A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY ENTITLED:-

BIOCHEMICAL ASPECTS OF CHRONIC HYPEROXIA IN MAMMALS

BY

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TO MY PARENTS

'We live in oxygen, the essential agent of respiration; oxygen bathes all the bodies and objects which surround us, and its great reactivity is well known. If then one were to effect by any means, a moderation or an excitation of the universal avidity of such an element, would there not result new perspectives in the chemistry of nature, living and dead?'

Moreau & Dufrais (1926)

ABSTRACT

The primary objective of the work described in this thesis was to investigate some of the effects of chronic oxygen toxicity in mammals. This thesis describes *in vivo* changes in certain metabolites and enzymes following the exposure of mice and rats to increased concentrations of oxygen at atmospheric pressure.

The introductory section reviews the historical background to oxygen toxicity, delineates its two forms and describes their aetiology. The physical and chemical properties of oxygen which contribute to its unusual reactivity are discussed, together with the reactive species of oxygen which may be responsible for its toxicity. To complete the introduction, the known effects of oxygen on metabolism are reviewed.

In order to pursue these studies it was necessary to design an apparatus capable of providing a controlled high oxygen atmosphere. Its construction and operation is described.

Results obtained indicated that the levels of the protective enzymes superoxide dismutase and catalase varied in lung, liver and brain of mice following exposure to increased oxygen levels. Overall protein levels were also found to vary, so an experiment involving a polyacrylamide gel separation was performed to reveal any qualitative differences. Another experiment monitored the changes in free amino acids in brain, liver, lung and muscle. A few possible deviations from normal were detected but these changes were not consistent between the tissues studied.

Results are presented of the changes in some gross physical parameters and the effect of 3 prostaglandin inhibitors on these *in vivo* changes. Other experiments revealed the changes in haematocrit, in retinol and in haemoglobin levels.

For comparative purposes some of the above measurements were also applied to samples of body fluids from men suffering a mild experimental oxygen toxicity.

The relevance of these results to the toxic effects of oxygen in mammals is discussed.

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ABBREVIATIONS

atm	atmosphere pressure (absolute)
CNS	Central Nervous System
DTU	Deep Trials Unit
EDU	Experimental Diving Unit
EPR	Electron Paramagnetic Resonance
GABA	γ-amino butyric acid
GAD	Glutamic acid decarboxylase
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
G6PDH	Glucose-6-phosphate dehydrogenase
GSH	Glutathione
Hb	Haemoglobin
LDH	Lactate dehydrogenase
LD_{50}	Median lethal dose
NPSH	Non-protein sulphydryl groups
OAP	Oxygen at atmospheric pressure
OHP	Oxygen at high pressure (greater than 1 atm)
6-OHDA	6-Hydroxydopamine
PDH	Pyruvate dehydrogenase
PG	Prostaglandin
-SH	Reduced sulphydryl groups
-SS-	Oxidised sulphydryl groups
SEM	Standard error of the mean

All other abbreviations are as used by the Biochemical Journal (see the Biochemical Society (1972) instructions to authors.

INTRODUCTION

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INTRODUCTION

1. OXYGEN TOXICITY: AN HISTORICAL PERSPECTIVE

Priestley (1775) is credited with publishing the first accounts of the isolation of oxygen. In his experiments on different kinds of air he showed that air was not elementary but a composition, and isolated a gas with the properties of the smaller portion of atmospheric air. Although his experiments were not performed in an orderly sequence and his results often inaccurate, he drew several startling inferences from his findings.

"From the greater strength and vivacity of the flame of a candle in this pure kind of air, it may be conjectured, that it may be peculiarly salutary to the lungs in certain morbid cases, when the common air would not be sufficient to carry off the phlogistic putrid effluvium fast enough. But, perhaps, we may also infer from these experiments that though pure dephlogisticated air might be very useful as a medicine, it might not be so proper for us in the usual healthy state of the body; for as a candle burns out much faster in dephlogisticated than in common air, so we might, as may be said, live out too fast, and the animal powers be too soon exhausted in this pure kind of air." On breathing oxygen he commented: "The feeling of it to my lungs was not sensibly different from that of common air; but I fancied that my breast felt peculiarly light and easy for some time afterwards. Who can tell but that, in time, this pure air may become a fashionable article in luxury."

Priestley was subsequently proven to be remarkably accurate in these conjectures.

Scheele (1777) independantly discovered oxygen at about the

same time as Priestley, but his work was not published until after Priestley's. Scheele's experiments were more orderly, logical, and accurate than Priestley's. Initially Scheele's aim was to elucidate the chemical nature of fire, but he soon found that the composition of the air was an integral part of the problem.

Scheele showed that air had two major components: vitiated air (nitrogen) and a component which was essential to combustion, living plants and animals, and which was absorbed by some oxidation processes. On discovering oxygen (fire air) he showed it to have the same properties as the smaller component of atmospheric air. However, when he tried to germinate peas in pure oxygen; he observed that they would put out roots but that they would not shoot. This is the first observation of the toxic effects of oxygen.

Both Priestley and Scheele were unable to see that the phlogiston theory of combustion was fallacious. It was left to the astute Lavoisier to recognise the real importance of oxygen. Lavoisier, who had narrowly missed discovering oxygen himself, used the discovery to overthrow the prevalent phlogiston theory, and to develop a new order to chemistry (French 1941). Lavoisier, investigating an observation communicated to him by Priestley autopsied guinea pigs which had died in oxygen. He noted that death had been caused by a "fievre ardent" and a "maladie inflammatoire". 'The flesh was a very red colour, the heart livid and turgid with blood, especially the right auricle and ventricle. The lungs were very flaccid, but very red even externally' (Bean 1945).

The toxic effects of oxygen were clearly exposed by Bert in 1878 in his classic work on barometric pressure which covered many aspects of high and low pressure physiology.

Bert's work was originally concerned with showing that the effects of pressures below atmospheric were almost exclusively due

to the low oxygen tensions and applying Dalton's law of partial pressures to physiology. In the course of his experiments he noted that high tensions of carbon dioxide were also capable of causing death. In persuing this work experimentally he conducted a series of experiments which revealed to him that increased partial pressures of oxygen were toxic; even when the carbon dioxide was removed using potash. His conclusion that this phenomenon "...can be called for convenience and in spite of the expression, a poisoning by oxygen", indicates that this result was unexpected. His scientific curiosity naturally led him to further experiments.

Using birds (mainly sparrows) and frogs, he observed the now well known convulsions that occurred when an oxygen tension in excess of 3·0 atm was employed. He further noted that animals which were removed from pressure soon after the convulsions began could survive, but that increasing oxygen tensions and prolonged exposures resulted in the death of the animals. Bert further showed that anaesthetics such as ether could prevent the onset of convulsions, but could not prevent the death of the animal, thus showing that death was not due to the convulsions *per se*.

Bert's experiments using dogs, permitting analysis of blood gases, have been severely criticised on the grounds that carbon dioxide was allowed to accumulate, and decompression was often rather rapid considering that superoxygenated air (usually about 60-90% oxygen) was employed rather than pure oxygen, and these defects have been said to invalidate his conclusions. However his earlier experiments using birds and frogs do not suffer from these experimental defects. In the experiments using dogs, the convulsions usually appeared before excessive amounts of carbon dioxide had accumulated and before decompression was performed. In addition, as pointed out by Bert, the effects of carbon dioxide were entirely different; carbon dioxide

induces a kind of anaesthesia, not convulsions.

That oxygen does not merely cause convulsions was demonstrated in his early experiments on asphyxia. In these experiments using superoxygenated air in closed vessels, his animals died well before the oxygen concentration had reached the theoretical minimum necessary for survival, even though the carbon dioxide was absorbed using potash. In later experiments in which silkworms, chrysalids, spiders, bees, mosquito and midge larvae, earthworms, snails, frogspawn, tadpoles, eels, carp, algae and higher plants were exposed to high tensions of oxygen at atmospheric pressure, all died when kept at a tension above 0·8 atm for several days. These experiments demonstrated not only the universal nature of oxygen toxicity, but also that even oxygen tensions slightly above normal were toxic.

Lorrain Smith (1899) attempted to use oxygen at high pressure as an antibacterial treatment, but found that oxygen above 1 atm produced pneumonia-like symptoms in healthy animals. Accordingly he carried out research to determine the effect of oxygen on healthy animals. In his experiments he tested the effects of a wide range of oxygen pressures varying from 0·4 to 0·5 atm and demonstrated the now well documented congestion, oedema, inflammation, haemorrhage and consolidation of the lungs which occurred when animals died in oxygen tensions of 0·7 atm or greater. This pathology due to oxygen at atmospheric pressure has often been referred to as the Lorrain Smith effect, while the convulsions due to high pressures of oxygen has been referred to as the Paul Bert effect, thus acknowledging the enormous contributions of these scientists to the study of oxygen toxicity.

The mice used in Lorrain Smith's experiments were found to show a large variation in their resistance to oxygen, but oxygen above 1 atm pressure was shown to be uniformly fatal. The greater the

tension of oxygen, the shorter the time taken for death to occur. Lorrain Smith confirmed the Paul Bert effect in birds (occurring at 3·0 atm) and in mice (occurring at 4·5 atm) and noted that these animals showed similar changes in the lungs to those exposed to oxygen at atmospheric pressure. He further noted that the effects of oxygen persisted on returning the animals to normal air and were frequently inconsistent with their recovery.

After the work of Lorrain Smith, investigations into hyperoxia lapsed for a few years. Later, with the development of diving in the mid-thirties to the mid-forties, oxygen toxicity once more became a problem, and physiological investigations were undertaken by Campbell, Behnke *et al* and Bean.

At about this time a biochemical explanation was sought, and work was performed concurrently on both sides of the atlantic. Stadie, Riggs and Haugaard in America, devised a Warburg apparatus in a pressure chamber which could be operated from the outside. Mann & Quastel, and Dickens working in Britain, used either pure oxygen at atmospheric pressure, or worked with oxygen inside a chamber of compressed air. These groups working independently on oxygen toxicity *in vitro* came to similar conclusions.

In the fifties a new surge of interest in hyperoxia developed with the increasing use of oxygen in medicine. The similarity of the effects of radiation and oxygen on tissues, led Gerschman *et al* (1954a) to propose a new mechanism of oxygen toxicity - the oxygen radical theory. The synergistic effect of oxygen and radiation was cited as supporting evidence. This theory did not meet with immediate approval. It was also proposed by Gerschman (1963) that oxygen toxicity did not begin at a certain concentration of oxygen, but that the effects of oxygen were continuous. Thus the normal life-span of an organism could be considered as the survival time for that organism exposed to 0·2 atm of oxygen (air at sea level).

Finally an interesting observation in fruit flies was reported by Fenn (1965), who showed that nitrogen at high pressures (20-30 atm) potentiated the effects of oxygen.

Medicinal Uses of Oxygen: The use of oxygen as a therapeutic agent was introduced soon after its discovery. Both Bert (1878a) and Bean (1945a) quote passages showing that there were early advocates of the use of oxygen in medicine and also that the irritant action of oxygen on the lungs was recognised. Later on, in the early 19th century, the therapeutic use of oxygen waned, and a new treatment, the use of compressed air, became popular among the rich. It is clear from accounts by Jacobson _et al_ (1965) and Bert (1878b) that many marvellous claims were made for this treatment, which usually involved increases in pressure of only about 0·2 to 1·0 atm, with very little evidence for the efficacy of the treatment. It appears that these practices were disappearing at about the turn of the last century.

After the second world war, oxygen was employed to aid premature babies suffering from respiratory difficulties. Usually 100% oxygen was administered by the use of incubators. As a result of this therapy two new ailments were reported: retrolental fibroplasia and hyaline membrane disease. Both hyaline membrane desease (Bruns & Shields 1951; 1954) and retrolental fibroplasia (Patz _et al_ 1952; Gerschmann _et al_ 1954c) were subsequently shown to arise as a consequence of the administration of high concentrations of oxygen.

Despite this rediscovery of the toxicity of oxygen by the medical profession, another new ailment appeared in the medical literature during the mid-sixties, 'respirator lung', showing remarkable similarities in its description to that of hyaline membrane disease. Not surprisingly this ailment was also found to be attributable to the tension of oxygen employed rather than by use

of the respirator *per se* (Nash *et al* 1967; 1971).

Although the toxicity of oxygen places serious constraints on its indiscriminate use, oxygen therapy still remains a valuable medical treatment provided that due care is exercised. Some of the wide ranging uses of hyperbaric oxygen therapy have been reported by Whipple (1965) and Smith (1978).

2. SYMPTOMATOLOGY OF OXYGEN POISONING

A. Delineation of 'Chronic' and 'Acute' Oxygen Toxicity

Oxygen toxicity can be arbitrarily divided into two conditions on the basis of the symptoms produced by different levels of toxicity. Firstly, a 'chronic' condition in which the symptoms are mainly of pulmonary origin, usually produced experimentally by exposure to increased partial pressures of oxygen or pure oxygen at atmospheric pressure for several days. Secondly, an 'acute' condition produced by pure oxygen at greater than 3 atm absolute pressure (or compressed air with an equivalent partial pressure of oxygen) where central nervous system involvement becomes prominent and the pulmonary symptoms are of lesser importance. The symptoms of this acute condition typically appear within a timespan of a few minutes to a few hours depending on the partial pressure of oxygen employed.

Chronic Oxygen Toxicity: In the chronic condition, using laboratory animals at 1 atm of pure oxygen, the first observations are that the animals become very active and restless eating their food greedily (Smith 1899). This is followed at about 48 hours after commencement of treatment, by lethargy, anorexia and sometimes vomiting. Dyspnoea becomes apparent and progressively more intense.

Terminal stages of toxicity (appearing normally after 3-4 days) are characterised by cyanosis, laboured or gasping respiration and frothing or bloody sputum. Throughout the period of toxicity the animals lose weight and condition due to the anorexia.

In experiments using human subjects under similar condition, subjective feelings have also been noted. Between 1 and 3 hours after commencement of oxygen breathing, impaired neuromuscular coordination and a waning power of attention are noted which require an increased effort to maintain these functions at the same level. After 3 hours a hyperpnoea may occur, followed at about 6 hours by substernal distress noted particularly on deep inspiration (Behnke _et al_ 1934). Soreness of the respiratory passages develop, beginning as a mild irritation which may induce a dry cough. These symptoms become progressively more intense until each inspiration is painful and coughing is uncontrollable.

Acute Oxygen Toxicity: A partial pressure of oxygen greater than 3 atm results in the occurrence of convulsions which in human subjects have been likened to a Grand Mal epileptic fit (Davies & Davies 1965). Using human subjects, 3 hours at 3 atm produced a progressive contraction of the visual field, dilatation of the pupils and some impairment of central vision. In the fourth hour at 3 atm there was an abrupt rise in both systolic and diastolic blood pressure, a rise in pulse rate, extreme pallor of the face, dizziness, and a feeling of impending collapse. At 4 atm oxygen, convulsions occurred in one subject and syncope in another after 45 minutes of exposure (Behnke _et al_ 1935).

In rats prior to convulsions local muscular twitching occurs especially about the eyes, mouth and forehead, small muscles of the forelimbs may also be involved. Restlessness, face washing movements and vigorous shaking as if drying their fur may also occur. These

symptoms increase in severity over a period of a few minutes to an hour, followed by an abrupt wave of excitation and the rigid tonic phase of convulsions lasting approximately 30 seconds. Clonic contractions then occur which become less violent over approximately 1 minute leading to a return of coordination. Respiration ceases, and loss of consciousness occurs at the tonic phase and does not return until muscular coordination is regained. The convulsions are followed by hyperpnoea due to the anoxic period (Davies & Davies 1965). Return to a normal partial pressure of oxygen does not necessarily result in an immediate return to normality. Animals may continue convulsing during and after the decompression even when the decompression is slow enough to ensure that no intravascular bubble formation occurs. After the decompression animals may show minor symptoms e.g. stupor, hyperexcitability, motor dysfunction and dyspnoea, either transiently, or for several days. Repeated exposure to oxygen at high pressure (OHP) over a period of days resulted in irreversible spastic motor paralyses which in mild cases involved just the forelimbs, but in the worst cases affected the entire body musculature (Bean & Siegfried 1945). Central nervous system lesions have been demonstrated pathologically in rats showing paralyses (Balentine 1977 and references therein).

Oxygen tensions under 3 atm do not usually result in convulsions; the death of the animals in these instances is usually attributable to the pulmonary pathology.

B. Pathological Changes Due To Oxygen At Atmospheric Pressure

Since the studies of Lorrain Smith in 1899 it has been recognised that oxygen tensions of about 1 atm over a period of days cause pneumonia-like symptoms in the lungs of healthy animals. The principal and most obvious finding is the swelling of the lungs, which become

inflamed and oedematous to such an extent that they may sink in fixing fluid. His microscopic observations also revealed extreme congestion of the lungs, and also, on occasions, a less pronounced congestion of the liver, spleen and kidneys.

These findings have been amplified by many authors (see Clark & Lambertsen 1971 and Bean 1945). An account of typical findings in these studies is presented below. The lungs show the greatest changes due to their direct contact with the respired gas and so are dealt with in some detail. For completeness, the effects on some other tissues are also noted.

i) Lung Pathology

In rats exposed to 0·7 atm of oxygen for 10 days the change in pathology was relatively small compared to slightly higher pressures of oxygen.

The cells affected most were the endothelial cells. The endothelium was thicker than normal and had the appearance of epithelium. The cell surface was wrinkled rather than smooth and the number of endothelial lining membranes were increased. In places the endothelium had lifted off the basement membrane, but the basement membrane was not denuded at any point.

In the interstitium the number and size of collagen bundles were increased, but the extracellular space was less dense than normal and the collagen bundles were separated in many places.

The alveolar lining was thicker than normal. This was mainly due to an increase in the number of type II granular pneumocytes; these are cuboidal or spherical instead of squamous like the type I pneumocytes. The alveolar capillaries were covered by a thin cytoplasmic extension from the type I cells which was considerably thickened after 10 days in oxygen. The cytoplasm of the type II cells

contained much rough endoplasmic reticulum as normal, but there were fewer dark granules containing dense lamellated material and these were less regularly arranged than in the controls. There was also an increase in the number of extracellular alveolar mambranous structures noted.

The alveolar capillaries were embedded more deeply into the septum rather than bulging into the alveolus, and in the septum, some small capillaries were found which had no direct contact with the alveolus (Schaffner _et al_ 1967).

Rats exposed to 0·85 atm oxygen for 5-7 days showed additional changes to the above.

Membranous pneumocytes showed cytoplasmic oedema as well as short villus like extensions on the alveolar surface. On infrequent occasions these cells also showed disruption, fragmentation and necrosis resulting in the denuding of the basement membrane. On the fifth day and after, the type II pneumocytes, which appear to be more resistant to the effects of oxygen, sometimes showed degenerative changes. These included large cytoplasmic vacuoles, elongated mitochondria (on day 6) and on the seventh day an increase in the number of mitochondria and moderate pleomorphism (cup shapes). Other mitochondrial changes noted were the increase in density and granular nature of the matrix. The cup-shaped mitochondria were often surrounded by endoplasmic reticulum showing signs of ribosome loss. Typically the cytoplasmic membrane was filled with free ribosomes. Once more the number of type II cells was increased.

In the alveoli sometimes an accumulation of oedema fluid, membranous-like material, lamellar whorls, myelin figures and conglomerations of lamellar bodies were present.

In the capillaries, aggregation of thrombocytes and leukocytes was common, and occasionally skeins of fibrin were seen, suggesting

disseminated intravascular coagulation (Rosenbaum et al 1969; Yamamoto et al 1970).

Using 0:98 atm oxygen, Kistler et al (1967) demonstrated similar changes again in rats. It was shown using morphometric methods, that there was a significant enlargement of the interstitial space after 48 hours and again at 72 hours, and that the air-blood barrier doubled in thickness over 72 hours; primarily due to the increase in thickness of the interstitium. There was also about a 50% decrease in capillary blood volume and capillary endothelial surface area. Further structural alterations in lung tissue were noted. Oedema that was taken up between the basement membranes at 48 hours became more granular after 72 hours and also contained numerous leukocytes and thrombocytes. Interstitial macrophages also appeared, together with cell debris from destroyed capillaries.

The endothelial cells suffered greater damage than in the previous less toxic regimes. Although at 48 hours the endothelial cells were mostly normal, at 72 hours a large proportion of these cells showed drastic changes. Tight junctions could no longer be distinguished, some cells had lost all their organelles and in others the cytoplasm was condensed and partially fragmented. In places, where the endothelial cells were completely destroyed, the basement membranes were covered with fibrin strands and in contact with erythrocytes, but the capillaries appeared to be lacking plasma.

Despite the destruction of the endothelium, most of the epithelium still remained intact and relatively normal. Only in rare areas was the entire air-blood barrier torn, forming a continuity between the blood vessels and alveoli, with fibrin masses extending from the tissue space into the exudate filled alveoli.

At 72 hours exposure, two thirds of all the alveoli were rendered useless with a heterogenous exudate containing fibrin strands,

cell debris and myelinated material forming highly ordered structures.

In a study where mice were exposed to 90% oxygen for 7 days, Adamson et al (1970) demonstrated similar changes to those expounded above. It is generally accepted that mice are more susceptible than rats to oxygen toxicity. Whereas most rats exposed to 0·85 atm oxygen for 1 week survive and are then able to 'tolerate' periods of 1 atm oxygen, in this study only about 10% of the mice survived. It was noted that the mice that survived showed a differing pathology to those that succumbed. The latter animals showed a gross lung pathology resembling hyaline membrane disease where the lungs are plum coloured, heavy, and solid with exudate. In these animals, although the type II pneumocytes were generally well preserved, the type I cells had become necrotic, and the basement membrane was covered with cell debris and fibrin strands which constituted a hyaline membrane. In the animals that survived, destruction of the type I cells did not occur. It has been suggested (Adamson & Bowden 1971) that the mucopolysaccharide sialomucin when present in sufficient quantity protects the epithelial cells and that those cells with a thinner layer of sialomucin are more susceptible to the cytotoxic effects of oxygen.

Kapanci et al (1969) and Kaplan et al (1969) monitored the effects of 1 atm oxygen on monkeys for periods up to 12 days and obtained similar results to Kistler et al (1967) working on rats, with a couple of notable exceptions.

The study on monkeys revealed a destruction of 50% of the lung capillaries similar to that reported for the rat. However in the monkey, morphometric measurements showed that the capillary volume and surface areas of capillaries were the same as control values: probably due to the opening of 'dormant' capillaries. Secondly, in the monkey, 90% of the alveolar type I epithelium was destroyed by the fourth day breathing 1 atm oxygen, with resultant formation of

hyaline membranes. Secondary to the destruction of the type I cells was a proliferation of type II epithelium between days 4 and 8 restoring the integrity of the alveolar epithelium and permitting a 50% survival of the monkeys.

Despite the general similarity of the pattern of findings between these studies, it is apparent that there exists between these species a considerable variation in their tolerance to oxygen, and the time course of their response. However, the true significance of a comparison between these studies is obscured by the different levels of toxicity employed by the different groups and the wide variation of response from animals of the same strain.

Finally, a contributory factor to the lung pathology has been reported by Berfenstam et al (1958b). One of the earliest changes in rabbits exposed to high concentrations of oxygen at atmospheric pressure is the disruption of the ciliary transport mechanism. The cilia become uncoordinated, with some patches beating transversely, some patches beating more slowly, and some not at all. This observation would, in part, help to account for the accumulation of alveolar debris due to the inability of the lung to clear it.

ii) Pathology of Other Organs

Lung tissue has received most of the attention in the field of oxygen toxicity being the tissue in direct contact with the respired air, and in chronic oxygen toxicity the first organ to fail in its function. However, the few studies performed on other tissues have shown that the toxic effects of oxygen are not confined to the lung.

Berfenstam et al (1958a) noted that abacterial myocarditis was a frequent finding in rabbits kept in 0·8 atm of oxygen. Caulfield et al (1972) investigated the effect of 1 atm oxygen on striated and cardiac muscle in guinea pigs, hamsters and rabbits. An exposure

of 48 hours, which causes minor pulmonary damage was shown to cause lesions in both striated and cardiac muscle. Electron microscopy revealed changes with as little as 24 hours exposure. In the biceps the changes were mainly mitochondrial and resembled the changes reported in type II alveolar cells after 6-7 days at 0·85 atm oxygen; mitochondrial swelling, loss of orientation of cristae, and in severe cases, replacement of mitochondrial sites with myelin figures. Sometimes the most severe mitochondrial changes were accompanied by sarcotubular swelling and loss of striations. The most badly affected muscles were the heart, diaphragm and the intercostals, indicating that perhaps these effects were mediated partially by diffusion of oxygen from the lungs in addition to the vesicular transport of oxygen. In the diaphragm and intercostals, deterioration of the filamentous array was noted which started with distortion of the Z bands and culminated in the total loss of Z and I bands leaving only myosin filaments in an organised array. Total loss of Z and I bands was often accompanied by loss of the plasma membrane and eventually the complete dissolution of the cell. An essentially similar pathology was presented of changes in heart muscle by Hughson et al (1977) using a hyperbaric exposure of 5 atm oxygen for up to 4 hours.

In an electron-micrographic study on rat livers exposed to 1 atm oxygen, a loss of glycogen, enlargement of mitochondria and development of bizarre mitochondrial shapes were reported after a 24 hour period (Schaffner & Felig 1965). These authors also cited a personal communication (W. Mautner) that similar changes had been observed in kidney. Further electron micrographic observations were made of the effect of 1 atm oxygen on livers of monkeys (Schaffner et al 1966). This study was continued for 15 days, with the most severe changes appearing between 3 and 9 days. Again the loss of

glycogen granules and rough endoplasmic reticulum was noted after 24 hours. Later changes were harder to delineate. These changes; appearance of autophagic vacuoles, increase of smooth endoplasmic reticulum and increase in size and change of shape of mitochondria after 3 days exposure were said to be of lesser importance due to the finding of occasional autophagic vacuoles and very variable mitochondrial shapes in controls. These authors commented that they believed the changes to be adaptive rather than degenerative. Examination of the photoelectronmicrographs for 3 and 9 days exposure presented in that paper revealed the endoplasmic reticulum to consist almost entirely of small vesicles rather than a competant internal structure. This feature, although difficult to define objectively or quantitate, would appear to be of consequence in the pathogenesis of oxygen toxicity.

Degeneration of testicular germinal epithelium has been reported in adult male hamsters exposed to 0·7atm oxygen for periods of 3-4 weeks (Gerschmann 1963). Various degrees of injury were recorded. In the worst cases the seminiferous epithelium was depleted of all but the sertoli system.

The effects of oxygen on the eye include visual cell death, retinal detachments, cytoid body formation, and in newborn animals retrolental fibroplasia (Nichols & Lambertsen 1969). Retrolental fibroplasia has been described as endothelial and glial cell proliferation, together with neovascularisation in the nerve fibre layer which extends from the retina towards the vitreous (Reese *et al* 1952).

Finally the effects of OHP on adrenal glands have been described by Bean and Johnson (1954). The gross effects are a change in colour and an increase in size and weight. Histologically a thickening of the zone fasciculata, a reduction of its lipid content and hypertrophy

of its constituent cells were noted. These changes were also seen to a lesser extent in the zona reticularis. Because these changes are also seen in the adrenals as a result of other types of stress, it is doubtful that they represent a direct toxic action of oxygen in that tissue.

3. OXYGEN AND ITS REACTIVE FORMS

Oxygen is an oxidising agent for many organic substances present on earth. Some of the more reactive organic molecules react spontaneously or explosively with oxygen. However, despite the fact that about 21% of our atmosphere is oxygen, many highly reduced organic molecules are not oxidised by oxygen at appreciable rates at normal temperature and pressure. It is this property of oxygen which allows living organisms to use reduced organic molecules as energy stores and oxygen as a terminal oxidising agent.

This dichotomy in the properties of oxygen show it to be a molecule potentially injurious to life. In order to see the reasons for this unusual reactivity of oxygen it is necessary to look at the electronic structure and physical properties of oxygen.

A. Electronic Structure and Physical Properties of Oxygen

Oxygen, atomic number 8, usually exists as a paramagnetic dimer in its ground state. This can be explained by looking at its molecular orbital description (figure 1). The molecular orbital description for oxygen can thus be written:

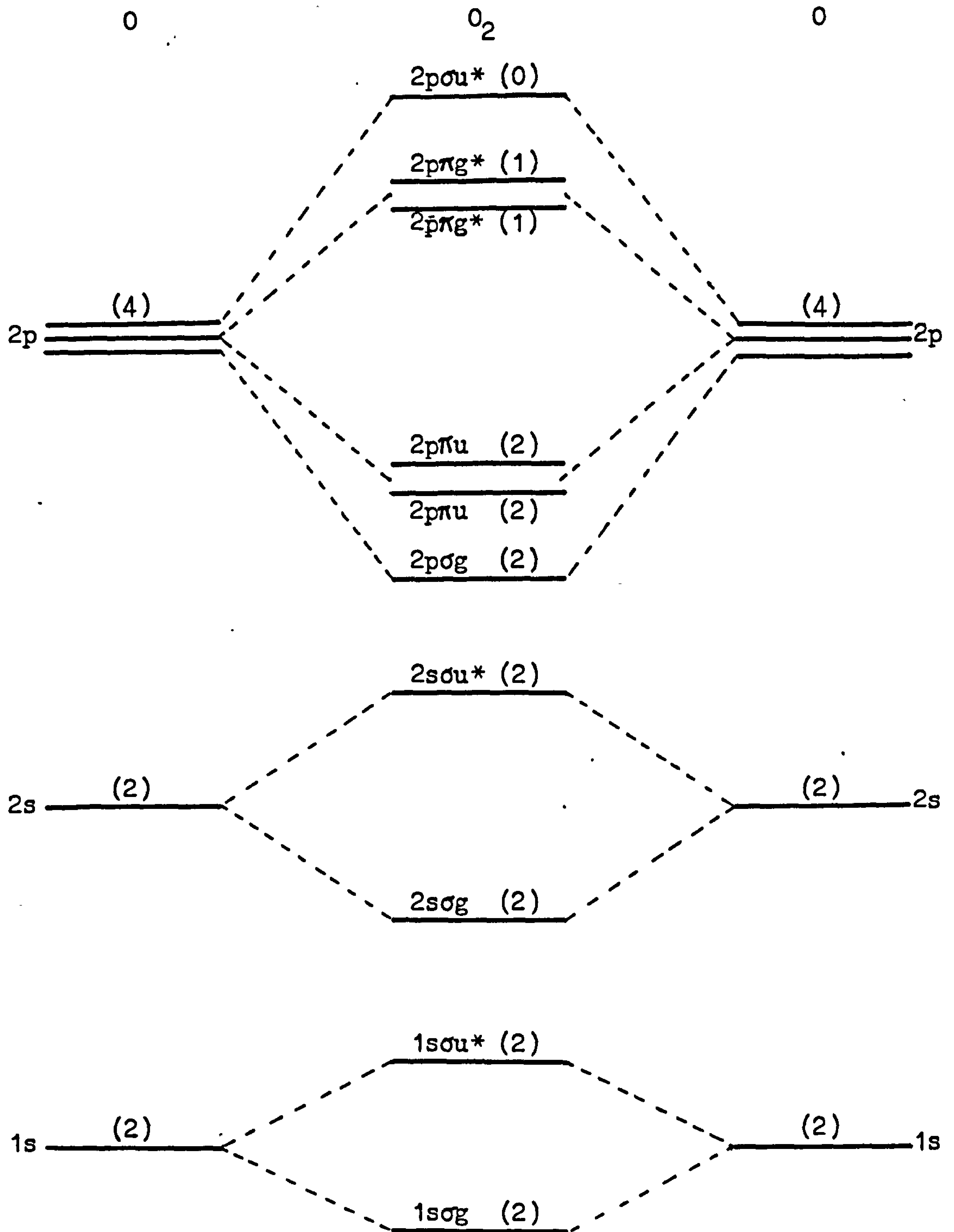
$$(1s\sigma g)^2(1s\sigma u*)^2(2s\sigma g)^2(2s\sigma u*)^2(2p\sigma g)^2(2p\pi u)^2(2p\pi u)^2(2p\pi g*)^1(2p\pi g*)^1$$

* indicates an antibonding orbital

g (gerade) means even) these terms relate to the symmetry
u (ungerade) means odd) of homonuclear diatomic molecules

The two electrons in the 2pπg* orbitals are unpaired and in the ground state have parallel spins to minimise electronic interactions. This accounts for the paramagnetism of ground state oxygen. It can

Figure 1: Molecular Orbital Diagram of Oxygen



figures in brackets denote number of electrons

also be seen that there are three bonding p orbitals and two 'half' antibonding p orbitals giving molecular oxygen its double bond character.

The $2p\pi g^*$ orbitals are doubly degenerate and taking electron spin into account (electron spin imparts angular momentum), there are six ways that the 2 electrons can be distributed into 2 orbitals, giving rise to 3 separate energy states. There are three equivalent distributions in the triplet ground state, 2 equivalent distributions in the lower singlet state and 1 distribution in the higher energy singlet state. However, the distribution of these electrons can be presented in the following simplified picture.

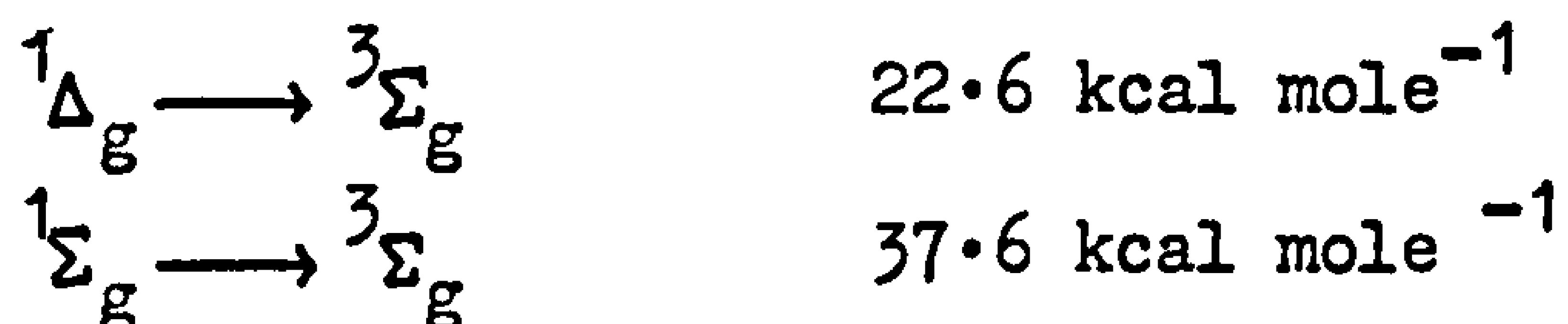
In the ground state each electron occupies a separate orbital and the electrons have parallel spins. This results in a zero net angular momentum, therefore it is a Σ state. It has a net spin (S) of 1, thus the multiplicity $M = 2S + 1 = 3$ - a triplet state. Ground state oxygen is thus designated a $^3\Sigma_g$ state.

In the next highest state the electrons occupy the same orbitals and have opposite spins, giving a net angular momentum of +2 or -2 and it is therefore a Δ state. The net spin is zero, $M = 2S + 1 = 1$, so it is designated a singlet, $^1\Delta_g$ state.

In the highest state both electrons occupy different orbitals with opposite spins. Again the net angular momentum is zero giving a Σ state, but the net spin is zero making this a singlet, $^1\Sigma_g$ state.

In most molecules the singlet state lies lowest and the triplet state highest. However due to Hund's rule which states that for a given configuration, the state of highest multiplicity lies lowest, in oxygen the triplet state is the lowest.

The energy differences between the three states described above are as follows (Kearns 1971).



$$^1\Delta_g \longrightarrow {}^3\Sigma_g \qquad 22{\cdot}6 \text{ kcal mole}^{-1}$$
$$^1\Sigma_g \longrightarrow {}^3\Sigma_g \qquad 37{\cdot}6 \text{ kcal mole}^{-1}$$

However singlet-triplet transitions are very highly forbidden in light molecules (composed of atoms from the first row of the periodic table). As most other molecules are singlet in the ground state, a reaction involving one atom of oxygen (a two electron transfer) would involve a spin forbidden transition. This is why generally oxygen is fairly unreactive. Reactions with oxygen therefore take place through either: an excited singlet oxygen state, an excited triplet sensitiser molecule, a radiationless transition (e.g. via the accessible d-levels in a transition metal) in which the spin-transition restrictions do not apply, or via a univalent oxidation.

Oxygen has two important features that contribute to its reactivity under the correct conditions. Firstly that it has a ground state with spin = 1, and the other is that it has low-lying excited states, all of which have the same orbital configuration and represent a double bond structure.

The importance of the non-zero spin is that it enables oxygen to facilitate other processes which involve a change of spin multiplicity. The presence of oxygen with S = 1 enables another molecule to pass from a triplet state to a singlet state without a change in the total spin of the pair.

Also although the two electrons in the $2p\pi g^*$ orbitals have their spins coupled quite strongly parallel, from a chemical point of view oxygen is a diradical and has been implicated as a chain intermediate and a chain terminator in free radical reactions.

Because of weak acceptor-donor interaction between the half filled π^* orbitals of molecular oxygen and the n and π orbitals of organic substrates, only molecules with very low redox potentials can react directly with oxygen. In most cases oxygenation of organic

molecules requires at least activation of either the organic molecules or molecular oxygen.

For general references for this section see Reid (1958); Griffiths (1963); Kasha & Khan (1970).

B. Reactive States of Oxygen

i) Transition Metal Complexes

Ground state oxygen can be activated by complexing to transition metals (Collman 1968; Walling 1975; Nishinaga 1977). The structure and the electronic state of the resulting oxygen complexes are dependant on the nature of the metal, the ligand in the complex and the nature of the coordination (which is solvent dependant). In these complexes it is not the activated oxygen which takes part in the reaction. The function of the oxygen complex is to activate the substrate, and ground state oxygen then reacts with the activated substrate. Certain cobalt and iron complexes have been shown to perform some oxygenations in this way in a manner similar to certain enzymic oxidations.

There is no evidence as yet that this is a mechanism for oxygen activation *in vivo* in an uncontrolled reaction.

ii) Singlet Oxygen

a) Reactivity

The occurrence and physical properties of singlet oxygen are quite well documented (see reviews by Kearns 1971; Kasha & Khan 1970). Singlet oxygen has the following radiative lifetimes: $^1\Sigma_g$ approximately 7 seconds, $^1\Delta_g$ approximately 45 minutes. However, the actual lifetimes of these species are determined in practice by quenching, and in aqueous solutions these lifetimes become: $^1\Sigma_g$

approximately 1 x 10^{-10} seconds, $^1\Delta_g$ approximately 2 x 10^{-5} seconds. Biologically the production of $^1\Sigma_g$ in aqueous solutions is not very important as it would not be expected to react significantly with anything at a lower concentration than 10^{-3}M.

Singlet oxygen has been shown to be fairly selective in its reactivity. Singlet oxygen is electrophilic and fails to react at all with many substrates. Only compounds with fairly heavily substituted double bonds or other electron-rich functions react at appreciable rates (Foote 1976).

The reactions of singlet oxygen with biological materials so far documented has come mainly from studies on and related to photooxidation which has been shown to proceed in most cases by a singlet oxygen mechanism. Various nucleotides, nucleosides and free purine and pyrimidine bases (Hallett et al 1970; Clagett & Galen 1971; Kubitschek 1971; Politzer et al 1971) and aliphatic amines (Smith 1972) have been shown to react with singlet oxygen. Of the amino acids only histidine, methionine, tryptophan and cysteine (Foote 1968; Spikes & Macknight 1970) react with singlet oxygen. Although tyrosine is photoxidised this is not by a singlet oxygen mechanism. Because of the reactivity of these amino acids, it is expected that any proteins containing these amino acid residues in important structural or catalytic positions will also be sensitive to singlet oxygen. Work performed using a photooxidation system has shown this to be the case, and of 50 enzymes studied, only horseradish peroxidase and a bacterial alkaline phosphatase were shown to be resistant to the effects of photooxidation. (Spikes & Macknight 1970). Finally singlet oxygen has been proposed as the initiator of lipid peroxidation which occurs in vivo (Rawls & Van Santen 1970)

b) Evidence for the Formation of Singlet Oxygen In Vivo

While the formation of singlet oxygen in well defined chemical mixtures and by physical methods is well known, the positive identification of singlet oxygen in *in vitro* biological studies or *in vivo* is more contentious. Singlet oxygen has been shown to arise from the spontaneous decomposition of secondary peroxide radicals (Howard & Ingold 1968) and the base catalysed decomposition of hydrogen peroxide (Smith & Kulig 1976). Both of these chemicals have been shown to occur *in vivo*. Singlet oxygen trapping reagents have been shown to inhibit prostaglandin E formation (Panganamala *et al* 1974a) implicating singlet oxygen production. Lipoxygenase (Chan 1971), and superoxide anion (Khan 1970) have been reported to produce singlet oxygen, but Nilsson & Kearns (1974) have demonstrated the opposite. There is some evidence for the formation of singlet oxygen in the microsomal NADPH dependent cytochrome P_{450} oxidation system (King *et al* 1975). Finally, it has been shown that xanthine oxidase acting on its substrates can cause lipid peroxidation. The lipid peroxidation has been variously attributed to singlet oxygen and/or hydroxyl radical formed from the superoxide anion produced by xanthine oxidase. There is contention as to which is the damaging species, but it appears that both of these reactive species are probably formed in this system and that their formation is dependent on the presence of both superoxide anion and hydrogen peroxide. The Haber-Weiss reaction which has been invoked to explain these experiments has now been shown to occur only at very low rates if at all (see section iii) c)). No other explanation for these observations has yet been put forward.

c) Protection Against Singlet Oxygen *In vivo*

It has been proposed that superoxide dismutase (SOD) deactivates singlet oxygen (Paschen & Weser 1973; Richter *et al* 1975; Weser & Paschen 1972; Agro *et al* 1972). However when Baird *et al* (1977) repeated

the work of Paschen & Weser (1973) they found the opposite. Similarly when Michelson (1974) examined the work of Agro et al (1972) he found no support for the hypothesis. The remaining evidence for SOD as a singlet oxygen deactivator comes from work using lipoxygenase and xanthine oxidase as singlet oxygen sources and monitoring the reaction by its luminescence (Richter et al 1975; Weser & Paschen 1972). Xanthine oxidase luminescence has been shown to be due to carbonate radical (Hodgson & Fridovich 1976) and lipoxygenase has been shown not to evolve singlet oxygen (Nilsson & Kearns 1974), casting severe doubts on this work. SOD, however has been shown not to deactivate singlet oxygen when authenticated singlet oxygen sources were used (Goda et al 1974; Schaap et al 1974; Mayeda & Bard 1974). Fridovich (1979) finally laid to rest the possibility of SOD as a singlet oxygen quencher. A calculation showed that SOD in the concentration present in cells would need a reaction rate of 10^{15} l $mol^{-1}sec^{-1}$ to compete with quenching by water. As this rate is faster than diffusion by several orders of magnitude, it is impossible for SOD to be an effective singlet oxygen quencher in vivo.

On the positive side, both ascorbic acid (Bodannes & Chan 1979) and tocopherols (Grams 1971; Grams & Eskins 1972; Grams & Inglett 1979) have been proposed as singlet oxygen scavengers. Both have been shown to scavenge singlet oxygen with a rate constant of approximately $10^{8} M^{-1} sec^{-1}$. The reactivity of the tocopherols with singlet oxygen is in the order $\alpha > \beta > \gamma > \delta$ and correlates well with their biological activities. Lucy (1972) postulates that tocopherols intercalate in membranes in association with the most unsaturated lipids which would be most prone to attack by reactive molecules.

It would seem therefore that the only protection against singlet oxygen in vivo is from singlet oxygen scavengers like ascorbic acid and the tocopherols.

iii) The Univalent Reduction of Oxygen

Univalent reduction is one of the mechanisms that permits ground state oxygen to circumvent the spin restriction which causes triplet oxygen to be a sluggish oxidant under physiological conditions. Oxygen is a four electron oxidant giving three intermediate reduced states, each of which has been characterised.

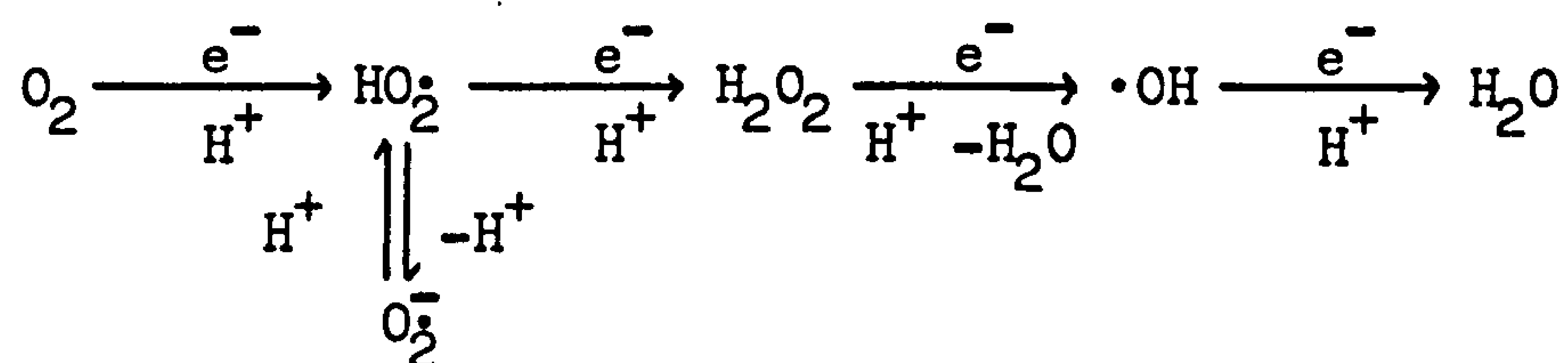


Figure 2 Univalent Reduction of Oxygen

The reason that this pathway can curcumvent the spin restriction is that a free radical is a doublet. The reaction of a triplet with a singlet to give two doublets is a spin allowed process. The unpaired electrons originally on the triplet, once separated in doublets, have no reference to their original spin and thus can pair to give singlet products.

Although the univalent reduction pathway is favoured kinetically, the first step, the formation of the perhydroxyl radical, is not thermodynamically favoured. The reaction below is endothermic by about 40-50 kcal mole^{-1} for most organic substrates.

$$RH + O_2 \longrightarrow R\cdot + HO_2^{\cdot}$$

H — O bond energy in $HO_2^{\cdot}$ is 47 kcal mole^{-1} (George 1965)

R — H bond energy is usually 90-100 kcal mole^{-1} (March 1968)

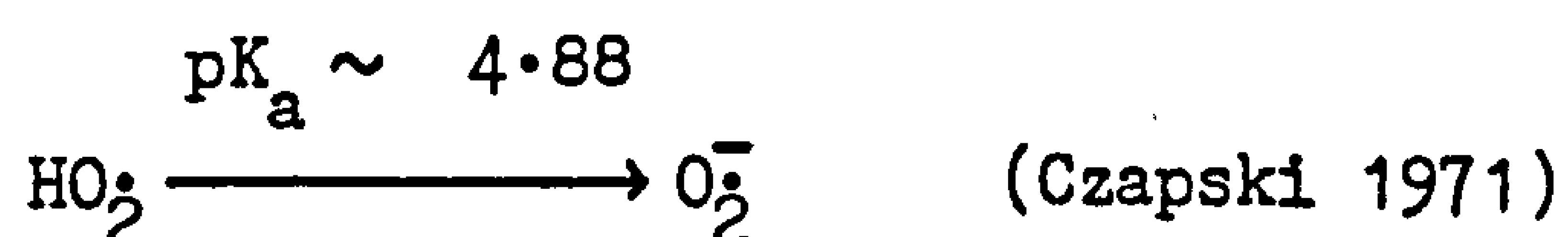
Only by the R — H bond energy being considerably less will this reaction proceed at physiological temperature and pressure. In all

such cases, the radical product is part of a conjugated system which is highly stabilised by resonance delocalisation.

a) Perhydroxyl Radical (Superoxide Anion Radical)

(i) Reactivity

Under physiological conditions (about pH 7) the perhydroxyl radical is ionised to the superoxide radical.



$$HO_2^{\cdot} \xrightarrow{pK_a \sim 4{\cdot}88} O_2^{\cdot -} \qquad \text{(Czapski 1971)}$$

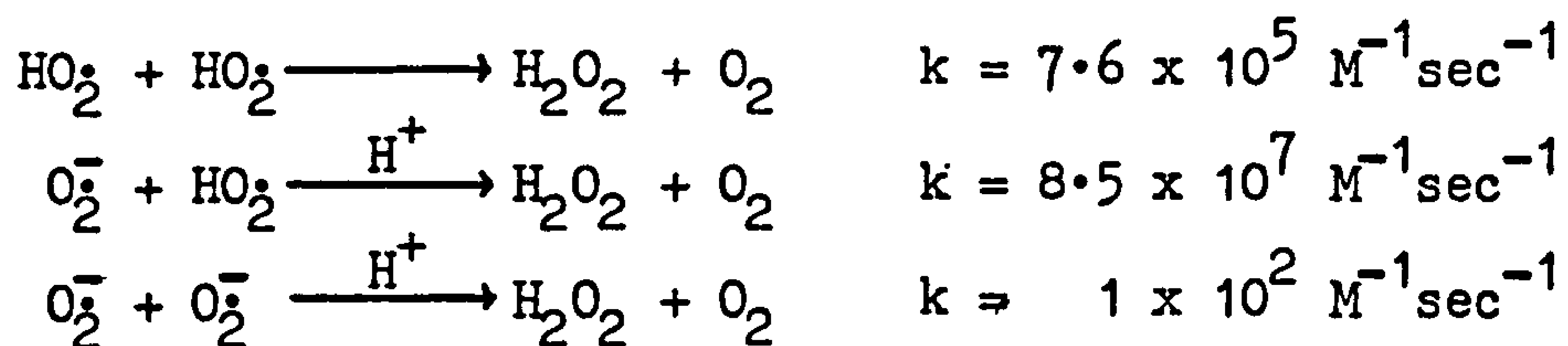
The superoxide anion and the perhydroxyl radical have similar reactivities, the main differences in reactivity being due to the negative charge of the superoxide anion. As expected they show radical characteristics in their reactions with organic compounds, but the reaction:

$$R\text{-}H + HO_2^{\cdot} \longrightarrow R\cdot + H_2O_2$$

is endothermic for most organic compounds. Therefore as in the reaction of triplet oxygen to form superoxide radical, superoxide radical only reacts with compounds giving stabilised free radical products.

The reactions of superoxide on organic solvents have been fairly well documented (Fee & Valentine 1977 and references therein). However very little work has been carried out on the reactions of superoxide radicals in aqueous solutions, and there are good reasons for believing that the reactivity varies greatly with the solvent. Firstly superoxide is relatively stable in aprotic solvents with decay rates as low as a few per cent per hour, but in aqueous solution at pH 7 superoxide dismutates with a rate constant of

$4{\cdot}5 \times 10^5\ M^{-1}\ sec^{-1}$. This results from a combination of the dismutation reactions below (Behar et al 1970).



$$HO_2^{\cdot} + HO_2^{\cdot} \longrightarrow H_2O_2 + O_2 \qquad k = 7{\cdot}6 \times 10^5\ M^{-1}sec^{-1}$$
$$O_2^{\overline{\cdot}} + HO_2^{\cdot} \xrightarrow{H^+} H_2O_2 + O_2 \qquad k = 8{\cdot}5 \times 10^7\ M^{-1}sec^{-1}$$
$$O_2^{\overline{\cdot}} + O_2^{\overline{\cdot}} \xrightarrow{H^+} H_2O_2 + O_2 \qquad k \Rightarrow 1 \times 10^2\ M^{-1}sec^{-1}$$

Secondly there is a considerable difference between the oxidation-reduction couple of $O_2/O_2^{\overline{\cdot}}$ in protic and aprotic solvents the latter being less negative. This shows the superoxide radical to be considerably more stable when solvated. The implication of this is that $O_2^{\overline{\cdot}}$ will not be formed in the hydrophobic regions of the cell due to a lack of reducing agents with sufficiently low redox potentials. The solvation of superoxide in water will also influence its nucleophilicity. Although superoxide has considerable nucleophilic character in non-aqueous systems, there is little available evidence for the nucleophilic properties in aqueous solution (Fee & Valentine 1977).

Superoxide is able to react as both a one electron reductant to form dioxygen or as a one electron oxidant to form hydrogen peroxide. In aqueous systems however these reactions are quite limited for the reasons given above.

The reaction of superoxide radicals with biological substrates in aqueous solutions so far reported are as follows. Superoxide radical oxidises ascorbate (Nishikimi 1975a; Greenstock & Miller 1975), and adrenaline (Misra & Fridovich 1972b). The oxidation of lactate dehydrogenase bound reduced nicotinamide adenine dinucleotide is initiated by superoxide radical and propagated by ground state oxygen (Bielski & Chan 1973;1976). Ferricytochrome c is reduced by superoxide radicals (McCord & Fridovich 1968;1969; Butler et al 1975). Superoxide radicals oxidise oxyhaemoglobin to methaemoglobin

(Lynch et al 1976; Sutton et al 1976) but also reduce methaemoglobin to oxyhaemoglobin. The concentrations of SOD observed in normal erythrocytes are reported to be sufficient to prevent the formation of excessive methaemoglobin (Sutton et al 1976).

Xanthine oxidase acting on its substrate xanthine is an authenticated source of superoxide anions and hydrogen peroxide. Fridovich (1974) reported that cysteine was oxidised by this system and that the oxidation of cysteine was inhibited partly by SOD and partly by catalase suggesting that cysteine is oxidised by both superoxide radical and hydrogen peroxide. Bielski & Shiue (1979) reporting work on the reactivity of amino acids with superoxide radical and hydroperoxyl radicals quote reaction rates of these radicals with cysteine as $15 \pm 2\ M^{-1}sec^{-1}$ and $601 \pm 85\ M^{-1}sec^{-1}$ respectively. This is not a particularly fast reaction although cysteine may undergo a chain reaction as well. The only other amino acids that showed any reaction with hydroperoxide or superoxide or superoxide radicals were histidine, tyrosine, tryptophan and possibly phenylalanine. These reactions proceded at relatively low rates. The authors of this paper assumed that the reactivity of superoxide and hydroperoxide radicals does not change dramatically towards amino acids which are integral parts of larger molecules, so that it can be said that superoxide and hydroperoxide also react with amino acids in proteins at similar low rates.

(ii) Evidence for the Production of Superoxide Radicals *In Vivo*

Superoxide radicals can be produced *in vitro* by the action of various enzymes on their substrates. The best documented of these enzymes is xanthine oxidase, in which 20% of the total electron flux through the enzyme can be accounted for in terms of the univalent reduction of oxygen (Fridovich 1970). Other enzymes which have been

shown to produce superoxide radicals are: aldehyde oxidase and dihydroorotic acid dehydrogenase (Handler et al 1964), NADPH cytochrome c reductase (Aust et al 1972), diamine oxidase (Rotilio et al 1970) and some flavoprotein dehydrogenases (Massey et al 1969). Spontaneous oxidations of tetrahydropteridines (Nishikimi 1975b; Heikkila & Cohen 1975), and reduced flavins (Misra & Fridovich 1972a; Ballou et al 1969) also produce superoxide radicals. Superoxide radical formation has been implicated in the autooxidation of haemoglobin (Misra & Fridovich 1972c; Brunori et al 1975) although this reaction is very slow.

Boveris & Chance (1973) and Loschen et al (1971) have demonstrated that actively respiring mitochondria produce hydrogen peroxide. Loschen et al (1974) propose that there are two sites of hydrogen peroxide formation and that in one of these sites a reactive oxygen species, probably superoxide radical, is an intermediate in the hydrogen peroxide formation. Cadenas et al (1977) using submitochondrial particles demonstrated that complex I and complex III both produced superoxide radical and that the production of hydrogen peroxide was essentially due to the dismutation of superoxide.

The production of superoxide radicals was shown to constitute 17% of the total oxygen consumption in crude lysates of Streptococcus faecalis in which the SOD had been precipitated by specific antibodies (Britton et al 1978). Although the lack of structural integrity of this preparation could affect the rate of formation of superoxide anions, this experiment provides the best estimate to date of *in vivo* superoxide radical production.

(iii) Protection Against Superoxide Radicals *In Vivo*

In vivo protection against superoxide radicals is provided by a class of heterogenous enzymes called superoxide dismutases which

catalyse the reaction:

$$O_2^{\cdot -} + O_2^{\cdot -} + 2\,H^+ \longrightarrow O_2 + H_2O_2$$

(McCord & Fridovich 1969; Michelson et al 1977). Superoxide dismutases are found in all organisms apart from obligate anaerobes thus demonstrating their role in the protection against the deleterious effects of oxygen.

Eucaryotes contain a cytosolic copper-zinc SOD which is sensitive to cyanide. Mitochondria contain a manganese SOD which is insensitive to cyanide and located in the matrix. In addition mitochondria contain the copper-zinc cytosolic SOD in their intermembrane space. Generally the superoxide dismutases are stable enzymes, particularly the copper-zinc enzyme, as would be expected of an enzyme whose function is to eliminate a reactive species such as superoxide radical. Human and primate livers appear to be unusual in that they contain larger amounts of manganese SOD than is usual and that the excess manganese SOD is located in the cytosol rather than the mitochondrial matrix (McCord et al 1977).

The requirement for an enzyme to catalyse a reaction which already proceeds at a rate of approximately 10^5 $M^{-1}sec^{-1}$ seems rather obscure. However this appears to be the only reaction catalysed by superoxide dismutases and the enzyme catalysed reaction has a rate constant of 2×10^9 under the same conditions as the uncatalysed reaction. In addition to the advantage of a faster rate constant, the cellular SOD concentration is probably four to five orders of magnitude greater than the steady-state concentration of superoxide radicals. This gives the enzyme catalysed reaction a gain by a factor of about 10^9 over the uncatalysed reaction (McCord, Crapo & Fridovich 1977). These figures indicate that it is probably advantageous to the cell

to maintain the steady state level of superoxide at a very low level.

Both copper II (Weser et al 1978) and iron II and III (Halliwell 1975) form complexes which have superoxide dismutase activity, but this is several orders of magnitude lower than the enzymic dismutations. It is difficult to see that these dismutation reactions are of great consequence in vivo.

Hirata & Hayaishi (1971; 1975) have shown that activity of intestinal indoleamine 2,3, dioxygenase is dependent upon superoxide radical as a substrate for its activity. Although indoleamine 2,3, dioxygenase is unlikely to have any significant effect on the steady state level of superoxide radicals in cells containing this enzyme, it is plausible that a change in the steady level of superoxide could produce metabolic alterations arising from a change in the activity of this enzyme.

From the reactions above it can be seen that as yet no reaction has been demonstrated in which the actions of superoxide per se are particularly cytotoxic or deleterious given the very low steady state concentration of superoxide radicals. The reactivity of superoxide radicals per se does not seem to justify the protection provided in vivo by superoxide dismutases. It seems likely that if superoxide is a cytotoxic species it is indirectly in the formation of a species of greater reactivity.

For a more detailed review of superoxide radicals and superoxide dismutases see the following reviews and references therein. Michelson, McCord & Fridovich (1977), Fridovich (1972; 1974 ; 1975), Hayaishi & Asada (1977) and Ciba Foundation Symposium (1979).

b) Hydrogen Peroxide

(i) Reactivity

Hydrogen peroxide in the absence of transition metal catalysts

is an unreactive molecule under physiological conditions despite the high exothermicity of its reactions. An exception to this is when it can act as a nucleophile. Peroxides do not cleave to radicals to produce free radical chain reactions unless the peroxide is heated, irradiated with high energy radiation or unless a transition metal in a lower valence state is present.

