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SOME OBSERVATIONS ON LIVER CELL PROLIFERATION IN THE ISOLATED PERFUSED RAT LIVER

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Summary.—Experiments were designed to test the hypothesis that in an isolated perfused system a regenerating liver is able to stimulate a normal liver to increase DNA replication after cross-circulation for one hour. The high degree of purity of DNA extracted by the " phenol " technique is demonstrated and the suitability of this technique for rat livers emphasized. The effect of chemiluminescence on tritiated thymidine is presented.

The results of experiments presented here, together with other evidence, indicate the improbability of a stimulus being able to induce increased DNA synthesis in a normal liver after an exposure of only one hour.

IN THE RAT, and in several other animal species, partial hepatectomy results in rapid compensatory hepatocyte proliferation in the remaining fragment. The mechanism by which this liver cell proliferation is initiated and controlled is not fully understood, although two distinct sources of the stimulus have been invoked, *viz.* a specific humoral agent, the concentration of which changes following partial hepatectomy, and the haemodynamic or metabolic load imposed on the remnant as a result of resection.

Involvement of a humoral factor is suggested by an increasing body of evidence: Portions of liver (5–10%) implanted in regions remote from portal blood proliferate in response to partial hepatectomy in the parent organ (Sigel, Acevedo and Dunn, 1963; Sigel, Dunn and Butterfield, 1963; Leong *et al.*, 1964; Virolainen, 1964). The distribution of newly formed hepatocytes (in response to partial hepatectomy) is dependent on the direction of intra-hepatic blood flow (Sigel *et al.*, 1967, 1968). There is an increase in DNA synthesis in the liver of the normal partner of a pair of parabiotic (Bucher, Scott and Aub, 1951; Christensen and Jacobson, 1950; Wenneker and Sussman, 1951), or cross-circulated rats (Moolten and Bucher, 1967; Bucher, Schrock and Moolten, 1969; Sakai, 1970; Fisher *et al.*, 1971) when a partial hepatectomy has been performed on the other partner. An increased rate of DNA synthesis in normal rat livers occurs after injection with serum or plasma from partially hepatectomized rats (Paschkis, 1958; Smythe and Moore, 1958; Adibi, Paschkis and Cantarow, 1959; Zimmerman and Cellozzi, 1960). Further, an accelerated growth of rat liver cells takes place in culture after the addition of serum from partially hepatectomized animals (Wrba *et al.*, 1962; Grisham, Kaufman and Alexander, 1967; Hays, Tedo and Matsushima, 1969), and finally the observation by Levi and Zeppa (1971) that there is an increase in DNA synthesis (using isolated perfused rat livers) in the normal liver when the other has undergone partial hepatectomy 22 hours previously, after cross-circulation for one hour only.

The last named effect was particularly striking in that proliferation could be demonstrated after exposure to the stimulus for only one hour, whereas all previous evidence indicated that in order to induce liver cell proliferation in a normal liver, the time of exposure to the stimulus had to be of the order of 20 hours (Moolten and Bucher, 1967; Sakai, 1970). Thus, not only would a study of liver cell proliferation in the isolated perfused liver enable the effect of haemodynamics or metabolic variables to be studied, but the brief time of exposure suggested the development of an assay system for an initiating agent or agents. It was with this latter objective in mind that we attempted to substantiate the work of Levi and Zeppa (1971). In this report, we describe experiments designed to test the hypothesis that in an isolated perfused system a regenerating rat liver is able to stimulate a cross-circulated normal liver to increase DNA replication in one hour.

MATERIALS AND METHODS

Male Wistar rats (200–250 g) were housed under controlled conditions of temperature, humidity and lighting and permitted water and a balanced diet *ad libitum*. Partial hepatectomies were performed under diethyl ether anaesthesia, with the removal of the median and left lateral lobes as described by Higgins and Anderson (1931).

Thymidine.—(Methyl ^{3}H) thymidine was provided by the Radiochemical Centre, Amersham, England with a specific activity of 23·7 Ci/mmol.

