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A MICROGEL ELECTROPHORETIC STUDY OF THE STABILITY OF THYROID IODOPROTEINS

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SUMMARY

The stability of the large iodoproteins in the rat thyroid gland was studied by polyacrylamide gel electrophoresis.

19S Thyroglobulin was stable in phosphate-buffered saline (0.01 *M* phosphate, 0.15 *M* NaCl, pH 6.8; PBS) containing 20% (w/w) sucrose. Reduction of the sucrose concentration by dilution with PBS resulted in the appearance of two 12S fractions and a band migrating with the front. After the same treatment of 27S iodoprotein, also stable in PBS + 20% sucrose, no large dissociation products appeared, only a band moving with the front.

Urea of 8 *M*, pH 6.8, and 2 *M* urea, pH 8.1, partially dissociated 19S thyroglobulin into three species and a band at the electrophoretic front. The main dissociation product probably represents a modified 12S protein. Both treatments further resulted in a reduced gel migration of undissociated thyroglobulin. After urea treatment of 27S iodoprotein, larger amounts of front species were found than after the same treatment of 19S thyroglobulin, where only trace amounts of large dissociation products were observed.

The main dissociation product in the front band of 19S thyroglobulin had a molecular weight of 82,000–86,000 (SDS gel electrophoresis). Four other distinct fractions were observed.

It was concluded that sucrose has a stabilizing effect on the large thyroid iodoproteins. The different dissociation patterns of 19S thyroglobulin and 27S iodoprotein indicate that the subunits of 27S, to a larger extent than those of 19S, are linked together by non-covalent bonds.

INTRODUCTION

In a previous paper from this laboratory, a microgel electrophoretic method for the separation of the large iodoproteins of the thyroid gland was described[1]. In a preliminary study of the protein composition of the colloid collected from single rat thyroid follicles, it was found that 19S thyroglobulin (TG) was the major fraction of the large iodoproteins and that more slowly migrating fractions (27S and/or prethyroglobulin) and 12S protein were present in varying relative amounts[2].

Native 19S TG is dissociated by heat, alkali and denaturating agents[3] and

the extent of conversion to slower sedimenting components is influenced by the ionic strength of the solvent[4]. The iodine-poor molecule dissociates more readily than the fully iodinated 19S TG[5–8]. It has also been demonstrated that low temperature (2°) influences rat 19S TG with the formation of a broad and asymmetrical TG sedimentation band in the ultracentrifugal gradient pattern[9].

It is therefore evident that TG (especially incompletely iodinated molecules) is very susceptible to alternations in the environment and the presence of 12S in the gel separation patterns in the previous studies from this laboratory indicated an instability of TG and/or 27S in the experimental system adopted. It was therefore deemed to be of importance to analyse the stability of the larger iodoproteins during experimental conditions mimicking the handling of the colloid samples. Because of the presence in the colloid samples of fractions that migrated faster than the 12S protein, the identification of the subunits of the TG and 27S molecules[10–12] in the microgel separation patterns was also considered to be of interest.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 250–400 g were used. The animals were reared under standardized temperature conditions (22°) and fed on standard pellets (Astra Ewos, Södertälje, Sweden) and tap water *ad libitum*. The intake of iodine was 50–100 μg per 100 g of body weight in 24 h. For the preparation of iodine-poor TG fractions, the animals were fed for at least 3 months on iodine-poor pellets (Ewos III, Astra Ewos, Södertälje, Sweden) containing 0.3 mg of iodine per kilogram of pellet.

Gel electrophoresis

The separation of the proteins was performed at room temperature by gel electrophoresis on 8 or 10 % polyacrylamide gels at pH 6.8. The gels were prepared from stock solutions previously described[1] and polymerized in 5-μl microcaps (Drummond Scientific Co., Broomall, Pa., U.S.A.). No spacer gel was used; instead, a 2-mm layer of buffered 20 % (w/w) sucrose solution was applied on the separating gel. The buffer, 0.01 *M* phosphate buffer containing 0.15 *M* saline (PBS), had an ionic strength of 0.17 at pH 6.8. The electrophoreses were run with a square pulse (75 V, 500 Hz and 25 % duty cycle) layered on an off-set potential of 25 V (IB Electronic, Gothenburg, Sweden). The run was interrupted when the tracking dye (bromophenol blue) had migrated 7 mm in the separating gel. After fixation in 80 % ethanol, the gels were stained in 0.5 % amido black in 7.5 % acetic acid and after destaining in 7.5 % acetic acid the separation patterns were recorded with a previously described microdensitometer[1]. Simultaneously with the densitometric recordings, the amounts of stain in the protein discs in the gels were determined by integration of the area of the protein peaks in the recordings and the relative amounts of stain bound to the protein fractions in the gels were calculated.

