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Synthesis, intracellular transport, and processing of the precursors for mitochondrial ornithine transcarbamylase and carbamoyl-phosphate synthetase I in isolated hepatocytes

(mitochondrial enzyme precursors/extramitochondrial pool/import and processing)

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ABSTRACT **The synthesis and intracellular transport of the mitochondrial matrix enzymes ornithine transcarbamylase (carbamoylphosphate: L-ornithine carbamoyltransferase, EC 2.1.3.3.) and carbamoyl-phosphate synthetase (ammonia) I [carbon-dioxide:ammonia ligase (ADP-forming, carbamate-phosphorylating), EC 6.3.4.16] were studied in isolated rat hepatocytes. In pulse experiments at 37°C, the larger precursors of the two enzymes appeared in the cytosol of the liver cells, where radioactivity levels of the precursors reached a plateau in 10–20 min after the pulse. The pulse-labeled mature enzymes appeared in the particulate fraction (containing mitochondria) after a time lag and increased almost linearly with time up to 40 min. The specific radioactivities of the precursors in the cytosol were much higher than those of the mature enzymes in the particulate fraction. In pulse–chase experiments, the labeled precursors disappeared from the cytosol with estimated half-lives of about 1–2 min. These results indicate that ornithine transcarbamylase and carbamoyl-phosphate synthetase I are initially synthesized as larger precursors and exist in a cytosolic pool from which they are transported into mitochondria and processed there to the mature enzymes concomitantly with or immediately after transport. Although the rates of synthesis, transport, and processing were decreased about 3-fold at 25°C (as compared to incubation at 37°C), the pool size of the precursors in the cytosol were somewhat larger at this temperature.**

Carbamoyl-phosphate synthetase (ammonia) I [CPSase; carbon-dioxide:ammonia ligase (ADP-forming, carbamate-phosphorylating), EC 6.3.4.16] and ornithine transcarbamylase (OTCase; carbamoylphosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3.) catalyze the first and second steps of urea biosynthesis. The former enzyme exists in a monomer–dimer equilibrium of the subunit of M_r 156,000–165,000 (1–7) and the latter enzyme consists of three identical subunits of M_r 35,300–39,600 (8–13). Both enzymes are localized in the liver mitochondrial matrix of ureotelic animals (14). The enzymes are coded by nuclear genes, synthesized on cytoplasmic ribosomes, and subsequently transported into the mitochondrial matrix.

Studies in our (15, 16) and other laboratories (17, 18) have shown that the enzymes are synthesized in larger molecular weight forms (precursors pCPSase and pOTCase) in heterologous cell-free protein-synthesizing systems. These precursors appear to exist in highly aggregated forms (19). We further have shown that pCPSase was converted to an apparently mature form by a mitochondrial membrane preparation of rat liver (15) and that pOTCase was transported into isolated mitochondria in association with the processing of pOTCase to the mature form of the enzyme (16). The processing of pOTCase appears to involve a neutral protease present in mitochondria (20). Raymond and Shore (21, 22) reported that pCPSase is synthesized on membrane-free polysomes and that the processing of pCPSase is inhibited by a protease inhibitor, *p*-aminobenzamidine. We have shown that pOTCase also is synthesized on free polysomes and is detectable in the cytosol of the liver cells (23). However, detailed kinetic studies of the synthesis, processing, and intracellular transport of pOTCase and pCPSase have not been reported. The present paper describes the results of such studies with isolated rat hepatocytes.

MATERIALS AND METHODS

Materials. [^{35}S]Methionine (>1000 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) was obtained from New England Nuclear, digitonin was from Wako Pure Chemical Industries (Tokyo, Japan), and microbial protease inhibitors (antipain, leupeptin, chymostatin, and pepstatin) were from the Peptide Institute (Osaka, Japan).

