AN INVESTIGATION OF THE EFFECTS OF THE ANTI-LEUKAEMIC DRUG MYLERAN ON THE REGENERATION OF RAT LIVER

A thesis submitted in part fulfilment of requirements for the degree of Doctor of Philosophy in the Faculty of Science of the University of London.

by

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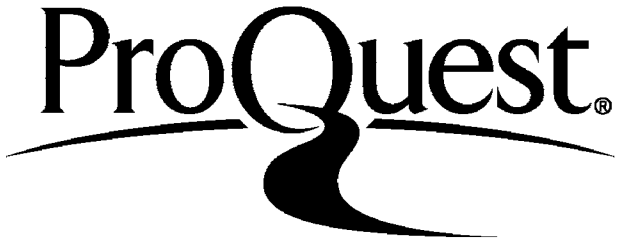

Dedication

To my parents.

Acknowledgements

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ABSTRACT

An investigation was undertaken into the effects of the anti-leukaemic drug Myleran on regenerating rat liver. The effects on DNA synthesis and the cell cycle were studied.

Random bred rats were subjected to partial hepatectomy and experimental animals given Myleran (1,4-dimethanesulphon-oxybutane) in dispersion. Controls were given dispersant lacking Myleran and analysed concurrently.

DNA synthesis was followed using radioactive thymidine. The specific activity of the DNA was measured over three days following partial hepatectomy. An investigation into 'apparent' thymidine kinase activity was undertaken in conjunction with the studies on DNA synthesis. Levels of nucleic acids were determined.

Regeneration in the weight of the liver remnant after partial hepatectomy was followed and a histological examination conducted in which the mitotic index and the incidence of mitotic abnormality were determined. The lengths of the individual mitotic phases and the phases of the cell cycle were found by the construction of mitotic labelling curves.

The drug was found to decrease the ability of the liver to regenerate and to partially inhibit DNA synthesis. These results are explained by a decrease in the 'apparent'

thymidine kinase activity and are discussed. No significant effects were found on the RNA or DNA contents of the liver remnant. The levels of mitotic abnormalities increased following Myleran treatment and were measured for the different phases of mitosis. Measurements of mitotic index over the first 26hr. following Myleran treatment gave variable results. The drug was found to shorten the length of mitosis, determined by summation of the individual mitotic phases or from the graphs of % labelled mitoses.

Analysis of the curves of labelled mitotic figures was complicated in the case of the Myleran-treated rats by the absence of a second wave of labelled mitoses; thus total cell cycle time (T_c) and the length of the G_1 phase could not be found. However the G_2 and S phases of the cell cycle were found to be lengthened for Myleran-treated rats; the significance of these findings is discussed.

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INTRODUCTION

1 LIVER REGENERATION

1.1 Early Work.

If we disregard the legend of Prometheus, the possibility of the liver to regenerate was first recognised by Andral in 1834 (see Milne, 1909). The first experimental production of liver regeneration was carried out in Italy by Colucci (1883) who injured guinea pig liver and noted the appearance of new hepatocytes. He thought, however, that these arose from leucocytes. Canalis (1885), followed by Podwyssozki (1886), removed wedges of liver and demonstrated proliferation of hepatic cells with the formation of new bile ducts. Podwyssozki observed that mitotic figures appeared near the wound within 36hr. In 1889 Ponfick (see Looney,1960) found that removal of as much as three-quarters of the liver of a rabbit still resulted in regeneration. Von Meister in 1894 (see Looney, 1960) was one of the earliest to observe that the liver would regenerate after repeated removal of tissue. The first major study of the quantitative aspects of liver regeneration was that of Higgins & Anderson (1931).

1.2 Factors affecting Liver Regeneration.

Normal liver, when subjected to the stimulus of partial hepatectomy, comprising removal of the left lateral and median lobes (Higgins & Anderson, 1931), undergoes compensatory hyperplasia, termed regeneration, of the two residual lobes (Bucher et al. 1969). Liver regeneration is the subject of an extensive literature dealing with the biochemical, physiological and metabolic activities during restoration to the original size (Bucher, 1963; Bengmark, 1970).

Various factors influence the restoration of the liver following partial hepatectomy and these factors must be standardized in studies on liver regeneration.

a) Resection of the left lateral and median lobes leads to a reduction of the liver mass by approximately 68% (Slonaker rats: 68.4%, Brues, Drury & Brues, 1936; Wistar rats: 68.3%, Bucher & Glinos, 1950; Sprague-Dawley rats: 68.3%, Bucher & Swaffield, 1964). The regenerative rate is related to the size of resected liver when either the incorporation of $[^{14}C]$ thymidine (Mac Donald, Rogers & Pechet, 1962) or increase in liver weight (Straube & Patt, 1961) are used as the criterion of regeneration.

b) The age of the animal influences the liver regeneration (Bucher & Glinos, 1950). The rise in the mitotic activity is delayed with age, as is the incorporation of $[2\text{-}^{14}C]$ thymidine

into the DNA (Bucher, Swaffield & Ditroia, 1964).

c) There is evidence for diurnal variation in the deposition of glycogen, synthesis of RNA, DNA and phospholipids, and in the time of mitosis in regenerating rat liver (Kelly et al. 1951; Halberg & Barnum, 1961; Halberg, 1960; Döring & Rensing, 1973).

d) Diet is capable of modifying the proliferative rate (Soberón & Sánchez-Q., 1961).

e) Sex of the animal appears to have no important effect on the regenerative process (Bucher, 1963). Pregnancy however, may slightly modify liver regeneration (Gershbein, 1958).

f) Surrounding temperature has a considerable effect on the changes in the liver of animals after partial hepatectomy (Chevillard, Hamon & Mayer, 1937). At 26°, near the temperature of minimum heat production for the rat (Herrington, 1940), the effects of small variations in temperature on the regeneration are minimised.

1.3 Liver Regeneration and the Reticuloendothelial System.

Anatomically the hepatic lobule consists of the tissue specific parenchymal cells (60-65%) and the smaller endothelial cells of the Kuppfer type (30-35%) which make up part of the reticulendothelial system (RES). Changes in this littoral or endothelial cell population are unreliable as an index of the regeneration after partial hepatectomy, as they constitute only

a part of the whole body response.

Animals given chronic pretreatment with endotoxin exhibited arrested DNA synthesis in response to regeneration (Agarwal, 1973), while animals given a prior injection of Celite, which is known to alter RES function (Agarwal & Berry, 1966), show enhanced DNA synthesis after resection (Simek et al. 1968). Both these results suggest the seeming involvement of the RES during the regenerative response.

1.4 Morphological Changes accompanying Regeneration.

Glycogen-free, fat-free vacuoles appear 5min after partial hepatectomy - they may be lysosomes (Aterman, 1952). In electron microscopy numerous fat droplets appear in the cytoplasm 1hr. after partial hepatectomy (Jordan, 1964) and osmophilic bodies are decreased proportionally.

From 30min to 8hr. after hepatectomy, basophilic bodies (RNA-protein complexes, Lagerstedt, 1953) start to disperse, first peripherally, then spreading centrally (Glinos, 1958). This is reflected in a disaggregation of the endoplasmic reticulum. 'Cloudy swelling' appears in some of the mitochondria (Bernhard, 1958).

Within 6hr. of partial hepatectomy one finds cytoplasmic inclusions, described as vacuoles by Aterman (1961) or

hyalin protein droplets by Weinbren (1959), within the liver. The glycogen content becomes very low by 10hr. and remains low for several days following partial hepatectomy (Harkness, 1957). By 12hr. there is an increase in the size of the cells, nuclei and nucleoli, which is maximal at 24hr. (Harkness, 1957; Weinbren, 1959) or 36hr. (Ekelund, 1971). Nucleoli are increased in number (St. Mironescu & Dragomir, 1966). At 16-18hr. following partial hepatectomy the endoplasmic reticulum, in contact with groups of mitochondria, starts to reform; first centrally then peripherally (Bernhard & Rouiller, 1956; Glinos, 1958). Normal structure is restored by 36-48hr. (Bernhard, 1958; Oberling, 1959).

1.4.1 Microbodies

According to Mochizuki & Tsukada (1970), the number of microbodies of regenerating rat liver cells per unit area of cytoplasm is decreased 1 day after partial hepatectomy and then increases gradually up to 14 days following the operation. Rouiller & Bernhard (1956) state that at 36hr. microbodies are larger, more abundant, polymorphic and more organised internally. They may be precursors of mitochondria or lysosomes (Rouiller, 1960).

1.4.2 Mitotic Activity

Mitotic activity follows DNA synthesis by 6-8hr.

(i.e. with a peak at 26-32hr.) and proliferation spreads inwards towards the central vein (Grisham, 1960). Mitotic figures appear to have a peak at 28hr. according to Cater et al. (1957) and Weinbren (1959), though Shima & Asashima (1971) placed the peak at 36hr.

1.4.3 Nuclearity and Ploidy

It is the diploid mononuclear cells that proliferate most actively (Uryvaeva & Marshak, 1969). Following mitosis the proportion of binuclear cells falls from 25-30% (Harrison, 1953) to 8-10% (St. Aubin & Bucher, 1952). The number of parenchymal nuclei is not restored to normal, though DNA is restored (Harkness, 1957) reflecting the increase in ploidy.

Diploid and tetraploid nuclei passing through the DNA synthetic (S) phase are labelled by $[^3H]$ thymidine while octaploid nuclei are unlabelled (Uryvaeva & Marshak, 1969). 70-80% of normal adult parenchymal nuclei are tetraploid and 1-2% octaploid. When regeneration is completed the proportion of tetraploid and octaploid nuclei has increased by 10% and 50% respectively (Post et al. 1960). The increased ploidy persists indefinitely (Himes et al. 1957).

1.5 Biochemical Changes accompanying Regeneration.

Morphological considerations suggest that any analyses

relating to cytoplasmic constituents will give values approximating to the composition of parenchymal cells, while those relating to nuclear constituents will reflect a composite of cell types.

1.5.1 Carbohydrate

The liver is the principal store of glycogen. During regeneration glycogen is mobilised with consequent decrease in liver glycogen content (Harkness, 1957). The blood glucose level is maintained and gluconeogenesis (the formation of glucose from non-carbohydrate sources) is increased (Camargo & Migliorni, 1971).

1.5.2 Lipid

The liver is a site of fat synthesis and the principal site of fatty acid oxidation. The principal site of biosynthetic functions associated with lipid is the microsomal fraction (Abraham et al. 1961).

There is a change in the relative proportions of the unsaturated fatty acids during the first 3 days (Johnson et al. 1954) and other lipids, mainly neutral fats, accumulate during the first few hours (Harkness, 1957). These lipids are derived from fat deposits rather than by *in situ* synthesis since :

a) if fat mobilisation is depressed by adrenalectomy, fatty infiltration does not occur (Brody et al. 1961);

b) synthesis of fatty acids from [^{14}C] acetate remains low for the first few days of regeneration (Johnson & Albert, 1960).

In glucose-injected, partially hepatectomized rats the mean increase in total lipid is only 50% of non-injected, partially hepatectomized animals (Bengmark, Olsson & Svanborg, 1965); this is presumably due to the stimulation of glucose oxidation both diminishing the release of depot free fatty acid (Dole, 1956) and lessening uptake of plasma free fatty acids (Spitzer & Mc Elroy, 1960). This is consistent with the post-hepatectomy increase of plasma free fatty acid found by Fex & Olivecrona (1968). Camargo, Cornicelli & Cardoso (1966), on the other hand, found no significant differences in levels of free fatty acid in 70% hepatectomized rats and sham-operated controls in the first 24hr. after operation.

At 18hr. the triglyceride content, the rate of esterification of fatty acids to triglycerides and phospholipids (determined by incorporation of [^{14}C] glycerol) are all elevated. Formation of compound lipids, such as inositol phosphatide and sphingomyelin (estimated by use of [^{14}C] acetate), are also increased. The peak values in phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine content however, occur later (Johnson & Albert, 1960). These results

agree with studies on the incorporation of ^{32}P into phospholipids. There is a linear increase in total phospholipid during the first 48hr. after operation, though little change in the relative concentrations of individual phospholipids. There is an increase of incorporation of ^{32}P into the phosphatidylserine and phosphatidylinositol fractions, though a lowered incorporation into phosphatidyl choline, in comparison with control unoperated rats (Fex, 1970).

1.5.3 Amino Acids

Six hours after operation there is a fall in hepatic glutamine and a rise in glutamate, aspartate and lysine. The latter three amino acids start to fall at 20-30hr. as other amino acids start to increase (Ferrari & Harkness, 1954).

1.5.4 Protein

Rates of protein synthesis are generally determined either *in vivo* using labelled amino acids, or *in vitro* using the microsomal fraction plus soluble fraction, labelled amino acid, an energy source and co-enzymes. *In vivo* studies are complicated by alterations of precursor pool size, penetration of precursors and differing turnover rates of different proteins, while *in vitro* studies measure only the activity of an isolated part of the system.

Net increment of protein is not easily detectable until nearly 12hr.; maximal protein synthesis has been found between 36 and 48hr. (Harkness, 1957 and Campbell, 1958, give reviews: see also Schreiber et al. 1971). Busanny-Caspari & Deimel (1963) found that initially liver protein content lagged behind, then increased linearly with, the weight of regenerated liver.

The mechanism of the increase in protein synthesis is, according to Van Lancker (1969) likely to result from increased synthesis of messenger and ribosomal RNA, rather than from an increase in their activity. Earl & Hindley (1971) propose the intracellular concentrations of amino acids or aminoacyl-tRNAs as rate limiting. Liew & Gornall (1973) suggest the acetylation of ribosomal proteins to be closely associated with the regulation of protein synthesis during regeneration.

Microsomes appear to be the site of protein synthesis in regenerating liver, since they incorporate labelled amino acids most rapidly in a cell-free system (Hoagland, 1961).

1.5.5 Histone

The nuclear histones have a synthetic peak at 26hr. with a lower peak at 56hr. In contrast, cytoplasmic proteins synthesize maximally at 56hr. with a smaller peak at 26hr.

(Evans, Holbrook & Irvin, 1962). In the regenerating liver of rat and mouse, nucleochromosomal protein synthesis resembles (in rate / time) DNA synthesis but precedes it by 4-12hr. (Sávina & Révich, 1972).

1.6 Enzymes.

1.6.1 General Enzyme changes after Partial Hepatectomy

According to Bengmark's review (1970), it appears that there is a decrease in activity of nearly all the enzymes in the early post-operative phase. Cytochrome oxidase (EC 1.9.3.1), acid phosphatase (EC 3.1.3.2) and glucose-6-phosphatase (EC 3.1.3.9), representative of the mitochondria, lysosomes and microsomes respectively, did not differ in their rates of restitution when studied over 72hr. after partial hepatectomy. These enzymes were restored in direct proportion to the total protein (Van Lancker & Sempoux, 1958). However, the subcellular fractions and enzymes of particular subcellular fractions often differ in their rates of restitution. Alterations in enzymes of microsomal and soluble cell components in general precede those in mitochondria (Harkness, 1957).

1.6.2 Microsomal Fraction

This fraction may be defined (Roodyn, 1967) as consisting of a mixture of ribosomes and elements of endoplasmic

reticulum. According to Reid (1967), microsomal enzymes are generally located in the membranes rather than in the ribosomes. Microsomal activities were increased in regenerating liver (Walkinshaw & Van Lancker, 1964). Amylase, found in the microsomal fraction (Bresemer & Rutter, 1961), increases in activity markedly in the first phase of regeneration (Albertini et al. 1966). The activity of acid phosphatase (EC 3.1.3.2) increases 18hr. after partial hepatectomy (Walkinshaw & van Lancker, 1964). The activity of glucuronidase (EC 3.2.1.31), 37-40% of which is in the microsomal fraction (Walker, 1952), starts to increase at 24hr. following partial hepatectomy (Walkinshaw & van Lancker, 1964; Ekelund, 1971), although a contrasting view is given by Albertini et al. (1966). Alkaline phosphatase (EC 3.1.3.1) which is 42% microsomal (Allard et al. 1952), increases markedly in activity after partial hepatectomy and there is also a progressive increase in its activity from the central lobular vein towards the periphery of the lobule (Tsuboi et al. 1954; Palma & Dominedo, 1963).

Regeneration has little effect on the specific activities of such microsomal enzymes as glucose-6-phosphatase, NADH and reduced NADPH, dehydrogenases (EC 1.6.99.3 and 1.6.99.2) and lipoamide dehydrogenase (EC 1.6.4.3) (protein disulphide reductase (EC 1.6.4.4)), while the capacity to perform certain oxidative demethylations and to bind carcinogenic amines falls rapidly after a lag of 12-14hr.

Cytochrome b_5 content is reduced by 50% at this time (Von der Decken & Hultin, 1960).

Microsomal drug-metabolizing enzymes are not affected to the same extent by regeneration (Fouts et al. 1961), though there is a generalized fall in their activities (Fouts, 1963).

1.6.3 Mitochondrial Fraction

The number of mitochondria is depressed during the first week of regeneration (Allard et al. 1952), although according to Gear (1965) partial hepatectomy induces a massive biogenesis of mitochondria. In normal liver mitochondria may turn over as complete functional units (Fletcher & Sanadi, 1961), but not all mitochondrial enzymes change at parallel rates during regeneration. This implies that, like the microsomes, these organelles are renewed in modified form (Gear, 1970). The specific activity of marker enzymes for matrix, inner membrane and inter-membrane remain constant or are altered synchronously. In contrast the specific activities of monoamine oxidase (EC 1.4.3.4) and kynurenine 3-hydroxylase (EC 1.14.1.2), both outer membrane markers, fall by 67% and 40%, respectively, from their control values 4 days after operation, and normalize after 3 weeks. The inner membrane and matrix seem to turn over as a unit, while the enzymes of inner and outer membranes are synthesized asynchronously (Gear, 1970).

Recent work (Ekelund,1971) on succinic dehydrogenase (EC 1.3.99.1) suggests that its activity increases following partial hepatectomy, in contrast to the earlier work of Pearson, Grove & Green (1959).

In contradiction to earlier observations (Wilczok,1961), the amounts of aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.6.1.2) have been shown to increase with the proliferative rate; an increase in the content of transminases 24hr. following partial hepatectomy, followed by a decrease at 24hr., has been reported (Sheid, Morris & Roth, 1965; Bengmark, Ekholm & Olsson, 1967; Nikiforov & Larionova, 1971).

1.6.4 Nuclei

Nuclear glycolytic activity is increased, becoming maximal 12hr. after operation (Siebert et al. 1961), though aldolase (EC 4.1.2.7) activity is decreased (Albertini et al. 1966). Stirpe & Aldridge (1961) have claimed that isolated nuclei synthesize NAD in decreasing amounts for 3 days after operation - an indication of decreased NAD pyrophosphorylase (EC 2.7.7.1) activity. This has been questioned by Ferris & Clark (1971), who have found that this enzyme and NMN pyrophosphorylase, (EC 2.4.2.12) remain at, or slightly above, the initial value for 12hr., then rise continuously till 48hr. after partial hepatectomy.

1.6.5 Other Fractions

In the phagolysosomes proteinase activity increases during the first 6hr. then falls till 60hr. Dipeptidase activity decreases, followed by a sharp rise at 48hr.(Hassan, Nardelli & Autori, 1962).

The number of microbodies per unit area of cytoplasm in regenerating liver cells decreases 1 day after partial hepatectomy then increases up to the 14th day in rats. The activity of catalase (EC 1.11.1.6) was proportional to the numerical change in microbodies (Mochizuki & Tsukada, 1970). The net rate of albumin synthesis in the ribosomes changes only slightly (Schreiber et al. 1971).

1.6.6 Enzymic Routes of DNA and RNA Synthesis

Reviews of nucleic acid enzymic routes are to be found in Bucher (1963) and Murray (1971). A composite diagram based on the above sources is shown in Fig. 1. The abbreviations and symbols used are those recommended in the Journal of Biological Chemistry and include:

PRPP	∝-5-phosphoribosyl pyrophosphate
OMP	orotidylic monophosphate
A,G,C,U,T	adenine, guanine, cytosine, uracil, thymine

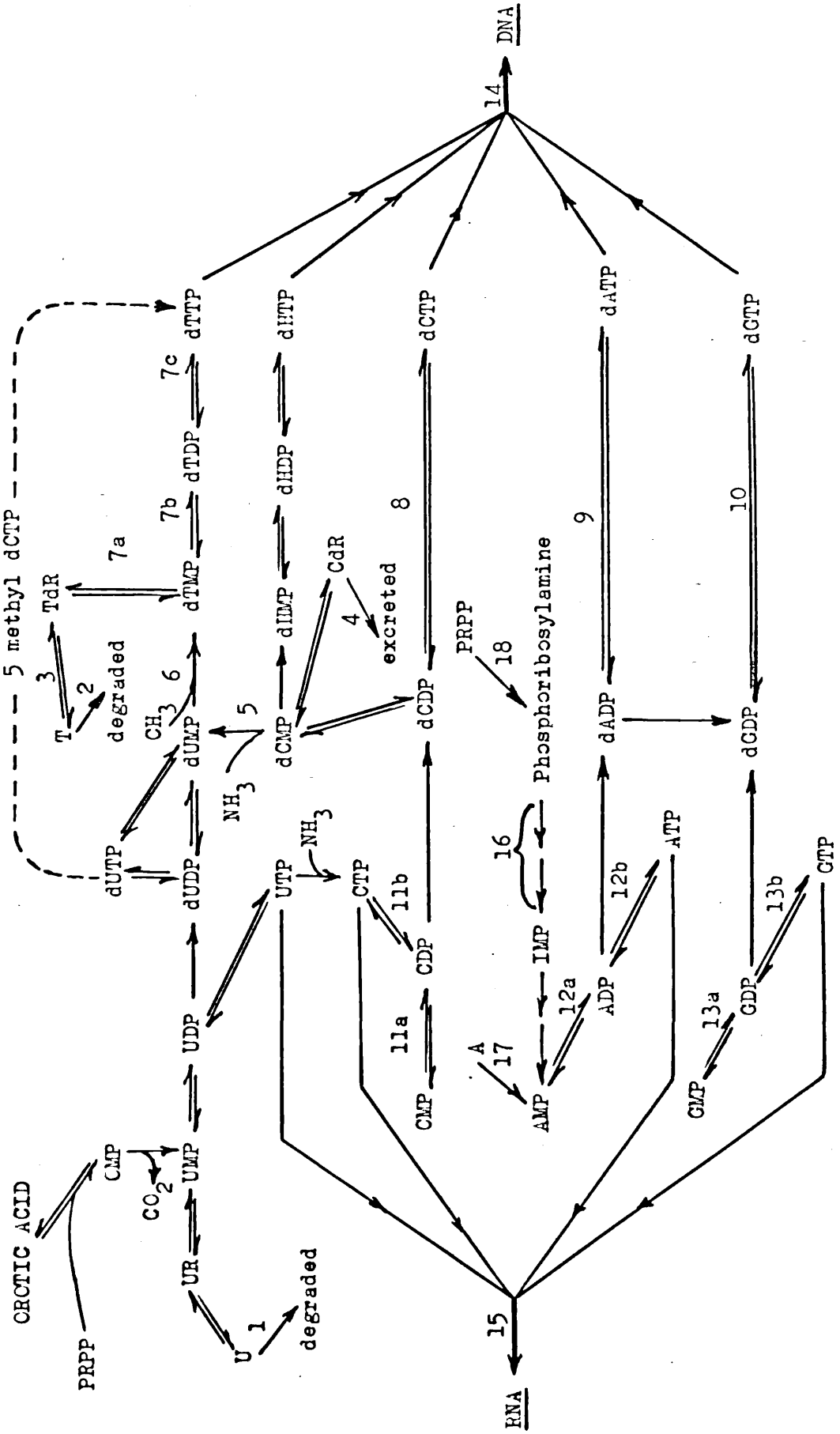


Fig. 1. The interrelationships of precursors of RNA and DNA synthesis.

AMP, GMP, IMP, CMP, UMP	5'-phosphate of ribosyl adenine, guanine, inosine, cytosine and uridine.
ADP etc.	5' (pyro)-diphosphate of adenosine, etc.
ATP etc.	5' (pyro)-triphosphate of adenosine, etc.
T	thymine
TdR	thymidine
dTMP, dHMP, etc.	5'-phosphate of 2'-deoxyribosyl thymine, 2'-deoxyribosyl-5-hydroxy-methyl cytosine, etc.
dTDP etc.	5' (pyro)-diphosphate of 2'-deoxy-thymidine etc.
dTTP etc.	5' (pyro)-triphosphate of 2'-deoxy-thymidine etc.
RNA	ribonucleic acid
DNA	deoxyribonucleic acid

The numbers on the diagram indicate enzyme reactions.

