METABOLISM OF *L*-THYROXINE DUE TO TRANS-AMINATION BY RAT KIDNEY MITOCHONDRIA

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Evidence strongly suggestive of the presence of a transaminase acting upon thyroxine (T_4) has been offered in a previous study (15) employing rat kidney homogenates as enzyme sources. The present paper describes a more extensive research with rat kidney mitochondria which provided satisfactory support for the previous assumption that the transaminase may be an important enzyme taking part in the conversion of T_4 to tetraiodothyropyrvic acid (KT_4) which may presumably be decarboxylated to tetraiodothyroacetic acid (TA_4). Since authentic KT_4 is not available at present and the compound is not yet detectable *in vitro* among metabolic products of T_4 , the production of TA_4 and the concurrent consumption of T_4 were employed as convenient measures of the activity of the transaminase.

On the other hand, interesting reports (1, 2, 14) concerning a highly active, DPN-linked enzyme system, which converts T_4 and triiodothyronine (T_3) to TA_4 and triiodothyroacetic acid (TA_3), led the present authors to undertake some comparative studies. The results have shown that DPN and some member compounds of the tricarboxylic acid cycle were less effective than pyridoxal phosphate and α -ketoglutarate, essential cofactors in transamination reaction.

MATERIALS AND METHODS

Materials and preparations. Kidneys of adult male rats of Wistar strain were used in all experiments. After homogenization with an equal volume of 0.25 M sucrose in an all-glass homogenizer under ice-cold conditions, mitochondria were isolated after Schneider and Hogeboom (9) by means of a refrigerated centrifuge at -1°C .

Reaction procedures. The basic composition of reaction mixtures (final volume 2 ml.) was as follows:

Mitochondria resuspended in 0.25 M sucrose	1.0 ml.
10^{-3} M Pyridoxal phosphate	0.2 ml.
I^{131} - <i>L</i> -Thyroxine dissolved in 0.01 N KOH	0.2 ml.
0.1 M Potassium phosphate buffer (p_H 7.4)	0.4 ml.
0.1 M α -Ketoglutarate (p_H 7.4)	0.2 ml.

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All mitochondrial preparations for a single experiment were derived from the same donor animal. Unless otherwise stated, in each case an amount of mitochondria representing 500 mg. of the fresh tissue was used as an enzyme source. In controls run simultaneously, 0.25 M sucrose was used in place of the mitochondrial suspension.

When some substances were used instead of pyridoxal phosphate and/or α -ketoglutarate for the comparison of their effect, and the total volume of the mixture did not reach to 2.0 ml., a suitable amount of an appropriate solvent was added. Reagents were all of commercial source except for oxalacetate supplied by Dr. K. Makino, Tōkyō Jikeikai School of Medicine, and pyridoxal phosphate by Dr. K. Ōike, Wakamoto Drug Lab. The amount of these will be given in the following description in terms of a final molar concentration.

Preparation of labeled thyroxine. T_4 labeled with a tracer amount of radioactive iodide was prepared after Roche *et al.* (7), whose "iodination method", however, required the following considerable modifications in order to adapt it to our laboratory conditions:

A minute volume of I^{131} (2 to 5 mc.) was brought to the laboratory in a glass container closed with a gum cover. Through the cover, H_2SO_4 , I_2 and T_3 solutions were added successively by a syringe. The amounts added were in the following proportion:

0.02 N H_2SO_4	0.07 ml.
I_2 solution (52 mg. of I_2 in 20 ml. of freshly redistilled ethanol)	0.7 ml.
3, 5, 3'- I - T_3 solution (30 mg. of T_3 in 18 ml. of 28% NH_4OH)	1.8 ml.

All solutions had been cooled with cracked ice prior to the addition. The mixture was kept at 0°C for 3 hours. Then the container was uncovered and the contents were transferred to a test tube and concentrated to approximately 0.5 ml. under reduced pressure by a vacuum pump.

