Synthesis of Carbamyl Phosphate Synthetase in Liver Slices From Thyroxine-treated Tadpoles*

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Metamorphosis of tadpoles into frogs is accompanied by the induction of the enzymes of the urea cycle in the liver (1), which leads to the transition from ammonotelism in tadpoles to ureotelism in the frog. Paik and Cohen (2) observed that thyroxine, which is known to promote anatomical metamorphosis (3, 4), induces a marked increase in the level of carbamyl phosphate synthetase activity in the liver of tadpoles before gross morphological changes are observed. It was established by Metzenberg, Marshall, Paik, and Cohen (9) in experiments in vivo that the thyroxine-induced increase in carbamyl phosphate synthetase activity represents net synthesis de novo of the enzyme. Evidence for this was based on L-leucine-14C incorporation into carbamyl phosphate synthetase which was precipitated by means of a specific antibody.

The present paper deals with the active incorporation of radioactive leucine into carbamyl phosphate synthetase in liver slices from thyroxine-treated tadpoles. The evidence from this study indicates that carbamyl phosphate synthetase can be synthesized de novo in liver slices of thyroxine-treated tadpoles.

EXPERIMENTAL PROCEDURE

Assays—Carbamyl phosphate synthetase activity was determined according to the procedure of Brown and Cohen (8). A unit of carbamyl phosphate synthetase is the amount of enzyme which produces 1 amole of citrulline in 15 minutes under the conditions of the assay. Protein was assayed by the method of Lowry, Rosebrough, Farr, and Randall (7) with bovine serum albumin as the protein standard.

Preparations—Carbamyl phosphate synthetase was prepared from the liver of bullfrogs (Rana catesbeiana) as described previously (8). Antibody, goat, and rabbit γ-globulin (5, 9), was obtained from Dr. M. Marshall of this laboratory. Pure rabbit antibody was isolated from the γ-globulin by the method of Marshall and Cohen (9).

Animals—Tadpoles of the giant bullfrog (Rana catesbeiana) in premetamorphic stages weighing 4 to 12 g were used. Maintenance of the tadpoles and thyroxine treatment were performed as described previously (2).

Incubation of Liver Slices—The livers were sliced by hand with a Stadie-Riggs type tissue slicer (10) to an average thickness of about 0.4 mm. A modified Krebs-Ringer bicarbonate solution was used for incubation medium, and contained the following constituents: NaCl, 9.58 × 10⁻² m; NaHCO₃, 1.67 × 10⁻２ m; KCl, 1.88 × 10⁻² m; NH₄Cl, 1.0 × 10⁻³ m; sodium phosphate at pH 7.4, 1.0 × 10⁻³ m; CaCl₂, 2.2 × 10⁻³ m; MgCl₂, 1.0 × 10⁻³ m; L-thyroxine (when added), 2.6 × 10⁻⁷ m; glucose, 5.6 × 10⁻⁵ m; amino acid mixture, 100 μg per ml. A solution containing the first three components was gassed with a mixture of 95% O₂ and 5% CO₂ before the addition of the other components. The completed medium was fully equilibrated with the same gas mixture, the final pHi being 7.38 to 7.40 at 30°C. The composition of the amino acid mixture is the same as that described by Borsok, Fischer, and Keighley (11) except for omission of L-hydroxyproline and the addition of L-asparagine. Uniformly labeled L-leucine-14C was obtained from Nuclear-Chicago Corporation. The slices were rinsed with medium, drained on filter paper, and weighed. Incubation was performed in a 125-ml Erlenmeyer flask containing a volume of medium equal to 10 times the weight of slices with an appropriate amount of L-leucine-14C at 30°C in an atmosphere of 95% O₂-5% CO₂. The maximum weight of slices in one flask was 1 g. The flasks were shaken 80 times per minute during incubation.

Isolation of Radioactive Carbamyl Phosphate Synthetase—After incubation, the flasks were cooled, and the slices were washed three times with cold 0.12 m NaCl and once with 0.15 m KCl by repeated suspension and low speed centrifugation. The washed tissue was then homogenized with a volume of medium equal to 10 times the weight of slices with an appropriate amount of L-leucine-14C at 30°C in an atmosphere of 95% O₂-5% CO₂. The maximum weight of slices in one flask was 1 g. The flasks were shaken 80 times per minute during incubation.

