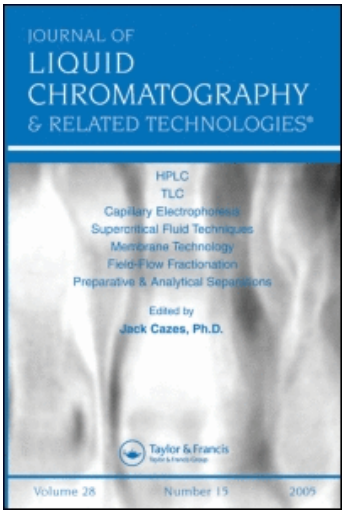

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USE OF A DUAL COULOMETRIC-AMPEROMETRIC DETECTION CELL APPROACH IN THYROID HORMONE ASSAY

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ABSTRACT

The development and application of a dual (in series) coulometric-amperometric thin-layer electrochemical detection cell system to the assay of thyroid hormones following reversed-phase chromatography is described. Using this approach the system was characterized by empirically determined electrolysis efficiencies, demonstrating adherence to the diffusion-layer approximation theory. When academic mixtures of the thyroid hormones were studied, the precision of both response and relative retention data was to within 4%. With the dual-cell approach, improvements by a factor of two in the limit of detection were noted over the same system using only amperometric detection. The electrochemical behavior of these analytes in the dual-cell system was also studied. These latter experiments demonstrated that, in principle, it is possible to assay the thyroid hormones with both cells set at a potential where mass transport control obtains, analyzing the oxidation products of the first cell at the amperometric cell.

INTRODUCTION

The use of two electrodes in series, or two thin-layer electrochemical (TLED) cells in sequence has previously been

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applied in several areas. Gerischer and coworkers (1) first introduced and discussed the arrangement of two closely spaced electrodes in a configuration where laminar flow obtained. The first electrode served as a "generator" electrode and the latter as the detection or "collector" electrode. This type of configuration would have similar advantages to that of the ring-disc system (2) for applications to electrode-kinetic studies, and in spite of theoretical rigor from several groups (3-5), the ring-disc system remains the more popular application.

In 1976 Blank (6) noted that with closely spaced channel electrodes placed in series within one cell body, it was possible to conveniently monitor a chromatographic effluent at two separate potentials. Using this approach, if coelution of electroactive components occurred, it was possible to determine the presence or absence of a given component by independently evaluating peak responses as a function of applied potential. More recently, studies involving dual channel electrodes in series have been carried out by several groups (7-12). Increased selectivity (7,10,12), on-line blank correction (8,9), electrochemical analysis of electrolysis products (10,12), removal of oxygen interferences (10,11) and increased sensitivity (10,12) have both suggested and further demonstrated the potential utility of this configuration in application to specific problems.

Other examples of the application of dual electrode systems of varying geometry and configuration have appeared in the literature. Both Fenn et al. (13) and Weber (14,15) have demonstrated the use of parallel opposed electrodes in a TLED cell configuration for purposes of improved detection limits, while Roston and Kissinger (16) have utilized this approach to evaluate phenolic constituents in commercial beverages. Schieffer (17) has demonstrated the use of a minimum volume packed bed coulometric detection cell placed on-line in front of an amperometric TLED cell for purposes of improving selectivity in this latter cell. Most recently Matson and co-workers (18) utilized two porous graphite coulometric electrodes in series to detect electroactive components at two different potentials, improving both qualitative and quantitative data and providing the ability to screen out unwanted signals from specific chromatographic systems.

In the current work on the thyroid hormones, discrimination against electroactive background was felt to be desirable in order to improve detection limits. In this approach, as opposed to the single-cell method previously reported (19), two cells were connected in series following the chromatographic column. The first served as a "scavenger" cell to remove electroactive background unimportant to the analysis, while the second cell was an amperometric cell the purpose of which was to detect and

assay the analytes of interest. The more efficient the first cell (i.e., approaching coulometric yields) the better the clean-up. Using low temperature isotropic carbon, LTIC (20), as a working electrode material in a thin-layer "sandwich cell" (21) geometry, it was hoped to accomplish this goal with a minimum of cell volume.

Applying the above approach to the assay of the thyroid hormones it was possible to characterize the dual-cell system through empirically determined electrolysis efficiencies. These data were, in turn, utilized to document the adherence of these cell types to the diffusion-layer approximation theory (15,22). Improvements in the limit of detection of the thyroid hormones in a dual-cell system were demonstrated and the electrochemical behavior of these analytes studied.

EXPERIMENTAL

Apparatus

The liquid chromatographic system used in this work was the same as that previously described (19). Detection was carried out with (i) a Waters Model 440 absorbance detector at 254 nm connected in series with a laboratory designed TLED cell, (ii) a single TLED cell following the chromatographic column or (iii) two TLED cells in series. The PAR 174A Polarographic Analyzer previously noted (19) and/or a Bioanalytical Systems Inc. Model DCV-4 voltammetry control unit were used as potentiostats in the

amperometric work. Heath-Schlumberger Model SR-204 strip-chart recorders were employed to record chromatograms at chart speeds of 0.1 in. min^{-1}. Electrode resistance measurements were made with a Fluke Model 8030A multimeter. An Orion Model 701A pH meter was used for pH measurements.

Construction of LTIC Cells

1. Amperometric cell: external TLED cell geometry - The cell design used in this work is the same as that previously described (19).

