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RNA SYNTHESIS DURING THE EARLY PERIOD OF RAT LIVER REGENERATION

by

DENNIS M. BAKEWICZ

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1977

Dennis Bakewicz

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

June 6, 1977 Date	Carolyn J. Burdick Chairman of Examining Committee Prof. C. Burdick
June 7, 1977 Date	Louis G. Moriber Executive Officer Prof. L. G. Moriber
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The City University of New York

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Abstract

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Adviser: Professor C. J. Burdick

Liver nuclei were isolated hourly, during the first four hours after partial hepatectomy, from regenerating liver remnants and from the corresponding 0-hour, resected liver segments. After four hours of regeneration had elapsed, polymerase activities I and II as well as total nuclear RNA polymerase activity were shown to increase by approximately four-fold in the regenerating liver remnants when compared to their respective control segments. Increases in polymerase II activity were observed to precede those for polymerase I as well as total nuclear polymerase activity in general.

Template activity of chromatin isolated from nuclei of 2 and 4 hour regenerating liver remnants exhibited respective 50% and 90% increases over control levels when transcription was performed with E. coli RNA polymerase. Chromatin reconstitution experiments indicated that the histone component of the chromatin

was responsible for the two-hour increase in template activity while the acidic chromosomal proteins (and possibly histone modification) was responsible for the four-hour peak. Polyacrylamide gel electrophoresis revealed what appeared to be extensive F1 phosphorylation, one hour after partial hepatectomy, which preceded a decline in F1 histone amount at two hours post partial hepatectomy. Analysis of the thermal melting profiles of native chromatin and of reconstituted nucleohistone complexes confirmed the presence of approximately 3.5% more DNA melting in the free DNA region at 2 hours post partial hepatectomy concomitant with the decrease in F1 histone amount and the two-hour peak in chromatin template activity.

CD spectral analysis of native chromatin revealed that, at 4 hours post partial hepatectomy, a decrease in histone α-helical content was shown to occur at the time of the second peak in chromatin template activity. Also observed at this time was an apparent decrease in multi-acetylated F2a1 species and an increase in the mono-acetylated form of this histone.

In summary, an increase in the amount of free DNA (or non-histone bound DNA) is believed to be responsible for the two-hour peak in template activity while changes in the conformation of chromosomal proteins are believed to cause the four-hour peak.

ACKNOWLEDGMENTS

Firstly, I would like to express my appreciation to my parents whose understanding and support were instrumental to the completion of this thesis. Secondly, I wish to express my thanks to Mr. and Mrs. Vincent Bakewicz for help in the preparation of the manuscript. Thirdly, thanks are due to my doctoral committee, Drs. C. J. Burdick, M. Himes, H. J. Li and J. Nishiura, for their unfailing help, advice and moral support. Lastly, I wish to express my thanks to the people of the City of New York from whose University I have received the totality of my graduate and undergraduate education.

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CHAPTER I

INTRODUCTION

General Introduction

The phenomenon of regeneration, no matter in what organism it is studied, tends by its very nature to raise numerous questions of paramount biological significance. Among the metazoa alone there are many examples of startling regenerative feats, some of the most amazing being manifested by the lowest forms. The demonstration that a bisected planarian can form two new planaria from its cut halves is indicative of the fact that some adult animals retain vast regenerative potential in the form of a labile organization not unlike that of the early embryo. In other instances, this potential is more limited and only a part of an organism that has been removed may be replaced, as in the case of amphibian limb regeneration. Lastly, there is the much more commonplace phenomenon of physiological regeneration, which encompasses those normal replacements of cell types (blood cells, the cells lining the gastrointestinal tract, etc.) that must be restored on a regular basis. It is the middle category, the replacement of lost parts, more commonly termed reparative regeneration or simply regeneration which is being dealt with here.

Among the mammals, examples of reparative regeneration are usually not as awesome as those seen in the lower vertebrates. Certainly one exception is mammalian liver regeneration. The ability of the mammalian liver to regenerate even exceeds that demonstrated by the livers of such lower vertebrates as urodeles. This regenerative capability was probably known to mankind even in very ancient times. In Greek legend, the myth of Prometheus as told by Hesiod and Aeschylus describes the unusual punishment to which this demigod was subjected. For his numerous affronts, it was decreed that Prometheus should suffer for three myriads (30,000 years). He was bound to a great rock and was afflicted by the frequent visitations of an eagle which tore his liver. The regular restoration of this organ insured that the punishment would be virtually unending (Rose, 1959).

Mammalian liver regeneration differs to a certain extent from other types of regenerative phenomena. After the performance of a partial hepatectomy, cell proliferation in the liver proceeds at a rate unmatched by any other mammalian organ and even exceeds that of most neoplasms. This is in spite of the fact that normal adult mammalian liver cells are long-lived and consequently only one mitosis is observable in approximately 20,000 cells (Bucher & Malt, 1971). Partial hepatectomy as described by Higgins and Anderson (1931) for rats refers to the removal of the left and medial lobes of the liver,

comprising approximately 68% of the liver's mass. Unlike amphibian limb regeneration, which proceeds from a blastema at the stump, in the liver, regeneration proceeds by enlargement of the residual right and caudal lobes as a result of repeated cellular divisions. In their original work, Higgins and Anderson choose to term the process resulting from partial hepatectomy "restoration" instead of regeneration. In any case, the end result of this process is the exact restoration of the amount of the liver removed.

The significance of using a system such as this is its obvious advantage in studying both the control of and the sequence of events in cellular division, which in this case can be sharply initiated by the operative procedure to convert an otherwise quiescent group of cells to an actively proliferating one. It is the aim of this thesis to investigate that sequence of events relating to various aspects of RNA synthesis during the first six critical hours of hepatic regeneration. First, however, it is necessary to describe the nature of the hepatic regenerative response with the aim of enumerating those factors which are of special importance here.

The Mammalian Liver

Although the liver is not a highly structured organ internally (like the kidney), its four lobes are subdivided

into polygonal lobules. The lobules are surrounded by connective tissue containing branches of the hepatic artery and the portal vein which bring blood to the lobular parenchymal cells. Trabaculae formed by these cells are separated by sinusoidal capillary beds from which blood drains into a central vein. This vascular arrangement is of some importance to the way in which cell proliferation within the lobule proceeds. Labeled nuclei and later mitotic figures can always be seen to spread synchronously in the direction from the periportal area to the central vein and never in the opposite direction (Grisham, 1962; Edwards and Koch, 1964).

Most of the past work on mammalian liver regeneration has been done with rats. With these animals are associated a decided number of advantages which are discussed in detail in the Materials and Methods Section of this thesis. The liver itself, as an organ with which to study cellular division, also offers a number of advantages for workers wishing to conduct studies on cells that have undergone or are preparing to undergo cellular proliferation in vivo.

The cellular composition of the liver, although not homogeneous, does have the distinction of being predominated by mainly one cell type -- the parenchymal cell. This cell type comprises approximately 90% of the liver by volume and about 60% of the total liver cell population (Bucher, 1963; Steiner et al., 1966). The other cell types present in the liver are the ductal

cells forming the bile ducts and the cholangioles and the littoral cells, some of which, like the Kupffer cells, exhibit phagocytic activity. Although these nonparenchymal cells do increase in number after partial hepatectomy by the same factor (3.37) as do the parenchymal cells, it is quite fortunate in terms of cellular proliferation studies, that the ductal and littoral cells lag behind the parenchymal cells by almost a full day in achieving a peak of mitotic activity (Grisham, 1962; Edwards and Koch, 1964). This fact insures that biochemical studies in the early stages of regeneration are being performed on a single class of proliferating cells. In addition, during the first day following partial hepatectomy, the greatest degree of parenchymal cell synchrony is maintained (Bucher and Malt, 1971). It appears, moreover, that increases in the number of other littoral cell types are not brought about by cell division in response to partial hepatectomy, but merely by cellular migration (Edwards and Koch, 1964). Lastly, studies further indicate that the ductal cells of the liver do not function as any kind of stem cell population from which the parenchymal cells arise (Edwards and Koch, 1964). In summary, it appears that studies of parenchymal cell proliferation are aided by the facts that (1) they comprise the overwhelming mass of the liver, (2) their cell cycle is distinct from and begins ahead of those of other cell types, and (3) within the first 24 hours post partial

hepatectomy they exhibit a good amount of natural synchrony.

The Parenchymal Cell Cycle

Studies of the liver parenchymal cell cycle have been done on both normally developing livers and on livers that have been stimulated to regenerate.

The time required for liver cells to pass through the different phases of the cell cycle in normally growing cells tends to vary depending upon the age of the animals. At three weeks of age and older, the cell cycle tends to stabilize in duration at 21.5 hours; G_1 and S both occupy 9.0 hours each with G_2 and M requiring approximately 2 hours and 1.5 hours, respectively (Grisham, 1973).

Also dependent upon age is the growth fraction, the proportion of hepatocytes that are actively involved in the replicative cycle. This fraction declines from a high of 36% on the first day after birth to 6% in young adult rats 6-8 weeks old (Fabricant, 1969; Post and Hoffman, 1964). Apparently, once formed, these adult hepatocytes are long-lived cells, and, barring no undue injury, have a "life span" of approximately 500 days (MacDonald, 1961).

After partial hepatectomy is performed, the growth fraction tends to increase dramatically to over 250-300 times that in sham-operated or nonhepatectomized adult rats. In spite of this remarkable change, the overall duration of the first cell cycle after partial hepatectomy

does not change with age and does not differ appreciably from that of normally growing three-week old rats (Grisham, 1973). The time, however, after partial hepatectomy at which the peak of DNA synthesis is reached, does tend to differ slightly with age; this occurs at 20 hours for weanlings, at 23 hours for young adults and at 28 hours in old adults (Bucher, et al., 1964). Succeeding cell cycles after the initial one following partial hepatectomy tend to decrease in duration to 16.5 hours with a G_1 of 5 hours, an S phase of 8 hours and a G_2+M of 3.5 hours (Fabricant, 1968). This shorter cell cycle is due essentially to a shorter G_1. In cells such as mature liver cells which are arrested in a long G_1 phase, sometimes referred to as G_0, it is believed that those functions associated with cellular division have deteriorated (Prescott, 1969). It has been reasoned that before such an arrested cell can successfully divide, it must first spend some time to resynthesize those materials necessary for cell replication; this has been offered as an explanation to account for the longer initial G_1 encountered after partial hepatectomy is performed. Those required synthetic events preceding the onset of DNA replication will be further described below and serve as the primary basis of investigation in this thesis.

Factors Affecting Regeneration

Although hepatic regeneration is in general a phenomenon that is superimposed upon the existing conditions present

within the animal, a voluminous literature has been amassed which indicates that many factors can affect this process. These factors cannot adequately be reviewed here but include: the hormonal state of the animal (as reviewed by Grisham, 1973); diet (Doljanski et al., 1966; Doyle et al., 1968; Vilchez et al., 1968; Peters, 1962); diurnal considerations (Linge and Mathyl, 1969; Günther et al., 1968; Glasser and Spelsberg, 1972; Barbiroli et al., 1973; Letnansky and Reisinger, 1972) and stress responses (Ove et al., 1966; Fujioka et al., 1963; Bucher et al., 1969; Chandler et al., 1968; Lieberman, 1969; Ove et al., 1967; Majumdar et al., 1967; Tsukada et al., 1968; Schrock et al., 1970; Davis and Hyde, 1966; Feller et al., 1967; Talarico et al., 1969; Simek et al., 1968; Moolten et al., 1970).

In addition to the factors mentioned above, which can affect the regenerative response in terms of both its magnitude and timing, of paramount importance is the relationship between the percentage of liver removed and the age of the rat. This relationship is of critical significance in determining the magnitude of the response (growth fraction), timing being essentially unaffected. Differences in age affect the critical mass of liver that must be removed in order to illicit a maximal response. In both weanling and old rats, only a 10% partial hepatectomy is required in order to stimulate a maximal

response; in young adults, however, a 40% partial hepatectomy is the minimal magnitude of resection that will constitute a stimulus toward regeneration. Work performed by Bucher et al., (1964) demonstrates the effect of the size of the resected mass upon the magnitude of the response in young adult rats. A 43% partial hepatectomy will cause approximately 6 times more tritiated thymidine to be incorporated into liver cell DNA than will a 9% partial hepatectomy; a 68% partial hepatectomy is shown to be approximately 14 times more effective.

Just as there appears to be a minimum percentage of partial hepatectomy needed to induce regeneration, so too, there is an upper limit to the amount of liver that can be removed. When partial hepatectomies exceeding 82% are performed, DNA synthesis and mitosis are delayed by 10 to 15 hours. In addition, a greater frequency of abnormal mitoses are observed (Weinbren and Woodward, 1964; Weinbren and Taghezadeh, 1965).

The Initiation of the Regenerative Response

Those changes, immediately following the excision of a piece of liver which are of possible critical significance to the triggering of the regenerative response, have been the subjects of much and varied careful experimental scrutinization. Although it is unfortunate that as yet a clear-cut description of the triggering mechanism cannot be given, it seems, in the light of recent work, that

numerous factors can be implicated.

An attempt will be made to describe the most important factors that have received attention as being possible triggers of the regenerative response.

1. Haemodynamic Factors.

A change that occurs immediately after partial hepatectomy is an increase in the portal blood pressure (Heikkinen and Larmi, 1968; Menghart and Simon, 1966). The importance of this as a triggering mechanism, however, is doubtful. The liver can in fact regenerate in the absence of portal blood flow (Bucher, 1963). Conversely, in normal livers, when blood flow is increased by the use of fistulas or by portal arteriolization, no increase in mitotic activity is observed (Rabinovici and Wiener, 1963; Fisher et al., 1962; Alston and Thomson, 1963; Becker, 1963).

2. Functional Demand.

The decline in liver functional capacity resulting from partial hepatectomy has been suggested as another possible triggering mechanism (Goss, 1964) but no conclusive experimental evidence for this has to date emerged. Studies in which the liver was subjected to biliary obstruction or to overloading with circulatory exretory products have not resulted in the onset of parenchymal cell proliferation (although biliary obstruction does result in the growth of ductular cells) (Bucher, 1963). The continuous infusion of protein hydrolysate stimulates increased protein and RNA synthesis and an increased mitotic rate in liver cells

from normal animals, but these events are not of the magnitude of those occurring in regenerating livers (Alston and Thomson, 1966). Indeed, it is conceivable that the existence of such a wide range of liver functions and combinations thereof might make the conclusive demonstration of a triggering mechanism related to functional demand impossible.

3. Humoral Factors.

In the ongoing search for factors which can trigger hepatic regeneration, humoral substances have received an overwhelming amount of attention. Experiments giving some indication of the role of humoral factors have involved parabiosis between normal and partially hepatectomized animals. The majority of these experiments failed to show increased mitotic activity in the normal partner, although some did (Bucher, 1963; Virolainen, 1967; Goss, 1964; Leduc, 1964; Fisher et al., 1963; Kim and Cohen, 1965; Pechet et al., 1963; Heimann et al., 1963; Virolainen, 1967; Wrba et al., 1967; Wrba and Rabes, 1967).

Also yielding equivocal results were experiments in which injections of plasma or crude liver extracts from partially hepatectomized rats were given to normal rats with the expectation of increasing mitotic activity. The opposite experimental design which sought to depress mitotic activity in regenerating rat livers produced equally enigmatic results (Bucher, 1963; Goss, 1964; Leduc, 1964).

Other experiments have been performed in which normal animals were given repeated blood transfusions from

partially hepatectomized rats. These transfusions resulted in a stimulation of hepatocyte proliferation. The opposite situation, however, in which partially hepatectomized rats received repeated transfusions from normal animals, only resulted in a delay in the start of regeneration (Grisham, 1969).

A technique which brings about a rapid equilibration of the blood between a partially hepatectomized and a normal partner is one involving a carotid-to-jugular cross circulation via cannulas. When carried out for at least 10 hours, the normal partner is stimulated to regenerate. This stimulation is more than doubled if the operated partner is subjected to an 85% partial hepatectomy. Of enigmatic significance is the fact that although the entire blood supplies are completely exchanged every three minutes, prolonged transfer times are still required, and, even then, DNA synthesis is still more pronounced in the operated partner (Alston and Thomson, 1963; Moolton and Bucher, 1967; Bucher et al.,(b) 1969; Bucher et al.,(c) 1969). Such observations have been interpreted as being indicative of either the presence of a cell division activator substance of rather short half-life (which equilibrates with difficulty between both animals) or of a long-lived inhibitor substance (which equilibrates well but takes time to degenerate).

Experiments employing blood exchange between the

abdominal aortae of paired rats have the advantage of an even quicker rate of blood transfer. In these experiments, the operated partner undergoes regeneration almost as if it were unpaired, while the normal partner also experiences a great stimulation in mitotic activity (Lieberman, 1969).

Another line of approach has been with experiments involving the dilution or concentration of substances in the blood. These studies in which the blood of an intact animal was diluted by plasma-phoresis have demonstrated the production of a single wave of mitotic activity in the liver (Glinos, 1967; Sudweeks and Hill, 1967). In the opposite situation when the blood plasma concentration was increased by fluid restriction, the partially hepatectomized animals were observed to have a decreased regenerative capability (Glinos, 1967). In the above cases, the changing concentration of albumin in the plasma was reported not to influence the results of these procedures. These experiments as well as the others mentioned in this section do emphasize the possible importance of both the presence of and the relative concentrations of blood borne humeral substances; unfortunately they cannot differentiate between the possibility of the presence of humoral activators or inhibitors.

Similar experiments to the above have been performed in culture. Liver cells cultured <u>in vitro</u> are stimulated to divide with serum from partially hepatectomized rats (Bucher, 1963). It is of interest that serum from normal

animals can also cause a similar response if greatly diluted (Bucher, 1963). A fact of additional significance which came from this type of study was that sera from partially hepatectomized animals of different species could still produce this response, whereas sera from animals with some other organ resection could not (Wrba and Rabes, 1967; Matusushima et al., 1967; Hays et al., 1969). The humoral factors involved, although species nonspecific, do appear to be organ specific. These characteristics were further confirmed by experiments with "confrontation cultures" in which regenerating and non-regenerating cultures were maintained in vitro on opposite sides of a membrane; stimulations in mitotic activity have been reported in the non-hepatectomized liver. Whatever humoral factors are involved, they appear to be of rather low molecular weight, since they apparently have the ability to pass through membranes of small pore size, and are thermostable (Wrba and Rabes, 1967).

Perhaps the most convincing evidence in support of a humoral influence upon regeneration, is derived from experiments with hepatic autografts. They demonstrate that when small pieces of liver are autotransplanted, the pieces are capable of undergoing the same rate and extent of regeneration as the intact regenerating remnant (Leong et al., 1964; Sigel et al., 1963(a); Sigel et al., 1963(b); Virolainen, 1964). In addition, after the grafts are

established with arterial blood entering through the portal vein and exiting out of the hepatic vein, the wave of mitoses occurs as normal, in the direction from the periportal area to the central vein within the lobule. When the blood flow is reversed, the mitotic wave is also reversed and travels from the central vein to the periportal area as if directed by a substance carried by the blood (Sigel et al., 1968).

4. Chalones.

Chalones are mitotic (or S phase) inhibitors that operate in a negative feedback manner (Bullough, 1973). When cells are injured or destroyed, the internal chalone concentration drops. The cells are then able to divide and the chalone concentration increases until cell division is again turned off. Although this explanation by Bullough is the simplest, it need not be, as cautioned by Bullough, the only appropriate explanation to account for the onset of cellular division.

The experiments described above thus far do allude to the existence of a humoral factor characterized by tissue specificity, species nonspecificity and low molecular weight. Chalones (Bullough, 1973) meet all of these specifications and have been isolated from liver extracts by Verly (1973). Verly demonstrated that when slices of 24 hr. regenerating liver remnant were incubated in vitro in flasks containing chalone extracts or in control

flasks which didn't contain these extracts, inhibition of DNA synthesis was shown to be greater than 90% in the chalone containing cultures. Moreover, this inhibition was found to be dose-dependent and was eliminated by prior pre-treatment of the chalone preparations with pronase (but not with RNase). Results such as these make it necessary to examine the mechanism of chalone action in reference to liver regeneration.

It is obvious that a negative feedback system must work differently for local damage, as in the case of the skin, than for large scale tissue replacement, as in the case of liver regeneration. In the former example, a local cut merely causes membrane damaged cells to lose chalones in addition to the fact that the decreased cell population in the area of the cut causes an overall decrease in the average chalone concentration. In the case of liver regeneration, the lobes that are resected (and have consequently lost chalones) are not the ones regenerated. The regenerative response tends to spread throughout the remaining cell mass which consists of undamaged cells. It has been hypothesized (Bullough, 1973) that critical to this process is the fact that cells in the liver synthesize chalones which are constantly lost to the outside where chalone concentration increases. Chalones can then move back into the cells of the liver. It can be seen that the intracellular chalone concentration is thus determined by the rate of chalone synthesis, plus

the rate of uptake from the outside, minus the rate of internal destruction and rate of loss to the outside. Once a partial hepatectomy is performed, the chalone synthetic capacity of the liver is diminished, the external chalone concentration drops and the overall gradient tends to favor the movement of liver chalones to the outside. Cell division then ensues until enough cells are replaced to restore the chalone synthetic capacity and to consequently reverse the gradient such that the intracellular chalone concentration is high enough once again to inhibit mitotic activity. The above explanation has been suggested by Bullough (1973) as a means of explaining the role of chalones in hepatic regeneration.

The lowering of an intracellular chalone concentration stimulates the movement of Ca^{++} into cells (Houck, 1973). This higher intracellular Ca^{++} concentration then leads to an increase in 1, 2-phosphodiesterase activity, which is in essence a cyclic AMPase activity. The net result is a decrease in cAMP; this condition is absolutely necessary for cellular division to occur.

5. Cyclic AMP

It appears that the intracellular level of this cyclic nucleotide (and possibly of others) is of paramount importance in the control of cellular division (Abell and Monahan, 1973). In a manner analogous to that of chalones, intracellular cAMP levels are determined by the interplay

of a number of factors: adenyl cyclase activity, phosphodiesterase activity and extracellular cAMP levels. Experimental procedures resulting in higher intracellular cAMP levels, whether brought about by increased adenyl cyclase activity, reduced phosphodiesterase activity, by the addition of exogenous cAMP or by combinations of these three, have all indicated that such increased levels of cAMP are sufficient to inhibit cellular division. This has been shown for PHA stimulated normal lymphocytes, for lymphocytes from patients with chronic lymphatic leukemia (Abell et al., 1970; Johnson and Abell, 1970), for HeLa and L cells (Ryan and Hendrick, 1968) and for fibroblasts (Bürk, 1968; Johnson et al., 1971; Sheppard, 1971).

In addition to the observations resulting from the above experimental manipulations of cAMP levels, correlations have been found to exist between intracellular cAMP levels and the rates of DNA synthesis in a number of cell lines. The generalized situation is indicative of the fact that an inverse relationship exists between the rate of DNA synthesis and intracellular cAMP levels. This was demonstrated by Otten et al. (1971) for 13 different cell lines.

Of particular interest is the fact that intracellular cAMP concentrations tend to vary during the course of the cell cycle. Sheppard and Prescott (1972), using synchronized Chinese hamster ovary cells, found that cAMP levels

increased slightly in early G_1 and then increased even more in late G_1. The intracellular cAMP concentration reached its peak during S phase and later fell to its lowest level during mitosis. These studies are indicative of the fact that fluctuations in cAMP concentration throughout the cycle may be necessary in order to exert very specific control over the entire process of cellular division.

The above observations were confirmed experimentally by the work of MacManus and Whitfield (1970). They demonstrated *in vitro* that rat thymus cultures of lymphocytes, when exposed to 10^{-8}M to 10^{-6}M concentrations of cAMP, were stimulated to undergo DNA synthesis. Higher concentrations were, however, decidedly inhibitory. These results were confirmed *in vivo* by Rixon *et al.* (1970) who injected cAMP intraperitonially into colchemid-treated rats. Low concentrations stimulated bone marrow mitotic activity while high cAMP concentrations proved to be inhibitory.

With correlations between cAMP levels and mitotic activity abundantly in evidence, the nature of the effect of cAMP on early G_1 events is of particular significance. Work by Cross and Ord (1971) and by Averner *et al.* (1972) with cultured lymphocytes has clearly indicated that cAMP stimulates RNA synthesis in much the same manner as does PHA stimulation of lymphocytes. The work of Cross and Ord (1971) has also indicated that histone kinase activity

is increased by cAMP treatment. Studies such as these point out the possibility that the ultimate effect of cAMP may be exerted through the action of kinases at the levels of transcription (Pastan and Perlmann, 1972) and/or translation (Barnett and Wicks, 1971; Wicks, 1969; Walter et al., 1971).

Many of the trends manifested by cAMP during cell division in the numerous cell types that have already been discussed would appear to be verified in the case of regenerating liver cells. MacManus et al.(1972) reported the existence of a biphasic increase in cAMP during hepatic regeneration similar to the pattern reported by Sheppard and Prescott (1972) for Chinese hamster ovary cells. After a 1-1/2 hr. lag, cAMP levels began to rise and reached a peak at 3 hrs. post partial hepatectomy. After returning to normal, a second peak was observed at 12 hrs. Moreover, the same biphasic patterns could be observed when cell division in liver cells was induced by means other than partial hepatectomy.

In addition to the changes in cAMP levels in liver cells observed by MacManus et al. (1972) during hepatic regeneration, Martello and Hirsh (1974) have demonstrated the existence in normal liver cells of a cAMP dependent kinase. This kinase was shown to be capable of phosphorylating RNA Polymerases I and II. What is of even greater importance, however, is the fact that the phosphorylated polymerase I demonstrated a threefold

increase in activity and polymerase II a 50% increase in activity. The effect of cAMP on transcription in the regenerating liver system thus appears to resemble quite well the effect of cAMP upon transcription already mentioned for the case of cultured lymphocytes.

6. Hormones.

The work, already described, dealing with chalones, Ca++ and cAMP, has the basis for its effect upon hepatic regeneration in a complex negative feedback system. However, as has been pointed out, certain inconsistencies and incongruities exist in any explanation of liver regeneration based solely upon such a system. To begin with, the results of confrontation culture experiments suggest the presence of stimulations by activator substances. Secondly, cell replication can be stimulated in normal livers by plasmaphoresis but not in ectopic autografts, suggesting that a factor from the portal blood is necessary. Finally, it is difficult to explain the fact that in efficient cross-circulation experiments between normal and massively hepatectomized partners, the deficient partner undergoes almost normal regeneration but the unoperated partner can never seem to attain quite the same degree of response. In short some factor(s) is still unaccounted for.

The critical importance of both insulin and glucagon for hepatic regeneration has been most effectively demonstrated by Bucher and Swaffield (1975). They

performed partial hepatectomies upon rats which were also subjected to evisceration entailing resection of the gastrointestinal tract, pancreas and spleen. These animals which were deprived of portal blood were maintained by continuous intravenous infusions of electrolytes, amino acids and glucose. After 24 hrs., these animals were observed to respond to partial hepatectomy by incorporating less than 1/5 the tritiated thymidine that was incorporated into the remnants of non-eviscerated partially hepatectomized animals. Bucher and Swaffield were able to show that appropriate replacement therapy by the infusion of both insulin and glucagon (but not of either hormone alone) into these eviscerated, partially hepatectomized animals was sufficient to restore the full response even after hormone infusion was begun as late as 7 hrs. after the partial hepatectomy. Observations of additional significance were that 1) the infusions of these hormones into normal rats were insufficient in and of themselves to elevate the rates of DNA synthesis, and that 2) following partial hepatectomy, non-eviscerated rats demonstrated an abrupt fall in portal vein insulin concentration which was gradually restored to normal. These findings indicate that insulin and glucagon, working together, are essential to the process of hepatic regeneration. It is clear, however, that these hormones do not in and of themselves control the process entirely.

Insulin and glucagon can affect both the production and release of cAMP by liver cells. Perfused liver systems have been used to assess the effect of these hormones on cAMP release. Kuster et al. (1973) reported that the infusion of glucagon at a concentration of 10^{-8}M resulted after 8 minutes in an 85 fold increase over basal levels in cAMP release. Insulin was found to lower the glucagon induced cAMP release, whereas insulin alone caused no significant cAMP release as compared with basal levels.

Further experiments by Mallette et al.(1973) with the perfused liver system have indicated that glucagon infusion results in a 10 fold increase in histone F_1 phosphorylation; the infusion of cAMP alone also yields similar results. These findings have been further verified by Langan (1969 a,b). These experiments thus establish a second parallel between the liver and lymphocyte systems. cAMP not only stimulates RNA polymerase activity in both systems but also histone kinase activity. It is important to point out, however, that in liver cells the cAMP dependent kinase results in the phosphorylation of a specific F_1 site whereas a cAMP dependent kinase later in the regenerative process results in a different F_1 phosphorylation event (see discussion).

7. Summary of Initiation.

In attempting to summarize this rather lengthy section on the initiation of the hepatic regenerative

response, it appears that both functional demand and portal blood pressure are of least importance. Of greater significance are the concentrations of various intracellular and blood borne substances. The effects of all of these components seem to be directed at exerting some influence upon intracellular cAMP concentrations which in turn have been implicated in stimulating various kinase activities needed for cellular control.

These effects upon intracellular cAMP concentrations occur in at least two phases. First, immediately after partial hepatectomy, the effect of glucagon in the near absence of insulin in the portal blood coincides with the 1-1/2 hr. rise in cAMP reported by MacManus (1972). Such relatively slight increases in cAMP are necessary to activate cAMP dependent kinases affecting histone F_1 and both RNA polymerases. Later in the cell cycle during M, the cAMP concentration drops to its lowest level. It has been demonstrated already in many systems that cAMP depletion is necessary in order for cells to divide. This later depletion of cAMP in liver cells may be regulated over a period of hours by changing chalone gradients as described by the negative feedback theory of Bullough (as well as by the return of insulin levels to normal). Upon partial hepatectomy, the chalone synthetic capacity of the liver is decreased; blood chalone levels will eventually drop, causing the formation of a gradient which allows chalones to leave the liver cells. The

net effect may be to increase intracellular Ca^{++} levels. Ca^{++} increases intracellular phosphodiesterase activities which leads to the decrease in cAMP necessary to permit the later stages of the cell cycle to occur. These stages were clearly demonstrated by Verly (1973) to be inhibited by the direct addition of putative liver chalones.

Certainly much has been learned about the initiation of the hepatic regenerative response. Cell division will not occur, however, unless certain synthetic events take place which provide the otherwise quiescent liver cells with those materials required for cell proliferation.

Synthesis of DNA

After the regenerative response has been triggered, a peak of DNA synthesis in the livers of young adult rats is usually attained after approximately 24 hours (Bucher et al., 1964). Liver cells, being long-lived and not being prone to undergo cellular division, exhibit the presence of exceedingly low levels of endogeneous deoxribonucleotides (Bucher, 1963; Bucher and Oakman, 1969). Moreover, the enzymatic activities of DNA polymerases and of those enzymes associated with the synthesis of DNA precursors are barely detectable. It is, however, unlikely that these enzymes control the onset and termination of DNA synthesis. This is verified by the facts that (1) DNA polymerase activity and the concentrations of DNA precursors reach high levels well

before the actual onset of DNA synthesis and that (2) after DNA synthesis has ceased, DNA polymerase activity levels, precursor concentrations and precursor synthetic enzymatic activities still remain elevated for many hours (Bucher, 1963; Bucher and Oakman, 1969; Larsson, 1969; King and Van Lancker, 1969; Gross and Rabinowitz, 1968; Fausto and Van Lancker, 1965; Labor et al., 1969; Giudice and Novelli, 1963; Ove et al., 1969). The increase in these enzymatic activities as well as the appearance of DNA synthesis initiator substances are an important part of the precise sequence of events necessary for the proper progression of events in the regenerative process.

It might first be asked how regenerating liver cells acquire the precursor materials necessary for DNA replication. It is known that between 12 and 24 hours post partial hepatectomy deoxyribonucleotide concentrations increase many fold (Bucher, 1963; Bucher and Oakman, 1969). This increase can be accounted for in three ways. The first is through the salvage pathways that retrieve breakdown products of leukocyte DNA. The retrieval mechanism functions by the breakdown of DNA in white blood cells to the nucleoside level. These nonphosphorylated substances, which can pass relatively easily across cell membranes into liver cells, are then phosphorylated to the triphosphate form (Bryant, 1962). The

second mechanism involves the conversion of ribonucleotides, which usually exist in high concentrations in liver cells and are made de novo from smaller compounds, to the appropriate deoxyribonucleotides by ribonucleotide reductase (King and Van Lancker, 1969; Larsson, 1969). The third mechanism results in an increase in the intracellular thymidine concentration. Thymidine is degraded rapidly in normal liver. However, with the onset of regeneration, this breakdown process is greatly diminished (Bucher, 1963).

In addition to the increased activities of DNA polymerase and ribonucleotide reductase, those enzymes which lead to the production of thymidine triphosphate all demonstrate increased activity as well. These include thymidine monophosphate synthetase, thymidine monophosphate kinase, thymidine kinase, deoxycytidine monophosphate deaminase and orotidylate pyrophosphorylase. Between 12 and 24 hrs. post partial hepatectomy, these enzymatic activities have been observed to increase by 7 to 15 fold over normal values (Bucher, 1963; Maley et al. 1965; Fausto and Van Lancker, 1965; Labor et al., 1969).

Of prime importance is the question of how these enzymatic activities (and others) are increased after the onset of regeneration. It cannot be denied that many of the aforementioned enzymes exhibit great instability in the absence of their substrates and that a large increase in activity can be attained by the mere infusion

of these substrates into normal rats (Bojarski and Hiatt, 1960; Hiatt and Bojarski, 1961). Nor can feedback control mechanisms be overlooked in view of the fact that deoxyribonucleotide triphosphates can inhibit many of the enzymes on the pathways leading to their own formation (Ives et al., 1963; Bresnick et al., 1964; Moore and Hurlbert, 1966; Reichard, 1968; Blakely and Vitols, 1968; Wyngaarden et al., 1969). These mechanisms, however, do not in and of themselves explain the great increases in the various enzymatic activities that follow partial hepatectomy. It is known that actinomycin D and X-irradiation do significantly block the attainment of these elevated levels of activity which reflect the importance of the synthesis of new RNA molecules in this process (see section on Template capacity).

Another factor of significance in the triggering of DNA synthesis during regeneration is the fact that concurrent protein synthesis of various as yet unspecified factors must accompany DNA replication. These are separate from DNA polymerase and the precursor producing enzymes mentioned above whose activities are elevated before and for hours immediately after DNA synthesis has occurred. That the above statement is true is illustrated by the fact that numerous protein synthetic inhibitors, given during S phase, greatly reduce DNA synthesis but have no immediate effect on polymerase or precursor producing enzyme activities (Gottleib et al., 1964; Brown et al., 1970). The above situation is highly

reminiscent of the situation in bacteria where specific, initiating, short-lived proteins must be produced in order for DNA replication to proceed. The low levels of these substances which do not accumulate are sufficient for one round of replication (Lark, 1969). The fact that gene products similar to those in bacteria may be involved is further supported by the fact that isolated nuclei from non-regenerating liver cells, when incubated with appropriate precursors and cytoplasmic extracts from regenerating liver cells, have demonstrated the onset of DNA synthesis (Thomson and McCarthy, 1968; De Bellis, 1969). It is thus apparent that DNA synthesis in regenerating liver cells requires both prior and concurrent S phase protein synthesis.

RNA Synthesis

In normal liver cells, 5% of the RNA is nuclear, 10-15% is tRNA, 80-85% is rRNA and only 1 to 3% is in the form of mRNA (Hirsch and Hiatt, 1966; Blobel and Potter, 1967; Harris, 1968). Regeneration is at least in part dependent upon RNA synthetic events. This includes a general increase in the rate of RNA synthesis and the production of some entirely new species of RNA required specifically for regeneration.

1. RNA Synthesis *In Vivo*

The synthesis of this important informational macromolecule undergoes extensive and almost immediate changes following partial hepatectomy. Within six hours

after the operation, a net gain in RNA of about 30% can be detected (Bucher et al., 1969a; Lieberman and Kane, 1965). Since 80% of liver RNA is rRNA, much of this new increment must be of the rRNA variety. This net increase in the amount of RNA is in part due to an increased rate of synthesis and is in part due to decreased degradation as will be discussed later.

Bucher et al., 1969b has established that in fed rats the in vivo rate of RNA synthesis increases 1-1/2 - 2 times normal by three hours after partial hepatectomy and remains elevated through hour 12. In rats that have been fasted prior to the operation, the maximal rate of RNA synthesis reaches two times normal by six hours post partial hepatectomy and also remains elevated through hour 12.

In summary, there are a variety of possible explanations which can account for the increased rate of RNA synthesis during regeneration. These include an increase in template capacity, increased amounts of RNA precursors, an increase in the amount of and/or activity of polymerases present or a combination of some or of all of these factors. In addition, a change in template capacity would allow for the production of the new kinds of mRNA molecules needed to produce those proteins that appear only when liver cells are preparing to divide.

2. RNA Precursors

Increased rates of RNA synthesis and the production of new varieties of RNA require that appropriate amounts of ribonucleotide precursors be present. Unlike DNA precursors, ribonucleotides are usually present in plentiful supply in liver cells. This is consistent with the fact that RNA synthesis is not correlated with the events of the cell cycle to the extent that DNA synthesis is. Moreover, there exists a general requirement in the liver for specific ribonucleotides to be present for lipid and glycogen synthetic processes. Nevertheless, the increased production of RNA associated with liver regeneration does necessitate the expansion of existing ribonucleotide pools. Such an expansion is brought about by increases in enzymatic activities associated with some of the critical enzymes leading to RNA precursor formation.

In the pyrimidine pathway, the enzymes aspartic transcarbamylase and dihydroorotase both undergo 200-300% increases in activity which are not affected by end product inhibition or repression in the presence of orotic acid (Hager and Jones, 1967; Nakanishi *et al.*, 1968; Bresnick *et al.*, 1968; Bucher and Swaffield, 1969). Probably the most important event leading to the formation of pyrimidine RNA precursors is that concerning orotidylate pyrophosphorylase following partial hepatectomy. This enzyme increases 3-4 fold in activity during regeneration.

Most other enzymes in the pathway leading to UTP formation increase in activity in response to the presence of orotic acid (Bresnick, 1965; von Euler et al., 1963). In addition, the enzymes in the salvage pathway for cytidine also increase in activity. The net result of these changes in enzymatic activities is that the levels of CTP and of UTP increase in regenerating liver cells by 1.5-2 times at the end of six hours of regeneration (Bucher and Swaffield, 1969b; Bucher and Swaffield, 1966; Bucher and Swaffield, 1969). This occurs at a time during the regenerative process at which similar increased rates of RNA synthesis also are observed in vivo. In contrast to this, it appears that ATP and GTP are present already in sufficient amounts to sustain the regenerative process without extensive alterations in enzymatic activities leading to the production of these purine RNA precursors (Ove et al., 1967; Mandel, 1967).

3. Template Capacity

As has been mentioned in previous sections, new species of RNA must be produced during the early stages of regeneration in order to account for the specific enzymatic activities upon which regenerating cells depend. Various inhibitor studies have attested to the facts that (1) newly appearing enzymatic activities are not merely the result of differential translation from pre-existing messengers and (2) these new transcripts must be produced within the first few hours of regeneration. X-irradiation

studies (Bollum et al., 1960) have demonstrated the importance of the timing of events during the regenerative process. Animals irradiated within the first 6 hours post partial hepatectomy fail to undergo regeneration and do not exhibit increased enzymatic activities. Animals irradiated after 16 hours, however, will undergo at least one round of cellular division and do demonstrate elevated enzymatic activities. Of a more specific nature, actinomycin D studies (Fujioka et al., 1963) have shown that injections of this drug, given early during regeneration, will prevent the onset of mitosis for as long as the injections are continued.

Hybridization and competition experiments performed by Church and McCarthy (1967a) provide further clear illustrations of the sequence of transcriptional events that occur during hepatic regeneration. Their work has indicated that the greatest efficiency of hybridization occurs when using unfractionated RNA extracted 1 hour after partial hepatectomy. In addition, competition experiments demonstrate that the greatest number of transcripts, different from those present in normal animals, is present at this time. In succeeding hours the population of RNA molecules present becomes progressively like that within non-regenerating cells. Of particular significance is the fact that 1 hour transcripts from regenerating cells were found to compete successfully with RNA extracted from embryonic mouse liver cells from animals at the 14th day of the gestational period (Church

and McCarthy, 1967b). At this time in development, the liver is in a state of rapid cellular proliferation. These results have been interpreted as being suggestive of the possibility that, during regeneration, gene sequences normally repressed in adult liver cells, are reactivated.

Other studies utilizing similar procedures have been performed by Church and McCarthy (1969) using separated nuclear and cytoplasmic RNA fractions. Their results indicate the presence in normal animals of certain varieties of HnRNA (heterogenous RNA) which never appear in the cytoplasm. During the course of regeneration, however, some of these nuclear transcripts were found to move into the cytoplasm. These results are indicative of the fact that in addition to the appearance of new transcripts during regeneration, there may also occur the phenomenon of selective transport of RNA molecules from the nucleus to the cytoplasm for possible utilization there.

Experiments with isolated liver nuclei (Pogo et al., 1966) have provided a further demonstration of template activation during the course of regeneration. Nuclei from regenerating liver cells were shown to incorporate several fold more H^3-UTP into RNA than did their non-regenerating counterparts. Moreover, mixtures of regenerating and non-regenerating nuclei yielded rates of incorporation midway between those for suspensions of regenerating or non-regenerating nuclei alone. This latter result was interpreted as an indication of the absence of the influence

of diffusible activators or inhibitors upon the rates of incorporation observed. Much of the RNA produced by non-regenerating nuclei was found to be of the ribosomal variety as indicated by a high GC content. When such nuclei, however, were treated with high concentrations of salt, the rate of incorporation was observed to increase sharply, approaching that for the regenerating nuclei. Moreover, salt treatment caused a change in the types of RNAs being made as indicated by the shift to an increased utilization of ATP and UTP, suggesting a change toward a greater production of non-rRNA transcripts. Treatment of regenerating nuclei with salt was much less effective in altering the rate of RNA synthesis. Such alterations in RNA synthesis were believed to result from structural changes in the chromatin involving the separation of various components which would thus give rise to a higher template capacity.

In order to establish that changes in RNA synthesis during regeneration were indeed due to alterations in chromatin structure and not simply to changes in the polymerases, E. coli RNA polymerase was used to assay the template capacity of control and regnerating nuclei (Pogo et al., 1966). Higher rates of incorporation were obtained only with nuclei isolated from regenerating livers even though the type and amount of polymerase used was the same in each case.

In a more direct manner, Thaler and Villee (1967)

have examined the role of the chromatin activation during the first 24 hours of regeneration. They assayed the template capacity of purified chromatin utilizing exogenous bacterial RNA polymerase. Their results indicated three phases of template activity: the first extending through hour six during which time template capacity doubles, the second from hours 6 through 12 in which template capacity remains constant, and, the third phase from hours 12 through 24 in which template capacity is tripled.

It is apparent from all of these studies that changes in the template capacity of the chromatin do occur and that they play an active part in the regenerative process.

4. RNA Polymerases

Template activation is but one means by which the overall production of RNA can be augmented. The activation of RNA polymerases by cAMP dependent kinases has already been discussed in the section on the initiation of the regenerative response. Numerous in vitro experiments have indicated that RNA polymerase activity increases following partial hepatectomy (Tsukada and Lieberman, 1964; Busch et al., 1962; Ro and Busch, 1967; Doly et al., 1965). A complication that arises is the question as to whether increased amounts of polymerase(s) are synthesized during regeneration and whether among these enzymes are included new varieties of polymerases.

In general, it should be stated at the outset that liver cells contain multiple forms of DNA-dependent RNA polymerases. (Symposia of Quantative Biology, 1970).

Roeder and Rutter (1969) have designated three forms of RNA polymerase (I, II, III) based upon their order of elution from DEAE-Sephadex columns. Alternatively, Seifart et al. (1972) have described three forms of RNA polymerase by letter using the convention of Chambon which distinguishes polymerases according to sensitivity to α-amanitin. Polymerase A is localized in the nucleolus, polymerase B in the non-nucleolar regions of the nucleus and polymerase C has been reported in the cytoplasm (Kedinger et al., 1971; Mandel and Chambon, 1971; Weaver et al., 1971; Chesterton and Butterworth, 1971; Schmuckler and Tata, 1971; Sajdel and Jacob, 1971).

Tsukada and Lieberman (1964) first reported increased specific activities for both the nuclear and nucleolar RNA polymerases that reached a plateau at approximately 12 hrs. post partial hepatectomy. In addition, they found that the half-life of RNA polymerase molecules from regenerating cells was 3 hours while that from non-regenerating cells was about 12 hours (Tsukada and Lieberman, 1965). The use of the protein synthesis inhibitor, puromycin, or high concentrations of para-fluorophenylalanine were shown to eliminate this elevated level of enzyme activity. Much lower levels of para-fluorophenylalanine could completely prevent this rise in activity if given between 0 and 2 hrs. post partial hepatectomy but not if given after four hours. Tsukada et al. (1964) have interpreted their results to suggest that a protein rich in phenylalanine, is

synthesized through hour four which may cause the switching on of polymerase genes. At hour four, some other event is believed to occur which then allows the polymerase genes to remain active in the absence of the original protein substance. Past four hours, only very high levels of para-fluorophenylalanine can affect polymerase activity.

Protein Synthesis

As indicated in previous sections, protein synthesis is essential for the process of hepatic regeneration. *In vivo* experiments using partially hepatectomized rats have revealed that net increases in protein amount first become detectable after 12 hours. Between 12 and 36 hours, the most rapid rates of protein synthesis are observed. Concomitant with this increased protein synthesis are elevated levels of amino acid anabolic activity as well as decreased rates of amino acid catabolism. This net increase in overall protein content is associated with the appearance of those new proteins that must be present in liver cells preparing to divide (Bucher and Malt, 1971).

The means by which this augmented protein production is achieved, although not totally established, seems to be related to changes in the amount and condition of RNA available to the ribosomes of the regenerating liver cells. (Bucher, 1963; Higashino and Lieberman, 1965; Staib and Miller, 1964). It is believed that the ribosomal population

from regenerating liver cells has access to more mRNA (Van Lancker, 1967) which, in this case, may be present in greater amounts and/or which may be "unmasked" to a greater extent for translational purposes (Henshaw, 1968). This hypothesis is supported by experiments showing that in the presence of excess poly U (as an artificial messenger) that differences in activity between microsome preparations from regenerating and nonregenerating cells are abolished (Cammarano et al., 1965; Campbell and Cooper, 1963). In addition, microsomes from non-regenerating liver cells exposed to trypsin show increased protein synthetic activity which may be indicative of an unmasking of the mRNA (Salb and Marcus, 1966). This line of investigation points strongly to the role of mRNA content and the condition of the messenger in regulating protein synthesis during regeneration.

Numerous experiments indicate the inhibitory effects on translation of RNase enzymes associated with the endoplasmic reticulum membranes of non-regenerating liver cells on translation. These enzymes are assumed to disappear or to become inactivated upon the advent of the regenerative process (Bucher, 1963; Cammarano et al., 1965; Scornik et al., 1967; Campbell et al., 1967; Tsukada et al., 1968). Experiments utilizing deoxycholate treated microsomes have shown that such microsomes obtained from non-regenerating liver cells exhibit rates of protein

synthesis comparable to those of regenerating microsomal preparations. It appears that the presence of RNase activities are particularly associated with the endoplasmic reticulum membranes of non-regenerating cells.

In normal liver cells, an acid ribonuclease, usually associated with the lysosomes, can be detected. An alkaline RNase activity, which may be responsible for the degradation of specific RNA molecules is also manifested (Roth, 1967). Although the acidic RNase activity increases with time after partial hepatectomy, the alkaline RNase activity (mainly in the form of RNase II) shows a definite decrease during the first 24 hrs. of the regenerative process after which it increases severalfold (Rahman et al., 1969; Maor and Alexander, 1968). Concomitant with the low initial alkaline RNase activity is an over 80% increase in ribonuclease inhibitor within the first critical 12 hours after partial hepatectomy (Shortmen, 1962; Chakravorty and Busch, 1967). These results correlate well with the net increase in RNA content at this time. It is also of particular interest that nuclear and nucleolar RNase activities seem to be increased after the onset of the regenerative process in order to handle the processing of RNA (Arora and deLamirande, 1967). All of this evidence, although circumstantial, points to the possibility that inhibition of ribosomal alkaline RNase during the regenerative process may play an important role in the regulation

of protein synthesis. This hypothesis seems to be upheld by observations from experiments in which the stability of purified polyribosomes, isolated by the sucrose gradient technique in the absence of detergent from regenerating liver cells, was determined. These polysomes from regenerating liver cells were found to be more stable than corresponding polysome preparations from normal liver cells. Of even greater importance, is the fact that these more stable preparations exhibited a higher rate of protein synthesis (Bont et al., 1967; Shortman, 1962; Tsukada et al., 1968).