Preparation of Isolated Hepatocytes. Male Wistar rats, weighing 200–250 g, were fed a commercial laboratory chow (crude protein content, 21%) ad lib and fasted for 24 hr before the experiments. Hepatocytes were isolated by a modification (24) of the method of Berry and Friend (25).

Incubation and Subcellular Fractionation of Isolated Hepatocytes. The cells (≈2.5 × 10^7 cells per ml) were incubated with [^{35}S]methionine (170–320 μCi/ml) at 37°C or 25°C with shaking at 115 oscillations per min under air in scintillation vials or test tubes in a medium (pH 7.6) described by Ries *et al.* (26) but modified as follows: 65 mM KCl/3.3 mM $MgCl_2$/2.5 mM $CaCl_2$/71 mM NaCl/6.2 mM sodium phosphate and a mixture of 19 amino acids free of methionine (0.1 mM each). Particulate components and the cytosol fraction of the cells were separated by the method of Zuurendonk and Tager (27) modified as follows. A portion of the cell suspension (10–100 μl) was mixed with a digitonin solution so that the final mixture (0.5 ml) was 20 mM potassium Hepes/0.25 M sucrose/3 mM EDTA/0.4 mM antipain/0.4 mM leupeptin/0.4 mM chymostatin/0.4 mM pepstatin containing 1 mg of digitonin per ml. After 2 min at 0°C, the mixture was rapidly centrifuged for 1 min at 14,000 × *g* in an Eppendorf 5412 Microfuge. The supernatant (0.5 ml)

Abbreviations: OTCase, ornithine transcarbamylase; CPSase, carbamoyl-phosphate synthetase (ammonia)I; pOTCase, precursor of OTCase; pCPSase, precursor of CPSase.

was mixed with 0.5 ml of 20 mM Tris·HCl/0.2% $NaDodSO_4$/0.2% Triton X-100/4 mM EDTA, pH 7.4. The pellet was dissolved in a solution (1.0 ml, pH 7.4) containing 10 mM Tris·HCl, 0.1% $NaDodSO_4$, 0.1% Triton X-100, 2 mM EDTA, and the four protease inhibitors (0.2 mM each). Insoluble material was removed by centrifugation.

When chase experiments were performed, the cell suspension was mixed with 3 vol of the medium described, but which contained 20 mM cold methionine instead of [^{35}S]methionine. After a chase for the indicated times at 37°C or 25°C, the cells were fractionated into the cytosol fraction and the particulate components.

Immunoprecipitations. The cytosol fraction from about 2.5 × 10^6 cells and the particulate extract from about 2.5 × 10^5 cells were subjected to immunoprecipitation with 10 μl of an antiserum to bovine liver OTCase and 100 μl of 10% *Staphylococcus aureus* cells as described (28). The cytosol fraction from about 2.5 × 10^5 cells and the particulate extract from about 2.5 × 10^4 cells were subjected to immunoprecipitation with 20 μl of an antiserum to rat liver CPSase and 100 μl of 10% (wt/vol) *S. aureus* cells.

Preparations and Other Methods. Antisera to rat liver CPSase and bovine liver OTCase were prepared as described (15, 16). Fixed *S. aureus* cells were prepared as described (16). $NaDodSO_4$/polyacrylamide (6% or 12%) slab gel electrophoresis (29) and fluorography (30) were performed by the cited methods. The diphenyloxazole-impregnated gel strips containing radioactive polypeptides were cut out and assayed for radioactivity in a toluene scintillant with an efficiency of 77% (28). Trichloroacetic acid-insoluble radioactivity was measured as described (31).

RESULTS

Isolated rat hepatocytes were fractionated into the cytosol and particulate fractions by the digitonin procedure of Zuurendonk and Tager (27). Under the conditions used, more than 80% of the activity of a cytosol enzyme, lactate dehydrogenase, was recovered in the cytosol fraction, whereas less than 5% of the OTCase activity appeared in the same fraction. When the cells were incubated with [^{35}S]methionine, the radioactivity was actively incorporated into the cytosol and particulate fractions (Fig. 1). The incorporation into both fractions was somewhat slower for the first 5–10 min and then increased almost linearly with time up to 40 min. No delay of the incorporation of [^{35}S]methionine into the particulate fraction compared to that into the cytosol fraction was seen. The incorporation into both fractions stopped almost instantly when the cells were chased by the addition of excess cold methionine.