The resultant mixture was applied on a filter paper of Tōyō-Roshi No. 50 (40 × 40 cm.), which was washed previously with ethanol-2 N NH_4OH (1:1). The paper was developed with a *n*-butanol-dioxane-2 N NH_4OH system (40:10:20) for about 10 hours. Then the paper was dried in air and a radioautogram was made. The paper was then cut at a place corresponding to I^{131} - T_4 on the autogram. From the paper strip T_4 was almost completely recovered by elution with ethanol-2 N NH_4OH (1:1) for about 24 hours. The material was concentrated and freed from both ammonia and ethanol by a vacuum pump for about 30 minutes, followed by dilution with 0.01 N KOH to make the T_4 concentration between 10^{-3} and 10^{-4} M. The accurate concentration of T_4 was calculated from the optical density at 327 m μ .

Preparing labeled T_4 by the "exchange method" of Taurog (12), a comparative test with that by the "iodination method" was made on the paper-chromatogram, which made clear that the "iodination method" is the better since the radioactivity staying at origin and contaminating the whole surface of the paper was far less in the case of I^{131} - T_4 prepared by this method. Consequently, preparations obtained by the "iodination method" were used throughout the present study.

Prior to use, this partly labeled T_4 was diluted with 0.01 N KOH to concentrations ranging from 10^{-4} to 10^{-5} M, because the radioactivity of this amount of T_4 was sufficient to permit identification of the main product as TA_4 .

In spite of the purification procedure mentioned above, some radioactivity appeared at the positions occupied by iodide and TA_4 (and TP_4). (TA_4 and TP_4 used as non-radioactive markers were the gifts of Dr. S. Tanaka of this university.) After development on filter papers, the composition of the preparations determined in 11 experiments was as follows: T_4 $82 \pm 3.0\%$, TA_4 $7 \pm 1.8\%$, I^- $5 \pm 3.7\%$, and the residual part $7 \pm 2.6\%$. No appreciable change of the composition was observable after it had been left standing for several days or even after incubation for 2 hours at 37.5°C , when the reaction mixture was lacking mitochondria. Therefore, any yield of TA_4 less than 10% is to be regarded as indicating that the enzyme source has little activity or none.

Assay procedures. The general procedures for the purpose of detecting the T_4 transaminase activity of rat kidney mitochondria were essentially the same as those described in details previously (15). After incubation for 2 hours at 37.5°C , 20 μl . of the reaction mixture was chromatographed directly with *n*-butanol-acetic acid-water (78:5:17) as a solvent system. Percent distribution of radioactivity among T_4 , TA_4 , I^- and the residual part was estimated in two ways; one was the same as described before (15), and the other as follows: The radioactivity of a filter paper was graphed with a continuous automatic recording scanner equipped with a GM counter (Nihon-musen Electro-Medical Lab. Ltd.). Thereafter, the paper was cut at places corresponding to the peaks on the record and the spots on a radioautogram, and the radioactivity of each piece was counted by means of a well-type scintillation counter. The results obtained by the two methods almost completely coincided.

RESULTS

Enzyme activity of homogenate and mitochondria. Whole homogenate and mitochondria, representing 500 mg. of fresh tissue each, from the same rat kidney were compared as to their enzyme activity under anaerobic conditions. Table 1 shows that the enzyme activity is far greater with mitochondria.

TABLE 1. Enzyme Activity of Homogenate and Mitochondria

Conditions	Percent distribution of radioactivity			
	TA_4	T_4	I^-	Resid.
Homogenate				
Fresh	12	72	7	9
Boiled	5	74	7	14
Mitochondria				
Fresh	37	44	8	11
Boiled	5	77	7	11

The percent radioactivity corresponding to TA_4 formed by mitochondria was 3 times that formed by homogenate.

When tissue preparations were boiled for 20 minutes, the enzyme activity disappeared completely.

