THE CIRCULATING THYROID HORMONES IN THYROTOXICOSIS

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The application of the technique of paper chromatography and the use of radioactive iodine resulted in the demonstration of thyroxine (T_4) as the major iodine-containing component in the plasma (Laidlaw, 1949; Taurog *et al.*, 1950). Thyroxine was also found to be the major circulating iodine compound in thyrotoxicosis by Rosenberg (1951). In 1952 Gross and Pitt-Rivers demonstrated the presence of 3-5-3'-L triiodothyronine (T_3) in the serum of four thyrotoxic patients and two patients with thyroid carcinoma. They suggested that there was no qualitative difference between the iodine compounds present in the circulation of normal and thyrotoxic subjects. Subsequent studies (Benua and Dobyns, 1955; Dinglede *et al.*, 1955; Arons and Hydovitz, 1959) have confirmed the findings of Gross and Pitt-Rivers in thyrotoxicosis. T_4 was found to be the major component, while T_3 was detected in a variable proportion of thyrotoxic patients after radioactive iodine (^{131}I) administration.

The presence of triiodothyronine in the plasma of both normal and thyrotoxic subjects was confirmed by MacLagan *et al.* (1957) using a chemical method of detection. This method made use of the very sensitive ceric-sulphate-arsenious-acid catalytic system for detecting iodine. Their finding eliminated the possibility that the presence of triiodothyronine might have been artifactual owing to radiation damage to the thyroid gland from the radioactive iodine employed in the earlier studies.

Farran *et al.* (1959) using "semi-tracer" doses of radioiodine, reported the presence of iodotyrosines in the plasma of approximately 50% of cases of thyrotoxicosis. The patients whose plasma contained iodinated tyrosines showed a greater mean gland weight and were more resistant to radioactive iodine therapy than those without iodinated tyrosines. The presence of iodotyrosines was confirmed by the use of chemical methods (Bird and Farran, 1960). This finding raised the possibility of an abnormal thyroid secretion in thyrotoxicosis. More recently still, two separate groups of investigators (Block *et al.*, 1960; Beale and Whitehead, 1960) have reported the presence of iodotyrosines in the blood of normal subjects accounting for 35-50% of the total organic blood iodine. This finding has not been confirmed in normal animals (Pitt-Rivers and Rall, 1961).

In view of these variable results and their importance for understanding the pathogenesis of thyrotoxicosis it was decided to reinvestigate the pattern of plasma hormones in normal and thyrotoxic patients. Chemical methods were used in studying plasma from normal subjects, but in thyrotoxic subjects both chemical and radioactive methods of detection were used whenever possible.

Methods

Observations were made in unselected euthyroid and hyperthyroid subjects; their thyroid status was assessed on the usual clinical criteria and confirmed by such tests as the plasma protein-bound iodine (P.B.I.) estimation performed according to the method of Acland (1957) and also the uptake of ^{131}I by the thyroid gland. The thyrotoxic subjects were divided into two groups; the first group received no treatment whatever, the second received therapeutic doses of ^{131}I two to four days before blood-sampling. Blood was collected, and the plasma separated under iodine-free conditions, using heparin as an anticoagulant.

Plasma in 6-ml. samples was extracted by a modification of the anion exchange method developed for tissues and serum by Galton and Pitt-Rivers (1959), full details of which are being published elsewhere (Wellby, 1962).

The iodinated compounds in the extract were separated by paper chromatographic methods, using at least two solvent systems; *n*-butanol saturated with 2N acetic acid and *n*-butanol:ethanol:2N ammonia (4:2:2) (B.E.A.). After the drying of the papers in a forced-air draught at 20° C. the iodine-containing components on the paper chromatograms were detected, using the method of Bowden *et al.* (1955) depending on the ceric-sulphate-arsenious-acid reaction which is catalysed by iodine. After staining, the papers were dried rapidly; a visual assessment was made of each chromatogram under ultra-violet light with peak emission at 3,660 Å.U., and finally permanent blue-print records were made. The chromatograms prepared from plasma from ^{131}I -treated subjects were scanned for radioactive peaks in a Nuclear Chicago "Actigraph II" with a Nuclear Chicago (D47) "Gas Flow" open detector. After scanning, the papers were assessed for ^{127}I content as above. A prominent feature of all chromatograms of plasma extracts performed using this method is a spot due to the chloride content of the plasma (Fig. 1). This spot which is not radioactively labelled following administration of ^{131}I to the patient prevents adequate chemical demonstration of mono-iodotyrosine (M.I.T.) and occasionally diiodotyrosine (D.I.T.) in the B.E.A. system but does not interfere in the butanol-acetic system. Although the resin removes all or nearly all the inorganic iodide from the plasma sample, staining due to iodide is seen on all chemical chromatograms of plasma (Fig. 1). It is derived from the trace contamination with iodide of the 50% acetic acid solution used for elution.

