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Ultrastructural and hormonal metabolic studies of rat liver maintained *in vitro* by perfusion at 30° C and 37° C: a time course study by TEM, SEM and RIA

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Summary. Isolated rat liver perfusion system has been extensively used for metabolic and functional studies. Results derived from the application of this system may reflect true biochemical changes but they may also be associated with some structural changes. This study was undertaken to correlate the cytological changes and functional integrity of isolated rat liver perfused in vitro at normal physiological temperature (37°C) and 30°C, using a non-recirculating system. The livers were perfused for 3 hours with modified Ham's F10 culture medium supplemented with thyroxine hormone (T4). The hepatocyte structural integrity was studied by light microscopy, transmission and scanning electron microscopy. The triiodothyronine (T3) and T4 hormones in the perfusion medium and the effluent fractions were assessed by radioimmunoassay. The livers perfused at 30°C remained morphologically intact at the ultrastructural level for 3 hours whilst at 37°C, hepatocytes in the centrilobular zone exhibited marked structural alterations. The percentage of T4 uptake was significantly higher ($P < 0.01$) in livers perfused at 30°C (50.8 ± 7.7% vs 38 ± 7.7%, 37°C), but the net T3 output (3.16 ± 1.04 μg) and the conversion of T4 to T3 (4 ± 0.62%) were significantly higher ($P < 0.001$) in livers perfused at 37°C in comparison to livers perfused at 30°C (1.61 ± 0.84 μg and 1.68 ± 0.76%, respectively). In conclusion, at 30°C the hepatic T4 uptake is not inhibited, but the rate of T4 to T3 conversion has decreased, additionally the livers remain morphologically well preserved throughout the experimental period. At 37°C, although T4 to T3 conversion is higher, structurally the livers could not be maintained intact for more than 2 hours. Therefore, isolated rat livers perfused in vitro at 30°C offer the best compromise for further morphological and metabolic studies.

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Introduction

Isolated rat liver perfused in vitro has been extensively used to study the hepatic metabolism of glucose (Buschiazzo et al., 1970), insulin (Misbin et al., 1976; Weiland et al., 1979), vasoactive hormones (Hems et al., 1976), radiotracer studies (Scheffel et al., 1986) and thyroid hormones (Hillier, 1972; Bartels and Sestoft, 1980; Hassan and Ramsden, 1981). Extra-thyroidal conversion of thyroxin thyrosine (T4) to triiodothyronine (T3) by the liver accounts for most of the daily production of T3 in rat and man (Schimmel and Utiger, 1977; Chopra et al., 1978). In spite of the extensive studies using this system, no simultaneous structural and functional assessment has been carried out. The recent morphological study (Al-Ali et al., 1987) showed that extensive cell death occurs at 3 and 4 hours in the isolated rat liver perfused with Krebs-Ringer solution. The present study was designed in an attempt to maintain the viability of the hepatocytes and to correlate the fine structural morphology and thyroxine hormone metabolism of the hepatocytes using isolated rat liver perfused with Ham's F10 culture medium in a non-recirculating system for 3 hours at 30° C and 37° C.

Materials and methods

Animals

Male, white Wistar rats, weighing 230-308 g, were used in the present study. They were maintained at a constant temperature (22° C) in an airconditioned quarter and fed standard laboratory chow ad libitum. A total of thirty rats were used. Fourteen and sixteen livers were perfused at 30°C and 37°C respectively for time-course morphological study and hormone metabolism.

Perfusion Medium

The perfusion medium (PM) was freshly prepared during the morning of the liver perfusion experiment. It consisted of: (1) Ham's F10 culture medium, purchased as a powder (Flow Laboratories) and stored at 4°C. 19.82 g (9.91 g/l) of the powder was dissolved in 1600 ml distilled water and 6 g (3 g/l total volume) sodium bicarbonate was added; the final volume was made up to 2 litres. (2) 10% Dextran solution of average molecular weight 40,000 in 0.9% sodium chloride; the solutions were stored unopened at room temperature. The PM was prepared by adding equal volumes of Ham's F10 culture medium and Dextran solution. The PM was supplemented with (2 g/l total volume) bovine serum albumin (Cohn fraction V, Sigma) and D (+) glucose (Dextrose) powder (10 g/l total volume). The osmolarity of the PM was 390 mOsm measured by an Advanced Digimatic Osmometer 3D11. The pH and the PO2 readings of the PM after oxygenation were 7.4 and 300 mm Hg respectively, and were kept constant throughout the experimental periods. The pH and PO2 were measured by an Automatic Gas Check AVL 940. Thyroxine (Sigma) used in the PM was prepared after dissolving the solid in a minimum volume of freshly prepared ammonium hydroxide (5 M). Stock solution (77.7 µg/ml) was prepared in PM and stored in aliquots at −20°C.

Liver Perfusion and Tissue Processing

The rats were anaesthetized with an intra-peritoneal injection of 1 ml 3.5% chloral hydrate per 100 g body weight. The technique was modified from Miller (1973) and described in detail (Al-Ali et al., 1987).

