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ORIGINAL ARTICLE

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The isolation of thyroxine (T4), the discovery of 3,5,3′-triiodothyronine (T3), and the identification of the deiodinases that generate T3 from T4: An historical review

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Introduction

The thyroid gland was first described in the 17th century. However, the concept that this organ might have a functional role in the body was not recognized until the 19th century, when cretinism and myxedema were associated with thyroid atrophy, surgical thyroidectomy was found to result in symptoms comparable to those seen in patients with myxedema [1] and Murray demonstrated that myxedema could be treated successfully with daily injections of sheep thyroid extract [2]. These findings suggested that the thyroid secreted an active principle(s) essential for the prevention of myxedema, and in 1914 Kendall succeeded in isolating from the thyroid the compound now recognized as thyroxine (T4).

The isolation of thyroxin

Edward C. Kendall, was born in 1886 in South Norwalk, Connecticut. He developed a keen interest in chemistry during high school and completed his undergraduate degree in chemistry at Columbia University, where he also received a MS degree in biochemistry and a PhD in chemistry. During his oral defense, he was asked about iodine metabolism and did not know that it was concentrated within the thyroid gland [3].

He was appointed to his first job on September 1st, 1910 in a chemical laboratory at Parke Davis and Co. in Detroit on a salary of $1350 per year. He was assigned to isolate the active principle from the thyroid gland, which reminded him of the question he was asked during his thesis defense. However, he felt isolated and was not pleased with the work environment of the pharmaceutical industry and, after four months, resigned, without an available job offer [3]. He returned to New York City and found an opening to head a lab at St. Luke's Hospital (a Columbia affiliate). There, at Columbia and St. Luke's Hospital, he became intrigued by the thyroid, in part because of the question he was unable to answer as a doctoral candidate, and was able to pursue his interest, and on February 1st, 1911 he began work on isolation of the active product of the thyroid gland [3].

By 1911, the biological and clinical effects of the loss of function of the thyroid gland were well described and it was clear that those effects could be reversed by administration of desiccated thyroid glands. But the nature of the active ingredient was only minimally understood. The most important clues came from Professor Eugen Baumann and colleagues at the University of Freiburg, Germany. In 1895 Baumann demonstrated that fusing dried thyroid gland with sodium hydroxide and then making the solution acidic with nitric acid liberated iodine from the mixture, demonstrating the iodine content from the gland. He next boiled the dried thyroid in 10% sulfuric acid and determined that the product was 9.3% iodine [4]. Later it was shown that the biological activity of the thyroid resided in that iodine containing fraction. The work in Freiburg unfortunately ended suddenly when Baumann died of heart disease less than a year after his discovery. This work however was the basis of pursuing iodine as the source of activity from the thyroid and served as the starting hypothesis for Kendall's purification [3].

This article does not contain any studies with human participants or animals performed by any of the authors.

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By 1913, Kendall had purified the active principle of the thyroid by 100-fold, with biological activity proven in animal models (a dog in a metabolism cage) and in patients suffering from myxedema. However, he felt that his research progress was under-appreciated at St. Luke's: the hospital administrator at St. Luke's sent Kendall a box of breakfast cereal with a letter directing him to analyze the cereal. "I threw the letter and cereal into the wastebasket". Kendall wrote: "The final decisive incident was…that my salary for 1914 would be the same as it had been in 1913". "And so ended my efforts to carry out research in biochemistry in an institution that was not interested in research" [3].

A friend arranged for an interview at the Rockefeller Institute with Dr. Simon Flexner. Kendall shared his progress on thyroid extracts and treatment of hypothyroidism. Flexner was not impressed and said: "Young man, it is not easy to get into Rockefeller Institute. When we want to have someone come with us we will do whatever is necessary to get him, but otherwise it is not possible to join this Institute." Kendall later wrote that "I did not like what Dr. Flexner said, and I disliked the way he said it" [3].

The early years of the 20th century found Rochester, Minnesota, to be the busiest center in the world for thyroid surgery. Dr. Charles H. Mayo and Dr. Henry S. Plummer had developed methods for safe thyroidectomy, especially for exophthalmic goiter, that reduced the mortality rate from levels that were as high as 20 percent in some hospitals to below 3 percent. This achievement brought thousands of patients to Mayo Clinic for treatment of thyroid diseases and goiter. In 1908, Drs. Mayo and Plummer reported their experience with 1000 thyroidectomies, and with 5000 only three years later. By 1911 thyroidectomy was the most commonly performed operation at Mayo Clinic. In part because of this great advance in the success of thyroid surgery, there was a great flood of new patients to Mayo. The institution was growing rapidly with patients, physicians, facilities and in reputation. The clinical laboratory also grew rapidly during this period. A decision to add research facilities was considered, including the addition of what we would now call basic scientists to the staff. Against some opposition, Drs. Mayo, Plummer, and Louis B. Wilson argued that 'pure research' was vital to the future success of the group and would prevent falling into routine based solely on present knowledge. An opportunity arose when in late 1913 a letter arrived from a young New York chemist interested in isolating the active ingredient of the thyroid gland and seeking a position [5, 6].

