DETERMINATION OF IODINE CONCENTRATION AND DISTRIBUTION IN RAT THYROID FOLLICLES BY ELECTRON-PROBE MICROANALYSIS

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ABSTRACT

The concentration and the distribution of iodine in various sized follicles of rat thyroid glands have been analyzed by electron-probe microanalysis. The results of the iodine analysis were grouped according to uncorrected lumen diameter size. No significant differences in iodine concentration were observed among the various size categories. When the results for all follicles from a given sample were pooled, the standard error of the mean was approximately 4%. Usually 40–50 follicles per animal were analyzed. The concentration of iodine ranged from 0.9 to 2.1% by weight among individual animals. Scanning pictures and step-scan analysis showed the iodine distribution to be quite uniform across the colloid area. Several techniques of sample preparation were used; they produced no significant differences in the iodine concentrations observed. Sodium concentration, also determined in all samples, was found to vary from 2 to 9% by weight. Because of the mobility of the sodium ion, its distribution was greatly affected by the method of sample preparation. The technique that best preserved the natural chemistry of the sample was that of freezing the tissue, sectioning, and then freeze-drying.

INTRODUCTION

The determination of iodine concentration in individual thyroid follicles is of interest in studies of rate of uptake and excretion of iodine as well as in thyroid dosimetry. In the mathematical treatment of uptake and excretion of radioiodine by the thyroid, and by individual follicles in particular, it has been necessary to assume the concentration of iodine to be the same in all follicles (Nadler et al., 1954). This has been done despite an early report in the literature (Gersh and Caspersson, 1940) of nearly 10-fold differences in concentration of iodine among individual follicles. If concentration differences of this order of magnitude do exist, they could present one possibility for the production of "hot-spots" (certain areas receiving higher doses) from the uptake of radioiodine. In the work of Gersh and Caspersson (1940) the concentrations of diiodotyrosine and thyroxine were determined by the ultraviolet absorption in 20-μ sections of tissue. More recently, Simon and Droz (1965) and Loewenstein and Wollman (1967), using ^{125}I and a method of isotopic equilibrium, have found the concentration of iodine to be essentially the same in all follicles examined. The present report deals with the analysis of iodine concentration and distribution in individual follicles by use of the technique of electron-probe microanalysis. This method enables one to determine directly the stable iodine concentration in each follicle.

MATERIALS AND METHODS

Thyroids were obtained from adult (age 4–7 months) male Long-Evans rats. Some rats were put on a low iodine diet and were maintained on such a diet for at least 9 wk prior to sacrifice. The thyroid tissue was sectioned on an IEC cryostat-microtome and analyzed on an Applied Research Laboratory (Sunland, California) electron microprobe. Stains and dyes were obtained from Allied Chemical Corp., National Aniline Div., San Francisco, California. The monoiodotyrosine and triiodothyronine were obtained from Calbiochem, Los Angeles, California. The fixative used contained 20% glutaraldehyde, 10% formalin, 10% acetic acid, and 60% methanol.

Immediately after the animals were sacrificed, thyroids of rats on normal and low iodine diets (Simonsen Laboratory Diets, Simonsen Co., Gilroy, California), were removed, frozen with Freon spray (E. I. du Pont de Nemours & Co., Inc., "Freon" Products Div., Organic Chemicals Dept., Wilmington, Delaware) and placed in the cryostat. Frozen sections 10–14 μ thick were then cut. The alternative methods of treatment were as follows.

(*a*) Frozen sections were placed on cold quartz discs and lyophilized. The sections were not allowed to thaw before lyophilization.

(*b*) Frozen sections were placed on cold quartz discs, fixed, and lyophilized. Again the sections remained frozen throughout fixation and lyophilization.

(*c*) Frozen sections were placed on quartz discs, allowed to warm to room temperature, fixed, and stained with hematoxylin and eosin.

(*d*) Frozen sections were placed on quartz discs, allowed to warm to room temperature, and fixed; but they were not stained.

A film of carbon, from 200 to 400 A, was evaporated onto all sections for analysis with the electron probe.

