Biochemistry and Regulation of Liver Regeneration.

By

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Abbreviations

Abbreviations used in this thesis are as laid down in the Biochemical Journal Instructions to Authors (1978) with the following additions.

BSP	Bromsulphalein
PMS	Phenazine Methosulphate
POPOP	1, 4-di-[2-(5-phenyloxazolyl)]-benzone
PPO	2, 5-diphenyloxazole.
PGE	Prostaglandins series E
PGF	Prostaglandins series F
PGH	Prostaglandins series H
PGA	Prostaglandins series A
PGB	Prostaglandins series B

CONTENTS

1. INTRODUCTION

1. INTRODUCTION

Loss of a large amount of liver tissue sets in motion a sudden burst of mitotic activity in the remnant, during which the incidence may be in the range of 200 to 300 per 10,000 cells (Abercrombie and Harkness, 1951; Grisham, 1962). This response is highly reproducible and specific, growth being undetectable in other organs (Bucher and Malt, 1971). By contrast, in normal liver, mitosis is rare: only one or two mitotic figures can be seen in 10,000 cells. The hepatocytes are long-lived and may survive throughout the adult life of the animal. The calculated average life-span of the rat hepatocyte is at least 150 days (Brues and Marble, 1937; Swick et al., 1956).

This process of compensatory growth is called "liver regeneration". This is a misnomer. If a liver lobe is excised, a new lobe does not form at the site of the wound. Instead, the other intact lobes enlarge by hyperplasia until the original mass of the liver is attained. The process can be best described as compensatory hyperplasia. However, since the term "liver regeneration" is deeply embedded in the literature it will be used in this thesis.

1.1 Historical

It was discovered as early as 1883 that surgical damage to the liver was followed by a process of repair (Colucci, 1883; Tizzoni, 1883). Von Podwyssozki (1886) removed small pieces of tissue from the livers of rats and rabbits. Within two and a half days of operation mitosis was observed in the hepatic cells adjacent to the wound and often some considerable distance away from it. Ponfick (1889, 1890) removed over two-thirds of the liver in rabbits and dogs. He reported a three-fold increase in the size of the remaining organ. These observations were confirmed by Von Meister (1894), who further discovered

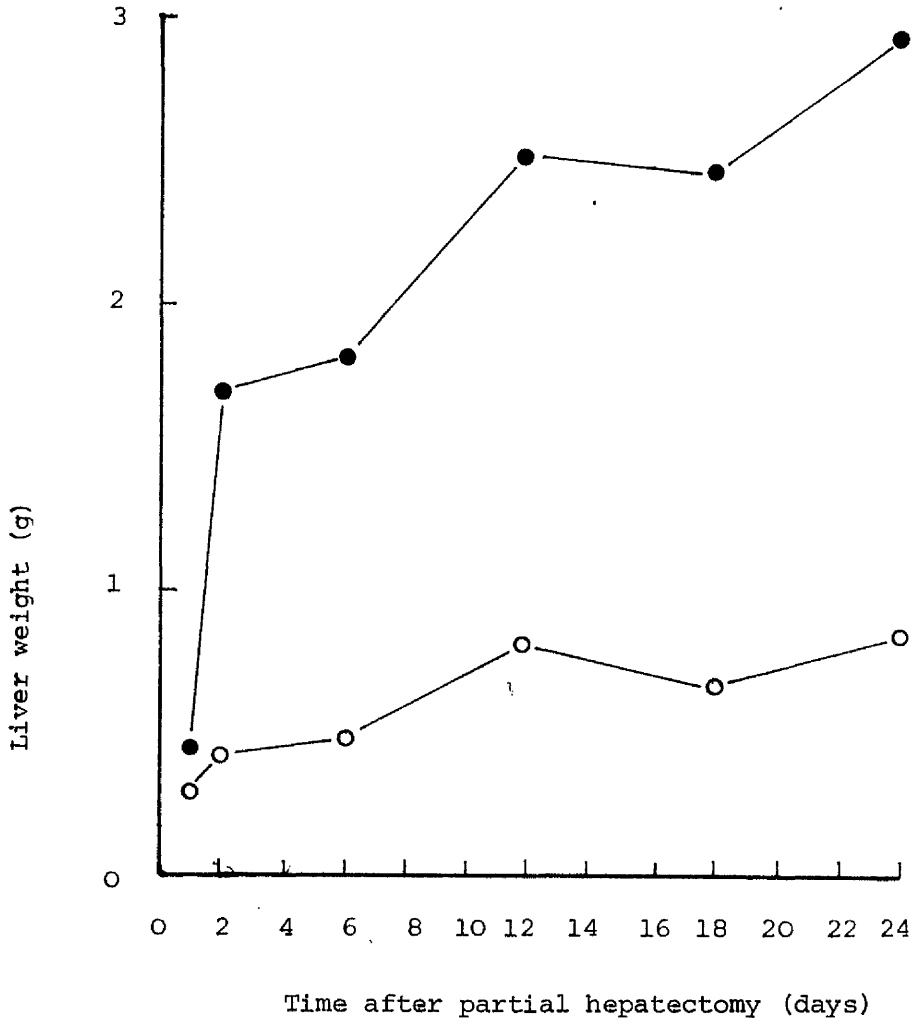
that an animal could be subjected to repeated partial hepatectomy and that on each occasion the residual liver would grow to the size of the original intact organ. Eventually the liver could be pared down to one lobe by repeated partial hepatectomy and only technical difficulties concerned with haemostasis seemed to prevent indefinite repetition of the process. Fishback (1929) performed similar experiments and obtained similar results. He reported that growth was more rapid when large amounts of liver were removed.

Chemical damage to the liver is followed by a similar sort of repair. So is infarction. Whipple and Sperry (1909) produced varying degrees of necrosis in the liver of dogs by prolonged chloroform anaesthesia. The undamaged cells multiplied rapidly and regeneration was usually complete within three weeks. Rous and Larimore (1920) showed that,in rabbits, ligation of the branch of the portal vein supplying one of the liver lobes caused atrophy of that lobe, and this was followed by hypertrophy of the other lobes.

Higgins and Anderson (1931) were the first investigators to make a systematic study of the rate of growth in the remaining liver fragments of rats after two-thirds hepatectomy. They found that the remaining fragment almost doubled in weight during the first two days. Thereafter there was a period of relatively slower growth which continued at a diminishing rate for about three weeks, by which time the liver remnant was about the size of the original intact organ (Figure 1). These findings were later confirmed by Brues et al., (1936).

By about 1940 therefore, it had become evident that damage to, or removal of, a substantial part of the liver was followed by a process of growth in the surviving liver tissue. Since then liver regeneration has been demonstrated in response to various forms of liver damage in several animal species. These include frogs, birds, dogs, some hibernating

Figure 1



Restoration of liver weight per one hundred g body weight following partial hepatectomy in rats (Dry weight O-O, wet weight ●-●; Redrawn from Higgins and Anderson, 1931).

ground squirrels, mice and even man (Williams, 1961; Pack, et al., 1962 Sigel, 1963, Leduc, 1964; Thomson and Clarke, 1965; Bade, 1967). The great majority of studies, however, have been carried out on rats using the Higgins and Anderson (1931) method of two-thirds hepatectomy.

1.2 Histological and Ultrastructural changes

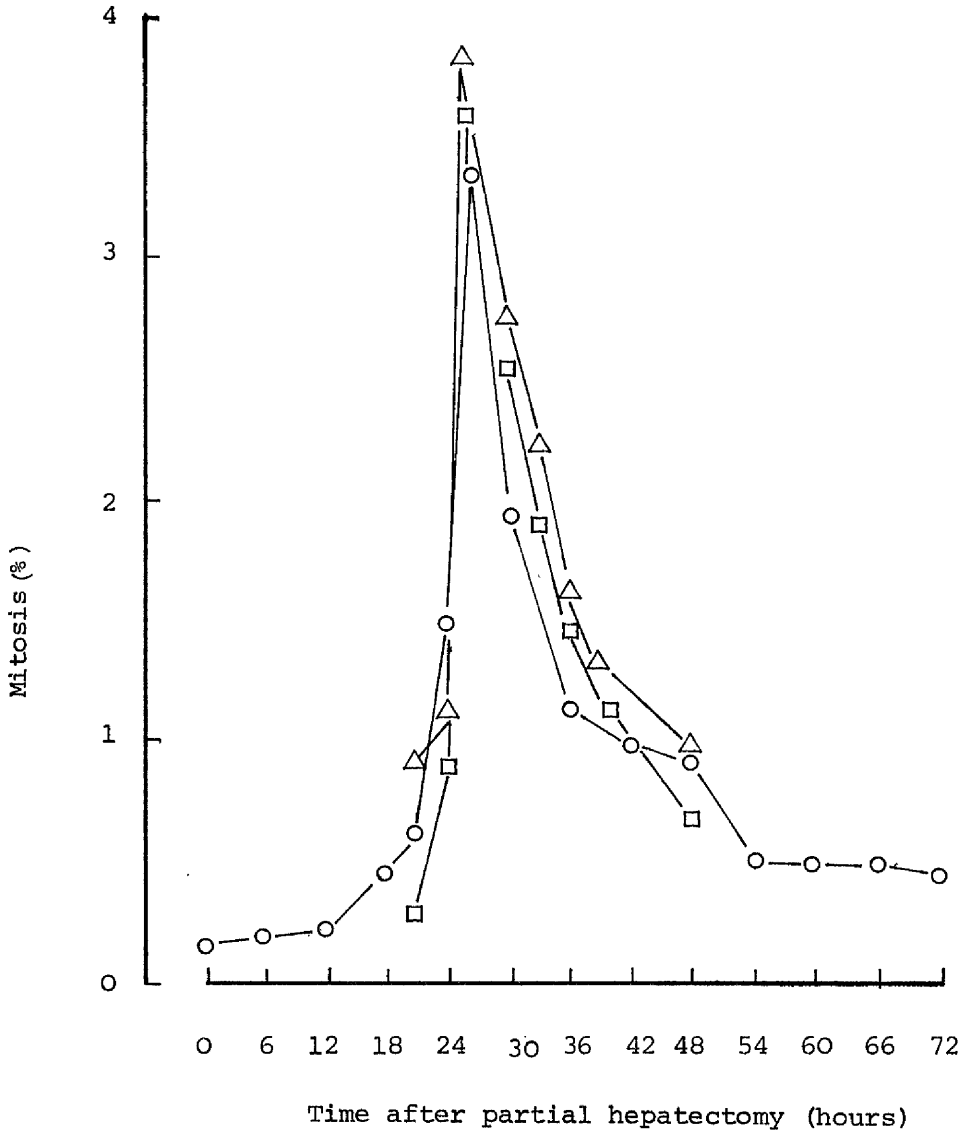
At microscopic level, the changes following partial hepatectomy are first seen in the parenchymal cells, which constitute 90 to 95 per cent of the total hepatic cellular volume but only 60 to 65 per cent of the total cell population (Abercrombie and Harkness, 1951; Daoust and Cantero, 1959; Steiner et al., 1966).

1.2.1 Mitosis

Growth in the liver remnant after partial hepatectomy is accomplished by cell division rather than cell enlargement. Initially, however, cell division lags about twelve to eighteen hours behind cell enlargement. Increased mitosis is first seen in parenchymal cells twenty-four hours after partial hepatectomy. The frequency of mitosis increases sharply to a maximum of about 300 to 400 per 10,000 cells around thirty hours. Thereafter it declines, rapidly at first, then slowly during the next several days (Figure 2; Abercrombie and Harkness, 1951; Grisham, 1962). Some workers have however reported that the peak of mitosis occurs at later times, but the patterns and frequency of mitosis is more or less the same (Johnson and Albert, 1952; Jaffe, 1954).

The increase in non-parenchymal cells lags behind the increase in parenchymal cells. Two days after partial hepatectomy an increase in the frequency of mitosis is seen in the littoral cells, bile duct cells, and in the mesothelial lining cells. Mitosis is also observed in the cells of the periportal connective tissue and of the endothelium of blood

Figure 2



Percentage of hepatocytes in mitosis following two-thirds hepatectomy. Redrawn to show sharp peaks and good agreement in the results of three different groups (Abercrombie and Harkness, 1951; △-△; Weinbren, 1959 □-□; Grisham, 1962 ○-○).

vessels other than the sinusoids (Abercrombie and Harkness, 1951; Grisham, 1962). Once liver regeneration is complete, the frequency of mitosis falls back to the normal of about 2 per 10,000 cells.

1.2.2 Ultrastructural changes

Within an hour of partial hepatectomy the endoplasmic reticulum, which normally appears as clumps in the cytoplasm, starts to disperse, and much of it disappears leaving an abundance of polyribosomes free in the cytoplasm. After nine to twelve hours, the residual endoplasmic reticulum begins to increase in amount and clusters of smooth endoplasmic reticulum begin to appear in close association with mitochondria. There is a tendency to form dilated vesicles instead of the flattened cisternae characteristic of normal liver. The ribosomes may attach to these later (Jordan, 1964; Bucher, 1967; Claude, 1967; Becker and Lane, 1968).

Meanwhile the mitochondria of the hepatocytes become swollen and pale. Some show unusual dumb-bell shapes with derangement of internal architecture, but only a small fraction of the population is affected and normal mitochondria can be seen in the same hepatocyte (Jordan, 1964; Claude, 1967). There is an activation of lysosomes, which increase in size and number and develop into autophagosomes. They are most prominent six to nine hours after partial hepatectomy. Thereafter they gradually return to their original appearance (Becker and Lane, 1965; 1966; Bucher, 1967).

Cytoplasmic inclusion bodies or vacuoles of variable sizes, as well as fat globules, begin to accumulate as early as two to three hours after partial hepatectomy. Histochemical studies show that the fat globules become increasingly conspicuous while the glycogen stores are rapidly depleted, falling to very low levels by ten hours and later reappearing only gradually (Stowell, 1948; Harkness, 1957; Jordan, 1964). Hepatocytes are normally not pinocytic cells but become so after partial hepatectomy (Mori and Novikoff, 1977).

The nuclei and nucleoli of hepatocytes start enlarging within six to twelve hours and have doubled in size by twenty-four hours. During this period the sinusoidal spaces become progressively diminished as the large fatty hepatocytes crowd in (Stowell, 1948; Harkness, 1952). The litoral, bile duct, and connective tissue cells remain relatively unchanged.

However, most of these morphological changes are not unique to partial hepatectomy, because they are produced also by hypoxia, ischaemia, toxic injury or trauma (Fawcett, 1955; Bernhard and Rouiller, 1956; Bassi et al., 1964; Herdson et al, 1964). They can even be seen occasionally in the hepatocytes of normal liver, especially in cells at the centre of the lobule (Bassi et al., 1964; Laud, 1968).

1.3 Biochemical Changes

Marked biochemical changes occur in the liver remnant as early as one hour after partial hepatectomy. These presumably correspond to the morphological changes in the hepatocytes because the other cell types show little morphological response in the early stages of regeneration.

1.3.1 Lipids

The most obvious biochemical change is the deposition of lipids in the liver remnant. Soon after partial hepatectomy there is a largely hormone-dependent mobilisation of fat from adipose tissue (Camargo et al., 1966). Plasma free fatty acid levels rise and neutral fat accumulates in the liver remnant, rising to a peak of more than three times its normal level within twelve to eighteen hours (Camargo et al., 1966; Glende and Morgan, 1968; Fex and Olivecrona, 1968a). During regeneration the uptake of free fatty acids by the liver remnant and their esterification increases, as does their synthesis (Fex and

Olivecrona, 1968a; 1968b; Neville et al., 1969). Total phospholipids remain about the same, but individual phospholipids undergo changes. Within twelve to eighteen hours of partial hepatectomy the levels of palmitic, palmitoleic, oleic and linoleic acids are three to five times the normal amount, whereas stearic and arachidonic acids appear to be unchanged (Gilliam et al., 1968; Glende and Morgan, 1968). The significance of lipid deposition in the liver remnant during regeneration is not clear. Continuous infusion of glucose for twenty-one hours into partially hepatectomised rats prevents lipid deposition in the liver remnant, with little effect upon its regenerative capacity as evidenced by DNA synthesis (Simek et al., 1968). Fatty infiltration of regenerating liver may also be considerably reduced by adrenalectomy without impairing the regenerative response (Camargo et al., 1966). This suggests that lipid accumulation might merely be a result of an increased supply of free fatty acids to the liver remnant.

1.3.2 Glycogen

Glycogen depletion occurs in parallel with lipid deposition. The depletion is fast, and within ten hours the glycogen concentration is reduced almost to zero. This is followed by an irregular rise and even at seventy-two hours the glycogen concentration is reported to be only about one-third normal (Gurd et al., 1948; Navikoff and Potter, 1948; Harkness, 1952). The depletion of glycogen may be prevented by continuous infusion of glucose for twenty-one hours (Simek et al., 1968). This suggests that it may either be due to the loss of two-thirds of the glycogen stores as a result of partial hepatectomy, or a consequence of the decrease in plasma insulin and the increase in plasma glucagon which follow partial hepatectomy (Bucher and Swaffield, 1975; Leffert et al., 1975).

1.3.3 DNA Synthesis

It might be anticipated that an enormous increase in mitosis would require a correspondingly increased DNA synthesis. Studies with labelled precursors show that this is indeed the case. About sixteen hours after partial hepatectomy the rate of DNA synthesis suddenly starts to increase. The increase is rapid and reaches a peak of more than forty-fold at about twenty-two to twenty-four hours. This is followed by an equally rapid decline which later slows down, though the rate of DNA synthesis remains elevated for more than two days before returning to normal (Grisham, 1962; Bucher, 1963; Bucher et al., 1964; Edwards and Koch, 1964). The number of hepatocytes involved in active DNA synthesis at its peak may be as high as thirty percent (Grisham, 1962). However if [^{3}H]-thymidine is repeatedly injected into rats for about sixty hours following partial hepatectomy, almost 95 per cent of the cells become labelled, thus providing direct evidence that almost all cells replicate DNA at least once (Fabrikant, 1969).

Mitochondrial DNA synthesis occurs independently of nuclear DNA synthesis but precedes it (Schneider and Kuff, 1965; Nass, 1967). The rate of labelling of mitochondrial DNA is maximal around sixteen to eighteen hours after partial hepatectomy, when it shows an elevation of about ten-fold . The mitochondrial DNA content is restored to normal within three days (Nass, 1967).

The total DNA content of the liver remnant following partial hepatectomy increases roughly in parallel with the increase in cell number (Thomson et al., 1953). However, during the most rapid period of growth, the average DNA content per nucleus shows a substantial but temporary increase, variously reported at 20 to 80 per cent (Price and Laird, 1950; Thomson et al., 1953; Ultman et al., 1953).

1.3.4 RNA Synthesis

The rate of RNA synthesis, as reflected by incorporation of labelled precursors, has been reported to increase soon after partial hepatectomy and to level off at twice the normal value five hours after the operation (Fujioka et al., 1963). Hecht and Potter (1956), on the other hand, reported a continuous increase reaching a maximum at twenty-four hours. The rates of synthesis of all forms of RNA are reported to increase. The synthesis of nuclear RNA increases at about the same rate as the bulk of the cellular RNA but nucleolar RNA synthesis is more rapid (Hecht and Potter, 1956; McArdle and Creaser, 1963; Tsukada and Lieberman, 1964; Muramatsu and Bush, 1965; Jacob et al., 1967). Changes in RNA species and their distribution in the cells are also reported to occur during regeneration. In hybridization experiments, RNA from fourteen-day embryos was found to compete effectively with RNA from early regenerating liver. The synthesis of new molecules of RNA not present in normal or sham-operated animals was shown to begin within one hour of regeneration, although the RNA synthesis characteristic of normal liver continued. Some RNA's that in normal livers are restricted to the nucleus were found in the cytoplasm soon after partial hepatectomy (Church and McCarthy, 1967; 1969).

Although the rate of synthesis of RNA increases soon after partial hepatectomy, its concentration seems to remain somewhat depressed until about twenty hours. This would suggest that during regeneration the rate of RNA turnover is considerably increased. The relation of these changes to the initiation and control of regeneration is not however established. Fujioka et al., (1963) have reported that amounts of actinomycin-D and p-fluorophenylalanine which have no effect on RNA synthesis in normal liver completely suppress the increase in RNA synthesis after partial hepatectomy. This suppression was associated

with a delay in the start of DNA synthesis. It may be, therefore, that the increase in RNA synthesis is in part a preparation for DNA synthesis.

1.3.5 Nucleotides

The levels of ribonucleotides do not show large variations after partial hepatectomy, perhaps because they are present in sufficient amount to cope with a several-fold increase in RNA synthesis. The levels of CTP and UTP (normally 100 and 350 n moles per gram respectively) increase 50 to 100 per cent within three to six hours of partial hepatectomy (Bucher and Swaffield, 1966; 1969). The level of adenine nucleotides (AMP + ADP + ATP) is normally around three thousand n moles per gram liver. It does not rise, but the rate of turnover, as measured by uptake of label, is elevated during regeneration (Ove et al., 1967). Mandel et al., (1963) have however reported the levels of all four ribonucleotides to fall during regeneration. The deoxyribonucleotides arise from the ribonucleotides. In normal liver they are hardly detectable, but their levels show a several-fold increase during regeneration (Bucher and Malt, 1971). Thymidine and its nucleotides (dTMP + dTDP + dTTP) in normal liver altogether total about four n moles per gram (Gross and Rabinowitz, 1968). In twenty-four hour regenerating liver the level of dTTP alone is reported to be between five to seven n moles per gram (Bucher and Oakman, 1969). The significance of these changes is difficult to interpret. A fall could be attributed to an increased conversion to polynucleotides, with consequent depletion of mononucleotides. An increase could be explained quite plausibly as evidence of increased synthesis in anticipation of an increased demand. The enhanced supply of precursors may be an important factor in the progression of events enabling DNA synthesis to begin, but their role in the control of regeneration is not settled.

1.3.6 Cyclic Nucleotides

The levels of c-AMP and c-GMP show triphasic increases after partial hepatectomy. The level of c-AMP increases within two to four hours and then returns to normal by about eight hours after partial hepatectomy. It increases again at about twelve hours and returns to normal by seventeen hours of operation. A third peak occurs at about twenty-two hours and perhaps coincides with the peak of DNA synthesis. On all three occasions the increase is reported to be around two to three-fold. It has been suggested that the first peak is associated with the increased activity of protein kinase and ornithine decarboxylase (MacManus et al., 1972; 1973; MacManus and Whitfield, 1974; Thrower and Ord, 1974). The level of c-GMP first increases as early as fifteen to twenty minutes after partial hepatectomy and declines again at about one hour. (Miura et al., 1976). It shows a second peak at about thirteen hours and a third peak at about twenty-four hours. On all three occasions the increase is about two-fold (Goridis et al., 1977) An increase in cyclic nucleotides commonly precedes mitosis in many types of cells and the increase which occurs during regeneration is presumably related to the initiation of DNA synthesis. MacManus et al., (1972) have reported that changes in c-AMP levels and an increase in DNA synthesis similar to those following partial hepatectomy can be brought about by infusion of a hormone mixture into intact rats. But the role played by c-AMP in initiating DNA synthesis was not established in these experiments. Cyclic nucleotides therefore may or may not play an important role in liver regeneration.

1.3.7 Protein Synthesis

The rate of total protein synthesis, as measured by the uptake of ^{14}C-arginine, increases in the liver remnant after partial hepatectomy and is three times normal at twenty-four hours (Janne, 1967). The rate

of synthesis of phosphoproteins increases sharply, reaches a peak at twenty hours after partial hepatectomy and then gradually declines (Johnson and Albert, 1952). Studies with ^{14}C-leucine show that the rate of synthesis of serum albumin and fibrinogen is elevated three to four-fold during liver regeneration (Majumdar el al., 1967). However these changes are not unique to partial hepatectomy, as protein synthesis is elevated in rats after acute stress such as electric shock or intraperitoneal injection of celite. (McKenzie et al., 1963; Majumdar et al., 1967). Accumulation of some blood proteins by liver has also been shown to follow myocardial infarction and injury (Losner et al., 1954; Weimer and Benjamin, 1965). Narayan et al., (1968) reported that, whereas the level of serum high density lipoproteins falls sharply after partial hepatectomy, the low density lipoproteins level increases. Leffert et al., (1976) reported, on the contrary, that they fell, the extent of the fall depending on the amount of liver removed. These changes, in whatever direction they take place, may be important. Leffert and Weinstein (1976) reported specific inhibition of initiation of DNA synthesis by very low density lipoproteins in primary monolayer cultures of foetal rat hepatocytes.

1.3.8 Histones

The earliest change in nuclear histones after partial hepatectomy seems to be acetylation of arginine-rich histones. Within one hour it has more than doubled and the rate of deacetylation is strikingly reduced. The peak acetylation occurs at three to four hours and is followed by an abrupt decline. It appears to precede the rise in RNA synthesis (Pogo et al., 1968; Allfrey, 1969; 1970). Acetylation and phosphorylation of histones also increase but only after sixteen hours, with a peak at about twenty-two hours (Ord and Stocken, 1968; Allfrey, 1969; 1970). Somewhat later the methylation of histones is also increased and reaches a peak at thirty hours. This may be related to

changes known to occur in the nucleus prior to mitosis, such as condensation of the chromatin and curtailment of nucleic acid synthesis (Tidwell et al., 1968).

The primary role of histones is far from being settled. Histones are linked with DNA by electrostatic bonds that do not confer specificity and bear no evident relation to the DNA informational content (Bonner et al., 1968). However Allfrey (1969) has suggested that some degree of specificity may exist in histone-DNA bonding such as the lysine rich histones preferentially combining with AT-rich regions of DNA. He has also pointed out that cells involved in active growth actively modify their histones. It seems therefore uncertain whether histones play an important role in the control of gene activation and thus regulate liver regeneration.

1.3.9 Concentration of Cations

Following partial hepatectomy there are increases in the concentrations of Na, K, Zn and Cu of 15, 20, 100 and 100 per cent respectively. The concentrations of Fe and Mn show a decrease of forty and thirty per cent respectively. The concentrations of Mo, Co, Rb, Sc, As, Mg and Cs seem to remain unaltered. There is no fundamental difference between the results in male and female animals. However comparable changes in cation concentration also occur following sham operation (Volm et al., 1974). It seems therefore that these changes may be due to nonspecific effect of operation.

1.4 Enzyme Changes

Numerous changes in enzyme activity occur during liver regeneration. For the purpose of simplicity they can be arbitrarily divided into the following: -

(a) Changes in enzymes involved in nucleic acid biosynthesis.

(b) Changes in enzymes involved in other metabolic pathways.

1.4.1 Changes in Enzymes Involved in Nucleic Acid Biosynthesis

The first enzyme in the de novo pathway of pyrimidine synthesis, aspartate carbamoyltransferase, begins to increase immediately after partial hepatectomy. It reaches a maximum, twice normal, after forty-eight hours and then gradually returns to normal within fifteen days. The next enzyme, dihydroorotase, behaves similarly. Its activity reaches a three-fold maximum by forty-eight hours and then declines. The third enzyme, dihydroorotic acid dehydrogenase, however, remains unaltered (Bresnick 1965; Bresnick et al., 1968). The fourth enzyme is orotic acid monophosphate pyrophosphorylase. Its activity increases soon after partial hepatectomy and reaches a maximum of three to four times normal by twenty-four hours. The remaining enzymes (orotic acid monophosphate decarboxylase, uridine monophosphate kinase, uridine diphosphate kinase) leading to the formation of UTP from OMP do not change appreciably. It may be that they are present in the normal liver in adequate amounts (Bresnick, 1965; Fausto, 1969).

The enzymes involved in the so-called "salvage pathway" leading to the synthesis of UMP,dUMP and CDP are also increased during liver regeneration. Uridine phosphorylase shows a three-fold increase between thirty-six to seventy-two hours and deoxyuridine phosphorylase increases four-fold at about forty-eight hours. Similarly uridine kinase increases five-fold between twenty four to thirty-six hours and deoxyuridine kinase increases almost ten-fold during the corresponding period (Skold, 1960). The cytidine phosphorylating enzymes however show relatively small increases. Their activity goes up by thirty per cent and remains at this elevated level from twelve to forty hours (Baugnet-Mahieu et al., 1967).

Ribonucleotides are converted into deoxyribonucleotides by the action of ribonucleotide reductase. The reduction of CDP to dCDP

increases after about forty-eight hours of partial hepatectomy. The activity of ribonucleotide reductase becomes maximum around fifty hours when it may be as high as twenty times normal (King and Vanlancker, 1969; Larsson, 1969).

In normal liver, dTTP-forming enzymes such as dTMP synthetase, dTMP kinase, dTDP kinase, dTDR kinase and dCMP deaminase are hardly detectable,but during regeneration they increase by seven to fifteen-fold . The initial rise, though difficult to pinpoint precisely because of the variability among rats, probably occurs at about the same time in all five enzymes, about twelve hours after partial hepatectomy with a peak between twenty-four and forty-eight hours (Maley and Maley, 1960; Weissman et al., 1960; Fausto and VanLancker, 1965; Maley et al., 1965; Labow, et al., 1969).

Like the dTTP-forming enzymes , the polymerases responsible for linking the nucleotides together in a chain also show increased activity during regeneration. The activity of RNA polymerase, as measured in liver extracts, increases by more than two-fold and remains so between twelve to twenty-four hours (Bush, et al., 1962; Tsukada and Lieberman, 1964b; Ro and Bush, 1967). The activity of DNA polymerase increases about the start of DNA synthesis and continues to increase for at least six hours. Its activity remains at this elevated level for almost three days and then gradually declines (Fausto and VanLancker, 1965; Labow et al., 1969; Ove et al., 1969).

Changes in the nucleases are uncertain. Ove et al., (1969) found an inverse relation between the rate of DNA synthesis and the activity of acid deoxyribonuclease. However earlier reports suggested that the activity of acid deoxyribonuclase increases soon after partial hepatectomy and remains elevated for about three days (Brody, 1958;

Brody and Balis, 1959; Adams, 1963). Alkaline ribonuclease activity remains unaltered or shows a small decrease during the first day and then increases (Shortman, 1962; Maor and Alexander, 1968; Rahman et al., 1969).

The enzymes degrading uracil and thymine (dihydrouracil hydrase, dihydrouracil dehydrogenase, β -ureidopropionase) all decrease rapidly to between 40 to 60 per cent of normal within twenty-four hours after partial hepatectomy. Dihydroprimidinease also shows a 25 per cent fall. All these enzyme activities gradually return to normal after four to six days (Canellakis et al., 1959; Fritzson, 1962).

The significance of these changes in enzyme activities is not established. Actinomycin-D, given before the rise in nucleic acid-forming enzymes, delays the onset of DNA synthesis more effectively than when given a few hours later, presumably by inhibiting enzyme synthesis. On the other hand, the enzyme activities remain elevated long after the most active phase of DNA synthesis has ended. They cannot therefore be the only factor controlling it.

1.4.2 Changes in Enzymes involved in other Metabolic Pathways

Various enzymes not involved in nucleic acid metabolism show altered activity during liver regeneration. The first detectable change occurs in the activity of c-AMP dependent protein kinase immediately followed by an increase in ornithine decarboxylase activity. Both these enzymes show peaks at four and again at fourteen hours after partial hepatectomy (Byus et al., 1977). Guanylate cyclase activity increases more than three-fold and is maximal at eight hours after partial hepatectomy. It returns to normal at the time of increased DNA synthesis, before the proliferative response is initiated (Goridis et al., 1977).

As early as 1948 Navikoff and Potter reported that the activities of a wide range of mitochondrial enzymes remain depressed in the twenty-four hours following partial hepatectomy. Similar results were later reported by Von der Decken and Hultin (1960). Various lysosomal hydrolases have also been studied (acid phosphohydrolase, β--glucosidase, β-glucuronidase, β-galactosidase). Their activities show a 100 per cent decrease (Verity et al., 1975). Catalase activity decreases to less than 50 per cent of its original level during the first three days of regeneration and gradually returns to normal in about two weeks (Stein et al., 1951). On the other hand, alkaline phosphatase activity increases more than three-fold within forty-eight hours, with a gradual decrease to normal in about nine days (Rosenthal et al., 1952). Similarly hyaluronidase, cathepsin-D and glutamic-oxaloacetic transaminase activities increase during the first twenty-four hours after hepatectomy (Grisham, 1962, Fiszer-Szarfarz et al., 1977). The activities of several other enzymes, such as transaldolase, transketolase, lactate dehydrogenase, cytochrome oxidase, (Na^+-K^+)ATPase, palmitoyl-CoA synthetase, glycerol phosphate acyltransferase and diglyceride acyltransferase, do not change appreciably.

The activities of hepatic microsomal drug-metabolizing enzymes is markedly reduced during regeneration. Full recovery occurs at about the same time as complete restoration of hepatic mass. The extent of the decrease and the rate of recovery vary according to the drug used. During the first few days of regeneration the remaining liver tissue shows a markedly reduced ability to metabolize hexobarbital, chlorpromazine, p-nitrobenzoic acid, codeine, aniline and p--nitroanisole (Fouts et al., 1961; Gram et al., 1968). Nonetheless regenerating liver, like foetal or neonatal liver, although exhibiting low levels of microsomal enzymes, has the capacity to respond to

enzyme-inducing agents such as phenobarbital and 3-methylcholanthrene (Fouts et al., 1961; Gram et al., 1968). The significance of these changes in relation to liver regeneration is obscure. However it appears that the metabolic capacity of the liver remnant after partial hepatectomy is reduced and the balance between catabolic and anabolic processes is shifted towards the latter, at least during the most rapid period of growth.

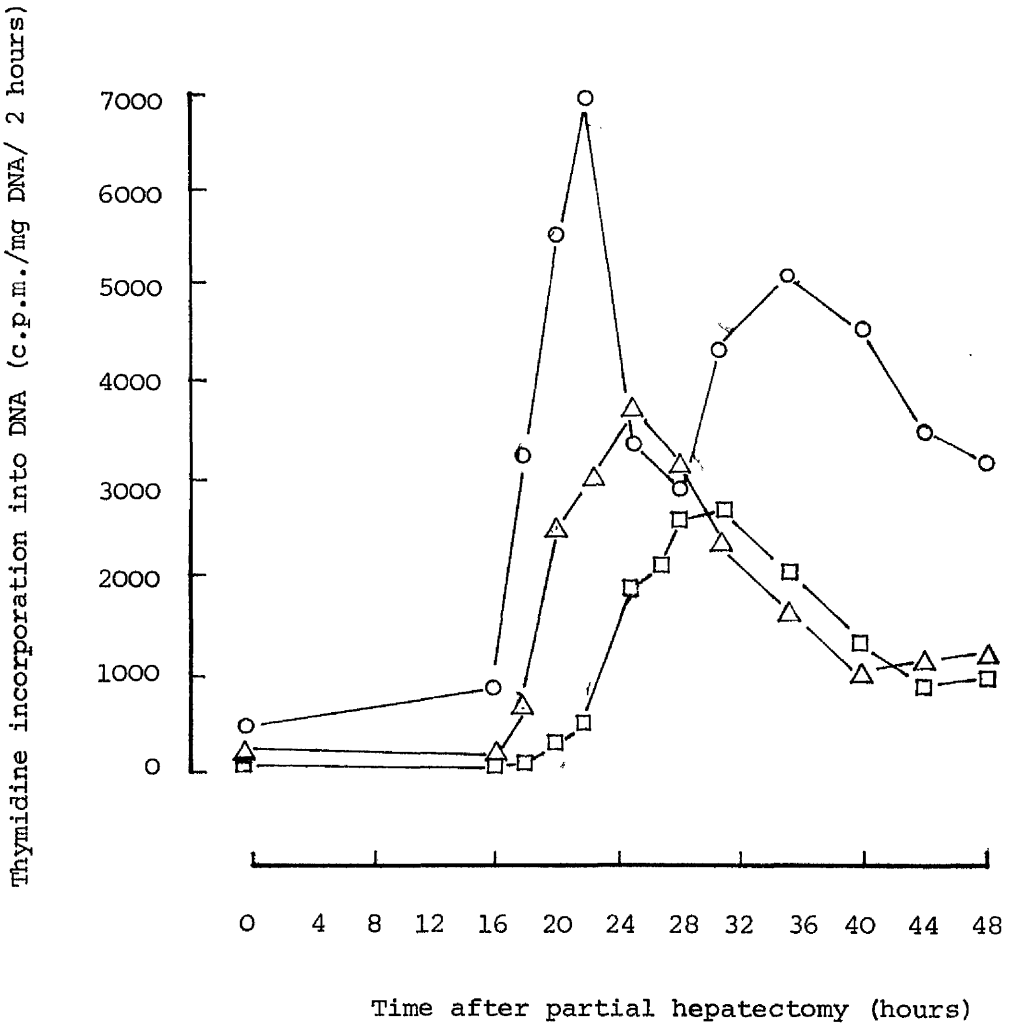
1.5 Modifying Influences

Under carefully controlled conditions the liver responds to partial hepatectomy in a well-defined manner. The sequence, the extent and the timing of almost all changes during regeneration are definite and predictable. However, a number of factors affect this response to varying degrees. These may explain some of the discrepancies in the results of different workers.

1.5.1 Age

The age of the experimental animal significantly modifies the regenerative response. Young rats restore tissue mass and cell population more rapidly than adults. Young adults and old rats restore tissue mass at similar rates but the old rats lag behind the young adults in new cell population (Bucher and Glinos, 1950; Post et al., 1957b; 1960). The rate of DNA synthesis, as measured by the uptake of labelled precursors, is also affected by the age of the animal. In weanlings the rate of DNA synthesis shows a biphasic increase with sharp peaks occuring at twenty and at thirty-five hours after partial hepatectomy. In young adults and old rats the peak of DNA synthesis is not only considerably smaller than in weanlings but it is also delayed and occurs only once, at twenty-five hours in young adults and, at thirty-two hours in old rats (Figure 3; Bucher, 1963).

Figure 3



Effect of age on DNA synthesis rate in regenerating rat liver (Weanlings O-O; young adults △-△; One year or more old rats □-□; Redrawn from Bucher, 1963).

1.5.2 Hormones

Liver regeneration can be modified considerably by the hormones of pituitary, adrenals, thyroid and especially pancreas but the general conclusion reached by Harkness (1957) that none of the endocrine glands are essential to the process still stands. Hypophysectomy delays the onset of DNA synthesis by fifteen hours but does not affect its rate (Doljanski and Novogrotzky, 1959; Rabes and Brandle, 1969). Administration of growth hormone results in a higher and more prolonged rise in the rate of DNA synthesis and a wave of mitosis several times greater than normal (Cater et al., 1957). Treatment with growth hormone also stimulates mitosis and nucleic acid synthesis during repair following carbon tetrachloride injury (Post et al., 1957a). Adrenalectomy retards restoration of tissue mass and protein and the deposition of lipids after partial hepatectomy but the mitotic response is normal or possibly somewhat elevated (Roberts, 1953; Tipton et al., 1959; Hemingway, 1960). Injection of glucocorticoids such as cortisone stimulates the incorporation of labelled precursors into RNA and proteins (Feigelson et al., 1962) but depresses the mitotic response (Einhorn et al., 1953). Cortisone treatment also results in the production of new species of RNA in the liver remnant (Drews and Brawerman, 1967). Injections of epinephrine during the first day of regeneration greatly delay the rise in DNA synthesis (Sakuma and Terayama, 1967). Removal of parathyroid glands prior to partial hepatectomy depresses and delays the onset of DNA synthesis. However this effect is only observed if the glands are removed twenty four-hours before partial hepatectomy. Removal twelve hours before partial hepatectomy neither diminishes nor delays the increase in DNA synthesis (Rixon, 1974). Pancreatectomy impairs the process of liver regeneration but the role of pancreatic hormones is controversial. Some workers have reported that

the impairment of regenerative response in pancreactomised rats could be reversed by insulin infusion (Ozawa et al., 1974; Starzl et al., 1975; 1976a; 1976b). Others have reported glucagon to be more potent in this respect (Whittemore et al., 1973; 1975; 1976; Price, 1976). Others again have failed to observe either of these effects and have suggested that portal blood probably carries some other hepatotrophic factor which influences regeneration (Chandler, 1976; Duguay and Orloff, 1976; Sakai et al., 1976).

1.5.3. Portal Blood

Portal blood is an important modifier of liver regeneration. Diversion of portal blood to only one of the liver lobes triggers a regenerative response in that lobe accompanying atrophy of those which have been deprived (Weinbren, 1967; Weinbren et al., 1972). Liver isografts fail to regenerate in response to partial hepatectomy or the response is greatly diminished if they are deprived of portal blood (Lee et al., 1969; Chandler et al., 1971; Starzl et al., 1975). Studies involving modification of the portal blood supply in which only some lobes of the liver receive portal blood while the others are supplied with systemic blood have shown that after partial hepatectomy the lobes which receive portal blood proliferate while those receiving systemic blood do not (Starzl et al., 1973). Further experiments in which some lobes received intestinal blood while the others received blood from the stomach, pancreas, spleen and duodenum suggested that pancreatic blood might be particularly important for liver growth (Starzl et al., 1975).

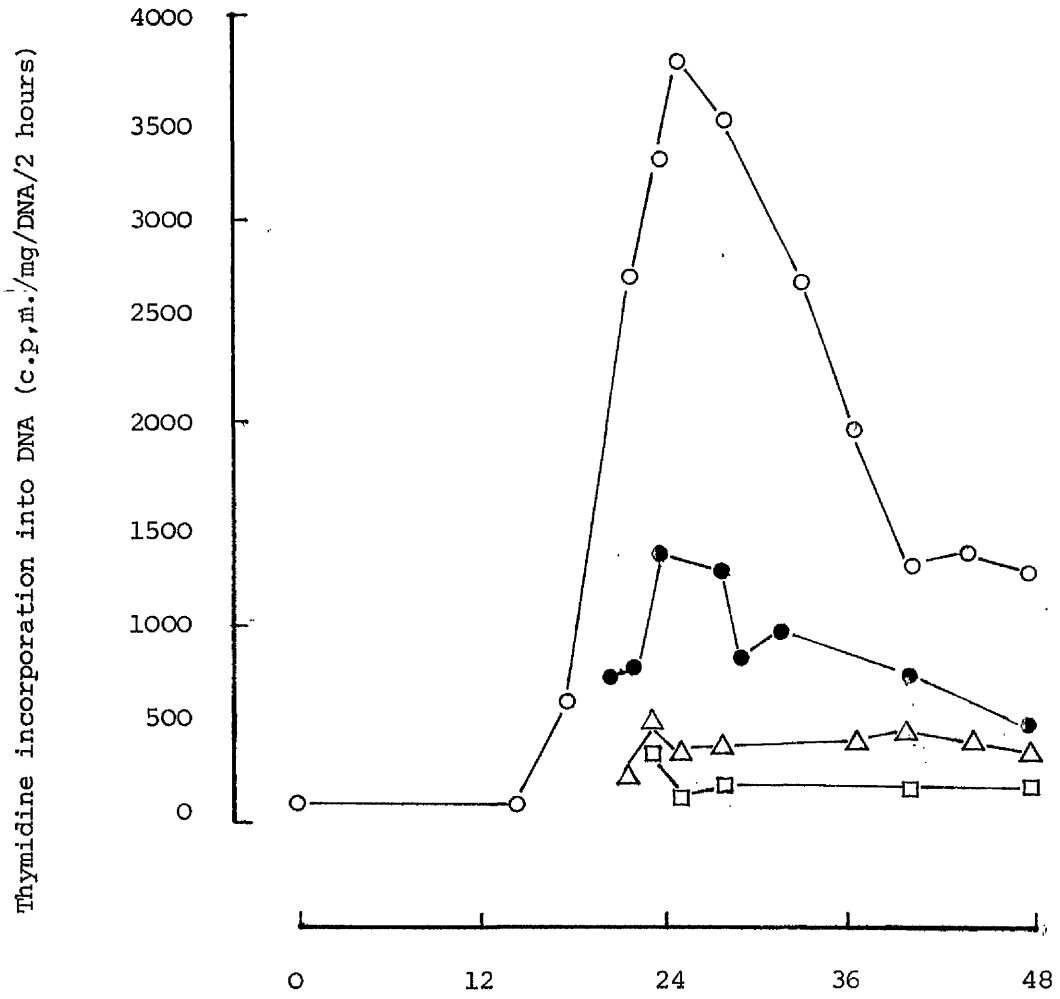
The results of experiments involving resection of visceral organs singly or in combination excluded the special implication of pancreas in liver regeneration (Sgro et al., 1973; Bucher and Weir, 1976). Bucher and Swaffield, (1975) reported that rats subjected to resection of gastrointestinal tract, pancreas, and spleen and maintained by continuous

intravenous infusion of electrolytes and glucose responded to partial hepatectomy with a significant rise in hepatic DNA synthesis, which was, however, greatly delayed and diminished compared to the controls. The activity was restored to normal by infusion of insulin and glucagon. Infusion of these hormones into rats with intact livers did not elevate DNA synthesis. These findings are consistent with the suggestion that pancreatic hormones are not the only modifiers of liver regeneration and portal blood presumably carries some other hepatotrophic factor(s) which initiate and modify the response.

1.5.4. Amount of Liver Removed

The rate of liver regeneration depends on the extent of the partial hepatectomy which elicts it. Excision of less than the usual two-thirds results in a smaller and more gradual response (Pack and Islami, 1956; Islami et al., 1959; Straube and Patt, 1961)with no change in the occurence of binucleate cells and degree of polyploidy (Sulkin, 1943). For vigorous liver regeneration liver deficiency must be above a certain threshold which for young adult rats is thirty percent. At or below this level, maximum incorporation of labelled precursors into DNA is only one to five times control value. Once the thirty percent limit has been exceeded, the response increases markedly. A forty per cent hepatectomy increases DNA synthesis seventeen-fold; 68 per cent hepatectomy increases it fifty-fold. Even after the peak has passed, DNA synthesis remains higher in animals which have had a more extensive hepatectomy (Figure 4). The mitotic response is very similar: slight if thirty percent or less of the liver is removed, but increasing sharply as the extent of the hepatectomy is increased beyond this threshold (Bucher, 1963; Bucher and Swaffield, 1964).

Figure 4



Effect of amount of liver removed on DNA synthesis rate in the remaining liver lobes (9% hepatectomy □ - □; 34% hepatectomy △-△; 43% hepatectomy ● - ●; 68% hepatectomy O-O; Redrawn from Bucher and Malt, 1971).

If more than the customary two-thirds of the liver is removed, the regenerative response is slower and less well marked. The maximum rate of DNA synthesis is reached twelve hours later and is lower than after the standard procedure. The mitotic response is also delayed, and depressed and the incidence of abnormal mitotic figures is increased (Weinbren and Taghizadeh, 1965; Woodward, 1967; Weinbren and Dowling, 1972). This depression of the regenerative response may perhaps be related to the profound hypoglycaemia produced by the removal of these large amounts of liver tissue.