After corresponding with Dr. Wilson, head of the Mayo Clinic laboratories, Dr. Kendall came to Rochester for an interview. He brought with him a small vial of his most pure thyroid extract to date, which reproduced the biological effects of the thyroid substance. He was hired in short order, specifically to pursue the project of isolating the active ingredient of the thyroid [3, 5, 6].

He started work in Rochester in February 1914, in a laboratory on the fourth floor of a newly constructed Mayo Clinic building, later known as the 1914 Building, in which he was the first resident. In an effort to isolate the active principle from the thyroid, Kendall used 6500 pounds of hog thyroid glands [6]. By December 1914 he had made considerable progress and had a powder that was 40 percent by weight iodine, but its volume was quite small. He decided, rather than risk losing more of his product through another round of barium hydroxide purification, to attempt to crystallize a pure substance from the current and already highly enriched mixture by alcohol extraction. After several rounds of heating in a water bath to evaporate the ethanol and discarding the byproducts, he found that the extract was now 60 percent iodine, much more pure than his previous best. On Christmas morning 1914, he dissolved the extract in ethanol and then added acid and observed crystals of pure thyroid hormone (TH) falling out of the solution [3].

Treatment of patients with the hormone of the thyroid gland in crystalline form produced astonishing results. "10-year-old girl 37" tall, weight 37 lbs., slowed somatic and mental growth—treated with crystalline TH and 6 months later she had grown 4" and was bright and responsive". Several other patients demonstrated similar dramatic responses. The work was presented to the scientific community with considerable fanfare at the Federation of American Societies for Experimental Biology meeting in New York in 1916, in a paper entitled "Isolation in Crystalline Form of the Iodine-Containing Compound of the Thyroid Gland" [7]. The chairman of the meeting was Dr. Simon Flexner, who had scoffed at Kendall's project and refused him a position in 1913. In 1918 he named the substance "thyroxin" because he believed it to be an indole in structure [3]. Subsequently Kendall abandoned work on the thyroid and focused on the adrenal gland. He was awarded the Nobel Prize in 1950, along with Philip Hench and Tadeus Reichstein for the discovery of cortisone.

Thyroxine: 1920s–1950

By 1924, Kendall's thyroxin had been shown to be one of the most important substances through which the thyroid exerts its control on basal metabolic rate. Eventually it became clear that it was the same substance as the thyroxine (T4) used today and thus Kendall must be credited as the first person to treat hypothyroidism with this hormone. However, he had concluded, erroneously, that thyroxin was an indole derivative; the name was a contraction of thyroxindole [7].

In 1926, this structure was challenged by Sir Charles Harington, who improved the method for the isolation of thyroxin from thyroid tissue, determined its chemical structure and then synthesized it [8, 9]. He showed that it was a tyrosine rather than an indole derivative and added the "e" to end of the name to reflect this [9]. Harington's synthetic T4 was tested by Murray Lyon in two patients with myxedema, and its effect in raising the BMR was found to be quantitatively similar to the T4 extracted from the thyroid [9]. Subsequently a commercial preparation of racemic T4 became available, but it was very expensive and there was little interest in using it as an alternative to dried thyroid preparations to treat myxedema. Indeed, James Howard Means, a highly respected endocrinologist, wrote in 1935 that T4 had no advantage over dried thyroid; in fact, given by mouth he considered T4 to be inferior. His reasoning for this was that "in the whole gland the hormone is in protein linkage as iodothyroglobulin; it is more soluble and is absorbed from the GI tract in a quantitative manner". He goes on to say that "dried thyroid by mouth is practically a perfect substitution therapy. It is the only one that needs to be employed in the treatment of myxedema" [10].

