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Effect of post-ischaemic recovery on albumin synthesis and relative amount of translatable albumin messenger RNA in rat liver

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In liver cells recovering from reversible ischaemia, total protein synthesis by postmitochondrial supernatant and membrane-bound and free polyribosomes is not different from that in sham-operated controls. However, the relative proportion of specific proteins is changed, since the incorporation of [^{3}H]leucine *in vivo* into liver albumin, relative to incorporation into total protein, as determined by precipitation of labelled albumin with the specific antibody, decreases by 40–50% in post-ischaemic livers. Cell-free synthesis by membrane-bound polyribosomes and poly(A)-enriched RNA isolated from unfractionated liver homogenate shows that the decrease in albumin synthesis in liver of rats recovering from ischaemia is due to the relative decrease in translatable albumin mRNA.

Nuclei and nucleoli isolated from liver after 60 min of ischaemia show a severe impairment of RNA synthesis. After restoration of the blood supply RNA synthesis not only returns to normal within 2 h, but later increases significantly, reaching a maximum between 8 and 16 h, and finally returns to control values at 48 h (Schiaffonati *et al.*, 1978). This increase in RNA synthesis is preceded by activation of ornithine decarboxylase, leading in turn to an increase in putrescine concentration (Ferioli *et al.*, 1980), and is essentially sustained by the enhanced activity of the engaged RNA polymerases, whereas free polymerases and chromatin template activity are unaffected (Cairo *et al.*, 1981). Moreover, in post-ischaemic liver, as no cell loss has occurred, enhanced RNA synthesis is not followed by thymidine incorporation into DNA and cell multiplication, suggesting that the recovery from non-necrogenic ischaemia can be interpreted essentially as a process of intracellular repair (Schiaffonati *et al.*, 1978).

In the present research we investigated whether the enhanced RNA synthesis in post-ischaemic liver is in some way correlated with quantitative and/or qualitative modifications of liver protein synthesis. Since albumin, the major protein product of vertebrate liver, has been shown to respond to a variety of physiological and pathological stimuli (Peters, 1975), in order to study possible changes in the synthesis of specific proteins we investigated whether post-ischaemic recovery leads to modifications of albumin synthesis and whether such changes are associated with variations in the amount of functional albumin mRNA. While this work was in progress, Krieg *et al.* (1980) demonstrated that a decrease of albumin-specific mRNA sequences occurs in regenerating liver, a condition which has been shown to share several analogies with post-ischaemic recovery (Ferioli *et al.*, 1980).

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate; poly(A)$^+$ RNA, poly(A)-containing RNA.

Materials and methods

Animals

Male albino rats (Wistar strain) weighing approx. 200 g were used throughout. They were maintained with alternating 12 h cycles of light and dark (light 07:00–19:00 h) and fed on a balanced diet *ad libitum*. Post-ischaemic rats were used 16 h after the end of 60 min ischaemia, induced as previously described (Bernelli-Zazzera & Gaja, 1964). Control rats were sham-operated.

Chemicals

L-[4,5-^{3}H]Leucine (sp. radioactivity > 100 Ci/mmol) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.).

Translation assays *in vitro* using reticulocyte lysate were performed by using a Translation Kit from N.E.N. Chemicals G.m.b.H., Dreieich, West

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Germany, with L-[3,4,5-^{3}H]leucine (sp. radioactivity > 110 Ci/mmol).

CNBr-activated Sepharose 4B was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Oligo(dT)–cellulose (type 3) was obtained from Collaborative Research, Waltham, MA, U.S.A. Pancreatic ribonuclease A (EC 3.1.27.5) from bovine pancreas and yeast rRNA were from Boehringer, Mannheim, West Germany. α-Amylase (EC 3.2.1.1) (type I-A, phenylmethanesulphonyl fluoride-treated, from pig pancreas) was from Sigma Chemical Co., St. Louis, MO, U.S.A.; 1 unit is the amount of enzyme that can liberate 1 μmol of maltose from starch/min at pH 6.9 and 20°C. The scintillation fluid contained 5.5 g of Permablend III (Packard Instrument Co.)/litre in toluene. Solutions for RNA extraction and assay were autoclaved before use. All glassware was heated to 150°C for 5 h. Non-disposable plastics were soaked with 5% (v/v) ethanol/0.5% (v/v) diethyl pyrocarbonate (Sigma).

Amino acid incorporation by postmitochondrial supernatant

Postmitochondrial supernatant was prepared as described by Blobel & Potter (1967). Incubation and determination of radioactivity incorporated into protein were performed as described by Ragnotti & Aletti (1975), with [^{3}H]leucine (0.5 μCi/sample).

