THE PRODUCTION AND CHARACTERIZATION OF MEDIUM AND HIGH SPECIFIC ACTIVITY ^{125}I-T_3 and ^{125}I-T_4 UTILIZING SUBSTITUTION AND EXCHANGE MECHANISMS

Max G. Reese and Richard H. Hales

Becton Dickinson Immunodiagnostics, Salt Lake City, Utah 84115
Received March 3, 1978
Revised April 17, 1978

SUMMARY

The reaction of ^{125}I with 3,5-diiodo-L-thyronine (T_2), 3,3',5-triiodo-L-thyronine (T_3), and L-thyroxine (T_4) have been studied under various pH and concentration conditions. It was found that when the pH is 7.5 and approximately equimolar amounts of thyronine substrate and ^{125}I are reacted, T_2 produces as the major product monolabeled T_3 of high specific activity (~3300 mCi/mg) and long term stability while T_3 produces as the major product approximately 30% dilabeled ^{125}I-T_4 with short term stability and a specific activity of ~3000 mCi/mg. Simple methods for purification of this product to make it useful are discussed. When the pH is raised to 8.7 and the molar ratio of ^{125}I to thyronine substrate is increased to 1:6 then T_3 produces as the major product T_3 of medium specific activity (~500 mCi/mg) and long term stability while T_4 produces very stable monolabeled T_4 of medium specific activity (~250 mCi/mg). The mechanisms of substitution and exchange, as they relate to the formation of these and other side products in these reactions, are discussed.

Key words: thyronine derivatives, iodination, substitution, exchange, stability.

INTRODUCTION

Thyroxine (T_4) and 3,3',5-triiodothyronine (T_3), the two most physiologically important iodinated thyronines secreted by the thyroid gland, display a pronounced influence on the rate of general metabolic activity of the body. In recent years the technique of radioimmunoassay (1,2,3), first developed by Berson and Yalow (4), has been applied to the analysis of both T_3 and T_4 in plasma and serum (5,6). The most basic and variable

0362-4803/78/0015-0555$01.00

component of the RIA system is the labeled antigen. Ideally, the labeled antigen should have high specific activity, high immunoreactivity (reacts like cold compound), purity, and long shelf life (7). The use of higher specific activity antigen facilitates the RIA test by improving sensitivity. Several groups of workers (8-12) have reported the synthesis of ^{125}I-T_3 and ^{125}I-T_4, but only Weeke and Örskov (8) and Kjeld et al. (11) reported high specific activity preparations. When we repeated their work we observed that the high specific activity T_4 had a very short shelf life. This paper presents the results from our efforts to produce both medium and high specific activity ^{125}I-T_3 and ^{125}I-T_4 with high purity and long shelf life.

MATERIALS AND METHODS

Materials

3,5-Diiodo-L-thyronine (T_2), 3,3',5-triiodo-L-thyronine (T_3), and L-thyroxine (T_4), were purchased from Sigma Chemical Co. and used without further purification. Carrier free ($\sim$17 Ci/mg), low pH $Na^{125}I$ of greater than 99% radionuclidic purity was obtained from New England Nuclear Corp. Chloramine T, trihydrate (11% active chlorine) was used as obtained from J. T. Baker Chemical Co. Sodium metabisulphite, analytical reagent, was purchased from Mallinckrodt Inc., and propylene glycol, U.S.P. grade, was purchased from Aldrich Chemical Co. Both were used without purification, as well as Sephadex G-25-80 from Sigma Chemical Co.

