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DISCUSSION OF HEPATOTROPHIC FACTORS AND REGENERATION

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Until about a decade ago, dogma supported by the publications of Child [1] and Fisher [2] and many others held in what has been termed the flow hypothesis, that the quantity of portal blood was the only important hepatotrophic influence, and that the quality of this portal blood was of no real significance in the maintenance or restoration of hepatic mass. As Schindler mentioned earlier this afternoon, this concept has been seriously weakened, if not overthrown, by a series of investigations with what can be loosely termed double liver fragment preparations, which models, incidentally, were originally evolved without any real consideration at all for studying regeneration. I would like to present this information to you now, with an apology for the lack of sophistication which it obviously has in relation to much of the other work you have heard about today. Yet, what I have to say undoubtedly has a relation to regeneration. In particular I am intrigued with the possible relationship of our studies to Professor Weber's presentation.

The first experiment was with auxiliary liver transplantations to immunosuppressed canine recipients, using portal revascularization with a technique (Fig. 1) by which the portal vein was given blood flow from the inferior vena cava [3]. Arterialization was from the aorta or iliac artery. The native liver retained its natural vascularization.

In spite of the fact that the blood flow to this kind of auxiliary transplant has been shown to be greater than to the native liver [4], the extra organs undergo fantastic atrophy [3]. The atrophy in turn can be prevented if the portal flow is given to the transplant, depriving the host liver of this source of blood supply [5].

Later, the more satisfactory model shown in Fig. 2 was used, dividing the animal's own liver into 2 components. Unlike auxiliary transplantation, the split liver preparation avoided the situation in which one of the liver fragments was under immunologic attack, whereas the other was not. The split liver technique consisted of detaching one of the portal branches and connecting it to the inferior vena cava, whereas the other liver

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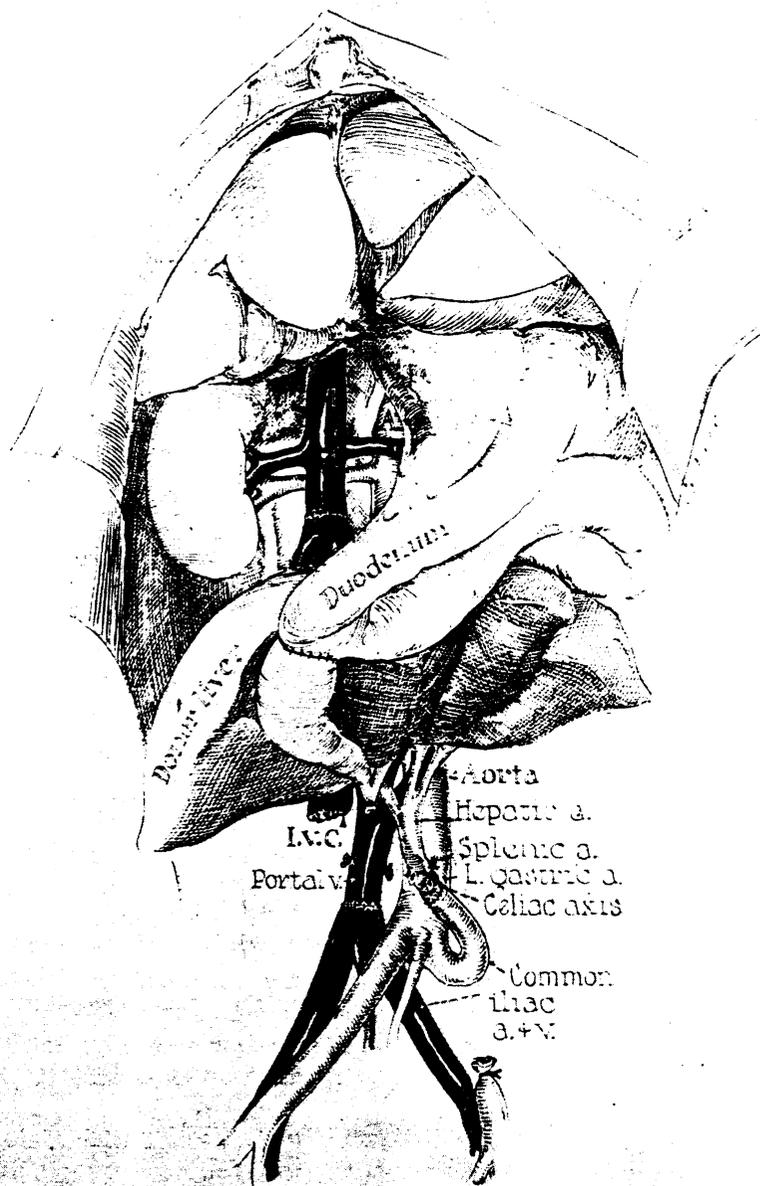


