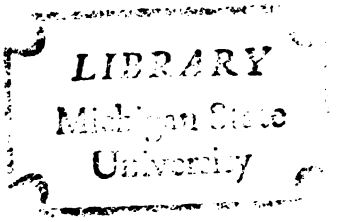
DNA SYNTHESIS IN REGENERATING LIVER IN VITRO

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
RALPH JOEL ROTHENBERG
1974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ABSTRACT

DNA SYNTHESIS IN REGENERATING LIVER *IN VITRO*

By

Ralph Joel Rothenberg

The adult rat liver is an organ in which almost all of the cells are in the non-proliferating (G_0) state. Following partial hepatectomy, there is a compensatory hyperplasia and the liver will regenerate to it's original mass within a few days. Among the features of regenerating liver is a synchronous wave of hepatocyte DNA synthesis which occurs at about 20 to 30 hours post-hepatectomy. Work done by others *in vivo* or *in vitro* has not yet succeeded in defining the regulation of liver regeneration. This may be due, in part, to the complexity of the interactions between organs and within tissues that occur in an organism. This thesis tests the hypothesis that slices of regenerating liver incubated *in vitro* have properties of liver regeneration similar to those *in vivo*, and therefore, this system could be a valuable tool in the study of the regulation of liver regeneration.

The following methods were used in this study. At various times following 70% partial hepatectomy of male adult rats, slices of regenerating liver were made and incubated *in vitro*. At specified times during the incubation (up to 24 hours) the slices were pulsed with radioactive DNA precursors and the resulting DNA specific activity of the

slices was determined and used to estimate rates of DNA synthesis in the slices. To begin the search for systemic factors which may regulate liver cell division, the effects of including various serums in the incubation medium on slice DNA syntheses were determined. Prior to partial hepatectomy, some rats were fed a diet containing the hepatocarcinogen N-2-fluorenylacetamide in order to see if exposure to this chemical would alter aspects of DNA synthesis.

The results of this study indicate that slices of regenerating liver incubated in vitro can be an important tool in studies of liver regeneration. When liver slices made from rats subjected to 70% partial hepatectomy 16 hours earlier are incubated in vitro, the times of the onset (16 hours) and peak (26 hours post-hepatectomy) DNA synthetic rates are similar to those seen in vivo. When liver slices are made at earlier times after partial hepatectomy, they do not appear to progress through a wave of DNA synthesis. This suggests that prior to 16 hours post-hepatectomy there is a critical period in vivo during which exposure to a systemic factor(s) commits cells to enter the S phase of the cell cycle. This may be an important period in the control of liver regeneration. Calf serum, fetal calf serum, and serum from rats partially hepatectomized 18 hours earlier do not alter DNA synthesis in slices made at any time but dialyzed calf serum does appear to decrease this rate in slices prepared at 16 hours post-hepatectomy. Although estimates of DNA synthesis obtained with the use of ^{14}C-formate

and ^{3}H-thymidine are similar *in vivo*, this is not true *in vitro*. This may be because the capacity of formate to be incorporated *in vitro* into the pool of proximate DNA precursors is a limiting factor, rather than DNA polymerase activity.

DNA synthesis in liver slices made from rats fed a diet containing the hepatic carcinogen N-2-fluorenylacetamide (0.05% w/w) for 14 days, followed by either 7 or 30 days on a control diet, and incubated beginning at 16 hours post-hepatectomy, is lower than normal and continually increases during a 25 hour incubation *in* vitro. This does not suggest a synchronous wave of DNA synthesis in these slices.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Pharmacology

1974

ACKNOWLEDGEMENTS

The author thanks Dr. J. I. Goodman, his major advisor, for guidance in his graduate study program, and for his valuable suggestions and assistance in the preparation of this thesis. The same appreciation is extended to Dr. T. M. Brody, the Chairman of the Department of Pharmacology, for his interest and to Dr. T. Tobin and Dr. J. Trosko for their generous willingness to sit in on his guidance committee.

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INTRODUCTION

1.1 Objective: The long range goal of this research is to develop an *in vitro* system to study the regulation of mammalian cell division. This could then be used to study the alterations in growth regulation induced by exposure to chemical carcinogens.

1.2 Background: In order to understand the control of cell division, one approach now being used in many laboratories is to identify and evaluate the role(s) of endogenous factors which alter rates of DNA synthesis and/or cell division in tissues. A lot of this work is being done in rat and mouse liver because the biochemistry of these species and this tissue have been extensively studied. In addition, the effect of partial hepatectomy, which has been carefully described by many authors,[1,2] is that liver cells normally not progressing through the cell cycle are converted into a group of cells which are synchronously going through the cell cycle. The ability to experimentally manipulate the liver so that this conversion occurs under defined conditions allows one to examine specific aspects of the cell cycle and its regulation.

Since the liver, following partial hepatectomy, eventually returns to the same percent of body weight as before the operation (which in the rat is about 4%[1]), control of liver regeneration is exercised. Factors in the systemic

circulation seem to influence this process because cross circulation experiments between a partially hepatectomized rat and a normal rat show that DNA synthesis is stimulated not only in the partially hepatectomized rat but also in the normal rat.[3] Also indicating that factors regulating liver regeneration are found in the blood is the observation that cells in implanted subcutaneous bits of liver will proliferate in a manner similar to that of the liver remenant when the rat is subjected to partial hepatectomy.[4]

It is important to understand those aspects of regenerating liver which form the basis, and some of the criticisms, of work in this area. The technique of partial hepatectomy, first quantified by Higgins and Anderson in 1931,[1] induces changes in the liver within one hour after the operation. Basophilic clumps (endoplasmic reticulum) in the hepatocyte cytoplasm disperse and fat globules soon accumulate.[5,6] Glycogen stores decrease to a low point at 10 hours and then slowly increase.[2] Approximately 12 hours after the partial hepatectomy, DNA synthesis begins, and it peaks at 20 to 30 hours after the operation.[2] DNA synthesis,[2] as determined by autoradiography with tritiated thymidine, occurs in a wave starting in the periportal part of the hepatic lobule and moves inward as time passes, though with steadily diminishing activity. By 48 hours, this wave of hepatocyte DNA synthesis has subsided and only random cells are labeled. Six to eight hours after peak DNA synthesis, hepatocyte mitoses can be observed.[2,7]

Nonparenchymal cell DNA synthesis lags behind hepatocyte DNA synthesis by almost a whole day,[2,7] and thus, experiments examining liver DNA synthesis which are limited to the first 30 hours after partial hepatectomy essentially examine a homogeneous synchronized hepatocyte population. In addition to the events that were mentioned above, one can detect changes in the biochemistry of the liver such as increased RNA synthesis by 6 hours post-hepatectomy, increased protein synthesis by 12 hours, and increased activities of enzymes which are needed for nucleotide synthesis and the assembly of DNA by 12 hours.[2] Examples of these changes are increased activities of cytidine kinase, RNA and DNA polymerase, asparagine synthetase, and increased histone synthesis, all of which occur at or before DNA synthesis begins. If one injects tritiated thymidine at a time corresponding to peak DNA synthesis in partially hepatectomized rats, most of the mitotic figures that are seen in regenerating liver during the wave of hepatocyte mitoses contain labeled DNA as determined by autoradiography.[7] This observation indicates that these cells synthesized DNA and progressed through the S phase of the cell cycle following the operation. Based on this observation, it appears that almost all adult rat hepatocytes are arrested in their progression through the cell cycle somewhere in the G_1 phase.

As a first step in the identification of the factor(s) which regulates liver cell DNA synthesis, Llanos[8,9] injected mouse plasma i.p. isolated under various conditions, to

intact and to partially hepatectomized mice. He noted that in intact mice, serum from partially hepatectomized mice stimulated DNA synthesis; that growth hormone also had this effect; and that there is a large release of growth hormone at a time corresponding to that when serum of partially hepatectomized mice was taken for use in the experiment. He noted that extracts of the pars distalis, which releases growth hormone, stimulated DNA synthesis in intact mouse liver only when the pars distalis was taken from partially hepatectomized mice and not from intact or sham operated mice.[10] This effect was mimicked by injecting pure growth hormone into the mice. Growth hormone may, therefore, be one factor involved in liver regeneration.

In contrast, growth hormone did not increase DNA synthesis in partially hepatectomized mice, and plasma from intact and partially hepatectomized mice inhibited DNA synthesis in partially hepatectomized mice.[8] Llanos concluded that at least two factors affecting liver DNA synthesis are present in plasma and that qualities of the system used allow one or the other to be active. In intact mice, where liver growth is not normally present, factors which stimulate DNA synthesis would be easiest to detect. In hepatectomized mice, where cell replication may already be maximal, the addition of stimulating factors would have no effect. Inhibitory factors, however, would be easily observed under these conditions. An alternative explanation, and one which is easily tested, is possible. The plasma that was injected

may have contained toxic breakdown products as a result of storage or bacterial growth because it was kept at only 0°C. In intact mice, these toxic products could injure liver cells and cause a compensatory increase in DNA synthesis by uninjured cells. In hepatectomized mice where most cells are already synthesizing DNA, the toxic effects on liver cells will show up as decreased DNA synthesis since there are no reserve cells for DNA synthesis which can cover up the decrease. On the other hand, storage at 0°C itself may not result in the production of factors decreasing DNA synthesis because none were observed in boiled serum in Morley's experiments,[11] which will be discussed below. In summary, it would appear that Llanos has observed a stimulating effect of growth hormone on DNA synthesis but has not necessarily shown that plasma normally contains any inhibiting factors. Although growth hormone may influence DNA synthesis, it is not an obligatory factor because liver regeneration can occur in hypophysectomized and partially hepatectomized animals.[12]

A word of caution must be introduced at this point about any conclusions based on DNA synthesis rates. These rates can only be estimated by measuring the incorporation of radioactive precursors into DNA. Apparent differences in DNA synthesis rates may really be differences in the availability of the labeled precursor for incorporation into DNA. The use of the term DNA synthesis in this section and throughout this introduction is used loosely and really

means DNA synthesis as estimated by the incorporation of the labeled precursor used into DNA.

Despite problems in interpreting Llanos's results showing inhibition of DNA synthesis by serum factors, the factors may actually be present. In support of this possibility, Onda[13] has isolated an alpha-1-globulin fraction from serum which inhibits the appearance of mitotic figures in regenerating rat liver. He states that this factor is hepatocyte-specific because it does not act on nonparenchymal liver cells. However, he has not examined the effects of this factor on cell division in parenchymal cells of other organs. Because of this, the conclusion that this factor acts only in the liver is not necessarily correct.

Because histological examination of regenerating liver shows that hepatocytes nearest portal blood areas are most likely to divide and are responsible for most of the liver regeneration,[14] a factor in portal blood has been suspected of controling liver cell division. In support of this belief, Fisher[15] was able to block liver regeneration by partial intestinal resection. In contrast, Price[16] eviscerated dogs and found a higher rate of DNA synthesis in these livers after partial hepatectomy than in dogs with partial hepatectomy alone. He noted that glucagon reduced the level of DNA synthesis of eviscerated and partially hepatectomized dogs to the level found in dogs only partially hepatectomized.

In an important breakthrough in the study of liver growth, Short[17] was able to induce liver DNA synthesis in intact rats by infusing a mixture into the tail vein for 3 hours which contained glucagon as well as triiodothyronine, amino acids, and heparin. Three consecutive days of this infusion induced a 40% increase in liver DNA content. A single infusion induced a pattern of incorporation of tritiated thymidine into DNA which was temporally similar to that following 70% partial hepatectomy. This mixture was chosen because the increased blood levels of amino acids and free fatty acids found in partially hepatectomized rats would be stimulated by administration of these compounds. Short[17] hoped that levels of circulating amino acids and free fatty acids would be significant factors in the control of liver cell division. One indication that the mixture of chemicals used results in a physiological stimulation of DNA synthesis is that this mixture causes a biphasic wave of increased cyclic-3',5'-adenosine monophosphate (cAMP) levels in rats which parallels the wave of cAMP changes seen after partial hepatectomy.[18] Short's mixture can, therefore, be used as an important tool to study the control of liver regeneration.

The effect of hormones other than polypeptides such as growth hormone, triiodothyronine, and glucagon on liver cell division is also being investigated. Noting that corticosteroids are known to inhibit the growth of a variety of normal and neoplastic tissues, Rizzo[19] examined the effects

of hydrocortisone on regenerating rat liver. He found that when the hydrocortisone was injected 19 hours after partial hepatectomy, the DNA synthesis normally seen at this time was inhibited and was detectable only after 30 hours, with a peak at 36 hours. Cortisone inhibits thymidine kinase and thymidylate kinase activity in regenerating liver, suggesting that cortisone may act by altering activities of enzymes important in DNA synthesis.[20] The observation by Rizzo[19] that orotic acid incorporation into DNA of partially hepatectomized and hydrocortisone-treated rats is decreased, as well as that of thymidine, supports this suggestion. Thus, adrenocorticosteroids as well as glucagon, growth hormone, and triiodothyronine may play a role in the regulation of liver DNA synthesis. The role is unclear, however, because both adrenalectomy and sham adrenalectomy result in higher than normal rates of DNA synthesis in partially hepatectomized rats.[21] In addition, the administration of growth hormone and cortisone to hypophysectomized rats which are partially hepatectomized results in a greater than normal mitotic activity while growth hormone or cortisone alone does not affect the mitotic response in partially hepatectomized rats which are hypophysectomized.[2]

The use of i.p. injections of various substances in order to evaluate their roles as physiological liver growth regulators is subject to misinterpretation. For example, any substance which causes an increase in the secretion of

hydrocortisone may appear to inhibit liver regeneration following partial hepatectomy. By the same token, it has been observed that substances with irritating properties will induce DNA synthesis in liver.[22] It is evident that work on factors affecting DNA synthesis *in vivo* is complicated by the interactions of organs and by inflammatory reactions that may occur when serum, or fractions isolated from serum, are injected into the test animal.

