analysis. It is hoped that an awareness of the advantages of immobilized enzyme activity as an analytical tool will lead to applications in clinical investigations or wherever there is a need for miniaturized continuous chemical analyzers.

S. J. UPDIKE
G. P. HICKS

Department of Medicine,
University of Wisconsin, Madison

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Regeneration of Rat Liver: Transfer of Humoral Agent by Cross Circulation

Abstract. *Carotid-to-jugular cross circulation between partially hepatectomized and normal rats, via polyethylene cannulas, stimulated incorporation of ^{14}C-thymidine into hepatic DNA in the normal partners when it was maintained for 19 hours at a flow rate of about 2 milliliters per minute. Cross circulation for 7 hours or less was ineffective.*

When part of a rat's liver is excised, the remaining hepatocytes proliferate until the original mass of the liver is regained. The signal for this response has been variously proposed to be overloading of the excretory function of the liver, changes in liver hemodynamics, or changes in the concentration of a hypothetical blood-borne ("humoral") agent that regulates growth of the liver (*1*). As a result of reports that partial hepatectomy in one member of a parabiotically united pair of rats stimulated mitosis in the normal partner, numerous attempts were made to stimulate liver growth by injecting normal rats with blood, serum, plasma, or liver preparations from partially hepatectomized donors, or to inhibit regeneration by injecting partially hepatectomized rats with similar materials from normal donors (*1*). Reports of success generally eluded confirmation, however.

The recent demonstrations (*2*) that partial hepatectomy stimulates mitosis and DNA synthesis in liver autografts remote from the site of hepatectomy have reinvigorated the search for a humoral agent. Believing that such an agent might be metabolized so rapidly that only its prolonged and continuous introduction into the test animal would demonstrate it, we reinvestigated the problem with cross circulation at high rates of flow between normal and partially hepatectomized rats.

We used Charles River COBS female rats, 37 to 42 days old, weighing 112 to 160 g, denied food from the start of the experiments, and kept in restraining cages during cross circulation. Fractions of liver excised were 34 percent (left lateral lobe) (*3*), 68 percent (median plus left lateral lobe) (*3*), or about 85 percent (all the liver except the caudate lobe and about one-fifth of the right lateral lobe). Cross circulation was started within 90 minutes of hepatectomy except that the 85-percent hepatectomized rats were permitted about 2 hours to recuperate. Rats were selected such that the partner to be hepatectomized was slightly heavier than the normal partner.

Blood circulated from the left carotid artery of each rat to the right jugular vein of its partner. The arterial cannula consisted of 2 cm of PE-50 polyethylene tubing (Clay-Adams; internal diameter, 0.058 cm) inserted into the artery; it led into 1.5 cm of Vivosil tubing (Becton-Dickinson; internal diameter, 0.064 cm; used for flexibility) which in turn was connected to another 6.5 cm of PE-50. The venous cannula was a 10-cm length of PE-50 tubing. The cannulas were filled with heparinized saline (100 units per milliliter) before insertion, and an additional 0.35 ml of heparinized saline was injected through the arterial cannula after its insertion. Each rat received 7 ml of isotonic saline subcutaneously before cannulation, and 1.5 to 2.0 ml of saline through the venous cannula immediately before cross circulation was established by joining the arterial and venous cannulas of the partners with 15-cm lengths of PE-90 polyethylene tubing (internal diameter, 0.086 cm). This circuit permits a flow rate of approximately 2 to 2.5 ml/min. At the end of the cross circulation, we checked the flow by placing the partners on a double-pan trip balance and timing the change in weight following brief clamping of one of the cannulas. Except as noted, cross circulation was maintained for about 19 (18 to 20) hours before the partners were separated.

To study DNA synthesis, we injected thymidine-2^{14}C (0.80 μc; specific activity, 30 μc/μmole) into the tail veins of the separated animals 20 hours after operation in the hepatectomized partner and 20.5 hours after the start of cross circulation in the normal partner; we killed the rats 1 hour later. DNA was extracted from the livers with hot 5 percent trichloroacetic acid (*4*), and the extract was assayed for DNA content by the diphenylamine procedure (*5*) and for radioactivity in a Packard Tri-Carb scintillation counter, model 314 EX.