2. Coulometric cell - The coulometric cell designed for use in this work is shown in Figure 1. The design follows the precedent set in previous designs by employing the hemi-cylindrical-yoke approach (19). The difference in this cell lies with the replacement of the platinum and/or silver-hoop electrode imbedded in the upper Kel-F half by a second LTIC plate electrode, M. Two holes, 0.1 cm in size, were drilled approximately 2.0 cm apart centrally in this plate using a diamond-tipped drill bit. The location of the two holes was such that when the plate was overlaid on the upper cell half, they were aligned with the entry, C, and the exit, L, ports previously drilled through the cell block (Figure 1). This plate was fixed in place using EPD-TEK 353 ND epoxy (Epoxy Technology Inc., Billerica, MA 01821). Contact to this second plate was made by means of the banana plug-stainless steel

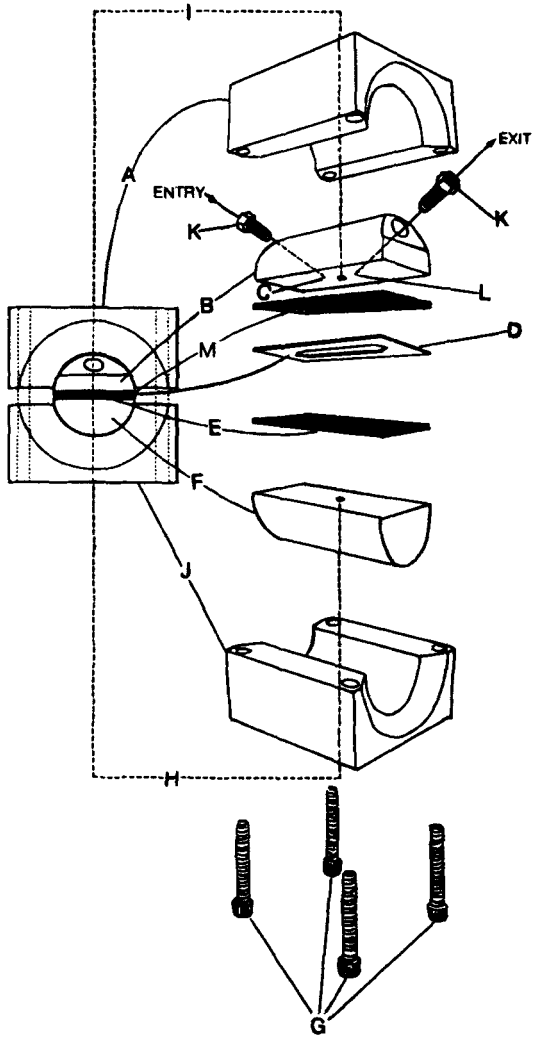

FIGURE 1. Hemicylindrical coulometric TLED cell, end view at left, exploded view at right, (A) and (J) upper and lower yokes machined from polyvinyl chloride for holding together hemicylindrical cell, (B) and (F) upper and lower cell halves machined from Kel-F, (C) entry port into cell's inner channel, (D) PTFE spacer, (E) LTIC plate working electrode, (G) stainless steel screws for holding yokes together, (H) electrical contact to LTIC plate working electrode, (I) electrical contact to combined reference-auxiliary LTIC plate electrode, (K) Altex 1/16 inch fittings, (L) exit from cell's inner channel, (M) LTIC plate combined reference-auxiliary electrode.

spring combination described elsewhere (19). Spacers were cut from 0.0025 cm PTFE material and the channel in the spacer provided a working electrode area of about 3.0 cm^2. This resulted in cell volumes of 7-8 μL. The cell was utilized in both a two-electrode and three-electrode short-circuited geometry mode (19). In the former configuration the upper LTIC plate served as a "reference" electrode to which the auxiliary electrode lead from the PAR 174A (or Bioanalytical Systems DCV-4 analyzer) had been shorted. In the three-electrode short-circuited configuration the upper LTIC plate was shorted to the external Ag/AgCl reference electrode. The stainless steel capillary served as both an exit conduit and auxiliary electrode.

Reagents

The structures of the various tyrosine and thyronine analogs used in this study are shown in Figure 2. The sodium salts of triiodothyronine (T_3) and tetraiodothyronine (T_4) as well as the compounds diiodothyronine (T_2), monoiodotyrosine (MIT), diiodotyrosine (DIT) and thyronine (T_0) were obtained from the Sigma Chemical Co. (St. Louis, MO 63178). The reverse triiodothyronine (rT_3) used was from the same lot of material provided earlier by Dr. Russel Saunders of the Nuclear Medical Laboratories. Stock standards were prepared at 1.00 mg mL^{-1} in 3% (v/v) concentrated NH_4OH in methanol (Fisher Scientific, Montreal, PQ). These materials were stored tightly capped at

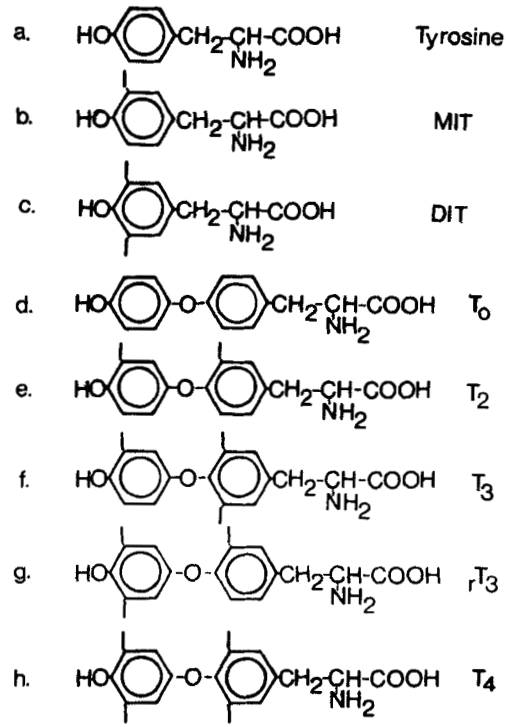

FIGURE 2. Structures of the various tyrosine and thyronine analogs. a. Tyrosine, b. Monoiodotyrosine, c. Diiodotyrosine, d. Thyronine, e. Diodothyronine, f. Triiodothyronine, g. "Reversed" triiodothyronine, h. Tetraiodothyronine.

-20°C. Fresh standards were prepared every six weeks. Working standards were prepared as needed in the appropriate chromatographic mobile phase.

Procedures

All glassware used was first washed with Fisher-brand (Sparkleen) detergent, followed by rinsing first in tap water

and finally in distilled water. These washed items were soaked overnight in 6 M HNO_3, rinsed again in tap water followed by distilled water, oven dried, cooled and silylated with 10% (v/v) chlorotrimethylsilane in toluene. This reagent was prepared from toluene which had been dried and stored over molecular sieves (American Chemicals Ltd., grade 564, type 3A, 8-12 mesh). The combined silane-toluene reagent was also stored over these same molecular sieves and reused up to ten times. All silylations were carried out by total immersion in the silylation bath for 15-30 min, followed by toluene, then methanol rinsing and finally overnight oven drying at 120°C. Glassware prepared in this manner was then stored dessicated at room temperature until used.