In summation then, with regard to protein synthesis, it appears that both the amount and condition of RNA available are of prime importance. The amount of RNA present seems to be affected both by the rates of synthesis and of degradation. The coordinated manipulation of these processes results in a net increase in the amount of RNA available for protein synthesis. Increased amounts of RNase inhibitor apparently decrease degradation. In addition, decreased levels of substances that can mask mRNA are associated with polysomes obtained from regenerating liver cells.

The Histones

As discussed in the previous section, the template capacity of rat liver chromatin changes dramatically during the course of the hepatic regenerative process. Eucaryotic

chromatin is composed of numerous components which have been implicated in the regulation of template activity. Among these components, the histones have received a great deal of attention.

Since the initial reports of Stedman and Stedman (1950; 1957), the histones have generally been regarded as repressive components of the chromatin, responsible for restriction of the eucaryotic genome. In general, approximately 5-15% of the DNA in eucaryotic chromatin is normally expressed (Allfrey and Mirsky, 1962). Numerous *in vitro* studies have correlated histone removal with increased rates of RNA synthesis (Allfrey *et al.*, 1963; Bonner and Huang, 1962) whereas the reassociation of histones with DNA has been shown to be generally repressive Bonner and Huang, 1962; Allfrey and Mirsky, 1962; Spelsberg and Hnilica, a,b 1971; Paul and Gilmour, 1968). Of the various histone fractions, the arginine-rich ones (F3 and F2a1) have been reported to be the most inhibitory in nature (Allfrey and Mirsky, 1963; Skalka *et al.*, 1966), and the very lysine-rich histones (F1) the least. The selective removal of F1 histones has been shown to exert almost a negligible effect on template efficiency (Spelsberg and Hnilica, a,b 1971; Seligy and Neelin, 1970; Murray, 1969; Jensen and Chalkley, 1968) although extensive changes in chromatin structure, solubility and the length of transcripts produced have been reported (Littau *et al.*, 1965;

Chalkley and Jensen, 1968; Jensen and Chalkley, 1968; Koslov and Georgiev, 1970). On the other hand, selective removal of histones other than the F1 fraction has been shown to result in large increases in template efficiency (Spelsberg and Hnilica, 1971 a,b; Seligy and Neelin, 1970; Murray, 1969; Marushige and Bonner, 1966; Samis et al., 1968; Kurashima et al., 1970; O'Meara and Herrmann, 1970).

In addition to altering the rate of transcription, histones have been shown to change the types of transcripts produced. The removal of histones from chromatin results in an alteration of the composition of the RNAs produced. A greater similarity in composition to that of DNA is observed (Allfrey et al., 1963; Liau et al., 1965). Hybridization and competition studies have tended to verify this result (Paul and Gilmour, 1968). Again, the removal of the F1 histones has been shown to be the least effective of the five major fractions in altering the types of RNAs produced (Spelsberg and Hnilica, 1971 a,b).

Histone Modifications

That histones have the ability to restrict genetic expression is apparent. However, if they are to perform this function in a physiologically relevant manner, they should be able to exert a variable type of restriction in accordance with cellular need. Chemical modifications of the histones have been thought to represent just such a mechanism. The major modifications, all of which have

been reported to occur during liver regeneration, are acetylation, phosphorylation, thiolation and methylation.

1. Acetylation

In general, histones can be acetylated terminally (Phillips, 1963; Phillips, 1968) or internally giving rise to ϵ-N-acetyl lysine residues (Gershey et al., 1968; Vidali et al., 1968; Allfrey, 1970; DeLange et al., 1969; DeLange et al., 1970; Liew et al., 1970). Recently it has been suggested (Ruiz-Carrillo et al., 1975) that N-terminal acetylation and other modifications occurring in the cytoplasm are associated with histone processing. Internal nuclear histone acetylation (and deacetylation) tend to correlate with alterations in the genetic activity of various cell types. It is the internal form of acetylation which will be considered here.

Labeled acetate derived from acetyl CoA, is known to be enzymatically incorporated into histones in the absence of amino acid uptake. This incorporation of acetate is reversible (Allfrey et al., 1964). Both in vivo and in vitro, the arginine-rich histones (F2a1 and F3) are the fractions which are most affected by internal acetylation (Lieu et al., 1970; Pogo et al., 1968; Byvoet, 1968; Jungman et al., 1970; Allfrey, 1970; Pogo et al., 1967; Ono et al., 1969; Takaka et al., 1969; Willhelm and McCarty, 1968; Cross and Ord, 1970; Wilhelm and McCarty, 1970; Allfrey et al., 1964; Vidali et al., 1968; Gallwitz and Sekeris, 1969; Nohara et al., 1966; Racey and Byvoet,

1971). The F3 histones are reported to be acetylated at lysines 9, 14, 18 and 23 (Dingman and Sporn, 1964; Marushige and Bonner, 1966) whereas the F2a1 fraction appears to undergo acetylation at residues 5, 18, 12 and 16 (Maio and Schildkraut, 1969). In addition, it seems that while some molecules of a particular histone fraction will accept acetate, other members of the same class will not. In the arginine-rich histone classes mentioned above, it is not uncommon to find different percentages of unacetylated, mono-, di-, tri- and tetraacetylated histone sub-fractions. The deacetylation of histones also appears to be under enzymatic control (Inoue and Fujimoto, 1969; Libby, 1970). The joint functioning of both acetylating and deacetylating enzymes in regulating the rate of acetate turnover and the types of acetylated species produced may be of profound importance in determining the way in which histones interact with DNA in chromatin.

The acetylation of histones is generally regarded as a means by which histone inhibition may be relieved. This is believed to occur through a lessening of the positive charge on histones which would therefore result in a decrease in the ionic interactions of histones with the negatively charged phosphate groups of DNA. Some direct evidence of this has been provided through the use of <u>in vitro</u> DNA directed RNA synthesizing systems. The addition of acetylated arginine-rich histones to such

systems inhibits RNA synthesis to a much lesser extent than the addition of the unacetylated variety of the same histone fraction (Allfrey et al., 1964).

Rapid rates of histone acetylation are characteristic of the morphologically diffuse, genetically active fractions of chromatin, whereas low rates of acetylation are associated with condensed, genetically inactive chromatin (Allfrey, 1968; Allfrey et al., 1964; Allfrey, 1965).

Much support for the idea that acetylated histones are important for template activation has been derived from in vivo studies correlating histone acetylation with alterations in the rates of DNA-dependent RNA synthesis. The histones of immature avian erythrocytes are more highly acetylated than their mature counterparts (Cameron and Prescott, 1963; Grasso et al., 1963; Scherrer et al., 1966; Ruiz-Carrillo et al., 1974); the young cells are more genetically active than the mature ones and contain less highly condensed chromatin. Similar correlations between histone acetylation and increased RNA synthesis have been observed in the appropriate target cells of various hormones: cortisol (Feigelson et al., 1962; Kenney and Kull, 1963; Greenman et al., 1965; Yu and Feigelson, 1969; Allfrey, 1966; Allfrey, 1965; Murthy et al., 1970), estradiol (Mueller et al., 1961; Hamilton et al., 1968; Libby, 1968) and hydrocortisone (Sluyser, 1966 a; Sluyser, 1966 b; Sunaga and Koede, 1967; Monder and Walker, 1970;

Wagner, 1970).

Of particular importance here are those studies performed on otherwise quiescent cells stimulated to undergo cellular division. In phytohaemagglutinin (PHA) stimulated human peripheral lymphocytes, the rates of arginine-rich histone acetylation increase immediately and are followed within 30 minutes by changes in both the rate of RNA synthesis and the types of RNAs produced (Rubin and Cooper, 1965; Monjardino and MacGillivray, 1970; Pogo et al., 1966). These events have been correlated with structural changes in the chromatin of these cells as evinced by the increased binding of both acridine orange and of actinomycin D to DNA within minutes after PHA stimulation (Killander and Rigler, 1965; Killander and Rigler 1969; Darzynkiewicz et al., 1969). In the opposite fashion, PHA treated polymorphonuclear leukocytes undergo increased rates of deacetylation and decreased rates of acetylation at times of reduced RNA synthesis (Pogo et al., 1967).

Information on histone acetylation and gene activation during liver regeneration agrees well with evidence derived from other systems. Pogo et al. (1968) demonstrated that in normal liver, tritiated acetate was maximally incorporated into histones 15 minutes after injection and was then rapidly lost. After 1 to 2 hours of regeneration, however, the rate of incorporation of acetate into histone was reported to begin to increase and was accompanied by

a decreased rate of deacetylation. As much as 70.7% of incorporated acetate is lost in one hour in non-regenerating liver while only 13.7% of incorporated acetate is lost in 2 hours in regenerating liver. In addition, the net amount of acetylated histone present was reported to reach a maximum between 3 and 4 hours post partial hepatectomy after which time a rapid decline in acetate content occurred. This peak of acetylation coincides with the approximate two fold increase in RNA synthesis reported *in vivo* by Bucher *et al.* (1969) for unfasted rats. Of all the fractions, the F2a1 fraction contained the greatest amount of acetate.

2. Phosphorylation

A second major histone modification is phosphorylation. Labeled phosphate has been shown to be incorporated into histones under both *in vivo* and *in vitro* conditions (Ord and Stocken, 1966; Kleinsmith *et al.*, 1966 a,b). The fraction reported to be most extensively affected is the F1 histone (Langan, 1968 a, b; Langan, 1969 a,b; Kleinsmith *et al.*, 1966 a, b; Stevely and Stocken, 1968; Buckingham and Stocken, 1970 a, b; Eltzina and Veresotskaya, 1969), although phosphorylation of other fractions has certainly been demonstrated (Sung *et al.*, 1971; Sung and Dixon, 1970; Ord and Stocken, 1966; Evans *et al.*, 1970; Gutierrez and Hnilica, 1967; Gutierrez-Cernosek and Hnilica, 1971; Shepherd *et al.*, 1971; Jungmann *et al.*, 1970; Gurley and Walters, 1971; Murthy *et al.*, 1970; Eltzina and Veresotskaya,

1969; Adams et al., 1970; Hayashi and Iwai, 1970). ATP is the donor of the phosphate group which is attached enzymatically by an ester bond predominantly to the hydroxyl groups of serine residues (Langan, 1968 a; Ord and Stocken, 1966; Kleinsmith et al., 1966 a) and, less frequently to those of threonine (Ord and Stocken, 1966). The site of F1 phosphorylation has been reported to exhibit extensive species and tissue specificity (Rall and Cole, 1971; Langan et al., 1971; Jergil et al., 1970). Phosphorylation is not affected by puromycin treatment (Kleinsmith et al., 1966 a; Marushige et al., 1969).

Histone phosphorylation has been shown to be affected by cAMP both in vivo and in vitro. The rate of phosphorylation of F1 was reported to increase 6 fold in the presence of low concentrations of cAMP; the net phosphate content, however, remained unchanged whether the cyclic nucleotide was present or absent (Langan, 1968 b; Langan, 1969 a,b; Walsh et al., 1968). This stimulation is known to be the result of the activation of histone kinase(s) (Cross and Ord, 1971). Yamamura et al. (1970) have been able to isolate two histone kinases from rat liver. The B1 kinase was shown to be cAMP dependent and essential for the phosphorylation of serine residue 40 of the F1 fraction. On the other hand, the cAMP independent B2 kinase was reported to catalyze the phosphorylation of the serine residue at position 106 in the F1 carboxyl terminal region (Siebert et al., 1971).

Like acetylation, the physiological role of F1

histone phosphorylation has been associated with the derepression of chromatin. Using highly phosphorylated F1 histones, Stevely and Stocken (1966) were able to demonstrate a less inhibitory effect on *in vitro* transcription than with minimally phosphorylated F1. In a separate study, phosphorylated F1 was also shown by circular dichroic analysis to affect DNA conformation less than dephosphorylated F1 histone (Adler *et al.*, 1971).

Some of the best studies linking F1 histone phosphorylation to gene activation have been performed with cells stimulated to proliferate. These studies have revealed an early cAMP dependent F1 phosphorylation event as well as a later cAMP independent F1 phosphorylation. Both events, as illustrated below, appear to be specific for different serine residues, modified by the appropriate cAMP-dependent or-independent kinases.

These modifications are well documented in lymphocyte cultures stimulated to divide by PHA or by dibutryl cAMP treatment (Abell and Monahan, 1973). An increase in the rate of F1 phosphorylation was reported to occur within 15 minutes after drug administration and was concomitant with template activation (Cross and Ord, 1971). It is of interest to note that at this time, during which increased RNA synthesis was reported, only the rate of F1 phosphate turnover increased and, only later in the process (at the time of DNA synthesis), did the net phosphate content increase under the action of a cAMP independent kinase.

A similar sequence of early and of late phosphorylation events has been reported in the case of rat liver regeneration. It should be mentioned at the outset that both glucagon and insulin have been reported to affect the rate of F1 phosphorylation at the primary site (serine residue 40). After partial hepatectomy, cAMP levels within the liver remnant are known to increase (section on initiation) and the B1 kinase of Yamamura is sensitive to these levels of cAMP. Extensive increases in the rates of histone phosphorylation (correlated with early increases in cAMP) after partial hepatectomy have been reported to occur at the time at which increased RNA synthesis and the appearance of new RNA species occur (Gutierrez-Cernosek and Hnilica, 1971; Church and McCarthy, 1967; Pogo et al., 1967; Thaler and Villee, 1967).

At approximately the time of DNA synthesis, the cAMP independent kinase was found to undergo a six fold increase in activity (Siebert et al., 1971). The increase in activity is believed to be accomplished by the dissociation of the cAMP dependent kinase from a tetrameric to a dimeric form which thus results in its conversion to a cAMP independent enzyme. At this time in regeneration the rate of histone phosphorylation increases, net histone phosphate increases and the additional F1 serine site is phosphorylated (Shepherd et al., 1970; Stevely and Stocken, 1968; Balhorn et al., 1971).

The role of late histone phosphorylation in liver regeneration is only speculative. It has been suggested that late F1 phosphorylation is associated with the turnover of this particular fraction and with its successful transport from the cytoplasm to the nucleus. It is important to stress that at this later time in the hepatic regenerative process, not only the F1 histones are phosphorylated but also the F2a2 (Sung and Dixon, 1970) and F2b (Gutierrez-Cernosek and Hnilica, 1971) histones as well. In the case of the F2a2 fraction, the phosphorylation was shown to be at the NH_2-terminal serine which was also acetylated. The possible role of acetylation and phosphorylation for the transport of histones and protamines has already been suggested (Ingles and Dixon, 1967; Jergil and Dixon, 1970; Marushige et al., 1969; Sung and Dixon, 1970).

In summary then, based upon evidence obtained from hepatic regeneration studies (and other systems) it appears that early phosphorylation events may be associated with gene activation while the later phosphorylations may be more directly related to histone transport.

3. Methylation

Numerous examples exist of histone methylation in different organisms and in different tissue types. These studies have revealed that lysine residues primarily in artinine-rich histone fractions are affected (Gershey et al., 1969; Murray, 1964; Hempel et al., 1968 a,b; DeLange et al.,

1969; Tidwell et al., 1968; Ogawa et al., 1969). Among the arginine-rich histones, specific methylations have been shown to occur at only one of many possible sites (DeLange et al., 1969; Ogawa et al., 1969; DeLange et al., 1970). Moreover, this phenomenon is complicated by the fact that the modified site of methylation can differ in methyl group content leading to the formation of ε-N-mono,-di- and trimethyl lysines (Paik and Kim, 1967; Hempel et al., 1968 a). Frequently, different percentages of all three forms as well as the unmodified condition can be found in a particular histone fraction usually with the ε-N-dimethyl lysine being predominant (Hempel et al., 1968 b; Tidwell et al., 1968; DeLange et al., 1970; Paik and Kim, 1967). The methyl donor is known to be methionine which donates its methyl group enzymatically after histone synthesis has already occurred (Murray, 1964; Tidwell et al., 1968; Allfrey et al., 1964; Paik and Kim, 1967; Comb et al., 1966).

Although no definite physiological function for histone methylation has been determined, changes in histone methylation are known to occur late in the cell cycles of various types of proliferating cells. Unlike acetylation and phosphorylation, increased methylation has been shown to occur at times during the cell cycle of low template activity when chromatin is maximally condensed. It has been pointed out (Allfrey, 1971) that methylation would

make the ϵ-amino groups of lysine more basic. The increase in positive change could thus bring about a tighter binding between histones and DNA and therefore possibly aid in achieving a more tightly coiled state in premitotic chromosomes.

Regenerating liver cells clearly illustrate this inverse relationship between methylation and template activity. Tidwell et al. (1968) have shown that the peak of methylation is a late event (approximately 30 hours post partial hepatectomy); occurring after the peaks of DNA and histone synthesis have already passed and the cells are dividing or are preparing to divide. The arginine-rich histones were the only fractions reported to be affected, and the ϵ-N-dimethyl lysine form appeared to be predominant. Moreover, higher histone methylase activities have been demonstrated in other types of rapidly dividing liver cells: embryonic and neoplastic (Turner and Hancock, 1970). A similar correlation between increased histone methylase activity and rapid cell division has also been observed in synchronously dividing HeLa cells (Borun et al., 1971), Chinese hamster ovary cells (Shepherd et al., 1971 a), and maturing avian erythrocytes (Gershey et al., 1969).

4. Thiolation

There have been numerous reports in the literature of cysteine residues being present in the F3 histone fraction;

approximately 70% of the thiol groups present in the nucleus are located in this fraction (Blazsek and Bukaresti, 1964; Phillips, 1965; Ord and Stocken, 1967; Hnilica and Bess, 1965). It appears that the F_3 histone of plants, rodents and the lower animals have one cysteine group whereas higher animals have two (Panyim et al., 1971; Panyim et al., 1970; Sadgopal and Bonner, 1970). Sequencing studies have indicated the cysteine to be present in the nonbasic area of the F_3 histone molecule (DeLange and Smith, 1971).

Although the functional significance of disulfide bond formation among F_3 molecules is unclear, like methylation, it appears to correlate well with chromatin condensation and with times of low template activity during the cell cycle. Studies have indicated high thiol content in interphase chromatin (Panyim et al., 1971; Sadgopal and Bonner, 1970). Lymphocytes stimulated by PHA were found to exhibit an increased thiol content at times of maximum DNA synthesis when the chromatin was most dispersed (Cross and Ord, 1970). Sadgopal and Bonner (1970) found a similar situation with synchronized HeLa cells. Experimental evidence has indicated that oxidized F_3 is effective in decreasing in vitro RNA synthesis (Hilton and Stocken, 1966; Stocken, 1966). Condensed chromatin has been shown to have a higher disulfide bond content whereas dispersed chromatin was found to contain a lower disulfide bond content (Frenster et al., 1963; Ueda et al., 1968).

No changes in disulfide bond formation have been reported in liver regeneration during early times of increasing template activity. In fact, histones from non-regenerating rat liver were found to exist in the thiol form and no changes were found to occur even after 30 hours of regeneration (Ord and Stocken, 1969).

Of those modifications enumerated, it is clear that, as in other proliferating cell types, liver cells, when stimulated to divide by partial hepatectomy, demonstrate many of the same correlations. Acetylation and one type of phosphorylation event are found to occur in the early part of the cell cycle when RNA synthesis is increasing. Conversely, methylation and in some systems oxidation events occur later in the cell cycle and appear to correlate well with the condensation of chromatin and the repression of template activity.

The Acidic Chromosomal Proteins

The acidic chromosomal proteins, as well as the histones discussed previously have also been implicated in playing a role in the specific regulation of DNA-dependent RNA synthesis. Unlike the histones, the acidic chromosomal proteins have high turnover rates (Daly *et al.*, 1952; Allfrey *et al.*, 1955; Steele and Busch, 1963), vary in amount from one cell type to another (Mirsky and Ris, 1951) and undergo changes that correlate with differing rates of tissue RNA synthetic capacity (Dingman and Sporn,

1964).

Numerous studies employing chromatin reconstitution and hybridization-competition techniques have indicated a direct role for the acidic chromosomal proteins in directing specific RNA synthesis. Paul and Gilmour (1968) were able to form artificial nucleohistone complexes by the in vitro reassociation of histones with DNA and found these complexes to be completely restricted as templates for RNA synthesis. When, however, the same procedure was performed using histones and dehistonized chromatin, the resulting reconstituted chromatin was found in their judgment, to be as qualitatively and quantitatively restricted as the original native chromatin. Even after all of the chromatin proteins had been salt dissociated, a reconstituted complex, similar in template capacity to the native chromatin, could be obtained by gradient dialysis in the presence of 5M urea. Paul and Gilmour were able to obtain the acidic chromosomal proteins from the pellicle formed after the high speed centrifugation of chromatin in 4M CsCl and to demonstrate that this component of chromatin must be present in the reconstitution mixture in order to re-establish the original specificity of association; these proteins could not be replaced by proteins such as serum albumin.

Further specific demonstrations of the role of the acidic proteins in directing RNA synthesis were provided by Gilmour and Paul (1970) and by Spelsberg et al. (1971). Both groups salt dissociated histones from chromatin

extracted from various eucaryotic tissue types and pelletted the DNA-acidic protein complexes by centrifugation. When "hybrid" reconstituted chromatins were formed by reassociating the histones from one tissue type with the DNA-acidic protein complex of another tissue type, the pattern of RNA synthesis obtained in each case was shown to be similar in nature to that of the tissue from which the DNA-acidic protein complex originated.

The above experiments have been criticized for a number of reasons. Firstly, they have employed hybridization-competition techniques developed for the analysis of RNAs of procaryotic organisms. These organisms have smaller genomes with RNAs present in sufficient concentration to hybridize to chromosomal DNA (cDNA) in relatively short periods of time. In eucaryotes, hybridization performed over short periods of time involves essentially the reiterated DNA sequences in chromatin (Britten and Kohne, 1966). In addition, mismatching can occur between RNA and DNA sequences which are similar but not identical. Therefore, the experiments described above more specifically demonstrate that repetitive DNA genes are specifically restricted by the acidic proteins in various animal tissues. This result may be of some significance, however, in view of the recent findings indicating that mRNAs contain both unique message sequences and repeated sequences (Dina et al., 1973).

Other criticisms have come from the use of heterologous *in vitro* systems employing *E. coli* RNA polymerase and from the method of reconstitution used. These criticisms have been countered by the use of cDNA probes, made by reverse transcriptase, for specific messengers. Gilmour and Paul (1973) using this type of probe have demonstrated that *E. coli* RNA polymerase could be used to transcribe chromatin from haemopoietic and from nonhaemopoietic tissue; only in the case of chromatin from haemopoietic tissue did the globin cDNA probe detect the presence of globin mRNA. Similar results were obtained by Axel *et al.* (1973). In this manner, it was demonstrated that a heterologous polymerase system could effectively produce mRNA molecules normally associated with chromatin from a specific tissue type. In a similar fashion, the validity of the process of chromatin reconstitution was confirmed. cDNA probes have been used to detect the presence of globin messenger when transcription was performed from native or reconstituted chromatin of foetal mouse liver (MacGillivray *et al.*, 1972) but not from native or reconstituted mature liver chromatin.

cDNA probes have also been used by Gilmour *et al.* (1973) to confirm earlier findings that the nature of the specificity of chromatin restriction could be attributed directly to the acidic proteins. They observed that if mouse brain chromatin was dissociated and then reconstituted in the presence of foetal mouse liver acidic chromosomal proteins,

the globin cDNA probe was able to detect the presence of globin mRNA when transcription was performed. This result did not occur after the transcription of brain chromatin alone. This experiment represents a more stringent proof of the original findings presented in the earlier works of Gilmour and Paul (1970).

Specificity of Interaction

As might be expected of proteins that function as regulators of gene activity, the acidic chromosomal proteins have been shown to exhibit specific binding to DNA.

Ultracentrifugal analysis of acidic protein-DNA complexes, formed by gradient dialysis, has indicated that a definite species specificity exists between these chromatin components. Binding of acidic proteins to homologous DNA was shown to occur in a specific fashion as evinced by polyacrylamide gel banding patterns (Teng et al., 1971; Teng et al., 1970) These ultracentrifugal studies have been confirmed by the use of affinity chromatography on DNA cellulose columns. Rat liver acidic proteins were shown to bind specifically to columns with rat DNA but not to columns with salmon or E. coli DNA (Kleinsmith et al., 1970). Similar results were obtained in other labs using other types of DNA columns (van den Broek et al., 1973; Kleinsmith, 1973). Furthermore, Allfrey (1974) and Allfrey et al., (1975)

have isolated acidic proteins from calf thymus and have run these proteins on aminoethyl cellulose columns with bound homologous DNA of different cot values. The elution profiles obtained indicate that the acidic proteins exhibit preferences for binding to DNAs of high, medium or low cot values. Not only does the specificity of interaction vary with the cot value of the DNA but also with the strandedness of the DNAs at the respective cot values utilized.

Correlations Between Changes in the Nuclear Acidic Proteins and Template Activity

In addition to the specificity of interaction with DNA and the direct role exhibited by the acidic proteins in influencing differential transcription, a wealth of information has been accumulated demonstrating correlations between template activity and the amounts and types of acidic proteins present.

In chromatin fractionation studies, the acidic proteins of the eu- and heterochromatic fractions have been compared. The transcriptionally active euchromatin fraction has been shown to have twice as much acidic protein as the corresponding heterochromatic fraction (Frenster *et al.*, 1963; Dolbeare and Koenig, 1970; Marushige and Bonner, 1971; Reeck *et al.*, 1972). Furthermore, Wang (1970) and Kamiyama and Wang (1971) have been able to add an acidic protein from euchromatin

to condensed heterochromatin and were able to observe the production of new transcripts from this formerly repressed material. Investigations, however, have not been successful in demonstrating differences in SDS acrylamide gel banding patterns for these acidic proteins extracted from euchromatic or heterochromatic chromatin preparations (Wilhelm et al., 1972; Gronow, 1972; Rickwood et al., 1973). This finding is in agreement with other reports which have indicated that great similarities exist in the SDS-gel electrophoresis banding patterns of acidic proteins extracted from chromatins of various fully differentiated tissues. Most studies, however, indicate that real differences do exist (Teng et al., 1971; Loeb and Cruezet, 1970; Platz et al., 1970; Teng et al., 1970; Chytil and Spelsberg, 1971; Spelsberg et al., 1972; Wang, 1971; Richter and Sekeris, 1972). In particular, it appears that problems arise in the interpretation of these various studies because of the fact that the isolation procedures for the acidic proteins have not been uniform. Those studies employing total acidic protein extraction have demonstrated little variation in banding patterns from one tissue to another. Other studies, utilizing partial extraction procedures, have revealed a great deal of acidic protein tissue heterogeneity. More recent evidence, employing immunological techniques, tends to verify, however, the claims of tissue specificity with regard to differences in acidic protein distribution (Chytil and

Spelsberg, 1971; Spelsberg et al., 1972; It has been suggested that this latter technique can successfully recognize the presence of acidic protein control components present in concentrations so low that they are not discernable on gels.

A number of examples can also be cited of changes in acidic protein content and/or type correlated with physiological changes in genetic activity. In Drosophilia polytene chromosomes, stimulated by ecdysone or by temperature shock, more acidic proteins have been shown to be present in the active puff areas than in the rest of these chromosomes; the excess acidic proteins apparently are the result of the accumulation and not the de novo synthesis of these proteins (Helmsing and Berendes, 1971). In response to hormonal treatments, numerous studies have demonstrated the production of specific nonhistone proteins correlated with gene activation. Such observations have been made on liver tissue after cortisol (Shelton and Allfrey, 1970) or glucagon administration (Enea and Allfrey, 1973) and on uterine tissue after estradiol administration (Teng and Hamilton, 1970). In other instances, changes in the rates of acidic protein synthesis have been correlated with the effects of insulin (Buck and Schandler, 1970), aldosterone (Swaneck et al., 1970) and the drug phenobarbitol (Ruddon and Rainey, 1970), respectively, on the appropriate target tissues.

In a similar manner, during the course of embryological development, changing acidic protein gel patterns have been observed with the progression from one developmental stage to another. These changes have been correlated with a progressive decrease in genetic activity as greater differentiation is achieved. In particular, both qualitative and quantitative differences have been reported during the early stages of sea urchin development (Cognetti et al., 1972). Whereas merely quantitative differences have appeared to be characteristic of the later stages of its development (Hill et al., 1971).

All of the above reports are indicative of the fact that changes in acidic proteins do indeed take place at physiologically relevant times.

The Acidic Proteins in Cellular Division

Numerous studies have focused upon the role of the acidic proteins in cellular division. Although great variations in results have been obtained due to the different systems, isolation procedures and synchronization techniques employed, various trends have begun to emerge which point to the importance of the acidic chromosomal proteins as regulators of cellular division.

Probably the first important piece of information to be gained about the behavior of the acidic proteins during cell division was in relation to their metabolism. Whereas histone synthesis is restricted to S phase, numerous studies have established that the acidic proteins are

synthesized continuously throughout the entire cell cycle. (Shapiro and Levina, 1967; Shapiro and Palikarpova, 1969; Crampton et al., 1955; Stein and Baserga, 1970; McClure and Hnilica, 1970).

In systems in which cells have been stimulated to divide, numerous observations have been made. Increases in the rate of synthesis of acidic proteins have been reported in developing and lactating mammary glands (Stellwagen and Cole, 1969) and in foetal mouse liver cells stimulated with erythropoeiten (Malpoix, 1971). These changes in acidic protein metabolism were associated with S phase.

Other reports have indicated that increased acidic protein synthesis occurs well before DNA replication beings. Specifically, Teng and Hamilton (1969) were able to observe an increase in the synthesis of one electrophoretic protein band in uterine cells after estrogen treatment. The protein in question, upon addition to chromatin from untreated uterine cells, was able to cause a marked increase in template capacity. A similar early G_1 alteration in acidic protein metabolism was reported by Chung and Coffey (1971) in ventral prostate cells stimulated by testosterone.

Still other studies indicate early G_1 increases in acidic protein synthesis followed by a shift in the types of acidic proteins produced as cells progress toward S phase. For example, in PHA stimulated lymphocytes,

immediate changes in metabolic activity have been characterized by increased synthesis of acidic proteins and by a change in the banding patterns of these proteins as the lymphocytes progress through G_1 to S phase. In particular, Weisenthal and Ruddon (1972) have observed a threefold accumulation of a middle molecular weight protein by the time these cells have begun the initiation of DNA synthesis. Similar observations by Jeter and Cameron (1974) were made on tetrahymena, synchronized by the starvation-refeeding method. Within minutes after refeeding, increased acidic protein synthesis occurred resulting in three times more protein present per nucleus. As the cells progressed toward S phase, a shift to middle molecular weight proteins was also observed.

An essentially bi-phasic nature of acidic protein synthesis has been demonstrated during cellular division. In various cell systems, such as isoproterenol-treated mouse salivary gland cells (Stein and Baserga, 1970), and WI-38 fibroblasts (Rovera and Baserga, 1971) early increases in acidic protein synthesis were shown to occur in G_1 followed by a leveling-off in activity as the cells approached S phase, and then, once again, acidic protein synthetic activity was observed to increase. Also in these studies shifts to increased amounts of middle molecular weight proteins were observed as the cells approached S phase. Probably of even greater significance, from these experiments came the observation that actinomycin D treatment did not affect early acidic protein synthesis which, however,

is absolutely necessary in order for the second stage of acidic protein synthesis as well as DNA replication. As might be expected, cycloheximide, administered early in G_1, not only affects the first phase of acidic protein synthesis but also the later stage and DNA synthesis as well. These observations have led Baserga's group to suggest that acidic protein synthesis early in G_1 is due to translation from stable long-lived mRNAs. These acidic proteins, produced in early G_1, when reassociated with DNA and histones by density gradient dialysis, were shown to stimulate greater template activity than that observed for acidic proteins from non-stimulated cell cultures (Stein et al., 1972). Acidic proteins obtained from M phase of the cell cycle, however, did not exhibit a stimulative effect when reconstitution experiments were attempted (Stein and Farber, 1972).

More precise studies of the acidic proteins during cellular division have been performed with various different types of synchronized cell cultures. In particular, the work of Salas and Green (1971), Fox and Pardee (1971), Becker and Stanners (1972) and Gerner and Humphrey (1973) have indicated that although few qualitative changes in the acidic proteins occur, quantitative differences in the rates of synthesis of these proteins are exhibited throughout the cell cycle. High molecular weight acidic proteins which turn over rapidly in resting and early G_1

cells demonstrate lower turnover rates as the cells progress through G_1 and to S. On the other hand, the rate of synthesis of the intermediate molecular weight acidic proteins tends to exhibit the opposite phenomenon and increases by late G_1 and S.

In addition, other studies have indicated that the net amounts of acidic proteins tend to increase during the cell cycle. The work of Mueller (1969) has demonstrated that all of the acidic proteins increase in amount as cells proceed through the cell cycle to S phase. More specifically, Karn et al. (1974) have studied the nuclear protein to DNA ratio at different times in the cell cycle. This ratio, they report, tends to vary greatly during the cell cycle is lowest at M, increases through G_1 to late S and then declines. Consistent with these results, the work of Borun and Stein (1972) suggests that not only the synthesis but also the transport into and out of the nucleus and the retention of the acidic proteins are variable throughout the cell cycle.

The changes in the amounts of the acidic proteins during the cell cycle are believed to be of importance with regard to their influence upon template activity. According to the work of McClure and Hnilica (1970) (as reported by Jeter and Cameron, 1974), the relationship between the net amount of acidic proteins per unit of chromatic and the template activity during the cell cycle is an inverse one. They have reported increases in protein

amounts which reach peaks in late G_1 and in S. High template activity, however, was reported in early G_1 and in G_2. This has led McClure and Hnilica to suggest a repressive role for acidic proteins which accumulate during the cellular cycle as opposed to a stimulatory one for those that turn over rapidly.

A different factor that may exert an influence upon the template activity of chromatin during the cell cycle is the state of phosphorylation of the acidic proteins. The work of Platz *et al.* (1973) with HeLa cells indicates an overall maximal rate of phosphorylation during G_1 and G_2, a depressed level during S and a low point of phosphorylation at M. It appears that a direct relationship may exist between phosphorylation and increased template activity.

The importance of acidic protein phosphorylation in altering genetic activity is indicated by numerous studies. These have been reviewed in detail by Kleinsmith (1974). Different acrylamide gel phosphoprotein banding patterns have been demonstrated for different tissue types as well as for the same tissue at different points in differentiation (Platz *et al.*, 1970; C. T. Teng *et al.*, 1970; C. S. Teng *et al.*, 1971; Vidali *et al.*, 1973). In general, euchromatin has been shown to have a higher phosphoprotein content than heterochromatin, and, genetically active cell types have a higher content of phosphoprotein than those relatively inactive transcriptionally (Kleinsmith and Allfrey, 1969 a,b). Moreover, specific correlations exist

demonstrating a direct relationship between acidic protein phosphorylation and transcriptional activity. The systems that show this include PHA stimulated lymphocytes (Kleinsmith *et al.*, 1966 a,b), *Physararum polycephalum* in different states of cell growth (Le Stourgeon and Rusch, 1971), synchronized HeLa cells at different stages of the cell cycle (Platz *et al*, 1973), and the sea urchin at different stages of development (Platz and Hnilica, 1973) and isoproterenol-treated rat salivary gland cells (Ishida and Ahmed, 1974). In addition, numerous hormones are known to stimulate acidic protein phosphorylation in their target tissues: insulin and prolactin (Turkington and Riddle, 1969), chorionic gonadotropin (Jungmann and Schweppe, 1972 a,b), and Testosterone (Ahmed, 1971; Ahmed and Ishida, 1971). Of greater significance are studies which demonstrate that the direct addition of phosphorylated acidic proteins to *in vivo* RNA synthesizing systems can stimulate RNA synthesis (Kamiyama and Dastugue, 1971; Kamiyama *et al.*, 1972; Kostraba and Wang, 1972 a,b; Teng *et al.*,1971; Rickwood *et al.*, 1972; Shea and Kleinsmith, 1973). Probably the best demonstration to date that phosphorylation of acidic proteins can alter genetic activity is that of Kleinsmith *et al.* (1976). This group showed that the removal of phosphate from HeLa cell acidic proteins (extracted during S phase of the cell cycle) caused these proteins to lose their ability to stimulate the transcription of histone mRNA when chromatin reconstitution was attempted. This was verified by cDNA probes.

In the regenerating rat liver system, a great deal of work has implicated the acidic chromosomal proteins as important components necessary for template activation. In general, the consensus of reports by Holbrock et al. (1962), Butler and Cohn (1963), Pogo et al. (1968) and Dastugue et al. (1971) is that the rates of synthesis of the acidic proteins are greatly altered during regeneration.

A biphasic pattern of acidic protein synthesis occurs during the regenerative process: increased rates of synthesis are observed through hour 12 followed by a slight decrease lasting until the initiation of DNA synthesis after which the maximal rate of synthesis is attained at 25 hours post partial hepatectomy (Jeter and Cameron, 1974).

In spite of these alterations in synthetic activity, Garrard and Bonner (1974) have reported the absence of overt qualitative as well as of any reproducible quantitative differences in acidic protein acrylamide gel banding patterns at different times in regeneration. The acidic proteins of regenerating liver cells exhibited an overall 42% increase in labeling over their non-regenerating counterparts by 2-1/2-4 hours post partial hepatectomy. In particular, acidic proteins in the molecular weight range of 45,000-55,000 demonstrated selective increases in their labeling pattern as compared with other acidic proteins.

The work of Kostraba and Wang (1973) has revealed a

specific relationship between the acidic proteins and differential gene activation in regeneration. When they reconstituted 6 hour regenerating acidic proteins with non-regenerating DNA, histones and residual proteins, they observed an increase in template activity. This increase was not obtained with reconstituted chromatin which contained non-regenerating acidic proteins. Moreover, the transcripts produced in the former case were hybridizable with DNA to 8.8% while the latter transcripts were hybridizable to DNA to the extent of 6% hybrid formation. These values were similar to those obtained from hybridization studies performed with transcripts from native 6 hour regenerating and non-regenerating chromatin, respectively.

It is apparent that the acidic proteins are important components not only of regenerating rat liver chromatin but also of the chromatins of a great many other cellular systems in the process of cellular proliferation. Although numerous studies have been performed, dealing with the possible functions of these proteins, still, as yet, a clear-cut picture as to their precise role during cellular proliferation has not emerged.

Chromosomal RNA

One last factor that should be discussed in dealing with the control of hepatic regeneration is chromosomal RNA (cRNA). This form of RNA has been implicated by Bekhor *et al.* (1969) and by Huang and Huang (1969) as

being necessary for the maintenance of the differentiated state. Their experiments have pointed to the fact that cRNA was the essential component without which chromatin could not be specifically reconstituted. It was further shown to be covalently attached to the nonhistone chromosomal proteins (Huang and Huang, 1969). This type of RNA is also particularly noted for its high content of dihydropyrimidine (Bonner et al., 1963) and for the fact that it hybridizes to repetitive DNA sequences (Sivolak and Bonner, 1971; Holmes et al., 1971). Moreover, heterogeneous RNA and cRNA share a large proportion of their nucleotide base sequences which suggests that the former may be the precursor of the latter (Holmes et al., 1971).

Mayfield and Bonner (1972) have studied cRNA during the process of rat liver regeneration. They have reported that at 1 hour post partial hepatectomy HnRNA synthesis reaches a maximum and that by 4 hours cRNA is observed to contain a maximum amount of nucleotide sequences different from those present in non-regenerating cRNA molecules. They have suggested that this sequence of events resembles the model proposed by Britten and Davidson (1969) for the control of the eucaryotic genome. This theory holds that at times of gene activation small size activator RNA molecules which have the ability to bind to repetitive areas of the genome are produced and exert control over differential gene expression. It is interesting to note that the greatest number of new cRNA molecules appear

during hepatic regeneration at a time when gene activation is indeed taking place.

Aims

From the preceding review of the essential aspects of hepatic regeneration with special emphasis upon those factors influencing RNA synthesis, it is clear that much has been learned about the sequence of events inherent in this process and in cellular proliferation in general. There are, however, many aspects of the regeneration process that either remain unexplored or require further attention. The aims of this thesis are manifold:

1. To examine the changes in enzymatic activity of RNA polymerases I and II after the onset of regeneration.

2. To examine the changing template capacity of native liver chromatic after partial hepatectomy.

3. To examine by the use of chromatin reconstitution the role of the histones and the acidic proteins in influencing differential gene activity during the first 6 hours of regeneration.

4. To examine by long gel polyacrylamide electrophoresis whether variations occur in the amounts of the different histone fractions relative to each other and whether modifications of these fractions are observable.

5. To examine by thermal denaturation the changes in the amounts of free DNA in chromatin as regeneration

progresses.

6. To examine by circular dichroism whether changes occur in the relative amounts of α-helical content of regenerating and non-regenerating histone molecules.

7. To correlate any structural and functional observations with existing information about the physiological sequence of *in vivo* events that are known to occur during the hepatic regenerative process.

CHAPTER II

MATERIALS AND METHODS

Animals, Maintenance, Operative Procedures and Controls

1. Selection of Animals.

The rat was the animal of choice for these studies on liver regeneration. The reasons for selecting this animal for experimentation were a) its availability in large numbers; b) its ability to withstand surgical procedures; c) the inherent resistance to infections; d) its low postoperative mortality rate; e) the ease of maintenance and handling; f) the ability to survive without special postoperative care; g) its yielding of a liver remnant of sufficient size for biochemical analysis and h) the existence of an already vast literature on rat liver regeneration to which one can refer.

2. Maintenance of Animals.

Hooded-white, Long Evans rats were obtained from Charles River Breeding, North Welmington, Massachusetts. These animals were placed in plastic cages usually at five rats per cage. The cages were kept in an air-conditioned room and the animals had access to Purina rat chow and water ad libitum. A small colony of rats was maintained in the above manner. Animals selected for study were either taken from this colony or were

acquired anew from Charles River Breeding whenever colony size dwindled sufficiently. In all cases, the rats utilized for experimental purposes were male rats approximately 2-3 months old.

3. Operative and Postoperative Procedures.

Partial hepatectomies were performed according to the method of Higgins and Anderson (1931) under light ethyl ether anesthesia between the hours of 12:00 and 6:00 P.M. In order to reduce diurnal effects, the operations were staggered in time so that all animals could later be sacrificed at the same time, after having undergone various desired periods of regeneration.

Of the four main lobes of a rat liver, the term partial hepatectomy as used throughout this thesis refers to removal of the medial and left lateral lobes. The excised lobes are not restored during the process of regeneration, but, the mass of the liver is returned to normal by an increase in size of the remaining right lateral and caudal lobes.

A 3-4 cm incision was made posteriorly from the xiphoid process of the sternum through the linea alba of the ether anesthetized rats. Through this opening, the medial and left lateral lobes, comprising 67% of the liver, were delivered, ligated with course linen thread and excised. The peritoneum and abdominal muscles were then sutured closed as was the integument.

Following the operations, the animals were returned to their cages where they had access to water ad libitum but

not to food. After having undergone various periods of regeneration, all animals were sacrificed at the same time by decapitation and were then exsanguinated.

4. Controls.

Essentially, two different types of controls were utilized in these studies. In the more conventional type of control, an ether anesthetized rat underwent a sham operation in which a laporatomy, consisting of the opening of the integument, abdominal muscles and peritoneum, was performed without ligation or resection of the liver. In other cases, the excised lobes of the liver of a partially hepatectomized rat were placed immediately into ice-cold isotonic sucrose (0.32 M sucrose, 3 mM $MgCl_2$) and were treated as 0-hour non-regenerating liver samples to which the regenerating remnants could later be compared. In this way, each animal could have its own control liver segment which served as a base from which changes resulting from the process of regeneration could be assessed.

In all cases, both regenerating remnants and control liver segments were placed in ice-cold, isotonic sucrose and were either used immediately or stored frozen overnight at -20°C.

Isolation of Nuclei

Nuclei were isolated as described by Pogo *et al.* (1966) utilizing a modification of the procedure of Chauveau *et al.*

(1956). Both regenerating and control liver segments were treated in the same manner and were carried through the following steps at the same time.

The liver segments were minced finely with scissors. The minced liver was added to a volume of ice-cold, isotonic sucrose equal to ten times the weight of the liver. The tissue was then blended for 2 minutes in a Sorvall stainless-steel omni-mixer at 6000 rpm. The resulting suspension was filtered through 3 layers of cheese-cloth and was then centrifuged at 800xg for 7 minutes. The supernatant was aspirated off and the pellet was mixed with a volume of 2.4 M Sucrose-1 mM $MgCl_2$ equal to 10 times the volume of the pellet. The suspension was blended again in the omni-mixer at 2000 rpm for 2 minutes.

This crude nuclear suspension was then placed into nitrocellulose centrifuge tubes and was centrifuged for 1.5 hours at 30,000 rpm in the #30 rotor of a Beckman Model L Ultracentrifuge. The nuclear pellets were resuspended in 30 times their volume of isotonic sucrose and were centrifuged at 0°C at top speed in a clinical centrifuge for 10 minutes. This washing procedure was repeated 2 times, at the end of which time a white nuclear pellet, free from cytoplasmic contamination, resulted.

Preparation and Extraction of Chromatin

1. Chromatin Preparation.

Chromatin was prepared as described by Shelton and

Allfrey (1970). Nuclei, washed 3 times with isotonic sucrose, were then washed twice with buffered saline (0.15 M NaCl, 0.01 M Tris-HCl, pH 715) to remove saline soluble proteins. The nuclei were centrifuged at 3000 x g for 5 minutes and then were dispersed in distilled water by homogenization in a teflon-to-glass homogenizer. Chromatin was precipitated by adjusting the salt concentration to 0.15 M NaCl. The chromatin was recovered by centrifugation in a Sorvall RC2B centrifuge at 13,000 rpm for 20 minutes at 4°C. The pellet was again redispersed in distilled water, precipitated and recovered as described. The composition of the final chromatin preparation was then assayed with regard to DNA, RNA and protein.

2. Histone Extraction.

The chromatin pellet was dispersed by homogenization in 10 ml of 0.2 N HCl at 4°C and histones were extracted with stirring for 30 minutes. The extracted chromatin was recovered by centrifugation for 20 minutes at 13,000 rpm and the extraction procedure was repeated an additional two times. The three supernatants were pooled and stored at -20° C for use at a later time.

3. Acidic Chromosomal Protein Extraction.

Acidic proteins were extracted and solubilized by a modification of the procedure of Vinuela *et al.* (1967) as described by Teng *et al.* (1971).

The dehistonized chromatin pellet was dispersed by

homogenization in buffer A (0.10 M Tris-HCl, pH 8.4, 0.01 M EDTA, 0.14 M 2-mercaptoethanol) to which an equal volume of buffer-A-saturated-phenol was added. This mixture was again dispersed by homogenization. The phenol layer was removed after centrifugation for 15 minutes at 4000 rpm. This procedure was repeated again after which an additional volume of phenol was added to the phenol extracts and the combined mixture was stored for at least 14 hours at 4°C. The pooled phenol extracts were concentrated to 1/5 their original volumes by dialysis against 100 volumes of 0.1 M acetic acid, 0.14 M 2-mercaptoethanol which was changed twice. The contents of the dialysis sacs were removed and the phenol layer was separated by centrifugation and then dialyzed against 100 volumes of 0.05 M acetic acid, 9.0 M urea, 0.14 M 2-mercaptoethanol overnight. A further 2 hour dialysis against 100 volumes of 0.1 M tris-HCl, pH 8.4, containing 8.6 M urea, 0.01 M EDTA, and 0.14 M 2-mercaptoethanol restored the phenol soluble proteins to the aqueous phase. These proteins were then either used immediately or stored at -20°C.

DNA Extraction

DNA was isolated by a modification of the Marmur (1961) procedure from nuclei washed 3 times with isotonic sucrose and 2 times with buffered saline (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.5).

The nuclei were first lysed in buffered 0.15 M saline

containing 0.1 M EDTA and 2% SDS, pH 8.0. Sodium perchlorate was added to a final concentration of 1 M. An equal volume of chloroform-isoamyl alcohol (24: 1 - v/v) was added to the lysed suspension and partial deproteinization was effected by shaking for 30 minutes. The upper, aqueous, nucleic acid-containing phase was separated by centrifugation in a Sorvall centrifuge for 5 minutes at 10,000 rpm, and the nucleic acids were precipitated gently with 2 volumes of ethyl alcohol. The precipitated DNA was then dissolved in 15 ml of dilute saline-citrate (0.015 M NaCl, 0.0015 M trisodium citrate, pH 7.0).

Pancreatic ribonuclease (Sigma) that had been previously heated at 60o C for 15 minutes (to inactivate DNase) was added to a final concentration of 50 ug/ml and incubation was carried out at 37° C for 30 minutes. Pronase (Signa), autodigested at 37° C for 2 hours, was then added to a final concentration of 50 ug/ml and protein hydrolysis was allowed to continue for 2 hours at 37° C. The solution was adjusted to standard saline-citrate concentration (0.15 M NaCl, 0.15 M trisodium citrate, pH 7.0) and was then shaken again with an equal volume of chloroform-isoamyl alcohol for 15 minutes. The mixture was separated and nucleic acids ethanol precipitated as before after which the deproteinization process was repeated until maximum protein removal was achieved as judged by biuret assay.

