LCM 2 (*Supplement—South African Journal of Laboratory and Clinical Medicine*)

THYROIDAL IODOPROTEINS IN A CONGENITAL GOITRE*

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In cattle with congenital goitre[1] the organic iodine content per weight of thyroid tissue is abnormally low, while the thyroidal iodine uptake and the serum protein-bound iodine (PBI) are greatly elevated.[2] The raised serum PBI is largely due to the presence of an iodo-albumin since approximately 70% of the serum proteins precipitate with antiserum active against bovine serum albumin. The goitre contains an abnormal thyroglobulin.[3]

Despite a high thyroidal iodotyrosine de-iodinase activity, mono- and di-iodotyrosines (MIT and DIT) appear in blood and urine and, despite an elevated thyroid acid-protease activity, iodoproteins occur in blood.[2] The origin of these iodo-amino acids and iodoproteins and their role in the aetiology of the goitre are unknown.

The present study was undertaken to locate the main sites of the dehalogenase and protease activities and to compare the iodine content of the particulate and soluble fractions in the goitre with that of bovine thyroid tissue. Since it also appeared from a previous study[3] that the abnormal thyroglobulin is a relatively large molecule which is less symmetrical than thyroglobulin, further experiments were performed to study the behaviour of the abnormal thyroidal iodoproteins during gel filtrations with a view to their partial purification and ultimate isolation.

MATERIAL AND METHODS

Stable iodine and nitrogen analyses on soluble and particulate thyroid fractions and *in vitro* experiments for the localization of thyroidal enzyme activities were performed on a thyroid gland (110 G) obtained from a stillborn calf (Fig. 1) belonging to a herd of Afrikander cattle selectively bred for congenital goitre at the Onderstepoort Veterinary Research Institute. A fresh young calf thyroid was collected from the Maitland Abattoir and served as control. Gel filtration was done on thyroid extracts prepared from a goitrous cow and a normal cow of the same strain. Both animals received intravenous injections of 400 μc ^{125}I 2 days, and 200 μc ^{131}I 2 hours before thyroidectomy.

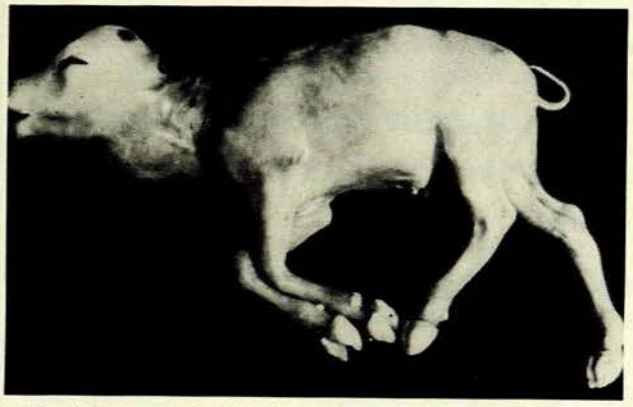

Fig. 1. Congenital goitre in a stillborn calf bred selectively for this defect at the Onderstepoort Veterinary Research Institute.

Thyroid glands were frozen on dry ice and sealed in cellophane bags which were packed in dry ice in a thermosflask and sent by air from Onderstepoort to Cape Town. They reached our laboratory within 6 hours after thyroidectomy.

Preparation of Particulate and Soluble Fractions

Equal weights of thyroid tissue from a stillborn, goitrous and a normal calf were homogenized in 0·25M sucrose at 4°C, and at pH 7·4. From these homogenates the following fractions were prepared by centrifugation at 4°C:

(*a*) a nuclear fraction, separated at 700 × g for 15 minutes,

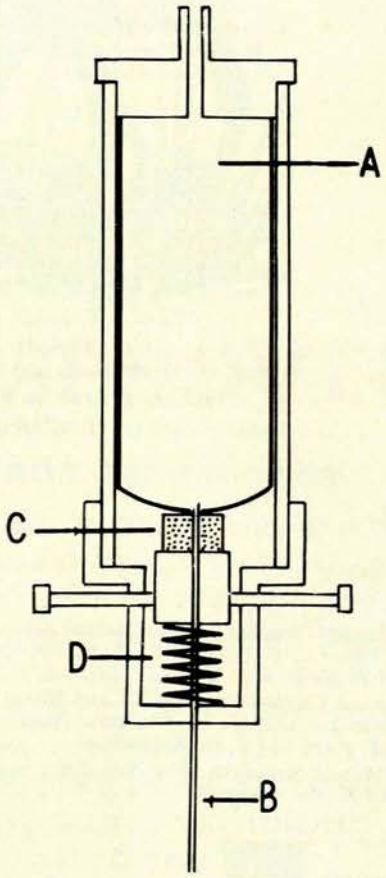

Fig. 2. Modified fractionator for linear sucrose gradient sampling. A = cellulose nitrate tube; B = needle; C = soft rubber gasket; D = spring-mechanism controlled by a trigger. Each drop contains 0·016 ml., thus yielding approximately 60 fractions for tubes used in the SW 25·1 swinging bucket.

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(*b*) a mitochondrial fraction, separated at 8,500 × g for 15 minutes,
(*c*) a microsomal fraction, separated at 105,000 × g for 60 minutes, and
(*d*) a soluble fraction which consisted of the supernatant of the microsomal fraction.

Each fraction was tested for its de-iodinase and protease activities as described previously and for its iodine nitrogen ratio (I/N) by making use of the iodine analysis as described by Wilson and Van Zyl[4] and nitrogen analysis according to Vogel.[5]

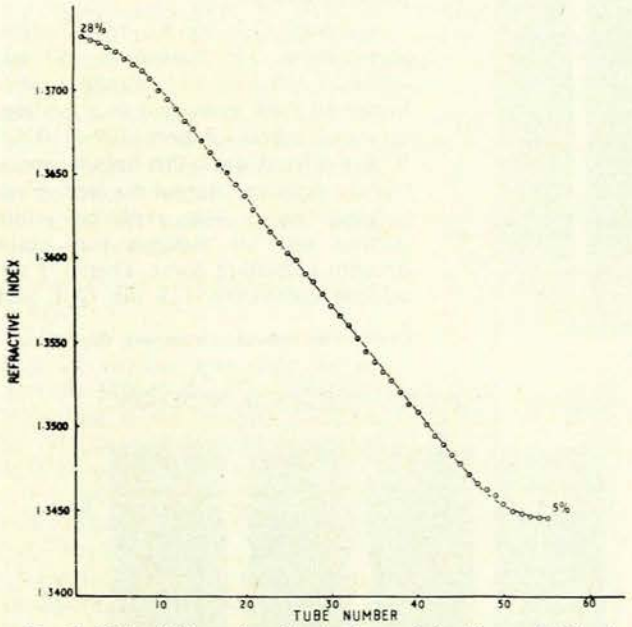

Fig. 3. Plot of the refractive indices of fractions obtained by 5-28% sucrose gradient preparation samples by the fractionator illustrated in Fig. 1.

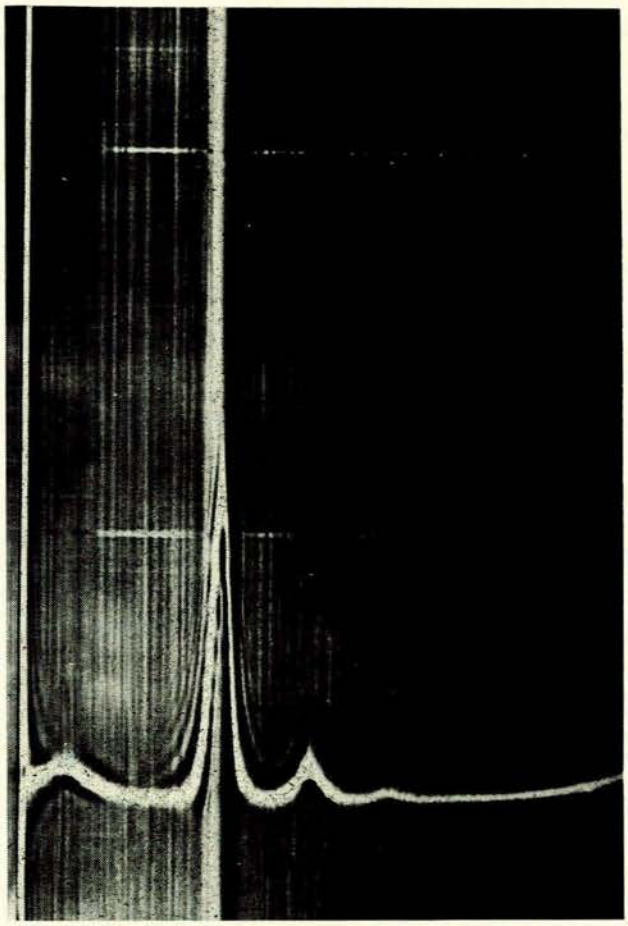

Fig. 5. Moving boundary ultracentrifugation from left to right of normal calf thyroid extract, indicating the same protein fractions as obtained by sucrose gradient analysis. Protein concentration 0·49%; speed 56,000 r.p.m.; bar angle 60°. S 19, S 27 and S 32 proteins are clearly visible.