1 Degradation path for uracil.

2 Degradation path for thymine.

3 Thymidine phosphorylase (EC 2.4.2.4).

4 Excretion of deoxycytidine.

5 Deoxycytidylate deaminase.

6 Thymidylate synthetase (EC 2.1.1.b).

7a	Thymidine kinase (EC 2.7.1.21).
7bc	Thymidylate kinase.
8	Deoxycytidylate kinase (EC 2.7.4.5).
9	Deoxyadenylate kinase.
10	Deoxyguanylate kinase.
11ab	Cytidylate kinase.
12ab	Adenylate kinase (EC 2.7.4.3).
13ab	Guanylate kinase (EC 2.7.4.8).
14	DNA polymerase (EC 2.7.7.7).
15	RNA polymerase (EC 2.7.7.6).
16	Enzymes of de novo purine biosynthesis.
17	Adenine phosphoribosyltransferase (EC 2.4.2.7).
18	PRPP amidotransferase (EC 2.4.2.14).

The main pathways for the introduction of pyrimidine bases are at the nucleotide level beyond orotic acid and do not normally include the free bases or nucleosides, except as a means for 'salvage' of degradation products (Kornberg, 1960). Thymidine is on the salvage pathway . Liver is probably the major source of purine available for salvage since it contains a high activity of PRPP amidotransferase (Casky, Ashton & Wyngaarden, 1964). Indirect evidence for significant purine salvage following partial hepatectomy is that the activities of adenine and hypoxanthine/guanine phosphoribosyltransferase are augmented following the operation (Murray,1966).

1.6.7 Enzymes Fluctuating with DNA level and Histone Phosphorylation

The earliest enzyme observed to change in activity after partial hepatectomy is ornithine decarboxylase (EC 4.1.1.17) (Russell, Medina & Snyder, 1970). The activity of this enzyme increases in parallel with the increase in DNA and RNA content, and preceeds the peak of DNA synthesis. The enzyme catalyses the formation of putrescine whose production is rate-limiting for the production of spermine. Spermine is essential for the assembly of increased numbers of polysomes after partial hepatectomy (Ord & Stocken, 1972).

The activities of NAD pyrophosphorylase (EC 2.7.7.1) and NMN pyrophosphorylase (EC 2.4.2.12) rise continually from 12hr. till 48hr. after operation (Ferris & Clark, 1971). There is positive correlation between DNA synthesis and NAD pyrophosphorylase activity (Haines et al. 1969).

The acidic proteins of chromatin are generally assumed to be specific regulators of gene activity, and histones to preserve nucleoprotein structure. Only the content of histone F2 shows a close correlation with the increased DNA content between 15hr. and 26.5hr. following partial hepatectomy (Evans, Holbrook & Irvin, 1962). According to Révich, Sávina & Bokhon'ko (1971), the maximum rate of synthesis of both F1 and F2b fractions occurs 16-18hr. after partial hepatectomy. Sung,

Dixon & Smithies (1971) found that 15hr. after partial hepatectomy only the 2b histone was phosphorylated. They regarded this as a possible specific control event. Histone F1 phosphokinase activity increases in the S period (Siebert, Ord & Stocken, 1971). Gutierrez-Cernosek & Hnilica (1971) measured synthesis of histones during liver regeneration using [^{14}C] lysine. They found that the peak of incorporation preceeds the peak of DNA replication. At the time of maximum DNA synthesis, arginine-rich F3 and lysine-rich F1 histones incorporate lysine several times more rapidly than F2a and F2b histones. There is intensive phosphorylation of F2b, F1 and F2a during the first 6hr. where the content of F3 histone does not increase. However, during the period of intensive DNA replication all the histones are phosphorylated in proportion to their rates of synthesis.

1.6.8 Enzymes involved in Purine and Pyrimidine Base Synthesis

Partial hepatectomy causes an increase in the flow of metabolites into the pyrimidine path in the early stages of liver regeneration (Fausto, 1972). Various enzymes associated with pyrimidine synthesis, such as carbamoylphosphate synthetase (EC 2.7.2.5), aspartate transcarbamylase (EC 2.1.3.2), dihydro-orotase (EC 3.5.2.3), dihydro-orotate dehydrogenase (EC 1.3.3.1) and uridine kinase (EC 2.7.1.48), increase in specific activity 12-24hr. after partial hepatectomy (Bresnick, 1965). Fig. 1 shows pyrimidine interconversions in detail, enzyme alterations

during regeneration are geared to producing thymidine triphosphates.

Dihydro-uracil dehydrogenase (EC 1.3.1.1), the rate limiting enzyme in the degradation of U or T, falls in regenerating liver to 74% of sham-operated controls at 24hr. after partial hepatectomy (Sherry, Morris & Weber, 1971). According to Fritzson (1964) this is due to a 24hr. delay in the synthesis of the enzyme from the initiation of liver growth. Stevens & Stocken (1963) showed that there is a correlation between activity of thymidine phosphorylase and DNA synthesis in regeneration.

One may compare the two major paths for biosynthesis of thymidine nucleotides:

1 UMP → dUMP → dTMP

2 UMP → CMP → dCMP → dUMP → dTMP

Crone & Itzhaki (1965) concluded from [6-^{14}C] orotate studies, using the technique of flooding with unlabelled cytidine, that dTMP is formed to the greatest extent by route 1. Deoxycytidylate deaminase (converting dCMP → dUMP) activity increases eight-fold 2 days after partial hepatectomy (Oda, Holtzer & Chiga, 1966). The specific activity of thymidylate synthetase (converting dUMP → dTMP) is barely detectable in normal liver but increases 4-10 fold in regenerating liver (Hartmann

& Heidelberger, 1961).

The activity of thymidine kinase has been reported to be very low in preparations of adult liver, though higher in foetal (Klemperer & Haynes, 1968) and regenerating liver (Bucher, 1963). According to Bianchi, Crathorn & Shooter (1962) thymidine kinase is present during the early stages of regeneration in rat liver. Activities of both thymidine and thymidylate kinases increase from 18-30hr. in regenerating rat liver (Ol'shanetskaya, Kavelina & Novikova, 1968; Beltz, 1962), that of thymidine kinase increasing to six times normal control values at 24.5hr. after partial hepatectomy. Mal'Kova et al. (1968) found that the activity of thymidine and thymidylate kinases of regenerating liver in the rat varied directly with mitotic activity.

1.6.9 RNA

53hr. after partial hepatectomy uridine and deoxyuridine phosphorylases, which control the production of RNA, increase in activity (Yamada, 1962). Both nuclear and nucleolar RNA polymerase rise quickly after 70% partial hepatectomy, apparently from de novo synthesis (Ro & Busch, 1967).

Suppression of the increase in the rate of RNA formation after partial hepatectomy results in delay in the appearance of DNA synthesis (Tsukada, 1969).

1.6.10 DNA

DNA synthesis increases with DNA polymerase activity and this in turn is essentially synchronous to thymidine incorporation in rat liver (Ove, Jenkins & Laszlo, 1969; Kizer & Howell, 1973).

2 THE CELL CYCLE

Quastler & Sherman (1959) first estimated parameters of the cell cycle. Both cell cycle and mitotic times may be estimated by use of $[^3H]$ thymidine and autoradiography (Post et al. 1963). The generative cycle may be compartmentalised into the four phases G_1, S (DNA synthetic phase), G_2, and M (mitosis) (Lajtha, Oliver & Ellis, 1954). A history of cell cycle analysis is given by Petersen, Tobey & Anderson (1969).

Biochemical parameters of the cell cycle have been reviewed by Mueller (1971); Mitchison (1969) has reviewed phase 'markers' in the cell cycle. Additional phase markers, in He La cells, (Marks, Paik & Borun, 1973) are commencement of F1 histone phosphorylation during S phase, and the physiochemical characteristics of ornithine - transaminase, which differ in G_1, S and G_2 (Volpe & Eremenko, 1971).

Actinomycin (an inhibitor of RNA synthesis) and puromycin (an inhibitor of protein synthesis) have been widely used in cell cycle analysis. It is generally agreed from study of a number of systems with these inhibitors that the RNA which is required for mitosis is synthesised during G_1 and S (Crippa, 1966; Monesi, Molinaro & Siracusa, 1969), while the proteins required for mitosis are synthesised during G_2 (Petersen, Tobey & Anderson, 1969; Messer, 1973). Both the RNA and protein, synthesised consecutively in the G_1 phase, are

prerequisite for cells to proceed into the S phase (Yamada & Hanaoka, 1973).

There appear to be genetic inducers and repressors synthesised during the cell cycle: extracts of liver cells which are in the G_1 phase inhibit DNA synthesis in cultured cells (Aujard, Chany & Frayssinet, 1973), and cells in DNA synthesis are capable of stimulating cells in the G_1 phase into the S phase (Dewey, Miller & Nagasawa, 1973).

3 THE ALKYLATING COMPOUNDS ACTIVE IN THE TREATMENT OF CANCER

The alkylating agents used in the treatment of cancer have been reviewed by a number of authors (Ross, 1962; Wheeler, 1967; Haddow, 1973). Those alkylating agents that have extensive biological activity have at least two alkylating groups in each molecule. The monofunctional 6-diazo-5-oxo-norleucine (DON) active biologically, reacts by a bimolecular mechanism, while the other alkylating agents react by SN_2 or SN_1 mechanisms (Ross, 1962).

Alkylating agents may be classified according to the blood response pattern following their administration. The 'Myleran type' drugs affect markedly the neutrophil and platelet counts, while the lymphocyte count is affected to a lesser extent. In contrast the 'nitrogen mustard type' of drugs, typified by chlorambucil ((di-2 chloroethylamino) phenylbutyric acid) bring about characteristic changes in neutrophil, platelet and lymphocyte counts (Dunn & Elson, 1970). In their effects on blood counts, chlorambucil and Myleran each partly mimic the effects of radiation. Only in the response of the platelets is Myleran truly radiomimetic; a rapid fall in platelet concentration occurs 6-8 days after a single dose, followed by a steady rise. It is the lymphocytes that are most affected by a single dose of chlorambucil in an analogous fashion to whole body x-irradiation (Elson, 1963).

The primary site of action of monofunctional and difunctional alkylating agents has been considered to be the mitotic apparatus (Brookes & Lawley, 1963; Koller, 1969). There is considerable evidence that DNA is alkylated both *in vivo* and *in vitro* (Trams, Nadkarni & Smith, 1961; Ross, 1962; Brookes & Lawley, 1963; Lawley & Brookes, 1965; Tomisek & Simpson, 1966). Bifunctional agents, such as the N-mustards, the diepoxides or the polyimines may cross-link the guanine and thymine bases in DNA. The most extensive alkylation occurs on N-7 of guanine (Lawley & Brookes, 1963a) and causes labilisation of the glycosidic link between base and deoxyribose and consequent depurination (Ross, 1962). This depurination may:

a) stop replication at this point,
b) cause 'skipping' of this unit,
c) introduce nonspecifically any available deoxynucleotide (Lawley & Brookes, 1963b).
d) labilise the deoxyribose-phosphate linkage, causing chain scission (Ross, 1962).

A study by Rhaese & Boetker (1973) of the molecular basis for the biological effects of alkylation using transforming DNA has indicated that base alkylations are mutagenic and depurination or triester breaks are lethal DNA alterations.

Brookes & Lawley (1965) conclude that cross linking of DNA is the main cytotoxic action of bifunctional alkylating agents. Using $[^{35}S]$ 'mustard gas' (2-dichloroethyl sulphide)

Brookes & Lawley (1963) detected bis (guanin-7-yl) derivatives in mouse ascites cells after in vivo treatment which they considered evidence for cross-linkage. Other workers using bifunctional agents such as the nitrogen mustards have also found evidence of cross-linkage (Rutman, Chun & Jones,1969; Verly & Brakier, 1970). Wheeler (1967) in a review and De Cosse & Gelfant (1970), using N-mustard on the Ehrlich ascites tumour, have both stressed the probability and importance of random cross linkages between DNA, RNA and protein. However that there is a disparity between the low alkylation of DNA by the alkylating agents and their large amount of cell damage is well documented (Biesele, 1963; Wheeler, 1967; De Cosse & Gelfant, 1970).

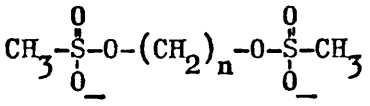
The multiplicity of the possible reactions of alkylating agents with biological materials makes it difficult to designate any single reaction as the cause of cytotoxicity. A mechanism of action involving attack at many sites, some probably of enzymic nature, rather than direct chemical action on the mitotic apparatus (thought to be affected only indirectly), is favoured by some authors (De Cosse & Gelfant, 1970).

There are conflicting reports concerning the phase(s) in the cell cycle at which the alkylating agents act (De Cosse & Gelfant, 1970; Wheeler, 1967). Wheeler (1967) and Lawley & Brookes (1963a) suggest that bifunctional alkylating agents affect DNA replication by forming interstrand cross-links

during the S period of the cell cycle; cells would then be held up in the G_2 phase when strand separation is futilely initiated as a prelude to mitosis. An alternative view has, however, been presented (Levis, Danieli & Piccinni, 1965). Verly & Brakier (1970) point out that if DNA interstrand cross-linkage is the main cause of cytotoxicity of the alkylating agents then the duration of the cell cycle is a crucial factor in the formation of the cross-links.

3.1 Myleran.

The dimethanesulphonyloxyalkanes have the general formula:



Myleran or Busulphan is the member in which n=4 and is the most potent biologically, based on neutrophil depression (Elson, 1958).

3.1.1 The Reactions of Myleran with DNA and related Cell Constituents

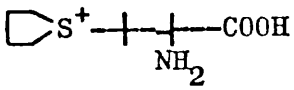
Myleran alkylated guanine in mouse L cells (Mitchell & Walker, 1972) though there was no evidence of cross linkage found on alkaline denaturation and renaturation of the DNA.

Lawley & Brookes (1963a) and Verly & Brakier (1969) both conclude that Myleran presents all the features of a monofunctional alkylating agent, and the latter authors attribute its effects to depurination and single strand breakage. Brookes & Lawley (1965) showed that Myleran was capable of binding not only to DNA but also to RNA and protein of the liver. Bergel'son (1971) showed that Myleran also interacts with lipid in regenerating liver. Myleran's injury to the mitotic apparatus, especially the chromosomes (Koller, 1969), could, however, be due to an indirect effect on the lysosomes (Allison & Paton, 1969; Davies, Lloyd & Beck, 1971).

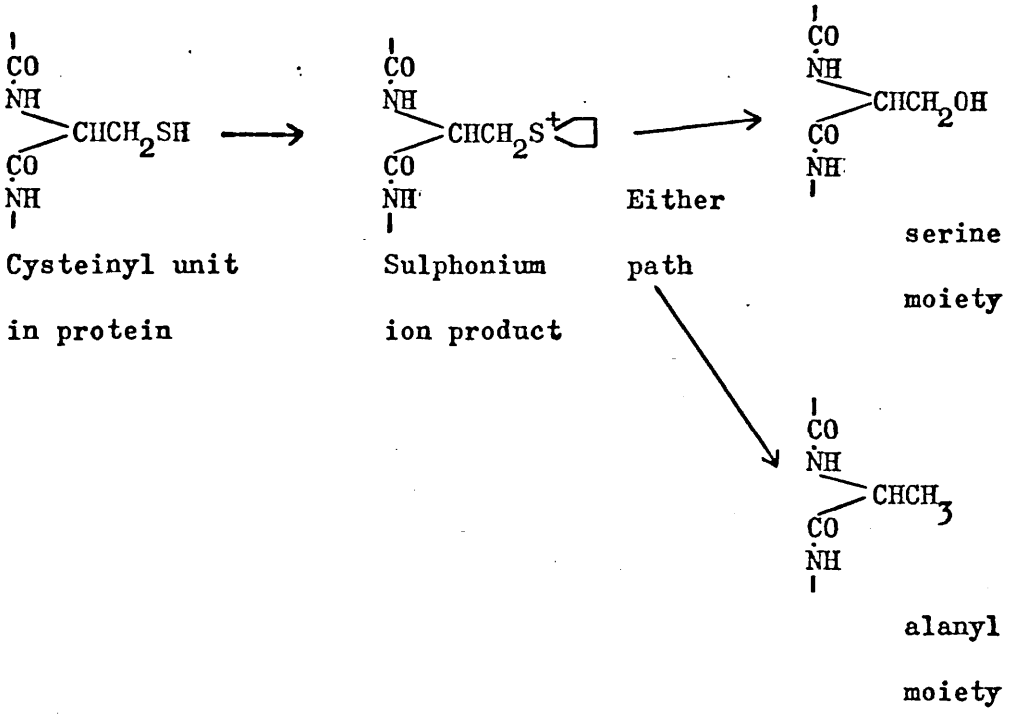
Trams, Nadkarni & Smith (1961) found stimulation of the incorporation of orotic acid into the DNA and RNA of leukaemic cells by Myleran and inhibition of incorporation of labelled P, adenine, formaldehyde and aspartate. Myleran increases the incorporation of $[^3H]$ thymidine <u>in vitro</u> in bone marrow (Niskanen, 1967a,b), but not into seminiferous epithelium (Kramer & De Rooij, 1970).

3.1.2 <u>The reaction of Myleran with Protein</u>

Roberts & Warwick (1959a) suggest that Busulphan may conjugate to SH groups of proteins to give:



based on analogy with ethyl methane sulphonate ($CH_3CH_2OSO_2CH_3$, 'half-Myleran'; Roberts & Warwick, 1958). They suggest reaction with cysteine containing compounds and indeed Butler & Crathorn (1958) have shown that Myleran and protein (of nucleoprotein) combine. The positively charged sulphonium ion may itself modify the function of the protein or it may be dethiolated to an alanyl or serine unit (Roberts & Warwick, 1959a,b; Parham & Wilbur, 1959) as below:



Danjic (1966) found that cysteamine and cystamine reduced the lethal action of Myleran in the rat. However, the hypothesis that the cytotoxicity of Myleran is mediated by thiol group alkylation is not supported by Addison & Berenbaum (1971), who found that sub-cutaneous injection of cysteine-HCl potentiated the action of Myleran. Shamrai (1969) found that Myleran decreased the incorporation of $[^{35}S]$ methionine

in the proteins of liver nuclei and mitochondria.

3.1.3 The Viability of Myleran in the Body

As regards the 'viability' of Busulphan it has a half-life of 11-12hr. both *in vitro* and *in vivo* (Fox et al. 1960). Myleran suffers little simple hydrolysis *in vivo* (Roberts & Warwick, 1957). It reacts by an SN_2 type reaction and its rate of reaction will depend upon the concentration of alkylatable centres (Ross, 1962).

3.1.4 The Clinical use of Myleran

The clinical use of Myleran was first described by Galton (1953). A review of the treatment of chronic myelogenous leukaemia by Myleran has been made by Galton (1956), its use in this disease being due to its selectivity of action on haemopoietic tissues (Elson, 1963). A review and interpretation of old and new data on the treatment of chronic myelogenous leukaemia has been made by Mueller (1963). Kaung et al. (1971) give more recent data on cases of chronic myelocytic leukaemia treated with Myleran therapy. The kinetics and cytodynamics of the proliferation of leukaemic cells and possible drug implications have been reviewed by Doermer (1973) and Fernandez-Ranada et al. (1972). It should be noted that the continued administration of Myleran in high doses causes neoplastic growth in normal liver (Kandori & Kurimoto, 1960).

3.1.5 The effect of Myleran on the Cell Cycle

The effect of Myleran on the cell cycle *in vitro* has been reviewed (Metcalf, 1973), and Nagao (1971) reviews the cell cycle and chemotherapy of infantile leukaemic cells. It has been proposed (Elson, 1958a,b) that the G_1 phase of the cell cycle shows the greatest response to the 'Myleran type' drugs. Grimes, Sallman & Frichette (1964) substantiated this in rat lens epithelium, concluding that Myleran acts in the G_1 phase in a manner that allows cells to initiate and complete DNA synthesis normally but prevents their subsequent mitotic division.

MATERIALS

All reagents used were of 'Analar' grade. The standard used for DNA assays was calf thymus sodium deoxyribonucleic acid (B.D.H. Chemicals Ltd., Poole, England). Solutions to be injected were sterilized following Millipore filtration (Millipore (U.K.) Ltd., London, England; 3 μM pore size).

4.1 Animals.

Mus norvegicus - Hooded Lister strain, male, random bred (Animal Suppliers (London) Ltd., Welwyn, England).

Rattus rattus - male, random bred (Anatomy Institute, Medical University of Debrecen, Debrecen, Hungary).

4.2 Instruments.

Beckman DB 1402 spectophotometer (Beckman Instruments Ltd., Glenrothes, Scotland).

Packard Tri-Carb liquid scintillation counter (Packard Instruments Co. Inc., Downers Grove, Ill., U.S.A.).

Microtomes: Beck rotary (Beck, London) and M.S.E. sledge (M.S.E.,London).

4.3 Hepatectomy and Injections.

Diethyl Ether; Ether Anaesthetic B.P. (May and Baker Ltd., Dagenham, England).

Rat Feed: Oxoid 41B Breeders Diet (Oxoid, London, England). Dextrose monohydrate B.P.

Myleran (Busulphan), 1,4-dimethyl sulphonyloxybutane, was kindly donated by Dr. D.M. Taylor, Institute of Cancer Research, Sutton, Surrey, England.

Dimethyl sulphoxide (B.D.H. Chemicals Ltd., Poole, England).

Arachis oil (E.F. Langdale Ltd., New Addington, England).

$[6\text{-}^{3}H]$ thymidine. 1mCi per ml of specific activity 20-30 mCi $mmol^{-1}$ in sterilized aqueous solution (Radiochemical Centre, Amersham, England).

4.4 Thymidine Kinase Estimations.

$[^{3}H_{3}C]$ thymidine. 1mCi per ml, specific activity 15-30 mCi $mmol^{-1}$ in sterilized aqueous solution (Radiochemical Centre, Amersham, England).

ATP (Sigma Chemical Company, Kingston-upon-Thames, England).

Diethylaminoethyl (DEAE) substituted paper circles (Whatman DE-81, Maidstone, England).

4.5 Liquid Scintillation.

Silica gel coarse (B.D.H. Chemicals Ltd., Poole, England).

Triton X-100 (iso-Octylphenoxypolyethoxyethanol containing approximately 10 moles of ethylene oxide) (B.D.H. Chemicals Ltd., Poole, England).

Toluene (B.D.H. Chemicals Ltd., Poole, England).

PPO: 2,5-Diphenyloxazole (Koch-Light Laboratories Ltd., Colnbrook, England).

POPOP: 1,4-Di 2-(5-phenyloxazolyl) benzene (Koch-Light Laboratories Ltd., Colnbrook, England).

Nitromethane (B.D.H. Chemicals Ltd., Poole, England).

4.6 Autoradiography.

Emulsions: Ilford K5 Nuclear Research Emulsion in Gel Form (Ilford Ltd., Ilford, England). Agfa-Gevaert 7.15 Nuclear Emulsion (Agfa-Gevaert, Belgium).

Developers: Kodak Microdol-X (Kodak Ltd., London). Agfa-Gevaert G170c (Agfa-Gevaert, Belgium).

Ilford Hypam Rapid Fixer (Ilford Ltd., Ilford, England).

Meyer's Haematoxylin comprised: 0.5g Haematoxylin (B.D.H.) 0.1g sodium iodate, 25g aluminium ammonium sulphate (ammonium alum), 0.5g citric acid and 25g chloral hydrate made up to 500ml.

DPX slice mountant (B.D.H. Chemicals Ltd., Poole, England).

METHODS

5.1 Animals and partial hepatectomy.

Four to six week old male rats, Mus norvegicus of the Hooded Lister strain, random bred and weighing between 80-150g were used. Rattus rattus of the same age, weight and sex were used only in the thymidine kinase experiments.

Partial hepatectomy of approximately two-thirds of the liver - median and left lateral lobes - was performed under ether anaesthesia by the method of Higgins and Anderson (1931). Partial hepatectomy was carried out between 6 a.m. and noon to minimise diurnal variation in regenerative ability (Bucher, 1963; Bade et al. 1966). Animals were allowed food and water ad libitum, 5% dextrose being substituted for water following operation. The rats were maintained at approximately 22°.

5.2 Thymidine Kinase Activity.

Thymidine kinase activity was determined in vitro by the method of Ives, Durham & Tucker (1969). In this method disks of anion-exchange paper are used to retain nucleotides (TMP, TDP and TTP) formed by the reaction, while the precursor nucleoside (TdR) is washed away. The use of the isotopically labelled precursor enables the formation of product to be followed by liquid scintillation counting.