Perfusate.—The perfusate consisted of: (1) 56 ml whole blood from rats the same weight and kept under the same conditions as those perfused; (2) 4 ml heparin (1000 u/ml—heparin injection *B.P.* (Evans Medical Ltd, Speke, Liverpool, England); (3) 23 ml Plasmalyte B (Baxter–Saphar Laboratories Ltd, Johannesburg, South Africa) containing g/100 ml: sodium chloride 0·6, potassium chloride 0·03, magnesium chloride 0·03 and sodium bicarbonate 0·23; (4) 2 ml of 4·2% sodium bicarbonate.

The final haematocrit was 25%.

Perfusions.—A perfusion cabinet designed to house isolated liver perfusions at constant temperature (37°) and humidity was built in the workshop of the Department of Medicine, University of Cape Town.

The livers were perfused as described by Hems *et al.* (1966). All perfusions were carried out between 10.00 a.m. and noon to avoid variations due to circadian rhythms.

The following indices were used as criteria of hepatic viability: (1) bile production and perfusate flow rate through the liver; (2) the absence of visible infarcts; (3) the absence of liver swelling; (4) stability of portal pressure; (5) glucose and urea production.

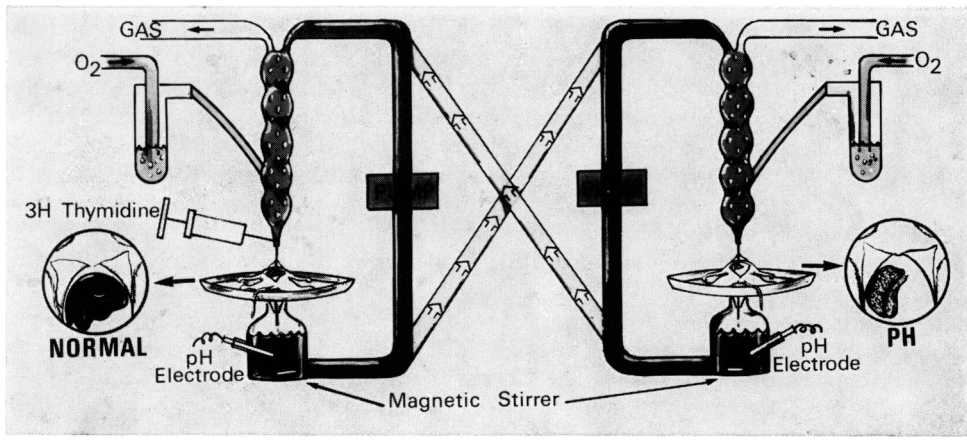

FIG. 1.—Circuit used for cross-circulation of isolated (*in situ*) liver perfusions. (Perfusion A).

The perfusate was maintained at physiological pH by the addition of aliquots of 4·2% sodium bicarbonate, and internal Astrups and pO_2 measurements carried out to ensure adequate oxygenation.

Four different perfusion systems were used: A. (*Cross-circulation*).—Isolated normal livers were perfused (*in situ*) with the effluent from either a liver subjected to partial hepatectomy 22 hours previously, or a normal liver (Fig. 1). After cross-circulation had been established, an interval of 20 min was allowed to ensure thorough mixing following which 380 μCi (methyl ^{3}H) thymidine was infused over a 60 min period. At the start of the infusion a bolus of 0·3 ml (methyl ^{3}H) thymidine (1 mCi/ml) was injected into the reservoir below the partially hepatectomized liver. At the end of the 60 min perfusion period the livers were immediately flushed with cold normal saline and homogenized.

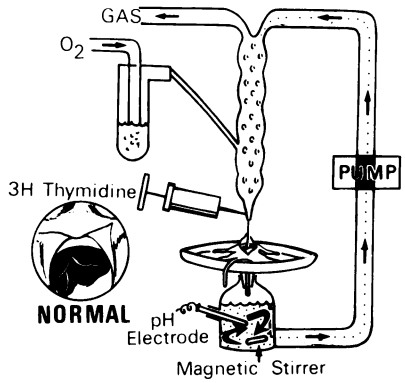

FIG. 2.—Circuit designed to reduce the perfusate volume by using a common lung for both livers. (Perfusion B).