1.5.5 Other Modifying Factors

Liver regeneration can be modified by a variety of other extraneous factors. Partial hepatectomy, chemical damage or infarction of the anterior lobes produce similar responses, but after chemical damage there is an early and high level of mitosis (Weinbren and Tarsh, 1964). Studies *in vitro* show that mitosis in hepatocyte cultures is influenced by the interval following partial hepatectomy at which cells are taken from the animal for cultivation (Hays, 1968). Apart from these, physical stress such as centrifugation or overcrowding, irradiation, various chemicals, vagotomy, splenectomy, all influence liver regeneration to varying degrees but their affects are rather small (Bucher, 1963; Bucher and Malt, 1971).

1.6 Theories of Liver Regeneration

A number of theories have been proposed to explain how growth in the liver remnant is stimulated and controlled after experimental injury.

1.6.1. Theory of Haemodynamic Control

The first theory to command general acceptance was put forward by Mann and his associates (1922; 1931; 1940). It was based on the fact

that the liver's main blood supply is obtained via the portal vein and is derived from the stomach, intestines, spleen and pancreas. Its volume is determined by the venous outflow from these organs and the liver is no more than a passive recipient. After partial hepatectomy, the remaining liver fragment still has to accept an undiminished portal blood supply. This presumably will produce a degree of congestion proportionate to imbalance between the blood flow and the remaining mass of liver tissue. Mann and his colleagues suggested that this congestion might be the stimulus to regeneration. This suggestion has the merit that, since liver growth will relieve the congestion, the regenerative process is self-regulating and self-limiting. The liver fragment grows until it can comfortably accommodate its portal blood supply, and its rate of growth is proportional to its inadequacy in this respect.

The evidence adduced in support of this theory consisted mainly of demonstrations that liver regeneration would only take place if portal blood flow was unimpaired. Thus Mann and Magath (1922) diverted the portal blood supply into the posterior vena cava in dogs by creation of an Eck fistula, and showed that subsequent partial hepatectomy was not followed by regeneration. Ten years later Stephenson (1932) showed that regeneration was diminished if partial hepatectomy was accompanied by constriction of the portal vein. Such experiments show that an unimpaired portal blood supply is a <u>prerequisite</u> of regeneration. They fall short of demonstration that portal blood flow <u>controls</u> regeneration. For this reason particular importance attaches to the experiments of Higgins, Mann and Priestley (1932). They took advantage of the fact that in birds the coccygeomesenteric vein forms a natural connection between the portal vein and the posterior vena cava. This means that, if the posterior vena cava is ligated between the kidneys and the liver, the blood returning from the kidneys and the posterior part of

the body is diverted to the liver, thus greatly increasing its supply of portal blood. Higgins et al., (1932) reported that liver regeneration in fowls was greatly increased if this ligation was performed before partial hepatectomy. They also reported that the ligation by itself caused liver growth. This last finding was unfortunately based on a very small number of fowls. But ,if it was correct, it constituted conclusive evidence that liver tissue is stimulated to growth when its portal blood supply is abnormally increased in relation to its mass.

The haemodynamic theory of liver regeneration, as it may conveniently be called, thus rested on a narrow and insecure basis of evidence. Nonetheless it provided a neat and attractive explanation of regeneration and it was generally accepted for about thirty years. It was first effectively undermined by Weinbren (1955) in a series of experiments which, unlike those of Higgins and his school, were carefully controlled. Weinbren demonstrated that, if one of the lobes of the liver was deprived of portal blood by ligation of the corresponding branch of the portal vein, it underwent a partial atrophy. If, however, an animal which had been subjected to this procedure was subsequently partially hepatectomised,the atrophic lobe participated in the regenerative response to the same extent as the normal lobes. These findings were confirmed by Fisher et al., (1962). They reported that, in dogs, diversion of the portal flow directly into the posterior vena cava caused considerable atrophy of the liver. But if an animal so treated was subsequently partially hepatectomised, the atrophic liver showed a regenerative response (in terms of mitotic activity and DNA synthesis) comparable to that of a liver with a normal blood supply. Further evidence against the hemodynamic theory was provided by Becker (1963). He demonstrated that severe impairment of blood flow to the liver, as produced by partial ligation of the portal vein, ligation of

the hepatic artery, or the combination of these, failed to prevent the characteristic increase in mitosis that follows partial hepatectomy. Severe anaemia likewise had no effect.

Thomson and Clarke (1965) and Thomson (1969) adopted a different approach. They suggested that, if the theory of haemodynamic control is right, an increased portal flow should produce growth in an intact liver. They reported that an increased blood supply to the intact liver, achieved by a reverse Eck fistula or splenoportal shunt, did not produce any signs of growth. Increased blood supply along with increased oxygen supply, achieved by aortoportal shunt, similarly failed to induce growth in an intact liver. It therefore seems established that liver regeneration is not controlled by the amount of blood traversing the liver from the portal vein.

1.6.2 Glinos' Theory of Inhibitory Factors

Glinos and Gey (1952), while studying the effect of sera on the growth of primary rat liver explants cultivated *in vitro*, observed that serum under certain conditions exerted an inhibitory effect on the outgrowth of tissue cells. A decrease of serum concentration resulted in a longer growth period. They noticed that a longer growth was also obtained with sera from partially hepatectomised rats. This led them to formulate a theory to explain the control of growth during liver regeneration. They proposed that liver regeneration was controlled by a feedback system involving inhibitory factors. Liver itself, they suggested, produced the inhibitory factors which checked its growth. Damage to, or removal of, a substantial part of the liver could result in a decrease in the production of the inhibitory factors, thus allowing the remnant to grow. When the liver was large enough to produce sufficient inhibitors again its growth would diminish and eventually stop.

In a series of later publications, Glinos elaborated his theory of inhibitory factors. He pointed out that the concentration of plasma proteins fell during liver regeneration. Plasmapheresis, which caused dilution of plasma proteins, not only enhanced mitosis in regenerating liver; it also stimulated mitosis in the intact livers of otherwise normal rats. Furthermore, concentration of plasma constituents, achieved by fluid deprivation , resulted in depressed mitotic activity in the livers of partially hepatectomised rats. On the basis of these findings, Glinos identified the inhibitory factors with plasma proteins, and proposed albumin to be the key substance in the negative feedback mechanism controlling liver regeneration (Glinos and Grey, 1952; Glinos, 1956; 1958; 1960).

Growth-inhibiting activity in the serum and liver of adult rats was reported by several other groups. Brues et al., (1940) reported that aqueous or alcoholic extracts of normal liver inhibited mitosis in cultured fibroblasts and explants of embryo liver or embryo heart. The potency of the extract in inhibiting growth was directly proportional to its concentration. Saetren (1956) injected macerates of normal liver into partially hepatectomised rats. He reported that in these rats the liver remnant failed to grow. Stich and Florian (1958) made a detailed study of the influence of serum and tissue homogenates on the mitotic activity of regenerating liver. They observed that serum and liver homogenates from normal rats depressed mitosis, whereas serum and liver homogenates from partially hepatectomised rats, as well as homogenates of heterologous tissues, showed no retarding effect. They concluded that some organ-specific inhibitor of mitosis was present in the serum and liver of the normal adult rat. Further evidence in favour of inhibitory factors was provided by Moya (1963). He reported that injections of serum from normal or partially hepatectomised rats into normal rats

failed to produce any increase in mitotic activity of intact liver; whereas similar injections into partially hepatectomised rats depressed the mitotic activity in the regenerating liver remnant. Normal serum was more potent in diminishing growth than serum from partially hepatectomised rats. Moreover, serum from normal rats was found to be inhibitory to growth of a culture of rat ascites hepatoma; whereas serum from partially hepatectomised rats was almost as effective as bovine serum in supporting growth.

Although the results of these experiments supported Glinos' theory of inhibitory factors, it was nevertheless open to criticism on several grounds. The increase in mitosis in vivo produced by plasmapheresis was very small compared to the increase after partial hepatectomy. Later experiments showed that plasmapheresis induced only a single wave of mitosis occuring in about three days, even when the dilution process was continued for a week (Virolainen, 1967). Moreover simple laparotomy causes a fall in plasma albumin, though slightly less pronounced than in partially hepatectomised animals. It follows a similar course but is not associated with a corresponding increase in mitosis. In addition, the albumin remains at almost equally low levels in sham-operated and partially hepatectomised animals for many days after mitotic activity in the latter has returned to normal (Bucher and Malt, 1971). The presence of inhibitory factors in normal rats serum and their dilution after partial hepatectomy was therefore questionable.

Hemingway (1961) modified Glinos theory and suggested that plasma protein influence on liver regeneration might be attributable, at least in part, not to the proteins themselves, but to the corticosteroids which they carry. Hemingway demonstrated, as Glinos had done, that, in rats, fluid deprivation (which tends to increase plasma protein concentration) diminishes the regenerative response to partial hepatectomy. But if the

animals had previously been adrenalectomised, fluid deprivation, instead of depressing regeneration, actually enhanced it. Although Hemingways's modification was an improved version of Glinos' theory, the objections described earlier were equally applicable to it.

1.6.3 Theory of Humoral Control

In parallel with the search for specific liver growth inhibitors described above, other evidence was accumulating which suggested that liver regeneration might be controlled by blood-borne hepatotrophic factors, though the possible nature of these factors remained obscure. This came first from Christensen and Jacobsen (1949), who set up pairs of parabiotic rats and four months later partially hepatectomised one partner in each pair. They reported that in the unoperated partner the mitotic activity was increased between 4 to 30-fold fifty-two hours after the operation. Similar results were reported by Wenneker and Sussman (1951). Bucher et al., (1951) extended this study to parabiotic pairs in which one partner was later partially hepatectomised and parabiotic triplets in which two partners were later partially hepatectomised. They reported that, at intervals of forty-eight or seventy-two hours following partial hepatectomy of the partner(s), the intact liver in the unoperated partner exhibited an elevated mitotic index. In parabiotic pairs the mean value was six times and in triplets fifty times that of controls.

Unfortunately, these promising early results could not be consistently repeated by later workers. Islami et al., (1959) carried out similar experiments and failed to observe any increase in mitotic index in the intact liver of the unoperated partner. However the mitotic index in the operated partner was depressed considerably compared to a single partially hepatectomised animal. Rogers et al., (1961) performed a carefully controlled series of experiments on parabiotic

pairs and triplets. They found that partial hepatectomy of one partner did increase DNA synthesis and mitosis in the liver of the other partner, but that an equal effect was produced if a liver biopsy was performed instead of hepatectomy.

Attempts were also made to detect blood-borne factor(s) influencing liver growth by injecting plasma or serum from partially hepatectomised rats into normal rats and vice versa. Friedrich-Freksa and Zaki (1954) reported that intraperitoneal injections of serum from partially hepatectomised rats caused a 40-fold increase in the mitotic activity in the livers of normal intact rats. Smythe and Moore (1958) reported that intravenous administration of plasma from partially hepatectomised rats enhanced the mitotic response in the livers of partially hepatectomised rats and also induced mitosis in the intact livers of normal rats. Injections of plasma from normal rats into partially hepatectomised rats resulted in diminished DNA labelling and slow increase in weight of the liver remnant, but mitotic activity remained undeminished. Similar results were also reported by Hughes (1960) and Zimmerman and Cellozzi (1960).

All these attempts to demonstrate control of liver regeneration by blood-borne factor(s), whether by parabiosis or by injection, were subject to the same limitations. In every case the effects reported, whether in terms of growth of the intact liver, or of inhibition of regeneration after partial hepatectomy, were small in comparison to the enormous differences in mitotic rate and DNA synthesis between regenerating liver and normal liver. It was not difficult to find plausible explanations why this should be so. In the usual parabiotic preparation, the exchange of blood between the partners, though quite variable, is never very great. If the supposed blood-borne factor(s) had a limited life, it might be destroyed almost as rapidly as it could

be transferred from the hepatectomised animal to its normal partner or vice versa. Similarly, the volume of serum or plasma which can safely be injected into an animal is necessarily limited. This limitation might be responsible for the small effects observed.

Clearly, therefore, the importance of blood-borne factor(s) in liver regeneration could only be reliably assessed if more effective means could be found for transferring it from partially hepatectomised rats to normal rats and vice versa. This problem was first tackled by Alston and Thomson (1963) who devised a novel form of parabiosis in which the partners were united by cannulae inserted into the common carotid artery of each animal and into the external jugular vein of its partner. By this means an exchange of 4 ml of blood per minute (equivalent to more than a quarter of the blood volume) was attained. If one animal in such a parabiosis was partially hepatectomised, it regenerated in the usual way, but little or no sign of liver growth was detected in the intact liver of its partner. Alston and Thomson (1963) concluded that, whatever might control liver regeneration, blood-borne factor(s) could not be important. Their experiments were, however, limited in number. Subsequently Moolten and Bucher (1967) undertook a much more extensive investigation on the same lines as Alston and Thomson, and were able to show that partial hepatectomy in one animal in the Alston-Thomson type parabiosis did produce a significant, although quite modest, increase in mitosis and DNA synthesis in the liver of its partner. However, prolonged exchange of blood (about twenty hours) was essential. If the exchange lasted less than twelve hours the expected DNA rise did not occur. They further reported that the response in the unoperated partner was dependent on the amount of liver removed from the operated partner. The DNA labelling in the unoperated partner was more than doubled when the other partner was subjected to 85 per cent

hepatectomy instead of the usual 68 per cent. Sakai (1970) performed similar experiments but achieved rapid exchange of blood through aorta-to-aorta cross-circulation. In these experiments the lower half of one rat received blood from the upper half of the other rat. He reported that partial hepatectomy of one partner resulted in a 7-fold increased DNA labelling in the liver of unoperated partner compared with the response produced by biopsy in the operated partner. But the DNA labelling in the liver remnant itself was reduced to half compared to partially hepatectomised single rats. Fisher et al., (1971) repeated Moolten and Buchers (1967) experiments and confirmed their results. They further reported that the response in the unoperated rat was maximal when total hepatectomy was performed in the partner. These experiments taken together indicated that regeneration was at least to some extent a response to changes in the composition of the blood. But they equally demonstrated that the blood-borne factor(s) concerned could only be transferred from one animal to another by very rapid exchange of blood. Presumably, therefore it must be remarkably short-lived.

The experiments with cross-circulated animals had two main disadvantages

(a) potential immunologic reaction between partners

(b) the difficulty in removing enough liver from only one partner to cause a maximal proliferative response.

To avoid these disadvantages, Sigel et al., (1963) studied the effect of partial hepatectomy on autotransplanted liver tissue. They reported that the liver autografts showed a moderate proliferative activity in response to partial hepatectomy. These results were later confirmed by Virolainen (1964). Leong et al., (1964) improved this technique and prepared liver autografts in which billary drainage was preserved. They demonstrated that in such autografts the proliferative activity was just as vigorous as in the liver remnant itself.

Together, the cross-circulation and liver autograft experiments provided convincing evidence that liver regeneration is initiated and controlled largely, and perhaps exclusively, by changes in the composition of the blood. But they could not distinguish between the two possible forms this might take. On the one hand, partial hepatectomy may elicit production of a factor(s) which stimulates liver growth and which disappears when the liver mass is restored to its original level. On the other hand, it is posible that partial hepatectomy may diminish production of a factor(s) which in the normal animal inhibit liver growth and which re-appear as the liver mass is restored. Sigel (1968) tried to find out which of these mechanisms was operating. He established liver autografts in the necks of dogs. These were supplied by arterial blood which either entered via the portal vein and left by the hepatic vein (normal flow) or vice versa (reversed flow). The dogs were then partially hepatectomised in the usual way. The autografts, as well as the residual fragment of the liver proper, showed evidence of regeneration, as indicated by increased labelling of DNA. In the autografts with "normal blood flow", as in the residual liver fragment, DNA labelling was seen first in the peripheral part of each lobule. In the autografts with "reversed blood flow" DNA labelling was seen first around the central vein. In other words, regeneration in each case occured in cells surrounding the points at which blood entered the autografts. Sigel interpreted this as meaning that regeneration was a response to some positive stimulant and not merely to the dilution of some agent which in the intact animal inhibited liver growth.

1.6.4 Work Hypertrophy Theory

Implicit in most of the attempts to find a humoral mechanism for liver regeneration there has been an assumption that the mechanism must

involve some special humoral agent, analogous perhaps to adrenocorticotrophic hormone. Belief in such a hormone is made more difficult by the mass of evidence from cross-circulation experiments indicating that it has an extraordinarily transient existence. This has led one author to question the necessity of postulating the existence of a specific hormone and to suggest that the liver fragment left after partial hepatectomy grows because it is obliged to undertake the metabolic functions of the original intact organ (Thomson, 1969).

There is nothing novel in this idea; it was propounded by Ponfick in 1889, though he adduced no evidence in its support. It was revived by Raus and Larimore (1920). They showed that ligation of a branch of portal vein resulted in atrophy of the lobes concerned and hypertrophy of the lobes whose portal circulation was intact. The hypertrophy was almost as rapid as if the tissue deprived of portal blood had been ablated. Raus and Larimore suggested that liver thrived on substances carried to it by portal blood. Local portal obstruction deprived that part of the liver of its normal opportunity to obtain many substances. Therefore it failed to carry out its proper share of functions and became useless to the animal, whereas the portion of liver with intact portal blood supply received an undue quantity of portal blood, and probably carried out all the functions normally performed by the intact organ.

The idea that the liver grew (or did not grow) depending on the functions it had to perform attracted little further attention. Solopaev (1957) reported that addition of dog bile in the diet of partially hepatectomised rats resulted in an enhanced regenerative activity of the liver remnant. Alston and Thomson (1968) demonstrated that injections of a protein hydrolysate at regular intervals for forty-eight hours caused a rise of twenty per cent in hepatic protein and forty percent in RNA content and also increased the frequency of mitosis in otherwise normal rats.

1.7 Cell Growth and Cell Division

Whatever may be the nature of the (presumably external) stimulus which brings about regeneration, there is a question also of the mechanism by which it acts within the residual liver fragment. It is well established that liver regeneration is brought about ultimately by cell division. When it reaches completion, the liver is quite different in gross structure from the original intact organ, but histologically it is virtually indistinguishable. Careful examination reveals a modest increase in the proportion of polyploid nuclei (about 10 per cent); otherwise nothing has changed. This is in striking contrast to the compensatory renal hypertrophy which follows unilateral nephrectomy. Though superficially this looks strikingly analogous to liver regeneration it is in fact brought about very largely by cell enlargement; there is relatively little cell division (Thomson, 1969).

While liver regeneration is so clearly a consequence of cell division, there remains the question whether the agency which brings it about acts as a direct stimulus to mitosis, or whether it merely brings about cell growth, which in turn brings about cell division. Mazia (1956) enunciated, as a general theory, that cell division is a consequence of cell growth. When a cell has enlarged to the point where it is too large for a single nucleus - Mazia called this the "critical mass" - it automatically undergoes mitosis. This theory was based on the observation that, in the amoeba, cell division can be postponed indefinitely by repeated removal of cytoplasm at a rate which balances cell growth. Johnson (1969) attempted to use the idea of critical mass to explain the observation that, in regeneration, cell growth (as evidenced by RNA and protein synthesis) precedes cell division by about twenty-four hours. Johnson believed that mathematical analysis of

hepatocyte growth and mitosis in partially hepatectomised mice fitted well with the predictions of Mazia's (1956) theory. Johnson's experiments were carried out on a small number of animals and the correctness of his mathematical model was subsequently criticised by Tangendorff (1975). But the fundamental objection to the applicability of the critical mass theory to liver regeneration is much simpler. As early as 1936 Brues et al., showed that, when fasting rats are partially hepatectomised, the volume of the liver cells decreases as a response to the lack of food, but the cells still divide more or less normally in response to the hepatectomy. Very much later, Weinbren et al., (1971) ligated the portal vein branch supplying the right lobe of the liver in rats. The lobe atrophied in response, and the mean volume of its hepatocytes fell to a little above half the normal value. Removal of the median and left lateral lobes resulted is some increase in hepatocyte volume in the atrophic lobe but the average was still only three-quarters that found in normal intact liver. Nevertheless these shrunken cells underwent mitosis in response to the hepatectomy.

1.8 The Situation in 1976

By 1976 a great deal of information had been accumulated about the process of liver regeneration. Its morphological aspects had been described in detail at every level from gross anatomy, through histology, to the electron microscopy of individual cells and their organelles. The accumulation of biochemical information was even more impressive; the levels of all the enzymes involved in DNA synthesis, and of many which are not, had been measured. But this great mass of information about what happens during regeneration could not be fitted together

because an essential piece of the puzzle was missing. In spite of forty years of effort, the mechanism initiating and controlling regeneration remained wrapped in obscurity.

A few generalizations had been established, mostly of a negative kind. The work of Weinbren (1955) and later of Thomson and Clarke (1965) seemed to demonstrate that regeneration was not the result of the diminished mass of liver tissue having to accommodate the same portal blood flow as the normal intact organ. Equally, the work of Moolten and Bucher (1967) and of Sakai (1970) showed that, at least in part, liver regeneration was evoked by some change in the composition of the blood passing through the liver. But the same experiments that established this showed that the factors involved must have a quite extraordinarily short half-life, of the order of seconds rather than minutes. The prospect of characterizing, let alone isolating, such transient entities was daunting. Finally, there was some evidence from Starzl's (1975) work that, while the volume of the portal blood supply might be unimportant, its composition might have a special significance; in particular, it might act as a route by which the pancreatic hormones could reach the liver without having to pass through the general circulation. As against this, Bucher's (1975) experiments with eviscerated rats appeared to show that regeneration could take place in total absence of pancreatic tissue.

1.9 The Technical Difficulties

The disappointing lack of progress in elucidating the mechanism controlling liver regeneration is perhaps attributable in some degree to technical difficulties. Among these is the problem of estimating the

rate at which the residual liver fragment is regenerating. Ideally, as a first step, one would like to compare the mass (and composition) of

a. the intact liver immediately before hepatectomy

b. the residual liver fragment immediately after hepatectomy

c. the residual liver fragment at various time intervals thereafter.

Unfortunately the first and second of these can be estimated only indirectly and approximately by assuming that the lobes removed in the standard operation represent a known fraction of the total liver substance, an assumption which is only very approximately correct. The third quantity, the size of the residual liver fragment after regeneration has been in progress for a pre-determined time, can be easily and precisely measured, but only at the cost of killing the animal. It can therefore only be measured once. These considerations mean that any attempt to plot the course of regeneration, even in the very simple terms of measuring the restoration of tissue mass, requires the use of very large numbers of animals.

In practice most investigators have been concerned, not with the extent of regeneration up to a given time, but rather with its rate at that time. This has most commonly been estimated by measuring mitotic frequency or DNA synthesis as reflected by [^{3}H]-thymidine incorporation. These are simpler measurements to make and interpret than estimates of tissue mass and composition, but they have the same disadvantage that they can be made once, and only once, in any given animal. A method of measuring regeneration with reasonable precision which did not involve sacrificing an animal, and which preferably could be carried out several times in the same animal, would offer a very great economy in labour and expense, and would hold out the hope of providing much more precise information than is at present available. Such a

procedure would almost certainly have to be based on some sort of measurement of liver function, and the earlier part of the experimental work described in this thesis was concerned with attempts to devise a suitable procedure along these lines.

A more fundamental difficulty concerns the identification of a humoral agent, which all the available evidence indicates is extraordinarily short-lived. A direct attack on this problem seems unlikely at present to have much chance of success. For this reason the latter part of the work described in this thesis was intended to provide an indirect approach by examining how the residual liver fragment, and the organism as a whole, adjust to the deficit of liver tissue produced by partial hepatectomy.

2. RESULTS AND DISCUSSION

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2.1 Bromsulphalein (BSP) clearance

Preliminary experiments by Alston and Thomson (unpublished work) had held out hope that the rate of BSP clearance from the blood stream might be proportional to liver mass.

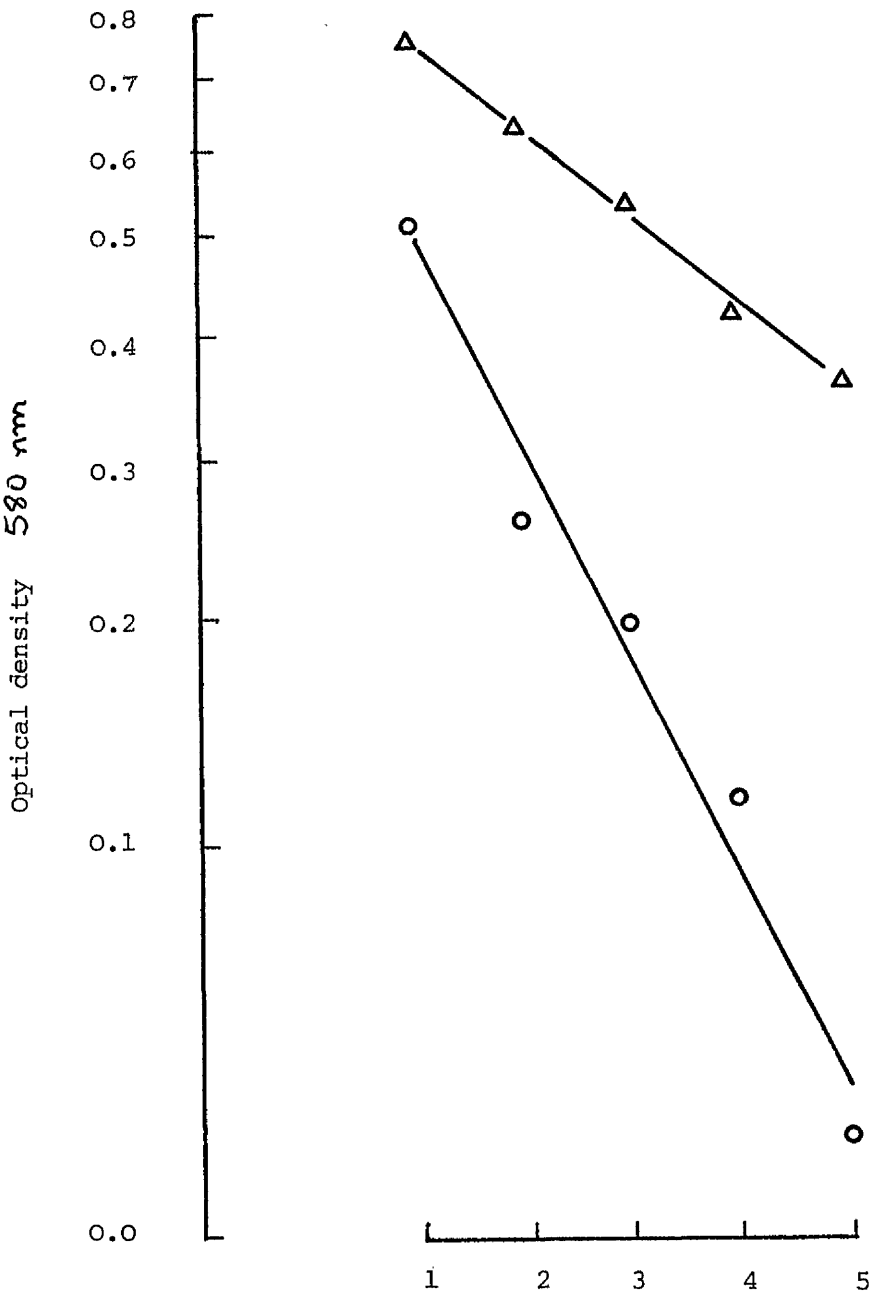
Figure 5 illustrates the clearance from the blood stream of an injected dose of BSP immediately before, and immediately after, partial hepatectomy. In both cases the process is approximately logarithmic, and it is clear that the rate of clearance is considerably decreased after the operation. Table 1 shows the results of several such experiments. It is evident that removal of two-thirds of the liver is followed by a proportional fall in rate constant. In other words, BSP clearance appears to be proportional to the mass of liver tissue.

These experiments are however open to objection.

a) The fall in BSP clearance after the operation is not necessarily to be attributed only to the loss of liver tissue. It might simply be a consequence of the trauma of operation.

b) Approximately 0.3 ml of blood must be withdrawn for each BSP estimation, and five estimations are needed to establish the clearance curve. Each curve therefore necessitates the removal of at least 1.5 ml out of a total blood volume of about 12 ml. This loss of blood may well affect the second clearance curve.

Figure 5



Bromsulphalein clearance from the blood-stream of a male rat (body weight approximately 220 g) immediately before (o) and immediately after (△) partial hepatectomy.

TABLE 1

Bromsulphalein clearance in four male rats (body weight approximately 220 g) immediately before and immediately after partial hepatectomy (see Figure 1).

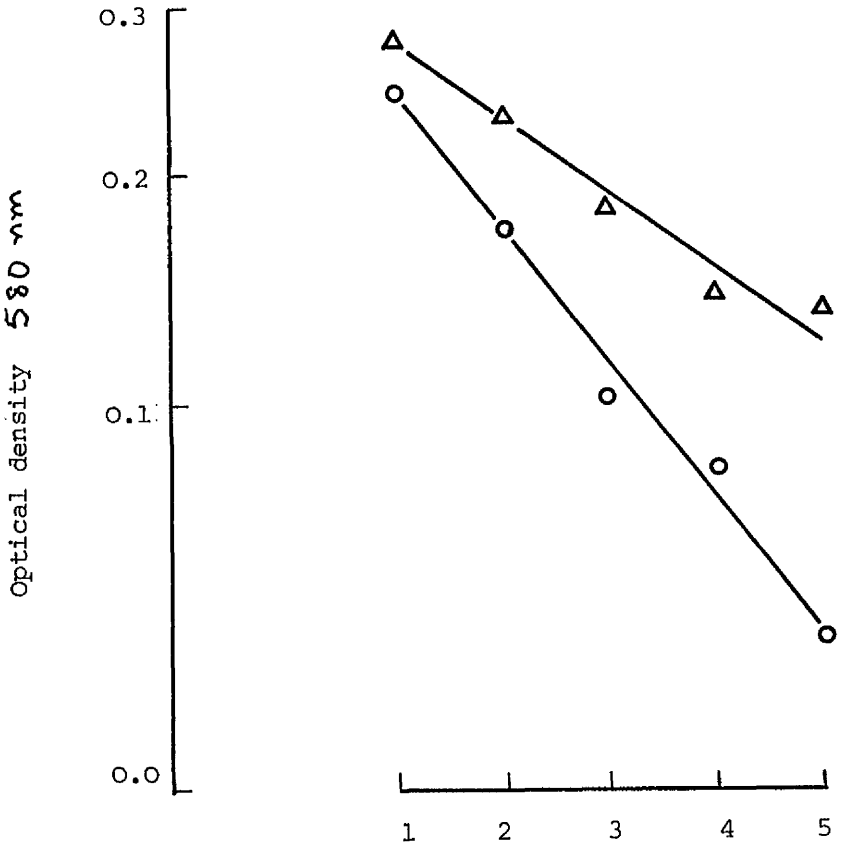
Rat No.	Rate constant of Bromsulphalein clearance		Post-operative rate constant as a percentage of pre-operative value
	Before operation minutes $^{-1}$	After operation minutes $^{-1}$	
1	0.330	0.111	33.6
2	0.229	0.080	34.9
3	0.263	0.097	36.9
4	0.260	0.090	34.6
Mean	0.271	0.095	35.0
$\pm$ S.d.	$\pm$ 0.043	$\pm$ 0.013	$\pm$ 1.36

c) Since two BSP clearance curves were made from the same animal within fifteen minutes, BSP had to be injected twice over a brief period. There was therefore the danger of overloading whatever mechanism removes BSP from the blood stream.

In order to investigate these possible sources of error an experiment was carried out in which two consecutive BSP clearance curves were obtained from the same intact animal at an interval of about ten minutes, but in which the total volume of blood removed on each occasion was reduced to 0.125 ml (instead of the 1.5 ml required in the standard procedure). Figure 6 shows a typical set of results. It is obvious from the figure that the second injection of BSP is cleared at a considerably slower rate than the first. Table 2 shows the results of several such experiments. The rate constant for the second BSP injection is approximately 40 per cent lower than the first. It is clear therefore that while the results in Figure 5 and Table 1 appear to show that removing two-thirds of the liver reduces BSP clearance by two-thirds, this result is quite fortuitous.

To eliminate the error inherent in carrying out consecutive clearance estimations in the same animal, the clearance of BSP was measured in rats which had undergone either two-thirds hepatectomy or removal of a liver biopsy amounting to no more than 10 per cent by weight of the whole organ. Clearance was estimated only once in each animal. The results of these experiments are listed in Table 3 and a typical set is shown in Figure 7. It is clear that BSP clearance is not affected by the removal of a biopsy but that it is diminished by partial hepatectomy. The effect of the latter - a 44 per cent reduction in rate constant - is, however, rather less than one might anticipate in view of

Figure 6



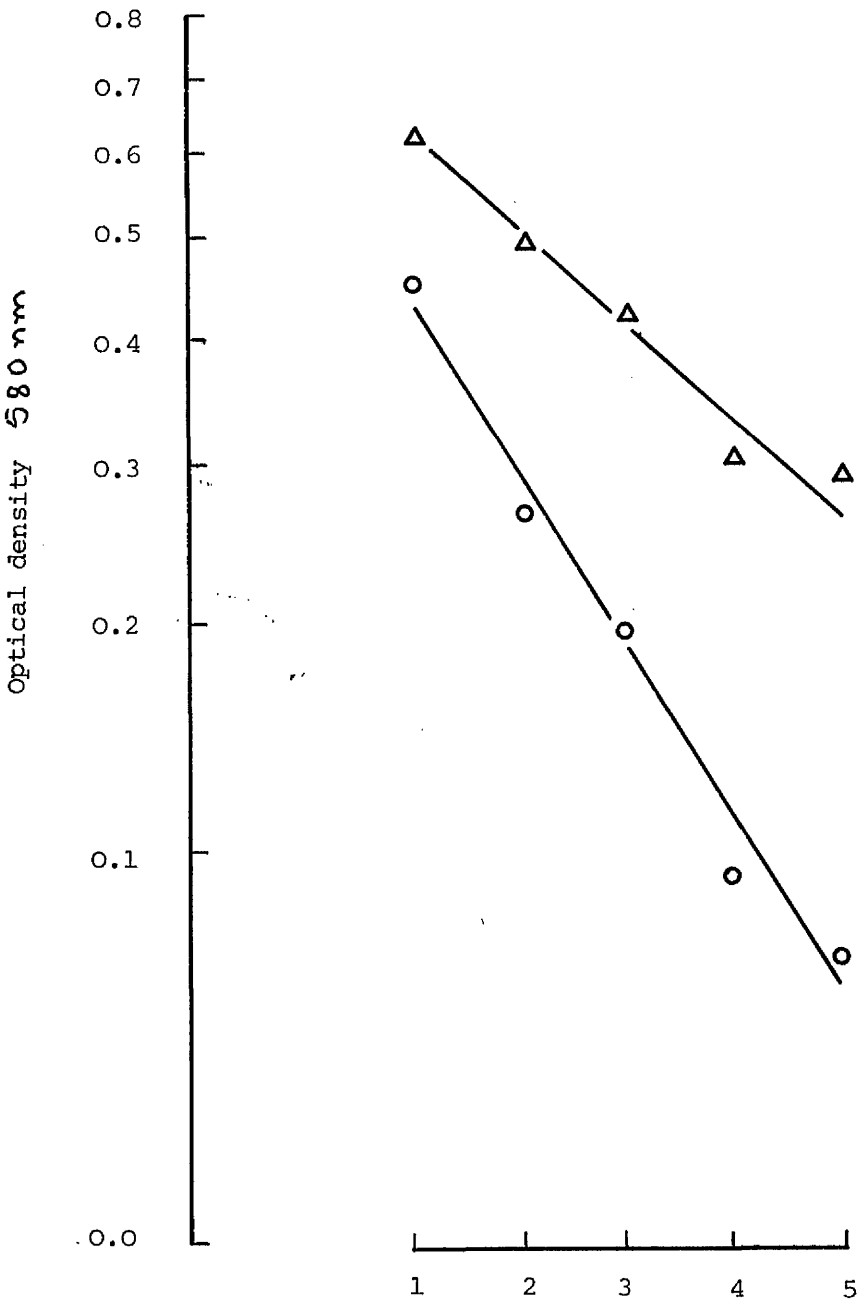
Bromsulphalein clearance from the bloodstream of a male rat (body weight approximately 220 g) with intact liver. The curves were obtained from the same animal at an interval of about ten minutes. The total volume of blood removed on each occasion was reduced to 0.125 ml instead of the 1.5 ml required in the standard procedure. First estimation (o); Second estimation (△)

TABLE 2

Bromsulphalein clearance in intact male rats (body weight approximately 220 g) determined twice in each animal, the second determination being performed approximately ten minutes after the first (see Figure 2).

Rat No.	Rate constant of bromsulphalein clearance		Second determination as percentage of first
	1st determination minutes $^{-1}$	2nd determination minutes $^{-1}$	
1	0.248	0.128	51.6
2	0.217	0.141	65.0
3	0.217	0.136	62.7
4	0.231	0.144	62.3
Mean	0.228	0.137	60.4
$\pm$ S.d.	$\pm$ 0.015	$\pm$ 0.007	$\pm$ 5.99

Figure 7



Bromsulphalein clearance from the bloodstream of two male rats (body weight approximately 220 g). In one case the estimation was made immediately after a liver biopsy (o) and in the other immediately after two-thirds hepatectomy (△).

TABLE 3

Bromsulphalein clearance in male rats (body weight approximately 220 g) after either removal of a small liver biopsy or two-thirds hepatectomy. There were four animals in each group. The results are shown as mean ± standard deviation.

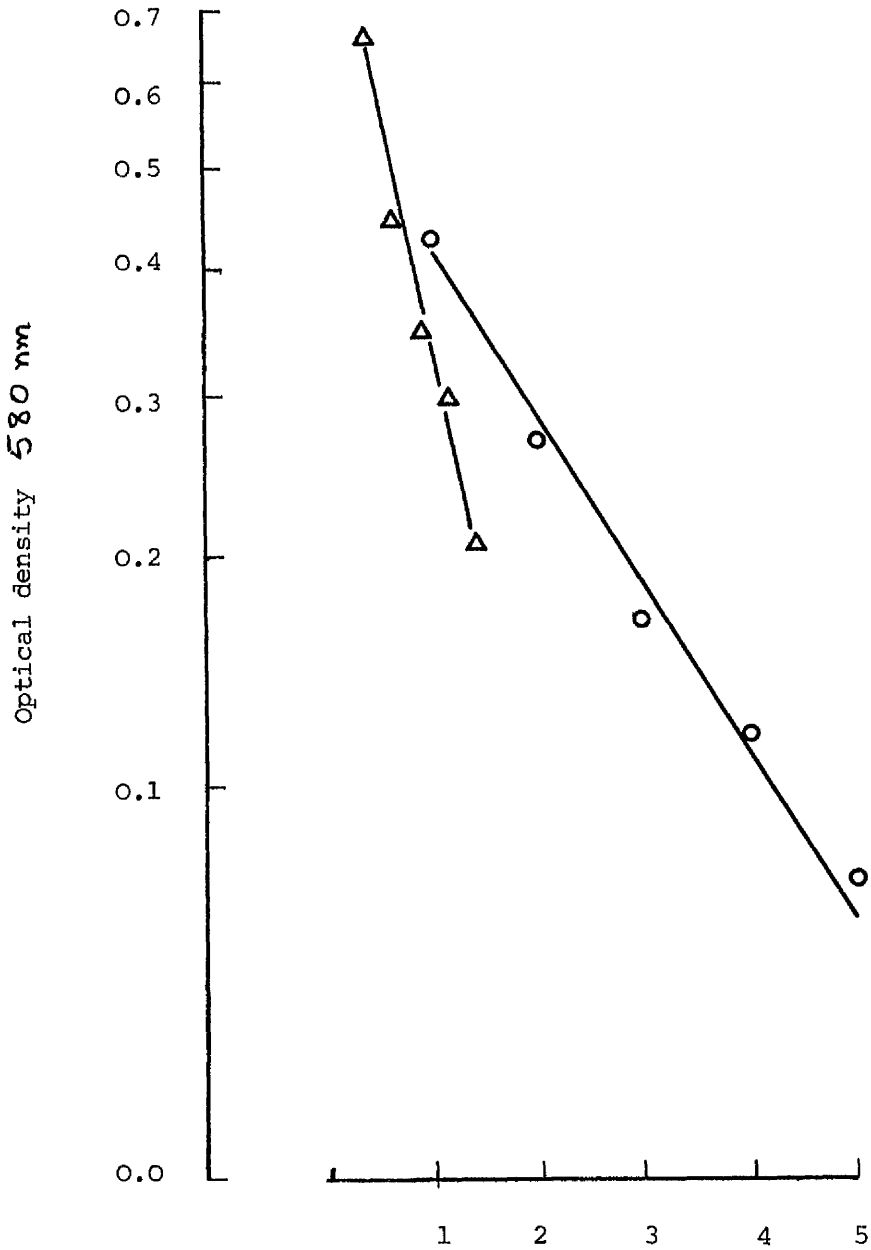
Condition of rats	Rate constant $minutes^{-1}$	Post-operative rate constant as percentage of controls
Intact controls	0.268 ± 0.050	
Immediately after a liver biopsy	0.258 ± 0.028	96.1 ± 10.28
Twenty-four hours after liver biopsy	0.263 ± 0.029	97.9 ± 10.72
Immediately after partial hepatectomy	0.153 ± 0.022	56.9 ± 8.27
Twenty-four hours after partial hepatectomy	0.148 ± 0.013	55.0 ± 4.69

the fact that the mass of liver tissue had been reduced by about 67 per cent. The same results are obtained if BSP clearance is measured immediately after the operation or after a lapse of twenty-four hours.

A possible explanation of this lack of proportionality may lie in the fact that the BSP clearance is exponential only if measured over a brief period. The clearance rates shown in Figures 5 to 7 and Tables 1 to 3 were determined by measuring plasma BSP concentrations at one minute intervals over the period from one minute to five minutes after the injection. During this period the plasma BSP concentration falls about four-fold in normal or biopsied animals but only about two-fold in hepatectomised animals. The initial concentration is also higher in the latter than in the former. In other words, in the hepatectomised animals the clearance process is being observed at an earlier stage than in the normal or biopsied animals. This would not matter if BSP clearance was a strictly logarithmic process throughout its entire course, but this may be only an approximation. Figure 8 shows BSP clearance determined in two normal rats, in one case over the period from one to five minutes after the injection and, in the other, over the period from thirty seconds to ninety seconds. It is obvious that the BSP clearance is much more rapid in the latter than in the former. It would appear therefore that BSP clearance is not a logarithmic process. The initial rate of clearance corresponding to a fall in optical density from about 0.7 to about 0.4 is more rapid than that observed in the latter part of the process when the optical density is falling from about 0.4 to about 0.1.

These results suggest that, although the BSP clearance rate is not proportional to the liver mass, there still exists a relationship between the two which perhaps could be applied to measure liver regeneration

Figure 8



Bromsulphalein clearance from the bloodstream of two male rats (body weight approximately 220 g) with intact livers. In one case, the estimation was made over the period from one minute to five minutes after the injection (o), and in the other over the period from thirty seconds to ninety seconds (△).

in vivo. But BSP clearance would only be a reliable measure of liver regeneration if it was not altered by any of the stresses which the animals might have to bear during the experiments. Table 4 and Figures 9 and 10 represent the results of a series of experiments in which the animals were subjected to various forms of stress. These show that the intact livers of alloxan-diabetic rats and of rats starved for twenty-four hours clear BSP at a remarkably slow rate as compared to normal rats, almost indeed as slowly as partially-hepatectomised rats.

The mechanism of BSP extraction by the liver is not fully understood. Goresky (1965) has suggested that it occurs in three steps.

i) Uptake of BSP into the hepatocytes by a membrane transport system of *high* capacity.

ii) Conjugation of BSP with glutathione within the hepatocytes.

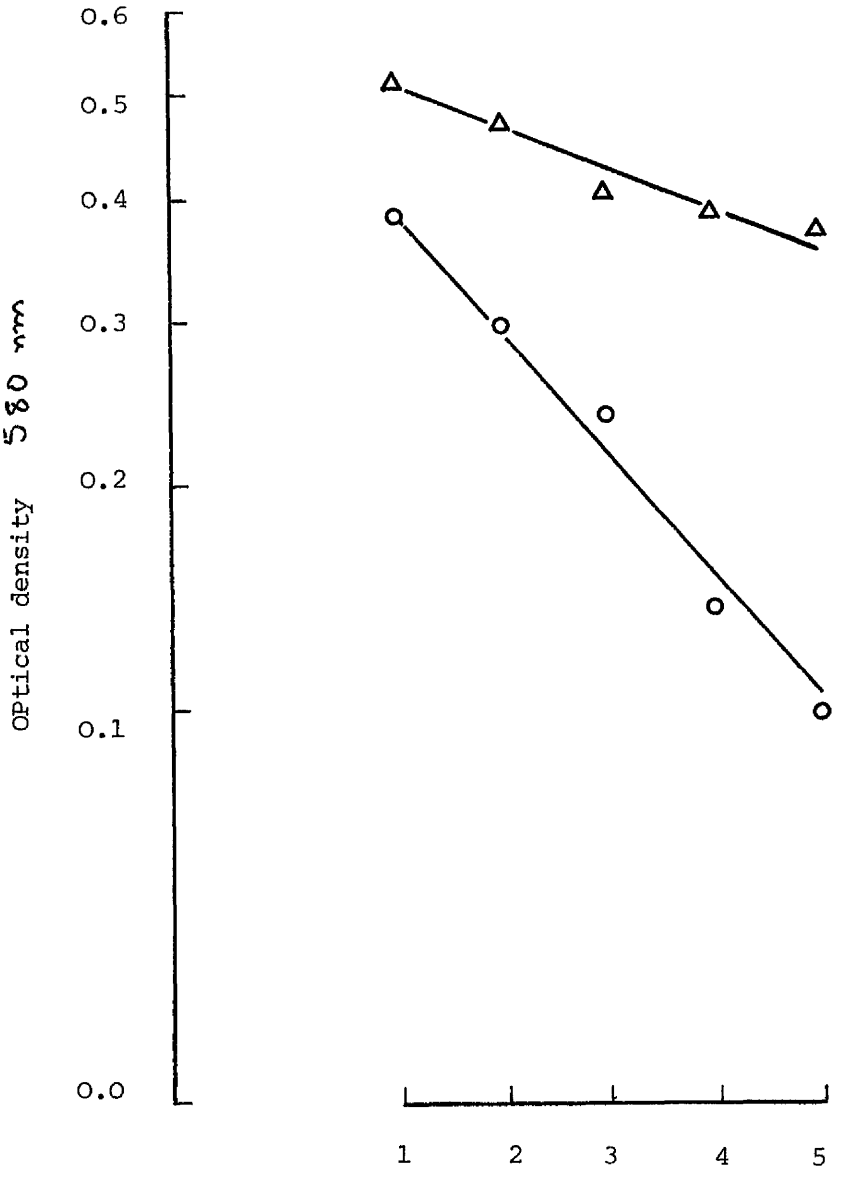
iii) Excretion of the conjugated substance into the bile by a second membrane system of *low* capacity.

