A STUDY OF THE BASEMENT MEMBRANE IN EXPERIMENTAL HYPERPLASIA OF THE RAT THYROID USING THE ELECTRON MICROSCOPE AND STEREOLOGICAL ANALYSIS

by

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required standard

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September, 1971

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ABSTRACT

Hyperplasia was induced by physical and chemical means. The resulting goitres were examined by electron microscopy, and the basement membranes were analyzed by stereological methods. The basement membrane in experimental colloid goitre was found to be significantly thinner than its control. A consideration of the course of the various goitres and of the chemical nature of basement membranes leads to the conclusion that the thinning of the basement membrane was the result of stretching.

Table Of Contents

List Of Tables

List Of Figures

List Of Figures, cont.

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Special mention must be made of the contribution of Stephen Borden and Frederick Glick to this thesis and to the field of stereology. Inspired, no doubt, by the non-ichthyo-ecological nature of the subject matter, they good-humouredly provided the stereological analysis used in this study.

I ask all these people to accept my thanks.

Introduction

The basement membrane is the moderately electron-dense muco-protein sheet that underlies all epithelial tissues. Histochemical reactions indicate its chemical composition. A positive reaction with periodic acid-Schiff where the basement membrane is very thick, and with periodic acid-silver-methenamine where it is thinner, demonstrate the carbohydrate moiety. Conjugated antibody reactions demonstrate a protein component.

The basement membrane is important to developing and mature tissues. For embryonic tissues, it provides a framework, giving strength and orientation in development (Menefee, 1957; Cohen, 1961); for mature tissues it provides a framework on which conformational integrity may be maintained (Pease, 1958). As the membrane lies between a tissue and the circulation, it also acts as a filter, and considerable work has been done to determine the size of molecules that can penetrate the basement membrane (Gelke *et al.*, 1966a, 1966b).

In certain pathological conditions, the basement membrane undergoes interesting changes. In experimental scurvy, basement membranes of synovial capillaries become thin and tenuous (Friederici *et al.*, 1966). In Hashimoto's thyroiditis, the basement membrane changes from the thick and seemingly multi-layered structure in the normal gland to a much thinner membrane in the diseased gland (Irvine and Muir, 1963). In nephrosis, the glomerular basement

membrane becomes very irregular with large deposits of similar material scattered along its length (Alousi et al., 1969).

Often, these basement membrane changes occur in a condition that features a concomitant hyperplasia of the affected tissue. The purpose of this study was to induce an experimental hyperplasia in some tissue in a laboratory animal and then investigate possible changes in the basement membrane of the altered tissue. The thyroid was chosen because it is a tissue in which hyperplasia can be easily induced. The thyroid is under the control of the pituitary hormone, thyrotropin (TSH). When thyroid hormone metabolism is inhibited, the level of TSH increases, causing the gland to increase in size. This aspect of thyroid metabolism can be effected by surgical removal of part of the gland or by administration of chemicals that interfere with the gland's production of iodinated proteins (Turner, 1971).

Materials and Methods

Male rats of the Wistar strain from the colony maintained in the U. B. C. vivarium were used in this study.

The animals were kept in separate pans in mesh-covered racks. Water and food (U. B. C. ration #66) were fed ad libitum.

Induction of Hyperplasia

Hyperplasia was induced by physical (surgical) and by chemical (goitrogenic) means.

Physical -- Hemithyroidectomy. The animals were anesthetized with sodium pentobarbital at 40 mg / kg of body weight, given i.p. The thyroid was exposed by central incision, the right lobe gripped with forceps, the tissue eased off the trachea with a needle, and the isthmus cut with scissors. The incision was closed with surgical (000) silk and smeared with an antibiotic ointment. The remaining lobe was taken for microscopy eight days later. Sham operations were performed to test the effect of the surgical procedure on the thyroid. The same procedure was followed, but no tissue was removed. These animals were maintained under the same conditions as the animals with hemi-thyroidectomies. Both lobes of the sham-operated thyroids were taken for microscopy eight days later. Controls (no treatment at all) were established to check the effects of fixation, embedding, and seasonal variation on the thyroid. The controls were maintained under laboratory conditions for eight

days prior to collection of tissue for microscopy.

Chemical -- Parenchymatous Goitre. Parenchymatous goitre was induced by feeding 5% potassium perchlorate ($KClO_4$) mixed with ground feed (U. B. C. ration #66). This mixture was allowed ad libitum. Tissue for microscopy was taken after 30 days of this treatment. Estimated average dose was 0.6 g $KClO_4$ / day. Control animals were given ground feed with no additives for 30 days, after which tissue was taken for microscopy.

Chemical -- Colloid Goitre. Colloid goitre was induced by first causing parenchymatous goitre (20 mg of 6-n-propylthiouracil (PTU) in olive oil, sub-cutaneously, per day for 30 days) and then leaving the animals untreated for 14 days. Some animals were sacrificed without a recovery period to check that PTU had induced a parenchymatous goitre. Tissue for microscopy was taken at these times (after 44 days for colloid and after 30 days for parenchymatous). Tissue from untreated animals was taken at 44 days and at 30 days.

Fixation and Embedding

The animals were anesthetized with an overdose of sodium pentobarbital (80 mg / kg body wt.), i.p. The thyroid was exposed and flooded with buffered 6% glutaraldehyde (pH 7.4). The portion of the trachea with the gland adhering was removed to a petri dish containing more fixative. The thyroid was dissected into cubes 1 mm^3 and placed in vials containing fresh glutaraldehyde.

The tissues, except for the tissue from the parenchymatous goitre and its control, were then post-fixed in osmium tetroxide (pH 7.4), after which they were dehydrated in graded ethanols and propylene oxide, and finally embedded in Epon 812.

Microscopy

Thick sections (1 u) for light microscopy were cut manually on an LKB microtome, affixed to glass slides by heating in a Bunsen flame, and stained with 1% methylene blue in sodium borate. The slides were examined and photographed on a Zeiss microscope. Measurements of follicle diameter and cell width were made on prints with a final magnification of 710 X.

Thin sections (less than 500 A) for electron microscopy were cut on an LKB microtome, mounted on neoprene-coated copper grids (Kushida and Fujita, 1964), and stained with uranyl acetate and lead citrate. The sections were examined in a Hitachi HU-11A electron microscope with accelerating voltage of 50 kV. Micrographs were taken at an initial magnification of 19,000 X. Measurements were made on prints enlarged to 53,000 X.

Light Microscopy

All measurements were made on prints with a final magnification of 710 X. Only those follicles that could be considered as having been sectioned near the middle of the follicle were measured. To be near the middle, the lumen must be visible, and the circle of cells must be a monolayer. This second criterion would be met by

a single row of nuclei and no changes in colour or texture of the cytoplasm that might indicate that two cells had been sectioned through at an angle. Frequency distributions of follicle diameter were plotted. Cell width (the distance between lateral cell membranes as judged by colour and texture differences) was measured at the narrowest part of the cell in each case. Several cells in several different follicles were measured and the means computed. Cell height (the distance from the base of the cell to the apex) was measured at four points on suitable follicles, at the points of maximum and minimum follicle diameter. Means were computed.

Electron Microscopy

Measurements of basement membrane thickness were made on prints with a final magnification of 53,000 X. Magnification on the microscope was verified by comparison with standard specimens. Twenty micrographs representing twenty different follicles were used. Measurements were taken at 1-micron intervals perpendicular to the basal cell membrane with a measuring magnifier that further magnified the membrane 8 X. Measurements were made to the nearest 0.05 mm. Means were calculated for each follicle, converted to angstroms, and ranked. Frequency distributions were plotted. Means, standard deviations, medians, and inter-quartile ranges (IQR) were calculated. T-tests were made to test for differences between the means of means for each condition and its control.

Results

Goitre Production

The usual method of judging the extent of hyperplasia in experimental goitre prior to histological examination is weighing the excised glands and comparing the weights with weights of control glands. This procedure was considered incompatible with satisfactory fixation for electron microscopy. Therefore, the goitres were judged subjectively before excision and were ranked. The severity of the goitre is described by a series of plus signs. Size and colour of gland were both used as indicators. The most severe goitre was chosen for electron microscopical examination. In that instance where two goitres appeared equally severe, the gland selected was from the animal that seemed healthier, on the basis of body weight, weight gained, food intake, and behaviour. These data are summarized in Table I. The gland selected for electron microscopy is marked by an asterisk.

Micrographs of typical portions of the various tissues are shown in Figs. 1 - 3.

Light Microscopy

All light microscopy was done on the same tissue that was chosen for electron microscopy.

All measurements for follicle diameter were taken along the longest axis of the follicle and were made on only those follicles that met the criteria previously described. No significant differences between experimental

Table I. Condition of Experimental Animals and Sizes of Goitres.

Experiment	Final body wt. (g)	Wt. gain (g)	Food intake (g/day)	Behaviour (subjective)	Goitre size (subjective)	
Hemithyroidectomy	190	33	18.5	normal	++	*
	180	22		"	++	
	170	15		"	+	
Sham operated	190	40	20.5	normal	0	
Control	240	50	25.5	good	0	*
	184	30		"	0	
Parenchymatous	150	75		nervous	++++	*
	116	41		"	+++	
	90	15		nervous & staggering	+++	
Control	235	160		good	0	*
	208	133		"	0	
	242	167		"	0	
Colloid Goitre						
Parenchymatous (15 days)	182	6	12.0	listless	+++	
	121	3	9.1	"	+++	
Parenchymatous (30 days)	160	53	11.4	listless	+++	
	224	55	16.4	"	+++	
Colloid	263	120	15.8	improved	++++	*
	309	127	19.3	"	+++	
Control	333	60	22.4	good	0	*

Fig. 1 Typical thyroid follicles -- hemithyroidectomy.

a. Hemithyroidectomy

b. Sham-operated

c. Control

Alkaline methylene blue. 710X

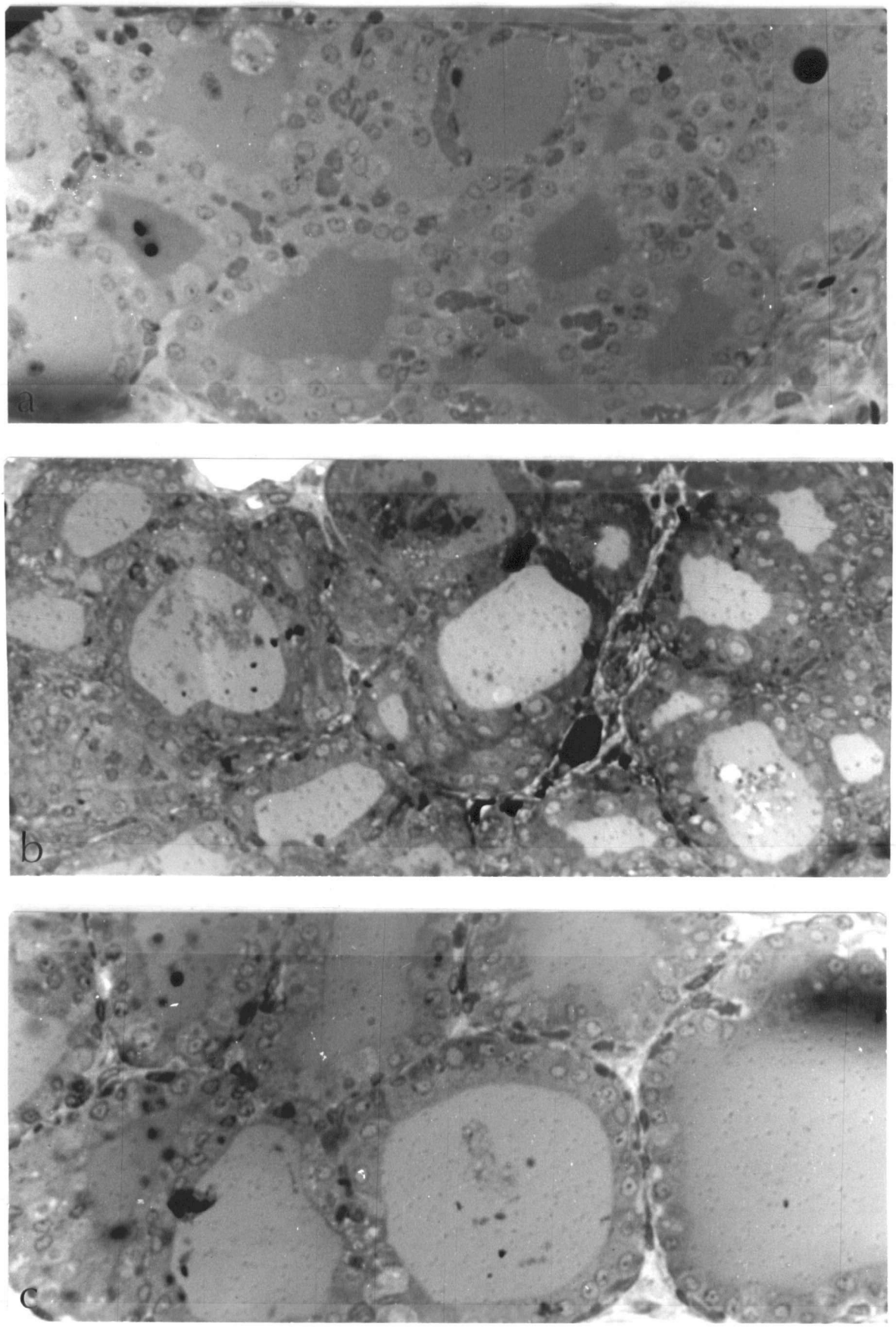

a
b
c

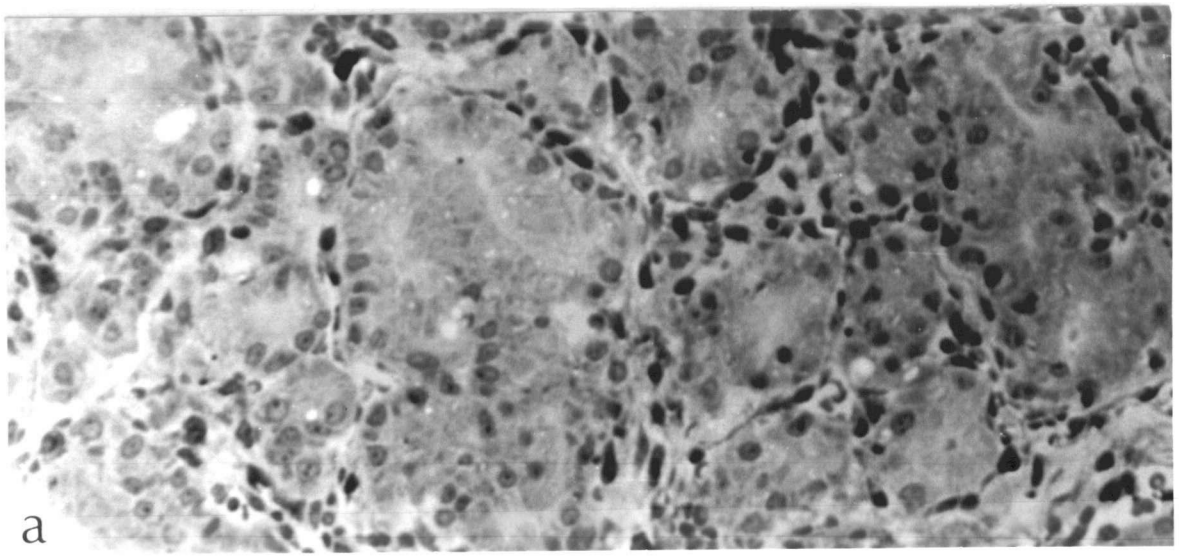
Fig. 2 Typical thyroid follicles -- parenchymatous goitre.

a. Parenchymatous goitre

b. Control

Alkaline methylene blue. 710X

Note: increased cell height in parenchymatous goitre (a)



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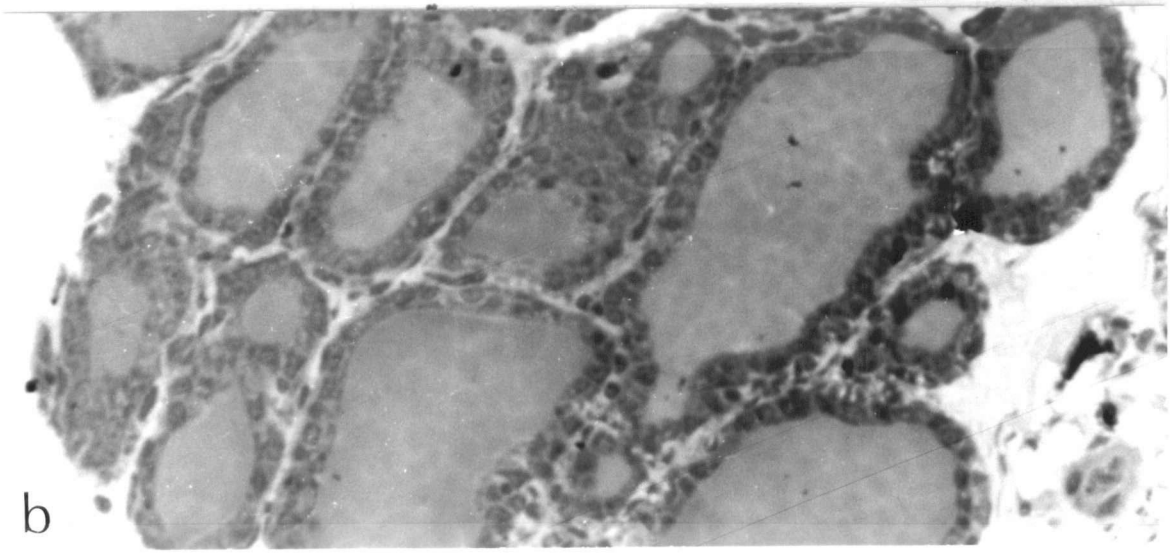


b

Fig. 3 Typical thyroid follicles -- colloid goitre.