In contrast to its slow reactions with organic reagents hydrogen peroxide reacts quite readily with transition metals and their complexes. The transition metal complexes of relevance *in vivo* are those formed with iron. Fentons reagent (Fenton 1894), iron II and hydrogen peroxide, has long been known as a powerful oxidant of organic substrates. Haber and Weiss in 1934 demonstrated that iron III and hydrogen peroxide had similar properties. These two reagents were postulated to act via formation of hydroxyl radicals (see section iii) (c)). Walling (1975) has further shown that iron III ions catalysed the decomposition of hydrogen peroxide when complexed with EDTA. In that pH region where the iron III-EDTA complex is formed it is a catalyst for the oxidation of organic substrates as well as hydrogen peroxide decomposition. The kinetics of this reaction are complex and it is not yet known whether this reaction procedes by a concerted complex mechanism or by production of the reactive hydroxyl radical. These reactions are relevant to physiological conditions because there is a low concentration (0·032 mmoles/Kg wet weight in liver) of free inorganic iron in cells (King *et al* 1975)

(ii) Production of Hydrogen Peroxide *In Vivo*

Hydrogen peroxide is produced *in vivo* from a class of oxidases which transfer 2 electrons from one molecule of oxygen to produce one molecule of hydrogen peroxide as a product e.g. d-amino acid

oxidase. Hydrogen peroxide is produced from the dismutation of superoxide, both in the catalysed and uncatalysed reaction. Hydrogen peroxide is also produced, as mentioned above, in mitochondria; probably as a consequence of superoxide formation. It is probable that hydrogen peroxide is always present at low concentrations in all aerobic cells.

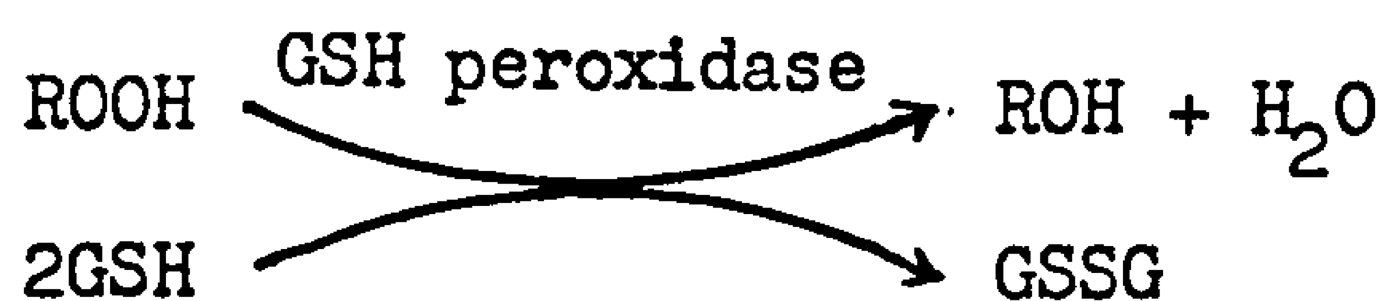
(iii) Protection Against Hydrogen Peroxide _In Vivo_

Hydrogen peroxide is eliminated from animals _in vivo_ mainly by the action of two enzymes: catalase and glutathione peroxidase. These enzymes are located in different fractions of the cell. Catalase is located in the peroxisomes together with the oxidases which produce hydrogen peroxide so they are compartmentalised within the cell. Catalase catalyses the following reaction:

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2}O_2$$

Catalase is a very efficient enzyme which becomes saturated only with extremely high substrate concentrations.

The other major enzyme for eliminating hydrogen peroxide appears to be glutathione peroxidase.



Under _in vitro_ conditions glutathione peroxidase will reduce a large number of hydroperoxide substrates in addition to hydrogen peroxide, but the only physiological reductant it can utilise for this reaction is reduced glutathione for which it is very specific. The compartments protected by glutathione peroxidase are the cytosol and the matrix

space. It has been suggested that the major role of glutathione peroxidase *in vivo* is the removal of toxic lipid hydroperoxides rather than the removal of hydrogen peroxide, but it remains to be demonstrated that it does react with these substrates *in vivo*.

Several other peroxidases are known which use hydrogen peroxide as oxidising agents (Nelson 1972):

$$AH_2 + H_2O_2 \xrightarrow{\text{peroxidase}} A + 2H_2O$$

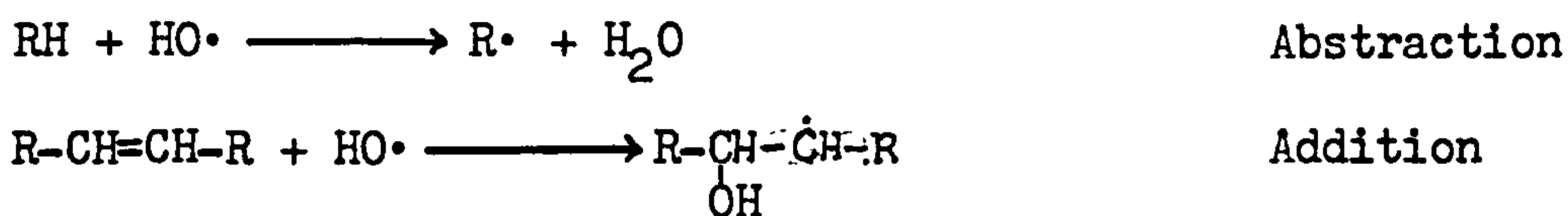
It seems unlikely that these enzymes afford significant protection from an excess production of hydrogen peroxide.

For a more detailed picture of the roles of hydrogen peroxide, glutathione peroxidase and catalase see Flohé (1979), Hamilton (1974), Oshino & Chance (1977), Kosower & Kosower (1976) and references therein.

c) Hydroxyl Radical

(i) Reactivity

The hydroxyl radical is a very reactive species, showing little selectivity in its reactions, and which will react with all known organic compounds. The two major reactions of the hydroxyl radical are addition to π systems and hydrogen atom abstraction:



$$RH + HO\cdot \longrightarrow R\cdot + H_2O \qquad \text{Abstraction}$$

$$R\text{-}CH\text{=}CH\text{-}R + HO\cdot \longrightarrow R\text{-}CH(OH)\text{-}\dot{C}H\text{-}R \qquad \text{Addition}$$

Both of these reactions are exothermic due to the fact that the H-O bond energy in water is higher than any known R-H bond energy (George 1965). The formation of free radicals after the reaction of organic substrates with hydroxyl radicals can lead to free radical chains propagated by molecular oxygen (Betts 1971 and references therein).

$$R\cdot + O_2 \longrightarrow RO_2$$

propagation

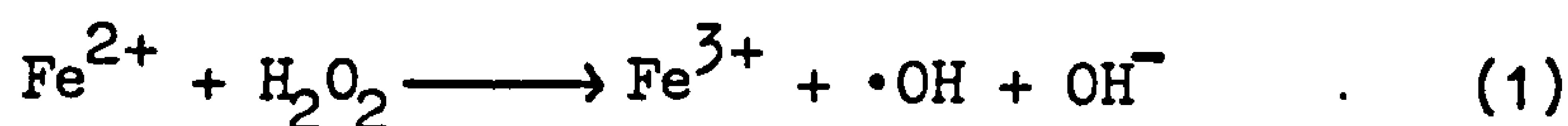
$$RO_2^{\cdot} + RH \longrightarrow RO_2H + R\cdot$$

termination $$2RO_2^{\cdot} \longrightarrow O_2 + \text{stable products}$$

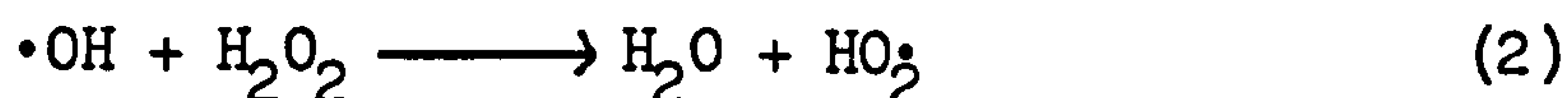
The hydroxyl radical can also react by electron transfer (Willson 1979). It is likely that if $\cdot OH$ is generated in a biological medium, its lifetime will be very short ($<1\mu sec$) and it will react very close to where it is formed.

(ii) Evidence for the Formation of Hydroxyl Radicals In Vivo

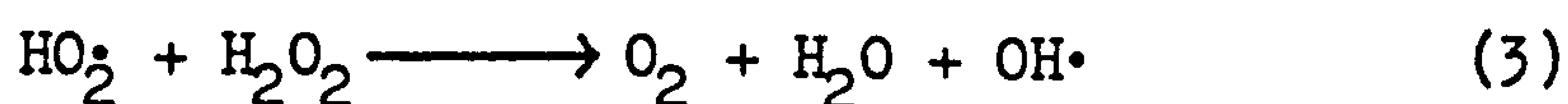
Haber and Weiss first proposed in 1934 that hydroxyl radicals were the active agent of Fentons reagent and in the catalytic decomposition of hydrogen peroxide by iron III salts. Reactions (1) to (4) were proposed to explain the action of Fentons reagent.



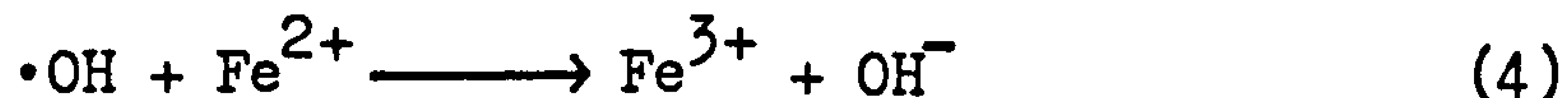
$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \cdot OH + OH^- \quad (1)$$



$$\cdot OH + H_2O_2 \longrightarrow H_2O + HO_2^{\cdot} \quad (2)$$

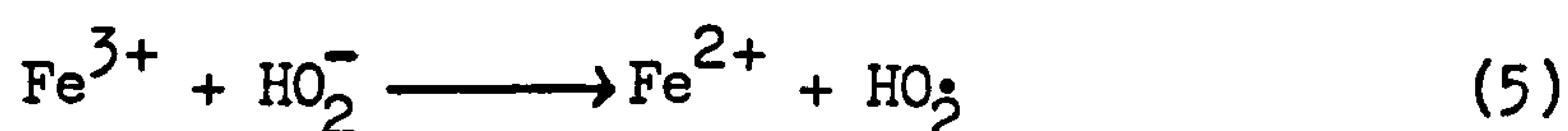


$$HO_2^{\cdot} + H_2O_2 \longrightarrow O_2 + H_2O + OH\cdot \quad (3)$$

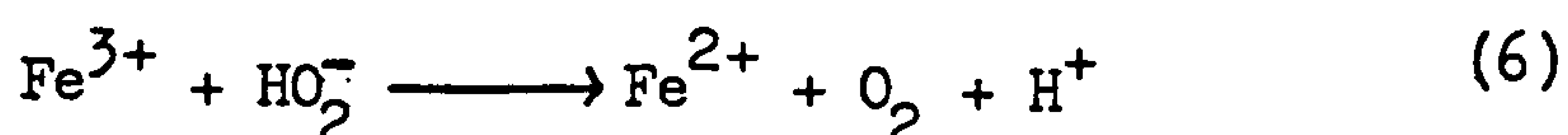


$$\cdot OH + Fe^{2+} \longrightarrow Fe^{3+} + OH^- \quad (4)$$

Two further reactions (5 and 6) were postulated by Haber & Weiss to explain the varying kinetics when iron III salts were present.

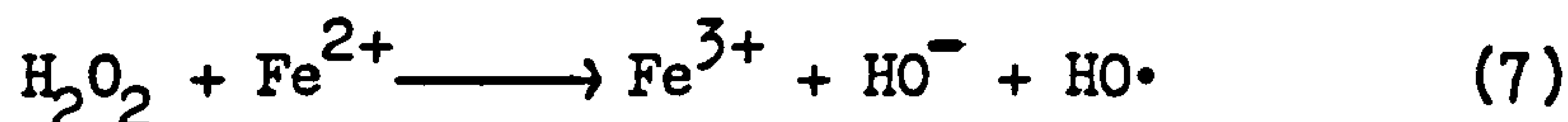


$$Fe^{3+} + HO_2^- \longrightarrow Fe^{2+} + HO_2^{\cdot} \quad (5)$$

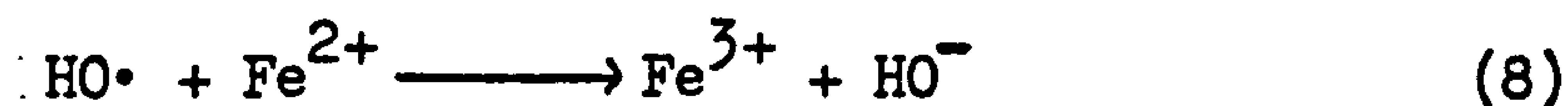


$$Fe^{3+} + HO_2^- \longrightarrow Fe^{2+} + O_2 + H^+ \quad (6)$$

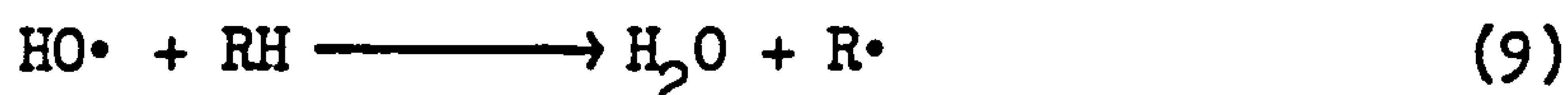
The Haber & Weiss scheme was modified by Merz & Waters (1949) and later by Walling (1975) to a new scheme (reaction 7-12) which eliminated reaction (3) for which there was little evidence.



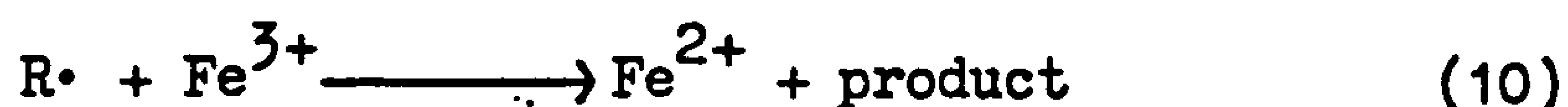
$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + HO^- + HO\cdot \qquad (7)$$



$$HO\cdot + Fe^{2+} \longrightarrow Fe^{3+} + HO^- \qquad (8)$$

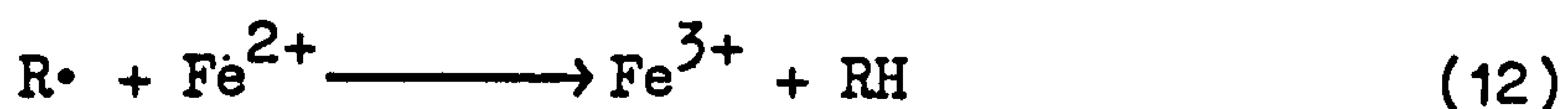


$$HO\cdot + RH \longrightarrow H_2O + R\cdot \qquad (9)$$



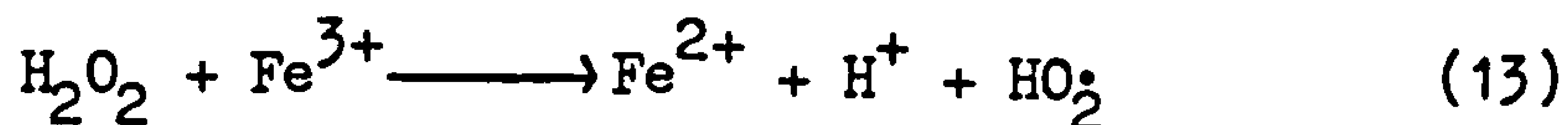
$$R\cdot + Fe^{3+} \longrightarrow Fe^{2+} + \text{product} \qquad (10)$$

$$2R\cdot \longrightarrow \text{product (dimer)} \qquad (11)$$

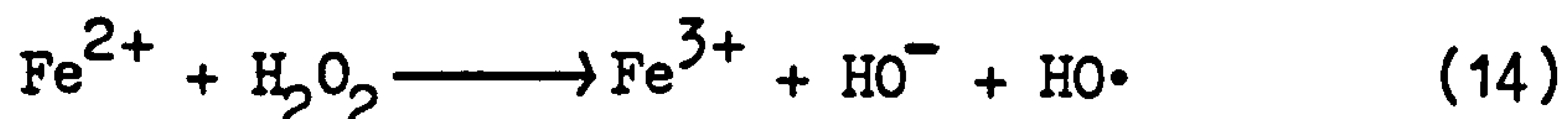


$$R\cdot + Fe^{2+} \longrightarrow Fe^{3+} + RH \qquad (12)$$

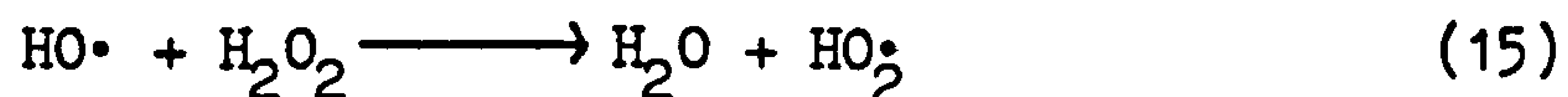
The second part of the Haber & Weiss paper dealt with the catalytic decomposition of hydrogen peroxide by iron III in dilute acid solution, for which Barb <u>et al</u> (1951) proposed a modified Haber-Weiss scheme (reactions 13-18).



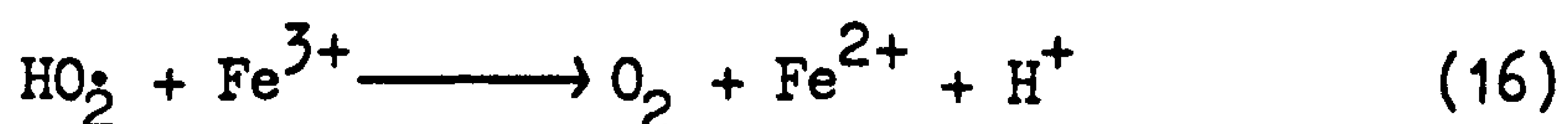
$$H_2O_2 + Fe^{3+} \longrightarrow Fe^{2+} + H^+ + HO_2\cdot \qquad (13)$$



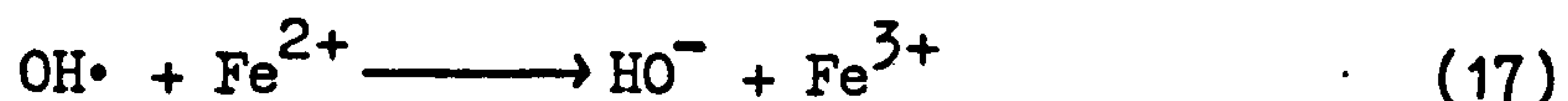
$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^- + HO\cdot \qquad (14)$$



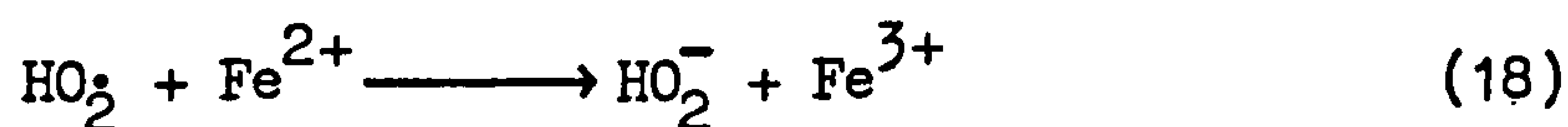
$$HO\cdot + H_2O_2 \longrightarrow H_2O + HO_2\cdot \qquad (15)$$



$$HO_2\cdot + Fe^{3+} \longrightarrow O_2 + Fe^{2+} + H^+ \qquad (16)$$

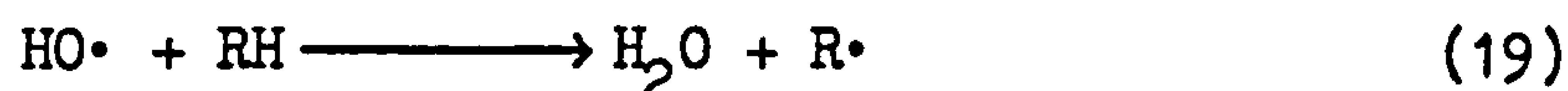


$$OH\cdot + Fe^{2+} \longrightarrow HO^- + Fe^{3+} \qquad (17)$$

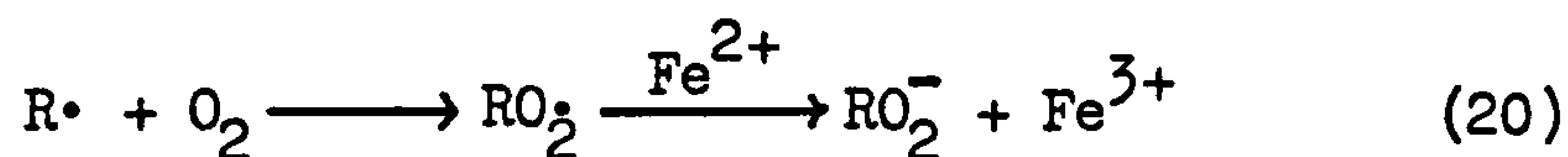


$$HO_2^{\bullet} + Fe^{2+} \longrightarrow HO_2^{-} + Fe^{3+} \qquad (18)$$

in which reactions (13) and (16) occur by complex formation and are pH dependent. This scheme was opposed by Kremer and Stein (1959) who postulated that the reaction occured by a non-radical non-chain mechanism. However organic substrates were known to retard the iron catalysed decomposition of hydrogen peroxide. It was proposed that the retardation was due to hydroxyl radicals being trapped by organic substrates. Walling & Goosen (1973) showed that if the chain termination equations (17) and (18) were replaced by (19) and (20) the kinetics of this overall scheme accounted for the remaining anomalies, thus establishing the free radical mechanism.



$$HO\bullet + RH \longrightarrow H_2O + R\bullet \qquad (19)$$



$$R\bullet + O_2 \longrightarrow RO_2^{\bullet} \xrightarrow{Fe^{2+}} RO_2^{-} + Fe^{3+} \qquad (20)$$

The original work of Haber & Weiss on Fentons reagent used neutral aqueous solutions and so these reactions are directly applicable to physiological solutions, and show the feasibility of generating hydroxyl radicals in vivo.

Direct evidence for the production of hydroxyl radicals and superoxide radicals in vivo is available from EPR spectroscopy on stimulated human neutrophils (Green et al 1979). The production of hydroxyl radicals was inhibited by SOD and hydroxyl radical scavengers but not by catalase suggesting that hydrogen peroxide is not involved in the reaction. This supports previous evidence that phagocytes produce hydroxyl radicals, demonstrated by hydroxyl radical trapping reagents and the production of ethylene from methional as indicators.(Tauber & Babior 1977 and Weiss et al 1977).

Beauchamp and Fridovich (1970) have also demonstrated the production of ethylene from methional by incubation of xanthine with xanthine oxidase under aerobic conditions where catalase, SOD and hydroxyl radical scavengers all inhibit ethylene production. Hydroxyl radical has also been postulated as the cause of lipid peroxidation by the microsomal NADPH dependent cytochrome c reductase (King *et al* 1975; Cederbaum *et al* 1977; Fong *et al* 1973). However Tyler (1975) and Pederson & Aust (1975) claim that the reaction requires complexed iron in solution and does not involve hydroxyl radicals. While Fong *et al* (1973) and King *et al* (1975) also show that the reaction depends upon the presence of iron in solution, Kellog & Fridovich (1975) state that iron is not required for maximal activity of their xanthine oxidase peroxidation system. The 'Haber-Weiss' reaction (3) has been cited by the latter authors as the means of hydroxyl radical formation.

Several investigators have shown that this reaction only occurs at very low rates if at all without transition metal catalysts (McClune & Fee 1976; Halliwell 1976; Rigo *et al* 1977). It has since been shown that this reaction does occur in the presence of iron chelates (McCord & Day 1978; Halliwell 1978). This work on the feasibility of the 'Haber-Weiss' reaction is supported by the theoretical studies of Koppenhol & Butler (1977) who state that it is unlikely that hydroxyl radicals can be formed directly from the reaction of superoxide and hydrogen peroxide but that the reaction involving iron catalysis is feasible. They also postulate that it is feasible that singlet oxygen is evolved in this reaction.

It would appear that it is possible that hydrogen peroxide, either in conjunction with superoxide ions and/or catalytic traces of iron produces hydroxyl radical or a strongly oxidising complex. These resultant reactive species are capable of causing lipid

peroxidation and probably many other cytotoxic reactions. It seems likely that these species together with singlet oxygen are responsible for the toxicity of oxygen although further work in this area will be necessary to determine more precisely which of these species is the active principle in each of the various systems investigated.

For further reading on hydroxyl radicals and their relevance *in vivo* see Hayaishi & Asada (1977), Hamilton (1974) and Ciba Foundation Symposium (1979).

4. BIOCHEMICAL CHANGES DURING HYPEROXIA: A REVIEW

A. Inhibition of Cell Division, DNA, RNA, and Protein Metabolism

The effect of oxygen on cell division was first noticed in tissue culture of mammalian cells. It was found that the optimum oxygen tension for the growth of cell cultures was between 0·1 and 0·35 atm. Cells could be persuaded to grow in a nitrogen atmosphere - a total lack of oxygen (Brosemer & Rutter 1961) - but that 95% oxygen 5% carbon dioxide had a powerful growth inhibitory effect leading to a total cessation of cell division within the space of 1 generation (Rueckart & Mueller 1960; Brosemer & Rutter 1961; Allison 1965). The inhibition is reversible if the cells are transferred to a lower concentration of oxygen within 48 hours of starting exposure to an oxygen atmosphere. If the oxygen tension is reduced after this length of time, cellular degeneration and irreversible changes take place so that the cells never recover. That this effect was not due to an accumulation of toxic products or deletion of essential nutrients was demonstrated by the lack of growth even after transferring the cells to a fresh medium. A reduction in the rate of DNA, RNA, and protein synthesis was also noted by Rueckart & Mueller (1960). In confirmation, Brosemer & Rutter (1961) showed that there was a reduction in the uptake of radioactive phosphorous into DNA, but there was no change in the ratio of total soluble phosphate to acid labile phosphate, or in the proportions of the intracellular adenine nucleotides. Interestingly, Rueckart & Mueller (1960) observed a transition to an anaerobic metabolism in these HeLa cancer line cells on exposure to 0·95 atm oxygen.

The effect of oxygen on lung cell division *in vivo* was determined directly by injecting tritiated thymidine into mice kept in oxygen at atmospheric pressure (OAP) one hour before sacrifice (Evans *et al*

1969). The number of dividing cells fell dramatically after 24 hours and showed a minimum at 48 hours. The largest decreases in cell division occurred in leucocytes, endothelial cells and type II alveolar cells. Further studies on the inhibition of DNA synthesis were carried out by measuring the incorporation of tritiated thymidine into DNA of lung slices of newborn mice kept in 0·96-1·0 atm oxygen (Northway et al 1972;1976). Between 24 and 96 hours, lung DNA synthesis was decreased, total lung DNA was diminished, and there was a decrease in the ratio of lung DNA to body weight. From 96-144 hours a reversal of inhibition of DNA synthesis occurred and thus there was an increase in total lung DNA. After 144 hours the surviving mice showed a return towards control levels of the above parameters. It should be noted in connection with these experiments that newborn animals show a much greater tolerance to the toxic effects of oxygen. In this study 50% of the mice survived, whereas almost total mortality would be expected if adult animals had been used.

Similar observations have been made of the effect of oxygen on protein synthesis (Massaro 1973; Gacad & Massaro 1973; Massaro & Massaro 1974) and on RNA synthesis (Gail & Massaro 1976). After 24 and 48 hours of exposure to oxygen at greater than 0·98 atm, the incorporation of ^{14}C-leucine into lung slices was diminished, with the incorporation into protein of the surface active fraction being reduced more than incorporation into total protein after 48 hours. At 96 hours of exposure, the incorporation of ^{14}C-leucine into protein rose above that of controls. These changes in protein synthesis were paralleled by the number of lamellar bodies (which synthesise the surface active material) and the amount of endoplasmic reticulum in granular pneumocytes. Similarly the lung RNA content increased in lung slices after exposure of rats to greater than

0·98 atm oxygen. The ratio of RNA to DNA was also increased showing that RNA synthesis inhibition is reversed more quickly than that of DNA.

B. Enzyme Inactivation and Inhibition By Oxygen

i) Oxygen Consumption and Carbohydrate Metabolism

The tenth paper published by Stadie & Haugaard (1946) in their classic series of experiments on the toxic effects of oxygen, showed that mice developed severe symptoms of toxicity within a few minutes of being exposed to 8 atm of pure oxygen. During the short period before the death of these animals, no detectable decrease of oxygen consumption occurred. Similarly, no significant changes in either oxygen uptake or respiratory quotient had been observed in brain slices prepared from animals killed by exposure to oxygen at high pressure (OHP) (Stadie *et al* 1945a). However, Gail & Massaro (1976) demonstrated that using oxygen at atmospheric pressure (OAP) *in vivo*, a decrease in oxygen consumption by rat lung of 30% was noted, but that after 96 hours the oxygen consumption was the same or higher than controls. In contrast to these reports, Bassett & Fisher (1979) presented data showing an increased respiration as measured by glucose utilisation, and lactate, pyruvate and carbon dioxide production in perfused lung ventilated with pure oxygen at 5 atm for 80 minutes.

In vitro experiments measuring the changes in respiration of tissue slices and homogenates exposed to oxygen present a different picture. Mann & Quastel (1946), Dickens (1946), Stadie *et al* (1945a), Haugaard *et al* (1957) and Thomas *et al* (1963) have all reported that oxygen causes a gradual decline in the respiration of tissue homogenates and slices. Dickens (1946) and Stadie *et al* (1945b) showed that all of the tissues tested showed this effect, but that

some tissues were more susceptible than others, i.e. brain > liver or kidney > muscle. There was disagreement between these authors whether liver or kidney was more susceptible. In these systems metal ions played a part in the inhibition of respiration. Cobalt, manganese, magnesium and calcium ions were found to be protective (Dickens 1946), but copper (Haugaard *et al* 1957) and ferrous ions (Williams & Haugaard 1970) were found to augment the action of oxygen. The effect of oxygen on respiration was reported to be irreversible. The inhibition of brain metabolism was the same with glucose, fructose, lactose or pyruvate as substrates, but the ability to oxidise succinate was much less poisoned (Dickens 1946; Mann & Quastel 1946). Although carbon dioxide exerts a potentiating effect on the toxicity of oxygen *in vivo*, a high tension of carbon dioxide (100mm Hg) had no effect on the respiration of brain tissue at 8 atm OHP (Stadie *et al* 1945a).

Various groups have attempted to explain the above inhibitions on the basis of inhibition of specific enzymes.

Total *in vivo* dehydrogenase levels have been determined by coupled oxidation of methylene blue. Jamieson & Brenk (1962) used 5 atm OHP to show that the rate of methylene blue oxidation was progressively decreased in lung tissue upon exposure until a 50% drop in activity had been recorded after 1 hours exposure. However no decrease was obtained with either brain or liver. Similarly Bardell & Fowler (1971) demonstrated a significant decrease in methylene blue oxidation by lung tissue after 2 hours exposure to OAP. This latter group also suggested that succinic dehydrogenase was affected less than other dehydrogenases. Attempts to obtain effects of this magnitude in specific dehydrogenases have not been so successful.

In glycolysis, the possibility that inhibition of glyceraldehyde-

3-phosphate dehydrogenase (G3PDH) and lactate dehydrogenase (LDH) occurs has been investigated. The inhibition of G3PDH was first studied by Dickens (1946) who showed that it was poisoned *in vitro* by oxygen, but only in the absence of its coenzyme NADH. Balazs (1959) demonstrated that the specific site of action of the 'Pasteur reaction' (the inhibition of glucose utilisation/lactate production upon a change from an anaerobic to an aerobic environment) was at G3PDH: either due to inhibition of G3PDH *per se* or indirectly by the lack of ATP produced at this step when no ADP was present in the system. Similarly Horn *et al* (1965) and Horn & Haugaard (1966) provided evidence that G3PDH was inhibited by OAP. They demonstrated an increase in fructose 1,6 diphosphate, dihydroxyacetone phosphate and glycerol-3-phosphate which could be reversed by the addition of either glutathione or G3PDH. However the work of Shaw & Leon (1970) showed that there was no decrease in retinal G3PDH activity after exposing rabbits to OAP for 4 days. This result has also been confirmed in liver, muscle and brain of rats exposed to 6 atm OHP *in vivo* (Bannister *et al* 1973). These latter reports indicate that inactivation of G3PDH does not occur *in vivo*.

The reports of the effects of oxygen on LDH are not all in accord. The earlier *in vitro* experiments of Mann & Quastel (1946) and Dickens (1946) demonstrated the resistance of LDH to inactivation by oxygen and were confirmed by a later report of *in vivo* experiments on rabbit retina (Shaw & Leon 1970). Another paper (Bannister *et al* 1973) agreed that liver and muscle LDH were unaffected *in vivo* by OHP (6 atm) but showed that brain LDH activity was significantly depressed, and that plasma LDH activity was significantly elevated. The elevated plasma levels were also noted in controls compressed to the same pressure using air, casting severe doubts on their significance as an effect of oxygen. Delarbré *et al* (1976)

published conflicting evidence showing approximately a 20% drop in liver, lung and kidney LDH activity after 18 hours _in vivo_ exposure to 2 atm OHP. During the same period plasma LDH activity was almost doubled. _In vitro_ experiments by this group showed inactivation by oxygen of LDH isolated from all the above tissues. Further confusion is added by a report (Tierney _et al_ 1975) that rat lung LDH activity was increased after 60 hours _in vivo_ exposure to 0·85 atm oxygen. It would appear on the whole that changes in LDH activity, when they occur, tend to be fairly small and thus would not be expected to contribute significantly to the pathogenesis of oxygen toxicity; particularly since _in vivo_, the function of LDH is merely to facilitate the attainment of equilibrium between pyruvate and lactate rather than providing a product for further metabolic conversion.

Pyruvate dehydrogenase (PDH) is another enzyme which has been suggested as a site of oxygen action. Barron (1936) found gonococcal PDH to be quite sensitive to air, and Mann & Quastel (1946) in their _in vitro_ study obtained evidence showing that the oxidation of pyruvate was inhibited in brain. Dickens (1946) also concluded that PDH was a likely site of oxygen inhibition. Thomas _et al_ (1963) in their _in vitro_ studies presented several lines of evidence in favour of inactivation of this enzyme. They showed using radioactively labelled substrates that glucose utilisation was decreased, lactate production was increased and that the production of $^{14}CO_2$ from 1-^{14}C and 2-^{14}C pyruvate was decreased. All these findings are compatible with inactivation or inhibition of PDH. Dickens (1946) and Mann & Quastel (1946), both demonstrated that lipoyl dehydrogenase, the enzyme responsible for the reoxidation of dihydrolipoamide to the coenzyme lipoamide was unaffected by oxygen _in vitro_.

Dickens (1946) reported malate dehydrogenase activity to be unaffected by oxygen _in vitro_ and Bannister _et al_ (1973) were unable

to demonstrate any changes in isocitrate dehydrogenase after in vivo exposure of rats to OHP. There are no further reports of changes in these two enzymes.

An irreversible inhibition in succinoxidase was noted in vitro by Dickens (1946), notably in the dehydrogenase part of the activity. This slow in vitro inactivation had been previously noted by Stadie & Haugaard (1945) who had stated that this inhibition could be reversed by the addition of glutathione or cysteine. However no difference from controls was detected in succinate dehydrogenase activity in animals killed by oxygen at 7 atm (Stadie & Haugaard 1945). In contrast Sanders & Hall (1966) using liver, kidney and brain from animals treated with OHP and Shaw & Leon (1970) using retina from rabbits exposed to OAP have both demonstrated a decrease in the activity of succinate dehydrogenase. Thomas et al (1963) also supported the inhibition of succinate dehydrogenase with evidence that $^{14}CO_2$ production from 1,4-$^{14}C_2$ succinate is reduced in vitro at 5 atm OHP. Similar evidence was presented by these workers for the inhibition of α-oxoglutarate dehydrogenase by oxygen. Williams & Haugaard (1970) confirmed the in vitro depression of α-oxoglutarate metabolism by OAP.

Jamieson & Chance (1966) used split beam spectrophotometry to monitor steady state changes in the oxidation states of constituents of the electron transport chain in mitochondria exposed to OHP. Their results indicated that there was an inhibition of the electron transport chain on the substrate side of cytochrome c, probably occurring at the dehydrogenase step. The rate of inhibition was similar to that observed in dehydrogenases by Jamieson & Brenk (1962). These results are in agreement with those previously obtained by Dickens (1946) who ascribed the main effects of oxygen toxicity on succinoxidase to the dehydrogenase. On purification, the NADPH

cytochrome c reductase was found to be extremely susceptible to inactivation by oxygen (Dixon et al 1960). Dickens also noted that cytochrome c was itself resistant to attack by oxygen, but that long periods of exposure did cause a slight inhibition to the cytochrome oxidase.

Bannister et al (1973) measured the in vivo changes in NADH cytochrome c reductase and cytochrome oxidase after exposure of rats to OHP. There were no significant changes in the NADH cytochrome c reductase of brain or muscle although their levels were slightly depressed in both brain and muscle after exposure to OHP, but the activity was also depressed to a similar level on exposure to hyperbaric air. This suggests an effect due to pressure per se rather than to oxygen. Rister & Baehner (1976) determined changes in NAD(P)H cytochrome c reductases after in vivo exposure to OHP in guinea pig alveolar macrophages and polymorphonuclear leucocytes obtained by lung lavage. In polymorphonuclear leucocytes the NAD(P)H cytochrome c reductase levels in the 16,000g pellet dropped throughout the exposure, but in the 100,000g pellet an initial drop was seen followed by a return towards normal levels. In alveolar macrophages the NADPH and NADH cytochrome c reductases showed different effects. The NADH cytochrome c reductase showed a rise in the 16,000g pellet but little change in the 100,000g pellet. The change in the NADPH cytochrome c reductase showed the opposite effect. The above results strongly indicate that the inactivation seen in vitro is not necessarily seen in vivo; any changes appear to be dependant on the tissue, the levels of toxicity employed and possibly the species.

ii) Glutathione Peroxidase, Glutathione Reductase and the Pentose Phosphate Pathway

Glutathione, glutathione reductase and glutathione peroxidase constitute a reducing system postulated to keep various cell constituents e.g. lipids, sulphydryl groups, in a fully reduced state. The reducing equivalents to keep glutathione functioning as a reducing agent are supplied by NADPH produced by the cytoplasmic pentose phosphate pathway. The effects of oxygen on this system have been investigated by several groups.

Bassett & Fisher (1979) using perfused lung exposed to OHP, obtained results which showed a nearly twofold stimulation of the pentose phosphate pathway and a stimulation of NADPH turnover. Tierney (1974) and Tierney et al (1975) also demonstrated a doubling of lung glucose-6-phosphate dehydrogenase (G6PDH) activity after in vivo exposure of rats to 0·85 atm OAP. Kimball et al (1976) found a quadrupling of lung G6PDH activity after similar in vivo exposures. However, determinations of rabbit retinal G6PDH showed a significant decline in activity and no significant changes were detected in 6-phosphogluconate dehydrogenase activity after 3 days at 1 atm OAP (Shaw & Leon 1970).

Again using rats, Kimball et al (1976) demonstrated an increase of 200% in glutathione reductase using the same conditions as above. Under similar conditions Frank, Bucher & Roberts (1978) managed to demonstrate a 29% rise in glutathione peroxidase activity in neonatal rats, but the change in adult animals was insignificant. Neonatal mice and rabbits gave even smaller increases, and in adult guinea pigs, mice rabbits and hamsters no significant rise in the activity of this enzyme was detected. Rister & Baehner (1976) demonstrated significant decreases in glutathione peroxidase in alveolar macrophages and polymorphonuclear leucocytes from guinea pigs subjected to 0·85 atm OAP. A 60% decrease in rabbit erythrocyte glutathione reductase was noted under similar conditions (Ray & Cherry 1977).

These results tend to show a variation between species, of the response of this enzyme system to oxygen. Rats in most of the studies have shown a moderate to large increase in the reducing capacity of this system after exposure to OAP, but the other species tested showed no change or a decrease in activity. The correlation between increases in the activity of this system after OAP and the acquired 'tolerance' of these animals to oxygen has been noted by several groups (Tierney 1974; Tierney et al 1975; Kimball et al 1976; Frank, Bucher & Roberts 1978).

iii) Neurotransmitter Synthesis and Metabolism

The effects of oxygen on neurotransmission have been investigated to attempt to determine the cause of the seizures associated with OHP; thus most of the work has centred on hyperbaric exposures. The effects of oxygen on neurotransmitter synthesis and metabolism are covered briefly in this section partly because of the minor neuronal symptoms reported for chronic hyperoxia, and partly for completeness.

Cholinergic Transmission: The effects of oxygen on choline acetyltransferase were first investigated by Stadie et al (1945e). Rat brain slices were found to be unaffected by OHP, but cell free preparations were rapidly inactivated by oxygen. Tunnicliff et al (1973) showed a small drop in activity of choline acetyltransferase, and Tunnicliff & Wood (1974) also supported the work of Stadie et al with similar in vitro evidence that both choline acetyltransferase and acetylcholinesterase from chick brain were unaffected by OHP treatment.

GABA Metabolism: Gaba Metabolism was implicated in oxygen toxicity when Wood & Watson (1963) discovered that GABA concentrations were reduced in brains of rats exposed to OHP in vivo, with the greatest decreases occurring in the most severely convulsed animals.

Exposure to OAP for a similar period of time was reported to produce no changes. Further experiments (Wood _et al_ 1963) showed that GABA injected i.p. had an ameliatory effect on the course of acute hyperoxia. Several subsequent reports have provided supporting evidence for the derangement of GABA metabolism by OHP _in vivo_ (Wood _et al_ 1966) and _in vitro_ in both chick (Wood & Watson 1964; Tunnicliff _et al_ 1973; Tunnicliff & Wood 1974) and human (Wood _et al_ 1975) brain homogenates. These reports were all in agreement that the enzyme synthesising GABA, glutamic acid decarboxylase (GAD), was markedly affected by oxygen toxicity. Approximately a 50% decrease in GAD activity was obtained _in vivo_ using 3 atm oxygen for 20 minutes. The enzyme metabolising GABA, glutamate-α-oxoglutarate aminotransferase, was unaffected by oxygen in these studies. In view of the role of glutamate as an excitatory neurotransmitter, GABA as an inhibitory neurotransmitter, and the role of GAD in helping to regulate the relative proportions of these two neurotransmitters (Bachelard 1974), these results would seem to have a significant bearing on the occurence of convulsions during OHP.

In contrast to the above reports, Schäfer (1978) reported an increase in brain GABA concentrations in mice exposed to OAP. The increase began 30 minutes after exposure with half maximum value at 2-4 hours and a maximum value at 50 hours, soon after which the GABA levels fell rapidly towards normal.

Biogenic Amines: Studies using OAP _in vivo_ have demonstrated that both catecholamines (Neff & Costa 1967) and serotonin (Diaz _et al_ 1968) show an increased turnover under these conditions. The findings of Diaz _et al_ indicated that it was the rate limiting step in the synthesis catalysed by tryptophan hydroxylase that was responsible for the increased turnover rather than an acceleration of 5-hydroxytryptophan decarboxylase activity. Fisher & Kaufman (1972)

showed that in vitro, oxygen caused an inhibition of phenylalanine and tryptophan hydroxylases when the natural cofactor was present, but that this decrease was not so marked employing the synthetic cofactor. However, in vitro studies supported the work of Neff & Costa and Diaz et al that the activity of tryptophan hydroxylase was increased by oxygen both in OAP (Nelson & Huggins 1976) and in OHP (Tunnicliff & Wood 1974). The latter authors also showed an increase in activity of tryptophan hydroxylase, but a decrease of the enzymes dopa decarboxylase, and 5-hydroxytryptophan decarboxylase of the synthetic pathway of catecholamine and serotonin synthesis. Nelson & Huggins (1976) however were unable to demonstrate any inhibition of 5-hydroxytryptophan decarboxylase. This difference of findings is not necessarily of consequence to oxygen toxicity. These enzymes are not the rate limiting step in the synthesis of biogenic amines, so as long as these activities do not decrease to limiting levels, no effect would be seen.

In the degradative pathways for biogenic amines, monoamine oxidase had been reported to increase in activity in vitro in OAP (Nelson & Huggins 1976) and in OHP (Tunnicliff & Wood 1974), but catechol-O-methyl transferase was unaffected. Despite the lack of in vitro evidence for inhibition of serotonin metabolism, Block & Fisher demonstrated that perfused lungs obtained from rats subjected to either OAP up to 48 hours (1977a) or OHP for 60 minutes at 4 atm (1977b) showed a decrease in their ability to clear serotonin from the perfusion fluid.

iv) Other Miscellaneous Enzymes

Dickens (1946) reported that phosphoglucomutase was sensitive to OHP in vitro but that this effect was only noted at certain stages of its purification. D-amino acid oxidase was reported to be

sensitive by this author, but Mann & Quastel (1946) revealed it was sensitive only in the absence of its substrate. Stadie et al (1945d) showed that D-amino acid oxidase was rapidly inactivated in homogenates but not in tissue slices. Xanthine oxidase was also reported to be sensitive (Mann & Quastel 1946; Stadie et al 1945d). The sensitivity of xanthine oxidase to oxygen had been noted previously during its purification (Dixon & Kodama 1926). Uricase was demonstrated to be unaffected by oxygen Stadie et al (1945d). Exposure of reaction mixtures containing fatty acid dehydrogenase to oxygen was reported to lead to a rapid inactivation of this enzyme (Shapiro & Wertheimer 1943).

Recent work by Brown et al (1978) has demonstrated that both quinolinate transferase and 5-phosphoribose pyrophosphokinase were extremely sensitive to OHP in E.coli. In view of the roles of these enzymes in NAD/NADP synthesis and RNA metabolism respectively, it is a matter of some interest whether the mammalian enzymes are as sensitive to oxygen as the E.coli enzymes. It would not be wise just to assume that the mammalian enzyme is as easily affected as the E. coli enzyme since yeast LDH is extremely labile (Dixon et al 1960) but the mammalian enzyme is relatively stable.

Pepsin and carbonic anhydrase have been shown to be insensitive to the effects of OHP (Stadie et al 1945c). Similarly, rat brain ATPase showed no changes after exposure in vitro to OAP (Williams & Haugaard 1970), but Koehler & Gottlieb (1972) and Hemrick & Gottlieb (1977) showed that oxygen in vitro could have stimulatory or inhibitory effects depending on the pressure. Nitrogen and helium also had different effects on ATPase at different pressures, suggesting that perhaps this effect was related to the physical properties of these gases on membranes rather than their chemical reactions. These findings however are consistent with explaining the strange

changes in ATP concentrations of rats poisoned by OHP recorded by Sanders et al (1966) (see section C. ii) below).

Various markers for cell or tissue damage have been assayed. Hall & Sanders (1966) monitored the levels of free cathepsin and acid phosphatase as markers for lysosomal enzyme release. The percentage free cathepsin in brain and liver was found to be significantly elevated after exposures to 1, 3 and 5 atm of oxygen with increasing levels up to 3 atm, but 5 atm showing the same change as 3 atm. In kidney, 1 atm caused a significant drop in percentage free cathepsin, 3 atm a significant rise and 5 atm was not significantly different from control. The percentage free acid phosphatase activities were not so clear cut. Liver showed a significant and equal rise at 3 atm and 5 atm oxygen, but kidney only showed significant rises at 1 and 5 atm with the 3 atm value being very similar to the control. In brain, the highest percentage free acid phosphatase levels were in the controls. These results were taken to indicate changes in cellular structure, particularly the lysosomal membrane; a conclusion shared by Allison (1965).

Bannister et al (1973) monitored the levels of blood alkaline phosphatase, aspartate transaminase and creatine kinase. The latter two enzymes showed a definate rise in exposed rats, with the greatest changes seen in minimally convulsed rats. This result was indicative of the occurrence of liver damage.

Both Dickens (1946) and Haugaard (1946) have pointed out that the class of enzymes most susceptible to the effects of oxygen are sulphydryl enzymes. However, the possession of sulphydryl groups by an enzyme is not a prerequisite for its inactivation by oxygen, nor are all sulphydryl groups susceptible or equally susceptible to oxidation. The inactivation of any specific sulphydryl enzyme will depend not only on the availability of any particular sulphydryl

group to oxygen, but also on the importance of that sulphydryl group to the structure and function of that enzyme.

It has also been frequently noted that the inactivation of an enzyme may be demonstrable *in vitro* or at a certain stage of purification e.g. phosphoglucomutase, but not necessarily *in vivo*. Conversely, the demonstration that an enzyme activity in a homogenate from an hyperoxic animal is not altered, does not preclude that enzyme from being activated or inhibited by a higher tension of dissolved oxygen e.g. tyrosine and tryptophan hydroxylases. In addition, many enzymes are present in excess quantities for the metabolic requirement, the metabolism being controlled by key enzymes in the various pathways. If the enzyme affected by oxygen is not a control enzyme, it must be demonstrated that this enzyme becomes limiting before that enzyme can be said to affect the course of oxygen toxicity at a molecular level. Finally the demonstration of no inhibition of an enzyme by oxygen does not necessarily mean that a lack of structural integrity of the cell is not affecting the useful function of that enzyme e.g. possibly electron transport.

These criteria must all be tested before any enzyme activity can without doubt be said to be a cause of cellular oxygen toxicity.

C. Changes In Cellular Constituents Other Than Enzymes

i) Reduced/Oxidised Pyridine Nucleotide Ratio

Direct evidence of the effects of oxygen on the ratio of reduced to oxidised nucleotides has been produced by Chance *et al* (1965). The surface fluorescence of organs from intact animals anaesthetised and exposed to oxygen was measured using a micro-fluorometer. These experiments showed that the fluorescence, which is proportional to the concentration of reduced pyridine nucleotides present, decreased with increasing oxygen concentration. The

relationship between oxygen pressure and fluorescence was hyperbolic in brain and kidney resulting in a plateau at a 25% decrease in fluorescence. In liver the relationship appeared to be almost linear, the fluorescence still decreasing at 12 atm OHP despite already having decreased by 75%. These effects were also seen _in vitro_ using mitochondria.

Indirect support for the decrease in reduced pyridine nucleotides was provided by Felig & Lee (1965). These authors demonstrated that lactate was a useful protective agent against the toxic effects of OAP by significantly extending the life of these animals. Other metabolites and buffers tested: pyruvate, acetate,bicarbonate or tris buffer had far smaller beneficial effects. This led these authors to conclude that lactate must be exerting its effect through an increase in NADPH produced by metabolism of lactate.

ii) ATP Concentration

Horn _et al_ (1965) and Williams & Haugaard (1970) have both reported decreases in ATP concentrations in heart and brain homogenates respectively in their _in vitro_ studies of oxygen toxicity. This effect has been confirmed using hyperbaric exposure of perfused lungs (Bassett & Fisher 1979). Despite the fact that this study showed increased glucose utilisation, and lactate pyruvate and carbon dioxide production, the amount of ATP produced was decreased as was the ATP/ADP ratio.

Larkin (1973) demonstrated decreased levels of erythrocyte ATP after exposure of active and hibernating ground squirrels to OHP, but the work of Sanders _et al_ (1966) on ATP levels after OHP exposure is somewhat confusing. Although hyperbaric exposure produced a significant decrease in brain ATP at 1 atm, 3 atm and 5 atm oxygen, the depression of ATP concentration was less at 3 atm than at either 1 atm or 5 atm. Liver and kidney ATP concentrations were

different again. The levels were normal at 1 atm oxygen, increased at 3 atm oxygen and decreased at 5 atm oxygen exposure.

iii) Coenzyme A Concentration

A single report has been presented of an *in vivo* decrease in coenzyme A (CoA) concentration (Bond *et al* 1967). In this study, a pure oxygen atmosphere at 259mm Hg pressure (only 0·34 atm oxygen) caused a 50% decrease in CoA of rats over a period of 4-5 weeks, which on further exposure returned to normal. A parallel decrease in the incorporation of ^{14}C-acetate into lipid was noted. Oxidation of CoA in 20 minutes at 17 atm oxygen was obtained (Barron 1955). In view of the considerable depletion of CoA at a concentration of oxygen which is generally considered not to be noticeably toxic, it would be interesting to see if a similar effect occurs, using oxygen at atmospheric pressure, which could be correlated with the time course of oxygen toxicity.

iv) Free Sulphydryl Groups

The oxidation of free sulphydryl groups by oxygen was investigated *in vitro* by Barron (1955). Using a stainless steel 'bomb', it was confirmed that sulphydryl compounds were perticularly susceptible to oxidation by high pressures of oxygen (typically 20 minutes at 17 atm oxygen) and that this was mediated by contact with the steel walls of the bomb, showing the importance of Fe ions in uncontrolled oxidations.

Using OHP a decrease in reduced sulphydryl groups was demonstrated in lung tissue; a resulting increase in oxidised sulphydryl groups led to a halving of the -SH/-SS- ratio (Jamieson *et al* 1963). Further experiments on sulphydryl groups (Harris & Brenk 1968) showed a considerable drop in lung non-protein sulphydryl (NPSH) groups (44%)

in animals convulsed by OHP, but not in animals exposed to but not convulsed by OHP. Liver showed a smaller loss of NPSH (25%), but this loss was the same whether convulsions occurred or not. A similar loss of liver NPSH could be produced by 18 hours starvation casting doubts on the relevance of this result. Determinations of retinal NPSH and blood glutathione levels (Shaw & Leon 1970) failed to reveal any significant changes between rabbits exposed to OAP and their controls, although blood glutathione levels tended to be increased. Contrary to the previous reports, Kimball *et al* (1976) using 0·9 atm OAP for 7 days observed an increase of lung glutathione to 195% of controls and NPSH levels to 365% of controls in rat lung. A possible explanation of the differences, is that OHP provides a very concentrated oxidant stress, but that OAP allows time for adaptive changes to occur. A variation of adaptive response between species probably further complicates the results.

v) Peroxide Formation

Joanny *et al* (1971) demonstrated an increase in brain malonaldehyde levels in rats which were exposed to OHP, and also that rats which convulsed in OHP showed higher levels than rats which had not convulsed. Plasma malonaldehyde levels were also elevated in rats exposed to OHP, but in erythrocytes there was no difference between exposed and control animals. *In vitro* studies were used to demonstrate a correlation between malonaldehyde levels and peroxide formation. Mengel & Kann (1966) showed an increase in lipid peroxidation after *in vivo* exposure to OHP in tocopherol deficient mice, but not in tocopherol deficient & supplemented rats or tocopherol supplemented groups. Similarly a rise in brain lipid peroxides was also only noted in vitamin E deficient mice (Jerrett *et al* 1973).

Nishiki *et al* (1976) provided indirect evidence for the

formation of lipid peroxides. Using perfused lung and liver preparations, it was shown that infusion of t-butyl hydroperoxide caused a release of glutathione into the perfusate and a concurrent oxidation of pyridine nucleotides occurred. In addition, an increase in hydrogen peroxide formation was estimated from the turnover of the catalase reaction. Measurements of these parameters, which are an index of lipid peroxidation, revealed a slight increase in lipid peroxide formation in these perfused tissues when subjected to OHP. Tocopherol deficient organs showed a marked increase in lipid peroxide formation as estimated by this technique.

vi) Ascorbic Acid

It has been reported (Gerschman & Fenn 1954) that both OHP and OAP treatment caused a significant decrease in the ascorbate content of adrenals, (a tissue which contains a high proportion of ascorbate). However similar changes in ascorbate were noted after pressurisation using air (Gerschman & Fenn 1952): this would tend to indicate that this decrease in ascorbate was a non-specific reaction due to stress.

vii) 2,3 Diphosphoglycerate

Decreases in erythrocyte 2,3 diphosphoglycerate (2,3 DPG) have been reported in mice exposed to OHP (Mengel 1972), active and hibernating ground squirrels exposed to OAP (Larkin 1973) and in 3-4 week old, but not older, rats exposed to OAP (Weissberg & Crapo 1976). The decrease in 2,3 DPG is obviously an adaptive response, but the effect of the response on tissue oxygen tensions is unlikely to greatly affect the pathogenesis of oxygen toxicity.

D. The Effect Of Hormones On Hyperoxia

The influence of hormones on the time course of oxygen toxicity

was investigated as long ago as 1937(a) by Campbell who published a report showing that both starvation and lowered body temperature ameliorated the effects of oxygen at high pressure (30 minutes at 6 atm oxygen). He also demonstrated (1937b) that thyroxine, adrenaline, insulin and pituitary extract were found to increase the toxic effects of oxygen. Further experiments reported in 1938 showed thyroidectomy and hypophysectomy to be effective in reducing the noxious effects of oxygen, but that hypophysectomy was not as effective as thyroidectomy. Using a similar level of toxicity, Grossman & Penrod (1949) confirmed the detrimental influence of the thyroid by showing that administration of thyroid powder augmented and propylthiourea (an antithyroid agent) reduced the toxic effects of OHP on rats. These authors further noted a very good correlation between the oxygen consumption of the animals and their survival time. These findings concerning the involvement of the thyroid in acute oxygen toxicity were extended to include OAP by Bean & Bauer (1952) and Smith _et al_ (1960). These latter experiments demonstrated that hypophysectomised animals were less susceptible to the effects of OAP than animals administered desiccated thyroid extract or thyroxine.

Although these results would appear to indicate that hyperoxia might be causing hyperfunction of the thyroid, this was demonstrated to be a fallacy (Galton 1978). Results were presented to show that hyperoxia caused a decrease in peripheral deiodination and deiodinative clearance of thyroxine, the serum thyroxine concentration and also a decrease in the thyroxine binding capacity of serum. These decreases in thyroid functions were accompanied by a decrease in whole body oxygen consumption and were attributed at least partially to a fall in the rate of secretion of pituitary thyrotropin.

Adrenalectomy has also been shown by many authors (Gerschman et al 1954b;1955; Taylor 1954; 1958) to have a beneficial effect on the susceptibility of animals to OHP. That adrenal medullation was not as effective in this respect was demonstrated by supplementation experiments. Adrenaline only partially reversed the beneficial effects of adrenalectomy, but when cortisone was also administered a complete reversal was obtained. Cortisone alone given in a sufficient dose could also reverse the beneficial effects of adrenalectomy (Gerschman et al 1954b; 1955). The results of Taylor (1954; 1958) were somewhat equivocal on the effects of supplemental therapy on adrenalectomised rats. Smith & Bean (1955) have extended these findings to chronic hyperoxia and shown that adrenalectomy both increased the survival time and reduced pulmonary damage. Injections of either cortisone or adrenaline caused the opposite effect. The detrimental effect of cortisone on oxygen therapy at atmospheric pressure was also noted by Warshaw et al (1952).

Moderation of the toxic response of animals to OHP by hypophysectomy has been confirmed by Bean (1952) and Bean & Johnson (1952). However, although the injection of ACTH reversed the effects of hypophysectomy it did not do so completely, suggesting that the pituitary control of hormones other than the adrenocortical hormones is of importance in hyperoxia. Bean & Smith (1953) have also shown that hypophysectomy was of beneficial effect during the administration of OAP.