Duplicate hydrodynamic voltammograms were run on chromatographic peaks following 20-μL injections of mixtures containing 25 ng each of T_2, T_3 and rT_3 and 50 ng of T_4; duplicate voltammograms were taken after independent 20-μL injections of 25 ng each of MIT, DIT and T_o. Voltammograms were run from a positive potential setting in the region of the hydrodynamic plateau (i.e., the potential invariant peak height) to a negative value where the signal was indistinguishable from the background. These voltammograms were plotted from averaged peak current values as a function of potential applied. Injection and retention precision data were obtained from 10 replicate injections of 25 ng each (50 ng in the case of T_4) of the mixtures noted

above. Relative retention data were determined using rT_3 as an internal standard.

Dual-cell, diffusion-layer approximation verification and coulometric-yield (electrolysis-efficiency) experiments were carried out with the amperometric-cell (or "following" TLED cell) potential set at 1.500 V and the coulometric (guard) TLED cell potential varied as necessary. MIT and DIT (at concentrations of 25 ng/20μL) were used as model compounds in electrolysis-recovery (efficiency) experiments. In these recovery experiments the applied potential of the guard cell was set on the experimentally determined hydrodynamic plateau for the particular analyte and the change in recovery for the compound was monitored by the amperometric cell as the guard cell was turned on and then off during separate runs. Recovery was then determined by taking the ratio of the peak current obtained at the following amperometric cell with the guard cell turned on, to that obtained with the guard cell turned off. When this ratio is multiplied by a factor of 100 percent recovery is obtained. If this percentage is subtracted from 100, the percent electrolysis efficiency for a given analyte under defined conditions at a given cell can be determined.

In dual cell experiments on the electrochemistry of the thyroid hormones, the guard cell potential was varied until the hydrodynamic plateau for a specific thyroid hormone was determined. The electrolysis products obtained from this oxida-

tion process were then monitored at the following amperometric cell at a potential of 1.500 V, as a function of analyte recovery (guard cell off, then on) and transport time between the guard and amperometric cell. By variation of the flow rate both analyte recovery of the cells studied and the transport time between cells could be varied. Flow rates used in these studies were 0.20, 0.50, 0.70, 1.00 and 1.50 ($\pm$0.02) mL min^{-1}. In these latter experiments all recoveries obtained as a function of the electrolytic removal of analyte by the guard cell, were normalized by the averaged recovery obtained from the model compounds MIT and DIT evaluated under the same conditions.

For all of these experiments the chromatographic columns were thermostatted at 60°C.

RESULTS AND DISCUSSION

Precision Studies

Representative precision data for the dual-cell TLED system were obtained from the chromatographic analysis of academic test mixtures of the thyroid hormones. These data are presented in Tables 1-3 and in Figure 3. Within-day and between-day retention data can be compared from Tables 2 and 3. As might be expected for both cases retention precision is best when based upon an internal standard (rT_3).

TABLE 1

Tabulated Peak Height Data from 20-μL Injections of a Mixture of T_2, T_3, rT_3 and T_4 Containing 25, 25, 25 and 50 ng, Respectively. Conditions of the chromatographic experiment were: applied potential of analytical cell, 1.500 V (short-circuited geometry); applied potential of guard cell 0.600 V (51% electrolysis efficiency); flow rate 1.00 mL min^{-1}; 50:50 aqueous 0.015 M phosphate buffer, pH 2:methanol; temperature of LC column, 60°C. Peak heights were measured in nA.

	T_2	T_3	rT_3	T_4
Mean	59.91	33.21	26.01	32.63
SD	0.728	0.413	0.402	0.449
RSD	0.0122	0.0124	0.0155	0.0138
n	10	10	10	10

TABLE 2

Within-Day Retention Time (RT) and Relative Retention Time (RRT) Data for a Mixture of T_2, T_3, rT_3 and T_4. Chromatographic conditions are the same as those in Table 1.

	RT, min				RRT[a]		
	T_2	T_3	rT_3	T_4	T_2	T_3	T_4
Mean	4.14	5.91	8.11	9.46	0.510	0.728	1.166
SD	0.0852	0.105	0.127	0.120	0.00864	0.0115	0.00989
RSD	0.0206	0.0178	0.0157	0.0127	0.0169	0.0159	0.00849
n	12	12	12	12	12	12	12

[a] Relative to rT_3

Evaluation of the Coulometric TLED Cell for Application in Dual-Cell Experiments

As mentioned previously, the coulometric TLED cell has many uses in liquid chromatography. One of these is to selectively serve as an on-line clean-up (or guard) device to

TABLE 3

Between-Day[a] Retention Time (RT) and Relative Retention Time (RRT) Data for a Mixture of T_2, T_3, rT_3 and T_4. Chromatographic conditions are the same as those in Table 1.

	RT, min				RRT[b]		
	T_2	T_3	rT_3	T_4	T_2	T_3	T_4
Mean	4.22	6.26	8.58	9.97	0.492	0.730	1.162
SD	0.126	0.321	0.434	0.523	0.0184	0.0132	0.0210
RSD	0.0300	0.0512	0.0505	0.0524	0.0374	0.0181	0.0181
n	11	11	11	11	11	11	11

[a] Collected over a two-week period
[b] Relative to rT_3.