The livers were perfused using a non-recirculating system, first with T4-free PM for 10 min, followed by PM containing T4 (77.7 µg/l). The flow rate was kept constant within the normal physiological range at 12-15 ml/min (ignoring the effluent medium in the first 10 min) and the effluent samples stored at −20° C until the time of hormone assay. During the perfusion, the medium and the chamber were kept either at 37° C or 30° C and the medium was oxygenated with a mixture of 95% oxygen and 5% carbon dioxide. At the end of each perfusion period (10 min, 1, 2 and 3 hour[s]), the livers were fixed by perfusion fixation with 1.5% glutaraldehyde for 2 min, this was followed by immersion fixation overnight in the same fixative. For glycogen histochemistry the periodic acid-Schiff (PAS) reaction was used in conjuction with diastase control technique. 1-2 µm Araldite sections were stained with toluidine blue for survey light microscopy. For transmission electron microscopy, ultrathin sections were cut, stained with uranyl acetate and lead citrate and examined under a Jeol 1200 EX transmission electron microscope. For scanning electron microscopy the liver slices were dried by the critical point method, coated with gold and examined under a Jeol GSM 840 scanning electron microscope.

Radioimmunoassay (RIA) and Calculations

The T4 and T3 were measured in the «starting» medium and at (0) time, i.e. after the 10 min washing period as well as in each effluent sample collected every 15 min. The T4 and T3 were assayed by RIA kit (Amersham). Standards and samples were assayed in duplicate and all tubes were counted until 10,000 counts has accumulated in the maximum binding tubes. Since flow rate remains constant, it is assumed that the amount of T4 entering the liver is directly proportional to the number of effluent fractions and to the length of time of perfusion. Total input of T4 at any given time was calculated by multiplying the T4 concentration in the «starting» medium by the volume of fluid which passes through the liver at that time. The amount of T4 not taken by the liver at any given time was calculated from the measured hormone concentration in the effluent medium by the fraction volume and the time at which the fraction was collected. The net T4 taken up by the liver was therefore the difference between input and output. Calculations were performed by VAX Computer.

Cumulative T3 production was calculated in the same fashion as T4. The starting medium contained a small amount of T3 introduced as a contaminant in the thyroxine solution. Net T3 ouput was calculated as the difference between T3 input and output. The above assumption and the description of the model have been previously reported (Hassan and Ramsden, 1981; Hassan, 1982). The T4 to T3 conversion is expressed as: net T3 output (µg)/net T4 uptake (µg) × 100.

All data are expressed as mean ±standard deviation. Comparison among groups was performed by unpaired t-test.

Results

Survey light microscopy showed that the hepatic plates, basophilia and glycogen contents of livers perfused at 30° C were comparable to the normal non-perfused livers throughout the experiment periods (Fig. 1a). At the ultrastructural level the hepatocytes of these livers exhibited well preserved cell organelles such as mitochondria, granular endoplasmic reticulum, ribosomes, glycogen granules plasma membrane and microvilli, in both centrilobular (around central veins) and periportal (around portal veins) zones (Figs. 1b, c).

Livers perfused at 37°C showed a reduction in the basophilia of the hepatocytes in the centrilobular zones after 2 hours (Fig. 2a). After 3 hours of perfusion at 37° C, the hepatocytes in the centrilobular zone showed loss of glycogen granules (Fig. 2b), loss of ribosomes, disruption of the granular endoplasmic reticulum, alteration in the shape of mitochondria (signet-ring or dumbell appearance), and clumping of the nuclear chromatin (Fig. 2c). The cells were round in shape and the surface exhibited bleb formation and depletion of normal microvilli (Fig. 2d), whilst the periportal zone remained intact throughout the experiment (Figs. 2a, b).

The effluent fraction pH was always slightly lower than that of the starting medium (pH 7.34 ± 0.04 and 7.4

EXP. NO.	T4 CONC. IN PM (µg/L)	ACTUAL T4 INPUT (µg)	NET T4 UPTAKE (µg)	% T4 UPTAKE	NET T3 OUTPUT (µg)	T3/T4 (%)	FLOW RATE (ML/MIN)	RAT WEIGHT (GMS)
3 hours perfusion at 37° C								
1	76.1	205.6	64.5	31.4	2.1	3.26	15	248
2	57.9	156.3	61.0	39.0	2.3	3.77	15	290
3	80.0	216.1	68.1	31.5	3.6	5.29	15	300
4	80.8	174.5	67.1	38.4	3.0	4.47	12	265
5	62.1	145.5	66.1	45.5	2.5	3.78	13	290
6	93.2	251.7	95.4	37.9	3.7	3.88	15	270
7	97.1	262.2	74.7	28.5	2.8	3.75	15	230
8	99.5	268.5	138.6	51.6	5.3	3.8	15	303
Mean ± SD	80.8 ± 15.4	210 ± 48	79.4 ± 26	38 ± 7.7	3.16 ± 1.04	4 ± 0.62	14.4 ± 1.19	274.5 ± 26
3 hours perfusion at 30° C								
1	82.4	222.2	109.6	49.3	3.22	2.94	15	300
2	67.0	176.2	70.2	39.9	1.5	2.14	14	265
3	62.2	167.8	76.8	45.8	1.09	1.42	15	254
4	82.4	192.7	101.0	52.4	1.69	1.67	13	308
5	84.7	328.7	125.8	55.1	1.24	0.99	15	270
6	58.3	157.3	97.7	62.1	0.91	0.93	15	280
Mean ± SD	72.8 ± 11.7	190.8 ± 29.3	96.9 ± 20.6	50.8 ± 7.7	1.61 ± 0.84	1.68 ± 0.76	14.5 ± 0.8	279.5 ± 20
P =	NS	NS	NS	< 0.01	< 0.001	< 0.001	NS	NS