Taken as a whole, these reports would appear to suggest that thyroid, adrenal medullary and adrenal cortical hormones all have a detrimental effect on the pathogenesis of oxygen toxicity, and that this effect is probably 'orchestrated' by the pituitary. However the observation of Campbell (1938), that thyroidectomy is more protective than hypophysectomy, indicates that the pituitary may

moderate the effects of some hormones in the response to oxygen toxicity.

To put these findings into perspective, it is worth emphasising the observation of Bean & Smith (1953), that although these hormonal factors may be responsible for augmenting the effect of the toxicity, they are not essential to the production of lung damage by oxygen.

5. GENERAL SUMMARY AND AIMS OF THE PROJECT

Almost since its discovery over 200 years ago, oxygen has shown its potential and actual toxicity to scientists working with it. At atmospheric pressure it causes changes mainly in the lungs. Pneumonia-like symptoms and pathological lung changes including oedema, haemorrhage, consolidation, atalectasis, thickening of alveolar walls, fibrin formation and necrosis of certain susceptible cell types have been consistently reported with OAP. Using OHP additional neurological changes become prominent. Degenerative changes have also been recorded in other organs in both OAP and OHP.

These changes are thought to be mediated through the chemical reactivity of oxygen which is due mainly to formation of radicals. The reactive species so far implicated are superoxide anion, peroxyl and hydroxyl radicals, the excited singlet state, and possibly the catalysis of hydrogen peroxide's potential reactivity via the vacant outer orbitals of transition metal ions. Of these possibilities, the excited singlet state, the hydroxyl radical and transition metal complexes are the most reactive, and there is little biological protection against the direct effect of the latter two chemical species.

The above reactive species produce damage at the molecular level on essential cellular components including enzymes, coenzymes,

metabolites and structural components such as lipid membranes. The most likely points of attack so far elucidated include inactivation of enzymes - mainly those containing sulphydryl groups. Pyruvate dehydrogenase, α-oxoglutarate dehydrogenase, succinate dehydrogenase, glutamic acid dehydrogenase, quinolinate transferase and 5-phosphoribose pyrophosphokinase appear to be particularly susceptible, but possibly activation of tyrosine and tryptophan hydroxylases and monoamine oxidase may also be contributory. A decreased production of reduced pyridine nucleotides and ATP also appears to occur. Other reactions of major importance are thought to be the formation of lipid peroxides and the general oxidation of sulphydryl groups. Lipid peroxidation could be particularly damaging because once it is initiated, a chain reaction ensues: lipids are intimately involved in the integrity of subcellular organisation essential to controlled cellular metabolism. In addition the lipid peroxides produced are themselves highly toxic.

The effects of oxygen are potentiated by the endocrine system and by physiological effects such as the constriction of blood vessels, changes in gaseous exchange rates and resultant carbon dioxide retention and acidosis.

There is no doubt that oxygen acts directly on many different aspects of metabolism and that there are also secondary consequences of these reactions.

The aims of this project were to investigate *in vivo* metabolic changes which could help to explain the pathogenesis of oxygen toxicity at a molecular level. The experimental work was carried out using chronic exposures of experimental animals at atmospheric pressure in order to avoid the complications attendant with working at pressures above atmospheric. The areas for study for this

project were chosen to be compatible with the eventual aim of attempting to confirm the results in larger mammals.

Initially an investigation into the levels of the protective enzymes SOD and catalase was pursued to determine whether mammalian cellular antioxidant defence systems respond to increased oxygen levels, typically 0·85 atm, or alternatively whether this defence system was overwhelmed by oxygen. Protein concentrations were also monitored in these experiments. It was thought that the changes in protein concentration which were noted might have been due to alterations in the water content of tissues. This question was investigated together with the question of whether the changes noted in protein were merely quantitative, or whether they were the sum of several different qualitative changes. Following the determination of changes in protein metabolism a survey of amino acid levels was conducted to see if it was possible that a deranged amino acid metabolism contributed to the changes noted in protein levels.

At the conclusion of the above section of work, investigations were directed towards further likely metabolic sites where oxygen might exert its toxic action. Exploratory work was performed on both the effects of oxygen on prostaglandin metabolism in relation to lung inflammation and also whether vitamin A was affected by the toxic action of oxygen.

A secondary aim of this project was realised towards the end of this work, when certain of these determinations were applied to blood samples from 'divers' exposed to a mild experimental hyperoxia to investigate whether any changes could be detected and correlated with the work on rodents.

MATERIALS AND METHODS

1. Design And Construction Of An Apparatus To Provide A Controlled High Oxygen Environment At Atmospheric Pressure
   - A. The Ambient Pressure Oxygen Chamber
   - B. Scrubbing System
   - C. Feedback Control System Of Oxygen Supply
2. Materials
   - A. Animals
   - B. Enzymes And Chemicals
   - C. Solvents
   - D. Water
   - E. Buffers
3. Methods
   - A. Enzyme Assays
   - B. Other Assays
   - C. Preparation Of Polyacrylamide Gels
   - D. Spectrophotrometry
   - E. Experimental Procedures

MATERIALS AND METHODS

1. DESIGN, CONSTRUCTION AND OPERATION OF AN APPARATUS TO PROVIDE A CONTROLLED HIGH OXYGEN ENVIRONMENT AT ATMOSPHERIC PRESSURE

The design of this apparatus was based upon a perspex ambient pressure chamber with a recirculating air supply and chemical scrubbers to remove metabolic waste products. A feedback control system permitted oxygen to be admitted to the system from a gas cylinder as it was respired in order to maintain the desired partial pressure of oxygen.

Initially a perspex box, oxygen analyser, mains controller and solenoid valve were provided by A.M.T.E. (PL) to set up a trial system. To complete the system a small capacity pump to feed the oxygen analyser, a large pump and the scrubbing assembly were added. Figure 3 shows the principle of operation and the relationship of the parts. The specification and function of each of the parts is described separately below.

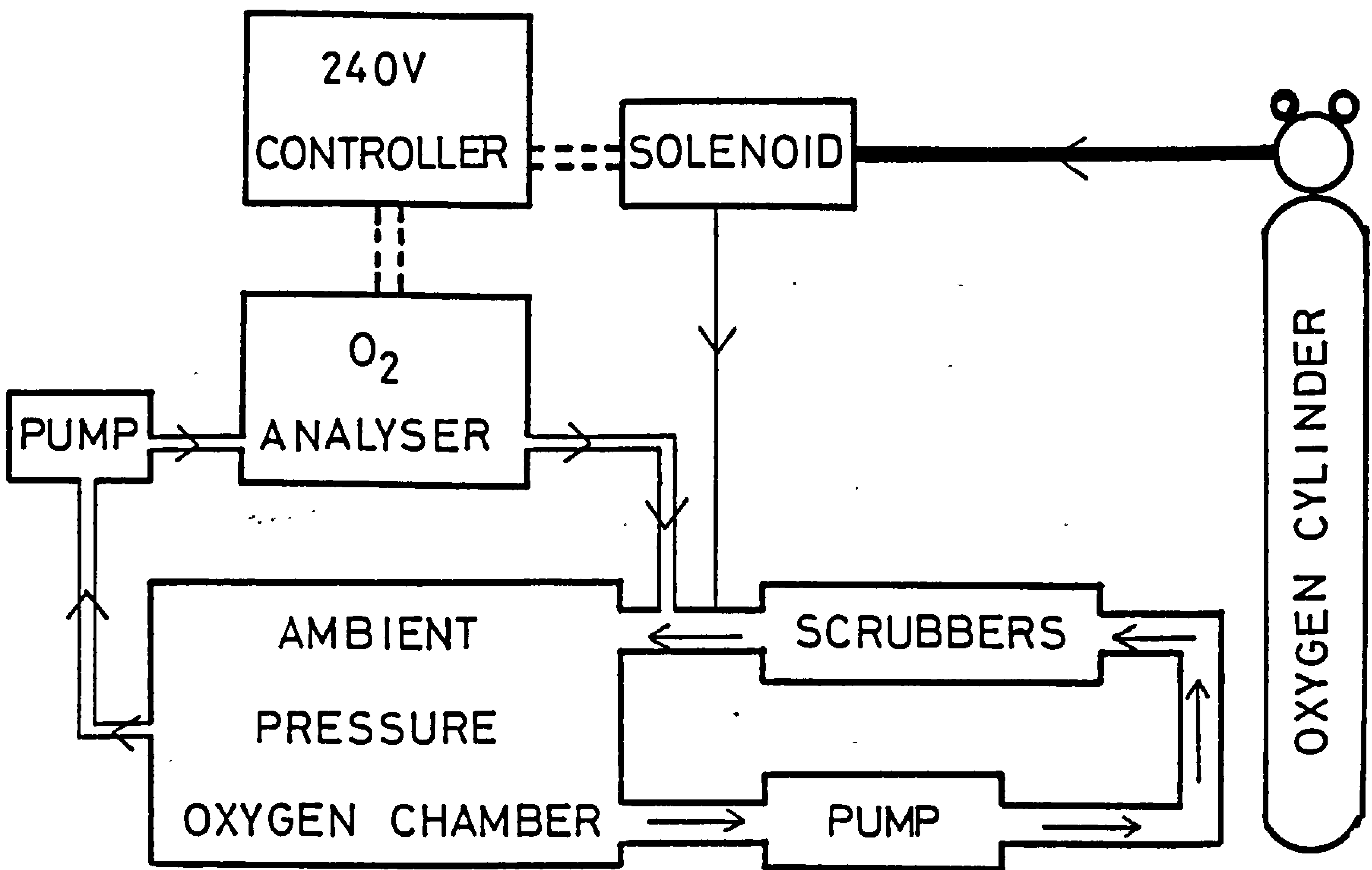
A. The Ambient Pressure Oxygen Chamber

This was initially a box of a basic design constructed of flat sheets of perspex glued along all edges of each side. The front was a single piece of perspex which was sealed onto a rubber gasket on flanges at the front by screws. The animal cage, which had a stainless steel grid in place of a solid plastic bottom, rested on two perspex ledges along the side of the chamber. The overpressure relief valve consisted of a perspex tube dipping into a tube of water. Incoming air was distributed by two perforated perspex tubes.

This chamber was found to suffer from several drawbacks during the relatively long periods of the experiments for which it was used.

FIGURE 3

DIAGRAM TO SHOW THE PRINCIPLE OF OPERATION OF THE AMBIENT PRESSURE OXYGEN CHAMBER



NARROW BORE PLASTIC TUBING

COPPER TUBING

WIDE BORE PLASTIC TUBING

STAINLESS STEEL TUBING

ELECTRICAL CONNECTIONS

ARROWS INDICATE DIRECTION OF GAS FLOW

The perspex seams were found to develop leaks at intervals which were difficult to reseal properly. It was not possible to replenish food or water without removing the entire front of the chamber, an operation which caused the partial pressure of oxygen to fall to almost 0·2 atm. Also as there was no provision for the collection of excreta the chamber became very messy after several days in operation. Finally the external connection tubes to the scrubbing system were found to be too narrow for the size of tubing commensurate with an adequate flow of air through the scrubbing system.

In order to reduce these problems to a more acceptable level, an improved version of the chamber was constructed of similar dimensions to the original. The new design used a large single piece of perspex with 3 right-angled bends and just one joint to form the longitudinal axis of the chamber. This single joint was then strengthened externally using a right-angled strip of perspex. The rear of the chamber was glued onto one end and the front was again removable, being screwed onto a flange. The seal on the front was again effected by a rubber gasket smeared with silicone high vacuum grease. However this time the front was in two pieces, the bottom portion allowed access to a tray and the upper portion permitted the cage to be removed. In addition, a sailing dinghy hatch-cover (RWO England) was fitted to the larger upper portion to allow food and water to be replenished. It was also possible to remove rats via this opening. The remaining fitments to the chamber were similar to the original version. The chamber had the dimensions 0·3m by 0·3m by 0·47m giving a volume of 42·3 litres. The design of the chamber is illustrated in figure 4. Part of the chamber was blacked out with a black plastic bag to minimise disturbance to the animals.

FIGURE 4: DETAILED PLAN OF THE AMBIENT PRESSURE OXYGEN CHAMBER

SCALE: 1:3

B. Scrubbing System

In order to provide a reasonally habitable environment, it was necessary to continuously remove all waste products of metabolism, namely:- carbon dioxide, ammonia, volatile amines and excess water vapour. To accomplish this the following filters were provided.

i) Acid Scrubber

Two wads of absorbent cotton wool were dampened with sulphuric acd (H_2SO_4:water, 1:2) and held in place in a glass tube by non-absorbent cotton wool. For the final experiments on blood chemistry this filter was improved by the addition of activated charcoal to further reduce unpleasant smells. The acid filter was changed when white crystals visibly appeared. In practice this occurred about every other day.

ii) Soda-lime Scrubber

Two glass tubes each containing approximately 250g of soda-lime held in place at each end by wads of non-absorbent cotton wool. The principal function of this scrubber was to prevent a build-up of carbon dioxide, but also it was positioned downstream of the acid scrubber to remove any acid vapour which may have passed over from that scrubber.

iii) Silica-gel Scrubber

This was a bulbous glass tube which held approximately 500g of silica-gel. Although this was sufficient to prevent the appearance of condensation for a 12 hour period when up to 16 mice were present, when 6 rats were in the chamber it was found necessary to add a second 500g container.

Both the silica-gel and soda-lime scrubbers were changed as

indicated by the contents of their respective tubes. The tubing connecting the scrubbers to the chamber was a 12mm internal diameter thick-walled polythene tube.

iv) Pump Supplying the Scrubbing System

This was a double reciprocating bellows pump supplied by the Williamson Manufacturing Company (London). It was an uprated model which operated with a stroke volume of 25ml and 250 (2 x 125) strokes per minute, giving a capacity to circulate 5 litres of air per minute. The total volume of air in the system (chamber plus scrubbers and tubing) was approximately 45 litres which would therefore pass through the scrubbers once every 9 minutes.

C. Feedback Control System of Oxygen Supply

i) Pump to Oxygen Analyser

A Watson-Marlow MHRK 55 flow inducer supplied the oxygen analyser with a constant output of air from the chamber. A narrow bore tube (2mm internal diameter) from the pump to the analyser permitted a response within 40 seconds of a change in oxygen concentration in the chamber.

ii) Control Equipment

A 240v solenoid (Dewrance Asco) controlled the supply of oxygen to the chamber. The solenoid was operated by a 240v controller (Controller 77, Luft Instruments, Lincoln, Mass.) in response to the signal from an oxygen analyser. The Oxygen analyser was a mains powered Servomex OA 250 analyser (Servomex Controls Ltd. Crowborough) fitted with an output socket and back-off control to provide a suitable signal to the 240v controller.

Under normal circumstances it was possible to control the level

of oxygen in the chamber to better than ±2% using the above system. The photograph in figure 5 shows the apparatus in operation.

Figure 5: Photograph Showing the Ambient Pressure Oxygen Chamber In Operation

2. MATERIALS

A. Animals

Male albino mice (strain BKI..A, 30-32g) and male albino rats (Sprague-Dawley strain 160-180g) were used throughout this investigation. Except for the mice used in the experiment described in chapter 3 all animals were bred in the department and kept as far as possible under identical conditions with respect to diet and temperature. Animals were fed on Dixons GR3EK diet and allowed water *ad libitum*. The remaining mice were of the same strain and weight as above but purchased from Banting and Kingman Ltd. Hull.

B. Enzymes and Chemicals

SOD (Superoxide dismutase E.C. 1.15.1.1.) from bovine blood was obtained as a lyophilised powder containing approximately 2-5% potassium phosphate salts.

6-Hydroxydopamine (2,4,5 Trihydroxyphenethylamine) was purchased as the crystalline hydrobromide salt from Sigma London Chemical Co. Poole and stored desiccated at -20°C. Due to its inherent instability in aqueous solution in an oxygen atmosphere, fresh solutions were prepared immediately before use in distilled water previously sparged with oxygen-free nitrogen for at least 5 minutes. Solutions were kept under an atmosphere of oxygen-free nitrogen for the duration of the assay.

Hydrogen peroxide was a strong stock solution, 30% w/v from BDH Chemicals Ltd. Poole, which was stored refrigerated. Buffered solutions of hydrogen peroxide for catalase assays were prepared fresh daily after monitoring the concentration of the stock solution spectrophotometrically at 240nm using $\varepsilon = 40{\cdot}0$ l. mole^{-1}cm^{-1}.

Retinol type X crystalline synthetic alltrans was purchased from Sigma London Chemical Co. Poole, stored at -20°C and purified

chromatographically before use.

Charcoal, granular activated for gas adsorption; soda-lime, carbosorb, large self indicating granules; and silica-gel, large self indicating granules for the scrubbing system were all obtained from BDH Chemicals Ltd. Poole.

Boehringer Chemicals Ltd. Lewes, supplied the 'Hemoglobin Test Combination'.

C. Solvents

Methanol A.R. quality was supplied by James Burroughs and Co. Ltd. London.

Absolute ethanol A.R. quality was supplied by James Burroughs and was redistilled over potassium hydroxide pellets. The fraction boiling between 78·8 and 79·0°C was collected.

Hexane pure, diethyl ether pure, and petroleum ether 60-80°C fraction pure, were purchased from Koch-light Laboratories Ltd. Colnbrook. Diethyl ether was made peroxide free (Dasler & Bauer 1946) by passing it through a column of activated alumina (neutral Brockmann grade 1) and redistilled, the fraction between 34·5-35·5°C being collected. Petroleum ether was redistilled the fraction coming over at 68·5-69·0°C being collected. Ethanol, petroleum ether, diethyl ether and hexane were all stored in the dark and were redistilled at approximately monthly intervals. The redistillations were necessary to convert these solvents to the purity required for fluorimetry.

D. Water

Double glass-distilled water was used to prepare aqueous reagents and buffers throughout this study.

E. Buffers

The pH of buffers was checked on either a Pye model 79 pH meter or a Corning PTI-6 universal digital pH meter previously calibrated with standard phosphate buffer pH 7·0 at room temperature.

3. METHODS

A. Enzyme Assays

i) Superoxide Dismutase

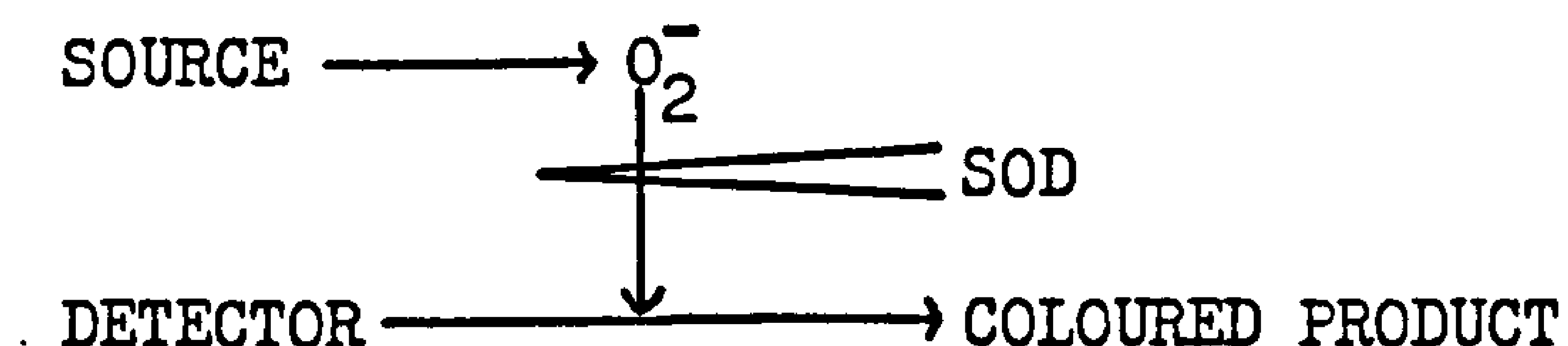
SOD catalyses the dismutation of superoxide anions which proceeds essentially as follows:

$$O_2^- + HO_2 + H^+ \longrightarrow H_2O_2 + O_2$$

This reaction also occurs uncatalysed by enzyme with a rate constant of approximately 1 x 10^5 $M^{-1}sec^{-1}$ at physiological pH (see introduction). Although it is possible to monitor the SOD catalysed disappearance of free radical generated by an electron pulse using a linear electron accelerator over a millisecond time scale (Klug et al 1972), this is obviously not an acceptable method for a routine laboratory determination. Consequently assays of SOD activity have had to rely on enzyme catalysed alterations in steady-state concentrations of superoxide.

All commonly used SOD assays depend on two components: a) a generator which produces the radical at a controlled constant rate, and b) a superoxide detector. In the absence of SOD, the radical rapidly achieves a concentration where its rate of reaction with the detector equals its rate of generation. If SOD is present it competes with the detector for superoxide resulting in a lower steady state concentration of radical. This lower steady state of superoxide is reflected in the reduced rate of reaction of radical with the detector. The degree of inhibition of the reaction of the detector

with superoxide is thus a measure of SOD concentration.



For this study the assay of Heikkila & Cabbat (1976) was adopted because of its rapid reaction allowing many samples to be handled quickly. It is performed at physiological temperature and pH, and its high sensitivity permits its use even on quite crude homogenates. Due to the constraints of equipment and space available the technique of Heikkila & Cabbat was modified as follows.

In their assay the reaction took place in an ehrlenmeyer flask, containing buffer and enzyme to which 6-hydroxydopamine (6-OHDA) was added to a final concentration of 10^{-4}M, in a shaker bath. The reaction was monitored at 490nm using a flow cell.

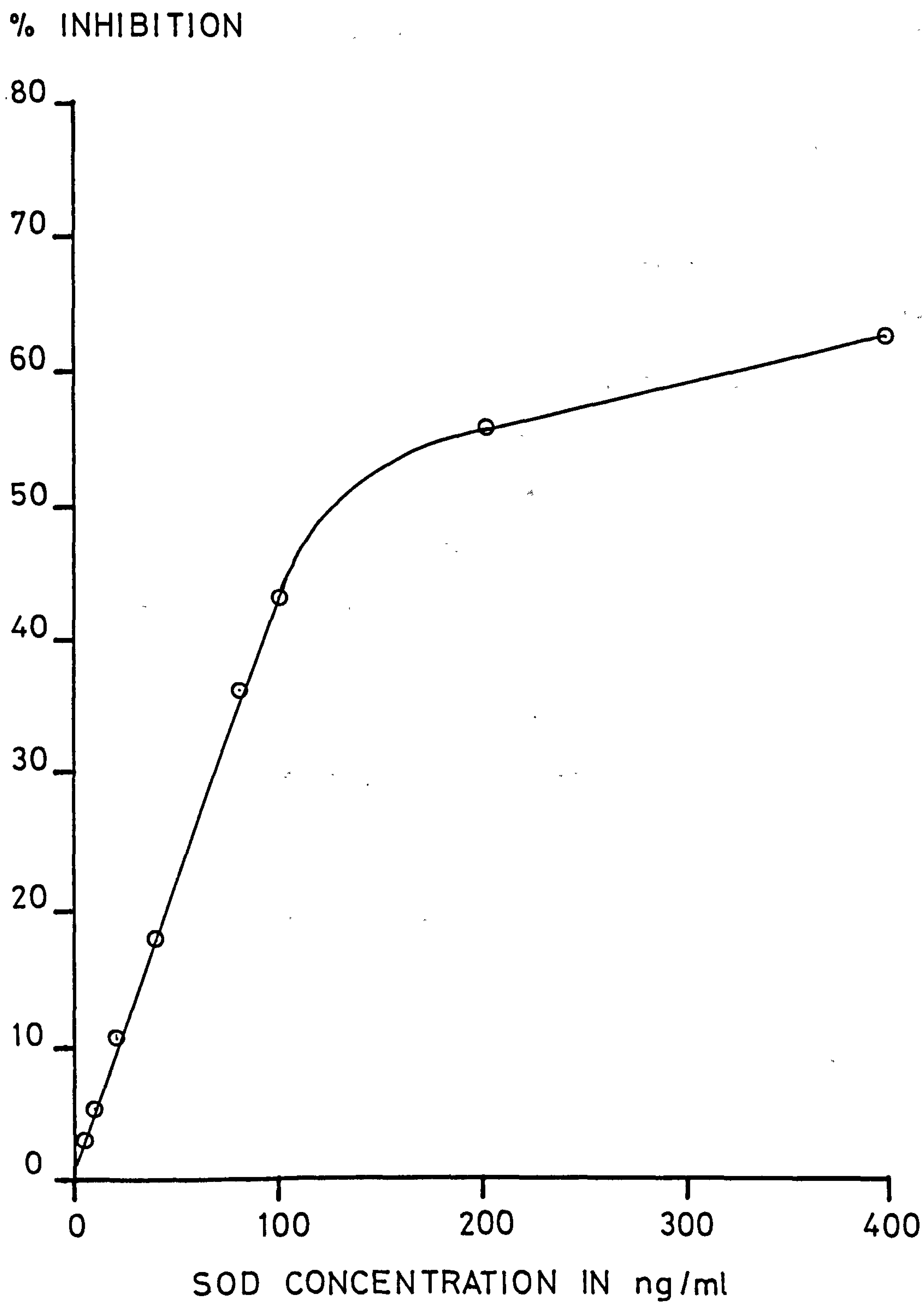
It was found that if the buffer was equilibrated by standing in a water bath and the final concentration of 6-OHDA was doubled to 2×10^{-4}M, the reaction could be carried out in a thermostatted spectrophotometer compartment thus dispensing with the need for a flow cell.

A water bath was heated to 37°C by a water heater and circulating pump (B.Braun Melsungen Apparatebau) which also circulated water to the spectrophotometer to maintain the cell holder at the same temperature as the water bath. The assay buffer, 50mM phosphate buffer pH 7·4 containing 0·1 mM EDTA, was equilibrated with the oxygen in the atmosphere for 1½-2 hours before commencement of the assay.

An aliquot of buffer (2·3ml) was carefully transferred to a plastic cuvette in the sample compartment followed by a small volume of enzyme extract (0·1ml). The solution was mixed with a flat-ended

FIGURE 6

% INHIBITION OF 6-OHDA AUTOXIDATION MONITORED AT 490nm AGAINST SOD CONCENTRATION

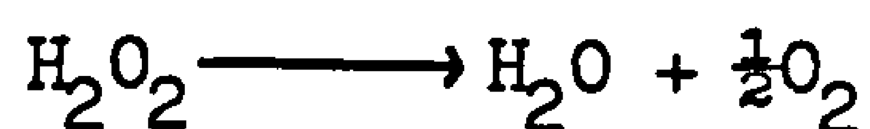


glass rod and the spectrophotometer was then zeroed on this mixture. A small volume of 6-OHDA(0·1ml) was added as the clock was started and the mixture rapidly mixed. The change in absorbance after 15 seconds was noted. A standard reaction against which the enzyme inhibited reaction could be compared was found by using distilled water in place of enzyme extract.

This modified assay was found to give an approximately linear time course for 15 seconds, and to give a linear percentage inhibition of reaction with increase in SOD concentration up to about 45% inhibition (see figure 6) as described by Heikkila and Cabbat (1976). One unit of enzyme was defined as the concentration of enzyme required to give a 40% inhibition of the standard reaction, and all SOD assays were adjusted so that between 10% and 40% inhibition was obtained. Note that the definition of a unit is a concentration and not an absolute amount of SOD. One unit of SOD was found to be approximately equivalent to 90ng/ml of a commercial preparation of SOD from bovine erythrocytes.

ii) Catalase

Catalase catalyses the destruction of hydrogen peroxide to water and oxygen.



$$H_2O_2 \longrightarrow H_2O + \tfrac{1}{2}O_2$$

Hydrogen peroxide absorbs light at 240nm, and thus this reaction can be monitored by the decrease in absorbance at this wavelength. The method of Aebi (1974) was used for this determination.

Enzyme extract, 0·5ml, was added to 2·5ml of 50mM phosphate buffer pH 7·0 containing 12mM hydrogen peroxide giving a final concentration of 10mM with respect to peroxide. The reaction was carried out at 37°C using 1 cm^2 silica cells in the water jacketed

cell compartment of a Cecil CE292 digital ultraviolet spectrophotometer connected to a Philips PM8100 flatbed recorder. The initial rate of reaction was determined from the chart recorder and expressed as the absorbance change for the initial 30 seconds reaction time. This value was substituted into the following equation:

$$k = \frac{2\cdot3}{\Delta t} \times \log \frac{E_1}{E_2}$$

where E_1 = absorbance at t=0, E_2 = absorbance at t=30 seconds and Δt=30 seconds. The first order rate constant, k, is then a measure of the catalase activity.

B. Other Assays

i) Protein

Protein was assayed by the method of Lowry *et al* (1951). A 0·2mg/ml solution of defatted grade IV bovine serum albumin freshly prepared for each experiment was used as the standard. Absorbance measurements were kept within the range corresponding to 0·02-0·12mg protein per ml in order to use a portion of the standard curve which was found to be almost linear and very reproducible.

ii) Haematocrits

Haematocrits were performed usign the Hawksley micro-haematocrit and read using vernier calipers accurate to 0·1mm.

iii) Haemoglobin

Haemoglobin levels were determined using the Boehringer 'Hemoglobin Test Combination'. This assay utilises the absorbance of cyanmethaemoglobin at 546nm.

iv) Urea Estimation

The assay of Archibald (1945) was employed for the estimation of urea in urine. This assay uses the reaction of α-isonitrosopropiophenone with carbamido compounds to produce a chromophore of unknown composition which absorbs light at 560nm.

v) Amino Acid Analyses

Amino acid analyses were performed on a Technicon amino acid analyser, the operation of which is based on the work of Moore et al (1958). The column was packed with Technicon Chromobeads B resin, a sulphonated polystyrene cation exchange resin. Amino acids were eluted by means of a gradient elution system based on that described by Schmidt (1966) with a column temperature of 60°C, a column pressure of 350-400psi and a running time of 12·5 hours. Norleucine was used as an internal standard and the identity of each amino acid was established by means of its position on the chromatogram against authentic standards. The amount present calculated on the basis of the area under each curve with appropriate corrections for differential colour yields and the results expressed as μmoles amino acid per g wet weight of tissue.

vi) Fluorometric Estimation of Retinol

The fluorometric analysis of retinol incorporating a correction formula devised by Thompson et al (1970) was used for this estimation.

Plasma (0·2ml) was homogenised for 30 seconds with 1ml water, 1ml ethanol and 5ml hexane in a 15ml glass centrifuge tube using a Rotamixer. After a brief centrifugation on a bench-top centrifuge, an aliquot of the hexane was removed and the fluorescence at 475nm was determined at excitation wavelengths of 330nm and 360nm. The fluorescence readings were taken on a Perkin-Elmer 3000 fluorometer

in the x10 scale using slit settings of 10. Calibration of the fluorometer for day to day variations was carried out using a solution of quinine sulphate in 0·1M H_2SO_4 previously calibrated against purified retinol. The concentration of purified retinol was determined in 1 cm^2 silica cells on a Unicam SP8-100 spectrophotometer at 325nm using $E^{1\%}_{1cm}$ = 1830. Retinol was purified by column chromatography as described by Thompson et al (1970). Absorbance and fluorescence readings of retinol to be substituted in to the correction formula and for the calibration of the quinine sulphate solution were carried out within an hour of finishing the purification. The quinine standard was recalibrated every 2-3 weeks.

The correction formula is presented below:

$$\text{Retinol } (\mu g/100ml \text{ plasma}) = \frac{P}{P-A} \frac{Q_1}{q_1} X - B - \frac{1}{P-A} \frac{Q_2}{q_2} Y - B \times 25\frac{c}{d}$$

where: $P = \frac{\text{fluorescence of phytofluene at 360nm excitation}}{\text{fluorescence of phytofluene at 330nm excitation}}$

$A = \frac{e}{d} = \frac{\text{fluorescence of retinol at 360nm excitation}}{\text{fluorescence of retinol at 330nm excitation}}$

Fluorescence of quinine standard at 330nm excitation = Q_1 and at 360nm excitation = Q_2, these readings taken when retinol was standardised.

Fluorescence of quinine standard at 330nm excitation = q_1 and at 360nm excitation = q_2, these readings taken at the time the unknowns were determined.

X = fluorescence of unknown at 330nm excitation

Y = fluorescence of unknown at 360nm excitation

B = fluorescence of blank at the respective excitation wavelength

c = concentration of retinol in μg/ml determined from the absorbance reading

All fluorescence measurements were taken at an emission wavelength of 475nm.

P was assumed to be 1·4 as reported in the paper of Thompson *et al*.

To eliminate stray fluorescence it was found necessary to wash all glassware with fluorometric grade hexane immediately prior to performing the assay. All fluorescence readings from plasma retinol samples were recorded within an hour of starting the organic extraction in order to minimise losses due to the breakdown of retinol in organic solvents. Tubes for the extraction of retinol were wrapped in aluminium foil to minimise degradation of retinol by ultraviolet light.

C. Preparation of Polyacrylamide Gels

Polyacrylamide gels (7%) were prepared according to the method of Cawley (1969) with the exception that only the lower gel was used; the spacer gel and upper gels being found unnecessary.

Protein was loaded onto the gels dissolved in 0·25M sucrose containing 2μl of bromophenol blue tracking dye. Buffer was layered carefully over the top of the sample using a syringe so as not to disturb the denser protein/sucrose layer. The gels were developed with a current of 0·2mA per tube.

The protein was fixed and stained immediately after running using a 0·2% solution of Brilliant blue R in methanol: glacial acetic acid: water, 25:7:58 for 1 hour. The gels were destained, using several changes of the above solution minus the dye, over a period of about 48 hours. The quantities of protein extract loaded onto the gels were: lung 15μl, liver 15μl, and brain 35μl

respectively. The destained gels were scanned using a Joyce-Loebl U.V. gel scanner.

D. Spectrophotometry

All spectrophotometric measurements during this study were made on a Cecil CE292 digital ultraviolet spectrophotometer unless otherwise stated.

E. Experimental Procedures

i) Measurements of SOD, Catalase and Protein in Lung, Liver and Brain Of Mice

EXPERIMENTAL FORMAT Batches of 20 animals supplied by the departmental animal house were divided ino 5 groups of four animals each. One group was set aside as zero time controls and the remaining sixteen animals were placed in the ambient pressure chamber. Initially the concentration of oxygen in the chamber was set at 0•21atm for 3 days. One group were sacrificed after 2 days to check for any variation in enzyme levels arising from the use of the apparatus. After 3 days, the concentration of oxygen was raised to 0•85 atm and was kept at that level for 7 days. During this time two groups of animals were sacrificed, one group after 3 days and a second group after 6 days for the experimental readings. The remaining group of mice were kept in the chamber a further 3 days at an oxygen concentration of 0•21 atm before sacrifice as post exposure controls.

EXPERIMENTAL PROCEDURE The animals were sacrificed by stunning followed by decapitation. Brain, lung and liver were rapidly removed from each animal and placed in ice-cold homogenising medium. When all tissues had been collected, each tissue was blotted dry, weighed and placed in 9 volumes of homogenising medium so that a

consistent 10% homogenate resulted. All tissues were disrupted in Potter-Elvehjem homogenisers on ice using 6 strokes. Lung and brain were homogenised in 15mm diameter tubes with a pestle clearance of 0·1mm for the former and 0·2mm for the latter. Liver was homogenised in a 25mm diameter tube with a pestle clearance of 0·3mm.

Due to the difficulties involved in assaying crude homogenates spectrophotometrically, a simple centrifugal separation procedure was devised so that the relevant enzymic activity could be concentrated into a solution containing a smaller amount of interfering protein. The separation scheme is outlined below and was carried out on a refrigerated MSE angle 18 centrifuge.

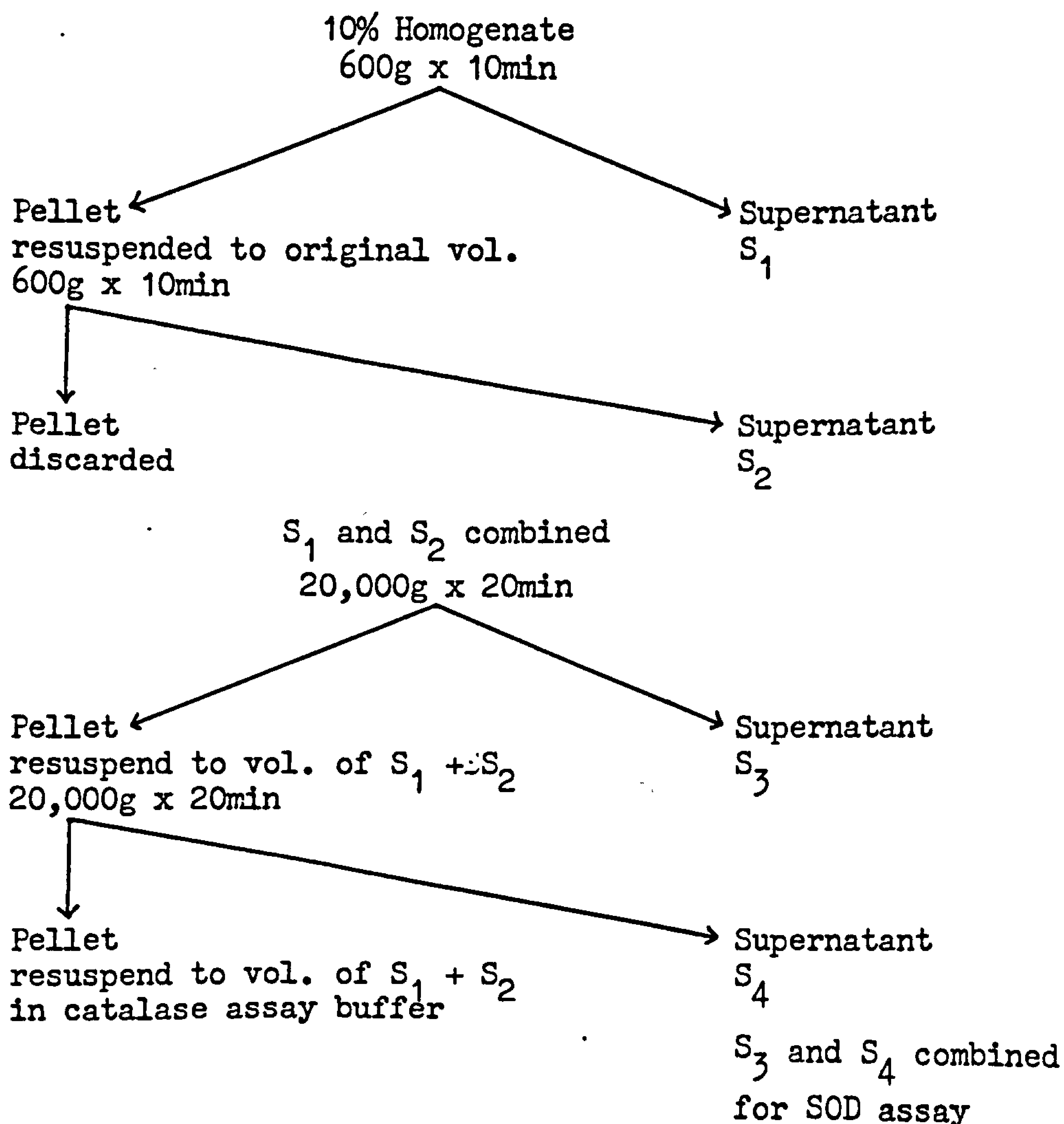


Figure 7 Centrifugation Scheme to Obtain Crude Enzyme Extracts of SOD and Catalase

All homogenisation and resuspension was carried out in 0·25M sucrose containing 0·1mM EDTA except for the final pellet which was resuspended in its assay medium 50mM phosphate buffer pH 7·0 containing 0·1mM EDTA

In the above procedure SOD and catalase partitioned approximately as follows in table 1.

Table 1 Distribution of SOD and Catalase Between Pellet and Supernatant Fractions

	Lung % activity	Liver % activity	Brain % activity
Pellet SOD	5	7	15-20
Supernatant SOD	95	93	80-85
Pellet Catalase	70-75	75	100
Supernatant Catalase	25-30	25	0

It was assumed that the proportion of each enzyme in either fraction would not change during exposure to oxygen, and that therefore the amount of enzyme activity contained in the richest fraction was proportional to the total enzyme activity present.

Overnight storage was found to substantially diminish the catalase activity present but had little effect on the SOD activity. For this reason catalase was assayed on the day the extracts were prepared and SOD was assayed on the following day after being kept on ice overnight. Catalase assays were performed in duplicate, and SOD and protein assays in triplicate.

ii) Determination of Tissue Water Content and Qualitative Changes in Protein

EXPERIMENTAL FORMAT This experiment followed the same format as the experiment of SOD, catalase and protein concentrations.

EXPERIMENTAL PROCEDURE A supernatant fraction was prepared by the same procedure employed in the SOD, catalase and protein concentration determination except that a small portion of each tissue was retained for the determination of % water content.

In order to permit a reasonably clean separation of protein on polyacrylamide gels it was found necessary to use acetone powders. To prepare the acetone powders, 2ml of the final combined supernatant were added with shaking to 18ml acetone previously cooled on crushed dry ice. The resulting suspension was spun in glass centrifuge tubes at 2,000 rpm (1,100g) for 10 minutes on a MSE Mistral centrifuge at -10°C. The acetone supernatant was poured off, the pellets warmed to 0°C and resuspended in 1·5ml 0·25M sucrose containing 0·1mM EDTA. After resuspension the samples were refrozen and stored for electrophoresis at a later date.

For the determination of % water content, tissue samples as nearly identical as possible were removed from each animal. From liver and lung a complete lobe of tissue, the same lobe in each case, was detached. A complete cerebral hemisphere was excised from each brain. Each portion of tissue was blotted dry on absorbent tissue paper and weighed to an accuracy of four decimal places on a Mettler H10 balance. Each tissue was dried in an oven at 85°C until no further loss of weight was detectable. This constant weight was noted as the dry tissue weight. Percentage water content of each tissue was calculated as:

$$\frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100$$

iii) Measurements of Amino Acid Levels in Lung, Liver Brain and Muscle of Mice

EXPERIMENTAL FORMAT For this experiment 32 mice were employed in two groups of sixteen mice each. One group, a control group, was kept in the chamber for 1 week at 0·21 atm and the second group, the experimental group, exposed to 0·85 atm oxygen for 1 week at a different date. Each group consisted of four sets of four mice. One set was sacrificed to serve as a zero time control as the other sets were placed in the chamber. The remaining three sets in each group were sacrificed after 1 day, 3 days and 7 days respectively in the chamber.

EXPERIMENTAL PROCEDURE Animals were again sacrificed by stunning and decapitation. Upon death, brain, lung, liver and a strip of muscle from a hind limb from each animal were excised and placed in ice cold sucrose containing 0·1mM EDTA until all tissues were ready. Each tissue was then blotted dry, weighed and transferred to a homogeniser tube containing a small quantity of extractant (80% methanol containing 0·01M HCl). Each tissue was then homogenised for about 15 seconds using an Ultra-Turrax homogeniser before transferring the suspension to a centrifuge tube. The homogenising equipment was washed with a further small volume of extractant. All tissues were left to extract for 2 hours at room temperature. The suspension was centrifuged at 5,000g for 20 minutes on a MSE angle 18 centrifuge. The supernatants were removed and kept, while the pellets were resuspended and extracted for a further hour. The suspension was recentrifuged and the supernatants combined.

For the determination of amino acid levels, an aliquot of extract as tabulated in table 2 was dried on a rotary evaporator, resuspended in a small quantity of the first elution buffer and loaded onto the column.

Table 2 Quantities of Extractant used at Each Stage of Amino Acid Extraction

Tissue	Approx. wt tissue g	Homog. ml	Washings ml	Resusp. ml	Total ml	Vol Loaded ml
Brain	0·4	3·0	1·0	2·0	6·0	0·9
Lung	0·2–0·4	2·5	1·0	1·5	5·0	1·0
Liver	0·9–1·8	5·0	4·0	3·0	12·0	0·7
Muscle	0·25	2·0	1·0	1·5	4·5	1·0

iv) The Effects of 3 Prostaglandin Synthesis Inhibitors on Oxygen Toxicity Determined by Monitoring the Changes in Certain Gross Physical Parameters

The format of this experiment essentially followed similar lines to those of the experiments to determine the effect of hyperoxic exposure on the levels of amino acids. Thirty two mice were taken and divided into two sets of sixteen mice, a control set which were kept in the animal house at a normal concentration of oxygen and the experimental set which was subjected to 0·95 atm oxygen in the chamber. Each group of sixteen mice were then subdivided into four matched groups of four mice. Each mouse was marked with a felt-tip pen on the tail so that it could be identified from the rest. Each mouse was injected daily subcutaneously with one dose (10µl) of a preprepared mixture according to its group. The preprepared mixtures are given below.

Group 1: Mepacrine in distilled water, 120µg per mouse (4mg/kg).

Group 2: Control; 87% olive oil + 13% absolute alcohol = carrier.

Group 3: Dexamethasone 17·4µg per mouse in carrier (0·58mg/kg).

Group 4: Indomethacin 52·2µg per mouse in carrier (1·74mg/kg).

Measurements were made of the total body weight of each mouse daily at the time of injection. Upon the death of experimental mice,

the time of death, total body weight, weight of liver and lungs were noted and the percentage water content of the lungs was determined as in a previous experiment. Control mice were sacrificed by stunning and cervical dislocation, at the time when the estimated LD_{50} of oxygen had been administered (5 days) and the same parameters measured.

v) Measurements of Haematocrit, Haemoglobin, Erythrocyte SOD and Plasma Retinol Levels in Rats

a) Initial Procedure

Rats (140-160g) were individually marked with felt tip pen on the tail for identification purposes and injected i.p. with 55 mg/kg sodium pentobarbitone. Seven minutes after injection, 0•8-1•0ml of blood was withdrawn by heart puncture into a syringe containing 0•1ml sodium heparin (5,000 units/ml). Upon recovery the animals were returned to the animal house to recuperate. After 1 week, 2 rats were sacrificed for zero time controls while the remaining 6 rats were placed in the chamber in 0•85 atm oxygen for periods up to 1 week. The animals were sacrificed by cervical dislocation and blood was withdrawn from the heart using a syringe containing 0•1ml heparin.

All blood samples were mixed thoroughly and placed on ice. Haematocrit and haemoglobin were measured and the blood was spun on an Eppendorf 5412 centrifuge for 4 minutes and plasma was removed for the assay of retinol.

b) Modified Procedure

In the second series of experiments no control samples of blood were taken using anaesthetic, so animals in the weight range 160-180g were used. Two rats were used for zero time controls and six rats were exposed to 0•85 atm oxygen for periods up to 1 week as in the

previous experiments. Animals were stunned and guillotined and the blood was collected in siliconised beakers containing 0·05ml heparin. Blood samples were treated as above except that 0·2ml of erythrocytes were washed in 4 volumes saline, recentrifuged, lysed in 4 volumes of distilled water and resuspended in 50mM phosphate buffer pH 7·4 containing 0·1mM EDTA for the measurement of erythrocyte SOD.

vi) Measurements Performed on Human Blood and Urine Samples from 'Divers' Undergoing a Recompression Procedure

The purpose of these dives was to test a modified recompression procedure to ascertain if it caused a mild oxygen toxicity. There was a series of 4 dives to test this hypothesis; one was conducted in the Deep Trials Unit (DTU) using 4 subjects, and three in the Experimental Diving Unit (EDU) using 2 subjects on each occasion. The recompression procedure involved a compression to 50m of sea water (1 atm = 10m s.w.) for 2 hours followed by a decompression using R.N. TABLE 73 (see figure 8). In the modified procedure 32·5% oxygen/67·5% nitrogen was breathed from a mask for 20 minutes on 10 minutes off during the 2 hour stop at 50m. Three of the subjects in the dive at DTU and one of the subjects in each of the dives at EDU breathed the oxygen enriched mixture while the remaining subjects breathed compressed air.

Blood samples (8ml) were collected from all subjects into heparinised tubes. The tubes were removed from the diving chamber in a steel pressure container which was subsequently decompressed at a rate of 1m s.w. per minute. An aliquot of the blood was centrifuged in a refrigerated MSE centrifuge to provide plasma for the determination of retinol and plasma amino acids, and erythrocytes for the assays of SOD and catalase. Two blood samples were taken 18-24 hours and ½ hour respectively before the dive to serve as

controls and 3 blood samples were taken during the dive as shown in the dive profile (figure 8). A final blood sample was taken 12 hours after the dive had finished in the case of the 3 dives at EDU.

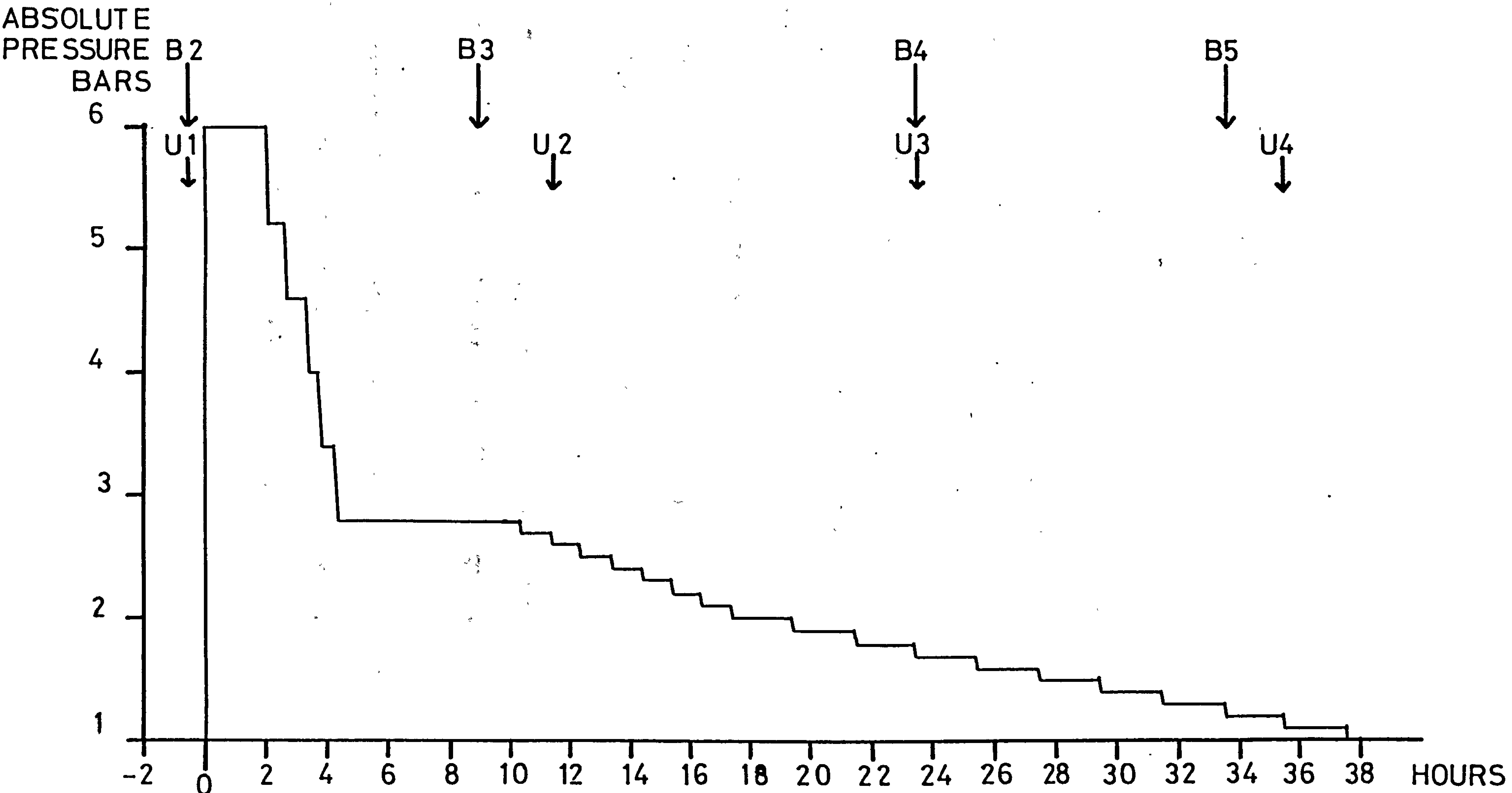
Urine samples were collected from the EDU dives in plastic bottles containing 10ml of 10% HCl as a preservative. A control sample was collected for the 18-24 hour period before the dive, three 12 hour urine samples were collected during the dive, and a final sample was collected from the 12 hour period immediately after the dive. The volume of each urine sample was recorded and a small sample was removed for analysis of urea and urine amino acids).

All samples for analysis were frozen in the ice compartment of a refrigator and then transferred to a freezer at -70°C. Samples were transported back to London in a large pre-frozen Dewar flask and stored at - 40°C until analysis was performed.

Plasma amino acids were extracted as follows. One ml of plasma was homogenised with 4ml of extractant (80% methanol containing 0·01M HCl) using an Ultra-Turrax homogeniser. The samples were extracted for 2 hours and then centrifuged in a bench-top centrifuge to remove precipitated protein. The supernatant was decanted, dried on a rotary evaporator, and resuspended in 1 ml of 0·01M HCl. The resuspended samples were recentrifuged in an Eppendorf 5412 centrifuge for 5 minutes to remove the remaining protein. Samples were then stored at - 20°C until they were analysed. 0·7ml of sample was used in the analysis.

All further details of methods are contained in the text of the results and discussion.

FIGURE 8: PROFILE OF DIVE AND DECOMPRESSION



RESULTS AND DISCUSSION

Chapter 1:

Quantitative Determination Of Changes In SOD, Catalase, Protein And Tissue Water Content: Qualitative Determination Of Protein In Lung, Brain And Liver Of Mice Exposed To 0·85 Atm Oxygen.

Chapter 2:

Determination Of Changes In Amino Acid Metabolism In Lung, Liver, Brain And Muscle Of Mice Exposed To 0·85 Atm Oxygen.

Chapter 3:

Determination Of The Effects Of Prostaglandin Inhibitors On Oxygen Toxicity, Monitored By Survival Time, Lung Water Content, Whole Body, Lung And Liver Weights.

Chapter 4:

Changes In Plasma Retinol, Haematocrit, Haemoglobin, And Erythrocyte SOD, In Rats Exposed To 0·85 Atm Oxygen.

Chapter 5:

Determination Of Haemoglobin, Retinol, Erythrocyte SOD And Catalase, Plasma Amino Acids, And Urine Urea Levels, In Humans During A Therapeutic Recompression Procedure.

Chapter 1: QUANTITATIVE DETERMINATION OF CHANGES IN SOD, CATALASE, PROTEIN AND TISSUE WATER CONTENT: QUALITATIVE DETERMINATION OF PROTEIN IN LUNG, BRAIN AND LIVER OF MICE EXPOSED TO 0·85 ATM OXYGEN.

A. Introduction

Superoxide anion, the first reactive intermediate in the univalent reduction of oxygen is produced in all actively respiring tissue. The defence mechanism of the body against superoxide radical is the enzyme superoxide dismutase (SOD) which maintains the steady state concentration of this radical at a low level. As a result of the dismutation of superoxide radical, hydrogen peroxide, a less reactive intermediate is produced, which in turn is removed by the action of catalase.

$$O_2^{\bar{\cdot}} + O_2^{\bar{\cdot}} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$$

In an oxygen rich atmosphere it would be expected that the resultant higher tissue tensions of oxygen would result in a greater production of superoxide anions and a consequent increase in the formation of hydrogen peroxide, both from the spontaneous and enzyme catalysed dismutation reactions.

Experiments were therefore designed to measure the levels of both SOD and catalase to determine whether any changes, either reductions or adaptive increases in levels of these enzymes occurred which could affect the course of oxygen toxicity. Protein levels were also determined as an internal marker. Because of reports that oxygen affected protein synthesis, the precaution was taken of

starting the preparation of enzyme extracts with an exact 10% homogenate (w/v) so that the activities of these enzymes could also be expressed directly as a concentration in each tissue. SOD, catalase and protein levels were measured concurrently in each sample.

Preliminary experiments revealed a large individual variation and suggested that the relationship between increasing periods of toxic exposure was not a simple one. For this reason the experiments reported in this chapter contained extra sets of controls. The experimental format was described in the materials and methods (section 3.D. i)).

The descriptions attached to each of the sample and control groups will be as follows. The animals which were sacrificed at the start of the experiment to determine the norm of the animals arriving direct from the animals house will be referred to as the 'initial' controls. At the time the 'initial' controls were sacrificed, the remaining animals were placed in the chamber at an oxygen tension of 0•21 atm for 3 days. During this period a second set of animals were sacrificed after 2 days to indicate any variations due to a change of environment or stress, and this group will be called the 'chamber' control. Collectively the first two sets of controls will be referred to as the 'pre-exposure' controls. After the period at normal oxygen tensions the experimental groups were subjected to a tension of 0•85 atm oxygen for a period of 7 days. The experimental animals sacrificed at 3 and 6 days will be referred to as the 3 day and 6 day exposure animals respectively. The final group of animals, which had been kept for the initial control period, then 7 days at 0•85 atm oxygen followed by a return to a tension of 0•21 atm oxygen for 3 days before sacrifice, will be referred to for convenience as the 'post-exposure' controls. It

should be noted, however, that this group was not a control in the true sense of the word because it has not previously been determined whether the parameters measured in this experiment return to normal after re-exposure to normal tensions of oxygen.

For all the quantitative determinations of SOD, catalase and protein carried out and reported in this chapter the results are the mean of four separate experiments where there were initially four mice in each group. For the qualitative protein determinations, and % water content determinations, the results presented were from a single experiment using four mice in each group.

B. Results

i) Observations on Behaviour and Symptoms of the Animals

During the initial control period at 0·21 atm oxygen, the animals appeared healthy, ate and drank heartily and kept themselves clean. On increasing the oxygen concentration to 0·85 atm, the animals appeared at first to be slightly more lively and continued to eat and drink heartily for about 24 hours. By the third day, food and water consumption was very much reduced and the animals began to lose condition. Cleaning was no longer as rigorous, breathing showed signs of being laboured and the mice tended to be less active. By the fourth and fifth days the animals appeared obviously emaciated with bones beginning to protrude. Breathing was definately laboured and often of a gasping nature, consumption of food was low, and many of the animals lay flat on the bottom fairly inactive instead of standing on their haunches. Hair on the body became erect so that instead of looking sleek the mice looked ragged and the skin was visible. Occasionally animals would walk with difficulty as if control of the hindlimbs was impaired; this symptom was also accompanied by an arched back. Ineffective

cleaning movements of the forelimbs on the face which gave the impression of an involuntary action were also common. The mice tended to show their worst symptoms at about day 5 with a tendency to a slight improvement on day 6 which did not persist into day 7.

On a return to normal oxygen levels (0·21 atm) a wide variety of response was seen. Most animals appeared to have embarrassed respiration to start with, sometimes resulting in death during the first 12 hours. After surviving this period some mice appeared to show a rapid gain in weight and a return to a normal healthy-looking condition together with an improvement in breathing. Other mice showed very little improvement either in condition, breathing, eating or gain of weight. The remainder showed a moderate improvement in all of these signs.

ii) Record of Changes Noted at Dissection

On dissection the control animals showed a high proportion of animals with already inflamed lungs. This usually took the form of oedema but occasionally portions of some lungs showed small patches indicative of haemorrhage. Only in one control animal was any consolidation noted and this only in a small portion of the lung. Lungs from animals after three days of exposure showed a general picture of inflammation, oedema and usually signs of haemorrhage. Occasionally consolidation was seen. Few of the animals at this exposure showed the gross inflammation, haemorrhage and liver-like consistency of the lungs which was a common finding after 6 days exposure. At this later exposure the heart generally appeared large and flaccid and the intestines and liver appeared small and shrunken.

A record of the initial and final body weights was kept together with the wet weight of lung, liver and brain of all the animals used

in this study. These results generally support the descriptions given above, and are presented in Table 3.