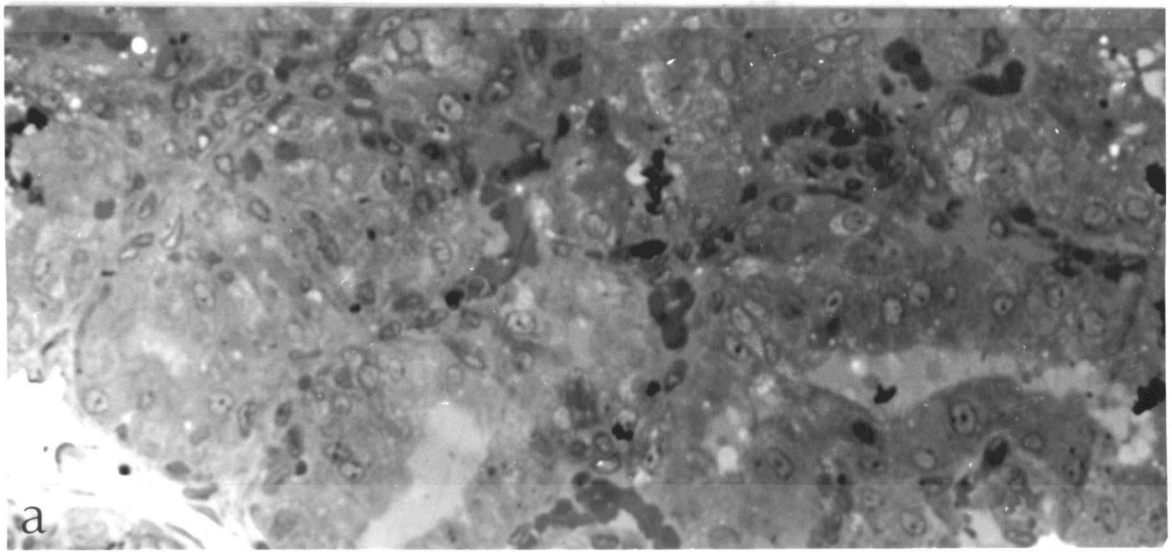
a. Parenchymatous goitre -- 30 days

b. Colloid goitre

c. Control

Alkaline methylene blue. 710X

Note increased cell height in parenchymatous goitre (a) and lower (cuboidal) cell height in colloid goitre (b)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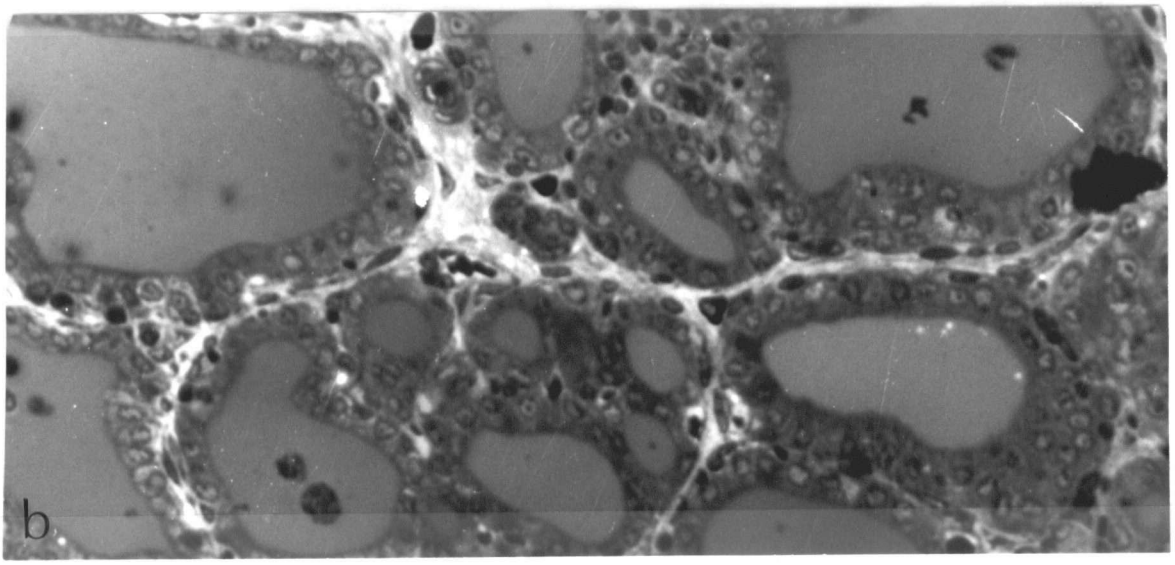
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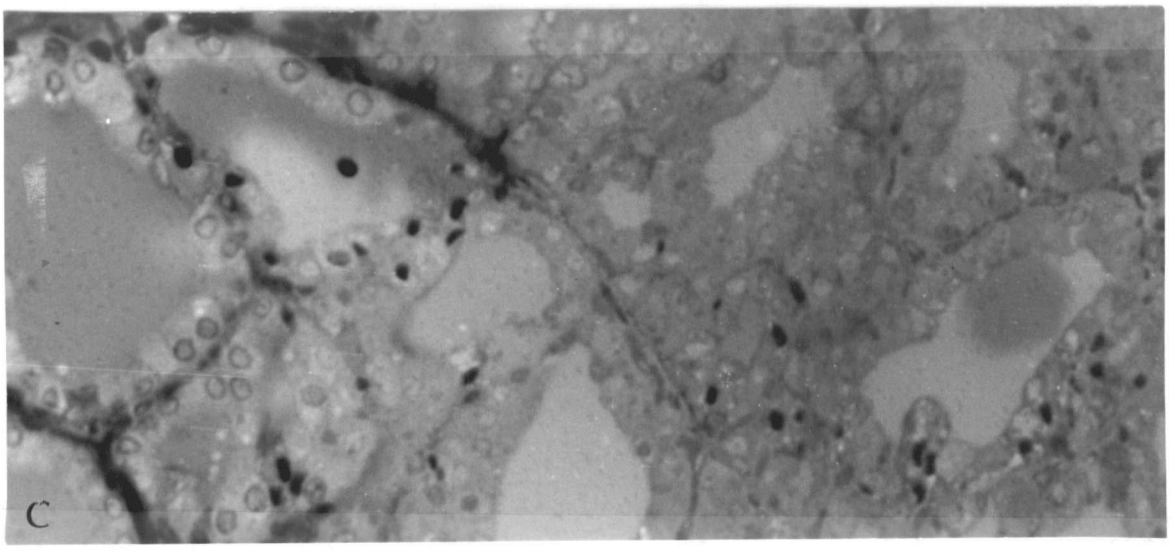

c

and control tissues were found, nor was there any difference between test tissues (the sham operations for hemithyroidectomy and parenchymatous goitre for colloid goitre) and their controls. The follicle diameter means, variances, and standard deviations are summarized in Table II. Frequency distributions of follicle diameters with a class size of 0.01 mm are shown in Figs. 4 - 11.

The cell heights give a good estimate of the severity of the goitres. These measurements are summarized in Table III. There is a definite increase in cell height (from cuboidal cells to columnar cells) for the ClO_4F (parenchymatous) goitre and for the parenchymatous goitre preceding colloid goitre. The cell height in colloid goitre can be seen to be similar to its control. Hemithyroidectomy did not make any significant difference in cell height in the remaining lobe. The sham operations had no effect on cell height.

Cell widths were obtained for the six tissues to be examined by electron microscopy. These measurements are summarized in Table IV.

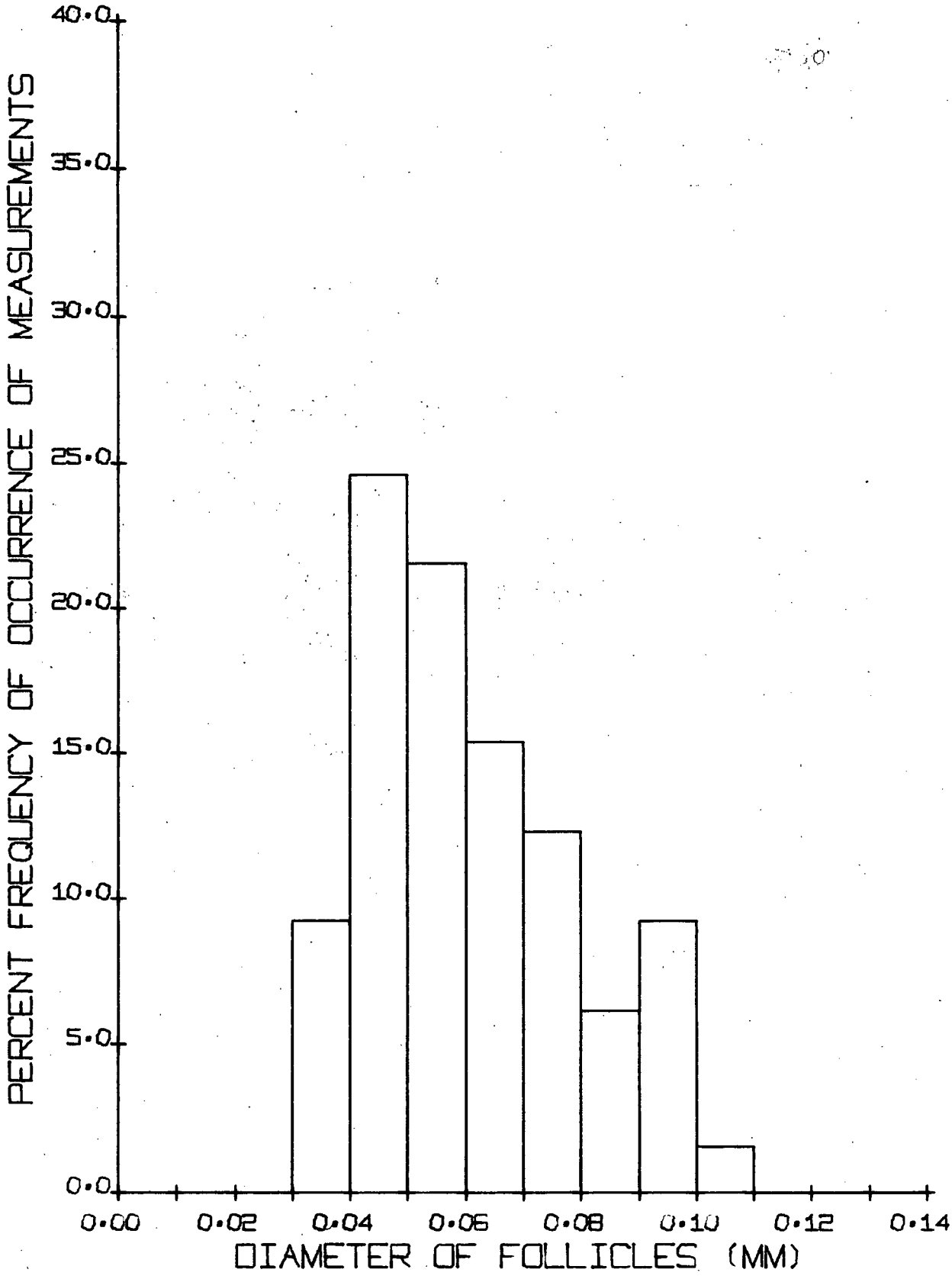
Electron Microscopy

Measurements of basement membrane thickness were obtained on 20 micrographs representing 20 different follicles. A mean basement membrane width was obtained from each micrograph. Table V summarizes these measurements and presents a mean of means for each tissue. Also shown in Table V are the results of t-tests done to

Table II. Means, Standard Deviations, and Variances for Follicle Diameters.

Treatment	No.	Mean (cm)	Variance	Standard deviation
Hemithyroidectomy				
Sham	111	0.0442	0.0002	0.0149
Rt. Lobe	65	0.0606	0.0003	0.0179
Control	57	0.0593	0.0003	0.0198
Parenchymatous Goitre				
ClO_4F	70	0.0368	0.0001	0.0104
Control	148	0.0434	0.0003	0.0186
Colloid Goitre				
Parenchymatous	139	0.0502	0.0003	0.0185
Colloid	431	0.0392	0.0002	0.0164
Control	75	0.0535	0.0003	0.0199

Fig. 4 Percent frequency distribution of follicle diameter -- hemithyroidectomy.


PERCENT FREQUENCY OF OCCURRENCE OF MEASUREMENTS
40·0
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0·0
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0·02
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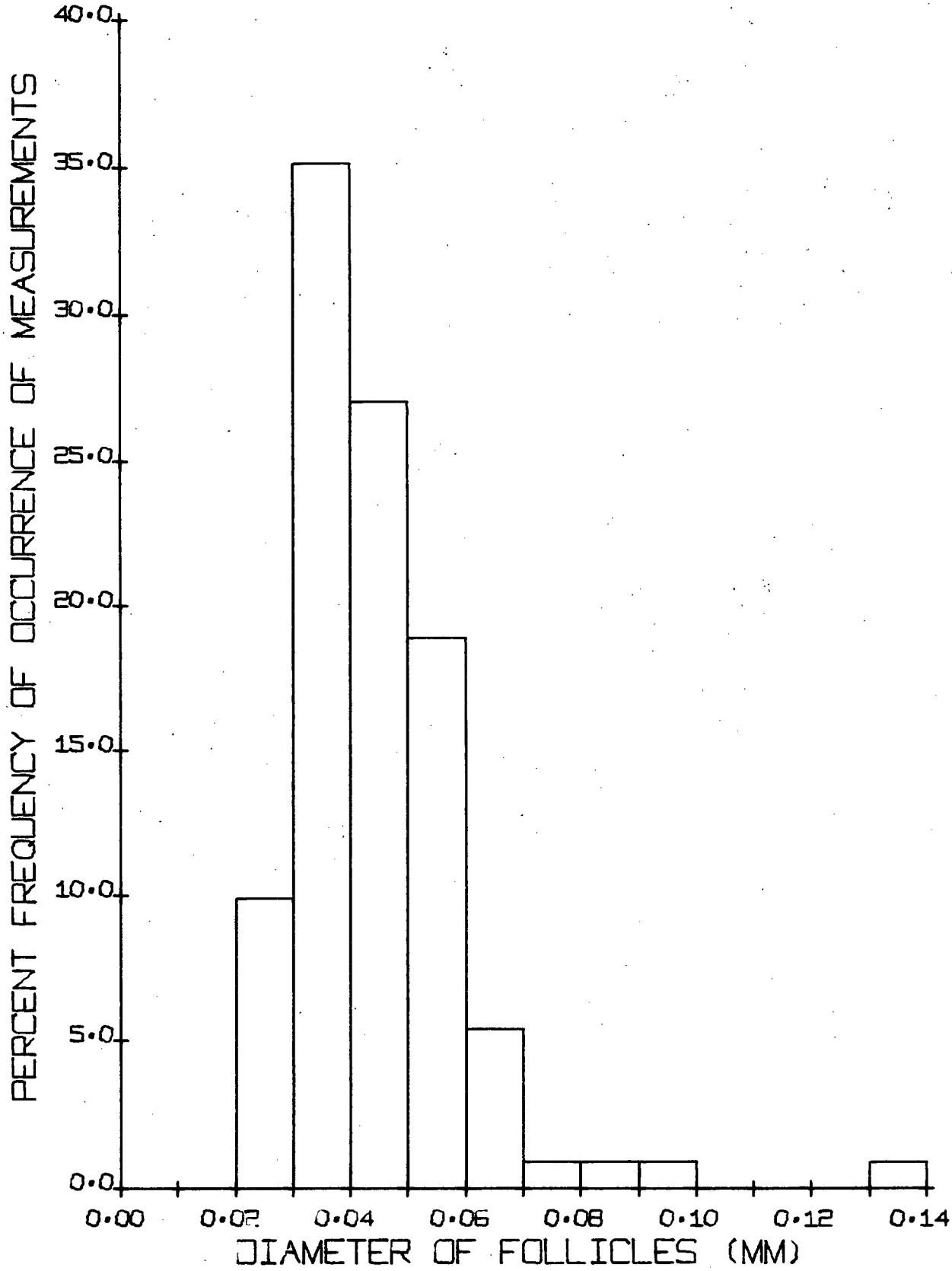
0·08
0·10
0·12
0·14
DIAMETER OF FOLLICLES (MM)

Fig. 5 Percent frequency distribution of follicle diameter -- sham-operated.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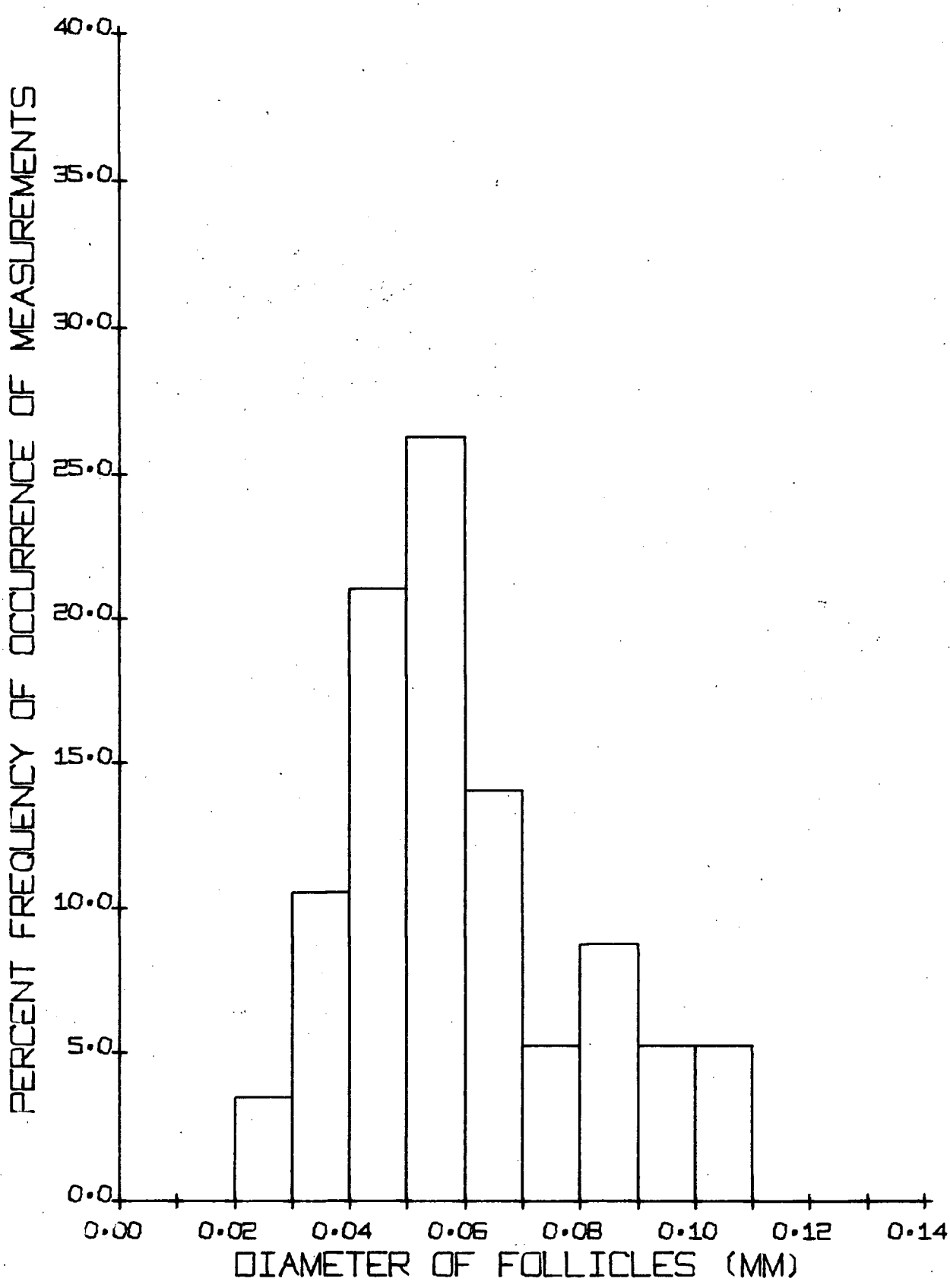
DIAMETER OF FOLLICLES (MM)