Various *in vitro* systems to study liver growth regulation have been devised to avoid these problems. The organ perfusion system that Levi[23] used is one of these techniques. He has found, when using this system, that normal liver increases its incorporation of tritiated thymidine into DNA when cross-circulated with livers removed 18 hours after partial hepatectomy but not when cross-circulated with liver removed 12 hours or earlier.[23] Levi[23] also observed that the perfusate from livers removed 18 hours after partial hepatectomy contains a substance which is not dialyzable and is stable at -70°C and which causes an increase in apparent DNA synthesis in normal rat liver. He concluded that liver not only has receptors for this factor but also is "the source of its production" since it could be collected from isolated, perfused, partially hepatectomized livers. It seems possible that while a factor stimulating incorporation of tritiated thymidine into liver DNA can be observed in a perfusate of an isolated, partially hepatectomized liver, no evidence in Levi's paper indicates that the liver

makes the factor. It is possible that the factor is synthesized somewhere else, is fixed to liver between 12 and 18 hours post-hepatectomy, and is slowly washed out during the perfusion. Morley's[11] observations of the appearance of a DNA synthesis stimulating-protein in blood and Llanos's[9] observations on the release of growth hormone at this time period support this possibility. However, Levi's observations of DNA synthesis stimulation in normal liver by perfusates of partially hepatectomized livers were not able to be duplicated in another laboratory.[24]

While organ perfusion experiments can yield important results, the liver contains several different cell types which make it difficult to pinpoint the site of action of any putative liver growth regulator. In addition, the technique of organ perfusion is technically more complicated and slower to use than some of the methods which are described below. Tissue culture is one method where the control of growth of a homogeneous cell population derived from liver is studied. Rutzy[25] reported that 10% serum from rats partially hepatectomized 48 hours earlier increased the rate of production of viable cells in the tissue culture. There are questions that can be raised about the significance of Rutzy's[25] observations to the control of liver growth and about tissue culture work in general. Remembering that the onset of hepatocyte mitosis in partially hepatectomized rats occurs at about 30 to 32 hours post-hepatectomy, one would expect that any factor

stimulating liver regeneration would be present at or before this time period. Serum collected at 48 hours does not necessarily contain the factor acting to initiate liver parenchymal DNA synthesis. Rutzy's[25] cells exhibited fibroblast-like qualities and littoral cells can differentiate into fibroblasts. Rutzy may be observing a littoral cell division stimulator. It, therefore, remains to be seen whether the factor stimulates hepatocyte cell division or whether more than one factor is active in the control of the various cell types in the liver during regeneration. In order to relate observations in this system to the control of liver regeneration *in vivo*, one must assume that the tissue culture response to factors affecting cell division is similar to that of intact liver. However, young tissue cultures in general do not exhibit the arrest of cell division, despite an apparently adequate environment, that the liver does *in vivo*. The only things that stop the growth of many cells in culture are lack of space to expand or the presence of medium exposed to cells for several days. (Since new medium can increase DNA synthesis, it is possible that some nutritional factor has been exhausted in the serum or toxic factors added by the cells.) When either is rectified, the cells begin to divide again. Thus, tissue cultures may not be good models for the control of liver regeneration. One cannot dismiss Rutzy's[25] work since factors affecting cells derived from the liver may in fact play a role in some of the processes of liver

regeneration. For example, Aujard[26] can detect inhibition of DNA synthesis in synchronized cells in cultures by liver extracts made in a manner similar to those which have been reported to be inhibitors of DNA synthesis when using *in vivo* and other *in vitro* bioassay systems.

As mentioned earlier, work *in vivo* is beset with many problems. Morley[13] was working on a factor stimulating liver DNA synthesis *in vivo* but great variability between rats limited the conclusions he could draw from the experiments. In an attempt to avoid this problem, Morley[13] developed a method to make mouse liver cell suspensions whose rate of DNA synthesis is higher when a 24 hour regenerating liver was used as the source of the cells than when an intact liver was used. This indicated that liver cell suspension DNA synthesis can reflect DNA synthesis rates *in vivo*. He found that the injection of growth hormone into an intact rat 24 hours before sacrifice resulted in cell suspensions made from the rat which had higher than normal rates of DNA synthesis. In addition, boiled serum (which inactivates any growth hormone in the serum) from partially hepatectomized rats also increased DNA synthesis. This is in contrast to observations made by Llanos,[8] but perhaps the differences in bioassay of the serum can account for the contradiction. In view of the ability of cell suspensions made from livers to show increases in apparent DNA synthesis due to growth hormone and also to boiled serum, Morley[13] concluded that he observed two factors which may

stimulate *in vivo* liver DNA synthesis. Characterization of the boiled serum factor indicates that it is liver specific and appears in the blood of partially hepatectomized rats after 12 hours. Since the factor appears just before stimulation of DNA synthesis *in vivo*, this factor may be important in liver regeneration. However, there is another possible explanation for the apparent stimulation of DNA synthesis. Enzymatic isolation of cells from a liver is a procedure which may have damaged the cells so that the suspension could not make DNA to its full capacity. Any factor which would somehow protect the cells during the isolation procedures would result in higher rates of DNA synthesis by suspensions and appear to be a stimulatory factor involved in the regeneration of the liver following partial hepatectomy. To support this possibility, it has been observed that liver plasma membranes have altered spectrofluorescence properties in growth hormone-treated rats.[27] Hypophysectomized rat plasma membranes in the presence of growth hormone have altered concentrations of various phospholipids and altered activities of Na-K-ATPase and 5'-nucleotidase.[28]

On a subcellular level, serum factors and heparin have been observed to increase DNA synthesis in isolated rat liver nuclei.[29,30] This system lacks the interactions between nucleus and cytoplasm and between cells, and as a result, the amount of information about the control of cell replication is limited. However, it certainly is not

irrelevant. The detection of heparin stimulation of DNA synthesis in nuclei for example, has also been reported *in vivo* by Short.[17]

A better method to study the control of liver regeneration may be the liver slice incubation system. The *in vitro* incubation avoids the complex *in vivo* interactions between organs and the indirect effects on liver by test substance actions on other organs. It is cheaper and less technically complicated than an organ perfusion system and allows many more tests to be performed in the same experiment since many slices can be incubated at the same time. It also avoids the problem of altered cell division properties seen in tissue and cell cultures. Furthermore, it has been observed that incorporation of tritiated thymidine into DNA of slices is qualitatively similar to what occurs in liver *in vivo*, suggesting that slices maintain at least some of the regenerative properties of liver *in vivo*.[31] The first thorough investigation of the properties of liver slices *in vitro* was by Hecht and Potter in 1958.[32] They noted that the initial rate of incorporation of precursors into DNA *in vitro* paralleled that seen *in vivo* in regenerating livers. However, they failed to observe changes in the rate of labeling of DNA with time as are seen *in vivo*. The presence of rat serum in the medium did not affect the incorporation of precursors into DNA. To explain the apparent lack of a wave of DNA synthesis in their slices, they suggested that the 4 hour incubation may have been too short to see any

changes in the rate of labeled precursor incorporation into DNA. In addition, possible other problems in their system were: 1) The incubation medium consisted only of Krebs-Ringer buffer and fructose; slices may have been deficient in nutrients needed to progress through the wave of DNA synthesis. 2) Labeled precursors were present throughout the 4 hour incubation and this might have allowed precursor concentration or specific activity changes to occur. To the extent that liver slice DNA synthesis is due to hepatocyte DNA synthesis, as it is in the first 30 hours post-hepatectomy in vivo, a favorable aspect of studies of DNA synthesis in slices is that a single cell type can be studied.

Using this type of system, Verly[33] reported on a factor in rat liver homogenates which inhibited DNA synthesis in slices from livers removed 24 hours after partial hepatectomy. He also showed that this homogenate, when injected into rats 21 hours after partial hepatectomy, decreased the incorporation of tritiated thymidine into DNA of liver slices prepared 24 hours post-hepatectomy. The factor appears to be liver specific because it did not inhibit DNA synthesis in rat spleen or kidney slices. A criticism of this work is that slices were exposed to this factor at a time of peak DNA synthesis. For an inhibitory factor to be important in the regulation of liver growth, one would expect it to prevent the onset of DNA synthesis, not to decrease DNA synthesis already occuring. The inhibition of DNA synthesis by a liver homogenate may be due to toxic

factors present in the homogenate.[34] However, Verly[35] tested his factor for toxicity in liver-derived tissue and cell cultures and did not detect any increase in lethality over that normally seen in the cultures as judged by Trypan blue staining of dead cells.

Chemicals known to cause cancer have been observed to interact covalently to macromolecules such as protein and nucleic acid.[36] Alkylating agents such as uracil mustard can covalently bind without prior modification of their structures to nucleophilic centers on these macromolecules.[37] In contrast, most other carcinogens appear to require metabolism to chemicals which can act as electrophiles before they can react with the nucleophilic centers of cellular macromolecules.[38] Examples of carcinogens which require metabolic activation are the aminoazodye 3'-methyl-4'-dimethylaminoazobenzene, the aromatic amide N-2-fluorenylacetamide, and the nitrosamines. Chemicals such as N-2-fluorenylacetamide are not carcinogenic in animals or tissues which fail to metabolize them to a compound capable of binding to nucleophiles.[36] An example is the lack of carcinogenicity of N-2-fluorenylacetamide in the guinea pig.[38] This suggests that binding of the carcinogen to macromolecules in a cell is an important step in transformation to a malignant state.

Alterations in cell biochemistry and morphology result from the administration of hepatocarcinogens to rats, perhaps as a result of carcinogen binding to proteins or to

DNA. For example,[39] N-2-fluorenylacetamide induces hepatic nodular hyperplasia and it is believed that hepatomas can arise from these nodules. Besides changes in liver architecture, there are also changes in certain liver enzyme activities. Areas of nodular hyperplasia in the liver have reduced activities of glucose-6-phosphatase and glycogen phosphorylase. In addition, glycogen levels change only slightly following glucagon administration. In view of the widespread changes seen in carcinogen-treated liver, it seems reasonable to postulate that there may be changes in the levels of, or response to, factors controling liver cell division. In support of this hypothesis are suggestions in the literature that factors inhibiting DNA synthesis in normal liver are less effective in chemical carcinogen-induced hepatomas. For example, the inhibitory activity of one of Verly's extracts, which is marked on normal liver slices, is lower on slices of a rat hepatoma induced by oral administration of the hepatocarcinogen p-dimethylaminoazobenzene.[35] Since cell division in hepatomas is not inhibited to the degree that normal liver is, this decreased response to Verly's inhibitory factor is consistent with a physiological role for the factor in the control of liver growth. This relative lack of inhibition of DNA synthesis in hepatoma tissue by factors known to be inhibitory in normal tissue has been observed in other systems by Terayama and Chany.[26]

1.3 Rationale: A good model for the study of the control of tissue growth is the liver since both a non-growing tissue, and by partial hepatectomy or perhaps by the use of Short's hormone mixture,[17] a synchronously dividing cell population can be studied in a controlled manner. This model can be related to the general case of regulation of cell division if one assumes that properties of the control of cell division in the liver are similar to those in all tissues; i.e., that there is in general a common basic mechanism(s) for the control of cell division in all mammalian tissues. A system to study this regulation of liver cell division is needed which avoids the complex interactions of factors and of organs which occurs *in vivo* but which retains properties of liver cell division seen in animals following partial hepatectomy. Specifically, what is desired is a system which can resemble both the G_0 state and the synchronized progression of cells through the cell cycle which can be observed *in vivo*. In this way, factors which influence liver cell division might be detected and studied in slices and the results might have important implications for the *in vivo* situation.

1.4 Specific Aims: One of the first steps in this study was to determine whether or not slices can progress through a wave of DNA synthesis in the absence of serum *in vitro*. A closely related experiment was to ask at what time the liver becomes committed to DNA synthesis following partial

hepatectomy. This was tested by determining how early after partial hepatectomy the liver remenant could be sliced, incubated, and still make DNA *in vitro*. To estimate the rate of DNA synthesis, DNA specific activity or percent labeled nuclei in an autoradiograph was measured following a pulse of ^{3}H-thymidine at various times during an incubation.

Another aspect of this project was to begin an examination of the effects of blood-borne factors on liver slice DNA synthesis. This was done by incubating slices in medium containing various types of serum and searching for alterations in the normally observed pattern of DNA synthesis when incubations were performed in the absence of serum.

METHODS AND MATERIALS

2.1 Animals: Male Sprague-Dawley rats, 150 to 300 gm, were used in all experiments. Rats used in any given experiment were received on the same day and were within a 25 gm weight range. The rats were kept in a room with a lighting cycle set so that it was light from 7 p.m. to 7 a.m. The method of Higgins and Anderson[1] was used to perform 70% partial hepatectomies under ether anesthesia. The time of partial hepatectomy in all cases was 6 p.m. $\pm$ 2 hours. Food and water were available to the rats at all times including the time after the partial hepatectomy and up to the time of sacrifice.

2.2 Liver Slice Incubation: At specified times after the partial hepatectomy the rats to be used in an experiment were decapitated and the livers perfused *in situ* with ice-cold incubation medium taken from the batch to be used in that day's experiment. The right lateral lobe of the liver was removed and sliced with a Stadie-Riggs microtome. Slices were placed in ice-cold incubation medium as they were cut and remained there until the incubation began. The first slice was normally discarded, except where noted.

The incubation medium contained Swim's S-77 medium, and also final concentrations of 0.05 mM cystine, 1.8 mM $CaCl_2$, 4 mM L-glutamine, 26 mM $NaHCO_3$, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of

amphotericin B. Into sterile 50 ml erlenmeyer flasks was placed 8 ml of incubation medium made up to 80% of its final volume. Then either 2 ml of a solution of 0.8% NaCl and 0.04% KCl or 2 ml of serum was added to the flask. Serums tested included fetal calf serum, calf serum, dialyzed calf serum, and serum from rats partially hepatectomized 18 hours earlier. To prepare rat serum, blood was obtained from a rat by cardiac puncture under ether anesthesia 18 hours after partial hepatectomy. After being allowed to clot for two hours in ice, the blood was centrifuged at 2000 x g x 10 minutes and the serum was then removed, pooled with serum from other rats similarly treated, and frozen at -20°C until used.

Flasks containing the medium to be used in an experiment were tightly closed with sterile silicone stoppers and the medium was allowed to equilibrate for 20 minutes at 37°C in an atmosphere of 95% oxygen and 5% CO_2. The liver slices were cut in half and one piece was placed in each of the flasks, followed by replentishment of the atmosphere. In experiments testing the pattern of ^{3}H-thymidine (^{3}H-dThd) into DNA as a function of time, the half-slices were randomly placed in flasks but in the other experiments, slices were placed in flasks so that one half-slice served as a control for the other corresponding half-slice. All incubations were done at 37°C and the platform on which the flasks rested was rotated at 100 rpm in a New Brunswick gyrorotator incubator.

At various times during the incubation 10^{-5}M ^{3}H-dThd, 20 μCi/μmole, and 5 x 10^{-4}M sodium ^{14}C-formate, 0.8 μCi/μmole, as specified, were added to the flasks, the oxygen-CO_2 atmosphere was replaced, and the flasks were allowed to incubate for one more hour. At the end of this time, the slice in each of the flasks pulsed with the labeled DNA precursors was immediately homogenized in 5% trichloracetic acid in a Potter-Elvejem homogenizer. The DNA was extracted by the method of Sneider and Potter[40] after lipids were removed with the use of solutions of 95% ethanol and 10% potassium acetate,[41] 100% ethanol, chloroform:methanol (1:2), and ether.[42] To analyze protein labeling, the pellet remaining after the DNA extraction was dissolved in 0.5 ml of tetraethylammonium, diluted to 2 ml with water, and the radioactivity of this solution was measured. The amount of DNA recovered was determined by the method of Blobel and Potter.[43] DNA radioactivity was determined by placing some of the extracted DNA in Multisol and analyzing it in a Packard model 3380 liquid scintillation counter. The calculated DNA specific activity resulting from the pulses of labeled DNA precursors were then used as estimates of DNA synthesis during the pulse periods. DNA synthesis rates were not due to bacterial or fungal DNA synthesis because no colonies grew when incubation medium was plated on saubaurad or tryptose blood agar base.