Fig. 1. Auxiliary liver transplantation in dogs. Note that the reconstituted portal blood supply is from the distal inferior vena cava. Cholecystoduodenostomy is performed. (By permission of *Ann Surg* 160:411, 1964.)

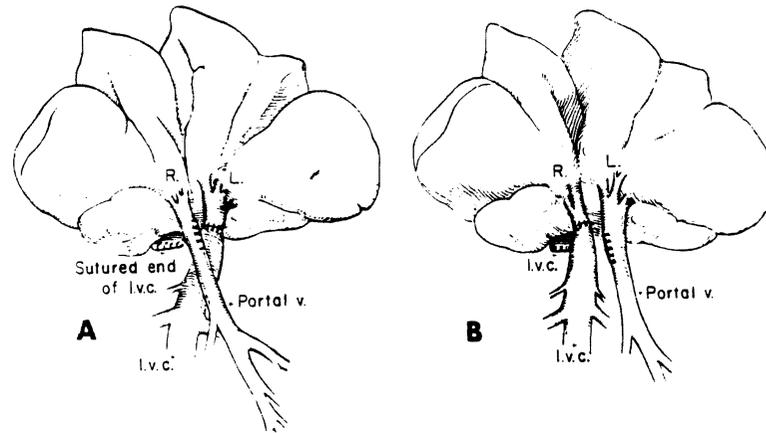


Fig. 2. Partial portacaval transposition. The entire vena caval flow is directed into either the left (A) or the right (B) portal venous branch, while the contralateral liver lobes continue to receive splanchnic venous blood. (By permission of Surg Gynec Obstet 137:179, 1973.)

fragment received a natural blood supply. Under these conditions, the side which was given vena cava blood flow, even though this flow was greater than on the other side, invariably underwent profound atrophy and was found to be in a state of relative hypoplasia and deglycogenation. The glycogenation, hypertrophy and hyperplasia on the side getting splanchnic venous blood were imputed to exposure of these hepatocytes to some unspecified component(s) of the splanchnic venous blood, which was (were) speculated to be largely removed by one passage through the liver [3, 5, 6, 7].

In an effort to learn the nature of these hepatotropic factors, we recently published [8] a series of observations with a canine preparation which divides the splanchnic venous return into two compartments (Fig. 3). One portion of the liver is perfused by intestinal venous blood going through a vein graft to either the right or left portal branch. The other liver lobes received blood from the pancreaticogastrroduodenosplenic area.

Liver fragments receiving the hormone-rich blood from the upper abdominal organs flourished. The glycogen content of these cells was greater. The size of the hepatocytes were increased, as could be quantitated with a technique developed by Dr. K. A. Porter of London, in which the liver cell images were cut out and weighed (Fig. 4).

In the deceptive original title of my discussion, particular reference was made to cyclic AMP. This phase of our investigation was designed to either support or deny the hypothesis I just implied, that trace quantities of pancreatic hormones were the principal hepatotropic substances in portal blood. This was done by measuring cyclic AMP concentrations and the production of cyclic AMP in the two sides of the liver fragments which had differing blood supplies, as shown in Fig. 2 and 3, under a variety of conditions. Numerous other biochemical determinations were also done by my colleague Dr. Francavilla of Bari, Italy, including