Fig. 6 Percent frequency distribution of follicle diameter -- control for hemithyroidectomy.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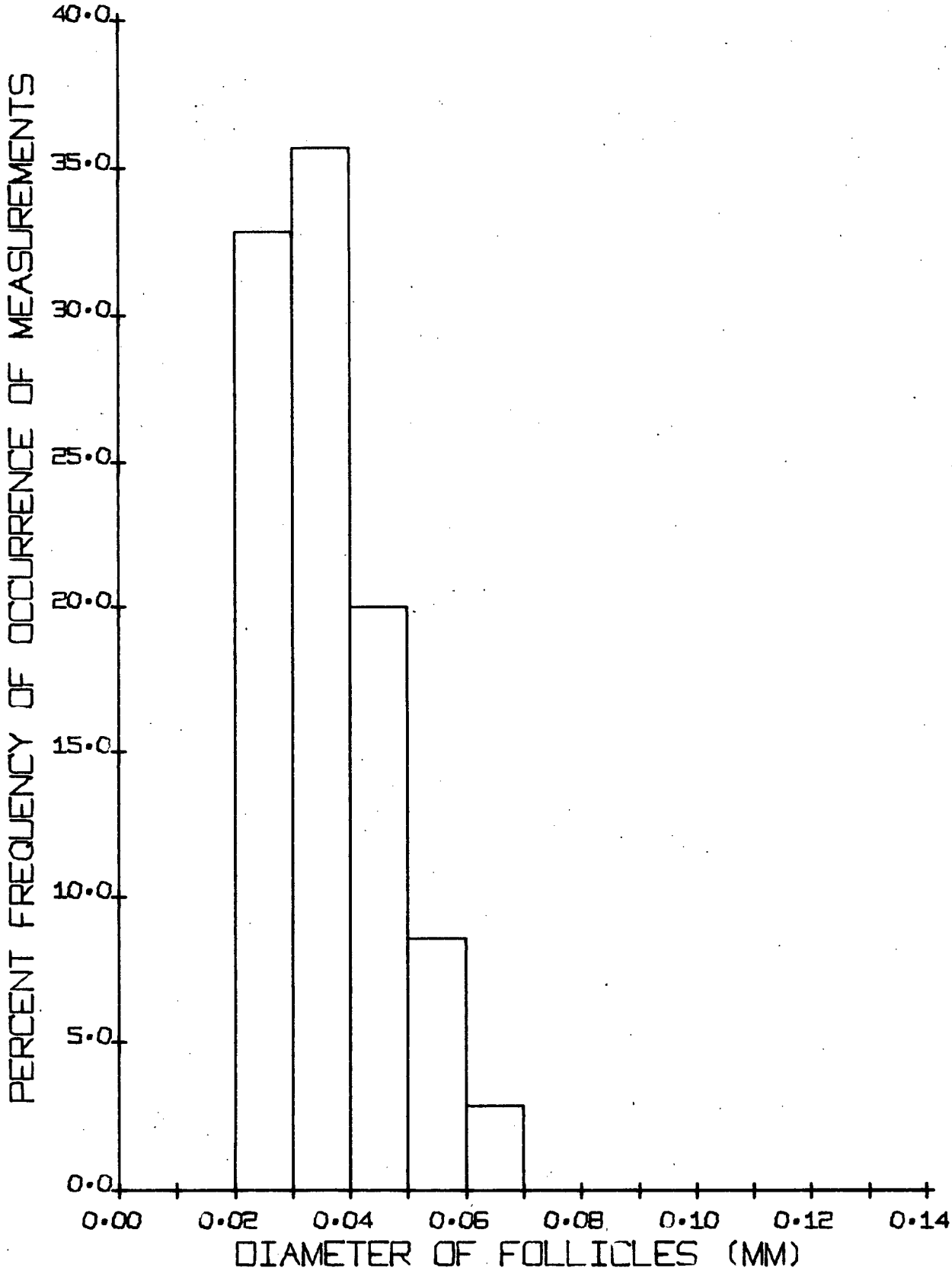
DIAMETER OF FOLLICLES (MM)

Fig. 7 Percent frequency distribution of follicle diameter -- parenchymatous goitre.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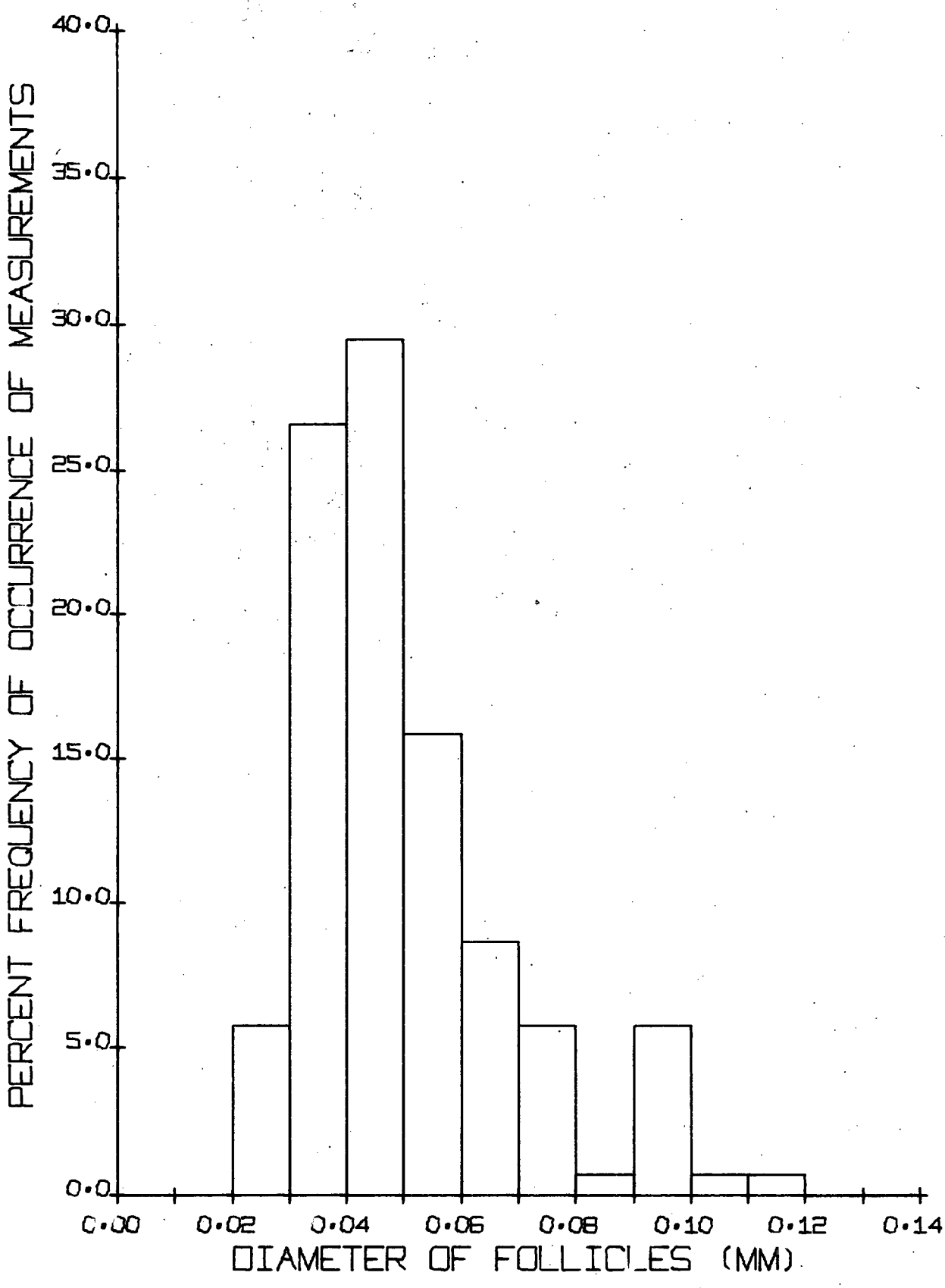
DIAMETER OF FOLLICLES (MM)

Fig. 8 Percent frequency distribution of follicle diameter -- control for parenchymatous goitre.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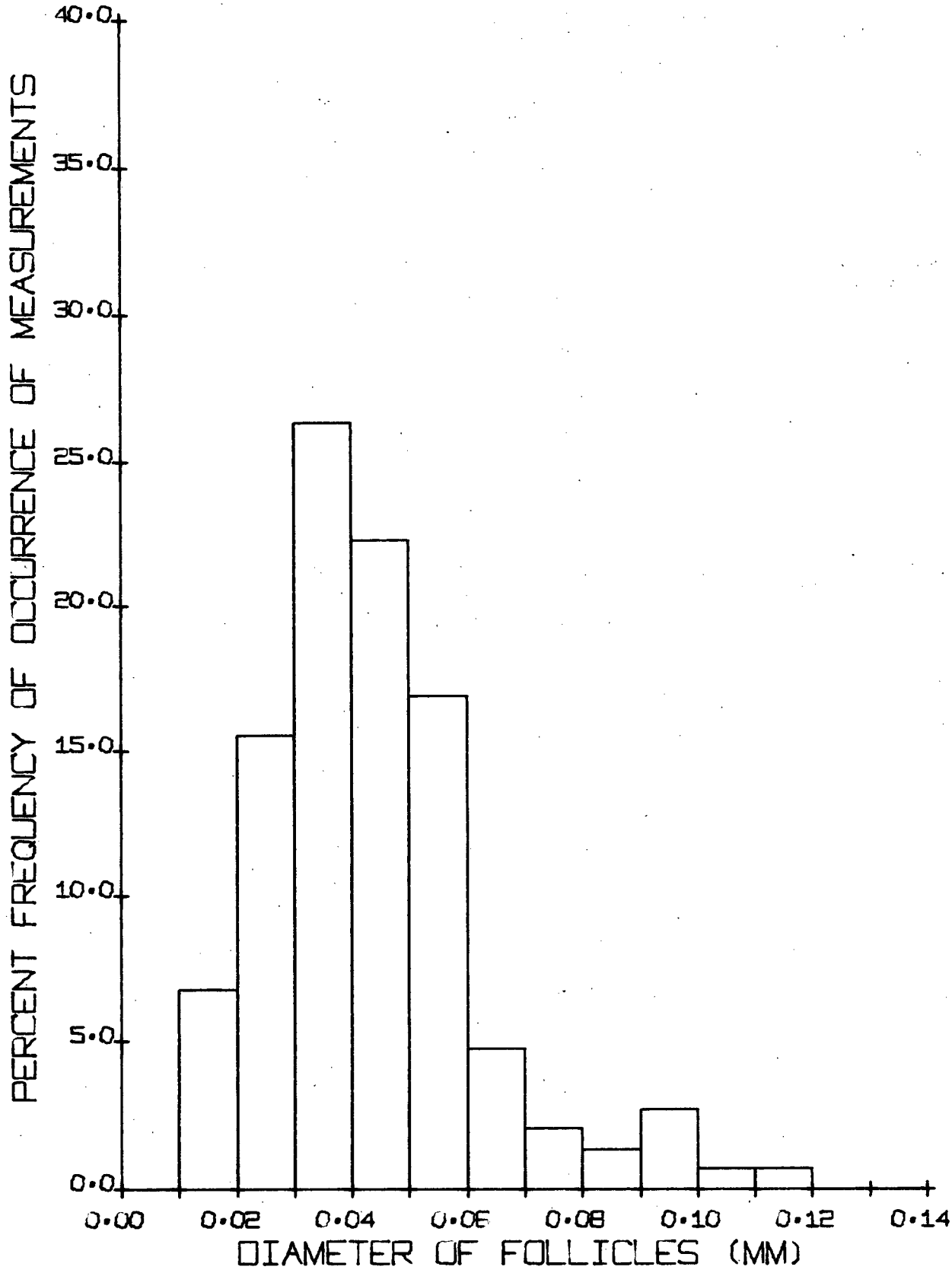
DIAMETER OF FOLLICLES (MM)

Fig. 9 Percent frequency distribution of follicle diameter -- parenchymatous goitre preceding colloid goitre.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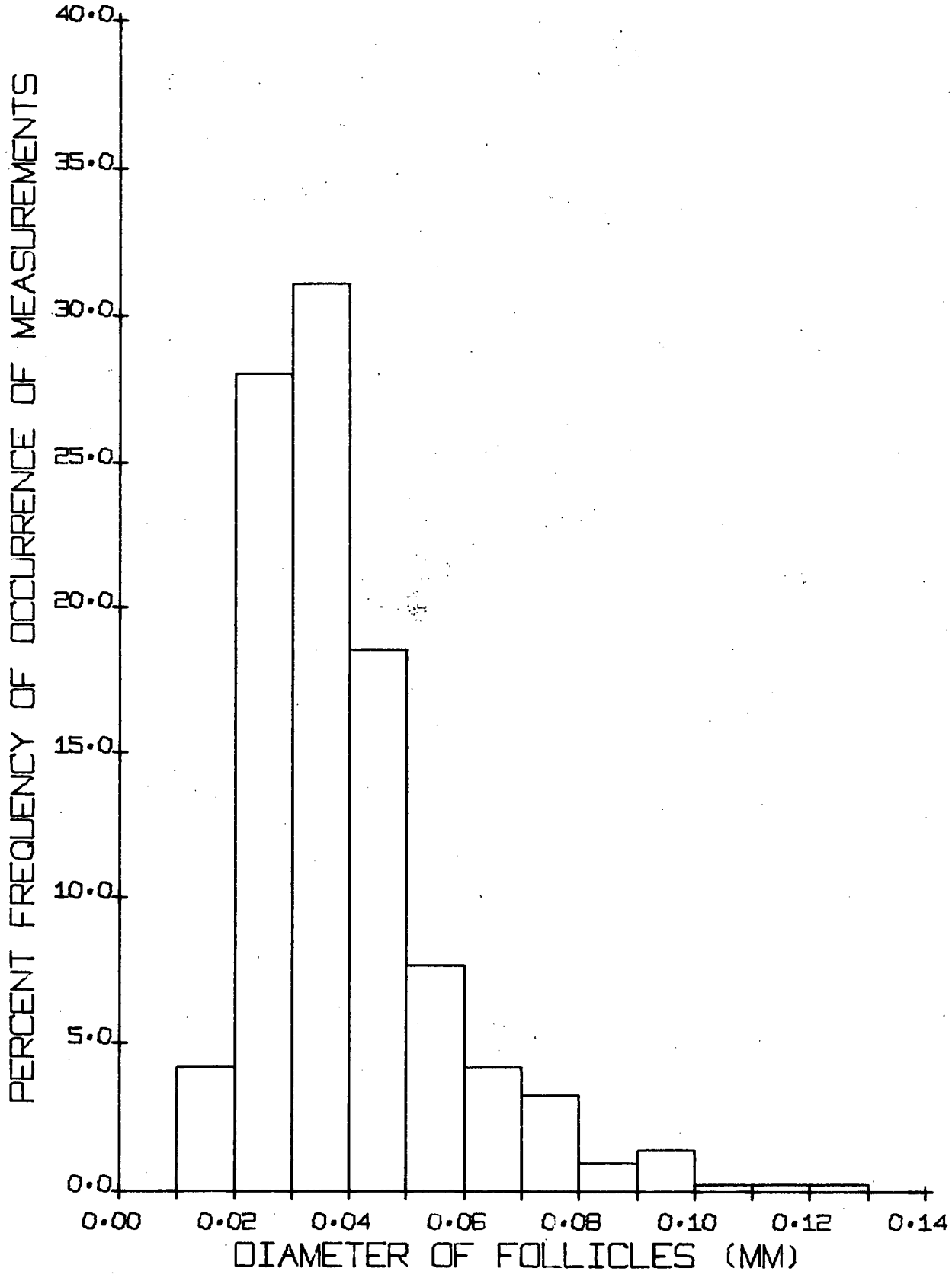
DIAMETER OF FOLLICLES (MM)

Fig. 10 Percent frequency distribution of follicle diameter -- colloid goitre.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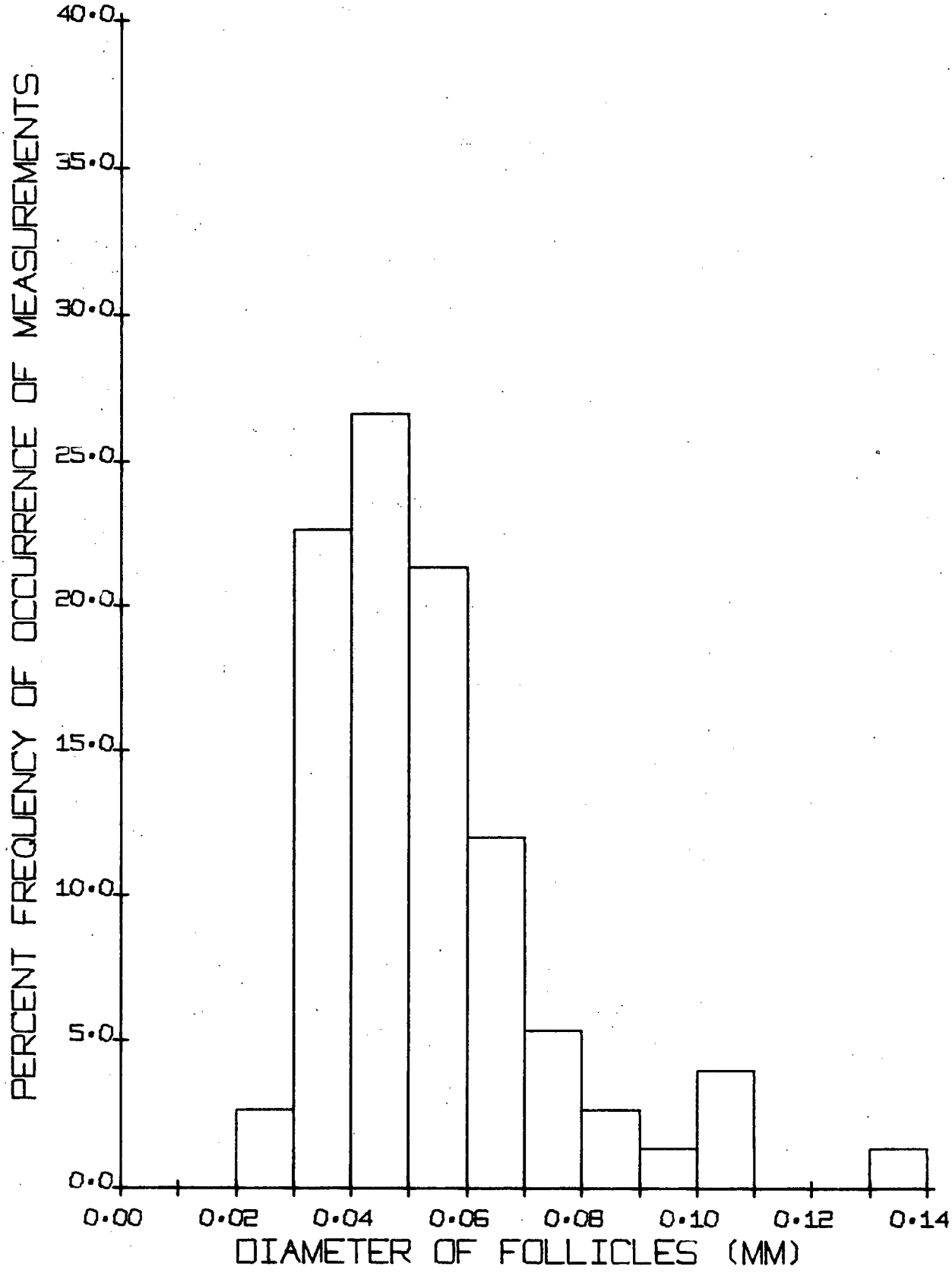
DIAMETER OF FOLLICLES (MM)

Fig. 11 Percent frequency distribution of follicle diameter -- control for colloid goitre.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0·12
0·14
DIAMETER OF FOLLICLES (MM)

Table III. Means, Variances, and 5% Confidence Intervals for Cell Heights.