Table 1. Thyroid hormone uptake and output in livers perfused with Ham's F10 perfusion medium at 37° C and 30° C over 3 hours period. (NS = not significant).

respectively) and remained constant during the experiment. There was no significant difference in the mean rat weight (274.5 ± 26 g) and flow rate (14.4 ± 1.19 ml/min) of livers perfused at 37°C in comparison to livers perfused at 30° C (279.5 ± 20 g and 14.5 ± 0.8 ml/min respectively) (Table 1). The actual T4 input in livers perfused at 30° C (190.8 ± 29.3 µg) was lower in comparison with livers perfused at 37° C (210 ± 48 µg), but the percentage uptake of T4 was significantly higher ($P < 0.01$) in the livers perfused at 30° C (50.8 ± 7.7%) compared with livers perfused at 37° C (38 ± 7.7%) (Table 1).

The cumulative net T4 uptake and cumulative net T3 output by livers perfused at 37° C are shown in Fig. 3, where the mean net T4 uptake was 79.4 ± 26 µg and the mean net T3 output was 3.16 ± 1.04 µg.

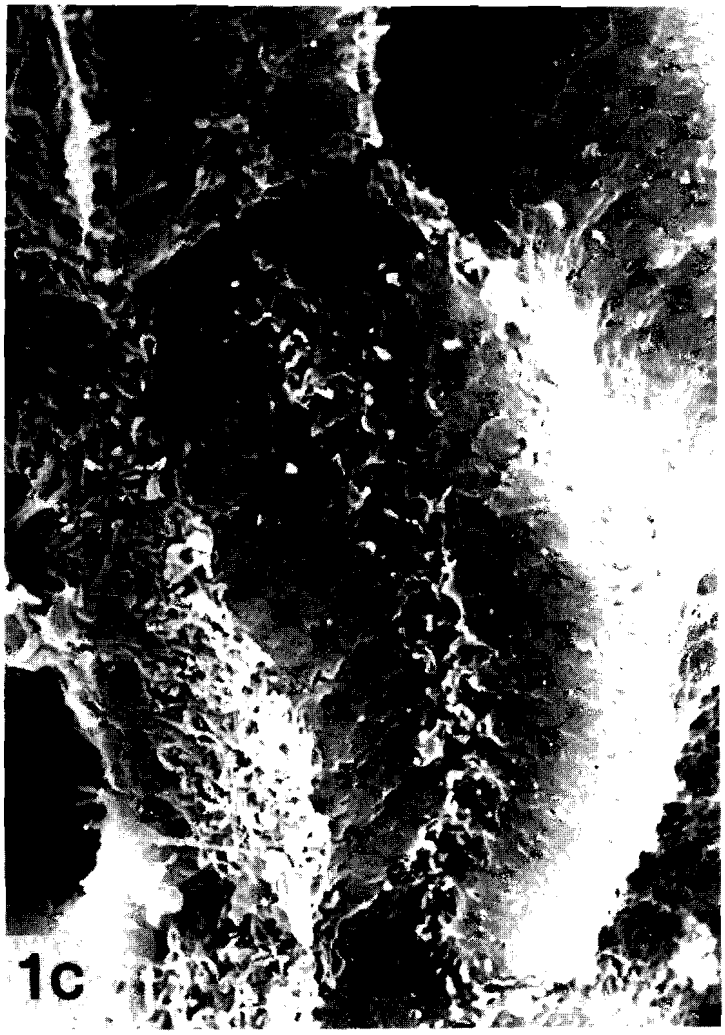
The mean cumulative T4 uptake and T3 output of livers perfused at 30°C are shown in Fig. 4, where the net T4 uptake was 96.9 ± 20.6 µg and the net T3 output was 1.61 ± 0.84 µg.

It is evident that the net T3 output (3.16 ± 1.04 µg) and the T4 to T3 conversion (4 ± 0.62%) in livers perfused at 37° C are significantly higher ($P < 0.001$) than those (1.61 ± 0.84 µg and 1.68 ± 0.76%, respectively) of livers perfused at 30° C (Table 1).

Discussion

The liver is an excellent system for organ perfusion study. It has been extensively used in carbohydrate metabolism and hormone studies (Buschiazzo et al., 1970; Hillier, 1972; Bartels and Sestoft, 1980; Kamada et al., 1980; Hassan, 1982). In liver perfusion, the medium can be delivered to the liver through the portal vein, following which the effluent perfusate can be either recirculated (Hesch et al., 1975; Scheffel et al., 1986) or collected in fractions in a non-recirculating system (Jennings et al., 1984; Al-Ali et al., 1987). It has been stated that the loss of glycogen and ribosomes, changes in the mitochondria and other organelles in isolated livers lead to inevitable cell death (Dixon , 1982). In our laboratory morphometry and ultrastructural investigation was performed on the isolated rat liver perfused with oxygenated Krebs-Ringer bicarbonate buffer solution for 1, 2, 3 and 4 hours at 37° C (Al-Ali et al., 1987). At 37° C about 40% and 55% cell death occurred in the centrilobular zone after 3 hours and 4 hours of perfusion respectively. In the present investigation a more nutritional tissue culture medium was used in comparison with our previous experiments; however, structural changes, in the centrilobular zone were observed after 2 hours in livers perfused at 37° C. These changes led us to lower the temperature from 37° C to 30° C. At this temperature the fine structure of the hepatocytes in all hepatic zones were uniformly well preserved throughout the experimental period. Perfusion of livers at very low temperature (below 4° C) results in total inhibition of enzymatic activity and some morphological damage to the hepatocytes (Kamada et al., 1980; Otto et al., 1986). At higher temperature (30° C - 32° C) the enzyme system (5' - deio-

