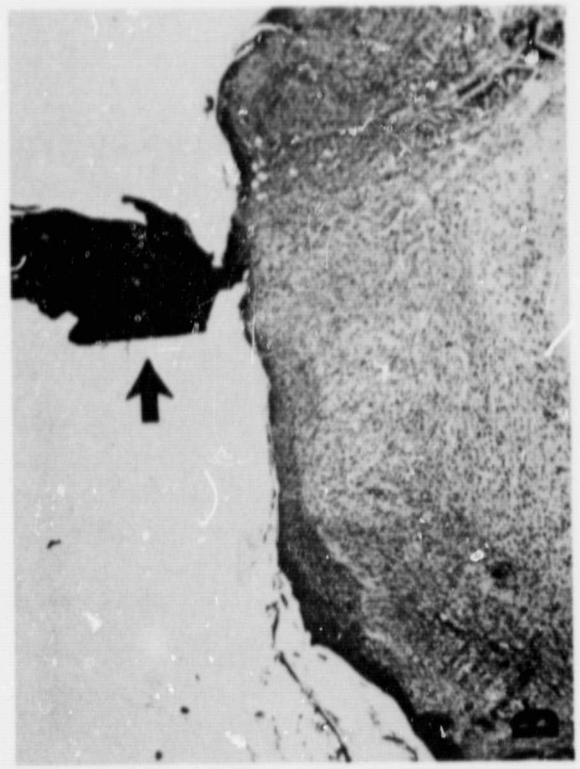
Figure 19 Photomicrographs of skin homografts.

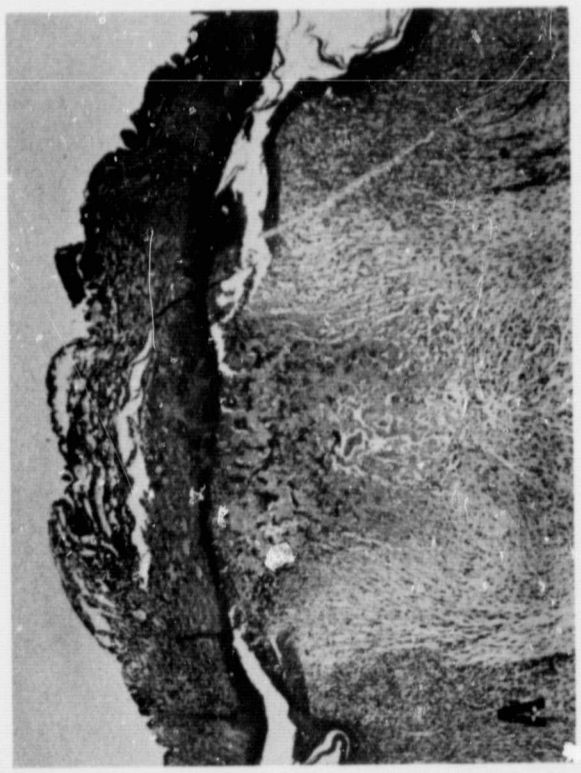
A. Control (unacclimated donor and unacclimated host) graft after 8-9 days. 10X

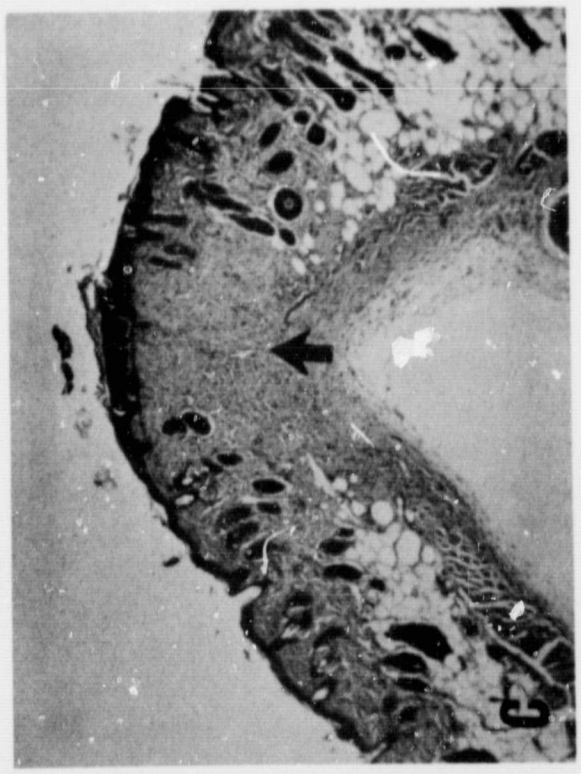
B. Experimental (acclimated donor and acclimated host) graft after 8-9 days. Arrow indicates junction of host and donor skin; the grafted skin is to the left. 10X

