The Conversion of Orotic Acid into Uridine 5'-Monophosphate by Isolated Perfused Normal and Regenerating Rat Livers

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(Received 14 April 1972)

The release of $^{14}\text{CO}_2$ from $[7\text{-}^{14}\text{C}]$ orotic acid was measured in isolated perfused normal and regenerating rat livers. With some limitations, the release of $^{14}\text{CO}_2$ from $[7\text{-}^{14}\text{C}]$ orotic acid can be used to estimate UMP synthesis in perfused livers. Isolated perfused livers rapidly pick up labelled orotic acid added to perfusate and convert most of it into UMP. Perfused regenerating livers produce approx. 2.5 times as much UMP/g of liver as do perfused normal livers. However, the absolute amount of orotic acid converted into UMP is higher in perfused normal livers than in perfused regenerating livers. Perfused regenerating livers do not differ in their orotic acid uptake and UMP synthesis from livers of comparable size in which regeneration is not taking place. The total amount of orotic acid taken up by the liver (rather than the rate of uptake) and the size of the liver appear to be the determining factors in UMP production. The results suggest that the decrease in liver size caused by partial hepatectomy may be in itself sufficient to account for an increase in the flow of metabolites in the pyrimidine pathway at the early stages of liver regeneration.

Among the complex metabolic events that follow partial hepatectomy in the rat it is difficult to select those that are a direct consequence of the regenerative stimulus. It is generally assumed that the increase in blood flow in the liver remnant after partial hepatectomy is not essential for the development of liver hypertrophy and hyperplasia (Bucher & Malt, 1971), but insufficient attention has been given to the possibility that some metabolic phenomena occurring in liver regeneration may derive from an altered relationship between blood flow and liver size. In metabolic pathways in which enzyme activity is not limiting it can be argued that an increase in the amount of some substrates in the liver cells, brought about by an accelerated flow of blood per unit of liver tissue after partial hepatectomy, will cause an increase in the end product of that pathway. It is also possible that a high influx of amino acids to the liver may play a role in the elevation of ornithine decarboxylase activity occurring in the first hour after partial hepatectomy (Fausto, 1969b, 1971). The decrease in the liver size may be sufficient to account for an increased flow of metabolites in biochemical pathways in the liver remnant, making it unnecessary to assume that a specific humoral factor unrelated to liver function is needed to drive some of the initial steps of liver regeneration (Thomson, 1969).

Bucher & Swaffield (1966, 1969) demonstrated that in studies of the synthesis of pyrimidine nucleotides from orotic acid, precursor uptake by the liver and the size of the organ have to be taken into account to obtain meaningful estimates of nucleotide metab-

olism. Such studies, however, are difficult to perform in the intact rat because of the technical problems involved in measuring blood flow in the liver and obtaining repeated blood samples from the animal. Moreover, the complex distribution of precursors to other organs, urinary excretion, hormonal influences etc. complicate the interpretation of the results.

In this paper I present a study of some aspects of pyrimidine nucleotide formation in isolated perfused normal and regenerating rat liver. Using appropriately labelled precursors I obtained an estimate of UMP synthesis in perfused rat livers and attempted to determine if factors other than a specific regenerative stimulus could be responsible for an increase in UMP synthesis during liver regeneration.

Materials and Methods

Male albino rats weighing approx. 190-250 g (Holtzman Co., Madison, Wis., U.S.A.) were maintained in temperature-controlled rooms under 12h light-dark cycles. The animals were starved for approx. 14h before the experimental procedures. Partial hepatectomies were performed under ether anaesthesia by the method of Higgins & Anderson (1931). Whole blood for the perfusions was obtained under sterile conditions from the aorta of rats weighing between 400 and 600 g (Holtzman Co.). Blood was collected not longer than 2h before the perfusion and was mixed with 1000 units of heparin/100ml. $[6\text{-}^{14}\text{C}]$ Orotic acid (specific radioactivity 8.15 mCi/mmol) and $[7\text{-}^{14}\text{C}]$ orotic acid (specific radioactivity

3.67 mCi/mmol) were obtained from New England Nuclear Corp. (Boston, Mass., U.S.A.). Amino acids were obtained from Schwartz-Mann Co. (Orangeburg, N.Y., U.S.A.).

