# *In vivo* Labelling of Extractable Iodotyrosines in Serum of Rat and Dog. (31910)

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The claim has been made that stable iodotyrosines constitute a significant percentage of the iodoamino acids present in acid butanol extracts of normal human serum(1,2). A major objection to acceptance of this concept has been the failure of efforts to label these materials isotopically(3,4). The present study was undertaken to investigate this issue further. Labelled iodotyrosine materials have been demonstrated in significant concentrations in extracts of rat sera, following radioiodine administration, daily, for prolonged periods of time; in dog sera after a single labelled pulse; but, with a possible single exception, not in man.

*Materials and methods.* Male Wistar rats, 300 ± 18 g, were fed "Purina Chow" diet. Each animal received 0.6 μc Na 125-I (Squibb) and 6 μg Na 127-I, daily, in distilled drinking water, for 50 days. From days 51-57 inclusive, those rats which were not autopsied received half these amounts, daily, because of a shortage of radioactive stock solution. Blood samples were obtained (under anesthesia) on days 50, 51 and 57 from the inferior vena cava, 3-6 rats being used per sample.

Five mongrel male dogs, (B,C,E,F, and G) weighing between 12-15 kg each, were placed on a "Casco" dog cereal diet. Two of the dogs had previously been used in an unrelated experiment. Each dog received a single injection of 100 μc Na 125-I i.v. Dog F was not otherwise treated and served as a control. Dog G, unoperated, and B, thyroidectomized, received triiodothyronine (T3), as Cytomel, 1.5 μg/kg in the diet per day, for 7 days before 125-I was injected; whereas dogs C, intact, and E, sham operated, received T3 in the same daily doses but starting 3 days after 125-I injection. Blood samples were obtained from the jugular vein.

Na 125-I was fed daily to 4 healthy, euthyroid male volunteers in their early twenties in an effort to achieve isotopic equilibrium. Two subjects (W.H. and R.M.) received 4 μc Na 125-I and 20 μg Na 127-I, daily, for 60 days. Two other subjects (R.S. and A.S.) received 8 μc Na 125-I with 10 μg Na 127-I, daily, for 90 days. Blood was obtained from the brachial vein.

An additional 4 male patients, 3 euthyroid on desiccated thyroid and 1 on L-sodium thyroxine (Synthroid) after total thyroidectomy for thyroid cancer, were fed inner-ring, 3:5-125-I labelled thyroxine as a label. One, J.W., was given 65 μc in a single dose by mouth. Two, J.S. and M.M., each received by mouth 175 μc together with 3.0 mg 127-I T4, as a single dose. The fourth, A.K., the patient maintained on sodium thyroxine (T4), as Synthroid, 0.2 mg daily, was fed 125-I T4, 0.6 μc together with 0.2 mg 127-I T4, daily.

125-I inner-ring labelled L-thyroxine (3,5-positions) was prepared by the method of Lissitzky and Chettel(5) starting from L-DIT-125-I (Nuclear Science and Engineering Corp.).

*Extraction procedures.* Method 1(M-1) of Block and coworkers (6,7) was followed, using acid butanol for extraction and chromatographic separation of iodocompounds in serum. A shorter modification (Method 4) was employed when unusually high levels of radioactivity in the serum sample required only 1.0 to 2.0 ml serum to be treated.

The method of Lissitzky and coworkers, with water as the eluant, was also employed (8), thereby avoiding quantities of salt which interfere with subsequent chromatographic separation. The results agree well with those from the use of ammonium acetate buffer for elution.

A column, 75 × 2.5 cm, was used for 30cc human serum, 70 × 1.5 cm for 3 cc rat se-

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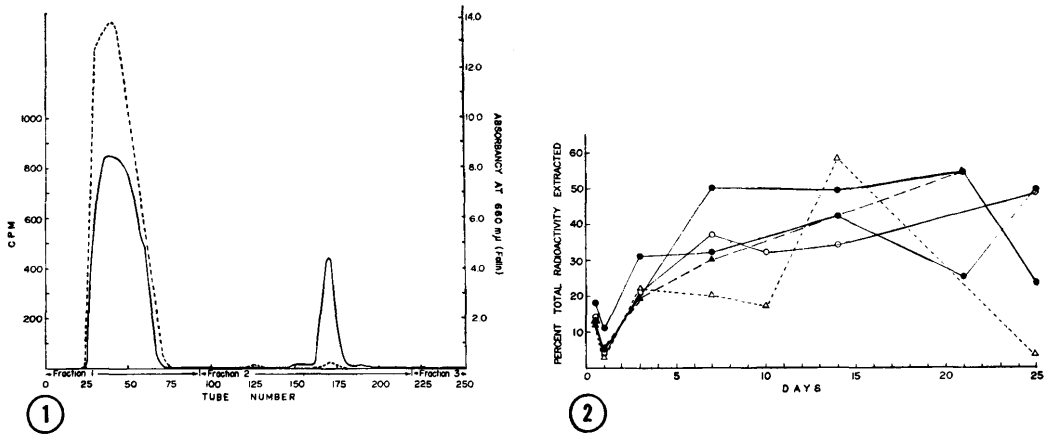