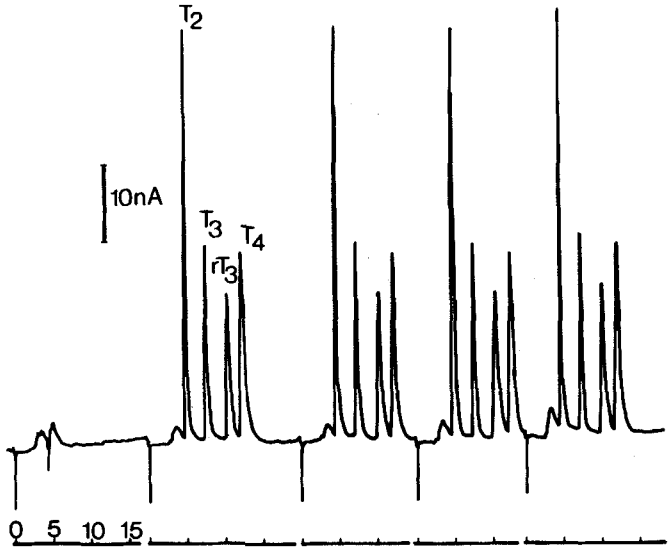

FIGURE 3. Representative chromatograms from repetitive 20-μL injections of an academic mixture of T_2, T_3, rT_3 (25 ng each) and T_4 (50 ng) into the chromatographic system containing the dual-cell detector. The guard cell potential was set at 0.600 V and the amperometric detection cell potential at 1.500 V. Chromatographic conditions are given in Table 1.

remove unwanted electroactive background either contained in the mobile phase or introduced into it during the chromatographic process. Conceptually, the principle of this use is straightforward; however, in the present context its implementation was of limited success. The inherent advantage of a coulometric cell is the complete conversion of the total amount of material available for the electrolysis process, as opposed to the rather small portions converted in amperometric cells. The practical disadvantage of the coulometric cell lies in the cell geometry required to achieve total conversion of an electroactive analyte. Historically this has meant using packed beds of conduction fibers or particles (17,23), larger surface area channel electrodes (13,15,24) or a metal-gauze or carbon-cloth electrode (25). It has been noted (21,23,24) that under these conditions the larger electrode surfaces resulted in proportionally larger background currents and hence similar signal-to-noise ratios. Additionally the argument can be made that with the increased electrode areas necessary to achieve total conversion come increased cell volumes which are potentially detrimental to chromatographic processes. These problems are due to larger extra-column dead volumes which facilitate band broadening.

By using LTIC plate electrodes and the cell design shown in Figure 1 it was hoped to obviate both problems mentioned above. Using the diffusion-layer approximation theory (15,22) and an

assumed flow rate of 1.00 mL min^{-1}, the cell efficiency was plotted against the working electrode area at the various PTFE template thicknesses available (see Figure 4). A close inspection of this figure demonstrates the dynamic aspect of varying the template thickness. For example, if a cell of 90% coulometric efficiency is required, a working electrode area of 1.7 cm^2 is needed with a 0.00064-cm spacer or an electrode area of 5.0 cm^2 with a 0.0025-cm spacer. This translates into cell volumes of 11 and 130 µL, respectively. Snyder and Kirkland (7) have previously observed that it is desirable to keep extra-column dead volumes to less than 100 µL and ideally less than 30 µL. With the advent of microbore chromatography (26) keeping

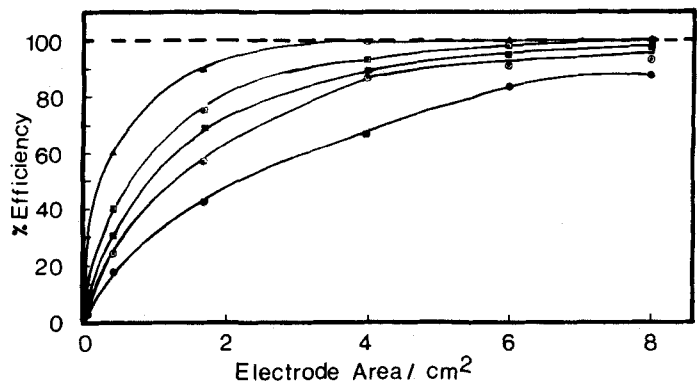

FIGURE 4. Plot of electrolysis efficiency vs. working electrode area as a function of spacer thickness. These data were obtained for a constant flow rate of 1.00 mL min^{-1} and with the working electrode width equal to the channel width. Calculations were based on the diffusion-layer approximation theory. Thicknesses are: Δ 0.00064, □ 0.0013, ■ 0.0019, O 0.0025, ● 0.0051 cm.

this parameter small becomes even more crucial. Examination of Figure 4 reveals that the easiest way to achieve the goal of smaller cell volumes is to decrease template thickness. The use of LTIC electrode material helped to realize this goal.

Preliminary experiments with two strips of plastic, 0.4 cm x 5.0 cm x 0.0006 cm thick placed along the edge between two LTIC plates, demonstrated that when these plates were squeezed together between the PTFE-insulated jaws of a vise, contact between the plates did not occur. Thus, in principle, any commercially available PTFE material of this or greater thickness could serve as a spacer in TLED cells.

Two plates which exhibited no contact under the above conditions were chosen as electrodes in the TLED cell. When constructed, however, it was quickly apparent that irrespective of the thickness of spacer material (0.00064-0.0051 cm), no TLED cell could be assembled that possessed an efficiency greater than 50% at a flow rate of 1.00 ± 0.01 mL min^{-1}. This was due to electrode contact problems and was independent of template thickness. More efficient electrolysis could be achieved at slow flow rates (maximum 86% at 0.20 ± 0.02 mL min^{-1}), the trade-off being greatly extended analysis times. No further studies on electrode material were undertaken.

The inability to fully achieve the promise offered by the LTIC materials was a disappointment. However it was felt that the failure was not with the material itself but rather with its

bonding to the Kel-F. In affixing the LTIC plates to the Kel-F hemicylindrical cell half with the EPO-TEK 353 ND epoxy, a curing temperature of 60°C was used (27). Although both the Kel-F (28) and the LTIC (29) materials are stable at this temperature, some expansion and contraction does occur due to the different thermal properties of the two materials. When this occurs, even the slightest "warping" of one or both surfaces can be expected which leads to contact problems such as those observed above. One might speculate that allowing these materials to cure gradually at room temperature might obviate this problem. Cells constructed recently (30) with a 3-5 day curing may yet fulfill the promise initially suggested for the LTIC product. In the present work it was decided to accept the electrolysis efficiencies that the "coulometric" cell would give at a flow rate of 1.00 mL min^{-1}.

