The Identification of 3,3',5,5'-Tetraiodothyroformic Acid within the Rat Liver

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(Received 26 March 1974)

Homogenized rat livers were extracted with organic solvents and the extracts converted into more readily volatile derivatives (trimethylsilylmethyl esters and permethyl esters). Combined g.l.c.–mass-spectrometric analysis identified the presence of tetraiodothyroformate. It is postulated that mitochondrial β -oxidation of tetraiodothyropropionate results in the formation of this apparently undescribed metabolite.

It has been known for some years that the body's major store of extrathyroidal iodine is the liver (Abelin & Scheinfinkel, 1925; Kendall, 1919; Krayner, 1928; Zawadowsky & Asimoff, 1927). Within this organ three principal pathways for the metabolism of thyroid hormones have been shown to exist. These are: (a) deiodination (MacLagan & Spratt, 1954); (b) conjugation with either glucuronic acid (Taurog *et al.*, 1952), or sulphate (Roche & Michel, 1957; Roche *et al.*, 1957); (c) oxidative decarboxylation via the pyruvic acid analogue (Myant, 1956; Roche *et al.*, 1954) to the acetic acid analogue (Galton & Pitt-Rivers, 1959).

The metabolites resulting from these various processes have been identified by relatively unsophisticated physicochemical techniques such as paper chromatography or t.l.c. The more recent developments in g.l.c. combined with mass spectrometry, however, have provided an extremely sensitive and discriminating tool for the identification of substances of biological origin. With the availability of this facility, we decided to reinvestigate the products of hepatic metabolism of thyroid hormones as a first step towards a more detailed understanding of the kinetics of these processes.

Experimental

Materials

Tetraiodothyroformic acid and tetraiodothyropropionic acid were obtained from K & K Rare Chemicals Ltd., c/o Kodak, Kirkby, Liverpool, U.K. Toluene-*p*-sulphonyl-*N*-methylnitrosamide was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Boron trifluoride (15%, w/v) in methanol and tetraiodothyroacetic acid were from Sigma (London) Chemical Co. Ltd., Kingston-upon-

Thames, Surrey, U.K. Deuteromethanol ($C^2H_5O^2H$) was from Miles–Seravac Ltd., Maidenhead, Berks., U.K. and *NO*-bis-(trimethylsilyl)acetamide was from Pierce Chemicals, Phase Separation R & D Chemicals Division, Queensferry, Flintshire, U.K. All other reagents were obtained from BDH Chemicals Ltd., Atherstone, Warwickshire, U.K. and were of 'AnalaR' grade.

Purification of benzene. Benzene was washed with conc. H_2SO_4 , decanted and stored over $NaHCO_3$ to remove excess of acid.

Diazomethane. This was made by the following method: toluene-*p*-sulphonyl-*N*-methylnitrosamide (2.14 g) was dissolved in diethyl ether (30 ml) and the solution cooled in ice. KOH (0.4 g) in aq. 90% (v/v) methanol (10 ml) was added to this solution and the mixture left for 5 min. The ethereal diazomethane was then distilled into an ice-cooled receiver by heating the reaction medium to 45–50°C.

Extraction of liver

The rat was anaesthetized under ether and the liver back-perfused *in situ* with sterile 0.9% NaCl via the inferior vena cava until fully blanched. The liver was then excised and homogenized with a hand-driven homogenizer for 15 min at 0°C. The homogenate thus obtained was freeze-dried and the residue extracted with anhydrous methanol containing 25% (v/v) HCl. The methanolic extract was evaporated to dryness and the residue re-extracted with benzene–methanolic HCl (10:1, v/v). This second extract was again evaporated to dryness and the residue dissolved in a small volume of benzene–acetic acid (20:1, v/v). The solution was placed on top of a short silica-gel column (thin-layer grade; 8 cm × 0.6 cm) and the column was eluted with benzene–acetic acid until the beginning of the first major pigment band emerged.

Eluate, collected from the column during this period, was evaporated to dryness and a derivative was made of the residue for g.l.c.-mass-spectrometric studies.

Derivative formation

(a) *Trimethylsilylmethyl ester derivatives.* Anhydrous methanol-25% (v/v) HCl was added to the sample and the mixture heated in a sealed tube for 15 min at 60°C. The resultant solution was evaporated to dryness and then treated with *NO*-bis-(trimethylsilyl)acetamide (0.2 ml) for 15 min at 60°C (Alexander & Scheig, 1968). Deuteromethanol analogues were obtained by methylating with anhydrous $C^2H_5O^2H$ -HCl, then silylating with *NO*-bis-(trimethylsilyl)-acetamide.