Perfusion technique

Two perfusion instruments were used in these experiments: the liver-perfusion apparatus developed by Miller *et al.* (1951, 1956) (Metaloglass Co. Inc., Boston, Mass., U.S.A.) and an Extracorporeal perfusion unit (Ambeck Industries, Boulder, Colo.,

U.S.A.). The techniques used were essentially those described by Miller *et al.* (1951, 1956). Normal or partially hepatectomized rats were anaesthetized with sodium pentobarbital (50 mg/kg). The small lobes of the liver were freed from connective tissue and the oesophagus was cut between two ligatures placed near the stomach. The common bile duct was cannulated with fine polyethylene tubing (Intramedic PE 10; Clay Adams, N.Y., U.S.A.) and the hepatic artery was ligated. The rat was injected with 500 units of heparin intravenously and the portal vein was cannulated with a plastic 15-gauge cannula containing a

Fig. 1. Acid-soluble radioactivity in the perfusate, liver and bile and ${}^{14}\text{CO}_2$ release after addition of $[6\text{-}^{14}\text{C}]$ orotic acid

At 2 h after the start of the perfusion, 5 mCi of $[6\text{-}^{14}\text{C}]$ orotic acid was injected in the cannula connected to the portal vein of isolated perfused normal livers. The amounts of labelled material remaining in the perfusate or excreted in the bile was determined by obtaining a sample of the blood and bile at the times indicated on the abscissa. Samples of blood (0.5 ml) and bile were precipitated with an equal volume of 5% (w/v) trichloroacetic acid, centrifuged and washed two more times with trichloroacetic acid. The supernatants of the centrifugations were combined and a sample was taken for the determination of radioactivity. The acid-soluble radioactivity in liver biopsies of approx. 200–300 mg was determined after trichloroacetic acid precipitation. The ${}^{14}\text{CO}_2$ was collected at the times indicated on the abscissa in a CO_2 -trap containing a mixture of ethylene glycol and ethanolamine. (a) Acid-soluble radioactivity, expressed in d.p.m./ml of perfusate; (b) acid-soluble radioactivity excreted in the bile, expressed in d.p.m. in 1 ml of bile/g of liver; (c) ${}^{14}\text{CO}_2$ release, expressed in d.p.m./g of liver; (d) acid-soluble radioactivity in the liver expressed as a percentage of the total radioactivity initially added to the perfusate. The abscissa indicates the times at which samples were obtained after the addition of labelled orotic acid. All results were corrected for counting efficiency. Averages and S.E.M. values obtained from four different experiments are presented.

tight-fitting 19-gauge needle (BN-1915; Sterilon Corp., Buffalo, N.Y., U.S.A.). The needle was removed and the cannula was tied to the portal vein through three ligatures. Immediately after the cannulation the portal vein was connected to a drip-bottle containing a small amount of the perfusing fluid to be used in the experiment. The thorax of the animal was open and a polyethylene cannula (Intramedic PE 260, Clay Adams, N.Y., U.S.A.) was inserted in the thoracic vena cava through the right atrium with the aid of a needle (Hems *et al.*, 1966). The vena cava was ligated in the abdomen above the right renal vein and the liver was freed from the animal and rapidly transferred to the perfusion unit with the drip-bottle still attached. The unit was maintained at 37°C and the circulating blood was oxygenated with O₂+CO₂ (95:5). The blood-flow rate was 2–2.5 ml/min per g of liver. Samples of liver were obtained without interfering with the perfusion by ligating and removing the small lobes of the organ. Bile was collected throughout the perfusions, (see legend of Fig. 1).

Perfusate

The perfusing fluid used in these experiments was whole rat blood, diluted to twice its volume with Krebs–Ringer bicarbonate solution (Krebs & Henseleit, 1932), containing a complete amino acid mixture (Jefferson & Korner, 1969), glucose (500mg/100ml of perfusate) and antibiotics (2000 units of penicillin and 2mg of streptomycin/100ml of perfusate). The total volume of the perfusate was 70ml.

Fig. 2. Release of ¹⁴CO₂ from [6-¹⁴C]orotic acid and [7-¹⁴C]orotic acid in isolated perfused normal livers

The labelled precursor (0.1mCi) was added 2h after the start of the perfusion. The ¹⁴CO₂ samples were collected every 10min after orotic acid addition, as indicated on the abscissa. Procedures are the same as in Fig. 1(c). The ordinate represents ¹⁴CO₂ released as a percentage of the total amount of radioactivity added to the perfusate. ●, ¹⁴CO₂ release from [7-¹⁴C]orotic acid; ▲, ¹⁴CO₂ release from [6-¹⁴C]orotic acid. Averages and s.e.m. values were calculated from four different experiments.

