Chemical Aspects of Compensatory Hyperplasia

by

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A C K N O W L E D G E M E N T S

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C O N T E N T S

INTRODUCTION

INTRODUCTION.

Section I.0 Historical.

It was discovered in the latter part of the last century by several workers that surgical damage to the liver was followed by a process of repair (Colucci 1883; Tizzoni 1883). For example von Podwyssozki (1886) removed small wedge-shaped areas from the livers of rats and rabbits. Within two and a half days of the operation, mitoses were observed in the hepatic cells adjacent to the wound and often some considerable distance away from it. Later workers excised complete lobes of the liver and discovered that this was followed by very rapid growth of the remainder. Ponfick (1889; 1890) succeeded in removing over two thirds of the liver in rabbits and dogs. Within a few weeks he observed a threefold increase in the size of the remaining fragment. He interpreted this as a response to the hepatic insufficiency which he had brought about. These observations were confirmed by von Meister (1894), who further discovered that a single animal could be subjected to repeated partial hepatectomy, and that on each occasion the residual fragment would grow to the size of the original intact organ. At a later date, Fishback (1929) was able to corroborate this finding in dogs. He also found that growth was more rapid when large amounts of

tissue were removed. Eventually the liver could be pared down to one lobe by repeated hepatectomy, and only technical difficulties concerned with haemostasis, seemed to prevent indefinite repetition of the process. The mitotic response which followed removal of an entire lobe or lobes, unlike that following removal of a small wedge, occurred diffusely throughout the liver.

Other forms of damage to the liver were followed by a similar sort of repair. Rous and Larimore (1920) showed that in the rabbit, ligation of the branch of the portal vein supplying one of the liver lobes caused atrophy of that lobe and that this was followed by hypertrophy of the other lobes the portal blood supply to which was unimpaired. Whipple and Sperry (1909) produced varying degrees of necrosis in the livers of dogs by prolonged chloroform anaesthesia. The necrosis was confined to the areas in the hepatic lobule around the central vein. Necrotic debris was carried off by wandering cells. The undamaged hepatic cells multiplied so rapidly by mitosis that regeneration was usually completed by the 11th to 21st day. In later experiments, Davis and Whipple (1919; 1921) studied the influence of diet on the rate of regeneration after chloroform-induced liver necrosis and found that diets rich in

carbohydrate or in protein were better than their standard diet, which in turn was better than a diet rich in fat. The poorest response of all was obtained in fasted animals.

By about 1921 therefore, it had become clear that, in general, damage to or removal of a substantial part of the liver is followed by a diffuse compensatory hyperplasia throughout the surviving liver tissue. This process of repair is usually called "liver regeneration", which is really a misnomer. If a lobe of the liver is removed, a new lobe does not form at the site of the wound. Instead, the other intact lobes all increase in size and cell number, to an extent necessary to make good the deficiency. This process can best be described as compensatory hyperplasia. However, the expression "liver regeneration" is so well established that it has become hallowed by usage and will be used throughout the following pages to denote the restoration process which follows partial hepatectomy or extensive liver damage.

Figure 1

The restoration of moist liver weight and dry liver weight per unit body weight following partial hepatectomy (from Higgins & Anderson, 1931).

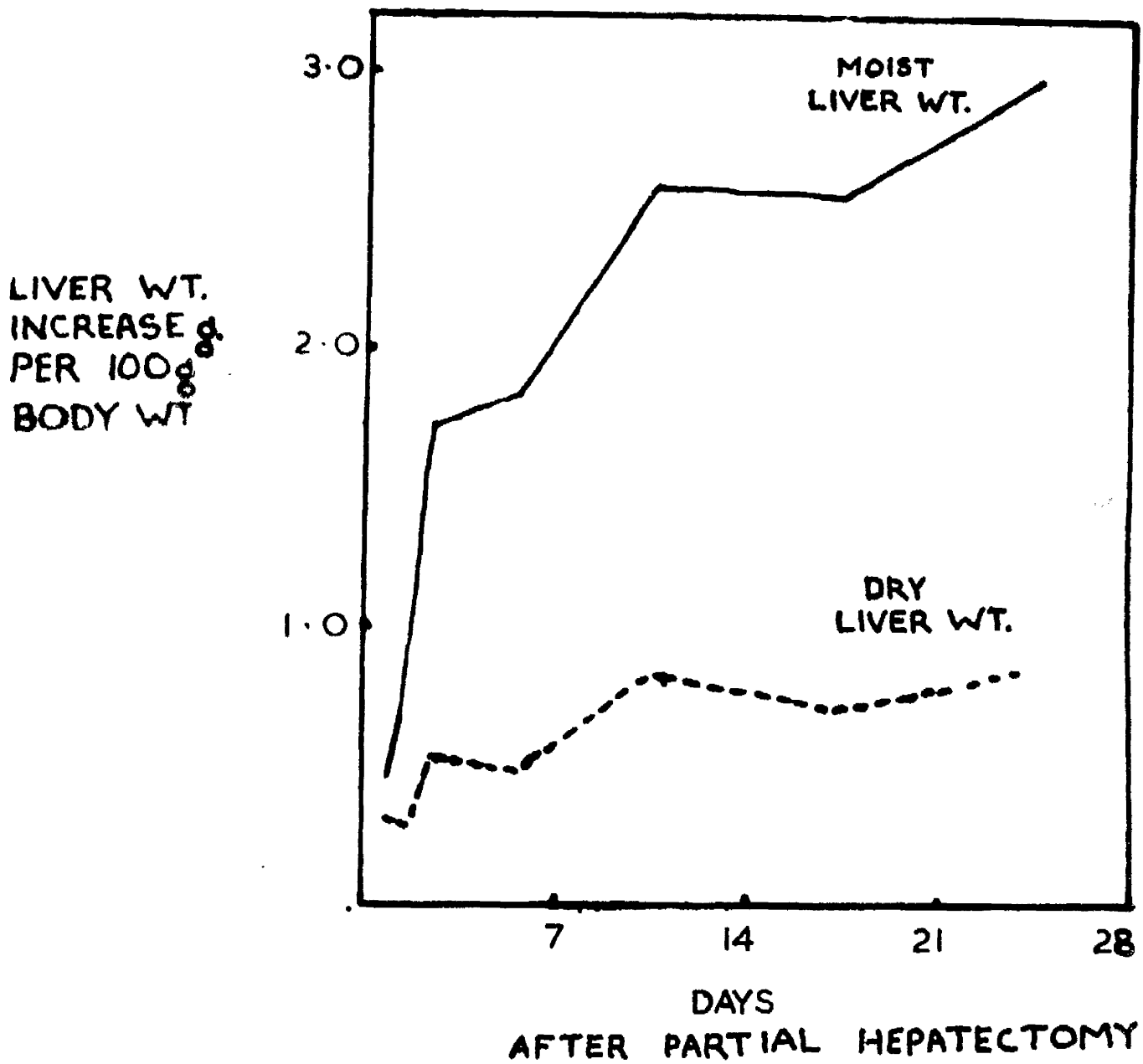

LIVER WT. INCREASE g. PER 100g BODY WT
3·0
2·0
1·0
MOIST LIVER WT.
DRY LIVER WT.
7
14
21
28
DAYS AFTER PARTIAL HEPATECTOMY

FIGURE 1

Section I.I Increase in size and cell number.

Higgins and Anderson (1931) were the first investigators to make a systematic study of the rate of growth in the remaining liver fragment of rats after two-thirds partial hepatectomy. They found that the 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 to four weeks, by which time the liver fragment was about the size of the original intact organ (Fig. 1.). Brues, Drury and Brues (1936) re-examined the process using slightly different methods. They noticed that during the first 10 days after partial hepatectomy the body weight of the animals fell by 10 to 15 per cent, and that this loss was subsequently regained. They corrected the increase in liver weight for variation in body weight and found that a much smoother curve was obtained than that obtained by Higgins and Anderson (Fig. 2.). Apart from the secondary increase in the second week, the curve of restoration of liver size followed the course of a process which begins at a maximal rate and is progressively retarded as the final size is reached.

In contrast to liver mass, cell number showed no

FIGURE 2

The restoration of liver mass and cell number following partial hepatectomy. The upper curve represents growth in liver mass (moist weight) corrected for changes in body weight i.e., liver growth expressed as a percentage of original weight regained. The lower curve represents growth in cell number expressed as a percentage of the original cell number regained (from Brues, Drury & Brues, 1936).

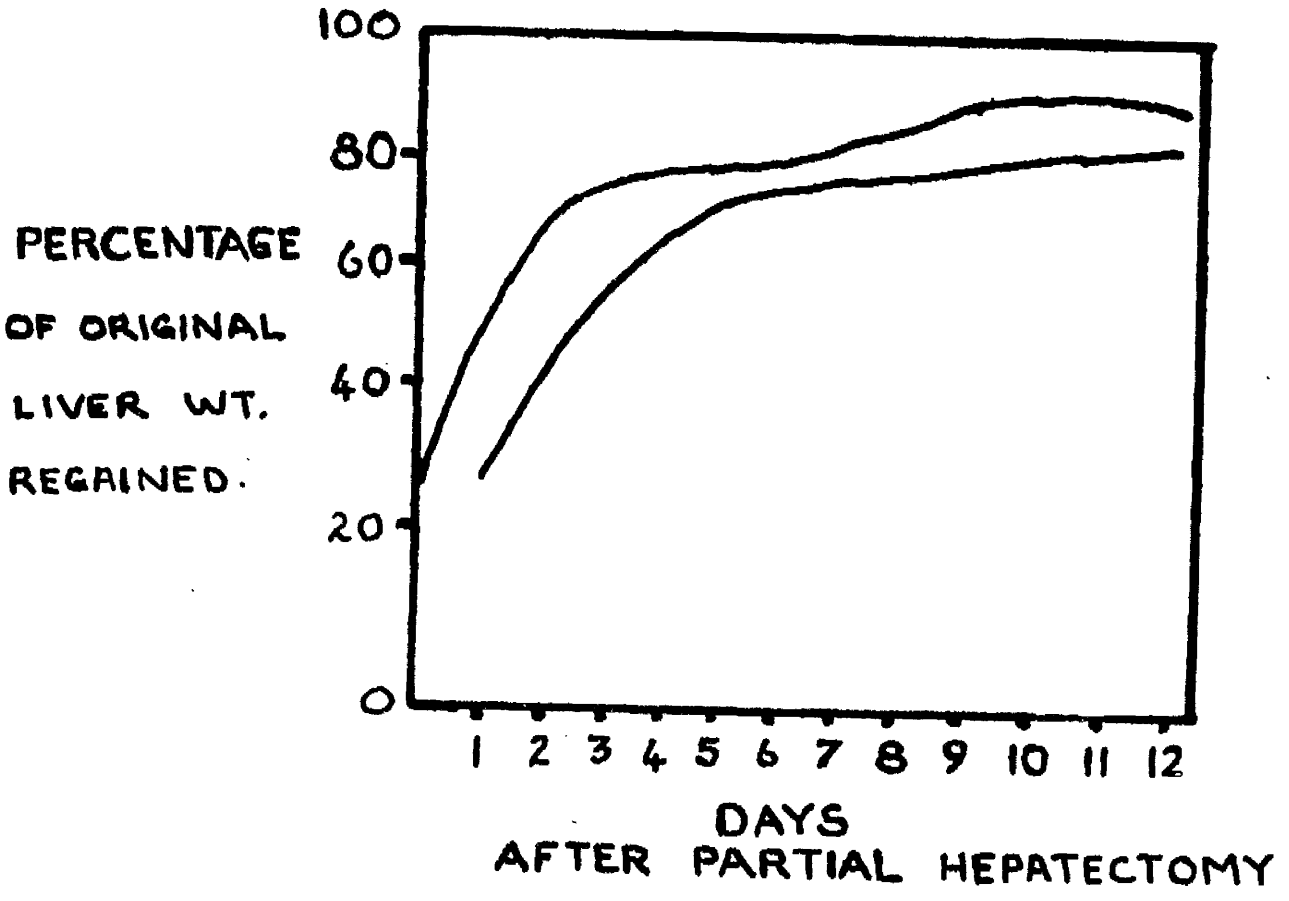

100
80
60
40
20
0
PERCENTAGE
OF ORIGINAL
LIVER WT.
REGAINED.
1 2 3 4 5 6 7 8 9 10 11 12
DAYS
AFTER PARTIAL HEPATECTOMY

FIGURE 2

by W.C. Alston, M.B.

Chemical Aspects of Compensatory Hyperplasia.

The mechanism controlling liver regeneration after partial hepatectomy has been investigated and the principal theories put forward by previous workers have been re-examined The theory that regeneration might be controlled by some "humoral" factor circulating in the blood stream was tested by injecting serum from partially hepatectomised rats into normal rats. This failed to produce any sign of growth in the livers of the recipients. Conversely, injection of normal serum into partially hepatectomised animals failed to inhibit liver regeneration. In an attempt to find a more satisfactory method of detecting the supposed humoral factor a new technique of cross-circulation was devised by means of which two rats exchanged approximately one-quarter of their blood volume per minute. When one partner in such a cross-circulation was partially hepatectomised its liver regenerated in the normal manner, but the liver of its unoperated partner showed no sign of regeneration. These experiments appear to exclude the possibility that liver regeneration is controlled by anything in the nature of a "humoral" factor.

An older theory that regeneration is a response to the residual liver fragment having to accommodate the same total blood flow as the original intact organ was tested

chiefly by surgical procedures which increased the blood flow through the livers of normal animals. None of them produced the sort of growth response in the liver which follows partial hepatectomy, so that this theory also seems to be disproved.

A third theory that plasma protein concentration might have an important role in liver regeneration, was tested by observing the fall in plasma protein level in rats after partial hepatectomy. This proved to be small and transient. On the other hand, when plasma protein levels in rats were substantially lowered by repeated plasmapheresis only a slight mitotic response was obtained in their livers. Conversely, liver regeneration in partially hepatectomised rats was not inhibited when they were supplied with extra plasma protein by intravenous infusion of normal rat plasma. It seems unlikely therefore that plasma protein levels are important in control of liver regeneration.

Investigation of the chemical changes in the remaining liver fragment after partial hepatectomy in rats showed a slight increase in sodium and water content within 10 minutes of the operation, a sharp fall in glycogen concentration at 2 hours, and accumulation of total lipid (but not phospholipid) at 6 hours and an increase in RNA per cell

at 12 hours. The rate at which the liver cleared injected bromsulphthalein was diminished in proportion to the amount of tissue removed in the hepatectomy and did not show any improvement within 48 hours of the operation.

If one partner in a pair of cross-circulating rats was partially hepatectomised, the lipid content of its liver and the RNA content per liver cell increased in the same way as in single partially hepatectomised rats but to a lesser extent. The liver of the unhepatectomised partner did not show an equivalent increase either in lipid or in RNA. On the other hand, the rate at which injected bromsulphthalein was cleared from the blood stream of a partially hepatectomise rat was substantially increased if it was linked in cross-circulation with a normal rat.

These results seemed consistent with the view that the stimulus to liver regeneration might be the inability of the remaining liver fragment to discharge the duties normally performed by the intact liver, and that cross-circulation with a normal rat is insufficient to make up for the deficienc An attempt was made to test this hypothesis by overloading the livers of normal intact rats by parenteral administration of amino acids. This produced a pronounced increase in the RNA content per cell in the liver, a less pronounced increase

in protein per cell and ultimately a substantial increase in mitotic activity. In so far as these changes resemble the changes associated with liver regeneration, they support the hypothesis that liver regeneration is a response to a functional overload.

Reference:

Alston, W.C., & Thomson, R.Y. (1963)
Cancer Res., 23 (6), 901.

change for the first 24 hours but thereafter it increased in parallel with liver mass. Brues et al., (1936) found that in starved animals, the liver hypertrophied during the first day and remained about the same size from 24 hours onward. However, the number of cells increased almost as rapidly as in animals which had been fed. Abercrombie and Harkness (1951) made a detailed study of the increase in number of different cell types in regenerating rat liver up to 21 days after partial hepatectomy. They confirmed that 24 hours after operation the total number of parenchymal cells was not significantly greater than normal, though the weight of the liver had increased considerably. Thereafter the number of parenchymal cells increased steadily, roughly in proportion to the weight increase. The increase in the total number of littoral cells, ran approximately parallel to that of the parenchymal cells during the first two post-operative days. By 21 days, the total populations of both parenchymal and littoral cells had returned practically to those of normal liver.

It might be anticipated that the increase in cell number would be associated with a high mitotic activity and this is indeed the case. In normal adult liver, the mitotic frequency is quite small; only one or two

mitotic figures can be detected per 20,000 nuclei (Brues & Marble, 1937). Abercrombie and Harkness (1951) found that this condition persisted for about 20 hours after partial hepatectomy after which there was a spectacular rise in parenchymal cell mitosis to a maximum of about 3 per cent at 24 hours. At this time interval, the mitotic rate of littoral and bile-duct cells was still very low. Two days later the mitotic rate in the parenchymal cells had dropped but it was now high in the littoral cells, bile-duct cells and also in the mesothelial lining cells. At this time, mitosis was also observed in the cells of the periportal connective tissue and of the endothelium of blood vessels other than the sinusoids.

Section I.2 Structural changes.

Since the increase in liver size during the first 24 hours after partial hepatectomy is not due to an increase in cell number, it must be attributable to an increase in mean cell mass or extracellular material. An increase in cell volume has in fact been demonstrated microscopically and shown to be associated with a larger increase in nuclear and more especially nucleolar volumes (Stowell, 1948; Harkness, 1952b). These changes were partly reversed once

increased cell division was set in train. The swelling of the parenchymal cells produced a diminution of the extra-vascular space from the normal figure of 21·2 ml./100 g., liver to a minimum of 16·8 ml./100 g., liver at 24 hours, followed by a return to normal within 48 hours. Vascular space diminished also, but the minimum was observed at 48 hours and it was still below normal at 72 hours (Harkness, 1952b).

Until quite recently, the commonly accepted view of liver structure was that the hepatocytes or parenchymal cells were arranged in more or less well-defined lobules around the central veins. It was therefore natural to enquire whether, during liver regeneration, the existing lobules simply increased in size or whether they were re-organized in such a way that their number was increased. Now that the liver is regarded rather as a muralium of cells, penetrated by portal tracts and central veins, this question has less significance (Elias, 1963). It is of interest, however, that a small increase in the number of bile ducts and centrilobular veins does occur (Abercrombie & Harkness, 1951). Collagen growth is very slow but the intralobular reticulin fraction regenerates rather faster (Harkness, 1957).

The increase in cell number in regenerating liver must however, be accompanied by some form of reorganization so that hepatocytes, fibrocytes, Kupffer cells and the other cell types are in the correct spatial relationship. It might be anticipated that this would be reflected in changes in the way in which these cells behave in tissue culture. Such changes have indeed been reported. Abercrombie and Harkness (1951) and Glinos (1949) observed a greater out-wandering of cells in explants from regenerating than in those from normal livers. A change in the electrophoretic mobility of liver cells has also been reported (Eisenberg, Ben-Or, & Doljanski, 1962).

Section I.3 Chemical changes in the liver fragment.

Section I.30 Introduction.

The chemical changes which occur in the liver fragment during regeneration are not easy to interpret. The difficulty arises partly from the fact that the liver consists of a variety of cell types. Parenchymal cells, which account for 90 per cent of the liver volume, constitute only 60 per cent of the cell number. Moreover, the liver contains substantial but highly variable amounts of glycogen and fat. Finally, marked changes occur in

vascular and extravascular space during liver regeneration (see Section I.2). It is evident, that a change in any of these quantities or in the properties of the different cell types might produce changes in the overall composition of the liver which would not necessarily have any immediate relationship to the regeneration process.

To some extent, the problem can be met by judicious selection of the form in which analytical results are expressed. The common practice of expressing the amount of some tissue component as so many mg., per unit wet or dry weight of tissue or per unit weight of protein nitrogen is informative only so long as one can be confident that wet or dry weight of tissue or protein content represent standards which will not be affected by the phenomena under investigation. This assumption is by no means a safe one. For example, starvation causes an increase in RNA concentration per mg., wet weight of liver. This change is not due to an increase in the total amount of RNA in the liver. In fact, in rats of initial body weight about 200 g., the total amount of RNA in the liver was observed to fall by approximately 27 per cent after 72 hours of starvation; but the liver weight fell almost 40 per cent in the same time interval; hence the misleading increase in RNA concentration (Thomson, Heagy,

Hutchison & Davidson, 1953). Of course, this sort of ambiguity does not arise if the total amount of the constituent in the entire organ can be determined. This is not always possible since it requires excising the entire liver and therefore killing the animal. If, for example, only a biopsy specimen is available, some other solution to the problem has to be found. One possibility is to make use of the well established observation that in any species the DNA content per set of chromosomes is constant for all nuclei.* Consequently, the DNA content of a tissue sample can be taken as a measure of the number of cells present and by relating other components to DNA one obtains a measure of the average amount per cell. Unfortunately, in liver the situation is complicated by the fact that a large proportion of the hepatocytes are tetraploid. This complication, however, is less serious in practice than might be anticipated since it seems likely that tetraploid hepatocytes are in any case twice the size of diploid hepatocytes (Harrison, 1953). A more fundamental limitation is that in any tissue growing as rapidly as liver does in the early stages of regeneration, there must necessarily be many cells synthesising DNA in preparation for cell division, so that the average DNA per nucleus over this period will be abnormally high. Never-

* Vendrely, R. (1955).

theless, in the absence of any more reliable guide, the use of DNA as a standard remains the most useful key to understanding the chemical changes which accompany regeneration.

Section I.3I Increase in DNA

The morphological work described in Section I.2 has made it clear that, in the end, liver regeneration is achieved by an increase in cell number not cell size. In agreement with this conclusion, Thomson et al., (1953) found that the total DNA content of the liver fragment increased roughly in parallel with the increase in cell number but that the average cell content of protein, RNA and lipid phosphorus, when regeneration was complete, was similar to that of normal liver. However, during the most rapid period of growth, the average DNA content per nucleus shows a substantial though temporary increase, variously reported at 20 to 80 per cent (Price & Laird, 1950; Thomson et al., 1953; Ultman, Hirshberg & Gellhorn, 1953). This synthesis of DNA in which large numbers of cells are participating, is manifestly due to a preparation for cell division. The process of DNA synthesis has been repeatedly demonstrated by radio-isotopic methods. Hecht and Potter (1956) using ^{14}C-orotate, showed that synthesis began 18 hours after

partial hepatectomy and reached a maximum at 24 to 30 hours. These conclusions were confirmed and extended by Grisham (1962) using ^{3}H-thymidine autoradiography, which made it possible to distinguish between different cell types. By this technique Grisham showed that synthesis in the hepatocytes began at 12 to 18 hours, reached a maximum at 20 hours, and had declined by 48 hours. The corresponding wave of synthesis in the littoral and ductal cells came 6 to 12 hours later and was spread over a longer period. These observations accord well with the morphological evidence that cell division begins in the hepatocytes and is observed in the other cell types only after an interval of some hours (see Section I.I).

Section I.32 Increase in RNA.

It has been known for many years that growth (presumably because it involves increased protein synthesis) is associated with increased concentrations of RNA (Brachet, 1941; Caspersson, 1940). In the case of liver regeneration, an increase in the total amount of RNA per cell was observed by Price and Laird (1950) as early as 12 hours after partial hepatectomy, rising to 62 per cent at 24 hours. The increase appeared to involve the microsomes and cell sap

rather than nuclei or mitochondria (Price & Laird, 1950; Von Der Decken & Hultin, 1958). On the other hand, Hecht and Potter (1956) found that 24 hours after partial hepatectomy incorporation of ^{14}C-orotate into RNA was initially much more rapid into the nucleus than into the cytoplasm. Much more recently, Tsukada and Lieberman (1964a) have measured the amount of RNA synthesis both spectrophotometrically and by means of ^{14}C-orotate incorporation and found evidence of very high synthetic activity in the nucleoli of regenerating liver. Fujioka, Koga and Lieberman (1963) have also shown that an increase in the rate of RNA synthesis was demonstrable in liver immediately after partial hepatectomy, and that it reached a plateau value equivalent to twice the normal rate by 5 hours. The increased RNA synthetic rate was observed in all subcellular fractions. It would appear therefore, that after partial hepatectomy an increase in RNA synthesis appears almost immediately, followed within 12 hours by an increase in the amount of RNA per cell; DNA synthesis starts after about 18 hours and cell division at about 24 hours. It would be very interesting to unravel the relationship between the events in this sequence of changes. Lieberman's group have shown that amounts of p-fluorophenylalanine and actinomycin D which

have no effect on RNA synthesis in normal rat liver, completely suppress the increase in RNA synthesis after partial hepatectomy. Moreover, this suppression is associated with a delay in the start of DNA synthesis. It may be therefore, that the increased RNA synthesis is, in part, a preparation for DNA synthesis.

Section I.33 Free nucleotides.

Since increased polynucleotide synthesis is quantitatively one of the most important chemical features of regeneration, it is not surprising that changes in concentration of the free mononucleotide precursors should have been sought. Unfortunately, the significance of such changes is ambiguous. A fall could be attributed tc an increased conversion to polynucleotide with consequent depletion of the mononucleotide pool. An increase would be explained quite as plausibly as evidence of increased mononucleotide synthesis in anticipation of an increased demand. In practice, the deoxyribonucleosides and their derivatives accumulate after partial hepatectomy reaching a maximum at the onset of mitosis. This increase is accompanied by a change in their relative proportions; in normal liver deoxycytidine accounts for 90 per cent of this material.

In regenerating liver, up to 33 per cent is in the form of nucleotides (Schneider & Brownell, 1957). On the other hand, the concentrations of the four ribonucleoside triphosphates (ATP, CTP, GTP, and UTP) which are the immediate precursors of RNA all fall during regeneration (Mandel, Wintzerith, Klein-Pete, & Mandel, 1963).

Section I.34 Changes in protein.

Since the term protein includes all the enzymatic and part of the structural components of the cell, it is evident that changes in total protein content are less easy to interpret. Two aspects of protein synthesis in regenerating liver have to be considered, since it has to produce more proteins than are needed for its own growth. In addition to the protein required for the construction of new tissue, it must also compensate for the depletion of plasma proteins which follows partial hepatectomy. The early investigators found that the total nitrogen concentration fell during regeneration (Brues, Drury & Brues, 1936). Gurd, Vars and Ravdin (1948) found also that protein concentration per unit wet weight of tissue diminished after partial hepatectomy. This fall in concentration is attributable to the accumulation of other cell constituents such

as fat. The total amount of protein in the liver fragment shows an increase starting from the first day after operation. This net increase in protein is reflected in an increased uptake of labelled amino acids which starts to increase 12 to 14 hours after partial hepatectomy and reaches a maximum value at about 30 hours, thereafter declining (Von Der Decken & Hultin, 1958). Incorporation of labelled amino acids into plasma albumin (which is of course synthesised in liver) was observed to increase only after 72 hours, but the increase was still apparent after 144 hours (Braun, Marsh & Drabkin, 1962).

Section I.35 Changes in glycogen and total lipid.

Among the earliest and most obvious changes in regenerating liver are alterations in the cytoplasmic storage products, glycogen and neutral fat. There is a rapid drop in glycogen concentration to almost zero at 10 hours, followed by an irregular rise; even at 72 hours the value is still only about one third of normal (Harkness, 1952b). Lipid concentration rises very rapidly in regenerating liver and reaches a maximum by about 10 hours after operation; the total amount in the liver fragment at this time is very close to that present in the liver preoperatively. Ludewig, Minor, and Hortenstine (1939) who studied lipid

fractions at later time intervals, found that they were all considerably elevated after 24 hours, the greatest elevation being in neutral fat, which only returned to control value by the 7th day.

Section I.36 Changes in water content during regeneration.

There is some disagreement about changes in water content of the liver fragment during regeneration. Higgins and Anderson (1931) and Szego and Roberts (1949) both found an appreciable drop during the first 24 hours. However, Gurd, Vars and Ravdin (1948) found no change in the water content of the liver fragment after 24 hours, and Harkness (1952b) obtained a value of 70·8 per cent at 10 hours after partial hepatectomy, the value for normal liver being 71 per cent. Harkness also found an increase in the water content of the regenerating liver fragment to a value of 73·9 per cent at 70 hours.

Section I.37 Summary.

The chemical features of regeneration may be summarised in the following way. A rapid fall occurs in glycogen and is accompanied by an accumulation of neutral lipid. These changes are probably indicative of hepatic

insufficiency, but soon after they become manifest, changes indicative of growth appear. Almost immediately after partial hepatectomy, RNA synthesis is accelerated; increase in protein synthesis follows at 12 to 14 hours, and finally at 18 hours DNA synthesis is under way. The whole process culminates in the outburst of mitotic activity which starts at about 24 hours. The later stages of the regenerative process are characterised by a gradual return to normal; the rates of synthesis of RNA, protein and DNA revert slowly to the resting levels. The accumulation of neutral lipid is gradually dispersed and a normal content of glycogen is recovered.

Section I.40 Enzyme changes in regeneration.

The studies on liver composition during regeneration, detailed above, have been paralleled by similar studies on the liver enzymes. It is convenient to subdivide the descriptive account into two groups,

a) those enzymes concerned with nucleic acid synthesis and degradation,

b) those enzymes concerned with other metabolic activities.

Section I.4I Enzymes synthesising and degrading nucleic acids.

Since the association between tissue growth and nucleic acid synthesis has been established for a decade and suspected for much longer, it is hardly surprising that a great deal of attention has been given to the enzymes of nucleic acid synthesis in regenerating liver. It is well known that in the liver of the normal intact adult rat, DNA synthesis is scarcely detectable but that within 18 hours of partial hepatectomy there is a sudden increase (Hecht & Potter, 1956). It might be expected that this sudden alteration in synthetic activity would be accompanied by a corresponding change in the enzymes catalysing the synthesis both of DNA and of its precursors. This expectation has been fulfilled. For example the first enzyme in the pathway of pyrimidine biosynthesis, aspartate carbamoyltransferase*(2.1.3.2) was found to increase to a maximum of twice normal after 50 hours and then to fall to control level by the 15th day. The enzymes leading to the synthesis of uridine monophosphate (UMP) and deoxyuridine monophosphate (dUMP) were also observed to increase during liver regener-

*The number in parenthesis after each enzyme is the Enzyme Commission number.

ation (Sköld, 1960). Uridine phosphorylase(2.4.2.3) which catalyses the conversion of uracil (U) to uridine (UR) increased threefold between 36 and 73 hours, deoxyuridine phosphorylase increased fourfold after 48 hours. The synthesis of uridine monophosphate from uridine, catalysed by uridine kinase(2.7.1.f) was accelerated fivefold between 24 and 36 hours, and the synthesis of deoxyuridine monophosphate from deoxyuridine (UdR) by deoxyuridine kinase was 10 times more rapid after 36 to 48 hours. On the other hand, the incorporation of labelled orotic acid into uridine monophosphate was found to increase five to sixfold about 48 hours after partial hepatectomy. However, since this information was obtained using cell extracts, it was not possible to assess the relative efficiencies of the orotic acid pathway and the uracil pathway <u>in vivo</u>. All these enzyme activities had reverted to that of normal liver after about 6 days. The formation of deoxyuridine monophosphate (dUMP) also occurs in regenerating liver from deoxycytidine monophosphate (dCMP) through the action of deoxycytidylic acid deaminase(3.5.4.b) (Maley & Maley, 1960). This enzyme which was scarcely detectable in the liver of the normal adult rat, suddenly increased in activity 12 hours after partial hepatectomy, rose to a maximum at 48 hours, and then

declined. Thymidylate synthetase which catalyses the formation of thymidine monophosphate (TMP) from deoxyuridine monophosphate (dUMP), also essentially absent from normal adult liver, appeared about 18 hours after the operation, rose to a maximum at 30 hours and gradually declined thereafter. The next stage in the sequence of synthetic reactions is the conversion of thymidine monophosphate (TMP) to thymidine triphosphate (TTP) catalysed by specific kinases. This sequence of reactions is greatly increased in regenerating liver in contrast to the kinases involved in the phosphorylation of deoxyadenosine monophosphate (dAMP), deoxyguanosine monophosphate (dGMP), and deoxycytidine monophosphate (dCMP), which have comparable activities in normal and regenerating liver (Bollum & Potter, 1959). By 24 hours, thymidine kinase(2.7.1.21) began to increase, reaching a maximum in about 30 hours. This was followed closely by thymidine monophosphate kinase(2.7.4.c) and thymidine diphosphate kinase(2.7.4.6) in that order, maximum activities being observed between 30 and 48 hours after partial hepatectomy (Weissman, Smellie & Paul, 1960). In view of the sequential appearance of this series of enzymes from deoxycytidylic acid deaminase to thymidine diphosphate kinase, it has been suggested that the process of enzyme induction

FIGURE 3

The biosynthesis of pyrimidine deoxyribonucleoside triphosphates in regenerating liver (see text).