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‡ Tadpoles were purchased from the Carolina Biological Supply Company, Elon College, North Carolina, and from the Lemberger Company, Osakis, Wisconsin.

The abbreviation used is: CTB, cetyltrimethylammonium bromide.
added to the extract (usually about 2 μmoles for each 1 ml of extract). The extract was equilibrated with 0.005 M sodium succinate, pH 5.9, by dialysis or by means of a column of Sephadex G-25 (12), and fractionated on a small column of phosphocellulose with a bed volume of 2 ml for each gram of original tissue slices, followed by chromatography on a column of DEAE-cellulose. A measured amount of purified frog liver carbamyl phosphate synthetase was added to the tadpole preparation before each of the two chromatographic procedures in some experiments. Proteins precipitated from the supernatant fraction were fractionated in the same way.

Precipitin Reaction for Radioactive Carbamyl Phosphate Synthetase—The precipitin reaction was carried out under varying conditions, depending on the enzyme concentration and the nature of buffer in the sample solution. Standard conditions were essentially the same as those described previously (9), based on the procedure of Heidelberger and Kendall as outlined by Kabat and Mayer (13). A volume of sample solution, usually 0.5 to 1.0 ml and containing 3 to 5 units of the enzyme, was adjusted so that the NaCl and Tris-HCl (pH 7.4) concentrations were 0.15 and 0.04 M, respectively, followed by the addition of 0.05 ml of 5 x 10⁻³ M nonradioactive L-leucine and an appropriate amount of antibody, in that order. The mixture was incubated at 37° for 15 minutes, kept overnight in a refrigerator, and washed as described below.

Preparation of Proteins for Measurement of Incorporated Radioactivity—The precipitate formed in the precipitin reaction from a radioactive preparation was washed three times in 0.15 M NaCl and then dissolved in 0.5 ml of 0.1 M NaOH containing 0.1 M nonradioactive L-leucine (5). The protein fraction was precipitated by the addition of 0.25 ml of 30% trichloroacetic acid. The protein was further treated with hot 5% trichloroacetic acid and organic solvent extraction (14). The resulting protein preparation was suspended in 1 ml of acetone and transferred to a vial for liquid scintillation counting. Acetone was completely removed by a stream of warm air from a hair dryer. The protein residue was dissolved in 1 ml of Hyamine 10-X (15), followed by the addition of 10 ml of a toluene solution of 2,5-diphenyloxazole and 1,4-bis-2’-(5’-phenyloxazolyl)benzene. Radioactivity was measured by means of a Packard Tri-Carb liquid scintillation counter. Quenching was determined with a benzoic acid-1,4-C standard. Radioactivity of aqueous solutions was measured with the aid of a naphthalene-dioxane solution described by Bray (16).

Proteins other than the antigen-antibody complex were purified by the procedure of Elckowitz (14) and counted as above.

Tryptic Digestion of Carbamyl Phosphate Synthetase and Separation of Peptides on Filter Paper—A salt-free solution of the purified enzyme was denatured by heating at 85° for 5 minutes. The solution was then cooled, and trypsin (2% by weight) and an amount of 1 M NH₄HCO₃ (pH 8) to give a final concentration of 0.2 M were added. Incubation was performed at 37° for 48 hours with constant stirring. A small amount of toluene was added to prevent bacterial growth. After the incubation was completed, the digest was frozen and stored. An aliquot containing the equivalent of 2.0 mg of the original enzyme was applied to a sheet (36 X 57 cm) of Whatman No. 3MM paper. Peptides were separated by ascending chromatography with pyridine-isomyl alcohol-water (35:35:30) as solvent (17) for 20 hours, followed by electrophoresis with a voltage gradient of 50 volts per cm in a modified Michl apparatus (18) with pyridine acetate buffer, pH 3.7 (19). Spots were located by means of a ninhydrin spray and cut out from the paper. The peptides were eluted with 3.0 ml of 0.1 M NH₄OH for each spot by immersion and shaking in test tubes at 37°. Radioactivity was measured on a 2.0-ml aliquot with the use of naphthalene-dioxane solution (16).

One-dimensional Starch Gel Immunoelectrophoresis—Double immunodiffusion with agar gel after separation of antigen by one-dimensional starch gel electrophoresis (20) was carried out essentially by the method of Poulak (21) except that smaller pieces of starch gel (9.0 x 2.0 x 0.3 cm) and a higher voltage (15 volts per cm) were employed for electrophoresis.