The precipitated nucleic acids were dissolved in 9 ml of dilute saline-citrate to which 1 ml of acetate-EDTA (3.0 M

sodium acetate, 0.001 M EDTA, pH 7.0) was added. A 0.54 volume of isopropyl alcohol was added with stirring to precipitate DNA, leaving (double-stranded) RNA and oligoribonucleotides behind. This final precipitate was then washed free of acetate in progressively increasing concentrations of ethyl alcohol (70% - 95%), after which it was stored under alcohol at -16° C. The concentration of DNA was determined by diphenylamine assay and by UV analysis at 260 mu. The absence of protein and RNA was confirmed by Lowry and orcinol assays, respectively.

RNA Polymerase Isolation

RNA polymerase was isolated from Escherichia coli by the method of Burgess (1969). The isolation was carried out through step IV starting with 200 grams, net weight, of cells.

E. coli were grown to maximum stationary phase at 37° C with vigorous shaking in media containing 16 g of Difco Bacto-Tryptone, 10 g of Difco yeast extract and 5 g of NaCl per liter. The cells were harvested at 4° C by centrifugation at 10,000 rpm for 15 minutes. The unwashed cells were stored at -20° C until ready for use.

The frozen pellet was broken into small pieces and disruption of the cells was brought about by blending in a pre-chilled Waring Blender to which 500 grams of cold Superbrite 100 glass beads (Minnesota Mining & Manufacturing) and 200 ml of buffer G (0.05 M Tris-HCl, pH 7.5, 0.01 M

$MgCl_2$, 0.2 M KCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol) were added. The homogenization was carried out in a 4° C coldroom at low speed for 5 minutes and then at high speed for an additional 10 minutes. DNA was hydrolyzed by incubation of the homogenate for 30 minutes at 4° C with an added 2 ml of a solution of DNase I (Sigma) in buffer G (1 mg/ml). All succeeding steps were carried out at 4° C. After the beads had settled, the supernatant was decanted off and an additional 100 ml of buffer G was mixed with the glass beads and gently drawn through them with suction through a funnel plugged with glass wool. This filtrate and the original supernatant comprised the 500 ml of fraction I.

Fraction II (320 ml) is an amber-colored supernatant devoid of cellular debris and ribosomes formed by the centrifugation of fraction I at 30,000 rpm for 2 hours in a Beckman Model L preparative ultracentrifuge.

Fraction II was brought to a concentration of 33% $(NH_4)_2SO_4$ and was stirred for 30 minutes. The precipitate formed was removed by centrifugation at 9000 rpm for 30 minutes in a Sorvall centrifuge and the supernatant was then made up to a $(NH_4)_2SO_4$ concentration of 50%. This was stirred and centrifuged as before and the resulting precipitate, containing the polymerase activity, was resuspended in 260 ml of 42% $(NH_4)_2SO_4$ in buffer A (0.01 M Tris-HCl, pH 7.9, 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol), was stirred for 45 minutes

and was centrifuged. The pellet was dissolved in 500 ml of buffer A and this solution comprised fraction III.

Fraction III was applied to a DEAE-Cellulose (Whatman DE 52 pre-swollen diethylaminoethyl cellulose) column with a 100 ml column volume (column dimensions - 3 x 13 cm) equilibrated with buffer A. The column was washed first with 50 ml of buffer A and then with 400 ml of buffer A + 0.13 M KCl. The polymerase was eluted from the column with 300 ml of buffer C (0.05 M Tris-HCl, pH 7.0, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol) + 0.23 M KCl. Fractions comprising 10 ml each were collected. These fractions were assayed for protein content by UV analysis at 280 mμ with a Gilford Spectrophotometer. Enzymatic activity was measured utilizing assay conditions described in section 9 with 10 μg of DNA as template; enzyme was added in 0.2 ml volumes. The total assay volume was 0.4 ml.

On figure 1, tubes 1-90 contain the flow through material and tubes 91-146 contain the buffer A + 0.13 M KCl protein peak. Tubes 147-166, which contain the polymerase activity eluted with buffer C + 0.23 M KCl, were pooled (fraction IV).

Fraction IV was precipitated with 1-1/2 volumes of saturated $(NH_4)_2SO_4$, stirred for 30 minutes and centrifuged for 30 minutes at 9,000 rpm. The resulting precipitate was then dissolved in storage buffer (0.01 M Tris-HCl,

pH 7.9, 0.01 M $MgCl_2$, 0.1 M KCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, 50% glycerol) at a final concentration of 5 µg/ml at -20° C until use.

Formation of DNA-Protein Complexes

The formation of DNA complexes with either histones, phenol soluble acidic chromosomal proteins, or both histones and acidic proteins was carried out in the presence of urea according to the method of Bekhor *et al.*, (1969) under conditions used for the study of chromatin reconstitution.

Solutions of histones, acidic proteins and DNA were dialyzed overnight against 2 M NaCl, 5 M urea and 0.01 M Tris-HCl, pH 8.0. The desired solutions were then mixed at various starting ratios (described in sections 7, 10 and 11). The salt concentration was then progressively lowered by stepwise dialysis against 1, 0.8, and 0.6 M NaCl, each for a 2 hour period, and then against 0.4 M NaCl overnight; all steps were carried out in the presence of 5 M urea, 0.01 M Tris-HCl, pH 8.0. The urea was removed and the salt concentration lowered by dialysis for 3 hours against 2 changes of 0.01 M NaCl, 0.01 M Tris-HCl, pH 8.0. The reassociated complexes were then further dialyzed for 2 hours against the appropriate *in vitro* assay buffer in preparation for transcription by RNA polymerase.

Determination of Final Histone to DNA Ratios

Histones from 2 hour regenerating or from corresponding 0-hour, non-regenerating liver segments and pure DNA were dialyzed against 5 M urea, 0.01 M Tris-HCl, pH 8.0, and were then added to dialysis sacs at ratios of 0.5, 1.0 and 2.0 of histone to DNA. The concentration of DNA within these sacs was 13.5 ug/ml. The histones were reassociated with DNA as described in section 6. After dialysis against 0.01 M NaCl, 0.01 M Tris-HCl, ph 8.0, the sacs were dialyzed against 0.15 M NaCl in order to precipitate complexes of histones and DNA. The sac contents were then centrifuged at 13,000 rpm for 30 minutes and both the pellet and supernatant were analyzed for DNA and protein content by diphenylamine assay and by biuret assay respectively. In addition, aliquots of the reassociated histone-DNA complexes, after 0.01 M salt dialysis, were further dialyzed against buffer for RNA polymerase and used for transcription by *E. coli* polymerase. Other aliquots were reserved for physical studies involving thermal denaturation.

RNA Polymerase Assays on Isolated Nuclei

1. Nuclei

Nuclei were isolated hourly for six hours as described in section 2. Nuclei were obtained from both the regenerating remnants and the 0-hour, non-regenerating

segments from each animal. Diphenylamine assays were performed upon the suspensions of washed nuclei, which were then diluted down to comparable DNA concentrations.

2. Assay Procedure

0.2 ml of nuclei were added to test tubes containing an equal volume of assay buffer. The final concentrations after this dilution (in a total assay volume of 0.4 ml) were 10 mM 2-mercaptoethanol, 67 mM Tris-HCl, pH 7.9, 0.12 mM ATP, 0.15 mM GTP, 0.18 mM CTP, 0.014 mM UTP, 7.5 μC (^{3}H)-UTP (Schwarz/Mann, Specific Activity 14 C/mmole), 3.4 mM $MnSO_4$, and 67 mM $(NH_4)_2SO_4$ ionic strength = 0.2). The $(NH_4)_2SO_4$ and Mn^{2+} concentrations used in these assays were those described by Seifart et al. (1972). Studies by these authors have indicated that isolated rat liver DNA-dependend RNA polymerase I exhibits an optimal activity in the presence of 20 mM Mg^{2+} in the absence of $(NH_4)_2SO_4$, whereas polymerase II exhibits its optimal activity in the presence of 3.4 mM Mn^{2+} at a high salt concentration (67 mM $(NH_4)_2SO_4$). (See figure 1 in Appendix B from Seifart et al. (1972). It was also noted by these authors that when assays were performed under conditions in which 3.4 mM $MnSO_4$ amd 67 mM $(NH_4)_2SO_4$ were present in the absence of 20 mM Mg^{2+}, both polymerases were operating at near optimal capacities; activity is maximum under these conditions for polymerase II and is only 7% less than maximum for polymerase I. Total RNA

polymerase activity from intact nuclei was assayed in this manner.

The assay tubes were incubated for 10 minutes at 37° C. Incorporation of H^3-UMP into RNA was linear for this time period. The tubes were placed on ice and 1 ml of ice-cold bovin serum albumin (3 mg/ml) was added to them. The reactions were then stopped and the cooled assay mixtures were processed by a modification of the procedure of Pogo et al. (1966) in which 0.5 ml of cold 10% TCA, and 0.04 M sodium pyrophosphate were added to each tube followed by the addition of 13 ml of cold 5% TCA, 0.02 M sodium pyrophosphate. The acid precipitatable material was centrifuged for 6 minutes at top speed in a clinical centrifuge and was washed 6 times, by resuspending in 5% TCA, 0.02 M sodium pyrophosphate. At the end of this procedure, the retention of absorbed nucleotide precursors was negligible.

0.3 ml of 0.3 N KOH was added to these TCA washed nuclear precipitates and RNA was hydrolyzed by incubation at 37° C for 1 hour.

The tubes were centrifuged at top speed in a clinical centrifuge for 6 minutes. 0.05 ml aliquots of the supernatant were added to scintillation counting vials containing 20 ml of counting fluid. This counting fluid was prepared by adding 4 grams of New England Nuclear Omniflour and 20 ml of Beckman Bio-Solv Solubilizer (BBC-3) to 1 liter of scintillation grade toluene. Samples

were counted in a Nuclear Chicago Unilux II Scintillation Counter. Counting efficiencies were determined for each vial and results were reported as picomoles of H^3-UMP incorporated into RNA/minute/mg of DNA.

3. α-Amanitin Inhibition

0.2 ml aliquots of nuclei, obtained from regenerating and corresponding 0-hour, non-regenerating liver segments for each hour through hour 4 were also incubated as described above in the presence of α-amanitin. This toxin from the toadstoool Amanita phalloides was first shown by Stirpe et al. (1967) to inhibit nuclear RNA synthesis. α-amanitin (Sigma) was present in the assay tubes at a final concentration of 5 µg/ml; by examination of figure 2 of Appendix B from Seifart et al. (1972) it can be seen that this concentration is sufficient to completely inhibit polymerase II activity. By subtracting the results of the α-amanitin inhibited assays for experimentals and controls from their corresponding uninhibited assay results, it was possible to determine the α-amanitin resistant and the α-amanitin sensitive incorporations in each case and thus to determine the change in activities of these enzymes during the course of the regenerative process.

Transcription of Chromatin

1. Chromatin

Native chromatin was transcribed with exogenous E. coli

RNA polymerase by a modification of the procedure of Pogo et al. (1966). Chromatin was isolated hourly for 6 hours as described in section 3 from regenerating and corresponding 0-hour, non-regenerating liver segments. The chromatin in these experiments was examined spectrophotometrically for the presence of aggregates; only solutions in which the ratio of the optical densities at 320/260 mu was less than 0.05 were used in assays of template activity. Analysis of the composition of this chromatin revealed a DNA:RNA: Protein ratio of 1.0:0.08:1.7 In all cases, chromatin was either prepared and assayed immediately after the isolation of nuclei or was stored at -20° C and assayed the next day. After diphenylamine analysis, these chromatin preparations were diluted to DNA concentrations of 135 µg/ml.

2. Assay Procedure

27 µg of DNA template in chromatin were standardly used in these assays. Periodically, the polymerase preparation was tested by monitoring transcription of DNA at different DNA: polymerase ratios. Exogenous polymerase was always used in these chromatin assays in saturating amounts. Chromatin samples were dialyzed against assay buffer prior to their addition to the assay tubes. The assays were performed in a total volume of 1 ml which contained final concentrations of 0.05 mM 2-mercaptoethanol, 16 mM Tris-HCl, pH 8.3, 6.8 mM $MgCl_2$, 20 mM KC l, 0.02 mM EDTA, 200 mM sucrose, 48 µM ATP, 63 µM GTP, 70 µM CTP, 5.4 µM

UTP and 7.5 μCuries of H^3-UTP (specific activity 14 Curies/mmole). Incubation was for 10 minutes at 37°C; the reaction rate was linear for this time period. The reactions were stopped and the transcripts were processed and counted as described in section 8.

Transcription of Histone-DNA Complexes

Histones were reassociated with DNA as described in section 6 at a histone to DNA starting ratio of 1.0 by weight. Histones were extracted hourly for a period of 6 hours, as described in section 3, from regenerating and corresponding 0-hour, non-regenerating liver segments. The concentration of complexed DNA in the assay tubes was 6.25 μg/ml. Transcription of the complexes with exogenous *E. coli* RNA polymerase and processing of the transcripts were performed as described in section 9.

The complexes described in section 7, formed at varied histone to DNA ratios utilizing 2-hour, regenerating histones and 0-hour, corresponding non-regenerating histones, were also transcribed in the same manner, however, the concentration of DNA in the assay tubes was 13.5 μg/ml.

Transcription of Reassociated Histone-Acidic Protein-DNA Complexes

In this group of experiments, histones and acidic proteins were extracted hourly for 6 hours during

regeneration as described in section 3. Histones and acidic proteins were also extracted in the same manner from control, sham-operated animals, described in section 1. In order to establish maximum conditions of histone inhibition of template activity, control histones and histones from different hours in regeneration were reassociated with DNA at a starting ratio of 2.0 (histone/DNA by weight) in the presence of acidic proteins from control animals in starting ratios of 0.6 (acidic protein/DNA by weight).

In another series of experiments, utilizing the same starting ratios of histone: acidic protein: DNA, control acidic proteins and acidic proteins extracted at different hours in regeneration were reassociated with DNA in the presence of histones extracted from control animals.

In a third series of experiments, acidic proteins alone, extracted from control animals and from animals at different times in regeneration, were reassociated with DNA at a starting ratio of 0.6 (acidic proteins/DNA by weight).

In the last of these series of experiments, histones alone, extracted from control animals and from animals at different hours in regeneration, were reassociated with DNA at a starting ratio of 2.0 (histone/DNA by weight).

In each of the above series of experiments, chromosomal proteins were reassociated with DNA as described in section 6. Transcription and processing of the transcripts were as

described in section 9. The concentration of complexed DNA in the assay tubes was 13.5 µg/ml.

High Resolution Polyacrylamide Gel Histone Electrophoresis

1. Sample Preparation

Histones, extracted as described in section 3, were obtained hourly from the pooled acid supernatants of pairs of rats whose livers had undergone various periods of regeneration; the corresponding 0-hour, non-regenerating excised liver pieces served as controls from which histones were also obtained from each pair of rats. The histones from controls and experimentals were precipitated by the addition of TCA (final concentration 18%, w/v), and were centrifuged for 30 minutes at 13,000 rpm. This was followed by washes with acetone. The histones were dissolved in 0.9 N acetic acid at a concentration of 3300 µg/ml. They were then dialyzed at 25° C for 18 hours against 0.5 M 2- mercaptoethanol, 8 M urea, 0.9 N acetic acid in order to break disulfide bonds formed in the F3 component which, if not broken, upon electrophoresis can give rise to a slow moving band at the expense of the F3 band. Histones were then dialyzed against 0.9 N acetic acid, pH 2.8, 2.5 M urea, 0.5 M 2-mercaptoethanol overnight and this final dialysate was brought to a sucrose concentration of 15%.

2. Gel Components

Electrophoresis was performed on 15% polyacrylamide gels

in 2.5 M urea, 0.9 N acetic acid, pH 2.8, according to the Panyim and Chalkly (1969) modification of the method of Reisfeld et al. (1962) utilizing Canalco reagents. These gels were formed from the following solutions: 2 parts solution A (60% acrylamide and 0.4% N, N'-bisacrylamide - w/v - in water), 1 part solution B (43.2% glacial acetic acid, v/v, and 4% N,N,N',N'-tetramethylethylene-diamine, w/v, in water), and 5 parts of solution C (0.2% $(NH_4)_2S_2O_8$, w/v, in 4 M urea, freshly prepared). Solutions A and B were stored at 0° C prior to use.

3. Gel Formation

In order to prevent the formation of bubbles in the gels as they polymerized, the three stock solutions were centrifuged at top speed in a clinical centrifuge for 20 minutes and then stored for 1 hour at room temperature in a vacuum desiccator. The gels were allowed to polymerize anaerobically for 90 minutes after overlayering with 3 M urea. 27 cm gels were cast in 30 cm tubes (0.4 cm inner diameter).

4. Pre-electrophoresis

All gels were pre-electrophoresed at 200 volts, utilizing an LKB constant voltage power supply, until the dye benzene-azo-α-naphthylamine had been completely eluted. This dye which carries a single positive charge migrates at the rate of 1.65 cm/hr. at 2 milliamps/tube and has a molecular weight greater than that of the other ions

present in the gels. The tray buffer consisted of 2.5 M urea, 0.9 M acetic acid, pH 2.8. Pre-electrophoresis was performed as a standard part of the technique in order to affect the removal of $(NH_4)_2S_2O_8$, the oxidant present in the gels, which can cause the formation of disulfide bonds in the F3 component.

5. Histone Electrophoresis, Gel Staining, and Scanning

20 µl of the sucrose containing histone dialysate (~100 µg) were applied to the pre-electrophoresed gels, and benzene-azo-α-naphthylamine which runs 1.5 times ahead of the F2a1 component, was added as a tracking dye. The histones were electrophoresed at 4° C at 250 volts for 26 hours, after which time the gels were cracked out of their tubes and were stained for 1 hour in 1% Fast Green, 7% acetic acid. Destaining was by diffusion against numerous changes of 7% acetic acid until background dye had been removed. The gels were then scanned with a Gilford Model linear transport scanning apparatus. These scans were used to assess qualitative changes in histone fractions, particularly changes in acetylation and phosphorylation of histone components. The scanned peaks were also cut out and weighed on a Mettler balance in order to determine whether quantitative changes had occurred between histones from regenerating and non-regenerating liver segments.

Thermal Denaturation and Circular Dichroism

In these studies, native chromatin and/or complexes

described in sections 7 and 10 were subjected to thermal denaturation, circular dichroism or both. These experiments were performed on samples from the hourly removed regenerating liver segments and upon their corresponding 0-hour, non-regenerating counterparts. All samples were dialyzed against 2.5×10^{-4} M EDTA, pH 8.0.

1. Thermal Denaturation

Thermal denaturation measurements were made using a Gilford Spectrophotometer, Model 2400-S, coupled to a temperature regulator with a constant heating rate of $\sim 2/3^{\circ}$ C per minute. The first derivative (dh/dT plot of the thermal denaturation profile (as employed by Li and Bonner, 1971) was used to describe the melting, where h (hyperchromicity) is the percent increase in absorbance with heating (with reference to the A_{260}) and T is the temperature in degrees centigrade. The area under these curves in the regions of free and of bound DNA could then be integrated and a comparison could then be made between the percentages of free melting vs. bound DNA present in experimental and control samples at each hour during the regenerative process.

2. Circular Dichroism

CD spectra were taken at room temperature on a Jasco Spectropolarimeter, Model J-20. They are reported as $\Delta\epsilon = \epsilon_L - \epsilon_R$, where ϵ_L and ϵ_R are molar extinction coefficients for the left and right-handed circularly polarized light.

The units of $\Delta\epsilon$ are $M^{-1}cm^{-1}$ in terms of nucleotide. The contributions of light scattering to the A_{260} were corrected for according to the method of Leach and Scheraga (1960). The $\Delta\epsilon_{220}$ readings were obtained at each hour for regenerating and non-regenerating samples. These values were plotted in terms of $\Delta\epsilon_{220}R / \Delta\epsilon_{220}N$ with time. In this way relative increases or decreases in secondary structure would be determined for R / N (regenerating/ non-regenerating) at each point during the regenerative process.

Assay Procedures for DNA, RNA, and Protein

1. DNA Assays

DNA content was measured in preparations of isolated DNA, nuclei and chromatin by the method of Burton (1952) and/or by UV analysis at 260 mμ.

2. RNA Assays

RNA content was measured in preparations of chromatin and isolated DNA by orcinol assay according to the method of Dische (1955).

3. Protein Assays

Protein content was measured diehter by the biuret assay of Crampton et al. (1955) by Lowry assay (Lowry et al. 1951) and/or by UV analysis at 280 mμ.

For the above assays bovine serum albumin (Sigma) was used as a standard.

CHAPTER III

RESULTS AND DISCUSSION

Transcriptional Activity of Isolated Nuclei During Regeneration

One of the aims of this thesis is to compare the *in vitro* transcriptional activity of regenerating liver cells at different times during the initial hours after partial hepatectomy.

The first group of experiments was performed utilizing isolated nuclei. Table 1 shows the H^3-UMP incorporation into RNA at various times after partial hepatectomy. Figure 2 is a graphic representation of these values. It can be seen that the rate of incorporation increased gradually over a period of three hours and then leveled off to form a plateau through hour 6. This is in good agreement with the *in vivo* results of Bucher *et al.* (1969). One point of difference to be noted is in the height of the plateaus reached in both cases. Under *in vivo* conditions, the rate of incorporation at the plateau is 1-1/2 - 2 times that of the non-regenerating liver cells. In Figure 2, the maximum rate of incorporation *in vitro* is shown to be approximately four and one-half times that of the 0-hour nuclei. This difference is easily explained by the fact that in these *in vitro* experiments optimum conditions for both polymerases I and II were employed.

Although studies of alterations in total nuclear transcriptional activity are of interest, eucaryotic cells are unique in that they possess multiple RNA polymerases each of which may respond differently to various physiological situations (Lewis, 1974). The localization of polymerase I in the nucleolus and of polymerase II in the nucleoplasm has been verified by both autoradiographic (Moore and Ringertz, 1973) and nuclear fractionation techniques (Roeder and Rutter, 1970 a,b). In addition, work done with isolated nuclei has indicated that polymerase I produces a product similar in composition to rRNA which competes well with rRNA whereas polymerase II produces a product similar in composition to DNA and competes well with transcripts of DNA (Lindell et al., 1970). Purified polymerase I has been found to prefer native DNA as a template while polymerase II prefers denatured DNA. The preference of polymerase II for native DNA tends to increase either when "crude" polymerase preparations are used or when factors obtained during the isolation of polymerase II are added back to the purified enzyme preparation (Lentfer and Lezius, 1972; Stein and Hausen, 1970; Sugden and Keller, 1973).

In order to gain a more in-depth look into the onset of the regenerative process, an attempt was made to study the individual roles played by these multiple RNA polymerases. Intact nuclear polymerase systems present within isolated nuclei were employed. The inhibitor α-amanitin

was used to distinguish between the α-amanitin sensitive and α-amanitin insensitive incorporations of H^3-UMP into RNA. The α-amanitin sensitive and insensitive incorporations were followed in regenerating liver nuclei during the first four hours post partial hepatectomy in order to ascertain whether these incorporations increase concomitantly or whether an increase in one precedes the other.

In Figure 3, the hourly percent changes in incorporation by nuclei isolated from regenerating liver remnants are expressed with reference to those of the corresponding incorporations for the 0-hr., non-regenerating resected liver segments. It can be seen in Figure 3 and in Table 2 that α-amanitin sensitive incorporation showed a 144.4% increase by one hour post partial hepatectomy. Total incorporation and α-amanitin insensitive incorporation, on the other hand, exhibited no observable changes this early in the regenerative process. After three hours post partial hepatectomy, all incorporations studied increased at about the same rate. It thus appears that although all respective incorporations increase rapidly after partial hepatectomy, increases in α-amanitin insensitive H^3-UMP incorporation into RNA occur ahead of the others. In view of the fact that polymerase II has been associated with the transcription of chromosomal DNA, it is of interest to note that at one hour post partial hepatectomy in mice, Church and McCarthy (1967) reported that the

maximum number of new RNA species different from those present in non-regenerating livers are shown to appear. The early increase in α-amanitin insensitive H^3-UMP incorporation is of interest in that it may explain part of the mechanism by which stretches of DNA which supply the information for the production of enzymatic activities required during regeneration are transcribed.

Strong evidence already exists that a product of the nucleoplasmic polymerase II is required for the continued functioning of the nucleolar polymerase I. Yu and Fiegelson (1972) have shown that cycloheximide affects nucleolar polymerase activity but not nucleoplasmic activity; actinomycin D was also shown to cause a decline in polymerase I. They concluded that both polymerase I and its messenger had rather short half-lives. Moreover, the work of Tata et al. (1972) and of Jacobs et al. (1970) have indicated that a direct relationship exists between polymerase II and polymerase I activities. These workers have demonstrated that only polymerase II activity is inhibited by α-amanitin in normal isolated nuclei. When, however, this toxin is injected into live animals, the nucleoplasmic enzyme and then approximately two hours later the nucleolar enzyme are affected.

It appears then that polymerase I activity may depend upon continued transcription by polymerase II. During regeneration, augmented polymerase I activity might in a similar manner also depend upon the prior increase in activity of polymerase II.

Template Activity of Regenerating Rat Liver Chromatin

In order to distinguish between the effects of the process of regeneration on the template capacity of chromatin as opposed to those effects exerted on the polymerases, chromatins, isolated hourly from regenerating liver remnants and from the corresponding non-regenerating resected segments, were transcribed with exogenous E. coli RNA polymerase. It should be noted that in the system used here for transcribing liver chromatin with the procaryotic enzyme, ionic conditions were employed under which chromosomal proteins are not removed and protein exchange is reduced to a minimum (Cedar and Felsenfeld, 1973; Clark and Felsenfeld, 1971). Reinitiation can occur (Jenkins et al., 1971; Chamberlin and Ring, 1972), and contributions to the observed template activity by exogenous polymerases are reduced to a minimum (in a low salt system in the absence of Mn^{++}(Cox, 1973). Any incorporation observed from chromatin in the absence of exogenous enzyme was subtracted from the results. It can thus be concluded that differences due only to chromosomal proteins will be investigated in this section.

Table 3 and Figure 4 show clearly that peak increases in template activity of regenerating chromatins over the respective controls occur at two different times. The three-hour point in Figure 4 was significantly different from both the 2 and 4 hour points. A 50.1% increase occurs at 2 hours and an 89.3% increase occurs at 4 hours post

partial hepatectomy. When chromatin template activities are displayed as percentages relative to purified rat liver DNA (Table 3), it can be observed that with the controls at 2 and 4 hours, respectively, these percentages are 6.5% and 12.1% whereas with the regenerating chromatins these percentages are 9.7% and 22.9%. It thus appears that at 2 and 4 hours post partial hepatectomy relatively more DNA is free for transcription by the bacterial enzyme thus accounting for the large increases in template activity at these two times in the regenerative process.

In view of the fact that the above *in vitro* results may be open to criticism because they were obtained using a procaryotic polymerase, a discussion of the relevance of the system employed will follow.

Many reports in the literature attest to the validity of using *E. coli* RNA polymerase to transcribe eucaryotic chromatin in order to produce a specific gene product. One method has involved the use of cDNA probes made with reverse transcriptase (as discussed in the Introduction). These probes have been used to demonstrate specifically that the procaryotic polymerase can successfully transcribe DNA sequences normally associated with various eucaryotic cell types. This has been established independently by Axel *et al.* (1973), Gilmour and Paul (1973); Steggles *et al.* (1974) and by Crouse *et al.* (1976) for the transcription of globin genes in chromatin of erythroid tissues but not by chromatin from other sources.

Of particular importance to the issue of the use of

E. coli polymerase for eucaryotic chromatin transcription are hybridization experiments in which unique SV40 viral genomes have been integrated into eucaryotic SV3T3 chromatin. Susan Astrin (1973) has shown that chromatin from transformed cells can be transcribed by *E. coli* RNA polymerase to yield early viral transcripts that hybridize to the correct "minus" strand (DNA strand that serves as a template for early RNA). Moreover, *in vivo*, only 70% of the "minus" strand is transcribed. The *same* degree of restriction was noted when the procaryotic polymerase was used to transcribe chromatin from transformed cells. The *E. coli* polymerase in this system has thus been shown to function faithfully by virtue of its ability to asymetrically transcribe the correct strand and to observe the appropriate degree of template restriction. Since the procaryotic polymerase will transcribe the entire "minus" strand from purified viral DNA, it is evident that the failure to transcribe all of this strand from chromatin *in vitro* cannot simply be due to the fact that *E. coli* polymerase and the eucaryotic polymerase recognize different initiation sites. Results similar to the above have been independently obtained by Shih *et al.* (1973) using SV40 and by Jacquet *et al.* (1974) with Avian Myelobastosis viral RNA sequences.

Additional evidence of the usefulness of *E. coli* polymerase with eucaryotic chromatin comes from experiments

utilizing intact nuclear transcriptional systems. Smith and Huang (1976) have observed that nuclei from myeoloma 66.2 tissue cultures can produce immunoglobin kappa light chain mRNA whereas nuclei from MOPC315 tissue cultures do not. *E. coli* RNA polymerase was shown to successfully produce this mRNA with chromatin from 66.2 but not with MOPC315.

One of the major objections to the use of *E. coli* RNA polymerase for eucaryotic transcription has been the claim that transcripts made with this enzyme are decidedly shorter than those produced *in vivo* (Maryanka and Gould, 1973). Using a sensitive test for RNase activity, Cox (1973) has shown that these discrepancies in size were due to a greater amount of RNase activity than had been suspected by past workers. In addition, by using high concentrations of heparin in his heterologous system, Cox was able to show that transcripts produced *in vitro* by *E. coli* RNA polymerase did not exhibit the small size that had been previously reported when such transcripts were compared to their *in vivo* counterparts.

The work of Cedar and Felsenfeld (1973) involved the use of *E. coli* RNA polymerase to transcribe eucaryotic chromatin as well as DNA. They observed that the number of polymerase binding sites capable of initiation was about 10 times greater with DNA than for chromatin. This result resembles the situation *in vivo*, in which approximately 10% of the genome is involved in making RNA.

Using the E. coli polymerase, Crouse et al. (1976) have been able to determine the percentage of the reticulocyte genome that undergoes transcription. Critical to this determination, however, was the problem of whether in vitro transcription of specific genes from chromatin demonstrates specificity or is the result of random genomic transcription. An important problem here is that endogenous chromatin globin RNA is indistinguishable from that formed in vitro when assayed by globin cDNA hybridization. This detriment was overcome by producing mercury-labeled in vitro transcription products which could be isolated by column chromatography. This technique was used to verify that one tenth as many globin RNA sequences were transcribed by the procaryotic polymerase from erthrocyte chromatin than could be produced from reticulocyte chromatin. Of particular relevance to this discussion is the fact that Crouse et al. (1976) were able to use the heterologous system described above to calculate correctly the percentage of the reticulocyte genome being transcribed. The 2% figure that they arrived at agreed well with the number of reticulocyte chromatin initiation sites expressed as a percentage of the number for DNA (2.5-3.5%). In particular, the results of Crouse et al. (1976) also agreed with the work of McConoughy and McCarthy (1972) who found 2% of the reticulocyte genome to be transcribed in vivo. Crouse et al. (1976) therefore concluded that "Although a definitive assessment will require measurements with the homologous enzyme, the observation that the erythrocyte chromatin supports so little, if any, globin synthesis compared

to reticulocyte chromatin strongly suggests that the essential specificity has been retained in the present experiment. . . ."

In spite of the work mentioned above, other investigators have come to the conclusion that heterologous systems are invalid by virtue of the fact that eucaryotic and procaryotic RNA polymerases appear to recognize and utilize different initiation sites. Such claims have been made by Butterworth *et al.* (1971) who observed that template activities for procaryotic and eucaryotic polymerases were additive and not competitive in nature on a particular template. They concluded that different localized areas on the template were being used by each enzyme and that different initiation sites must exist for each. Meilhoc and Chambon (1973) came to the same conclusion as a result of work indicating that different rifamycin derivative AF/013 protection sites existed for procaryotic RNA polymerase when transcribing calf thymus DNA than for purified RNA polymerase II when transcribing this same template. They did point out, however, that there need not necessarily be any equivalence between protection sites and initiation sites. Work by Reeder (1973) (described in the Introduction) has also indicated that transcription of rRNA genes by *E. coli* RNA polymerase may be abberant. Lastly, enzymological experiments performed by Keshgegian and Furth (1972) have indicated that *E. coli* RNA polymerase binds equally well to DNA or to chromatin but that more

polymerase molecules will engage in transcription on the DNA template (Km was the same with both templates but Vmax was different). The eucaryotic polymerase II was shown to bind in greater amounts to DNA than to chromatin but it was capable of transcribing any region to which it was bound (Km was different for DNA and chromatin but Vmax was the same for both).

All of the experiments discussed which have led to the conclusion that the procaryotic and eucaryotic polymerases are decidedly different from each other suffer from a common fault. The purified eucaryotic enzymes used in these studies may well be mere artifacts of the isolation procedure. Firstly, it is known that purified polymerase II has a preference for denatured DNA. Numerous groups have shown that the addition of a factor removed during purification or that the use of a less purified polymerase preparation will increase the preference of polymerase II for native DNA (Lentfer and Lezius, 1972; Stein and Hausen, 1970; Sugden and Keller, 1973). Secondly, information exists that purified polymerase II may be lacking specific initiation factors (Chuang and Chuang, 1975). Thirdly, it is now becoming increasingly clear that eucaryotic polymerases are highly complex in that they contain many minor subunits (Sklar et al., 1975). Evidence exists that the loss of even one or two of these subunits can drastically change the functioning of these enzymes (Huet et al., 1975). It thus appears that workers who make claims about purified

eucaryotic polymerases must first demonstrate that they are indeed dealing with legitimate enzymes.

Of particular importance with regard to determining the usefulness of E. coli RNA polymerase in transcribing eucaryotic chromatin is the fact that in systems in which it does not function effectively, neither will the purified homologous enzyme. In these cases, the equal ineptitude of both enzymes stresses the general inadequacy of the particular system and does not imply that one enzyme is any better than another. This is exemplified by the work of Wilson et al. (1975). These investigators, by the use of cDNA probes complimentary to anti-strand globin sequences, have demonstrated that unique globin sequences are transcribed asymetrically in vivo. In vitro transcription of erythroid chromatin by either purified eucaryotic or procaryotic polymerase, however, yielded symetrical transcription. Similar results have been reported in vivo for the ribosomal genes of X. laevis which have been shown both by electron microscopic and by hybridization studies to be transcribed asymetrically. Honjo and Reeder (1974) have found that rRNA genes are transcribed symetrically from chromatin templates in vitro by the purified eucaryotic or procaryotic enzymes.

The only well documented restricting influences on the ability of E. coli polymerase to utilize eucaryotic chromatin seem to be chromosomal proteins. Shih and Bonner (1970) have demonstrated that DNA complexed with

polyarginine or protamine is quantitatively blocked from acting as a template in support of RNA synthesis. They concluded that only the free DNA portion of such preparations can serve as a template. A similar situation has been shown to exist for chromatin (Marushige and Bonner, 1966). Under these circumstances, E. coli RNA polymerase can therefore serve as a probe in attempting to determine whether different chromatin preparations are relatively more or less template active by virtue of their restriction by chromatin proteins.

In this thesis a probe such as this was used to determine the template activity of liver chromatin after the onset of hepatic regeneration. It is apparent from the work already mentioned that in every case reported E. coli RNA polymerase has been able to transcribe specific DNA sequences normally transcribed by eucaryotic polymerases in particular cell types. In view of this, the results in this and in other sections obtained using this enzyme should reflect the degree of restriction imposed by chromosomal proteins on transcription.

Reconstituted DNA-Chromosomal Protein Complexes

In an attempt to gain some insight into the reasons for the two peaks of template activity at 2 and 4 hours post partial hepatectomy, reconstitution experiments were performed. Although this technique has been criticized in the past (Chae et al., 1975), reconstituted chromatin has proven to be remarkably similar to native chromatin

in many respects. Quantitative and qualitative aspects of gel profiles of chromosomal proteins, binding of reporter molecules, extractability of histones, numbers of initiation sites, CD spectra, free DNA ends and availability of histone genes for transcription are parameters which have all been found to be quite alike in both native and reconstituted chromatin (Kleinsmith *et al.*, 1976).

The first group of reconstitution experiments were performed in order to determine the degree of inhibition of template activity obtained with nucleohistone complexes of varying histone to DNA ratios. As can be seen in Table 4 and Figure 5, the formation of DNA-histone complexes with increasing amounts of histone leads to an increasingly more restricted template when transcription is performed with *E. coli* RNA polymerase. The actual amounts of histone bound in the final complexes at various starting ratios are presented and are very similar to those reported by Paul and More (1972 and 1973). At physiological 1:1 starting ratios by weight of histone to DNA, the nucleohistone complexes exhibited 62.6% of the template efficiency of free DNA. When, however, the starting histone to DNA ratio was raised to 2:1, the complexes appeared to be virtually inactive in directing RNA synthesis. Based on these results, succeeding experiments were performed utilizing nucleohistone complexes reconstituted at 1:1 starting ratios by weight of histone to DNA which thus allow transcription to occur. Other experiments (discussed later) were performed in which histones, acidic chromosomal

proteins and DNA were reassociated. In these experiments the purpose was to determine whether phenol soluble acidic proteins extracted at different hours during regeneration could derepress chromatin containing histones extracted from sham-operated, non-regenerating livers. In this case, reconstitution was performed utilizing starting weight ratios of 2:0.6:1 of histone: acidic protein: DNA, respectively. In this way, the ability of physiological amounts of acidic chromosomal proteins from regenerating livers to derepress chromatin reconstituted in the presence of maximally inhibitory amounts of histone could be determined.

Table 5 shows the results of experiments in which the template activities of regenerating and non-regenerating complexes of nucleohistones were assayed. The starting ratio of histone to DNA was 1.0. Each template activity is expressed as a percentage relative to that of purified DNA. Figure 6 is a bar graph of the template activities of the complexes formed with histones extracted hourly after partial hepatectomy; the template activities of the complexes formed with the corresponding 0-hour histones are also presented. From this figure and from Figure 7 it can be seen that the largest percent increase in template activity was obtained with 2 hour regenerating histones. No four-hour peak in activity is observable.

Table 6 shows the results of two experiments in which DNA-histone-acidic protein complexes were formed utilizing

starting weight ratios of 1.0:2.0:0.6, respectively. The histones were obtained from regenerating rat liver nuclei isolated hourly after partial hepatectomy. The phenol soluble acidic chromosomal proteins in all of the complexes were obtained from the same sham-operated control rat for each experiment. Figures 8 and 9 show that the greatest incorporation of H^3-UMP into RNA was obtained with complexes containing histones extracted at 1.5-2.0 hours post partial hepatectomy. Figures 10 and 11 indicate that this time period also shows the largest percentage increase in template activity over control values. It can be seen in the third column of Table 6 that the templates were highly restricted to values of approximately 5.0% of those for free DNA except at or near the time period mentioned at which activation was taking place. Once again, it is evident that no four-hour peak was obtained. It thus appears that histones extracted at 1.5-2.0 hours post partial hepatectomy do not restrict template activity to the same extent as histones obtained at other hours. This result could be explained by the presence of a co-extracted non-histone activator substance formed at this time, by histone modification, by the presence of a DNase that produces more free ends, or, more probably, by the presence of a histone degrading proteolytic enzyme which is either activated or synthesized <u>de novo</u> during the period of increased template activity. The last possibility mentioned appears more plausible in view of the fact that

a protease, although present with the 1.5-2.0 hour histones, at what appears to be a physiologically relevant time, would have an opportunity during the long reconstitution procedure to degrade sizable amounts of histone proteins and thus produce an unphysiologically high peak of template activity. This is indicated by comparison of the large percentage increases (842% and 662%) in activity that occurred in the presence of the phenol-soluble acidic chromosomal proteins with those increases observed with native chromatin (50.0%) in Table 3 and with nucleohistone complexes (32.1%) in Table 5 both extracted at 1.5-2.0 hours post partial hepatectomy. In the case of the native chromatins, there was no prolonged period of reconstitution, and, in the case of the nucleohistone complexes, the reconstitution time was exactly the same as that for the DNA-histone-acidic protein complexes. In both of these latter instances, however, the percentage increases were much smaller. Nevertheless, it is still possible that the unphysiological height of the peaks observed in Figures 8 and 9 could be explained by the presence of a histone degrading enzyme normally associated with histones obtained 1.5-2.0 hours after the onset of hepatic regeneration. One may also speculate that the presence of only the phenol-soluble acidic chromosomal proteins may have caused the histones to become more accessible to proteolytic attack. This is most evident in the case of the nucleo-histone complexes where no phenol-soluble acidic chromosomal

proteins were present and consequently the smallest increase in template activity was observed (and therefore possibly the lowest amount of histone degradation occurred) (Himes et al., 1969). In the case of the native chromatins where less phenol soluble acidic protein was present when compared with the reassociated DNA-histone acidic protein complexes, thereto a smaller peak of template activity was observed. The hypothesis regarding the effect of the presence of a histone proteolytic enzyme during regeneration is further explored in other sections of this thesis.

One additional point that should be mentioned is the fact that the exposure of such a proteolytic enzyme to a denaturing agent like 5 M urea, necessary during the reconstitution procedure, need not denature the enzyme. As pointed out by Allfrey (1974), carbonic anhydrases B and C regain almost complete activity after urea treatment. Likewise, other instances of this phenomenon include chymotrypsin A and bovine serum albumin which regain their respective enzymatic activity and immunological properties after exposure to phenol (Carlsson et al., 1973; Pusztai, 1966).

Additional investigations were carried out to examine the nature of the four-hour peak of template activity. Table 7 shows the results of two reconstitution experiments in which DNA-histone-acidic protein complexes were formed using the same starting ratios and conditions of transcription as described for the experiments in Table 6. In

this case, however, the phenol soluble acidic proteins were extracted hourly after partial hepatectomy while the histones were extracted from the same sham-operated control animal for each experiment. Figures 12 and 13 clearly show a peak of incorporation of H^3-UMP into RNA by complexes containing acid proteins extracted at four hours post partial hepatectomy. Figures 14 and 15 indicate that the greatest percentage increase in activity over control values occurred with acidic proteins from hour 4. Column 3 of Table 7 shows that when template activity is expressed as a percentage relative to that for purified DNA, in both experiments, the percentage at hour 4 was approximately double that obtained for the other hours studied. Also, conspicuously evident is the absence of a 15-2.0 hour peak of template activity. Since only control extracted histone was used here, there may be no histone proteolytic enzymatic activity present.

In order to further investigate the nature of the 4 hour peak in template activity, another experiment was performed in which complexes were formed with DNA and phenol soluble acidic proteins hourly extracted during regeneration. The results in Table 8 illustrate a phenomenon first reported by Teng *et al.* (1971). They showed that with DNA-acidic protein complexes, template activities in all cases were greater than that for DNA alone. Figure 16 indicates that the ability of the non-histone proteins, extracted at different hours post partial hepatectomy, to enhance template activity is rather uniform.

Figure 17 shows the percentage increase of the regenerating complexes relative to their controls. As indicated in Figure 16 the apparent peak with the 4 hour proteins is not significant.

The 4 hour peak in template activity observed with native chromatin could thus be explained by the ability of the acidic proteins to relieve template restrictions imposed by the histones and/or by the positive control mechanism mentioned above. When the template activity of the 0-hour DNA-acidic protein complex, expressed as a percentage relative to DNA, is subtracted from that of the 4 hour DNA-acidic protein complex a difference of 16.4% results. When the template activity of 0-hour native chromatin (Table 3), expressed as a percentage relative to DNA, is subtracted from that of its 4 hour regenerating counterpart, a difference of 10.8% is obtained. It thus appears that the positive control mechanism alone could account for the peak in template activity occurring at 4 hours. It would not be inconsistent with the results presented above, however, to postulate a mechanism involving a combination of both relief of histone inhibition plus enhancement of transcription by positive acidic protein control factors.

Of particular interest with regard to both the 2 and 4 hour peaks of template activity is a paper by Mayfield and Bonner (1972) concerning the presence of cRNA during hepatic regeneration. As described in the Introduction, numerous reports exist in the literature of cRNA, covalently bonded to acidic proteins, being involved with gene regulation. HnRNA has been considered to be a possible precursor for cRNA (Holmes et al., 1971). The report by Mayfield and Bonner (1972) describes an increase at 2 hours post partial hepatectomy in HnRNA sequences not found in normal livers. At 4 hours post partial hapatectomy, they discovered the presence of increased amounts of cRNA also containing sequences not present in cRNA of normal livers. They suggested the existence of a precursor-product relationship between HnRNA at 2 hours and the cRNA at 4 hours post partial hepatectomy. In addition, Holmes and Bonner also suggested that cRNAs present at 4 hours might function as activator molecules for gene regulation as implied in the Britten-Davidson Hypothesis (1969). If such cRNA activator molecules are co-extracted with 4 hour regenerating acidic proteins by virtue of their covalent attachment, then, they could possibly account for the ability of the phenol soluble acidic proteins to alter template activity in the manner described in this section.

Electrophoresis of Histones Extracted at Different Hours Post Partial Hepatectomy

In the preceding section, histone degradation has been suggested as the reason for the 2 hour peak in template activity. In order to establish in a more direct way whether this hypothesis is correct and if all histones or particular histone fractions are affected, histones were extracted hourly from regenerating liver remnants and from the corresponding 0-hour non-regenerating resected segments. The histones were then electrophoresed on long polyacrylamide gels and quantitated by desitometric analysis.

The hourly percentage changes of all histone fractions relative to their respective control values are presented in Figure 18 and Table 9. With regard to the F1 fraction, Figure 20 and Table 9 reveal that F1 histone extracted from the chromatin of nuclei isolated from 2 hour regenerating liver remnants comprises 11% of the total histones. The F1 histones extracted from the chromatin of nuclei isolated from the corresponding 0-hour resected segments comprise, however, 24% of the total histones. This 13% decline in the F1 fraction 2 hours post partial hepatectomy constitutes a 54% decrease when compared with the 0-hour control (Figure 18A). If the F1 fraction is taken to comprise approximately one-fifth of the total histone compliment, then a 13% decline in this fraction should be consistent with an approximate 2.6% increase in unbound

DNA normally associated with the very lysine-rich component. Reference to Table 3 indicates that with 2 hour regenerating native chromatin, a difference of approximately 3.2% more template activity was obtained when template activity relative to that for DNA is compared. It thus appears that the increase in template activity observed at 1.5-2.0 hours may have resulted from a corresponding decline in F1 histone at this time in regeneration.

These electrophoretic results differ from those reported by Garrard and Bonner (1974). They reported a 12.5% increase in the rate of F1 histone turnover in regenerating livers but did not detect differences in the amounts of the five major histone fractions relative to each other. They detected a 7% decrease in the histone to DNA mass ratio which they attributed to an overall effect exerted upon each of the histone fractions. Chong et al. (1974), however, in a later publication have reported the isolation of a proteolytic activity specific for the F1 histone fraction when present in chromatin. This protease, on the other hand, attacks all histones quite readily when they are not attached to DNA with the exception of the F1 histone which in this case undergoes the least amount of degradation. The discovery of this F1 specific neutral protease may explain the degradation effects mentioned here.

A possible mechanism by which the F1 degradation during hepatic regeneration observed here might be brought about is suggested by the work of Chong et al. (1974) who

state that in trout testes the transformation from nucleohistone to nucleoprotamine is preceded by acetylation and phosphorylation of the histones. They suggest that these modifications might render the histones susceptible to digestion. This hypothesis appears to be consistent with the results presented here. Figure 19 shows what appears to be extensive phosphorylation of the 1 hour regenerating F1 histone fraction in comparison to its non-regenerating counterpart. This modification was detected a full hour ahead of the 2 hour decrease in F1 associated with the concomitant surge in template activity.

As discussed in the Introduction, rat liver has been shown to contain two kinases which are capable of phosphorylating the F1 histone but at different sites. One of these is cAMP dependent and brings about an increased rate of phosphorylation early in the cell cycle whereas the cAMP independent kinase causes increased amounts of phosphate to accumulate on F1 later in the cell cycle. The influence of hormones on cAMP production at the start of hepatic regeneration has also been discussed in the Introduction. Although some investigators have associated F1 phosphorylation with chromosome condensation and mitosis (Bradbury et al., 1974; Balhorn et al., 1972), others (Gorovsky and Keevert, 1975) have suggested that phosphorylation of F1 might be a mechanism for gene regulation. This latter hypothesis appears to be

consistent with the results presented here pertaining at least to the start of the cell cycle.