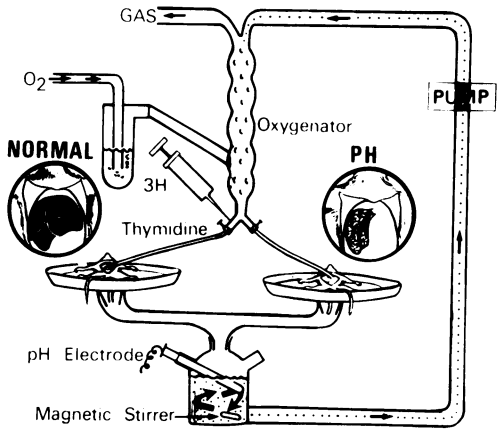

FIG. 3.—Diagrammatic representation of apparatus used in perfusions C and D.

B. *Common reservoir.*—Either an isolated liver of a rat which had been subjected to partial hepatectomy 22 hours previously or a normal liver was perfused (*in situ*) on the right-hand limb and simultaneously a normal liver was perfused (*in situ*) on the left-hand limb (Fig. 2). Twenty min after the perfusions were established (methyl ^{3}H) thymidine was introduced into the system as described in A and after the 60 min period the livers were treated as in A.

C. *Twenty-two hour partially hepatectomized Donors.*—Six male rats (200–250 g) were subjected to partial hepatectomy; 22 hours later the blood drawn from these animals was

used to make up the perfusate to perfuse a normal isolated liver (*in situ*). Controls were normal livers perfused with blood from normal donors. (Methyl ^{3}H) thymidine was introduced and the liver processed as in A (Fig. 3).

D. *Three livers in sequence using common perfusate.*—The liver of a rat which had been subjected to partial hepatectomy 22 hours earlier was perfused in the isolated state (*in situ*), as in Fig. 3 for one hour. At the end of the hour, the liver was removed from the system and discarded, being replaced on the same system with another 22-hour partially hepatectomized liver, which was thus perfused with the same perfusate that had been perfusing its predecessor. At the end of a second hour this liver, too, was discarded, being replaced by a normal liver. Controls were 3 normal livers perfused in sequence.

Thymidine was introduced into the system for 60 min, as described in A and the liver treated as in A.

DNA extraction

In the preparation of DNA, a fixed method cannot be applied indiscriminately to all tissues and species (Frick, 1954; Jones and Marsh, 1954). Kirby (1959) showed that the p-amino-salicylate phenol method was ideally suited to rat liver.

It has been shown by Morley and Kingdon (1972) that when (methyl ^{3}H) thymidine is used as label, some of the counts which are *not* associated with DNA may appear in the " DNA fraction ". They therefore recommend the adoption of the " phenol " method for DNA extraction in experiments that employ tritiated thymidine for measurements of DNA synthesis, especially with hepatocytes. Hence our selection of the " phenol " technique for DNA extraction and the employment of other methods (described in 2 and 3 below) to scrutinize our DNA more closely, ensuring the absence of stray counts.

Method 1.—A modification of the method of Kirby (1959) was used as follows: Liver tissue (3 g) was homogenized rapidly in 6 mmol Tris ph 7·5 (10 ml) in a glass homogenizer at 0°. An equal volume of sodium p-amino salicylate 12% (w/v) was added and the mixture homogenized further. Samples (6·0 ml) of this solution were transferred to a centrifuge tube, an equal volume of phenol 90% (w/w) added and shaken vigorously. The solutions were centrifuged for 20 min at 15,000 rev/min in a Sorval centrifuge at 4°. The upper aqueous layer was removed and the DNA precipitated by addition of an equal volume of 2-ethoxyethanol with whirli-mixing. The DNA strands were washed 3 times with 2-ethoxyethanol and hydrolyzed by incubation (15 min, 70°) in 10% perchloric acid (PCA). It was observed that unless the DNA strands were washed 3 times, they remained contaminated with sodium p-amino salicylate, which interfered with the diphenylamine reaction described below.

Method 2.—The same extraction procedure was carried out as in 1, with the exception that the DNA strands were suspended in 2·0 ml of a solution of 5 mol urea and 2 mol NaCl pH 8·5 for 30 min at 0°. The DNA was then washed 3 times with 2-ethoxyethanol and dissolved as in Method 1.

