single displacement mechanisms for enzymic substitution reactions recently discussed by Koshland (1954).

SUMMARY

1. The ability of a number of methylsulphonium and ethylsulphonium salts related to dimethylthetin to act as substrates for partially purified preparations of thetin-homocysteine transmethylase from rat liver has been examined. Dimethylthetin is the most effective methyl donor so far investigated.

2. Trimethylsulphonium chloride acts as a substrate for the enzyme, and gives rise to a linear reaction rate and a linear enzyme activity/concentration relationship. This compound has therefore been considered as a suitable substrate for the manometric assay of the transmethylase.

3. Homocysteine was the only effective methyl acceptor among the thiols tested, although some evidence was obtained to suggest that L-cysteine might be acting as a substrate.

4. None of the metal ions which were tested activated the enzyme; several heavy-metal ions and potassium ions were inhibitory. Several other commonly-used inhibitors had no effect on the enzyme.

5. Possible mechanisms for the participation of the enzyme in the transmethylation reaction have been proposed.

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Further Observations on the Oxidation of Di-iodotyrosine Derivatives

By ROSALIND PITT-RIVERS AND A. T. JAMES

National Institute for Medical Research, Mill Hill, London, N.W. 7

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It has already been shown by one of us (Pitt-Rivers, 1948) that the conversion of di-iodotyrosine into thyroxine *in vitro* is enhanced when both the amino and carboxyl groups of di-iodotyrosine are blocked; the first compound of this nature to be studied was *N*-acetyl-DL-di-iodotyrosyl-L-glutamic acid, which gave rise to the corresponding thyroxine derivative in yields up to 35%, calculated on amount of di-iodotyrosine derivative lost. In the present paper, the formation *in vitro* of several new thyroxine derivatives is described. At the same time, the side chain lost from one di-iodotyrosine molecule during the coupling reaction has been identified.

EXPERIMENTAL

The acetyl-DL-di-iodotyrosyl-amino acid derivatives are all formed from 4-(3:5-di-iodo-4-acetoxybenzyl)-2-methyl-oxazolone and the required amino acid, as in the synthesis

of *N*-acetyl-DL-di-iodotyrosylglutamic acid (Pitt-Rivers, 1948) and will not, except for *N*-acetyl-DL-di-iodotyrosyl- ϵ -*N*-(α -*N*-acetyl)-lysine, be described in detail; the aerobic incubation of this compound, which gave the highest yields of the thyroxine derivative, was extensively studied and led to the recognition of the C_5 residue split off from the di-iodotyrosine molecule.

N-Acetyl-DL-di-iodotyrosyl- ϵ -*N*-(α -*N*-acetyl)-L-lysine

4-(3:5-Di-iodo-4-acetoxybenzyl)-2-methyl-oxazolone (20 g.) was dissolved in dry acetone (170 ml.) and chilled in an ice bath. α -*N*-Acetyl-lysine (Neuberger & Sanger, 1943) (6 g.) dissolved in *N*-NaOH (32 ml.) was added and the reaction was allowed to proceed for 14 hr. Most of the acetone was then removed under diminished pressure, the solution was diluted with water, and acidified with HCl. After chilling, the product was collected, washed with water and crystallized from 50% (v/v) ethanol. The compound crystallized in thin leaflets, m.p. 170-171°; the yield was 88% of the theoretical (Found: N, 6.4; I, 39.4. $C_{19}H_{28}O_8N_2I_2$ requires N, 6.5; I, 39.4%).

*Incubation of N-acetyldi-iodotyrosyl-
ε-N-(α-N-acetyl)-L-lysine*

The above compound (12.9 g., 0.2 mole) was suspended in 100 ml. of 0.2 M-borate buffer (pH 7.5) and dissolved by addition of 35 ml. of *N*-NaOH. Saturated Na₂SO₄ solution (35 ml.) was added, since it was found to produce filterable crystals of the sodium thyroxine derivative. The solution was kept at 38° for 10–11 days in an open Erlenmeyer flask (700 ml.) without shaking, after which a heavy crystalline deposit of the sodium salt of *N*-acetyl-DL-thyroxyl-ε-*N*-(α-*N*-acetyl)-L-lysine had separated. This was filtered off, washed with borate buffer and decomposed by acidification of its alkaline solution. The thyroxine derivative was recrystallized from 70% (v/v) ethanol, and separated in fine needles, which had an indeterminate m.p. with final decomposition at 200° (Found: C, 30.1; H, 3.0; N, 4.3; I, 51.0. C₂₅H₂₇O₇N₃I₄ requires C, 30.3; H, 2.7; N, 4.25; I, 51.4%).