SDS gel electrophoresis

Two TG standard preparations were treated with 8 *M* urea for 3 h and then dialysed against 1.0 % sodium dodecylsulphate and 1.0 % mercaptoethanol in 10 m*M* phosphate buffer of pH 7.0 for 48 h. Electrophoretic separation of the proteins

in the dialysates was carried out in 10 % gels in the SDS electrophoretic system of SHAPIRO *et al.*[13], mainly as described by WEBER AND OSBORNE[14]. The electrophoreses were run with 1 mA per gel for 30 min and 6 mA per gel for 3 h.

After treatment of the gels with 20 % sulphosalicylic acid for 15 h and staining with 0.02 % Coomassie brilliant blue (R 250, I.C.I. Ltd.) in 12.5 % TCA for 5 h[15], the gels were stored in 10 % TCA and photographed. The mobilities of the proteins were calculated and by comparison with standard curves correlating mobility and molecular weight, the molecular weights of the protein fractions in the TG–SDS–ME mixtures were estimated.

Preparation of standard samples of 19S thyroglobulin and 27S protein

Three rats were generally used for each preparation of the standard protein samples. In order to avoid contamination of the soluble thyroid proteins with serum proteins, the animals were perfused prior to the excision of the glands. The perfusion was performed through the aorta with 500 ml of PBS (pH 6.8, 22°) or 500 ml of PBS containing 20 % (w/w) sucrose.

After perfusion, the thyroids were excised and homogenized in PBS containing 5 % sucrose at pH 6.8. The homogenate was centrifuged at 105,000 *g* for 90 min and the supernatant was used as source of the soluble thyroid proteins.

The major soluble thyroid proteins were separated by layering 0.7 ml of the supernatant on a 4.5-ml 10–25 % sucrose density gradient, prepared with PBS of pH 6.8, and centrifuging at 65,000 *g* for 16 h. From each gradient, about 35 fractions were collected. Every other fraction was diluted with 0.8 ml of PBS and the optical density at 280 nm was determined. The protein composition in the *undiluted* gradient fractions of the 27S and TG peaks was analysed by gel electrophoresis and only fractions containing single protein bands in the 27S and TG regions[1] of the gels were used for the subsequent studies. The sucrose concentrations in the undiluted gradient fractions that contained TG and 27S protein were about 20 % as determined with a refractometer. The protein concentration in the TG standard samples varied between 1.2 and 1.4 $^0/_{00}$ and in the 27S preparations between 0.03 and 0.13 $^0/_{00}$.

Stability analyses

(*A*) *Dilution of the 27S and TG standard preparations with PBS of pH 6.8 and ionic strength 0.17*. PBS of pH 6.8 was added (1:8) to the TG and 27S standard preparations, giving final TG concentrations of 0.15–0.20 $^0/_{00}$, 27S concentrations of 0.004–0.02 $^0/_{00}$ and sucrose concentrations of about 2.5 %. The PBS solution was cooled (0–4°) and the diluted standard preparations and the non-diluted controls were stored in ice during the experiments. The protein composition of the diluted standard preparations of TG and 27S were analysed at intervals from 10 min to 24 h. Undiluted standard preparations were run in parallel with the test samples. In some experiments, the TG and 27S standard preparations were diluted with cold PBS of pH 6.8 containing 20 % (w/w) sucrose. Nine and four standard preparations were used for the analyses of TG and 27S, respectively.

(*B*) *8 M urea*. TG and 27S standard preparations were mixed with 10 *M* urea in separate glass capillaries (100-μl microcaps). The final urea concentration was 8 *M*. The mixtures were incubated for 3 h at 0–4° and then transferred to the electrophoresis capillaries. Three standard preparations of 27S and TG were used for these analyses.

(*C*) *0.10 M Tris, pH 8.1, + 2 M urea.* Standard preparations of TG and 27S were dialysed at 4° against 0.10 *M* Tris of pH 8.1 containing 2 *M* urea. After 3 and 24 h, test samples were directly transferred to the electrophoresis capillaries. Three standard preparations of 27S and TG were used for these analyses.

RESULTS

The TG standard preparation and PBS (A)

When the TG standard preparation was diluted with cold PBS of pH 6.8, a protein fraction appeared within 10 min in the 12S region of the gel (Fig. 1). Generally, there was a shoulder on this peak in the densitometric recordings and in some separations two distinct fractions could be discerned (Fig. 2). Parallel with the appearance of a band in the 12S region of the gel, more slowly migrating components were