The 8-μL volume added by the "coulometric" cell had a negligible influence on chromatographic parameters. In contrast, although Schieffer (17) also found negligible effects with the addition of a packed-bed coulometric cell to a chromatographic system in one application, in related work (31) using a technically much less complicated packed-bed cell an overall 12% loss in chromatographic efficiency was observed. The use of plate electrodes and thinner spacers ultimately offers not only the advantages of decreased cell volumes, but the possibility of applying "super-coulometric" techniques (14,15,32) in analyses

where the potential of one plate is controlled at an oxidizing potential and that of the facing plate at a reducing potential. This is not possible with packed-bed cells.

Finally the dual-cell approach was used to reduce background current. Employing the dual LTIC plate electrolysis guard cell with an electrolysis efficiency of 50.0 ± 2.0% (determined over a two-week period) the potential of the working electrode was set at 0.600 V, the foot of the wave for the most readily oxidized thyroid hormone (rT_3). Background currents were measured. Background currents recorded at the amperometric (8.6 ± 0.1% efficient) analysis cell (potential of the working electrode set at 1.500 V) ranged from 75-200 nA with the guard cell off. When the guard cell was operating, the reduction in background currents at the amperometric cell ranged from 25-50 nA. For the dual-cell approach this translates to a reduction of 25-33%. Since this reduction reflects losses in the faradaic component of the background current, the range in values is due to the quality variation of the mobile phase which was prepared on a daily basis. More important, when detection limits for a given day were compared for the dual-cell approach and the single amperometric TLED cell (19), lower detection limits were seen with the dual cell (see Table 4). These results, using the same cells, templates, chromatographic conditions and calibration techniques, suggest that if faradaic components of the background are minimized by their electrolytic removal from the

TABLE 4

Limit of Detection[a] Determined as the Amount of Analyte[b] Necessary Under Chromatographic Conditions[c] to Generate a Signal Twice the Size of the Peak-to-Peak Noise for Both the Single Amperometric Cell and Dual-Cell Systems.

	Single cell[d], ng	Dual cell[e], ng	Ratio single/dual
T_3	0.12	0.047	2.6
rT_3	0.096	0.061	1.6
T_4	0.18	0.096	1.9

[a] Determined as sensitivities in nA ng^{-1} and twice the p-p noise value in nA from amperometric cell data.
[b] Using academic standards of T_3, rT_3 and T_4.
[c] 1.00 mL min^{-1}; 50:50 (v/v) aqueous 0.015 M phosphate buffer, pH 2:methanol; column temperature, 60°C.
[d] Amperometric TLED cell (8.6 ± 0.1% efficient) short-circuited geometry, applied potential 1.00 V.
[e] TLED guard cell, 0.600 V applied potential, 50.0 ± 1% efficient, amperometric TLED cell as in (d).

mobile phase, flow-dependent noise will in turn be minimized with appropriate improvements in the limits of detection. Except for T_3, the improvement seen in the ratio of dual-cell: single-cell response reflects a similar improvement in the limit of detection for similar electrode processes. The deviation of the T_3 value from the other values seen in Table 4 is probably artifactual and due to chromatographic factors involving bands eluting close to the void volume and extra column band broadening.

Determination of TLED Cell Efficiencies

The dual-cell approach was used to empirically determine specific TLED cell efficiencies. The cell of interest served as

the first in a two-cell sequence and its effect on the removal of a test analyte (MIT and DIT) from the flow stream was determined. In these experiments the first cell was utilized in the "two-electrode" mode, with either the platinum disc (19) or the upper LTIC plate (Figure 1) serving as a combined auxiliary-reference electrode. With the second cell functioning as the analytical cell, the ratio of peak heights for repetitive injections of the model compounds as a function of the first cell being turned off or on, would represent the electrolysis efficiency of the first cell. In these studies, 3 to 5 injections of each compound were generally made and the average of results for MIT and DIT were compared. The precision of these measurements for both peak height and retention were consistent with the data in Tables 1-3; agreement between the averaged MIT and DIT values was within 2%. Typical hydrodynamic tracings as a function of the potential applied to the first cell (or cell being tested) are in Figure 5. The response of the test and the analytical cell are shown in 5a and 5b, respectively. These plots are both determined as averaged peak currents vs. the potential applied to the guard cell. It was noted that with the analysis cell held essentially at infinite potential, the hydrodynamic voltammograms obtained are qualitatively the mirror image of those generated at the test cell. Plateaus are achieved both prior to and following the electrolysis process occurring in the test cell. The relative magnitude of current

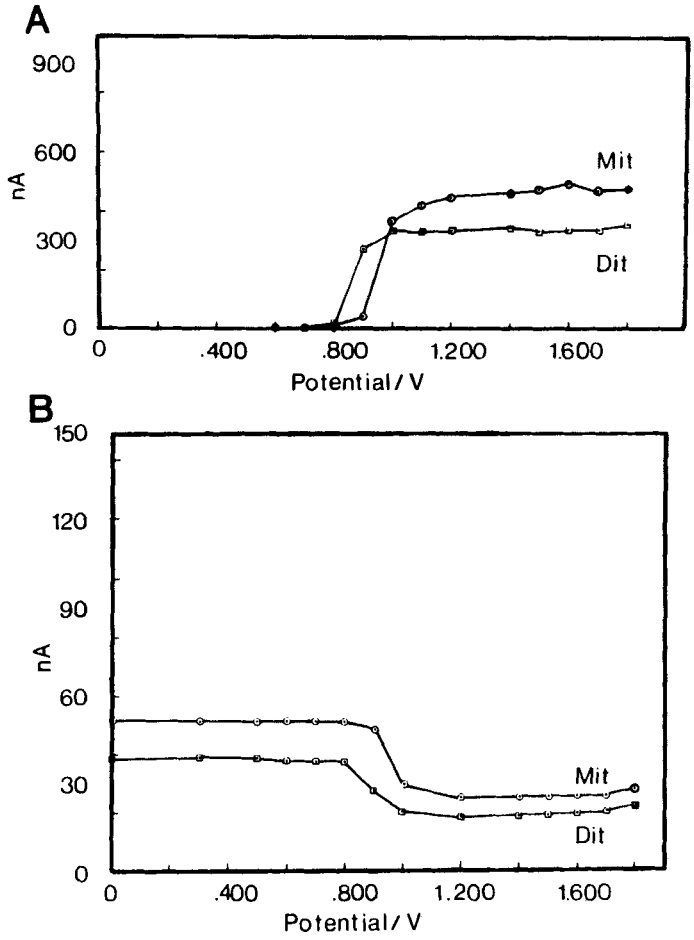

FIGURE 5. Hydrodynamic voltammograms for MIT and DIT as a function of the guard cell potential in the dual-cell system. A. Voltammogram from the guard cell. B. Voltammogram from the amperometric TLED cell. Applied potential of the amperometric cell was set at 1.500 V, using short-circuited geometry. Guard cell was in two-electrode mode. Each point is the average of duplicate determinations. Chromatographic conditions are given in Table 1.

