THE BINDING OF IODOTYROSINES WITH SERUM PROTEINS

A. VAN ZYL, M.SC. (STELL.), PH.D. (RAND), AND B. WILSON, B.SC. (HONS.) (CAPE TOWN), *Department of Physiology, Medical School, University of Cape Town*

The various serum proteins show different affinities for different iodinated amino acids. Thus, although 3:5:3′-tri-iodothyronine (T_3) has an affinity for thyroxine-binding globulin (TBG),[1] it is only about one-third of that of thyroxine (T_4)[2] and can be displaced by T_4. TBG has no affinity for 3:5- di-iodotyrosine (DIT).[3, 4] The affinity of albumin for T_3 and T_4 is far less than for TBG; T_3 and T_4 are about equally bound by serum albumin.[5, 6] A pre-albumin fraction with high tryptophan content was found by Robbins and Rall[7] and recognized by other workers as a T_4 carrier[8] with no affinity for T_3.

Iodoproteins appear in blood and other body fluids under special circumstances. Lymph contains iodoproteins after TSH [thyroid-stimulating (thyrotrophic) hormone] treatment.[9, 10] Sodium-hydroxide hydrolysis of such iodoproteins yields iodotyrosines. Iodoproteins have been found in blood after large doses of radio-iodine[11-15] in subacute thyroiditis,[16] chronic thyroiditis,[17, 18] congenital goitre,[19-21] and functioning carcinoma,[22, 23] and in humans treated with large doses of inorganic iodine.[24] In some of these conditions the iodoprotein was thyroglobulin, in others the iodoproteins after hydrolysis revealed a preponderance of mono-iodotyrosine (MIT),[22, 23] whereas others contained mainly DIT.[20, 25] In these iodoproteins the protein-bound iodine (PBI) could not be dissolved in butanol to the same extent as when the serum iodine is in the form of T_4; the iodoprotein had an electrophoretic mobility comparable to that of serum albumin and was more soluble in phosphate buffer than thyroglobulin.[3]

Until recently it was believed that iodotyrosines were not normally secreted by the thyroid, since they could not be demonstrated by ^{131}I-techniques in blood. However, with the ceric sulphate and arsenious acid reaction,[26] Werner and Block[27] found that 30 - 70% of the ^{127}I was in the iodotyrosine region. This was confirmed by Dimitriadou *et al.*[28] It is believed that no significant binding of iodotyrosines occurred with serum proteins.[4, 5, 29]

If thyroidal secretion of plasma protein binding of iodotyrosines takes place, it would appear to be similar to the situation in which thyroidal iodoproteins are released into the blood. This would occur particularly in abnormal conditions, where the thyroid biosynthesis proceeds only to the stage of MIT and DIT formation. This study reports evidence of the binding of MIT and DIT by distinctly separate proteins of normal human, ox and frog serum.

MATERIALS AND METHODS

^{131}I-labelled MIT and DIT were prepared endogenously by injecting 200 μc. of ^{131}I into rats. The thyroids were removed after 24 hours and hydrolysed in a 1% pancreatin solution buffered with tris-maleate at pH 8·4. The digest was applied to Whatman no. 1 filter paper and chromatographed in butanol:dioxan:2N-NH_4OH;4:1:5 (BDA). The MIT and DIT fractions were identified after radioautography. The radioactive spots were eluted with methanol:ammonia (3:1 v/v), the eluates were concentrated

under vacuum (<45°) and re-chromatographed in butanol, acetic acid, and water (4:1:5) (BAW). The re-chromatographed samples were again eluted, dried, and dissolved in 0·1% Na_2CO_3, of which a portion was used for specific radioactivity determinations by the stable-iodine method.[30]

Serum samples were incubated with ^{131}I-labelled MIT and DIT at 39°C for ½ hr before electrophoresis in veronal or tris-maleate buffers (pH 8·6 and 8·4 respectively) or before dialysis against isotonic saline.