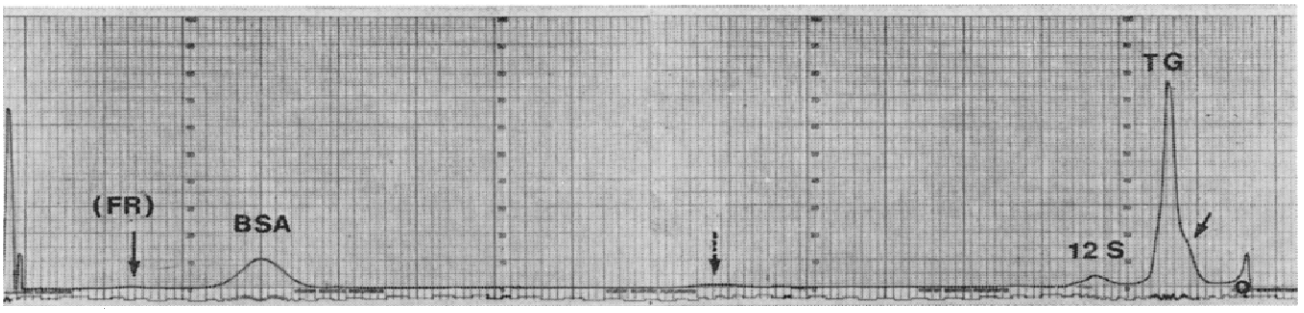

Fig. 1. Densitometric recording of the protein separation pattern of a 19S TG standard preparation following a reduction of the sucrose concentration from 20% to 2.5%. TG dissociates into a more slowly migrating component (→) and the 12S component. In some preparations, a minute amount of protein was observed at the front (FR). BSA = added bovine serum albumin and the dimer of this protein (- - →). O = origin.

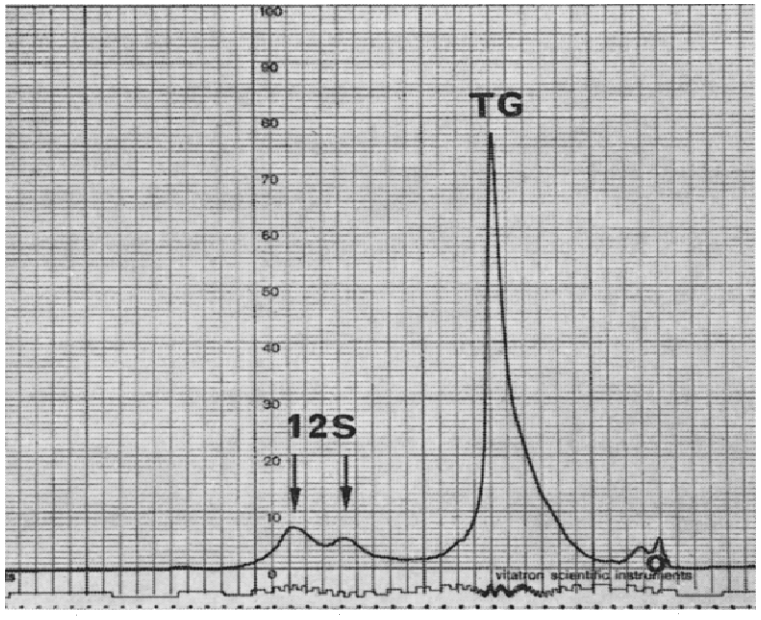

Fig. 2. Densitometric recording of the protein separation pattern of a 19S TG standard preparation following reduction of the sucrose concentration from 20% to 2.5%. In some separation patterns, two distinct bands in the 12S region of the gels were observed (→). O = origin.

observed as shoulders of the TG peak (Fig. 3). In rare instances, a minute protein band, FR, was observed which moved with the tracking dye in the 10 % separation gel (Fig. 1). The relative amounts of the fractions in the protein separating patterns are listed in Table I. It was found that about 80 % of the TG molecules remained stable whereas about 10 % appeared in protein bands migrating to the 12S region of the gels and less than 10 % appeared in the shoulders of the TG peak.

In controls, run in parallel, of the undiluted standard preparations, no new components appeared in the separation patterns within 24 h (Fig. 4a). Neither did dilution of the TG standard preparation with PBS containing 20 % sucrose result in the appearance of new fractions in the separation pattern (Fig. 4b).

The 27S standard preparation and PBS (A)

The 27S protein appeared more resistant to the effects of PBS dilution than the TG standard. After 2 h a slight shouldering of the 27S peak and a band at the front were the only signs of 27S fragmentation (Fig. 5).

The TG standard preparation and 8 M urea (B)

When TG standard preparations were incubated for 3 h with 8 *M* urea, there was a noticeable broadening of the base of the major protein peak and the top of this

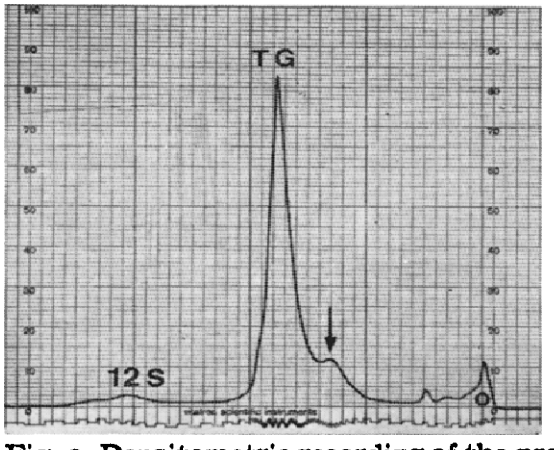

Fig. 3. Densitometric recording of the protein separation pattern of a 19S TG standard preparation following reduction of the sucrose concentration from 20 % to 2.5 %. In some separation patterns, a slower migrating fraction formed a distinct shoulder on the TG peak (→). O = origin.