As shown in Table 1, the yield of the thyroxine derivative varied from 40 to 50%, calculated on the recovered di-iodotyrosine derivative; the latter after recrystallization from 50% ethanol plus charcoal could be used for further incubation experiments.

Compounds giving rise to thyroxine derivatives

In Table 1 are shown the di-iodotyrosine derivatives which give rise to thyroxine derivatives and the net yields obtained after 10–11 days' aerobic incubation at pH 7.5 and 38°, in open Erlenmeyer flasks (700 ml.).

As is seen, the yields were variable and in most instances the reaction mixtures at the end of the experiment had a brownish colour, indicating decomposition of the di-iodotyrosine derivative. This was not so with the lysine peptide, where the reaction mixture remained a pale-straw colour throughout the incubation. For this reason it was chosen for the investigation of other products of the reaction.

*Other products formed during the incubation of
N-acetyldi-iodotyrosyl-ε-N-(α-N-acetyl)-lysine*

In the initial incubation experiments, a thorough search was made in the reaction mixture for serine, alanine and oxalic acid. In no case could any of these be detected,

although it was shown that recovery of all these compounds when added to the reaction mixture was good.

Acetamide. A little free NH₃ was shown to be present in the reaction mixture, and it was therefore suspected that acetamide might be present, resulting from oxidative deamination of *N*-acetyldi-iodotyrosine. Ammonia and acetamide were determined according to the method of Pucher, Vickery & Leavenworth (1935). In two experiments, where the amount of thyroxine derivative obtained was 0.51 and 0.57 m-mole, the NH₃ plus acetamide was found to be 0.52 and 0.585 m-mole respectively. The free NH₃ represented less than 10% of the NH₃ lost. It therefore appears that in these experiments the formation of thyroxine is accompanied by an equivalent loss of NH₃ (as acetamide) from the side chain of di-iodotyrosine. This led to a search for a carbonyl derivative which might be quantitatively isolated.

The carbonyl derivative from the di-iodotyrosine side chain. Early attempts to isolate the carbonyl derivative with 2:4-dinitrophenylhydrazine were unsatisfactory and *p*-bromophenylhydrazine was substituted. After removal of unchanged di-iodotyrosine peptide by acidification, the reaction mixture was treated with an amount of *p*-bromophenylhydrazine molecularly equivalent to the amount of thyroxine peptide obtained. After a short time, a pale-yellow crystalline precipitate appeared; separation was complete after 3 days at 4°; further addition of *p*-bromophenylhydrazine did not increase the precipitate. Chromatographic analysis of the yellow material in butanol-dioxan-2*N*-NH₃ (4:1:5, by vol.) showed that the major component was colourless, and absorbed in u.v. light (substance *A*). There was also present a bright-yellow component which fluoresced in u.v. light (substance *F*). These two compounds were not satisfactorily separated by crystallization, but *A* was soluble in hot water whereas *F* was not. The crude material was therefore extracted with water in a Soxhlet apparatus and the crystalline substance *A* so obtained was recrystallized from 30% (v/v) methanol, from which it separated in glistening plates, m.p. 160–162° (Found: C, 46.0; H, 5.0; N, 12.6; Br, 18.0%).

This analysis agrees with that required for the *p*-bromophenylhydrazone of ε-*N*-hydroxypyruvoyl-α-*N*-acetyl-lysine, which has C, 46.0; H, 5.0; N, 12.65; Br, 18.1%. The amounts of the bromophenylhydrazone obtained after incubation varied between 80 and 90% of the amounts

Table 1. *Di-iodotyrosine derivatives giving rise to thyroxine derivatives during aerobic incubation*