RESULTS

Fig. 1 demonstrates the proteins in human and ox sera associated with MIT and DIT when tris-maleate was used as the buffer during electrophoresis. In both samples DIT

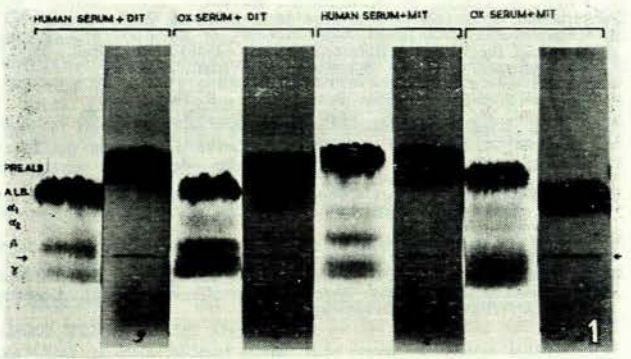

Fig. 1. The association of mono- and di-iodotyrosine with human and ox serum proteins electrophoresed in tris-maleate buffer, pH 8·4.

was exclusively associated with the pre-albumin fraction, whereas MIT ran together with an inter α_1-albumin fraction.

When veronal buffer was used, DIT coincided exactly with albumin and MIT with the α_1-protein fraction (Fig. 2).

Human serum differs from frog serum in the number of protein fractions, in their electrophoretic mobilities, and in their binding capacities for T_4.[31] Because of these differences the experiment was repeated in veronal buffer in which human serum was compared with frog serum at

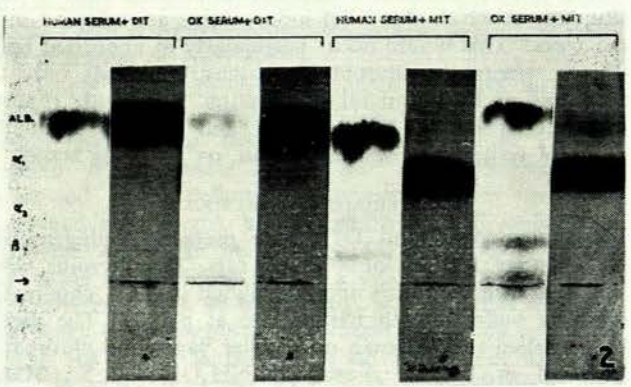

Fig. 2. The association of mono- and di-iodotyrosine with human and ox serum proteins electrophoresed in veronal buffer, pH 8·6.

increasing concentrations of MIT (20 μg./100 ml. and 185 μg./100 ml. serum) and of DIT (20 μg./100 ml. and 95 μg./100 ml. serum). At the same time, MIT and DIT were electrophoresed separately without serum on Whatman 3 MM paper and on the same paper treated previously with 1% phenylalanine.[32] The results are shown in Figs. 3A and 3B, from which it is clear that frog albumin has a greater mobility than human albumin and that frog serum only shows three distinct bands in veronal buffer at pH 8·6. It can also be seen that MIT and DIT have electrophoretic mobilities of their own and that the presence of serum proteins changes their mobilities. Thus, the serum proteins retard the electrophoretic migration of DIT, while that of MIT is increased. The mobilities of both MIT and DIT are retarded by treating the paper with phenylalanine before electrophoresis.

In human serum DIT was again associated with albumin (Fig. 3A) and MIT with the α_1-globulins (Fig. 3B), whereas

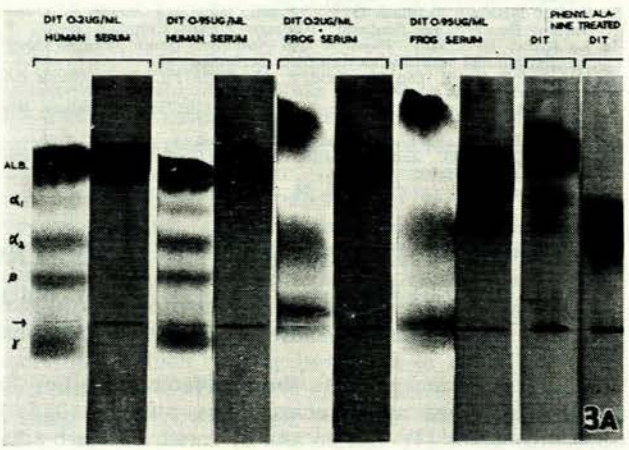

Fig. 3A. Electrophoretic migration of human and frog serum samples incubated with 0·2 μg./ml. and 0·95 μg./ml. of di-iodotyrosine, compared with the electrophoretic migration of di-iodotyrosine alone and with di-iodotyrosine on phenylalanine-treated filter paper.

in frog serum DIT ran behind albumin and MIT on the β-globulins. No 'spilling over' effect was observed with either MIT or DIT when their concentrations were increased to 185 and 95 μg./100 ml. serum respectively. Such a 'spilling over' effect can readily be demonstrated in normal human serum with 20 μg.T_4/100 ml. serum, but not in frog serum (Fig. 4).

