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Isolated Rat Liver Needs Calcium to Make Bile (39797)

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Heparin, the conventional anticoagulant for isolated rat liver perfusions, interferes with coagulation factor assays. It can be neutralized or removed by protamine, platelet factor 4, or barium sulfate, but all these techniques themselves create problems. Citrate as anticoagulant is preferred because assays can be done directly on the citrated plasma perfusate. However, earlier studies (1), with the isolated rat liver and generation of the vitamin-K-dependent factors, revealed that biliary flow diminished when the perfusing blood was anticoagulated with citrate, oxalate, or EDTA.

This is a report of the citrate effect.

Methods. Livers from normal, adult, male Sprague–Dawley rats were isolated and perfused by the Brauer *et al.* technique (2). This method has been found satisfactory in our laboratory for studying the metabolism of various amines, amino acids, and copper. Approximately 100 ml of blood were pooled from normal rats and heparinized with 10 to 15 units of heparin/ml (porcine intestinal mucosa, Fellows Medical Mfg. Co.). Twenty milliliters of 0.15 *M* NaCl were placed in the perfusion apparatus, and the blood was added to a total volume of about 120 ml. The plasma–saline volumes were about 80 ml, with hematocrit levels of about 35%.

The effect of citrate on liver perfusions was evaluated by adding 0.13 *M* sodium citrate in the conventional proportions of 1 vol of citrate (about 13 ml) to 9 vol of blood (about 120 ml). This volume of citrate is described as "full strength." "Half-strength," "one-fourth strength," and so on, are proportionately smaller volumes of citrate solution added to the heparinized blood.

For standard coagulation testing, a volume of 0.02 *M* $CaCl_2$ is added to an equal volume of citrated plasma. To reduce the volume of calcium solution, one-tenth the plasma volume of 0.1 to 0.2 *M* $CaCl_2$ was used instead in these liver perfusions. Comparable volumes and concentrations of $MgCl_2$ also were used.

Degradation of citrate was estimated by serial citrate assays (3) on the plasma throughout the perfusion.

In order to evaluate liver function, coagulation factor 7 was measured as a reference, and the conversion of L-[U-^{14}C]alanine to trichloroacetic acid (TCA)-precipitable ^{14}C was assessed. For these determinations, red cells were removed from plasma and separated from platelets by centrifugation. The red cells were frozen and, after thawing, were suspended in Tyrode's solution containing bovine albumin to a concentration of 6% (4). No heparin was added, but citrate in various volumes was added to assess its influence on generation of factor 7 and TCA-precipitable ^{14}C from [^{14}C]alanine.

Biliary flow was measured in calibrated, glass capillary tubes. One microliter of fluid occupied 3.9 mm of length in these tubes. The biliary volume was measured for 60-sec periods.

Temperatures were taken from a thermistor placed in the blood reservoir of the perfusion apparatus.

A total of 29 perfusions, including 6 on two livers, was performed.

Results. When a normal rat liver was perfused with heparinized normal rat blood, optimal blood flow rate was often not achieved for about an hour, and the biliary flow rate was essentially constant after the first 20 min (Fig. 1); but even at the start of perfusion, the blood flow rate exceeded the 1 ml/g of liver/min rule imposed by Brauer *et al.* (2).

Full-strength citrate promptly diminished the flow of bile, and the flow decreased to

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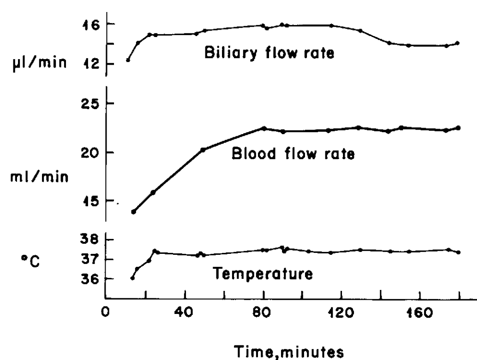

FIG. 1. Normal liver perfusion: 100 ml of heparinized, pooled, normal rat blood, plus 20 ml of saline, were perfused through a normal rat liver. Temperature was fixed at 37.2 to 37.6°.

one-tenth its normal rate (Fig. 2). An almost complete recovery followed the addition of calcium chloride. The addition of oxalate again reduced biliary flow, but this reduction was not reversed by the addition of calcium; blood flow decreased drastically.

When full-strength citrate was added in one-fourth increments, modest reductions of biliary flow rate accompanied the first two additions, but the third addition virtually abolished biliary flow (Fig. 3).

Similarly, when full-strength citrate was added, followed by the addition of calcium in one-fourth increments, the incremental effect of the calcium chloride on biliary flow rate was apparent (Fig. 4).