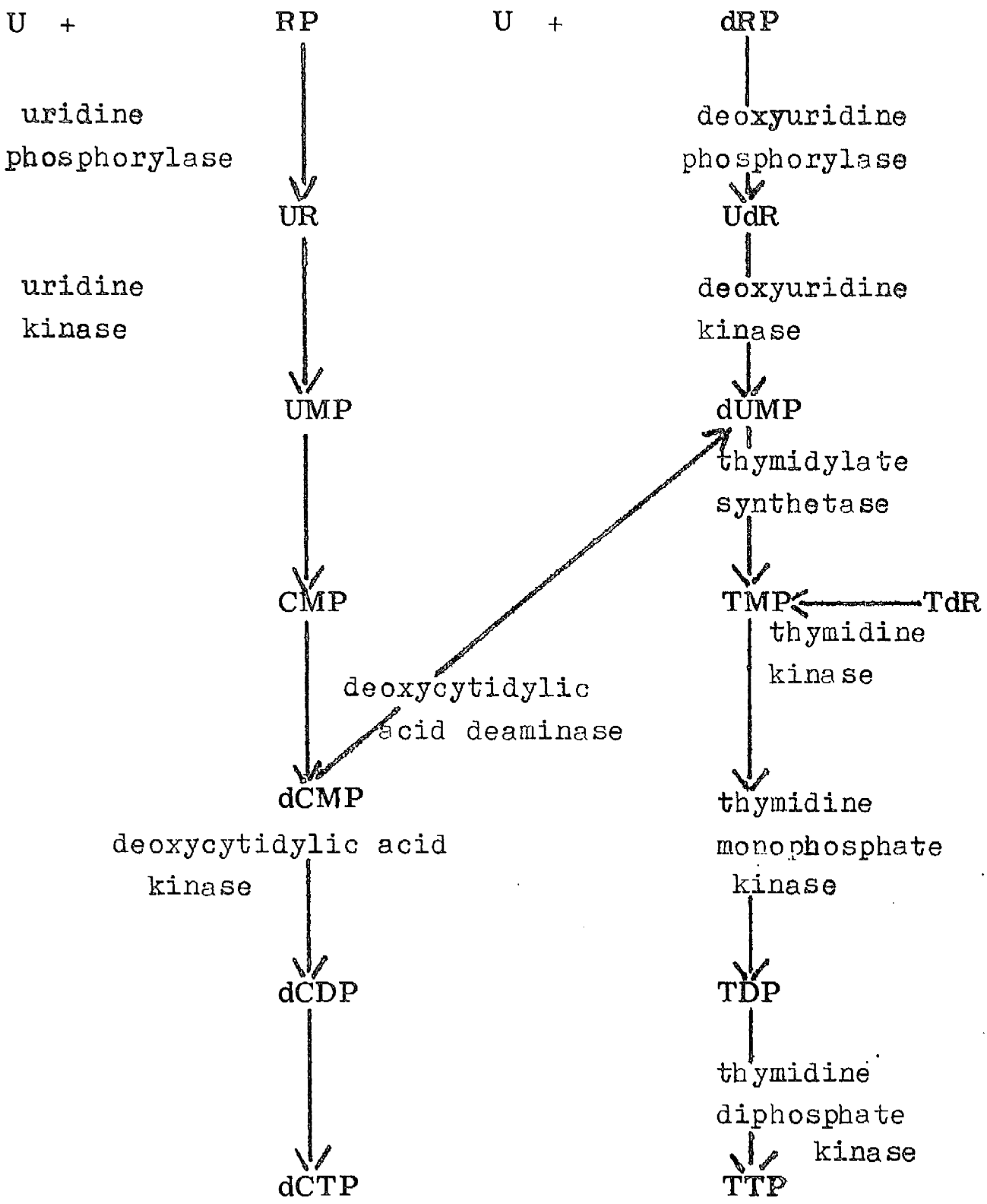

U +
RP
uridine
phosphorylase
UR
uridine
kinase
UMP
CMP
deoxycytidylic
acid deaminase
dCMP
deoxycytidylic acid
kinase
dCDP
dCTP
U +
dRP
deoxyuridine
phosphorylase
UdR
deoxyuridine
kinase
dUMP
thymidylate
synthetase
TMP
TdR
thymidine
kinase
thymidine
monophosphate
kinase
TDP
thymidine
diphosphate
kinase
TTP

FIGURE 3

might be operating in regenerating liver (Maley & Maley, 1960; Weissman, Smellie & Paul, 1960). Thus deoxyuridine monophosphate (dUMP) the product of action of deoxycytidylic acid deaminase, could induce the formation of thymidylate synthetase. The product of action of the latter enzyme thymidine monophosphate (TMP), could then effect the formation of thymidine monophosphate kinase, as previously found by Hiatt and Borjarski (1960), and so on (Fig. 3.).

That enzyme concentrations alone cannot be the sole factor regulating DNA synthesis in regenerating liver in vivo, however, was demonstrated by Bollum and Potter (1959). Using a technique for comparing DNA synthesis in regenerating rat liver in vivo and in cell-free supernatant fractions from the same animal in vitro, they found that enzymatic incorporation of thymidine into DNA appeared at the same time (18 hours after operation) as DNA synthesis in vivo but continued to increase over the period 18 to 30 hours while DNA synthesis in vivo was decreasing (24 to 30 hours). The same investigators found that DNA nucleotidyl transferase (2.7.7.7) the final enzyme in the pathway of synthesis of deoxyribopolynucleotide, started to increase at about 18 hours and remained elevated even at 72 hours.

These changes in the enzymes associated with

nucleic acid synthesis described by Maley and Maley, Weissman et al., and Bollum and Potter involved the assay of activity in soluble supernatant fractions. Busch, Chambon, Mandel and Weill (1962) however, studied RNA nucleotidyltransferase(2.7.7.6) in nuclei. These authors found that its activity started to increase 6 hours after partial hepatectomy, rose sharply to a maximum at 12 hours and then declined to normal by 48 hours. Tsukada and Lieberman (1964b) re-investigated RNA nucleotidyltransferase activity of liver nuclei and nucleoli, using enzyme preparations which had been rendered soluble by treatment with deoxycholate. The activity of RNA nucleotidyltransferase in both nuclear and nucleolar preparations started to increase immediately after the operation; it increased linearly with time and at 12 hours was about twice the initial one. No further change occurred by 20 hours.

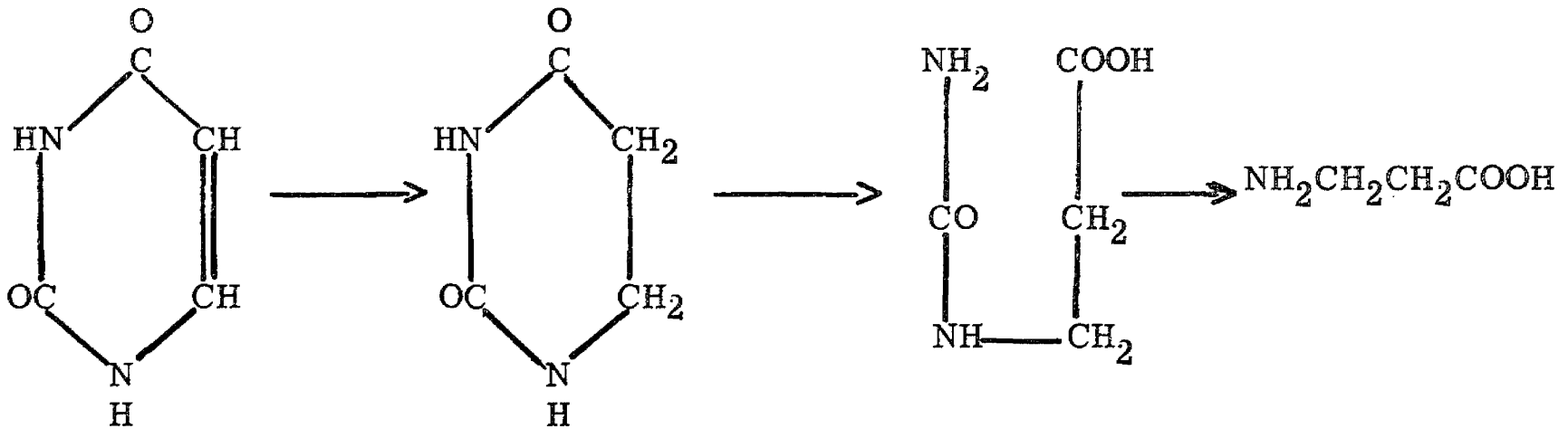
The increases in the enzymes of nucleic acid synthesis described above are of course all consistent with the process of growth. However, they are accompanied by an increase in acid deoxyribonuclease activity(3.1.4.5), the significance of which is not quite clear. There is some evidence that such an increase may be a feature of non-malignant growth in other tissue also (Brody, 1953a,&b, 1958,

Brody & Thorell, 1957). Brody and Balis (1959) observed that acid deoxyribonuclease activity increased as early as 12 hours after partial hepatectomy and reached a maximum between 36 and 41 hours. Adams (1963) on the other hand, found a maximum increase in activity at 14 to 20 hours. The suggestion of Brody and Balis that the function of the enzyme might be to terminate DNA synthesis seems to be invalidated by the finding of Adams that maximal total activity coincided with the rapid phase of DNA synthesis. In view of this observation, it seems more likely, as Adams suggests following Keir and Aird (1962) that free acid deoxyribonuclease activity stimulates DNA synthesis by attacking the DNA primer, making more 3'-hydroxyl groups available on the deoxyribose residues as starting points for DNA synthesis (Adams, 1963; Keir, 1962; Keir & Aird, 1962).

It has been proposed by Reichard, Canellakis, and Canellakis, that DNA synthesis takes place most readily at critical concentrations of the deoxyribonucleoside triphosphates, and that these compounds are components of a homeostatic mechanism (Reichard, Canellakis, & Canellakis, 1960). If this is so changes in nucleic acid precursors might also be anticipated during regeneration. As already stated above, an accumulation of free deoxyribosidic and a

FIGURE 4

The pathway of uracil degradation.
(From Davidson, J.N., "The Biochemistry of the Nucleic Acids", 5th Ed., Methuen & Co. Ltd., London, 1965).



uracil → dihydrouracil → β-ureido-propionic acid → β-alanine

FIGURE 4

decrease of free ribosidic compounds occur in the liver fragment after partial hepatectomy. It is evident that the amount of these nucleic acid precursors present must depend upon the balance of enzymes catalysing their destruction and those effecting their synthesis. The question naturally arises as to how enzymatic activities are altered in favour of synthesis during regeneration. Fritzson has proposed that the deoxyribosidic precursors act as repressors of uracil catabolic and inducers of anabolic enzymes (Fritzson, 1962). As evidence for this hypothesis, he has shown that after partial hepatectomy, a very striking decrease occurs in the activity of three enzymes catalysing the degradation of uracil (Fig. 4.). The amounts of dihydrouracil dehydrogenase(1.3.1.2) and β-ureidopropionase(3.5.1.6) decreased rapidly within 24 hours of partial hepatectomy and were maintained at 60 per cent of the normal level for the next day. Both activities returned towards normal values between the 4th and 7th days. Dihydropyrimidinase(3.5.2.2) activity decreased to about 75 per cent of the normal level in the first day and maintained this level for the next 6 days before returning to normal. The net effect of these changes would be to increase the pool of uracil derivatives available for polynucleotide synthesis immediately after partial hepatectomy.

Section I.42 Changes in other enzymes.

The changes in activity of the other enzymes during regeneration are less easy to interpret. In general, there is a slight decrease in the activities of the microsomal enzymes relative to microsomal protein. Comparing microsomes from regenerating with normal liver on the basis of equal protein content, Von Der Decken and Hultin (1960) observed that the activities of glucose-6-phosphatase(3.1.3.9) $NADPH_2$-cytochrome c(1.6.2.3), and $NADH_2$-cytochrome c-reductases (1,6,2,1), $NADPH_2$-(1,6,99.1) and $NADH_2$-(1.6.4.3) diaphorases all decreased about 15 to 20 per cent between 15 and 40 hours after partial hepatectomy. The slight general decrease observed in these enzymes during the course of regeneration may be due to a changed proportion between the membranous material and the nucleoproteins in the microsomal preparations.

Following partial hepatectomy, there is a depression also in the activities of mitochondrial enzymes. Novikoff and Potter (1948) found decreased activity during the first two days of succinate dehydrogenase(1.3.99.1) malate dehydrogenase(1.1.1.40), oxaloacetate decarboxylase (4.1.1.3) and cytochrome reductase. In a study of several mitochondrial enzymes, including succinate dehydrogenase, $NADH_2$-cytochrome c reductase, rhodanese(2.8.1.1), ATPase

(3.6.1.4), amine oxidase(1.4.3.4), and glutamate dehydrogenase(1.4.1.3), Greenbaum, Greenwood, and Harkness (1954) found that they were diminished relative to tissue mass, and that the rate of recovery of activity was less than the rate of restoration of tissue mass.

A decrease in activity after partial hepatectomy also occurs in catalase(1.11.1.6) and tryptophan oxygenase (1.99.2.c) which fall to half the normal value and slowly recover (Thomson & Moss, 1955: Stein, Skavinski, Appleman & Shugarman, 1951). An increase in alkaline phosphatase (3.1.3.1) activity occurs reaching a maximum between one and two days in liver and between two and three days in plasma (Oppenheimer & Flock, 1947). This elevation of activity however is probably due to an impairment of the process of elimination due to reduction of the total functioning liver mass, the enzyme seems to be produced mainly in the intestine absorbed into the blood stream, taken up by the liver and secreted into the bile (Rosenthal, Fahl & Vars, 1952). Aspartate aminotransferase(2.6.1.1) increases in activity in the regenerating liver fragment at a rate greater than the restoration of liver mass (Greenbaum, Greenwood & Harkness, 1954). Arginase(3.5.3.1), on the other hand, remains essentially unaltered in activity throughout the period of hepatic

regeneration (Thomson & Moss, 1955).

Section I.50 Function in regenerating liver.

One of the most remarkable features of the liver is that even when as much as 75 to 80 per cent of the organ is removed from an experimental animal no obvious signs of hepatic insufficiency develop (Bollman & Mann, 1936). In such circumstances, the only striking deviation from normality was an elevation in the concentration of uric acid in the blood and in its excretion in the urine. Clinical and pathological observations have also indicated that in human patients liver damage must be very extensive before the clinical signs of hepatic insufficiency develop (Milne, 1909). These pieces of evidence have led to the view that the normal liver possesses a large reserve capacity.

A detailed examination of the composition of the blood after partial hepatectomy supports this generalisation. For example, little change has been observed in the blood glucose*(Reinecke et al 1948). Total plasma protein concentration begins to fall only after about 16 hours after partial hepatectomy, reaches a minimum at 24 hours and then returns gradually to normal (Roberts & White, 1949). Bile pigments tend to rise, but the increase is short-lived

* Reinecke, Rudolph, Bryson & Samuels (1948)

(Royer & Bogetti, 1939a,& b). Some changes in plasma lipids have also been reported, including a rise in free cholesterol (Chanutin & Ludewig, 1936; Royer & Bogetti, 1939a,& b; Chanutin & Gjessing, 1949a,& b).

The rate of urea formation in the partially hepatectomized animal has been reported to be virtually normal (Bollman & Mann, 1936; Thomson & Moss, 1955). At 24 hours, the liver fragment was producing 65 per cent more urea per unit time than would be expected on the basis of its size. However, the concentration of free amino nitrogen in the liver fragment was increased considerably between 20 and 30 hours after partial hepatectomy even though no change in the free amino nitrogen of the plasma was observed. As early as 6 hours after the operation, glutamic and aspartic acids, lysine and ethanolamine phosphoric ester concentrations in the liver were all increased. Except in the case of ethanolamine phosphoric ester these changes were less marked at 24 hours and there was an increase in glutathione (Ferrari & Harkness, 1954).

The surviving liver fragment might well be expected to have an increased requirement for energy. In addition to performing its normal function, it is discharging some of the duties normally performed by the excised lobes (e.g.,

urea production) and it is also in a process of growth or preparation for growth. The oxygen consumption of liver slices from partially hepatectomized rats has indeed been shown to be higher than that of normal liver throughout the period of regeneration (Perkinson & Irving, 1956). Although, as noted in Section 4.2 the level of the mitochondrial repiratory enzymes is actually lowered during regeneration, it is still high enough to support a level of respiration many times greater than that actually found in the intact cells (Schwartz & Barker, 1957).

One of the main difficulties in studying liver function during regeneration is the alteration in blood flow produced by partial hepatectomy. More than two thirds of the blood flow to the normal intact liver is provided by the portal venous blood coming from the splanchnic viscera. It is fairly well established that portal venous flow rate is regulated largely by conditions in the prehepatic splanchnic bed where 90 per cent of the pressure drop in the system artery-portal vein-vena cava occurs (Fig. 5.). The liver, on the other hand, offers only a very low resistance to the passage of portal blood, and therefore tends to accept passively whatever volume is supplied through the portal vein and hepatic artery. Partial hepatectomy does not greatly affect the volume of blood traversing the liver.

FIGURE 5

The pressure drop in the system artery-portal vein-inferior vena cava (see text).

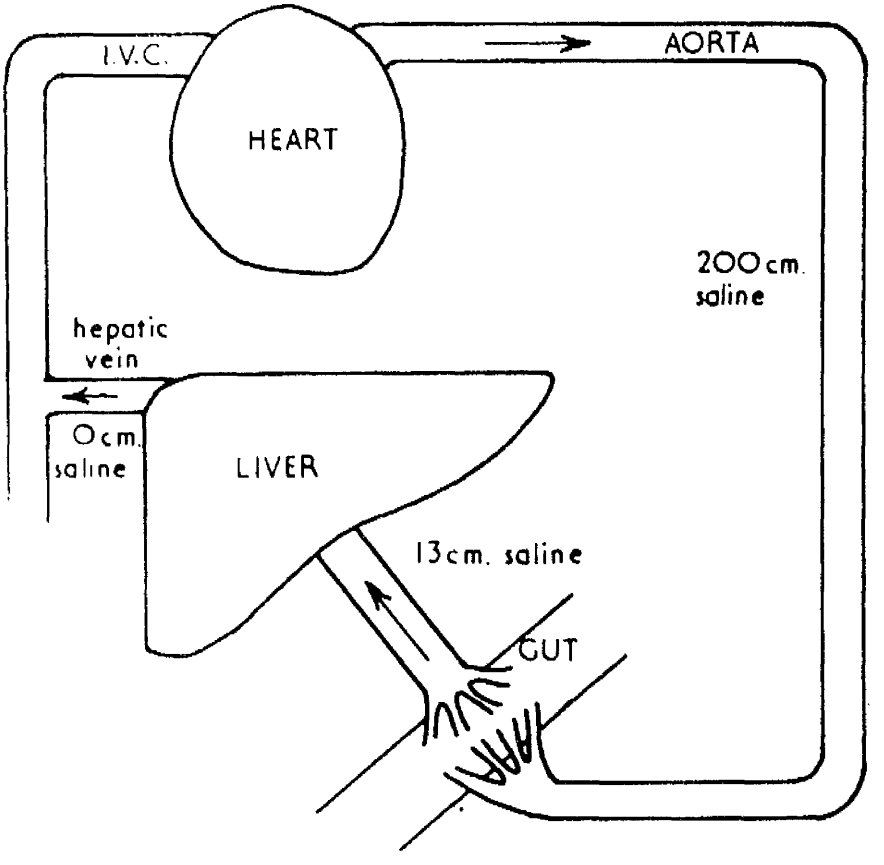

I.V.C.
AORTA
HEART
200 cm.
saline
hepatic
vein
0 cm.
saline
LIVER
13 cm. saline
CUT

FIGURE 5

Even though greatly diminished, the liver is still able to accommodate the blood flow reaching it. However, it follows that the blood flow per unit weight of liver tissue is greatly increased. This increase is initially almost threefold control level (Benacerraf, Bilbey, Biozzi, Halpern, & Stiffel, 1957) In order to avoid this difficulty, Leong, Pessoti and Brauer (1959) used isolated perfused liver to study the uptake of colloidal chromium phosphate (which is a measure of Kupffer cell activity) and the specific rate of bile flow. By this means they showed that 24 hours after partial hepatectomy the remaining liver tissue began to show an improved capacity for colloid uptake. This reached a peak at 72 hours and then declined very slowly so that even after 3 months, the colloid uptake efficiency was still well above normal. The capacity of liver tissue to secrete bile also began to increase at 24 hours, reached a maximum after 72 hours and thereafter declined slowly to reach the normal value by 14 days. At its peak, 3 days after the operation, it was found that the bile flow per unit weight of tissue was more than 50 per cent greater than in normal liver. This transient increase in bile flow rate followed a course parallel to the changes in mitotic frequency but 40 hours later. It has been observed by many workers that during

the process of regeneration mitoses first appear in the peripheral zone of the liver lobule and only later become evident in the middle and central zones (Milne, 1909; Harkness, 1952a; Grisham, 1962). Moreover, it has been shown that many of the cells in the peripheral zone undergo more than one division while many of the central zone cells do not divide at all (Grisham, 1962). These observations strongly suggest that the stimulus to hyperplasia following partial hepatectomy acts primarily upon the peripheral zone cells. At any rate, it is evident that in the process of regeneration a relatively greater increase occurs in the cells derived from the peripheral zone. There is evidence indicating that bile secretion is a function in particular of peripheral zone cells (Forsgren, 1918; Novikoff, 1959). Brauer has pointed out that if this latter conclusion is correct, then changes in bile secretion rate and cell proliferation indicate that during regeneration the liver becomes for a time a predominantly "peripheral zone" liver (Brauer, 1963).

Section I.60 The mechanism of control of liver regeneration.

Although the remarkable capacity of liver tissue to restore itself rapidly after partial removal or destruction by hepatoxic agents has long been recognised, the mechanism by which growth is stimulated and controlled is still imperfectly understood.

Section I.61 The work hypertrophy theory.

Ponfick (1889) who was the first investigator to describe hepatic restoration after removal of complete lobes, attributed it to functional stimulation arising from physiological lack. This view was supported by Milne (1909) in a review of clinical and experimental aspects of the problem. Evidence in favour of this view was obtained by Rous and Larimore (1920). These authors demonstrated that compensatory growth of liver tissue could be induced by ligation of a branch of the portal vein. This procedure resulted in atrophy of the lobes supplied by the branch concerned, together with hypertrophy of the others whose portal circulation was unimpaired. The atrophy was simple (no degeneration or increase of connective tissue was involved) and was conditional, since it did not occur when hypertrophy in the other lobes was checked by ligation of their respective bile ducts.

Section I.62 The blood-flow theory.

A new phase in the study of the phenomenon of liver restoration was initiated by Mann and Magath (1922). These investigators were concerned to produce chronic hepatic insufficiency in dogs for experimental purposes. They appreciated that the difficulty in producing such a condition by partial hepatectomy was due to the enormous regenerative power of liver. Therefore, they sought to produce conditions which would prevent this repair. It had previously been demonstrated by Whipple and Hooper (1917) in dogs, that when the portal blood is diverted from the liver through an Eck fistula into the posterior vena cava, the volume of the liver is reduced by half. Despite the reduction, with proper diet and management, these animals maintained apparent good health (Mann, Fishback, Gay & Green, 1931). Mann and Magath (1922) performed 70 per cent partial hepatectomies on a series of such animals and found that little or no regeneration occurred. In these animals little immediate change was observed. After a few weeks, however, they gradually lost weight, strength, appetite and became emaciated.

The effect of impairment of blood supply to regenerating liver was studied in rats by Stephenson (1932).

He found that by combining partial hepatectomy with partial ligation of the portal vein, it was possible to prevent, to some extent, the restoration that follows simple partial removal of the liver. By constricting the portal vein to about half its normal calibre, he found that, over a period of 4 weeks, the livers of a series of rats did not return to more than 76 per cent of the expected weight. The body weights of the animals were also below the pre-operative level.

These results suggested that the prevailing view of liver function required revision. They suggested that:

a) since dogs with Eck fistulae and rats with partially ligated portal veins can exist quite comfortably with smaller livers than normal, the volume of the liver in any normal animal must be functionally greater than actually required. Clinical and pathological studies seemed to reinforce the conclusion that the liver possesses a large reserve power and that an organism may function adequately with greatly reduced parenchyma (Milne, 1909; Bollman & Mann, 1936):

b) the volume of blood delivered to the liver per unit time must be considered as a factor in controlling the extent of hepatic restoration. Anderson (1932) pointed out that he had always noted marked congestion of the remnant of the liver during the first few days after two thirds partial hepatectomy.

This last observation, taken together with the known inhibitory effect of deprivation of portal blood already described, suggested to Anderson that liver restoration after partial hepatectomy might be associated with the increased volume of blood passing through the residual fragment. He envisaged that the "sinusoids distend in order to make a capillary bed available that is adequate to handle the pre-operative portal blood volume". He believed "that this distention induces coincident hypertrophy of the hepatic cell which, with the occasional mitosis, may largely explain all that is involved in hepatic restoration."

Higgins, Mann and Priestley (1932) carried this argument a stage further. They reasoned that "if the volume of blood that entered the lobe of a liver largely determined the extent of restoration that occurred after partial removal of the organ, then conversely, an increase

of blood delivered to the hepatic remnant should induce an increased amount of new hepatic parenchyma". In order to test this hypothesis they used the domestic fowl because in birds there is an anastomosis between the inferior mesenteric vein and the iliac veins. Thus there is a direct venous connection between the portal and postcaval vascular systems (the circle of Jacobson). In the fowl, the liver is divided into two main lobes. The left lobe, which is about 40 per cent of the total organ, may be removed surgically. In a series of fowls treated in this way, the remaining liver fragment showed some increase in weight, but even after two or three months it did not attain the original pre-operative weight of the intact organ. Higgins et al., (1932) suggested that the reason why complete restoration did not occur was that some of the portal blood was diverted through the anastomosis of the inferior mesenteric and iliac veins into the posterior vena cava thus reducing the volume of portal blood delivered to the remaining right lobe of the liver.

In another series of fowls the posterior vena cava was ligated anterior to the kidney, thus increasing the flow of blood through the liver. Two or three weeks later, the left lobe of the liver was surgically removed. In these

birds Higgins and his colleagues discovered that restoration of the liver always occurred, was usually complete and often greatly in excess of the tissue removed. Finally, a group of four fowls was subjected to ligation of the posterior vena cava above the kidney, without subsequent partial hepatectomy. When they were sacrificed after an interval of four months, their livers were very much larger than those in controls. These experiments however, did not indicate whether the stimulus causing the restoration was the portal blood itself, or the presence in it of some particular constituent. Mann (1940) recognized that his original experiments were open to criticism on three grounds,

a) the portal blood comprises about two thirds of the liver blood supply and in its absence there may not be enough blood for restoration,

b) failure of restoration may be due to damage to the liver cell,

c) perhaps the stimulus causing restoration is in sufficiently high concentration to act only in portal blood (it may be an absorption product from the intestinal tract or one of the organs draining into the portal system).

To meet these criticisms Mann performed a series of experiments in dogs in which he established a portocaval anastomosis without ligation of the portal vein or vena cava, thus permitting blood to pass freely between the two vessels, depending upon the difference in pressure in them. In such dogs, subsequent partial hepatectomy does not result in the liver fragment having to afford passage to the portal blood normally passing through the liver tissue removed i.e., the portal hypertension which follows partial hepatectomy is prevented. In a series of dogs with such portocaval anastomoses, Mann performed partial hepatectomy, removing one third to one half of the total hepatic tissue. Judging by the increase of liver weight, little restoration occurred in the remaining liver fragment compared to that of normal controls. Mann interpreted this result to mean that restoration of hepatic tissue following partial removal depended primarily on the portal blood flow, and that restoration occurred to establish an adequate portal pathway. Grindlay and Bollman (1952) showed in dogs that following partial hepatectomy there is indeed a considerable elevation of portal vein pressure which lasts for about 10 days.

Mann's conclusion that the volume of portal blood delivered to the liver after partial hepatectomy is the primary stimulus to liver growth was generally accepted until challenged by Weinbren (1955). This author ligated the portal vein branch to the right posterior lobe of the liver in a series of rats. This led to a considerable atrophy of the right posterior lobe. However, when a standard partial hepatectomy was performed on such an animal, the usual regenerative changes were evoked in this lobe (although deprived of its portal blood supply). These regenerative changes i.e., increase in weight, cellular and lobular size and mitotic rate were apparent when such a lobe was compared with the right posterior liver lobe of control rats similarly treated but without hepatectomy.

The possibility that liver regeneration might depend on some special ingredient in portal blood was considered as being very unlikely by Mann (1940) following his failure to produce regeneration in dogs with portocaval anastomosis, as already described. However, it was definitely disproved by the demonstration of liver regeneration in dogs which had previously undergone portocaval transposition (Child, Barr, Holswade & Harrison, 1953). Liver regeneration was also shown to follow partial

hepatectomy in dogs with an arterialised portal supply (Fisher, Russ, Updegraff, & Fisher, 1954; Fisher, Russ, & Bluestone, 1955). The arterialized dog liver was found to attain a slightly greater than normal equilibrium weight, although the hepatic blood flow was almost three times as large as normal.

By about 1955, therefore, it seemed unlikely that the blood flow theory of liver regeneration could be correct at least in the simple form in which it was originally propounded.

Section I.63 The "humoral" theory.

a) The parabiotic experiments

The possibility that the control of liver growth might be exercised by a specific humoral factor was suggested independently by three groups of workers. Christensen and Jacobsen (1949) set up parabiosis between three pairs of young rats of the same litter and sex. Four months later one parabion of each pair was partially hepatectomised, and all animals killed 52 hours later. In the hepatectomised parabions the mitotic index of the regenerating liver at death was in the range 160 to 730 per 100,000 nuclei. In the sunoperated parabions however, it was 4 to 33 per

100,000 nuclei which they reported as being above the normal level. Wenneker and Sussman (1951) set up 8 pairs of rats in parabiosis, and performed partial hepatectomy on one parabion of each pair after an interval of only 5 to 6 days. As controls they used 16 single partially hepatectomised rats. The animals were killed at various intervals up to 14 days following partial hepatectomy. They obtained the usual increase of wet weight in the liver fragment of the partially hepatectomised parabion. However, in each of the unhepatectomised parabions the livers were larger than normal. Moreover, the mitotic index in the livers of the unhepatectomised parabions was greatly increased over controls, especially in the first three days when it attained values in the range 60 to 220 per 100,000 nuclei. Bucher, Scott and Aub (1951) performed partial hepatectomies on one partner of each of fourteen parabiotic pairs, and on two partners of each of three sets of parabiotic triplets. The interval which elapsed between parabiosis and partial hepatectomy varied from 7 weeks to 5 months. At intervals of 48 to 72 hours after partial hepatectomy, the intact livers of the unhepatectomised partners all exhibited a higher mitotic rate than the control livers removed at the time of hepatectomy. In parabiotic

"twins" the mean value was 6 times and in "triplets" it was 50 times that of controls.

These results were interpreted as evidence of a blood-borne "humoral" factor, which, when present in sufficient quantity stimulates liver cell mitosis. The humoral agent was believed to be carried from the operated to the unoperated twin or triplet. Christensen and Jacobsen's (1949) conclusions were based on the results of only three pairs of animals and the degree of mitotic stimulation obtained was very slight. Wenneker and Sussman (1951) however, obtained a much greater mitotic stimulation in the liver of the unoperated parabion of eight pairs. However, unlike Christensen et al. and Bucher et al., they partially hepatectomised the rats only 5 to 6 days after establishment of the parabiosis. This enhanced response may well represent the result of the combined operations. Bucher, Scott and Aub (1951) pointed out that a correlation existed between the amount of tissue removed and the degree of mitotic stimulation achieved. If 68 to 80 per cent of the liver is removed from one of a parabiotic pair, the total liver mass removed from both is only 30 to 40 per cent, whereas 80 per cent hepatectomies performed on two out of three means that the total mass removed from the trio is

about 53 per cent. Differences in experimental technique may account to some extent for the great variation in results between the three groups of workers.

Unfortunately, these early promising results could not be consistently repeated by later workers. Hurowitz and Studer (1960) obtained an increased mitotic index in the liver of the unoperated partner of a parabiotic pair after partial hepatectomy of the other, in 62 per cent of the intact animals. Islami, Pack and Hubbard (1959) however, observed no increase in mitotic index in the unoperated livers of parabiotic rats 48 to 72 hours after one partner had been partially hepatectomised. Finally, Rogers, Shaka, Pechet and MacDonald (1961) in an extensive and carefully controlled series of experiments on parabiotic rats, failed to obtain any evidence for the existence of a humoral mechanism controlling regeneration. As criteria of growth, they used both mitotic activity and DNA synthesis as measured by autoradiographic counts of nuclear incorporation of ^{3}H-thymidine. Sham operation alone was found to increase liver mitosis and DNA. They found that the effect of partial hepatectomy of one partner of twins on hepatic mitosis and DNA synthesis in the intact partner was no greater than sham operation alone. Also, in triplets, partial hepatectomy

of two lateral members induced higher but not significantly higher mitosis and DNA synthesis in the intact middle partner than in similar sham-operated triplets, but less than in control, un-operated triplets.

b) Factors in serum

If liver regeneration is brought about by a humoral mechanism as indicated by the early parabiotic experiments, it might be anticipated that the humoral factors concerned would be demonstrable in the blood plasma. Accordingly, several investigators tested the effect of injecting serum from partially hepatectomised rats into normal ones. Friedrich-Freksa and Zaki (1954) reported that intraperitoneal injections of serum from rats hepatectomised 23 to 72 hours previously into normal intact rats caused a forty fold increase in liver mitotic activity. Similar results were obtained by Hughes (1960). On the other hand Smythe and Moore (1958) observed no significant increase in liver weight after five injections of serum removed from rats 24 hours after partial hepatectomy, into normal rats, but did notice a slight increase in mitotic activity. Laquerriere and Laumonier (1960) claimed that a similar procedure caused an increase in the DNA content of the liver nuclei.

Zimmerman and Celozzi (1960) found increased incorporation of ^{3}H-thymidine and ^{14}C-orotic acid into liver DNA of intact rats that received injections of serum from partially hepatectomised animals. These experiments all seemed to indicate that the serum of hepatectomised animals had the property of stimulating liver growth in normal animals.

Other workers tested the effect of injections of normal serum into partially hepatectomised rats for its possible growth-controlling capacity. Smythe and Moore (1958) found that intravenous injection of normal plasma into partially hepatectomised recipients depressed $^{32}PO_4$ uptake into DNA and retarded liver weight gain, but the mitotic activity of the liver was not diminished. Stich and Florian (1958) reported depression of mitotic activity 48 hours after partial hepatectomy in rats injected 15 to 16 hours previously with normal serum, intraperitoneally. Kohn (1958) injected normal serum at 8 hour intervals intraperitoneally into partially hepatectomised rats and sacrificed them at 72 hours. Doses of 1·2 or 2·0 ml., every 8 hours decreased the rate of liver regeneration as determined by weight. Weinbren (1959) injected normal serum into rats 10 hours after partial hepatectomy and found a 50 per cent reduction in mitotic activity 29 hours post operatively. Bucher (1958)

however, was unable to obtain any effect on liver regeneration after intravenous injection of normal serum at four-hour intervals for 30 hours.

Finally, some workers have tested the effect of injections of serum from partially hepatectomised rats into others similarly operated. Following such treatment, Stich and Florian (1958) and Smyth and Moore (1958) observed increased proliferation in regenerating livers. Abidi, Paschkis and Cantarow (1959) compared the effects of serum derived from the general circulation with that taken from the hepatic viens of rats partially hepatectomised 24 hours previously. They found that both sera stimulated proliferation in the regenerating liver and that hepatic vein serum was twice as potent as that derived from the general circulation. Taken together, these results seemed to indicate that plasma from partially hepatectomised rats had a stimulatory effect on liver growth and normal plasma had a depressing effect.

Unfortunately, this clear and simple picture was soon complicated by the results of later workers. Glinos (1952, 1958) and Peters (1962) independently repeated the experiments of Friedrich-Freksa and Zaki and failed to find any effect on liver mitotic frequency. Also MacDonald and

Rogers (1961) using mitotic frequency and DNA synthesis measured by autoradiographic estimation of ^{3}H-thymidine incorporation as indices of liver growth, tested the three combinations of serum injections described above and noted no effect in any of the combinations.

Section I.64 Glinos theory.