Table 1 shows that labeling of hepatic DNA remains at a low level in normal rats when they are cross-circulated with each other, but rises appreciably in normal rats cross-circulated with 68- or 85-percent hepatectomized partners. The effect is dose-related; labeling is not increased in partners of 34-percent hepatectomized rats compared with members of normal pairs, whereas it increases threefold in partners of 68-percent hepatectomized rats and more than sevenfold in partners of 85-percent hepatectomized rats. The latter increase is in the range obtainable in single rats by excision of 30 to 40 percent of the liver (*3*).

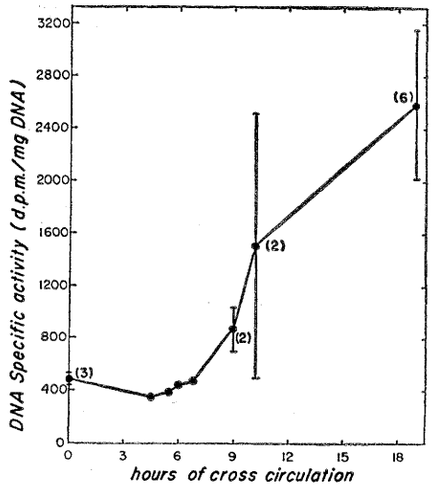

Fig. 1. Specific activity of hepatic DNA from normal members of 85 percent hepatectomized–normal pairs as a function of the duration of cross circulation. Rats were killed 1 hour after intravenous injection of thymidine-2-^{14}C in separated animals 20.5 hours after the start of cross circulation. Hepatectomies were done 2 hours before cross circulation began, except for the 4.5- and 5.5-hour points on the graph, for which operation preceded cross circulation by 7.5 and 6.5 hours, respectively (see text). For points covering more than one rat, vertical bars show standard errors of the means; numbers of rats appear in parentheses.

Figure 1 shows that prolonged cross circulation is needed to demonstrate this effect; less than about 8 hours appears insufficient, and, although the data are too few to yield a precise figure, considerably longer may be needed for maximum response. To determine whether this requirement merely reflects the time necessary for a humoral agent in the hepatectomized partner to reach a "critical" concentration, rather than the need for prolonged exposure to such a factor, we set up two pairs in which the 85-percent hepatectomies were done 7.5 and 6.5 hours in advance of cross circulation, which was maintained for 4.5 and 5.5 hours, respectively. Figure 1 shows that cross circulation of this brevity did not stimulate labeling of DNA despite the extra time afforded the hepatectomized partner before cross circulation.

By analogy with partially hepatectomized single rats (*1*), we presumed that increased labeling reflected increased synthesis of DNA preparatory to mitosis, and that it involved primarily hepatocytes. To strengthen this presumption, we examined sections of liver, stained by the Feulgen procedure or with hematoxylin and eosin, from normal rats cross circulated with 85-percent hepatectomized partners for 20 hours, and killed 8 hours later to permit DNA synthesis to be translated into mitosis (*1*). Mitoses were limited almost entirely to hepatocytes and were increased in frequency (72 and 135 per 20,000 hepatocyte nuclei, compared with 5 and 18 per 20,000 hepatocyte nuclei in two normal single rats of the same age). Inflammatory cells, bacteria, or other abnormal acquisitors of labeled DNA were absent.

Although the observed changes in DNA labeling clearly suggest a humoral mechanism, they do not distinguish between accumulation of a stimulatory agent and loss of an inhibitor. In either case, one may suppose that, if cross circulation obliterated differences between normal and partially hepatectomized rats in the concentration of the agent, it would also obliterate differences in DNA labeling. Table 1 shows, however, that DNA labeling is always greater in the partially hepatectomized partner; this fact implies that the agent, whether stimulatory or inhibitory, is renewed so fast that the excess in one partner cannot be completely dissipated despite rapid removal by way of the cannulas.

The question of whether a slight reduction of DNA labeling occurs in partially hepatectomized partners of normal rats is unanswered by our data. Table 1 does suggest such a reduction if 68-percent hepatectomized single rats are selected as controls, whereas an increase is suggested if 68-percent hepatectomized pairs are selected; neither difference, however, attains statistical significance in the face of the highly variable DNA labeling in 68-percent hepatectomized rats. Furthermore, neither control may be entirely appropriate. The reduction in DNA labeling that occurs in 68-percent hepatectomized rats when they are cross-circulated with each other indicates a suppressive effect of the procedure, but this effect may not occur in partially hepatectomized-normal pairs, since 85-percent hepatectomized rats appear to benefit from cross circulation. Usually listless after operation, they regain their alertness when cross circulation begins. Moreover, labeling of their hepatic DNA is considerably greater than would be predicted from Weinbren and Woodward's demonstration in single rats that 82-percent hepatectomy greatly delays DNA synthesis, relative to 67-percent hepatectomy (*6*). The crucial variable may be the presence of a partner having an intact vascular bed capable of protecting against stresses to the partially hepatectomized partner.