Di-iodotyrosine derivative	Thyroxine derivative formed	Net yield of thyroxine derivative (%)
<i>N</i> -Formyl-L-di-iodotyrosine	<i>N</i> -Formyl-L-thyroxine	20
<i>N</i> -Propionyl-L-di-iodotyrosine	<i>N</i> -Propionyl-L-thyroxine	20
<i>N</i> -Acetylglucyl-L-di-iodotyrosine	<i>N</i> -Acetylglucyl-L-thyroxine	30
<i>N</i> -Acetyl-DL-di-iodotyrosylglycine	<i>N</i> -Acetyl-DL-thyroxylglycine	30
<i>N</i> -Acetyl-DL-di-iodotyrosyl-β-alanine	<i>N</i> -Acetyl-DL-thyroxyl-β-alanine	10
<i>N</i> -Acetyl-DL-di-iodotyrosyl-L-valine	<i>N</i> -Acetyl-DL-thyroxyl-L-valine	10
<i>N</i> -Acetyl-DL-di-iodotyrosyl-L-proline	<i>N</i> -Acetyl-DL-thyroxyl-L-proline	15
<i>N</i> -Acetyl-DL-di-iodotyrosyl-ε- <i>N</i> -(α- <i>N</i> -acetyl)-L-lysine	<i>N</i> -Acetyl-DL-thyroxyl-ε- <i>N</i> -(α- <i>N</i> -acetyl)-L-lysine	40–50
4-Hydroxy-3,5-di-iodophenylacetic acid*	Tetraiodothyroacetic acid	15–20

* Although not a derivative of di-iodotyrosine, this compound has been included since it gives considerable yields of the thyroxine analogue.

expected from the yields of the thyroxine derivative obtained.

Reduction of substance A. The purified bromophenylhydrazone, *A*, was suspended in water and shaken with sodium amalgam at room temperature (cf. Sprinson & Chargaff, 1946). When hydrogen ceased to be evolved the aqueous solution was neutralized and then further acidified to 2*N* with HCl. After boiling under reflux for 2½ hr., the solution was concentrated and chromatographed on Whatman No. 3 paper in *m*-cresol-water in an atmosphere of NH₃. This system has been shown to separate glycine, alanine, serine and lysine. The last-named amino acid, after 4 days' descending chromatography, is washed off the paper. The *R_F* of the reduction product of *A*, after removal of lysine, is the same as that of serine; no alanine was found. It was therefore concluded that the whole of the di-iodotyrosine side chain had been converted into hydroxypyruvic acid.

Purification of substance F. The water-insoluble yellow material left after aqueous extraction of *A* was crystallized from ethanol, from which it separated in fine yellow needles having m.p. 225–227° (decomp.) (Found: C, 45.6; H, 4.6; Br, 26.0%). This analysis agrees with that required for the *p*-bromophenylosazone of ϵ -*N*-hydroxypyruvoyl- α -*N*-acetyllysine, which has C, 45.1; H, 4.3; Br, 26.2%.

The osazone was only formed in very small amounts, and was considered to be a by-product of the reaction.

Incubation of *N*-acetyl-L-di-iodotyrosine

This compound has been shown (Pitt-Rivers, 1948) to give rise to *N*-acetyl-L-thyroxine during aerobic incubation. Repeated attempts to characterize the side chain which is split off during the reaction have been made, by using *p*-nitrophenylhydrazine, 2,4-dinitrophenylhydrazine and *p*-bromophenylhydrazine for the isolation of the carbonyl derivative, but no pure compounds could be isolated.

Identification of the side chain after incubation of 3:5-di-iodo-4-hydroxyphenylacetic acid

This compound was incubated in borate buffer for 10 days at 37°, and, after removal of tetraiodothyroacetic acid and unchanged starting material, the solution was treated with 2:4-dinitrophenylhydrazine. The yellow precipitate so obtained was shown by chromatographic analysis in butanol saturated with 3% aqueous NH₃ to have the same *R_F* values as those of the dinitrophenylhydrazones of glyoxylic acid (probably obtained from a mixture of the *cis* and *trans* forms of the acid).

Di-iodotyrosine derivatives and related compounds which do not give rise to the corresponding thyroxine analogue

The following compounds gave no thyroxine derivative after aerobic incubation at pH 7.5 for 10–14 days: DL-di-iodotyrosyl-L-di-iodotyrosine, *N*-acetyldi-iodotyrosyldi-iodotyrosine, benzoyl-L-di-iodotyrosine, phenacetyl-L-di-iodotyrosine and 4-hydroxy-3:5-di-iodophenylbenzoic acid.

