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ANALYSIS OF THYROID PROTEINS BY MEANS OF MICRO DISC ELECTROPHORESIS

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SUMMARY

A micro disc electrophoresis technique was used to separate the soluble thyroid proteins at a neutral pH. Samples of 27 S, 19 S and 12 S proteins were prepared by centrifugation in sucrose density gradients and the electrophoretic separation patterns of these proteins were recorded by direct densitometry of the gels after staining with Amido Black.

The relations between the amount of 27 S, 19 S and 12 S and the optical densities of the stained protein discs were analysed.

The contribution of serum proteins to the 3–8 S peak in the density gradient centrifugation pattern was studied by electrophoresis of the thyroid proteins from perfused and non-perfused glands. It was found that the "heavy" part of the 3–8 S peak from non-perfused glands mainly represents serum proteins. However, after perfusion the 3–8 S region still contains four electrophoretically distinct bands in addition to the 19 S and 12 S bands.

The colloid extracted by micropuncture from one rat thyroid follicle was analysed by the method described. The migration rate of the single recorded protein was very similar to that of 19 S thyroglobulin.

INTRODUCTION

It is a well established fact that the major protein component of the thyroid gland is 19 S thyroglobulin. It is also generally accepted that the thyroglobulin is stored in the follicular lumen and a large number of observations indicate that thyroglobulin is the predominant constituent of the colloid. For example, the spectral absorbance properties of the colloid have been found to be the same as those of thyroglobulin solution[1–3] and fluorescein-coupled thyroglobulin antiserum has been observed to stain the colloid[4,5]. Furthermore, light and electron microscopic autoradiographic observations strongly indicate that the iodoproteins synthesized in the follicular cells are stored in the follicular lumen[6–8].

However, none of the methods used is specific for 19 S thyroglobulin. For example, both larger and smaller soluble thyroid iodo-proteins exert thyroglobulin antigenecity[9]. Furthermore, autoradiographic studies with labeled iodine have low

pecificity since not only thyroglobulin but also its 12 S precursor protein can be odinated[10–12].

Proteins other than iodoproteins are also probably present in the follicular umen. Proteolytic activity has been found in extracts obtained by micro-puncture of single follicles of rat thyroids[13]. According to BALFOUR *et al.*[14], sera from patients vith Hashimoto's and other thyroid diseases contain antibodies against an antigen of the colloid distinct from thyroglobulin.

Thus, the colloid evidently contains thyroglobulin-like proteins and probably ther substances as well but detailed information of the composition of the colloid s lacking. This must be considered as a notable deficiency in our knowledge of hyroid physiology. For example, studies on thyroglobulin synthesis in this[15,16], and ther laboratories[17,18], strongly indicate that the 19 S thyroglobulin formation is ompleted intracellularly, but without data on the composition of the colloid it is ot possible to determine if precursor proteins are also secreted into the follicular umen. Also having a bearing on the colloid composition is the question whether any ydrolysis of thyroglobulin occurs in the follicular lumen or whether the hormone elease is the result of exclusive intracellular thyroglobulin degradation by lysosomal nzymes[19–23].

Against this background, it was deemed important to obtain information on the composition of the colloid in the follicular lumen. The only way to isolate the colloid, without substantial contamination by extrafollicular or intracellular components, seemed to be by micropuncture of the thyroid follicles. This, in turn, made it necessary to adapt a micromethod capable of separating the proteins extracted from a single or a few follicular lumina.

This paper presents the results, obtained by a micro disc electrophoresis technique at a neutral pH, of a separation and identification of the soluble thyroid proteins. The reliability of a quantitative determination of the protein by direct densitometry of the gels is analysed. The usefulness of the technique for the analysis of colloid from a single follicle is also illustrated. A more comprehensive account of the protein composition of the follicular colloid will be presented in another paper.

MATERIAL AND METHODS

Micro disc electrophoresis

The electrophoresis technique used is a microscale modification[24] of the original disc electrophoretic method of ORNSTEIN[25] and DAVIS[26].

The inner walls of 5 μl glass capillaries (Microcaps, Drummond Sci. Co., Broomall, Pa., U.S.A.) were coated with 0.07% methyl cellulose (Methocel, Dow Chem. Co., Midland, Mich., U.S.A.) in 30% formic acid–formaldehyde solution (1:5)[27].

Stock solutions. Stock A: pH 6.8, 8.69 g Tris + 0.63 ml N,N,N′,N′-tetramethylethylenediamine + 3.6 *N* H_2SO_4 + H_2O to 100 ml and a pH of 6.8.

Stock B: 10 g acrylamide (Eastman Organic Chemicals) + 200 mg N,N′-methylenebisacrylamide + H_2O to 37.5 ml.

Stock C: 1 mg riboflavin + 50 ml H_2O.

Stock D: pH 6.8, 5.98 g Tris + 0.46 ml N,N,N′,N′-tetramethylethylenedi-

amine + 1.0 *N* HCl + H_2O to 100 ml and a pH of 6.8.

Stock E: 3 mg riboflavin + 50 ml H_2O.

Preparation of the lower gel. One ml of (0.5 ml Stock A + 1.5 ml Stock B) + 1.0 ml Stock C gives a 10% solution.

Preparation of the upper gel. One ml of (0.5 ml Stock D + 1.5 ml Stock B) + 1 ml H_2O. One ml of this solution is added to 1 ml Stock D. One ml of this solution is added to 0.8 ml H_2O + 0.2 ml Stock E. The final concentration of the upper gel will be 2.5%.

Electrode buffer, pH 8.3. 3.0 g Tris + 14.4 g glycine + H_2O to 500 ml. A drop of Bromphenol Blue is added.

The electrophoresis was carried out at +4° and the voltage gradient over the gel was kept at 20 V and 40 V during the stacking and separation phases, respectively. The run was terminated when the Bromphenol Blue band had migrated 7 mm into the separation gel.