TABLE I

PROTEIN FRAGMENTS FORMED AFTER DIFFERENT INCUBATIONS OF 19S THYROGLOBULIN

The figures represent the relative amounts (%) of stain bound to the proteins.

Solution	*Protein fragments*						
	19S	*17S?*	*12S*	*Component I*	*Component II*	*Component III*	*FR*
PBS, pH 6.8	80	10	10				Traces
8 *M* urea, pH 6.8		65–75		20–30	Traces	Traces	Traces
Dialysis, 0.1 *M* Tris, pH 8.1–2 *M* urea		55–65		25–35	Traces	Traces	Traces

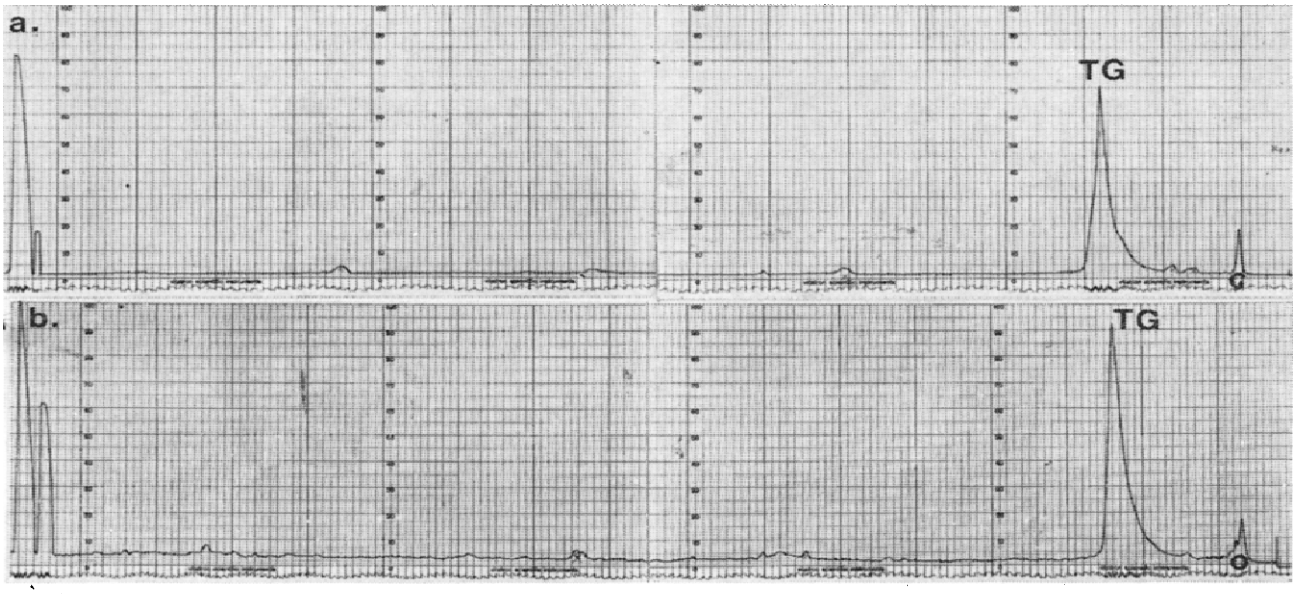

Fig. 4. Densitometric recordings of the protein separation patterns of 19S TG standards (a) after 24 h in 20% sucrose and (b) after dilution (1:8) with 20% sucrose in PBS. O = origin.

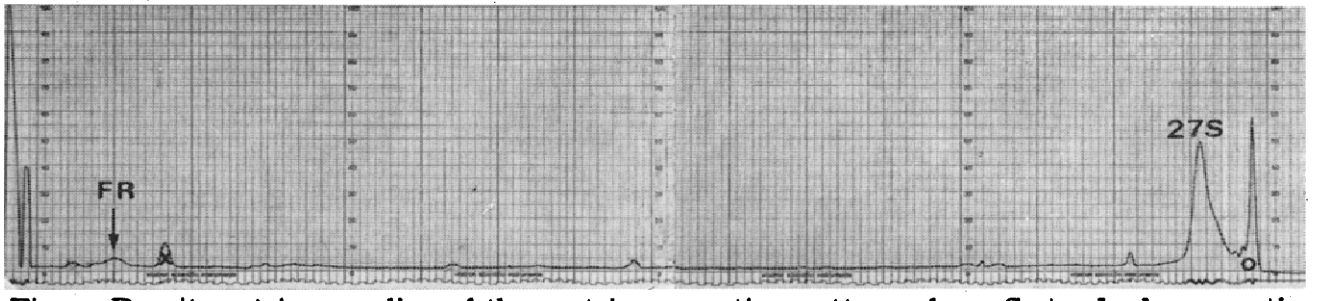

Fig. 5. Densitometric recording of the protein separation pattern of a 27S standard preparation following reduction of the sucrose concentration from 20% to 2.5%. A small amount of protein migrates with the front (FR) and the 27S peak shows a slight broadening. X = artefacts; O = origin.