Fig. 1. Sephadex G-25 gel filtration of *in vivo* labelled rat serum. Effluent diagram showing distribution of radioactivity (solid line), and distribution of proteins (broken line). Distilled deionized water was used to elute fractions 1 and 2 and tertiary amyl alcohol saturated with 2N ammonia to elute fraction 3.

Fig. 2. Radioactive iodotyrosine concentrations in sera of dogs at various time intervals after injection, 100 μc Na125I i.v. solid circle, solid line, Dog F, untreated; hatched circle solid line, Dog G, unoperated, received Cytomel, 1.5 μg/kg per day for 7 days prior to injection of Na125I; solid triangle broken line, Dog B, thyroidectomized, was similary treated; open circle solid line Dog C, unoperated, was put on Cytomel 1.5 μg/kg per day 3 days after the radioactive iodide was administered; open triangle broken line, Dog E, sham operated, received the same treatment as Dog C.

rum. Four fractions were removed from the column(8) and fractions 2 and 3 combined for rat serum but not for human serum. Corresponding fractions from 3 of the smaller columns were pooled to provide sufficient material for stable iodine determination. Protein concentration of each fraction was determined by a modified Folin-Cicocalteau reaction(9). Radioactivity was counted in a Packard well-type gamma scintillation counter. Samples were withdrawn from each fraction for paper electrophoresis in 0.075 M veronal buffer, pH 8.6. Fractions were reduced to a small volume on a Rotovac *in vacuo* at low temperature, extracted for iodocompounds, and extracts chromatographed on paper in one or two dimensions(10,11).

The distribution of radioactivity among the individual iodocompounds on the chromatograms was determined by standard procedure (M-1), 2-dimensional chromatograms being first analyzed by autoradiography. The amount of stable iodine was determined from the same chromatographic strips after elution. A ceric sulfate-arsenious acid procedure was employed with continuous colorimetric recording but in a few instances a modification of the Barker "wet ash" method was used (12).

*Results.* Isotopic labelling of serum iodotyrosines was accomplished in the rat and dog, not in man.

*Rats.* The concentrations of radioactivity and of protein from a typical column separation of serum are shown in Fig. 1. The rats received daily doses of NaI, constant specific activity, for 50 days. Most of the radioactivity and most of the proteins were concentrated in Fraction I. Two small protein peaks and some radioactivity as iodide were present in Fraction 2. Fraction 3 contained no detectable protein and not enough counts to permit chromatography.

After electrophoresis on paper, the proteins in Fraction 1 were distributed as follows: albumin 55.1%, $\alpha_1$-globulin 8.1%, $\alpha_2$-globulin 7.7%, $\beta$-globulin 16.1%, and $\gamma$-globulin 13%. In acidic butanol extracts of Fraction 1, labelled iodotyrosines, iodothyronines and inorganic iodide were present. Traces of these compounds and most of the iodide were identified in extracts of Fraction 2; no iodotyrosines but small amounts of iodide, iodothyronines, and unidentified compounds

TABLE I. Distribution of *in vivo* 125-I Labelled Iodinated Compounds (per cent distribution of total butanol extractable iodine) in Fractions 1 and 2 of Rat Sera from Sephadex G-25 Columns.

