

CHANGES IN THE ACTIVITY OF SOME ENZYMES IN  
THE RAT HEMIDIAPHRAGM HYPERTROPHYING AS A  
CONSEQUENCE OF UNILATERAL PHRENICECTOMY

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Editorial comment (in author's absence) by his father.

This copy has had page numbers pencilled in after binding to agree with the other.

The text covers pp 1-309, the prelims and references are unnumbered but both copies are identical.

Page 3 appears after p 4.

P 27 is back to front.

Pp 41 & 42 also back to front, also p 98.

Table 4 occupies four pairs of sheets, numbered 119 to 123, No. 121 is omitted. Pp 119, 120 and 122 might have been better filed as verso-recto broadsides, as p 123 which appears after p 124, instead of as extended recto sheets

P 124 precedes p 123 as above.

P 199 From here onwards, where a caption appears on a separate sheet to its relevant graph, both carry the same page number. See 203, 216, 231, 234, 235, 238 and 241 in same fashion.

P 210 is perhaps back to front.

P 256 is repeated, p 256a overcomes the duplication.

P 299 is omitted, presumably in error. Nothing appears to be missing.



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CHANGES IN THE ACTIVITY OF SOME ENZYMES IN THE RAT HEMIDIAPHRAGM  
HYPERTROPHYING AS A CONSEQUENCE OF UNILATERAL PHRENICECTOMY

By

Leslie Victor Turner B.Sc.

Being a thesis submitted to the University  
of St. Andrews as a requirement  
for the degree of Doctor  
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## ABSTRACT

The post-denervation hypertrophy of the rat hemidiaphragm has been studied for up to 15 days after nerve section. The denervated tissue increases in wet weight to a maximum at 5 days of 40 % over its initial weight; by the 15th day the tissue has atrophied to below the control value.

Measurements have been made throughout the hypertrophy period of the activities of some enzymes characteristic of particular aspects of muscle metabolism. In addition, concentrations of glycogen from fed, and overnight fasted rats; of myoglobin; and of free amino acid concentrations in the denervated tissue have also been studied.

Methods are described for the use in rats of the anaesthetic Halothane so as to preserve muscle glycogen concentrations and to prevent stress-mediated activation of phosphorylase a levels.

Unilateral phrenicectomy causes a decrease in glycogen concentration and content; a decrease of total glycogen phosphorylase activity is also found, but total content of phosphoglucomutase increases slightly. Hexokinase and phosphorylase a contents increase so that their concentrations are maintained. The validity of the hexokinase/phosphorylase ratio as an indicator of fibre composition in pathological tissues is questioned.

Phosphohexoisomerase activity remains constant for 1 week after denervation, then decreases, but glyceraldehyde phosphate dehydrogenase & lactate dehydrogenase demonstrate increased contents at 7 days before they decrease; the responses of the dehydrogenases may be related to the reported proliferation of the sarcoplasmic reticulum. No significant change is observed in the lactate dehydrogenase isoenzyme proportions until 7 days when a decrease of H-type subunits is indicated; 3 days later though control proportions are regained.

Content of malate dehydrogenase, NAD-, & NADP-specific dehydrogenases demonstrate rapid decreases after nerve section to roughly half the initial levels at 3 days after nerve section. Glutamate

dehydrogenase concentration also decreases in the early stages of the hypertrophy, but later increases when protein catabolism becomes a significant process. These decreases are in accord with the reported fragmentation of the mitochondria.

Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase concentrations increase dramatically to a peak within the first few days after nerve section. The increased enzyme capacity could be responsible for the provision of pentose phosphates for nucleotide and nucleic acid synthesis.

Total creatine kinase activity remains constant for up to 5 days before a decline is observed; adenylate kinase & adenylate deaminase show increased contents. The responses are interpreted in terms of possible involvement in increased adenine nucleotide production. The validity of the adenylate kinase/creatinase activity ratio for the identification of physiologically distinct muscles is questioned.

Content of glutathione reductase shows two small peaks of increased activity at 3 and 10 days after denervation. Thus unlike other denervated or dystrophic muscles, the responses of NADP-linked dehydrogenases are not similar.

Myoglobin content increases slowly during the hypertrophy only reaching a peak of 20 % over control levels at 10 days. Concentration in the early stages is thus decreased but rises after 5 days. The response, as well as the changes in lactate dehydrogenase isoenzyme proportions, is interpreted in terms of an increased blood flow through the denervated tissue.

Total free amino acid concentration in the denervated tissue is increased; responses of the individual species are interpreted in terms of possible modes of metabolism in the tissue.

The responses in the denervated hemidiaphragm are interpreted in terms of a hypertrophy of the "red" &/or "intermediate" fibre types; suggestions are made as to possible causes for the responses.

### DECLARATION

I hereby declare that the following thesis is based upon work carried out by myself, that the thesis is my own composition, and that no part of it has previously been submitted for a higher degree.

The research was conducted in the Department of Biochemistry, University College, London, under the direction of Dr., now Professor, K. L. Manchester.

I hereby certify that Leslie Turner has spent nine terms in research work under my supervision and that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

### ACADEMIC RECORD

I matriculated at St. Andrews University in October 1964 and graduated with the degree of Bachelor of Science, Second Class Honours in Biochemistry in June 1967.

In October 1967 I matriculated as a research student in the Department of Biochemistry, University of St. Andrews; from April 1968 I undertook research work under the supervision of Dr. K.L. Manchester at University College, London.

### ACKNOWLEDGEMENTS

Sincere thanks are due to my two supervisors: in St. Andrews, Professor G.H. Tristram, and at University College, London, Professor K.L. Manchester, for all their help and encouragement during the execution of this work.

Thanks are also extended to: Dr. E.J. Harris of the Biophysics Dept. at University College, for the demonstration of the denervation technique, and for many stimulating discussions about the metabolism of denervated muscle; to Dr. D.L. Bloxam for advice on the amino acid analysis and on the use of the anaesthetic Halothane; and to all my colleagues at University College, London, who in one way or another, helped to make this project that much easier to accomplish.

From October 1967 until September 1970 I was in receipt of a Medical Research Council Scholarship for Training in Research Methods, for which I am indeed grateful.

Finally, I wish to thank Mrs. Colley for her endurance in typing the manuscript.

"There must be some kind of way out of here...  
There's too much confusion."

Dylan (1967)

## Trivial Names of Enzymes

- AMP Deaminase (AMP aminohydrolase) E.C. 2.3.4.6
- Adenylate Kinase (ATP:AMP phosphotransferase) E.C. 2.7.4.3
- Creatine Kinase (ATP: creatine phosphotransferase) E.C. 2.7.3.2
- Glucose 6-phosphate Dehydrogenase (D-Glucose-6-phosphate: NADP oxidoreductase) E.C. 1.1.1.49
- Glutamate Dehydrogenase (L-Glutamate: NAD(P) oxidoreductase (deaminating)) E.C. 1.4.1.3
- Glutathione Reductase (reduced-NAD(P): oxidized glutathione oxidoreductase) E.C. 1.6.4.2
- Glyceraldehydephosphate Dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating)) E.C. 1.2.1.12
- Glycogen Phosphorylase ( $\alpha$ -1,4-Glucan:orthophosphate glucosyltransferase) E.C. 2.4.1.1
- Hexokinase (ATP: D-hexose 6-phosphotransferase) E.C. 2.7.1.1
- Isocitrate Dehydrogenase, NAD-linked (threo-Ds-Isocitrate: NAD oxidoreductase (decarboxylating)) E.C. 1.1.1.41
- Isocitrate Dehydrogenase, NADP-linked (threo-Ds-Isocitrate:NADP oxidoreductase (decarboxylating)) E.C. 1.1.1.42
- Lactate Dehydrogenase (L-lactate:NAD oxidoreductase) E.C. 1.1.1.27
- Malate Dehydrogenase (L-malate:NAD oxidoreductase) E.C. 1.1.1.37
- Phosphoglucomutase ( $\alpha$ -D-Glucose-1,6-diphosphate: $\alpha$ -D-glucose-1-phosphate phosphotransferase) E.C. 2.7.5.1
- 6-Phosphogluconate Dehydrogenase (D-Glucose-6-phosphate:NADP oxidoreductase (decarboxylating)) E.C. 1.1.1.44
- Phosphohexoisomerase (D-Glucose-6-phosphate ketol-isomerase) E.C. 5.3.1.9

## I N T R O D U C T I O N

In contrast to the usual atrophic response of skeletal muscle deprived of its motor innervation, section of the phrenic nerve to one half of the diaphragm (unilateral phrenicectomy) initiates a pronounced, although transient, hypertrophy of the muscle on that side. The hypertrophy, manifest as an increase in tissue wet weight, but also paralleled by similar changes in dry weight, was first observed by Martin & Sola (1948) in the rat; the phenomenon has also been demonstrated in mice and rabbits (Stewart & Martin, 1956) and may well occur in other species.

In the rat, the species for which the response to phrenicectomy is most documented, the hypertrophy is found to vary in magnitude and duration according to the age of the experimental animal (Stewart, 1968). In rats of 100 g body weight such as were used in this investigation, the hypertrophy reaches its maximal extent some five to seven days after phrenicectomy, at which time an increase in wet weight of the tissue of between 30 - 40% is

typical. Thereafter the mass of the denervated tissue declines so that by the 14th day after nerve section, the hypertrophy can no longer be detected, and, after the elapse of further time, atrophy of the tissue is apparent. Rats which are considerably heavier than 100 g at the time of the operation do not show such a pronounced hypertrophy, and the subsequent atrophic weight loss of the tissue is more rapid. On the other hand, the denervated tissue from neonatal rats demonstrates a less pronounced hypertrophy when compared to the contralateral tissue because of the almost equally rapid increase in weight of the latter during the first few weeks after birth. The paralysed tissue, though, continues to increase in weight, albeit at a reduced rate, for a considerable time after the operation. Atrophy, however, is apparent in the sense that the tissue, although not losing weight, fails to grow as rapidly as it would if still innervated. This graduation in response to unilateral phrenicectomy according to the age of the experimental animal is undoubtedly the result

by the non-paralyzed contralateral hemidiaphragm.

Morphologically, each hemidiaphragm may be readily demarcated into three distinct regions, the lateral costal, the ventral sternal, and the dorsal vertebral regions (see Fig. 1). Feng & Lu, (1965), investigating the changes in wet weight of these regions following unilateral phrenic-ectomy, discovered that it was only the lateral portion of the hemidiaphragm that demonstrated the hypertrophic response. The smaller ventral region was found to atrophy, whilst the dorsal region maintained, approximately, its normal weight. It is apparent, therefore, that any theory proposed to account for "the hypertrophy of the hemidiaphragm", implying the lateral region, must take into consideration the regional differences that occur following phrenic nerve section. Since there is evidence that the three regions of the hemidiaphragm differ appreciably in a number of aspects, for example, nerve and blood supply, histological fibre type, and

of a number of interacting factors, possibly not the least important of which is the greater dependence upon diaphragmatic respiration in neonatal and juvenile animals, (Sweiber, 1968).

The diaphragm, of course, being bilaterally symmetrical, is supplied by two phrenic nerves, one to each hemidiaphragm, with no apparent crossover of nerve impulses during the normal function. Section of either phrenic nerve produces a corresponding hypertrophy in the appropriate half of the diaphragm; there appears to be no difference in response to denervation of either hemidiaphragm, even though, in the rat, at least, the right hand tissue weighs some 10% more than that of the left side. If both phrenic nerves are cut, however, then no resultant hypertrophy is found in either tissue (Feng & Lu, 1965). Clearly, hypertrophy of the unilaterally phrenic-ectomized hemidiaphragm therefore, depends not only the severance of motor innervation, but also upon the influence exerted over the denervated tissue

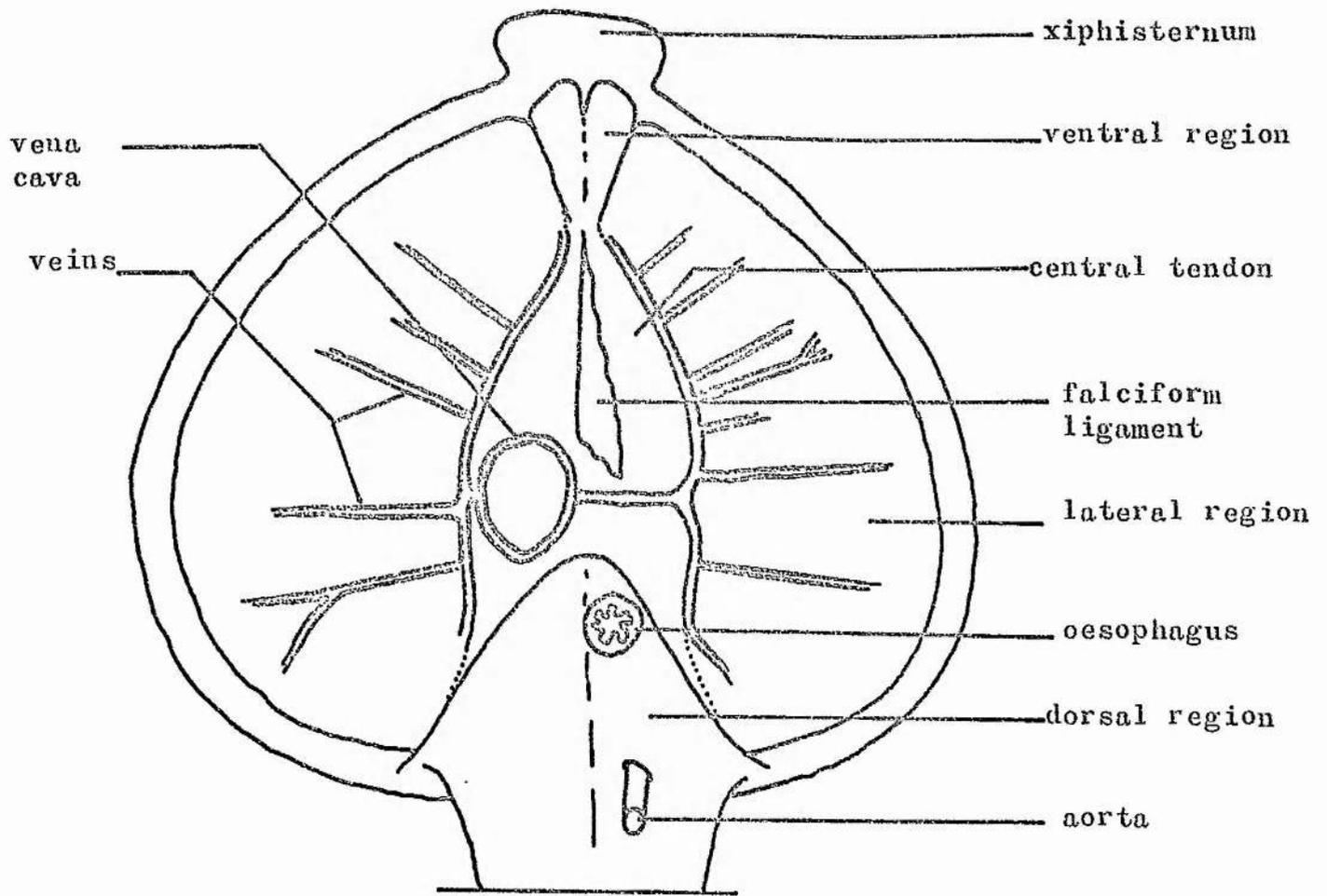


Figure 1. Generalised Diagram of the Rat Diaphragm, viewed from the Abdominal Side.

physiological properties, an adequate understanding of the interplay of the many possible contributory factors can only be obtained by a thorough examination of the tissue in the intact rat. The following sections will deal with these aspects.

GENERAL ANATOMY, HISTOLOGY AND PHYSIOLOGY  
OF THE RAT DIAPHRAGM

Of the three regions of the hemidiaphragm, only the lateral region has been examined at all thoroughly; the ease with which it may be dissected out with its intact phrenic innervation has rendered the preparation a favourite one for the study of certain neuro-muscular interations. Rarely, however, has the whole tissue been the object of study, and there are only a few instances in which comparisons have been made between the properties of the three regions. Unavoidably, therefore, information relating to the physiology and biochemistry of the regions is sparse, a situation hardly representing the most prudent foundation for conjecture as to their differential response to denervation.

Gross Anatomy. As will be seen from Figure.1, the bulk of the diaphragm is composed of the muscle fibres, orientated radially from the central tendon, through which passes the inferior vena cava. The

oesophagus penetrates the diaphragm at the edge of the dorsal region, whilst the aorta actually passes through this portion of the tissue, which forms a funnel shaped structure, of irregular thickness, adhering to the back wall of the abdominal cavity. The lateral and ventral portions of the diaphragm are flat sheets of muscle fibres, approximately 20 cells in thickness (Creese, 1954; Feng & Lu, 1965) inserted at the central tendon and the periphery of the rib cage. A considerable number of the muscle fibres of the rat diaphragm in fact span the whole length of the muscle (Barstad, Gjone & Löwlie (1956). Compared to the total muscle content of the diaphragm, the relative wet weights of the three regions are: ventral, 6%; lateral 54% and dorsal 40% (Feng & Lu, 1965). As mentioned previously, the right hand side of the diaphragm weighs some 10% heavier than the left. Both the peritoneal and pleural surfaces of the diaphragm are covered by mesothelial membranes (Oder, 1954; Peterson, Beatty & Bocek, (1961), have shown that these mesothelial coverings possess a

distinct and separate metabolism compared to that of the muscle fibres. Similarly, it may be expected that other non-muscular cells, for example, connective tissue and cells of the blood vascular system, will contribute distinctly to the properties of the whole tissue, a fact that ought to be borne in mind when dealing with homogenates of "muscle".

Blood supply to the lateral region of the diaphragm comes mainly from the inferior phrenic arteries, which arise as branches of the abdominal aorta (Beck & Baxter, 1960). At the periphery of this region, small branches of the posterior intercostal arteries supplement the supply. The ventral region is vascularized by branches of the internal mammary artery, whilst the oesophageal branches of the inferior phrenic artery supply blood to the dorsal region. Venous drainage of the tissue is by anterior, middle, posterior and accessory posterior phrenic veins; the major vessels lie just within the confines of the central tendon, emphasizing the point of the muscle

insertion, and displaying a distinct bilateral symmetry.

The principal efferent innervation of the diaphragm originates in the phrenic nerve, which, in the rat, derives from cranial nerves IV and V, and cervical VII, (Greene 1935; Wilson, 1968). Passing posteriorly from the brachial plexus, through the thoracic cavity, the phrenic nerve enters the pleural surface of the diaphragm at a point approximately in the middle of the lateral region. Here, it branches and extends intramuscularly as a much ramified diaphragmatic plexus in both dorsal and ventral directions. Histochemical staining with acetylthiocholine to demonstrate the location of the motor end plates shows them to be restricted to a narrow strip, 1 - 2 mm on either side of the main intramuscular branches, about midway between the central tendon and the peripheral attachment of the diaphragm. Examination of both fresh and osmic acid stained preparations of whole diaphragm shows that practically all of the fine terminal branches of the phrenic nerve are located in this strip (Hebb, Krnjevic & Silver, 1964), and it is only in this zone that miniature end

plate potentials can be recorded in the lateral region (Krnjevic & Miledi, 1958). This end plate zone extends from the ventral region as far as the dorsal edge of the lateral region, but there seems to be no continuity with the dorsal region. Acetylthiocholine however, reveals a distinct zone of motor end plate activity in the dorsal region. Since the pneumogastric nerve actually passes through the dorsal region (Greene, 1935) it does not seem inconceivable that innervating branches might arise from it, although as far as the author is aware, direct experimental verification for this supposition is lacking. Nevertheless, a separate innervation for the dorsal region seems likely, and would explain the lack of response of this region to unilateral phrenicectomy.

According to Wilson (1968) there are three categories of nerve fibre that are derived from the phrenic nerve and supply structures within the diaphragm of the rat. Approximately two-thirds of the branches of the diaphragmatic plexus consist of myelinated fibres, 10 - 16  $\mu$  in diameter directed

centrifugally; a few millimeters from the main fasciculi of the plexus, these fibres form irregular dilations, termed "preterminal expansions" peripheral to which the fibre is reduced in size, the myelin sheath becomes no longer visible, and the fibres terminate in structures that resemble motor end plates. Such fibres are considered to be somatic efferent components. Most of the remaining one third of the nerve branches of the plexus comprise myelinated fibres, 6 - 10  $\mu$  in diameter, which are longer and more tortuous than the efferent fibres, and which ramify throughout the musculature, central tendon and serosal coverings of the tissue. As no indentifiable end organs were seen in relation to these fibres, their appearance and mode of termination as free nerve endings suggest a somatic afferent function. The remaining fibres derived from the phrenic nerve are unmyelinated and, on emerging from the plexus as small bundles, accompany vascular structures towards the periphery of the tissue, ramifying at irregular intervals of the vessel wall; these fibres are considered to be vasomotor autonomic nerves.

The phrenic nerve, however, is not unique in supplying the diaphragmatic plexus. Wilson (1968) also describes contributions made by the direct branches of the lower six intercostal nerves, i.e. thoracic 7 - 12, directed centripetally into the muscle and ramifying within a few millimeters of the peripheral attachment of the lateral region. The most numerous of these small fasciculi resembled closely the paravascular vasomotor fibres derived from the phrenic, with which they merged. Afferent components arising from the intercostal nerves were also found; these tortuous, myelinated fibres, 6 - 10  $\mu$  in diameter, were observed to ramify within the serosal coverings of the periphery of the diaphragm. In addition, a small number of myelinated fibres, 10 - 14  $\mu$  in diameter, extended from the periphery to enter the diaphragmatic plexus. Such fibres were especially prominent in the posterolateral portion of the tissue, and some were seen to possess preterminal expansions typical of the efferent fibres of the phrenicus. However, definitive identification as efferent components cannot be made since these intercostal fibres possessed no

demonstrable specialized end terminals, and it therefore remains to be established as to whether the fibres possess any motor function, or whether they are efferent fibres of neuromuscular spindles.

A histological search for these latter structures throughout the rat diaphragm, however, regularly revealed only three such spindles in each hemidiaphragm (Barstad, Kristoffersen, Lilleheil & Staaland, 1965). One was almost always found in the dorsal region of the tissue, the other two usually occurring in the lateral region, although, more rarely, one might be found in the ventral region instead. Nevertheless, these authors found that stretching the muscle in order to stimulate these spindles to fire produced characteristic afferent discharge which could be recorded in the phrenic nerve.

Electrophysiological evidence in support of an "accessory motor innervation" for the diaphragm has been obtained for a number of species (Rosen -

bleuth, Alanis, & Pilar, 1961). Although section of the phrenic nerve was found to reduce drastically the magnitude of the diaphragmatic inspiratory action potentials, it was found that they were rarely eliminated completely. Instead, two separate bursts of activity were commonly found, one coincident with the contractile activity of the inspiratory muscles of the rib cage, and the other coincident with the expiratory contraction. Peripheral stimulation of selected intercostal nerves, and in particular nerves 7,8, & 9, produced a definite mechanical and electrical response in the diaphragm, though not as pronounced as occurred on stimulation of the phrenic nerve.

The presence of efferent intercostal fibres within the diaphragm is scarcely surprising considering the similar embryological development of both the diaphragm<sup>g</sup> and the rib cage musculature from thoracic somites (Wells, 1954), and also in view of the need for co-ordination of intercostal and diaphragmatic activity during respiration. In addition, it should be remembered that the diaphragm is not

exclusively a respiratory muscle; it contracts in many non - respiratory actions, e.g. coughing, vomiting, defaecation, parturition, etc. The presence of the intercostal innervation provides possible efferent pathways for some of these actions not requiring motor stimuli from the respiratory centre in the brain. The degree of involvement of these intercostal fibres in the hypertrophic response following unilateral phrenic-ectomy is, of course, open to conjecture. Certainly, the contribution that they make to the respiratory activity of the intact diaphragm is small, the phrenic nerve undoubtedly representing the major motor pathway to the tissue.

Physiology. The functional unit of contraction in striated muscles is the "motor unit", consisting of a single motoneurone, its axon, and the group of contractile muscle fibres innervated by this single axon. Physiologically, three different types of motor unit have now been recognized in mammalian muscles (Close, 1967), and there is strong evidence to support the concept that muscle fibres of a particular motor unit all possess identical contractile properties (Edström & Kugelberg, 1968). Thus, each type of motor unit may be characterized by its speed of contraction, and the response to repetitive stimulation of its muscle fibres. In addition, it has been shown that the speed of conduction of nerve impulses along the motor axon is also a characteristic of the motor unit type (Eccles, Eccles & Lundberg, 1958). Thus, slow conducting motor axons innervate muscle fibres that, in response to a single stimulus, contract in a slow twitch (not to be confused with the sustained contracture of vertebrate "slow" muscle fibres, (Hess, 1970)), and, on repetitive stimulation, the twitches summate to

establish a state of tetanus at relatively low frequencies. Fast conducting motor neurones, on the other hand, are found to supply muscle fibres, which characteristically produce fast intermittant twitches, and can only be tetanized at higher stimulatory frequencies. The third type of motor unit has properties intermediate between the extremes of these two previous types. For convenience, the three types of motor unit will be referred to, in a physiological sense, as "slow", and "fast" and "intermediate" motor units, respectively.

The characteristic innervation received by each type of muscle fibre is of interest since it seems to represent an adaption towards greater efficiency in muscle contraction. For example, a high frequency discharge of impulses from a fast conducting nerve to a slow contracting motor unit would not result in any greater contractile tension, and would merely serve to fatigue the muscle, whilst the lower frequency discharge from a slow conducting motor neurone to a fast motor unit would be inefficient

in fusing the individual twitch responses to give an effective tetanic contraction (Eccles et al. 1958).

Skeletal muscles may possess varying proportions of these three types of motor unit, the summation of the properties of which accounts for the wide variability in the contractile behaviour of different whole muscles (McPhedran, Wuerker & Henneman, 1965). In general, it is found that motor units extend over a relatively large cross-sectional area of a given muscle (Edström & Kugelberg, 1968), there being a considerable overlap of neighbouring motor units and an extensive scatter of the individual muscle fibres of separate motor units throughout the body of the muscle; this has been found to be especially true for rat diaphragm (Krnjevic & Miledi, 1958). In all probability, this intermingling arrangement of motor units represents an adaption towards greater efficiency in the integration of contraction of several separate motor units, and, thereby, aiding contraction of the muscle as a whole. Recruitment of motor units

in the muscle, as a means of increasing the contractile tension, would thus spread the work load equally over the whole body of the muscle during all stages of tension development.

The average sizes of motor units may be judged from the innervation ratio, i.e. number of contractile fibres in the muscle/number of innervating motoneurons; typically, innervation ratios of 100 or more are found, and sometimes even maybe in excess of 1000 (Clark, 1931; Feinstein, Lindegard, Nyman & Wohlfart, 1955; Wuerker, McPhedran & Henneman, 1965). Rat diaphragm, however has an innervation ratio of only about 25 (Krnjevic & Miledi, 1958); it is not known why this should be so.

In terms of its contractile properties, rat diaphragm is neither a fast nor a slow contracting muscle (Ritchie, 1954; Hajek, Hanikova & Gutmann, 1967; Miledi & Slater, 1969), but rather must be termed "intermediate". Similarly, the average conduction velocity of nerve impulses along the

phrenic nerve is found to be intermediate between those of slow and fast conducting axons (Lewis, 1968; Wuerker et al. 1965a, 1965b). This is not to say, of course, that the tissue is composed solely of intermediate motor units; there is no evidence in support of such an assumption. Rather it would seem, from histological evidence, that rat dia - phragm is composed of a mixture of different motor unit fibre types and that the overall summation of their individual responses accounts for the contractile properties of the whole muscle.

Histology It has long been recognized that there are two main histological types of contractile fibre in mammalian muscles (Needham, 1926). Termed "red" and "white" according to their content of myoglobin, the two types have been the subject of numerous investigations, both histochemical and biochemical, carried out with the purpose of characterizing their respective mode of metabolism. Such studies soon established that the "red" fibre possessed a higher mitochondrial content (Paul & Sperling, 1952) and depended largely upon aerobic metabolism (Domonkos & Latzkovits 1961), whilst the "white" fibres relied chiefly upon anaerobic glycolysis for energy production (Domonkos, 1961). In addition, the "red" fibre was found to have a smaller cross sectional area, and thus possessed a greater surface area to volume ratio for metabolite exchange. In keeping with this, it was discovered that the blood capillary density around the "red" fibre was greater than around the "white" fibre (Romanul, 1964), and, as a consequence, blood flow through muscles composed

largely of "red" fibres was found to be greater than that through muscles containing predominantly "white" fibres (Reis, Wooten & Hollenberg, 1967).

Physiologically, such "red" muscles were found to have a slower contractile behaviour than "white" muscles (Ranvier, 1874) so that the "red" fibre thus became equated with the "tonic" or slow contracting fibre, whilst the "white" fibre was assumed to be fast contracting, since it predominated in fast, "tetanic" muscles. But because of the technical difficulties involved the contractile properties of individual "red" and "white" fibres have never been investigated, these conclusions having been derived from studies on whole muscles with varying proportions of "red" and "white" fibres.

Recent investigations, however, cast serious doubt upon this central tenet of neuromuscular physiology, and it is now proposed that there are three main types of extrafusal fibre found in

mammalian tissues, discriminated by histochemical criteria, and possessing characteristic contractile behaviour. Fortunately, the rat diaphragm is one tissue that has been intensively studied from the point of view of identifying the three main fibre types, so that a great deal of information has been gained concerning the fibre population of the tissue and the ultrastructural details of the fibre types within the tissue.

Discrimination of the three fibre types was first made by Stein & Padykula (1962) using histochemical criteria to examine the leg muscles of the rat: one of the fibre types was found to correspond to the classical "white" fibre, whilst the other two types were both "red" fibres. Of particular interest was the observation that the rat soleus muscle, previously thought to be homogenously "red", was, in actual fact, composed of the two types of "red" fibre. Gauthier (1969) has also found in the rat semitendinosus that the third type of fibre, as well as the "red" fibre appears to reflect "redness" in skeletal muscle.

Further histochemical investigations by Romanul (1964), Dawson & Romanul (1964), Kugelberg & Edstrom (1968) Nystöm (1968) & Pearse (1970), have confirmed and extended the observations of Stein & Padykula. Table.1. summarises the histochemical methods used by various workers to identify the three fibre types. It will be seen that differences in mitochondrial enzyme activity has formed the most consistent rationale for cytochemical discrimination, a fortuitous situation since it allows ultrastructural identification of the fibre types under the electron microscope. As will become apparent later, the two types of "red" fibre possess morphological and cytochemical properties which, although similar, are distinctive in that, to a large extent, they differ quantitatively from each other; for this reason, the three fibre types found in mammalian muscle will be referred to as "red", "white" and "intermediate" fibres.

On the basis of differences in mitochondrial content, the three fibre types have been identified

Table 1. The Histochemical Discrimination of Skeletal Muscle Fibres.

Histochemical Reaction	Degree of Staining Intensity with respect to Fibre Type*			Reference
	"Red"	"Intermediate"	"White"	
Fixed ATPase	+++	+	+	Stein & Padykula (1962)
Unfixed ATPase	+++	+	+++	
Esterase	+++	+++	+	
Glycolytic enzymes	+	+++	+++	Romanul (1964)
Mitochondrial enzymes	+++	+++	+	
Mitochondrial ATPase	+++	++	+	Henneman & Olsen (1965)
Succinate dehydrogenase	+++	++	+	Kugelberg & Edström (1968)
Phosphorylase	+	++	+++	
Myosin ATPase	+++	+	+++	Edgerton & Simpson (1969)
αGPDH	+++	+	+++	
Phosphorylase	+++	+	+++	
NADH diaphorase	+++	++	+	
Malate DH	+++	++	+	
Succinate DH	+++	++	+	
Myosin ATPase	+++	+	+++	
NADH diaphorase	+++	++	+	

\*Staining intensity is graded: +++ = high, ++ = medium, + = low.

with the electron microscope and examined at the ultrastructural level in the adult albino rat diaphragm (Gauthier & Padykula, 1966; Padykula & Gauthier, 1963). The predominant fibre type in this muscle is the "red" fibre, which has an average diameter of 27  $\mu$  and is rich in mitochondria, in which the cristae are typically packed closely together. A broad layer of large mitochondria is interspersed between the plasma membrane and the contractile fibrils; these peripheral mitochondria are often spherical. In addition, the "red" fibre also possesses chains of mitochondria that run longitudinally among the myofibrils. Branches from these mitochondria encompass the myofibrils at the region of the I band to form the characteristic mitochondrial doublets of mammalian extra-fusal fibres. Numerous triglyceride droplets occur amongst the mitochondria of the "red" fibre suggestive that fat utilization plays a significant role in energy metabolism.

The "white" fibre comprises 20% of the popu-

lation of diaphragm muscle fibres, is the largest in diameter (about  $44\ \mu$ ), and tends to store glycogen rather than lipid. In contrast to the "red" fibre, subsarcolemmal aggregations and interfibrillar chains of mitochondria are inconspicuous or absent; practically, all the mitochondria are those associated with the I band, and only a very few small mitochondria occur in a perinuclear position. Cristae in the mitochondria of the white fibre are distinctly sparse. A further distinction between "red" and "white" fibres at the ultrastructural level may be made on the basis of the configuration of the sarcoplasmic reticulum in the region of the H band; "red" fibres possess an elaborate network of narrow tubules, whilst in "white" fibres, there is more compact arrangement of broad more or less parallel tubules (Gauthier, 1969).

The "intermediate" fibre is intermediate in diameter (about  $34\mu$ ) as well as in mitochondrial content when compared to the "red" and "white"

fibres, although at first glance the "intermediate" fibre appears similar to the "red" fibre this is not so, for the subsarcolemmal accumulations of mitochondria and interfibrillar chains are less conspicuous, consisting of smaller mitochondria with less tightly packed cristae. This is especially well seen in the electron micrographs of Gauthier & Padykula (1967) (Figs. 4A & 4B), in which "red" and "intermediate" fibres are seen side by side. The sarcoplasmic reticulum of the "intermediate" fibre most closely resembles that of the "red" fibre (Gauthier. 1969). Whilst it is generally true that "red" fibres tend to be of smaller diameter than "white" ones, especially within a given muscle, such measurements cannot be relied upon as good criterion for identification since it has been shown that diameters of muscle fibres may vary considerably among different muscles of a particular species, and also among similar muscles of different species of animal (Gauthier & Padykula 1966). Recently it has been suggested that the width of the Z-line may be used for positive fibre identification; in the rat diaphragm and semitendinosus muscles the width of

the Z-line for each of the three fibre types is:  
"red" =  $6340 \pm 310$  nm (s.d.), "intermediate" =  
 $4330 \pm 390$  nm, and "white" =  $3390 \pm 300$  nm  
(Padykula & Gauthier, 1970).

In addition, it has been found that there are distinct ultrastructural differences in the neuromuscular junctions of the three muscle types in the rat diaphragm. In the "red" fibre, the neuromuscular relationship presents the least sarcoplasmic and axoplasmic surface at each contact, the points of contact being relatively discrete and separate, with small elliptical axonal terminals. The junctional folds are relatively shallow, sparse, and irregular in arrangement. Axoplasmic vesicles are moderate in number, although post synaptic vesicles in the sarcoplasm are scarce. In the "white" fibre the long flat axonal terminals present a considerable axoplasmic surface, with a vast sarcoplasmic surface area created by long, branching closely spaced junctional folds that may merge with folds of adjacent contacts to occupy a more continuous and widespread area. Both axoplasmic and sarcoplasmic vesicles

are numerous. The "intermediate" fibre has axonal endings that are larger and deeper than those of either the "red" or the "white" fibre, and are associated with the most widely spaced and deepest junctional folds of the primary cleft. In addition, these folds are relatively straight and unbranched. As in the "white" fibre, post synaptic sarcoplasmic vesicles are conspicuous near the sarcolemma of the deeper portions of the junctional folds (Padykula & Gauthier, 1970). Thus it is clear that there is a considerable variation in the ultrastructural detail of the neuromuscular junction of the three fibre types; in view of the known heterogeneity of motor unit types, it is reasonable to suppose that these ultrastructural differences represent functional adaptations related to the contractile properties of the individual muscle fibre types. Unfortunately, at the present time, unequivocal assignation of the three histological fibre types, "red", "white" and "intermediate" to the three physiological types of motor unit "fast", "slow" and "intermediate" has yet to be made, but it seems, on the basis of the evidence

to be presented below, that the traditional concept "red" = "slow" and "white" = "fast" may no longer be tenable:

(i) Close (1967) identified the three kinds of motor unit in rat extensor digitorum longus muscle and soleus muscle. Assuming that the properties of the whole muscle were an exact summation of the individual motor units, that the motor units examined were a representative sample, and that there was no branching of motor axons peripheral to the point of stimulation, Close calculated the average number of motor units for each of these two muscles as 40 for EDL and 30 for soleus. In the latter it was estimated that 27 of the motor units were "slow" contracting, whilst the remaining 3 were "intermediate" motor units, and that each type overall contributed 88% and 12% respectively to the maximum isometric tetanic tension for the whole muscle. The estimated size of the "intermediate" and "slow" motor components corresponds approximately with the size of the histologically "red" and "intermediate" fibre components of Stein & Padykula, (1962), who found that 30% of the muscle fibres present in the rat soleus were "red" fibres and that

these occupied approximately 20% of the total cross sectional area. In addition, they reported higher sarcoplasm/fibril ratios for "red" fibres than for "intermediate" fibres. It thus seems probable that the tetanic tension developed per unit cross sectional area is less for "red" fibres, than it is for the histologically "intermediate" fibre. The "red" fibre therefore probably contributes less than 20% and the "intermediate" fibre component more than 80% of the maximum isometric tetanic tension of the rat soleus muscle.

(ii) Fast contracting leg muscles taken from guinea pigs that had been chronically exercised by treadmill running for up to 18 weeks were found to have a greater concentration of mitochondrial protein per gram of muscle than unexercised muscles, whilst the  $Q_{O_2}$  of the muscles were also found to be significantly increased. Histochemical examination of the muscle using techniques capable of discriminating between the three fibre types showed an increase in the proportion of "red" fibres in the muscles from trained animals, However, no significant differences

were observed in the contractile properties of the trained muscles as compared to the untrained ones, despite the increased proportion of "red" fibres present (Barnard, Edgerton & Peter, 1970a, 1970b).

(iii) The cricothyroid muscle of the bat is believed to control the tension of the two laryngeal membranes involved in the production of the characteristic emissions of ultrasound by this species. The muscles are thought to be responsible for affecting the frequency modulation of the emission, so that it seems likely from the frequency characteristics of the ultrasound that contraction of the muscle may be as rapid as 200 times a second, reaching its maximum state of tension in about 4 msec. and relaxing its tension in 1 msec. It is thus an extremely fast contracting muscle. Ultrastructural examinations, however, (Revel, 1962) reveal that the fibres of this muscle possess features that resemble those of known "red" fibres of the bat diaphragm, e.g. muscle fibres of the cricothyroid have a diameter

of 15 to 30  $\mu$ , whilst those of the bat diaphragm have an average diameter of about 22  $\mu$ , and stain characteristically as "red" fibres (Gauthier & Padykula, 1966). Revel also found numerous densely packed subsarcolemmal aggregations of mitochondria, as well as interfibrillar chains of these organelles, whilst the neuromuscular junction depicted in Fig.13, most closely resembles that typical for "red" fibres. On the other hand, the sarcoplasmic reticulum consists of dilated cisternal-type tubules, more typical of the "white" fibre, although it is difficult to decide, whether they form a network or parallel tubules.

(iv) The thyroarytenoid muscle of the rabbit larynx was found to have an isometric contraction time consistently below 10 msec and usually in the region of 6.5 msec. The contraction time of the rabbit cricothyroid muscle was found to lie between 24 and 30 msec; the former muscle thus is considerably faster than the cricothyroid which has a contraction time more closely comparable to that of a typical fast limb muscle such as the rabbit tibialis anterior. Histochemically, the

cricothyroid of the rabbit was found to be composed of a spectrum of fibres typical of a fast mammalian limb muscle, and including all three histochemical types. The thyroarytenoid muscle, however, was found to possess an exclusively homogenous population of fibres resembling the "red" type (Hall - Craggs, 1968).

(v) Wuerker, McPhedran & Henneman(1965) analysed the fibre conduction velocities of the motoneurons to the gastrocnemius muscle of the adult cat; two main populations of conduction velocity were found, each showing approximately normal distribution, the smaller group having mean conduction velocity approximately one third of that of the faster group. Accepting the equivalence of  $1 \mu$  of fibre diameter to 6 m/sec of conduction velocity (Hursh, 1939), the spectrum of conduction velocities compares well with the histological study of nerve fibre diameters made by Eccles & Sherrington (1930). The proportion of the slow conducting fibres with respect to the total number in the nerve was 32%. On the basis of studies of the

contractile properties of individual motor units in gastrocnemius and soleus, Henneman & Olson (1965) advanced the hypothesis that the functional properties of motor units depend upon the size of motor neurones that innervate them. Histological examination of the adult cat gastrocnemius muscle revealed the following composition: "white" = 54%, "intermediate" = 30%, "red" = 16%. Assuming that Henneman & Olson's hypothesis is correct, it seems that the "intermediate" fibre corresponds to the slow contracting motor unit type. Nystrom (1968) has also analysed the fibre spectrum of cat gastrocnemius according to the tripartite classification; similar figures were obtained, whilst again 31% of the fibres stained histologically as the "intermediate" type.

(vi) A comparative investigation of histological fibre types in the mammalian diaphragm has revealed a correlation between certain aspects of muscle fibre metabolism and functional activity (Gauthier & Padykula, 1966). In small mammals, such as the shrew or the bat, with high metabolic and fast ventilatory rates, the diaphragm is found to consist of a homogenous population

of "red" fibres, whilst in large mammals, such as the pig or the cow, the diaphragm is composed exclusively of "white" fibres; such animals typically have lower metabolic and breathing rates. Mammals with intermediate body size have diaphragms consisting of a mixture of all three histological types. Similarly, the investigations of George & Talesara (1961), and of Lasiewski, Galey & Vasquez (1965), have demonstrated that in the pectoralis muscle of birds the "red" fibres are predominant in those species with high wing beat frequencies, such as humming birds, whilst in soaring or flightless birds, the "white" fibre is most common. Thus it seems that the "red" fibre with its relatively high complement of mitochondria reflects a high degree of metabolic activity and is associated with a high frequency of contraction of muscle. Certainly from a functional point of view, the large numbers of mitochondria arranged between the myofibrils will facilitate rapid provision of "metabolic energy" to the contractile apparatus. In addition, the greater surface area in relation to volume, together with the greater capillary density round "red" fibres (Olson & Swett, 1966),

favours efficient supply of nutrients and removal of metabolic waste materials. It is probably for this reason that the "red" fibres of small mammals are of smaller diameter than those of larger animals (Gauthier & Padykula, 1966).

It is on the basis of evidence such as that given above that Edgerton & Simpson(1969) have suggested that histochemically "intermediate" fibres are physiologically slow, whilst "white" fibres are fast, and "red" fibres only moderately slow or even fast contracting. Similar thoughts were hinted at by Olson & Swett, (1966) and Romanul (1964); there appear to be no reports in the literature to dispute directly the supposition.

In one or two cases an additional fibre type has been described in rat diaphragm; the chief distinguishing characteristic of this type is that it possesses no M line (Forssmann & Matter, 1966; Miledi & Slater, 1969). A possible explanation for this observation though lies in the finding of Knappeis and Carlson (1968) that in frog muscle the appearance of the M line varies according to the degree of stretch applied to the muscle before fixation.

Since muscle fibres constitute by far the highest proportion of cells in the diaphragm, it is to be expected that the metabolism of the tissue will reflect the properties of the muscle fibre types in the various regions of the tissue. It should be pointed out that in the studies of Gauthier & Padykula, (1963, 1966), the portion of the diaphragm investigated consisted of a well defined strip taken from the lateral region of the right hemidiaphragm, and the possibility must certainly be entertained that the proportions of fibre types vary over the extent of the whole muscle. Indeed, Susheela & George (1963) have determined the respiratory quotient for the three component regions of the rat diaphragm, and found them to differ appreciably: dorsal region 0.75, ventral region 0.94, and lateral region 1.14, clearly indicative of utilization of different metabolites in the three regions. Histologically, the dorsal region possesses a high fat content and high lipase and succinate dehydrogenase activities, all criteria suggestive of a preference for fat oxidation (George & Susheela, 1961). The ventral

region contains a still higher fat content and a high lipase activity, but has the lowest level of oxidative capacity in terms of succinate dehydrogenase activity; it is suggested that this indicates a capacity for storage of fat in excess of that utilized. The lateral regions on the other hand show specialization for carbohydrate metabolism in which glycogen forms the fuel for muscular contraction; the glycogen concentration of this region was nearly a third greater than that of the dorsal region, and over twice as much as the concentration in the ventral region. The very high succinate dehydrogenase activity, even higher than that seen in the fat-loaded dorsal and ventral regions, indicates a high oxidative metabolism, and Susheela & George (1963) suggest that the lateral region is capable of oxidising both fat and carbohydrate depending upon which is available at the time. No doubt this capacity is related to the requirement of the diaphragm for continual contraction throughout the rat's lifetime. However, even the lateral region itself seems to be non-homogeneously distributed with respect to fibre type: Frunder (1954) showed that the  $\dot{Q}_{O_2}$  of the dorsal

part of the lateral region of the rat diaphragm was significantly greater than that of the ventral portions, whilst conversely they contained significantly less glycogen. The proportion of the three fibre types recorded by Gauthier & Padykula (1966) are thus not constant even throughout the lateral region.

A further complication is evidence to suggest that the fibre composition of rat diaphragm changes with respect to age of the animal. Davidson (1968) showed, in contradistinction to the usual parabolic relationship, that the weight of the diaphragm is linearly related to the body weight of the rat. The only other organ to possess a similar weight dependency in the rat is the heart. Such a finding seems to imply that the work of breathing is closely correlated with the amount of body tissue requiring oxygenation. The  $\dot{Q}_{O_2}$  of the rat diaphragm is inversely related to body size (Bertalanffy & Pyrozynski, 1953; Frunder, 1954). Thus as the animal gets older its oxygen consumption decreases, so that by infer-

ence one would expect an increasing proportion of white fibres in the tissue, just as is found for different species with lower ventilatory rates (Gauthier & Padykula, 1966); Gesell & Atkinson (1943) have similarly shown physiologically in a variety of animals that frequency of twitching of the diaphragm is inversely related to body size.

Partial confirmation of this supposition comes from the work of Lewis (1968) in his determination of the average conduction velocity of the phrenic nerve with respect to the weight of the rat from birth to the age of six months. It was found that, after an initial postnatal period of 10 days, the average conduction velocity was linearly to the weight of the rat. Thus maturation of the phrenic motoneurons was associated with an increase of the average conduction velocity which, by implication suggests an increase in the average speed of diaphragmatic contraction with age. In addition, Burleigh & Shimke (1969) have shown an increase in the activities of typical white fibre glycogenolytic

enzymes: glycogen phosphorylase, fructose diphosphate aldolase and phosphohexoisomerase, that increase from birth and reach adult levels between three and eight months of age, whilst myoglobin similarly increases during the first three months of life; conversely total hexokinase, high levels of which are considered to be characteristic of red muscles (i.e. those containing "red" and "intermediate" fibres) is found to decrease during postnatal development. Succinate oxidase activities, on the other hand, remain more or less constant during this period. It is therefore apparent that as well as the possibility of an increase in the proportion of "white" fibres in the diaphragm as the animal matures, an alternative might be an increase in the proportion of both "red" and "white" fibres at the expense of the "intermediate" fibres.

## THE EFFECTS OF DENERVATION OF THE METABOLISM

### OF THE DIAPHRAGM

Physiological Effects. The most immediate, and perhaps the most obvious, effect of unilateral phrenic nerve section on the activity of the diaphragm is that the ipsilateral portion of the tissue ceases to contract. Concurrently, an increase in the net blood flow through the tissue takes place; Anrep, Cerqua & Samaan, (1934), using a hot wire anemometer to study the arterial inflow in perfused muscles showed that during the respiratory contractions of the diaphragm, blood flow through the tissue was considerably reduced. As the muscle relaxed, so the flow rate increased. Section of the phrenic nerve caused immediate disappearance of all respiratory fluctuations in the flow of perfusate through the tissue, which remained at a rate comparable to that in the relaxed muscles: net flow through the tissue is therefore increased.

A similar conclusion has been reached by other workers (Buse & Buse, 1959, 1961), from studies of Xylose penetration into the denervated tissue, and of

the binding of  $I^{131}$ -insulin to the tissue:d-xylose, a non-metabolizable sugar, administered in vivo, accumulates to a greater extent in denervated hemidiaphragm, 1 - 3 days after nerve section, than it does in the contralateral control muscle. The difference in accumulation, however, is not apparent if the tissues are incubated in a xylose containing medium in vitro. Similarly, in vivo administration of  $I^{131}$ -insulin results in twice as much radioactivity being bound to the denervated than to the control tissue, whilst identical quantities of the hormone are bound in in vitro incubation studies.

Thus it seems that blood flow rate through the tissue is increased at least up to three days after denervation; beyond this time, however, changes in the flow rate are a matter for conjecture, for, not only does section of the phrenic nerve terminate neuromotor impulses to the muscle fibres, but it also interrupts vasomotor fibres whose function is to exert neuronal control over vascular resistance in the tissue. It may, for example, be the case that at denervation such vasoconstrictors initially are dilated, as in the

case during heavy rhythmic exercise (Folkow, Gaskell & Waaler, 1969); later on they could possibly go into a state of contracture and so reduce blood flow. Information on the rate of blood flow after some time has elapsed after denervation is scarce.