After the run, the gels were immediately pushed out of the capillaries into 80% ethanol. The proteins were stained for 5 min in 0.5% Amido Black solution in 7.5% acetic acid. After staining, the gels were destained in 7.5% acetic acid and stored in 7.5% acetic acid containing a small amount of Amido Black. The extinction of the storage solution was about 0.15 at 620 mμ.

Densitometry

The optical density of the stained protein discs in the gel was recorded after destaining in the storage solution for 1 hour and 45 minutes. A Schnell Photometer II (Zeiss, Jena, East Germany) equipped with a modified UR 400 Linear Logarithmic Integrator Recorder (Vitatron N.V., Dieren, Holland) was used for the recording[33]. For densitometric measurements the gel was mounted in a slit (20 × 0.5 mm) in a brass plate which was placed between two glass plates. By means of a motor, the gel was scanned at a speed of 2.17 mm/min, and the recording ratio between the paper and gel was 92. The gel was magnified 32 times on the photometer screen. An adjustable slit permitted the recording of the entire width of the gel image or a fraction of it. A red filter, Wratten No. 25, was inserted in the light path.

Photographs of the gels were taken using a Liesegang enlarger with parallel light and Ilford N-50 half tone backed plates.

Preparation of the thyroid proteins

The thyroid protein samples used as standards were prepared as shown in Fig. 1. Thyroid glands were excised from guinea pigs and rats after thorough perfusion of the anaesthetized animals with large volumes of phosphate buffered saline (PBS). The glands were pooled in cold PBS, weighed and minced. Homogenisation was performed in a glass homogenizer equipped with a tightly fitting Teflon pestle. The homogenate was centrifuged at 700 × *g* for 10 min and the resulting supernatant was centrifuged at 105 000 × *g* for 90 min.

An aliquot of the supernatant from the second centrifugation (S II) was stored at −20° until used for electrophoresis and protein determination. The proteins in the remaining S II supernatant were separated by centrifugation on either a 4.5 ml or a 14 ml continuous sucrose density gradient, 10–25%, prepared with PBS in 5 ml or 15 ml Spinco tubes respectively. After centrifugation at 75 000 × *g* for 16 h (5 ml

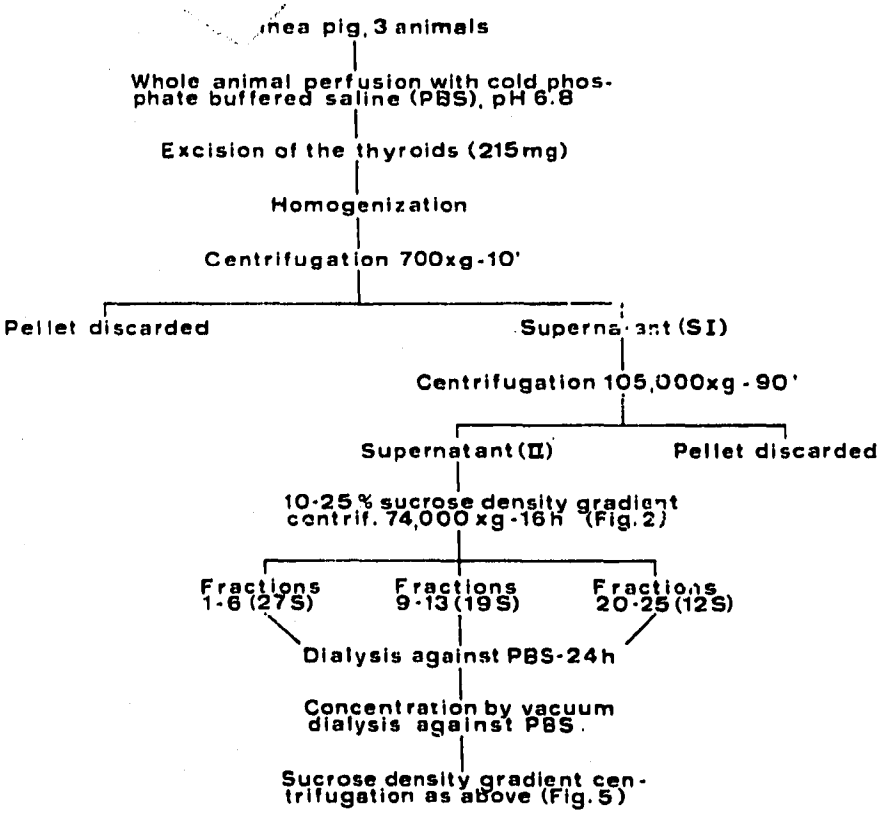


Fig. 1. Scheme for the preparation of the soluble proteins from guinea pig and rat thyroids.

tube) or at 150 000 × *g* for 14 h (15 ml tube) the tube was punctured and about 40 fractions were collected. Every second fraction was diluted with 0.8 ml PBS and the optical density at 280 mμ was measured. Every second, non-diluted fraction was analysed by disc electrophoresis. The samples used for electrophoresis were diluted in such a way that they all contained approximately the same amount of protein as judged from the absorption figures at 280 mμ.

In the guinea pig series, fractions were pooled from three regions of the density gradient. These fractions are referred to as 27 S, 19 S and 12 S (see Fig. 2). The pooled fractions were dialysed against PBS for 24 h and concentrated by vacuum dialysis at +4°. In the rat series, fractions from the 19 S region of the density gradient were pooled and handled in parallel with the guinea pig series. The pooled fractions from both series were further purified by another sucrose density gradient centrifugation as described above. The density gradient patterns were recorded at 280 mμ and the top fractions from each peak were analysed by electrophoresis.

Extraction of colloid

The extraction of colloid was performed by a micropuncture technique similar to that described by DE ROBERTIS[13] and SELJELID[31].