The enzyme was extracted by preparing a 1/5 (g liver / v 0.25M sucrose) homogenate. The homogenates from control and Myleran-treated rats were assayed for thymidine kinase activity at 1/5, 1/25 and 1/250 (w/v) dilution of liver / 0.25M sucrose, as follows:

An equal volume of reaction mixture, containing Tris-HCl (pH 8.0_{37^o}), 50mM; ATP, 5mM; $MgCl_2$, 2.5mM and $\left[{}^3H_3C\right]$ thymidine, 0.08mM was added to the homogenate. The mixture was then incubated at 37° for 30 min. The reaction was halted by placing the ends of the test-tubes containing the mixture into a water bath at 100°. After centrifugation 20 µl aliquots of the supernatant were applied to 23mm diameter disks of DEAE substituted paper. After standing for 10 min the disks were washed for 20 min in 0.001M ammonium formate (30 ml : per disk). This procedure was repeated and the disks given an additional three rinses in water. The disks were then placed in glass scintillation vials. 1.0 ml of aqueous HCl/KCl (0.1/0.2M) was added and the vials were gently shaken for 15 min to elute the labelled nucleotides. After elution was complete 10 ml of Triton-toluene scintillation solvent was added and the radioactivity of the samples determined. Control rats were assumed to have 100% kinase activity and the thymidine kinase activity in rats given Myleran was expressed as a % of the control value.

5.3 Drug and Thymidine Injections.

Myleran (4.4mg /Kg body weight) was dissolved by grinding with 1 part of dimethyl sulphoxide in an agate mortar. Nine parts of arachis oil were then added with further grinding both to allow prolongation of drug action and reduce peritonitis associated with intraperitoneal administration of the solution (Elson et al. 1970; Dunn & Elson, 1970). 0.5 ml doses of Myleran emulsion, or drug free emulsion for controls, were given intraperitoneally before completion of suturing except where otherwise indicated.

For measurement of DNA synthesis and the labelling index partially hepatectomized rats were injected at intervals of approximately 6hr. following the operation with $[6\text{-}^{3}H]$ thymidine (0.5 μCi /g body weight, made up to 0.5 ml with normal saline (0.15M sodium chloride)). They were killed 45 min (Frayssinet et al. 1968) after administration of the isotope. In the case of rats being used for the determination of the fraction of labelled mitoses 0.5 ml $[6\text{-}^{3}H]$ thymidine was injected 16hr. following partial hepatectomy and rats were killed at hourly intervals. The dose of isotope administered was selected as giving good autoradiographs within a 2 week exposure time without causing radiation injury (Post & Hoffman, 1961; Grisham, 1960).

The control rats used were of the same age, weight,

sex and strain and were treated in the same manner as their experimental counterparts. Experimental animals were given intraperitoneally 0.5 ml of a mixture of dimethyl sulphoxide and arachis oil (1:10, v/v) containing 4.4mg Myleran /Kg body weight, and later 0.5 μCi [6-^{3}H] thymidine /g body weight intraperitoneally, made up to 0.5 ml with 0.15M saline. Controls were given the same injections, but without Myleran.

5.4 Nucleic Acid Extraction.

Nucleic acids were extracted by a modification of the Schmidt-Thannhauser procedure as detailed by Munro (1966): this procedure requires no lipid extraction prior to that of nucleic acids. Excised liver was immediately transferred to iced water, weighed and a 1.5/20 (w/v) homogenate prepared using a Potter-Elvehjem tissue homogeniser. A 1hr. incubation at 37° in 0.3M potassium hydroxide solubilized RNA and three 30 min extractions at 70° with 1.0M perchloric acid were used to extract DNA. The extractions were carried out in 10 ml Pyrex homogenisation tubes capable of withstanding centrifugation at 3,000 r.p.m. thus facilitating washing and preventing errors in transference. All procedures were carried out at 0°.

5.4.1 Assay of Nucleic Acids

RNA was determined spectrophotometrically using quartz cells with a 1cm light path in a Beckman DB 1402 spectrophoto-

meter. The concentration of RNA was calculated from the formula:

$C_{RNA} = 3.40\ A_{260nm} - 1.44\ A_{232nm}$ (Fleck & Begg, 1965)

where:

C_{RNA} = concentration of RNA in μg RNA-P per ml of solution.

A_{260nm} and A_{232nm} = absorbance of the solution measured at 260 and 232nm respectively.

DNA was estimated by Giles and Myers' (1965) modification of the Burton diphenylamine method as the latter method gave unacceptably high blank readings. One volume of test solution, one volume of 4% diphenylamine and 0.05 volumes of acetaldehyde containing 1.6 mg ml^{-1} in aqueous solution were mixed, incubated at 30° for 16hr. and the absorption at 595 and 700nm read against a suitable blank. The difference in absorption (595 - 700nm) was used to determine the DNA content of samples by comparison with a standard solution of calf thymus DNA. The phosphorus content of the standard calf thymus DNA solution was measured by the method of Allen (1940). Results were expressed in terms of μg DNA-P /g liver remnant.

5.5 Percentage Regeneration.

Percentage regeneration was calculated from weights of wet liver excised at time of operation and at death.

$$\% \text{ Regeneration} = \frac{\text{Increase in mass of liver after hepatectomy}}{\text{Mass of liver removed at partial hepatectomy}} \times 100$$

The fraction of liver excised at operation was established at 70.2% by direct measurement. This value together with the mass excised was used to calculate the weight of liver remaining in experimental animals following partial hepatectomy.

5.6 Liquid Scintillation.

Assay of radioactivity in the DNA fractions was carried out with a Packard Tri-Carb liquid scintillation counter by the emulsion technique of Patterson and Greene (1965). Scintillant was prepared by mixing 1 volume of Triton X-100, purified by stirring with 1/10th part by volume of silica gel, to 2 volumes of toluene containing 0.4% PPO and 0.01% POPOP. Quench curves were prepared by the addition of 8-10 μl of nitromethane to vials containing 10 ml of scintillant and 1 ml of 1.0M perchloric acid containing a known amount of tritiated thymidine. One ml of the 1.0M perchloric acid DNA extract mixed with 10 ml of scintillator mixture was counted with an efficiency of about 27%. Thymidine incorporation is expressed as the specific activity of the DNA fraction in d.p.m. / μg DNA-P.

5.7 Histological Preparations.

It has been shown in resting and regenerating liver

that proliferative cell populations are distributed uniformly throughout the lobes of the liver remnant (Fabrikant, 1964). For all studies the right lateral lobe was used and considered representative.

Animals were killed by heavy ether anaesthesia and exsanguination. Portions of liver 1-2mm thick were placed in fixative (20 parts 70% ethanol, 2 parts 10% formalin ($CaCO_3$ in excess) and 1 part glacial acetic acid) for 24hr., transferred to 70% ethanol, and after dehydration in increasing alcohol strengths, cleared in toluene and mounted in histological wax by vacuum embedding after thorough infiltration. Microtome sections 4 to 5 micrometers thick were obtained using a Beck rotary or MSE sledge microtome.

5.8 Autoradiography.

The emulsion dipping procedure for high resolution autoradiography was selected (Bogoroch, 1972; Lord, 1963; Baserga & Malamud, 1969). The liquid photographic emulsion used was Ilford K5 Nuclear (Gevaert Scientia 7.15 was also used) with grain size of the order 0.2 micrometers. After being brought to water by passage through toluene (1hr.) and decreasing strength alcohols, tissue sections were dipped in emulsion, diluted 1:1 (v/v) with 2% glycerol at 43°, and dried vertically at 25° and 75% relative humidity to minimise background. Slides were stored at 4° in light-tight black

boxes in the presence of silica gel. A dry atmosphere maintained in this manner was found to be essential for proper development.

A 'cold' and 'hot' standard were present in each box. The cold standard was a non-radioactive section. A heavily labelled slice, taken from the regenerated liver of an animal injected 16hr. after partial hepatectomy with 0.5 μCi /g body weight of tritiated thymidine and killed 22hr. after partial hepatectomy, close to the mitotic labelling peak, was used as the hot standard. The cold and hot standards were used to evaluate the background labelling and reliability of the method. The cold standard had generally less than 1 grain per cell. Batches of slides with a cold standard having greater than this amount were discarded. For the hot standard parameters investigated were:

i) the average label per mitosis.

ii) the number of labelled nuclei per hundred cells.

Batches of autoradiographs in which the hot standard had less than 18 grains per mitosis and less than 20 labelled nuclei per 100 nuclei were discarded. Additionally individual slides having a high background were discarded.

All histological preparations were examined using an oil immersion lens (magnification 1300x) on a Vickers Patholux binocular microscope.

The exposure time selected was two weeks. Slides were developed at room temperature with Kodak Microdol-X (and in some instances with Gevaert G170c, with comparable results) for 3 min, washed in fresh 1% acetic acid for 10 sec, fixed in 1:4 (v/v) Hypam /water for 2 min and washed in running distilled water. Sections were stained with Meyer's Haematoxylin and eosin (Bogoroch, 1972) and then dehydrated and cleared to minimise artifacts as follows (Bogoroch, 1972): 2 min in 95% ethanol, 4 min in absolute ethanol, 1hr. in 1:1 (v/v) absolute ethanol /cedarwood oil, 1hr. in 1:1 (v/v) toluene / DPX. After mounting in DPX, slides were dried at room temperature. Parenchymal cells were scored as labelled if an arbitrary lower limit of 5 grains per nucleus was exceeded.

5.9 Determination of Cell Cycle Phases by the Method of Labelled Mitoses.

The times of the G_1, S, G_2 and M phases and T_c were determined by the technique of labelled mitoses (Fabrikant, 1968a). The fraction of labelled parenchymal cell mitoses was determined at hourly intervals after injection of tritiated thymidine by the method of Quastler & Sherman (1959).

The time of prophase was obtained from prophase and metaphase labelling curves, while the times of the other mitotic phases were obtained from the ratios:

$$\text{Metaphase time} = \frac{\text{Prophase time}}{\%\text{Prophase population}} \times \%\ \text{Metaphase population}$$

$$\text{Anaphase time} = \frac{\text{Prophase time}}{\% \text{ Prophase population}} \times \% \text{ Anaphase population}$$

$$\text{Telophase time} = \frac{\text{Prophase time}}{\% \text{ Prophase population}} \times \% \text{ Telophase population}$$

(Post et al. 1963).

Partially hepatectomized rats were injected with 0.5 μCi /g body weight [6-^{3}H] thymidine 16hr. after operation. They were killed at hourly intervals and the excised livers added to fixative. After blocking and slicing, as described previously (5.7), a minimum of four slides were studied autoradiographically (5.8). One hundred mitoses were scored for each rat.

The means and standard deviations of G_1, S and G_2 phase durations were also estimated by plotting the fraction of the labelled mitoses on probit paper by the method of Okumura, Onozawa, Morita & Matsuzawa (1973). This method assumes the frequency distribution of the duration of each phase to follow a normal distribution. The values were compared with the values found from a Monte Carlo programme (Barrett, 1966); this requires iteration of a simulated labelled mitoses curve until simulated and experimental curves are consistent.

5.10 Mitotic Index and Abnormal Mitotic Figures.

The mitotic index was found by counting the number of

fields necessary to score 100 mitoses and from a determination of the number of cells /field. The mitotic index was corrected for the increasing mass of the liver (Fabrikant, 1968). The corrected mitotic index may be defined as the total number of parenchymal cells proliferating in the liver remnant at the time of observation expressed as a fraction of the number of cells in the remnant at the time of partial hepatectomy (Fabrikant, 1968).

Concurrently with the determination of mitotic index the proportion of abnormal mitotic figures was noted. The following criteria of abnormal mitotic figures were taken (Horst & Rudnicki, 1962):

a) Prophase: the dominating feature was taken as an abnormally formed chromatin network which appeared homogeneous and without the typical filamentous structure, with occasionally final nuclear dissolution and irregular chromatin clumps in the cytoplasm.

b) Metaphase: abnormal figures were characterised by interrupted connection of some chromosomes with the spindle, clumping of chromosomes into a homogeneous circular formation or chromosomal fragmentation.

c) Anaphase: irregular distribution of chromosomes, fragmentation and chromosomal bridges.

d) Telophase: pseudo amitosis, acentric fragments and micronuclei.

5.10.1 Corrected Mitotic Index

The mitotic index gives the proportional number of cells in mitosis. In order to provide a measure of the total number of cells in division the mitotic index must be corrected for the increasing mass of the liver remnant on regeneration (Fabrikant, 1968). The estimate of the liver size, taking the size of the liver remnant at partial hepatectomy as unity, was based on the hypothesis that at the end of each 2hr. period a number of cells equal to the number in mitosis during this period was added to the cell population. The number of cells in mitosis was determined from the mitotic index at the end of the 2hr. period and the estimated number of cells at the beginning of the period.

$$CMI_t = MI_t \times H_t$$

where:

MI_t = observed mitotic index; fraction of cells in mitosis at time t.

CMI_t = corrected mitotic index at time thr. after partial hepatectomy.

H_t = number of cells in the liver at time t expressed as a fraction of the number of cells in the liver remnant at the time of partial hepatectomy.

For a mitotic length of 1hr. (Fabrikant, 1968) after nhr.:

$$H_t = (MI_t \times H_{t-1}) + (MI_{t-1} \times H_{t-2}) \ldots + (MI_{t-(n+2)} \times H_{t-(n+1)}) + (MI_{t-(n+1)} \times 1) + 1.$$

RESULTS

6.1 DNA Synthesis in Regenerating Liver.

The rate of DNA synthesis is generally measured by the incorporation of radioactive tracers, thymidine usually being used for this purpose. Maximal incorporation of radioactive thymidine into the acid soluble nucleotide fraction and DNA of regenerating liver occurs within 10 min of intraperitoneal injection (Chang & Looney, 1965). The use of labelled thymidine to measure DNA synthesis has been criticised because thymidine has been found to bind to proteins in hepatocytes (Morley & Kingdon, 1972) and to a DNase resistant part of the mouse cell cytoplasm (Bryant, 1966). The thymidine used in both the above studies was labelled in the methyl group, which is perhaps more labile than the 6 labelled thymidine used in the present study. The controls, injected with labelled thymidine but without Myleran eliminate this source of error.

The rate of DNA synthesis was estimated at various times following partial hepatectomy by the incorporation of $[6\text{-}^{3}H]$ thymidine into the regenerating liver remnant. The $[6\text{-}^{3}H]$ thymidine was injected intraperitoneally 45 min before sacrifice and its incorporation into DNA measured by liquid scintillation counting (5.6).

Incorporation of 6-^{3}H thymidine in the regenerating liver per g wet weight of liver for control and Myleran-treated rats is shown in Fig. 2; the resultant specific activity of the thymidine labelled DNA (d.p.m./μg DNA-P)is shown in Fig. 3. Table 1 summarises the effect of Myleran on the DNA synthetic rate at the peaks of DNA synthesis, as shown in Fig. 3.

For each rat the DNA content /g wet liver was determined in duplicate, for two liver samples. The specific activity of extracted DNA was also measured in duplicate for each of the liver samples. The standard deviation at each point (σ) was calculated from the formula (Beyer, 1968):

$$\sigma = \pm \sqrt{\frac{(X_{1 \rightarrow n} - \bar{X})^2}{n - 1}}$$

where:

$X_{1 \rightarrow n}$ = d.p.m. /g wet liver (Fig. 2) or specific activity of the DNA fraction (Fig. 3) for each of the values $1 \rightarrow n$.

$\bar{X}$ = the mean of the X values.

n = the number of rats used at each point.

Three peaks of DNA synthesis, at 18hr., 30hr. and 58hr., were found during the first 72hr. of liver regeneration. Myleran does not appear to alter the times at which DNA synthesis is maximal, with measurement of the rate of DNA synthesis

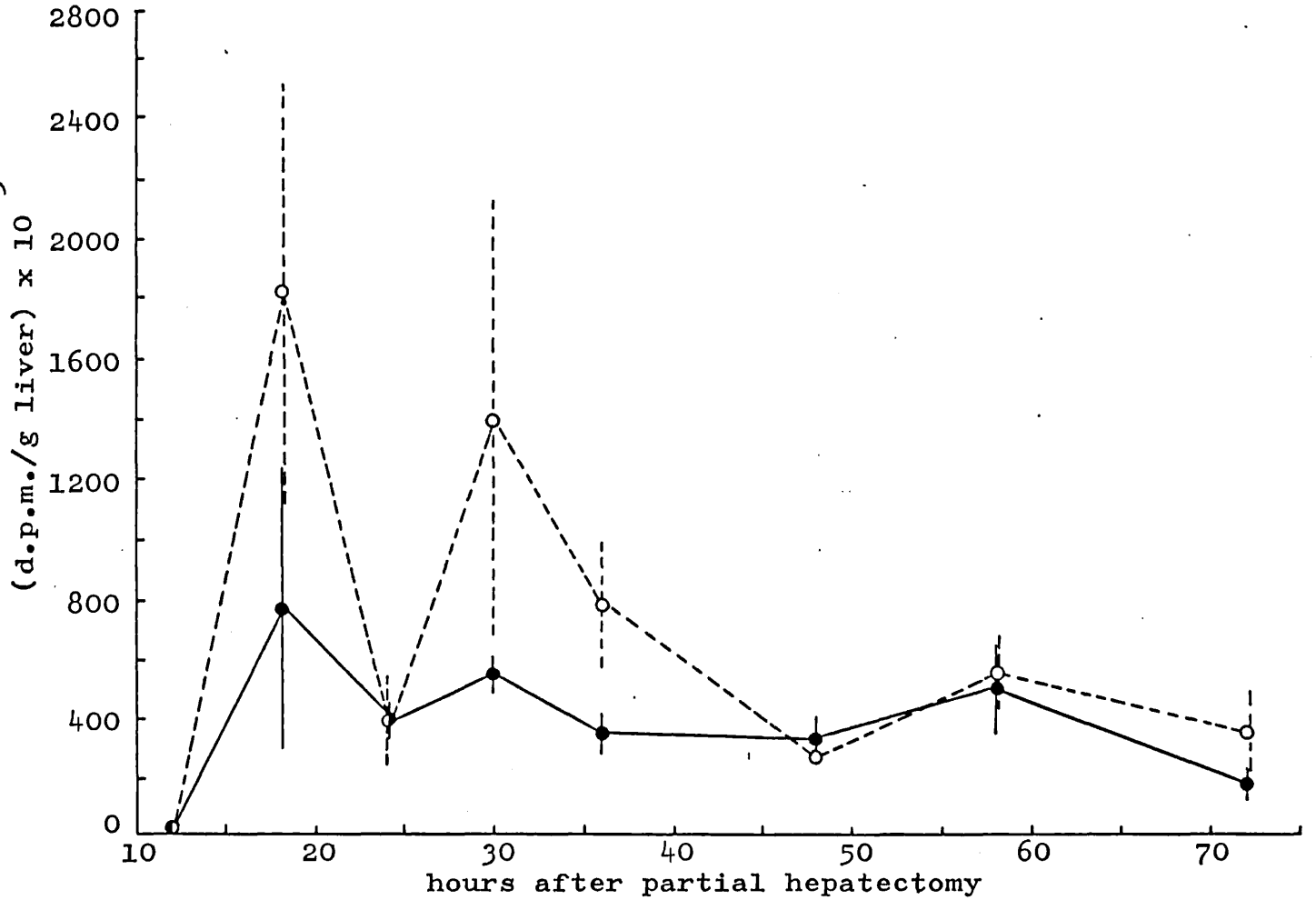


Fig. 2. The incorporation of [6-^{3}H] thymidine relative to wet weight regenerating rat liver.

Rats were either (●) given Myleran directly following operation ; or were (○) controls. Both groups were injected intraperitoneally with [6-^{3}H] thymidine 45 min before death. Vertical bars represent the standard deviation. Each point represents the average determined using three rats.

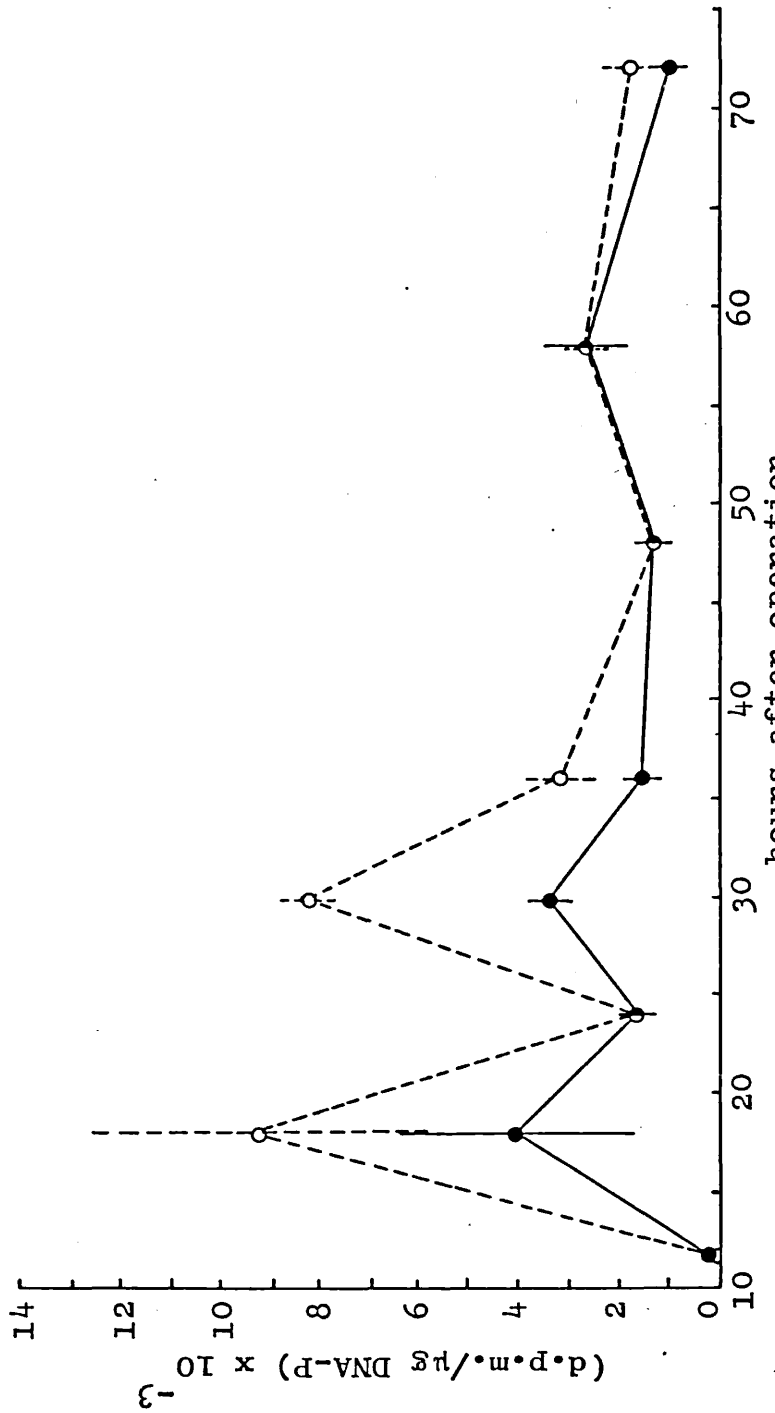


Fig. 3. The effect of Myleran on specific activity of the DNA fraction.

Regenerating liver, DNA fraction, in rats (●) given Myleran directly following operation, and (○) in controls. Both groups were injected with [6-^{3}H] thymidine intraperitoneally 45 min before death. Vertical bars represent the standard deviation. Each point is representative of an average value obtained from three rats.

Table 1. The Effect of Myleran on DNA synthesis in Regenerating Rat liver.

Position of DNA synthesis maxima (hr.)	Time (hr.) between DNA synthetic peaks	[1]% difference in peaks (control - Myleran)
18		55
	1st & 2nd 12	
30		58
	2nd & 3rd 28	
58		3

Myleran-treated rats were injected with 4.4mg Myleran /Kg body weight at operation and $[6\text{-}^{3}H]$ thymidine 45 min before death. Control rats were untreated with Myleran.

[1]These values obtained from Fig. 3 (DNA specific activity) were the differences between control and Myleran peaks of the thymidine incorporation as a % of the respective control peaks.

made at intervals of 6hr. for the first two peaks and 14hr. for the third peak (Fig. 3). A shift in the position of the peaks of DNA synthesis of <3hr. for the first two maxima and <7hr. for the third maximum, would not be observable for these experiments.

The estimate of the DNA synthetic rate obtained from the slope between the points of Fig. 3 is an approximate value, due to the large time gap between experimental values. Myleran lowers the incorporation of thymidine into DNA, in comparison with the rate of incorporation shown by the control rats. There is a decrease to 45% at 18hr. 42% at 30hr. and 97% at 58hr., Table 1. Myleran was injected immediately after operation, when the liver parenchymal cells were in the G_o phase of the cell cycle. Myleran exerts its greatest effect on incorporation of $[6\text{-}^3H]$ thymidine, as seen in Table 1 and Fig. 3, between 24hr. and 36hr. following partial hepatectomy.

The areas under the bands of DNA synthesis shown in Fig. 3 are proportional to the amount of DNA synthetized during their duration. The total DNA synthetized over 12-72hr. following partial hepatectomy in Myleran-treated rats and controls was estimated from the weight of cardboard cut-outs of the graphs of specific activity of the DNA (Fig. 3). The total DNA synthetized between 12-72hr. for the Myleran-treated rats was found to be 60.3% of that of the control animals. Fig. 3 was used to calculate these values since it is more

accurate than Fig. 2, which depicts the incorporation of $[6\text{-}^{3}H]$ thymidine /g wet weight regenerated liver, due to the difficulty of obtaining an accurate weight for the liver remnant.