Treatment	$\bar{X}$ (mm)	s^2	5% C. I.
Hemithyroidectomy			
Sham	0.005343	0.002874	0.004889 0.005797
Right Lobe	0.0069749	0.0048748	0.0063455 0.0076044
Control	0.006571	0.003635	0.005921 0.007221
Parenchymatous Goitre			
ClO_4F	0.009789	0.007770	0.0087599 0.018196
Control	0.004503	0.003125	0.004103 0.004903
Colloid Goitre			
Parenchymatous	0.011516	0.009486	0.016868 0.012346
Colloid	0.004797	0.002577	0.0043488 0.005244
Control	0.0066325	0.004986	0.005916 0.007349

Table IV. Cell Widths.

Treatment	Mean Cell Width (u)	±	Standard Deviation
Hemithyroidectomy	4.4		0.8
Control	6.4		1.1
Parenchymatous Goitre	4.2		1.0
Control	7.2		1.2
Colloid Goitre	7.4		1.1
Control	4.9		0.9

Table V. Measurements of Basement Membrane Widths.

Ranked means and t-tests for difference between means of means.

Hemithyroidectomy		Control	
304.27	395.04	298.69	417.86
307.69	400.15	305.66	422.90
349.78	425.31	319.70	445.01
370.32	427.56	350.57	445.75
371.09	427.79	360.20	446.54
375.18	436.55	375.26	451.88
377.35	443.39	392.45	479.41
381.78	447.52	399.09	499.99
388.17	451.57	405.65	503.42
388.45	474.97	415.09	567.48

Mean = 397.20
Variance = 2076.679

Mean = 415.13
Variance = 4868.311

Probability = 0.31723
Difference between means of means is not significant.

Colloid Goitre		Control	
262.73	346.55	332.76	424.03
279.68	352.42	338.05	432.69
283.87	358.57	359.97	433.95
285.30	366.40	372.23	441.54
291.02	373.58	376.02	444.62
309.19	380.78	381.44	448.11
314.68	400.94	383.99	464.74
317.21	436.36	399.71	488.78
329.63	437.29	402.48	496.46
331.27	479.76	419.44	575.47

Mean = 346.89
Variance = 3453.773

Mean = 420.82
Variance = 3401.060

Probability = 0.00028
Difference between means of means is highly significant.

Table V. cont.

Parenchymatous Goitre		Control	
350.48	486.49	383.41	512.26
356.13	486.59	425.53	520.83
376.60	489.44	443.84	538.78
387.69	503.88	445.71	547.16
394.50	504.22	447.83	553.06
436.79	540.56	451.92	561.69
438.67	553.39	464.95	561.89
440.25	578.44	472.32	578.01
444.12	637.09	473.69	581.43
475.47	653.23	499.99	596.91

Mean = 476.70
Variance = 7350.061

Mean = 503.06
Variance = 3665.678

Probability = 0.26842
Difference between means of means is not significant.

test for differences between the means of means for each experimental tissue and its control.

Frequency distributions of the means were plotted and are shown in Figs. 12 - 17. The class size of 100 A is that generally used in this type of work.

No difference in the basement membrane width between the hemithyroidectomy and its control was found, nor was there any difference in parenchymatous (ClO_4F) goitre. A highly significant difference was found for colloid goitre ($p = 0.00028$). No other change in ultrastructure was found; the basement membrane had remained a continuous and fairly uniform sheath throughout. Electron micrographs of representative conditions are shown in Figs. 18 - 20.

Stereological Analysis

The basement membrane that is considered in this study is essentially the coating of a sphere. The most accurate measurement of this basement membrane would be made on a section that cut through the exact middle of the follicle. However, finding the exact middle would require serial sections and such an approach would be impractical. Therefore, random sections were used in this study. Most of these sections will not pass through the middle of the follicle, and the true width of the basement membrane will be masked not only by error but also by bias, the effect of sectioning away from the middle of the follicle. It is essential to get an estimate of bias in order to make an accurate inference of the true width of the basement membrane.

Fig. 12 Frequency distribution of means of basement membrane widths -- hemithyroidectomy.

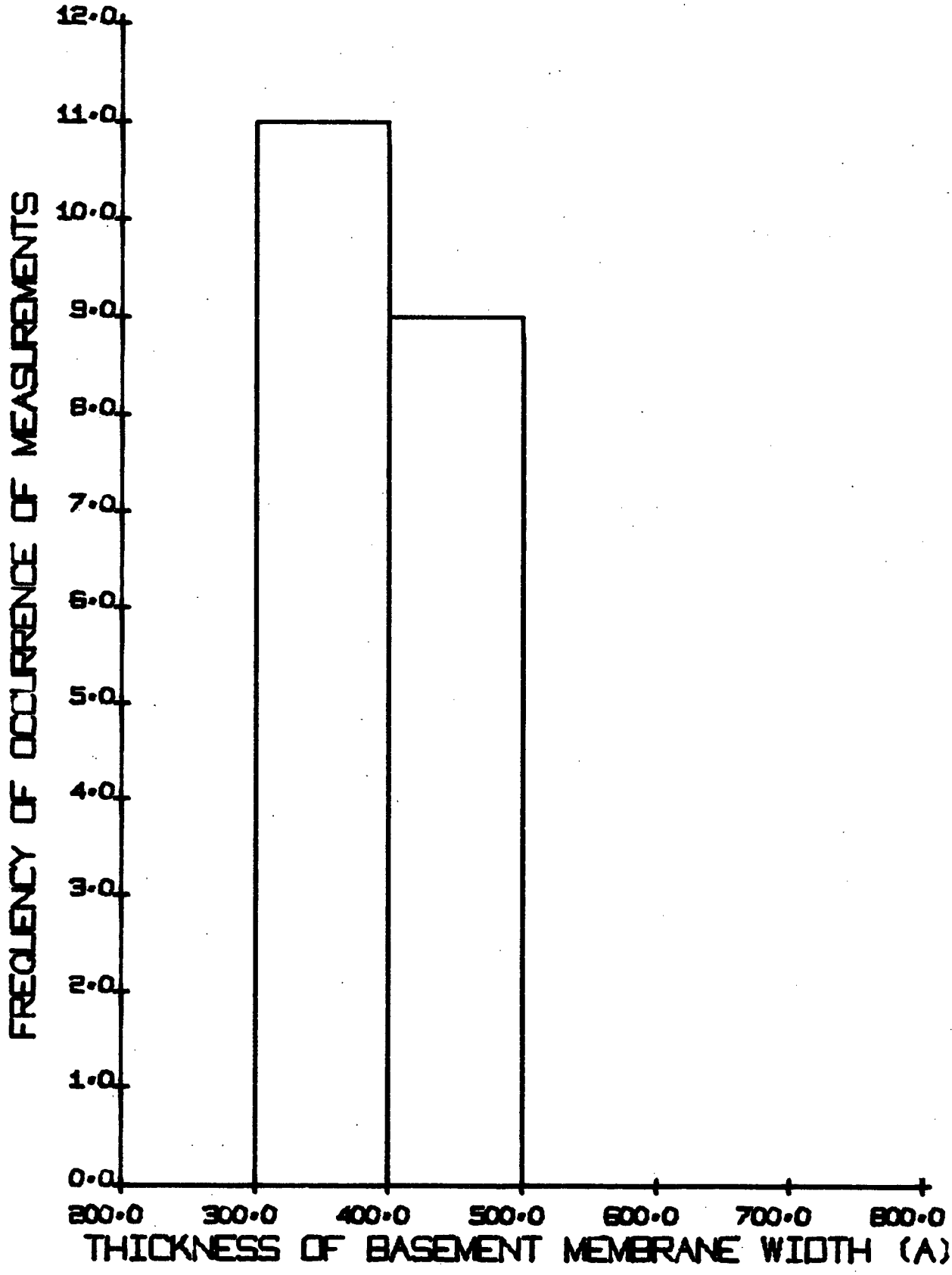

FREQUENCY OF OCCURRENCE OF MEASUREMENTS
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
200.0
300.0
400.0
500.0
600.0
700.0
800.0
THICKNESS OF BASEMENT MEMBRANE WIDTH (A)

Fig. 13 Frequency distribution of means of basement membrane widths -- control for hemithyroidectomy.

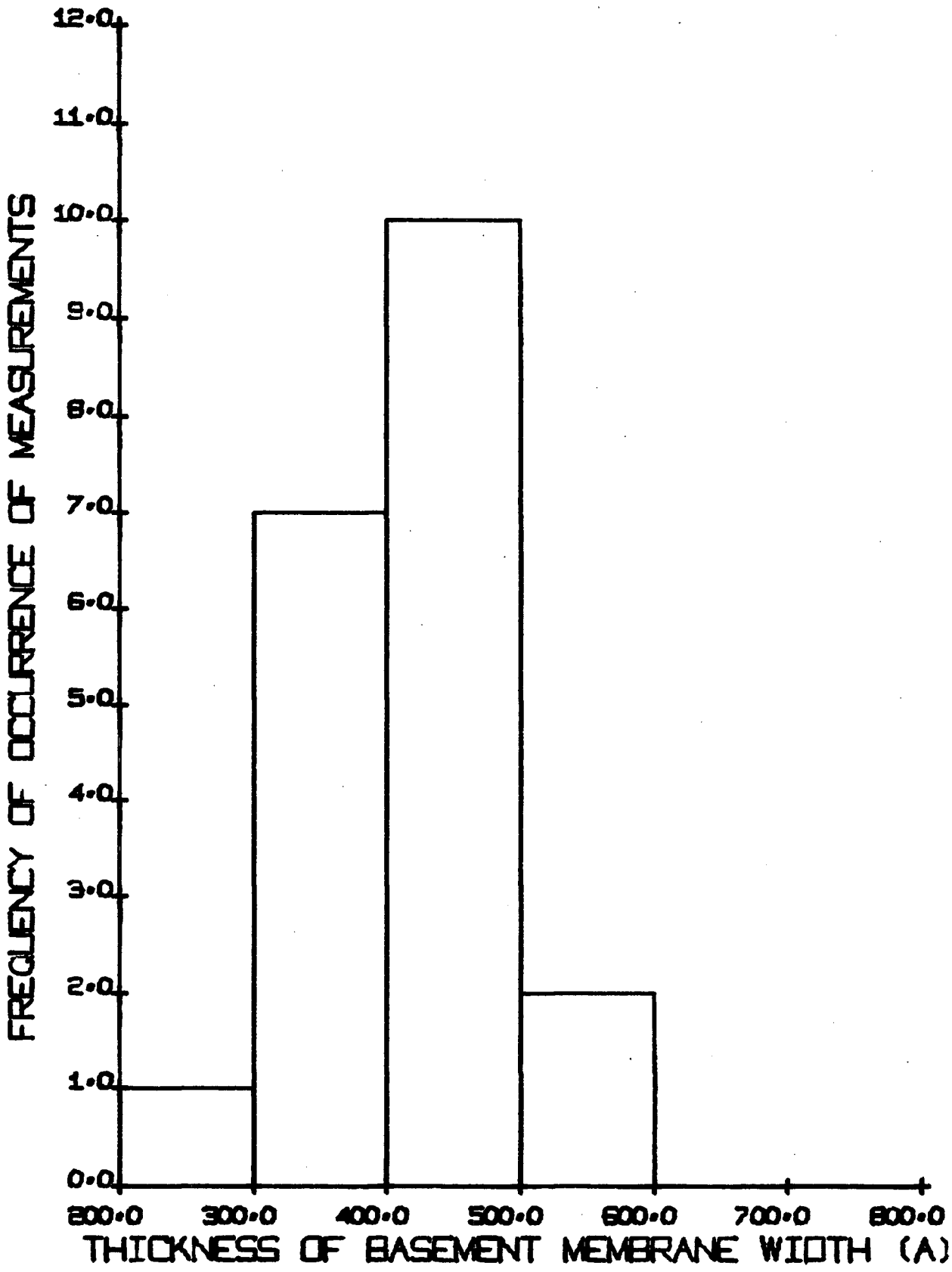

12·0
11·0
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·0
FREQUENCY OF OCCURRENCE OF MEASUREMENTS
200·0
300·0
400·0
500·0
600·0
700·0
800·0
THICKNESS OF BASEMENT MEMBRANE WIDTH (A)

Fig. 14 Frequency distribution of means of basement membrane widths -- parenchymatous goitre.

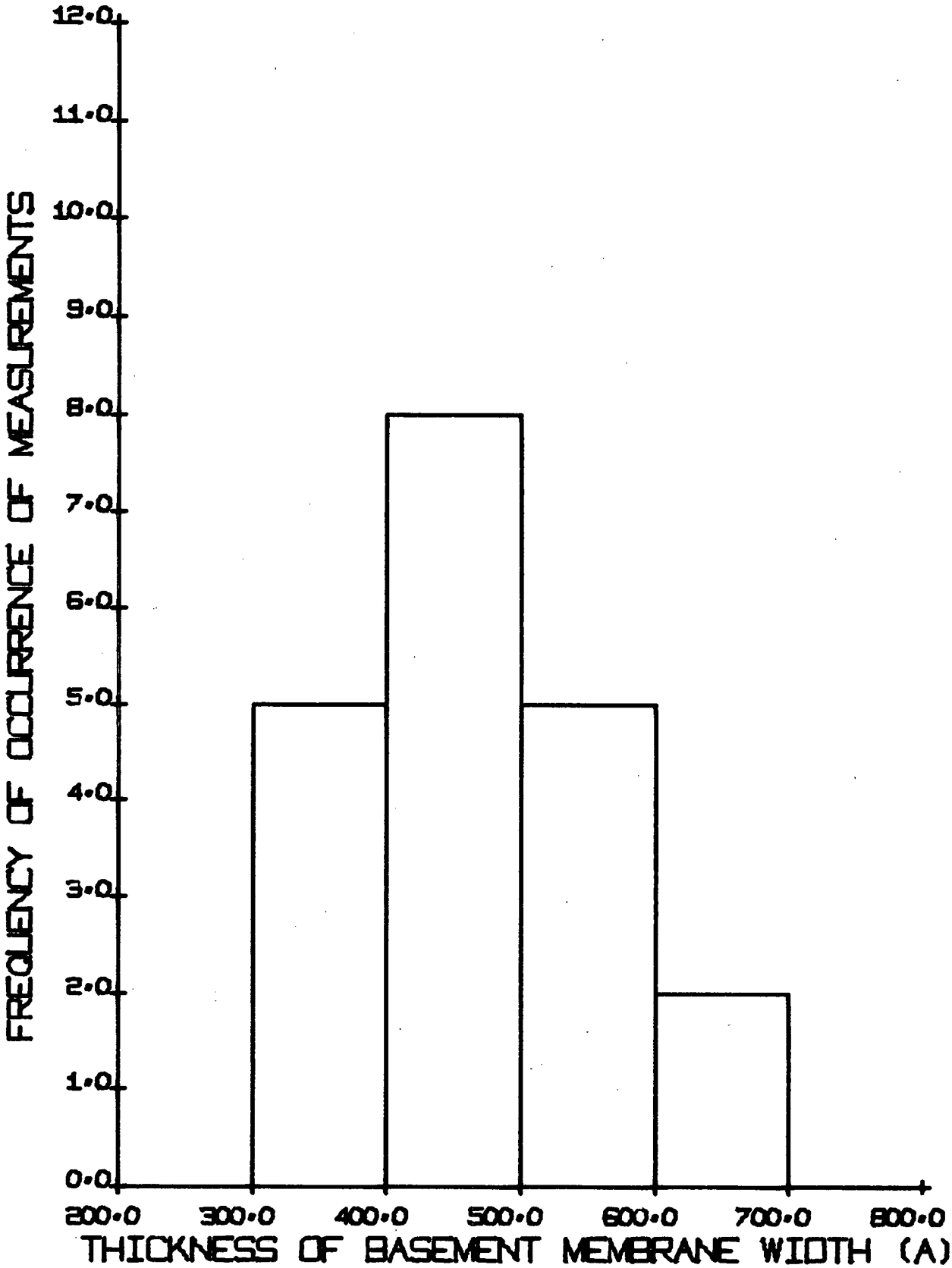

12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
FREQUENCY OF OCCURRENCE OF MEASUREMENTS
200.0
300.0
400.0
500.0
600.0
700.0
800.0
THICKNESS OF BASEMENT MEMBRANE WIDTH (A)

Fig. 15 Frequency distribution of means of basement membrane widths -- control for parenchymatous goitre.