Since the capacities of the two transport systems are disproportionate there is a transient accummulation of BSP in the liver. Wheeler et al., (1960) have shown that BSP is concentrated several fold (about ten-fold in dogs and four-fold in man) in the liver in comparison with plasma. It appears therefore that the measurements of the entire clearance process from uptake to excretion might be necessary to determine the size of liver. This would involve analysis of the liver tissue and the animal would have to be killed.

2.2 Ethanol clearance

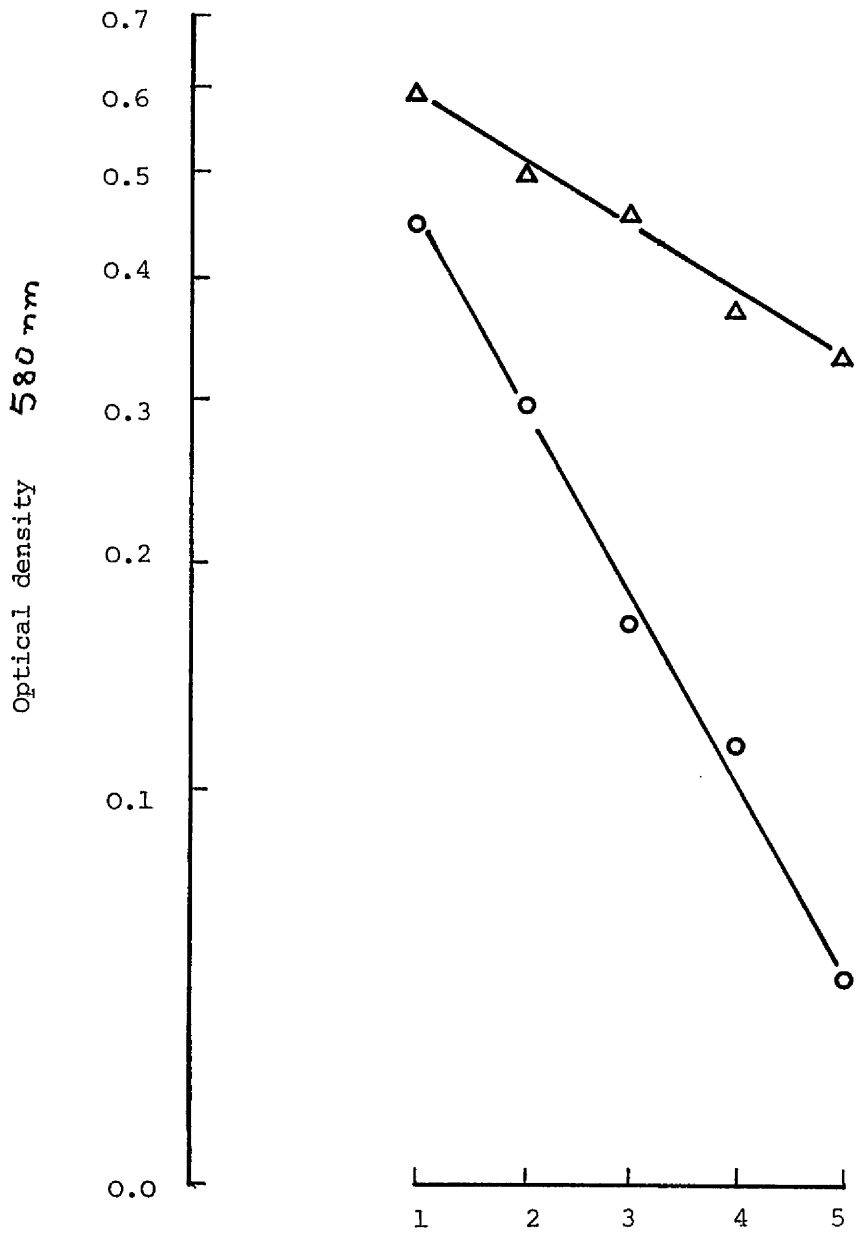
An alternative means of estimating liver function might be found in the clearance of alcohol. The liver is the organ primarily concerned

Figure 9



Bromsulphalein clearance from the bloodstream of two male rats (body weight approximately 220 g) with intact livers. One rat was normal (o); the other had alloxan diabetes (△).

Figure 10



Bromsulphalein clearance from the bloodstream of two male rats (body weight approximately 220 g) with intact livers. One rat was normal (o); the other was fasted for twenty-four hours before the estimation (△).

TABLE 4

Bromsulphalein clearance in male rats (body weight approximately 220 g) with intact liver under various experimental conditions. The results are shown as mean ± standard deviation.

Condition of Rats	Number of rats used	Rate constant $(minutes)^{-1}$	Rate constant as percentage of controls
Normal controls	8	0.25 ± 0.03	
Alloxan diabetic	4	0.16 ± 0.04	63.0 ± 17.09
Fasted for twenty-four hours	4	0.15 ± 0.05	59.0 ± 19.15
Immediately after establishment of a porto-caval shunt	4	0.06 ± 0.03	23.0 ± 11.02

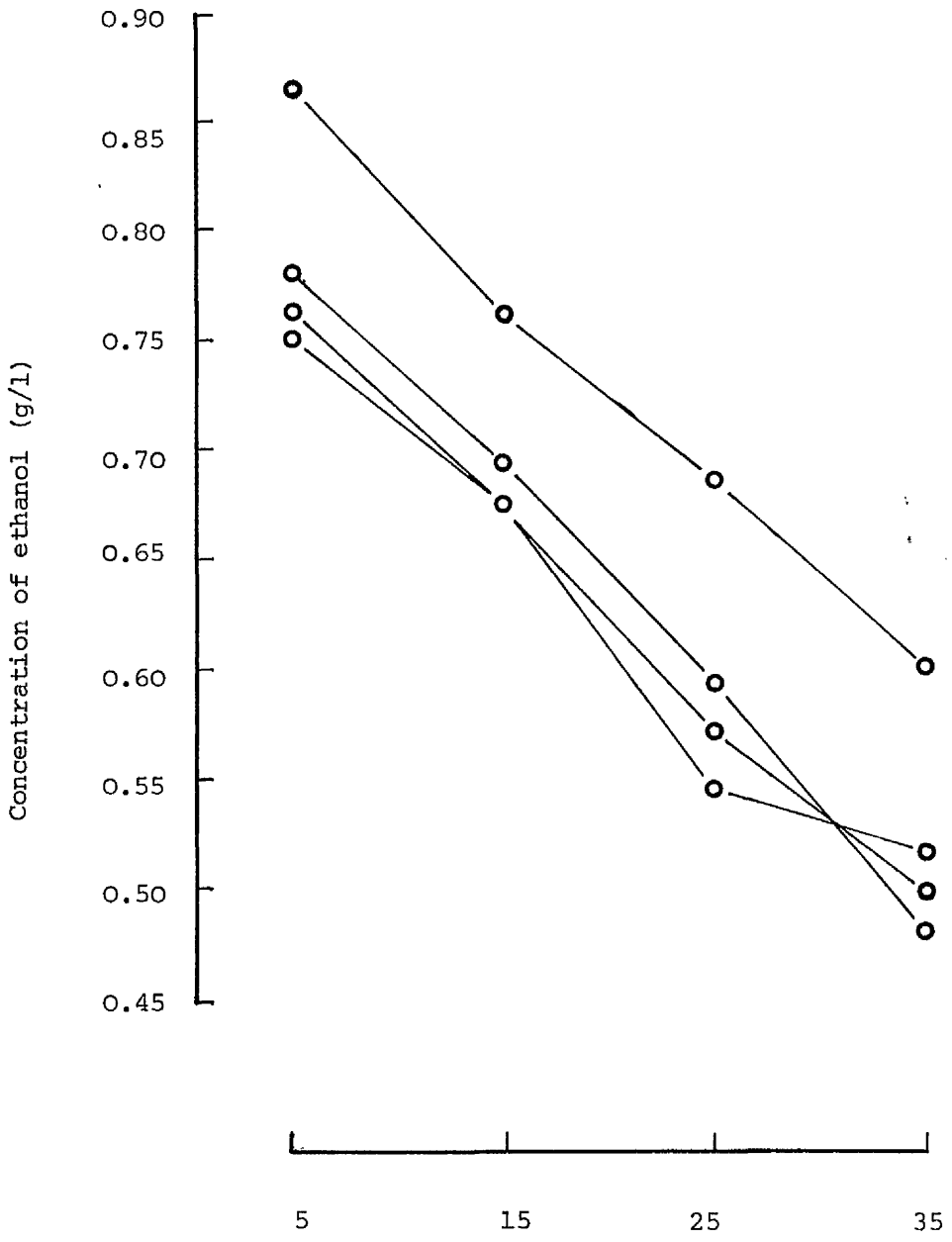
in the oxidation of ethanol. Mirsky and Nelson (1939) had reported that in rabbits partial removal of liver tissue diminished the capacity for ethanol oxidation in proportion to the amount of liver removed. Figure 11 shows the clearance of an injected dose of ethanol from the bloodstream of normal rats. The ethanol disappears at a steady rate of approximately 0.14 mg/min/g wet weight of liver. Figure 12 shows the clearance of a similar dose of ethanol immediately after a liver biopsy. It is clear that the ethanol is cleared virtually at the same rate as in intact rats. Obviously, therefore, the trauma of operation has no effect on the capacity of rats liver to oxidize ethanol.

Figure 13 shows the clearance of an injected dose of ethanol from the blood stream of rats immediately after partial hepatectomy. For the first fifteen minutes this proceeds at the same rate as in controls: but at that point it appears to cease. Exactly the same result is obtained if the experiment is carried out twenty-four hours after partial hepatectomy (Figure 14). It seems therefore that the capacity of the liver to oxidize ethanol is drastically reduced as a result of partial hepatectomy and remains reduced for at least the following twenty-four hours.

A few reports have appeared in the recent past concerning the rate of ethanol oxidation. Poso and Poso (1977a; 1979b) have reported the rate of ethanol oxidation by normal rats which is about twice as high as that shown in Figures 11 and 12. Since their experimental design is significantly different from ours a detailed comparison is difficult. Nevertheless, measured in terms of ethanol removed per minute per 100 g body weight, their results also show a substantial decrease in ethanol oxidation following partial hepatectomy.

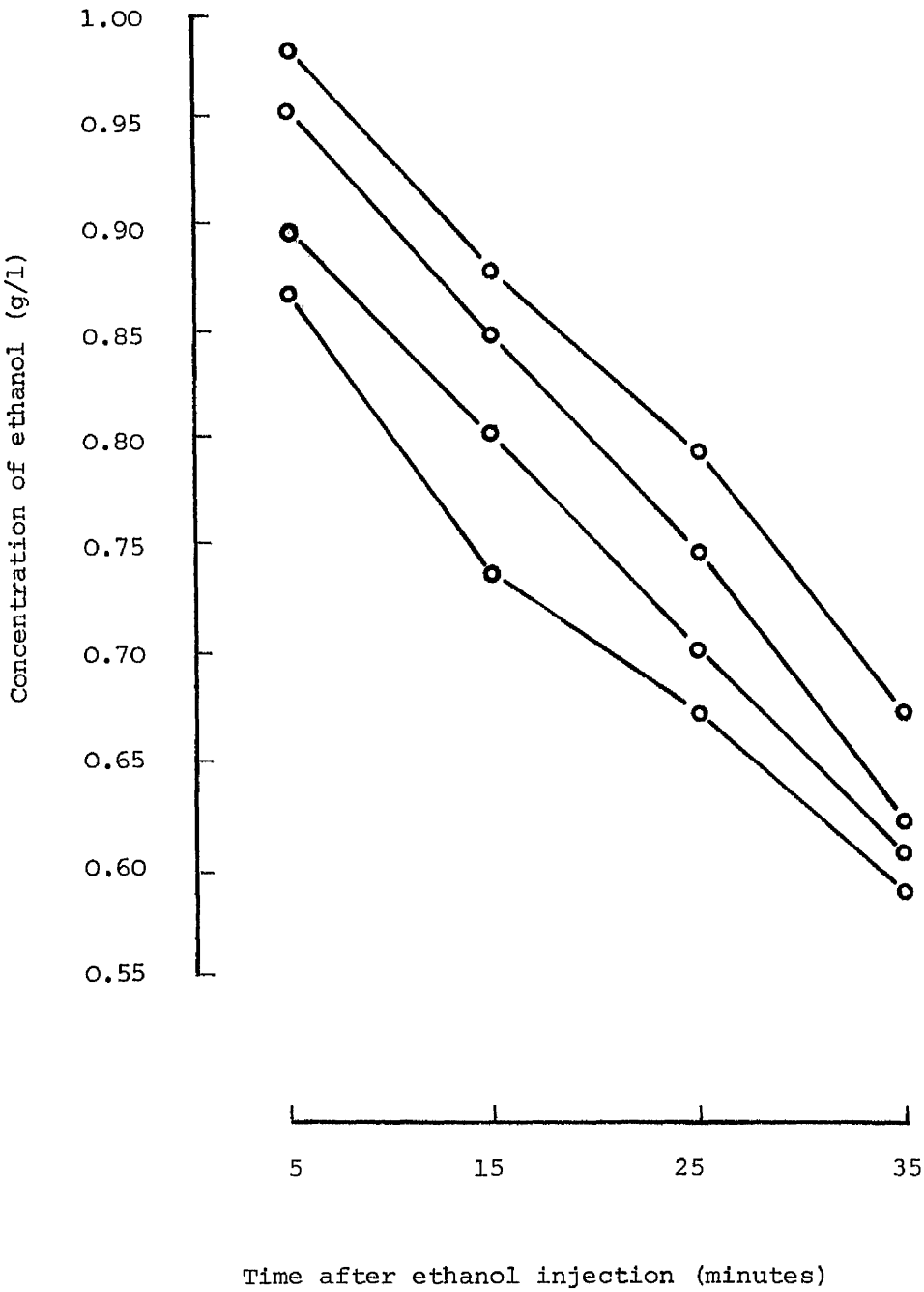
There is considerable uncertainty as to the overall mechanism of ethanol metabolism. The only enzyme known to act upon ethanol is

Figure 11



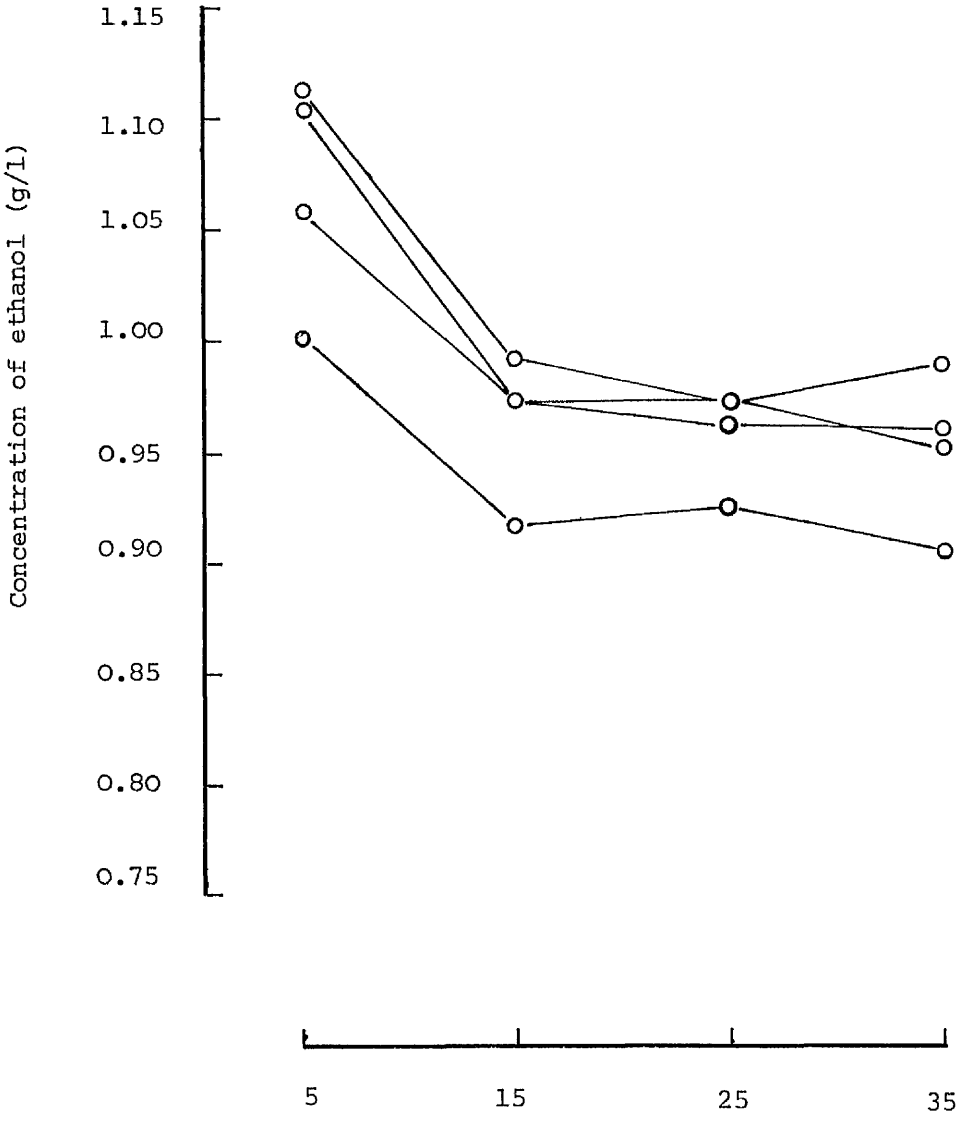
Clearance of an injected dose of ethanol (0.5 ml of 30 per cent v/v in physiological saline) from the bloodstream of normal rats. All rats were male and approximately 220 g in body weight.

Figure 12



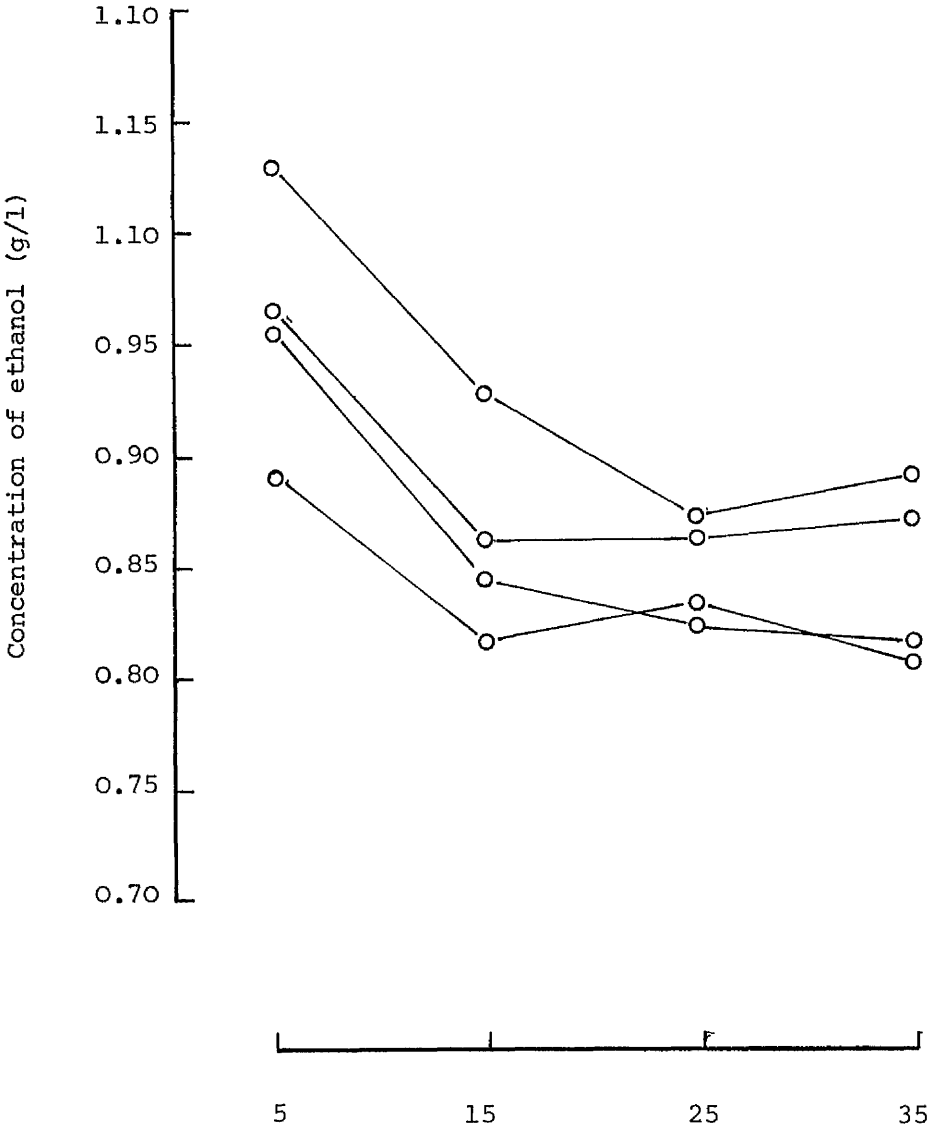
Clearance of an injected dose of ethanol (0.5 ml of 30 per cent v/v ethanol in physiological saline) from the bloodstream of rats immediately after a liver biopsy. All rats were male and approximately 220 g in body weight.

Figure 13



Clearance of an injected dose of ethanol (0.5 ml of 30 per cent v/v ethanol in physiological saline) from the bloodstream of rats immediately after partial hepatectomy. All rats were male and approximately 220 g in body weight.

Figure 14



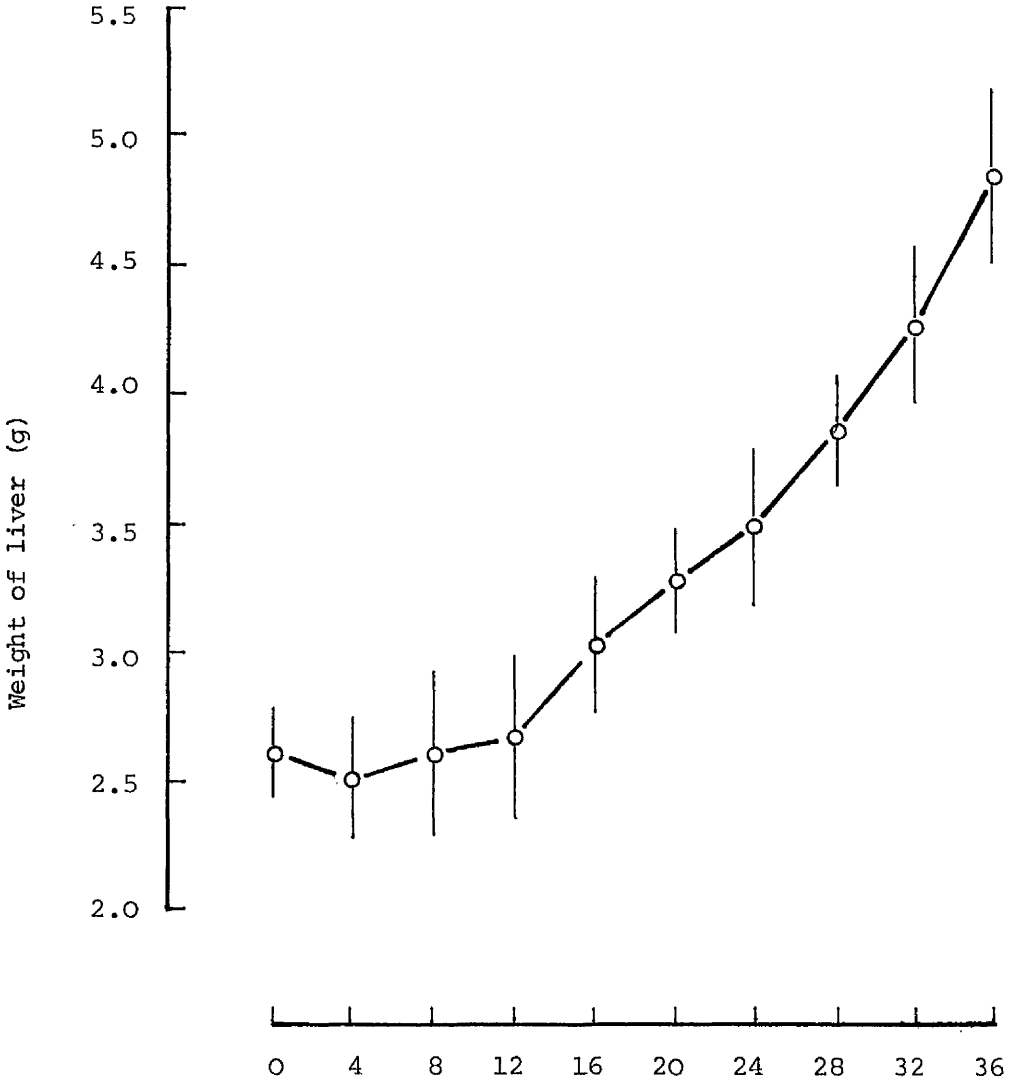
Clearance of an injected dose of ethanol (0.5 ml of 30 per cent v/v ethanol in physiological saline) from the bloodstream of rats twenty-four hours after partial hepatectomy. All rats were male and approximately 220 g in body weight.

alcohol dehydrogenase, which is almost exclusively present in the liver (West et al., 1966). This enzyme converts ethanol to acetaldehyde, which is converted to acetate and then to acetyl CoA. Our experiments do not provide any evidence regarding the limiting factor(s) in ethanol oxidation after partial hepatectomy. However, it seems clear that the liver remnant after partial hepatectomy is unable to oxidize ethanol effectively up till at least twenty-four hours after partial hepatectomy. Since the rate of ethanol oxidation becomes negligibly small and remains so for at least twenty-four hours following partial hepatectomy, it could not be reliably used as a measure of liver size in the study of liver regeneration.

2.3 The growth of the remaining liver fragment

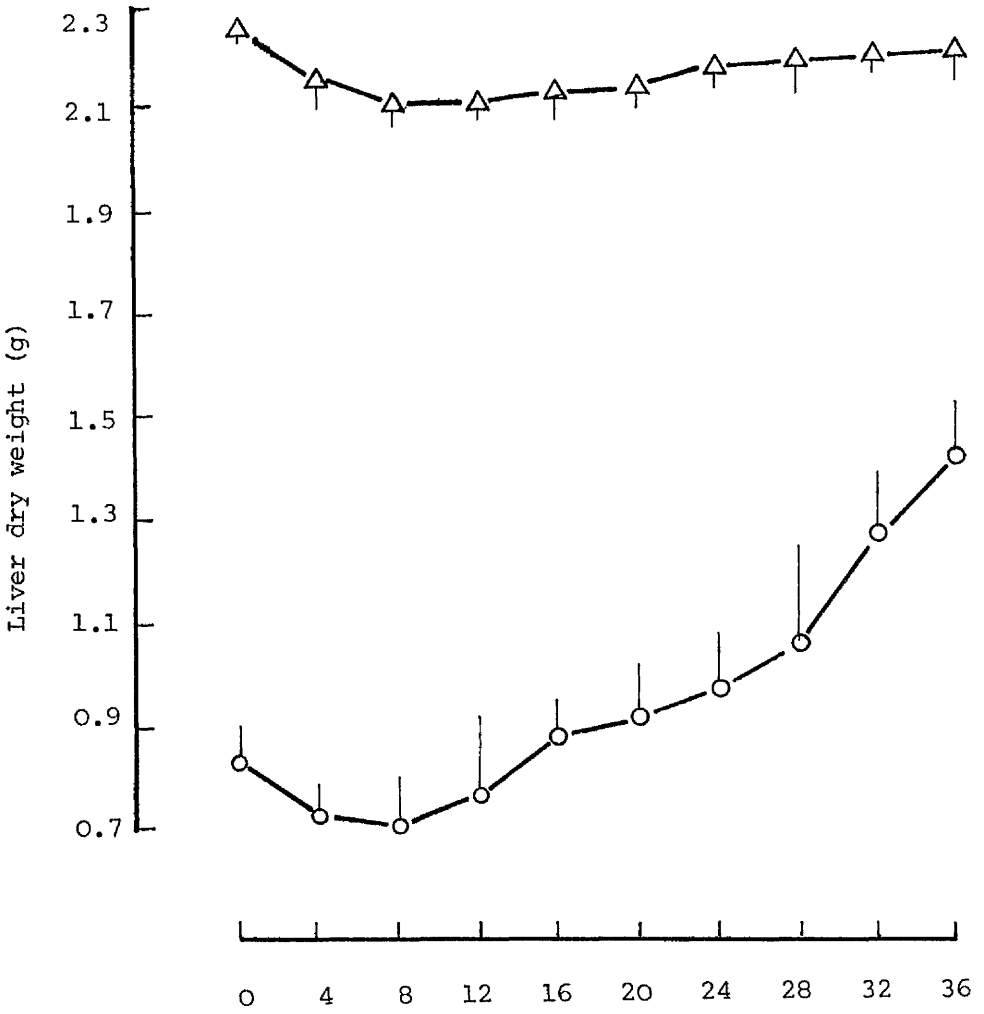
Figure 15 shows the increase in wet weight of the liver lobes left behind at partial hepatectomy. It is clear that for the first twelve hours there is no increase, but thereafter growth is rapid and continuous. The dry weight (Figure 16) follows a similar pattern as does the total protein (Figure 17). The total DNA content of the lobes seems to respond somewhat more slowly. It shows no significant alteration for the first sixteen hours and only then increases, roughly in parallel with wet and dry weight and total protein (Figure 18). The overall pattern is of an initial lag phase, lasting about twelve hours, then of an increase of tissue mass followed at a gap of four hours by an increase in DNA which presumably reflects preparation for cell division. This interpretation is in accordance with the rate of DNA synthesis as reflected in [^{3}H]-thymidine incorporation (Figure 19) which remains very low for the first sixteen hours and then increases dramatically.

Figure 15



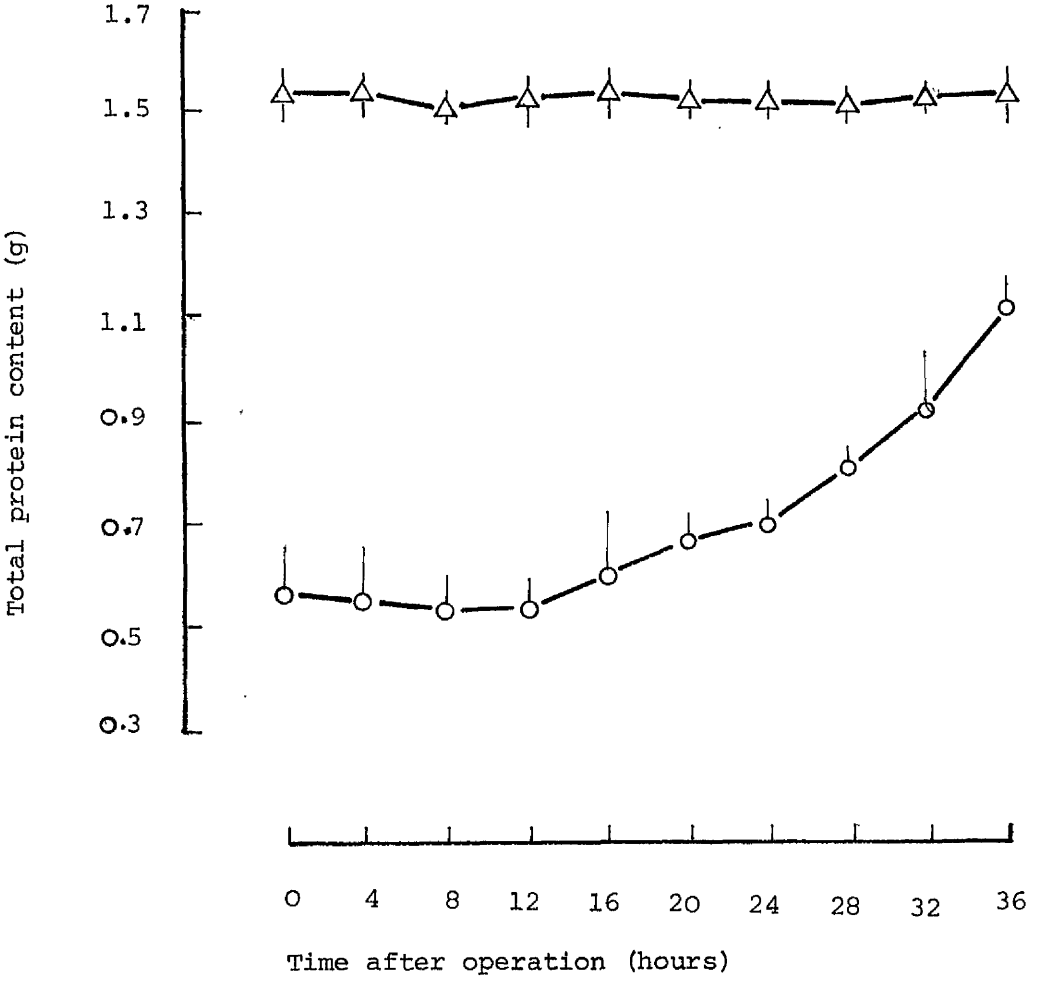
Increase in the wet weight of liver lobes remaining after partial hepatectomy. At least eighteen male rats (body weight approximately 220 g) were used for each estimation. The results are shown as mean ± standard deviation.

Figure 16



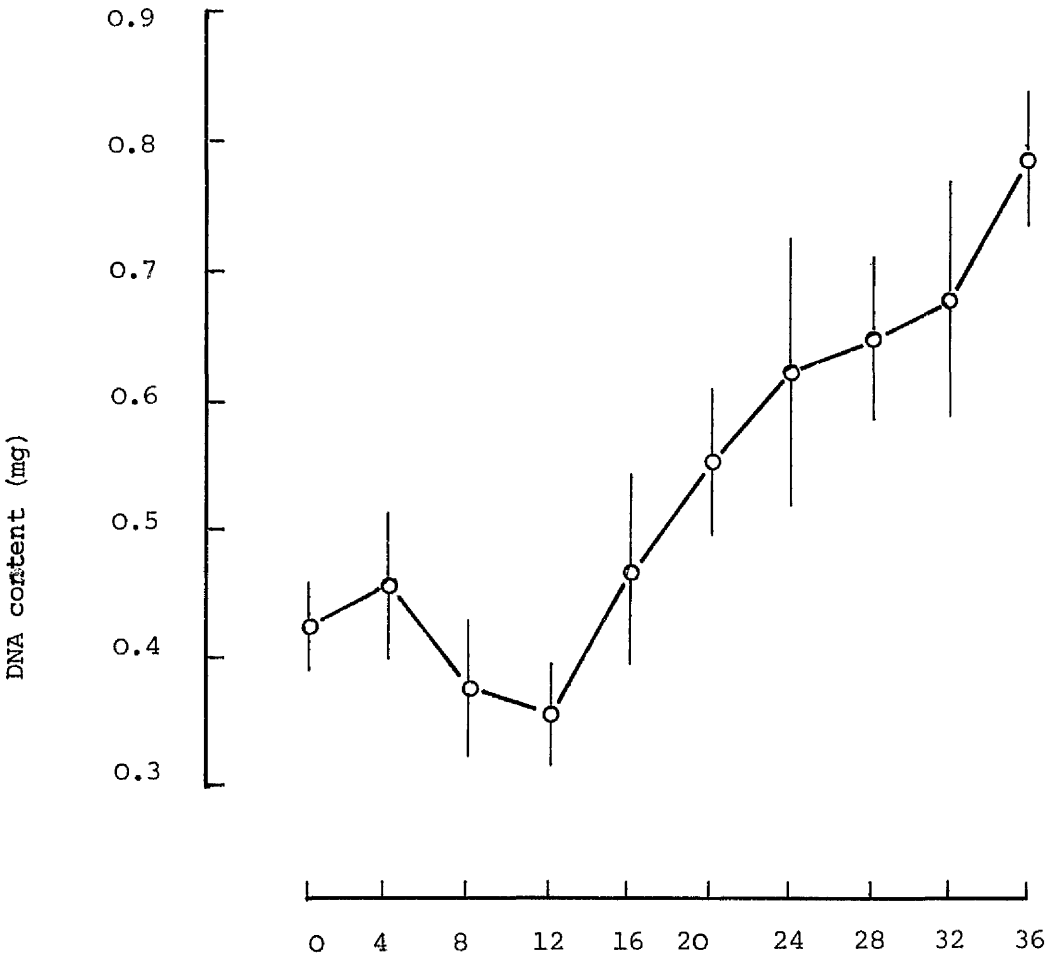
Changes in the dry weight of liver lobes remaining after either liver biopsy (△) or partial hepatectomy (o). Six male rats (body weight approximately 220 g) were used for each estimation. The results are shown as mean ± standard deviation.

Figure 17



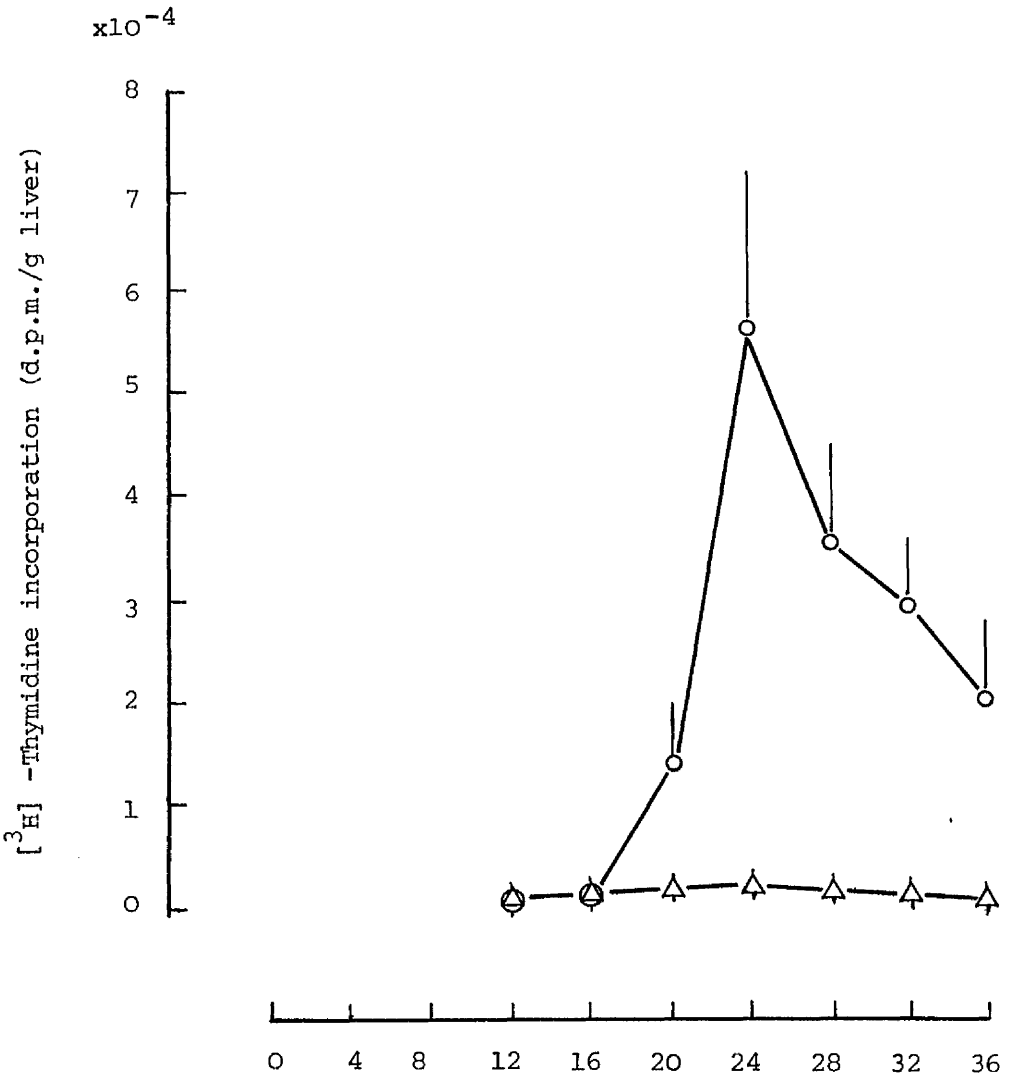
Changes in the total protein content of liver lobes remaining after either liver biopsy (△) or partial hepatectomy (o). At least six male rats (body weight approximately 220 g) were used for each estimation. The results are shown as mean ± standard deviation.

Figure 18



Increase in the total DNA content of liver lobes remaining after partial hepatectomy. Eighteen male rats (body weight approximately 220 g) were used for each estimation. The results are shown as mean ± standard deviation.

Figure 19

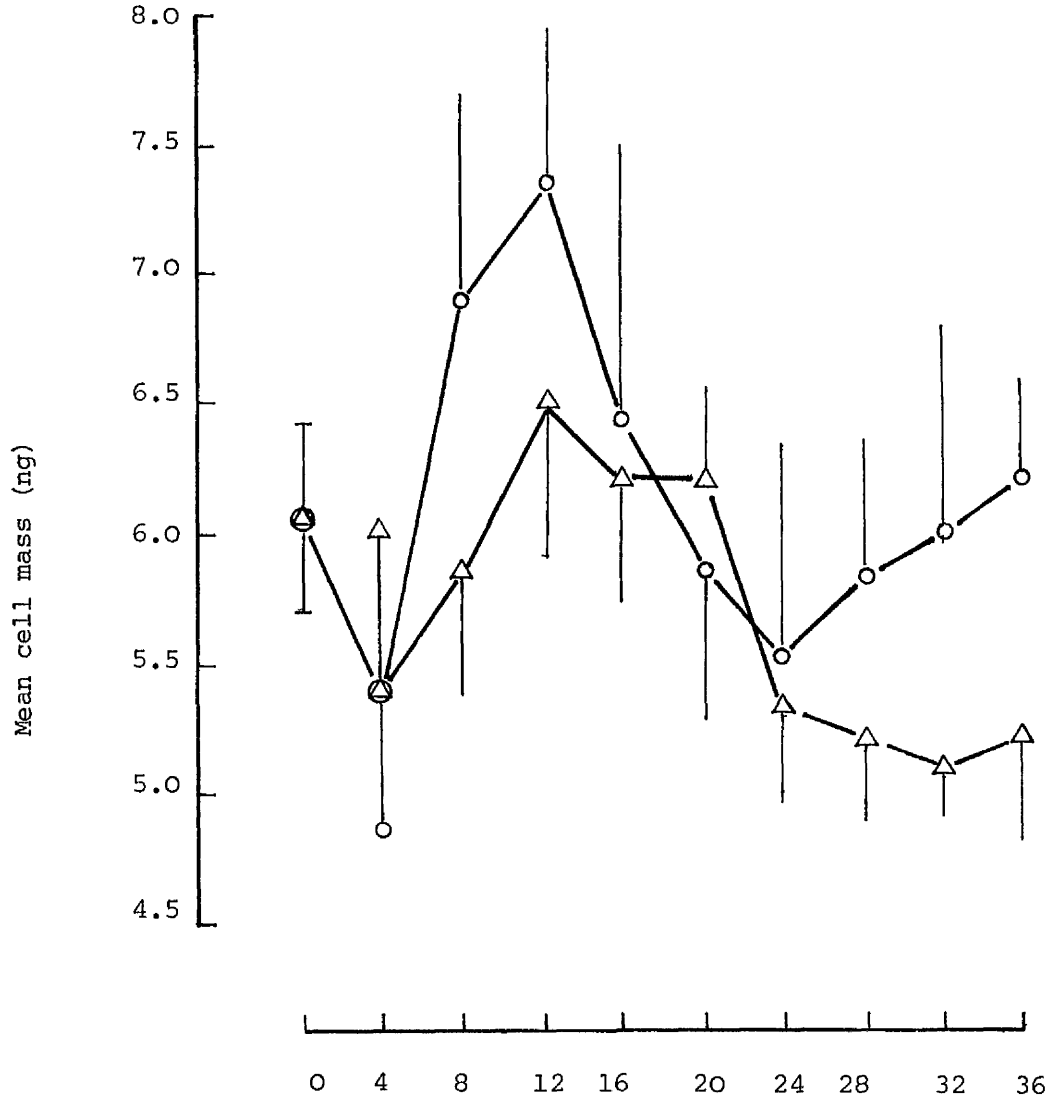


The rate of DNA synthesis following <u>either</u> liver biopsy (△) <u>or</u> partial hepatectomy (o) as reflected in the increase in uptake of [^{3}H]-thymidine by the liver lobes. Six male rats (body weight approximately 220 g) were used for each estimation. The results are shown as mean ± standard deviation.

Figure 20 shows the changes in mean cell mass calculated on the assumption that the mean DNA per cell is constant, an assumption which seems safe for the twelve to sixteen hours immediately following the operation when DNA synthesis had not yet started and which is at least approximately true thereafter. For purposes of comparison, Figure 20 shows the changes which follow removal of a biopsy as well as partial hepatectomy. The effects of the two operations are remarkably similar. There is an initial fall four hours after operation, succeeded by a rise which reaches a peak at twelve hours, the peak being higher after partial hepatectomy. From twelve to twenty-four hours the patterns diverge. In biopsied animals there is little further change. In hepatectomised animals there is a second significant increase between twenty-four and thirty-six hours.