Table 3: Whole Body and Wet Tissue Weights of Mice Exposed to 0·85 atm Oxygen

	0·21 atm		0·85 atm		0·21 atm
Days	0	2	3	6	+3
n=	16	16	16	15	11
Initial Body Weight	30·3 ±0·3	30·3 ±0·2	30·3 ±0·3	30·2 ±0·3	30·0 ±0·3
Final Body Weight	30·3 ±0·3	30·9 ±0·5	30·1 ±0·7	26·1 ±0·5	29·0 ±1·3
Wet Lung Weight	0·322 ±·022	0·299 ±·018	0·351 ±·018	0·383 ±·018	0·412 ±·032
Wet Brain Weight	0·419 ±·006	0·401 ±·005	0·416 ±·005	0·399 ±·006	0·401 ±·009
Wet Liver Weight	1·609 ±·037	1·781 ±·028	1·541 ±·060	1·139 ±·045	1·620 ±·097

All weights are in grams ± s.e.m..

iii) Quantitative Determination of Protein Levels

The determination of protein levels was originally intended as an internal standard against which to measure the change in enzyme levels. However the changes in protein levels were such that this intention was thwarted, but that instead, questions were raised that this might itself be a change attributable to oxygen toxicity.

Protein levels were determined in both supernatant and pellet fractions obtained during the extraction procedure (figure 7, materials and methods, section 3.D. i)). In all tissues the

supernatant fraction was the major protein containing fraction but in brain and liver the pellet fraction appears the largest because the supernatant is in fact a 2x dilution.

The changes in protein levels in all these fractions are depicted in figures 9 to 11.

Lung and brain showed similar alterations in protein levels after exposure to 0·85 atm oxygen but the changes shown by liver were totally different. In each tissue, the pattern of changes shown in the pellet was different to that shown in the supernatant.

In lung and brain supernatant the 'pre-exposure' controls were in good agreement. At 3 days exposure a significant rise in the protein levels was noted in both lung and brain. In brain tissue the rise in protein level was significantly different only from the 'chamber'control, but in lung tissue the 3 day exposure level was significantly different from both of the 'pre-exposure' controls. This rise was followed at 6 days exposure by a fall in protein levels below that of both 'pre-exposure' controls but in neither case was there a significant difference from either of these controls. In both lung and brain the 'post-exposure' control showed a value very similar to that of the two 'pre-exposure'.controls. In the liver the supernatant protein levels showed a significant decrease between the 'chamber' control and the 'initial' control. After 3 days exposure the protein level was similar to the 'chamber' control, but at 6 days it had risen significantly to the level of the 'initial' control. This picture was complicated by the 'post-exposure' value falling again to a value close to the chamber control.

In the pellet fractions these changes were less pronounced. In both lung and brain pellet fractions the 'chamber' control, the 3 days exposure to oxygen and the 'post-exposure' control all had similar values. The 6 day exposure level showed a slight decrease

Legend to Figures 9 to 11

Changes In Protein Concentration In Lung, Liver And Brain Of Mice Exposed To 0·85 atm Oxygen

In each figure, the upper graph depicts the protein concentration changes in the supernatant fraction, and the lower graph, the changes in pellet protein concentration. The supernatant and pellet fractions were prepared as in figure 7 (materials and methods section 3.D. i)).

The x axis has the units of days and is divided by the long vertical divisions into three sections: the pre-exposure period, the experimental period and the post-exposure period, which are labelled with the relevant oxygen concentration. Day 0 refers to the 'initial' control, day 2 to the 'chamber' control, day 3 and 6 to the 3 day and 6 day exposure groups respectively and +3 to the 'post-exposure' control.

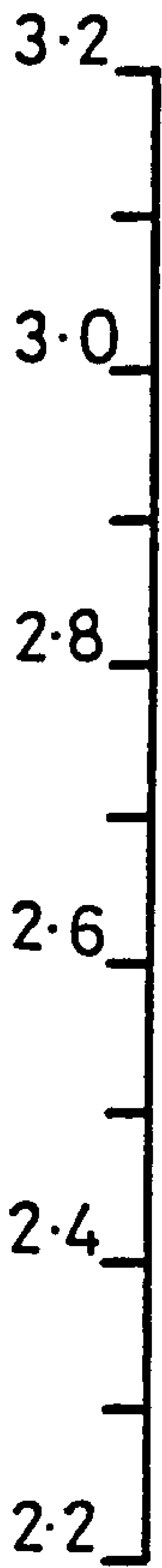
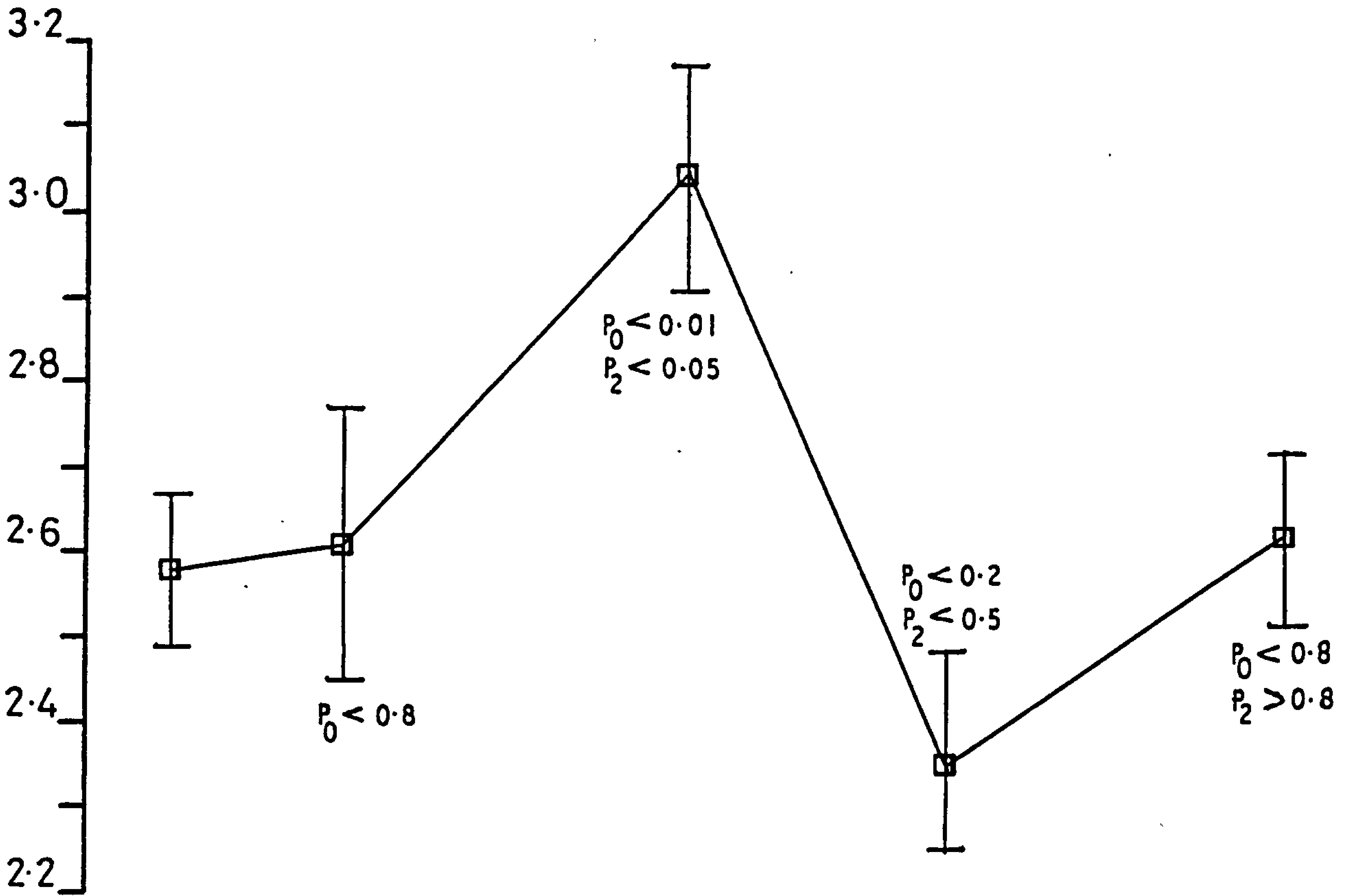
n= Refers to the number of animals used for that particular point in both supernatant and pellet samples except in day 0 figure 11 where different figures are given for each graph.

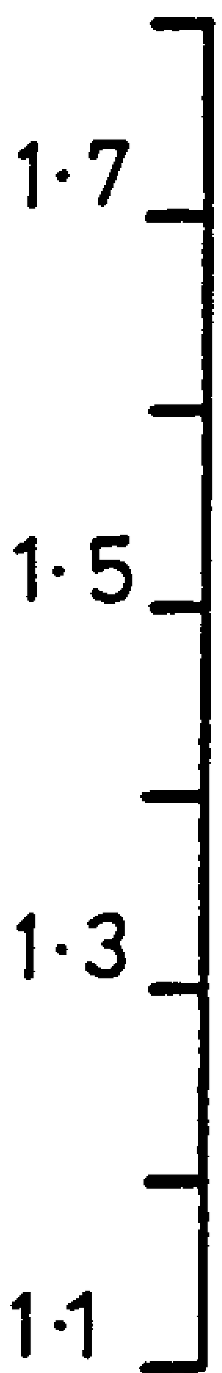
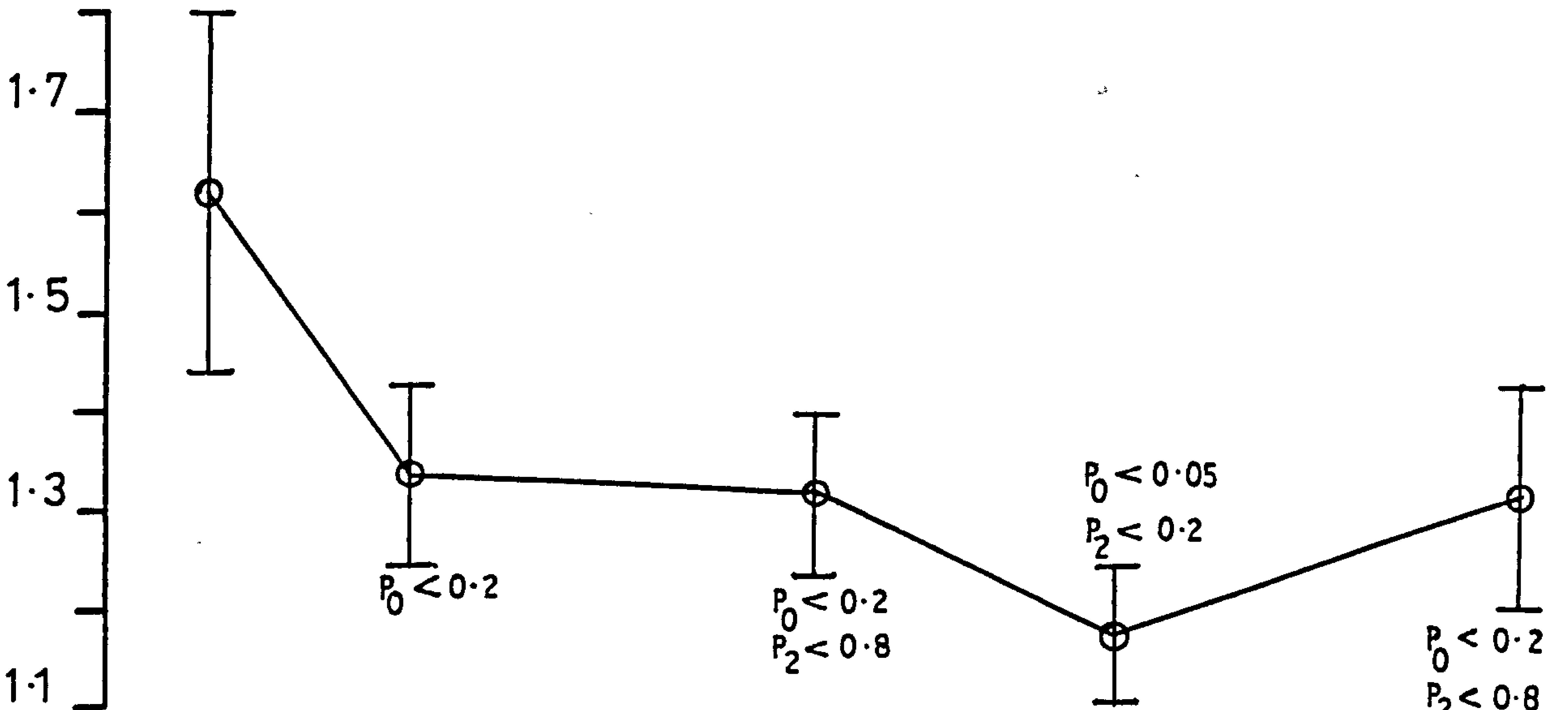
All points given are the mean of n samples ± SEM. The P values of Student's 't' test (two tailed) are given for each point. P_0 is the probability that a particular value is identical to the 'initial' control and P_2 is the probability that it is identical to the 'chamber' control.

Each point represents the determinations on samples from 4 separate experiments.

FIGURE 9: LUNG PROTEIN

mg PROTEIN/ml

DAYS	0	2	3	6	+3
O_2	21%		85%		21%
n =	16	16	16	15	11

FIGURE 10: BRAIN PROTEIN

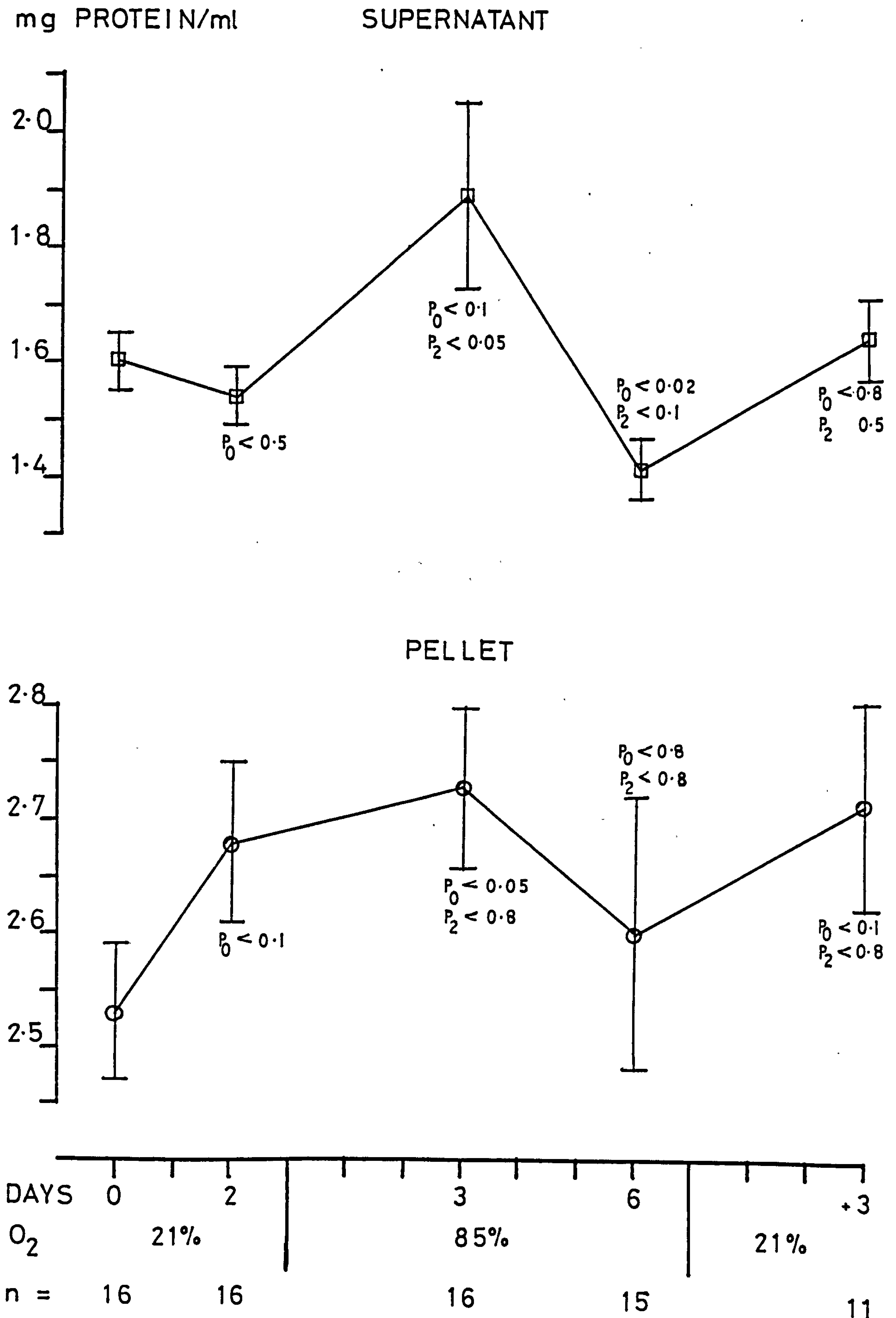
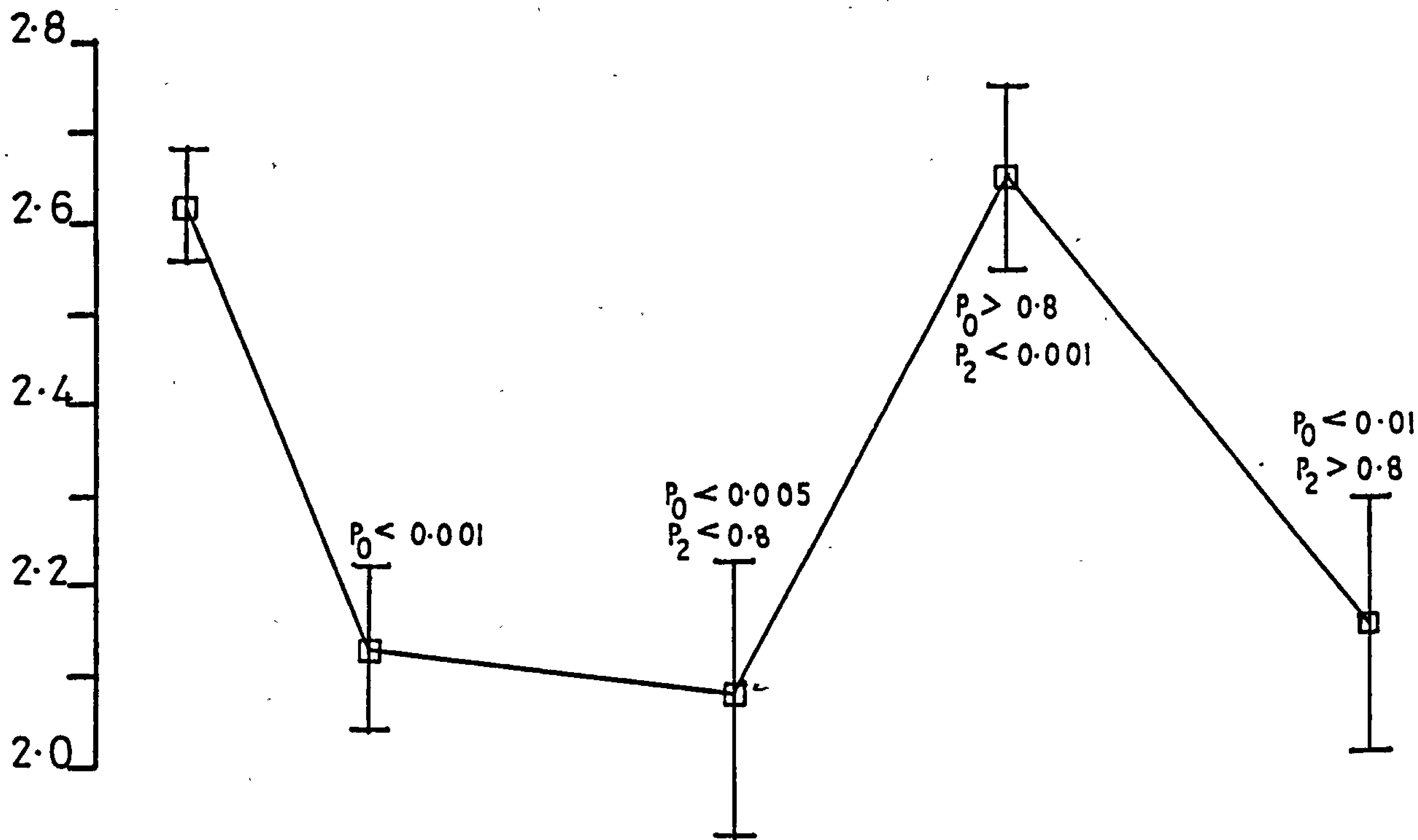
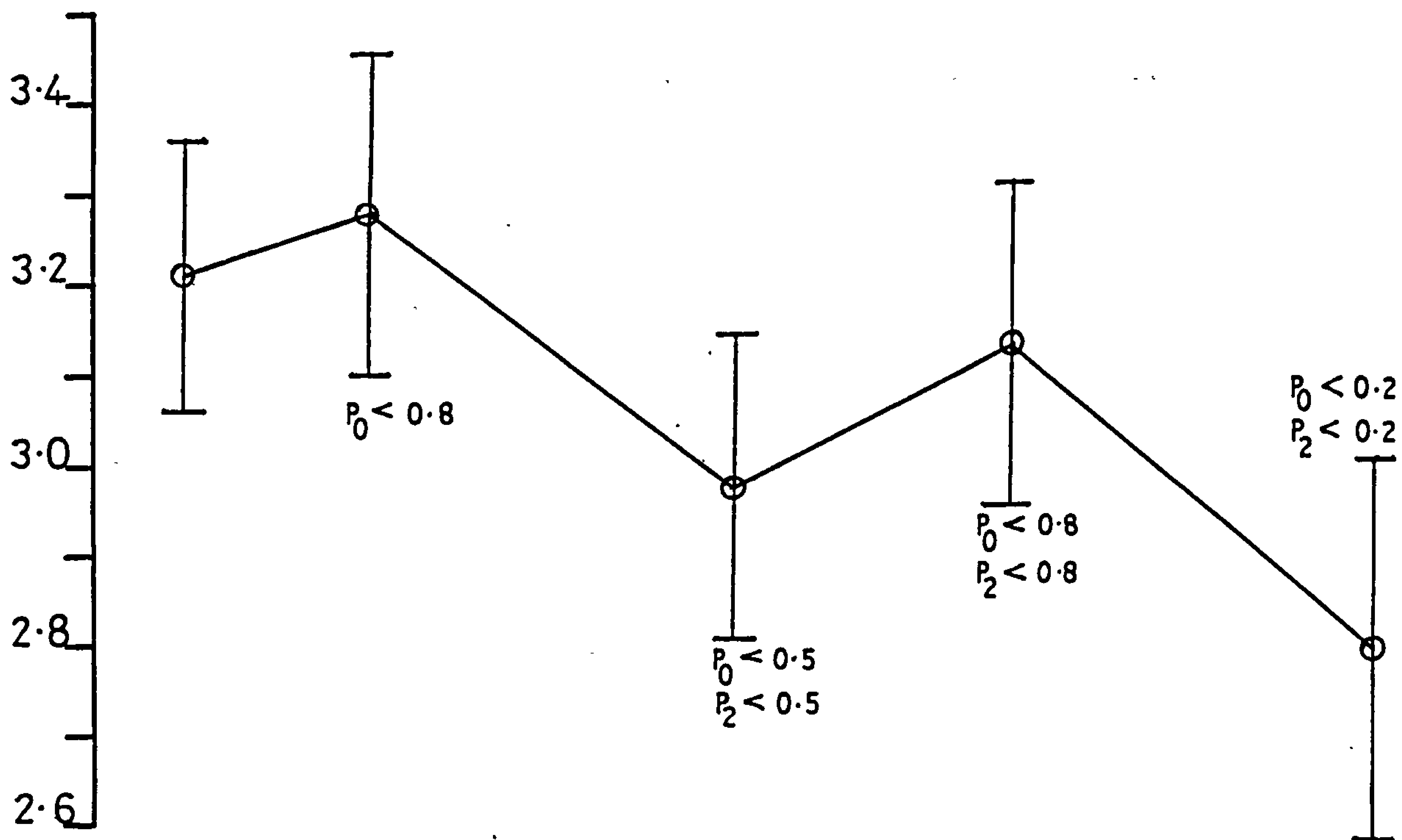


FIGURE 11: LIVER PROTEIN





DAYS	0	2	3	6	+3
O_2	21%		85%		21%
n =	16 S 12 P	16	16	15	11

which was not significantly different from these values. In the lung the 'initial' control level showed a higher protein level than the other protein levels, whereas in the brain the 'initial' control level was below the other levels. The only differences significant at the P = 0·05 level in these extracts were the 6 day protein level from the 'initial' control in the lung, and the 3 day protein level from the 'initial' control value in the brain. In the liver pellet the 'initial' control, 'chamber' control and the 6 day exposure levels were similar but the 3 day exposure and 'post-exposure' control showed a slight decrease below these levels which were not significant; partly as a result of the large variation shown in this fraction.

As a result of the experimental procedure employed, the total protein in the lungs was different from the protein concentrations discussed above. Values for total protein were obtained by multiplying the protein concentration by the wet tissue weight in g x 10. Note that the supernatant was in fact double this amount due to combining supernatants S3 and S4 (see figure 7). This calculation is presented in Table 4.

Table 4: Calculation of Total Lung Protein

	0·21 atm		0·85 atm		0·21 atm
Days	0	2	3	6	+3
Lung Weight g	0·322	0·299	0·351	0·383	0·412
Protein (P) mg/ml	1·62	1·34	1·32	1·18	1·32
Protein (S) mg/ml	2·58	2·61	3·04	2·36	2·62
Lung Protein (P) mg	5·22	4·01	4·63	4·52	5·44
Lung Protein (S) mg	16·61	15·60	21·36	18·08	21·60
Total Lung Protein mg	21·83	19·61	25·99	22·60	27·04

Table 4 shows that the average total protein per lung did not show the same variation as the protein concentrations discussed above. A rise in the total protein was indicated after 3 days exposure, similar to the rise in supernatant protein concentration (protein (S) mg/ml) at that time. However, after 6 days exposure the table shows that the total lung protein was similar to but slightly higher than the 'pre-exposure' controls, whereas the 6 day supernatant and pellet concentrations (protein (S) and protein (P) mg/ml) were both lower than their respective 'pre-exposure' control. There was also a difference in the 'post-exposure' control. The total protein in the 'post-exposure' control was much higher than the 'pre-exposure' controls, but the protein concentrations were very similar to their control values.

Table 5: Calculation of Total Liver Protein

	0·21 atm		0·85 atm		0·21 atm
Days	0	2	3	6	+3
Liver Weight g	1·609	1·781	1·541	1·139	1·620
Protein (P) mg/ml	3·21	3·28	2·98	3·14	2·80
Protein (S) mg/ml	2·62	2·13	2·08	2·65	2·16
Liver Protein (P) mg	51·6	58·4	45·9	35·8	45·4
Liver Protein (S) mg	84·3	75·9	64·1	60·4	70·0
Total Liver Protein mg	135·9	134·3	110·0	96·2	115·4

The liver wet weight, like the lung wet weight, did not remain constant throughout the experiment. There was a dramatic loss in the wet weight of the liver at 6 days exposure, followed by a return to nearly normal in the 'post-exposure' control. As a consequence, the total liver protein also differed from the changes in liver

protein concentration, as occurred in the lung. Another calculation, this time for liver, revealed the changes in total liver protein (table 5).

These results showed that total liver protein declined during exposure to oxygen but that on return to air, a recovery occurred.

Note that in the supernatant, the difference between the 'initial' control and the 'chamber' control was less after accounting for the change in the weight of the liver, and that the apparent change was largely due to a change in the distribution of protein between the two fractions.

iv) Changes in Superoxide Dismutase Concentration

Examination of figures 12 to 14 showing the change in SOD concentrations expressed per ml of homogenate revealed that although the general trend in the changes of the 3 day and 6 day exposure values was the same in each tissue studied, the controls behaved differently in every case. In lung tissue, the 'chamber' control was significantly greater than the 'initial' control, but after 3 days exposure to 0·85atm oxygen, the SOD concentration had decreased to a level significantly less than either of the 'pre-exposure' controls. After 6 days exposure, the SOD level had recovered to approximately that of the 'chamber' control, but during the post-exposure period fell to a level similar to that of the 'initial' control. In the brain, the two 'pre-exposure' controls were similar. Three days exposure to oxygen resulted in a fall in the brain SOD concentration to less than that of both 'pre-exposure' controls but by 6 days exposure it had increased to a level greater than the 'pre-exposure' control levels, and the 'post-exposure' control remained at a similar value to that shown at 6 days exposure. The 2 experimental points and the 'post-exposure'

Legend to Figures 12 to 14

Changes in SOD Concentration in Lung, Liver and Brain of Mice Exposed to 0·85 atm Oxygen

In each figure the upper graph depicts the concentration of SOD expressed per ml of supernatant fraction, and the lower graph the specific SOD activity per mg protein in the supernatant fraction. The supernatant fraction was prepared as in figure 7 (materials and methods section 3.D. i)).

The x axis has the units of days and is separated by the long vertical divisions into three sections: the pre-exposure period, the experimental period, and the post-exposure period, which are labelled with the relevant oxygen concentrations. Day 0 refers to the 'initial' control, day 2 to the 'chamber' control, day 3 and 6 to the 3 day and 6 day exposure groups respectively, and +3 to the 'post-exposure' control.

n= Refers to the number of animals used for that particular point in both the concentration and specific activity graphs.

All points given are the mean of n samples ± SEM. The P values of Student's 't' test (two tailed) are given for each point. P_0 is the probability that a particular value is identical to the 'initial' control, and P_2 is the probability that it is identical to the 'chamber' control.

Each point represents the determinations on samples from four separate experiments.

FIGURE 12: LUNG SUPEROXIDE DISMUTASE ACTIVITY

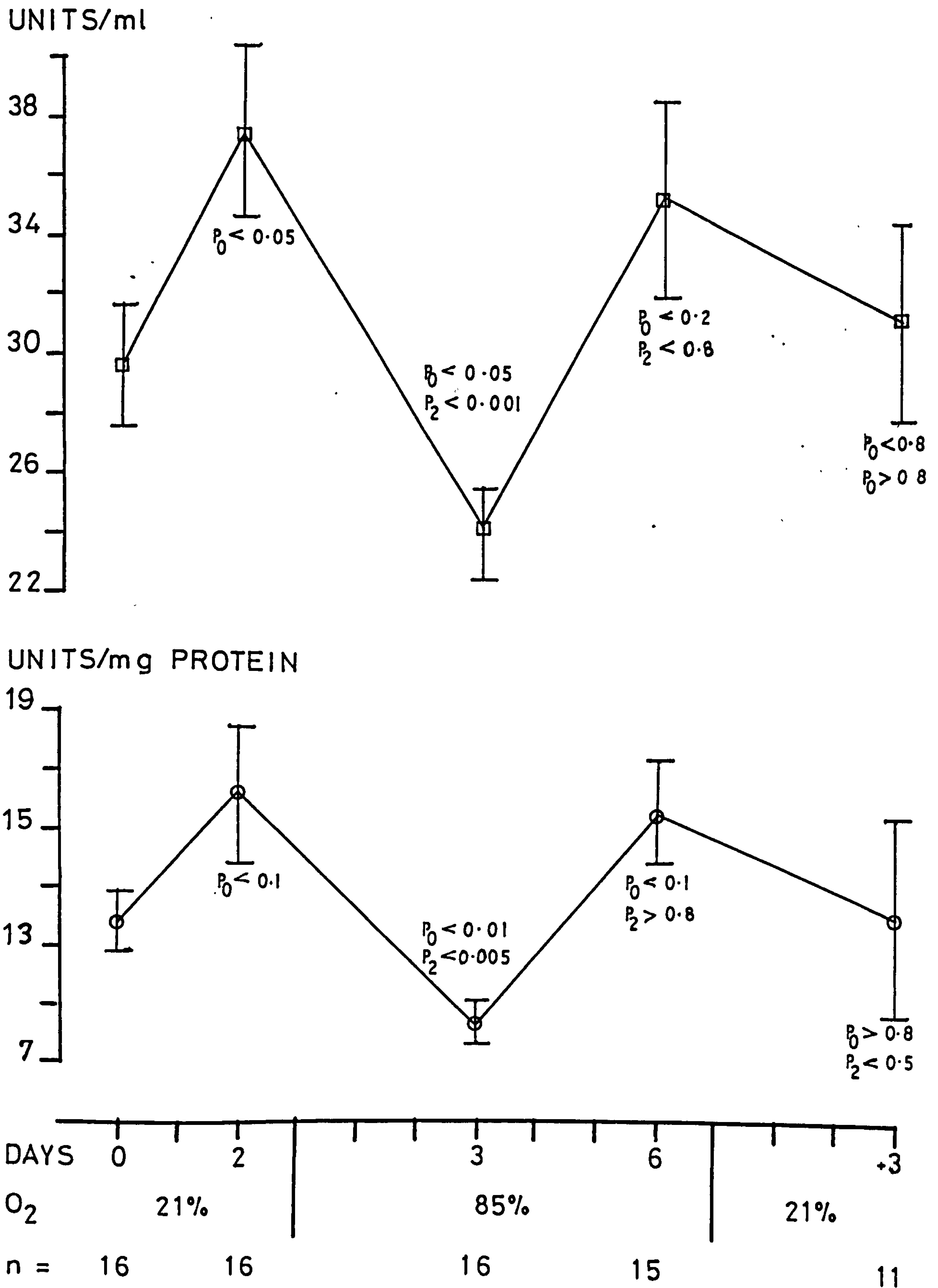


FIGURE 13: BRAIN SUPEROXIDE DISMUTASE ACTIVITY

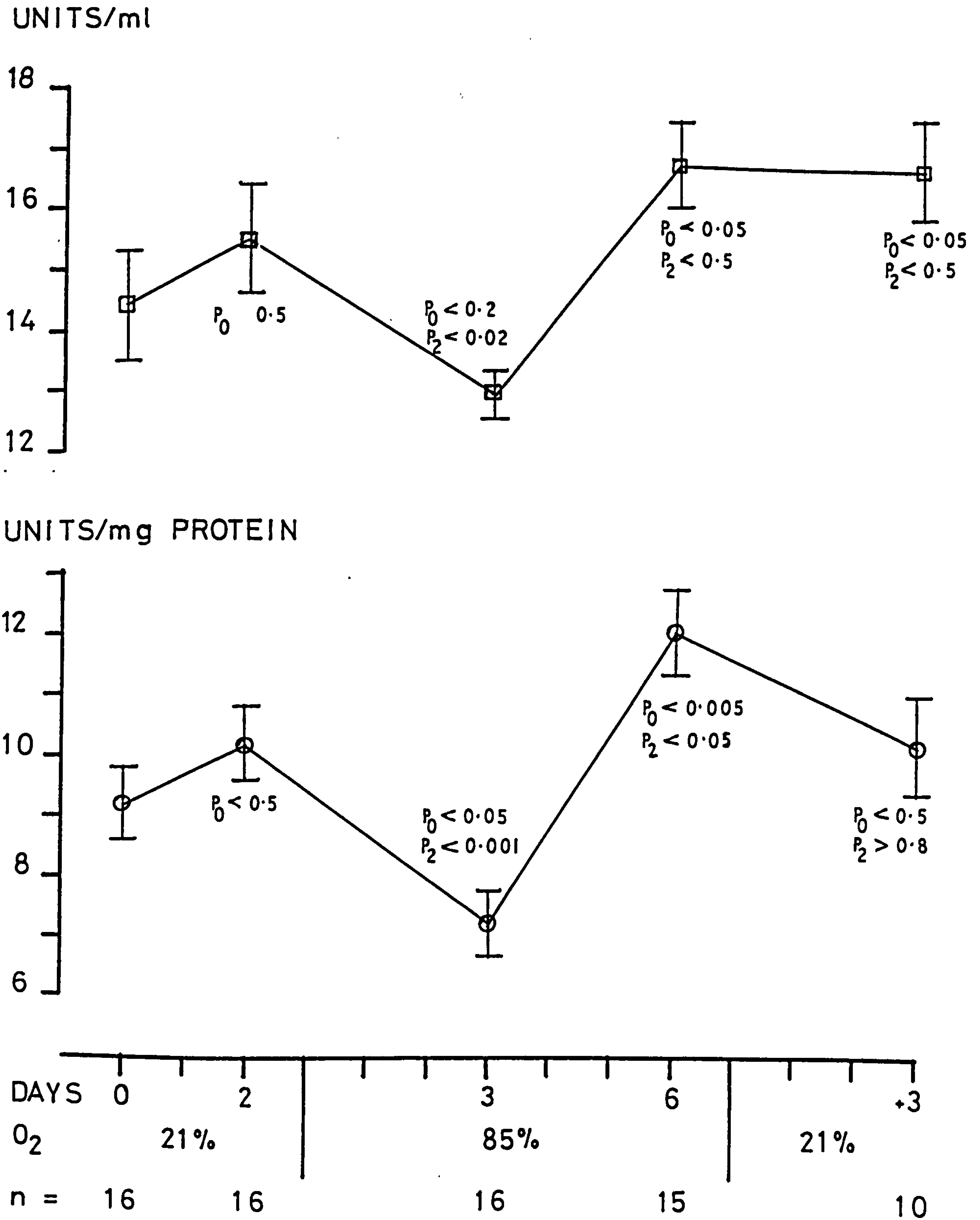
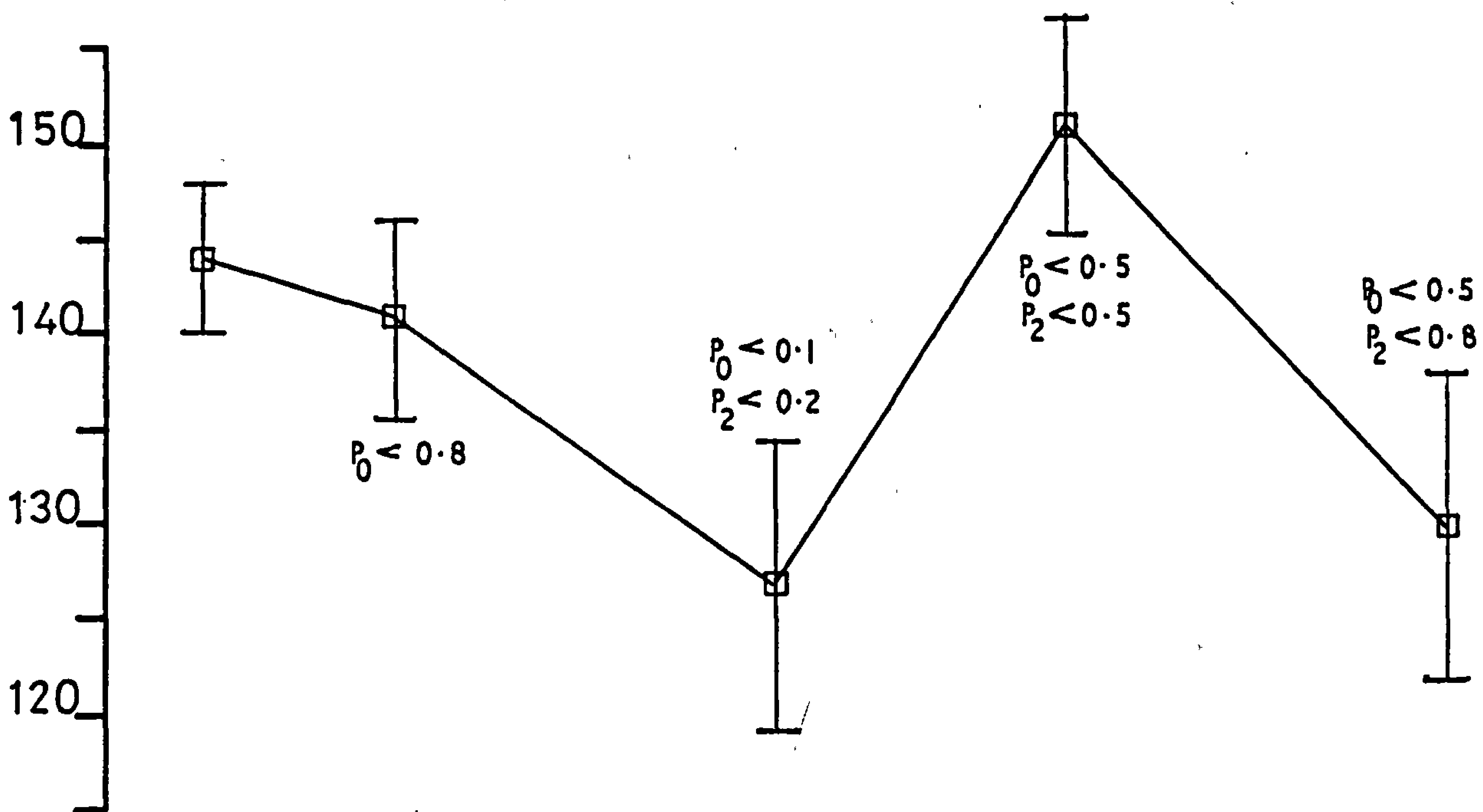
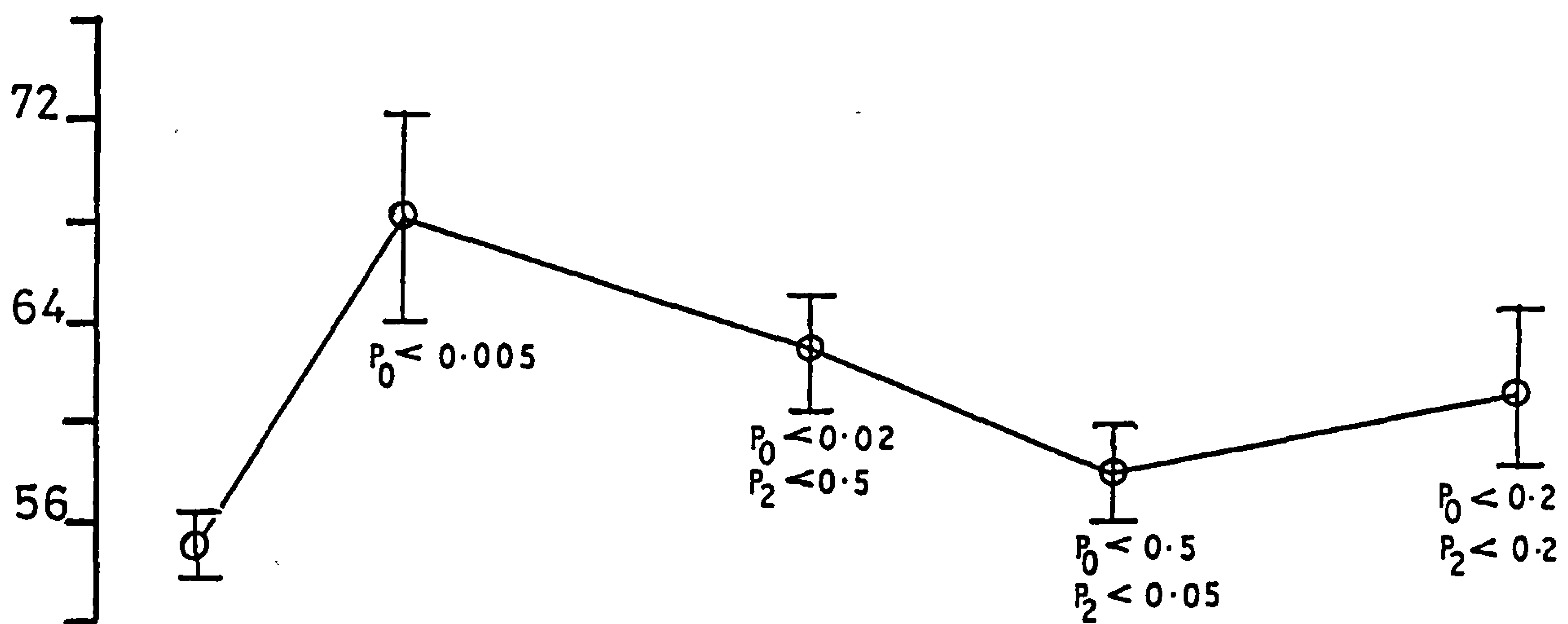


FIGURE 14: LIVER SUPEROXIDE DISMUTASE ACTIVITY

UNITS/ml



UNITS/mg PROTEIN



DAYS	0	2	3	6	+3
O_2	21%		85%		21%
n =	16	16	16	15	11

control were each only significantly different compared to one of the 'pre-exposure' controls. In the liver the trends shown were similar to those seen in the brain, except that the 'post-exposure' control SOD concentration was slightly below the 'pre-exposure' controls. Due to the large variation in the results from the liver however, none of the changes were statistically significant.

By expressing the above results per mg of protein, it can be determined whether these effects follow the changes in protein due to a deranged protein metabolism.

In the supernatant fraction of the lung and brain, the changes in protein were the reverse of the changes in SOD concentration, so that the fall and rise in SOD specific activity on days 3 and 6 respectively were accentuated. In liver the changes in supernatant protein levels were similar to the changes in SOD concentration, so that SOD activity expressed per mg of protein showed the largest change between the two 'pre-exposure' controls, with all subsequent values falling between these control values.

v) Changes in Catalase Concentrations

The catalase levels compared as a tissue concentration (i.e. units per ml of homogenate) showed a similar trend downwards in all of the tissues examined (see figures 15 to 17). In lung tissue the catalase concentration was significantly reduced compared to both 'pre-exposure' controls by 6 days exposure to oxygen. In the brain a significant decrease in catalase concentration to below the 'chamber' control level was noted after both 3 and 6 days exposure, and in the 'post-exposure' control. However none of these values were also significantly different from the 'initial' control. In liver the results showed a significant decrease in catalase concentration at 3 days and 6 days exposure and in the 'post-exposure' control compared with both of the 'pre-exposure' controls.

Legend to Figures 15 to 17

Changes in Catalase in Lung, Liver and Brain of Mice Exposed
To 0·85 atm Oxygen

In each figure the upper graph depicts the concentration of catalase expressed per ml of pellet fraction, and the lower graph the specific catalase activity per mg protein in the pellet fraction. The pellet fraction was prepared as in figure 7 (materials and methods section 3.D. i)).

The x axis has the units of days and is separated by the long vertical divisions into three sections: the pre-exposure period, the experimental period, and the post-exposure period which are labelled with the relevant oxygen concentrations. Day 0 refers to the 'initial' control, day 2 to the 'chamber' control, day 3 and 6 to the 3 day and 6 day exposure groups respectively, and +3 to the 'post-exposure' control.

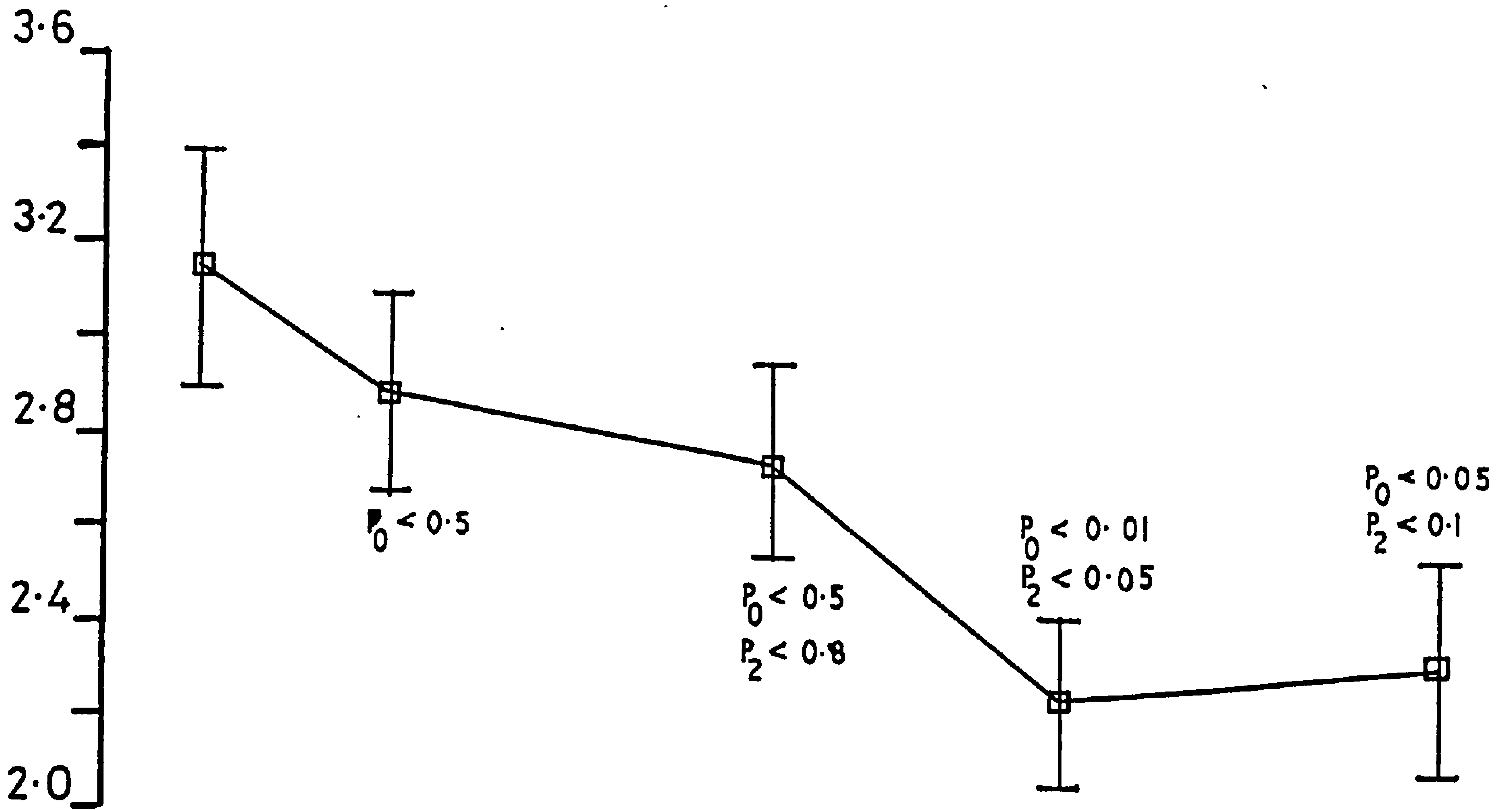
n= Refers to the number of animals used for that particular point in both concentration and specific activity graphs, except day 0 in figure 17 where different figures are given for each graph.

All points given are the mean of n samples ± SEM. The P values of Student's 't' test (two tailed) are given for each point. P_0 is the probability that a particular value is identical to the 'initial' control, and P_2 is the probability that it is identical to the 'chamber' control.

Each point contains the determinations from samples from four separate experiments.

FIGURE 15: LUNG CATALASE ACTIVITY

UNITS = k×10²



UNITS/mg PROTEIN

2·6
2·4
2·2
2·0
1·8
1·6

P0 < 0·5

P0 < 0·8
P2 < 0·8

P0 < 0·5
P2 < 0·01

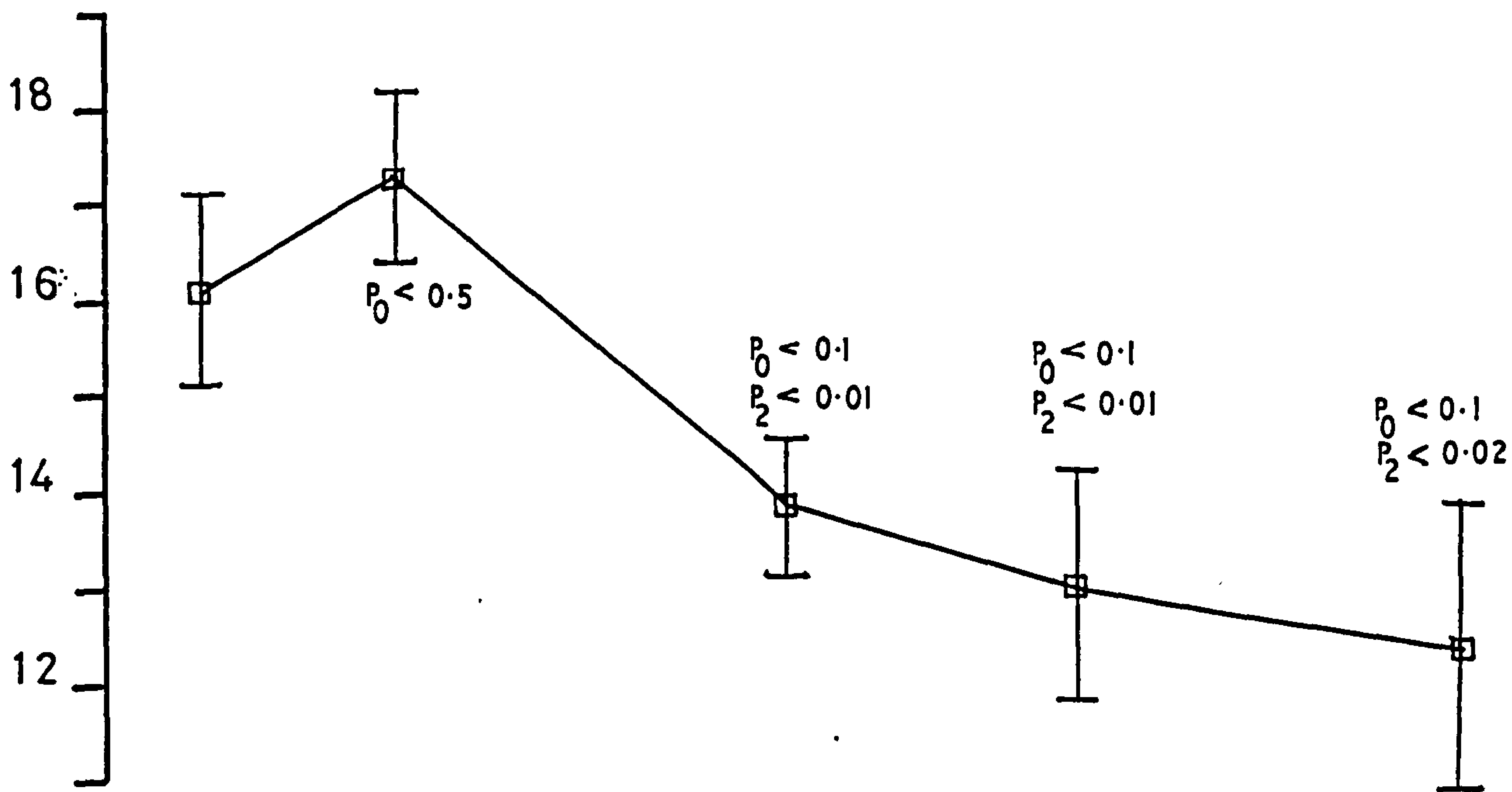
P0 < 0·5
P2 < 0·05

DAYS	0	2	3	6	+3
O_2	21%		85%		21%
n=	16	16	16	15	11

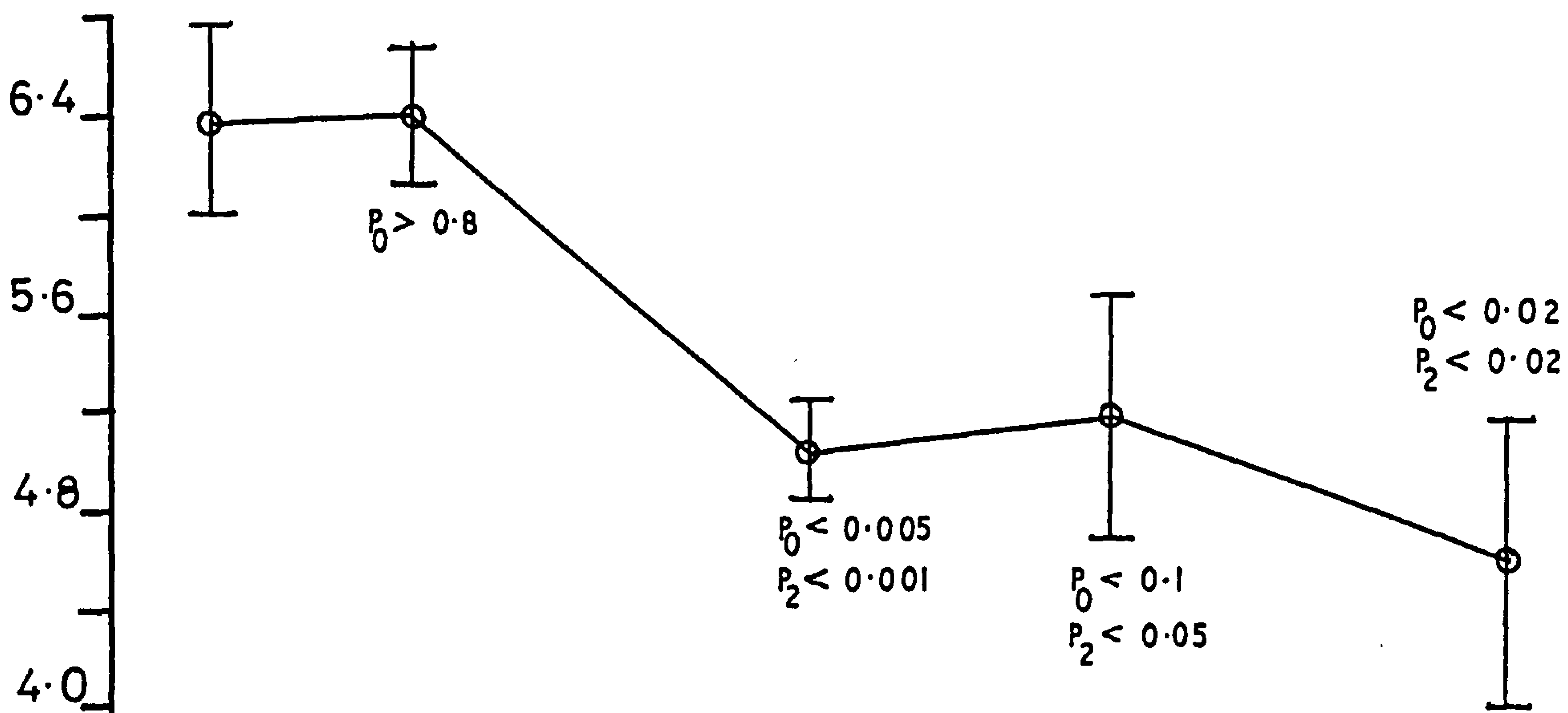
FIGURE 16: BRAIN CATALASE ACTIVITY

UNITS = k×10^3

UNITS/ml



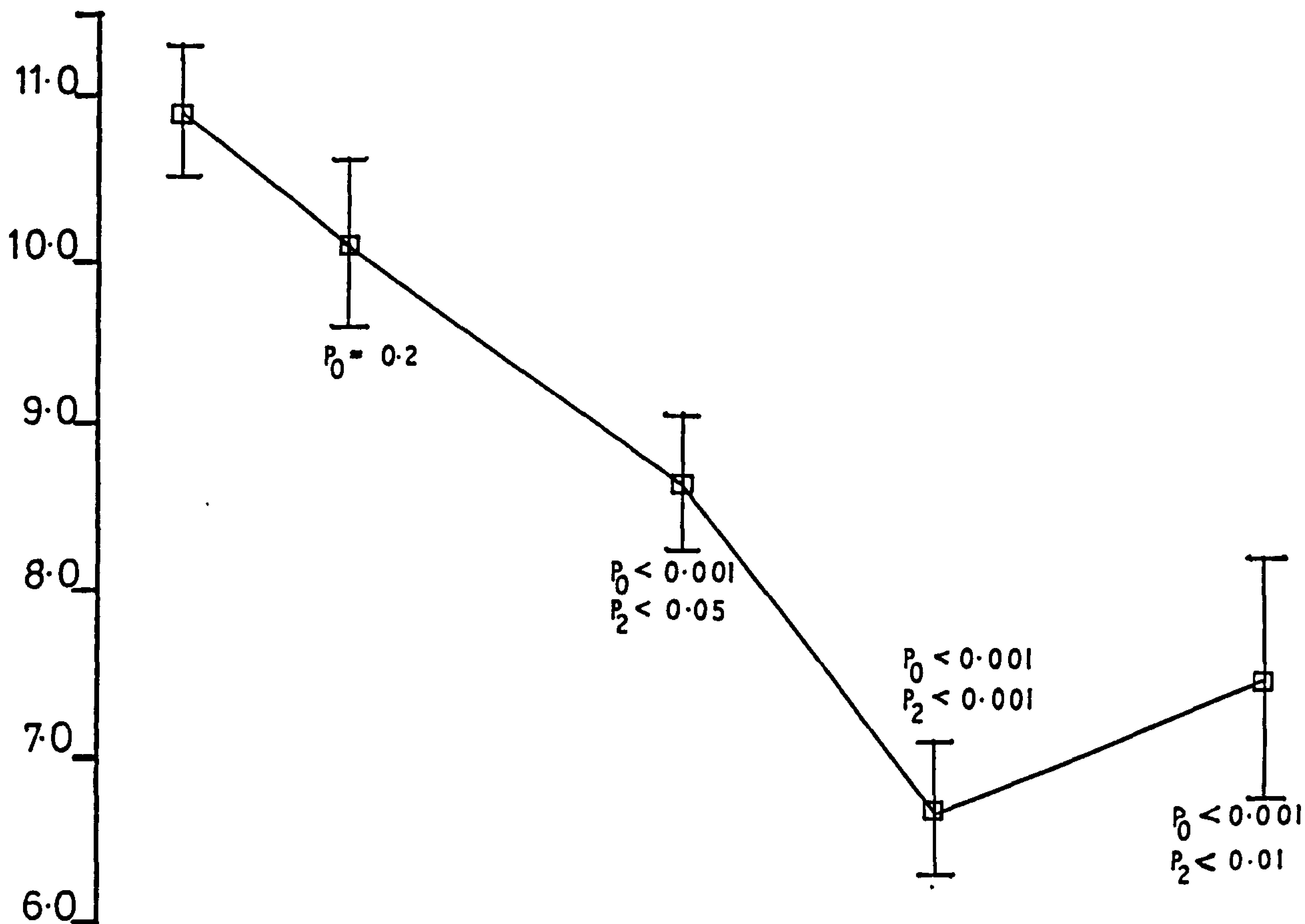
UNITS/mg PROTEIN



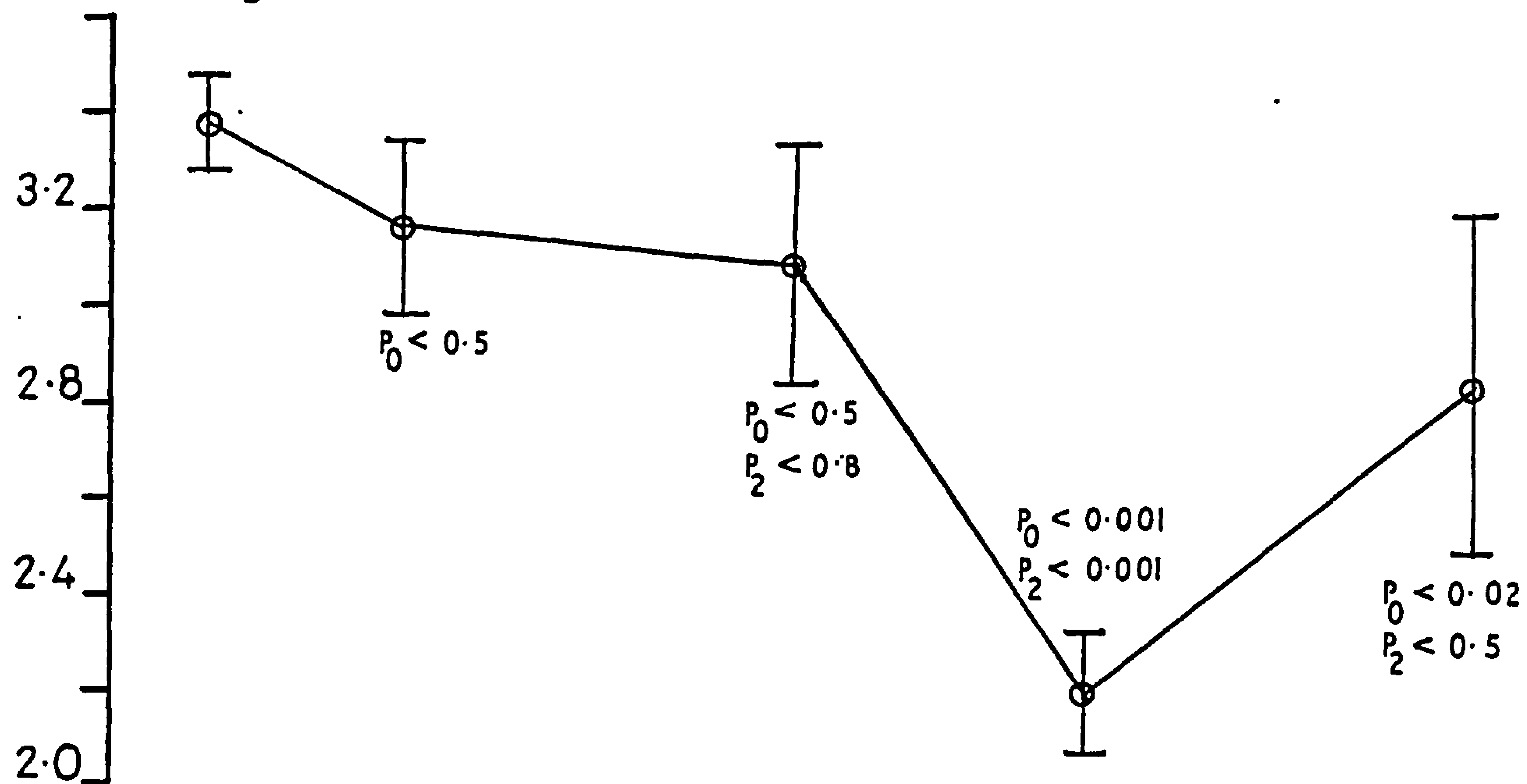
DAYS	0	2	3	6	+3
O_2	21%		85%		21%
n=	16	16	16	15	11

FIGURE 17: LIVER CATALASE ACTIVITY

UNITS/ml UNITS = k×10



UNITS/mg PROTEIN



DAYS	0	2	3	6	+3
O_2	21%		85%		21%
n =	16/ml 12/mg	16	16	15	11

The changes in pellet protein levels were not the same as the changes in catalase, and due to the slightly varying changes in catalase concentrations between tissues, each tissue showed different changes in catalase specific activities, to the changes in catalase concentrations. In the lung, the large variation of the 'initial' control precluded any statistically significant changes, although the trend was for a decrease in catalase specific activity at 6 days exposure. The 'post-exposure' control still showed significantly reduced catalase specific activity compared to the 'chamber' control. In the brain the catalase levels were very similar whether expressed per ml of homogenate or per mg protein, but the 3 days exposure and the 'post-exposure' control values showed a significant difference against both of the 'pre-exposure' controls when expressed in the latter way. In the liver, the decreases in catalase levels were almost eliminated when expressed per mg protein. The only change which was then significant against both the 'pre-exposure' controls was the decrease in catalase specific activity after 6 days exposure. This change was considerable; the 6 day value was only between 65-69% of the 'pre-exposure' controls. The 'post-exposure' control was still significantly different from the 'initial' control, although an upward trend was noted.