The literature does contain reports that indicate a possible role for proteolytic enzymes in exposing stretches of DNA necessary for cellular proliferation. Miyamoto et al. (1973) have attempted to determine the effects of powerful proteolytic inhibitors on hepatic regeneration. By using leupeptin and pepstatin, protease inhibitors of cathepsins B and D, respectively, Miyamoto et al. (1973) were able to demonstrate a decline (in cells stimulated to proliferate) to control levels of RNA synthesis, DNA synthesis and the number of mitotic figures in partially hepatectomized rats. These results appear to be consistent with the data presented here for the 2 hour decrease in F1 which in turn may be responsible for the increase at this time in free DNA and in template activity. Of additional significance, the protease inhibitors TLCK (N-α-p-Tosyl-L-lysine chlormethyl ketone HCl) and TAME (p-Tosyl-L-arginine methyl ester HCl) have been shown by Collard and Smets (1974) to reduce the growth of SV40 transformed mouse fibroblasts. Ishida and Ahmed (1974) have used isoproterenol to stimulate cellular proliferation of the cells of the submandibular glands of rats. They reported a reduction in the total chromatin protein content at 2 hours after the administration of isoproterenol.

One other significant finding from these electrophoresis experiments remains to be discussed. Extensive acetylation

of the F3 and F2a1 histone fractions is discernable in Figures 19-24 for both regenerating and for non-regenerating histones. Since the conditions utilized here allowed effective resolution of only the F2a1 acetylation bands, these were quantitated and are discussed below. Past work by Pogo et al. (1968) has indicated that after partial hepatectomy, acetate incorporation increases while deacetylation decreases, resulting in a net acetate accumulation 3-4 hours post partial hepatectomy; the F2a1 fraction was reported to have acquired the greatest acetate content. With these results in mind it is of interest to examine the densitometric profiles of the F2a1 histones obtained at 4 hours post partial hepatectomy. Figures 22 and 25 and Table 10 clearly show that after 4 hours of regeneration there is a redistribution of F2a1 acetate groups such that mono-acetylated molecules are the predominant acetylated form (48.6%) while di-(2.6%) and tri-acetylated (3.0%) forms had declined relatively to their respective control values. The percentage change in each of these forms is shown in Figure 25. Also to be noted in Figure 25 and in Table 10 is the fact that at 2 hours post partial hepatectomy there is the greatest percentage increase in di-acetylated F2a1.

Recent work by Adler et al. (1974) has indicated that mono-acetylated F2a1 causes the least distortion in DNA conformation when reassociated with DNA. In contrast to this, unacetylated F2a1 was shown to cause an increase in the positive circular dichroism (CD) and a blue shift of

the spectrum when compared to the CD spectrum for DNA alone. In the opposite manner, multi-acetylated F2a1 histones when reassociated with DNA also were shown to produce a distorted CD spectrum but this was in the form of a decreased positive signal and a red shift when compared to the spectrum for DNA alone. The authors pointed out that just as phosphorylation of F1 has been shown to reduce the distortion present in an F1-DNA complex, so too, the mono-acetylated form of F2a1 when complexed with DNA yields a conformation most similar to that of free DNA. They concluded that such a situation might therefore allow more DNA to be available for template activity. It can be seen in Table 3 that the highest percentage increase in template activity was obtained using 4 hour regenerating native chromatin. This chromatin in addition to being stimulated by some effect mediated by the acidic proteins is also associated with having the highest percentage increase in mono-acetylated F2a1 histones and the highest percentage decreases in the di- and tri-acetylated forms. It appears that a combination of the influences of both the acidic proteins and of acetylation phenomena acting concomitantly might be responsible for the peak in template activity observed at 4 hours post partial hepatectomy.

In this section, no experiments dealing with the electrophoresis of the acidic proteins have been reported. An experiment of this sort has already been performed by Garrard and Bonner (1974) using regenerating rat livers.

They detected no major differences in gel profiles at different hours during regeneration nor have they found there to be a change in the mass-ratio of acidic proteins to DNA. Consistent with the results presented in this thesis, they have, however, observed differential increases in the rates of turnover of the acidic proteins during the period of 2-1/2-4 hours post partial hepatectomy as judged by the incorporation of labeled amino acids into these acidic proteins. This coincides with the data presented in previous sections indicating that a change has occurred in the behavior of the acidic protein fraction, apparently enabling it to bring about an increase in template activity. Whatever this change in the acidic proteins is, it seems to involve an increase in the shuttling of these proteins onto and off of chromatin at 2-1/2-4 hours during regeneration (Garrard and Bonner, 1974).

Thermal Denaturation of Regenerating Rat Liver Chromatin and Nucleohistone Complexes

Data has already been presented indicating that two peaks of template activity are obtained with 1.5-2 and 4 hour native chromatins extracted from regenerating liver remnants. The larger 4 hour peak has been associated with the influence of the acidic chromosomal proteins in altering template activity and with a change in histone acetylation patterns. The smaller 1.5-2 hour peak of activity, on the other hand, has been associated with decreased amounts of F1 histone and with an increase in

di-acetylated F2a1. These results imply that the smaller peak occurs at a time when more free-DNA is available for transcription whereas the larger peak occurs at a time when the chromosomal proteins (histones and acidic proteins) appear to be altered or changed in various ways. Thermal denaturation was performed on both native chromatins and on reconstituted nucleohistone complexes in order to establish directly whether changes take place in the amounts of DNA (free DNA and non-histone bound DNA) melting in the free DNA region (30°C-60°C) after the onset of hepatic regeneration.

In Table 11 are displayed the percentages of free melting DNA present in native chromatins extracted hourly from regenerating liver remnants and from the corresponding 0-hour resected segments. At 2.0 hours post partial hepatectomy, the regenerating chromatin contained 3.2% (R-N) more free melting DNA than the controls. The magnitude of this difference is seen to decrease slightly at 3 hours, and, by 4 hours, the amounts of free melting DNA were the same for the experimentals and for the controls. Figure 26 shows the changes in amounts of free melting relative to controls at each hour. Figures 27-32 show the individual derivative plots of the melting curves for each hour studied. Figure 29 illustrates the fact that the 2 hour curve for regenerating chromatin is above its respective control in the free melting DNA region. Figure 33 is a repeat of the 2-hour thermal melt with chromatin from a different rat.

It can be seen once again that the curve for the chromatin from the 2-hour regenerating remnant is above its respective control in the free melting DNA region. Approximately 2% more free DNA was present in this regenerating chromatin sample.

In view of the results obtained with native chromatin, similar experiments were performed with reconstituted nucleohistone complexes. Figure 34 shows the derivative plots of the thermal melts obtained with complexes of DNA and histones extracted from non-regenerating rat livers. It can be seen that as the ratio of histone to DNA is increased, the amount of DNA melting in the free DNA region is decreased. Figure 35 shows the derivative plots of the thermal melts obtained with complexes of DNA and histones extracted from 2-hour regenerating liver remnants and of histones extracted from the corresponding 0-hour resected segments. In this case, only the more physiological 1:1 histone to DNA ratio was used. It was found that the 2-hour regenerating histone-DNA complex had 5.3% more free melting DNA than did its respective control complex. This data is shown in Table 12 (Experiment I). Experiment II in the same table is a repeat of Experiment I with histones obtained from a different rat at 2 hours post partial hepatectomy; 3.3% more free melting DNA was directed in the nucleohistone complex containing the histones extracted from the regenerating remnant.

The results obtained with both native chromatin and with nucleohistone complexes clearly indicate that

approximately 3.5% more free melting DNA is present as compared with the controls after 2 hours of hepatic regeneration. In addition, from Figure 26 it can be seen that there is no percentage increase in the detectable amount of free melting DNA present at 4 hours post partial hepatectomy. It thus appears that the 3.2% increase in template activity observed in Table 3 for 2-hour regenerating native chromatin correlates well with the amount of free melting DNA directly detected by the analysis of thermal melting curves. The increase in free melting DNA also correlates well with the observed decrease in F1 histone reported at this time.

At the time of the 4-hour peak in template activity, the absence of any additional free melting DNA would lead to the conclusion that some other mechanism must be in operation at this time in order to permit the template activation that was observed. This will be discussed further in the next section.

The results presented here thus suggest that the freeing of regions of DNA for transcription might be one mechanism by which template activity could be regulated. Evidence of this has been reported in the literature. McConaughy and McCarthy (1972), also using thermal denaturation experiments, found that chromatin from chick erythrocytes contained 2.5% free DNA whereas chromatin from more active liver cells had 15% free DNA. Chromatin fractionation experiments revealed that most of the transcripts

produced *in vivo* by erythrocyte and liver cells hybridized to the low melting fractions of erythrocyte and liver chromatins, respectively. Moreover, other workers also have been able to fractionate chromatin into extended transcriptionally active fractions and into condensed inactive ones. It is of interest that the major compositional differences between these fractions are (1) the depletion of F1 histone in the extended chromatin fraction and (2) differences in the nonhistone chromosomal protein distribution (Simpson and Reeck, 1973; Reeck *et al.*, 1972).

In the case of 1.5-2.0 hour regenerating liver chromatin, the results presented here suggest that histone degradation may be the mechanism by which increased amounts of DNA are freed and transcriptional regulation is achieved.

Circular Dichroism Analysis of Regenerating Rat Liver Chromatin

In previous sections, results have been presented indicating that histone degradation with the attendant freeing of additional regions of DNA was not responsible for the increased template activity observed at hour 4. Moreover, the reconstitution experiments discussed earlier have implicated the phenol-soluble non-histone chromosomal proteins as being at least in part responsible for the events occurring at 4 hours post partial hepatectomy. The other notable changes reported at that hour were the

increase in mono-acetylated F2a1 histone and the decrease in multi-acetylated members of this fraction. CD spectra for chromatins extracted hourly from regenerating liver remnants and for the corresponding non-regenerating resected segments were taken.

Figure 36 is a CD spectrum of rat liver DNA. Comparison of this with Figures 37-42, CD spectra of chromatins, reveals that the positive CD signal for each chromatin in the pair is less than that for DNA alone. Also of interest are the negative CD signals obtained at 220 mμ which are indicative of the relative amounts of protein secondary structure present.

In Table 13, the amounts of histone α-helical secondary structure in chromatin are given in terms of the $\Delta\epsilon$ at 220 mμ. These are presented for the chromatins extracted hourly from regenerating liver remnants (R) and for their respective controls (N). Inspection of Table 13 and Figure 43 indicates that the lowest ratio for R/N is observed at hour 4. This result indicates that the greatest loss of histone α-helical secondary structure occurred at hour 4. This is illustrated in Figures 37-42 by the fact that in each case the broken line at 220 mμ is below the unbroken one except in Figure 40 where the positions are reversed. Table 14 and Figures 44 and 45

illustrate a repeat of this decrease in secondary structure from hour 3 to hour 4 for another group of partially hepatectomized rats.

The results presented above and in other sections may be significant within the general framework of recent models that have been proposed for the structure of chromatin. The modern conception of chromatin structure first began to evolve with the X-ray diffraction studies of Pardon et al. (1967) who believed chromatin to be arranged into a regular supercoil of 100-120 Å pitch. The reflections indicating this were not obtained with either DNA or histones alone. A different view as to the reason for the 100 Å X-ray reflections was put forth by Olins and Olins (1974). On the basis of their electron microscopic work, they suggested that the regular repeating unit was not due to the pitch of a supercoil but to regular repeated chromatin particles (ν-bodies) approximately 80 Å in diameter joined together by thin 15 Å filaments. These dimensions have been substantiated by other laboratories (Woodcock, 1973; Griffith, 1975; Oudet et al., 1975). Moreover, the action of both exogenous and endogenous nucleases verifies the existence of this chromatin subunit (Hewish and Burgoyne, 1973; Burgoyne et al., 1974; Clark and Felsenfeld, 1971; Clark and Felsenfeld, 1974; Axel et al., 1974; Rill and van Holde 1973; Sahasrabuddhe and van Holde, 1974; Noll,

1974; Oosterhof et al., 1975). Electron micrographs have demonstrated the actual production of ν-bodies after nuclease treatment or sonication of chromatin (van Holde et al., 1974; Senior et al., 1975; Gottesfeld et al., 1975).

The chromatin subunits have been reported to consist of approximately 200 base pairs (Hewish and Burgoyne, 1973; Noll, 1974; Kornberg, 1974). Other groups, however, have reported the subunit to be composed of 140 base pairs with a 60 base pair spacer (Shaw et al., 1976). Shaw et al., (1976) have suggested that the 140 base pair (beads-on-a-string) particles may be produced from the 200 base pair particles (beads). The isolated particles sediment at approximately 11S (Sahasrabuddhe and van Holde, 1974) and represent a packing ratio of 7:1 (Kornberg, 1974; Griffith, 1975; Oudet et al., 1975).

It appears that the 140 base pairs represent all that is needed in order to associate with an eight molecule histone complex lacking F1 (Shaw et al., 1975). Such an octamer of histones can be isolated free in solution. In addition, crosslinking studies with dimethyl suberimidate established that this unit exists within chromatin in a manner such that 2 or more contacts of each histone are made with others within the octameric unit (Thomas and Kornberg, 1975). Weintraub (1975) has suggested that the DNA in these particles is folded into hairpin loops held together by the conserted crosslinking action of the 8

histone molecules which form a trypsin resistant core on the inside of the nucleosome. These histones are believed to be arranged with the N-termini predominantly along one side of a loop and their C-termini along the other side of each loop. A more specific model for the internal structure of the nucleosome has been proposed by Li (1977).

Many attempts have been made to establish the fundamental role of the nucleosome. Gottesfeld et al. (1975) have fractionated chromatin into active and inactive regions. The nuclease-resistant particles obtained from active chromatin were shown to contain RNA and nonhistone proteins as well as histones whereas the nuclease-resistant particles obtained from inactive chromatin had only histones associated with their DNA. Kuo et al. (1976) have demonstrated that most repetitive as well as single copy sequences in mRNA are present in chromatin subunits. Consistent with this, the work of Axel et al. (1975), using hybridication techniques, have found all classes of genomic sequences to be present within -body DNA. The work of all of these groups appears to indicate that -body formation may be random with respect to DNA sequences and that the inclusion of a DNA sequence into the subunit need not prevent its transcription in vivo.

Regardless of which of the nucleosome models (beads-on-a-string or beads) is correct, the tertiary structure of DNA appears to be associated with round, repeated subunits, about 100 Å in diameter comprising a nucleofilament in

which the packing ratio is 7:1.

Recently, various reports have implied the existence of a quaternary level of organization of DNA in which the nucleosomal filament is wound into a supercoil or solenoid. Electron microscopic work by Finch and Klug (1976) has revealed the existence of a supercoiled chromatin structure when 0.2 mM Mg^{++} is present. This supercoil is reported to be approximately 300 Å in diameter with a pitch of 110 Å and to consist of approximately six nucleosomes per turn. What is also of significance is the fact that F1 histone is apparently necessary for the stabilization of this supercoiled structure; the F1 histone is presumably present in the central hole of the solenoid which, it has been suggested, might also accommodate nonhistone proteins. Noll and Kornberg (as reported by Finch and Klug, 1976) have noticed that F1 removal causes a marked reduction in the sedimentation constant of chromatin, the latter result implying the formation of a less compact structure from a more compact one.

Evidence for the existence of 300 Å fibers has also come from electron microscopic studies by Davies et al. (1974) and Davies and Haynes (1975) who have demonstrated the presence of thread-like units in condensed interphase chromosomes and chromatin bodies. In addition, Bram et al. (1975) employing neutron scattering techniques on chromatin, have also come to the conclusion that a supercoil of chromatin consisting of 8 nucleosomes per turn exists. The

diameter of the supercoil they propose is also of the order of 300 Å. They further have suggested that the DNA compacted into such a structure would be sterically "inaccessible to recognition proteins and polymerases." Whereas the packing ratio of DNA in nucleosomes is 7:1, that envisioned for the solenoidal structure would be on the order of 40:1.

It appears that if the quaternary structure of chromatin is stabilized by the F1 histone, then the degradation of F1 reported at 1.5-2.0 hours post partial hepatectomy may be merely a means by which accessibility of either polymerases and/or other control components might be gained. Once this is accomplished, however, control molecules would then have to exert their respective effects upon the exposed tertiary structure of DNA present in the nucleosomal filament.

Some light has been shed upon DNA-histone-acidic protein interactions at the tertiary level by the work of Wilhelm et al. (1974 a,b). These workers have studied the variations in both O. D. and C. D. with temperature using F1 and acidic protein depleted chromatin. Their thermal melting curves were biphasic in character. The first transition is believed to be due to regions weakly stabilized by histones while the second is attributed to DNA regions fully covered by histones. They performed CD spectra at 280 mμ and at 227 mμ during the melting process in order to ascertain whether conformational changes

affecting DNA and histones, respectively, were taking place.

In terms of the effects on DNA the CD 280 spectra revealed an increase in positive elepticity at 40°C (a premelting phenomenon) and another increase in positive elepticity at 65°C occurring before the actual melting of DNA regions fully covered by histones. With respect to the 227 CD scans, Wilhelm et al. (1974a) demonstrated that histone α-helical content exhibited a gradual decrease reaching its low point just before 65°C. The three effects observed by Wilhelm's group--premelting phenomena, the 65°C CD peak, and the decrease in histone α-helical content--are extremely important in view of the work presented in this thesis. A comparison will be made between both sets of results where relevant.

The premelting effect reported above was shown in a separate report (Wilhelm et al., 1974b) to be due to the absence of the acidic chromosomal proteins since this effect is not observed with native chromatin but is observed with acidic protein depleted chromatin. The premelting effect is believed to occur when substances affecting the stability of AT base pairs are removed. Wilhelm et al. (1974b) associate the premelting phenomenon with the dynamic state ("breathing") of AT regions and suggest that this might be a mechanism by which acidic proteins can by virtue of the opening and closing of AT regions exert a form of transcriptional control. The CD

scans on regenerating rat liver chromatin were performed at room temperature and thus could not demonstrate premelting phenomena. It is, however, significant to recall that the 4-hour peak of transcriptional activity appeared in part to be due to the acidic proteins present at that hour. The acidic chromosomal proteins in general repress premelting phenomena. If any change were brought about in these proteins by the regenerative process as is implied by the 4-hour data, then AT areas might be effectively destabilized.

In the data reported here and in that reported by Wilhelm et al. (1974a), it can be seen that both liver chromatin and F1-acidic protein depleted chromatin of Wilhelm et al. (1974a) exhibit a lower positive elepticity at 280 than that associated with naked DNA. This is due to the presence of both B and C forms of DNA in chromatin whereas the B conformation is present only in naked DNA. The second increase in positive elepticity reported by Wilhelm et al. (1974a) at 65°C (just before the firmly histone bound DNA regions melt) is attributed to a change in conformation in DNA from the C to the B form. However, just prior to this change in DNA secondary structure, they observed a decrease in α-helical secondary structure of the histones which in turn they assert results in a change in histone tertiary structure. To summarize these events that occur during thermal denaturation:

1) the premelting destabilization of AT base pairs occurs just before weakly histone bound DNA melts.

2) histone α-helical structure decreases resulting in a change in histone tertiary structure and failure of the helical regions of histone molecules to interact with each other.

3) DNA secondary structure is then changed from the C to B conformation.

4) Firmly histone bound DNA regions can then begin to melt.

(See reproduction of the scheme from Wilhelm et al. (1974a) in Figure 46)

The CD data presented here indicate that at 4 hours post partial hepatectomy the α-helical content of the histone molecules decreases. It appears that this event may indicate the point during regeneration when, as a result of histone secondary structural changes, the tertiary structural features of histone molecules are altered. This alteration may thus bring about the C→B conformational change in DNA structure which must precede the opening up of firmly histone bound DNA sequences.

CHAPTER IV

SUMMARY

The central concern of this thesis has been to investigate alterations in transcriptional activity that begin after the performance of a partial hepatectomy and that are associated with the ensuing early hours of hepatic regeneration. Among the salient features of RNA synthesis, the main points of inquiry investigated here involve elucidation of 1) the role of the nuclear DNA-dependent RNA polymerases during regeneration and 2) the role of chromatin and its specific components (primarily histones and non-histone proteins) in regulating DNA availability as a template for RNA synthetic purposes during hepatic regeneration.

The rate of total nuclear incorporation of H^3-UMP into RNA was shown to increase linearly through hour 3 and then remained level through hour 6 at a rate of incorporation which was approximately 4 times that of the control. This situation resembles that reported *in vivo* with regard to the time required in order to reach the plateau. The *in vivo* rate of incorporation at 3 hours, however, is only reported to be 1-1/2 - 2 times greater than that for the controls; this difference is attributed to the fact that in the intact nuclear systems,

optimal conditions for both polymerases were employed. Of possible significance with regard to the control of hepatic regeneration is the fact that the 3 hour plateau of polymerase activity is attained at a time when intracellular cAMP levels have been shown by MacManus et al. (1972) to reach a peak. The work of Martelo and Hirsh (1974) has led to the suggestion that cAMP, by virtue of its effect on kinase enzymes, may, by way of polymerase phosphorylation, be responsible for altered RNA polymerase activity. A mechanism for polymerase activation such as the one suggested above may be of particular relevance in the light of a recent report by Bucher and Swaffield (1975). These investigators have reported that immediately after the performance of a partial hepatectomy, the ratio of insulin/glucagon is decreased in the portal blood. Perfusion experiments (Kuster et al., 1973) have indicated that just such an alteration in hormonal balance would be sufficient to raise the intracellular cAMP levels which are suggested to ultimately effect RNA polymerase activity.

The toxin α-amanitin was used in an intact nuclear RNA synthesizing system in order to investigate the incorporation of H^3-UMP into RNA by polymerases I and II, respectively. At one hour post partial hepatectomy, polymerase II was found to have undergone a 144.4% increase in activity relative to control levels. Only

after a lag of more than an hour after the operation could polymerase I activity be shown to begin to increase and by hour 4 was observed to parallel polymerase II activity. Of possible relevance to the control of hepatic regeneration is the fact that polymerase II activity begins to increase at the same time that MacManus *et al.* (1972) have reported initial increases in intracellular cAMP levels. In addition, a possible relationship, suggested by the literature, may exist between polymerase II and polymerase I; alterations in activity of the former may be required in order to exert control over the latter. The lag period reported here lends some support to the hypothesis.

Irrespective of changing polymerase activities, chromatin from regenerating hepatic cells must also function in a physiologically relevant manner to insure the appearance at the correct times in regeneration of the appropriate enzymatic activities. In order to ascertain at which times post partial hepatectomy major template activation was occurring, chromatin extracted hourly during regeneration was transcribed with saturating amounts of *E. coli* RNA polymerase. The relevance of such a heterologous system has been reviewed in the Results and Discussion section. A recent report received since preparation of this thesis suggests, however, that one possible major drawback to the use of this enzyme is its ability to extend RNA chains already present on

isolated chromatin (Shih et al.,1977). Native chromatin was shown to demonstrate a 50.1% increase in template activity over control levels at 2 hours post partial hepatectomy and an 89.3% increase at 4 hours. Both of these percentage increases in template activity are significantly different from the 3 hour time point. The fact that the curves for chromatin template activity and for total nuclear polymerase activity are not superimposible, can be explained by the fact that reports in the literature suggest that during hepatic regeneration the polymerases and the template are affected in a distinct and separate manner. In the nuclear studies, both of these factors are being studied while the chromatin data reflects only changes in template capacity.

Chromatin reconstitution was employed to determine which components of chromatin were responsible for the 2 and 4 hour peaks of template activity. Complexes were formed of DNA and non-histone proteins extracted from control chromatin and of histones extracted from chromatin of livers at different hours in regeneration. Transcription of these complexes with exogenous RNA polymerase demonstrated the presence of only a 2 hour peak of template activity. A similar 2 hour peak of template activity was also obtained when E. coli RNA polymerase was used to transcribe nucleohistone complexes composed

of DNA and of histones extracted hourly from chromatin of regenerating livers. Both results imply that the 2 hour peak observed with native chromatin might be mediated by alterations in the histone components of the chromatin.

Reconstituted complexes were also formed with DNA and histones extracted from control livers and with non-histone proteins extracted hourly during regeneration. Transcription of these complexes revealed the presence of only a 4 hour peak of template activity, the implication here being that the 4 hour peak of template activity in native chromatin may have been mediated by alterations in the non-histone components of chromatin. The non-histone proteins may serve to relieve the repressive effects of histones at 4 hours post partial hepatectomy and/or to function as positive control agents in stimulating template activity. Experiments reported here indicate the latter possibility to be highly probable. When complexes composed of DNA and of non-histone proteins from different hours in regeneration were formed, all yielded template activities higher than that for DNA alone when transcribed with exogenous polymerase. Wilhelm *et al.* (1974) have proposed that the non-histone proteins, observed to be involved with premelting phenomena, could regulate the initiation of RNA synthesis by controlling the "breathing" associated with stretches of AT base pairs in promoter regions.

Electrophoresis was performed on histones extracted hourly through hour 6 of hepatic regeneration in order to monitor any qualitative or quantitative changes in the histone components that occurred relative to histones from control livers. At one hour post partial hepatectomy, the F1 histone component appeared to undergo extensive modification possibly of the phosphorylation variety. At 2 hours post partial hepatectomy, this same fraction was observed to decrease in amount by approximately 50% relative to control levels. The prior modification of histone F1 might render it susceptible to proteolytic attack as discussed in the Results and Discussion section. The decrease in the amount of F1 reported here might be responsible for freeing stretches of DNA which were either 1) covered directly by F1 or 2) inaccessible to RNA polymerase by virtue of their presence within a quaternary DNA configuration stabilized by F1 as suggested by Finch and Klug (1976). In any case, the amount of F1 destruction encountered here would be consistent with an approximate 3% increase in free DNA, which would be sufficient to account for the 2 hour peak of template activity obtained with native chromatin and with reconstituted nucleohistone complexes. Derivative thermal melting profiles obtained for 2 hour regenerating native chromatin and for reconstituted nucleohistone complexes, composed of DNA and of histones extracted 2 hours post partial hepatectomy,

yielded approximately 3% more DNA melting in the free DNA region of the curves. This may correspond to either more free DNA and/or more non-histone bound DNA being present at this time in regeneration. Although the increases in free melting DNA obtained by thermal denaturation were indeed small, they fit in well with what would be expected based on the results obtained from the transcriptional and electrophoretic experiments.

Both electrophoretic and CD experiments were used to explore further the nature of the factors involved in the production of the 4 hour peak of template activity. Electrophoretic profiles of histones extracted 4 hours post partial hepatectomy revealed the presence of greater amounts of mono-acetylated F2a1 when compared with control levels for this histone. As discussed in the Results and Discussion section, CD analysis has revealed that mono-acetylated F2a1 is capable of causing the least amount of distortion of DNA away from the beta conformation. The shift to greater amounts of the monoacetylated form of the F2a1 histone at a time in regeneration concomitant with the second, higher peak of template activity observed with native chromatin is indeed suggestive of a relationship between this modification and augmented template activity.

CD analysis of native chromatin extracted hourly during the first six hours of regeneration revealed a decrease in histone α-helical content when compared with

controls. The significance of such an effect with regard to template activity has been discussed by Wilhelm et al. (1974). They proposed that nucleosomes may be held in a closed configuration by interactions between α-helical regions of histone molecules. A decrease in α-helical content might reduce these interactions and therefore be responsible for the opening up of the nucleosomes for purposes of transcription. The decrease in α-helical content encountered at 4 hours post partial hepatectomy correlates well with the large 4 hour peak in template activity and suggests a possible relationship between the two events.

In summary, the results of this thesis suggest that an increase in the amount of free DNA (and/or non-histone bound DNA) may be responsible for the two-hour peak in template activity observed while changes in the conformations of chromosomal proteins may cause the four-hour peak.

APPENDIX A

TABLES AND FIGURES

TABLE 1.--Incorporation of H^3-UMP Into RNA of Isolated Rat Liver Nuclei

Standard reaction conditions as described in "Materials and Methods" were used. The incorporation obtained at each hour is an average of the results, given in parenthesis, from two separate rats.

Hrs. after partial hepatectomy	Polymerase activity[a]	% change in incorporation relative to 0-hr.
0	47.4 (55.1, 39.7)	-
1	48.7 (49.6, 47.8)	3.0
2	105.0 (108.6, 101.4)	121.5
3	176.3 (184.0, 168.6)	271.9
4	89.4 (81.0, 97.7)	88.6
5	190.1 (187.3, 192.9)	301.1
6	185.7 (209.0, 162.4)	291.8

[a]Expressed as mpicomoles of H^3-UMP incorporated into RNA per mg of DNA per minute.

Fig. 1. DEAE-cellulose column profile. E. coli RNA polymerase fraction 3 protein was applied to a DEAE-cellulose column and eluted as described in Materials and Methods. Fractions of 10 ml each were collected. Tubes 1 to 90 contained the flow through material. Tubes 91 to 130 contained the Buffer A + 0.13M KCl peak. Tubes 131 to 150, the Buffer C + 0.23M KCl step, contained the polymerase activity.

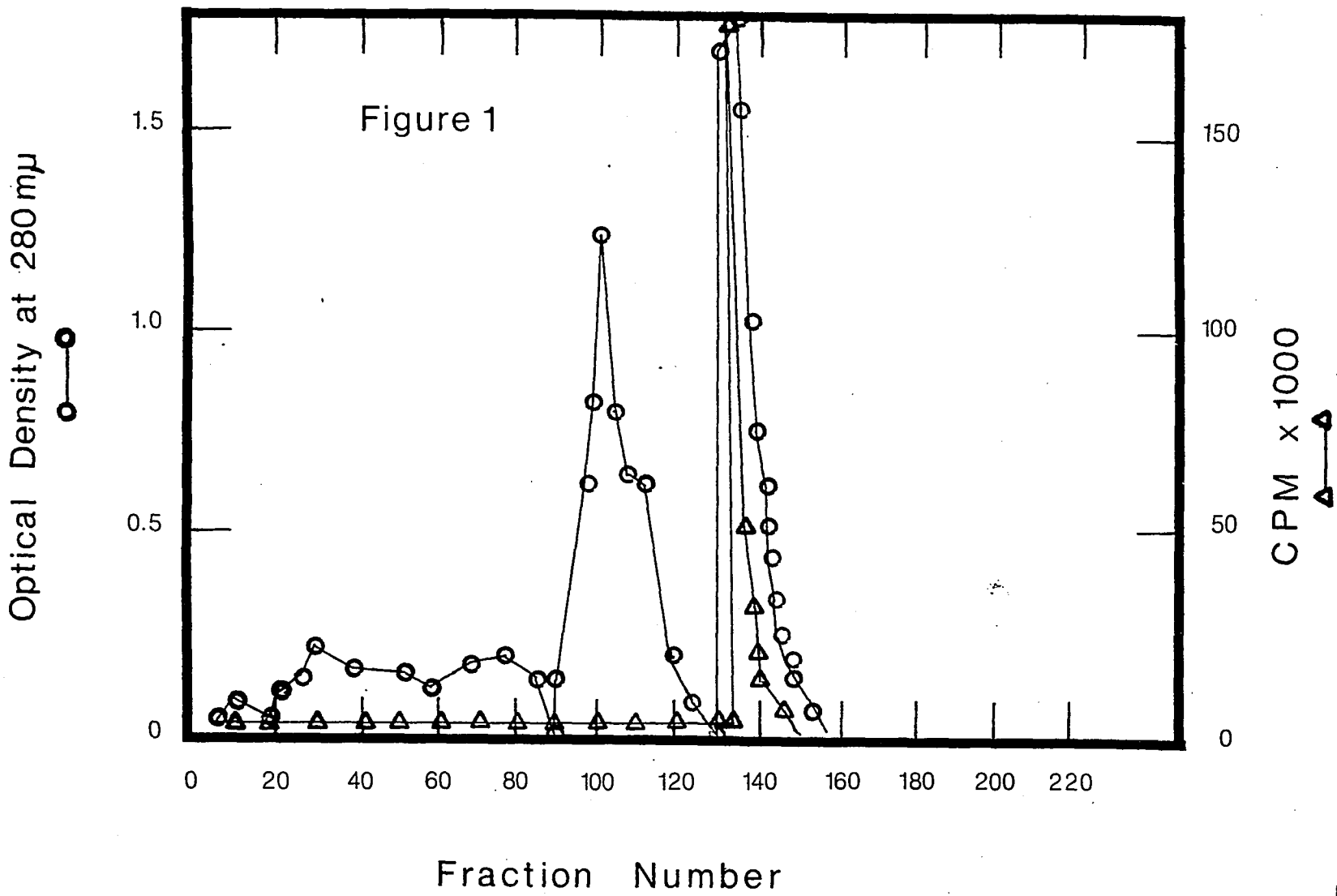


Figure 1
Optical Density at 280 mμ
CPM x 1000
1.5
1.0
0.5
0
150
100
50
0
0 20 40 60 80 100 120 140 160 180 200 220
Fraction Number

Fig. 2. Incorporation of H^3-UMP into RNA of isolated rat liver nuclei plotted against time after the performance of the operation (data from Table 1).

Figure 2

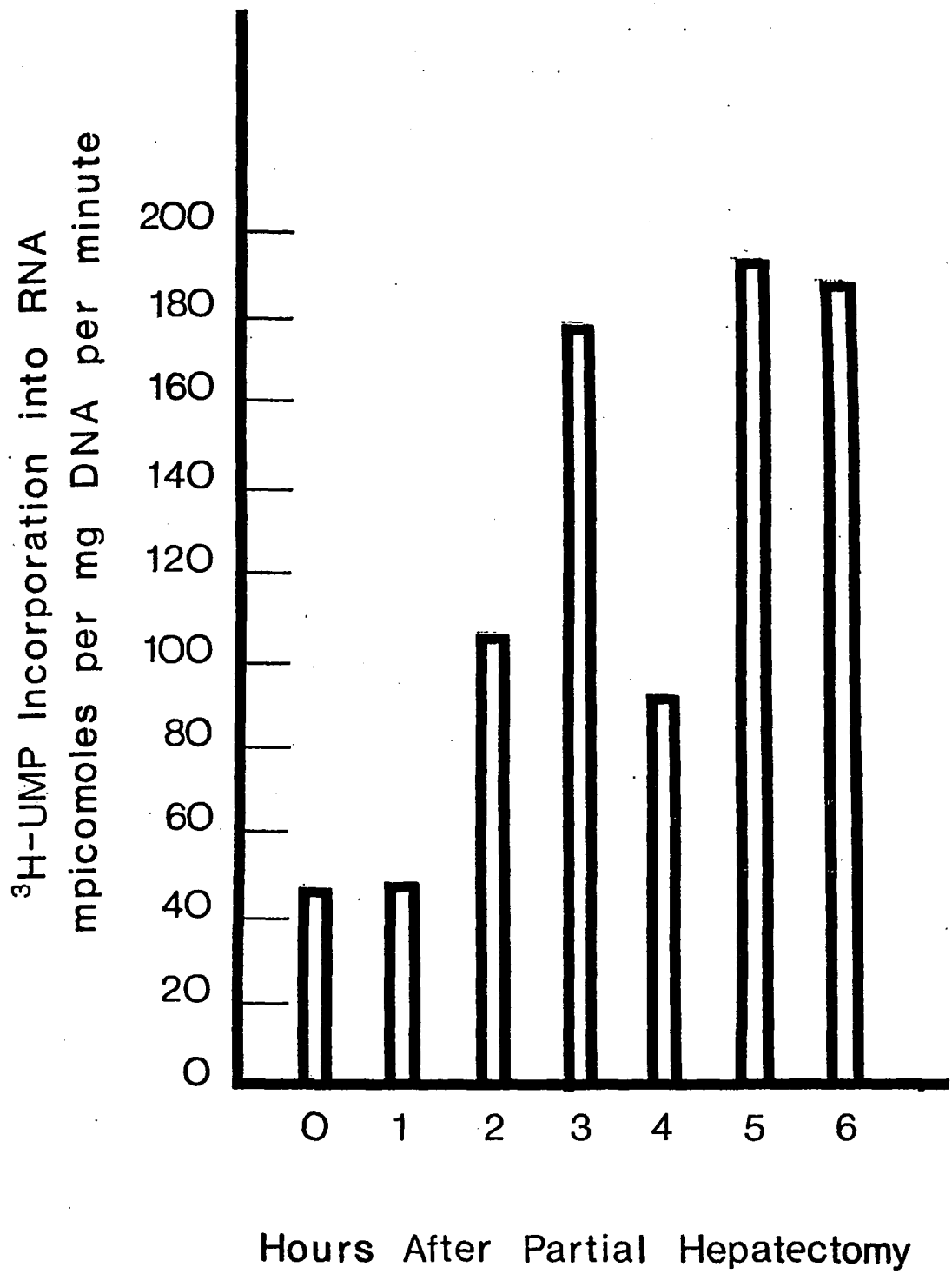

^{3}H-UMP Incorporation into RNA
mpicomoles per mg DNA per minute
200
180
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
Hours After Partial Hepatectomy

Fig. 3. The percent change in H^3-UMP incorporation into RNA of isolated rat liver nuclei in the presence and in the absence of α-amanitin plotted against time. The percent change in incorporation by nuclei isolated hourly from regenerating liver remnants is expressed relative to the incorporation for nuclei isolated from the corresponding 0-hr, resected liver segments. (data from Table 2).

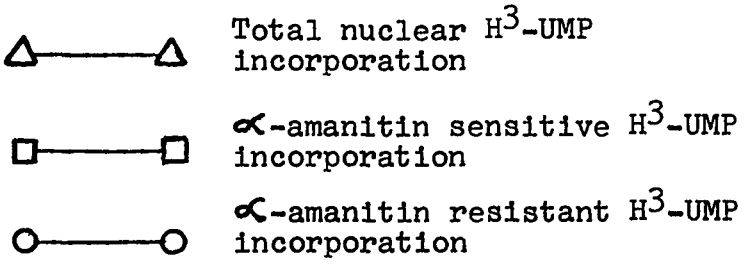


Figure 3

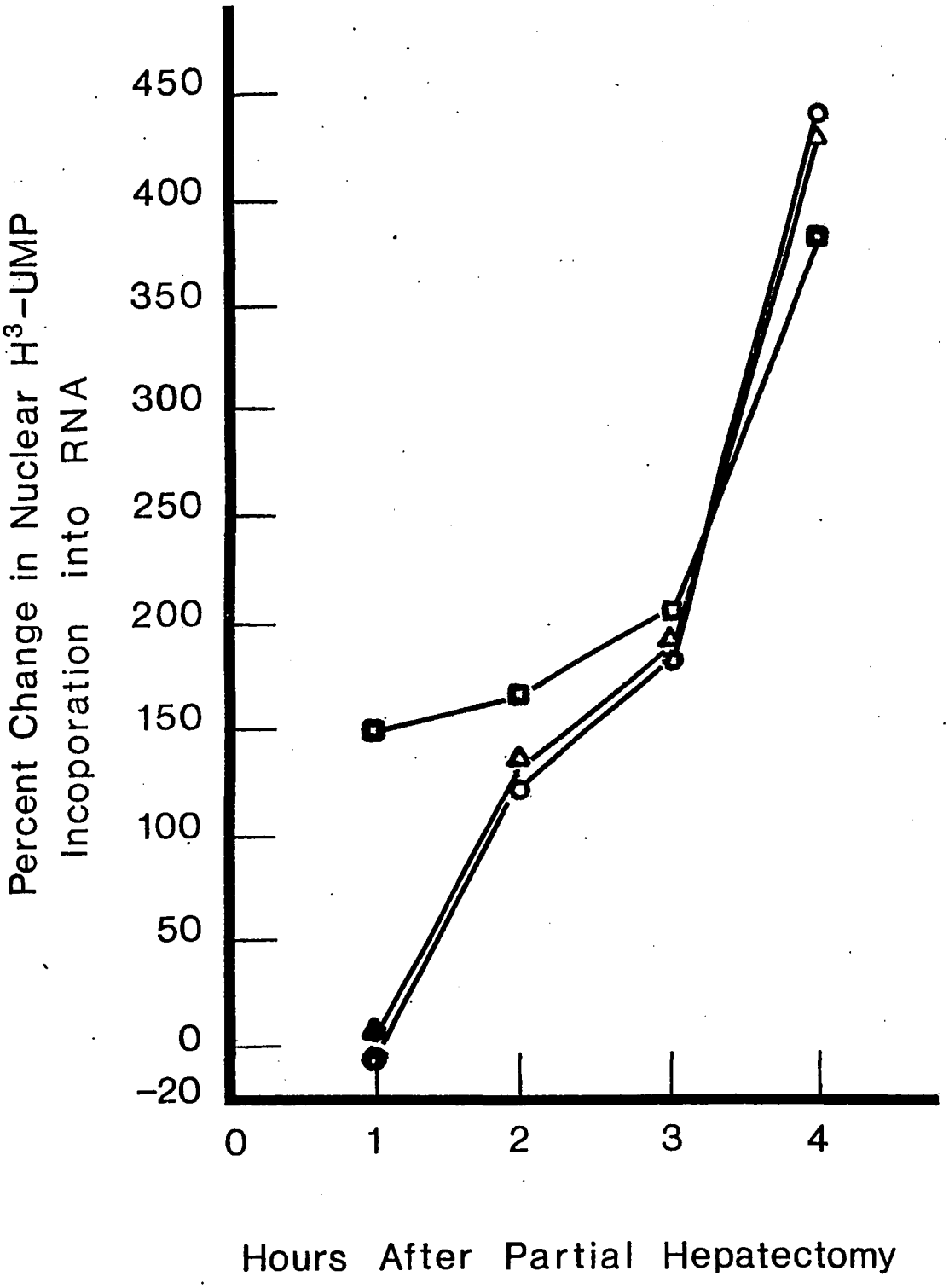

Percent Change in Nuclear H3-UMP Incoporation into RNA
450
400
350
300
250
200
150
100
50
0
-20
0
1
2
3
4
Hours After Partial Hepatectomy

TABLE 2.--Incorporation of H^3-UMP Into RNA of Isolated Rat Liver Nuclei in the Presence and in the Absence of α-amanitin

Assay conditions and the concentration of the inhibitor were as described in "Materials and Methods". The incorporation obtained for each hour is an average of the results, given in parenthesis, from two separate rats.

Hrs. after partial hepatectomy[b]	Total nuclear H^3-UMP incorporation[a]	% change in R relative to N	α-amanitin resistant H^3-UMP nuclear incorporation[a]	% change in R relative to N	α-amanitin sensitive H^3-UMP nuclear incorporation[a]	% change in R relative to N
1R	48.7(49.6,47.8)	2.7	39.9(43.0,36.8)	-8.9	8.8	144.4
N	47.4(55.1,39.7)		43.8(49.8,37.8)		3.6	
2R	105.0(108.6,101.4)	128.8	77.5(75.7,79.3)	119.5	27.5	159.4
N	45.9(52.6, 39.1)		35.3(35.7,34.9)		10.6	
3R	176.3(184.0,168.6)	182.2	119.5(119.8,119.1)	174.0	56.8	197.5
N	62.7(78.1,47.2)		43.6(53.9,33.2)		19.1	
4R	89.4(81.0,97.7)	429.0	76.4(63.7,89.2)	438.0	13.0	381.5
N	16.9(14.1,19.6)		14.2(16.0,12.4)		2.7	

[a]Expressed as mpicomoles of H^3-UMP incorporated into RNA per mg of DNA per minute.

[b]R refers to nuclei isolated from liver remnants at different hours after partial hepatectomy and N to nuclei isolated from the corresponding 0-hr., resected liver segments.

TABLE 3.--Template Activity of Regenerating and Non-regenerating Native Rat Liver Chromatin

Transcription was performed with E. coli RNA polymerase under conditions as described in "Materials and Methods". R refers to chromatin extracted from nuclei isolated from liver remnants at different hours after partial hepatectomy and N to chromatin extracted from nuclei isolated from the corresponding 0-hour resected liver segments.

Animal number	Template activity[a] of non-regenerating chromatin (N)	Template activity of N as % of that for DNA[b]	Template activity[a] of regenerating chromatin (R)	Template activity of R as % of that for DNA[b]	% change in R relative to N
			1-1.5 Hours of Regeneration		
1	9.86	4.49	7.93	3.61	-19.6
2	8.14	3.71	9.57	4.36	17.6
3	2.97	1.35	19.8	9.01	-33.0
4	54.1	24.6	36.1	16.5	-33.2
5	38.3	17.4	36.0	16.4	-6.00
Total no. of animals	Mean[d] ± S.E.	Mean	Mean[d] ± S.E.	Mean	Mean
5	27.6 ± 8.48	10.3	21.8 ± 8.60	9.96	-14.8

Table 3 - continued

Animal number	Template activity[a] of non-regenerating chromatin (N)	Template activity of N as % of that for DNA[b]	Template activity[a] of regenerating chromatin (R)	Template activity of R as % of that for DNA[b]	% change in R relative to N
			1.5-2 Hours of Regeneration		
1	7.43	3.38	14.0	6.37	88.5
2	12.7	5.79	17.8	8.10	40.0
3	8.50	3.87	11.1	5.07	30.1
4	8.50	3.87	11.8	5.37	38.6
5	24.6	11.2	28.3	12.9	13.1
6	23.5	10.7	44.8	20.4	90.5
Total no. of animals	Mean[c] ± S.E.	Mean	Mean[c] ± S.E.	Mean	Mean
6	14.2 ± 2.89	6.46	21.3 ± 5.16	9.70	50.1

Table 3 - continued

Animal number	Template activity[a] of non-regenerating chromatin (N)	Template activity of N as % of that for DNA[b]	Template activity[a] of regenerating chromatin (R)	Template activity of R as % of that for DNA[b]	% change in R relative to N
			3 Hours of Regeneration		
1	9.21	4.19	10.1	4.62	10.1
2	23.9	10.9	29.4	13.4	23.0
3	36.9	16.8	47.5	21.6	28.6
Total no. of animals	Mean[d] ± S.E.	Mean	Mean[d] ± S.E.	Mean	Mean
3	23.7 ± 7.97	10.6	29.0 ± 10.7	13.2	20.6

Table 3 - continued

Animal number	Template activity[a] of non-regenerating chromatin (N)	Template activity of N as % of that for DNA[b]	Template activity[a] of regenerating chromatin (R)	Template activity of R as % of that for DNA[b]	% change in R relative to N
			4 Hours of Regeneration		
1	23.3	10.6	42.6	19.4	82.8
2	29.7	13.5	58.1	26.5	95.7
Total no. of animals	Mean[d] ± S.E.	Mean	Mean[d] ± S.E.	Mean	Mean
2	26.5 ± 3.18	12.1	50.4 ± 7.74	22.9	89.3
			5 Hours of Regeneration		
1	8.50	3.87	7.86	3.58	-7.60
2	24.7	11.3	25.1	11.4	0.80
Total no. of animals	Mean[e] ± S.E.	Mean	Mean[e] ± S.E.	Mean	Mean
2	16.7 ± 7.74	7.59	16.5 ± 8.42	7.49	-3.40

Table 3 - continued

Animal number	Template activity[a] of non-regenerating chromatin (N)	Template activity of N as % of that for DNA[b]	Template activity[a] of regenerating chromatin (R)	Template activity of R as % of that for DNA[b]	% change in R relative to N
			6 Hours of Regeneration		
1	10.0	4.55	9.93	4.52	-0.70
2	30.2	13.6	23.9	10.9	-20.9
3	40.6	18.7	28.8	13.1	-29.0
Total no. of animals	Mean[e] ± S.E.	Mean	Mean[e] ± S.E.	Mean	Mean
3	26.9 ± 8.89	12.3	20.9 ± 5.17	9.50	-16.9

[a]Expressed as picomoles of H^3-UMP incorporated into RNA per μg of DNA per minute $x10^4$.

[b]Template activities of R and N were expressed as percentages of the template activity of DNA which was 219.7 picomoles of H^3-UMP incorporated into RNA per μg of DNA per minute $x10^4$. This value was the average of six assays.

[c]The difference between the means at this hour was significant ($P<0.001$).

[d]The differences between the means at these hours were not significant ($0.3>P>0.1$).

[e]The difference between the means at this hour was not significant ($0.5>P>0.3$).

Fig. 4. The percent change in template activity of native rat liver chromatin isolated hourly from regenerating liver remnants expressed relative to the template activity of chromatin isolated from the corresponding 0-hr., resected liver segments and plotted against time. The standard error of the mean is indicated by the bars at each point.

