Humoral Factors Involved in the Induction of Liver Regeneration in the Rat.*† (19643)

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In previous work the relationships between liver regeneration in rats, age and carcinogenesis with azodyes were investigated(1,2). This is a report on further investigations with the emphasis on the mechanism controlling liver regeneration.

In vitro experiments. In order to investigate the possibility of the existence of humoral factors involved in liver regeneration, blood sera from partially hepatectomized and control rats were compared with respect to their action: a) on the initial growth response of primary rat liver explants cultivated *in vitro*, and b) on the growth period of *in vitro* cultures of a strain of normal rat fibroblasts. In the first series 24 male rats, 6 to 10 months of age grouped in pairs, were used. The experimental procedure employed on each pair was as follows: A partial hepatectomy consisting of the removal of the two main lobes of the liver was performed on one of the partners and a sham operation consisting of a laparotomy and manipulation of the liver on the other. Since hepatectomy reduces food uptake, the diet of the control was reduced in order that his food uptake would not exceed that of the hepatectomized rat. Twenty-four hours after the operation the animals were bled and the sera obtained were tested within 48 hours. Liver from a male rat 3 weeks of age was used as test tissue. A small piece of the tissue was cut up in the fluids to be used as culture medium and explanted directly on the glass wall of roller tubes without the use of plasma or embryo extract. Six tubes containing 6 fragments each were set up for each experiment. In 3 tubes the medium consisted of 50% serum from the hepatectomized animal plus 20% human placental cord serum, and in the other three, 50% serum from the control animal plus 20% human placental cord serum. The diluent was balanced salt solution (Gey). The tubes were allowed to stand in the incubator for 45 minutes in a vertical position in order to obtain good adherence of the fragments to the glass and then incubated in the rotating drum. Observations were made every 24 hours for a period of 6 days without renewal of the medium. The growth activity was estimated using as a criterion the fibroblastic outgrowth which always precedes epithelial outgrowth in such primary explants. Four grades of activity were recognized and defined as follows: Grade 0 activity was characterized by migration of macrophages and a few isolated fibroblasts without the formation of a network; Grade 1+ activity by the formation of a loose fibroblastic network involving only a limited area of the periphery of the fragment; Grade 2+ activity by the formation of a loose fibroblastic network involving a larger area of the periphery of the fragment; and Grade 3+ activity by the formation of a denser fibroblastic network involving most of the periphery of the fragment. Migration of liver cells accompanied the fibroblasts, but was not consistent enough to allow classification. Each group of cultures was graded on the basis of maximum activity found within the group.

The results are summarized in Fig. 1. In 11 out of 12 cases the activity of the cultures containing serum from the hepatectomized animals was greater than in the controls. In 7 cases the difference was striking since the controls showed no activity. In only one case was the activity in the control above Grade 1.

In the second series the action of sera from partially hepatectomized and control rats on

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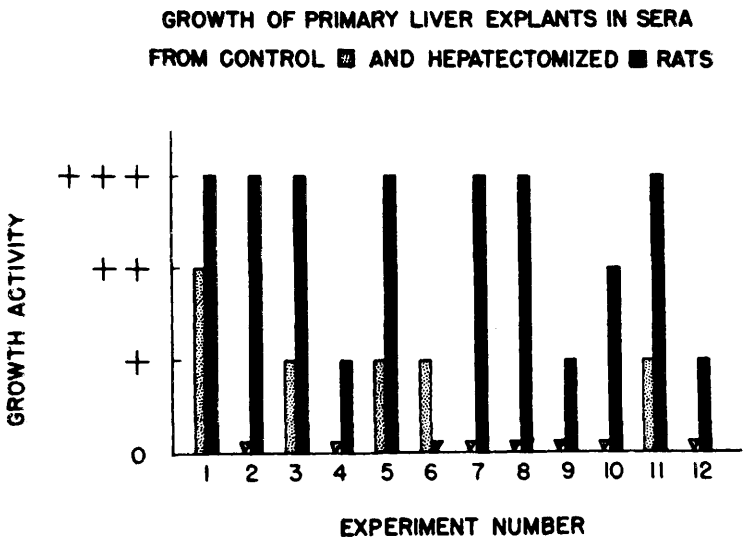