With this method, the lowest concentration of T_4 and D.I.T. detectable chemically was 0.5 $\mu\text{g.}/100\text{ ml.}$ plasma. The recovery of M.I.T. was more variable. Enrichment of plasma to 12.5 $\mu\text{g.}$ of $T_4/100\text{ ml.}$ yielded no detectable amounts of T_3 or D.I.T. Enrichment of plasma with radioactive T_4 and T_3 also failed to produce D.I.T. after extraction and chromatography. Full details on the performance of the method have been published elsewhere (Wellby, 1962).

Results in Normal Subjects

Plasma from 30 subjects without clinical evidence of thyroid disease was examined. This group consisted of 18 females and 12 males, with an average age of 29 years. The mean plasma P.B.I. level was 5.8 $\mu\text{g.}/100\text{ ml.}$ ranging from 4.2 to 9.0 $\mu\text{g.}$

In each plasma, T_4 was the main component detected (Fig. 1); T_3 was detected in 16 of the 30 subjects but when found was present in a quantity much smaller than that of T_4 .

FIG. 1.—This and other similar figures are untouched photographic reproductions of the blue-print records of the chemical chromatograms. The photographic reproduction does not always reproduce the more faintly stained spots. This figure shows chemical chromatograms of plasma from a normal subject (a man aged 31) in two solvents showing spots due to chloride, iodide, and T_4 . The plasma P.B.I. level was 6.0 $\mu\text{g./100 ml.}$ O=Origin. F=Front.

The mean plasma P.B.I. level in those subjects in which T_3 was detected was 6.3 $\mu\text{g./100 ml.}$, whereas it was 5.3 $\mu\text{g./100 ml.}$ in those subjects in which only T_4 was found. This was a significant difference— $P < 0.05$ using a rank test (White, 1952).

D.I.T. was found in the plasma of five subjects, and in one of these M.I.T. also. In the five subjects in which D.I.T. was detected the plasma P.B.I. levels were 5.6, 6.4, 7.0, 7.5, and 8.1 $\mu\text{g.}$ respectively.

Results in Thyrotoxic Subjects

Untreated.—The mean plasma P.B.I. level for this group was 12.8 $\mu\text{g./100 ml.}$ and the average age of the group, which consisted of 10 males and 32 females, was 36 years (range 16–74 years). In the 42 plasma samples from this group, T_4 was detected in each case and was the main fraction found (Figs. 2 and 3). In all cases but one (in which the plasma P.B.I. level was only 7.5 $\mu\text{g.}$) T_3 was found also and appeared as a larger spot than that found in the plasma of normal subjects (Fig. 2). In some chromatograms in the B.E.A. solvent system a lighter staining spot with an R_f value just greater than T_3 ($>T_3$) was noted (Fig. 2). In the butanol-acetic solvent system D.I.T. was found in 32 cases (Fig. 3), and in 9 of these M.I.T. was also found. The identification of D.I.T. was based on the following criteria: the spots were found to have R_f values coinciding with those of known D.I.T. in three solvents; plasma from hyperthyroid subjects was enriched with known D.I.T., and chromatography showed that the known D.I.T. was superimposed on the suspected D.I.T. spots in two solvents; further evidence that this

component was D.I.T. was deduced from its elution from the anion exchange column not only at a pH of 1.4 but also at a pH of 2.2, which is known to recover

FIG. 2.—Chemical chromatograms in two solvents of plasma from an untreated case (a woman aged 20) of hyperthyroidism in which no D.I.T. was detected. The chromatograms in B.E.A. demonstrate T_4 , T_3 , and the $>T_3$ area, as described in the text. The plasma P.B.I. level was 9.1 $\mu\text{g./100 ml.}$

D.I.T. and not the iodothyronines (Galton and Pitt-Rivers, 1959).

Treated with ^{131}I .—Plasma from 20 subjects following ^{131}I therapy was chromatographed. The average age of this group of 20 (of which 18 were females and 2 males) was 51 years. With the chemical assessment T_4 was found in all cases, T_3 in 17 cases, and in the butanol-acetic solvent D.I.T. was detected in 14 and M.I.T. in 6 out of the 14 cases showing D.I.T. In 15 of the above plasma samples and one other plasma taken from a hyperthyroid subject 48 hours after ^{131}I therapy (not appearing in the chemically assessed group) the chromatograms were scanned for ^{131}I content. T_4 was found in all these cases, T_3 in all cases but one (Fig. 4), D.I.T. in 12 cases out of the 16, and M.I.T. in 7 of the

FIG. 3.—Chemical chromatograms of plasma samples from two untreated cases of hyperthyroidism, demonstrating D.I.T. in both solvent systems, in addition to the thyronines.