C. Control 14-15 days after grafting. The graft has been sloughed. Arrow indicates area previously occupied by graft. 10X

D. Experimental graft 14-15 days after grafting. Arrow indicates partially sloughed graft. 10X







DISCUSSION

The present study demonstrated that exposure of mice to hypoxia results in an involution of the thymus, a decrease in the size of lymph nodes, and a moderate hypertrophy of the spleen. Histologic analyses of lymphatic organs suggest that hypoxia stimulates mobilization of thymic lymphocytes from cortical and medullary regions of the thymus to the spleen and lymph nodes where they appear as densely packed aggregates in primary nodules. These observations agree with findings of several workers, who, using radioautographic measures of tritium labeled cells, demonstrated that lymphocytes released by the thymus are later found in the spleen and lymph nodes (Diderholm and Fichtelius, 1959; Miller et al., 1963; Murray and Murray, 1964; Murray and Woods, 1964). Hypoxia then acts as a powerful stimulus causing the release of thymocytes into the circulation. Since it is established that circulating thymic lymphocytes do not re-enter the thymus (Gowans, 1964), one would expect a persistence of the involuted state which occurs during the acute period of exposure. This proved to be the case. The extent of thymic involution in both two and three week exposed groups was similar to that of one week exposed mice. Some reduction in number of mature splenic and nodal lymphocytes was evidenced in the first week of exposure, suggesting that there is probably a general

release of lymphocytes from all lymphatic organs during the onset of acclimation. On the other hand, the greatest loss was observed in the thymus. Two and three week exposed mice exhibited more mature lymphocytes in the spleen and nodes. This suggests a generalized lymphatic mobilization from all lymphatic organs during the first week, and a repopulation of the spleen and nodes with thymic cells in the second and third weeks of exposure.

The above interpretation was reinforced by data on changes in DNA profiles of lymphatic organs. A shift favoring increased nuclear DNA in lymphatic cells was evidenced in one week exposed mice, but not in two and three week exposed groups. It is quite possible that the observed shift in cellular DNA profile of the three organs studied is due to an increase in the relative number of immature blast cell types which were observed to be more abundant in one week exposed animals relative to controls or two to three week exposed mice. Since such cells would be in the DNA synthesis phase of the mitotic cycle, any appreciable increase in the number of dividing cells would shift the DNA profile to the right. Because of the increased presence of blast cell types in one week exposed animals, it is likely that the observed shift in DNA profiles of lymphatic organs is due to increased mitotic activity and not to functional changes of these organs as has been found by several other workers (Roels, 1954; Viola-Magni, 1965, 1966).

Thus, cytophotometric data coupled with cytological analyses indicate that the onset of hypoxia acclimation is characterized by a mobilization of lymphocytes from all lymphatic organs to the

circulation and the lymphatic organs respond with increased production of lymphocytes.

Although one function of the spleen is the production of antibodies, the results suggest that this function may be suppressed during hypoxia acclimation because of the decrease in size of the primary nodules of the white pulp. However, the increase in spleen size can be attributed to a marked increase in erythropoiesis which serves to increase the oxygen-carrying capacity of the blood (Clark and Otis, 1952). The increase in erythropoiesis is commonly found during the first two weeks of hypoxia exposure (VanLiere and Stickney 1963), and it is significant that the greatest increase in spleen weight occurs during this time. Furthermore, the increased hematocrit that occurs during hypoxia acclimation, and which has been reported by others (VanLiere and Stickney, 1963; Nelson et al., 1963, Godish, 1967), reached 95 to 99% of its final level after the first two weeks of hypoxia acclimation.

In the present study, the homograft response was used to evaluate the immunological competence of acclimated and non-acclimated mice. It was observed that acclimated mice retain homografts for a longer period than unacclimated mice. Since the homograft reaction is an antigen-antibody response (Snell, 1967), at least two possible mechanisms could account for the increased tolerance of acclimated mice to homotransplants: (a) exposure of mice to hypoxia may result in delayed transplant vascularization which is prerequisite for the initiation of antibody formation

(Medawar, 1948); and (b) the immune system of acclimated mice may be suppressed allowing for increased graft survival.