Even when normal partners are present we have found that cross circulation may be markedly suppressive when certain experimental conditions are varied; these conditions include the age of the rat, the degree of restraint or sedation (we used no sedation), the rate of flow through the cannulas, and derangements of hydration. Neglect of such factors may account for the failure of Alston and Thomson, using a similar technique, to stimulate mitosis in intact partners of partially hepatectomized adult rats (*7*). The profound depression of hepatic synthesis of DNA, recently reported in partially hepatectomized rats exchange-transfused from normal rats (*8*), also may hinge on procedural details since we have repeatedly failed to confirm these results.

Our findings, added to the conflicting results (in our hands, uniformly negative) from single or repeated injections or transfusions, suggest that control of liver regeneration resides in a humoral mechanism almost instantaneously responsive to changes in effective mass of liver. Perhaps relevant to the rapidity of this response is the well-known preferential localization of DNA synthesis and mitosis in regenerating liver to the parts of the lobule that are first exposed to inflowing blood. Normally these are the portal areas (*1*), but DNA of centrally located hepatocytes has recently been shown to be labeled preferentially by tritiated thymidine when flow of blood was reversed by perfusion of dog-liver autografts through the hepatic vein (*9*). These patterns are consistent with physiologically significant changes in the concentration of a humoral agent during a single passage of blood through a hepatic sinusoid.

Table 1. Specific activity of hepatic DNA determined 1 hour after intravenous injection of 0.8 μc of thymidine-2-^{14}C into normal or partially hepatectomized single rats, or into separated members of cross-circulated pairs. Thymidine was injected 20 hours after hepatectomy (20 to 23 hours for 85-percent hepatectomized rats), or 20.5 hours after the start of cross circulation in normal rats. Single rats were denied food but were not restrained or cannulated. Data are expressed as mean specific activities ± S.E. of means; numbers of rats appear in parentheses. Normal partners of 68-percent hepatectomized rats and normal partners of 85-percent hepatectomized rats both differ significantly from normal rats cross-circulated with each other ($p < 0.01$, rank test); dpm, disintegrations per minute.

Rats	Mean specific activity, DNA (dpm/mg)	
	Normal partners	Partially hepatectomized partners
Normal single	489 ± 47 (3)	
Normal–normal pairs*	331 ± 27 (8)	
Normal–34 percent hepatectomized pairs	309 ± 44 (3)	991 ± 245 (3)
Normal–68 percent hepatectomized pairs	1056 ± 413 (5)	11860 ± 1720 (5)
Normal–85 percent hepatectomized pairs	2591 ± 568 (6)	4566 ± 1710 (6)
68 percent–68 percent hepatectomized pairs		6580 ± 841 (4)
Single, 68–percent hepatectomized		15005 ± 3783 (4)

* Including two sham-operated rats.

FREDERICK L. MOOLTEN
NANCY L. R. BUCHER
John Collins Warren Laboratories of Huntington Memorial Hospital of Harvard University, Massachusetts General Hospital, Boston

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Imine-Bonding in Membrane Transport of Monosaccharides: Invalidity of Kinetic Evidence

Abstract. *The proposition that carrier mediation of sugar transport may involve formation of imine complexes with specific cell membrane proteins has recently been advanced. However, the primary data presented accord more quantitatively with the presumption of a nonspecific reaction and furnish no evidence for the existence of the high-affinity glucose-binding sites which are essential to the proposed interpretation.*

