Metabolism of 3,3'-Diiodothyronine and 3'-Monoiodothyronine, and Effect of Potassium Cyanide and Dinitrophenol on Glucuronidation of Thyroxine in Cultured Rat Hepatoma Cells

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Abstract

Rat hepatoma cells (R117-21B) metabolized 3,3'-diiodothyronine and 3'-monoiodothyronine by sulfation and glucuronidation. Glucuronidation of iodothyronines (a mixture of 3,3'-diiodothyronine, ~90%, and 3'-monoiodothyronine, ~10%) was very rapid and 83% of the total ^{125}I in the medium was their glucuronides after 8 hr incubation with the cells at 37°C. Iodothyronine sulfates (~9% of the total ^{125}I) were also detected. No clear increase in free iodide was observed during the 8 hr incubation.

The effect of KCN or dinitrophenol on glucuronidation of thyroxine was investigated. Both drugs inhibited glucuronidation, and dinitrophenol was more effective than KCN. Dinitrophenol accelerated the liberation of $^{125}\text{I}^-$ in the metabolism of thyroxine in the cultured rat hepatoma cells.

In the metabolism of the thyroid hormones, 3,3'-diiodothyronine (3,3'- T_2)¹ which is formed by monodeiodinations of 3,3',5-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (rT_3) would be one of the metabolites. In fact, 3,3'- T_2 was detected in the rat kidney after injection of the thyroid hormones (Roche *et al.*, 1956; Roche *et al.*, 1959) and in serum (Wu *et al.*, 1976; Burman *et al.*, 1977; Burgar and Sakoloff, 1977; Gavin *et al.*, 1978). Furthermore, 3,3'- T_2 conjugates were identified in bile, urine or plasma (Flock *et al.*, 1960; 1961; 1963). Recently, it was reported that monkey hepatocarcinoma cells (NCLP-6E) produced 3,3'- T_2 from T_3 and rT_3 , and that 3,3'- T_2 was converted to its sulfate

(Sorimachi and Robbins, 1977). The homogenates of this cell culture (Sorimachi and Robbins, 1979) and of the normal rat liver (Visser *et al.*, 1978; Chopra *et al.*, 1978) also formed 3,3'- T_2 from rT_3 .

Experiments using tissue homogenates are not suitable for the investigation of the dynamic metabolism of the thyroid hormones. On the other hand, cell culture systems are useful for this kind of study, if cell lines have some enzymes related to the metabolism, although it is very difficult to find these cells. However, since we found that rat hepatoma cells (R117-21B) metabolized the thyroid hormones in a similar fashion to the normal liver *in vivo* (Sorimachi *et al.*, 1980a), 3,3'- T_2 and 3'- T_1 which are deiodinated products of the thyroid hormones were investigated in detail in this study. Furthermore, the effect of dinitrophenol and KCN on glucuronidation of thyroxine was studied, since cell culture systems easily elucidate those effects *in vitro*.

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Abbreviations used are: T_4 , thyroxine; T_3 , 3,3',5-triiodothyronine; rT_3 , 3,3',5'-triiodothyronine; 3,3'- T_2 , 3,3'-diiodothyronine; 3,5- T_2 , 3,5-diiodothyronine; 3',5'- T_2 , 3',5'-diiodothyronine; 3'- T_1 , 3'-moniodothyronine.

Materials and Methods

Labeled compounds

3, [3'-¹²⁵I]diiodo-L-thyronine and [3'-¹²⁵I]monoiodo-L-thyronine were prepared by following procedures: 3, 5, [3'-¹²⁵I]triiodo-L-thyronine (1.2 mCi/ μ g) purchased from New England Nuclear was incubated with monkey hepatocarcinoma cells ($\sim 10^7$ cells) for 24 hr at 37°C (Sorimachi and Robbins, 1977). After incubation the medium was concentrated with a rotary evaporator under reduced pressure, and then the sulfates were isolated and purified by ion-exchange column chromatography using AG50W-X4 (Sorimachi and Ui, 1975). The sulfates were treated with sulfatase (Sorimachi and Robbins, 1977), and the liberated iodothyronines were desalted and concentrated by a small column (Sorimachi and Cahnmann, 1977).

[3', 5'-¹²⁵I]thyroxine (1.8 mCi/ μ g) was obtained from England Nuclear and used without further purification. This preparation contained $\sim 3\%$ free iodide.

Unlabeled compounds

KCN and dinitrophenol were purchased from Nakarai Chemical Ltd. (Kyoto). Other chemicals were commercial products of reagent grade.