From gross observations of the vascularization of skin grafts, it was observed that development of vascular anastomoses at the graft edges is not slower in acclimated mice as compared to controls. In fact, grafts appeared to be more readily vascularized in acclimated mice than in control mice. This is in keeping with reports of other workers who have demonstrated that acclimation is associated with a general hyperplasia of the entire cardiovascular system (Valdivia, 1956; Korner, 1959; Oster and Anthony, 1966). As a consequence, one would expect the epidermal capillary bed to be more extensive in acclimated animals than in controls.

It was tentatively concluded that increased tolerance to skin grafts was probably related to an alteration of the immunological potential of mice rather than to changes associated with vascularization of the transplant.

Since it is known that the lymphatic system is intimately involved in homograft reactions (Miller, 1965) and also that the homograft reaction is a cellular response (Brent et al., 1962a; Billingham, 1967; Wilson, 1967; Hildemann, 1967), it can be postulated that acclimation to hypoxia affects the lymphatic system to such an extent that a longer time is required for initiation of transplant rejection.

The data on weight and histological changes in the thymus and lymph nodes, and on the histological changes of the white pulp of the spleen, support this interpretation. Histological and

cytophotometric analyses confirmed a reduction in mature lymphocytes in all lymphatic organs during the first week of exposure, and even though this was not evidenced in two and three week exposed animals, weights of lymphatic organs of these groups were significantly lower than controls after three weeks of exposure to reduced pressure.

Histological analyses of skin grafts showed that infiltration of the graft with mononuclear leucocytes was delayed in transplants between acclimated mice. In acclimated mice, infiltration of grafts did not occur until 11 to 12 days after grafting while in unacclimated mice homografts became infiltrated with mononuclear leucocytes five to six days after grafting. Of importance here are two reports, one by Dunne (1966) and the other by McCluskey et al. (1963). Dunne observed that when mice acclimated to reduced pressure were returned to ambient pressure, there was a reversal of thymic involution and that thymic weight and histological appearance returned to control levels in about six days. McCluskey et al., using H^3 thymidine and radioautography, demonstrated that almost all (about 90%) of the lymphocytes involved in hypersensitivity reactions are cells which have recently proliferated. The function of the thymus is to produce and liberate lymphocytes that pass to the spleen and lymph nodes and there settle down and mature into cells that look after the integrity and security of the body (Miller et al., 1963). Since acclimated animals were not returned to reduced pressure after grafting, it is significant that lymphocytes appeared in grafts between acclimated animals about 12 days after grafting because in

this amount of time the thymus would have regained its control status and would then be able to produce enough cells for the homograft reaction to proceed. It thus appears that increased retention of skin homografts in acclimated mice may be due to a hypofunction of the acclimated thymus.

Also of importance is the fact that transplants from unacclimated hosts to acclimated donors and acclimated hosts to unacclimated donors were retained for a longer time than were control (unacclimated donor to unacclimated host) homografts. In the first case, unacclimated donor to acclimated host, increased retention could be brought about by the fact that the immune system of the host had been suppressed and therefore could not react to the graft. The second case, acclimated donor to unacclimated host, however, is difficult to explain. Mallette (1962) obtained similar results when thyroid implants from acclimated rats were put into the anterior eye chamber of unacclimated rats. Recently several workers (Manson et al., 1963; Brent et al., 1962b) have reported the association of transplantation antigens with lipoprotein complexes. It may be possible that hypoxia acclimation not only affects the immune mechanism but also the production of a substance which acts as the antigen in homograft reactions. If this is the case, then prolonged graft retention in transplants between acclimated animals may be due to both a hypofunction of the immune system and a shortage of antigen necessary to elicit a response.

In any event there is no question that hypoxia acclimation alters the function of the lymphatic system. This is supported by

weight and histological changes which occur during acclimation. Furthermore, the cytophotometric data indicate that the acclimation process causes changes not only at the tissue but at the cellular level. Finally, results obtained with homografts indicate that the immune response, at least in the case of homografts, is suppressed in hypoxia acclimated mice.

SUMMARY

Histologic and Feulgen-DNA cytophotometric analyses were made on lymphatic organs of A/J and C57BL/6J strain mice exposed to reduced barometric pressure (380 mm Hg) for one, two, or three weeks. Erythropoietic responses were assessed using hematocrit changes during the three week experimental period. Skin homografts were used as an indication of the immune potential of mice acclimated to moderate hypoxia. Two hundred sixty-four adult male mice (132 A/J and 132 C57BL/6J) were used in this study.

The major results are as follows. First, after three weeks of hypoxia exposure both strains of mice exhibited a decrease in the amount of functional lymphatic tissue. Second, DNA profiles, as determined by two-wavelength cytophotometry of Feulgen stained lymphatic cells, shifted in the direction of increased DNA in all cases after one week of exposure. Restoration of control profiles occurred in all three lymphatic organs by week two of exposure. Third, skin homografts between acclimated animals and in cases where either the donor or recipient were acclimated were retained for a longer time than skin transplants between unacclimated mice.