General Procedure

All labeling reactions were carried out in a fume hood in the opened Combi-V-Vial in which the $Na^{125}I$ had been received. Addition of reagents and solutions to the reaction container was effected with Drummond disposable micro-pipettes. The general procedure was conducted by first adding 50 µl of 0.05 M phosphate buffer, pH 7.1-7.5, to 5 mCi (2.27 nmoles) of $Na^{125}I$ in the Combi-V-Vial. This was followed by addition of 2-10 µg of thyronine substrate dissolved in 10-40 µl of dilute ammonium hydroxide, pH 10.5. Immediately afterwards, 90 µg of chloramine-T in 25 µl of 0.05 M phosphate buffer, pH 7.5, was added and the reaction allowed to proceed for 15 seconds. The reaction

was quenched by adding 240 µg of sodium metabisulphite in 100 µl of 0.05 M phosphate buffer, pH 7.5.

Low pH reaction

The order of addition of reagents was the same during this reaction. However, to arrive at the final pH of 5.8 it was necessary to initially add phosphate buffer of pH 4.6 to the $Na^{125}I$ and then use pH 6.0 phosphate buffer for the chloramine-T and sodium metabisulphite solutions.

Separation of Products

The reaction products were separated by adsorption chromatography on a Sephadex G-25-80 column (1.1 x 14 cm). The elution buffer used was 0.05 M phosphate buffer of pH 7.5. Approximately eighty fractions of 6.0 mls each were collected and stored in dim light. The fractions containing similar products were pooled, diluted to 50% with propylene glycol and stored in the dark at 4°C.

Characterization of Products

The specific activity was either calculated where the product was obvious, or else determined by RIA methods or referenced to previously published results.

RESULTS

Various thyronine derivates were reacted with $^{125}I^-$ which was oxidized to molecular iodine by chloramine-T under different pH conditions. In all cases radioactively labeled thyroxine and 3,3',5-triiodothyonine were obtained. The unreacted $^{125}I^-$ was eluted from the Sephadex column before fraction 10 while T_3 and T_4 were eluted between fractions 30-50 and 50-70 respectively. Only traces of T_1 and T_2 were ever observed in any of these reactions. In all cases, efficient separation of T_3 and T_4 labeled products was achieved. They were found to be stable, except for doubly labeled T_4, for periods up to 5 months when stored in a 50:50 mixture of propylene glycol and 0.05 M phosphate buffer at pH ranging from 4.9 to 10.0. These reactions are summarized in Table 1.

When T_2 was reacted with ^{125}I (run 1), essentially following the procedure of Weeke and Ørskov (8), the radioactivity ratio of ^{125}I-T_4 to ^{125}I-T_3 produced

was 2:3. In this case all the T_4 produced must be diiodinated. Since

TABLE 1

Reaction of Various Thyronine Derivatives with ^{125}I by the Chloramine-T Method

Run	Reactants			Oxidation	Products
		mole ratios		pH	^{125}I-T_4:^{125}I-T_3
	Thyronine	Substrate	^{125}I		
1	T_2	1.68	1	7.5	2:3
2	T_3	1.35	1	7.5	2:1
3	T_3	1.35	1	5.8	2.7:1
4	T_3	6.77	1	8.6	1:2
5	T_4	5.67	1	8.7	28:1

carrier free $Na^{125}I$ is used the specific activity of the T_3 is calculated to be on the order of 3300 mCi/mg, or near the maximum theoretical value, while that for the dilabeled T_4 was estimated to be approximately 5600 mCi/mg. The T_3 was observed to be stable for ten months while the T_4 was very unstable, remaining usable for only a week or two. These data are tabulated in Tables 2 and 3.

TABLE 2

Antigenic Stability of Labeled Thyronine Products

Run	^{125}I-T_3	^{125}I-T_4
1	10 months	1-2 weeks
2	10 months	2-3 weeks
3	10 months	2-3 weeks
4	10 months	> 2 months
5	_____	> 5 months

TABLE 3

Specific Activity* of Labeled Thyronine Products

Run	$^{125}I\text{-}T_3$	$^{125}I\text{-}T_4$
1	~3300	~5600
2	~1200	> 3000
3	< 1200	> 3000
4	~500	~2800
5	____	~250