1a



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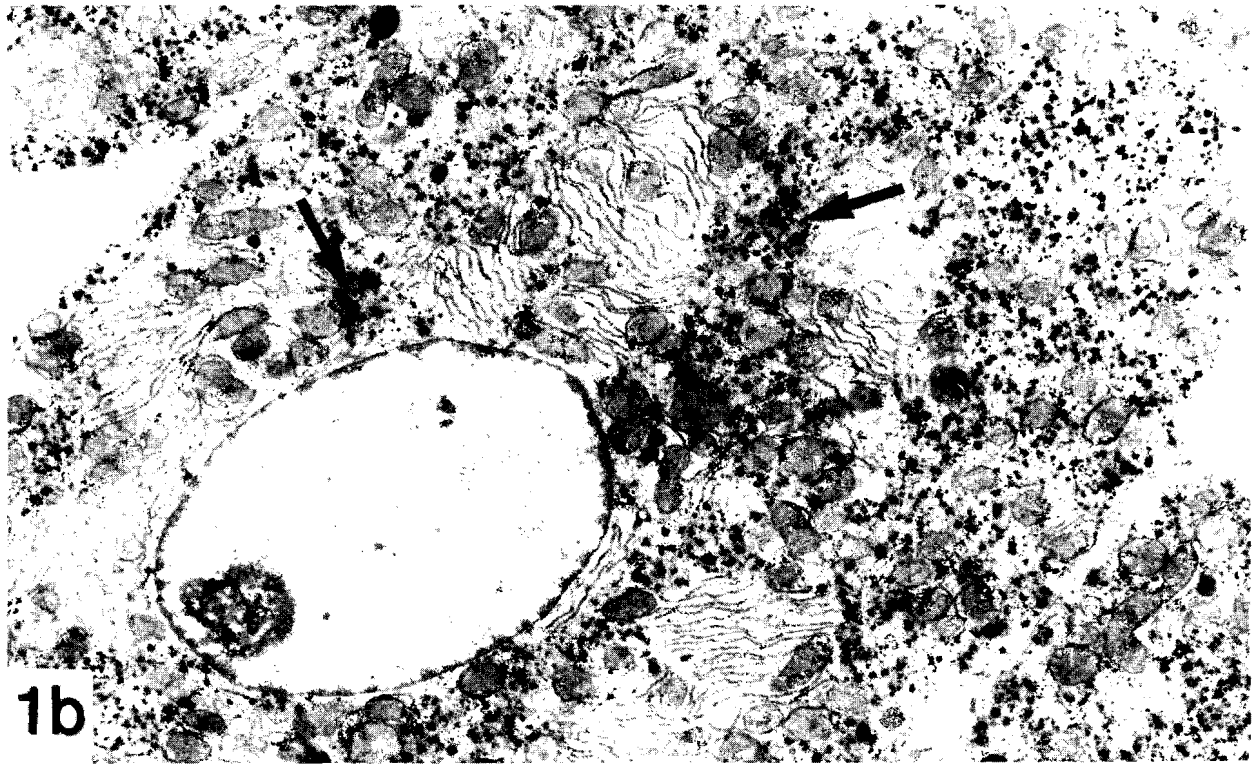

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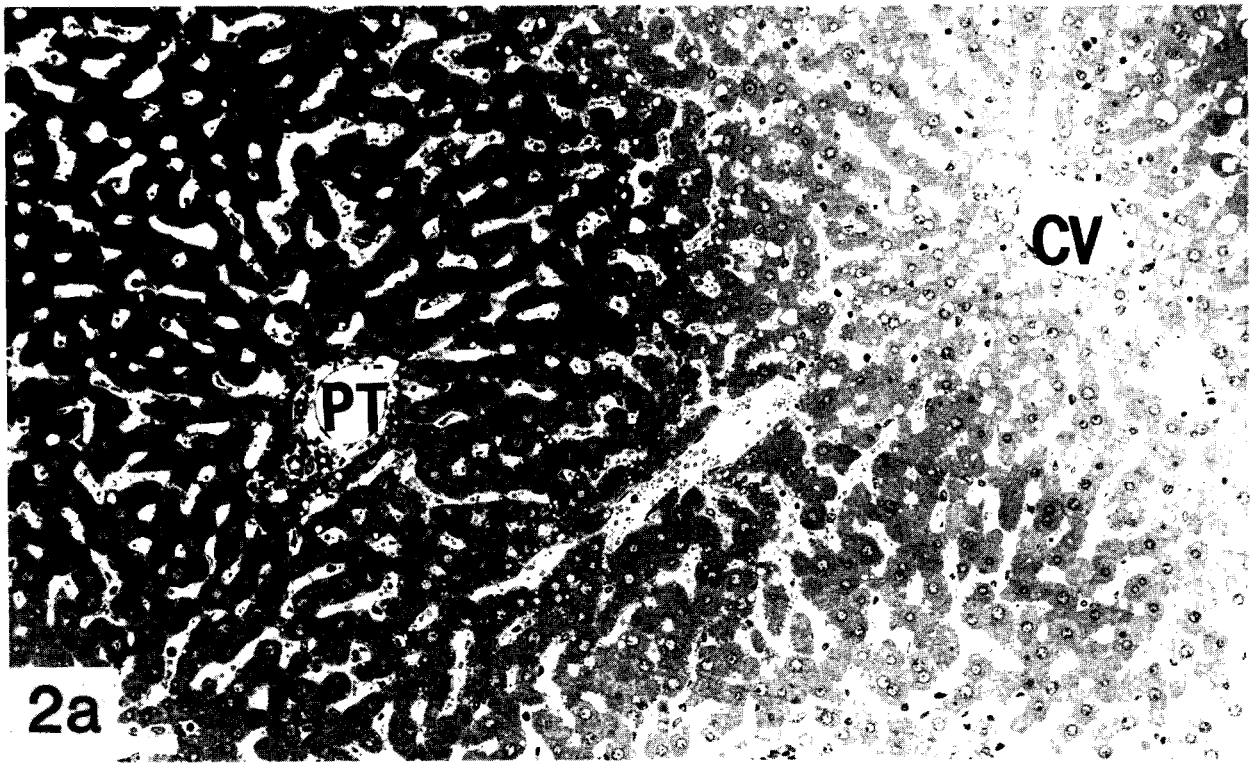