The subcellular fractions were subjected to immunoprecipitation with anti-OTCase, and the immunoprecipitates were analyzed by $NaDodSO_4$ gel electrophoresis and fluorography. The larger precursor, pOTCase, was found exclusively in the cytosol fraction, and the radioactivity in pOTCase increased linearly for the first 10 min and then more slowly until a plateau (30–40 dpm/10^6 cells; 1 dpm = 16.7 mBq) was reached in 10–20 min of the pulse (Fig. 2). On the other hand, the labeled mature enzyme was found exclusively in the particulate fraction. The radioactivity in the mature enzyme appeared after a lag time of a few min and then increased steeply and almost linearly with time up to 40 min. The radioactivity in the mature enzyme was several times higher than that in pOTCase even at 5 min of chase and was 20 times higher at 20 min. The pulse-labeled pOTCase disappeared almost completely from the cytosol in 10 min of the subsequent chase, and the radioactivity in the mitochondrial mature form increased during the chase period. When the gel was stained with Coomassie blue, the OTCase protein was clearly seen at the position of the labeled mature enzyme, whereas no protein band was detected at the position of pOTCase. Thus, the specific radioactivity of pOTCase was much higher than that of the mature enzyme.

Similar kinetics were obtained with CPSase. The labeled enzyme precursor (pCPSase) appeared in the cytosol fraction, and the radioactivity reached a maximum after about 20 min of pulse and then decreased somewhat (Fig. 3). On the other hand, the radioactivity in the mature enzyme was first detected after

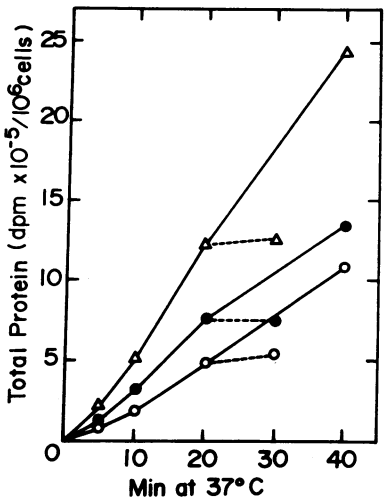

FIG. 1. Incorporation of [^{35}S]methionine into proteins of the cytosol and particulate fractions of isolated hepatocytes. The hepatocytes (2.7 × 10^7 cells) were incubated at 37°C in 0.8 ml of the medium containing 150 μCi of [^{35}S]methionine. Aliquots (100 μl) were removed at indicated times during the pulse, and the trichloroacetic acid-precipitable radioactivities of the cytosol (○), of the particulate (●), and of the cytosol plus particulate fractions (△) were measured. After 20 min of the incubation, an aliquot (100 μl) was chased for 10 min at 37°C in the presence of excess cold methionine (----).