In a series of papers, Weiss (1947, 1955, 1957) elaborated a general theory to account for the phenomenon of organ regeneration. According to this theory, each organ secretes into the circulating blood a growth-inhibitory factor specific for itself. Damage or removal of part of an organ would result in a diminished production of its specific inhibitory factor, thus allowing it to grow more rapidly until it would be large enough to produce sufficient of the factor to inhibit growth once again.

Glinos (1958) influenced by the theory of Weiss, proposed a mechanism of control of liver regeneration based on the concept that liver cell growth is regulated by such an inhibitor. He supported this theory with two lines of evidence,

a) if explants of adult liver cells are cultured in plasma, their outgrowth is more rapid if

the plasma is taken from a hepatectomised animal (Glinos & Gey, 1952),

b) it is possible to stimulate the mitotic frequency in the liver of a normal intact rat by plasmapheresis (a procedure which causes dilution of the plasma constituents). Moreover, an increase in the level of plasma protein achieved by means of fluid restriction was found to inhibit cell mitosis in regenerating liver.

Glinos envisaged a system of automatic self-regulation involving the liver itself as being the site of synthesis of the inhibitory factors. He identified these inhibitory agents with the plasma proteins most of which are synthesised in liver. According to his theory the plasma proteins, especially albumin, in the interstitial fluid bathing the parenchymal cells inhibit cell growth and division. Consequently, any means of reducing the concentration of plasma proteins in this region might lead to a release of this inhibition with subsequent parenchymal cell growth and mitosis. According to Glinos, following partial hepatectomy two mechanisms would tend to effect this change. First, during the initial 24 hours or more after the operation,

the elevation of portal blood pressure would cause increased transudation of the non-colloid fraction of the plasma into the interstitial space with consequent dilution of the plasma proteins in this space. This effect, which would be almost immediate, would provide the initial stimulus to liver growth. Secondly, after a time it would be reinforced by a fall in plasma protein concentration due to the inability of the liver fragment to synthesise plasma proteins at the same rate as the intact liver. As the liver fragment regenerated, the portal pressure and plasma protein level would return to normal so that the regeneration process would be self regulating. The increased transudation of the non-colloid fraction of the plasma would be most pronounced in the region of the cells nearest the portal areas in view of the parallel, radial arrangement of the liver sinusoids, so the theory would be able to account for the early, initial burst of mitosis in the periportal zones of the liver.

This theory, although attractive, has several serious disadvantages. The actual changes in plasma protein concentration which follow partial hepatectomy in well nourished rats seem too small to cause the massive proliferative response of the liver. Changes of the same order produced by plasmapheresis in the experiments reported by

Glinos gave rise to increases in liver mitotic rate which scarcely exceed those reported in relation to diurnal cycles (Halberg, Barnum, Silber, & Bittner, 1958; Jaffe, 1954). Glinos proposal of an early diminution of interstitial fluid plasma proteins in the periportal zones caused by elevated portal vein pressure is also open to objection for two reasons,

a) such a mechanism obviously cannot apply to the littoral cells, in which mitosis occur plentifully, and

b) the proposed mechanism cannot be reconciled with present knowledge of extracellular fluid formation in the liver, and of the net countercurrent flow of lymph and blood dictated by anatomical relations for the livers of most mammals (Brauer, 1963).

Hemingway (1961) has proposed a modification of Glinos theory. Plasma proteins have been shown to carry corticosteroids (Sandberg, Slaunwhite & Antoniades, 1957; Petersen, 1959) and Hemingway suggest that the plasma protein influence on liver cell mitosis may be attributable, at least in part, to the associated corticosteroids, changes of protein levels automatically altering the corticosteroid inhibition. Evidence for this view was obtained by repeating Glinos

experiment on the effect of fluid restriction on rats after partial hepatectomy. In a control group of fluid-deprived partially hepatectomised rats he confirmed that regeneration was depressed; but in adrenalectomized fluid-deprived animals this did not happen. Indeed the adrenalectomized fluid-deprived animals showed even more regeneration than the animals allowed unrestricted access to fluid. Interesting as this experiment is, the objections to Glinos' theory already described, are equally applicable to Hemingway's modification of Glinos' theory.

The situation in 1962.

At the beginning of the present investigation, the situation could be summarised as follows. The "blood-flow" theory proposed by Mann and his colleagues was obviously open to grave objections. On the other hand, the alternative "humoral" theory lacked conclusive proof and even if it were accepted the nature and mode of action of the postulated humoral agent were quite obscure. Finally Glinos' theory, which combined some of the features of the other two was still largely speculative. The time seemed ripe for a fresh attack on the whole problem.

R E S U L T S S E C T I O N

RESULTS SECTION.

Section 2.1 Introduction to the problem and the parabiotic phase.

At the outset of this investigation the humoral theory seemed to provide a much more attractive explanation of the control of liver regeneration than either of its rivals. It seemed reasonable in the light of the available evidence to assume that the organism produced a specific 'hormone' or 'humoral' factor(s) which either stimulated or inhibited liver growth. The results of the parabiotic experiments suggested that this 'humoral' agent was transferable. Therefore the most fruitful approach to the problem seemed to be an attempt to isolate and characterise the 'humoral' agent. As a preliminary to this investigation, it was obviously essential to try to demonstrate its existence unequivocally and to devise an assay for it.

As already detailed in the Introduction (Section I.63) belief in the existence of the 'humoral' factor was based mainly on the results of parabiotic and serum injection experiments. Because the latter type of experiment is technically so much easier than the former, it seemed a good starting point for the present project; also since it was

Table 1

The effect of repeated injections of rat blood serum on the frequency of mitoses in the livers of normal rats. The treated animals were given 6 intravenous injections each of 2 ml., at 12-hour intervals. The animals were killed 12 hours after the last injection.

Treatment	No. animals	Mitoses per 100,000 nuclei (Mean ± standard error)
Nil	4	18 ± 6
Normal serum	4	9 ± 2
Serum from partially hepatectomised animals	4	5 ± 2

The differences between the means for the three groups are not significantly different ($P > 0.05$).

uncertain whether the humoral agent stimulated growth or inhibited it, both possibilities had to be investigated. The first experiment was designed to test whether the serum of hepatectomised rats stimulated liver growth in normal rats. The main defect of all previous experiments had been the relative smallness of the response produced. In the present experiments therefore, an attempt was made to overcome this defect. First it was decided to inject the serum intravenously rather than intraperitoneally. Secondly, to augment any stimulatory effect of the serum, multiple injections were performed, six 2 ml., amounts being administered at 12 hour intervals. The animals were killed 12 hours after the last injection. With these improvements of technique it was hoped to obtain results at least comparable to those of Friedrich-Freska and Zaki (1954). However, contrary to expectation, it is evident from Table I that serum from hepatectomised donor rats did not produce any increase in liver mitotic rate in the recipients. This experiment, therefore, did not support the view that the serum of partially hepatectomised rats contains a 'humoral' factor which stimulates liver cells to divide. These results are quite contrary to those of Friedrich-Freska and Zaki (1954) who gave a single injection of 1 to 5 ml., of regenerating serum,

Table 2

The effect of repeated injections of blood serum from normal rats on the frequency of mitoses in the livers of partially hepatectomised rats. A total of 6 x 2 ml., injections was given at 12-hour intervals starting 24 hours after hepatectomy. The animals were killed 24 hours after the last injection.

Treatment	No. animals	Mitoses per 100,000 nuclei (Mean ± standard error)
Controls (given injection of physiological saline)	6	246 ± 89
Injections of normal serum	6	131 ± 22

The difference between the means for the two groups is not statistically significant ($P > 0.05$).

killed the animals 24 to 72 hours later and reported a 40 fold increase in mitotic frequency. They are contrary also the the results of Hughes (1960) and Moore (1958); but they are in harmony with the negative findings of MacDonald and Rogers (1961) (see Section I.63b).

Table 2 presents the results of an experiment to test the effects of normal serum administration on the frequency of mitosis in the livers of hepatectomised animals. It is clear that the normal serum did not significantly depress the mitotic frequency below the level found in control animals given injections of saline. This experiment, therefore, gave no support to the view that normal serum contains a factor which inhibits liver cells from dividing. Again these results are in sharp contrast to the positive responses reported by Stich and Florian (1958), Kohn (1958), and Weinbren (1959) but in agreement with the negative results of Bucher (1958), and MacDonald and Rogers (1961).

The results of these two experiments do not disprove the humoral theory. It is possible that the supposed humoral factor may have a very short half life <u>in vivo</u>. In that case, the limited amount present in the volume of serum injected might be destroyed before it had time to produce its effect. Clearly increasing the number, or frequency of serum

injections would not provide an answer. There is obviously a limit to the amount of serum which can be injected without fatally overloading the animal's circulation. The problem, essentially, was to transfer sufficient of the supposed humoral agent from a hepatectomised animal to a normal or vice versa. The obvious alternative to multiple injections was to try to exchange plasma or whole blood between a normal and a partially hepatectomised animal. Provided injection and withdrawal were performed at a suitable rate, there would be no theoretical limit to the extent or number of such exchanges. The procedure of multiple withdrawals and injections, however, poses technical problems. The obvious method would be to make the injections and withdrawals from a suitable vein using a syringe and needle. The superficial veins of the tail are a fairly convenient site for injection or for withdrawal of small quantities of blood. However, repeated puncture tends to provoke either collapse or thrombosis of the vein. The external jugular vein presents less difficulty in these respects but easy access to it can only be obtained by surgical exposure, which virtually excluded very frequent injections or withdrawals from this site.

In view of the cumulative problems associated with multiple venepunctures, the use of an indwelling catheter had

to be considered. Such a procedure implied some form of immobilization of the experimental animal, which in turn inevitably meant sedation of the rat almost to the level of surgical anaesthesia. In view of the difficulty in withdrawal from veins, it was decided to use an arterial cannula for withdrawal and a venous cannula for injection. To avoid the labour involved in repetitive transfers of blood from one rat' to another, it was decided to connect up the venous cannula of one rat to the arterial of its partner and vice versa. Initially, it was intended to use some sort of pump (hand or automatic) to effect exchange and to ensure that there was no net tranfer of blood from one animal to the other. Transfer by hand pumping, however, seemed tedious and the development of a mechanical pump would have been time-consuming. The possibility of using the arterio-venous pressure difference instead of a pump for the transfer of blood seemed attractive. Therefore a trial experiment was set up with cannulae joining the carotid artery of one partner to the jugular vein of the other and vice versa. Initially a three-way metal stopcock was interposed on each cannula to regulate the flow, but clotting inevitably occurred inside and eventually it was found satisfactory to omit it. It was necessary to prevent clotting in the cannulae, to heparinize the animals. With

scrupulous attention to surgical technique, haemostasis and dosage of anaesthetic employed, it was possible to keep cross-circulated rats alive for periods up to 48 hours.

Provided the exchange of blood between the two partners can be made so rapid and extensive that they virtually share a common circulation, cross-circulation should provide a certain means of detecting a humoral mechanism. In the experiments, the theoretical rate of flow (v) in each cannula can be calculated (provided the blood flow in the cannula is non-turbulent) from the formula:-

$$v = \frac{\pi P r^4}{8 l n}$$

where P = pressure difference between the carotid artery and the jugular vein (assumed to be 90 mm., of mercury i.e., 120×10^3 dynes/sq. cm.)

r = internal radius of cannula (0·0375 cm.)

n = viscosity of blood (assumed to be 0·04 poise)

l = length of cannula (30 cm.).

Using the formula, it can be calculated that under the conditions described, the value of v is 4 - 5 ml./minute, which is equivalent to an exchange per minute of about a quarter of the blood volume of each animal. To ascertain whether this extraordinarily high figure was realized in practice, a cross-circulation of the type described above was

set up between two normal rats. One of the animals was given an intravenous injection of approximately 0·2 ml., of a saline suspension of erythrocytes previously labelled with ^{51}Cr. Blood samples (0·02 ml.,) were then withdrawn at intervals from the left jugular veins of both animals and assayed for radioactivity. If it is assumed that the blood which flows through the cannula from one animal to another is complete and instantaneously mixed with the blood of the second animal, the difference $\underline{x}$ in specific activity between the two animals will diminish with time according to the equation:-

$$\frac{-dx}{dt} = 2kx$$

where k is the fraction of the total blood volume exchanged between the two animals per minute. Substituting for k the value 0·25 calculated above, the difference in specific activity between the two animals after 10 minutes may be calculated:-

$$-\frac{dx}{dt} - = 2kx$$

integrating,

$$x = x_o e^{-2kt}$$

and

$$2kt = \ln \frac{x_o}{x} = 2{\cdot}303 \log \frac{x_o}{x}$$

Table 3

The Specific Activity of blood withdrawn from a pair of parabiotic rats at varying time intervals after injection of labelled erythrocytes into one of them.

Time after injection (min.)	Specific Activity (counts/min./ml. blood) Partner given injection	Partner given injection
6	4260	
9	4500	4340
19	4270	4340
39	4130	4210

$$\log \frac{x_o}{x} = \frac{2k\ t}{2{\cdot}303} = \frac{2 \times 0{\cdot}25 \times 10}{2{\cdot}303}$$

therefore

$$\log \frac{x_o}{x} = 2{\cdot}171$$

and

$$\frac{x_o}{x} = 148{\cdot}2$$

therefore at 10 minutes from the start of the experiment the difference in specific activities between the red cells in the circulations of each rat $= \frac{100}{148{\cdot}2} = 0{\cdot}67$ per cent

This prediction is in good agreement with the experimental results shown in Table 3. It therefore seems safe to proceed on the assumption that the calculated value for the rate of exchange between the two animals is at least approximately correct. An exchange as rapid as this approaches the ideal situation in which the two partners share a common circulation. Any humoral agent produced in either animal must be rapidly transferred to the other. If, therefore, liver regeneration is initiated and controlled solely by humoral factors, it should follow that partial hepatectomy of one partner should result in an almost equal degree of compensatory hyperplasia in both partners. Table 4 shows

Table 4

The frequency of mitoses in the livers of parabiotic rats after partial hepatectomy of one partner. In experiments 1-6 inclusive, parabiosis was established approximately 30 minutes after hepatectomy. In experiment 7 the corresponding interval was 48 hours.

Exp. No.	Duration of parabiosis (hours)	Mitoses per 100,000 nuclei	
		Hepatectomised partner	Partner not operated upon
1	48	671	2
2	48	709	20
3	48	276	27
4	48	902	14
5	36	175	4
6	28	140	14
7	23	267	36

the results of a series of such experiments. Within 36 to 48 hours of partial hepatectomy the livers of the hepatectomized partners showed the same high mitotic frequency found in single animals subjected to the same operation. The livers of the unhepatectomized partners, on the other hand, showed only the low mitotic frequency found in the livers of normal adult rats. These results give no support to the view that the control of liver regeneration is effected by means of (a) humoral agent(s).

Section 2.2 The blood-flow phase.

The apparent overthrow of the humoral theory described in the previous section prompted a reconsideration of the alternative and much older "blood-flow" theory. As already indicated (Section I.62) the work of Weinbren (1955) argued strongly that it could not by itself account for the regenerative process, but the possibility that it might make some contribution had to be considered. It has also been mentioned in the Introduction (Section I.62) that the strongest evidence in favour of the vascular theory was the series of experiments using fowls carried out by Higgins, Mann and Priestley, (1932). These workers claimed that an increase in the volume of portal blood delivered to the liver

by ligation of the postcaval vein in fowls produced a definite increase in the wet weight of the intact liver. The experiments in which these results were obtained included only a small number of fowls. Moreover, little or no information was given about the histology or composition of the hypertrophied organ; and the experiments did not indicate whether the hypertrophy had occurred with the explosive rapidity characteristic of liver regeneration or whether it had been a gradual process over several weeks or months. If the blood-flow theory was to be seriously reconsidered therefore, the obvious starting point would be a repetition of the fowl experiment on a larger scale and using post-operative mitotic activity as the criterion of growth.

The possible form of partial hepatectomy in any species is dictated largely by the architecture of the liver. Small biopsis amounting to only a few per cent of the liver mass can easily be obtained. However, easy surgical removal of a large part of the liver is only possible where the organ is lobulated. In the domestic fowl the liver is divided into a right and a left lobe. The former accounts for about 60 per cent of the total mass of the organ, but because the gall bladder is closely attached to its undersurface it cannot easily be excised without disturbing the normal flow

Table 5

The frequency of mitoses in the livers of fowls before and after partial hepatectomy.

Time between partial hep. and death (days)	Mitoses per 100,000 nuclei at operation	Mitoses per 100,000 nuclei at death
1	6	10
2	-	167
3	5	465
3	5	125
4	14	106
5	9	14
5	5	38
6	8	15
7	14	10
7	4	53

of bile. Consequently, partial hepatectomy has to take the form of removal of the left lobe, which accounts for about 40 per cent of the organ. The operation may easily be performed through an incision in the left flank, the left liver lobe being ligated and excised by the same technique as in the rat. The operation is attended by a slight blood loss, but this is not usually serious.

Table 5 shows the effect of this operation on the frequency of mitosis in the remaining liver fragment. There is a pronounced although irregular increase for the first four days, followed by a decline. These findings suggest that a regenerative response does occur in fowls; admittedly less dramatic than in the rat, but this may simply be due to the fact that the proportion of the organ excised is much smaller. Table 6 illustrates the effect on liver mitotic index of ligation of the postcaval vein (Fig. 6). This operation, though technically rather difficult because of the inaccessibility of the vein was not attended by haemorrhage or other complications. For the period 2 to 7 days after the operation the mitotic activity was certainly increased, though to a variable degree, over the low resting value found in unoperated animals (Table 5). It is evident, however, that sham operation produced an almost similar response

Table 6

The frequency of mitoses in the livers of fowls after ligation of the postcaval vein or sham operation.

Time between operation and death (days)	Mitoses per 100,000 nuclei at death	
	sham operation	postcaval vein ligation
2	5,6,10,6	12,20,34,144
3	8,26,16,37	31,30,14,54,18
5	10,11,17,26,57	78,29,16,42,24
7	23,10,10	63,9,12,7

Each figure represents a single animal. An analysis of variance indicates that the two treatments were significantly different at the 5 per cent but not at the 2·5 per cent level ($0{\cdot}05 > P > 0{\cdot}025$).

FIGURE 6

Ligation of the postcaval vein in the fowl. Blood from the postcaval vein below the ligature is diverted through the portal-systemic anastomosis (circle of Jacobson) into the portal system.

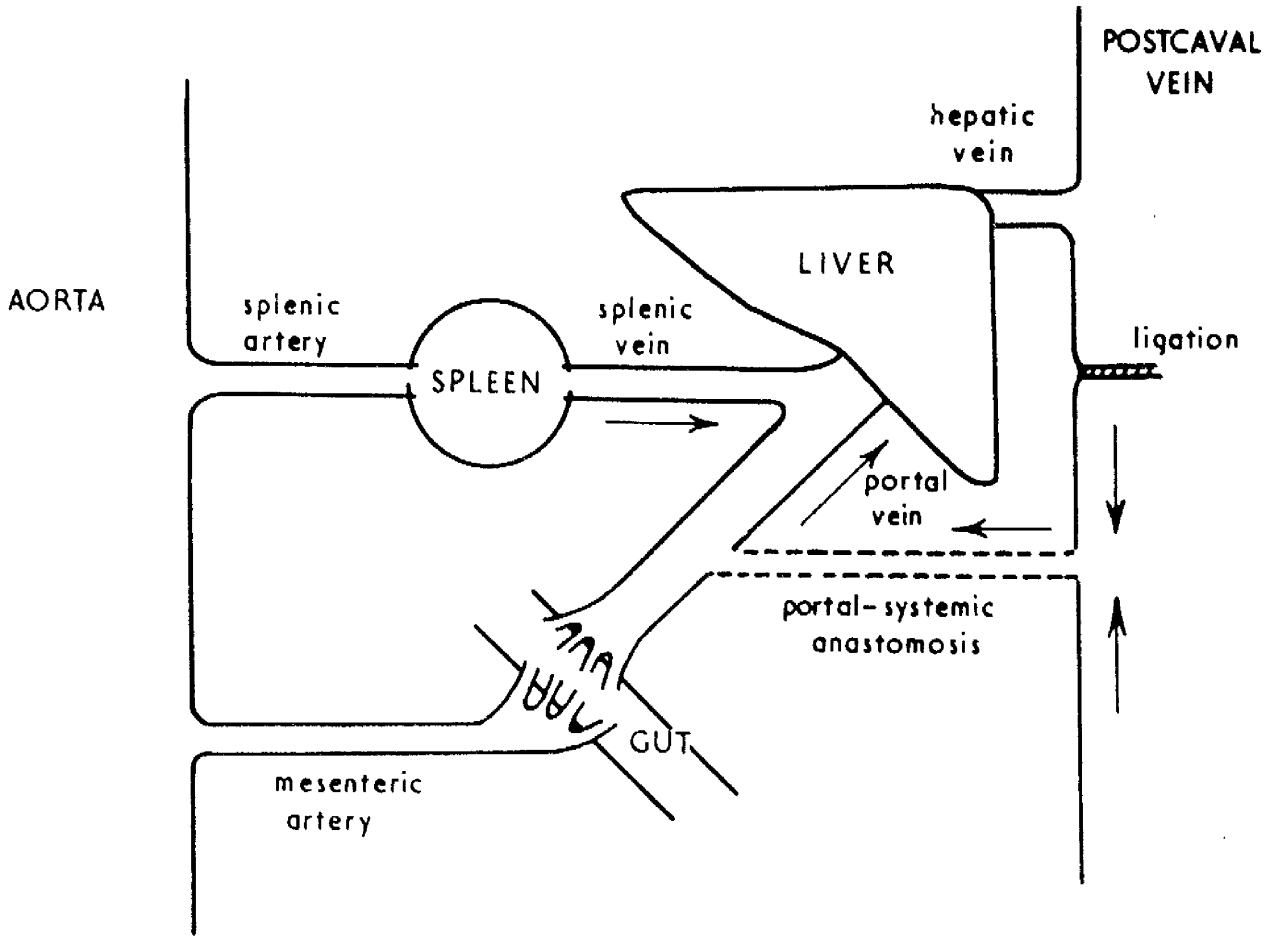

POSTCAVAL
VEIN
hepatic
vein
LIVER
AORTA
splenic
artery
splenic
vein
SPLEEN
ligation
portal
vein
portal-systemic
anastomosis
GUT
mesenteric
artery

FIGURE 6

(Table 6). It appears therefore, that ligation of the post-caval vein does not produce in fowls a mitotic response comparable to that produced in rats by partial hepatectomy.

The one unequivocal piece of evidence for the blood-flow theory was thus seriously weakened. It may be objected however, that the results in Tables 5 and 6 are too variable and ambiguous to constitute adequate grounds for rejecting the theory outright rather than regarding it as unproven. There was an obvious need for a more critical test. It was clearly desirable to perform this test in some mammal in which it was known that partial hepatectomy was followed by liver regeneration of the classical type. As in the case of the fowls, the most suitable form of experiment would be to increase the portal blood flow to the liver in an otherwise normal animal. These requirements were met by using the dog as experimental subject. The increase in the portal blood flow was achieved by performing a reverse Eck fistula (side-to-side anastomosis of the portal vein and the inferior vana cava, with ligation of the latter above the level of the anastomosis) (Fig. 8 - compare with the normal in Fig. 7). This operation was generally well tolerated and the dogs remained fairly normal throughout the experimental period.

FIGURE 7

The normal splanchnic vascular system in the dog.

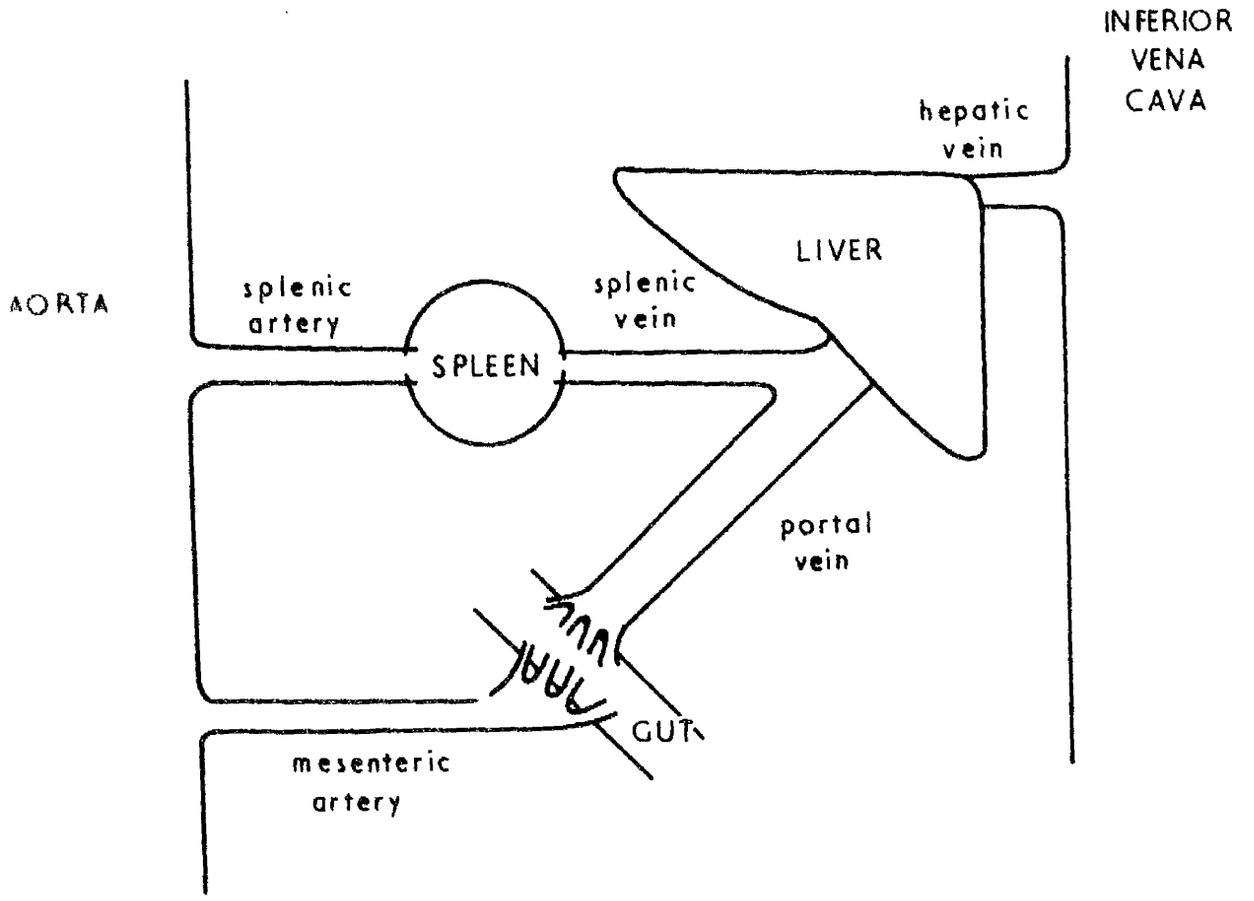

INFERIOR
VENA
CAVA
hepatic
vein
LIVER
AORTA
splenic
artery
splenic
vein
SPLEEN
portal
vein
GUT
mesenteric
artery

FIGURE 7

FIGURE 8

The Reverse Eck Fistula in the dog i.e., side-to-side anastomosis of the portal vein and inferior vena cava with ligation of the inferior vena cava above the level of the ligature.

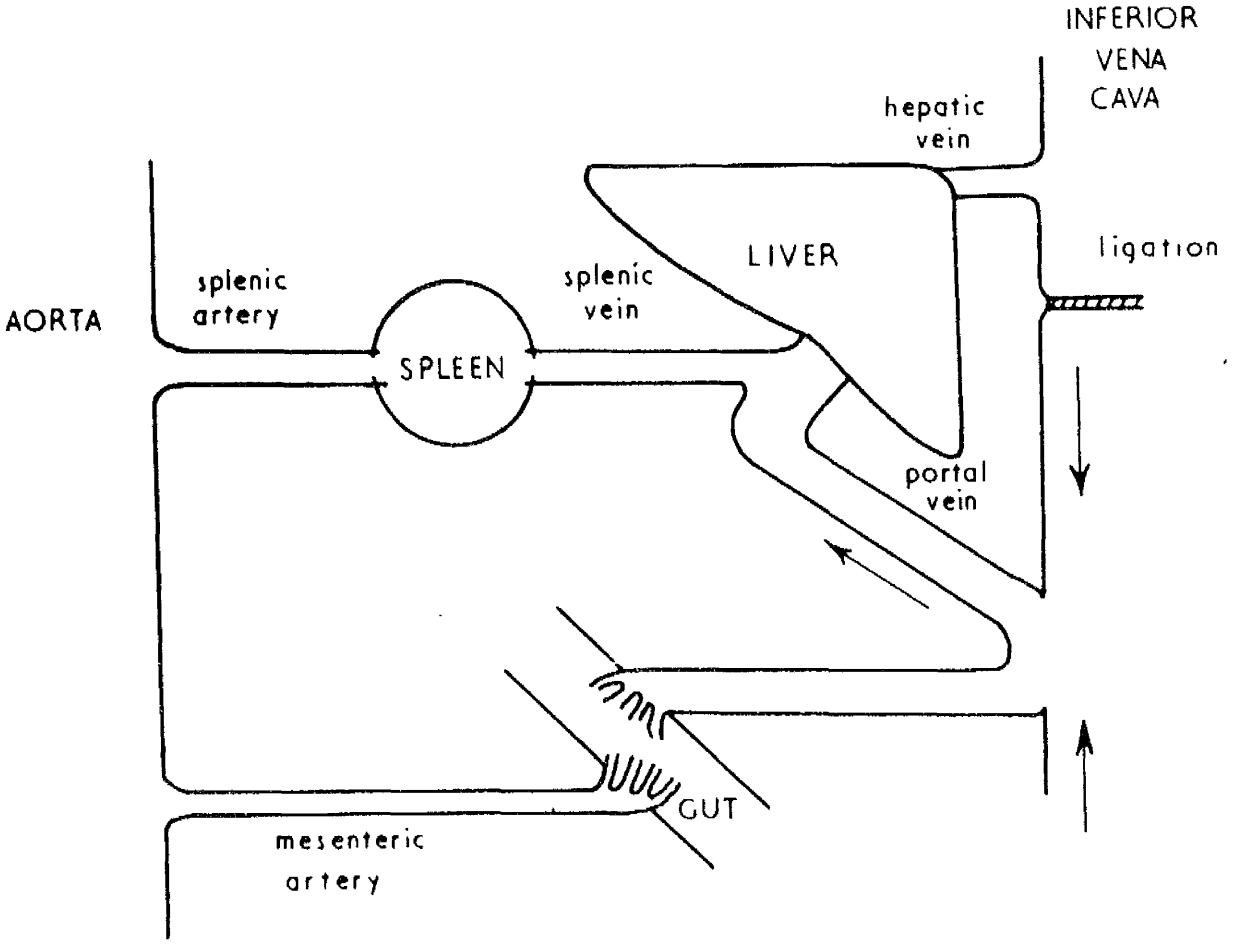

INFERIOR
VENA
CAVA
hepatic
vein
LIVER
ligation
splenic
artery
splenic
vein
AORTA
SPLEEN
portal
vein
GUT
mesenteric
artery

FIGURE 8

Table 7

The effect of Reverse Eck Fistula and partial hepatectomy on mitotic frequency in the liver of the dog.

Animal No.	Operation	Mitoses per 100,000 nuclei			
		before operation	3 days later	6 days later	9 days later
1	Reverse Eck Fistula	5	6	-	-
2	-do-	55	13	-	-
3	-do-	3	7	-	-
4	-do-	18	0	-	-
5	-do-	7	-	6 + 529*	-
6	-do-	47	-	34	-
7	-do-	3	-	10	-
8	-do-	3	-	-	15
9	-do-	5	-	-	15
10	-do-	7	-	-	8
11	Partial hepatectomy	3	382	198	381

* biopsy from left lobe (see text)

Table 7 shows the effects of this procedure on the mitotic frequency in the livers of dogs. The time intervals of 3, 6 and 9 days were selected because, in the dog, mitotic activity resulting from partial hepatectomy is apparent on the 2nd day after the operation and increases to a maximum between the 4th and the 10th day (Fishback, 1929). A single partial hepatectomy gave in our hands the same type of response (Table 7). It is quite clear from Table 7 that, in general, the shunt produced no significant increase in mitotic frequency. There was, however, one exception. In general, when the animals were under anaesthesia before being sacrificed, the livers were a dark bluish colour with rounding of the normally sharp edges. At death the liver of animal number 5 did not have the usual homogeneous dark colour. The left central and left lateral lobes were a bright red in contrast to the bluish colour of the rest of the liver. This colour difference disappeared after death when the liver was excised. The mitotic index of the red lobes was extremely high and of the order of that seen following partial hepatectomy (Table 7). These results clearly indicate that increased portal blood flow per se does not lead to hyperplasia of the liver. This conclusion is not affected by the anomalous result given by dog number 5 in Table 7. It may be that in this particular

FIGURE 9

End-to-side anastomosis of the splenic artery to the portal vein with concomitant removal of the spleen in the dog.