FIG. 1. Degrees of growth activity defined in text. The small triangles indicate 0 activity.

the growth period *in vitro* of cultures of a normal rat fibroblastic strain, *14pf*, was investigated. This strain has been isolated and grown *in vitro* in this laboratory since 1938 (3). The growth period is defined here as the interval between the beginning of cellular outgrowth of a newly transferred culture and the cessation of growth with degeneration under conditions which provide no renewal of the medium. The degenerative process characterizing the end of the growth period is such that no morphologically recognizable living cells can be seen within the growth zone of the colony (Fig. 2,3). Twenty-eight rats were used. The experimental procedure was essentially the same as in the first series except that no human placental cord serum was used. In each experiment 4 roller tubes of strain 14pf were set up with only the serum from the hepatectomized animal and 4 tubes with serum from the control. In each group of 4 tubes 2 contained 20% serum and the other two 50%. Thus the experimental and control sera were each represented in 2 concentrations. There was no renewal of the medium and observations were carried out every 24 hours as before. The results are summarized in Fig. 4. In the large majority of cases the growth period was longer in the experimental than in the control serum. In all cases the growth period was longer in the 20% than in the 50% serum concentration. Analysis of variance was the statistical procedure employed to evaluate the significance of these results(4). The difference between control and experimental sera was significant with P less than 0.01. The difference between the 2 serum concentrations was also significant with P less than 0.001.

Comment. These data indicate that rat serum under certain conditions exhibits an inhibitory effect on the outgrowth of tissue cells *in vitro*, since a decrease of the serum concentration in the medium resulted in a longer growth period. A longer growth period was also obtained with sera from partially hepatectomized rats. If these 2 findings are related the effect of the serum from partially hepatectomized rats could be mediated through the decrease of certain constituents of the serum following partial hepatectomy. These constituents at the concentrations normally present in the blood would be considered to exert a growth-inhibitory effect. It is known that partial hepatectomy induces marked alterations in the composition of blood serum as a result of the decreased amount of functioning hepatic tissue. Serum proteins, for example, are known to undergo a decrease up to 20% during the 24 hours following partial hepatectomy in the rat(5). In such a case should the decrease of serum constituents

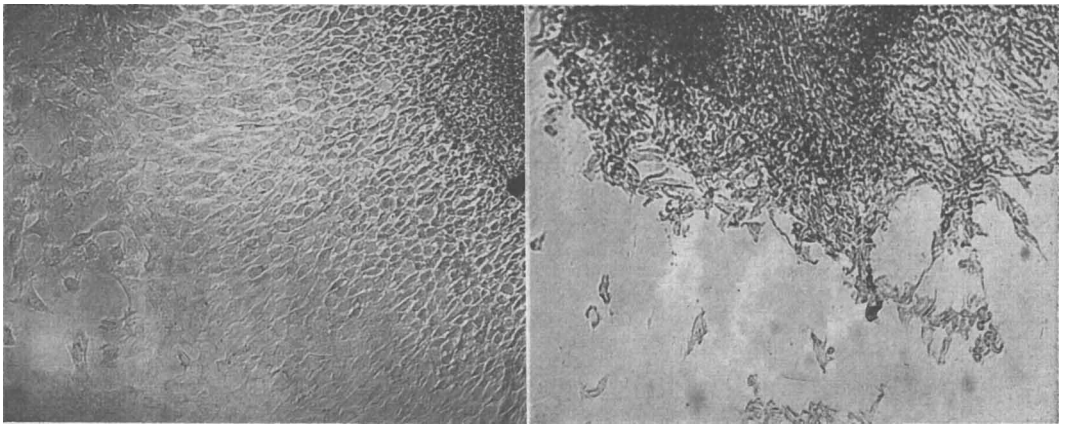

FIG. 2. 24-hr-old culture of strain 14pf in 50% normal rat serum. Beginning of growth period. Growth zone composed entirely of living cells.

FIG. 3. 6-day-old culture of strain 14 pf in 50% normal rat serum. End of growth period. No morphologically recognizable cells within the growth zone.

be involved in the initiation of the regenerative process in the liver then a dilution of the serum *in vivo* through plasmapheresis might be expected to initiate mitosis in the intact liver of a rat.

