CHROM. 6091

MICRO-SCALE PROTEIN SEPARATION BY ELECTROPHORESIS IN CONTINUOUS POLYACRYLAMIDE CONCENTRATION GRADIENTS

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(Received April 17th, 1972)

SUMMARY

This report describes a micro-scale method for preparing continuous concentration gradient rods of polyacrylamide gel.

Polyacrylamide gradients (5–25%) were used to separate proteins of molecular weights ranging from about 10^6 to less than 7×10^4.

INTRODUCTION

The resolution of the electrophoretic separation of proteins is enhanced by carrying it out in supporting media that exert a molecular sieving effect. In such separation systems, the importance of the variations in the free electrophoretic mobilities is reduced and the resulting separation pattern is due to a large extent to the size and configuration of the proteins. Polyacrylamide gels of low and high concentrations are used extensively for the separation of large and small proteins, respectively, and gels of adequate concentrations are easily chosen for specified separation purposes. By using continuous concentration gradients of polyacrylamide gels, the separation power of gel electrophoresis can be further increased and more information can be obtained on the protein composition of samples that contain a large number of proteins. Such separation systems were first introduced by SLATER[1] and methods for the macro-scale preparation of polyacrylamide slabs or rods of continuously increasing concentration of the gel were described by MARGOLIS and co-workers[2–4].

In this laboratory, a micro-scale separation system based on a continuous concentration gradient of the polyacrylamide gel was developed to make it possible to analyse the minute samples of colloid that are obtained by the micro-puncture of single rat thyroid follicles. These samples, about 0.1–1 nl in volume, were found to contain a number of proteins with greatly differing sizes, their molecular weights ranging from 1,200,000 (27S iodoprotein) to less than 70,000 ("prealbumin"). In the polyacrylamide system adopted for the separation of these thyroid proteins (10% polyacrylamide gel in 5-μl micro-caps[5]), the large iodoproteins, 27S, 19S and 12S, migrate with R_F values of less than 0.18 and the prealbumin fractions near the front.