Enzyme activity of mitochondria and supernatant. Mitochondria and supernatant, representing 500 mg. of fresh tissue each, were compared under both aerobic and anaerobic conditions. Table 2 shows that the enzyme is present mainly in the mitochondrial fraction. The supernatant, including in this case microsomal fraction also, shows far less activity. Aerobic conditions seem to be favorable for the enzyme activity. In general, however, the difference is rather slight, as will be shown later.

TABLE 2. Enzyme Activity of Mitochondria and Supernatant

	Enzyme sources	Percent distribution of radioactivity			
		TA_4	T_4	I^-	Resid.
Aerobic	Mitochondria	38	46	4	12
	Supernatant	17	60	12	11
Anaerobic	Mitochondria	23	59	11	8
	Supernatant	9	77	8	5

Amount of mitochondria and the enzyme activity. An aerobic experiment was carried out employing various amounts of mitochondria, representing 500 mg. to 15 mg. of fresh tissue. Fig. 1 shows that the amount of TA_4 formed approximately parallels the amount of mitochondria below the 250 mg. level. Even with the least amount of mitochondria, the enzyme activity was demonstrable. Percentages of radioactivity calculated as TA_4 were 35, 36, 33, 22, 19, 13 for amounts of mitochondria of 500, 250, 125, 60, 30, 15 mg. respectively. In a control without the addition of mitochondria, TA_4 production was 5%.

Under anaerobic conditions, a similar result was obtained and it was not listed here.

Oxygen pressure and the enzyme activity. Under anaerobic conditions, the enzyme activity of mitochondria was to some extent lower than under aerobic conditions. Table 3 shows the difference observed in repeated experiments. It may be also observable that the enzyme activity varies considerably from experiment to experiment, probably on account of variations in rat kidney mitochondrial preparations.

Potassium chloride and sucrose as homogenizing and suspending media. Mitochondrial fractions were isolated from homogenates prepared with 0.15 M KCl and 0.25 M sucrose and resuspended in the same media respectively. Under anaerobic conditions these two preparations, each representing 50 mg. of fresh tissue, showed exactly the same activity (fig. 2). Calculated TA_4 production was 18% in each case.

FIG. 1. Conversion of T_4 to TA_4 and the amount of rat kidney mitochondrial enzyme. The amounts of mitochondria, expressed in mg. of fresh tissue, are in *A* 500, *B* 250, *C* 125, *D* 60, *E* 30, *F* 15. *G*: control without mitochondria. O, I^- , T_4 and TA_4 indicate the positions of origin, inorganic iodide, thyroxine and tetraiodothyroacetic acid on paperchromatogram.

TABLE 3. Enzyme Activity under Aerobic and Anaerobic Conditions

	Percent distribution of radioactivity			
	TA ₄	T ₄	I ⁻	Resid.
Aerobic	17	67	12	4
	26	63	3	8
	26	65	3	6
	29	53	11	7
	33	44	14	9
	36	47	6	11
	38	46	4	12
	46	35	6	14
Mean±S.D.	31±7.4	52±10.7	7±4.3	9±3.1
Anaerobic	17	63	9	11
	18	65	10	6
	22	64	5	10
	23	59	11	8
	26	54	12	8
	31	48	13	8
	34	40	12	14
	35	47	6	12
Mean±S.D.	26±6.5	55±8.7	10±2.7	10±2.5

FIG. 2. Enzyme activity and the homogenizing and suspending media. A: with 0.25 M sucrose. B: with 0.15 M KCl. Symbols are the same as in fig. 1.

Influence of lack of pyridoxal phosphate (PP) and α -ketoglutarate (KG). Under either aerobic or anaerobic conditions, the lack of the two cofactors of transaminase resulted in a marked reduction of the TA_4 production (table 4). In some cases, no enzyme activity was detectable.