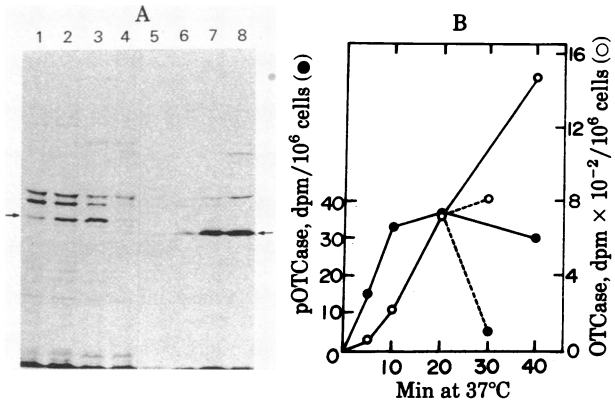

FIG. 2. Synthesis of pOTCase and the mature enzyme in isolated hepatocytes. The cells were those of Fig. 1. (*A*) Cells were pulsed with [^{35}S]methionine for 5 min (lanes 1 and 5), 10 min (lanes 2 and 6), and 20 min (lanes 3 and 7) or were pulsed for 20 min and then chased for 10 min (lanes 4 and 8). Whole samples of the cytosol fractions (lanes 1–4) and 10% of the particulate extracts (lanes 5–8) were subjected to immunoprecipitation, followed by $NaDodSO_4$/12% N (wt/vol) polyacrylamide gel electrophoresis and fluorography (10 days exposure at −80°C). Arrows, positions of pOTCase and the mature enzyme. (*B*) Radioactivities of pOTCase in the cytosol fraction (●) and of the mature enzyme in the particulate extract (○) were measured as described. ----, Data of the chase experiments.

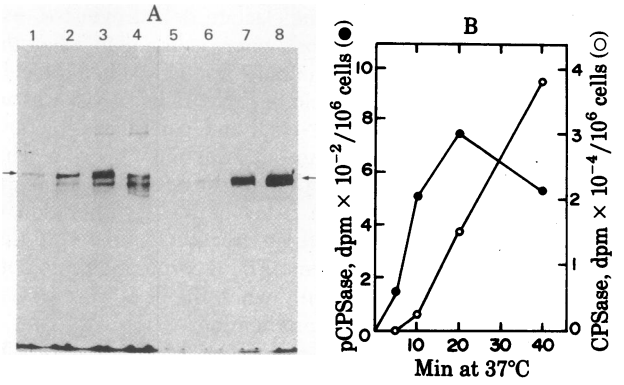

FIG. 3. Synthesis of pCPSase and the mature enzyme in isolated hepatocytes. (*A*) Hepatocytes (2.2×10^6 cells) were incubated at 37°C in 0.1 ml of the medium containing 30 μCi of [^{35}S]methionine. Aliquots (10 μl) were removed at 5 min (lanes 1 and 5), 10 min (lanes 2 and 6), 20 min (lanes 3 and 7), and 40 min (lanes 4 and 8) of pulse. Whole samples of the cytosol fractions (lanes 1–4) and 10% of the particulate extracts (lanes 5–8) were subjected to immunoprecipitation, followed by $NaDodSO_4$/6% polyacrylamide gel electrophoresis and fluorography (10 days). Arrows, positions of pCPSase and the mature enzyme. (*B*) The radioactivities of pCPSase in the cytosol fraction (●) and of the mature enzyme in the particulate extract (○) were measured as described. Radioactivities in total protein (cytosol fraction plus particulate extract) at 5, 10, 20, and 40 min of the pulse were 1.02, 2.21, 4.40, and 6.72×10^6 dpm per 10^6 cells, respectively.

10 min in the particulate fraction and then increased linearly up to 40 min. The ratio of the radioactivity in the mature enzyme to that in pCPSase at 20 min of the pulse was 10:20 (see also Fig. 5), the value being similar to or a little lower than that for OTCase. The radioactive polypeptide that moved faster than pCPSase (Fig. 3*A*, lanes 1–4) and was slightly smaller than the mature enzyme (see also Fig. 5*A*) appeared to be a degradation product of pCPSase; the band was always very intense after a long incubation. Protein staining of the gel showed that the specific radioactivity of pCPSase was much higher than that of the mature enzyme.