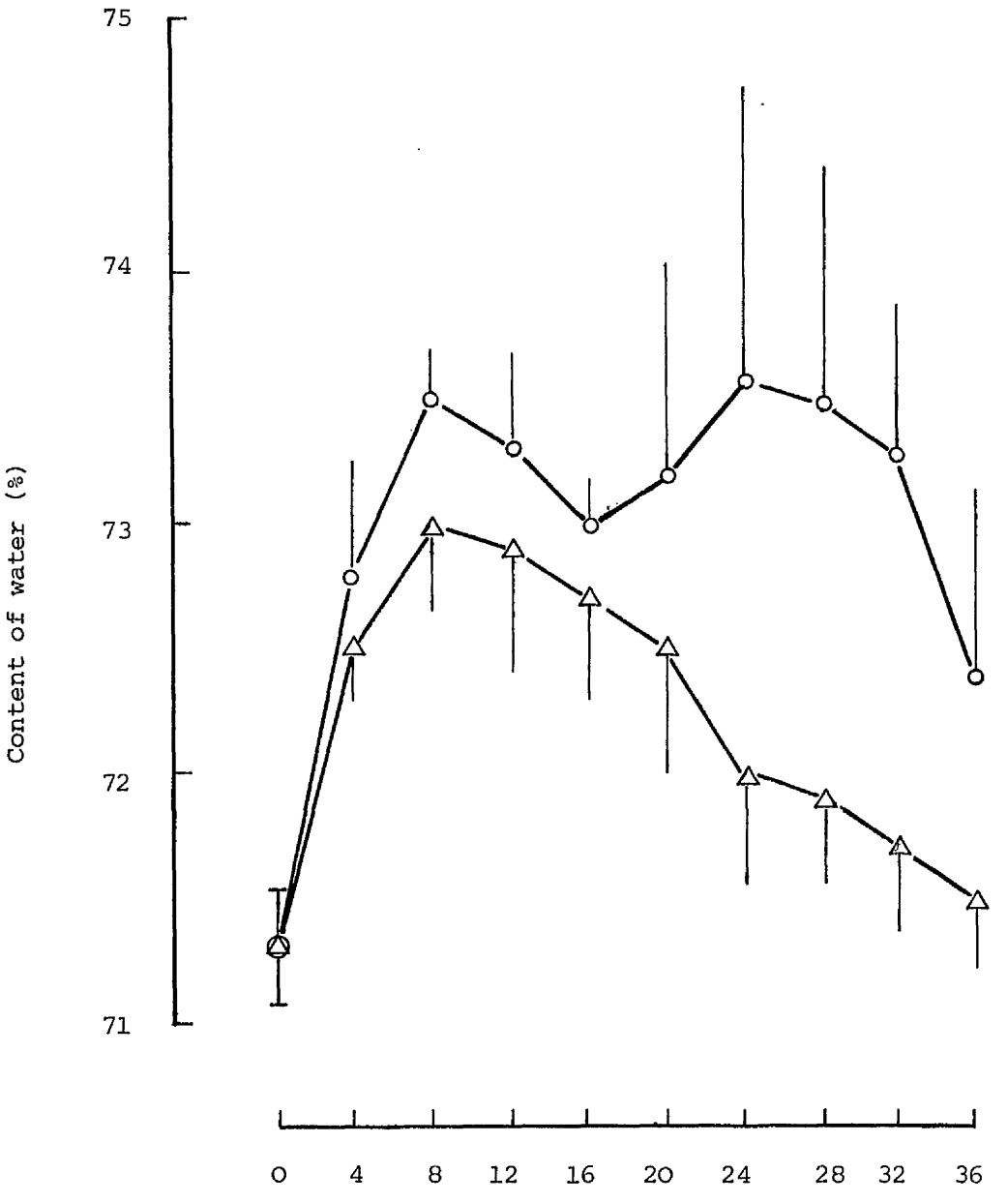
Figure 21 shows the changes in water content in the remaining liver after either partial hepatectomy or liver biopsy. These, though significant, are quite small. Initially both operations are followed by an increase in water content which reaches a peak at eight hours. Thereafter in biopsied animals there is a progressive return towards the preoperative level, which is attained at thiry-six hours. In hepatectomised animals, however, there is a second influx of water between sixteen and twenty-four hours, and even after thirty-six hours the water content of the cells is still significantly above the preoperative level. The trend on the whole therefore is similar after either operation but the changes are more pronounced in partially-hepatectomised rats and take longer to reverse. A plausible explanation for these observations may be that after the initial four hours of either liver biopsy or partial hepatectomy there is a net increase in water content as well as the dry weight, but since the water

Figure 20



Changes in mean cell mass following either liver biopsy (△); or partial hepatectomy (o). Eight rats were used for each time interval in the case of liver biopsy and eighteen rats at each time interval in the case of partial hepatectomy. All rats were male and approximately 220 g in body weight. The results are shown as mean ± standard deviation.

Figure 21



Changes in the per centage water content in the liver lobes remaining after either liver biopsy (△) or partial hepatectomy (o). Six male rats (body weight approximately 220 g) were used for each estimation. The results are shown as mean ± standard deviation.

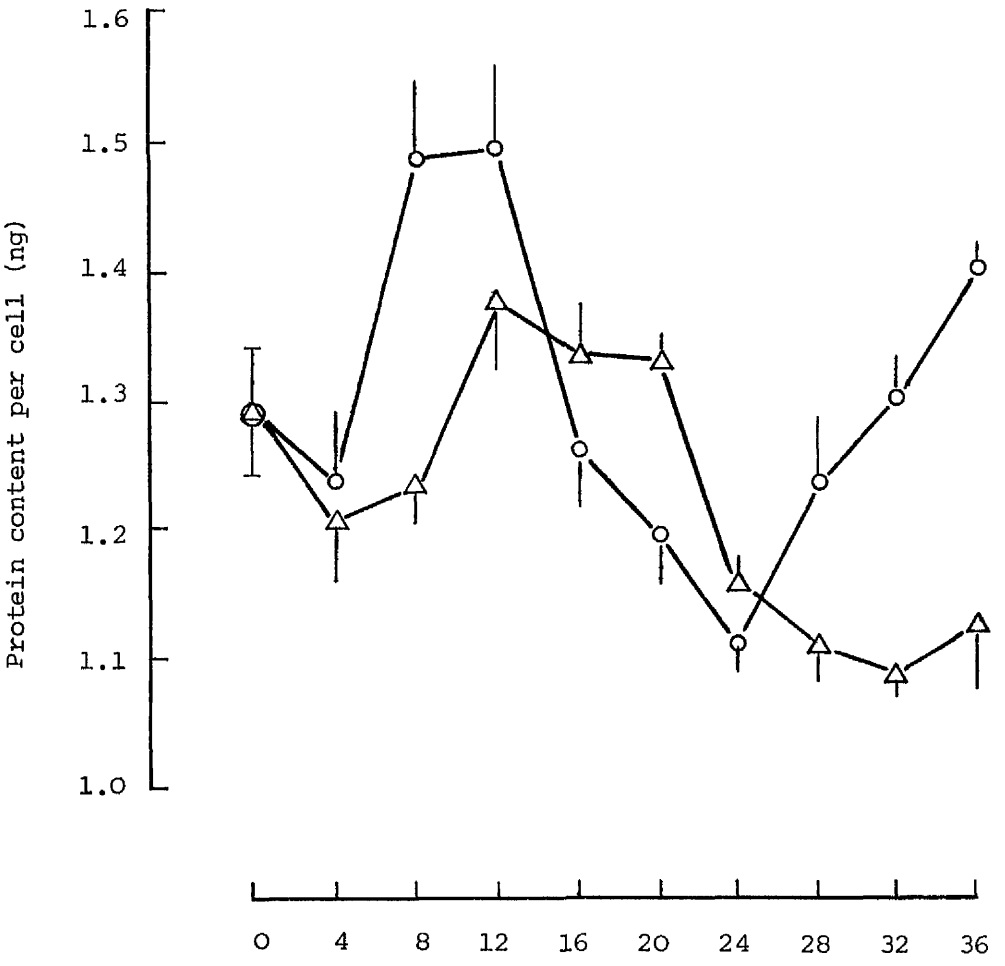
content shows a proportionately greater increase the dry weight percentage appears to reduce, thus giving an erroneous impression of decrease in dry weight. The analysis of individual cell constituents also supports this explanation.

Figure 22 shows the content of protein per cell following either liver biopsy or partial hepatectomy. In both cases, after an abrupt initial fall, this increases to a peak around twelve hours after the operation (higher and earlier after partial hepatectomy) and then falls back towards the pre-operative level at twenty-four hours. Thereafter there is a divergence. In biopsied animals there is little further change up to thirty-six hours, but in hepatectomised animals there is a renewed increase.

Figure 23 shows the changes in total lipids following either liver biopsy or partial hepatectomy. In biopsied animals the total lipid content per cell does not show appreciable change and, except at twelve hours, does not alter significantly during the first day of operation. In partially-hepatectomised rats, however, the total lipid content per cell starts to increase immediately after the operation and is more than twice normal by twelve hours. Thereafter it decreases progressively towards the normal level, which is reached at twenty-eight hours.

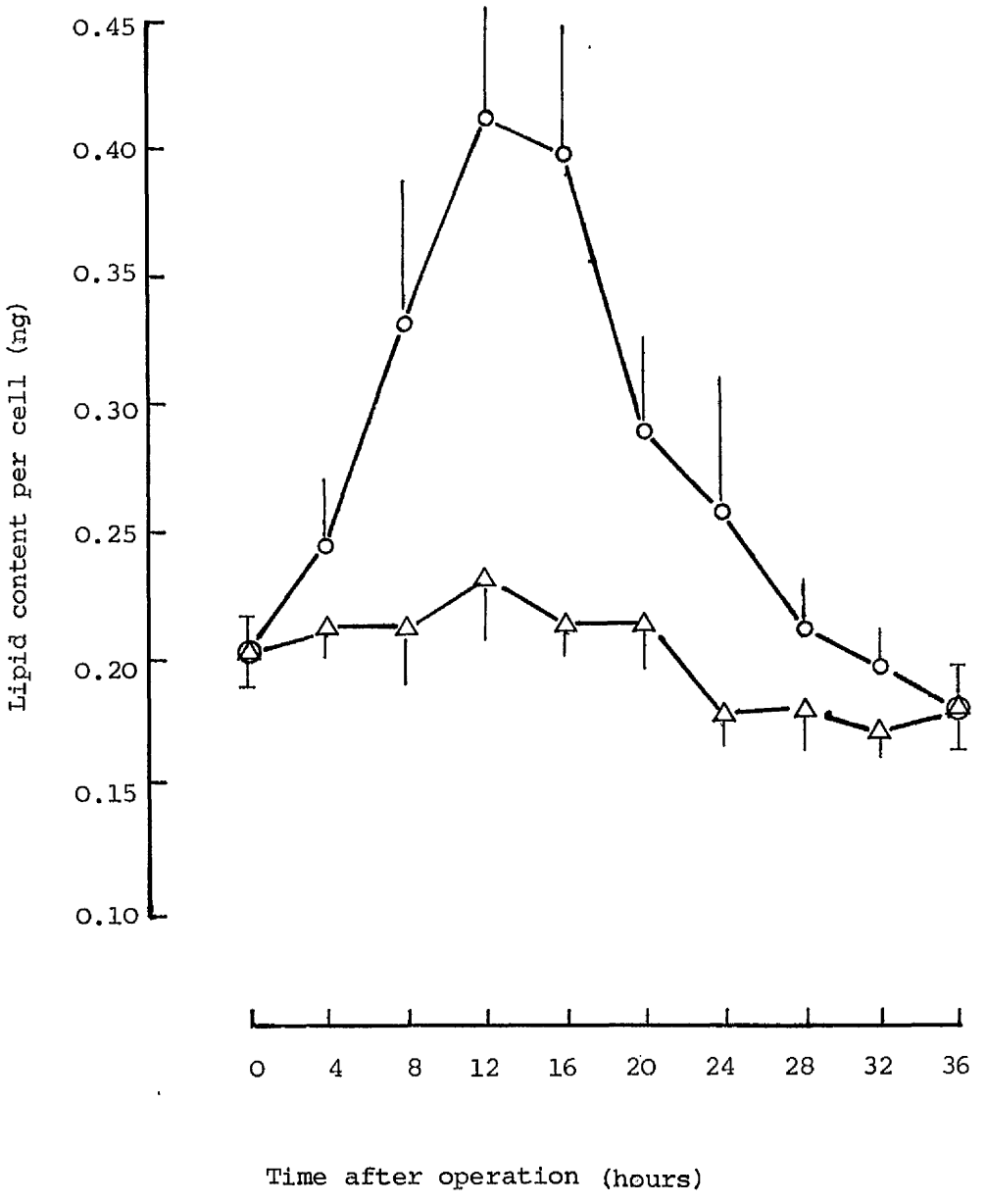
Figure 24 shows the changes in glycogen content per cell after partial hepatectomy and liver biopsy. In both cases it decreases sharply immediately after the operation. Thereafter there is a slow but steady recovery, which is still incomplete at thirty-six hours. The fall is marginally but significantly ($p < 0.05$) greater in the case of partial hepatectomy and the recovery is much slower. Figure 25 shows the changes in dry weight per cell after partial hepatectomy and liver biopsy. After either operation there is an immediate and substantial

Figure 22



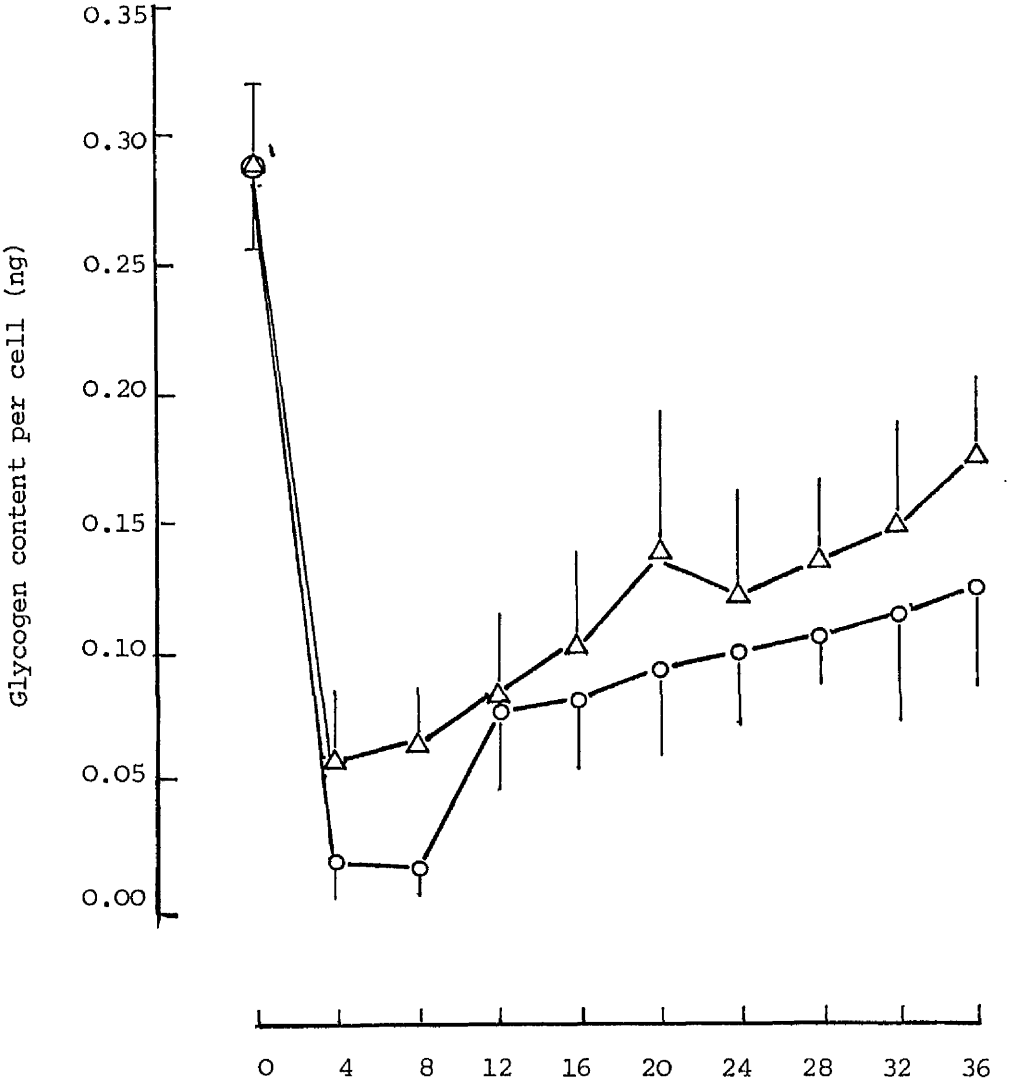
Changes in protein content per cell following either liver biopsy (△) or partial hepatectomy (o). At least six male rats (body weight approximately 220 g) were used for each estimation. The results are shown as mean ± standard deviation.

Figure 23



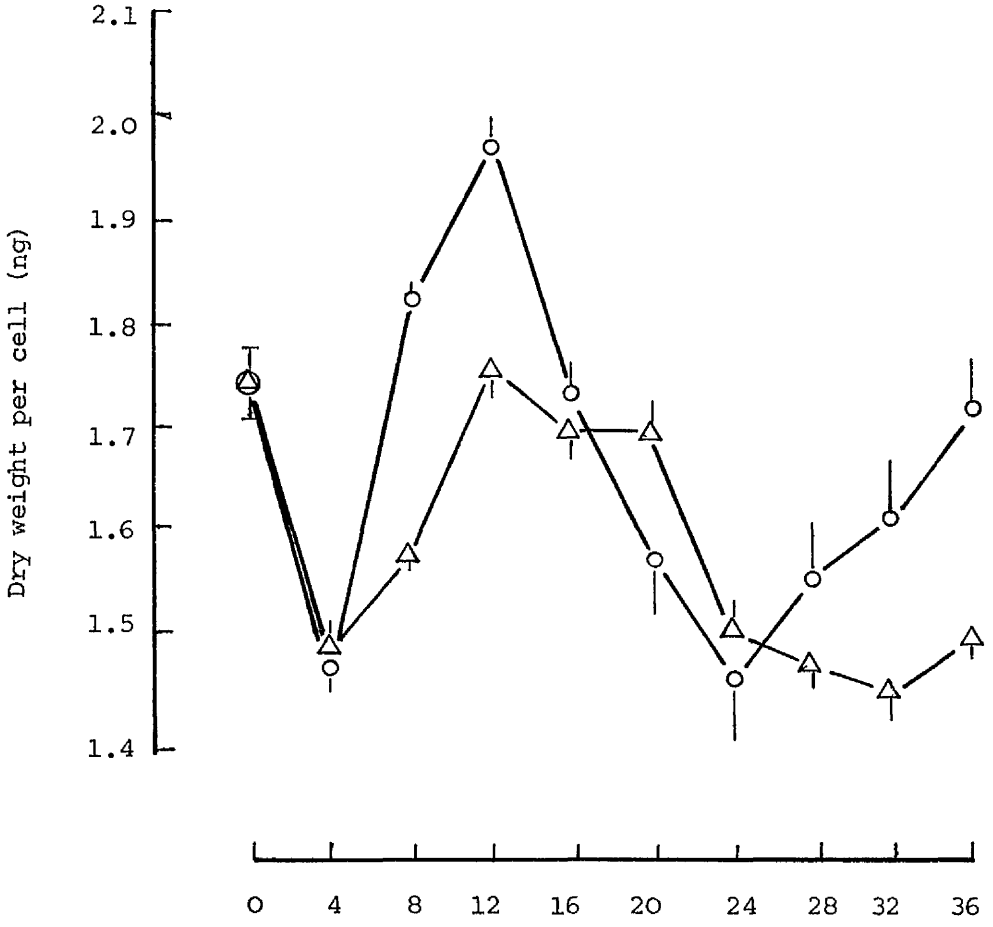
Changes in total lipid content per cell following either liver biopsy (△) or partial hepatectomy (o). Six male rats (body weight approximately 220 g) were used for each estimation. The results are shown as mean ± standard deviation.

Figure 24



Changes in glycogen content per cell after either liver biopsy (△) or partial hepatectomy (o). Six male rats (body weight approximately 220 g) were used for each estimation. The results are shown as mean ± standard deviation.

Figure 25



Changes in dry weight per cell after either liver biopsy (△) or partial hepatectomy (o). Six male rats (body weight approximately 220 g) were used for each estimation. The results are shown as mean ± standard deviation.

fall, reflecting presumably the loss of glycogen. Thereafter there is a recovery, more marked in the hepatectomised animals and attaining a maximum around twelve hours. In biopsied animals this is followed by a decrease to a steady level below that found before operation. But in hepatectomised animals the decrease between twelve and twenty-four hours is succeeded by a second increase between twenty-four and thirty-six hours. Comparison of Figures 22 and 25 shows that the trend in the two figures is similar. This suggests that the changes reflected in Figure 25 are largely determined by the changes in total protein content of cells, although the changes in other cell constituents modify the values of dry weight.

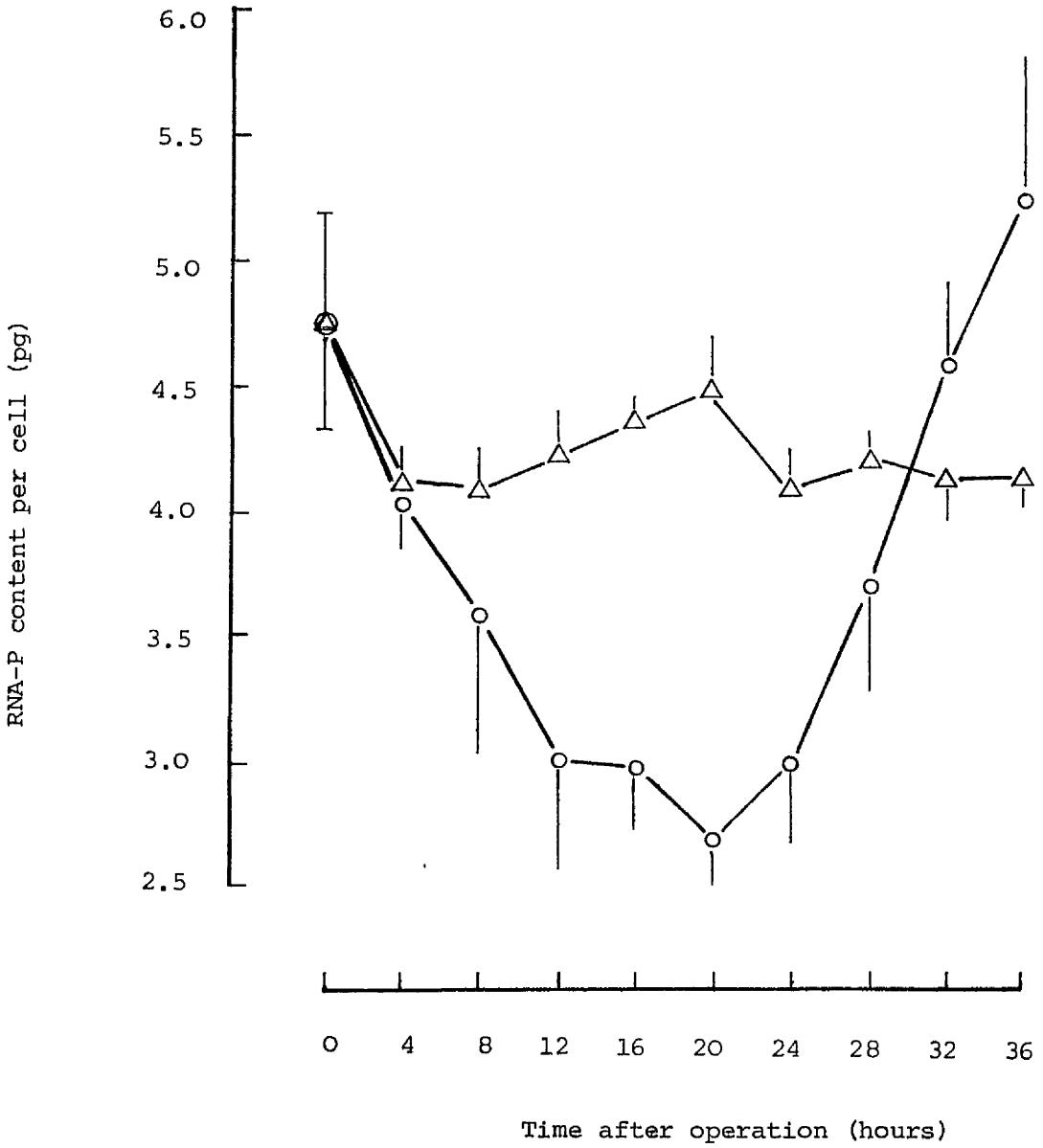
Figure 26 shows the amount of RNA per cell both after partial hepatectomy and liver biopsy. Soon after either operation the concentration of RNA decreases. In biopsied rats the decrease is relatively small but significant ($P < 0.05$) and there is little further change up to thirty-six hours. In partially-hepatectomised rats, however, the fall is substantial and reaches a minimum at twenty hours. Thereafter there is a progressive recovery with the preoperative value being attained at thirty-two to thirty-six hours.

2.4 Some suggested explanations of liver regeneration

At the time when present investigation was started there were in the literature a number of suggested explanations for liver regeneration supported to varying degrees by experimental evidence but as yet neither clearly established nor conclusively disproved. It seemed worthwhile to re-examine these to see whether any of them were worth further investigation. These were as follows.-

A) A report that the regenerative response could be evoked by a brief ligation of the lobes customarily excised at hepatectomy (Lieberman et al., 1967).

Figure 26



Content of RNA-P per cell following either liver biopsy (△) or partial hepatectomy (o). Eight male rats (body weight approximately 220 g) were used for each estimation. The results are shown as mean ± standard deviation.

B) A claim that hyperplasia could be induced in the intact liver by repeated intraperitoneal injections of a protein hydrolysate (Alston and Thomson, 1968).

C) A series of experiments by Starzl and his collaborators which appeared to indicate that insulin plays some role in regeneration (Starzl et al., 1975, 1976a, 1976b).

D) The evidence, mainly from the early experiments by Mann and his colleagues, that the haemodynamic changes consequent on partial hepatectomy might provide part of the stimulus for regeneration (Mann et al., 1931).

The results of reinvestigating each of these are reported below.

2.4.1 Experiments with ligated livers

Lieberman et al., (1967) reported that ligation of two-thirds of the liver for only ten minutes induced a regenerative response, as indicated by mitotic activity in the remaining lobes, comparable to the response produced after partial hepatectomy. On the basis of these results, they suggested that the stimulus for regeneration is produced immediately after partial hepatectomy and, once produced, it is not reversed. In these experiments they assumed that ligation for ten minutes was unlikely to cause death of hepatocytes. Table 5 shows the results of our similarly designed experiments. No increase in the rate of DNA synthesis was apparent in livers ligated for ten minutes. Livers which were ligated for fifteen minutes showed a small increase in the rate of DNA synthesis but it was not statistically significant and the ligated lobes showed considerable atrophy.

Table 5 also shows the concentration of nucleic acids in the unligated lobes of the liver in these rats. It is clear from the table that the concentration of RNA-P, DNA-P and the RNA-P: DNA-P ratio are not

TABLE 5

The effect of temporary ligation of the median and left lateral lobes of the liver on the concentrations of nucleic acids in the remaining lobes and, on the uptake of [^{3}H]-thymidine in these lobes. There were three male rats (body weight approximately 200 g) in each group. The results are shown as mean ± standard deviation.

Observations	Condition of Rats		
	Sham operated controls	Two-thirds liver ligated for ten minutes	Two-thirds liver ligated for fifteen minutes
RNA-P (ug/g wet weight of liver)	541 ± 97	461 ± 75	564 ± 82
DNA-P (ug/g wet weight of liver)	177 ± 15	147 ± 30	202 ± 22
RNA-P : DNA-P ratio	3.04 ± 0.3	3.15 ± 0.14	2.8 ± 0.35
[^{3}H]-thymidine uptake per g liver (dpm)	995 ± 214	1020 ± 89	6217 ± 4700

statistically different in the three groups. This suggests that the hepatocyte structure in the unligated lobes remains unaltered by temporary ligation of other lobes. Clearly such ligation does not produce any regenerative response comparable to that seen after partial hepatectomy. Our findings therefore do not support the suggestion of Lieberman and his associates. It appears from our results that the stimulus for liver regeneration either arises much later than they thought or that any stimulation produced could be reversed.

2.4.2 Protein hydrolysate injections

Alston and Thomson (1968) reported that infusion of a protein hydrolysate induces regenerative response in the intact livers of normal rats. We performed similar experiments to confirm this finding. In these experiments 4 ml doses of Vamin (chick albumin hydrolysate) were injected intraperitoneally at four hour intervals for forty-eight hours into rats which had been biopsied twenty-four hours before the first injection. In total every rat received twelve injections. These were well tolerated. The results are shown in Tables 6 and 7. The first of these shows a comparison between a liver biopsy taken twenty-four hours before the vamin injections were started and the remainder of the liver at death. It is apparent that the Vamin has not altered the concentrations of either RNA-P or DNA-P (Table 6). Table 7 shows a comparison of the nucleic acid concentrations in the livers of Vamin-treated rats with those of saline-injected controls. There is no difference in body weight or liver weight between the two groups and the apparent difference in total DNA content is not significant. But the Vamin-injected group have a lower concentration of RNA-P per gram wet weight than the controls and a higher concentration of DNA-P, i.e. Vamin treatment appears to reduce mean cell mass and mean content of RNA per cell, which is the reverse of Alston and Thomson's (1968) findings.

TABLE 6

Effect of multiple injections of "Vamin" on the concentrations of nucleic acids in rat liver. Five male rats (body weight approximately 200 g) were biopsied and then subjected to a course of twelve intraperitoneal injections of Vamin (4 ml on each occasion) at four hour intervals, starting twenty-four hours after the operation. All rats were killed forty-eight hours after the first injection. The results are shown as mean ± standard deviation. The composition of Vamin is given in Materials and Methods.

Observations	Concentration		Difference in concentration
	At biopsy	At death	
RNA-P ug/g wet weight of liver	440 ± 69	474 ± 54	-30 ± 16.30
DNA-P ug/g wet weight of liver	268 ± 16	270 ± 15	- 5.76 ± 4.41
RNA-P : DNA-P ratio	1.646 ± 0.352	1.695 ± 0.278	- 0.345 ± 0.116

TABLE 7

Changes in liver weight and nucleic acid concentrations in male rats following a series of injections of either saline or "Vamin". The rats were biopsied and then subjected to a course of twelve intraperitoneal injections (4 ml on each occasion) at four-hour intervals starting twenty-four hours after the operation. All rats were killed forty-eight hours after the first injection. The results are shown as mean ± standard deviation. The composition of Vamin is given in Materials and Methods.

Observations	Injected with Saline	Injected with Vamin
No. of animals	5	10
Body weight at death (g)	190 ± 6	186 ± 5
Liver weight (g)	6.98 ± 0.29	7.13 ± 0.45
RNA-P (ug/g wet liver weight)	596 ± 59	495 ± 72
DNA-P (ug/g wet liver weight)	217 ± 20	246 ± 21
RNA-P : DNA-P ratio	2.75 ± 0.17	2.02 ± 0.24
Total DNA content (mg)	16.97 ± 1.43	18.41 ± 2.04

It seems unlikely therefore that the growth response obtained by Alston and Thomson (1968) was a response to the administration of amino-acids as such. It may have been produced by something else in the protein hydrolysate they used.

2.4.3 Liver regeneration in diabetic rats

Starzl and associates (1975, 1976a, 1976b) using elaborate surgical techniques found evidence for insulin being the major factor involved in maintaining hepatocyte integrity and stimulating regeneration. To test the role of insulin as a stimulator of liver regeneration we attempted to elicit liver regeneration in the absence of insulin. The diffuse nature of the pancreas in rats makes pancreatectomy very difficult. To overcome this, chemical destruction of β-cells of the pancreas (which are responsible for the production of insulin) was attempted by administration of alloxan (0.2 ml of 60 mg alloxan per ml in physiological saline) one day before partial hepatectomy. In each case blood sugar levels in excess of 250 mg per 100 ml were obtained.(The normal value in the rat is about 60 mg per 100 ml). The results of these experiments are given in Tables 8 and 9. Table 8 compares the regenerative response five days after partial hepatectomy in alloxan-diabetic rats with that in normal rats. The diabetic rats lost weight post-operatively while the normal controls showed an increase; but the weight of the liver remnant at death bore the same relation to the weight of the lobes excised in both groups. And,while the diabetic animals showed at death a lower mean cell mass,the DNA content of the liver remnant in both groups was the same. Clearly,therefore, regeneration is unimpeded even when insulin production is seriously impaired.

Table 9 compares the effect of partial hepatectomy with those of liver biopsy in alloxan-diabetic rats three days after operation. The

TABLE 8

Liver weight and nucleic acid concentrations five days after partial hepatectomy in normal and alloxan diabetic rats. Diabetes was induced by a single injection of alloxan (0.2 ml of 60 mg alloxan per ml in physiological saline) twenty-four hours before partial hepatectomy. All rats were male and approximately 220 g in body weight. The results are shown as mean ± standard deviation.

Observations	Normal	Diabetic
No. of rats	3	4
Change in body weight since operation (g)	+ 6.67 ± 0.99	-13.38 ± 8.71
Weight of lobes excised (g)	4.94 ± 0.51	4.11 ± 1.24
Weight of liver remnant at death (g)	5.61 ± 1.59	5.08 ± 0.64
Ratio of liver remnant weight to excised liver weight	1.133 ± 0.285	1.234 ± 0.352
RNA-P (ug)g wet weight of liver)	768 ± 36	708 ± 54
DNA-P (ug/g wet weight of liver)	342 ± 59	389 ± 33
RNA-P/DNA-P ratio	2.25 ± 0.54	1.82 ± 0.24
DNA content of liver remnant (mg)	19.77 ± 3.53	21.06 ± 4.49

TABLE 9

Liver weight and nucleic acid concentrations in alloxan diabetic rats subjected to liver biopsy or partial hepatectomy. Diabetes was induced by a single injection of alloxan (0.2 ml of 60 mg alloxan per ml in physiological saline) twenty-four hours before operation. All rats were male and approximately 220 g in body weight. The results are shown as mean ± standard deviation.

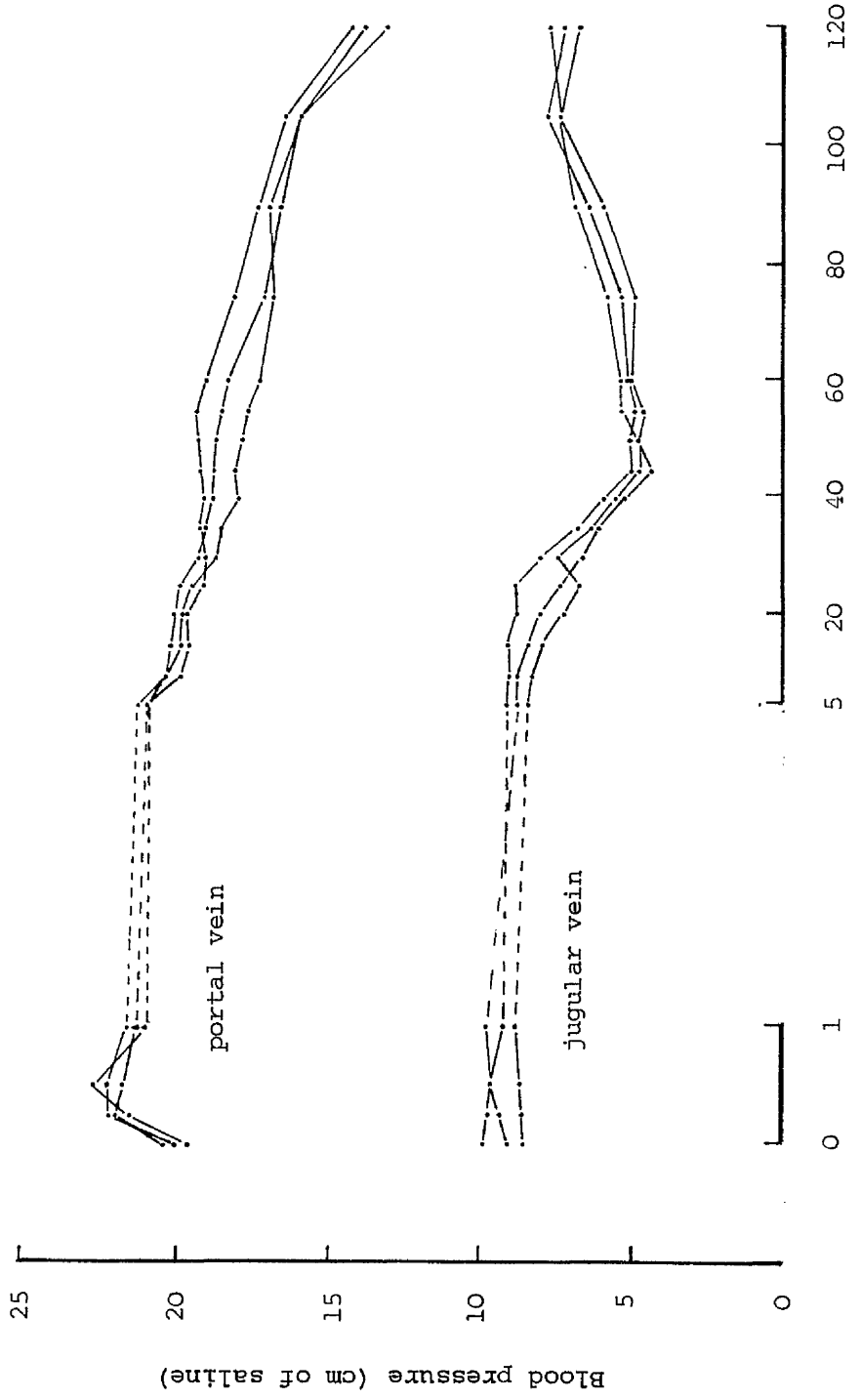
Observations	Biopsied rats	Partially hepatectomised rats
No. of rats	4	4
Change in body weight since operation (g)	-28 ± 9	-28 ± 5
Weight of liver excised (g)	0.51 ± 0.24	4.41 ± 1.36
Weight of liver at death (g)	7.39 ± 0.88	4.78 ± 1.48
RNA-P (ug/g wet weight of liver)	648 ± 183	712 ± 156
DNA-P (ug/g wet weight of liver)	300 ± 64	344 ± 30
RNA-P : DNA-P ratio	2.23 ± 0.16	2.11 ± 0.31

blood sugar in both groups again showed levels in excess of 250 mg per 100 ml. Diabetes caused a decrease in the body weight in both groups. The RNA-P and DNA-P concentrations were not significantly different in the two groups nor was the RNA-P : DNA-P ratio. Once again, however, although partial hepatectomy would remove about two-thirds of the liver mass, the liver remnant at death in the hepatectomised animals was slightly larger than the lobes excised at operation. Clearly therefore, although loss of insulin production has marked effects on the size and composition of the liver, it does not impair the capacity for regeneration.

2.4.4 Portal vein blood pressure

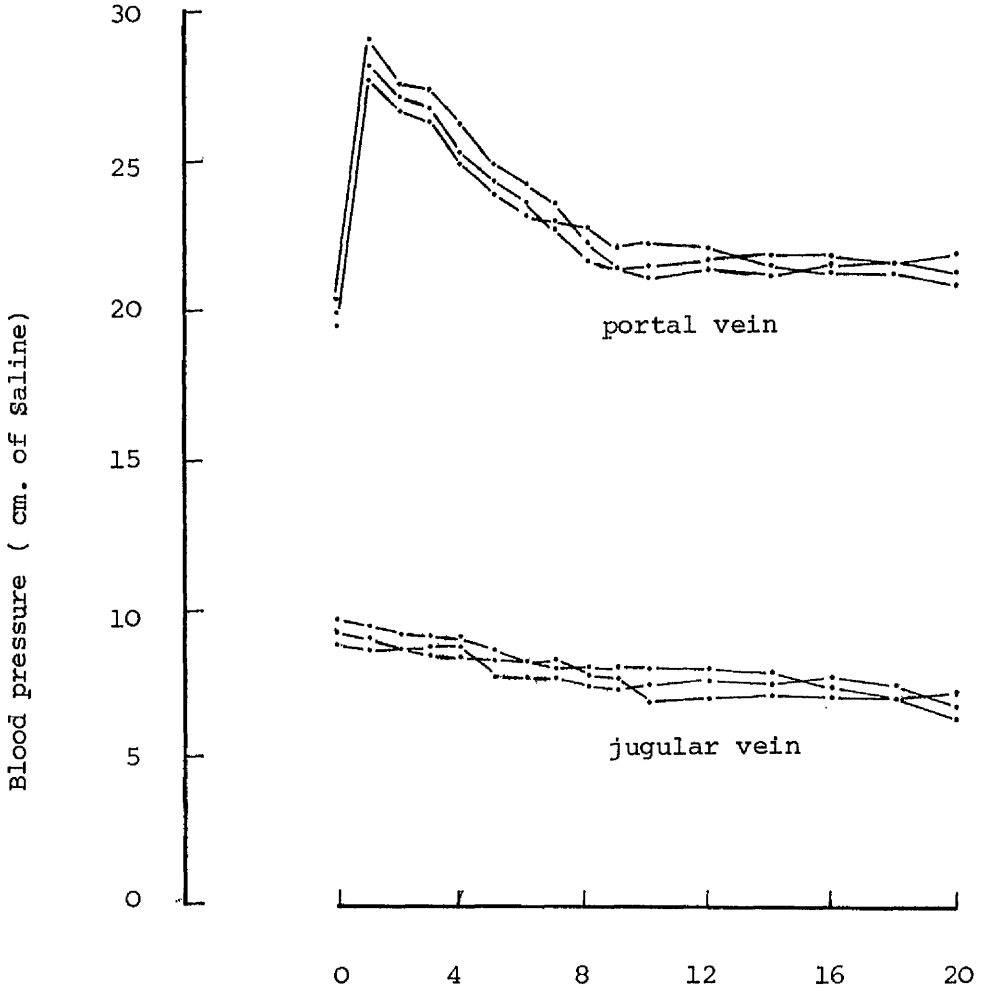
The theory, once generally accepted, that portal blood flow plays an important role in liver regeneration has now been generally abandoned. But the results of Thomson and Clarke (1965)do not entirely rule out the possibility that it may have some influence and it seemed worthwhile to look again at the haemodynamic changes following partial hepatectomy. Figures 27 and 28 show the results of experiments to estimate the blood pressure in portal vein following partial hepatectomy (two-thirds hepatectomy) and sub-total hepatectomy (four-fifth hepatectomy) respectively. Since the blood pressure may vary with the posture of the animal, the blood pressure in the left external jugular vein was also recorded. It is clear from Figure 27 that when two-thirds of the liver is removed the blood pressure in the portal vein rises only slightly for a brief period immediately after the operation and returns to normal within ten minutes. It later remains normal for at least one hour and thereafter decreases gradually. This may be an effect of prolonged anaesthesia. Figure 28 shows that the portal blood pressure following a four-fifths hepatectomy increases considerably more compared to its rise after partial hepatectomy. But this increase is also

Figure 27



Changes in blood pressure in the portal vein and left external jugular vein following two-thirds hepatectomy. All rats used for the experiment were male and approximately 220 g in body weight.

Figure 28



Changes in blood pressure in the portal vein and left external jugular vein following four-fifth hepatectomy. All the rats used for the experiment were male and approximately 220 g in body weight.

short-lived and the portal blood pressure returns to within normal limits by about ten minutes. It seems clear therefore that following both operations neither the liver nor the portal vein is subjected to excessive hemodynamic pressure. Obviously therefore the hemodynamic pressure cannot trigger the regenerative response in the liver.

2.5 BLOOD ANALYSIS

2.5.1 Introduction

The most plausible theory of liver regeneration is still that partial hepatectomy produces some unspecified change in the composition of the blood and that this is, directly or indirectly, the stimulus which causes the remaining liver fragment to grow. Hitherto, attempts to demonstrate such supposed changes in blood composition have been designed to demonstrate that blood from a hepatectomised rat is capable of stimulating growth in normal liver. As has been shown above (1.6.3.) this has proved remarkably difficult. It seemed therefore worth while to tackle the problem from the other end by trying to establish in what respects and to what degree the liver fragment remaining after partial hepatectomy functions differently from the original intact organ. The obvious way to do this is to try to determine what the liver adds to, or removes from, the blood passing through it. This would be a formidable undertaking since it would necessitate measurements of the blood flow to the liver contributed by the portal vein and hepatic artery and comparisons of the composition of arterial and portal venous blood with that of the blood leaving the liver by the hepatic veins. But as a first step it seemed that a much less ambitious experimental design might

be made to yield useful information. It has been shown by Blumgart (1971) that the flow of blood through the liver fragment after partial hepatectomy is approximately equal to that through the original intact organ (i.e. the blood flow per gram of tissue increases approximately three-fold). This is, of course, consistent with our own finding that partial hepatectomy does not produce any sustained increase in portal venous pressure. Such an increase would be expected if the liver fragment was unable to accommodate the same blood flow as the original intact organ.

If one measures the concentration of a metabolite in the blood entering the liver (say, glucose) and measures also the concentration of the same metabolite in the blood leaving the liver, the difference will be proportional to the amount added to, or subtracted from, the blood by the liver. If the difference is diminished after partial hepatectomy, it follows that (since the blood flow is unaltered) the liver fragment is adding or subtracting less of the metabolite than the original intact liver did. Again, for the purposes of a first approximation, it seemed permissible to neglect the contribution of the hepatic artery to total liver blood flow since it is so much smaller than that of the portal vein and, with the important exception of oxygen, a proportionately less important source of metabolites. The experimental results which follow therefore take the form of comparisons between the compositions of portal and hepatic venous blood. Even so, the technical difficulties of obtaining such comparisons are considerable, and their consequent limitations and liability to error merit discussion. Practical and humanitarian considerations make it necessary to obtain samples of portal and hepatic venous blood by opening the abdomen under anaesthesia rather than by the use of indwelling catheters. What one observes therefore

reflects the performance of the liver under anaesthesia in an animal subjected to the trauma of laparotomy. Acquisition of hepatic venous blood presents particular problems in the rat. There is no single hepatic vein. The liver invests the posterior vena cava and the hepatic veins of the individual lobes empty directly into it. It is possible to pass a catheter from the external jugular vein through the right atrium and anterior vena cava into the hepatic vein of one of the major lobes, but the operation is difficult and attended by serious risk of laceration of the lobe. A safer and simpler expedient is to ligate the posterior vena cava just anterior to its junction with the renal veins, thus in effect turning it into a drain for the hepatic veins. A sample drawn form the vena cava immediately anterior to the ligature consists therefore exclusively of hepatic venous blood. Quite clearly, the tying of such a ligature, and consequent obstruction of venous return from the kidneys and hind limbs, must constitute a serious interference with the cardiovascular system. It is obviously essential that the hepatic venous blood samples must be withdrawn before this interference has time to influence the blood supply to the liver. At the same time,if the sample is withdrawn too rapidly, it might be contaminated with blood drawn back through the right atrium from the anterior vena cava. Since the normal blood flow through the liver is approximately 20 ml per minute and the sample required is only 0.5 ml, contamination from this source is unlikely if the sample is withdrawn over a period of, say, ten seconds.

It is a reasonable assumption that these technical difficulties and the errors to which they may give rise will affect normal and hepatectomised animals equally. Consequently, while the absolute measurements one might attempt might well be inaccurate, differences between normal and hepatectomised animals can safely be regarded as

significant. So also can differences between animals examined at different time intervals after operation. The results which follow are subject to these limitations.