Nuclei were isolated at the end of a pulse of ^{3}H-dThd by a modification of the method described by Goodman.[42] The

slice was homogenized in 5 ml of a solution containing 0.05 M TRIS-HCl, pH 7.5 and 0.25 M sucrose, filtered through cheesecloth and mixed with Triton X-100 to give a final concentration of 2% and centrifuged at 1000 x g x 10 minutes. The pellet was resuspended and recentrifuged twice in 0.25 M sucrose before suspending it in the TRIS-sucrose solution. This suspension was spread on microscope slides and allowed to air dry. Slides were then dipped in nuclear track emulsion (Kodak NTB-3) and exposed for 23 days. They were developed (Kodak D-19 developer), fixed (Kodak acid fixer), and were stained with Harris hematoxylin. The autoradiographs were analyzed by scoring the percent labeled nuclei (5 silver grains or more) in random fields throughout each slide.

2.3 Experiments In Vivo: In vivo DNA synthesis rates were estimated by injection of 29.8 nmoles, 200 μCi/kg ^{3}H-dThd and 20 μmoles, 40 μCi/kg sodium ^{14}C-formate i.p. at various times after partial hepatectomy. The rats were sacrificed one hour later and the livers were perfused with a solution containing 0.05 M TRIS-HCl, pH 7.5, 0.025 M KCl, and 0.005 M $MgCl_2$. The right lateral lobe of the liver was then removed and the DNA specific activity was determined as described above.

2.4 Experiments Using N-2-Fluorenylacetamide: In experiments examining the effect of the hepatocarcinogen

N-2-fluorenylacetamide (FAA) on DNA synthesis in slices *in vitro*, rats were fed a powdered diet for 14 days which contained 0.05% FAA mixed in Farber basal carcinogenic diet[44] supplemented with para-aminobenzoic acid, inositol, vitamin E acetate, and USP salt mix XIV. Following this period of time, rats were switched to a pelleted control diet for at least 7 days, as specified in the results. Procedures for 70% partial hepatectomy and for incubation were the same as described for normal rats. Rats weighed 150 to 175 gm when placed on the carcinogenic diet.

2.5 *Materials*: Sprague-Dawley rats were obtained from Spartan Farms, Haslett, Michigan. Swim's S-77 medium, calf serum, antibiotics, dialyzed calf serum, and fetal calf serum were purchased from Grand Island Biological Company, Grand Island, New York. Multisol was obtained from Isolab Incorporated, Akron, Ohio, and the ^{3}H-thymidine (^{3}H-methyl), 6.7 Ci/mmole and sodium ^{14}C-formate, 3 mCi/mmole, was purchased from New England Nuclear, Boston, Massachusetts. The basal carcinogenic diet was obtained from General Biochemicals, Chagrin Falls, Ohio.

RESULTS

3.1 Experiments In Vivo: The data in Figure 1 show the rate of incorporation of ^{3}H-dThd and ^{14}C-formate into DNA of the right lateral lobe of the liver *in vivo* following partial hepatectomy. Points are plotted at the time of sacrifice of the rats. The onset of a wave of apparent DNA synthesis in this liver lobe occurs at some time between 12 and 22 hours post-hepatectomy and peaks at 26 hours. These observations are consistent with the observations of others,[2,7,45] who studied the total regenerating hepatic remnant. This suggests that the pattern of DNA synthesis of this liver lobe in experiments *in vivo* is a valid reflection of what occurs throughout the regenerating liver and that this lobe can be used to study the general aspects of liver regeneration *in vitro*.

3.2 Experiments Using ^{3}H-thymidine: Figure 2 shows the typical pattern of incorporation of ^{3}H-dThd into DNA as a function of time in liver slices made from the right lateral lobe when the rats were sacrificed at 14 or 16 hours after partial hepatectomy. Each point represents slices pulsed with ^{3}H-dThd for one hour. The mean specific activity of DNA at the end of the pulse is plotted in this figure. At 14 hours there has never been a wave of ^{3}H-dThd incorporation into DNA during the incubation period. At 16 hours there usually was a wave of incorporation which peaked at

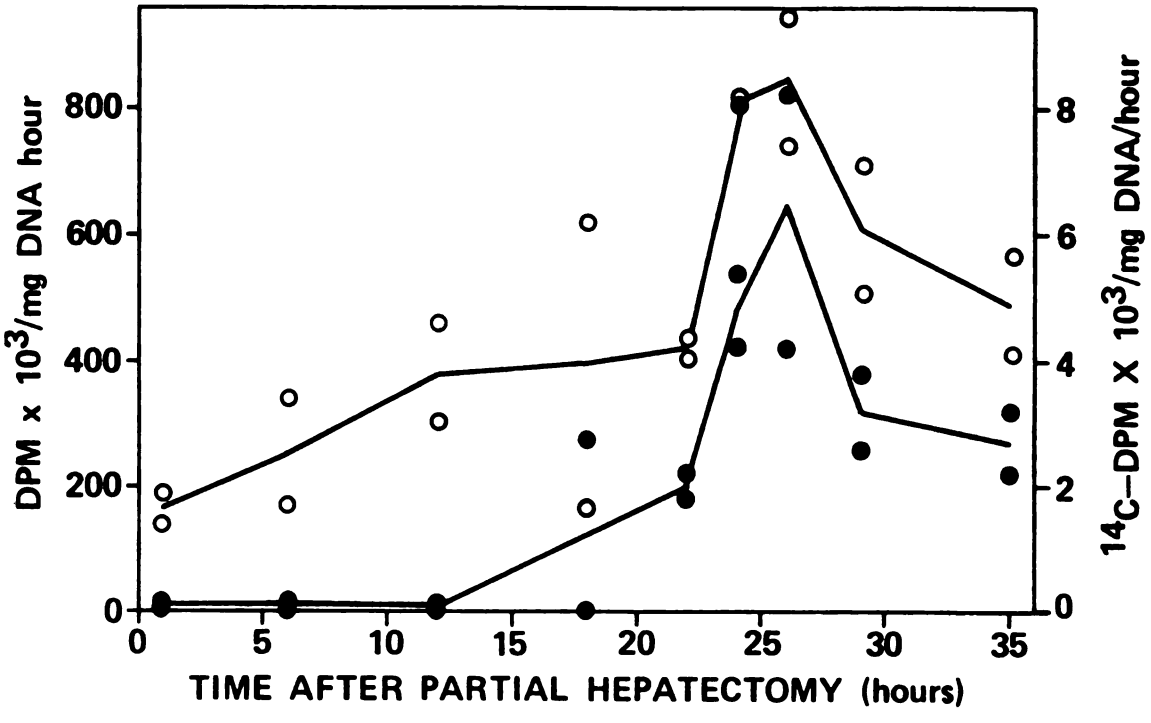

Figure 1. A comparison of ^{3}H-dThd and ^{14}C-formate incorporation into DNA of regenerating liver *in vivo*. ^{3}H-dThd, 29.8 nmoles, 200 µCi/kg. ●—● , and ^{14}C-formate, 20 µmoles, 40 µCi/kg. o—o , were injected i.p. into rats at various times after 70% partial hepatectomy. Rats were sacrificed one hour later and the specific activity of DNA in the right lateral lobe was determined. Each point, plotted at the end of the one hour pulse, represents one rat.

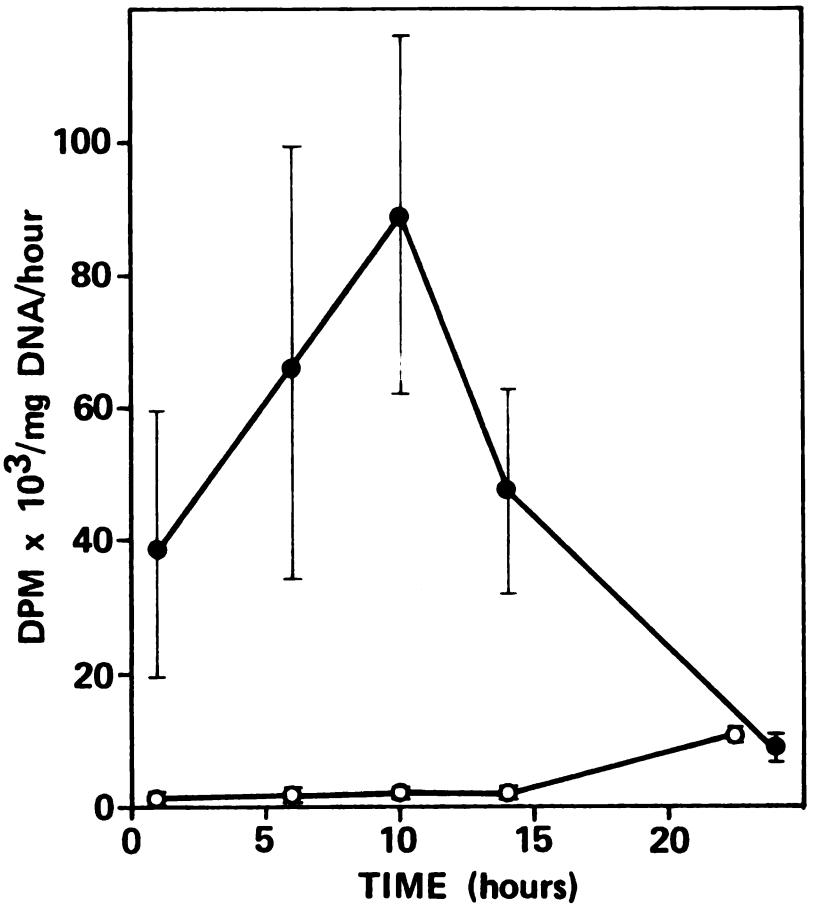

Figure 2. A comparison of DNA specific activity as a function of time in liver slices made and incubated starting at 14 or 16 hours post-hepatectomy. Following 70% partial hepatectomy, slices incubated beginning at 14 hours o—o, and at 16 hours ●—● post-hepatectomy were pulsed for one hour with ^{3}H-dThd, 10^{-5}M, 20 µCi/µmole, at various times during the incubation. Each point, plotted at the end of the pulse, represents the mean of 4 slices except for the last point which represents the average of 2 slices. Each curve represents liver slices pooled from 3 rats.

about 10 hours after the start of the incubation; i.e., at 26 hours after partial hepatectomy. Those slices which failed to show this wave had a pattern similar to that shown at 14 hours post-hepatectomy, as did slices from rats not subjected to partial hepatectomy. One can note that the peak effect is at the same time as that seen *in vivo* in the right lateral lobe of the rat liver (Figure 1). At 18 hours after partial hepatectomy (not shown) a wave of incorporation of labeled thymidine into DNA has always been observed. This suggests that liver slices prepared from the right lateral lobe of rats sacrificed at 16 hours post-hepatectomy can progress through the S phase of the cell cycle *in vitro* independent of systemic influences and in a manner similar to that of the whole liver lobe *in vivo*. Slices from rats made 14 hours post-hepatectomy do not appear to synthesize DNA *in vitro* (Figure 2). No major differences in these patterns were observed when rat weight ranged from 150 to 300 gm.

Presented in Figure 3 are the results of an experiment which compared DNA specific activities and percent labeled nuclei in slices from a liver removed 16 hours post-hepatectomy. Figure 3a shows the pattern of incorporation of ^{3}H-dThd into DNA in slices from one rat which showed a wave of incorporation while Figure 3b is of a rat whose liver slices failed to progress through this wave. (While slices made 14 hours post-hepatectomy or earlier always failed to progress through a wave of ^{3}H-dThd incorporation into DNA, this also

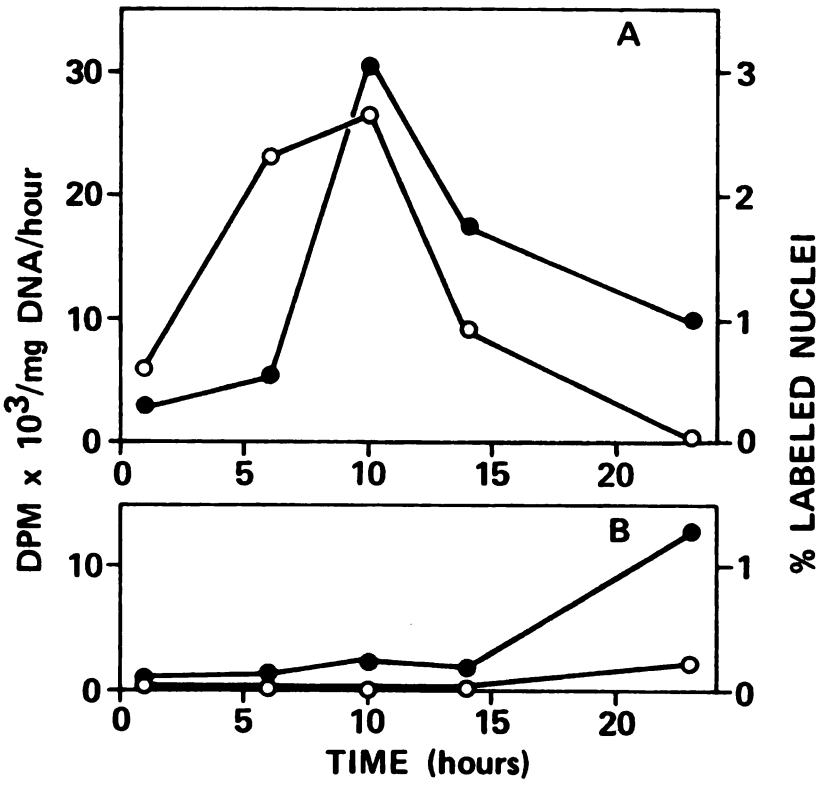

Figure 3. A comparison between liver DNA specific activity and the percentage of labeled nuclei at various times in slices made and incubated in vitro from one rat starting at 16 hours after 70% partial hepatectomy. Slices were cut in half and at indicated times each half was separately pulsed for one hour with ^{3}H-dThd, 10^{-5}M, 20 µCi/µmole. In one half ●—●, DNA specific activity was determined, while in the other half ○—○, the nuclei were isolated and the percentage of labeled nuclei was determined by autoradiography. A and B represent the incorporation of ^{3}H-dThd into DNA in slices made from different rats.

occasionally occurred at 16 hours.) It is important to remember that the number of grains over a nucleus had no influence on the decision that it was labeled (as long as there are at least 5 grains present). Therefore, changing rates of DNA synthesis in the slice are not reflected in the resulting plot of the percentage of labeled cells as a function of time. Because the plot of DNA specific activity in the slice as a function of time is similar to that of the percentage of labeled cells, this suggests that changes in DNA specific activity reflect changes in numbers of cells making DNA. At 6 hours after the start of the incubation, the percentage of labeled cells is near its peak while DNA specific activity at this time is only slightly increased (Figure 3a). While the cause for this discrepancy has not been determined, a similar high percentage of labeled cells and a low DNA specific activity a few hours before peak levels of incorporation of ^{3}H-dThd into DNA are reached has been observed in experiments studying liver regeneration in vivo.[45]

In addition to what is shown in Figure 3, one slice from the rat liver represented in Figure 3a was incubated in the presence of ^{3}H-dThd throughout the 23 hour incubation period. The percentage of labeled nuclei in this slice, an indication of the percentage of cells which progressed through the wave of ^{3}H-dThd incorporation into DNA seen in Figure 3a was 5.70% (95% confidence interval = 4.33 to 7.07%).

