Ribonucleic Acid Synthesis in Porphyric Rat Liver Induced by 3,5-Dicarbethoxy-1,4-dihydrocollidine

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1. Administration of 3,5-dicarbethoxy-1,4-dihydrocollidine to rats caused a marked increase in the activity of δ -aminolaevulinic acid synthetase as well as a slight net increase in RNA in the livers. 2. 3,5-Dicarbethoxy-1,4-dihydrocollidine primarily stimulated the synthesis of RNA in the nucleus of the liver cell. 3. The decrease in RNA synthesis after administration of this drug resulted in a rapid decrease in the activity of this induced enzyme. This was also confirmed by treatment with actinomycin D. 4. From kinetic experiments on synthesis of RNA and δ -aminolaevulinic acid synthetase *in vivo* the induction mechanism of this enzyme was discussed.

δ -Aminolaevulinic acid synthetase, the initial and rate-limiting enzyme in the pathway of porphyrin biosynthesis, is induced by various chemicals such as 3,5-dicarbethoxy-1,4-dihydrocollidine (Granick & Urata, 1963), allylisopropylacetamide, hexachlorobenzene and griseofulvin. The chemically-induced increase in δ -aminolaevulinic acid synthetase activity is due to increased synthesis of the enzyme *de novo* (Marver *et al.*, 1966b).

Hepatic δ -aminolaevulinic acid synthetase has been generally considered to exist exclusively in mitochondria. Hayashi *et al.* (1969) demonstrated that considerable amounts of δ -aminolaevulinic acid synthetase also accumulate in the soluble fraction of chemically induced porphyric rat liver and suggested that δ -aminolaevulinic acid synthetase is synthesized originally in the microsomal fraction and subsequently is transferred into mitochondria.

These findings strongly suggest the importance of the specific action of chemical inducer on RNA synthesis for the induction mechanism of δ -aminolaevulinic acid synthetase in porphyric rat liver. The present studies are directed at the kinetics of δ -aminolaevulinic acid synthetase induction with regard to RNA synthesis in the porphyric rat liver after administration of 3,5-dicarbethoxy-1,4-dihydrocollidine.

Materials and Methods

Treatment of animals

Male albino rats of the Wistar King strain (150–200 g), maintained on MF rat diet, were starved for 12 h after which the crystalline suspension of 3,5-dicarbethoxy-1,4-dihydrocollidine (1.5 g/kg body wt.)

in 0.9% NaCl was administered via a stomach tube every 24 h. The animals were killed 24 h after the administration of this drug unless otherwise stated.

Reagents

3,5-Dicarbethoxy-1,4-dihydrocollidine was supplied by Daiichi Chemical Co. (Tokyo, Japan). Actinomycin D was obtained from Merck, Sharp and Dohme Co. (West Point, Pa., U.S.A.). α -Oxoglutaric acid, glycine and acetyl-CoA were purchased from Wako Chemical Co. (Tokyo, Japan), Daiichi Pure Chemicals Co. (Tokyo, Japan) and Dainippon Chemical Co. (Tokyo, Japan), respectively.

[32 P]P_i and [14 C]orotic acid were obtained from the Japan Radioisotope Association, Tokyo, Japan). Other reagents were obtained commercially.

Analysis of nucleic acids and protein

The animals were decapitated without anaesthesia. The livers were quickly removed and chilled immediately in 5 vol. of ice-cold 0.25 M-sucrose. The livers were weighed and minced with scissors in 5 vol. of ice-cold 0.02 M-Tris-HCl (pH 7.4) containing 1 mM-EDTA and 0.9% NaCl. The livers were homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was filtered through four layers of cheesecloth. The samples were fractionated by Schneider's (1945) procedure, and the amounts of DNA, RNA and protein were measured by the methods of Burton (1956), Ceriotti (1955) and Lowry *et al.* (1951) respectively with calf thymus DNA, yeast RNA and bovine serum albumin as each standard.