2.5.2 Glucose

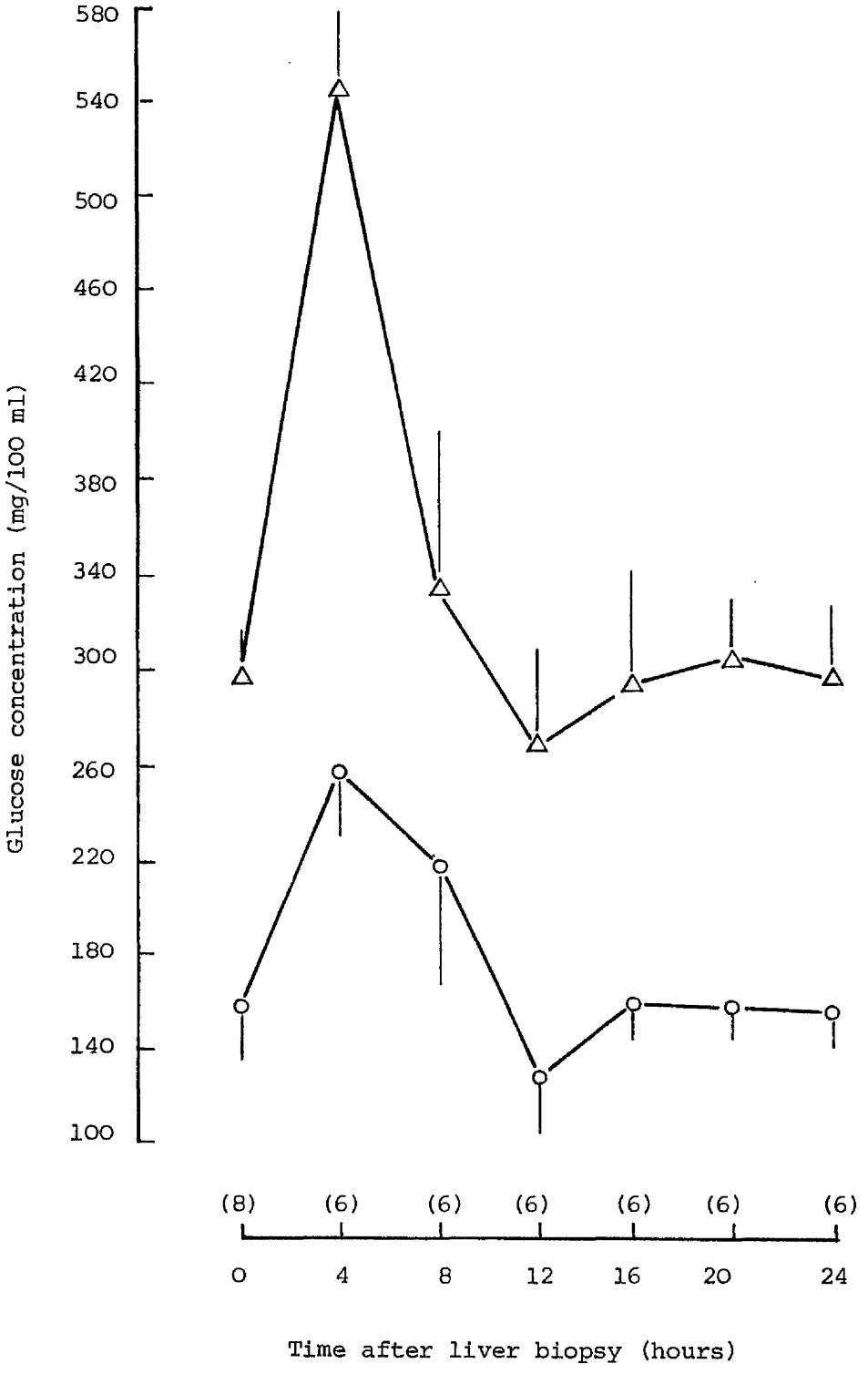
Figure 29 shows the concentration of glucose in the plasma of portal and hepatic veins of rats after liver biopsy. Two generalizations can be made from this Figure.

(i) At all time intervals, the concentration of glucose was much higher in the hepatic than in the portal venous blood, i.e. under the conditions of the experiment the liver was producing glucose rather than taking it up.

(ii). The production of glucose by the liver (as reflected in the different concentrations in the portal and hepatic veins) had increased sharply four hours after the operation (Figure 31) and diminished thereafter to the preoperative level.

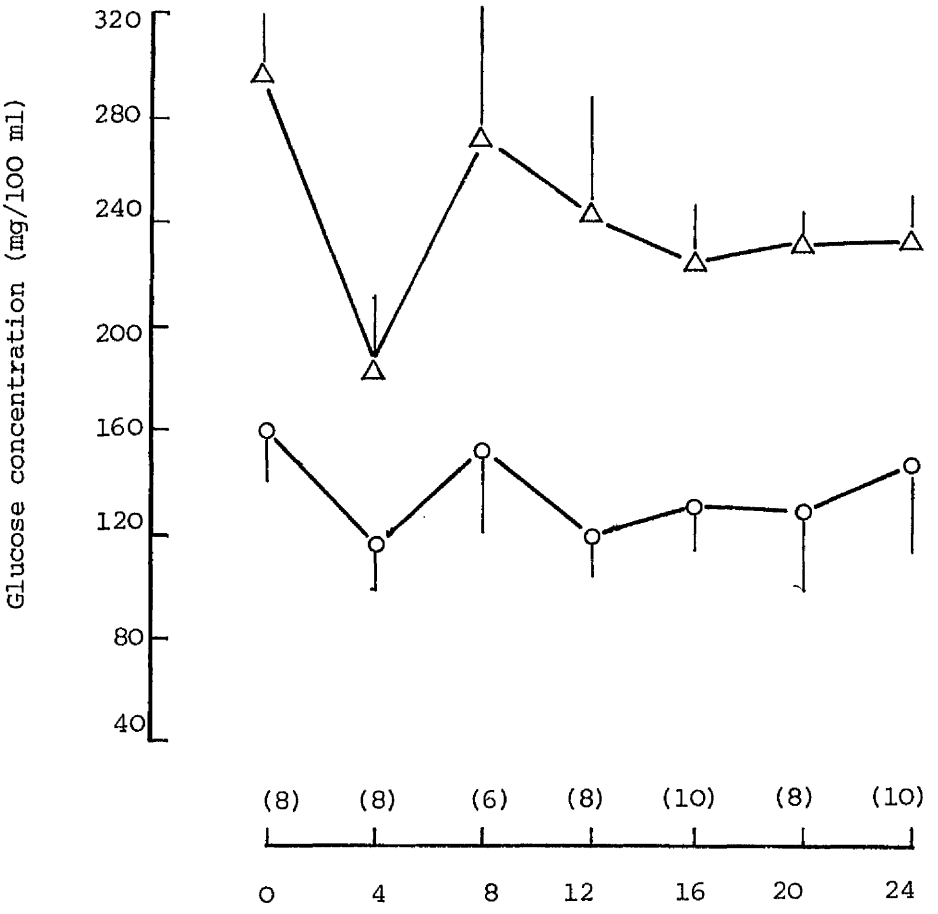
Figure 30 shows the corresponding results obtained after partial hepatectomy. As after liver biopsy, the concentration of glucose in the hepatic vein is always greater than in the portal vein. The liver fragment therefore is, in spite of constituting only one-third of the original liver mass, supplying glucose to the blood passing through it. But the spectacular increase in glucose output seen four hours after liver biopsy does not follow partial hepatectomy (Figure 31). These observations are most easily interpreted on the assumption that the trauma of operation stimulates release of glucose from the intact liver but that the fragment of liver remaining after partial hepatectomy is too small to respond in similar fashion.

Detailed comparison of Figures 29 and 30 reveals another difference: although both show that blood in the hepatic veins has a consistently higher concentration of glucose than that in the portal



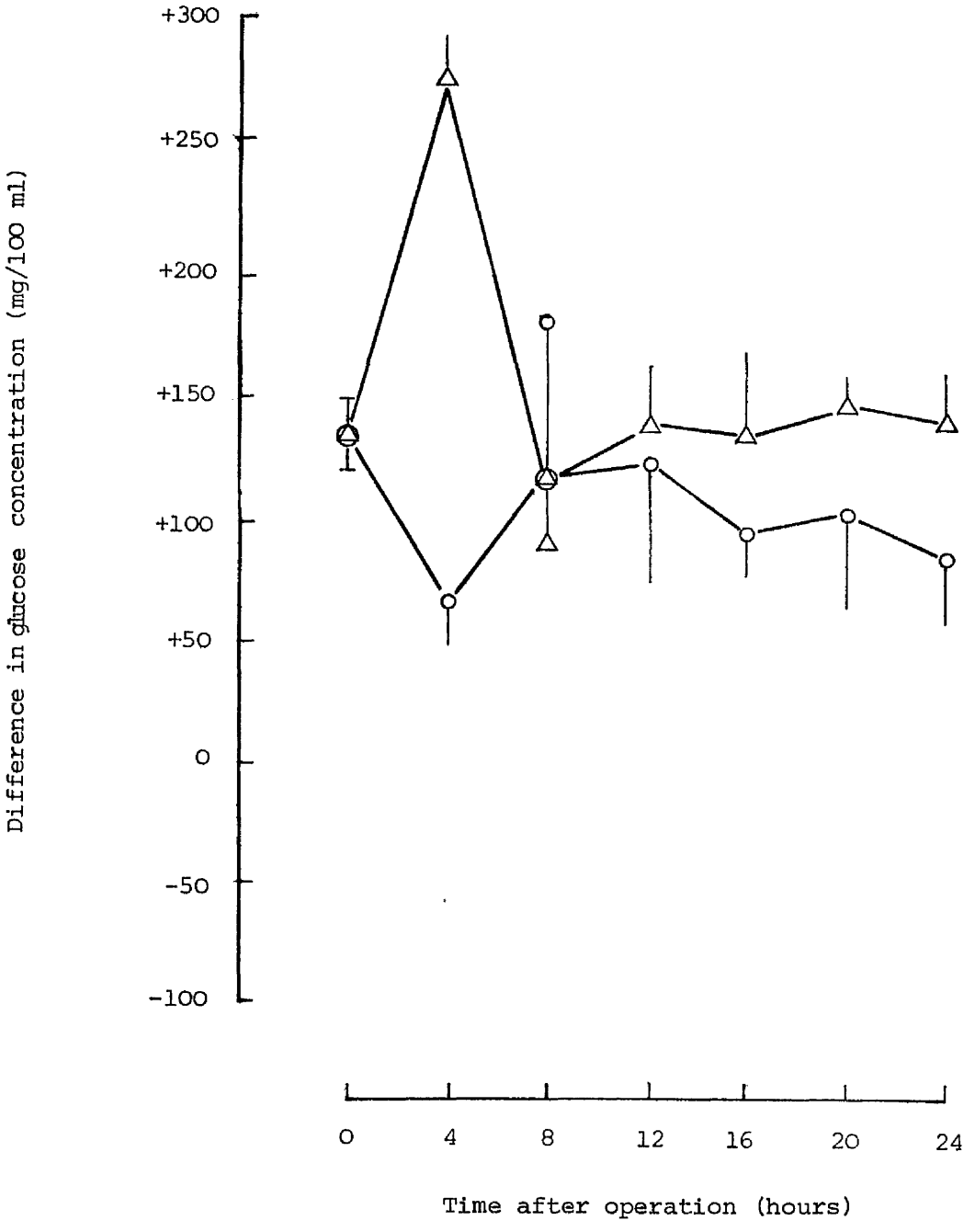
Concentrations of plasma glucose in portal (o) and hepatic (△) venous blood of rats at various time intervals after liver biopsy. All rats were male and approximately 220 g in body weight. The number of rats used at each time interval is given in parenthesis. The results are shown as mean ± standard deviation.

Figure 30



Concentrations of plasma glucose in portal (o) and hepatic (Δ) venous blood rats at various time intervals after partial hepatectomy. All rats were male and approximately 220 g in body weight. The number of rats used at each time interval is given in parenthesis. The results are shown as mean ± standard deviation.

Figure 31



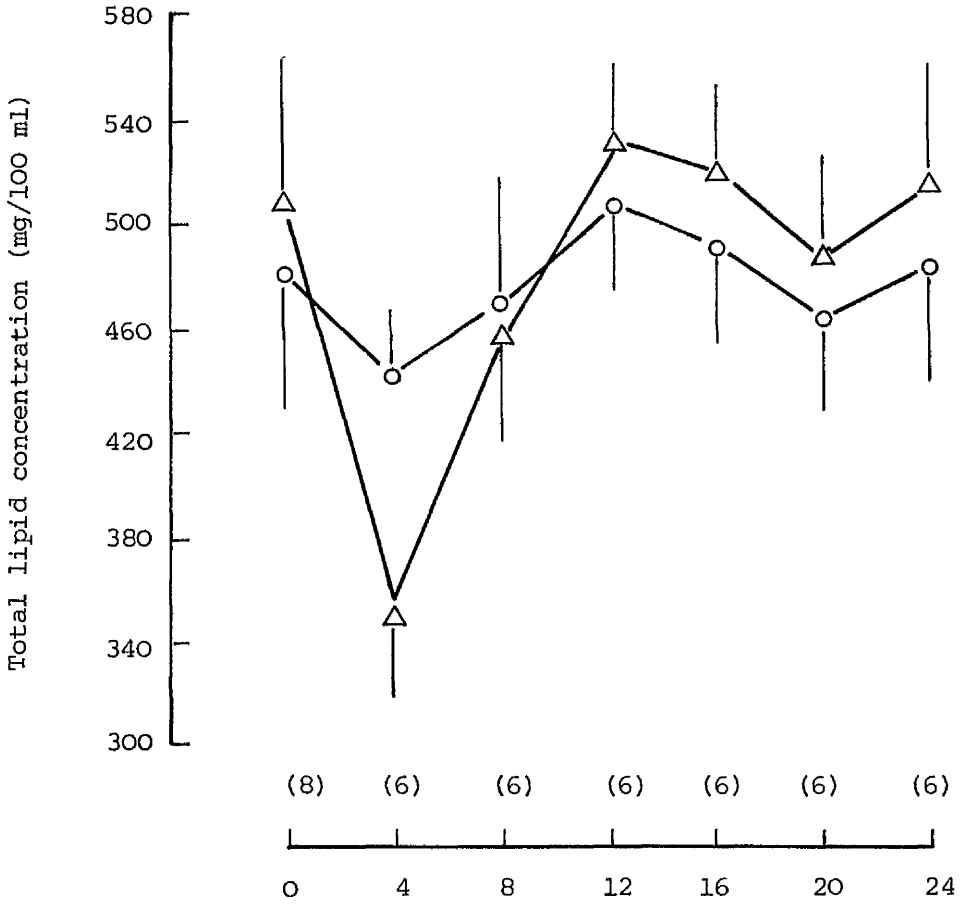
Differences in glucose concentration between hepatic and portal venous blood plasma of rats at various time interval after either liver biopsy (△) or partial hepatectomy (o). The values shown in this figure are derived from Figures 29 and 30. The results are shown as mean ± standard deviation.

veins, the difference is (except at eight hours after the operation) consistently smaller in animals which have been partially-hepatectomised (Figure 31). It would appear, therefore, that though the liver fragment left after partial hepatectomy supplies glucose to the blood passing through it, it does so more slowly than the essentially intact liver left after biopsy. It should be noticed, however, that although the liver fragment remaining after partial hepatectomy is only one-third the size of that remaining after liver biopsy, its apparent glucose release from twelve to twenty-four hours after operation is more than one-third that of the biopsied liver. In other words, the fragment of liver remaining after partial hepatectomy does appear to be capable of releasing more glucose than might be expected from it size.

2.5.3. Total plasma lipids

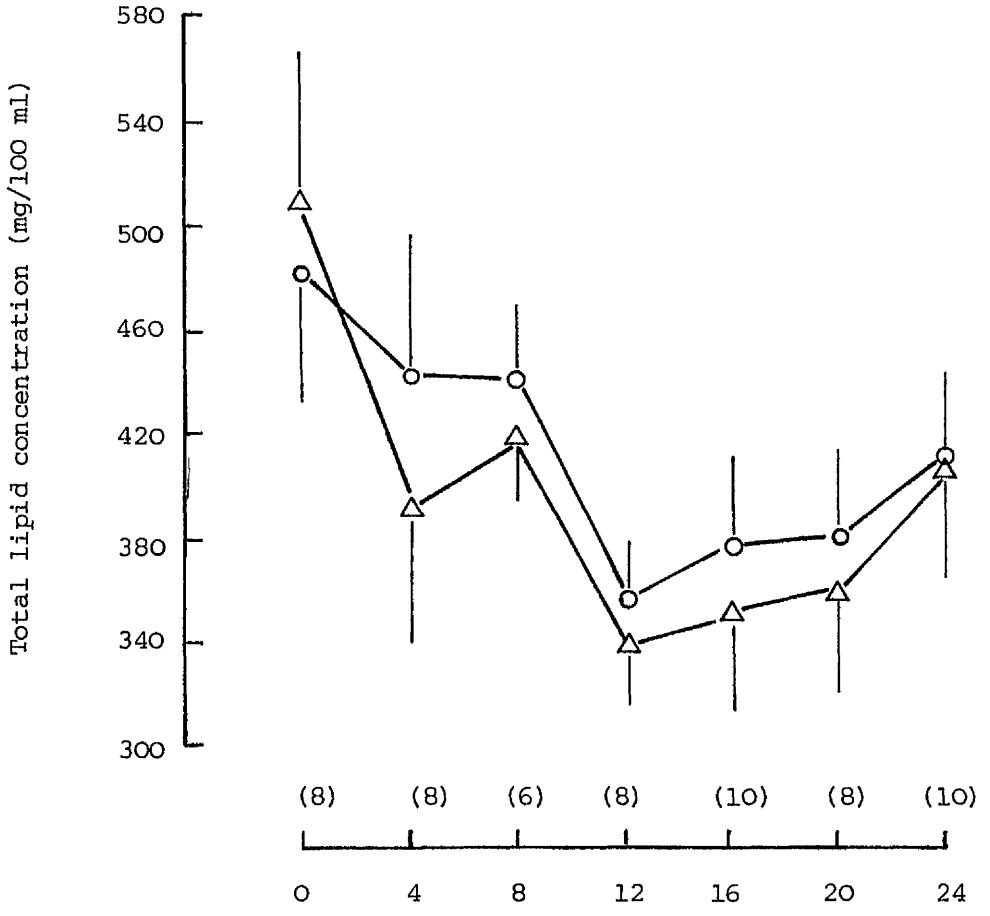
Figure 32 shows the concentration of total plasma lipids in the portal and hepatic veins of rats after liver biopsy. Before operation, the lipid concentration is higher in the hepatic vein than in the portal vein. The liver therefore is supplying lipid to the blood. Soon after biopsy there is a sharp fall in the lipid concentration in the hepatic vein accompanied by a smaller fall in the portal vein. The latter now contains more lipids than the former. Presumably, therefore, the liver during this period extracts lipids from the blood. This situation persists only for the initial eight hours. Thereafter the concentration of total lipids returns to more or less normal in both veins. Figure 33 shows the corresponding results obtained after partial hepatectomy. As after liver biopsy, there is a fall in the total lipid concentration in both the portal and hepatic veins and the liver starts to extract lipid from the blood instead of supplying it. The lipid concentrations in both the veins keep falling till about twelve hours after partial hepatectomy. Thereafter they show an upward trend but remain

Figure 32



Concentrations of plasma total lipids in portal (o) and hepatic (△) venous blood of rats at various time intervals after liver biopsy. All rats were male and approximately 220 g in body weight. The number of rats used at each time interval is given in parenthesis. The results are shown as mean ± standard deviation.

Figure 33



Concentrations of plasma total lipids in portal (o) and hepatic (△) venous blood of rats at various time intervals after partial hepatectomy. All rats were male and approximately 220 g in body weight. The number of rats used at each time interval is given in parenthesis. The results are shown as mean ± standard deviation.

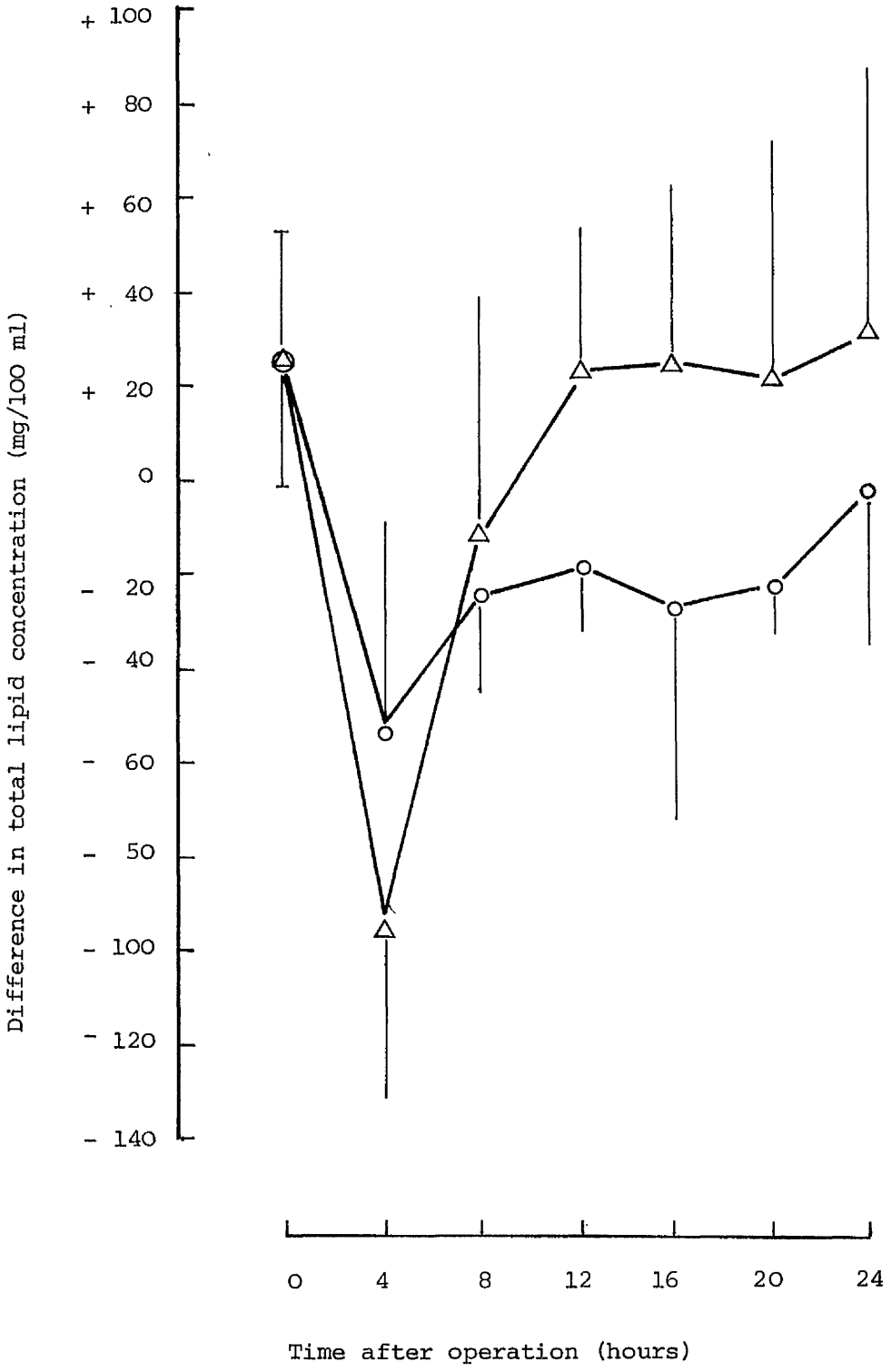
significantly below normal even twenty-four hours after the operation. The portal vein consistently contains more lipid than the hepatic vein. Presumably, therefore, the liver remnant left after partial hepatectomy extracts lipids from the passing blood.

Figure 34 shows the hepatic-portal difference in lipid concentration. There is an obvious qualitative similarity in the response to the two operations in that the liver stops releasing lipid into the blood and instead starts to take it up. In biopsied rats this phase of lipid uptake lasts for only eight hours. Twelve hours after the operation the livers normal role as a net exporter of lipids is regained. The response to partial hepatectomy is quantitatively different. The liver fragment does not initially take up lipid as rapidly as the intact organ. But uptake continues for at least twenty-four hours, whereas with an intact liver it lasts for only eight to twelve hours.

2.5.4 Plasma phospholipids

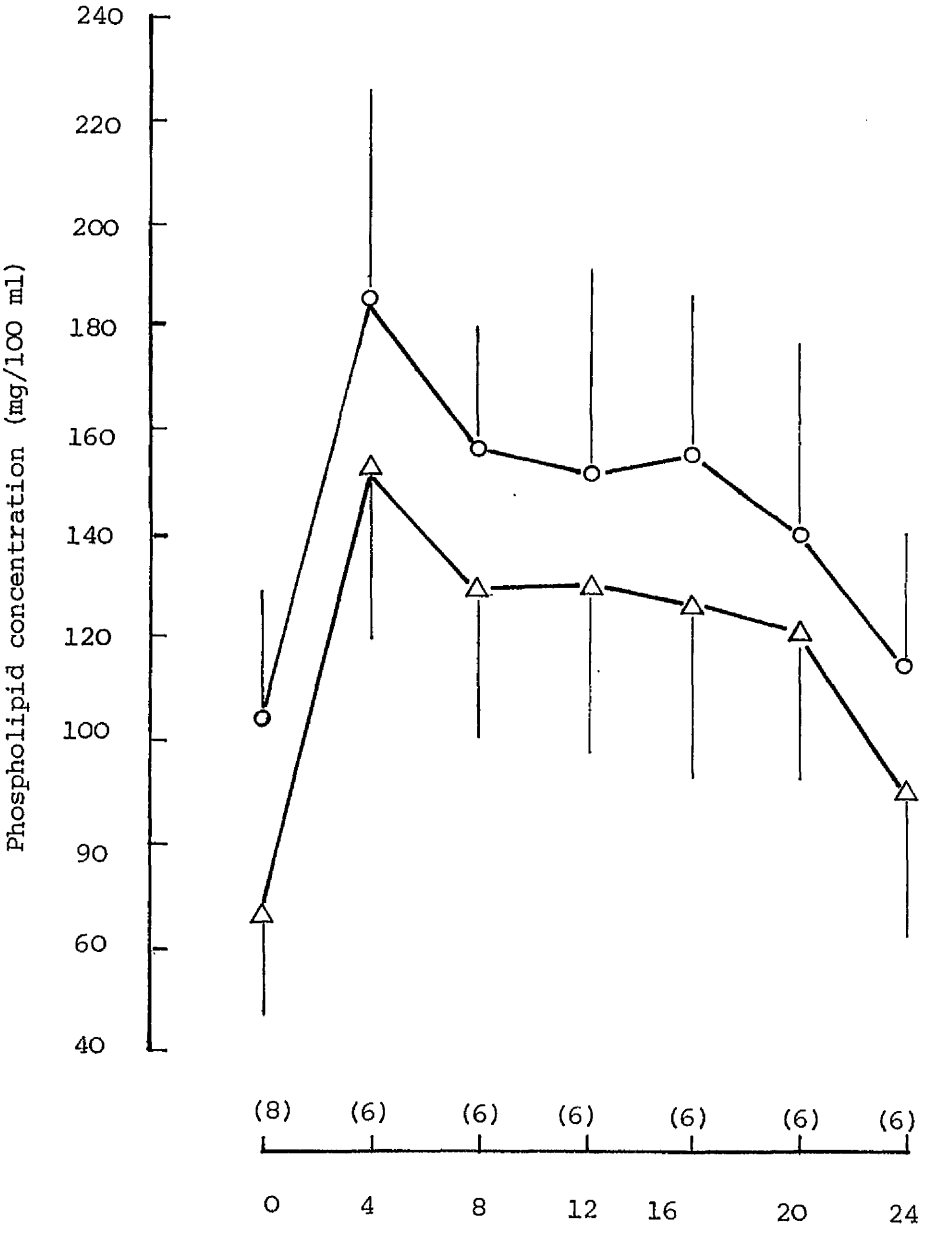
Figure 35 shows the concentration of plasma phospholipids in the portal and hepatic veins of rats after liver biopsy. Before operation the phospholipid concentration is higher in the portal vein than in the hepatic vein. The liver therefore is extracting phospholipids from the blood. After biopsy the phospholipid concentrations in both veins show a sharp increase and thereafter gradually return almost to normal within twenty-four hours after the operation. Throughout the experiment the concentration in the portal vein is consistently greater than that in the hepatic vein. The pattern seen after partial hepatectomy (Figure 36) is less clear cut. The concentrations in both veins rise, but the increase is less dramatic than that seen after biopsy and the difference between the portal vein and the hepatic vein is sharply diminished. It is not

Figure 34



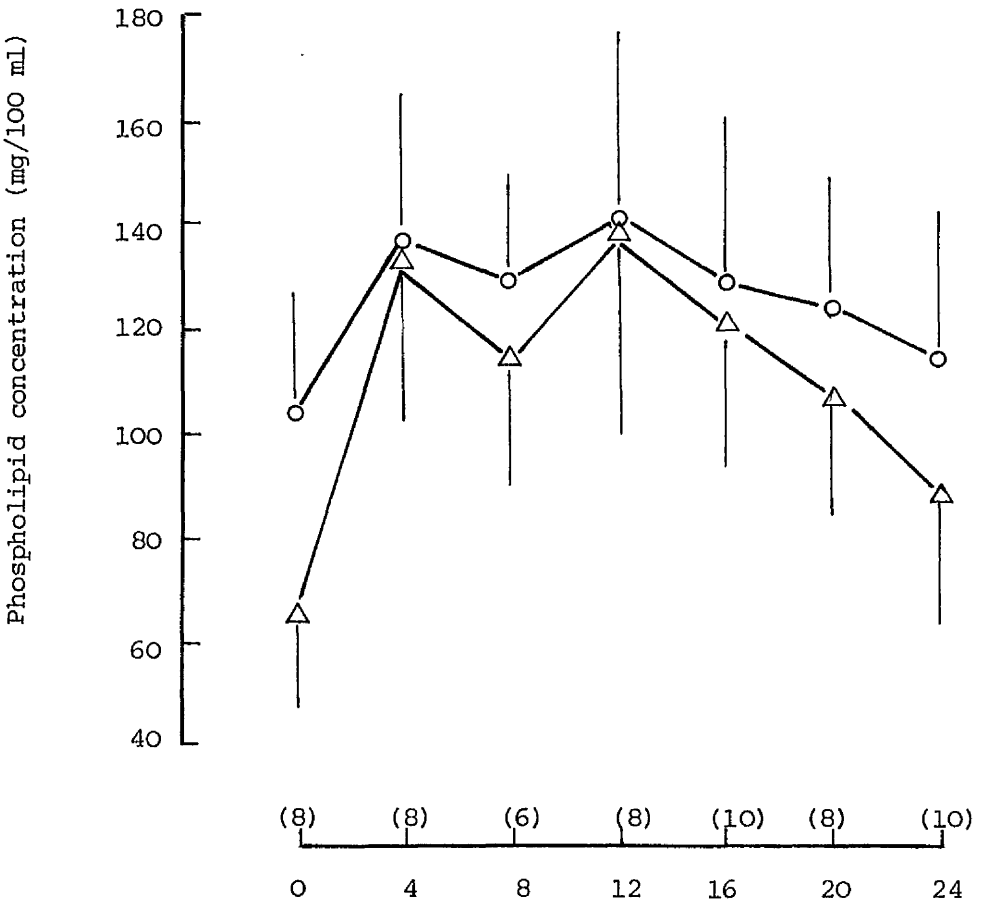
Differences in total lipid concentration between hepatic and portal venous blood plasma of rats at various time intervals after either liver biopsy (△) or partial hepatectomy (o). The values shown in this figure are derived from Figures 32 and 33. The results are shown as mean ± standard deviation.

Figure 35



Concentrations of plasma phospholipids in portal (o) and hepatic (△) venous blood of rats at various time intervals after liver biopsy. All rats were male and approximately 220 g in body weight. The number of rats used at each time interval is given in parenthesis. The results are shown as mean ± standard deviation.

Figure 36



Concentrations of plasma phospholipids in portal (o) and hepatic (△) venous blood of rats at various time intervals after partial hepatectomy. All rats were male and approximately 220 g in body weight. The number of rats used at each time interval is given in parenthesis. The results are shown as mean ± standard deviation.

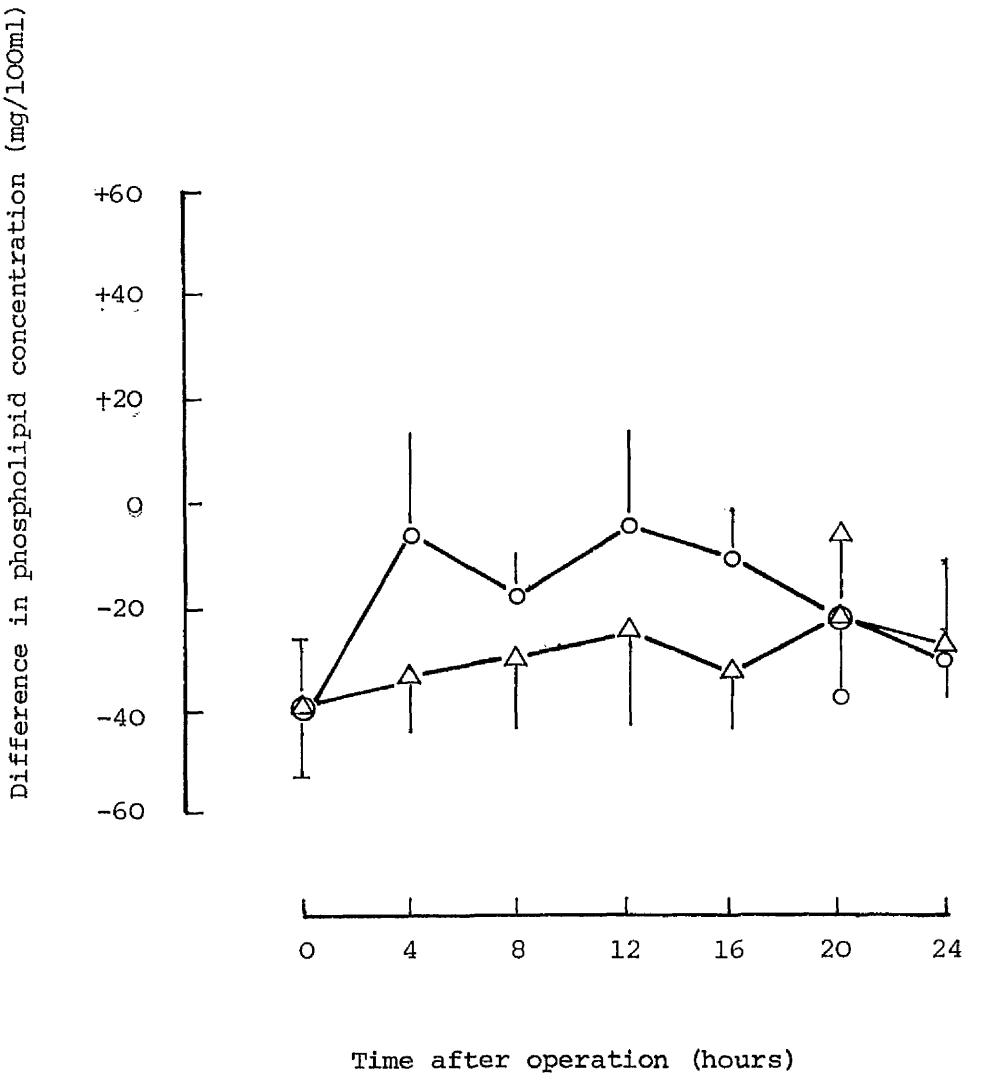
until twenty hours after the operation that this difference is re-established. The distinction between the responses is illustrated in Figure 37. Biopsy clearly has little effect on extraction of phospholipids by the liver; partial hepatectomy, by contrast, diminishes it sharply.

2.5.5 Total plasma protein

Figure 38 shows the concentration of total plasma protein in the portal and hepatic veins of rats after liver biopsy. The figure shows that the concentration of proteins in the portal vein is higher than in the hepatic vein. Therefore the liver is consistently extracting proteins from the passing blood. After biopsy, the protein concentration increases significantly in both the veins. It shows a peak at four hours and thereafter gradually declines towards normal. In the portal vein the protein concentration becomes normal by twenty-four hours but in the hepatic vein it is still significantly higher than normal.

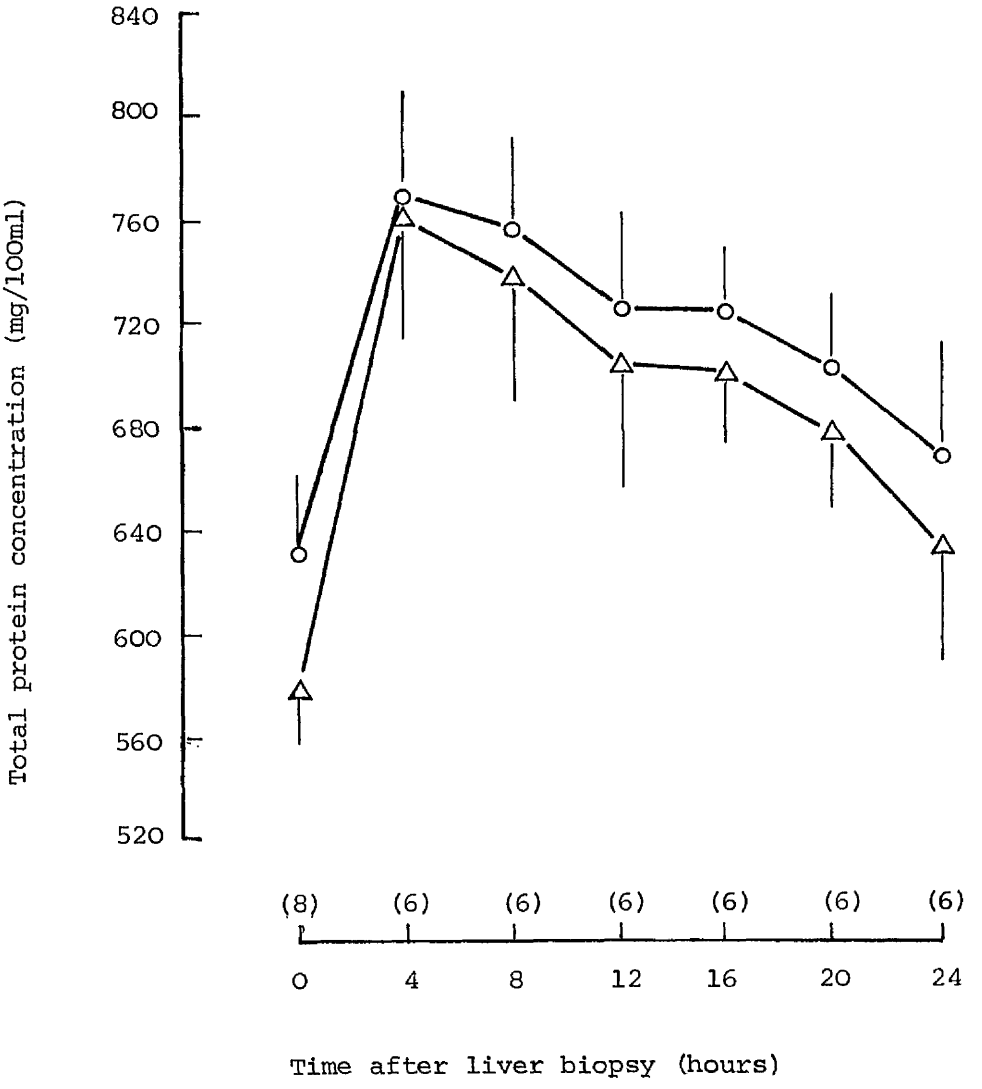
The response to partial hepatectomy is dramatically different (Figure 39). In the first four hours there is a modest increase in protein concentration in the hepatic vein, none at all in the portal vein. Thereafter the concentration in the hepatic vein falls sharply to a minimum at sixteen hours before showing a modest recovery. The concentration in the portal vein shows an approximately parallel sequence of changes but of lesser magnitude. The difference in the response to the two operations is brought out in Figure 40. Biopsy sharply diminishes protein uptake by the liver, but this effect progressively wears off over the twenty-four hours following the operation. Partial hepatectomy also causes an initial diminution in protein uptake, but within eight to twelve hours this has been reversed and thereafter, up to twenty-four hours, protein uptake by the liver is far above the preoperative level.

Figure 37



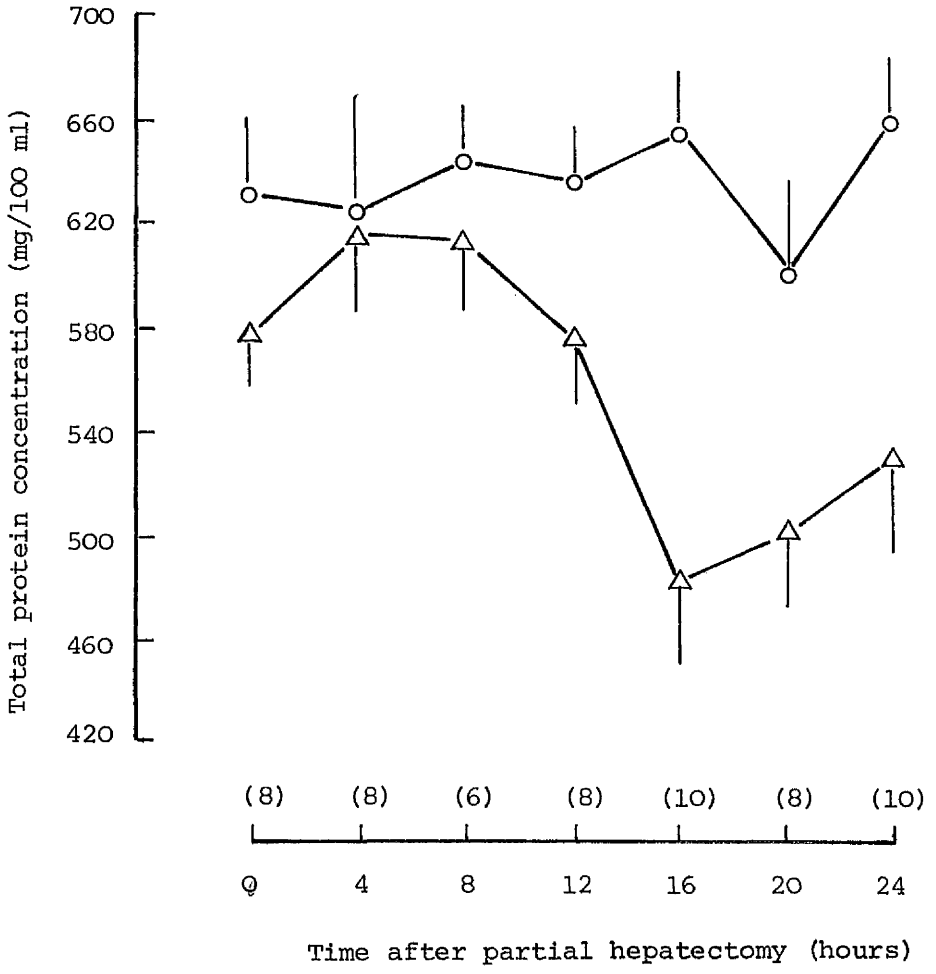
Differences in phospholipid concentration between hepatic and portal venous blood plasma of rats at various time intervals after either liver biopsy (△) or partial hepatectomy (o). The values shown in this figure are derived from Figures 35 and 36. The results are shown as mean ± standard deviation.

Figure 38



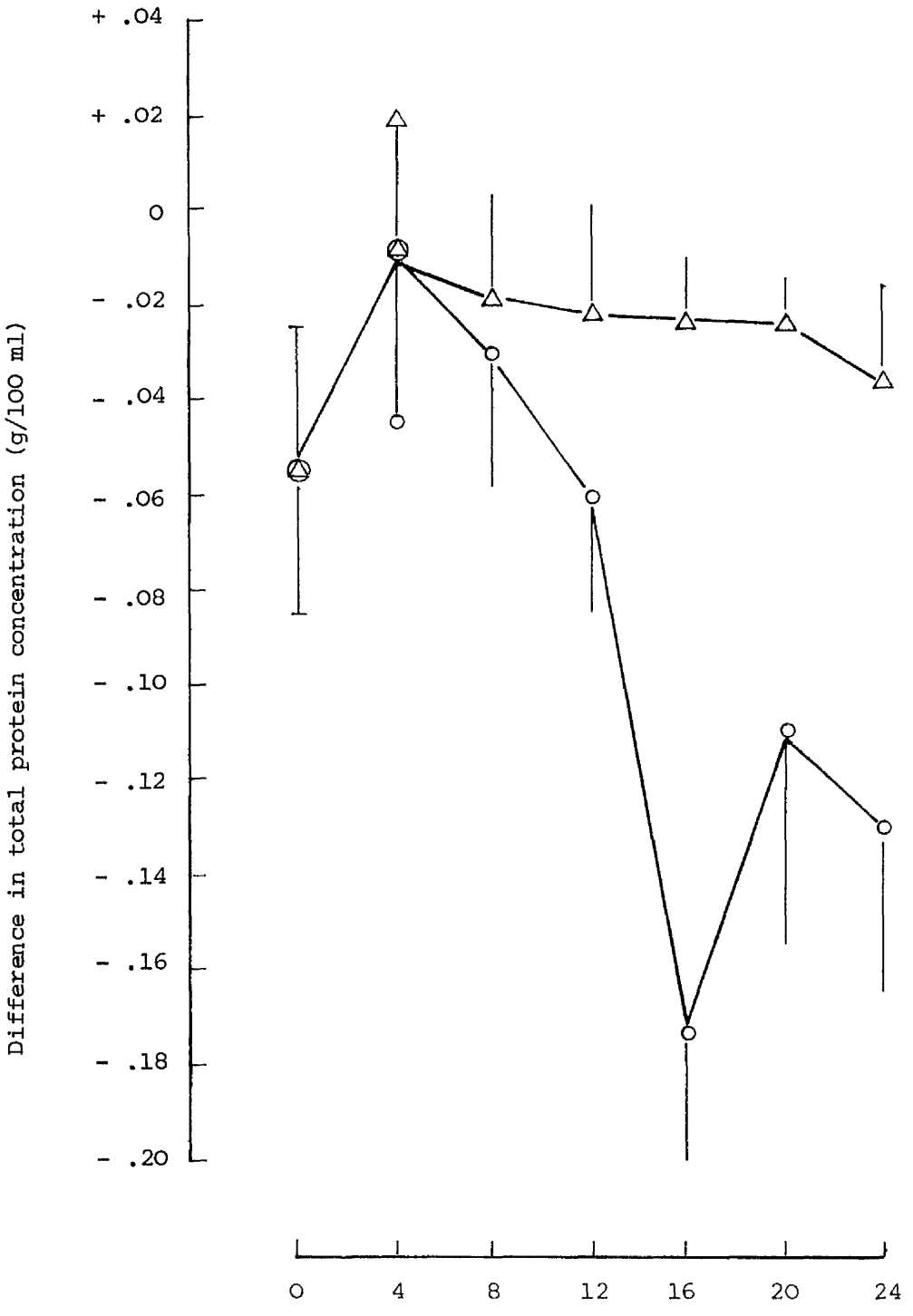
Concentrations of plasma total protein in portal (o) and hepatic (△) venous blood of rats at various time intervals after liver biopsy. All rats were male and approximately 220 g in body weight. The number of rats used at each time interval is given in parenthesis. The results are shown as mean ± standard deviation.

