Studies of the Congenitally Goitrous Sheep

IODOPROTEINS OF THE GOITRE

By IAN R. FALCONER, I. M. ROITT, R. F. SEAMARK AND G. TORRIGIANI

Department of Applied Biochemistry and Nutrition, University of Nottingham School of Agriculture, Sutton Bonington, Loughborough, Leics., U.K., Department of Immunology, The Middlesex Hospital Medical School, London, W1P 9PG, U.K., and Department of Animal Physiology, Waite Agricultural Research Institute, Glen Osmond, S. Austral. 5064, Australia

(Received 14 November 1969)

1. Congenitally goitrous thyroid tissue was obtained from South Australian Merino sheep. Ultrastructural studies of the secretory cells in this tissue showed active cells of normal appearance, containing apical protein droplets. 2. ^{125}I -labelling *in vivo* of goitre tissue was used to investigate the iodoproteins, in which the major proportion of ^{125}I appeared in the cell protein fraction soluble in 0.9% sodium chloride (average 62% in goitres from untreated sheep). 3. Ammonium sulphate fractionation showed two clear peaks of iodoprotein precipitation, one at 35-40% saturation and the other at 50-55% saturation. Both iodoprotein fractions contained iodotyrosines and iodothyronines, which were identified chromatographically after enzymic hydrolysis of the protein. 4. Polyacrylamide-gel electrophoresis at pH 9.4, at either 7.5 or 5.0% acrylamide concentration, was used to characterize the iodoproteins. Two major fractions were observed, the fastest-migrating fraction coincident with serum albumin, and a slower-migrating, less-well-defined zone. This fraction migrated in 7.5% acrylamide gel, which excluded normal thyroglobulin. 5. Density-gradient (10-40% sucrose) centrifugation was used to determine the approximate sedimentation coefficients of the iodoproteins, which showed major components at $s_{20,w}$ 8-9S and $s_{20,w}$ <5S. 6. Immunoprecipitation with rabbit anti-(sheep thyroglobulin) failed to sediment ^{125}I -labelled proteins from goitre extracts. 7. Ouchterlony-type double diffusion in agar plates demonstrated immunoprecipitation lines between rabbit anti-(sheep thyroglobulin) and both the concentrated goitre extract and its Sephadex G-200-excluded fraction, which were confluent with that obtained on reaction with purified normal thyroglobulin. 8. It was concluded that both major iodoprotein fractions were capable of supplying thyroid hormones to the animal, and that the fraction of $s_{20,w}$ <5S was iodinated serum albumin. As ^{125}I -labelled thyroglobulin was not detected in goitre tissue from untreated or thyroxine-treated animals, it was possible that the genetic defect causing goitre resulted in an abnormal thyroglobulin, incapable of being iodinated but immunologically reactive.

Earlier studies of the congenital goitre occurring in Merino sheep established that the primary cause was in the defective biosynthesis of thyroglobulin in these animals (Falconer, 1965, 1966a,b). Young lambs suffered a greatly diminished viability as a consequence of inadequate thyroid hormone biosynthesis, but in adult animals the large goitres supplied sufficient hormone for normal wool growth and reproduction (Rac, Hill, Pain & Mulhearn, 1968). Measurements of the concentration of circulating thyroid hormones in these adult goitrous animals showed adequate quantities, with a normal rate of hormone utilization (Rac *et al.* 1968; Falconer, 1966a).

As no normal thyroglobulin had been recorded in these congenital goitres, the sources of the iodothyronines detected in the goitres and in the blood of these animals must be other iodinated proteins (Falconer, 1965). The present paper reports the results of investigations into the nature of the iodoproteins of the goitres, and the changes in iodoproteins occurring as a result of the goitrous animals receiving thyroxine treatment.