*mCi/mg

When T_2 was replaced by T_3 and the reaction repeated using approximately the same molar ratio of ^{125}I to T_3 (run 2, Table 1) and the same pH, the radioactivity ratio of $^{125}I\text{-}T_4$ to $^{125}I\text{-}T_3$ became 2:1. The $^{125}I\text{-}T_3$ produced in this reaction was estimated from the work of Kjeld et al. (11) to have a specific activity of approximately 1200 mCi/mg and was found to be stable for ten months. The $^{125}I\text{-}T_4$ produced was found to be stable for only a few weeks and have a specific activity >3000 mCi/mg. Since these results were contrary to those reported by Weeke and Örskov for $^{125}I\text{-}T_4$ obtained under the same conditions (8) a further examination of this product was made. When a sample of the $^{125}I\text{-}T_4$ product from run 2 (Table 1) was rechromatographed on a 5 mm x 20 mm Sephadex G-25-80 column two days after the initial reaction, only a small amount of free $^{125}I^-$ (or non-T_4 ^{125}I labeled material eluting concurrently with free $^{125}I^-$) was observed (Figure 1). However, when this separation was repeated 60 days after the reaction, the free $^{125}I^-$ peak was quite large and the $^{125}I\text{-}T_4$ peak was reduced to approximately two-thirds the original amount. Also, when a sample of the $^{125}I\text{-}T_4$ product was reacted with an excess of cold KI, oxidized by chloramine-T under these same reaction conditions, all of the label was released from the T_4 and eluted as free $^{125}I^-$. A double antibody assay was used to monitor the antigenic properties of the high specific activity $^{125}I\text{-}T_4$ produced in this reaction. It can be seen from Figure 2 that the labeled T_4 suffers a relatively rapid decrease in antigenic