Figure 39



Concentration of plasma total protein in portal (o) and hepatic (△) venous blood of rats at various time intervals after partial hepatectomy. All rats were male and approximately 220 g in body weight. The number of rats used at each time interval is given in parenthesis. The results are shown as mean ± standard deviation.

Figure 40



Differences in total protein concentration between hepatic and portal venous blood plasma of rats at various time intervals after either liver biopsy (△) or partial hepatectomy (o). The values shown in this figure are derived from Figures 38 and 39. The results are shown as mean ± standard deviation.

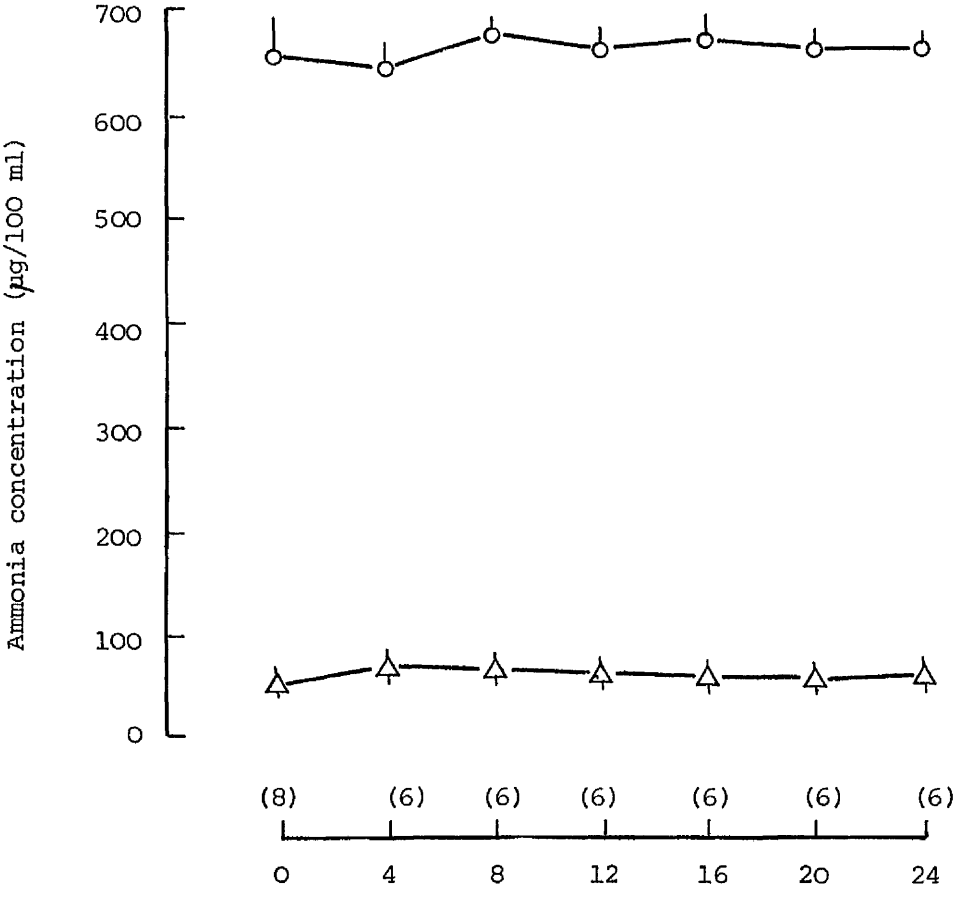
2.5.6 Ammonia

Figure 41 shows the concentration of ammonia in the portal and hepatic veins of rats after biopsy. Two facts are immediately apparent. The concentration of ammonia in the hepatic venous blood is only a small fraction of that in the portal vein; and this state of affairs is quite unaffected by biopsy. Clearly the liver is very efficient at removing ammonia from blood passing through it, and its efficiency is quite unaffected by anaesthesia and the stress of operation. The consequences of partial hepatectomy are quite different (Figure 42). The operation is followed by a steady, inexorable increase in the concentration of ammonia in the hepatic vein. Clearly the remaining liver fragment cannot remove ammonia from the blood as effectively as the intact organ. In the meantime, the concentration of ammonia in the portal vein first falls, then rises and then falls again and stabilises at the pre-operative level. The significance of these fluctuations is not clear. But if one looks at the hepatic-portal concentration differences (Figure 43) it is apparent that, although the liver after partial hepatectomy is, as we have seen, incapable of bringing the concentration of ammonia in the hepatic venous blood down to the very low level achieved by the intact liver, nonetheless the absolute amount of ammonia which it extracts is not much inferior to that removed by the intact liver.

2.5.7 Oxygen

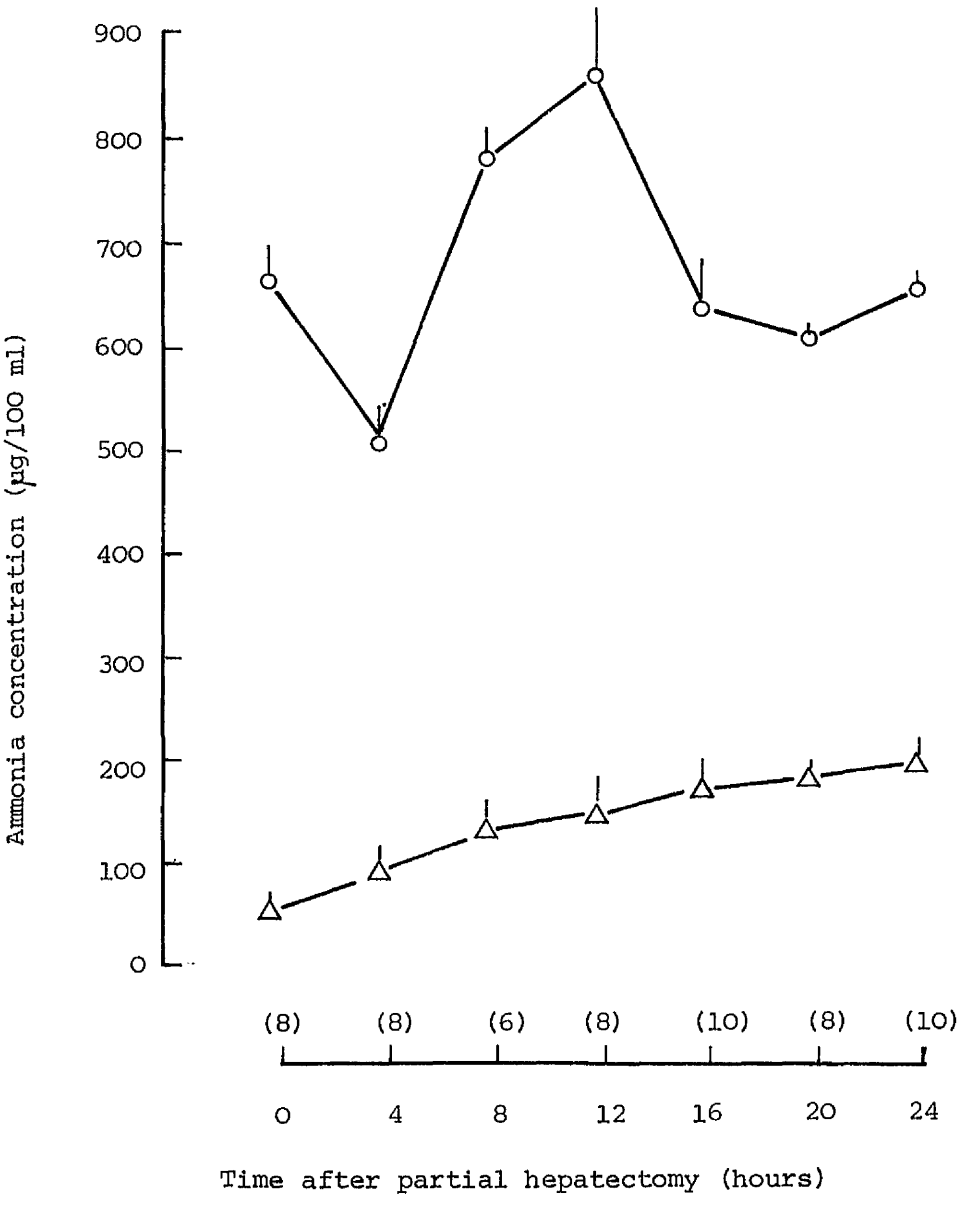
The final aspect of liver metabolism investigation by comparison of the compositions of portal and hepatic venous blood was oxygen uptake. There is a complication here in that the blood supplied by the hepatic artery, though in terms of volume much less than the portal venous supply, may be of major importance as a source of oxygen. In an

Figure 41



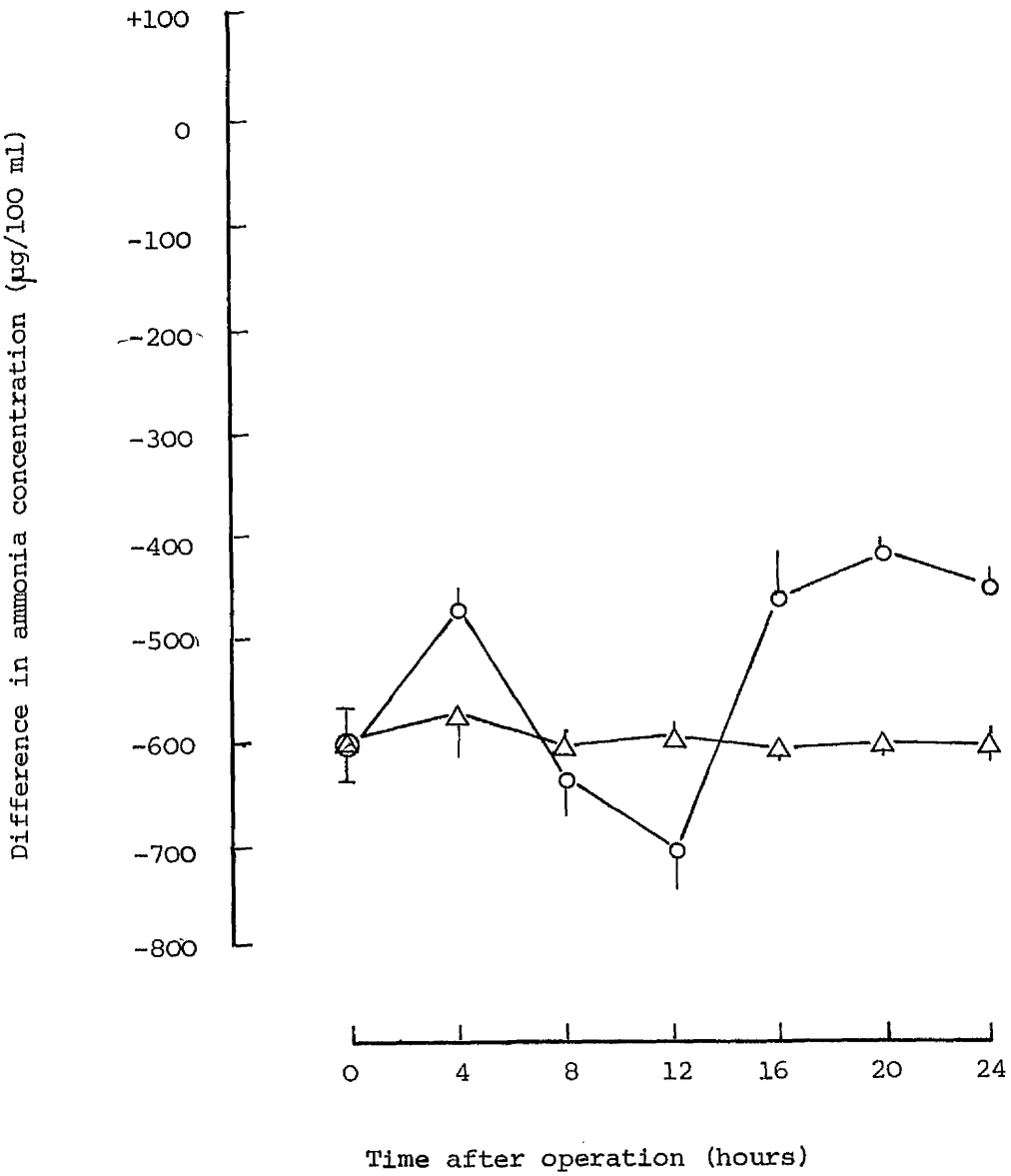
Concentrations of ammonia in portal (o) and hepatic (△) venous blood of rats at various time intervals after liver biopsy. At least six male rats, approximately 220 g in body weight were used at each time interval. The results are given as mean ± standard deviation.

Figure 42



Concentrations of ammonia in portal (o) and hepatic (△) venous blood of rats at various time intervals after partial hepatectomy. At least six male rats, approximately 220 g in body weight were used at each time interval. The results are given as mean ± standard deviation.

Figure 43

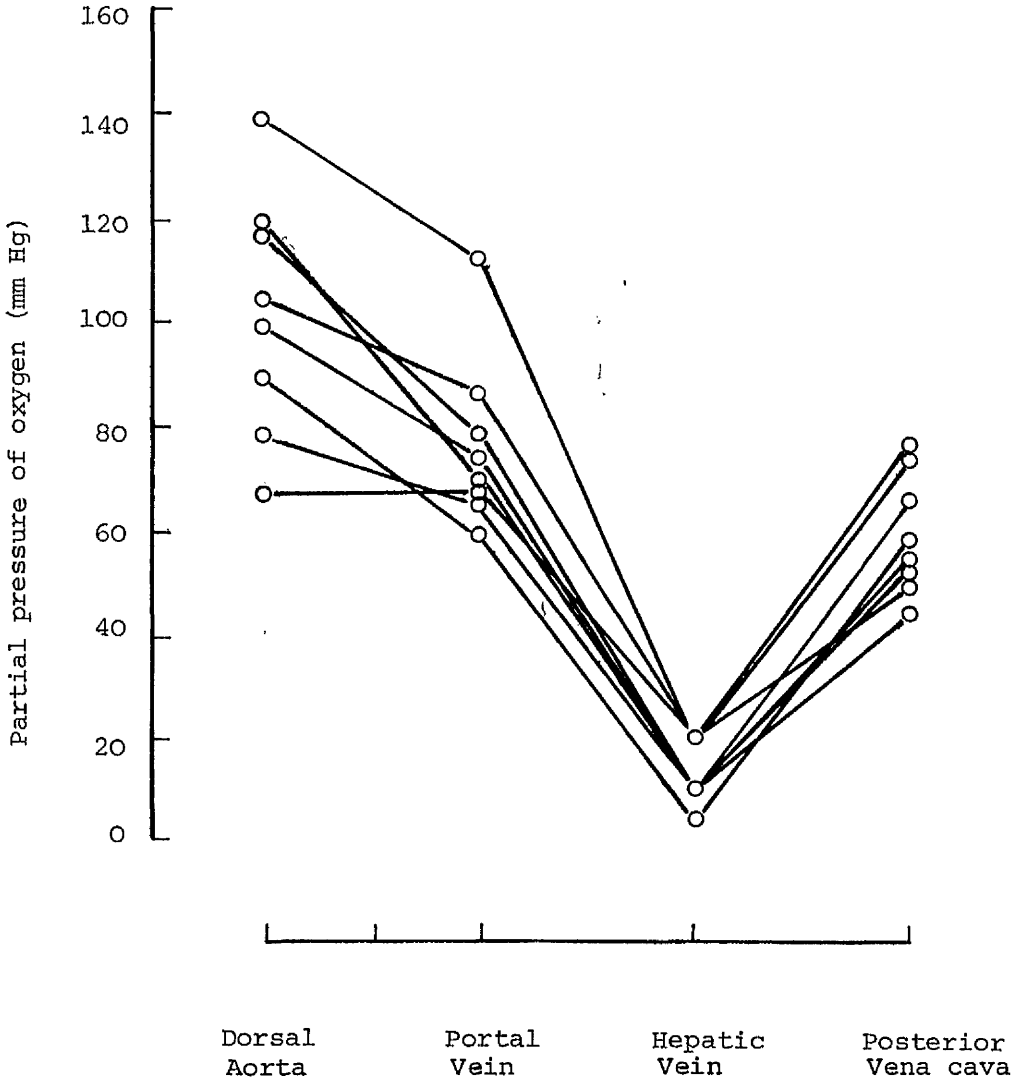


Differences in ammonia concentration between hepatic and portal venous blood plasma of rats at various time intervals after either liver biopsy (△) or partial hepatectomy (o). The values shown in this figure are derived from Figures 41 and 42. The results are shown as mean ± standard deviation.

animal as small as the rat it is difficult to compare the hepatic artery and portal vein in terms either of blood flow or of provision of oxygen. It is however quite easy to ligate the hepatic artery and observe whether the pO_2 in either the portal vein or hepatic veins show any change in consequence.

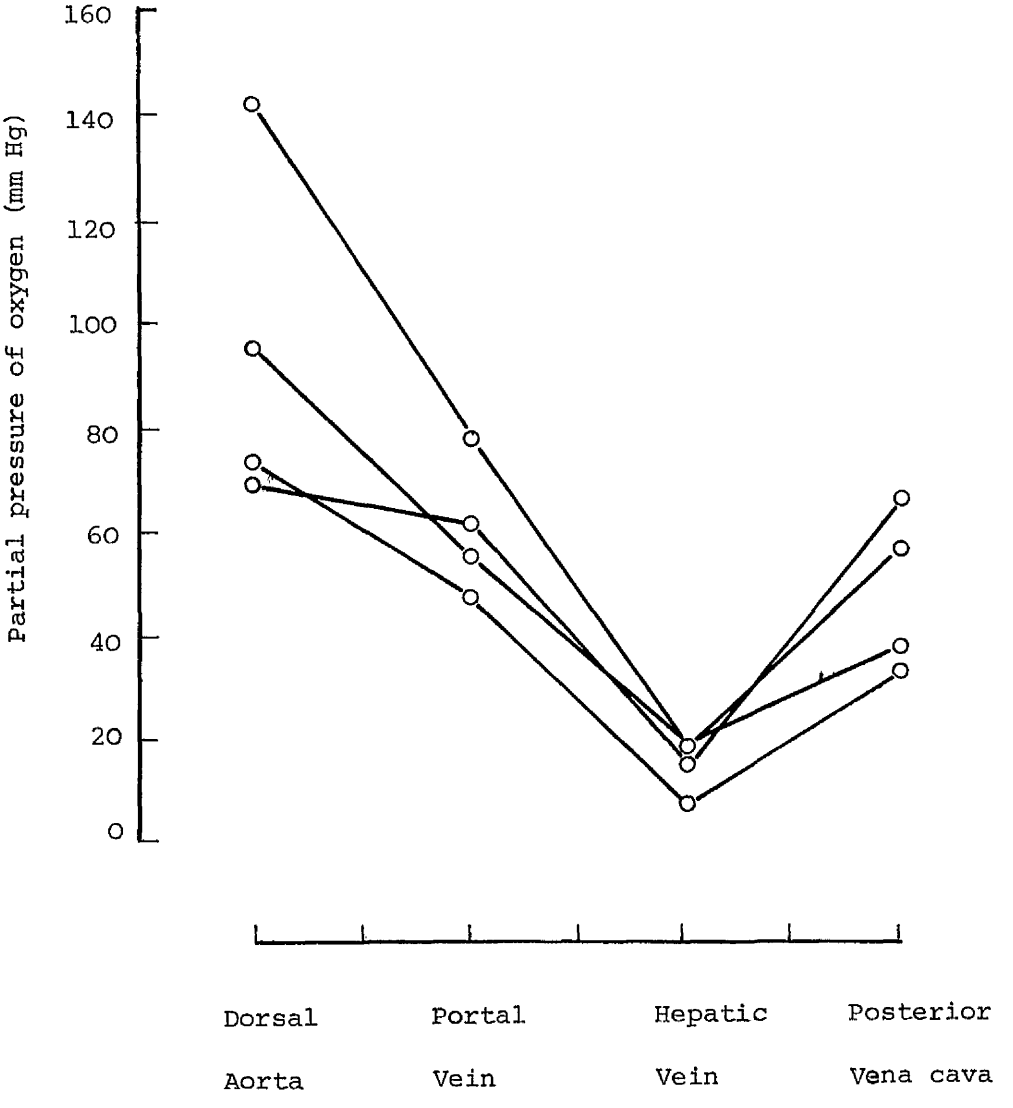
Figures 44, 45, 46 and 47 show the pO_2 in the aorta, portal vein, hepatic vein and posterior vena cava in a series of rats subjected to various procedures. Figure 44 shows the values obtained from control animals. It is immediately apparent that they fall into a consistent pattern: the pO_2 in the portal vein is, in general, not much lower than that in the aorta and clearly a little higher than that of mixed caval blood. On the other hand, the pO_2 of hepatic venous blood is very low indeed. The sigmoid shape of the oxygen saturation curve of haemoglobin and the degree to which it is affected by pH and pCO_2 makes it dangerous to argue from pO_2 to oxygen uptake. Nonetheless the pattern of differences is very striking. Figure 45 shows the corresponding pattern in rats in which the hepatic artery had been ligated. The results here are more scattered than in Figure 44, but basically not very different. In particular, the difference in pO_2 between portal venous and hepatic venous blood is no greater than in the controls. In the absence of arterial blood, the liver does not appear to be extracting more oxygen from the blood supplied by the portal vein. Figure 46 shows the results of an experiment in which an attempt was made to enhance the importance of the hepatic artery as a source of oxygen by administering pure oxygen. This as might be expected, produced very high pO_2 levels in the aorta (and presumably in the hepatic artery) but the general pattern of pO_2 values was not otherwise affected. It would appear, therefore that the hepatic artery in the rat does not have any very significant influence on the pattern of pO_2 levels in the portal and hepatic veins.

Figure 44



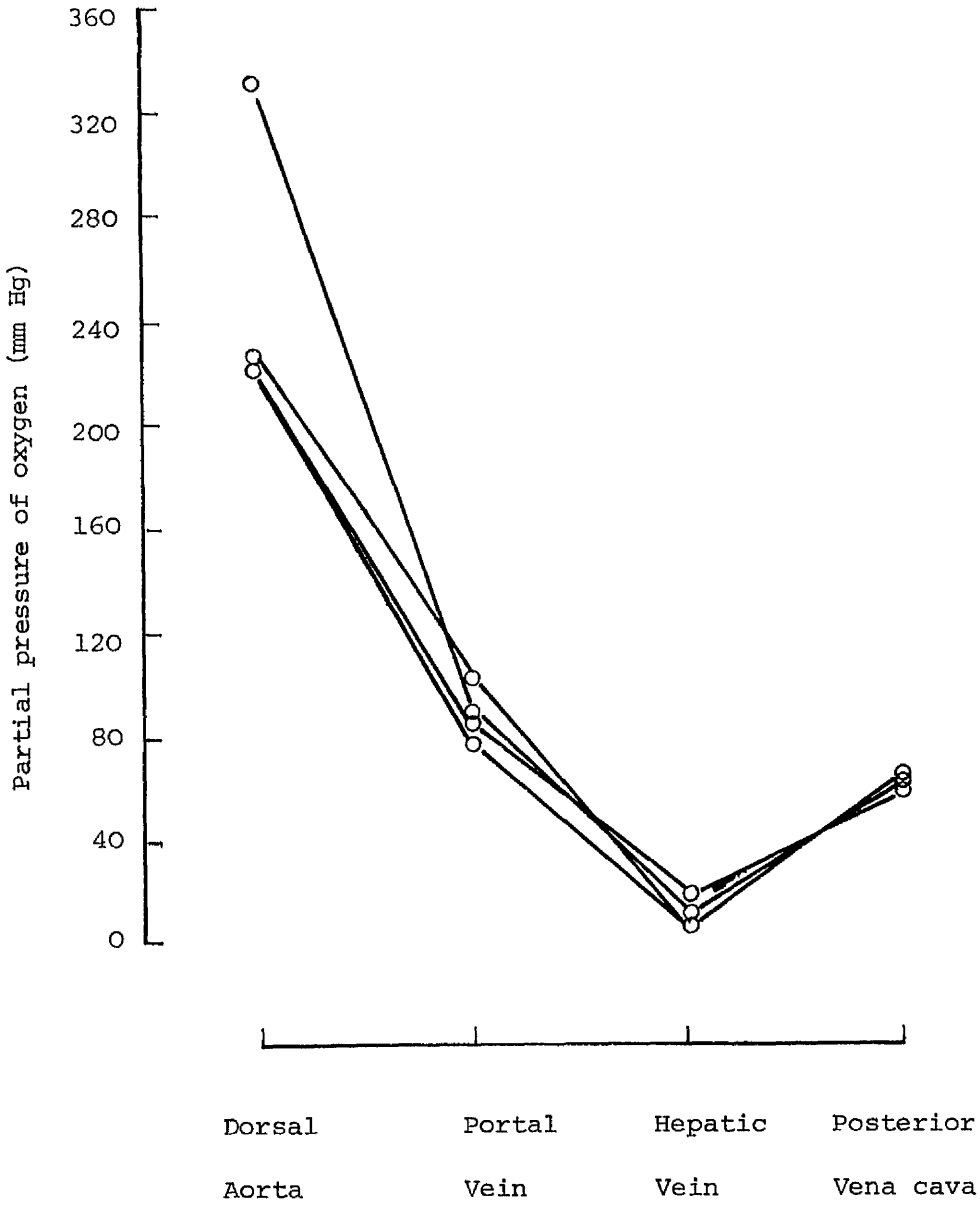
Partial pressure of oxygen in blood from dorsal aorta, portal vein, hepatic vein and posterior vena cava of normal rats. All the rats were male and approximately 220 g in body weight.

Figure 45



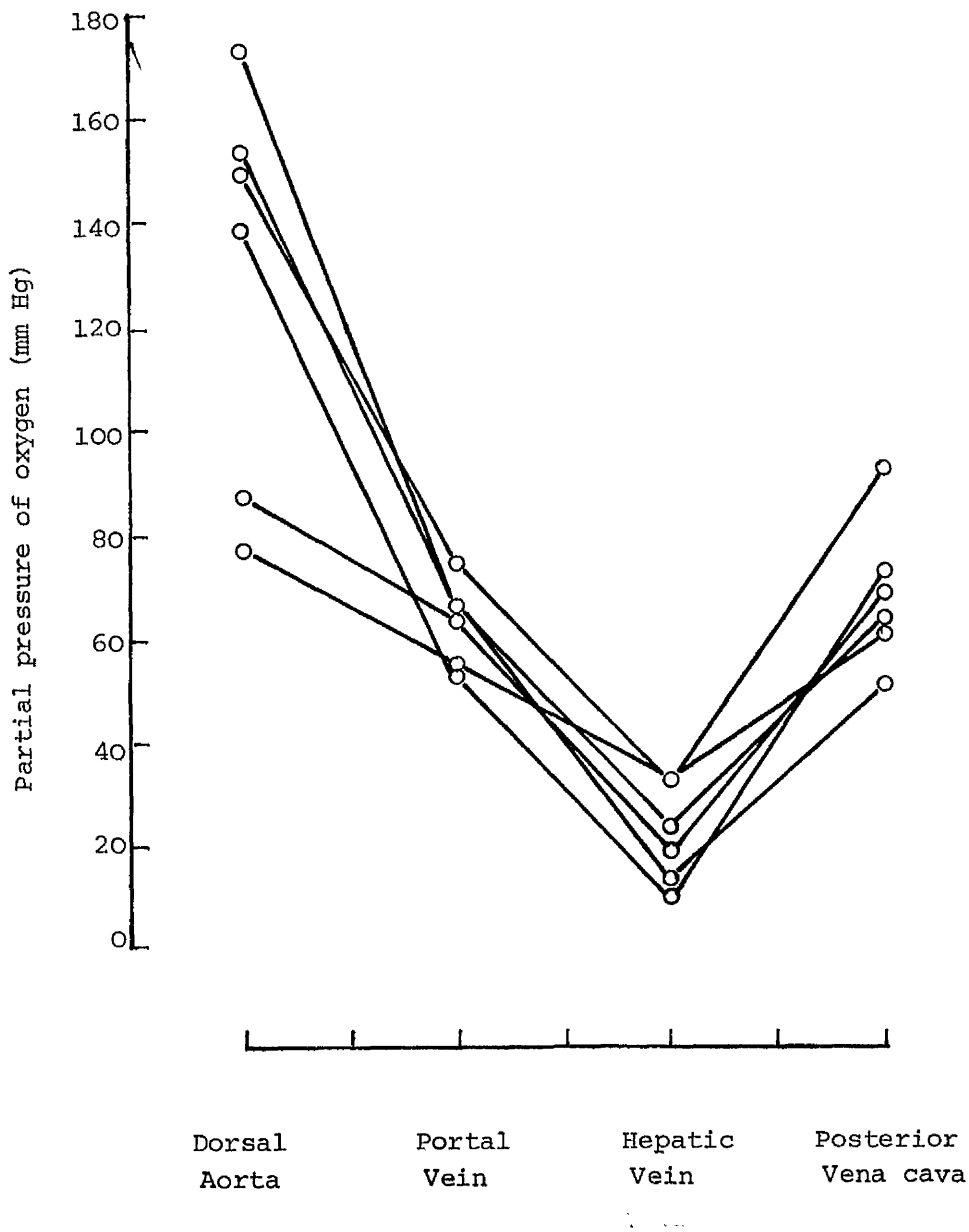
Partial pressure of oxygen in blood from dorsal aorta, portal vein, hepatic vein and posterior vena cava of rats in which the hepatic artery was ligated immediately prior to blood sampling. All the rats were male and approximately 220 g in body weight.

Figure 46



Partial pressure of oxygen in blood from dorsal aorta, portal vein, hepatic vein and posterior vena cava of rats breathing pure oxygen. All the rats were male and approximately 220 g in body weight.

Figure 47



Partial pressure of oxygen in blood from dorsal aorta, portal vein, hepatic vein and posterior vena cava of rats immediately after partial hepatectomy. All the rats were male and approximately 220 g in body weight.

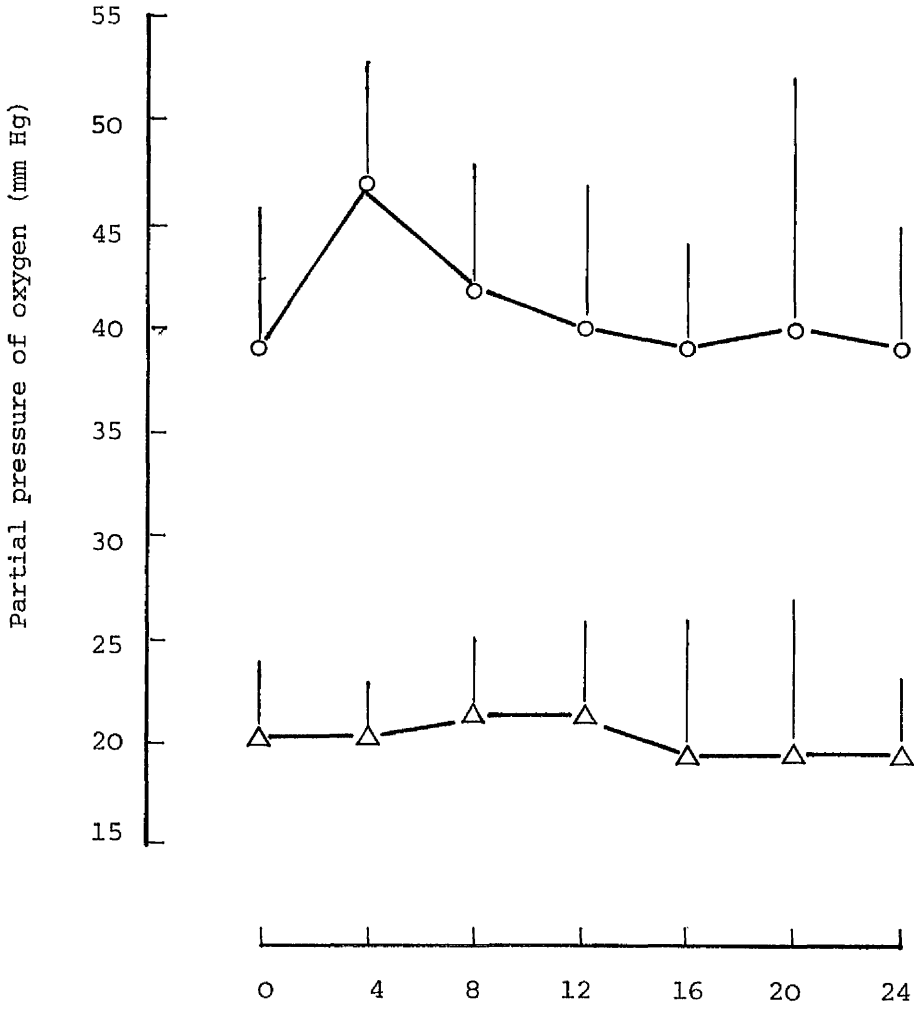
Figure 47 shows the pattern of pO_2 levels found immediately after partial hepatectomy. Clearly it differs little, if at all, from that obtained from the normal animals shown in Figure 44. Apparently, therefore, the liver remnant is removing virtually as much oxygen from the portal blood as did the intact liver. This conclusion is confirmed by the results shown in Figures 48, 49 and 50 which are directly comparable to Figures 44 - 47. It is apparent from Figure 50 that liver biopsy produces a slight and quite temporary increase in the pO_2 in portal vein without any corresponding alteration of the pO_2 in the hepatic vein. A rather more complex pattern of changes is seen after partial hepatectomy, but perhaps the important point is that the changes are transient and relatively slight, as is apparent also from Figure 50, which shows the difference in each case between the portal vein and the hepatic vein. Clearly, neither biopsy nor partial hepatectomy has any substantial effect on the amount of oxygen extracted.

2.5.8 Summary

It may be convenient at this point to try to summarize the results shown in Figures 29 to 50.

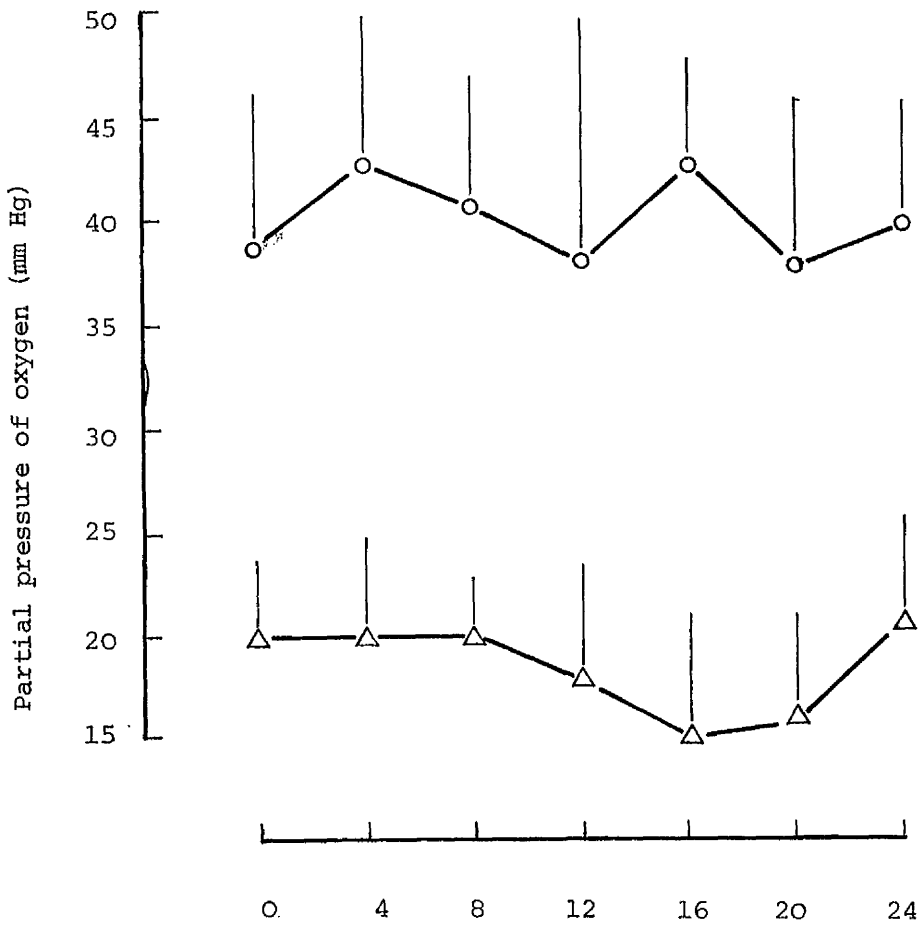
In normal rats, anaesthetized with ether and laparotomized between 2.00 p.m. and 4.00 p.m., the liver is releasing glucose into the blood passing through it. The extent of this release appears to be considerable: the hepatic venous blood has a glucose concentration approximately twice that of portal venous blood. The liver may also be releasing lipid, though on a much less dramatic scale. The hepatic venous blood has a total lipid content only a few per cent above that of portal venous blood and the difference is of doubtful significance. Phospholipid, as opposed to total lipid, is being taken up by the liver; the concentration in the hepatic veins is 40 per cent lower than in the

Figure 48



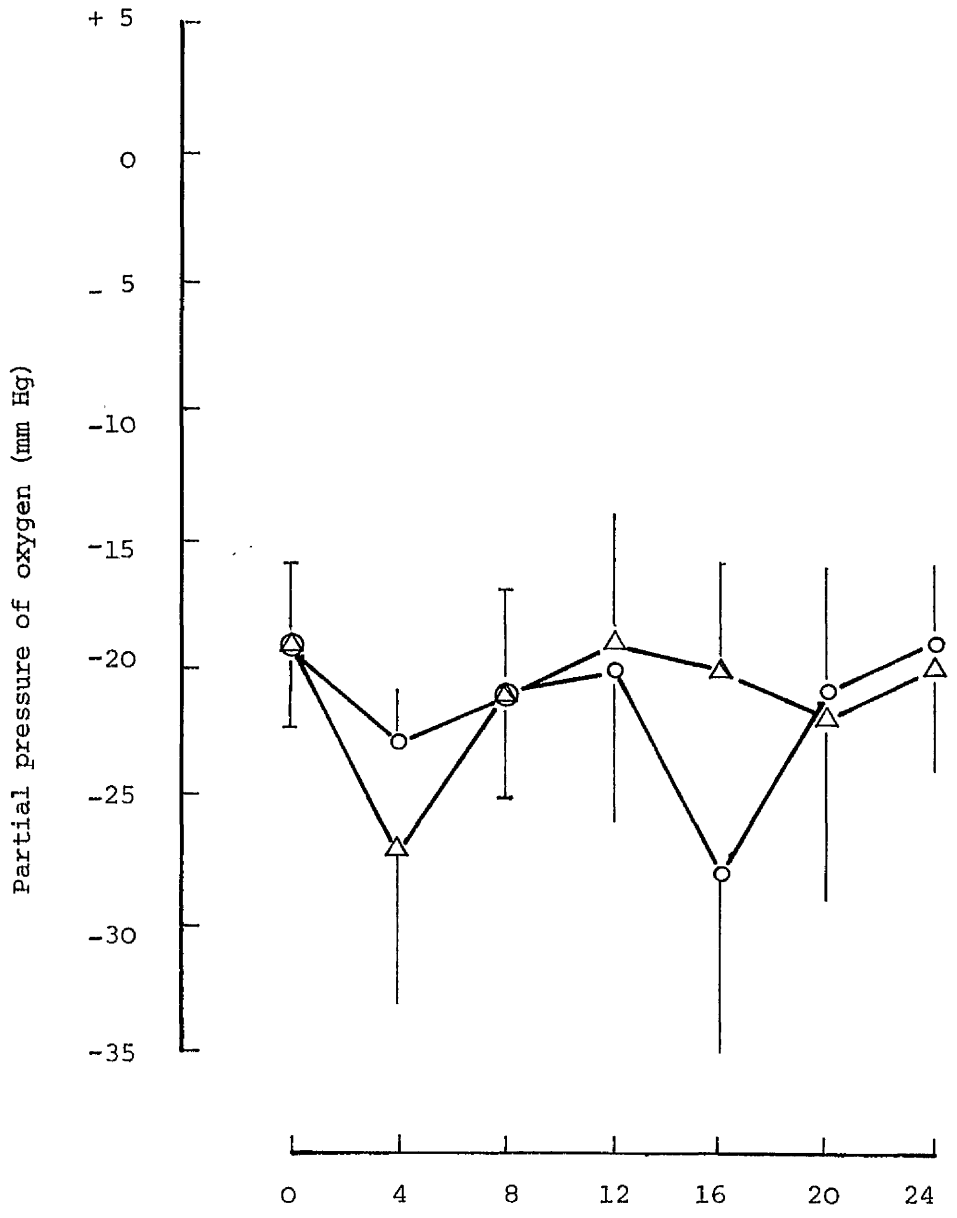
Partial pressure of oxygen in portal (o) and hepatic (△) venous blood of rats at various time intervals after liver biopsy. At least six male rats, approximately 220 g in body weight were used at each time interval. The results are shown as mean ± standard deviation.

Figure 49



Partial pressure of oxygen in portal (o) and hepatic (△) venous blood of rats at various time intervals after partial hepatectomy. At least six male rats, approximately 220 g in body weight were used at each time interval. The results are shown as mean ± standard deviation.

Figure 50



Differences in partial pressure of oxygen between portal and hepatic venous blood of rats at various time intervals after either liver biopsy (△) or partial hepatectomy (o). The values shown in this figure are derived from Figures 48 and 49. The results are shown as mean ± standard deviation.

portal vein. Protein is also being taken up though to a less marked degree; the hepatic-portal difference, though significant, is only of the order of ten per cent. Ammonia is being removed almost completely. Finally, a substantial proportion of the oxygen in the portal blood is removed by the liver, although in the absence of information about pH and pCO_2, this cannot accurately be assessed.

The effects of the trauma of biopsy can be enumerated as follows.

a) There is an immediate and dramatic increase in the release of glucose by the liver, reaching a peak four hours after the operation, with a return to the pre-operative situation at twelve hours.

b) The slow (and doubtfully significant) release of lipid is dramatically reversed and four hour after the operation the liver is taking up lipid on a substantial scale. This is, however, a temporary state of affairs; between eight and twelve hours there is a return to the pre-operative pattern.

c) The uptake of phospholipids is not obviously affected, although there is a marked increase in concentration in both the portal and hepatic vein lasting for twenty to twenty-four hours.

d) Uptake of protein diminishes sharply within four hours of operation and there is a concomitant (and presumably consequent) increase in protein concentration in both the portal and hepatic veins. Thereafter there is a slow return to normal both in respect of the concentrations in the two veins and of the difference between them.

e) The uptake of ammonia is quite unaffected.

f) The pO_2 in the portal vein shows a slight elevation four hours after the operation, but this is of doubtful significance. From eight hours onwards the situation is that obtained before the operation.

The response to partial hepatectomy differs from that elicited simply by biopsy in the following respect

a) The dramatic increase in glucose release found after biopsy does not occur after partial hepatectomy. Instead the rate of glucose release falls sharply and significantly four hours after the operation. This fall, like the increase seen after biopsy, is transient, and the situation at eight hours or twelve hours is similar to that seen before the operation. But the recovery seems only temporary. At sixteen, twenty and twenty-four hours after operation the liver fragment releases glucose at a rate significantly and substantially below that achieved by the (essentially) intact liver at the corresponding times after biopsy.

b) The pattern of total lipid metabolism is more complex. It will be recalled that after liver biopsy there is a dramatic but transient uptake of lipid by the liver. There is a rather similar uptake after partial hepatectomy, but in this case it lasts longer. Even at twenty hours the liver fragment is still taking up lipid to a significant extent.

c) Uptake of phospholipids, which is little affected by liver biopsy, is substantially, though temporarily, diminished by partial hepatectomy.

d) Uptake of protein is initially sharply diminished, as it is after biopsy. But whereas in biopsied animals there is a gradual return to the pre-operative situation, after hepatectomy the initial fall in uptake is sharply reversed and it is still well above the pre-operative level at twenty-four hours.

e) Uptake of ammonia, which is quite unaffected by biopsy, shows a curious sequence of changes after partial hepatectomy. There is a marked impairment at four hours, followed by a return to

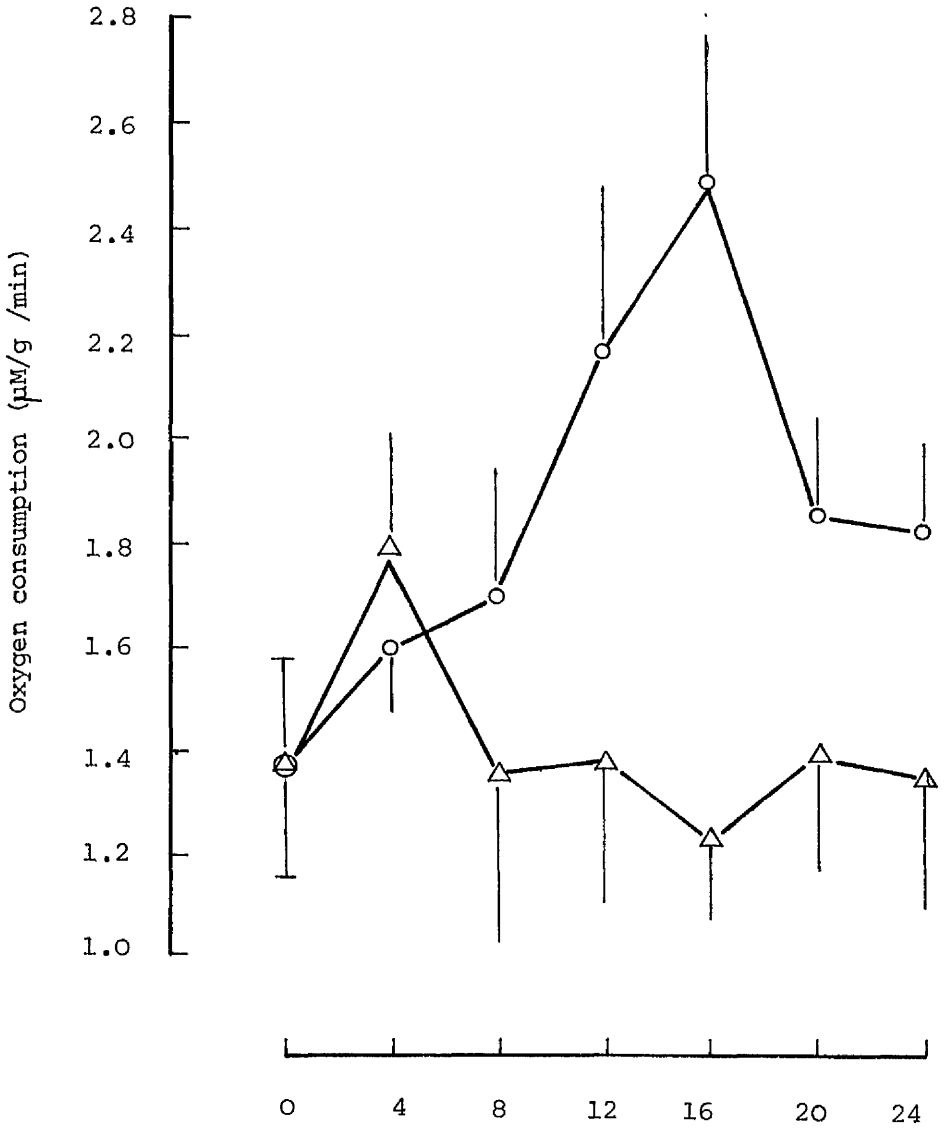
normal or perhaps better than normal between eight and twelve hours, followed in turn by second phase of impaired uptake which continues up to, and perhaps beyond twenty-four hours.

f) Oxygen uptake after partial hepatectomy is little if at all different from that seen after biopsy.