seen at each cell in series is a reflection of the approximate factor of 5 difference in the electrode areas between the two cells, the cell geometries and potential applied to the guard cell. The relative difference in the magnitude of current between MIT and DIT for a similar electron transfer process is due to the fact that all voltammograms in this work were obtained under chromatographic conditions and MIT elutes before DIT. If voltammograms such as these are generated, the plateau currents observed in the amperometric analysis-cell tracing (Figure 5b) can be used directly to determine electrolysis efficiency.

Demonstration of Adherence to the Diffusion-Layer Approximation Theory

It is graphically possible to display a logarithmic electrolysis efficiency parameter for channel electrodes as a function of a logarithmic cell geometry parameter (15,22). This latter parameter, r_L, is a combination of volume flow rate, cell thickness, cell width, channel width, length of electrode and diffusion coefficient for the analyte of interest. The relationship is:

$$r_L = \frac{DLW_c}{\bar{u}b}$$

where D is the diffusion coefficient of the analyte in cm^2 sec^{-1}, L is the electrode length in cm, W_c is the channel width in cm, $\bar{u}$ is the volume flow rate in cm^3 sec^{-1} and b is the cell

thickness in cm. W_c is equal to the electrode width, W_e, with r_L being expressed as a function of the normalized (by cell thickness, b) diffusion layer, $\bar{\delta}_L$, determined at the end of the electrode in the direction of flow (15,22).

It has been shown previously (15) that r_L is the ratio of analyte residence time within the cell to the time necessary for the analyte to diffuse to the electrode surface prior to the electron-transfer process. The shorter the cell residence time relative to the diffusion time, the less efficient the cell. This is seen in Figure 6 by observing the decrease in cell efficiency as r_L becomes smaller.

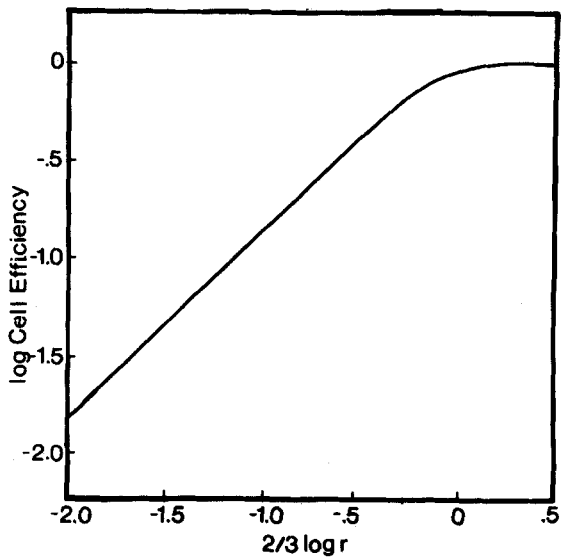

FIGURE 6. Plot of the logarithm of cell efficiency vs. a logarithmic function of 4, the dimensionless ratio of intercell analyte residence time to the time required for the analyte to diffuse to the working electrode surface prior to electron transfer (15,22).

Adherence to this relationship can be demonstrated by determining steady-state currents obtained at infinite potential for given analytes at specific concentrations and comparing the experimentally obtained values to those predicted for TLED cells of known geometries. This was essentially the approach used in the original verification of the diffusion-layer approximation theory applied to channel electrodes in a flowing stream (15,22). In the present work however, by using empirically determined cell efficiency values (obtained as discussed above) and the graphic relationship of Figure 6, it is possible to empirically determine a specific cell geometry constant based upon the fixed geometrical parameters for a given cell and upon knowledge of the diffusion coefficient of the analyte of interest and the flow rate. The value of this cell geometry constant is readily obtained from the above equation.

In principle, for a given analyte, TLED cell and PTFE template, this value should remain constant. In practice, exact knowledge of the diffusion coefficient is unnecessary for conditions of constant temperature, mobile-phase composition and analyte composition as this value will also be constant and become part of the "cell constant" for a given system. Once this "cell constant" value has been determined, r_L can be varied as a function of flow rate and the predicted cell efficiency (expressed as percent recovery) may be compared to the empirically determined cell efficiency (expressed in a like

manner). A plot of this relationship should ideally be a straight line at a 45° angle through the origin. For the TLED cell used in the present work this relationship is plotted in Figure 7 (values to within 1%). As is readily seen adherence occurs up through a flow rate of 1.00 mL min^{-1}. Above this flow rate deviation from the diffusion-layer approximation theory is observed. Since it is known that the diffusion-layer approximation theory has been validated at flow rates up to 3.0 mL min^{-1} (15,22) and the flow rates utilized were measured to within $\pm$ 0.02 mL min^{-1}, the deviation is within the cell constant parameter. Because the same temperature (to within $\pm$ 0.1°C), mobile phase and standard solution were used in this determination, the variance would be expected to be in the geometric parameters of this constant. This deviation may, in fact, be due to the expansion of the PTFE template at increased

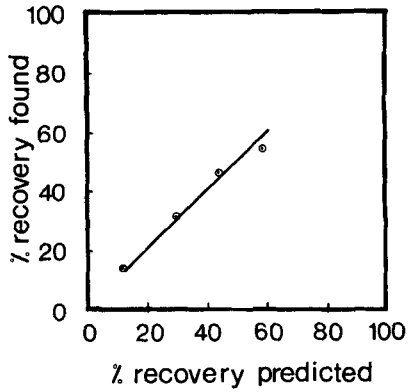

FIGURE 7. Plot of percent model compound found (recovered) vs. percentage expected for TLED cells from the diffusion-layer approximation theory (15,22).

flow rates and/or a change in the flow pattern within the cell which influences the transport of analyte to the electrode surface. In either event, all experiments were carried out at a flow rate of 1.00 ± 0.01 mL min^{-1} or less assuring a region in which adherence to the diffusion-layer approximation theory obtained for the cell utilized. Use of the dual-cell approach provides a way in which experimental data can be generated for cells in which laminar flow does not obtain prior to crossing the surface of the working channel electrode. Although not applied in the present work, it would follow that with knowledge of specific "cell constant" values (obtained from data using analytes of known diffusion coefficients under defined conditions) in regions where the diffusion-layer approximation theory applied, steady-state currents could be predicted and/or diffusion coefficients for other analytes empirically determined.