12 showing D.I.T. (Fig. 5). A broad radioactive peak, corresponding in R_f value with the chemical spot $>T_3$, was noted in some chromatograms (Figs. 4 and 5).

Fig. 4

Fig. 5

FIGS. 4 and 5.—Photographic records of radioactive scans of chromatograms in two solvents. The symbols M.I.T., D.I.T., T_4 , etc., are included for the identification of the peaks on the figure, and do not represent the extent of chemical markers. The peaks corresponded in R_f values with the spots obtained by chemical staining after scanning. N.W.: Received 30 mc. of ^{131}I orally 24 hours before blood-sampling. Chromatograms demonstrate T_4 and T_3 , but no M.I.T. or D.I.T. The peak $>T_3$ corresponds in R_f value with the chemical spot $>T_3$ in Fig. 2. A.S.: Received 5 mc. of ^{131}I orally 48 hours before blood-sampling. The chromatograms demonstrate M.I.T. and D.I.T. in two solvent systems, though complete resolution of them was not achieved in the butanol-acetic solvent.

Discussion

The results of the chromatographic investigation in normal subjects indicate that T_4 is the predominant thyroid hormone in the blood, that T_3 can be detected frequently (16 out of 30 cases) but in much smaller quantities than T_4 and that the iodotyrosines are not usually present.

It is not possible to estimate the contribution of the total organic iodine due to T_3 from this qualitative study, although it was apparent that T_3 accounted for only a small percentage. Using a similar visual assessment, MacLagan *et al.* (1957) found T_3 in the plasma in seven out of eight normal subjects, and with a semi-

quantitative method calculated that T_3 amounted to 10% of the iodothyronine concentration. Pind (1957) found an even smaller amount of T_3 in human subjects. In two other studies in rats, values of 10–20% (Feuer, 1959) and 5% (Pitt-Rivers and Rall, 1961) have been given. The possibility that the detection of T_3 in the present study is due to the breakdown of T_4 to T_3 during extraction has been excluded by chromatography of radioactive T_4 -enriched plasma (Wellby, 1962).

In both groups of hyperthyroid subjects it appeared that there was more T_4 and T_3 in the plasma compared with the amounts in the normal subjects. The increase in the T_3 appeared to be more pronounced, however, although this conclusion must be provisional in the absence of a precise quantitative estimation. From the results in the untreated hyperthyroid group the possibility that the appearance of T_3 is related to radiation damage and not to the condition of hyperthyroidism, a point which concerned the earlier workers (Dingledine *et al.*, 1955) is obviously ruled out. The chemical findings reported here are consistent with those reported in a radioactive study (Arons and Hydovitz, 1959). It may well be that the symptoms and clinical effects of hyperthyroidism may be related to the increase in the T_3 secretion.

The chemical spot and radioactive peak designated $>T_3$ was not identified. It may be identical with "substance 3" of Feuer and Vekerdi (1958) found in chromatograms prepared from rat plasma and considered by them to be an acetyl derivative of an iodothyronine. There are other possibilities, however. The spot has the same R_f value as triiodothyroacetic acid. Furthermore, interference from artifacts in this area has been reported in chromatograms in alkaline solvents, including spots due to iodinated lipids (Taurog, 1957) and pigments (Wilkinson, 1957).

A striking observation in the present study was that D.I.T. was found in the plasma of 32 out of 42 (76%) untreated hyperthyroid subjects. Furthermore, D.I.T. was found in the ^{131}I -treated group with the same degree of frequency with both ^{127}I and ^{131}I methods. An earlier study of the chromatographic pattern of the plasma thyroid hormones in hyperthyroidism reported that M.I.T. and D.I.T. were found in most of the sera examined, but it was thought that the D.I.T. in the blood was due to radiation damage within the thyroid gland (Benua and Dobyns, 1955). The present finding confirms that of Bird and Farran (1960) as well as an earlier study from this laboratory (Wellby and Hetzel, 1960), in demonstrating the presence of D.I.T. in plasma in the absence of radiation exposure. However, in the present study, in comparison with that of Bird and Farran (1960), D.I.T. has been found to be a more consistent plasma constituent in thyrotoxic patients. Furthermore, the presence of D.I.T. could not be correlated with any clinical finding in these cases.