When the full-strength solutions of citrate and calcium were added simultaneously, a slight transient decrease in biliary flow rate occurred (Fig. 5). When the calcium was added 10 min after the citrate, the reduction in biliary flow rate was more pronounced but was reversible. However, when the time between the addition of citrate and calcium solutions was 60 min, only a partial recovery of the suppressed biliary flow rate occurred.

When a solution of magnesium chloride was added to the perfusate after the citrate effect had been established, biliary flow remained decreased (Fig. 6). The subsequent addition of calcium chloride overcame the citrate toxicity.

The temperature was carefully controlled in all experiments because of the exquisite sensitivity of the bile-generating mechanism to temperature. A reduction of the temperature of the perfusate by only 2 to 3° reduced the biliary flow rate by half.

The degradation of citrate was measured in four perfusions. Citrate was added to fi-

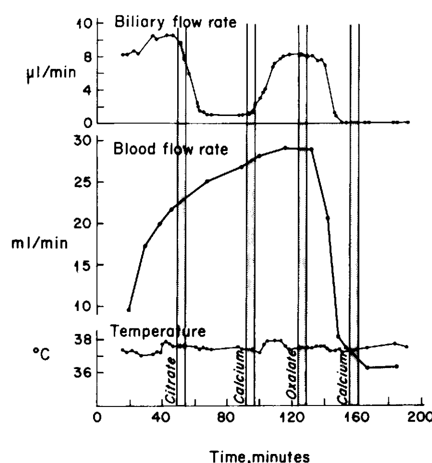

FIG. 2. Effect of citrate, oxalate, and calcium on biliary flow rate: 96 ml of heparinized, pooled, normal rat blood, plus 20 ml of saline, were perfused through a normal rat liver. At 49 min, 10 ml of 0.13 *M* citrate solution was added over a 5-min period. At 92 min, 10 ml of 0.1 *M* $CaCl_2$ was added over a 5-min period. At 124 min, 10 ml of 0.1 *M* sodium oxalate was added over a 5-min period. $CaCl_2$ again was added at 156 min.

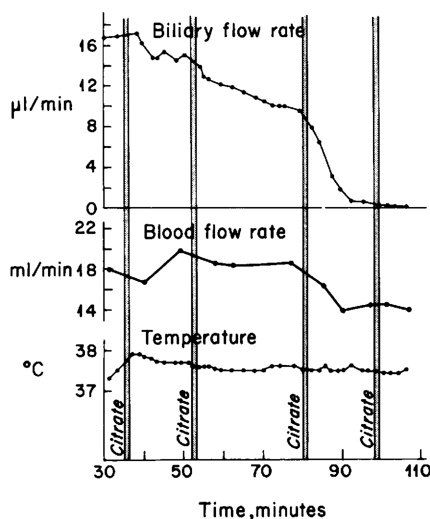

FIG. 3. Effect of multiple small additions of citrate on biliary flow rate: 96 ml of heparinized, pooled, normal rat blood, plus 20 ml of saline, were perfused through a normal rat liver. Since 13 ml of 0.13 *M* citrate represented "full-strength" citrate, one-fourth volumes (3.25 ml) were added at 35, 52, 80, and 98 min.

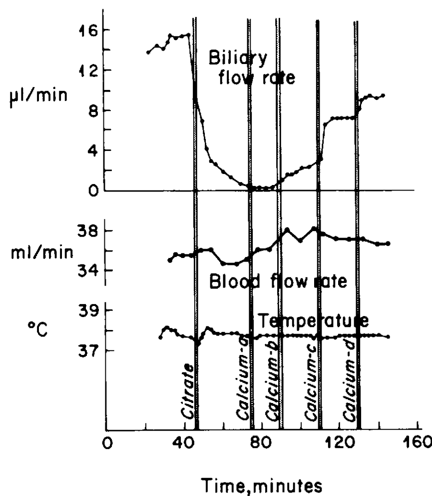

FIG. 4. Effect of multiple small additions of calcium chloride on the citrate inhibition of biliary flow: 80 ml of heparinized, pooled, normal rat blood, plus 20 ml of saline, were perfused through a normal rat liver. At 46 min, 10 ml of 0.13 *M* sodium citrate was added. At 74, 89, 108, and 129 min, 2.5 ml of 0.2 *M* $CaCl_2$ was added (total volume 10 ml of $CaCl_2$).