| | | % of total labelled iodocompounds in fractions 1 & 2 | | | | | |
|---|---|---|---|---|---|---|---|
| Sample of serum | Fraction* | DIT | MIT | I† | $T_4$ | $T_3$ | X (front) |
| 50 days | 1 | 4 | 9 | 32 | 47 | 3 | 0 |
| | 2 | <1 | 0 | 1 | 2 | <1 | 0 |
| | Total | 4 | 9 | 33 | 49 | 3 | 0 |
| 51 " | 1 | 2 | 11 | 7 | 71 | 2 | 5 |
| | 2 | 0 | 0 | 2 | 0 | 0 | 0 |
| | Total | 2 | 11 | 9 | 71 | 2 | 5 |
| 57 " | 1 | 5 | 5 | 14 | 60 | 9 | 0 |
| | 2 | 0 | 0 | 6 | 1 | <1 | 0 |
| | Total | 5 | 5 | 20 | 61 | 9 | 0 |

* Fraction 3 contained too few counts for analysis.
† Iodide in Fraction 1 mainly from deiodination; low iodide values in Fraction 2 mainly from losses in the steps after elution.

travelling close to the solvent front of the paper chromatograms were present in extracts of Fraction 3.

The distribution on the chromatograms of the individual labelled iodocompounds in Fractions 1 and 2 is shown in Table I. The difference in counting rates between the iodotyrosine areas and the adjacent areas is significant by the Student "t" test, $p<0.001$. Counts in Fraction 3 were too low to permit calculation of percentage distribution.

To confirm the identity of the extractable iodotyrosines, normal rat serum was passed through a Sephadex G-25 column. After elution, paper chromatography was conducted. The various areas of the chromatogram were eluted, and iodine content determined. There was 0.50 $\mu$g iodine in the DIT area and 0.75 $\mu$g iodine in the MIT area per 100 cc original serum. The PBI after correction for losses in the procedure was found to be 4.72, 4.64, and 4.23 $\mu$g in different serum samples. A PBI value of 4.90 $\mu$g% was obtained directly in an aliquot of the original serum of the first of these, by Dr. Melvin Schwartz, Memorial Hospital. On average, 23% of the original radioactivity was present on paper chromatograms of Fraction 1; 2% in Fraction 2. However, 47% and 64% of the total radioactivity in Fraction 1 was butanol insoluble, and was treated as a loss in the corrected PBI determinations above.

*Dogs.* A suggestive peak of radioactivity was observed in the iodotyrosine areas of the chromatogram from the serum sample obtained shortly after 125-I administration (Fig. 2). This peak, containing 12-18% of the total radioactivity, was present in all instances in the earliest sample drawn (12 hr.). Iodotyrosine radioactivity then dropped to a minimum, 3-11% at 24 hours, followed by a rise to a higher plateau. The time interval from low to later high values was very variable.

Identity of the iodotyrosines, primarily MIT, with corresponding stable standards was established by co-chromatography in one or two dimensions. Extracts were used of dog sera obtained 12 or 24 hours after 125-I administration. Fig. 3 shows a significant radioactive MIT spot on the radioautogram obtained after 2-dimensional chromatography of an extract of dog G's 12 hour serum. Low levels of radioactivity made it impractical to employ this procedure for later blood samples.

*Men.* Efforts at labelling the extractable iodotyrosine materials in human serum were unsuccessful except possibly in 1 patient. No more than 3% of the total radioiodine in serum appeared in the iodotyrosine areas of the chromatograms, regardless of whether Na 125-I or 3,5-125 I T4 was employed, except in the one patient: chromatograms showed 7% radioactivity in the MIT area. This patient had received 125-I T4 in daily doses and had a slow rise of radioactivity in the MIT area, to a maximum level in the last blood sample at 42 days. Not enough blood was drawn to permit further identification.

*Discussion.* Between 11-13% of the total

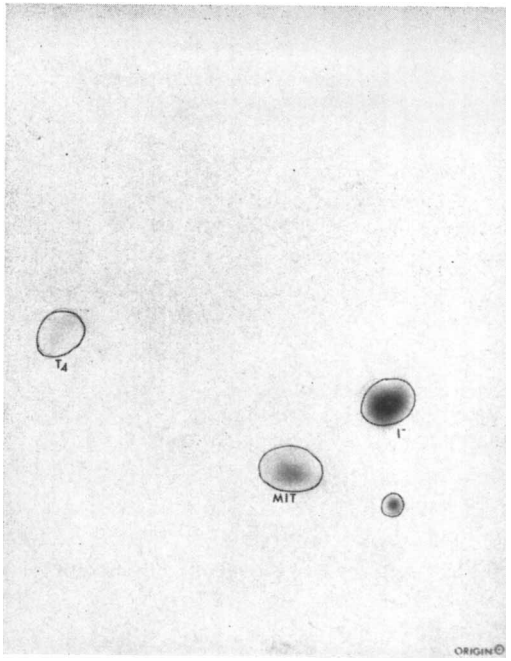


Fig. 3. Radioautogram of the two-dimensional chromatagram of an extract of Dog G's 12 hour serum. The chromatogram was developed in the vertical direction in 2-butanol-3.3% ammonia (3:1, v/v) an in the horizontal direction n-butanol-gl. acetic acid-water (9:1:2.5 v/v/v).

organically bound radioactivity present on the chromatograms of rat serum was in the form of iodotyrosines after 125-I was fed daily for 50 days. These compounds were found in the protein fraction, Fraction 1, obtained by column chromatography with Sephadex.