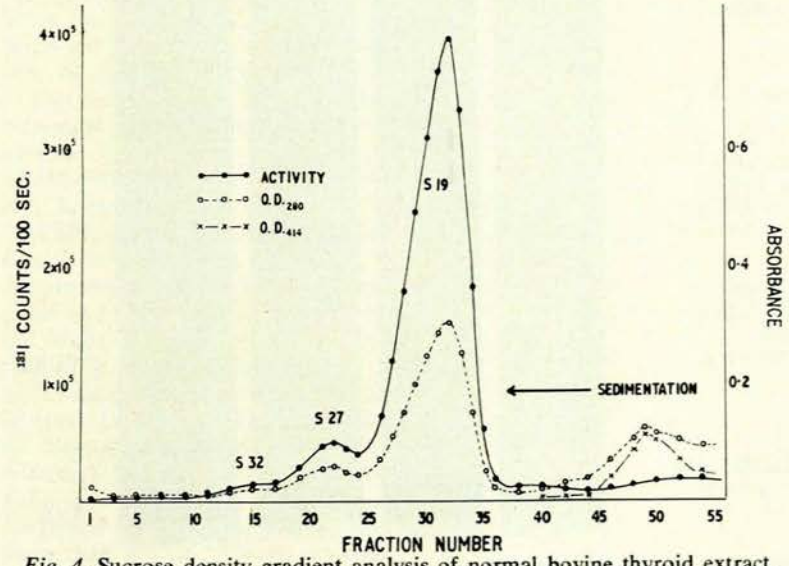

Fig. 4. Sucrose density gradient analysis of normal bovine thyroid extract, showing thyroidal iodoproteins S 19 (thyroglobulin), S 27 and S 32. Arrow indicates the direction of sedimentation.

Preparation of Soluble Proteins for Exclusion Chromatography Analysis

Frozen thyroid tissue was partially thawed out and while in a semi-solid state it was sliced with a Stadie-Riggs tissue-slicer.

The slices were suspended immediately in a 0·1M KCl-0·02M PO_4 buffer, pH 7·4, at a ratio of 2 ml. buffer/1 G tissue, for a period of between 1 and 6·5 hours, depending on the time of slicing. During this period the beaker containing the slices and buffer was immersed in a container with ice, thus controlling the temperature at approximately 4°C. Subsequently the material was extracted for 2 hours by mechanical stirring in a cold room at 4°C and centrifuged in a Sorvall apparatus at 31,500 × g for 30 minutes. It was then filtered through glass wool which removed most fatty material. The supernatant was again centrifuged as before. The material was finally cleared in a Spinco Model L Ultracentrifuge for ½ hour at 105,000 × g before

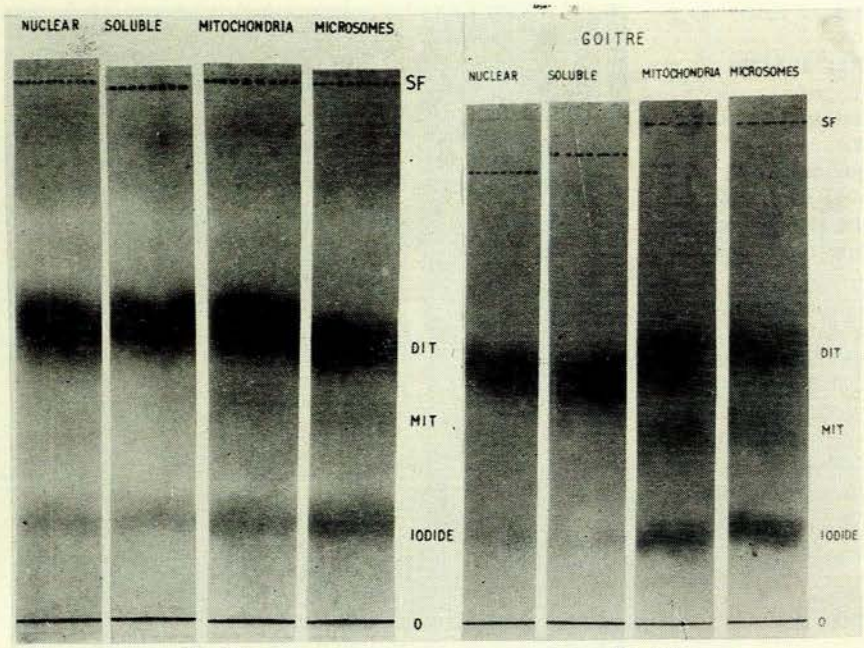

Fig. 6(a) *Fig. 6(b)*

Fig. 6. Di-iodotyrosine de-iodinase activity in the nuclear, soluble, mitochondrial and microsomal fractions of normal (*a*) and goitrous (*b*) thyroid preparations. SF = solvent front; DIT = di-iodotyrosine; MIT = mono-iodotyrosine; O = origin.

it was dialysed against fresh KCl-PO_4 buffer used for column chromatography.

Preparation of Soluble and Insoluble Thyroid Fractions for the Measurement of Radioactive Iodine Distribution

For the distribution of radioactive iodine in soluble and insoluble thyroid fractions from normal and goitrous cows which received ^{125}I and ^{131}I, 'representative slices' were analysed, for which purpose 2·28 G of sliced goitre and 0·60 G of normal tissue were used. The procedure followed is depicted in Table I:

TABLE I. PREPARATION OF THYROID FRACTIONS

Thyroid Slices
Stirred in KCl-PO_4 pH 7·35 for 2 hours (2 ml./G), centrifuged (15,000 r.p.m. for ½ hr)

Sediment 1 — *Super 1*
Re-suspended in KCl-PO_4, stirred for 2 hours. Centrifuged (15,000 r.p.m. for 20 min.)

Sediment 2 — *Super 2*
Homogenized. Centrifuged (15,000 r.p.m. for ½ hr)

Sediment 3 — *Super 3*
Re-suspended and washed with KCl-PO_4. Centrifuged (15,000 r.p.m. for 20 min.)

Sediment 4 — *Super 4*

All fractions were brought to the same volume and counted in a Packard auto-gamma counter.

Preparation of Agarose-beads for Chromatography

Basically the method of Hjerten* was followed. After several unsuccessful attempts using Tween 61 (polyoxyethylene sorbitan monostearate) as emulsifier, batches of beads were prepared as follows:

Agarose* 7·5 - 25 G for 3 - 10% preparations was heated in 250 ml. water in a 2 litre wide-mouth round-bottomed flask immersed in a boiling, saturated saline solution (109 - 110°C). It was stirred until the homogeneous viscous solution started to boil. Evaporated water was replaced while stirring and the mixture was again brought to boiling point. Then 375 ml. toluene containing 125 ml. CCl_4 and

*Supplied by Seravac Laboratories, Cape Town.