The significance of these findings was discussed in relation to the immune potential of acclimated mice. One conclusion supported by the present study is that hypoxia stimulates the release of

lymphocytes from the thymus to the spleen and lymph nodes. Furthermore, during the acute phase of hypoxia acclimation a generalized lymphocytic release occurs in all lymphatic organs. A second conclusion is that lymphatic organs of exposed mice respond to the release of lymphocytes with increased production of lymphocytes. The large number of blast cells and the shift of DNA profiles of one week exposed animals indicate increased cell production in lymphatic organs during the first week of exposure. A third conclusion, supported by data on skin homografts is that the immune response of acclimated mice is suppressed. In summary, the present study indicates that functional changes in lymphatic organs occur during early weeks of hypoxia acclimation which probably reduce the ability of an animal to react to an immunological challenge.

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APPENDIX

Summary Tables of Weight Data

TABLE 3

WEIGHT ANALYSES OF THYMUS, SPLEEN, AND LYMPH NODES OF A/J MICE EXPOSED TO REDUCED BAROMETRIC PRESSURE (380 mm Hg)[a]

Weeks of Exposure	n	Mean Organ Weights mgs ± S.E.			
		Thymus	Spleen	Brachial Lymph Nodes	Inguinal Lymph Nodes
0	12	21.3 ± 1.1	81.3 ± 8.4	11.3 ± 0.9	4.4 ± 0.3
1	12	10.1 ± 2.1	72.8 ± 6.5	7.3 ± 0.6	2.9 ± 0.6
2	12	10.9 ± 0.6	95.6 ± 14.5	7.6 ± 0.4	3.4 ± 0.2
3	12	13.0 ± 1.2	63.3 ± 5.9	6.1 ± 0.3	2.9 ± 0.2

Weeks of Exposure	n	Mean Organ Weights mgs/100 gms Body Weight ± S.E.			
		Thymus	Spleen	Brachial Lymph Nodes	Inguinal Lymph Nodes
0	12	79.7 ± 5.7	306.9 ± 2.3	38.8 ± 2.8	16.0 ± 1.5
1	12	39.9 ± 9.2***	342.4 ± 29.1	34.0 ± 1.2**	14.5 ± 3.2
2	12	51.2 ± 1.9**	446.2 ± 50.4**	35.6 ± 2.2	15.8 ± 1.5
3	12	63.1 ± 3.6**	306.2 ± 17.5	29.6 ± 0.5**	13.5 ± 0.9*

[a]Asterisks are used to designate confidence levels of 0.5 (*), 0.2 (**), and .01 (***), using Student's t-test.

TABLE 4

WEIGHT ANALYSES OF THYMUS, SPLEEN, AND LYMPH NODES OF C57BL/6J MICE EXPOSED TO REDUCED BAROMETRIC PRESSURE (380 mm Hg)[a]

Weeks of Exposure	n	Mean Organ Weights mgs ± S.E.			
		Thymus	Spleen	Brachial Lymph Nodes	Inguinal Lymph Nodes
0	12	39.1 ± 2.2	64.5 ± 2.8	11.4 ± 0.7	5.7 ± 0.4
1	12	17.9 ± 0.9	151.4 ± 11.9	8.9 ± 0.6	5.2 ± 0.3
2	12	17.2 ± 2.1	107.5 ± 11.0	7.8 ± 0.6	4.3 ± 0.2
3	12	11.3 ± 1.1	80.6 ± 9.5	6.1 ± 0.8	3.0 ± 0.3

Weeks of Exposure	n	Mean Organ Weights mgs/100 gms Body Weight ± S.E.			
		Thymus	Spleen	Brachial Lymph Nodes	Inguinal Lymph Nodes
0	12	133.3 ± 13.9	292.2 ± 10.4	44.1 ± 3.3	27.6 ± 1.4
1	12	75.3 ± 5.0**	632.1 ± 38.5***	37.3 ± 2.8	21.8 ± 0.6**
2	12	74.0 ± 8.5**	464.5 ± 36.9**	33.7 ± 2.1*	18.6 ± 0.7**
3	12	54.5 ± 3.1***	426.0 ± 42.1**	28.8 ± 1.7**	16.0 ± 1.1**

[a]Asterisks are used to designate confidence levels of .05 (*), .01 (**), and .001 (***), using Student's t-test.