Messenger RNA levels of plasma proteins following fasting

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1. The effect of fasting on hepatic mRNA levels of seven plasma proteins was examined in the rat.
2. The levels of mRNA were measured directly in cytoplasmic extracts by hybridization to specific ^{32}P-labelled cDNA probes.
3. Following a 48 h period of fasting, the mRNA levels of apolipoprotein E, apolipoprotein A-IV, albumin, transferrin, and transthyretin decreased by 15–30 %, while apolipoprotein A-II decreased by 78 % compared with non-fasted control rats. The mRNA for apolipoprotein A-I increased by 33 %.
4. These findings suggest that mRNA levels in the liver following fasting are regulated independently and variations in these levels may be due to differences in transcription rates or mRNA stability.

Investigations into the effects of starvation on the content of ribosomes (Webb *et al.* 1966; Henshaw *et al.* 1971; Wilson *et al.* 1967), DNA (Ramsey & Steele, 1976), RNA (Henderson, 1970) and protein (Enwonwu & Munro, 1970; Ramsey & Steele 1976) in rat liver have been reported. It has been shown that RNA polymerase activity decreases markedly during starvation (Henderson, 1970), and refeeding increases RNA synthesis and RNA polymerase activity following fasting (Kosterlitz, 1947; Laird *et al.* 1955; Kirsch *et al.* 1968; Rothschild *et al.* 1968; Henderson, 1970; Vesley & Cihak, 1970). Decreases in protein synthesis in the liver following fasting parallel decreases in ribosome content, decreases in polyribosome activity and a disaggregation of polyribosomes (Hirsche & Hiatt, 1966; Reid *et al.* 1970; Henshaw *et al.* 1971; McNurlan *et al.* 1972; Yap & Hafkenscheid, 1978). Studies have demonstrated that mRNA is necessary for the structural integrity of the polyribosome (Warner *et al.* 1962; Noll *et al.* 1963; Staehelin *et al.* 1963; Trakatellis *et al.* 1964). The hepatic mRNA levels for albumin have been studied in animal models following fasting by a number of investigators (Pain *et al.* 1978; Yap *et al.* 1981; Goldman *et al.* 1985, Howard *et al.* 1986).

In the present paper, the relative mRNA levels for albumin, as well as transferrin, transthyretin, apolipoprotein (Apo) A-I, Apo A-II, Apo A-IV and Apo E were measured by hybridization techniques using specific cDNA probes in rats fasted for 2 d.

METHODS

Animals and materials

Male Buffalo rats from an inbred strain, weighing 230–280 g, were fed on a standard laboratory rat chow (GR2+ Ration; Clark King and Co., Allied Mills Industries Pty Ltd, North Melbourne) containing (g/kg) 200 protein, 20 crude fat and 30 crude fibre, and were fed *ad lib.* or fasted for 48 h. Rats were housed in a constant-temperature room at 22° with a 12 h light period from 08.00 to 20.00 hours and weighed before and after the 48 h experimental period. All experimental procedures were performed between 09.00 and 12.00 hours.

Nitrocellulose filters were purchased from Schleicher and Schull, Dassel, West Germany, ribonuclease A from Boehringer, Mannheim, West Germany and [α-^{32}P]dATP and [α-^{32}P]dCTP (specific radioactivity > 3000 Ci/mmol) from Amersham International plc, Amersham, Bucks. All reagents were of analytical grade.