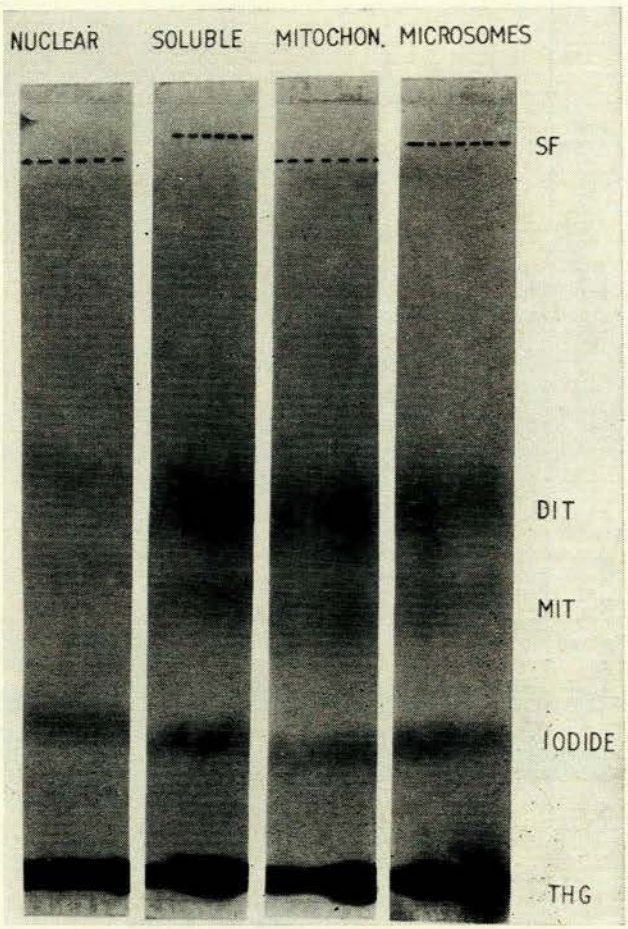

Fig. 7. The release of iodide, mono-iodotyrosine (MIT) and di-iodotyrosine (DIT) from ^{131}I-labelled thyroglobulin (THG) after 1 hour incubation at pH 5·3 of nuclear, soluble, mitochondrial and microsomal preparations obtained from a goitrous calf thyroid.

5 - 15 G Emulphor EL† as stabilizer was rapidly added, with the simultaneous emptying of the salt-water bath. Stirring at a full speed was started immediately, using a 5/16 inch silver-steel rod with a 2 inch stainless-steel propeller blade. Stirring was continued for 1 minute at elevated temperature before the mixture was rapidly cooled in iced water. Only when the mixture was at room temperature was the stirring stopped. The mixture was transferred to 250 ml. centrifuge cups, suspended in methanol (laboratory grade) and centrifuged at 2,000 r.p.m. for 5 minutes. The washing with methanol was repeated until the supernatant gave no milkiness upon treatment with water in a test-tube. As the organic solvents and stabilizer were removed the pearls changed in colour from white-opaque to yellowish-translucent. The diameter of the beads lay between 30 and 200 μ; approximately 60% were between 50 and 100 μ. All pearls had a spherical form. Preparations were suspended in de-ionized water and packed in columns by sedimentation under reduced pressure.

Linear Density Gradient Ultracentrifugation

Density gradient ultracentrifugation in sucrose was done in the Spinco Model L Ultracentrifuge according to the method described by Martin and Ames[7] and Salvatoré *et al.*[8]

For sampling of fractions after centrifugation, the tube containing the material and gradient is placed into a holder, supplied by a spring-mechanism and trigger‡ so that a No. 20 gauge needle (0·9 mm.) is shot into the bottom of the cellulose nitrate tube without upsetting the gradient or increasing the pressure as may happen when a screw mechanism is required to force the needle through the bottom of the tube (Fig. 2).

This technique was tested out by refractive indices on a 5 - 28% sucrose gradient in 0·1M KCl - 0·02M PO_4 and gave reproducible and linear gradients (Fig. 3).

When applied to a crude extract of calf thyroid, S 19, S 27 and S 32 protein peaks (Fig. 4), corresponding to the Schlieren pattern of the Model E analytical ultracentrifuge (Fig. 5), were obtained.

^{121}I-labelled thyroglobulin and ^{131}I-labelled DIT were prepared from rat thyroids as described earlier.[2] Although the radioactive thyroglobulin was a crude preparation, it contained no inorganic iodine. Not more than 4% of ^{131}I were released in blank analyses during incubation at 37°C in Tris maleic buffer, pH 5·2, for periods up to 7 hours.

†Badische Anilin and Soda Fabr. AG.
‡Designed and constructed by Mr L. Soskolski, Workshop, Karl Bremer Hospital.

RESULTS

Thyroidal Iodotyrosine De-iodinase and Thyroglobulin Acid-Protease Activities

The 4 fractions prepared from thyroid tissue of a stillborn calf with congenital goitre and a normal calf were tested for their de-iodinase and protease activities. Tubes were incubated at 37°C while being constantly shaken and 20 μl. samples were applied to Whatman No. 1 chromatography paper after incubation periods of 10 min., 1 hour, 3 hours and 7 hours. The samples were chromatographed in butanol equilibrated with 2N acetic acid by the ascending technique for 16 hours.

Nuclear and soluble fractions of both normal and goitrous thyroid tissue contained no de-iodinase activity.

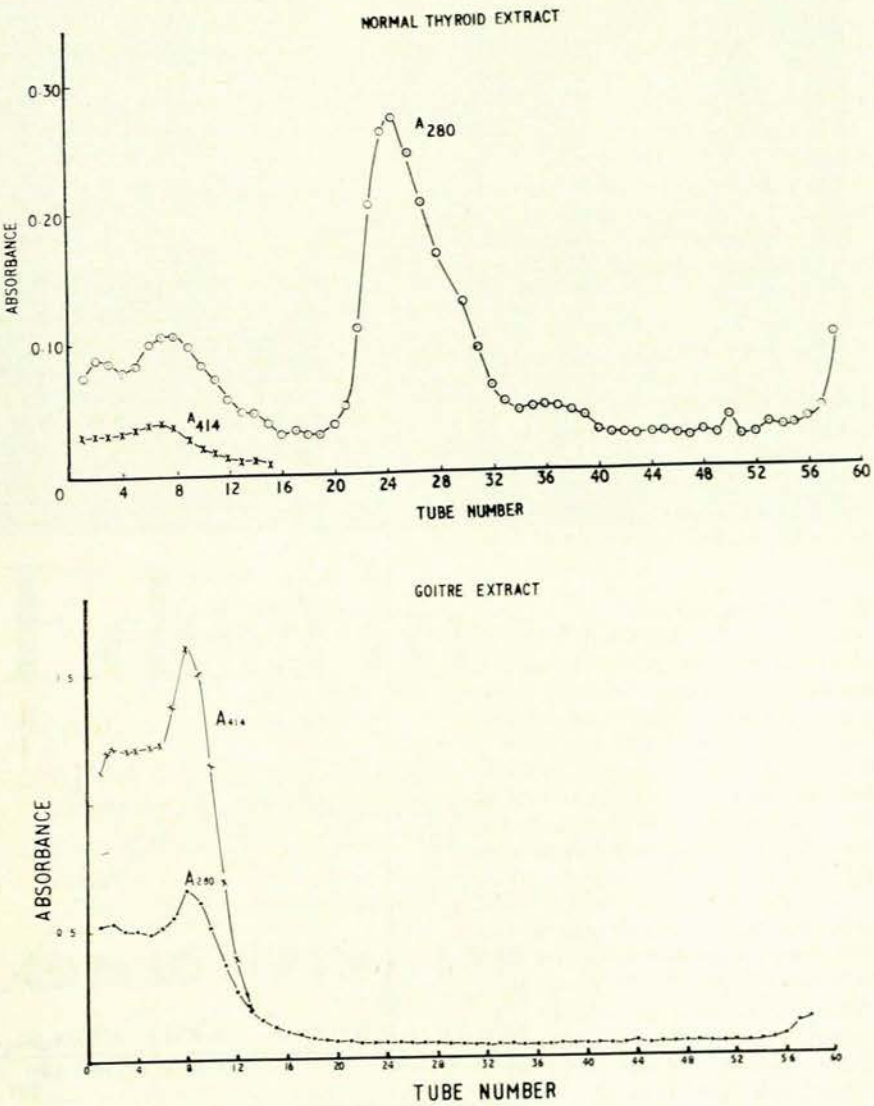

Fig. 8. Sucrose gradient analysis of normal calf thyroid extract (*above*) as compared with a foetus goitre extract (*below*), indicating the position of haemoglobin (OD_{414}) and the lack of thyroglobulin in the goitre.

Even after an incubation period of 7 hours the percentage radioactivity in the iodide band did not exceed that of the blank (Fig. 6(*a*) and (*b*)).