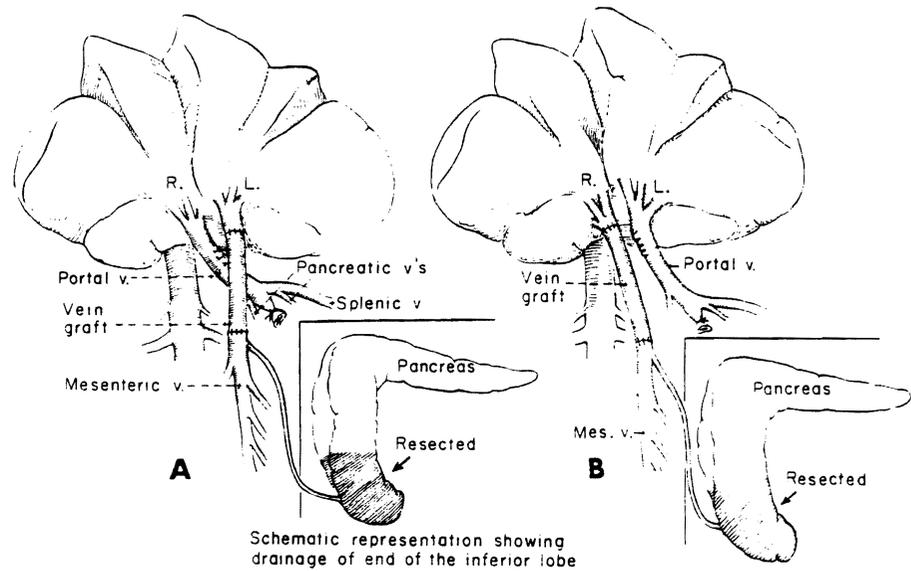


Fig. 3. Technique of division of splanchnic venous flow into a pancreatico-gastro-duodenal-splenic compartment and an intestinal compartment. Blood from these respective sources is directed into the right or left lobes. The tail of the inferior lobe of the pancreas was resected since it drains separately into the mesenteric vein. (By permission of *Surg Gynec Obstet* 137:179, 1973.)

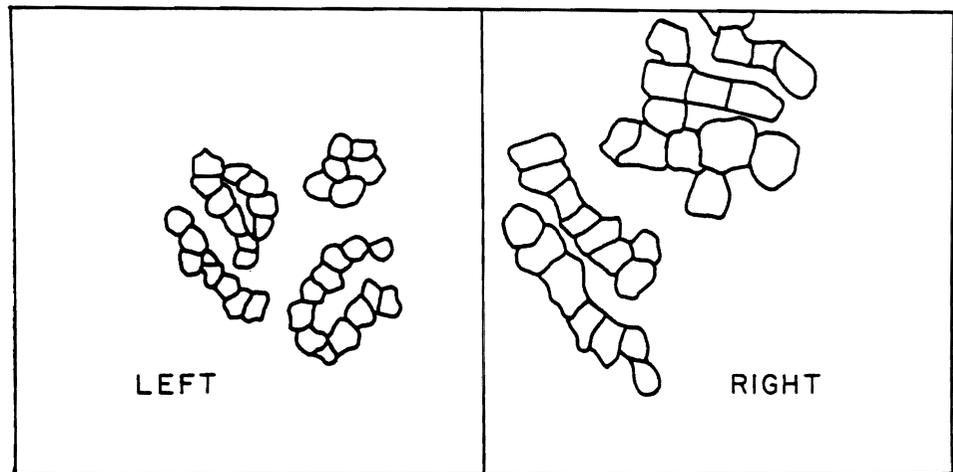


Fig. 4. Hepatocyte shadows traced during histopathologic examination. These were later cut out on standard paper and weighed as an index of hepatocyte size. The specimens depicted were from the experimental Group 2 (see Fig. 3A). The right lobes with the large hepatic cells received venous blood from the pancreas, stomach, duodenum and spleen. The relatively shrunken left lobes with the small hepatocytes received intestinal blood. (By permission of *Surg Gynec Obstet* 137:179, 1973.)

quantitative glycogen, phosphorylase, glucokinase and protein synthesis, just to mention a few. I will not bother you with the details of our results which, as I mentioned, have been published [8], except to say that there was a major biochemical, apparently hormone directed, dissociation between the two sides of the liver. The preparation I would like to briefly allude to is the one (Fig. 2) in which the entire splanchnic flow goes to one side and the vena caval flow to the other side of the liver. In Fig. 5 is shown a pilot experiment done on normal dogs. These animals were given intravenous tolbutamide, which generates a marked increase in insulin concentration in the portal vein, but has very little change in the systemic venous insulin levels. Hepatic cyclic AMP is essentially unchanged. Since a