Several reviews of the technique of electron-probe microanalysis are available. Those of Castaing (1960), Wittry (1963), and Anderson (1967) are general reviews, of which the latter discusses some methods for biological application. Therefore, only a very brief description of the technique will be given here.

The analytical technique used in electron-probe microanalysis is that of X-ray fluorescence. The excitation source of an electron probe is a finely focused beam of electrons produced by an electron gun in much the same manner as an electron beam is formed in an electron microscope. These primary electrons interact with the sample and cause the loss of electrons in various energy shells within the atoms present. The K, L, and M shells are of primary interest in X-ray fluorescence analysis. As the electrons from one energy shell in the atom move to fill a vacancy in a lower energy shell, the excess energy is released in the form of X-rays which are characteristic of the element from which they are emitted (the energy of the characteristic X-ray for each element is proportional to the square of the atomic number). Energy discrimination (therefore, elemental selection) is accomplished by crystal dispersion according to Bragg's law and by pulse-height selection. The X-ray fluorescence technique is essentially free from interference by other elements. When some interference is possible the effect is generally small; this can be predicted by theory, and corrections can be applied, which is not true for most other techniques for elemental analysis. There is a continuous background produced as a result of deceleration of the electrons; however, line to background ratios were of the order of 20:1 for the I analysis. In a first approximation in quantitative electron-probe microanalysis the counting rate versus concentration is a linear function. Corrections for nonlinearity do have to be applied for absorption and matrix differences between the sample and the standard. If the matrix of the standard can approximate the biological matrix of the sample, the magnitude of these corrections is minimized.

The diameter of the electron beam determines the resolution of the analysis. In biological material the minimum beam diameter, and therefore the best resolution, is about 1–2 μ, which means that the elemental content and elemental distribution of individual cells and cell nuclei can be determined. The beam diameter can be increased to approximately 300 μ. The energy of the electrons can be varied from 2 to 35 kv, and the sample current is variable over a wide range. The detection limit varies with the element and the matrix, but for many cases it is about 0.01% or 100 p.p.m. However, one is analyzing only a few cubic microns of volume, so that the absolute amounts are of the order of 10^{-12}–10^{-14} g. Analysis of elements from atomic number 8 to atomic number 92 can be accomplished with our microprobe system.

The beam can be used either in the static mode or in the scanning mode (Cosslett, 1962). In the scanning mode, the beam is swept in a raster across the sample surface by electrostatic deflection plates immediately above the sample. A beam sweeps an oscilloscope screen simultaneously and in synchrony with the beam sweep across the sample. The output of one of the detectors is used to modulate the CRT sweep and thereby gives a display of the distribution of any desired element in the scanned sample area.

A visible light microscope is built into the system to allow visual localization and observation of the sample prior to and during bombardment.

The iodine $L\alpha_1$ characteristic X-ray line was used for detection and analysis of iodine. The $K\alpha$ lines were used for sodium and phosphorus. The accelerating potential used throughout the experiments

TABLE I

Concentrations of Iodine, Total and by Lumen Diameter, in the Thyroids of Male Long-Evans Rats. Values Listed are Concentration in Weight Per Cent, ± Standard Error of Mean, with Number of Follicles Analyzed (in parenthesis)

Animal	Sample preparation‡	Lumen diameter§ (μ) 10–40	41–69	70–100	>100	All follicles
1 (Li)*	A	0.82 ± 0.04 (13)	0.90 ± 0.03 (18)	0.99 ± 0.10 (7)	1.13 ± 0.30 (3)	0.91 ± 0.03 (41)
2 (Li)	A	0.90 ± 0.04 (12)	0.92 ± 0.05 (14)	0.96 ± 0.06 (9)	0.88 ± 0.05 (4)	0.92 ± 0.02 (39)
3 (Li)	C	1.07 ± 0.19 (7)	1.10 ± 0.09 (7)	1.25 ± 0.13 (7)	1.01 ± 0.10 (3)	1.12 ± 0.07 (24)
4 (Li)	C	1.17 ± 0.11 (6)	1.32 ± 0.08 (35)	1.23 ± 0.06 (3)		1.30 ± 0.06 (44)
5	B		1.08 ± 0.06 (23)	1.14 ± 0.06 (10)		1.10 ± 0.05 (33)
6	B	1.91 ± 0.15 (18)	2.17 ± 0.19 (27)	2.36 ± 0.29 (13)	2.10 ± 0.41 (4)	2.12 ± 0.11 (62)
7	B	1.75 ± 0.08 (6)	1.73 ± 0.06 (26)	1.81 ± 0.06 (16)	1.54 ± 0.25 (2)	1.75 ± 0.04 (50)

* Low iodine diet.