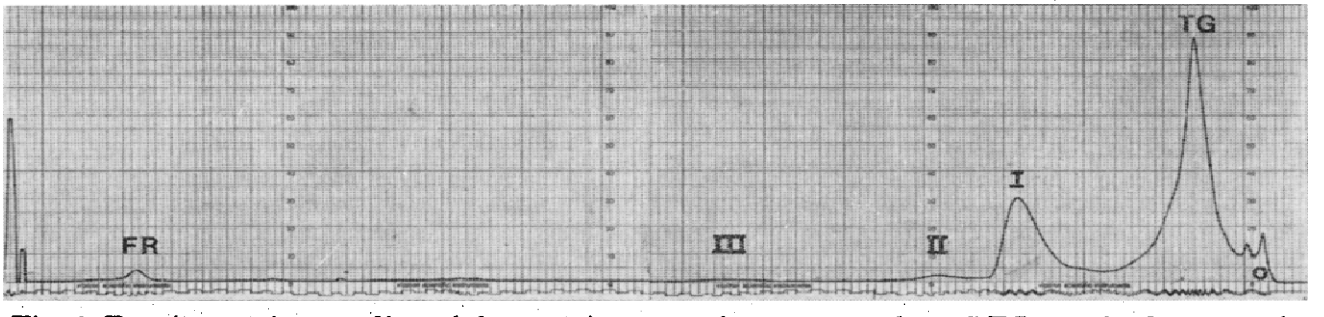

Fig. 6. Densitometric recording of the protein separation pattern of a 19S TG standard preparation kept in 8 *M* urea for 3 h (0–4°). The major protein band was retarded compared with a 19S TG standard. Four faster migrating fractions, components I–III and FR, appeared in the gel. O = origin.

peak was slightly retarded compared with the migration of the TG standard (Fig. 6) and 65–75 % of the stain was found in this band (Table I). Four faster migrating fractions (components I, II, III and FR) were observed. The major new fraction (component I) migrated slightly faster than a 12S standard and bound 20–30 % of the stain where-

as components II and III and a protein migrating with the front, FR, together contributed only a few per cent to the total stain.

The 27S standard preparation and 8 M urea (B)

On incubation in 8 *M* urea at 0–4° for 3 h, the 27S protein generally reacted with the formation of a fraction of varying size migrating with the front (Fig. 7). In addition, some separation patterns contained faint bands in the 19S and 12S regions of the gel.

Dialysis of the TG standard preparation against 0.10 M Tris buffer, pH 8.1, and 2 M urea (C)

After dialysis for 3 h, two peaks were observed in the TG region of the gel. The faster migrating peak corresponded to the TG standard (Fig. 8). After dialysis for 24 h only the more slowly migrating fraction remained in the separation pattern but the base of the peak was broad, similar to that of the double peak in the 3 h pattern. Four faster migrating fractions were observed. The migration rates and the relative amounts of these fractions were similar to those observed after treatment of TG with 8 *M* urea (Table I).

Dialysis of the 27S standard preparation against 0.10 M Tris buffer, pH 8.1, and 2 M urea (C)

After dialysis of the 27S standard against 0.10 *M* Tris buffer of pH 8.1 and

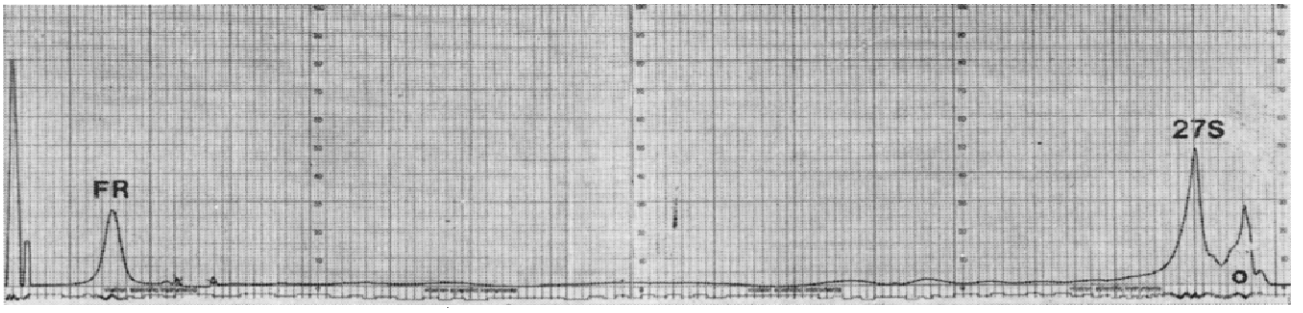

Fig. 7. Densitometric recording of the protein separation pattern of a 27S standard preparation incubated in 8 *M* urea for 3 h (0–4°). A large amount of small components (FR) migrates with the front. O = origin.