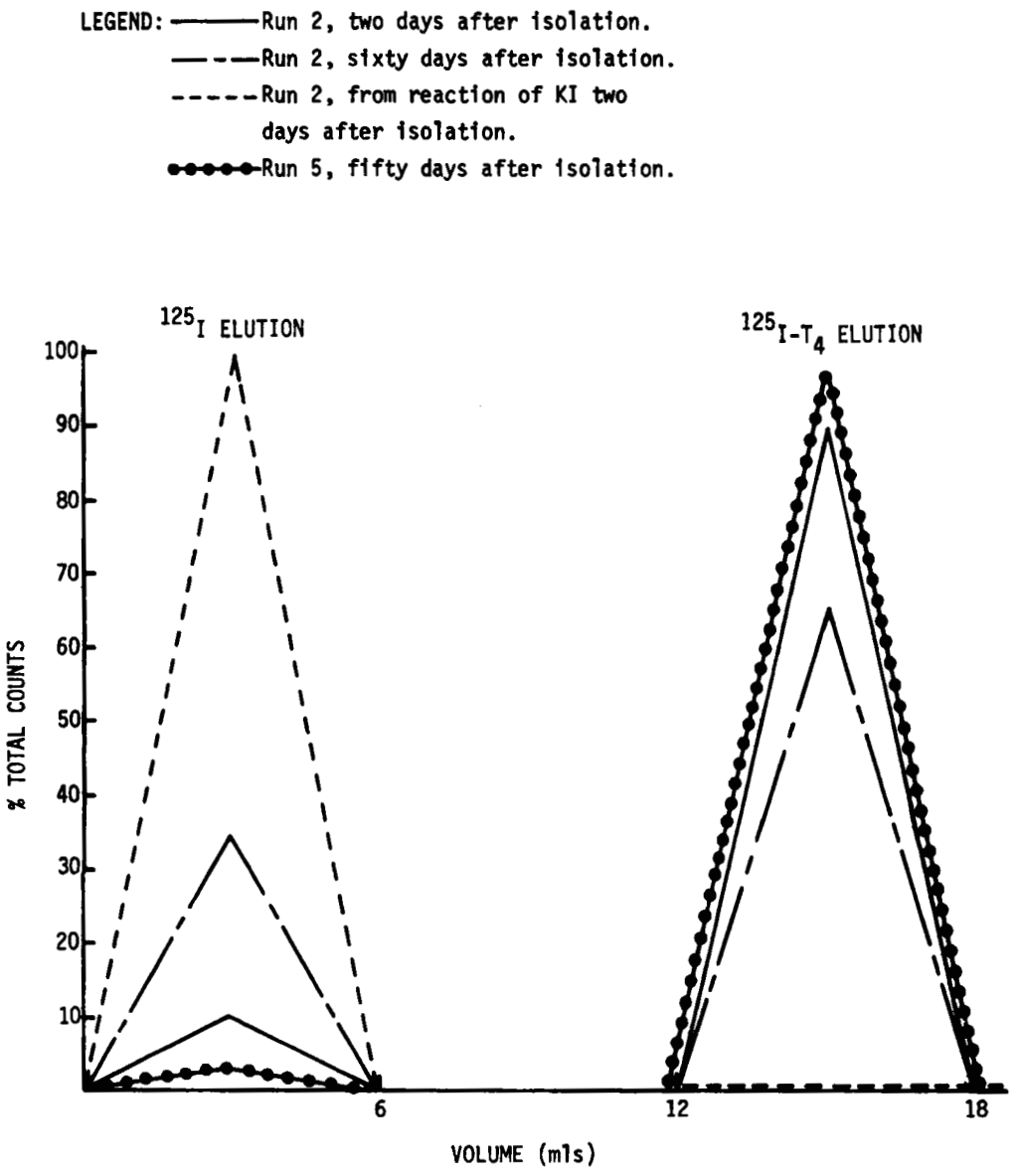


Figure 1. Chromatography of ^{125}I-T_4 products on Sephadex G-25-80.

properties during the first 60 days of storage followed by a much slower change.

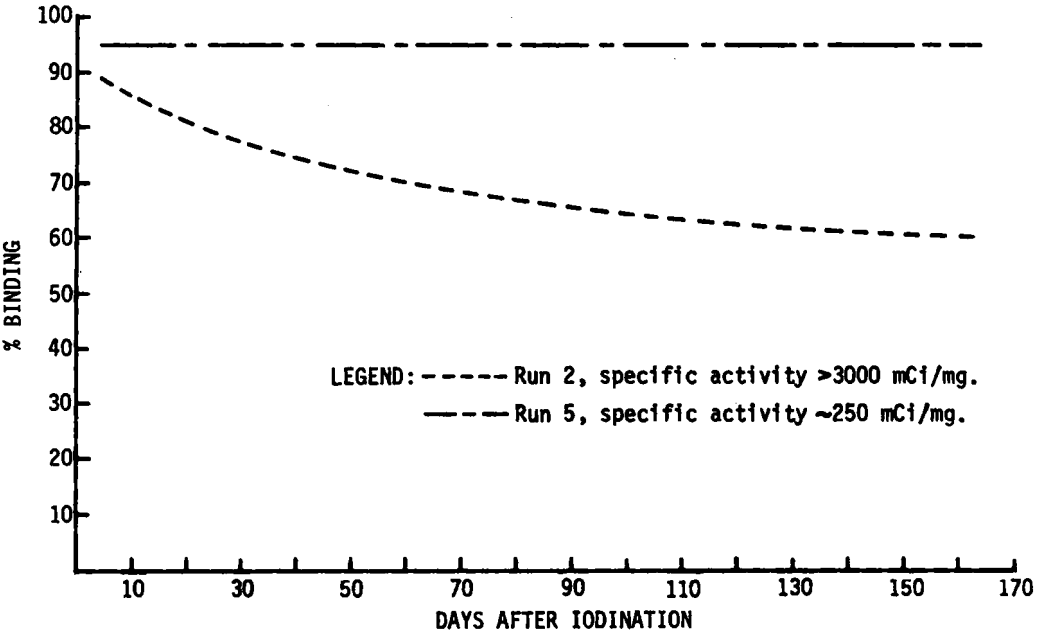


Figure 2. Antigenic properties of $^{125}I\text{-}T_4$ products monitored by double antibody RIA.