^{131}I-labelled T_4, DIT, and MIT, were incubated for ½ hr with normal human serum, and the serum proteins were then precipitated with $ZnSO_4$ and the precipitate counted after each washing of the precipitate. The precipitates were washed with de-ionized water. From Fig. 5 it is clear that in every instance more than 70% of the radioactivities of T_4, DIT and MIT were retained in the proteins and could not be washed off.

Subsequently ^{131}I-labelled MIT and DIT were incubated with normal human serum for 30 min. and then dialysed against isotonic saline and compared with the rate of dialysis of MIT and DIT alone in the absence of serum.

Fig. 6 demonstrates the results of such an experiment for DIT in which the saline was renewed every 10 min. After

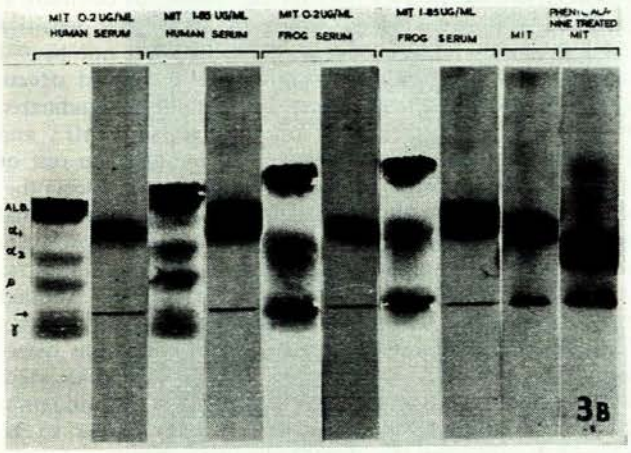

Fig. 3B. Electrophoretic migration of human and frog serum samples incubated with 0·2 μg./ml. and 1·85 μg./ml. of mono-iodotyrosine, compared with electrophoretic migration of mono-iodotyrosine alone and with mono-iodotyrosine on phenylalanine-treated filter paper.

55 min. 85% of the DIT that was previously incubated with serum was retained, whereas about 90% of DIT was lost during dialysis in the absence of serum.

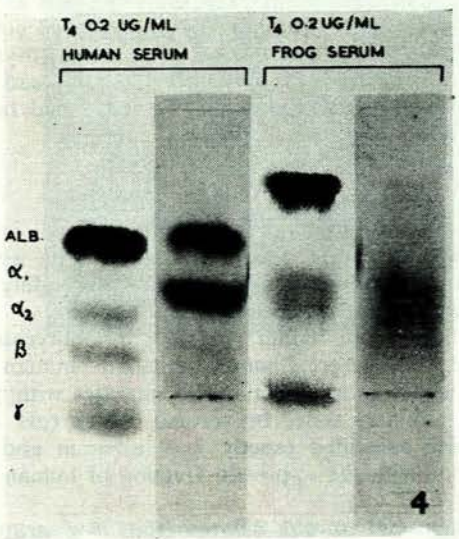

Fig. 4. A comparison of human and frog serum samples incubated with 0·2 μg. ^{131}I-labelled thyroxine/ml. serum.

When the experiment was repeated without changing the dialysing medium (Figs. 7 and 8), both MIT and DIT were retained again in the presence of serum proteins, but well over 50% of their activities were lost within 6 min. of dialysis in the absence of serum proteins. This binding effect of serum proteins on MIT and DIT is illustrated in Table I.

The serum proteins responsible for binding MIT and DIT have a greater carrying capacity for the iodotyrosines