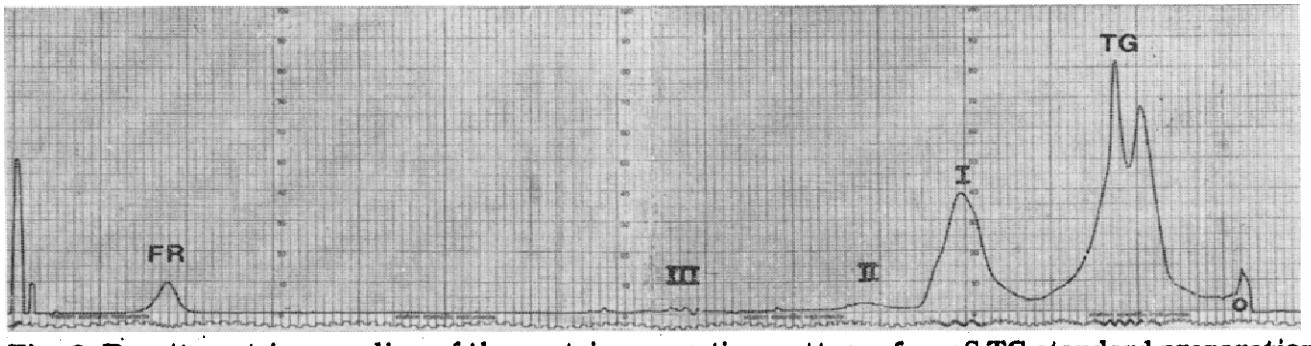

Fig. 8. Densitometric recording of the protein separation pattern of a 19S TG standard preparation dialysed against 2 *M* urea at pH 8.1 for 24 h (0–4°). The separation pattern shows a large split fraction, one component of which migrates more slowly than 19S TG, and four distinct peaks (components I–III and FR). *Cf.* Fig. 6. O = origin.

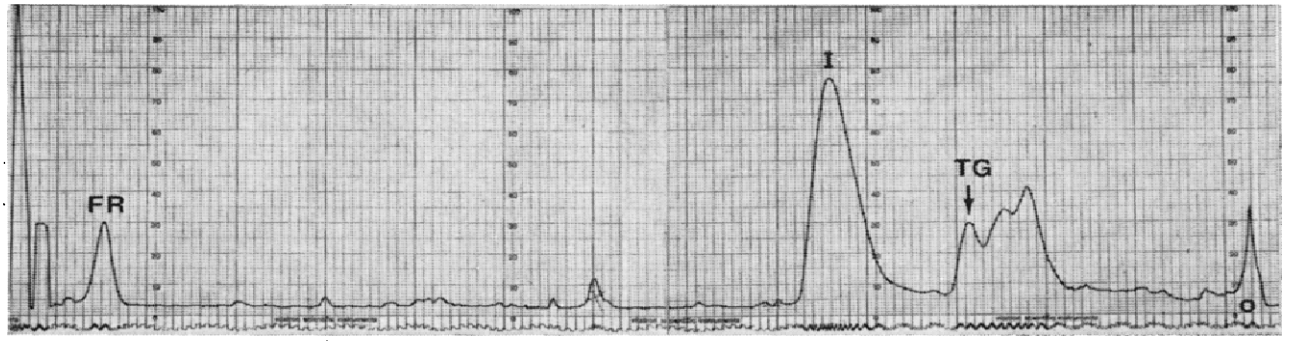

Fig. 9. Densitometric recording of the protein separation pattern (8% gel, pH 6.8) of an iodine-poor TG standard preparation incubated in 8 *M* urea for 3 h (0–4°). The separation pattern shows one large fraction (I) migrating faster than TG, a fairly large fraction at the front and two fractions slower than TG. O = origin.