Figure 4

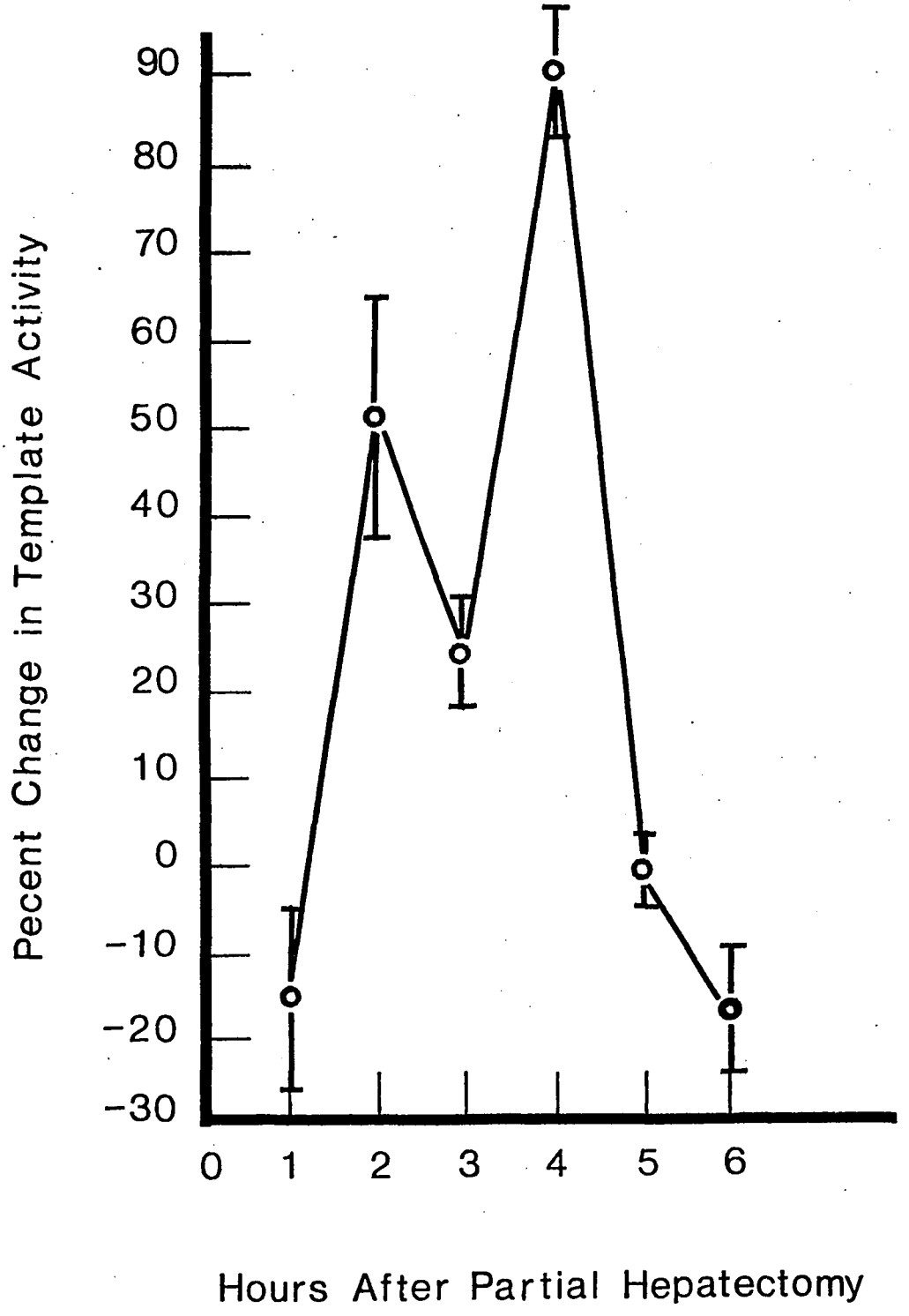

Pecent Change in Template Activity
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
0
1
2
3
4
5
6
Hours After Partial Hepatectomy

TABLE 4.--Template Activity of Reconstituted DNA-Histone Complexes

Assay conditions with the E. coli RNA polymerase and the method of reconstitution were as described in "Materials and Methods". The incorporation obtained for each hour is an average of the results, given in parenthesis, from two separate rats.

Starting ratio of histone to DNA	Final ratio of histone to DNA	Template activity[a]	Percent incorporation relative to DNA
DNA alone (no enzyme)	0.00	0.33(0.38, 0.28)	0.54
DNA	0.00	61.9 (70.1, 53.7)	100.0
0.5	0.14	59.8 (62.1, 57.6)	96.6
1.0	0.84	38.7 (37.0, 40.5)	62.6
2.0	1.76	1.53(1.31, 1.75)	2.47

[a]Expressed as mpicomoles of H^3-UMP incorporated into RNA per μg of DNA per minute.

Fig. 5. Template activity of reconstituted rat liver nucleohistones transcribed with E. coli RNA polymerase and final histone/DNA ratios plotted against starting histone/DNA ratios (data from Table 4).

■ Template Activity

□ Final Ratio of Histone/DNA

Figure 6

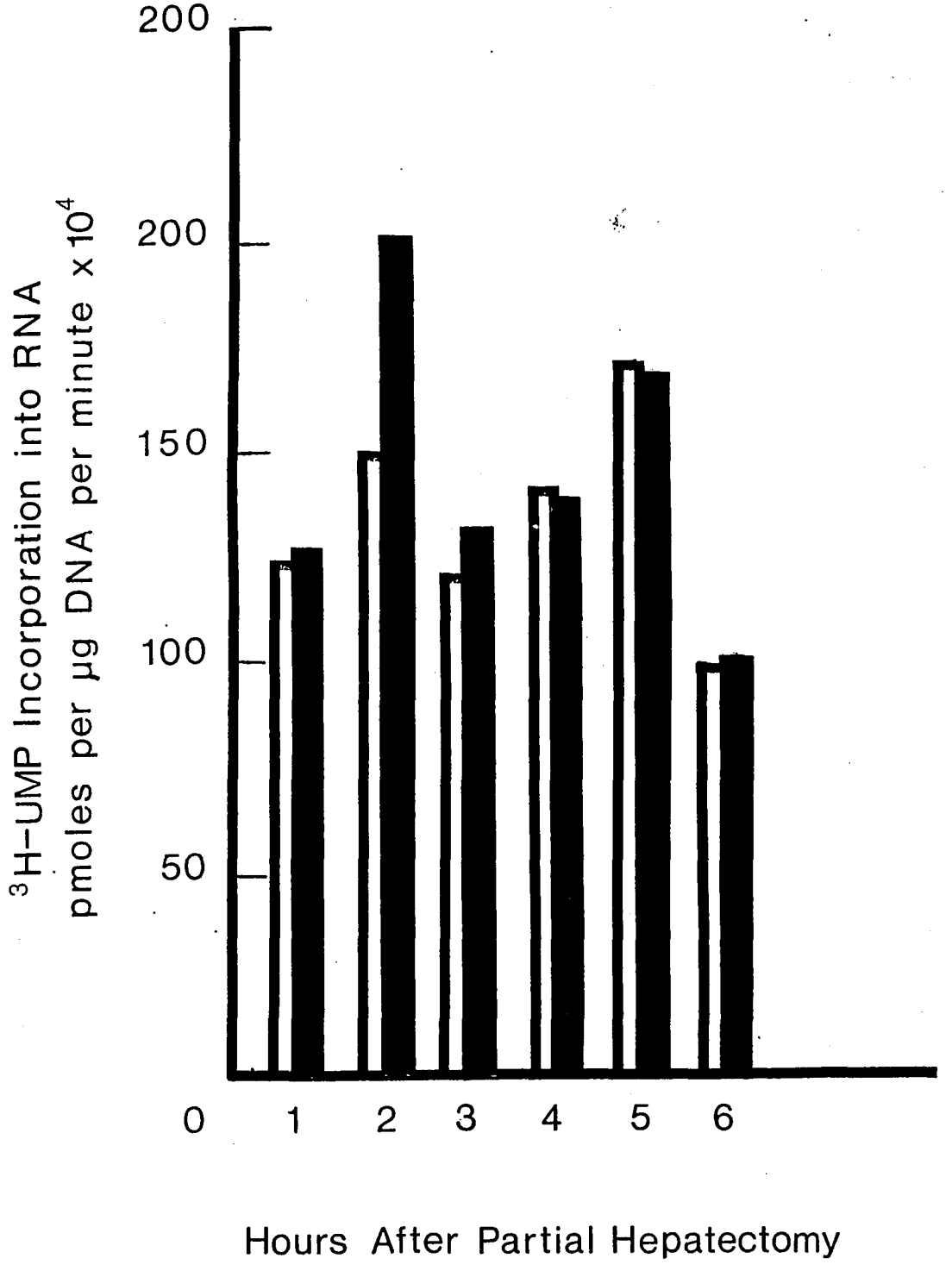

200
150
100
50
0
1
2
3
4
5
6
^{3}H-UMP Incorporation into RNA
pmoles per µg DNA per minute x 10^4
Hours After Partial Hepatectomy

TABLE 5.--Template Activity of Reconstituted Regenerating and Non-regenerating Rat Liver Nucleohistones

The assay conditions for transcription with E. coli RNA polymerase and the method of reconstitution were as described in "Materials and Methods". The starting ratio of histone to DNA was 1.0. R refers to nucleohistone complexes containing histones extracted from chromatin of nuclei isolated from liver remnants at different hours of regeneration. N refers to nucleohistone complexes containing histones extracted from chromatin of nuclei isolated from the corresponding 0-hour resected liver segments.

Animal number	Template activity[a] of non-regenerating nucleohistone complexes (N)	Template activity of N as % of that for DNA[b]	Template activity[a] of regenerating nucleohistone complexes (R)	Template activity of R as % of that for DNA[b]	% change in R relative to N
			1-1.5 Hours of Regeneration		
1	104.8	37.3	102.1	36.4	-2.50
2	97.29	34.7	109.0	38.8	12.0
3	141.1	50.3	168.8	60.1	-19.6
4	186.9	66.6	155.9	55.5	-16.6
5	93.8	33.4	106.4	37.9	13.4
Total no. of animals	Mean[g] ± S.E.	Mean	Mean[g] ± S.E.	Mean	Mean
5	124.8 ± 17.6	44.4	128.4 ± 13.9	45.7	-2.66

Table 5 - continued

Animal number	Template activity[a] of non-regenerating nucleohistone complexes (N)	Template activity of N as % of that for DNA[b]	Template activity[a] of regenerating nucleohistone complexes (R)	Template activity of R as % of that for DNA[b]	% change in R relative to N
			1.5-2 Hours of Regeneration		
1	110.9	37.5	151.2	53.9	36.4
2	208.6	74.3	252.4	89.9	21.0
3	81.71	29.1	121.9	43.4	48.0
4	250.5	89.2	322.1	115	28.6
5	174.7	62.2	216.6	77.1	24.0
6	80.71	28.7	135.3	48.2	40.3
Total no. of animals	Mean[c] ± S.E.	Mean	Mean[c] ± S.E.	Mean	Mean
6	151.2 ± 29.1	53.9	199.8 ± 32.0	71.2	33.1

Table 5 - continued

Animal Number	Template activity[a] of non-regenerating complexes (N)	Template activity of N as % of that for DNA[b]	Template activity[a] of regenerating nucleohistone complexes (R)	Template activity of R as % of that for DNA[b]	% change in R relative to N
			3 Hours of Regeneration		
1	95.14	33.9	108.5	38.6	14.0
2	109.8	39.1	118.7	42.3	8.10
3	153.9	54.8	166.7	59.4	8.40
Total no. of animals	Mean[d] ± S.E.	Mean	Mean[d] ± S.E.	Mean	Mean
3	119.6 ± 17.6	42.6	131.3 ± 18.2	46.8	10.2

Table 5 - continued

Animal number	Template activity[a] of non-regenerating nucleohistone complexes (N)	Template activity of N as % of that for DNA[b]	Template activity[a] of regenerating nucleohistone complexes (R)	Template activity of R as % of that for DNA[b]	% change in R relative to N
			4 Hours of Regeneration		
1	246.6	87.8	231.4	82.4	-0.10
2	99.71	35.5	105.4	37.5	5.70
3	123.4	44.0	122.8	43.7	-0.50
4	89.40	31.8	99.00	35.3	10.7
Total no. of animals	Mean[e] ± S.E.	Mean	Mean[e] ± S.E.	Mean	Mean
4	139.8 ± 36.3	49.7	139.7 ± 31.0	49.7	3.95

Table 5 - continued

Animal number	Template activity[a] of non-regenerating nucleohistone complexes (N)	Template activity of N as % of that for DNA[b]	Template activity[a] of regenerating nucleohistone complexes (R)	Template activity of R as % of that for DNA[b]	% change in R relative to N
			5 Hours of Regeneration		
1	219.1	78.0	227.1	80.9	3.70
2	118.4	42.2	105.6	37.6	-10.8
Total no. of animals	Mean[f] ± S.E.	Mean	Mean[f] ± S.E.	Mean	Mean
2	168.8 ± 50.4	60.1	166.4 ± 60.8	59.2	-7.10
			6 Hours of Regeneration		
1	104.3	37.2	103.0	36.9	-1.20
2	88.90	31.7	92.6	33.0	4.20
Total no. of animals	Mean[f] ± S.E.	Mean	Mean[f] ± S.E.	Mean	Mean
2	96.58 ± 14.5	34.4	97.79 ± 6.33	34.8	3.00

Table 5 - continued

[a]Expressed as picomoles of H^3-UMP incorporated into RNA per µg of DNA per minute x 10^4.

[b]Template activities of R and N were expressed as percentages of the template activity of DNA which was 280.9 picomoles of H^3-UMP incorporated into RNA per µg of DNA per minute x10^4. This value was the average of ten assays.

[c]The difference between the means at this hour was significant ($P < 0.001$).

[d]The difference between the means at this hour was significant ($0.05 > P > 0.01$).

[e]The difference between the means at this hour was not significant ($0.1 > P > 0.05$).

[f]The differences between the means at these hours were not significant ($0.3 > P > 0.1$).

[g]The difference between the means at this hour was not significant ($0.7 > P > 0.5$).

Fig. 6. Template activity of regenerating and non-regenerating reconstituted nucleohistone complexes transcribed with E. coli RNA polymerase plotted against time after the operation (data from Table 5).

■ Template activity of nucleohistone complexes containing histones extracted from regenerating liver remnants

□ Template activity of nucleohistone complexes containing histones extracted from corresponding non-regenerating, resected liver segments

Figure 6

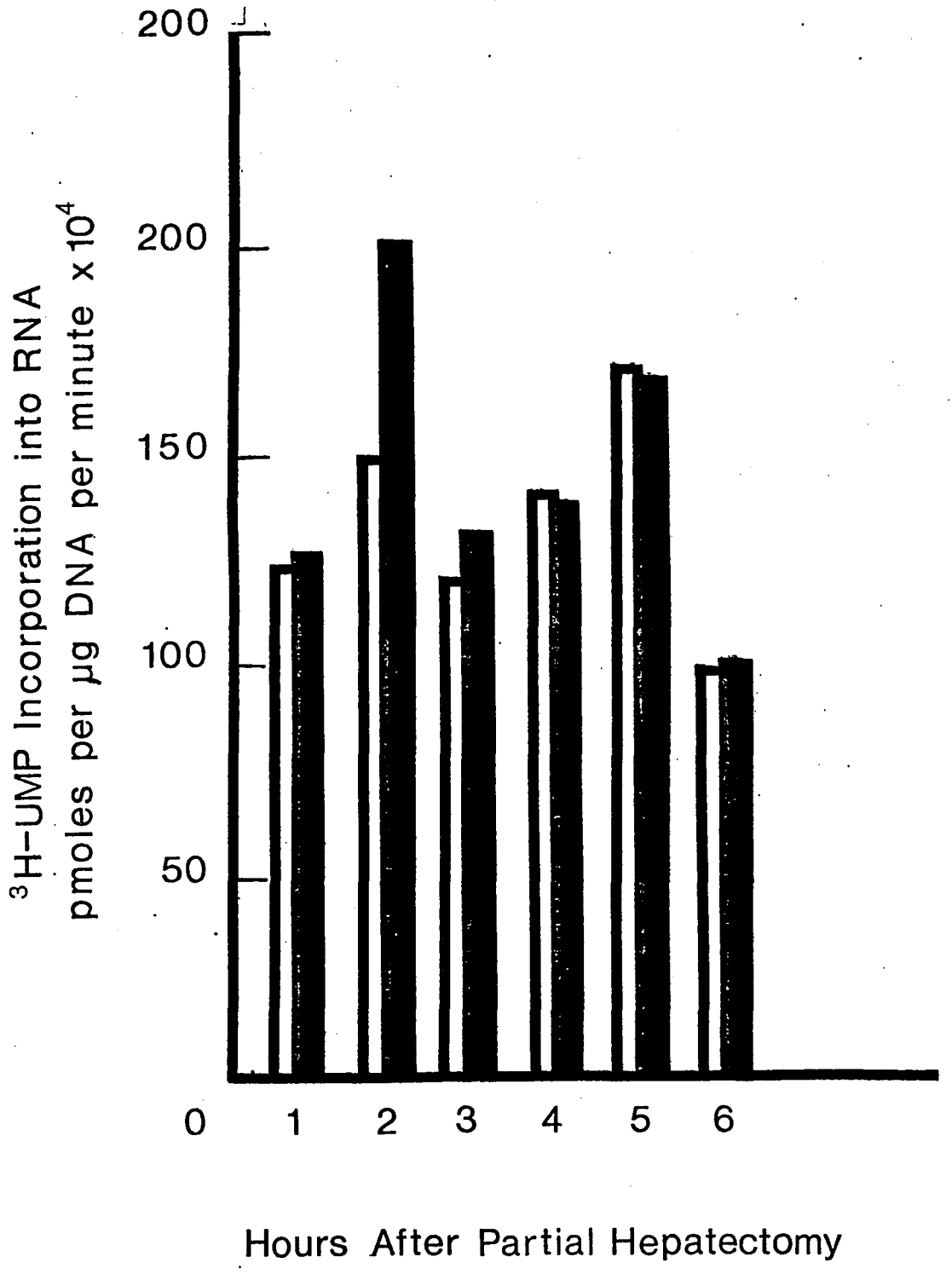

200
200
150
100
50
0
1
2
3
4
5
6
^{3}H-UMP Incorporation into RNA
pmoles per µg DNA per minute x 10^4
Hours After Partial Hepatectomy

Fig. 7. The percent change in template activity of reconstituted nucleohistone complexes containing histones isolated hourly from regenerating liver remnants expressed relative to the template activity of nucleohistone complexes containing histones isolated from the corresponding 0-hr, resected segments and plotted against time. The standard error of the mean is indicated by the bars at each hour.

Figure 7

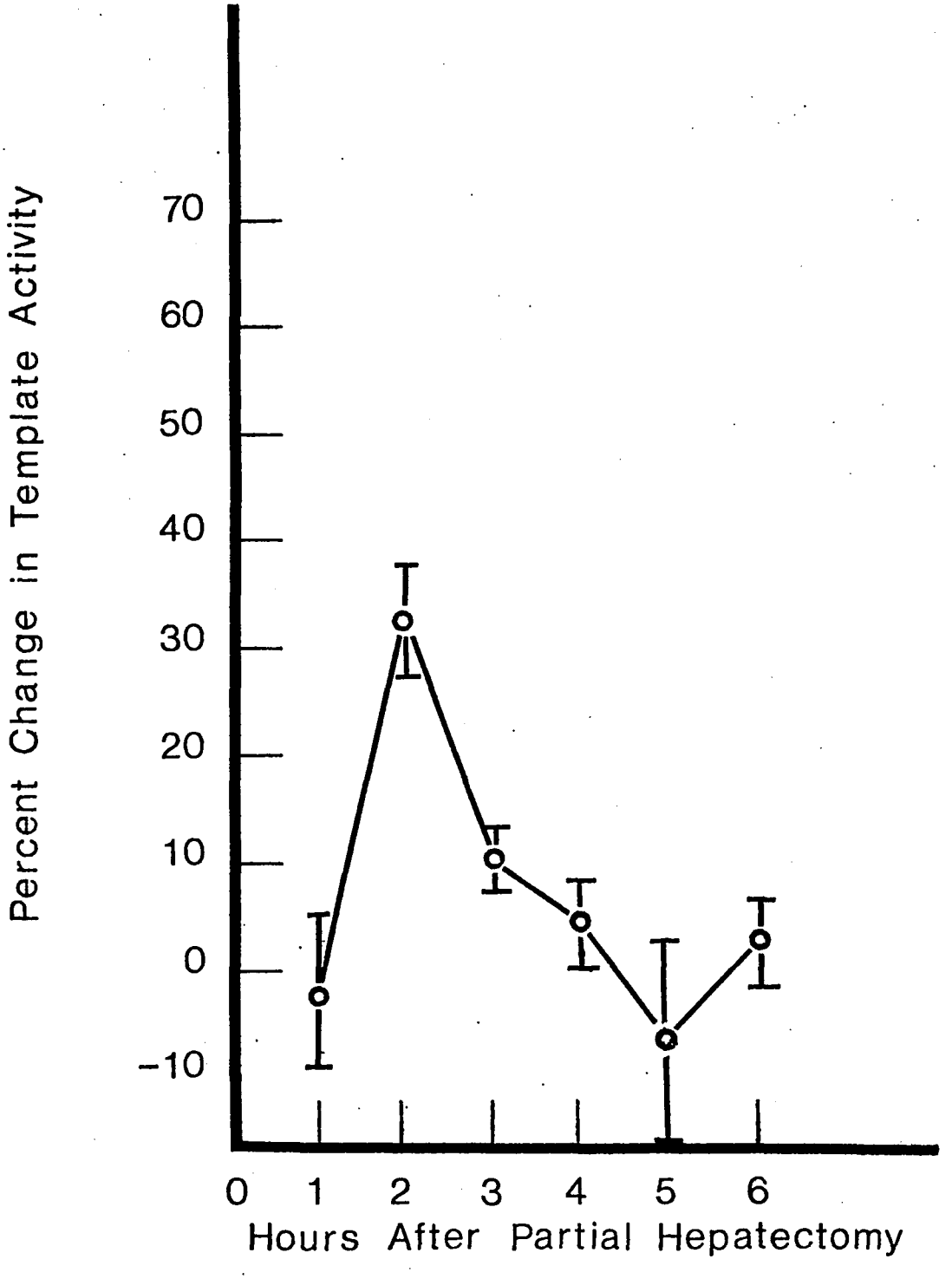

Percent Change in Template Activity
70
60
50
40
30
20
10
0
-10
0
1
2
3
4
5
6
Hours After Partial Hepatectomy

TABLE 6.--Template Activity of Reconstituted Complexes Composed of DNA and Acidic Proteins from Non-Regenerating Livers and of Histones Extracted from Livers at Different Hours During Regeneration

Transcription with E. coli RNA polymerase and the method of reconstitution were as described in "Materials and Methods". The ratio of histone:acidic protein:DNA was 2.0:0.6:1.0.

Hrs. after partial hepatectomy	Template activity[a]	Template activity as % of DNA	% change in template activity relative to 0-hr.
Control (no enzyme)	3.33	0.65	-
DNA	517.87	100.0	-
		Experiment I	
0[b]	21.13	4.10	-
1	49.67	9.60	135.0
2	161.20	31.10	662.9
3	73.33	14.20	247.1
4	28.87	5.60	36.6
6	20.53	4.00	-2.8
		Experiment II	
0[b]	40.13	7.70	-
1.5	378.40	73.1	842.9
3	93.73	18.1	133.6
4	16.40	3.2	-59.1
5	22.33	4.3	-44.4
6	23.87	4.6	-40.5

[a]Expressed as picomoles of H^3-UMP incorporated into RNA per μg of DNA per minute x 10^4.

[b]Sham-operated control.

Fig. 8. Template activity of reconstituted complexes composed of DNA and acidic proteins from non-regenerating livers and of histones extracted from livers at different hours during regeneration plotted against time (data from Table 6: Experiment I).

Figure 8

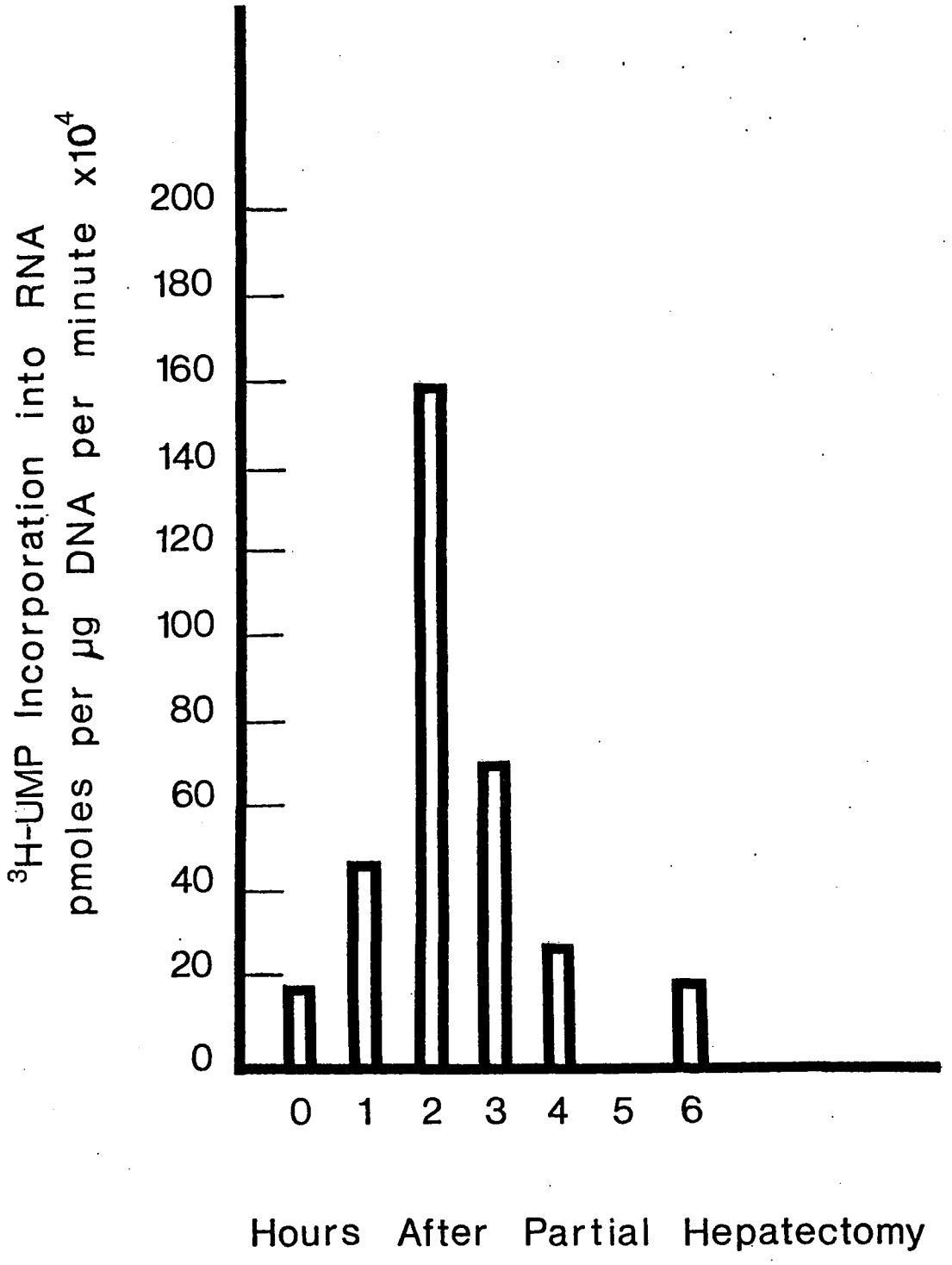

^{3}H-UMP Incorporation into RNA
pmoles per µg DNA per minute x10^4
200
180
160
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6
Hours After Partial Hepatectomy

Fig. 9. Template activity of reconstituted complexes composed of DNA and acidic proteins from non-regenerating livers and of histones extracted from livers at different hours during regeneration plotted against time (data from Table 6:Experiment II).

Figure 9

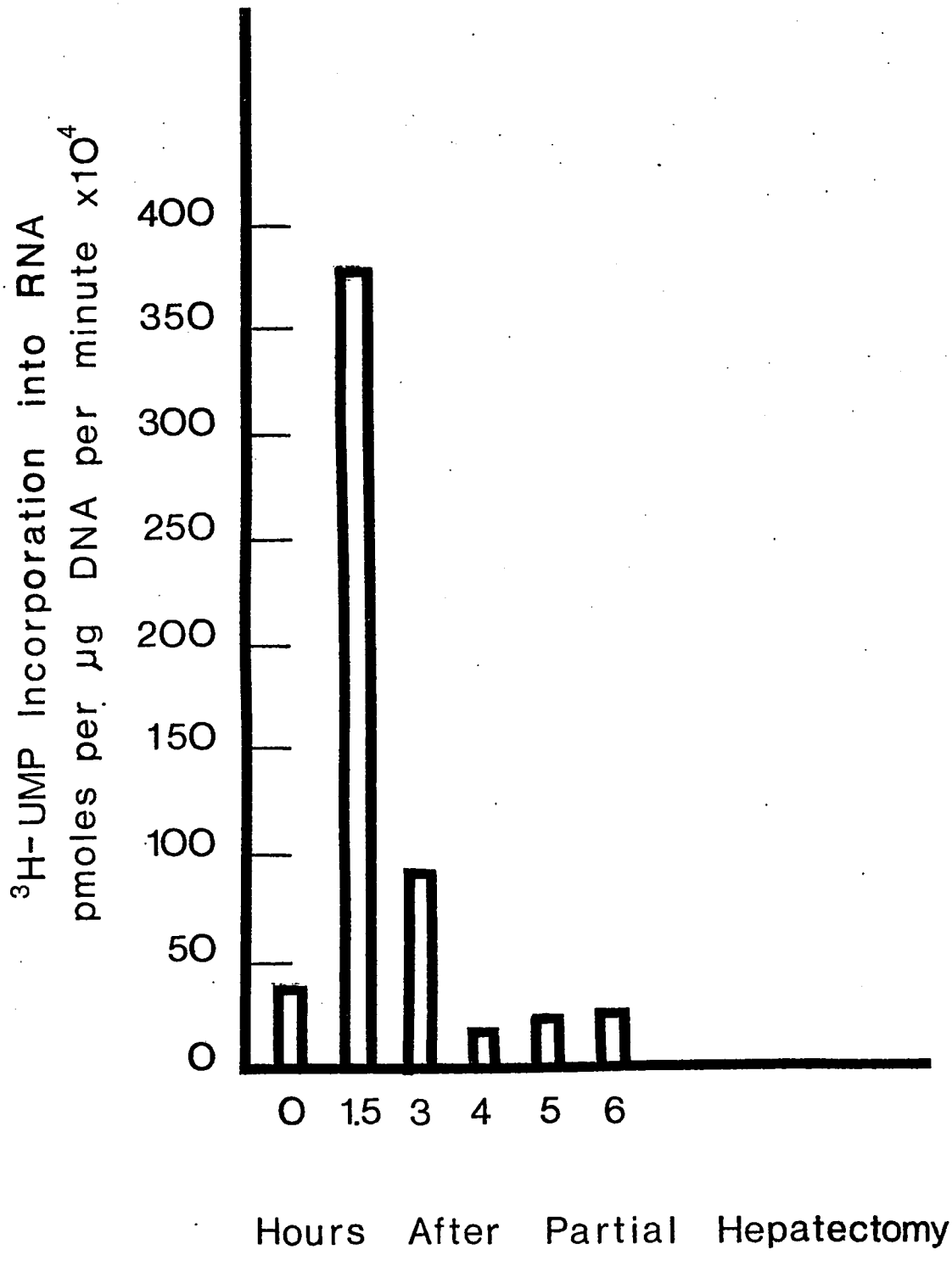

^{3}H-UMP Incorporation into RNA
pmoles per µg DNA per minute x10^4
400
350
300
250
200
150
100
50
0
0
1.5
3
4
5
6
Hours After Partial Hepatectomy

Fig. 10. The percent change in template activity of reconstituted complexes of DNA and acidic proteins from non-regenerating livers and of histones extracted from livers at different hours during regeneration expressed relative to 0-hr. template activity and plotted against time.

Figure 10

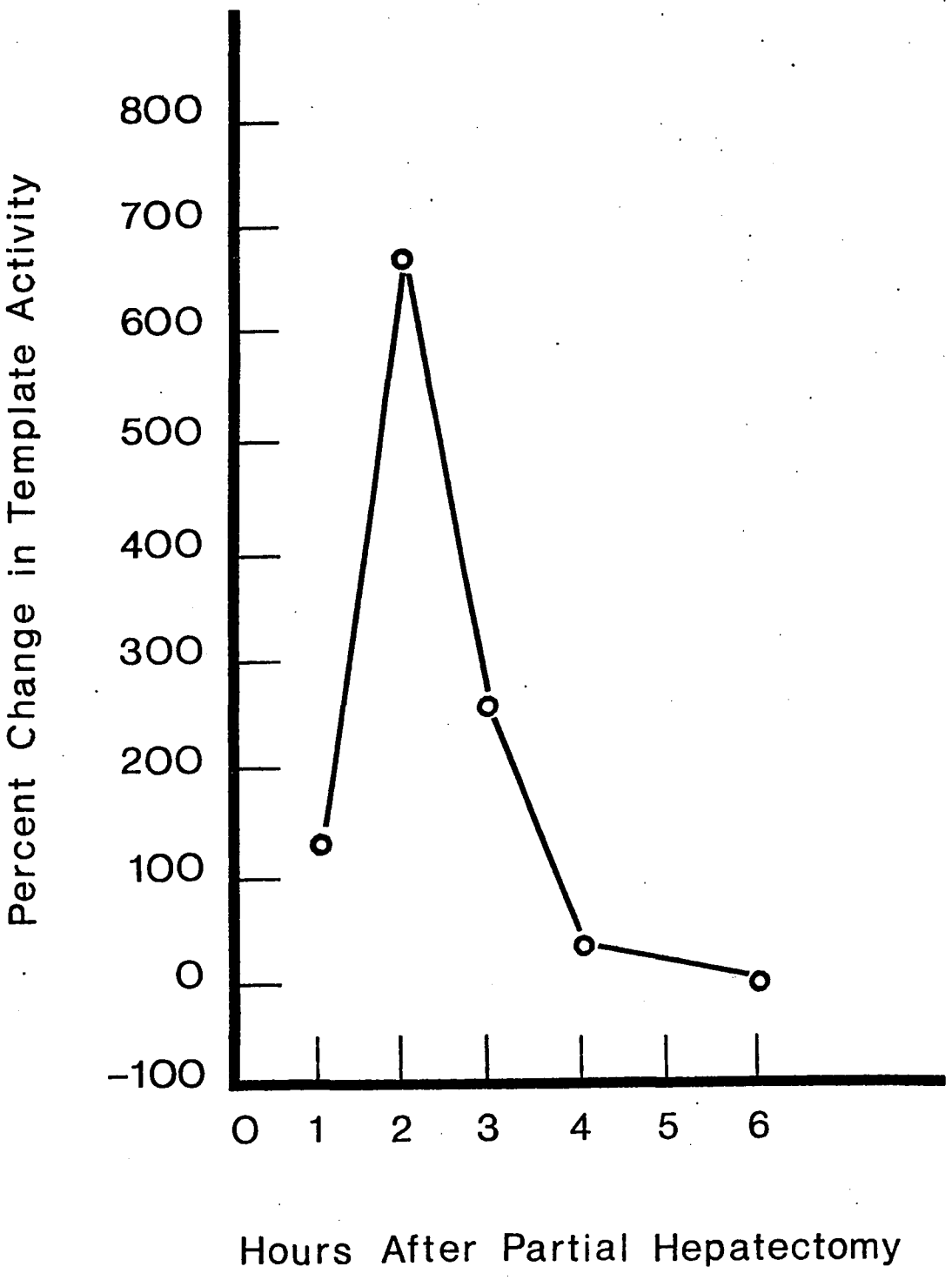

Percent Change in Template Activity
800
700
600
500
400
300
200
100
0
-100
0 1 2 3 4 5 6
Hours After Partial Hepatectomy

Fig. 11. The percent change in template activity of reconstituted complexes composed of DNA and acidic proteins from non-regenerating livers and of histones extracted from livers at different hours during regeneration expressed relative to 0-hr. template activity and plotted against time.

Figure 11

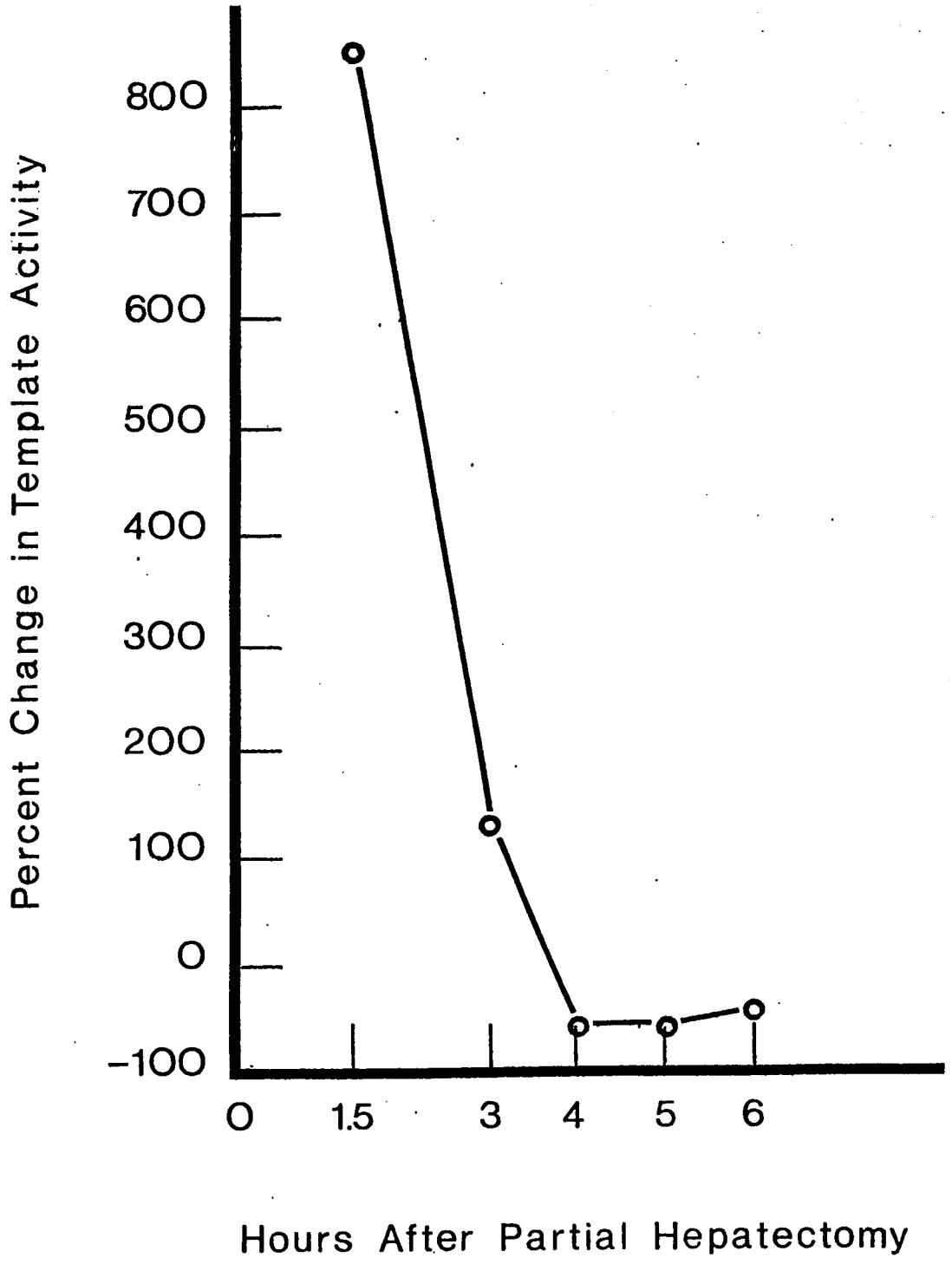

Percent Change in Template Activity
800
700
600
500
400
300
200
100
0
-100
0
1.5
3
4
5
6
Hours After Partial Hepatectomy

TABLE 7.--Template Activity of Reconstituted Complexes Composed of DNA and Histones from Non-regenerating Livers and of Acidic Proteins Extracted from Livers at Different Hours During Regeneration

Transcription with E. coli RNA polymerase and the method of reconstitution were as described in "Materials and Methods". The ratio of histone: acidic protein: DNA was 2.0:0.6:1.0.

Hrs. after partial hepatectomy	Template activity[a]	Template activity as % of DNA	% change in template activity relative to 0-hr.
Control (no enzyme)	3.33	0.65	-
DNA	517.87	100.0	-
		Experiment I	
0[b]	40.13	7.60	-
1	45.80	8.80	14.1
2	34.73	6.70	-13.5
3	43.80	8.50	9.1
4	91.53	17.80	128.1
6	55.20	10.70	37.6
		Experiment II	
0[b]	131.27	25.30	-
1.5	136.20	26.30	3.8
3	158.87	30.60	21.0
4	261.33	50.50	99.1
5	135.67	26.20	3.3
6	178.47	34.40	36.0

[a]Expressed as picomoles of H^3-UMP incorporated into RNA per μg of DNA per minute x 10^4.

[b]Sham-operated control.

Fig. 12. Template activity of reconstituted complexes composed of DNA and histones from non-regenerating livers and of acidic proteins extracted from livers at different hours during regeneration plotted against time (data from Table 7: Experiment I).

Figure 12

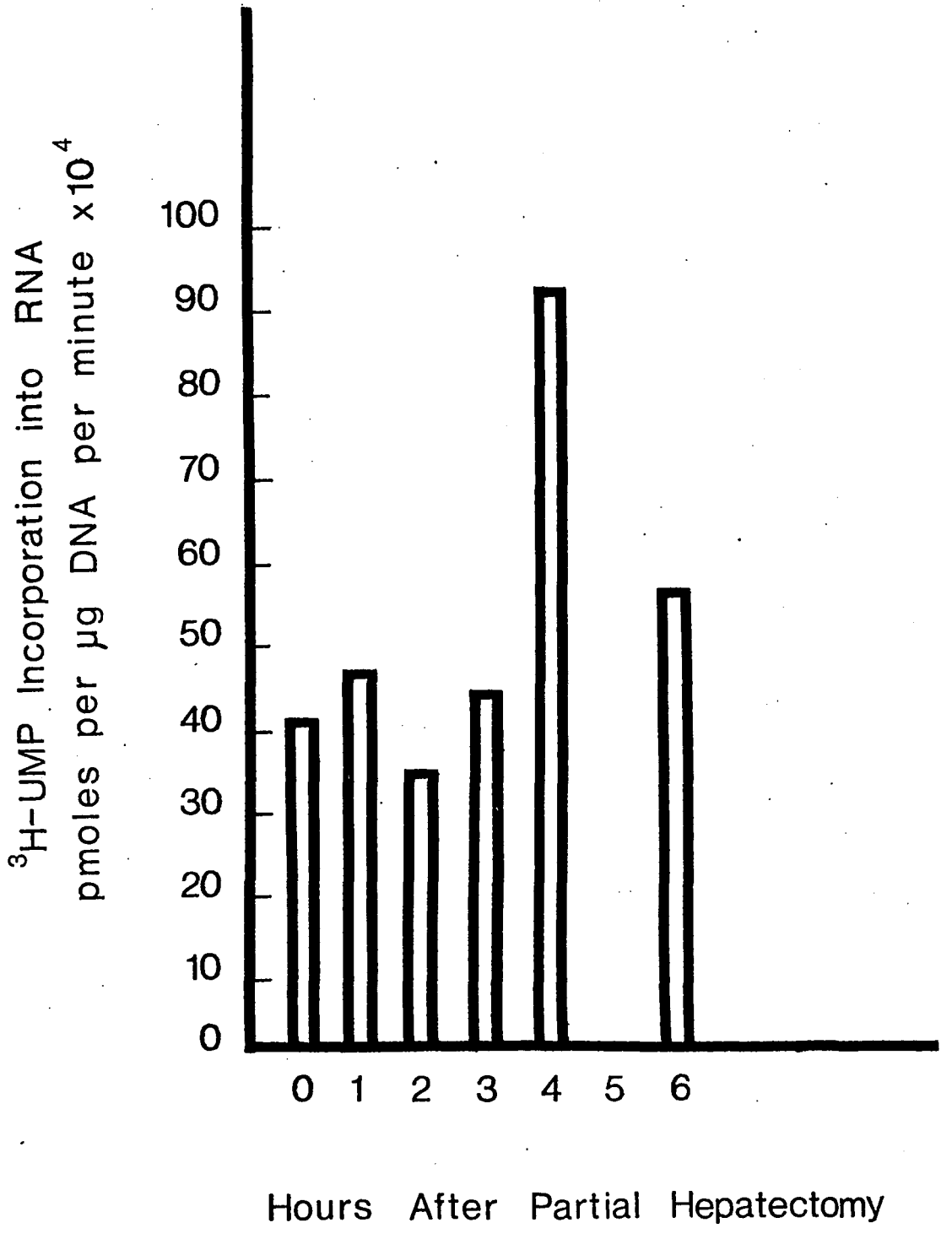

^{3}H-UMP Incorporation into RNA
pmoles per µg DNA per minute x10^4
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6
Hours After Partial Hepatectomy

Fig. 13. Template activity of reconstituted complexes composed of DNA and histones from non-regenerating livers and of acidic proteins extracted from livers at different hours during regeneration plotted against time (data from Table 7: Experiment II).

Figure 13

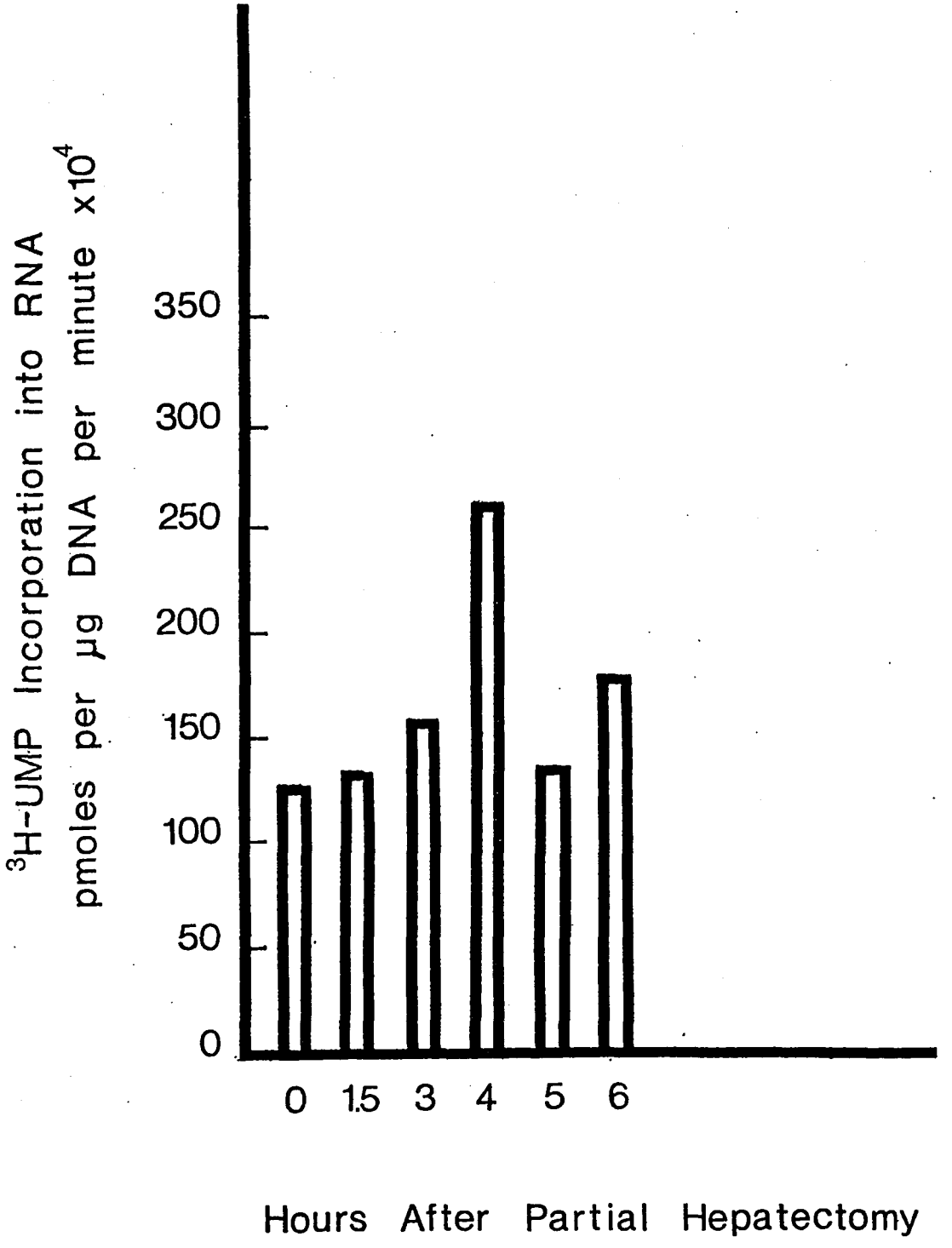

^{3}H-UMP Incorporation into RNA
pmoles per µg DNA per minute x10^4
350
300
250
200
150
100
50
0
0
1.5
3
4
5
6
Hours After Partial Hepatectomy

Fig. 14. The percent change in template activity of reconstituted complexes composed of DNA and histones from non-regenerating livers and of acidic proteins extracted from different hours during regeneration expressed relative to 0-hr. template activity and plotted against time.

