Thymidine Metabolism in Regenerating Rat Liver One to Two Hours after Practial Hepatectomy

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1. When [³H]thymidine was injected intravenously into rats in amounts up to 40mg/kg body wt. and the ³H radioactivity in the livers measured at 30min, saturation kinetics for thymidine uptake were not found. If the animals were examined 3 min after intravenous injection, saturation could be attained in normal rats with 12mg of thymidine/kg and in partially hepatectomized rats with 4mg/kg. At concentrations of thymidine close to saturation, no differences were found in rate or amount of uptake/g of liver between normal and partially hepatectomized rats 1–2h after operation. 2. Perfusion techniques were used to compare thymidine uptakes in the two sets of rats at concentrations up to 40 μM-thymidine. Uptakes with tracer amounts of thymidine after 30min were identical *in vivo* and in the perfusion studies and were twice as great in livers from partially hepatectomized rats with concentrations up to 40 μM-thymidine. 3. At 1.5h after operation there was nearly twice as much β-aminoisobutyrate present per g of liver from partially hepatectomized as compared with normal rats.

Alterations in the pattern of thymidine metabolism are well established after partial hepatectomy but are not detectable before about 12h after operation. There is an immediate increase in the amount of intracellular ³H radioactivity of the acid-soluble fraction of livers after the administration of [³H]-thymidine to partially hepatectomized rats (Ord & Stocken, 1972, 1973), which may be important in evoking the changes in thymidine metabolism. The conditions under which the increased uptake of ³H radioactivity into partially hepatectomized livers occurs have now been examined kinetically. Nucleoside uptake is thought to be by facilitated diffusion (Plagemann & Roth, 1969) so that the requirements for saturation of the thymidine transporters in liver have been investigated. Thymidine is catabolized by liver to β-aminoisobutyrate; concentrations of this metabolite in normal and regenerating liver were determined.

Methods

Animals

Males (body wt. 200g) from this laboratory's strain of Wistar rats, maintained under controlled lighting conditions, were partially hepatectomized (Higgins & Anderson, 1931) under ether anaesthesia between 09.00 and 12.00h. No differences in thymidine uptake were observed between normal and sham-operated rats. Adrenalectomized rats were given 0.9% NaCl to drink and were used 3–8 days later. Through the courtesy of the late Professor G. W. Harris, hypophysectomies were kindly performed by Mr. C.

Graham (Department of Human Anatomy, Oxford, U.K.). The removal of glands was checked at death.

Uptake of ³H into liver and muscle

The animals were injected intravenously with 2.5 μCi of [¹⁴C]inulin plus 5.0 μCi of ³H-labelled precursor/100g body wt. Plasma was collected at death and deproteinized with 10% (w/v) trichloro-acetic acid, and the supernatant was used to establish the ³H/¹⁴C ratio and ³H radioactivity of the extra-cellular fluid. In later experiments the ³H radioactivity of 50 μl of plasma was determined without prior deproteinization. The small left-hand liver lobe was weighed and homogenized in 10% trichloroacetic acid. In the perfusion studies small portions of other lobes were also analysed. From the total radioactivity of the acid-soluble supernatant and the plasma results, the acid-soluble intracellular ³H radioactivity/g wet wt. of liver could be calculated. Similar procedures were used to determine [³H]thymidine uptake by psoas muscle.

Perfusion techniques

The procedure was based on that of Hems *et al.* (1966) and is similar to that of Hems *et al.* (1972). Rats were anaesthetized with ether and perfused with 1 vol. of washed and dialysed rat erythrocytes diluted with 3 vol. of dialysed synthetic plasma [3.9% (w/v) bovine albumin (fraction V; Sigma Chemical Co., St. Louis, Mo., U.S.A.) in a NaHCO₃-based saline

(Krebs & Henseleit, 1932)] containing 2.78 mM-glucose. In some experiments 1–3 vol. of dialysed rat plasma replaced some or all of the synthetic plasma. No differences in [³H]thymidine uptakes were observed in the presence or absence of dialysed rat plasma. Amino acids were not added; preliminary experiments established that by 30 min amino acid concentrations in the circulating fluid were almost restored to those normally present in plasma. Partially hepatectomized rats were prepared 1–2 h before perfusion.