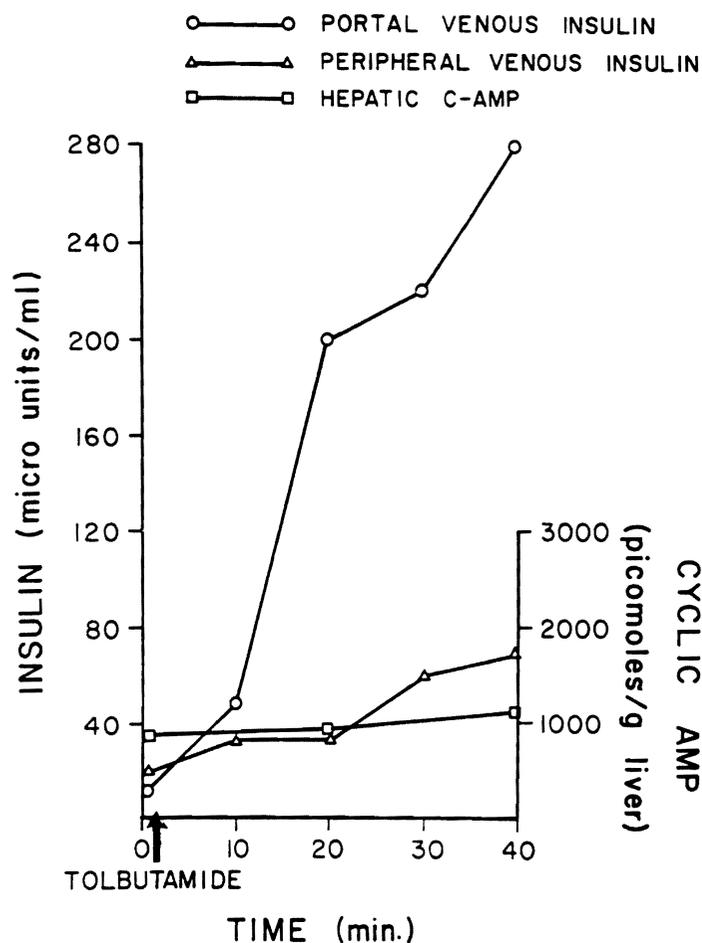


Fig. 5. Changes in peripheral and portal venous insulin and hepatic cyclic 3', 5'-adenosine monophosphate, cyclic AMP, occurring in a normal dog infused with tolbutamide. Note that the peak insulin response in the portal blood occurred 25 to 40 minutes after infusion and that no significant alterations in hepatic cyclic 3', 5'-adenosine monophosphate were caused acutely by the tolbutamide itself. (By permission of Surg Gynec Obstet 137:179, 1973.)

small decrease in hepatic cyclic AMP would be impossible to measure biochemically, the effect of the tolbutamide-induced insulin on cyclic AMP was magnified by also giving glucagon, which, unopposed, produces a tremendous increase in liver cyclic AMP, but when opposed by insulin, gives a cyclic AMP pattern similar to control animals. When the preparations shown in Fig. 2 were studied with the tolbutamide-glucagon test, the lobes receiving splanchnic blood (Fig. 6—panels on the left) had a response similar to normal dogs, whereas the contralateral lobes supplied by vena caval blood (Fig. 6—panels on the right) showed a rapid accumulation of cyclic AMP. From this and other experiments, we have concluded that the hepatotropic factors in splanchnic venous blood are trace quantities of hormones, the master anabolic hormone being insulin, counterbalanced to an unknown extent by catabolic hormones including glucagon, epinephrine and probably others as well.