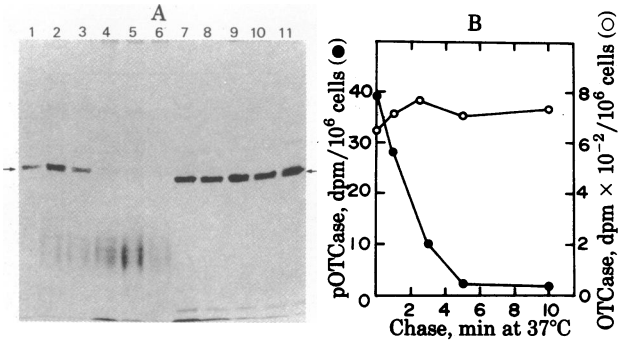

FIG. 4. Kinetics of pOTCase disappearance from the cytosol in pulse–chase experiments. (*A*) Hepatocytes (2.2×10^7 cells) were pulsed with 120 μCi of [^{35}S]methionine for 20 min at 37°C in 0.7 ml of the medium and were chased by the addition of 2.1 ml of the medium containing 20 mM cold methionine. Aliquots (400 μl) were removed at 0 min (lanes 2 and 7), 1 min (lanes 3 and 8), 3 min (lanes 4 and 9), 5 min (lanes 5 and 10), and 10 min of chase (lanes 6 and 11) at 37°C, and the cytosol fractions (1.0 ml) and the particulate extracts (1.0 ml) were prepared. Portions (0.9 ml) of the cytosol fractions (lanes 2–6) and 90-μl portions of the particulate extracts (lanes 7–11) were subjected to immunoprecipitation, followed by $NaDodSO_4$/12% polyacrylamide gel electrophoresis and fluorography (9 days). Lane 1, pOTCase synthesized in a rabbit reticulocyte lysate system (16). Arrows, positions of pOTCase and the mature enzyme. (*B*) The radioactivities of pOTCase in the cytosol fractions (●) and of the mature enzyme in the particulate extracts (○) were measured as described.

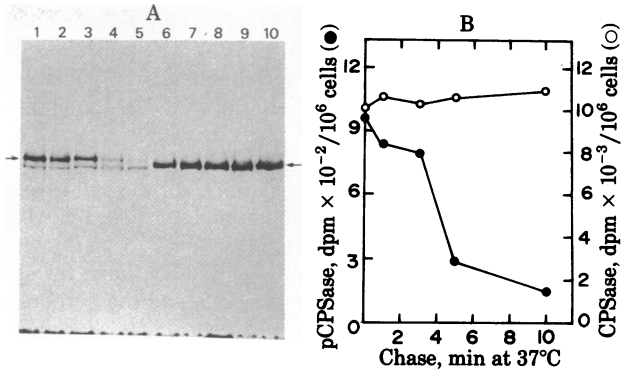

FIG. 5. Kinetics of pCPSase disappearance from the cytosol in pulse–chase experiments. The cytosol fractions of the hepatocytes and the particulate extracts were those in Fig. 4. (*A*) Aliquots (0.1 ml) of the cytosol fractions (lanes 1–5) and 10-μl aliquots of the particulate extracts (lanes 6–10) were subjected to immunoprecipitation, followed by $NaDodSO_4$/6% polyacrylamide gel electrophoresis and fluorography (9 days). Chase period was for 0 min (lanes 1 and 6), 1 min (lanes 2 and 7), 3 min (lanes 3 and 8), 5 min (lanes 4 and 9), and 10 min (lanes 5 and 10). Arrows, positions of pCPSase and the mature enzyme. (*B*) The radioactivities of pCPSase in the cytosol fractions (●) and of the mature enzyme in the particulate extracts (○) were measured as described.

Kinetic aspects of pOTCase disappearance in pulse–chase experiments are shown in Fig. 4. The pulse-labeled pOTCase disappeared from the cytosol with an apparent half-life of 2 min. The actual half-life of pOTCase is presumably somewhat shorter because completion of the labeled nascent peptide of pOTCase would continue during the chase period. The radioactivity of the mature enzyme showed a substantial increase during the chase.