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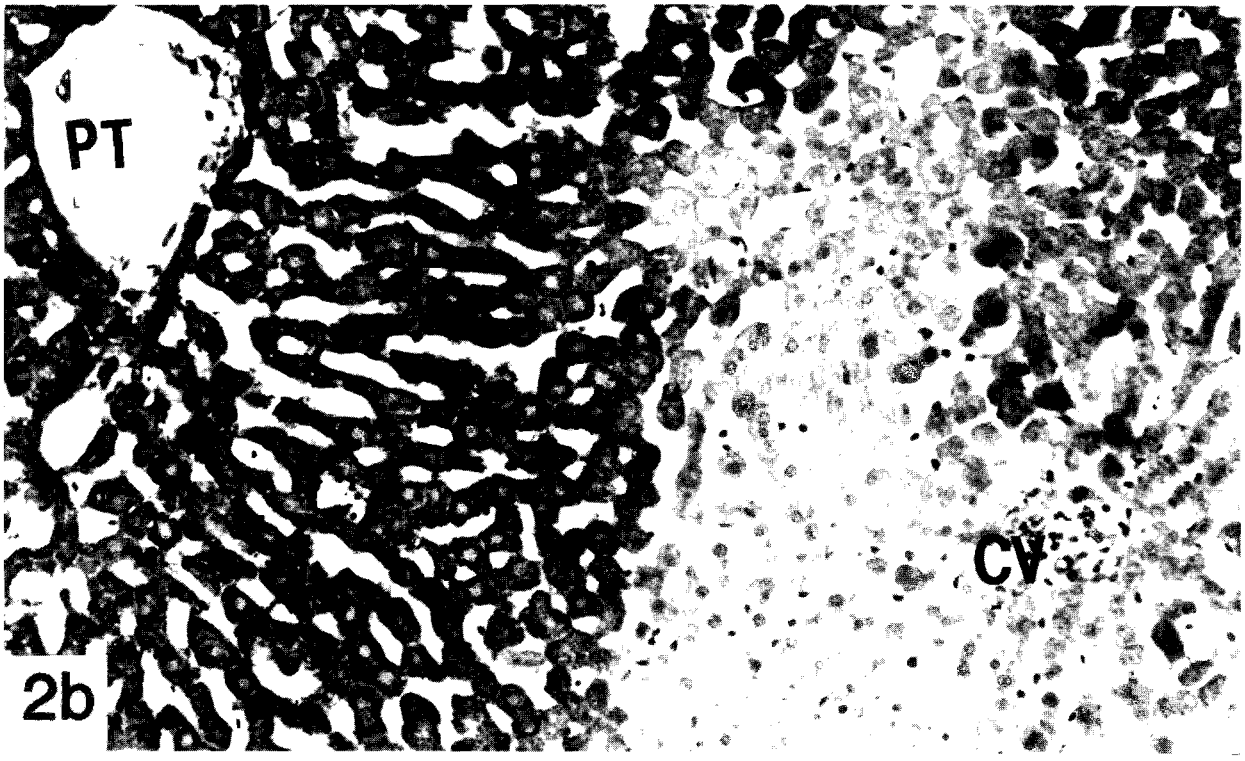