Table 1. Release of ¹⁴CO₂ from [7-¹⁴C]orotic acid in a perfusion circuit bypassing the liver

Normal livers were perfused with blood for 2h. At the end of this period a short segment of tubing was inserted in the perfusion circuit so as to bypass the liver and exclude it from the circulation. Then 5mCi of [7-¹⁴C]orotic acid was added to the blood. Samples of the recirculating blood and of CO₂ were collected every 10min for the first 1h after the addition of the labelled precursor. The values for radioactivity have been corrected for quenching with an internal standard. The results presented are the total amounts of radioactivity in the blood and CO₂ at each sampling time. Numbers in parentheses indicate the amount of ¹⁴CO₂ released as a percentage of the amount of [7-¹⁴C]orotic acid added to the blood.

Time after orotic acid addition (min)	Radioactivity in blood (d.p.m.)	Radioactivity in CO ₂ (d.p.m.)
0	9500000	
10	8745100	66511 (0.70%)
20	8740355	93240 (0.98%)
30	8710260	107643 (1.13%)
40	8707455	137640 (1.45%)
50	8651630	142064 (1.50%)
60		151848 (1.60%)
		Total (7.35%)

Biochemical methods

Orotidylate (OMP) pyrophosphorylase (EC 2.4.2.10) and OMP decarboxylase (EC 4.1.1.23) activities, measured in biopsies of the perfused liver and at the end of the perfusion, were determined by methods previously described, by using [7- 14 C]orotic acid as a substrate (Fausto, 1969a). Protein was determined by the method of Lowry *et al.* (1951), with bovine albumin as standard.

Collection of $^{14}\text{CO}_2$

Radioactive CO_2 was collected in a CO_2 trap (Metaloglass Inc.) connected to a side arm of the oxygenator of the Miller liver perfusion apparatus. The collected CO_2 was passed through a desiccant and bubbled into a solution (1:2, v/v) of ethanol-amine and ethylene glycol monomethyl ether (Jeffay & Alvarez, 1961; Fausto, 1969a,b). At 10 min intervals this solution was collected and fresh ethanol-amine-ethylene glycol mixture was introduced into the CO_2 -collecting flask. A sample (0.5 ml) of the $^{14}\text{CO}_2$ -containing solution was added to a radioactivity-counting vial, diluted with 4 ml of ethylene glycol monomethyl ether and mixed with 10 ml of Bray's (1960) scintillation fluid. The samples were counted for radioactivity in a Nuclear-Chicago liquid-scintillation counter and corrected for efficiency.

Determination of pyruvate, lactate and transaminase activity

Pyruvate and lactate concentrations in the perfusate were determined spectrophotometrically by the methods described by Bücher *et al.* (1965) and by Hohorst (1965). Glutamate-oxaloacetate transaminase (EC 2.6.1.1) activity was determined by the method described by Bergmeyer & Bernt (1965) with malate dehydrogenase (EC 1.1.1.37) as indicator enzyme.

Results

Evaluation of liver integrity during perfusion experiments

Histology. Histological examination of the liver at the end of 4 h perfusion experiments showed no significant areas of cell necrosis. The only morphological alteration detected by light microscopy was a widening of sinusoids and some oedema of the periportal areas.

Lactate/pyruvate ratio. Scholz & Bücher (1965) have shown that the lactate/pyruvate ratio of the perfusate is a sensitive indicator of the metabolic condition of the isolated perfused liver. Under certain conditions this ratio reflects the NADH/NAD $^+$ ratio in the cytoplasm of the perfused liver (Schimassek,

1962). In a series of five perfusion experiments the lactate/pyruvate ratios (physiological range 10–20) were 7.5 ± 1 at the beginning, 10.6 ± 0.8 after 4 h and 14.3 ± 1.2 after 7 h of perfusion.