(b) *Permethyl derivatives.* The sample was treated with an excess of a mixture of diazomethane and methylene chloride (2:1, v/v) for 30 min at room temperature (Zimmerer & Grady, 1971). The reaction mixture was then evaporated to dryness and the residue redissolved in benzene.

G.l.c.-mass spectrometry

Analysis was carried out on a Varian Aerograph 2700 gas chromatograph coupled via a Watson-Biemann twin-stage separator to a Varian-Mat 731 double-focusing mass spectrometer. Either a 0.3 m or a 2.0 m 3% OV-1-packed glass column with a helium flow rate of 20 ml/min was used for g.l.c. The injector, carrier gas separator and transfer lines were maintained at 290°C and the samples were ionized by electron impact (70 eV).

When a complete mass spectrum of a g.l.c. peak was desired, data from the spectrometer were processed by a Varian 100MS Spectro System, which gave normalized spectra in the form of bar graphs. For selective ion monitoring the peak-matching unit of the spectrometer was used, and although the switching range of this is limited (10% of low-mass channel) and there are only two channels, further ions within this range were monitored in a single g.l.c. run by changing the ratio of the decade resistances.

Trimethylsilylmethyl ester derivatives of rat liver homogenate were injected on the column together with carrier compounds, deuteromethyl *O*-trimethylsilyl 3,3',5,5'-tetraiodothyroformate, deuteromethyl *O*-trimethylsilyl 3,3',5,5'-tetraiodothyroacetate and deuteromethyl *O*-trimethylsilyl 3,3',5,5'-tetraiodothyropropionate. No carrier compounds were employed with the permethyl derivatives.

Results

Although rat liver is known to contain complex mixtures of thyroid-hormone metabolites, it was decided to concentrate on the acidic analogues initially, as it is easier to form derivatives of these. Because

of the high temperatures involved in g.l.c. and the attendant risk of deiodination only compounds containing four iodine atoms were investigated, thus avoiding the generation of deiodinated metabolites as artifacts. From the list of known metabolites there appeared to be only one acidic (tetraiodinated) compound; 3,3',5,5'-tetraiodothyroacetate. Initially the trimethylsilylmethyl ester derivatives were used as these had been shown to be suitable for selective ion monitoring (Hoffenberg *et al.*, 1973).

Tetraiodothyroformate and tetraiodothyropropionate have not previously been described as metabolites of thyroxine and therefore it was thought that one of these compounds might act as a suitable internal standard for the extraction and derivative-formation steps when added to rat liver homogenates. However, it was thought advisable first to establish the absence of these compounds from rat liver extracts. To this end, the effluent from the gas chromatograph was scanned for ions of *m/e* 820, 834 and 848 (molecular ions of methyl *O*-trimethylsilyl 3,3',5,5'-tetraiodothyroformate, methyl *O*-trimethylsilyl 3,3',5,5'-tetraiodothyroacetate and methyl *O*-trimethylsilyl 3,3',5,5'-tetraiodothyropropionate) when rat liver extract plus 50 ng of each of the trimethylsilyl deuteromethyl esters were injected on the column. These latter derivatives had been shown to

Fig. 1. G.l.c.-mass-spectrometric trace of rat liver extract by using the trimethylsilylmethyl ester technique of derivative formation

A, B and C represent the ions of *m/e* ratio 820, 834 and 848 and are equivalent to the molecular ions of trimethylsilylmethyl ester derivatives of 3,3',5,5'-tetraiodothyroformate, tetraiodothyroacetate and possibly tetraiodothyropropionate respectively. The gas chromatographic run was performed isothermally at 250°C by using a 0.3 m column of 3% OV-1 with a flow rate of 20 ml of helium/min.

be suitable as carrier compounds and give rise to minimum interference with the determination of their hydrogen analogues. A typical result of such a scan is shown in Fig. 1. 3,3',5,5'-Tetraiodothyroacetate was usually by far the most dominant metabolite. A total of ten rat livers was investigated by the technique of making the trimethylsilylmethyl ester derivative. An ion of m/e ratio equivalent to the molecular ion of the trimethylsilylmethyl ester derivative of 3,3',5,5'-tetraiodothyroacetate and possessing the same retention time was the major metabolite in all but two livers; 3,3',5,5'-tetraiodothyroformate was the major metabolite in two livers and was present in a further eight.