Cells

Monkey hepatocarcinoma cells (NCLP-6E), used in the previous study (Sorimachi and Robbins, 1977), and rat hepatoma cells (R117-21B) (Niwa *et al.*, 1979), established from the Reuber hepatoma cells (H4-II-E) (Pitot *et al.*, 1964), were serially cultured in rubber-stoppered glass culture flasks with a 25 cm² bottom area in an experimental medium at 37°C. This medium is identical with Eagle's minimum essential medium except that glutamine is replaced with glutamic acid, and biotin (25 ng/ml) is added. After autoclaving, penicillin (100 units/ml) and streptomycin (100 μ g/ml) were incorporated in the medium. For the growth of the cells the medium was supplemented with 0.5% fetal calf serum. Monkey hepatocarcinoma cells were used to prepare 3, 3'-T₂ and 3'-T₁, as described above. Rat hepatoma cells (R117-21B) for further experiments were then grown in 5 cm diameter plastic culture dishes (NUNC, Denmark) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Usually, when the cells had nearly reached confluency, labeled iodothyronines were incubated with the cells. The incubation of iodothyronine with the cells was carried out in the absence of serum. Aliquots (0.1 ml) of the medium were withdrawn at the stated intervals for the analysis of the labeled compounds.

Ion-exchange column chromatography

In order to analyze the metabolites in the medium,

ion-exchange column chromatography using AG50W-X4, 0.9 $\times$ 10 cm (Sorimachi and Ui, 1975) was used. The column was initially equilibrated with 0.04 M ammonium acetate, pH 4.7, containing 30% ethanol, and then a linear gradient of 0.7 M ammonia containing 30% ethanol was performed. In some experiments, a long column (0.9 $\times$ 20 cm) was used and the elution for the conjugates was carried out only with 0.04 M ammonium acetate, pH 4.7, containing 30% ethanol, since iodothyronine sulfates and iodothyronine glucuronides were eluted with the initial solution.

Hydrolysis of iodothyronine conjugates

The hydrolysis of sulfates was performed according to the same procedure reported elsewhere (Sorimachi and Robbins, 1977). Iodothyronine glucuronides were treated with glucuronidase (Lot. 1318515) obtained from Boehringer Mannheim GmbH in 50 mM phosphate buffer, pH 6.0, overnight at 37°C. After hydrolysis, the radioactive components were extracted with ethanol from the hydrolysates, and then were applied on ion-exchange column chromatography.

Results

Monkey hepatocarcinoma cells (NCLP-6E) metabolized T₃ by nonphenolic ring deiodination and by sulfation (Sorimachi and Robbins, 1977). Figure 1A shows the elution profile of the hydrolysate of iodothyronine sulfates, obtained from the metabolism of T₃ in monkey hepatocarcinoma cells, on ion-exchange column chromatography. 3, 3'-T₂ ($\sim 90\%$) and 3'-T₁ ($\sim 10\%$) were liberated from the sulfates by the sulfatase treatment, and this is consistent with our previous results (Sorimachi and Robbins, 1978a).

When 3, 3'-T₂ and 3'-T₁ were incubated with the rat hepatoma cells (R117-21B) at 37°C for 24 hr, the peaks due to free iodothyronines disappeared on column chromatography (Figure 1B). On the other hand, a huge peak appeared after iodide which is not retained with this column. The elution profile suggests that minor components were eluted between iodide and the huge peak. Therefore, a long column was used to get better separation of the minor

Fig. 1. Ion-exchange column chromatography of iodothyronines and the medium containing iodothyronine metabolites produced by the cells. (A), 3, 3', 5-triiodothyronine (0.5 nM) was incubated with monkey hepatocarcinoma cells ($\sim 10^7$ cells) for 24 hr at 37°C and the isolated sulfates were hydrolyzed by sulfatase treatment. The liberated iodothyronines were applied on ion-exchange column chromatography. (B), Iodothyronines were incubated with rat hepatoma cells ($\sim 2 \times 10^6$ cells) and an aliquot of the incubation medium was analyzed by ion-exchange column chromatography. (C), The incubated medium was concentrated and applied on a long column (0.9 $\times$ 10 cm) and the elution was carried out only with 0.04 M ammonium acetate, pH 4.7.