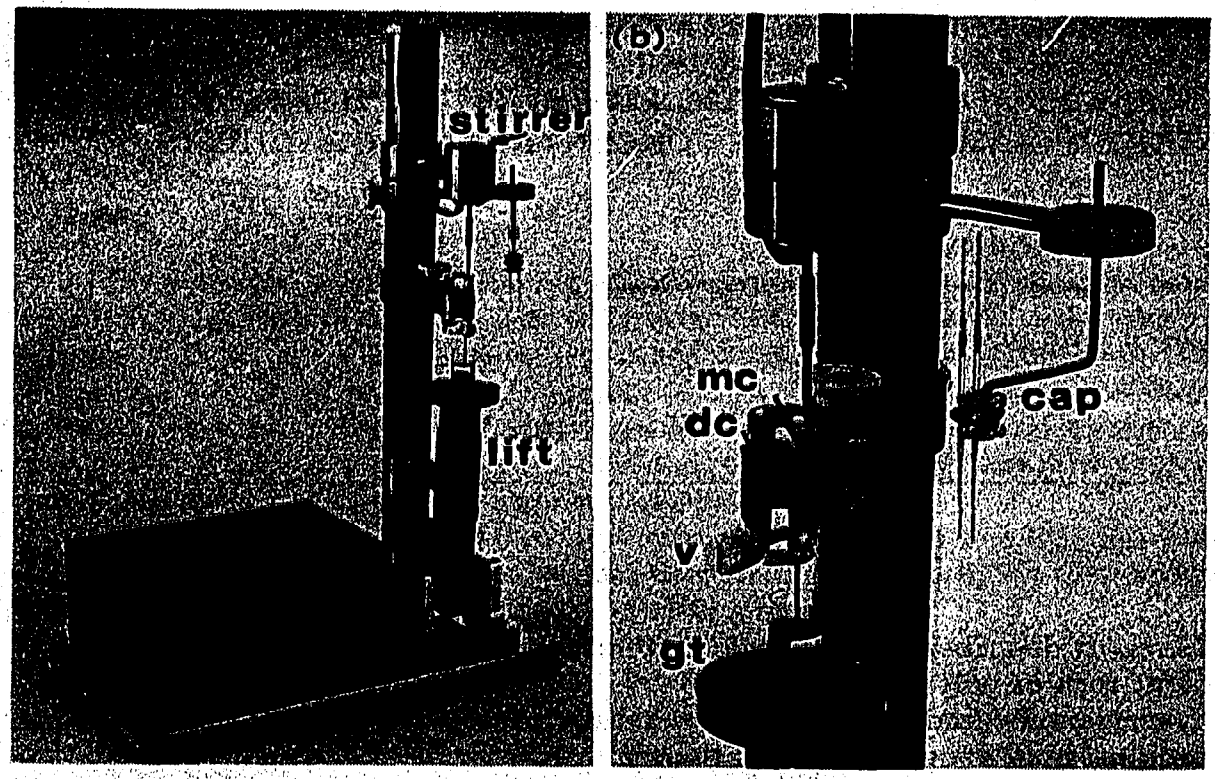

Fig. 1. Apparatus for preparing micro-gels. The gradient tube (gt) was mounted on the lift and partially filled with sealing gum and a concentrated sucrose solution. The dense chamber (dc) and the mixing chamber (mc) of the gradient mixer were connected by a valve (v). The outlet from the mixing chamber was positioned over the sucrose solution in the gradient tube. The stirrer was inserted into the mixing chamber. After preparation of the gradient, the gradient mixer and the stirrer were re-positioned, the capillaries in the capillary holder (cap) were placed above the surface of the gel solution and the lift was started.

Hence it was necessary to develop a system for the analysis of small and virtually indivisible samples that contain proteins with a wide range of molecular size.

MATERIALS AND METHODS

The apparatus for preparing the micro-gels is demonstrated in Fig. 1a. A lift can be elevated (2 to 20 mm/min) by an excentre-plate connected to a motor with stepwise speed control. A small plastics tube (diameter 13 mm for the 5- and 50-μl gradients and 10 mm for the 100-μl gradient) is mounted on the lift and partially filled with sealing gum, on top of which a 70% sucrose solution (2 mm layer) is placed.

The gradient mixing device (Fig. 1b) consists of two cylindrical chambers, the "dense chamber" and the "mixing chamber", 5.5 × 40.0 mm, near to each other in a transparent plastics block. The chambers are interconnected by a channel, diameter 1.0 mm, provided with a valve. The bottom of the "mixing chamber" is pierced by a steel tube with a bore of 0.25 mm and a length of 45 mm. The whole gradient mixing device is mounted on a rigid stand but can be moved in all directions. A stirrer is fixed to the same stand as the gradient mixer and is connected to an electric motor that has a continuously controlled speed.

Micro-caps of volume 5, 50 and 100 μl (Drummond Scientific Co., Bromall, Pa.,

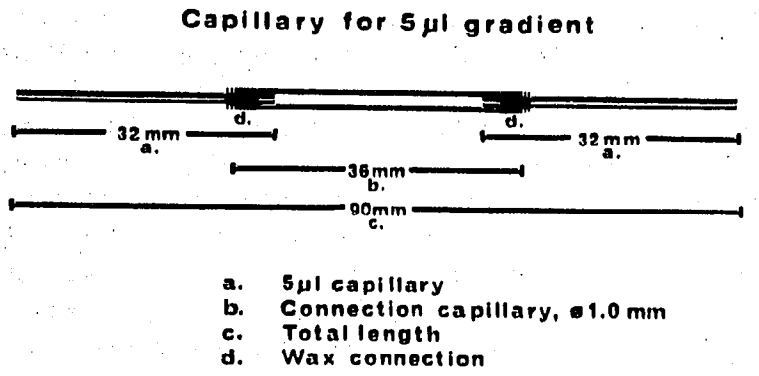

Fig. 2. Schematic representation of the arrangement for water equilibration in the 5-μl capillaries.