EXPERIMENTAL

General chemicals. All reagents and solvents were of A. R. grade or equivalent unless otherwise specified, except for $\text{Ce}(\text{SO}_4)_2 \cdot 4\text{H}_2\text{O}$ (low in other rare earths) from

British Drug Houses Ltd., Poole, Dorset, U.K. For gel electrophoresis, acrylamide and *NN'*-methylenebisacrylamide were obtained from Kodak Ltd., Kirkby, Liverpool, U.K., and *NNN'*-tetramethylethylenediamine from Canaco, Bethesda, Md., U.S.A. L-3-Monoiodotyrosine and L-3,5-di-iodotyrosine were obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., L-3,3',5-tri-iodothyronine was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and L-thyroxine was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Goitre samples. South Australian Merino rams and ewes were provided by Mr C. Mulhearn of the South Australian Department of Agriculture. During experimentation the animals were kept in partially roofed pens on a constant diet of hay chaff and oats. Goitre samples were obtained by surgical excision from animals given an intramuscular injection of 250 μ Ci of [125 I]iodide 50 h earlier. Thyroxine-treated goitrous animals received 200 μ g of L-thyroxine intramuscularly three times a week for 1 month before goitre excision. On excision the goitres were immediately thoroughly perfused with 0.9% NaCl at 0°C, and then frozen in liquid N₂. The goitres were transported in insulated containers packed with solid CO₂, and stored at -15°C. Histology of goitrous tissue was carried out by formol-saline fixation, dehydration and paraffin-embedding, sectioning and staining with haematoxylin and eosin.

Goitre samples for electron microscopy were removed by biopsy under Nembutal (Abbott Laboratories, Queenborough, Kent, U.K.) anaesthesia, and fixed in potassium permanganate by the method of Luft (1956). The fixed tissue was embedded in Araldite (Luft, 1961) and the sections were post-stained with lead citrate (Reynolds, 1963).

Extracts. For the immunochemical studies, goitre extract was prepared by disrupting the tissue in an all-glass Potter-Elvehjem homogenizer with 2 vol. of cold 0.85% NaCl buffered with 50 mM-potassium-sodium phosphate, pH 7.2, followed by centrifugation at 15000g to give a clear supernatant.

For the biochemical studies, goitre samples were homogenized with 5 vol. of chilled 0.9% NaCl in an MSE top-drive homogenizer (Measuring and Scientific Equipment Ltd., Birmingham, U.K.). Subcellular fractions were collected as precipitates after separation of the homogenate in an MSE Superspeed 40 centrifuge at 1000g for 15 min, 16000g for 20 min, 60000g for 30 min and 100000g for 60 min. For studies on the soluble iodoproteins the homogenate was centrifuged only at 100000g for 60 min and the supernatant collected. For total precipitation of proteins from this soluble supernatant 20% (w/v) trichloroacetic acid was added to a final concentration of 5% (w/v) and the precipitate collected after 15 min at 3000g at room temperature in an MSE angle-head centrifuge.

Ammonium sulphate fractionation of iodoproteins. The soluble supernatant was dialysed before use against three changes of 10 vol. of 0.9% NaCl for 24 h at 3°C. Saturated (NH₄)₂SO₄ solution at pH 7 was added to a series of samples of dialysed supernatant to produce final concentrations of 0-75% saturation with (NH₄)₂SO₄ in 5%-saturation steps. After standing for 20 min the samples were centrifuged for 15 min at 3000g at room temperature,

and *E*₂₈₀ values of the supernatants measured in a Unicam SP.500 spectrophotometer (Unicam Instruments Co., Cambridge, U.K.). Difference spectra of the supernatant samples were recorded on a double-beam spectrophotometer (Unicam SP.800). Radioactivity of the supernatant was measured by recording the time for 10000 counts to be detected in an automatic well-type scintillation counter (type 6052; Isotope Developments Ltd., Reading, Berks., U.K.).

The presence of 125 I-labelled iodothyronines in the goitre iodoproteins was detected after hydrolysis of (NH₄)₂SO₄ precipitates with 1% (w/v) trypsin-1% (w/v) pancreatin at pH 6.8 in 0.1 M-potassium phosphate buffer for 20 h, followed by chromatographic separation of the free amino acids on Whatman no. 1 paper with a descending solvent of butan-1-ol-ethanol-2 M-ammonia (2:1:1, by vol.). Strips (1 cm) of chromatograph paper were serially cut for radioactivity measurement, and iodine was detected by the CeSO₄-As₂O₃ reaction (MacLagan, Bowden & Wilkinson, 1957).