Taken overall these changes indicated that exposure to oxygen caused a decrease in tissue catalase concentration which was small after 3 days exposure but significant after 6 days exposure. Liver was the only tissue which showed evidence of a recovery of catalase levels during the post-exposure period, with a rise in catalase specific activity of the 'post-exposure' control compared to the 6 day value.

vi) Changes in Tissue Water Content

In view of the very obvious oedema and inflammation of the lungs, and the changes in the supernatant protein levels after exposure to oxygen, it was thought that changes in the percentage water content of the lungs might provide a partial explanation for some of these results. Although changes were expected only in the lung, brain and liver percentage water contents were also determined.

The results obtained from this experiment (see figure18) were somewhat surprising. Small increases in tissue water content were observed in lung and brain after 3 days exposure, but the only statistically significant change was the increase in tissue water content in the liver after 6 days exposure to oxygen. Any increase in water content would, in the event of no other change, be expected to result in a concomitant decrease in protein concentration of the supernatant fraction. It was of interest however, that the increases in water content in each tissue coincided with the increases in supernatant protein concentration. In the lung the decrease in percentage dry tissue weight from the 'initial' control was approximately 9% at 3 days exposure whereas the increase in supernatant protein was 18%. In the brain the decrease in percentage dry tissue weight from the 'initial' control was approximately 5% but the increase in protein concentration was 18%. Similarly in the liver the decrease in the percentage dry tissue weight from the 'chamber' control(this control was thought to be a better guide in this tissue) and the supernatant protein level at 6 days was approximately 6% and the increase in protein concentration was again 18%.

These figures strongly indicated that the changes in protein concentration occurred in spite of an increase in tissue water content rather than partly as a result of a decrease in tissue water content.

Legend to Figure 18

Changes in % Water Content of Lung, Brain and Liver of Mice
Exposed to 0·85 atm Oxygen

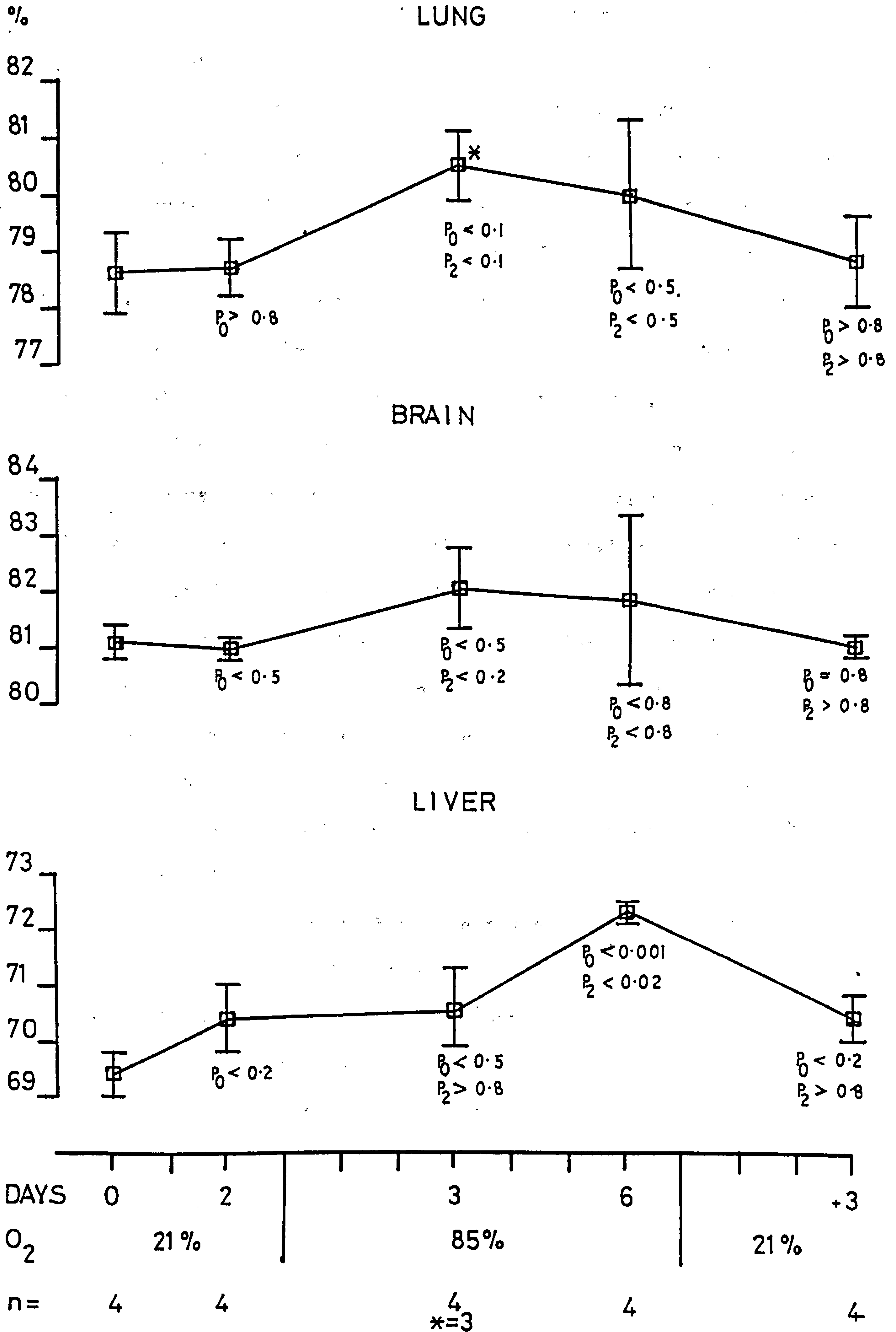
The upper graph depicts the changes in lung, the middle graph the changes in brain, and the lower graph the changes in liver, of the % tissue water content.

The x axis has the units of days and is separated by the long vertical divisions into three sections: the pre-exposure period, the experimental period and the post-exposure period which are labelled with the relevant oxygen concentrations. Day 0 refers to the 'initial' control, day 2 to the 'chamber' control, day 3 and 6 to the 3 day and 6 day exposure groups respectively, and +3 to the 'post-exposure' control.

n= Refers to the number of animals used for that particular point in all graphs except for one point marked by an asterisk. The P values of Student's 't' test (two tailed) are given for each point. P_0 is the probability that a particular value is identical to the 'initial' control, and P_2 is the probability that a particular value is identical to the 'chamber' control.

Each point represents the determinations of four samples from a single experiment.

FIGURE 18:
% WATER CONTENT OF LUNG BRAIN AND LIVER



vii) Qualitative Determination of Protein Content

Because of the changes seen in the levels of protein content, particularly in the supernatant, an attempt was made to see if any qualitative changes in the protein present could be discerned. Employing a supernatant separation on polyacrylamide gel, qualitative changes should be reflected either in an extra protein band or bands during an increase of protein, or a missing band or bands during a decrease in protein upon electrophoresis, if specific changes were taking place at a gross level. Because of the experimental procedure adopted, any qualitative changes in protein should be referable to the quantitative changes in protein previously noted.

The developed and stained polyacrylamide gels were each scanned and the patterns recorded using a chart recorder which was adjusted so that all of the scans showed similar peak heights. This was found to be necessary due to the range of intensity given by different acetone powder extracts. There were four different samples of each tissue from each experimental point. Each set of four scans was examined separately, and one scan from each group was selected which showed the greatest resemblance to a hypothetical scan showing an average of the features of the group. These selected scans can be seen in figures 19 to 21.

In the lung the largest change seen was that between the two 'initial' controls. After 3 days exposure to oxygen when any specific changes should be represented by extra peaks, the major additions were an extra peak to the cathode side of the large multiple peak, and a general increase in the background staining at the negative pole which must represent higher molecular weight proteins. At 6 days exposure when a loss of protein was apparent, this latter higher molecular weight protein had largely disappeared, but the major peak had larger shoulders. The 'post-exposure' control appeared to be very similar to the 'initial' control.

Legend to Figures 19 to 21

Electrophoretogram Scans Showing Qualitative Protein Changes In Lung, Liver and Brain of Mice Exposed to 0·85 atm Oxygen

Each figure consists of five scans, one from each of the control and experimental points from one particular tissue. OD refers to the 'initial' control, 2D to the 'chamber' control, 3D and 6D to the 3 day and 6 day exposures respectively, and +3 to the 'post-exposure' control. Each scan is the best average representative scan selected from four scans, each in turn made from a separate sample prepared during the same experiment. For each scan presented the anode is at the right and the cathode at the left.

The polyacrylamide gels were prepared as outlined in the materials and methods section C., and the acetone powders as described in materials and methods section D. ii).

FIGURE 19:

POLYACRYLAMIDE GEL ELECTROPHORETOGRAM SCANS OF MOUSE LUNG PROTEIN

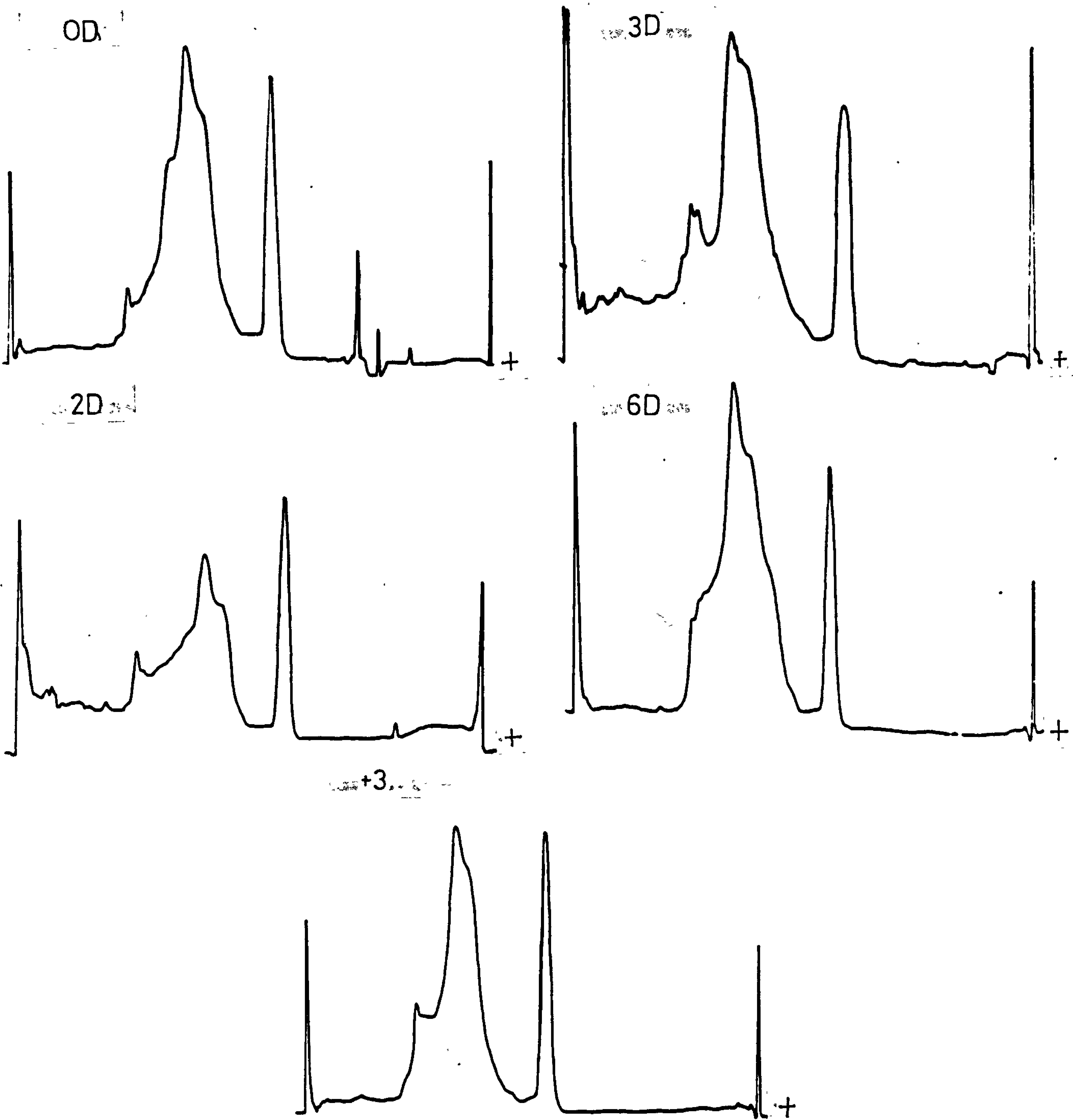


FIGURE 20:

POLYACRYLAMIDE GEL ELECTROPHORETOGRAM SCANS OF MOUSE BRAIN PROTEIN

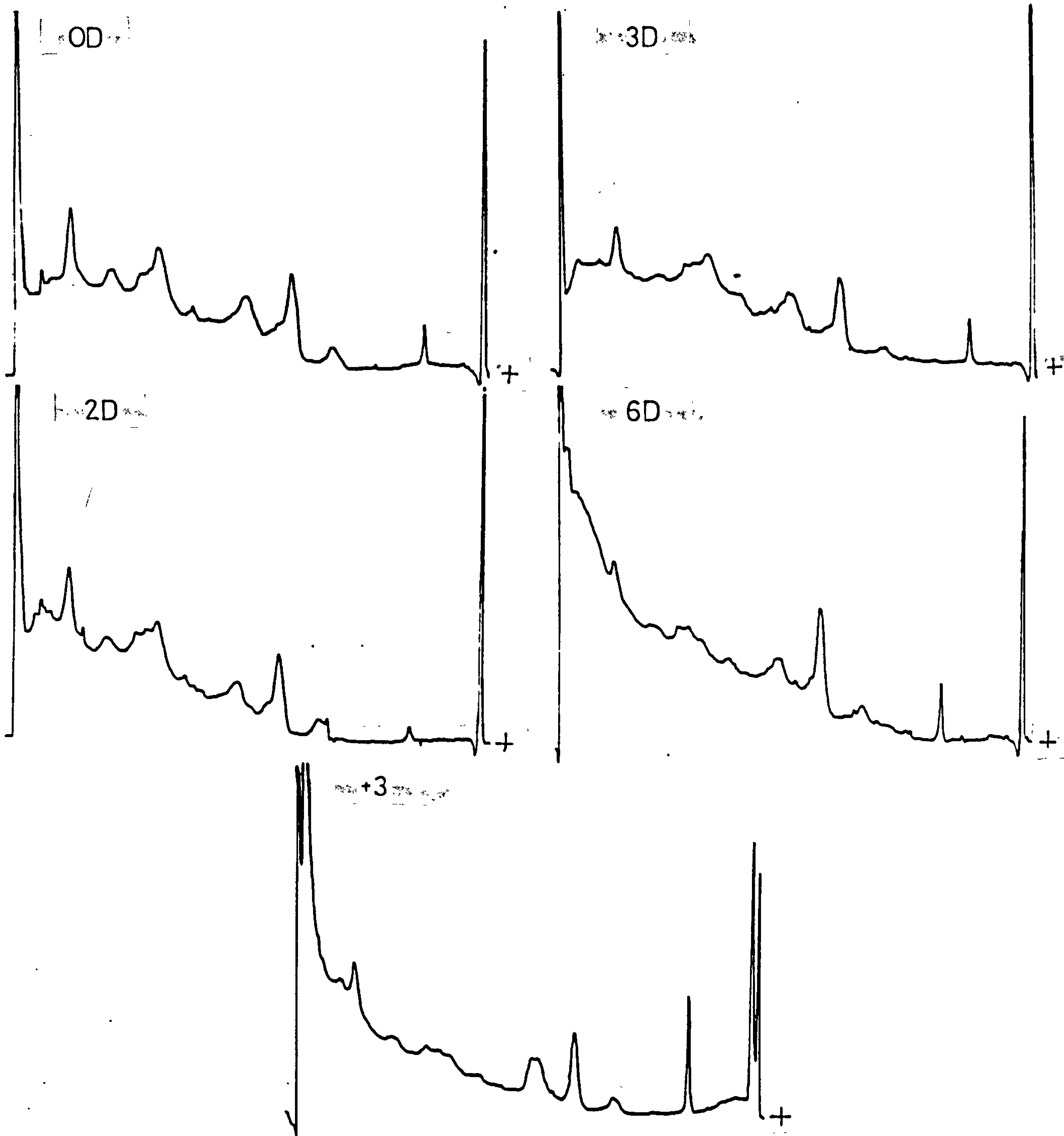
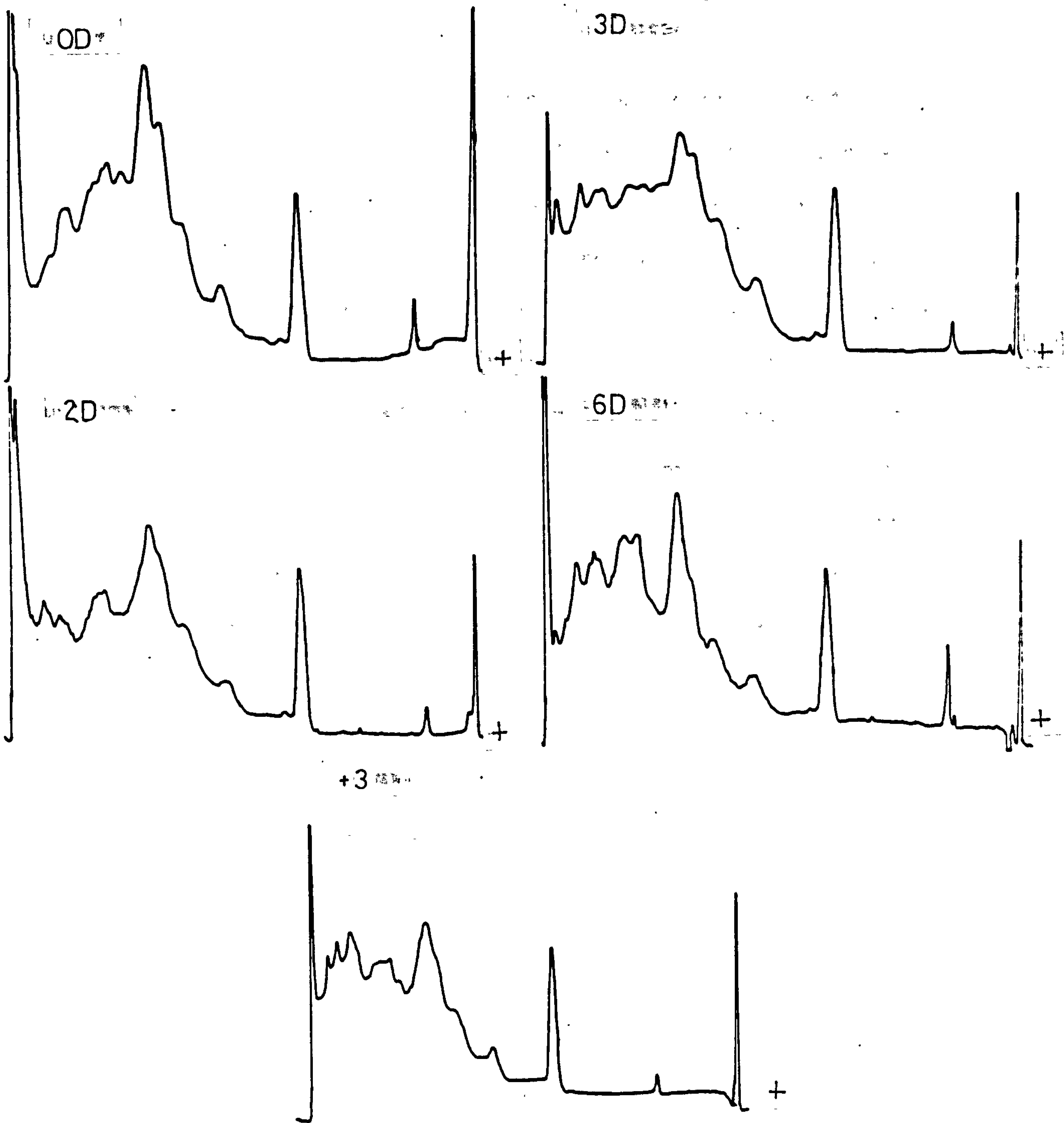


FIGURE 21:

POLYACRYLAMIDE GEL ELECTROPHORETOGRAM SCANS OF MOUSE LIVER PROTEIN



In the brain protein scans, the only notable difference between any of the gels was the appearence after 6 days exposure, and in the 'post-exposure' control of an increase in the amount of high molecular weight protein.

In the liver, very little change in the separation pattern between the 'initial' control and the 'chamber' control could be seen despite the decrease in protein concentration which occurred previously. However after 3 days exposure to oxygen the appearance of a moderate amount of higher molecular weight material filling in between the peaks could be seen despite little change in the overall protein concentration noted in the previous experiment. The scan from 6 days exposure to oxygen shows either some clearance of the higher molecular weight protein or a general increase in the level of more specific protein bands compared to the 3 day exposure, the latter being more likely in view of the increase in protein concentration which has been shown to occur at this time.

C. Discussion

The changes in the behaviour of mice subjected to 0·85 atm oxygen in the present investigation appeared to be very similar to observations previously reported in the literature, with the exception that the mice used in this study appeared to be slightly less susceptible to the effects of oxygen. In the experiments described in this chapter, 96 mice were employed, of which 32 mice were exposed to 0·85 atm oxygen for a full 7 day period. Of these 32 mice, only 2 died during the exposure, the remaining 4 fatalities occurring on return to atmospheric oxygen levels. Other studies using mice have reported a higher mortality rate under similar conditions. It was observed that a large proportion of the control mice lungs were inflamed and oedematous. Frank, Yam & Roberts

(1978) have shown that treatment of rats with bacterial endotoxin before exposure increased the tolerance of rats to oxygen. Similarly, Lorrain-Smith (1899) noted that previous exposure to oxygen sufficient to induce inflammation of the lungs prolonged survival on a second exposure to hyperbaric oxygen. Wright et al (1966) noted that germ-free rats and mice tended to die sooner and within a narrower time span than conventional animals. It is possible, therefore, that inflammation of the lungs hindering gaseous exchange, or an exposure to infection, may have thus contributed to the slow response of the mice used in this study to the effects of oxygen. Alternatively, the strain of mice used in this study may have been more resistant to the toxic effects of oxygen.

It was also noted that the average wet lung weight of the mice decreased during the control pre-exposure period. This may have been due to a beneficial effect of the isolation of the mice and the filtered atmosphere in which they were kept.

Lung protein synthesis has been shown to be inhibited by 1 atm oxygen for the first 2 days but after 4 days was greater than control levels (see introduction section 4.A.). While a similar effect may in part account for the protein changes occurring in the liver, it is not in the least consistent with explaining the changes in lung and brain protein. It is possible that the increase in protein in the lung at 3 days exposure may have arisen partly as a result of exudation of plasma protein into the lung caused by hypertension (Wood & Perkins 1970; Wood et al 1972; Välimäki et al 1974), but this cannot have been the case in brain where no significant increase in weight was shown over this period. Neither do the above results account for the decrease demonstrated in total lung protein on the sixth day, relative to the third day, of exposure to oxygen. The only remaining explanation for these results

is that there was an inhibition of protein degradation at this time which was greater than the inhibition of protein synthesis. The decrease in protein from day 3 to day 6 could then be explained by a release of protein degradation from this inhibition before the inhibition of protein synthesis was relieved. This proposal could be tested by determination of oxygen on the half-life of protein.

The protein concentration changes in the liver were probabaly more a reflection of the loss of the weight of the animals due to anorexia and the resulting biochemical adaptation to starvation.

An increase in the total lung protein was noted in rats exposed to oxygen (0·85 atm) by Crapo & Tierney (1974). However in their study, the change was shown to be larger. No information was presented whether this increase occurred gradually over the whole period or was alinear as in the results presented above.

The qualitative protein determinations were found to be not sensitive enough to detect any significant changes in specific proteins. This could be an indication that the quantitative changes in protein did not lead to a gross qualitative change in protein. The only consistent finding in this section of work was the appearance of background high molecular weight protein. In view of its diffuse nature and lack of resolution into specific bands, this material could conceivably represent polymerised or otherwise denatured proteins.

In all of the tissues studied, the trend was for a decrease of SOD concentration after 3 days exposure, despite a large general rise in the level of protein in lung and brain. This result was indicative that SOD was degraded more rapidly, or synthesised more slowly than the other protein in these tissues. As the latter possibility is less likely, it would appear that SOD was inactivated *in vivo* more rapidly than most proteins. However by the sixth day of

exposure, the general protein levels had fallen, but the levels of SOD had risen, indicating that a specific induction of SOD occurred to return the concentration of SOD to normal, or in the case of the brain, marginally above normal. Contrary to reports of changes in SOD in rats (Crapo & Tierney 1974; Kimball et al 1976; Crapo & McCord 1976), guinea pig polymorphonuclear leucocytes and alveolar macrophages (Rister & Baehner 1976), and in neonatal mice, rats and rabbits (Frank, Bucher & Roberts 1978), there was little evidence from this study to show that an adaptive response to oxygen, in the production of SOD above normal levels, occurs in adult mice. However Frank, Bucher & Roberts 1978) found no increase in SOD in adult animals and Crapo & Tierney (1974) found only a small increase in mouse lung SOD levels. The latter authors expressed their results as SOD per whole lung. If the results of this study are expressed in a like manner viz.:

	0·21 atm		0·85 atm		0·21 atm
Day	0	2	3	6	+3
SOD/Whole Lung (units)	9·53	11·2	8·53	13·5	12·8

an increase of similar magnitude is seen to that reported by Crapo & Tierney (1974). As superoxide anion concentration is due to an equilibrium of formation versus uncatalysed plus catalysed dismutation, it is the concentration of SOD that is the important factor, not the absolute amount. Unless it can be shown that the newly synthesised SOD is distributed specifically where it is most effective, an overall increase in the quantity of SOD in an organ which increases proportionally in size and weight during exposure to oxygen is of no real advantage in vivo.

In liver and lung the catalase activities expressed per mg

protein were little different between 3 days exposure and control values, whereas a decrease in activity was noted between 3 days and 6 days exposure. In the brain the reduction occurred at 3 days exposure and this level was maintained at 6 days exposure. This pattern of results was not entirely consistent either with the effect of oxygen toxicity on catalase activity being proportional to its effect on protein in general, nor to an enhanced action of oxygen on catalase. One possible explanation, is that on exposure to oxygen, catalase activity was dissociated from the pellet fraction due to subcellular structural damage. Further experiments would be required to reveal whether or not this was the true explanation.

Overall these results appeared to show that catalase activity expressed as a concentration (units per ml of homogenate) was diminished _in vivo_ during oxygen toxicity. There are only two reports of the effects of oxygen on catalase activities _in vivo_. Frank, Bucher & Roberts (1978) found no significant changes in the catalase activities of adult animals exposed to oxygen, but small increases in the activity of this enzyme in neonatal mice, rabbits and rats. Rister & Baehner (1976) found a decrease in activity in catalase from polymorphonuclear leucocytes and alveolar macrophages obtained from guinea pigs subjected to oxygen toxicity.

The results from the present investigation on the effects of oxygen on the enzymes catalase and SOD lead to the conclusion that in mice, there was no increase in the concentration of these enzymes. On the contrary, it appeared that a reduction in the concentration resulted during the exposure. SOD appeared to be induced to regain its normal concentration, but catalase was not induced.

The changes noted in water content were somewhat surprising. In view of the visible oedema noted in the lungs a large increase in the percentage water content would be expected. However, lung and

liver showed increases of a similar magnitude in percentage water content (9%), with brain showing a slightly smaller increase (5%). Neither do the changes in water content help to explain the changes in protein levels. From the current work it is not possible to differentiate between the increase in tissue water content being due to an intracellular or extracellular accumulation of water.

Chapter 2: DETERMINATION OF CHANGES IN AMINO ACID METABOLISM IN LUNG, LIVER, BRAIN AND MUSCLE OF MICE EXPOSED TO 0·85 atm OXYGEN

A. Introduction

Amino acid metabolism, although of great consequence to the total cellular metabolism in respect of both protein synthesis and degradation, and the urea cycle, has been largely neglected in investigations into oxygen toxicity. Few studies have so far persued the effects of oxygen on amino acid metabolism. Huggins & Nelson (1975) examined free amino acid levels in brains of mice exposed to OHP, Boehme et al (1979) showed that E. coli exposed to OHP needed supplements of certain amino acids to continue growing, and Yusa et al (1979) investigated the effect of OHP on the metabolic balance of amino acid pools in plasma of fasted rabbits following injections of carbohydrate solutions. Wood & Watson (1963) demonstrated a decrease in GABA levels as a result of exposure to OHP, and have subsequently reported an inhibition of the synthesising enzyme glutamic acid dehydrogenase (see introduction section 4.B. iii)). Schäfer investigated alterations of GABA, glutamate and glutamine in brains of mice exposed to OAP, but as yet, no other studies have been performed on the changes in amino acid pools in mammalian tissues exposed to OAP.

Because of this gap in research into oxygen toxicity it was decided to conduct a study into amino acid metabolism during chronic hyperoxia. Due to the universal nature of oxygen toxicity to all living organisms and all tissues, and the severity of the toxicity, it was expected that any changes due to defects in metabolism should be relatively large. A survey was therefore executed using a small number of samples from four different tissues: brain, lung,

liver and muscle. Brain was chosen because of its involvement with convulsions at higher tensions of oxygen, lung because it is the tissue in direct contact with the excess tension of oxygen, liver because it is a metabolically active tissue, with a good blood supply and which is the best example of an 'unspecialised' tissue, and finally muscle because it is a tissue which in caged animals would not be expected to be particularly active.

B. Results

This experiment followed a simpler format than the experiments reported in the previous chapter. Only a single control group was sacrificed as the experimental animals were put into the chamber, but in addition a complete set of comparative controls were used which were subjected to identical conditions to the experimental animals in the chamber, but at 0·21 atm oxygen at a later date. Each group originally contained four animals which were all exposed to the same conditions at the same date. Samples were taken at 1, 3, and 7 days exposure. No fatalities were recorded during this experiment, but two of the muscle samples were lost during their preparation.

The results of these amino acid analyses are presented in tables 6 to 9, and are expressed as µmoles/g wet weight tissue; means $\pm$ SEM. The number of samples is 4 unless otherwise indicated. OT refers to the zero time control and 1D, 3D and 7D to the respective number of days exposure to the indicated oxygen tension.

A close examination of the results in these tables revealed that not only was there a large variation between different samples of the same sample group, but that also there was a larger variation between the sample groups in the controls than in the experimental animals. In addition all the amino acids in any one sample generally

Table 6:

LUNG FREE AMINO ACIDS IN 0·21 ATM OXYGEN

Units are μmoles/g wet weight tissue

Amino Acid	OT	1D	3D	7D
Alanine	0·73 ± 0·04	0·55 ± 0·12	1·01 ± 0·21	0·63 ± 0·09
Arginine	0·13 ± 0·01	0·09 ± 0·02	0·12 ± 0·02	0·10 ± 0·01
Aspartate	1·60 ± 0·35	0·84 ± 0·12	1·56 ± 0·20	1·02 ± 0·10
Cysteic acid	0·55 ± 0·20	0·38 ± 0·20	0·52 ± 0·16	0·30 ± 0·03
Glutamate	1·77 ± 0·18	1·03 ± 0·18	1·90 ± 0·21	1·44 ± 0·14
Glycine	3·31 ± 0·41	2·11 ± 0·41	4·20 ± 0·65	3·04 ± 0·22
Histidine	0·09 ± 0·01	0·09 ± 0·01	0·12 ± 0·02	0·08 ± 0·02
Isoleucine	0·06 ± 0·01	0·04 ± 0·01	0·08 ± 0·01	0·05 ± 0·01
Leucine	0·12 ± 0·02	0·09 ± 0·01	0·16 ± 0·03	0·11 ± 0·02
Lysine	0·22 ± 0·02	0·21 ± 0·03	0·27 ± 0·04	0·21 ± 0·07
Ornithine	0·28 ± 0·02	0·25 ± 0·04	0·37 ± 0·05	0·28 ± 0·08
Phenylalanine	0·08 ± 0·02	0·06 ± 0·01	0·10 ± 0·03	0·06 ± 0·01
Serine	0·25 ± 0·02	0·19 ± 0·02	0·32 ± 0·07	0·15 ± 0·03
Taurine	8·36 ± 1·67	5·55 ± 0·74	9·47 ± 1·77	6·98 ± 0·65
Threonine	0·28 ± 0·04	0·20 ± 0·04	0·35 ± 0·08	0·22 ± 0·34
Tyrosine	0·08 ± 0·01	0·07 ± 0·01	0·12 ± 0·05	0·08 ± 0·03
Valine	0·15 ± 0·02	0·10 ± 0·01	0·15 ± 0·02	0·13 ± 0·02

Table 6:

LUNG FREE AMINO ACIDS IN 0·85 ATM OXYGEN

Units are μmoles/g wet weight tissue

Amino Acid	OT	1D	3D	7D
Alanine	0·63 ± 0·08	0·64 ± 0·11	0·67 ± 0·02	0·93 ± 0·25
Arginine	0·10 ± 0·01	0·10 ± 0·01	0·09 ± 0·01	0·08 ± 0·02
Aspartate	1·46 ± 0·20	1·09 ± 0·12	1·24 ± 0·08	1·17 ± 0·19
Cysteic acid	0·41 ± 0·12	0·37 ± 0·04	0·38 ± 0·18	0·43 ± 0·11
Glutamate	1·49 ± 0·20	1·27 ± 0·19	1·35 ± 0·02	2·18 ± 0·21
Glycine	2·69 ± 0·48	3·16 ± 0·46	3·02 ± 0·24	1·95 ± 0·34
Histidine	0·08 ± 0·02	0·10 ± 0·02	0·13 ± 0·02	0·11 ± 0·02
Isoleucine	0·06 ± 0·01	0·04 ± 0·01	0·08 ± 0·01	0·06 ± 0·01
Leucine	0·13 ± 0·01	0·11 ± 0·01	0·15 ± 0·01	0·14 ± 0·02
Lysine	0·15 ± 0·03	0·20 ± 0·01	0·25 ± 0·02	0·24 ± 0·03
Ornithine	0·25 ± 0·03	0·27 ± 0·08	0·39 ± 0·07	0·24 ± 0·05
Phenylalanine	0·07 ± 0·05	0·08 ± 0·01	0·10 ± 0·01	0·07 ± 0·02
Serine	0·27 ± 0·02	0·25 ± 0·03	0·27 ± 0·05	0·33 ± 0·08
Taurine	7·28 ± 0·76	7·18 ± 0·73	6·56 ± 0·62	7·03 ± 0·41
Threonine	0·30 ± 0·04	0·26 ± 0·04	0·25 ± 0·06	0·33 ± 0·04
Tyrosine	0·06 ± 0·01	0·07 ± 0·01	0·10 ± 0·01	0·07 ± 0·02
Valine	0·13 ± 0·03	0·11 ± 0·01	0·14 ± 0·01	0·12 ± 0·03

Table 7:

BRAIN FREE AMINO ACIDS IN 0•21 ATM OXYGEN

Units are µmoles/g wet weight tissue

Amino Acid	OT	1D	3D	7D
Alanine	0•67 ± 0•05	0•75 ± 0•08	0•71 ± 0•11	0•71 ± 0•08
Arginine	0•16 ± 0•01	0•20 ± 0•02	0•16 ± 0•02	0•18 ± 0•01
Aspartate	2•65 ± 0•13	2•57 ± 0•09	2•16 ± 0•12	2•02 ± 0•11
Cysteic acid	0•33 ± 0•02	0•34 ± 0•01	0•24 ± 0•02	0•26 ± 0•02
GABA	1•93 ± 0•11	2•17 ± 0•16	1•77 ± 0•06	1•80 ± 0•05
Glutamate	7•34 ± 0•64	7•05 ± 0•69	7•24 ± 0•48	6•50 ± 0•34
Glycine	1•10 ± 0•06	1•53 ± 0•13	1•19 ± 0•12	1•26 ± 0•15
Histidine	0•13 ± 0•01	0•13 ± 0•02	0•10 ± 0•01	0•11 ± 0•01
Isoleucine	0•02 ± 0•01	0•05 ± 0•01	0•04 ± 0•01	0•02 ± 0•01
Leucine	0•05 ± 0•01	0•08 ± 0•01	0•07 ± 0•01	0•07 ± 0•01
Lysine	0•31 ± 0•01	0•35 ± 0•04	0•29 ± 0•02	0•29 ± 0•01
Ornithine	0•23 ± 0•01	0•29 ± 0•06	0•21 ± 0•04	0•27 ± 0•02
Phenylalanine	0•07 ± 0•01	0•10 ± 0•01	0•07 ± 0•02	0•06 ± 0•01
Serine	1•20 ± 0•12	1•14 ± 0•17	1•32 ± 0•27	1•20 ± 0•13
Taurine	7•91 ± 0•14	9•21 ± 0•57	6•34 ± 0•51	7•05 ± 0•43
Threonine	0•44 ± 0•03	0•56 ± 0•10	0•39 ± 0•07	0•37 ± 0•01
Tryptophan	0•10 ± 0•01	0•13 ± 0•02	0•11 ± 0•03	0•10 ± 0•01
Tyrosine	0•10 ± 0•02	0•14 ± 0•02	0•12 ± 0•02	0•09 ± 0•01
Valine	0•07 ± 0•01	0•10 ± 0•01	0•07 ± 0•01	0•10 ± 0•02

BRAIN FREE AMINO ACIDS IN 0·85 ATM OXYGEN

Units are µmoles/g wet weight tissue

Amino Acid	OT	1D	3D	7D
Alanine	0·64 ± 0·08	0·71 ± 0·02	0·63 ± 0·05	0·60 ± 0·08
Arginine	0·21 ± 0·02	0·12 ± 0·04	0·13 ± 0·01	0·15 ± 0·02
Aspartate	2·72 ± 0·34	2·03 ± 0·11	1·68 ± 0·04	1·95 ± 0·27
Cysteic acid	0·33 ± 0·07	0·29 ± 0·02	0·24 ± 0·02	0·31 ± 0·02
GABA	2·14 ± 0·19	1·95 ± 0·03	1·98 ± 0·05	1·93 ± 0·09
Glutamate	7·38 ± 0·96	6·90 ± 0·16	6·56 ± 0·43	5·99 ± 0·32
Glycine	1·60 ± 0·17	1·49 ± 0·37	1·17 ± 0·24	1·18 ± 0·07
Histidine	0·10 ± 0·01	0·12 ± 0·01	0·13 ± 0·01	0·10 ± 0·02
Isoleucine	0·04 ± 0·01	0·03 ± 0·01	0·04 ± 0·01	0·03 ± 0·01
Leucine	0·08 ± 0·01	0·09 ± 0·01	0·09 ± 0·01	0·08 ± 0·01
Lysine	0·36 ± 0·05	0·32 ± 0·02	0·33 ± 0·02	0·35 ± 0·03
Ornithine	0·23 ± 0·03	0·17 ± 0·03	0·29 ± 0·02	0·23 ± 0·05
Phenylalanine	0·09 ± 0·01	0·10 ± 0·01	0·09 ± 0·01	0·08 ± 0·01
Serine	0·91 ± 0·09	1·08 ± 0·10	0·97 ± 0·09	0·79 ± 0·09
Taurine	7·34 ± 0·44	7·91 ± 0·67	6·47 ± 0·32	6·43 ± 0·38
Threonine	0·46 ± 0·08	0·35 ± 0·06	0·49 ± 0·09	0·33 ± 0·07
Tryptophan	0·13 ± 0·02	0·16 ± 0·01	0·14 ± 0·01	0·12 ± 0·01
Tyrosine	0·11 ± 0·01	0·10 ± 0·01	0·12 ± 0·02	0·10 ± 0·01
Valine	0·11 ± 0·02	0·09 ± 0·01	0·10 ± 0·01	0·08 ± 0·01

Table 8:

LIVER FREE AMINO ACIDS IN 0·21 ATM OXYGEN

Units are µmoles/g wet weight tissue

Amino Acid	OT	1D	3D	7D
Alanine	2·69 ± 0·15	2·99 ± 0·40	2·72 ± 0·14	2·75 ± 0·07**
Aspartate	0·35 ± 0·05	0·48 ± 0·07	0·33 ± 0·05*	0·47 ± 0·09
Cysteic acid	0·55 ± 0·10	0·51 ± 0·08	1·08 ± 0·33	0·84 ± 0·26
Glutamate	1·45 ± 0·15	1·66 ± 0·12	1·44 ± 0·15	1·44 ± 0·16
Glycine	3·33 ± 0·99	1·88 ± 0·39	1·51 ± 0·17	1·71 ± 0·29
Histidine	0·31 ± 0·01	0·45 ± 0·05	0·42 ± 0·02	0·49 ± 0·05
Isoleucine	0·18 ± 0·01	0·17 ± 0·01	0·14 ± 0·01	0·18 ± 0·02
Leucine	0·35 ± 0·02	0·24 ± 0·01	0·29 ± 0·04	0·33 ± 0·05
Lysine	0·49 ± 0·09	0·33 ± 0·05	0·34 ± 0·01	0·54 ± 0·08
Ornithine	0·45 ± 0·06	0·34 ± 0·06	0·33 ± 0·03	0·55 ± 0·07
Phenylalanine	0·19 ± 0·04	0·14 ± 0·02	0·11 ± 0·01	0·18 ± 0·03
Serine	0·80 ± 0·14	0·42 ± 0·12	0·53 ± 0·08	0·69 ± 0·20
Taurine	7·95 ± 1·37	9·10 ± 0·64	10·8 ± 0·72	10·2 ± 0·26*
Threonine	0·26 ± 0·04	0·39 ± 0·09	0·73 ± 0·14	0·45 ± 0·04
Tyrosine	0·13 ± 0·02	0·13 ± 0·01	0·14 ± 0·03	0·15 ± 0·01
Valine	0·32 ± 0·02	0·20 ± 0·01	0·22 ± 0·01	0·32 ± 0·03

* n = 3

** n = 2

LIVER FREE AMINO ACIDS IN 0·85 ATM OXYGEN

Units are μmoles/g wet weight tissue

Amino Acid	OT	1D	3D	7D
Alanine	2·33 ± 0·22	2·48 ± 0·12	2·80 ± 0·51	2·94 ± 0·36
Aspartate	0·42 ± 0·04	0·43 ± 0·06	0·57 ± 0·06	0·62 ± 0·16
Cysteic acid	0·41 ± 0·05	0·65 ± 0·10	0·55 ± 0·14	0·41 ± 0·08
Glutamate	1·49 ± 0·16	1·32 ± 0·10	1·48 ± 0·30	1·86 ± 0·43
Glycine	2·25 ± 0·18	2·02 ± 0·44	2·08 ± 0·75	1·61 ± 0·19
Histidine	0·34 ± 0·03	0·39 ± 0·01	0·45 ± 0·01	0·47 ± 0·06
Isoleucine	0·19 ± 0·01	0·12 ± 0·01	0·13 ± 0·01	0·09 ± 0·02
Leucine	0·33 ± 0·03	0·23 ± 0·02	0·26 ± 0·02	0·22 ± 0·01
Lysine	0·49 ± 0·02	0·37 ± 0·02	0·46 ± 0·05	0·46 ± 0·04
Ornithine	0·55 ± 0·04	0·46 ± 0·06	0·48 ± 0·11	0·45 ± 0·06
Phenylalanine	0·15 ± 0·01	0·14 ± 0·02	0·13 ± 0·01	0·12 ± 0·01
Serine	0·64 ± 0·19	0·56 ± 0·03	0·73 ± 0·12	0·43 ± 0·09
Taurine	10·6 ± 1·89	11·2 ± 1·55	10·0 ± 2·20	9·5 ± 1·91
Threonine	0·47 ± 0·04	0·34 ± 0·03	0·26 ± 0·06	0·53 ± 0·27
Tyrosine	0·14 ± 0·02	0·10 ± 0·01	0·12 ± 0·01	0·13 ± 0·03
Valine	0·33 ± 0·02	0·22 ± 0·02	0·25 ± 0·02	0·19 ± 0·03

Table 9:

MUSCLE FREE AMINO ACIDS IN 0·21 ATM OXYGEN

Units are μmoles/g wet weight tissue

Amino Acid	OT	1D	3D	7D
Alanine	1·28 ± 0·11	1·72 ± 0·17	1·60 ± 0·17	1·71 ± 0·14
Arginine	0·32 ± 0·09	0·32 ± 0·08	0·47 ± 0·09	0·37 ± 0·07
Aspartate	0·40 ± 0·04	0·30 ± 0·07	0·34 ± 0·05	0·33 ± 0·07
Glutamate	0·72 ± 0·12	0·58 ± 0·03	0·45 ± 0·13	0·65 ± 0·11
Glycine	1·98 ± 0·17	2·04 ± 0·20	2·32 ± 0·18	2·59 ± 0·29
Histidine	0·16 ± 0·02	0·19 ± 0·02	0·17 ± 0·02	0·19 ± 0·03
Isoleucine	0·07 ± 0·01	0·06 ± 0·01	0·10 ± 0·02	0·07 ± 0·01
Leucine	0·12 ± 0·02	0·10 ± 0·01	0·16 ± 0·01	0·13 ± 0·01
Lysine	0·67 ± 0·22	0·72 ± 0·13	0·89 ± 0·20	0·83 ± 0·17
Ornithine	0·40 ± 0·07	0·26 ± 0·06	0·19 ± 0·04	0·31 ± 0·06
Phenylalanine	0·10 ± 0·01	0·08 ± 0·01	0·12 ± 0·02	0·13 ± 0·02
Serine	0·24 ± 0·01	0·37 ± 0·06	0·33 ± 0·05	0·31 ± 0·04
Taurine	15·9 ± 0·75	17·0 ± 1·53	20·8 ± 1·40	17·5 ± 1·04
Threonine	0·28 ± 0·05	0·29 ± 0·03	0·30 ± 0·04	0·30 ± 0·04
Tryptophan	0·66 ± 0·09	0·64 ± 0·03	0·73 ± 0·08	0·86 ± 0·05
Tyrosine	0·09 ± 0·01	0·10 ± 0·01	0·14 ± 0·02	0·13 ± 0·02
Valine	0·14 ± 0·02	0·14 ± 0·02	0·21 ± 0·02	0·17 ± 0·02

MUSCLE FREE AMINO ACIDS IN 0·85 ATM OXYGEN

Units are μmoles/g wet weight tissue

Amino Acid	0T	1D**	3D	7D
Alanine	1·56 ± 0·08	1·60 ± 0·17	1·39 ± 0·12	1·34 ± 0·16
Arginine	0·45 ± 0·10	0·55 ± 0·21	0·31 ± 0·08	0·22 ± 0·02
Aspartate	0·38 ± 0·03	0·44 ± 0·07	0·37 ± 0·03	0·30 ± 0·04
Glutamate	0·70 ± 0·06	0·66 ± 0·15	0·64 ± 0·03	0·68 ± 0·27
Glycine	2·75 ± 0·33	2·35 ± 0·39	1·99 ± 0·31	1·40 ± 0·34
Histidine	0·13 ± 0·01	0·21 ± 0·01	0·16 ± 0·02	0·15 ± 0·03
Isoleucine	0·06 ± 0·01	0·08 ± 0·01	0·08 ± 0·01	0·07 ± 0·01
Leucine	0·08 ± 0·03	0·12 ± 0·01	0·15 ± 0·02	0·11 ± 0·01
Lysine	1·08 ± 0·31	1·23 ± 0·04	0·75 ± 0·17	0·87 ± 0·10
Ornithine	0·23 ± 0·03	0·24 ± 0·05	0·26 ± 0·01	0·30 ± 0·05
Phenylalanine	0·06 ± 0·01	0·08 ± 0·03	0·10 ± 0·01	0·08 ± 0·01
Serine	0·25 ± 0·04	0·39 ± 0·07	0·29 ± 0·05	0·36 ± 0·09
Taurine	17·7 ± 0·37	18·8 ± 0·05	19·0 ± 0·60	19·6 ± 1·46
Threonine	0·36 ± 0·06	0·38 ± 0·01	0·24 ± 0·01	0·34 ± 0·10
Tryptophan	0·59 ± 0·09	1·11 ± 0·20	0·70 ± 0·06	0·85 ± 0·12
Tyrosine	0·08 ± 0·01	0·13 ± 0·01	0·13 ± 0·02	0·09 ± 0·02
Valine	0·16 ± 0·02	0·21 ± 0·02	0·15 ± 0·01	0·15 ± 0·02

**n = 2

followed the same trend, but that the trend was not necessarily the same in all samples from any one group. These observations suggested that the major errors lay in changes of sample concentration before analysis. A consideration of the procedure employed suggested that these effects could result from either a differential evaporation of the volatile extractant during the lengthy extraction, a differential evaporation of extractant during storage before the samples were analysed, or differential losses during the evaporation of methanol and resuspension of samples before loading.

In view of these possible defects a correction was applied to these results to normalize them. It was assumed that the majority of amino acids did not alter during exposure to oxygen or that if so, not all of them altered in the same manner. In support of this assumption, Yusa et al (1979) reported that total amino acid levels did not change on exposure to OHP in rabbits. Each amino acid was then expressed as a percentage of the sum of the amino acids from that sample, and the mean percentage of 'total' amino acids ± SEM was calculated. For the summation of 'total' amino acids, the amino acid taurine, and ammonia and urea were excluded because of their high levels compared to other amino acids, and the large variations of these amines between samples. The corrected results appear in tables 10 to 13. The results of the determinations of urea and ammonia are not included in either set of tables because of their extremely large variation. Also, due to being at the limit of detection, methionine was not detectable in all samples and has therefore been omitted.