Figure 14

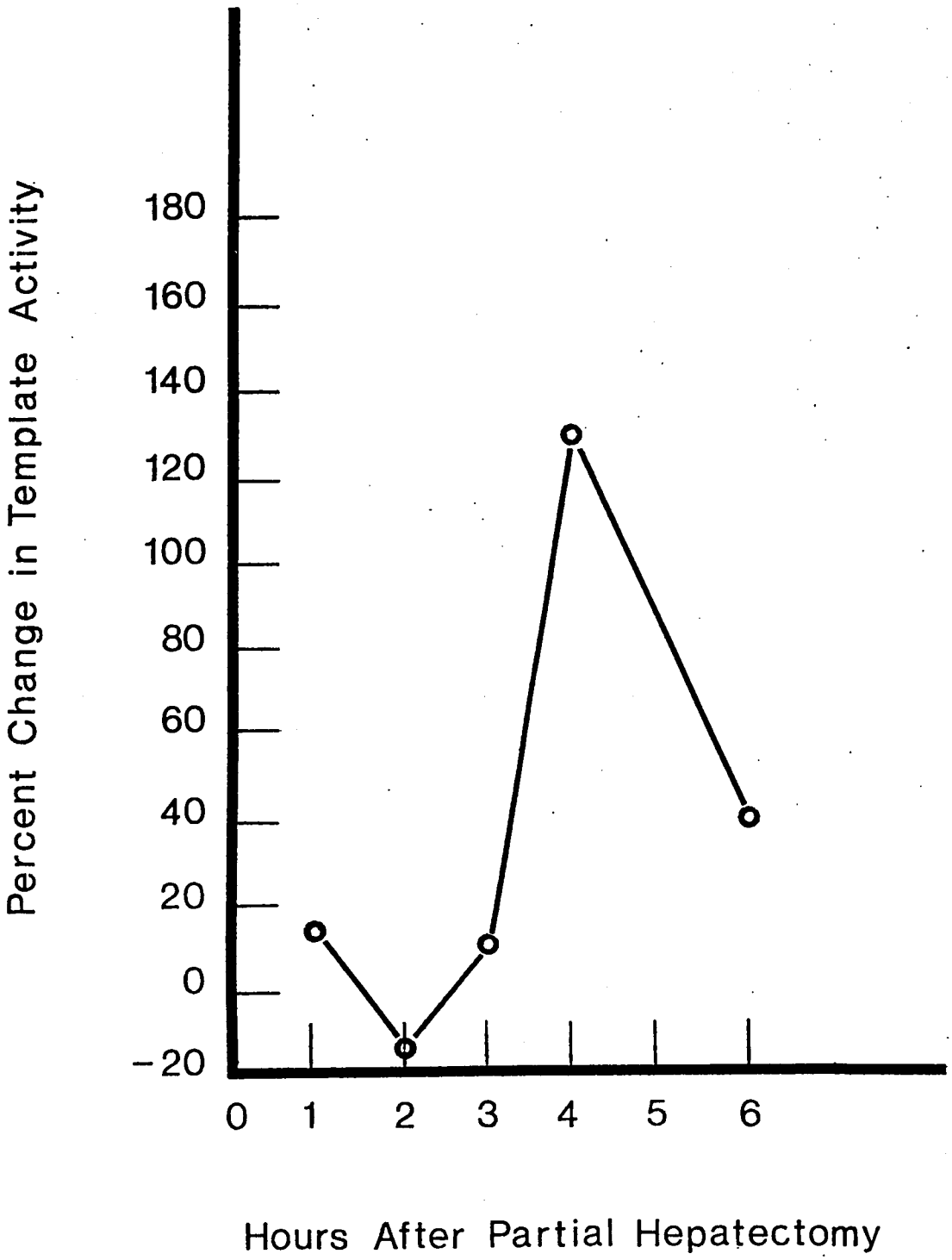

Percent Change in Template Activity
180
160
140
120
100
80
60
40
20
0
-20
0
1
2
3
4
5
6
Hours After Partial Hepatectomy

Fig. 15. The percent change in template activity of reconstituted complexes composed of DNA and histones from non-regenerating livers and of acidic proteins extracted from livers at different hours during regeneration expressed relative to 0-hr. template activity and plotted against time.

Figure 15

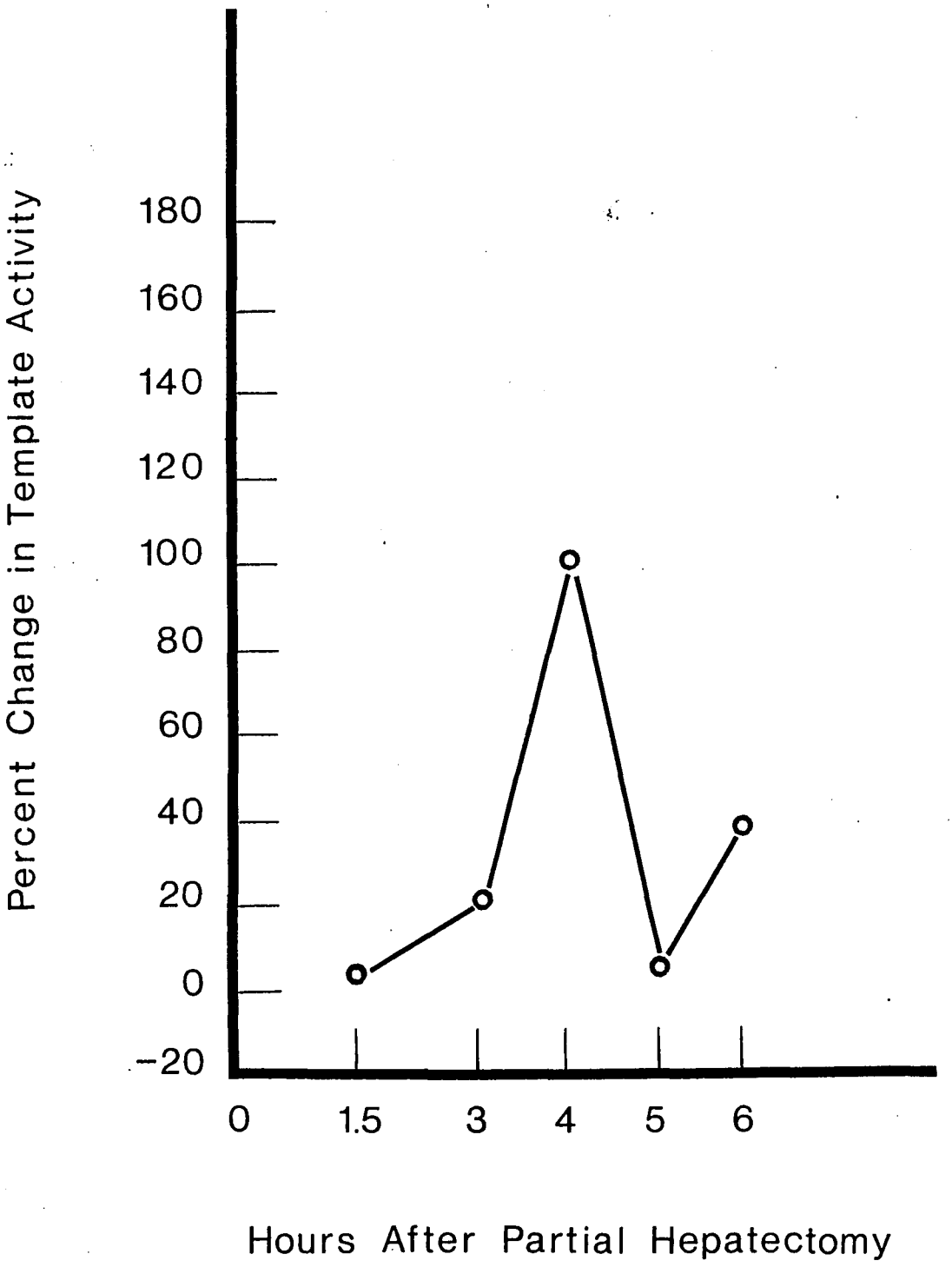

Percent Change in Template Activity
180
160
140
120
100
80
60
40
20
0
-20
0
1.5
3
4
5
6
Hours After Partial Hepatectomy

TABLE 8.--Template Activity of Reconstituted Complexes Composed of DNA from Non-regenerating Livers and of Acidic Proteins Extracted from Livers at Different Hours During Regeneration

Transcription with E. coli RNA polymerase and the method of reconstitution were as described in "Materials and Methods". The ratio of acidic protein: DNA was 0.6:1.0.

Hrs. after partial hepatectomy	Template activity[a]	Template activity as % of DNA	% change in template activity relative to 0-hr.
Control (no enzyme)	3.33	0.65	-
DNA	517.87	100.0	-
0[b]	670.09	129.5	-
1.5	669.93	117.8	-0.1
3	747.80	144.4	11.5
4	755.60	145.9	12.6
5	657.00	126.9	-2.1
6	668.53	129.1	-0.3

[a]Expressed as picomoles of H^3-UMP incorporated into RNA per μg of DNA per minute x 10^4.

[b]Sham-operated control.

Fig. 16. Template activity of reconstituted complexes composed of DNA from non-regenerating livers and of acidic proteins extracted from livers at different hours during regeneration plotted against time (data from Table 8).

Figure 16

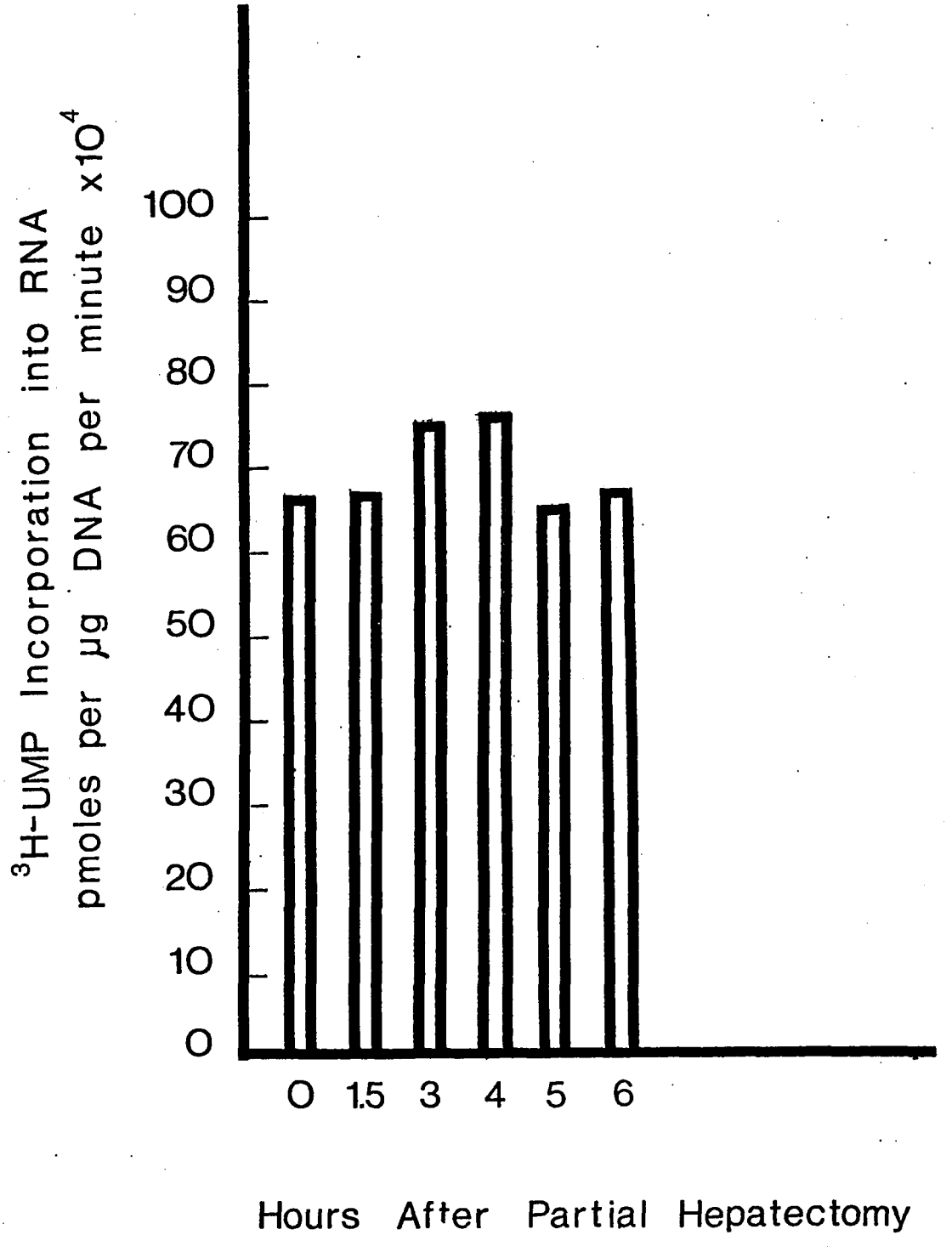

^{3}H-UMP Incorporation into RNA
pmoles per µg DNA per minute x10^4
100
90
80
70
60
50
40
30
20
10
0
0
1.5
3
4
5
6
Hours After Partial Hepatectomy

Fig. 17. The percent change in template activity of reconstituted complexes composed of DNA from non-regenerating livers and of acidic proteins extracted from livers at different hours during regeneration expressed relative to 0-hr. template activity and plotted against time.

Figure 17

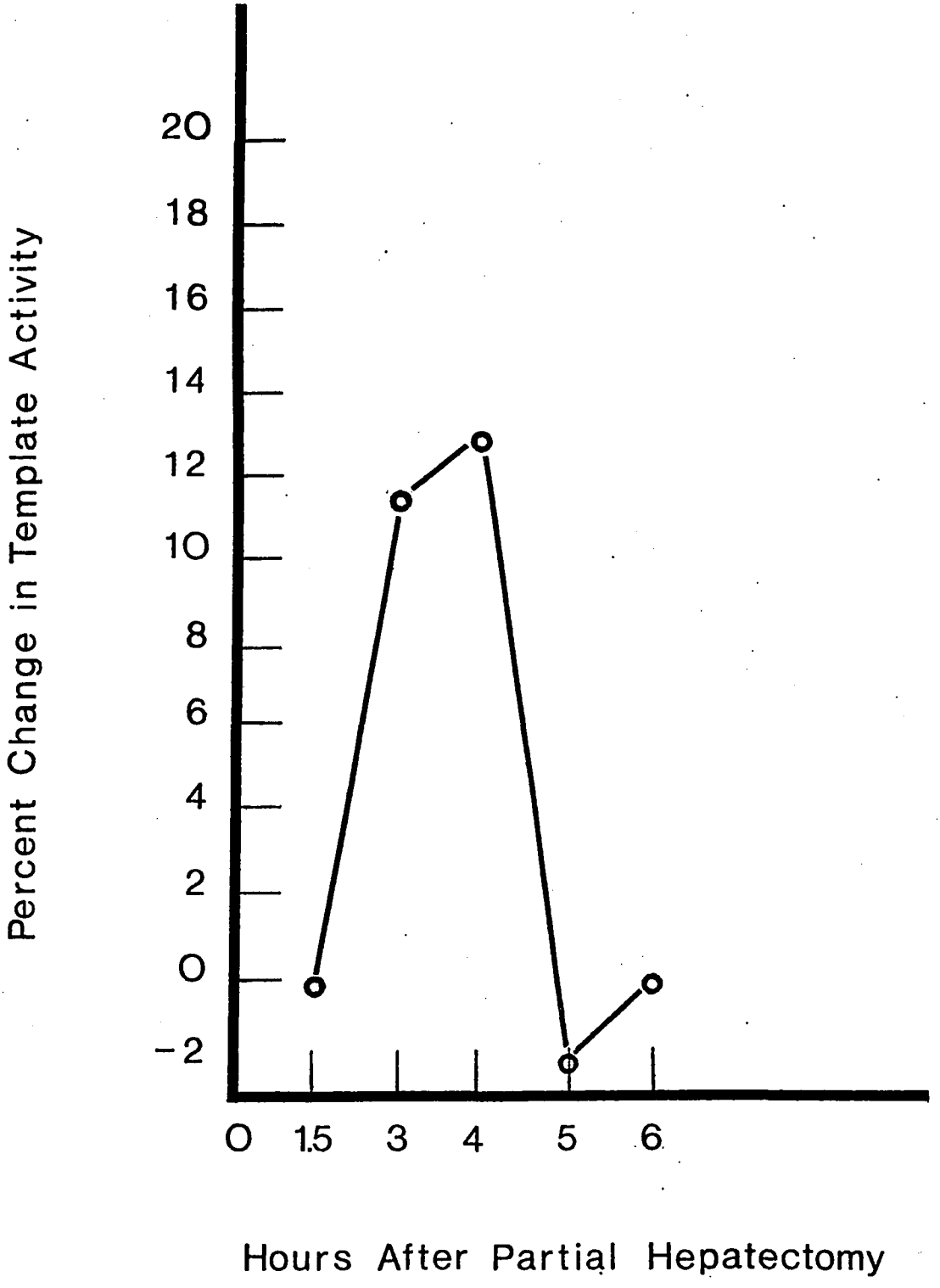

Percent Change in Template Activity
20
18
16
14
12
10
8
6
4
2
0
-2
0
1.5
3
4
5
6
Hours After Partial Hepatectomy

Fig. 18. Percent change in amount of the five major histone fractions plotted against the time after the operation (computed from the data in Table 9). (A) F1, (B) F3, (C) F2b, (D) F2a2, (E) F2a1.

Figure 18

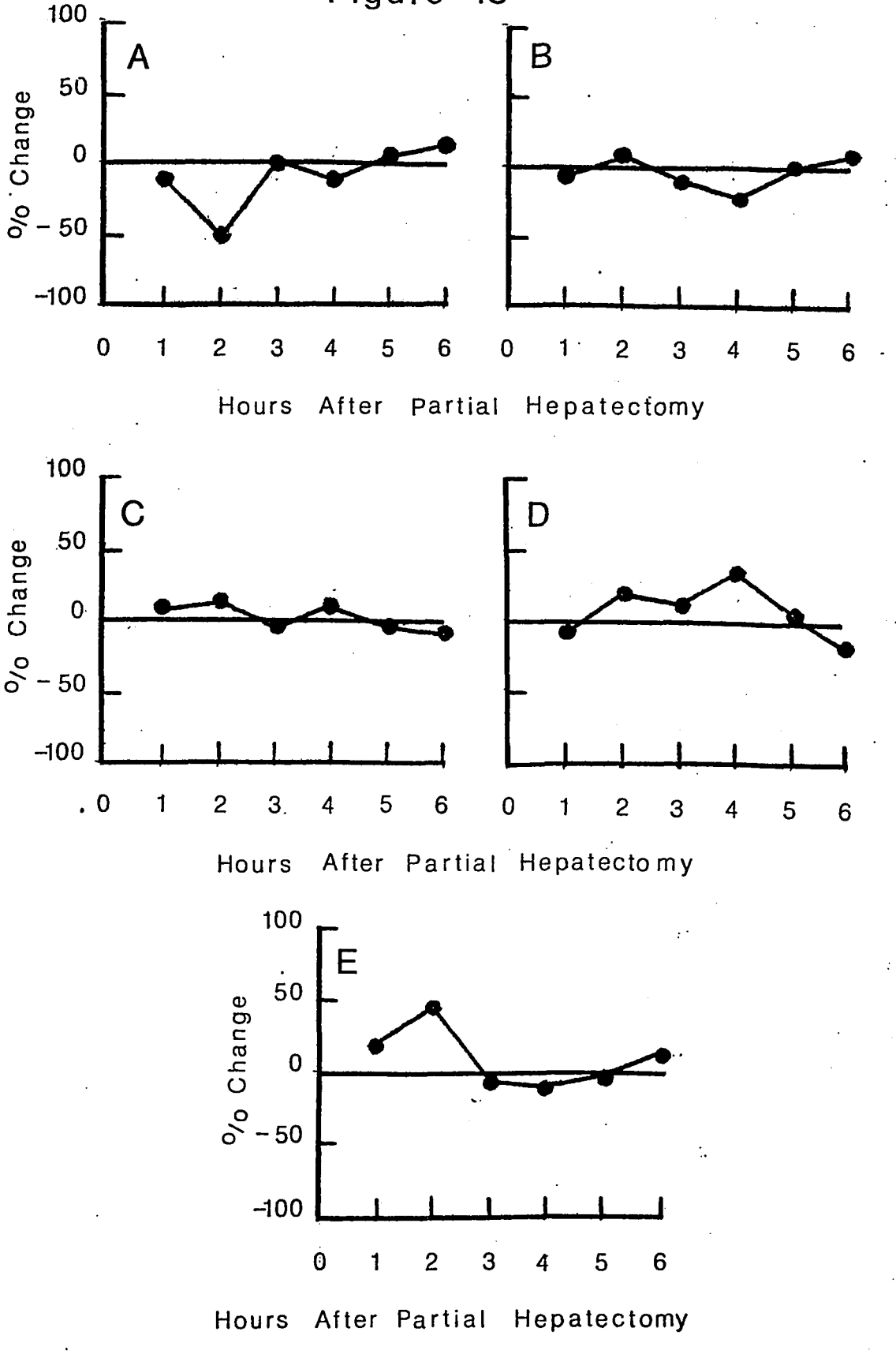

A
B
C
D
E
100
50
0
-50
-100
% Change
0 1 2 3 4 5 6
Hours After Partial Hepatectomy

TABLE 9.--Polyacrylamide gel Scanning Quantitation of the Five Major Histone Fractions During Regeneration

The Percentages of the five histone fractions were determined for chromatins from liver remnants (R) at different hours during regeneration and from the corresponding 0-hr. resected segments (N). The conditions for electrophoresis and for gel scanning were as described in "Materials and Methods".

Hrs. after partial hepatectomy	F1		F3		F2b		F2a2		F2a1	
	N	R	N	R	N	R	N	R	N	R
1	14.2	12.7	22.4	20.7	28.2	30.4	20.5	19.0	14.6	17.2
2	24.2	11.1	16.5	17.5	27.8	30.9	18.6	22.0	12.8	18.6
3	19.8	20.1	18.8	16.9	30.0	29.2	14.0	15.7	19.4	18.3
4	17.6	15.9	21.1	16.5	28.5	31.4	14.6	19.6	18.2	16.5
5	20.3	21.7	16.5	16.4	30.9	29.2	14.9	15.6	17.4	17.1
6	19.0	21.3	17.2	18.5	29.8	27.1	16.9	13.9	17.1	19.2

Fig. 19. Long polyacrylamide gel scans of histones extracted from 1 hr. regenerating liver remnants and from the corresponding 0-hr. resected segments. The numbers represent the percentages of the five major histone fractions (data from Table 9).

Figure 19

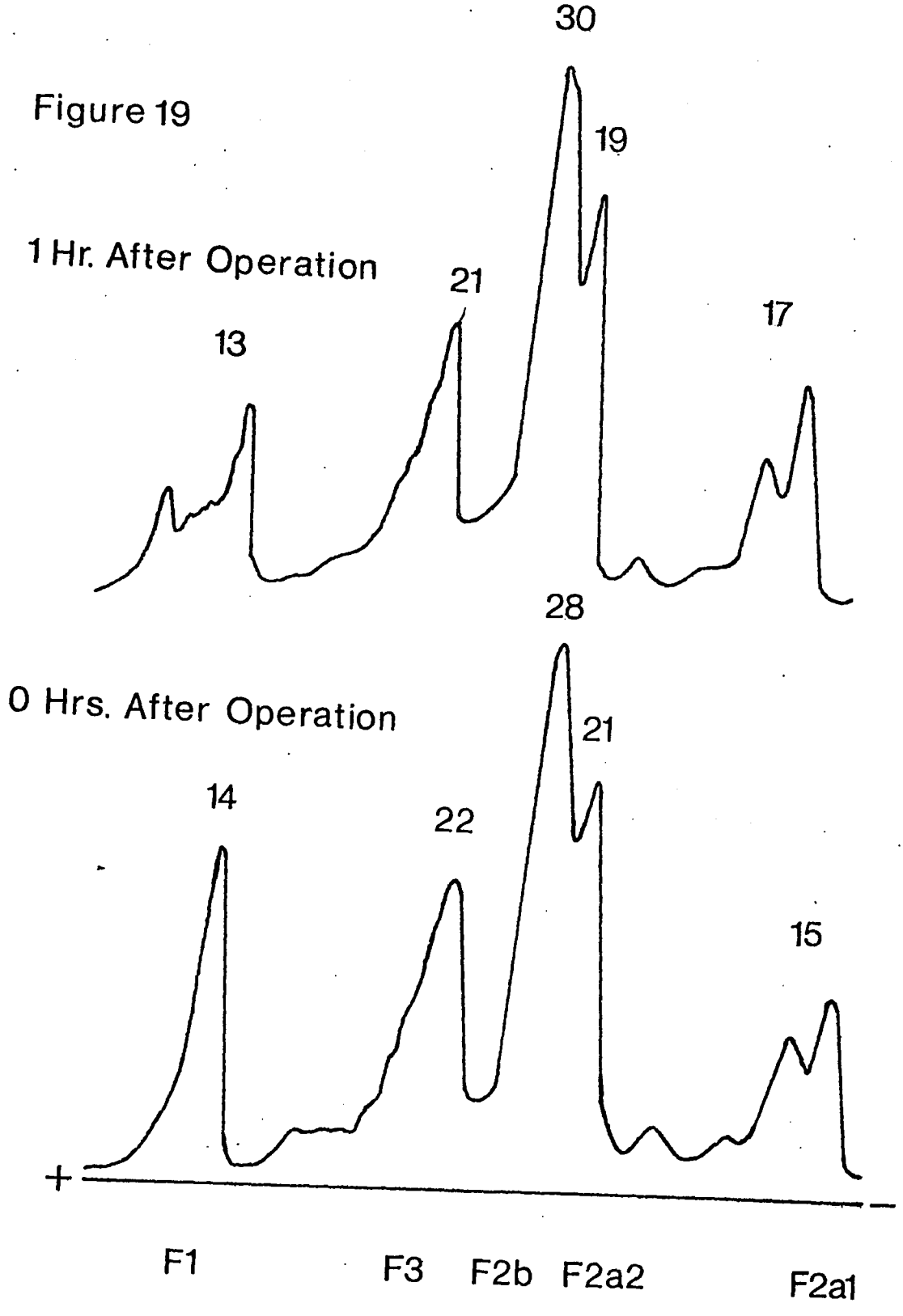
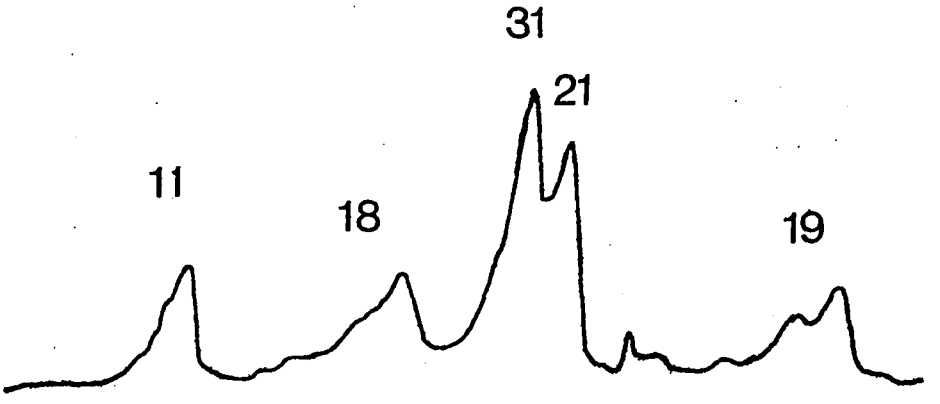

30
19
1 Hr. After Operation
21
17
13
28
0 Hrs. After Operation
21
14
22
15
+
F1
F3
F2b
F2a2
F2a1

Fig. 20. Long polyacrylamide gel scans of histones extracted from 2 hr. regenerating liver remnants and from the corresponding 0-hr. resected segments. The numbers represent the percentages of the five major histone fractions (data from Table 9).

Figure 20

2 Hrs. After Operation



0 Hrs. After Operation

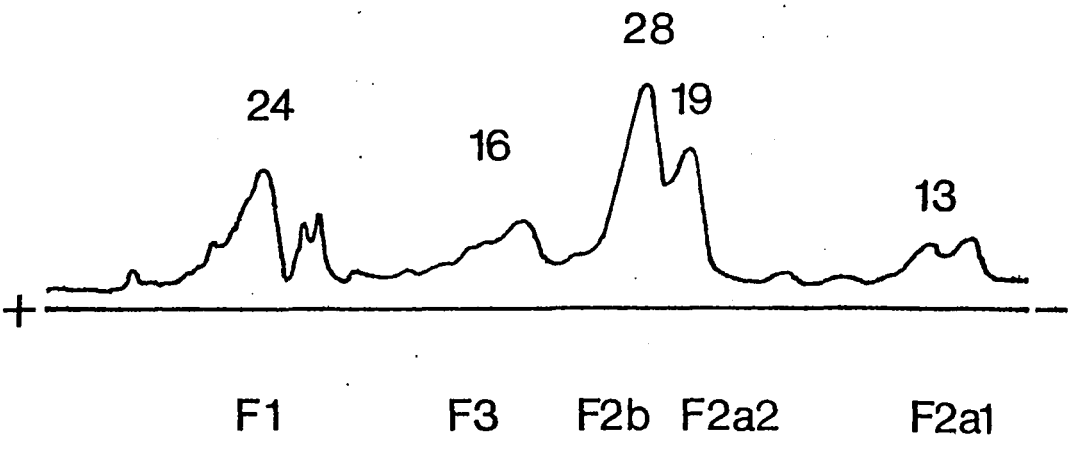


Fig. 21. Long polyacrylamide gel scans of histones extracted from 3 hr. regenerating liver remnants and from the corresponding 0-hr. resected segments. The numbers represent the percentages of the five major histone fractions (data from Table 9).

Figure 21

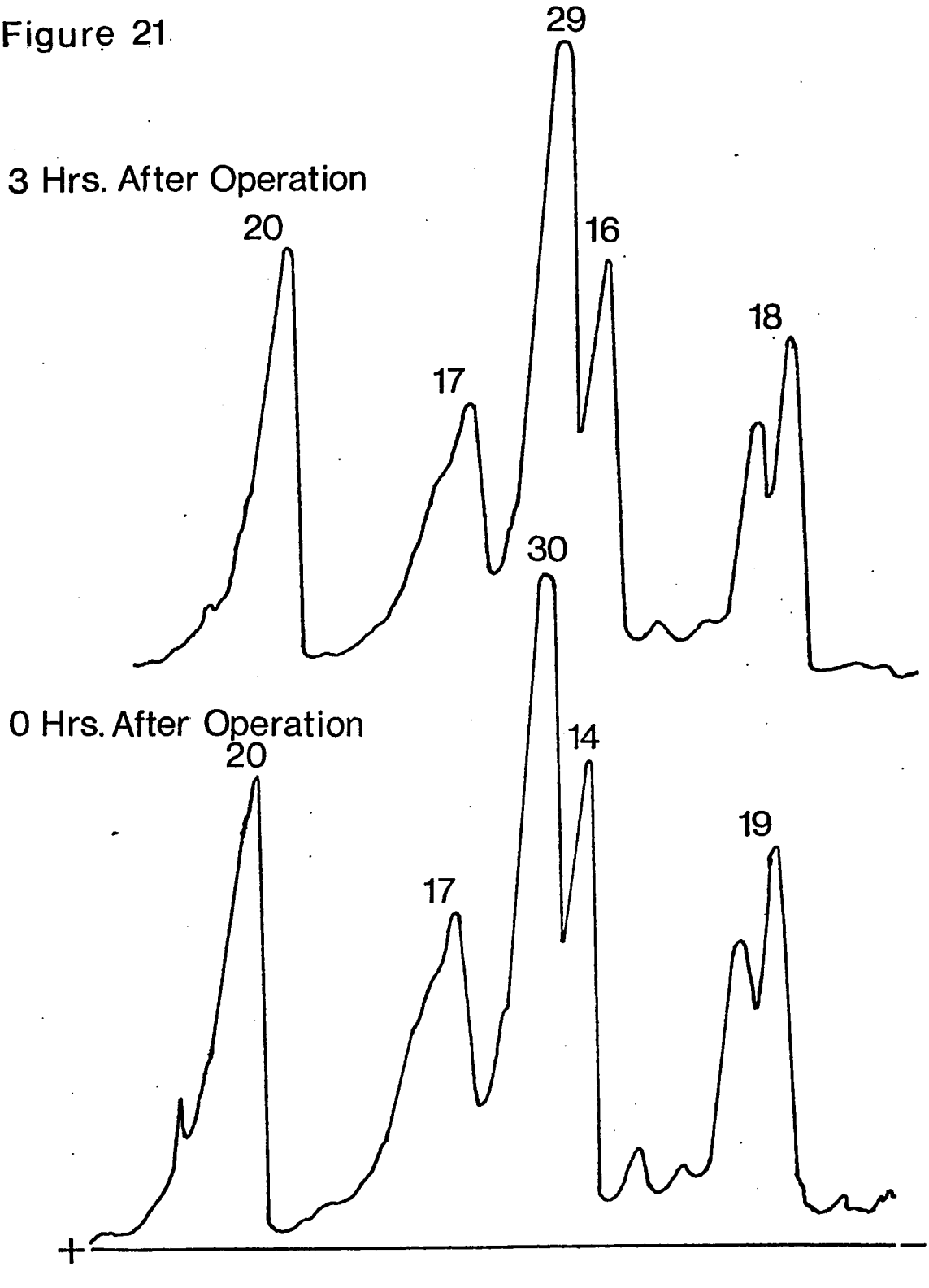

3 Hrs. After Operation
20
29
16
18
17
30
0 Hrs. After Operation
20
14
19
17
+
F1
F3
F2b
F2a2
F2a1

Fig. 22. Long polyacrylamide gel scans of histones extracted from 4 hr. regenerating liver remnants and from the corresponding 0-hr. resected segments. The numbers represent the percentages of the five major histone fractions (data from Table 9).

Figure 22

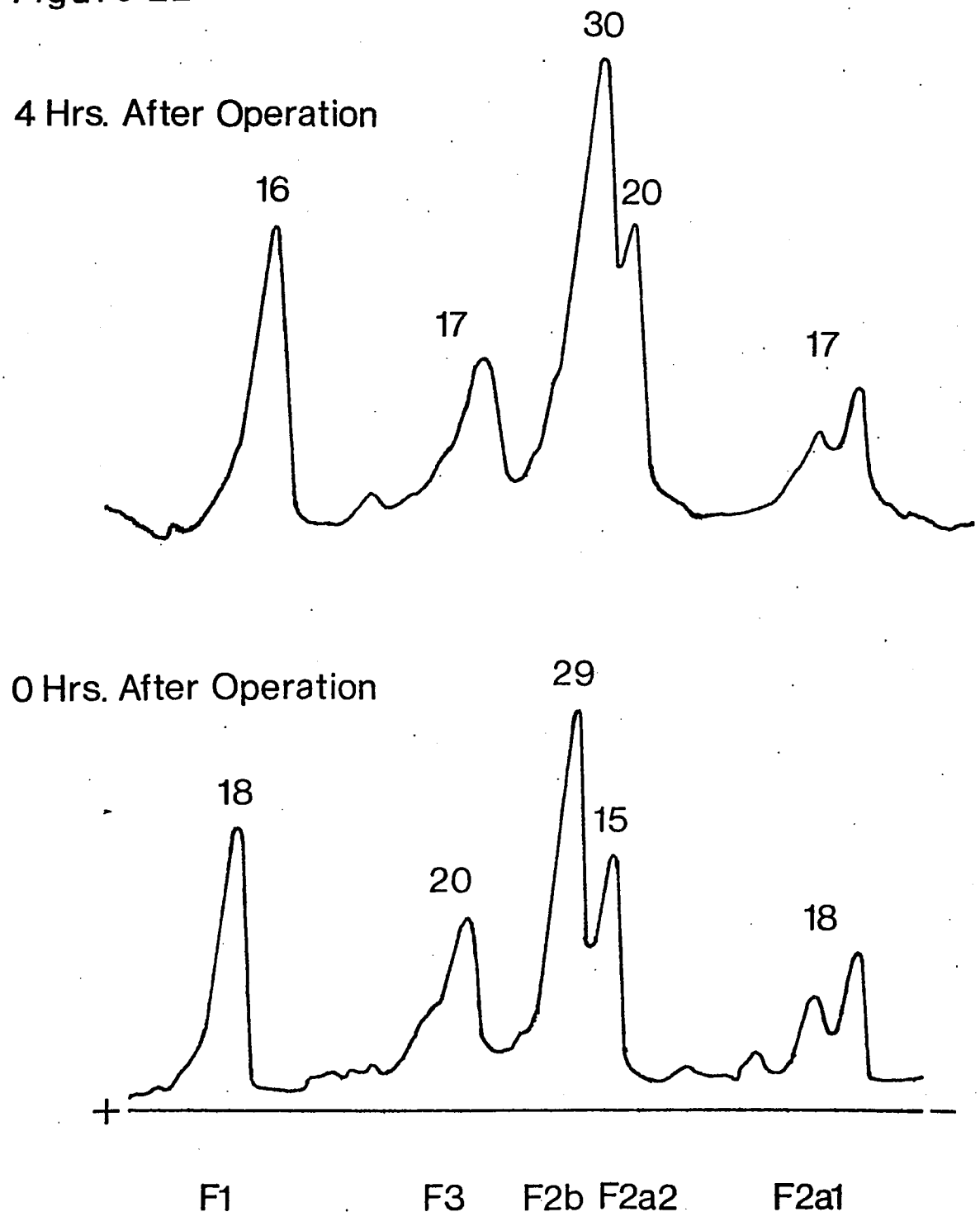
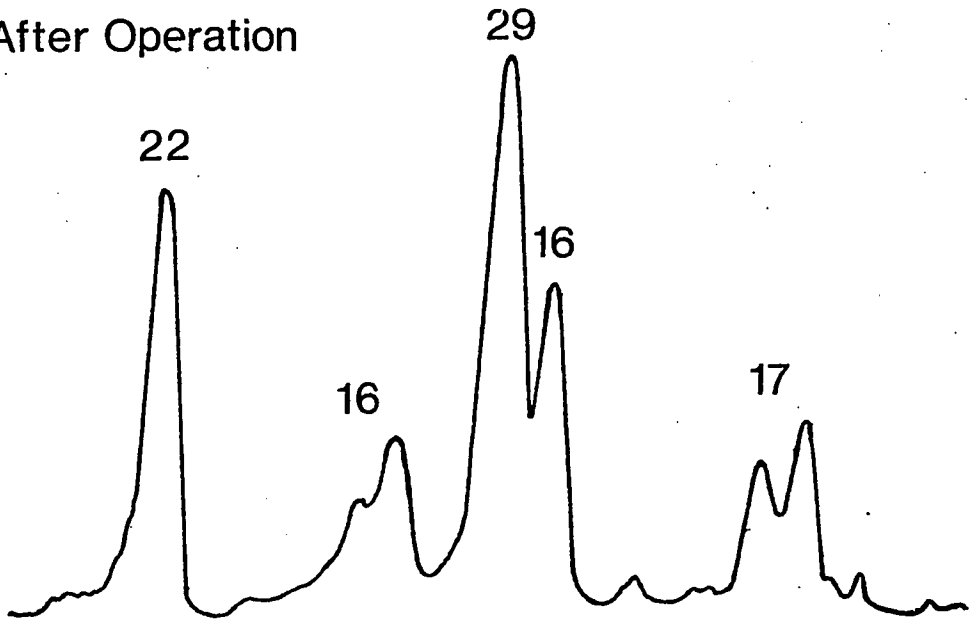

4 Hrs. After Operation
30
16
20
17
17
0 Hrs. After Operation
29
18
15
20
18
+
−
F1
F3
F2b
F2a2
F2a1

Fig. 23. Long polyacrylamide gel scans of histones extracted from 5 hr. regenerating liver remnants and from the corresponding 0-hr. resected segments. The numbers represent the percentages of the five major histone fractions (data from Table 9).

Figure 23

5 Hrs. After Operation



0 Hrs. After Operation

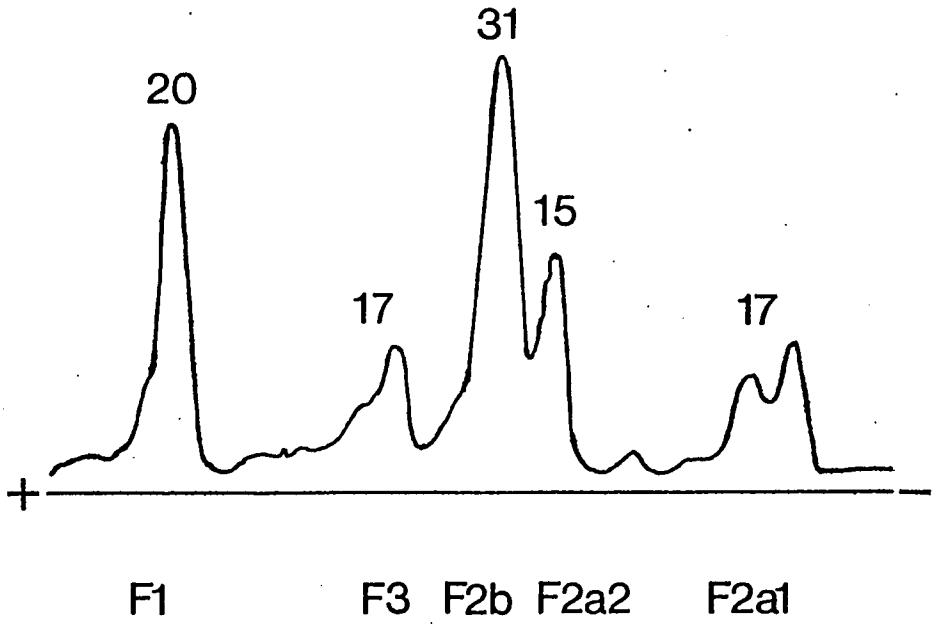


Fig. 24. Long polyacrylamide gel scans of histones extracted from 6 hr. regenerating liver remnants and from the corresponding 0-hr. resected segments. The numbers represent the percentages of the five major histone fractions (data from Table 9).

Figure 24

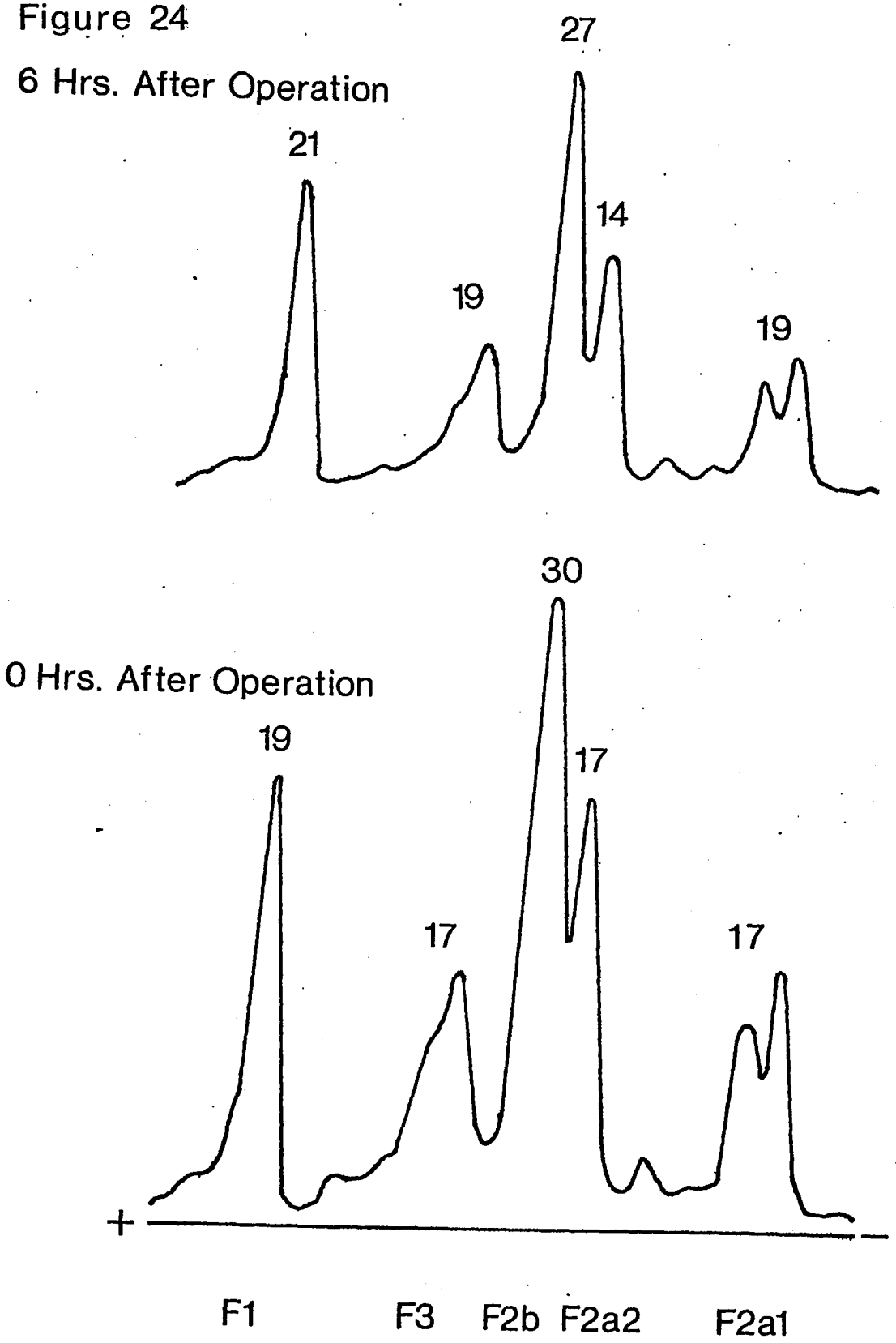
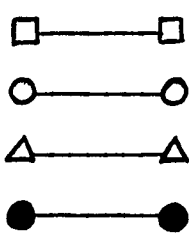

6 Hrs. After Operation
21
19
27
14
19
0 Hrs. After Operation
19
17
30
17
17
+
F1
F3
F2b
F2a2
F2a1

Fig. 25. Percent change in amounts of F2a1 acetylated subspecies plotted against the time after the operation.



monoacetylated

diacetylated

triacetylated

unacetylated

Figure 25

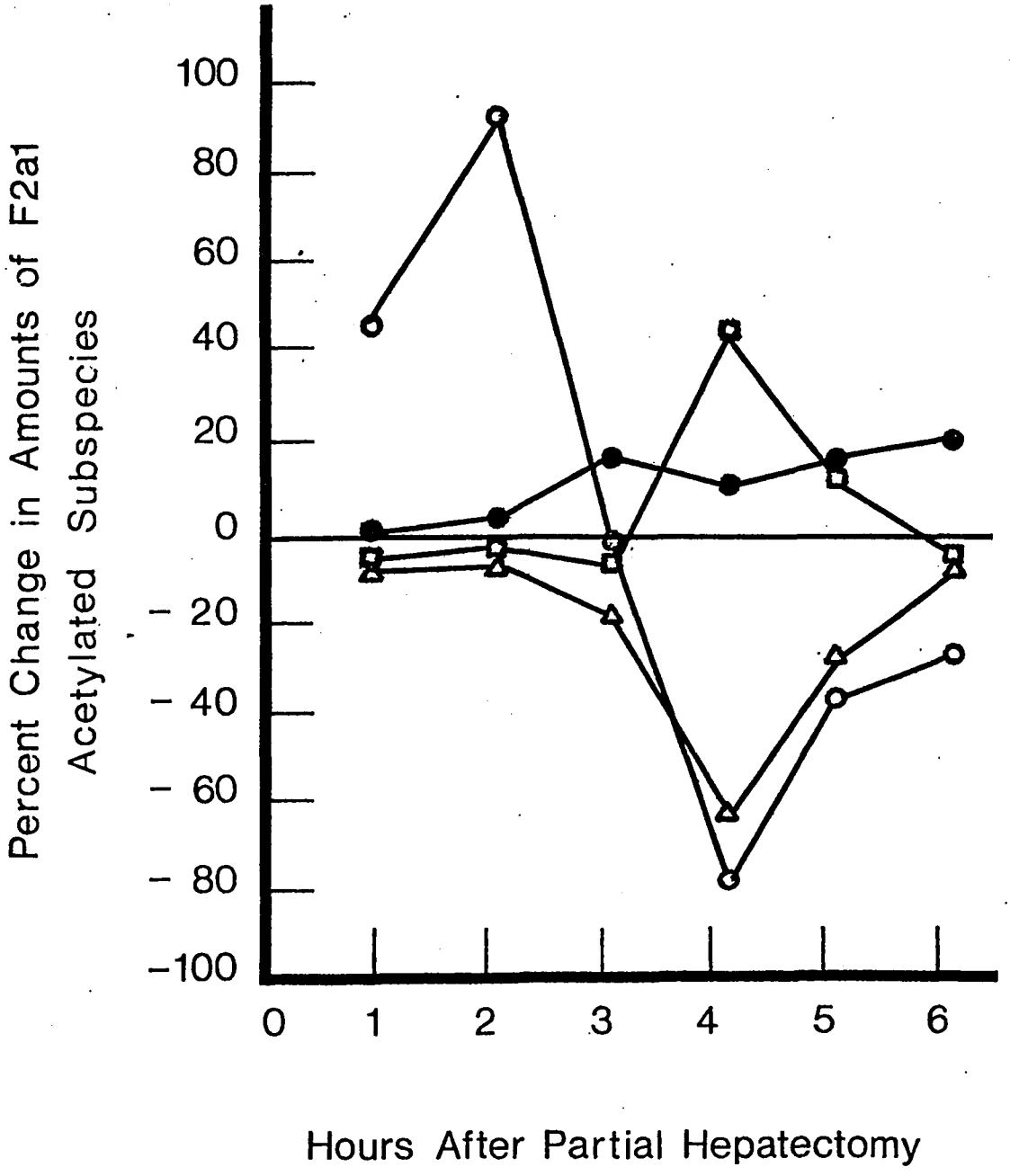

Percent Change in Amounts of F2a1
Acetylated Subspecies
100
80
60
40
20
0
- 20
- 40
- 60
- 80
-100
0
1
2
3
4
5
6
Hours After Partial Hepatectomy

TABLE 10.--Polyacrylamide Gel Scanning Quantitation of the F2a1 Histone Acetylated Subspecies During Regeneration

The percentages of the F2a1 histone fractions were determined for chromatins extracted from liver remnants (R) at different hours during regeneration and from the corresponding 0-hr. resected segments (N). The conditions for electrophoresis and for gel scanning were as described in "Materials and Methods".