6.2 Thymidine Kinase Activity.

It was felt that the effects of Myleran would be most clearly shown at the time when thymidine kinase activity was maximal. Ol'shanetskaya, Kavelina & Novikova (1968) and Beltz (1962), found thymidine kinase activity to increase from 18–30hr. in regenerating rat liver. Thymidine kinase activity was therefore determined at 30hr. following operation; the synthesis of DNA, dependant upon the activity of thymidine kinase, is still proceeding at a high rate at this time (6.1).

6.2.1 Analysis of Thymidine Kinase Activity

At 30hr. after operation thymidine kinase was extracted and its activity determined by the method of Ives, Durham & Tucker (1969), as detailed previously (5.2). Two rats were used for each determination. The value for the activity of the enzyme was found to be dependant upon the dilution of the homogenate used for the assay. The enzyme activity was first determined at a dilution of 1/5 (g liver /v 0.25M sucrose) and this value taken as unity. The activity found for the 1/25 (g liver /v 0.25M sucrose) and 1/250 (g liver /v 0.25M sucrose) diluted rat liver homogenates was 8.3 and 73.1 of the activity of the original 1/5 (g liver /v 0.25M sucrose) diluted homogenate. These values are summarised in Table 2.

Experimental rats were injected with Myleran directly

after operation. The values of thymidine kinase activity for experimental animals were expressed as a % of the activities found for control rats killed at the same time. Thymidine kinase activity in the 1/5 (g liver/ v 0.25M sucrose) diluted homogenate was lower in those rats given Myleran by an average of 36%. The values of thymidine kinase activity determined for control and Myleran-treated rats using a 1/25 (g liver/ v 0.25M sucrose) diluted liver homogenate were both greater than the values found for a 1/5 (g liver/ v 0.25M sucrose) diluted liver homogenate obtained from control rats. At a 1/250 (g liver/ v 0.25M sucrose) dilution, the values of thymidine kinase activity obtained from control and Myleran-treated rats were both greater than the value of the 1/25 (g liver/ v 0.25M sucrose) homogenate from control rats. At this dilution the activity of thymidine kinase was 12.5% lower for rats treated with Myleran than that of the 1/25 (g liver/ v 0.25M sucrose) diluted control; while at 1/250 (g liver/ v 0.25M sucrose) dilution, activity was 25% greater for Myleran-treated rats than the control value. Table 3 summarises the effect of Myleran on the 'apparent' thymidine kinase activity.

Table 2. Rat Liver 'Apparent' Thymidine Kinase Activity on Dilution of Homogenate for Control Animals 30hr. following Partial Hepatectomy.

Dilution of Homogenate	'Apparent' Thymidine Kinase Activity as a fraction of activity of 1/5 diluted homogenate
1/5	1
1/25	8.3
1/250	73.1

The activity of thymidine kinase was determined as detailed before (5.2).

Table 3. Rat Liver 'Apparent' Thymidine Kinase Activity 30hr. after Partial Hepatectomy and Myleran injection.

1/5 dilute homogenate Activity (% control)	1/25 dilute homogenate Activity (% control)	1/250 dilute homogenate Activity (% control)
61	87.5	125
67		

Myleran-treated rats were given 4.4mg Myleran /Kg body weight at operation. The control value found in each instance was assigned 100% thymidine kinase activity. Two rats were used for each determination.

6.3 The DNA content of Regenerating Liver.

Fig. 4 depicts the DNA content per g wet weight of regenerated liver, for control and Myleran-treated animals over the first 72hr. following partial hepatectomy. The DNA was estimated (5.4.1); each point on the graph representing the average from determinations on three rats. Myleran was injected intraperitoneally immediately after partial hepatectomy.

It is not possible to decide by inspection of Fig. 4 whether there are significant differences between the values for DNA-P/ g wet liver for control and Myleran-treated rats, since the standard deviations overlap. Student's t-distribution for probability was applied to the results. A % probability that a difference exists between the mean values for the two sets of results at any time point is found from tables of T versus υ , where:

$X_n = \sigma_n^2/n_n$, σ being the standard deviation of a set of values (n) and n their number.

$$T = \frac{|MEAN_1 - MEAN_2|}{\sqrt{X_1 + X_2}}$$

$$\upsilon = \frac{(X_1 + X_2)^2}{\left(\frac{X_1^2}{(n_1 - 1)} + \frac{X_2^2}{(n_2 - 1)}\right)}$$

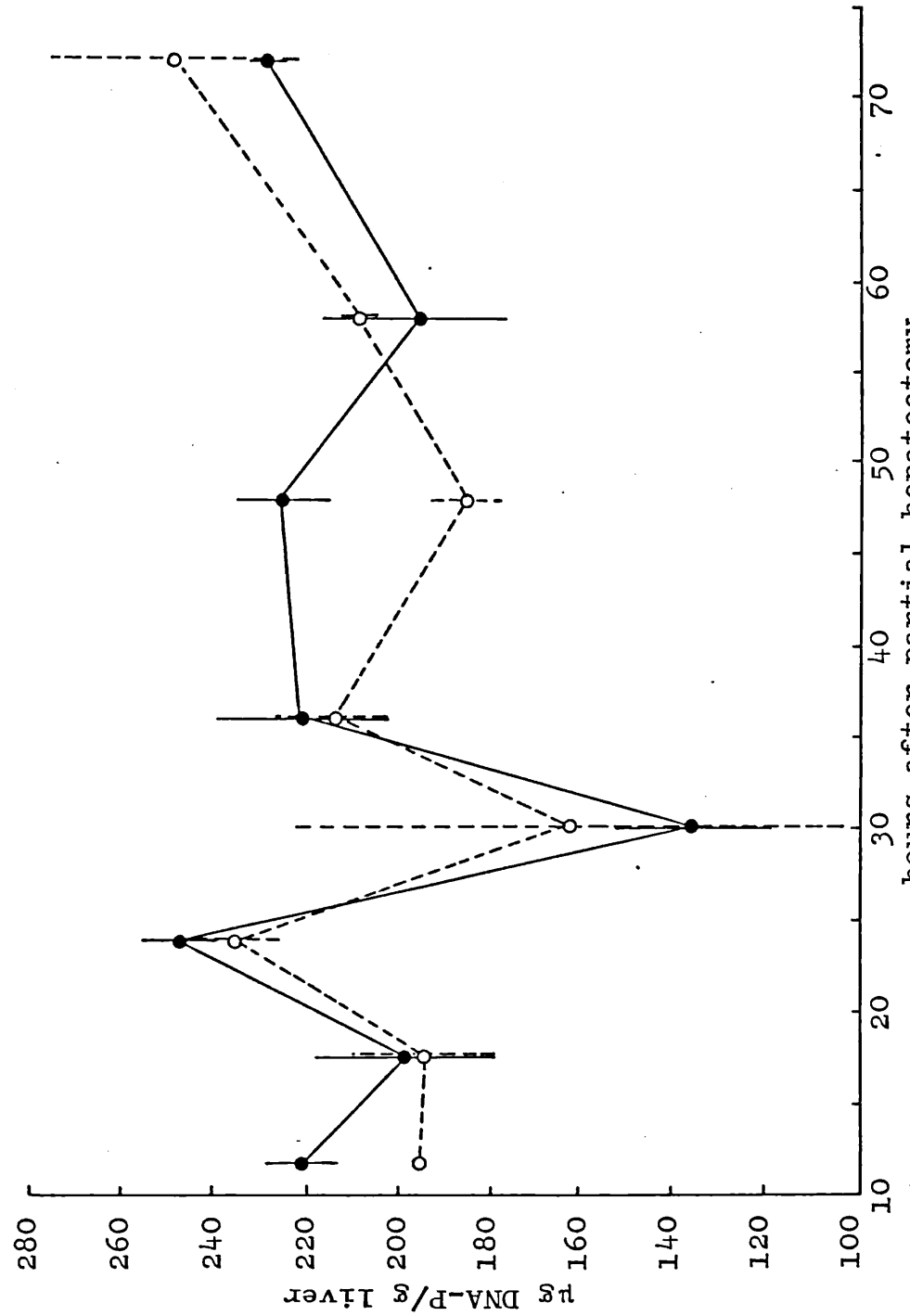


Fig. 4. DNA content of regenerating liver as a function of time after partial hepatectomy.

DNA content per g wet weight regenerated liver measured over 72hr. following partial hepatectomy, in rats (●) treated with Myleran immediately after operation, and (○) in controls. Vertical bars represent the standard deviation. Each point represents the average of determinations on three rats.

The results from Student's t-distribution indicated, at the least probability (at 30hr. and 36hr.), a >60% probability for difference between the two sets of results. The DNA-P/g wet liver from Myleran-treated rats is approximately 9% above the mean level for control rats between 12-24hr. and 36-48hr. following partial hepatectomy; while the value of DNA-P/g wet liver for the experimental animals is approximately 10% below that of the control animals at 30hr. and between 58-72hr. These results are summarised in Table 4.

Over the complete 12-72hr. period following operation DNA-P/g wet liver in Myleran-treated rats was found to be 101.3% in comparison with controls.

Table 4. DNA-P/g wet Regenerated Liver in Myleran-treated Rats compared to the Control Values.

Time (hr.) after partial hepatectomy.	% difference between Myleran-treated and control rats in DNA-P/g wet liver.	[1]% Probability for true difference between values for Myleran-treated & control rats.
18	3	60-75
24	5	80-90
30	-17	60-75
36	3	60-75
48	22	97.5-99
58	-6	75-90
72	-8	70-75

[1]The % probability that there was a difference between the two sets of values, that for Myleran-treated and that for control rats, was determined using Student's t-distribution (6.3).

6.4 The RNA Content of Regenerating Liver

The change in RNA-P/ g regenerated liver over the first 72hr. following partial hepatectomy is shown in Fig. 5 : each point represents the average value for three rats. The RNA-P content for both control animals and those treated with Myleran immediately after operation (5.3) was greatly elevated from 25hr. and remained elevated up to 72hr. A peak in the RNA-P content occurred at 36hr. with a gradual fall to 72hr.

RNA-P/g wet liver is raised in Myleran-treated rats, in comparison with those values found for the control rats, by 9% at 12hr. and 30hr. and 10% and 8% at 48hr. and 58hr. respectively; while the value is 4% lower than that found for controls at 18hr. after operation. The probability for there being a difference between control values and values for those rats that were treated with Myleran was found by calculation using Student's t-distribution to be $>70\%$ at each of these times. Probability values indicated that at other times after partial hepatectomy there was no difference in the values for RNA-P/g wet liver between control and Myleran-treated rats. Table 5 summarises these findings. Over the complete 12-72hr. period following partial hepatectomy RNA-P/g wet liver in Myleran-treated rats was found to be 104% of the control value.

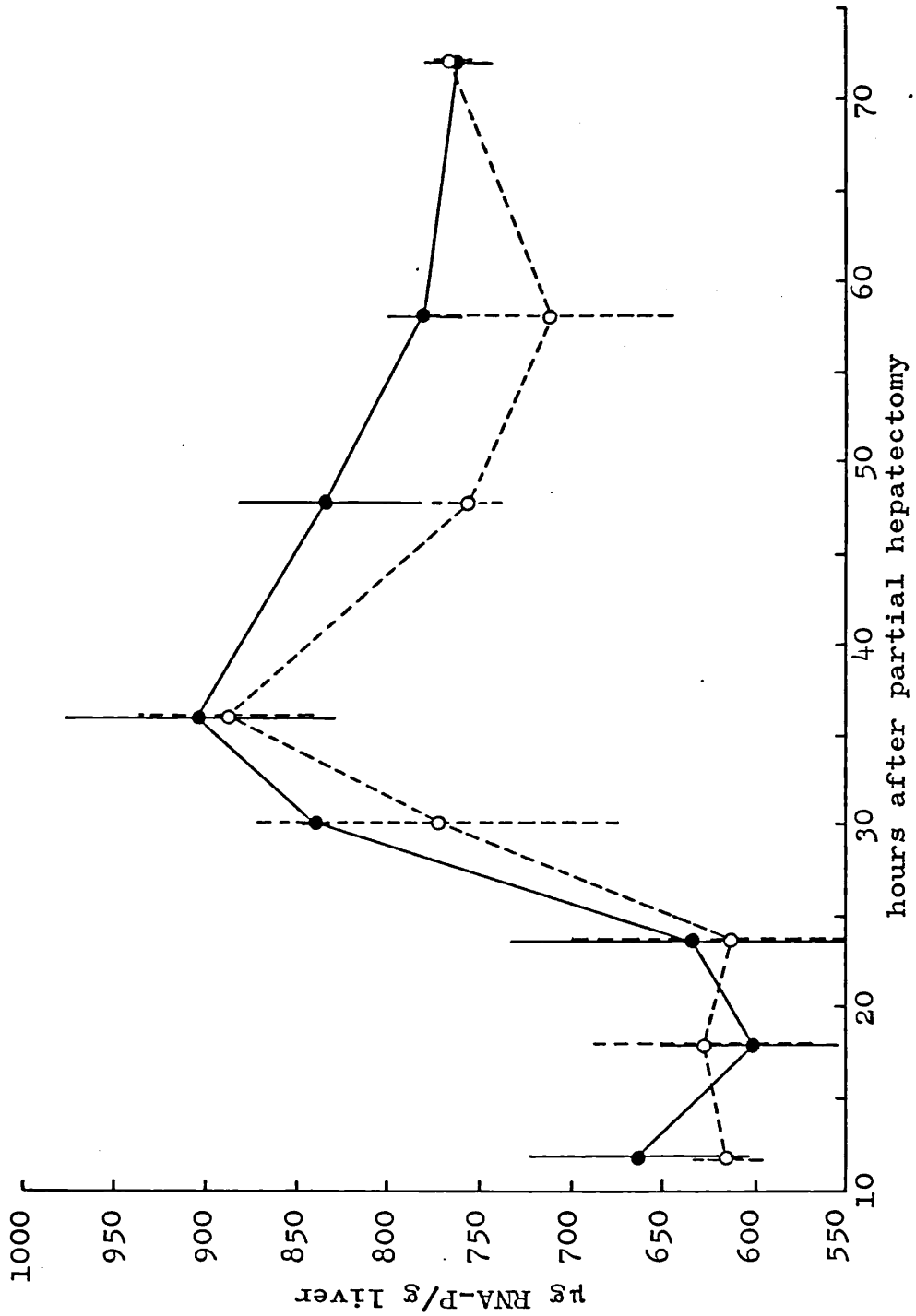


Fig.5. RNA content per g wet weight of regenerated liver as a function of hr. following partial hepatectomy.

(●) Rats treated with Myleran immediately after operation, and (○) controls. Vertical bars represent the standard deviation. Each point represents the average of determinations on three rats.

Table 5. RNA-P/g wet weight Regenerated Liver in Myleran-treated Rats compared to the Control Values.

Time (hr.) after partial hepatectomy.	% difference between Myleran-treated and control rats in RNA-P /g wet liver.	[1]% Probability for true difference in values for Myleran-treated & control rats.
12	9	75 - 90
18	-4	70 - 75
24	0	≤60
30	9	70 - 75
36	0	≤60
48	10	97.5 - 99
58	8	75 - 90
72	0	≤60

[1]The % probability that there was a difference between the two sets of values, that for Myleran-treated and that for control rats, was determined using Student's t-distribution (6.3).

The initial (12hr.) and final (72hr.) determinations of RNA content in the control rats agree with published values: 608 µg RNA-P/g wet liver before regeneration and 749 µg RNA-P/g wet liver after regeneration (Pai, 1964).

6.5 Restoration of Liver Weight

Fig. 6 shows restoration of liver weight in Myleran-treated and control rats over the first 72hr. following partial hepatectomy. Rate of regeneration, represented by the slope of the curve (Fig. 6) is most marked during the periods 12-30hr. and 36-48hr. following operation. It can be seen that the control curve of Fig. 6 has large standard deviations for each mean value, overlapping the standard deviations found for the values for Myleran-treated rats, even though three to sixteen rats were used at each time interval. By inspection therefore, it is not possible to see if the two curves of Fig. 6 differ. Student's t-distribution however, shows there to be a $>80\%$ probability for difference between the mean values for the Myleran-treated and control rats at 12hr. and between 24-58hr. Myleran-treated rats being 68% and 34% less restored in weight, compared to the controls, at these respective periods. At 72hr. Myleran-treated rats are 9% less regenerated in weight in comparison with untreated rats: that the difference is significant is shown by the probability value of $>70\%$. Measurements of liver weight regeneration at 18hr. for the drug-treated animals are not significantly different from those of the control rats when these values are compared using Student's t-distribution. These results are detailed in Table 6. By comparing the area under the curves of Fig. 6 between 12-72hr., which gives a measure of total regeneration in weight of the liver, Myleran-treated rats were found to be 73% regenerated in comparison with the control value.

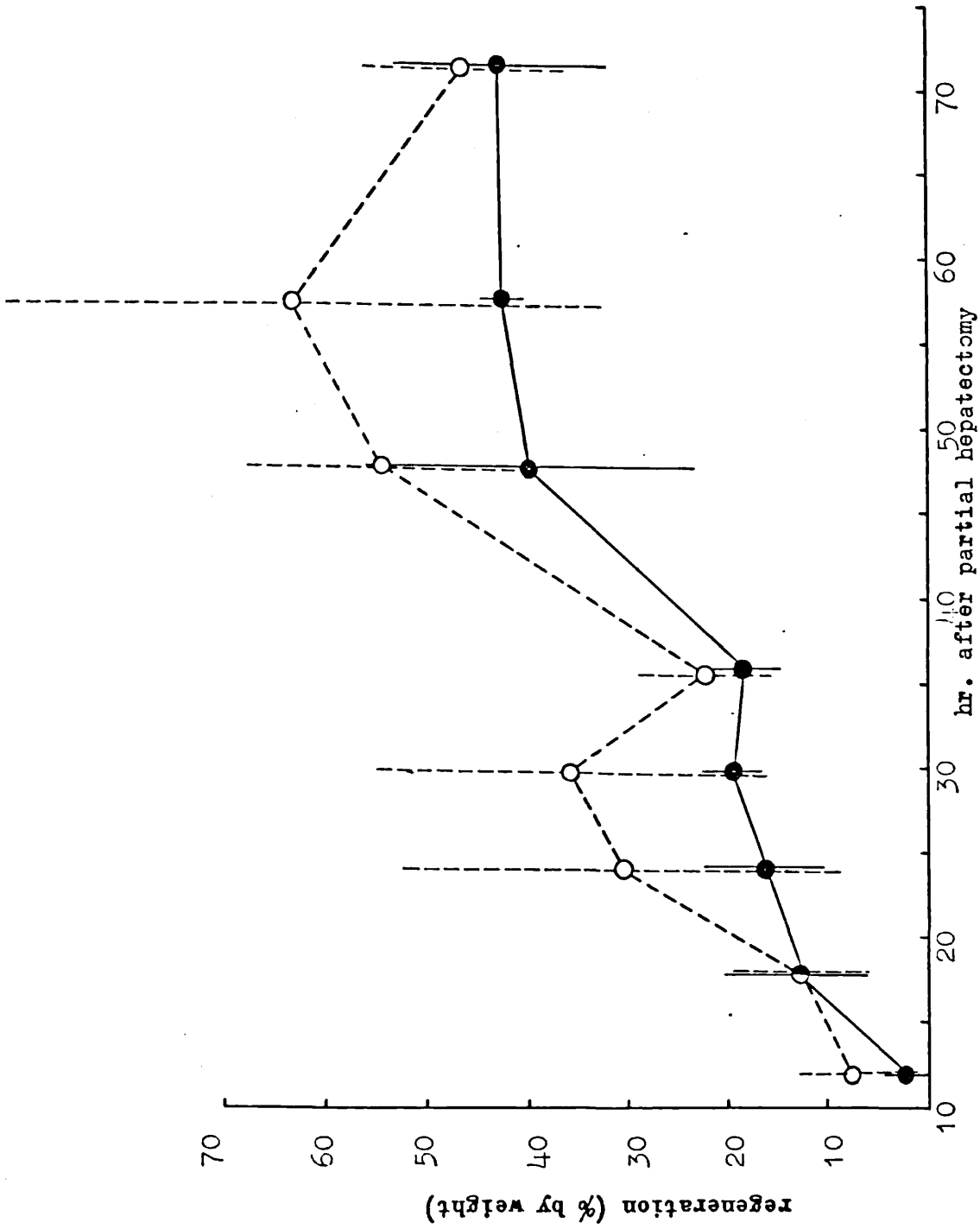


Fig. 6. The restoration of the liver by weight following partial hepatectomy.

(●) Average values for weight regeneration for rats treated with Myleran immediately following operation, and (○) controls. Vertical bars represent the standard deviation of the determinations. The % regeneration of the liver was calculated from the wet weight of liver removed at operation and that removed at death (5.5).

Table 6. Restoration of Liver Weight between 12-72hr. after Partial Hepatectomy in Myleran-treated rats as a % of Control.

Time (hr.) after operation.	Restoration of Myleran-treated livers (% control).	[1]% Probability of difference between Myleran-treated & control values being real.
12	33	80 - 90
18	100	$\leqslant$60
24	55	95 - 97.5
30	56	85 - 90
36	83	80 - 85
48	68	90 - 95
58	68	90 - 95
72	92	70 - 75

[1]The % probability that there was a difference between the two sets of values, that for Myleran-treated and that for control rats, was determined using Student's t-distribution (6.3).

The rate of regeneration (% regeneration/time) for Myleran-treated rats as a % of the rate shown by control rats is shown in Table 7. The rate of regeneration, as measured from the slope of Fig. 6, is decreased in the Myleran-treated animals to 36% of the control rate between 12-30hr. and 43% of the control rate between 36-48hr. Myleran does not affect the rate of regeneration over 48-58hr. There is a less sharp decline in the regenerative rate for Myleran-treated rats between 30-36hr. than observed for the control rats. Between 58-72hr. Myleran-treated rats have a very low regenerative rate.

The rate of regeneration found in the present work using liver weight measurements for control rats, over the first 30hr. after partial hepatectomy, agrees well with the following values found by Fabrikant (1964): 10, 18, 31 and 38% regeneration at 12, 18, 24 and 30hr. respectively after partial hepatectomy.

Table 7. Rate of Regeneration (% regeneration / time) of Liver from Myleran-treated Rats, as a % of control between 12-58hr. after Partial Hepatectomy.

Time (hr.) between which rate was measured.	Rate of Regeneration of liver from Myleran-treated rats as a % of the rate shown by untreated controls.
12 - 30	36
30 - 36	[1]-13
36 - 48	43
48 - 58	100

[1] Between 30-36hr. there was a negative rate of regeneration for both control and Myleran-treated rats.

6.6 The Lengths of the Mitotic Phases in Regenerating Rat Liver.

Partially hepatectomized rats treated with Myleran immediately after operation and untreated controls were given a 'pulse-label' of $[6\text{-}^3H]$ thymidine at 16hr. after operation (5.3). The rats were sacrificed at hourly intervals from 3-12hr. after the injection of $[6\text{-}^3H]$ thymidine. Sections of the liver remnant were prepared and examined by the emulsion dipping procedure for autoradiography (5.8). After autoradiography those cells that had incorporated the tritiated thymidine were seen to be labelled by the presence of silver grains in the overlying emulsion. Total mitoses and cells in prophase, metaphase, anaphase or telophase were counted and scored as either labelled or unlabelled.

Mitotic labelling curves were drawn for each of the four mitotic phases, for both control and Myleran-treated rats, between 16hr. and 28hr. after partial hepatectomy, Fig. 7 and Fig. 8. The prophase and metaphase % labelling curves are probably more reliable than the anaphase and telophase curves, because of the longer time taken for these stages of mitosis; this results in an increase in the number of prophase and metaphase stages seen under the microscope. Fig. 9 shows the relative % of cells in prophase, metaphase and telophase, for Myleran-treated and control rats, between 19-28hr. after partial hepatectomy.