Animals were used without prior starvation; glucose was therefore released from their livers immediately after the start of the perfusion. A preliminary period of 30 min was used, after which the circulating fluid was replaced and the nucleoside-uptake studies were commenced. In some experiments [³H]nucleoside was added to the fresh perfusion fluid and its radioactivity followed through the next 30 min, after which the ³H radioactivity of the livers was determined. In other short-term experiments, the perfusion fluid was not recirculated during the period when radioisotope uptake was being studied. In experiments where the effects of increasing concentrations of thymidine on uptake into the liver were to be investigated, the appropriate concentrations of thymidine were added to the 'plasma' and this and the cells were dialysed together overnight against bicarbonate medium (Krebs & Henseleit, 1932) to which glucose and thymidine had been added. Glucose concentration during the experimental periods were between 5.55 and 7.8 mM.

A hydrostatic pressure of 1–2 kPa (10–20 cm of H_2O) was used to obtain a flow rate of 2–2.5 ml/min per g of liver. The higher pressures were required for the short-term experiments with partially hepatectomized livers. No differences were observed in inulin space between intact and partially hepatectomized livers; any livers in which inulin spaces rose above 10 % (average value *in vivo* 8 %; Ord & Stocken, 1972) were discarded.

Amino acid determination

Amino acid concentrations were determined in supernatants from liver which had been deproteinized in 10 % trichloroacetic acid and the acid was subsequently removed with ether. Analyses were performed on a Locarte amino acid analyser, a single column being used for the acidic, neutral and basic amino acids. By extending the programme for the elution of amino acids around phenylalanine (see Tolstoshev & Wells, 1973) β-alanine and β-aminoisobutyrate could be separated and determined.

Deoxyribose determination

Concentrations in plasma were assayed microbiologically as described by Schneider (1962).

Identification of thymidine and its catabolites

Liver extracts obtained after injection of [³H]thymidine were analysed by t.l.c. The method of Shimizu *et al.* (1969) was used to separate the free bases, nucleosides and nucleotides; in this procedure the phosphorylated compounds remained at the origin. Recoveries of ³H radioactivity and non-radioactive markers were better than 80 %. When ³H₂O in liver plasma was to be determined, ³H radioactivity in deproteinized extracts was measured before and after evaporation of ³H₂O *in vacuo*.

Radioisotopes

All radioisotopes were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The specific radioactivities were: [*carboxy*-¹⁴C]inulin, 11.4 mCi/mmol (mol.wt. 5175 ± 95); [6-³H]thymidine, 23.3 Ci/mmol.

Measurement of radioactivity

³H and ¹⁴C radioactivity was measured by scintillation counting in a Beckman CPM 2000 liquid-scintillation counter. The scintillant contained 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (5 g) and Triton X-100 (300 ml) in 1 litre of toluene. Sufficient counts were recorded to give an accuracy of ±3 %; the efficiency of counting was about 50 % for ³H and about 95 % for ¹⁴C.

Results

Thymidine uptake into liver in vivo: saturation conditions

Our earlier studies (Ord & Stocken, 1972, 1973) on thymidine uptake by rat livers after partial hepatectomy used mainly tracer amounts of [³H]thymidine. To characterize the thymidine transporters it was necessary to investigate the behaviour of the liver when amounts of thymidine were given that saturated the transport mechanism. Experiments were performed with intact rats given intravenous injections of [³H]thymidine of constant specific radioactivity but with increasing amounts of thymidine/g body wt. With animals killed 30 min after injection and with doses of thymidine up to 40 mg/kg the amount of ³H radioactivity in the livers was linearly related to the amount of thymidine injected. No signs of saturation were detected. When the plasma concentrations were examined it was noted that with doses of thymidine exceeding 10–12 mg/kg the ³H radioactivity in the plasma was less than expected. Uptake of thymidine into tissues other than liver is limited (Ord & Stocken, 1973), and as that into kidney was low it was assumed that doses above 10 mg/kg produced con-