The assumption made in these experiments is that the liver is homogeneous; that is, that slices taken from various depths in the liver, as well as sections from the same slice, are comparable to each other. Table 1 shows an experiment in which the depth of the slices and specific activities in the two halves of a slice were examined. All the values represent slices made from a single rat liver 16 hours post-hepatectomy and incubated 9 hours plus a one hour pulse period. This time for the pulse was chosen because the incorporation of ^{3}H-dThd into DNA of the slices should be at a peak, as indicated by Figure 2. There was no significant (analysis of variance, $F > 0.05$) difference between corresponding halves of a slice or between slices made throughout the liver lobe. However, inspection of the table indicates that a given half-slice specific activity may vary by a factor of two from other slices or from its corresponding half-slice. This variability accounts for the large standard errors shown in experiments in this report.

3.3 Experiments Using ^{14}C-formate: Although the incorporation of ^{14}C-formate into liver DNA *in vivo* paralleled that of ^{3}H-dThd (Figure 1), this was not the case in slices *in vitro*. Figures 4a, 4b, and 4c represent experiments where the incorporation of labeled precursors into DNA in slices was measured when the livers were sliced 18, 16, and 14 hours post-hepatectomy, respectively. As a result of the different times of slice preparation, three different

Table 1. The relationship between DNA specific activity of half-slices and of slices made at different depths in the liver. One rat was sacrificed 16 hours post-hepatectomy. Consecutive slices were made from the right lateral liver lobe. Each slice was cut in half, and each piece was then separately incubated *in vitro* for 9 hours. Following a subsequent one hour pulse of ^{3}H-dThd, 10^{-5}M, 20 µCi/µmole, the DNA specific activity of the tissue was determined. Slice #1 is normally discarded.

	DNA Specific Activity (DPMX10^{3}/mg DNA/hour)	
Slice #	Half-slice	Half-slice
1	29.69	21.50
2	29.31	22.22
3	24.81	24.36
4	21.45	40.19
5	20.86	42.44
6	51.39	24.72
7	45.74	27.93
8	58.33	16.40
9	4.28	38.38

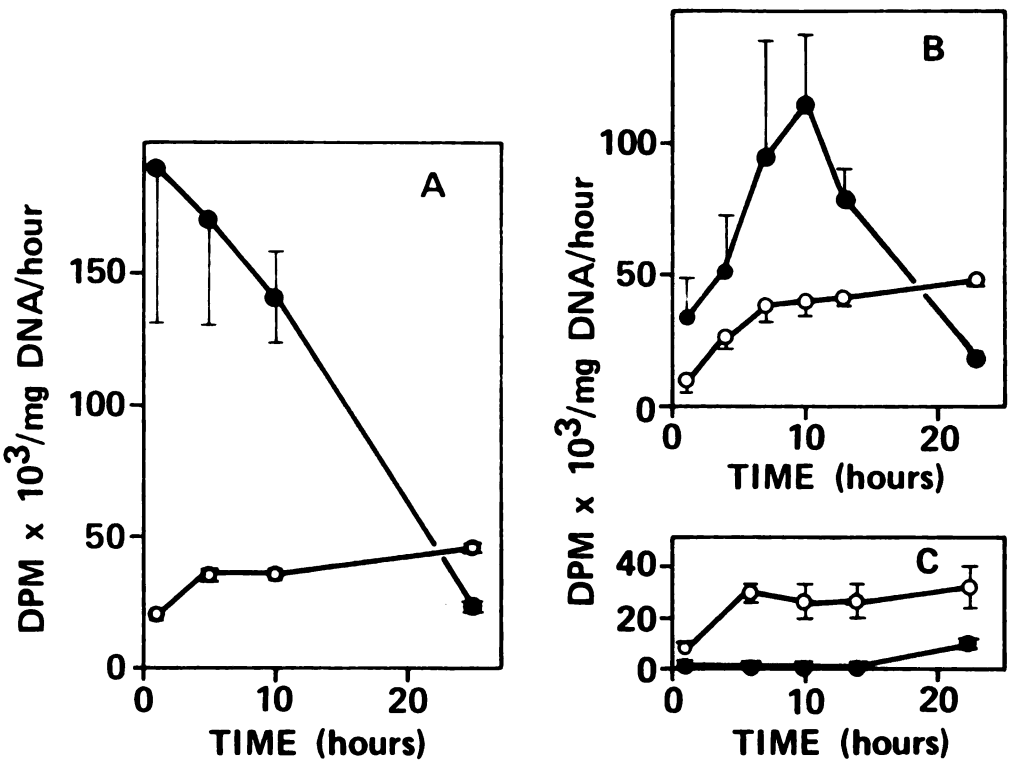

Figure 4. A comparison of ^{3}H-dThd and ^{14}C-formate incorporation into DNA at various times in liver slices made and incubated in vitro 18, 16, or 14 hours after 70% partial hepatectomy. Slices were pulsed for one hour with ^{3}H-dThd, 10^{-5}M, 20 µCi/µmole ●—●, and ^{14}C-formate, 5 x 10^{-4}M, 0.8 µCi/µmole o—o. Points are plotted at the end of the pulse.

A. Slices made at 18 hours post-hepatectomy; slices from 2 rat livers were pooled and were distributed so that each point represents the mean of 4 slices except for the first point which is the average of 2 slices.

B. Slices made at 16 hours post-hepatectomy; slices from 3 rat livers were pooled and distributed so that each point represents the mean of 5 slices.

C. Slices made 14 hours post-hepatectomy; slices from 3 rat livers were pooled and distributed as described in Figure 2.

patterns of ^{3}H-dThd incorporation into DNA are displayed but only a single pattern of ^{14}C-formate incorporation occurred at these times. The incorporation of ^{14}C-formate (5×10^{-4}M, 0.8 μCi/μmole) into DNA, therefore, appears to be independent of the time of sacrifice of the rats used in the experiment and dependent only on the length of the incubation. No wave of incorporation of ^{14}C-formate is apparent at the times tested.

The question immediately arose as to whether the ^{14}C-formate incorporation into DNA affects the conclusions about liver slice properties as determined by ^{3}H-dThd incorporation since many of the experiments used both ^{3}H-dThd and ^{14}C-formate in the pulses. This question was approached by examining the effects of formate concentration on the incorporation of ^{3}H-dThd into DNA as shown in Figure 5. In this experiment, the amount of labeled formate was kept constant while cold formate was added to obtain the specific final concentrations. The activities at the three concentrations of formate tested, 2, 6.5, and 10×10^{-4}M, are 2, 0.62, and 0.4 μCi/μmole, respectively. In Figure 5a, one can see that DNA specific activity decreases as the formate concentration is increased in slices incubated beginning at 15 hours post-hepatectomy and pulsed at either the start of the incubation or 12 hours after the start of the incubation. One can note that while the specific activity of DNA in the slices decreases by a factor of five, the incorporation of ^{14}C-formate into DNA decreases by only a factor of two at

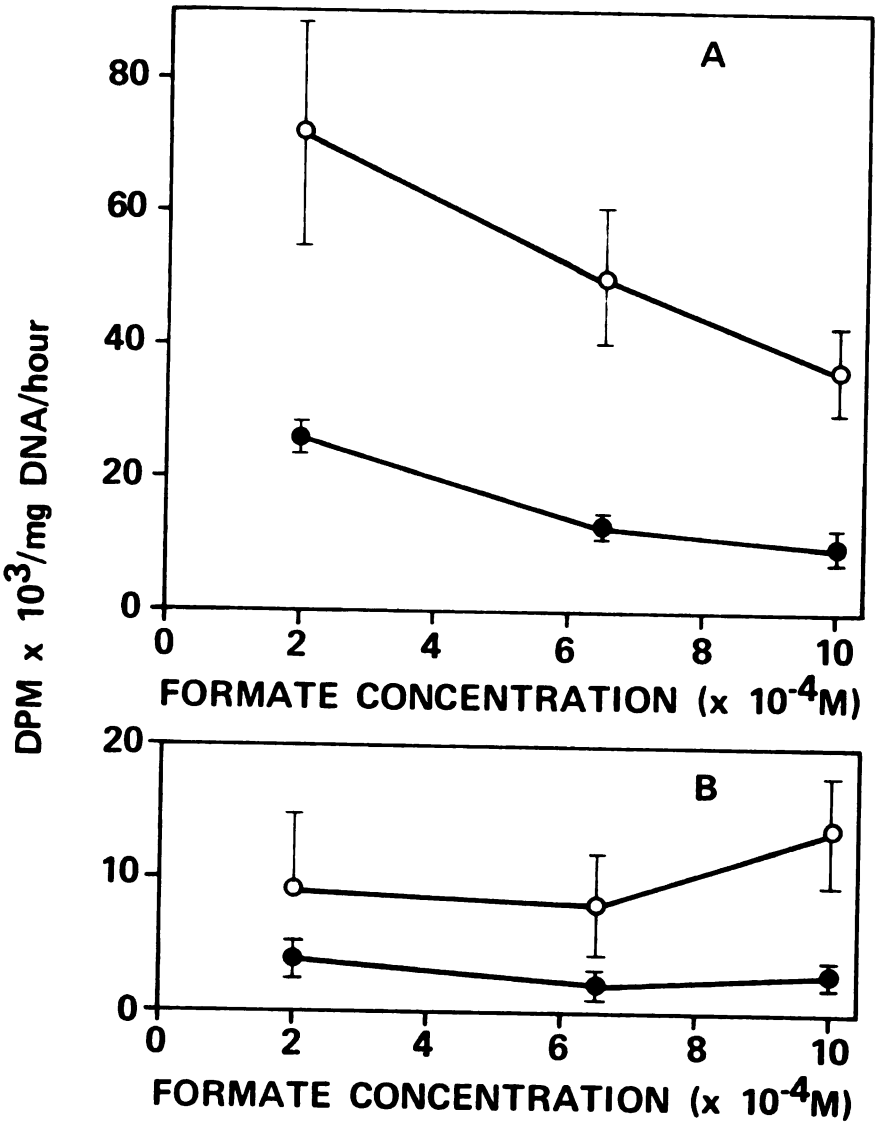

Figure 5. The effect of formate concentration on the incorporation of ^{14}C-formate and ^{3}H-dThd into DNA of liver slices made and incubated in vitro starting at 15 hours after 70% partial hepatectomy. The ^{3}H-dThd concentration in the medium in all cases was 10^{-5}M, 20 µCi/µmole. ^{14}C-formate specific activity at formate concentrations of 2, 6.5, and 10 x 10^{-4}M are 2, 0.62, and 0.4 Ci/ mole respectively. Slices from 2 rat livers were pooled and distributed so that each point represents the mean of 5 slices. Points are plotted at the end of a one hour pulse which began at the start of the incubation ●—●, or at 12 hours after the start of the incubation, o—o.

A. The incorporation of ^{14}C-formate into DNA as a function of formate concentration.

B. The incorporation of ^{3}H-dThd into DNA as a function of formate concentration.

both pulse times. This indicates that the concentrations of formate are not saturating for DNA synthesis since the decrease should be a factor of 5 if it was. It seems that factors other than formate concentration alone play a role in determining the incorporation of ^{14}C-formate into DNA, especially in view of the lack of saturation of ^{14}C-formate incorporation into DNA at the high formate concentrations used in this experiment. Figure 5b shows a relatively constant incorporation of ^{3}H-dThd into DNA despite increases in formate concentration in the medium. The slices in this experiment were incubated beginning at 15 hours post-hepatectomy and were pulsed at the start or at 12 hours after the start of the incubation. It is, therefore, evident that the concentrations of formate tested in this experiment do not alter the incorporation of ^{3}H-dThd into DNA. This suggests that the apparent lack of a relationship between ^{14}C-formate incorporation into DNA of slices from regenerating liver incubated *in vitro* and the time at which rats were sacrificed probably do not affect the conclusions drawn through the use of ^{3}H-dThd to estimate DNA synthesis.