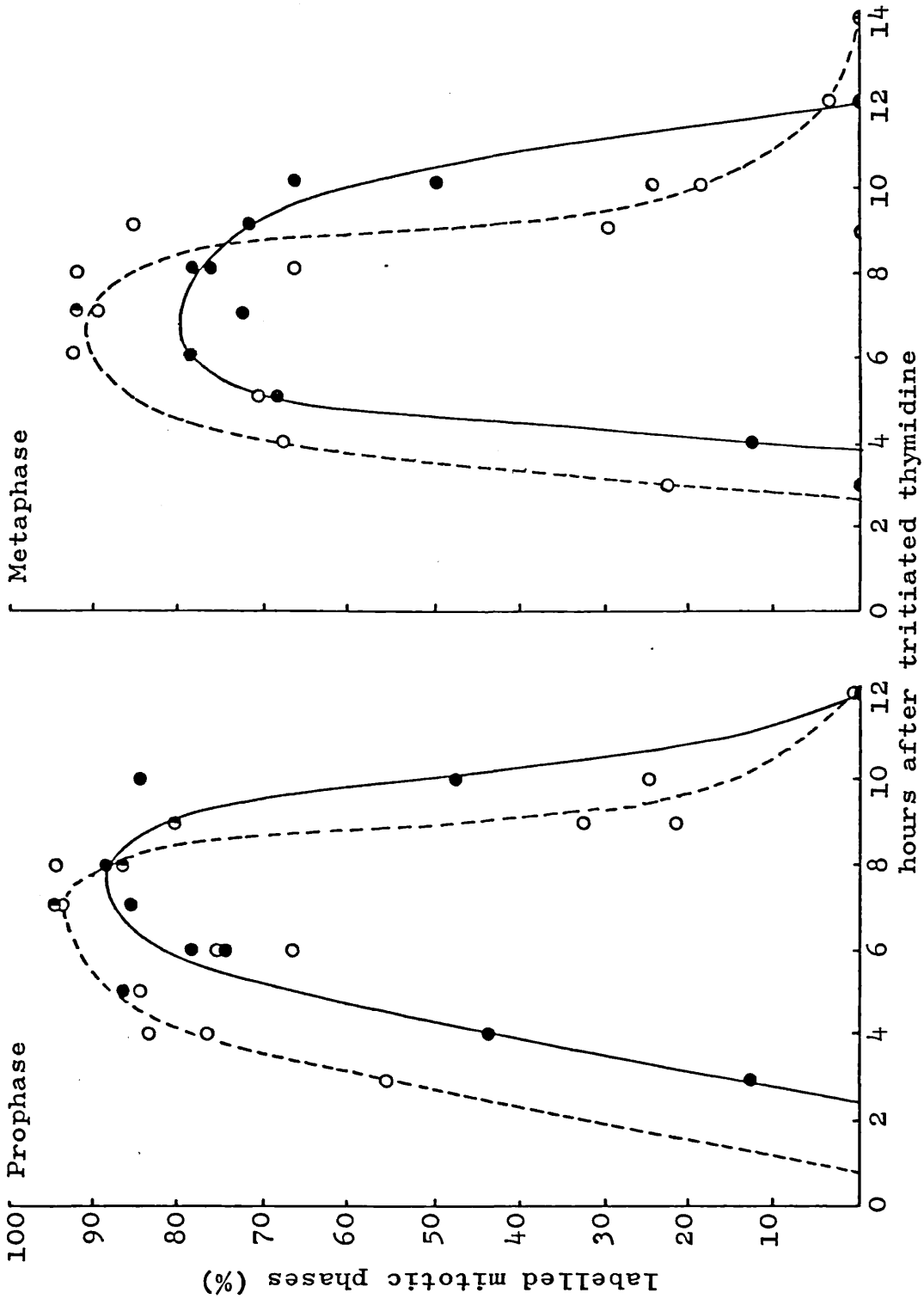


Fig. 7. The fraction of mitoses labelled in prophase and metaphase after partial hepatectomy. (●) Experimental rats given Myleran directly after operation, and (○) controls. Both groups were given [6-^{3}H] thymidine 16hr. after operation, corresponding to the origin of the abscissa.

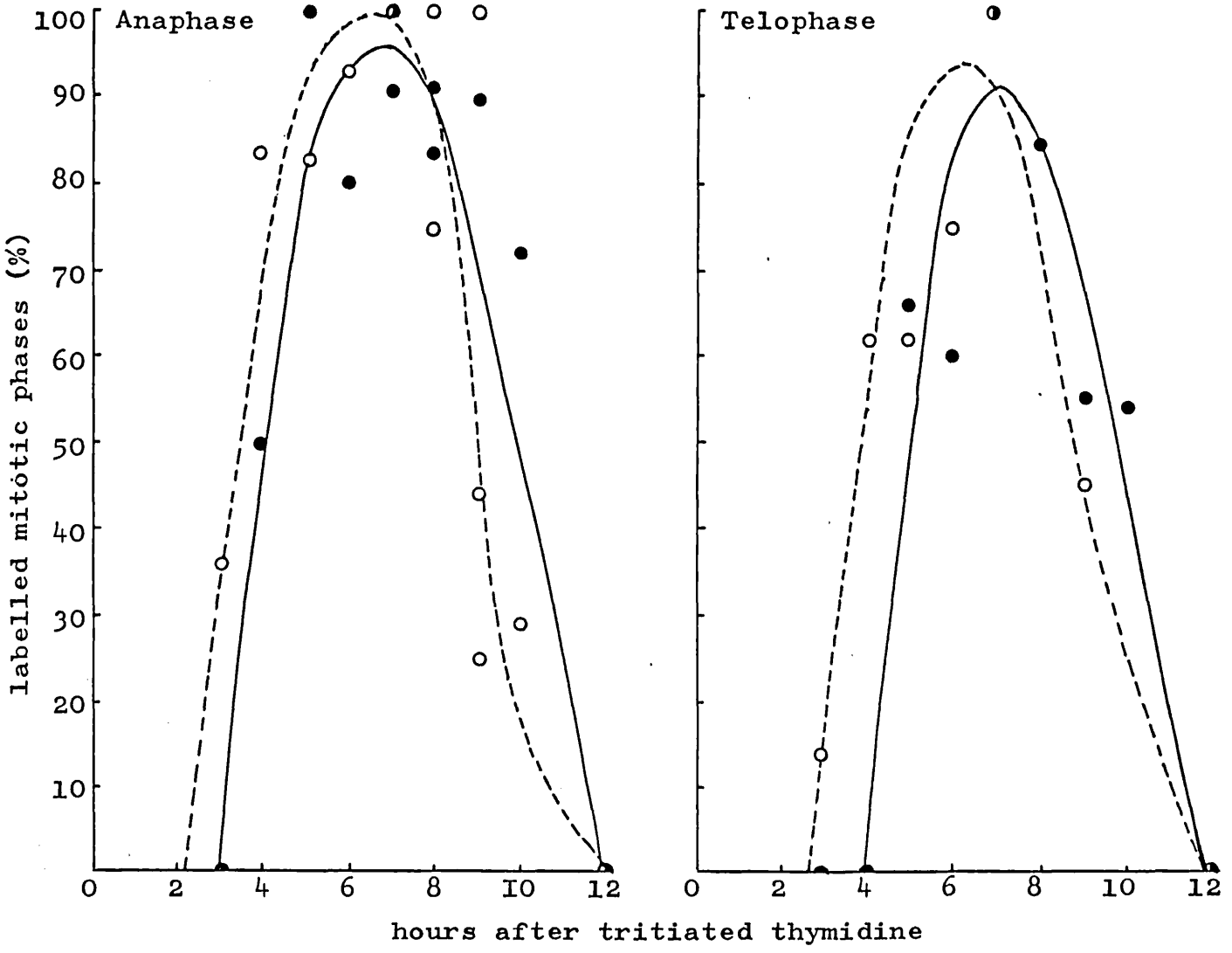


Fig. 8. The fraction of mitoses labelled in anaphase and telophase after partial hepatectomy.

(●) Experimental rats given Myleran directly after operation, and (○) controls. Both groups were given [6-^{3}H] thymidine 16hr. after operation, corresponding to the origin of the abscissa.

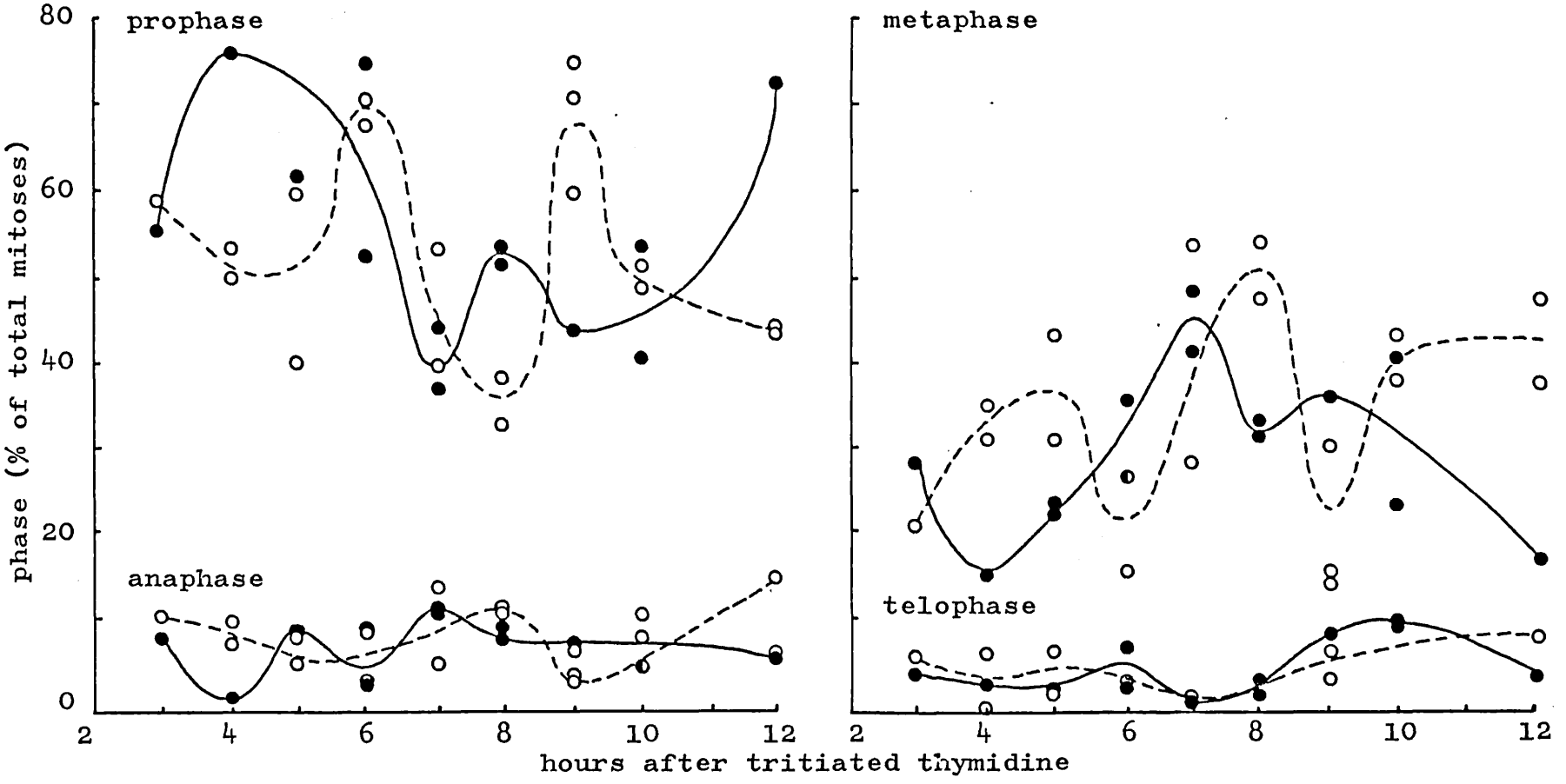


Fig. 9. The % of cells in differing mitotic phases following partial hepatectomy.

(●) Experimental animals, given Myleran directly following partial hepatectomy, and (○) controls. The origin of the abscissa corresponds to 16hr. after partial hepatectomy, at which time [6-^{3}H] thymidine was injected into both control and Myleran-treated rats.

Prophase and metaphase nuclei constituted the predominant mitotic population and led to the greater accuracy of the % labelling curves for these phases (Fig. 7). For each rat used for the determination of the % labelled mitotic phases (Fig. 7 and Fig. 8), one hundred mitoses were scored. Up to sixteen microscope slides, or four thousand fields (5×10^4 cells) were examined for each rat.

The time occupied in prophase was found by the difference in time between the first appearance of labelled metaphase figures and the first appearance of labelled cells in prophase. The respective times were found from the intercepts of the ascending limbs of the % labelled metaphase and prophase curves of Fig.7. It was not possible to find the times of metaphase or anaphase in this manner as the intercept on the abscissa of the ascending limb of the anaphase curve (Fig.8) was identical to that of the metaphase curve (Fig.7), for both Myleran-treated and control rats. The use of this method would give a value of zero for the time spent in metaphase and consequently an inaccurately long time spent in anaphase. The lengths of metaphase, anaphase and telophase were calculated, using the premise that the time interval occupied by a class of mitoses is proportional to the frequency of occurrence (5.9). The number of cells in a phase being calculated over 19-28hr. after operation.

The lengths of prophase, metaphase, anaphase and

telophase were 1.1, 0.65, 0.18 and 0.09hr. for Myleran-treated rats and 1.3, 0.94, 0.21 and 0.06hr. for controls, respectively. These values are given in Table 8. A total mitotic time of 2hr. for Myleran-treated rats and 2.5hr. for the controls is obtained on summation of the values found for the lengths of the individual mitotic phases.

The lengths of prophase and metaphase found in control rats (Table 8) correspond closely with the 1.3hr. and 1hr., respectively, found by Post et al. (1963) in neonatal rat liver.

Table 8. Estimation of the Length of Individual Mitotic Phases in Regenerating Rat Liver.

Phase of Mitosis	Control rats: Time (hr.) in phase.	Myleran-treated rats: Time (hr.) in phase.
PROPHASE	1.3	1.1
[1]METAPHASE	0.94	0.65
[1]ANAPHASE	0.21	0.18
[1]TELOPHASE	0.06	0.09

[1]Metaphase, anaphase and telophase times were estimated from equations relating the population of cells in a phase to the length of the phase (5.9).

6.7 The Incidence of Mitotic Abnormality in Regenerating Liver.

Sections of liver were prepared for Myleran-treated and control rats 20-48hr. following partial hepatectomy (5.7) and examined for mitotic abnormalities (5.10). In general, for both Myleran-treated rats and controls two animals were used at each time interval. For each rat up to eight microscope slides or two thousand fields (2.5×10^4 cells) were examined and at least fifty mitoses were scored. The mitoses were differentiated as either normal, or abnormal; the abnormal phases were distinguished as being in prophase, metaphase, anaphase or telophase. The type of abnormality within a particular phase of mitosis was not recorded.

The total number and % of abnormal mitotic cells in each phase for the period 20-48hr. following partial hepatectomy are shown in Fig. 10, 11, 12, and 13 for normal and Myleran-treated rats. Prophase and metaphase nuclei constituted the predominant mitotic population (Fig. 9); the greater frequency of their occurrence allowed a better estimate of their % abnormality to be made than for the abnormalities of anaphase and telophase.

There are a large number of abnormal prophase cells for both Myleran-treated rats and control animals (Fig. 10).

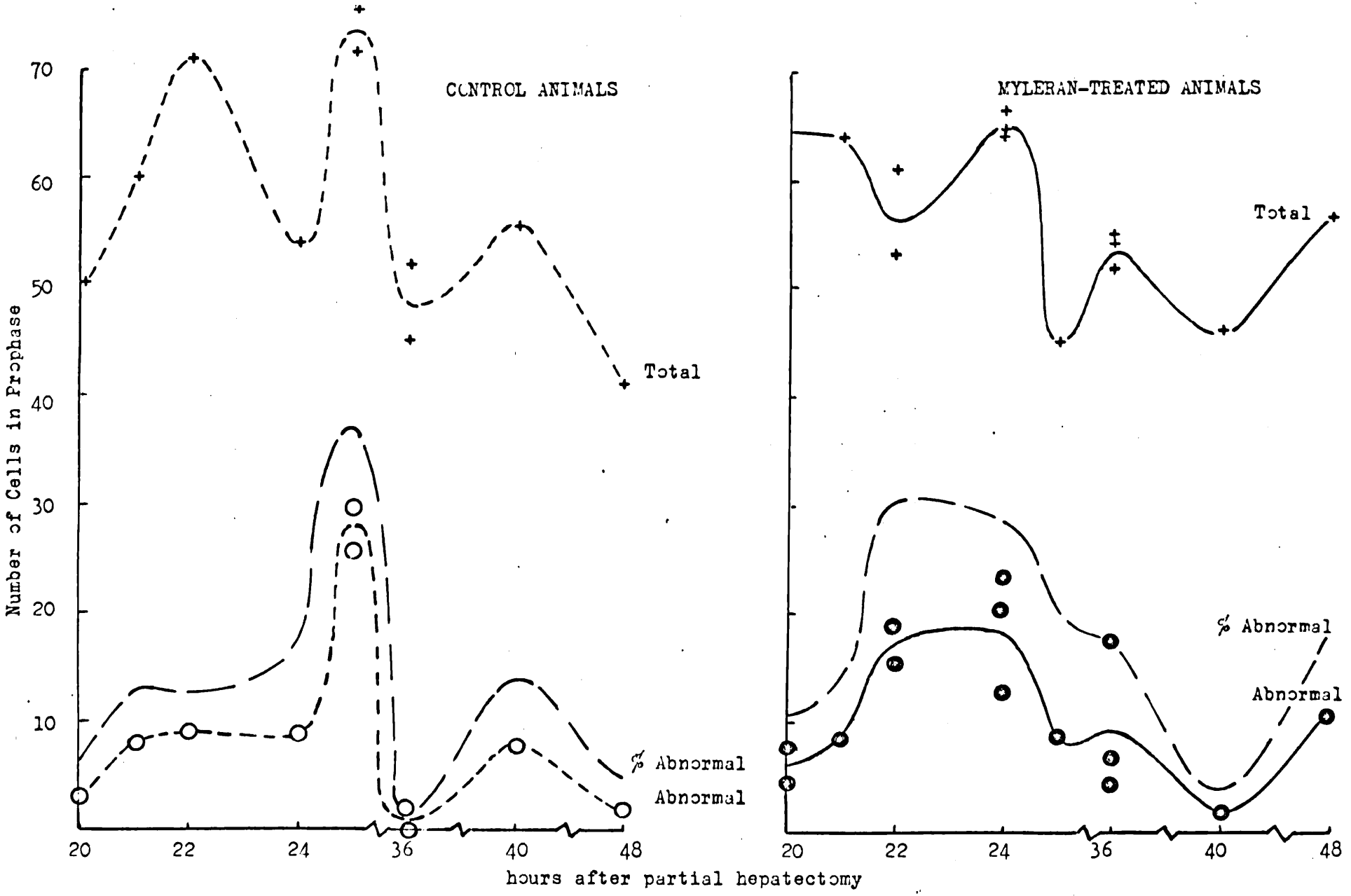


Fig.10. The number of normal and abnormal cells in prophase in rat liver, as a function of time after partial hepatectomy.

Total number of prophase cells, in a mitotic population of 100, and the number and % abnormal figures, over 20-48hr. following operation. (O) Control animals and (●) those treated with Myleran directly following operation.

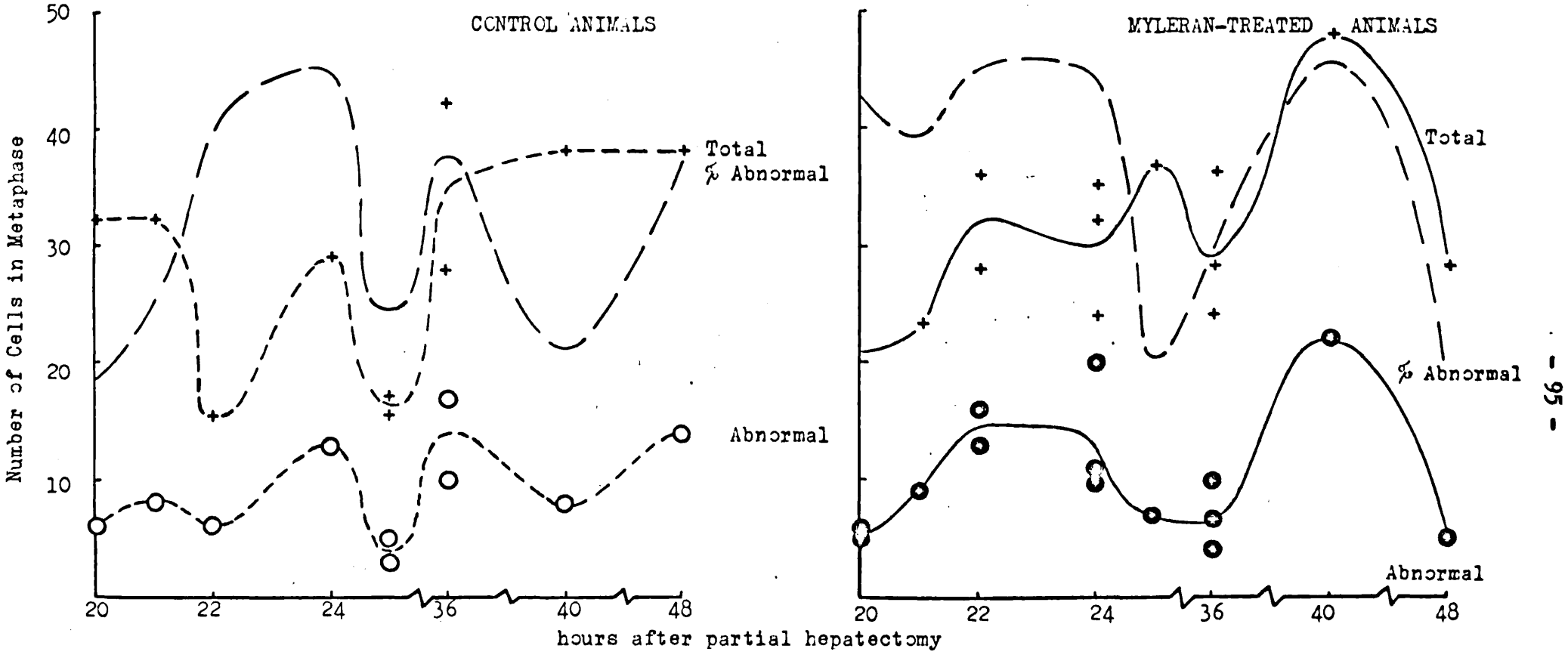


Fig. 11. The number of normal and abnormal cells in metaphase in rat liver, as a function of time after partial hepatectomy.

Total number of metaphase cells, in a mitotic population of 100, and the number and % abnormal metaphase figures over 20-48hr. following partial hepatectomy. (O) Control animals and (●) those treated with Myleran directly following operation.

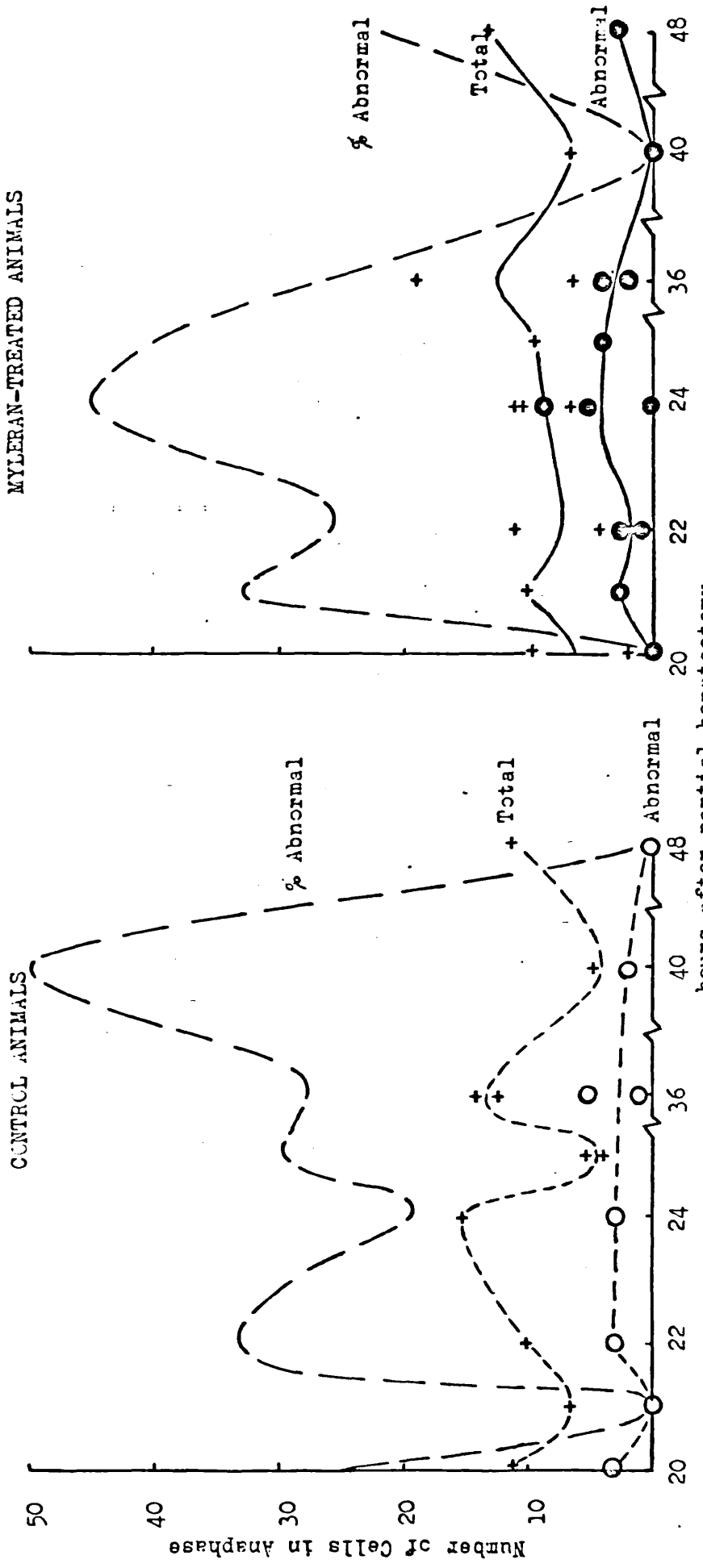


Fig. 12. The number of normal and abnormal cells in anaphase in rat liver, as a function of time after partial hepatectomy.