RESULTS AND DISCUSSION

Incorporation of Radioactivity into Carbamyl Phosphate Synthetase—Chromatograms of elution from a DEAE-cellulose column (the final step of enzyme purification) of the samples from thyroxine-treated and nontreated tadpoles are shown in Figs. 1 and 2, respectively. As can be seen, there is a good coincidence of (a) the locations of the peaks of radioactivity, (b) of protein, and (c) of enzyme activity. Although the radioactivity in the peak is not strictly a proportional measure of total radioactivity of the enzyme originally present in the CTB extract, a marked increase in the case of liver slices from thyroxine-treated tadpoles is obvious as regards radioactive L-leucine incorporation into the enzyme. To confirm this, the enzyme was precipitated with antibody from the fractions of highest specific activity. More than 90% of the radioactivity in the eluate of thyroxine-treated samples was recovered in the antigen-antibody complex (672 c.p.m. out of 731 c.p.m. in Fractions 4 to 7; see Fig. 1). In the same way, 36 c.p.m. out of 100 c.p.m. were recovered from the eluate of the preparation from nontreated tadpoles (Fig. 2). From these data, the total radioactivity of carbamyl phosphate synthetase in the initial tissue extract was calculated taking into account the amount of carrier enzyme and recovery of enzyme at each step of purification. The results are shown in Table I along with the radioactivity distribution among the other protein fractions. Although most of carbamyl phosphate synthetase in the tadpole liver, if not all, is located in the mitochondrial fraction, no attempts were made in this experiment to fractionate the cellular components, because of the possibility of the presence of highly radioactive enzyme precursors or enzyme attached to microsomes. The data in Table I show that the remarkable increase in the radioactive leucine incorporation into carbamyl phosphate synthetase is a highly specific response to thyroxine treatment without any significant increase in incorporation into the other protein fractions. These results are in good agreement with those obtained on whole animals by Metzenberg et al. (5) and also with the findings of DeGroot and Cohen (22), who showed that the levels of amino acid-activating enzymes in tadpole livers remained relatively constant during thyroxine-induced metamorphosis. These findings differ from those of Sokoloff and Kaufman (23), who reported that administration of L-thyroxine in vivo, or its addition in vitro, stimulated amino acid incorporation into proteins in cell-free rat liver homogenate. The different results may at least in part be due to the different levels of L-thyroxine used. In the present experiment, tadpoles were kept in water containing 2.0 x 10⁻⁴ M L-thyroxine (2). This concentration is more than 100 times lower than that reported (23) to be necessary for a response in the rat liver cell-free homogenate.
As seen in Table I, there was some carbamyl phosphate synthetase activity in the supernatant fraction. However, it was found in later experiments that most, if not all, of the enzyme in the supernatant fraction is the result of a "leak" from the particulate fractions after tissue homogenization with 0.15 M KCl. Use of 0.25 M sucrrose instead of 0.15 M KCl prevented leakage of carbamyl phosphate synthetase.

**Fingerprinting of Tryptic Digests of Radioactive Carbamyl Phosphate Synthetase**—In order to determine whether the radiolabeled leucine incorporation into the enzyme represented synthesis de novo of peptides in the protein molecule, or some other process such as transpeptidation reported by Suttie (24) in isolated rat liver mitochondria, radioactive carbamyl phosphate synthetase was subjected to tryptic digestion. The peptides obtained were separated on filter paper and the distribution of radioactivity among the peptides was examined. About 60 peptide spots were revealed by a ninhydrin spray, and of these more than 40 were found to contain significant radioactivity. The sum of the radioactivity recovered accounted for 80% of the radioactivity originally applied on paper. These findings indicate that the peptide chain(s) of the enzyme molecule were newly synthesized from amino acids in the liver slices.

**Time Study of Incorporation**—After an initial lag period, the liver slices maintained their ability to incorporate L-leucine-\(^{14}\text{C}\) into carbamyl phosphate synthetase for at least 4 hours under the conditions used (Fig. 3).