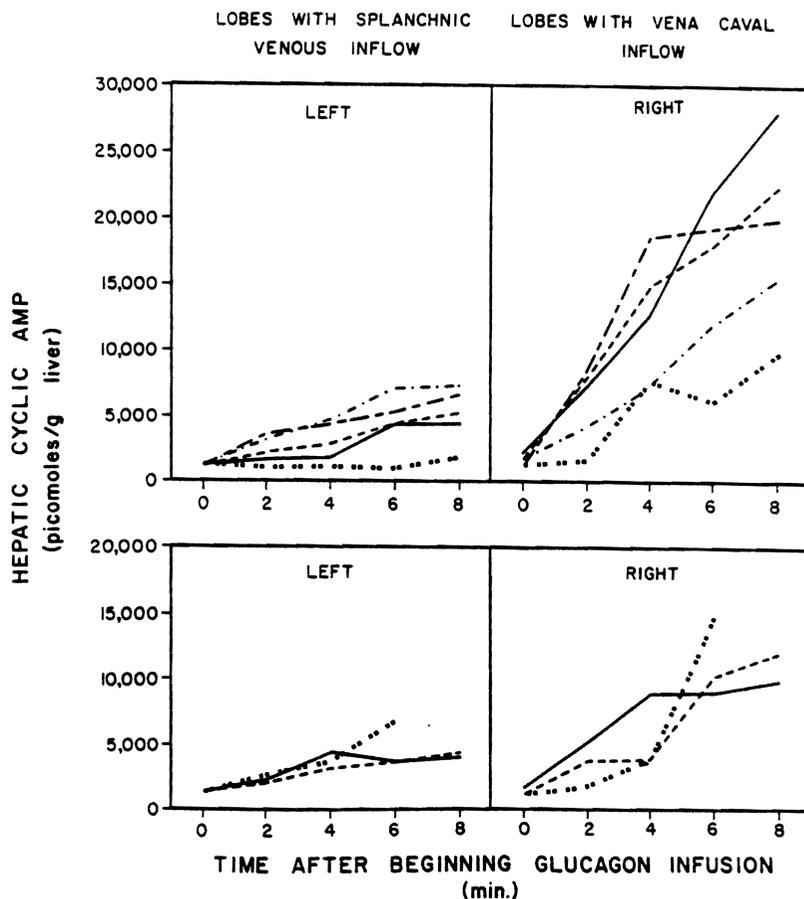


Fig. 6. Results of tolbutamide-glucagon tests in eight dogs with partial portacaval transposition (Fig. 2), demonstrating the effect of endogenous insulin in the lobes receiving splanchnic venous blood. These insulin-controlled lobes had a restrained cyclic 3', 5'-adenosine monophosphate response to the exogenous glucagon, whereas the response in the other lobes was uninhibited. (By permission of Surg Gynec Obstet 137:179, 1973.)

What has this to do with regeneration? From the old-fashioned measures of mitotic indices in our early publications, we have thought for almost a decade that the hepatotrophic factors subserve hyperplasia as well as hypertrophy and are, therefore, central to a full understanding of regeneration. These contentions have been bolstered by confirmatory data with DNA synthesis, as measured by thymidine incorporation in rats, by Chandler, Lee and Orloff [9] of San Diego about 5 years ago, and, more recently, by Fisher [10]. We ourselves have made thymidine incorporation studies in our split liver preparations. When all the splanchnic flow goes to one side and the vena cava flow to the other side, a few days after the operation, there is invariably a greater thymidine uptake in the splanchnic-fed lobes. After some 60 or 70 days, this differential goes away, but can be restored with a 40% resection of the liver. It is interesting that the differential can be eliminated or blunted by a total pancreatectomy. With the procedure dividing the splanchnic flow into intestinal and pancreatic components (Fig. 3), the differential favors the pancreatic side, as opposed to the liver lobes receiving intestinal blood.

These findings are consistent with the hypothesis that pancreatic hormones are important hepatotrophic factors and are related to regeneration. This conclusion is, of course, directly contrary to the claims by Price et al. [11] that hypertrophy and hyperplasia, as affected by splanchnic hepatotrophic factors, are in inverse relation to each other; it is also contrary to the recent opinions of Fisher [12], who has claimed that the hepatotrophic factors are of intestinal origin and are probably related to folic acid or vitamin B₁₂ from the ileum.

Be that as it may, it seems probable that the hepatotrophic concept is a central but incompletely recognized fact of liver physiology, which, when completely worked out, should help reconcile a number of previously divergent opinions about such diverse matters as the explanation for liver regeneration (as we have heard from Professor Weber today), the origin of portacaval syndromes, optimum conditions for auxiliary liver transplantation and the reasons for the dramatic benefits of portal diversion procedures for patients with glycogen storage disease or idiopathic type II hyperlipoproteinemia, to cite only a few examples.