TABLE 4. Enzyme Activity with and without Addition of Pyridoxal Phosphate and α -Ketoglutarate

Exper. No. Conc. of T_4	Conditions	Percent distribution of radioactivity			
		TA_4	T_4	I^-	Resid.
1 3×10^{-6} M	Aerobic				
	With PP and KG	26	63	3	8
	Without PP and KG	13	79	2	6
	Anaerobic				
2 10^{-6} M	With PP and KG	22	64	5	10
	Without PP and KG	8	79	1	12
	Aerobic				
	With PP and KG	36	47	6	11
3 10^{-5} M	Without PP and KG	11	72	7	10
	Anaerobic				
	With PP and KG	17	63	9	11
	Without PP and KG	5	82	4	9
4 10^{-5} M	Aerobic				
	With PP and KG	14	80	3	4
	Without PP and KG	4	92	1	3
	Anaerobic				
4 10^{-5} M	With PP and KG	31	48	13	8
	Without PP and KG	11	63	17	9

Effect of oxalacetate and pyruvate. As amino acid acceptors in the transamination reaction, the effect of oxalacetate and pyruvate upon the enzyme activity was tested under anaerobic conditions (table 5). Oxalacetate (10^{-2} M) was proved to be as active as KG in increasing the production of TA_4 . When PP was lacking, the effect decreased considerably. Pyruvate (10^{-2} M) appeared to be less effective.

TABLE 5. Effect of Oxalacetate and Pyruvate upon Enzyme Activity

Experimental conditions	Percent distribution of radioactivity			
	TA_4	T_4	I^-	Resid.
KG+PP	35	47	6	12
Oxalacetate+PP	38	48	5	9
Oxalacetate	27	60	6	8
Pyruvate+PP	13	75	3	9
Control	5	87	2	5

Influence of glutamate and aspartate. For the purpose of detecting the inverse transamination reaction, that is, transamination from glutamate or aspartate to KT_4 , the following experiments were carried out. If such a reaction took place, the conversion of T_4 to TA_4 , through KT_4 as an intermediate product, would be reduced and the decrease of formed TA_4 and the increase of remaining T_4 could be predicted.

Fig. 3 shows precisely such a predicted effect of glutamate. Percent radioactivity of TA_4 was 32 without glutamate, whereas it was 21 with 10^{-2} M glutamate in the presence of 10^{-2} M KG; 32 and 17 without and with 1.5×10^{-2} M glutamate in the presence of 5×10^{-3} M KG.

Table 6 indicates a similar type of, but less marked, effect of aspartate in the presence of oxalacetate.

FIG. 3. Influence of glutamate upon enzyme activity. A: without glutamate and B: with 10^{-2} M glutamate in the presence of 10^{-2} M α -ketoglutarate. C: without glutamate and D: with 1.5×10^{-2} M glutamate in the presence of 5×10^{-3} M α -ketoglutarate. Symbols are the same as in fig. 1.

TABLE 6. Influence of Aspartate upon Production of TA_4 and Consumption of T_4 .

Experimental conditions	Percent distribution of radioactivity			
	TA_4	T_4	I^-	Resid.
Oxalacetate (10^{-2} M)+PP	33	56	5	7
Without aspartate	29	59	6	6
With aspartate (10^{-2} M)				
Oxalacetate (5×10^{-3} M)+PP	33	55	5	7
Without aspartate	28	60	6	7
With aspartate (1.5×10^{-2} M)				

Effect of DPN. Effect of DPN upon the conversion of T_4 to TA_4 was compared with that of PP and KG. Table 7 indicates that DPN (10^{-3} M) is considerably effective under aerobic conditions, though the effect was much less than that of PP and KG. It is interesting that DPN is without effect under anaerobic conditions. Experiment 3 shows that DPN does not enhance the formation of TA_4 in the presence of PP and KG under aerobic conditions.