Polyacrylamide-gel electrophoresis. This was carried out in 7.5 and 5.0% polyacrylamide rods, pH 9.4, polymerized by ammonium persulphate-tetramethylethylenediamine solution with tris-glycine buffer in the electrode reservoirs (Davis, 1964). The equipment used was a standard disc-electrophoresis apparatus (Shandon Scientific Co. Ltd., London N.W.10, U.K.). After electrophoretic separation the polyacrylamide rods were stained, photographed and cut into discs (2 mm thick) for measurement of radioactivity.

Density-gradient centrifugation. Linear gradients of 10-40% (w/v) sucrose solution in 10 mM-potassium phosphate buffer, pH 6.8, were used. Samples (0.3 ml) of the soluble supernatant from goitre homogenates were each carefully layered on to 4 ml gradients in polypropylene centrifuge tubes (5 ml capacity), and centrifuged in an MSE 3×5 ml swing-out rotor at 35000 rev./min (105000 *g*_{av.}) for 20 h in an MSE Superspeed 40 centrifuge at 15°C. After centrifugation the tubes were pierced and 4-drop fractions collected for radioactivity determination and protein-concentration measurements by the biuret method (Gornall, Bardawill & David, 1949).

Calibration of the density-gradient separations was carried out with thyroglobulin (*s*_{20,w} 19.4S; mol.wt. 680000) obtained from sheep thyroid glands, haemoglobin from lysed sheep erythrocytes (*s*_{20,w} 4.8S; mol.wt. 68000) and bovine liver catalase (British Drug Houses Ltd.) [*s*_{20,w} 11.4S; mol.wt. 240000 (Samejima & Yang, 1963)]. Measurements of the distribution of thyroglobulin and haemoglobin between fractions were done by the biuret reaction. Catalase was assayed from its enzymic activity in catalysing the reduction of 25 mM-H₂O₂ in 25 mM-potassium phosphate buffer, pH 6.8, during 40 min incubation at 20°C. Residual H₂O₂ was titrated with 0.1 M-KMnO₄ in the presence of 0.5 M-H₂SO₄.

Antisera. Antisera to sheep thyroglobulin prepared from normal thyroids by (NH₄)₂SO₄ precipitation (Derrien, Michel & Roche, 1948) were raised in rabbits by intramuscular injection of 5 mg of protein in complete Freund's adjuvant followed by a further 5 mg of alum-precipitated antigen given as five intravenous injections over the fourth and fifth weeks; blood sample was collected from each rabbit after a further week. For the production of antisera against the abnormal protein

contained in the goitrous sheep thyroid, 4 ml of goitre extract from a sheep that had received [^{125}I]iodide was separated on a Sephadex G-200 column and the radioactive fractions forming a sharp peak were pooled and injected with complete Freund's adjuvant into two rabbits. The animals were 'boosted' three times at weekly intervals with antigen in incomplete Freund's adjuvant and killed 1 week after the last injection.

RESULTS

Cellular and subcellular structure. Light-microscopic examination of the goitrous tissue revealed densely hyperplastic glands, with infolding of the secretory epithelial cells into the space occupied by colloid in normal thyroid tissue (Plates 1a and 1b). Electron-microscopic examination of goitrous and normal tissue showed that the secretory epithelial cells from the goitrous gland were virtually identical with cells from highly active normal tissue. Islands of an abnormal vacuolated cell also have been reported in these congenital goitres (Rac *et al.* 1968).

Near the apex of normal thyroid secretory cells is an accumulation of protein-containing vesicles, which are considered to be mainly droplets of colloid undergoing hydrolysis for the liberation of free iodothyronines (Plate 1c). Some of the vesicles may, however, contain newly synthesized thyroglobulin, before liberation into the colloid occurs (Ekholm & Smeds, 1966). Similar vesicles were also seen in the secretory cells of goitrous thyroids (Plate 1d), suggesting that a protein secretion was also being produced and hydrolysed in these cells. The absence of follicular colloid in the goitres was

indicative of the rate of hydrolysis of secreted protein being in excess of the rate of secretion, so that no accumulation occurred in the follicles.