Transaminase activity. The liver is a rich source of transaminase and damage to parenchymal cells leads to an increase in transaminase activity in the serum. Glutamate-oxaloacetate transaminase activity in the perfusate was practically unchanged throughout the

Fig. 3. OMP pyrophosphorylase and OMP decarboxylase activities in isolated perfused normal livers

Livers were perfused for 4 h. Biopsy samples were taken at 1 and 2 h after the start of the perfusion. The biopsy samples as well as a lobe of the liver obtained at the end of the perfusion period were homogenized in 0.25 M-sucrose and centrifuged for 60 min at 105 000g. The supernatant fractions were assayed for OMP pyrophosphorylase and OMP decarboxylase activities. The incubation mixture contained in a final volume of 1.4 ml: 0.2 ml of 0.1 M-potassium phosphate buffer, pH 7.2; 8 μ mol of MgCl_2 ; 8 μ mol of phosphoglyceric acid; 0.8 μ mol of ATP; 4.5 μ mol of ribose 5-phosphate; 0.45 μ mol of [7- 14 C]orotic acid and 0.2–0.6 ml of the supernatant fraction. The $^{14}\text{CO}_2$ was collected in a mixture of ethylene glycol-ethanol-amine (2:1, v/v). Enzyme activity is expressed in c.p.m. in the collected CO_2 . The amount of supernatant-fraction protein used for the assay is shown on the abscissa. ●, Enzyme activity in liver biopsy obtained 30 min after the start of perfusion; ▲, enzyme activity in liver biopsy obtained 2 h after the start; ■, enzyme activity in liver lobe obtained at the end of the perfusion experiment (4 h).

course of the perfusion. When the flow rate of the perfusate was lowered from 2 to 0.4 ml/min per g of liver, the lactate/pyruvate ratio increased to approx. 35, and transaminase activity in the perfusate was significantly elevated after the first hour of perfusion.

Metabolism of orotic acid in perfused livers

I determined the distribution of radioactivity in the blood, bile and CO_2 as well as the amount of acid-soluble radioactivity in the perfused liver after the addition of labelled orotic acid to the perfusate. In this manner it was possible to calculate the percentage of orotic acid uptake by the liver, the amount of degradation of labelled pyrimidines and the overall recovery of radioactive material. At 2 h after the start of the experiment, 5 mCi of $[6\text{-}^{14}\text{C}]\text{orotic acid}$ was added to the perfusate (see legend of Fig. 1). Blood, bile and CO_2 samples were taken every 10 min during the following hour, assayed for radioactivity and corrected for efficiency of counting. Liver biopsies were taken 10, 20 and 40 min after addition of the precursor. The biopsies as well as a sample of the liver obtained at the end of the perfusion were processed for the measurement of acid-soluble radioactivity.

Fig. 1 shows the distribution of acid-soluble radioactivity in the perfusate (Fig. 1a), liver (Fig. 1d), bile (Fig. 1b), and the production of $^{14}\text{CO}_2$ (Fig. 1c), at various times after the addition of $[6\text{-}^{14}\text{C}]\text{orotic acid}$ to the perfusate. The radioactivity in the perfusate decreases rapidly to approx. 30% of the initial value after 10 min (Fig. 1a). Approx. 10% of the labelled material is left in the perfusate 60 min after the addition of $[6\text{-}^{14}\text{C}]\text{orotic acid}$.

The amount of radioactivity recovered in the acid-soluble fraction of the liver (Fig. 1d) increases progressively after the addition of $[6\text{-}^{14}\text{C}]\text{orotic acid}$ to the perfusate. At 20 min after orotic acid administration 55% of the total amount of label was present in the acid-soluble fraction of liver homogenates and at 40 min, radioactivity in the liver acid-soluble fraction corresponded to 80–90% of the labelled material added to the perfusate. The amount of radioactivity found in the bile (Fig. 1b) reached a maximum 10 min after addition of orotic acid to the perfusate and decreased sharply in the next 20 min. Release of $^{14}\text{CO}_2$ (Fig. 1c), a product of the degradation of pyrimidines by the liver (see below), was detected 15 min after the administration of orotic acid and increased linearly for the next 25–30 min.

Fig. 4. Release of $^{14}\text{CO}_2$ from $[7\text{-}^{14}\text{C}]\text{orotic acid}$ in perfused normal and regenerating livers

At 2 h after the start of perfusion labelled orotic acid (0.1 mCi) was added to perfused normal and 3 h regenerating livers. The $^{14}\text{CO}_2$ was collected every 10 min as described in Fig. 1(c). Results are expressed in: (a) d.p.m. in CO_2 produced/min per g of liver (cumulative basis); and (b) total $^{14}\text{CO}_2$ (d.p.m.) produced by the whole liver for each 10 min period. ▲, $^{14}\text{CO}_2$ release by 3 h regenerating livers; ●, $^{14}\text{CO}_2$ release by normal livers. Averages and S.E.M. values were calculated from three different experiments.