DISCUSSION

From these findings it appears that aerobic incubation of acetyldi-iodotyrosine peptides gives rise to the following products: the corresponding thyroxine derivative, acetamide and hydroxypyruvoylpeptide; these last are both present in amounts approximately equivalent to the amount of thyroxine found. It has already been suggested (Pitt-Rivers, 1948) that oxidation on the β -carbon atom of the di-iodotyrosine side chain probably occurs during oxidation by hydrogen peroxide since 3:5-di-iodo-*p*-hydroxybenzaldehyde was

Fig. 1

obtained as a by-product. However, in the milder conditions used in the present experiments the further breakdown of the side chain does not take place, and the reaction may be shown to follow quantitatively the scheme shown in Fig. 1. It must again be emphasized that, under the conditions of these incubations, the thyroxine derivative, acetamide and the hydroxypyruvoyl-lysine derivative are the only compounds obtained. Thus we have been unable to confirm the claim by Johnson & Tewkesbury (1942) that pyruvic acid was formed during alkaline incubation of di-iodotyrosine; nor have we been able to demonstrate the presence of serine, which Sela (1956) found among the products obtained from the alkaline incubation of iodinated polytyrosine.

The fact that the hydroxypyruvic acid derivative can be isolated at all is probably due to the blocking of its carboxyl group. Attempts to form a *p*-bromophenylhydrazone from solutions of hydroxypyruvic acid prepared according to Sprinson & Chargaff (1946) led to gummy decomposition products. The latter authors have also demonstrated the instability of hydroxypyruvic acid in alkaline solution. This may account for the failure to detect it when the carboxyl group of di-iodotyrosine is not blocked, i.e. after the incubation of *N*-acetyl-di-iodotyrosine.

It seems worth commenting on the fact that a di-iodo-*p*-hydroxyphenyl derivative with a C_2 side chain can also give rise to a tetraiododiphenyl ether. The formation of tetraiodothyroacetic acid from 4-hydroxy-3:5-di-iodophenylacetic acid occurred in yields as good as those of acylated thyroxines obtained from the acylated di-iodotyrosines; it may be that an essential feature

during the formation of tetraiododiphenyl ether is oxidation of the side-chain carbon atom adjacent to the ring (Fig. 2). Certainly in the absence of this methylene group no condensation occurs, i.e. as with 4-hydroxy-3:5-di-iodobenzoic acid.

A final comment may be made about other di-iodotyrosine derivatives which do not give a thyroxine derivative during mild alkaline oxidation, namely benzyl- and phenacetyl-di-iodotyrosine, di-iodotyrosyldi-iodotyrosine, *N*-acetyldi-iodotyrosyldi-iodotyrosine and 4-hydroxy-3:5-di-iodophenylpyruvic acid. The marked instability of the last compound in alkaline solution probably leads to its complete breakdown, as evidenced by the green colour rapidly obtained during incubation. With the other compounds steric hindrance may be the predominant cause for the failure to couple; in each, the amino group of di-iodotyrosine is attached to a group containing another phenyl residue. Table 1 shows that only di-iodotyrosine derivatives acylated with completely aliphatic groups will give rise to thyroxine.

SUMMARY

1. The formation of thyroxine peptides from di-iodotyrosine peptides has been further investigated.

2. *N*-Acetyldi-iodotyrosyl- ϵ -*N*-(α -acetyl)-lysine has been shown to give rise to high yields of the corresponding thyroxine derivative during aerobic incubation at pH 7.5.

3. The side chain lost during incubation of this peptide has been characterized through its *p*-bromophenylhydrazone as ϵ -*N*-hydroxypyruvoyl- α -*N*-acetyl-lysine.

4. The side chain lost from *N*-acetyl-L-di-iodotyrosine during oxidative coupling has not been satisfactorily characterized. It has not been possible to demonstrate the presence of pyruvic acid.

5. The side chain lost during the formation of tetraiodothyroacetic acid from 4-hydroxy-3:5-di-iodophenylacetic acid has been identified as glyoxylic acid.

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Fig. 2