Albumin synthesis in vivo

Rats were injected intraperitoneally with 300–500 μCi of [^{3}H]leucine and killed after 12 min. Livers were excised, homogenized in 3 vol. of 0.25 M-sucrose at 0°C and adjusted to 1% (v/v) Triton X-100 and 1% (w/v) sodium deoxycholate. The homogenate was centrifuged for 10 min at 2°C and 10000 *g* ($r_{av.}$ 6.04 cm). Incorporation into total protein was determined by spotting 5 μl samples of the supernatant fraction on Whatman 3MM paper followed by washing through the hot-trichloroacetic acid procedure of Mans & Novelli (1961). Some 95% of all the acid-insoluble radioactivity in the whole homogenate was recovered in the detergent-treated supernatant.

For electrophoresis of total protein synthesized *in vivo*, postmitochondrial supernatant was treated as described by Sippel *et al.* (1976).

The relative proportion of albumin synthesis was determined by precipitation of labelled albumin with specific antibody. Antibody reactions were performed with several different ratios of liver postmitochondrial supernatant to anti-albumin to assure that immunoprecipitation was quantitative.

Polyribosome isolation

Bound and free ribosomes were prepared from rat liver as described by Ramsey & Steele (1976), but the homogenate was incubated with hog pancreas α-amylase (10 units/ml) for 15 min at 4°C with slow mixing as described by Ramsey & Steele (1979). Free and membrane-bound polyribosomes were harvested by suspension in a buffer containing 10 mM-Hepes, pH 7.4, 75 mM-KCl, 5 mM-$MgCl_2$ and 3 mM-reduced glutathione and used either for translation assay or for determination of RNA content.

Polyribosome-dependent translation in vitro

Bound and free ribosomes were incubated in a cell-free protein-synthesizing system containing cell-sap fraction from rat liver or S_{30} wheat-germ supernatant as a source of enzymes, factors and tRNA. Cell sap was prepared as described by Ragnotti *et al.* (1970), and S_{30} as described by Roberts & Paterson (1973). The translation mixture contained in 100 μl: 100–150 μg of rRNA, determined by the A_{260} as described by Ragnotti (1971), 30 μl of cell sap or S_{30} fractions, 10 μCi of [^{3}H]leucine, 2.5 mM-magnesium acetate, 90 mM-KCl, 2 mM-dithiothreitol, 19 essential amino acids (except leucine) (final concn. 25 μM each), 1 mM-ATP, 20 μM-GTP, 8 mM-phosphocreatine and 12 mM-Hepes buffer, pH 7.6. Incubation was at 29°C for 1 h. After incubation, radioactivity incorporated into total peptides was measured by the hot-trichloroacetic acid procedure of Mans & Novelli (1961). To measure the relative proportion of albumin synthesis, 10 μg of unlabelled rat serum albumin was added to the reaction mixture. Ribosomes were removed by centrifugation for 1 h at 2°C at 105000 *g* ($r_{av.}$ 5.9 cm), and the postribosomal fluid was adjusted to 25 mM-Na_2EDTA, pH 7.4, and incubated with pancreatic ribonuclease A (20 μg/ml) for 15 min at 37°C. Radioactivity in albumin was determined with enough antibody to precipitate the albumin carrier.

RNA isolation

Total cellular RNA was isolated from unfractionated liver homogenates by phenol/chloroform extraction as described by Keller & Taylor (1976).

Purification of poly(A)$^+$ RNA

To purify poly(A)$^+$ RNA, total cellular RNA was dissolved in 0.5 M-NaCl/10 mM-Tris/HCl (pH 7.4)/0.5% (w/v) *N*-lauroylsarcosine and bound to oligo(dT)–cellulose. Bound RNA was eluted with 10 mM-Tris/HCl (pH 7.4)/0.5% *N*-lauroylsarcosine, adjusted again to 0.5 M-NaCl and rechromatographed. Recovery was about 1% for both control and post-ischaemic preparations. The RNA had a typical A_{260}/A_{280} ratio of 2.0–2.1.

Translation of RNA in vitro

Cell-free protein synthesis was performed in a total volume of 25 μl containing 10 μCi of [^{3}H]-leucine and various amounts of poly(A)$^+$ RNA with

a reticulocyte translation assay system. Samples were removed and the incorporation into total protein was measured by the hot-trichloroacetic acid procedure of Mans & Novelli (1961). The reaction mixture was then treated with 25mM-Na_2EDTA and pancreatic ribonuclease A (20μg/ml) to release the nascent chains. For electrophoresis of the translation products, samples were concentrated by trichloroacetic acid precipitation as described by Sippel *et al.* (1976).