Another reason for caution in using T4 therapeutically was that the nature of the circulating TH was not known. Although it was clear that T4 accounted for much of the biological activity of the thyroid, there was considerable doubt that it was the circulating hormone for several reasons. First, it was not possible to account completely for the biological activity of thyroid extracts by its T4 content alone. Second, T4 has a long latent period of action when injected into animals; at the time it was thought that the effects of biologically active compounds occurred shortly after their administration. Third, T4 was inactive in most in vitro systems. In 1935, Harington, on the basis of his extensive studies concerning the iodine compounds in the thyroid gland, postulated that the circulating TH was a peptide consisting of T4 and diiodotyrosine [11]. However, by 1944 he had re-evaluated the evidence and hypothesized that the circulating hormone was in fact T4 [12]. The breakthrough, aided by several advances in technology, came in 1948 when Taurog and Chaikoff obtained strong evidence that most, if not all, of the organic iodine in the circulation consisted of T4 bound reversibly to plasma proteins [13]. Subsequently, using radioactive iodine (^{131}I) to label the iodinated compounds synthesized in the thyroid, and analysis by paper chromatography, it was demonstrated unequivocally that the majority of this protein-bound iodine fraction in serum did indeed comprise T4 [14, 15]. In 1949, a commercially feasible process for the economical synthesis of L-T4 sodium was developed and Hart and Maclagan showed that it was consistently effective in treating hypothyroidism when given by mouth [16]. They concluded that although they could not claim that T4 was any better than preparations of dried thyroid, they felt that it was equally satisfactory. They also considered that tablets containing synthetic T4 were more reliable therapeutically than preparations of dried thyroid, for which there was no defined efficacy standard. T4 tablets were included in the British Pharmacopeia in 1958. However, T4 still did not achieve widespread clinical use. The reluctance to use synthetic T4 to treat hypothyroid patients at that time is understandable. Even when it became known that T4 was the major circulating hormone, only an estimate of its level in serum could be obtained by measuring the level of protein-bound iodine (PBI). The possibility existed that other active compounds were present in the circulation. Most clinicians found dried thyroid preparations to be satisfactory and saw no compelling reason to switch. In fact, thyroid preparations, mostly desiccated thyroid, continued to be the treatment of choice for hypothyroidism well in to the 1970s. However, by the mid 1970s, a switch began and by 1980, the use of oral T4 exceeded desiccated thyroid, and since the late 1980s L-T4 has been the treatment of choice [17].

Discovery of 3,5,3′-triiodothyronine

In the early 1950s a second TH was discovered. Gross et al. were attempting to identify, using two-dimensional paper chromatography, the various iodinated amino acids present in plasma and in an extract of hydrolyzed thyroid gland obtained from rats injected with carrier-free ^{131}I. Using the corresponding synthetic compounds as carriers, monoiodotyrosine and diiodotyrosine, T4 and iodide were identified in the thyroid extract, and T4 was clearly evident in plasma. In addition, several other compounds were present in the thyroid extract, and one of them, designated Unknown 1, was also present in plasma [15]. In 1950, Dr. Jack Gross joined the laboratory of Dr. Rosalind Pitt-Rivers at the National Institute for Medical Research in London. The Director of the Institute was Sir Charles Harington. The primary goal of Gross and Pitt-Rivers, with input from Sir Charles, was the identification and characterization of Unknown 1. First, they demonstrated the presence of Unknown 1 in the plasma of humans who had received therapeutic doses of ^{131}I [18]. Then they synthesized 3,5,3'-triiodothyronine (T3) from 3,5-diiodothyronine [19] and used it to demonstrate that the Unknown 1 present in human plasma and in hydrolyzed thyroid tissue from rats given ^{131}I was indistinguishable chromatographically from synthetic T3 [19]. Finally, they isolated T3 from ox thyroid tissue and demonstrated that its X-ray crystallographic characteristics were identical to those of synthetic T3 [19].

Concurrently with the investigations of Gross et al., Roche, Lissitsky, and Michel, in France, also synthesized T3 and used it to identify radioactive T3 in hydrolyzed

thyroid tissue from rats given ^{131}I [20]. Although somewhat different methods were used in the two studies, the results obtained in each study provided mutual confirmation.