‡ A = frozen section, warmed, fixed with glutaraldehyde, stained. B = frozen section, lyophilized. C = frozen section, fixed with glutaraldehyde, lyophilized.

§ Diameters listed are uncorrected diameters; true diameters were not calculated, due to the consistency of iodine concentration observed among all follicles.

TABLE II

Iodine Concentrations in Eight Thyroid Samples Taken from the Same Animal and Prepared by Different Techniques. Values Listed are Concentrations in Weight Per Cent, ± Standard Error of Mean, with Number of Follicles Analyzed (in parenthesis)

Sample	Method of preparation*	Lumen diameter‡ (μ) 10–40	41–69	70–100	100	All follicles
1	E	1.73 ± 0.18 (6)	1.72 ± 0.06 (23)	1.90 ± 0.08 (16)	2.20 ± 0.25 (5)	1.83 ± 0.05 (50)
2	F	1.75 ± 0.08 (6)	1.73 ± 0.06 (26)	1.81 ± 0.06 (16)	1.54 ± 0.25 (2)	1.75 ± 0.04 (50)
3	B		1.93 ± 0.09 (24)	1.92 ± 0.07 (19)	1.80 ± 0.13 (6)	1.89 ± 0.06 (49)
4	B	1.74 ± 0.20 (7)	1.89 ± 0.08 (17)	2.36 ± 0.30 (18)	1.99 ± 0.14 (8)	2.05 ± 0.12 (50)
5	A		1.74 ± 0.11 (6)	1.83 ± 0.09 (17)	1.80 ± 0.08 (7)	1.80 ± 0.06 (30)
6	E		2.53 ± 0.20 (23)	1.96 ± 0.17 (19)	1.91 ± 0.34 (5)	2.23 ± 0.14 (47)
7	A	1.20 ± 0.09 (4)	1.48 ± 0.10 (18)	1.41 ± 0.06 (7)	1.26 ± 0.15 (2)	1.41 ± 0.06 (31)
8	A	1.78 ± 0.15 (6)	1.94 ± 0.06 (23)	1.97 ± 0.10 (18)	1.90 ± 0.14 (3)	1.93 ± 0.05 (50)

* A = frozen section, warmed, fixed with glutaraldehyde, stained. B = frozen section, warmed, fixed with glutaraldehyde, unstained. E = frozen section, fixed with glutaraldehyde, lyophilized. F = frozen section, lyophilized.

‡ Diameters listed are uncorrected diameters; true diameters were not calculated, due to the consistency of iodine concentration observed among all follicles.

was 10 kv, and the sample currents were 0.05–0.065 μamp. A beam diameter from 4 to 9 μ was used for the quantitative analysis, and a minimum spot size of approximately 1–2 μ was used for the scanning pictures.

Each follicle was counted for 5 min (300 sec) in 1-minute intervals. The standards were counted in a similar manner. Several follicles and the standards were counted for extended periods of time (10–15 min) to see whether the intensity was affected by continual bombardment. In this time interval no significant changes in intensity occurred. Background measurements were made by counting one degree off of the Bragg angle for both the follicles and the standards.

The standards used for the determination of iodine concentration in thyroid follicles were pellets pressed from purified powders of mono-iodotyrosine (weight per cent = 41.3) and the sodium salt of triiodothyronine (weight per cent = 56.25). Both compounds are components of the thyroglobulin within thyroid follicles. Any number of iodinated compounds, which approximate the biological matrix of the follicle, could have been used as standards (such as iodouracil, diiodotyrosine, etc.). These two were chosen because they were stable under bombardment by the electron beam, they contained known amounts of iodine, and they were homogeneous on a micron scale.