Method 3.—Four 1·0 ml samples of DNA hydrolysate were taken from a perfusion, prepared as in Method 1 above. Three were incubated with chymotrypsin, bovine albumin and 0·06 mol/l Tris pH 7, 6 thus: (a) 1·0 ml DNA + 1·0 ml chymotrypsin (500 μg/ml); (b) 1·0 ml DNA + 1·0 ml bovine serum albumin (500 μg/ml); (c) 1·0 ml DNA + 1·0 ml 0·06 mol/l Tris pH 7·6, and the fourth (d) was not treated at all. (a), (b) and (c) were incubated for one hour at 37° with agitation and the whole samples were then dialysed for 2 days against 200 volumes 0·06 mol/l Tris pH 7·6.

The DNA specific activity of all 4 samples was determined as described below.

Method 4.—This method was a modification of that used by Marmur (1961) as cited by Levi and Zeppa (1971). Approximately 3 g liver were homogenized in 9·0 ml of saline sodium citrate (0·15 mol/l NaCl; 0·015 mol/l sodium citrate; 4% sodium dodecyl sulphate) and incubated at 60° for 20 min. An equal volume of chloroform isoamylalcohol (24 : 1) was added and the mixture shaken at room temperature for 30 min. The resulting emulsion was centrifuged at 2000 rev/min for 30 min at 4° and the aqueous layer removed. The nucleic acids were extracted into an appropriate volume of 0·5 N PCA by incubation at 80° for 40 min.

Estimation of specific activity

Radioactivity of the samples was measured in a Beckman liquid scintillation system. DNA samples were placed in counting vials containing 10 ml of scintillation fluid (PPO, 5 g/l; POPOP 0·1 g/l; BBS-3 10%). Correction for quenching was carried out by internal

standardization using n-hexadecane-1,2-^{3}H (The Radiochemical Centre, Amersham, England).

DNA concentration was measured by the diphenylamine technique of Burton (1956) as modified by Giles and Myers (1965) using Bovine DNA supplied by Sigma Chemical Co., St Louis, Mo., U.S.A.) as standard.

Results were expressed as specific activity = d/min/μg DNA. We found that chemiluminescence affected the tritiated thymidine and that our samples had to be kept in the dark for 10 hours before counting in order to reduce the background counts.

RESULTS

As seen in Table I, with perfusion system A there was no significant difference in DNA synthesis after cross-circulation between 2 normal livers or between a normal liver and a partially hepatectomized one. Since the perfusate volume in this model (Fig. 1) is 6 times the total blood volume of the size rat we were using, dilution was an obvious factor to be considered.

The apparatus was re-designed (Fig. 2) in such a way that the perfusate volume was halved, thus reducing any dilution factor by 50%. This, too, failed to demonstrate any increased DNA synthesis in the normal liver after cross-circulation with a partially hepatectomized one (Table I).

TABLE I.—*Specific activity (d/min/μg DNA)*

	Control	Experimental	P*
Cross-circulation (Perfusion A)	716·1±267·9 (4)	910·4±273·4 (4)	N.S. (>0·5)
Common reservoir perfusions (Perfusion B)	932·2±474·1 (4)	739·1±143·3 (4)	N.S. (>0·7)
Perfusate from 22 hour P.H.† donors (Perfusion C)	529·9±110·7 (5)	340·6± 63·3 (4)	N.S. (>0·1)
Three livers in sequence using common perfusate (Perfusion D)	311·3±123·9 (3)	189 ± 59·1 (3)	N.S. (>0·4)

* Student's t test.
† Partial hepatectomy.

In a further modification, designed to completely eliminate dilution, the perfusion was carried out as in " C " (Fig. 3). Again no increased DNA synthesis in the normal liver took place (Table I).

Method " D " was another variation designed to increase the concentration of any circulating agent that might be present. There was no increase in DNA synthesis in the normal liver (Table I).

As mentioned above, there is a very real possibility that counts not associated with DNA may be collected in the " DNA fraction " and contribute to erroneous results. Since there was a possibility that any increase in specific activity (reported by others) might be due to incorporation of (methyl ^{3}H) thymidine into nucleoproteins but being counted as DNA, we scrutinized our DNA for purity as described in Methods 2 and 3.