Table 1. *Content of thymidine and/or thymidine metabolites in liver and plasma from normal and partially hepatectomized rats 3 or 6 min after intravenous injection of various amounts of thymidine*

'Thymidine' contents were calculated from the specific radioactivities of the injected material and are expressed as μg/ml of plasma or μg/g of liver. No corrections were made for the distribution of ^3H radioactivity in plasma or for the complete conversion of all ^3H radioactivity from thymidine in the liver into β-aminoisobutyric acid and ^3H_2O. The amount of injected thymidine is given as mg/kg body wt. When the standard errors are given, four to six rats were used per group; otherwise the results are from two animals, and the differences between them were not more than $\pm 5\%$.

		'Thymidine' contents							
		Normal rats				Partially hepatectomized rats			
		3		6		3		6	
Dose	Time (min) ...	Plasma	Liver	Plasma	Liver	Plasma	Liver	Plasma	Liver
14		32.2	19.1	25.6	31.5	—	—	—	—
12		28.9 ± 6.6	19.2 ± 3.2	22.5	29.9	—	—	—	—
10		25.4	17.7	20.0	28.5	—	—	—	—
8		—	—	14.7	19.3	24.8	19.8	—	—
6		—	—	—	—	19.5	20.0	15.2	30.3
4		5.0	9.0	—	—	11.5 ± 3.3	14.2 ± 4.1	9.9	31.4
1		1.3	3.2	—	—	1.8	5.1	—	—
0.5		1.0 ± 0.4	1.9 ± 0.3	—	—	1.7 ± 0.4	2.7 ± 0.7	—	—

centrations of thymidine in the plasma which exceeded the renal threshold, with consequent loss of thymidine into the urine.

The experiments were then repeated with the animals killed at much shorter times. With rats killed either 3 or 6 min after intravenous injection the ^3H radioactivity in the plasma was related to the amount of thymidine administered with doses up to 14 mg/kg in the intact rats (Table 1). The radioactivity in the liver was then identical with 12 or 14 mg of thymidine administered/kg, indicating that under these conditions saturation could be achieved. The same amount of radioactivity/g of liver could be obtained in partially hepatectomized rats given 4–6 mg of thymidine/kg. Saturation with 12 and 4 mg/kg was found if the rats were killed at 6 min after injection.

At saturation the uptakes of radioactivity/g of liver between 3 and 6 min for both control and partially hepatectomized rats were equal, supporting many earlier reports showing no changes in utilization of thymidine 1 h after partial hepatectomy. Analysis of the ^3H radioactivity in the liver extracts 5 min after giving 24 and 12 mg of thymidine/kg to intact and partially hepatectomized rats showed no detectable [^3H]thymidine, suggesting that the capacity of the liver to metabolize thymidine was not limiting the transport mechanism, and that with doses up to 12 mg/kg all thymidine entering the liver was oxidized.

Experiments were next performed comparing ^3H radioactivity in the livers of normal and partially hepatectomized rats 3 min after intravenous injection

of non-saturating amounts of thymidine (Table 1). If the plasma ^3H radioactivity at 3 min was plotted directly against the amount of thymidine injected a linear relationship was found but the ^3H radioactivity in the plasma was 1.3 times higher in the partially hepatectomized rats than in normal animals. When the reciprocals of the liver contents were plotted against the reciprocals of the plasma concentrations of thymidine found at 3 min after injection all the results fell on the same straight line. This contrasts markedly with results with tracer amounts of thymidine (Ord & Stocken, 1972, and see below).

Calculations of the amount of thymidine taken up by the livers showed that 3 min after intravenous injection approximately 9% of the injected radioactivity was present in the livers of intact rats, whereas in partially hepatectomized rats the livers took up 5–6% of the radioactivity. In further experiments it was found that by 30 min livers from intact animals contained 20% of the radioactivity, with slightly lower recoveries in livers from partially hepatectomized rats. The smaller overall uptake by livers 3 min after partial hepatectomy indicates that other tissues must be taking up relatively more label. Part of this was due to increased amounts of ^3H radioactivity in muscles of partially hepatectomized rats (Table 2), which make up about 25% of body weight. T.l.c. analyses showed that thymidine accounted for 70% of the ^3H radioactivity in the muscles 3 min after injection; by 30 min the total radioactivity in muscle had decreased and about 30% of the radioactivity was now present as ^3H_2O. The results suggested that

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Table 2. [^3H]Thymidine distribution and metabolites in plasma, liver and psoas muscle from sham-operated and partially hepatectomized rats 3 and 30 min after intravenous injection of 0.5 mg of thymidine/kg body wt.