Subcellular fractionation. Fractionation of homogenates of goitrous tissue was carried out as described above. The results obtained for the distribution of ^{125}I radioactivity from untreated goitrous tissue, and tissue from goitres of sheep treated for 1 month with thyroxine, are shown in Table 1. These results show that no major redistribution of ^{125}I resulted from thyroxine treatment, and that goitres from both groups of animals contained most of their radioactive iodine in organically bound macromolecules soluble in 0.9% sodium chloride, but precipitated by the presence of trichloroacetic acid at 5% (w/v). The increased ^{125}I soluble in 5% trichloroacetic acid in the goitres from thyroxine-treated sheep may indicate a decrease in the activity of these glands in converting iodide into organically bound iodine. It would be expected that the activity of enzymes on the normal pathway for thyroid hormone biosynthesis would be decreased as a result of thyroxine treatment in these animals, as a consequence of suppression of thyroid stimulating hormone release from the pituitary.

Fractional precipitation. The soluble supernatants from normal thyroid and goitre homogenates were subjected to differential precipitation by ammonium sulphate as described above. The results of representative experiments (Fig. 1) are expressed as the change in E_{280} of the supernatant for a 5% increase in saturation of ammonium sulphate solution, and

Table 1. *Subcellular fractionation of ^{125}I radioactivity in goitre tissue obtained from untreated sheep, and sheep treated with 200 μg of L-thyroxine intramuscularly three times a week for 4 weeks*

Fractionation procedure and radioactivity measurement were as described in the text. All radioactivity values are expressed as percentages of the total radioactivity of that goitre sample.

	1000g, 15 min (Debris, nuclei)	16000g, 20 min (Mitochondria)	60000g, 30 min (Microsomes)	100000g, 60 min (Ribosomes)	Fraction soluble in 0.9% NaCl insoluble in 5% trichloroacetic acid	Fraction soluble in 5% trichloroacetic acid
Goitre A (untreated)	24.9	6.3	1.9	3.1	52.5	11.2
Goitre B (untreated)	16.1	5.3	1.2	2.4	65.7	9.4
Goitre C (untreated)	9.0	6.0	3.6	3.1	67.8	10.5
Mean	16.7	5.9	2.2	2.9	62.0	10.4
Goitre R ₁ (thyroxine-treated)	15.2	11.9	4.4	4.5	39.8	24.0
Goitre R ₂ (thyroxine-treated)	3.6	8.5	3.4	6.8	68.8	15.9
Goitre E ₁ (thyroxine-treated)	4.3	4.0	1.8	3.7	61.5	17.1
Mean	7.7	8.1	3.1	5.0	56.7	19.0

as the change in supernatant radioactivity for the same change in saturation.

The distribution of radioactivity showed a marked difference between goitre extracts from the untreated and thyroxine-treated sheep (Fig. 1). Goitre C (untreated) shows iodinated macromolecules precipitated mainly at 35–50% saturation with ammonium sulphate, with the largest proportion of radioactivity being precipitated at 35–40% saturation. Goitre E (thyroxine-treated sheep) by contrast showed a substantial peak of radioactivity precipitated at 50–55% saturation, as well as radioactivity precipitated earlier. Normal thyroglobulin is precipitated at 35–40% saturation with ammonium sulphate (Fig. 1a).

The u.v. difference spectra of supernatants from

Fig. 1. Ammonium sulphate fractionation of soluble proteins from normal and goitrous thyroid glands. (a) Proteins from normal thyroid. (b) Goitre proteins from untreated sheep. (c) Goitre proteins from thyroxine-treated sheep. The results are expressed as the change in E_{280} (—), and the change in supernatant radioactivity (---), for a change in saturation of $(\text{NH}_4)_2\text{SO}_4$ of 5%.

ammonium sulphate fractionation indicated that nucleic acids as well as proteins were being precipitated from extracts of normal and goitrous glands, with most of the nucleic acids being precipitated at below 45% saturation.