Hrs. after partial hepatectomy	un-acetylated		mono-acetylated		di-acetylated		tri-acetylated	
	N	R	N	R	N	R	N	R
1	36.8	37.0	42.6	40.1	7.4	10.6	13.2	12.3
2	41.9	40.4	36.6	37.9	6.7	12.6	14.8	9.1
3	39.1	45.4	44.5	40.1	6.8	6.4	9.6	7.4
4	42.4	45.8	34.1	48.6	13.7	2.6	10.0	3.0
5	40.0	44.0	39.6	43.0	8.9	5.2	11.5	7.8
6	41.9	48.3	37.5	34.8	7.6	5.2	13.1	11.7

TABLE 11.--Amount of Free DNA[a] Present in Native Rat Liver Chromatin at Different Hours During Regeneration

Thermal denaturation was performed as described in "Materials and Methods". The % of free DNA in chromatin is taken as $(\Delta h/h_{max}) \times 100$ where Δh is the difference in hyperchromicity between 30°C and 60°C.

Hrs. after partial hepatectomy	% free DNA in chromatin from 0-hr. resected setments (N)	% free DNA in chromatin from regenerating liver remnants (R)	R-N	% change in R relative to N
1	13.4	6.9	-7.5	-49.2
1.5	12.9	10.8	-2.1	-16.5
2	7.9	11.1	3.2	41.2
3	5.6	7.4	1.8	32.1
4	4.4	4.5	0.1	2.5
6	12.0	12.0	0.0	0.0

[a]Free DNA is that which melts in the free DNA region.

Fig. 26. The percent change in amount of free DNA present in native rat liver chromatin isolated hourly from regenerating liver remnants expressed relative to the amount of free DNA present in chromatin isolated from the corresponding 0-hr. resected segments and plotted against time.

Figure 26

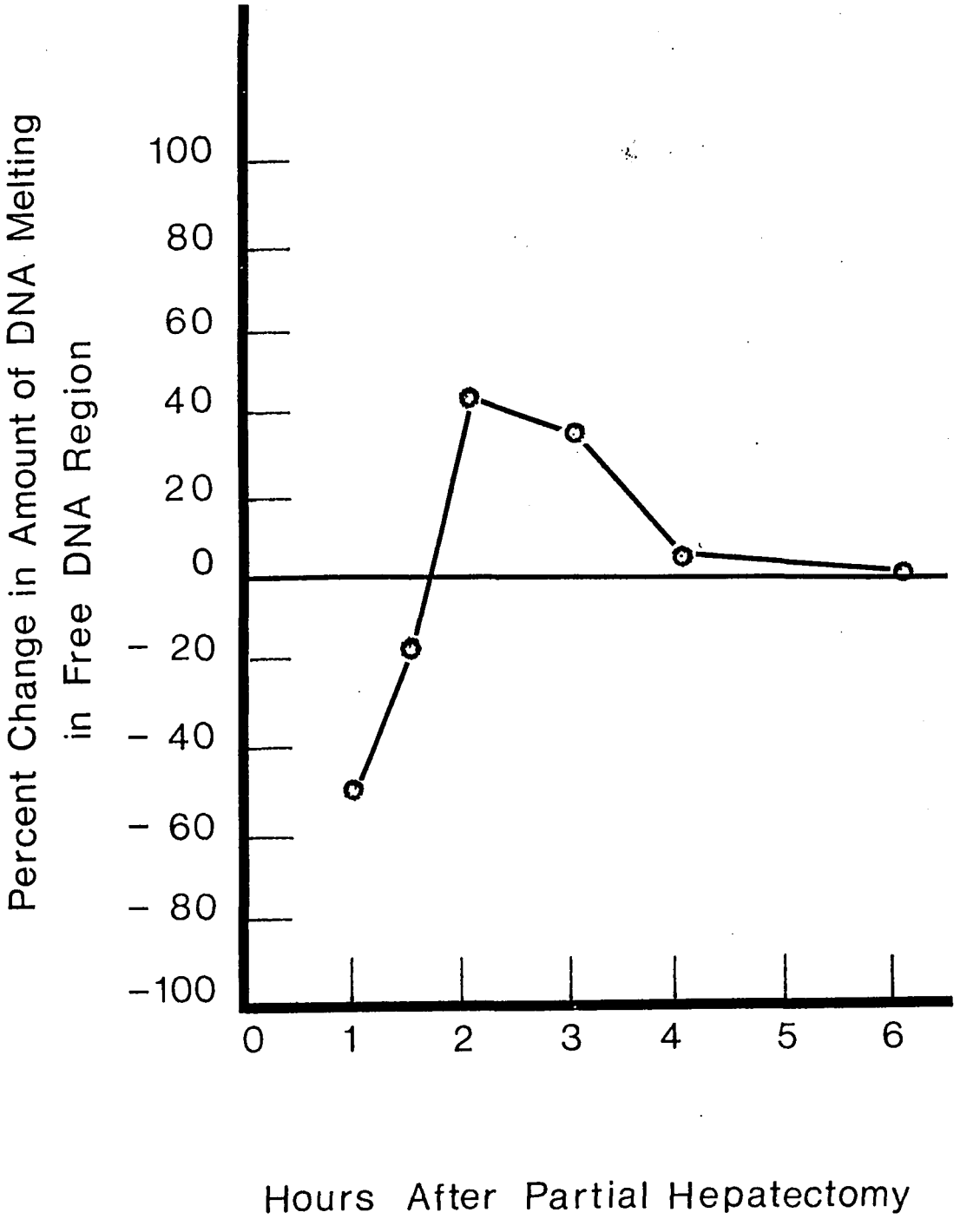

Percent Change in Amount of DNA Melting
in Free DNA Region
100
80
60
40
20
0
- 20
- 40
- 60
- 80
-100
0
1
2
3
4
5
6
Hours After Partial Hepatectomy

Fig. 27. Derivative melting profiles obtained for native chromatin extracted from liver remnants after 1.25 hrs. of regeneration and from the corresponding 0-hr. resected segments. The derivative plot for rat liver DNA is also included.

Figure 27

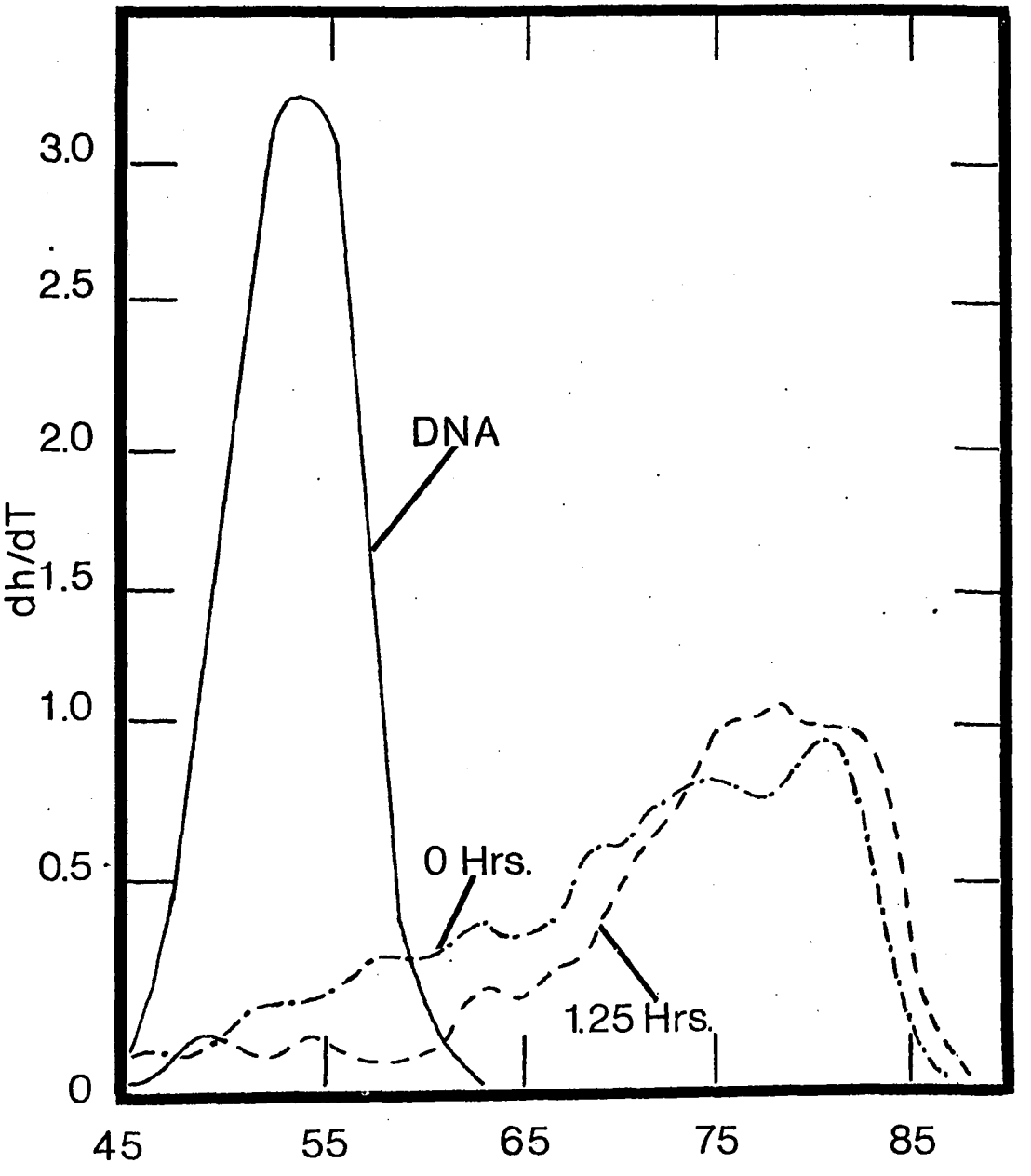

3.0
2.5
2.0
1.5
1.0
0.5
0
dh/dT
DNA
0 Hrs.
1.25 Hrs.
45
55
65
75
85
Temperature (°C)

Fig. 28. Derivative melting profiles obtained for native chromatin extracted from liver remnants after 1.5 hrs. of regeneration and from the corresponding 0-hr. resected segments. The derivative plot for rat liver DNA is also included.

Figure 28

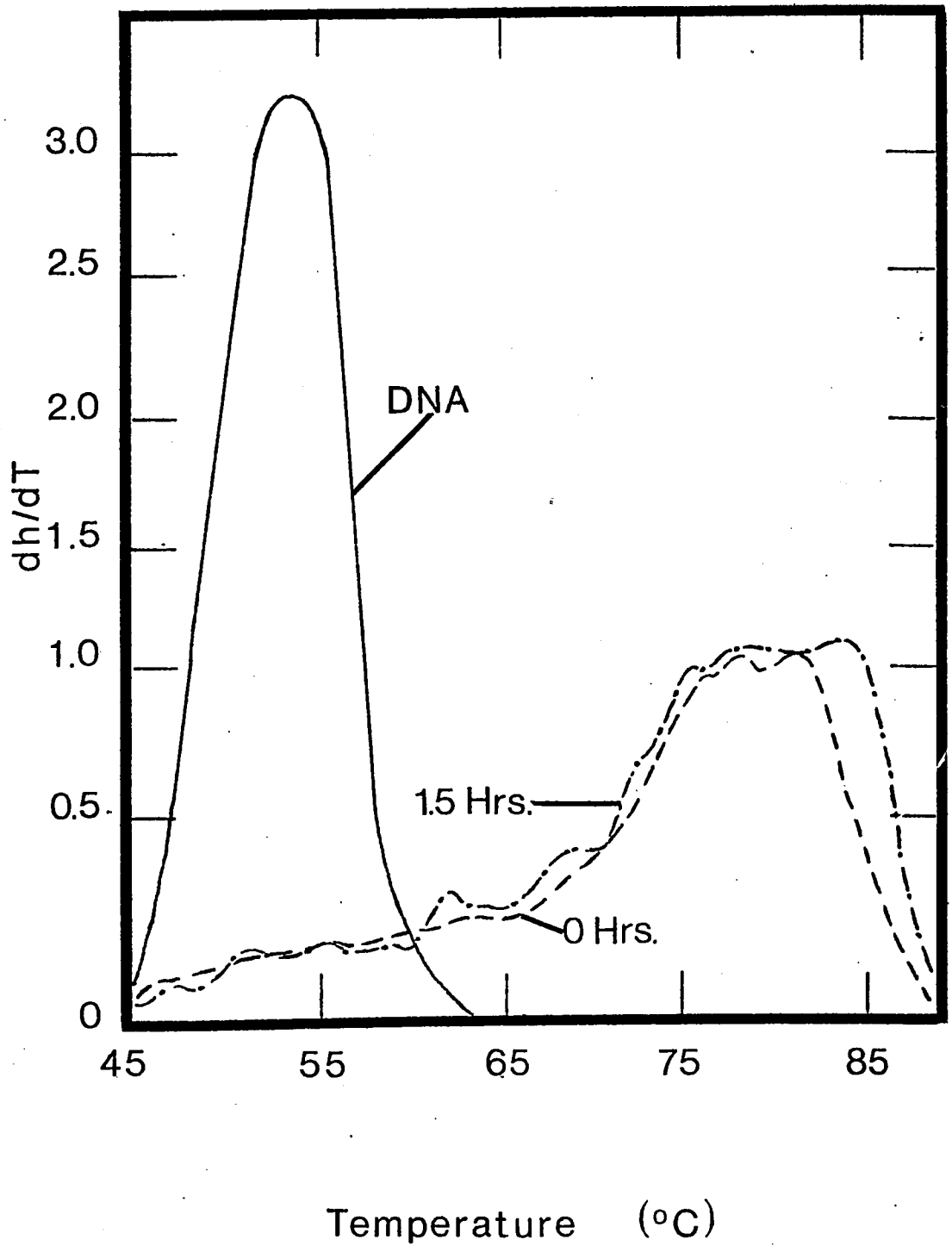

3.0
2.5
2.0
1.5
1.0
0.5
0
dh/dT
DNA
1.5 Hrs.
0 Hrs.
45
55
65
75
85
Temperature (°C)

Fig. 29. Derivative melting profiles obtained for native chromatin extracted from liver remnants after 2 hrs. of regeneration and from the corresponding 0-hr. resected segments. The derivative plot for rat liver DNA is also included.

Figure 29

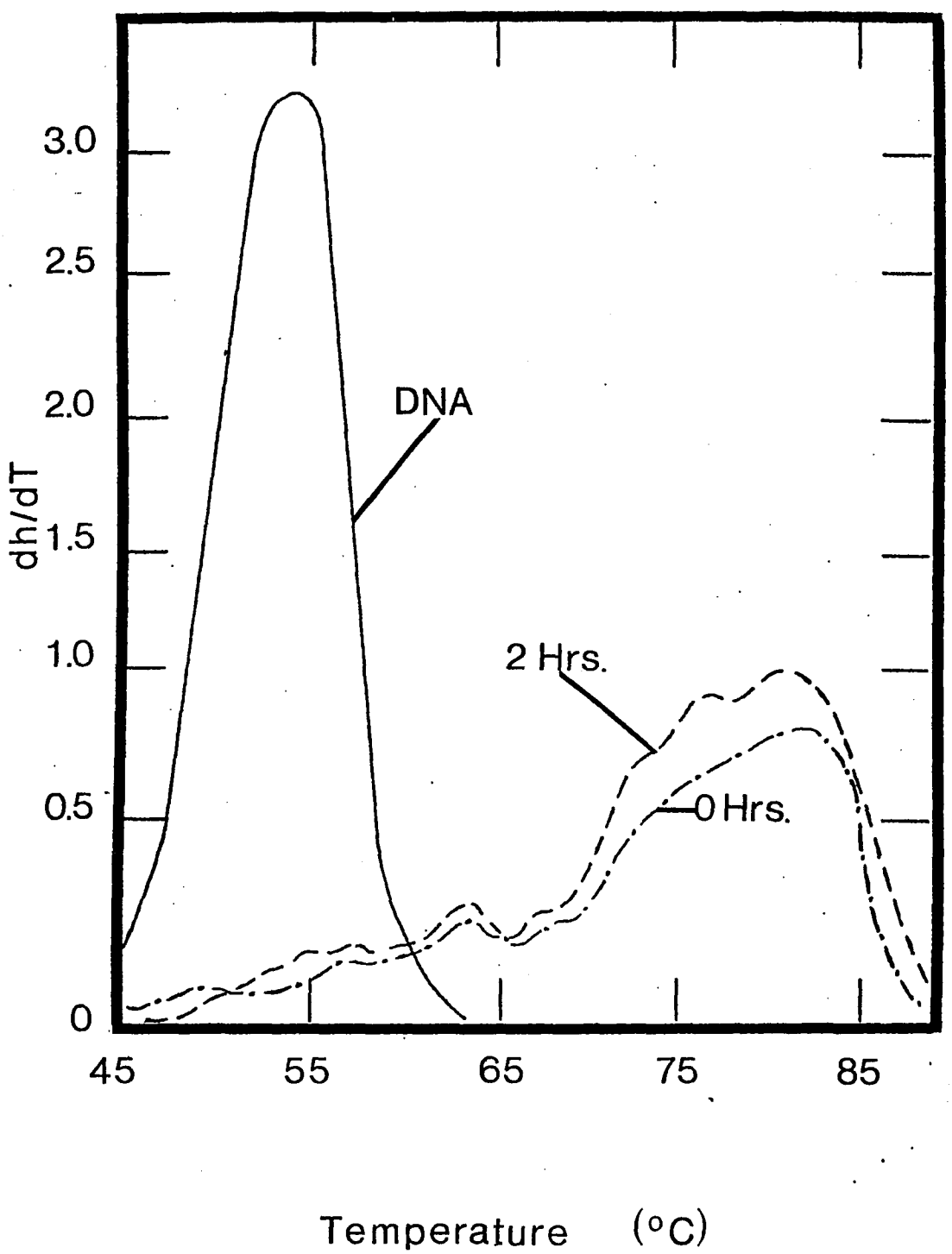

3.0
2.5
2.0
1.5
1.0
0.5
0
dh/dT
DNA
2 Hrs.
0 Hrs.
45
55
65
75
85
Temperature (°C)

Fig. 30. Derivative melting profiles obtained for native chromatin extracted from liver remnants after 3 hrs. of regeneration and from the corresponding 0-hr. resected segments. The derivative plot for rat liver DNA is also included.

Figure 30

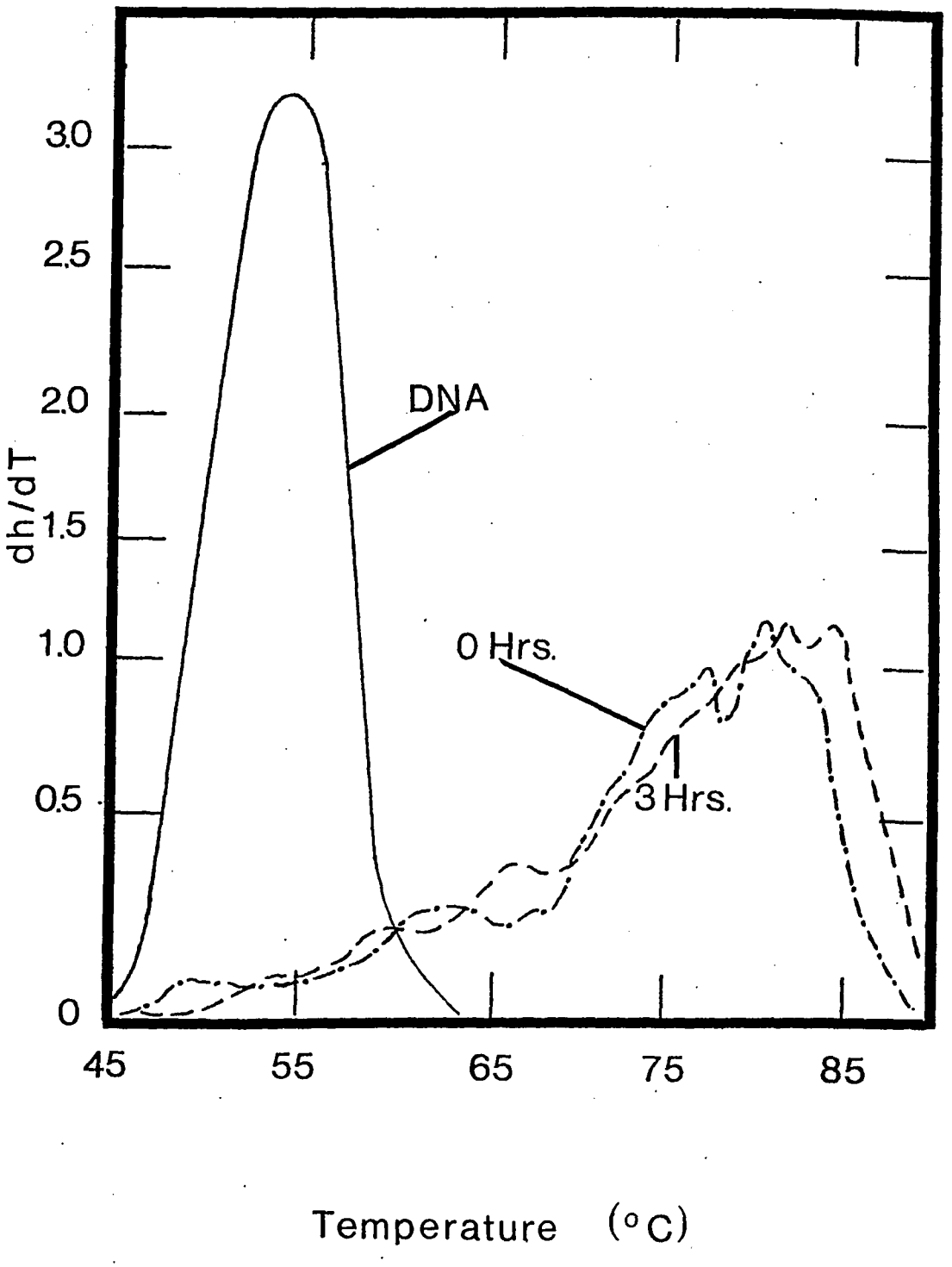

3.0
2.5
2.0
1.5
1.0
0.5
0
dh/dT
DNA
0 Hrs.
3 Hrs.
45
55
65
75
85
Temperature (°C)

Fig. 31. Derivative melting profiles obtained for native chromatin extracted from liver remnants after 4 hrs. of regeneration and from the corresponding 0-hr. resected segments. The derivative plot for rat liver DNA is also included.

Figure 31

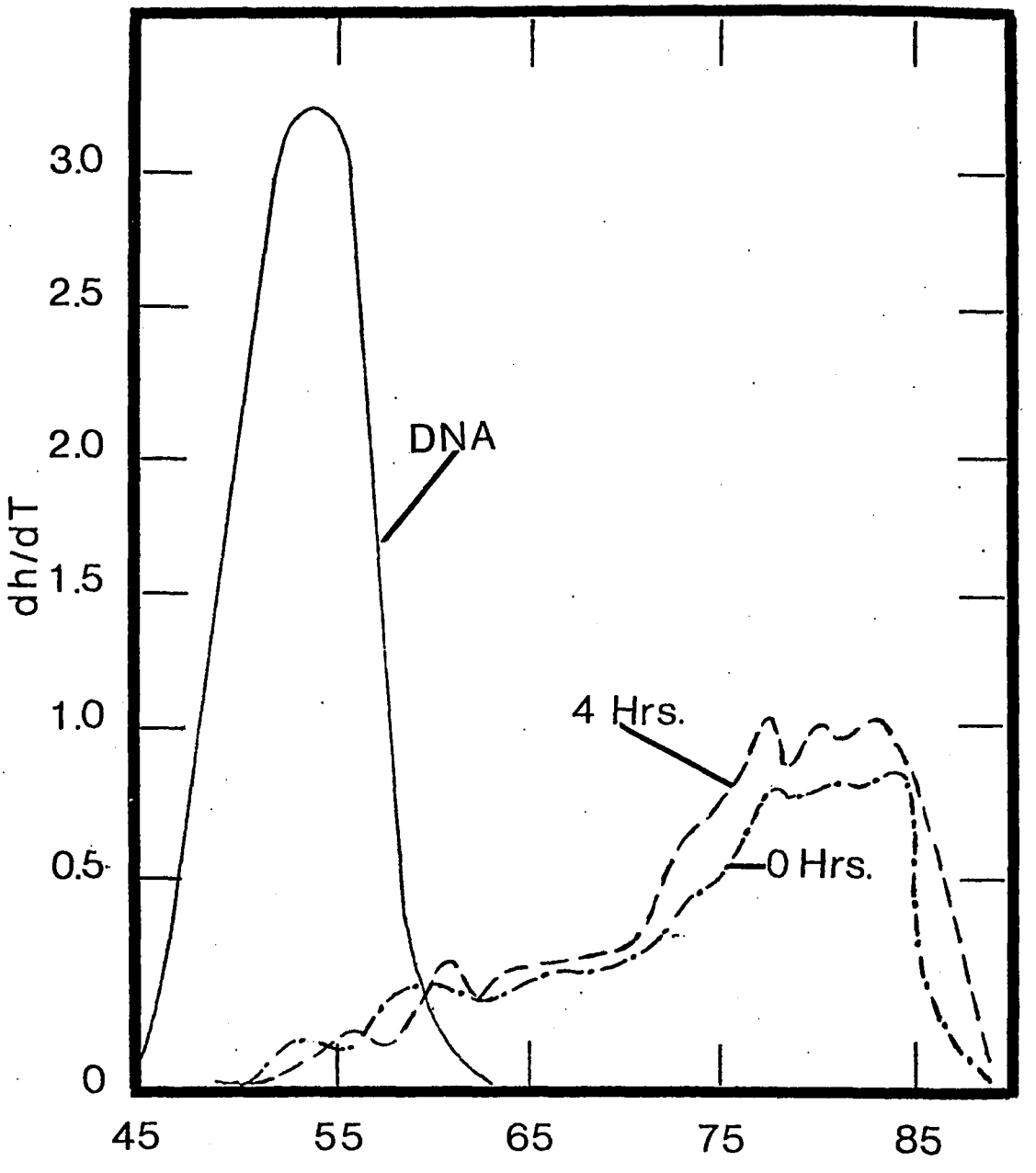

3.0
2.5
2.0
1.5
1.0
0.5
0
dh/dT
DNA
4 Hrs.
0 Hrs.
45
55
65
75
85
Temperature (°C)

Fig. 32. Derivative melting profiles obtained for native chromatin extracted from liver remnants after 6 hrs. of regeneration and from the corresponding 0-hr. resected segments. The derivative plot for rat liver DNA is also included.

Figure 32

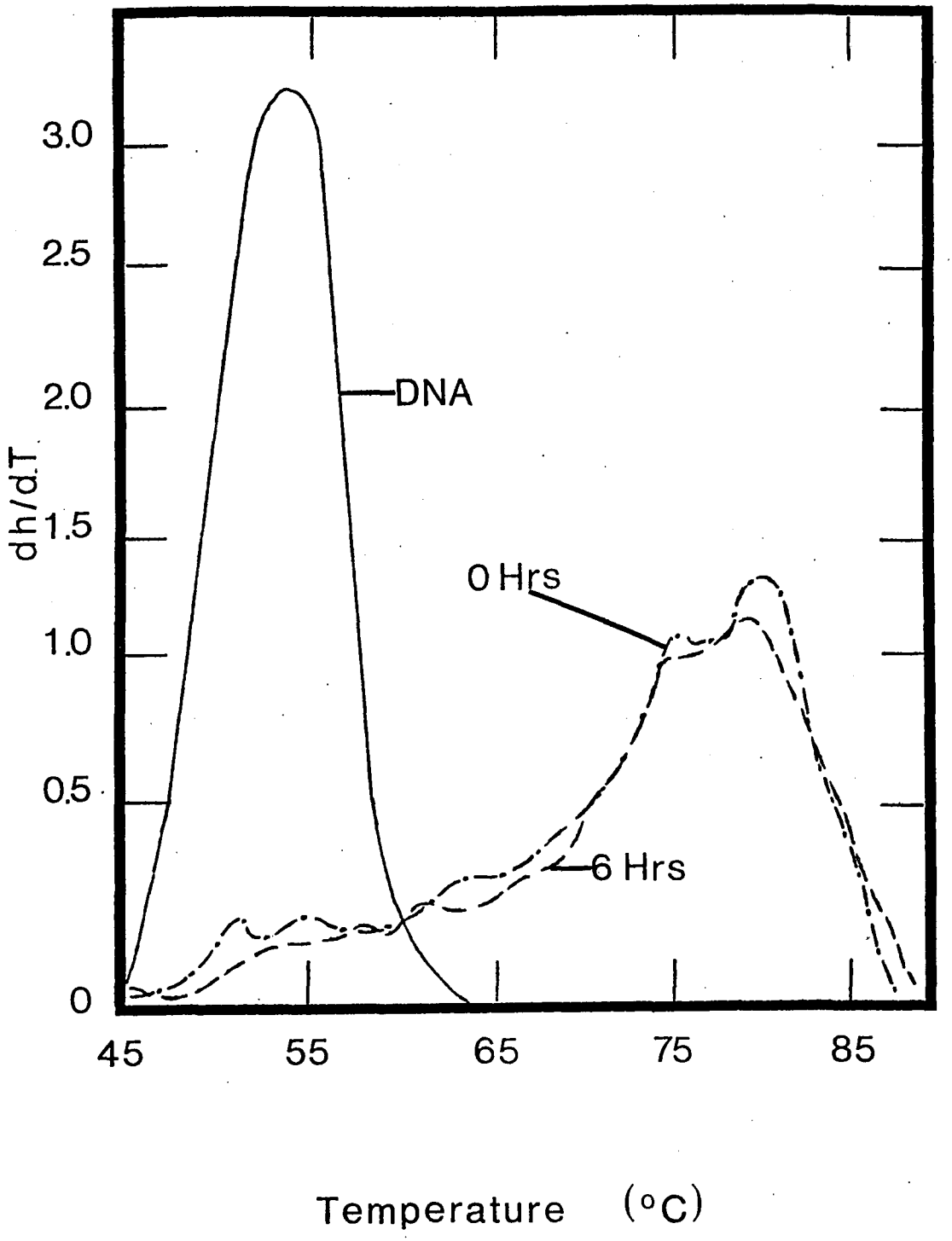

3.0
2.5
2.0
1.5
1.0
0.5
0
dh/d.T
DNA
0 Hrs
6 Hrs
45
55
65
75
85
Temperature (°C)

Fig. 33. Derivative melting profiles obtained for native chromatin extracted from liver remnants after 2 hrs. of regeneration and from the corresponding 0-hr. resected segments.

Figure 33

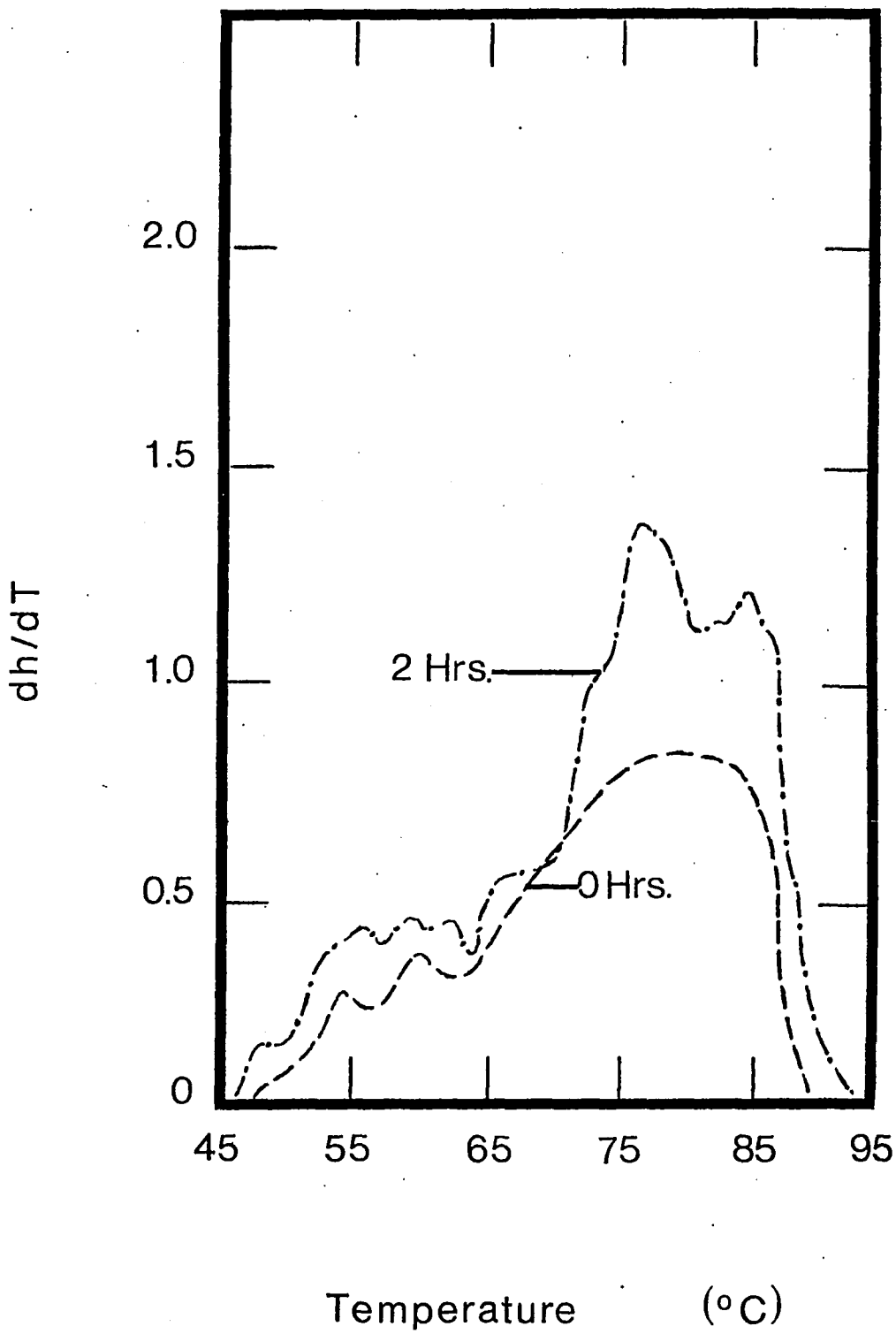


Fig. 34. Derivative melting profiles obtained from nucleohistone complexes of DNA and of histones extracted from non-regenerating livers. Ratio by weight of histone/DNA: (A) 0.0, (B) 0.5, (C) 1.0, (D) 2.0.

Figure 34

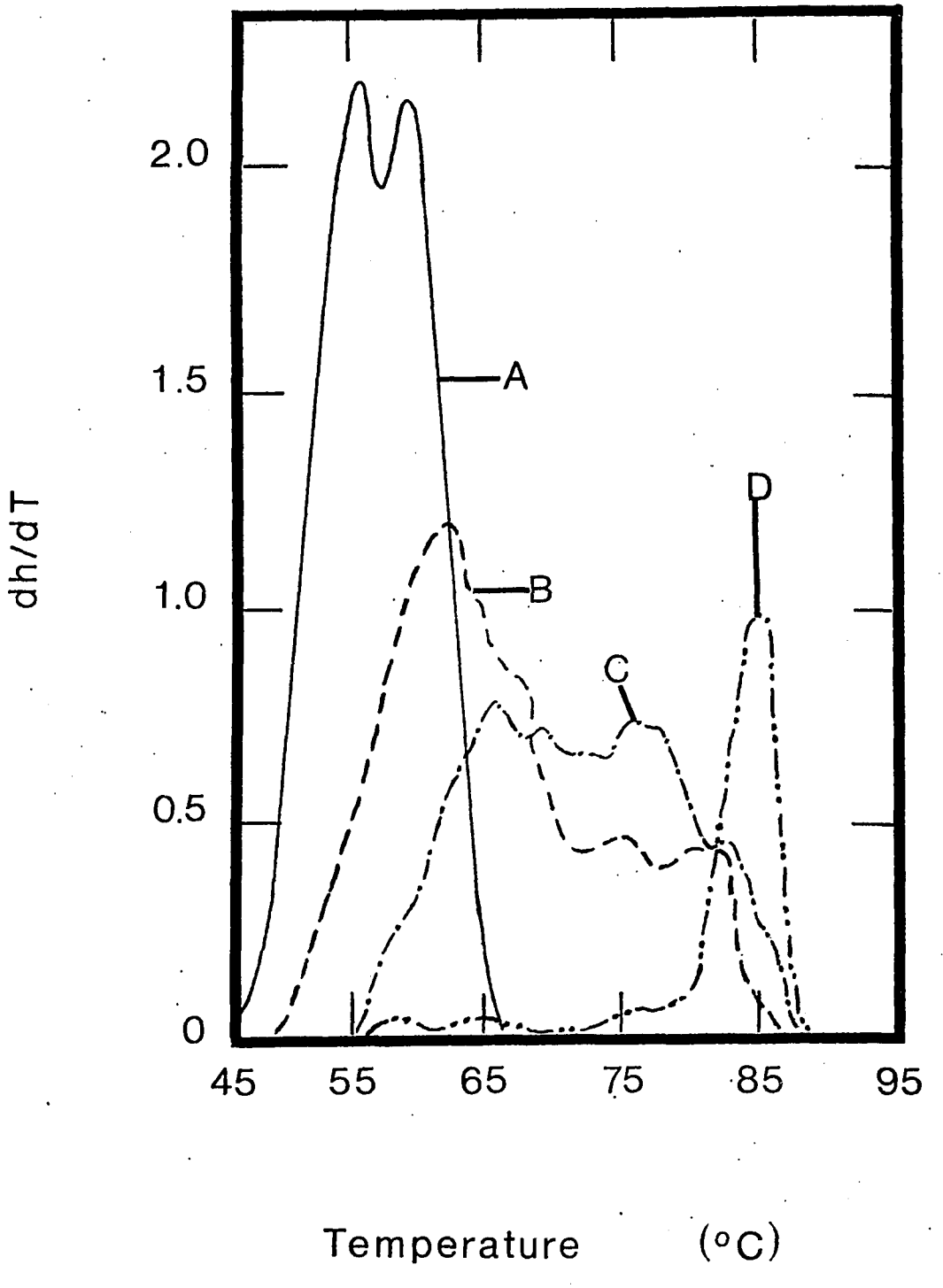


Fig. 35. Derivative melting profiles obtained from nucleohistone complexes of DNA and histones extracted from liver remnants after 2 hrs. of regeneration and from the corresponding 0-hr. resected segments. The ratio by weight of histone/DNA was 1.0.

Figure 35

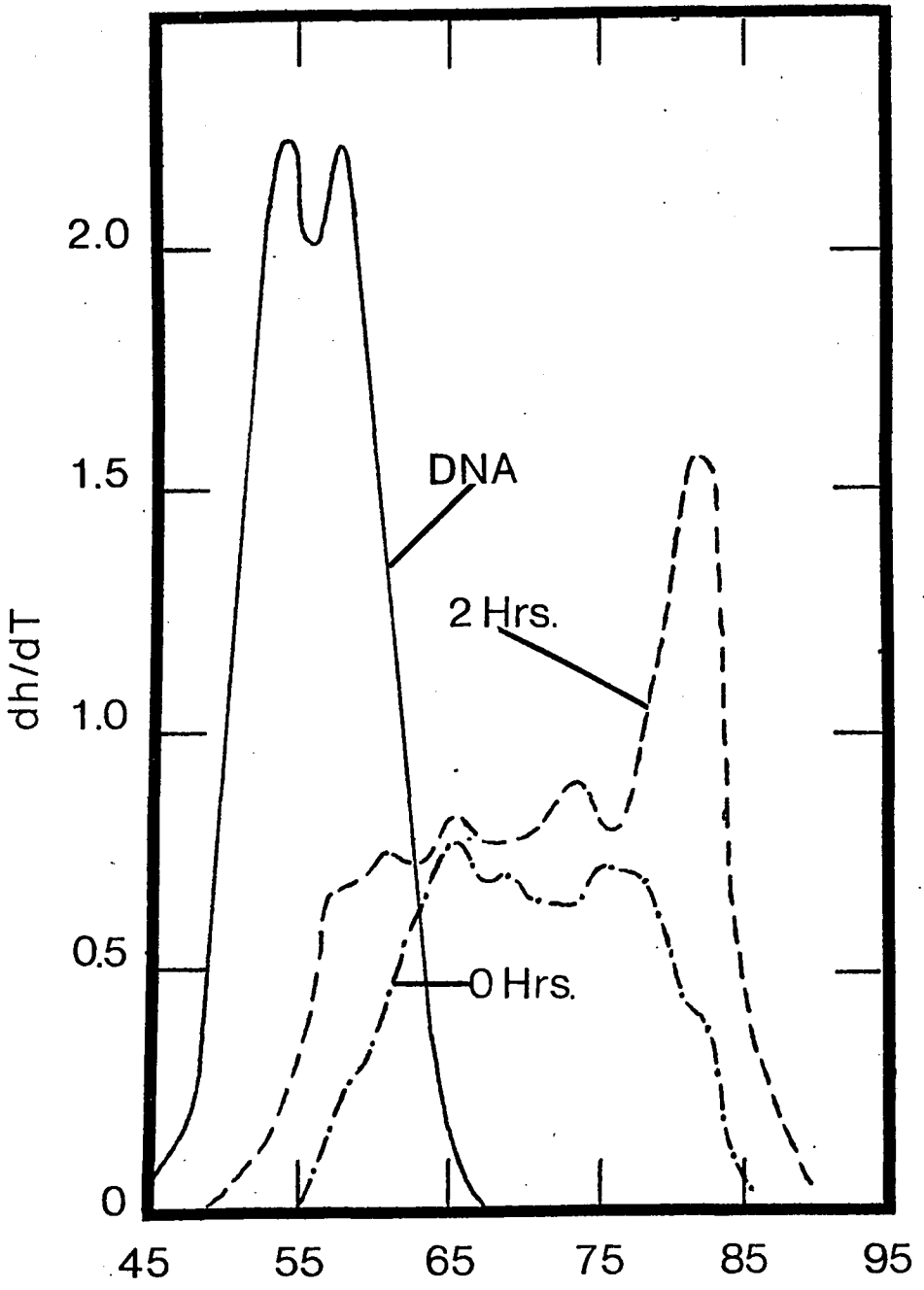


TABLE 12.--Amount of Free DNA[a] Present in Reconstituted Nucleohistone Complexes of DNA and Histones from Regenerating Livers

The procedures for thermal denaturation and for reconstitution were as described in "Materials and Methods". The percentages of free DNA in the nucleohistone complexes were taken as $(\Delta h/h_{max}) \times 100$ where Δh is the difference in hyperchromicity between 30°C and 60°C.

Hrs. after partial hepatectomy	% free DNA in chromatin from 0-hr. resected segments (N)	% free DNA in chromatin from regenerating liver remnants (R)	R-N	% change in R relative to N
		Experiment I		
2	21.7	27.0	5.3	24.4
		Experiment II		
2	10.7	14.0	3.3	31.0

[a]Free DNA is that which melts in the free DNA region.

Fig. 36. CD spectrum of rat liver DNA.

Figure 36

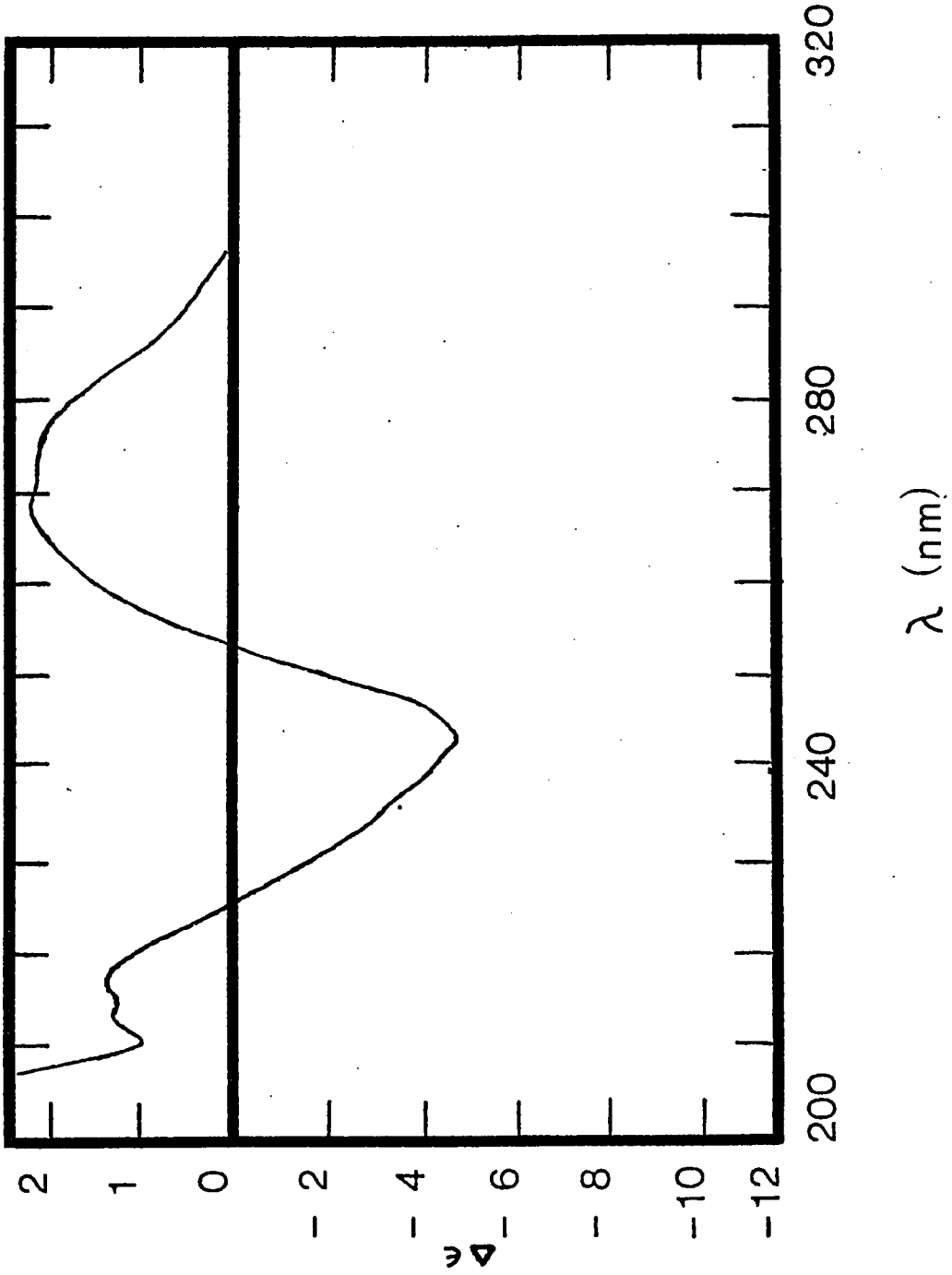


Fig. 37. CD spectra of native rat liver chromatin.