The influence of passive stretching on the flow rate through the tissue is another factor that must be taken into account. Gimlette & Nasrallah (1969), showed that stretching the gastrocnemius of the dog reduced the blood flow rate, whilst on releasing the stretch, flow rate returned to normal. Following denervation, the paralysed hemidiaphragm is subjected to a passive rhythmic stretching caused by the opposing respiratory contractions exerted by the rib cage and the intact half of the diaphragm. Whilst the effect of stretch thus does not appear to impede blood flow a few days after nerve section, only experimental investigation will decide whether net blood flow rate alters as further time elapses.

Whilst unilateral phrenicectomy produces immediate changes in the vascular resistance of the tissue, alterations in neuromuscular transmission properties take a

little longer to come about. Obviously, centrally generated nerve impulses cannot be transmitted to the muscle, but, in an electrophysiological study (Miledi & Slater (1968) found that the transmission of electrical stimuli along the peripheral nerve stump could continue for about 10 hours after section of the phrenic nerve. During this time, release of acetylcholine quanta took place, manifest as miniature end plate potentials. Soon after, however, some of the nerve terminals cease to function, and the proportion of end plates showing min.e.p.ps. fell rapidly, so that after 15 - 20 hours no response was obtained to phrenic nerve stimulation, and there was no sign of spontaneous min.e.p.ps. Slater (1966) found a linear relationship between the length of the peripheral nerve stump and the time at which failure of min.e.p.ps. occurred: for every centimeter of intra - or extra - muscular phrenic nerve remaining, failure was delayed by about 40 minutes. It seems therefore that the maintenance of functional neuromuscular transmission activity of isolated motor end plates is related to the proportion of remaining motor neurone cytoplasm. A similar sort of effect has been reported by Gutmann (1960) who observed

in the denervated anterior tibialis muscle of the rat a different capacity to synthesize glycogen depending upon whether the muscle was left with a long or a short nerve stump. Possibly such effects are related to axoplasmic flow, (see later).

The contractile properties of the diaphragm are found to change on denervation, (Hajek, Hanikova & Gutmann, 1967; Miledi & Slater, 1969). Contraction times, i.e. the time elapsing from the first observable tension development to the peak of the twitch, in response to a single supramaximal stimulus, is found to increase after denervation, but at a slightly decreasing rate as time progresses. Half relaxation times, i.e. the times from the peak of the twitch to one half maximum amplitude, are found to increase rapidly; by two days after denervation a 75% increase is observed, and by 4 days the half relaxation time has doubled to reach a plateau at which it remains for at least a further four days. Maximal tetanic tension in response to repetitive stimulation decreases rapidly. Two days after denervation tension development is only one half of that of the control tissue, and remains close to this value for up to 8 days after the operation. These

experiments demonstrate a considerable and rapid reduction in the speed of contraction and tension development of the muscle.

A reduction in the resting membrane potential of denervated diaphragm muscle fibres has also been observed (Thesleff, 1963); Lullmann & Pracht (1957), found that the membrane potential, measured by means of intracellular microelectrodes, decreased exponentially from 87 mv at 4 days after nerve section to 67 mv at 80 days. Thus the decrease in membrane potential during the period that the tissue is hypertrophied is only slight, and in accordance with this observation, alterations in ionic compositions are similarly found to be of small magnitude. Indeed, the  $\text{Na}^+$  content of the muscle remains essentially unchanged after three days denervation (Harris & Manchester, 1966) and after 9 - 11 days (Lullmann, 1958; Adamic, 1968), whilst less than a 5% decrease in the  $\text{K}^+$  content of the tissue was observed three days after denervation (Harris & Manchester, 1966). On the other hand Creese, El-Shafie & Vrbrova (1968) found no change in  $\text{K}^+$  content of hemidiaphragms denervated 8 days previously, whilst Gutmann et al (1966)

observed an increase in  $K^+$  content following denervation.

Although denervation does not appear to markedly alter  $Na^+$  and  $K^+$  contents in the tissue, it does affect the permeability properties to these ions. Klaus, Lullman, and Muscholl (1960) attributed the decrease in membrane potential to an observed reduction in the  $K^+$  permeability of the denervated fibre membrane. Similarly Ademic (1968) using a kinetic method to determine  $Na^+$  ion movements, reported a  $Na^+$  influx nearly 50% less than in the control tissue, which again was attributed to greater impermeability of the membranes. Creese et al (1968), however, using a steady state technique, found that increased rates of exchange between intracellular and extracellular  $Na^+$  took place with the denervated tissue; the derived value for the muscle permeability constant to  $Na^+$  was twice as high for the 8 days denervated tissue as for the normal hemidiaphragm. It seems likely that the discrepancy between the conclusions of Ademic and Creese et al, lies in the validity of the assumptions that must be made for each of the two methods for determining flux rates of  $Na^+$ .

Further changes in the membrane properties of the denervated hemidiaphragm are suggested by the response of the tissue to acetyl choline. Iontophoretic application of this chemical to innervated muscle fibres causes contractile twitches and depolarization of the fibre membrane. In slow contracting muscles such as the rat soleus sensitivity to acetylcholine, as demonstrated by the ability of the chemical to cause membrane depolarization, extends usually over the whole surface of the fibre, with a peak of sensitivity coinciding with the end plate (Miledi & Zelena, 1966). In fast contracting muscles such as the rat extensor digitorum longus, the depolarization is restricted to the region of the neuromuscular junction and its immediate surroundings. In this respect fibres of the rat diaphragm are typical of fast contracting muscles, for the length of fibre over which acetylcholine produced detectable depolarization was some 500  $\mu$ ; centred around the motor end plate region, and representing approximately 0.8% of the total fibre length (Miledi, 1960; Elmquist and Thesleff, 1960).

The response of chronically denervated muscle, however, to the application of acetyl choline is to shorten and go into a state of contracture. With the denervated rat diaphragm no contractures are produced by acetylcholine until about 40 hours after nerve section. Subsequently, dose dependent contractures may be obtained in response to the drug, reaching its maximum rate of isotonic shortening about seven days after phrenicectomy. By the use of iontophoretic microapplication of acetyl choline and simultaneous recording of the resulting membrane depolarization, Elmqvist & Thesleff (1960), were able to estimate the size of the acetylcholine sensitive surface in single muscle fibres at various days after nerve section. By two days after denervation, the length of the sensitive region had increased to 5% of the total fibre length, extending towards the fibre ends from the end plate region. After about 4 days, half the fibre length was sensitive to applied acetyl choline, and within 7 days, 90% of the entire fibre length responded to the chemical. Lullman & Pracht, (1957). measured the extent of depolarization produced by a constant quantity of acetyl choline for up to 80 days after denervation of

the hemidiaphragm; they found that an average decrease in the membrane potential of 9mv occurred, irrespective of the "resting" membrane potential of the denervated tissue, which, it will be recalled, decreases exponentially throughout this period. The depolarization is associated with an increase in the permeability to potassium ions (Klaus, Kuchinsky, Lullman & Muscholl, 1959), there being both on increased influx and efflux of this ion; a similar effect is also observed for sodium ions. Influx of calcium ions is markedly increased during the depolarization, but only small effects on chloride ion flux can be detected (Jenkinson & Nicholls, 1961). These latter authors suggested that it was the massive influx of calcium ions that caused the contracture to take place; they were unable to observe contracture in calcium-free medium, even though there was no corresponding reduction in the membrane permeability. The response of chronically denervated diaphragm to acetyl choline, thus appears in general to resemble that of the post synaptic membrane. Evidence from studies involving alteration of the ionic environment

of the denervated tissue in vitro appears to confirm the similarity (Freeman & Turner, 1969); as suggested by del Castillo & Katz (1955) for the post synaptic membrane, but seemingly also applying to denervated diaphragm, acetyl choline "short circuits" the excitable membrane, allowing ionic currents to flow according to their gradients.

Morphological Effects. Apart from one or two isolated observations, most of the information available regarding the morphological effects of phrenic nerve section comes from the work of Hajek et al (1967) and Miledi & Slater (1968, 1969). These latter authors have carried out a particularly extensive ultrastructural investigation of the denervated diaphragm, and much of that which now follows is taken from their work.

As soon as one day after denervation the lateral region of the paralysed tissue is visibly paler than its contralateral, and by the second day "has a turgid, glistening appearance not normally associated with this tissue" (Manchester & Harris, 1968). By five days after nerve section, the time at which fibrillation is observable, the tissue is considerably paler, so that in comparison, the blood-filled veins of the muscle are seen to stand out particularly distinctly. Later on, as the tissue begins to atrophy, the red colour returns to the muscle fibres.

The only histochemical examination of the hypertrophying tissue (Feng & Lu, 1965) has revealed that the hypertrophy of the lateral region is a function of small fibres of the tissue. i.e. those rich in lipid and succinate dehydrogenase activity. As soon as four days after denervation, the increase in size of the small fibre was "usually quite evident", but according to these authors "reached a maximum around ten days". At the same time the histochemical staining properties of these fibres was found to be somewhat poorer. The large fibres, by contrast underwent atrophy as a result of denervation but appeared to stain rather better. Thus, the two initially distinct fibre types of the diaphragm became less easy to differentiate from each other as a result of nerve section. In the ventral region of the tissue, it was found that both types of muscle fibre atrophied. One particular aspect of this work however demands special emphasis: despite the fact that Feng & Lu observed a certain proportion of fibres in the hypertrophying tissue with intermediate staining properties, the histochemical criteria used by these authors would not have positively

discriminated between "red" and "intermediate" fibre types. It is not clear therefore, and the possibility must certainly be entertained, whether either or both of these fibre types are involved in the hypertrophy process.

Ultrastructural changes in the denervated hemidiaphragm are evident as soon as 24 hours after nerve section: Miledi & Slater (1968) observed that in contrast to the tortuous and sometimes branched cylindrical mitochondria seen in transverse sections of muscle fibres, the mitochondria of denervated fibres are greatly reduced in size, tending to become globular rather than cylindrical. Many elongated mitochondria were still found running parallel to the myofibrils, but in transverse sections almost all of the mitochondria had a round profile. Qualitatively, it was also found that within a short time after denervation, the total volume of muscle fibre occupied by mitochondria was reduced.

That synthesis of myofibrils takes place during the period of hypertrophy is apparent from the elec-

tron micrographs of Miledi & Slater (1969). Nine days after denervation (corresponding to a time just past the peak of the hypertrophy, since these authors were using 150 - 250 g rats) the average cross sectional area of the denervated fibre was 1.94 times that of the normal ones. The percentage of the total fibre area occupied by myofibrils was 85% in innervated fibres, but only 69% in denervated fibres. Thus, although each denervated fibre has a lower percentage area occupied by myofibrils, the total amount of space occupied in denervated fibres was about 160% of that in control fibres. Since there appeared to be no great detectable change in the mean area occupied by the separate myofibrillar bundles, the number of myofibrils must increase after denervation. This would account for the increased myofibrillar space that is found after nerve section, although, when the denervated muscle fibre is viewed in transverse section, the impression is of a much more loosely packed fibre (See Fig.7. Miledi & Slater, 1969).

In addition it was found that during the early period after denervation, i.e. up to 16 days after nerve section, there was no significant alteration in the myofibrillar filament spacing in the hypertrophied fibres, and the length of the A-filaments also remain unchanged. Denervation hypertrophy thus involves the synthesis of new myofilaments which are possibly added to a small extent to pre-existing myofibrils, but more probably arranged into new, distinct myofibrils.

In keeping with the observation of Muscatello & Aloisi (1965) for atrophying denervated muscle, it has been found that phrenic nerve section causes a proliferation of the sarcoplasmic reticulum in denervated diaphragm (Hajek et al, 1967); one especially marked feature in the hypertrophying tissue was the enlargement and dilation of the longitudinally orientated tubules and of the transverse system, especially at the level of the Z-line. In addition, the Z-line was irregular.

Although they were not commented upon, these changes may also be observed in the electron micrographs of Miledi & Slater (1968).

Further ultrastructural changes are seen in grossly atrophic hemidiaphragm (Miledi & Slater, 1969) but, apart from the observation that muscle fragmentation and myofibril disintegration takes place, the majority of these observations are obscure and difficult of interpretation. Since they are unlikely to be directly relevant to an understanding of the causes of the hypertrophy, they will not be dealt with further.

Biochemical Effects. The changes in the dry weight of the hypertrophying hemidiaphragm correlate closely with those of the tissue wet weight. Nevertheless, increases of a few per cent in the water content of the denervated muscle have been observed (Harris & Manchester, 1966; Creese et al, 1968) and are found to be statistically significant. According to Buse, McMaster & Buse (1965), tissue water content is unchanged on the first two days following phrenicectomy, but by the third day, and thereafter, for up to one month after denervation, an increase in water content was observed.

It is conceivable that this small, but nevertheless, consistent increase in water content could be due to increased capacitance of the blood vascular system of the tissue, following section of the sympathetic fibres of the phrenic nerve. Studies of the distribution of extracellular space markers in the denervated tissue, lend support to this proposition: Buse & Buse (1961) observed in vivo an increase

of the thiosulphate space that paralleled the increase in water content, three days after denervation. Conversely though, in vitro studies demonstrate a decrease in the thiosulphate space of the isolated "cut" diaphragm preparation (Buse & Buse, 1961), and a slight, but not statistically significant decrease in the insulin space of the "intact" diaphragm preparation, (Harris & Manchester, 1966) that, it was suggested, was indicative of expansion of the intracellular fluid. Buse & Buse, (1961), however reported a distinct increase in the water content of the tissue following the incubation procedure, whilst thiosulphate spaces determined in vitro were nearly half as great again as that found in vivo if glucose was absent from the medium, but were still some 30% greater if the sugar was present. There is evidence (Midrio, Corsi & Granata, 1968; Lesh & Rothe, 1969) that in vivo section of sympathetic innervation governing the vascular resistance of muscle causes an increase in the blood flow through the tissue due to dilation of the vascular system.

In addition, it is well established that control over muscle vascular resistance is also exerted by circulating catecholamines, which presumably are required in fixed concentrations in the blood for their effect to become apparent. Since no attempt is made in in vitro experiments to maintain or control the "tone" of the contractile vessels, it seems that the difference observed in the distribution of extracellular space markers in vivo and in vitro are the result of extraneous parameters entering into the experimental design.

That extra synthesis of protein takes place in the hypertrophying tissue is apparent from studies of the relative composition of the denervated muscle. Stewart (1955) observed increases in the total protein nitrogen that paralleled closely the change in wet weight of the tissue, and, on fractionation into the major protein categories of muscle, i.e. sarcoplasmic, contractile and connective tissue proteins, found that the

proportions were unchanged up to 15 days after denervation. Later on, as the tissue atrophied, a relative increase was observed in connective tissue but this was not an absolute increase, since by this stage decreased amounts of the other fractions were found. The constancy of protein composition during the hypertrophy phase has also been confirmed by other workers (Gutman et al., 1966; Zak, Grove & Rabinowitz, 1969):

Since it is apparent that the hypertrophic weight changes of the denervated muscle are the result of the interaction of the protein synthetic and degradative processes of the tissue, it is convenient to consider separately the changes that occur in each of these systems following denervation.

(i) Synthetic Processes: In view of the substantial increase in the protein content of the denervated tissue, it is hardly surprising that there should also be pronounced changes in the nucleic acid

content of the muscle (Gutmann et al, 1966; Manchester & Harris, 1968; Zak, Grove & Rabinowitz, 1969). DNA content rises rapidly after nerve section to a peak value some 65% greater than control values at three or four days postoperative, and a day or so before the maximum protein content is observed. The subsequent decline of total DNA flattens out by the 9th day to a value from 15-20% above control levels. Concentration of DNA per unit weight, therefore, shows a biphasic response: a peak concentration of approximately 30% above control level is reached by the third or fourth day, and because of the continual increase in protein content for 1 or two days afterwards, concentration of DNA then falls. After 6 or 7 days, when the weight of the tissue begins to drop, whilst total DNA remains constant and above normal, a second increase in concentration occurs.

During the initial phase of the hypertrophy, increased rates of incorporation of labelled DNA precursors into the polymer are observed even as

soon as one day after denervation. Maximal rates of incorporation are observed 3 days after nerve section, and even at six days when the content of DNA has begun to decline, the specific activity of extracted DNA is still appreciably greater than control values, thus admirably demonstrating the interplay between synthetic and degradative processes. By 10 days decreased rates of incorporation were found. Zak et al (1969) have also demonstrated increased DNA polymerase activity in a cell-free extract within 24 hours and at three days after nerve section.

Whilst it is clear that increased rates of DNA synthesis occur within the denervated muscle, less certain is the identity of the nuclei in which this process is taking place. Zak et al (1969) found that 2 days after denervation, the tissue contained more nuclei than control tissues, and that after administration of  $^3\text{H}$ -thymidine, the individual

nuclei of the hypertrophying tissues were more heavily labelled. However, the labelled nuclei appeared to be localized almost exclusively in the interstitial connective tissue space between the muscle fibres, and no labelled nuclei could unambiguously be indentified as muscle cell nuclei, which, themselves, appeared to be no more numerous than in control tissues. Quite frequently, though, groups of from 3 to 6 contiguous muscle nuclei were seen in the denervated tissue, and occasionally mitoses observed.

It seems therefore that until an autoradiographic electron microscopic investigation is carried out, it must be presumed that the DNA synthesis is taking place in connective tissue fibrocytes or else in muscle satellite cells; Moss & Leblond (1970) have recently demonstrated that in young, growing animals, satellite cells are capable of undergoing mitosis followed by the incorporation of one or both daughter nuclei into the associated muscle fibre.

Enhanced synthesis of RNA is also apparent after unilateral phrenicectomy (Gutmann et al , (1966) Manchester & Harris, 1968). By one day after nerve section a slight increase in RNA content was observed, which by the third day, had become quite substantial, being some 50 per cent in excess of control values. Concentration of RNA at this time was thus nearly doubled in the denervated tissue. At seven and ten days after denervation, the concentration of RNA was still approximately 20% above control values; it seems therefore, from these limited observations, that the concentration of RNA does not follow the same course as that for DNA in the later stages of the hypertrophy.

Sucrose gradient analysis of phenol-extracted RNA from control and denervated tissues three days after nerve section demonstrates that it is ribosomal RNA that is synthesised after phrenicectomy; in comparison with the "4S" peak, the "18 S" and "28 S" peaks of the denervated tissue

RNA were clearly greater (Manchester, Turner, & Harris, 1970). Presumably the enhanced synthesis of protein in the denervated tissue is to some extent facilitated by increased synthesis of ribosomes.

Increased rates of incorporation of labelled RNA precursors into the polymer are also observed after denervation (Manchester & Harris, 1968). The specific activity of RNA extracted from tissues incubated in vitro with  $^{14}\text{C}$ -adenine or  $^{14}\text{C}$ -uracil was found to be some 20% in excess of control values as soon as one day after nerve section. On the other hand, incorporation of  $^{14}\text{C}$ -orotate, when expressed in terms of radioactivity incorporated into RNA per milligram of tissue, was found to be unaffected by denervation at this time, whilst in terms of the specific activity of the extracted RNA, a decrease was observed in the paralysed tissue. Two and three days after denervation total incorporated radioactivity from  $^{14}\text{C}$ -adenine was still enhanced, but by 10 days had returned to control values; specific

activity, however, declined so that by three days, a 20% decrease was found that, presumably, remained constant until up to 10 days after phrenicectomy.

Conversion of  $^{14}\text{C}$ -orotic acid and its subsequent incorporation into RNA appears to be accomplished with an overall efficiency rather less than that for adenine incorporation. Among many possible explanations, this might be indicative of dilution of the orotic acid pool in the denervated muscle, or even of selective drainage of orotic acid from the pool in favour of alternative pathways for the utilization of the precursor. Certainly, total incorporation of the labelled orotate into the muscle RNA is not so enhanced in the denervated tissue at two days as is the case for adenine: only an 11% increase is observed for orotic acid, compared to the 42% increase in denervated hemidiaphragm when adenine is used. By three days, however,

even this slight increase had been turned into an 18% decrease in incorporation; in terms of the specific activity of extracted RNA, a 12% decrease in incorporation at 2 days, and a 44% decrease at three days were observed. A possible explanation for this apparent drop in incorporation, when measured in terms of specific activity, is that it arises from a constant rate of synthesis of the polymer constituting a decreasing proportion of a rising quantity. Whether this explanation is still applicable to tissues 10 days after denervation, when there would appear to be no further increase in net RNA synthesis, is debatable.

Since accumulation of the nucleotide precursors, adenine and orotic acid, into the tissue water shows no appreciable change in extent at 3 days after nerve section (Manchester et al, 1970), whilst that for thymidine shows a decrease in the denervated tissue, it is clear that further experimentation must

be carried out before an adequate understanding of the situation is achieved.

As might be expected from such a system as the denervated diaphragm that is synthesizing relatively large quantities of protein within a relatively short period of time, enhanced rates of incorporation of labelled amino acids into the tissue proteins may be demonstrated, both in in vitro (Buse, McMaster & Buse, 1965; Harris & Manchester, 1966) and in vivo (Gutmann et al, 1966). Significantly greater rates of incorporation into the denervated muscle proteins have been observed in vitro using  $^{14}\text{C}$ -leucine, one to three days after denervation (Buse et al, 1965); with  $^{14}\text{C}$ -glycine or  $^{14}\text{C}$ -phenylalanine, three days after nerve section (Harris & Manchester, 1966) or with  $^{35}\text{S}$ -methionine at three days (Gutmann et al, 1966). Tissues denervated eight to 14 days before incubation, however do not show enhanced rates of incorporation into muscle protein, compared to controls.

In contrast, Gutmann et al (1966) found that in vivo administration of  $^{35}\text{S}$ -methionine permitted observation of elevated rates of incorporation into denervated tissue protein from three days until up to 14 days after nerve section. Four to six weeks after phrenicectomy, however, greatly increased rates of  $^{14}\text{C}$ -leucine incorporation into protein are found with the in vitro technique (Buse et al, 1965). It is worth considering though, that by this stage the tissue is grossly atrophic and very much thinner than the contralateral control hemidiaphragm, it is quite possible, for example, that the actual rates of protein synthesis, per se, in the control and experimental tissues are similar, but that the latter, being atrophic, presents fewer permeability barriers to the path of the radioactive amino acid. In fact, an elevated rate of incorporation of labelled amino acid into protein could result from one or more of the following circumstances.

(i) The mechanism of synthesis of peptide bonds is more rapid in the denervated tissue than in the control.

(ii) Penetration of labelled amino acid into the cells and access to the protein synthesizing machinery is more rapid in the denervated tissue.

(iii) The intracellular pool of amino acid is smaller in denervated muscles than in controls, or alternatively, a restrictive compartmentation of the pool exists so that effectively the pool from which amino acids are taken for protein synthesis is smaller.

In the latter two cases, the intracellular pool of amino acid under consideration would contain a larger proportion of the labelled amino acid, which would then result in a greater density of labelling in the newly synthesized proteins.

Whilst the first of these postulates could in principle, be readily tested by means of a cell-free in vitro incorporation system, the remaining two are less easy to investigate. Available evidence would seem to suggest that compartmentation of amino acid pools occurs in muscle to a significant extent: Kipnis, Reiss & Helmreich (1961) found a linear rate of incorporation of  $^{14}\text{C}$ -proline into protein of the rat diaphragm under conditions in which the specific activity of the amino acid pool was still rising, prior to reaching a steady state level. Similarly, Manchester & Wool, (1963) observed a comparable phenomenon for the amino acids leucine and arginine; it would seem therefore that for these amino acids, at least, the pool from which the amino acids are taken for incorporation into protein is not continuous with and rapidly mixing with the total free amino acid pool. In this context Guroff & Udenfriend(1961) found that only a small fraction of the tyrosine of the isolated diaphragm was available for

isotopic exchange, whilst Hider, Fern & London, (1969), found that labelled amino acids taken up from the incubation medium are incorporated into protein before they can mix with the total cell pool.

In addition, it should be remembered that in the case of the denervated diaphragm in which increased protein degradation is taking place (see later), then not only would newly synthesized labelled protein be degraded, thus reducing the measurable "synthesized protein", but also the specific activity of the intracellular pool would be decreased, so that if proteins are synthesized from amino acids of this pool, their labelling would be less dense. Quite possibly, the different response in vitro and in vivo in the later stages of the hypertrophy could arise from an increased rate of protein degradation occurring in the in vitro system. Manchester (1961), for example, has shown that during incubation of the diaphragm preparation, there is a leakage of free amino acids

from the cells into the medium. Buse et al (1965) observed no difference in the leakage from control or denervated hemidiaphragm for up to eight days after nerve section, but from eight to ten days a significantly greater efflux was observed from the denervated tissue. It should be mentioned, though, that this effect, possibly indicative of increased protein catabolism taking place in the tissue at this time, was not observed after 14 days had elapsed since denervation.

It should be apparent from this discussion, therefore, that mere measurement of increased incorporation of labelled amino acid into protein is not sufficient evidence to be able to conclude unequivocally that the rates of peptide bond synthesis are altered on denervation.

Since increased incorporation of labelled amino acids into protein could be explained on the basis of increased amino acid uptake, it is pertinent to an investigation of protein synthesis to

consider whether denervation affects the capacity of the hemidiaphragm to accumulate amino acids. Buse, McMaster & Buse (1965) found no difference in the accumulation ratio, (i.e. the ratio of non-incorporated radioactivity contained per ml of tissue water to the amount of radioactivity per ml of medium) of  $^{14}\text{C}$ -leucine up to three days after phrenicectomy; in both control and denervated hemidiaphragm the tissue appeared to concentrate the label to the same extent against a gradient since the accumulation ratio always exceeded unity. In one experiment, eight days after denervation, the paralysed tissue accumulated significantly less  $^{14}\text{C}$ -leucine from the medium, a result probably related to the fact that at this time the denervated tissue is releasing more amino acids into the medium than is the control tissue. Four to six weeks after denervation, accumulation ratios for leucine in the control and experimental tissues were again comparable. In contrast, the in vitro accumulation of the synthetic, non-metabolizable

amino acid: L-aminoisobutyric acid (AIB) was found to be significantly increased up to three days after nerve section; a similar result was observed by Harris & Manchester (1966) using the "intact", but denervated diaphragm preparation. These latter authors also noted an increased accumulation of  $^{14}\text{C}$ -glycine at three days, but by 10 days after nerve section, the accumulation ratio was lower in the denervated tissue and not significantly different from unity, suggestive of an elimination of permeability barriers to this amino acid in the paralysed muscle at this time. Phenylalanine, on the other hand, was not accumulated by control muscles, the ratio being less than unity, and, after three days of denervation, there was found to be no significant change in the accumulation ratio. Thus it appears that in the early stages of the hypertrophy the paralysed tissue is capable of accumulating glycine and AIB, but not leucine or phenylalanine. Amino acid analysis of the free amino acid content of diaphragm muscles (Turner & Manchester, unpublished observations;

Manchester, 1970) show that the concentration of glycine in the tissue water is more than 15 times as great as that of either phenylalanine or leucine. Since the incorporation of both  $^{14}\text{C}$ -leucine and  $^{14}\text{C}$ -phenylalanine is known to be enhanced upon denervation, the possibility exists that an increased rate of protein synthesis is obscuring an effect of amino acid accumulation. AIB, by this argument, would thus be found to accumulate because it is not incorporated into protein, whilst the enhancement of glycine accumulation would be observable because the overall rate of protein synthesis limited by the availability of essential amino acids such as leucine and phenylalanine, would be insufficient to reduce the size of the glycine pool to obscure the accumulation taking place. Recently, Manchester (1970) has suggested the use of cycloheximide for in vitro studies of amino acid accumulation to overcome the complicating influence of protein synthesis draining amino acids away from the intracellular pools.

The involvement of lipid metabolism in the hypertrophy of the denervated diaphragm is indicated by the work of Kouvelas & Manchester (1968), who studied the incorporation of  $^{14}\text{C}$ -acetate into lipid fractions of the tissue. As in the control muscle, considerably greater quantities of radioactivity were found to be incorporated into the neutral lipid fraction of the denervated tissue than into its phospholipid fraction; denervation, itself, appeared to cause a slight rise in the incorporation from one to four days, but by 10 days there was no sign of any increase in either fraction. Since the concentration of neutral lipid extractable from the diaphragm remained the same after denervation, it is clear that an increase in the specific activity of this fraction must have taken place, whilst the increase in tissue content thus implies a massive deposition of lipid during the early stages of the hypertrophy.

(ii) Catabolic Processes: Very little information is available concerning the degradative

changes occurring in the diaphragm muscle after unilateral phrenicectomy, although it is clear that such changes play an equally significant part in the chronology of the hypertrophy. Gutmann et al (1966) recorded an 18% increase in the activity of leucine amino peptidase activity in the denervated muscle at three days, whilst at seven days a 39% increase was observed. From this it would appear that section of the phrenic nerve not only increases protein synthesis, but also protein catabolism quite soon after denervation, and that a greater increase in peptidase activity is associated with the eventual decline of the hypertrophy. It must, of course be remembered that leucine amino-peptidase is but one of many peptidases present in muscle, and that the observed response may not be typical of all such enzymes.

Alterations in the activities of nucleases within the denervated tissue have received little attention, despite the fact that there are decreases in the contents of both DNA and RNA. Manchester,

however, has observed a moderate decrease in ribonuclease activity at three days after denervating the muscle (Unpublished observations); the possibility, therefore, exists that the increased stability of "messenger" RNA is involved in the hypertrophic process, although the action of the ribonuclease upon transfer RNA or even the ribosomes themselves cannot be ruled out.

## Some Aspects Of Energy Metabolism In The

Denervated Rat Hemidiaphragm. Basal uptake of glucose by the rat hemidiaphragm, measured in vitro, is unaffected by denervation if expressed in terms of unit weight of tissue (Buse & Buse, 1959). Similarly, the non-metabolizable pentose d-xylose was found in vitro to be accumulated to comparable extents in both control and denervated tissues. In in vivo studies, however, a different picture emerged: on the first day after denervation the concentration of d-xylose was smaller in the denervated tissue than in the control. On the second and third days, more d-xylose accumulated in the paralysed muscles than in the controls. One possible explanation to account for this effect is that blood flow through the tissue could become greater on the denervated side so that between the time that the xylose was administered and the death of the animal, more of the sugar had accumulated in the two or three day denervated tissue. A similar argument has been put forward to explain the difference in  $^{131}\text{I}$ -insulin binding to denervated muscle in vivo and in vitro

Buse & Buse, 1959, 1961). On the other hand, the observation of Smith, Randle & Battaglia (1961) that enhanced rates of sugar entry into muscle is induced by anoxia might be put forward to explain the discrepancy between the in vitro and in vivo observations on sugar uptake: in the in vitro system, the tissues are incubated in isotonic salt media saturated with 95% O<sub>2</sub>; 5% CO<sub>2</sub>, with the presumed consequence that the adequate oxygenation of both control and experimental tissues causes identical sugar uptakes. In vivo, though, the greater uptake in the denervated tissue would be considered to be indicative of anoxia, and probably therefore of reduced blood flow. If this is the case, it becomes extremely difficult to advance a plausible explanation for the different rates of <sup>131</sup>I-insulin binding.

Whilst basal glucose uptake thus apparently remains unchanged on denervation, the concentration of glycogen decreases in the paralysed tissue to about 60% of the control value in fed rats between

two and four days after the operation (Buse & Buse, 1959). Possibly this is indicative of decreased capacity to synthesize glycogen from the glucose entering the cell, as suggested by Gutmann et al (1966), but it could also indicate a greater energy demand than could be satisfied by glucose entry alone. On fasting for 16 hr, though, it was found that the two to four day denervated muscle contained more glycogen than in the control tissue; degradation of glycogen thus appears to be impeded in the denervated hemidiaphragm, so that it seems likely that the decreased glycogen concentration found in the fed animal results from an inability to synthesize the polysaccharide. Amongst many possible reasons, this could be due to an effectively reduced enzymic capacity of glycogen synthetase, or even a selective drainage from the glucose 6-phosphate pool for modes of metabolism other than the storage of glycogen; The synthesis of nucleic acids, for example, would be expected to require the ester in large quantities for the reactions of the pentose phosphate pathway.

Oxidation of  $^{14}\text{C}$ -acetate by the unilaterally phrenicectomized diaphragm is found to decrease in the paralysed tissue; by four days, the rate of  $^{14}\text{CO}_2$  production in the denervated muscle, on a unit weight basis, is only half that of the innervated side, and is consistent with the observed fragmentation of mitochondria (Miledi & Slater, 1969).

The Effect Of Insulin On The Metabolism Of

The Denervated Rat Diaphragm. The response of the denervated tissue to the presence of insulin, although inexplicable in terms of mechanism, is particularly interesting. Insulin stimulated the in vitro incorporation into protein of labelled amino acids by the normal innervated tissue (Buse et al, 1965; Harris & Manchester, 1966) and also accelerates the accumulation of certain amino acids into the intracellular water (Manchester 1970). Similarly, the presence of insulin increases the rate of uptake of glucose in vitro and of d-xylose penetration both in vitro and in vivo (Buse et al, 1959, 1961). Insulin is thus considered to exert an anabolic effect on muscle (Manchester, 1970).

On denervation, however, the effect of insulin on all these aforementioned parameters becomes reduced within a few days, though possibly an effect of the hormone is regained to a limited extent at

about eight to ten days after denervation (Buse et al. 1959, 1961, 1965; Harris & Manchester, 1966). Whether this lack of an insulin effect is due to increased sensitivity of the denervated tissue to the hormone is not certain; there seems to be no impairment in the binding of  $^{131}\text{I}$ -insulin to the paralysed tissue (Buse et al. 1959). The most likely explanation is as follows: with the exception of the in vitro uptake of glucose and d-xylose, the effect of denervation is to increase the uptake and assimilation of certain amino acids and sugars; most studies have shown that by three days after denervation the increase has reached an upper limit, similar to that due to the presence of insulin, and possibly indicative of the maximal capacity of the tissue. The lack of the response to insulin during the early stages of the hypertrophy possibly thus arises because of the inability of the tissue to demonstrate an increase in excess of this apparently maximum limit. Later on, when degeneration of the tissue becomes evident, and the rates of uptake and assimilation are reduced in the

denervated tissue, the insulin effect, though diminished, is once again observed. At this stage, however, the enhancement of these activities due to denervation, and the effect of insulin are reduced, and presumably indicate that degenerative changes have reduced the maximum capabilities of the tissue.

## AXOPLASMIC FLOW

Studies using radioisotopes have revealed that substances believed to be synthesized in the cell bodies of neurones are carried down inside the axon in a process termed "axoplasmic flow". Two distinct rates of passage of such material are now recognized (Ochs, Johnson & Ng, 1967); the injection of  $^{32}\text{P}$  into ganglia containing motoneurone cell bodies reveals a relatively slow axoplasmic flow rate for labelled material passing down the nerve: 4.5mm/day (Ochs, Dalrymple & Richards, 1962), whilst the injection of  $^3\text{H}$ -leucine demonstrates a more rapid flow rate, the crest of radio-actively labelled materials being displaced down the nerve at a rate of  $401 \pm 35$  mm/day (Ochs et al, 1967).

Analysis of the phosphate labelled material of the nerve showed that only the phosphoprotein fraction, which contained some 7% of the total axonal phosphorus, took part in the axoplasmic flow (Samuels, Boyarsky, Gerard, Libet & Brust, 1951). Gel chromatography of the  $^3\text{H}$ -labelled material showed it to consist largely of soluble proteins of high molecular weight (450,000 & 68,000),

polypeptides of molecular weight 5,000 and a small amount of free leucine (Ochs et al, 1967). Studies using inhibitors of protein synthesis, pulse loading experiments and a ligature technique have demonstrated, for the fast axoplasmic flow at least, that the labelled material is synthesized in the soma of the neurone and that a somal mechanism of propulsion is not responsible for the flow. Since the form of the peak of activity moving down the nerve suggests that diffusion is unlikely to be responsible, Ochs & Ranish (1970) have proposed that the mechanism of fast transport is locally present in the axons, and infer from the linear displacement of the activity crest with respect to time that the mechanism is present all along the length of the fibre.

If this is the case then one might reasonably expect axoplasmic flow to continue in the peripheral portion of the sectioned nerve for a finite time after the section had taken place. It is of some interest therefore that Slater (1966) detecting the failure of miniature end plate potentials in denervated rat diaphragm fibres, recorded that for each extra centimetre of peripheral phrenic nerve

remaining failure was delayed by 40 minutes.

Calculation shows that the failure is delayed by 360 mm/day, in excellent agreement with the findings of Ochs and his co-workers for the fast axoplasmic flow rate. It is possible therefore that such axoplasmic flow is involved in the maintenance of activity of nerve terminals.

Buller, Eccles & Eccles (1960) on the basis of the results of cross re-innervation experiments postulated the existence of a substance that "passes down the axons of motoneurons, crosses the neuromuscular junction and traverses the muscle fibres, where it exerts its effect over the process of fibre differentiation". Gutmann (1960, 1969) has amassed a considerable quantity of evidence which he considers to be indicative of the existence of a "trophic" substance present in nerve and exerting its effect on muscle. Typically such evidence consists of a small but nevertheless significant change observed in a variety of parameters measured in denervated muscle depending upon whether the muscle was left with a long or a short nerve stump. As far as the present author is aware such a trophic substance has yet to be

isolated, although Samuels & Gorevic (1968) claim to have demonstrated the retardation of denervation atrophy following the injection of brain proteolipids; the evidence however is not convincing as it stands and appears not to have been repeated.

POSSIBLE CAUSES OF THE DENERVATION HYPERTROPHY OF  
THE RAT HEMIDIAPHRAGM.

The post denervation hypertrophy of the rat hemidiaphragm is a complex process. Of the many explanations that have been put forward to account for the phenomenon perhaps the most plausible is that the hypertrophy is caused by the passive rhythmic stretching which is experienced by the paralysed muscle whilst the animal respire (Sola & Martin, 1953). Thus diaphragms that are bilaterally phrenicectomized (Feng & Lu, 1965) or unilaterally phrenicectomized but also costotomized (Gutmann et al, 1966) fail to hypertrophy because the stretch effect is reduced. Further evidence in support of this suggestion comes from the observation that the resting metabolism of isolated muscle increases during passive stretch (Feng, 1932) and that rates of in vitro protein synthesis are increased when the muscle is kept under tension (Buresova, Gutmann & Klicpera, 1969). Stewart (1968) found that tenotomy of denervated limb muscles enhances the atrophic response only when the limb bones are rapidly elongating, i.e. the passive stretch of the denervated muscle that is released on tenotomy seems

to exert a trophic influence on the muscle. Increased tension has also been suggested as a cause for the compensatory hypertrophy of limb muscles following tenotomy of the synergist; the phenomenon occurs independently of innervation and points to a non-neural factor as a causative agent (Schiaffino & Hanzlikova, 1970).

A particular feature of the denervation hypertrophy of the rat hemidiaphragm is that the hypertrophy does not take place in all the fibre types present in the muscle (Feng & Lu, 1965). Available evidence suggests that the "white" fibres begin to atrophy immediately after nerve section and that the "red" and/or "intermediate" fibre types are the ones that undergo the hypertrophy. Schiaffino & Hanzlikova (1970) showed that compensatory hypertrophy occurs to an almost equal extent in "red" & "white" muscles, the hypertrophy of the rat soleus being only fractionally greater than that of the plantaris. In the hypertrophying denervated diaphragm therefore some factor other than passive rhythmic stretch must account for the differential fibre response.

An increased blood flow has been suggested as being an early consequence of denervation. Schreiber and his co-workers (Schreiber, Oratz & Rothschild, 1966; Schreiber, Evans, Oratz & Rothschild, 1967) have demonstrated in the isolated perfused working rat heart that increased ventricular perfusion rate or a rise in aortic pressure resulted in an increased rate of incorporation of radioactive amino acid into protein. This effect could also be observed when the normal perfusion medium was made hyperbaric with respect to oxygen tension; increased oxygen availability thus appeared to stimulate protein synthesis in the working rat heart. Romanul (1965) has shown that in mixed skeletal muscles the capillary density around mitochondrial-rich fibres is appreciably greater than that around the "white" muscle fibres. The fibre selective hypertrophy of the denervated diaphragm may thus be a manifestation of the greater capillary supply to the non-white fibre types, the increased blood flow permitting a greater availability of oxygen to the red fibres and thus stimulating protein synthesis. Carrow, Brown & van Huss (1967) subjected rats to a lengthy exercise schedule sufficient to cause hypertrophy of the

hind limb muscles. "Red" muscle fibres hypertrophied to a greater extent than did the "white" fibres, but nevertheless a statistically significant increase in fibre diameter was found for the "white" fibres after the enforced exercise. More surprisingly it was found that the number of capillaries available to each fibre type increased more for the "white" fibres as a result of the exercise than was the case for the "red" fibres. Thus under conditions in which hypertrophy of white fibres takes place an augmentation of the capillary supply to these fibres is observed. If it is a necessary condition for the hypertrophy of the white fibre that there be an increased blood supply then the situation occurring in the denervated rat hemidiaphragm, in which possibly all the fibre types are stimulated to hypertrophy by the stretch effect, will act adversely against the white fibres because of the inability within the time available to augment the capillary supply to these fibres. Of course the situation is probably complicated still further by the extent that control over blood flow through the tissue still remains to the vasoconstrictors.

On the other hand, the differential response of the various fibre types may be due to some intrinsic property of the fibres themselves. Bajusz (1964) found that in denervation atrophy the "white" fibres decreased in size earlier than did the "red" fibres and therefore proposed the greater dependence of the "white" fibres on neuromuscular integrity, since he found that if muscles were immobilized (in plaster casts, or by tenotomy) both "red" and "white" fibres atrophied at the same rate. In support of this concept is the finding of Brust (1966) that in mice with muscular dystrophy the percentage reduction in weight of the soleus muscle was only half that of the gastrocnemius, whilst the contraction characteristics of the soleus were much less severely affected by the dystrophy. Goldberg (1967) has demonstrated that the uptake of  $^{14}\text{C}$ -AIB, and incorporation of  $^{14}\text{C}$ -leucine into muscle protein occurs to a greater extent in red skeletal muscle than in white muscle. Unfortunately these results could be interpreted as indicating that red muscles have a greater blood flow than white ones, but recently studies of the protein synthetic capacity of individual fibres in vitro have confirmed Goldberg's conclusion (Short, 1969).

## PURPOSE OF THE INVESTIGATION.

It is apparent from the foregoing that the hypertrophy of the denervated rat hemidiaphragm is poorly understood, at least from a mechanistic viewpoint; that information that is available is often of a fragmentary, and in some cases, conflicting nature. Nevertheless, it is possible to generate an overall impression of how denervation effects the metabolism of the diaphragm: it is of a system which once activated to hypertrophy, increases its anabolic metabolism towards its maximum capabilities. During the hypertrophy, though, the adverse effects of denervation begin to exert their increasingly greater influence, with the result that degeneration of the tissue eventually takes place. As yet, the causative agents for the anabolic and catabolic responses are unknown.

It has been the aim of this investigation to study the activities of selected enzymes, characteristic of various aspects of muscle metabolism, in the denervated tissue, and using them as diagnostic indicators, to try to gain information as to the causes of the hypertrophy and a greater understanding of the changes that take place after phrenic nerve section.

## MATERIALS AND METHODS.

Operative Procedure For The Denervation. Left unilateral phrenicectomy was routinely performed using an intrathoracic approach. Male albino rats, bred in the department and of the appropriate body weight were anaesthetized with ether, the fur trimmed from the left ventral surface of the thorax and a thoracotomy incision made. Using a glass hook to manipulate the phrenic nerve out through the incision, a portion of the nerve, usually about 2 to 3 mm in length, was cut out with scissors; reinnervation was thus considered to be impossible since the proximal and distal portions of the nerve could not now come into contact with each other inside the thoracic cavity. Penicillin powder ("Crystapen" Benzyl penicillin, sodium salt, B.P.; Glaxo Laboratories Ltd, Greenford) was applied to the exposed surfaces of the wound which was then closed with silk sutures. Within about 10 minutes of the completion of the operation the rat would be fully

recovered from the effects of the ether and seemed unaffected by the paralysis of one half of its diaphragm.

Rats which had been denervated for up to 15 days before sacrifice were used in this investigation; the period of study thus covered the hypertrophy of the tissue and the beginning of the atrophy phase. Since many enzymes are found to vary in concentration according to the age of the animal from which the particular muscle was taken (Burleigh & Shimke, 1969), it was considered advisable that at the time of sacrifice all the rats should be of comparable body weights, i.e. approximately 100 g. The post denervation hypertrophy of the hemidiaphragm of the rat of this body weight is reported to be the most pronounced (Stewart, 1968) at least when compared to the contralateral tissue of the same rat. Accordingly it was necessary to operate on animals often of considerably lower body weight so that by the time of sacrifice the rats would have grown to the required tare. Table 2 presents the body weight ranges of rats at the time of denervation that were found to be appropriate for

Table 2. Approximate body weight range of rats at the time of unilateral phrenicectomy that were required in order that the rats should weigh close to 100 g at the time of sacrifice.

No. of days between time of operation & time of sacrifice.	Approximate body weight range (g)
15	35 - 40
10	60 - 65
7	75 - 80
5	85 - 90
3	90 - 95
1	95 - 100
0	100

adequate weight gain before slaughter. Rats, which are referred to in the tables as being denervated for 0 days, were not operated upon at all.

Routinely, rats were killed by decapitation. On opening up the thoracic cage, prior to removal of the hemidiaphragms, a check was made to ensure that the nerve to the left hemidiaphragm was indeed sectioned. In cases where an anaesthetic was used and it was possible to turn the animal onto its back, the asymmetry of the respiratory movements also served to confirm that denervation was complete. As a rule the two halves of the diaphragm were removed separately so that they might not be confused. Except where specifically indicated, the two hemidiaphragms were rinsed separately in isotonic (0.15 M) sodium chloride solution, blotted upon Whatman No.42 filter paper, trimmed free of excess fat and connective tissue, weighed in air on a torsion balance, and then placed in the appropriate homogenization medium.

Anaesthesia. For the measurement of certain parameters in the denervated muscle, i.e. glycogen levels

and the activities of glycogen phosphorylase a, it was necessary to induce anaesthesia in the animal before removal of the tissue, so that stress-mediated release of catecholamines might not affect the values of the parameters concerned. Halothane (1,1,1-fluoro-2,2-bromochlorethane) was considered to be the most suitable anaesthetic for this situation.

Anaesthesia was induced in rats, one at a time, by placing them in a cage enclosed within a polythene outer covering, through which a gaseous mixture of oxygen and halothane was passed; the mixture was produced by bubbling the oxygen through a Dreschel bottle containing the liquid anaesthetic. On removal of the anaesthetised rat from the cage, anaesthesia could easily be maintained by blowing oxygen through a loosely fitting mask soaked in halothane and placed over the animal's snout. By manipulating the position of the mask, it was possible to adjust the depth of anaesthesia such that the "pinch-withdrawal" reflex of the hind leg was just absent. Preliminary trials showed that at this stage it was possible to decapitate and exsanguinate the rat without it showing any signs of "voluntary" muscular activity. The hemidiaphragm could then be dissected

out rapidly for the analysis of glycogen.

In experiments in which it was required to obtain resting levels of phosphorylase a, the following procedure was found to be convenient in practice: as gently as was possible, each rat was removed from the keeping cage and placed in the anaesthetising cage which had been gassed for a few minutes previously with the oxygen/halothane mixture. The rat was quickly anaesthetized and, provided that adequate concentrations of the oxygen/halothane mixture were also present, would remain quiescent for long periods of time. Preliminary studies showed that about 10 min of anaesthesia was adequate to obtain low (approx 30%) levels of phosphorylase a, but in actual practice a period of 20 min of anaesthesia was routinely adopted. The extended time period was used in order to take advantage of the observation of Piras & Staneloni (1969) that the return to normal values of an elevated phosphorylase a level took approximately 5-10 min. That the 20 min period was adequate for this purpose is shown by the excellent agreement of the values so obtained with other values quoted in the literature for "intact" preparations (see discussion). During this 20 min period, a check was maintained on the rat's breathing rate. At

the end of this time the rat was decapitated, the body cavity rapidly opened, and the diaphragm exposed. Ice cold buffer (50 mM potassium phosphate buffer, 20 mM NaF, & 5 mM EDTA, final pH 6.9) was immediately rinsed over the surface of the muscle to cool it and to allow the NaF/EDTA inhibitors to take effect as soon as possible. A small hole was then cut in the diaphragm, just below the xiphisternum, and the pleural cavity was filled with more of this ice-cold buffer. The hemidiaphragms were then dissected out and soaked in the same buffer for about 5 min prior to weighing.

pH. In an attempt to minimize adverse conditions that might alter the stability of the enzymes during their extraction and estimation, it was decided to maintain the pH of all the solutions used in the process at a value comparable to the overall intracellular pH ( $\text{pH}_i$ ) of rat muscle. In addition, the assay of all enzymes at a constant pH value makes for easier comparisons between extractable activities than if each enzyme were assayed at its "optimum" pH, a situation which it was hoped might give a clearer indication of the metabolic status of the muscle cell.

Several methods have been reported in the literature for the determination of muscle pH; the closely similar estimates obtained by each of the methods lends credibility to the value of each as a means of determining  $pH_i$ . A recent review (Waddell & Bates, 1969) summarizes the available methods for the determination of  $pH_i$  in muscle and discusses the advantages and disadvantages of each technique. Table 3 collects together reported values for overall  $pH_i$  for rat skeletal muscle, and clearly shows that  $pH_i$  is close to 6.9, the value finally chosen for these studies. Use of this pH also has the advantage that it happens to be close to the optimum pH for activity for a number of characteristic muscle enzymes, e.g. creatine phosphokinase (Rosalki, 1967); adenylate kinase (Bowen & Kerwin, 1956); myosinfree  $Mg^{++}$  activated ATP'ase (Kielley & Meyerhof, 1948); glycogen phosphorylase (Cori & Cori, 1940); the I form of glycogen synthetase (Villar Palasi, Perez, Hizukuri, Huijing & Larner, 1966); and so on.