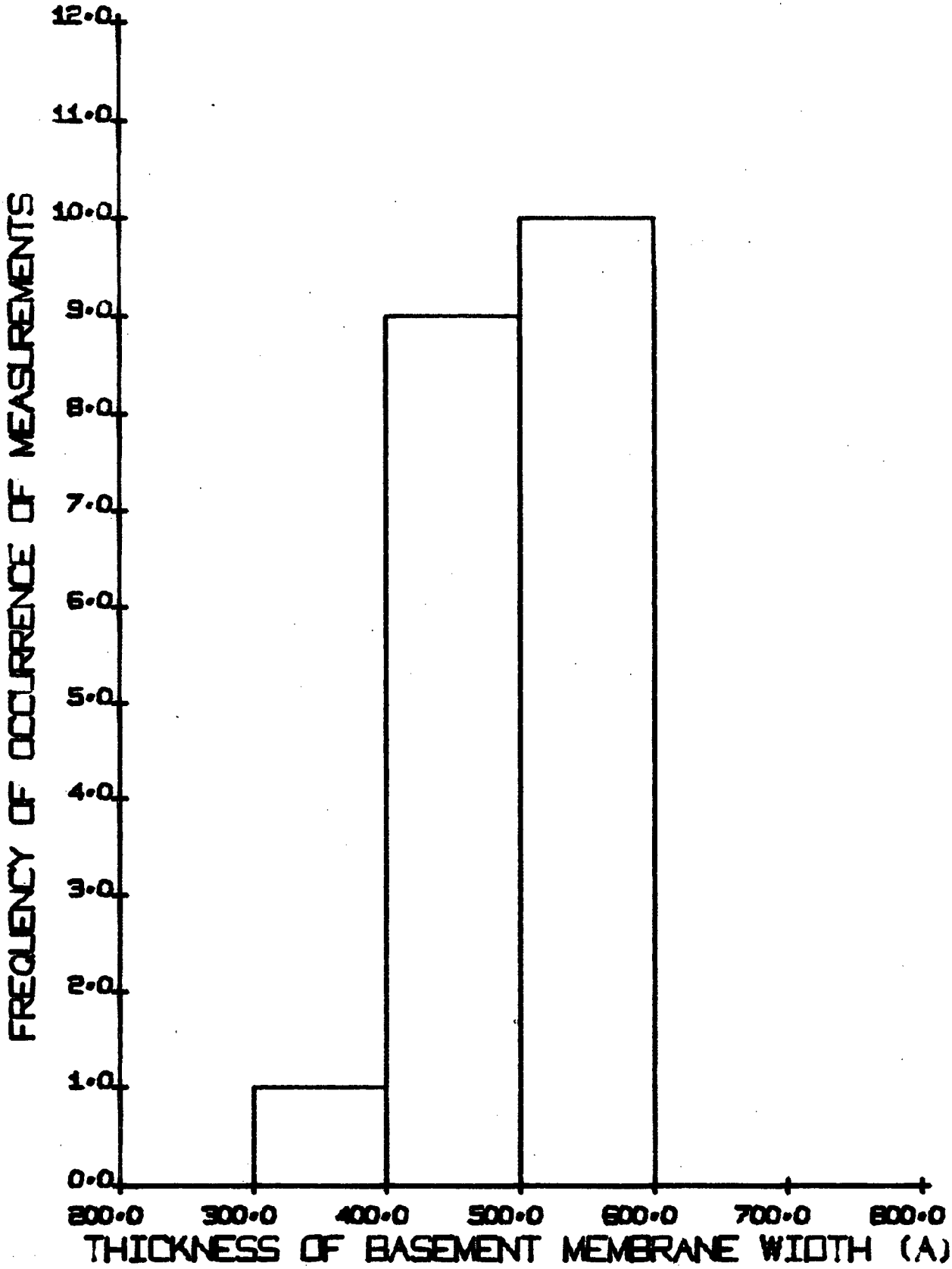

FREQUENCY OF OCCURRENCE OF MEASUREMENTS
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
200.0
300.0
400.0
500.0
600.0
700.0
800.0
THICKNESS OF BASEMENT MEMBRANE WIDTH (A)

Fig. 16 Frequency distribution of means of basement membrane widths -- colloid goitre.

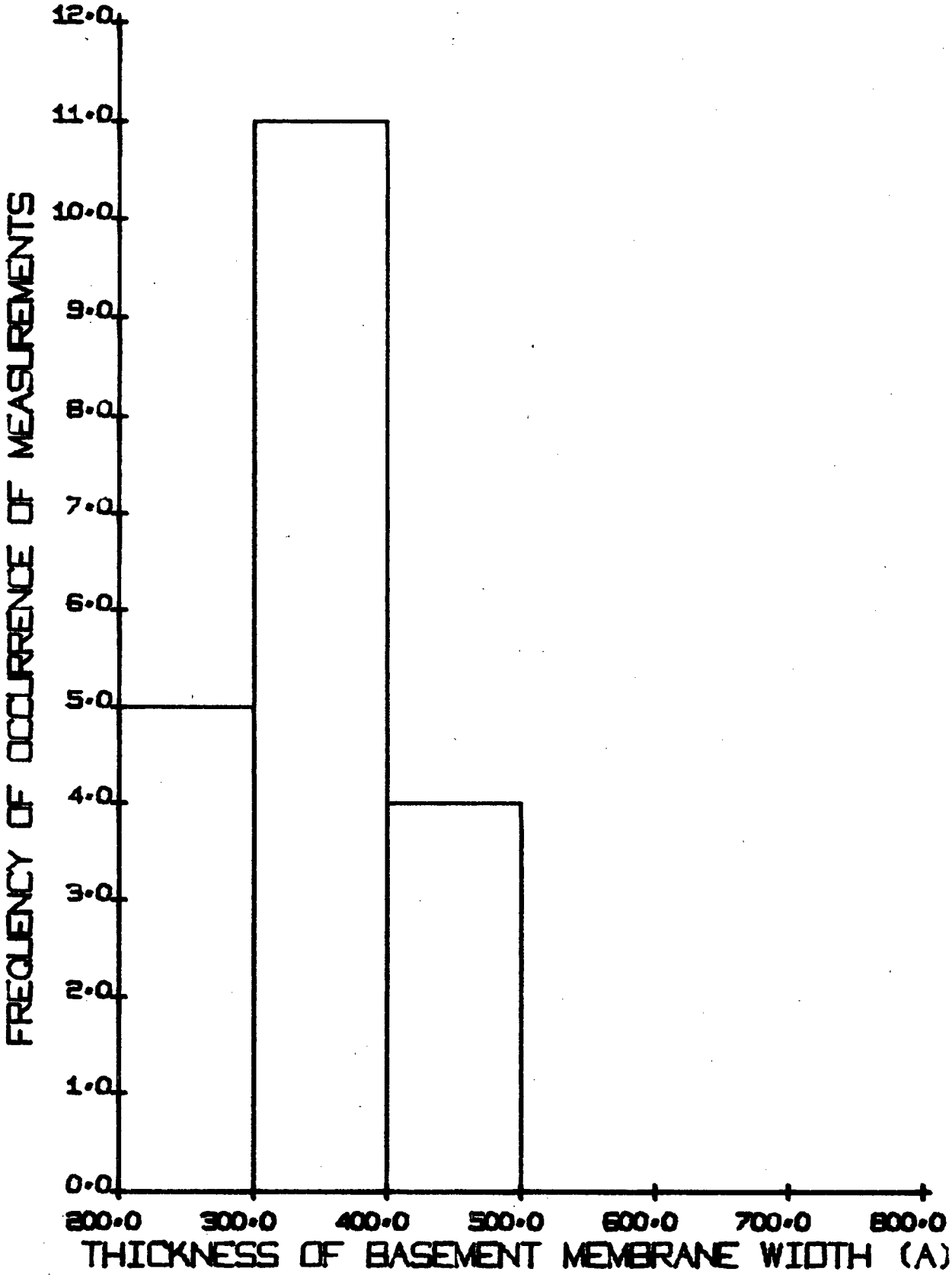

FREQUENCY OF OCCURRENCE OF MEASUREMENTS
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
200.0
300.0
400.0
500.0
600.0
700.0
800.0
THICKNESS OF BASEMENT MEMBRANE WIDTH (A)

Fig. 17 Frequency distribution of means of basement membrane widths -- control for colloid goitre.

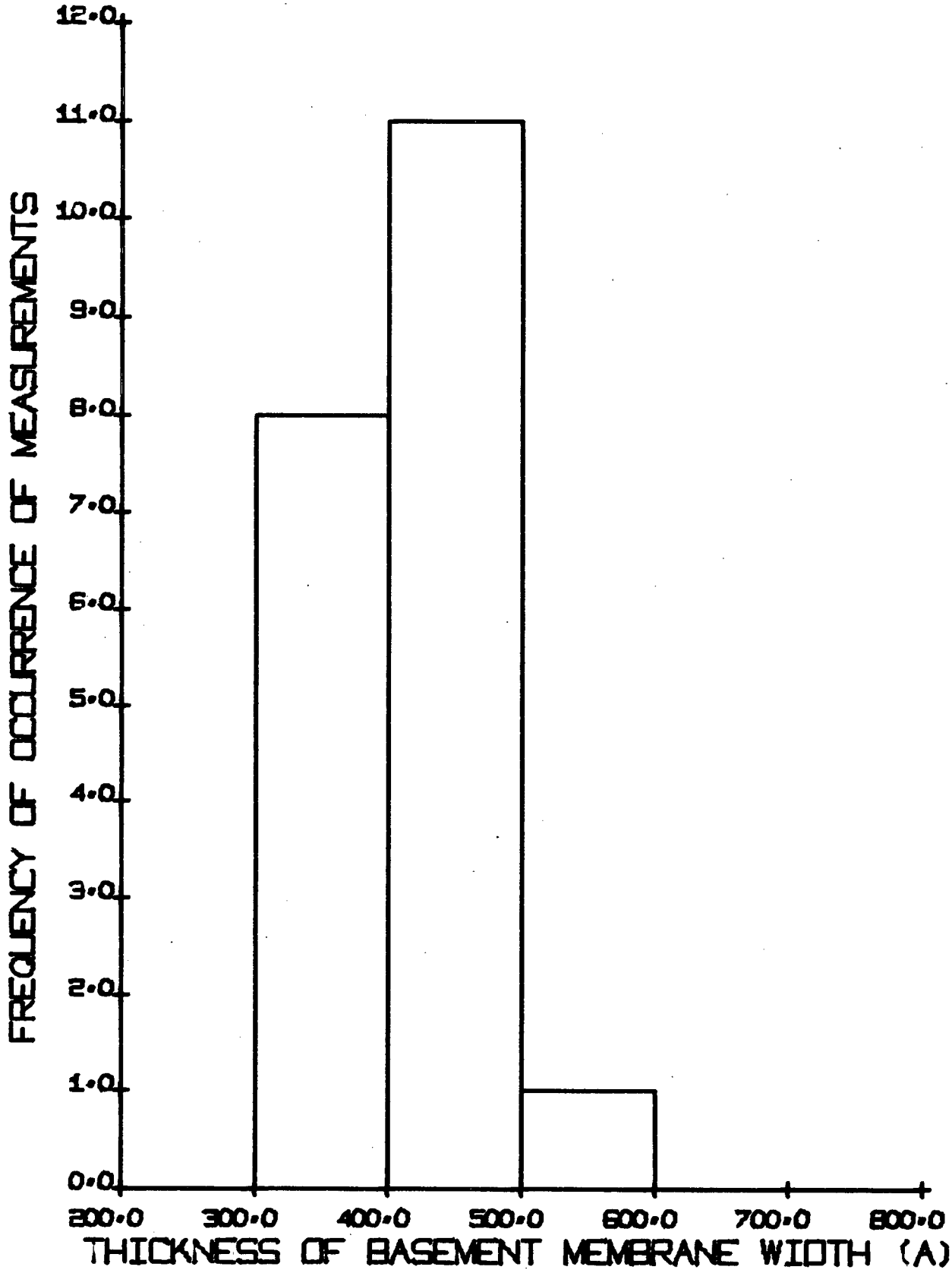

FREQUENCY OF OCCURRENCE OF MEASUREMENTS
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
200.0
300.0
400.0
500.0
600.0
700.0
800.0
THICKNESS OF BASEMENT MEMBRANE WIDTH (A)

Fig. 18 Typical portion of thyroid cell showing basement membrane.

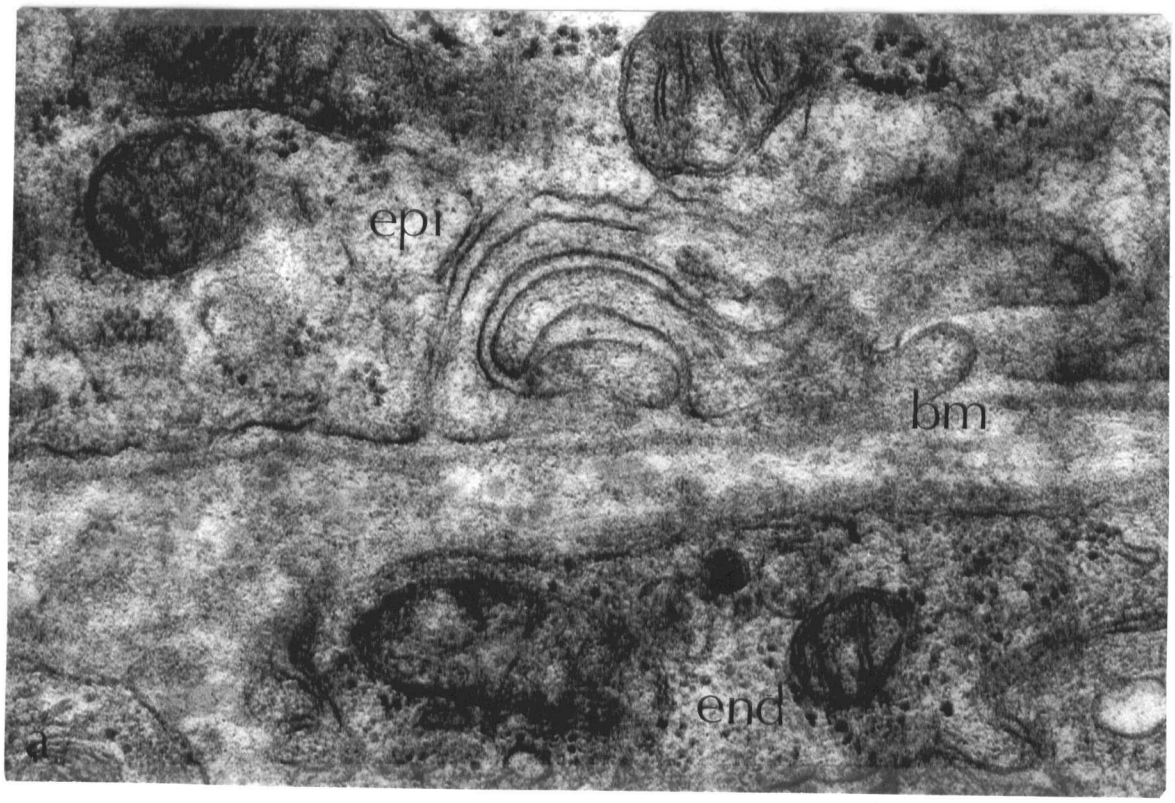
a. hemithyroidectomy

b. control for hemithyroidectomy

epi = thyroid epithelium

bm = epithelial basement membrane

end = endothelium


epi
bm
end
a

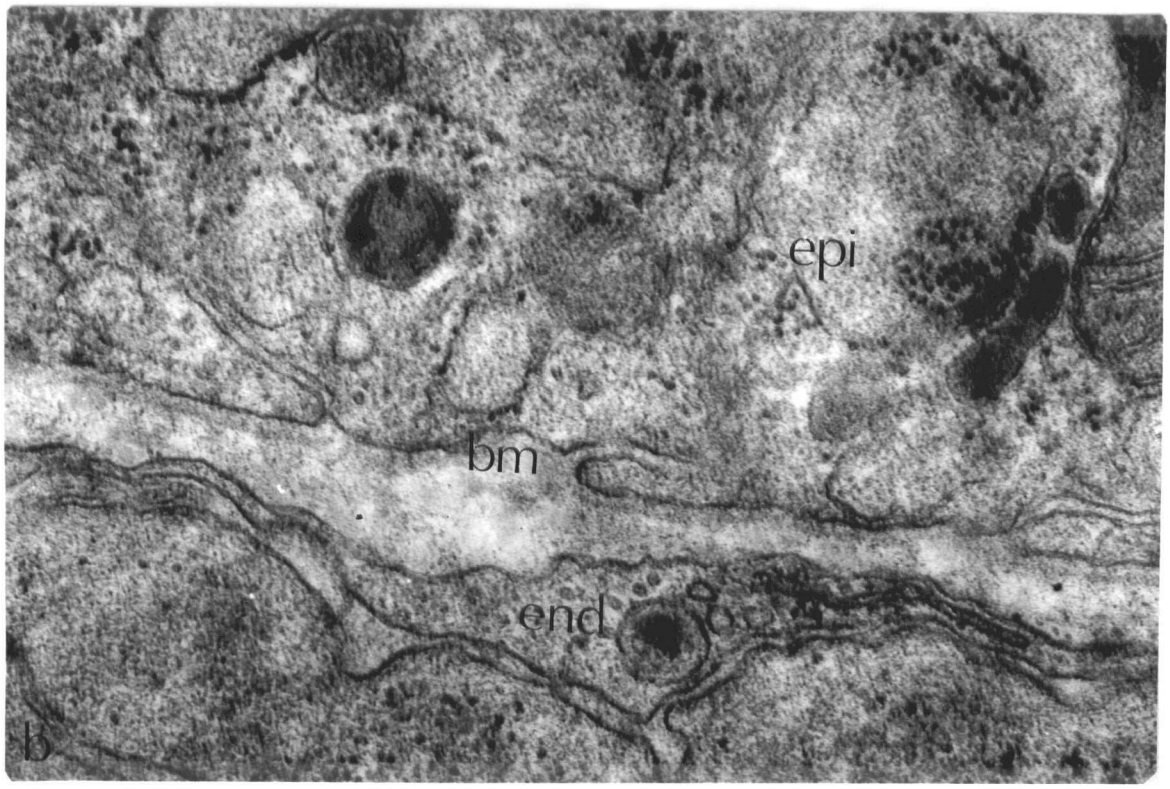

epi
bm
end
b

Fig. 19 Typical portion of thyroid cell showing basement membrane.