Identity of the extractable iodotyrosines in normal human serum with standard MIT and DIT has been recently established in this laboratory by a procedure employing re-chromatography to constant specific activity, with the appropriate labelled compound(12).

Successful labelling of extractable iodotyrosines in rat plasma has also recently been reported by Rhodes and Wagner(13), using 129-I with a half life of $1.6 \times 10^7$ years(14) as the label. They confirmed the identity of the labelled material in the iodotyrosine areas of the chromatogram by thermal neutron activation and measurement of the resultant radionuclide, 130-I. MIT and DIT were present in all samples of plasma analyzed. However, amounts equalled only 1 to 2% of the value obtained for thyroxine iodine, unlike the larger values of the present experiments.

In our experiments with dogs, labelling of a significant amount of extractable iodotyrosines were also accomplished: 12-18% of the total radioactivity was present in this form within 12 hours. Even though attempts were made to block the thyroid gland by administration of triiodothyronine, labelling took place. The meaning of the high plateau of iodotyrosine radioactivity which occurred after 24 hours needs further evaluation, particularly in view of the irregular time of appearance.

Efforts to label iodotyrosines in man have been uniformly unsuccessful. Ermans and coworkers(15) found no labelled iodotyrosines in chromatograms of sera from euthyroid patients with simple goiter given 5 $\mu$c 125-I daily until isotopic equilibrium. Equally, no labelled iodotyrosines were detected after 16 days when 3:5 131-I labelled L-thyroxine was fed to 5 patients by Dunn and Werner (4).

However, in the present experiment, patient A.K. was found to have 7% of the total radioactivity on paper in the MIT area, after he received daily doses for 42 days of inner ring labelled 125-thyroxine. Whether this is meaningful or not has not been established.

One explanation for failure of labelling in man could be the need for greater amounts of isotope than can permissibly be administered. The serum albumin appears to be the source in human serum from which the iodotyrosines are derived(12). To provide enough radioactivity to label the serum albumin probably would require dangerously large radioiodine doses.

The difference between our results in the rat and those of Pitt-Rivers and Rall(16) need to be explained. The latter coworkers found from K 131-I equilibrium studies in normal rats on a low iodine diet, that only 0-4% of the total radioactivity appeared in the combined MIT, DIT fractions of the serum. A Dowex $1 \times 2$ resin column was used for the separation. They considered this amount of radioactivity to be insignificant. However, they did not separate out an organic fraction. Moreover, careful studies(17) have

shown that the resin procedure employed entails considerable loss of iodotyrosine material. The same criticism can be made of Rhodes and Wagner's(13) method of extraction to account for their low estimates.

Lissitzky and Bismuth(3) reported finding no labelled MIT or DIT in rats fed 125-I for 50 days. However, they employed a Sephadex G-25 column for iodoaminoacid separation and determined only the free compounds but did not extract the protein fraction as was done in the present experiment. It is of interest that Zeidler and Graul(18) gave 131-I labelled DIT to a rabbit and reported that the material in plasma became protein bound within an hour.

*Summary.* Extraction was made of the protein fraction, Fraction 1, (after passage through a Sephadex G-25 column) of serum of rats fed 125-I for 50-57 days. The presence of labelled iodotyrosines, 11-13% of the organic labelled iodine, was established. In dogs injected once with 125-I, 12-18% of total serum radioactivity was extractable as iodotyrosines by 12 hours after labelling. In man, iodotyrosines in serum were not found to be labelled, with one possible exception.

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*Erratum*

**Volume 123, pp. 875-880: Gimeno *et al.*, "Effect of Glucose, Pyruvate, Lactate and Starvation on Contractibility of Isolated Rat Atria." The concentration of arsenite for curves 1 and 2 in legend of Fig. 6 should read 0.1 mM instead of 1 mM.**