Table 3. Reported Values of  $pH_i$  for Rat Skeletal Muscle

Method of Determination	$pH_i$	Reference
Glass microelectrode	6.98	Carter (1961)
Tissue $CO_2$ distribution	6.98	Gardner, MacLachlan & Berman (1952)
" " "	7.11	Eckel, Botschner & Wood (1959)
" " "	6.92	Hudson & Relman (1962)
" " "	7.03	Miller, Tyson & Relman (1963)
" " "	7.06	Sanslone & Muntwyler (1968)
" " "	7.20	Nichols (1958)
" " "	6.66	Koch & Woodbury (1960)
DMO* distribution	6.94	Irvine, Saunders, Milne & Crawford (1961)
" "	6.89	Miller, Tyson & Relman (1963)
" "	6.89	Adler, Roy & Relman (1965)
" "	7.01	Irvine & Dow (1966)
" "	6.91	Sanslone & Muntwyler (1966)
" "	6.83	Schloerb, Blackburn & Grantham (1967)
" "	6.89	Sanslone & Muntwyler (1968)
" "	6.72	Irvine, Farelly & Fraser (1964)
" "	6.85	
" "	6.92	Saunders, Irvine, Crawford & Milne (1960)
" "	6.99	Sanslone & Muntwyler (1967)
" "	6.81	
" "	6.62	
" "	6.82	Wilson & Simmons (1970)
" "	6.80	

\*DMO = 5,5-dimethyl-2,4-oxazolidinedione

## Analytical Methods.

(1) Myoglobin Determination The extraction and analytical procedure for the estimation of myoglobin was essentially that of Reynafarje (1963), modified to cope with the small quantities of pigment obtainable from the rat hemidiaphragm: each tissue was placed in an ice-chilled glass homogenization vessel of the Potter-Elvehjem type, where it was minced finely with scissors before being homogenized in 0.04 M potassium phosphate buffer, pH 6.6, in the proportion of 9.25 ml buffer per g wet weight of tissue. Throughout the homogenization period the containing vessel was surrounded by ice. The resultant homogenate was centrifuged at 28,000 g for 50 min at 2°C. The clear supernatant was collected by syringe and transferred to a microtonometer, which had been made by securing a small glass bowl onto the top of a 0.5 ml volume glass microcuvette, using "Araldite" epoxy resin. The aperture at the top of the bowl was made airtight with a rubber bung through which two shortened syringe needles had been thrust to allow for gassing; gas flow was controlled by means of a gate clip serving two short pieces of rubber tubing which had been sealed over the plastic couplings of

of the syringe needles. Tests demonstrated that this arrangement was gas-tight and that it could be comfortably accommodated within the cell chamber of the Unicam SP 600 spectrophotometer.

The 28,000 g supernatant was first degassed by connecting the microtonometer to a vacuum line, and then pure carbon monoxide (Air Products, Ltd) was passed through for about 8 min. At this point the flow of gas was interrupted and a few crystals of dry sodium dithionite were added to the solution in the tonometer to ensure complete reduction of the pigments. Carbon monoxide was then passed through for a further two minutes. Extinctions were determined for the two wavelengths 538 and 568 nm. Myoglobin concentration was calculated from the expression:

$$\text{Myoglobin (mg/g wet weight)} = (E_{538} - E_{568}) \times 58.65$$

(ii) Glycogen Determination "Fed" rats were allowed free access to food and water up to the time of death; "Fasted" rats were starved by withdrawing food from their cages some 16 - 18 hr beforehand.

The rats were anaesthetized with halothane as described previously; tissue glycogen was estimated by the method of Good, Kramer & Somogyi (1933), the glucose produced from the acid hydrolysis of the glycogen being assayed by the o-tolidine linked glucose oxidase method of Marks (1959). Glycogen concentrations are expressed as  $\mu$ moles glucose per g wet weight.

(iii) Analysis of Free Amino Acids and Other Ninhydrin Reacting Substances. Amino acid analysis was carried out on hemidiaphragm extracts pooled from groups of 5 animals (non-denervated, 10 days and 15 days) or groups of 6 animals (1, 3, & 7 days after denervation). Rats were killed by decapitation, the hemidiaphragms removed individually, trimmed and blotted free of blood between filter papers, before each was homogenized in 0.75 ml ice cold 3% (w/v) sulphosalicylic acid containing 0.1 mM nor-leucine as an internal standard as suggested by Walsh & Brown (1962). The homogenization was carried out with the containing vessel surrounded by ice to prevent breakdown of glutamine, which would produce erroneously high values for glutamate and ammonia.

The homogenates were centrifuged, producing clear supernatants, 0.2 ml portions of which were pooled to give the extracts for analysis; these were stored at  $-15^{\circ}\text{C}$  until used. Between 0.8 and 1.0 ml of the extracts, depending upon the expected amino acid content, were loaded onto the column of a Technicon Amino Acid Analyser (20 hr system, Technicon Handbook No.2). The column was kept at room temperature for the loading of the samples, then the temperature was turned up immediately to  $45^{\circ}\text{C}$ . When the glutamine peak had passed through the column, the temperature was raised to  $60^{\circ}\text{C}$  for the rest of the analysis. This procedure minimizes the breakdown of glutamine (D.L.Bloxam, unpublished observations). Colour yields used in the calculations were those determined in the department for the particular analysis system used. The exceptions were those for ethanolamine, phosphoethanolamine and glutathione for which a colour yield of unity was assumed. For the calculations of the intracellular concentrations, expressed as  $\mu\text{moles}$  per 100 ml tissue water, the water of the diaphragm was taken to be 75% of the wet weight of the tissue samples (Ottaway, 1961).

(iv) Enzyme Activity Determinations.

Measurements of enzyme activities were made by a continuous spectrophotometric method; in order therefore to avoid problems due to turbidity in the reaction cuvette, the muscle homogenates were centrifuged and the assays made using the clearer supernatant fluid. Particular attention was thus made to ensure that each particular enzyme was efficiently solubilized by the extraction medium employed. In general, a number of different extraction media were tested for each enzyme, the composition of the solutions being based upon those which had been reported as being satisfactory in the literature. The extraction media were buffered using either HEPES (N-2-hydroxyethylpiperazine-N<sup>+</sup>-2-ethansulphonic acid) or potassium phosphate buffer, in each case adjusted to pH 6.9 by the addition of KOH; both these buffers possess good buffering capacity at this pH. In many cases 1 mM EDTA was included to overcome possible inhibition by heavy metal ions, whilst those enzymes that were known to require reduced sulphhydryl groups for optimum activity were extracted and assayed

in media containing a suitable thiol reagent.

Tissues were homogenised in 1.5 or 2.0 ml of extraction buffer using a "Silverson" laboratory mixer-emulsifier, the homogenate being surrounded by ice during this operation; a homogenization period of 15 sec, even at quite low homogenization speeds was found to be adequate for such small quantities of muscle, i.e. of the order of 100 mg, whilst no appreciable rise of temperature of the medium occurred. A single centrifugation step was then performed according to the requirements of the subsequent assays: coarser particles, fibrous material, etc were spun down at 2,200  $g_{av}$  for 15 min, whilst to produce a supernatant fluid essentially free of mitochondrial contamination, where the presence of such particles caused appreciable interference with the assay, a centrifugation of 30,000  $g_{av}$  for 30 min was used. In both cases, centrifugation was carried out at temperatures close to 0°C.

Dilution of the supernatant extract before assay was usually carried out using the same medium as was used for homogenization; glyceraldehyde-3-phosphate dehydrogenase

was found to be unstable on dilution under these conditions, but a special diluent containing dithiothreitol was found to maintain the initial activity for a period more than sufficient to assay the enzyme, i.e. for several hours. All enzyme extracts were kept on ice prior to assay, and were assayed only on the day that they were prepared.

Enzyme assays were performed with a Unicam SP 800 recording ultraviolet spectrophotometer, with accessories that permitted the use of a multiple cell carriage with constant temperature cell housing, constant wavelength and scale expansion facilities and a subsidiary chart recorder. Assays, which were all performed in duplicate, were made at 25°C, the temperature being maintained by water circulated from a water bath through the constant temperature cell housing.

Table 4 sets out in detail the methods used for the preparation and assay of the individual enzymes measured in this study. Preliminary experiments were carried out in all cases to ascertain that zero order kinetics

were being followed, and that concentrations of substrates and cofactors were optimal for the conditions employed. Unless stated to the contrary, the reaction was initiated by the addition of the enzyme extract and was followed for up to 10 min, every reaction rate being linear for at least this period. In cases where a discrepancy between the activities of extracts from control and denervated muscles was apparent, random checks were made to ensure that the combined enzyme activities of both extracts were additive; in no case was the converse found to be true, presumably because dilution of the enzyme in the assay mixture was such as to effectively remove activators or inhibitors that may have been present.

Enzyme activity concentrations are expressed in terms of  $\mu\text{moles product}/\text{min}/\text{mg}$  wet weight of tissue, measured at pH 6.9 and  $25^{\circ}\text{C}$ .

Table 4. Methods used for the Estimation of Enzyme Activities

Enzyme	Composition of extraction buffer & volume of buffer per headlapphragn	Comments	Assay Medium	Reference upon which assay medium based.
Hexokinase	0.3 M sucrose 0.8 M KCl 1 mM EDTA 20 mM HEPES Final pH = 6.9 2.0 ml/tissue	High ionic strength buffer required for solubilization of mitochondriai (?) bound hexokinase. Homogenate kept on ice for minimum of 60 min to permit this to occur. Centrifugation at 30,000 g <sub>av</sub> for 30 min	20 mM HEPES 10 mM MgCl <sub>2</sub> 5 mM β-mercaptoethanol 5 mM ATP 25 mM glucose 0.55 mM NADP excess G6PDH Final pH = 6.9	Dipietro & Weinhouse (1960)
Phosphohexo- isomerase	0.3 M sucrose 20 mM HEPES 1 mM EDTA Final pH = 6.9 2.0 ml/tissue	Homogenate centrifuged at 30,000 g <sub>av</sub> for 30 min. Supernatant diluted 50 fold in extraction buffer before assay	20 mM HEPES 20 mM MgCl <sub>2</sub> 2 mM fructose 6-P 0.2 mM NADP excess G6PDH Final pH = 6.9	Wu & Racker(1959)
Phosphoglucos- utase	0.3 M sucrose 20 mM HEPES 1 mM EDTA Final pH = 6.9 2.0 ml/tissue	Homogenate centrifuged at 30,000 g <sub>av</sub> for 30 min. Supernatant diluted 50 fold in extraction buffer before assay. EDTA activates the enzyme.	40 mM Imidazole 4 mM MgCl <sub>2</sub> 4 mM glucose 1-P 0.2 mM NADP excess G6PDH Final pH = 6.9	Hashimoto, Sasaki & Yoshikawa (1967)
Glycogen phosphorylase	20 mM HEPES 5 mM EDTA 20 mM NaF 5 mM cysteine-HCl Final pH = 6.9 2.0 ml/tissue	Homogenate centrifuged at 30,000 g <sub>av</sub> for 30 min. Supernatant diluted 10 fold in 50 mM potassium phosphate buffer, 1 mM EDTA, 10 mM NaF, pH 6.9 Interconversions of phosphorylase a & b prevented by EDTA & NaF.	50 mM X-phosphate 1 mM EDTA 10 mM NaF 2.5 mM MgCl <sub>2</sub> 5 mM cysteine-HCl 0.5 % rabbit muscle glycogen 0.4 mM NADP 0.2 mg/ml fructose 1,6 di-P ± 1 mM AMP excess PGM, G6PDH. Final pH = 6.9	Burleigh & Schimke (1968)
Glucose 6-P dehydrogenase	0.3 M sucrose 20 mM HEPES 1 mM EDTA Final pH 6.9 2.0 ml/tissue	Homogenate centrifuged 30,000 g <sub>av</sub> for 30 min.	20 mM HEPES 20 mM MgCl <sub>2</sub> 2 mM glucose 6-P 0.25 mM NADP excess G6PDH Final pH = 6.9	Glock & McLean(1953)

Table 4 continued.

Enzyme	Composition of extraction buffer & volume of buffer per hemidiaphragm	Comments	Assay Medium	Comments	Reference upon which assay medium based.
6-Phosphogluconate dehydrogenase	0.3 M sucrose 20 mM HEPES 1 mM EDTA Final pH 6.9 2.0 ml/tissue	Homogenate centrifuged at 80,000 g <sub>av</sub> for 30 min.	20 mM HEPES 20 mM MgCl <sub>2</sub> 2 mM G-phosphogluconate 0.25 mM NADP Final pH = 6.9	50 $\mu$ l undiluted supernatant per assay. Total volume = 3.05 ml	Glock & McLean (1953)
Creatine phosphokinase	0.3 M sucrose 20 mM HEPES 1 mM EDTA Final pH = 6.9 2.0 ml/tissue	Homogenate centrifuged at 20,000 g <sub>av</sub> for 30 min. Supernatant diluted 300 fold with extraction buffer prior to assay	20 mM HEPES 6 mM MgCl <sub>2</sub> 20 mM glucose 5 mM cysteine-HCl 1 mM ADP 10 mM AMP 0.8 mM NADP 10 mM creatine phosphate excess hexokinase, G6PDH Final pH = 6.9	50 $\mu$ l 300-fold diluted supernatant used per assay. Total vol. = 1.05 ml 10 ml AMP included in assay to inhibit adenylate kinase.	Rosaliki (1967)
Adenylate kinase	0.3 M sucrose 20 mM HEPES 1 mM EDTA Final pH = 6.9 2.0 ml/tissue	Homogenate centrifuged 30,000 g <sub>av</sub> for 30 min. Supernatant diluted 50 fold with extraction buffer prior to assay.	20 mM HEPES 6 mM MgCl <sub>2</sub> 1 mM ADP 20 mM glucose 0.8 mM NADP 5 mM cysteine-HCl excess hexokinase, G6PDH Final pH = 6.9	50 $\mu$ l 50-fold diluted supernatant used per assay. Total volume = 3.05 ml	Oliver (1955)
Adenylate deaminase	0.3 M sucrose 20 mM HEPES 1 mM EDTA 0.8 M KCl Final pH = 6.9 2.0 ml/tissue	High ionic strength buffer required to prevent adsorption of enzyme to myofibrils. Homogenate centrifuged 30,000 g <sub>av</sub> for 20 min.	20 mM HEPES 100 mM KCl 2 mM AMP Final pH = 6.9	50 $\mu$ l undiluted supernatant used per assay, total vol. = 3.05 ml KCl activates enzyme	Raggi, Ronca-Testoni, & Ronca (1960)
Glutathione reductase	100 mM K-phosphate 1 mM EDTA Final pH = 6.9 1.5 ml/tissue	Homogenate centrifuged 2,200 g <sub>av</sub> for 15 min.	50 mM K-phosphate 1 mM EDTA 0.2 % oxidized glutathione 0.2 mM NADPH	50 $\mu$ l undiluted supernatant used per assay. Total volume = 3.05 ml	Racker (1956)

Table 4 continued.

Enzyme	Composition of extraction buffer and volume required per hemidiaphragm	Comments	Assay Medium	Reference upon which assay medium based.
Malate dehydrogenase	100 mM K-phosphate 1 mM EDTA Final pH = 6.9 1.5 ml/tissue	Phosphate buffer ruptures mitochondria. Homogenate centrifuged 2,200 g <sub>av</sub> for 15 min. Supernatant diluted 100 fold in extraction buffer prior to assay	50 mM K-phosphate 1 mM EDTA 0.2 mM oxaloacetate 0.5 mM NADH Final pH = 6.9	Delbrück, Zebe & Bucher (1958)
NAD-specific isocitrate dehydrogenase	100 mM K-phosphate 1 mM EDTA Final pH = 6.9 1.5 ml/tissue	Phosphate buffer ruptures mitochondria. Homogenate centrifuged 2,200 g <sub>av</sub> for 15 min	20 mM HEPES 1 mM EDTA 2 mM ADP 5 mM dl-isocitrate 1.5 mM NaCl <sub>2</sub> 0.6 mM NAD <sup>+</sup> 0.375 g/l Triton X-100 Final pH = 6.9	Chen & Picot (1963)
NADP-specific isocitrate dehydrogenase	100 mM K-phosphate 1 mM EDTA Final pH = 6.9 1.5 ml/tissue	Phosphate buffer ruptures mitochondria. Homogenate centrifuged 2,200 g <sub>av</sub> for 15 min.	20 mM HEPES 1 mM EDTA 10 mM MgCl <sub>2</sub> 2.6 mM dl-isocitrate 0.3 mM NADP 0.375 g/l Triton X-100 Final pH = 6.9	Delbrück, Zebe & Bucher (1958)
Glutamate dehydrogenase	100 mM K-phosphate 1 mM EDTA Final pH = 6.9 1.5 ml/tissue	Phosphate buffer bursts mitochondria. Homogenate centrifuged 2,200 g <sub>av</sub> for 15 min.	50 mM K-phosphate 1 mM EDTA 60 mM NH <sub>4</sub> Cl 3 mM $\alpha$ -oxoglutarate 0.25 mM NADH 0.03125 g/l Triton X-100 Final pH = 6.9	Delbrück, Zebe & Bucher (1958)
Glyceraldehyde phosphate dehydrogenase	100 mM K-phosphate 1 mM EDTA Final pH = 6.9 1.5 ml/tissue	Homogenate centrifuged 2,200 g <sub>av</sub> for 15 min. Supernatant diluted 100 fold in 20 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, pH 6.9	20 mM HEPES 1 mM EDTA 3.3 mM MgCl <sub>2</sub> 10 mM 3-phosphoglycerate 0.25 mM NADH 1.5 mM ATP 2.4 mM reduced glutathione 75 mM semicarbazide-HCl 100 $\mu$ g 3-phosphoglycerate kinase Final pH = 6.9	Bass, Brdiczke, Eyer, Hofer & Pette, (1969)

## Presentation and Statistical Evaluation of Results.

Results obtained for control and denervated tissues, the stated number of days after nerve section are expressed as the mean  $\pm$  standard error of the mean (S.E.M.), followed in parenthesis by the number of samples estimated. Tables also include as derived values the paired differences ( $\pm$  S.E.M.) of estimates obtained from control and denervated tissues from each rat, together with values expressing the particular parameter in the denervated tissue as a percentage ( $\pm$  S.E.M.) of the corresponding value in the control muscle.

In addition each parameter investigated is expressed in two concentration terms, viz: units/unit wet weight, and units/hemidiaphragm. It is to be understood that the terms "concentration" and "content" respectively will be used throughout this work to refer to these terms.

Statistical analysis was performed by Student's 't' test; statistical significance is indicated if  $P < 0.05$ .

Table 4 continued.

Enzyme	Composition of extraction buffer and volume required per hemidiaphragm	Comments
Lactate dehydrogenase	100 mM K-phosphate 1 mM EDTA Final pH = 6.9 1.5 ml/tissue	Homogenate centrifuged 2,200 for 15 min. Supernatant diluted 100 fold in extraction buffer

N.B. All reactions followed at 340 nm, except for:

(i) malate dehydrogenase (366nm)

(ii) Adenylate deaminase (285nm, production of inosinic acid)

Assay Medium	Comments	Reference upon which assay medium based.
100 mM K-phosphate 10 mM or 0.33 mM pyruvate 0.15 mM NADH Final pH = 6.9	50 $\mu$ l 100-fold diluted supernat- ant used per assay. Total vol = 3.05 ml Ratio of activity with high pyruvate concentration to that with low pyruvate concentration is indicative of LDH isoenzyme proportion	Wilson, Kahn & Kaplan (1963)

## SOURCES OF MATERIALS.

### Rats.

Male rats of a Wistar strain, bred in the department.

### Enzymes.

Peroxidase, Fermcozyme, Hughes & Hughes (Enzymes) Ltd.

All other subsidiary enzymes from Boehringer Corporation  
(London)Ltd.

### Substrates.

Glucose, rabbit liver glycogen (containing less than  
0.2% N),  $\alpha$ -oxoglutarate, D,L-isocitric acid lactone  
from British Drug Houses, Ltd.

All other substrates were obtained from Boehringer  
Corporation.

### Nucleotides & Coenzymes.

With the exception of chemically reduced NADPH,  
obtained from Sigma Ltd., and of ATP from Kyowa Hakko  
Kogyo Co. (Tokyo), all nucleotides and coenzymes were  
obtained from the Boehringer Corporation.

### HEPES.

N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid,  
A grade, containing less than 1ppm heavy metals, was

obtained from Calbiochem Ltd.

Sulphydryl Reagents.

Dithiothreitol: Calbiochem Ltd.

$\beta$ -mercaptoethanol: Koch-Light, Ltd.

L-cysteine hydrochloride: British Drug Houses, Ltd.

All were stored under refrigerated conditions.

Miscellaneous.

Penicillin Powder: "Crystapen" benzyl penicillin (sodium) B.P. Sterile; Glaxo Laboratories, Ltd. Greenford.

All other chemicals were of analytical reagent grade where available; otherwise the best quality that could be obtained was used.

All solutions were made up in distilled, deionized water that had been passed through a mixed bed ion exchange resin (Elgastat, The Elga Group of Companies, Buckinghamshire). Solutions containing  $MgCl_2$  or  $CaCl_2$  were made up from stock kept in the cold room and which had been accurately standardised by titration against a standard EDTA solution, using Eriochrome Black T as indicator.

## R E S U L T S

### Observations on the wet weights of the tissues.

Table 5 & Fig.2. show the effect of left unilateral phrenicectomy on the wet weights of the two hemidiaphragms taken from rats of approximately 100 g body weight. It will be seen that a statistically significant difference is found between the two populations, the left hand tissue being some 10% lighter than the right. Three days after nerve section the position has reversed itself, whilst at 5 days the peak of the hypertrophy is observed. A gradual decline over the next 5 days is followed by a more rapid decrease in wet weight of the left hemidiaphragm, culminating in a value at 15 days lower than that of the unoperated rat; atrophy of the tissue has thus taken place.

The body weights of the rats used in this investigation, of course, were not all identical but were distributed around the 100 g mark. Linear regression analysis demonstrated that in unoperated rats there is a correlation between the wet weights of the control hemidiaphragms and those of the carcass; this to be expected considering the observation of Davidson (1968). Statistical significance tests demonstrated that the slope of the regression line in denervated rats was not

Table 5. The effect of left unilateral phrenicectomy upon the wet weights of the two rat hemidiaphragms.

Each value is the mean  $\pm$  S.E.M. of the number of observations in parenthesis.

Time (days) elapsed since phrenicectomy	Wet weights of the hemidiaphragms (mg)			Paired differences	Percentage
	Innervated Controls	Denervated hemidiaphragms			
0	88.7 $\pm$ 1.2 (71)	79.5 $\pm$ 1.0 (71)		9.1 $\pm$ 0.8	90 $\pm$ 1
		P < 0.001			
1	92.2 $\pm$ 1.1 (71)	88.4 $\pm$ 1.1 (71)		3.8 $\pm$ 0.9	96 $\pm$ 1
		P < 0.05			
2	98.5 $\pm$ 1.8 (8)	107.3 $\pm$ 3.8 (8)		-8.7 $\pm$ 3.7	109 $\pm$ 4
3	89.5 $\pm$ 1.1 (66)	103.1 $\pm$ 1.2 (66)		-13.7 $\pm$ 0.9	116 $\pm$ 1
		P < 0.001			
5	89.8 $\pm$ 1.2 (52)	116.0 $\pm$ 1.8 (52)		-26.2 $\pm$ 1.5	130 $\pm$ 2
		P < 0.001		P < 0.05	
7	91.5 $\pm$ 1.4 (62)	113.4 $\pm$ 1.6 (62)		-21.8 $\pm$ 1.2	123 $\pm$ 2
		P < 0.001		P < 0.05	
10	90.5 $\pm$ 1.6 (82)	107.6 $\pm$ 1.6 (82)		-17.1 $\pm$ 1.2	120 $\pm$ 2
		P < 0.001		P < 0.05	
15	92.4 $\pm$ 2.2 (59)	74.3 $\pm$ 2.5 (59)		18.0 $\pm$ 1.7	80 $\pm$ 2
		P < 0.001			

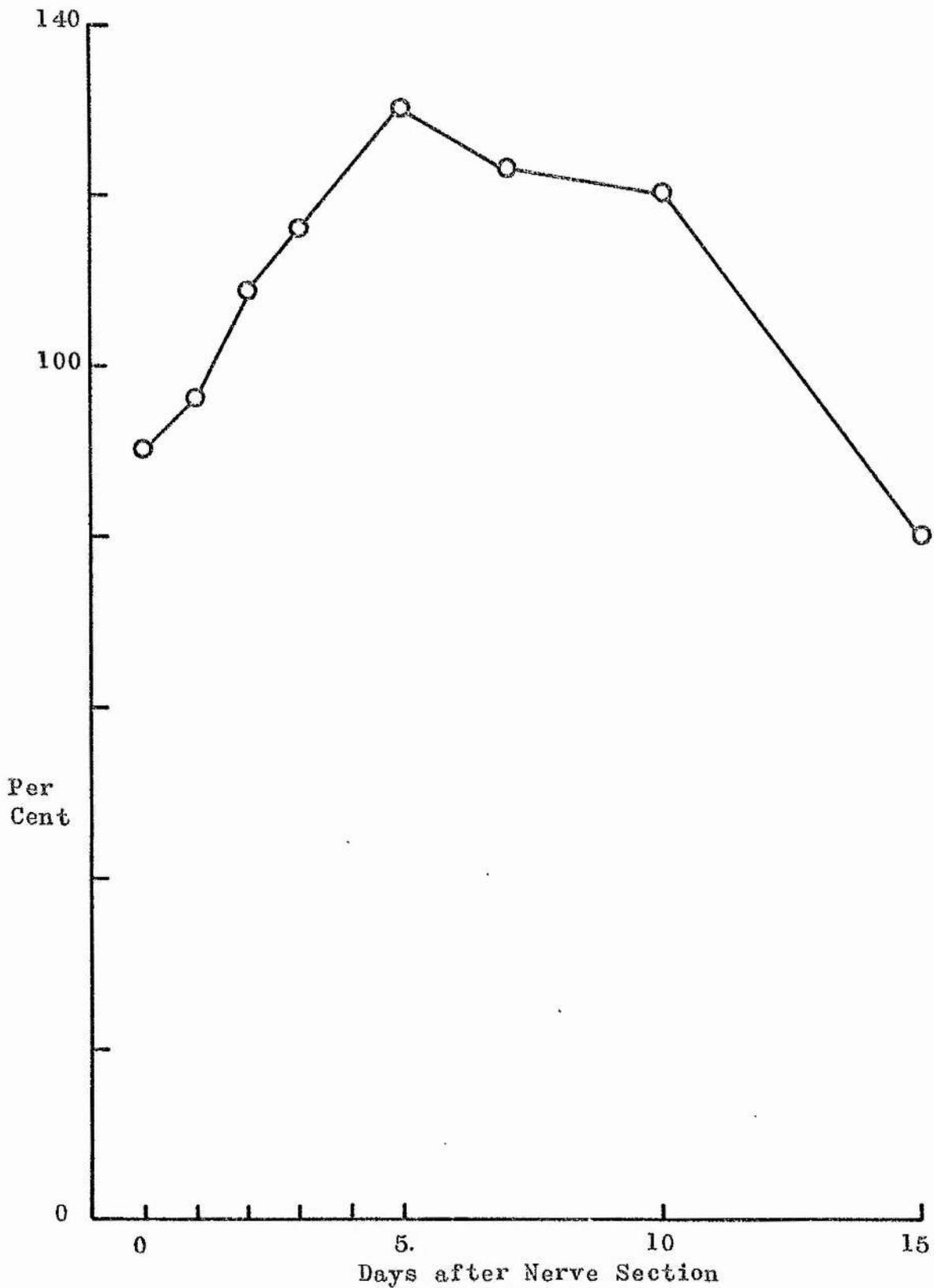


Figure 2. The Effect of Unilateral Phrenicectomy upon the Wet Weight of the Left Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.

significantly different from that in unoperated rats, at least over the range of values recorded in this study; the values have therefore been pooled to obtain the following relationship:

Wet wt of control hemidiaphragm (mg)=

$$16.7. + 0.796(\text{Carcass wt. in g.})$$

The correlation coefficient,  $r = 0.741$ , for 466 observations, was found by Student's t test to be highly significant ( $P < 0.001$ ). It may be concluded that over the range of reported values, the wet weight of the right hemidiaphragm is proportional to the body weight of the rat, and that denervation of the left hemidiaphragm does not cause any significant alteration in this relationship. A compensatory phenomenon in the control tissue thus seems unlikely.

Regression analysis also indicated a linear relationship between the weight of the left hemidiaphragm with respect to the right, in unoperated rats (see first line in Table 6). The finite value of the intercept demonstrates, if these values are applicable over a wider range of values,

Table 6. Regression analysis of the effects of denervation upon the relationship between the wet weights of the two hemidiaphragms.

The relationship is of the form  $y = a + bx$ , where  $x$  is the wet weight in mg of the control hemidiaphragm and  $y$  is the wet weight of the denervated hemidiaphragm, also expressed in mg.

Days elapsed since nerve section	n	a	b	Correlation coefficient, r	Significance of r, P
0	71	23.804	0.628	0.776	< 0.001
1	71	31.796	0.614	0.651	< 0.001
2	8	48.433	0.597	0.282	n.s.*
3	66	39.031	0.716	0.681	< 0.001
5	52	40.334	0.842	0.585	< 0.001
7	62	42.133	0.779	0.689	< 0.001
10	82	39.552	0.749	0.747	< 0.001
15	59	- 1.735	0.823	0.731	< 0.001

\* n.s. = not significant

that the relative proportions of the two "halves" of the diaphragm change with respect to the weight of the rat. The effect of unilateral phrenicectomy upon the regression relationship between the wet weights of the two hemidiaaphragms is shown in Table 6. With the exception of the value for the slope obtained at 2 days after denervation, the slope of the line is found to be not significantly different from that of the unoperated rats; it may be presumed therefore that denervation does not profoundly affect the correlation between the wet weights of the two hemidiaphragms, whilst the observed differences in intercepts are indicative of the hypertrophic process. The corollary of this finding is that younger rats demonstrate a greater hypertrophy following denervation.

Glycogen. The effects of unilateral phrenicectomy upon the muscle glycogen in fed rats (Table 7 & Fig. 3.,) is not apparent until after 3 days have elapsed since nerve section, at which time the glycogen content of the denervated tissue is some 60% of that in the control tissue. After a further 4 days, the normal content is regained, but then declines, gradually at first and then more rapidly. In terms of the concentration of the polysaccharide per unit wet weight of tissue, the decrease to 50% of control values at 3 days is hardly recovered by 10 days, after which a slight decline is observed.

Starvation for 16 to 18 hours before sacrifice reduces the glycogen concentration in unoperated animals to approximately 40% of the level in fed animals. The effect of such a period of fasting on the muscle glycogen of denervated rats is shown in Fig. 4, whilst the absolute values are presented in Table 8. It will be seen that in rats denervated for up to 7 days, the concentration of muscle glycogen in the paralysed tissue remains unaffected by the period of starvation; for rats denervated 10 to 15 days before assay the concentration in the denervated tissue from fed rats was

Table 7. The effect of unilateral phrenicectomy upon the concentration and content of glycogen in the hemidiaphragms of 'fed' rats.

Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis. 'Fed' rats were allowed free access to food until the time of sacrifice.

Time (days) elapsed since phrenicectomy	Glycogen Concentration ( $\mu$ moles glucose/g wet wt.)			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	32.1 $\pm$ 2.7 (17)	32.3 $\pm$ 2.7 (17)	-0.2 $\pm$ 0.5	101 $\pm$ 2
1	30.8 $\pm$ 2.2 (13)	29.1 $\pm$ 1.3 (13)	1.6 $\pm$ 1.9	98 $\pm$ 6
3	31.7 $\pm$ 3.8 (7)	15.4 $\pm$ 1.0 (7)	16.4 $\pm$ 3.9	52 $\pm$ 7
		P < 0.01		
7	32.3 $\pm$ 2.0 (7)	20.1 $\pm$ 0.7 (7)	12.2 $\pm$ 1.6	63 $\pm$ 2
		P < 0.001	P < 0.05	
10	32.4 $\pm$ 2.1 (13)	21.3 $\pm$ 0.7 (13)	11.1 $\pm$ 2.1	69 $\pm$ 4
		P < 0.001		
15	34.6 $\pm$ 2.3 (7)	18.5 $\pm$ 0.8 (7)	16.1 $\pm$ 2.6	55 $\pm$ 4
		P < 0.001		

Table 7 continued.

Time (days) elapsed since phrenicectomy	Glycogen Content ( $\mu$ moles glucose/hemidiaphragm)			Percentage
	Innervated control	Denervated Hemidiaphragms	Paired differences	
0	2.74 $\pm$ 0.24 (17)	2.46 $\pm$ 0.20 (17)	0.28 $\pm$ 0.07	91 $\pm$ 2
1	2.94 $\pm$ 0.26 (13)	2.67 $\pm$ 0.13 (13)	0.28 $\pm$ 0.19	95 $\pm$ 5
3	2.74 $\pm$ 0.32 (7)	1.54 $\pm$ 0.11 (7)	1.21 $\pm$ 0.34	60 $\pm$ 8
	P < 0.01			
7	2.64 $\pm$ 0.24 (7)	2.36 $\pm$ 0.11 (7)	0.28 $\pm$ 0.15	92 $\pm$ 5
10	2.85 $\pm$ 0.34 (13)	2.30 $\pm$ 0.15 (13)	0.55 $\pm$ 0.28	88 $\pm$ 7
15	2.95 $\pm$ 0.39 (7)	1.20 $\pm$ 0.17 (7)	1.75 $\pm$ 0.27	41 $\pm$ 4
	P < 0.01			

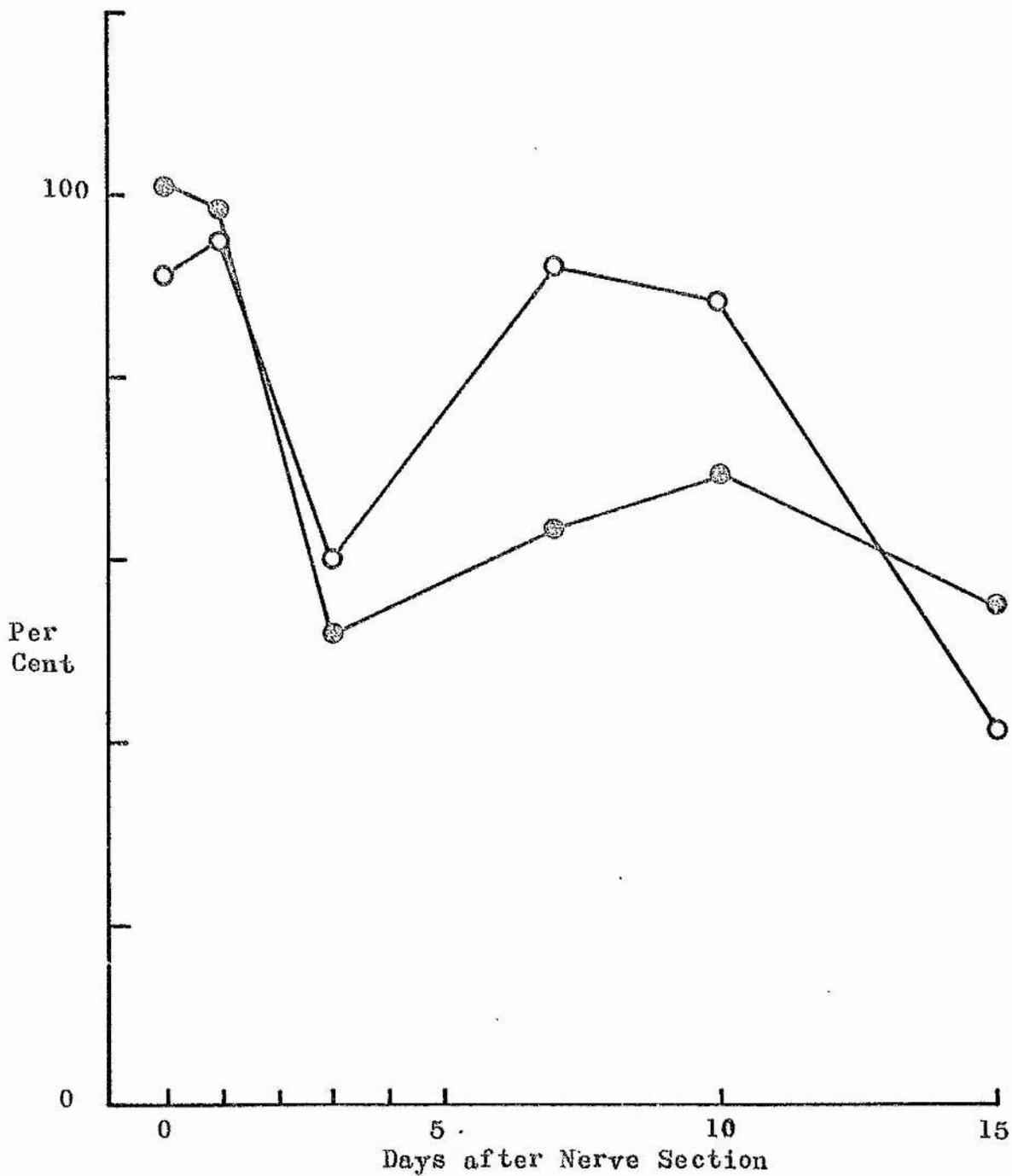


Figure 3. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Glycogen in the Hemidiaphragms from Fed Rats, expressed as a Percentage of the Contralateral Control Values.

Table 8. The effect of unilateral phrenicectomy upon the concentration and content of glycogen in the hemidiaphragms of 'fasted' rats.

Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis. 'Fasted' rats were without food in their cages for 16 - 18 hours before sacrifice.

Time (days) elapsed since phrenicectomy	Glycogen Concentration ( $\mu$ moles glucose/g wet wt)			Paired differences	Percentage
	Innervated controls	Denervated Hemidiaphragms			
0	13.4 $\pm$ 1.2 (8)	13.7 $\pm$ 1.3 (8)		-0.2 $\pm$ 0.5	102 $\pm$ 4
1	14.4 $\pm$ 0.9 (7)	29.1 $\pm$ 1.9 (7)	$P < 0.001$	-14.8 $\pm$ 1.6	206 $\pm$ 15
3	11.9 $\pm$ 1.2 (8)	15.0 $\pm$ 1.2 (8)		-3.1 $\pm$ 1.1	133 $\pm$ 13
7	16.8 $\pm$ 1.6 (8)	18.7 $\pm$ 1.0 (8)		-1.9 $\pm$ 1.0	115 $\pm$ 7
10	14.1 $\pm$ 1.6 (12)	15.6 $\pm$ 0.6 (12)		-1.5 $\pm$ 1.6	124 $\pm$ 12
15	13.0 $\pm$ 2.4 (7)	14.2 $\pm$ 0.8 (7)		-1.2 $\pm$ 1.9	126 $\pm$ 16

Table 8 continued

Time (days) elapsed since phrenicectomy	Glycogen Content ( $\mu$ moles glucose/hemidiaphragm)			Percentage
	Innervated controls	Denervated hemidiaphragms	Paired differences	
0	1.20 $\pm$ 0.13 (8)	1.08 $\pm$ 0.11 (8)	0.13 $\pm$ 0.05	91 $\pm$ 4
1	1.30 $\pm$ 0.08 (7) $P < 0.001$	2.73 $\pm$ 0.18 (7)	-1.43 $\pm$ 0.15 $P < 0.05$	212 $\pm$ 15
3	1.00 $\pm$ 0.11 (8) $P < 0.05$	1.49 $\pm$ 0.13 (8)	-0.49 $\pm$ 0.12	158 $\pm$ 17
7	1.48 $\pm$ 0.17 (8) $P < 0.05$	2.06 $\pm$ 0.15 (8)	-0.58 $\pm$ 0.11	147 $\pm$ 11
10	1.06 $\pm$ 0.10 (12) $P < 0.01$	1.56 $\pm$ 0.08 (12)	-0.50 $\pm$ 0.14	164 $\pm$ 18
15	0.82 $\pm$ 0.14 (7)	0.83 $\pm$ 0.09 (7)	-0.01 $\pm$ 0.10	113 $\pm$ 15

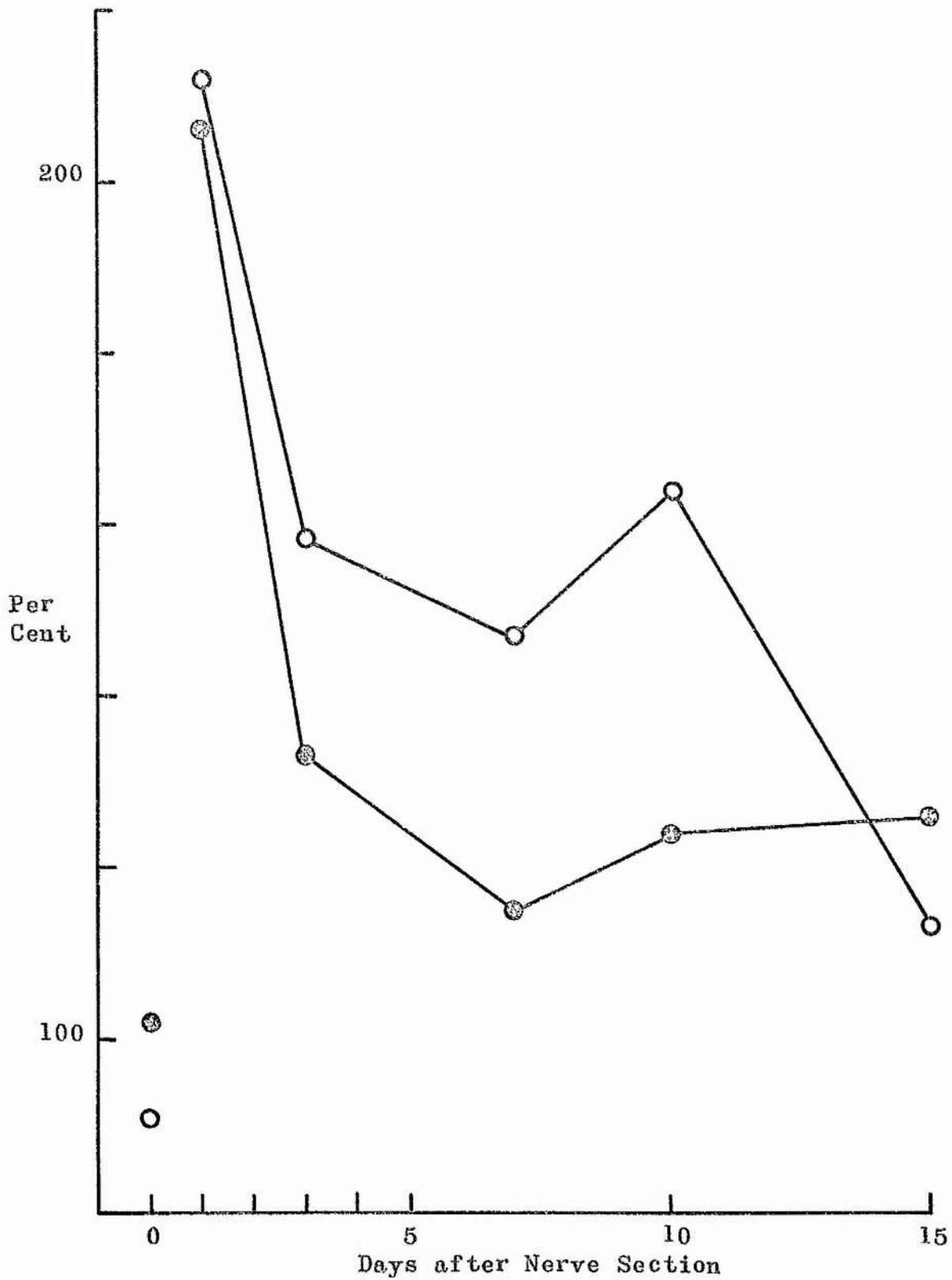


Figure 4. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Glycogen in Hemidiaphragms from Fasted Rats, expressed as a Percentage of their Contralateral Control Values

significantly higher ( $P < 0.001$ ) than that in the muscle from starved rats, which themselves were not significantly different from the concentration of glycogen found in unoperated rats starved for a similar period of time.

Enzymes of Glucose and Glycogen Metabolism: Hexokinase, Phosphoglucomutase & Glycogen Phosphorylase.

(i) Hexokinase In preliminary studies undertaken to investigate the extraction of hexokinase from rat diaphragm muscle it was found that it was essential to separate off the mitochondrial fraction from the muscle extracts in order to obtain linear reaction kinetics. Since it is now well established that the hexokinase activity of mammalian cells is to be found in both the high speed supernatants and in the mitochondrial fraction of sucrose homogenates, it thus became of importance to test the efficiency of various extraction buffers in releasing bound hexokinase into the supernatant fraction. Jervell, Walaas & Walaas (1955) found 82% of rat diaphragm hexokinase activity could be extracted as the soluble enzyme, with some 10% of the total activity being associated with a 15,000 g particulate fraction. Borrebaek, Abraham & Chaikoff (1964), on the other hand, claimed 100% recovery in the supernatant fraction for rat thigh muscle hexokinase. Similarly, Burleigh & Schimke (1968) state that the rat muscle enzyme does not sediment, although they did find that 40 - 60% of the rabbit muscle enzyme could be centrifuged down at

30,000 g for 20 min.

Hernandez & Crane (1966) reported that high ionic strength solutions could be used to release bound porcine heart muscle hexokinase from mitochondria, whilst Rose & Warms (1967) working with Ascites tumour cells, and later Spydevold & Borrebaek (1968) using rat adipose tissue reported that glucose 6-phosphate could cause an almost quantitative release of the mitochondrial bound enzyme. Experiments showed that the high ionic strength buffer containing KCl, based upon that reported by Hernandez & Crane (1966) and described in the methods section (Table 4) produced the greatest solubilizing effect, liberating 15% more enzyme activity into the 30,000 g supernatant than did a comparable buffer lacking the KCl. Tests further showed that this effect was not due to activation of the extracted enzyme by the ionic composition of the medium. No evidence, however, was found for an effect of glucose 6-phosphate in releasing bound hexokinase in rat diaphragm extracts. Hexokinase solubilized by the high ionic strength KCl, containing buffer is considered in this study to represent

"total hexokinase"; the activity recorded compares favourably, if perhaps on the more active side, with that found by Burleigh & Schimke (1969) for the rat diaphragm using Triton X-100 to ensure solubilization, if the age of the rat and the conditions of assay are taken into account. The similarity of the activity concentration of hexokinase in rat hemidiaphragm reported in this study to that recorded for the rat heart (Burleigh & Schimke, 1969; Shonk & Boxer, 1964) is noted and is considered to emphasize the aerobic nature of the respiratory muscle.

There is no effect of unilateral phrenicectomy upon the hexokinase concentration in the hypertrophying tissue until 10 days after denervation, when a statistically significant decrease is found. Tissue content therefore rises in the hemidiaphragm reaching a peak at 5 days and thereafter declining (Table 9 & Fig.5). Net synthesis of the enzyme is indicated.

(ii) Phosphoglucomutase. The activity of phosphoglucomutase is that determined after activation by EDTA, included in the extraction medium, and assayed using imidazole buffer. Harshman, Robinson, Bocchini & Najjar (1965) have shown a  $Mg^{++}$  -

imidazole complex to activate the enzyme presumably by stabilization of an active conformation. The use of a substrate, glucose 1-phosphate, containing 1% of glucose 1,6-diphosphate (British Drug Houses, Ltd.) did not result in any significant activation of the enzyme; presumably the quantity of diphosphate present in the Boehringer ester preparation was sufficient to initiate catalytic activity.

The concentration of phosphoglucomutase activity was found to decrease until 5 days after nerve section, after which the level rose again reaching the control values by the 15th day. In terms of tissue content, therefore, a slight increase takes place reaching a peak between 7 and 10 days, but by the 15th day the denervated tissue contains significantly lower content than the control hemidiaphragm (Table 10 & Fig.6).

(iii) Glycogen Phosphorylase. The use of halothane for the determination of phosphorylase activities is discussed in a separate section. Phosphorylase a was estimated in the absence of added AMP, total phosphorylase in the presence of 1 mM AMP. Quantities of phosphorylase b are calculated by difference. The activity of total

Table 9. The effect of unilateral phrenicectomy upon the extractable activity of hexokinase in the rat diaphragm, expressed in terms of concentration and content.

Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

Time (days) elapsed since phrenicectomy	Hexokinase Concentration (nmole/min/mg wet wt, 25°C)			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	4.34 $\pm$ 0.21 (12)	4.42 $\pm$ 0.22 (12)	-0.07 $\pm$ 0.06	102 $\pm$ 1
1	5.04 $\pm$ 0.22 (12)	4.66 $\pm$ 0.20 (12)	0.38 $\pm$ 0.06	93 $\pm$ 1
3	4.52 $\pm$ 0.16 (12)	4.46 $\pm$ 0.18 (12)	0.06 $\pm$ 0.11	99 $\pm$ 2
5	4.57 $\pm$ 0.17 (11)	4.50 $\pm$ 0.12 (11)	0.12 $\pm$ 0.08	99 $\pm$ 2
7	5.42 $\pm$ 0.22 (11)	5.21 $\pm$ 0.14 (11)	0.32 $\pm$ 0.19	97 $\pm$ 3
10	4.53 $\pm$ 0.11 (12)	3.73 $\pm$ 0.11 (12)	0.80 $\pm$ 0.06	82 $\pm$ 1
		P < 0.001	P < 0.01	
15	4.71 $\pm$ 0.17 (12)	3.65 $\pm$ 0.08 (12)	1.07 $\pm$ 0.17	79 $\pm$ 3
		P < 0.001		

Table 9 continued.