Dual-Cell Electrochemistry of the Thyroid Hormones

The electrochemical oxidation of the thyroid hormones in all probability emulates the model compound chosen for this group, thyronine, T_o. T_o is initially oxidized in a two-electron step with increasing positive potential; subsequently a homogeneous hydrolysis step occurs in which a quinone and tyrosine residue are produced (see Figure 8). Tyrosine upon further increase in potential is itself oxidized in an overall two-electron step. It has been suggested that the thyroid hormones

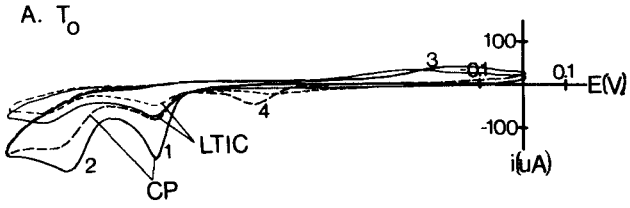

FIGURE 8. Cyclic voltammograms of T_o determined on carbon paste (CP) and LTIC stationary electrodes (20). Concentration of T_o is 2.0 x 10^{-4} g mL^{-1} in 1.0 M H_2SO_4. 1: 2-electron oxidation of T_o, 2: 2-electron oxidation of the hydrolytically generated tyrosine, 3: 2-electron reduction of the generated p-quinone, 4: subsequent 2-electron oxidation of p-hydroquinone.

follow a similar path (33); this is not refuted by the present work. A cursory search of the literature has not yielded information of the rate of hydrolysis to form quinone and tyrosine; however it has been suggested that the determination of these constants would be difficult (33). The dual-cell approach as described above would seem to provide a useful way in which to explore these constants in a qualitative if not a quantitative fashion.

In the current work using the dual-cell system, the guard cell was set such that the most difficult to oxidize analyte MIT and DIT were electrolyzed essentially at infinite potential (i.e., 1.500 V, Figure 5). Likewise, the following amperometric cell was set to a potential of 1.500 V. In principle at this potential setting any MIT or DIT residue generated as a function of hydrolysis of a given thyroid hormone would be available for oxidation at the following amperometric cell. Hence, the

electrochemical reaction products focused on in this evaluation were the iododinated residues MIT and DIT.

In studying the dual-cell electrochemical behavior of the thyroid hormones under these conditions, it was felt that if the rates of hydrolysis of these materials were fast compared to the intercell residence time within the guard cell (≈ 0.5 sec for an 8-μL cell volume), then the electrolysis recoveries obtained would correspond to those of the iodinated tyrosine residues (MIT and DIT) used as model compounds in the present work. However, when the experiment was performed, the recoveries found were quite different from those for the model compounds (see Table 5).

TABLE 5

TLED Percent Recovery Using the Dual-Cell System.[a] Each value in the table represents the average of at least two determinations[b] of the thyroid hormones obtained on three separate days.

	Day 1 % recovery	Day 2 % recovery	Day 3 % recovery
T_o	74.6	76.8	76.5
T_2	64.4	68.7	64.4
T_3	68.8	73.3	74.4
rT_3	80.6	81.4	83.3
T_4	80.1	83.4	86.4
MIT	51.4	49.8	48.8
DIT	49.1	49.7	49.6

[a] Guard cell potential set at 1.500 V using two-electrode mode, amperometric TLED cell potential set at 1.500 V using short-circuited geometry.

[b] Determined chromatographically (chromatographic conditions as in Table 4) with the guard cell off, then turned on.

The size of the recoveries demonstrate that under the conditions noted, either thyroid hormones are not being removed at the guard cell in an overall process to the same extent as the model compounds, the interceding homogeneous electrolysis step is indeed not instantaneous, or most likely, the interceding homogeneous and heterogeneous chemical processes following the initial 2e oxidation of the thyroid hormones in this system are more complex than expected. In the first instance, if electrolytic removal of the various analytes were preceding to a smaller extent one might reasonably expect that at the potential applied the hydrodynamic waves of the thyroid hormones at the guard cell had not been achieved. Inspection of the voltammograms on Figure 9 however, demonstrates that at 1.500 V mass transport control in every case does obtain and that hydrodynamic plateaus are well established, hence, the potential applied is essentially "infinite". As before, superimposed upon the system, other relative differences in current magnitudes at the two electrochemical cells for similar overall electrode processes include those of electrode area and the determination of the voltammograms under chromatographic conditions (elution order MIT, DIT, T_2, T_3, rT_3 and T_4).