From the corrected results, it can be seen that in many cases the large variation between the 0·21 atm control groups has been greatly diminished and that the variation in each group has been substantially reduced. These corrections have been particularly

Table 10:

LUNG FREE AMINO ACIDS IN 0·21 ATM OXYGEN

Corrected as % 'total' amino acids

Amino Acid	OT	1D	3D	7D
Alanine	7·83 ± 0·95	8·54 ± 0·76	8·68 ± 0·65	7·89 ± 0·70
Arginine	1·44 ± 0·33	1·95 ± 0·02	1·02 ± 0·32	1·17 ± 0·17
Aspartate	15·9 ± 1·6	13·5 ± 0·81	14·0 ± 0·66	13·1 ± 1·09
Cysteic acid	5·29 ± 1·29	5·26 ± 1·59	3·71 ± 0·61	4·46 ± 0·52
Glutamate	18·1 ± 1·30	16·4 ± 0·60	17·3 ± 1·10	18·1 ± 0·33
Glycine	34·0 ± 0·64	33·4 ± 1·80	36·6 ± 0·54	38·6 ± 0·74
Histidine	0·95 ± 0·11	1·54 ± 0·16	1·05 ± 0·04	1·02 ± 0·21
Isoleucine	0·64 ± 0·11	0·69 ± 0·11	0·71 ± 0·11	1·06 ± 0·28
Leucine	1·27 ± 0·25	1·44 ± 0·16	1·42 ± 0·05	1·37 ± 0·20
Lysine	2·36 ± 0·31	3·35 ± 0·13	2·08 ± 0·20	2·73 ± 0·37
Ornithine	2·98 ± 0·19	3·96 ± 0·30	3·47 ± 0·53	3·39 ± 0·69
Phenylalanine	0·79 ± 0·15	0·95 ± 0·18	0·86 ± 0·12	0·71 ± 0·09
Serine	2·61 ± 0·16	3·13 ± 0·53	2·71 ± 0·27	1·79 ± 0·18
Taurine	84·1 ± 6·6	90·3 ± 6·9	82·0 ± 4·6	88·4 ± 5·5
Threonine	2·89 ± 0·16	3·49 ± 0·86	3·03 ± 0·28	2·80 ± 0·41
Tyrosine	0·81 ± 0·15	1·11 ± 0·12	1·01 ± 0·31	0·94 ± 0·22
Valine	1·59 ± 0·28	1·55 ± 0·10	1·33 ± 0·14	1·61 ± 0·25

LUNG FREE AMINO ACIDS IN 0·85 ATM OXYGEN

Corrected as % 'total' amino acids

Amino Acid	OT	1D	3D	7D
Alanine	7·62 ± 0·57	7·82 ± 0·89	7·86 ± 0·45	10·5 ± 1·60
Arginine	1·22 ± 0·15	1·18 ± 0·18	1·06 ± 0·20	0·87 ± 0·14
Aspartate	17·6 ± 1·84	13·5 ± 0·67	13·2 ± 1·46	13·7 ± 0·46
Cysteic acid	4·70 ± 0·98	4·56 ± 0·10	4·28 ± 1·98	4·96 ± 0·69
Glutamate	17·8 ± 1·20	15·5 ± 0·55	15·7 ± 0·73	26·3 ± 1·44
Glycine	32·2 ± 3·40	30·8 ± 3·10	34·8 ± 1·43	22·8 ± 2·50
Histidine	1·02 ± 0·19	1·17 ± 0·25	1·45 ± 0·27	1·33 ± 0·17
Isoleucine	0·75 ± 0·16	0·54 ± 0·08	0·88 ± 0·09	0·74 ± 0·11
Leucine	1·55 ± 0·16	1·33 ± 0·10	1·76 ± 0·06	1·66 ± 0·18
Lysine	1·83 ± 0·34	2·53 ± 0·27	2·88 ± 0·22	2·90 ± 0·09
Ornithine	3·06 ± 0·19	3·19 ± 0·61	4·73 ± 0·86	2·98 ± 0·58
Phenylalanine	0·90 ± 0·14	1·04 ± 0·14	1·18 ± 0·24	0·80 ± 0·12
Serine	3·33 ± 0·41	3·08 ± 0·45	3·19 ± 0·68	4·11 ± 1·30
Taurine	88·1 ± 4·34	89·3 ± 3·27	75·4 ± 4·91	85·8 ± 7·00
Threonine	3·69 ± 0·49	3·16 ± 0·35	2·89 ± 0·74	3·51 ± 0·45*
Tyrosine	0·81 ± 0·16	0·93 ± 0·18	1·17 ± 0·16	0·79 ± 0·12
Valine	1·67 ± 0·52	1·40 ± 0·21	1·68 ± 0·16	1·34 ± 0·24

Table 11:

BRAIN FREE AMINO ACIDS IN 0·21 ATM OXYGEN

Corrected as % 'total' amino acids

Amino Acid	OT	1D	3D	7D
Alanine	3·98 ± 0·31	4·22 ± 0·26	4·29 ± 0·51	4·64 ± 0·52
Arginine	0·95 ± 0·06	1·10 ± 0·05	0·83 ± 0·08	1·17 ± 0·07
Aspartate	15·7 ± 0·52	14·7 ± 0·92	13·2 ± 0·33	13·2 ± 0·92
Cysteic acid	2·01 ± 0·21	1·91 ± 0·13	1·50 ± 0·10	1·67 ± 0·20
GABA	11·5 ± 0·33	12·3 ± 0·58	10·8 ± 0·24	11·7 ± 0·50
Glutamate	43·2 ± 1·28	39·5 ± 1·73	44·3 ± 1·03	42·0 ± 1·02
Glycine	6·58 ± 0·53	8·69 ± 0·80	7·44 ± 1·16	8·09 ± 0·79
Histidine	0·74 ± 0·02	0·70 ± 0·09	0·60 ± 0·08	0·71 ± 0·07
Isoleucine	0·16 ± 0·01	0·28 ± 0·04	0·24 ± 0·03	0·15 ± 0·03
Leucine	0·28 ± 0·05	0·46 ± 0·02	0·39 ± 0·09	0·48 ± 0·04
Lysine	1·85 ± 0·09	2·04 ± 0·38	1·77 ± 0·16	1·90 ± 0·08
Ornithine	1·37 ± 0·07	1·70 ± 0·42	1·37 ± 0·37	1·77 ± 0·10
Phenylalanine	0·45 ± 0·04	0·57 ± 0·02	0·41 ± 0·08	0·41 ± 0·08
Serine	7·08 ± 0·51	6·33 ± 0·54	7·97 ± 1·45	7·72 ± 0·76
Taurine	47·2 ± 3·06	52·1 ± 1·50	39·0 ± 2·79	45·6 ± 2·61
Threonine	2·60 ± 0·11	3·14 ± 0·45	2·42 ± 0·40	2·37 ± 0·08
Tryptophan	0·58 ± 0·08	0·76 ± 0·11	0·70 ± 0·15	0·64 ± 0·03
Tyrosine	0·56 ± 0·10	0·79 ± 0·12	0·78 ± 0·13	0·43 ± 0·13
Valine	0·44 ± 0·01	0·58 ± 0·01	0·44 ± 0·05	0·62 ± 0·09

BRAIN FREE AMINO ACIDS IN 0·85 ATM OXYGEN

Corrected as % 'total' amino acids

Amino Acid	OT	1D	3D	7D
Alanine	3·60 ± 0·17	4·53 ± 0·19	4·22 ± 0·13	4·11 ± 0·28
Arginine	1·18 ± 0·05	1·18 ± 0·25	0·84 ± 0·05	1·03 ± 0·11
Aspartate	15·5 ± 1·11	12·8 ± 0·47	11·0 ± 0·55	13·4 ± 0·87
Cysteic acid	1·80 ± 0·24	1·87 ± 0·11	1·58 ± 0·11	2·18 ± 0·19
GABA	12·2 ± 0·34	12·4 ± 0·27	13·1 ± 0·31	13·5 ± 0·52
Glutamate	41·6 ± 2·60	43·9 ± 0·89	43·0 ± 1·53	41·8 ± 0·90
Glycine	9·05 ± 0·20	9·43 ± 2·37	7·70 ± 1·63	8·27 ± 0·37
Histidine	0·59 ± 0·08	0·77 ± 0·06	0·83 ± 0·08	0·73 ± 0·14
Isoleucine	0·20 ± 0·07	0·21 ± 0·05	0·25 ± 0·05	0·21 ± 0·03
Leucine	0·46 ± 0·13	0·57 ± 0·10	0·49 ± 0·07	0·55 ± 0·03
Lysine	2·01 ± 0·09	2·04 ± 0·07	2·19 ± 0·10	2·43 ± 0·06
Ornithine	1·36 ± 0·20	1·06 ± 0·21	1·91 ± 0·14	1·57 ± 0·30
Phenylalanine	0·49 ± 0·09	0·62 ± 0·08	0·62 ± 0·06	0·54 ± 0·08
Serine	5·25 ± 0·41	6·87 ± 0·68	6·35 ± 0·59	5·03 ± 0·27
Taurine	42·4 ± 2·82	50·3 ± 4·45	42·5 ± 1·50	44·9 ± 2·32
Threonine	2·58 ± 0·37	2·25 ± 0·39	3·21 ± 0·57	2·27 ± 0·34
Tryptophan	0·72 ± 0·09	1·01 ± 0·07	0·94 ± 0·08	0·88 ± 0·09
Tyrosine	0·62 ± 0·09	0·65 ± 0·04	0·76 ± 0·08	0·69 ± 0·09
Valine	0·67 ± 0·14	0·55 ± 0·03	0·64 ± 0·06	0·55 ± 0·07

Table 12:

LIVER FREE AMINO ACIDS IN 0·21 ATM OXYGEN

Corrected as % 'total' amino acids

Amino Acid	OT	1D	3D	7D
Alanine	26·0 ± 1·52	30·1 ± 3·81	26·1 ± 0·44	34·6 ± 5·54*
Aspartate	3·38 ± 0·40	4·97 ± 0·62	3·29 ± 0·62*	4·81 ± 0·90
Cysteic acid	5·42 ± 1·11	5·13 ± 0·78	10·1 ± 2·68	8·67 ± 2·79
Glutamate	14·0 ± 1·25	16·6 ± 0·53	13·7 ± 1·32	14·6 ± 1·48
Glycine	31·4 ± 8·43	18·6 ± 3·49	17·8 ± 2·41	17·6 ± 2·85
Histidine	3·58 ± 0·09	4·44 ± 0·36	4·01 ± 0·21	5·00 ± 0·47
Isoleucine	1·68 ± 0·09	1·22 ± 0·05	1·31 ± 0·09	1·81 ± 0·25
Leucine	3·33 ± 0·07	2·45 ± 0·11	2·81 ± 0·33	3·37 ± 0·47
Lysine	4·62 ± 0·67	3·29 ± 0·41	3·30 ± 0·19	5·51 ± 0·73
Ornithine	4·31 ± 0·50	3·46 ± 0·54	3·17 ± 0·26	5·68 ± 0·72
Phenylalanine	1·76 ± 0·34	1·41 ± 0·23	1·02 ± 0·08	1·60 ± 0·04
Serine	7·76 ± 1·29	4·19 ± 1·16	5·03 ± 0·67	7·13 ± 2·11
Taurine	76·3 ± 11·9	91·8 ± 7·21	105 ± 8·4	124 ± 18·6
Threonine	2·47 ± 0·34	4·01 ± 0·98	7·03 ± 1·52	4·61 ± 0·45
Tyrosine	1·19 ± 0·14	1·29 ± 0·09	1·33 ± 0·31	1·49 ± 0·12
Valine	3·05 ± 0·06	2·04 ± 0·05	2·13 ± 0·19	3·30 ± 0·30

* n = 3

LIVER FREE AMINO ACIDS IN 0•85 ATM OXYGEN

Corrected as % 'total' amino acids

Amino Acid	OT	1D	3D	7D
Alanine	21•9 ± 1•40	25•3 ± 0•57	25•2 ± 2•18	28•1 ± 2•76
Aspartate	4•04 ± 0•44	4•41 ± 0•21	5•73 ± 0•76	4•69 ± 1•24
Cysteic acid	3•96 ± 0•51	6•59 ± 0•89	5•28 ± 1•03	4•14 ± 1•10
Glutamate	14•2 ± 1•72	12•0 ± 0•74	15•2 ± 2•30	17•1 ± 2•60
Glycine	21•3 ± 1•58	20•1 ± 2•99	16•7 ± 2•93	15•4 ± 1•47
Histidine	3•20 ± 0•37	3•91 ± 0•20	4•48 ± 0•25	4•67 ± 0•96
Isoleucine	1•78 ± 0•07	1•14 ± 0•11	1•31 ± 0•08	0•93 ± 0•22
Leucine	3•08 ± 0•27	2•35 ± 0•23	2•61 ± 0•23	2•09 ± 0•21
Lysine	4•64 ± 0•19	3•91 ± 0•26	4•57 ± 0•53	4•41 ± 0•33
Ornithine	5•21 ± 0•27	5•03 ± 0•65	4•60 ± 0•73	4•27 ± 0•14
Phenylalanine	1•44 ± 0•09	1•32 ± 0•14*	1•23 ± 0•04	1•19 ± 0•09
Serine	5•95 ± 1•59	5•75 ± 0•37	7•21 ± 1•67	4•19 ± 0•99
Taurine	101 ± 18•0	114 ± 15•4	100 ± 19•6	92 ± 21•3
Threonine	4•44 ± 0•48	3•47 ± 0•27	2•59 ± 0•49	4•51 ± 1•71
Tyrosine	1•30 ± 0•13	0•96 ± 0•14*	1•21 ± 0•10	1•18 ± 0•18
Valine	3•14 ± 0•29	2•23 ± 0•25	2•48 ± 0•15	1•85 ± 0•20

Table 13:

MUSCLE FREE AMINO ACIDS IN 0·21 ATM OXYGEN

Corrected as % 'total' amino acids

Amino Acid	OT	1D	3D	7D
Alanine	16·6 ± 1·41	21·6 ± 0·55	18·7 ± 0·74	18·8 ± 0·45
Arginine	4·13 ± 1·18	3·88 ± 0·66	5·58 ± 0·98	3·98 ± 0·52
Aspartate	5·24 ± 0·63	3·78 ± 0·72	3·90 ± 0·44	3·58 ± 0·63
Glutamate	9·26 ± 1·40	7·39 ± 0·49	7·97 ± 1·03	7·00 ± 0·51
Glycine	25·6 ± 2·20	25·7 ± 1·20	27·4 ± 1·00	28·6 ± 2·30
Histidine	2·10 ± 0·23	2·34 ± 0·18	2·04 ± 0·15	2·07 ± 0·30
Isoleucine	0·89 ± 0·12	0·79 ± 0·07	1·22 ± 0·18	0·81 ± 0·07
Leucine	1·60 ± 0·21	1·27 ± 0·17	1·67 ± 0·21	1·40 ± 0·14
Lysine	8·58 ± 2·81	8·79 ± 0·92	10·2 ± 1·84	7·77 ± 1·82
Ornithine	5·25 ± 1·02	3·21 ± 0·49	2·37 ± 0·60	3·37 ± 0·50
Phenylalanine	1·31 ± 0·19	1·07 ± 0·10	1·36 ± 0·17	1·28 ± 0·29
Serine	3·06 ± 0·09	4·63 ± 0·50	3·71 ± 0·34	3·38 ± 0·33
Taurine	205 ± 13	215 ± 8	246 ± 13	205 ± 7
Threonine	3·60 ± 0·75	3·61 ± 0·26	3·49 ± 0·35	3·28 ± 0·24
Tryptophan	8·44 ± 0·99	8·26 ± 0·74	8·35 ± 0·93	9·61 ± 0·88
Tyrosine	1·17 ± 0·15	1·30 ± 0·22	1·67 ± 0·17	1·44 ± 0·09
Valine	1·78 ± 0·19	1·74 ± 0·20	2·40 ± 0·12	1·91 ± 0·12

MUSCLE FREE AMINO ACIDS IN 0·85 ATM OXYGEN

Corrected as % 'total' amino acids

Amino Acid	OT	1D**	3D	7D
Alanine	17·6 ± 0·86	16·3 ± 0·45	17·9 ± 0·59	18·5 ± 1·50
Arginine	5·02 ± 1·07	5·47 ± 1·70	3·91 ± 0·80	3·03 ± 0·12
Aspartate	4·29 ± 0·42	4·54 ± 1·02	4·83 ± 0·60	4·17 ± 0·45
Glutamate	7·94 ± 0·39	6·85 ± 2·00	8·44 ± 0·88	8·68 ± 2·89
Glycine	33·7 ± 3·8	23·7 ± 2·1	25·2 ± 2·3	18·9 ± 3·6
Histidine	1·44 ± 0·21	2·13 ± 0·10	2·12 ± 0·25	2·06 ± 0·40
Isoleucine	0·71 ± 0·06	0·86 ± 0·18	0·97 ± 0·02	0·99 ± 0·11
Leucine	0·90 ± 0·28	1·26 ± 0·15	1·99 ± 0·22	1·55 ± 0·17
Lysine	12·1 ± 3·15	12·3 ± 2·68	9·32 ± 1·68	11·8 ± 0·34
Ornithine	2·52 ± 0·25	2·51 ± 0·70	3·93 ± 0·52	4·18 ± 0·72
Phenylalanine	0·70 ± 0·13	0·76 ± 0·20	1·30 ± 0·16	1·15 ± 0·11
Serine	2·82 ± 0·31	3·99 ± 0·36	3·62 ± 0·37	4·87 ± 0·95
Taurine	202 ± 15	192 ± 10	248 ± 18	276 ± 29
Threonine	3·38 ± 0·56	3·35 ± 0·29	3·16 ± 0·19	3·49 ± 0·15*
Tryptophan	6·67 ± 1·02	11·6 ± 2·91	9·43 ± 1·45	11·9 ± 1·59
Tyrosine	0·83 ± 0·08	1·36 ± 0·23	1·69 ± 0·12	1·19 ± 0·17
Valine	1·83 ± 0·13	2·14 ± 0·03	1·96 ± 0·20	2·02 ± 0·11

** n = 2

* n = 3

effective in the lung samples where the results were the most variable. In examining the results from this experiment, it was decided that for any amino acid to show a notable change at any point, that point should be different not only from its initial control and its respective control exposed to 0·21 atm oxygen, but also from any other control point from the 0·21 atm exposure. On this basis the amino acids showing evidence of changes have been expressed graphically in figure 22 to 31. These amino acids were the branched chain amino acids valine, leucine and isoleucine in liver, glycine in lung and muscle, glutamate in lung and GABA in brain. For all of these results both the corrected and uncorrected results have been presented to demonstrate the effects of the correction procedure.

The changes in the branched chain amino acids valine, leucine and isoleucine shown in figure 22 all appear only after 7 days exposure to oxygen. In each case the 7 day values showed a decrease, and were significantly different from the initial controls and from the 7 day control, but not the 1 day and 3 day controls. However, in each case the close agreement between the controls and their respective experimental points lent greater credence to the divergence occurring between the controls and experimental points at day 7. Also in this instance, the uncorrected results in figure 23 were very similar to the corrected values in figure 22.

The change in lung glutamate (figure 24) also occurred on day 7 but in this case there was an increase. This was the only change which was statistically significant against all control points in the corrected results. In the uncorrected results (figure 27) the experimental points showed a similar trend, but the variation in the control points was such that the significance of this change was almost completely obscured.

Legend to Figures 22 to 31

Changes in Free Amino Acid Pools in Lung, Liver, Brain and Muscle of Mice Exposed to 0·85 atm Oxygen

The amino acid levels were determined as described in the materials and methods section 3.D. iii).

The x axis has units of days and the y axis has the units, % of 'total' amino acids, in figures 22, 24, 25, 26, and 30, and units of umoles/g wet weight tissue, in figures 23, 27, 28, 29, and 31. Each graph shows the results of both the 0·85 atm experimental points (Ɨ) and the 0·21 atm control points (Ɨ) for that amino acid: the lines joining the former are thick and the lines joining the latter points are thin. Both the corrected and uncorrected results are presented as means ± SEM. The lines connecting each point are positioned correctly with respect to the x axis, but in some cases the points and error bars have been slightly displaced laterally for clarity. The number of observations is 4 for each point except where indicated otherwise in figures 26 and 29. The P values show the probability that the value indicated is identical to the 7 day 0·85 atm value for that graph using a Student's 't' test (two tailed).

FIGURE 22

FREE AMINO ACIDS: CORRECTED AS A % OF TOTAL FREE AMINO ACIDS

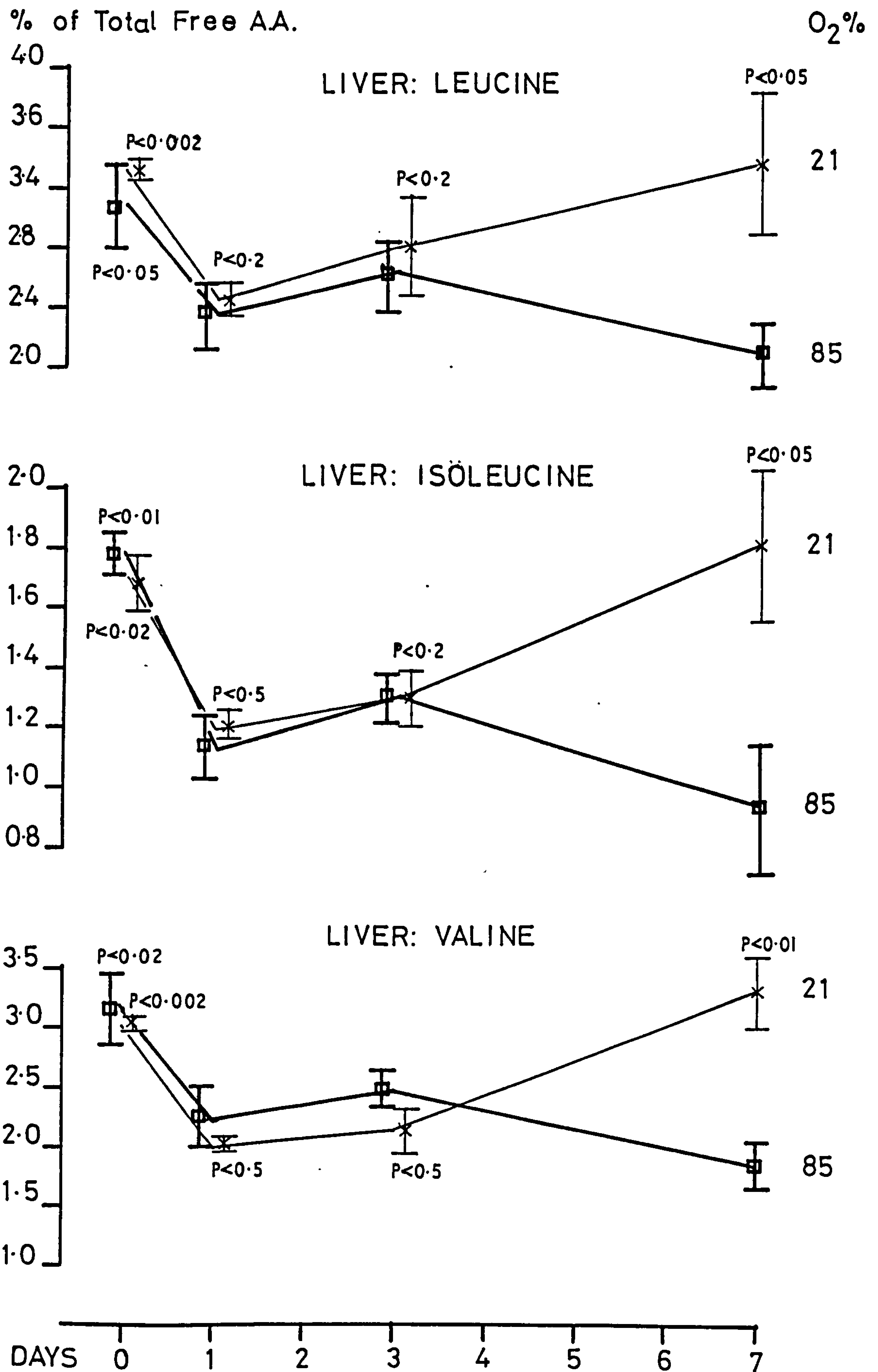
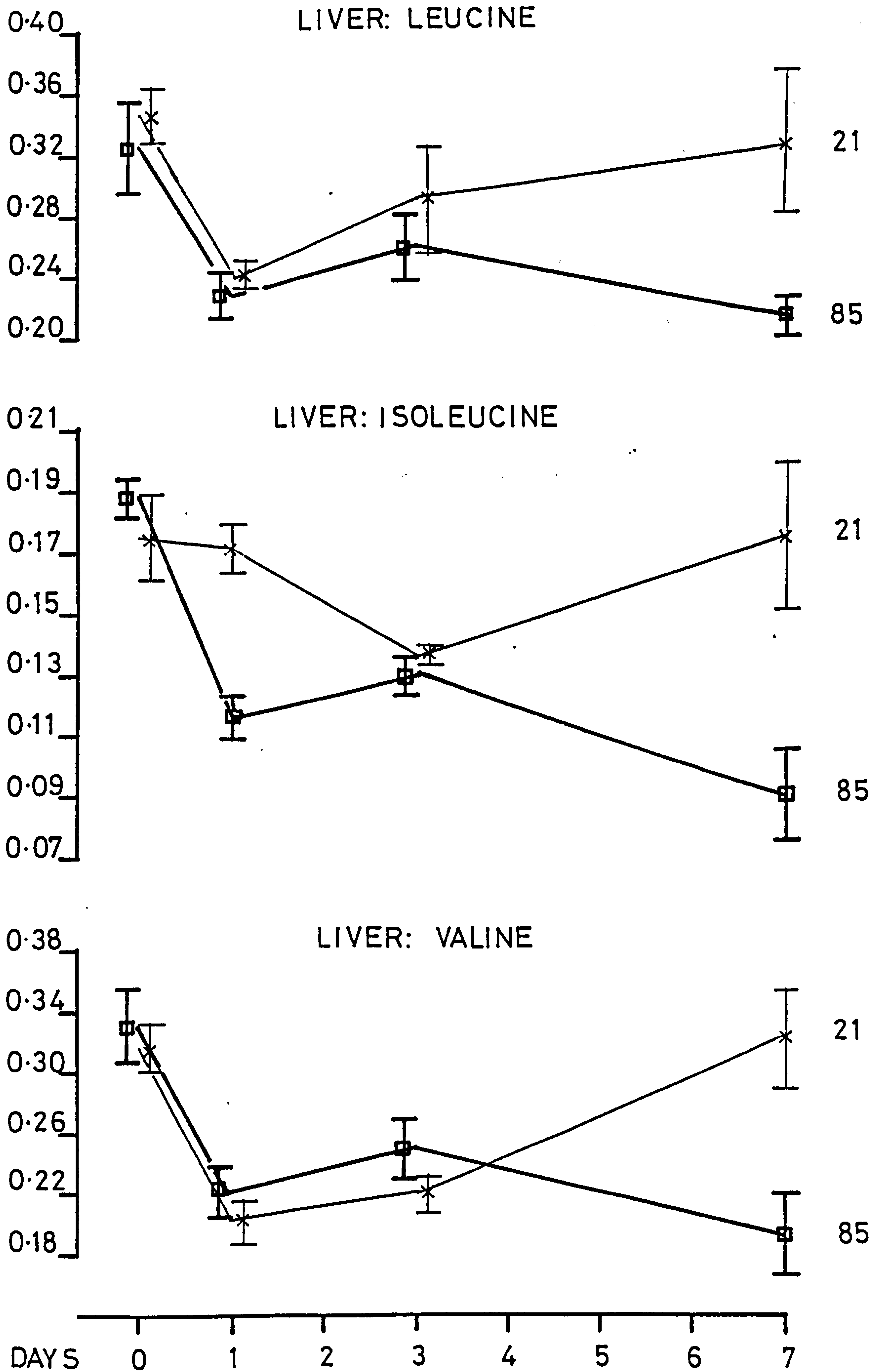
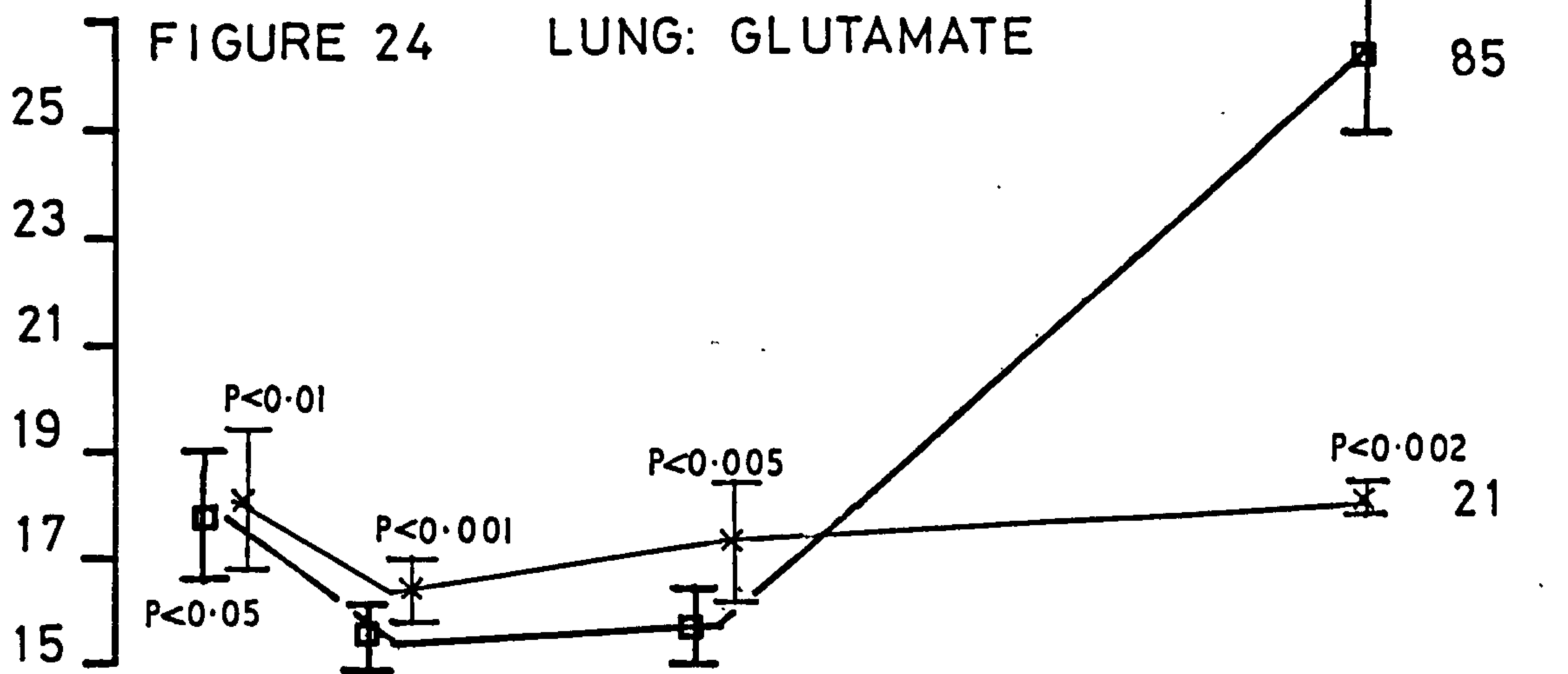
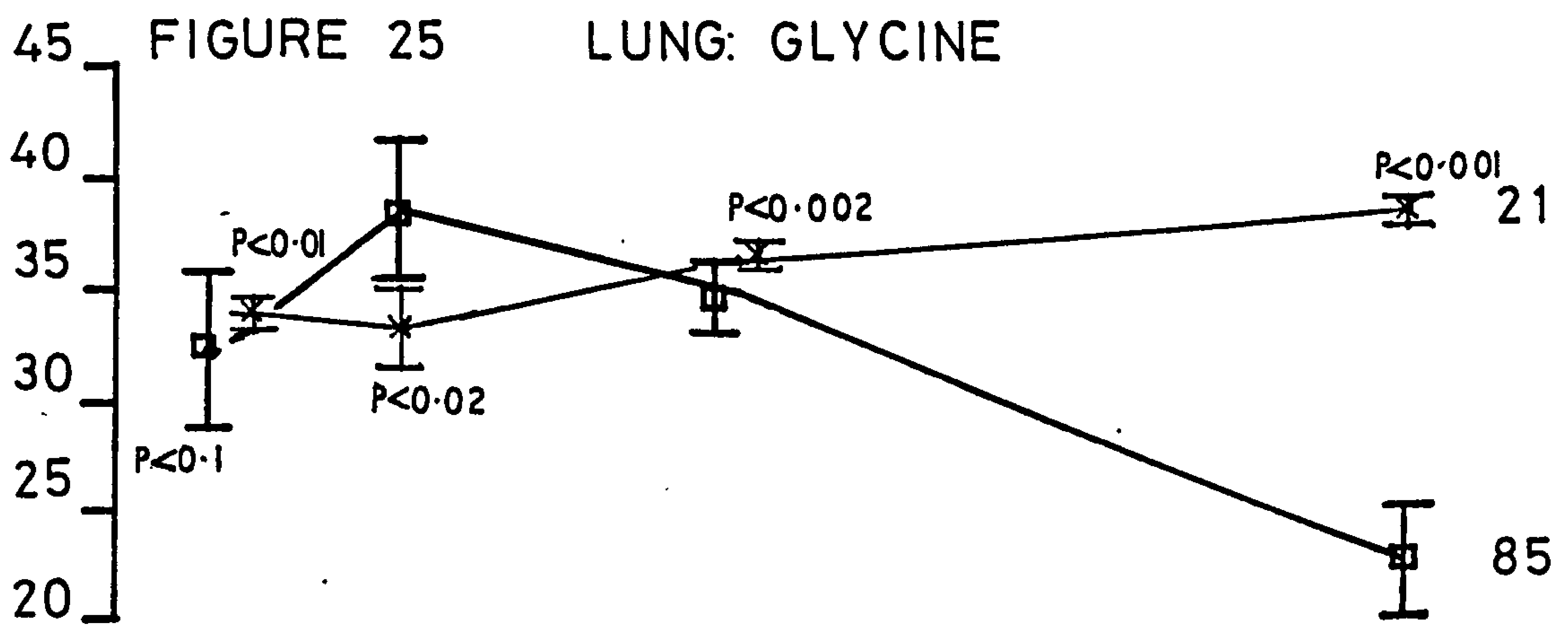


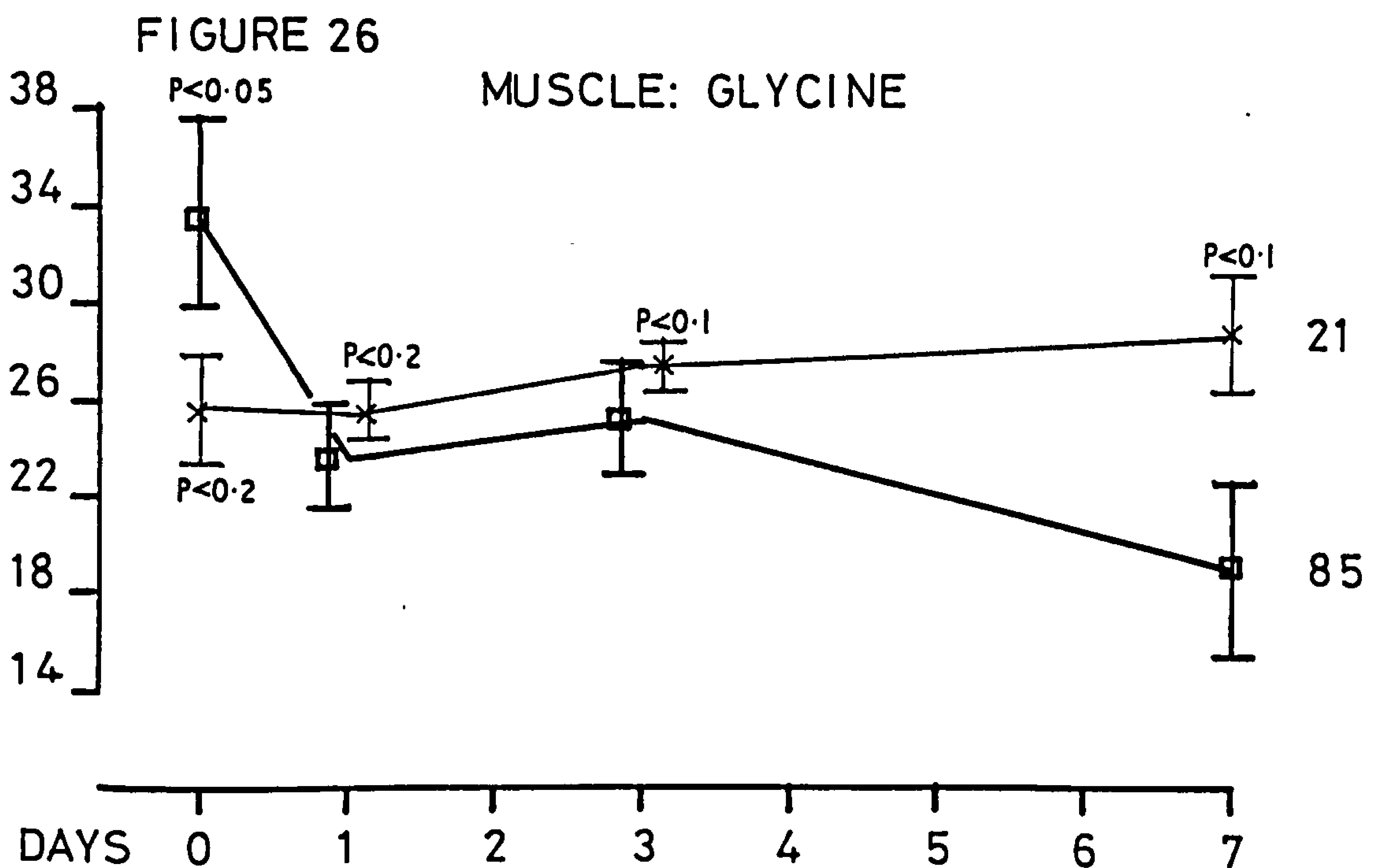
FIGURE 23



FREE AMINO ACIDS: CORRECTED AS A % OF TOTAL FREE AMINO ACIDS

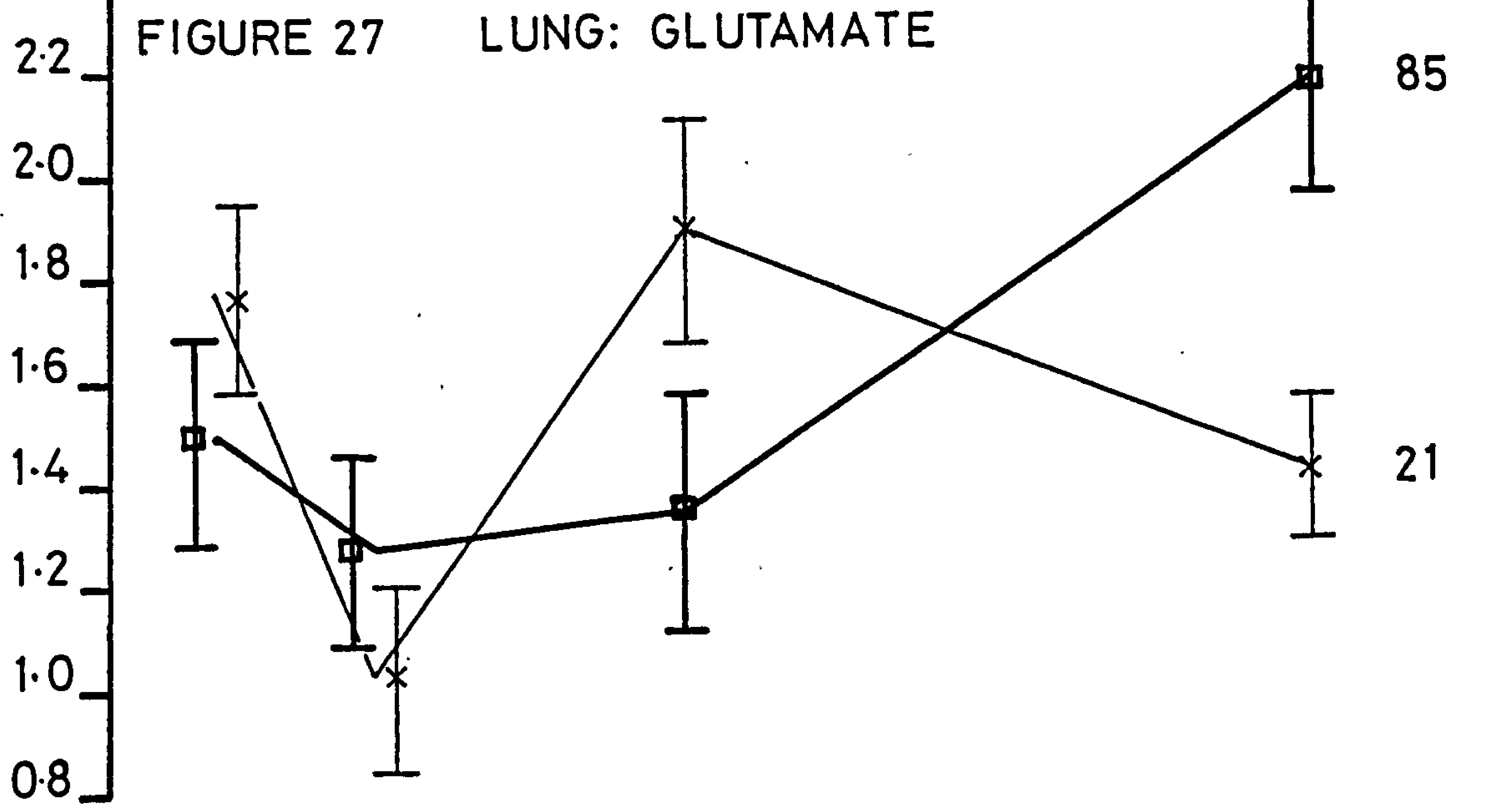


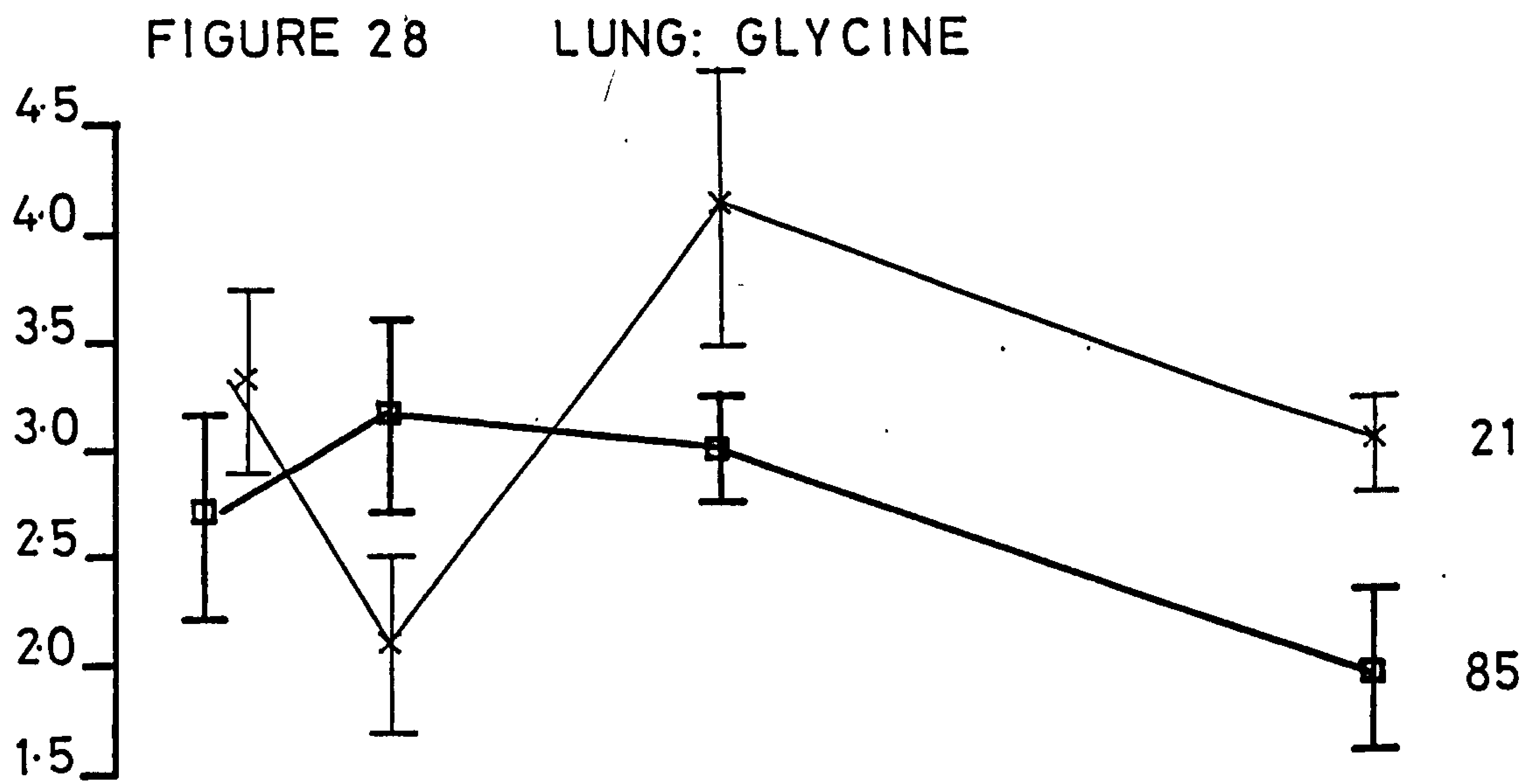


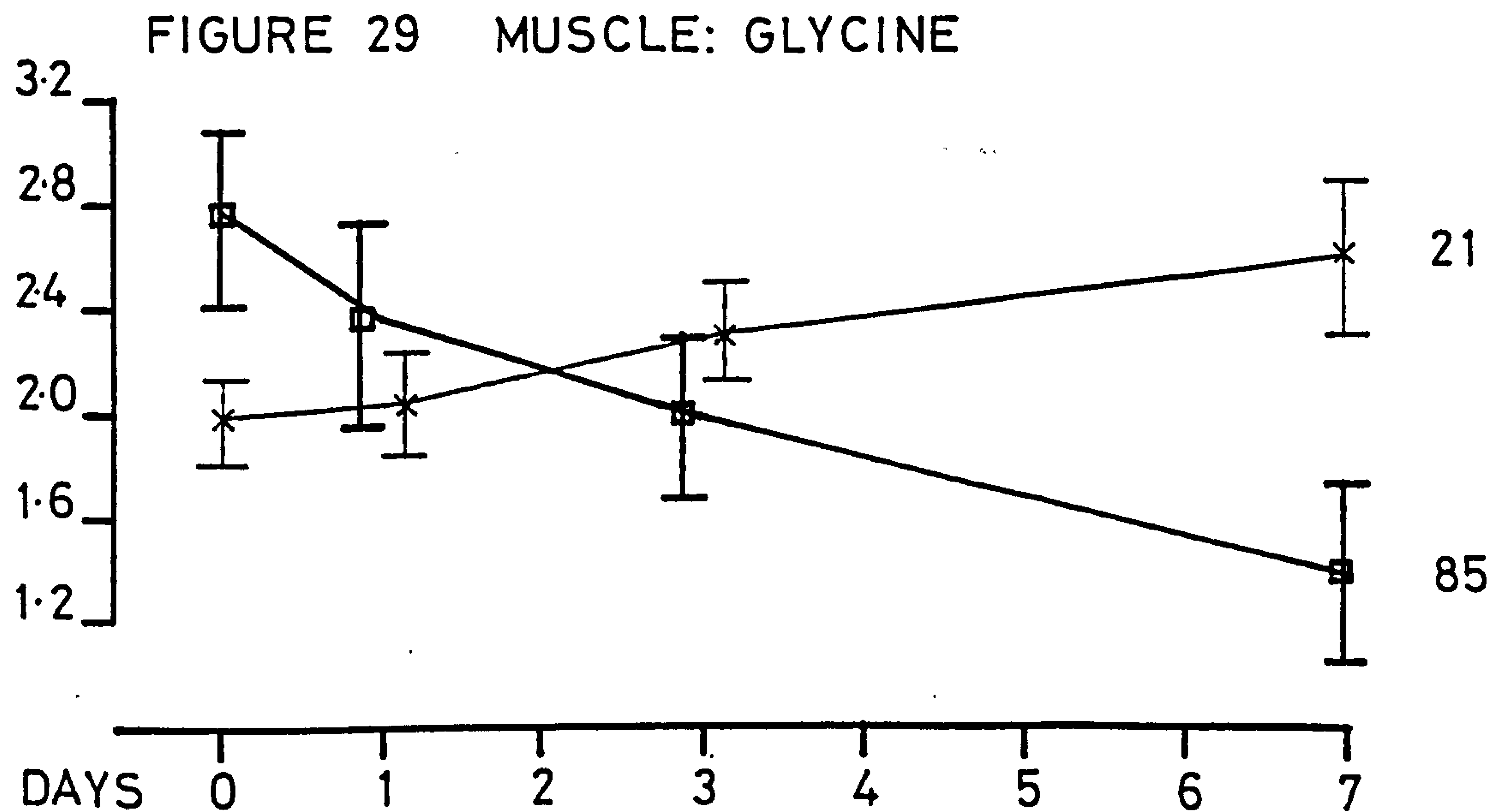


FREE AMINO ACIDS: UNCORRECTED

µmoles/g Wet Wt. Tissue O_2%







FREE AMINO ACIDS: BRAIN GABA

FIGURE 30

CORRECTED AS A % OF TOTAL FREE AMINO ACIDS

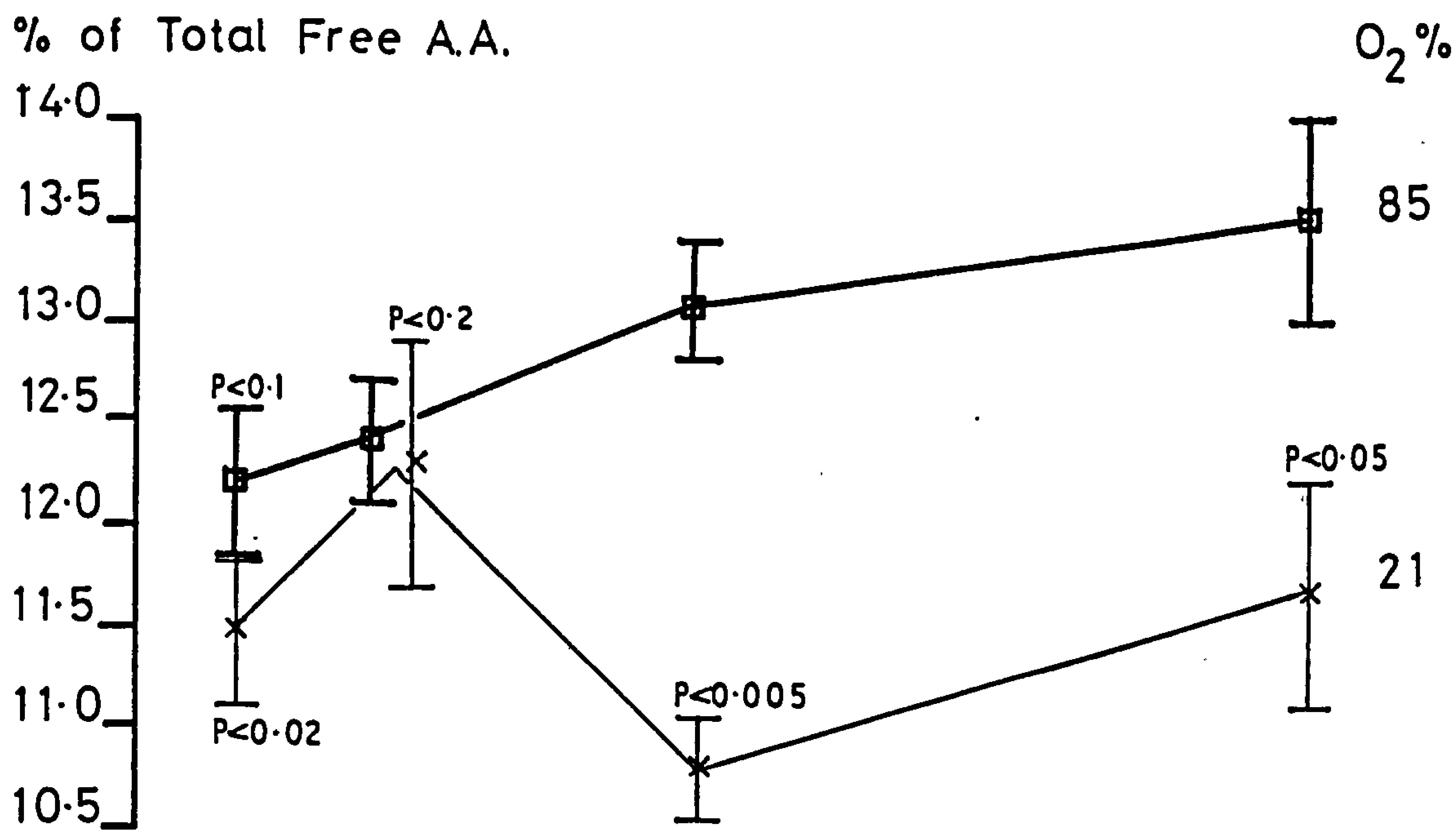
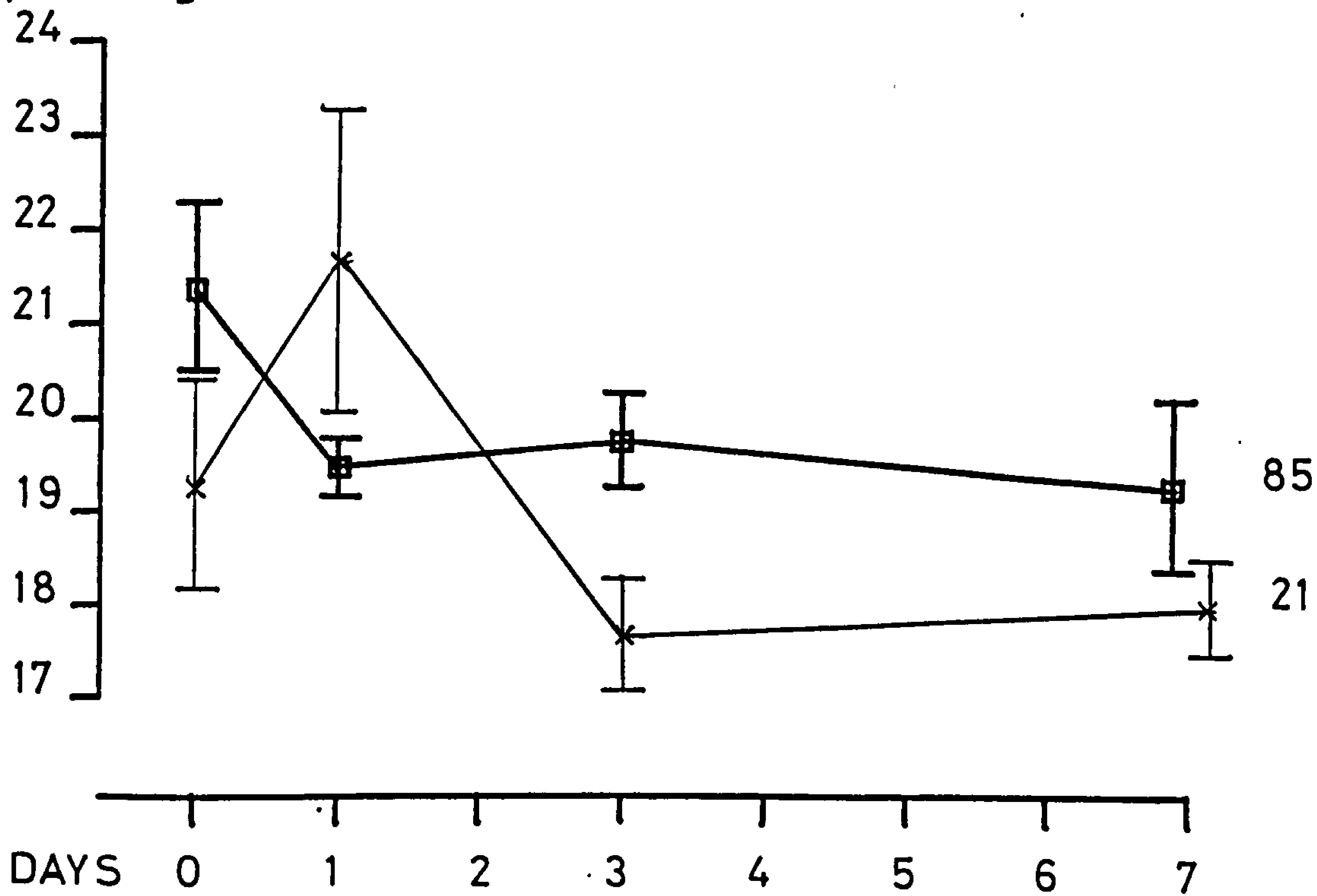


FIGURE 31 UNCORRECTED



Changes were also seen in lung and muscle glycine (figures 25 and 26): these changes were again only seen on day 7 and were again decreases. The change in lung glycine was statistically significant against all groups in the 0·21 atm controls but did not reach statistical significance at the P = 0·05 level against the 0·85 atm zero time control. The change seen in muscle glycine (figure 26) followed a similar trend to that in lung glycine, however, statistical significance was achieved only against the 0·85 atm zero time control. The uncorrected results of lung and muscle glycine shown in figures 28 and 29 followed the same trend as the corrected results in the experimental groups, but the variation, particularly in the controls, was again large.

The final change noted was that GABA levels in the brain (figure 30) increased on exposure of the animals to 0·85 atm oxygen in the corrected results. Although the final reading taken after 7 days exposure was significantly different from most of the 0·21 atm controls, both the 1 day 0·21 atm control and the 0·85 atm zero time control were not statistically different from this value. Also, this amino acid did not show an underlying trend in the uncorrected experimental results (figure 31) towards an increase in GABA concentration.

C. Discussion

The branched chain amino acids are all essential amino acids, therefore a decrease of these amino acids in liver must represent either an inadequate dietary supply, an activation of the enzymes catalysing their degradation, or a change in protein metabolism (either an increased synthesis or a decreased breakdown of proteins containing these amino acids). Although the mice used in this study ate very little after about the third day, similar decreases in the

branched chain amino acids were not seen in other tissues, neither were there decreases in other essential amino acids. Enzyme activation due to oxygen seems an unlikely mechanism, suggesting a decrease in protein breakdown. In view of the emaciated condition of the animals, the decrease in the size of the liver by 7 days exposure to oxygen, and the changes in protein metabolism reported in the previous chapter, this explanation appears to be the most likely.

A decrease in glycine levels would be more likely to arise from a reduction rather than an increase in glycine formation, due as noted above, to the unlikelihood of an enzyme activation by oxygen. Although glycine oxidase could conceivably be activated by oxygen, this is a liver enzyme, and the changes in glycine were noted only in muscle and lung. The decrease in glycine could result from a decrease in the degradation of serine or threonine, or the transamination of glyoxalate to glycine utilising glutamate or alanine. Because of the low levels of serine and threonine in both lung and muscle, the former two possibilities seem less likely, but in the latter case the decrease could arise either from an inactivation of glycine aminotransferase or a reduced availability of glyoxalate.

The increase in lung glutamate noted after 7 days exposure could again arise from either an increased synthesis or a decreased utilisation. In the case of glutamate, most of its reactions are as a result of its central position in amine transfer from its α-amino group. The amino acids proline, ornithine, citrulline and arginine which are derived from the carbon skeleton of glutamate have a relatively small concentration compared to the change recorded in glutamate concentration. A more likely reason for the increase in glutamate would be a blockage in the citric acid cycle at or beyond the α-oxoglutarate dehydrogenase step, resulting in an accumulation of α-oxoglutarate and consequent transamination to glutamate.

The changes seen in brain GABA concentration in the corrected results were only significant against 3 out of the 5 controls and were not indicated in the uncorrected results. In addition, for the assay of a compound which has been proposed as a neurotransmitter (Krnjević 1970), the brain tissue should have been frozen rapidly using liquid notrogen to obtain true values of the *in vivo* concentrations. In view of these comments there must be some doubt as to the authenticity of this result. However, despite the decrease in GABA concentration reported when rats were subjected to OHP (Wood & Watson 1963) an increase in GABA has been reported in mice exposed to 1·0 atm oxygen (Schäfer 1978). The study by Schäfer showed a significant increase in GABA levels occurred within 30 minutes of exposure continuing until a maximum value was reached at 50 hours, followed by a rapid decrease to a plateau which was about 11% higher than control values. Although the results reported in this chapter do not follow the same trends reported by Schäfer, it is interesting to note that the corrected values obtained after 3 and 7 days exposure were about 11% above the 0·85 atm control values. It has been reported that both anoxia and hypoxia increase levels of GABA in the brain (Folbergrova *et al* 1974; Thorn *et al* 1973), but how this relates to an increase in GABA caused by hyperoxia is unknown.

Schäfer and Citoler (1978) have reported that mice exposed to 1·0 atm oxygen for 50 hours show a rapid development of severe pulmonary dysfunction which correlates with an impairment of pulmonary diffusing capacity, a steep decrease in blood oxygen tension and an increased carbon dioxide accumulation. In the present study a slightly lower oxygen concentration was employed and the gross alterations in lung appearance did not occur so quickly, but were apparent before 7 days exposure. The results of Schäfer & Citoler applied to the present study would therefore tend to indicate that the changes seen

in amino acid levels which all occurred after 7 days exposure, arose not as a result of hyperoxia but hypoxia. The slight recovery of experimental animals at about day 6 followed by a deterioration the following day might therefore indicate the point at which animals pass from a hyperoxic to a hypoxic condition. If this was indeed the case, it could be said that mammalian amino acid metabolism is relatively unaffected by chronic hyperoxia. This explanation may also be of relevance to the observation of Huggins & Nelson (1975) that the branched chain amino acids leucine and valine increased in mouse brain after OHP exposure, whereas in this study a decrease in liver was demonstrated.