Time (days) elapsed since phrenicectomy	Hexokinase Content (nmole/min/hemidiaphragm, 25°C)			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	397 ± 18 (12)	345 ± 19 (12)	52 ± 6 P < 0.05	87 ± 2
1	435 ± 19 (12) P < 0.05	375 ± 14 (12)	60 ± 9	87 ± 2
3	406 ± 24 (12)	441 ± 21 (12)	-35 ± 11	110 ± 3
5	393 ± 22 (11) P < 0.01	518 ± 23 (11)	-125 ± 9 P < 0.01	133 ± 3
7	479 ± 35 (11)	559 ± 36 (11)	-80 ± 25	118 ± 5
10	460 ± 11 (12) P < 0.05	417 ± 12 (12)	43 ± 11	91 ± 2
15	509 ± 27 (12) P < 0.001	342 ± 21 (12)	167 ± 23	68 ± 4

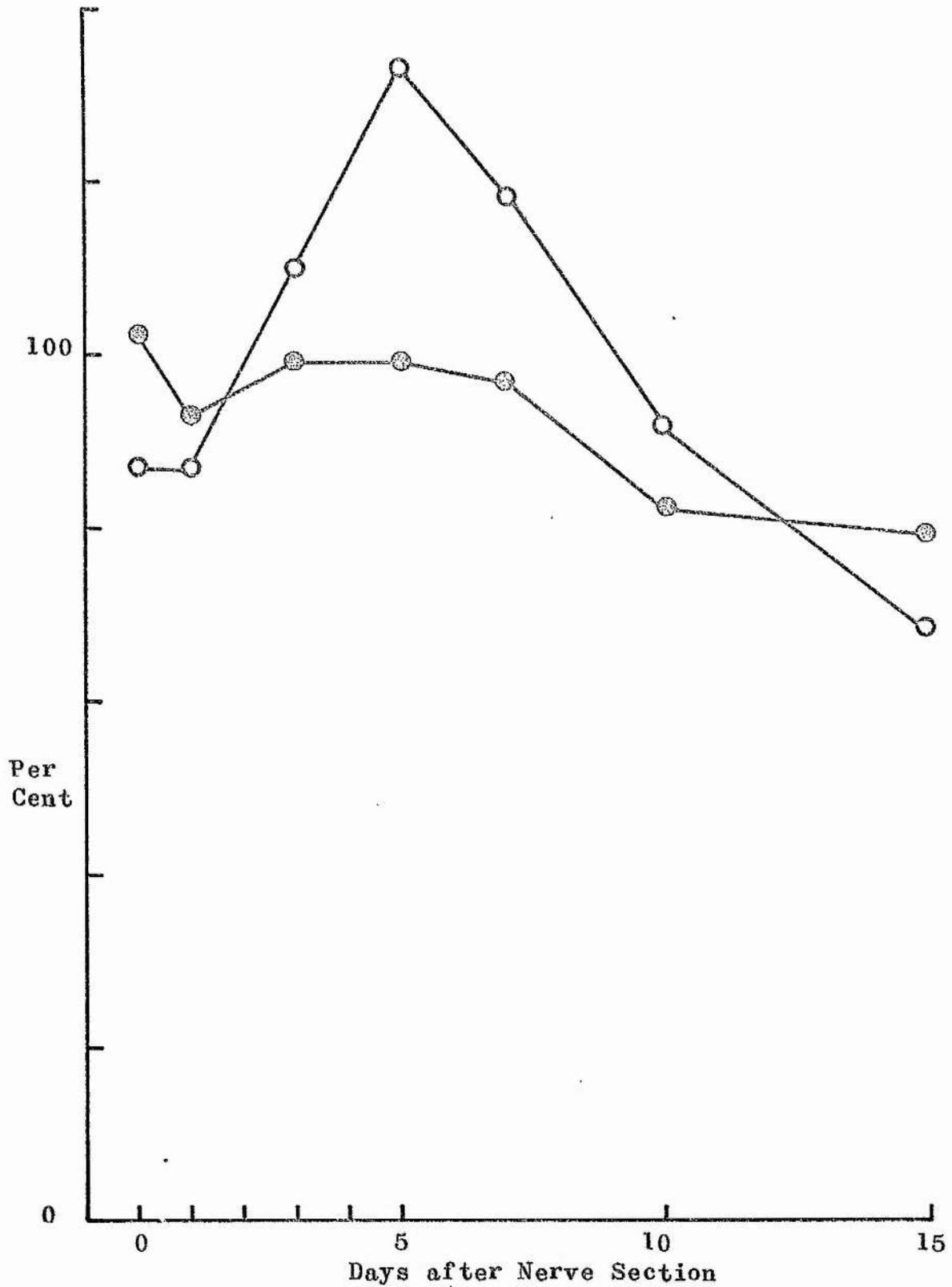


Figure 5. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Hexokinase in the Rat Hemidiaphragm, expressed as a Percentage of their Contralateral Control Values.

Table 10. The effect of unilateral phrenicectomy upon the concentration and content of the phosphoglucomutase activity extractable from rat diaphragm.

Each value is the mean  $\pm$  S.E.M. of the number of observations in parenthesis.

Time (days) elapsed since phrenicectomy	Enzyme Concentration ( $\mu$ mole/min/mg wet wt., at 25°C)			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	115 $\pm$ 3 (6)	111 $\pm$ 5 (6)	4 $\pm$ 2	96 $\pm$ 2
1	126 $\pm$ 3 (6)	115 $\pm$ 3 (6)	11 $\pm$ 4	92 $\pm$ 3
	P < 0.05			
3	116 $\pm$ 4 (6)	97 $\pm$ 3 (6)	19 $\pm$ 4	84 $\pm$ 3
	P < 0.01			
5	128 $\pm$ 4 (9)	102 $\pm$ 2 (9)	26 $\pm$ 3	80 $\pm$ 2
	P < 0.001		P < 0.05	
7	123 $\pm$ 5 (9)	102 $\pm$ 4 (9)	21 $\pm$ 3	83 $\pm$ 2
	P < 0.001			
10	111 $\pm$ 3 (6)	107 $\pm$ 3 (6)	4 $\pm$ 2	96 $\pm$ 2
15	131 $\pm$ 4 (6)	131 $\pm$ 2 (6)	0 $\pm$ 4	100 $\pm$ 3

Table 10 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content ( $\mu\text{mole}/\text{min}/\text{hemidiaphragm}$ , at $25^{\circ}\text{C}$ )			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	$10.6 \pm 0.6$ (6)	$9.2 \pm 0.6$ (6)	$1.4 \pm 0.5$	$87 \pm 5$
1	$11.8 \pm 0.6$ (6)	$10.4 \pm 0.7$ (6)	$1.4 \pm 0.5$	$87 \pm 4$
3	$10.1 \pm 0.7$ (6)	$9.5 \pm 0.5$ (6)	$0.7 \pm 0.3$	$95 \pm 3$
5	$11.6 \pm 0.6$ (9)	$11.7 \pm 0.5$ (9)	$-0.1 \pm 0.3$	$102 \pm 3$
7	$11.4 \pm 0.5$ (9)	$12.2 \pm 0.3$ (9)	$-0.7 \pm 0.5$	$108 \pm 4$
10	$9.7 \pm 0.9$ (6)	$11.1 \pm 0.6$ (6)	$-1.4 \pm 0.6$	$118 \pm 8$
15	$11.8 \pm 0.6$ (6)	$8.4 \pm 0.7$ (6)	$3.5 \pm 0.5$	$70 \pm 4$
	$P < 0.01$		$P < 0.05$	

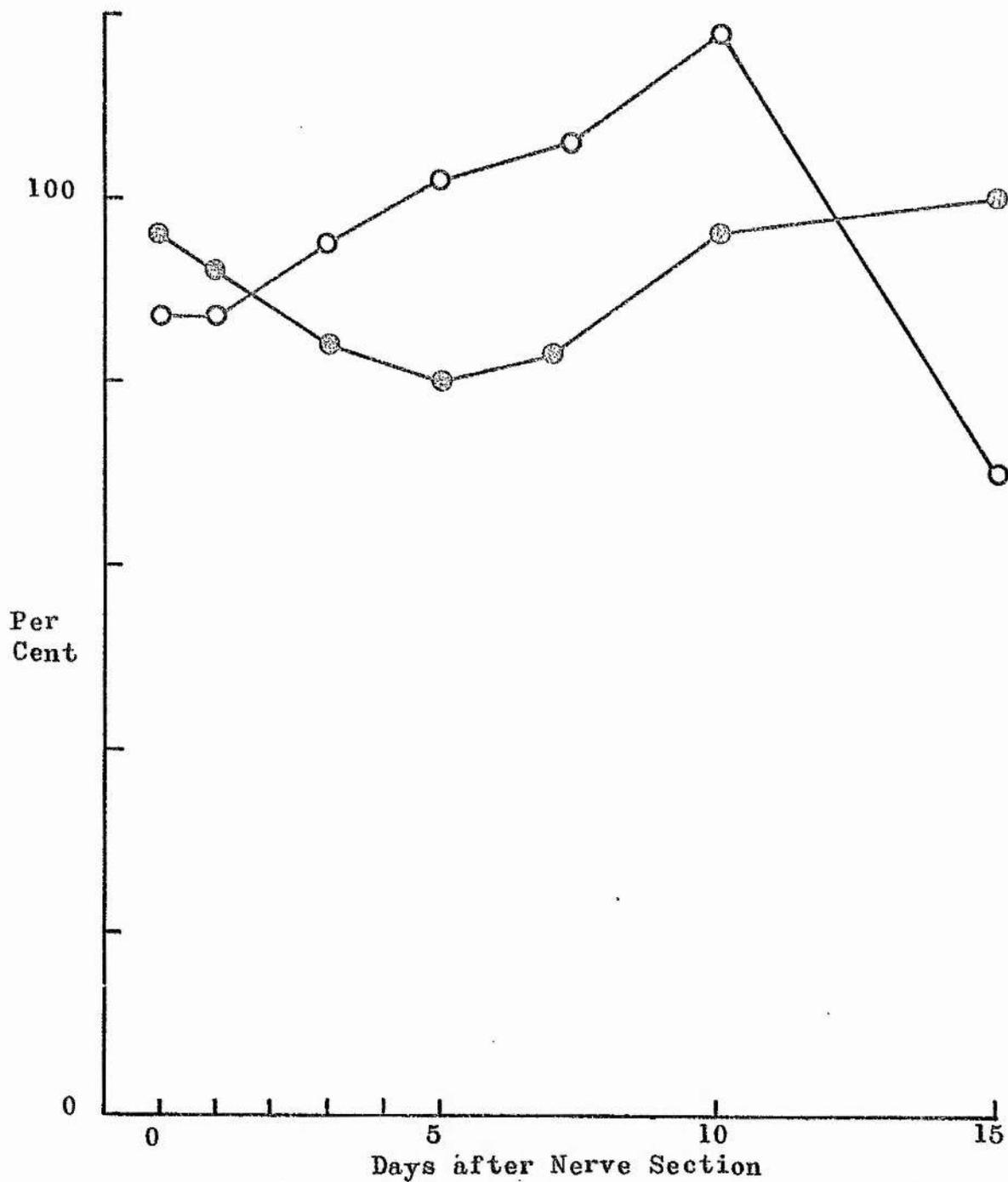


Figure 6. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Phosphoglucomutase Activity in the rat hemidiaphragm, expressed as a Percentage of the Contralateral control values.

phosphorylase reported in this study compares well with other values reported in the literature, provided due allowance is made for the age of the rat (see Burleigh & Schimke, 1969) and the direction and conditions of the assay (Holmes & Mansour, 1968; Bass, Brdiczka, Hofer & Pette, 1969). The activity of phosphorylase a compares extremely favourably with other in vivo values (see separate section, and Table. 31 ).

Phrenic nerve section has the immediate effect of causing a statistically significant decrease of the content of total phosphorylase activity 1 day after nerve section; phosphorylase a is unaffected at this stage. Two days after denervation, the content of total enzyme is marginally higher than that in the control tissue, indicating that net synthesis of the enzyme had taken place. Thereafter the total enzyme content decreases dramatically. Phosphorylase a content increases in activity reaching a peak at 5 days and decreasing to below control values at 15 days. (Tables 11. 12. 13; Fig. 7. 8. & 9).

Table 11. The effect of unilateral phrenicectomy upon the extractable activity of glycogen phosphorylase a in rat diaphragm muscle, expressed as concentration and content.

Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

Time (days) elapsed since phrenicectomy	Enzyme Concentration (nmole/min/mg wet wt., at 25°C)			
	Innervated control	Denervated hemidiaphragm	Paired differences	Percentage
0	1.88 $\pm$ 0.19 (6)	1.91 $\pm$ 0.26 (6)	-0.03 $\pm$ 0.12	101 $\pm$ 6
1	1.68 $\pm$ 0.09 (9)	1.64 $\pm$ 0.06 (6)	0.04 $\pm$ 0.08	99 $\pm$ 5
2	1.79 $\pm$ 0.14 (8)	1.90 $\pm$ 0.18 (8)	-0.11 $\pm$ 0.07	106 $\pm$ 4
3	1.70 $\pm$ 0.19 (7)	1.80 $\pm$ 0.24 (7)	-0.10 $\pm$ 0.11	105 $\pm$ 7
5	1.88 $\pm$ 0.24 (6)	2.08 $\pm$ 0.25 (6)	-0.20 $\pm$ 0.06	111 $\pm$ 4
7	1.45 $\pm$ 0.09 (6)	1.60 $\pm$ 0.10 (6)	-0.15 $\pm$ 0.10	112 $\pm$ 7
10	1.72 $\pm$ 0.15 (6)	1.54 $\pm$ 0.05 (6)	0.18 $\pm$ 0.10	92 $\pm$ 5
15	1.63 $\pm$ 0.08 (5)	1.16 $\pm$ 0.07 (5)	0.47 $\pm$ 0.06	71 $\pm$ 3

P < 0.01

Table 11 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content (nmoles/min/hemidiaphragm, at 25°C)			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	177 ± 22 (6)	163 ± 24 (6)	14 ± 10	92 ± 5
1	160 ± 11 (9)	142 ± 7 (9)	18 ± 7	90 ± 4
2	177 ± 14 (8)	203 ± 18 (8)	-27 ± 9	116 ± 5
3	157 ± 17 (7)	201 ± 27 (7)	-45 ± 13	127 ± 8
5	176 ± 24 (6) P < 0.05	282 ± 33 (6)	-106 ± 13 P < 0.05	161 ± 6
7	146 ± 12 (6) P < 0.05	202 ± 13 (6)	-56 ± 16	142 ± 11
10	171 ± 8 (6)	182 ± 5 (6)	-12 ± 12	109 ± 8
15	153 ± 8 (5) P < 0.01	105 ± 9 (5)	48 ± 7 P < 0.05	68 ± 4

Figure 7. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Glycogen Phosphorylase a activity in the rat hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.

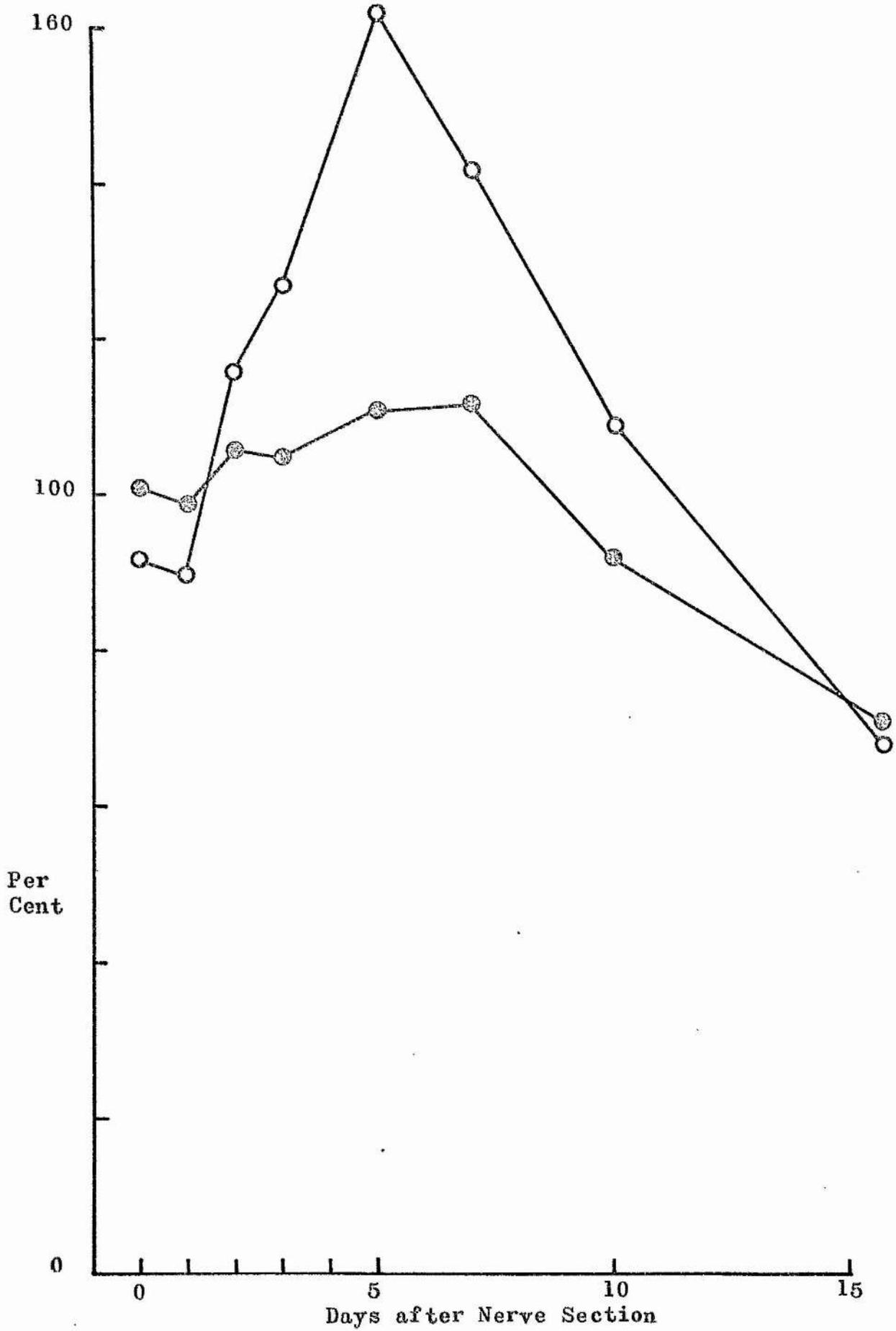


Table 12. The effect of unilateral phrenicectomy upon the extractable activity of glycogen phosphorylase b in rat diaphragm muscle, expressed as concentration and content.

Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

Time (days) elapsed since phrenicectomy	Enzyme Concentration (nmole/min/mgm wet wt., at 25°C)			
	Innervated controls	Denervated Hemidiaphragms	Paired differences	Percentage
0	4.47 $\pm$ 0.53 (6)	4.16 $\pm$ 0.56 (6)	0.31 $\pm$ 0.36	94 $\pm$ 8
1	4.14 $\pm$ 0.43 (9)	2.72 $\pm$ 0.35 (9)	1.42 $\pm$ 0.27	67 $\pm$ 6
		P < 0.05		
2	4.13 $\pm$ 0.47 (8)	3.79 $\pm$ 0.37 (8)	0.34 $\pm$ 0.20	94 $\pm$ 5
3	3.77 $\pm$ 0.37 (7)	2.64 $\pm$ 0.24 (7)	1.13 $\pm$ 0.32	72 $\pm$ 7
		P < 0.05		
5	4.17 $\pm$ 0.27 (6)	1.13 $\pm$ 0.19 (6)	3.04 $\pm$ 0.29	27 $\pm$ 5
		P < 0.001		
			P < 0.01	
7	5.56 $\pm$ 0.39 (6)	1.34 $\pm$ 0.10 (6)	4.22 $\pm$ 0.44	25 $\pm$ 3
		P < 0.001		
			P < 0.05	
10	5.20 $\pm$ 0.33 (6)	0.88 $\pm$ 0.09 (6)	4.32 $\pm$ 0.26	17 $\pm$ 1
		P < 0.001		
			P < 0.01	
15	3.92 $\pm$ 0.61 (5)	0.31 $\pm$ 0.03 (5)	3.61 $\pm$ 0.59	8 $\pm$ 1
		P < 0.01		

Table 12 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content (nmole/min/hemidiaphragm, at 25°C)			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	419 ± 53 (6)	355 ± 50 (6)	64 ± 26	86 ± 6
1	394 ± 44 (9)	233 ± 27 (9)	160 ± 28	62 ± 5
	P = 0.01			
2	412 ± 52 (8)	407 ± 42 (8)	5 ± 24	103 ± 7
3	349 ± 35 (7)	295 ± 27 (7)	53 ± 32	87 ± 8
5	387 ± 20 (6)	154 ± 29 (6)	232 ± 30	40 ± 7
	P < 0.001		P < 0.05	
7	555 ± 40 (6)	171 ± 17 (6)	384 ± 48	32 ± 5
	P < 0.001		P < 0.05	
10	532 ± 59 (6)	107 ± 15 (6)	426 ± 46	20 ± 2
	P < 0.001		P < 0.05	
15	352 ± 54 (5)	27 ± 2 (5)	325 ± 53	8 ± 1
	P < 0.001			

Figure 8. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Glycogen Phosphorylase b activity in the Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.

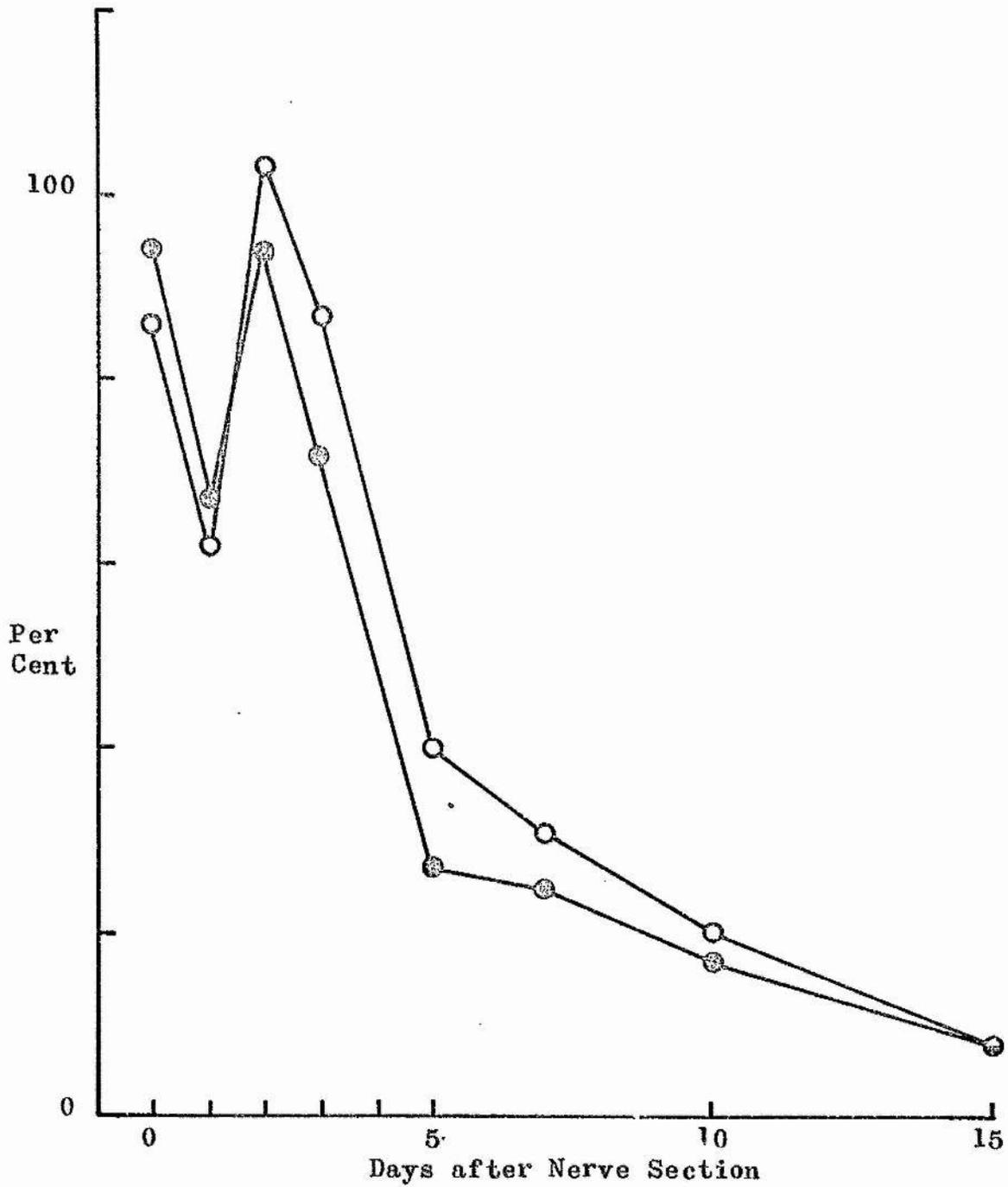


Table 13. The effect of left unilateral phrenicectomy upon the extractable activity of total glycogen phosphorylase of rat diaphragm muscle, expressed as concentration and content.

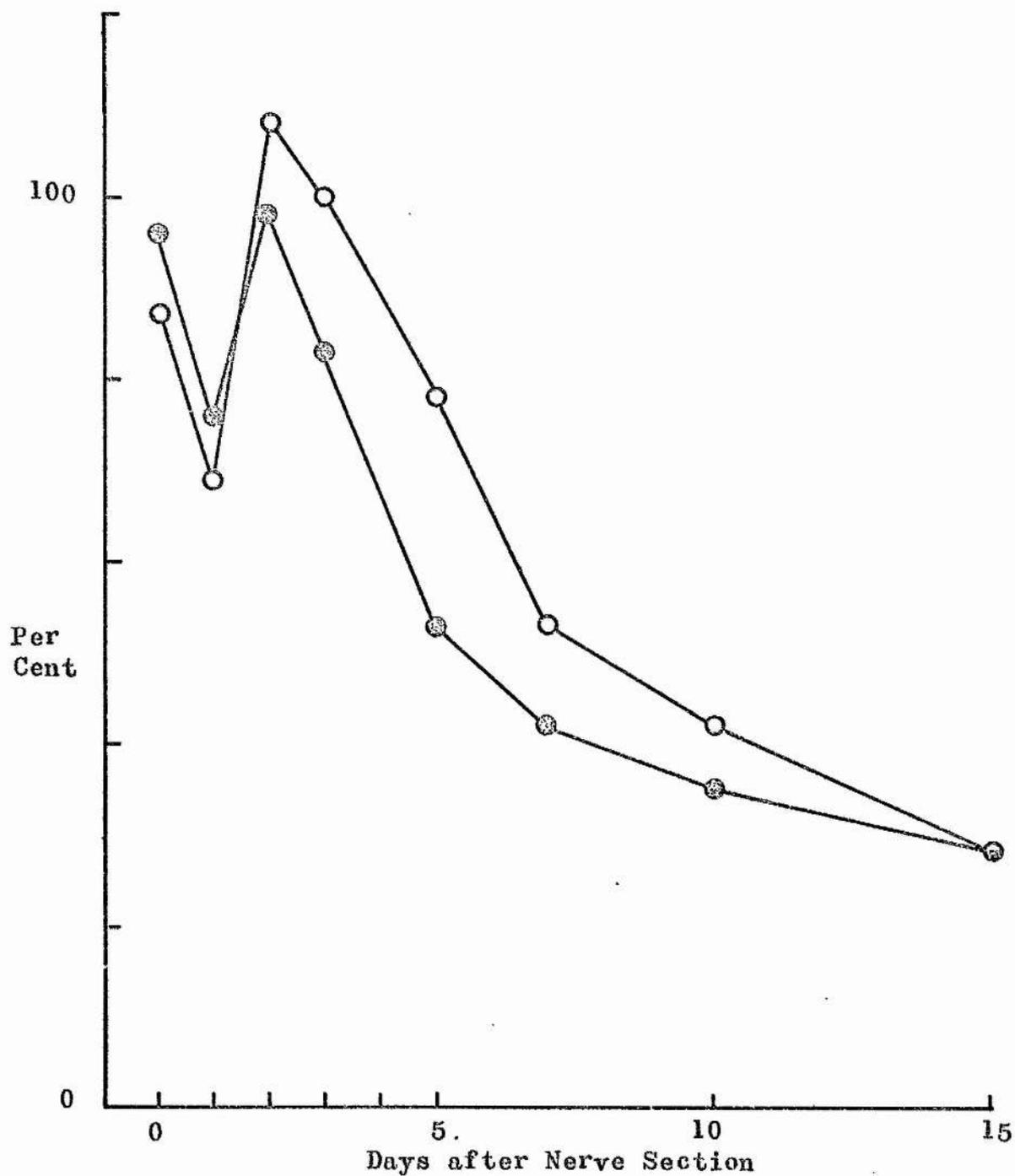
Each value is the mean  $\pm$  S.E.M. of the number of observation shown in parenthesis.

Time (days) elapsed since phrenicectomy	Enzyme Concentration (nmole/min/mg wet wt., at 25°C)			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	6.34 $\pm$ 0.61 (6)	6.07 $\pm$ 0.70 (6)	0.37 $\pm$ 0.37	96 $\pm$ 6
1	5.82 $\pm$ 0.45 (9)	4.36 $\pm$ 0.36 (9)	1.46 $\pm$ 0.26	76 $\pm$ 4
		P < 0.05		
2	5.92 $\pm$ 0.52 (8)	5.69 $\pm$ 0.39 (8)	0.23 $\pm$ 0.22	98 $\pm$ 4
3	5.47 $\pm$ 0.44 (7)	4.44 $\pm$ 0.34 (7)	1.03 $\pm$ 0.36	83 $\pm$ 5
5	6.05 $\pm$ 0.35 (6)	3.21 $\pm$ 0.19 (6)	2.84 $\pm$ 0.29	53 $\pm$ 3
		P < 0.001	P < 0.01	
7	7.01 $\pm$ 0.37 (6)	2.94 $\pm$ 0.09 (6)	4.07 $\pm$ 0.35	42 $\pm$ 2
		P < 0.001	P < 0.05	
10	6.92 $\pm$ 0.27 (6)	2.42 $\pm$ 0.07 (6)	4.50 $\pm$ 0.24	35 $\pm$ 1
		P < 0.001	P < 0.001	
15	5.54 $\pm$ 0.52 (5)	1.46 $\pm$ 0.04 (5)	4.08 $\pm$ 0.56	28 $\pm$ 3
		P < 0.001	P < 0.05	

Table 13 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content (nmole/min/hemidiaphragm, at 25°C)			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	596 ± 66 (6)	518 ± 64 (6)	78 ± 25	87 ± 4
1	554 ± 49 (9)	376 ± 28 (9)	178 ± 29	69 ± 3
	P < 0.05			
2	589 ± 59 (8)	610 ± 45 (8)	-22 ± 29	108 ± 6
3	506 ± 41 (7)	497 ± 37 (7)	35 ± 29	99 ± 6
5	562 ± 31 (6)	436 ± 34 (6)	126 ± 25	78 ± 4
	P < 0.05			
7	701 ± 41 (6)	372 ± 21 (6)	328 ± 42	54 ± 4
	P < 0.001		P < 0.05	
10	702 ± 57 (6)	289 ± 19 (6)	413 ± 42	42 ± 2
	P < 0.001		P < 0.05	
15	504 ± 47 (5)	132 ± 7 (5)	372 ± 53	28 ± 4
	P < 0.001		P < 0.05	

Figure 9. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Total Glycogen Phosphorylase Activity of Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.



Glycolytic Enzymes: Phosphohexoisomerase, glyceraldehyde phosphate dehydrogenase & lactate dehydrogenase.

The concentrations of the extractable activities of these three glycolytic enzymes from the rat hemidiaphragm compare favourably with other values for rat skeletal muscle reported in the literature (Shonk & Boxer, 1964; Bass et al, 1969; Burleigh & Schimke, 1969). The activities observed for the concentration of the two dehydrogenases are typical of those found for "red" muscles or for heart (Bass et al, 1969). Estimation of lactate dehydrogenase at two levels of pyruvate concentration, i.e. 0.33 & 10 mM permits an evaluation to be made of the relative proportions of the LDH isoenzyme subunits, i.e. the H- and the M-types, since the H-type is inhibited to a greater extent by high concentrations of pyruvate (see discussion later). The ratio (LDH activity at low pyruvate concentration)/(LDH activity at high pyruvate concentration) thus increases linearly the greater the proportion of H-type subunits present in the enzyme preparation. In preliminary studies the following LDH activity ratios were obtained for some rat tissues:

heart 4.15; diaphragm 2.50; gastrocnemius 1.30; liver 1.94. Thus diaphragm, being a "red" muscle possesses proportionately more H-type subunits than a white muscle such as gastrocnemius; rat liver has recently been shown to contain almost entirely M-type subunits (Fritz, Vesell, White & Pruitt, 1969), so that it would seem that in the gastrocnemius extract used here the proportion of H-types was low indeed.

All three glycolytic enzymes exhibit a reduced concentration following denervation, but the response in each case is different (Tables 14 - 16 & Figs 10 - 12). Content of phosphohexoisomerase remains more or less constant until 7 days after denervation after which a decline takes place such that by the 15th day the total amount of enzyme in the denervated tissue is only 40% of that in the controls. Glyceraldehyde phosphate dehydrogenase concentration shows a very slight increase 1 day after denervation but by three days the levels had dropped to below 90% of the control value, and decreases in an approximately linear manner. Content of this enzyme rises erratically to a peak of 106%

Table 14. The effect of left unilateral phrenicectomy upon the concentration and content of the extractable activity of phosphohexoisomerase of the rat diaphragm.

Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

Time (days) elapsed since phrenicectomy	Enzyme Concentration (nmoles/min/mg wet wt., at 25°C)			
	Innervated Controls	Denervated Hemidiaphragms	Paired Differences	Percentage
0	88.8 $\pm$ 5.2 (6)	88.8 $\pm$ 5.1 (6)	0.0 $\pm$ 1.0	100 $\pm$ 1
1	84.3 $\pm$ 3.1 (6)	79.2 $\pm$ 3.9 (6)	5.1 $\pm$ 2.4	94 $\pm$ 3
3	80.4 $\pm$ 2.7 (6)	63.5 $\pm$ 2.5 (6)	16.9 $\pm$ 2.7	79 $\pm$ 3
	P < 0.01			
5	76.0 $\pm$ 2.6 (8)	57.3 $\pm$ 2.1 (8)	18.7 $\pm$ 1.9	76 $\pm$ 2
	P < 0.001		P < 0.05	
7	79.1 $\pm$ 1.6 (9)	55.2 $\pm$ 1.3 (9)	23.9 $\pm$ 1.5	70 $\pm$ 2
	P < 0.001		P < 0.001	
10	82.2 $\pm$ 3.3 (6)	53.7 $\pm$ 2.6 (6)	28.4 $\pm$ 3.0	66 $\pm$ 3
	P < 0.001		P < 0.05	
15	69.4 $\pm$ 2.1 (6)	38.9 $\pm$ 0.8 (6)	30.5 $\pm$ 1.9	56 $\pm$ 2
	P < 0.001		P < 0.01	

Table 14 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content ( $\mu\text{mole}/\text{min}/\text{hemidiaphragm}$ , at $25^{\circ}\text{C}$ )			
	Innervated Controls	Denervated Hemidiaphragms	Paired Differences	Percentage
0	$8.17 \pm 0.40$ (6)	$7.37 \pm 0.49$ (6)	$0.80 \pm 0.27$	$90 \pm 4$
1	$7.86 \pm 0.48$ (6)	$7.18 \pm 0.67$ (6)	$0.82 \pm 0.32$	$91 \pm 5$
3	$7.04 \pm 0.54$ (6)	$6.24 \pm 0.32$ (6)	$0.80 \pm 0.27$	$90 \pm 3$
5	$6.80 \pm 0.26$ (8)	$6.55 \pm 0.21$ (8)	$0.25 \pm 0.11$	$97 \pm 1$
7	$7.33 \pm 0.24$ (9)	$6.56 \pm 0.17$ (9)	$0.83 \pm 0.23$	$90 \pm 3$
		$P < 0.05$		
10	$8.29 \pm 0.68$ (6)	$6.35 \pm 0.50$ (6)	$1.95 \pm 0.38$	$77 \pm 4$
15	$6.20 \pm 0.32$ (6)	$2.43 \pm 0.16$ (6)	$3.77 \pm 0.33$	$40 \pm 3$
		$P < 0.001$	$P < 0.01$	

Figure 10. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Phosphohexoisomerase Activity in the Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.

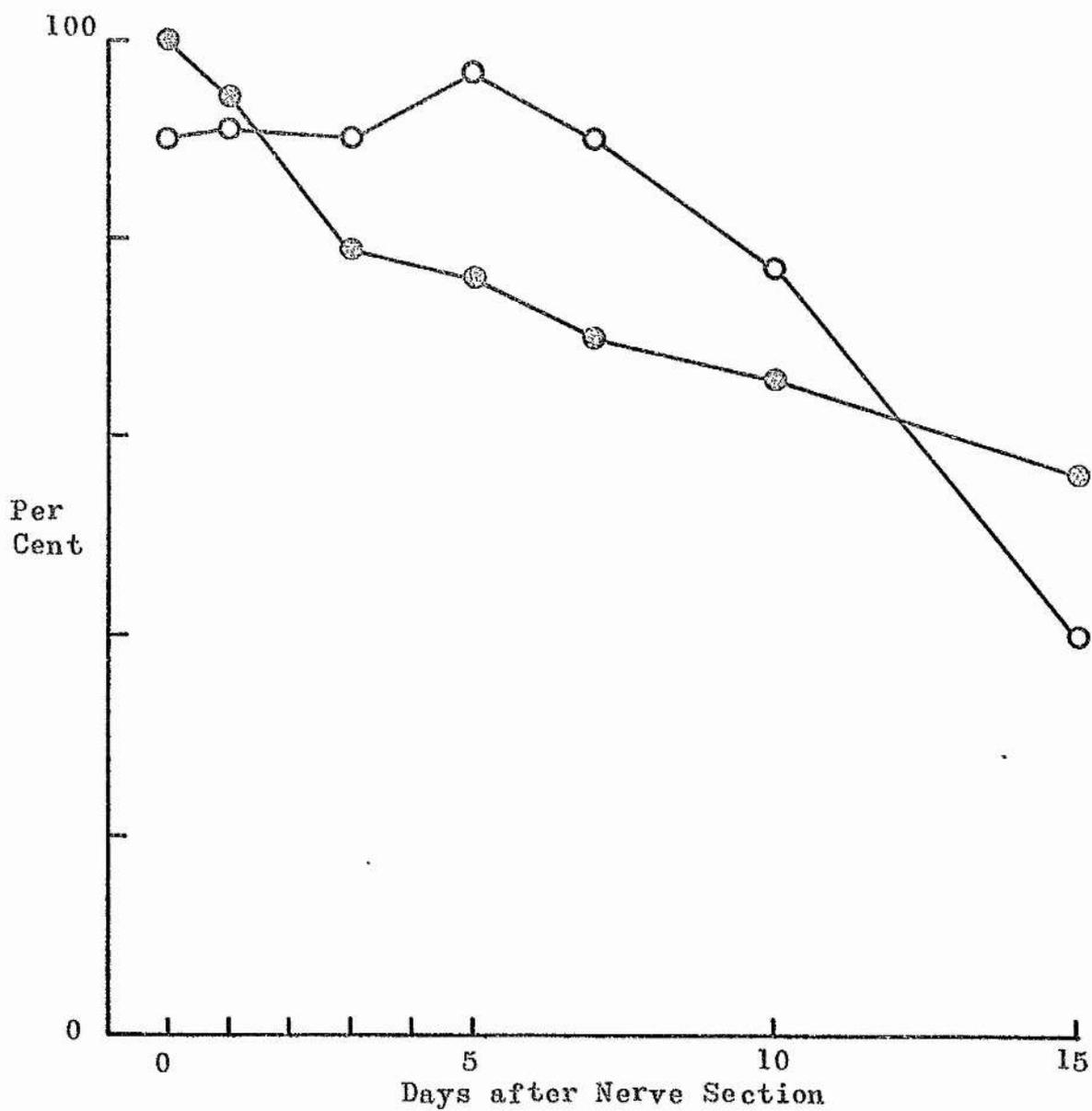


Table 15. The effect of left unilateral phrenicectomy upon the concentration and content of the extractable activity of glyceraldehyde 3-phosphate dehydrogenase of rat hemidiaphragm.

Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

Time (days) elapsed since phrenicectomy	Enzyme Concentration (nmole/min/mg wet wt., at 25°C)			
	Innervated controls	Denervated Hemidiaphragms	Paired Differences	Percentage
0	205 $\pm$ 8 (12)	210 $\pm$ 6 (12)	-5 $\pm$ 4	103 $\pm$ 2
1	175 $\pm$ 7 (12)	186 $\pm$ 7 (12)	-11 $\pm$ 6	107 $\pm$ 4
3	210 $\pm$ 13 (12)	184 $\pm$ 11 (12)	26 $\pm$ 5	88 $\pm$ 2
5	207 $\pm$ 7 (12) P < 0.01	175 $\pm$ 4 (12)	33 $\pm$ 7	85 $\pm$ 3
7	222 $\pm$ 10 (12) P < 0.01	178 $\pm$ 8 (12)	44 $\pm$ 6 P < 0.05	80 $\pm$ 2
10	190 $\pm$ 9 (12) P < 0.01	148 $\pm$ 6 (12)	42 $\pm$ 6	79 $\pm$ 3
15	249 $\pm$ 6 (6) P < 0.001	154 $\pm$ 6 (6)	95 $\pm$ 5 P < 0.001	62 $\pm$ 2

Table 15 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content ( $\mu\text{mole}/\text{min}/\text{hemidiaphragm}$ , at $25^{\circ}\text{C}$ )			
	Innervated Controls	Denervated Hemidiaphragms	Paired Differences	Percentage
0	$18.9 \pm 0.8$ (12)	$16.0 \pm 0.5$ (12)	$2.9 \pm 0.6$	$86 \pm 3$
	$P < 0.05$			
1	$16.5 \pm 1.0$ (12)	$15.7 \pm 1.0$ (12)	$0.7 \pm 0.4$	$96 \pm 3$
3	$18.6 \pm 1.5$ (12)	$18.1 \pm 1.2$ (12)	$0.6 \pm 0.5$	$98 \pm 3$
5	$18.7 \pm 0.7$ (12)	$20.0 \pm 0.8$ (12)	$-1.3 \pm 0.6$	$107 \pm 3$
7	$21.9 \pm 1.0$ (12)	$20.0 \pm 1.0$ (12)	$1.9 \pm 0.8$	$92 \pm 4$
10	$18.6 \pm 1.2$ (12)	$16.5 \pm 1.0$ (12)	$2.1 \pm 0.9$	$90 \pm 5$
15	$23.4 \pm 1.5$ (6)	$9.54 \pm 0.7$ (6)	$13.7 \pm 1.0$	$41 \pm 2$
	$P < 0.001$		$P < 0.01$	

Figure 11. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Glyceraldehyde Phosphate Dehydrogenase Activity of Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.

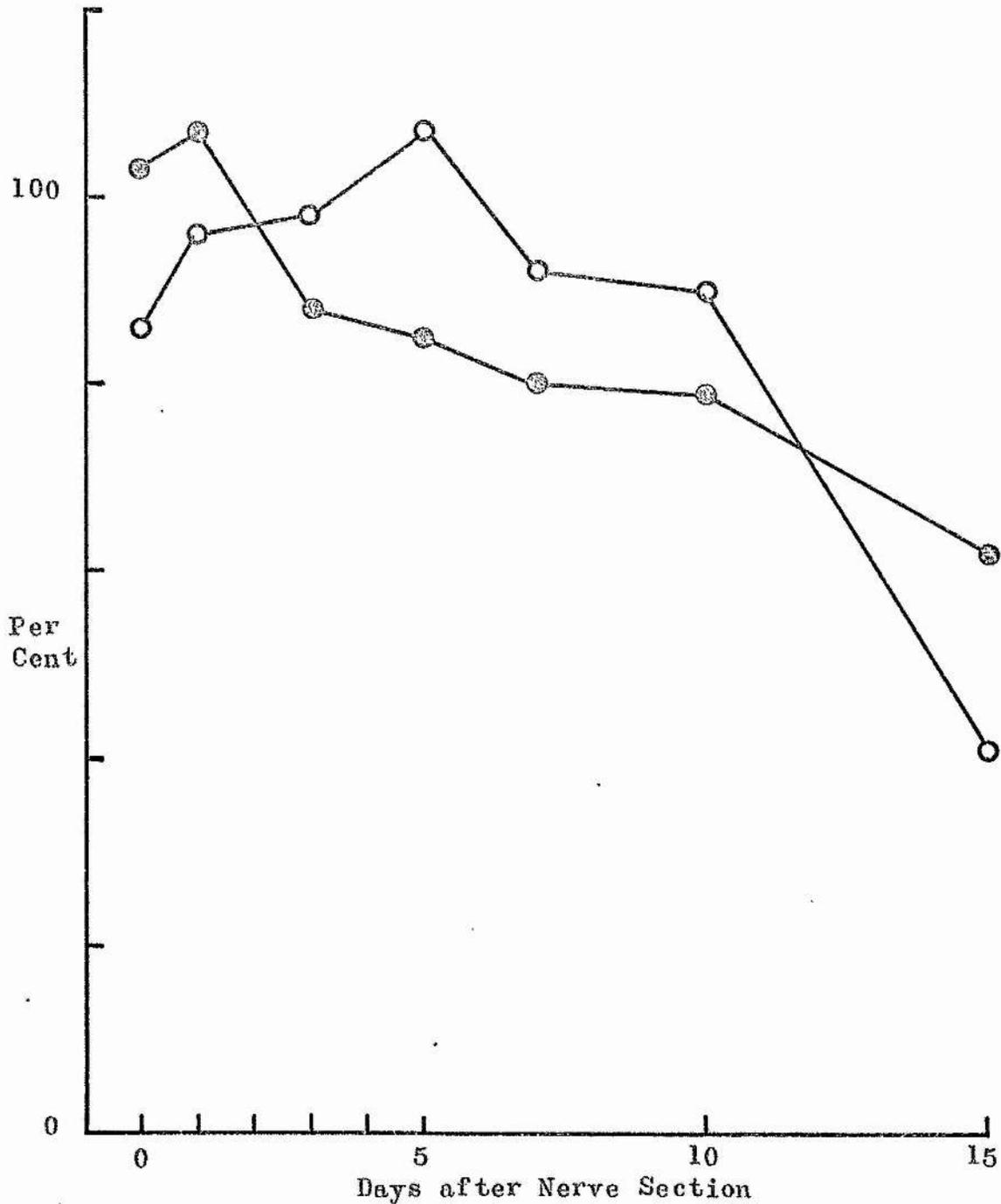


Table 16. The effect of left unilateral phrenicectomy upon the concentration and content of the extractable activity of lactate dehydrogenase of rat hemidiaphragm, measured at low (0.33 mM) pyruvate concentration.