Although de-iodinase activity is present in both normal and goitrous mitochondrial and microsomal preparations, particulate fractions from the goitrous animal showed greater activity than those in normal thyroid glands. However, this difference between foetal calf thyroid and normal calf is not so extensive as was previously observed in older animals.[2] These results confirm that the congenital defect cannot be ascribed to a thyroidal de-iodinating system defect and that the iodotyrosine de-iodinase activity is in excess of that which is observed in normal bovine thyroid tissue, even at birth.

Acid-protease activities were demonstrated in all fractions of goitrous thyroid preparations after 1 hour incubation with ^{131}I-labelled thyroglobulin although the nuclear fraction was least active (Fig. 7).

In the nuclear and soluble fractions obtained from normal thyroid tissue ^{131}I-labelled MIT and DIT were not released from thyroglobulin even after 7 hours of incubation. However, in the mitochondrial and microsomal fractions ^{131}I, MIT and DIT were released in both the 4-hour and 7-hour incubation periods.

It is of importance to note that, whereas normally thyroid-protease activity seems to be located in the mitochondria and microsomes alone, in goitrous thyroids thyroglobulin degradation could be demonstrated in particulate as well as in soluble fractions.

^{127}*Iodine:Nitrogen Ratios*

Earlier results[2] showed that thyroidal ^{131}I uptake was grossly elevated in the goitrous thyroid, yet the stable iodine per unit weight of thyroid tissue of goitrous cattle was only a fraction of that of control animals. However, since the total thyroidal PBI of the goitre in general was higher than that of the normal bovine thyroid, it was concluded that no block in thyroidal iodination exists in the goitre and that thyroidal hypertrophy more than compensated for the relatively low ^{127}I per weight of tissue.

From a comparison of the ^{127}I: nitrogen ratios in the particulate and soluble fractions in the goitre of a stillborn calf with those of a normal calf thyroid (Table II), it is concluded that the iodine content was highest in the soluble fraction of the normal animal, but lowest in the soluble fraction of the goitre. This may be due to the low thyroxine content of the abnormal thyroglobulin or may indicate a fast turnover rate of iodoprotein in the goitre so that the organic iodine is not stored in the colloid. On the other hand, per unit weight of tissue, the nuclear fraction of the goitrous thyroid contained more nitrogen than that of the normal. This is probably a function of a greater nuclear mass and an indication that mitotic activity during compensatory hypertrophy is already firmly established at birth. The high

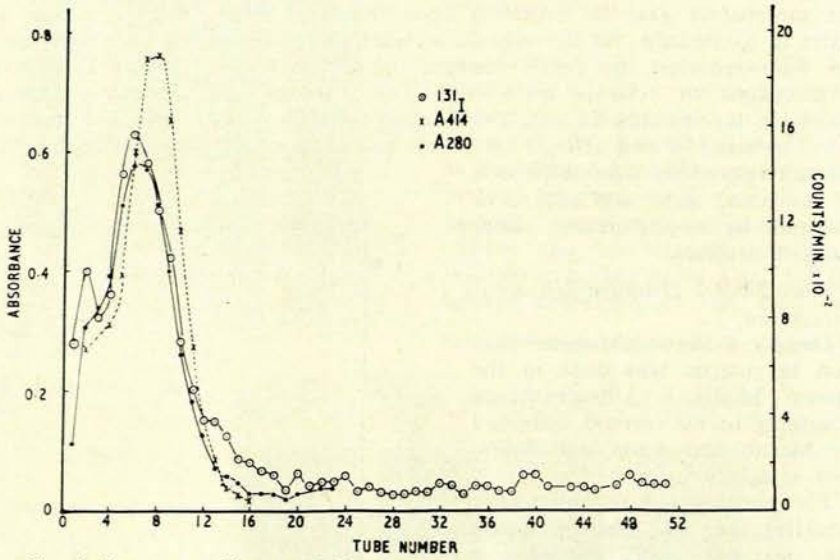

Fig. 9. Sucrose gradient analysis of a thyroid extract prepared from thyroid slices of a stillborn goitrous calf incubated with ^{131}I, showing different sedimentation values for the OD_{280} and OD_{414} peaks.

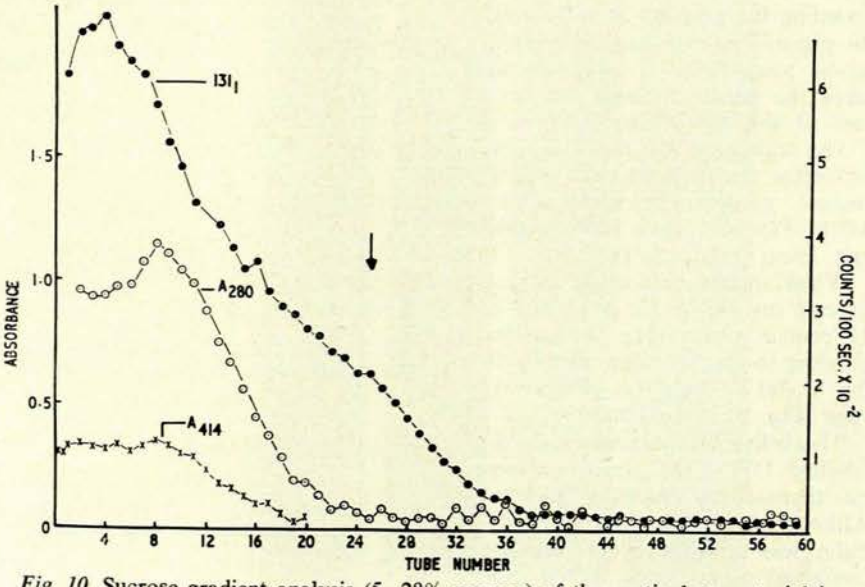

Fig. 10. Sucrose gradient analysis (5 - 28% sucrose) of the particulate material in a goitrous thyroid of a foetus calf. Thyroid slices were incubated with ^{131}I. Particulate material was rendered soluble with deoxycholate. The arrow indicates the position of thyroglobulin.

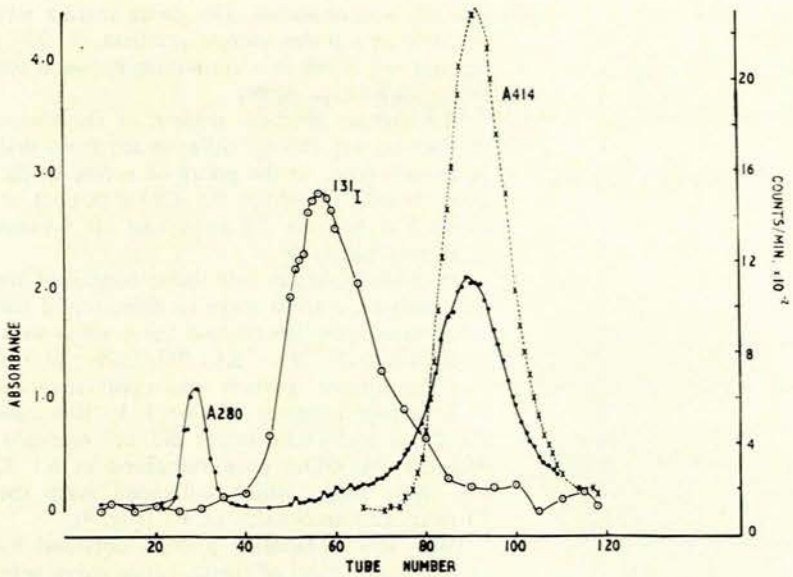

Fig. 11. 3% agarose gel filtration of a thyroid extract obtained from a goitrous calf. ^{131}I-labelled guinea-pig thyroglobulin was added to the thyroid extract to indicate the position of thyroglobulin.

nuclear mass per unit weight of tissue is also due, at least in part, to decreased colloid stores.

In both normal and goitrous thyroid tissue, the highest I/N ratios were found in the mitochondrial and microsomal fractions, whereas the lowest ratios were observed in the soluble and nuclear fractions. In all the fractions the I/N ratios obtained from goitrous thyroid tissue were lower than those of the normal thyroid by a factor of at least 10. The highest I/N ratio differences between the normal and goitrous thyroid fractions obtained in those compartments where no de-iodinase activity could be demonstrated, i.e. in the nuclear and soluble fractions.