Enzymic hydrolysis of precipitates collected by 20–40% and 40–60% saturation with ammonium sulphate showed the liberation of ^{125}I -labelled mono- and di-iodotyrosine, thyroxine and tri-iodothyronine. The relative proportions are shown in Table 2.

Polyacrylamide-gel separation. To obtain more effective resolution of the soluble protein constituents of goitre tissue, separations were carried out in polyacrylamide-gel columns as described above. It was found that thyroglobulin, of molecular weight about 680000, would not enter gels containing 7.5% acrylamide, but would migrate into gels of 5.0% acrylamide (Fig. 2a). By the use of both concentrations of acrylamide it was therefore possible to separate thyroglobulin, if present, from other iodoproteins in the goitres.

To obtain an adequate concentration of iodoproteins, preliminary fractionation by ammonium sulphate precipitation was carried out. The results of polyacrylamide-gel separations of the iodoproteins of a goitre from a thyroxine-treated animal are shown in Figs. 2(b), 2(c), 2(d) and 2(e).

The major difference between the polyacrylamide-gel patterns of untreated and thyroxine-treated goitre extracts was the lack of a clear peak of radioactivity in the zone occupied by serum albumin in extracts of untreated goitres (Figs. 3a and 3b). It is apparent from these results that two major iodinated protein fractions can be distinguished, one a rather heterogeneous fraction of mobility lower than that of albumin, and the other a single peak at the front of the plasma albumin zone. The radioactivity in the albumin zone is strongly evident only in extracts from goitres removed after treatment of the animal with thyroxine. Since the slower-migrating iodoprotein fraction will migrate in 7.5% acrylamide gel, it must have an appreciably lower molecular weight than thyroglobulin. This concentration of acryl-

Table 2. ^{125}I -labelled amino acids liberated by enzymic hydrolysis of ammonium sulphate precipitates from the soluble supernatant fraction of goitre homogenates

Results are expressed as the percentages of the total radioactivity of the hydrolysate $\pm$ S.E.M. (no. of glands in parentheses).

Ammonium sulphate fraction	Radioactivity remaining at the origin	Monoiodotyrosine	Di-iodotyrosine	Iodide	Thyroxine	Tri-iodothyronine
20–40% ppt.	40.1 $\pm$ 4.1 (4)	18.8 $\pm$ 1.6 (4)	28.9 $\pm$ 4.0 (4)	8.1 $\pm$ 0.7 (4)	1.9 $\pm$ 1.0 (4)	2.2 $\pm$ 0.5 (4)
40–60% ppt.	46.8 $\pm$ 4.3 (3)	14.4 $\pm$ 0.4 (3)	18.9 $\pm$ 2.4 (3)	11.8 $\pm$ 3.6 (3)	4.2 $\pm$ 0.6 (3)	4.0 $\pm$ 1.1 (3)

EXPLANATION OF PLATE I

Microscopic anatomy of normal and congenitally goitrous thyroid tissue. (a) Light microscopy of normal thyroid tissue showing colloid-filled follicles. Magnification $\times 600$. (b) Congenitally goitrous thyroid tissue showing collapsed follicles and lack of colloid. Magnification $\times 700$. (c) Electron microscopy of the apex of a normal thyroid secretory cell adjacent to the colloid. Magnification $\times 30\,000$. (d) Apices of secretory cells in congenitally goitrous thyroid tissue. Magnification $\times 30\,000$. Arrows mark vesicles of protein near the cell apices in both types of tissue.

I. R. FALCONER, I. M. ROITT, R. F. SEAMARK AND G. TORRIGIANI

(Facing p. 420)

EXPLANATION OF PLATE 2

Reaction of identity of thyroglobulin with goitre extract and its high-molecular-weight fraction in a double-diffusion precipitation test in agar. I, Rabbit anti-(sheep thyroglobulin). II, Concentrated goitre extract. III, Sheep thyroglobulin (0.1 mg/ml.) IV, Sephadex G-200-excluded material from goitre extract. After 2 days the agar was washed in 0.9% NaCl for a further 2 days, dried and stained (the distortion of the wells occurred during the drying process).