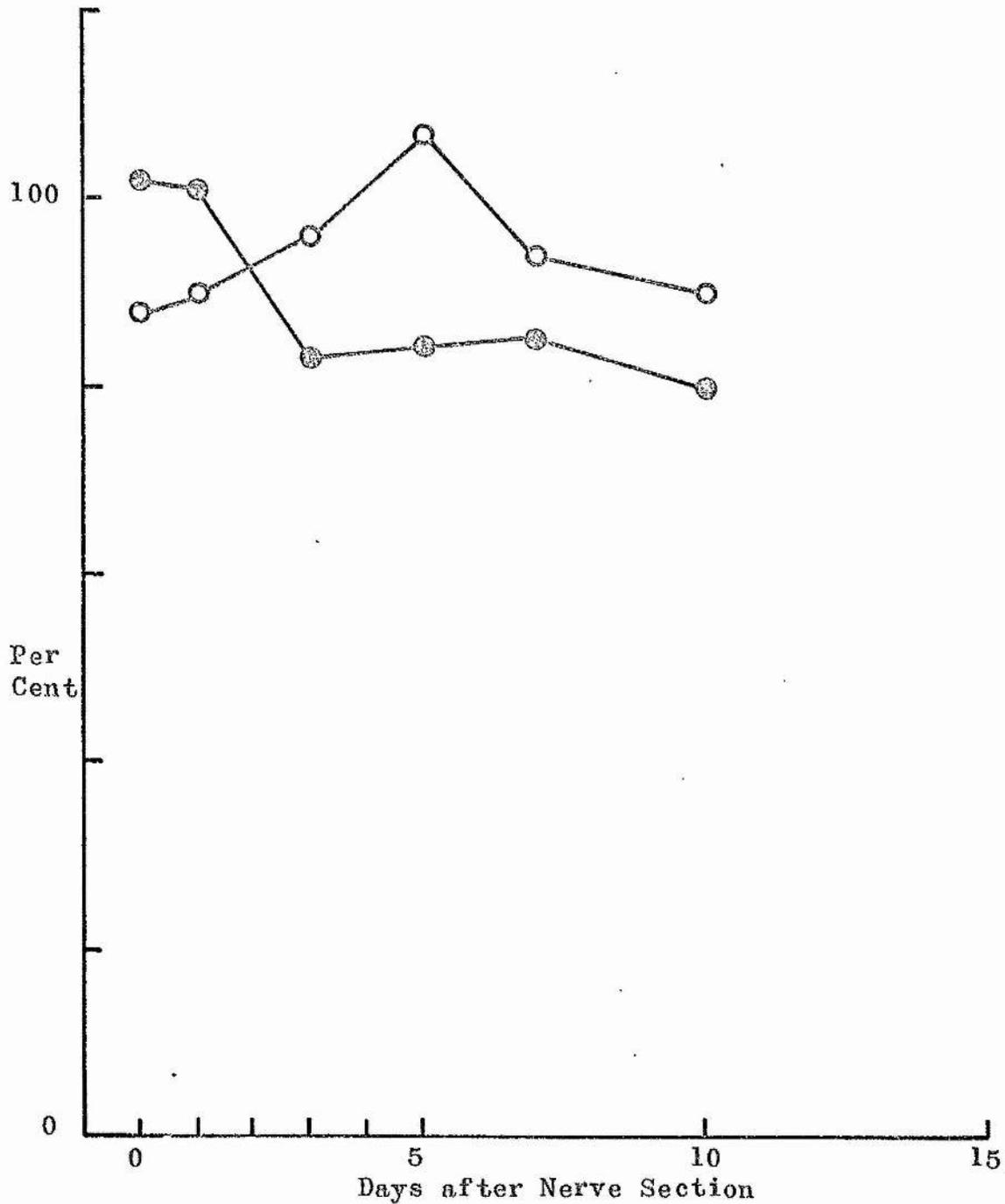
Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

Time (days) elapsed since phrenicectomy	Enzyme Concentration (nmole/min/mg wet wt., at 25°C)			
	Innervated controls	Denervated hemidiaphragms	Differences	Percentage
0	239 $\pm$ 2 (6)	242 $\pm$ 10 (6)	-4 $\pm$ 10	102 $\pm$ 4
1	204 $\pm$ 7 (6)	205 $\pm$ 6 (6)	-1 $\pm$ 9	101 $\pm$ 4
3	260 $\pm$ 8 (6) P < 0.01	215 $\pm$ 9 (6)	45 $\pm$ 7 P < 0.05	83 $\pm$ 3
5	218 $\pm$ 6 (6) P < 0.001	182 $\pm$ 2 (6)	35 $\pm$ 7	84 $\pm$ 3
7	257 $\pm$ 12 (6) P < 0.05	215 $\pm$ 4 (6)	42 $\pm$ 12	85 $\pm$ 4
10	254 $\pm$ 14 (6) P < 0.01	201 $\pm$ 4 (6)	53 $\pm$ 11	80 $\pm$ 3

Table 16 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content ( $\mu\text{mole}/\text{min}/\text{hemidiaphragm}$ , at $25^{\circ}\text{C}$ )			
	Innervated Controls	Denervated Hemidiaphragms	Paired Differences	Percentage
0	$21.7 \pm 0.3$ (6)	$19.0 \pm 0.8$ (6)	$2.9 \pm 0.8$	$88 \pm 4$
	$P < 0.05.$			
1	$18.4 \pm 0.7$ (6)	$16.5 \pm 1.0$ (6)	$1.9 \pm 0.7$	$90 \pm 3$
3	$21.0 \pm 0.9$ (6)	$20.1 \pm 0.8$ (6)	$0.9 \pm 0.4$	$96 \pm 2$
5	$20.4 \pm 0.9$ (6)	$21.7 \pm 0.9$ (6)	$-1.3 \pm 0.6$	$107 \pm 3$
7	$26.4 \pm 1.8$ (6)	$24.1 \pm 1.0$ (6)	$2.3 \pm 1.9$	$94 \pm 8$
10	$24.6 \pm 1.8$ (6)	$21.8 \pm 1.1$ (6)	$2.8 \pm 1.4$	$90 \pm 4$

Figure 12. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Lactate Dehydrogenase Activity of the Rat Hemidiaphragm, measured at low pyruvate concentration, and expressed as a Percentage of the Contralateral Control Values.



at 5 days and declines just as erratically to 40% in the denervated tissue at 15 days. The response of the lactate dehydrogenase activity shows remarkable similarity to that of the triose phosphate dehydrogenase: 1 day after denervation the concentration of the former enzyme is identical to that of the control muscle but by 3 days a decrease of nearly 20% is found, whilst thereafter very little change in activity is observed. Content of the enzyme rises to a peak at 5 days, though the response is not as erratic as for the triose phosphate dehydrogenase. No significant difference in the lactate dehydrogenase activity ratios measured at two pyruvate concentrations, between the denervated muscles and their contralaterals are observed until 7 days after denervation at which time the paralysed tissue has a significantly lower ratio ( $P < 0.05$ ) indicative of a relative diminution of H-type subunits in the muscle. Ten days though after nerve section, this effect was no longer apparent. (Table 17).

Table 17. The effect of left unilateral phrenicectomy upon the activity ratio of lactate dehydrogenase concentration; the ratio is defined as the activity measured at 0.33 mM pyruvate concentration divided by the activity measured at 10 mM pyruvate.

Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

Time (days) elapsed since phrenicectomy	Lactate dehydrogenase activity ratio		
	Innervated Controls	Denervated Hemidiaphragms	Paired Differences
0	3.02 $\pm$ 0.08 (6)	3.01 $\pm$ 0.08 (6)	0.01 $\pm$ 0.07
1	3.38 $\pm$ 0.09 (6)	3.40 $\pm$ 0.12 (6)	-0.02 $\pm$ 0.06
3	3.24 $\pm$ 0.07 (6)	3.38 $\pm$ 0.12 (6)	-0.13 $\pm$ 0.17
5	4.25 $\pm$ 0.16 (6)	3.87 $\pm$ 0.06 (6)	0.38 $\pm$ 0.17
7	3.04 $\pm$ 0.04 (6)	2.82 $\pm$ 0.07 (6)	0.22 $\pm$ 0.06
	P < 0.05		
10	3.00 $\pm$ 0.05 (6)	2.91 $\pm$ 0.04 (6)	0.09 $\pm$ 0.04

Enzymes associated with the Tricarboxylic Acid Cycle:

NAD- & NADP specific isocitrate dehydrogenases, malate dehydrogenase & glutamate dehydrogenase.

There are technical difficulties associated with the fractional extraction procedure described by Pette (1966) when applied to such small quantities of tissue as are furnished by a single rat hemidiaphragm. For this reason no attempt was made to determine the distribution of the activities of those enzymes associated with the citric acid cycle, i.e. NAD- and NADP-specific isocitrate dehydrogenase, malate dehydrogenase and glutamate dehydrogenase. Moreover, because two at least of the enzymes are known to occur in appreciable quantities in the extramitochondrial compartment, i.e. malate dehydrogenase and NADP-linked isocitrate dehydrogenase, it was decided to assay the total enzyme activity at the pH of the overall intracellular environment, i.e. pH 6.9. The review by Waddell & Bates (1969) summarizes the results of the estimates reported in the literature for the "internal" pH of isolated mitochondria, and although the values produced by the various techniques do not agree closely, it may<sup>be</sup> seen that apart from a few instances, the internal pH of the mitochondrion does not vary

greatly from that of its suspension medium. Estimation of mitochondrial enzymes under the conditions used in this study presumably does not expose them to too alien an environment; indeed, Wolfe & Niellands (1956) have demonstrated that the maximum turnover of pig heart malate dehydrogenase occurs at pH 6.9.

The extraction technique used in this study (homogenisation in 0.1 M potassium phosphate buffer containing 1 mM EDTA; see Pette, 1966) may thus be considered to solubilize the entire muscle complement of the citric acid cycle associated enzymes. However, there may be difficulties in comparing the activities of such extracts with other values reported in the literature, for in at least one case, i.e. malate dehydrogenase, it is known that the properties of the cytoplasmic located enzyme differ from those of the enzyme found in the mitochondrion. High concentrations of oxaloacetate i.e. 1.9 mM, have been shown to have no inhibitory effect on the "cytoplasmic" enzyme of beef heart (England, Siegel, & Brieger, 1960) whilst the "mitochondrial" enzyme was appreciably inhibited by concentrations of oxaloacetate as low as 0.27 mM (Siegel & England, 1960).

In this study optimal activity of malate dehydrogenase was observed with an oxaloacetate concentration of 0.2 mM. It is possible therefore that inhibition of the mitochondrial enzyme may be taking place and that the full capacity of the tissue is not realized. Shonk & Boxer (1964) reported that the inclusion of 2.7 M ethanol in the reaction mixture renders unstable and therefore inactivates the extramitochondrial malate dehydrogenase; thus a comparison of the activity assayed with and without the presence of ethanol may be used to determine the proportions of the enzyme in the two locations. Unfortunately, even when assayed at a number of different oxaloacetate concentrations, the inclusion of ethanol in the reaction mixture was found not to alter the measurable enzyme activity of the extracts of rat diaphragm muscle. Since Pette (1966) has demonstrated that in rat muscles the NAD-linked isocitrate dehydrogenase, and glutamate dehydrogenase enzymes are confined to the mitochondrion, it is clear that determination of the total activities of these enzymes in the denervated tissue will provide information on the effect of phrenic nerve section on the muscle mitochondria.

It is difficult to make a comparison of the activity of malate dehydrogenase determined in this study with values reported in the literature; it may be noted however that the enzyme activity expressed as a concentration lies between that for rat heart and skeletal muscle, and most closely resembles that for the extra-mitochondrial enzyme from rat heart (Shonk & Boxer, 1964). This having been said, it is of some interest to note that the relative activities of the two isocitrate dehydrogenases differ by an order of magnitude, and thus correspond exactly to the proportions reported by Pette (1966) for rat heart; the quadriceps muscle of the rat however has equal proportions of these two enzymes. Rat diaphragm may thus be considered as being heart-like in character.

Of further interest is the finding that in non-operated rats, the left hemidiaphragm may possess a 10% higher concentration of these citric acid cycle associated enzymes than is found in the right hemidiaphragm. In contrast, those enzymes usually considered to be located in the muscle cytosol have concentrations in the left hemidiaphragm less than or equal to those of the right. This

point is discussed in greater detail in a subsequent section.

The effect of unilateral phrenicectomy is to cause a rapid decrease in enzyme content; by one day after nerve section the denervated muscles have significantly lower enzyme contents than the contralateral control tissues ( $P < 0.05$ ). Perhaps the most clear cut picture is shown by NAD-linked isocitrate dehydrogenase (Table 18, & Fig.13). The decrease continues for a further 2 days and then levels out at about 50% of the control content; after 10 days a second decrease in content is observed, but at this time the concentration of the enzyme is maintained suggesting a generalised decrease of all muscle proteins. Similar responses are shown by the NADP-linked enzyme and by malate dehydrogenase (Figs 14,15, & Tables 19 & 20). Glutamate dehydrogenase on the other hand demonstrates a different behaviour (Fig.16 & Table 21); a decrease in concentration takes place for the first 7 days after nerve section reaching a minimum of about 60% of control values. Subsequently an increase is observed so that by the 15th day, concentration is slightly below 80% of the control values. In terms of enzyme content though, an overall decline is observed (Fig.16), upon which is superimposed two increases of activity coming to maxima at 3 and 7 days after denervation.

Table 18. The effect of left unilateral phrenicectomy upon the concentration and content of the extractable activity of NAD-specific isocitrate dehydrogenase of rat diaphragm.

Each value is the mean  $\pm$  S.E.M. of the number of observation shown in parenthesis.

Time (days) elapsed since phrenicectomy	Enzyme Concentration (nmole/min/mg wet wt., at 25°C)			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	1.55 $\pm$ 0.08 (6)	1.78 $\pm$ 0.10 (6)	-0.24 $\pm$ 0.07	116 $\pm$ 5
1	1.37 $\pm$ 0.08 (6)	1.26 $\pm$ 0.08 (6)	0.11 $\pm$ 0.09	93 $\pm$ 7
3	1.64 $\pm$ 0.08 (6) P < 0.001	0.82 $\pm$ 0.08 (6)	0.82 $\pm$ 0.05 P < 0.001	50 $\pm$ 3
5	2.16 $\pm$ 0.11 (6) P < 0.001	0.96 $\pm$ 0.11 (6)	1.20 $\pm$ 0.07 P < 0.001	44 $\pm$ 3
7	2.38 $\pm$ 0.09 (6) P < 0.001	1.00 $\pm$ 0.03 (6)	1.38 $\pm$ 0.10 P < 0.01	42 $\pm$ 2
10	2.41 $\pm$ 0.22 (6) P < 0.001	0.97 $\pm$ 0.11 (6)	1.44 $\pm$ 0.18 P < 0.05	40 $\pm$ 4
15	2.06 $\pm$ 0.15 (6) P < 0.001	0.77 $\pm$ 0.08 (6)	1.30 $\pm$ 0.09 P < 0.01	37 $\pm$ 2

Table 18 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content (nmole/min/hemidiaphragm, at 25°C)			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	147 ± 8 (6)	142 ± 7 (6)	5 ± 7	97 ± 5
1	144 ± 9 (6) P < 0.05	114 ± 6 (6)	29 ± 12	82 ± 8
3	155 ± 6 (6) P < 0.001	84 ± 8 (6)	71 ± 6 P < 0.01	54 ± 4
5	208 ± 11 (6) P < 0.001	105 ± 9 (6)	103 ± 13 P < 0.05	51 ± 5
7	226 ± 8 (6) P < 0.001	113 ± 4 (6)	113 ± 7 P < 0.01	50 ± 2
10	234 ± 16 (6) P < 0.001	110 ± 14 (6)	123 ± 14 P < 0.05	47 ± 5
15	191 ± 9 (6) P < 0.001	48 ± 3 (6)	145 ± 8 P < 0.001	24 ± 2

Figure 13. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of NAD-linked Isocitrate Dehydrogenase Activity of Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.

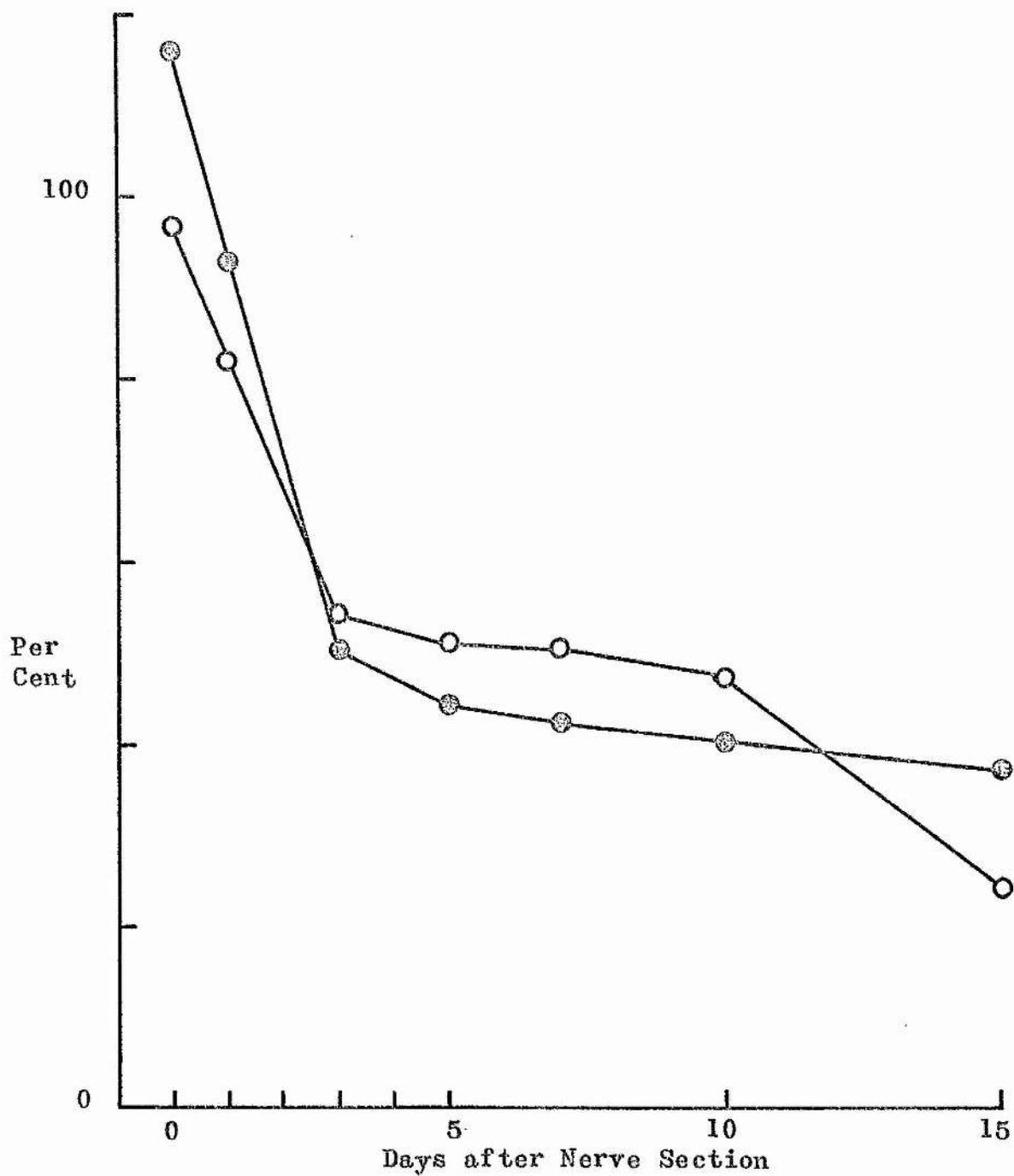


Table 19. The effect of left unilateral phrenicectomy upon the concentration and content of the extractable activity of NADP-specific isocitrate dehydrogenase of rat hemidiaphragm.

Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

Time (days) elapsed since phrenicectomy	Enzyme Concentration (nmole/min/mg wet wt., at 25°C)			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	14.6 $\pm$ 0.3 (6)	15.9 $\pm$ 0.5 (6)	-1.3 $\pm$ 0.5	109 $\pm$ 3
1	9.4 $\pm$ 0.6 (6)	8.3 $\pm$ 0.5 (6)	1.1 $\pm$ 0.4	89 $\pm$ 5
3	13.2 $\pm$ 0.3 (6) P < 0.001	6.9 $\pm$ 0.3 (6)	6.2 $\pm$ 0.3 P < 0.001	53 $\pm$ 2
5	14.0 $\pm$ 0.6 (6) P < 0.001	6.0 $\pm$ 0.3 (6)	7.9 $\pm$ 0.5 P < 0.01	43 $\pm$ 2
7	14.7 $\pm$ 0.4 (6) P < 0.001	5.5 $\pm$ 0.2 (6)	9.2 $\pm$ 0.4 P < 0.001	37 $\pm$ 1
10	16.0 $\pm$ 1.5 (6) P < 0.001	6.1 $\pm$ 0.3 (6)	10.0 $\pm$ 1.2 P < 0.05	39 $\pm$ 2
15	12.6 $\pm$ 0.6 (6) P < 0.001	4.2 $\pm$ 0.3 (6)	8.4 $\pm$ 0.5 P < 0.001	33 $\pm$ 2

Table 19 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content ( $\mu\text{mole}/\text{min}/\text{hemidiaphragm}$ , at $25^{\circ}\text{C}$ )			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	$1.36 \pm 0.02$ (6) $P < 0.05$	$1.27 \pm 0.03$ (6)	$0.09 \pm 0.03$	$94 \pm 2$
1	$0.91 \pm 0.05$ (6) $P < 0.05$	$0.72 \pm 0.05$ (6)	$0.19 \pm 0.04$	$80 \pm 4$
3	$1.25 \pm 0.03$ (6) $P < 0.001$	$0.71 \pm 0.04$ (6)	$0.54 \pm 0.04$ $P < 0.01$	$57 \pm 3$
5	$1.22 \pm 0.05$ (6) $P < 0.001$	$0.66 \pm 0.02$ (6)	$0.56 \pm 0.04$ $P < 0.01$	$54 \pm 2$
7	$1.40 \pm 0.05$ (6) $P < 0.001$	$0.62 \pm 0.03$ (6)	$0.77 \pm 0.04$ $P < 0.001$	$45 \pm 1$
10	$1.55 \pm 0.11$ (6) $P < 0.001$	$0.68 \pm 0.29$ (6)	$0.87 \pm 0.09$ $P < 0.01$	$44 \pm 2$
15	$1.17 \pm 0.04$ (6) $P < 0.001$	$0.26 \pm 0.01$ (6)	$0.92 \pm 0.04$ $P < 0.001$	$22 \pm 1$

Figure 14. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of NADP-linked Isocitrate Dehydrogenase Activity, expressed as a Percentage of the Contralateral Control Values.

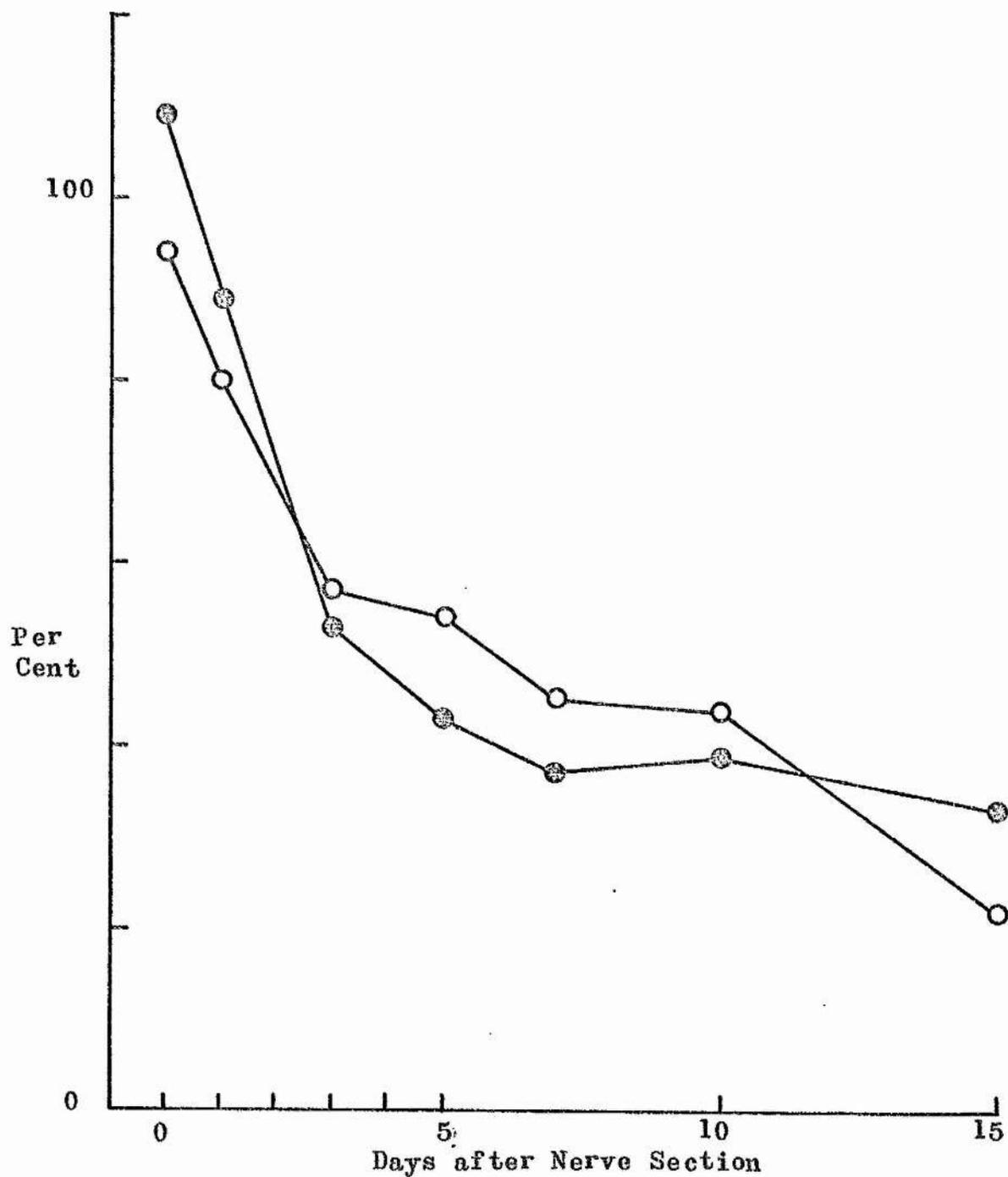


Table 20. The effect of left unilateral phrenicectomy upon the concentration and content of the extractable activity of malate dehydrogenase of rat hemidiaphragm tissue.

Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

Time (days) elapsed since phrenicectomy	Enzyme Concentration (nmole/min/mg wet wt., at 25°C)			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	781 $\pm$ 38 (6)	858 $\pm$ 50 (6)	-77 $\pm$ 46	110 $\pm$ 6
1	726 $\pm$ 32 (6)	644 $\pm$ 25 (6)	82 $\pm$ 22	89 $\pm$ 3
3	986 $\pm$ 26 (6) P < 0.001	591 $\pm$ 22 (6)	394 $\pm$ 33 P < 0.01	60 $\pm$ 3
5	870 $\pm$ 63 (6) P < 0.001	449 $\pm$ 32 (6)	421 $\pm$ 52 P < 0.05	52 $\pm$ 4
7	916 $\pm$ 26 (10) P < 0.001	511 $\pm$ 19 (10)	405 $\pm$ 25 P < 0.01	56 $\pm$ 2
10	884 $\pm$ 18 (6) P < 0.001	447 $\pm$ 17 (6)	437 $\pm$ 28 P < 0.01	51 $\pm$ 3
15	643 $\pm$ 28 (6) P < 0.001	338 $\pm$ 10 (6)	306 $\pm$ 24 P < 0.01	53 $\pm$ 2

Table 20 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content ( $\mu\text{mole}/\text{min}/\text{hemidiaphragm}$ , at $25^{\circ}\text{C}$ )			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	$71.2 \pm 3.4$ (6)	$67.5 \pm 3.9$ (6)	$3.7 \pm 3.6$	$95 \pm 5$
1	$64.7 \pm 3.5$ (6) $P < 0.05$	$51.7 \pm 3.1$ (6)	$13.0 \pm 2.0$ $P < 0.05$	$80 \pm 3$
3	$79.7 \pm 2.9$ (6) $P < 0.001$	$55.7 \pm 3.2$ (6)	$24.0 \pm 3.7$ $P < 0.05$	$70 \pm 4$
5	$81.7 \pm 7.4$ (6) $P < 0.01$	$53.0 \pm 3.3$ (6)	$28.7 \pm 5.5$	$66 \pm 5$
7	$91.8 \pm 4.3$ (10) $P < 0.001$	$57.9 \pm 2.8$ (10)	$34.0 \pm 4.1$ $P < 0.05$	$64 \pm 3$
10	$85.3 \pm 2.7$ (6) $P < 0.001$	$48.2 \pm 0.8$ (6)	$37.0 \pm 2.1$ $P < 0.001$	$57 \pm 1$
15	$60.5 \pm 4.6$ (6) $P < 0.001$	$20.8 \pm 1.2$ (6)	$39.7 \pm 3.6$ $P < 0.01$	$35 \pm 1$

Figure 15. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Malate Dehydrogenase Activity of Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.

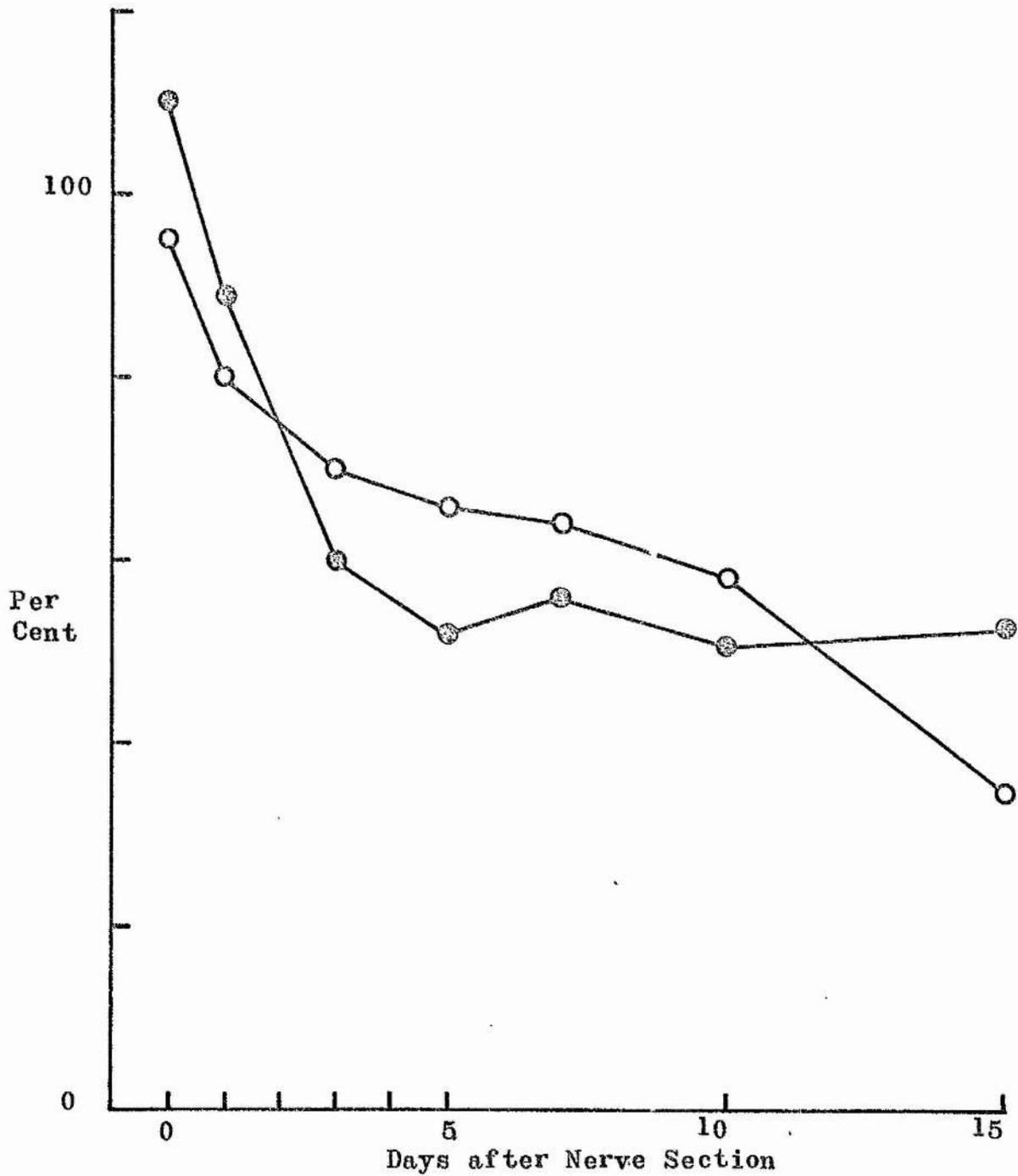


Table 21. The effect of left unilateral phrenicectomy upon the concentration and content of the extractable activity of glutamate dehydrogenase of rat hemidiaphragm.

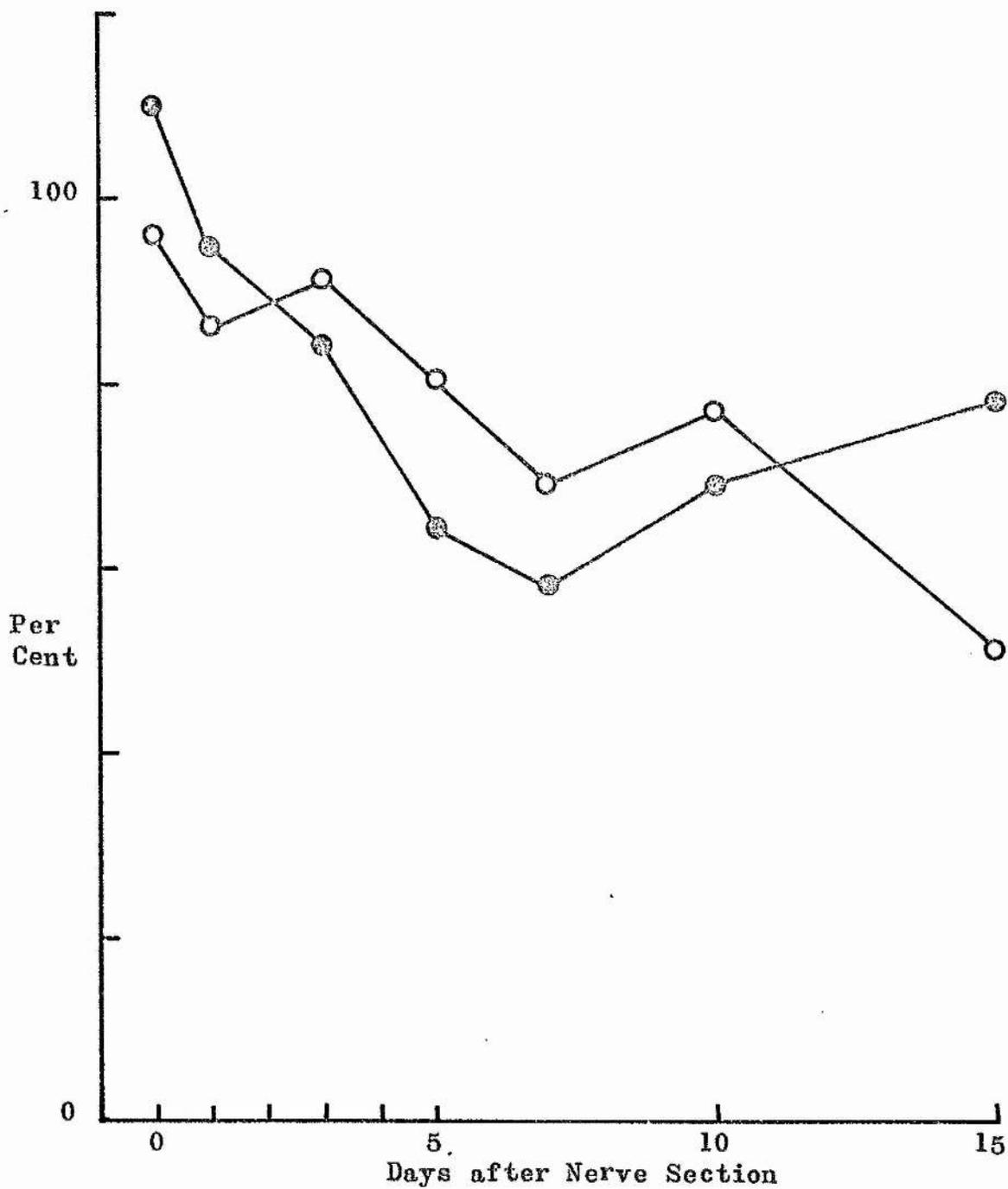
Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

Time (days) elapsed since phrenicectomy	Enzyme Concentration (pmole/min/mg wet wt., at 25°C)			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	878 $\pm$ 32 (6)	974 $\pm$ 43 (6)	96 $\pm$ 23	111 $\pm$ 3
1	824 $\pm$ 37 (6)	783 $\pm$ 44 (6)	41 $\pm$ 40	95 $\pm$ 5
3	864 $\pm$ 20 (6) P < 0.01	727 $\pm$ 18 (6)	137 $\pm$ 23	84 $\pm$ 2
5	969 $\pm$ 28 (6) P < 0.001	614 $\pm$ 17 (6)	355 $\pm$ 25 P < 0.01	64 $\pm$ 2
7	987 $\pm$ 42 (6) P < 0.001	571 $\pm$ 14 (6)	415 $\pm$ 37 P < 0.01	58 $\pm$ 2
10	674 $\pm$ 40 (6) P < 0.001	459 $\pm$ 13 (6)	215 $\pm$ 32 P < 0.05	69 $\pm$ 3
15	840 $\pm$ 29 (6) P < 0.01	651 $\pm$ 26 (6)	189 $\pm$ 25 P < 0.05	78 $\pm$ 3

Table 21 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content (nmole/min/hemidiaphragm, at 25°C)			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	81.6 ± 3.1 (6)	77.9 ± 2.8 (6)	3.6 ± 2.1	96 ± 2
1	80.0 ± 3.8 (6) P < 0.05	68.3 ± 2.5 (6)	11.7 ± 3.5	86 ± 3
3	81.8 ± 1.5 (6) P < 0.05	74.1 ± 2.4 (6)	7.7 ± 3.1	91 ± 4
5	84.7 ± 2.0 (6) P < 0.001	67.6 ± 1.9 (6)	17.1 ± 3.3	80 ± 3
7	93.5 ± 3.7 (6) P < 0.001	64.5 ± 1.7 (6)	29.0 ± 2.8 P < 0.01	69 ± 2
10	67.5 ± 1.6 (6) P < 0.01	51.6 ± 2.1 (6)	15.8 ± 2.6	77 ± 4
15	78.8 ± 4.5 (6) P < 0.001	40.1 ± 2.1 (6)	38.6 ± 2.9 P < 0.01	51 ± 2

Figure 16. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Glutamate Dehydrogenase Activity of Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.



NADP-linked Dehydrogenases: Glucose-6-phosphate  
Dehydrogenase, 6-Phosphogluconate Dehydrogenase  
& Glutathione Reductase.

For reasons which will become apparent later (see discussion) it is more convenient to consider together these NADP-linked dehydrogenases rather than to deal with them in isolation.

The effect of unilateral phrenicectomy upon the extractable activities of the pentose shunt dehydrogenases is recorded in Tables 22, 23 & Figs. 17 & 18. As soon as 1 day after denervation an increased content of both enzymes is observed, maximal content of the glucose phosphate dehydrogenase being found at 3 days, whilst the peak for the gluconate dehydrogenase probably occurs between 3 and 5 days. Later the contents of the two enzymes decrease equally as rapidly as they rose, such that by 15 days approximately normal contents of the enzymes are found. Because of the overall decrease of tissue mass at this time a slight increase in concentration is indicated.

Table 22. The effect of unilateral phrenicectomy upon the extractable activity of glucose 6-phosphate dehydrogenase in the rat hemidiaphragm, expressed in terms of concentration and content.

Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

Time (days) elapsed since phrenicectomy	Enzyme concentration (nmole/min/g wet wt., at 25°C)			
	Innervated controls	Denervated hemidiaphragms	Paired Differences	Percentage
0	228 $\pm$ 22 (6)	232 $\pm$ 28 (6)	- 4 $\pm$ 16	101 $\pm$ 7
1	353 $\pm$ 9 (6) P < 0.01	567 $\pm$ 43 (6)	213 $\pm$ 39	160 $\pm$ 10
3	303 $\pm$ 33 (5) P < 0.01	622 $\pm$ 70 (5)	319 $\pm$ 38 P < 0.05	206 $\pm$ 5
5	342 $\pm$ 25 (9) P < 0.01	497 $\pm$ 30 (9)	-155 $\pm$ 16 P < 0.05	148 $\pm$ 8
7	326 $\pm$ 47 (9)	375 $\pm$ 37 (9)	- 49 $\pm$ 16	120 $\pm$ 7
10	258 $\pm$ 15 (6)	280 $\pm$ 11 (6)	- 22 $\pm$ 11	109 $\pm$ 4
15	228 $\pm$ 17 (6)	290 $\pm$ 20 (6)	- 63 $\pm$ 8 P < 0.05	128 $\pm$ 4

Table 22 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content ( $\mu\text{mole}/\text{min}/\text{hemidiaphragm}$ , at $25^{\circ}\text{C}$ )			
	Innervated control	Denervated hemidiaphragm	Paired differences	Percentage
0	$21.4 \pm 2.8$ (6)	$19.4 \pm 2.8$ (6)	$2.0 \pm 2.1$	$91 \pm 8$
1	$33.0 \pm 1.9$ (6) $P < 0.01$	$51.0 \pm 4.5$ (6)	$-18.1 \pm 3.6$	$154 \pm 10$
3	$28.1 \pm 3.9$ (5) $P < 0.05$	$66.0 \pm 9.4$ (5)	$-37.9 \pm 5.7$ $P < 0.05$	$235 \pm 8$
5	$29.3 \pm 1.8$ (9) $P < 0.001$	$55.8 \pm 2.9$ (9)	$-26.5 \pm 2.6$ $P < 0.01$	$194 \pm 13$
7	$30.2 \pm 4.3$ (9) $P < 0.05$	$44.3 \pm 4.1$ (9)	$-14.1 \pm 2.5$	$157 \pm 13$
10	$25.8 \pm 2.0$ (6)	$33.3 \pm 2.8$ (6)	$-7.5 \pm 1.5$	$129 \pm 6$
15	$20.8 \pm 2.2$ (6)	$19.1 \pm 1.8$ (6)	$1.6 \pm 2.1$	$96 \pm 10$

Figure 17. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Glucose-6-phosphate Dehydrogenase Activity of Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values

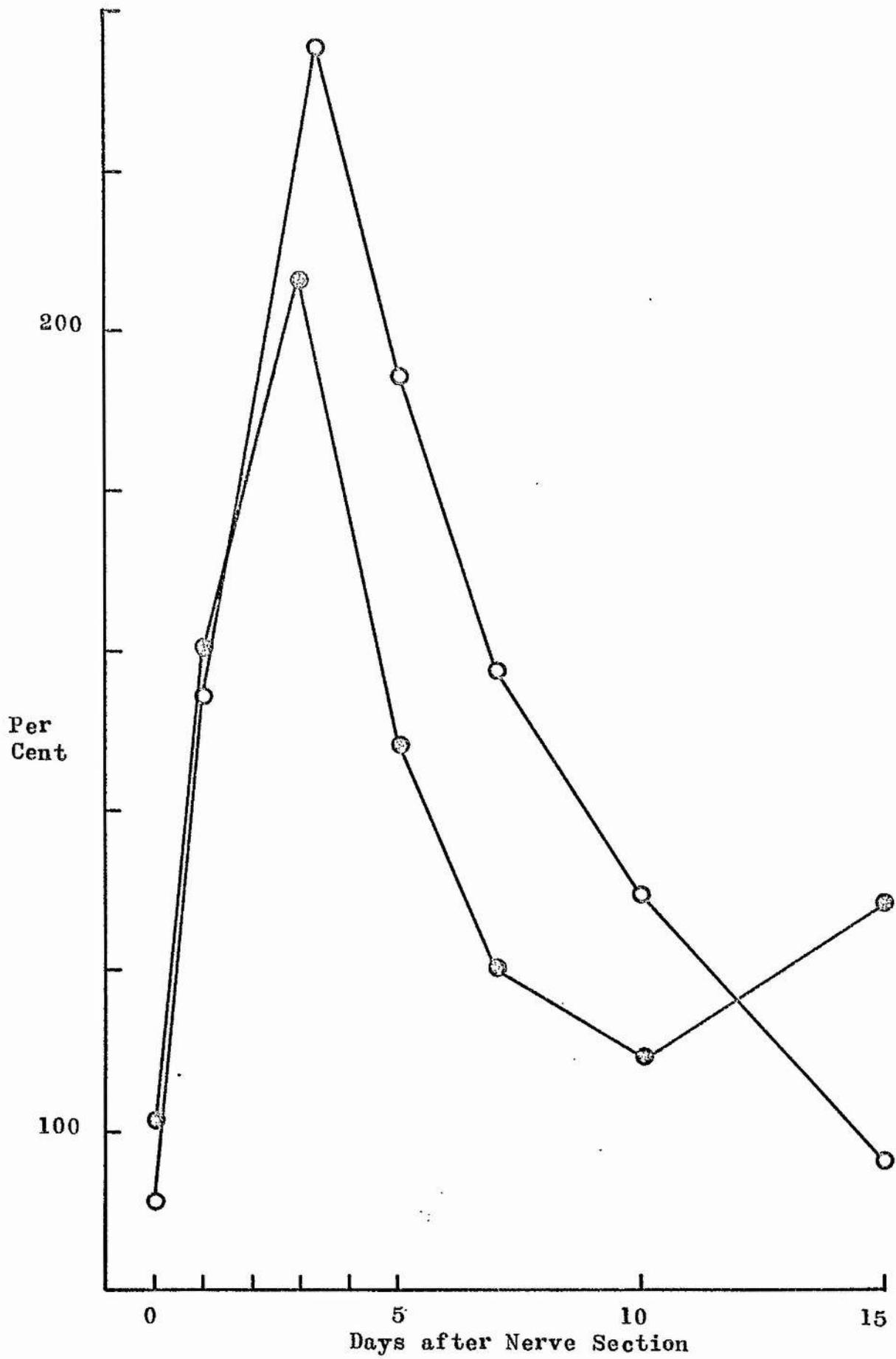


Table 23. The effect of unilateral phrenicectomy upon the extractable activity of 6-phosphogluconate dehydrogenase from rat hemidiaphragm, expressed as concentration and content.

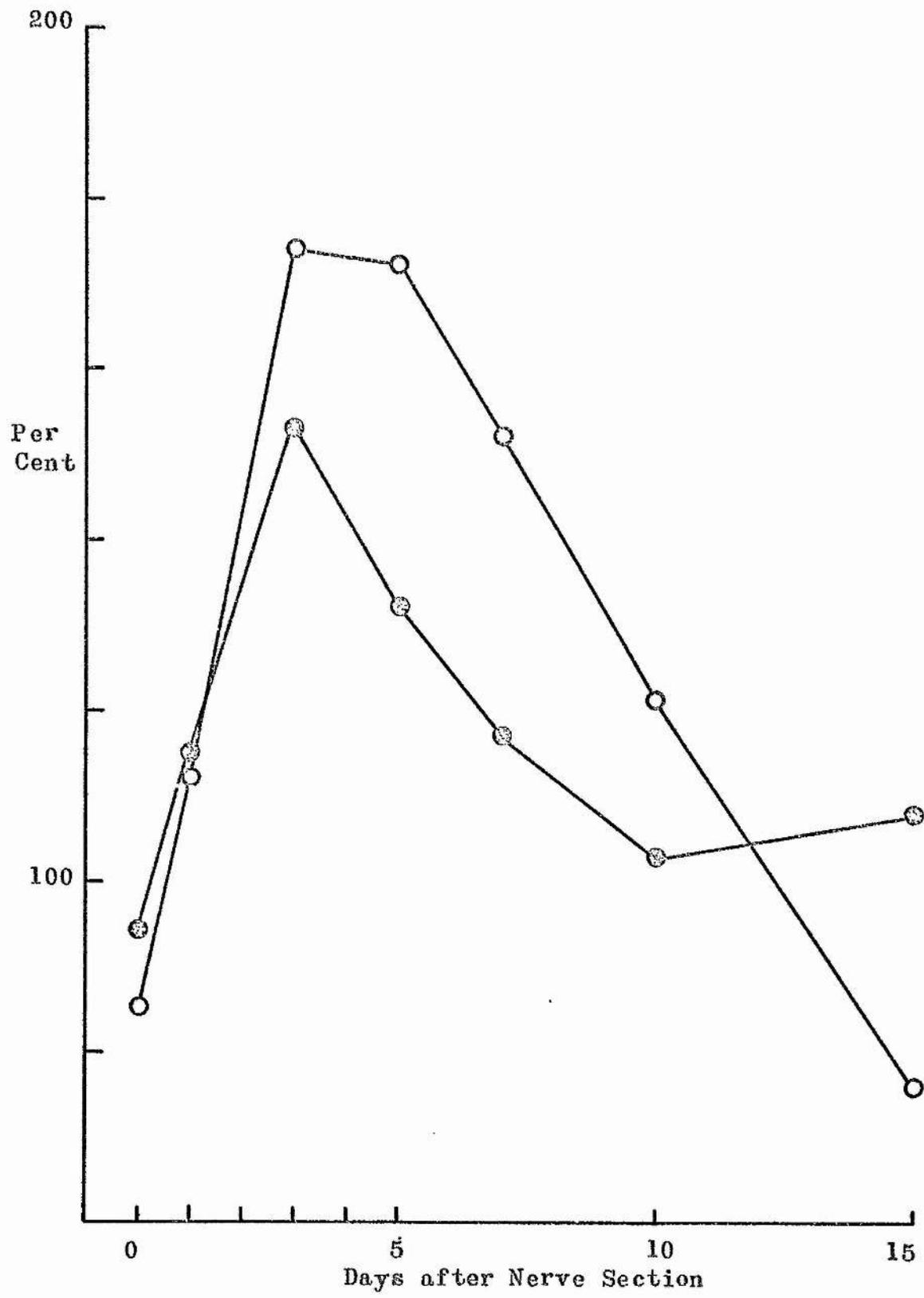
Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

Time (days) elapsed since phrenicectomy	Enzyme Concentration ( nmole/min/g wet wt., at 25°C)			
	Innervated Controls	Denervated Hemidiaphragms	Paired Differences	Percentage
0	223 $\pm$ 14 (6)	209 $\pm$ 18 (6)	14 $\pm$ 16	94 $\pm$ 7
1	327 $\pm$ 22 (6)	374 $\pm$ 23 (6)	-46 $\pm$ 20	115 $\pm$ 7
3	280 $\pm$ 11 (6) P < 0.001	426 $\pm$ 14 (6)	-146 $\pm$ 11 P < 0.01	153 $\pm$ 5
5	287 $\pm$ 13 (9) P < 0.01	375 $\pm$ 20 (9)	-89 $\pm$ 16	132 $\pm$ 7
7	282 $\pm$ 30 (9)	321 $\pm$ 27 (9)	-39 $\pm$ 8	117 $\pm$ 5
10	291 $\pm$ 15 (6)	297 $\pm$ 9 (6)	-6 $\pm$ 11	103 $\pm$ 4
15	210 $\pm$ 14 (6)	225 $\pm$ 14 (6)	-14 $\pm$ 16	108 $\pm$ 7

Table 23 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content ( $\mu\text{mole}/\text{min}/\text{hemidiaphragm}$ , at $25^{\circ}\text{C}$ )			
	Innervated Controls	Denervated Hemidiaphragm	Differences	Percentage
0	$20.9 \pm 2.2$ (6)	$17.3 \pm 1.8$ (6)	$3.6 \pm 1.6$	$85 \pm 8$
1	$30.6 \pm 2.6$ (6)	$34.0 \pm 3.6$ (6)	$-3.4 \pm 2.5$	$112 \pm 7$
3	$24.5 \pm 2.0$ (6) $P < 0.01$	$42.6 \pm 3.9$ (6)	$-18.1 \pm 2.2$ $P < 0.05$	$174 \pm 7$
5	$24.7 \pm 0.9$ (9) $P < 0.001$	$42.7 \pm 3.2$ (9)	$-18.0 \pm 2.7$	$172 \pm 10$
7	$26.1 \pm 2.8$ (9) $P < 0.01$	$37.2 \pm 2.5$ (9)	$-11.6 \pm 1.6$ $P < 0.05$	$152 \pm 11$
10	$29.4 \pm 2.5$ (6)	$35.3 \pm 2.6$ (6)	$-5.9 \pm 1.2$	$121 \pm 5$
15	$19.2 \pm 2.3$ (6)	$14.0 \pm 1.0$ (6)	$5.3 \pm 1.8$	$76 \pm 6$

Figure 18. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of 6-Phosphogluconate Dehydrogenase Activity of Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.