The values are the means obtained with two to five rats per group; variation between the values was not greater than ±5%. The amounts of thymidine were calculated from the specific radioactivity of thymidine administered and are expressed as μg/ml of plasma or per g tissue wet wt.

Time (min) ...	3		30	
	Sham-operated rats	Partially hepatectomized rats	Sham-operated rats	Partially hepatectomized rats
Plasma				
Amount	0.6	1.5	0.3	0.5
% present as: ^3H_2O	5	0	43	29
Thymidine	42	49	26	31
β-Aminoisobutyrate	7	0	0	7
'Origin'	30	28	3	13
Liver				
Amount	2.0	2.6	3.0	3.3
% present as: ^3H_2O	7	2	8	7
Thymidine	1	2	1	1
β-Aminoisobutyrate	74	92	71	76
'Origin'	2	3	2	0
Muscle				
Amount	0.2	0.3	0.15	0.2
% present as: ^3H_2O	11	4	39	23
Thymidine	66	75	—	36
β-Aminoisobutyrate	0	0	0	8
'Origin'	11	7	0	2

thymidine diffused into muscle in amounts proportional to the concentration in the plasma; by 30 min, when at least 20% of the injected thymidine had been catabolized by the liver, about half the thymidine present at 3 min had been released from muscle.

When the distribution of ^3H radioactivity in plasma was examined (Table 2) with 0.5 mg of thymidine administered/kg 30% of the ^3H was located on the origins of the t.l.c. plates. The proportion diminished to about 10% at 30 min, indicating that this ^3H radioactivity could be taken up into tissues. Its identity was not determined. Over 99% of the label migrated as [^3H]thymidine, but if rat blood was incubated with [^3H]thymidine (6 mg/ml of blood) for 30 min at 37°C, 50% of the ^3H radioactivity in the deproteinized plasma was retained at the origin on the plates. If this plasma was dialysed overnight against 5 vol. of 0.9% (w/v) NaCl at 4°C the dialysate then contained the non-migrating ^3H radioactivity.

Thymidine uptake in vivo: tracer amounts of thymidine

Schneider (1955) has shown that virtually all the deoxyribose content of rat plasma is deoxycytidine. The concentration (40 μM) is unchanged 1 h after partial hepatectomy (Ord & Stocken, 1972, where the material was incorrectly described as thymidine). Since with concentrations of circulating thymidine substantially exceeding the amount normally present in plasma no differences in uptake between control and partially hepatectomized rats occurred, it was decided to extend earlier studies with tracer amounts of radioisotope (Ord & Stocken, 1973). The disappearance of ^3H radioactivity from the plasma at 10–30 min after intravenous injection was linear with time for both groups of rats, the rate of removal being slightly faster in the partially hepatectomized animals (Table 3). As observed above (Table 1) the ^3H radioactivity in the plasma of the partially hepatectomized rats was slightly higher than in the controls. Uptake into the livers was also linear with time, the increase per 10 min in the partially hepatectomized animals being approximately three times as great per g of liver as into the intact livers. If the ^3H radioactivity in the liver was expressed relative to that in plasma, the ratio at 30 min was identical with that found in the series of experiments (Ord & Stocken, 1973) when the radioisotope was given intramuscularly.

The results confirm that if only tracer amounts of [^3H]thymidine are given partially hepatectomized

Table 3. [^3H]Thymidine uptake into livers of normal and partially hepatectomized rats 1 h after operation; studies in vivo with intravenous injections of tracer amounts of [^3H]thymidine

Injections of 5 μCi of [^{3}H]thymidine and 2.5 μCi of [^{14}C]inulin were given intravenously (0.1 ml per 100 g body wt.) and the radioactivity in liver and plasma was determined. The mean ^{3}H radioactivity in plasma is expressed as c.p.m./μl of plasma. That of the intracellular acid-soluble fraction of the liver is given as c.p.m./mg of liver, with the number of experiments given in parentheses. The ratio of the values is calculated $\pm$ s.e.m.