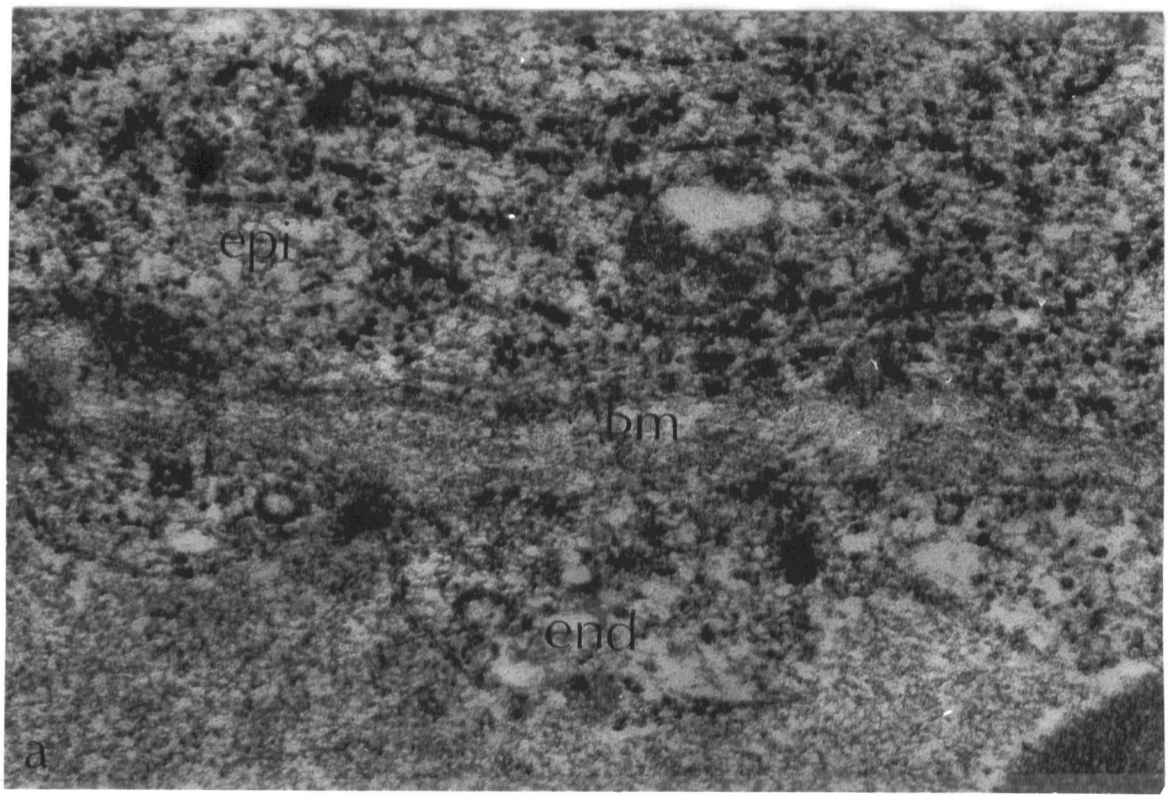
a. parenchymatous goitre

b. control for parenchymatous goitre

epi = thyroid epithelium

bm = epithelial basement membrane

end = endothelium


epi
bm
end
a

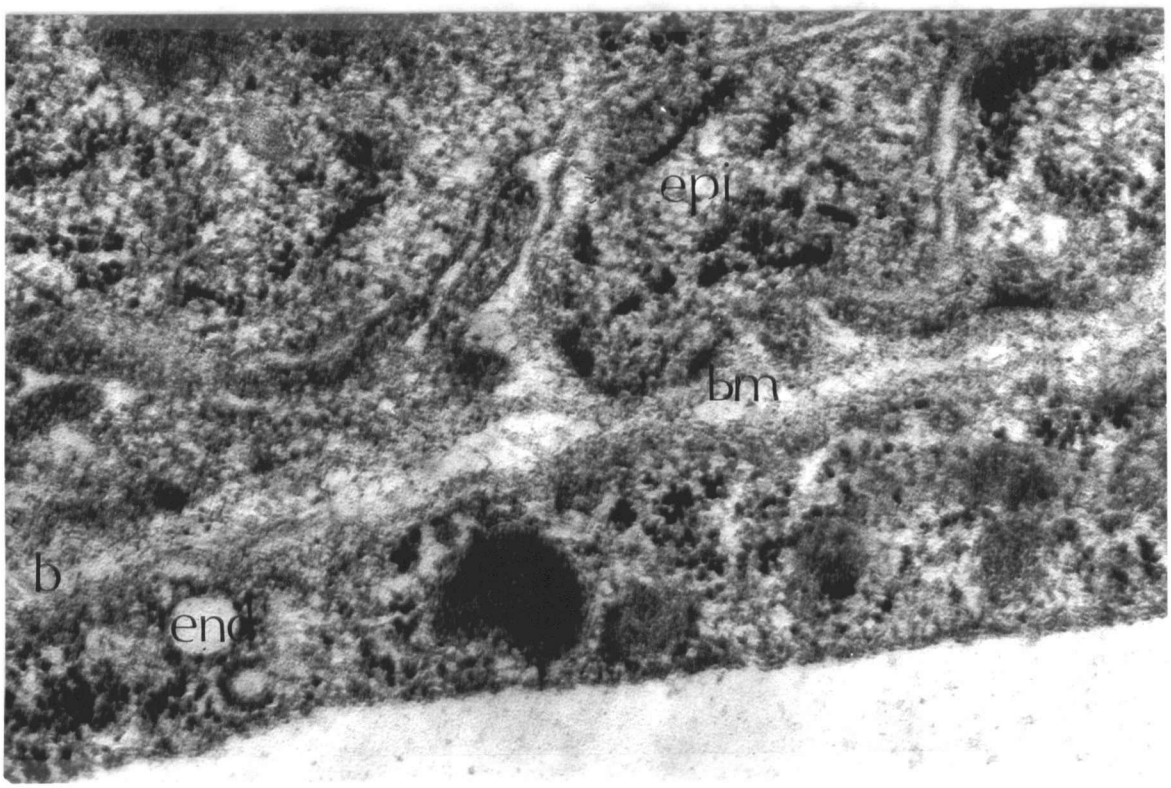

epi
bm
b
end

Fig. 20 Typical portion of thyroid cell showing basement membrane.

a. colloid goitre

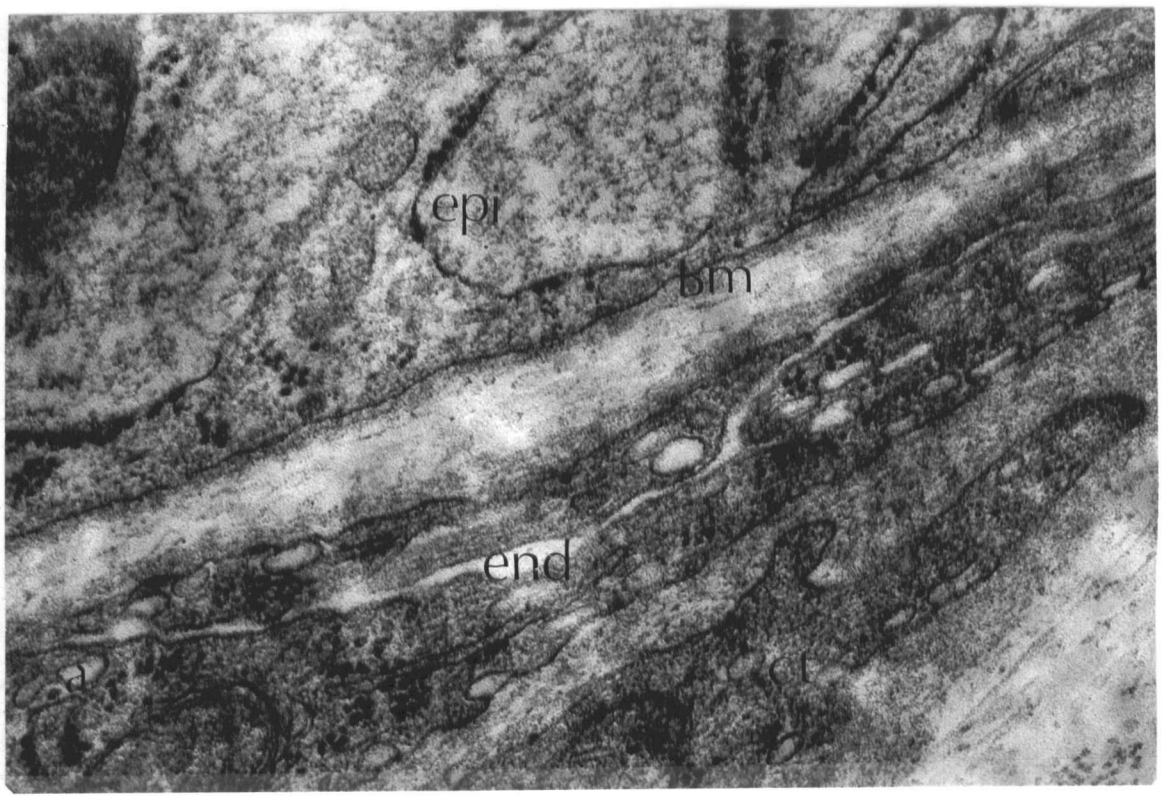
b. control for colloid goitre

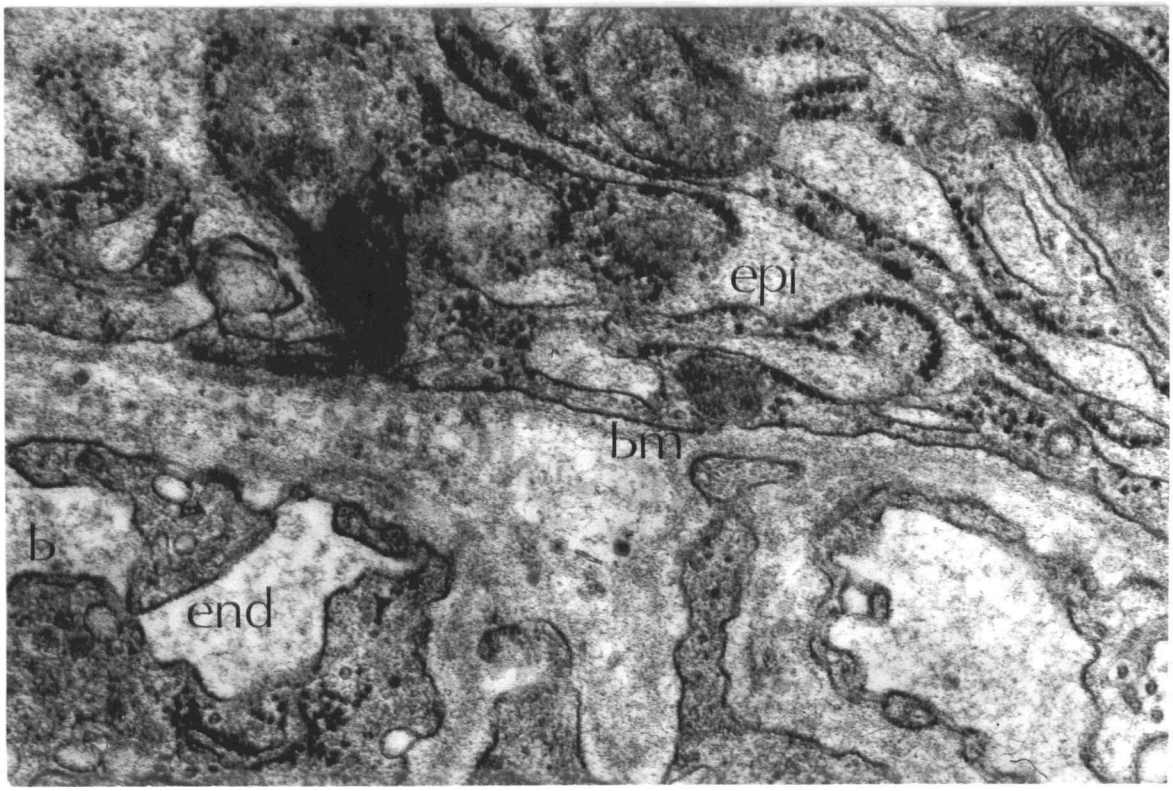
epi = thyroid epithelium

bm = epithelial basement membrane

end = endothelium

ct = connective tissue cell


epi
bm
end
a


epi
bm
b
end

The bias can be calculated knowing the measured values of basement membrane thickness and the size of thyroid follicles. An equation which will predict the effect of bias can be derived as follows:

T is the real thickness of basement membrane.

t is the measured thickness -- the best possible estimate since average values of measurements must be used.

To find the average value of t, the following data are needed:

the several values of t that were found,

the frequency with which the values were found, and

the range over which the values were found.

Thus, to find the average value of t, $E\{t\}$, the function of the frequency of the occurrence of an event (the frequency of being able to find t), f(x), is multiplied by the function of which the average is being found, t(x), and then the product is integrated over all possible values of x (i.e. all possible places where t can be found). This gives the equation:

$$E\{t\} = \int t(x)\ f(x)\ dx \qquad (1)$$

However: this is a uniform distribution and x has equal chances of occurring over its range.

Therefore: f(x) is a constant and is equal to 1/length where x occurs.

Therefore: f(x) may be withdrawn from the integral and

t(x) integrated over the range where t(x) may be found.

Equation (1) becomes: $E\{t\} = {}^{1}/_{x} \int_{0}^{x} t(x)\, dx$ (2)

From Fig. 21, it can be seen that x = D - T, so:

$$E\{t\} = {}^{1}/_{D-T} \int_{0}^{D-T} t(x)\, dx \quad (3)$$

Now: $t = d_o - d_i$ (See Fig. 21)

and $d_o^2 = D^2 - X^2$

$d_i^2 = (D - T)^2 - X^2$

Therefore: $t = (D^2 - X^2)^{\frac{1}{2}} - ((D - T)^2 - X^2)^{\frac{1}{2}}$ (4)

Substituting the value of t from equation (4) into equation (3), and putting D - T = DT, equation (4) becomes:

$$E\{t\} = {}^{1}/_{DT} \int_{0}^{DT} (D^2 - X^2)^{\frac{1}{2}}\, dx - \int_{0}^{DT} (DT^2 - X^2)^{\frac{1}{2}}\, dx \quad (5)$$

This equation can be integrated and simplified:

$$E\{t\} = {}^{1}/_{DT} \frac{X(D^2 - X^2)^{\frac{1}{2}}}{2} + \frac{D^2}{2} \sin^{-1} \frac{X}{D} - \frac{X(DT^2 - X^2)^{\frac{1}{2}}}{2}$$

$$- \frac{DT^2}{2} \sin^{-1} \frac{X}{DT} \Bigg]_{0}^{DT}$$

$$= \frac{1}{2DT} DT\,(D^2 - DT^2)^{\frac{1}{2}} + D^2 \sin^{-1} \frac{DT}{D} - DT^2 + \frac{\pi}{2}$$

$$= \frac{1}{2} (D^2 - DT^2)^{\frac{1}{2}} + \frac{D^2}{DT} \sin^{-1} \frac{DT}{D} - DT \frac{\pi}{2}$$

$$= \frac{1}{2} (D^2 - D^2 + 2(D)(T) - T^2)^{\frac{1}{2}} + \ldots$$

Fig. 21 Diagram of the cytological origin of the symbols used in the stereological analysis.

D = radius of follicle

T = true width of basement membrane

D-T = radius of thyroid follicle (cells only)

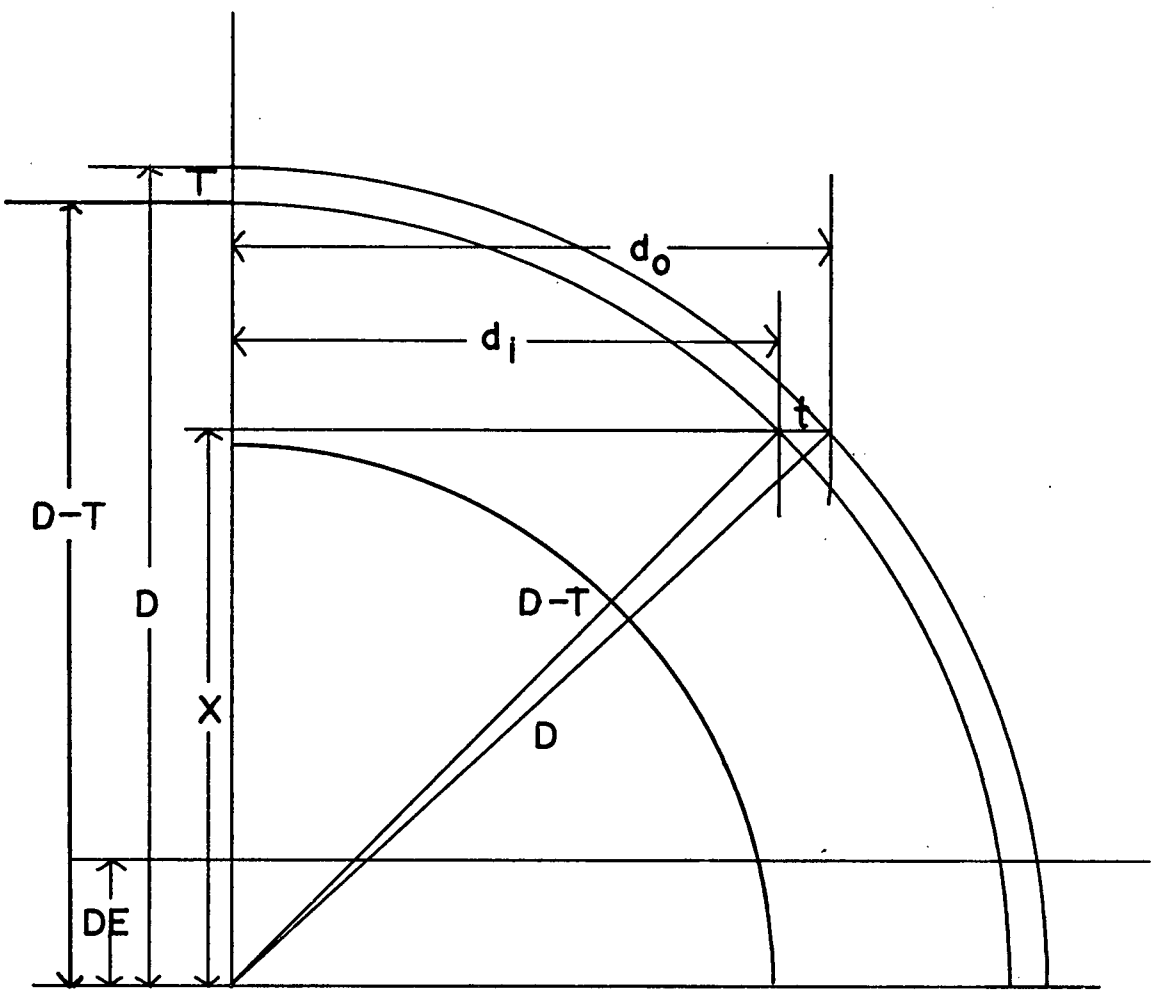
t = apparent thickness of basement membrane

d_o = estimate of D in the section where t is found

d_i = estimate of D-T in the section where t is found

x = any portion of follicle used in analysis

DE = zone of acceptability


T
d_o
d_i
t
D−T
D
X
D−T
D
DE

$$\frac{E\ (t)}{T} = \frac{1}{2}\ \left(\frac{2D}{T} - 1\right)^{\frac{1}{2}} + \frac{D^2}{DT(T)} \sin^{-1} \frac{DT}{D} - \frac{DT}{T} \frac{\pi}{2} \qquad (6)$$

Now, this equation allows for t to be measured anywhere in the follicle. However, certain portions of the follicle that give less accurate measurements can be eliminated by inspection. The nearer the measurements are to the centre of the follicle, the more closely will the measurements approach the real value, and as a result, the less will be the error. All sections that are obviously not near the centre are eliminated. Such sections would be those that, while showing the lumen, show also at least one transection of a lateral cell membrane. Only those sections showing a perfect monolayer would be acceptable. If this "zone of acceptability" is designated as D - E, or DE, (Fig. 22) an equation predicting bias is obtained:

$$E\ (t)_{DE} = \frac{1}{DE} \frac{X(D^2 - X^2)^{\frac{1}{2}}}{2} + \frac{D^2}{2} \sin^{-1} \frac{X}{D} - \frac{X(DT^2 - X^2)^{\frac{1}{2}}}{2}$$

$$- \frac{DT^2}{2}\ \sin^{-1} \frac{X}{DT}\Bigg]_0^{DE}$$

$$= \frac{1}{2DE}\ DE(D^2 - X^2)^{\frac{1}{2}} + D^2\ \sin^{-1} \frac{DE}{D} - DE(DT^2 - DE^2)^{\frac{1}{2}}$$

$$- DT^2\ \sin^{-1} \frac{DE}{DT} \qquad (7)$$

If the graph of this equation is plotted with various portions of the radius of the follicle as D - E, the graph shown in Fig. 23 is obtained.