Chapter 3: DETERMINATION OF THE EFFECTS OF PROSTAGLANDIN INHIBITORS ON OXYGEN TOXICITY, MONITORED BY SURVIVAL TIME, LUNG % WATER CONTENT, WHOLE BODY, LUNG, AND LIVER WEIGHTS

A. Introduction

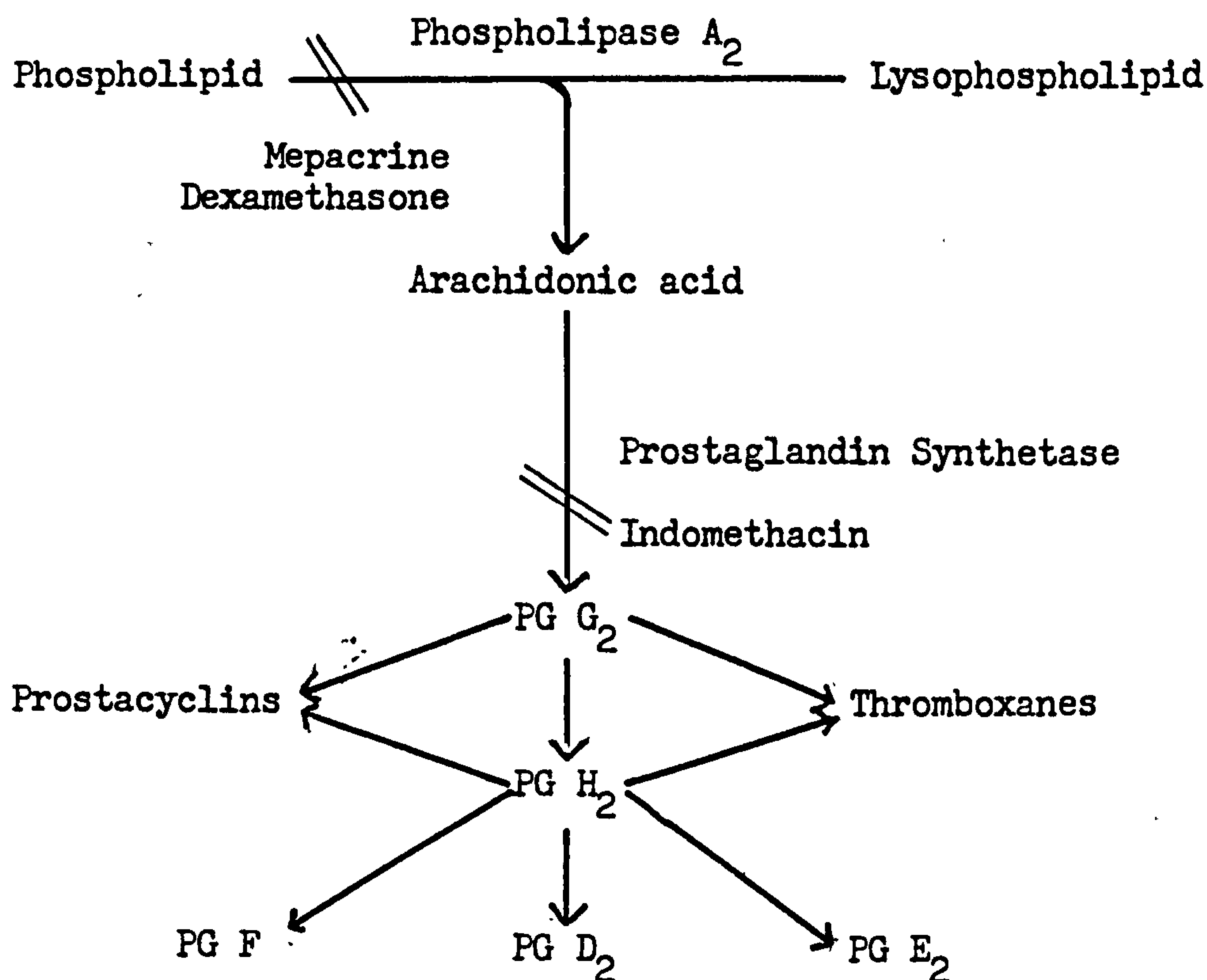
Prostaglandins are synthesised and metabolised in the lung, and their physiological activity can be modified during passage through the pulmonary circulation. Prostaglandin (PG) $F_{2\alpha}$ is the predominant prostaglandin in the lung but E series prostaglandins have also been noted. Prostaglandins of the F series appear to constrict pulmonary bronchial and smooth muscle whereas those of the E series tend to relax these muscles (Fanburg 1973). The importance of the lung in prostaglandin metabolism is shown by the study of Piper *et al* (1970) where it was shown that prostaglandins were inactivated at all concentrations which might be encountered *in vivo* in a single passage through the lungs. Klein *et al* (1978) similarly showed that an average of 93% of prostaglandins at a concentration of 5 nM were metabolised in a single passage through an isolated perfused lung.

In addition to the role of the lung in both prostaglandin metabolism and oxygen toxicity, there is some evidence that prostaglandin synthetase, the enzyme catalysing the formation of PG G_2 from arachidonic acid, acts by an oxygen radical mechanism (Yoshimoto *et al* 1970; Panganamala *et al* 1974b; Marnett *et al* 1974). These findings suggested that prostaglandin metabolism may be affected by oxygen toxicity.

A simple experiment was therefore devised to investigate whether a reduction of the prostaglandin levels would affect the health and survival of mice kept in a toxic oxygen environment. The three prostaglandin synthesis inhibitors mepacrine, dexamethasone

and indomethacin were employed, all of which exert their effects by a different mechanism, so that any side effects should only be seen from one of the groups, whereas any effects arising from alterations in prostaglandin metabolism should occur in all three groups.

Figure 32: Simplified Scheme Showing Synthesis of Prostaglandins



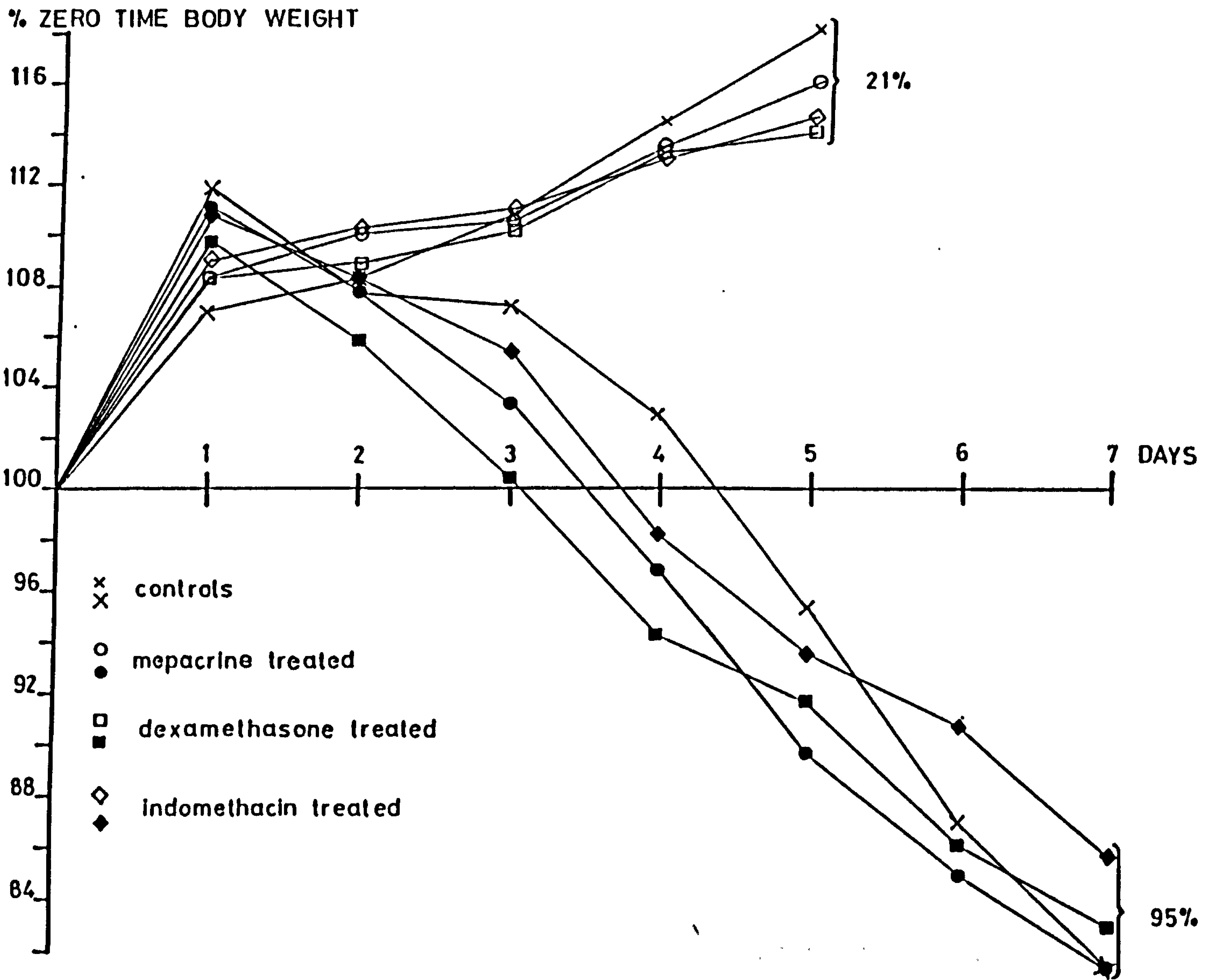
Mepacrine and dexamethasone both inhibit prostaglandin synthesis at the phospholipase A_2 step where arachidonic acid is liberated from phospholipids. Although both of these inhibitors affect the same metabolic point, they do not function by the same mechanism. Mepacrine inhibits arachidonic acid release in both whole tissue and disrupted tissue whereas dexamethasone will inhibit in whole tissue, but fails to show an effect in cell free homogenates (Flower 1978). Indomethacin inhibits the prostaglandin synthetase complex Flower & Blackwell 1976). See figure 32 for the relationships of these reactions to prostaglandin synthesis.

B. Results

The format of this experiment, described in the materials and methods section 3.D. iv), follows essentially that of the previous experiment but with the exception that the 0·21 atm controls were monitored at the same time as the experimental mice and were kept in the animal house, not the oxygen chamber. The pressure of oxygen employed in this experiment was 0·95 atm not 0·85 atm as in all other experiments. The reason for this choice, was to increase the severity of the toxicity so that the lifespan of the mice was reduced, facilitating the use of 'survival time' as an indicator of the effect of the prostaglandin inhibitors. Each mouse was injected once daily with its prescribed mixture at the same time of day. It was necessary to remove the experimental animals from the chamber to inject them and therefore this period was kept to a minimum: usually 30 to 45 minutes. In discussing the results of this chapter, the animals kept at 0·21 atm will be referred to as the 'control' animals, and those kept at 0·95 atm as the 'experimental' animals. The groups (both control and experimental) injected with the control carrier mixture will be referred to as the 'untreated' group, and the groups injected with any of the prostaglandin inhibitors will be designated as 'treated' groups.

During the course of this experiment, it was noted that many of the animals were thrown into a convulsive fit immediately prior to their death. Typically the fit lasted approximately 15 to 30 seconds. The most common period when deaths occurred was at the time the mice were removed from the chamber for injection, although deaths did occur at other times. Notes were made on the appearance of the lungs of all 'experimental' and 'control' animals at death or sacrifice. It was revealed that, with only two exceptions, all the control lungs were of a pink, healthy appearance, both the exceptions occurring in

FIGURE 33: GROWTH PROFILE OF MICE LIVING IN 95% AND 21% OXYGEN



the dexamethasone 'treated' group, one pair of lungs showing a minor abnormality in a small lobe, and the other showing several bloody patches and being of a brownish colour instead of pink. Half of the 'experimental' animals had lungs of a liver-like consistency upon death, two in each of the 'untreated' and indomethacin 'treated' groups, three in the mepacrine 'treated' group and only 1 in the dexamethasone 'treated' group. The remaining animals all showed gross abnormalities of the lungs, usually oedema, patches of haemorrhage and often fluid in the lungs. All other observations of behaviour etc. were very similar to those reported in chapter 1. The control mice all appeared healthy and vigorous throughout the experimental period.

It was originally intended that the control mice should be sacrificed, and the measurements on these mice performed when approximately 50% of the 'experimental' animals had succumbed to the effects of the oxygen. This was expected to occur after about 4 days exposure. After 5 days exposure, this point had still not been reached, but many of the mice appeared to be in very poor condition, and were not expected to survive much longer, therefore the 'control' mice were sacrificed at this time. In fact, the time taken for 50% of the 'experimental' animals to die occurred during the following day.

The growth profile of both 'experimental' and 'control' groups is presented in figure 33, and was constructed from the means of the % difference of total body weight at those points from the initial total body weight. It was seen from this figure that the 'experimental' animals gained more weight during the first 24 hours than the 'control' animals, but whereas the control groups continued to grow steadily during the rest of the experiment, the 'experimental' animals showed a linear decline in total body weight from that point

onwards. It should be noted that among both the 'experimental' and 'control' animals the 'untreated' groups fared the best in terms of total body weight. The reading from the 'experimental' animals shown in figure 33 started to become distorted after day 5 due to the increasing number of deaths in each group.

Figure 34 shows the lifespan of the mice in each of the 'experimental' groups, each step of the bar graph representing one mouse. The time of death of the three mice dying overnight, was given as the average of the time last seen alive and the time found dead. It can be seen from this graph that all of the experimental 'treated' groups had a mean survival time of about 165 hours, whereas the 'untreated' group showed a mean survival time of about 135 hours. Due to the large variation between individuals and the small number of animals in each group, the difference between the 'untreated' and 'treated' groups was not statistically significant in any case.

The differences in the total body weights of animals subjected to 0·95 atm oxygen were very significantly different from the total body weights of animals kept in 0·21 atm oxygen (figure 35). There were only very small differences between the 'untreated' and 'treated' 'control' groups, but all of the 'experimental' 'treated' groups had lower total body weights than the 'experimental' 'untreated' group. This difference is only statistically significant between the mepacrine 'treated' group and the 'untreated' group. Although figure 35 demonstrated the differences in whole body weight at death, it should be noted that the 'treated' groups survived longer than the 'untreated' group, and that this accounts for some of the difference.

It was noted in previous experiments, that in addition to a decline in total body weight, there was also a decline in liver weight. The changes in liver weight were also determined in this

FIGURE 34:
LIFESPAN OF MICE IN 95% OXYGEN

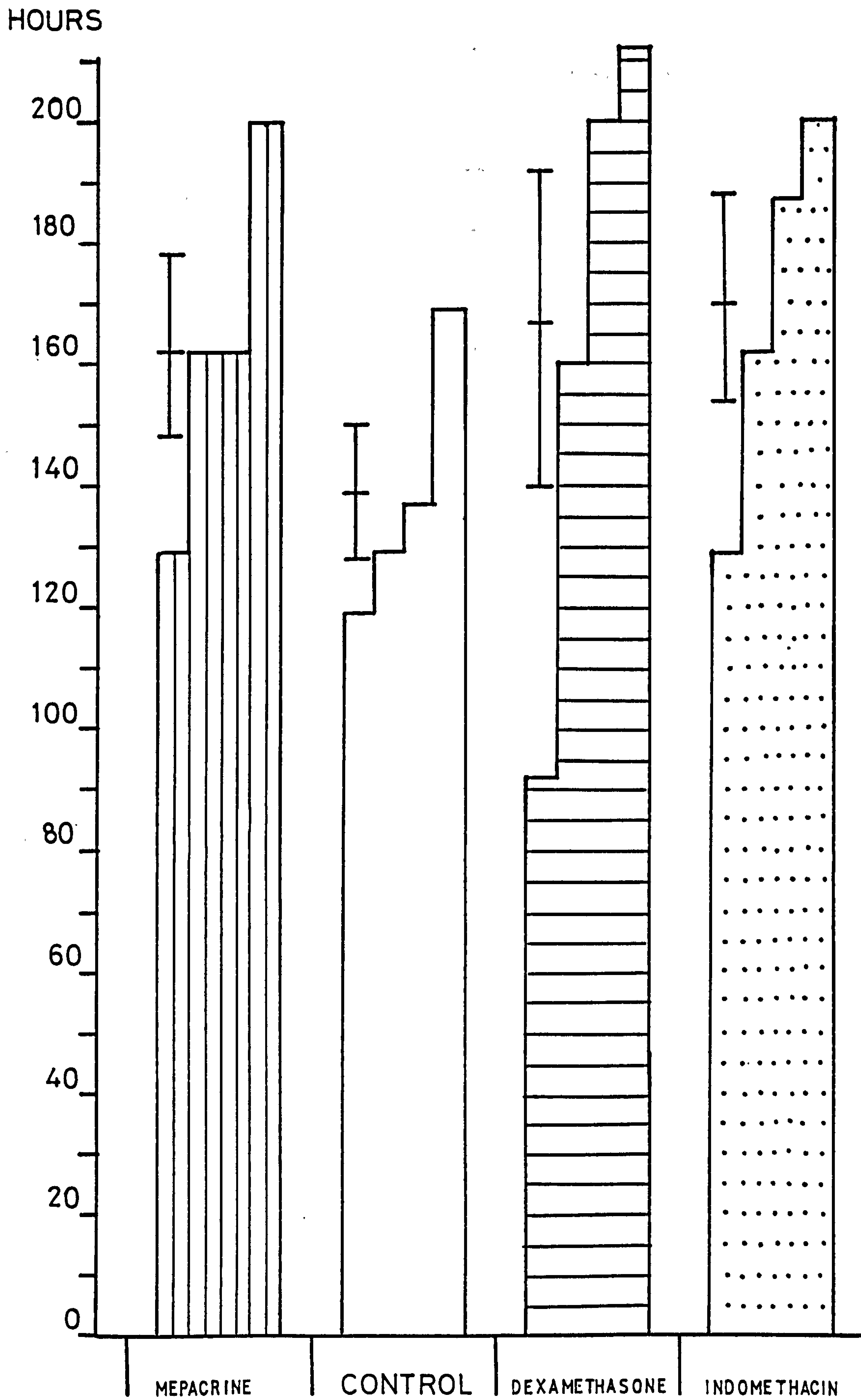
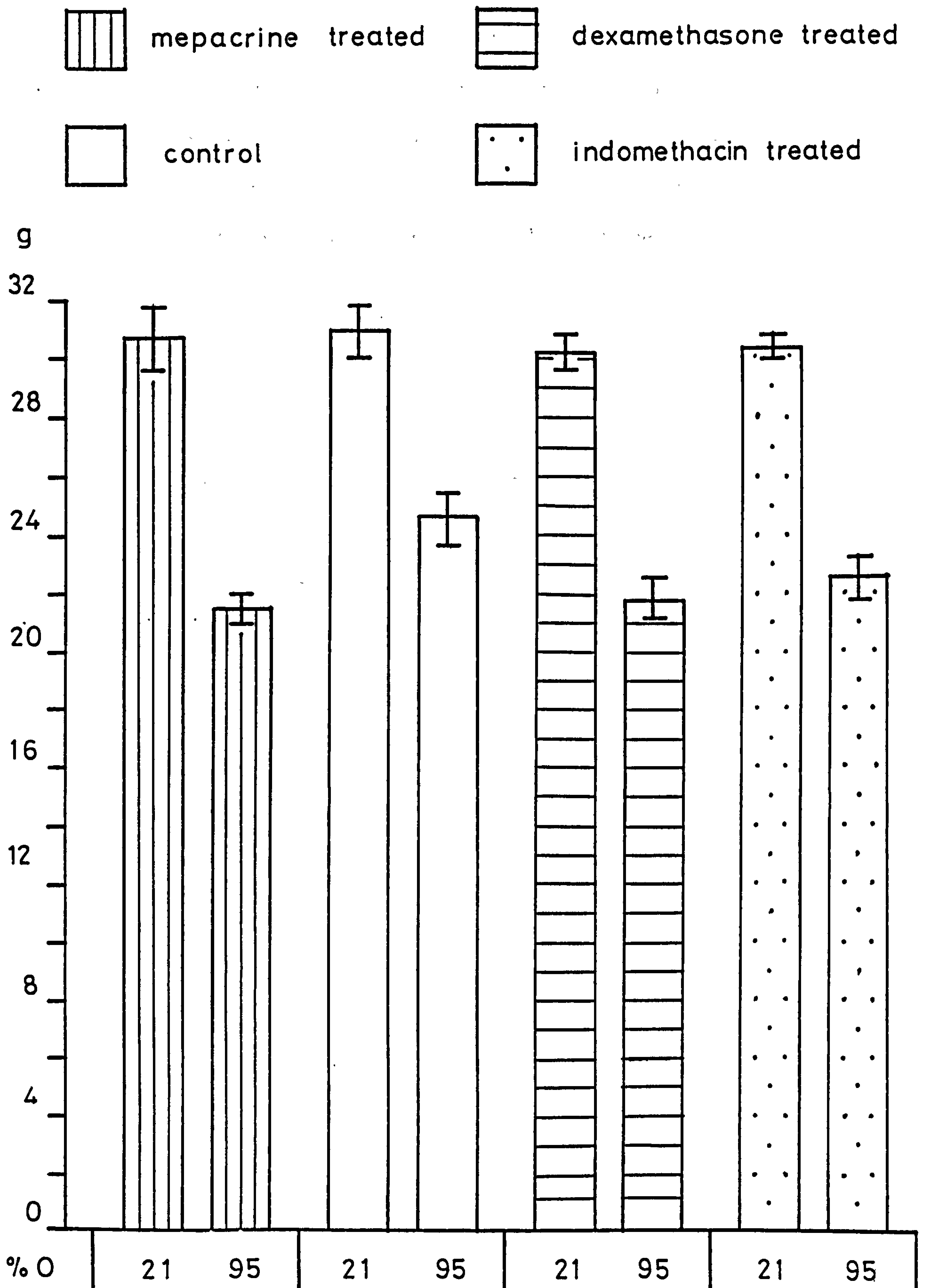


FIGURE 35:
BODY WEIGHT OF MICE IN 21% AND 95% OXYGEN AT END OF EXPERIMENT



experiment, the results for which are presented in figure 36. The mepacrine and dexamethasone 'treated' 'control' animals gave very similar values to the 'untreated' 'control', but the indomethacin 'treated' group showed a slightly lower liver weight. This difference was not statistically significant. All of the 'experimental' groups exposed to high oxygen tensions showed very significant differences from their respective 'controls'. None of the 'treated' 'experimental' groups showed significant differences from the 'untreated' 'experimental' group although all showed a slight decrease.

Because of the large difference in total body weight between the 'control' and 'experimental' animals, the changes in liver were also expressed as a % of total body weight. From the data presented in figure 37, it was seen that although there was a slight decrease in the size of the liver as a proportion of total body weight between the 'untreated' 'experimental' and 'control' animals, this was not significant. The indomethacin 'treated' groups showed very little difference in this ratio, but the mepacrine and dexamethasone 'treated' groups showed a greater difference between the 'control' and 'experimental' values than that which occurred between the two 'untreated' groups. Although the difference between the mepacrine 'treated' groups was not significant, that between the dexamethasone 'treated' groups was ($P = 0{\cdot}01$ between dexamethasone 'treated' 'control' and dexamethasone 'treated' 'experimental' animals).

Figure 38 demonstrates the increase in wet lung weight of the 'experimental' animals, which was statistically very significant between all 'experimental' groups and their respective 'control' groups. Although the mepacrine and indomethacin 'treated' 'experimental' groups did not show quite such large increases as the 'untreated' 'experimental' group, these differences were not

FIGURE 36:
LIVER WEIGHT OF MICE IN 21% AND 95% OXYGEN

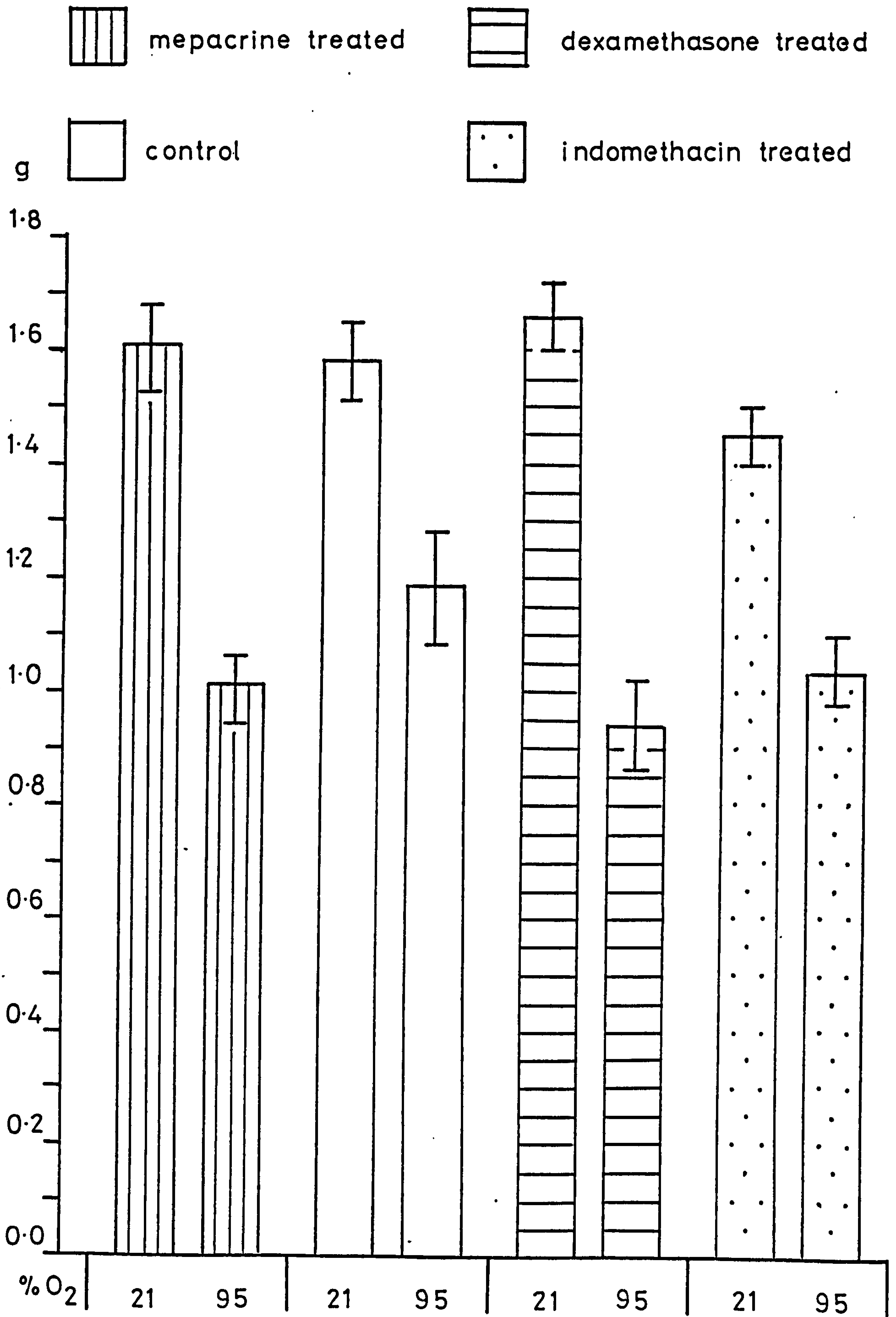
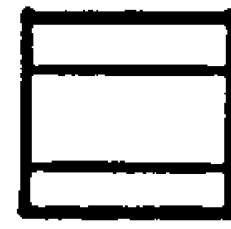
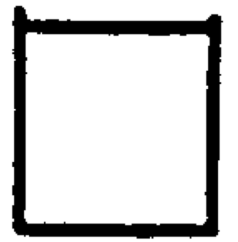


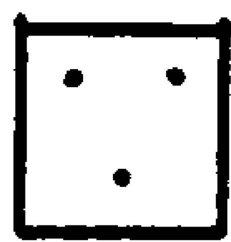
FIGURE 37:

LIVER WEIGHT EXPRESSED AS A % OF BODY WEIGHT OF MICE IN 21% AND 95% OXYGEN

mepacrine treated

 dexamethasone treated

 control

 indomethacin treated

%

5

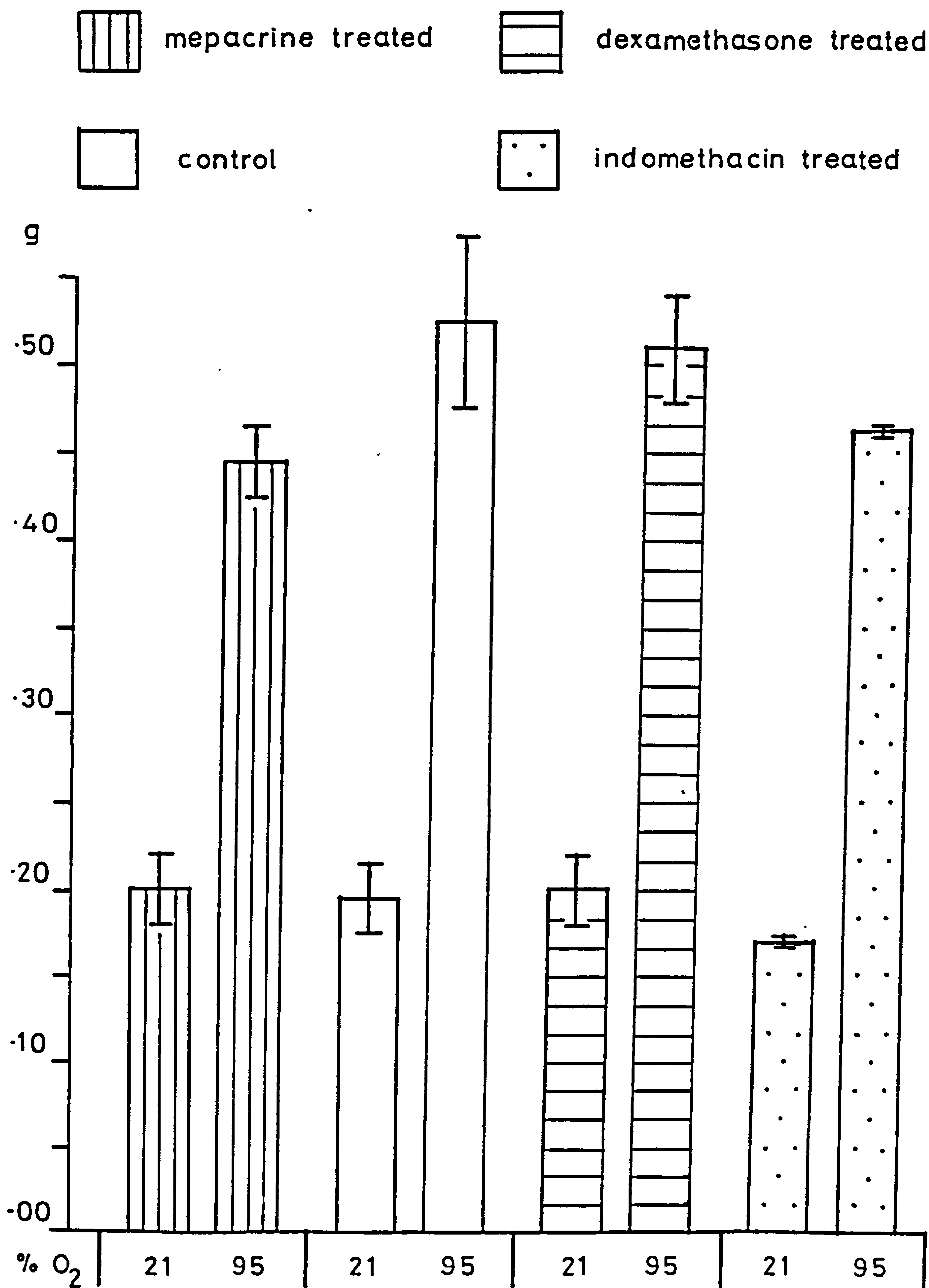
4

3

2

% O_2 | 21 | 95 | 21 | 95 | 21 | 95 | 21 | 95

FIGURE 38:
WET LUNG WEIGHTS OF MICE IN 21% AND 95% OXYGEN



statistically significant, nor was the decrease between the indomethacin 'treated' and 'untreated' 'control' group.

Figure 39 shows that there was also a very significant difference between the dry lung weights of mice exposed to oxygen compared to their respective controls. With one exception the 'experimental' and 'control' 'treated' animals had very similar dry lung weights to their respective 'untreated' 'controls'. The exception, the indomethacin 'treated' 'control' group in which there was a lower dry lung weight than in the 'untreated' 'control', did not show a statistically significant difference. When the % water contents were calculated from the wet and dry lung weights it was seen (figure 40) that the 'treated' groups had very similar water contents to the 'untreated' groups, both in the 'control' animals and the 'experimental' animals. Each 'experimental' group was very significantly different from its respective 'control' group.

C. Discussion

It was noted in this experiment, as in the experiments reported in chapter 1 that the mice subjected to increased oxygen, were less susceptible to oxygen toxicity than those reported in the literature. In the previous chapter it was thought possible that the inflammation noted in the lungs of control mice, or perhaps recent infection in these mice, had contributed to the increased resistance to oxygen toxicity. In this experiment however, the mice had been purchased commercially and were very healthy; most of the lungs from the control animals showing no signs of inflammation. It therefore appears that the increased resistance of the mice used throughout this study was probably due to the genetic disposition of the strain rather than to environmental or health reasons.

A further observation noted during this experiment, was the

FIGURE 39:
DRY LUNG WEIGHT OF MICE IN 21% AND 95% OXYGEN

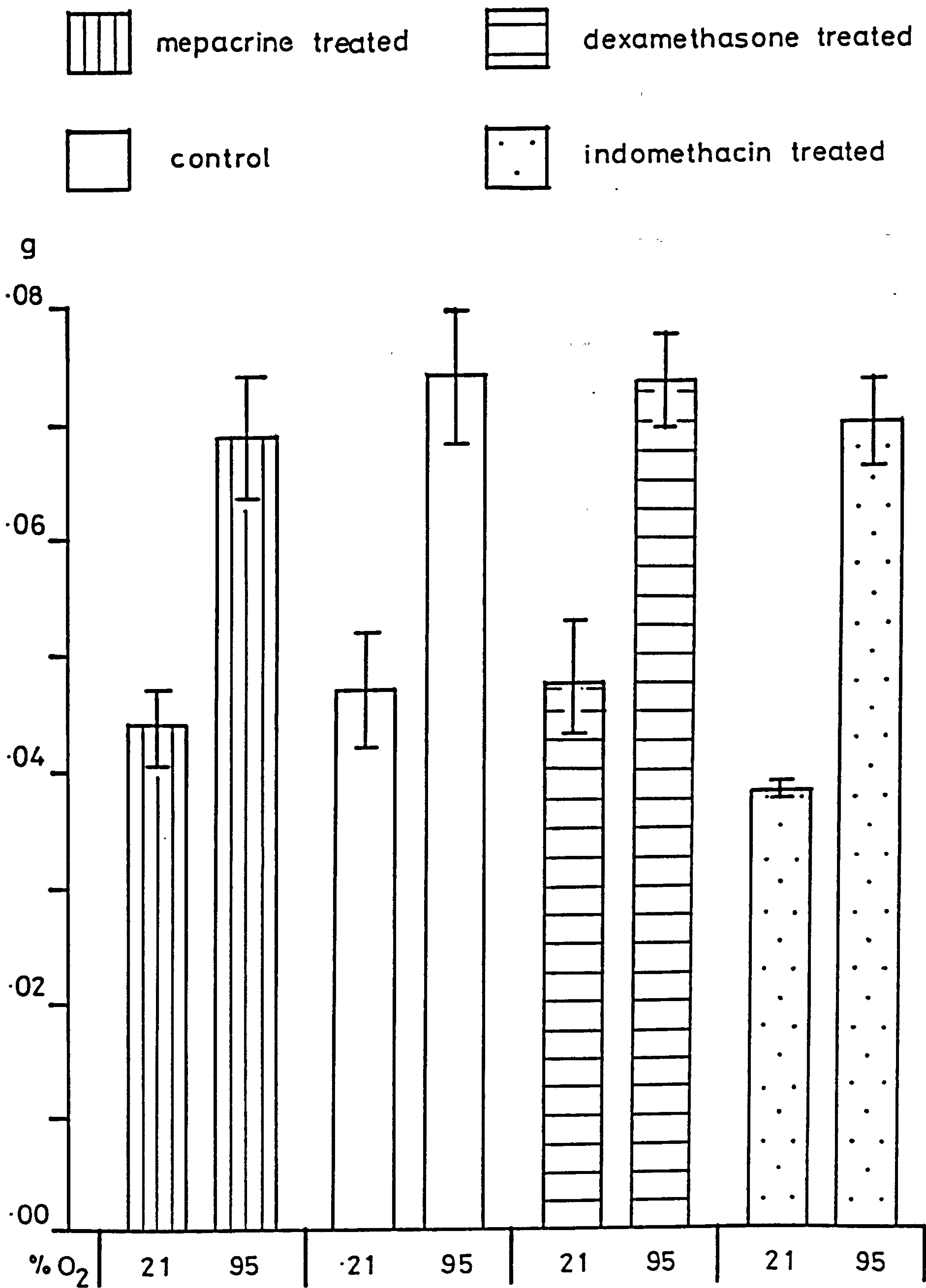
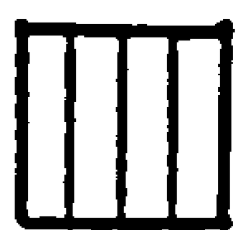
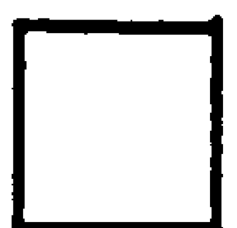


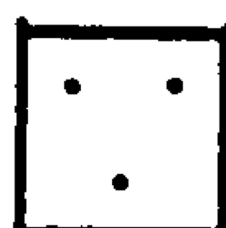
FIGURE 40:

% WATER CONTENT OF LUNGS OF MICE
IN 21% AND 95% OXYGEN

 mepacrine treated

dexamethasone treated

 control

 indomethacin treated

%

80

60

40

20

%O 21 95 21 95 21 95 21 95

convulsive death throes of the mice. The convulsions, occurring as they did, when the animals concerned experienced great difficulty in breathing, and often during a brief return to 0·21 atm oxygen, are unlikely to be due to an excess of oxygen in the CNS. Convulsions due to hypoxia have been reported in which the onset of the convulsions was paralleled by a sudden drop in brain ATP concentration (Sanders et al 1970). It would appear that hypoxia is a possible explanation for the convulsions noted in this study.

From the quantitative measurements reported in this chapter, it has been shown that oxygen toxicity causes: a decrease in total body weight and liver wet weight, and an increase in wet lung weight, dry lung weight and tissue water content, all of which are highly significant. However, when the liver wet weight was expressed as a percentage of total body weight it was seen that the liver retained its approximate proportion to the body size. This experiment also demonstrated that the increase in lung % water content as a result of exposure to oxygen at 0·95 atm was highly significant, whereas in chapter 1 using 0·85 atm oxygen, the increase in this parameter was not statistically significant. This was partly due to the control animals showing a lower average % water content than in the previous experiment, as a result of the healthier and less inflamed state of the lungs; in addition a higher % water content was shown in the experimental animals due, presumably to a more toxic dose of oxygen.

It was determined that most of the effects of the three prostaglandin inhibitors on the experimental animals were minimal in comparison to the 'untreated' group. One significant difference occurred in the liver weight expressed as a % of total body weight, in the dexamethasone 'treated' group, where administration of oxygen caused a large decrease compared to 'control' animals. This

alteration, as it occurred in only one of the 'treated' groups, is likely to be a side effect of that drug. Dexamethasone has corticosteroidal activity. In the introduction, section 4.D. it was reported that adrenalectomy had a beneficial effect, and that the administration of cortisone had a detrimental effect on the course of oxygen toxicity. It is probable that the difference noted above arose as a result of the adverse effects of corticosteroids on oxygen toxicity.

The only other effects shown by the prostaglandin inhibitors were the increase in survival time, and the decrease in total body weight. In view of the linear decrease in body weight with time of exposure to oxygen, these two factors must be considered to be linked. Figure 33 demonstrates that if all the 'untreated' and 'treated' groups were considered at the same point in time, e.g. day 5 of the exposure, the 'treated' groups showed a slight decrease in total body weight over the 'untreated' group. However, a difference of only 5% of the initial body weight (approximately 1·5g) separated all four groups at this point: this difference was about equal to the SEM of any of the four groups, and was therefore not significant.

Taking into account the similarity of the condition of the lungs of the 'experimental' mice both 'treated' and 'untreated' at death, and the other factors discussed above, a likely explanation for the results of this experiment is as follows. The prostaglandin inhibitors slightly reduced the inflammation of the lungs of the 'treated' animals, thus delaying the onset of the eventual hypoxia resulting from the inflammation, and accounting for the small average increase of survival of the 'treated' animals. In the middle stages of the toxicity, the slightly lower inflammation in the lungs of the 'treated' animals postulated above, might also permit a better diffusion of oxygen, thus increasing the cellular toxicity and leading to a more rapid drop in body weight. It must be

concluded from these results that the effect of prostaglandin inhibitors in delaying death from oxygen toxicity was not great. It is possible that a slightly higher dose administered more frequently might produce a greater protective effect. However, as with the effect of hormones on oxygen toxicity outlined in the introduction, prostaglandins may have a small effect on the rate of production of toxic symptoms, but do not appear to have much influence on oxygen toxicity at the cellular level.

In studies on the therapeutic effects of drugs on OAP, certain prostaglandin inhibitors have been tested. Yam & Roberts (1979) reported that both meclofenamate (which inhibits prostaglandin synthetase) and dexamethasone failed to alter the survival of rats to 96-98% oxygen, and further that dexamethasone treatment induced greater lung damage. However, Frank et al (1980) reported that although when pregnant rats were given injections of dexamethasone and the pups subjected to 96-98% oxygen they showed greater decreases in lung weight, lungweight/bodyweight, and lung DNA compared to untreated rat pups, they had slightly improved survival in hyperoxia. As reported in the present study, these experiments indicated that the effects of prostaglandin inhibitors were only marginal.

More detailed studies have been performed on the effect of exposure to oxygen of prostaglandin metabolism. After 24 hours exposure to OAP, no difference was noted in PG E_2 metabolism (Klein et al 1978), however perfused lungs from rats exposed to oxygen for 36 and 48 hours showed a decreased ability to metabolise PG E_2 (Klein et al 1978; Bakhle et al 1979). Similarly exposure of guinea pigs to OAP for 48 hours inhibited the lung metabolism of PG $F_{2\alpha}$. A kinetic analysis in this latter study suggested that this decrease in metabolism was due to destruction or inactivation of prostaglandin dehydrogenase.

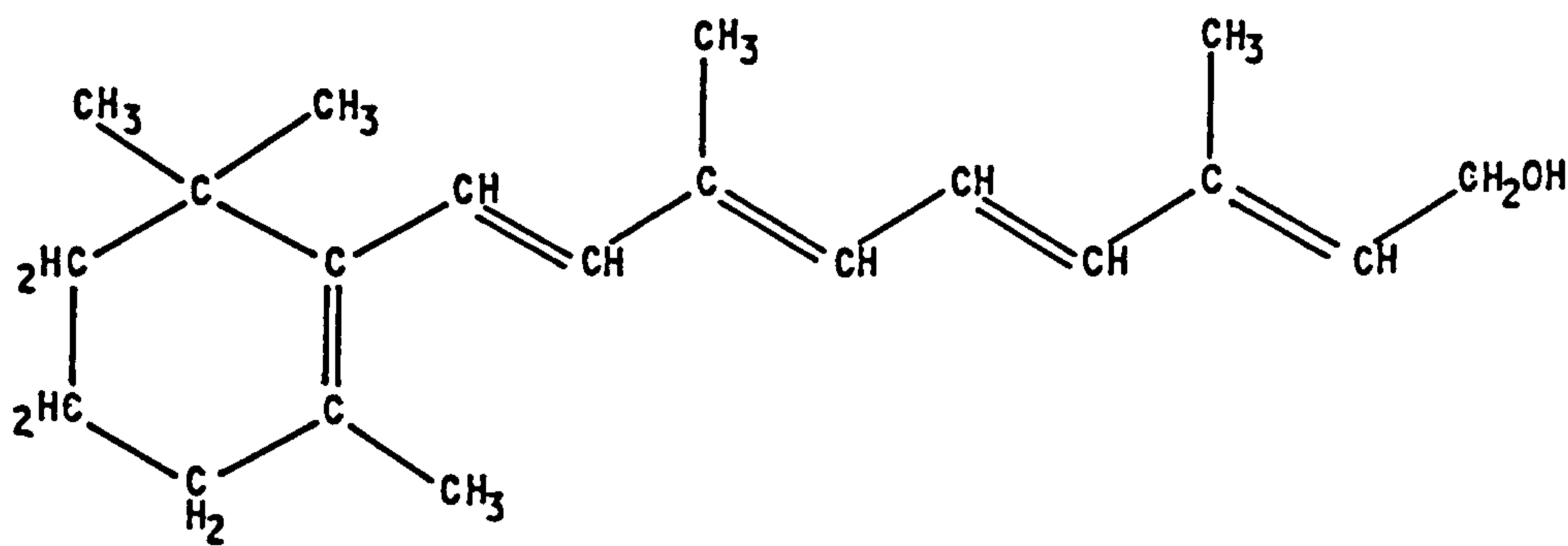
These reports demonstrate that there is an impairment of prostaglandin metabolism by oxygen at atmospheric pressure which in theory should be alleviated at least partially by the administration of prostaglandin inhibitors. It remains to be determined however, what effect this defect in prostaglandin metabolism would have on the metabolism and health of otherwise normal animals, and thus by implication, its contribution to oxygen toxicity.

Chapter 4: CHANGES IN PLASMA RETINOL, HAEMATOCRIT, HAEMOGLOBIN AND ERYTHROCYTE SOD, IN RATS EXPOSED TO 0·85 ATM OXYGEN

A. Introduction

Vitamin A occurs in living organisms either as the free alcohol retinol, the free aldehyde retinal, retinyl esters, or bound to protein. The role of retinol in vision is well defined, but the systemic function of vitamin A is obscure at the present time. Vitamin A has been proposed to have roles in sulphuration, steroid hormone biosynthesis, stimulation of the protein synthetic mechanism of the intestine resulting in the production of a specific protein, and alteration of the structural integrity of biological membranes. A role in electron transport has also been suggested (Wasserman & Corradino 1971).

Retinol has a long conjugated system of double bonds and is therefore very sensitive to both ultraviolet light and oxygen.



Vitamin A_1 (Retinol)

In view of this reactivity it is possible that retinol may be affected by hyperoxia. In support of this supposition, certain symptoms have been observed in preceding chapters that are also symptoms of vitamin A deficiency (Moore 1957), namely: cessation of growth, failure of appetite and decline in body weight, untidy hair, incoordination and paresis. In addition, other workers have noted

degeneration of testes (Gerschman _et al_ 1963), loss of peripheral vision (Behnke _et al_ 1935) and changes in electroretinogram (Shaw & Leon 1970) which are also found in vitamin A deficiency. Although these observations do not comprise a complete list of the symptoms of vitamin A deficiency, nor are they necessarily symptoms restricted to vitamin A deficiency, taken as a whole they suggest a possible line of fruitful research.

The major reserve of vitamin A in mammals is contained in the liver which can store sufficient of the vitamin to last months or years, but the other organs of the body contain only small amounts. While it is unlikely that during a chronic exposure to oxygen the liver stores would be totally depleted, it is possible that if a disruption of vitamin A transport occurred then deficiencies might occur in certain organs. Although the measurement of small amounts of vitamin A in tissues other than liver is difficult, an accurate and relatively convenient assay for the determination of retinol in small quantities of blood has been devised (Thompson _et al_ 1970). An experiment was therefore designed to assess the effect of chronic hyperoxia on vitamin A transport. Haematocrit, haemoglobin and SOD were also determined for reasons given below.

B. Results

In previous experiments, where a wide variation between individual animals, and large numbers of controls have been used, the experiments have been lengthy lasting up to 13 days, and the results sometimes difficult to interpret. This section of work, using blood, appeared to present an ideal opportunity to use each animal as its own control and thus alleviate many of the former problems.

In the first series of experiments, rats of an equivalent age

to the mice used in the other experiments were employed. The experimental format of the first series of experiments followed the initial procedure given in the materials and methods section 3.D. v) (a). Each animal was marked so that upon sacrifice after a predetermined exposure to oxygen, the changes arising from that exposure could be accurately determined by reference to the previous control determination. In the first series of experiments, haematocrit and haemoglobin were determined primarily as a check that the animals had recovered fully from the control sampling of blood under pentobarbital anaesthesia. The second blood sample from each rat was withdrawn from the heart with a syringe after sacrifice and opening of the thorax. Rats were sacrificed at zero time, 1 day 3 days and either 6 or 7 days exposure to oxygen to obtain the experimental samples. Although in theory the use of animals as their own controls solved many problems, in practice severe difficulties were encountered. There was a high mortality rate arising from the combined use of pentobarbital and the removal of approximately 1 ml of blood from such small animals. Usually only 8 out of 12 animals survived this procedure. Secondly, difficulty was encountered in taking the sample of blood from the heart after sacrifice. Although this was not usually too difficult for the zero time controls and 1 day exposure samples, after 6 or 7 days exposure, not only was the heart very flaccid and beating poorly making withdrawal of blood difficult, but that also the blood of these animals was very thick and clotted quickly thus blocking the needle before the required blood sample was obtained. In addition small clots were found in the syringe despite the large amount of heparin used. Low haematocrit and haemoglobin values were obtained, presumably as a result of the clotting either preventing a withdrawal of whole blood, or causing improper mixing of the sample. In addition there was some doubt as

to whether the pentobarbital interfered with the response of the animals to oxygen.

Problems were also encountered with the retinol assay. When dealing with the larger numbers of control samples (usually 12), an unidentified fluorescence was found randomly distributed among these samples leading to widely disparate results even among duplicate samples. In addition, the control samples often bore little resemblance to the experimental samples: even those taken at zero time. The retinol assays performed on the experimental samples two at a time did not appear to suffer this problem. Similarly a set of standard samples obtained by guillotining 8 unanaesthetised unexposed rats did not suffer from problems of extraneous fluorescence. It was noted that the values obtained from the zero time controls from the 3 series I experiments ($\bar{x}$ = 27•8, SEM = 2•1, n = 6) agreed very well with the standard samples ($\bar{x}$ = 27•7, SEM = 1•8, n = 8). In view of this the experimental results were presented graphically without reference to the control samples (figure 41) to determine if this investigation was worth persuing further. Figure 41 shows that after 1 day exposure there was virtually no difference in the retinol levels from the zero time control, but that at 3 days exposure a slight but statistically insignificant decrease had occurred. At 6 days, there was a very significant decrease to less than half the zero time control value, and a slight but variable recovery on day 7. However, the small number of samples used at day 6 and day 7 do not allow great importance to be attached to these results considered in isolation.

Due to the promising nature of the results presented, a second series of experiments was planned. In this series the use of each animals as its own control was abandoned in favour of a very simple format employing only zero time controls. This choice was due in

Legend to Figures 41 and 42

Changes in Plasma Retinol of Rats During Exposure to
0·85 atm Oxygen

The x axis is days and the y axis µg retinol/100ml plasma. Each point is shown as the mean ± SEM, and n the number of samples for each point is given below the x axis. P_0 gives the probability that the indicated point is the same as the zero time control using Student's 't' test (two tailed). In figure 41, the results are a composite of 3 experiments, and in figure 42 of 2 experiments. Retinol was assayed as detailed in the materials and methods section 3.B. vi).

FIGURE 41:
CHANGES IN PLASMA RETINOL OF RATS DURING EXPOSURE TO 85% OXYGEN [SERIES I]

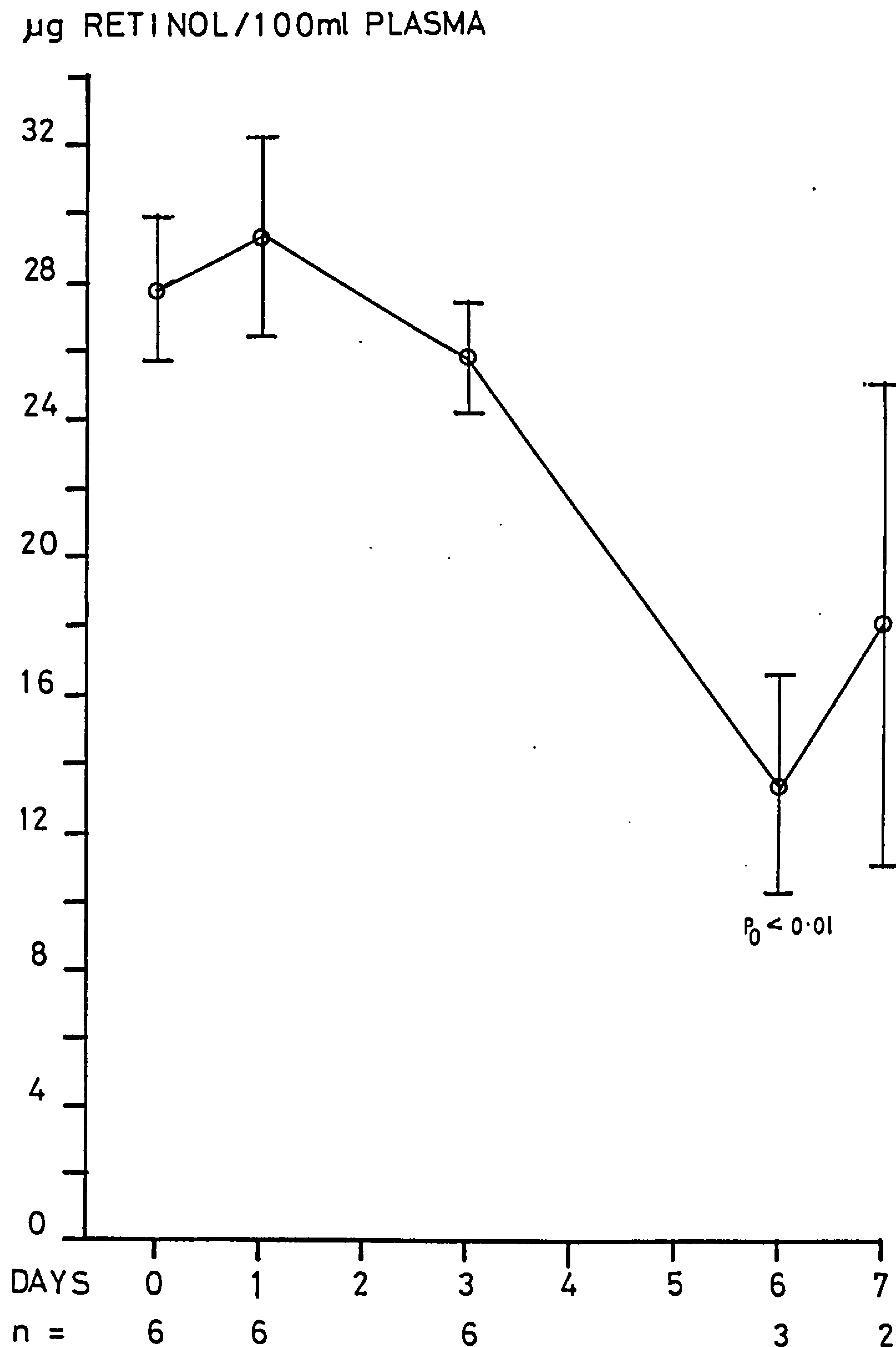
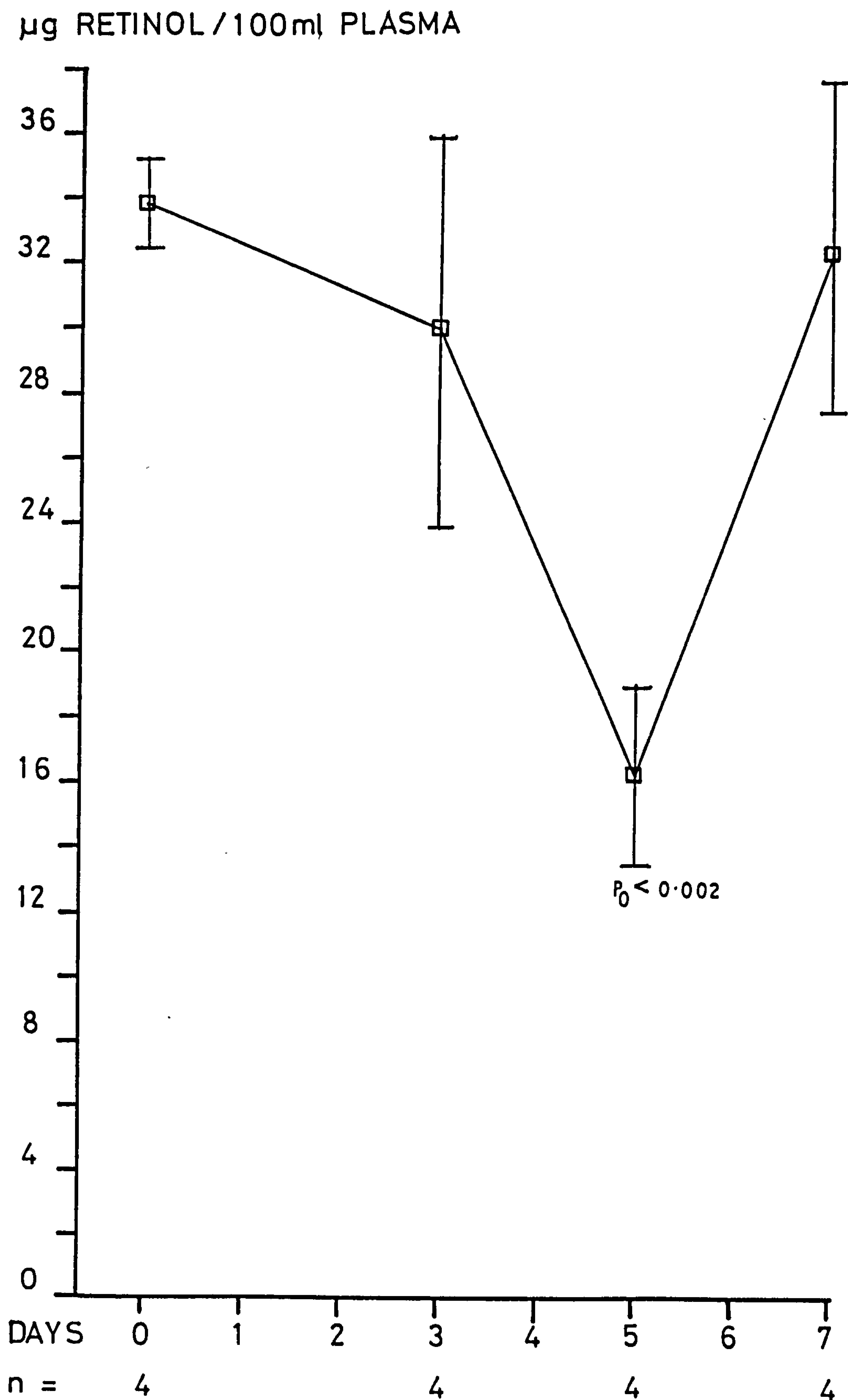


FIGURE 42:
CHANGES IN PLASMA RETINOL OF RATS DURING EXPOSURE TO 85% OXYGEN [SERIES II]



part to the small number of rats which could be accommodated in the chamber at one time (6) and also the lack of time available for experimentation. Because of the previous difficulty of obtaining sufficient blood, the animals in this experiment were sacrificed by stunning and guillotine, permitting a rapid collection of ample blood in small beakers containing only a small amount of heparin (see materials and methods section 3.D. v) (b)). Haematocrits and haemoglobin estimations were carried out in this series as a result of the observations on the changes in blood during the previous series. Erythrocyte SOD determinations were also performed to correlate the results from chapters 1 and 6.