As a standard methyl *O*-trimethylsilyl 3,3',5,5'-tetraiodothyroformate preparation was used to focus the low-mass channel of the peak-matching unit before biological specimens were investigated and all trimethylsilylmethyl ester derivatives tend to be absorbed by the column, it was thought that these results might still have been artifacts, even though great care had been taken to clean the column with repeated injections of a carrier solution of the trimethylsilyl deuteromethyl ester derivatives of 3,3',5,5'-tetraiodothyroformate, 3,3',5,5'-tetraiodo-

thyroacetate and 3,3',5,5'-tetraiodothyropropionate and *NO*-bis(trimethylsilyl)acetamide. To overcome this objection a second set of derivatives (permethyl) were used which have a much lower tendency to be absorbed. Also a standard preparation of dimethyl 3,3',5,5'-tetraiodothyroacetate was used to focus the high-mass channel of the peak-matching unit, which had been set with the appropriate ratio on the decade resistances to monitor the molecular ion of dimethyl 3,3',5,5'-tetraiodothyroformate on the low-mass channel. In this way no 3,3',5,5'-tetraiodothyroformate derivative was introduced into the instrument before injection of the permethylated rat liver extract. Fig. 2 shows a typical result of such an injection when scanning at m/e 762 and 776, then 780, equivalent to the molecular ions of dimethyl 3,3',5,5'-tetraiodothyroformate, dimethyl 3,3',5,5'-tetraiodothyroacetate and dimethyl 3,3',5,5'-tetraiodothyropropionate respectively. Six rat liver extracts were investigated by using the permethyl derivative formation technique; 3,3',5,5'-tetraiodothyroformate was detected in four samples.

Attempts were made to obtain a complete spectrum of the dimethyl 3,3',5,5'-tetraiodothyroformate. These were unsuccessful owing to its relatively low concentration in most liver extracts. A complete spectrum of dimethyl 3,3',5,5'-tetraiodothyroacetate, which was usually present in much higher concentrations, was obtained and this was identical with that obtained from the standard preparation of this derivative.

Discussion

The previously recognized pathways of thyroxine metabolism within the liver are deiodination, conjugation and oxidative decarboxylation. Although Tata *et al.* (1957) postulated that deamination might give rise to the propionic acid analogue the presence of this compound has not been positively demonstrated. None of these pathways, however, would account for the presence of 3,3',5,5'-tetraiodothyroformate. Nevertheless, the evidence for the presence of this compound is very strong; when derivatives of extracts of rat liver homogenates were formed by either one of two independent methods, a compound was present which had the same g.l.c. retention times as a standard preparation of the appropriate 3,3',5,5'-tetraiodothyroformate derivative and which gave rise to ions of m/e ratio equivalent to its molecular ion.

Evidence that 3,3',5,5'-tetraiodothyropropionate was also an hepatic metabolite was much less conclusive. Although a compound possessing the correct retention time, and giving rise to an ion equivalent to the molecular ion of methyl *O*-trimethylsilyl 3,3',5,5'-tetraiodothyropropionate, was detected in a number of rat liver extracts by the first method of derivative formation, this could not be repeated with

Fig. 2. G.I.c.-mass-spectrometric trace of permethylated rat liver extract

A and B represent ions of m/e ratio 762 and 776 and are equivalent to dimethyl 3,3',5,5'-tetraiodothyroformate and dimethyl 3,3',5,5'-tetraiodothyroacetate. The gas chromatographic run was performed isothermally at 260°C by using a 2m column of 3% OV-1 with a flow rate of 20ml of helium/min.

Scheme 1. *Proposed metabolic pathway for the formation of 3,3',5,5'-tetraiodothyroformate from thyroxine via the pyruvate and lactate analogues*

A similar pathway has been described for the formation of *p*-hydroxybenzoate from tyrosine (Olsen, 1966; Trumpower *et al.* 1972).

the second method of derivative formation (per-methylation).

To account for the presence of 3,3',5,5'-tetraiodothyroformate within the rat liver, we propose an alternative second step for an already recognized pathway, oxidative decarboxylation, which normally proceeds from the amino acid via the pyruvate to the acetic acid analogue. This step would require conversion of the pyruvate into the lactate analogue, which then loses water to form the acrylic acid analogue. This latter compound, being an unsaturated fatty acid, would undergo microsomal β -oxidation ultimately to give the formic acid analogue (Scheme 1). Although this pathway appears somewhat complex, nevertheless it is analogous to a recognized pathway of tyrosine metabolism within the rat liver, by which tyrosine is converted into ubiquinone via *p*-hydroxybenzoate (Olsen, 1966; Trumpower *et al.*, 1972).

P. J. R. is the recipient of a Medical Research Council Scholarship.

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