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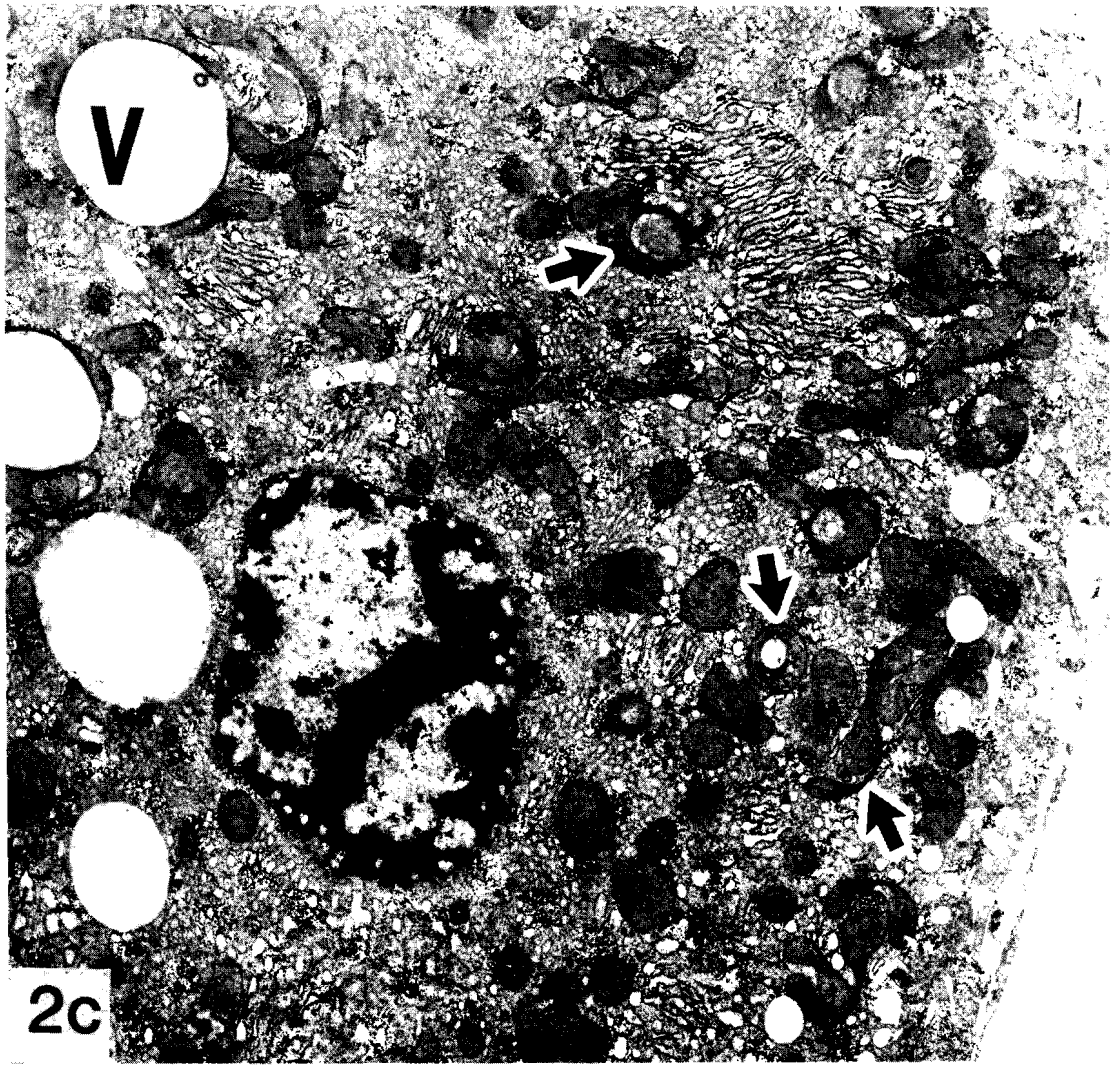
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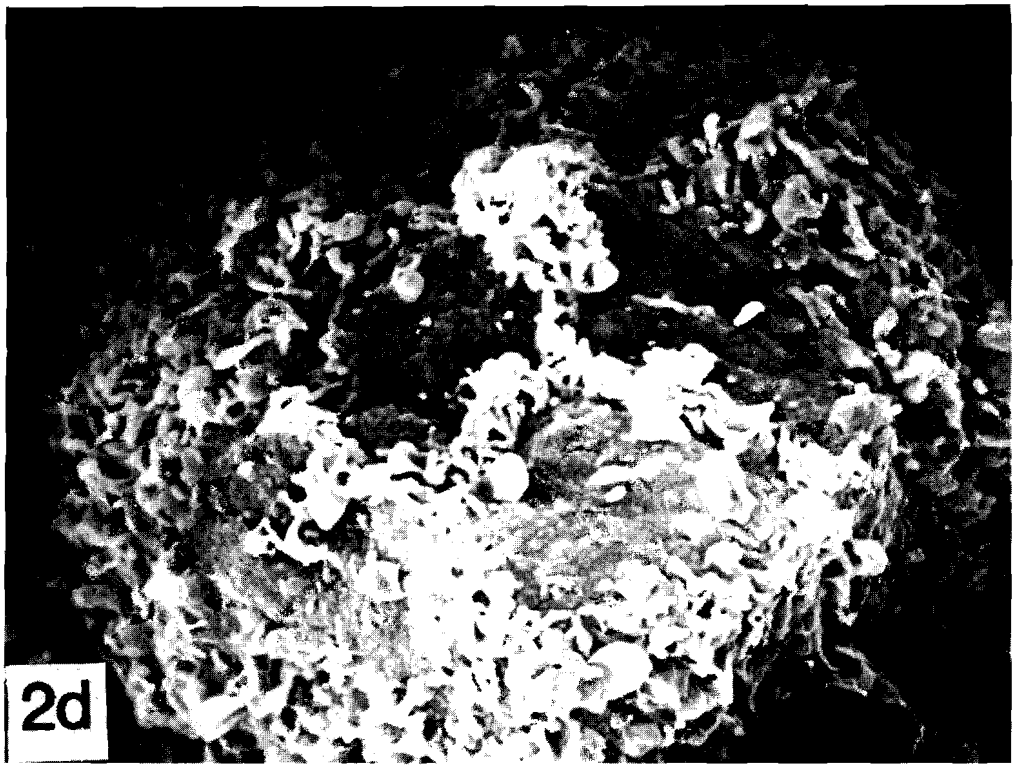
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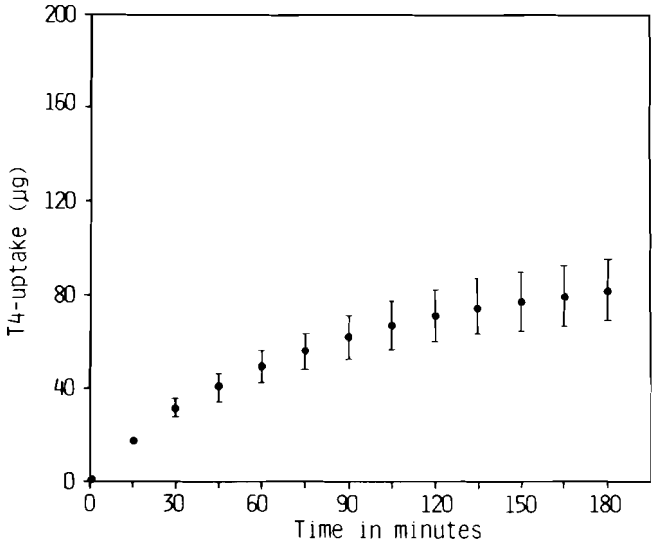