Assay of δ -aminolaevulinic acid synthetase activity

The activity of δ -aminolaevulinic acid synthetase was assayed by the modified method of Marver *et al.* (1966a) as follows. Liver was homogenized in 2 vol. of 0.9% NaCl solution containing 0.5 mM-EDTA and 0.01 M-Tris-HCl (pH 7.4). The reaction mixture contained 120 μ mol of glycine, 20 μ mol of EDTA, 4 μ mol of α -oxoglutaric acid, 0.2 μ mol of pyridoxal phosphate, 150 μ mol of Tris-HCl (pH 7.4) and 0.5 ml of homogenate in a final volume of 2 ml. The incubation was carried out with shaking at 37°C for 60 min and terminated by addition of 0.5 ml of 25% (w/v) cold trichloroacetic acid. The δ -aminolaevulinic acid formed was measured by the non-chromatographic method of Granick (1966).

Determination of P_i and [32 P]RNA

Carrier-free [32 P] P_i dissolved in 0.9% NaCl (0.5 μ Ci/g body wt.) was injected into the femoral vein of rats, which were anaesthetized lightly with ether. The rats were killed at appropriate time-intervals after the injection. The livers were quickly removed without perfusion and homogenized individually in 2 vol. of 0.9% NaCl, 1 mM-EDTA and 0.02 M-Tris-HCl (pH 7.4). The homogenate was rapidly acidified by addition of trichloroacetic acid to give a final concentration of 8% (w/v) and centrifuged at 600g for 5 min. P_i in the resulting supernatant was extracted and determined by the method of Fiske & SubbaRow (1925). The [32 P]RNA fraction in each precipitate after centrifugation at 600g for 5 min was prepared by the methods of Smellie *et al.* (1955) and Schmidt & Thannhauser (1945). The 32 P radioactivity was measured in a Kobe Kogyo Geiger-Mueller counter. Relative specific radioactivity of [32 P]RNA was calculated as follows: $\{([^{32}\text{P}]\text{RNA}/\mu\text{g of RNA})/(^{32}\text{P}/\mu\text{g of } P_i)\} \times 100$.

Preparation of nuclei and polyribosomes

[14 C]Orotic acid was injected into the femoral vein of rats at a dose of 10 μ Ci/100 g body wt. under light anaesthesia with ether. Livers were excised after rapid perfusion with ice-cold 0.25 M-sucrose–3.3 mM- CaCl_2 and homogenized in a glass-Teflon homogenizer in 5 vol. of 0.25 M-sucrose containing 0.05 M-Tris-HCl (pH 7.4), 0.1 M-KCl and 12 mM- MgCl_2 (Tris- K^+ - Mg^{2+} buffer). The homogenates were filtered through four layers of cheesecloth and centrifuged at 700g for 15 min. The sediments containing the nuclei were resuspended by homogenization in 2.3 M-sucrose–1 mM- MgCl_2 of half the volume of the original homogenates and centrifuged at 50000g for 70 min for the preparation of nuclei.

The resulting supernatants were centrifuged twice at 10000g for 15 min to remove mitochondria and cellular debris. The post-mitochondrial supernatants

were treated with sodium deoxycholate at a concentration of 1% (w/v) and stirred for 10 min. The mixture was layered over 0.5 M-sucrose containing Tris- K^+ - Mg^{2+} buffer, which was superposed over 2 M-sucrose containing the same buffer, the relative proportion of the three layers being 2:1:1 (by vol.) respectively. The tubes were centrifuged at 105000g for 4 h for the preparation of polyribosomes.

Extraction of RNA from nuclei and polyribosomes

The extraction of RNA from the nuclear fraction was performed by the procedure of Muramatsu *et al.* (1968). The RNA of polyribosomal fraction was extracted in a manner similar to that for nuclear RNA except that phenol extraction was carried out at room temperature.

Sucrose-density-gradient centrifugation of RNA

RNA was dissolved in a solution containing 0.4 M-NaCl and 50 mM-sodium phosphate buffer (pH 7.2). A 1 ml portion of this was layered on a 30 ml linear 10–40% (w/v) sucrose-density gradient prepared in the solution containing 0.1 M-NaCl–1 mM-EDTA–0.01 M-sodium acetate (pH 5.1). The gradients were centrifuged in a Spinco SW25.1 rotor at 25000 rev./min for 18 h at 4°C and then fractionated into 1 ml fractions by piercing the bottom of the tube. After appropriate dilution, the E_{260} of each fraction was measured with a Shimadzu DU spectrophotometer. Samples (0.1 ml) were pipetted into glass vials and 10 ml of Bray's (1960) scintillation solution were added. The radioactivity was measured in a Beckman liquid-scintillation counter.