One explanation for the pattern of formate incorporation into DNA is that some of the labeled protein made during the incubation is extracted in the DNA fraction of the procedure for DNA isolation.[40] Figure 6 compares the total incorporation at various times during the incubation. Since the rates of incorporation appear to be similar, protein hydrolysis could account for some of the radioactivity since the

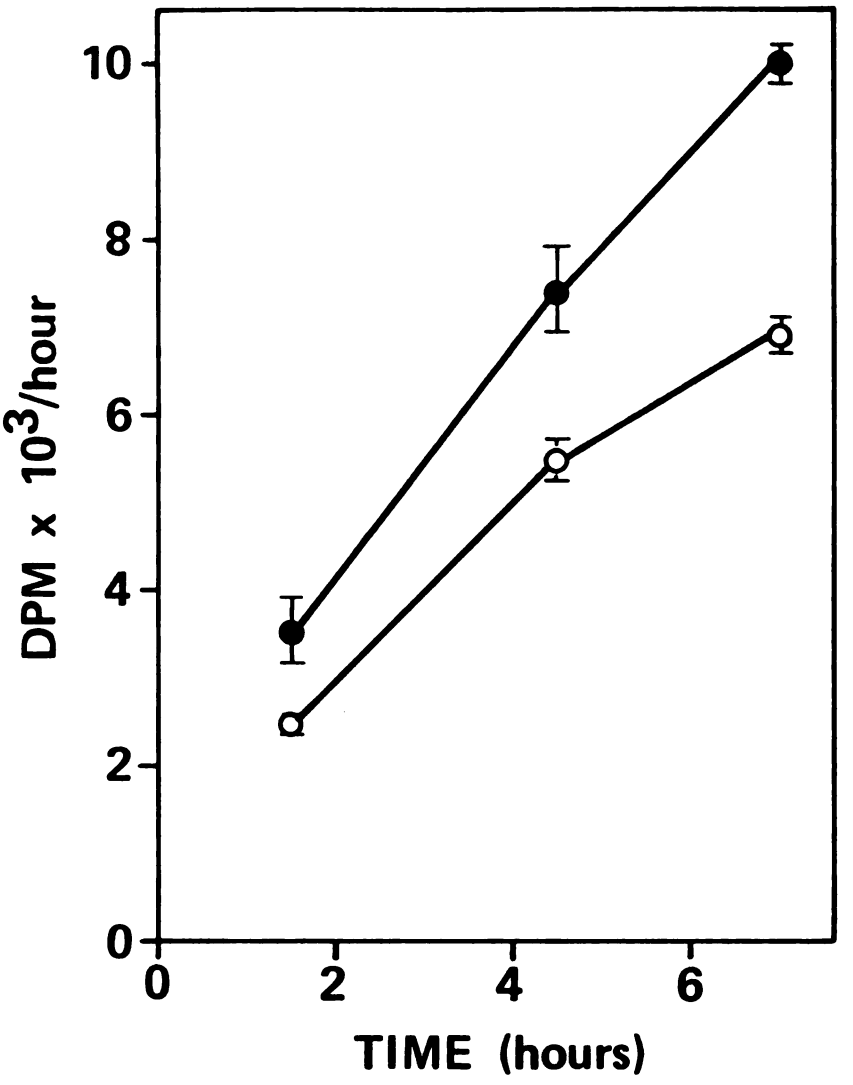

Figure 6. A comparison of ^{14}C-formate incorporation into DNA and into protein as a function of time in liver slices made and incubated in vitro starting at 18 hours after 70% partial hepatectomy. Slices were pulsed for one hour with ^{14}C-formate, 2 x 10^{-4}M, 2 µCi/µmole. Total radioactivity incorporation into DNA o–o, and into protein ●–●, in a slice is plotted at the end of the one hour pulse. Slices from 2 rat livers were pooled and distributed so that the middle point represents 4 slices while the other points represent 5 slices.

DNA fraction in a slice contains 30 to 40% of the total radioactivity measured in the DNA and protein fractions, as can be calculated from the data in Figure 6.

3.4 Serum Effects on Slice DNA Synthesis: Figure 7 shows the apparent lack of effect (paired Student's t-test, $p > 0.05$) of 20% calf serum on slices incubated beginning at 16 (Figure 7a) and 14 (Figure 7b) hours post-hepatectomy. Although points are displayed as grouped means, each half-slice incubated in medium plus serum was compared to its other half of the slice which was in control medium and was pulsed at the same time as the test half-slice. There was a similar lack of effect when 20% fetal calf serum (Figure 8), or 5% serum from rats partially hepatectomized 18 hours earlier (Figure 9) was tested. Because of these observations, we must conclude that factors affecting DNA synthesis are not present in the serums tested or that this liver slice incubation system was not able to detect the presence of these factors.

It still seemed possible that stimulatory factors were present but that unlabeled thymidine in the serum diluted the ^{3}H-dThd specific activity and caused the stimulation to be obscured. Accordingly, dialyzed calf serum was tested to see if it stimulated DNA synthesis in 16 hour post-hepatectomy liver slices. To our surprise, the dialyzed serum decreased the incorporation of ^{3}H-dThd into slice DNA, as shown in Figure 10. One explanation of this observation

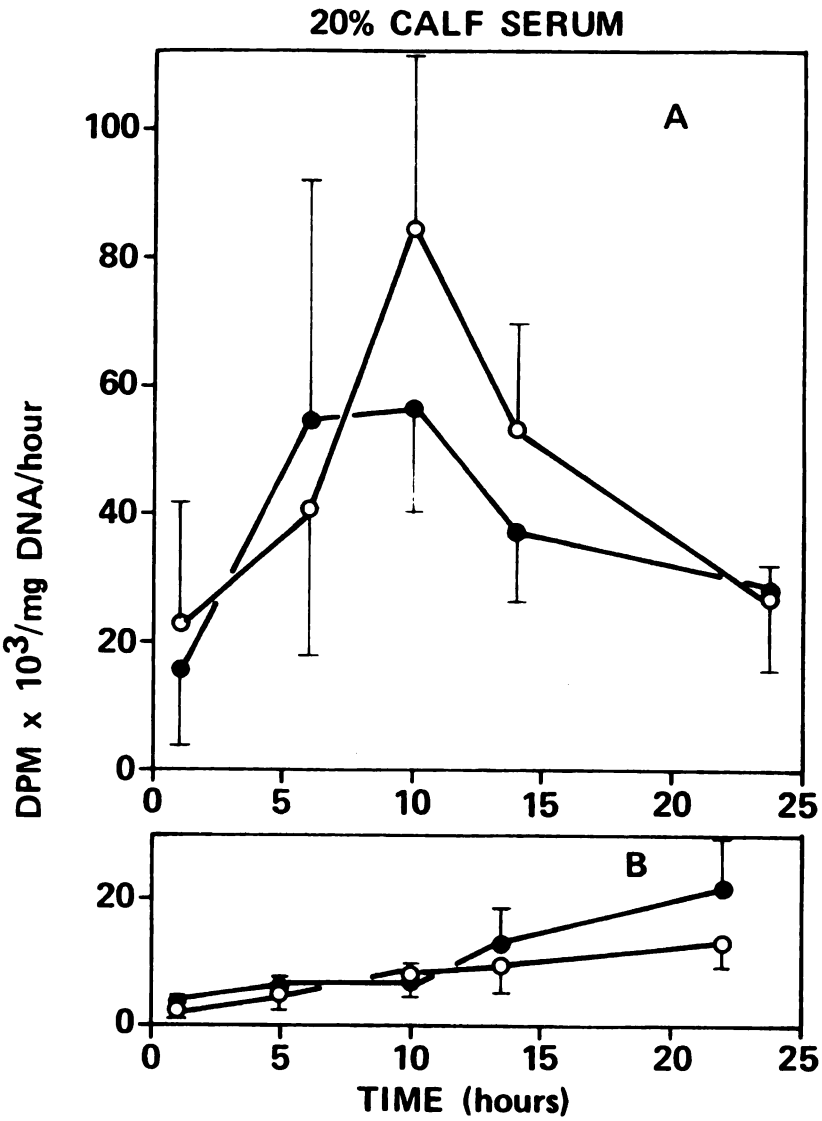

Figure 7. The effect of 20% calf serum on the incorporation of ^{3}H-dThd, 10^{-5}M, 20 µCi/µmole, into DNA at various times in liver slices made and incubated in vitro starting at 16 or 14 hours after 70% partial hepatectomy. Slices per point are as described in Figure 2. Points are plotted at the end of the one hour pulse. o—o - No serum present in the incubation medium. ●—● - 20% calf serum present in the incubation medium.
A. Slices made from 3 rat livers were pooled and were incubated beginning at 16 hours post-hepatectomy.
B. Slices made from 2 rat livers were pooled and were incubated beginning at 14 hours post-hepatectomy.

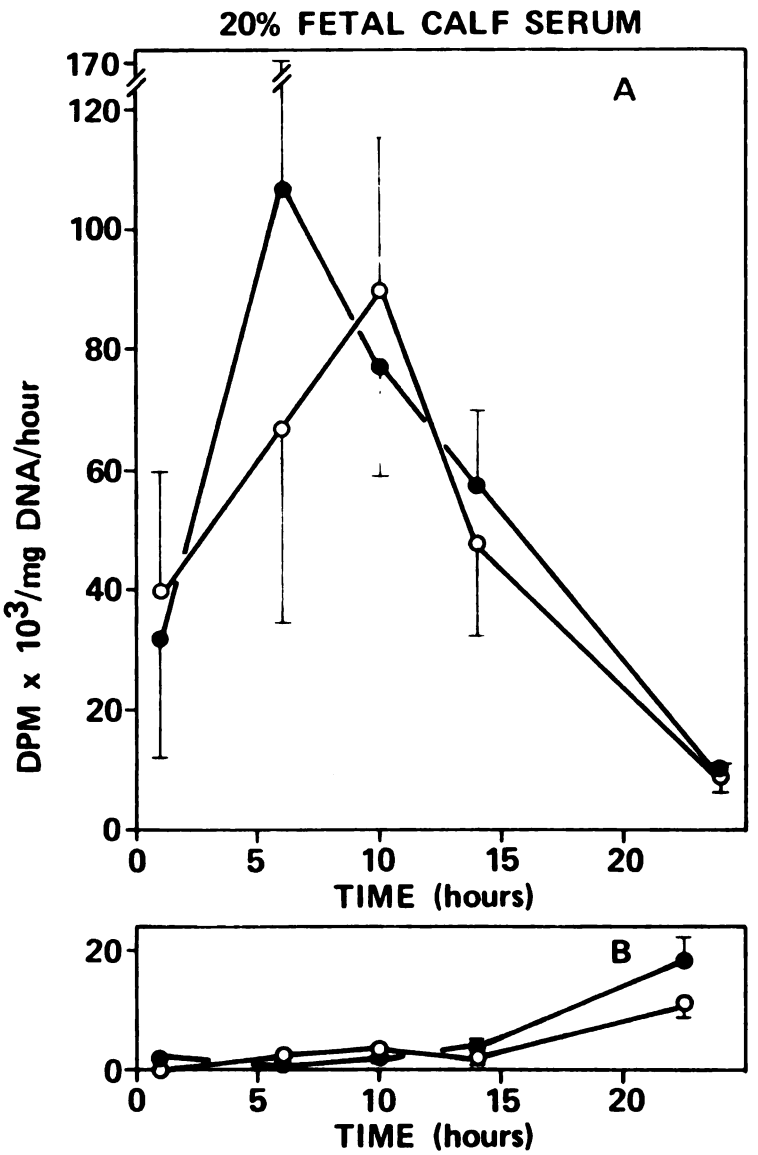

Figure 8. The effect of 20% fetal calf serum on the incorporation of ^{3}H-dThd, 10^{-5}M, 20 µCi/µmole, into DNA at various times in liver slices made and incubated <u>in vitro</u> starting at 16 or 14 hours after 70% partial hepatectomy. In each case, slices from 3 rat livers were pooled and distributed as described in Figure 2. Points are plotted at the end of the one hour pulse. o—o - No serum present in the incubation medium. ●—● - 20% fetal calf serum present in the incubation medium.
A. Slices incubated beginning at 16 hours post-hepatectomy.
B. Slices incubated beginning at 14 hours post-hepatectomy.

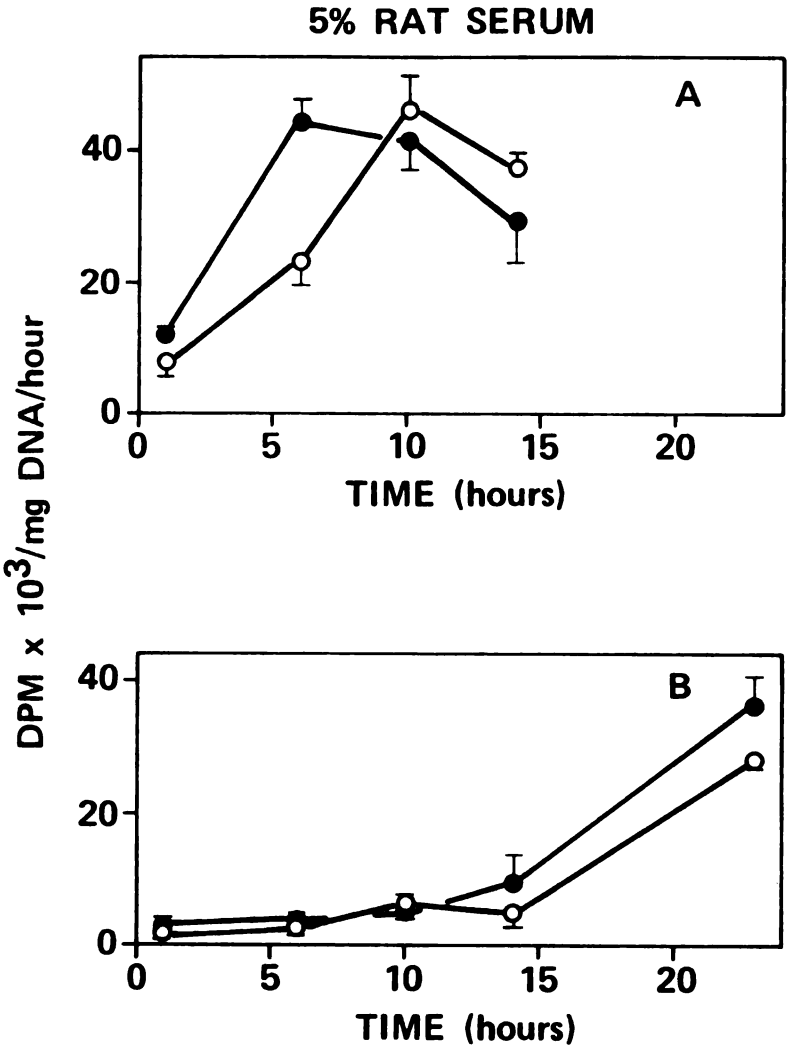

Figure 9. The effect of 5% serum from rats partially hepatectomized 18 hours earlier on the incorporation of ^{3}H-dThd, 10^{-5}M, 20 µCi/µmole, into DNA of liver slices made and incubated in vitro starting at 16 or 14 hours post-hepatectomy. Slices were pooled from 4 rat livers and distributed as described in Figure 2. Points are plotted at the end of the one hour pulse. o—o - No serum present in the incubation medium. ●—● - 20% fetal calf serum present in the incubation medium.