Preparation of cytoplasmic extracts and total RNA from livers

Livers were removed from rats under diethyl ether anaesthesia and homogenized in a cold solution of 10 mM-Tris hydrochloride (pH 7·0), 1 mM-EDTA, Nonidet P-40 (5 g/l; 10 ml/g liver) with a 30 ml Potter Elvehjem homogenizer. The homogenate was centrifuged at 4° (15 000 g, 5 min) and portions of the supernatant fractions taken. To 1 ml supernatant fraction, 600 μl of 20 × standard saline citrate (3 M-NaCl, 0·3 M-trisodium citrate) and 400 μl 37% (w/v) formaldehyde were added. The mixture was incubated at 60° for 15 min and centrifuged at 4° (15 000 g, 15 min). Control samples were prepared by incubation of 500 μl of the supernatant fraction from the tissue homogenate with ribonuclease A (EC 3.1.27.5; 0·04 mg/ml final concentration) and 400 μl cold 10 mM-Tris hydrochloride, pH 7·0, 1 mM-EDTA, Nonidet (5 g/l) for 1 h at 37°. After centrifugation at 4° (15 000 g, 15 min) standard saline citrate and formaldehyde were added and processed as before. All processed extracts were stored at −70°. The tissue extracts were diluted 1:20 (v/v; 1:10 for ribonuclease-treated controls) with 1·2 M-sodium chloride, 0·112 M-trisodium citrate before spotting on to nitrocellulose filters using a Biorad 'Biodot' apparatus. Total RNA was prepared by homogenizing liver with a Potter Elvehjem homogenizer in 5 M-guanidinium thiocyanate containing 50 mM-sodium citrate (pH 7·0), 0·1 M-β-mercaptoethanol and n-laurylsarcosine (1 g/l; 10 ml/g liver). The homogenate was layered over a cushion of 1 ml 5·65 M-CsCl. The homogenate was overlayered in turn by 5 ml sterile H_2O. The pellet obtained after centrifugation for 18 h at 185 000 g was resuspended in 2 ml 10 mM-Tris hydrochloride (pH 7·6) containing 10 mM-EDTA. Protein was extracted by adding 4 ml of a mixture of chloroform–butanol (4:1; v/v) followed by rigorous shaking. After separation of the phases by a short centrifugation, the aqueous phase was removed and the organic phase was extracted again with 1 ml 10 mM-Tris-hydrochloride (pH 7·6), containing 10 mM-EDTA. The aqueous phases were combined and RNA was precipitated by adding 2 vols ethanol and 0·1 vol. 3 M-sodium acetate (pH 5·0), followed by incubation for 2 h at −20°. The precipitated RNA was resuspended in 0·03 ml sterile H_2O. The total liver RNA was subjected to 'Northern' analysis (Thomas, 1980).

Synthesis of radioactively-labelled probes

Specific cDNA clones for plasma proteins in plasmid pBR325 or bacteriophage λgt11-Amp3 were used to obtain probes for measuring mRNA levels. Characteristics of the clones for albumin (Schreiber et al. 1986), transferrin (Aldred et al. 1984), transthyretin (Dickson et al. 1985), Apo E (de Jong et al. 1984), and Apo A-I, Apo A-II and Apo A-IV (Guo-Fen et al. 1987) have been described elsewhere. Inserts from clones were isolated and nick-translated (Maniatis et al. 1982) to produce ^{32}P-labelled probes. These probes were then allowed to hybridize to their respective mRNA bound to nitrocellulose filters (Thomas, 1980). Following autoradiography on Kodak XRP film at −70° with an intensifying screen, the areas on the nitrocellulose filters with hybridized cDNA were cut out and the bound radioactivity was quantified by liquid-scintillation spectrophotometry in toluene containing 5 g 2,5-diphenoloxazolel. Relative mRNA levels for plasma proteins were calculated as radioactivity bound to mRNA on the filters (minus background) as a percentage of the value for normal rats, corrected for decreases in body-weight and liver weight during fasting.

Statistical evaluation

Five animals were used for each time-point. Means with their standard errors were calculated. Statistical evaluation of body-weight and liver weight was performed using Student's t test, $P < 0.05$ being considered as statistically significant.

RESULTS

Cytoplasmic extracts were prepared from rat liver and mRNA levels were measured by hybridization to specific α-^{32}P-labelled cDNA for albumin, transferrin, transthyretin, Apo A-I, Apo E, Apo A-II and Apo A-IV. As an example, Plate 1 shows hybridization of cDNA obtained for rat serum albumin (RSA), Apo A-I and Apo E to RNA in extracts from livers of control and 48 h fasted rats. As seen in lane A of Plate 1, hybridization signals obtained from cytoplasmic extracts increased in intensity when increased amounts of extract were added. In the quantitative analysis of radioactivity bound to excised dots by liquid-scintillation spectrometry, a linear relation existed between bound radioactivity and amount of extract in the dots.

Fasting for 48 h resulted in a decrease of body-weight compared with non-fasted controls (251 (SE 10, n 5) v. 221 (SE 9, n 5) g; $P < 0.05$), and a decrease in liver weight compared with non-fasted controls (10·3 (SE 0·5, n 5) v. 6·3 (SE 0·2, n 5) g; $P < 0.001$). The average liver weight of control animals was 3·96% of body-weight, whereas after 48 h fasting the liver weight was only 2·85% of total body-weight. Table 1 shows quantitative mRNA levels as a percentage of control values for the seven proteins studied before and after correction for the disproportionate loss of liver weight. As the results obtained for each time-point were determined for a fixed amount of liver, it is necessary to account for the greater decrease in liver weight relative to body-weight in order to compare the synthesis of different mRNAs by the whole liver. To eliminate the effect of differences in rat size the change in liver weight as a percentage of initial body-weight was used to calculate the results presented in Table 1 (corrected mRNA levels):

$$\text{mRNA(\% control)} \times \frac{\text{\% experimental liver weight}}{\text{\% control liver weight}}.$$

After correcting for liver weight loss, the amount of mRNA for albumin, transferrin, Apo A-IV and Apo E in the whole liver were seen to decrease 15–30% following the 48 h fasting period. Apo A-I mRNA levels increased 33% compared with control levels. The largest change was observed for Apo A-II, where mRNA levels fell to 22% of control values.