Fig. 2. Separation of soluble iodoproteins from a goitre of a thyroxine-treated sheep (E) by polyacrylamide-gel electrophoresis. (a) Proteins precipitated between 20% and 40% saturation with $(\text{NH}_4)_2\text{SO}_4$ separated on 5% acrylamide gel, pH 9.4. (b) Proteins precipitated between 40% and 60% saturation with $(\text{NH}_4)_2\text{SO}_4$ separated on 5% acrylamide gel, pH 9.4. (c) Proteins as in (a) separated on 7.5% acrylamide gel, pH 9.4. (d) Proteins as in (b) separated on 7.5% acrylamide gel, pH 9.4. (e) Soluble proteins of normal thyroid separated on 5% acrylamide gel, pH 9.4. Abbreviation: Alb, albumin.

amide would retard, to 50% of the free mobility, proteins of mol.wt. 150 000 (Tombs, 1965), so it may be assumed that the molecular weight of the migrating protein was of this order.

Density-gradient centrifugation. Calibration of the 10–40% (w/v) sucrose gradient was carried out as described above, and the results of such a calibration are shown in Fig. 4. Separations of soluble extracts of four goitres from sheep previously treated with thyroxine are shown in Fig. 5, to illustrate the differing proportions of the major components between glands. Fig. 5(a) shows that the major radioactive peak lies in the region of $s_{20,w}$ 8–9S, and Figs. 5(b), 5(c) and 5(d) show that radioactivity also occurred at, and lower than, $s_{20,w}$ 5S.

Immunochemical studies. The possibility that the labelled iodoproteins in the goitre extracts might be related in some way to thyroglobulin was investigated by using immunological techniques.

Successive addition of rabbit anti-(sheep thyroglobulin) followed by a precipitating goat anti-(rabbit immunoglobulin G) failed to bring down more radioactivity than did controls containing

normal rabbit serum (Table 3). Negative results were also obtained when the experiment was repeated with sera from rabbits injected with purified radioactive material from the goitre extracts.

In further studies the six thyroid extracts from the sheep that had received $[^{125}\text{I}]$ iodide were incubated at 37°C for 1 h with normal rabbit serum, with an antiserum to sheep thyroglobulin or with the two antisera obtained after the injection of the goitrous protein. The mixtures were then subjected to electrophoresis on paper (Daniel, Pratt, Roitt & Torrigiani, 1967) and the electrophoretograms were scanned for radioactivity. There was no difference in the mobility of the radioactivity after incubation with the different sera.

Although these experiments failed to detect any appreciable labelling of proteins immunologically related to thyroglobulin, conventional gel-diffusion studies were carried out to see whether the goitre extracts contained any thyroglobulin-like material. By using double diffusion in Ouchterlony-type agar plates, a weak but definite line of precipitate was observed between two wells containing goitre

Fig. 3. Separation of soluble iodoproteins from goitres of untreated sheep by polyacrylamide-gel electrophoresis. Proteins precipitated between 40% and 60% saturation with $(\text{NH}_4)_2\text{SO}_4$ were separated on 5% acrylamide gels at pH 9.4. (a) Goitre B; (b) goitre A. Abbreviation: Alb, albumin.

Fig. 4. Calibration of 10-40% (w/v) sucrose density gradient, with sheep thyroglobulin ($s_{20,w}$ 19.4S), ox liver catalase ($s_{20,w}$ 11.4S) and sheep haemoglobin ($s_{20,w}$ 4.8S). The methods used are described in the text. ■, Concn. of protein; ×, catalase activity.

extract and a potent rabbit anti-(sheep thyroglobulin). The stronger line given by normal sheep thyroglobulin (1.0 mg/ml) in an adjoining well gave a 'spur' with that formed by the extract. When the experiment was repeated with 10-times-concentrated goitre extract placed in a larger well, a line of complete identity with thyroglobulin was obtained (Plate 2), showing that the 'spur' formation was an

artifact resulting from gross disparity in the concentrations of antigens in adjoining wells. The thyroglobulin-like material in the extract was recovered in the breakthrough peak on Sephadex G-200 consistent with the behaviour of thyroglobulin derived from normal glands. These studies further indicated that the concentration of 'thyroglobulin' in the extract was of the order of 10 $\mu\text{g}/\text{ml}$.