In rats, the isthmus of the thyroid was used for the puncture, in guinea pigs the right lobe was the most suitable part of the gland. Under light sodium pentobarbital anaesthesia the gland was surgically exposed under a dissecting microscope and gently freed from surrounding connective tissue. The extraction of colloid was performed through a glass cannula with a 10–15 μ wide, tapered tip and adapted to

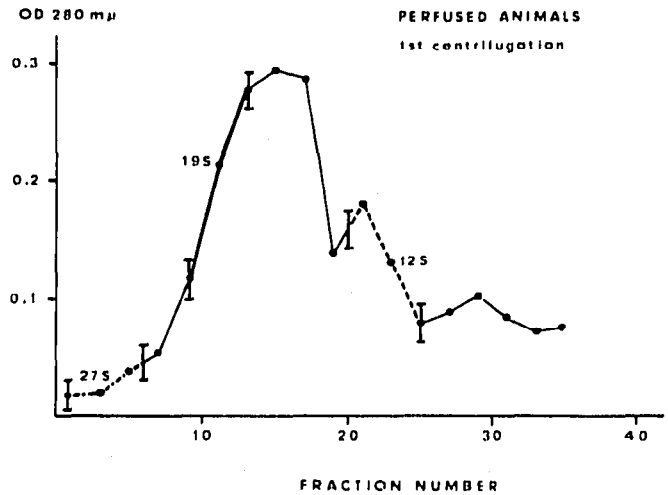


Fig. 2. Density gradient centrifugation pattern of the soluble proteins from guinea pig thyroids. The fractions were pooled and recentrifuged from the three regions (27 S, 19 S and 12 S) of the gradient indicated.

a Fonebrune micromanipulator. The colloid was extracted by negative pressure over the tip of the cannula produced by a glass syringe connected to the cannula *via* a low-elastic tube-system. The extracted colloid samples were immediately poured out into 5 or 10 μl of cold PBS.

RESULTS AND DISCUSSION

The main component of a sample of soluble thyroid proteins is the 19 S thyroglobulin molecule, which has a molecular weight of near 2/3 million. In addition to this molecule the heavier 27 S molecule and the lighter 12 S, 7 S and 4 S components are found in different amounts[9]. The wide range of molecular weights makes it difficult to design a separation system which gives optimal separation of all the proteins which could be expected in the colloid.

Under the separation conditions found to be most satisfactory (as regards voltage, separation time etc.) separation gels of 5.5 to 7.5% acrylamide were suitable for the separation of the larger molecules, 27 S and 19 S, while the smaller units, 12 S to 4 S, were preferably separated on 15 to 25% gels. As the amount of protein extracted from a single follicle is too small to be divided into several portions for analysis in different systems a 10% gel was chosen as a compromise for the survey analysis of the colloid proteins.

Since the resistance, and consequently the heat production, increases rapidly during the stacking of the proteins in the spacer gel[25], the voltage gradient was kept low during this phase of the electrophoresis (20 V). When the reference substance (Bromphenol Blue) was visible in the separation gel as a distinct band, the voltage gradient was increased to 40 V. This augments the speed of the separation and reduces the effect on the separation pattern of the time-dependent free diffusion.

In an alkaline environment, thyroglobulin is easily degraded into 12 S subunits[28]. To eliminate the effect of the basic pH's originally described for the method (pH 9.8, ref. 26, and 8.8, ref. 24) the pH of the separation gel was adjusted to 6.8.