Total number of anaphase cells, in a mitotic population of 100, and the number and % abnormal anaphase figures over 20-48hr. following partial hepatectomy. (○) Control animals and (●) those treated with Myleran directly following operation.

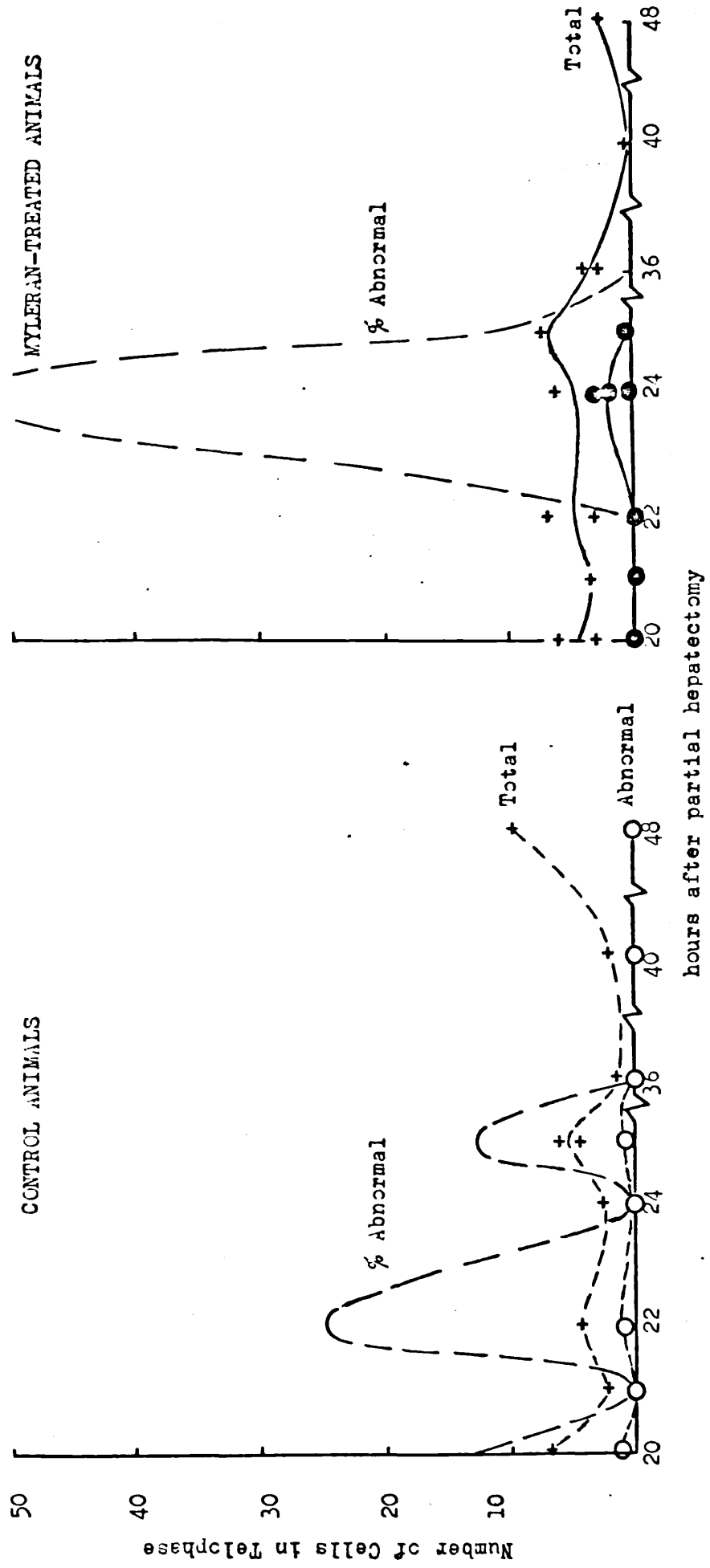


Fig. 13. The number of normal and abnormal cells in telophase in rat liver, plotted as a function of time after partial hepatectomy.

Total number of telophase cells, in a mitotic population of 100, and the number and % abnormal telophase figures over 20-48hr. following partial hepatectomy. (○) Control animals and (●) those treated with Myleran directly following operation.

The number of prophase figures throughout the period 20-48hr. after partial hepatectomy are not markedly depressed following Myleran administration, although a maximum in the number of prophase figures, found at 25hr. following operation in controls, is absent for Myleran-treated rats. Between 20-24hr. after operation the % of abnormal prophases is increased for Myleran-treated rats. Abnormal metaphase figures (Fig. 11) are, similarly, higher for Myleran-treated rats than controls between 20-24hr. following partial hepatectomy. No consistent effect of Myleran on the % of abnormal prophase and metaphase figures is seen later than 24hr. after operation and Myleran injection (Fig. 10 and 11).

An elevation in the number of abnormal anaphase figures is seen at 24hr. after operation in Myleran-treated rats though no significant effect of Myleran is apparent in the % of abnormal anaphases over the whole 20-48hr. period (Fig.12). The large value for the % abnormal anaphase cells at 25 and 40hr. is based on a low number of anaphases. The incidence of abnormal telophase figures (Fig. 13) was negligible for both Myleran-treated rats and control animals except at 24hr. A high % of abnormal mitotic cells in telophase at this time was based on an average of only four mitoses in this phase for each of the three rats examined.

Examples of normal and abnormal mitotic figures found in the liver remnant of rats injected with Myleran immediately following partial hepatectomy are shown in Fig. 14,15, 16 and 17.

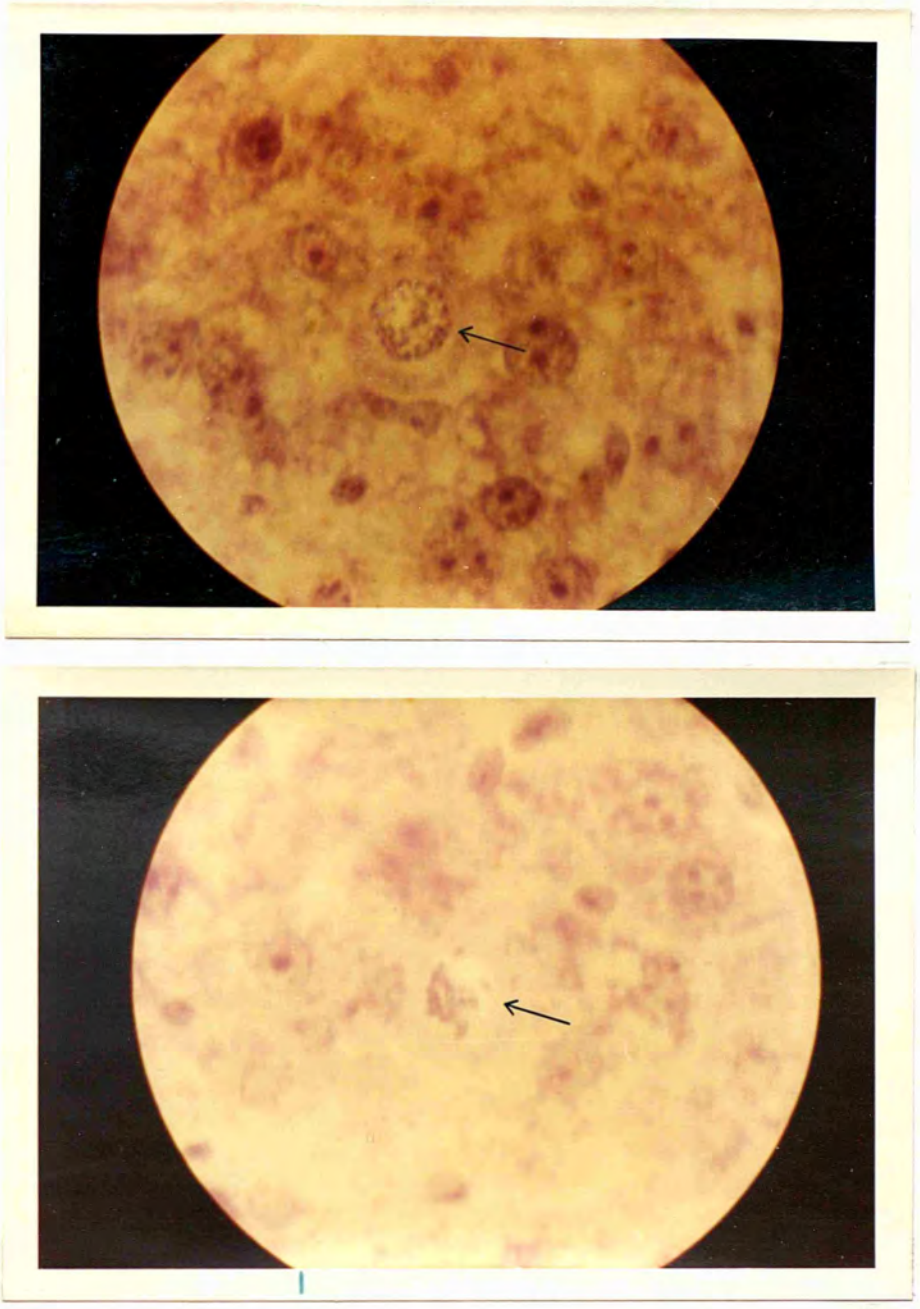


Fig. 14. Examples of abnormal prophase figures in the liver remnant following partial hepatectomy and Myleran injection.

In the upper photograph the chromosome network is homogeneous, while in the lower there is nuclear dissolution.
Magnification: Approximately 3000x.

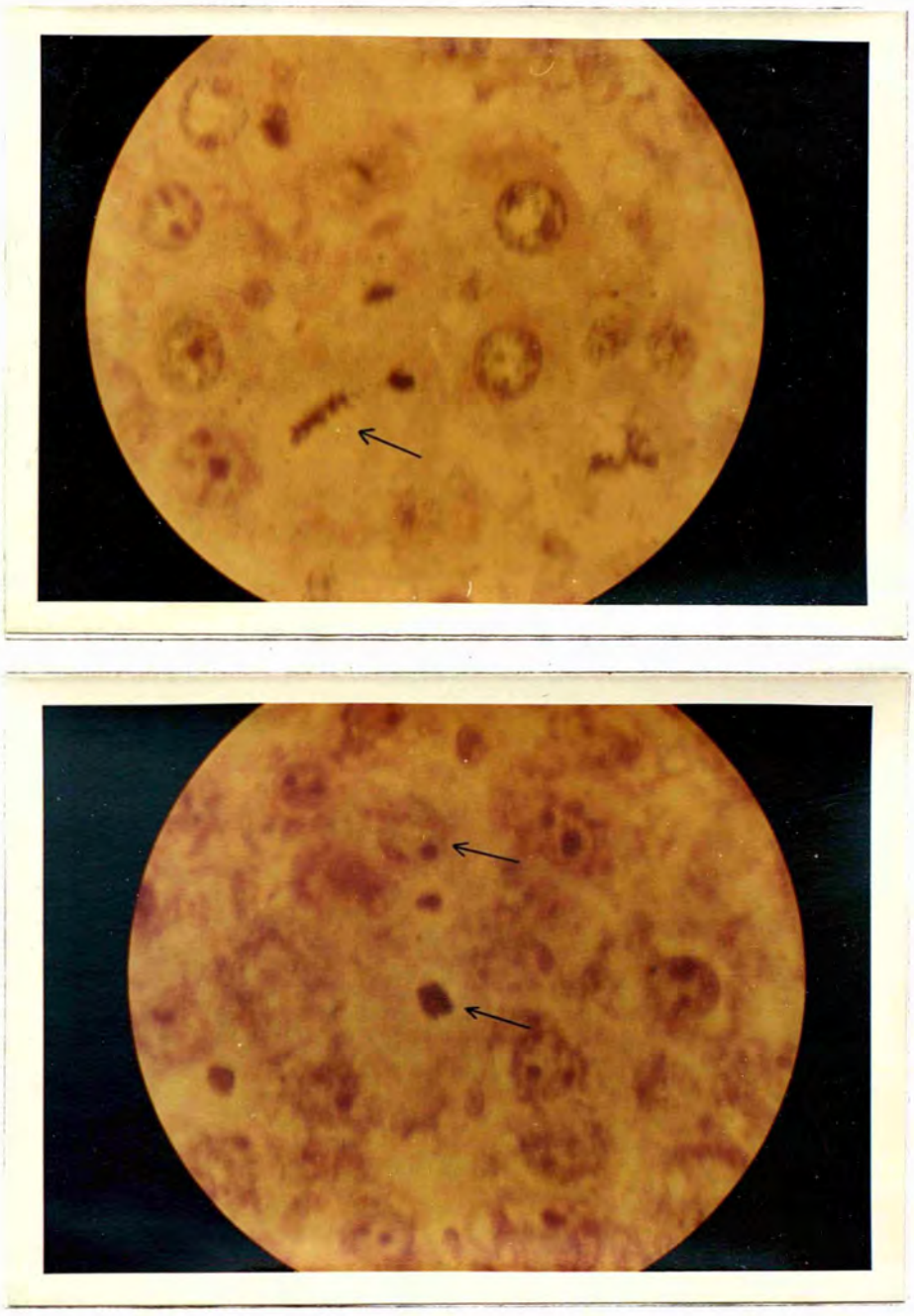


Fig. 15. Examples of normal and abnormal metaphase figures in the liver remnant following partial hepatectomy and Myleran injection.

The upper photograph shows a normal metaphase cell in which the chromosomes are arranged centrally on the spindle. The lower photograph shows an abnormal metaphase figure in which the chromosomes are clumped, while immediately above it is a normal metaphase, in which the chromosomes attached to the spindle are drawing to opposite sides of the cell.

Magnification: Approximately 3000x.

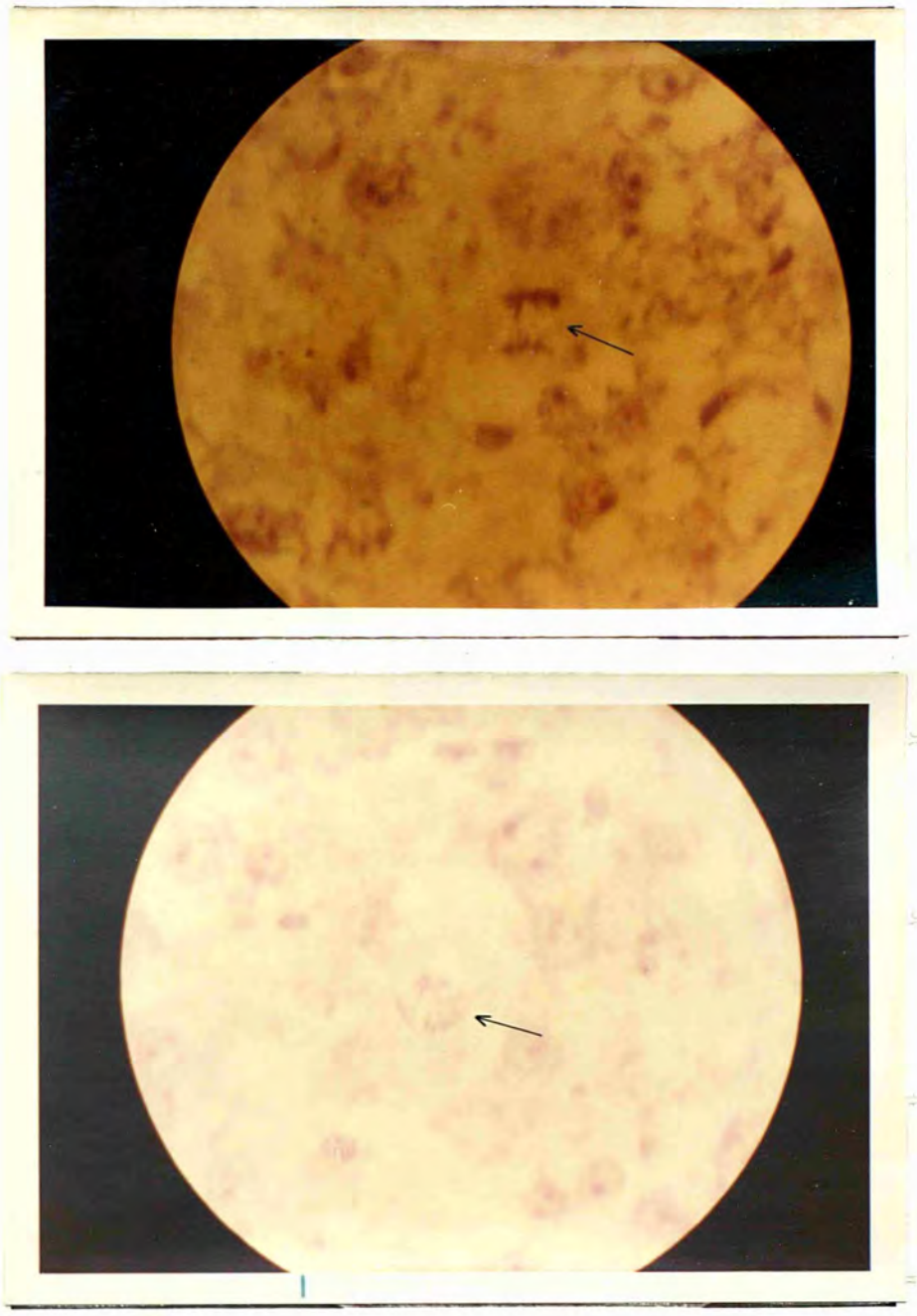


Fig. 16. Examples of abnormal anaphase figures in the liver remnant after partial hepatectomy and Myleran injection.

The upper photograph shows centrally an abnormal anaphase cell in which there is irregular distribution of the chromatin to one pole of the spindle. The lower photograph shows chromosomal interconnections, even though the chromosomes have reached the poles of the spindle.

Magnification: Approximately 3000x.

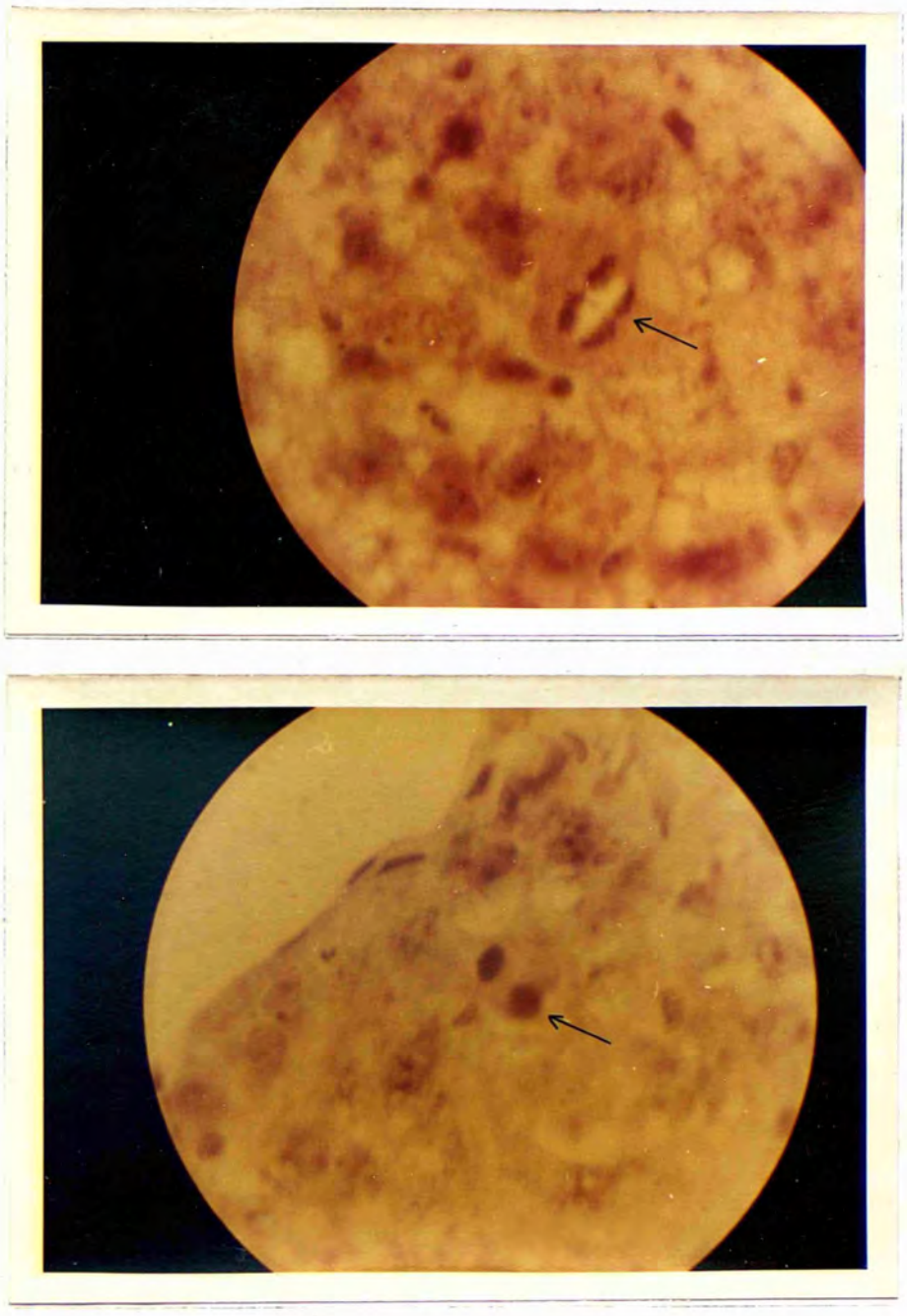


Fig. 17. Examples of abnormal telophase cells in the liver remnant after partial hepatectomy and Myleran injection.

The upper photograph shows a polyploid cell in telophase with both an enlarged nucleus and an increased number of chromosomes. The lower photograph shows pseudo amitosis.
Magnification: Approximately 3000x.

6.8 The Mitotic Index during Liver Regeneration.

The % mitotic index was determined at hourly intervals between 20-26hr. after partial hepatectomy for rats given Myleran directly after operation. For control rats the % mitotic index was determined at hourly intervals between 20-26hr. and two hourly intervals between 26-30hr. after partial hepatectomy. The % mitotic index for rats treated with Myleran directly after operation and controls are shown in Fig. 18. The standard deviations at each point were derived from measurements on at least two rats; for each rat 100 mitoses were found by examination of up to 16 microscope slides or four thousand fields (5×10^4) cells. The derived curves (5.10.1) which express % mitotic index relative to the number of cells in the liver remnant at the time of partial hepatectomy are also given in the figures.