components contained in the incubation medium, and the elution was carried out with only 0.04 M ammonium acetate, pH 4.7, containing 30% ethanol (Figure 1C). Two small peaks clearly separated from iodide and the huge peak. Judging from our previous data (Sorimachi and Robbins, 1977; Sorimachi and Cahnmann, 1979), these unknown peaks would be iodothyronine conjugates. From the first peak (Peak I) eluted just after iodide, iodothyronines were liberated by the sulfatase treatment, and 3, 3'-T₂ and 3'-T₁ were identified (Figure 2). Peak II and III were converted to 3'-T₁ and 3, 3'-T₂, respectively, by the glucuronidase treatment. These results show that the rat hepatoma cells metabolized

Fig. 2. Ion-exchange column chromatography of hydrolysates of iodothyronine conjugates. Peak I (see Fig. 1C) was treated with sulfatase, and Peak II and III were treated with glucuronidase before column chromatography.

3, 3'-T₂ and 3'-T₁ by sulfation and by glucuronidation.

The production of the metabolites and the disappearance of iodothyronines (3, 3'-T₂ and 3'-T₁) in the rat hepatoma cell culture medium were analyzed with ion-exchange column chromatography (Table 1). The disappearance of iodothyronines was very rapid, and they were almost completely converted to their conjugates after 8 hr incubation at 37°C. However, no clear increase in labeled iodide was observed during 8 hr incubation of the cells with 3, 3'-T₂ and 3'-T₁, although a minute amount of ¹²⁵I⁻ was liberated from 3, 3'-T₂ in the rat hepatoma cell homogenate experiments (unpublished data).

We showed that KCN and dinitrophenol inhibited the sulfation of iodothyronines (Sorimachi and Robbins, 1978b). Therefore, the effect of these chemicals on glucuronidation was investigated with T₄, since only glucuronidation was a main reaction in the

Table 1. Time course of the metabolism of 3,3'-diiodothyronine and 3'-monoiodothyronine in cultured rat hepatoma cells.

Time (hr)	I ⁻	S	T ₁ G	T ₂ G	T ₁ +T ₂
1	3.1±0.4	3.9±0.8	7.5±1.7	25.6±1.7	59.9±3.4
2	4.0±0.9	6.6±1.5	11.0±0.9	45.4±6.2	32.7±8.4
4	3.3±0.9	7.8±0.5	13.4±1.7	61.7±3.4	10.4±3.2
8	3.5±0.4	8.8±0.6	12.2±1.0	71.1±1.1	4.4±0.4

Iodothyronine (40 pM) was incubated with the cells ($\sim 2 \times 10^6$ cells) at 37°C. Aliquots of the medium were analyzed by ion-exchange column chromatography. The values are percent ages of the total radioactivity and the mean±standard deviation is given for three culture dishes.

Abbreviations used are: S, 3,3'-T₂ and 3'-T₁ sulfates; T₁G, 3'-T₁ glucuronide; T₂G, 3,3'-T₂ glucuronide.

Table 2. Inhibitory effect of KCN and dinitrophenol on glucuronidation of thyroxine in rat hepatoma cells.

Time (hr)		I ⁻	Unknown ^a	T ₄ G	T ₄
3	Control	4.6±1.7	10.5±0.6	14.8±2.2	70.1±4.4
	DNP	8.9±3.6	10.0±2.0	5.5±1.2	75.8±4.3
	KCN	6.2±1.0	11.4±2.3	4.7±0.8	77.7±3.3
8	Control	5.0±1.2	9.2±1.2	34.6±8.1	51.7±9.8
	DNP	10.4±2.3	9.1±1.0	13.4±1.0	67.1±4.0
	KCN	5.7±2.5	9.9±2.8	9.3±3.7	75.0±8.9
24	Control	5.4±1.1	8.0±3.1	77.5±2.6	9.1±4.4
	DNP	15.1±1.0	10.9±1.8	22.3±0.9	51.7±1.8
	KCN	4.9±0.8	9.4±0.9	51.3±1.6	34.3±2.1

Thyroxine (72 pM) was incubated with the cells in the absence or presence of KCN or dinitrophenol. The concentrations of KCN and dinitrophenol were 10 mM and 0.5 mM, respectively. The values are percent of the total radioactivity and the mean±standard deviation is given for three culture dishes.

(a), Unknown material, eluted between iodide and T₄ glucuronide on ion-exchange column chromatography, would not be a metabolite, since this component was observed even at 0 time and its quantity was independent of the incubation.