In vivo experiments. Ten adult male rats, 10 to 12 months of age grouped in pairs, were used in a preliminary study and the experimental procedure employed on each pair was as follows: Under ether anesthesia and with aseptic conditions the left femoral vein of one of the partners was exposed and 5.0 ml of blood withdrawn through a silicone-treated syringe and needle containing 0.2 ml of 0.3% heparin solution. The skin wound was then washed with a 1000 u/ml solution of penicillin

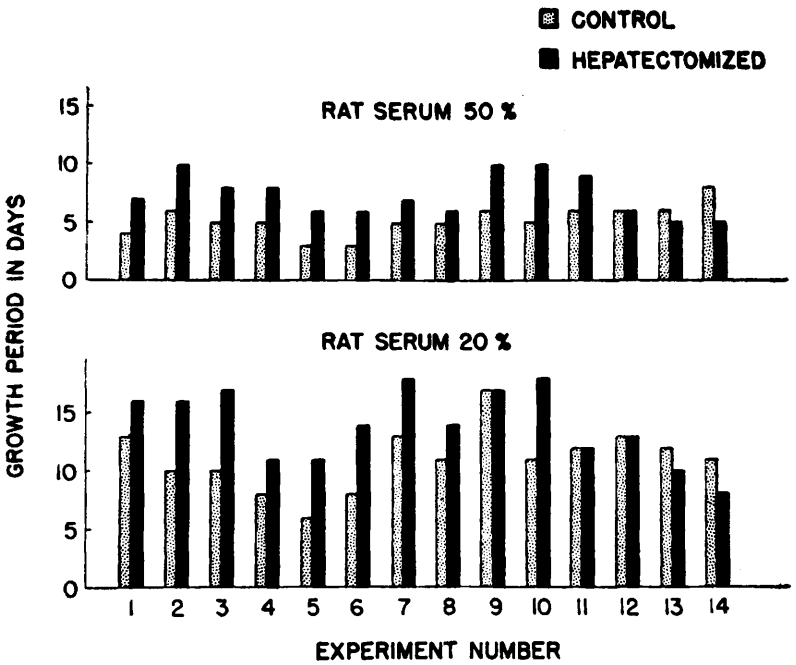

FIG. 4. Growth period of connective tissue strain 14pf in sera from partially hepatectomized and control rats.

and closed with the aid of Michel clips. The animal was returned to his cage and allowed food and water *ad libitum*. The same procedure was repeated on the other partner of the pair but the blood was only withdrawn and reinjected immediately. The blood from the first animal was centrifuged, the plasma decanted, and its specific gravity measured by the copper sulfate method(6). The red cells were then washed twice in balanced salt solution and finally resuspended to a volume of 4.0 ml. Twelve hours later the right femoral vein was exposed under the same conditions as before and 7 ml of blood withdrawn from the experimental animal followed by reinjection of the previously prepared red cell suspension. The same procedure was used on the control rat but with immediate reinjection of the whole blood. The blood obtained from the experimental rat was centrifuged and again a red cell suspension made. The same procedure was then repeated (at 12 hour intervals from 24 to 84 hours), alternating the site of venipuncture. The amount of blood withdrawn at times was reduced to 5 ml and no reinjection of red cells was given. The animals were then sacrificed by exsanguination 12 hours after the last bleeding, a final determination of the specific gravity of the blood was made, and the livers removed and fixed for histological examination.