Kinetic aspects of pCPSase disappearance in pulse–chase experiments are shown in Fig. 5. The pulse-labeled pCPSase disappeared from the cytosol slowly for the first 3 min of the chase and then more rapidly. The pattern of disappearance differed from that of pOTCase. The difference is probably due to the difference in the subunit molecular weight of the two precursors (165,000 and 39,400 for pCPSase and pOTCase, respectively). Elongation and completion of the radiolabeled nascent peptide of pCPSase would proceed for a longer time during the chase compared to that of pOTCase, resulting in the apparent delay in the disappearance of the labeled pCPSase. The half-life of pCPSase, estimated from the values after 3 min of the chase, was about 2 min. The radioactivity of the mature enzyme in the particulate extract increased significantly during the chase period.

In an attempt to accumulate the precursors in the isolated hepatocytes, pulse–chase experiments were carried out at 25°C. [^{35}S]Methionine incorporation into total protein at 25°C was about 35% of that at 37°C. The rate of incorporation of radioactivity into pOTCase and into mature enzyme was decreased compared with the rate at 37°C, but the pattern was otherwise similar to that observed at 37°C (Fig. 6). The maximal radioactivity in pOTCase at 25°C was substantially higher than that at 37°C. Pulse-labeled pOTCase disappeared from the cytosol in 10 min of chase.

[^{35}S]Methionine incorporation into pCPSase in the cytosol took place more slowly at 25°C, and the radioactivity in pCPSase did not reach a plateau in 40 min of pulse (Fig. 7). The labeled mature enzyme appeared in the particulate fraction with a lag time of 20 min and then increased. The appearance of the putative degradation product of pCPSase was less evident at 25°C.

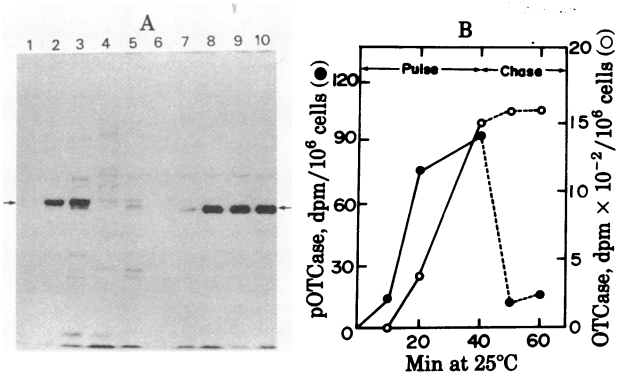

FIG. 6. Synthesis of pOTCase and the mature enzyme in isolated hepatocytes at 25°C. (*A*) The hepatocytes (1.8×10^7 cells) were incubated at 25°C in 0.7 ml of the medium containing 220 μCi of [^{35}S]methionine. Aliquots (0.1 ml) were removed at 10 min (lanes 1 and 6), 20 min (lanes 2 and 7), and 40 min of pulse (lanes 3 and 8). After 40 min of pulse, 0.1-ml aliquots were chased for 10 min (lanes 4 and 9) and 20 min (lanes 5 and 10) at 25°C in the presence of excess cold methionine. Whole samples of the cytosol fractions (lanes 1–5) and 10% of the particulate extracts (lanes 6–10) were subjected to immunoprecipitation, followed by NaDodSO$_4$/12% polyacrylamide gel electrophoresis and fluorography (9 days). Arrows, positions of pOTCase and the mature enzyme. (*B*) The radioactivities of pOTCase in the cytosol fraction (●) and of the mature enzyme in the particulate extract (○) were measured as described. ----, Data of the chase experiments. Radioactivities in total protein (cytosol fraction plus particulate extract) at 10, 20, and 40 min of pulse and at 40 min of pulse plus 10 and 20 min of chase were 0.29, 1.11, 3.56, 3.60, and 3.45×10^6 dpm per 10^6 cells, respectively.