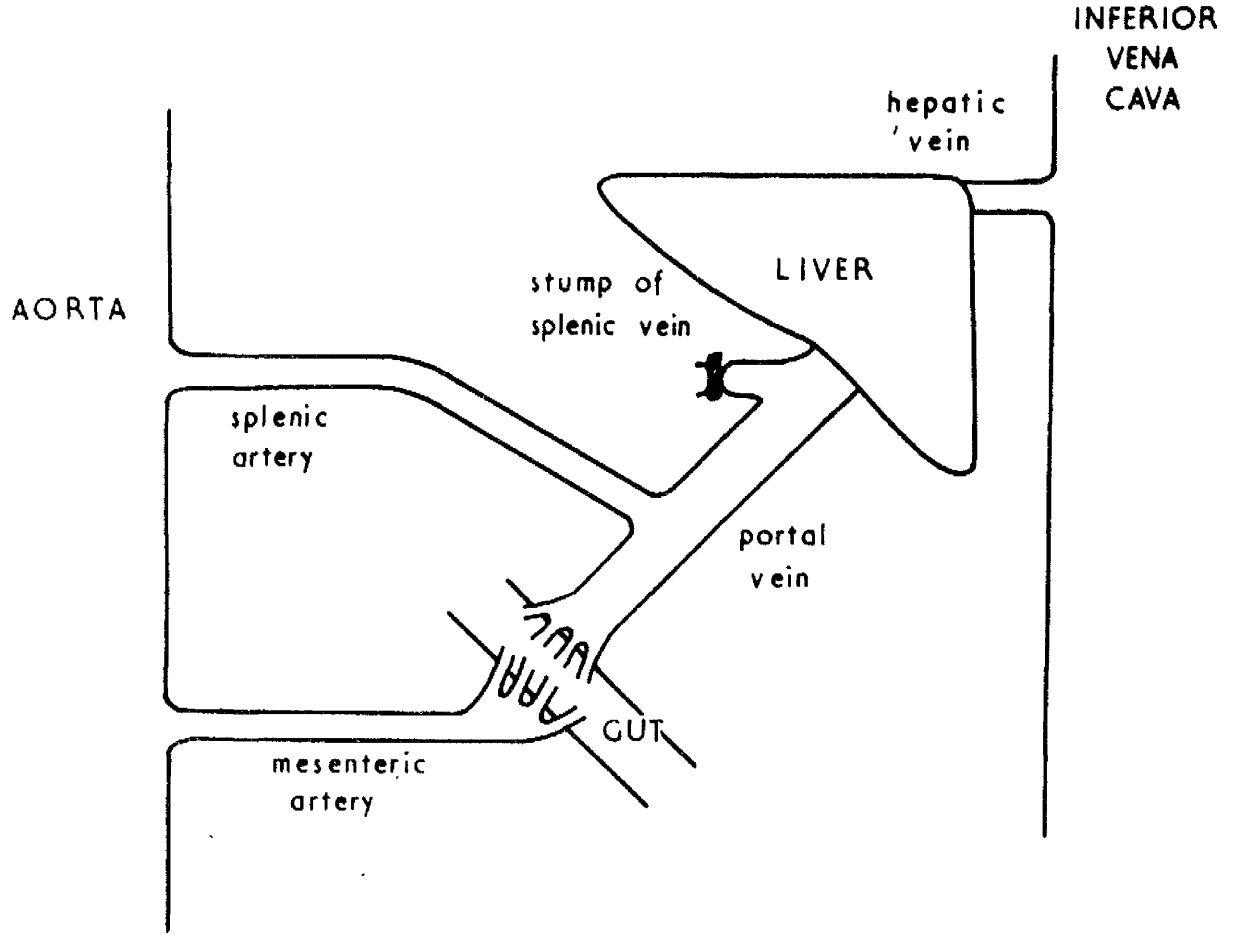

INFERIOR
VENA
CAVA
hepatic
vein
LIVER
stump of
splenic vein
AORTA
splenic
artery
portal
vein
GUT
mesenteric
artery

FIGURE 9

FIGURE 10

Splenoctomy in the dog.

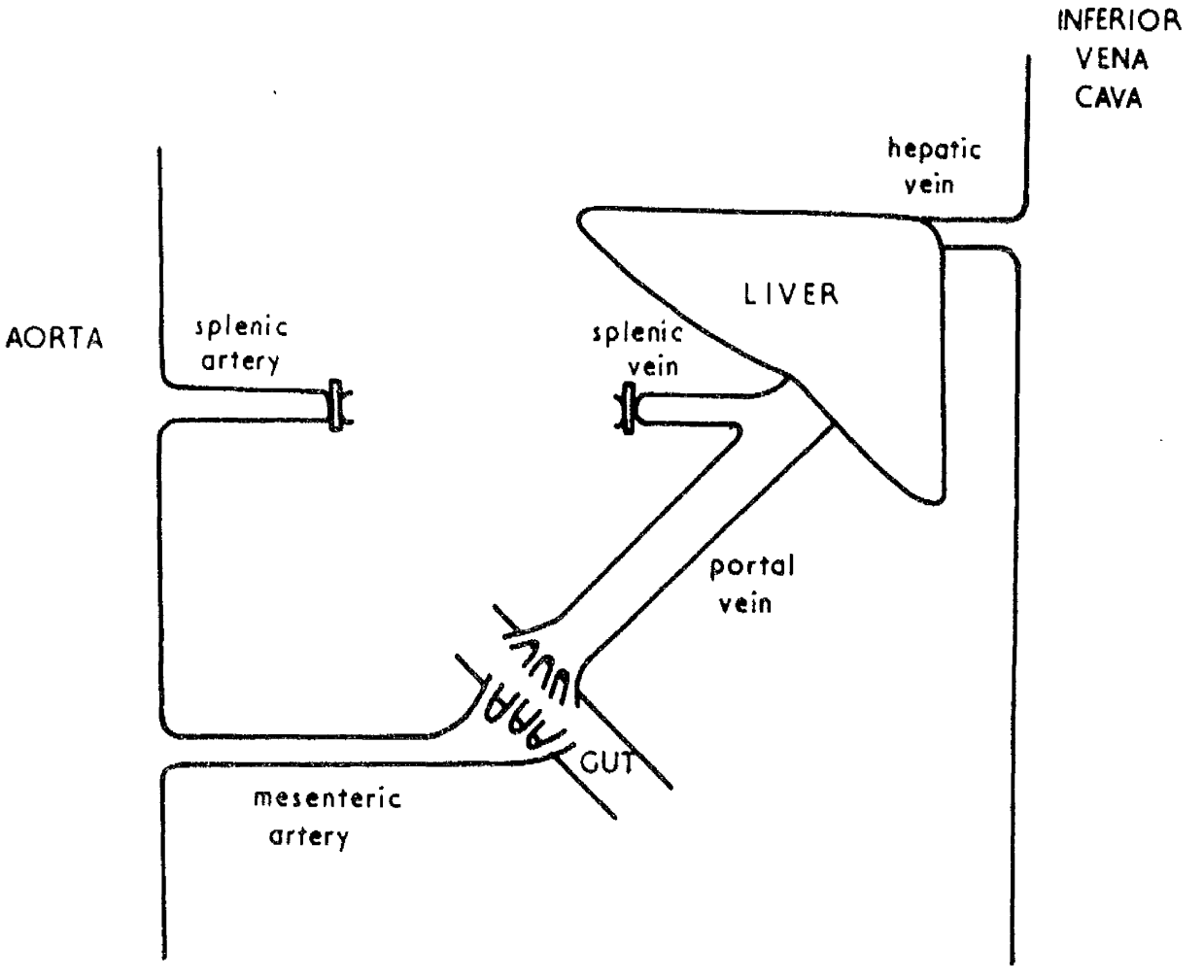

INFERIOR
VENA
CAVA
hepatic
vein
LIVER
AORTA
splenic
artery
splenic
vein
portal
vein
GUT
mesenteric
artery

FIGURE 10

Table 8

The portal pressure in the dog after various surgical operations. Each figure represents a mean for the number of animals shown in parenthesis. Since the portal pressure varies from minute to minute, no attempt has been made to calculate standard deviations.

Operation	Portal pressure (cm. saline)				
	Control (pre-operative)	Time after operation			
		immediately after	3 days	6 days	9 days
Spleno-portal shunt	12·8 (5)	14·7 (3)	15·7 (3)	16·5 (2)	19·5 (4)
	13·1 (12)		17·0 (12)		
Splen-ectomy	14·4 (4)	15·7 (3)	13·7 (3)	13·5 (4)	
Aorto-portal shunt	11·1 (2)	18·1 (2)	21·3 (2)	22·3 (2)	22·5 (2)

Table 9

The effect of a splenic artery-portal vein shunt on the mitotic frequency in the liver of the dog.

Dog No.	Mitoses per 100,000 nuclei			
	before operation	after 3 days	after 6 days	after 9 days
1	4	10	-	5
2	0	7	-	10
3	0	-	10	-
4	2	7	-	10
5	2	-	6	16
6	4	-	5	-
7	2	-	4	21
8	3	3	-	-
9	2	-	0	-
10	5	-	19	-
11	0	-	5	6
12	0	-	26	-

Table 10

The effect of splenectomy on the mitotic frequency in the liver of the dog.

Dog No.	Liver mitoses per 100,000 nuclei			
	before operation	after 3 days	after 6 days	after 9 days
1	0	12	6	-
2	3	10	16	-
3	4	16	19	-
4	11	22	3	-
5	9	9	-	5
6	0	36	-	4
7	1	-	9	22
8	0	-	7	51

case the liver haemodynamics was disturbed in such a way that the left lobes were being perfused by an increased proportion of hepatic arterial blood. This finding suggested the possibility that in a situation where delivery of high pressure hepatic arterial blood to a liver fragment is preferred to portal blood, the resultant increase in oxygen saturation of sinusoidal blood might be a stimulus to liver regeneration.

In order to test this possibility, it was necessary to arterialise the portal vein. Accordingly, a series of dogs was subjected to an end-to-side anastomosis of the splenic artery to the portal vein, with concomitant removal of the spleen (Fig. 9). Table 8 shows that this procedure was followed by a small but progressive increase in portal pressure whereas splenectomy had no effect. It can be seen from Table 9 that this type of shunt did produce a very slight increase in liver mitotic index. However, it is evident from Table 10 that splenectomy alone gave an increase of comparable size (Fig. 10). In view of these negative results a more vigorous form of arterialisation was desirable. Therefore, in another series of dogs a shunt between the abdominal aorta and the portal vein was created by means of a surgical grafting operation (Fig. 11). This procedure

FIGURE 11

The synthetic shunt created between the abdominal aorta and the portal vein in the dog (aorto-portal shunt).

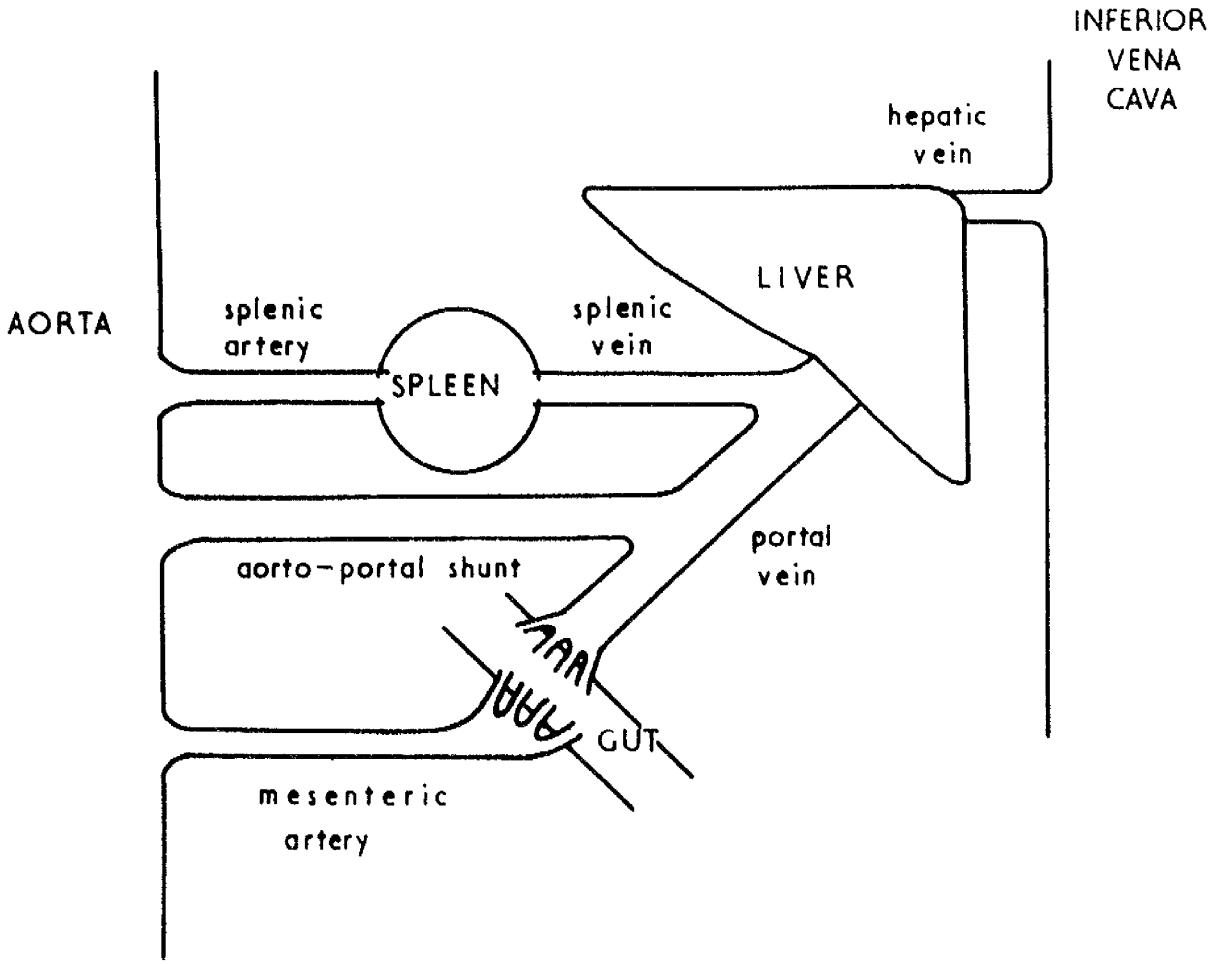

INFERIOR
VENA
CAVA
hepatic
vein
LIVER
AORTA
splenic
artery
splenic
vein
SPLEEN
portal
vein
aorto-portal shunt
GUT
mesenteric
artery

FIGURE 11

Table 11

The effect of aorto-portal shunt on the mitotic frequency in the liver of the dog.

Dog No.	Liver mitoses per 100,000 nuclei			
	before operation	after 3 days	after 6 days	after 9 days
1	1	50	45	10
2	0	5	16	3
3	26	108	138	-

almost doubled the portal pressure. Table 11 shows its effect on liver mitotic index. There is evidently a variable increase, on the average slightly greater then in the spleno-portal shunt. Dog number 3 has given a sizeable response, but in this case the resting mitotic rate was quite high. On the whole, the results may be considered negative. In spite of its drastic nature, the operation has not produced the sort of mitotic response found in liver regeneration. It may be concluded therefore, that neither an increased portal blood flow, nor an increase in the partial pressure of oxygen in the liver provide an adequate explanation of liver regeneration.

Section 2.3 Glinos theory.

The demonstration of the inadequacy of the humoral and blood-flow theories did not exclude the possibility that liver regeneration might be the result of the two mechanisms acting in concert. Such a theory had already been proposed by Glinos (1958). An account of Glinos' view and of the evidence which he brought forward to support it has been given in the Introduction (Section I.64). It will suffice here to restate his central idea, which was that partial hepatectomy would result first in an increase in portal

pressure, and then in a fall in plasma protein. Each of these factors would tend to bring about a fall in extracellular protein concentration in the liver, and this, according to Glinos is the immediate stimulus to cell division. Clearly such a theory is amenable to experimental test. If it is correct it should be possible to cause liver growth in an otherwise normal animal either by increasing the portal pressure or by diminishing the plasma protein concentration. Furthermore, the changes in portal pressure or plasma protein concentration necessary to produce this effect should not be any larger than the corresponding changes produced by partial hepatectomy.

The question of pressure may conveniently be considered first. In the previous section a description was given of three types of experiment designed to increase portal blood flow to the liver of the dog,

1) the reverse Eck Fistula,

2) spleno-portal shunt,

3) aorto-portal shunt.

The actual increases in portal pressure produced by these operative procedures are shown in Table 8. As might be expected, much the largest increase was produced by the aorto-portal shunt (almost a twofold increase). As is

FIGURE 12

The change in portal pressure following partial hepatectomy in the rat. On each occasion, the difference between the portal and jugular vein pressure is recorded.

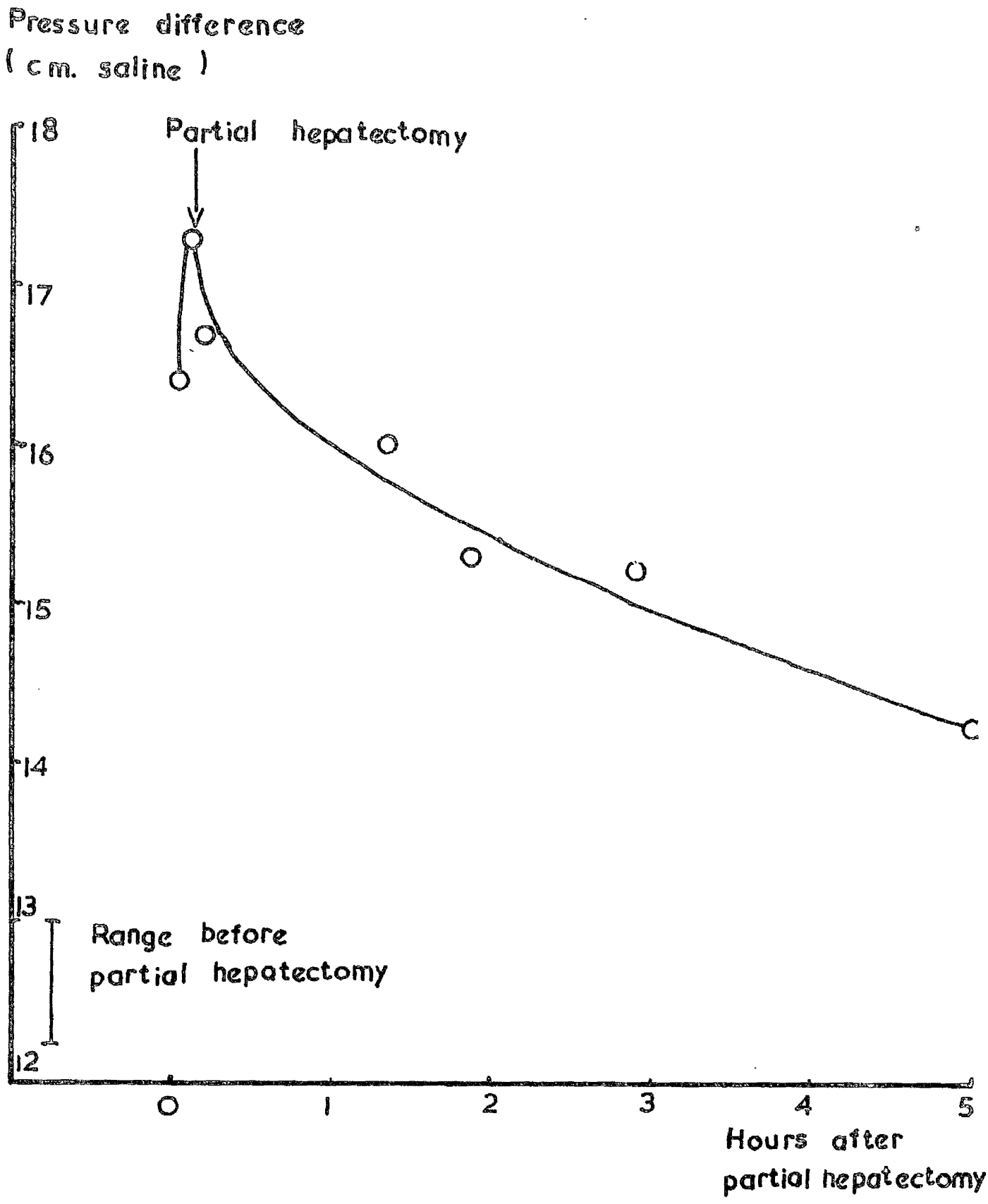

Pressure difference
(c m. saline)
18
17
16
15
14
13
12
Partial hepatectomy
Range before
partial hepatectomy
0
1
2
3
4
5
Hours after
partial hepatectomy

FIGURE 12

Table 12

The difference in pressure between the portal and jugular veins in the rat before and after partial hepatectomy. Since the portal pressure fluctuated slightly from minute to minute the results for each group are shown as a range.

Condition of animals	Pressure difference (cm. of saline)
Normal	12-14
24 hours after partial hepatectomy	14-15
48 hours after partial hepatectomy	14-15
72 hours after partial hepatectomy	12-14

evident from Tables 9, 10 and 11, none of these procedures produced anything comparable to the mitotic response observed in liver regeneration. The changes in portal pressure in partially hepatectomised rats are shown in Figure 12 and Table 12. Although there is a very pronounced initial elevation immediately after operation there is a rapid decline in the ensuing 4 to 5 hours. However, even after 48 hours it is still slightly elevated. Taking the evidence of the dog and rat experiments together, it seems safe to conclude that an increase in portal pressure of the magnitude found after partial hepatectomy could not by itself account for liver growth.

The second factor to be considered in Glinos' theory is the fall in plasma protein concentration after partial hepatectomy. Previous work performed by Roberts and White (1949) on this topic had indicated that although a fall in circulating plasma protein begins about 16 hours after partial hepatectomy it does not become pronounced until 24 hours. However, the results reported in Table 13 would seem to indicate that a substantial fall in both total plasma protein and albumin is not evident until 48 hours after partial hepatectomy. Since the most rapid growth rate in terms of both mitotic index and liver weight occurs during

Table 13

The plasma protein level in the rat after partial hepatectomy.

Animal No.		Plasma protein concentration (g./100 ml. plasma)		
		before operation	after 24 hours	after 48 hours
1	albumin	3·03	3·10	2·37
	total	4·95	4·60	4·42
2	albumin	2·37	3·10	2·50
	total	5·25	5·17	4·72
3	albumin	3·15	2·95	2·50
	total	5·85	5·35	5·00
4	albumin	2·77	2·95	2·22
	total	5·95	5·55	4·52
5	albumin	2·75	2·50	2·05
	total	6·13	5·40	4·75

the first 48 hours, it is difficult to reconcile these findings with Glinos' theory.

The contention that plasma protein concentration may diminish in the immediate environment of the hepatic parenchymal cells as a consequence of increased portal pressure is difficult to test directly. Glinos did not attempt to do so but based his conclusion on the effects of plasmapheresis on liver mitotic frequency. It was decided, therefore, to re-examine this question and to determine if any relationship could be observed between the lowering of circulating plasma protein and liver mitotic frequency. Table 14 shows the results of a pilot experiment indicating extent and duration of plasmapheresis, the levels of total plasma protein and plasma albumin attained and the resulting mitotic index. There is no obvious relationship. Figure 13 presents the results of a more detailed investigation. Again there is no obvious relationship between the fall in plasma protein and mitotic index. In general, the mitotic activities obtained by plasmapheresis are much lower than those found in regenerating liver.

Table 15 presents the results of the converse experiment, i.e., the attempt to inhibit or depress liver cell mitosis after partial hepatectomy by artificially

TABLE 14

The effect of repeated plasmapheresis on the plasma protein level and liver mitotic frequency in rats. The lower section of the table indicates the amount of blood which was exchanged on each occasion, i.e., 4 x 1 means that 4 ml., of blood was withdrawn, centrifuged and the plasma exchanged for physiological saline.

Table 14

Animal	Plasma protein concentrations at each plasmapheresis (g./100 ml. plasma)					
	Day 1	Day 2 a.m.	Day 2 p.m.	Day 3	Day 4	Mitoses per 100,000 nuclei
1 albumin	3·2	3·1	2·9	3·5	3·9	136
total	5·8	6·0	4·6	4·9	6·1	
2 albumin	2·4	1·9	3·2	3·0	3·1	50
total	4·5	3·7	4·5	5·0	5·6	
3 albumin	3·3	2·1	2·4	2·7	-	98
total	6·8	4·3	4·5	5·4	-	
4 albumin	3·6	2·4	3·3	2·7	-	19
total	5·9	4·0	4·7	4·7	-	
						Day of Sacrifice
1	4 x 1	4 x 1	4 x 1	4 x 1		4th
2	4 x 1	4 x 1	4 x 1	4 x 1		4th
3	5 x 1	5 x 2	5 x 2	-		3rd
4	5 x 1	5 x 2	5 x 2	-		3rd

FIGURE 13

The relationship between the fall in total plasma proteins and the mitotic index in rat liver.

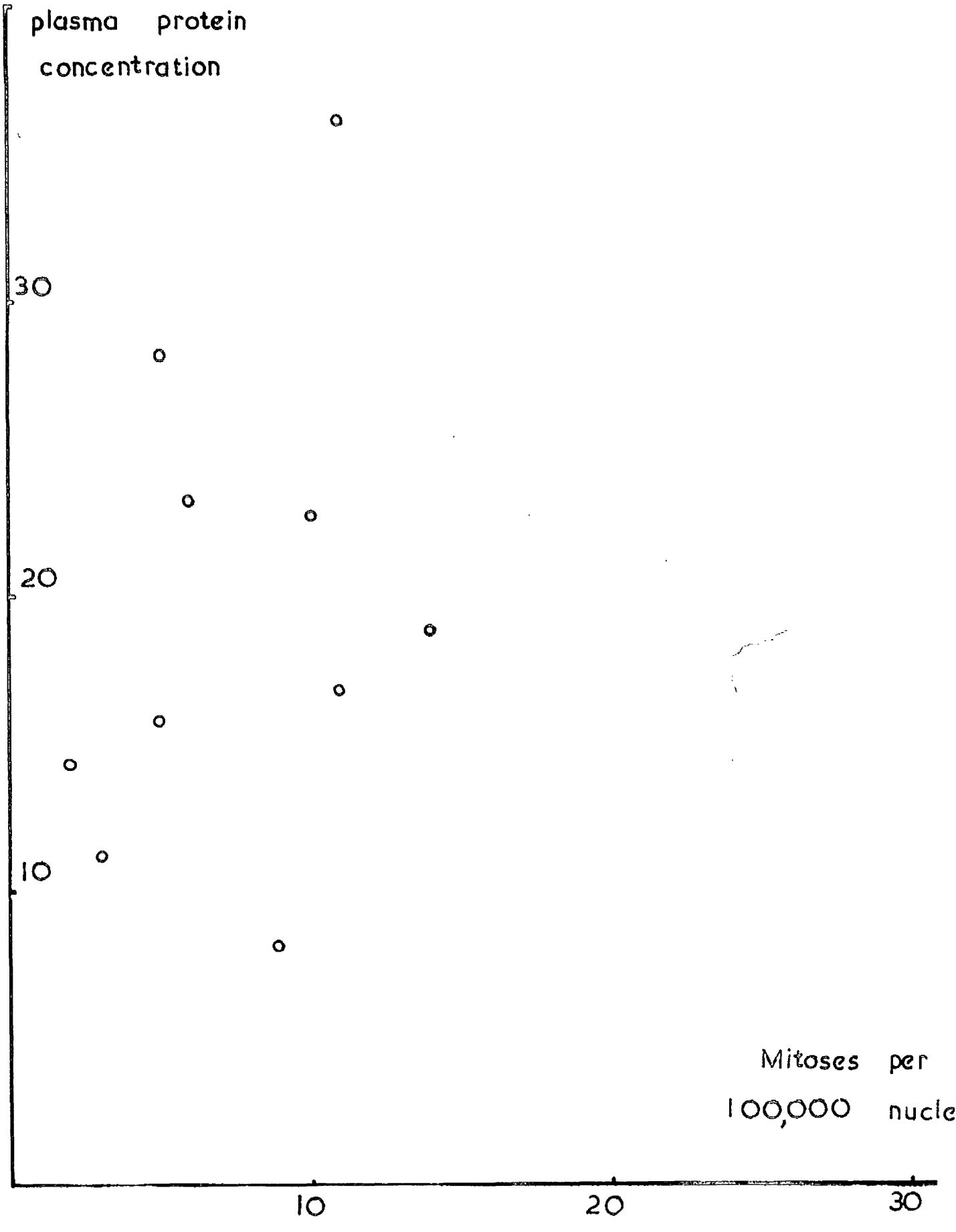

plasma protein
concentration
30
20
10
Mitoses per
100,000 nucle
10
20
30

FIGURE 13

TABLE 15

The effect of intravenous injections of normal rat plasma or saline on the mitotic index in regenerating rat liver. Three groups of rats were partially hepatectomised at the start of the experiment. One group was treated with plasma, one with saline and one was untreated. 41 hours after partial hepatectomy all three groups were sacrificed and liver biopsies taken for histology.

Table 15

Animal No.	Treatment	Number of mitoses per 100,000 nuclei
1	2 ml., normal plasma	166
2	intravenously every	123
3	8 hours: first	30
4	injection immediately	115
5	after operation.	57
6	2 ml., physiological	16
7	saline intravenously	81
8	every 8 hours:	21
9	first injection	82
10	immediately after	55
11	operation.	43
12	None	49
13	-do-	51
14	-do-	55
15	-do-	75
16	-do-	66

An analysis of variance of the three groups of mitotic indices shows that there was no significant difference between the treated and untreated groups ($P > 0.05$)

increasing the level of circulating plasma protein. It is quite clear that the results are again negative.

In view of the failure to stimulate mitotic frequency in liver much beyond the normal range even by vigorous plasmapheresis, and the complete failure to inhibit or suppress the normal proliferative response following partial hepatectomy by intravenous plasma infusion, it seems safe to conclude that plasma protein concentration is unlikely to be the main factor in controlling liver cell growth and division.

Section 2.4 Changes in chemical composition of regenerating liver.

The apparent failure of the three existing theories to account for the mechanism of liver regeneration necessitated a new approach to the problem. It was surmised that a careful study of the early chemical changes occurring in the liver fragment following partial hepatectomy might indirectly shed light on the mechanism of growth control. After partial hepatectomy a latent period of 14 to 16 hours elapses before DNA synthesis begins. Presumably it is during this latent period that the stimulus to regenerate begins to act on the liver fragment. Some understanding of

its nature and mechanism of action might therefore be gained by looking for chemical changes in the residual fragment before DNA synthesis gets under way.

Changes in glycogen concentration.

The rapid disappearance of glycogen is one of the earliest and most dramatic features of regenerating liver (see Introduction section I.35). The mechanism of this change has not been specifically investigated. However, since glycogen is being continuously synthesised and degraded in liver, its decrease must represent an imbalance between these two processes. Whether a change in its rate of synthesis might occur during regeneration is unknown, but it might be reasonable to expect an increased breakdown to maintain blood sugar. Obviously, to perform this latter function the liver fragment, having only one third the quantity of glycogen, will have to degrade its store three times as fast to maintain the normal blood sugar level, not to mention the increased requirements following the stress of operation. The role of the liver in the homeostatic regulation of blood sugar has long been appreciated. Mann (1927) for example, found that while the level of blood sugar decreased in animals with reduced hepatic tissue, a level was

Table 16a

The effect of partial hepatectomy on liver glycogen concentration.

Animal No.	Glycogen concentration (g./100 g. wet wt. of liver)		
	at operation	2 hours later	per cent fall
1	2·34	1·19	49
2	4·58	0·58	87
3	2·89	1·61	44
4	5·18	4·05	22
5	3·88	3·08	21
6	0·75	0·104	86
7	1·01	0·56	46
8	4·02	1·36	66
		average fall in liver glycogen	53

Table 16b

The effect of liver biopsy on liver glycogen concentration.

Animal No.	Glycogen concentration (g./100 g. wet wt. of liver)		
	at operation	2 hours later	per cent fall
1	4·43	1·39	69
2	5·1	0·96	82
3	5·48	1·67	69
4	5·57	4·64	17
5	2·23	1·68	25
6	3·25	2·07	36
7	1·25	0·87	30
8	2·61	1·31	50
		average fall in liver glycogen	47

finally reached at which the blood sugar remained constant, regardless of the further decrease in hepatic tissue. He concluded that the liver stubbornly maintains an adequate level of blood sugar even though greatly incapacitated by the loss of much of its substance.

The extent and rapidity of the process of glycogen disappearance in regenerating liver is shown in Table 16a. It is clear, however, that over the short time interval of 2 hours, a virtually equivalent fall in glycogen takes place if only a small liver biopsy is removed instead of the full two thirds (Table 16b). It seems reasonable to assume that in this latter case, the loss of glycogen is simply a response to stress. In view of the 'labile' nature of this glycogen store and its responsiveness to stress, it would obviously be difficult to relate changes in its concentration in liver to the regeneration process.

Changes in lipid concentration.

Lipid infiltration of the residual liver fragment is one of the earliest and most striking changes in liver regeneration. Although this phenomenon has been investigated from various points of view neither its underlying cause not its physiological significance has been fully elucidated.

Table 17

The concentration of total lipid and phospholipid P in the liver fragment at time intervals after partial hepatectomy.

Animal No.	Time interval between partial hepatectomy and sacrifice (hr.)	Liver Total lipid (g./100g.wet wt.)		Liver Phospholipid P (mg./100g.wet wt.)	
		before operation	at death	before operation	at death
1	2	2·56	4·74	123	176
2	2	3·42	4·58	148	158
3	4	3·59	4·97	182	164
4	4	4·02	6·26	178	181
5	8	4·67	7·94	177	173
6	8	4·02	8·05	162	189
7	10	4·17	9·08	140	232
8	10	4·83	5·17	173	153
9	12	4·88	8·01	157	201
10	12	2·03	10·06	82	196
11	24	4·12	11·73	160	170
12	24	3·84	7·91	163	163

Table 18

The concentration of total lipid and phospholipid P in the liver and plasma at time intervals after liver biopsy.

Animal No.	Time between biopsy & death (hr.)	Plasma		Liver			
		Phosphol. P. mg/100ml.	Total lipid mg/100ml.	phospholipid P. mg/100g.		Total lipid g./100g.	
				at oper.	at death	at oper.	at death
1	6	9·63	285	83	171	3·50	3·72
2	6	8·34	260	110	170	4·01	3·98
3	12	8·89	278	-	178	3·87	3·95
4	12	7·85	285	-	161	2·73	3·29
5	24	6·96	226	-	159	2·98	5·16

Harkness (1952b) found that lipid concentration was already maximal 10 hours after the operation although Szego and Roberts (1949) reported that the maximum came later. The results of a re-examination of the extent of lipid deposition at intervals after partial hepatectomy is shown in Table 17. An increase in total lipid is already apparent at 2 hours and increases steadily from a control value of 3 to 4 per cent to reach 8 to 12 per cent between 12 and 24 hours. Since very little change occurred in the phospholipid fraction, the major part of this increase must have been due to non-phospholipid. This is in agreement with the finding of Ludewig, Minor and Hortenstine (1939) that the accumulated lipid is mainly neutral fat. Removal of a small biopsy from the liver also caused a small increase in non-phospholipid the effect being most pronounced after 24 hours (Table 18). A more detailed examination of liver lipid concentration 6 hours after surgical biopsy (Table 19b) shows that although there is some variation of response, a definite increase is evident, the values ranging from 3 to 30 per cent. By comparison, a similar series, previously partially hepatectomised, gave a much larger increase after the same time, the values ranging from 29 to 81 per cent (Table 19a).

Table 19a

The concentration of total lipid in rat liver before and 6 hours after partial hepatectomy.

Animal No.	Total lipid g./100 g. liver (wet wt.)		per cent increase
	before	after	
1	3·06	5·50	80
2	3·04	5·50	81
3	2·94	3·78	29
4	2·23	3·45	55
5	2·78	3·72	34
		average increase	55·8

Table 19b

The concentration of total lipid in rat liver before and 6 hours after liver biopsy.