The response of glutathione reductase concentration is more strange (Table 24 & Fig.19). A slight increase in concentration takes place for the first 3 days, but 2 days later a decrease is observed in the denervated muscles; thereafter the concentration slowly rises reaching normal values by the 10th day, and being 20% in excess by the 15th day after nerve section. In terms of total enzyme in the tissue, the response seems more consistant: a 30% increase in content over the first 3 days is followed by a slight (10%) decrease, but by the 10th day the increase had been regained; after 15 days a slightly lower than normal content of the enzyme is found in the denervated tissue.

Table 24. The effect of unilateral phrenicectomy upon the extractable activity of glutathione reductase of rat hemidiaphragm, expressed as concentration and content.

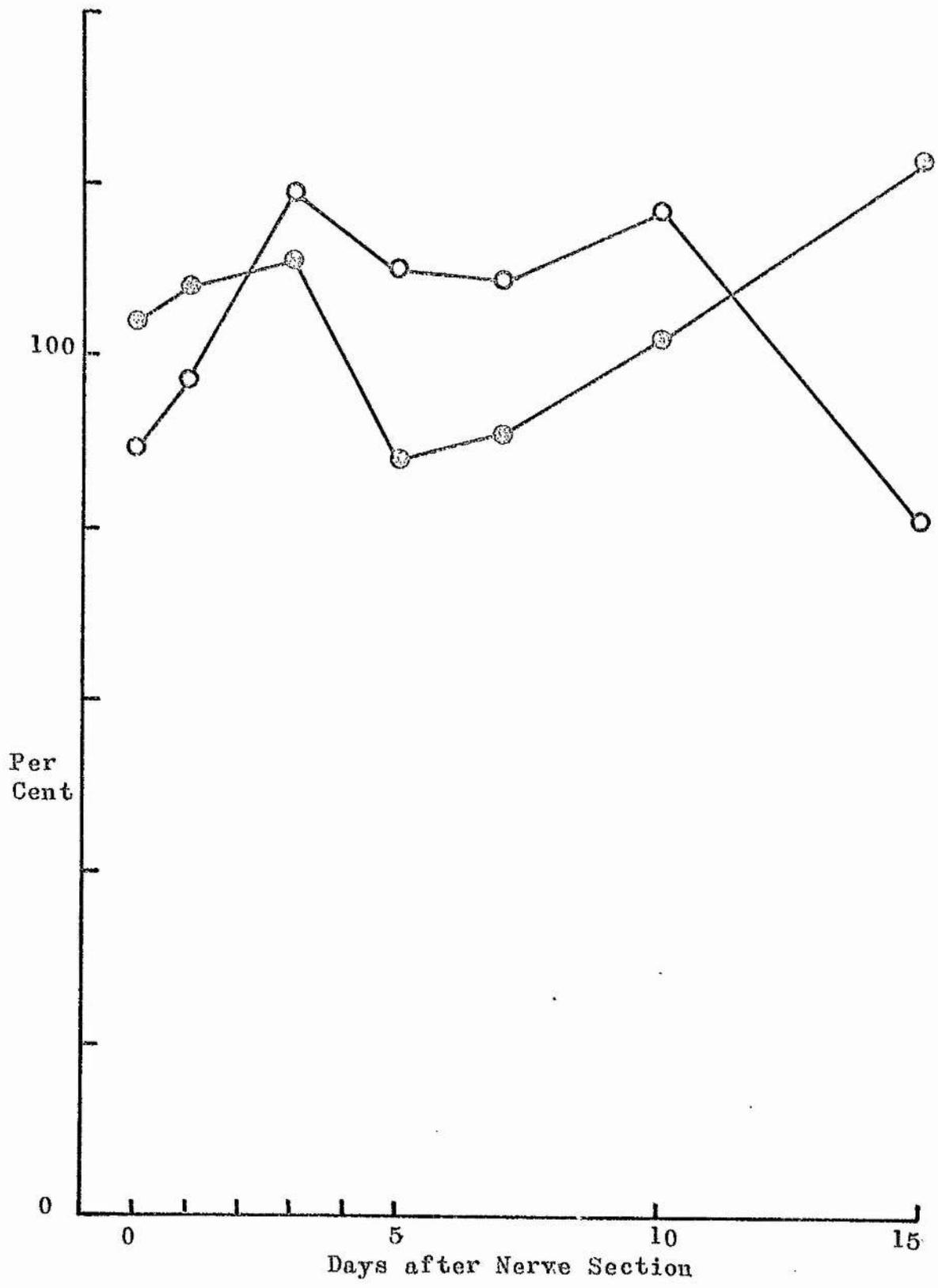
Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

Time (days) elapsed since phrenicectomy	Enzyme Concentration (nmole reduced glutathione/min/mg)			
	Innervated Controls	Denervated Hemidiaphragms	Paired Differences	Percentage
0	640 $\pm$ 26 (6)	664 $\pm$ 23 (6)	- 24 $\pm$ 11	104 $\pm$ 2
1	564 $\pm$ 15 (6)	607 $\pm$ 18 (6)	-43 $\pm$ 22	108 $\pm$ 4
3	614 $\pm$ 27 (6)	675 $\pm$ 21 (6)	- 62 $\pm$ 18	111 $\pm$ 4
5	913 $\pm$ 33 (6)	797 $\pm$ 15 (6)	116 $\pm$ 24	88 $\pm$ 2
	P < 0.05			
7	824 $\pm$ 30 (6)	753 $\pm$ 32 (6)	71 $\pm$ 17	91 $\pm$ 2
10	558 $\pm$ 12 (6)	567 $\pm$ 25 (6)	-9 $\pm$ 21	102 $\pm$ 4
15	688 $\pm$ 25 (6)	844 $\pm$ 28 (6)	-155 $\pm$ 20	123 $\pm$ 3
	P < 0.01		P < 0.05	

Table 24 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content ( $\mu$ mole reduced glutathione/min/hemid.)			
	Innervated Controls	Denervated Hemidiaphragms	Paired Differences	Percentage
0	59.6 $\pm$ 2.8 (6)	53.2 $\pm$ 2.1 (6)	6.4 $\pm$ 1.5	89 $\pm$ 2
1	54.9 $\pm$ 2.0 (6)	53.4 $\pm$ 2.6 (6)	1.5 $\pm$ 1.8	97 $\pm$ 3
3	58.1 $\pm$ 2.7 (6)	69.0 $\pm$ 3.4 (6)	-10.9 $\pm$ 2.8	119 $\pm$ 5
		P < 0.05		
5	78.9 $\pm$ 2.5 (6)	87.8 $\pm$ 2.1 (6)	-8.0 $\pm$ 2.2	110 $\pm$ 3
7	78.5 $\pm$ 4.6 (6)	85.3 $\pm$ 4.9 (6)	-6.8 $\pm$ 1.8	109 $\pm$ 2
10	54.6 $\pm$ 2.1 (6)	63.6 $\pm$ 2.8 (6)	-9.0 $\pm$ 1.6	117 $\pm$ 3
		P < 0.05		
15	65.0 $\pm$ 5.2 (6)	52.4 $\pm$ 4.2 (6)	12.6 $\pm$ 2.1	81 $\pm$ 3

Figure 19. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Glutathione Reductase activity of Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.



Enzymes of Nucleotide Metabolism: Creatine Kinase,  
Adenylate Kinase, Adenylate deaminase.

The extractable activities of the enzymes creatine kinase, adenylate kinase and adenylate deaminase from rat diaphragm are all well within the ranges of values reported for rat skeletal muscles, provided that the pH, temperature and direction of assay are all taken into account (Kendrick-Jones & Perry, 1967; Volfin, 1970; Oliver, 1955, Raggi, Ronca-Testoni & Ronca, 1969). The enzyme activities determined for rat hemidiaphragm do not, however, agree with the scheme of Raggi et al (1969) which proposes that white muscles have higher levels of these enzymes than do red muscles. Table 25 reports the extractable activities of the enzymes obtained from different muscles of the rat hind limb, and clearly demonstrates that the activities found in diaphragm, thought to be a red muscle, are quite comparable to those of known white muscles. In addition, the ratio of activities adenylate kinase/creatine kinase, considered by Golisch, Pette & Pichlmaier (1970) to be a discriminative ratio capable of determining whether a given muscle

Table 25. The extractable activities of the enzymes creatine kinase, adenylate kinase & adenylate deaminase from different muscles of the rat hind limb compared to those obtained for the diaphragm.

With the exception of the figures relating to the enzyme activities for rat diaphragm, which are taken from the values obtained from unoperated rats included in Tables 26, 27 & 28, each value represents the mean of 2 assays, performed in duplicate, of muscle samples pooled from a group of 3 rats. Enzyme activity is expressed as  $\mu\text{mole product formed/min/mg wet weight of muscle}$ , at  $25^{\circ}\text{C}$ .

Muscle	Creatine Kinase	Adenylate Kinase ( $\times 10^{-1}$ )	Adenylate De-aminase ( $\times 10^{-1}$ )
Soleus	0.56	0.51	0.40
Peroneus longus	0.96	0.76	0.36
Plantaris	1.11	0.92	0.77
Extensor Digitorum Longus	1.11	0.99	0.53
Diaphragm	1.13	1.50	0.77
Gastrocnemius (red interior)	1.22	1.11	0.65
Anterior Tibialis (white exterior)	1.37	1.04	0.55

is to be thought of as "fast" or "slow" contracting muscle, is clearly not discriminative when applied to rat hind limb muscles. Probably this lack of an effect is due to the differences in the assay conditions.

The effect of unilateral phrenicectomy on the extractable activities of these enzymes in the diaphragm of the rat is probably best considered by an examination of the changes observed in total content of each enzyme. Content of creatine phosphokinase in the hypertrophying tissue remains constant, for up to 5 days after denervation; it may be concluded that net synthesis of the enzyme does not take place during this time, whilst it seems likely that no loss of the enzyme into the blood occurs. After 5 days the content of this enzyme is seen to fall in a more or less linear fashion, such that by 15 days after denervation, a content of 33% of control values is reached. In contrast, adenylate kinase and adenylate deaminase both demonstrate increases in content in the hypertrophied tissue reaching a maximum between 5 & 7 days after nerve section, the latter enzyme showing

the greatest increase. Net synthesis of these enzymes thus occurs after denervation. Two weeks after nerve section the content of myokinase shows a significant difference between control and denervated tissues, being some 60% lower in the paralysed muscle. Content of AMP deaminase at this time has returned to normal values (Tables 26 - 28, & Figs. 20 - 22).

Table 26. The effect of unilateral phrenicectomy upon the extractable activity of creatine phosphokinase of rat hemidiaphragm, expressed as concentration and content.

Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

Time (days) elapsed since phrenicectomy	Enzyme Concentration ( $\mu$ mole/min/mg wet wt., at 25°C)			
	Innervated Controls	Denervated Hemidiaphragms	Paired Differences	Percentage
0	1.16 $\pm$ 0.04 (6)	1.11 $\pm$ 0.06 (6)	0.05 $\pm$ 0.03	94 $\pm$ 3
1	1.19 $\pm$ 0.03 (6)	1.07 $\pm$ 0.04 (6)	0.12 $\pm$ 0.03	90 $\pm$ 3
3	1.08 $\pm$ 0.03 (6) P < 0.001	0.77 $\pm$ 0.03 (6)	0.30 $\pm$ 0.03 P < 0.01	72 $\pm$ 2
5	1.08 $\pm$ 0.03 (6) P < 0.001	0.75 $\pm$ 0.02 (6)	0.33 $\pm$ 0.02 P < 0.001	70 $\pm$ 1
7	1.18 $\pm$ 0.02 (6) P < 0.001	0.68 $\pm$ 0.01 (6)	0.50 $\pm$ 0.02 P < 0.001	58 $\pm$ 1
10	1.18 $\pm$ 0.05 (6) P < 0.001	0.63 $\pm$ 0.02 (6)	0.55 $\pm$ 0.04 P < 0.01	54 $\pm$ 2
15	1.07 $\pm$ 0.05 (6) P < 0.001	0.50 $\pm$ 0.03 (6)	0.57 $\pm$ 0.04 P < 0.01	47 $\pm$ 2

Table 26 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content (umole/min/hemidiaphragm, at 25 <sup>o</sup> C)			
	Innervated Controls	Denervated Hemidiaphragms	Paired Differences	Percentage
0	108 ± 7 (6)	92 ± 7 (6)	15 ± 4	86 ± 4
1	111 ± 7 (6)	97 ± 9 (6)	13 ± 4	87 ± 4
3	100 ± 2 (6) P < 0.001	85 ± 2 (6)	15 ± 2 P < 0.05	85 ± 2
5	99 ± 4 (6) P < 0.05	84 ± 3 (6)	16 ± 2 P < 0.01	84 ± 1
7	110 ± 4 (6) P < 0.001	85 ± 3 (6)	26 ± 6	77 ± 4
10	119 ± 9 (6) P < 0.01	74 ± 4 (6)	45 ± 6 P < 0.05	63 ± 2
15	96 ± 6 (6) P < 0.001	31 ± 2 (6)	65 ± 7 P < 0.05	33 ± 4

Figure 20. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Creatine Kinase in the Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.

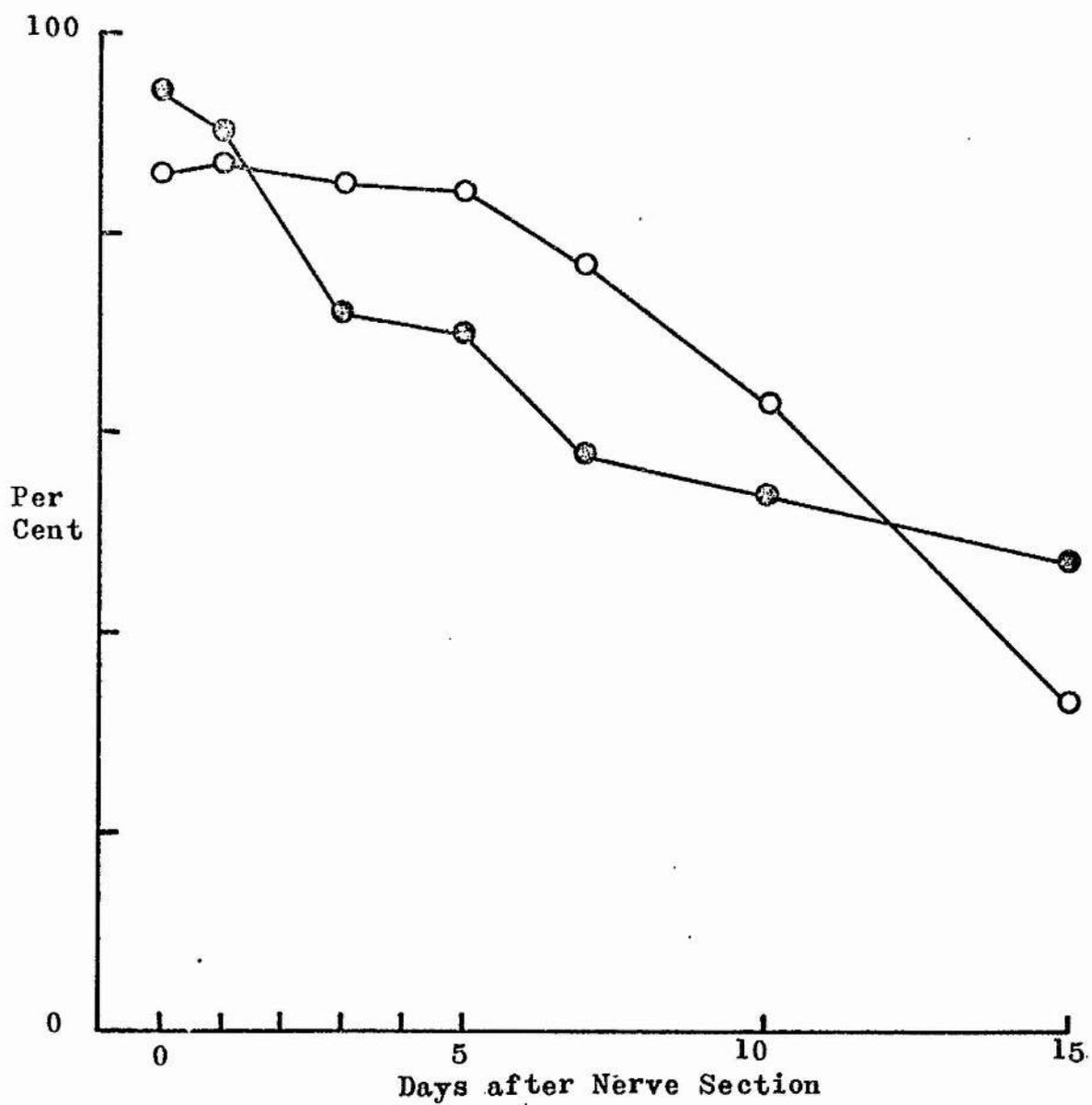


Table 27. The effect of unilateral phrenicectomy upon the extractable activity of adenylyate kinase of rat hemidiaphragm, expressed in terms of concentration and of content.

Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

Time (days) elapsed since phrenicectomy	Enzyme Concentration (umole/min/mg wet wt., at 25 <sup>o</sup> C)			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	155 $\pm$ 29 (6)	146 $\pm$ 29 (6)	9 $\pm$ 7	95 $\pm$ 4
1	152 $\pm$ 30 (6)	142 $\pm$ 29 (6)	10 $\pm$ 3	92 $\pm$ 2
3	210 $\pm$ 14 (6)	167 $\pm$ 13 (6)	43 $\pm$ 3 P < 0.01	79 $\pm$ 2
5	166 $\pm$ 27 (6)	133 $\pm$ 22 (6)	33 $\pm$ 5	79 $\pm$ 1
7	146 $\pm$ 15 (6)	105 $\pm$ 11 (6)	41 $\pm$ 5 P < 0.05	72 $\pm$ 2
10	167 $\pm$ 12 (6) P < 0.05	116 $\pm$ 9 (6)	51 $\pm$ 4 P < 0.01	69 $\pm$ 1
15	151 $\pm$ 30 (6)	85 $\pm$ 18 (6)	65 $\pm$ 12	56 $\pm$ 1

Table 27 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content ( $\mu\text{mole}/\text{min}/\text{hemidiaphragm}$ , at $25^{\circ}\text{C}$ )			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	$14.4 \pm 2.9$ (6)	$12.1 \pm 2.5$ (6)	$2.3 \pm 0.8$	$85 \pm 5$
1	$13.8 \pm 2.4$ (6)	$12.5 \pm 2.3$ (6)	$1.3 \pm 0.3$	$89 \pm 3$
3	$19.6 \pm 1.4$ (6)	$18.5 \pm 1.5$ (6)	$1.1 \pm 0.3$	$94 \pm 2$
5	$15.0 \pm 2.1$ (6)	$14.4 \pm 2.2$ (6)	$0.6 \pm 0.2$	$96 \pm 1$
7	$13.8 \pm 1.5$ (6)	$13.0 \pm 1.3$ (6)	$0.8 \pm 0.5$	$94 \pm 3$
10	$16.8 \pm 1.8$ (6)	$13.8 \pm 1.7$ (6)	$3.0 \pm 0.6$	$82 \pm 3$
15	$12.9 \pm 1.9$ (6)	$5.1 \pm 1.0$ (6)	$7.8 \pm 1.2$	$39 \pm 3$

$P < 0.05$

Figure 21. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Adenylate Kinase Activity of Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.

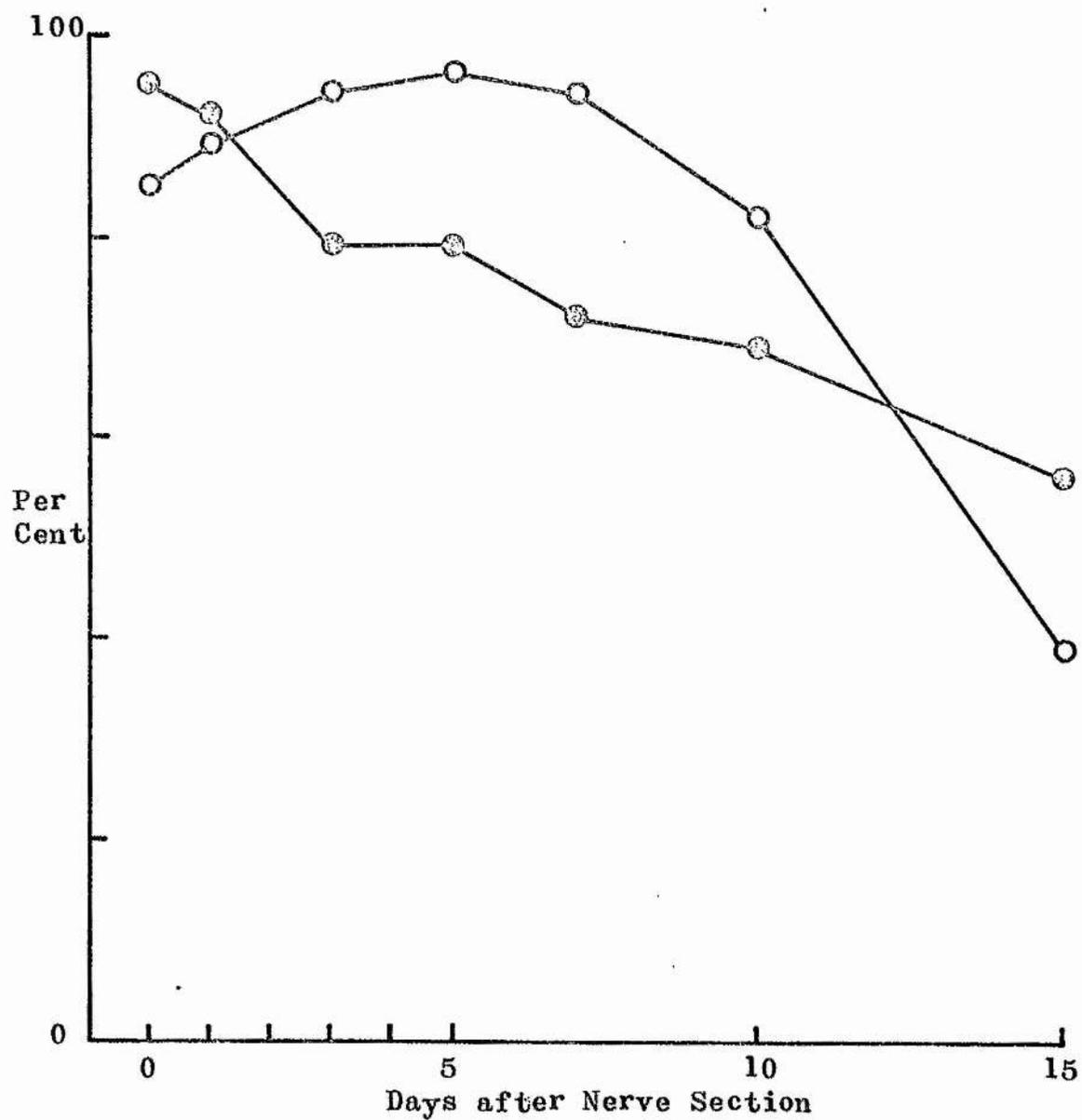


Table 28. The effect of unilateral phrenicectomy upon the extractable activity of adenylic acid deaminase in the rat hemidiaphragm, expressed as concentration and as content.

Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

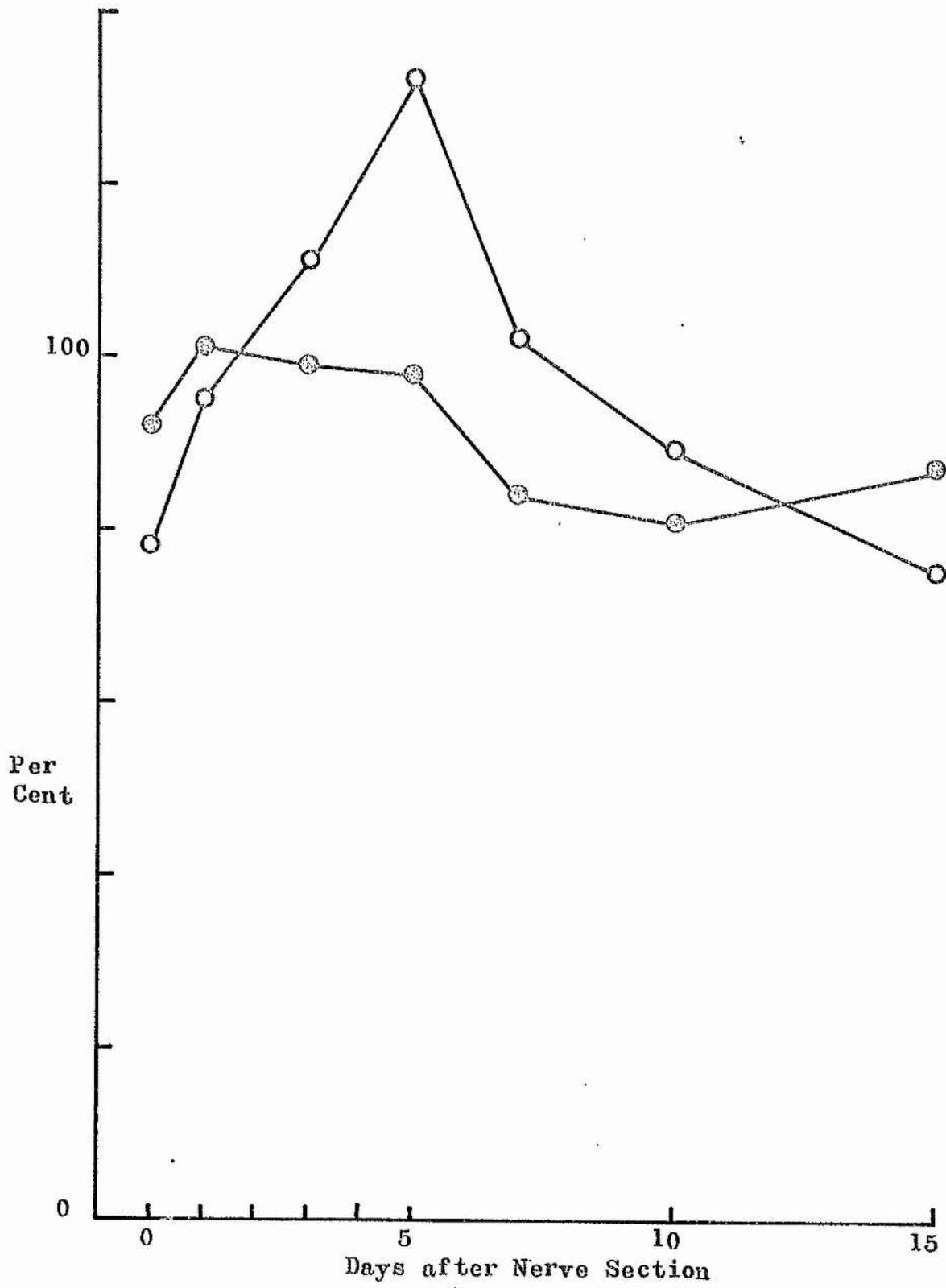
Time (days) elapsed since phrenicectomy	Enzyme Concentration (nmole/min/mg wet wt., at 25°C)			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	79.5 $\pm$ 3.5 (12)	73.8 $\pm$ 4.7 (12)	5.7 $\pm$ 1.8	92 $\pm$ 2
1	64.6 $\pm$ 4.6 (12)	65.3 $\pm$ 4.7 (12)	-0.8 $\pm$ 2.6	101 $\pm$ 4
3	63.6 $\pm$ 3.6 (11)	62.0 $\pm$ 3.9 (11)	1.6 $\pm$ 4.5	99 $\pm$ 7
5	58.4 $\pm$ 2.4 (11)	56.3 $\pm$ 1.9 (11)	2.1 $\pm$ 2.7	98 $\pm$ 5
7	76.0 $\pm$ 4.4 (11)	63.7 $\pm$ 3.6 (11)	12.3 $\pm$ 2.4	84 $\pm$ 3
10	79.9 $\pm$ 3.0 (12)	64.3 $\pm$ 2.7 (12)	16.3 $\pm$ 2.4	81 $\pm$ 3
		P < 0.01		
15	74.8 $\pm$ 3.5 (12)	63.8 $\pm$ 1.9 (12)	11.1 $\pm$ 3.0	87 $\pm$ 4
		P < 0.05		

Table 28 continued.

Time (days) elapsed since phrenicectomy	Enzyme Content ( $\mu\text{mole}/\text{min}/\text{hemidiaphragm}$ , at $25^{\circ}\text{C}$ )			
	Innervated controls	Denervated hemidiaphragms	Paired differences	Percentage
0	$7.32 \pm 0.42$ (12)	$5.79 \pm 0.43$ (12)	$1.53 \pm 0.19$	$78 \pm 3$
		$P < 0.05$	$P < 0.05$	
1	$5.61 \pm 0.46$ (12)	$5.28 \pm 0.41$ (12)	$0.33 \pm 0.25$	$95 \pm 4$
3	$5.88 \pm 0.43$ (11)	$6.22 \pm 0.40$ (11)	$-0.34 \pm 0.53$	$110 \pm 10$
5	$5.00 \pm 0.27$ (11)	$6.44 \pm 0.22$ (11)	$-1.44 \pm 0.27$	$131 \pm 6$
		$P < 0.001$		
7	$6.69 \pm 0.54$ (11)	$6.83 \pm 0.56$ (11)	$-0.14 \pm 0.21$	$102 \pm 4$
10	$8.13 \pm 0.36$ (12)	$7.22 \pm 0.35$ (12)	$0.91 \pm 0.26$	$89 \pm 3$
15	$8.12 \pm 0.53$ (12)	$6.03 \pm 0.44$ (12)	$2.09 \pm 0.33$	$75 \pm 4$
		$P < 0.01$		

4. 3/1

Figure 22. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of AMP Deaminase activity of the Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.



Myoglobin. The concentration of myoglobin found in unoperated rats in this study compare favourably with the values reported for rat diaphragm by Burleigh & Schimke (1969), providing allowance is made for the age of the rat at the time of assay. Phrenic nerve section has the effect of causing a very gradual increase in content of the pigment in the denervated muscle reaching a peak of 120% of control values at 10 days, and thereafter declining. Myoglobin concentration decreases in the paralysed muscle within 3 days of nerve section; after 5 days however, the concentration rises such that by the 10th day normal values are regained and by the 15th day, a 20% increase is found (Table 29. & Fig. 23).

Table 29. The effect of unilateral phrenicectomy upon the concentration and content of myoglobin in the rat hemidiaphragm.

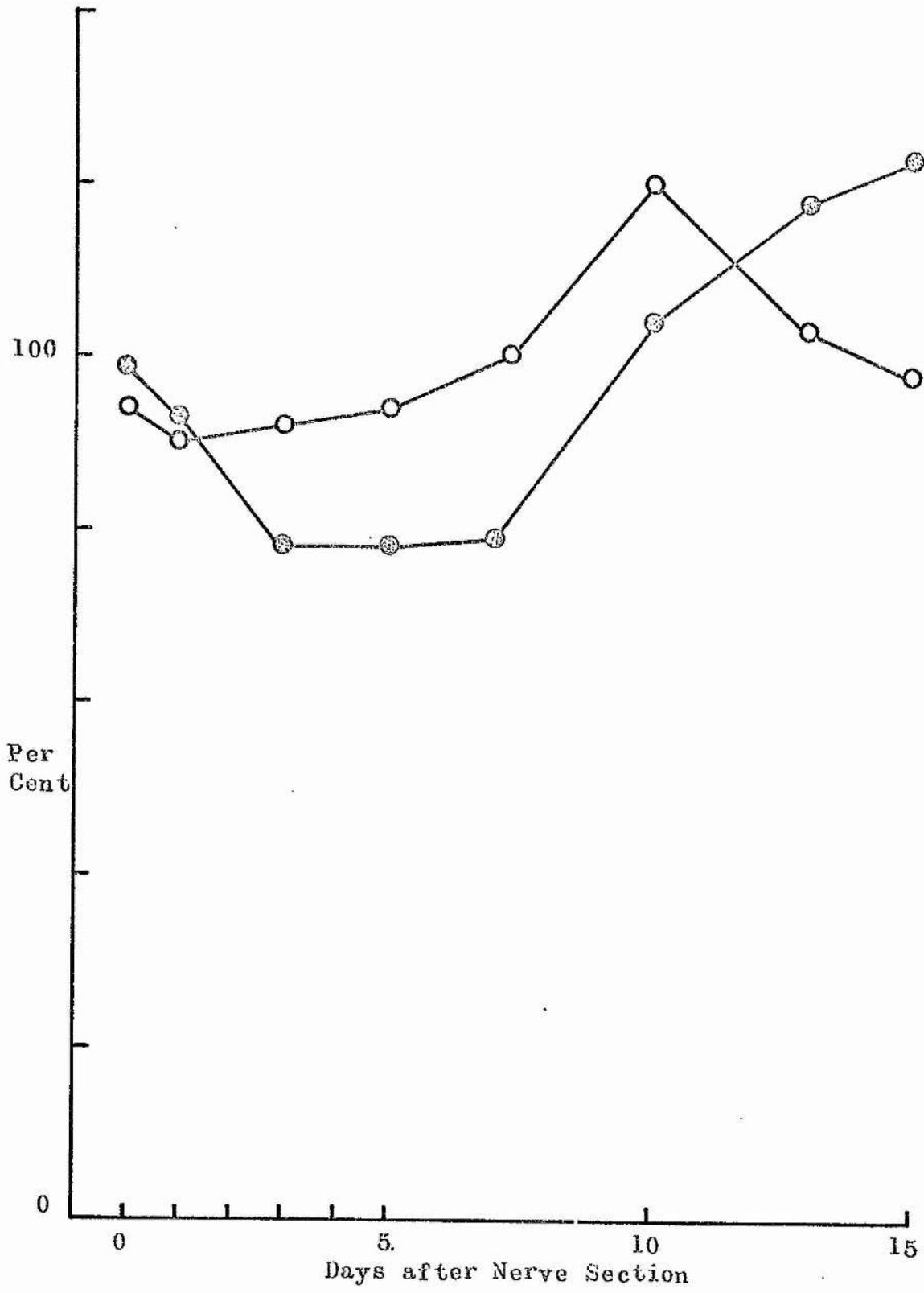
Each value is the mean  $\pm$  S.E.M. of the number of observations shown in parenthesis.

Time (days) elapsed since phrenicectomy	Myoglobin Concentration (mgm/gm wet wt.)			Percentage
	Innervated controls	Denervated hemidiaphragms	Paired Differences	
0	1.79 $\pm$ 0.05 (11)	1.76 $\pm$ 0.03 (11)	0.03 $\pm$ 0.04	99 $\pm$ 2
1	1.75 $\pm$ 0.07 (11)	1.62 $\pm$ 0.08 (11)	0.15 $\pm$ 0.08	93 $\pm$ 4
3	1.69 $\pm$ 0.08 (11)	1.30 $\pm$ 0.08 (11)	0.43 $\pm$ 0.06	78 $\pm$ 5
		P < 0.01		
5	1.79 $\pm$ 0.05 (8)	1.38 $\pm$ 0.04 (8)	0.41 $\pm$ 0.07	77 $\pm$ 3
		P < 0.001		
7	1.72 $\pm$ 0.10 (10)	1.34 $\pm$ 0.08 (10)	0.38 $\pm$ 0.08	79 $\pm$ 4
		P < 0.01		
10	1.62 $\pm$ 0.08 (9)	1.68 $\pm$ 0.11 (9)	-0.06 $\pm$ 0.08	104 $\pm$ 5
13	2.32 $\pm$ 0.15 (8)	2.71 $\pm$ 0.15 (8)	-0.39 $\pm$ 0.14	118 $\pm$ 6
15	1.85 $\pm$ 0.13 (11)	2.19 $\pm$ 0.09 (11)	-0.29 $\pm$ 0.15	123 $\pm$ 8
		P < 0.05		

Table 29 continued.

Time (days) elapsed since phrenicectomy	Myoglobin Content ( $\mu\text{g}/\text{hemidiaphragm}$ )			Percentage
	Innervated controls	Denervated hemidiaphragms	Paired differences	
0	156 $\pm$ 5 (11)	145 $\pm$ 5 (11)	10 $\pm$ 5	94 $\pm$ 3
1	157 $\pm$ 9 (11)	139 $\pm$ 9 (11)	18 $\pm$ 8	90 $\pm$ 5
3	150 $\pm$ 9 (11)	136 $\pm$ 9 (11)	14 $\pm$ 7	92 $\pm$ 6
5	167 $\pm$ 8 (8)	154 $\pm$ 8 (8)	13 $\pm$ 11	94 $\pm$ 6
7	156 $\pm$ 12 (10)	153 $\pm$ 12 (10)	3 $\pm$ 11	100 $\pm$ 6
10	143 $\pm$ 8 (9)	163 $\pm$ 9 (9)	-20 $\pm$ 10	120 $\pm$ 8
13	247 $\pm$ 19 (8)	248 $\pm$ 18 (8)	1 $\pm$ 16	103 $\pm$ 8
15	184 $\pm$ 16 (11)	169 $\pm$ 7 (11)	15 $\pm$ 16	98 $\pm$ 8

Figure 23. The Effect of Unilateral Phrenicectomy upon the Concentration (—●—) and Content (—○—) of Myoglobin in the Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.



Amino Acid Analysis. Table 30 records the content and concentration of ninhydrin reacting material of low molecular weight that may be extracted from the hemidiaphragm of the rat. It will be seen that some 40% of the ninhydrin-positive material of unoperated rat muscle is taurine, the oxidation product of cysteine metabolism; the function of this compound in the metabolism of muscle is still obscure. A further 40% of the material is represented by the amino acids alanine, aspartate, glutamate, glutamine, glycine and serine; since studies in this department have shown that asparagine is eluted concurrently with serine it is possible that the high concentration of serine reported here is due to the presence of asparagine. Scharff & Wool (1964), have similarly observed high serine concentrations in rat diaphragm muscle that they attributed to an "as yet unidentified substance of similar chromatographic properties". Unlike these authors though this present study records considerable lower levels of ammonia whilst it is also found that there is an appreciable concentration of glutamine (and by implication asparagine) in the tissue; precautions were taken during the isolation procedure to minimize the degradation of glutamine, and it is likely that the improved

technique accounts for this. By and large, the present amino acid levels compare favourably with those reported by Scharff & Wool, in spite of the doubt as to whether their values can be taken as representing the in vivo concentration; the preliminary soaking in ice cold buffer that these authors gave the excised tissue would be expected to bring about leakage of the amino acids from the muscle cells, since the maintenance of the amino acid gradients is known to be energy dependent and therefore sensitive to temperature. In order to minimize such leakage effects, the tissues used in this study were not rinsed after removal from the animal; instead, particular care was taken to prevent contamination of the tissue by blood, and the hemidiaphragm was merely blotted on filter paper prior to homogenization.

The effects of denervation upon the concentration of free amino acids are probably best considered by collecting together groups of similar amino acids, or those showing similar responses; it must of course be remembered that this grouping does not necessarily imply a functional relationship.

Table 30. The effect of unilateral phrenicectomy upon the concentration and content of free amino acids and other acid soluble ninhydrin reacting substances in the rat hemidiaphragm.

The values were determined using extracts pooled from 5 or 6 animals for each time interval after denervation as described in the methods section, and were calculated assuming a tissue water content of 75 % of the wet weight.

Substance	Days after Denervation	Concentration ( $\mu$ mole/100 ml)		Content (mmole/hemidiaphragm)	
		Control	Denervated	Control	Denervated
Alanine	0	386	441	30.4	33.3
	1	457	431	43.1	41.3
	3	472	699	43.5	75.3
	7	374	469	30.9	51.4
	10	355	379	28.2	38.4
	15	537	410	50.0	29.0
Ammonia	0	484	587	38.1	44.3
	1	423	286	39.9	27.4
	3	387	293	35.6	31.6
	7	463	303	38.2	33.2
	10	485	308	38.6	31.2
	15	522	436	48.6	30.9
Arginine	0	40.4	37.5	3.18	2.83
	1	35.4	27.3	3.34	2.61
	3	31.5	26.9	2.90	2.90
	7	34.9	47.6	2.88	5.22
	10	54.1	61.7	4.30	6.26
	15	30.5	43.7	2.84	3.09

Table 30 continued.

Substance	Days after Denervation	Concentration ( $\mu$ mole/100 ml)		Content (mmole/hemidiaphragm)	
		Control	Denervated	Control	Denervated
Aspartate	0	199	195	15.7	14.7
	1	275	192	25.9	18.4
	3	199	224	18.3	24.1
	7	256	170	21.1	18.6
	10	283	195	22.5	19.8
	15	346	197	32.2	13.9
Carnosine	0	10.1	8.3	0.796	0.627
	1	9.4	8.7	0.836	0.833
	3	11.1	7.2	1.022	0.775
	7	9.6	7.7	0.793	0.845
	10	12.5	9.2	0.994	0.933
	15	23.1	12.6	2.15	0.892
Citrulline	0	68.1	71.4	5.37	5.39
	1	51.8	54.5	4.88	5.22
	3	51.6	64.6	4.75	6.96
	7	72.2	68.5	5.96	7.51
	10	61.3	53.1	4.87	5.38
	15	75.8	58.1	7.06	4.11
Ethanolamine	0	0.6	0.6	0.047	0.045
	1	1.6	0.4	0.151	0.038
	3	0.4	0.5	0.037	0.054
	7	0.3	0.8	0.025	0.088
	10	0.5	1.1	0.040	0.111
	15	1.0	0.9	0.093	0.064

Table 30 continued.

Substance	Days after Denervation	Concentration ( $\mu\text{mole}/100\text{ ml}$ )		Content ( $\text{mmole}/\text{hemidiaphragm}$ )	
		Control	Denervated	Control	Denervated
Glutamate	0	431	452	34.0	34.1
	1	448	330	42.2	31.6
	3	424	540	39.0	58.2
	7	482	457	39.8	50.1
	10	401	441	31.9	44.7
	15	591	439	55.0	31.1
Glutamine	0	405	406	31.9	30.6
	1	605	708	57.0	67.8
	3	658	1070	60.6	115.0
	7	705	1330	58.2	146.0
	10	697	969	55.4	98.3
	15	578	971	53.8	68.7
Glutathione (reduced)	0	5.7	5.2	0.449	0.393
	1	9.7	11.1	0.915	1.063
	3	10.4	13.7	0.968	1.475
	7	12.5	16.3	1.032	1.788
	10	11.3	17.5	0.898	1.774
	15	10.8	11.4	1.005	0.807
Glycine	0	271	284	21.3	21.4
	1	288	321	27.2	30.7
	3	336	551	30.9	59.3
	7	290	405	23.9	44.4
	10	305	407	24.2	41.3
	15	268	344	24.9	24.3

Table 30 continued.

Substance	Days after Denervation	Concentration ( $\mu$ mole/100 ml)		Content (nmole/hemidiaphragm)	
		Control	Denervated	Control	Denervated
Histidine	0	47.6	54.4	3.75	4.11
	1	63.6	36.8	6.00	3.52
	3	64.6	37.4	5.95	4.03
	7	75.1	50.9	6.20	5.58
	10	72.4	45.6	5.75	4.63
	15	68.0	60.8	6.33	4.30
Isoleucine	0	10.5	8.2	0.827	0.619
	1	6.8	12.6	0.641	1.207
	3	8.8	5.2	0.810	0.560
	7	9.4	10.1	0.776	1.108
	10	10.5	8.6	0.835	0.872
	15	14.1	15.5	1.313	1.100
Leucine	0	15.2	16.2	1.20	1.22
	1	12.9	21.6	1.22	2.07
	3	14.4	19.5	1.33	2.10
	7	15.0	17.9	1.24	1.96
	10	15.7	19.2	1.25	1.95
	15	20.7	25.1	1.93	1.78
Lysine	0	71.9	69.5	5.67	5.25
	1	76.3	53.2	7.19	5.10
	3	34.9	30.2	3.21	3.25
	7	47.3	58.9	3.91	6.46
	10	173	205	13.7	20.8
	15	57.9	83.1	5.39	5.88

Substance	Days after Denervation	Concentration ( $\mu$ mole/100 ml)		Content (mmole/hemidiaphragm)	
		Control	Denervated	Control	Denervated
Methionine	0	14.3	11.2	1.13	0.85
	1	15.4	19.3	1.45	1.85
	3	10.0	13.8	0.92	1.49
	7	14.9	12.6	1.23	1.38
	10	13.9	14.7	1.10	1.49
	15	10.6	13.1	0.99	0.93
Ornithine	0	13.7	13.4	1.08	1.01
	1	12.3	8.2	1.16	0.79
	3	13.6	16.8	1.25	1.81
	7	10.8	16.2	0.89	1.78
	10	13.1	18.0	1.04	1.82
	15	8.7	18.2	0.81	1.29
Phenylalanine	0	15.1	15.4	1.19	1.16
	1	16.3	16.2	1.54	1.55
	3	43.6	15.5	4.02	1.67
	7	10.5	17.3	0.87	1.90
	10	9.8	15.1	0.78	1.53
	15	13.4	19.5	1.25	1.38
o-Phospho- ethanolamine	0	5.8	5.6	0.457	0.423
	1	5.4	7.5	0.509	0.718
	3	3.3	8.0	0.304	0.862
	7	5.2	6.8	0.429	0.746
	10	6.6	8.3	0.525	0.842
	15	4.7	8.0	0.438	0.566

Table 30 continued.

Substance	Days after Denervation	Concentration ( $\mu$ mole/100 ml)		Content (mmole/hemidiaphragm)	
		Control	Denervated	Control	Denervated
Serine	0	228	266	18.0	20.1
	1	232	270	21.9	25.9
	3	225	353	20.7	38.0
	7	227	416	18.7	45.6
	10	300	434	23.8	44.0
	15	212	269	19.7	19.0
	Taurine	0	2020	1980	159
1		1770	1490	167	143
3		1610	1860	148	200
7		1940	2110	160	231
10		1920	2570	153	261
15		1600	2240	149	159
Threonine		0	57.9	57.2	4.56
	1	74.3	76.1	7.01	7.29
	3	42.1	43.1	3.88	4.64
	7	56.7	51.5	4.68	5.65
	10	113	96.5	8.98	9.78
	15	42.2	43.6	3.93	3.09
	Tyrosine	0	18.9	17.9	1.49
1		19.0	19.6	1.79	1.88
3		16.3	24.1	1.50	2.60
7		16.3	18.9	1.35	2.07
10		14.6	17.7	1.16	1.79
15		20.0	23.2	1.86	1.64

Table 30 continued.