The suspension of the DNA preparation in 5 mol/l urea (Method 2) reduced contamination with nucleoproteins and the incubation of the DNA preparation with chymotrypsin (Method 3) ensured the removal of more strongly bound proteins.

TABLE II.—*Suspension of DNA in 5 mol/l Urea: 2 mol/l NaCl*

DNA samples	Specific activity d/min/μg DNA: Method 1 (phenol)	Specific activity d/min/μg DNA: Method 2 (phenol and urea)	Wilcoxon test for two samples
1	346	241	$T_1 = 107$
2	818	705	
3	543	409	$T_2 = 101$
4	546	428	
5	72	79	$N_1 = N_2 = 10$
6	93	90	
7	26	38	$\therefore P < 0{\cdot}01$
8	27	45	
9	874	1291	
10	383	343	

TABLE III.—*Incubation of DNA in Chymotrypsin*

DNA sample	Treatment	Specific activity d/min/μg DNA	Diff+	P*
a	Incubation with chymotrypsin	224·1	57·4	
b	Incubation with BSA	262·3	19·2	
c	Incubation with Tris	222·0	59·5	N.S. $> 0{\cdot}05$
d	None	281·5		

+ a, b and c individually compared with d.
* Student's *t* test.

As seen in Tables II and III, there is a high degree of correlation between the specific activities obtained by these methods and our "phenol" method, thus indicating a high degree of purity in our DNA preparation and that the presence of nucleoprotein was not affecting our results.

Method 4, after Marmur (1961), that used by Levi and Zeppa (1971), was used to compare their DNA extraction technique with ours and, as Table IV shows, there is a high degree of correlation between the two.

TABLE IV.—*Comparison of DNA Extraction Techniques*

DNA samples	Specific activity d/min/μg DNA: Method 1 (phenol)	Specific activity d/min/μg DNA: Method 2 (Marmur)	Wilcoxon test for two samples
1	543	371	$T_1 = 44$
2	466	569	$T_2 = 34$
3	626	464	$N_1 = N_2 = 6$
4	72	65	
5	26	18	$\therefore P < 0{\cdot}01$
6	27	23	

DISCUSSION

There is no evidence from our experiments that there is a substance in the cross-circulated perfusate from 22 hour regenerating livers which causes an increase in DNA synthesis in normal rat livers in one hour.

The first factor we considered when attempting to account for the discrepancy between our findings and those of Levi and Zeppa was the DNA extraction technique but, as demonstrated here, this was excluded as a possible source of error.

The other factor that might be masking the action of the hypothetical humoral agent was dilution. The variety of perfusions described were designed to eliminate this, but in spite of these, as Table I shows, there was no increase in DNA synthesis in the normal livers.

Mayfield and Bonner (1972) have pointed out that the first observable response to partial hepatectomy is the production in the liver nuclei of rapidly-labelled high-molecular weight RNA of sequence not produced by normal liver. This is followed, *with a lag of about one hour*, by the appearance of increased (above normal) amounts of chromosomal RNA, again of sequences not produced by normal liver. *With a lag of another hour*, the template activity in support of RNA synthesis of the liver chromatin increases substantially. These events occur *before* initiation of DNA synthesis in the cells of the regenerating liver.

In vivo cross-circulating experiments indicated that in order to induce increased DNA synthesis in a normal liver, it must be cross-circulated with a partially hepatectomized rat for approximately 20 hours (Moolten and Bucher, 1967; Sakai, 1970). Work with tissue culture appears to confirm this (Paschkis, 1958).

In a recent abstract, Compagno and Grisham (1973) using similar techniques to those used by Levi and Zeppa (1971), report findings similar to ours and point out that to enter the S phase within one hour, non-cycling hepatocytes of intact livers must compress the 10–14 hour G_1 period to one hour. They have not found any situation in which non-cycling mammalian cells can be made to enter the S phase in less than 10–12 hours.

In the face of this evidence, together with our findings, we consider it improbable that increased DNA synthesis in a normal liver can be induced after exposure to the stimulus or stimuli for only one hour.

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