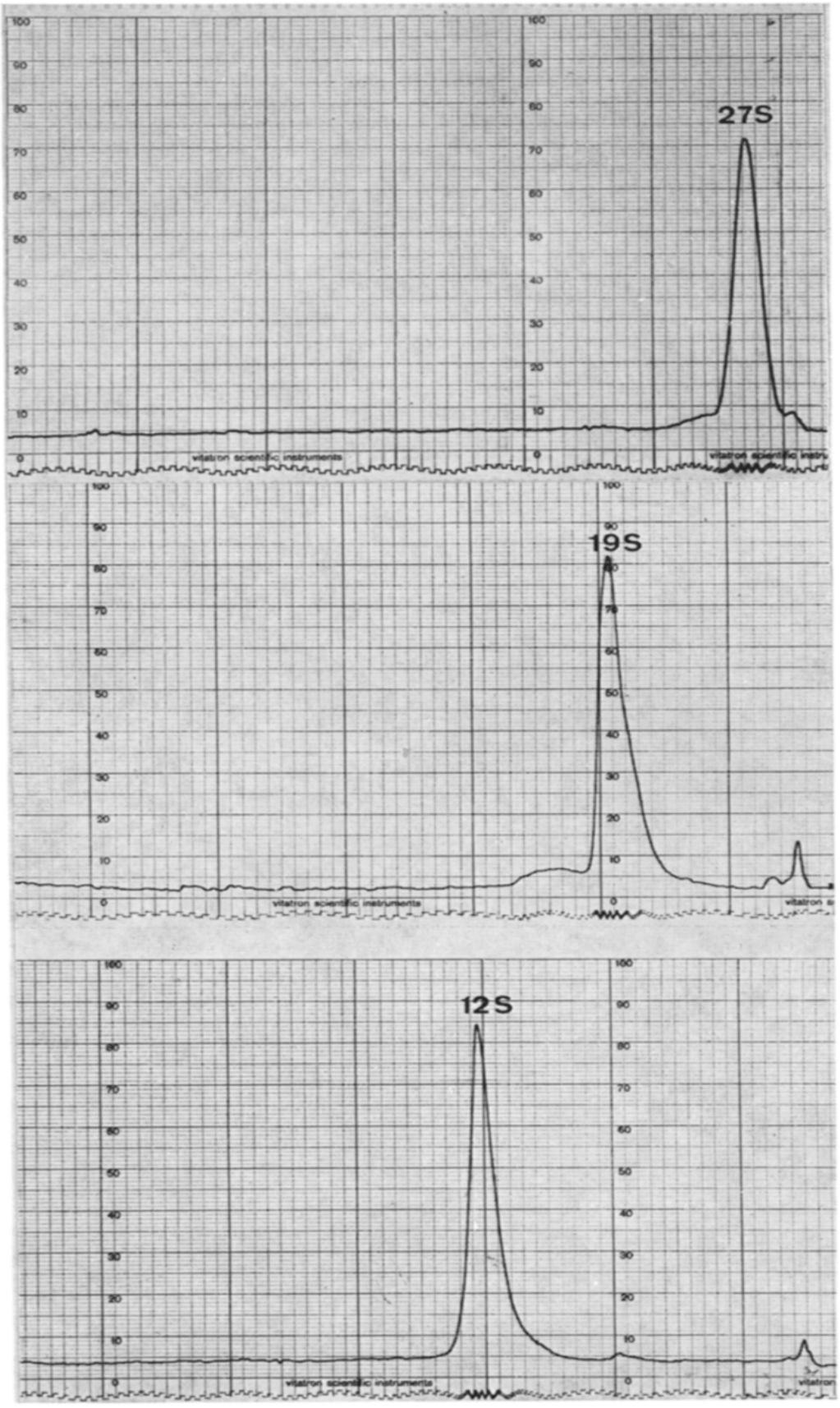


Fig. 3. Densitometric recordings of the electrophoretic separation patterns for 27 S, 19 S and 12 S proteins.

In this system no degradation of 27 S, 19 S or 12 S components was observed during the electrophoresis (Fig. 3).

Qualitative analysis of the electrophoretic separation patterns

The soluble thyroid proteins from guinea pigs and rats were used. Guinea pigs were chosen because their thyroids contain considerable amounts of stable 12 S protein[29]. In order to eliminate the disturbing influence of serum proteins, the animals were perfused with cold PBS prior to the excision of the glands. This was considered important owing to the fact that one of the 4 S components extracted from the thyroid gland has very similar electrophoretic properties to those of serum albumin[9].

The density gradient centrifugation pattern of the soluble guinea pig thyroid proteins is shown in Fig. 2. After electrophoretic analysis it was obvious that the peaks of the density gradient pattern did not represent single protein components (Fig. 4). Therefore the proteins of each one of the 27 S, 19 S and 12 S regions of the gradient were further purified by a second density gradient centrifugation.

Recentrifugation of the fractions in the 27 S region gave no distinct peak in the optical density pattern of the gradient (Fig. 5). However, on disc electrophoresis

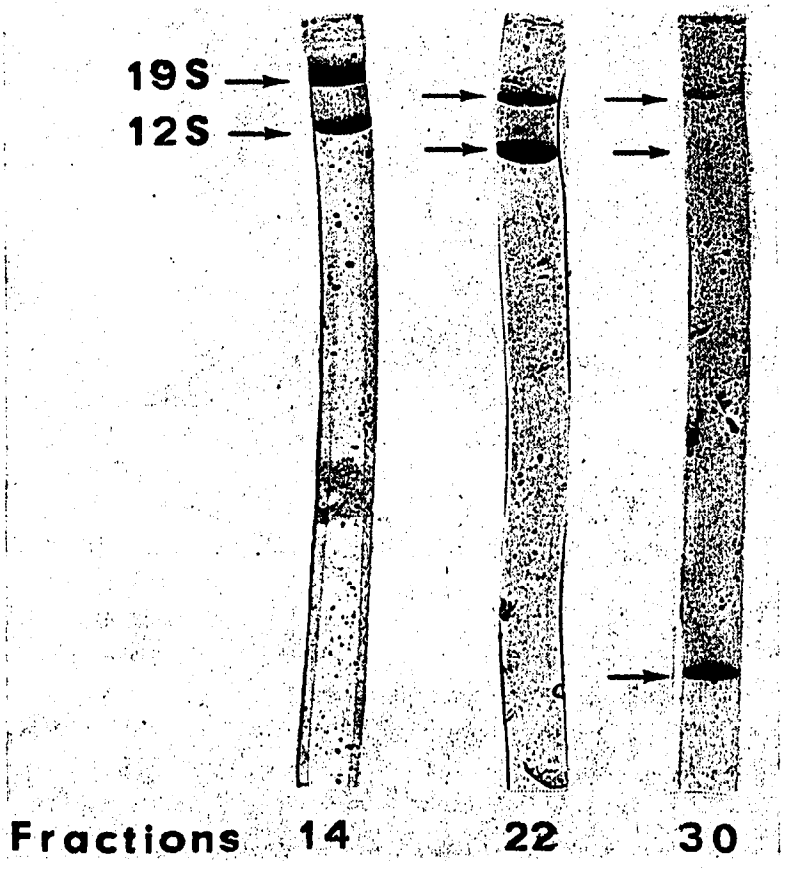
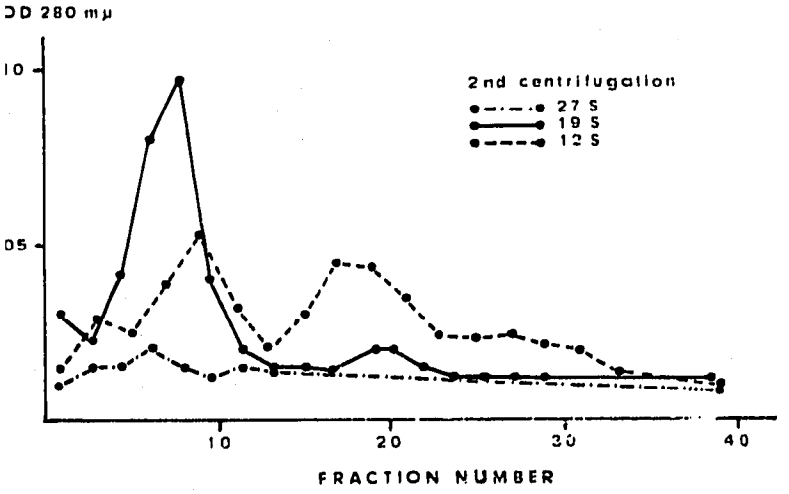