Absolute concentration values in the rat thyroid follicles were determined by using the average value of the intensity for the follicles in conjunction with intensity values and known concentrations of the standards. The mathematical relationship is $C_F = C_S \frac{I_F}{I_S}$ where C_F is the iodine concentration in the follicle, C_S is the known iodine concentration of the standard in weight per cent (i.e., $\frac{g}{100g}$); I_F is the intensity obtained from a follicle, and I_S is the intensity obtained from the standard. The concentration values calculated in this manner were then corrected for absorption differences between the sample and the standard by using the Duncumb and Shields (1966) modification of the Philibert (1962) technique. Atomic number corrections were not attempted in that the atomic number effect should be small, due to the biological molecules used as standards and in view of the fact that satisfactory data are not available for such corrections on biological material.

RESULTS

The results of the iodine analyses of thyroid samples from seven different animals are presented in Table I. The method of treating the initial frozen section is given along with the number of follicles analyzed in each of the size ranges listed. The last column in the table gives the value for all of the follicles combined. 40–50 follicles were analyzed for most of the sample sections, and the range of the lumen diameters was roughly 10–130 μ. Most lumen diameters were 20 μ or greater, however. The results are given for the follicles in each size category as concentrations of iodine in weight per cent with the associated standard errors of the means. The lumen diameters listed are uncorrected diameters. 50 follicles were analyzed at random from a section of each gland so that a considerable distribution in real follicle size was sampled. No attempt was made to correct the crude lumen diameter to its true diameter when it was observed that there was little difference in the stable iodine concentration among all of the follicles analyzed (SEM approximately 4%). Also, a statistical analysis of the probability of sectioning spheres at different diameters, such as explained by Nadler et al. (1954), shows that with the size categories listed (10–40 μ, 41–69 μ, 70–100 μ, and >100 μ), the greatest percentage of crude lumen diameters found in any given category would be contributed by follicles with true lumen diameters within that range.

The average iodine concentrations and the standard errors of the means are nearly the same for all size ranges for any given animal. When the Mann-Whitney U test (Siegel, 1956) was applied to the means for the different follicle size groups for any one animal, there were no significant differences ($0.1 < P < 0.4$). When the Spearman correlation coefficient (Siegel, 1956) was calculated for the follicle size vs. iodine concentration, the results indicated no correlation for all samples ($0.05 < P < 0.5$). For a sample from any given rat, the extremes of the range differed usually by only a factor of two, and these extremes usually represented only a few follicles of all those analyzed. For most follicles the range was much less, as can be seen by the standard error of the means. In two animals the three follicles that had the lowest concentration and the three follicles that had the highest concentration differed by a factor of three. Concentrations of all other follicles were within a factor of two. The other animal that showed a range differing by more than a factor of two had one follicle at the upper limit and one at the lower limit which differed by a factor of six. The next 3 follicles at the upper and lower limits

differed by a factor of three, and the other 42 follicles within a factor of two.

The iodine concentrations of several sections from the same animal prepared by different techniques are shown in Table II. As in Table I, the mean iodine concentrations are listed with the associated standard errors of the means for the four size ranges and for all analyzed follicles combined. The $\bar{Z}$ test and Mann-Whitney U test for differences in means indicated no significant differences except in one sample; this was sample No. 7 at the lower extreme of the range, which tested significantly different from all the other samples.

Sodium intensities, obtained at the same time as the iodine intensities for all of the follicles, were much more variable, ranging from approximately 2 to approximately 9% by weight in the frozen sections. In the sections that were warmed, fixed, and stained in aqueous solutions, all of the sodium was removed although the iodine concentration was unaffected.

A typical distribution pattern of iodine in a follicle (Fig. 1 *a*) shows the iodine apparently uniformly distributed over the entire colloid area. Fig. 1 *b* shows the scanning picture for phosphorus, which would be indicative of the epithelial cells lining the colloid. This allows one to locate the colloid material. After a phosphorus picture was taken, the beam was placed in static mode and stepped across a follicle as indicated by the white dots in Fig. 1 *c*. The corresponding iodine intensity (white trace) at each point was then plotted. The iodine concentration is fairly constant across the entire colloid area (approximately a 15% variation between upper and lower values).