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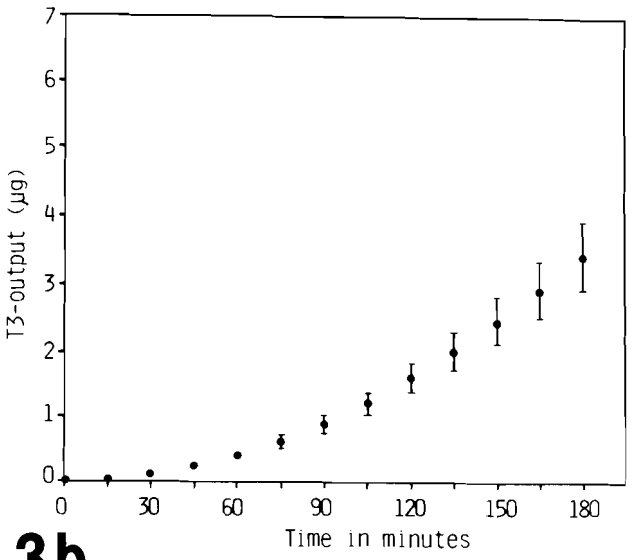

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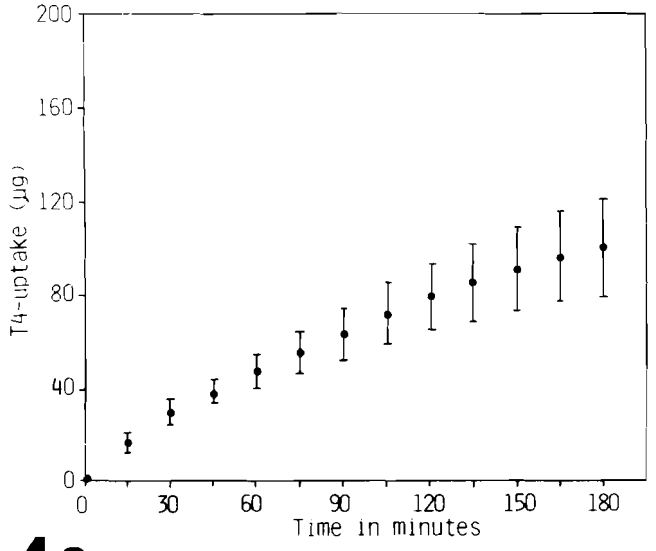

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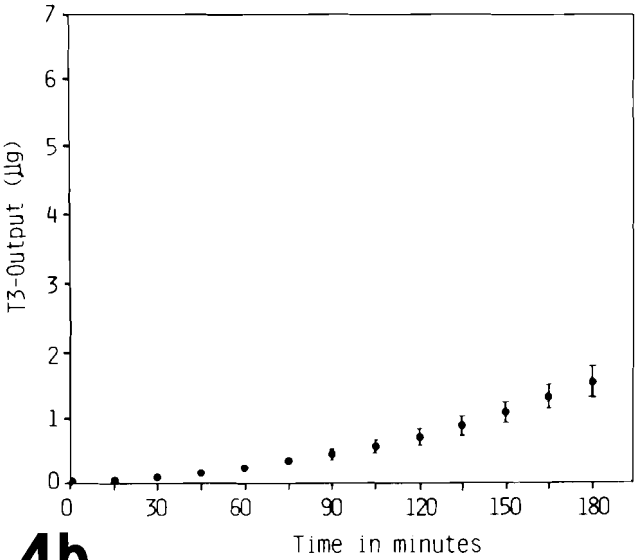