Fig. 4. Photographs of the gel separation patterns resulting from electrophoresis of fractions No. 14, No. 22 and No. 30 in Fig. 2. The part migrating most slowly is the major component in fraction No. 14, from the 19 S peak, and the second, faster migrating band predominates in fraction No. 22, from the 12 S peak. In fraction No. 30 a very fast migrating band predominates, but very faint bands corresponding to 19 S and 12 S are also visible.



ig. 5. Density gradient patterns of the recentrifuged 27 S, 19 S and 12 S samples indicated in ig. 2. Fraction No. 4 from the 27 S gradient, fraction No. 9 from the 19 S gradient and fraction o. 18 from the 12 S gradient were subjected to electrophoretic analyses.

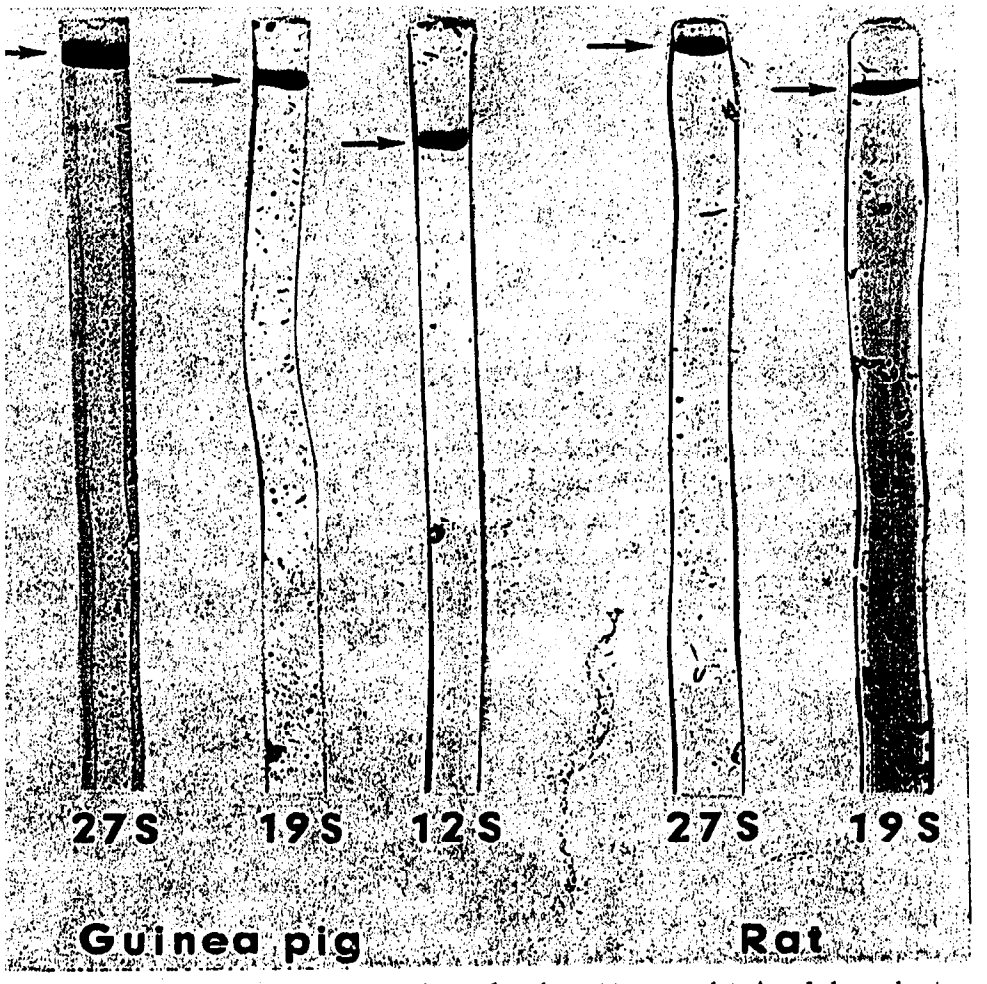


Fig. 6. Left: Three photographs of gel patterns obtained by electrophoresis of purified guinea pig thyroid proteins: fraction No. 4, No. 9 and No. 18 in Fig. 5. Right: The electrophoretic separation patterns of similarly purified rat thyroid proteins from the 27 S and 19 S density gradient regions.

the protein of the first four fractions of the 27 S gradient migrates as one band, distinct from the 19 S particles (Fig. 6).

The optical density pattern of the second 19 S gradient shows two peaks (Fig. 5). Samples from the faster sedimenting larger peak display only one electrophoretic band, most probably 19 S thyroglobulin (Fig. 6).

The extinction pattern obtained from the recentrifugation of the 12 S proteins shows two peaks (Fig. 5). The components of the larger, slowly sedimenting peak move in a single band on electrophoresis and very probably constitute 12 S protein (Fig. 6).

Density gradient centrifugation of the soluble rat thyroid proteins, which was run simultaneously with that of the guinea pig proteins, resulted in a small extinction peak in the 27 S region and a large peak in the 19 S region but no visible 12 S peak. Electrophoretic separation of the proteins in these peaks resulted in a single band from each peak, moving at the same rate as the 27 S and 19 S components of the guinea pig proteins (Fig. 6).