Animal No.	Total lipid g./100 g. liver (wet wt.)		per cent increase
	before	after	
1	2·80	3·03	8
2	2·42	3·15	30
3	3·05	3·83	26
4	2·03	2·53	25
5	2·82	3·62	28
6	2·94	3·03	3
		average increase	24

The two population means of tables 19a and 19b are significantly different. ($P < 0.05$)

The question of the origin of this increased non-phospholipid occurring in the liver after both partial hepatectomy and biopsy remained to be answered. Chanutin and Gjessing (1949a, b) observed an elevated serum level of total lipid in rats at one and two days after partial hepatectomy. Janion and Szenberg (1959) obtained evidence for the view that fatty infiltration occurring in the early stages of liver regeneration is partly due to transfer of reserve fat from fat depots to the liver. It is clear from Figures 14a and 14b that an elevation of plasma total lipid does follow partial hepatectomy and that it is most pronounced in the first 4 hours. It is also clear that very little change occurs in the plasma phospholipid, so that the increased lipid concentration must be ascribed to the other fractions. However, as can be seen from Figure 14a and Table 18 there is also a substantial, sustained rise in plasma total lipid after sham operation.

Tables 20 and 21 present the results of two series of experiments to test whether in a cross-circulation the liver of an intact partner (Table 20), or of a biopsied partner (Table 21) was able to compensate for the loss of liver substance in the partially hepatectomised partner at least in its ability to metabolise lipid. It is however,

FIGURE 14 a

The effect of sham operation and partial hepatectomy on the level of total lipid in the circulating blood plasma of the rat. Each experiment was performed on one animal.

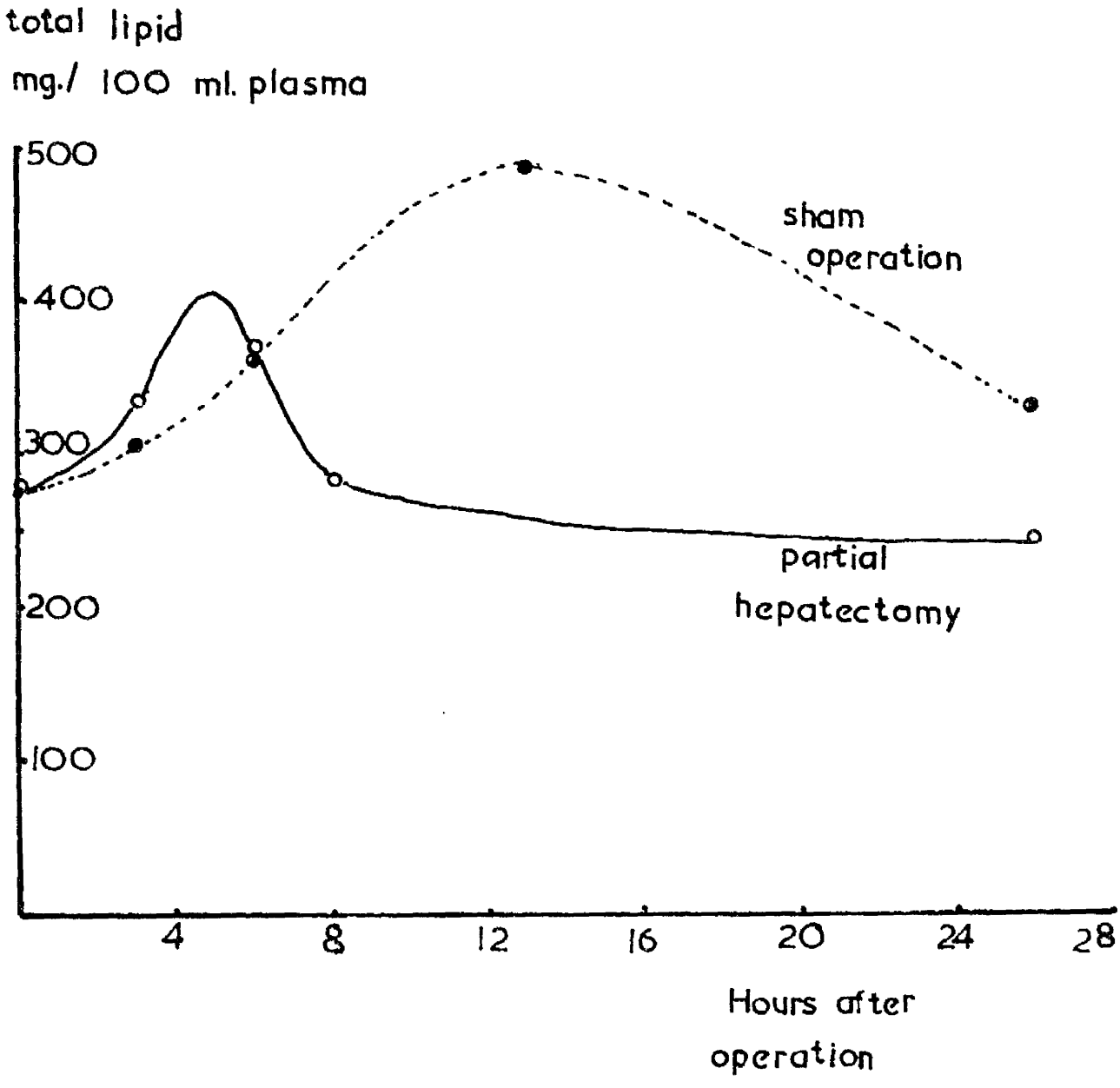

total lipid
mg./ 100 ml. plasma
500
400
300
200
100
sham
operation
partial
hepatectomy
4
8
12
16
20
24
28
Hours after
operation

FIGURE 14 a

FIGURE 14 b

The effect of partial hepatectomy on the levels of total lipid and phospholipid P in the circulating blood plasma of the rat. One animal was used for each point.

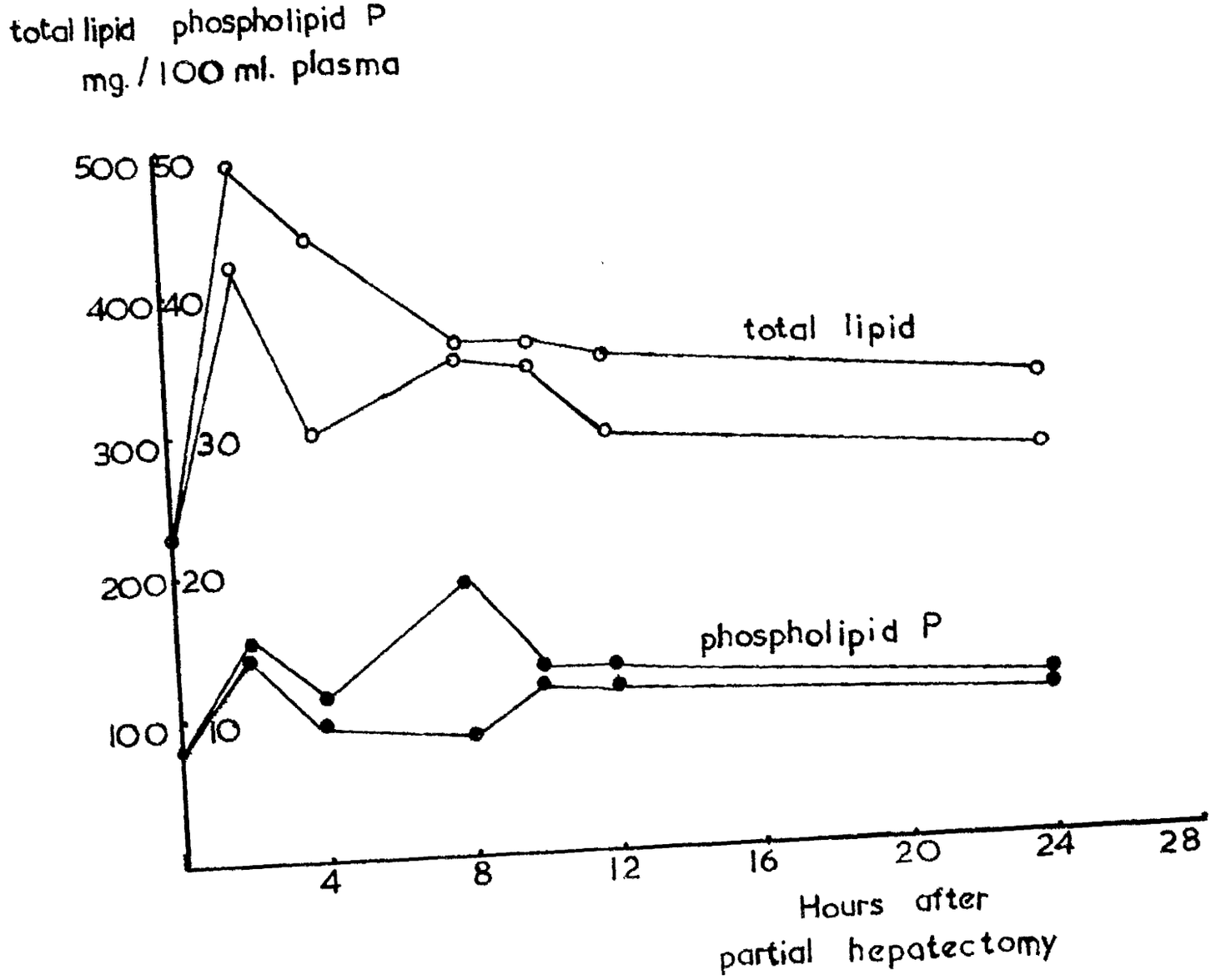

total lipid phospholipid P
mg. / 100 ml. plasma
500
400
300
200
100
50
40
30
20
10
total lipid
phospholipid P
4
8
12
16
20
24
28
Hours after
partial hepatectomy

FIGURE 14 b

clear that after cross-circulation for 6 hours, the liver of the unoperated partner has a substantially lower lipid concentration than the residual liver fragment of the hepatectomised partner (Table 20). The cross-circulation therefore had not abolished the difference between the two partners in this respect. On the other hand, the concentration in the unoperated partner at the end of the cross-circulation was uniformly higher than that in the partially hepatectomised partner at the beginning of the experiment. This suggested that there had been some accumulation of lipid in the liver of the unoperated partner. This conclusion is confirmed by the results shown in Table 21. In this experiment both the partially hepatectomised and biopsied partners showed, on average, an increase in lipid content. However, the increase was significantly larger in the partially hepatectomised animals. It may be concluded, then, that the liver of the normal partner was able to compensate, though not completely, for the deficiency of liver in the partially hepatectomised one with regard to lipid metabolism.

Table 20

The effect of a 6-hour corss-circulation on total lipid concentration in the liver of both the normal intact and the partially hepatectomised rat.

Experiment No.	Liver total lipid conc. (g./100 g. wet wt.) hepatectomised partner before cross-circulation	hepatectomised partner at sacrifice	intact partner at sacrifice
1	2·87	4·17	3·20
2	3·05	5·98	3·52
3	3·12	6·30	3·93
4	2·65	4·73	3·50
5	2·36	5·26	3·41
6	3·21	5·57	4·56
7	3·38	4·23	4·22
8	3·35	4·45	4·60
average conc.	3·00	5·09	3·88

The difference between the mean values of the liver lipid concentration in the two animals at the end of the experiment is highly significant. ($P < 0{\cdot}01$)

Table 21

The effect of a 6-hour cross-circulation on total lipid concentration in the liver of both the biopsied and partially hepatectomised rat.

Experiment No.	Liver total lipid conc. (g./100 g. wet wt.)					
	hepatectomised partner		per cent change	biopsied partner		per cent change
	before	after		before	after	
1	3·01	4·16	38·2	3·83	3·83	0
2	3·23	3·70	14·5	2·92	3·33	14·0
3	2·73	3·28	20·1	2·76	2·64	- 4·3
4	2·84	3·65	28·5	3·03	3·45	13·9
5	2·58	3·52	36·5	2·64	3·09	17·0
	average increase		27·5	average increase		8·12

The difference between the mean lipid increase for the two animals after the cross-circulation is significant. ($P < 0{\cdot}02$)

The mechanism of liver lipid deposition after partial hepatectomy.

That the liver plays a significant role in the intermediary metabolism of lipids has long been appreciated. Since this organ is concerned not only with the oxidation of fats but also their synthesis and retransport in lipoprotein form, a change in liver lipid concentration may theoretically be due to a change in any of these activities. The results reported in this section concerning elevated plasma lipid levels support the view that increased lipid deposition is partly due to an increased delivery of lipids to the liver. Even a normal rate of delivery of lipid to a liver one third normal size might be expected to exceed its capacity to metabolise it. The attainment of a liver lipid concentration of about three times control value after about 12 hours suggests that there is a homeostatic control of the total amount of lipid in the liver. This conclusion is, of course, complicated by the fact that the proportions of the various fractions are altered, there being a relatively greater increase in neutral lipid.

Recently Steinberg (1963) has demonstrated that in dogs with intact livers, an increased deposition of neutral lipid can be produced by an intravenous infusion of nor-

adrenaline and also of free fatty acids (FFA). That noradrenaline was not mediating its action by hepatotoxic effect but by a FFA mobilizing action was indicated by the finding that intraportal infusion caused no elevation of liver lipid but that switching the infusion to a peripheral vein caused the usual increase in liver triglycerides. It is known from the work of Frederickson and Gordon (1958) that free FFA complexed to serum albumin are a major transport form of fat. Further it was discovered that serum FFA is under the controlling influence of hormones (Langdon, 1960). Adrenocorticotrophic hormone, adrenaline, noradrenaline, Growth Hormone and Thyroid Stimulating Hormone all increase, whereas insulin lowers the level of serum FFA. On the basis of his results on dogs, Steinberg (1963) concluded that elevation of serum FFA concentrations per se was an adequate basis for the development of fatty liver. He further proposed that an "excessively rapid mobilization of FFA to the liver, even though the latter is functioning normally, can exceed the capacity of the liver to dispose of FFA by oxidation or resecretion in ester form via lipoproteins".

According to this theory, fatty liver would be expected to occur on the basis of an imbalance between the rate of uptake of FFA by the liver and the capacity of the

liver to dispose of FFA by,

a) oxidation,

b) esterification and retransport in lipoprotein form.

Provided there is no alteration in the rate of oxidation or esterification of FFA in the liver fragment after partial hepatectomy, a similar explanation might account for the accumulation of neutral fat which occurs in the early stages of regeneration. In this case, although the elevation of total lipid in plasma was found to be marked only for the first few hours after operation, perhaps a normal rate of delivery of lipid in the form of FFA to a liver reduced to one third of control size would be sufficient to account for the accumulation.

<u>The physiological significance of lipid accumulation in regenerating liver.</u>

An obvious explanation of lipid accumulation is that it represents a state of relative hepatic insufficiency i.e., the liver fragment is being functionally overloaded. Evidence which supports this concept is available from the results of the cross-circulation experiments already described (Tables 20, 21). The liver of the intact or biopsied partner

of a cross-circulated pair was able to assist, to some extent, the liver fragment of the partially hepatectomised partner in its ability to metabolise lipid.

Indirect support for this view is provided by the observations of other workers. MacKay and Carne (1938) found that both adrenalectomy and infusion of glucose markedly reduced the amount of the lipid deposition in the liver remnant of rats after partial hepatectomy. Ferrari and Harkness (1954) also noted the inhibitory effect of adrenalectomy on lipid deposition after partial hepatectomy. Both of these effects can now be understood in terms of the theory of Steinberg (1963). It has been shown that both insulin and glucose act by inhibiting the release of FFA from adipose tissue. It is probable that an elevated level of glucose in vivo stimulates the endogenous secretion of insulin. Moreover, according to Steinberg (1963), adrenocortical steroids condition the metabolic state of adipose tissue to permit maximum response to catecholamines e.g., adrenaline. It seems likely, then, that the effect of glucose infusion and adrenalectomy in diminishing lipid accumulation in liver after partial hepatectomy is mediated by their known actions on the serum level of FFA.

A second possibility is that the increased con-

contration of lipid in the liver fragment after partial hepatectomy might facilitate regeneration by providing more energy for the synthesis of nitrogen containing components i.e., proteins, RNA and DNA. The work of Campbell and Kosterlitz (1948), Munro and Naismith (1953), Calloway and Spector (1955) has demonstrated that the protein content of rat liver varies with variations in energy intake. The general conclusion derived from this work is that the protein content of the liver is sensitive to caloric intake when the diet contains adequate amounts of protein. Munro and Naismith (1953) investigated the effect of dietary addition of carbohydrate and fat to the protein nitrogen content of rat liver. They found that each nutrient had similar effects in promoting increase of protein nitrogen content. These authors found moreover, that although most of the nitrogen retained as a result of increasing caloric intake was deposited in the carcass, because of the small size of the liver relative to the carcass, the effect on liver protein content was much more dramatic than on carcass protein content. It seems probable that energy intake influences utilization of amino acids for all synthetic processes.

Although these conclusions regarding the beneficial effect of increased carbohydrate and lipid intake on nitrogen

Table 22

Comparison of the RNAP and DNAP content of median, left lateral and right lateral lobes of the liver of a rat. The results for each lobe are in duplicate.

Lobe	μg./g. wet wt. liver		
	RNAP	DNAP	$\frac{\text{RNAP}}{\text{DNAP}}$
Median	903·6	286·0	3·16
	955·2	298·0	3·20
Left lateral	847·6	266·0	3·18
	866·8	256·0	3·38
Right lateral	801·2	224·0	3·56
	801·2	252·0	3·18

retention by the tissues have been derived from dietary studies, it seems reasonable to apply them to the regenerating liver fragment. Since there is an almost threefold increase in the perfusion rate of the liver fragment after partial hepatectomy, the supply to the liver of glucose, fatty acids and amino acids is presumably also trebled.

Changes in RNA and protein concentration.

One of the earliest manifestations of regeneration in the liver is an increase in RNA concentration (Harkness, 1957). Before investigating this change, however, it was obviously desirable to make sure that the different parts of the liver were indeed chemically uniform with respect to RNA concentration. The results of analyses of the median, left lateral and right lateral lobes of the liver of a single rat are presented in Table 22. From this it can be seen that within the limits of experimental accuracy, the concentration of RNA and DNA do not vary from one lobe to another. Hence, it is reasonable to assume that they are fairly constant throughout the whole liver. The next step in the investigation was to measure the changes in RNA and DNA which took place in the first 24 hours after partial hepatectomy. From the results in Table 23, it can be seen that, while there is

Table 23

The change in RNAP/DNAP ratio 24 hours after partial hepatectomy.

Animal No.	$\frac{\text{RNAP}}{\text{DNAP}}$ before operation	$\frac{\text{RNAP}}{\text{DNAP}}$ after operation	per cent increase
1	3·15	3·80	20·6
2	2·74	3·80	38·6
3	3·02	3·85	27·5
4	3·58	4·28	19·6
5	3·48	4·07	16·0
6	3·57	4·17	15·8
7	2·64	3·75	42
8	2·53	3·70	46·2
9	3·03	3·73	23·1
10	4·32	4·33	0
11	3·28	4·43	35·0
		average increase	25·9

Table 24

The change in RNAP/DANP ratio 12 hours after partial hepatectomy.

Animal No.	RNAP/DNAP before operation	RNAP/DNAP after operation	per cent increase
1	4·00	4·7	17·5
2	4·02	5·30	31·8
3	4·01	4·45	10·97
4	3·82	3·94	3·14
5	3·69	4·75	28·7
6	3·62	4·82	33·1
	average change		20·9

Table 25

The change in protein/DNAP ratio 12 hours after partial hepatectomy.

Animal No.	Protein/DNAP before operation	Protein/DNAP after operation	per cent change
1	358	337	- 5·87
2	332	356	7·22
3	315	317	0
4	200	222	11
5	363	308	-15·2
6	366	409	11·8
		average increase	1·5

considerable variation from one animal to another, the concentration of RNA consistently increased after the operation. Even more significant was the fact that the RNA/DNA ratio, which represents the average RNA content per cell, showed a mean increase of 26 per cent. Table 24 presents the corresponding results for an interval of 12 hours after the operation. While again there is a considerable variation in the response of individual rats, there is a mean increase in RNA/DNA ratio of about 21 per cent. Table 25 shows changes in protein/DNA ratio (the average protein content per cell) after 12 hours. The change in this ratio as a result of the operation varies greatly between animals, but on the average it is very small.

Although the average increase in RNA/DNA ratio of 21 per cent at 12 hours is not very great, it seemed large enough to be used as an indication of regeneration. It was necessary, however, to ensure that it was in fact a response to the partial hepatectomy and not merely to the shock of the operation. Accordingly, the experiment was repeated, but in this case only a small biopsy sample of the liver was removed. The procedure consists in passing a loop of coarse linen thread around the base of the left segment of the cleft median lobe, and ligating it firmly (see Appendix on Methods).

Table 26

The change in RNAP/DNAP ratio 12 hours after biopsy of the liver.

Animal No.	$\frac{RNAP}{DNAP}$ before operation	$\frac{RNAP}{DNAP}$ after operation	per cent change
1	4·02	4·00	0
2	3·74	3·77	0
3	3·72	4·06	9·1
4	4·20	4·14	- 1·4
5	4·19	4·58	9·3
6	3·03	3·23	6·6
		average change	3·9

The average changes in RNAP/DNAP ratio 12 hours after liver biopsy and partial hepatectomy (Table 24) are significantly different. ($P < 0{\cdot}02$)

The liver segment distal to the ligature may be cleanly removed with a scalpel. It constitutes less than one tenth of the liver substance. The results in Table 26 clearly show that the removal of such a biopsy did not significantly alter the RNA/DNA ratio. It may therefore be concluded that the change in the corresponding ratios shown in Table 24 must be a response to the substantial loss of liver substance and not merely to the stress of the operation. These findings are in harmony with the results of Fujioka et al.,(1963) who demonstrated that more than 10 per cent of the liver must be removed before increased incorporation of ^{14}C-orotate into liver RNA occurs.

Table 27 shows the results of a series of cross-circulations in which one of the partners was submitted to partial hepatectomy and the other to excision of a liver biopsy immediately before the cross-circulation was established. It is quite clear that there is a distinct difference between the two partners. On the whole, the biopsied partners show no change or a slight fall in RNA/DNA ratio. On the other hand, the hepatectomised partners show an increase in RNA/DNA although this increase was slightly less than that found in single partially hepatectomised rats at the same time interval (Table 24). These results show that in a cross-

Table 27

Changes in the RNAP/DNAP ratio in the liver of a partially hepatectomised compared to that of a liver-biopsied rat after they had been cross-circulated for 12 hours.

Exp. No.	RNAP/DNAP Biopsied rat			RNAP/DNAP Partially hep., rat		
	before	after	per cent change	before	after	per cent change
1	3·48	3·30	- 5·17	2·68	2·93	9·32
2	3·28	2·97	- 9·45	3·26	3·56	9·20
3	3·56	3·40	- 4·49	2·92	3·52	20·54
4	3·56	3·70	3·93	3·20	3·41	6·56
5	3·52	3·30	- 6·25	3·40	3·07	- 9·7
6	4·04	3·89	- 3·71	3·76	3·92	4·25
7	3·93	3·56	- 9·41	3·98	4·10	3·01
8	3·38	3·19	- 5·62	3·62	3·89	7·45
9	3·67	3·70	0·82	3·81	4·85	27·2
10	2·99	3·24	8·36	3·80	3·90	2·63
11	2·92	3·32	13·7	2·30	2·70	17·39
	average change		- 1·57	average change		8·90

The mean values of the two population increases are significantly different ($P < 0{\cdot}02$).

circulation, partial hepatectomy of one partner does not produce identical regenerative changes in the livers of both partners. Whatever the stimulus may be which causes RNA to accumulate in the residual liver fragment of the hepatectomised animal, it is not effectively transferred to the biopsied partner. This result confirms the negative results obtained in the 48 hour cross-circulation experiments in which mitotic index was used as an index of growth (Section 2.1). Like the latter it is difficult to reconcile with the existence of a humoral mechanism.

Physiological significance of changes in RNA.

The increased synthesis of RNA which occurs in regenerating liver is presumably an integral part of the cellular preparation for cell growth and division. Two pieces of evidence support this point of view.

1) A threshold amount of liver tissue (corresponding to 10 per cent of the total organ) must be removed before an increased incorporation of precursors into liver RNA is observed (Fujioka, Koga & Lieberman, 1963). The removal of a similar amount of tissue, reported as 9·4 to 12·3 per cent, must be removed to effect a

significant stimulation of DNA synthesis (MacDonald, Rogers, & Pechet, 1962).

2) If p-fluorophenyalanine or actinomycin D is injected into partially hepatectomised rats in concentrations which will not effect normal RNA synthesis, the increase in the rate of synthesis produced by partial hepatectomy can be completely suppressed (see Section I.32). In rats treated in this way, a delay occurs in the initiation of DNA synthesis (Fujioka, Koga, & Lieberman, 1963).

If this point of view be accepted, then the absence of increased synthesis of RNA in the liver of the biopsied rat cross-circulated with a partially hepatectomised partner, confirms that no growth changes have been evoked in this liver. It is interesting however, that in these cross-circulation experiments the RNA increase in the livers of the partially hepatectomised partners was, on the average, somewhat less than in single partially hepatectomised animals after the same time interval. This observation suggests that the liver of the normal partner was compensating to some degree, for the deficiency in the partially hepatectomised rat (in other words, it may be that the normal increase in

RNA synthesis after partial hepatectomy may be a response to some aspect of hepatic insufficiency.)

An interesting analogy to this phenomenon, is offered by the response of the remaining kidney of a rat which has been unilaterally nephrectomised. It has been recently demonstrated by Halliburton and Thomson (1965) that although the mitotic response of such a kidney is quite small, the amount of RNA per cell increases dramatically to reach a plateau value of about 30 per cent above control value by 24 hours after operation. Moreover, they have further shown that feeding a high protein diet to rats can cause an RNA increase per cell of the same order. A high urea diet fed to rats also caused an increase of RNA per cell and protein per cell but to a lesser degree. These authors however, think that the increase of RNA per kidney cell in animals on a high protein diet is not entirely due to increased urea production. At any rate, it is possible that some of the increase after uninephrectomy may be attributed to functional overload or work hypertrophy of the remaining kidney.

Table 28

The concentration of soluble sodium, and potassium, in the rat liver before and 10 min., after partial hepatectomy.

Rat No.	Milliequivalents/kg. wet wt. liver			
	Na^+		K^+	
	before	after	before	after
1	32·3	35·5	97·5	88
2	29·7	30	89	100
3	24·8	33·5	92·2	92·8
4	31·2	39·2	97	87·5
5	30·3	33	95	87·8
6	29	35·3	95	97·8
7	32·7	38·3	96·2	86·5

Table 28 (contd.)

The concentration of soluble inorganic phosphorus and water in the rat liver before and 10 min., after partial hepatectomy.

Rat No.	Milliequivalents/ kg. wet wt. liver P_i		Per cent water	
	before	after	before	after
1	14·1	14·2	72·2	75·2
2	12·9	14·3	71	74
3	8·0	9·2	70	73·5
4	14·2	12·8	72	73·8
5	8·3	8·9	68·1	70·1
6	9·3	10·7	68·2	69·8
7	8·9	7·1	69·2	70·3

Table 29

The concentration of soluble sodium and potassium in the rat liver before and 10 min., after sham operation.

Rat No.	Milliequivalents/kg. wet wt. liver			
	Na^+		K^+	
	before	after	before	after
1	39	28·4	105	100
2	31·3	30	100	102·5
3	18·3	22·1	99·5	94·5
4	37·2	31·1	95·0	111·7
5	26·4	26·2	100	99
6	34·7	39·3	87	97

Table 29 (contd.).

The concentration of soluble inorganic phosphorus and water in the rat liver before and 10 min., after sham operation.

Rat No.	Milliequivalents/ kg. wet wt. liver P_i		Per cent water	
	before	after	before	after
1	11·2	8·6	68·3	68·4
2	8·15	9·35	69·3	68·8
3	8·95	10·0	70·8	68·8
4	9·05	11·2	69·8	70
5	8·6	9·6	70·0	69·4
6	9·4	10·6	71·0	71·2

Changes in water and electrolytes.

The changes in the concentrations of RNA, lipid and glycogen in the liver fragment which follow partial hepatectomy are detectable within a few hours after the operation. Presumably therefore the stimulus to regeneration is likely to begin its action very soon after the operation. In this connection it is of particular interest that Lieberman, Gingold, Kane and Short (1965) found a significant increase in sodium and inorganic phosphate concentration in the liver within minutes of partial hepatectomy. Such rapid changes might perhaps be the trigger of the entire regeneration process. Table 28 presents the results of a study of sodium, potassium, inorganic phosphate and water content of the liver, before and 10 min., after partial hepatectomy. As a control, the same changes were studied in liver before and 10 min., after biopsy (Table 29). It is evident that following sham operation, there was no change in any of these elements. However, following partial hepatectomy, there was a consistent though variable increase in sodium, and an increase in water content of 1 to 3 per cent (Table 28). Potassium and phosphate showed no change. Lieberman et al. (1965) however, think that these changes are probably unrelated to the subsequent enzymatic changes

associated with regeneration e.g., the increase in RNA nucleotidyltransferase activity. The significance of these changes in water and ionic concentration remain to be determined.

Section 2.5 Liver function during regeneration.

In view of the known large functional reserve of the liver it might be expected that when two thirds are removed, the remaining third would be able to work at almost three times the normal rate and thus at least partly compensate for the deficiency. Unfortunately, the functions of the liver are numerous and varied and some of the most important, e.g., its role in carbohydrate metabolism and in urea synthesis, are not easy to measure. However it seemed worthwhile to try one simple test of liver function before and after the loss of hepatic tissue. The dyestuff bromsulphthalein is removed from the circulating blood almost exclusively by the liver and excreted in the bile (Hoffman, 1955). The rate of removal of this test substance from the circulating blood per unit of time may therefore be used as an index of functional liver tissue.

Previously, it was believed that the process of removal of bromsulphthalein sodium (BSP) from the plasma of

FIGURE 15

The change in the concentration of bromo-sulphthalein in the blood of two normal rats following intravenous injection of the dyestuff.

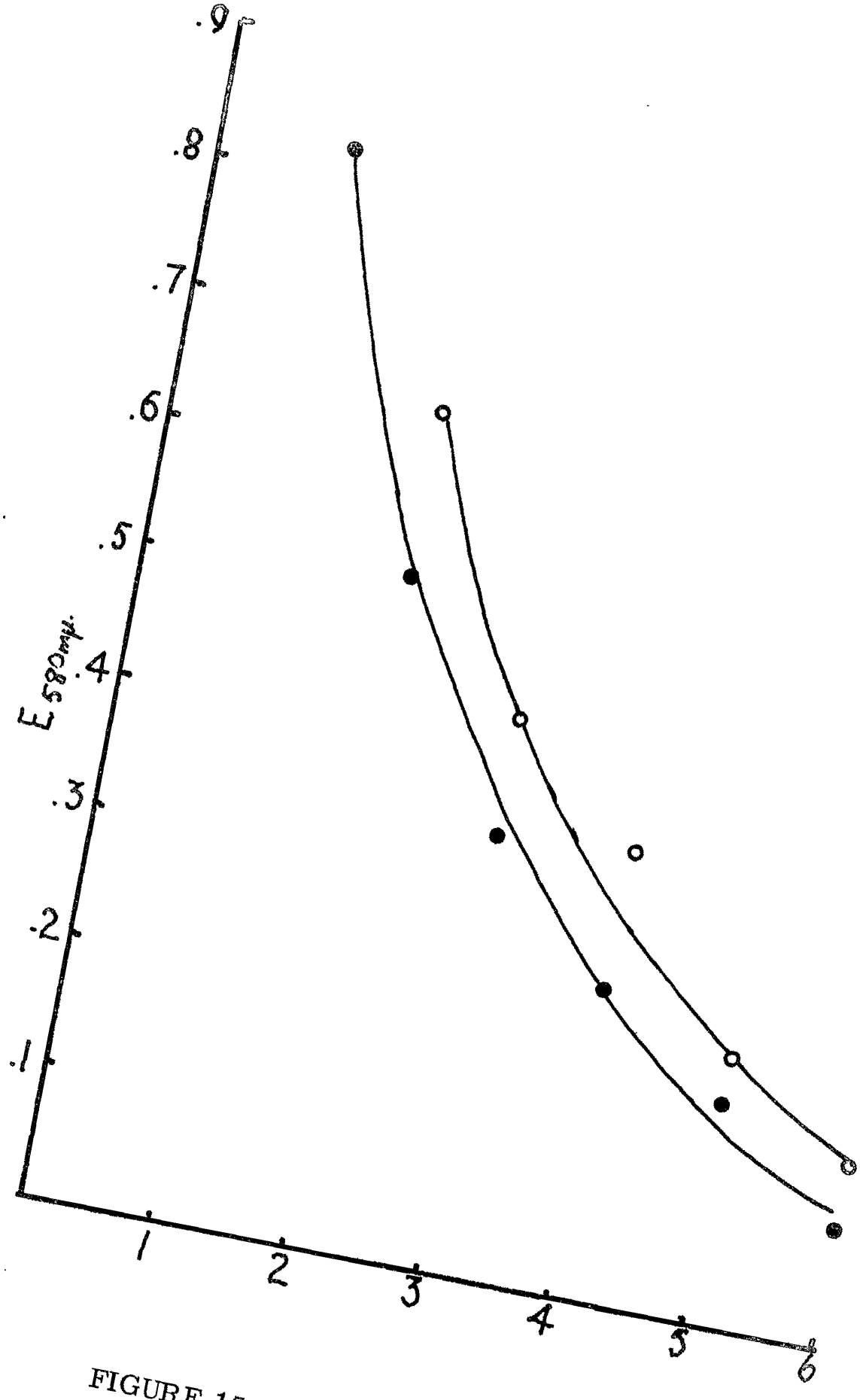


FIGURE 15

FIGURE 16

Clearance of bromsulphthalein from the rat. Two experiments on the same animal at an interval of 37 min. Open circles represent the first experiment with a half-life of 1·65 min., and closed circles represent the second experiment with a half-life of 1·60 min.