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DISCUSSION

WEINBREN: I noticed that in your technical preparation the blood coming from the intestine always went through a graft to one side of the liver, and the blood coming from the pancreas did not; it went directly to the liver. Knowing how delicate the portal pressures are, I was wondering perhaps if the graft caused some obstruction to flow and that part of the liver, as a result, was disfavored. The atrophy was related to the fact that the blood went through the graft and the hypertrophy on the other side was related to the atrophy that was produced by the blood going through the graft. It seems to me that in order to avoid this criticism, perhaps the graft should have been alternated between the two sides, that is the intestinal and pancreatic sources. At the moment, I cannot see how the one hypothesis is better than the other one, although you may well be right.

STARZL: I'm sorry that that paper (Starzl, T.E., Francavilla, A., Halgrimson, C.G., Francavilla, F.R., Porter, K.A., Brown, T. and Putnam, C.W.: The origin, hormonal nature and action of portal venous hepatotropic substances. *Surg Gynec Obstet* 137:179, 1973) hasn't gotten over here yet, but we did it both ways, precisely for the reason you mentioned. No matter which way the vein graft went (right versus left), the results were as I described.

HOLZER: Was this mentioned in your abstract here?

STARZL: No, it was not.

WEBER: I have a piece of information which might be helpful to Dr. Starzl. If you measure thymidine metabolism in the diabetic rat, there is a dramatic decrease of incorporation of thymidine into DNA and also, in a minor degree, of the degradation of thymidine. When you treat diabetic rats with insulin, there is, as you know, an enlargement of the liver, and

there is a very marked rise in the incorporation of thymidine into DNA. In 2 to 3 days, this becomes a very marked overshoot, the type of thing you observe in glucose 6-phosphate dehydrogenase. The catabolic pathway climbs up rather sluggishly. There is one other paper on this matter. This was published by Weber et al. in *Advances in Enzyme Regulation*, Vol. 10, pp. 39-62, 1972 and I refer to it in the *Israeli Journal of Medicine*, (8:325-342, 1972) at the Insulin Conference and the 50th anniversary of its discovery, so this was 1972. There is another paper which you should also keep in mind, Younger et al. (*Cancer Res* 26:1408-1414, 1966), on the action of insulin on stimulating the incorporation of thymidine into DNA. Younger and his groups counted mitotic figures, which were increased after insulin administration. They also determined DNA polymerase; these two lines of evidence favor insulin having a role in this process, along with many other things.

STARZL: I'm a little hesitant to talk about preliminary work, but we have, of course, gone ahead with other experiments. We are now working with alloxan diabetic dogs and dogs submitted to total pancreatectomy and followed chronically. It has seemed to me that there is a rather different effect of total pancreatectomy, compared to the insulinopenic effect of alloxan diabetes in animals with the split transposition (see Fig. 2) or splanchnic division (Fig. 3). Two months after total pancreatectomy, the two liver sides are quite different. The side that is deprived of all splanchnic flow becomes grossly waxy and is laden with fat. Such changes are less marked in animals with alloxan diabetes. Our conclusion has been that there is a very major difference between a mere insulinopenia and the more complex loss of total pancreatectomy. This would suggest that the hepatotrophic substances are multiple interlocking factors.

I would also like to comment on the question of both flow and oxygen content. The flow in these preparations, in which there has been withering of the nonsplanchnic side, has usually been shown to be greater than in the other side, at least early after operation. We have also carried out an experiment in which we arterialized one branch of the portal vein, so that the flow to that side was not only many times greater than to the other side, but it also had greater oxygen content. These advantages did not save the liver fragment deprived of splanchnic venous blood, which underwent atrophy.

Parenthetically, it has been accepted in the literature for many years that portal venous blood has a high oxygen content compared to vena caval blood. I don't know the origin of this belief. We published a small paper a few years ago, in which we examined precisely this question. In awake dogs, after we placed in-dwelling catheters in the portal vein and suprarenal vena cava, there was no difference in oxygen concentration between the portal venous and vena caval blood (Hermann, T.J., Taylor, P.D., Marchioro, T.L. and Starzl, T.E.: Oxygen and CO₂ content in the splanchnic and nonsplanchnic blood of dogs with portacaval transposition. *Surgery* 60:1229, 1966).