	^{3}H Radioactivity					
	Normal rats			Partially hepatectomized rats		
Time after injection (min)	Plasma	Liver	Ratio of intracellular radioactivity in liver to that in plasma	Plasma	Liver	Ratio of intracellular radioactivity in liver to that in plasma
10	89.5	124	1.4 ± 0.1 (4)	112	197	1.8 ± 0.2 (3)
20	68.0	143	2.1 (1)	77	273	3.1 ± 4.0 (2)
30	41	174	4.2 ± 0.5 (7)	46	381	8.3 ± 1.1 (5)

Table 4. Perfusion studies with normal rat livers and with livers 1–2 h after partial hepatectomy

The livers were perfused with recirculating blood for a preliminary period of 15–30 min. After this time they were perfused for 3 min with fresh blood containing 5 μCi of [^{3}H]thymidine and 2.5 μCi of [^{14}C]inulin/100 ml, with added unlabelled thymidine as indicated, and with no recirculation. The effluent blood was collected for 20 s periods through 3 min. Alternatively, to obtain the uptakes for 30 min, after the preliminary period the perfusion fluid was replaced with 60 ml of fresh blood to which 5 μCi of [^{3}H]thymidine and 2.5 μCi of [^{14}C]inulin were added, and this was recirculated for 30 min. The uptake of radioactivity is expressed as c.p.m./mg of liver for experiments without added thymidine and as μg/mg of liver with added thymidine. Uptake from 80 to 180 s was calculated from the 20 s collection periods and is expressed as c.p.m./mg of liver. All values are corrected to a mean plasma ^{3}H radioactivity of 100 c.p.m./μl of plasma. Unless otherwise stated the values were obtained from four to six rats per group and are given as means $\pm$ s.e.m.

	^{3}H uptake/mg of liver					
Amount of added thymidine (μg/ml)	Intact			Partially hepatectomized		
	80–180 s	3 min	30 min	80–180 s	3 min	30 min
0	43 ± 9	63 ± 17	161 ± 12	69 ± 14	99 ± 12	443 ± 75
2	0.7, 0.9	0.7, 0.8	—	1.4, 2.2	1.2, 1.3	—
5	2.2, 2.2	2.3, 2.7	—	3.1	2.8, 3.0	—
10	4.2 ± 0.5	4.7 ± 0.7	—	11.2	7.2	—

rats have a greater uptake of ^{3}H radioactivity into their livers than do control animals, the effect continuing through a 30 min pulse.

Although these data suggest an immediate action on thymidine transporters in the livers of partially hepatectomized rats they are difficult to interpret because of uncertainties about the plasma values. Release of [^{3}H]thymidine from muscle into plasma has been demonstrated above, and also the presence of ^{3}H$_2$O presumably derived from the catabolism of thymidine (see Baugnet-Mahieu & Goutier, 1972). It is also difficult to compare the behaviour of the livers when the ^{3}H radioactivity in the plasma of partially hepatectomized rats always exceeds that in the control animals after an identical dose of [^{3}H]-thymidine, since exposure to higher amounts of circulating [^{3}H]thymidine inevitably leads to higher uptake by the livers.

It was therefore decided to use perfusion techniques so that circulating concentrations of ^{3}H radioactivity and the flow of blood/g of liver could be more precisely controlled.

Thymidine uptake in vitro

Two types of experiment were performed (Table 4). In the first, after a preliminary perfusion period of 30 min, [^{3}H]thymidine and [^{14}C]inulin were added to

fresh perfusing fluid ($5 \mu Ci$ of ^3H/60ml of fluid), which was recirculated for a further 30min, superficially similar to the conditions *in vivo*. Radioactivity disappearing from the plasma equalled that taken up into the livers, about 20% of the administered radio-isotope being used. The ^3H radioactivity/g of liver was indistinguishable from amounts found after 30min *in vivo*.