Results

Effect of 3,5-dicarbethoxy-1,4-dihydrocollidine on the activity of δ -aminolaevulinic acid synthetase and the net increase in nucleic acids and protein

Activities of hepatic δ -aminolaevulinic acid synthetase increased almost linearly with time and reached a maximum value 3 days after the initial administration of 3,5-dicarbethoxy-1,4-dihydrocollidine to the rats. The maximum activity of this enzyme was about 30 times that of the control. Further daily administration of this drug caused a rapid decrease in the enzyme activity to a value four or five times that of the control (Fig. 1).

In 4 days, as shown in Table 1, the rats lost about 20 g of body weight, but the wet weight of the liver increased 1.5-fold. As shown in Fig. 2, daily administration of 3,5-dicarbethoxy-1,4-dihydrocollidine caused a net increase of RNA by 10–30%, whereas there was no net increase in DNA and protein compared with the control.

Fig. 1. Time-courses of δ -aminolaevulinic acid synthetase activity and relative specific radioactivity of [^{32}P]RNA after daily administration of 3,5-dicarbethoxy-1,4-dihydrocollidine

δ -Aminolaevulinic acid synthetase activities and the relative specific radioactivities of [^{32}P]RNA in the liver homogenates were determined as described in the Materials and Methods section. $\circ$, δ -Aminolaevulinic acid synthetase activity; $\bullet$, relative specific radioactivity of [^{32}P]RNA. Results are averages for four rats, bars representing the S.D.

Table 1. Net increase in nucleic acids and protein in rat liver after administration of 3,5-dicarbethoxy-1,4-dihydrocollidine

At 4 days after administration of 3,5-dicarbethoxy-1,4-dihydrocollidine, the amounts of DNA, RNA and protein in the liver were determined as described in the Materials and Methods section. Each value in the table is the average $\pm$ S.D. for four rats.

	Control	4 days after 3,5-dicarbethoxy- 1,4-dihydrocollidine
Body wt. (g)	171.0 $\pm$ 8.0	151.0 $\pm$ 10.0
Liver wt. (g)	5.3 $\pm$ 0.1	8.2 $\pm$ 0.2
DNA/liver (mg/g)	2.5 $\pm$ 0.1	2.7 $\pm$ 0.3
RNA/liver (mg/g)	12.5 $\pm$ 0.1	15.3 $\pm$ 0.1
Protein/liver (g/g)	0.28 $\pm$ 0.01	0.30 $\pm$ 0.01

3,5-Dicarbethoxy-1,4-dihydrocollidine stimulation of ^{32}P incorporation into RNA fraction

[^{32}P]P_i in 0.9% NaCl was injected into the femoral vein of rats 4h after administration of 3,5-dicarb-

ethoxy-1,4-dihydrocollidine. The animals were killed at 30, 60, 120 and 240min after ^{32}P injection. The relative specific radioactivities in RNA fractions of non-treated control livers increased linearly within 2h

Fig. 2. Time-courses of 3,5-dicarbethoxy-1,4-dihydrocollidine-induced increase in liver weight and the net increase in nucleic acids and protein in the livers

The determinations of RNA, DNA and protein are described in the Materials and Methods section. Results (per g of liver) of the treated group (averages for four rats) are plotted as percentages of the corresponding values for the control group (six rats). $\circ$, Wet wt. of liver; $\bullet$, DNA; $\blacktriangle$, RNA; $\square$, protein. Bars represent the s.d.

Fig. 3. ^{32}P incorporation into RNA fractions of the livers of normal and 3,5-dicarbethoxy-1,4-dihydrocollidine-treated rats

Relative specific radioactivities were calculated as described in the Materials and Methods section. Each value is the average for four rats. $\circ$, Control; $\bullet$, 4h after 3,5-dicarbethoxy-1,4-dihydrocollidine administration.