Total RNA was prepared from livers of control and 48 h fasted rats and subjected to 'Northern' analysis. As an example, Plate 2 shows cDNA for Apo A-I, Apo A-II, Apo A-IV, Apo E and albumin hybridizing to specific RNA species. The intensities of bands obtained from hybridization also shows a decreased level of mRNA for Apo A-II, and an increase in mRNA for Apo A-I following the 48 h fasting period.

DISCUSSION

The results obtained by the technique of cytoplasmic dot hybridization used to measure changes in specific mRNA levels of albumin, transferrin, transthyretin, Apo A-I, Apo A-II, Apo A-IV and Apo E in rat liver during fasting are consistent with the assumption that these levels are regulated independently during fasting.

Nutritional status has a profound effect on the activity of hepatic enzymes and the rate

Table 1. *Liver mRNA levels* in rats after 48 h fasting as a percentage of control values*

(Mean values with their standard errors for five animals)

Protein	Uncorrected (counts/min per g liver) mRNA level (% control value)		Corrected (counts/min per whole liver) mRNA level (% control value)	
	Mean	SE	Mean	SE
Albumin	136	11	86	7
Transferrin	105	13	67	8
Transthyretin	107	10	68	6
Apo A-I	209	17	133	11
Apo A-II	35	4	22	3
Apo A-IV	114	11	72	7
Apo E	135	12	86	8

Apo, apolipoprotein.
* mRNA levels determined as described on p. 82.

of protein synthesis. In studies where fasted mice (pretreated with actinomycin D) were tube fed on tryptophan, elevated cytoplasmic mRNA levels, increases in polysomal aggregates, and an increase in protein synthesis were observed (Murty & Sidransky, 1972). Later studies revealed that tryptophan may act to stimulate the transport of mRNA into the cytoplasm of the liver (Murty *et al.* 1976). These studies indicate that amino acids, in particular tryptophan, play a part in the regulation of protein synthesis. However, other experiments show that amino acid concentrations in liver cytosol are unchanged following fasting (Yap & Hafkenscheid, 1981). These and other studies suggest that liver protein synthesis may be regulated by transcriptional or post-transcriptional mechanisms during starvation and not amino acid availability (Princen *et al.* 1983).

Nuclear RNA containing the primary transcript exists in association with proteins in the form of ribonucleoprotein particles (mRNP). Transport of the mRNP complex into the cytoplasm and incorporation into polysomes has been postulated (Spirin, 1966; Henshaw, 1968). Increases in free ribosomes following starvation have been reported in cultured mouse and human cells (Hogan & Korner, 1968; Lee *et al.* 1971; Vaughan *et al.* 1971; van Venrooij *et al.* 1972). The mRNA released by polysomes during starvation is in the untranslated ribonucleoprotein form (mRNP) which is stored in the cytoplasm and is readily available for uptake into the polysomes (Lee *et al.* 1971; Sonenstein & Brawerman, 1977; Yap *et al.* 1978). Addition of nutrients to starved cells results in rapid polysome formation (Lee *et al.* 1971; Sonenstein & Brawerman, 1977).

When the effect of starvation on translatable mRNA for liver-specific proteins was tested it was found that overall template activity of mRNA/μg RNA in both polysomal and total RNA was slightly decreased (Howard *et al.* 1986). In the same study, translatable albumin mRNA (from both RNA populations) was reported to decrease 20–40 %. These results agree with decreases in albumin mRNA observed by Pain *et al.* (1978) following protein depletion. Other studies show no changes in hepatic albumin mRNA levels following fasting. Goldman *et al.* (1985) reported that the synthesis of hepatic albumin mRNA was little affected in ducklings starved for 2 d. Results obtained by Yap *et al.* (1978) demonstrate that although albumin mRNA levels decrease in membrane-bound ribosomes they accumulate in postpolysomal ribonucleoprotein fractions following a short-term fast (24–30 h). In studies reported by Wilson & Hoagland, (1967) and Wilson *et al.* (1967) a decrease in polysomes and total RNA synthesis was observed in the livers of rats starved

for 5 d and treated with actinomycin D. In the same studies an increase of albumin relative to other proteins was demonstrated suggesting that albumin mRNA was more stable than other mRNA in liver.