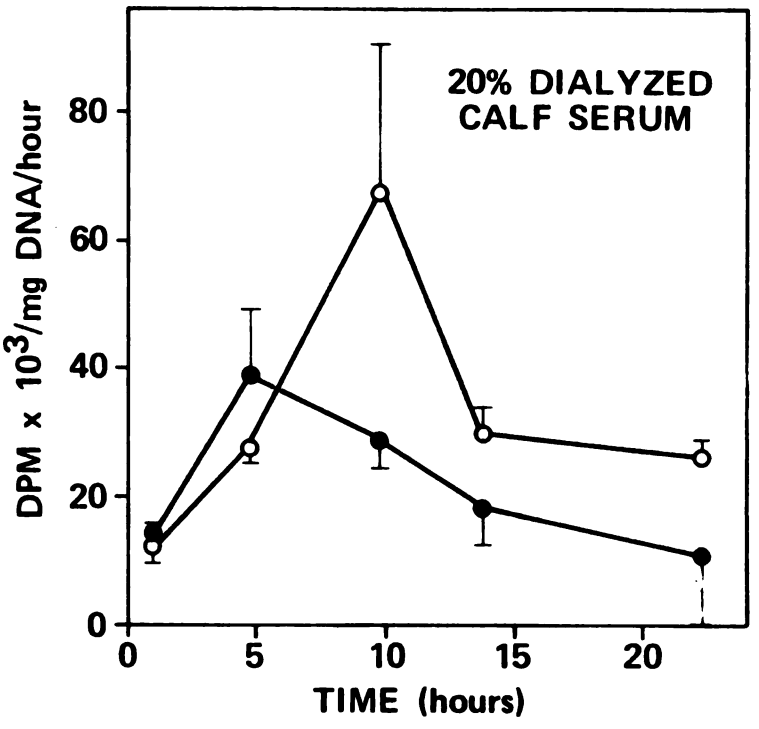

Figure 10. The effect of 20% dialyzed calf serum on the incorporation of ^{3}H-dThd, 10^{-5}M, 20 µCi/µmole, into DNA at various times in liver slices made and incubated in vitro starting at 16 hours after 70% partial hepatectomy. Slices were pooled from 4 rat livers and were distributed as described in Figure 2. Points are plotted at the end of the one hour pulse. o—o - No serum present in the incubation medium. ●—● - 20% dialyzed calf serum present in the incubation medium.

is that serum contains low molecular weight stimulators of DNA synthesis[17,29] and high molecular weight inhibitors[13] whose effects are equal in whole serum. However, another explanation is that inhibitory factors are produced during dialysis.

3.5 N-2-Fluorenylacetamide Effects on Slice DNA Synthesis: Factors important in the normal control of liver growth might be altered by the effects of a hepatocarcinogen such as FAA. It seems reasonable that an alteration in the control of liver growth should occur before the gross appearance of liver cancer. (At later times, secondary changes might occur such as when chromosomes are lost from tumor cells.) Therefore, a test of the importance of a change in the properties of regenerating liver at 16 hours post-hepatectomy (as evaluated by the onset of a wave of DNA synthesis in liver slices incubated beginning at this time), and of the importance of the synchronous wave of DNA synthesis, to the control of normal liver cell division would be to see if FAA alters the ability of regenerating liver slices to make DNA from that seen normally at a time before the gross appearance of liver cancer.

After 4 weeks on a diet containing 0.05% FAA, rats were observed to have gross evidence of hepatic cirrhosis. In addition, 2 out of 21 rats had an abnormal mass, in one rat in the lower thoracic cavity and in the other a subcutaneous abdominal mass. Two weeks of feeding on a diet containing FAA was chosen as a starting time to examine precancerous

effects of FAA on slice DNA synthesis *in vitro*. Rats exposed to FAA for 14 days, followed by seven days on a control diet (to decrease or eliminate the acute toxic effects of FAA in this study) were partially hepatectomized and their livers used in an experiment 16 hours later. Figure 11 shows that there was an almost continual increase in DNA specific activities with time rather than the wave of incorporation that was seen in normal rats. This suggests asynchronous DNA synthesis in the slices.

To determine if the alteration in DNA synthetic rate is due to acute or to chronic effects of FAA, rats were kept on the control diet for 30 days after 14 days eating FAA. In this experiment (Figure 12), the rats were again sacrificed 16 hours post-hepatectomy but the pattern of the incorporation of ^{3}H-dThd into DNA with time did not duplicate that shown in Figure 11. Instead, it resembled that of 14 hour post-hepatectomy slices; namely the pattern of DNA specific activity seen when a liver is removed from a rat before the critical period has passed in the animal (Figure 2). This suggests that the presence of a critical period in rats exposed to FAA and allowed to recover from FAA acute effects is unchanged from that seen in normal rats or that there is a decreased response to partial hepatectomy in these rats. Unfortunately, no conclusions can be drawn from this experiment about the stability of changes in incorporation of ^{3}H-dThd into DNA that were observed in Figure 11.

Since slices from livers removed before the critical period sometimes show a small gradual increase in DNA

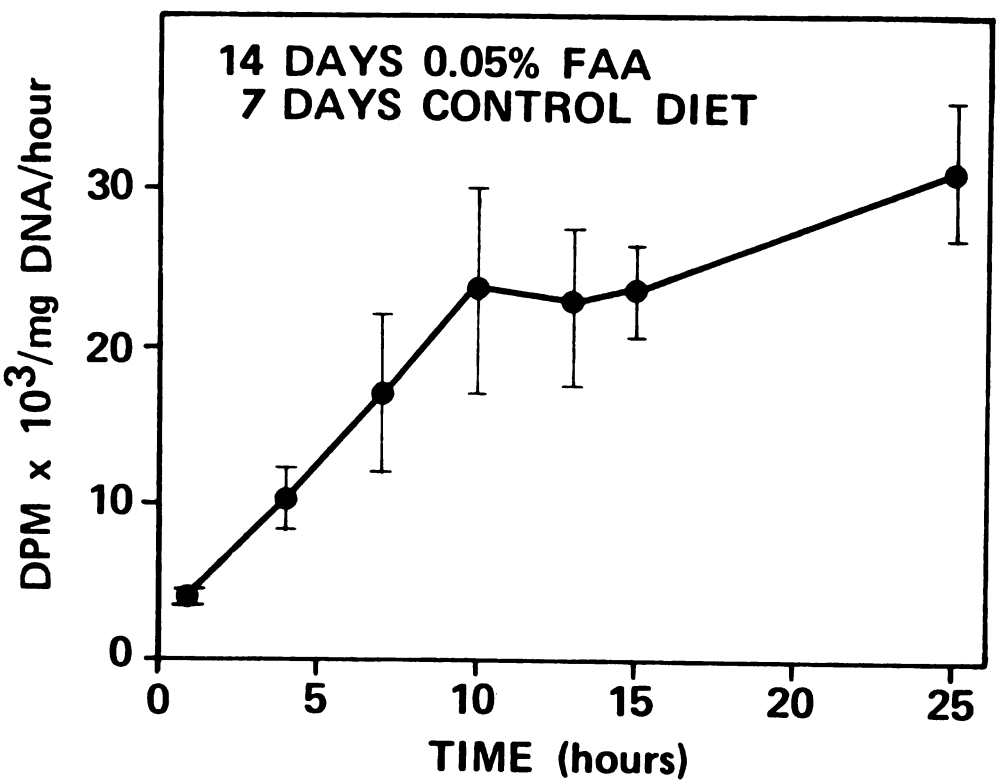

Figure 11. The effect of feeding a diet containing 0.05% FAA for 14 days followed by a control diet for 7 days on the incorporation of ^{3}H-dThd, 10^{-5}M, 20 µCi/µmole, into DNA at various times in liver slices made and incubated in vitro starting at 16 hours after 70% partial hepatectomy. Slices were pooled from 2 rat livers and each point, plotted at the end of the one hour pulse, represents 5 slices except for the last point where 4 slices were used.

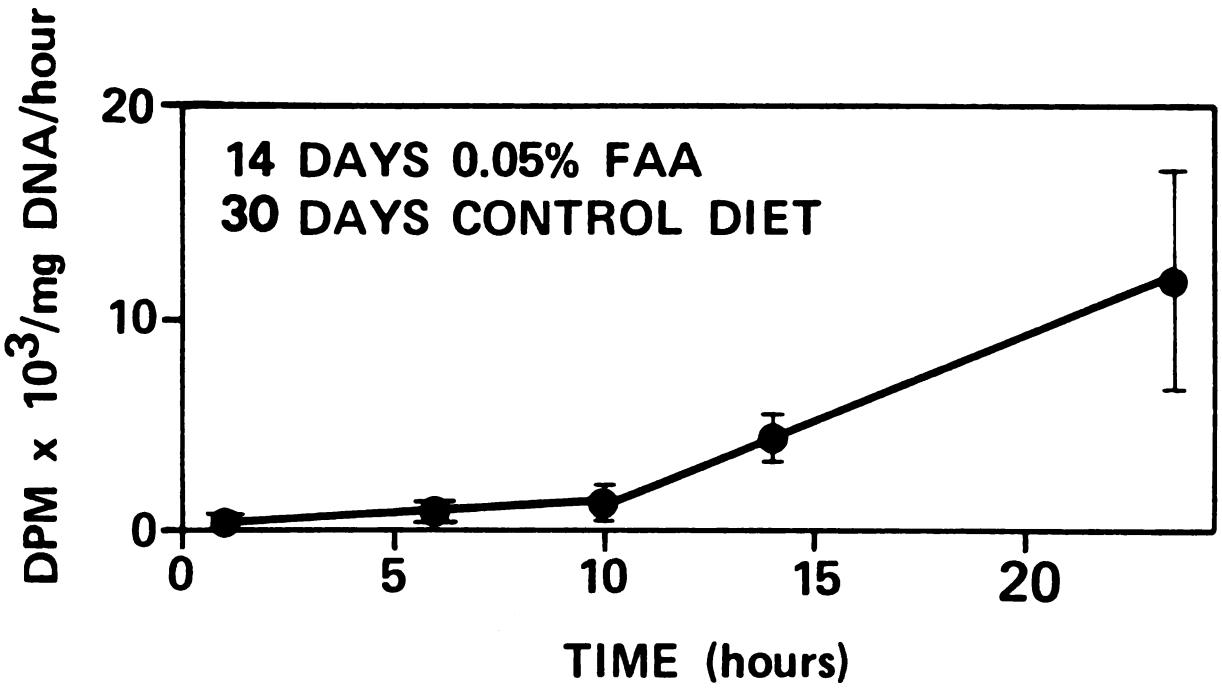

Figure 12. The effect of feeding a diet containing 0.05% FAA for 14 days followed by a control diet for 30 days on the incorporation of ^{3}H-dThd, 10^{-5}M, 20 µCi/µmole, into DNA at various times in liver slices made and incubated in vitro starting at 16 hours after 70% partial hepatectomy. Slices from 2 rat livers were pooled and distributed so that points on the graph represent 3,3,4,4, and 2 slices, respectively. Points are plotted at the end of the one hour pulse.

specific activities with time (Figure 4), the pattern seen in Figure 11 may be representative of the same type of DNA synthesis but in an increased manner. To test this, rats exposed to FAA for 14 days and to a control diet for 7 days were partially hepatectomized and sacrificed 18 hours later. The results, as depicted in Figure 13, indicate the same manner of ^{3}H-dThd incorporation into DNA as that seen in Figure 11. In view of the lack of a wave of incorporation of ^{3}H-dThd at 16 hours post-hepatectomy after treatment with FAA, and the presence of a wave in all normal livers removed at this time, one can conclude that pre-treatment with FAA alters the pattern of ^{3}H-dThd incorporation into DNA of slices from livers removed after the critical period at around 16 hours post-hepatectomy.

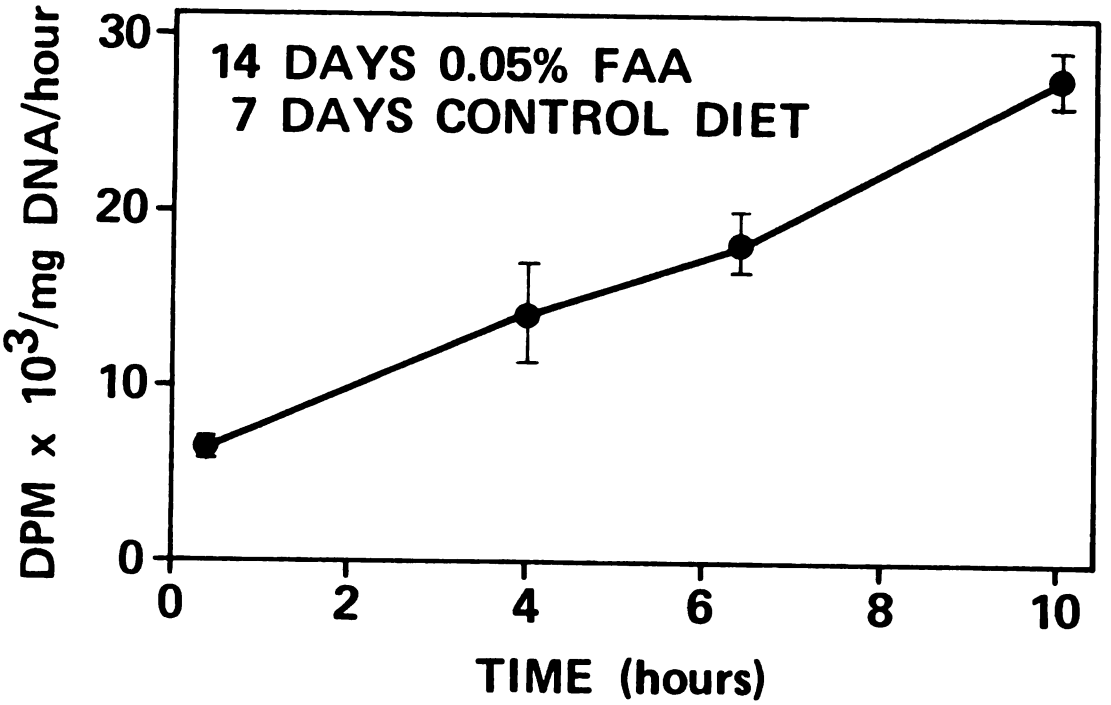

Figure 13. The effect of feeding a diet containing 0.05% FAA for 14 days followed by a control diet for 7 days on the incorporation of ^{3}H-dThd, 10^{-5}M, 20 µCi/µmole, into DNA at various times in liver slices made and incubated *in vitro* starting at 18 hours after 70% partial hepatectomy. Slices from one rat were used in this experiment and are distributed as described in Figure 2.

DISCUSSION

4.1 Slice DNA Synthesis: These studies are the first, to our knowledge, to show that slices of rat liver from the right lateral lobe are capable of progressing through a wave of incorporation of ^{3}H-dThd into DNA *in vitro* when the liver is removed 16 hours or later after partial hepatectomy. The observation that the peak rate of ^{3}H-dThd incorporation into DNA *in vitro* is seen at the same time as is seen *in vivo* suggests that this wave represents progression of liver slice cells through the S phase *in vitro* in a manner reflecting that seen *in vivo*. This, in turn, indicates that liver slice cells *in vitro*, when taken from livers after 16 hours post-hepatectomy, are capable of maintaining a physiological regulation of progression through the S phase independent of any putative systemic factors which may be required to control liver cell division.

Autoradiography of slices made 16 hours post-hepatectomy (Figure 3) resulted in a peak of 2.65% labeled nuclei. Since this number represents the percentage of all nuclei in the slices, the labeling of hepatocytes (which make up 60% of liver cells) was probably about 4.3%. (Only hepatocytes make DNA during the first 26 hours post-hepatectomy *in vivo* and assuming that this also holds true for the first 10 hours in an *in vitro* incubation of 16 hour post-hepatectomy slices.) Reports in the literature indicate that peak percentages of labeled cells seen in regenerating liver *in vivo*

range from 15% to 44%.[7,13,45,46] One must note that the peak specific activity observed in Figure 3 was lower than that seen in other experiments in this report so that in those experiments the percentage of hepatocytes participating in DNA synthesis might have been higher. In addition, some cell death without nucleus breakdown might occur either in the preparation of the slices or during the incubation itself. Therefore, the percentage of hepatocytes in liver slices that make DNA during the incubation could be a fairly good reflection of the percentage of hepatocytes that participate in DNA synthesis in regenerating liver *in vivo*.