TABLE II. IODINE/NITROGEN (I/N) RATIOS IN SOLUBLE AND PARTICULATE FRACTIONS PREPARED FROM THE THYROID GLANDS OF A STILLBORN CALF WITH CONGENITAL GOITRE AND A NORMAL CALF

Fraction	*Animal*	*Iodine (µg./100 ml.)*	*Nitrogen (mg./100 ml.)*	*I/N ratio (µg.^{127}I/mg.N)*
Nuclear	Normal	58·8	31·68	1·86
	Goitre	8·5	91·67	0·09
Mitochondrial	Normal	59·1	11·28	5·25
	Goitre	10·6	20·08	0·53
Microsomal	Normal	39·1	6·29	6·21
	Goitre	3·6	6·90	0·52
Soluble	Normal	88·0	37·02	2·38
	Goitre	1·65	17·73	0·09

Radioactive (^{131}I and ^{125}I) Distribution between Soluble and Insoluble Thyroid Fractions

One normal and one goitrous cow received 400 µc ^{125}I 2 days and 200 µc ^{131}I 2 hours before thyroidectomy. The distribution of radio-iodine in the goitre and normal thyroid tissue is given in Table III, from which it is clear that the particulate iodine is higher in the goitre (~ 11%) than in the normal (~ 3%), but this difference is less than previously reported.[3] The iodine uptake/G of tissue was approximately the same at 2 hours; i.e. for ^{131}I it was 4,761 c.p.s./G in the normal and 5,350 c.p.s./G in the goitre. However, the 48-hour uptake (i.e. the ^{125}I uptake) was far greater in the normal (1,498,000 ^{125}I c.p.s./G) than in the goitre (392,900 ^{125}I c.p.s./G).

As it can be expected that within a period of 2 hours the process of iodine concentration will exceed subsequent hormonal metabolism and secretion, Table III indicates that the ^{131}I uptake per gram of tissue in the goitre was not defective since it was within the range of that of the normal thyroid. The greater 48-hour ^{125}I uptake of the normal thyroid is indicative of a slower iodine turnover rate in the normal thyroid. However, since the total thyroid weight of the normal animal was 25 G and that of the goitre 338 G, the total 2-hour ^{131}I uptake in the goitre was 18 times greater than that of the normal and 3½ times higher at 48 hours than that of the normal.

Sucrose Linear Density Gradient Analysis

Thyroid tissue of a normal calf and the goitre of a foetus calf were homogenized in 0·1M KCl-0·02M PO_4 buffer at pH 7·4 and cleared in a centrifuge at 105,000 × g. Ten mg. of the soluble proteins were layered on a preformed 5 - 28% sucrose gradient and centrifuged in a SW-25·1 rotor at 23,000 r.p.m. for 22 hours. Total protein

TABLE III. DISTRIBUTION OF RADIOACTIVE IODINE BETWEEN SOLUBLE AND INSOLUBLE FRACTIONS OF NORMAL AND GOITROUS BOVINE THYROID TISSUE 48 AND 2 HOURS AFTER INTRAVENOUS INJECTIONS OF ^{125}I AND ^{131}I, RESPECTIVELY (TOTAL WEIGHT OF NORMAL THYROID WAS 25 G AND OF GOITRE 338 G)

	% of total c.p.s.									
	Goitre		*Normal*		*^{125}I c.p.s./G*		*^{131}I c.p.s./G*		*^{125}I/^{131}I*	
	^{125}I	*^{131}I*	*^{125}I*	*^{131}I*	*Goitre*	*Normal*	*Goitre*	*Normal*	*Goitre*	*Normal*
Super 1 (equivalent to preparation of whole gland)	72·3	74·0	65·7	68·0						
Super 1 + 2 (total slice extract)	84·5	85·4	91·1	92·2	333,000	1,360,000	4,610	4,390	72·2	310
Super 3 + 4 (homogenate super)	3·5	3·2	6·0	5·5	13,800	90,000	167	262	82·7	343
Sediment 4 (particulate iodine)	11·7	10·9	3·3	2·5	46,100	48,000	573	109	80·3	404

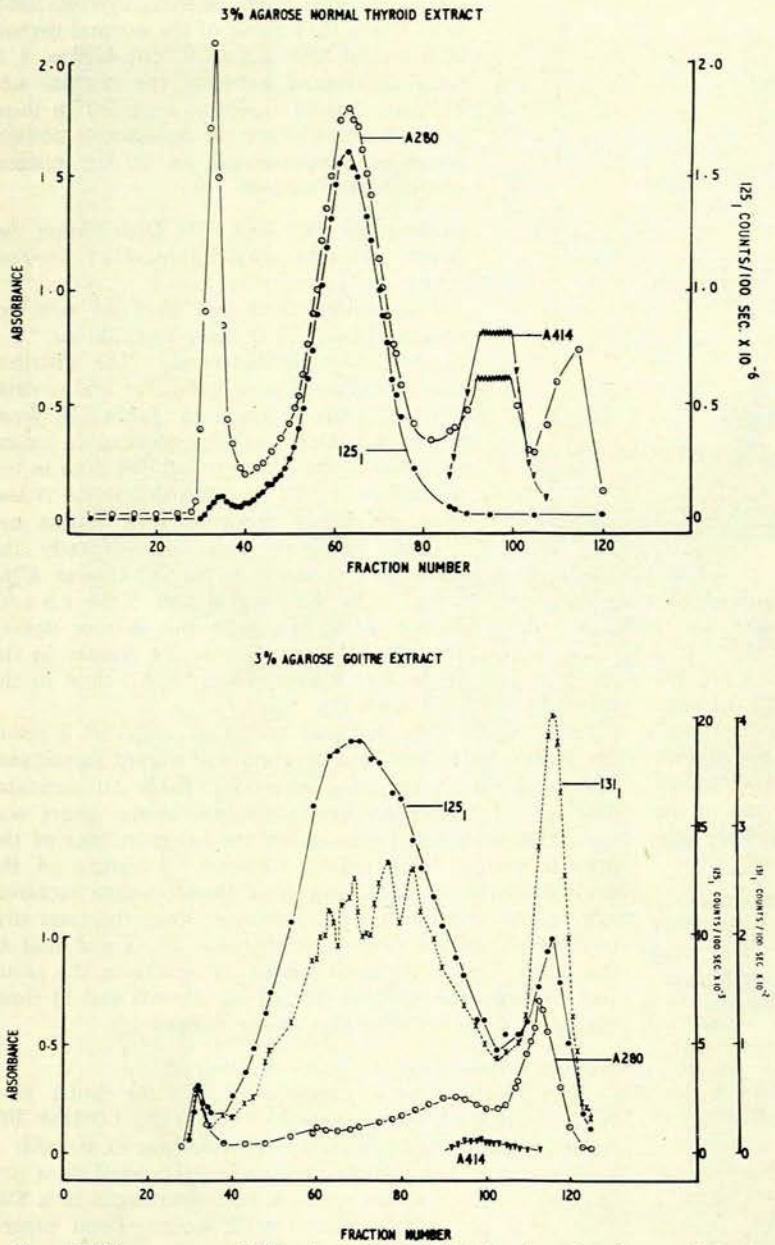

Fig. 12. 3% agarose gel filtration of normal (*above*) and goitrous (*below*) thyroid extract of adult cows.

concentrations were calculated from optical density readings by making use of a specific extinction coefficient of 10·5 at 280 mμ or of 207 at 210 mμ. Approximate sedimentation coefficients were calculated as follows:

$$S = \frac{\text{fraction number of peak} \times 19}{\text{fraction number of the thyroglobulin peak.}}$$

The pattern of the normal calf thyroid extract in Fig. 8 (*above*) shows the major peak as thyroglobulin (S 19) and a protein with greater sedimentation (S 27). The protein (OD_{280}) coinciding with haemoglobin (OD_{414}) had an S-value of 6·1. In the goitre extract (Fig. 8 (*below*)), the OD_{280} peak coincided with that of haemoglobin. No thyroglobulin was observed. The goitre extract was repeated in a lower sucrose gradient (5 - 20%) but did not result in a resolution between the OD_{280} and OD_{414} peaks.

The sucrose gradient pattern of the foetus thyroid extract did not differ much from that observed earlier in the goitre of a cow of the same breed,[3] in which the OD_{280} peaked at about S 6 but the ^{125}I peak had an S-value of approximately 9.