Gross et al. demonstrated that administration of T3 to hypothyroid patients restored their basal metabolic rates and plasma cholesterol levels to normal [21]. They also found that T3 was three times more potent that T4 in a goiter prevention assay in rats, a finding that led them to speculate that T3 was the active form of the TH [22]. In an expanded study, they demonstrated T3 to be more active than T4 in a wide variety of tests and again they suggested that T3 was the active hormone, and T4 the precursor [23]. Although this hypothesis was an attractive one, its confirmation proved to be challenging. Indeed, throughout the 1950s and 1960s there was disillusionment and confusion with respect to the concept that T3 was the active form of the TH. Since there were no techniques sensitive enough for the accurate measurement of T3 in serum, studies of T4 metabolism during this period were carried out using T4 labeled with ^{131}I. Arguably the most significant problem was that, despite several attempts, the presence of [^{131}I]T3 following injection of [^{131}I]T4 into athyreotic humans could not be demonstrated unequivocally [23–25]. Attempts to demonstrate T4 to T3 conversion in vitro were also largely unsuccessful. Although Albright et al. did obtain some conversion of [^{131}I]T4 to [^{131}I]T3 in both rat and human kidney slices [26, 27], a plethora of other groups were unable to demonstrate [^{131}I]T3 production from [^{131}I]T4, despite the fact that deiodination of T4, as evidenced by the release of ^{131}I, clearly occurred [28]. It was not until 1970 that Braverman et al., using highly sensitive TH displacement technology, demonstrated unequivocally that T4 to T3 conversion occurred in vivo in athyreotic humans [29]. In the same year, Sterling et al. provided additional proof by demonstrating the production of [^{14}C]T3 in euthyroid human subjects injected with [^{14}C]T4. They estimated that as much as a third of the extrathyroidal T4 that was metabolized was converted to T3 [30]. The finding that T3 is produced from T4 in vivo laid to rest the major issue that countered the theory that T3 was the active form of T4.

Although the finding that T4 can be converted to T3 in vivo is certainly compatible with the concept that T3 is the active form of the TH, it did not prove it. However, once the TH receptors were characterized it became evident that T3 is responsible for the majority of TH action in peripheral tissues. These receptors, located in the nucleus, were first reported in 1972 by Oppenheimer et al. [31, 32]. and were shown to have a much higher affinity for T3 than for T4 [33, 34]. The finding that more than 80% of the total iodothyronine bound to the nuclear receptors in liver and kidney from euthyroid rats was T3, provided strong additional support for the concept that T3 is responsible for the majority of TH action in the tissues [35].

Discovery of the deiodinases

Confirmation that T4 could be converted to T3 in vivo prompted a renewed interest in identifying and characterizing the enzyme(s) that catalyzes the process using in vitro systems. Between 1955 and 1976, the study of T4 deiodination in vitro had been severely hampered, especially in broken cell preparations, because considerable nonspecific, heat-stable deiodination occurred in a reaction that did not produce detectable amounts of T3 [28]. However, the problem was resolved in 1976 by the demonstration that thiol groups are essential cofactors for enzymatic deiodination, and thiol-containing compounds, such as dithiothreitol (DTT), needed to be included in broken cell preparations to support in vitro enzymatic deiodination. The presence of these compounds also greatly reduced the non-enzymatic breakdown of T4. Thus, in 1976 it was shown that, in the presence of DTT, subcellular fractions of rat liver readily convert T4 to T3 [36], and work began in earnest to determine the characteristics and tissue distribution of the responsible enzyme.

It was soon recognized that there were in fact two enzymes capable of catalyzing the conversion of T4 to T3. In a series of studies reported in the late 1970s, the ability of T4 to suppress thyrotropin (TSH) secretion in rats was found to be dependent on its conversion to T3 within the pituitary per se. Of particular interest was the finding that the effect was not compromised by pretreatment of the rats with 6-n-propyl-2-thiouracil (PTU), a compound known to block at least 70% of peripheral 5'deiodination (5'D) in rats [37]. In contrast, pretreatment with another inhibitor of 5'D, iopanoic acid (IOP), greatly reduced both 5'D activity and the amount of T3 produced from injected T4, and TSH secretion was not significantly decreased. IOP did not interfere with the suppressive effect of T3 on TSH secretion [38]. These seemingly contradictory findings were resolved in 1982 when kinetic evidence was obtained suggesting that two distinct mechanisms for 5'D were present in rat pituitary [39] and cerebral cortex [40]. One of them exhibited a substrate preference for 3,3',5'-triiodothyronine (rT3) over T4, followed "ping-pong" type kinetics with DTT as co-substrate, and was inhibited by PTU. The 5'-deiodinase in liver and kidney exhibited properties comparable to this deiodinase. The second type of 5'deiodinase preferred T4 to rT3 as substrate, was insensitive to PTU, although not to IOP, and followed "sequential" reaction kinetics. The activity of this second type of deiodinase was elevated in tissues from hypothyroid rats. The two enzymes are now referred to as the Type1 (D1) and Type 2 (D2) deiodinases, respectively. The D1 can also catalyze inner-ring or 5-deiodination (5D), in particular that of iodothyronines that have undergone sulfate conjugation of their hydroxyl group on the outer ring. D1 activity, as measured in vitro, is