It was found that at the end of the plasmapheresis the specific gravity of the plasma of the experimental animals fell below 1.015. That of the controls remained above 1.019. Microscopic examination of the sections of the livers of the experimental animals showed a variable but marked increase in cell and nuclear size, an increased basophilia mostly in the juxtanuclear area of the cytoplasm, and numerous mitotic figures in all stages. Several fields containing no more than 100 nuclei exhibited 2 or even 3 mitotic figures (Fig. 5). It is known that in normal adult liver the ratio of dividing to resting nuclei is no greater than 1 to 10,000(7). The overall histological picture of the livers of the experimental rats showed a striking resemblance to the picture seen in the regenerating liver at 48 hours after partial hepatectomy(8). The microscopic examination of the sections of the livers of the

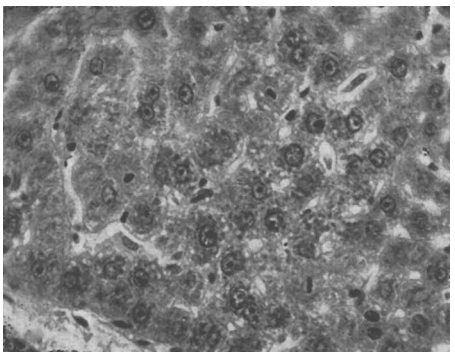

FIG. 5. Liver of an adult male rat submitted to plasmapheresis for 48 hr. Note large cells, variability of nuclear size, and deep basophilia of the cytoplasm. Three mitotic figures: one metaphase and two prophases. × 250.

control animals showed some increased basophilia of the cytoplasm of liver cells. In all other respects the histological picture of these livers was similar to the normal. Further work is now in progress aimed at standardizing the procedure of plasmapheresis in order to analyze quantitatively the mitotic activity thus induced and to establish any relationships with the degree of decrease in specific gravity of the plasma, and the length of time this decrease is maintained.

Discussion. The growth response *in vitro* of primary liver explants and of a connective tissue strain was shown to be significantly greater in a medium containing serum from partially hepatectomized rats than in a medium containing normal adult blood serum from control animals. This indicates that following partial hepatectomy the composition of the blood serum is altered and that this alteration is possibly related to the initiation of liver regeneration. The results with varying concentrations of sera further indicated that certain constituents of blood serum exert a growth inhibitory effect *in vitro*. The decrease of the concentration of those constituents by dilution of the serum had an enhancing effect on the growth response of tissue cells *in vitro* similar to the one obtained with sera from partially hepatectomized rats. Likewise, by effecting a dilution *in vivo* of the blood serum of normal adult rats through plasmapheresis, a growth response in the intact liver similar to the one obtained by partial hepatectomy was induced. These last 2

findings suggest that the alteration in the composition of the blood serum following partial hepatectomy may in effect be the decrease of the concentration of certain constituents which at their normal concentrations exert a growth inhibitory action. Such a decrease, then, could account both for the enhancing effect of such serum on the growth response of tissue cells *in vitro* and the initiation of liver cell proliferation *in vivo* which follows partial hepatectomy. Further work is in progress in order to test in a direct way the validity of this hypothesis.

In their work on regeneration in parabiotic rats, Bucher, Scott, and Aub(9) suggested that chemical entities in the blood when present in sufficient quantities may cause the proliferative response in the liver. An elevated concentration of such substances was considered by them to be the stimulus initiating liver cell division following partial hepatectomy(9). The findings of the present work do not provide evidence in support of such a concept. On the other hand, their experimental findings concerning the induction of mitotic activity in the intact liver of one of the partners of a parabiotic pair by partial hepatectomy performed on the other are in complete agreement with the findings of the present work and can readily be interpreted on the basis of the hypothesis previously discussed.

Summary. On the basis of a comparison of the action of blood sera from partially hepatectomized and control rats on the growth response of tissue cells *in vitro* an hypothesis was formulated with regard to the induction of the regenerative process in the liver which follows partial hepatectomy. According to this hypothesis certain constituents of normal blood serum exert a growth inhibitory action at their normal concentrations. Partial hepatectomy reduces the amount of functioning hepatic tissue thereby resulting in a decrease of the serum concentration of these constituents. This in turn results in the initiation of regenerative growth in the liver. Further evidence supporting this hypothesis was obtained by the induction of cell division in the intact liver of adult rats by decreasing the concentration of serum constituents *in vivo* through plasmapheresis.

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Pricking Pain Threshold in Different Body Areas.* (19644)

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Although pain threshold measurements are customarily made using the forehead as the test surface, it is often desirable to use other body areas for this purpose. Measurements of cutaneous hyperalgesia or hypalgesia in a particular locality should properly include control measurements on the opposite side or have reference to measurements made on

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