Because of the difficulties with contaminating fluorescence in the last series of experiments, detailed attention was paid to the retinol assay. An examination of procedure for this assay revealed that an incorrect volume of water had been added in the retinol extraction procedure during the series I experiments resulting in a slightly reduced extraction of retinol and a variable extraction of a fluorescent impurity. This problem was eliminated in the second series, and greater attention was also paid to purification of solvents, washing and rinsing of glassware, rinsing glassware with organic solvents, covering samples at all times when not being manipulated, and keeping the assay time to a minimum. These small changes in procedure led to a significant improvement in the reliability of the assay. From the insight gained during the first series, rats in the series II experiments were sacrificed on days 0, 3, 5 and 7 of the exposure.

In this series of experiments it was noted that on the 5 and 7 days exposure the blood was still very thick, and usually only 2-3 ml could be obtained from each rat, whereas at earlier points the animals yielded about 5 ml. The blood was again very dark at 7 days

exposure. Observation of the rats in this experiment revealed similar symptoms to the experiments using mice, but with the following exceptions. The impeded neuromuscular actions of the rear limbs and attendant arched back often noted in the mice were not observed, but the rats did appear to make the involuntary face washing movements more often. The breathing of the rats in the later stages of toxicity, although gasping, was not so laboured as that of the mice, and after 6-7 days of exposure the rats appeared generally less affected than the mice.

The results of the series II experiments are presented in figure 42. This figure again revealed a slight drop in mean plasma retinol levels on day 3, but because of the enormous variation after this exposure the difference was not significant. After 5 days exposure the plasma retinol levels decreased to below 50% of the zero time controls as occurred in series I at day 6. By day 7 the mean plasma retinol levels had returned almost to normal, but the variation was much greater than in the controls. At first sight the pattern of results in the series II experiments appeared somewhat different to those in series I. However when both sets of results were expressed as a % of the zero time control (necessary because of the slight difference in the efficiency of retinol extraction) and plotted on the same axes, they were seen to be complimentary (figure 43). The major differences noted between the two series of results, were the greater variation of the series II points at day 3 and the greater recovery of the series II points on day 7.

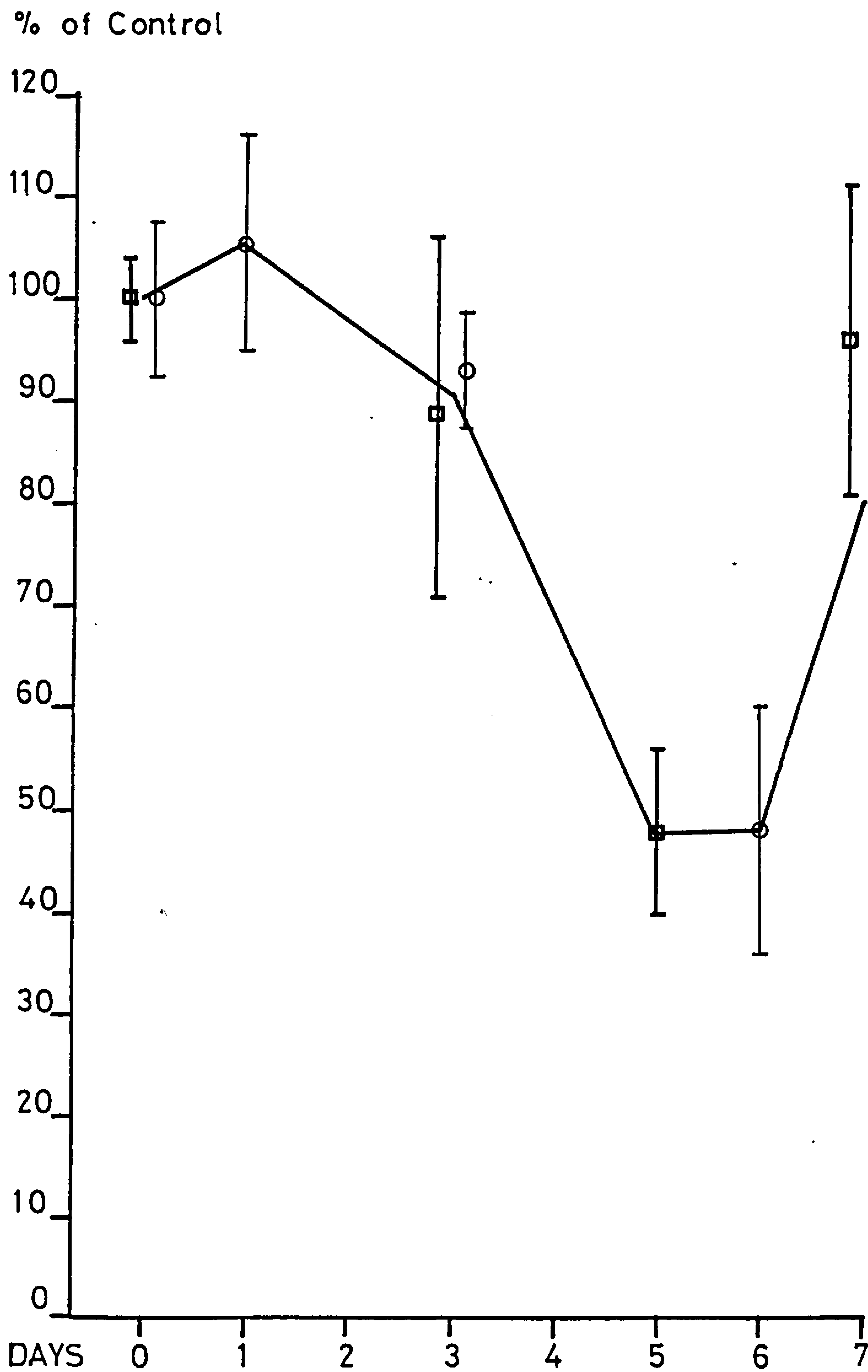
The results from the haematocrit and haemoglobin determinations made in the series II experiments are presented in figures 44 and 45 respectively. Both the haematocrit and haemoglobin showed a parallel increase in values from the zero time controls to the 3 day exposure and then remained at that level during the course of the toxicity.

Legend to Figure 43

Changes in Plasma Retinol of Rats During Exposure to
0·85 atm Oxygen (Series I & II)

Figure 43 shows the results of figures 41 and 42 expressed as a % of the respective zero time controls (day 0). Each point is shown as a mean ± SEM. The number of samples for each point can be found by reference to figures 41 and 42. In some cases the points are slightly displaced laterally for clarity. The series I points are shown (Φ), and the series II points (Φ).

FIGURE 43:
CHANGES IN PLASMA RETINOL OF RATS DURING EXPOSURE TO 85% OXYGEN [SERIES I+II]



The change in haemoglobin levels was statistically significant at all experimental points from the zero time controls, but the haematocrit only showed significance from the zero time controls at 3 days exposure to oxygen. The ratio haemoglobin/haematocrit was calculated, and the results presented in figure 46. From this figure it can be seen that, despite the apparently parallel increase in these two values, there was a change in the ratio. At 5 days exposure the ratio was significantly depressed compared to the zero time control. At 3 and 7 days exposure the ratio was not quite so depressed as at 5 days exposure, and in addition there was considerable variation between samples at these points.

Erythrocyte SOD was also determined as described in the materials and methods section 3.D. v), and the results presented in table 14.

Table 14: Changes in Erythrocyte SOD of Rats Exposed to 0·85 atm Oxygen

	Day 0	Day 3	Day 5	Day 7
n	4	4	4	4
$\bar{x}$	4·84	5·14	5·09	4·87
SEM	0·26	0·36	0·22	0·08

Mean SOD values are expressed as units per mg haemoglobin and represent the results from two separate experiments.

From these results, it can be seen that there was very little variation in erythrocyte SOD levels. Although the results showed a tendency towards an increase on days 3 and 5 of the exposure, this was small compared to the variation of the samples.

Legend to Figures 44 to 46

Changes in Rat Blood Haematocrit and Haemoglobin During Exposure to 0·85 atm Oxygen

Haematocrit and haemoglobin were determined as outlined in materials and methods section 3.B. iii). Each figure was compiled from the results of two experiments. Each point on each graph depicts a mean ± SEM, the number of determinations being 4 in each case. P_0 is the probability that the indicated value is the same as its respective zero time control using Student's 't' test (two tailed).

CHANGE IN RAT BLOOD HAEMATOCRIT AND HAEMOGLOBIN DURING EXPOSURE TO 85% OXYGEN

FIGURE 44: HAEMATOCRIT

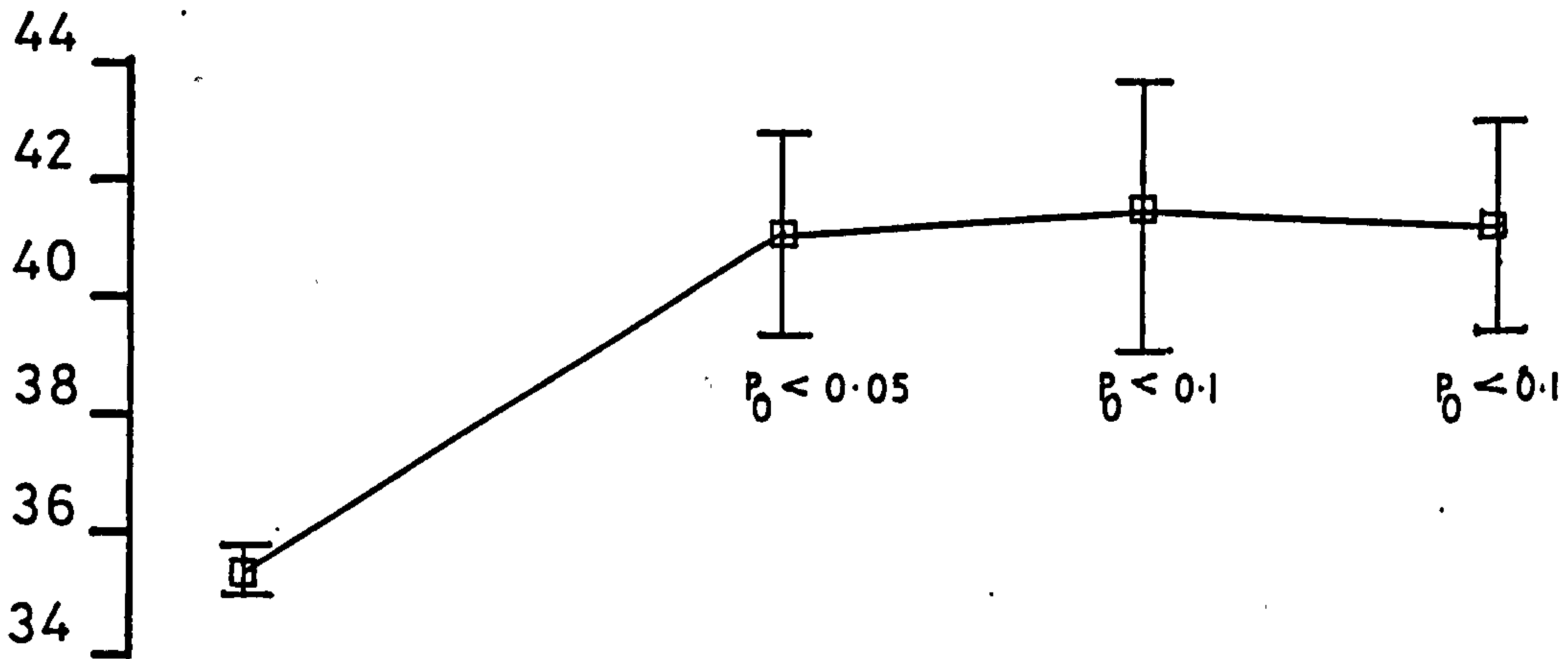


FIGURE 45:

gHb/100ml HAEMOGLOBIN

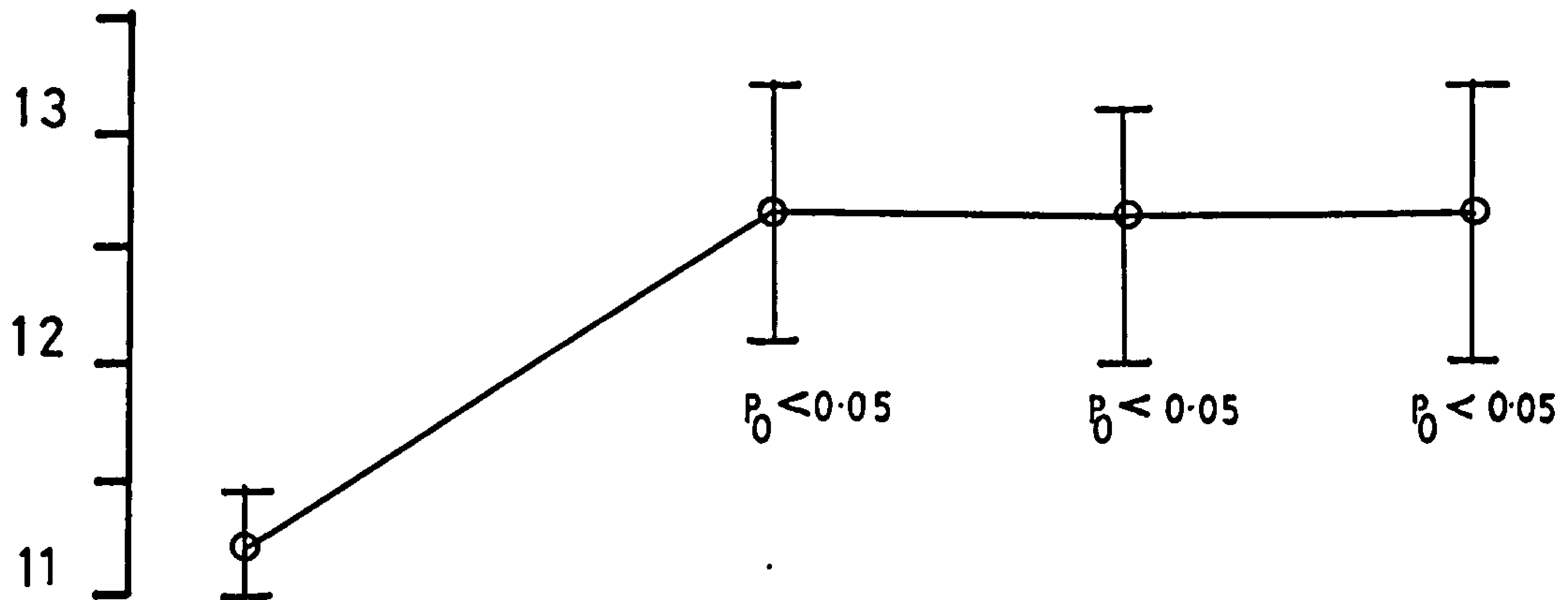
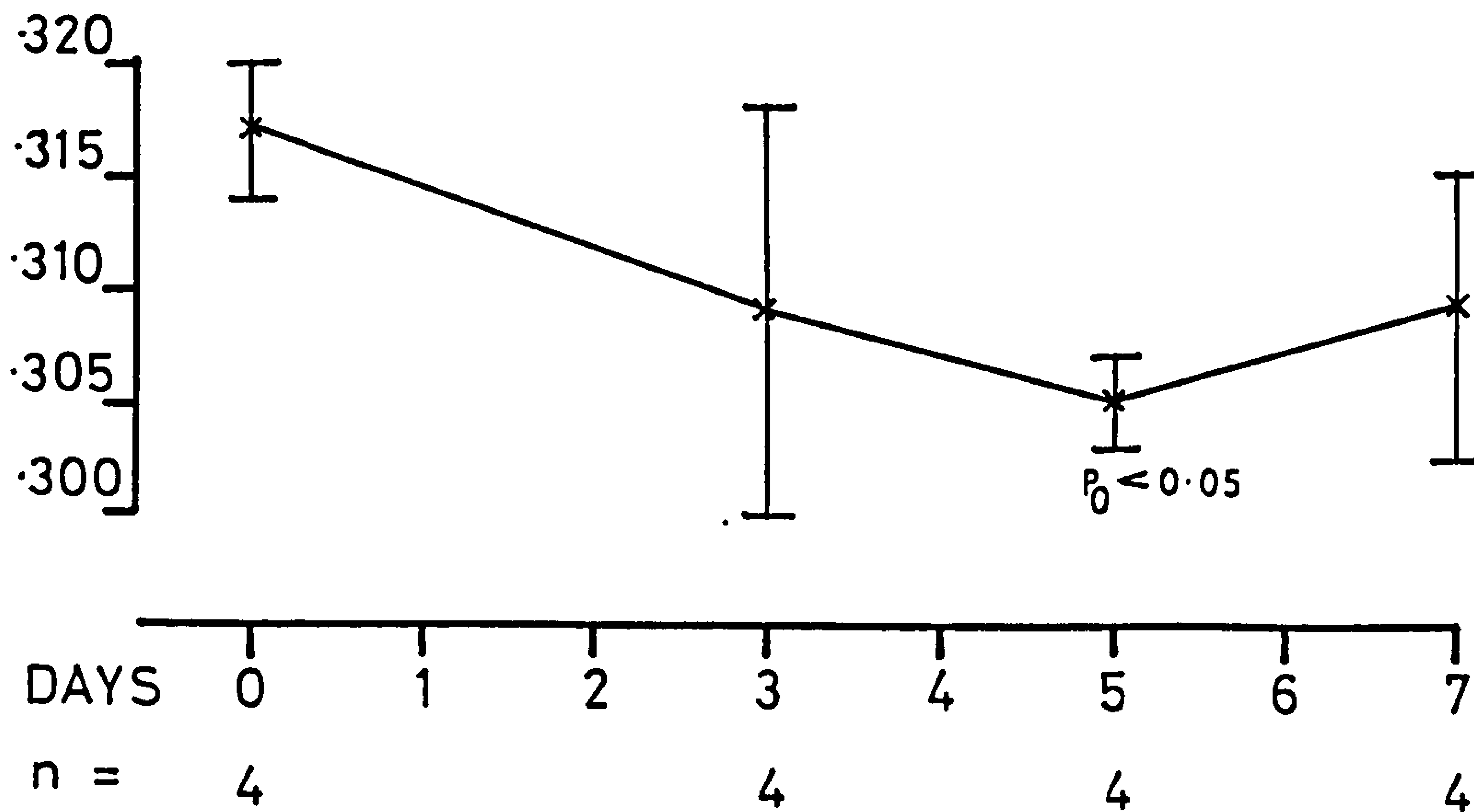


FIGURE 46: HAEMOGLOBIN/HAEMATOCRIT



C. Discussion

In the experiments reported in this chapter it was observed that several changes occurred in the blood as a result of exposure to oxygen. The change in colour of the blood after 6-7 days exposure was unexpected. Animals suffering from hyperoxia would be expected to have bright red blood, and although this was observed in some cases after short exposures, the later samples were invariably dark in colour. Some of the difference may be accounted for in terms of the increased haemoglobin demonstrated in figure 45, but the shade of red observed probably indicated either that the blood was not well oxygenated, or that some formation of methaemoglobin, or a combination of the two had occurred.

The second observation on the blood was the increase in viscosity and the ease and rapidity of clotting of blood from rats exposed to oxygen. The viscosity increase was probably mainly due to the increased haematocrit demonstrated in figure 44, but there was also an increased tendency for clotting of the blood to occur, observed in both series of experiments described. Increased clotting has been described in rats exposed to 4 atm oxygen (Kiesow 1977) where consumptive coagulopathy and disseminated intravascular coagulation preceded the death of the animals. The clotting was ascribed to an activation of coagulation factor XII (Hageman).

The increased haematocrit in animals exposed to OAP reported in this chapter has been previously described in both rabbits (Shaw & Leon 1970) and in rats (Brashear 1977). The % increase haematocrit reported in the present study fell between the values reported in the two previous studies. The biochemical significance of this change is not immediately obvious. The increase in haemoglobin demonstrated in figure 45 is similar to the increase shown in haematocrit. These two changes could represent either an increased

production of erythrocytes or a decrease in plasma volume. The effect of oxygen toxicity on lung protein synthesis described in the introduction would suggest that the latter alternative is more likely. Despite the apparently parallel rise of haematocrit and haemoglobin, a calculation of the haemoglobin/haematocrit ratio, which is a measure of the amount of haemoglobin per erythrocyte, showed a decrease of this ratio from zero time to 5 days exposure which was statistically significant. A large variation in this value was shown after 3 and 7 days exposure, and this was taken to indicate that a change in this parameter was occurring in the population as a whole, but that this change was occurring at different times in different animals. The decrease in the haemoglobin/haematocrit ratio either suggests an adaptive response to the higher levels of oxygen, or alternatively it could indicate an increase in erythrocyte volume.

The investigation of plasma retinol levels demonstrated that oxygen toxicity caused a marked decrease in this parameter at the time when observations revealed the animals to be in their worst physical condition. A rise in the level at day 7 from the minimum at days 5 and 6 was noted, and this was thought to be due to the animals suffering from hypoxia and not hyperoxia at this point. The large variations noted at days 3 and 7 can be attributed as above, to the speed at which individuals responded to the toxic effects of oxygen. As determinations were only made of retinol levels in plasma, it is difficult to relate the change recorded to cellular oxygen toxicity. Retinol binding protein is synthesised de novo for the transport of vitamin A, so the drop in plasma retinol levels could be merely due to an effect of oxygen on protein synthesis, however, this is not necessarily the case and it may be due to insufficient vitamin A available for transport. The latter possibility seems less

likely in view of the return towards normal plasma retinol levels seen on day 7. A similar decrease in vitamin A transport occurred in patients with liver desease and this has also been attributed to a lack of synthesis of carrier protein (Smith & Goodman 1971). The effect of the decrease in transport of vitamin A on other tissues requiring this vitamin are also unknown. A determination of the change in tissue retinol levels would be required before the action of oxygen on vitamin A could be evaluated.

The erythrocyte SOD level did not appear to change significantly during exposure to oxygen.

Although the work described in the present chapter suggests a number of promising possibilities, further determinations on both experimental and control animals are required to place the above results on a more secure footing and to further delineate the extent of the changes. Further studies would then be required to determine whether the decrease in plasma retinol levels i) arose from a failure of retinol binding protein synthesis or some other cause e.g. depletion of liver retinol stores as a result of peroxidation; and ii) had any effect on the retinol status of other tissues in the body e.g. lung or brain.

Chapter 5: DETERMINATION OF HAEMOGLOBIN, RETINOL, ERYTHROCYTE SOD AND CATALASE, PLASMA AMINO ACIDS, AND URINE UREA LEVELS IN HUMANS DURING A THERAPEUTIC RECOMPRESSION PROCEDURE

A. Introduction

In a previous series of experiments on a therapeutic recompression precedure, an increase in pulmonary tissue volume and the appearance of substernal distress indicated that the subjects suffered from a mild oxygen toxicity (Winsborough & McKenzie 1977).

The present series of experiments, is a modification of this procedure. A mixture of 32·5% oxygen/67·5% nitrogen was breathed intermittantly from a mask at an equivalent depth of 50m s.w. (6 atm absolute pressure), and subjects were similarly assessed by physiological tests for signs of mild oxygen toxicity.

In addition, blood samples were collected from all subjects for the determination of haemoglobin, retinol, and erythrocyte SOD and catalase: urine samples were collected from some subjects for the determination of urea. The biochemical determinations were performed to see whether any changes occurred in the above parameters as a result of such a mild hyperoxia, and if so to correlate the changes with those previously noted in rodents arising from a much more toxic exposure to oxygen.

B. Results

There were 4 experiments performed on the modified therapeutic recompression procedure. The first experiment took place in a large chamber in the Deep Trials Unit (DTU). In this experiment there were 4 subjects, three breathing the increased oxygen mixture at depth, and the remaining subject as a 'reference' breathing compressed air. In the DTU experiment it was not possible to obtain a post-exposure

blood sample from the subjects, neither were urine samples collected. The remaining experiments took place in a smaller chamber in the Experimental Diving Unit (EDU). In each of these three experiments there were two subjects: one breathing the oxygen enriched mixture through a mask and the other subject breathing chamber air. In view of the differences between these two sets of experiments the results from the first experiment are presented singly, and the results from the remaining three experiments are presented together.

The times of the blood and urine sample collections are marked on figure 8, materials and methods section 3 D. iv). In all of the experiments, the first two blood samples were pre-exposure controls, and in the EDU experiments the sixth blood sample was a post-exposure control. For the urine samples, only the first sample was a pre-exposure control, the remaining samples being taken over 12 hour periods, and the last urine sample being a post-exposure control.

All the results presented are expressed both as the actual measurement, and also as a percentage of the respective pre-exposure controls. In most cases there were 2 pre-exposure controls, so the mean of these two values was used. Although there were only 3 subjects in each group, the means and SEM's of the percentage pre-exposure control values were calculated and presented to give an indication of the variation among the subjects. Those subjects not breathing the enriched oxygen mixture have been called 'reference' subjects. It should be noted that these subjects were also breathing increased partial pressures of oxygen, and so any changes from the pre-exposure control due to oxygen toxicity should, in theory, show up in these subjects, but the changes in the 'experimental' subjects - those receiving the enriched oxygen mixture - should be greater.

From the results presented in tables 15a and 15b showing the

Table 15: Haemoglobin Determinations On Human Blood Samples

a) DTU Experiment

Experimental Samples

Subject	1	mean	2	3	4	5
1						
g/100ml	16•2	15•8	15•4	18•5	15•3	14•8
%	103	100	97	117	97	94
2						
g/100ml	15•3	17•2	19•0	18•4	16•4	15•4
%	89	100	111	107	96	90
3						
g/100ml	15•7	16•5	17•2	16•7	17•2	16•6
%	95	100	105	102	105	99
Mean %	96	100	104	109	99	95
SEM		4•1		4•4	2•8	3•2

Reference Sample

Subject	1	mean	2	3	4	5
4						
g/100ml	14•9	15•8	16•7	15•8	15•0	13•8
%	94	100	106	100	95	87

All haemoglobin determinations were performed in duplicate.

Table 15: Haemoglobin Determinations On Human Blood Samples

b) EDU Experiments

Experimental Samples

Subject	1	mean	2	3	4	5	6
5							
g/100ml	16•2	16•4	16•6	15•9	16•6	15•0	15•6
%	99	100	101	97	101	91	95
7							
g/100ml	16•2	16•0	15•7	15•9	16•1	15•9	17•3
%	102	100	98	100	101	100	108
9							
g/100ml	15•9	16•2	16•4	15•5	15•3	15•4	15•3
%	98	100	102	96	98	95	99
Mean %	100	100	100	98	98	95	99
SEM		1•2		0•6	2•5	2•6	4•3

Reference Samples

Subject	1	mean	2	3	4	5	6
6							
g/100ml	16•3	16•6	16•9	17•1	16•6	16•7	16•8
%	98	100	102	103	100	101	101
8							
g/100ml	14•2	14•9	15•6	18•0	14•9	15•3	15•3
%	95	100	105	121	100	95	99
10							
g/100ml	15•1	15•5	15•9	14•8	14•9	15•3	15•3
%	97	100	103	95	96	99	99
Mean %	97	100	103	106	98	97	100
SEM		0•9		7•7	1•2	1•2	0•4

haemoglobin determinations, it can be seen that there was some variation in individual samples from their pre-exposure control values, but when the results were examined collectively, the differences from the controls were very small.

Unfortunately the fixed timing of the dive programme dictated the dates on which the samples could be collected, and although the known instability of retinol made it imperative that the retinol assays were performed at the earliest opportunity, at the time blood samples were collected, the problems associated with the retinol assay had not been fully eliminated. Plasma samples were originally set aside for the assay of retinol, but when these were assayed, a problem arose in many of the samples of a non-specific fluorescence, which had not been encountered on small trial runs. In order to perform further retinol assays it was necessary to use whole blood samples obtained at the same times. Determinations on fresh human blood were made to confirm that erythrocytes did not interfere chemically with the retinol determinations, but ideally, a correction needed to be made for the volume occupied by erythrocytes. As haematocrits had not been determined in this series of experiments, it was assumed that they remained constant at a value of 50% during the period of the dives. In the assay for retinol, 0·4ml of whole blood was therefore substituted for 0·2ml of plasma.

The fluorescence readings when substituted in the correction formula resulted in a retinol content about 2-3 times the expected value. Further experiment revealed that blood samples kept in the refrigerator gave increased fluorescence and thus apparently increased 'retinol' levels. The large retinol values measured are therefore presumably due to the extraction of a fluorescent pigment associated with ageing of the sample. This factor was assumed to be constant for the samples in each experiment. The presence of this fluorescence

implies that any percentage changes in retinol content due to hyperoxia would be smaller than if the fluorescence was not present.

The results of the retinol determinations are presented in table 16a and 16b. In table 16a, the DTU experiment, the retinol results showed a slight rise in the reference subject and one of the experimental subjects, but a slight decrease in samples 4 and 5 in the other two experimental subjects. Overall the changes in the experimental subjects were cancelled out to show an approximately constant level. In the EDU reference subjects (table 16b), 1 of the 3, showed a slight decrease at samples 5 and 6. All the EDU experimental subjects showed a decline in the values of samples 5 and 6, and in sample 4 in subject 9 as well. Overall these results suggest a possible reduction in samples 5 and 6 (P = 0·1 compared to the pre-exposure values, using a Student's 't' test (two tailed)).

Tables 17a and 17b showed the effect of the recompression procedure on SOD levels. Although the mean experimental results showed a slight increase, this was not statistically significant.

The catalase results are presented in tables 18a and 18b. These results were also variable from subject to subject, and so a comment will only be made on the mean values. These showed a tendency towards a reduction at samples 3 and 4 of the experimental subjects, but a slight rise in the 'reference' samples above the pre-exposure controls. Again in view of the variability of the samples, it is not possible to draw any clear cut conclusions.

An examination of table 19 revealed that, for most of the plasma amino acids determined, the SEM's were greater than most of the apparent changes. It is therefore considered that there were no significant changes arising in plasma amino acids as a result of this recompression procedure.

Urine samples were only collected from the 3 EDU experiments.

Table 16: Retinol Determinations On Human Blood Samples

a) DTU Experiment

Experimental Samples

Subject	1	mean	2	3	4	5
1						
µg/100ml	168	172	176	174	189	190
%	98	100	102	101	110	111
2						
µg/100ml	156	153	150	148	142	149
%	102	100	98	96	92	97
3						
µg/100ml	177	171	166	173	156	166
%	103	100	97	101	91	97
Mean %	101	100	99	99	98	102

Reference Sample

4						
µg/100ml	131	133	134	130	143	139
%	99	100	101	98	108	105

All retinol determinations were performed in duplicate.

Table 16: Retinol Determinations On Human Blood Samples

b) EDU Experiments

Experimental Samples

Subject	1	mean	2	3	4	5	6
5							
µg/100ml	160	152	145	141	151	145	141
%	105	100	95	92	99	95	92
7							
µg/100ml	156	162	167	160	167	151	154
%	97	100	103	99	104	94	95
9							
µg/100ml	92	94	96	99	83	83	83
%	98	100	102	105	88	88	88
Mean %	100	100	100	99	97	92	92
SEM		2•5		3•8	4•7	2•2	2•0

Reference Samples

Subject	1	mean	2	3	4	5	6
6							
µg/100ml	174	178	181	173	166	187	177
%	98	100	102	98	94	106	100
8							
µg/100ml	142	150	158	157	151	149	142
%	95	100	105	104	100	99	94
10							
µg/100ml	95	98	100	98	93	90	89
%	97	100	103	101	96	92	92
Mean %	97	100	103	101	97	99	95
SEM		0•9		1•7	1•8	4•0	2•4

Table 17: SOD Determinations On Human Erythrocyte Samples

a) DTU Experiment

Experimental Samples

Subject	1	mean	2	3	4	5
1						
U/mg Hb	6•50	6•27	6•05	5•69	5•74	7•20
%	104	100	96	91	92	115
2						
U/mg Hb	6•29	6•37	6•47	6•68	6•86	6•67
%	99	100	101	105	108	105
3						
U/mg Hb	6•34	6•49	6•60	6•16	6•22	6•64
%	98	100	102	95	96	102
Mean %	100	100	100	97	99	107
SEM		1•9		4•2	4•8	3•9

Reference Sample

4						
U/mg Hb	5•68	5•75	5•82	6•24	5•86	6•06
%	99	100	101	109	102	105

All SOD determinations were performed in triplicate.

Table 17: SOD Determinations On Human Erythrocyte Samples

b) EDU Experiments

Experimental Samples

Subject	1	mean	2	3	4	5	6
5							
U/mg Hb	9•40	9•57	9•74	9•86	9•58	9•41	10•9
%	98	100	102	103	100	97	114
7							
U/mg Hb	5•58	5•49	5•40	6•65	5•82	5•78	6•38
%	102	100	98	121	106	105	116
9							
U/mg Hb	4•00	4•07	4•13	3•50	3•88	4•14	4•07
%	98	100	102	86	95	102	100
Mean %	99	100	101	103	100	101	110
SEM		1•3		10•1	3•2	2•3	5•0

Reference Samples

Subject	1	mean	2	3	4	5	6
6							
U/mg Hb	10•4	9•5	8•60	8•34	8•86	8•58	7•68
%	109	100	91	88	93	90	81
8							
U/mg Hb	6•33	6•52	6•71	6•63	6•60	6•04	6•48
%	97	100	103	102	101	92	99
10							
U/mg Hb	3•24	3•63	4•02	3•22	4•02	3•44	3•76
%	89	100	111	89	111	95	104
Mean %	98	100	102	93	102	92	95
SEM		5•8		4•5	5•2	1•5	7•0

Table 18: Catalase Determinations On Human Erythrocyte Samples

a) DTU Experiment

Experimental Samples

Subject	1	mean	2	3	4	5
1						
k/g Hb	60•3	57•0	53•6	50•6	48•5	49•8
%	106	100	94	88	85	88
2						
k/g Hb	72•8	68•6	64•3	64•3	60•4	61•4
%	106	100	94	94	88	90
3						
k/g Hb	41•7	42•4	43•1	38•9	41•7	47•5
%	98	100	102	92	98	112
Mean %	103	100	97	91	90	97
SEM		2•7		1•8	3•9	7•7

Reference Sample

4						
k/g Hb	45•8	50•5	55•2	70•1	64•9	71•0
%	91	100	109	139	129	141

All catalase determinations were performed in triplicate.

Table 18: Catalase Determinations On Human Erythrocyte Samples

b) EDU Experiments

Experimental Samples

Subject	1	mean	2	3	4	5	6
5							
k/g Hb	100	91•1	82•1	81•7	93•4	80•2	93•7
%	110	100	90	90	103	88	103
7							
k/g Hb	65•3	65•3	65•3	68•0	68•5	72•8	73•5
%	100	100	100	104	105	111	113
9							
k/g Hb	59•7	60•0	60•3	49•1	56•6	54•4	55•5
%	100	100	100	82	94	91	93
Mean %	103	100	97	92	101	97	103
SEM		3•3		6•4	3•4	7•2	5•8

Reference Samples

Subject	1	mean	2	3	4	5	6
6							
k/g Hb	72•8	81•1	89•4	95•8	77•4	94•1	86•2
%	90	100	110	118	95	116	106
8							
k/g Hb	67•7	67•4	67•0	68•0	71•6	74•0	75•2
%	101	100	99	101	1=6	110	112
10							
k/g Hb	51•2	51•9	52•6	46•8	50•5	51•7	54•4
%	99	100	101	90	97	100	105
Mean %	97	100	103	103	99	109	108
SEM		3•4		8•1	3•4	4•7	2•2

Table 19: Amino Acid Determinations On Human Plasma Samples

Reference Samples

A.A.	1	2	1&2	3	3	4	4	5	5	6	6
	%	%	SEM	%	SEM	%	SEM	%	SEM	%	SEM
Ala	96	104	4	104	26	135	39	114	19	136	54
NH_3	104	96	8	103	15	114	7	151	54	158	25
Arg	105	95	6	86	16	102	6	122	13	108	26
Asp	116	84	9	84	9	91	17	156	31	97	13
Cys	113	87	8	81	6	167	55	70	24	130	28
Glu	104	96	4	87	5	109	12	104	7	124	21
Gly	100	100	2	107	21	116	4	112	5	93	25
His	104	96	6	96	2	118	2	115	9	97	40
Ileu	102	98	6	78	3	103	5	115	5	89	22
Leu	104	96	4	80	6	90	26	114	9	121	37
Lys	97	103	7	94	13	93	23	120	21	100	40
Met	116	84	9	70	18	71	19	90	12	112	15
Orn	110	90	4	81	24	75	14	97	24	77	28
Phe	101	99	1	71	16	93	12	93	17	85	21
Pro	113	87	13	83	11	95	25	92	28	111	43
Ser	108	92	2	126	26	106	4	109	20	86	30
Tau	81	119	27	74	19	102	10	104	7	97	14
Thr	97	107	4	82	19	95	14	105	5	122	29
Tyr	99	101	6	96	17	105	25	97	9	119	21
Val	107	93	10	85	5	102	4	109	7	94	23

(Cys = cystine)

Column headings 1 to 6 refer to the sample numbers in figure 8.

All values are the mean of 3 subjects: each sample of each subject was calculated as a % of the mean of samples 1&2.

Table 19: Amino Acid Determinations On Human Plasma Samples

Experimental Samples

A.A.	1	2	1&2	3	3	4	4	5	5	6	6
	%	%	SEM	%	SEM	%	SEM	%	SEM	%	SEM
Ala	87	113	17	80	12	108	20	66	6	92	26
NH_3	88	112	3	112	12	133	20	110	25	121	26
Arg	103	97	20	75	18	112	39	86	7	134	37
Asp	99	101	10	70	12	84	25	101	43	87	31
Cys	98	102	11	119	21	148	45	128	16	208	74
Glu	94	106	17	91	10	98	4	86	7	121	17
Gly	99	101	14	98	19	101	14	78	3	126	24
His	105	95	7	100	16	112	34	91	11	99	21
Ileu	98	102	8	96	17	102	16	83	14	88	5
Leu	122	78	11	162	34	147	69	109	28	169	66
Lys	109	91	5	131	40	140	57	126	16	177	87
Met	99	101	18	85	12	66	7	95	16	115	36
Orn	100	100	8	84	2	89	14	84	11	85	38
Phe	99	101	8	99	17	99	11	93	13	125	20
Pro	94	106	8	88	11	91	21	87	12	104	32
Ser	93	107	17	109	18	89	17	138	24	78	16
Tau	90	110	19	127	16	130	23	111	33	123	34
Thr	94	106	14	63	10	72	6	71	27	87	8
Tyr	111	89	4	96	19	114	18	80	13	131	35
Val	81	119	9	93	8	94	12	78	10	112	14

Table 20: Urea Determinations On Human Urine Samples

(Units = mmoles urea/12 hr period)

Experimental Samples

Subject	1	2	3	4	5
5					
Units	352	8	189	284	331
%	100	25	54	81	94
7					
Units	114	150	111	202	47
%	100	134	97	172	41
9					
Units	60	339	158	57	-
%	100	565	263	95	-
Mean %	100	241	138	118	68
SEM		165	64	28	27

Reference Samples

Subject	1	2	3	4	5
6					
Units	574	509	245	216	158
%	100	89	43	38	28
8					
Units	302	145	116	200	114
%	100	48	38	66	38
10					
Units	71	261	134	103	-
%	100	272	189	145	-
Mean %	100	136	90	83	33
SEM		69	47	33	5

In the third EDU experiment, the post-exposure urine samples were not collected from either subject due to a misunderstanding. The results of the urine analyses are presented in table 20. In both the experimental and reference subjects there were very large variations of response, and this variation was reflected in the SEM's of the mean results. Although the mean, percentage of control, results indicated an increase in urea excretion which was greater in the experimental than the reference samples, the SEM's were so large that no statistical significance could be attached to these results.

C. Discussion

Despite the use of each subject as his own control, all of the results presented showed considerable variation, so that in most cases any changes observed were unlikely to be significant in terms of hyperoxia.

Haemoglobin and erythrocyte SOD showed no changes in the present experiments. Erythrocyte SOD when subsequently monitored in rats, similarly showed no significant changes, although a small but significant change in rat blood haemoglobin was noted as a result of the subsequent experiment using an increased exposure to oxygen.

The erythrocyte catalase levels monitored in this present chapter indicated that there might be a decrease in experimental subjects in sample 3. While no experiments have yet been performed on erythrocyte catalase in rodents, it is worth noting that in experiments reported in chapter 1 of this thesis, tissue catalase decreased in mice exposed to 0·85 atm oxygen for several days.

Plasma amino acids were found to be particularly variable in the present work, and no clear cut trends were noted outside this background variation. This result was in accord with the small changes noted previously in mouse tissues as a result of toxic exposure

to oxygen, and which were not detected until 7 days exposure.

An increase in urea levels was noted in some subjects. Carlyle et al (1978) found that a compression on a saturation dive to 420m s.w. resulted in a rise in urea levels during the compression which returned to normal at the end of the decompression. Although the compression was much lower in this dive, a similar effect cannot be ruled out. A change in the urea excretion could also be accounted for by alterations in the rate of protein breakdown. Because of the large variation of the results it is not possible to attribute the changes to an effect of the increased partial pressure of oxygen.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Despite many years of research into the causes of oxygen toxicity, little progress has been made in the determination of a lesion (or lesions) either physiological or biochemical which both precedes and can account for the overt symptoms of the toxicity.

One of the earliest suggested mechanisms for the toxicity of oxygen, supported by several workers, was the accumulation of carbon dioxide. This theory proposed that during OHP the haemoglobin in venous blood was fully saturated because there was sufficient oxygen dissolved in plasma to supply the tissue oxygen requirements. As a result of the permanent saturation of haemoglobin by oxygen, carbon dioxide transport was affected to such an extent that a massive build-up of tissue carbon dioxide and decreased plasma buffering capacity occurred resulting in acidosis. Work by Lambertsen *et al* (1953) on oxygen and carbon dioxide transport effectively ruled out the possibility that carbon dioxide plays a major role in the pathogenesis of oxygen toxicity. This line of research has been thoroughly reviewed and discussed in the above paper.

Dickens (1946) and Haugaard (1946) after their numerous *in vitro* experiments, postulated that the effects of oxygen may arise as a result of the oxidation of sulphydryl groups on certain enzymes. However the enzyme inactivation theory suffers from several major inconsistencies. Some enzymes were only inactivated at certain stages of purification (e.g. phosphoglucomutase; Dickens 1946), some enzymes were inactivated in the absence, but not in the presence of substrates or cofactors (e.g. G3PDH; Dickens 1946), some enzymes were inactivated in homogenates but not in tissue slices (e.g. choline acetyltransferase; Stadie *et al* 1945e, and D-amino acid oxidase Stadie *et al* 1945d). With some enzymes (e.g. succinate

dehydrogenase; Stadie & Haugaard 1945), the inactivation was reversible upon the addition of glutathione or cysteine. In addition to the inconsistencies in the _in vitro_ work, there were also problems in relating this work to oxygen toxicity _in vivo_. The inactivation of enzymes was studies after the observation that OHP _in vitro_ caused a decrease in the oxygen consumption of tissue slices. However, the time taken for appreciable reduction of the respiration of tissue slices was considerably longer than the latent period before convulsions due to oxygen occurred (Stadie _et al_ 1945a). Dickens was also forced to conclude that if poisoning of brain respiration was responsible for the convulsions produced by OHP, the clinical effects of oxygen toxicity resulted when only a small percentage inactivation of brain enzymes had occurred. Further to these observations, Stadie & Haugaard (1946) demonstrated that mice subjected to 8 atm OHP developed convulsions within minutes of reaching pressure, but showed no measurable decrease in oxygen consumption. Also that brain slices from animals killed by OHP showed no changes in oxygen consumption or respiratory quotient compared to controls when subsequently assayed at 1 atm oxygen. Stadie & Haugaard (1946) were thus forced to conclude that on the basis of their observations that 'hyperoxic anoxia' was not the cause of the acute phase of oxygen toxicity. While it has not been entirely ruled out that certain enzymes, either sulphydryl or non-sulphydryl, may be inactivated by, and cause the symptoms of hyperoxia, this has yet to be demonstrated. It appears very unlikely that oxygen toxicity is caused by a failure of carbohydrate metabolism.

A more general theory of the cause of oxygen toxicity was suggested by Gerschman _et al_ (1954a). These authors proposed that the toxic action of oxygen resulted from the formation of reactive

oxygen radicals. Although this theory was greeted with some scepticism at first, indirect evidence is now accumulating in favour of this theory, which must now be considered as the probable basis for the toxic action of oxygen. Physiological factors may be contributory towards oxygen toxicity in mammals, but oxygen is toxic to all living organisma, and also mammalian cells in tissue culture, so there must also be a common underlying mechanism. From a logical standpoint, the basic mechanism of oxygen toxicity must occur either through a physical or chemical effect of oxygen. While some of the effects of oxygen may be physical: e.g. possibly the effect of oxygen on ATPases (Koehler & Gottlieb 1972; Hemrick & Gottlieb 1977), most of the effects of oxygen must arise by oxidation processes of some description. This being the case, oxygen must react according to strict chemical principles, and as explained in the introduction, the reactivity of oxygen is mediated mainly by radical mechanisms. Singlet oxygen (a species which until recently was only of passing interest to astrophysicists; Kasha & Khan 1970), may possibly be involved, but there is as yet little direct evidence to connect this species with oxygen toxicity. Complexes of transition metals or natural chelates with reactive intermediates from the univalent reduction pathway, or the reactive intermediates *per se* may also be involved in oxygen toxicity. While all of the reactive intermediates discussed above and in the introduction, at present, appear the most likely species responsible for the toxicity, direct evidence for their involvement has yet to be demonstrated.

Some evidence compatible with the view that oxygen toxicity resulted from the action of reactive oxygen radicals, was provided by Fridovich and co-workers following the discovery of SOD and its function. Fridovich (1975) reported that SOD was not found at all in obligate anaerobes, but was present in aerotolerant and aerobic

microorganisms. Moreover SOD was induced in Streptococcus faecalis, E.coli B, and Saccharomyces cerevisiae due to molecular oxygen and other substances which increased the intracellular superoxide concentrations. All of these results were compatible with the view that superoxide was involved in the propagation of the noxious effects of oxygen on microorganisms. It seemed likely that superoxide would play a similar role in more complex forms of life. Further investigations were therefore undertaken on the induction of SOD and other enzymes postulated to be involved in the systemic defence against oxygen toxicity in mammals (Crapo & Tierney 1974; Kimball et al 1976; Crapo & McCord 1976; Rister & Baehner 1976; Frank, Bucher & Roberts 1978). The work of these above authors demonstrated that varying induction of SOD took place. The few determinations which had been performed on mice were reported by Crapo & Tierney (1974) as part of a survey on several mammals. Publications concerning the effect of chronic hyperoxia on *in vivo* alterations on catalase levels were similarly sparse.

The results of the present study indicated that there was no increase in concentration of either of these enzymes in mice following exposure to 0·85 atm oxygen. The level of SOD appeared to decrease by the third day of exposure and then return to a normal concentration. The increase in SOD coincided with a decrease in protein suggesting that SOD was specifically induced to restore the levels to normal. This result is in accord with most of the other studies which reported either that the levels of SOD remained approximately constant or showed a slight rise in SOD. the remaining study by Kimball *et al* (1976) reported a large rise in SOD as a result of a hyperoxic exposure of rats. An induction of SOD in mammals further supports the theory that superoxide is involved in oxygen toxicity. It should be noted however, that these results are

not proof of the involvement of superoxide anion, nor do they necessarily infer that the superoxide anion *per se* is the species directly responsible for the biochemical lesions of oxygen toxicity.

The present study indicated that catalase concentration decreased throughout the exposure in all tissues investigated. The *in vitro* experiments of Dickens (1946) and Stadie *et al* (1945c) failed to show any effect of OHP on catalase, suggesting that the decrease in catalase was due to cellular control rather than to a direct action of oxygen. However, catalase activity was found to diminish on overnight storage (personal observation), and Aebi (1974) also states that catalase activity rapidly declines in dilute solution, suggesting that catalase is sensitive to oxygen. If SOD is a protective enzyme and is specifically induced, and catalase is also a protective enzyme, why is catalase not also induced? Dickens (1946) noted that the catalase activity of a tissue was not proportional to i susceptibility to cellular oxygen toxicity as determined by the rate of decrease of oxygen consumption by tissue slices exposed to OHP. Also the subcellular distribution of catalase is different to that of SOD. The function of catalase may therefore be to remove hydrogen peroxide produced as a result of certain compartmentalised reactions rather than the general removal of hydrogen peroxide from all parts of the cell. Glutathione peroxidase is also capable of disproportionating hydrogen peroxide to water and oxygen in addition to the reduction of hydroperoxides (Flohé *et al* 1979) and has a subcellular distribution which is complementary to that of catalase.

An increase in glutathione peroxidase was noted in rats exposed to OAP (Kimball *et al* 1976) and in neonatal rats but not adult rats or other adult rodents (Frank, Bucher & Roberts 1978). Bassett & Fisher (1979) reported a two-fold stimulation of the pentose phosphate pathway and a stimulation of NADPH turnover in perfused

rat lung. The pentose phosphate pathway results in NADPH formation, postulated to keep glutathione in a fully reduced state. A paper by Burk <u>et al</u> (1978) reported the involvement of two glutathione peroxidases, one selenium dependant and one selenium independent, in the removal of hydrogen peroxide and hydroperoxides. The selenium independant glutathione peroxidase was much more efficient in reducing organic peroxides, whereas the selenium dependant enzyme utilised either hydrogen peroxide or organic hydroperoxides as substrates. Infusion of control livers with hydrogen peroxide resulted in a proportional release of oxidised glutathione, but selenium deficient livers released no oxidised glutathione in response to hydrogen peroxide infusion, and also had an increased catalase content. Both selenium deficient and control livers released oxidised glutathione in response to infusion of hydroperoxides. These findings suggest that glutathione peroxidase also plays a part in the cellular defence system against oxygen toxicity, with respect to the removal of hydrogen and organic peroxides, and is probabaly more important than catalase.

While the effects of OAP on SOD and catalase were similar to previous reports, the changes noted in protein are relatively novel. The only studies reported on protein metabolism as yet are those of Gacad & Massaro (1973) and Massaro & Massaro (1974), who demonstrated that in lungs of rats exposed to OAP, protein synthesis, monitored by the incorporation of ^{14}C-leucine, was diminished after 24 and 48 hours exposure but had risen to greater than control levels after 96 hours. In the present study, protein determinations showed that in lung, total protein rose after 72 hours exposure, had decreased from this level by 6 days, but was still greater than control values. The difference between the present study and those of Massaro and co-workers can be explained either by exudation of plasma proteins

into the lung (Välimäki *et al* 1974) or by concurrent changes in protein catabolism. A similar trend in protein levels was also noted in mouse brain, which must be assumed to result from an increase in synthesis/catabolism up to day 3, and the reverse from day 3 to day 6 of the exposure. The changes seen in liver were different to those in lung and brain, and were thought to arise mainly as a result of the anorexia of the mice, and resultant use of the liver as a store. These results would tend to indicate that in chronic exposures to oxygen, there are changes in protein metabolism which are not necessarily consistent from organ to organ, and that determination of changes in protein synthesis and protein catabolism as a result of oxygen toxicity would prove instructive.

The investigation of tissue amino acid levels in the present study showed that there were few if any changes occurring in amino acid metabolism before the seventh day of exposure. There appeared to be a slight rise in brain GABA levels by day 3 which continued to the seventh day of exposure, a result consistent with the work of Schäfer (1978) who also noted an increase in brain GABA levels in rats subjected to OAP. This increase in GABA is difficult to reconcile not only with the decrease in brain GABA demonstrated by Wood & Watson (1963) in rats subjected to OHP, but also with subsequent work by Wood *et al* (1966; 1975); Wood & Watson (1964); Tunnicliff *et al* (1973; 1974). These authors demonstrated an inactivation of glutamic acid decarboxylase, the enzyme synthesising GABA, by oxygen *in vitro* but could show no effects of oxygen on GABA α-oxoglutarate aminotransferase, the enzyme concerned with GABA degradation.

The remaining amino acids showing any changes did not do so until the seventh day of exposure. The branched chain amino acids valine, leucine and isoleucine showed a decrease in liver which was

thought to indicate a decrease in protein degradation. The rise in lung glutamate was thought to be due to an impairment of the citric acid cycle, while the decreases in glycine noted in lung and muscle were thought to be possibly due to a reduced transamination of glyoxalate to glycine. In view of the lack of similar changes from organ to organ and the late occurrence of the few changes noted, it is thought that oxygen toxicity causes relatively little disruption of mammalian amino acid metabolism. This is in contrast to the situation in microorganisms, where Brown (1977) had demonstrated using minimal media that the biosynthesis of 10 amino acids were inhibited by OHP. None of these amino acids are synthesised in mammals: they are all essential dietary requirements.

The study of the action of prostaglandin inhibitors on the course of oxygen toxicity revealed only a small effect in extending the life of the mice used in this study, and that at the death of the experimental animals, no gross differences were observed between the treated and untreated animals. However, this experiment did provide some limited quantitive support for the observations recorded during earlier experiments, both in the apparently beneficial initial effects from oxygen, and the subsequent deterioration in condition. Exposure to oxygen caused significant increases in dry lung weight, wet lung weight, percentage lung water content, and substantial decreases in liver weight and total body weight. Studies by Klein _et al_ (1978); Bakhle _et al_ (1979) showed that oxygen inhibits prostaglandin dehydrogenase, thus delaying clearance of prostaglandins by the lung. While it is known that prostaglandins have inflammatory, vasoconstrictive and vasodilative actions, the contribution of this lesion to oxygen toxicity can only be guessed at. Should a specific inhibitor for this dehydrogenase be found, the effects of this inhibition could then be determined.

Many studies into oxygen toxicity have reported physiological and pathological changes in the cardiovascular system. The present study is no exception. The increase noted in haematocrit suggested that either there was a considerable increase in the number of erythrocytes circulating in the body, or that the raised haematocrit reflected a decrease in plasma volume. In view of the previously reported effect of oxygen on lung protein metabolism (Gacad & Massaro 1973; Massaro & Massaro 1974), and the changes in tissue water content reported in the present study, the latter possibility appears more likely, Increases in haematocrit after OAP exposures were similarly noted by Shaw & Leon (1970) and Brashear (1977). These observations suggest that the distribution of body fluid should be investigated, and the changes in the proportions of intracellular/interstitial/intravascular water compartments determined, as any changes in fluid balance will affect measurements of other metabolite concentrations. In addition to the rise in haematocrit, there was a small but significant decrease in the ratio of haemoglobin/haematocrit (a measure of the amount of haemoglobin per erythrocyte). This change could represent either an adaptive measure of the body to regulate the amount of oxygen carried by the blood, or alternatively an increase in erythrocyte volume.

The most promising determinations performed in the present study, were those undertaken on plasma retinol levels which had shown a highly significant decrease (to 52% of control values) within 5 days of exposure to 0•85 atm oxygen. In the second series of experiments, the experimental values at 5 days exposure ranged from 36% to 71% of control values, while the control range was 90-107% of the mean. It is possible that the change might occur at an earlier time in some animals, and that if, as suggested above, there is a haemoconcentration this would tend to make the values

appear higher than they actually are. This is one of the largest *in vivo* biochemical alterations yet recorded as a result of toxic exposure to oxygen, and paralleled the physical condition of the group of animals under study. Since these results were obtained towards the end of this project, the determinations have, as yet, been performed on only a small number of animals and at relatively few points on the time course. Much work still needs to be performed therefore, to more accurately determine the time course of the changes and to correlate them with another physiological parameter representative of the toxicity e.g. decrease in body weight, or increase in pulmonary tissue volume.

At the present times it is not possible to say whether the decrease in the vascular transport of retinol arose as a result of liver malfunction, lack of synthesis of retinol binding protein, or interference with liver retinol stores. Determination of the retinol status of other tissues e.g. lung, brain or eye, should also be instructive.

Unsaturated lipids are potentially reactive to oxygen and the resultant formation of peroxides. While peroxide formation has been investigated chemically in relation to rancidity of fats and oil based products, research into lipid peroxidation in relation to living organisms is relatively new, with very little research as yet directed towards possible effects of *in vivo* peroxidation on metabolic processes and cellular organisation.

Several researches suggest that lipid peroxidation should be investigated in relation to oxygen toxicity. The most compelling evidence was provided by electron micrographic studies of the pathology of oxygen toxicity (see introduction section 2.B. for details and references). In these studies, one of the principal effects seen was the loss and distortion of subcellular structure.

As most of the subcellular structure is provided by lipid membranes, this indicated alterations in either lipid structure or composition had occurred. In vitro biochemical researches by Chio & Tappell (1969); Roubal & Tappell (1966a; 1966b) showed that in systems where lipids were undergoing peroxidation, proteins and amino acids were denatured, and turned into firmly crosslinked polymeric lipoprotein products which showed close similarity to fluorescent 'age-pigments' (Chio & Tappel 1969). A study by Cortesi & Privett (1972) revealed that when ozonides and peroxides of methyl linoleate were injected intravenously (0·07 mmol/100g), death of the animals resulted within 24 hours, the principal pathology being inflammation and oedema of, and accumulation of fluid in the lungs. Significant changes occurred in the lipid composition of serum and the lung, but only small quantities of peroxides were detected, indicating that the injected ozonides and hydroperoxides were destroyed in the tissues. Despite this evidence in favour of the occurrence of lipid peroxidation, the studies of Joanny et al (1971) and Mengel & Kann(1966) in which lipid peroxides were assayed after exposure to OHP, only small increases were noted. This was probably due to the toxicity of the peroxides and their rapid clearance.

Oxygen is 7-8 times more soluble in non-polar media than in polar ones. The only non-polar region of the cell is the hydrophobic midzone of the cell's membranes, and that is the region where the cell constituents most likely to undergo damage, i.e. the unsaturated fatty acids, are located. Average tissue pO_2's may therefore be a misleading indication in predicting the type of reaction which may occur and how the reaction is terminated (Demopoulos 1973).

The above evidence suggests that further research into the effect of oxygen on increased rates of lipid peroxidation resulting from exposure to hyperoxia may be very fruitful in providing answers to the problems of cellular mechanisms of oxygen toxicity.

This study has reviewed the chemical basis for the reactivity of oxygen, the symptoms and pathology of the toxic action of oxygen and the biochemical changes noted as a result of toxic exposures to oxygen.

The experiments reported in the present work have indicated that the protective enzyme SOD maintained its cellular concentration, but that the cellular concentration of catalase was reduced as a result of exposure to oxygen. That there are indications of alterations in protein metabolism and water balance, but that amino acid metabolism does not appear to be greatly affected by oxygen in mammals. The largest effect seen was the effect of oxygen on the levels of plasma retinol which were markedly reduced. No significant changes were noted in any of these parameters in body fluid samples from human subjects exposed to a mild hyperoxia.

The experimental work presented, and the literature reviewed suggest that, in the absence of suitable changes in other parts of metabolism to explain the cellular toxicity of oxygen, that lipid peroxidation and its resultant effects on cell structure and function, is the most promising area for further research.

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