The relative proportion of albumin synthesis was determined by precipitation of labelled albumin with sufficient antibody to precipitate 10μg of unlabelled albumin added as carrier.

Preparation of antibody and immunoprecipitation

Antibodies were raised in rabbits against rat serum albumin prepared from rat serum as described by Taylor & Schimke (1973). The final albumin preparation was shown to be homogeneous by SDS/polyacrylamide-gel electrophoresis. Anti-albumin was immunopurified by affinity chromatography on an albumin–Sepharose 4B column (Taylor & Schimke, 1974). Antibody specificity was checked by the method of double radial immunodiffusion in agarose gels (Ouchterlony & Nilsson, 1973).

Immunoprecipitation reactions were performed in a final volume of 0.5ml containing 1% (v/v) Triton X-100, 10mM-leucine and phosphate-buffered saline (10mM-sodium phosphate/150mM-NaCl, pH7.4) for 1h at 37°C and overnight at 4°C. The final reaction mixture was layered over 0.5ml of 1M-sucrose in phosphate-buffered saline containing 1% (v/v) Triton X-100 and 10mM-leucine, centrifuged for 3min at 10000*g* in an Eppendorf Microfuge in the cold and washed twice with phosphate-buffered saline. Radioactivity in albumin was determined by filtration of immunoprecipitates, suspended in 10% (w/v) trichloroacetic acid, through glass-fibre filters as described by Ragnotti *et al.* (1969).

Gel electrophoresis

Both immunoprecipitates and trichloroacetic acid precipitates were resuspended in 125mM-Tris/HCl (pH6.8)/2% (w/v) SDS/1% (v/v) glycerol/5% (v/v) 2-mercaptoethanol, boiled for 5min and subjected to SDS/polyacrylamide-gel electrophoresis as described by Laemmli (1970) on 10%-acrylamide slab gel. Gels were then processed for fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975), exposed to Kodak X-Omat XR-5 films and scanned with a Joyce Loebl Mark IIIC microdensitometer.

Determination of RNA

The RNA content in homogenate, free and bound polyribosomes and in the fractions assayed for protein synthesis was determined by the orcinol method (Mejbaum, 1939), with hydrolysed yeast rRNA as a standard.

Determination of radioactivity

Acid-insoluble radioactivity in total protein and immunoprecipitates was determined by liquid-scintillation spectrometry in a Packard Tri-Carb spectrometer model 3365 with an efficiency of 20% and a background of 15c.p.m.; the standard error was 5% or better.

Statistical treatment

The difference of the means was tested for statistical significance by the analysis of variance.

Results and discussion

Protein synthesis and RNA content in various subcellular fractions

Amino acid incorporation into protein by post-mitochondrial supernatant is thought to be the assay *in vitro* most closely reflecting the situation *in vivo* (Richardson *et al.*, 1971). Accordingly, we investigated the protein-synthetic activity of liver post-mitochondrial supernatant from control and post-ischaemic rats. At 16h after the re-establishment of blood supply, [^{3}H]leucine incorporation into protein per μg of RNA by post-ischaemic-liver postmitochondrial supernatants was not significantly different from that of controls (Table 1, part A). In addition, the synthetic activity per μg of RNA of bound and free polyribosomes in a cell-free protein-synthesizing system, with S_{30} wheat-germ supernatant as a source of soluble factors, was similar in control and post-ischaemic animals (Table 1, part A). Analogous results were found with homologous cell sap (results not shown). Protein synthesis is assumed to be dependent not only on the efficiency of the protein-synthesizing machinery but also on the amount of cellular RNA. Since the RNA content/g of fresh liver in the subcellular fractions used for incubation *in vitro* was the same for control and post-ischaemic rats (results not shown), we can conclude also that total protein synthesis is not altered during post-ischaemic recovery. However, changes in the relative synthesis of specific proteins cannot be ruled out.

Relative rate of albumin synthesis

To detect alterations in the relative proportions of specific proteins, we investigated the effect of post-ischaemic recovery on the incorporation *in vivo* of [^{3}H]leucine into liver albumin relative to the incorporation into total protein. The relative proportion of albumin synthesis was determined by precipitation of labelled albumin with specific antibody. For this reason the specificity of the antibody for rat liver albumin was previously investigated.