Release of $^{14}\text{CO}_2$ from labelled orotic acid added to the perfusate

In the synthesis of UMP, CO_2 is released from the orotic acid molecule by the action of the enzymes OMP pyrophosphorylase and OMP decarboxylase. Since the carbon atom released in the reaction is the carboxyl carbon (C-7) of the orotic acid molecule it is possible to obtain a continuous estimate of UMP synthesis in perfused livers by measuring the amount of $^{14}\text{CO}_2$ released when $[7\text{-}^{14}\text{C}]$ orotic acid is added to the perfusate. Such estimates will be reliable only if the rate of $^{14}\text{CO}_2$ generation by the degradation of pyrimidines (reduction to dehydropyrimidines, hydrolysis of the ring and decarbamylation) is negligible (Rabkin *et al.*, 1962). Estimates of pyrimidine breakdown and UMP production can be obtained by using $[6\text{-}^{14}\text{C}]$ orotic acid and $[7\text{-}^{14}\text{C}]$ orotic acid as precursor. The $^{14}\text{CO}_2$ generated by $[6\text{-}^{14}\text{C}]$ orotic acid indicates breakdown of pyrimidines, including UMP, whereas the $^{14}\text{CO}_2$ released from the $7\text{-}^{14}\text{C}$ -labelled molecule indicates the synthesis of UMP (Rabkin *et al.*, 1962; Whittle & Potter, 1968; Fausto, 1969a; Jones, 1971).

Fig. 2 shows the amount of $^{14}\text{CO}_2$ produced when $[6\text{-}^{14}\text{C}]$ orotic acid or $[7\text{-}^{14}\text{C}]$ orotic acid is added to the perfusate. The total amount of $^{14}\text{CO}_2$ released after administration of the $6\text{-}^{14}\text{C}$ -labelled molecule corresponds to a maximum of 5% of the total labelled material added to the perfusate. The small amount of final degradation product detected is an indication that most of the orotic acid either is being further metabolized to UMP or is being incompletely degraded by the perfused livers. From the rates of $^{14}\text{CO}_2$ released from $[7\text{-}^{14}\text{C}]$ orotic acid shown in Fig. 2, it can be concluded that the first possibility is the correct one and that the perfused livers efficiently convert orotic acid into UMP. Approx. 70–90% of the total radioactivity added initially as $[7\text{-}^{14}\text{C}]$ orotic acid is released as $^{14}\text{CO}_2$ in the course of 1 h, indicating that most if not all of the orotic acid that enters the perfused liver is converted into UMP. It could be argued, however, that the erythrocytes contained in the perfusate might synthesize a significant proportion of UMP. To investigate this possibility the perfusate was incubated in the perfusion apparatus without entering the liver. Table 1 shows the amount of $^{14}\text{CO}_2$ released in this experiment at various times after the addition of $[7\text{-}^{14}\text{C}]$ orotic acid. The total amount of $^{14}\text{CO}_2$ collected corresponds to a maximum of 5% of the total radioactive material added to the perfusate.

OMP pyrophosphorylase and OMP decarboxylase activities in isolated perfused livers

The conversion of orotic acid into UMP is catalysed by these two enzymes. For studies of UMP synthesis in perfused livers it is essential to determine

if the activities of these enzymes remain stable during perfusions. I measured the activities of OMP pyrophosphorylase and OMP decarboxylase in liver biopsies obtained during the perfusion and in a sample of the liver at the end of the experiment. The enzyme activities were determined *in vitro* in liver supernatant fractions obtained after high-speed centrifugation. Fig. 3 shows that OMP pyrophosphorylase and OMP decarboxylase activities are stable throughout the perfusion period and that the different lobes of the liver from which biopsies were taken during the perfusion have almost identical enzyme activities. OMP pyrophosphorylase and OMP decarboxylase activities were not detected in the diluted blood used as perfusate.