In both Myleran-treated rats and controls (Fig. 18) there is a base level of approximately 0.13% of cells in mitosis 20hr. after partial hepatectomy, rising to a first peak at 22hr. of 2% of the cells in mitosis. At 23hr. Myleran causes a fall in the mitotic index from a value of 1.7% in controls to zero. The mitotic index at 24hr. after operation is 3.7% in control rats but 9.6% in Myleran-treated rats. The rise in mitotic index in the Myleran-treated rats at this time is accompanied by a large increase in the % of anaphase and telophase abnormalities (Fig. 12 and Fig. 13). Student's

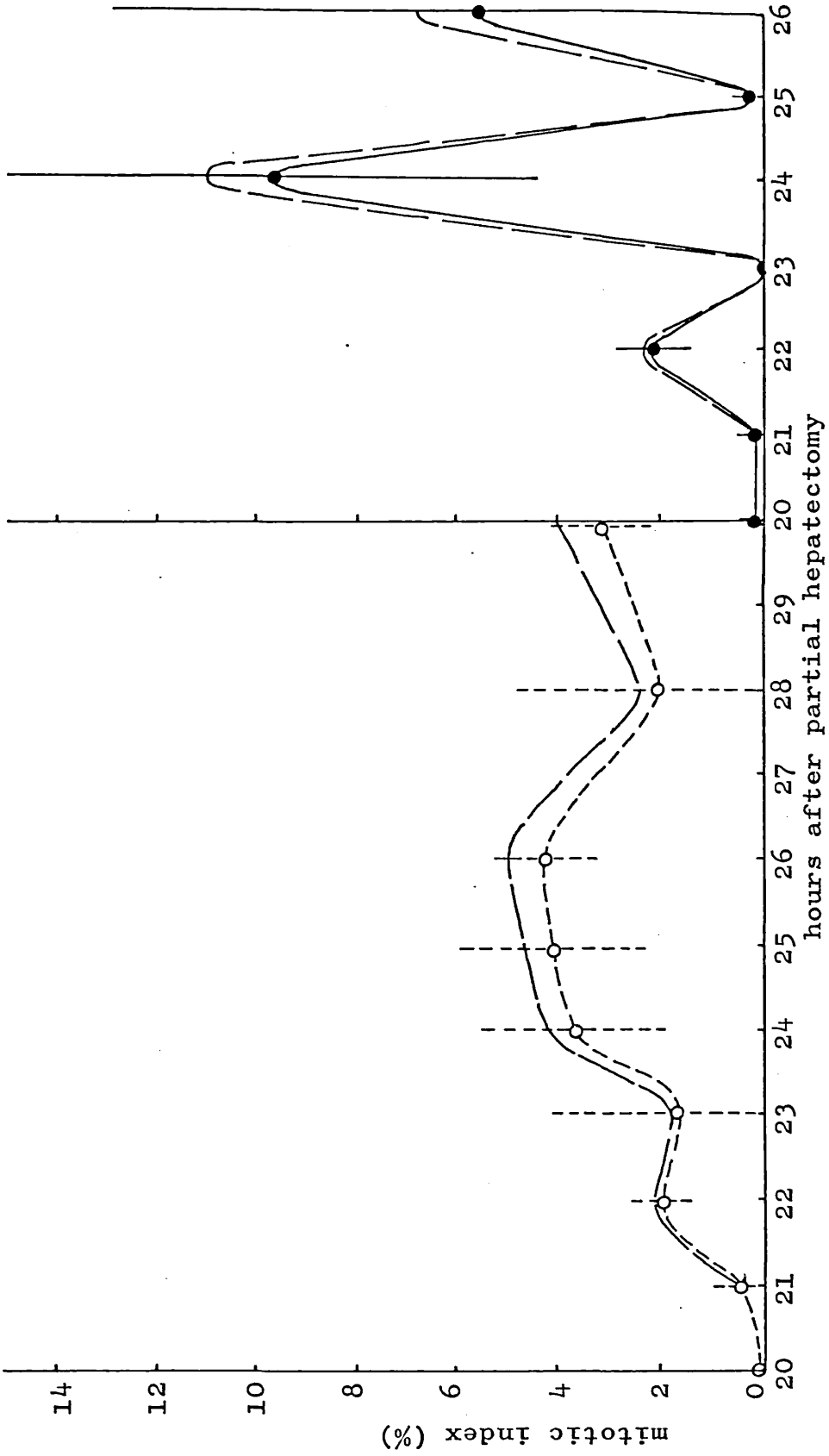


Fig. 18. The mitotic index after partial hepatectomy.

Rats were either (●) treated with Myleran directly after operation, or were (○) controls; vertical bars represent the standard deviation. The broken line shows the corrected mitotic index.

t-distribution (6.3), gives a probability of 60-75% for difference between the mean values for control and Myleran-treated rats at 23hr. after partial hepatectomy and an 85-90% probability for difference between the means at 24hr. Myleran decreases the % mitotic index at 25hr. to 0.3% compared with 4% in controls; the probability for difference of the means at this time is 75-90%. There is no difference between mean values of mitotic index at 26hr. since Student's t-distribution gives the probability for difference between the means as $<60\%$.

Two maxima occur in the mitotic indices of control rats; there is a small rise in the value at 22hr. and the maximal value occurs at 26hr. after partial hepatectomy. Maxima at 22, 24 and 26hr. were found for Myleran-treated rats (Fig. 18). Two peaks of mitosis in normal regenerating rats were found by Fabrikant (1967); in addition to a larger peak at 26hr. he found a small peak at 22hr. after operation.

6.9 The Effect of Myleran Injected at Different Times Following Partial Hepatectomy

Rats were partially hepatectomized and injected with Myleran, or solvent for control, either at the time of operation or at 11½hr., 17½hr. and 20hr. following operation. All rats were killed 26hr. after operation and mean percentage labelled mitoses scored using autoradiography on slices of the liver remnant.

When Myleran was injected at the time of the operation the value for % labelled mitoses was 57%, compared with the control value of 31%. A value of 18% labelled mitoses was found when Myleran was injected 11½hr. after operation. A value of 34% labelled mitoses, almost unchanged from the control value of 31% was found when Myleran was injected 17½ and 20hr. following operation. Thus the greatest effect of Myleran on mitotic labelling observable at 26hr. is shown if it is injected immediately after operation. These results are summarised in Table 9.

Table 9. The Effect of Myleran on % Labelled Mitoses when Injected at Different Times following Partial Hepatectomy.

Injection	Time of injection (hr.) after operation.	Mean labelled mitoses (%) at 26hr. Number of rats ( ).
MYLERAN	0	57 (2)
	$11\frac{1}{2}$	18 (2)
	$17\frac{1}{2}$	34 (2)
	20	34 (2)
CONTROL	as Myleran-treated	31 (5)

Myleran was injected at different times after partial hepatectomy and [6-^{3}H] thymidine injected at 16hr. after operation. All rats were killed at 26hr. after operation, corresponding to 10hr. on the graphs of labelled mitoses (Fig. 19). The mean labelled mitoses were scored (5.9) and values averaged.

6.10 The Lengths of the Phases of the Cell Cycle in Hepatocytes during Regeneration

Partially hepatectomized Myleran-treated and control rats were pulse-labelled with [6-^{3}H] thymidine 16hr. after operation and killed at hourly intervals (5.3). Histological preparations were made from the excised liver remnants (5.7) and examined using high resolution autoradiography (5.8). The times occupied in the differing phases of the cell cycle were estimated by preparing a graph of the % of mitoses labelled by the radioactive thymidine. At least four slides were examined for each rat. A minimum of 100 mitoses for each rat were examined for the curves of % labelled mitoses for both Myleran-treated and control rats (Fig. 19).

The time taken during the DNA synthetic period, or S phase of the cell cycle, was calculated from the first wave of labelled mitoses in Fig. 19. The S period was represented by the interval between the 50% points on the ascending and descending limbs of the curve. The duration of the S phase was also determined from the interval between the 37% points on the rising and falling limbs of the first wave of labelled mitoses. If the times of leaving and entering S phase were symmetrically distributed, the distance between the 50% points on the first wave of labelled mitoses would accurately represent the mean duration of the S phase. In populations of cells so far studied, the times of leaving and entering

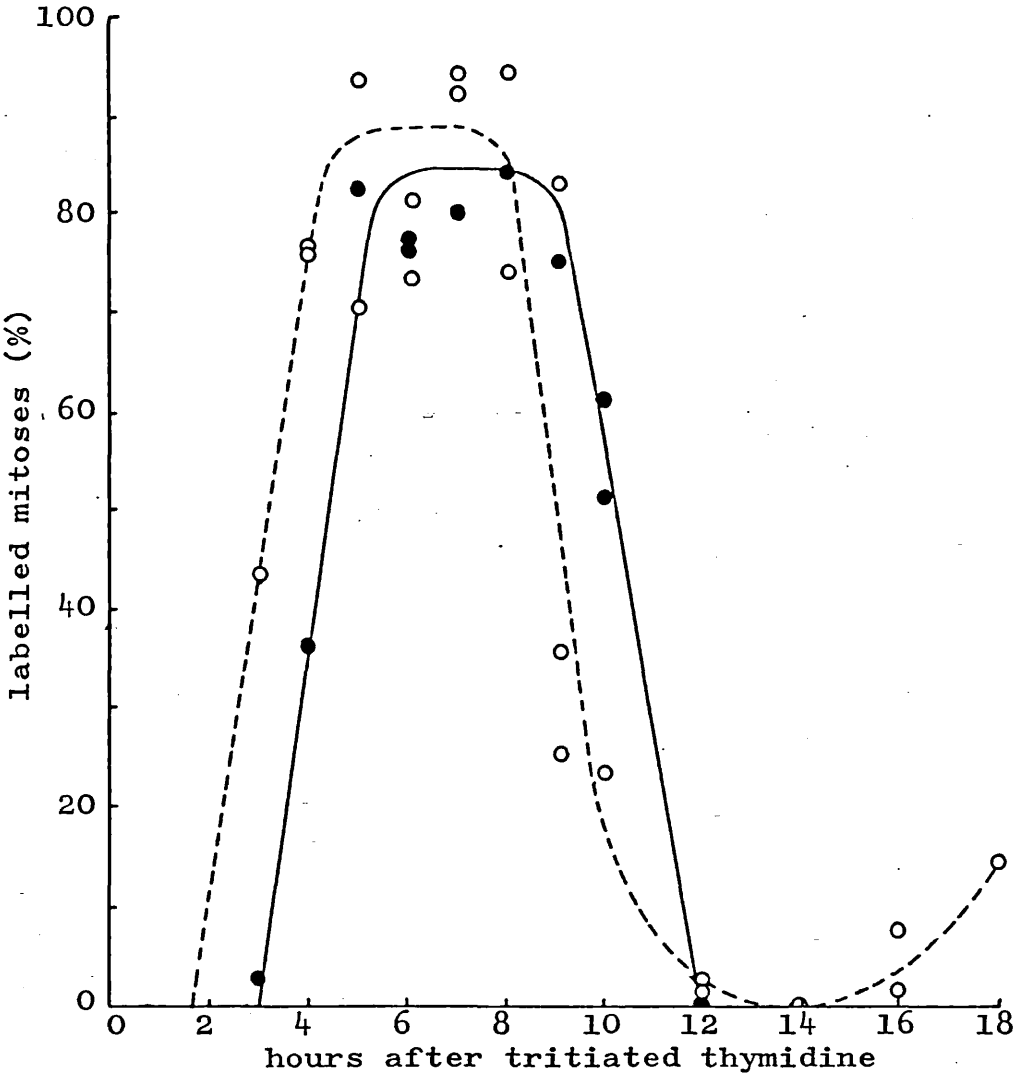


Fig. 19. The fraction of labelled mitoses found in regenerating liver.

(●) Animals treated with Myleran directly following operation and (○) controls. Both groups were injected with [6-^{3}H] thymidine at 16hr. after partial hepatectomy, which corresponds to the origin of the graph.

S phase are asymmetric. The shapes of the curves of labelled mitoses are such that the time interval represented by the width on the graph between the 37% points gives a fairly unbiased estimate of S (Lamerton, Fry & Quastler,1963).

The minimal time needed for completion of the premitotic G_2 phase of the cell cycle is the period between injection of $[6\text{-}^3H]$ thymidine and the first appearance of labelled mitoses. The average duration of the G_2 phase is given by the interval between injection of $[6\text{-}^3H]$ thymidine and 50% labelling of the mitotic figures. The time taken for the ascending limb of the curve of labelled mitoses to rise to 100% gave an approximate value for the mitotic phase time M. The total time taken for the complete cell cycle (T_c) was estimated in Fig. 19 from corresponding points on following waves of labelled mitoses. This was only possible in the case of the controls which manifested a second mitotic labelling wave for the parenchyma. It was difficult to detect a second wave of labelled parenchymal cells since most cells fail to divide a second time. There was also a loss of phase coherence because of spread in the duration of the G_1 and G_2 phases (the standard deviation G_2= 1.1hr.) for control rats (Table 10). The length of the G_1 phase of the cell cycle was calculated from the equation:

$$G_1 = T_c - S - G_2 - M. \quad . \quad . \quad . \quad . \quad (1)$$

Table 10. The Length of the Phases of the Cell Cycle in Regenerating Rat Liver.

Phase		Phase Length by FLM graph (hr.)		Phase Length by Probit Analysis (hr.)	Standard Deviation σ (hr.)	Coefficient of Variation $(\frac{\sigma}{T})$
G_1	M	-		-	-	-
	C	1.1		-	-	-
S	M	5.9	6.7[1]	6.0	1.4	.23
	C	5.7	6.6[1]	5.7	1.2	.21
G_2	M	4.3	2.9[2]	4.2	0.7	.17
	C	3.2	1.5[2]	3.2	1.1	.34
M	M	2.5		-	-	-
	C	3.0		-	-	-
T_c	M	-		-	-	-
	C	13.0		-	-	-

(M) rats treated with Myleran directly following operation and (C) controls.

[1]The values estimated for the S phase from the 37% points on the first wave of labelled mitoses.

[2]The minimal length of the G_2 phases is given after the mean duration of the G_2 phase.

From the curves of mitotic labelling (Fig. 19) the length of the S phase for Myleran treated regenerating liver was found to be 5.9hr., using the interval between the 50% points on the labelling wave, or 6.7hr. using the 37% interval. The corresponding values for the controls gave S values of 5.7hr. and 6.6hr. The length of the G_2 phase was found to vary between 2.9 - 4.3hr. in Myleran-treated rats while the control value varied between 1.5 - 3.2hr. The length of mitosis in Myleran-treated rats was 2.5hr. while in controls it was 3.0hr. The total cell cycle time (T_c) for the controls was approximately 13hr. Total cell cycle time (T_c) was not obtainable for the Myleran-treated rats as they showed only one wave of mitotic labelling. The length of the G_1 phase was also unobtainable for Myleran-treated rats, since the T_c parameter was unknown in equation (1) relating G_1 to the other parameters of the cell cycle. For the control rats the length of the G_1 phase was found to be 1.1hr. from equation (1). The above results are summarised in Table 10.

The curves of labelled mitoses (Fig. 19) were analysed further by means of probit analysis (5.9). In this method % labelled mitoses were plotted directly on to probability paper. Fig. 20 and Fig. 21 show labelled mitoses plotted on probit paper for control and Myleran-treated rats respectively. A normal distribution when plotted on probit paper, gives a straight line. It was found that straight lines could be

drawn through the slopes. The time at the 50% probability level and the period between 16% and 50% probability levels of the first two straight lines plotted in Fig. 20, in chronological order from the origin, correspond to the following means and standard deviations for the controls:

1st straight line	G_2	σ_{G_2}
2nd straight line	$G_2 + S$	$\sqrt{\sigma_{G_2} + \sigma_S}$

where G_2 and S correspond to the times spent in these phases and σ_{G_2} and σ_S are their respective standard deviations. Identical equations were applied for the fraction of labelled mitoses plotted on probit paper for Myleran-treated rats (Fig. 21).

Analysis of the labelled mitotic curves in the above manner gave the length of the S phase for Myleran-treated and control rats as 6.0hr. and 5.7hr. respectively, the corresponding standard deviations being 1.4hr. and 1.2hr. The values of coefficient of variation (σ_S / time spent in the S phase), showed the determination of the length of S phase for the Myleran-treated sample to be less accurate than that for the control animals. The values for the length of S phase determined by the probit method matched those determined using the 50% points on the two limbs of the wave of labelled mitoses of Fig. 19.

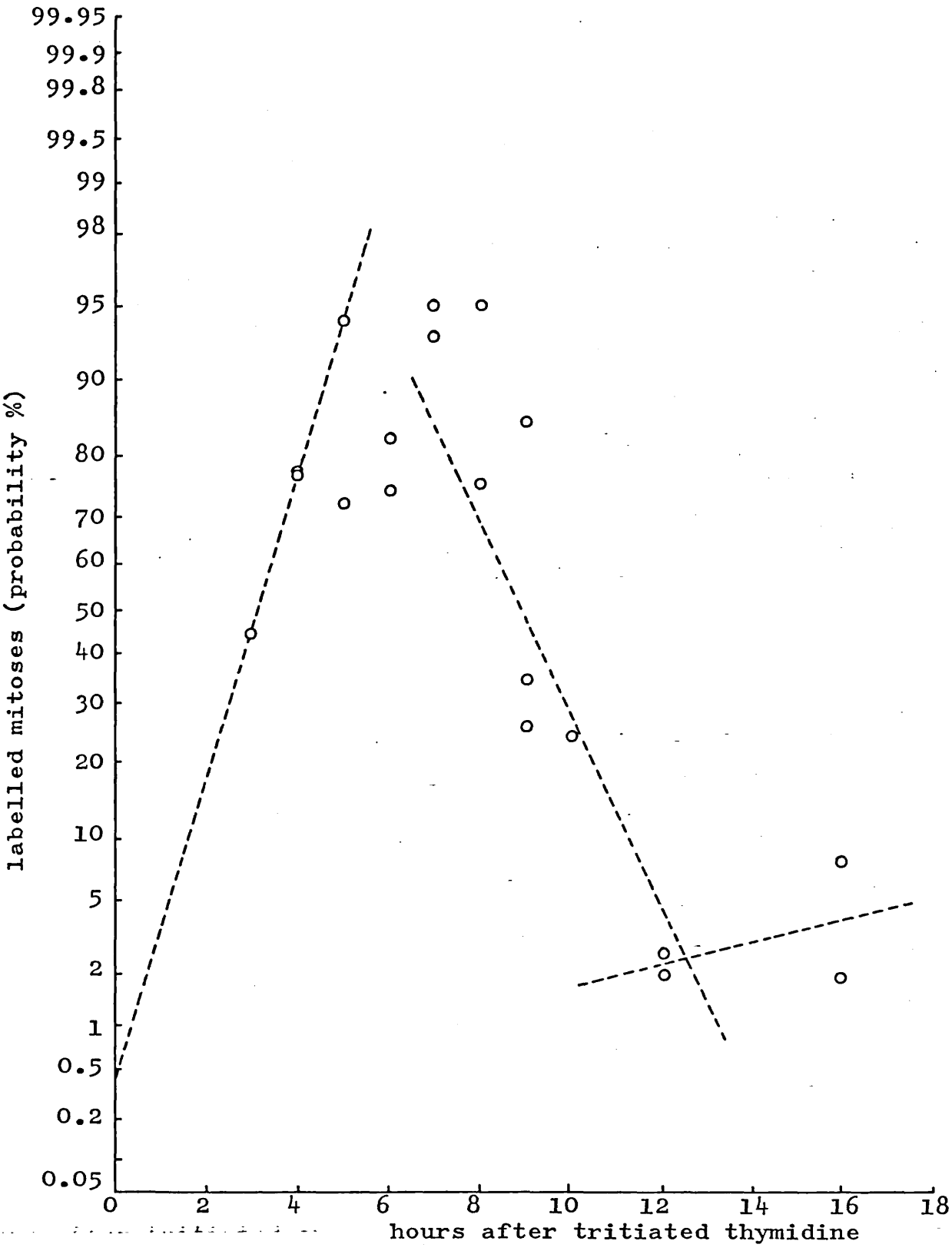


Fig. 20. The fraction of labelled mitoses plotted on a probit scale for control rats.

The origin of the abscissa corresponds to 16hr. after operation when [6-^{3}H] thymidine was injected.

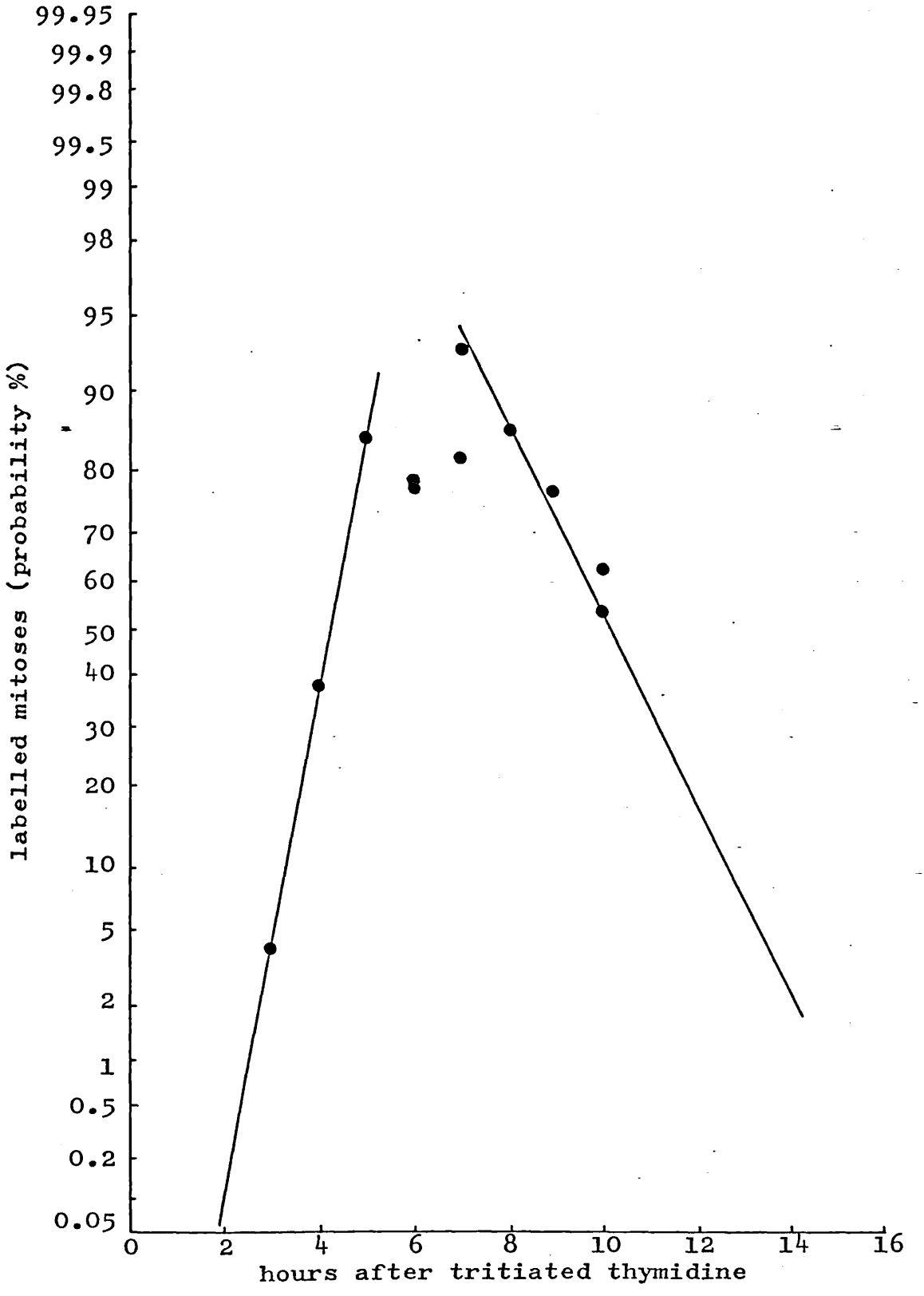


Fig. 21. The fraction of labelled mitoses plotted on a probit scale for rats treated with Myleran directly after partial hepatectomy.

The origin of the abscissa corresponds to 16hr. following partial hepatectomy when [6-^{3}H] thymidine was injected.

The lengths of the G_2 phases for Myleran-treated and control rats calculated using probit analysis gave times of 4.2hr. and 3.2hr. These values matched those read as the co-ordinates of the time axis for the 50% points on the first limbs of the waves of labelled mitoses of Fig. 19. The standard deviations for the G_2 phase times were 0.7hr. for the Myleran-treated rats and 1.1hr. for the controls. The coefficients of variation (σ_{G_2} / duration of the G_2 phase) for these values of G_2 were 0.17 and 0.34, respectively. The values for coefficient of variation compare the accuracy of determination of the given phase times with respect to the length of the phase.

The results obtained by probit analysis are compared, in Table 10, to those found by inspection from the curve of % labelled mitoses (Fig. 19). An examination of the lengths of the cell cycle phases found for Myleran-treated and control rats, given in Table 10, shows Myleran does not affect the length of the S phase; this is approximately 6hr. for both control and Myleran-treated animals. The length of the mitotic phase (M) is slightly shortened, from 3.0hr. in the controls to 2.5hr. in Myleran-treated rats. The duration of the G_2 phase was altered from 3.2hr. in controls to 4.3hr. in Myleran-treated rats; the standard deviation of the determination was less for the Myleran-treated animals (0.7hr.) than controls (1.1hr.) The results were considered significant; Student's t-distribution applied to the results gives a probability of 75-90% for the

validity of the difference between the two values of G_2.

The curves of labelled mitoses (Fig. 19) were smooth and symmetrical waves with a plateau of 85% and 89% labelled mitoses between 6-9hr. and 5-8hr. after intraperitoneal injection of $[6\text{-}^3H]$ thymidine for the Myleran-treated rats and controls, respectively. The shape of the curves was similar to those found for regenerating liver by Fabrikant (1964) and Stöcker & Pfeifer (1967) and for other cell systems, such as the ascites tumour (Okumura, Onozawa, Morita & Matsuzawa, 1973). The times of the cell cycle phases compare broadly with those given by Fabrikant (1964). These values, and those of other workers, for normal, neonatal and regenerating liver, are compared with the values found in the present work in Table 11.

Table 11. Cell Cycle Times (hr.) in the Liver of Rat as found by Different Authors.