Since the goitrous calf tissue contained no radioactive iodine to serve as indicator of the iodoprotein, goitrous thyroid tissue slices were incubated with ^{131}I in KCl-PO_4 buffer at pH 7·4. The soluble fraction was again analysed in a sucrose gradient (5 - 28%). In this case the OD_{280} and OD_{414} peaks did not coincide. Whereas the OD_{414} peak remained at 6·1 S, the OD_{280} peak, which coincided with the ^{131}I peak, had an S-value of 4·3 (Fig. 9).

Thus, the ^{131}I-labelled protein obtained by *in vitro* incubation of foetus goitre slices with ^{131}I differed in its sedimentation properties from those of thyroglobulin and haemoglobin. It also differed from the 9 S value observed earlier in an adult cow which received ^{125}I 24 hours before thyroidectomy.

Because of an approximately even distribution of ^{125}I in the goitrous thyroid between the soluble and particulate fractions observed earlier,[3] the particulate material of the goitre was rendered soluble with deoxycholate and with digitonin according to Nunez *et al.*[9] and dialysed against KCl-PO_4 buffer. The soluble particulate material showed sedimentation in a sucrose gradient over a broad zone which peaked at 3 S and overlapped with that of thyroglobulin. The OD_{280} and OD_{414} curves coincided and peaked at 6 S (Fig. 10).

Using sucrose gradient analysis and OD_{280} or radioactive iodine or both as indicators, different S-values have been observed in the thyroids of goitrous cattle, namely 9 S material when ^{125}I was administered *in vivo*,[3] a 4 S component in the soluble proteins and, when the thyroid of the stillborn goitrous calf was incubated with ^{131}I, a 3 S fraction was observed in the particulate proteins rendered soluble.

Exclusion Chromatography of Soluble Proteins (Agarose Gel Filtration)

The ability of various concentrations of agarose beads (3 - 10%) to fractionate thyroidal iodoproteins of different species was examined previously. In these analyses it was observed that a 3% agarose concentration can be used to prepare thyroidal iodoproteins in a one-step procedure, thus separating them from serum proteins (unpublished data). In all animal thyroids examined, a 'prethyroglobulin' peak appeared with the exclusion volume when 3% agarose was used.[10]

Since high concentrations of agarose should theoretically yield better separations of proteins smaller than

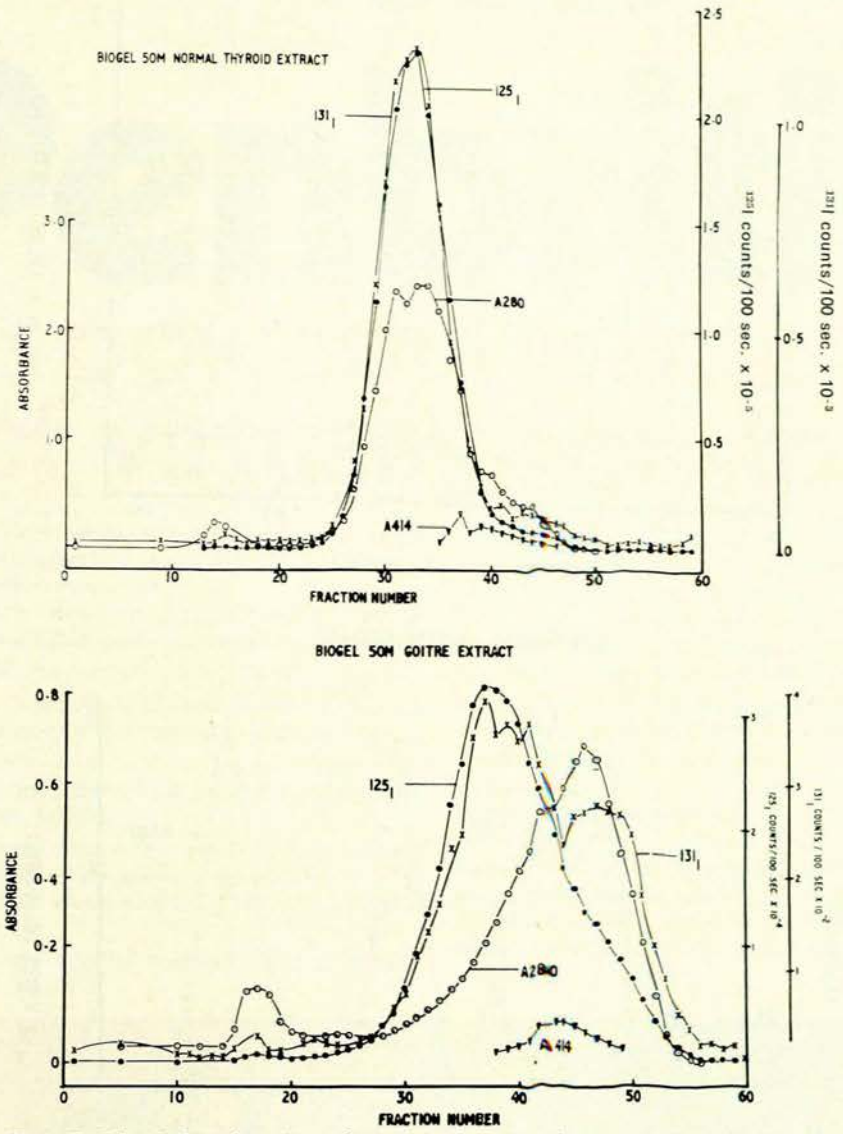

Fig. 13. Biogel 50M filtration of normal (*above*) and goitrous (*below*) thyroid extracts.

thyroglobulin, and since earlier results on abnormal thyroglobulin in goitrous cattle indicated more than one iodoprotein component, it was of interest to study the behaviour of normal and abnormal thyroidal iodoproteins in 3% and 10% agarose bead concentrations or their equivalents of other gels.

Gel Filtration in 3% Agarose and Biogel 50M (Equivalent to 2% Agarose)

When the goitre extract of the foetus was chromatographed on a 3% agarose column, the material separated into an OD_{280} peak which appeared with the exclusion volume (prethyroglobulin peak) and a second OD_{280} peak which coincided with haemoglobin (OD_{414}). In order to indicate the position of thyroglobulin in the eluate, 25 μg. of ^{131}I-labelled guinea-pig thyroglobulin was added to the goitrous thyroid extract. This concentration of labelled thyroglobulin was too low to influence the OD_{280} pattern of the fractions but high enough in activity to use the counts as indicators of the position of thyroglobulin. The mixture was subsequently chromatographed again in 3% agarose (Fig. 11).

The large amount of blood in the thyroid of the foetus is indicated by the difference in height between the OD_{414} and OD_{280} peaks. The OD_{414} peak, as in the case of sucrose gradient analysis (Fig. 9), did not exactly coincide with that of the main OD_{280} peak; the latter appeared slightly ahead of haemoglobin, indicating that the behaviour of the thyroidal iodoprotein in the goitrous thyroid was very similar to that of haemoglobin in 3% agarose (i.e. exclusion volumes between 178 and 184 ml.), while the exclusion volume of labelled thyroglobulin was 114 ml.