When the pH of the T_3 reaction was lowered to 5.8 in an effort to increase the yield of $^{125}I\text{-}T_4$ over $^{125}I\text{-}T_3$ (run 3, Table 1), the radioactivity ratio for $^{125}I\text{-}T_4$ to $^{125}I\text{-}T_3$ was found to be 2.7:1. However, the total yield of labeled products was reduced to about one half that obtained at neutral pH. The specific activity and stability of the products in this reaction were found to be similar to that of the $^{125}I\text{-}T_3$ and $^{125}I\text{-}T_4$ produced in run 2.

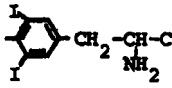
An interesting result was noted when the iodination of T_3 was repeated at pH 8.6 using a five fold molar increase of T_3 (run 4, Table 1). In this case the major product was $^{125}I\text{-}T_3$ and not $^{125}I\text{-}T_4$, as previously observed for T_3 reactions. The radioactivity ratio for $^{125}I\text{-}T_3$ to $^{125}I\text{-}T_4$ was 2 to 1. Also, it was observed in this case that the $^{125}I\text{-}T_4$ was stable for more than two months. It's specific activity was approximately 2800 mCi/mg. The

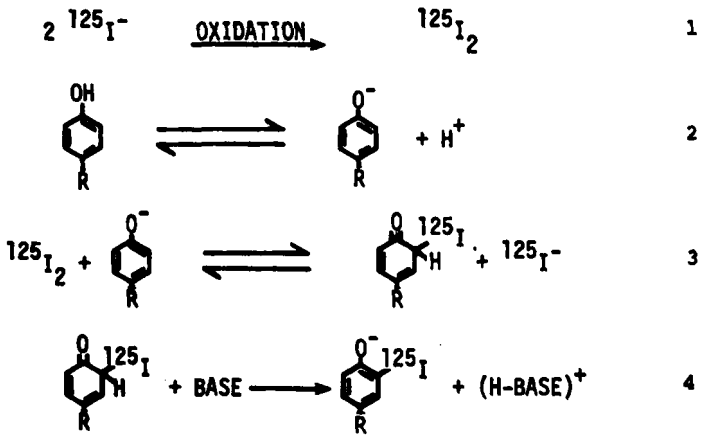
labeled T_3 product was found to have a specific activity of approximately 500 mCi/mg and remained antigenic for more than ten months.

When the pH 8.6 iodination reaction was repeated using a similar five fold molar increase of T_4 (run 5, Table 1), greater than 96% of the labeled product was ^{125}I-T_4 with only a trace of ^{125}I-T_3 being formed. The labeled T_4 was found to be stable for more than five months and have a specific activity of approximately 250 mCi/mg. When a sample of this product was rechromatographed on a 5mm x 20mm Sephadex G-25-80 column 50 days after the iodination reaction had taken place, only a very small free iodide peak was observed (Figure 1). The labeled T_4 was also monitored by the double antibody assay technique. Figure 2 shows the constant antigenic properties of this product with time.