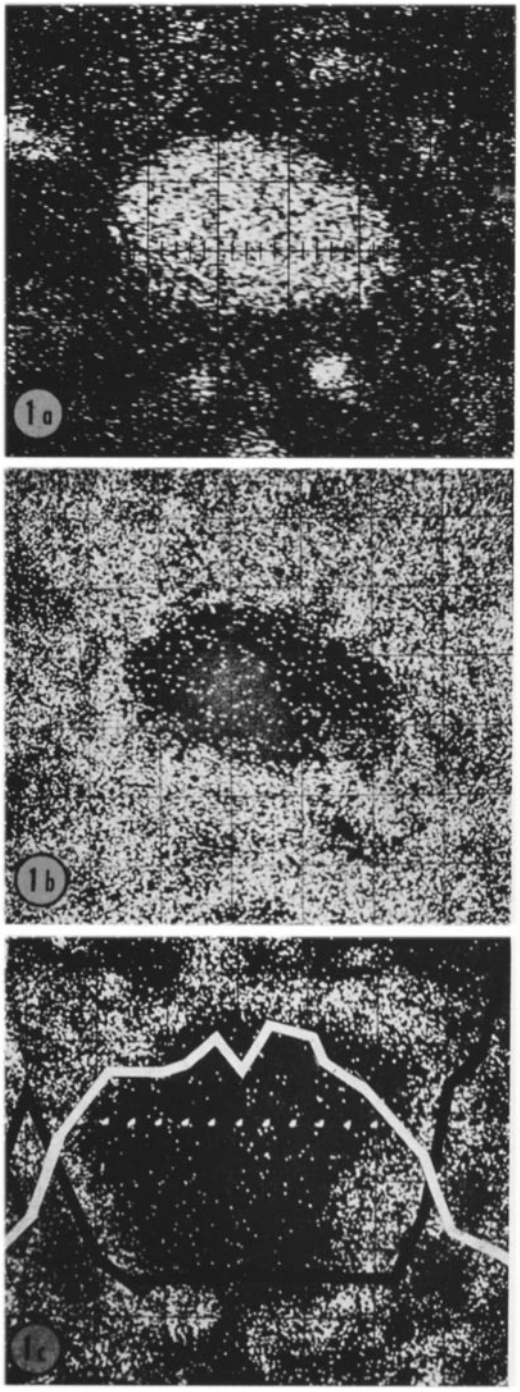

FIGURE 1 *a* Scanning picture of iodine in section of rat thyroid. One large follicle and two smaller ones are observable in the scanned sample area. White dots indicate a pulse from the detector produced by an I Lα X-ray or background radiation. × 500.

FIGURE 1 *b* Phosphate scan of same sample area as 1 *a*. Phosphate is indicative of epithelial cells lining the colloid. White dots indicate a pulse from the detector produced by a P Kα X-ray. The dots in the colloid containing region for this phosphate scan are due primarily to background radiation. × 500.

FIGURE 1 *c* Phosphate scan of a large follicle with the beam then put in static mode and stepped across the follicle (the white dots indicate beam position). The white trace is the iodine intensity corresponding to each beam position, and the dark trace is the phosphate intensity at each beam position. The resolution of the method can best be seen in the phosphate intensity. The iodine resolution near the cells is not as sharp due to underlying colloid material. × 500.

The dark trace is the phosphorus intensity corresponding to each beam position.

DISCUSSION

Microanalysis with the electron probe for iodine in thyroid glands provides a direct method for determining concentrations and distributions within individual follicles. The average iodine concentrations in a given gland varied little among the many follicles analyzed (SEM for 50 follicles is 4%) for animals that were either on high or on low iodine diets, even though the thyroid gland, when on low iodine supply, is in a much different metabolic state than is the case when a high supply of iodine is present. The upper and lower limits of concentration of iodine in the follicles of most animals differed by only a factor of two, although there were three exceptions. The majority of the follicles had iodine concentrations with a narrower range. Simon and Droz (1965) using isotopic equilibrium techniques analyzed about 30 follicles in two different animals and found a maximum difference of roughly a factor of two in the iodine concentration. Loewenstein and Wollman (1967) have stated that a difference of only a factor of two was observed in their isotopic equilibrium studies, except for some very small follicles (approximately 1%) which showed very intense radioautographic images. The concentration of iodine in these follicles relative to the other follicles was not stated. We did find the three cases where the range of concentration differed by more than a factor of two. However, we did not consistently find follicles with very high concentrations of iodine relative to other follicles. The maximum difference in concentration that was observed was a factor of six, but this was only one case.