Table 1. *Total protein (A) and albumin synthesis (B) by various subcellular fractions from control (C) and 16 h-post-ischaemic (PI) livers*

Radioactivity incorporated into total protein of postmitochondrial supernatants was determined in preparations incubated *in vitro*; percentage of radioactivity incorporated into albumin was determined in postmitochondrial supernatants prepared from livers labelled *in vivo* as described in the Materials and methods section. Other fractions were prepared and incubated *in vitro* as specified in the Materials and methods section. Radioactivity incorporated into total protein and into albumin was determined on the same sample. In all subcellular fractions, radioactivity into total protein was measured by the hot-trichloroacetic acid procedure, and the relative proportion of albumin synthesis was determined by precipitation of labelled albumin with specific antibody as described in the Materials and methods section. Values represent means ± S.E.M. for four experiments.

	(A) Radioactivity incorporated into total protein (c.p.m./μg of RNA incubated)		(B) Radioactivity incorporated in albumin (%)	
	C	PI	C	PI
Postmitochondrial supernatant	47.6 ± 5.8	40.3 ± 1.6	16.5 ± 3.7	9.5 ± 1.3
Bound ribosomes	29400 ± 1450	25400 ± 1150	5.0 ± 0.6	2.9 ± 0.4
Free ribosomes	35900 ± 2320	39000 ± 2100	—	—
Retyculocyte lysate with total cellular poly(A)$^+$ RNA	16205 ± 2078	16031 ± 2999	8.4 ± 0.4	4.5 ± 0.5

Fig. 1(*a*) represents the electrophoretic pattern of total peptides and immunoprecipitable material from [^{3}H]leucine-labelled postmitochondrial supernatant of control and post-ischaemic livers: the immunoprecipitate co-migrates as a single band, at the same position as homogeneous rat serum albumin. Albumin accounts for 16.5% of total protein in control animals; recovery from ischaemia decreases this to 9.5% of total protein (Table 1, part B). Incorporation of [^{3}H]leucine into both albumin and total liver protein was determined after a 12 min pulse *in vivo*. Labelled protein is not secreted within the 12 min labelling period (Peters, 1962), and the leucine content of albumin is similar to that of total protein (Peters, 1975; Schreiber *et al.*, 1971); therefore radioactivity in albumin, relative to that in total protein, approximates to the relative rate of albumin synthesis (Peavy *et al.*, 1981). In addition, the calculation of the rate of albumin synthesis in relation to the rate of total protein synthesis normalizes possible differences in [^{3}H]leucine specific radioactivity among different animals as well as minor variations in the absorption of the amino acids by the liver (Keller & Taylor, 1976).

Quantification of translatable albumin mRNA

Data in part B of Table 1 show a diminished synthesis of albumin *in vitro* relative to total protein by membrane-bound ribosomes from post-ischaemic liver cells incubated in the presence of S_{30} supernatant from wheat germ. Protein synthesis by bound polyribosomes has been interpreted as being dependent on polyribosome-bound mRNA, since Yap *et al.* (1977) demonstrated that 98% of liver polyribosomal albumin mRNA is found in the membrane-bound polyribosomal fraction. The absolute value of albumin synthesis by bound polyribosomes *in vitro* is lower than that *in vivo* in both control and post-ischaemic liver, and this may be explained by the fact that the extent of polypeptide release *in vitro* declines rapidly with increasing polypeptide molecular weight (Ramsey & Steele, 1976); the percentage decrease in albumin synthesis is of the same magnitude. Since the liver contains a substantial amount of poly(A)$^+$ RNA not bound to polyribosomes (Atryzek & Fausto, 1979), in order to detect whether the decreased rate of albumin synthesis was associated with decreased functional albumin mRNA, we used a translational assay for quantifying albumin mRNA in total cellular extracts. Part B of Table 1 shows the decrease in the amount of translatable albumin mRNA relative to total poly(A)$^+$ mRNA in liver of rats recovering from ischaemia. The translational assay was characterized with respect to kinetics, ionic requirement and RNA dependence for incorporation into total proteins. Different mRNA species may be translated with different efficiencies in the system *in vitro* (Tse & Taylor, 1977; Williams *et al.*, 1978). It seemed reasonable to us to assume that RNA isolated from either control or post-ischaemic liver should not vary greatly in translational efficiency. A difference between the amounts of proteins synthesized *in vivo* and *in vitro* by using mRNA from control rats has been found by some investigators (Esumi *et al.*, 1980; Towle *et al.*, 1980) and by us (Table 1, part B), and may be due to the fact that translation conditions *in vitro* may not have reflected