Cell system	Lengths (hr.) of the Phases					Reference
	G_1	S	G_2	M	T_c	
Neonatal	9.0	9.0	0.5	3.0	21.5	Post et al. (1963)
Normal	-	8.2	-	0.8	-	Edwards & Koch (1964)
Regenerating	-	8.5	-	0.9	-	"
"	3.5	7.5	2.5	1.1	14.5	Fabrikant (1964)
"	-	7.2	2	1-1.5	-	Stöcker & Pfeifer (1967)
"	1.1	6	3.2	3.0	13.0	The present work (C)
"	-	6	4.3	2.5	-	" (M)

In the present work listed above (C) refers to control and (M) refers to experimental rats given Myleran directly after partial hepatectomy.

DISCUSSION

The liver is a conditional cell renewal system (Patt & Quastler, 1963) which in response to partial hepatectomy undergoes compensatory hypertrophy and hyperplasia. Its regeneration in the rat is dependent upon age, enviromental temperature, diurnal time of excision and nutrition. These factors were standardised in the investigation of the effect of Myleran upon regeneration.

The Effect of Myleran on DNA Synthesis in the Regenerating Liver.

The specific activity of the DNA (d.p.m./µg DNA-P) in Myleran-treated partially hepatectomised rats and controls, after 'pulse-labelling' with tritiated thymidine shows three maxima at 18, 30 and 58hr. Bucher et al. (1961) in contrast found two peaks of synthesis in weanling rats at 22hr. and 35hr. The earlier (by 4 and 5hr.) position of the first two peaks, found in this study, may be accounted for by the younger age of the rats used. Bucher et al. (1961) found only one peak of DNA synthesis, occurring at 25hr. in four month old rats. In one year old rats the peak was shifted to 30-32hr. The peaks are sharply delineated and symmetrical in the case of the weanling rats and in the present study.

In Myleran-treated rats the maximal rates of DNA

synthesis, as shown by the incorporation of $[6\text{-}^{3}H]$ thymidine into the DNA, were significantly reduced in comparison with controls (Table 1). From consideration of the time gaps between values for specific activity of the DNA a shift in the maxima of <3hr., for the first two maxima, and <7hr., for the third maximum, would not be observable. Shifts in the position of an incorporation band would, however, be noticeable by differences in the values found at the bases of the maxima, provided the time of the S period of the cell cycle remains constant. There was no significant difference in the length of S phase after Myleran treatment, thus the decrease in values found for thymidine incorporation in the Myleran-treated rats in comparison with controls are reliable.

DNA synthesis during regeneration, measured by tritiated thymidine incorporation, was lowered to 60% of control values over 12-72hr. following Myleran treatment. The DNA-P/g wet regenerated liver over this period however, was the same in both control and Myleran-treated rats. The decrease in the incorporation of labelled thymidine found for Myleran-treated rats may be due to:

a) decreased thymidine uptake by the lymphatics;

b) decreased thymidine absorption from the blood in the liver sinusoids into the liver cells;

c) an increase in the size of the thymidine 'pool', so 'diluting' the label;

d) a decrease in the activity of thymidine kinase or of other enzymes on the pathway between thymidine and DNA (Fig. 1; 1.6.6).

e) an increase in the activity of thymidine phosphorylase (Fig. 1; 1.6.6).

A decrease in thymidine uptake by the lymphatics, or decreased absorption of thymidine from the liver sinusoids into the liver parenchyma seems unlikely. Chang & Looney (1965) have found tritiated thymidine to be incorporated from the blood into the nucleotide fraction and DNA of cells within 10 min , so that passage of tritiated thymidine across the sinusoid and cell walls must be facile. As regards fluctuation in size of nucleotide pools, Bucher & Swaffield (1969) found RNA synthesis to increase the precursor pool size. Endogenous UTP and CTP pools were expanded by 50% at 3hr. after partial hepatectomy and the rate of RNA synthesis rose by 50-100% at 3-6hr. after operation. The nucleotide pools are enlarged as a result of the stimulus of partial hepatectomy and Myleran is unlikely to enlarge the pools further. An effect of Myleran on thymidine kinase, the immediate enzyme on the pathway leading to incorporation of thymidine into DNA was investigated and is dealt with below.

'Apparent' Thymidine Kinase Activity.

The 'apparent' activity of thymidine kinase was

investigated at 30hr. after partial hepatectomy (6.2). In the crude enzyme homogenate obtained from the liver, thymidine kinase, the thymidylate kinases, which convert TMP to TDP and TTP, thymidine phosphorlase and the 5' - nucleotidases, as well as non-specific phosphatases, will be present. The higher nucleotides TDP and TTP, as well as TMP, will be adsorbed on to the ion-exchange paper. Thus the 'apparent' thymidine kinase activity is the summation of the synthetic and catabolic activities of a number of thymidine metabolising enzymes.

The variation in the 'apparent' activity of thymidine kinase with the dilution of the homogenate used for the assay of enzyme activity may be explained by dilution of inhibitors of the enzyme; in rat liver this appears to be specific and competitive inhibition by TTP (Breitman, 1963). At the lowest liver/sucrose dilution of the homogenate (1:5, w/v), which is closest to the situation existing in the animal, the 'apparent' thymidine kinase activity in Myleran-treated rats was only 64% of controls. A graph of % activity /dilution of homogenate was constructed to find the 'apparent' thymidine kinase activity <u>in vivo</u> after Myleran treatment (Fig. 22). Extrapolation of Fig. 22 to zero dilution gave an approximate value of 40-45% for 'apparent' thymidine kinase activity in Myleran-treated rats in comparison with controls. The lower rate of tritiated thymidine incorporation (42% of controls) at 30hr. found from 'pulse-labelling' <u>in vivo</u> for Myleran-treated

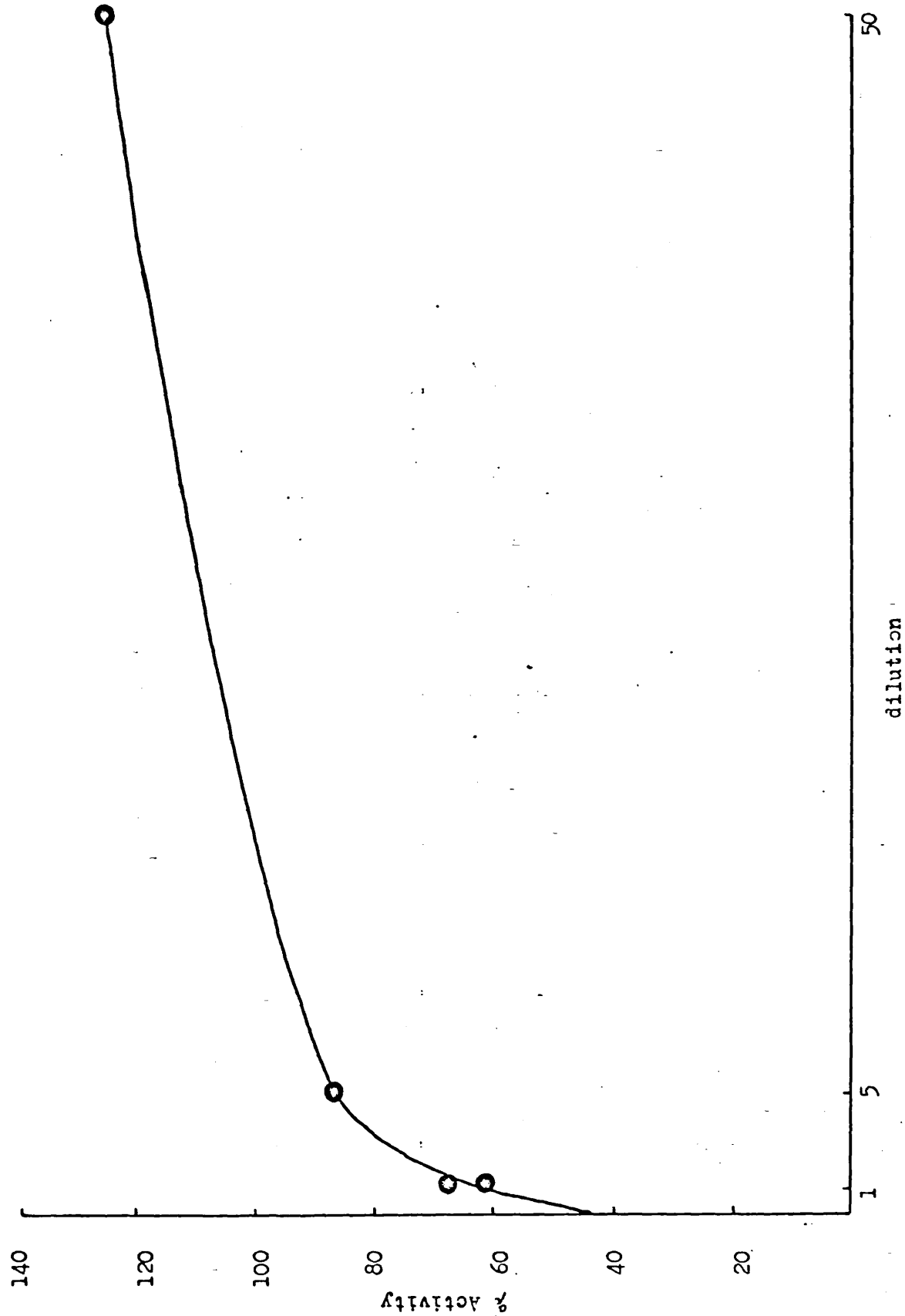


Fig. 22. The 'apparent' thymidine kinase activity for a liver homogenate from rats treated with Kyleran immediately after operation, as a function of the dilution of the homogenate.

rats (6.1) falls within this range of 'apparent' thymidine kinase activity. The increase in activity on dilution of the homogenate for the assay of thymidine kinase may be explained by an effect of Myleran on DNA polymerase. Inhibition of this enzyme would cause TTP to accumulate with consequent inhibition of thymidine kinase. Since TTP is a competitive inhibitor, high dilution would release inhibition.

Myleran, when injected in the G_0 phase of the cell cycle, immediately following operation, has its greatest effect on the second DNA synthetic peak at 30hr. following partial hepatectomy (Table 1). Thymidine kinase has highest activity at this time (Beltz, 1962; Ol'shanetskaya, Kavelina & Novikova, 1968); this lends support to an effect, suggested in the present study to be indirect, of Myleran on thymidine kinase. Nakai et al. (1966) found a decrease in thymidine kinase activity in leukocytes from leukaemic patients after Myleran therapy; they did not however, investigate TTP levels and the possibility of the effect being indirect.

The DNA Content of Regenerating Liver.

Three minima in DNA-P/g wet liver were found in partially hepatectomised Myleran-treated rats at 18, 30 and 58hr. (Fig. 4), which correspond to the times of maximal DNA synthesis (6.1). Control rats show similar minima at 18 and 30hr.; the displacement of the third minimum from 58 to 48hr.

would perhaps be refuted if more rats were analysed at these times.

These minima in DNA content are due to large increases in cell constituents other than DNA, both before and during the periods of rapid DNA synthesis. Triglyceride content and compound lipids are elevated, as well as total lipid, over the first 48hr. (1.5.2). Amino acid content rises after partial hepatectomy (1.5.3) and there is a net increase in protein and of the protein synthesising microsomes, from 12hr. until 14 days after partial hepatectomy (1.5.4). Concurrent with increase of protein content protease activity falls. Nucleochromosomal protein synthesis and nuclear histone synthesis precede DNA synthesis (1.5.5) and there is an increase in pyrimidine synthesis between 12-24hr. after operation (1.6.8). RNA and protein are both synthesised in the G_1 phase of the cell cycle preparatory to the DNA synthetic phase (S) (2). All contribute to the weight increase of the liver remnant and decrease the proportional DNA content.

No difference between control and experimental values of DNA-P/g liver were found (6.3). This constancy indicates the synthesis of other components is greatly impaired.

The RNA Content of Regenerating Liver.

Church & McCarthy (1967a,b) showed that normally

repressed embryonic liver RNAs and new short-lived RNA molecules are synthesized following partial hepatectomy in the mouse; the latter may partially mediate liver regeneration and presumably account for the increased RNA content of the liver. High molecular weight RNA molecules which may be rapidly labelled with radioactive precursors are also produced after partial hepatectomy (Mayfield & Bonner, 1972).

Myleran increases RNA-P/g wet liver over the first 72hr. after partial hepatectomy by 4%. This value is probably not significant and indicates little effect on RNA synthesis.

The Effect of Myleran on Regeneration of Liver Weight.

Measurement of weight of restored liver does not always give a reliable indication of the actual hepatocyte regeneration. For different animals the weight may vary depending on the glycogen content of the liver and the amount of blood present in the excised liver, which may be as high as 50% of the weight.

Most of the increase in weight of the liver during the first three days of regeneration may be due to increased fluid uptake (Higgins & Anderson, 1931). This is consistent with the data of Harkness (1952), who found liver cells to increase in size but not number during the first 24hr. after partial hepatectomy. In Myleran-treated rats DNA synthesis is

depressed by 40% and only 73% of the control weight of liver is restored over the first 72hr. There is no difference in the % regeneration in weight for Myleran-treated rats and controls up to 18hr., nor in DNA/g wet liver over the whole 72hr. period. These results suggest that Myleran has little effect on the fluid balance after partial hepatectomy, and that the lower weight restoration must be due to decreased DNA synthesis.

The Effects of Myleran on the Lengths of the Mitotic Phases in Regenerating Liver.

The lengths of the mitotic phases (prophase, metaphase, anaphase and telophase) for Myleran-treated and control rats were found to be markedly dependent upon the method used for their determination. The estimate of the length of prophase obtained from prophase and metaphase labelling curves was considered most reliable. The appearance of labelled cells in prophase and metaphase after pulse-labelling could be clearly distinguished as these phases constitute the larger part of the population.

The length of prophase estimated by the above procedure was used to find the lengths of the other three mitotic phases by substituting in formulae which relate the frequency of occurrence of mitotic figures to population size (5.9). It was necessary to use this method to find the length of metaphase, anaphase and telophase, since the interval between the first

appearance of labelled metaphase and anaphase figures was indeterminate, hence invalidating values obtained for anaphase. The frequency of occurrence method however, suffers from the disadvantage that changes in the number of cells in a phase, due to blockage in that or a previous phase, will be interpreted as a change in length of that phase. It is seen from the % of cells in the four mitotic phases, Fig. 9, that blockage does not occur in any particular phase after Myleran treatment.

Myleran has been shown to damage the chromosomes and especially the mitotic apparatus (Koller, 1969). The damage to the mitotic apparatus is probably of a general nature since the effects of Myleran found in the present study are not confined to a single phase.

The Effect of Myleran on the Incidence of Mitotic Abnormality in Regenerating Liver.

Abnormalities in all phases of mitosis were found in both control and Myleran-treated rats. The large number of abnormal figures in controls is unusual. Whereas most previous studies have considered only anaphase and telophase abnormalities, since abnormalities in these phases are easier to recognise and less equivocal, fewer abnormal phases were found after partial hepatectomy; Fabrikant (1967) gives a figure of 10-15%, while Albert (1958) and Horst & Rudnicki (1962)

give between 2-7% abnormal mitotic figures. It should be emphasised that recognition of a mitotic cell as abnormal is necessarily highly subjective, even though the criteria of abnormality (5.10) were strictly adhered to in the identification of abnormal cells. Dimethyl sulphoxide, used in both control and experimental groups is unlikely to have given chromosome abnormalities. On injection of 0.4 ml intravenously into rats it was shown to damage hepatocytes (Shilkin, Papadimitriou & Walters, 1966) but chromosomal damage was not noted.

Slight increases in the % of abnormal prophase and metaphase cells were seen in the period up to 24hr. after operation and Myleran injection, but no general increase in abnormal anaphase or telophase figures was found. A high % of abnormal anaphase and telophase cells was found in the mitoses of Myleran-treated rats at 24hr. Interference with mechanisms controlling the orderly process of mitosis, could explain the shortened length of the mitotic phases and the higher incidence of abnormality.

Myleran has previously been found to be mutagenic in barley, wheat and Drosophila (Lundquist et al. 1968; Mackey, 1968; Svetlov & Vorsakova, 1968).

The Effect of Myleran on Mitotic Index during Liver Regeneration.

In adult liver only .01% of the parenchymal cells are generally in mitosis (Bucher, 1963). Thus the onset of a large number of mitoses is a very sensitive indication of regeneration. Mitotic indices can be a misleading guide to regeneration, since procedures such as laparotomy or anaesthesia cause a 10-20 fold increase in the mitotic index in liver with no accompanying increase in liver mass (Servis & Kennedy,1963). Further disadvantages of the mitotic index as an index of growth are its subjectivity, sampling errors and the short length of the mitotic peak.

The high values of mitotic index for Myleran-treated rats have a large standard deviation making these values uncertain, the values at low mitotic index are less variable and significantly depressed compared with controls.

The Effect of Myleran on the Lengths of the Cell Cycle Phases of Regenerating Liver Cells.

A curve of % labelled mitoses was used to find the lengths of the four cell cycle phases in the present study as it is the only method generally applicable in vivo. Other methods using double or continuous labelling, and the use of drugs to 'block' cells at a particular point in the cell cycle,

are often better applicable to cells in culture, or are limited to the determination of the length of one phase.

Three methods are available to analyse curves of labelled mitoses. The first is direct measurement from the curve. Secondly, a simulated curve may be constructed by iteration using a Monte Carlo programme until simulated and experimental curves are consistent (Barrett, 1966). This method was not used as it is cumbersome and has been superseded by the method of Okumura, Onozawa, Morita & Matsuzawa (1973), using an improvement of Barrett's equation for a curve of labelled mitoses. Cell cycle parameters may be estimated from the straight lines found by plotting % labelled mitoses on probit paper. This method was used in the present work as it is simple and gives results in good agreement with those found using the Monte Carlo programme. Measurements made directly from the curves of % labelled mitoses yielded comparable results. Values for the lengths of G_1 and M phases and T_c for control rats were obtained by direct measurement from the curve of % labelled mitoses; these parameters were unobtainable from the plots of labelled mitoses on probit paper for either control or Myleran-treated rats.

A level of 85% labelled mitoses reached in the case of the Myleran-treated rats, compares with the 89% level found for controls (Fig. 19). The curves of labelled mitoses did not reach 100% labelling, partly because the cells were not

synchronous when the 'pulse-label' of $[6\text{-}^{3}H]$ thymidine was injected. Lack of synchrony is due to variation in length of the cell cycle phases. Hepatocytes are not a homogenous population and have different cell cycle times in different parts of the liver lobule (Rabes et al. 1972), The length of the S phase estimated for Myleran-treated rats was found to have a larger standard deviation (σ), or range of values, than that found for controls (Table 10). The standard deviation for the G_2 phase (σ_{G_2}), was less in Myleran-treated rats than the control value. The coefficient of variation relates standard deviation to the length of the phase under consideration and corresponds to the degree of asynchrony of the cells in the phase. The coefficient of variation for the S phase was slightly greater for Myleran-treated rats, though for the G_2 phase the coefficient of variation after Myleran treatment was half that of the control value. A decrease in synchrony for cells in the S phase but an increase in synchrony for cells in the G_2 phase was thus an outcome of Myleran treatment.

Another cause for the lower ($<100\%$) proportion of labelled mitoses is that partial hepatectomy induces a flow of parenchymal cells from a polyploid G_2 population directly into division. These cells do not pass through S and consequently do not take up labelled thymidine; this occurs especially amongst parenchymal cells around the central vein of the liver lobule (Brauer, 1963; Fabrikant, 1964). The effect of Myleran on this population was not studied in the

present work. An additional reason for the mitotic labelling curves not reaching 100% may be that a proportion of labelled cells were taken as unlabelled (false negatives), due to self-absorption of the β particles or latent image fading. The lowered $[6\text{-}^{3}H]$ thymidine incorporation found after Myleran injection may have increased the proportion of false negatives compared with controls, and may account for the small decrease observed between the labelling maxima found for control and Myleran-treated rats.

That the S phase was only slightly increased in duration in the Myleran-treated rats was predictable from the similar pattern of labelled thymidine incorporation (Fig. 3) for control and Myleran-treated rats. An increased length of S phase is consistent with the decreased rate of DNA synthesis found after Myleran injection. The increase in length of the G_2 phase may be related to the effect of Myleran on one or more of a number of events occurring in this phase. Peterson, Tobey & Anderson (1969) found, in <u>in vitro</u> experiments with Chinese hamster ovary cells, evidence for three temporal markers subdividing the G_2 phase. One marker was represented by the end of essential RNA synthesis, another was the synthesis of a protein allowing the cell to enter mitosis and the third was the synthesis of a protein allowing the cell to complete mitosis. The decreased protein synthesis which must result from the decreased DNA synthesis found after Myleran injection, may delay synthesis of the requisite protein(s)

allowing liver cells to divide. This delay would result in a longer G_2 period.

Different times for the duration of the mitotic (M) phase of the cell cycle were found depending on the method of determination. Metaphase, anaphase and telophase times were estimated from the average % of these cells determined hourly over a 9hr. period; only prophase was estimated graphically from the time between the first appearance of labelled prophase and metaphase cells. The mitotic time obtained from summation of prophase, metaphase, anaphase and telophase, was considered to be more reliable than estimation of the M periods from the times of ascent of the first mitotic waves on the curves of labelled mitoses (Fig. 19). The ascending limbs of the curves of % labelled mitoses were drawn by eye as curves of best fit. Both methods however, show that Myleran shortens the mitotic period. The mechanism whereby M phase is shortened is unclear. A shortening of the mitotic period has also been found for regenerating liver subjected to irradiation before partial hepatectomy (Fabrikant, 1968a). The lowered mitotic time found in the present work may be a compensatory mechanism for the decreased DNA synthesis.

The total cell cycle time (T_c), may be estimated as the interval between corresponding points on successive mitotic labelling waves. T_c was not obtainable, for Myleran-treated rats, from the graph of % labelled mitoses (Fig. 19) because

the second wave of labelling was too low to be followed. The maxima of DNA synthesis (Fig. 3), however, are at similar times for Myleran-treated rats and controls and it may be therefore inferred that T_c is not greatly altered after Myleran injection.

CONCLUSIONS

1. The decrease in DNA synthesis consequent upon Myleran injection is postulated to be due to inhibition of DNA polymerase.

2. Inhibition of DNA polymerase would cause an increase in TTP concentration. TTP has previously been shown to inhibit thymidine kinase. Its steady build up might explain the decreased activity of 'apparent' thymidine kinase, which was measured *in vitro*, and whose decrease was of the same order of magnitude as the decrease in DNA synthesis. The 'apparent' enzyme activity was de-inhibited on dilution of the homogenate.

3. Minima are found in proportional DNA-P content of the liver in controls and Myleran-treated rats which correspond to the times of maximal DNA synthesis. The values are suggested to be the result of large increases in cell constituents other than DNA which take place both before and during the periods of rapid DNA synthesis.

4. Myleran has little effect on RNA content.

5. Myleran probably has little effect on fluid balance in the liver after partial hepatectomy. A slower regenerative rate than that found in controls must be due to decreased DNA synthesis.

6. Damage to the mitotic apparatus caused by Myleran is thought to be of a general nature since effects are not confined to a single mitotic phase.

7. Interference with mechanisms controlling the orderly process of mitosis could explain the shortened length of the mitotic phases and the higher incidence of mitotic abnormality found after Myleran injection.

8. Both high and low values of mitotic index are found after Myleran treatment; the low values however, have associated low deviations and are significantly depressed compared with controls.

9. The greatest effect of Myleran is found when it is injected in the G_0 phase.

10. Cell cycle times are estimated by direct measurement from curves of % labelled mitoses and by plotting these curves on probability paper. The two methods give comparable results. The S phase is almost unchanged in length, while an increase in synchrony of cells in G_2 phase is an outcome of Myleran treatment. The G_2 phase is increased in length, perhaps as a result of the decreased protein synthesis which must result from the decreased DNA synthesis found after Myleran injection: the requisite protein(s) allowing liver cells to divide may be

synthesised tardily so lengthening the G_2 phase. The M phase is found to be decreased in length. This may partially compensate for decreased DNA synthesis by 'speeding up' the cell cycle. The total length of the cell cycle is probably not greatly altered after Myleran injection.

11. Myleran is thought to act at many sites since it is capable of binding to DNA, RNA and protein of the liver; damages the mitotic apparatus and interacts with lipid in regenerating liver (3.1.1).

FUTURE WORK

The system of regenerating liver seems suitable for the investigation of the mode of action of drugs on cells and tissues. Other chemotherapeutic compounds used in the treatment of cancer such as chlorambucil could be investigated in a similar manner to that in the present study.

The effect of Myleran on the precursor nucleotide 'pool' sizes and on RNA synthesis, not investigated in the present work, would be of interest. A study of the individual enzymes involved in the incorporation of thymidine into DNA may help to localise the enzyme(s) most affected by Myleran. It is intended to investigate DNA polymerase activity.

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