Equation (7) can also be used to gauge the effect

Fig. 22 Diagrammatic representation of the determination of the zone of acceptability.

D = radius of follicle (cf Fig. 21)

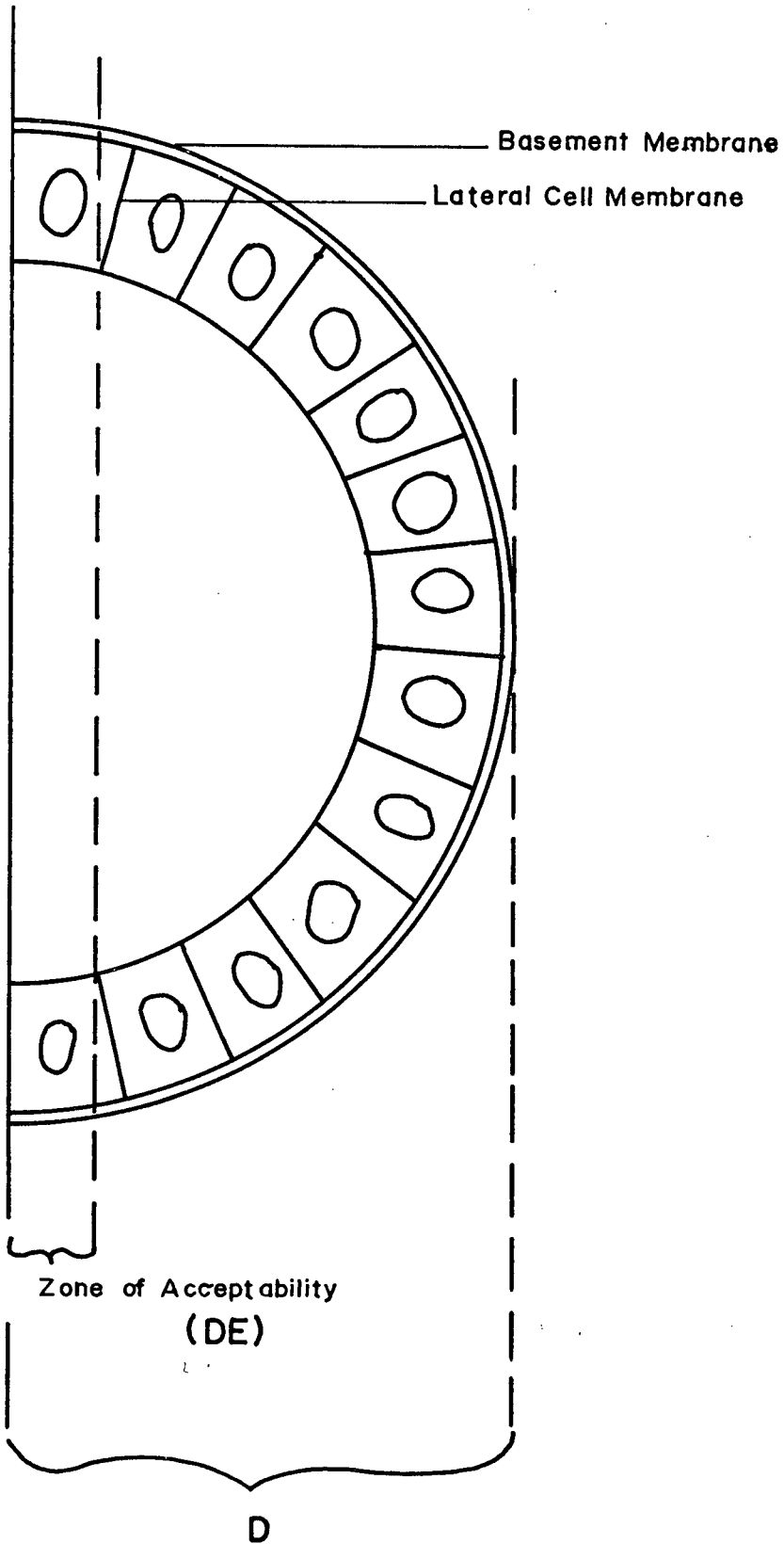
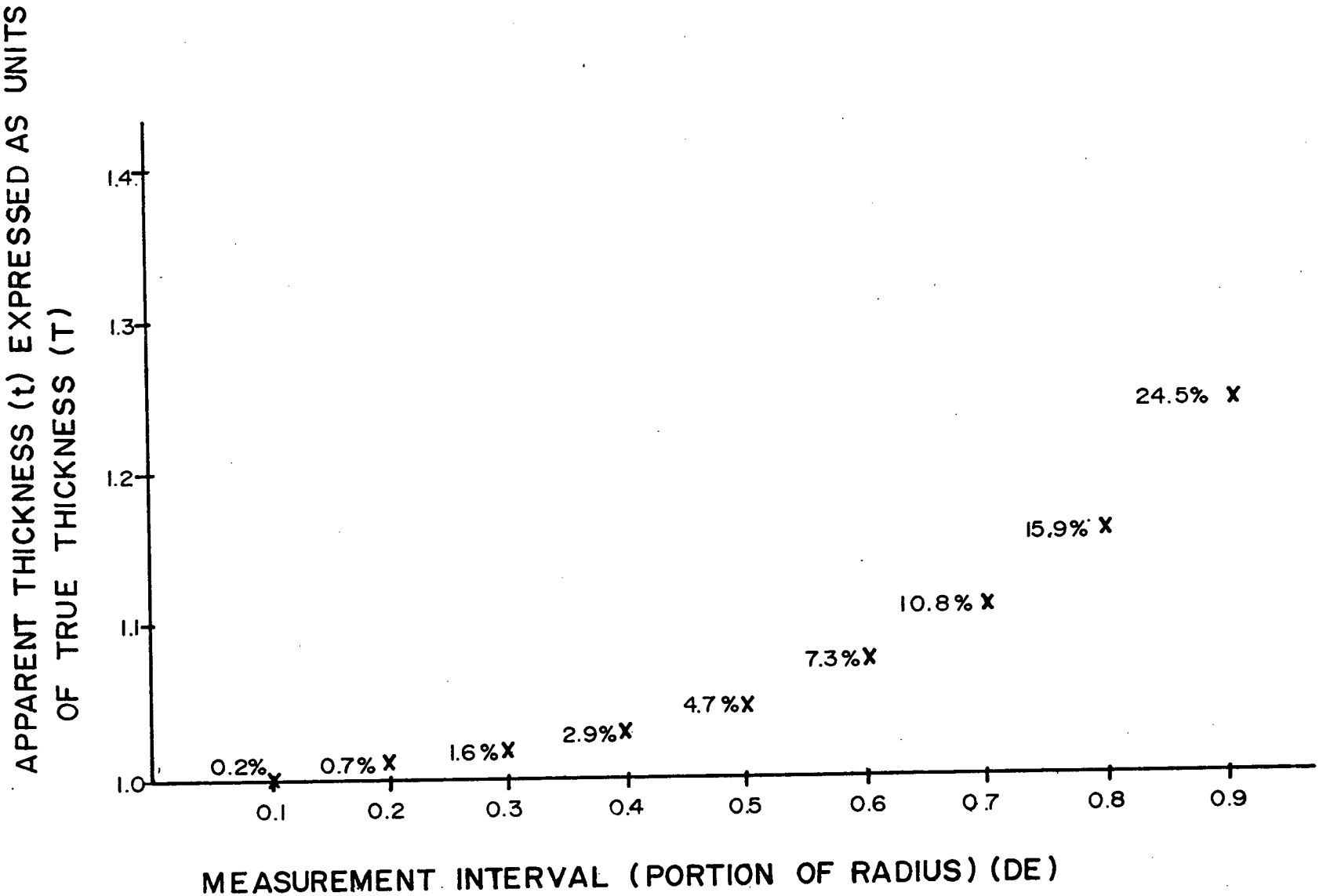

Basement Membrane
Lateral Cell Membrane
Zone of Acceptability
(DE)
D

Fig. 23 A graph of apparent thickness of basement membrane as a function of the interval over which measurements are made.


APPARENT THICKNESS (t) EXPRESSED AS UNITS OF TRUE THICKNESS (T)
1.4
1.3
1.2
1.1
1.0
0.2%
0.7%
1.6%
2.9%
4.7%
7.3%
10.8%
15.9%
24.5%
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
MEASUREMENT INTERVAL (PORTION OF RADIUS) (DE)

of follicle size on the accuracy of measurements. While follicle size is variable (Jackson, 1931), the methods of sampling follicle size emphasize this variability. We need to be sure that the variability does not invalidate the measurements by changing the bias through the range of follicle sizes. But, if a given value of DE and a range of values for D are substituted into equation (7), a range of values is obtained for t/T, which is the ratio of measured thickness to true thickness. These values are summarized in Table VI. It can be seen that the t/T ratios do not change as the T/D ratios (ratios of basement membrane thickness to follicle radius) change until DE becomes quite substantial, and then the t/T ratios change only slightly.

The effect of bias for each of the 6 tissues can be estimated using cell widths and follicle diameters. The cell widths give an idea of the size of the "zone of acceptability". No section was acceptable that showed a transection of a lateral cell membrane, so the zone of acceptability is limited by the cell widths (i.e. it is one cell wide). The zone will, of course, be different in different tissues. In Fig. 24 zones of acceptability for the 6 tissues are shown as per cent from centre. These zones were calculated by dividing the mean cell width by one-half the mean follicle diameter $\pm$ one-half follicle diameter standard deviation. The brackets in Fig. 24 enclose the range of the possible extremes of

Table VI. Relationship of t/T and T/D Ratios to DE

(Portion of Follicle Examined)

DE	T/D ($\times 10^{-3}$)	t/T ($\times 10^{-1}$)
0.1	1.000	0.1002
	3.162	0.1002
0.2	1.000	0.1007
	3.162	0.1007
0.3	1.000	0.1016
	3.162	0.1016
0.4	1.000	0.1029
	3.162	0.1029
0.5	1.000	0.1047
	3.162	0.1047
0.6	1.000	0.1073
	3.162	0.1073
0.7	1.000	0.1108
	3.162	0.1108
0.8	1.000	0.1159
	3.162	0.1160
0.9	1.000	0.1245
	3.162	0.1246
0.95	1.000	0.1320
	3.162	0.1322
0.985	1.000	0.1421
	3.162	0.1426

Fig. 24 Bias limits for treatments.

CONTROL = control for hemithyroidectomy

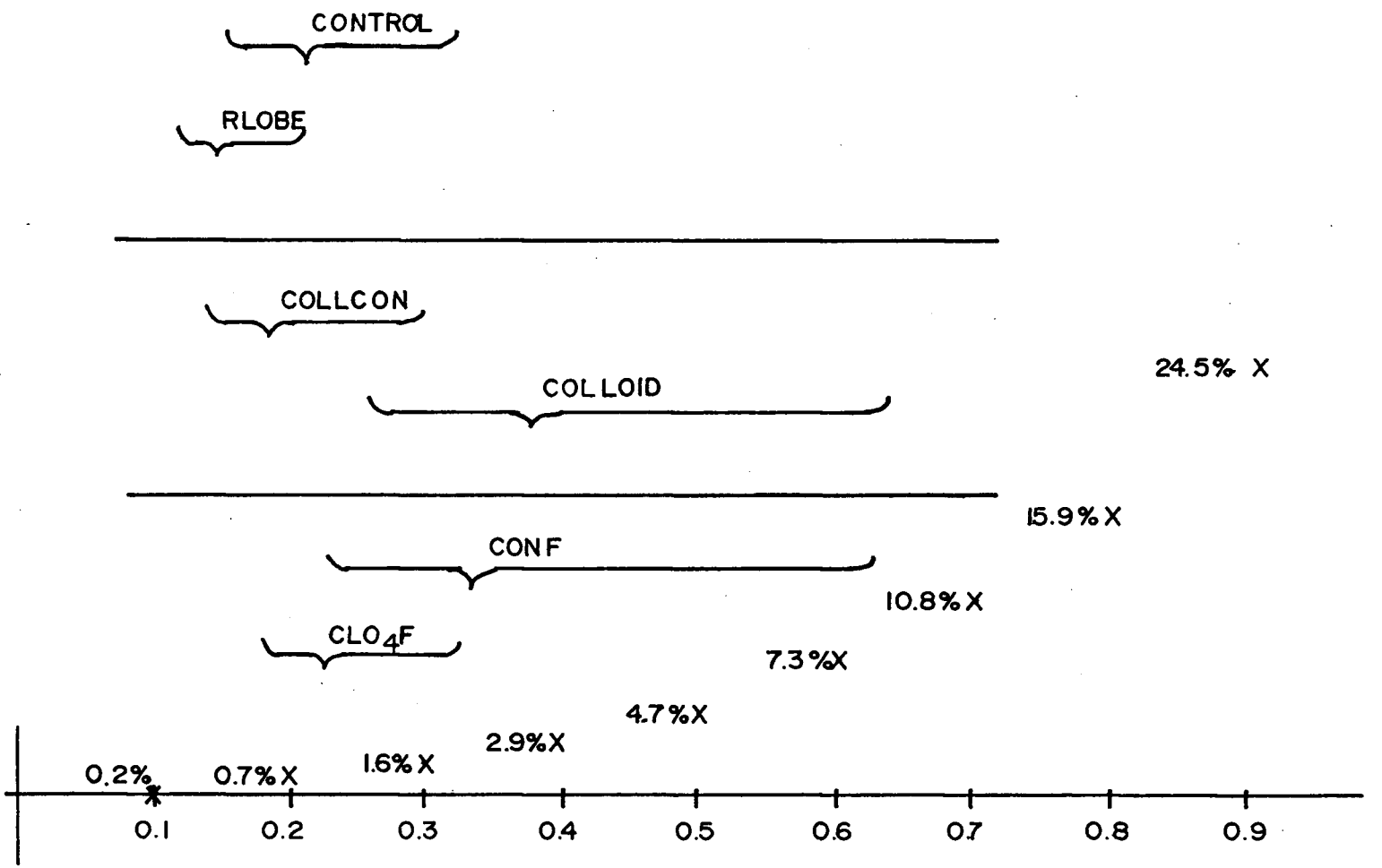
RLOBE = hemithyroidectomy

COLLCON = control for colloid goitre

COLLOID = colloid goitre

CONF = control for parenchymatous goitre

CLO_4F = parenchymatous goitre


CONTROL
RLOBE
COLLCON
COLLOID
CONF
CLO4F
24.5% X
15.9% X
10.8% X
7.3% X
4.7% X
2.9% X
1.6% X
0.7% X
0.2%
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
MEASUREMENT INTERVAL (PORTION OF RADIUS) (DE)

the zones of acceptability.

It must be remembered that not all measurements were taken at the worst possible bias, but were randomly distributed and had equal chances of occurring anywhere from the centre up to the limits of worst possible bias. However, in order to test the surety of the t-test probabilities, the bias limits can be used to modify the means of basement membrane widths. If, when the means are modified and the t-test is repeated, the probabilities still stand, then the validity of the t-test is enhanced. The t-tests for modified means for colloid goitre and ClO_4F goitre are shown in Table VII. Essentially what has been done is that the means have been divided by a factor that accounts for bias. This factor is obtained (from Fig. 24) by taking the inverse of the sum of the amount of bias calculated to be present at the extreme limit of the zone of acceptability and the effect of no bias (100%). For colloid goitre, the factor is 0.93, and it is applied to the mean for the experimental tissue. As can be seen in Table VII, this has the effect of pushing the means for experimental and control closer together, but the t-test shows that there is still a significant difference. For ClO_4F goitre the factor is 0.98, and it is applied to the mean for the control tissue. Applied this way, the factor tends to push the means farther apart. A t-test done after this modification shows that there is still no significant difference.

Table VII. Effect of Bias Modifications on t-tests on Parenchymatous Goitre and Colloid Goitre.