2.6 Oxygen consumption by liver slices in vitro

The errors inherent in the attempts to assess the affects of partial hepatectomy on hepatic oxygen uptake *in vivo* by the methods described in 2.5.7. above made it desirable to obtain confirmatory evidence for the view that the remaining liver fragment consumes oxygen at the same rate as the intact organ. Figure 51 shows the oxygen uptake *in vitro* of liver slices taken from rats at various time intervals after partial hepatectomy and biopsy. Biopsy was followed by a slight (but significant ($P < 0.02$) increase in oxygen uptake per gram wet weight four hours after the operation but at eight hours there was a return to the pre-operative level and this was maintained up to twenty-four hours. In the case of the hepatectomised animals there was a steady increase in oxygen uptake per gram reaching a peak of double the pre-operative value at sixteen hours and then falling back towards something like one-and-a-half times the pre-operative value at twenty and twenty-four hours. It appears therefore that the liver fragment remaining after partial hepatectomy does increase its oxygen uptake to an extent which must bring it fairly close to that achieved by the intact organ, a conclusion which is consistent with the results obtained *in vivo* (Fig. 48 to 50).

Figure 51



Consumption of oxygen by the liver slices *in vitro* taken from rats at various time intervals following *either* liver biopsy (△) *or* partial hepatectomy (o). At least six male rats (body weight approximately 220 g) were used for each estimation. The results are shown as mean ± standard deviation.

2.7 The effect of fasting on liver regeneration

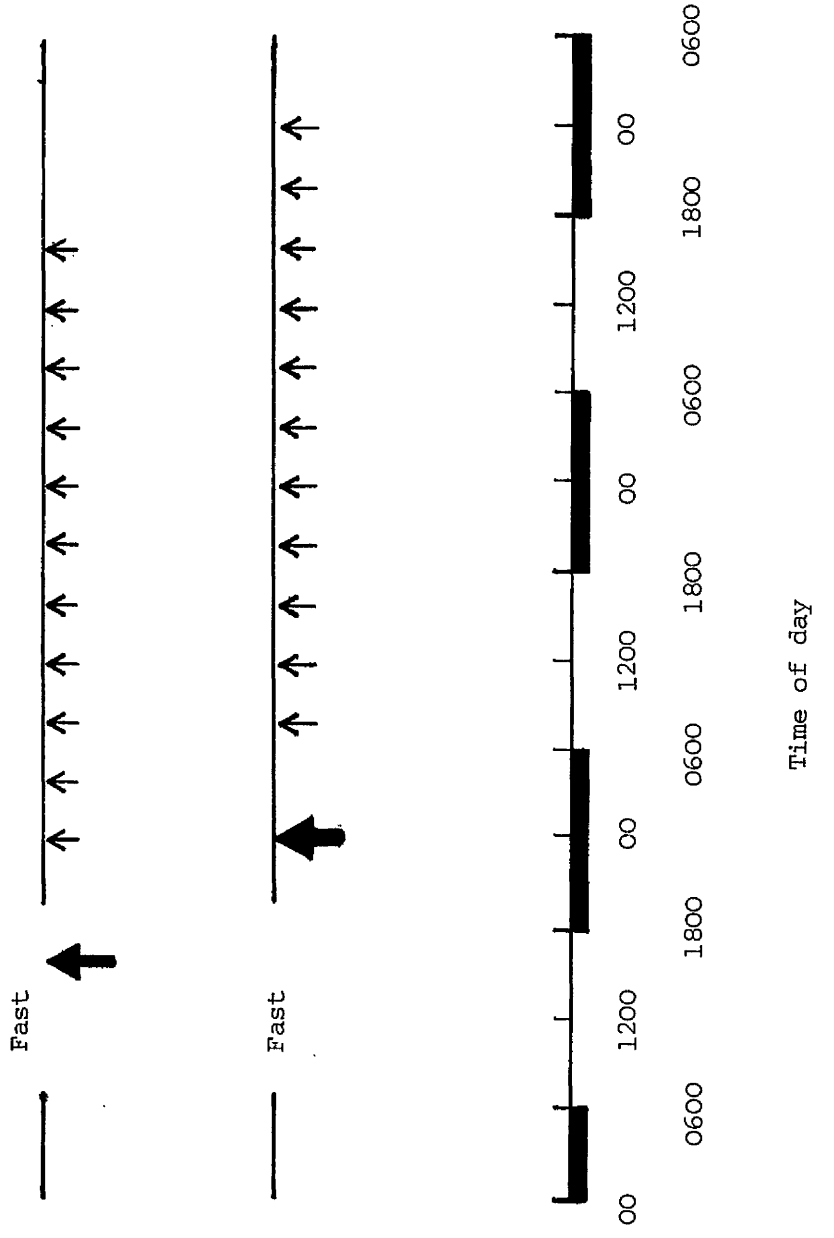
Whatever may be the mechanism controlling liver regeneration, it seems likely that the portal blood supply plays an important role. As long ago as 1920 Rous and Larimore showed that if the branch of the portal vein supplying about half the liver was tied off, the lobes it supplied atrophied and the other lobes enlarged. Comparable results were obtained much later in the rat by Weinbren (1967, 1972) and in the dog by Starzl et. al.,(1973, 1975). In conformity with these observations, it has been shown that the regenerative response in liver autografts after partial hepatectomy is greatly diminished or even abolished if they are deprived of portal blood (Sigel et al., 1963).

The portal blood is derived partly from spleen and pancreas, but chiefly from the gut. Its composition will therefore vary depending on whether the gut is full after a meal or empty in the post-absorptive state. Insofar as the liver is the first tissue which absorbed nutrients encounter, its metabolic load will vary depending on whether absorption is in process or not. It seemed of interest to find out whether this variation had any effect on regeneration. This was investigated by carrying out two parallel experiments. Their plan is illustrated in Figure 52. Rats normally feed by night and sleep for a great part of the day, so the absorption of nutrients should be maximal in the late evening and minimal around noon. But to reinforce this in the present experiments all the animals were fasted from 7 a.m. till 9 p.m. at the start of the experiments. In the first experiment, partial hepatectomy was performed nine hours after the start of the fast i.e. when there would be no absorption. In the second, it was performed three hours after the fast ended i.e. when absorption of nutrients should

Figure 52

Schematic representation of the design of experiments involving starved rats. The base line represents time of the day with periods of light and darkness in which the rats were kept. The straight lines represent the availability of food. The break in this line represents the period of fast. In these experiments partial hepatectomy was performed either at 4 p.m. or at 1 a.m. represented in the figure by large arrows (⬆). All rats were killed later at regular time intervals following partial hepatectomy represented by small arrows (↑).

Figure 52

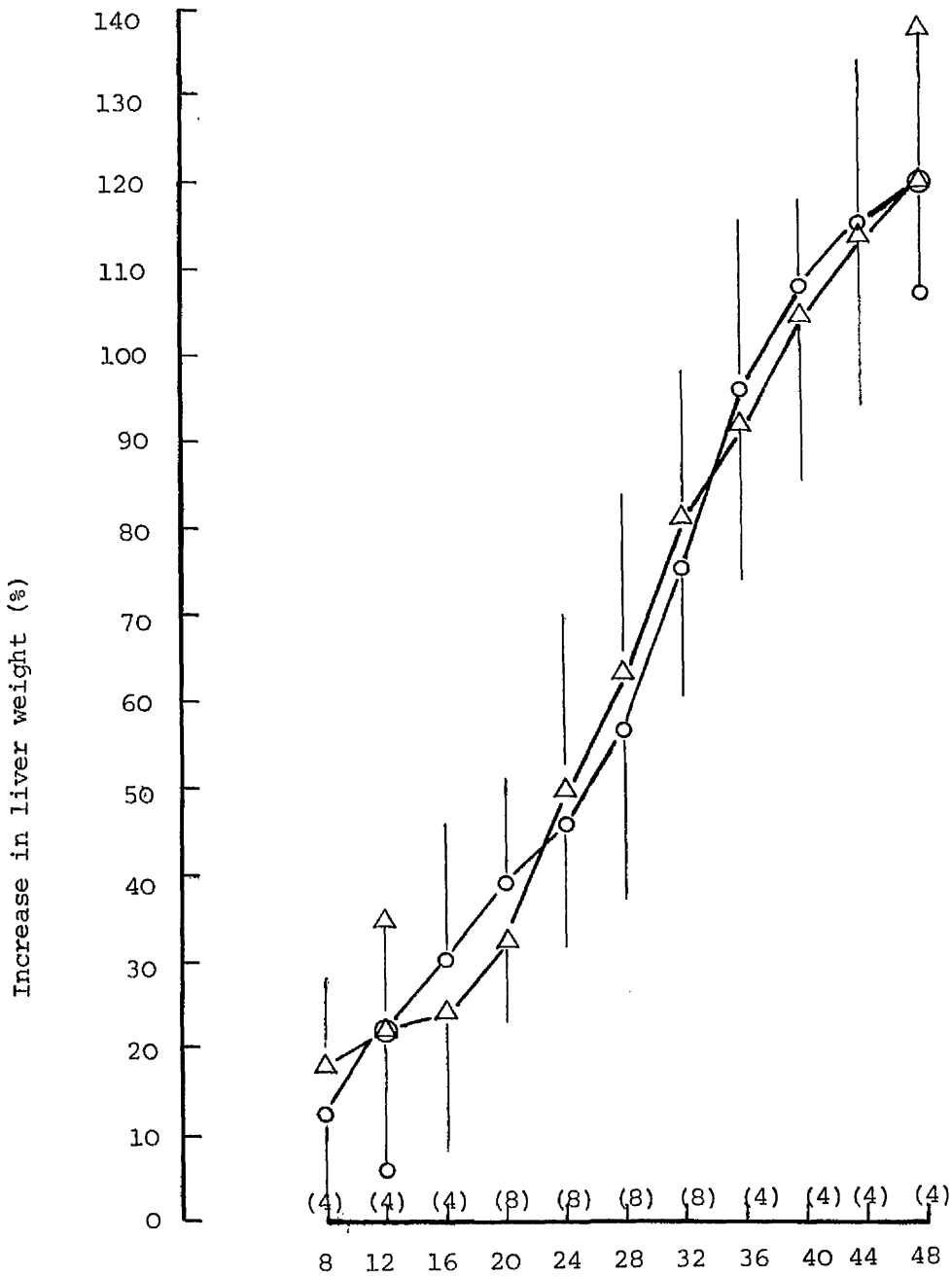

Fast
Fast
00
0600
1200
1800
00
0600
1200
1800
00
0600
1200
1800
0600
Time of day

have been maximal. Groups of rats were killed at various time intervals after hepatectomy, 20 μci [^{3}H] thymidine being injected thirty minutes before sacrifice.

Figure 53 shows the progress of regeneration in the two experiments in terms of increase in wet weight of the liver remnant. It is immediately apparent that, within the limits of experimental error, the two experiments give identical rates of increase. Both, moreover, are indistinguishable from the results obtained in a previous experiment (Figure 15, section 2.3.). Clearly, therefore, the presence or absence of absorbed nutrients in the portal blood has no effect on the regenerative process as measured by this criterion.

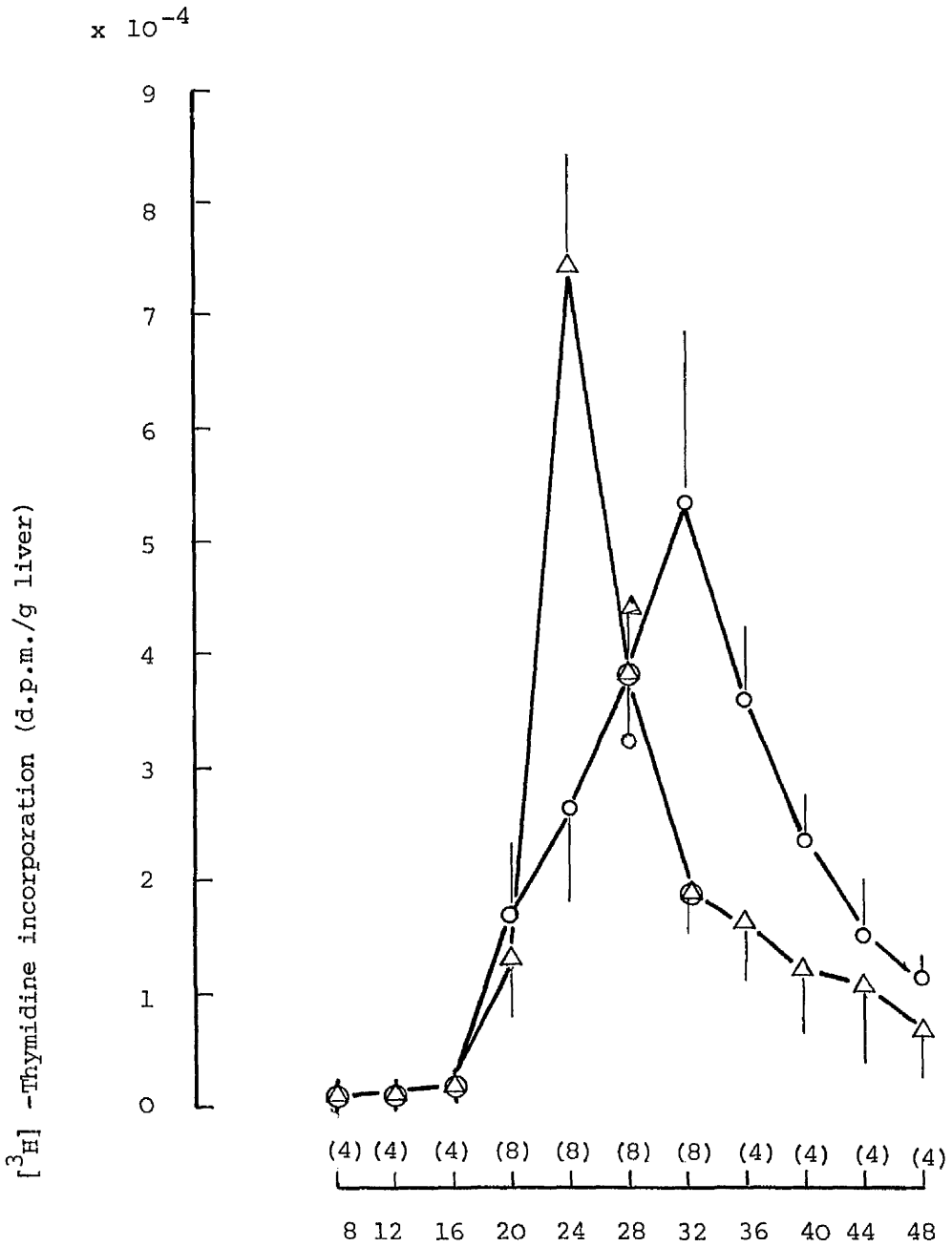
Figure 54 shows the rates of DNA synthesis in the two experiments. In both, there is a latent period for sixteen hours after the operation. At twenty hours both show equal rates of DNA synthesis, but in the animals hepatectomised after the fast the rate of synthesis accelerates more rapidly and reaches a higher peak earlier than in the animals hepatectomised during the fast. In both cases DNA synthesis diminishes after the peak almost as quickly as it increased earlier. It is interesting that, although the time which elapse between the operation and the peak of DNA synthesis differs by eight hours between the two experiments, the peak of DNA synthesis is achieved at the same time of day - midnight - in each case. This is readily apparent from Figure 55 in which DNA synthesis is plotted not against time elapsed since the operation but against time of day. This Figure, in which the two peaks are coincident, demonstrates another difference between the two experiments. When hepatectomy is carried out after the end of the fast, DNA synthesis occurs much more rapidly than when hepatectomy is performed during the fast. In the former case the change from resting level to

Figure 53



Increase in weight of the liver remnant in two groups of rats starved for fourteen hours and partially hepatectomised. In one case partial hepatectomy was performed nine hours after the start of the fast (o). In the other case it was performed four hours after the end of the fast (△). All rats were male and approximately 220 g in body weight. The number of rats killed in each group is given in parenthesis. The results are shown as mean ± standard deviation.

Figure 54

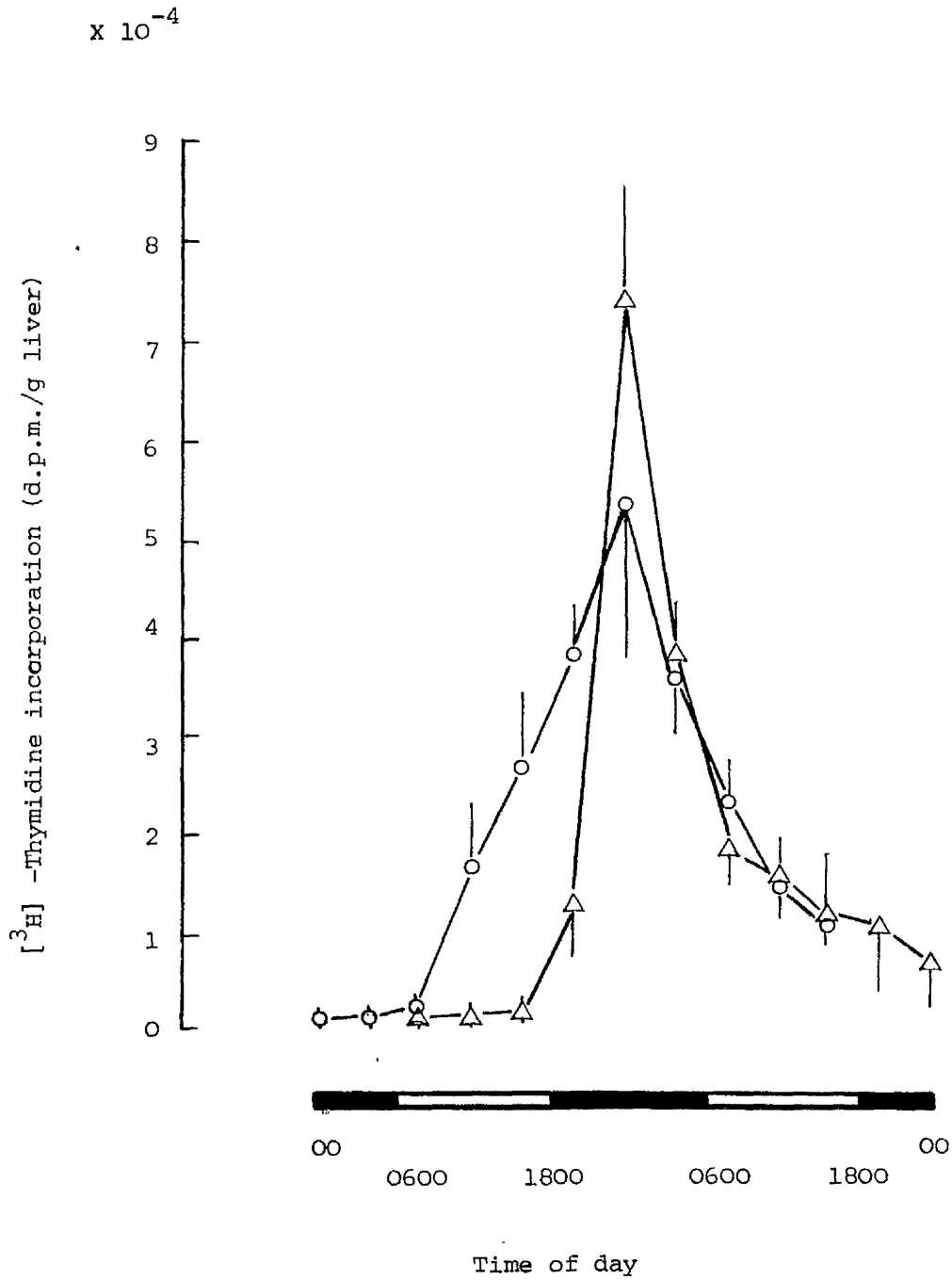


Incorporation of [^{3}H]-thymidine in the liver remnant of two groups of rats starved for fourteen hours and partially-hepatectomised. In one case partial hepatectomy was performed nine hours after the start of the fast (o). In the other case it was performed four hours after the end of the fast (△). All rats were male and approximately 220 g in body weight. The number of rats killed in each group is given in parenthesis. The results are shown as mean ± standard deviation.

Figure 55

Incorporation of [^{3}H] -thymidine in the liver remnant of two groups of rats starved for fourteen hours and partially hepatectomised. In one case partial hepatectomy was performed nine hours after the start of the fast (o). In the other case it was performed four hours after the end of the fast (Δ). All rats were male and approximately 220 g in body weight. The number of rats killed in each group is given in Fig. 54. The results are shown as mean $\pm$ standard deviation. The values in this figure are the same as in Figure 54 but the incorporation of thymidine is plotted not against time after operation but against the time of the day.

Figure 55


x 10^-4
9
8
7
6
5
4
3
2
1
0
[3H] -Thymidine incorporation (d.p.m./g liver)
00
0600
1800
0600
1800
00
Time of day

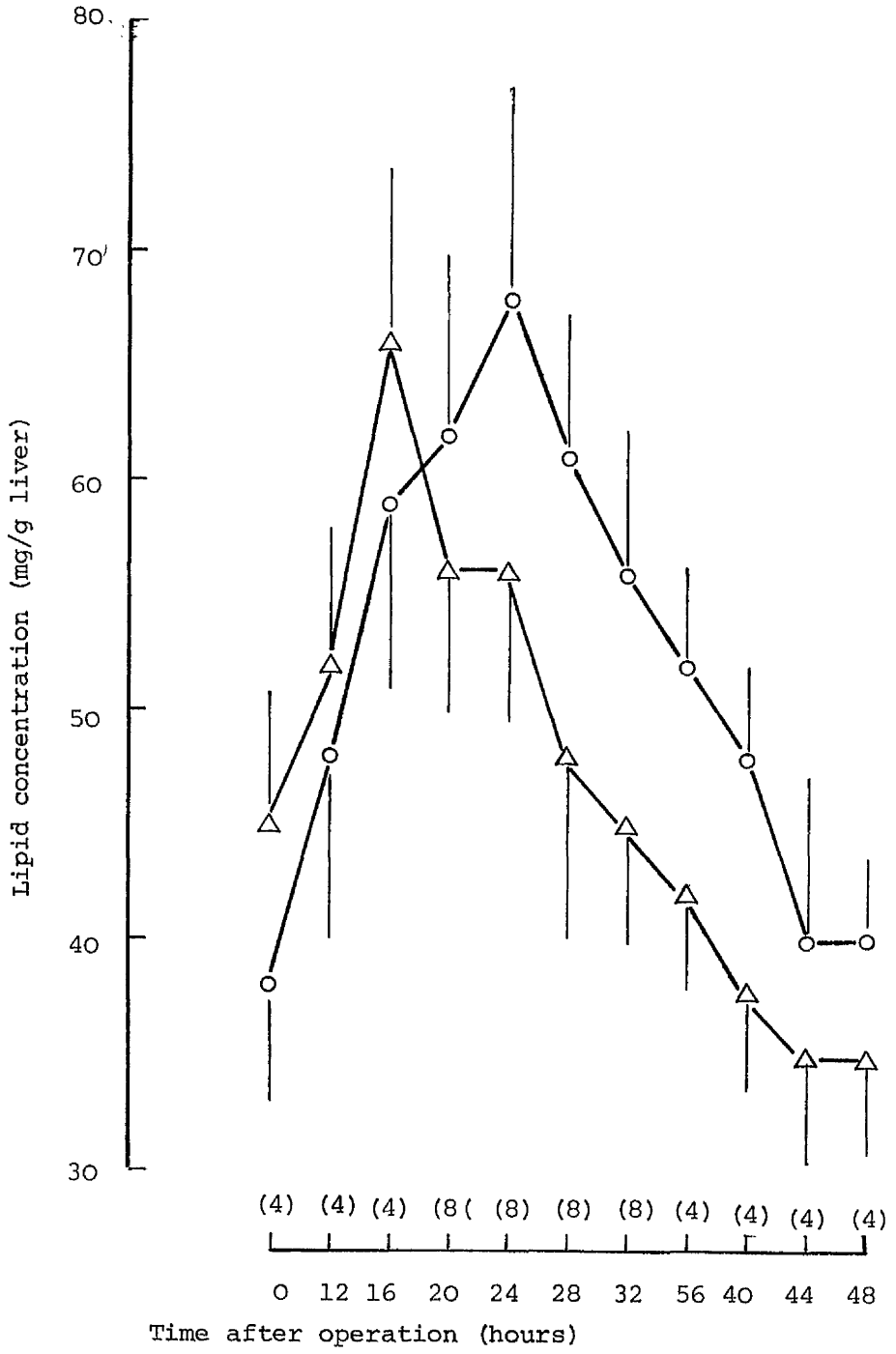
maximum is achieved in eight hours; in the latter it requires sixteen hours. But the decline of DNA synthesis after the peak is the same in both experiments.

Figure 56 shows the concentration of total lipids in the liver in these experiments. In both cases, as expected, it increases sharply after operation and subsequently falls back to normal. But the timing is different. The peak is attained eight hours earlier if hepatectomy is performed in the fed state. In other words in each case it occurs about the same time of day, around 4 p.m.. Concentration of phospholipids in both experiments show no significant change (Figure 57).

Figure 58 shows the changes in protein concentration in the liver in the two experiments. The patterns observed are strikingly different. The animals hepatectomised after fasting show no significant change; this is in agreement with the results of the earlier experiments shown in Figure 22. But in the animals hepatectomised while fasting there is a marked increase in protein concentration, reaching a peak at twenty-four hours after the operation. It is difficult to explain this observation. Protein concentration is, of course, at the mercy of many variables: it would be affected to a greater or lesser degree by the concentrations of other constituents. A fall in lipid concentration, for example, would produce an increase in protein concentration; so would dehydration. But there is no evidence for anything of the sort in the present instance.

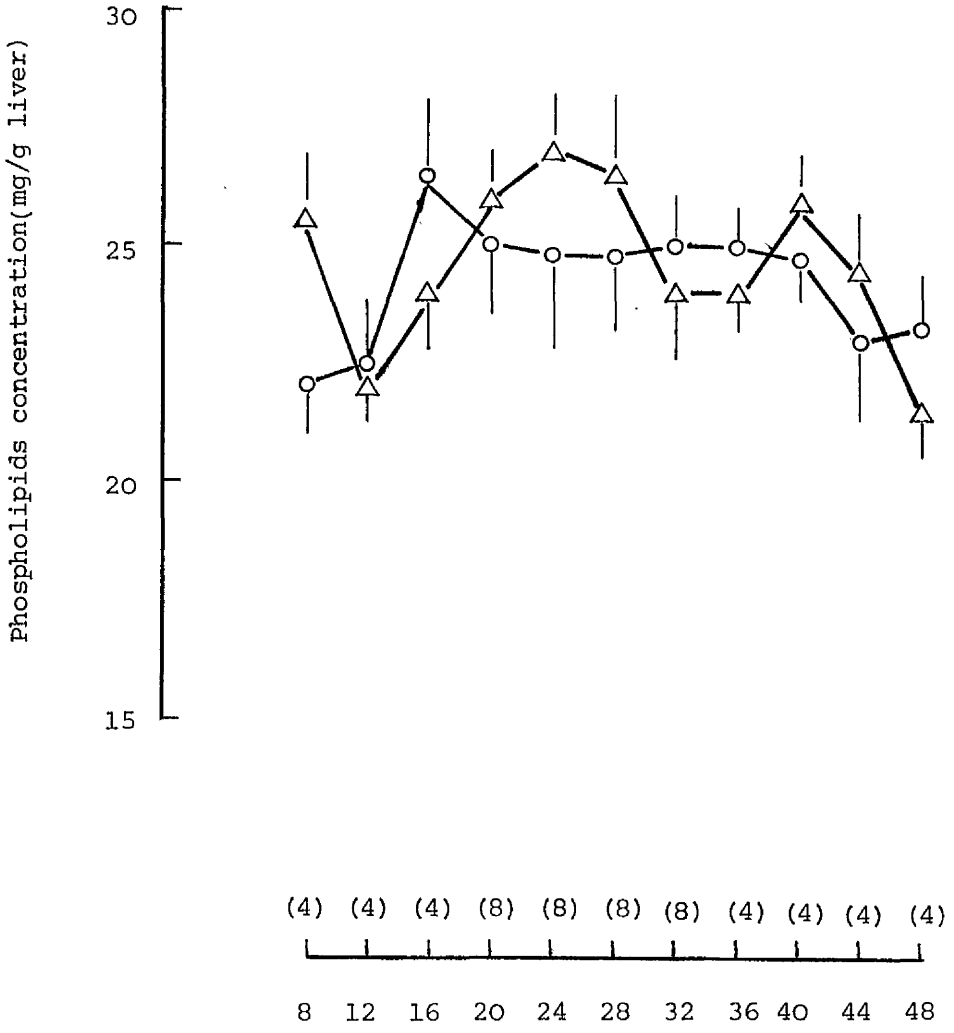
In summary, it is clear that the timing of partial hepatectomy, whether it is performed at a time when the animal is feeding or fasting, does affect some, but not all, aspects of the regenerative response. It does not affect the increase in liver mass, but it does alter the timing of the mitotic response and of the accumulation of fat, with the

Figure 56



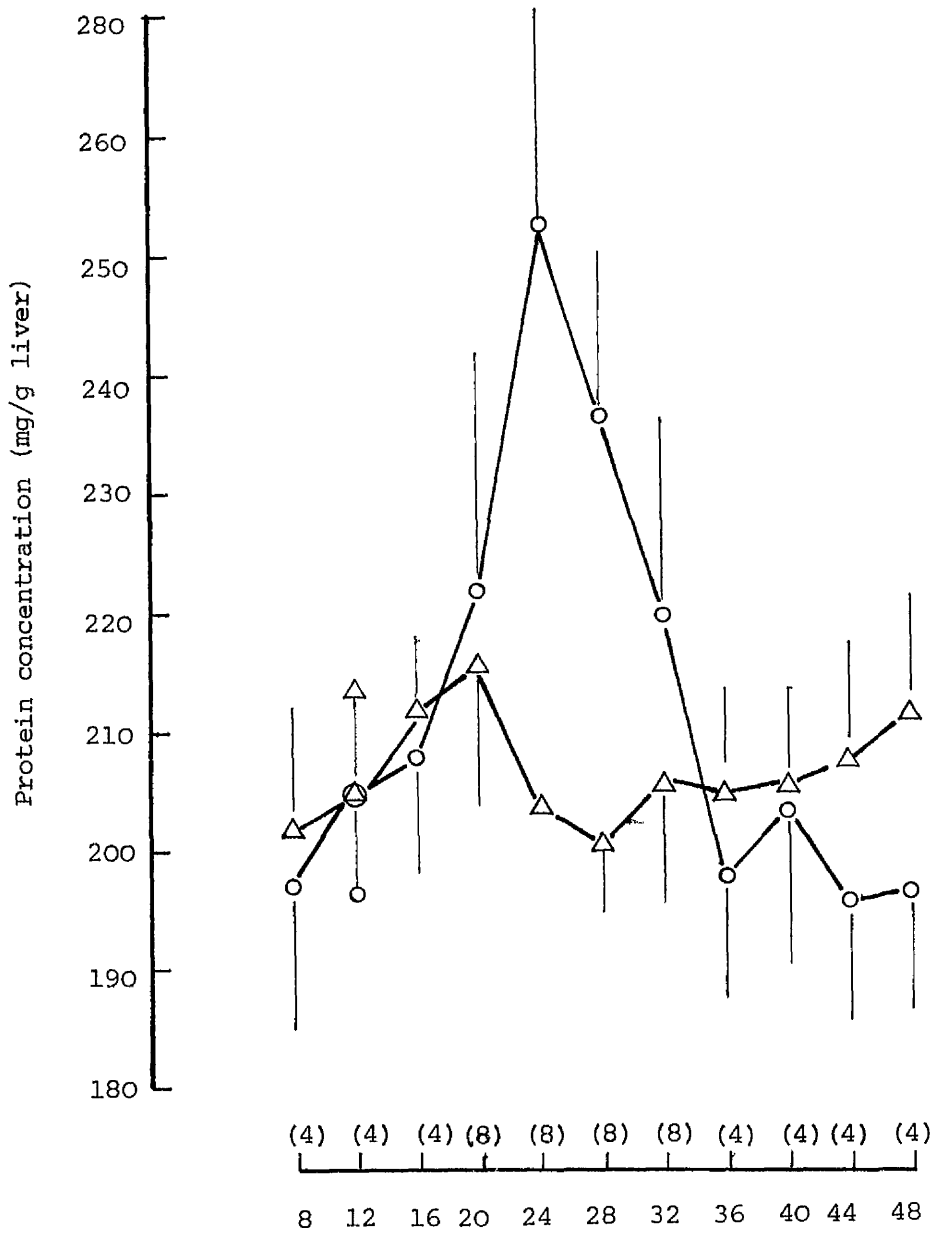
Concentration of lipids in the liver remnant of two groups of rats starved for fourteen hours and partially hepatectomised. In one case partial hepatectomy was performed nine hours after the start of the fast (o). In the other case it was performed four hours after the end of the fast (△). All rats were male and approximately 220 g in body weight. The number of rats killed in each group is given in parenthesis. The results are shown as mean ± standard deviation.

Figure 57



Concentration of phospholipids in the liver remnant of two groups of rats starved for fourteen hours and partially hepatectomised. In one case partial hepatectomy was performed nine hours after the start of the fast (o). In the other case it was performed four hours after the end of the fast (△). All rats were male and approximately 220 g in body weight. The number of rats killed in each group is given in parenthesis. The results are shown as mean ± standard deviation.

Figure 58



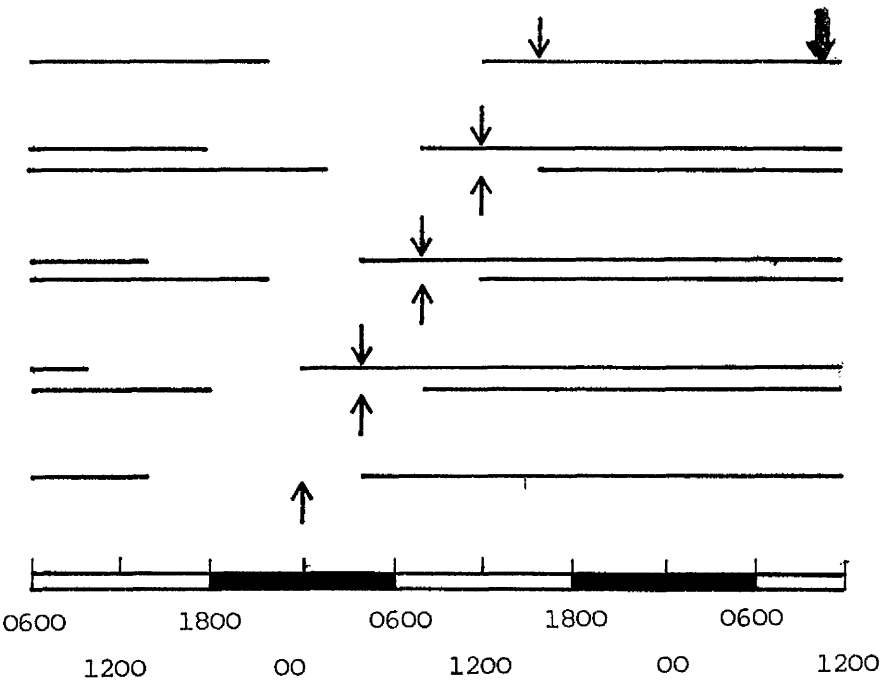
Concentration of protein in the liver remnant of two groups of rats starved for fourteen hours and partially-hepatectomised. In one case partial hepatectomy was performed nine hours after the start of the fast (o). In the other case it was performed four hours after the end of the fast (△). All rats were male and approximately 220 g in body weight. The number of rats killed in each group is given in parenthesis. The results are shown as mean ± standard deviation.

consequence that both of these reach a peak at the same time of day, midnight for the mitotic response and 4 p.m. for fat accumulation. And hepatectomy performed while the rat is fasting is followed by a transient increase in protein concentration not seen when the operation is performed when the animal is fed.

The results of these experiments show in broad terms that the response to partial hepatectomy, and more particularly the timing of that response, vary depending on whether the operation is carried out during daylight hours, when rats are normally asleep and fasting, or during the hours of darkness when they are awake and feeding. Not all aspects of regeneration are affected. The increase in wet weight is not (Figure 53). But the peak of lipid accumulation (Figure 56) and perhaps more important, the peak mitotic activity (Figure 54 and 55) seem to be determined as much by the time of day as by the time after operation. To separate the affects of time of day from nutritional state, we repeated the study with a modified experimental design, the details of which are shown in Figure 59. In this experiment the time of fast and partial hepatectomy was varied while the time of sacrifice in both groups of rats was kept constant between 10 and 11 a.m. The rest of the experimental procedure was essentially the same as in the previous experiment. The results are shown in Figures 60 and 61.

Figure 60 shows the increase in weight of the liver remnant. It is clear that this is virtually identical whether partial hepatectomy was performed when the animals were fed or fasting. Figure 61 shows the uptake of [^{3}H]-thymidine by the liver remnant. In rats hepatectomised in the fed state the peak incorporation occurs at twenty-four hours after the operation, while in rats hepatectomised during the fast the peak is attained eight hours later. These results are in exact agreement with the earlier observation (Figure 54). It seems clear, therefore, that DNA synthesis is affected by nutritional status, not by the time of day.

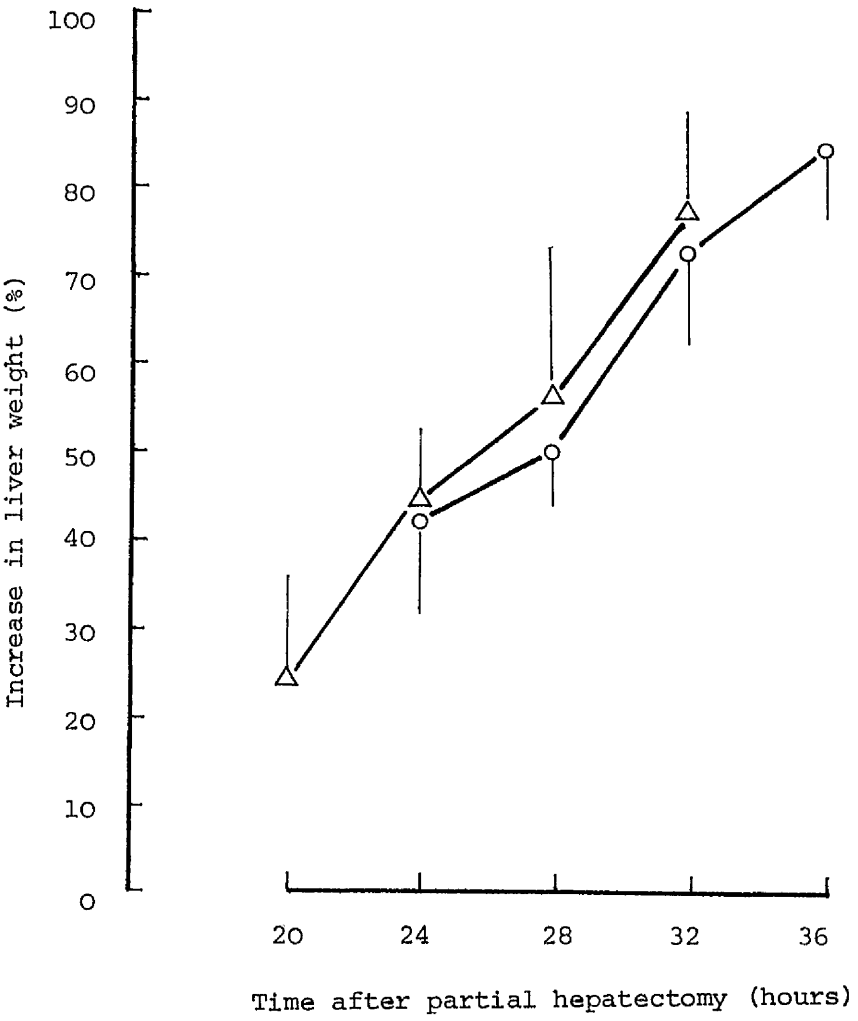
Figure 59



Time of day

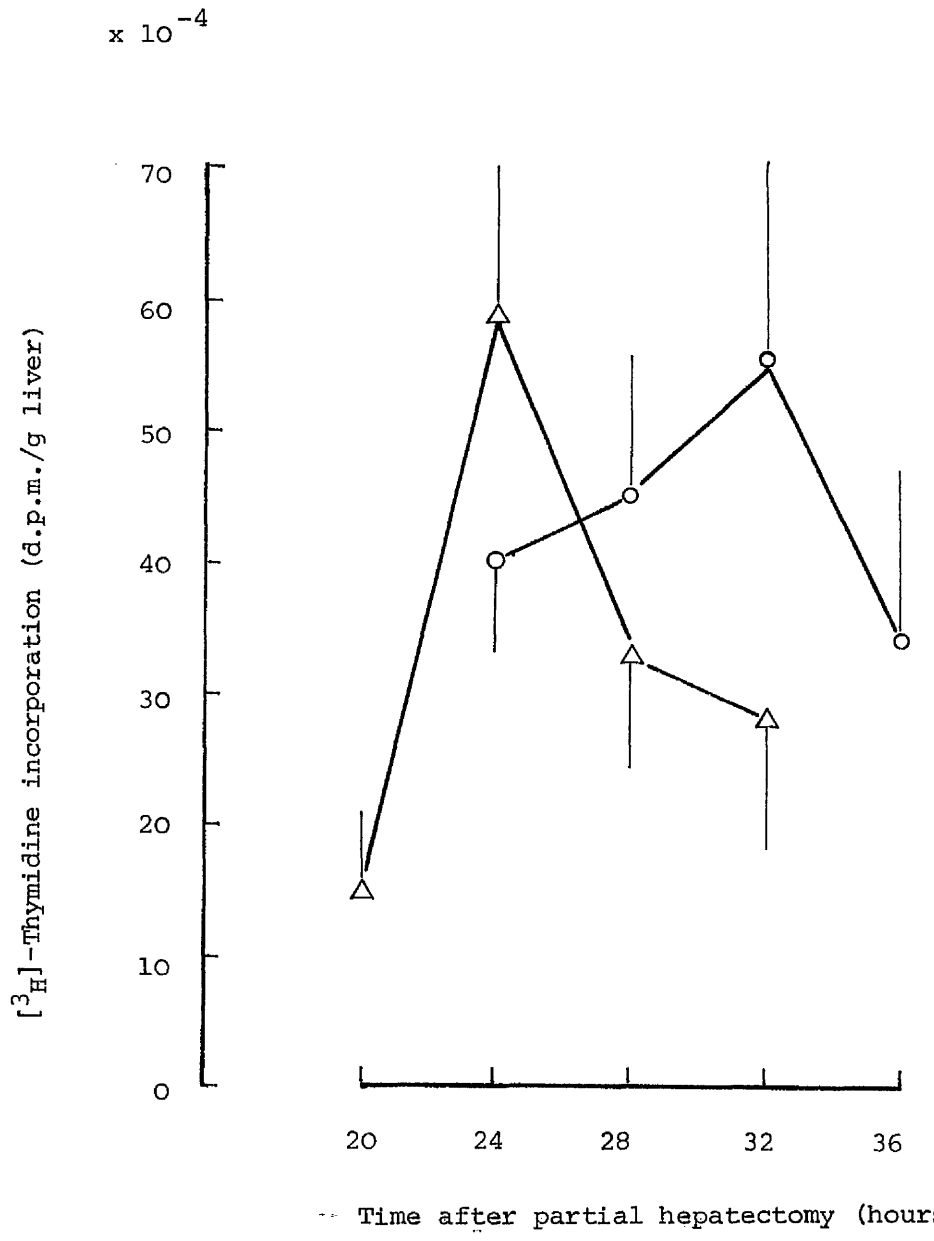
Schematic representation of the design of experiments involving starved rats. The base line represents the time of the day with periods of light and darkness in which the rats were kept. The straight lines represent the availability of food. The break in this line represents the period of fast. In these experiments partial hepatectomy was performed at various times of the day represented in the figure by small arrows (↑ and ↓). All rats were killed later at regular time intervals following partial hepatectomy but at the same time of the day (around 10 a.m.) represented in the figure by large arrow (⬇).

Figure 60



Increase in weight of the liver remnant in two groups of rats starved for fourteen hours and partially hepatectomised. In one case partial hepatectomy was performed nine hours after the start of the fast (o). In the other case it was performed four hours after the end of the fast (△). There were four male rats, approximately 220 g in body weight at each time interval. The results are shown as mean ± standard deviation.

Figure 61



Incorporation of [^{3}H]-thymidine in the liver remnant in two groups of rats starved for fourteen hours and partially hepatectomised. In one case partial hepatectomy was performed nine hours after the start of the fast (o). In the other case it was performed four hours after the end of the fast (△). There were four male rats, approximately 220 g in body weight at each time interval. The results are shown as mean ± standard deviation.