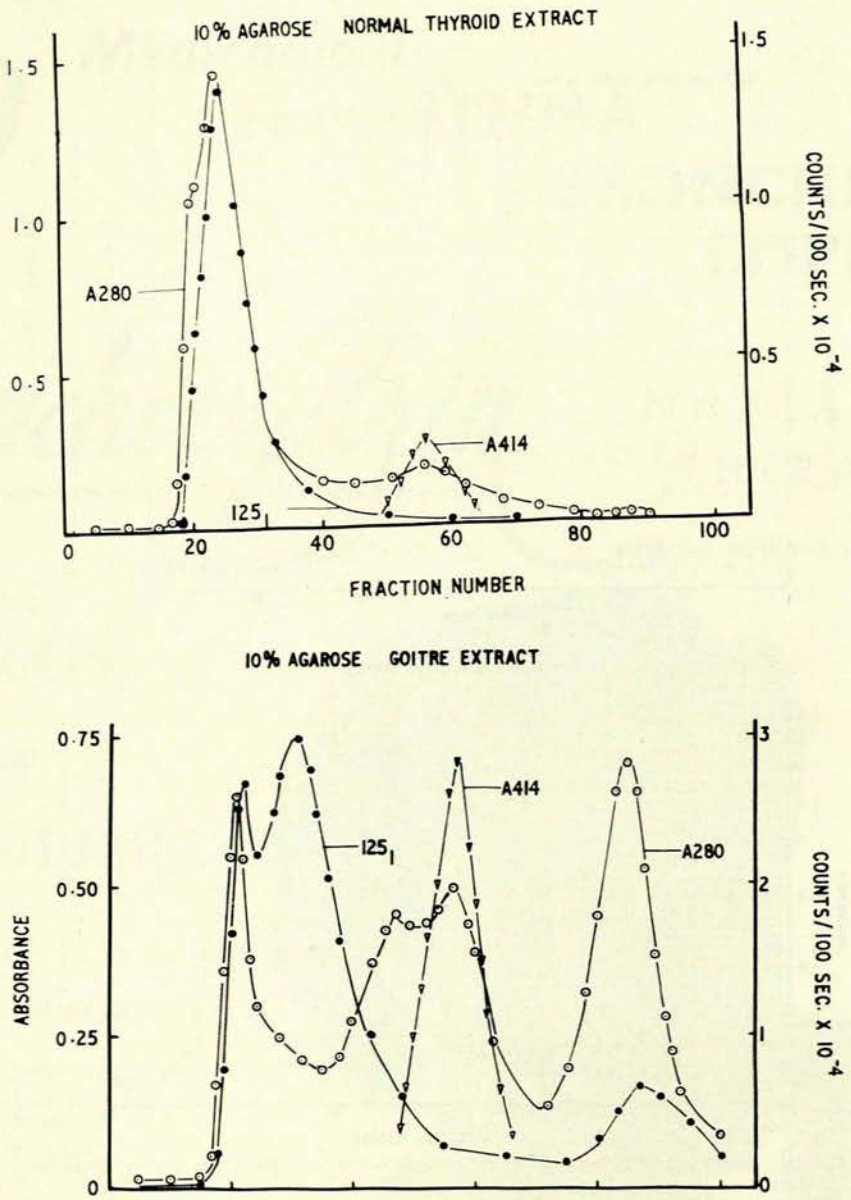

Fig. 14. 10% agarose gel filtration of normal (*above*) and goitrous (*below*) thyroid extracts of adult cattle.

Thyroid extracts of adult normal and goitrous animals which received ^{125}I and ^{131}I 48 hours and 2 hours before thyroidectomy respectively, were also chromatographed in 3% agarose and in Biogel 50M. The 3% agarose filtration of normal thyroid extract (Fig. 12 (*above*)) seems to be useful in so far as it purifies thyroglobulin from aggregates and from smaller proteins such as serum proteins. Although the goitre of the adult animal contained less haemoglobin than that of the foetus thyroid, the indications are that 3% agarose partially differentiates between the abnormal thyroglobulin and serum proteins (Fig. 12 (*below*)). In the pattern obtained from the normal thyroid the ^{125}I curve was identical with that of ^{131}I. However, in the goitre the ^{131}I (given 2 hours before thyroidectomy) was incorporated into the albumin region to a greater extent than ^{125}I (given 48 hours before thyroidectomy). The relatively greater incorporation of ^{131}I into the serum protein region than into the abnormal thyroglobulin peak and the greater ^{125}I activity of the abnormal thyroglobulin peak is evidence of a slower iodine turnover in the abnormal protein. The activity and OD_{280} peaks in the posthaemoglobin region are likely to be due to iodination of albumin, and are consistent with the high serum PBI in goitrous animals, which consists mainly of iodo-albumin and is probably of thyroidal origin. The high specific activity of the abnormal thyroglobulin shows that 3% agarose is useful for an extensive degree of purification of the protein.

In general, Biogel 50M did not show any advantage

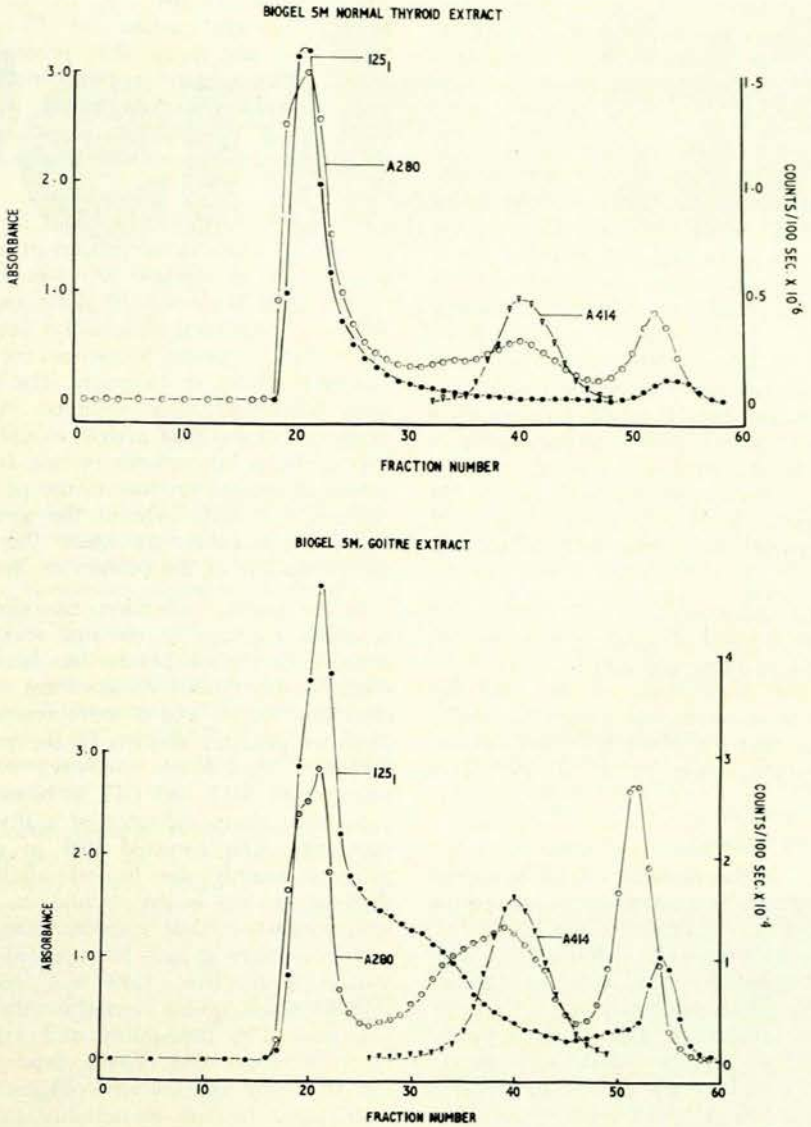

Fig. 15. Biogel 5M filtration of normal (*above*) and goitrous (*below*) thyroid extracts of adult cattle.

over 3% agarose in the separation of abnormal thyroglobulins from the serum proteins. Prethyroglobulin peaks were observed in the Biogel 50M chromatogram patterns of both normal and goitrous thyroid extracts (Fig. 13).

Similar to the findings in 3% agarose with normal thyroid extract, filtered through Biogel 50M (Fig. 13 (*above*)), the ^{131}I distribution coincided with that of ^{125}I, whereas in the goitre (Fig. 13 (*below*)) the ^{131}I peak was shifted towards smaller protein fractions and showed an extra peak in the albumin region. The separation was not so complete as in 3% agarose. Although the flow-rates in the Biogel 50M columns were not identical for the normal and goitre runs, the normal and goitrous peaks are quite similar relative to haemoglobin. The main differences between them were the breadth and asymmetry of the abnormal thyroglobulin peak and the fact that it extends through the 'albumin' area. In fact, it seems as if there is ^{125}I-labelled albumin just as there is ^{131}I-labelled albumin, but the amount is lower and thus forms a tail on the main peak rather than a separate peak.

Filtration through 10% Agarose and Biogel 5M (Equivalent to 6% Agarose)

In both normal and abnormal thyroid extracts filtered through 10% agarose or Biogel 5M, no prethyroglobulin peaks were observed. In all patterns the activity shifted towards the right of the OD_{280} exclusion peaks. This therefore shows that neither 10% agarose nor Biogel 5M can be used for thyroid extracts to separate and purify normal and abnormal iodothyroproteins from non-iodinated aggregates. The exclusion peaks obtained from 10% agarose and Biogel 5M can be further purified by sending them

through 3% agarose or Biogel 50M columns. Previous experiences (unpublished data) showed that 4 - 5% agarose concentrations only separate protein aggregates partially from thyroglobulin and that such a separation is complete in agarose concentrations of 3% agarose or less.