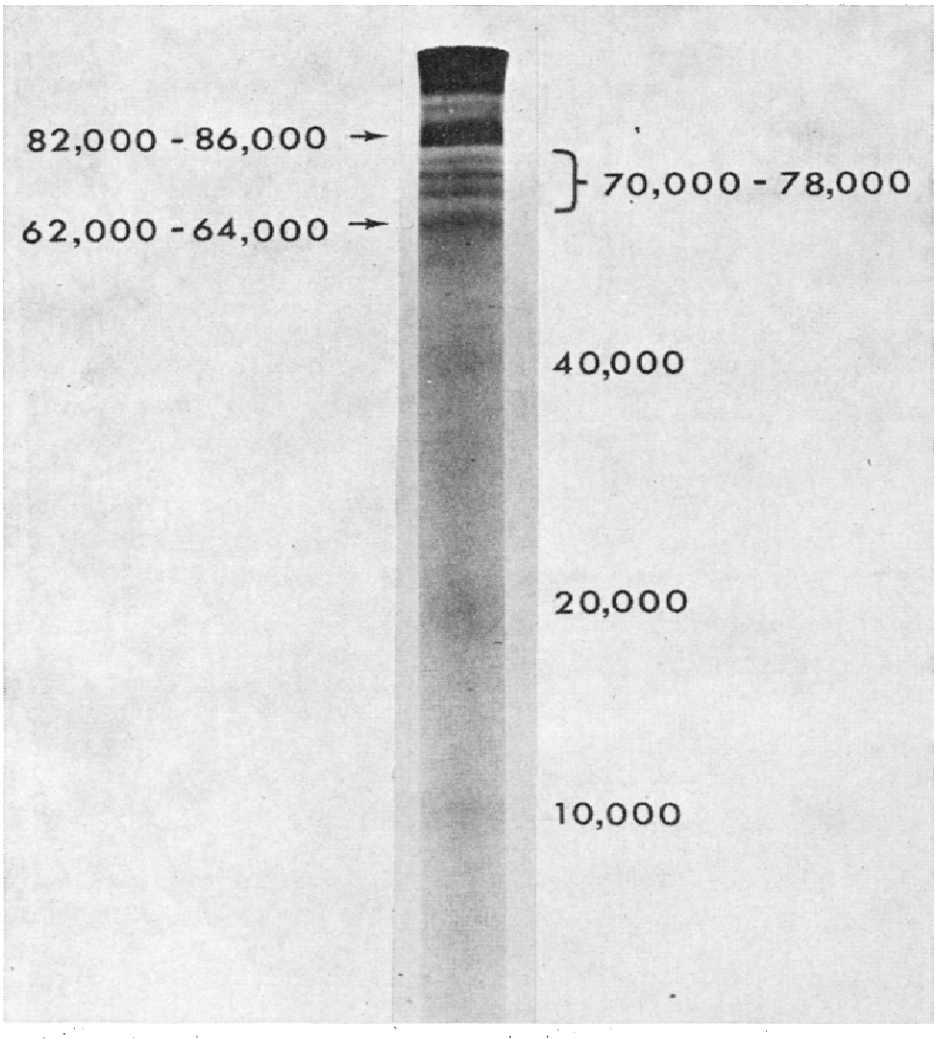

Fig. 10. SDS polyacrylamide gel electrophoresis of 19S TG, 2–4 mg/ml, 25 µl per gel column (0.4 × 12 cm). The 19S fraction was treated with 8 *M* urea for 3 h (0–4°) and dialysed against 1.0% sodium dodecylsulphate and 1.0% mercaptoethanol in 10 m*M* phosphate buffer of pH 7.0 for 48 h. The polyacrylamide monomer concentration was 10%. Calculated molecular weights of the split products are indicated.

2 *M* urea for 24 h, essentially the same separation patterns were observed as after treatment of 27S with 8 *M* urea.

The iodine-poor TG standard preparation and PBS (A)

The iodine-poor TG standard reacted in 8 *M* urea with formation of one main fraction that migrated similarly to component I. TG and two more retarded fractions were observed nearer the origin of the gel and one band was observed at the front (Fig. 9). There were no signs of the components II and III.

SDS gel electrophoresis

The TG standard preparations reacted with the formation of a number of protein fragments (Fig. 10). The largest and main dissociation product observed in the 10% SDS gel was a fraction with a molecular weight of 82,000–86,000. Three distinct protein bands with calculated molecular weights of 70,000, 74,000 and 78,000 were observed; the middle fraction was the most prominent. The fourth clearly visible dissociation product had a molecular weight of 62,000–64,000. In addition, more diffuse bands representing proteins with calculated molecular weights of about 20,000 and 10,000 were observed.

DISCUSSION

This series of experiments was performed to study the stability of the large soluble thyroid iodoproteins under conditions that were similar to those under which the proteins in samples of the colloid collected from single rat thyroid follicles were stored and separated[12]. Because of the presence of protein fractions in the colloid that migrated faster than TG and 12S protein, it was further deemed to be of importance to analyse the protein separation patterns that resulted from degradation of TG and 27S protein.