present at a relatively high levels in liver, kidney, and thyroid. D2 activity is notably present in pituitary, brain, and brown adipose tissue (BAT). However, both enzymes are expressed, albeit often at a low level in many other tissues. On the basis of evidence obtained by the late 1980s it was widely accepted that the primary function of the D1 was to generate T3 from T4 for the circulation and that of the D2 was to generate T3 from T4 for local use within the same cell or tissue. However, the deiodinases are low abundance membrane-bound proteins and attempts to purify them to study their structure and biochemical properties have been largely unsuccessful.

By the turn of the 20th century there was compelling evidence that both the D1 and the D2 contribute significantly to the daily T3 production, as much as 80% and 50% in euthyroid iodine-sufficient humans and rats, respectively. The remaining fraction comprises T3 secreted from the thyroid gland. In rats there is evidence that D1 and the D2 contribute equally to peripheral generation of T3, in the human the D2 may be responsible for the majority [41].

Given the apparent importance of the 5'-deiodinases in TH economy, it was predicted that the absence of one or both of these enzymes would severely compromise TH metabolism resulting in undesirable consequences. By the end of the 1990s the cDNAs for the D1 [42] and the D2 [43, 44] had been cloned, sequenced, and found to be selenoenzymes. These advances made it possible to create transgenic mice completely deficient in one or both of the 5'-deiodinases.

Unexpectedly, none of the 5'-deiodinase-deficient models exhibited an appreciable gross phenotype; the mice appeared healthy, growth was essentially normal and reproductive capacity was seemingly unimpaired. Furthermore, the serum T3 level was unaffected in all these models including the mouse lacking both the D1 and D2 [45–47]. In the latter model the only source of T3 was the thyroid gland. In the absence of the D1, this T3 must have been derived directly from hydrolysis of thyroglobulin in the thyroid gland. Clearly, maintenance of the serum T3 level was not dependent on the presence of these enzymes at least in this species under controlled circumstances. A notable feature of models lacking the D1 was the marked elevation of the circulating rT3. In view of the preference of the D1 for rT3 over T4 as a substrate for 5'D, the possibility that the 5'D of rT3 is a major function of the D1 must be considered.

Other findings in these models are consistent with the concept that the D2 is important for the local conversion of T4 to T3 in peripheral tissues, most notably in brain, pituitary, and BAT. Observations made in the mouse deficient in both the D1 and the D2 have shown that at postpartum day 15, the T3 content in various regions of the brain is reduced by approximately 50%. It had been estimated previously that at least 50% of the receptor-bound T3 in rat brain was generated predominantly by the D2 [48]. This D1/D2KO mouse also exhibited an almost two-fold increase in *both* the serum T4 and TSH levels. The likely explanation for this is that TSH suppression as a result of the elevated serum T4 level cannot occur due to the absence in the pituitary of the D2 required to convert the T4 to T3.

A third deiodinase, known as the Type 3 deiodinase (D3), catalyzes inner-ring, or 5D and thus it inactivates both T4 and T3. In 1977, 5D of the two hormones was demonstrated in cultured monkey hepatocarcinoma cells [49]. D3 activity is most abundant in placenta and pregnant uterus in rats [50] and the human uteroplacental unit [51], where it is thought to limit the exposure of the developing fetus to the circulating maternal T4 and T3. It is present at much lower levels in many fetal and neonatal tissues, most notably in the brain, where it is thought to protect fetal and neonatal brain from excessively high levels of T3 [52]. In the non-pregnant adult mammal, D3 activity is found predominantly in the brain and skin. Sequencing of cDNAs for the D3 have revealed that it is also a selenoenzyme [53, 54]. In contrast to mice deficient in the 5'-deiodinases, transgenic mice completely deficient in D3 activity exhibit significant embryonic and perinatal mortality, impaired growth, and fertility and neonatal thyrotoxicosis followed later by central hypothyroidism that persists throughout life [55].

In summary, there is abundant evidence to substantiate the concept that T3 is responsible for the majority of TH action in the tissues, and that the major function of deiodinases, especially the D2 and the D3, is to maintain the intracellular content of T3 at a level appropriate for a given peripheral tissue or cell. However, the possibility that one or more of them has other important functions cannot be excluded.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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