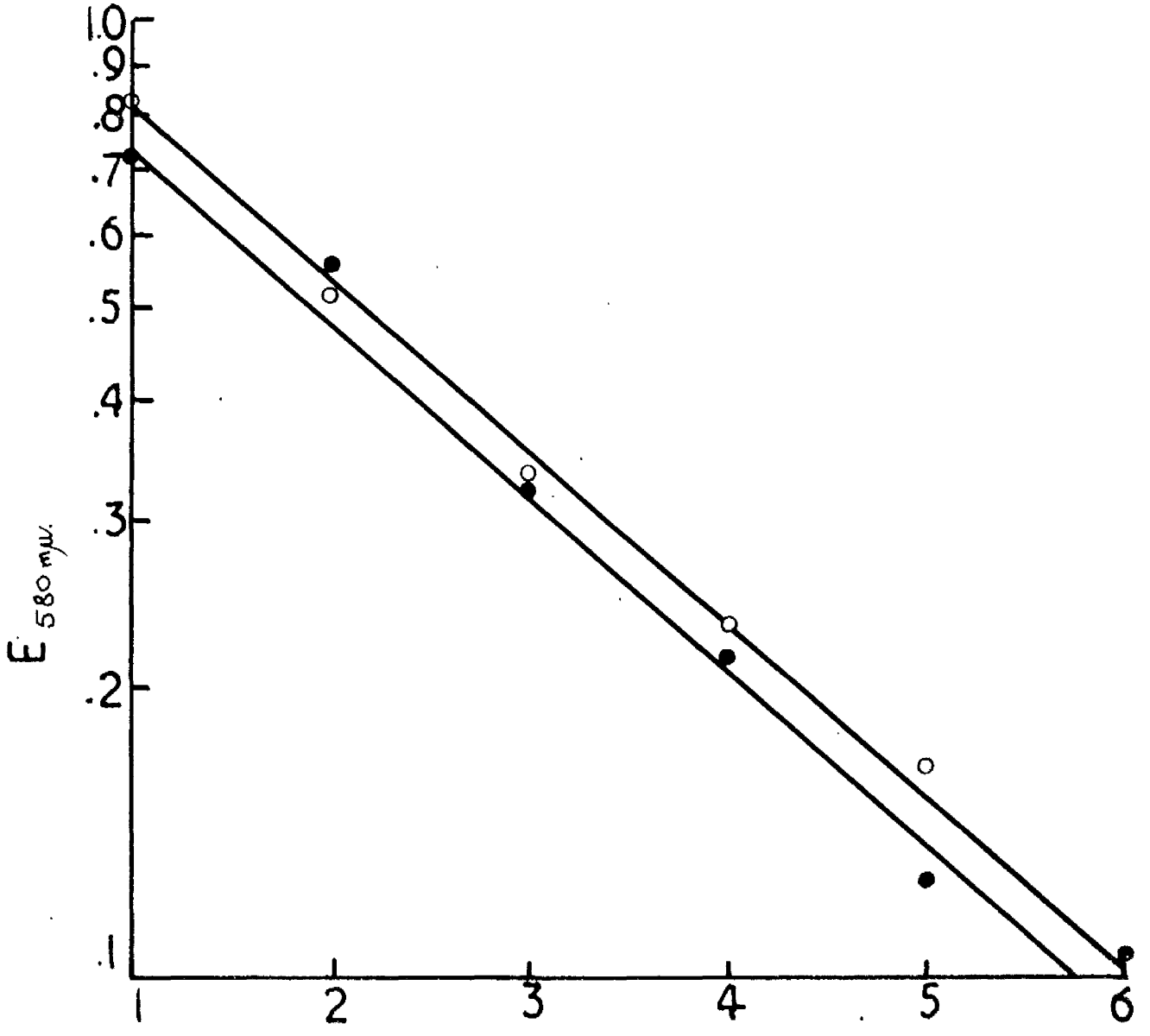


FIGURE 16

FIGURE 17

Clearance of bromsulphthalein from the rat before partial hepatectomy (open circles); half-life 2·10 min., and after partial hepatectomy (closed circles); half-life 6·40 min.

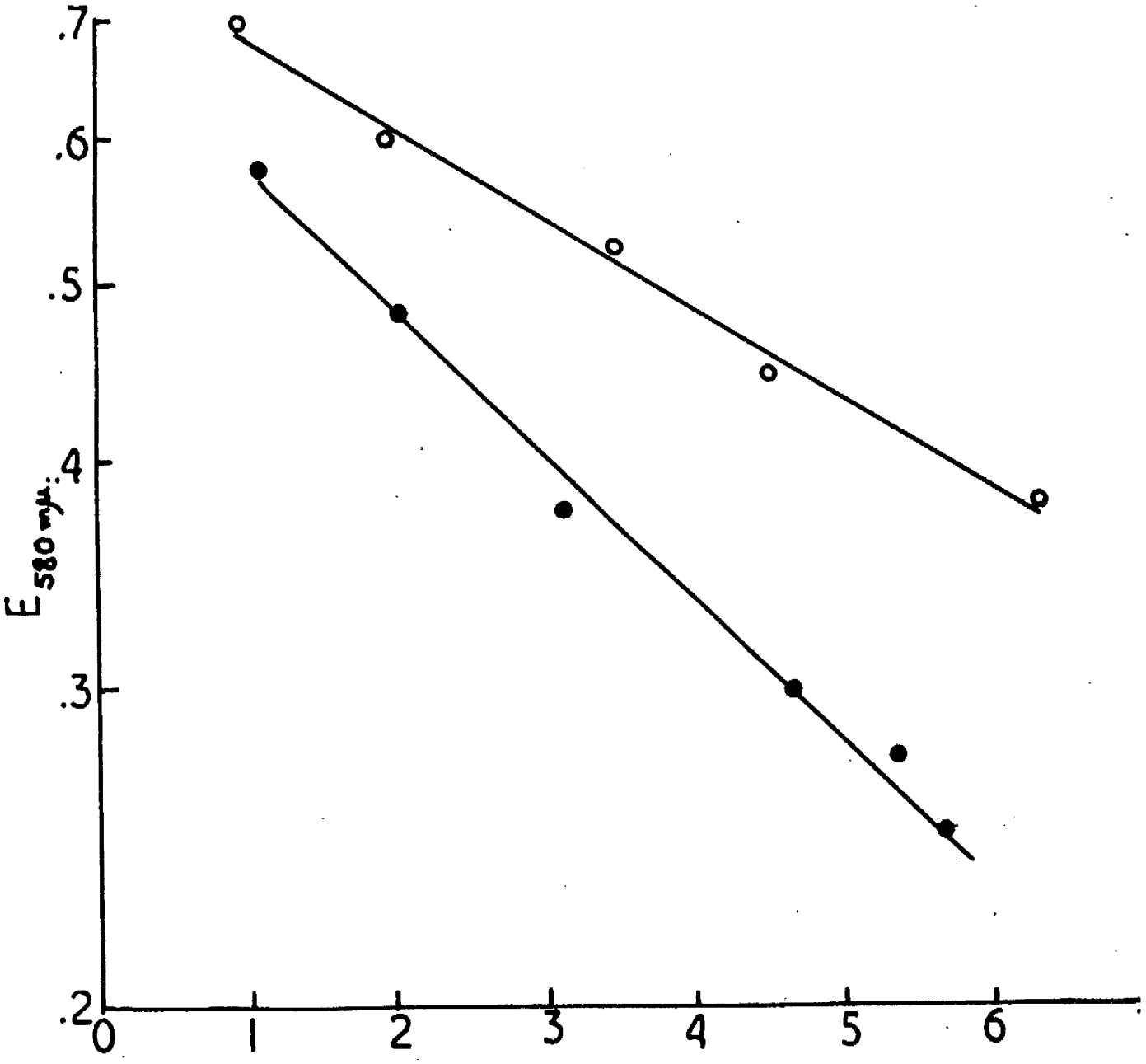


FIGURE 17

animals follows a conventional first-order time course i.e., the rate of disappearance of dye from the blood is proportional to the concentration of dye in the blood (Ingelfinger, 1946; Ingelfinger, Bradley, Mendeloff & Kramer, 1948). This conclusion was derived from work on the human subject within certain restrictions of dose and time of observation. It is not true that plasma clearance of BSP follows first-order kinetics in mammalian species in general (Krebs, 1956). Under the experimental conditions described in the Methods Appendix, however, injected BSP does disappear from the circulation in an exponential fashion, at least within the first 6 min., of observation (Fig. 15). Its disappearance may therefore be plotted on semi-logarithmic graph paper and the rate of disappearance may be represented by a single figure, the half-life. For a normal intact rat the half-life of injected BSP is approximately 2 minutes, and for a given animal could be reproduced after subsequent injections of the dye (Fig. 16); the BSP half-life for any given animal, under similar conditions, seems then to be constant. Partial hepatectomy, involving removal of about two-thirds of the liver, increased the half-life approximately threefold (Fig. 17). Therefore the capacity to excrete the dyestuff

FIGURE 18

Clearance of bromsulphthalein from the rat immediately after partial hepatectomy (open circles); half-life 5·25 min., and again 48 hours later (closed circles); half-life 4·30 min.

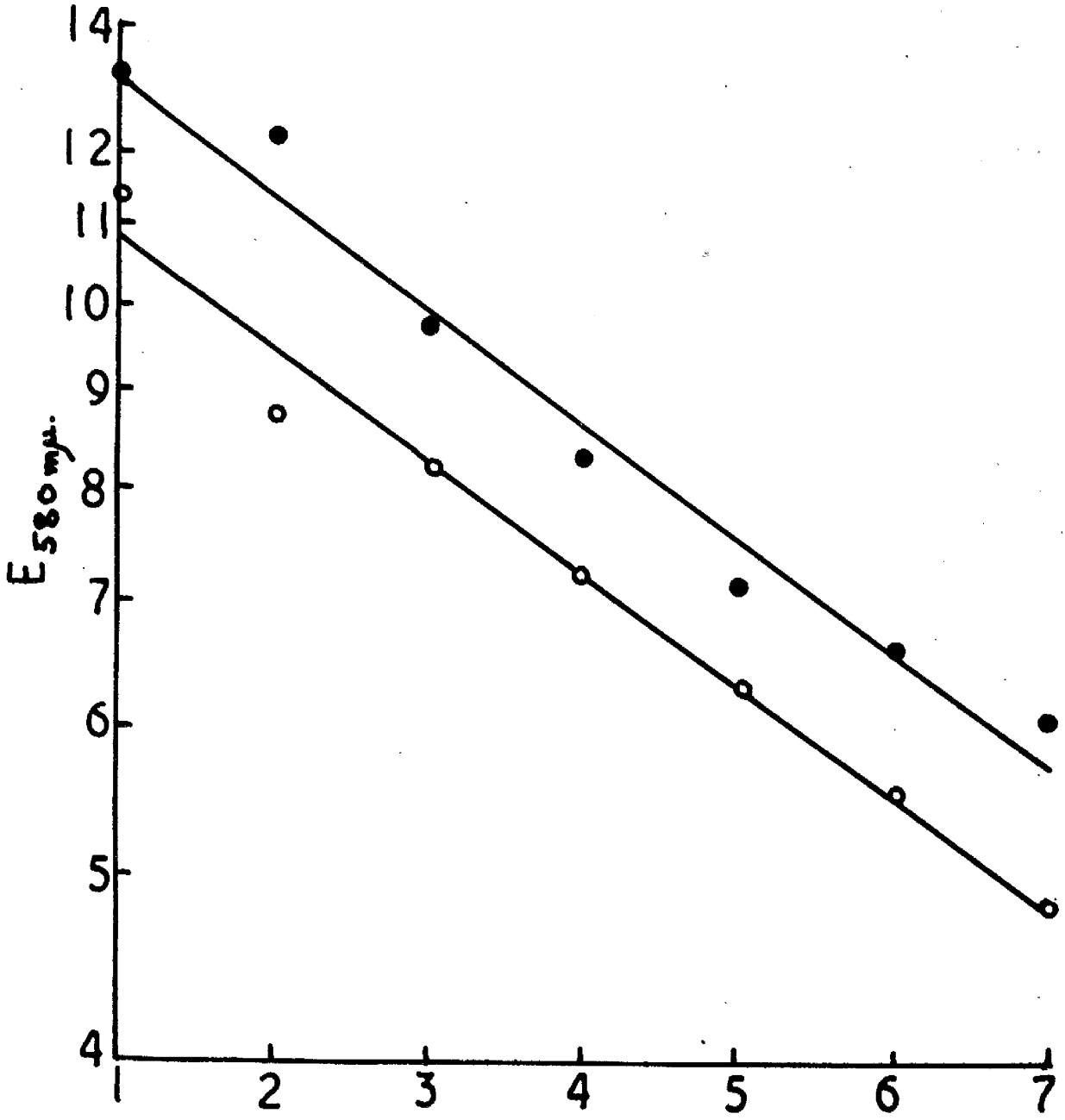


FIGURE 18

FIGURE 19

Clearance of bromsulphthalein from the rat before biopsy (open circles); half-life 2·50 min. and in the same animal 15 min. after biopsy (closed circles); half-life 2·75 min.

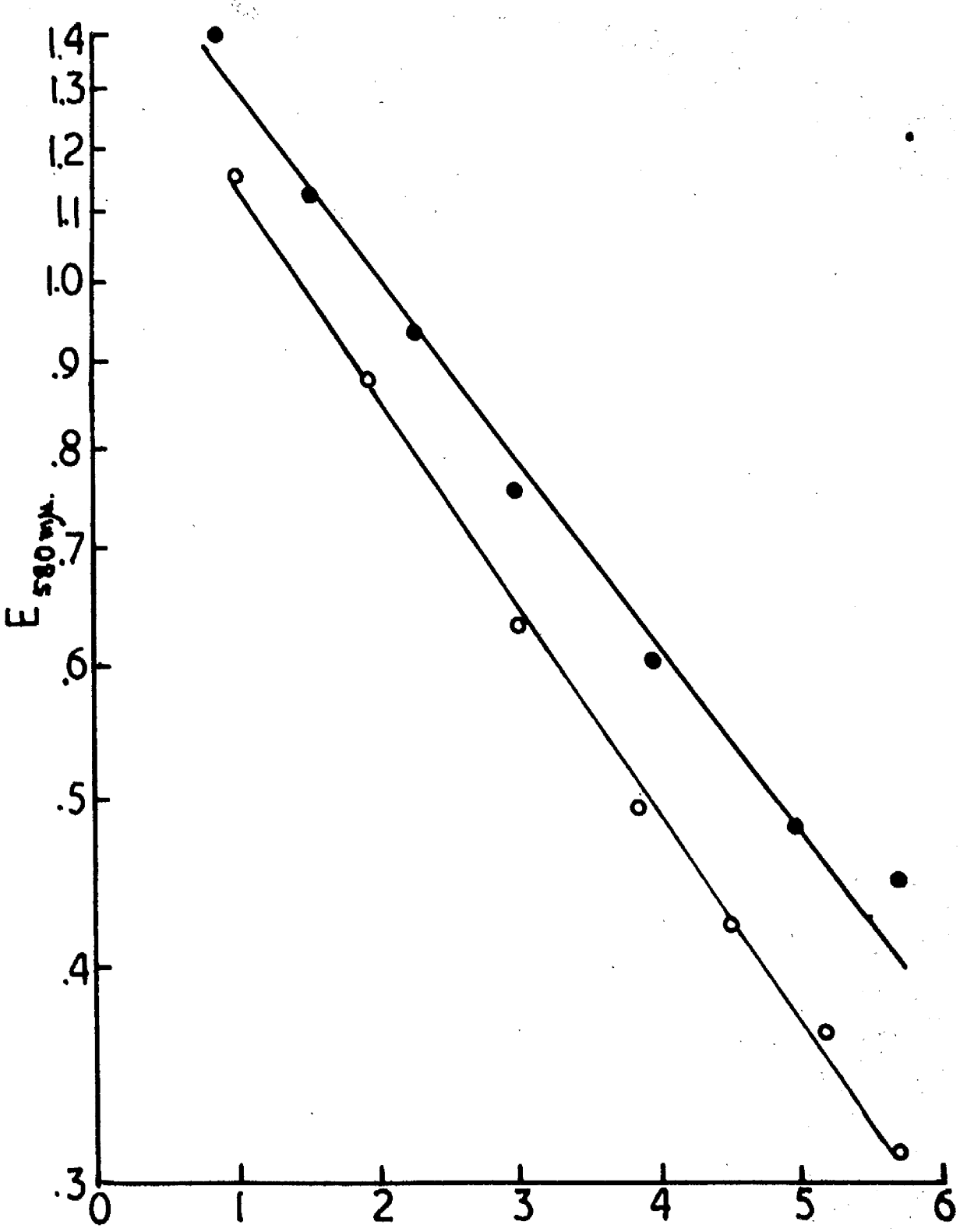


FIGURE 19

may be used as a rough index of functional liver tissue. BSP clearance was determined in partially hepatectomised rats immediately after the operation and after 48 hours (Fig. 18). It is quite clear that little change occurred in the half-life of BSP excretion during this period. With regard to this function, it did not appear that the liver fragment could compensate for the missing lobes by operating three times as efficiently as normal.

It is interesting that liver biopsy, a procedure involving the removal of about 10 per cent of liver substance, caused a barely detectable increase in the half-life of excretion of the dyestuff (Fig. 19). It would seem then that the liver was able to compensate almost completely for a loss of up to 10 per cent of its substance. As already referred to in the previous section, this quantity also represents the threshold amount of tissue which must be removed to initiate increased RNA synthesis, (Fujioka et al. 1963) and to initiate DNA synthesis in liver (MacDonald et al. 1962).

The experiment involving the injection of ^{15}Cr-labelled erythrocytes confirmed that the cross-circulation of rats gave an exchange of blood of the order of one quarter of each animal's blood volume per minute (Section 2.1). It

FIGURE 20

Clearance of bromsulphthalein from two normal cross-circulated rats after injection of the dye-stuff into one of the partners (open circles). The closed circles represent the non-injected partner.

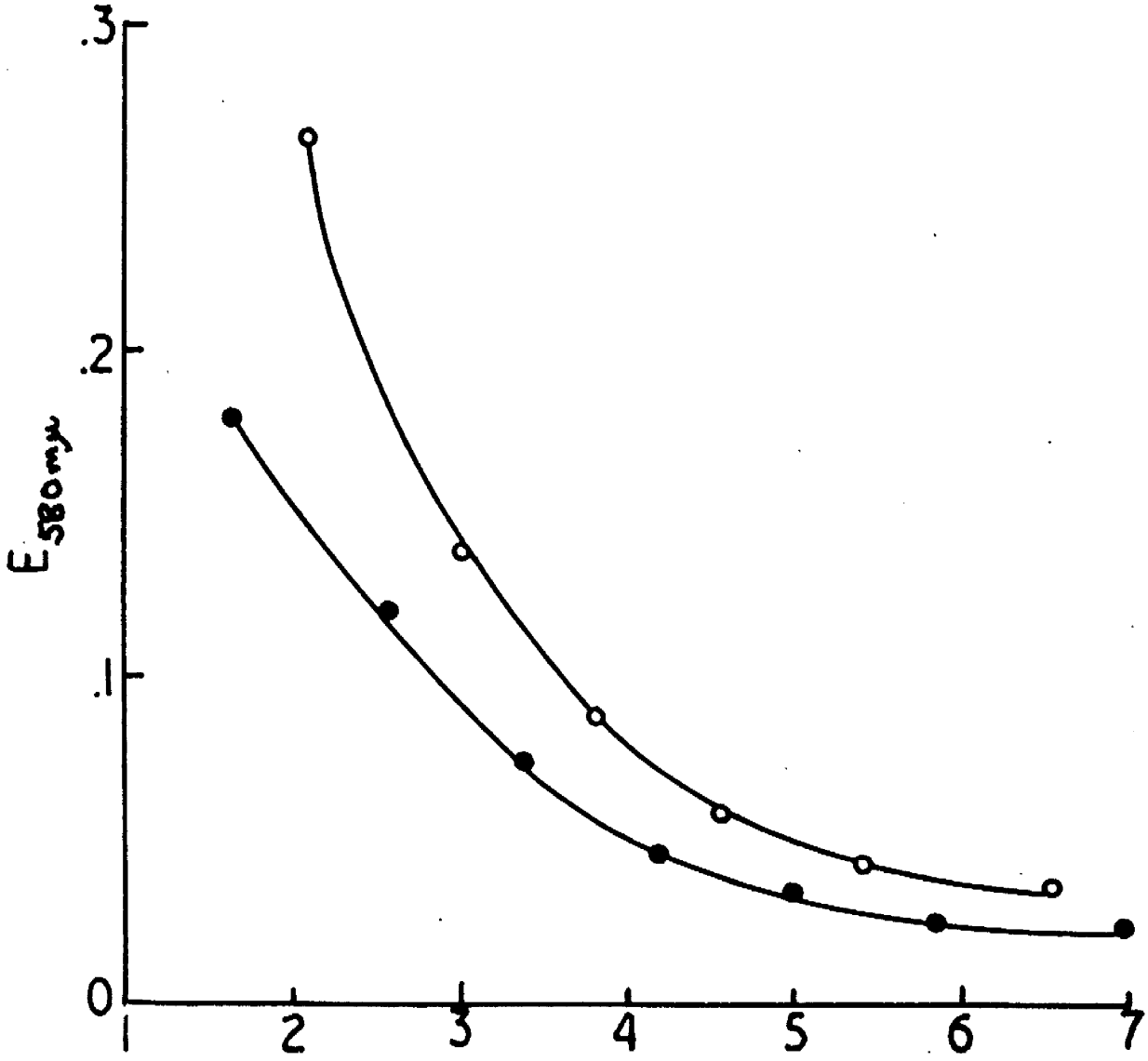


FIGURE 20

FIGURE 21

Clearance of bromsulphthalein from two cross-circulated rats. One partner was partially hepatectomised and injected with the dyestuff (open circles). The closed circles represent the non-injected, normal partner.

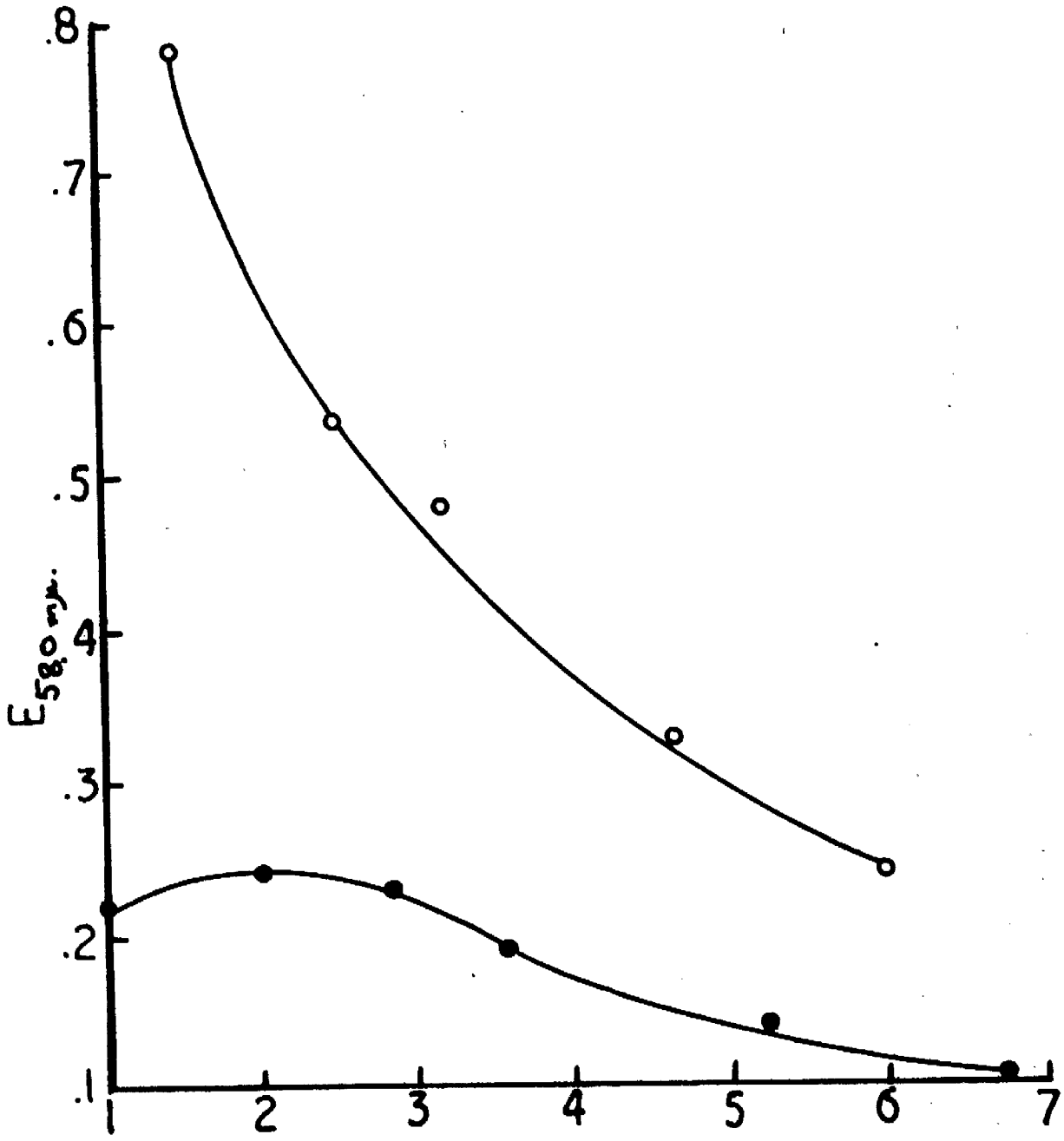


FIGURE 21

seemed worthwhile to demonstrate this exchange by another entirely different method. It was decided to investigate whether a rapidly metabolised compound would get from one partner to the other in a cross-circulation. BSP seemed to lend itself to this purpose since, as shown above, it is removed from the circulation of a normal animal with a half-life of about 2 minutes. Fig. 20 shows the result of one such experiment in which a cross-circulation was established between two normal animals and the usual quantity of BSP was injected into one of them. It is evident, that although rapidly removed from the circulation it is transferred rapidly from the injected rat to its partner. Fig. 21 shows a similar experiment in which one partner was hepatectomised.

Finally Fig. 22 shows the effect of rate of clearance of BSP from a partially hepatectomised rat before and after the establishment of a cross-circulation. In the first case the BSP half-life was 6·2 minutes; cross-circulation reduced this figure to 3·6 minutes. Clearly, therefore in a cross-circulation the intact partner can make a substantial contribution toward compensating the partially hepatectomised partner for its hepatic insufficiency, at least so far as the excretory function is concerned.

FIGURE 22

Clearance of bromsulphthalein from a partially hepatectomised rat before (open circles) and after (closed circles) establishment of cross-circulation with a normal partner. The half-life of clearance before cross-circulation was 6·2 min. and after cross-circulation was 3·6 min.

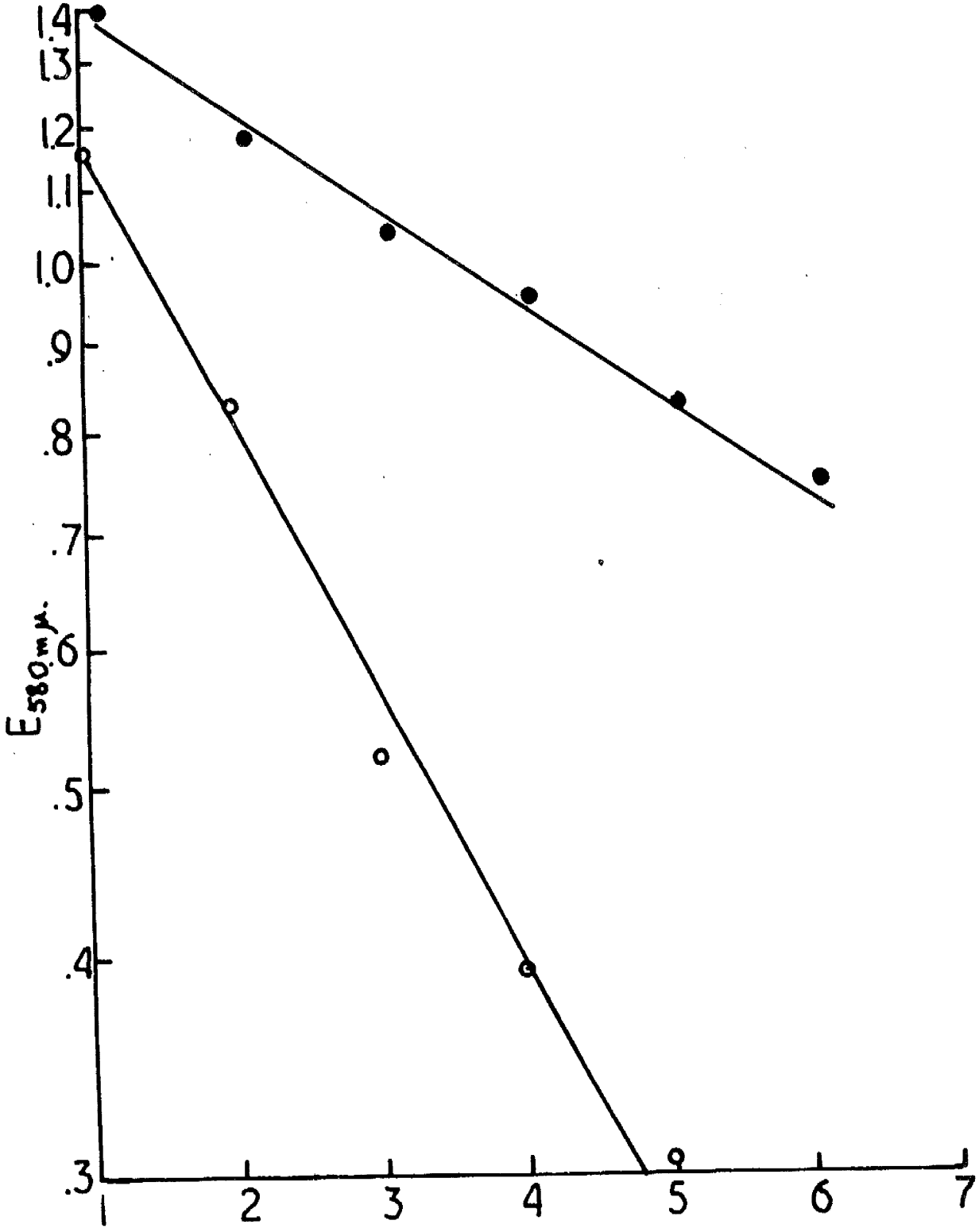


FIGURE 22

Section 2.6 Changes in free amino acid nitrogen in blood and in the liver fragment after partial hepatectomy.

In view of the established association of increased concentrations of amino acids with the growth of several types of tissues e.g., yeasts (Roine, 1946), bacteria (Gale, 1947) and also the key role played by the liver in their metabolism, it seemed natural to look for changes in their concentration in the liver fragment and blood after partial hepatectomy. If the liver fragment is overloaded with amino acids this may be partly responsible for the stimulus to compensatory growth.

Regarding the changes in free amino acids in the circulating blood, different results have been obtained by different workers. For example, Des Marais and Dugal (1948) found a rise in plasma free amino acids in the first day after partial hepatectomy. On the other hand, Christensen, Rothwell, Sears and Streicher (1948) obtained only a very small increase 24 hours after partial hepatectomy and Ferrari and Harkness (1954) found no significant change during the same period of time. In the present investigation it was found that sham operation (Table 30) (delivery of the median and left lateral lobes through an abdominal incision

Table 30

Comparison of the effect of cannulation and cannulation plus partial hepatectomy on the blood soluble amino acid nitrogen in rats.

Time interval after operation (min.)	µg amino acid nitrogen/ml. blood	
	sham operation	intact control
0	46•4	46•8
15	58•8	48•0
30	52•2	48•0
60	41•5	49•0
90	47•4	49•5
120	42•7	49•0

Table 31

Comparison of the operations of sham hepatectomy and partial hepatectomy on the blood soluble amino acid nitrogen in rats.

Time after operation (min.)	Sham operation	Partial hepatectomy
Short term experiment		
0	52·2	50·3
15	48·5	59
30	54·0	62·2
45	45·6	55·8
60	52·8	61·2
90	60	60
120	71·5	59·5
250	52·2	80
Long term experiment		
(hours)		
0	52·5	57
4	42	53·5
8	48	57
11	55	66

followed by their replacement and suture of the wound) caused a temporary elevation of the blood level of free amino acids. Table 31 compares changes in the blood level of free amino acids after partial hepatectomy and after sham operation. In the short term experiment, it is clear that, with the exception of the value at 250 minutes, the elevation which occurred was of the same order in both animals. Again, in the long term experiment little difference was observed between the test and control animal. These results agree with the findings of Ferrari and Harkness (1954). The changes observed seemed too small and irregular to justify further investigation along these lines.

However, the possibility still had to be considered that even though little or no change occurred in the blood free amino acids, after partial hepatectomy, the liver fragment might be taking up or releasing more amino acids than before. Consequently, a change in the free amino acid pool might occur in this way. Table 32 presents results which are suggestive that, at least for a short time after partial hepatectomy, the liver fragment may be taking up more amino acids than previously. Unfortunately, owing to technical difficulties, this experiment was difficult to repeat.

Table 32

Comparison of the effect of sham hepatectomy and partial hepatectomy on the blood soluble amino acid nitrogen in the portal and hepatic veins in rats.

Time interval after operation (min.)	Source of blood	Sham operation	Diff.	Partial hepatectomy	Diff.
0	portal v.	56•5		60	
			5•5		0•2
	hepatic v.	51		59•8	
15	portal v.	57•6		63	
			1•4		12•0
	hepatic v.	56•2		51	
30	portal v.	50•5		47•5	
			-0•5		1•3
	hepatic v.	51		46•2	

It is clear from Table 33 that no consistent change in the concentration of amino acids of the liver fragment from the control level could be detected after 6 and also after 12 hours of regeneration. These findings agree with Ferrari and Harkness (1954) who observed a change only after 24 hours, though Braun, Marsh and Drabkin (1962) obtained an increase after 14 hours.

Although no increase in total free amino acids in liver was detectable before 14 hours after partial hepatectomy indirect evidence suggests that their uptake from the blood plasma relative to the liver size is greatly increased. As already described in the Introduction (Section I.50), the production of urea per unit weight of liver is greatly augmented after partial hepatectomy (Thomson & Moss, 1955). If, as maintained by Christensen (1964) "the liver is geared to destroy amino acids constantly, and to destroy them even faster when they are furnished in large amounts," it may be supposed that even though the flow of amino acids to the liver increased after partial hepatectomy, its capacity to oxidise them and convert the amino nitrogen to urea might prevent the appearance of increased amounts of free amino acids, at least for a time. The increased concentration in the liver fragment at 6 hours of glutamic and aspartic acid

Table 33

The effect of partial hepatectomy on the soluble amino acid nitrogen in rat liver, before and after 6 and 12 hour intervals.