In the 10% agarose filtration of the goitre extract (Fig. 14 (*below*)), the radioactivity separated into two non-dialysable peaks in the globulin region and one dialysable peak in the postalbumin zone. Of the two peaks in the abnormal thyroglobulin region, the one with a higher exclusion volume had greater specific activity. This may indicate the existence of an abnormal thyroglobulin component which is either smaller or more symmetrical than other components, so that it is able to enter the pores more readily. This component also seems to be the major iodinated compound and should have a slower sedimentation value than the iodocompound with smaller exclusion volume. Although the results with 10% agarose (Fig. 14) are somewhat similar to those observed with Biogel 5M (Fig. 15), the former seems to be superior to Biogel 5M for use in order to separate the components of the abnormal thyroglobulin.

Analytical ultracentrifugal analysis of the peaks obtained from the agarose and Biogels under investigation showed that none of these gels can be successfully employed for a one-step purification of the abnormal thyroglobulin components in cattle with congenital goitre. They indicate, however, that the abnormal thyroglobulin is heterogeneous in nature, consisting of at least three components.

DISCUSSION

The first demonstration of the presence of an abnormal thyroglobulin was provided by a study of a congenital goitre in an inbred herd of Afrikander cattle. So far no specific defect has been demonstrated in the iodine metabolism of the goitre except for the apparent absence of normal thyroglobulin. In density gradient ultracentrifugation the abnormal thyroglobulin did not resemble the known thyroglobulin sub-units. The radio-iodine in the goitre extract showed an approximate 9 S value although it showed sedimentation over a broad zone which overlapped the ⁓ 6 S and 19 S zones.[3] The present investigation confirms the presence of several iodoprotein components and extends our information on this point. In view of our earlier findings, some of these at least are immunologically related to thyroglobulin.[3] Thyroid slices of the goitre iodinated *in vitro* gave a ⁓ 4 S component in sucrose gradient centrifugation which falls within the 3 - 8 S components considered to be precursors of the 12 S[11,12] and the 5 S component characterized as the smallest sub-unit appearing during the biosynthesis of rat thyroglobulin.[13] The presence of known thyroglobulin sub-units in the goitre therefore cannot be excluded.

Several authors[14-17] investigated thyroglobulin degradation by proteases in normal thyroids and concluded that it is an intracytoplasmic process. The present study in the goitre demonstrates that the soluble and particulate fractions had proteolytic activity. Of these fractions the nuclear preparation was least active. Whereas the soluble fraction of normal bovine thyroid contained no proteolytic activity at pH 5·3, in the goitre this fraction had a powerful action on ^{131}I-labelled thyroglobulin. This does not mean that protease is present in the colloid. Protease activity is in general greater in the goitre and therefore easier to detect in the soluble extract. Furthermore, the protease may have been attached to intracellular particles which are more readily disrupted in the goitre.

The low iodine/nitrogen ratios in the goitre may be due to a partial block in the process of iodination or to greater protease and de-iodinase activities. However, 2 hours after ^{131}I treatment there was no difference between the *in vivo* degree of iodination in goitrous and normal thyroids per unit weight of tissue. Moreover, the total thyroidal iodine uptake is greater in the goitre. The highest iodine/nitrogen ratio differences were observed in the soluble fraction where no de-iodinase activity could be detected. Since in thyroglobulin biosynthesis protein formation and the iodination of tyrosyl residues in the protein are separate procedures,[12,18-20] and since in the goitre the trapping and iodination processes are intact,[2] the defect seems to lie in the formation of the protein or in its rapid degradation.

In the goitre, iodination, de-iodination and proteolytic processes continue at elevated levels. Of these processes only the iodination process has been investigated with respect to the tyrosyl environment in proteins.[21] It is not clear how mono- and di-iodotyrosines can appear in body fluids of goitrous animals in the presence of a powerful thyroidal de-iodinase activity. Nevertheless, the appearance of MIT and DIT in blood and urine cannot be considered as an indicator of a thyroidal de-iodinase abnormality. The elevated PBI in cattle with congenital goitre is mainly due to iodo-albumin in serum. When thyroxine is low in the circulation, increased TSH output will stimulate iodide trapping and its oxidation. Under such conditions it may be expected that the high concentration of reactive iodine will iodinate proteins in the thyroid which, under normal conditions, are not normally iodinated. The probability and extent of iodination of tyrosyl residues in a protein depend upon the concentration of iodine and the environment of the tyrosyl residue with respect to their accessibility, degree of ionization and the degree whereby water is excluded from their environment.[22] In the goitre the MIT/DIT ratio is high and the thyroxine concentration very low.[2,3] Natural thyroglobulin is a highly compact, symmetrical protein, devoid of internal hydration. One of the principal forces stabilizing this natural form of the globular protein is the so-called hydrophobic bond.[21] Gel-filtration experiments indicate that the abnormal thyroglobulin consists of several components which are of asymmetrical nature or are smaller than thyroglobulin. S-values obtained with density-gradient sucrose analyses do not exclude the possibility that at least some of these components represent sub-units of thyroglobulin in the goitre. In such cases the hydrophobic nature and the accessibility of the tyrosyls may, to some extent, explain the elevated MIT/DIT ratio and the low thyroxine content.

Methods of fractionation of proteins which are based on molecular size may not be the most useful for separating protein components of asymmetrical nature and there-

fore should be employed together with other techniques for the ultimate purification of the abnormal components.*

SUMMARY

Nuclear, mitochondrial, microsomal and soluble fractions in the thyroid of a stillborn calf with congenital goitre were analysed for their iodotyrosine, de-iodinase and thyroglobulin protease activities and for their iodine/nitrogen ratios compared with those of a normal calf. Nuclear and soluble fractions of normal and goitrous thyroid tissue contained no de-iodinase activity. Both normal and goitrous mitochondrial and microsomal preparations were rich in iodotyrosine de-iodinase activity. The particulate fractions of the goitre had greater de-iodinase activity than those of the normal gland.

Acid-protease activity was demonstrated in all fractions of the goitre although the nuclear fraction was least active in the goitre. In the normal thyroid fractions the nuclear and soluble fractions contained no proteolytic activity.

In both normal and goitrous thyroid tissue the highest I/N ratios were observed in mitochondrial and microsomal preparations, whereas the lowest were observed in the soluble and nuclear fractions. The I/N ratios in all fractions of normal thyroid were at least 10 times greater than those obtained from the goitre.

In adult animals the radioactive iodine uptake per unit weight of thyroid tissue was about the same in the goitre after 2 hours but was less in the goitre after 48 hours than in the normal.

Sucrose gradient analysis confirmed previous findings of an iodoprotein with slower sedimentation than that of thyroglobulin.

The behaviour of the abnormal thyroglobulin in the goitre was studied in various concentrations of agarose and in Biogel 5M and Biogel 50M. The results confirm previous observations that the abnormal thyroglobulin consists of several components which are partially fractionated by 10% agarose and Biogel 5M. These gels, however, did not separate serum proteins from thyroidal iodoproteins. Biogel 50M and 3% agarose gels separate thyroidal iodoproteins from serum proteins and from protein aggregates. None of these gels was suitable for a successful one-step purification of the abnormal thyroglobulin components.

*Salting-out techniques and DEAE chromatography have been employed in attempts to isolate the abnormal thyroglobulin components. Recent findings indicate an extensive degree of purification whereby 3 components are observed with approximate S-values of 3, 6 and 12.

We are indebted to the South African CSIR and the Atomic Energy Board for grants which enable us to continue with this study. We wish to thank Dr B. C. Jansen, Director of the Onderstepoort Research Institute, for his keen interest and support of this investigation; Messrs Eli Lilly and the South African Lilly Professorship Committee, who made it possible for Dr J. Robbins to visit our laboratories in order to continue collaboration in the study of congenital goitre; Messrs A. D. and B. W. Gordon of the Cape Offal Company, Cape Town, for fresh thyroid material; and Mrs E. Coetzee for the line drawings and assistance in preparation of this study for publication.

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