UMP synthesis in isolated perfused normal and regenerating livers

Using the amount of $^{14}\text{CO}_2$ released from $[7\text{-}^{14}\text{C}]$ orotic acid added to the perfusate as an estimate of

Fig. 5. Release of $^{14}\text{CO}_2$ from $[7\text{-}^{14}\text{C}]$ orotic acid in perfused normal, regenerating and small non-regenerating livers

Labelled orotic acid (0.1 mCi) was added to perfused normal, 3h-regenerating livers and livers obtained from partially hepatectomized animals operated immediately before the start of the perfusion. The $^{14}\text{CO}_2$ was collected every 10 min as described in Fig. 1(c). Results are expressed as d.p.m. (cumulative) in $^{14}\text{CO}_2$ produced during the 60 min experimental period. ●, $^{14}\text{CO}_2$ produced by normal livers perfused with blood from intact donors; ▲, $^{14}\text{CO}_2$ produced by 2h-regenerating livers perfused with blood from intact donors; ■, $^{14}\text{CO}_2$ produced by small non-regenerating livers perfused with blood from intact donors; ◆, $^{14}\text{CO}_2$ produced by 3h-regenerating livers perfused with blood from partially hepatectomized rats. Averages were calculated from two different experiments.

Fig. 6. Effect of partial hepatectomy during liver perfusion on the release of $^{14}\text{CO}_2$ from $[7\text{-}^{14}\text{C}]\text{orotic acid}$

At 1 h after the start of the experiment 0.1 mCi of $[7\text{-}^{14}\text{C}]\text{orotic acid}$ was added to a perfused normal liver (9.3 g) and $^{14}\text{CO}_2$ release was measured for 1 h ($\Delta, \triangle$). When the radioactivity in the blood became negligible a partial hepatectomy was performed in the perfused liver, resulting in the removal of the median and left lateral lobes. Immediately after that a second dose of $[7\text{-}^{14}\text{C}]\text{orotic acid}$ (0.1 mCi) was added to the perfusate and the amount of $^{14}\text{CO}_2$ released was measured for the next 1 h ($\blacksquare, \square$). The abscissa shows the time in minutes after the addition of orotic acid. The ordinates show the total amount of $^{14}\text{CO}_2$ produced by the livers: (--- $\triangle$ ---), first dose of orotic acid; (--- $\square$ ---), second dose of orotic acid; or the amount of $^{14}\text{CO}_2$ released/g of liver (— $\blacktriangle$ —), first dose; (— $\blacksquare$ —) second dose of orotic acid.

UMP synthesis, I compared the rates of UMP synthesis in isolated perfused normal or regenerating livers. The regenerating livers were obtained from rats partially hepatectomized 3 h before the liver was placed in the perfusion apparatus. Fig. 4(a) shows the production of $^{14}\text{CO}_2$ by normal and regenerating livers in a period of 60 min after the addition of 0.1 μCi (0.027 μmol) of $[7\text{-}^{14}\text{C}]\text{orotic acid}$ to the perfusate. The regenerating livers produce approx. 2.5 times as much $^{14}\text{CO}_2/\text{g}$ of liver as do the normal livers. The increased UMP production/g of liver shown by the regenerating livers could be the result of a more efficient handling of the precursor by the regenerating livers. This is not so, however. Measurement of the absolute amount of $^{14}\text{CO}_2$ produced/10 min period by normal or regenerating perfused livers (Fig. 4b) shows that the normal livers synthesize a larger amount of UMP than do the regenerating livers. At the end of the 60 min period after addition of the labelled precursor the normal livers convert approx. 90% of the initial radioactivity into $^{14}\text{CO}_2$ whereas regenerating livers transform 70–80% of $[^{14}\text{C}]\text{orotic acid}$ into $^{14}\text{CO}_2$. The doses of orotic acid used in these experiments are certainly not a limiting

factor. Both normal and regenerating livers can handle doses at least 20 times greater without any changes in the shapes of the $^{14}\text{CO}_2$ -production curves.