Electrophoretic analyses of the guinea pig thyroid proteins in the fractions from the 3–8 S region of the gradient display a marked heterogeneity. The protein

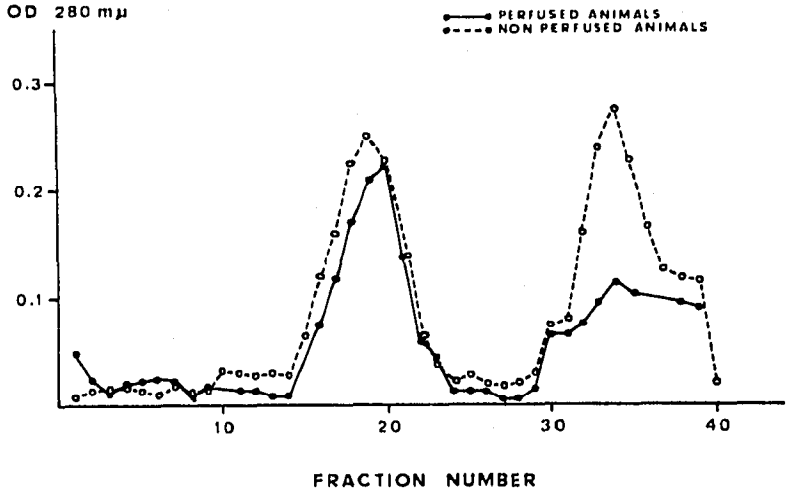


Fig. 7. Density gradient separation patterns representing the distribution of the soluble proteins in thyroid homogenates from perfused (●—●) and non-perfused (○— — —○) guinea pigs.

components of the fastest fractions of the 3–8 S region (Fig. 7, fractions 30–34), split up into a group of four bands which do not separate completely on 15% gel (Fig. 8a). In addition to this group of proteins the electrophoretic pattern also shows a smaller amount of faster migrating proteins which form a broader zone without discrete bands. The latter group of proteins are, however, the major protein components in the 3–8 S region of the density gradient pattern produced by proteins extracted from non-perfused thyroids (Fig. 8b). The later fractions from the 3–8 S peak (Fig. 7, fractions 36–40) display one major component which migrates just behind the reference substance in the 10% separation gel (Fig. 9).

Small amounts of 12 S and 19 S proteins were found in all the fractions from the 3–8 S peak.

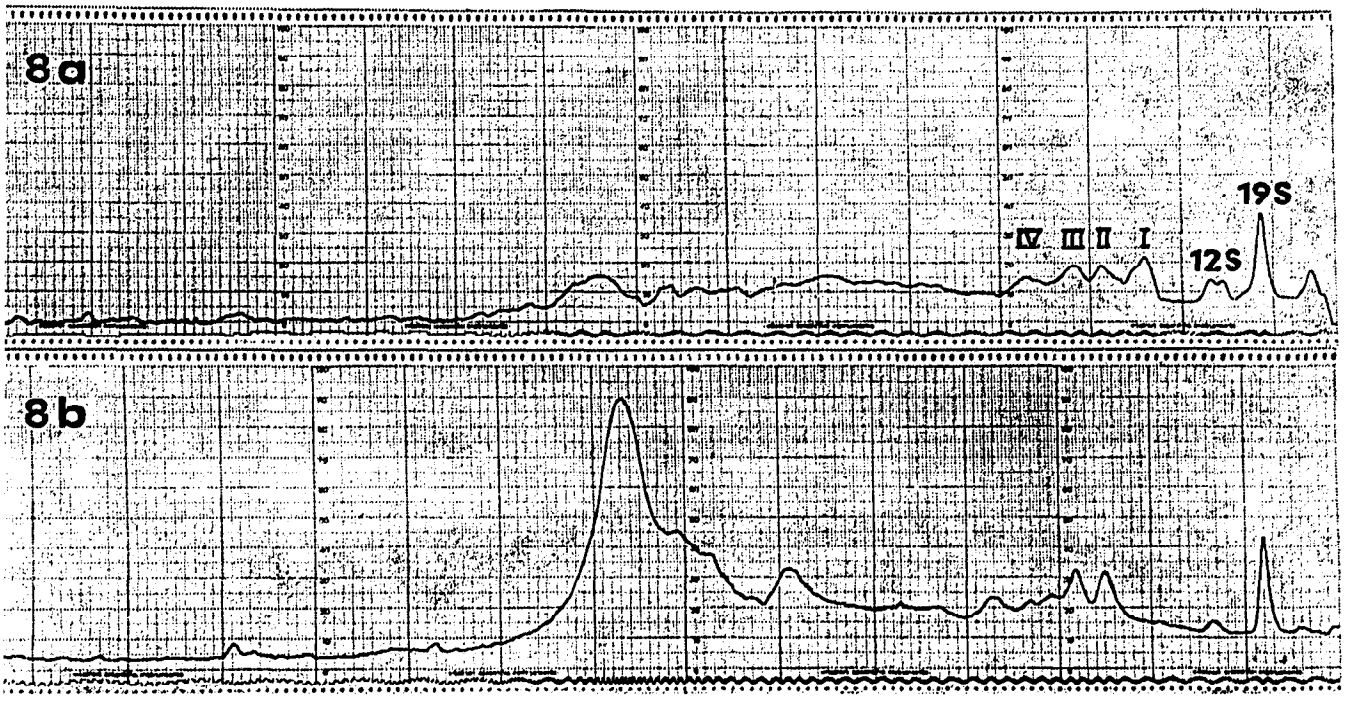


Fig. 8. Densitometric recordings of the gel separation pattern of fractions in the 3–8 S region of the gradients in Fig. 7. (8a) Perfused animals. Fraction No. 32, analysed on 15% separation gel. 19 S and 12 S particles form the two slowest migrating bands. The 12 S band is preceded by four bands (I–IV) of unknown nature. Faster migrating proteins are spread over a zone without discrete bands. (8b) Non-perfused animals. Fraction No. 32, analysed on 15% separation gel. The fastest migrating bands are the predominant constituents of this fraction. These proteins probably represent serum proteins.