RABES: I have just a short remark on the action of insulin in vitro. We succeeded in cultivating liver cells of new-born rats: they grow very fast if insulin is added to the medium.

MOLIMARD: I would like to know if you investigated the possibility of modifying the external pancreatic secretion, because the absorption in the upper part of the gut is normally very high and the blood from the pancreas and the upper part of the intestine might normally convey to the liver some nutriment it has to cope with. Did you investigate the possibility?

STARZL: I think you have raised two possible questions. First, do the upper intestinal organs emit blood with a higher oxygen concentration than the lower ones? That is probably true, but I do not think that the oxygen is the critical hepatotropic factor for the reasons I mentioned a few moments ago. Second, as to their being other possibilities than insulin, I have already conceded that, and indeed believe that interrelationships between multiple hormones and, even nutrients, explain the hepatotropic effect. Certainly, however, insulin is a key hepatotropic factor, as both Professor Weber and Prof. Rabes have suggested. In this connection, I would like to call your attention to a nice paper by Reaven, E.P., Peterson, D.T. and Reaven, G.M.: The effect of experimental diabetes mellitus and insulin replacement on hepatic ultrastructure and protein synthesis (*J Clin Invest* 52:248-262, 1973), in which is described the very striking depletion of rough endoplasmic reticulum in hepatocytes and other major changes, which occur very quickly after the creation of the state of insulinopenia. Their controls showed that it wasn't the alloxan *per se* that was responsible, but rather, the insulin deprivation.

With your permission, may I tell you about one other extremely interesting observation that has been made in humans a few months ago. This concerns the treatment of a 12-year-old child with homozygous type II idiopathic hyperlipoproteinemia, a disease that responds poorly to medical therapy. Our patient had a serum cholesterol concentration of almost 1000 mgm%. We speculated that the diversion of the pancreatic hormones and other hepatotropic effects away from the liver might turn off lipoprotein synthesis. We performed end-to-side portacaval shunt as a last resort, because the child had had a myocardial infarction and was in intractable heart failure from aortic stenosis, apparently due to xanthoma in the valve. After the operation, the visible superficial xanthoma, which characterize the disease, began to flatten within a few days, and her angina disappeared. Her heart problem went away and the serum cholesterol dropped from 1000 down to about 230 mgm%. She had a liver biopsy 6 months after the shunt operation, and the changes I described in Reaven's study were found in the liver. The profound benefit from the portacaval shunt again illustrates the specificity of the portal venous blood and the striking metabolic effects of its diversion in certain disease states. The report of this case was published (Starzl, T.E., Chase, H.P., Putnam, C.W. and Porter, K.A.: Portacaval shunt for the treatment of hyperlipoproteinemia. *Lancet* 2:940, 1973).

DATTA: I have understood that portal blood, when diverted, acts as a promoter of regeneration, and it has been postulated that possibly something coming from the pancreas, an insulin-like material, is operating here. Now my question is, even if we are diverting the blood, the insulin or the insulin-like materials still go into systemic circulation, i.e. the hepatic artery, which supplies the hepatocytes. Hence, diversion of blood should not make much difference. In other words, are we saying that only the portal side is important for this phenomena?

The second clinical observation we made was with extra hepatic portal vein obstruction, in which it is assumed that the portal vein is more or less occluded and the blood supply is from the arterial side. Even in this situation, the liver, at least in our Chandigarh series, had been of normal weight.

STARZL: To answer his question in a summary, I would say that when one uses the so-called double liver fragment models, it is possible to demonstrate that the two fragments live in different chemical worlds and that those worlds are, as far as we can tell, dominated mainly by hormone influences. The use of the double liver fragment model was the main breakthrough, technically making it possible to study hepatotrophic substances. The reason was exactly that stated by the gentlemen from India. If you divert portal blood in an animal with a single liver, the changes that are created in this liver, while real, are very subtle, and they are difficult to study because there is the recirculation effect that was just described. This is why Eck fistula has been the cause of so much controversy and why Child's so-called portal-caval transposition has created more confusion than it has shed light. In Child's transposition, the recirculation to the liver would occur roughly in proportion to total hepatic blood flow. In turn, this would explain why the transposition procedure protects the liver, because it recirculates more effectively than is the case with Eck fistula. But as I have already stated, while the effects of portal diversion may be subtle because of this recirculation, they are real, even when there is a single liver, and even when there is recirculation, as in the astonishing outcome of our child with hyperlipidemia indicates.