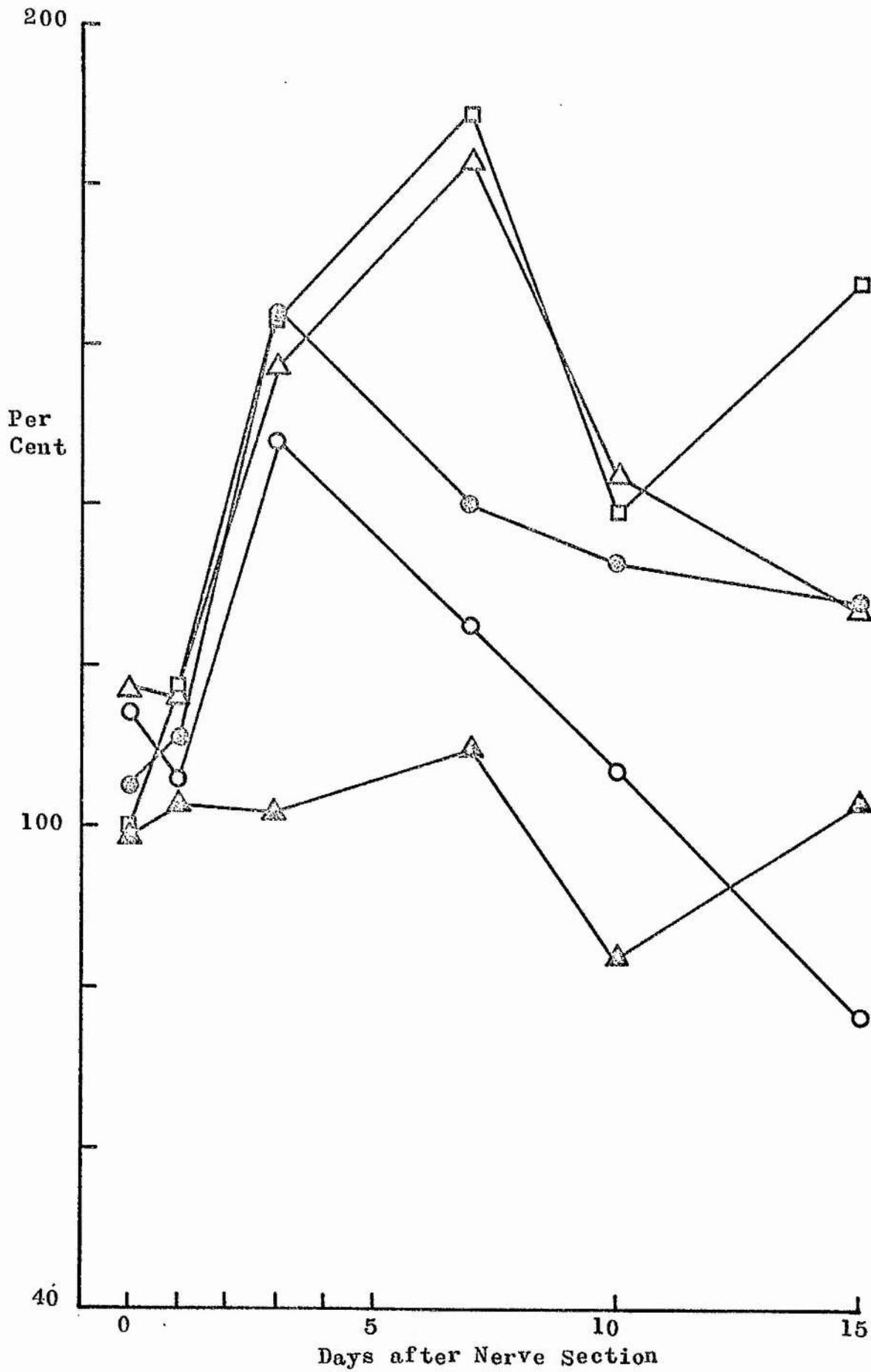
Substance	Days after Denervation	Concentration ( $\mu$ mole/100 ml)		Content (mmole/hemidiaphragm)	
		Control	Denervated	Control	Denervated
Valine	0	18.9	16.6	1.49	1.25
	1	16.9	18.8	1.59	1.80
	3	18.2	21.3	1.68	2.29
	7	18.3	20.7	1.51	2.27
	10	20.8	19.6	1.65	1.99
	15	23.4	27.4	2.18	1.94

(i) Glycine, alanine, glutamine, serine/asparagine, threonine: (Fig. 24). With the exception of threonine, unilateral phrenicectomy causes a rapid and pronounced increase in concentration, reaching a peak between 3 and 7 days and then falling, though in the case of glutamine a secondary increase is also found. The similarity between the response for glutamine and serine lends weight to the supposition that the latter compound may have included an appreciable proportion of asparagine, as one might think that the two amines, glutamine and asparagine, would behave similarly. Threonine, which might be expected to duplicate the serine response, shows no such dramatic changes. It is conceivable that the true serine response is masked.

(ii) Aspartate, glutamate: (Fig. 25). An initial decrease 1 day after denervation is followed by an increase to a peak at three days, with a second smaller peak occurring at 10 days.

(iii) Arginine, lysine, histidine, ornithine & citrulline: (Fig. 26). With the exception of citrulline, the response of the basic amino acids is essentially similar: an initial decrease 1 day after denervation is

Figure 24. The Effect of Unilateral Phrenicectomy upon the Concentration of Free Amino Acids in the Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values. Key: Glycine (—●—); Alanine (—○—); Threonine (—▲—); Serine (—△—); Glutamine (—□—).



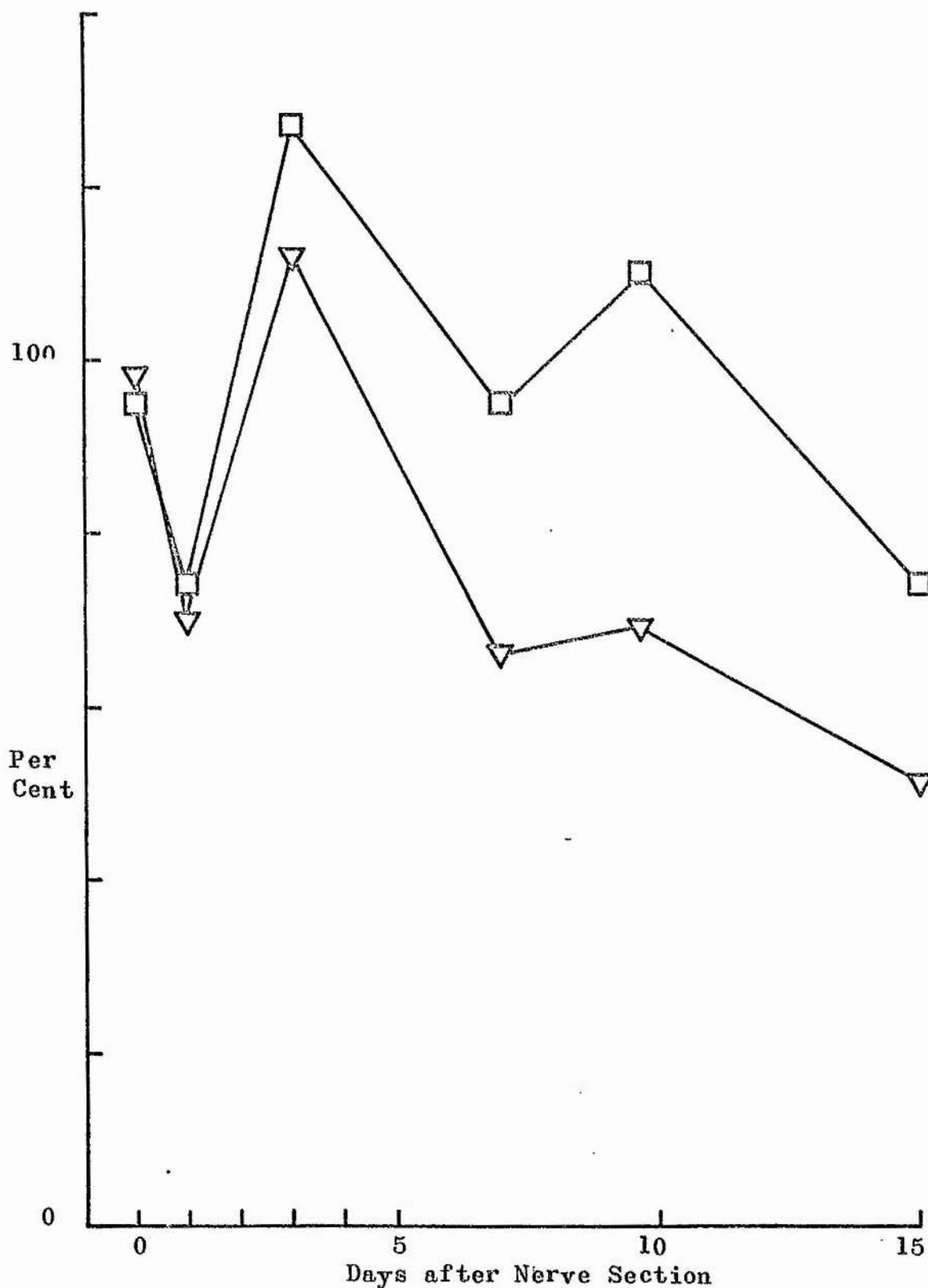


Figure 25. The Effect of Unilateral Phrenicectomy upon the Concentration of Free Glutamate (—□—) and Aspartate (—▽—) in the Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values

followed by an increase to a peak at 7 days, which, after a slight fall, is followed by a further rise. In the case of histidine this secondary increase is almost sufficient to regain the control value, whilst in the case of arginine, lysine and ornithine the concentration in the denervated tissue may be appreciably greater than in the control tissue. Citrulline is atypical in showing an initial increase, followed by a decrease to below control values, (Fig 28).

(vi) Valine, leucine & isoleucine: (Fig.27). With similar structures and being known to undergo similar processes of metabolism, it might be thought that the branched chain amino acids would at least demonstrate similar responses: this is not the case though. Whilst each shows an initial increase, the response thereafter bears no resemblance. For valine the increase is, but for one point, maintained; for leucine a decrease to a plateau concentration some 20% above control values, and for isoleucine erratic fluctuations about the initial control level, with the impression that the fluctuations are dampening down with time.

Figure 26. The Effect of Unilateral Phrenicectomy upon the Concentrations of Free Amino Acids in the Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values. Key: Lysine (—●—); Histidine (—○—); Arginine (—△—); Ornithine (—□—).

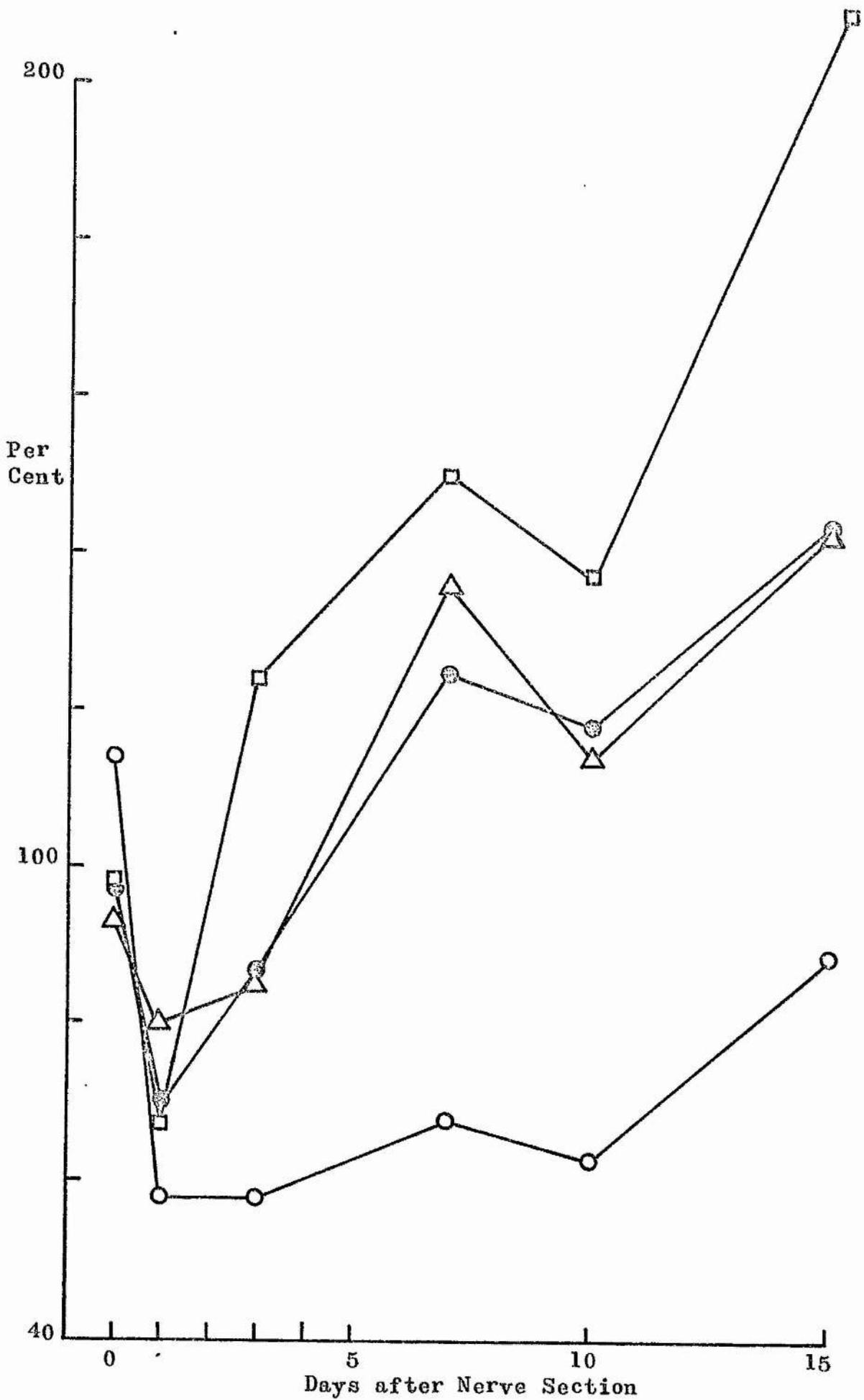
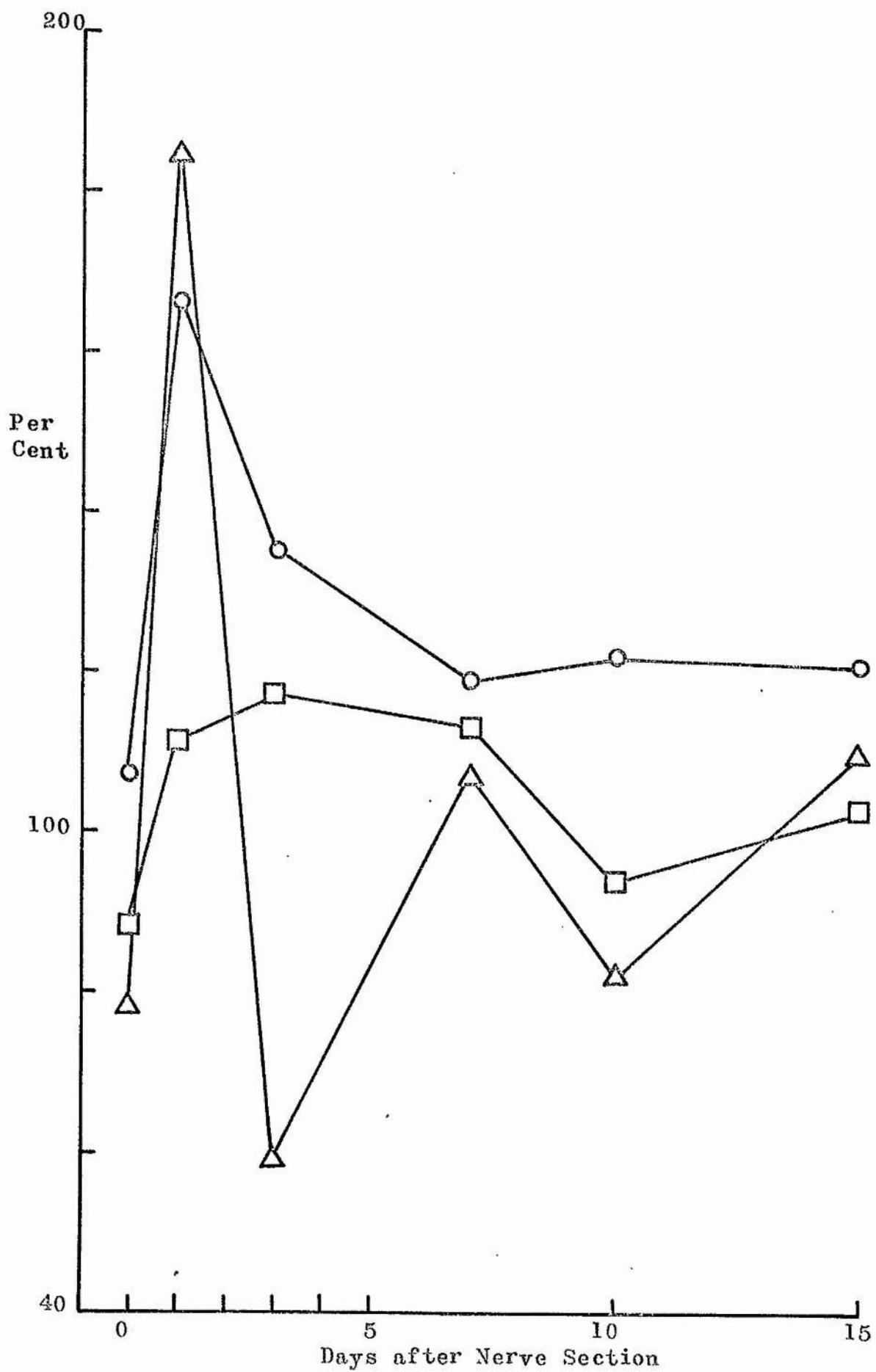


Figure 27. The Effect of Unilateral Phrenicectomy upon the Concentrations of Free Valine (—□—), Leucine (—○—), & Isoleucine (—△—) in the Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.



(v) Tyrosine, phenylalanine: (Fig. 28). Whilst tyrosine demonstrates an early increase in concentration, this is probably masked in the case of phenylalanine, due to an abnormally high control value at 3 days after nerve section. Each amino acid demonstrates an increased concentration after 7 days have elapsed.

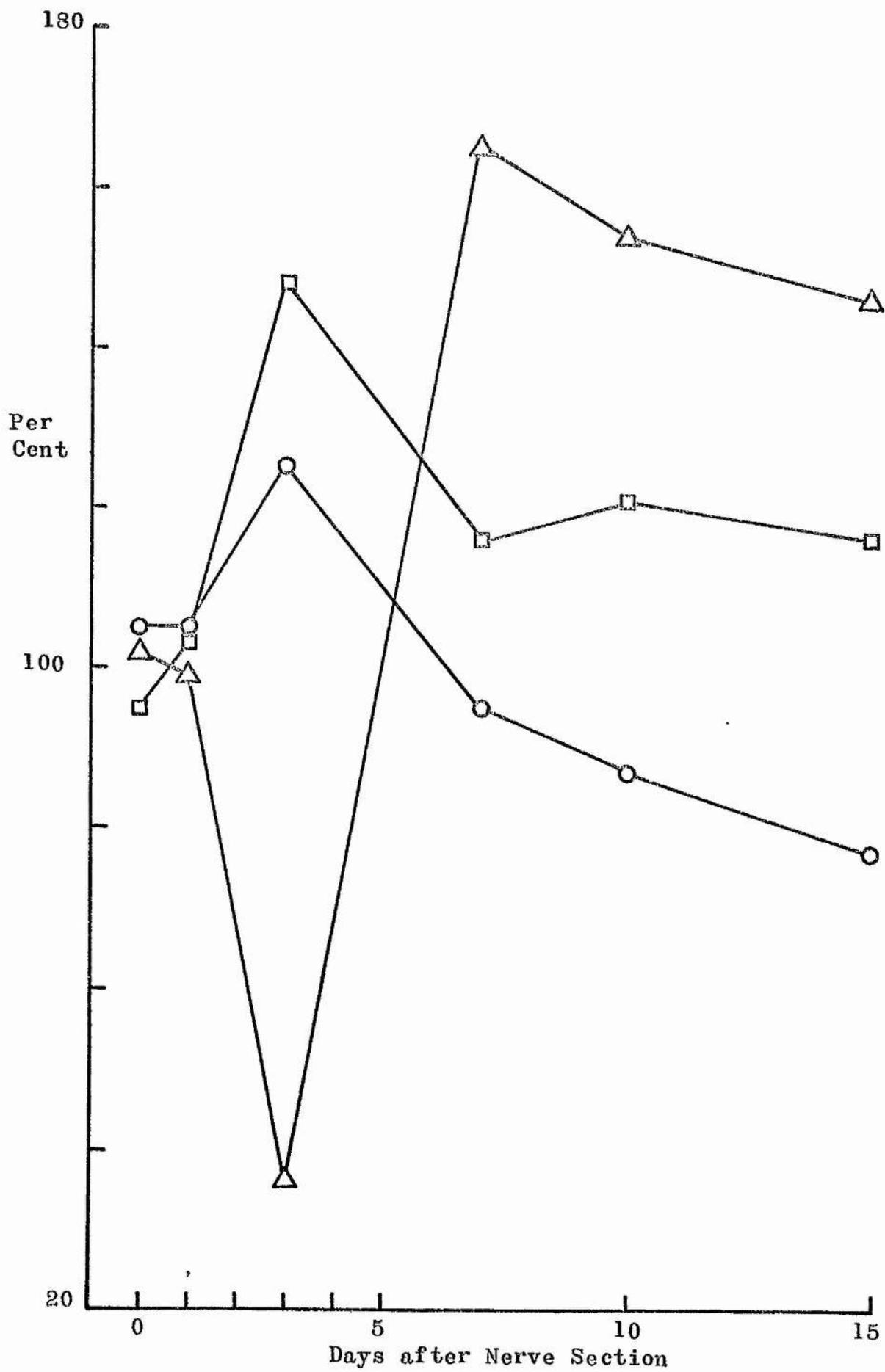
(vi) Methionine: (Fig. 29). The initial increase is restored to normal by 7 days after which there follows a slower increase.

(vii) Other ninhydrin reacting substances: ammonia, carnosine, taurine, ethanolamine, phosphoethanolamine & reduced glutathione. (Figs. 29 - 31). Ammonia and carnosine essentially show decreases on denervation, whilst ethanolamine and taurine demonstrate increases. O-Phosphoethanolamine shows a biphasic response: a peak of nearly 250% of control values at 3 days falls to approach the normal level at ten days, but then rises again. The similarity of the response to that of serine and glycine, its possible precursors, is noted. Reduced glutathione demonstrates a progressive increase in concentration throughout the first 10 days and is then

followed by a massive decline. Oxidized glutathione was not found except for a very small quantity in the extract from 3 day denervated tissues; assuming a color yield equal to twice that for reduced glutathione, the concentration was calculated to be 0.2  $\mu$ moles/100 ml tissue water for the oxidized compound.

Overall the concentration of ninhydrin reacting, acid soluble material rises on denervation reaching a peak at 3 days and thereafter declining (Fig. 32).

Figure 28. The Effect of Unilateral Phrenicectomy upon the Concentration of Free Phenylalanine ( $\text{---}\Delta\text{---}$ ), Tyrosine ( $\text{---}\square\text{---}$ ), and Citrulline ( $\text{---}\circ\text{---}$ ) in the Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values



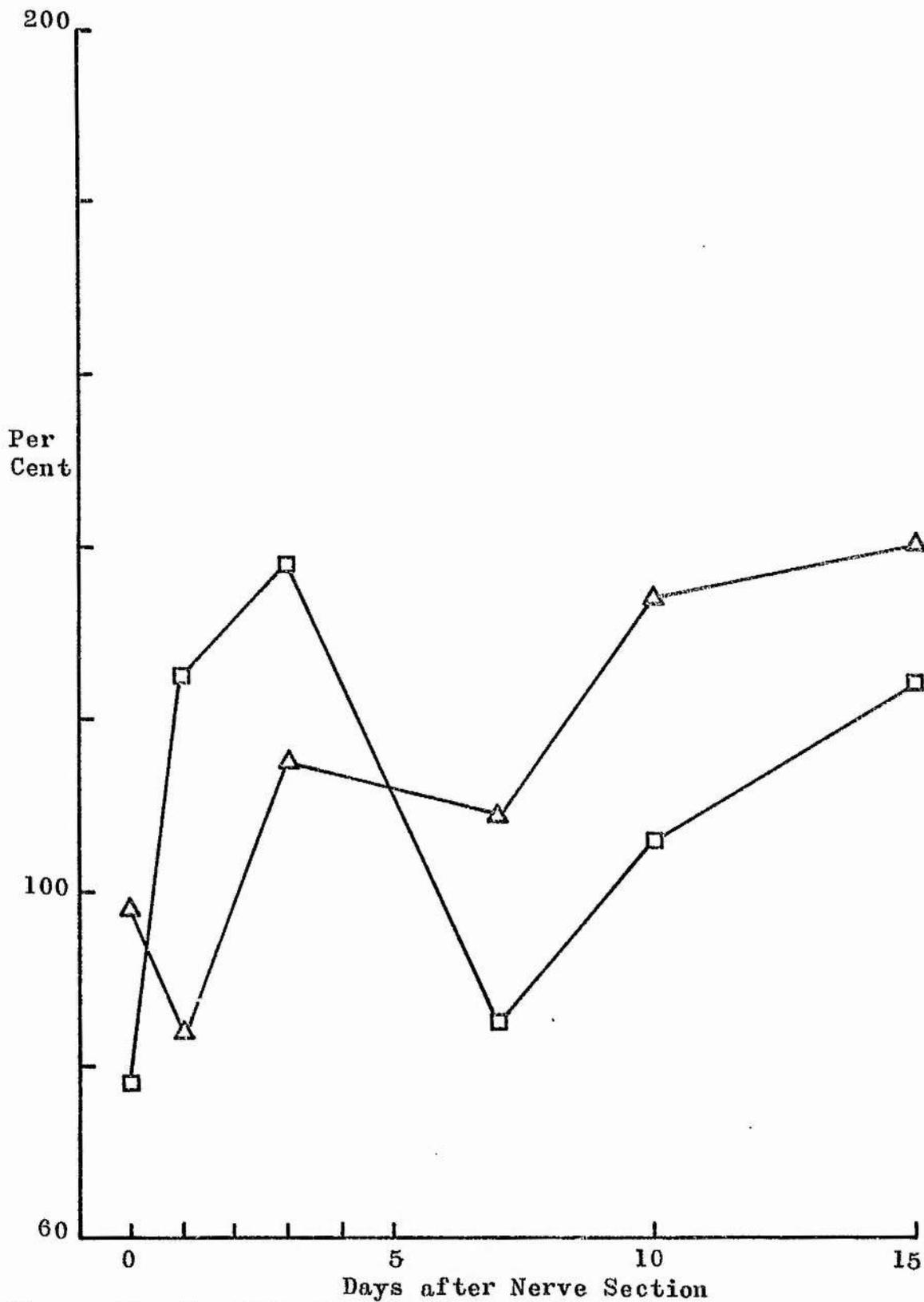


Figure 29. The Effect of Unilateral Phrenicectomy upon the Concentration of Free Methionine (—□—) and Taurine (—△—) in the Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.

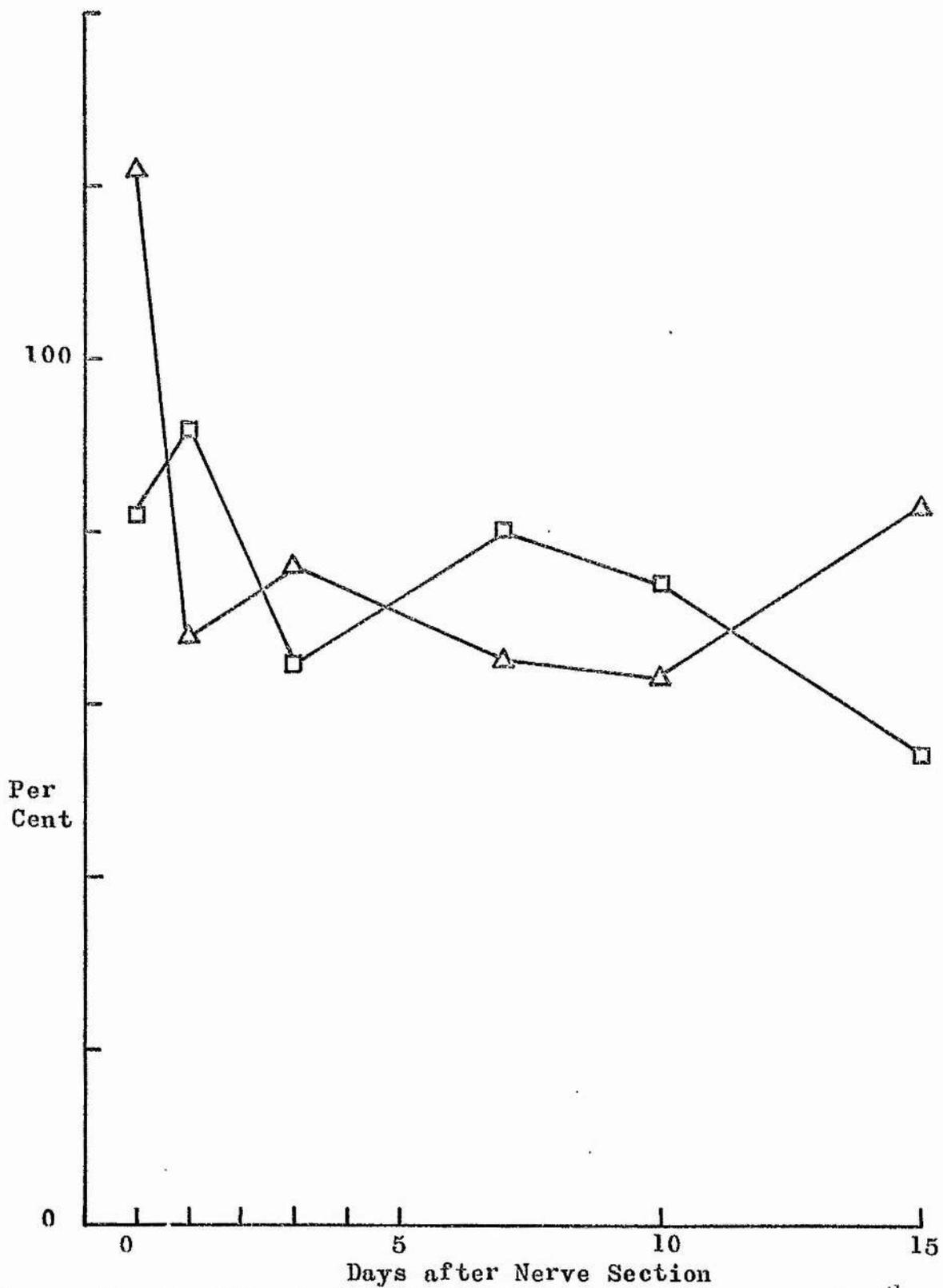
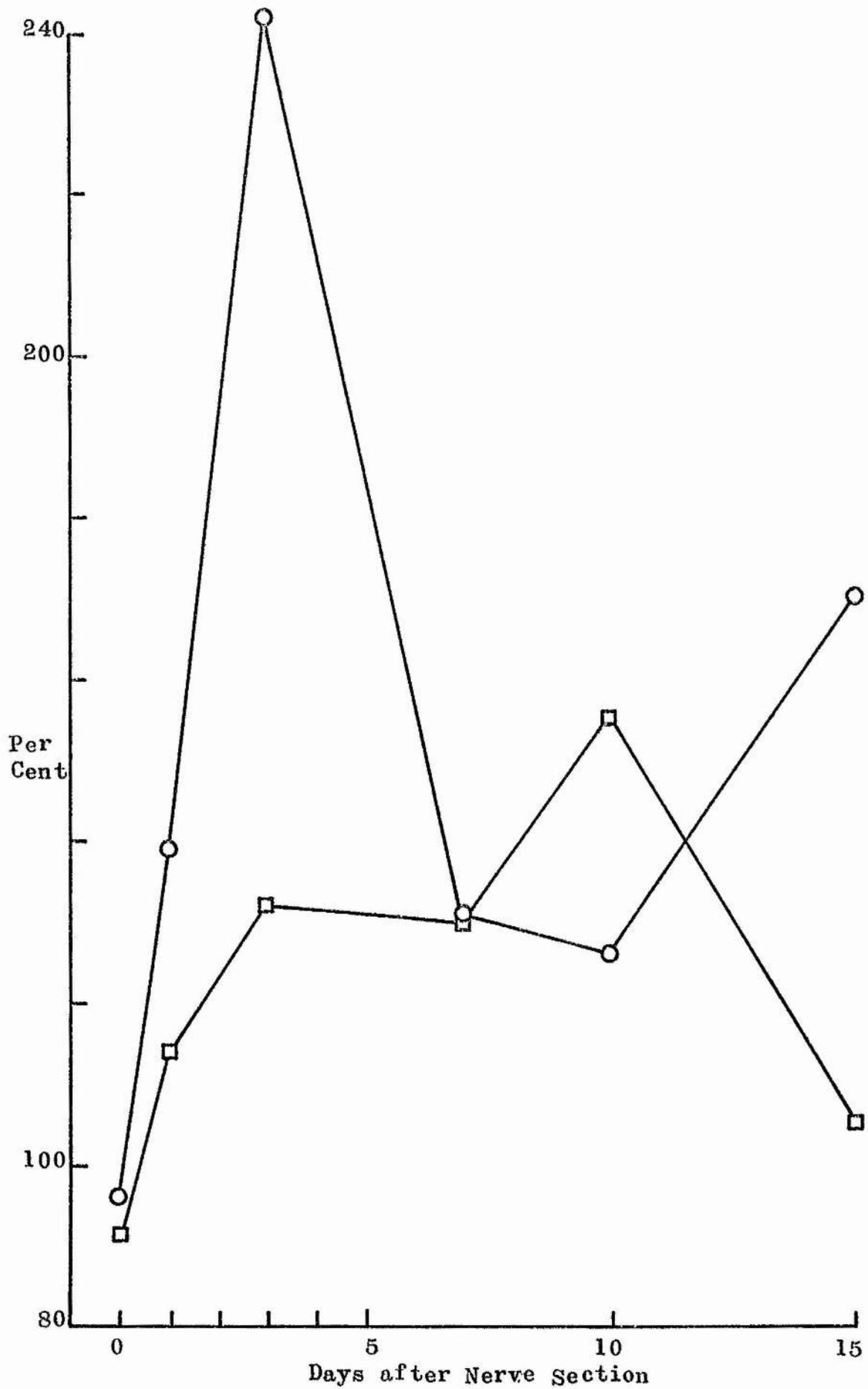


Figure 30. The Effect of Unilateral Phrenicectomy upon the Concentration of Ammonia (—△—) and of Carnosine (—□—) in the Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.

Figure 31. The Effect of Unilateral Phrenicectomy upon the Concentration of Reduced Glutathione (—□—) and of O-Phosphoethanolamine (—○—) in the Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.



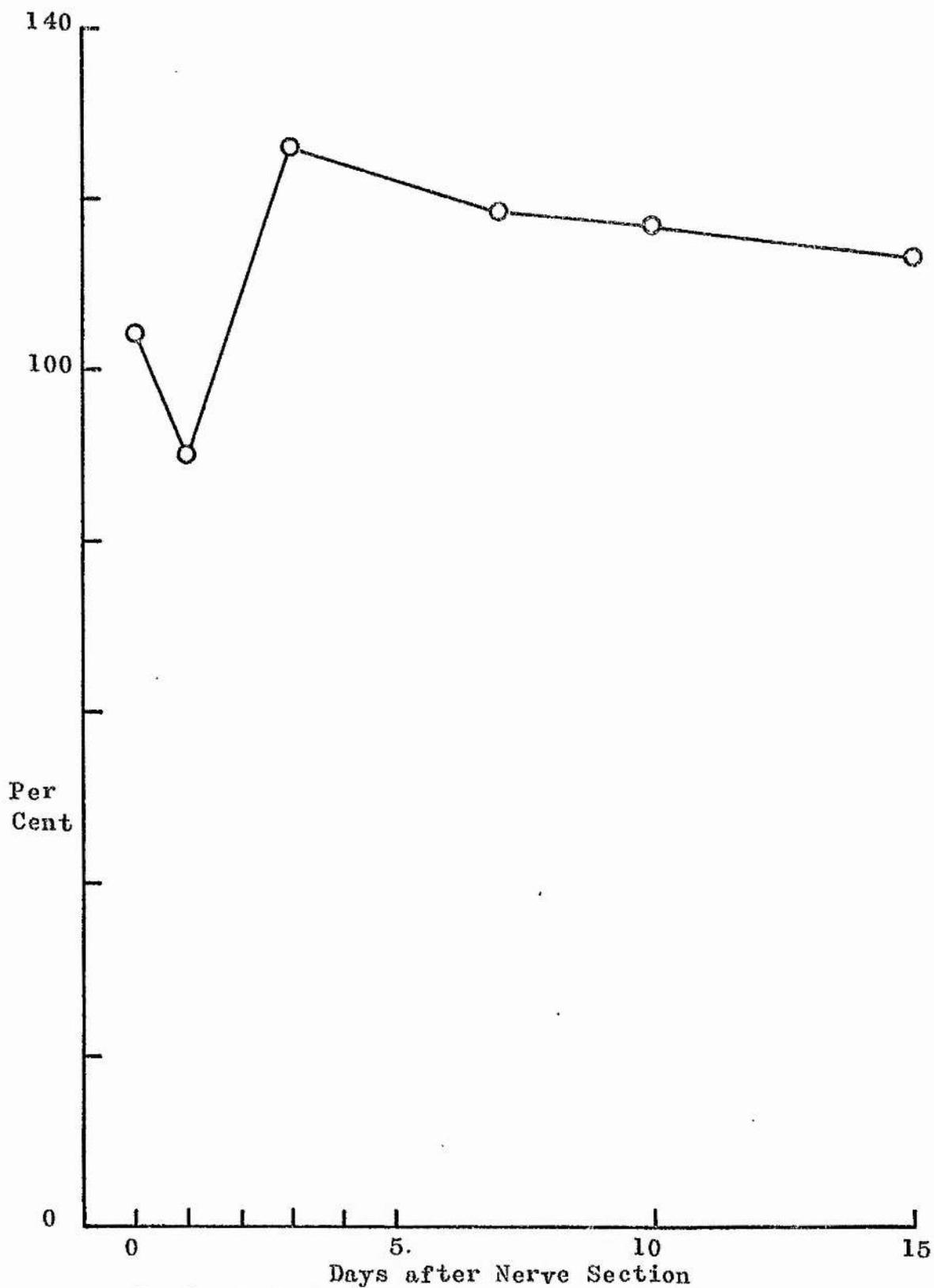


Figure 32. The Effect of Unilateral Phrenicectomy upon the Concentration of Total Acid Soluble Ninhydrin Reacting Substances in the Rat Hemidiaphragm, expressed as a Percentage of the Contralateral Control Values.

## D I S C U S S I O N

For the purpose of the present investigation it was decided that rats weighing 100 g at the time of the assay should be used, and that the effects of denervation on various parameters in the paralysed tissue could be usefully followed for up to 15 days after nerve section. This period thus covers the duration of the hypertrophic weight gain and the subsequent loss in weight of the left hemidiaphragm to values slightly lower than in the unoperated rat. The 100 g rat, however, is still growing and it is well documented that profound changes in the activity of some muscle enzymes take place during the growth phase (Burleigh & Schimke, 1969). Considerable effort was therefore made to ensure that the rats had comparable body weights in order to minimize this growth effect on enzyme levels, thereby aiding comparisons between groups of rats. To this end, it was necessary to operate on rats which, if they were to grow for up to 15 days before sacrifice, could be as little as 35 to 40 g body weight; clearly the initial response to denervation takes place in tissues of

different degrees of maturity. Since in the new born rat, maturation of both nerve and muscle is as yet incomplete, it might be argued that denervation in such young animals takes place at a time prior to full development of the neuromuscular apparatus, and that effects which may be subsequently observed result from an incapacity of the system to reach full maturation rather than representing deviations from the mature state as a consequence of denervation. In this context, Engel & Karpati (1968) found that denervation of the gastrocnemius or soleus of the neonatal rat prevented any subsequent characteristic fibre differentiation from taking place.

In the new born rat, as in some other mammalian species, skeletal muscle fibres are not fully differentiated into "fast" and "slow" types; instead the presumptive "red", "white" and "intermediate" fibres have properties characteristic of slow contracting muscles. The process of maturation and fibre differentiation takes place during the first few weeks after birth, (Denny-Brown, 1929; Buller, Eccles & Eccles, 1960). From histological evidence Dubowitz (1963) concluded

that maturation of rat muscle fibres took place between 7 and 10 days of age, depending to some extent upon the particular muscle concerned; by 14 days after birth, rat muscle was judged to be fully differentiated. Physiological maturation, however, appears to occur one week after this time: Close (1964), studying the contractile properties of rat soleus and extensor digitorum longus muscles from birth to 100 days of age, came to the conclusion that physiological differentiation could be explained solely in terms of an increase in the speed of shortening of the fast muscle. By about 20 days after birth, contraction and half relaxation times for this muscle were found to be typical of values for the adult tissue.

Similarly, a biochemical study of the effects of denervation on rat gastrocnemius muscle in early post natal life suggests maturation to take place also at about this time (Martinek & Mikulas, 1954): three days after sciatic nerve section both young and adult rats were found to have a lower glycogen content in the denervated muscle than in the contralateral control. One

day after denervation, however, adult rats have a significantly higher glycogen level in the paralysed side, whereas rats younger than 14 days have lower glycogen values. In the 14 day old rat, denervated for 1 day, glycogen in the paralysed muscle approximates to that in the control muscle. The authors concluded that the relationship between nervous control and metabolism reaches the adult level in the rat at about 14 days after birth.

These studies refer, however, to the rat hind limb muscles; after birth this species remains relatively inactive in the nest until about two weeks of age. The diaphragm though must function in conjunction with the thoracic musculature with a high degree of efficiency as soon as the umbilical connection with the placenta is severed. Kendrick-Jones & Parry (1967) showed, in contrast to the gradual post natal increase of characteristic muscle enzymes, such as creatine kinase, or adenylate deaminase, that takes place in the rat or rabbit leg muscle, that in the diaphragm there is a massive prenatal

increase of adenylate deaminase, which after birth declines to the adult value. Possibly such a response is indicative of earlier maturation of the diaphragm muscle, as suggested by the work of Sweiber (1968).

The evidence therefore suggests that in limb muscles of the rat full differentiation into fibre types and the possession of adult contraction characteristics is accomplished by about 20 days after birth, whilst in the diaphragm there is the possibility that this process occurs earlier, perhaps even prenatally. The strain of rats used in this investigation are about 25 to 30 days of age when they weigh 35 to 40 g. It seems likely therefore that the observation recorded for the various parameters of rat diaphragm muscle are the result of denervation rather than of failure of the processes of differentiation and maturation, although, of course, it must be remembered that the initial effects of denervation do take place on tissues of different ages. Whether this latter point is of significance cannot be ascertained from the present data.

Differences Between The Left And Right Hemidiaphragms  
Of The Rat.

It is usually assumed that the two halves of the rat diaphragm are identical and that they perform similar functions. That the relative proportions of the two hemidiaphragms should change with the age/body weight of the rat is a surprising finding and difficult to interpret; teleology is hard to avoid, but there appears to be no satisfactory explanation that can be put forward to account for the observation.

Further evidence for a difference between the two parts of the tissue comes from a consideration of the enzyme levels found in the undenervated rats. An identical composition for each of the hemidiaphragms would be confirmed by identical concentrations of all the enzymes measured, even though the relative weights of the two tissues may be different. Whilst this is so for those enzymes normally considered to be present in the muscle cytosol, this is not the case for the dehydrogenases of the tricarboxylic acid cycle, which are found to be about 10% higher in

concentration in the left hemidiaphragm than in the right. A systematic error in the extraction procedure seems improbable. The evidence therefore suggests a higher mitochondrial concentration in the left hemidiaphragm, and by implication, though not necessarily obligatory, fewer "white" fibres. Nevertheless it is unlikely that the effects of denervation found in the left hemidiaphragm differ from those that would be found in the right, or that the right hemidiaphragm is an unsatisfactory control to use when studying the effects of denervation in the left.

Comparing groups of rats denervated for different intervals of time, the variation of a particular parameter measured in the control tissues was, in most cases, reasonably small; in a few instances, e.g. hexokinase, myoglobin, glycogen phosphorylase, the fluctuations were greater. Burleigh & Schimke (1969) have shown that in rats corresponding to 100 g body weight, parameters such as the three mentioned above are increasing dramatically to the adult<sup>level</sup>; the wider variation thus seems inevitable. In spite of

this, there was no evidence of any systematic compensatory phenomenon occurring in the control hemidiaphragm of the various groups of denervated rats. In any case, provided that comparisons for any given parameter are only made on tissues from the same groups of rats, age effects as a confusing influence can be avoided.

The data of table 6 demonstrates that unilateral phrenicectomy induces a hypertrophy, the magnitude of which is dependent upon the age/body weight of the rat. Although statistical analysis revealed that the slope of the regression line relating the weight of the right hand hemidiaphragm to the weight of the left was not significantly different from that of unoperated rats, it is apparent from the table that the slope does increase in value reaching a maximum at the time of the peak of the hypertrophy. It appears therefore that whatever this correlation means in functional terms, denervation has but a slight effect upon it.

Suitability Of Halothane Anaesthesia For The Determination  
Of Glycogen And Phosphorylase Activities.

The stress induced release of catecholamines from the adrenal medulla into the blood has the effect of rapidly promoting glycogenolysis and an increase in the phosphorylase a/b ratio. It is essential, therefore, to minimize stress in an experimental animal if values determined for the concentration of glycogen and the activity of the phosphorylases are to reflect the in vivo situation. Anaesthesia is the usual method of overcoming the stress complication: Marquez-Julio & French (1967) demonstrated significantly lower glycogen levels in muscle taken from rats killed by decapitation than were found in muscle from rats that had been anaesthetized before sacrifice.

The choice of anaesthetic, obviously, is of importance; ether anaesthesia is probably unsatisfactory since it has been shown that diethyl ether not only induces activation of the sympatho-adrenal system, thereby increasing the concentrations of circulating catecholamines (Li, Laasberg & Etsten, 1964; Price, 1957; Ngai & Papper, 1962 ) but it

also induces glycogenolysis in the isolated rat diaphragm, as well as causing a significant decrease in the uptake of glucose (Brunner, 1969).

Gaseous anaesthetics have several advantages over the barbiturates, not the least of which is the relative ease of administration, involving a minimum handling of the animal, and the ability to vary the dose at anytime according to the appearance of the animal. Halothane was considered to be preferable for this situation for, not only does it induce anaesthesia quickly, and permit careful control of the anaesthetic dose, but it also with rats has been found to cause no mucus secretion, and to depress the ventilatory rate much less than do anaesthetic doses of barbiturates (Luschei & Mehaffley, 1957). The primary advantage of halothane is that during anaesthesia there is found to be no significant induced rise in the concentration of plasma catecholamines (Price, Linde, Jones, Black & Price, 1959; Davenport, Auld, Sekelj, Jegier & McGregor, 1964; Li, Laasberg & Etsten, 1964). In addition, Shimoji, Asari, Nioh Kawamoto, Takeshita, Morioka & Inamoto (1968) have shown that halothane, unlike diethyl ether, has no depressant effect

upon neuro-muscular transmission, even at the deepest stage of anaesthesia, so that it may be assumed that the nerve muscle interaction remains relatively unaffected as a result of the use of halothane.

Preliminary studies on the glycogen concentration of hemidiaphragms from rats killed without anaesthesia by decapitation produced values close to 25  $\mu$ moles glucose per g wet weight; the use of halothane appears therefore to preserve the in vivo glycogen levels, since reference to Table 7 shows the control level to be approximately 30  $\mu$ moles glucose/ gm wet weight.

The literature is in dispute as to the proportion of phosphorylase a present in resting muscle; initial experiments, using methods likely to preserve the proportion during the extraction and assay procedure showed that the level of phosphorylase a was only a few per cent of the total enzyme activity in the muscle fibre, whilst more recently it has come to be accepted that approximately 30% of the phosphorylase in resting muscle is in the a form. An examination of Table 31 shows that for intact resting

Table 31. The proportion of glycogen phosphorylase a in skeletal muscles as reported in the literature.

A. In vivo, intact preparations:

Tissue	Special Conditions	Proportion of phosphorylase <u>a</u>	Reference
Rat gastrocnemius	Pentobarbital anaesthesia, frozen <u>in situ</u>	29 %	Drummond, Harwood & Powell (1969)
Rat gastrocnemius	Amytal anaesthesia, frozen <u>in situ</u>	31 %	Cori (1956)
Rat gastrocnemius	Pentobarbital anaesthesia, frozen <u>in situ</u>	16 % 10 %	Posner, Stern & Krebs (1965)
Rat "thigh" muscles	Adrenalectomized 12 - 14 hr prior to assay. Pentobarbital anaesthesia; sciatic nerve sectioned to prevent central impulses transmitted to leg muscles. Frozen <u>in situ</u> .  "Muscles from rats with abnormal control values were discarded"	22 %	Piras & Staneloni (1969)
Rat gastrocnemius	Experiments carried out in early morning to ensure high glycogen levels. Pentobarbital anaesthesia; frozen <u>in situ</u> .	11 %	Schaeffer, Chenoweth & Dunn (1969)
Guinea pig adductor	Pentobarbital, then ether anaesthesia. Frozen <u>in situ</u>	34 %	Stubbs & Blanchaer (1964)
Guinea pig quadriceps femoris	As above	28 %	Stubbs & Blanchaer (1964)
Human, various muscles	Local lidocaine anaesthesia, biopsy samples frozen rapidly in Freon 12.	33 %	Hess (1965)
Rabbit, back & hind leg muscles	Nembutal anaesthesia	32 %	Fischer & Krebs (1955)

Table 31 continued.