TABLE 7. Effect of DPN upon Enzyme Activity

Exper. No. T_4 conc.	Exper. conditions	Percent distribution of radioactivity			
		TA_4	T_4	I^-	Resid.
1 10^{-6} M	Aerobic PP+KG DPN	29 13	53 68	11 8	7 10
	Anaerobic PP+KG DPN	18 6	65 76	10 10	6 8
2 10^{-6} M	Aerobic PP+KG DPN	33 19	44 61	14 12	9 8
	Anaerobic PP+KG DPN	26 9	54 71	12 10	8 10
3 10^{-6} M	Aerobic PP+KG	58	21	8	13
	PP+KG DPN	43	37	7	13
	PP+KG+DPN	52	27	8	13

Effect of succinate and malate. The effect of succinate (10^{-2} M) and malate (10^{-2} M) was compared with that of PP and KG (table 8). Experiments 1 and 2 show that succinate is far less active than PP and KG under aerobic conditions. Under anaerobic conditions, its effect appears negligible. Experiment 3 shows that succinate enhances the action of DPN but not that of PP and KG under aerobic conditions. Experiment 4 indicates the effect of malate similar

TABLE 8. Effect of Succinate and Malate upon Enzyme Activity

Exper. No. T ₄ conc.	Exper. conditions	Percent distribution of radioactivity			
		TA ₄	T ₄	I ⁻	Resid.
1 10 ⁻⁶ M	Aerobic PP+KG Succinate	29 9	53 75	11 9	7 7
	Anaerobic PP+KG Succinate	18 4	65 80	10 9	6 7
2 10 ⁻⁶ M	Aerobic PP+KG Succinate	33 28	44 51	14 13	9 8
	Anaerobic PP+KG Succinate	26 11	54 74	12 8	8 7
3 10 ⁻⁶ M	Aerobic PP+KG	58	21	8	13
	PP+KG+Succinate	47	29	8	16
	DPN	43	37	7	13
	DPN+Succinate	52	26	7	15
4 3 × 10 ⁻⁶ M	Aerobic Succinate	37	51	3	10
	Succinate+Malonate	27	55	6	11
	Malate	37	48	5	10
	Malate+Malonate	35	49	6	10

to that of succinate. The same experiment shows an inhibitory action of malonate (10^{-2} M) upon the effect of succinate but not that of malate.

DISCUSSION

The present study made clear that the conversion of T₄ to TA₄ by rat kidney mitochondria was greater than that by whole homogenate and that by supernatant including microsome, in the presence of PP and KG in each case. The activity was not detectable after boiling the tissue preparations for 20 minutes at 100°C. These observations indicate the existence of an enzyme system, probably including a transaminase, in rat kidney especially associated with the mitochondrial fraction.

As has been stated before (15), it is widely accepted that thyroid hormones are broken down by deamination. It then becomes necessary to make clear which of two enzymes, *L*-amino acid oxidase and transaminase, plays the main role in the deamination. Some serious difficulties are anticipated to be inherent in dealing with the problem. First of all, the immediate deamination products of thyroid hormones are the same keto acid analogues, whether the reaction is mediated by *L*-amino acid oxidase or transaminase. The difficulty is increased by the fact that the keto acid derivatives, thyroxyruvic acids in this case, are not available at present for *in vitro* experiments. In addition,

these compounds have never been detected in *in vitro* experiments, though they were reported to have been found in minute amount *in vivo* (6, 8). Other than these obstacles, usual procedures to discriminate transaminase from *L*-amino acid oxidase do not appear useful in cases of thyroid hormones. For example, the ammonia production due to *L*-amino acid oxidase, and not to transaminase, probably could not be tested on account of the extremely low solubility of thyroid hormones. The assay of amino acid resulting from transamination of the hormones is likewise unpromising for the same reason.