DISCUSSION

In man several cases of congenital goitre have been recorded in which the goitrous gland contains little or no thyroglobulin, but does contain other iodinated proteins (Stanbury, 1966; Lissitzky, Codaccioni, Bismuth & Depieds, 1967). Iodinated serum albumin has been identified in congenital goitres and its origin from the circulating albumin of the blood established (Lissitzky, Bismuth, Codaccioni & Cartouzou, 1968). The iodinated albumin was shown to contain both iodotyrosines and iodothyronines, and was therefore capable of providing a source of thyroid hormones in these patients (Lissitzky *et al.* 1967).

In the congenital goitre in sheep the results presented for polyacrylamide-gel electrophoresis (Fig. 2d), and also for density-gradient centrifugation (Fig. 5), indicate an iodinated protein of electrophoretic migration and sedimentation coefficient equal to those of serum albumin. When the goitrous animals were treated with thyroxine an accumulation of labelled iodoprotein was observed in the fraction (precipitate from 50-65% saturation with ammonium sulphate) containing iodinated serum albumin. Under these conditions of diminished hydrolytic activity in the goitres the increase of iodinated albumin indicated that a continual hydrolysis of this protein was occurring in the untreated goitre tissue. It is therefore suggested that the iodination of serum albumin, and its subsequent hydrolysis, may act as a source of thyroid hormones in these sheep.

The ultrastructural investigations of thyroid cells from goitrous sheep showed apparently normal active tissue with protein droplets in the cell apices similar to those found in thyroid cells from normal sheep (Plate 1). Earlier studies on this tissue demonstrated active protein biosynthesis, but with a markedly lower amino acid incorporation into thyroglobulin-like protein than occurred in normal tissue (Falconer, 1966b). No clear evidence of ^{125}I -labelled thyroglobulin in these goitres has been obtained, by physicochemical or immunochemical techniques; however, very small amounts of a protein immunologically identical with thyroglobulin were demonstrated, although no significant radioactivity could be detected (Plate 2).

Fig. 5. Sucrose-density-gradient separations of soluble proteins from thyroxine-treated goitres. (a) Goitre E₁. (b) Goitre R₁. (c) Goitre R₂. (d) Goitre R₃. Methods were as indicated in Fig. 4. ■, Protein concentration; ▲, radioactivity; x, sedimentation calibration.

Table 3. Measurement of radioactive thyroglobulin in goitre extracts by immunological co-precipitation

Fixed volumes of each extract (giving approx. 800 counts/s) were incubated for 30 min at 37°C with 0.1 ml of a 1:20 dilution of a precipitating rabbit anti-(sheep thyroglobulin) serum and with each of two rabbit anti-(goitre extract) sera; control tubes contained a similar amount of normal rabbit serum. An excess (0.3 ml) of precipitating goat anti-(rabbit immunoglobulin G) was added; after incubation at 37°C for 30 min and 4°C for 16 h the precipitate was collected, washed twice and the radioactivity counted.

Extract from goitre	Radioactivity of supernatant (counts/s)	Radioactivity of precipitate (counts/s)			
		Anti-thyroglobulin	Anti-goitre extract (A)	Anti-goitre extract (B)	Normal rabbit serum
A	320	3.2	3.7	3.1	3.5
B	710	4.2	4.0	4.3	4.5
C	150	2.1	2.0	2.5	2.2
R ₁	700	4.1	3.2	5.2	4.1
R ₂	830	8.2	8.1	8.6	7.9
R ₃	500	4.1	5.6	5.4	3.0

Also present in these goitres was a heterogeneous iodoprotein component, migrating behind serum albumin in 7.5% acrylamide gel, and having a

sedimentation coefficient of 8–9S. This component was precipitated at 40–50% saturation with ammonium sulphate, and was present as the major

labelled iodoprotein in untreated goitres. It was not immunologically related to thyroglobulin and was not antigenic in rabbits. It was therefore not related to the abnormal thyroglobulin recorded in congenitally goitrous cattle, which also had a sedimentation coefficient of about 9S, as the iodoprotein from cattle formed a complex with anti-thyroglobulin (Robbins, Van Zyl & Van der Walt, 1966). Enzymic hydrolysis of this iodoprotein showed both iodotyrosines and iodothyronines present.