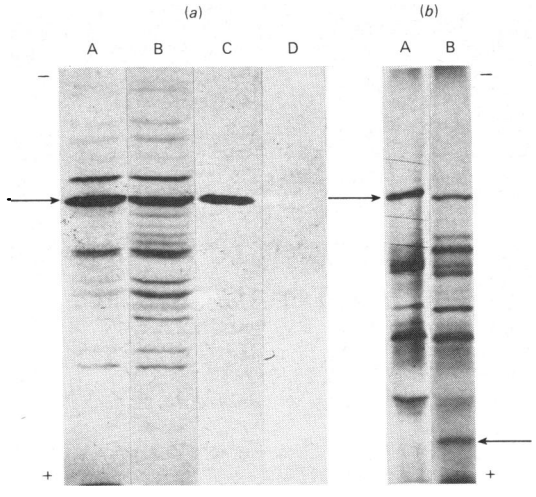

Fig. 1. *Fluorograms of SDS/polyacrylamide-gel electrophoresis (a) of total protein and immunoprecipitable material from rat liver postmitochondrial supernatant labelled in vivo and (b) of total translation products*

(*a*) Total proteins and immunoprecipitates were prepared as described in the Materials and methods section. Lanes: A, total protein from control rat liver; B, total protein from post-ischaemic liver; C, immunoprecipitate of total proteins from control rat liver with anti-(rat serum albumin) monospecific antibody; D, as in C, except that immunoglobulin G from preimmune serum was used. (*b*) Total proteins were synthesized in a rabbit reticulocyte-lysate cell-free system primed with poly(A)$^+$ RNA and prepared for electrophoresis as described in the Materials and methods section. Lanes: A, translation products of hepatic mRNA from control rat; B, translation products of hepatic mRNA from post-ischaemic rat. Slab-gel electrophoresis and fluorography were performed as described in the Materials and methods section. Arrows on the left of each gel represent the migration position of homogeneous rat serum albumin run in parallel slot of each gel. Arrows on the right of Fig. 1.(*b*) indicates the additional polypeptide band (see the text). Similar amounts of peptides labelled *in vivo* or *in vitro* were applied in each of the gel slots.

conditions *in vivo* accurately. For the reason reported above, this should not influence the observed decrease in active albumin mRNA molecules found in liver recovering from ischaemia. Moreover the decrease has the same magnitude when either polyribosomes or total cellular poly(A)$^+$ RNA are used to direct cell-free translation, thus making unlikely the possibility of release to the cytosol of polyribosome-bound mRNA for albumin during the post-ischaemic recovery.

Presence of abnormal proteins

Fluorograms of SDS/polyacrylamide-gel electrophoresis of total protein synthesized by normal and post-ischaemic liver poly(A)$^+$ RNA show a polypeptide peak, banding at low molecular weight, which seems to be present only in the product obtained with post-ischaemic poly(A)$^+$ RNA (Fig. 1*b*). This polypeptide deserves further characterization, but its presence suggests that post-ischaemic liver might synthesize proteins not detectable in the normal tissue.

α-Foetoprotein is a foetal protein whose synthesis is resumed in adult livers during regeneration after partial hepatectomy and in tumours such as hepatomas and teratocarcinomas (Rouslahti & Seppala, 1979). As albumin synthesis is diminished in most of the processes where α-foetoprotein is re-expressed, we decided to investigate if α-foetoprotein synthesis was resumed in post-ischaemic livers. However, sera from our post-ischaemic rats, tested for the presence of α-foetoprotein in Professor Sell's laboratory, failed to show significant increases over the normal very low α-foetoprotein content. Values (means ± S.E.M. of five experiments) were $1.43 \pm 0.16\,\mu g/100\,ml$ at 8h and 0.98 ± 0.10 at 16h post-ischaemia, compared with 1.10 ± 0.24 in sham-operated controls. The data presented above show that in rats recovering from ischaemia overall protein synthesis does not change, but qualitative differences exist, as shown by a 40–50% decrease in the relative rate of synthesis of albumin at this stage of post-ischaemic recovery. The defect can be ascribed to a relative decrease in the functionally active albumin mRNA. We hope that complementary-DNA/mRNA hybridization experiments will give a more definite indication whether the number of albumin-mRNA sequences is actually diminished in post-ischaemic livers. A decrease in albumin synthesis, accompanied by an unchanged total protein synthesis, indicates that the production of other proteins, probably 'housekeeping' proteins for the internal use of the cell, becomes more important in the post-ischaemic liver. This is easy to understand in cells recovering from a period of ischaemia and repairing the damage inflicted on them by the interruption of the blood supply.

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