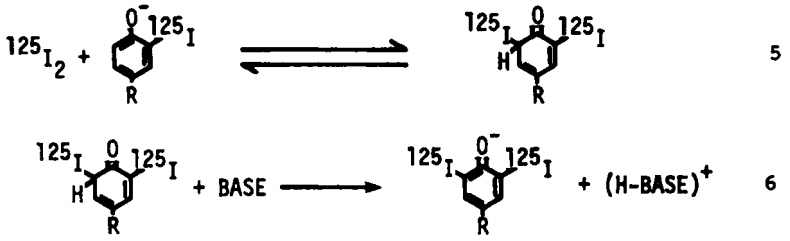
DISCUSSION

The synthesis of labeled tracers of high purity, high specific activity, and long shelf life is a prime requirement for any RIA test. Other workers have recently reported their results for the iodination of thyronine derivatives by the chloramine-T method (8, 10-12). Burger and Ingbar (10) reported a specific activity of 245 mCi/mg for their ^{125}I-T_4 produced from T_3 while a specific activity of 51 mCi/mg was found when T_4 was the substrate. The specific activity of ^{125}I-T_3 produced from T_3 was a low 31 mCi/mg. Thurlow and Puxley (12) iodinated T_2 and T_3 and obtained specific activities as high as 500 mCi/mg for ^{125}I-T_3 and 350 mCi/mg for ^{125}I-T_4. However, their procedure included more complex separation and purification steps. Both Weeke and Örskov (8) and Kjeld et al. (11) reported the production of very high specific activity ^{125}I-T_3 and ^{125}I-T_4; Weeke and Örskov claimed ~3000 mCi/mg for T_3 but were vague when referring to T_4, and Kjeld et al. reported that the use of T_2 as substrate gave ^{125}I-T_3 with specific activity of 2400 mCi/mg and ^{125}I-T_4 with specific activity of 5200 mCi/mg while the use of T_3 as substrate gave ^{125}I-T_3 with specific activity of 1200 mCi/mg and ^{125}I-T_4 with specific activity of 4000 mCi/mg.

When we repeated the procedure of Weeke and Örskov, which is essentially that used by Kjeld et al., we found similar results for the iodinations of T_2 and T_3 (runs 1 and 2, Table 1). Starting from T_2, both ^{125}I-T_3 and ^{125}I-T_4 are produced by substitution of either one or both hydrogen atoms ortho to the phenolic OH group. Obviously, any labeled T_4 produced would have two radioactive atoms and hence the extremely high specific activities observed by us and Kjeld et al. The mechanism most likely by which ^{125}I-T_3 would be formed may be represented by equations 1, 2, 3 and 4 where R represents  . Mayberry and Hacket (13)

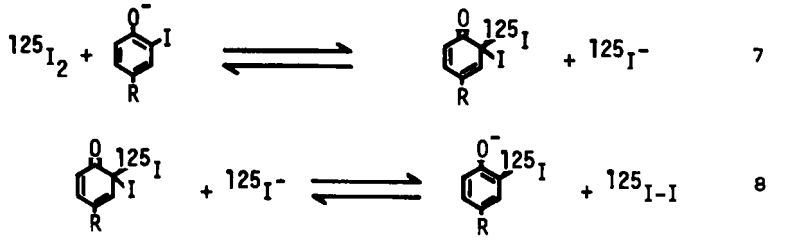


have shown that the iodination of tyrosine and its derivatives proceeds by way of the bimolecular reaction between molecular iodine and the phenoxide ion to produce the intermediate cyclohexadienone. The "rate-limiting" step was viewed as the proton removal in step 4. However, more recently Dobáš et al. (14) have shown that the reaction of I_2 with para substituted phenolate ion (equation 3) becomes rate-determining when the concentration of iodide ion is low. ^{125}I-T_4 would be formed as shown in equations 5 and 6 which are essentially a repeat of equations 3 and 4. The fact that more labeled T_3 than T_4 is produced when T_2 is the starting material is explained by the electron withdrawing inductive effect of the iodine atom. The presence of an iodine atom on the phenolic ring will reduce the nucleophilic character of the ring and make attack from the



iodine more difficult. It has been shown that tyrosine, for example, is iodinated at a rate about 20 times that of monoiodotyrosine (13).