Animal No.		Soluble amino acid nitrogen µg/g. liver (wet wt.) 6 hours	difference	12 hours	difference
1	before	300	100	261	1
	after	400		262	
2	before	286	100	338	60
	after	386		398	
3	before	308	- 3	300	- 29
	after	305		271	
4	before	365	- 57	369	22
	after	308		391	
5	before	340	- 48	265	22
	after	292		287	
6	before	305	11	247	160
	after	316		407	

The mean alterations in amino acid nitrogen concentration both at 6 and 12 hours are not significantly different from zero ($P > 0.05$).

and the decrease in glutamine may be related to this increased urea production. The significance of the increase in liver lysine concentration after partial hepatectomy reported by Ferrari and Harkness (1954) and confirmed by Fujioka et al (1963) is not so easy to explain. The former authors suggested that it may be related to the fact of its metabolic inertness, i.e., that it does not undergo transamination like the other amino acids. Since the flow of amino acids to the liver must be increased after the operation, it would be expected that those amino acids which are metabolised slowly would accumulate. Another factor which may contribute to the increase in liver free amino acid concentration is a diminution in the rate of amino acid catabolism which has been reported during the first post-operative day (Burke, 1962).

The capacity of the liver to take up amino acids in increased quantities from the blood was demonstrated in dogs by means of intra-arterial infusion (Van Slyke & Meyer, 1913a,b &c)The increase in amino acid concentration in liver was greater than muscle as a result of the infusion but the major quantitative site of deposition was muscle owing to the fact that it represented 40 per cent of the body weight as opposed to 3 per cent for liver. These authors concluded

therefore, that muscle is a major reservoir in the homeostasis of amino acid metabolism.

Gurd, Vars and Ravdin (1948) studied the regeneration of liver protein after partial hepatectomy both in protein fed and protein depleted rats. They found that not only was liver protein regenerated independent of dietary protein, but also that in terms of percentage increase, the rate of appearance of new liver protein was greater in the protein-starved than in the casein-fed series. These authors suggested that this indicates a more potent stimulus to liver protein recovery in the animals with a more severe reduction of liver substance. The liver fragment after partial hepatectomy in protein depleted rats, being smaller in size in relation to body weight, will experience a greater inflow of endogenous amino acids from the tissues.

Braun, Marsh and Drabkin (1962) have pointed out the analogy between the responses of the liver after partial hepatectomy and in the nephrotic state. In the severly nephrotic rat a large proportion of the circulating plasma albumin is lost daily in the urine. To meet this emergency Marsh and Braun (1958) have demonstrated that amino acids are mobilized mainly from muscle to the liver where they are channelled into the production of plasma proteins

all of which are presumably synthesised at greatly accelerated rates (Marsh & Drabkin, 1960). These changes are accompanied by an increase liver size, RNA and DNA (Marsh & Drabkin, 1958). This suggested to these authors that liver regeneration after partial hepatectomy and liver hypertrophy in nephrosis are both conditioned by the amount of metabolic work the tissue is called upon to perform. They found that in nephrotic rats no consistent change could be observed in the concentration of free amino acids in plasma but that there was a consistent fall in muscle (1962).* They assumed that the increased rate of removal of amino acids from blood to liver and their increased rate of incorporation into plasma proteins in liver may make plasma changes difficult to demonstrate. In this connection, it is interesting that Christensen et al. (1948) found a slight decrease in muscle α-amino nitrogen 18 to 20 hours after partial hepatectomy. The two conditions differ in that while in nephrosis amino acids are used almost entirely for the synthesis of plasma proteins, in the early stages of regeneration a greater increase occurs in the synthesis of total liver protein.

* Drabkin, Marsh & Braun (1962)

Section 2.7 Induction of regenerative changes in normal intact rat liver.

The previous sections have described the results of investigations of the regenerating liver fragment from various points of view. On the basis they provided, an attempt had to be made to formulate a new experimental approach to the problem of the regulation of liver cell growth in vivo. As shown in Section 2.2 the blood flow per se could not account for alterations in the rate of liver cell growth. It was a reasonable assumption that the factor controlling liver cell growth was some unspecified chemical change in the circulating blood, but this change was apparently due neither to humoral factors nor to plasma proteins. That oxygen partial pressure in the circulating blood was not a limiting factor was proved by the experiments on arterialisation of the portal vein.

The studies on changes in cell composition, function and amino acid metabolism in the regenerating liver fragment proved more fruitful. In general, they indicated that the residual liver fragment following partial hepatectomy was probably being subjected to a functional overload or "work hypertrophy", and that this condition continued until relieved by restoration of the normal "functional mass" of

the organ. To some extent, it seemed that the situation might be illuminated by comparing regenerating liver with another rapidly growing system i.e., bacterial cells in the logarithmic phase of growth. Step-up cultures of Escherichia Coli cells i.e., cells subcultured from a minimal to an enriched medium show some adaptational changes in cell composition analogous to those occurring in liver cells after partial hepatectomy. Kurland and Maaløe (1962) in "balanced growth" experiments observed an immediate acceleration of RNA synthesis following transfer of a culture of E. Coli from a minimal to an amino acid supplemented medium, and conversely an immediate halt to RNA synthesis upon transferring a culture from a rich to a minimal medium. Moreover, Stent and Brenner (1961) have presented evidence that in the normal auxotrophic bacterial cell, RNA synthesis is under strict control by the intracellular pool of amino acids. They proposed that the intervention of amino acids in the regulation of RNA synthesis allows the bacterium to adjust its steady-state level of RNA in accordance with the nutritional limitations imposed on the overall rate of protein synthesis.

It was clear that if functional overstimulation of the liver fragment by the blood nutrients i.e., fatty acids, glucose, and amino acids was the "stimulus" to liver restoration, then

TABLES 34 a & b.

The effect of intravenous infusion of 10 per cent Aminosol on the chemical composition of the liver of normal rats (previously biopsied). The injections were made through a catheter directly into the jugular vein at quarter-hour intervals for 12 hours, 24 ml., <u>in toto</u> being administered to each rat.

A control group of rats was treated with an isocaloric amount of 10 per cent glucose, each rat being given a total quantity of 26 ml., also over 12 hours at quarter-hour intervals. Throughout the duration of the experiment the rats were kept anaesthetised.

Table 34 a

Animal No.	Treatment	$\frac{PNAP}{DNAP}$ before treatment	$\frac{PNAP}{DNAP}$ after treatment	per cent change
1	Aminosol	3·14	3·76	19·8
2	"	2·48	3·11	25·3
3	"	3·16	3·81	20·6
4	"	3·73	4·31	15·6
5	"	3·28	3·66	11·6
6	"	3·43	3·76	9·6
		average change		17·1
7	Glucose	3·50	3·80	8·6
8	"	4·18	3·96	- 5·2
9	"	3·84	3·92	2·1
10	"	2·68	2·90	7·6
11	"	2·95	2·98	1·0
		average change		2·81

The average changes for the two treatments are significantly different ($P < 0{\cdot}02$).

Table 34 b

Animal No.	Treatment	protein / DNAP before treatment	protein / DNAP after treatment	per cent change
1	Aminosol	816	862	5·6
2	"	352	410	16·5
3	"	650	650	0
4	"	605	725	19·8
5	"	576	585	1·6
6	"	567	609	7·4
		average change		8·39
7	Glucose	497	494	- 0·6
8	"	595	557	- 0·6
9	"	727	630	-13·4
10	"	468	425	- 9·2
11	"	468	435	- 7·1
		average change		- 6·19

The average changes for the two treatments are not significantly different ($P > 0{\cdot}05$).

it should be possible to stimulate growth in the liver of a normal intact rat by overnourishing it. As already indicated, one of the earliest and most conspicuous changes in regenerating liver is an increase in RNA concentration per cell. Since in bacterial cells RNA synthesis is normally under the control of the intracellular amino acid pool, it seemed pertinent to examine whether RNA synthesis in liver cells of a normal intact rat

a) could be stimulated by increasing the amount of free amino acids delivered to the liver per unit of time, and

b) if this be the case, whether it would be followed by increased cell growth and division.

The first possibility was tested by infusing a 10 per cent solution of amino acids (Aminosol) intravenously by means of a catheter into the jugular vein in an anaesthetised rat. It is evident from the results reported in Tables 34a and b that infusion of amino acids equivalent to 2·4 g., of casein, caused a significant increase in RNA and also protein concentration per cell in liver, of the order of 17 and 8 per cent (mean values) respectively. Infusion of an iso-caloric amount of glucose caused very little increase of RNA and a decrease in protein, the respective mean values being 2·8 and and -6·2 per cent. The glucose effects may be accounted for

TABLES 35 a, b & c.

The effect of intraperitoneal injections of 10 per cent Aminosol on the chemical composition of the liver of normal rats (previously biopsied). The rats used were within the body weight range 200 g., ± 10. They were divided into two groups, test and control. Each animal in the test group was injected with 4 ml., Aminosol every 4 hours until sacrifice after 48 hours. Each animal in the control group was similarly treated with 0·9 per cent sterile physiological saline and sacrificed after 48 hours.

Table 35 a

Animal No.	Treatment	$\frac{RNAP}{DNAP}$ before treatment	$\frac{RNAP}{DNAP}$ after treatment	per cent change
1	Aminosol	2·83	4·72	66·8
2	"	2·93	3·95	34·8
3	"	2·96	3·39	14·5
4	"	2·67	3·65	36·7
		average change		38·2
5	Saline	3·85	3·77	- 2·1
6	"	2·88	3·38	17·4
7	"	2·77	2·91	4·8
8	"	2·41	2·58	7·1
		average change		6·8

The average changes for the two treatments are significantly different ($P < 0·05$).

Table 35 b

Animal No.	Treatment	protein / DNAP before treatment	protein / DNAP after treatment	per cent change
1	Aminosol	410	567	38·3
2	"	457	476	3·9
3	"	389	403	3·6
4	"	362	459	26·8
		average change		18·2
5	Saline	569	520	- 8·6
6	"	421	430	2·1
7	"	398	442	11·1
8	"	351	366	4·3
		average change		2·2

The average changes for the two treatments are not significantly different ($P > 0{\cdot}05$).

Table 35 c

Animal No.	Treatment	Mitoses per 100,000 nuclei		
		before treatment	after treatment	change
1	Aminosol	24	133	109
2	"	27	272	245
3	"	73	344	271
4	"	9	521	512
5	Saline	10	8	- 2
6	"	17	15	- 2
7	"	3	16	8
8	"	55	34	-21

by supposing that elevation of blood sugar causes an increased secretion of insulin. One of the known effects of insulin is to stimulate amino acid uptake especially into muscle and also enhance protein synthesis there (Munro, 1964). Hence the effect of glucose infusion will be to divert a larger proportion of the plasma amino acids into muscle and diminish the supply to liver.

To test the second possibility that increased RNA synthesis would be followed by an increased mitotic rate it was necessary to administer the amino acid solution over a longer period of time. In view of the possibility of serious metabolic upset it was obviously undesirable to prolong the period of anaesthesia beyond 12 hours. Therefore it was decided to inject the Aminosol solution at intervals of 4 hours intraperitoneally over a period of 2 days. The rats were allowed water and food ad libitum at all times. Table 35a, b and c show that after 48 hours of such treatment the cell content of RNA showed a mean increase of 38 per cent and of protein per cell of 18 per cent. These chemical changes were accompanied by a dramatic increase in mitotic activity. Saline injected controls over the same period showed a mean RNA change per cell of 7 per cent and mean protein change per cell of 2 per cent. Mitotic activity in this group was not significantly affected.

TABLES 36 a, b & c.

The effect of intraperitoneal injections of 10 per cent Aminosol on the chemical composition of the liver of normal rats (previously biopsied). The rats used were within the body weight range 185 g. ± 5. They were divided into two groups, test and control. Each animal in the test group was injected with 4 ml., Aminosol every 4 hours until sacrifice after 44 hours. Each animal in the control group was similarly treated with 0·9 per cent sterile saline and sacrificed after 44 hours.

Table 36 a

Animal No.	Treatment	RNAP/DNAP before treatment	RNAP/DNAP after treatment	per cent change
1	Aminosol	2·64	4·36	65
2	"	3·31	4·80	45
3	"	-	4·25	-
4	"	2·82	4·00	42
5	"	2·84	3·61	27
6	"	2·02	3·62	79
		average change		51·6
7	Saline	2·04	2·49	22
8	"	2·58	2·71	5
9	"	2·05	2·51	22·5
10	"	2·12	2·62	23·5
11	"	2·16	2·40	11
		average change		17

The average changes for the two treatments are significantly different ($P < 0·01$).

Table 36 b

Animal No.	Treatment	protein/DNAP before treatment	protein/DNAP after treatment	per change change
1	Aminosol	601	679	13
2	"	623	548	-12
3	"	-	588	-
4	"	558	607	9
5	"	746	815	9·3
6	"	718	1006	40
		average change		11·4
7	Saline	464	541	16·6
8	"	659	549	-16·7
9	"	432	447	3·5
10	"	466	460	0
11	"	405	436	7·7
		average change		2·2

The average changes for the two treatments are not significantly different ($P > 0{\cdot}05$).

Table 36 c

Animal No.	Treatment	Mitoses per 100,000 nuclei		
		before treatment	after treatment	change
1	Aminosol	6	196	190
2	"	2	732	730
3	"	2	477	475
4	"	33	372	339
5	"	26	833	807
6	"	16	126	110
7	Saline	24	28	4
8	"	20	25	5
9	"	12	8	- 4
10	"	13	259	246
11	"	26	74	48

Tables 36a, b and c present results from a similar experiment extending over 44 hours. It is evident that the changes obtained here were of the same order. However, in the saline injected controls one of the rats gave a mitotic index increase of 246, quite out of line with the other figures reported. This latter change cannot be accounted for. Macroscopic examination of the livers of the rats at death indicated no unusual appearances in the control groups. In the Aminosol-treated groups, however, the livers were obviously larger in some cases than at biopsy 44 to 48 hours previously. Moreover, the lobes were dome-shaped, fleshy in consistency and showed rounding of the edges. Also the colour had altered from the normal deep red to a pale brown. These changes were reminiscent of those observed in liver in the early stages of regeneration.

In view of these encouraging results it was decided to study the effect of Aminosol treatment over a period of two to three days on liver RNA and protein per cell, mitotic index and liver/body weight ratio. The object of this experiment was to determine if, by these criteria, the liver of the normal intact rat (previously biopsied) could be demonstrated to grow and to continue growing until a new equilibrium of liver to body weight ratio was attained. It was surmised that this new equilibrium size would be adjusted to the artificially

FIGURE 23

The relationship between moist liver weight and total body weight in the rat.

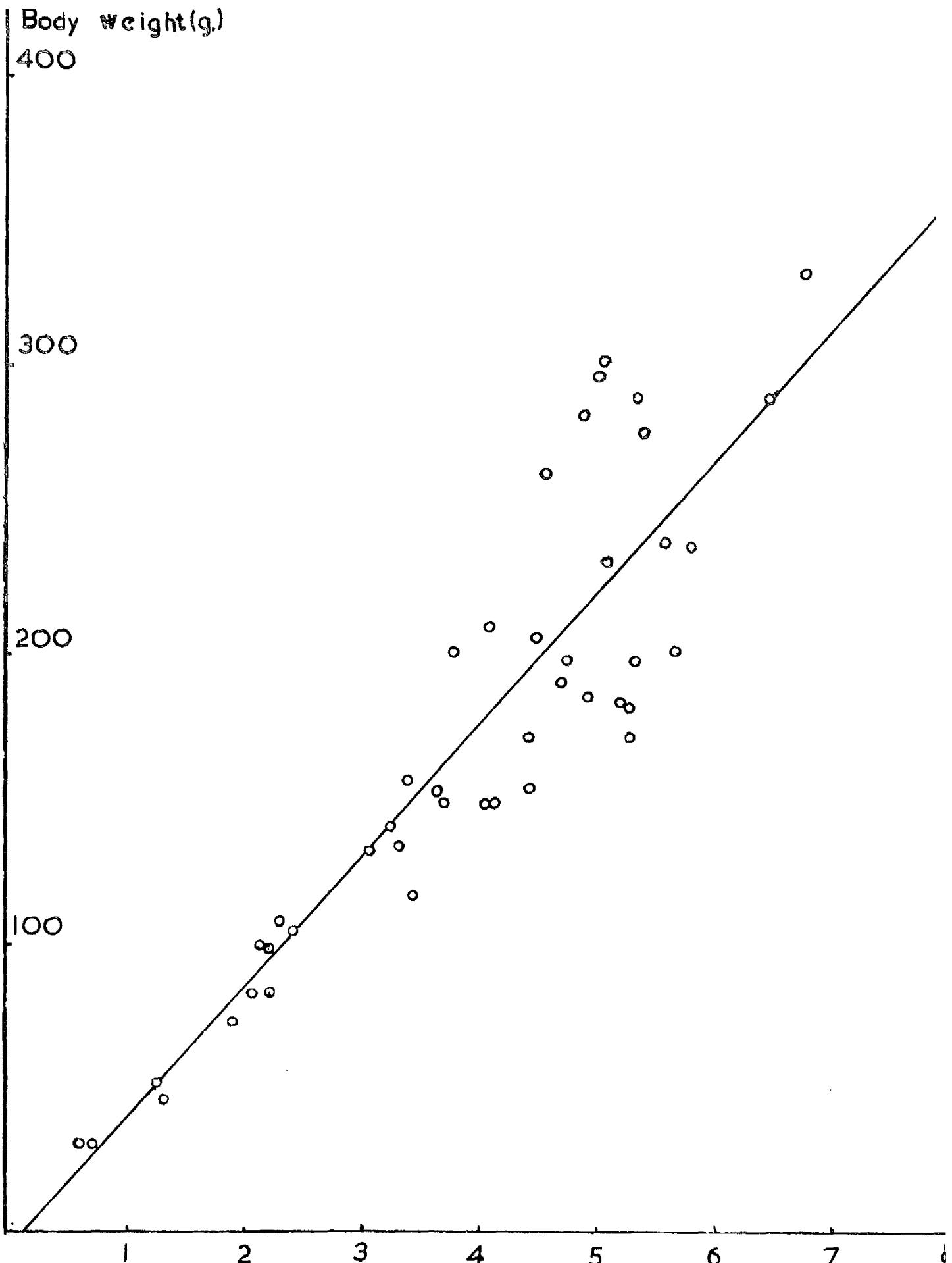


FIGURE 23

raised level of nutrition. Before embarking on this experiment, a study was made of the normal liver to body weight ratio the results of which are shown in Fig. 23. A linear regression line was calculated to fit the best line of points. Using this relationship it was then possible to calculate the per cent deviation from normal, of the liver of each rat at sacrifice.

From the results in Table 37a it is seen that there is considerable variation among the individual values for liver to body weight change. In the early stages of the experiment, perhaps partly owing to the previous biopsy, the ratio is below normal. However, from 36 hours onwards, there is a gradual upward tendency in the range of values until 60 hours indicating continuous liver growth. It would seem then, that the time course of the experiment was too short to observe a flattening out of the curve of liver weight increase. On the whole, there is an upward trend in the mitotic index but the scatter of values is too great to permit any further conclusions. Tables 37b and c show the changes in RNA and protein content per cell during the course of the experiment. Again the individual values at each time point show considerable variation. An increase in the cellular content of each component would be expected to occur until cell division got under way when a decline should result. The results reported in the

TABLES 37 a, b & c.

The effect of Aminosol treatment on the chemical composition and mitotic index of the liver of normal rats (previously biopsied). A group of 22 rats of body weight 180 g. ± 10 was subjected to liver biopsy. After allowing a day for the animals to recover from the operation each rat was given 4 ml., Aminosol intraperitoneally every 4 hours. All rats were permitted free access to water and food at all times. The rats were then killed in groups of 3 at time intervals, 18, 24, 30, 36, 42, 48 and 60 hours after the beginning of the experiment. At the 60 hour period 4 rats were killed. The rats' body and total liver weights were determined at sacrifice.

Table 37 a

Animal No.	Time interval between start of exp. & death (hr.)	before treat.	after treat.	change	Per cent change of liver/body wt. ratio from calculated normal value.
A1	18	8	6	- 2	- 5·87
A2	18	9	95	86	17·28
A3	18	7	20	13	-13·16
B1	24	2	19	17	- 5·95
B2	24	47	99	52	- 9·72
B3	24	0	12	12	15·13
C1	30	18	19	1	- 9·93
C2	30	0	101	101	-15·58
C3	30	0	43	43	- 4·05
D1	36	13	18	5	15·07
D2	36	19	23	4	8·56
D3	36	18	25	7	9·86
E1	42	2	240	238	2·53
E2	42	10	33	23	6·08
E3	42	3	18	15	12·00
F1	48	0	63	63	16·23
F2	48	24	97	73	27·27
F3	48	31	8	-23	0·98
G1	60	15	18	3	1·23
G2	60	0	17	17	3·90
G3	60	2	122	120	35·85
G4	60	6	11	5	22·95

Table 37 b

Animal No.	Time interval between start of exp. & death (hr.)	RNAP/DNAP before treatment	RNAP/DNAP after treatment	per cent change
A1	18	2·93	3·71	27
A2	18	4·07	4·48	10
A3	18	3·67	4·38	19
B1	24	3·51	4·52	29
B2	24	4·48	5·76	29
B3	24	4·87	5·10	5
C1	30	4·22	5·25	24
C2	30	4·38	4·82	10
C3	30	4·10	4·48	9
D1	36	4·58	6·00	31
D2	36	4·05	4·47	10
D3	36	4·26	6·17	45
E1	42	3·44	5·27	53
E2	42	4·10	4·06	0
E3	42	3·81	4·92	30
F1	48	4·00	4·79	20
F2	48	3·55	4·18	18
F3	48	3·86	5·03	30
G1	60	4·14	4·66	13
G2	60	4·10	5·35	31
G3	60	4·00	4·50	11
G4	60	4·41	4·74	8

Table 37 c

Animal No.	Time interval between start of exp. & death (hr.)	protein / DNAP before treatment	protein / DNAP after treatment	per cent change
A1	18	738	738	0
A2	18	872	800	- 8·3
A3	18	819	827	1·0
B1	24	760	922	21·3
B2	24	1020	1035	1·5
B3	24	1015	988	- 2·7
C1	30	944	1050	11·2
C2	30	875	917	4·8
C3	30	838	738	-12·0
D1	36	955	1135	18·8
D2	36	873	892	2·2
D3	36	982	1140	16·1
E1	42	700	883	26·1
E2	42	839	766	- 8·7
E3	42	755	952	26·1
F1	48	942	984	4·5
F2	48	777	862	11·0
F3	48	805	833	3·48
G1	60	1204	1280	6·3
G2	60	1008	1135	1·3
G3	60	1193	1282	7·5
G4	60	975	907	- 7·0

Tables 37b and c show an upward trend in the cellular content of both RNA and protein, but again the scatter of values was too great to permit any further conclusions.

It is evident that this latter experiment has several shortcomings. In the first place the range of biological variation in response to the treatment is quite considerable. This difficulty can only be circumvented by the use of a much larger number of animals so that the statistical error involved in sampling becomes reduced to permit more reliable analysis. It would be desirable to use at least five animals at each time point so that an averaging of results could be effected. In the second place, the treatment involving frequent intraperitoneal injections produced considerable stress in the rats and it is well known that adrenal corticosteroids are potent inhibitors of liver cell mitosis (Hemingway, 1960). In practice, then the response obtained may represent a balance of effects between amino acids on one hand and adrenal corticosteroids on the other. In order to obviate the effect of stress, it would be desirable to accustom the rats to the injection procedure for some time before the start of the experiment.

In spite of these drawbacks, the results obtained demonstrate quite unequivocally that it is possible to effect growth in the liver of an intact rat beyond its normal size by elevating the level of blood amino acids.

D I S C U S S I O N

DISCUSSION.

3.0 In mammals the capacity for restoration and repair varies greatly from one tissue to another. In general, it is most pronounced in the epithelial tissues e.g., skin, gut mucosa, and also in bone marrow and lymphatic tissue. Swann (1958) has pointed out that an inverse relationship exists between the cell's state of differentiation and its capacity for division. The most highly specialised cells, e.g., neurones and skeletal muscle cells, have little or no capacity to divide, and hence repair after damage or removal in such tissues is very limited.

The phenomenon of regenerative growth has been investigated in many tissues both in the adult and the embryo (Needham, 1952). However, the most intensively investigated systems with regard to the underlying mechanisms have been,

1) liver regeneration following partial hepatectomy,
2) bone marrow proliferation following haemorrhage or exposure to hypoxia,
3) compensatory growth of the remaining kidney following unilateral nephrectomy.

Compensatory growth may occur by an increase in cell size (hypertrophy), by an increase in cell number (hyperplasia),

or by both means. Restoration of red blood cells after loss occurs entirely by an increased rate of division of precursor cells (pure hyperplasia) (Linman & Bethell, 1960). Compensatory growth of liver after damage or partial removal is mainly by hyperplasia and to a much smaller extent by hypertrophy (Harkness, 1957). Compensatory renal growth after unilateral nephrectomy is mainly by hypertrophy and to a much lesser extent by hyperplasia (Simpson, 1961).

It has been an underlying assumption in much of the research into the nature of liver regeneration that following partial hepatectomy the organism is informed of the deficit of liver tissue. It is surmised that the organism then 'directs' the restoration of the deficient organ until it attains the previous equilibrium size. The nature of this process by means of which the body becomes aware of the liver 'deficiency' and 'stimulates' the restoration process has given rise to a great deal of speculation and indeed provides the impetus to current research devoted to elucidating the mechanism of growth control <u>in vivo</u>. Although suspected for a long time, conclusive evidence of the presence of the growth controlling factors in the circulating blood has only very recently been demonstrated, by the ingenious experiments of Leong, Grisham, Hole and Albright

(1964). These workers succeeded in transplanting the median lobe of the rat's liver to the abdominal subcutaneous tissue by a two stage procedure. Stage one consisted of a pedicle transfer of the median lobe through the midline abdominal incision to the subcutaneous situation. Stage two, performed after an interval of two weeks involved ligation with a steel ligature of the pedicle and its blood vessels, but excluding the effluent branch of the bile duct from the lobe. This technique, therefore, left the autograft dependent on a collateral blood supply from the subcutaneous tissues but preserved its normal bile drainage. In such a preparation Leong et al., were able to demonstrate that partial hepatectomy of the main part of the liver, one to three months after establishment of the autograft, produced DNA synthesis and mitosis in the graft as well as in the residual liver. However, the authors pointed out that the nature, origin, and manner of action of the blood-borne mediator of liver regeneration were not indicated by their investigation.

The present investigation has shed some light on this problem. The cross-circulation experiments (Section 2.1) seem to exclude the possibility that the supposed blood-borne factor is a stable hormone or humoral agent capable of being transferred from animal to another. The experiments

on plasmapheresis (Section 2.3) make it unlikely that the factor takes the form of plasma proteins. On the other hand, the changes in chemical composition of the residual liver fragment (Section 2.4) and the experiments with bromsulphthalein (Section 2.5) are consistent with the view that the liver fragment after partial hepatectomy is subject to a functional overload. Finally, the experiments on amino acid administration (Section 2.7) suggest that the functional overload may take the form partly of an abnormally large supply of amino acids. The normal intact liver may be regarded as analogous to bacterial cells growing very slowly on a minimal medium. When the liver is "subcultured" into a "richer medium" either,

a) by increasing the level of circulating nutrient especially amino acids, or

b) by altering the proportion of liver tissue present to the amount of "endogenous nutrient" i.e., by partial removal of liver substance,

the remaining liver cells will start to grow and divide quite spontaneously until a new equilibrium is attained between circulating nutrient to functioning liver tissue.

Regarded in this light, growth limitation of liver cells is by the amount of nutrient presented to the functional liver substance per unit of time. This value will determine the limit of the size to which the liver will grow, but the actual process of growth itself is spontaneous. This view, it will be appreciated, gives sense to the fact that a threshold amount of liver tissue has to be removed before the process of regeneration is set in train, whether the criterion of this process be DNA synthesis, RNA synthesis (Fujioka et al., 1963) or mitotic index (MacDonald et al., 1962). The same interpretation may be made of the findings of Lieberman and Short (1965) that ligation of the branch of the portal vein to the median and left lateral lobes of the liver in rats caused atrophy of these lobes together with increased synthesis first of RNA and then of DNA in the residual lobes at a rate identical with that following partial hepatectomy.

The actual value for this threshold amount of liver tissue to be removed to initiate the process of regeneration varies according to the investigator and criteria used. Thus MacDonald et al. (1962) obtained a value of 9·4 to 12·3 per cent before DNA synthesis is increased. Weinbren and Tarsh (1964) however, found that removal of the caudate lobe (a procedure involving the removal of 8·6 per cent of the

liver) caused a significant burst of mitoses in the residual liver after 72 hours. Goss (1964a) suggests that regenerative growth in liver probably follows the removal of even smaller amounts of tissue but by the usual criteria, the growth response remains undetected. Grisham (1962) has pointed out that in the normal intact liver cellular replacement occurs uniformly from all parts of the lobule. The same pattern is seen after partial hepatectomy if only a small proportion of the organ is excised. If two thirds are removed, mitoses are initially confined to the periportal and midzonal areas. This "restricted" distribution persists only for the first 36 hours of regeneration. Thereafter cellular replacement occurs from all parts of the lobule. Moreover evidence exists that the appearance of a restricted type of proliferation, in which cell mitoses occur in the periportal and midzonal areas, may be related to the amount of liver removed or destroyed and that at least 20 to 30 per cent must be removed before this happens. It may be that removal or destruction of a critical amount of tissue is required, before the level of endogenous nutrient supplied to the residual fragment is increased sufficiently to cause a detectable growth response. It is equally evident that this theory could account for the established fact that the rate of regeneration following

partial hepatectomy is more rapid the more tissue is removed (Fishback, 1929; MacDonald et al., 1962).

It will be noted that this theory disposes of the need for a "specific mitotic" or humoral agent stimulating liver cell division. The question may be raised however, how does this theory account for the "organ specific nature" of the stimulus? In other words, how is it that only liver cells respond so dramatically to partial hepatectomy, and not cells of other tissues. Glinos (1958), for example, studied the effect of partial hepatectomy on the mitotic frequency in parotid gland and pancreas in the rat, and found that no effect was detectable in these tissues. However, the fact that the stimulus is not so specific, as formerly believed, was demonstrated by Paschkis, Goddard, Cantarow, and Adibi (1959). These latter authors found that partial hepatectomy in the rat caused a significant elevation of mitotic index in the cornea 4 days after the operation. Partial hepatectomy also increased the compensatory enlargement of the residual kidney after unilateral hephrectomy, and also significantly increased epiphyseal width of the proximal tibia in hypophysectomized rats. The same authors also found that growth of certain transplanted rat tumors is enhanced by partial hepatectomy.

When it is conceded that other tissues may participate in the growth process which follows partial hepatectomy, it still remains necessary to account for the more dramatic response of the residual liver tissue. The answer to this question may reside in the fact that the liver is especially sensitive and responsive to the level of circulating endogenous nutrients. Considering the key role of the liver as the central organ or "clearing house" of intermediary metabolism, e.g., the regulation of blood sugar, the oxidation and synthesis of amino acids, the formation of proteins for the regulation of the function and nutrition of all tissues, the theory seems apt. That liver tissue is sensitive to the levels of circulating nutrients is well known. The classical work of Addis, Poo and Lew (1936) showed that during a fast, the liver loses protein more rapidly, and on refeeding with a 74 per cent casein diet, regains protein more rapidly than almost any other tissue. Also a similar loss of protein, RNA and phospholipid from liver was demonstrated to occur in rats fasted or on a protein-free diet (Kosterlitz, 1947). In this context also, it may be recalled that Gurd et al., (1948) found a more rapid regeneration of liver protein after partial hepatectomy in protein depleted rats, indicating that the "smaller" liver fragment was responding more

dramatically or that a more potent stimulus to liver protein recovery occurred in the animals with a more severe reduction of liver substance.

Another important objection to be accounted for, in terms of this theory, concerns the experiments of Leong et al. (1964) on the heterotopic liver transplant. It may well be asked, how does the transplant experience the necessary "functional overload" of circulating nutrient. This is clearly a problem which can not easily be explained. Since the blood levels of free amino acids and glucose do not alter greatly after partial hepatectomy, the only way an increased delivery could occur would be by an increased rate of perfusion. Moreover, it is difficult at present to envisage how partial removal of the main organ could cause haemodynamic changes in the liver transplant. However, in view of the transplant's less well defined blood supply, it would be expected that any response to a functional overload would be attenuated, which is what Leong et al., have found.

Finally, it may be urged in support of this theory relating liver cell growth to the functional demands made by the tissues, that it has the merit of being physiologically appropriate. It is fairly well established that the fundamental controlling factor in the regulation of

erythropoiesis is the oxygen tension in the circulating blood (Linman & Bethell, 1960). It seems likely that at least part of the stimulus to renal hypertrophy following unilateral nephrectomy is due to the increased excretory load, since it has been shown that an increased intake of sodium chloride increases renal mitoses in normal intact rats (Goss, 1964b) and also increasing the excretory load of acid by administration of ammonium chloride substantially increases renal cell mitoses (Lotspeich, 1965). Therefore a relationship of liver cell growth to function seems both reasonable and natural from the experimental evidence.

S U M M A R Y

SUMMARY OF EXPERIMENTAL RESULTS.

4.0 1. The mechanism controlling liver regeneration after partial hepatectomy has been investigated in the rat, the dog and the domestic fowl, and the principal theories proposed by previous workers have been re-examined.

2. The theory that regeneration might be controlled by some "humoral" factor circulating in the blood stream was tested by injecting serum from partially hepatectomised rats into normal rats. This failed to produce any sign of growth in the livers of the recipients. Conversely, injection of normal serum into partially hepatectomised animals failed to inhibit liver regeneration. In an attempt to find a more satisfactory method of detecting the supposed humoral factor, a new technique of cross-circulation was devised by means of which two rats exchanged approximately one-quarter of their blood volume per minute. When one partner in such a cross-circulation was partially hepatectomised, its liver fragment regenerated in the normal manner, but the liver of its unoperated partner showed no sign of regeneration. These experiments appear to exclude the possibility that liver regeneration is controlled by anything in the nature of a "humoral" factor.

3. The theory that regeneration is a response to the residual liver fragment having to accommodate the same total blood flow as the original intact organ was investigated in rats, dogs and domestic fowls. In rats, it was found that the pressure in the portal vein did rise sharply after partial hepatectomy but that it returned almost to the normal level within the first 24 hours. In dogs three surgical operations were performed in an attempt to produce a substantial increase in blood flow through the liver. These were: side-to-side porto-caval anastomosis with ligation of the inferior vena cava above the level of the anastomosis (reverse Eck fistula); spleno-portal arteriovenous shunt; and aorto-portal arteriovenous shunt. Each of these operations produced some increase in portal pressure. The latter two operations would be expected also to increase oxygen tension in the liver. None of them, however, produced the sort of growth response in the liver which follows partial hepatectomy. In the fowl, increased blood flow through the liver was obtained by ligating the postcaval vein and thus diverting caval blood via the porto-systemic anastomosis (circle of Jacobson) into the portal vein. In this case also there was no dramatic indication of liver growth. Taken all together, these results appear to exclude the possibility that the residual liver fragment remaining after partial

hepatectomy is stimulated to grow because it has to accept more than its normal blood flow.