exclusive primary control to agents specifically provided by portal blood now seems at least open to question. On the other hand, since regeneration is delayed, and possibly somewhat diminished in the absence of portal blood and portal splanchnic organs, portal blood factors must at the very least have a facilitating or enhancing role; whether insulin and glucagon, as proposed by Dr. Starzl, and other portal blood components as well, can still exert primary control over the growth process at the reduced levels prevalent in eviscerated animals seems questionable, but cannot be excluded without further study. We have not yet studied glucagon sufficiently to warrant any discussion of its effects.

DISCUSSION

WEBER: I would like to comment on the paper by Dr. Bucher, because I think what she presented is of major importance for liver regeneration and for our own studies. I think that the elegant method that Dr. Bucher developed indeed opened the door to decisive experiments to determine factors that play a role in unleashing the regenerating liver process. I do think that she might have eliminated the insulin quite effectively. There is one paper which might be consulted that you might have already looked at. This is by Carl Morgan, who examined induction of insulinase in the liver by insulin over period of time. I believe this would confirm your suggestion that the circulating insulin, but not necessarily the liver-bound insulin, would be removed effectively from the animal. In that case, you would have an insulin-free animal. So I think what you are reporting is of very great importance, indeed.

BUCHER: Thank you, Dr. Weber, for your kind remarks. We think that the circulating insulin is probably very low at the time of the partial hepatectomy in the eviscerated animals. This is confirmed in a preliminary way by measurements of portal blood insulin, but they are still too few to be really significant. There remains the possibility that insulin could be bound by the liver in a cumulative fashion. Dr. Leffert, who works with liver cell cultures in Dr. Holley's laboratory at the Salk Institute, mentioned, at a Gordon Conference last summer, that large scale hepatic binding of insulin occurs within minutes after a partial hepatectomy. Obviously, we must look into that further before I can make any positive assertions; we are simply presenting this information as an attempt to eliminate certain variables, and to obtain a system that I hope will be useful to ourselves and others.

STARZL: I would just like to congratulate Dr. Bucher on a marvelous study, some of which she was kind enough to send me several months ago. I think it might be an error to consider that insulin, or anything else for that matter, is not an hepatotrophic factor on the basis that its absence does not cause death or that its absence does not cause a complete cessation of DNA synthesis. Doctor Bucher's skepticism about hepato-

trophic factors is based on the finding that regeneration can occur after evisceration. Yet, to me, what is really significance about her data is that the response to hepatectomy is thereby so greatly reduced, and very probably delayed as well. To these striking effects, insulinopenia and other factors doubtless contributed.

In searching for implications of these findings, let me mention one which might be of interest to those interested in oncology. It can be illustrated by a paper by Heuson and Legros (*Cancer Res* 32:226, 1972) which confirms many previous similar publications about the effect of insulinopenia on tumor growth. What was done in Heuson and Legros' study was to evaluate what alloxan diabetes did to the expected growth of a chemically induced rat mammary carcinoma. The diabetes inhibited the growth of the tumor.

Heuson and Legros also did a corollary study in which they determined which of those animals carried tumors that were dependent on insulin for vigorous growth in tissue culture (not merely survival but vigorous growth). They found that the tumors from animals that were responders to alloxan diabetes, in that growth was inhibited, were the same tumors that required insulin in the tissue culture for vigorous growth.

This situation may be analogous to that of hepatic regeneration in that insulin may play an important permissive role. In either situation, an all or none attitude about factors which promote or permit cell replication does not seem justified, nor should factors be dismissed as trivial merely because their absence fails to cause total cessation of cell division.

BUCHER: I did not mean to imply that insulin has no function in liver growth. I am sure it does; we all know that it is necessary for survival of the animal, and that probably applies to the liver cells as well. The question we are asking is whether insulin can serve as the single dominant agent regulating liver growth, or, alternatively, whether it has a mere permissive or enhancing role, acting in conjunction with some other agents. The evidence so far favors the nondominant status. This may be an oversimplification. No doubt there are many factors here; we are simply attempting to sort them out.