It was observed that TG remained stable for at least 24 h when stored in PBS of pH 6.8 containing 20% sucrose. However, if PBS without sucrose was added to the TG standard preparation, resulting in a decreased sucrose and protein concentration, dissociation of the TG molecule occurred and 12S protein as well as small but significant amounts of a more slowly migrating fraction appeared in the electrophoretic separation patterns. Occasionally, a minute fraction migrating at the front was also observed. Dilution of TG standard preparations with PBS containing 20% sucrose, giving the same reduced final protein concentration, did not result in this dissociation pattern, indicating that it was the decreased sucrose concentration and not the reduced protein concentration that caused the dissociation. The observation that the TG molecule is susceptible to alteration of the sucrose concentration is of relevance to previous investigations from this laboratory on the protein composition of the colloid collected from single rat thyroid follicles. In a preliminary study[2] in which the colloid was collected and stored in PBS without sucrose, it was observed that a 12S protein was present in almost all colloid samples and constituted about 20% of the total proteins. After the introduction of sucrose in the storage medium, a 12S protein has been detected in only a small percentage of the colloids and has not exceeded about 7% of the sample protein. It should further be noticed that alteration of the sucrose concentration in the TG and 27S standard preparations did not result in

the appearance of any dissociation product migrating in the albumin region of the gel, as an albumin-like fraction was found in a large proportion of the colloid samples in normal thyroid follicles[16].

The presence of two peaks in the 12S region of the gel after dissociation of the TG molecule suggests that rat thyroglobulin is composed of two slightly different 12S subunits. As the difference in migration rates depends on the size, net charge density and conformation of the molecules, the reason for the observed heterogeneity of the 12S fraction could not be elucidated. The present observation, however, is in accord with previous studies which indicated that TG is composed of two different 12S molecules[17] and that two different peptide chains form the 12S subunits[18–21].

The reduced rate of migration of the TG molecule in the presence of dilute alkali and 2 *M* urea as well as 8 *M* urea is probably due to increased friction between the protein molecule and the gel matrix. Under similar conditions, the analytical ultracentrifugation pattern reveals a component with a sedimentation coefficient of 17S. The reduced sedimentation coefficient suggests a change in the conformation of the molecule that implies increased friction against the surrounding sedimentation medium, and this particle is therefore regarded as being a partially unfolded form of the 19S TG molecule[22,23]. The reduced rate of migration in gel electrophoresis could be due to a similar change in conformation and it is suggested that the band observed in the present electrophoretic separation pattern represents the 17S species. This protein fraction was not observed in the TG standard preparations but a component appeared in the same region in a relatively small amount, about 10%, when the sucrose concentration was decreased. This component could be a similarly unfolded 19S molecule.

The results of previous investigations on the subunits of TG are not in agreement: the number of subunits observed has been reported to be four[7,24], five to six[25] and eight[18]. Subunits of about one-tenth of the native molecules have also been observed in the presence of urea, guanidine or detergents[23]. In the present work, it was demonstrated that the rat TG constantly reacted on 8 *M* urea and on 2 *M* urea of pH 8.1 in essentially the same way and with the formation of four components that migrated faster than TG and 12S. The main dissociation product, component I, was the most retarded and probably represents the largest of these dissociation products. A large fraction that migrated very similarly to component I was formed by dissociation of iodine-poor TG. As it has been shown that the main dissociation product of urea-treated 19S TG is the 12S molecule[22] and that this product is more readily formed from iodine-poor TG[5–8], it seems reasonable to assume that component I is a modified 12S molecule.

The origin of the components II, III and FR has not been determined. They could represent split products of component I or dissociation directly from the thyroglobulin molecule.

To analyse the proteins in the front fraction, FR, the molecular weights of which are in the region of 80,000–90,000 and lower, urea- and SDS-treated 19S TG standards were analysed by SDS gel electrophoresis. The main dissociation product, of molecular weight 82,000–86,000, was in the region of the molecular weight of the smallest TG subunit reported by LISSITZKY *et al.*[18] and possibly represents this TG subunit. The nature of the smaller fragments is not clear.

The 27S protein was fairly stable in PBS at low sucrose concentrations. On

treatment with 8 *M* urea and 2 *M* urea at slightly alkaline pH, the 27S molecule reacted to a greater extent than did 19S with the formation of fragments that migrated at the front, and only very faint bands could be observed in the 19S and 12S regions of the gel. These large amounts of protein at the front in some dissociation patterns indicate that the 27S molecule is composed of a number of small peptides linked to each other by bonds which are broken in the absence of reducing agents. This suggests that non-covalent linkages bind a number of the subunits in the 27S molecule.

It has been proposed that the 27S molecule is a dimer of TG or a polymer of TG subunits[10]. Hence the amino acid composition seems essentially identical and immunochemical studies indicate that the two species contain common antigenic determinants[10]. The content of iodine, however, is higher in 27S than in TG and it has been proposed that this difference depends on a higher number of thyroxyl residues in the 27S molecule[10]. The present observations do not contradict the hypothesis that 27S is formed by association of two 19S molecules, but indicate that bonds other than those present in 19S thyroglobulin link the subunits in the 27S molecule.

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