Effect of liver size on UMP production by perfused livers

Before concluding that UMP production/g of liver is increased in isolated perfused regenerating livers as a consequence of the regenerative stimulus it is necessary to examine the effect of liver size on UMP synthesis in perfused normal and regenerating livers as well as the kinetics of orotic acid uptake. Since there is a large difference in weight between normal and regenerating livers used in any experiment, I used as a control a liver of small size in which regeneration was not occurring. These 'newly hepatectomized' livers were obtained by performing a partial hepatectomy immediately after the cannulation of the portal vein, and so less than 5 min before the transfer of the liver from the animal to the perfusion apparatus. Fig. 5 indicates that these non-regenerating livers of small size produce almost exactly the same amount of UMP as a 3 h-regenerating

Fig. 7. Uptake of [7- 14 C]orotic acid by normal, regenerating and small non-regenerating livers

At 2h after the start of the perfusion 0.1 μ Ci of [7- 14 C]orotic acid was added to perfused livers. Samples of the perfusate were collected every 10min and analysed for radioactivity as described in the legend of Fig. 1(a). Results are expressed as d.p.m. in the perfusate. ●, Acid-soluble radioactivity in the perfusate from normal liver; ▲, acid-soluble radioactivity in the perfusate from 2h regenerating liver; ■, acid-soluble radioactivity in the perfusate from small non-regenerating liver. Averages and S.E.M. values were calculated from three different experiments.

liver. Production of $^{14}\text{CO}_2$ by these small livers is higher than that of normal livers when expressed on a per-gram basis, but is considerably below the amounts released by the normal livers when the absolute amount of $^{14}\text{CO}_2$ generated is calculated. No changes in the rate of CO_2 production by normal or regenerating livers was observed when livers were perfused with blood obtained from partially hepatectomized rats at various times after the operation. A further demonstration that the rate of synthesis of UMP/g of liver is a function of the size of the organ and not of regeneration is presented in Fig. 6. In this experiment an initial dose of [7- 14 C]orotic acid was added to the perfusate and the amounts of $^{14}\text{CO}_2$ produced were recorded. When most of the radioactive precursor had been transformed into UMP a partial hepatectomy (identical with that usually performed *in vivo*) was performed without interrupting the perfusion. A second dose of [7- 14 C]orotic acid was added and the production of $^{14}\text{CO}_2$ measured for the next 1h. It is clear that the liver of small size produces more UMP/g of liver than does the large liver. Since it is most unlikely that re-

generation has been initiated under the conditions of the experiment, it appears that the rate of UMP production/g of liver increases because of a change in the size of the liver independently of regeneration.

UMP production and kinetics of orotic acid uptake

The increased UMP production/g of liver shown by small livers (regenerating or non-regenerating) could be a consequence of the kinetics of orotic acid uptake from the perfusate. In all experiments presented the blood flow/g of liver was constant at approx. 2.5ml of blood/min per g of liver. Under these conditions the rate of uptake of orotic acid from the perfusate is quite different for normal liver and livers obtained from partially hepatectomized rats, as indicated in Fig. 7. At similar blood-flow rates perfused normal livers pick up orotic acid from the blood much more rapidly than do regenerating livers. The same slow orotic acid uptake is shown by small non-regenerating livers. To determine if the rate of orotic acid uptake by the livers influences UMP synthesis, I measured the production of $^{14}\text{CO}_2$ from [7- 14 C]orotic acid by 3h-regenerating livers (2.47g) and 5h-regenerating livers (3.11g). In these experiments the blood-flow rate was adjusted to maintain identical orotic acid uptake rates, as shown in Fig. 8. In spite of the same uptake rate the production of UMP/g of liver was higher in the liver of smaller size. The same results were obtained when small non-regenerating livers were used.

Discussion

In this study I estimated the synthesis of UMP by perfused normal and regenerating livers by measuring the amounts of $^{14}\text{CO}_2$ released from carboxyl-labelled orotic acid added to the perfusate. In this manner it was possible to study some factors that may account for the increase in UMP synthesis from orotic acid in regenerating livers. The $^{14}\text{CO}_2$ method used in the present studies has been utilized for measurements of UMP synthesis in liver extracts, intact rats and human subjects (Rabkin *et al.*, 1962; von Euler *et al.*, 1963; Howell & Krooth, 1967; Whittle & Potter, 1968; Fausto, 1969a). This method is a reliable procedure for the estimation of UMP synthesis in liver extracts *in vitro* (Fausto, 1969a), since degradation of orotic acid through a reversal of the dihydro-orotate dehydrogenase step or the release of $^{14}\text{CO}_2$ from OMP without UMP formation have not been detected in the liver. The results presented here indicate that the release of $^{14}\text{CO}_2$ from [7- 14 C]orotic acid can be used as a valid estimate of UMP synthesis in perfused livers provided that the following limitations of the method are taken into consideration: (a) approx. 5% of the radioactivity released as $^{14}\text{CO}_2$ represents UMP formation by erythrocytes; (b)