Recent work has established that the congenital goitre in these sheep acts as a single autosomal recessive character, with no detectable manifestations in heterozygous animals (Mayo & Mulhearn, 1969). It is therefore possible that an error in the structural gene coding for thyroglobulin is the basic defect present, with a consequential inadequacy of thyroid hormone production. This would lead to an excessive secretion of thyroid-stimulating hormone and glandular hypertrophy. It would also be expected to lead to the iodination of serum albumin, which occurs in other types of thyroid hypertrophy (Triantaphyllidis, Thompson, Barnaby & Jasani, 1969). The presence of an apparently uniodinated protein reacting with anti-thyroglobulin may also be due to the biosynthesis of abnormal protein coded by a defective gene for thyroglobulin. No clear explanation can be given for the appearance of the highly iodinated protein with $s_{20,w}$ 8–9S in these goitres. In the goitrous glands both the iodoproteins $s_{20,w}$ 8–9S and iodinated albumin are potential sources of thyroid hormones.

We thank Mr J. R. Jones and Mrs C. Bradburn for their valuable technical assistance and Mr C. J. Mulhearn of the South Australian Department of Agricul-

ture for the goitrous animals. Acknowledgement is made of the award of a Wates Fellowship to G. T.

REFERENCES

- Daniel, P. M., Pratt, O. E., Roitt, I. M. & Torrigiani, G. (1967). *Immunology*, **12**, 489.
- Davies, B. J. (1964). *Ann. N.Y. Acad. Sci.* **121**, 404.
- Derrien, Y., Michel, R. & Roche, J. (1948). *Biochim. biophys. Acta*, **2**, 454.
- Ekholm, R. & Smeds, S. (1966). *J. Ultrastruct. Res.* **16**, 71.
- Falconer, I. R. (1965). *Nature, Lond.*, **205**, 978.
- Falconer, I. R. (1966a). *Biochem. J.* **100**, 190.
- Falconer, I. R. (1966b). *Biochem. J.* **100**, 197.
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
- Lissitzky, S., Bismuth, J., Codaccioni, J.-L. & Cartouzou, G. (1968). *J. clin. Endocr.* **28**, 1797.
- Lissitzky, S., Codaccioni, J.-L., Bismuth, J. & Depieds, R. C. (1967). *J. clin. Endocr.* **27**, 185.
- Luft, J. H. (1956). *J. biophys. biochem. Cytol.* **2**, 799.
- Luft, J. H. (1961). *J. biophys. biochem. Cytol.* **9**, 409.
- MacLagan, M. F., Bowden, C. H. & Wilkinson, J. H. (1957). *Biochem. J.* **67**, 5.
- Mayo, G. M. E. & Mulhearn, C. J. (1969). *Aust. J. agric. Res.* **20**, 533.
- Rac, R., Hill, G. M., Pain, R. W. & Mulhearn, C. J. (1968). *Res. vet. Sci.* **9**, 209.
- Reynolds, E. S. (1963). *J. Cell Biol.* **17**, 208.
- Robbins, J. A., Van Zyl, A. & Van der Walt, K. (1966). *Endocrinology* **78**, 1213.
- Samejima, T. & Yang, J. T. (1963). *J. biol. Chem.* **238**, 3256.
- Stanbury, J. B. (1966). In *The Metabolic Basis of Inherited Disease*, 2nd ed., pp. 215–257. Ed. by Stanbury, J. B., Wyngaarden, J. B. & Fredrickson, D. S. New York: McGraw-Hill Book Co.
- Tombs, M. P. (1965). *Analyt. Biochem.* **13**, 121.
- Triantaphyllidis, E., Thompson, B. D., Barnaby, C. F. & Jasani, B. (1969). *J. Endocr.* **44**, 467.