4. The theory that plasma protein concentration might play an important role in liver regeneration was tested by observing the fall in the plasma protein level in rats after partial hepatectomy. This proved to be small and transient. On the other hand, when the plasma protein level in normal intact rats was substantially lowered by repeated plasmapheresis, only a slight mitotic response was obtained in their livers. Conversely, liver regeneration in partially hepatectomised rats was not inhibited when they were supplied with extra plasma protein by intravenous infusion of normal rat plasma. It seems unlikely, therefore that plasma protein levels are important in the control of liver regeneration.

5. Investigation of the chemical changes in the remaining liver fragment after partial hepatectomy in rats showed a slight increase in sodium and water content within 10 minutes of the operation, a sharp fall in glycogen concentration at 2 hours, and accumulation of total lipid (but not phospholipid) at 6 hours and an increase in RNA per cell at 12 hours. The rate at which the liver cleared injected bromsulphthalein was diminished in proportion to the amount of tissue removed in the hepatectomy and did not show any improvement within 48 hours of the operation.

6. If one partner in a pair of cross-circulating rats was hepatectomised, the lipid content of its liver and the RNA content per liver cell increased in the same way as in single partially hepatectomised rats, but to a lesser extent. The liver of the unhepatectomised partner did not show an equivalent increase either in lipid or in RNA. On the other hand, the rate which injected bromsulphthalein was cleared from the blood stream of a partially hepatectomised rat was substantially increased if it was linked in cross-circulation with a normal rat.

7. The results described in paragraphs 5 and 6 seemed consistent with the view that the stimulus to liver regeneration might be the inability of the remaining liver fragment to discharge the duties normally performed by the intact liver, and that cross-circulation with a normal rat is insufficient to make up for the deficiency. An attempt was made to test this hypothesis by overloading the liver of normal intact rats by parenteral administration of amino acids. This produced a pronounced increase in the RNA content per cell in the liver, a less pronounced increase in protein content per cell and ultimately a substantial increase in mitotic activity. In so far as these changes resemble the changes associated with liver regeneration, they support the hypothesis that liver regeneration is a response to a functional overload.

EXPERIMENTAL

APPENDIX ON EXPERIMENTAL METHODS.

5.1 Animals.

Male and female albino rats from the Departmental Colony weighing 150 to 250 g., were used except where otherwise stated. The experiments on liver lipid deposition were carried out on male and female hooded rats in the same weight range and from the same source. All rats were maintained on a conventional diet of "rat-cubes", and were given water and food ad libitum.

The fowls used were pullets of the "Sussex legbar" strain from the West of Scotland College Poultry School approximately 26 weeks old and weighing about 1200 g.

The dogs used were mongrels weighing approximately 20 kg., from the Wellcome Laboratory of the University.

5.2 Surgical procedure.

5.21 Experiments with fowls.

Partial hepatectomy was performed through an incision on the left side parallel to the edge of the sternum and about 1 cm., above it, and extending for the full length of the liver. The entire left lobe of the liver was ligated and excised. The operation was tolerated well and there was no mortality.

Ligation of the postcaval vein was achieved by making an incision in the right flank, dissecting down to the vein and ligating it with a stout cotton thread. Provided the vein was not accidently torn, there was no mortality and the birds appeared quite normal after the operation. The wounds were in each case closed in two layers with interrupted sutures.

5.22 Experiments with dogs.

Partial hepatectomy in dogs was performed by the method of Sigel (1963). The technique is based on careful dissection and individual closure of the blood vessels and bile ducts supplying the portion of liver to be resected. Under general anaesthesia the abdomen was opened through a vertical midline incision. The branches of the hepatic artery, portal vein to and the bile duct from the left and central divisions of the liver were carefully dissected out, ligated and divided. The hepatic vein from the left division was clamped and divided. The stump was closed with an arterial silk ligature. The hepatic vein from the central division was also ligated and divided. The liver tissue to be resected was elevated out through the operative incision and a ligature applied around the isthmus of the

papillary process in an area of viable tissue. The tissue bridge was then sectioned and the left and central divisions removed. The abdomen was closed in two layers. The resected specimen represented 71 per cent of the whole liver.

The Reverse-Eck fistula (porto-caval anastomosis) was performed by the technique described by Markowitz (1954). Under general anaesthesia the abdomen was opened through a large right subcostal incision. The portal vein and inferior vena cava were dissected free, and a ligature placed around the vena cava but not tied. The posterior aspects of the two vessels were approximated by a continuous 40 braided nylon suture. A 20 braided nylon suture on a straight round-bodied needle was then passed through the portal vein close to the suture line and returned through the vena cava (the cutting suture). The closed anastomosis was completed anteriorly so that it included the cutting suture, and the fistula was opened by sawing through the vessel walls with the cutting suture. Bleeding from the suture line was easily controlled by pressure from a swab. The operation was completed by ligation of the inferior vena cava. After the selected period of time the dogs were re-anaesthetised, and the anastomosis excised between clamps and examined

closely. Unless the fistula was unequivocally open, the animal was not included in the study. Liver biopsies were taken before establishment of the fistula and at intervals of time during the course of the experiment.

The spleno-portal arterio-venous anastomosis. Under general anaesthesia, through a midline abdominal incision, the splenic artery was mobilised and implanted end-to-side into the portal vein near its origin. Splenectomy was performed. Liver biopsies were taken before establishment of the anastomosis and at intervals of time during the course of the experiment.

The aorto-portal arterio-venous anastomosis. Under general anaesthesia a right thoraco-abdominal incision was made. Using a jugular vein graft, a shunt was made between the lower thoracic aorta and the portal vein. Again liver biopsies were taken before the creation of the shunt, and at intervals during the course of the experiment.

5.23 Experiments with rats.

Partial hepatectomy. This was carried out under ether anaesthesia by the method of Higgins and Anderson (1931). A midline abdominal incision was made from about 1 cm., above the xiphoid process downwards to about 2 cm., below. Using gentle pressure on the lower part of the thorax and upper abdomen, the median and left lateral lobes were delivered through the incision. A loop of linen thread was passed around these lobes and secured in position. The ligature was then tightened so that the median and left lateral lobes could be cleanly excised. The abdominal wound was closed with interrupted sutures.

Liver biopsy. Two methods were used.

a) Method 1. The left lateral lobe of the liver was delivered through a midline incision and a small triangular piece of tissue (weighing about 100 mg.,) excised from its anterior edge. The cut edges of the liver were brought together and secured by means of two sutures. The abdominal wound was closed with interrupted sutures.

b) Method 2. The median and left lateral lobes were delivered through a midline abdominal incision, as in the operation of partial hepatectomy. A loop of linen thread was passed around the left radicle of the median lobe: a secure ligature was made at the pedicle or isthmus joining the radicles, and the left radicle subsequently excised. This fragment represents less than 10 per cent of the total liver substance. The abdominal wound was closed with interrupted sutures.

Cross-circulation experiments. Cross-circulation was established between pairs of rats of the same sex and body weight as follows. Both animals were premedicated with nembutal (10 mg./kg., body weight). Under ether anaesthesia the first animal was partially hepatectomised. A midline skin incision was then made from its chin to the anterior end of the sternum. Through this incision the right external jugular vein was located and gently dissected free from connective tissue for 5 - 10 mm., distal to its junction with the axillary vein. It was then cannulated in the usual

manner (Markowitz, 1954) with a 30 cm., nylon cannula (bore, 0·75 mm.; external diameter 0·94 mm.) previously filled with physiological saline containing 5 mg./ml., heparin and clipped about halfway down its length with a small Dieffenbach's bull-dog clamp. Through the same incision the right common carotid artery was located by blunt dissection deep between the sterno-mastoid and sterno-cleoid muscles and similarly cannulated. By the same procedure the free ends of these cannulae were introduced into the corresponding blood vessels of the second animal, so that the carotid artery of each animal was connected to the jugular vein of its partner. Both animals were then laid side by side on a framework over a warm table and the Dieffenbach's clamps opened to allow exchange of blood between them. Both animals usually recovered consciousness within 20 minutes of the operation. Cross-circulation was continued for up to 48 hours. During this period both animals were sedated with phenobarbitone and kept heparinized. The following schedule gave satisfactory results. All injections were made subcutaneously.

First day:

10:00 A.M. - partial hepatectomy; cross-circulation established.

11:30 A.M. - phenobarbitone, 30 mg./kg.

5:00 P.M. - heparin, 5 mg./kg.; phenobarbitone, 70 mg./kg.

Second day:

9:00 A.M. - heparin, 3 mg./kg.

5:00 P.M. - heparin, 3 mg./kg.; phenobarbitone, 20mg./kg.

Third day:

10:00 A.M. - both animals killed.

From time to time during the cross-circulation both animals were allowed to drink water containing 10 per cent glucose ad libitum. In agreement with observations of other workers on dogs (Firor, 1931; Greenfield & Horvath, 1959; Robertson & Horvath, 1958) cross-circulation appeared to produce no ill effects.

For the 6 hour cross-circulation experiments reported in Section 2.4, it was considered more satisfactory to keep the animals fully anaesthetised. In order to control the depth of anaesthesia satisfactorily, each external jugular vein was cannulated in the way described. One cannula was led into the carotid artery of its partner. To the end of the other cannula was inserted a needle attached to a 2 ml., syringe with a solution of physiological saline containing phenobarbitone 2 mg./ml. By this means, it was possible to

keep a constant surveillance over the state of each animal.

Blood pressure measurement. Portal pressure was estimated under ether anaesthesia by introducing a No. 14 hypodermic needle attached to a nylon cannula into a branch of the splenic vein and measuring the pressure by means of a manometer containing heparinized saline. Pressure in the jugular vein was measured directly with a similar cannula. Since the absolute values of both these pressures vary with the posture of the animal and the position of the manometer, only the difference between them is shown on Table 12 and Fig. 12.

Aminosol administration. Aminosol Vitrum 10 per cent (Manufacturer: Vitrum, Stockholm, Sweden) consists of amino acids and low-molecular weight peptides obtained by enzymatic hydrolysis of adequate proteins, and also contains Na ion in a concentration of 160 mEq./litre and K ion in the concentration 0·5 mEq./litre.

a) For administration of Aminosol intravenously over a period of 12 hours, the rat had to be kept under anaesthesia. The animal was premedicated and prepared

in the same way as described for cross-circulation. Both external jugular veins were exposed and cannulated. Injections of Aminosol (test series) or glucose (control series) were performed through one cannula from an attached 2 ml., syringe, at 15 minute intervals for a period of up to 12 hours. Injections of a saline solution of phenobarbitone (2 mg./ml.) were made through the other cannula as needed.

b) In order to study the effect of Aminosol over a longer period of time, it had to be administered intraperitoneally into conscious animals. Usually about 4 to 5 ml., of Aminosol (test series) or saline (control series) were injected at 4 or 5 hourly intervals for a period of 44 to 48 hours.

Bromsulphthalein (BSP) clearance. Under anaesthesia with phenobarbitone and ether, 1 ml., of 0·5 per cent (w/v) bromsulphthalein (sodium salt-Koch-Light Laboratories) in 0·9 per cent (w/v) sodium chloride was injected via either the left external jugular vein or the tail vein, and samples

of blood removed from the right external jugular vein at one minute intervals, starting one minute after injection. 0·2 ml., of each sample was added to a centrifuge tube containing 4 ml., of 0·9 per cent saline, and the tube spun at 1,500 r.p.m., for five minutes. The supernatant was pipetted off into a clean test tube and 0·2 ml., of N NaOH added. The colour developed was then read on the Unicam SP500 spectrophotometer at 580 mμ. As a blank, 0·2 ml., of N NaOH was added to 4 ml., 0·9 per cent NaCl.

Serum injection. Serum for injection was obtained by pooling blood withdrawn by syringe from the inferior vena cava of donor rats under ether anaesthesia, allowing it to clot and then centrifuging. It was stored at -15°c. Injections of serum were made via a tail vein under light ether anaesthesia.

Plasmapheresis. Under ether anaesthesia, the jugular vein was exposed by a skin incision and blunt dissection. For the operation a 5 ml., syringe fitted with a No. 14 hypodermic needle was used. The barrel of the syringe was moistened with heparinised saline to prevent clotting. The needle was carefully inserted into the vein

and about 3·5 to 4·5 ml., of blood withdrawn. The syringe was inverted several times to ensure adequate mixing, and the blood then delivered into a 15 ml., centrifuge tube and the cells spun down, (2,000 r.p.m. for about 5 min.,). The supernatant serum was removed and preserved for plasma protein estimation. The cells were subsequently made up to the same volume with sterile (physiological) saline and reinjected into the vein. This procedure was repeated over 2 or 3 days after which the animals were sacrificed and samples of liver taken for histology.

5.30 Analytical Methods.

5.31 Histological methods and mitotic counts.

Specimens of liver were taken from all animals at death and fixed in Bouin's fixative. Sections were cut approximately 7 μ thick and stained with haemalum and eosin. The mitoses in an entire section were counted. The area of the section was then determined by projecting its image with a photographic enlarger at a known magnification and measuring the area of the image with a planimeter. The number of nuclei per sq.mm., of the section was estimated by counting the number of nuclei in ten oil-immersion fields taken at random. From these measurements the number of mitoses per 100,000 nuclei was calculated.

5.32 Removal and storage of tissues.

The animals were killed by cervical dislocation or by exsanguination under ether anaesthesia. Where total liver wet weight was required, the organ was carefully dissected out, blotted free of excess blood and weighed on a torsion balance. A small piece of tissue was fixed for histological examination, and about 0·5 g., was accurately weighed out for whole tissue analysis. Tissues were preserved by freezing in a mixture of solid carbon dioxide and ethanol and stored at -75°c.

5.33 Extraction of lipid from blood plasma.

The method described by Folch, Ascoli, Lees, Meath and LeBaron (1951) was used. The reagents were as follows:

A Absolute methanol (A.R.)

B Chloroform (A.R.)

C Chloroform-methanol mixture 2:1 (v/v)

1 ml., of plasma was added to 20 ml., of the chloroform-methanol (2:1) reagent and the mixture thoroughly shaken in a measuring cylinder. The mixture was then transferred to a volumetric flask, the measuring cylinder being rinsed with three successive portions of solvent-reagent which were combined with the mixture. After mixing, and

making up to volume, the flask contents were filtered into a glass-stoppered vessel. A beaker of slightly larger capacity than the volume of extract to be washed, was submerged in a larger beaker 9/10ths full of water. The aliquot of extract, or total extract was then delivered into the small beaker from a volumetric pipette. The system was allowed to stand overnight. The following day, the water phase was removed as completely as possible without disturbing the interfacial fluff between the phases, a layer of water only 3 or 4 mm., thick being left. Methanol was added to the chloroform solution, fluff and water in the beaker until a single phase was formed. This was transferred to a volumetric flask, the beaker being rinsed with three successive aliquots of chloroform-methanol mixture and the flask contents made up to volume.

5.34 Extraction of lipid from liver tissue.

The method described by Folch, Lees and Sloan Stanley (1957) was used. The reagents were as follows:

A Chloroform-methanol mixture 2:1 (v/v)

B Pure solvents "upper phase" and "lower phase". Chlorofrom, methanol and aqueous 0·58 per pent NaCl were mixed in a separatory

funnel in the proportions 8 : 4 : 3 by volume. After standing a biphasic system was obtained; the two phases were collected separately and stored in glass bottles.

About 0·5 g., liver tissue was homogenised with about 10 ml., of the chloroform-methanol mixture (2:1) using a Waring blendor, 3 minutes usually being adequate. The total extract and the washings from the homogenising vessel were filtered through a filter paper into a measuring cylinder. The filtered extract was mixed thoroughly with 0·2 volume of 0·29 per cent NaCl by shaking thoroughly in the cylinder. The mixture was then allowed to separate into two phases, without interfacial fluff, by standing. As much as possible of the upper phase was removed by siphoning, and removal of its solutes was completed by rinsing the interface three times with small amounts of pure solvents "upper phase" in such a way as not to disturb the lower phase. Finally, the lower phase and remaining rinsing fluid were made into one phase by the addition of methanol, and the resulting solution diluted to the desired volume by the addition of 2:1 chloroform-methanol mixture.

5.35 Estimation of total lipid.

A modification of Bloor's method (Paul, 1958) was used for estimating total lipid. The reagent was as follows:

A Chromic Acid. This reagent was prepared by dissolving 8·5 g., of potassium dichromate in a minimum volume of water and making up to a final volume of 500 ml., with concentrated H_2SO_4 (ordinary grade).

An aliquot of the chloroform-methanol extract was transferred to a boiling tube and evaporated to dryness in a 70°c. water bath, 3 ml., of the chromic acid reagent were added to the residue obtained, and the boiling tube placed in a boiling water bath for 15 minutes. The tube was removed, cooled, 3 ml., of water added and then the green colour measured in a Unicam SP500 spectrophotometer at 620 mμ. Stearic acid dissolved in methanol was used as a standard.

5.36 Estimation of phosphorus in lipid extracts.

Analysis of the lipid residues for phosphorus was performed according to a modification of the method of Allen (1940). The reagents used were as follows:

A 10 N sulphuric acid

B 100 vol., hydrogen peroxide (M.A.R.)

C Amidol reagent. 1 g., amidol (2:4-diaminophenol hydrochloride) dissolved in 100 ml., of a stock 20 per cent (w/v) solution of sodium metabisulphite.

D 8·3 per cent (w/v) ammonium molybdate solution containing a few drops of ammonium hydroxide.

An aliquot of the chloroform-methanol extract, estimated to contain between 20 and 150 µg of phosphorus, was evaporated to dryness in a boiling tube which was placed in a water bath at 70°c. The dried residue was then digested with 1·2 ml., 10 N sulphuric acid and a few drops of 100 vol., hydrogen peroxide in a graduated micro-Kjeldahl flask until the residue became colourless. When digestion was complete the flask was allowed to cool and about 5 ml., distilled water were added followed by 2 ml., amidol reagent and 1 ml., 8·3 per cent (w/v) ammonium molybdate. The solution in the flask was then made up to a total volume of 25 ml., with distilled water. The intensity of the blue colour which developed was measured between 10 and 30 minutes after the addition of the reagents, in an SP500 spectrophotometer at 720 mµ. A calibration curve for the method

was prepared using serial dilutions of a standard solution of potassium dihydrogen phosphate.

5·37 Extraction and estimation of glycogen in liver.

The method used was that of Carroll, Longley, and Roe (1956). The reagents used were as follows:

A Anthrone reagent. This is a solution made by adding 50 mg., anthrone and 10 g., thiourea to make a total volume of 1000 ml., with 72 per cent aqueous sulphuric acid. Complete solution is effected by warming the mixture gently to 80°c to 90°c. occasionally shaking the flask to mix the contents.

B 5 per cent trichloracetic acid

C 95 per cent ethanol

D Glucose standard.

a) Stock solution - 100 mg., of dry, glucose in 100 ml., of saturated benzoic acid solution.

b) Working standard - 5 ml., of the stock solution made up to 100 ml., with

saturated benzoic acid solution. 2 ml., of this solution containing 0·1 mg., of glucose were used as standard.

A piece of tissue weighing about 0·5 g., was homogenised in about 5 ml., of trichloracetic acid for 3 minutes. The homogenate was centrifuged and the supernatant filtered into a graduated cylinder. The residue was then transferred quantitatively to the blendor with an appropriate volume of trichloracetic acid and homogenised again for one minute. The mixture was again centrifuged and the supernatant fluid poured through the same filter. Two more extractions were made in the same way, and trichloracetic acid added to the extract in the measuring cylinder to give a final volume containing 10 to 200 µg. of glycogen per ml. 1 ml., of the trichloracetic acid filtrate was pipetted into a 15 ml., pyrex centrifuge tube. Duplicate samples of each unknown were analysed. To each tube were added 5 volumes of 95 per cent ethanol with careful mixing. The tubes were then capped, and placed in a water bath at 40°c. for 3 hours. After precipitation was complete, the precipitate in the tubes was spun down at 3,000 r.p.m. for 15 minutes. The clear liquid was gently decanted from the packed glycogen and the tubes

were allowed to drain in an inverted position for 10 minutes. The glycogen was then dissolved by addition of 2 ml., of distilled water. 2 ml., water were used as reagent blank and 2 ml. of standard glucose solution (containing 0·1 mg., of glucose) were used as standard. 10 ml., of anthrone reagent was then delivered into each tube with vigorous blowing, and the tubes covered with metal caps. The tubes were then placed on a cold water bath until they had reached the temperature of the cold water. They were then immersed in a boiling water bath for 15 minutes, after which they were removed and cooled to room temperature. The contents of each tube were read at 620 mμ in an Unicam SP500 spectrophotometer.

5.38 Extraction of RNA and DNA from liver tissue.

These tissue components were extracted and RNA estimated by a modification of the method of Schmidt and Thannhauser (1945) (Fleck & Munro, 1962; Hutchison & Munro, 1961). DNA was estimated by the method of Ceriotti (1952; 1955) The reagents used for extraction and separation were as follows:

A 0·3 N potassium hydroxide

B 0·6 N perchloric acid

C 0·2 N perchloric acid

The liver tissue was homogenised in 20 vols., ice-cold distilled water in a cooled Nelco Blendor for 5 minutes. 5 ml., of this homogenate (≡250 mg., wet weight tissue) were pipetted into a centrifuge tube and 2·5 ml., of ice-cold 0·6 N perchloric acid added. After thorough agitation the mixture was allowed to stand 10 minutes in an ice bath. The mixture was then centrifuged, the supernatant discarded, and the precipitate washed twice with ice-cold 0·2 N perchloric acid. Following the second wash, the excess acid was drained off by inverting the tube briefly over filter paper. After this treatment, 4 ml., 0·3 N potassium hydroxide were added to the sediment and the mixture incubated in an air oven at 37°c. for one hour. The tube and contents were then cooled in ice, 5 ml., 0·6 N., perchloric acid added, and after standing 10 minutes in the cold, the mixture was then centrifuged. This precipitate was washed twice with 5 ml., 0·2 N perchloric acid and the supernatant and washings added to a 100 ml., cylinder. 10 ml., 0·6 N perchloric acid were added to the cylinder, and the solution was made up to 100 ml., with water. This gives the RNA fraction in 0·1 N perchloric acid.

The precipitate was dissolved in 5 ml., 0·3 N potassium hydroxide and transferred to a 50 ml., cylinder,

with the addition of 12 ml., 0·3 N potassium hydroxide. The volume was made up to 50 ml., with water. This gave the DNA fraction in 0·1 N potassium hydroxide.

5·39 Estimation of RNA in the extract.

RNA was estimated in the perchloric acid extract by its extinction at 260 mμ; extinction of 1·00 = 2·94 μg. RNAP/ml.

5.40 Estimation of DNA in the extract.

The DNA fraction of the extract was estimated for DNA by the method of Ceriotti (1952; 1955). The reagents used were as follows:-

A Indole. 0·04 per cent in distilled water.

B concentrated hydrochloric acid (S.G. 1.19-Analar).

C chloroform (anaesthetic grade).

D DNA standard. Calf thymus DNA prepared by the method of Kay, Simmons and Dounce (1952) was used. About 20 mg., DNA were dissolved in 50 ml., distilled water with the aid of a little NaOH to give a stock

standard solution. 2 ml., of this stock standard solution were diluted to 50 ml., with water to give a standard solution for routine use (16 µg DNA/ml.).

2 ml., of the DNA solution, 1 ml., indole reagent and 1 ml., concentrated hydrochloric acid were thoroughly mixed in a ground glass stoppered test tube and placed in a boiling water bath for 10 minutes. The tube was then cooled in running water. A standard DNA solution and a blank consisting of water, were similarly treated. Each test solution was examined in duplicate. The resulting solution was then extracted three times with 4 ml., of chloroform using a Pasteur pipette and discarding the chloroform layer on each occasion. On the last extraction, the mixture was centrifuged at 500 r.p.m. for 5 minutes to obtain clear separation of the phases. The colour in the aqueous phase was then read in a Unicam SP500 or Beckman DB spectrophotometer at 490 mµ.

5.41 Estimation of the phosphorus content of DNA.

The method used was that of Griswold, Humoller and McIntyre (1951). The reagents were as follows:

A 10 N sulphuric acid

B 4 N perchloric acid

C potassium dihydrogen phosphate standard (2·193 g., in 500 ml., of distilled water). Before use the standard was diluted 1 in 500 with distilled water to give a final concentration of 2 μg P/ml.

D reducing agent; 13·6 g., sodium meta-bisulphite, 1 g., sodium sulphite (N (Na_2SO_3.6 H_2O) and 0·25 g., 2-naphthol-1 amino-4 sulphonic acid (BDH) in 250 ml., distilled water.

E aqueous 2·5 per cent (w/v) ammonium molybdate.

An aliquot of the aqueous DNA solution was added to a graduated test tube which was then placed in a sand bath at 100°c. to evaporate off the water. To the evaporated residue of DNA in the graduated test tube were added 0·5 ml., 10 N sulphuric acid and 0·5 ml., 4N perchloric acid and the mixture digested until the solution was clear. 1 ml., of

the diluted standard solution and 1 ml., of water were treated in the same way to give standard and blank readings. The tubes were cooled and the solutions diluted to approximately 3 ml. To this 0·5 ml., reducing agent was added followed by 0·5 ml., 2·5 per cent (w/v) ammonium molybdate with careful mixing after each addition. The solutions were made up to 5 ml., and the tubes heated in a boiling water bath for 10 minutes. The intensity of the colour was read in the Unicam SP500 spectrophotometer at 820 mμ against a blank solution.

5.42 Estimation of plasma proteins.

Plasma proteins were estimated by the method of Gornall, Bardawill and David (1949). The reagents were as follows:

A 0·9 per cent (w/v) sodium chloride

B 22·6 per cent (w/v) sodium sulphate (stored at 37°c)

C ether

D biuret reagent. 1·5 g., cupric sulphate ($CuSO_4.5\ H_2O$) and 6·0 g., sodium potassium tartrate ($NaKC_4H_4O_6.4H_2O$) were dissolved in about 500 ml., water; 300 ml., of 10 per

cent (w/v) NaOH were then added with constant mixing and the volume made up to 1000 ml., with distilled water. The reagent was stored in a paraffin-lined bottle.

0·5 ml., plasma was diluted with 9·5 ml., of 22·6 per cent sodium sulphate. 2·0 ml., of the solution were pipetted off for the estimation of total protein. 3·0 ml., ether were added to the remainder of the solution. The two phases were mixed for 30 seconds by flicking the tube and then separated by centrifugation. 2·0 ml., of the aqueous phase were pipetted off for the estimation of albumin. Total protein and albumin were estimated by adding 8 ml., of biuret reagent to each and measuring the colour developed at 540 mμ after standing for 30 minutes at room temperature. A standard solution of bovine serum albumin was used to calibrate the method.

5·43 Estimation of liver total protein.

Estimation of total liver protein was by the method of Lowry, Rosebrough, Farr and Randall (1951). The reagents used were as follows:

A 2 per cent (w/v) sodium carbonate in 0·1 N sodium hydroxide.

B 0·5 per cent (w/v) cupric sulphate ($CuSO_4.5\ H_2O$) in 1 per cent sodium or potassium tartrate.

C alkaline copper solution; 1 ml., of reagent B was added to 50 ml., of reagent A.

D diluted Folin reagent; Folin-Ciocalteau reagent (The British Drug Houses Ltd., Poole, England) was titrated with NaOH using phenolphthalein as indicator. On the basis of this titration, it was diluted to make it 1 N with respect to acid.

E a standard solution of bovine serum albumin in water was used to calibrate the method.

1 ml., of the test solution (containing 25 to 500 µg. protein) was added to a test tube followed by 5 ml., of alkaline copper solution. After 10 minutes 0·5 ml., diluted Folin reagent was added rapidly and the solutions mixed immediately. After standing 30 minutes or longer, the colours of duplicate samples were read in the Beckman DB spectrophotometer at 500 or 750 mµ. A calibration curve

for the method was made using the standard bovine serum albumin solution.

5.44 Extraction of free amino acids from liver.

The method used was a modification of that described by Tallan, Moore and Stein (1954). The reagents were as follows:

A 1 per cent picric acid (w/v)

B Dowex 2 resin (purchased from Calbiochem Ltd., Log Angeles, U.S.A.)

A weighed amount of liver tissue (usually about 0·4 g.,) was homogenised in a Nelco blendor with 10 volumes of 1 per cent picric acid. The precipitate was promptly removed by centrifugation. To remove excess picric from the protein-free tissue extracts, the solutions were passed through columns of Dowex 2 (0·8 cm., in diameter); a bed 2 cm., high was used for 10 ml., of extract. The walls of the chromatograph tube and the resin bed were washed 5 times with 0·4 ml., quantities of 0·02 N HCl. The colourless effluent was collected into 15 ml., graduated centrifuge tubes and made up to volume with distilled water, a final volume of 10 ml., being obtained from 0·4 to 0·5 g., liver tissue.

5.45 Extraction of free amino acids from blood.

The method used was a modification of that described by Tallan, Moore and Stein (1954). The reagents were the same as those described in the last section. 0·2 ml., of blood was added to 0·8 ml., 1 per cent (w/v) picric acid. The mixture was thoroughly mixed by flicking the tube several times and then centrifuged at 2,000 r.p.m. for 5 minutes. The supernatant was then passed through a column of Dowex 2 (0·8 cm., in diameter); a bed 1 cm., high was used. The precipitate was washed 5 times with 0·2 ml., quantities of 0·02 N HCl and the washings used to wash the column. The effluent extract was collected in graduated centrifuge tubes and made up to a total volume of 4 ml. Appropriate aliquots of the extracts were taken for the estimation of total free amino nitrogen.

5.46 Estimation of total amino acid nitrogen.

The method used was a modification of that described by Yemm and Cocking (1955). The reagents were as follows:

A 0·2 N citrate buffer, pH 5; 21·008 g. citric acid ($C_6H_9O_7.H_2O$) were dissolved in 200 ml., of distilled water, 200 ml., of N sodium hydroxide were added and the mixture was diluted to 500 ml.

B 0·01 M potassium cyanide

C potassium cyanide-methyl cellosolve solution. 5 ml., of 0·01 M potassium cyanide diluted to 250 ml., with methyl cellosolve.

D methyl cellosolve-ninhydrin solution. 5 per cent (w/v) solution of ninhydrin in methyl cellosolve.

0·1 ml., clear liver extract (Section 5.44) was pipetted into a test tube; this amount was found to contain not more than 5·6 µg., of amino acid nitrogen. To this were added 2·9 ml., of citrate buffer pH, 0·4 ml., of the methyl cellosolve-ninhydrin solution and 2 ml., of the potassium cyanide-methyl cellosolve solution. A blank solution was made up omitting the amino acid extract and using 3·0 ml., of citrate buffer. Each test was performed in duplicate. After mixing well, the solutions were heated at 100°c for 15 minutes, and then cooled in running tap water for 5 minutes.

In the case of the blood, 0·25 ml., extract was used, with 2·75 ml., citrate buffer.

The colour developed was read in the Beckman DB spectrophotometer at 570 mµ. A calibration curve was constructed using serial dilutions of a standard solution of glycine.

5.47 Estimation of the water content of liver tissue.

At sacrifice, a very small piece of liver tissue was layered on to a weighed, clean watch-glass and weighed. The watch-glass and tissue were then incubated in a hot air oven at 70°c for 24 hours and reweighed. The water content was obtained by difference.

5.48 Estimation of sodium and potassium in liver tissue.

Estimation of sodium and potassium was made in a 5 per cent trichloracetic acid extract of liver tissue. The reagents used were as follows:

A Standard sodium chloride. A solution containing 1·27 g., sodium chloride per litre was used as standard. For use, this solution was diluted 1 in 100 with distilled water to give a concentration of 5 mg., sodium ion/litre.

B Standard potassium chloride. A solution containing 960 mg., potassium chloride per litre was used as standard. For use, this solution was diluted 1 in 50 with distilled water to give a concentration of 10 mg., potassium ion/litre.

A piece of liver tissue, usually about 0·5 g., (wet weight) was homogenised in 10 volumes of 5 per cent trichloracetic acid at 0°c in a Nelco blendor for 3 minutes. The mixture was then centrifuged and the supernatant poured over a filter paper in a funnel, into a measuring cylinder. The precipitate was washed twice with 5 volumes 5 per cent trichloracetic acid, and the washings also filtered into the measuring cylinder. The extract was made up to 10 ml. 1 ml., quantities of this extract were used for inorganic phosphorus estimation. A 1 in 25 dilution of the extract in water was made for sodium and potassium estimation. As a blank for the latter estimations a 1 in 25 dilution of 5 per cent trichloracetic acid was used. Before each estimation the flame photometer calibration was checked using the appropriate standard solution. After each estimation, the instrument was flushed through with distilled water until the zero position was again attained.

5.49 Estimation of inorganic phosphorus.

Inorganic phosphorus estimation was made in a 5 per cent trichloracetic acid extract of liver tissue. The reagents used were as follows:

A molybdic acid solution: 25 g. ammonium molybdate were dissolved in 300 ml. distilled water. A cooled mixture of 75 ml., concentrated sulphuric acid and 125 ml., distilled water was added and the solution made up to a final volume of 500 ml.

B hydroquinone solution: 5·0 g. hydroquinone were dissolved in 1 litre distilled water and 10 drops concentrated sulphuric acid were added.

C sodium sulphite: 200 g. crystalline sodium sulphite ($Na_2SO_3.7\ H_2O$) were dissolved in distilled water and the solution made up to a final volume of 1 litre.

D standard phosphate solution: 0·0439 g. potassium dihydrogen phosphate in 1 litre distilled water (2 ml. = 0·02 mg. Phosphorus).

A 5 per cent trichloracetic acid extract of liver tissue was made in the way described in the previous Section (5·48). 1 ml., of this extract was pipetted into a 10 ml., graduated cylinder followed by 2 ml., molybdic acid, and after mixing, 1 ml., sodium sulphite and 1 ml., hydroquinone. The mixture was made up to a final volume of 10 ml. A blank was prepared in the same way using water in place of the tissue extract solution. A standard was prepared using 2 ml., standard phosphate solution (containing 0·02 mg., phosphorus) instead of the tissue extract. After allowing to stand for 30 minutes, the colours were read in a Unicam SP500 spectrophotometer at 720 mμ.

Tests of Statistical Significance

In the experiments in which statistical analysis was performed, two types of approach have been used. Where three groups of data are compared simultaneously they were analysed using an analysis of variance (Snedecor, 1946). Where only two groups of data are compared, they were analysed by the Students' t test. The expressions $P < 0{\cdot}05$ and $P < 0{\cdot}01$ are used in the conventional sense to indicate significance at the 5 per cent and 1 per cent levels respectively.

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