The fact that the iodine concentration seems to be nearly the same for all follicles is of interest in connection with radioautographic data. The assumption (Nadler et al., 1954) that the total iodine concentration is the same in all follicles has been shown to be a good approximation. Therefore, it seems valid to relate the grain count from radioautographic data directly to specific activity over a given follicle. Observations from radioautographs that the iodine seems to be uniformly distributed in the follicle are confirmed by the electron-probe microanalysis.

With respect to possible hot-spot production (certain areas receiving higher doses) from the uptake of radioiodine by the thyroid gland (Clayton, 1953), a stable iodine concentration difference of nearly a factor of 10 in various follicles would be of considerable significance. This could lead to similar concentration differences in radioiodine upon chronic exposure to this radionuclide. If a threshold dose for radiation effects on the thyroid gland does exist, this would be extremely important. However, the present results show that the production of hot-spots would not occur from radioiodine uptake due to large stable iodine concentration differences per se. Differences in rate of uptake and release of radioiodine by different size follicles (Loewenstein and Wollman, 1967) would be a different problem.

Our absolute concentration values for dry colloid of 0.9–2% by weight can be compared indirectly with other values reported in the literature. Taurog et al. (1951) reported total iodine values for rats of 90 mg per cent wet weight. If one uses a concentration factor of 3–5 for drying, since this is the range observed for thyroid tissue and is typical of other tissues also (Anspaugh, L. R., unpublished data), and assuming that the colloid makes up 30–40% of the gland (40% is the maximum observed in mouse thyroids), then one can calculate that the iodine content of the colloid on a dry weight basis would be from 0.7 to 1.5%. The procedures employed in Taurog's study required the isolation of the colloid material. Values reported in the literature for the iodine content of thyroglobulin (19S), which requires centrifugation in several solutions, salting out procedures, and considerable dialysis for isolation, range from 0.2 to 1.1% by weight (Roche et al., 1968; Rolland et al., 1966). Only the results of one animal were reported in most cases, and in the one case where results from two animals were reported there was a difference of nearly a factor of two (0.66–1.13%). The fact that the electron-probe technique is the first analytical method that has allowed one to directly analyze the colloid material, thereby eliminating any prior isolation procedures, might account for the slightly higher values we report.

Individual animals exhibited a considerable variability in iodine concentration in thyroid follicles. The amount of iodine accumulated by the thyroid gland is known to be very dependent upon the environment and the health state of the animal. Although these animals were raised under similar conditions, a variation was still observed.

The influence of sample preparation on the sodium results points up one of the cautions that

must be observed in electron-probe microanalysis of biological samples. A bound ion (as, for example, iodine in this study) is in many cases not affected by the technique of sample preparation. However, considerable care must be taken to preserve the distribution of such mobile ions as sodium, potassium, and chlorine. Freezing and lyophilization of sections seems to be the technique of choice for such mobile ions.

In the quantitative aspects of electron-probe work, the usual method is to use as a standard a sample of the pure metal of the element being analyzed. Several corrections are then applied for differences in absorption and over-all matrix effects between the sample and the standard. However, in this study biological molecules containing a known quantity of iodine in their structure were used as standards. In this case the purified monoiodotyrosine and triiodothyronine are actual components of the colloid material. Therefore, the matrices of the sample and the standard are more nearly alike and the necessary corrections are considerably smaller. Also, these biological molecules with the element stoichiometrically involved in their structure provide one of the necessary requirements for an electron-probe standard, that is, homogeneity on a micron scale. Some molecules are more stable than others to bombardment by the electron beam. Many purified molecules and macromolecules exist and could serve as standards in biological analysis for such elements as P, Na, Cl, K, S, Mg, Cu, and Zn.

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