------- Chromatin extracted from 1.5 hr. regenerating liver remnants

_____ Chromatin extracted from the corresponding 0-hr. resected liver segments

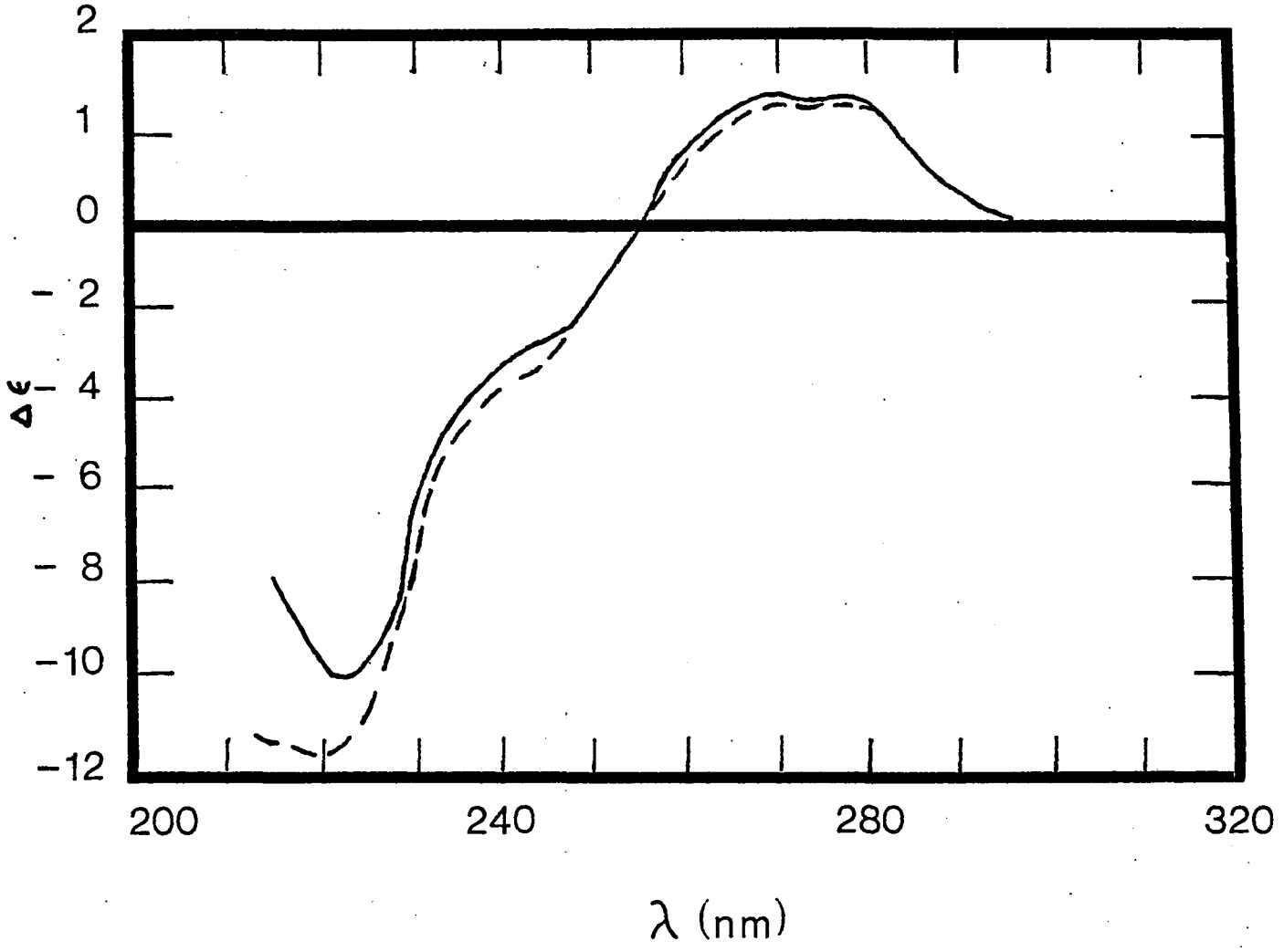


Figure 37

Fig. 38. CD spectra of native rat liver chromatin.

------- Chromatin extracted from 2.5 hr. regenerating liver remnants

——— Chromatin extracted from the corresponding 0-hr. resected liver segments

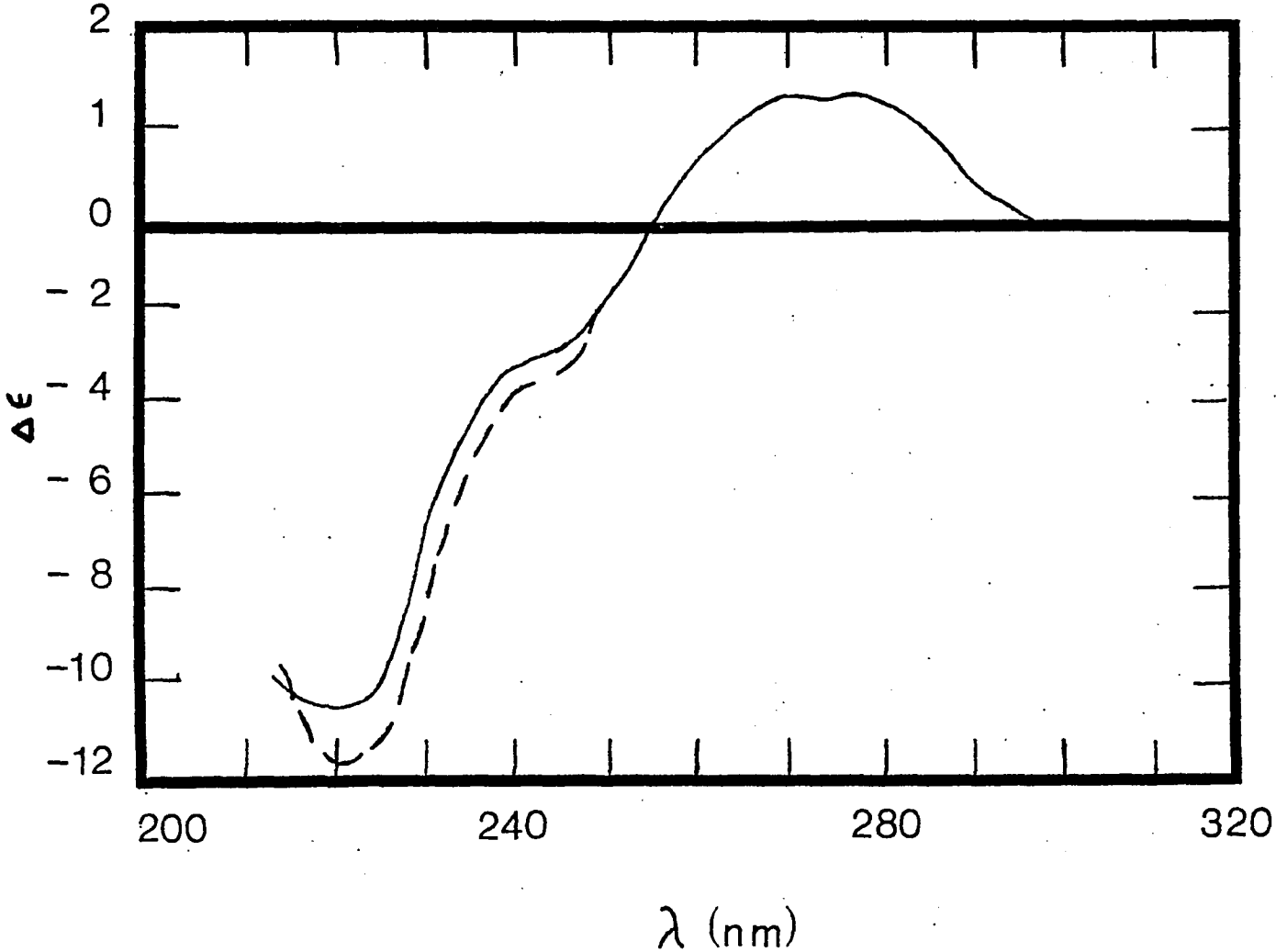


Figure 38

Fig. 39. CD spectra of native rat liver chromatin.

------ Chromatin extracted from 3 hr. regenerating liver remnants

——— Chromatin extracted from the corresponding 0-hr. resected liver segments

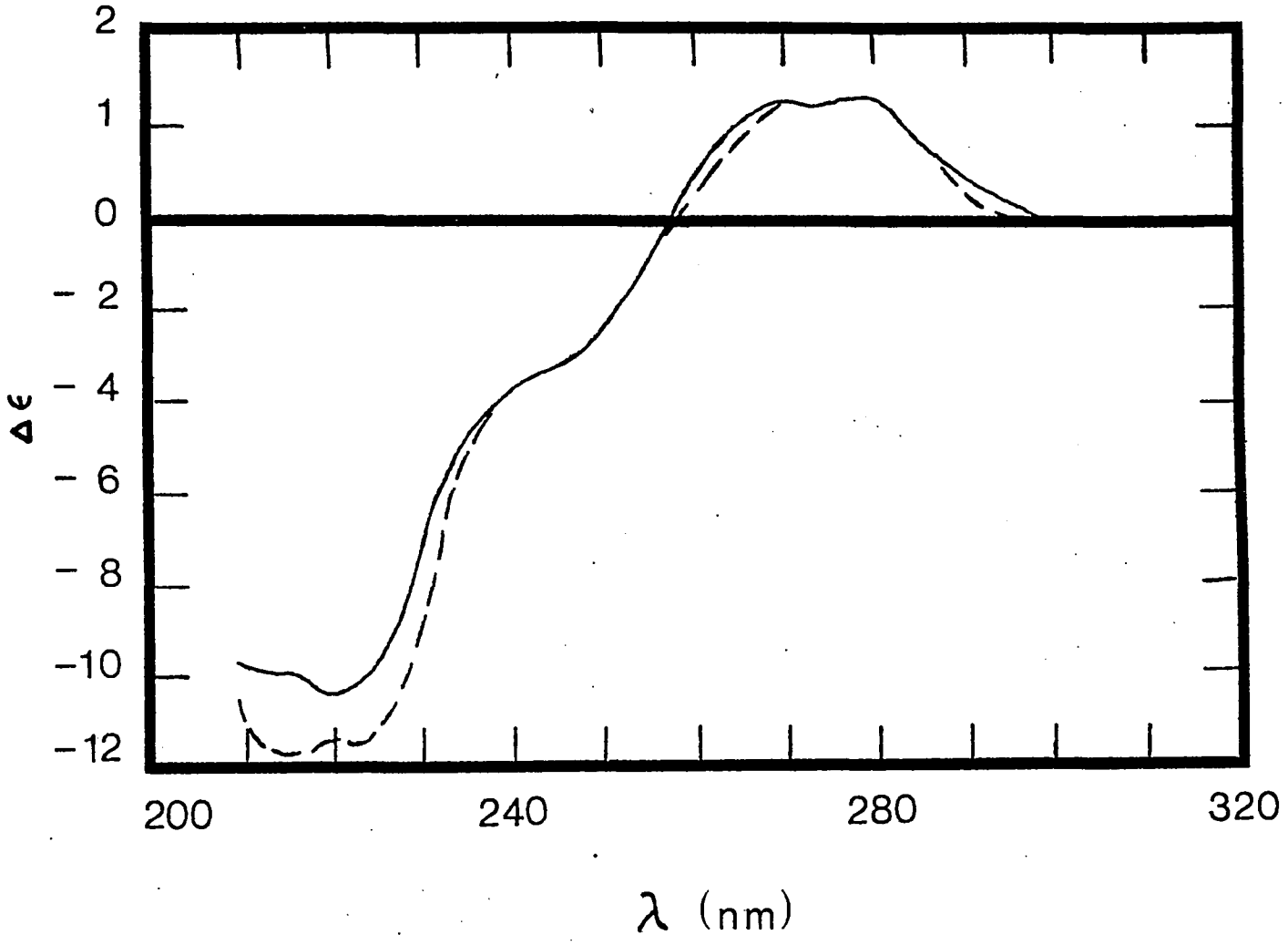


Figure 39

Fig. 40. CD spectra of native rat liver chromatin.

------- Chromatin extracted from 4 hr. regenerating liver remnants

_____ Chromatin extracted from the corresponding 0-hr. resected liver segments

Figure 40

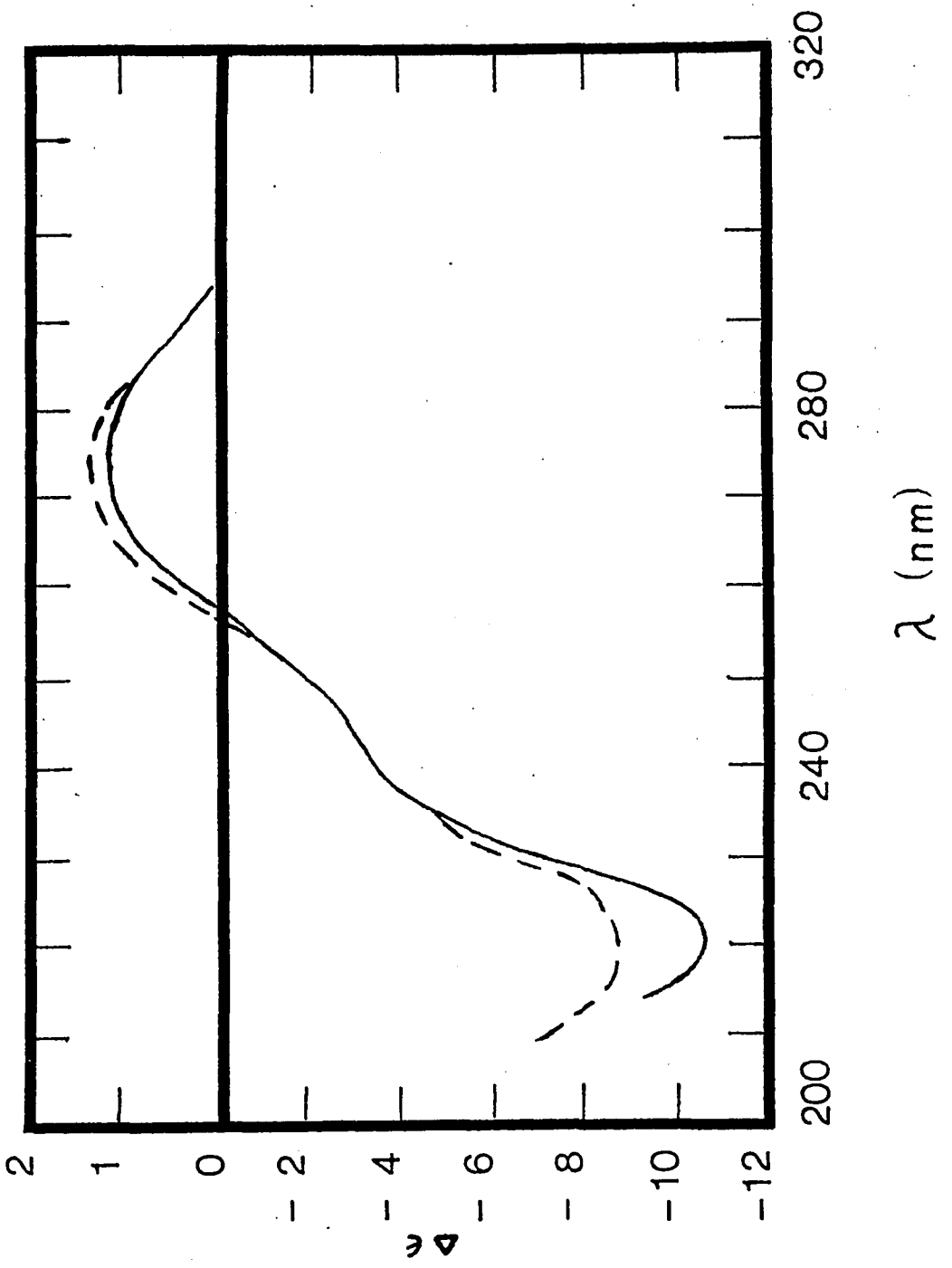


Fig. 41. CD spectra of native rat liver chromatin.

----- Chromatin extracted from 5 hr. regenerating liver remnants

——— Chromatin extracted from the corresponding 0-hr. resected liver segments

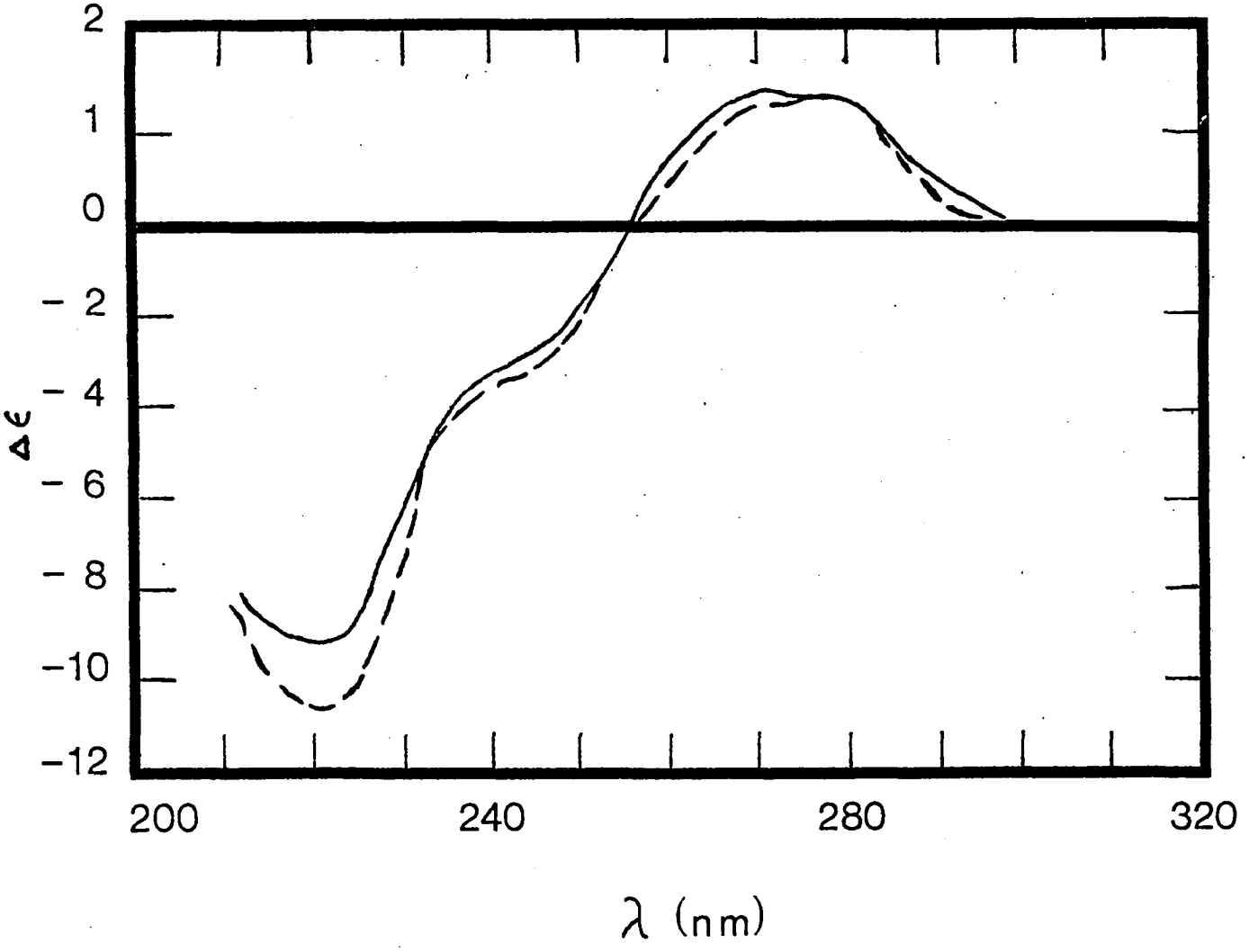


Figure 41

Fig. 42. CD spectra of native rat liver chromatin.

------- Chromatin extracted from 6 hr. regenerating liver remnants

——— Chromatin extracted from the corresponding 0-hr. resected liver segments

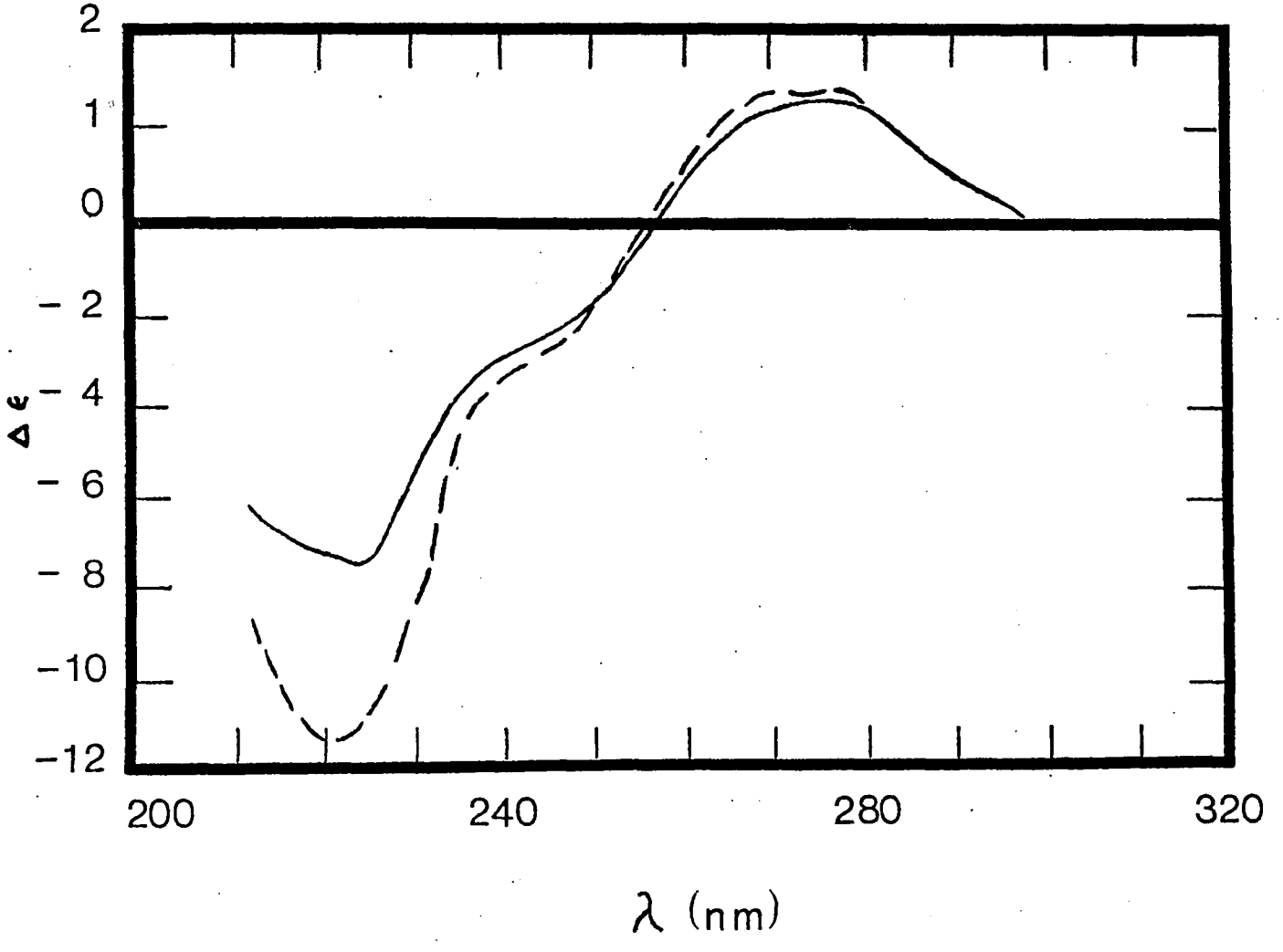


Figure 42

TABLE 13.--Amount of Histone α-helical content Present During Regeneration

Circular dichroism spectra were obtained as described in "Materials and Methods".

Hrs. after partial hepatectomy	$\Delta\epsilon$ 220 for histones of chromatin from 0-hr. resected segments (N)	$\Delta\epsilon$ 220 for histones of chromatin from regenerating remnants (R)	Ratio of R/N
1.5	-10.0	-11.6	1.16
2.5	-10.5	-11.5	1.10
3	-10.3	-11.6	1.13
4	-10.4	-8.6	0.83
5	-9.1	-10.5	1.15
6	-7.7	-11.2	1.45

Fig. 43. Ratio of α-helical content of histones present in chromatins extracted from liver remnants at different hours in regeneration (R) to that for the corresponding chromatins from the 0-hr. resected liver segments (N).

Figure 43

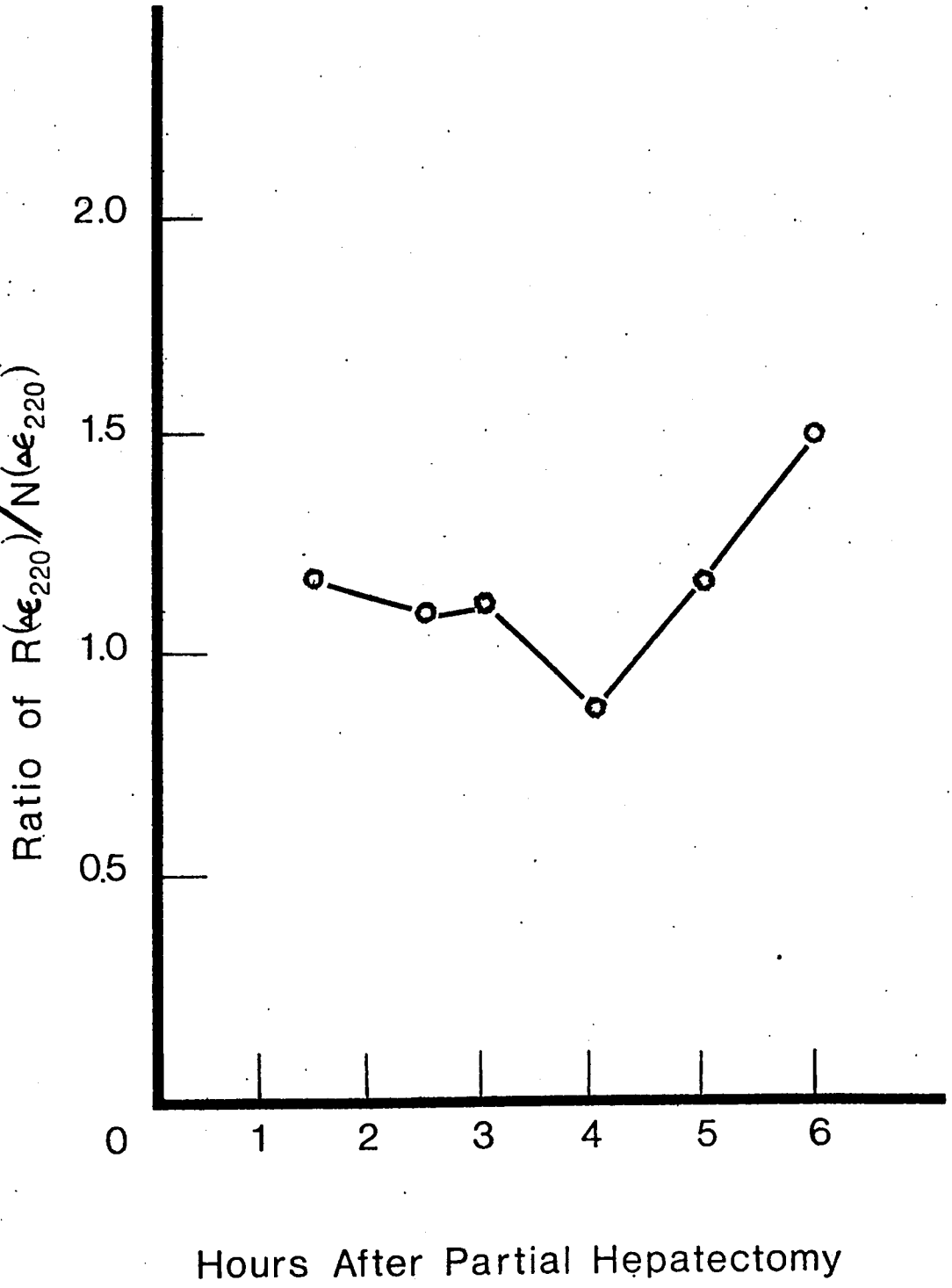

Ratio of R(Δε220)/N(Δε220)
2.0
1.5
1.0
0.5
0
1
2
3
4
5
6
Hours After Partial Hepatectomy

TABLE 14.--Amount of Histone α-helical Content Present During Regeneration

Circular dichroism spectra were obtained as described in "Materials and Methods".

Hrs. after partial hepatectomy	$\Delta\epsilon$ 220 for histones of chromatin from 0 hr. resected segments (N)	$\Delta\epsilon$ 220 for histones of chromatin from regenerating remnants (R)	Ratio of R/N
3	-10.5	-9.8	0.93
4	-10.1	-8.0	0.79

Fig. 44. CD spectra of rat liver DNA and chromatin.

----- Chromatin extracted from 3 hr. regenerating liver remnants

——— Chromatin extracted from the corresponding 0-hr. resected liver segments

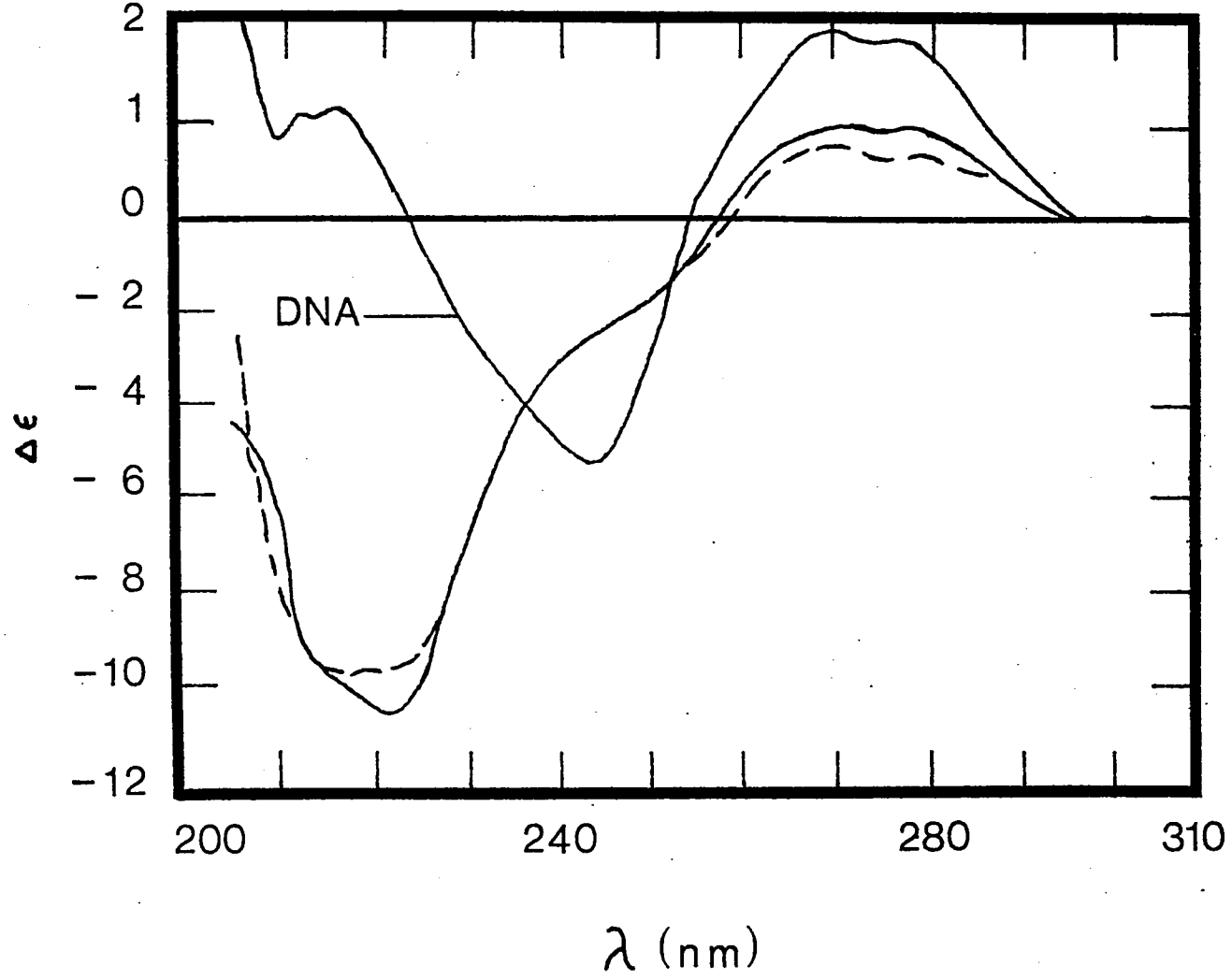


Figure 44

Fig. 45. CD spectra of rat liver DNA and chromatin.

----- Chromatin extracted from 4 hr. regenerating liver remnants

——— Chromatin extracted from the corresponding 0-hr. resected liver segments

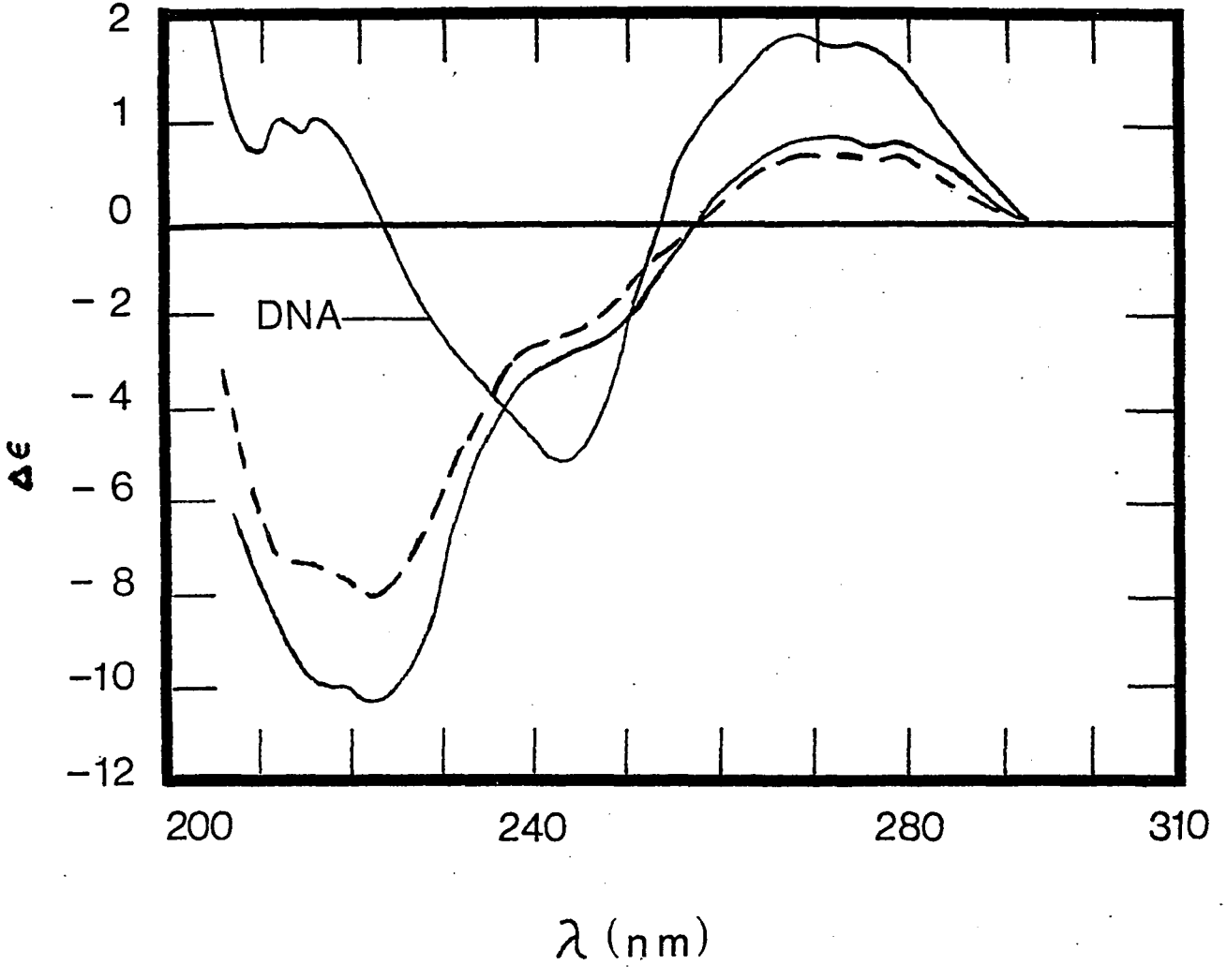


Figure 45

Fig. 46. A tentative model of the structural changes of nucleoprotein during denaturation. Native nucleoprotein: (a) at room temperature strong interactions occur between the α-helical regions of the histones  . The histone-bound DNA segments assume a C conformation  . The nucleoprotein is supercoiled. (b) Upon heating up to 65°C the histone-histone interactions are released. The histone-bound DNA segments assume a B conformation and the nucleoprotein supercoil is destroyed. DNA segments free of histones are denatured  . (c) After cooling, histone-free DNA segments renature. Histone segments cannot occur. The supercoil is irreversibly destroyed. (d) Denatured nucleoportein. The two strands of DNA are separated. (From Figure 13(I) of Wilhelm et al., 1974)

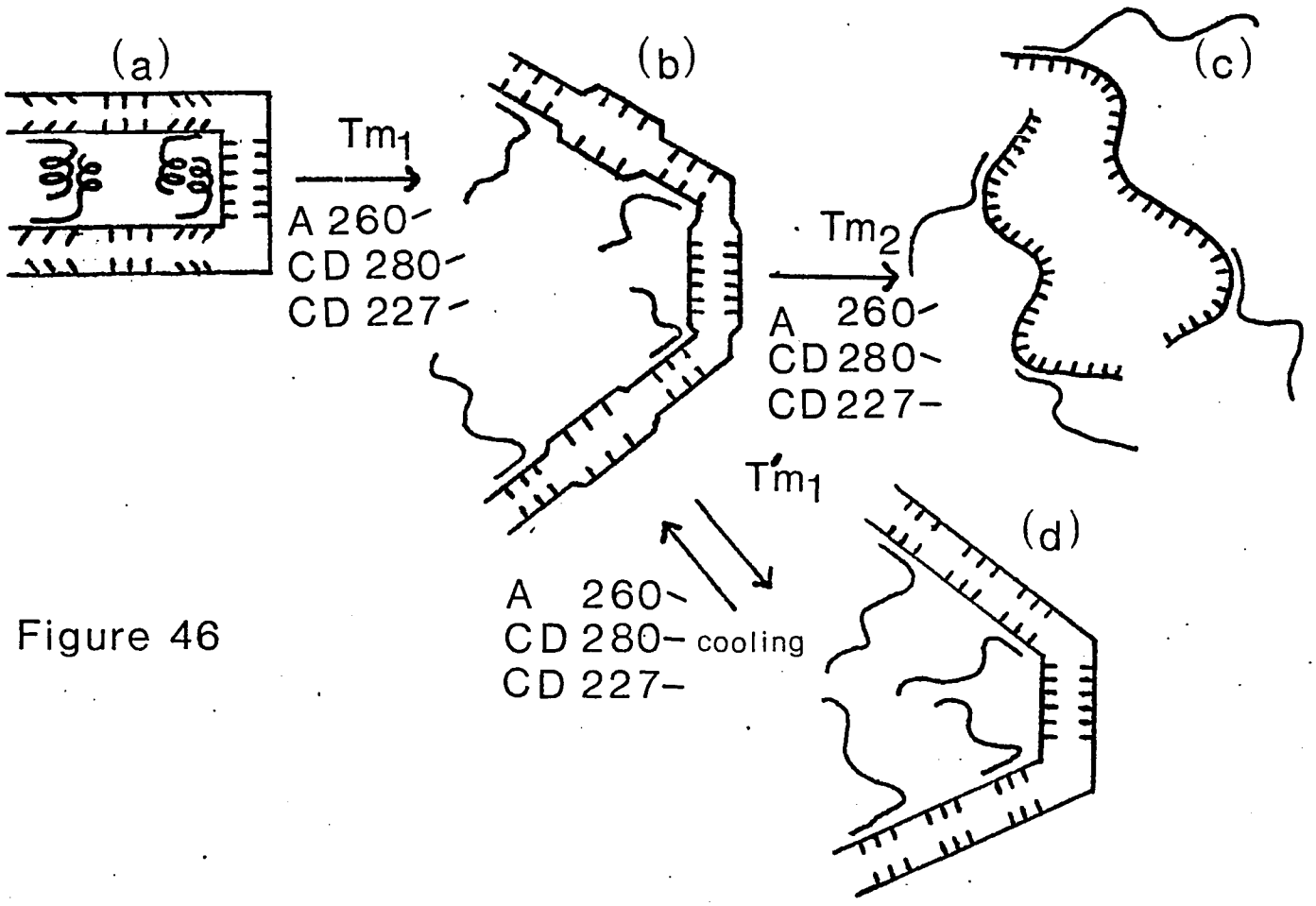

(a)
(b)
(c)
(d)
Tm_1
A 260-
CD 280-
CD 227-
Tm_2
A 260-
CD 280-
CD 227-
Tm_1
A 260-
CD 280-
CD 227-
cooling

Figure 46

APPENDIX B

SUPPLEMENTAL INFORMATION

Fig. 1. Effect of Mn^{2+} (3.4 mM) or Mg^{2+} (20 mM) cations on the activity of RNA polymerases A and B in the presence (67mM) or absence of $(NH_4)_2SO_4$ (data from Figure 10 of Seifart et al., 1972).

Figure 1

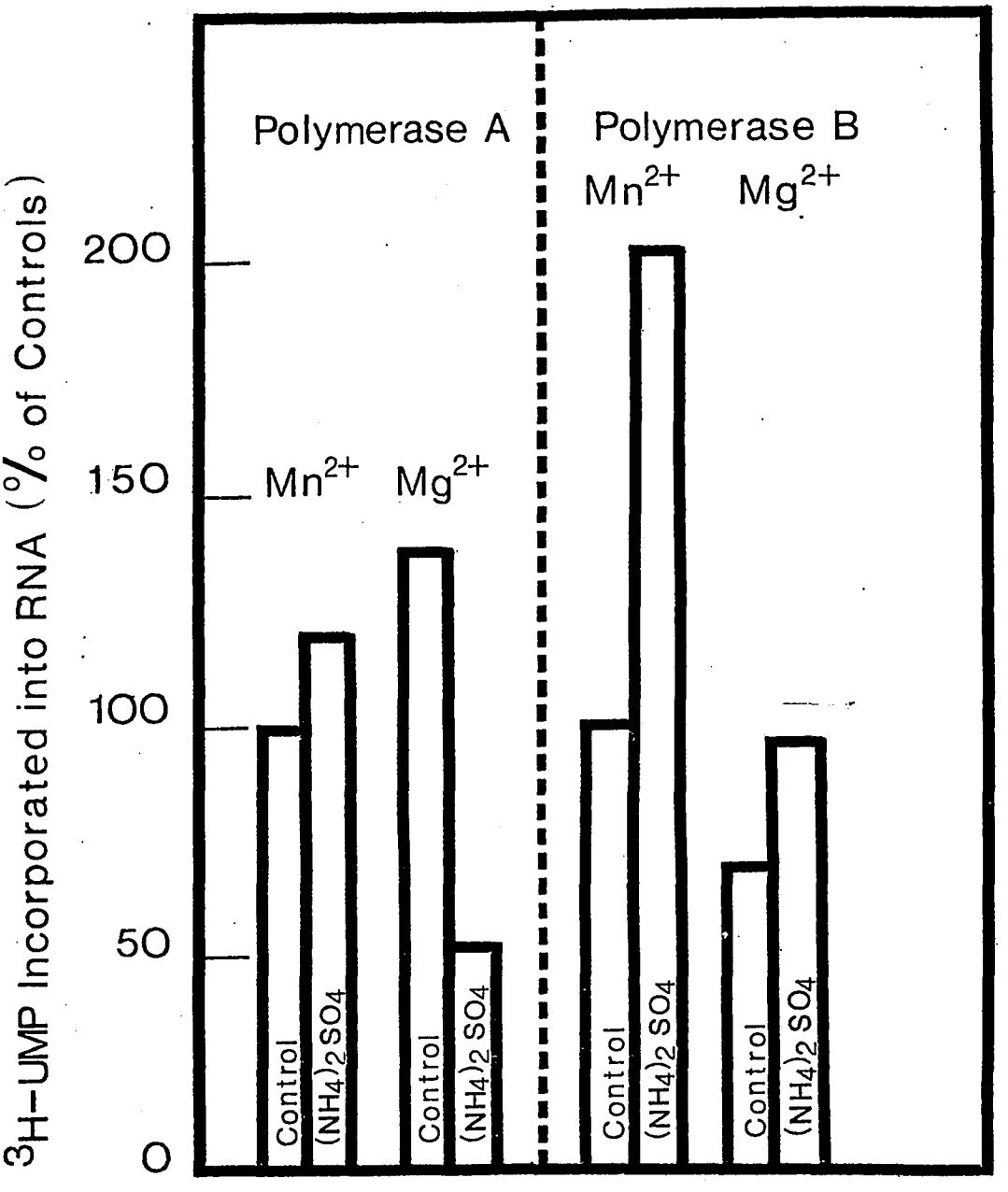
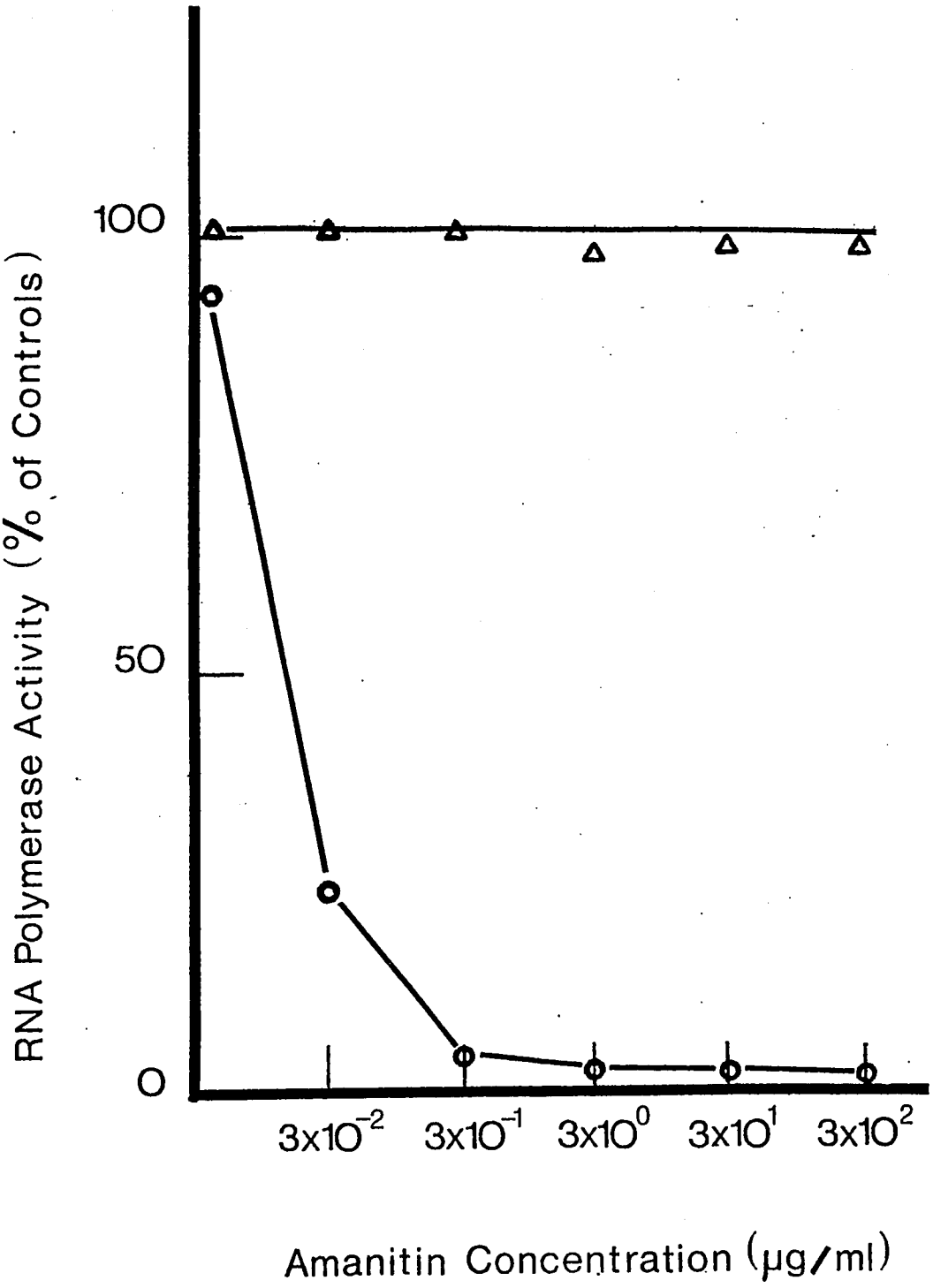

Polymerase A
Polymerase B
Mn2+
Mg2+
Mn2+
Mg2+
200
150
100
50
0
3H-UMP Incorporated into RNA (% of Controls)
Control
(NH4)2SO4
Control
(NH4)2SO4
Control
(NH4)2SO4
Control
(NH4)2SO4

Fig. 2. The effect of α-amanitin concentrations on the activity of RNA polymerase species A and B (data from figure 8 of Seifart et al., 1972). Amanitin concentrations reflect final concentrations in the assay.

Figure 2


100
50
0
RNA Polymerase Activity (% of Controls)
3x10^-2
3x10^-1
3x10^0
3x10^1
3x10^2
Amanitin Concentration (µg/ml)

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