When T_3 is the substrate, both ^{125}I-T_3 and ^{125}I-T_4 are formed. Obviously, in this case, the radioactive T_3 must be formed through an exchange mechanism which may be represented by equations 7 and 8. Based on the specific



activities of their labeled T_3 and T_4 products, Kjeld et al. (11) concluded that substitution was taking place more readily than exchange in their reactions. We find that which one of these reactions predominates depends upon the ratio of ^{125}I to thyronine substrate. When T_3 is the substrate and the ratio of ^{125}I to T_3 is 1:1.35 (run 2, Table 1), twice as much labeled T_4 as compared to labeled T_3 is produced and substitution would seem to be the dominant reaction. However, when the ratio of ^{125}I to T_3 is increased to 1:6.77 (run 4, Table 1) then the major product by a two to one margin is ^{125}I-T_3 over ^{125}I-T_4 and exchange is the major reaction. These results may be better understood by a closer examination of the mechanism of the two reactions. Exchange of ^{125}I for ^{127}I (equations 7 and 8) is an equilibrium process. An increase of ^{125}I relative to T_3 will not facilitate formation of ^{125}I-T_3.

On the other hand, the replacement of a hydrogen atom by an iodine atom (equations 3 and 4) is not an equilibrium reaction and an increase in the relative amount of starting $^{125}I_2$ will drive the reaction to completion thereby reducing the pool of I_2 available for exchange. The probability that exchange is rapidly taking place is also shown by the fact that when the ^{125}I-T_4 was reacted with excess cold I_2, all of the radioactivity was lost from the ring (Figure 1). Other workers (9,15) have reported that when radioactive I_2, produced by a different oxidation method, was reacted with T_3 or T_4, exchange was more rapid than substitution and was the primary reaction taking place.

Weeke and Örskov (8) reported the rapid formation of iodinated breakdown products from the dilabeled T_4 synthesized from diiodothyronine. At the same time they infer that stable monolabeled T_4 of high specific activity is produced when T_3 is the substrate. On the other hand Kjeld et al. (11) observed the formation of doubly labeled T_4 from T_3 and saw approximately 20% loss of radioactivity after 50 days from material stored in 50% propylene glycol. We likewise observed a large loss of radioactivity from the labeled T_4 product (run 2) when it was rechromatographed on Sephadex 60 days after isolation (Figure 1); also, there is approximately a 30% loss of antigenicity of labeled T_4 after 60 days of storage time as shown by our double antibody assay (Figure 2). However, when the monolabeled T_4 of medium specific activity produced by exchange in run 5 was monitored with time we observed no loss of radioactivity after 50 days (Figure 1) and complete stability of antigenic properties for up to five months (Figure 2). We conclude that monolabeled T_4 is stable and that the loss in radioactivity in the ^{125}I-T_4 produced from T_3 substrate by both Kjeld et al. and us is due to the presence of unstable doubly labeled T_4. It has been proposed (16) that the radioactive decay of ^{125}I to the stable nuclide ^{125}Te results in deiodination of T_4 since tellurium dissociates from the hormone. This most likely takes place by a rupture of the aromatic ring as postulated by Jiang et al. (15) with either loss of the second ^{125}I atom as free iodide or formation

of a labeled non-antigenic compound which elutes from Sephadex concurrent with free iodide (Figure 1).

The doubly labeled T_4 produced at pH 7.5 with T_3 as substrate (run 2) may have two sources. In theory it could be produced from substitution of a second ^{125}I atom on ^{125}I-T_3 formed from T_3 by exchange, or it could be formed by exchange of ^{125}I with the ^{127}I atom on the ^{125}I-T_4 produced from the T_3 substitution. The latter case is most likely when considering pH effects. At pH 7.5, T_4 with a phenolic pK of 6.73, will be more highly ionized than T_3 (pK = 8.45). Since both substitution and exchange take place more rapidly on the phenoxide ion (13) it seems likely that the major pathway for the formation of doubly labeled T_4 is by means of exchange on monolabeled T_4.