These limitations had led the present authors to carry out experiments, employing TA_4 production as an indirect measure of transamination, which may allow a decision, if not a final one, as to the nature of the enzyme. Results favorable for the transaminase obtained with rat kidney homogenate have been given in a preceding paper (15). Evidence obtained with kidney mitochondria in this study is as follows:

1. Dilution effect was not detectable on the enzyme in question, contrary to the case of *L*-amino acid oxidase.
2. Anaerobic conditions only slightly influenced the enzyme activity.
3. Pyridoxal phosphate and α -ketoglutarate, both being essential cofactors to transaminases, and not to *L*-amino acid oxidase, were strikingly effective in increasing the enzyme activity.
4. Oxalacetate and pyruvate were similarly active to α -ketoglutarate as amino group acceptors.
5. Finally, the influence of glutamate and aspartate, to which much importance has been attached in this work, was the most interesting. These compounds were proved to be effective in decreasing the break-down of T_4 and the production of TA_4 . This fact should be interpreted as showing that these compounds serve as amino group donors, and KT_4 as soon as formed may receive amino group from these donors and return again to T_4 . TA_4 formation decreases on account of the unsufficient availability of KT_4 , the precursor. This inverse transamination experiment has been suggested by Tomita (13), to whom the authors are grateful.

All of these experimental results indicate that the enzyme concerned in the deamination of T_4 is a transaminase rather than *L*-amino acid oxidase, at least in the kidney.

Reports have been accumulated concerning the conversion of thyroid hormones *in vitro* to their acetic acid derivatives by tissue preparations, such as the kidney (5), liver (5), skeletal muscle (10) and brain (11). Concerning the enzymes involved in the conversion process, however, few reports have been available up to the present. Employing a soluble enzyme extracted from rat kidney mitochondria, Tomita *et al.* (14) and Albright, Tomita and Larson (2) demonstrated that the conversion of T_4 to TA_4 and of T_3 to TA_3 was enhanced markedly by the addition of DPN under aerobic conditions. They further reported that the addition of succinate and the other intermediate compounds of the tricarboxylic acid cycle were also effective, though succinate enhanced no more the conversion in the presence of DPN. The nature of the enzyme remained obscure.

The present comparative data obtained with rat kidney mitochondria showed that DPN is consistently much less active than PP and KG under aerobic conditions and almost without effect under anaerobic conditions.

Succinate and malate were likewise less effective than PP and KG, and without effect under anaerobic conditions. The inhibitory effect of malonate upon the enhancing action of succinate appears to suggest that the effect of succinate is, at least in part, due to the production of oxalacetate. The same may be the case with malate.

In apparent contrast to the report that the soluble DPN-linked enzyme became inactive after treatment with 0.15 M KCl (14), the mitochondrial transaminase was fully active in 0.15 M KCl in the present experiment.

Another interesting study was made by Nakano *et al.* (4) who observed that *l*-amino acid oxidase derived from snake venom deaminated actively thyroid hormones. Later, thyroxyruvic acids formed were identified with authentic compounds synthesized by the same author (3).

Considering the data presented here together with those reported previously (15), it may be stated that a transaminase acting upon thyroxine exists in rat kidney and the enzyme is the most important enzyme involved in the deaminative breaking down of the hormone.

SUMMARY

The conversion of thyroxine to tetraiodothyroacetic acid was studied employing rat kidney mitochondria as an enzyme source. The conversion was only slight without the addition of pyridoxal phosphate and α -ketoglutarate. The effect of these two cofactors of transaminases was much greater than that of any other compounds tested, such as DPN, succinate and malate. The enhancing effect of pyridoxal phosphate and amino acid acceptors (α -ketoglutarate, oxalacetate and pyruvate), only a slight influence of anaerobiosis, the lack of "dilution effect", and a marked inhibitory effect of amino group donors (glutamate and aspartate) upon the conversion—all of these things indicated that the enzyme involved is of the nature of a transaminase rather than *l*-amino acid oxidase. The transaminase may be the main enzyme involved in the deaminative breaking down of thyroxine, even if other enzymes are present and active. The transaminase exists mainly within mitochondria and is inactivated by boiling for 20 minutes.

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