4b

Fig. 1. Isolated rat liver perfused *in vitro* for 3 hours at 30° C. **a.** Hepatic cell plates showing intracellular deposits of glycogen granules throughout the hepatic lobule. Portal tract (PT), central vein (CV). PAS stain, × 180. **b.** Electron micrograph representing the hepatocyte in the liver lobule. The fine-structure of the cell and glycogen granules (arrows) were well preserved. Lead citrate and uranyl acetate stains, × 9,800. **c.** Scanning electron micrograph of a hepatocyte in the centrilobular zone showing well preserved plasma membrane and normal appearance of microvilli. × 6,500

Fig. 2. Isolated rat liver perfused in vitro at 37° C. **a.** After 2 hours of perfusion the hepatic plates in the centrolobular zone showing reduction in the basophilia and vacuolation of the hepatocytes, whilst the periportal zones was well preserved. Portal tract (PT); central vein (CV). 1 µm Araldite section, stained with toluidine blue. × 180 **b.** Hepatic cell plates in the zone around the central vein (CV) showing loss of the glycogen, whilst liver plates in the zone around the portal tract (PT) exhibit glycogen deposits, 3 hours of perfusion. PAS stain, × 180. **c.** Electron micrograph of a hepatocyte in the zone around the central vein. The cell exhibiting clumping of the nuclear chromatin, loss of glycogen, disruption of the granular endoplasmic reticulum, signet-ring or dumbell appearance of mitochondria (arrows), and presence of clear vacuoles (V). 3 hours of perfusion. Lead citrate and uranyl acetate stains, × 11,400. **d.** Scanning electron micrograph of a hepatocyte in the centrilobular zone showing round appearance of the cell, depletion of microvilli, and bleb formation, after 3 hours of perfusion. × 9,000

Fig. 3. Mean cumulative net thyroxine uptake (a) and net triiodothyronine output (b) of 8 livers perfused with Ham's F10 perfusion medium at 37° C plotted against time.

Fig. 4. Mean cumulative net thyroxine uptake (a) and net triiodothyronine output (b) of 6 livers with Ham's F10 perfusion medium at 30° C plotted against time.

dinase) remains intact in vitro (Hillier, 1972; Hesch et al., 1975). Therefore, we assessed thyroxine metabolism at 30° C and compared it with that of the normal body temperature of 37° C in isolated livers. The exposure of animals to low temperature elevates thyroid releasing hormone and thus thyroid stimulating hormone (Jackson and Reichliun, 1977), as well as stimulating the sympathetic nervous system and the release of catecholamines. Furthermore, it is suggested that Beta-adrenergic activity in cold-acclimated rats may accelerates the deiodination of T4 (Scammell et al., 1980). In a non-recirculating isolated rat liver perfusion system both the hypothalamic pituitary thyroid axis and the catecholamine effects have been eliminated. The advantage of this system is that any alteration on hepatic T4 uptake and T4 to T3 conversion (T3-neogenesis) could not be attributed to the effect of released liver metabolites which accumulated in the recirculating medium. Furthermore, no correction is required for the net T3 output due to the further deiodination of T3 by the liver. There was a significant lag-time of 30 min for the start of net T3 output in livers perfused at 30° C and 37° C. This could be explained as the time required to saturate the existing intracellular binding sites, since rat liver cells contain two saturable binding systems for T3 and T4 (Krenning et al., 1981). However, at the subcellular level, conversion of T4 to T3 was detected with the homogenate and the microsomal fraction after 6 min (Hesch et al., 1975). This is consistent with our previous study using isolated rat liver perfused with radio-labelled thyroxine or radio-labelled reverse triiodothyronine, where free radioactive iodide was detected in the effluent perfusate after 6-10 min (Hassan, 1982).

The results showed that up to 2 hours there was no significant difference in the T4 uptake at 30° C and 37° C. The drop of T4 uptake at 37° C after 2 hours might be due to the faster loss of the energy-dependent process of T4 uptake. This was well correlated with the morphological evidence of ribosome and glycogen depletion. This may suggest that glycolysis initially supports ATP levels (up to 2 hours) but fails later because of glycogen depletion.

For T3 output the difference appears significant at 1.5-2 hours, where at 30° C, the conversion rate is slower. The decrease in net T3 output and T4 to T3 conversion in livers perfused at 30° C, when compared with livers perfused at 37° C, could be due to one or more of the following reasons:

1. An impairment of the secretory process of T3 from liver cells after conversion of T4 to T3, since the secretory process through the cell membrane is not yet fully understood.

2. A slowness of metabolism at low temperature may consequently lead to slow rate of peripheral conversion of T4 to T3.

3. Despite the use of a high glucose concentration in PM to inhibit glycogenolysis, we observed loss of glycogen granules in livers perfused at 37° C. Therefore, the increased available intracellular glucose at 37° C may generate more sulphydryl co-factor involved in the conversion of T4 to T3.

4. The significant increase in T3 release at 37° C may partly be attributed to the subcellular damage of hepatocytes observed in the centrilobular zone.

The results indicate that, at 30° C the hepatic T4 uptake is not inhibited, but the rate of T4 to T3 conversion has decreased, additionally the livers remain morphologically well preserved throughout the experimental period. At 37° C, the rate of T4 to T3 conversion is higher, but the livers could not be maintained intact for more than 2 hours. From the above evidence, isolated rat livers perfused with modified Ham's F10 culture medium at 30° C offers the best compromise for further metabolic and morphological studies.

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