Abbreviations used are: T₄G, T₄ glucuronide; DNP, dinitrophenol.

metabolism of T₄ (Sorimachi *et al.*, 1980a), and since labeled T₄ is commercially available. Both KCN and dinitrophenol inhibited also glucuronidation of T₄ (Table 2), and the latter was a better inhibitor of this reaction than the former. Furthermore, dinitrophenol accelerated the liberation of labeled iodide. This means that dinitrophenol affected the phenolic ring deiodination of T₄ in the rat hepatoma cells.

Discussion

The phenolic ring deiodination of T₃ was very slow either in rat liver homogenates or in the rat hepatoma cell (R117-21B) homogenates (unpublished data), even though the phenolic ring deiodinase activity is quite high. This means that the production of 3,5-T₂ might be very small. However, the conversion of rT₃ to 3,3'-T₂ easily took place in these homogenates (Visser *et al.*, 1978; Chopra *et al.*, 1978; Sorimachi *et al.*, 1980b). In monkey hepa-

tocarcinoma cells (NCLP-6E), 3, 3'-T₂ was rapidly produced not only from rT₃ by phenolic ring deiodination but also from T₃ by nonphenolic ring deiodination (Sorimachi and Robbins, 1977). Even though monkey hepatocarcinoma cells were very active in carrying out nonphenolic ring deiodination, the 3',5'-T₂ produced from rT₃ by nonphenolic ring deiodination was a very small amount (Sorimachi and Cahnmann, 1979). These data suggest that 3, 3'-T₂ would be a main deiodination metabolite in the metabolism of the thyroid hormones, and that 3',5'-T₂ and 3,5-T₂ would be minor metabolites.

Various iodothyronines had the inhibitory effects of different potency on the deiodinations of labeled iodothyronines (T₄, T₃, rT₃ and 3,5-T₂) in the intact cells (Sorimachi, 1980) and in their homogenates (Sorimachi and Robbins, 1979). A similar result was obtained in the rat hepatoma cell homogenates (Sorimachi *et al.*, 1980b). These results suggest that the analogs of thyroxine control the formation of T₃ or rT₃ from T₄. Recently, Coiro *et al.* (1980) showed the pharmacological effect of rT₃ on the conversion of T₄ to T₃. On the other hand, 3, 3'-T₂ was a good substrate for nonphenolic ring deiodination which produces rT₃ from T₄ (Sorimachi and Robbins, 1977).

Our previous data (Sorimachi and Robbins, 1977; Sorimachi and Cahnmann, 1979) revealed that 3, 3'-T₂ and 3,-T₁ were the best substrates for sulfation among iodothyronines, and both recent (Sorimachi *et al.*, 1980a) and present studies showed that these two iodothyronines were better substrates for glucuronidation than T₄, T₃ or rT₃. Furthermore, the cellular uptake of iodothyronine sulfates was extremely low or absent not only in the cultured cells but also in isolated rat hepatocytes (Sorimachi and Robbins, 1978b), and that of iodothyronine glucuronides was also very low (unpublished data). It seems, therefore, that glucuronidation and sulfation are important

processes in secreting the analogs of the thyroid hormones (particularly 3, 3'-T₂ and 3'-T₂) as well as the hormones from the cells.

Flock *et al.* (1960; 1961; 1963) detected 3, 3'-T₂ conjugates in bile, urine or plasma after administration T₄ or T₃. Therefore, the formation of 3, 3'-T₂ is involved in the metabolism of the thyroid hormones *in vivo*. However, the serum level of 3, 3'-T₂ was extremely low (Wu *et al.*, 1976; Burman *et al.*, 1977; Burger and Sakoloff, 1978; Gavin *et al.*, 1978). Judging from these data, even if a large amount of 3, 3'-T₂ is produced by monodeiodinations of T₃ or rT₃, this metabolite is immediately converted to its conjugates.

KCN and dinitrophenol inhibited glucuronidation of iodothyronines. Both drugs also inhibited sulfation of iodothyronines in monkey hepatocarcinoma cells (Sorimachi and Robbins, 1978b). It is therefore clear that glucuronidation and sulfation are related to a high energy requiring reaction. Dinitrophenol accelerated phenolic ring deiodination of T₄. A similar result was obtained in the metabolism of thyroxine in monkey hepatocarcinoma cells (Sorimachi and Robbins, 1978b). These dinitrophenol effects might be due to a change in the free concentration of the substrate by this drug (Wolff *et al.*, 1961).

The present results strongly suggest that the rat hepatoma cell cultures (R117-21B) are useful for further investigation of the metabolism of the thyroid hormones *in vitro*.

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