To use our ^{125}I-T_4 product of high specific activity (>3000 mCi/mg) produced from the reaction of T_3 with ^{125}I one needs only to apply a short purification step before use in order to remove radioactive non-antigenic contamination. We have done this by either of two simple techniques, i.e. separation on a small Sephadex column or affinity chromatography. After approximately two months, purification by either of these two methods will produce ^{125}I-T_4 which will maintain antigenicity and specific activity at the theoretical maximum ($\sim$2800) and require no further purification.

The fact that the labeled T_4 of medium specific activity produced by the exchange reaction (run 5) is very stable is proof that it is monolabeled. This should be the method of choice for production of medium specific activity T_4. It is anticipated that even higher specific activity monolabeled T_4 could be produced by adjusting the ^{125}I and T_4 concentration to more nearly a 1:1 ratio. Obviously when too much iodine becomes available then diiodination will begin to take place. Kjeld et al. reported that they obtained a very poor yield of labeled T_4 when using T_4 as substrate (11). Their results may very well be due to the lower pH of their reaction. We have found that pH is critical in the T_4 exchange reaction due to T_4 precipitation.

The method of choice for production of high specific activity ^{125}I-T_3 is by substitution starting from T_2 (run 1). Monolabeled T_3 is produced which has very high (near theoretical maximum of ~3300 mCi/mg) and constant specific activity. Purification is not needed after storage, as was noted with monolabeled T_4, because only non-antigenic breakdown products are produced. Monolabeled T_3 of medium specific activity is best produced from T_3 by exchange using a ratio of ^{125}I to T_3 of approximately 1:5 (run 4).

The method of choice for production of ^{125}I-T_4 of high specific activity is from the reaction of T_3 with ^{125}I (run 2) with frequent purification by either chromatography on Sephadex or affinity chromatography until a relatively constant specific activity is reached. Medium specific activity T_4 is best produced by the exchange reaction using a ratio of ^{125}I to T_4 of approximately 1:5 (run 5).

REFERENCES

1. Skelley D.S., Brown L.P., Besch P.K. - Clin. Chem. 19: 146 (1973)
2. Goldsmith S.J. - Sem. in Nucl. Med. 5: 125 (1975)
3. Kirkham K.E. and Hunter W.M. (Eds.) - Radioimmunoassay Methods, Churchill Livingstone, Edinburgh, 1971
4. Berson S.A. and Yalow R.S. - Nature 184: 1648 (1959)
5. Larsen P.R., Dockalova J., Sipula D. and Wu F.M. - J. Clin. Endocrinology and Met. 37: 177 (1973)
6. Cheung M.C. and Slaunwhite Jr. W.R. - Clin. Chem. 22: 299 (1976)
7. Chervu L.R. and Murty D.R.K. - Sem. in Nucl. Med. 5: 157 (1975)
8. Weeke J. and Örskov H. - Scand. J. Clin. Lab. Invest. 32: 357 (1973)
9. Hallaba E. and El-Shaboury G. - J. Nucl. Med. 15: 270 (1974)
10. Burger A. and Ingbar S.H. - Endocrinology 94: 1189 (1974)
11. Kjeld J.M., Kuku S.F., Diamant L., Fraser T.R., Joplin G.F. and Mashiter K. - Clin. Chem. Acta 61: 381 (1971)
12. Thurlow V.R. and Puxley H.J. - Ann. Clin. Biochem. 13: 364 (1976)

13. Mayberry W.E. and Hockert T.J. - J. Biol. Chem. 245: 697 (1970) and references cited therein

14. Dobáš I., Choulik A., Štěrbo V. and Večeřa M. - J. Chem. Soc. Perkin A 1972: 1189

15. Gleason G.I. - J. Biol. Chem. 213: 837 (1955)

16. Latham K.R., Ring J.C. and Baxter J.D. - J. Biol. Chem. 251: 7388 (1976)

17. Jiang V.W., Krohn K.A. and Welch M.J. - J. Am. Chem. Soc. 97: 6551 (1975)