DISCUSSION

The studies described here present a detailed kinetic study of the synthesis of two mitochondrial matrix proteins and their precursors in higher animals and their subcellular localizations. The results show most clearly that rat liver OTCase and CPSase are synthesized in the cytosol as larger precursors, which are transported from the cytosol into the mitochondria where they are processed to the mature enzymes. The extramitochondrial pools of the precursors are small and their half-lives at 37°C are estimated to be 1–2 min. Raymond and Shore (22) reported a similar half-life of pCPSase (about 2 min) in rat liver explants. The short half-life of pOTCase is in accord with our earlier observation with rat liver slices (23) that the pulse-labeled pOTCase disappeared rapidly from the cytosol during the chase period. The half-life values can well explain the ratios of the radioactivity levels of the mature enzymes to those of their precursors, which are of the order of 10:20 at 20 min of pulse. The proteolytic processing of the precursors is thought to occur concomitantly with or immediately after the transport because no precursor was detected in the particulate fraction. When pulse-labeling experiments were carried out at 25°C, the rates of the precursor transport (processing) and of their synthesis were slowed down about 3-fold, whereas the pool sizes of the pulse-labeled precursors were somewhat larger at this temperature. Thus, these conditions may be useful for studies of the molecular mechanisms involved in the intracellular transport and processing of the precursors. The present results are in sharp contrast to those reported by Yamauchi *et al.* (32) for δ-aminolevulinate synthetase of rat liver, another mitochondrial matrix enzyme. Under conditions where the enzyme is extensively induced, a large amount of the enzyme precursor accumulates in the cytosol fraction and is subsequently transferred to the mitochondria.

The events involved in the synthesis, translocation, and processing of the mitochondrial matrix proteins in rat liver appear to be very similar to those that are known to operate in lower eukaryotes (33–35). Although it was reported that isolated hepatocyte preparations could not be used for such studies (22), our results demonstrate the usefulness of such preparations.

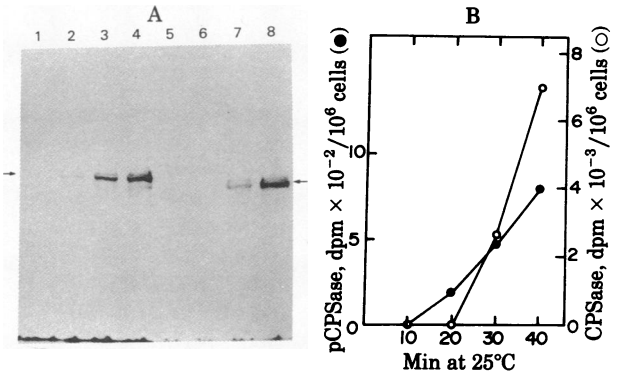

FIG. 7. Synthesis of pCPSase and the mature enzyme in isolated hepatocytes at 25°C. (*A*) The hepatocytes (2.2×10^6 cells) were incubated at 25°C in 0.1 ml of the medium containing 30 μCi of [^{35}S]methionine. Aliquots (10 μl) were removed at 10 min (lanes 1 and 5), 20 min (lanes 2 and 6), 30 min (lanes 3 and 7), and 40 min of pulse (lanes 4 and 8). Whole samples of the cytosol fractions (lanes 1–4) and 10% of the particulate extracts (lanes 5–8) were subjected to immunoprecipitation, followed by NaDodSO$_4$/6% polyacrylamide gel electrophoresis and fluorography (12 days). Arrows, positions of pCPSase and the mature enzyme. (*B*) The radioactivities of pCPSase in the cytosol fraction (●) and of the mature enzyme in the particulate extracts (○) were measured as described. Radioactivities in total protein (cytosol fraction plus particulate extract) at 10, 20, 30, and 40 min of the pulse were 0.45, 0.97, 2.02, and 2.47×10^6 dpm per 10^6 cells, respectively.