B. In vitro preparations:

Tissue	Special Conditions	Proportion of phosphorylase <u>a</u>	Reference
Rat diaphragm	Pentobarbital anaesthesia. Tissue incubated in Krebs-Ringer bicarbonate for 30 min.	23 % 27 %	Holmes & Mansour (1968)
Rat diaphragm	As above but incubated in buffer containing 16 mg/ml glucose for 30 min.	13 %	Holmes & Mansour (1968)
Rat levator ani	Pentobarbital anaesthesia. Excised muscle incubated in Krebs-Ringer bicarbonate, 37°C "to reduce proportion of <u>a</u> produced by extraction of muscle"	25 %	Turner & Leonard (1969)
Rat rectus femoris	As above	37 %	Turner & Leonard (1969)
Rat diaphragm	Tissue preincubated in Gey & Gey buffer before freezing with isopentane	21 %	Craig, Rall & Larner (1969)
Rat diaphragm	Preincubated in Gey & Gey buffer before freezing	32 %	Craig & Larner (1964)
Rat diaphragm	Rat decapitated and tissue attached to rib cage incubated in Tyrode's solution (contains 0.1 % glucose) for 15 min.	5 %	Drummond, Harwood & Powell (1969)
Rat diaphragm	Rat decapitated, tissue preincubated in saline buffer containing 10 mg glucose/ml	4 %	Torres, Marechal Bernard & Belicopitow (1968)
Mouse ant. tibial muscle	Ether anaesthesia; tissue incubated in Krebs-Ringer bicarbonate for 1 hr. prior to freezing with isopentane.	18 %	Rulon, Schottelius & Schottelius (1961)
Rat diaphragm	Rat decapitated, tissue preincubated in ice-cold phosphate-NaCl buffer	4 %	Hess, Aronson, Hottenstein & Karp (1969)

mammalian muscles, frozen in situ, or else very rapidly soon after excision, the proportion of phosphorylase a is close to 30%, whilst it is those experiments in which the muscle is incubated in vitro prior to the enzyme assay that demonstrate the very low phosphorylase a level. Possibly related to this distinction is the observation of Holmes & Mansour (1968) that incubation of isolated muscle in the presence of glucose results in a significantly lower level of phosphorylase a, as compared to control tissues incubated without glucose. However, not all the in vitro experiments reported in Table 31 included glucose in the incubation medium, so possibly some other effect is at work. For example, Holmes & Mansour (1968) concluded that the effect of glucose in lowering the phosphorylase a level was to activate phosphorylase phosphatase; glucose 6-P and glycogen also proved to be effective activators of the phosphatase. Presumably therefore, if muscle contains high levels of glycogen or of glucose 6-P then depression of phosphorylase a occurs, mediated by phosphorylase phosphatase.

An alternative explanation for the presence of low levels

of phosphorylase a in in vitro preparations resides in the knowledge that in vivo muscles never really relax completely; there is always a proportion, albeit small, of motor units that are active in maintaining the "tone" of the muscle. The neurostimulatory activity associated with this phenomenon would be expected therefore to induce a given level of activity to the phosphorylase system. Complete section of the nerve for the preparation of the in vitro system would thus be expected to eliminate this source of stimulation, provided all other parameters, e.g. AMP concentration, remain constant. Similarly adrenalectomy, by reducing to negligible levels the concentration of circulating catecholamines, might be expected to lower the activity of phosphorylase a; however, Schaeffer, Chenoweth & Dunn (1969) reported that adrenalectomy does not alter the proportion of phosphorylase a in rat muscle. Unfortunately though, the level of phosphorylase a that they found in muscle from normal rats was only 11%, so that it may be the case that no effect of adrenalectomy was observed because of the very low initial levels. These authors carried out their experiments "on fed animals at 7.00.a.m. to insure adequate muscle glycogen levels".

Using halothane anaesthesia as described in the methods section the proportion of phosphorylase a found in non-denervated hemidiaphragms and including the values obtained for the control tissues of operated rats was  $29.4 \pm 1.0\%$  for 59 observations.

Effects Of Unilateral Phrenicectomy Upon Glycogen  
Metabolism.

The effect of phrenic nerve section upon the glycogen content of hemidiaphragms from fed rats is typical, at least in the initial stages, of the changes observed in other denervated muscles (see for example Humoller, Griswold & McIntyre, 1950; Hines & Knowlton, 1935). Little change is observed until 3 days have elapsed since denervation, at which time values close to 60% of the control levels are obtained. The effect of fasting upon the glycogen content of rats whose phrenic nerve had been cut 1 day previously demonstrates that in denervated muscle there may be an impairment in glycogen mobilization, which presumably is not permanent since an appreciable decrease is found after three days. Such a temporary impairment may explain the rapid and transient increase in glycogen concentration reported to occur very soon after denervation: a reduced capacity to mobilize glycogen in the denervated tissue would lead to higher concentrations of glycogen found in the paralysed muscles if the animal experienced stress before sacrifice. It is

significant therefore that among these cases reported in the literature where the method of sacrifice was specifically stated, the transitory increase in glycogen content one day after denervation was not observed if an anaesthetic had been used before killing the animal, whereas in those cases where decapitation had been employed (e.g. Graff, Hudson, & Strickland, 1965; Carafoli, 1964) the transitory increase was observed.

That the concentration of glycogen in the denervated hemidiaphragm remains the same in the fasted animals as in the fed ones, at least for as long as 7 days after nerve section, suggests that the impairment in glycogen metabolism remains for this period of time. Alternatively, it may mean that the period of fasting for 16 to 18 hours, which in normal unoperated rats is long enough to halve the muscle glycogen concentration, is <sup>in-</sup> sufficient to do so in paralysed muscles; the denervated muscle after all does not need to expend metabolic energy for contractile purposes, and it is possible that the glucose and/or fatty acid uptake is adequate to supply the tissue's needs. The decreased

glycogen concentration observed at 3 days could thus be explained in terms of a reduced capacity to synthesize the polysaccharide, due either to some defect of the glycogen synthetase system, or else to a selective deployment of glucose entering the cell for purposes other than glycogen synthesis; Gutman et al (1966) have demonstrated a considerable loss of the ability to synthesize glycogen in the 7 day (?) denervated hemidiaphragm, whilst Carafoli (1964) showed a similar effect in the denervated pigeon pectoralis muscle in which the incorporation of  $^{14}\text{C}$ -glucose into the muscle glycogen was reduced to about 20% of the control values 2 days after nerve section. A reduction of glycogen synthesis thus seems apparent in denervated muscle.

Robbins, Traut & Lipmann (1959) found that for synthesis of glycogen from radioactive glucose or glucose 6-phosphate in pigeon homogenate, the presence of ATP-synthesizing mitochondria were obligatorily required; possibly the requirement was necessary for the production of UTP for the synthesis of UDP-glucose, without which metabolic synthesis of glycogen does not take place. Whether this is the case or not, the reduction of mitochondrial content in denervated

muscle (Garafoli, Margreth & Buffa, 1962, 1964; this study) so soon after nerve section may be of significance with respect to the observed decrease in glycogen content, if only because of the reduction of a major ATP generating system of the muscle fibre.

During the first seven days of denervation, the hypertrophying hemidiaphragm is synthesizing large quantities of nucleic acid for which ribose must be derived from glucose 6-phosphate via the pentose phosphate pathway. The question arises as to whether the decrease in glycogen content observed between 1 and 7 days could account for the pentose content of the nucleic acid synthesized during this period. Between 1 and 3 days after nerve section, the denervated hemidiaphragm synthesizes some 120  $\mu\text{g}$  DNA (Zak, Grove & Rabinowitz, 1969) and approximately 120  $\mu\text{g}$  RNA (Manchester & Harris, 1968) which amounts to a total of 105  $\mu\text{g}$  of pentose, assuming that the percentage of ribose in nucleic acid is 44% by weight. During the same period, the glycogen content of the denervated hemidiaphragm decreases by 1.2  $\mu\text{mole}$  glucose. Thus this is more than

adequate to account for the 0.7  $\mu$ moles pentose required for the synthesis of the nucleic acid.

Ten to 15 days after nerve section, a period of fasting significantly lowers the glycogen concentration ( $P < 0.001$ ) compared to that in the "fed" denervated hemidiaphragm. This may be indicative of a new drain upon the metabolic energy stores which, in the starving rat cannot be met from extracellular sources.

The Effect Of Unilateral Phrenicectomy Upon The Measurable Activities Of Some Enzymes Of Glucose And Glycogen Metabolism: Hexokinase, Phosphoglucomutase And Glycogen Phosphorylase.

(a) Hexokinase. The concentration of hexokinase within the hypertrophying hemidiaphragm hardly changes within the first seven days after denervation, but by 10 days has decreased by 20% and remains at this level for a further 5 days. Content of the enzyme therefore increased markedly during the hypertrophy indicating that synthesis of the enzyme had taken place. The constant enzyme concentration is in accord with the finding of Buse & Buse (1959) that the in vitro glucose uptake expressed per unit weight of the tissue is unaffected by denervation. Glucose presumably therefore remains of importance as an energy substrate for the denervated tissue, possibly even more so in the later stages of the hypertrophy when the decrease in the content of mitochondrial enzymes has taken place.

(b) Phosphoglucomutase. The initial decrease of phosphoglucomutase concentration in the denervated tissue is typical of the response seen for a number of other enzymes,

e.g. phosphohexoisomerase, adenylate kinase, creatine phosphokinase. The mutase activity differs however in that after 5 days the concentration returns to normal values; in terms of enzyme content, therefore net synthesis of the enzyme is implied, apparently beginning within the first two or three days after denervation. It is at this time that the massive decrease in glycogen content is observed, whilst later on 10 days after denervation when the content of glycogen has returned to normal, the content of phosphoglucomutase has reached its maximum; it seems highly probable that the changes in phosphoglucomutase levels within the tissue are functionally related to the changes in glycogen content.

(c) Glycogen Phosphorylase. The decrease in total phosphorylase activity 1 day after nerve section is an interesting observation since although at this time there is little decrease in glycogen content, the effects of fasting for 16 to 18 hours demonstrate a possible inability to mobilize the polysaccharide. Phosphorylase a, however, remains unchanged one day after denervation; further studies are clearly required to resolve this difficulty and establish

whether a period of fasting of 16 to 18 hours is adequate to cause depletion of glycogen in a non-contracting muscle.

Two days after denervation an increase of total phosphorylase activity is observed whilst phosphorylase a content also rises; the increases coincide with the time at which the content of glycogen decreases within the denervated tissue. Phosphorylase a continues to increase in content until 5 days after nerve section, after which it declines, but the total enzyme activity demonstrates a pronounced decrease immediately after the peak at 2 days. This rapid decrease seems to be in line with the observations of other workers that muscle phosphorylase activity may be rapidly depleted in a number of different states, e.g. after exercise (Kugelberg & Edstrom, 1968 ; Edgerton, Simpson, Barnard & Peter, 1970) or pathological conditions (Smith, 1965) and suggests that genetic regulation of glycogen phosphorylase levels in muscle may play a highly significant role in the overall control of glycogen metabolism.

(d) Phosphorylase/Hexokinase Activity Ratio.

Changes in the ratio of activities of glycogen phosphorylase and hexokinase have in the past been considered to be indicative of a changing proportion of fibre types within a muscle, the rationale being that "red" fibres are rich in hexokinase activity, but poor in phosphorylase activity, whilst the converse is true for "white" fibres (Burleigh & Schimke, 1969; Golische et al ,1970). Since Feng & Lu (1965) concluded the hypertrophy of the red fibres took place after nerve section, it is to be expected that, providing hypertrophy or atrophy of the appropriate fibres proceeds with a proportionate change of the two enzymes, a decrease in the ratio will be found. Comparing the contents of hexokinase and total phosphorylase such a decrease of course is observed, but it is clear that (i) proportionate changes of the two enzymes concerned have not taken place, and (ii) the involvement of the "intermediate" fibre type has been ignored. The ratio may be valid for the conclusion that the muscle shows a decreased capacity to mobilize glycogen and an increased capacity to phosphorylate glucose, but it is not permissible to conclude that a change in fibre proportions has taken place, except in non-pathological muscles.

The Effect Of Unilateral Phrenicectomy Upon The Extractable  
Activities Of Some "Glycolytic" Enzymes: Phosphohexoisomerase,  
Glyceraldehyde Phosphate Dehydrogenase And Lactate

Dehydrogenase. A decreasing proportion of white fibre metabolism in the hypertrophied hemidiaphragm would be expected to result in lower concentrations of enzyme activity for those enzymes typical of glycolysis; such is the case for the glycolytic enzymes determined in this study. i.e. phosphohexoisomerase, glyceraldehyde phosphate dehydrogenase and lactate dehydrogenase. Probably a similar situation also exists for phosphofructokinase, as suggested by the results of Manchester et al (1970). The response to denervation of each of these enzymes, though, is different. Total phosphohexoisomerase remains constant until 5 days after the operation, whilst both dehydrogenases demonstrate a slight increase reaching a peak coincident with the time of maximum hypertrophy. The similarity between the response of the two glycolytic dehydrogenases is interesting, for recently Fahimi & Karnovsky (1966) have unambiguously demonstrated by means of histochemically stained formaldehyde fixed sections studied under the electron microscope that both these dehydrogenases are to be found in the sarcoplasmic reticulum in the hypertrophying hemidiaphragm;

the observed increase of these two dehydrogenases may well be related to this phenomenon. Pette and his co-workers (Pette, 1966; Bass et al, 1969) have established that for both red and white skeletal muscles a constant proportion relationship exists between the activities of the "phosphotrioseglycerate-phosphate group" of enzymes, i.e. glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, triose phosphate isomerase, phosphoglyceromutase and enolase, whilst the comparable ratio of activities of lactate dehydrogenase/glyceraldehyde phosphate dehydrogenase in "red" and "white" muscles demonstrates that the capacity of lactate production is correlated quantitatively with glycolysis. Postnatal differentiation studies in red and white muscles of the chicken (Bass et al, 1970) have suggested a co-ordinated synthesis of the constant proportion groups of enzymes. In the light of this information it would be interesting to know whether the sarcoplasmic reticulum is the location of the entire constant proportion group or whether compartmentation divides, and thus could exert control over, the sequence of enzymic reactions.

As mentioned previously, the lactate dehydrogenase enzyme exists as a polymer which may be composed of two types of monomeric subunits, termed the H- or the M-type which differ appreciably in the extent to which they are inhibited by excess concentrations of substrate. The H-type subunit is significantly inhibited by high concentrations of pyruvate, whereas the M-type subunit is only slightly affected. This difference in substrate inhibition has been suggested as a basis for the physiological roles of the two extreme types of lactate dehydrogenase; the H-type isoenzyme is thought to function as a lactate dehydrogenase in heart, whereas the M-type may be geared to operate as a pyruvate reductase, as in striated muscle or in liver (Everse, Berger & Kaplan, 1970). Studies with "high physiological" concentrations of the enzymes using a stopped flow technique have suggested that a ternary abortive complex forms between pyruvate,  $\text{NAD}^+$  and the enzyme, and that the dissociation constant of the complex is lower for the H-type LDH than for the M-type, thus providing a mechanistic explanation for the differing activity (Everse et al, 1970). These authors propose that the M-type of lactate dehydrogenase evolved to overcome the abortive complex formation that would occur in skeletal

muscle where nearly all the nicotinamide adenine dinucleotide would be in the oxidized form. On the other hand, Wuntuck, Chen & Vessel (1970) have shown that commonly occurring intracellular proteins such as glyceraldehyde phosphate dehydrogenase, or malate dehydrogenase, may protect the lactate dehydrogenase from the ternary complex formation by successfully competing for the  $\text{NAD}^+$ ; these latter authors consider this point to render effectively unphysiological the model of Everse and his co-workers.

Nevertheless, although the physiological significance of the various proportions of the LDH isoenzymes is still in question, it is a fact that the assay procedure for the enzymes utilizing two levels of pyruvate concentration can be used as a diagnostic tool to determine easily the proportions of the H- and the M-type subunits, in a given tissue. Further it is also established that those tissues that are considered to be metabolically aerobic are the ones that contain the higher proportion of H-type subunits (Kaplan & Goodfriend, 1964). In this respect Hellung-Larson & Anderson (1970) have demonstrated in cultured human lymphocytes that changes in the LDH isoenzyme proportions are

related to variations in the  $pO_2$  of the aeration medium. It is possible therefore to use the substrate activity ratio as an indicator of the probable extent that aerobic metabolism is of importance in a given tissue.

Following nerve section, the proportion of lactate dehydrogenase isoenzymes in denervated muscle changes; red muscles, such as pigeon breast muscle (Muscatello & Patriarca, 1968) or rabbit soleus (Dawson & Kaplan, 1965) demonstrate a decrease in the proportion of the H-type enzyme, whereas in white muscles an increase is observed (Dawson & Kaplan, 1965; Hogan et al., 1965). These changes correlate well with the variations of pigment, probably mainly mitochondrial, and therefore indicative of potential aerobic metabolism (Romanul & Hogan, 1965) and with the reported changes in oxygen consumption of denervated red and white muscles (Sheves & Ryamina, 1956).

In the denervated hemidiaphragm there is no significant change in endogenous oxygen consumption at three or four days after denervation (K.L. Manchester, unpublished observations) which is in accord with the finding in this study that there

is no significant difference in the LDH activity ratio of control and denervated muscles until 7 days after nerve section, when a decrease is observed ( $P < 0.05$ ); this decrease is taken to be indicative of a decreased proportion of H-type subunits within the muscle, and thus presumably of a greater predominance of anaerobic metabolism. This would also be expected from a knowledge that a decreased content of mitochondrial enzymes was to be found in the tissue. Interestingly the decreased activity ratio is absent at 10 days after nerve section, the time at which the content of myoglobin in the tissue has reached its peak. Possibly the greater pigment content facilitates a higher rate of oxygen transfer from the blood, thereby permitting a greater degree of aerobic metabolism to take place, which is reflected in the LDH activity ratio.

The Effect Of Unilateral Phrenicectomy Upon The  
Extractable Activities Of Some Dehydrogenases  
Associated With The Tricarboxylic Acid Cycle:  
NAD- and NADP-linked Isocitrate Dehydrogenases,  
Malate Dehydrogenase and Glutamate Dehydrogenase.

A reduction in the number of mitochondria in denervated muscle, being one of the few really definitive changes observed soon after nerve section, has been suggested (Carafoli, Margreth & Buffa, 1964) as one possible cause of the eventual atrophy of the tissue. These authors using denervated pigeon breast muscle as their source of mitochondria, found that three to five days after nerve section, the yield of mitochondria obtainable from the denervated tissue began to decline. Moreover, distinct reductions in the biochemical capabilities of the organelle were observed, e.g. a decreased capacity to oxidize pyruvate or  $\alpha$ -oxo-glutarate; whilst ATP'ase and cytochrome oxidase activities were both reduced.

An electron microscopical study of the same system (Muscatello & Patriarca, 1968) shows a rapid and progressive diminution in number and volume of the mitochondria from the

early stages after denervation, i.e. they were recognized as soon as 3 days after nerve section. The reduction in volume and number of mitochondria was paralleled by the appearance in the fibre of mitochondria significantly modified in their inner structure; the number of cristae might be greatly reduced and unusually spaced in the matrix, which itself appears to be increased in amount, but with an electron density which was significantly decreased as compared to the contralateral control. It was further noted that although the relative proportions between the matrix and the membranous components in the same mitochondria were greatly modified, the membrane structure itself, as judged in the electron microscope appeared normal. This may account for the finding that whilst cytochrome oxidase activity and the ability to oxidise keto acids is reduced in mitochondria, expressed per unit weight of mitochondrial protein, there is no alteration in the same mitochondrial preparation in the capacity to couple oxidation to phosphorylation, a property known to require the maintenance of the normal organization of the membrane system.

(Carafoli et al, 1964).

Other authors have also provided evidence for a reduction in the number of functional mitochondria in denervated muscle (Pellegrino & Franzini, 1963; Nachmias & Padykula, 1958; Smith, 1965; Hearn, 1959; Romanul & Hogan, 1965; and Cherian et al, 1966).

The observed fragmentation of the mitochondria of the hypertrophying hemidiaphragm muscle within one day off nerve section now has biochemical support; the decrease in tissue content of the exclusively mitochondrial NAD-specific isocitrate dehydrogenase (Pette, 1966) appears to begin immediately after nerve section as evidenced by the strictly linear percentage decrease, illustrated in (Fig.13). Shortly after three days the decrease is terminated. Why the decrease should take place, whether the decrease is observed in one or all of the fibre types, or why it should halt so abruptly is unknown. Nevertheless, a decrease in mitochondrial content must presumeably rank as one of the earliest effects of denervation (the content of the enzyme in denervated tissues is significantly different from that in the contralateral controls as soon as one day after nerve section) and may be a useful parameter to investigate

with the aim of finding the ultimate cause of trophic changes in denervated muscles. Clearly though, with such a substantial loss of mitochondria as is indicated by the changes in the NAD-linked dehydrogenase, and with the substantial hypertrophy taking place at the same time, it is perhaps unreasonable to conclude that failure of mitochondrial capacity in the tissue could lead to denervation atrophy through limitation of ATP production (Carafoli et al, 1964).

With good agreement, similar decreases in activity content are also shown by the other dehydrogenases of or associated with the tricarboxylic acid cycle: malate dehydrogenase, NADP-linked isocitrate dehydrogenase and glutamate dehydrogenase, so that it seems that a generalized reduction of the capacity to operate the T.C.A. cycle takes place after denervation. Glutamate dehydrogenase produces a slightly deviant response, possibly related to the role that the enzyme plays in the provision of glutamate and/or NADPH for synthetic purposes, or else possibly to the role that it plays in deamination processes. Of interest is the observation that the two peaks of activity

of this enzyme, expressed in terms of concentration, coincide with the two peaks of glutamate concentration observed in the amino acid analysis; such evidence however is too fragmentary to give any information as to the physiological role that the enzyme plays in the denervated muscle. However, with a decreased ammonia content, for which the  $K_m$  of this enzyme is about 60mM (Bergmeyer, 1963), probably reduced levels of  $\alpha$ -oxoglutarate due to the reduction of the TCA cycle capacity, and an excess of glutamate (see amino acid analysis) we may surmise that the enzyme operates to produce reduced nicotinamide nucleotides for synthetic purposes. The increase in content of this enzyme in the later stages of the hypertrophy may thus be related in this respect to the increased catabolism of protein.

A decrease of functional activity of mitochondria in the hypertrophied tissue correlates well with the results of Kouvelas & Manchester (1968) indicating a decreased production of  $CO_2$  from acetate, but does not agree with the finding of Manchester et al (1970) that the tissue levels of citrate remain unaltered. However this may be because of

an incapacity in the cell to utilize the citrate present.

Pette (1966) demonstrated in red and white muscles a constant proportion relationship between the activities of the mitochondrial enzymes and that of glyceraldehyde phosphate dehydrogenase, stated by him to be "the key enzyme of the constant proportion group of the glycolytic pathway". This present work, as well as a recent paper of Pette and his co-workers (Golische et al, 1970) demonstrates that constant proportion relationships can have little meaning in muscles in which neuronal continuity is interferred with, and should be interpreted only with extreme caution.

The decrease in content of mitochondria immediately after nerve section would seem to be the result of a specific degradation process, since at the time of the hypertrophy all other structures in the cell seem to be proliferating. Gutmann et al (1966) has observed increased proteolytic activity in the tissue 3 days after nerve section; it would be interesting to know whether this activity was generalized or directed specifically towards mitochondrial proteins. One possibility is that

production of metabolites that are capable of causing mitochondrial swelling within the denervated muscle may induce sufficient swelling that they become vulnerable to proteolytic attack. Onishi, Buchner, Zittel & Thermann (1969) observed mitochondrial swelling in myocardial cells of the dog following aortic stenosis, a procedure that leads to heart hypertrophy. In addition they observed lightening of the electron density of the matrix and cristolysis, these changes taking place within 20 minutes of the stenosis being applied.

Amongst the potential mitochondrial swelling agents active in vitro and known to be present in muscle are reduced glutathione and L-ascorbate; Lehninger (1965) reports that swelling initiated by these agents may be accompanied by the formation of lipid peroxides and may proceed to actual lysis of the organelles. Apparently, the swelling causes exposure of the double bonds of the unsaturated fatty acids of membrane phospholipids to oxygen and their subsequent autooxidation. Graff, Hudson & Strickland (1965) found in denervated rat gastrocnemius muscles that the concentration of L-ascorbate rose

dramatically, reaching 209% at three days and 250% of the control at 5 days, a level close to which it remains for a further 10 days.

In this investigation a rapidly increasing content of reduced glutathione was found in the hypertrophying hemidiaphragm, reaching a peak at 10 days (Fig. 31 ). It would be interesting to know whether glutathione or ascorbate so produced could gain access to mitochondria, or whether it was compartmentalized to prevent mitochondrial swelling.

The second decrease in mitochondrial content observed after 10 days, suggested by the decreasing enzyme content, seems to be a non-specific decrease as evidenced by the maintenance of the concentration of the characteristic dehydrogenases despite a rapid fall in tissue weight. There seems to be no need to postulate a specific degradation system for this decrease, the autolytic processes already rampant at this time presumably being adequate.

A decrease in mitochondrial content in the denervated rat hemidiaphragm is not consistent with a hypertrophy of the "red" fibres unless one also makes the proviso that degradation of mitochondria takes place concurrently. An alternative explanation would be that the hypertrophy involves the "intermediate" fibre types whilst the red fibres also lose mitochondria. The methods used by Feng & Lu (1965) when they identified the red fibres as the hypertrophying component would not have allowed positive discrimination of "red" and "intermediate" fibre types; both hypotheses are thus consistent with the experimental evidence to date.

The Effect Of Unilateral Phrenicectomy Upon The  
Extractable Activities Of Some Nucleotide Metabolizing  
Enzymes: Creatine Phosphokinase, Adenylate Kinase &  
Adenylate Deaminase.

It is difficult to reconcile the observed activities of the enzymes creatine kinase, adenylate kinase and adenylate deaminase in rat hind limb muscles with the proposition (Raggi et al, 1969) that "white" fibres have higher enzyme activities than "red" fibres. Diaphragm muscle is seen to have an activity equally as great as, for example, extensor digitorum longus, which is known to consist of almost 100% fast contracting fibres (Close, 1967). If however it is considered that it is those muscles with a high proportion of "intermediate" type fibres that are slow contracting, then the difficulty seems to resolve itself. Diaphragm, with only a low proportion of "intermediate" type fibres (Gauthier & Padykula, 1966) would thus be expected to be reasonably fast contracting and therefore have higher activities of these nucleotide metabolizing enzymes.

If this is the case, and if hypertrophy of the rat diaphragm involves hypertrophy of the red fibres as suggested by Feng & Lu (1965) with presumably an enhanced synthesis of enzymes characteristic of red muscle fibres, then one might reasonably expect an increase in the content of these enzymes to take place. This is in fact observed for the adenylate kinase and deaminase activities, but the content of creatine kinase remains the same for up to 5 days after nerve section. The result is disquieting for an examination of Table 25 shows that in rat muscles the differential in activity observed for "slow" contracting muscles compared to "fast" contracting is greater for creatine phosphokinase than for adenylate deaminase. Hypertrophy of red fibres would be expected to produce a greater increase of creatine kinase than adenylate deaminase, the reverse of what is found. More likely therefore the enzyme changes are indicative of a metabolic change than of fibre types; an examination of the supposed functions of these enzymes may reveal an answer to the effects of denervation.

It is generally believed that the energy for muscular contraction derives in the first place from ATP; the Lohmann reaction catalysed by creatine phosphokinase being responsible for replenishing the muscle content of this nucleotide so that levels of ATP remain constant and presumably the muscle can operate with maximum efficiency (Davies, 1965 ). Creatine phosphokinase thus seems to be of importance only in the contracting muscle. The hypertrophying hemidiaphragm does not contract and so presumably has no need to synthesize further molecules of the enzyme. Adenylate kinase, with adenylate deaminase apparently acting in conjunction, is believed to function to conserve "high energy phosphate bonds" thereby allowing utilization of the free energy of hydrolysis of the pyrophosphate bond of ADP. AMP deaminase seems to act to prevent AMP inhibition of adenylate kinase (Ronca-Testoni, Raggi & Ronca, 1970). ATP, of course, is not only used for muscle contraction and there is no doubt that during the hypertrophic process the turnover of the ATP molecules may well be enhanced. Adenylate kinase and adenylate deaminase may thus be seen as enzymes increasing the efficiency of ATP turnover, and so their rise in content in the hypertrophying muscle is reasonable.

## The Effect Of Unilateral Phrenicectomy Upon Myoglobin Levels.

A recent review by Wittenberg (1970) has presented the considered role that myoglobin plays in facilitating oxygen transport into the muscle fibre; much of what follows derives from this excellent article.

Myoglobin is believed to act in vivo by speeding the inward diffusion of oxygen into red skeletal muscle fibres, the pigment thus serving to meet a sustained demand for oxygen. Observations on working muscles show that myoglobin functions in a state of partial oxygenation (averaged over the volume of the muscle fibre), implying a steep gradient of myoglobin bound oxygen from the periphery to the centre of the fibre (see for example Millikan, 1937). This appears to be a necessary condition for myoglobin to contribute to a large extent to the inward diffusion of oxygen in respiring red muscle fibres. Wittenberg maintains that working red muscles operate in steady state conditions, in which oxygen demand and supply are in instantaneous balance, an equilibrium assured by the control of the oxygen pressure gradient through manipulation of capillary blood flow.

In the light of this information it is possible to speculate as to the cause of the response of myoglobin concentration in the denervated hemidiaphragm, given the possibility that blood flow and/or content may be increased, there presumeably being a loss of fine control of capillary flow as a result of phrenic nerve section, and given the probability that functional mitochondria, the major sites of oxygen consumption in muscle, are reduced in number. The evidence suggesting increased blood flow was obtained for hypertrophying diaphragm muscle up to about 3 days after nerve section; it does not necessarily follow that it remains unaltered for longer period (see introduction), Mitochondrial content, as evidenced by the content of typical dehydrogenases, drops rapidly within the first few days. In addition the muscle is now no longer contracting and will have a considerably reduced demand for oxygen for catabolic purposes within the tissue. It seems likely that at this time the tissue has little difficulty in obtaining sufficient oxygen for its reduced(?) capabilities; as a consequence, we may surmise that the hypertrophy involving either/both the "red" and the "intermediate" fibre types, both of which are known to contain

significant concentrations of myoglobin (James, 1968) does not involve extra synthesis of myoglobin, presumably because sufficient is already present for the requirements of the muscle. The observed increase in content later on, reaching a peak at 10 days, would, by this argument be due to a reduction in the blood flow/content of the tissue, since it seems hardly likely that increased mitochondrial activity would take place.

Alternatively, the observed changes of myoglobin content may merely mean that myoglobin synthesis lags behind production of other muscle proteins, such as those of the myofibrils.

The response of myoglobin concentration in the denervated hemidiaphragm seems to be in accord with that found by other workers for some other denervated muscles; Hogan et al (1965) found a decrease in myoglobin concentration in denervated soleus and gastrocnemius muscles 7 days after sciatic nerve section, whilst after 14 days there was a progressive increase in concentration. Histochemical examination of the two muscles showed an increasing

pallor in the fibres of the soleus and the red portions of the gastrocnemius, whilst a slight enhancement was observed in the redness of the gastrocnemius white portions (Romanul & Hogan, 1965). Tower (1959) and Langley & Hashimoto (1918) also comment on the colour of the denervated atrophic muscle, both red and white muscles tending to become less distinct in terms of their pinkness. Since the concentration of myoglobin after denervation of both soleus and gastrocnemius was found to follow a similar pattern, Romanul & Hogan (1965) attribute the loss of colour difference to changes in the concentration of other porphyrin containing compounds. Indeed, these authors (Hogan, et al 1965) showed that a much smaller decrease of isocitrate dehydrogenase concentration in the gastrocnemius took place than occurred for the soleus muscle whilst after 14 days the white muscle enzyme actually showed a transitory increase in concentration. If these observed changes in isocitrate dehydrogenase activity are being taken as being indicative of the concentration of mitochondria in the tissues, then it may be expected that the concentration of cytochromes in the denervated white muscle decreased at a slower rate than is found in red muscles, and may contribute to the observed colour changes.

The Effect Of Unilateral Phrenicectomy Upon The Activities  
Of Some NADP-linked Dehydrogenases.

Increases in the activities of the enzymes glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase as a result of denervation of muscle have been frequently observed, both histochemically (Cherian & George, 1966) and by quantitative assay of homogenate extracts (Garcia-Bunuel & Garcia Bunuel, 1967; Hogan et al, 1965). Elevated levels of activity of these enzymes are also observed in muscles from animals with hereditary muscular dystrophy (McCaman, 1960; Pennington, 1963) whilst in muscle fibres regenerating from a number of causes, e.g. corticosteroid myopathy; inflammation due to virus invasion; and simple trauma due to crushing; increased activity of the glucose 6-phosphate dehydrogenase is observed (together with an increase in NADP-specific isocitrate dehydrogenase) (Smith, 1965).

Slow contracting muscles have higher activity concentrations of the pentose shunt dehydrogenases than do fast contracting muscles, but the response to denervation does not appear to differ markedly between the two types of muscle, at least for the first two weeks after denervation; thereafter the increase in the slow contracting

muscles is less pronounced (Hogan et al, 1965; Garcia-Bunel & Garcia-Bunel, 1967). McCaman (1960, 1963) showed that in the muscle of mice with hereditary muscular dystrophy or in those that had been denervated, the elevated levels of the pentose shunt dehydrogenases were associated with increased activity of other NADP-linked dehydrogenases such as glutathione reductase and NADP-specific isocitrate dehydrogenase, whilst NAD-linked dehydrogenases, such as lactate dehydrogenase and  $\alpha$ -glycerophosphate dehydrogenase were significantly less than in the controls.

Garcia-Bunuel & Garcia-Bunuel (1967) reported that increases of collagen content were associated with the elevated activities of the pentose shunt dehydrogenases, and concluded that the increased enzyme activities were due to proloferation of connective tissue within the muscle. Smith (1965), in her observations of regenerating muscles (in which fibrosis is a frequent occurrence) reported the increased enzyme activity to be within the muscle fibres themselves rather than in fibrocytes, so that it seems that the participation of the connective tissue cells in the regenerative process does not necessarily account for the increased enzyme activities.

Unilateral phrenicectomy causes a massive increase in the activity of the hexose phosphate dehydrogenases, observable 1 day after denervation; this is sooner than has hitherto been observed for denervated muscles and seems likely to be related to the abnormal, hypertrophic response of the tissue, since an equally rapid decrease in content is observed after 5 days. The muscle at this time is synthesizing large quantities of nucleic acids, which would involve these particular enzymes for the production of pentose phosphates, but it must also be recognised that a supply of NADPH would presumably be required for the hypertrophic processes and the increased enzyme activity, could equally well be related to this function. Stewart(1955) found no detectable increase in collagen content in the denervated hemidiaphragm at this time. The apparent increase of enzyme activity between 10 and 15 days after phrenic nerve section might be associated with an increase in collagen concentration, since at this time atrophy of the non-collagenous protein is observed, so that, relatively, collagen concentration rises. A causal relationship however is not indicated.

A small but nevertheless significant increase in content of glutathione reductase is observed 3 days after denervation, and thus coincided with the increase of the pentose shunt dehydrogenases, but at 10 days a second peak of activity is also observed. The significance of the second peak is obscure; possibly it is related to the observed decrease of reduced glutathione content that takes place between 10 and 15 days after nerve section.

All of the three NADP-linked dehydrogenases dealt with so far are known to be found in erythrocytes in high concentration; the question thus arises: could the increases observed in the denervated hemidiaphragm be reasonably accounted for if an increase of blood content in the tissue was to take place, as suggested previously (see Introduction). The evidence presented suggests an increase of blood content in the hypertrophied hemidiaphragm that is unlikely to exceed 5%; the observed increase in the NADP-linked enzymes are very much in excess of this value, so that it seems likely that increased erythrocyte content in the muscle homogenates could not account for the changes. However if nerve section were to set up an inflammatory response that resulted in invasion of the tissue by antibody synthesizing plasma

cells and lymphocytes, then possibly a larger increase of pentose shunt dehydrogenase would be observed. Whether this would account for the recorded changes is difficult to determine in the absence of information relating to the enzyme content of the invading cells.

The decrease of NADP-linked isocitrate dehydrogenase, following closely the changes observed for other tricarboxylic acid cycle enzymes assayed in this investigation, demonstrates that denervated hemidiaphragm exhibits a substantially different behaviour, at least as regards the NADP-linked dehydrogenase activities, compared to other denervated muscles, regenerating muscles or to muscles from dystrophic animals.

The Effects Of Unilateral Phrenicectomy Upon The Quantities  
Of Acid Soluble Ninhydrin Positive Compounds Found In Rat  
Hemidiaphragm.

Whilst many of the amino acids of skeletal muscle show pronounced changes in concentration after denervation, it is not possible to indentify with any certainty the processes by which the changes have been brought about. Clearly the intracellular concentration of the free amino acid within the muscle fibre depends upon the equilibrium between those processes supplying free amino acids: i.e. the uptake of amino acids from the plasma; the intracellular synthesis of dietary non-essential amino acids; and the liberation of amino acid from protein catabolism; and those processes that cause depletion of the amino acid pool: i.e. protein synthesis; synthesis of non-protein nitrogenous compounds such as purines and pyrimidines; the oxidation of amino acids; and the loss of amino acids into the plasma. Changes in intracellular amino acid concentration, consequent of denervation, may thus arise through the mediation of one or a number of factors which may be operative, in both the spatial and temporal sense, in series and in parallel. Obviously the identification of causal relationships with respect to amino acid metabolism is an almost insurmountable task which requires

considerably more information than is furnished by mere measurement of amino acid concentrations. Nevertheless there are a number of points of interest which will be dealt with further as plausible consequences of denervation.

Whilst phrenic nerve section causes an increase in the concentration of total acid soluble ninhydrin reacting material in the hypertrophied muscle, it is clear that the concentration changes for the individual molecular species are not coincident, so that the changes presumably reflect the metabolic status within the cell at the various times after denervation. Although protein synthesis is undoubtedly the major synthetic process in which the free amino acids of muscle participate, the concentration of the precursors do not seem to reflect the amino acid composition of the total muscle protein. For example, Hider, Fern & London (1969) found that in rat skeletal muscle protein the content of glycine was identical to that of leucine, whilst it was clearly not the case with regard to the concentration of the free amino acids. Six species of amino acids account for nearly 75% of the total free amino acids of diaphragm muscle, and it is of some interest to speculate as to the roles that these six may play in non-protein synthetic reactions. Thus glycine, glutamine, and aspartate are involved in the

synthesis of purines and pyrimidines, compounds of paramount importance in muscle. Serine, and, by interconversion, glycine, is considered to act as a precursor for ethanolamine, phosphoethanolamine and choline for phospholipid synthesis. In contrast aspartate, glutamate and alanine, by transamination, are all converted to keto acids of considerable importance for energy generation in aerobic muscle. Following unilateral phrenicectomy synthesis of nucleic acids and membrane phospholipids is enhanced; the amino acid concentrations of glycine, serine and glutamine are all very much elevated during that period of the hypertrophy which it might be thought that these synthetic processes would require higher concentrations of the precursors.

Glutamate and aspartate essentially show a similar response, with two peaks of increased concentration superimposed upon an overall decrease. It is of interest that the timing of the peaks for glutamate coincide with the observed increased levels of glutamate dehydrogenase content, whilst it seems possible that the overall decrease of these two amino acids may be related to the demise of the mitochondria. It would be of considerable interest to know if a parallel decrease in the activity of the enzymes glutamate

pyruvate transaminase and glutamate oxaloacetate transaminase was observed on denervation. The increase of the alanine concentration may also be speculatively related to the decreased mitochondrial content in the hypertrophying tissue in as much as since fewer mitochondria are available for pyruvate oxidation and since there will be an overall requirement for NADH ultimately for synthetic purposes, thereby decreasing the likelihood of lactate dehydrogenase being operational, the production of alanine may be a mechanism by which the muscle removes pyruvate from the environment so that glycolysis may proceed without hindrance.

The oxidation of the branched chain amino acids, which in muscle is a significant process, also takes place in the mitochondria, and might thus be expected, all other things being equal, to lead to elevated concentrations of these amino acids; whilst this is so for leucine and to some extent valine, the interpretation of the response of isoleucine seems somewhat less tangible. Being essential amino acids and present in low concentrations, it is clear that a restriction upon the rate at which these amino acids are oxidized could be of importance in determining the overall rate of protein synthesis.

Concentrations of the basic amino acids in muscle are related to the  $K^+$  concentration; Christensen, Riggs, Fischer & Palatine (1952) showed that basic amino acids act as intracellular cations, and in cases of  $K^+$  deficiency are found in increased concentrations in muscle (Iacobellis, Muntwyler & Dodgen, 1956; Eckel, Pope & Norris, 1954). Gutmann et al (1966) reported an increase in  $K^+$  content following unilateral phrenicectomy, observable as soon as one day after nerve section, and reaching a maximum between 3 and 7 days after denervation. It might be expected therefore that the initial decrease of the basic amino acids reflects this increased  $K^+$  concentration; however, it is clear that the overall response of the basic amino acids does not mirror the  $K^+$  increase so that it seems likely that other processes are at work.

Of the non-amino acid ninhydrin positive compounds determined in this study the di- and tri-peptides carnosine ( $\beta$ -alanylhistidine) and reduced glutathione ( $\gamma$ -glutamyl-cysteinylglycine) are of more than passing interest. Aonuma, Hama, Tamaki & Okumura (1969) have established that orotic acid serves as the  $\beta$ -alanine precursor for anserine and carnosine biosynthesis. This being the case the rapid decrease of carnosine concentration at 3 days after nerve

section could be related to the requirement of the tissue to direct orotate towards pyrimidine synthesis. On the other hand, Manchester & Harris (1968) found that incorporation of  $^{14}\text{C}$ -orotate into RNA was only slightly elevated in the denervated tissue 2 days after nerve section whilst at 3 days a decrease was found. Incorporation of  $^{14}\text{C}$ -orotate into DNA though was greatly enhanced so that a shortage of orotate for incorporation into RNA or carnosine, unless compartmentation is significant, seems unlikely. As biosynthesis of carnosine also requires histidine the overall decline of the dipeptide may also be related to the decreased concentration of that amino acid. Carnosine has been suggested as being a physiological activator of myosin ATP'ase (Avena & Bowen, 1969) which it will be recalled decreases after phrenic nerve section (Hajek et al, 1967). Davey (1960) has suggested that the role of the dipeptide carnosine may be to act as a physiological buffer for muscle;  $\text{pK}_2$  for the compound is 6.8, very close to the intracellular pH of rat skeletal muscle. According to Davey, the concentration of carnosine is highest in those muscles that have low respiratory activity, so that during anaerobic glycolysis the buffering action will help prevent a decrease in intracellular pH. Atrophy of the "white" fibres in the hypertrophying hemidiaphragm may thus explain the decreased carnosine concentration.

The rapid and pronounced rise in content of reduced glutathione within the denervated tissue is perhaps not so surprising when it is considered that elevated levels of the tripeptide are correlated in some way with the processes of growth (Gregory, 1939). That the compound is found, except in one instance, exclusively as the reduced tripeptide argues strongly in favour of the maintenance of the reducing environment of the denervated muscle cell. The one exception, 3 days after nerve section, when less than 5% of the total glutathione was found as the oxidized compound, coincides with the first peak of glutathione reductase content, but more probably represents an **artifactual** error. The possibility of the increased glutathione concentration being responsible for the specific degradation of the mitochondria has been considered in a previous section.

The synthesis of phospholipids for membranous material has been mentioned earlier when it was suggested that serine might be of importance. The increased content of the amino acid could be related to the increased content of glyceraldehyde phosphate dehydrogenase, as they show essentially the same chronology, the reactions presumeably taking place

between 1:3-diphosphoglyceric acid and serine via 3-phosphoglycerate, hydroxypyruvate phosphate and phosphoserine, as suggested by Ichihara & Greenberg (1957). Cultured fibroblasts from embryonic chick heart muscle have been shown to be capable of synthesizing serine from  $^{14}\text{C}$ -glucose (Fischer, Fischer, Landschutz, Ehrensvarð, Rafelson & Stjernholm (1952)). Serine is also implicated in the synthesis of phosphatidyl ethanolamine by decarboxylation of phosphatidyl serine (Kennedy, 1961) but it may also be synthesized from CDP-ethanolamine and diglyceride as elucidated by Kennedy & Weiss (1956). CDP-ethanolamine is derived from the condensation of ethanolamine phosphate and CTP with the elimination of pyrophosphate. The amino acid analysis has demonstrated (Fig. 31 ) that the concentration of free o-phosphoethanolamine rises dramatically to a peak at 3 days after denervation and then falls, but after the 10th day rises again. The similarity to the response of the pentose shunt dehydrogenases may be more than just coincidental; the enzymes could provide a significant proportion of the NADPH of the muscle cell for synthetic purposes and it seems quite likely that the processes of production and utilization of synthetic precursors would be integrated.

## CONCLUSION.

The hypertrophy of the denervated rat hemidiaphragm is a complex process. To draw specific conclusions regarding individual enzyme levels from mere measurements of the activities of those enzymes is a hazardous procedure; further information is required before it is possible to describe with any certainty the metabolic behaviour of the denervated muscle fibre. Nevertheless, measurement of enzyme activity is a relatively easy procedure and the results can indicate those areas of metabolism that might be expected to yield fruitful results of further investigation. Obviously there is no point in repeating the speculations included in the discussion section; in most cases a straight forward measurement of metabolite levels ought to be sufficient to clarify any uncertainties. In other cases flux measurements might be necessary. Possible areas where useful information might be forthcoming include glycogen and mitochondrial metabolism since as soon as one day after denervation there are statistically significant decreases in enzyme activities associated with these areas. The logical next step in the investigation would be to see if it was possible to detect significant decreases of

glycogen phosphorylase or of NAD-linked isocitrate dehydrogenase in a time interval shorter than 24 hours; obviously inter rat variation will become a more significant factor when endeavouring to detect smaller decreases but with care it may well be possible to record a decrease that is statistically significant and for which the number of animals required is not unduly great. If such a decrease can be observed it would be of interest to compare the chronology of the decrease with the observation of Slater (1966) that failure of spontaneous miniature end plate potentials in the denervated neuromuscular junction takes up to 16 hours to reach completion and may well be related to axoplasmic flow.

It is of some interest to discriminate between those responses taking place in the denervated hemidiaphragm that may be considered to be the result of denervation per se and those responses that are associated directly with the hypertrophic process. Thus the loss of mitochondrial function and of glycogenolytic capacity may be ascribed to denervation since a similar response for both capabilities is observed in other denervated muscles that do not hypertrophy, although in the former example the loss of function, as demonstrated by the decrease of the T.C.A. cycle dehydrogenases, seems to take place earlier than in the other cases.

If a decay of glycogenolytic capacity, as evidenced by the decline of total phosphorylase activity is considered a denervation effect, then the maintained concentration of hexokinase can be considered as being a hypertrophy-associated response. This being so, it is clear that the use of the ratio of hexokinase to total phosphorylase activity to provide information regarding the proportions of fibre types present in a muscle population is not a valid discriminatory procedure. Neither can the activity ratios of creatine kinase and adenylate kinase be used for this purpose; recently Goldspink, Harris, Park & Pennington (1970) have also shown that for rat extensor digitorum longus and soleus muscles the ratio of activities: adenylate kinase/creatin kinase is not discriminatory for these two physiologically different muscles. Further information is necessary before it is possible to state whether the response of the nucleotide metabolizing enzymes is related to the hypertrophy, the denervation, or both.

During the hypertrophy of the denervated hemidiaphragm a proliferation of the sarcoplasmic reticulum has been observed (Hajek et al, 1967) and possibly associated

with this response are the increases found in the total activities of lactate dehydrogenase and glyceraldehyde dehydrogenase, both enzymes now known to be located within the sarcotubular system (Fahimi & Karnovsky, 1966). It would be interesting to ascertain whether the proliferation of the sarcoplasmic reticulum was observed in all fibres present in the muscle population, or whether it was restricted to one particular fibre type. Obviously similar considerations apply also to a study of the degradation of the mitochondria.

A change in the lactate dehydrogenase isoenzyme proportions in denervated muscles towards the "foetal" type has been considered as being evidence for a "de-differentiation" process. In the denervated hemidiaphragm, no significant change in the lactate dehydrogenase activity ratio was observed until 7 days after nerve section, and even then the alteration was not maintained. Recently Schiaffino & Settembrini (1970) found that the post natal differentiation of the sarcotubular system in developing rat skeletal muscle was not impaired by neonatal denervation, evidence the authors argued, against the de-differentiation process. It is the present author's

opinion that it is not necessary to postulate de-differentiation as a consequence of denervation; the changes that are observed following nerve section could equally well be explained by alternative means. For example in the denervated hemidiaphragm, the lactate dehydrogenase activity ratio and the myoglobin content could just as easily be related to the oxygen tension within the tissue as to de-differentiation. From an experimental point of view, it is probably considerably easier to test a hypothesis based upon physical or chemical causes than one depending upon genetic retrogression.

The increased activity of the pentose shunt dehydrogenases in the early stages of the hypertrophy can be rationalised as a response to provide ribose phosphates for nucleotide biosynthesis; the decreased glycogen content at this time has been suggested as being a possible source of hexose for this purpose. Obviously an investigation of the flux of radiolabelled glucose through the glycolytic and pentose shunt pathways would be necessary to throw light upon this point. In contrast to other denervated and dystrophic muscles, the activities of other

NADP-linked dehydrogenases do not show the same response (see McCaman, 1963). The isocitrate dehydrogenase closely follows the behaviour of the other T.C.A. cycle dehydrogenases and thus must be considered a denervation response. The changes in the activity of glutathione reductase after unilateral phrenicectomy are, to put it bluntly, obscure. Possibly it is related to the glutathione content of the hypertrophying tissue and which may be responsible for the degradation of the mitochondria.

Obviously in covering such a wide spectrum of enzymes only a smattering of knowledge can be gained at each point of metabolism, whilst the amount of speculation that arises is seemingly boundless. Speculation and uncertainty can only be eliminated by further investigation. The denervated diaphragm of the rat is a convenient and interesting preparation with which to work and it is hoped that further study of this system will contribute to the sum of understanding of muscle processes. The difficulty as with any study of this nature is to know where to begin and which particular lines of investigation are likely to be rewarding and thus deserve special emphasis. Perhaps the most

difficult point of all though is how to establish a causal relationship, when there is an unknown number of responses between the causative agent and the response that can be measured.

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