2.8 Liver regeneration in indomethacin injected rats

The fact that the stimulus for liver regeneration is so short-lived and so difficult to transfer from one animal to another (1.6.3.) is consistent with the idea that prostaglandins, which are notoriously short-lived and which appear to mediate so many physiological control processes (Higgins and Braunwald, 1972; Oesterling et al., 1972; Silver and Smith, 1975), may be involved here.

To investigate this possibility, a series of experiments was undertaken to see whether indomethacin, a well known inhibitor of prostaglandin biosynthesis (Flower, 1974), had any effect on liver regeneration as reflected either in DNA synthesis or as increase in the mass of the liver fragment remaining after partial hepatectomy. The results are shown in Table 10 (DNA synthesis) and Table 11 (increase in tissue mass).

Table 10 shows the effect of indomethacin on DNA synthesis, as measured by [^{3}H]-thymidine incorporation. Administration of indomethacin four hours before partial hepatectomy brought DNA synthesis at twenty-four hours down to a little more than a tenth of its control level (Groups 1 and 2 in Table 10). But at thirty-six hours the effect appeared to have worn off and the rate of DNA synthesis had recovered (Group 3 in Table 10). This recovery could, however, be prevented by administering a second dose of indomethacin eight hours after the operation (Group 4 in Table 10).

Table 11 shows the corresponding effects on the increase of tissue mass. Reference back to Figure 15 will show that in male rats of around 220 g the liver fragment left at operation weighs around 2.7 g and increases to around 3.6 g twenty-four hours after hepatectomy. The

TABLE 10

Effect of injections of indomethacin on [^{3}H]-thymidine incorporation in the liver remnant in partially hepatectomised male rats (body weight approximately 220 g). The indomethacin was dissolved in propane-1,2-diol. Pre-operative injections contained 10 mg indomethacin per animal and post-operative 5 mg. Where appropriate, control injections of 1.0 ml and 0.5 ml of propane diol were given.

Group No.	Interval between hepatectomy and sacrifice hours	No. of rats used	Indomethacin injected	weight of liver remnant at sacrifice (grams)
1	24	8	None	70085 ± 15971
2	24	8	4 hours before operation	7523 ± 3877
3	36	8	4 hours before operation	53377 ± 27567
4	36	8	4 hours before and 8 hours after operation	10197 ± 3795

TABLE 11

Effect of injections of indomethacin on the weight of the liver remnant in partially-hepatectomised male rats (body weight approximately 220 g). The indomethacin was dissolved in propane-1,2-diol. Pre-operative injection contained 10 mg indomethacin per animal and post operative 5 mg. Where appropriate, control injections of 1.0 ml and 0.5 ml of propane diol were given. The results are shown as mean ± standard deviation.

Group No.	Interval between hepatectomy and sacrifice (hours)	No. of rats used	Indomethacin injected	weight of liver remnant at sacrifice (grams)
1	24	8	None	4.20 ± 0.526
2	24	8	4 hours before operation	3.35 ± 0.332
3	36	8	4 hours before operation	3.77 ± 0.359
4	36	8	4 hours before operation and 8 hours after operation	3.44 ± 0.352
5	80	4	None	5.76 ± 0.428
6	80	6	4 hours before operation and 24, 48 and 72 hours after operation	3.38 ± 0.282

control rats (Group 1) in Table 11 have done rather better than this: at twenty-four hours their liver remnants weigh about 4.2 g. In rats injected with indomethacin four hours before partial hepatectomy (Group 2 in Table 11) the liver fragment is much smaller, only about 3.3 g twenty-four hours after the operation. Even at thirty-six hours, when the liver fragment would be expected to weigh 4.8 g (Figure 15), rats injected with indomethacin pre-operatively have liver fragments weighing only 3.76 g (Group 3 in Table 11) and in rats injected both pre-operatively and post-operatively the liver fragments weigh only 3.44 g. Clearly, therefore, over these time intervals indomethacin effectively inhibits increase in tissue mass. And this inhibition can be prolonged by additional indomethacin injections. Animals injected with indomethacin four hours before the operation and at twenty-four hours, forty-eight hours and seventy-two hours post-operatively have liver fragments weighing only 3.38 g at eighty hours compared to 5.76 g in uninjected controls. Taken together Tables 10 and 11 demonstrate that indomethacin is an effective inhibitor of the process of regeneration. It seems reasonable therefore to conclude that prostaglandins play an essential role in the process.

3. DISCUSSION

3.0 DISCUSSION

In trying to elucidate the sequence of events in liver regeneration it is important to distinguish changes specific to partial hepatectomy from changes produced by the anaesthetic and the trauma of operation. Thus the precipitate fall in glycogen content and its subsequent slow recovery are qualitatively similar after partial hepatectomy and after liver biopsy (Figure 24). So also are the changes in water content (Figure 21), mean cell mass (Figure 20), mean cell dry weight (Figer 25) and total protein per cell (Figure 22). The only change peculiar to partial hepatectomy is the transient accumulation of lipids (Figure 23).

But even where partial hepatectomy and sham operation elicit the same type of response, a quantitative difference is sometimes apparent. Glycogen content is a case in point. Both operations cause a similar loss of glycogen but recovery is slower after partial hepatectomy (Figure 24). Similarly both operations produce a sharp increase in water content, but the subsequent return to the normal level is slower and less complete after partial hepatectomy (Figure 21). And again the changes in cell mass (Figure 20) dry weight (Figure 22) and protein (Figure 25), though qualitatively similar after the two operations, are more marked after partial hepatectomy. A feature of all these quantitative differences in response is that they manifest themselves only after a delay of around eight hours. They may therefore reflect not so much a difference in the direct response to the two operations, but rather a difference in ability to re-establish the pre-operative status quo. Both operations, in other words, produce the same metabolic upsets but rectification of the upsets is more rapid and complete if the liver is intact.

To some extent at least, the metabolic changes after the two operations resemble the changes in liver composition. In most cases both operations produce much the same initial response: output of total lipid changes to a (more rapid) uptake (Figure 34); uptake of protein virtually stops (Figure 40); the level of phospholipids in the blood increased sharply (Figure 35, 36). It is only after this initial response that any divergence manifests itself: the postoperative uptake of lipid reverses itself twelve hours after biopsy but continues for twenty-four hours after partial hepatectomy (Figure 34); uptake of protein returns gently to normal after biopsy but after partial hepatectomy it increases far beyond the pre-operative level (Figure 40); plasma phospholipids levels return slowly to normal after biopsy and phospholipids uptake by the liver remains virtually constant, but after partial hepatectomy the return to normal plasma levels is more erratic. There is one important exception to these generalizations: biopsy elicits an immediate though transient increase in the output of glucose by the liver; partial hepatectomy produces a similarly transient decrease. By about eight or twelve hours after operation glucose output has in both cases returned to about the pre-operative level though it remains higher in the biopsied animals than in those partially hepatectomised.

But the most striking feature of the observations summarized in Figures 29 to 38 is that the medium term metabolic consequences of partial hepatectomy are quite modest in relation to the amount of liver tissue excised. This is particularly well exemplified in the case of glucose. Once the initial reactions to the anaesthetic and the trauma of operation have passed, the liver remnant's output of glucose is about two-thirds that of the biopsied liver rather than, as might have been anticipated, only one-third (Figure 31). Even in the case of ammonia

extraction, the liver fragment left after partial hepatectomy is more efficient than might be expected in view of its size (Figure 42). The overall impression is that the liver fragment shows an increased activity which, to a substantial degree, compensates for the loss of the lobes removed at operation. This conclusion fits well with the estimates of oxygen uptake shown in Figures 44 to 47 and 48 to 50 which appear to show that after partial hepatectomy the liver fragment takes up virtually the same quantity of oxygen as the intact organ before operation.

Clearly therefore, following two-thirds hepatectomy, the animals body in general and the liver remnant in particular would experience some metabolic stress. Is this likely to be the primary stimulus for regeneration? Our results and evidence from other laboratories suggests that it might be so. Our experiments with starved rats show that the increase in the rate of DNA synthesis occurs earlier and is better synchronized if partial hepatectomy is performed at a time when the intestines are full rather than when they are empty (Figure 54). Clearly therefore, the contents of stomach and intestines exert some modifying influence on the growth of the liver remnant left after two-thirds hepatectomy. These results are in agreement with the results of Barbiroli and Potter (1971). These authors, working with rats on controlled feeding schedules, reported that the rhythms of food intake are the main synchronizers of liver cell proliferation. The idea of functional stress determining the size of the organ is neither new nor unique to liver. Thus, high protein intake results in an enlargement of the kidney (Dowben, 1969) and the salt glands of marine birds change size with the amount of salt injected (Dowben, 1969). As long ago as 1958, Kennedy, Pearce and Parrott reported that during the second and third weeks of lactation the liver in the female rats hypertrophed to approximately twice its normal size and that there was a smaller, but

significant, increase in the DNA content, indicating an increase in cell number. In another series of experiments Kennedy and Pearce (1958) reported that an increased food intake in the adult rats, caused by hypothalamic lesions, had the same effect on the liver as very rapid somatic growth. In such hyperphagic rats, both the rate of regeneration after partial hepatectomy and the ultimate size and composition of the liver remnant were determined by the caloric intake.

The mechanism by which functional stress may bring about cell proliferation in the liver remnant is obscure. Substantial evidence has accumulated in favour of humoral control of liver regeneration (1.6.3). It seems therefore that metabolic stress (determined by caloric intake and the composition of blood) might either stimulate some existing hormone or provoke the production of a new humoral factor(s) to bring about growth in the liver remnant. Since insulin plays an important role in numerous physiological processes and its release is induced by food injestion (Newsholme and Start, 1973), several workers have suggested it as the regulator of liver regeneration. However the results of our experiments with alloxan-diabetic rats show that absence of insulin does not affect liver regeneration (Table 8 and 9). Our results agree with those of Bucher (1975) who reported that vigorous liver regeneration occurs after partial-hepatectomy in totally eviscerated rats. Clearly, therefore, insulin cannot be the sole factor initiating liver regeneration. All the other known hormones have been found to play only a marginal role in liver regeneration (1.5.2). Therefore it seems reasonable to presume that metabolic stress provokes the production of some new humoral factor(s) which brings about growth in the liver remnant left after two-thirds hepatectomy.

It seems clear from the literature that this hepatotrophic factor(s) has a very short life and it fails to equilibrate in the blood

of cross-circulated animals. Prostaglandine seem to be the only known humoral agents which have the same properties as the hypothetical humoral agent(s) is supposed to have these are very short-lived, they may be produced by most tissues (including liver) and act as local hormones elicting numerous physiological responses. Our results with indomethacin-treated rats (Table lO and ll) suggest an important role for prostaglandins in liver regeneration. This conclusion was also reached by MacManus and Braceland (1976) who reported that the concentration of PGE equivalents in rats liver in vivo was increased during liver regeneration, and the ability of homogenates of regenerating liver to synthesise prostaglandins from arachidonate in vitro was also increased. Indomethacin prevented these prostaglandin changes and the subsequent increase in DNA synthesis.

To prove the role of prostaglandins unequivocally it is necessary to show that the effect of indomethacin can be reversed by prostaglandin administration. However the difficulty of providing such evidence may be appreciated by the fact that there are about 14 prostaglandins known to occur naturally which elicit a wide range of biological responses when tested in vivo and in vitro animal systems (Bergstrom et al., 1968; Weeks 1972). Other than their extreme potency and numerous pharmacological effects, few generalizations can be made because of diversity of effect shown by various prostaglandins e.g. PGEs and PGHs were shown to be potent vasodilators whereas PGFs were vasoconstrictors (Bergstrom et al., 1964; 1967; Nakona 1968). Similarly PGEs, PGAs and PGBs were found to relax human uterine strip whereas PGF compounds cause contraction of this strip (Bygdoman, 1964; Sandberg et al., 1965). Moreover, significant differences in potency exist within the members of the same prostaglandin series. The diversity of effects of prostoglandins is so great that not only different prostaglandins but

also the same prostaglandin may show opposed effects when tried *in vivo*. For example PGE at high concentration is a potent inhibitor of lipolysis and c-AMP synthesis but at low concentrations it increases lipolysis and c-AMP concentration (Bergstrom et al., 1966). Apart from these difficulties, the situation seems further complicated in indomethacin injected rats since indomethacin may inhibit both prostaglandin synthesis as well as prostaglandin action.

It may be concluded therefore that our experiments suggest that liver biopsy as well as partial hepatectomy causes metabolic changes in the animal as a whole. These changes are reversed in about 12 hours after biopsy but persist much longer after partial hepatectomy. The liver remnant left after two-thirds hepatectomy compensates at least partially for the lost-tissue by increasing its metabolic activity. This metabolic stress presumably provokes the synthesis of prostaglandins which in turn provoke growth in the liver remnant left after two-thirds hepatectomy.

4. SUMMARY

4.0 SUMMARY

1. Attempts have been made to use clearance of bromsulphalein or of alcohol to estimate the growth of the liver fragment after partial hepatectomy.

2. The liver's ability to clear an injected dose of bromsulphalein is not affected by biopsy but is diminished to about 45 per cent of the control value after partial hepatectomy. A similar reduction is produced by fasting or induction of diabetes by administration of alloxan.

3. The liver's ability to clear an injected dose of ethanol is not affected by biopsy but is reduced to almost zero fifteen minutes after partial hepatectomy.

4. A comparison has been made of the composition and metabolism of the liver remaining after either two-thirds hepatectomy or removal of a small biopsy.

5. The wet weight, dry weight and total protein content of the remaining liver fragment show no change for the first twelve hours after partial hepatectomy. Thereafter they increase together at a rate which remains roughly constant for the next twenty-four hours. DNA synthesis, measured by [^{3}H]-thymidine incorporation, starts to increase sixteen hours after the operation, reaches a peak at twenty-four hours and thereafter declines sharply. The total DNA content of the liver fragment remains unaltered for sixteen hours after the operation but thereafter increases steadily for the next twenty hours.

6. The mean cell mass (wet and dry) and the protein content per cell fall slightly in the first four hours after partial hepatectomy then rise to a maximum at about twelve hours, returning to the pre-operative level at twenty hours. Biopsy produces similar but less marked changes.

7. Both partial hepatectomy and liver biopsy result in a precipitate loss of glycogen from the remaining liver cells followed by a slow recovery. The loss is slightly greater, and the recovery slightly slower, after partial hepatectomy. The lipid content per cell increases markedly after partial hepatectomy and the RNA per cell falls. Neither of these is affected by removal of a biopsy.

8. In the anaesthetized laparatomized rat the liver secretes glucose and lipids into the blood flowing through it. At the same time it takes up phospholipids, proteins, ammonia and oxygen.

9. The immediate effects of biopsy are to increase glucose output, change output of lipids to rapid uptake, decrease the uptake of proteins and increase the uptake of oxygen by the liver. There is no effect on the uptake of ammonia and phospholipids. These changes however are soon reversed, and by eight to twelve hours the uptake and release of metabolites by the liver is much as it was before the operation.

10. The effects of partial hepatectomy differ from those of biopsy in the following respects.

a) The output of glucose decreases instead of increasing and, after returning to normal between eight to twelve hours, it decreases again.

b) The uptake of lipids is significantly less, but the return to normal is much slower.

c) The uptake of phospholipids is less.

d) The uptake of proteins (after the initial decrease at four hours) is very much greater.

e) The uptake of ammonia shows erratic changes; but there is a steady increase in the ammonia concentration in the hepatic venous blood.

f) The uptake of oxygen by the liver fragment is as great as that of the liver after biopsy, in spite of the fact that the liver fragment amounts to only one third of the intact organ.

11. The consumption of oxygen <u>in vitro</u> by liver slices has been measured. Slices from the fragment remaining after partial hepatectomy show a substantially greater uptake than slices from normal liver. The uptake is maximal (twice normal) at sixteen hours and thereafter starts to decline. Slices from a liver from which a biopsy has been taken show only a small increase in uptake at four hours and thereafter return to normal.

12. If partial hepatectomy is performed on a fasted rat, the increase in lipid concentration in the liver and the peak of DNA synthesis occur about eight hours later than is the case if the operation is performed on a fed animal.

13. The response to partial hepatectomy, in terms of increase in liver remnant weight, increase in lipid concentration and increase in rate of DNA synthesis, is not modified if the time of day at which the operation is carried out is varied.

14. Intraperitoneal injections of a commercial amino-acid mixture supplemented with glucose into rats produce a small decrease in the mean cell mass and the content of RNA per cell in the liver.

15. Partial hepatectomy performed on rats made diabetic by previous administration of alloxan is followed by liver regeneration comparable, in terms of increase in liver mass and DNA content, to that elicited in healthy animals.

16. A single intraperitoneal injection of indomethacin four hours before partial hepatectomy results in a delay of the peak of DNA synthesis and a reduction in the increase of liver remnant weight. Multiple injections of indomethacin effectively inhibit both.

5. MATERIALS AND METHODS

5. MATERIALS AND METHODS

5.1 Animals

Male albino rats of the Wistar strain, aged between nine to twelve weeks were used. They were obtained from the departmental colony and were maintained on conventional rat feeding pellets (manufactured by Labsure, Christopher Hill Group). The rooms in which they were maintained were illuminated by fluorescent lighting from 0600 hours to 1800 hours daily. Food and water were available ad libitum unless otherwise stated.

5.2 Surgical procedures

5.2.1 Partial hepatectomy

This was performed by the method of Higgins and Anderson (1931), under ether anaesthesia. A midline abdominal incision was made from about 1 cm above the xiphoid process to about 2 cm below. Using gentle pressure on the lower part of the thorax and the upper part of the abdomen, the median and left lateral lobes of the liver were delivered through the incision. A loop of stout linen thread was passed around these lobes and secured in position. The ligature was tightened by double knots and the lobes were cleanly excised. The abdominal wound was closed in one layer with interrupted sutures. The animals were allowed to recover from the anaesthetic. They were fully conscious within ten minutes. The operation was well tolerated with rare mortality. This procedure effects the removal of approximately two-thirds of the liver.

5.2.2 Liver biopsy

The procedure was similar to that of partial hepatectomy, but the loop of thread was passed around the left radicle of the median lobe at the pedicle joining the radicles. The ligature was tightened and the left radicle excised. The remaining liver was returned to the body cavity and the wound closed in one layer by interrupted sutures. This effected the removal of less than 10 per cent of the total liver tissue.

5.2.3 Ligation of liver lobes

Ligation of the liver lobes was achieved by the same procedure as partial hepatectomy except that the ligated lobes were not excised. The body cavity was opened and the liver delivered through the opening. A loop of stout thread was passed round the lobes to be ligated and the ligature tightened. This effectively caused the physiological disconnection of these lobes from the remainder of the liver. Slip knots were used to tighten the ligature so that they could easily be undone. After 10 or 15 minutes the knots were undone, the thread removed and the liver returned to the body cavity. The wound was closed in one layer with interrupted sutures.

5.2.4 Ligation of veins

The required vein was carefully exposed by dissection under ether anaesthesia. A loop of thread was then passed round it and knotted tightly.

5.2.5 Blood sampling

Blood was collected under ether anaesthesia. For samples from the tail vein the tail of the rat was immersed in water at about 45^{o} c for a few minutes to produce vasodilatation. Gentle pressure was then applied at the base of the tail to make the veins prominent. A 25-gauge hypodermic needle was used to penetrate the vein. The pressure at the base of the tail was released and the blood gently drawn in the syringe.

To obtain samples from the external jugular vein, an anterio-posterior incision, about 2 cm long,was made over the vein, which was then carefully exposed. A 21-gauge hypodermic needle was used to penetrate the vein and the blood gently drawn into a syringe.

To obtain samples from the portal vein, the posterior vena cava, and the dorsal aorta, the abdomen was opened, the vessels were exposed by blunt dissection and blood withdrawn using hypodermic needles varying from 25 gauge to 18 gauge. To obtain samples of hepatic venous blood, the posterior vena cava was ligated just above the level of the renal veins so that beyond the ligature it drained only the liver. A 21-gauge needle was immediately inserted into this part of the posterior vena cava and the required sample withdrawn without delay.

For simultaneous sampling of blood from different veins, hypodermic needles connected to nylon cannulae were used. The cannulae were attached to syringes which were strapped together in such a way that both plungers could be worked simultaneously.

5.2.6 Injections

Intravenous injections were made under light ether anaesthesia either into the external jugular vein or the tail vein. The procedure for inserting the hypodermic needles was the same as that for blood sampling. Intraperitoneal injections were given without anaesthetic. A 25-gauge hypodermic needle was used. The needle was inserted diagonally from the lower left quadrant of the abdomen upwards and the injection completed.

5.2.7 Blood pressure measurement

Portal blood pressure was measured under ether anaesthesia with the rat lying on its back. The portal vein and the external jugular vein were exposed by dissection. The pressure in the two veins was

measured using a manometer consisting of two nylon cannulae of 0.45 mm internal diameter, filled with heparinized saline and fitted with a vertical scale. The cannulae were attached to 21-gauge hypodermic needles which could be inserted into the veins. The levels of saline in the cannulae represented the pressure in the veins. Since the absolute portal blood pressure varies with the posture of the animal, the pressure in both veins was measured.

5.2.8 Porto-caval shunt

This was established using a nylon cannula of 1.25 mm internal diameter. The cannula was filled with heparin and connected to 18 gauge hypodermic needles at either end. The abdomen was opened and the portal vein and the posterior vena cava were exposed by blunt dissection. The cut edges of skin and muscles were clamped to stop bleeding. One needle was inserted in the posterior vena cava in the direction of blood flow and the other was inserted in the portal vein against the blood flow. The portal vein was then ligated tightly over the needle.

5.2.9 Removal and storage of liver tissue

Portions of liver were removed under ether anaesthesia, quickly blotted free of excess blood and immediately frozen over dry ice. The procedures were clean but not aseptic. If analysis were not performed the same day, the tissue was stored at $-70^{\circ}C$ until analysed.

5.3 Tissue analysis

5.3.1 Estimation of liver weight

To determine wet liver weight the liver tissue was cleanly excised, quickly blotted free of excess blood and either weighed immediately or frozen over dry ice and weighed in the frozen state.

To determine dry liver weight, fresh liver was chopped up and a few pieces, each of about 1 g, weighed and placed in a hot oven at 100°C for twenty-four to forty-eight hours and the dry residue weighed. At least three to five pieces were treated from each liver and the results averaged.

In some cases it was desirable to obtain some estimate, however approximate, of the wet liver weight in the living animal. Two methods of calculating this were employed.

a) Where animals were partially hepatectomised, it was assumed that the weight of the entire liver was 1.5 times the weight of excised lobes.

b) In the case of intact animals it was assumed that the liver accounted for 3.35 per cent of the body weight.

5.3.2 Extraction of RNA and DNA from liver

The procedure used to extract these tissue components was as described by Fleck and Munro (1962).

Reagents:

Perchloric acid	0.6M and 0.2M
Potassium hydroxide	0.3M
Chloroform/ethanol mixture	60:40 (v/v)

About 1 g of liver was homogenized in twenty volumes of ice-cold distilled water in a chilled homogenizer for about three minutes. 5 ml of this homogenate (equivalent to 250 mg wet liver weight) was mixed well with 2.5 ml of 0.6 M perchloric acid. The mixture was allowed to stand for ten minutes in an ice-bath and then centrifuged for ten minutes at 3000 r.p.m. The supernatant was discarded and the precipitate washed twice with 5 ml of ice-cold 0.2M perchloric acid and twice with 5 ml of chloroform/ethanol mixture. After the second wash, the tubes were briefly inverted over a filter paper to drain off the remaining chloroform/ethanol mixture. After this treatment, 4 ml of potassium

hydroxide were added to the sediment and the mixture incubated in a water bath at 37°C for two hours. The tubes and their contents were then cooled in an ice-bath and 5 ml of ice-cold 0.6M perchloric acid were added. After ten minutes in the ice-bath, the mixture was centrifuged and the precipitate washed twice with 5 ml of 0.2M perchloric acid. The supernatant and the two washings were combined and 10 ml of 0.6M perchloric acid were added. The volume was then made up to 100 ml with distilled water. This gave the RNA fraction in 0.1M perchloric acid.

The precipitate was dissolved in 5 ml of potassium hydroxide and transferred to a 50 ml cylinder. After the addition of 12 ml of potassium hydroxide, the volume was made up to 50 ml with distilled water. This gave the DNA fraction in 0.1M potassium hydroxide.

5.3.3 Estimation of RNA in the extract

This was estimated in the perchloric acid extract by the method described by Hutchison and Munro, (1961) and Fleck and Munro (1962). The extinction of the solution was measured at 260 nm in a Unicam SP500 spectrophotometer. An extinction of 1.00 = 2.94 ug RNA - phosphorus/ml.

5.3.4 Estimation of DNA in the extract

The DNA content of the DNA fraction was estimated by the method of Ceriotti (1952; 1955).

Reagents:

Indole reagent	0.04 per cent in distilled water
Hydrochloric acid	concentrated, specific gravity 1.19
Chloroform	Analytical reagent grade
DNA standard	16 ug/ml. Stock standard solution was prepared by dissolving 20 mg of calf thymus DNA in 50 ml distilled water with the aid of a little NaOH. This solution was then diluted twenty-five fold to give a standard solution for routine use.

2 ml of the DNA fraction, 1 ml of indole reagent and 1 ml of hydrochloric acid were thoroughly mixed in a ground glass stoppered test-tube and placed in a boiling water bath for ten minutes. The tubes were then cooled in running water and the mixture extracted three times with 4 ml of chloroform. The chloroform layer was discarded and the extinction of the aqueous layer was read at 490 nm in a Unicam SP500 spectrophotometer. A standard and blank were also prepared in the same way using standard DNA solution and distilled water.

5.3.5 Estimation of DNA-phosphorous in the DNA fraction

This was determined as described by Griswold et al., (1951).

Reagents:

Sulphuric acid	10M
Perchloric acid	4M
Ammonium molybdate	2.5 per cent w/v
Phosphate standard	2 µg P/ml This was prepared by dissolving439 mg of KH_2PO_4 in 100 ml of distilled water. This solution was diluted 500 fold to give a standard solution for routine use.
Reducing agent	5.44 g sodium metabisulphite, 0.4 g sodium sulphite and 0.1 g 2-napthol-1-amino-4-sulphonic acid in 100 ml distilled water.

An aliquot of the aqueous DNA fraction was evaporated to dryness at 100 °C. To the evaporated residue were added 0.5 ml of sulphuric acid and 0.5 ml of perchloric acid. The mixture was digested in a

heater block until it was completely clean and colourless. 1 ml of standard DNA solution, 1 ml of phosphate standard and 1 ml of water were treated the same way to give standard and blank readings. The tubes were then cooled and diluted to 3 ml. To this dilute solution 0.5 ml of reducing agent and 0.5 ml of ammonium molybdate were added with thorough mixing after each addition. The solutions were made up to 5 ml with water and placed in a boiling water bath for ten minutes. The extinction was then read at 820 nm in a Unicam SP500 spectrophotometer.

5.3.6 Estimation of [^{3}H]-thymidine incorporation into DNA

Reagents:

Sucrose solution	0.25M
Magnesium Chloride	1.0 mM
Perchloric acid	0.6M and 0.2M
Potassium hydroxide	0.3M
Ether/ethanol	40:60 (v/v)
Scintillant	Triton and toluene were mixed in 1:2 (v/v) ratio and 8 gm/l 2,5 diphenyl oxazole and 0.8 gm/l 1,4-di[2-(5-phenyloxazolyl)]-benzone were added to this mixture.

Liver tissue (about 3 to 5 g) was homogenised in twenty volumes of a 1:1 (v/v) mixture of sucrose and magnesium chloride in a chilled homogenizer for about three minutes. 5 ml of this homogenate was mixed well with 2.5 ml of ice-cold 0.6M perchloric acid and after standing in an ice-bath for ten minutes the mixture was centrifuged at 3000 r.p.m. for ten minutes. The supernatant was discarded and the precipitate was washed twice with 5 ml of 0.2M perchloric acid and then twice with 5 ml of ether/ethanol mixture. After this treatment the precipitate was

dissolved in 4 ml of potassium hydroxide and the mixture was incubated at 37° c for two hours. The tubes were then cooled in an ice-bath and 5 ml of ice-cold 0.6M perchloric acid was added. After ten minutes the tubes were centrifuged at 3000 r.p.m. for ten minutes. The precipitate was dissolved in 2.5 ml of potassium hydroxide. 1.0 ml of this solution was added to 3 ml of scintillant and the radioactivity was measured in a Phillips liquid scintillation analyser over four minutes.

5.3.7 Extraction of lipids from liver

Lipids were extracted from the liver tissue by the method of Folch et al., 1957).

Reagents:

Chloroform/Methanol mixture	2:1 (v/v)
Pure solvent upper phase (PSUP)	This was prepared by mixing chloroform, methanol and water in 8:4:3 (v/v) ratio respectively in a separating funnel. The mixture was allowed to stand to obtain a biphasic system. The two phases were collected separately and stored in glass bottles.

The liver tissue (about 2 g) was homogenised in ten volumes of chloroform/methanol mixture. After standing for a few minutes the mixture was centrifuged at 3000 r.p.m. for ten minutes. The supernatant was transferred to another tube and the precipitate was washed twice with half the original volume of chloroform/methanol mixture. The washings were added to the supernatant and the precipitate was discarded. This procedure gave a crude lipid extract in chloroform/methanol mixture.

The crude extract was mixed thoroughly with one-fifth of its volume of water and the mixture allowed to separate into two phases. As much of the upper phase as possible was removed by siphoning. The interface was rinsed three times with small amounts of PSUP without disturbing the lower phase. The lower phase and the remaining rinsing fluid was made into one phase by the addition of methanol. This constituted the lipid extract.

5.3.8 Estimation of total lipids in the extract

These were determined by the method described by Paul (1958).

Reagents:

Chromic acid	8.5 g $K_2Cr_2O_7$ was dissolved in the minimum amount of water and the volume made up to 500 ml with concentrated H_2SO_4.
Lipid standard	10 mg stearic acid was dissolved in 10 ml of chloroform/methanol 2:1 (v/v) mixture.

0.5 ml of lipid extract or lipid standard w evaporated to dryness in a 70°C water bath. 3.0 ml of chromic acid was added to the residue and the mixture placed in a boiling water bath for fifteen minutes. The tubes were then cooled and 3 ml of water added. The extinction of each solution was read in a Unicam SP500 spectrophotometer at 620 nm against a blank prepared in the same way using chromic acid and water.

5.3.9 Estimation of phospholipids in the extract

These were determined by the method of Allen (1940).

Reagents:

Sulphuric acid	10M
Hydrogen peroxide	30 per cent (v/v)
Ammonium molybdate	8.3 per cent (w/v)
Phosphate standard	2 ug P/ml (KH_2PO_4)
Amidol reagent	1 g of 2-4 diaminophenol hydrochloride was dissolved in 100 ml of 20 per cent (w/v) solution of sodium metabisulphite

0.5 ml of lipid extract was evaportated to dryness at 70^{o} c. To the residue 1.2 ml of sulphuric acid and .25 ml hydrogen peroxide were added. The mixture was heated in a heater block at 200^{o}c until completely clear and colourless. 5 ml of water, 2 ml of amidol reagent and 1 ml of ammonium molybdate were added. After thorough mixing, the solution was made up to 25 ml with distilled water. The extinction of this solution was read against a blank in a Unicam SP500 spectrophotometer at 720 nm.

A standard curve for phosphate estimation was prepared by using serial dilutions of standard phosphate solution and treating them the same way.

5.3.10 Extraction and estimation of liver glycogen

Liver glycogen was extracted and estimated by the method of Carroll et al., (1956).

Reagents:

Trichloroacetic acid	5 per cent (w/v)
Ethanol	95 per cent (v/v)
Glucose standard	50 mg/l glucose in saturated benzoic acid.

Anthrone reagent	50 mg anthrone and 10 g thiourea were dissolved in 1 litre of 72 per cent (v/v) sulphuric acid.

Liver tissue (about 2 g) was homogenised in twenty volumes of trichloroacetic acid and after standing for five minutes the homogenate was centrifuged. The supernatant was decanted and the pellet washed three times with half the original volume of trichloroacetic acid. The mixture was centrifuged and the washings were added to the supernatant. To 1 ml of this mixture 5 ml of ethanol were added and after standing for three hours at 37^{o} the precipitate was centrifuged down at 3000 r.p.m. for fifteen minutes. The supernatant was drained and the tubes were left inverted over a filter paper for a few minutes. The glycogen pellet was then dissolved in 2 ml of distilled water. 10 ml of anthrone reagent were added with vigorous shaking. The tubes were placed in a boiling water bath for fifteen minutes and then cooled in running water. 2 ml of glucose standard and 2 ml of water were treated the same way to give standard and blank. The extinction of all solutions was read against the blank at 620 nm in a Unican SP500 spectrophotometer.

5.3.11 Estimation of total protein in liver

Liver protein concentration was determined by the method of Lowry et al., (1951).

Reagents:

Folin-Ciocalteu reagent	Diluted with water to give a solution 1N in acid.
Standard protein	Bovine serum albumin 1 mg/ml
Alkaline copper solution	This was prepared by mixing the following two solutions in the ratio of one volume of

solution (a) to fifty volumes of solution (b) (v/v).

(a) 5 per cent (w/v) cupric sulphate in 1 per cent (w/v) sodium tartrate.

(b) 2 per cent (w/v) sodium carbonate in 0.1M sodium hydroxide.

A weighed sample of liver (about 0.5 g) was homogenized in fifty volumes of ice-cold distilled water. This homogenate was diluted five-fold with ice-cold distilled water. 0.1 ml of the diluted homogenate was mixed well with twenty volumes of alkaline copper solution and allowed to stand for ten minutes. 0.1 ml of Folin-Ciocalteu reagent was then pipetted rapidly into the mixture with thorough agitation. An aliquot of distilled water was treated in the same way to give a reagent blank. The extinction of the samples was read against the blank at 750 nm in a Unicam SP500 spectrophotometer. A standard curve was prepared using serial dilutions of standard protein and treating them in the same way.

5.3.12 Estimation of oxygen consumption by liver

A water-jacketed oxygen electrode (Rank Bros Bottisham, Cambridge, U.K.) was used.

Reagents:

Phenazine methosulphate(PMS)	0.5 mg/ml
NADH	3.23 mM/100 ml
Catalase	52000 u/ml, 867 Kat/ml
Krebs enriched medium	Composition given as appendix IV.

All estimations were made at 37^{o} c in Krebs enriched medium at pH7.2. The electrode was attached to a chart recorder and calibrated by using 3.8 ml Krebs medium, 1 ml PMS, 75 µl catalase and 25 µl NADH. The following reactions take place.

i) $PMS + NADH + H^{+} \longrightarrow PMSH_2 + NAD^{+}$

ii) $PMSH_2 + O_2 \longrightarrow PMS + H_2O_2$

iii) $H_2O_2 \longrightarrow H_2O + {}'/2\ O_2$

$NADH + H^{+} + {}'/2O_2 \longrightarrow NAD^{+} + H_2O$

Since one molecule of NADH is oxidized for each atom of oxygen reduced to water it is possible to estimate the oxygen content of Krebs enriched medium and calibrate the chart using NADH.

A small piece (about 200 to 300 mg) of liver was cleanly excised under light ether anaesthesia, quickly weighed, squeezed to 0.4 mm thickness (by placing between two glass plates one of which had a o.4mm deep groove) and placed in the electrode chamber which already contained 4 ml of Krebs medium. The medium was constantly stirred by magnetic stirrer. The change in oxygen concentration in the medium was recorded. At least three estimations were made simultaneously in separate electrods for each liver and the mean of these was considered to be the rate of oxygen consumption by that liver.

5.4. Blood plasma analysis

With the exception of bromsulphalein all plasma analysis was performed using enzymatic kits supplied and prepared by Boehringer Mannheim Corporation (London) Ltd.

5.4.1 Estimation of bromsulphalein in plasma

Reagents:

Sodium hydroxide	1 M
Sodium chloride	0.9 per cent (w/v)

A 0.1 ml aliquot of blood was mixed with 4 ml of 0.9 per cent saline and the mixture centrifuged at 2000 r.p.m. for ten minutes. The supernatant was carefully decanted and 0.2 ml of sodium hydroxide was added. The extinction of this solution was read at 580 nm in a Unicam SP500 spectrophotometer. 0.2 ml of sodium hydroxide in 4 ml of saline was used as blank.

5.4.2 Estimation of alcohol in plasma

This was determined enzymatically by the method of Bucher and Redetzki (1951).

Reagents:

Buffer solution containing	
Phosphate buffer	75 mM pH 8.7
Semicarbazide	75 mM
Glycine	21 mM
NAD	24 mM
Alcohol dehydrogenase	133 μkat/ml
Perchloric acid	0.33 M
Ethanol standards	0.5, 1.0, 2.0, 3.0 mg ethanol/ml.

0.5 ml of plasma or standard was mixed with 4 ml of perchloric acid and centrifuged at 3000 r.p.m. for five minutes. 0.1 ml of supernatant was mixed with 4.8 ml of buffer solution, 0.1 ml of NAD and 0.02 ml of alcohol dehydrogenase. The mixture was incubated for twenty-five minutes at 37° c and the extinction measured at 340 nm in a Unicam SP500 spectrophotometer. 0.1 ml of perchloric acid was treated the same way to give a reagent blank.

5.4.3 Estimation of ammonia in plasma

This was determined by the method of Da Fonseca-Wollheim (1973).

Reagents:

Buffer/substrate mixture containing

Triethanolamine buffer	0.15 M pH 8.6
Oxoglutarate	15 mM
ADP	1.5 mM
NADPH	0.12 mM
Glutamate dehydrogenase	13 μkat/ml

0.5 ml of plasma was mixed well with 2.5 ml of buffer/substrate mixture and after standing for ten minutes the extinction (A_1) was measured at 365 nm in a Unicam SP500 spectrophotometer. 0.02 ml of glutamate dehydrogenase was then added and after a further ten minutes the extinction (A_2) was again read at 365 nm. A reagent blank was prepared by adding 0.02 ml of glutamate dehydrogenase and 2.5 ml of buffer/substrate solution. The concentration of ammonia in the sample was calculated as follows:-

$A_1 - A_2 = \Delta A$;

(ΔA sample - ΔA blank) x 1633 = concentration μg/100 ml.

5.4.4 Estimation of glucose in plasma

This was determined enzymatically by the method of Werner et al., (1970).

Reagents:

Buffer/enzyme/chromogen mixture containing

Phosphate buffer	100 mM pH 7.0
Peroxidase	13 nKat/ml
Glucose oxidase	167 nKat/ml
Di-ammonium, 2-2' azino-bis(3-ethylbenzothiazoline-6-sulphonate)	1 mg/ml
Standard glucose	0.5 mM
Uranyl acetate	0.16 per cent (w/v) in saline.

An aliquot of plasma was deproteinized by the addition of ten volumes of uranyl acetate. After five minutes the mixture was centrifuged at 2000 r.p.m. for ten minutes. 0.1 ml of the supernatant was mixed with 2.5 ml of buffer/enzyme/chromogen mixture and after standing for twenty to fifty minutes at room temperature, the extinction was measured at 610 nm in a Unicam SP500 spectrophotometer. 0.1 ml of glucose standard and distilled water were also treated the same way to give standard and blank readings.

5.4.5 Estimation of total protein in plasma

These were determined by the method described by Weichselbaum (1946).

Reagents:

Biuret reagent

Sodium hydroxide	0.1 M
Sodium potassium tartrate	16 mM
Potassium iodide	15 mM
Cupric sulphate	6 mM
Standard protein	6 per cent (w/v)

Reagent blank

Sodium hydroxide	0.1 M
Sodium potassium tartrate	16 mM

0.1 ml of plasma or standard was mixed well with 5 ml of biuret reagent and incubated for thirty minutes at 20^{o} to 25^{o} c. The extinction of the mixture was measured against the reagent blank at 560 nm in a Unicam SP500 spectrophotometer.

5.4.6 Estimation of total lipids in plasma

These were determined by sulphophosphovannillin reaction as described by Zollner and Kirsch (1962).

Reagents:

Colour reagent containing	
Phosphoric acid	14 M
Vanillin	13 mM
Sulphuric acid	concentrated, Analytical reagent grade
Lipid standard	10 mg/ml

0.5 ml of plasma or lipid standard was thoroughly mixed with 2 ml of sulphuric acid in a test-tube, heated in a boiling water-bath for ten minutes and then cooled in running water. 0.1 ml of this digest was mixed with 2.5 ml of colour reagent. 0.1 ml of concentrated sulphuric acid was similarly mixed with 2.5 ml of colour reagent to provide a blank. After standing for thirty minutes at room temperature the extinction was measured against blank in a Unicam SP500 spectrophotometer at 530 nm.

5.4.7 Estimation of phospholipids in plasma

These were determined by the molybodate/vanadate reaction as described by Zilversmit et al., (1950).

Reagents:

Vanadate solution containing	
Ammonium vanadate	21 mM
Nitric acid	0.28 M
Molybdate solution containing	
Ammonium molybdate	40 mM
Sulphuric acid	2.5 M
Phosphorus standard	5 mg /100 ml
Trichloroacetic acid	1.2 M
Perchloric acid	70 per cent
Hydrogen peroxide	30 per cent v/v

0.1 ml of plasma or standard was mixed well with 2 ml of trichloroacetic acid and after standing forten minutes centrifuged at 3000 r.p.m. for ten minutes. The supernatant was discarded and 0.5 ml of perchloric acid and 0.2 ml of hydrogen peroxide were added to the precipitate. The mixture was digested in a heater block at 180^{o} to 200^{o} c for fifteen minutes or until the digest was clear. After the digest had cooled,2 ml of distilled water, 1 ml of vanadate and 1 ml of molybdate solution were added. Ten minutes later the extinction was measured in a Unicam SP500 spectrophotometer at 405 nm against a blank. The blank was prepared in the same way by using 0.1 ml of distilled water instead of plasma.

5.4.8 Estimation of partial pressure of oxygen in blood

This was estimated using a Corning Eel pH/ Blood Gas Analyser, Digital Model 160. The electrode consists of a silver/silver chloride reference element which acts as the anode and a fine platinum wire sealed into a glass tube which acts as the cathode (in the cell reaction). A constant voltage of 0.7 volts is maintained across these elements. Oxygen from the test sample diffuses through a polypropylene membrane and comes into contact with the platinum cathode causing a flow of electrons through the cell. The magnitude of this current is directly proportional to the partial pressure of oxygen and is displayed in digital form directly in units of mM Hg pO_2.

Appendix I

Description of solutions for injections

Bromsulphalein (BSP): 2.5 per cent w/v BSP in physiological saline. 0.5 ml of this solution was injected in the left external jugular vein.

Alloxan: 60 mg alloxan per ml in physiological saline. 0.2 ml of this solution was injected into the tail vein.

Ethanol: 3 ml of absolute alcohol was mixed with 7 ml of physiological saline. 0.5 ml of this solution was injected into the left external jugular vein.

Vamin: Vamin is a hydrolysate of chick albumin (complete chemical composition is attached as appendix III). 4 ml Vamin was injected intraperitoneally every four hours. Each rat received twelve injections.

Indomethacin: 5 mg (w/v) indomethacine in propane 1,2 diol. The injections were given intraperitoneally.

Appendix II

List of Reagents.

The following reagents were obtained from British Drug Houses, Ltd., England:

Alloxan, 2-4-di-Aminophenol hydrochloride, Ammonium molybdate, Antherone, Cupric sulphate, Folin-Ciocalteu, Glucose, Hydrochloric acid, Hydrogen peroxide, Indole, Magnesium chloride, Magnesium sulphate, 2-Napthol-1-amino-4-sulphonic acid, Perchloric acid, Potassium chloride, Potassium dichromate, Potassium dihydrogen orthophosphate, Potassium hydroxide, POPOP, Sodium bicarbonate, Sodium carbonate, Sodium chloride, Sodium dihydrogen orthophosphate, di-sodium orthophosphate, Sodium fumarate, Sodium glutamate, Sodium hydroxide, Sodium metabisulphite, Sodium tartrate, Stearic acid, Sucrose, Sulphuric acid, Thiourea, Trichloroacetic acid and Uranyl acetate.

The following reagents were obtained from Sigma Chemical Company, England: Bromsulphalein, Deoxyribonucleic acid, Indomethacin and Phenozine methosulphate.

The following reagents were obtained from May and Baker Ltd., England:

Chloroform, Ethanol, Ether and Methanol.

The following reagents were obtained from the sources listed:

Appendix II (cont'd.)

Reagents	Source
Bovine serum albumin	Pentex Incorporated.
Catalase	Worthington Biochemical Corp., Freehold, New Jersey.
Heparin	Evans Medical Ltd.
NADH	Boehringer Mannheim Corp., Ltd.
PPO	Koch Light Laboratory Ltd.
Toluene	Koch Light Laboratory Ltd.
Triton	Rohm and Haas Ltd.
[^{3}H]-thymidine	Radiochemical Center, Amersham.

Appendix III

Composition of Vamin

Contents	Concentration
L-Alanine	3.00 g/litre
L-Arginine	3.30 "
L-Aspartic acid	4.05 "
L-cysteine and L-cystine	1.40 "
L-Glutamic acid	9.00 "
Glycine	2.10 "
L-Histidine	2.40 "
L-Isoleucine	3.90 "
L-Leucine	5.25 "
L-Lysine	3.85 "
L-Methionine	1.90 "
L-Phenylalanine	5.45 "
L-Proline	8.10 "
L-Serine	7.50 "
L-Threonine	3.00 "
L-Tryptophane	1.00 "
L-Tyrosine	0.50 "
L-Valine	4.25 "
Glucose	100.00 "
Nitrogen	9.4 "
Sodium ions	50 mmol
Potassium ions	20 "
Calcium ions	2.5 "
Magnesium ions	1.5 "
Chloride ions	55 "

Manufactured by Vitrum, Stockholm, Sweden.

Kabivitrum Ltd., Ealing W5 2TH, PL0022/0030.

Appendix IV

Composition of Krebs enriched medium

This was prepared by mixing the following reagents in the proportions stated.

Concentration per cent	Substance	Content
0.9 (w/v)	NaCl	83 parts by volume
1.15 "	KCl	4 "
2.11 "	KH_2PO_4	1 "
3.82 "	$MgSO_4 \cdot 7H_2O$	1 "
1.3	$NaHCO_3$	3 "
[1.78 "	$Na_2H\ PO_4 \cdot 2H_2O$ (100 parts)]	18 parts
[1.38 "	$Na\ H_2PO_4 \cdot H_2O$ (25 parts)]	by volume
1.41 "	Sodium pyruvate	4 parts by volume
1.16 "	Sodium fumarate	7 "
3 "	Sodium L-glutamate	4 "
5.4 "	glucose	5 "

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6. BIBLIOGRAPHY

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