The results in the normal group in this study show that D.I.T. is not a usual constituent of normal plasma. However, Block *et al.* (1960) reported from a chemical study that D.I.T. accounts for up to 40% of the blood iodine in normal subjects but were unable to confirm this finding with ^{131}I . They suggested that this was due to D.I.T. being involved in an intrathyroidal metabolic pool with a slower turnover rate than that of T_4 . The present study does not support this view, in that after ^{131}I administration to hyperthyroid subjects, both the D.I.T. and the T_4 spots were labelled with a specific

activity of the same order within 48 hours of the dose, as assessed by the magnitude of the radioactive peak compared with the size and intensity of the chemical spot.

The M.I.T. fraction was found with a frequency of about one-third to one-half that of D.I.T. in each of the hyperthyroid groups, and when detected appeared as a smaller spot than the D.I.T. spot. This may be a reflection of the relative proportions of M.I.T. and D.I.T. within the thyroid gland (Pitt-Rivers and Rall, 1961). However, some M.I.T. is lost from the resin column in the preliminary wash (pH 3.6) (Wellby, 1962).

The mechanism of this release of iodotyrosine from the thyroid gland in thyrotoxicosis is not clear, but it may well be that it is due to a conditioned deficiency of the thyroidal iodotyrosine deshalogenase. That an absolute deficiency of this enzyme does not exist is suggested by direct studies on hyperthyroid glands removed surgically (Carr *et al.*, 1959).

The association of the more constant appearance of T_3 with a higher plasma P.B.I. in the normal subjects and the definite increase which is evident in most cases of hyperthyroidism suggests that this hormone is produced in greater quantities by the stimulated gland. In fact, there appears to be a gradual transition between the hormone pattern of the normal and that characteristic of hyperthyroidism. It is of interest also that the presence of D.I.T. is associated with levels of plasma P.B.I. in the upper half of the normal range as well as in hyperthyroidism when the P.B.I. is definitely elevated. It is tempting to postulate a connexion between these two phenomena: the appearance of increased amounts of T_3 in the plasma may even be promoted by a loss of D.I.T. from the gland. This possibility is further supported by the results of studies after thyrotropic hormone stimulation which have been briefly reported (Wellby and Hetzel, 1962). In these studies the administration of T.S.H. to 11 normal subjects was followed by the appearance of iodotyrosines within four hours except in one subject in whom iodotyrosines were not detected until 72 hours after T.S.H. administration. The appearance of iodotyrosines was associated with a definite increase in T_3 .

These findings indicate that the plasma pattern of circulating thyroid hormones in thyrotoxicosis can be reproduced by the administration of thyrotropic hormone. This suggests that the thyrotoxic gland secretion could be due to endogenous T.S.H. stimulation. There is evidence both for (Hetzel *et al.*, 1960) and against (Purves and Adams, 1960) this view. The recently demonstrated long-acting thyroid stimulator in the blood of thyrotoxic patients may or may not be of pituitary origin (Purves and Adams, 1960; McKenzie, 1961). Whatever the nature of the abnormal controlling mechanism operating in thyrotoxicosis the present findings indicate a pattern of thyroid secretion consistent with that produced by thyrotropic hormone stimulation.

Summary

The plasma pattern of the iodine-containing amino-acids in normal and thyrotoxic subjects has been investigated with paper chromatographic methods. Plasma extracts were prepared with an anion exchange resin and the components located by chemical staining using the ceric-sulphate-arsenious-acid reaction. In extracts from some thyrotoxic subjects the chemical assessment was compared with radioactive scanning.

In 30 normal subjects thyroxine (T_4) was the major constituent found. Small amounts of triiodothyronine (T_3) were found in 16 subjects. The mean plasma P.B.I. level in those showing T_3 was significantly higher than in those without T_3 . In five normal subjects with plasma P.B.I. levels in the upper half of the normal range diiodotyrosine (D.I.T.) was also found.

In 42 untreated hyperthyroid subjects increased amounts of T_3 and T_4 were detected; D.I.T. was found in 32 subjects, 6 of whom also showed M.I.T. Chemical methods of detection in 20 ^{131}I -treated subjects demonstrated T_4 in all, T_3 in 17, and D.I.T. in 14, in 6 of whom M.I.T. was found. With radioactive methods in 16 subjects T_4 was always found. T_3 occurred in 15, D.I.T. in 12, and M.I.T. in 7 of those showing D.I.T.

The possibility that the detection of T_3 and D.I.T. was due to the breakdown of T_4 during the extraction and chromatography was excluded. The detection of T_3 and D.I.T. chemically in untreated cases of hyperthyroidism indicates that their appearance is not due to radiation damage.

In conjunction with other evidence it is concluded that these findings indicate a pattern of thyroid secretion consistent with that produced by thyrotropic hormone stimulation.

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