Parenchymatous Goitre		Control	
$\bar{x}$	s^2	$\bar{x}$	s^2
476.701	7350.061	503.060	3665.678

Probability = 0.26842 ; Not significant

		(Adjusted by factor = 0.98)	
476.701	7350.061	513.326	3816.797

Probability = 0.12943 ; Not significant

Colloid Goitre		Colloid Control	
$\bar{x}$	s^2	$\bar{x}$	s^2
346.886	3453.773	420.823	3401.060

Probability = 0.00028 ; Highly significant

(Adjusted by factor = 0.93)			
372.995	3993.229	420.823	3401.060

Probability = 0.01737 ; Significant

Discussion

The purpose of this investigation was to study the ultrastructure of the basement membrane in an experimentally induced hyperplasia. From Table I it can be seen that the treatments did indeed cause the thyroid gland to enlarge by what is considered to be hyperplasia (Voitkevich, 1964; Turner, 1971). When the basement membrane was measured and corrections for bias made in each of the enlarged glands and then compared to the basement membrane in the control for each experimental condition, it was found that:

1. There was no change in basement membrane width in hemithyroidectomy.
2. There was no change in basement membrane width in parenchymatous goitre.
3. There was a change in basement membrane width in colloid goitre -- the basement membrane was significantly thinner when compared with its control.

As Friederici (1966), Alousi *et al.* (1969), and Irvine and Muir (1963) have observed, a change in the thickness of basement membrane is one of the most striking features of certain pathological states. Thus, basement membrane thickness is considered to be one of the more important aspects of the pathological conditions induced in this study. However, merely observing a thinning or a thickening is not sufficient

to evaluate a pathological state. Stereological methods are required if an accurate estimate of basement membrane thickness is to be obtained.

Several ways to measure basement membranes have already been devised. Waterhouse and Squier (1969) have been interested in the basement membrane of oral epithelium and, thus, in the best way to measure the basement membrane when it is present as an undulating sheet. Their measurements, when plotted as a histogram, show a decidedly skewed distribution which, according to these two workers, indicates that most of their measurements are too large and that the median and I. Q. R. (inter-quartile range) are, therefore, the best estimates of thickness and error respectively. The difficulty here lies in the lack of clues as to orientation; there is no way of knowing if the measurement axis is exactly perpendicular to the basement membrane.

Other researchers have been studying the cylinders that are capillary basement membranes, especially in muscles and the glomeruli. Osawa et al. (1966), working on the glomerulus, measured from the cell membrane of the endothelial cells to that of the epithelial foot processes, excluding all sites that were "unsuitable", sites where the cell membrane was not sharp, indicating tangential sectioning, sites bordering mesangial cytoplasm, or areas of tortuosity. Means and standard deviations were used as estimates of thickness and error.

Jorgensen and Bentzon (1968) devised a different method to study glomerular basement membrane, measuring only in the peripheral portions and eliminating those areas where one cell's membranes were not sharp. They measured the area of selected portions of basement membranes and related the area to the length of the portions (measured at the midpoints of the basement membrane) to find the thickness. The thicknesses were expressed as logarithms to give a normal distribution, with the geometric mean as an estimate of thickness and three different variances to estimate error.

Basement membrane of muscle capillaries is of interest to workers in the field of diabetes research. Vracko and Strandness (1967), using a planimeter, took the area occupied by the basement membrane and related it to the area of the whole vessel. This method eliminated several difficulties such as choosing a site at which to measure and an angle at which to measure. It also eliminated variation due to functional shrinkage and distension of the vessel, or differences in caliber of capillary, and permitted sections to be taken at an angle.

Siperstein et al. (1968), also working on muscle capillary, used a grid to choose measurement sites. They eliminated those sites that crossed pericytes and insisted on a minimum of 10 measurements per capillary. Their estimate of thickness and error is expressed as the mean and standard error.

When the thickness of thyroid basement membrane is to be discussed, a certain amount of difficulty is encountered. In cross-section, the basement membrane appears as a circle concentric with a circle of thyroid cells, not unlike those surrounding capillaries, and it would seem easy enough to just measure across it. However, the thyroid follicle is a sphere and a random section through the sphere may not pass through the best region to give the best estimate of basement membrane thickness. The ideal section from a thyroid follicle would cut through the exact middle. This would give the thinnest and most accurate measurement of basement membrane thickness. Finding the middle of the follicle would require serial sections and such a systematic approach would be laborious, expensive of time, and yet not very accurate. Therefore, random sections must be used, and these sections will probably not be through the centre. The true width of the basement membrane will be masked by error plus bias, and it is essential to get an estimate of these statistics.

The estimate of bias was calculated as outlined previously and the bias values plotted on the graph in Fig. 23. This graph shows, for example, that if all measurements are made within 40% of the distance from the centre of the follicle to the edge, the measured thickness of the basement membrane is affected by a maximum bias of less than 3%. By employing certain criteria, all sections

obviously not near the centre can be eliminated (see "Results" section). There will remain a zone of acceptability straddling the middle of the follicle, that can be used to estimate worst possible bias limits for each of the tissues. The bias limits are shown in Fig. 24 superimposed on the graph from Fig. 23. It can be seen that in only two cases did the bias limits exceed 3% (colloid goitre and parenchymatous goitre control), and that these were limited to a maximum bias of less than 8%. Therefore, the manner of sampling ensures small bias.

Since there was considerable variation in follicle size within each treatment, it was necessary to ascertain the effect of follicle size on bias. Table VI summarizes the ratios of measured thickness to true thickness of the basement membrane for various values of DE, the portion of the follicle studied. The t/T ratios for large follicles remain the same as the t/T ratios for small follicles until DE exceeds 80% (i.e. until the section taken is far from the centre). Fig. 24 shows that DE never exceeds 6.5% and, therefore, bias is independent of follicle size in this study and the basement membrane may be confidently measured on any follicle.

T-tests were used to test for differences between treatment means. The bias can be used to strengthen the t-tests. Since the strengthened t-tests provided the same results as the original tests, confidence in the accuracy of the measurements is reinforced. (See Table VII)

The mean was considered an accurate estimate of basement membrane width, even though the distribution was truncated at both ends. At one end, the distribution stops because the basement membrane does not become infinitely thin. At the other end, the distribution does not continue outside the zone of acceptability. Within these limits, the distribution can be considered normal, influenced by the sampling method and by the shape of the thyroid follicle in the region of examination.

The major finding in this study was that one of the treatments resulted in a thinner basement membrane. An examination of the physiological changes associated with each treatment is necessary to evaluate the significance of this finding.

The thyroid is a bilobed, endocrine gland that lies on the trachea just below the larynx. The gland synthesizes iodine-containing hormones, principally thyroxine and triiodothyronine, and they are produced at a rate influenced by the pituitary hormone, thyrotropin (TSH). As the thyroid hormone level drops in the peripheral circulation, TSH levels increase and act on the thyroid, stimulating both rate of release and rate of synthesis of the hormones. If peripheral hypothyroidism cannot be alleviated by the normal cybernetic mechanisms, the thyroid responds by becoming hyperplastic (Astwood, 1970). These changes are observed in both naturally occurring and experimentally induced goitres.

The removal of one lobe of the thyroid causes the gland to regenerate. This growth is in response to reduced thyroid hormone levels and is not merely an effect of trauma (Voitkevich, 1964). The thyroid fragment shows an increased iodine-trapping ability and an increased rate of radio-iodine turnover (Reichlin, 1958). The growth of the remaining tissue is fairly rapid and by eight days, the I^{131} uptake is 90% of normal (Logothetopoulos and Doniach, 1955). These observations result from increased TSH levels since sham-operated animals (Reichlin, 1958) and thyroxine treated animals (Voitkevich, 1964) do not react in the same manner as hemi-thyroidectomized animals. Removal of more than one-half of the gland leads to a more severe hypothyroidism, and the stress on the thyroid fragment leads to cellular hypertrophy and hyperplasia. But, if only half of the gland is removed, new follicles are formed and cell heights approximate normal cell heights (Voitkevich, 1964). In this study, no significant difference was found between cell heights of control and experimental tissue, nor was there any difference in follicle diameters. There was an increase in size of the remaining lobe, so it is considered that the number of follicles must have increased. However, the animals' requirements for thyroid hormone would not cause the gland to increase in size in excess of "normal". Nor is there any pathological condition present, and the hormone metabolism of the gland itself has not been altered. The response to increased

TSH levels is mild and, thus, no alterations in ultrastructure would be expected. Indeed, there was no significant difference between the widths of the basement membranes for the experimental and control tissues.

Interference with hormone metabolism has a much more striking effect on the thyroid than does partial thyroidectomy. If the dosage of a blocking agent is sufficient, the gland will synthesize no hormone at all, the peripheral hypothyroidism will be severe, and the TSH levels will be high. A marked hyperplasia should result.

The perchlorate ion, usually administered as a sodium or potassium salt, is a hydrated mono-valent anion similar in size to iodide, and it acts on the thyroid by interfering with the uptake of iodide ion. It has the effect, then, of "starving" the thyroid for iodine, a form of competitive inhibition which can be neutralized by the administration of excess iodide (Astwood, 1970). Its use as a drug to combat hyperthyroidism is much curtailed by the prevalance of side-effects (skin and gastric irritations, and lymphadenopathy).

In this study, a relatively large dose of perchlorate ion was fed, and, as expected, the gland greatly increased in size. The gross pathological changes included a more uniform distribution of follicle size than the controls. Cell heights increased drastically, causing the lumen to all but disappear. However, no significant change in basement membrane thickness was found, and bias corrections

did not produce significant differences. The lack of significant differences is almost certainly due to the large variance associated with the treatment (7350.061 as compared to 3665.678 for the controls), since there was a considerable difference in the means. This variance is probably not the result of side-effects of ClO_4F. Irritation caused by the ingestion of the drug may well stimulate the secretion of ACTH, which interferes with the production of TSH (D'Angelo, 1953; Carriere and Isler, 1959), but if TSH secretion is hampered, the effect is not likely to be observed in some thyroid follicles and not in others.

The variance in basement membrane widths is more likely to be influenced by the manner in which the number of follicles is increased. The thinner basement membranes might be found surrounding newly-budded follicles while the thicker ones may be found on older follicles in a dormant stage. The tissue examined here is still under stimulation and is still undergoing hyperplasia, and it is not likely that all follicles present are in the same stage of growth.

Propylthiouracil is an anti-thyroid agent that inhibits the formation of thyroid hormone by interfering with the binding of iodine into an organic form. Propyl-thiouracil produces a parenchymatous goitre similar to that produced by the perchlorate ion. The mechanism of action of propylthiouracil would seem to be competitive

inhibition of a peroxidase system that oxidizes the iodide ion and effects the coupling of mono- and di- iodotyrosines to tri-iodothyronine and thyroxine (Astwood, 1970; Morris and Hager, 1966; Maloof and Soodak, 1964; Bjousksten, 1966).

A PTU-induced parenchymatous goitre can become transformed into a colloid goitre (Follis, 1959) by removing the anti-thyroid agent. In this study the administration of PTU induced a parenchymatous goitre as judged by cell heights (Table III). The removal of PTU resulted in a lowering of cell heights.

The formation of colloid goitre from experimental parenchymatous goitre may be brought about in the following manner. All unused iodine has been excreted previously. Once the block is removed, there is an immediate demand for all available iodine, but there is not sufficient iodine to satisfy the requirements. TSH levels remain high and all iodine present is utilized. TSH continues to stimulate growth of the thyroid, causing colloid accumulation in the lumen (Greer, et al., 1967; Ryan, 1968). The period following goitrogen withdrawal is marked by a rebound in I^{131} uptake and altered mono- / di- iodotyrosine and tri-iodothyronine / thyroxine ratios (Studer and Greer, 1967). All features return to normal after a prolonged recovery period.

The course of colloid goitre is considerably different from that of parenchymatous goitre. The growth of the thyroid in parenchymatous goitre is gradual while that of

colloid goitre is almost explosive. It has already been shown that regeneration and the growth of the gland in parenchymatous goitre do not significantly alter the width of the basement membrane. If basement membranes are produced principally by their associated tissues, it would be reasonable to assume that the rate of growth of the gland in colloid goitre is responsible for the basement membrane change.

Considerable evidence has accumulated that epithelial tissues are the prime contributors to the basement membranes that underlie and support them. This evidence has been collected with such varied techniques as conjugated antibodies, silver nitrate stains, and tritiated glucose. (Pierce et al., 1962, 1963; Pierce et al., 1964; Mukerjee et al., 1965; Kurtz and Feldman, 1962; Nadol and Gibbons, 1970). Any alteration or renewal of thyroid basement membrane would be the major "responsibility" of the appropriate synthetic mechanisms in the thyroid gland. The change in basement membrane thickness in colloid goitre could be due to a failure of the gland to synthesize additional basement membrane material when the gland was growing most rapidly, the rapid growth causing the basement membrane to stretch. If stretching is a tenable hypothesis, then examination of the chemical nature of the basement membrane must show a composition or structure that would permit stretching to occur.

The chemical composition of basement membranes has

been studied by Mukerjee et al. (1965) and by Misra and Berman (1966, 1968). Protein is the major constituent (about 80%) and the carbohydrates are primarily reducing sugars and amine sugars. Diseased glomerular basement membrane has an increased cholesterol content and the glycoproteins are much altered, probably through the linkages of sialic acid to other constituents with the glycoprotein. However, the chemical changes were not enough to explain all the changes in kidney physiology. Misra and Berman (1968) speculate that the disease also brings about changes in the arrangements of constituents. Certainly, in experimental nephrosis, there are changes in both electrophoretic mobility and the diffraction patterns of glomerular basement membrane, probably due to loss of phospholipid and increased ordering within the crystal lattice (Kalant et al., 1966). Gang et al. (1970) confirm the loss of phospholipids in experimental nephritis, this loss coinciding with the onset of albuminuria and the permeability of the glomerular basement membrane to lanthanum hydroxide, a small, electron-dense particle. The basement membrane would seem to be fairly labile. Friederici (1965) observed that capillary basement membrane would repair a gap formed by the extrusion and budding off of a projection of endothelium. Whole lymphocytes have been found within the boundaries of basement membrane of normal thyroid, and yet no breaks were observed in the basement membrane (Irvine and Muir, 1963; Toujas et Guelfi,

1969). The different filtration properties of glomerular basement membrane of diseased kidney are thought to be due to a less cross-linked structure (Huang et al., 1967; Gang et al., 1970; Farquhar and Palade, 1960; Misra and Kalant, 1966). Larger molecules can pass through the basement membrane in nephrosis and nephritis (ferritin - Farquhar and Palade, 1960; labelled glucose and urea - Gelke et al., 1966a, 1966b; lanthanum hydroxide - Gang et al., 1970). These filtration changes are not attributed solely to chemical changes (Misra and Kalant, 1966; Gang et al., 1970).

Friederici et al. (1966), in studies on experimental scurvy, found a much thinner basement membrane surrounding affected capillaries. Affected capillaries are characterized by increased cell size, and increased synthetic and secretory apparatus. These authors cannot explain why the capillary cells are active and yet there is a reduction in basement membrane material, when the capillary presumably produces much of its own basement membrane. In scurvy, the size of the cells is much increased, all but obliterating the lumen. Friederici et al. (1966) give no measurements, but it is possible that the increased cell size also increases the circumference of the capillaries. The basement membrane would thus be stretched.

Another instance of a thinner basement membrane is found in Hashimoto's thyroiditis (Irvine and Muir, 1963). This is not the result of growth rate, as Hashimoto's

thyroiditis develops over several years. The basement membrane in normal human thyroid is a very unusual structure, much reduplicated and multilayered. Its like is not seen in other tissues. The reduplication and multilayeredness is not a fixation artifact, else Irvine and Muir would not have found it only in normal tissue, nor can it be reproduced by deliberate poor fixation (Mackenzie, 1966). There are no micrographs available showing how the basement membrane may change from what is found in normal thyroid to the thinner, single-layered basement membrane in Hashimoto's thyroiditis.

Hashimoto's thyroiditis is one of several naturally occurring thyroid disorders in humans. From only two of these disorders can there be recovery. One is the goitre due to dietary iodine deficiency, which is corrected by the addition of iodine to the diet. The other is adolescent idiopathic colloid goitre. This goitre occurs at the onset of puberty and spontaneously regresses later in adolescence (Nilsson, 1966).

Many goitres can be mimicked in laboratory animals, but when the experimental stress on the thyroid is removed, the animals recover. In humans, a high incidence of thyroid disorders within families indicates that there is a genetic predisposition toward goitres. Inborn errors in structure or metabolism will not be observed until there is some stress on the thyroid (Nilsson, 1966; Fraser, 1969). Experimental animals are probably able to recover from

induced thyroid disorders because all such errors would have been selected against and there would be no predisposition remaining in the stock. When goitre is induced, the lability of the basement membrane would prevent total disorganization during hyperplasia. Maintenance of gland integrity would aid recovery when the stress was removed.

Ultrastructural changes in the basement membrane, other than reduced thickness, were not observed in this series of experimentally induced hyperplasias. Those reports that described thickening or structural changes dealt with naturally occurring pathological conditions for which genetic predisposition, also, was often evident. It is likely that these authors were describing syndromes, and the changes they observed were not due directly to to a single causative factor. Indeed, Siperstein et al. (1968) examined animal models for thickening of basement membrane in muscle capillaries. Chinese hamster with spontaneous non-diabetic hyperglycemia did not show any increase in thickness of basement membrane, such as is found in diabetes.

It is, therefore, unlikely that interrupting only one phase of thyroid metabolism would induce spectacular changes in the structure of the basement membrane. While correlation does not necessarily imply a cause and effect relationship, the close correlation between rate of growth and basement membrane change make the hypothesis of basement membrane stretching a very tenable one. This hypothesis

is also consistent with the changes found in perchlorate goitre where rate of growth is more variable.

Summary

1. Hyperplasia was induced experimentally in rat thyroid to study the effect of hyperplasia on the basement membrane.
2. Hyperplasia was induced by hemithyroidectomy, by feeding perchlorate ion (parenchymatous goitre), and by injecting propylthiouracil and allowing a recovery period (colloid goitre).
3. The enlarged thyroids were examined by electron microscopy and the basement membrane width subjected to stereological analysis.
4. There was no significant change in basement membrane ultrastructure in hemithyroidectomy or in parenchymatous goitre. A significantly thinner basement membrane was found in colloid goitre.
5. The correlation between fast growth rate and thinner basement membrane in colloid goitre suggest that the basement membrane had been stretched. Existing knowledge of structure and chemical composition of basement membrane lends support to this conclusion.

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