Fig. 4. Effect of actinomycin D on 3,5-dicarbethoxy-1,4-dihydrocollidine-induced increase in relative specific radioactivity of ^{32}P RNA and δ -aminolaevulinic acid synthetase activity in rat liver

The relative specific radioactivities of ^{32}P RNA and δ -aminolaevulinic acid synthetase activities in the livers were determined as described in the Materials and Methods section. $\bullet$, Plus actinomycin D (administered at time shown by arrow); $\circ$, no actinomycin D. Results are averages for four rats, the bars representing the s.d.

after ^{32}P injection and then reached a plateau. When rats were treated with 3,5-dicarbethoxy-1,4-dihydrocollidine, the relative specific radioactivity was raised to 1.5 or 3 times that of the controls (Fig. 3).

To correlate the synthesis of δ -aminolaevulinic acid synthetase and RNA in porphyric rat liver, the enzyme activity and the relative specific radioactivity of [^{32}P]RNA at 2 h after ^{32}P injection were measured in the same liver samples. As shown in Fig. 1, the relative specific radioactivities were increased within 2 days and returned to the initial value at 3 days when the enzyme activity was at a maximum value. Further daily administration of 3,5-dicarbethoxy-1,4-dihydrocollidine caused the relative specific radioactivity to fall below the initial value.

Effect of actinomycin D on RNA synthesis and induction of δ -aminolaevulinic acid synthetase

Rats were given the ^{32}P injection and 3,5-dicarbethoxy-1,4-dihydrocollidine was administered simultaneously. At 1 h after ^{32}P injection and 3,5-dicarbethoxy-1,4-dihydrocollidine administration one group of rats was injected with actinomycin D (1.5 mg/kg body wt.) intraperitoneally. As shown in Fig. 4(a), the relative specific radioactivities of ^{32}P in RNA fractions after treatment with this antibiotic remained almost at the same value after 1 h as when it was injected into the rats, and then decreased fairly rapidly. Meanwhile, the activity of δ -aminolaevulinic acid synthetase increased at a similar rate to that of the control for 1 h, and later decreased (Fig. 4b).

These results indicate that the declines in hepatic activity of the induced δ -aminolaevulinic acid synthetase after treatment with actinomycin D are due to the antibiotic inhibiting RNA synthesis in the livers. From the results in Fig. 4(b), it can be shown that the half-life of δ -aminolaevulinic acid synthetase appears to be about 60 min, although the more detailed study on the enzyme activity after actinomycin D treatment reveals the decline to be composed of several components.

Incorporation of [^{14}C]orotic acid into nuclear and polyribosomal RNA

The kinetics of RNA synthesis in the nuclei and in the polyribosomes were studied by injecting [^{14}C]orotic acid into the rats and measuring the radioactivity incorporated into RNA fractions. At 4 h after the single administration of 3,5-dicarbethoxy-1,4-dihydrocollidine to the rats, [^{14}C]orotic acid (10 $\mu\text{Ci}/100\text{g}$ body wt.) was injected into the femoral vein. The animals were killed at 30, 60, 120 and 180 min after the injection, and the livers were fractionated to prepare the nuclei and polyribosomes.

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The specific radioactivities in the nuclear fraction of 3,5-dicarbethoxy-1,4-dihydrocollidine-treated rat livers were 3 times as high at 30 min and 2.5 times as high at 60 min as in non-treated rat livers, whereas no increase in the specific radioactivity of RNA in polyribosomal fractions of 3,5-dicarbethoxy-1,4-dihydrocollidine-treated rat livers was observed (Fig. 5). The decline in specific radioactivity of RNA in nuclear fractions after 60 min follows first-order kinetics.

The E_{260} profile of polyribosomal RNA after sucrose-density-gradient centrifugation showed the three peaks corresponding to 4S, 18S and 28S (Fig. 6). There was a tendency with time for the radioactivity to shift toward the heavier region. However, no significant changes were observed in the synthesis of the three components of polyribosomal RNA be-

Fig. 5. Time-courses of the incorporation of [^{14}C]orotic acid into nuclear and polyribosomal RNA from normal and 3,5-dicarbethoxy-1,4-dihydrocollidine-treated rat livers

The radioactivities of nuclear and polyribosomal RNA isolated from the livers were measured as described in the Materials and Methods section. Each value is the average for two rats. ●, Nuclear RNA from 3,5-dicarbethoxy-1,4-dihydrocollidine-treated rat liver; ○, nuclear RNA from control rat liver; ▲, polyribosomal RNA from 3,5-dicarbethoxy-1,4-dihydrocollidine-treated rat liver; △, polyribosomal RNA from the control rat liver.