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1. Guthöhrlein, G. & Knappe, J. (1978) *Eur. J. Biochem.* **7**, 119–127.
2. Virden, R. (1972) *Biochem. J.* **127**, 503–508.
3. Marshall, M. (1976) in *The Urea Cycle*, eds. Grisolia, S., Báguena, R. & Mayor, F. (Wiley, New York), pp. 133–142.
4. Clarke, S. (1976) *J. Biol. Chem.* **251**, 950–961.
5. Mori, M. & Cohen, P. P. (1978) *J. Biol. Chem.* **253**, 8837–8839.
6. Lusty, C. J. (1978) *Eur. J. Biochem.* **85**, 373–383.
7. Pierson, D. L. & Brien, J. M. (1980) *J. Biol. Chem.* **255**, 7891–7895.
8. Marshall, M. & Cohen, P. P. (1972) *J. Biol. Chem.* **247**, 1641–1653.
9. Clarke, S. (1976) *Biochem. Biophys. Res. Commun.* **71**, 1118–1124.
10. Pierson, D. L., Cox, S. L. & Gilbert, B. E. (1977) *J. Biol. Chem.* **252**, 6464–6469.
11. Kalousek, F., François, B. & Rosenberg, L. E. (1978) *J. Biol. Chem.* **253**, 3939–3944.
12. Lusty, C. J., Jilka, R. L. & Nietsch, E. H. (1979) *J. Biol. Chem.* **254**, 10030–10036.
13. Hoogenraad, N. J., Sutherland, T. M. & Howlett, G. J. (1980) *Anal. Biochem.* **101**, 97–102.
14. Gamble, J. G. & Lehninger, A. L. (1973) *J. Biol. Chem.* **248**, 610–618.
15. Mori, M., Miura, S., Tatibana, M. & Cohen, P. P. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5071–5075.
16. Mori, M., Miura, S., Tatibana, M. & Cohen, P. P. (1980) *J. Biochem. (Tokyo)* **88**, 1829–1836.
17. Shore, G. C., Carignan, P. & Raymond, Y. (1979) *J. Biol. Chem.* **254**, 3141–3144.
18. Conboy, J. G., Kalousek, F. & Rosenberg, L. E. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5724–5727.
19. Miura, S., Mori, M., Amaya, Y., Tatibana, M. & Cohen, P. P. (1981) *Biochem. Int.* **2**, 305–312.

20. Mori, M., Miura, S., Tatibana, M. & Cohen, P. P. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7044–7048.
21. Raymond, Y. & Shore, G. C. (1979) *J. Biol. Chem.* **254**, 9335–9338.
22. Raymond, Y. & Shore, G. C. (1981) *J. Biol. Chem.* **256**, 2087–2090.
23. Morita, T., Mori, M., Tatibana, M. & Cohen, P. P. (1981) *Biochem. Biophys. Res. Commun.*, **99**, 623–629.
24. Zahlten, R. N., Kneer, N. M., Startman, F. W. & Lardy, H. A. (1974) *Arch. Biochem. Biophys.* **161**, 528–535.
25. Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520.
26. Ries, G., Hundt, E. & Kadenbach, B. (1978) *Eur. J. Biochem.* **46**, 83–88.
27. Zuurendonk, P .F. & Tager, J. M. (1974) *Biochim. Biophys. Acta* **333**, 393–399.
28. Mori, M., Miura, S., Tatibana, M. & Cohen, P. P. (1981) *J. Biol. Chem.* **256**, 4127–4232.
29. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
30. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88.
31. Roberts, B. E. & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2330–2334.
32. Yamauchi, K., Hayashi, N. & Kikuchi, G. (1980) *J. Biol. Chem.* **255**, 1746–1751.
33. Hallermayer, G., Zimmermann, R. & Neupert, W. (1977) *Eur. J. Biochem.* **81**, 523–532.
34. Harmey, M. A., Hallermayer, G., Korb, H. & Neupert, W. (1977) *Eur. J. Biochem.* **81**, 533–544.
35. Schatz, G. (1979) *FEBS Lett.* **103**, 203–211.