Slices made from livers removed 14 hours or earlier after partial hepatectomy failed to progress through a wave of incorporation of ^{3}H-dThd into DNA. This indicates that there is a critical period at around 16 hours after partial hepatectomy when certain properties of the liver change so that slices made after this time can progress through a wave of ^{3}H-dThd incorporation into DNA *in vitro*. It is possible that this critical period, corresponding to a time near the beginning of DNA synthesis *in vivo*, may represent a control point in the regulation of cell division in the liver. This may be a time when blood-borne factors act to initiate a change from the normal G_0 state of liver cells to a state of active progression through the cell cycle beginning near the end of G_1. An alternative is that this critical period is one of stabilization of intracellular factors needed for DNA synthesis and that these factors are labile and sensitive to

disturbances such as the preparation of slices before 16 hours post-hepatectomy. This situation would be similar to models of heat shock synchronization of cell division in Tetrahymena,[47] although in the case of Tetrahymena, cell division, but not DNA synthesis, is delayed. If this possibility is correct, then the time at which specific steps are initiated which lead to DNA synthesis may be earlier than 16 hours post-hepatectomy. Evidence suggesting this transition of cells in G_0 to the early G_1 phase includes the increased levels of ribosomes and RNA seen by 6 hours post-hepatectomy and increased protein content and activities of enzymes important in DNA synthesis by 12 hours post-hepatectomy. A factor might act before 6 hours post-hepatectomy and a sequence of events would be initiated which affect levels of protein and RNA and eventually DNA synthesis. As an alternative, there may be several control points, including ones for increased protein and ribosome levels in a cell, one for increased activities of enzymes needed for DNA synthesis, and one for the critical phase observed in this report which occurs just before the onset of DNA synthesis. It has been pointed out that the onset of DNA synthesis in regenerating liver *in vivo* is dependent on the time of day at which the partial hepatectomy is performed.[45] In this study, the time of operation was relatively constant ($\pm$ 2 hours) and rats sacrificed at 14 or 16 hours post-hepatectomy were operated on randomly during the time period at which all operations were done. No relationship between

time of operation and the ability of liver slices to progress through a wave of ^{3}H-dThd incorporation into DNA was observed within the limited time period evaluated. Furthermore, Figure 3 is an example of an experiment where the partial hepatectomy of one rat (Figure 3b) was completed 17 minutes after the first rat (Figure 3a) and yet a wave of incorporation of ^{3}H-dThd into DNA of liver slices occurred in one rat (Figure 3a) but not in the other (Figure 3b), indicating that the onset of a wave of DNA synthesis in liver slices is not dependent on time of partial hepatectomy within the period tested. In summary, there are three possible explanations for the presence of the critical phase in regenerating liver. The first is that G_0 is located in late G_1. A factor acts during the critical phase to cause the G_0 cell to reenter the cell cycle and to begin to make DNA. To explain the changes in protein and RNA observed before the critical period, one need only to hypothesize that this represents a nonspecific response to hepatic insufficiency without a commitment to DNA synthesis. The second possibility is that the critical phase is an artifact, due to the preparation of slices before 16 hours post-hepatectomy. The third possibility is that the critical phase is only one of several control points which occur in early G_1 and throughout the G_1 phase and which must be passed before DNA synthesis can begin.

4.2 <u>The G_1 Phase of the Cell Cycle</u>: In an attempt to understand the regulation of cell division in chicken fibroblasts,

Temin examined the serum requirements of these cells.[48] He found that the G_1 phase could be divided into three segments. In the absence of serum, cells stop progressing through the cell cycle in a phase Temin named G_{1a}. Serum was required (G_{1b}) for these cells to enter G_{1c}, a phase where serum is no longer required and where cells are committed to DNA synthesis but have not yet begun to make DNA. In view of the requirement for serum in most cell and tissue cultures, one can suspect that liver also requires serum for cell division despite the apparent lack, in this system, of whole serum effects on liver slice incorporation of ^{3}H-dThd into DNA. This lack of effect of serum might be explained by postulating that the slices are made after the serum requirement has passed, or that stimulating and inhibiting factors in the serum are exactly balanced. Slices made from livers 16 hours post-hepatectomy or later appear to be in late G_1 (Figure 3a) or in early S phase (Figures 2,4,5,7-10) as judged by the apparent initial rates of DNA synthesis in these experiments. Since slices made earlier than 16 hours post-hepatectomy do not appear to enter the S phase even in the presence of serum, something more than a generalized serum requirement may be needed for liver to enter the S phase. (Temin[48] showed that bovine serum could substitute for chicken serum in the chicken fibroblast system so the requirement of his tissue culture for serum was not specific.) The time during which this factor would act is represented by the phase G_{1d} in Figure 14. This phase would be the

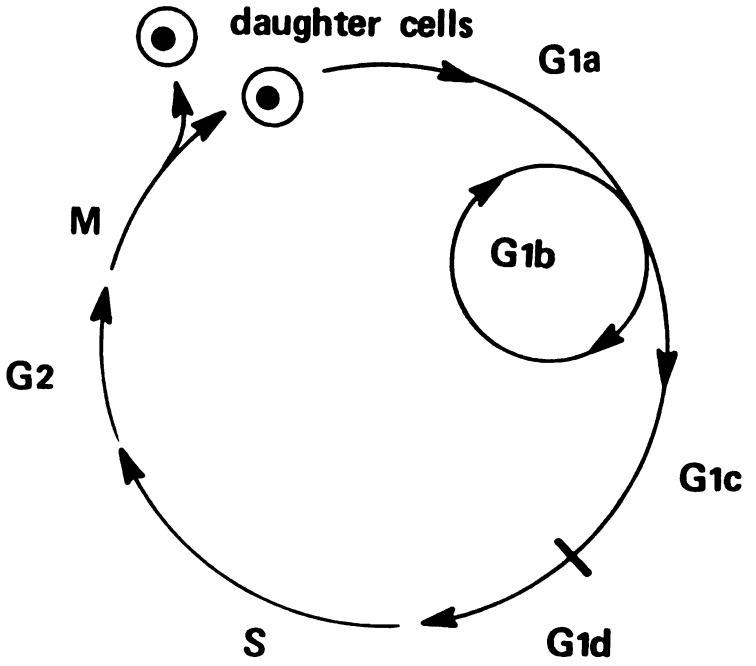

Figure 14. Serum requirements of cells progressing through the G_1 phase of the cell cycle.
G_{1a} - Cells stop progressing through the cell cycle here in the absence of serum.
G_{1b} - A period of time when serum is required for cells to enter G_{1c}.
G_{1c} - Serum is no longer required in order to enter the S phase. Cells are committed to make DNA but have not yet begun to do so.
G_{1d} - The critical phase observed in the liver slices. Certain properties of the liver change so that slices will make DNA in vitro only after the liver progresses through this phase in vivo.
S - DNA synthetic phase
G_2 - Premitotic phase
M - Mitotic phase

critical phase observed in the liver slices. Aside from the species difference, Temin's fibroblasts[48] are in a state of constant growth except when serum is removed from the medium or when serum becomes aged and has a decreased ability to support cell division due to the presence of the cells over a period of time. The plateau of cell division in the cultures may be due to a nutritional deficiency which develops as the serum ages. This nondividing state of the fibroblasts is not the same as is found in the liver *in vivo*, where cells are in a G_0 state despite apparently adequate nutrition. Thus, the conversion of the G_0 state to active progression through the cell cycle in the liver may not be comparable to the serum requirement seen in tissue or cell culture. It may be that the G_0 conversion of liver to active cell division does not require a generalized serum presence at all but rather something else or perhaps something in addition to serum.

In view of the lack of DNA synthesis in slices made before the critical period, one can conclude that there probably is a positive factor acting at this time to initiate DNA synthesis. Should an inhibitory factor have been present which prevented liver progression through the cell cycle and which disappeared at a time corresponding to the onset of DNA synthesis, one would have expected all slices to progress through a wave of DNA synthesis *in vitro* since this factor would not be present in the medium. This does not eliminate the possibility that inhibitory factors control DNA

synthesis at different times in the regeneration of liver than those examined here.

4.3 ^{14}C-Formate DNA Synthesis: The use of ^{14}C-formate as a precursor for DNA in slices was intended as a way to examine whether the de novo pathway for DNA synthesis was under similar control as the salvage pathway to DNA synthesis as determined by ^{3}H-dThd. While the experiments comparing ^{3}H-dThd incorporation into DNA and the autoradiography of nuclei provide strong evidence that ^{3}H-dThd incorporation into DNA reflects the true rate of DNA synthesis, it is still possible that real DNA synthetic rates may vary from the rate estimated by the use of ^{3}H-dThd. This would occur if the de novo DNA synthesis rate varied with time from the rate of ^{3}H-dThd incorporation into DNA via the salvage pathway. The use of a de novo precursor of DNA might have ruled out this possibility. It was, therefore, disappointing to find that ^{14}C-formate was not a good precursor for slice DNA synthesis.

Although ^{14}C-formate incorporation paralleled ^{3}H-dThd incorporation in vivo, it was much less sensitive than ^{3}H-dThd. In Figure 1, the peak incorporation of ^{14}C-formate into DNA was only 4 times as high as at early times during the incubation while the incorporation of ^{3}H-dThd into DNA increased 60-fold. In vitro, ^{14}C-formate incorporation did not parallel ^{3}H-dThd incorporation into DNA (Figure 4), although it did parallel formate incorporation into protein

(Figure 6). During the extraction of DNA, small amounts of protein are hydrolyzed and appear in the DNA extraction as oligopeptides and amino acids.[40] However, this hydrolysis is not sufficient to explain most of the radioactivity in the DNA fraction since 30 to 40% of the total radioactivity measured in the DNA and protein fractions appears in the DNA fraction. The data in Figure 5 indicate that the rate of incorporation of ^{14}C-formate into DNA is not saturated at concentrations as high as 10^{-3}M. This suggests that the capacity of formate to be incorporated into the pool of proximate DNA precursors is a limiting factor in the incorporation of ^{14}C-formate into DNA, rather than DNA polymerase activity. There are several possible steps in DNA precursor synthesis where this might occur. Formate is used to make N^{10}-formyltetrahydrofolate in an ATP requiring reaction. Two steps later this compound has been converted to N^5,N^{10}-methylenetetrahydrofolate. This second step is coupled with the oxidation of NADPH. Purine and thymidine synthesis require these tetrahydrofolate compounds so that the limiting step could be either in the steps of the tetrahydrofolate pathway just described, in nucleotide synthesis, or in the phosphorylation of the nucleotides to triphosphates. One possible explanation for the lack of saturation of ^{14}C-formate incorporation into DNA is that the amino acids serine, histidine, tryptophane, and glycine can donate one carbon units to the tetrahydrofolate pathway and thus compete with formate for incorporation into nucleotides. This

competitive inhibition of formate incorporation could be overcome at high formate concentrations and this would mean that ^{14}C-formate incorporation into DNA would increase at high concentrations rather than appear to be saturating as it might if the amino acid competition for incorporation into proximate DNA precursors was not present. This hypothesis could be tested by adding adenosine, guanosine, and cytidine to the medium and by removing the amino acids named above from the incubation medium to see if formate incorporation into DNA increases.

4.4 Serum Effects on Slice DNA Synthesis: Calf serum, fetal calf serum, and serum from rats subjected to partial hepatectomy 18 hours earlier did not alter the pattern of incorporation of ^{3}H-dThd into DNA at any time tested (Figures 7-9). Bovine serums were tested because of reports using other systems and other tissues that factors in serums affecting DNA synthesis were not species specific.[33,46] The rat serum was tested because of reports that factors stimulating liver DNA synthesis appear 12 to 18 hours post-hepatectomy.[11,23] The apparent lack of serum effects suggests that factors regulating rat liver DNA synthesis are never present in the blood, that factors with equal and opposite effects may be present, or that the slice incubation system is not sensitive to the serums tested. Another possibility to explain the lack of stimulatory effects of serum on 14 hour post-hepatectomy slices is that the control point

for DNA synthesis may occur earlier in time than 16 hours post-hepatectomy and that liver is sensitive to factors only at this earlier time. If serum is required for DNA synthesis to occur and does act at 14 hours post-hepatectomy, it may be that slice preparation had destabilized other factors needed for DNA synthesis and thus, serum would appear not to have a role in stimulating DNA synthesis at this time as judged by *in vitro* assay of serum effects on slice DNA synthesis. Of course, one may also theorize that factors controlling liver DNA synthesis are present in plasma but are removed or inactivated during blood clotting. This would explain the lack of effect of the serums on DNA synthesis. To test this,one would like to use plasma in the incubation but problems exist with this approach. To prevent clotting, either calcium must be removed from the medium, a condition judged not to be physiological and thus not acceptable, or heparin would have to be added to the medium. Heparin, however, is known to stimulate DNA synthesis both *in vivo* and in isolated nuclei.[17,30]

The inhibitory effect of dialyzed calf serum was puzzling in view of the lack of effect of calf serum in ^{3}H-dThd incorporation into DNA. Perhaps low molecular weight stimulatory factors[17,29] were removed from the serum by the dialysis, allowing inhibitory factors of high molecular weight[13] to decrease the incorporation of ^{3}H-dThd into DNA. It is also possible that the inhibition was due to factors introduced during the dialysis.

Verly[33] has reported that a factor in a liver homogenate can inhibit incorporation of ^{3}H-dThd into DNA in a two hour incubation of liver slices from rats partially hepatectomized 24 hours earlier. He believes that this factor circulates in the blood and inhibits liver cell DNA synthesis when it binds to the liver. One wonders whether his inhibitory factor is a physiological regulator of hepatic DNA synthesis because liver cells in G_0 appear to be blocked before the S phase rather than in its middle since DNA content is always an even multiple of two.[2] Since most of the cells committed to DNA synthesis have begun to make DNA before 24 hours, it seems that this inhibitor isolated by Verly is acting at a phase that is not normally inhibited *in vivo*. Unless this factor can block the onset of DNA synthesis in liver cells, one must question the biological significance of Verly's[33] observations.