Fig. 6. Sucrose-density-gradient analysis of polyribosomal RNA from normal and 3,5-dicarbethoxy-1,4-dihydrocollidine-treated rat livers

The polyribosomal RNA used here was the same material as that in Fig. 5. The analysis of polyribosomal RNA by sucrose-density-gradient centrifugation is described in the Materials and Methods section. The S values indicated in the figure are assumed from the published report of Darnell (1968). (a) Non-treated livers; (b) livers 4 h after intubation of 3,5-dicarbethoxy-1,4-dihydrocollidine. RNA was isolated from the polyribosomal preparations at the times indicated in parentheses after the injection of [^{14}C]orotic acid. $\circ$, E_{260} ; $\bullet$, radioactivity.

tween 3,5-dicarbethoxy-1,4-dihydrocollidine-treated and non-treated rat livers.

Discussion

The mechanism for the induced increase in hepatic δ -aminolaevulinic acid synthetase has been investi-

gated by using a number of compounds that interfere with different steps in the synthesis of nucleic acids and protein. These observations suggest that certain compounds induce the synthesis of δ -aminolaevulinic acid synthetase by affecting the transcriptional process and others by affecting the translational process. Sassa & Granick (1970) suggested that 3,5-dicarb-

ethoxy-1,4-dihydrocollidine might act at the transcriptional level, possibly by depressing an operon to permit the synthesis of this enzyme.

It is well known that the treatment of animals with various kinds of drugs results in the increase in the activities of microsomal enzymes (Orrenius, 1965). 3,5-Dicarbethoxy-1,4-dihydrocollidine, as reported by Wada *et al.* (1968), induces hepatic δ -aminolaevulinic acid synthetase more specifically than drug-induced microsomal enzymes.

The present study showed that 3,5-dicarbethoxy-1,4-dihydrocollidine, a potent porphyria-inducing agent, primarily stimulated the synthesis of nuclear RNA and caused the induced increase in formation of δ -aminolaevulinic acid synthetase in the rat livers. From the kinetic studies in our experiments, it seems probable that 3,5-dicarbethoxy-1,4-dihydrocollidine stimulates more specifically the production of nuclear RNA with a regulating function. Further, this can be interpreted to imply that 3,5-dicarbethoxy-1,4-dihydrocollidine acts at the transcriptional level as proposed by Sassa & Granick (1970). At the dosage of actinomycin D used here (1.5 mg/kg) there is reported to be both selective inhibition of RNA synthesis and retention of normal liver histology (Revel *et al.*, 1964). If in the present study actinomycin D blocks the synthesis of the mRNA for δ -aminolaevulinic acid synthetase, the decrease in hepatic activities of this enzyme after administration of the antibiotic would be a function of the degradation rate of both the specific mRNA and the enzyme. The decline in the activity of δ -aminolaevulinic acid synthetase after administration of actinomycin D could be explained on the basis of a relatively unstable mRNA for the enzyme, although the bulk of rat liver mRNA is assumed to be stable for at least 40h (Revel & Hiatt, 1964).

Rapidly labelled nuclear RNA could be divided into two distinct classes, one of which is the nucleolar precursor of ribosomal RNA, the other being the heterogeneous DNA-like RNA of extranucleolar nuclear origin. This heterogeneous DNA-like RNA is potentially mRNA and is transferred to cytoplasmic polyribosomes (Henshaw, 1968). It could be inferred that the 3,5-dicarbethoxy-1,4-dihydrocollidine-induced increase of rapidly labelled nuclear RNA reflects the stimulation of the synthesis of heterogeneous DNA-like RNA in the nuclei.

It is generally accepted that δ -aminolaevulinic acid

synthetase exists exclusively in mitochondria. Hayashi *et al.* (1969) demonstrated that δ -aminolaevulinic acid synthetase also exists as a soluble enzyme in the cytoplasm of the liver cell, and the mitochondrial enzyme has a higher molecular weight than the enzyme in soluble fractions, suggesting that δ -aminolaevulinic acid synthetase is synthesized originally in the microsomal system. Our observations may be also interpreted along the line of the hypothetical induction mechanism of δ -aminolaevulinic acid synthetase proposed by Hayashi *et al.* (1969) and Sassa & Granick (1970).

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