**Effect of Addition in Vitro of L-Thyroxine**—The incorporation of leucine into carbamyl phosphate synthetase in liver slices from thyroxine-treated tadpoles proceeded at an identical rate whether in the presence or absence of L-thyroxine (2.6 X 10\(^{-7}\) M) in the incubation medium. The addition of L-thyroxine (2.6 X 10\(^{-7}\) M) to liver slices from nontreated tadpoles had no stimulatory effect on the leucine incorporation into carbamyl phosphate synthetase.

**Effect of Administration in Vivo of High Dose of L-Thyroxine**—The induction of carbamyl phosphate synthetase during thyroxine-induced metamorphosis has a lag period of a few days (2, 5). However, there is a possibility that administration in vivo of a high dose of L-thyroxine can eliminate or shorten that lag period. If this did occur, this could be detected more easily by radio-

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**TABLE I**

| Distribution of radioactivity in carbamyl phosphate synthetase and other protein fractions |
|-----------------------------------------------|------------------|
| Thyroxine-treated | Nontreated \(\text{c.p.m./g slice}\) |
| Particulate fraction | CTB-extractable |
| Carbamyl phosphate synthetase | 19,900 | 890 |
| Other protein | 31,900 | 28,800 |
| Residue | 724,000 | 816,000 |
| Supernatant fraction | Carbamyl phosphate synthetase | 4,550 | 505 |
| Other protein | 215,000 | 257,000 |
| Total | 965,350 | 1,103,285 |

Radioactivity added to the medium as L-leucine-\(^{14}\text{C}\) (1.04 \(\mu\text{moles}\)) \(7,500,000\ \text{c.p.m.}\) 7,500,000 \(\text{c.p.m.}\)
active amino acid incorporation into the enzyme. To test this possibility, tadpoles were given injections of L-thyroxine (10 µg per g of body weight) and kept for 24 hours at 23°. Liver slices were prepared and analyzed for radioactive leucine incorporation into carbamyl phosphate synthetase as well as into the other proteins. It was found that the incorporation into the enzyme was 49% higher in the slices of the thyroxine-treated tadpoles than in the slices from the controls which were treated with 0.7% NaCl. However, the incorporation into the other proteins was also 40% higher in the thyroxine-treated animals. Therefore, the increase in the leucine incorporation into the enzyme can be interpreted to represent only a part of a general increase in protein metabolism caused by treatment with a high dose of thyroxine as reported by Sokoloff and Kaufman (23).

Specific Radioactivity of Carbamyl Phosphate Synthetase Precipitated with Antibody Before and After Enzyme Purification—
There was a considerable fluctuation in the value of specific radioactivity of the enzyme when isolated with antibody directly from a crude CTB extract. In order to eliminate possible contaminants, enzyme purification was carried out before the precipitation reaction. Specific radioactivity of the enzyme was obtained at each step of the purification procedure. Results of an experiment presented in Table II. As can be seen, there is a considerable fall in specific activity after chromatography of the CTB extract on a phosphocellulose column. There is no significant decrease after rechromatography on DEAE-cellulose. Antibody used in this experiment was a γ-globulin fraction from goat serum which has been shown to be specific (5, 9). Its specificity is actually greater than that of the rabbit antibody fraction (9). Further, it was shown by immunoelectrophoresis in starch gel that carbamyl phosphate synthetase is the only protein among the proteins in the CTB extract that forms a flocculation line with the antibody. This observation excludes the possible presence of some protein which can cross-react with the antibody. Precipitation of denatured protein under the conditions used for the precipitin reaction in the experiment shown in Table II was found to be negligible. Thus, the erratically high value of specific radioactivity of the enzyme from a crude CTB extract could be due to nonspecific coprecipitation of other radioactive proteins. Similar findings were reported by von der Decken and Campbell (25) for serum albumin synthesis in a cell-free system of rat liver nucleoprotein particles, and by Helmreich, Kern, and Eisen (26) in their studies of secretion of γ-globulin by isolated lymph node cells.

**SUMMARY**

Active incorporation of radioactivity into carbamyl phosphate synthetase was observed when liver slices from thyroxine-treated tadpoles were incubated with L-leucine-14C. The enzyme was isolated by chromatography on ion exchange cellulose columns followed by precipitation with specific antibody. Both steps were necessary to eliminate contaminating radioactivity. The radioactivity in the enzyme was found to be distributed among most of the peptides obtained by tryptic digestion of the enzyme, indicating that the radioactive leucine incorporation represents synthesis de novo of the enzyme protein.

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