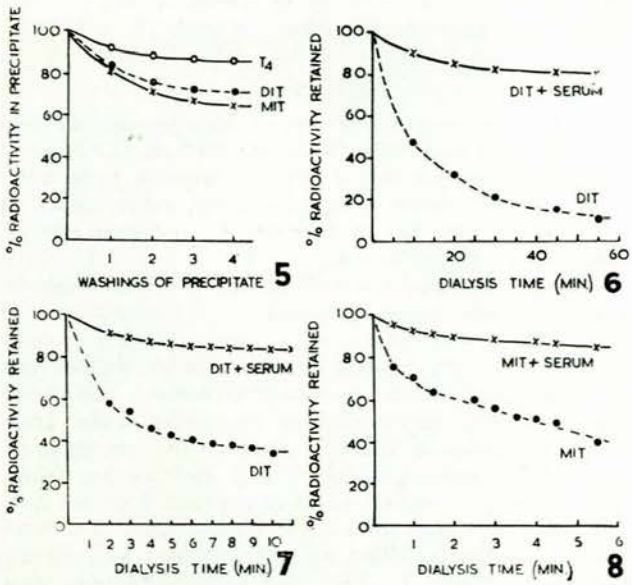

Fig. 5. The percentage radioactivity retained in the protein precipitate of serum incubated with ^{131}I-labelled T_4, DIT, and MIT, at stages during acid $ZnSO_4$-NaOH precipitation and washing of the protein precipitate with de-ionized water.

Fig. 6. The dialysis of ^{131}I-labelled di-iodotyrosine in the presence and in the absence of serum proteins (dialysis time extended to 55 min.).

Fig. 7. Dialysis of ^{131}I-labelled di-iodotyrosine in the presence and in the absence of serum proteins (dialysis time, 10 min.).

Fig. 8. Dialysis of ^{131}I-labelled mono-iodotyrosine in the presence and in the absence of serum proteins (dialysis time, 5·5 min.).

TABLE I. A COMPARISON OF THE DIALYSIS OF ^{131}I-LABELLED MONO-IODOTYROSINE AND OF ^{131}I-LABELLED DI-IODOTYROSINE IN THE ABSENCE AND IN THE PRESENCE OF HUMAN SERUM PROTEINS

Experiment	*Time (min.)*	*% Dialysable*
MIT + human serum	5·5	14·0
MIT	5·5	56·0
DIT + human serum	10	13·0
DIT	10	65·8
DIT + human serum	55	23·0
DIT	55	92·9

than TBG has for thyronines. Thus, loading the serum MIT and DIT to the extent of 500 μg./100 ml. made no difference to their sites of binding and did not result in a 'spilling over' effect onto other protein fractions; in every instance only one band of radioactivity was observed under the conditions of the experiments. When MIT and DIT did not separate well during chromatography, two radioactive bands appeared on the electrophoretograms, which were very similar to that of serum incubated with an excess of ^{131}I-labelled T_4.

An experiment was devised to ascertain quantitatively the concentrations of MIT and DIT in normal human serum. ^{131}I-labelled MIT and DIT were incubated with human serum and afterwards extracted quantitatively with butanol. The stable-iodine content of the butanol extracts

were then compared with that of the original ^{131}I-labelled MIT and DIT and found to be somewhat higher. This experiment requires confirmation.

DISCUSSION

Three different techniques—protein precipitation, dialysis, and electrophoresis—all indicate that MIT and DIT, when present in serum, are bound to serum proteins. In fact, the affinity of the serum proteins for the iodotyrosines is more specific than that of TBG for T_4, and their binding capacity exceeds that of TBG for T_4.

According to thyroid nomenclature, reference is usually made to 'specific binding proteins' or to 'iodoproteins' in blood. The former indicates the various serum proteins responsible for the binding of iodinated thyronines and their analogues, under normal conditions. The latter usually refers to serum proteins containing amino acids which are resistant to butanol extractions and, on digestion, yield various iodinated amino acids such as are found under abnormal conditions. Human serum does not normally contain a large amount of iodotyrosines. The term protein-bound iodine (PBI) includes iodinated amino acids other than T_4 and T_3. The only iodinated amino acids known to exist, apart from the iodinated thyronines, are MIT, DIT, and iodohistidine (MIH). If MIT and DIT are secreted into the blood stream and binding occurs with serum proteins, circulating MIT and DIT would add to the serum-PBI value. Because they are extracted with butanol, the PBI should under physiological conditions be the same as the butanol-extractable iodine except for the possible MIH content of serum. It is not known how MIH behaves with respect to butanol extractions and serum-protein binding.

Some animals, like the adult frog, may secrete more iodinated tyrosines into their blood than humans. In frogs that received ^{131}I, butanol extractions yielded a low recovery of organic ^{131}I, and the organic ^{131}I present was more difficult to extract than that of rat blood. It is possible that MIT and DIT bound to serum proteins are more resistant to butanol extractions than bound T_4 or T_3. The binding capacity of frog serum for T_4 is greater than that of human serum.[31] The strong affinity and the high binding capacity of frog serum proteins for T_4 may tend to keep the thyroid hormone in the circulation, giving rise to the relatively high serum PBI[33] and accounting for the low metabolic rate of adult frogs.