4.5 N-2-Fluorenylacetamide Effects on Slice DNA Synthesis: Among the changes in liver resulting from the administration of FAA is an altered control of growth in the liver. Two components of growth in normal slices that have been observed in this study are the pattern of incorporation of ^{3}H-dThd into DNA and the critical period seen at about 16 hours post-hepatectomy. An alteration in either of these aspects of DNA synthesis in livers of rats exposed to FAA might indicate that that aspect of DNA synthesis is important in the control of cell division and would suggest that a change in

that factor is important in the mechanism of chemical carcinogenesis due to FAA. Although the study of FAA effects on liver slice DNA synthesis is not complete, the results depicted in Figures 11 and 12 suggest that the change in certain liver properties at around 16 hours post-hepatectomy that allows a wave of DNA synthesis to occur *in vitro* still exists and occurs at the same time as in normal slices, or that there is a decreased response to partial hepatectomy in FAA-treated rats.[49] The pattern of incorporation of ^{3}H-dThd into DNA indicates that an asynchronous wave of DNA synthesis occurs in Figure 11. Of course, it is always possible that the use of ^{3}H-dThd as a precursor for DNA in FAA treated liver does not reflect the real rate of DNA synthesis. It may also be possible that the alteration of the pattern of DNA synthesis in FAA treated liver slices is not due to the carcinogenic actions of FAA but rather to the hepatotoxic effects of this chemical.[50] If so, then hepatotoxic chemicals such as chloroform[51] should be able to reproduce this pattern. One piece of evidence suggesting that the altered pattern of ^{3}H-dThd incorporation into DNA in liver slices may be a significant change is that female rats treated with FAA and partially hepatectomized show a similar pattern of incorporation of ^{3}H-dThd into DNA *in vivo* to that seen in Figure 11.[49] Caution in interpreting these results is needed because female rats are less sensitive to the hepatocarcinogenic effects of FAA than the male rats used here to make the liver slices.[52]

4.6 Significance: It seems possible that the liver slice incubation system will be an important tool in the study of the control of cell division. It can be used as a bioassay in the search for factors active in various phases of liver regeneration. Because the control of liver growth is altered by the administration of hepatic carcinogens, it is reasonable that alterations in the control of cell division induced by these chemicals may be detected in this system before the gross appearance of cancer. Should this be the case, then the liver slice incubation system would have potential as a screen for hepatic carcinogens and as a tool to aid in the understanding of the mechanism(s) of action of hepatic carcinogens.

SUMMARY

Liver DNA synthesis in the right lateral lobe, as estimated by the incorporation of ^{3}H-dThd into DNA, began at some time between 12 and 22 hours after 70% partial hepatectomy and peaked at about 26 hours. When regenerating slices were made from this lobe and incubated *in vitro* beginning at 16 hours post-hepatectomy, there was a wave of DNA synthesis which peaked 10 hours after the start of the incubation; i.e., at 26 hours after partial hepatectomy. Autoradiography of slices in one experiment at this time indicated a peak of 2.65% labeled nuclei and thus, an estimated 4.3% of hepatocytes, a fairly good reflection of the percentage of labeled hepatocytes that participate in DNA synthesis in regenerating liver *in vivo*. These observations suggest that liver slices prepared from the right lateral lobe of rats sacrificed at 16 hours post-hepatectomy can progress through the S phase of the cell cycle *in vitro* independent of systemic influences and in a manner similar to that of the whole liver lobe *in vivo*.

Slices made at 14 hours post-hepatectomy failed to progress through a wave of DNA synthesis. This indicates that *in vivo* there is a critical period at around 16 hours after partial hepatectomy when certain properties of the liver change so that slices made after this time can progress through a wave of DNA synthesis *in vitro*. It is possible that this critical period, corresponding to a time near the

beginning of DNA synthesis *in vivo*, may represent a control point in the regulation of cell division in the liver.

Although estimates of DNA synthesis obtained with the use of ^{14}C-formate and ^{3}H-thymidine were similar *in vivo*, this was not true *in vitro*. In contrast to a pattern of incorporation of ^{3}H-dThd into slice DNA which was related to the time of sacrifice of a rat, ^{14}C-formate incorporation into DNA was similar in slices made from rats sacrificed at any time tested and appeared to be dependent only on the length of the incubation. This may be because the capacity of formate to be incorporated into the pool of proximate DNA precursors is a limiting factor in the incorporation of ^{14}C-formate into DNA of regenerating liver slices, rather than DNA polymerase activity.

To begin an examination of the effects of blood-borne factors on liver DNA synthesis, the effects of various serums on regenerating liver slice DNA synthesis were evaluated. Calf serum, fetal calf serum, and serum from rats subjected to partial hepatectomy 18 hours earlier did not appear to alter DNA synthesis in regenerating liver slices at any time tested. In contrast, dialyzed calf serum decreased slice DNA synthesis. One possibility suggested to explain these observations was that various opposing factors were present in whole serum and that dialyzed serum contained predominantly inhibitory ones.

Aspects of regenerating liver slice DNA synthesis important in the control of normal liver cell division were

altered by prior exposure of the liver to FAA *in vivo*. Rats were fed a diet containing 0.05% FAA for 14 days followed by a control diet for 7 days or 30 days. In one experiment, DNA synthesis in regenerating liver slices made from rats treated in this manner and incubated beginning 16 hours post-hepatectomy was somewhat lower than normal and continually increased. This suggests that an asychronous DNA synthesis occurred in those slices. In another experiment, the rate of DNA synthesis appeared to be very low and similar to that seen in normal liver slices incubated beginning at 14 hours post-hepatectomy. This suggests that the change in certain liver properties at around 16 hours post-hepatectomy that allows a wave of DNA synthesis to occur *in vitro* still exists in livers treated with FAA or that these livers have a decreased response to partial hepatectomy.

The liver slice incubation system described here may become an important tool in the study of the control of cell division. As described above, exciting new information has been learned about the control of cell division in regenerating liver.

LIST OF REFERENCES

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1. Higgins, G. M. and Anderson, R. M.: Experimental pathology of the liver. Archives of Pathology 12:186-202, 1931.

2. Bucher, N. L. R. and Malt, R. A.: Regeneration of Liver and Kidney. Little, Brown, and Co., Boston, 1971.

3. Moolten, F. L. and Bucher, N. L. R.: Regeneration of rat liver - Transfer of humoral agent by cross circulation. Science 158:272-274, 1967.

4. Leong, G. F., Grisham, J. W., et al.: Effect of partial hepatectomy on DNA synthesis and mitosis in heterotopic partial autographs of rat liver. Canc. Res. 24:1496-1501, 1964.

5. Glinos, A. D.: Control of Cellular Growth in Adult Organisms. Teir, H. and Rytomaa, T., eds. Academic Press, New York, 1967, pp. 41-53.

6. Virolainen, M.: Mitotic response in liver autograft after partial hepatectomy. Exp. Cell Res. 33:588-591, 1964.

7. Grisham, J. W.: Morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating liver: Autoradiography with thymidine-^{3}H. Canc. Res. 22:842-849, 1962.

8. Badran, A. F., et al.: Assay of plasma from intact and hepatectomized mice on mouse regenerating liver DNA synthesis. Virchows Arch. Abt. B. Zellpath 10: 176-178, 1972.

9. Echave Llanos, J. M., et al.: Growth hormone release after hepatectomy. Experientia (Basal) 25:574-581, 1971b.

10. Badran, A. F., Echave Llanos, J. M., and Surur, J. M.: The pars distalis of the pituitary as a source of liver growth stimulating factors during liver regeneration. Virchows Arch. 13:79-85, 1973.

11. Morley, C. D. G. and Kingdon, H. S.: The regulation of cell growth. I. Identification and partial characterization of a DNA synthesis stimulating factor from the serum of partially hepatectomized rats. Biochim. Biophys. Acta. 308:260-275, 1973.

12. Weinbren, K.: Regeneration of liver. Gastroenterology 37:657-668, 1959.

13. Onda, H.: Presence of hepatocyte-specific mitotic inhibitor in normal rat plasma. Gann. 64: 139-149, 1973.

14. Grisham, J. W.: Cellular proliferation in the liver. Recent Progress in Cancer Res. 17:28-48, 1968.

15. Fisher, B.: Discussion of the paper by Levi and Zeppa. Ann. Surg. 174:369-370, 1971a.

16. Price, J. B., et al.: Glucagon as the portal factor modifying hepatic regeneration. Surgery 72:74-82, 1972.

17. Short, J., et al.: Induction of deoxyribonucleic acid synthesis in the liver of the intact animal. J. Biol. Chem. 247:1757-1766, 1972.

18. MacManus, J. P.: Increases in rat liver cyclic AMP concentrations prior to the initiation of DNA synthesis following partial hepatectomy or hormone infusion. Biochem. Biophys. Res. Comm. 49:1201-1207, 1972.

19. Rizzo, A. J., et al.: Temporal changes in DNA and RNA synthesis in the regenerating liver of hydrocortisone-treated rats. Canc. Res. 31:876-881, 1971.

20. Sakuma, K. and Terayama, H.: Effects of adrenal hormones upon DNA synthesis in regenerating rat liver and tumors. J. Biochem. 61:504-511, 1967.

21. Moolten, F. L., Oakman, N. J. and Bucher, N. L. R.: Accelerated response of hepatic DNA synthesis to partial hepatectomy in rats pre-treated with growth hormone or surgical stress. Canc. Res. 30:2353-2357, 1970.

22. Nadel, C.: Synchronization of baby rat hepatocytes: A new test for the detection of factors controling DNA synthesis in rat hepatic cells. Cell Tissue Kinetics 6: 437-446, 1973.

23. Levi, J. U. and Zeppa, R.: Source of the humoral factor that initiates hepatic regeneration. Ann. Surg. 174:364-370, 1971.

24. Compagno, J. and Grisham, J. W.: Do regenerating liver cells release a substance that shortens G_1? Fed. Proc. 32:837abs, 1973.

25. Rutzy, L. P., et al.: In vitro stimulation of rat liver cells by homologous partial hepatectomy serum. In Vitro 7:1-5, 1971.

26. Aujard, C., et al.: Inhibition of DNA synthesis of synchronized cells by liver extracts acting in G_1 phase. Exptl. Cell Res. 78:476-478, 1973.

27. Rubin, M. S., Swislocki, N. I. and Sonenberg, M.: Alteration of liver plasma membrane protein conformation by bovine growth hormone in vivo. Arch. Biochem. Biophys. 157:252-259, 1973.

28. Idem: Modification by bovine growth hormone of liver plasma membrane enzymes, phospholipids, and circular dichroism. Arch. Biochem. Biophys. 157:243-251, 1973.

29. Morioka, K., et al.: Serum factors stimulating DNA synthesis in the isolated nuclear system from rat liver. Biochem. Biophys. Res. Comm. 51:451-460, 1973.

30. Cook, R. T. and Aikawa, M.: The effects of heparin on endogenous DNA polymerase activity of rat liver nuclei and chromatin fractions. Exptl. Cell Res. 78:257-270, 1973.

31. Rothenberg, R. J. and Goodman, J. I.: DNA synthesis in regenerating rat liver in vivo and in slices in vitro. The Pharmacologist. 15:204, 1973.

32. Hecht, L. I. and Potter, V. R.: Nucleic acid metabolism in regenerating rat liver. V. Comparison of results in vivo and in tissue slices. Canc. Res. 18:186-192, 1958.

33. Verly, W. G., et al.: The hepatic chalone. I. Assay method for the hormone and purification of the rabbit liver chalone. Can. J. Biochem. 49:1376-1383, 1971.

34. Bardos, T. J., et al.: A systematic investigation of the presence of growth-inhibitory substances in animal tissues. Canc. Res. 28:1620-1630, 1968.

35. Lenfant, M., et al.: Thymidine as one of the factors in a beef liver extract decreasing ^{3}H-thymidine incorporation into DNA. Can. J. Biochem. 51:654-665, 1973.

36. Miller, J. A.: Review of chemical carcinogenesis. Canc. Res. 30:559-576, 1970.

37. Price, C. C., et al.: Mechanism of action of alkylating agents. Ann. N. Y. Acad. Sci. 163:593-598, 1969.

38. Miller, E. C., Miller, J. A. and Enomoto, M.: The comparative carcinogenicities of 2-acetylaminofluorene and its N-hydroxy metabolite in mice, hamsters, and guinea pigs. Canc. Res. 24:2018-2032, 1964.

39. Epstein, S., et al.: Cellular analysis of liver carcinogenesis: The induction of large hyperplastic nodules in the liver with 2-fluorenylacetamide or ethionine and some aspects of their morphology and glycogen metabolism. Canc. Res. 27:1702-1711, 1967.

40. Sneider, T. W. and Potter, V. R.: Methylation of mammalian DNA. Studies on Novikoff hepatoma cells in tissue culture. J. Mol. Biol. 42:271-284, 1969.

41. Steel, W. J., Okamura, N. and Busch, H.: Prevention of loss of RNA, DNA, and protein into lipid solvents. Biochim. Biophys. Acta 87:490-492, 1964.

42. Goodman, J. I. and Potter, V. R.: Evidence for DNA repair synthesis and turnover in rat liver following injection of 3'methyl-4-dimethyl-aminoazobenzene. Canc. Res. 32:766-775, 1972.

43. Blobel, G. and Potter, V. R.: Distribution of radioactivity between the acid soluble pool and the pools of RNA in the nuclear, nonsedimentable and ribosome fractions of rat liver after a single injection of labeled orotic acid. Biochim. Biophys. Acta 166:48-57, 1968.

44. Farber, E.: Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylaminofluorene, and 3'methyl-4-dimethylaminoazobenzene. Canc. Res. 16:142-148, 1956.

45. Hopkins, H. A., Campbell, H. A., et al.: Thymidine kinase and deoxyribonucleic acid metabolism in growing and regenerating livers from rats on controlled feeding schedules. Biochem. J. 136:955-966, 1973.

46. Fabrikant, J. I.: The kinetics of cellular proliferation in regenerating liver. J. Cell. Biol. 36:551-565, 1968.

47. Mitchison, J. M.: The Biology of the Cell Cycle. Cambridge at the University Press, New York, 1971.

48. Temin, H. M.: Stimulation by serum of multiplication of stationary chicken cells. J. Cell. Phys. 78:161-170, 1971.

49. Jackson, C. D. and Irving, C. C.: Effects of 2-acetylaminofluorene on liver cell proliferation after partial hepatectomy of female rats. Canc. Res. 33:397-401, 1973.

50. Jackson, C. D. and Irving, C. C.: Sex differences in cell proliferation and N-hydroxy-2-acetylaminofluorene sulfotransferase levels in rat liver during 2-acetylaminofluorene administration. Canc. Res. 30:577-595, 1970.

51. Price, H. L. and Drips, R. D.: General anesthetics. In, The Pharmacological Basis of Therapeutics, 4th edition. Goodman, L. S. and Gilman, A. G., eds. The MacMillan Co., New York, 1970.

52. Sidransky, H., Wagner, B. P. and Morris, H. P.: Sex differences in liver tumorigenesis in rats ingesting N-2-fluorenylacetamide. J. Nat. Canc. Inst. 26:151-187, 1961.