Quantitative determination of the thyroid proteins

The Zeiss photometer employed in this study has previously been used for quantitative estimation of electrophoresis separation patterns[32]. The Vitatron integrator unit was modified in such a way that the inverse extinction, caused by the stained band against the light background could be recorded[33].

The contrast between the stained band and the gel was increased about 2-fold by the red filter. In addition, the contrast could be increased by about 10% by narrowing the adjustable slit in the image plane of the photometer since the central

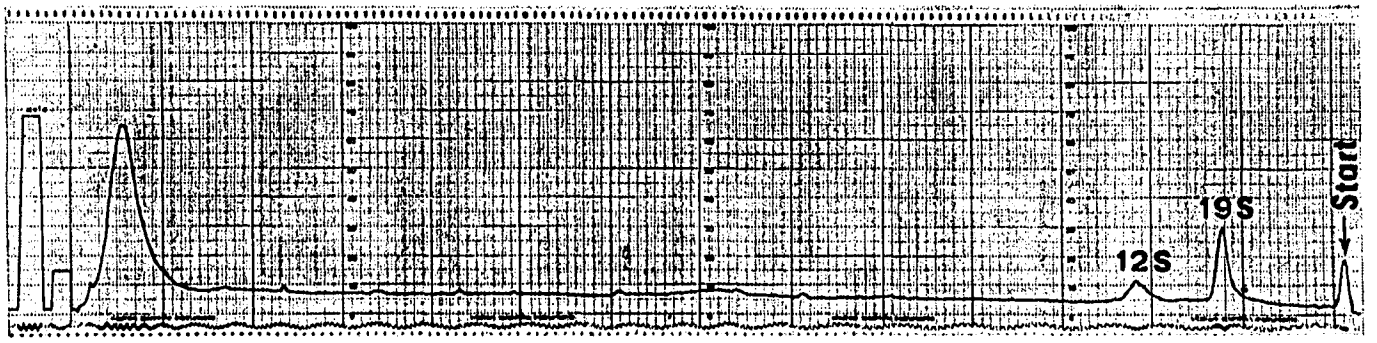


Fig. 9. Densitometric recording of the gel separation pattern of proteins in fraction No. 36 in Fig. 7. The proteins were analysed on 10% separation gel.

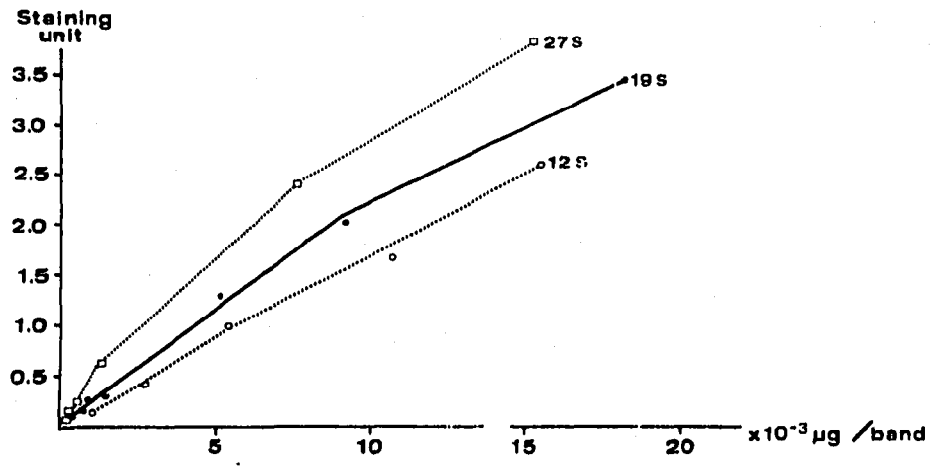


Fig. 10. Diagram showing the relation between the amount of 27 S, 19 S and 12 S particles (μg) and the optical densities of the resulting protein discs after electrophoresis in a 10% separation gel. The staining unit was calculated from S.U. $= a \cdot 1/b \cdot E$ where a is the number of integration impulses; b, the height of the external standard which varies with the sensitivity adjustment; E the fixed values of the external standard (1.0 and 0.2).

part of the stained band has a higher optical density than the lateral parts of the band, provided that the stain is evenly distributed in the protein disc.

In order to calibrate the staining against the amount of protein in a band, electrophoretically homogeneous samples of the 27 S, 19 S and 12 S proteins were prepared. The protein concentration of the samples was determined from their absorption at 280 mμ and at a neutral pH. The extinction coefficient employed was