Alternatively, after preliminary perfusion, the tracers were added to fresh fluid, which was available in excess and not recirculated. Effluent from the livers was collected for 3min through 20s periods. Amino acids were not added and it was not expected that in this time the concentrations would become physiological. In this way the uptake of the liver during the 3min pulse was measured and the removal of ^3H radioactivity from the perfusate followed. Contents reached in the livers after 3min pulses were proportionally higher than with the first procedure (Table 4), which suggested that both here and in the studies *in vivo* initial rates of thymidine uptake might be significantly higher.

In the first 20s collection of the perfusate, blood from the initial perfusion period was being washed out, but in the next interval from 20 to 40s considerable uptake occurred. From 80 to 180s disappearance of ^3H radioactivity from the plasma/20s was approximately constant, and when calculated per mg of liver corresponded fairly well with that determined directly in the liver at the end of the 3min perfusion period (Table 4). Between 80 and 180s 20% of the radioactivity of the plasma was removed into intact livers. Under these conditions, when fresh blood of constant specific radioactivity was available for the whole experimental period, uptake into the livers of partially hepatectomized rats was again about twice as great as in the controls, although the blood-flow rate/g of liver was in the same range for the two groups.

When thymidine was added to the perfusing medium uptake by the intact livers increased linearly with the amount of thymidine added from 2 to $10 \mu g$/ml, and again was doubled in the livers from partially hepatectomized rats compared with controls in this range of thymidine concentrations.

Accumulation of β-aminoisobutyrate in partially hepatectomized rats

Since at the normal plasma concentration of thymidine the rate of uptake into the livers of partially hepatectomized rats was doubled, the catabolites of thymidine might be expected to accumulate in livers from the operated animals. Earlier studies (Ord & Stocken, 1972) on amino acid accumulation in livers 1.5h after partial hepatectomy had shown an apparent and rather anomalous increase in phenylalanine

concentration, when in contrast with the other amino acids there was a fivefold increase in the ratio of intracellular acid-soluble phenylalanine in the livers to that in plasma. This was unaccompanied by any change in [^3H]phenylalanine entry into the liver (Ord & Stocken, 1973). When the programme of the amino acid analyser was changed to decrease the rate of elution of amino acids around phenylalanine, β-aminoisobutyrate and β-alanine were separated from phenylalanine and could be determined. With this procedure no changes were now found in the concentration of phenylalanine in the liver, and that of β-aminoisobutyrate in normal livers was found in a group of five rats to be $18.1 \pm 2.0 \mu mol$/100g wet wt. of liver. In five partially hepatectomized rat livers 1.5h after operation the content had risen to $31.5 \pm 7.5 \mu mol$/100g. Contents of β-alanine, which could be partially derived from uridine catabolism, were much smaller and were unaffected by partial hepatectomy.

Discussion

The experiments in which the thymidine concentrations in plasma are varied *in vitro* show that at concentrations up to $40 \mu M$ partial hepatectomy produced an immediate doubling of thymidine transport into the liver. At higher concentrations, close to those that saturate the transporters, no differences between partially hepatectomized and normal rats were observed, so that the effect of the partial hepatectomy on [^3H]thymidine uptake is comparable with a decrease in the K_m of a thymidine-transporter complex.

The post-absorptive concentration of circulating thymidine is very low, but β-aminoisobutyrate is a normal constituent in the intracellular fraction of liver, suggesting that appreciable catabolism of thymidine occurs in intact rats. The indication is therefore that the potential capacity to take up thymidine is not being fully utilized in normal rats, thus conserving deoxyribonucleoside so that its uptake by dividing tissues is favoured. At 1.5h after partial hepatectomy the increase in β-aminoisobutyrate concentration in the residual liver lobes suggests that the greater uptake of [^3H]thymidine in these rats is of physiological importance. The rapidity with which the effect of partial hepatectomy is demonstrable (Ord & Stocken, 1973) raises the possibility that it may be associated with ionic changes. The importance of Ca^{2+} (see Rasmussen *et al.*, 1972) or other ions in the response of liver to partial hepatectomy is still to be determined.

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