In the present study we observed that the levels of mRNA for albumin in the whole liver decreased by only 10–20 % in rats fasted for 48 h. These observations may reflect the increased levels of postpolysomal albumin mRNA observed by others (Yap *et al.* 1978) as well as the stability of albumin mRNA (Wilson & Hoagland, 1967; Wilson *et al.* 1967). It should be noted that our results, as well as the results obtained by Yap & Hafkenscheid (1981) and Goldman *et al.* (1985), are based on molecular hybridization techniques. These techniques rely on the assumption that all the mRNA transcripts can be detected and are fully functional.

During fasting the concentration of plasma transthyretin (prealbumin) in the rat has been reported to decrease to about 50 % of normal levels (Felding & Fex, 1983). The results presented here suggest that this may be due to decreased hepatic mRNA levels for this protein. Similar changes in plasma transthyretin and hepatic mRNA levels (Dickson *et al.* 1985) have been reported in response to acute inflammation.

In our studies Apo A-II mRNA levels were shown to change the most, decreasing to 22 % of normal levels. Decreases in lipolytic activity during fasting have been shown to reduce liver lipid synthesis (Burton *et al.* 1969). Hepatic triglyceride lipase activity in the rat has been reported to decrease following a period of fasting for 24 h (Elkeles & Hambley, 1977). It has been demonstrated that Apo A-II may play a role in vivo in the activation of hepatic lipase activity and high-density lipoprotein metabolism (Jahn *et al.* 1983). This decrease in hepatic lipase activity following fasting is consistent with diminished mRNA levels of activator, Apo A-II.

In the present study, the independent alterations of specific mRNA levels described following fasting may be caused by: (1) changes in rates of transcription, (2) differences in the rates in delivery of mRNA from the nucleus to the cytoplasm, or (3) differences in mRNA stability.

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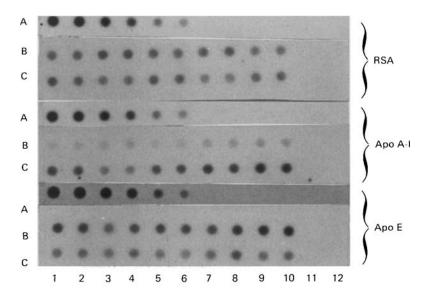Wilson, S. H. & Hoagland, M. B. (1967). *Biochemical Journal* **103**, 556–566.

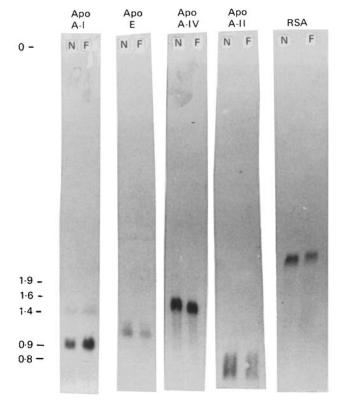Yap, S. H. & Hafkenscheid, J. C. M. (1981). *Annals of Nutrition and Metabolism* **25**, 158–164.

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EXPLANATION OF PLATES

Plate 1. Rat mRNA levels following fasting. Cytoplasmic mRNA extracts spotted on to nitrocellulose paper and hybridized to specific cDNA for rat serum albumin (RSA), apolipoprotein A-I (Apo A-I) and apolipoprotein E (Apo E). Autoradiography was at −70° using Kodak XRP film and an intensifying screen was used for 24 h. Autoradiographs for RSA, Apo A-I and Apo E are from identical filters. Lane A shows hybridization to RNA obtained for cytoplasmic extracts from (columns 1–6 respectively) 2500, 1000, 500, 250, 100 and 50 μg liver pooled from five control rats. Lane B (columns 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, in duplicate) shows results obtained from 100 μg liver from five individual control rats respectively. Lane C, same as lane B, except from five 48 h-fasted rats. Columns 11 and 12 (lanes B and C) contain similar material as rows 9 and 10 next to them, but extracts were incubated with ribonuclease A (*EC* 3.1.27.5) before formaldehyde treatment.

Plate 2. 'Northern' analysis of mRNA from 48 h fasted rat livers. Autoradiographs obtained from the analysis of apolipoprotein (Apo) A-I, Apo E, Apo A-IV, Apo A-II and rat serum albumin (RSA). Total RNA (5 μg: lanes Apo E and RSA) and total RNA (10 μg: lanes Apo A-I, Apo A-IV and Apo A-II), isolated from the liver of control rats (N) and from 48 h-fasted rats (F), was subjected to electrophoresis in a 1·2 % agarose gel containing formaldehyde. Transfer of the RNA to nitrocellulose filters and hybridization with radiolabelled cDNA for Apo A-I, Apo E, Apo A-IV, Apo A-II and RSA was performed as described by Maniatis *et al.* (1982). Autoradiography was at −70° using Kodak XAR film and an intensifying screen for 3 d.

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