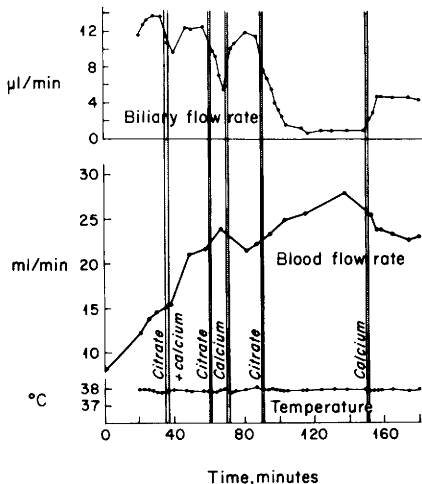

FIG. 5. Effect of variable interval between addition of citrate and calcium solutions: 80 ml of heparinized, pooled, normal rat blood, plus 20 ml of saline, were perfused through a normal rat liver. At 35 min, 10 ml of 0.13 *M* sodium citrate solution and 8 ml of 0.2 *M* $CaCl_2$ were added together. At 60 min, 10 ml of citrate was added, and at 70 min, 8 ml of calcium chloride was added. At 90 min, 10 ml of citrate was added, and at 150 min, 8 ml of calcium solution was added.

nal concentrations that varied from 65 to 350 mg/dl of plasma. The concentration decreased in all four experiments by 5 to 37% (mean 25%) of the initial value in 5-hr perfusions. It seems unlikely that anticoagulation is endangered by these modest reductions in the concentration of citrate.

When liver function was estimated by the addition of [^{14}C]alanine to the perfusate and the ^{14}C in a TCA precipitate of the perfusate was measured after 5 hr of perfusion, no effect of citrate could be detected. With no liver in the perfusion equipment, only a trace of TCA-precipitable ^{14}C emerged. When heparinized whole blood, without citrate, was perfused through liver, 4.8% of the initial ^{14}C was precipitable after 5 hr. When 2.5 to 12 ml of citrate solution were added to frozen–thawed red cells suspended in Tyrode's albumin solution (five experiments), the precipitability of the ^{14}C ranged from 3.4 to 5.1% after 5 hr of liver perfusion (mean 4.3%). These conversions occurred despite the expected reduction in biliary flow rate when the larger volumes of citrate were added.

Similarly, factor 7 was generated normally regardless of the amount of citrate added to red cells in Tyrode's albumin solution (120-ml vol) or of the reduction in biliary flow rate: When no citrate was added, the final factor 7 concentration after 5 hr of liver perfusion averaged 21% of normal rat plasma (range 16 to 30%; n =

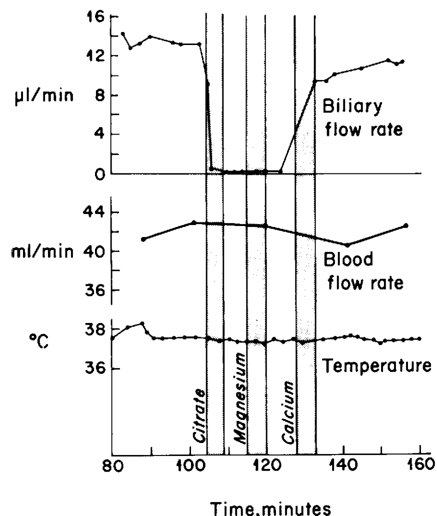

FIG. 6. Magnesium versus calcium to counteract citrate toxicity: 100 ml of heparinized, pooled, normal rat blood, plus 20 ml of saline, were perfused through a normal rat liver. Ten-milliliter volumes of the following were added: 0.13 *M* sodium citrate at 104 min, 0.1 *M* $MgCl_2$ at 115 min, and 0.1 *M* $CaCl_2$ at 128 min.

4); when 2.5 ml of citrate was added, the mean was 17% (range 11 to 21%; $n = 3$); when 5 ml of citrate was added, the mean was 14% (range 5 to 20%; $n = 5$); with 6 ml of citrate, the mean was 24% (range 21 to 30%; $n = 3$); with 9 ml of citrate, the mean was 15% (range 13 to 17%; $n = 3$); and with 12 ml of citrate, the mean was 20% (range 17 to 23%; $n = 2$).

Discussion. Calcium is normally present in bile and therefore might well be a necessary ingredient for the generation of bile, as was actually found in this study. Whether calcium deprivation interferes with the creation of bile was not explored. The hepatocyte seemed to be uninjured because the release of proteins ([^{14}C]alanine conversion to TCA-precipitable forms) and of one specific protein (coagulation factor 7) seemed uninhibited by the lack of calcium. The integrity of canalicular membranes could be examined by measurement of such membrane-associated enzymes as 5′-nucleotidase or Na^+K^+–ATPase. It might be noted that Berry and Friend (5) found that separation of rat liver parenchymal cells was facilitated by a calcium-free perfusion medium.

Summary. When normal rat livers were perfused with heparinized normal rat whole blood by the Brauer *et al.* technique, between 10 and 15 μl of bile were excreted per minute. This amount was drastically reduced by the addition of sodium citrate to the blood. When calcium chloride was subsequently added, bile flow returned to normal if the interval between the additions of citrate and calcium was not over an hour. Magnesium chloride had no such corrective effect. Two tests of liver function, generation of factor VII and conversion of [^{14}C]alanine to TCA-precipitable ^{14}C, were unaffected by the addition of citrate to plasma-free perfusions.

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