The second and third explanations for the observed differences in recovery are interrelated and a result of the complexities of the specific system studied. One might expect however, that if the hydrolysis rate of the interceding reaction was slow

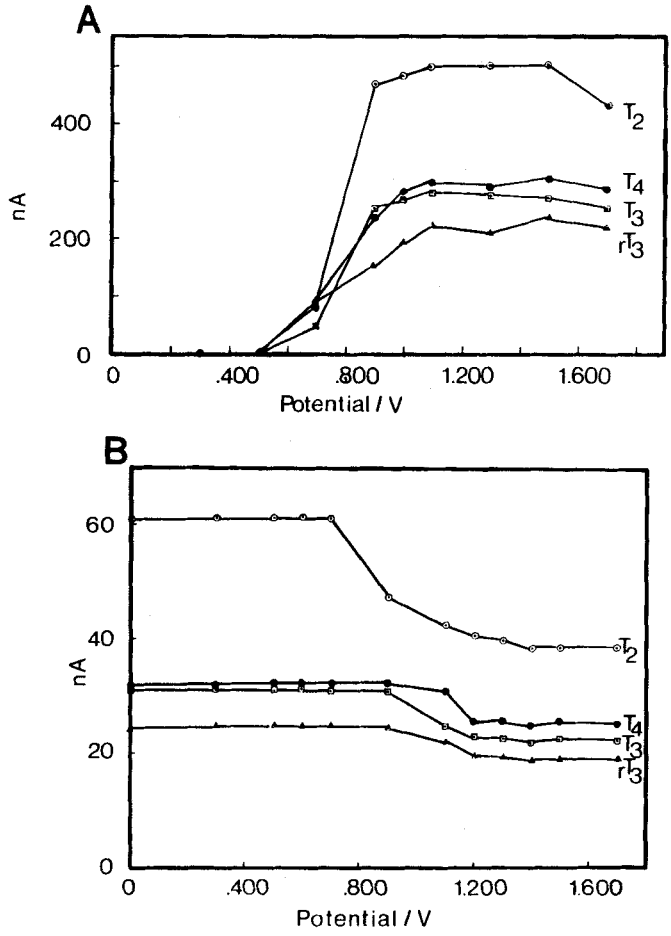

FIGURE 9. Hydrodynamic voltammograms as a function of guard cell potential for the electrolysis of T_2, T_3, rT_3 and T_4 (25 ng of each analyte, respectively, except for T_4 which was 50 ng, in a 20-μL injection). Points are the average of duplicate values. A. Voltammogram from guard cell using two-electrode model. B. Voltammogram from amperometric TLED cell using short-circuited geometry and a constant applied potential of 1.500 V.

(i.e., > 0.5s), its measurement would be readily made by existing electroanalytical methods (28). In the context of the current work with a parallel opposed two electrode circuit in the guard cell present the possibility for additional heterogeneous reductive electrochemical reactions does exist further complicating interpretation of these results.

Further experiments were undertaken to determine the influence of increasing the time it takes the analyte to flow from the first to the second cell. If electroactive material is being generated as a function of time, an increase in the time between cells would be expected to result in an increased recovery relative to the model compounds MIT and DIT. This experiment was performed and the results are presented in Figure 10, where normalized recovery (normalized to the averaged MIT-DIT recovery) is plotted as a function of the transit time between the guard and amperometric TLED cells. As can be seen, the recovery relative to the averaged model compounds does increase as a function of time. Interestingly, when these results are extrapolated back to zero time, it is seen that the efficiencies for the electrolysis of the thyroid hormones equal those of the model compounds. This infers that within the residence time of the first cell an overall electrochemical process does occur that is as efficient (i.e., equal recovery) as that for the model compounds. Subsequent to this initial oxidation process the product(s) that have formed interact

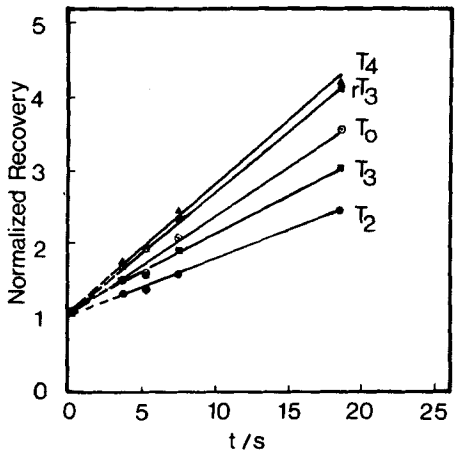

FIGURE 10. Plot of the normalized (to the averaged MIT, DIT recovery values) electrolysis recovery of T_0, T_2, T_3, rT_3 and T_4 as a function of transport time (in sec) between the guard cell (potential setting 1.500 V) and the amperometric TLED cell (potential setting 1.500 V) in a dual-cell experiment.

further through heterogeneous interactions and/or homogeneous interactions to produce additional electroactive analytes that are readily oxidized at the following amperometric cell. As can be seen from the relative slopes in Figure 10, the overall rates of these processes are not uniform. The greatest similarity seems to occur between rT_3 and T_4, molecules in which the outer ring (see Figure 2) is doubly iodinated. By contrast, molecules with a single iodinated outer ring (T_2 and T_3) seem to demonstrate this influence at a slower rate.

The importance of these findings in the current system lie in the utility of being able to set the guard cell at a higher potential allowing for greater removal of faradaic background

from the mobile phase, while still generating electroactive products derived from the thyroid hormones under investigation. Better understanding of the nature of the process of production of these derivatives awaits further study.

CONCLUSIONS

The use of the dual-cell approach, although not totally successful in demonstrating coulometric yields, did show that reductions in background were possible. In addition, improvement was observed over previously evaluated amperometric methods in the limit of detection for thyroid hormones. The failure to achieve coulometric yield with the dual LTIC electrode cell design is due to manufacturing problems and not with the design or materials.

Using the dual-cell approach it is possible to study both the electrolysis of the thyroid hormones and the nature of the hydrolysis constant associated with the electrochemical mechanism. Additional work in this area may permit quantitative evaluation of the constants involved. Also, the use of the dual-cell approach in validating adherence to the diffusion-layer approximation theory for thin-layer cells and in empirically evaluating specific cell electrolysis efficiencies has been demonstrated. These are reliable tools which can be applied in a predictable fashion.

The ultimate utility of the dual-cell approach may yet be with coulometric and supercoulometric cell configurations. A

system can be envisioned which has a guard cell set at "infinite potential" for the electrolysis of the thyroid hormones, removing other faradaic background present in the mobile phase subject to electrolysis at these potentials. After sufficient time delay the effluent stream would then proceed to a super coulometric cell which would be set to electrochemically recycle an electrochemically reversible couple (i.e., the iodinated quinone-hydroquinone couple), amplifying the signal accordingly. In theory (15,22,32), sufficient signal could be generated to analyze levels of free circulating T_3 and T_4, which indeed may be the ultimate avenue for thyroid function evaluation by laboratory methods (34,35).

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