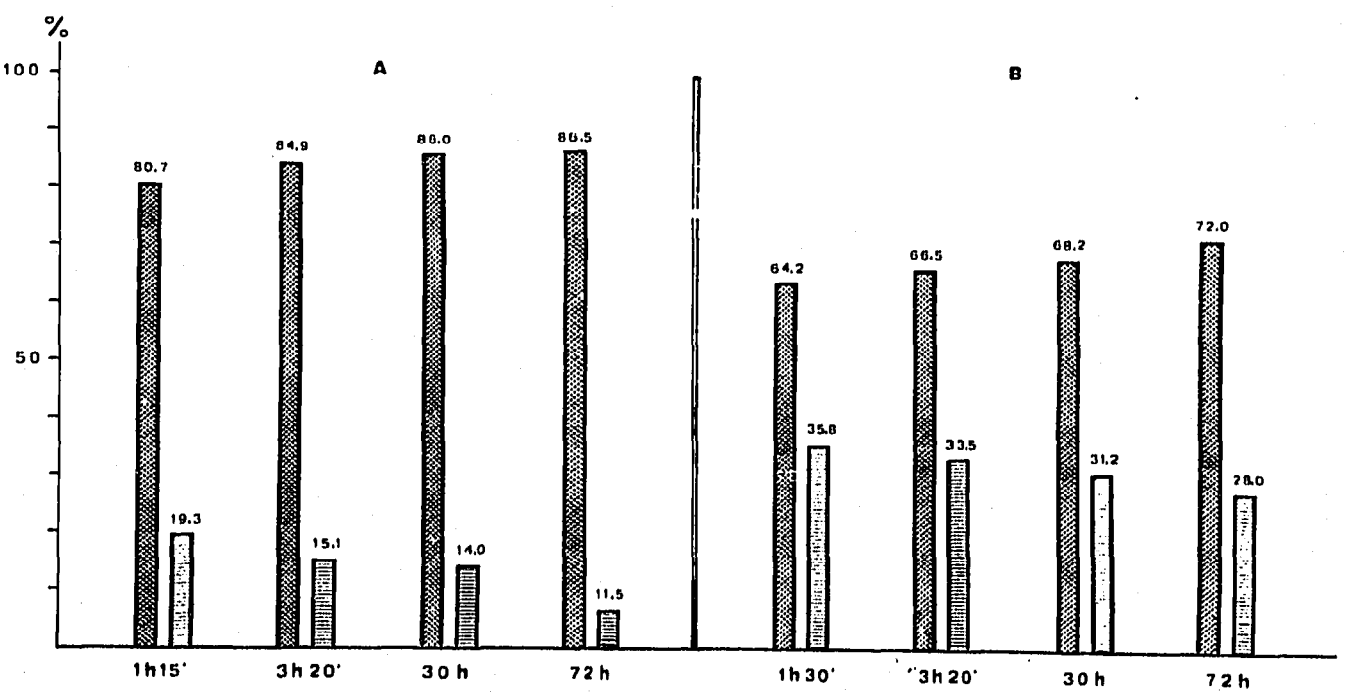
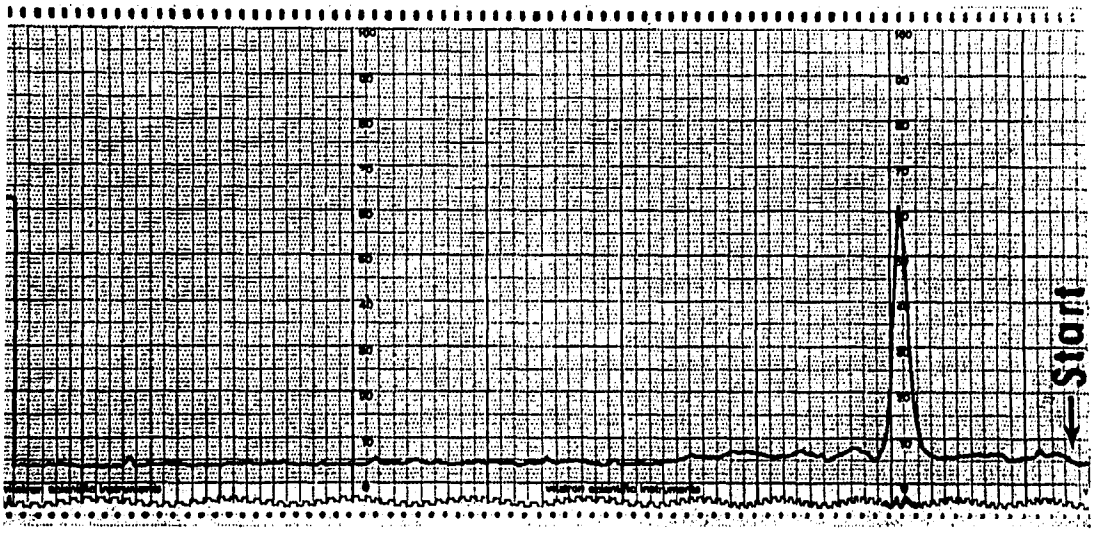


Fig. 11. The relative distribution of stain between a 19 S and a 12 S protein disc in the same gel at increasing destaining periods. In gel A and gel B the same trend of the stain distribution is visible.

$E^{1\%}_{1cm}$ = 10.4 (ref. 30). (This value of the extinction coefficient is an approximation ; it has been determined on a mixture of proteins extracted from hog thyroids.) fter electrophoresis and staining the optical density was recorded on the densitom- er and a staining unit was calculated on the basis of the number of integration ıpulses/band and the sensitivity adjustment of the recorder (Fig. 10).

The interrelation between the amount of protein and the optical density of the ained protein in a band is linear for all three species at low concentrations of protein. t higher concentrations of protein/band the quotient between the optical density ıd the protein concentration decreases.

In order to assess to what degree the densitometric recordings are affected by ıe diffusion rate of the staining molecules from the protein particles, a series of ‹periments was performed in which the percentage distribution of the stain between



ig. 12. Densitometric recording of the separation pattern of the colloid extracted from one arge follicle of the rat thyroid. This colloid was analysed in a 10% separation gel. The migration ate of the band is very similar to that of 19 S particles of the reference samples. No other com- ponents could be recorded in this gel.

ı 19 S and a 12 S band in the same gel was calculated after different destaining ntervals. It was found that the relative amount of stain in the 19 S band increases luring the destaining. This is the case both when the initial concentration of 19 S s high relative to 12 S (Fig. 11, A) and when there is a more even distribution of the :wo molecules in the sample (Fig. 11, B). After destaining for 1 h and 45 min, how- ever, there is practically no shift in the per cent distribution of stain between the 19 S and 12 S bands. Thus, this destaining artefact does not reduce the validity of :he densitometric recordings.

Disc electrophoresis of the colloid

Fig. 12 shows an example of an electrophoretic separation pattern of the colloid extracted from one follicle in the isthmus of a rat thyroid. The migration rate of the single visible band corresponds to that of 19 S thyroglobulin in the reference samples.

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