Considerable study is being directed toward identification of the physicochemical reactions underlying the carrier-mediated transport of monosaccharides through cell membranes. An exciting and original lead in this connection has come from Langdon and Sloan's findings (*1*) that one or more proteins in the membranes of human erythrocytes or rat adipose cells can become associated with sugars by formation of (Schiff's base) imines, demonstrable by the production of stable secondary amines upon reduction with sodium borohydride. A possible role of this process in the transport events was suggested, with the corollary implication of one of the protein participants as the sugar carrier which has long been postulated from various kinetic characteristics of sugar permeation in these and other cells. The essential basis for this suggestion lies in the purported contrast between the kinetics of the imine formation in systems capable of such transport and that observed with nonspecific proteins. Thus, when mixtures of glucose-C^{14} at varying levels with indifferent proteins (such as serum albumin or hemoglobin) were incubated for a brief fixed period with an excess of the borohydride, and when the extent of the reaction was estimated by determination of the label incorporated into the subsequently isolated protein, the pattern of such incorporation as a function of the sugar concentration suggested, for such proteins, only an apparently homogeneous, low affinity for the sugar. But when similar experiments were conducted with either intact erythrocytes or their ghosts, the patterns observed led Langdon and Sloan to conclude that there was an additional, relatively small component of much higher apparent affinity (of the same order as estimated for the transport function in the same cells). However, I now point out that the figures offered by Langdon and Sloan (as representative of their data in support of this contention) do not corroborate the latter conclusion.

The points in each panel of Fig. 1 have been taken from Fig. 1 of Langdon and Sloan's report (*1*, p. 403). It shows the incorporation of uniformly labeled D-glucose-C^{14} into the membrane protein of intact human erythrocytes, during incubation at 37°C for varying periods up to 2 minutes, in the presence of 45.5 m*M* borohydride (*1*). In accord with their presumption that the glucose binds with two pools of sites—one (P_T) of low affinity (expressed in terms of a sugar-protein complex dissociation constant, K_p), and the second (C_T) consisting of specific (carrier?) sites of higher affinity (dissociation constant, K_C)—Langdon and Sloan applied to these data the equation:

$$PC^{14} = C_T\left\{1 - \exp\left(-\frac{k_r\,[BH]\,[G]t}{K_C + [G]}\right)\right\} + P_T\left\{1 - \exp\left(-\frac{k_r\,[BH]\,[G]t}{K_p + [G]}\right)\right\} \quad (1)$$

where PC^{14} is the amount of protein-bound labeled sugar, [G] and [BH] are the glucose and borohydride concentrations during the incubation for time t, and k_r is a rate constant for the reduction of the complex by the borohydride (which is taken to be rate-limiting, the imine-complex formation being essentially at equilibrium). By successive approximation in application of this equation to the points in Fig. 1, they calculated the following values as most suitable for the five constants: C_T and P_T, respectively, 5 and 95 nmole per milligram of protein; K_C and K_p, respectively, 20 and 1000 m*M*; and $k_r = 6.5 \cdot 10^{-5}$ liters per millimole-second. But the very poor fit of these values with the data is illustrated in Fig. 1a. Conceivably, this incongruity might have arisen from a trivial miscalculation for [BH] (in that this figure was given in the report only indirectly, by way of the recipe for the incubation mixture); and it is true that if the constant exponential factor, k_r[BH], is arbitrarily increased by about 16 percent so as to force the agreement shown in the original figure with the highest data point (for 100 m*M* glucose at 120 seconds), the reasonably satisfactory fit illustrated in Fig. 1b is achieved. In fact, this revised pattern does not differ radically from the picture originally given by Langdon and Sloan.

However, in spite of the fair accord with the observations that is thus possible with the suggested equation, there is no basis here for serious adoption of this particular combination of values for the five constants. For example, the systematic overshooting of the points at the lower glucose levels in the upper two curves of Fig. 1b suggests an underestimation of K_C; and if, accordingly, the specific-site dissociation constant is taken as 30 m*M* instead of 20 m*M* (and k_r is again adjusted slightly to provide coincidence with the highest point), the fit is substantially improved (Fig. 1c). But the satisfactoriness of this fit is not at all unique: with suitable compensatory juggling of the values assigned to almost any pair of the constants, various solutions of approximately equivalent congruity with the observations can be proposed. Thus, it is clear that with these five independent parameters, simple fitting of the data by successive approximation leaves a very large uncertainty in the assignment of an optimum combination of values; and no experimental effect on any one parameter can be genuinely distinguished unless the situations are decidedly divergent.

Far more disturbing to the suggested interpretation, however, is the fact that the distribution of the points in Fig. 1 can be still more adequately accounted for if the alleged high-affinity sites are altogether dismissed from consideration. Thus, if C_T is set at zero (as in Langdon and Sloan's treatment of