The most likely explanation for the phenylalanine effect on the electrophoretic mobility of T_4 is that it blocks the thyroxine-binding sites on the paper, so that T_4, which does not move from the point of application in veronal buffer at pH 8·6 (1 - 4 mA), will move on phenylalanine-treated paper to a position corresponding to the inter α-zone.[32] Whereas T_4 remains at the origin in the absence of phenylalanine and of serum proteins, MIT travels a distance of about 5 cm. and DIT about 12 cm. under the same conditions. On phenylalanine-treated paper their mobilities are retarded to about $3\frac{1}{2}$ and 6 cm. respectively. Because phenylalanine mobilizes T_4 electrophoretically and restricts movement of the tyrosines it must exert an influence other than blocking the binding sites on paper. The explanation of this finding probably lies in differences in the isoelectric points of phenylalanine, MIT and DIT in relation to the pH of the buffer. Normally only minute amounts of MIT and DIT are secreted by the thyroid; hence the demonstration of MIT and DIT in serum by the highly sensitive ceric sulphate and arsenious acid reaction but not by ^{131}I techniques. If tyrosines are secreted into the blood stream it may be expected that greater amounts of non-iodinated tyrosine are secreted than of iodotyrosine, since MIT and DIT are continuously used for T_4 formation. The rest of the iodotyrosines, which do not participate in T_4 biosynthesis, are subjected to thyroidal deiodinase. Under abnormal conditions MIT and DIT could appear in serum owing to a lack of the coupling enzyme, thus resulting in intra-thyroidal accumulation of iodinated tyrosines. Another possibility is that the deiodinase may be absent, resulting in the secretion of iodinated tyrosines. In conditions where iodination of tyrosines is blocked, MIT could be secreted. By virtue of the closeness of its locus to TBG a radioautograph of the electrophoretic pattern may appear to be normal, because the ^{131}I of MIT could be mistaken for the ^{131}I of T_4 in the inter α position. If, on the other hand, thyroidal deiodinase is absent, both MIT and DIT may be secreted. The radioautographs of their electrophoretograms may show a 'spilling over' effect due to mistaken identity of MIT at the α_1 position and DIT on the albumin. Such double bands on radioautographs were often observed when electrophoresed serum was incubated with ^{131}I-labelled MIT or DIT that did not separate well in chromatography, as in cases when BDA only was used as solvent. Lastly, if DIT alone was secreted in abnormal conditions by virtue of its higher concentration in the thyroid, its association with the serum proteins would lead to the formation of albumin-bound DIT, which could be misinterpreted as an iodoprotein.

SUMMARY

1. ^{131}I-labelled mono- and di-iodotyrosine incubated with human, ox and frog sera were found to be associated with distinctly different serum proteins, depending on the buffer used for electrophoresis.

2. In tris-maleate buffer (pH 8·4) di-iodotyrosine was associated with the pre-albumin fraction of human and ox serum, whereas mono-iodotyrosine migrates with proteins in the α_1-albumin zone. In veronal buffer (pH 8·6) di-iodotyrosine coincided exactly with albumin and mono-iodotyrosine with the α_1-protein fraction of human and ox serum.

3. Human and ox sera differed from frog serum in the number of protein fractions, in their electrophoretic mobilities, and in their binding capacities for thyroxine. Frog albumin has a greater mobility than human albumin and shows only three distinct bands in veronal buffer. In frog serum di-iodotyrosine is not associated with albumin as in human serum, and mono-iodotyrosine is associated with the β-globulins.

4. Increasing the mono- and di-iodotyrosine concentrations to 500 μg./100 ml. serum did not result in a 'spilling over' effect of the iodotyrosines onto other serum proteins in human or frog sera.

5. Mono- and di-iodotyrosine in the absence of serum proteins migrated electrophoretically under the experimen-

tal conditions. The migration of both iodotyrosines was significantly retarded on treatment of the paper with 1% phenylalanine.

6. Three different techniques—electrophoresis, protein precipitation, and dialysis—all indicate that mono- and di-iodotyrosine were truly bound to the proteins after incubation with serum proteins.

7. Preliminary experiments on stable-iodine analyses indicate the presence of small quantities of iodotyrosines in human serum.

8. The implications of these findings are discussed.

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