Fig. 8. Effect of orotic acid uptake on ${}^{14}\text{CO}_2$ production by perfused regenerating livers

A 3h-regenerating liver (2.47g) or a 5h-regenerating liver (3.11g) was perfused for 1h. Then 0.1mCi of $[7-{}^{14}\text{C}]$ orotic acid was added to the perfusate and the amount of ${}^{14}\text{CO}_2$ released and blood radioactivity were measured for the next 1h. The blood flow was adjusted to provide similar uptake rates of orotic acid from the perfusate. The abscissa shows the time in minutes after the addition of orotic acid; the ordinates show the amount of acid-soluble radioactivity in the blood (a) or the amount of ${}^{14}\text{CO}_2$ released/g of liver (b) by a 3h- (Δ) or a 5h-regenerating liver (O).

about 10% of the total radioactive material added to the perfusate cannot be accounted for and is likely to represent labelled material lost in the experimental manipulations and ${}^{14}\text{CO}_2$ diffusion through the liver surface (Miller *et al.*, 1951); and (c) although precursor dilution by a non-radioactive pool of variable size does not occur because free orotic acid or OMP are not found in the liver, I cannot exclude the possibility that labelled orotic acid added to the perfusate is metabolized in a non-equilibrium situation with the orotic acid generated in the liver cells. In this situation ${}^{14}\text{CO}_2$ release from labelled orotic acid might not represent the overall UMP synthesis in the liver.

Approx. 80–90% of the orotic acid added to the perfusate is taken up by the liver in about 1h. Some 85–90% of the acid-soluble radioactivity found in the liver is actually converted into UMP by the perfused livers and some of the acid-soluble material is excreted in the bile. It appears that there are no restrictions in the conversion of orotic acid into UMP by the perfused livers with the dosages of precursor

used in most of these experiments ($0.027\mu\text{mol}$) or with dosages 20 times higher. Taking into consideration that the activities of OMP pyrophosphorylase and OMP decarboxylase are not rate-limiting for UMP synthesis at the onset of liver regeneration and that the activities of these and other enzymes involved in pyrimidine synthesis increase only many hours after the major changes in RNA metabolism in regenerating livers have occurred (Skold, 1960; Bresnick, 1965; Bresnick *et al.*, 1968; Fausto, 1969a), it appears that any elevation in UMP synthesis in regenerating liver is a consequence of an increased influx of substrates through the pyrimidine pathway.

Experiments with perfused livers indicate that regenerating livers, although showing a high rate of UMP synthesis/g of liver, are not more efficient than normal livers in converting orotic acid into UMP and do not in any way differ from non-regenerating livers of comparable size. If the capacity of the liver to convert orotic acid into UMP is to a large extent unrestricted it is plausible to suggest that the high rate of UMP synthesis/g of liver shown by the small

perfused livers (regenerating and non-regenerating) is a consequence of the formation of approximately the same amount of UMP by a smaller number of cells and is thus a function of liver size rather than the expression of a regenerative response. It is also apparent that the rate of uptake of orotic acid by the liver does not determine the total amount of UMP produced but may slightly change the shape of the $^{14}\text{CO}_2$ production curves, a slow uptake being associated with a small delay in maximum $^{14}\text{CO}_2$ -production rates.

Bucher & Swaffield (1969) have used 'newly hepatectomized' rats as controls in their studies of liver pyrimidine pools during liver regeneration. In both fed and starved animals the rate at which a unit of liver tissue converts labelled orotic acid into UMP is identical for 'newly hepatectomized' and 12h-regenerating livers. The experiments suggest that of the two mechanisms proposed to explain a higher rate of UTP labelling during regeneration *in vivo* (Bucher & Swaffield, 1966, 1969), namely slow uptake of orotic acid by regenerating livers or a high affinity of liver cells for orotic acid coupled with a constant rate of conversion of orotic acid into UMP, the second explanation is the more likely one.

I thank Dr. Robert Dresdale for his work in the initial stages of this study and Mrs. Paula Kelly and Miss Ann DiPippo for technical assistance. This work was supported by grants NP-52B from the American Cancer Society and R01 AM 14706-01 from the NIH.

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