U.S.A.) were used. All the capillaries were filled with and kept in distilled water and when required for use they were slowly removed from the water in a vertical position to eliminate the capillary action. The 5-μl capillaries were too short for this equilibration and therefore two 5-μl capillaries were inserted 5 mm into a slightly wider glass tube (100-μl micro-caps) giving a total length of 90 mm (Fig. 2). The connections between the three glass tubes were sealed with wax. The capillaries were inserted into a holder, connected to the stand and positioned over the surface of the acrylamide gradient (gt, Fig. 1b).

The main steps involved in preparing the micro-gels are as follows:

(1) Preparation of a concentration gradient.
(2) Filling previously equilibrated capillaries with the acrylamide gradient.
(3) Sealing the bottom of the capillaries, emptying the gradient tube and light polymerization of the acrylamide gradient in the capillaries.

The polyacrylamide gradient was prepared from 5% and 25% acrylamide solutions, prepared as described in Table I. The volumes of the 5% and the 25% acrylamide solutions were 0.50, 0.75 and 0.65 ml for the 5-, 50- and 100-μl gradients, respectively, and the heights of the gradients were 7.0, 12.5 and 15.0 mm, respectively.

Before the preparation of the gradient, the gradient mixing device was positioned with the drainage tube of the mixing chamber about 1 mm above the surface of the

TABLE I

REAGENTS FOR PREPARING 5–25% POLYACRYLAMIDE GRADIENTS

The stock solutions A, B and C were mixed in the proportions 1:3:4.

Reagent	*A (pH 6.8)*	*A (pH 8.8)*	*B (5%)*	*B (25%)*	*C*
Tris[a]	8.6 g	8.6 g			
Temed[b]	0.63 g	0.63 g			
H_2SO_4 (3.6 *N*)	to pH 6.8	to pH 8.8			
Acrylamide			5 g	25 g	
Bis[c]			200 mg	200 mg	
Riboflavine					1 mg
Total volume	100 ml	100 ml	37.5 ml	37.5 ml	50 ml

[a] Trishydroxymethylaminomethane.
[b] N,N,N′,N′-Tetramethylethylenediamine.
[c] N,N′-Methylenebisacrylamide.

70% sucrose solution in the gradient tube. The 25% acrylamide solution was passed into the "dense chamber", and into the connecting channel by opening the valve. The valve was then closed and the bottom of the mixing chamber was covered with a small amount of 46% sucrose solution in water. The 5% acrylamide solution was layered on the top of the sucrose and the stirring rod was inserted into the mixing chamber. The sucrose solution on the bottom of the mixing chamber started to flow through the drainage tube and the interface between the sucrose solution and the light acrylamide solution sank slowly in the mixing chamber. When this interface was at the level of the connecting channel, the valve was opened and the stirrer started. When the mixing chamber was almost empty, 0.7 ml of a 30% acrylamide solution was poured into the dense chamber and allowed to follow the gradient and form a cushion between the gradient and the 46% sucrose solution.

The whole procedure was carried out shielded from daylight and at room temperature. Before the capillaries were filled, the gradient was allowed to stand for 10 min in darkness.

The equilibrated capillaries were placed 2–3 mm above the surface of the gradient and the lift was started (2 mm/min). All four layers of fluid in the gradient tube were passed by the capillaries, the lower ends of which were finally pushed into the sealing gum at the bottom of the gradient tube. All fluid in the gradient tube was withdrawn with a syringe. The polymerization of the acrylamide was accelerated by illumination of the capillaries for 16–20 h.

The gel cushion was added to the system mainly as a buffer between the gradient and the steel rod used for pushing the gel out of the electrophoresis capillary. A relatively large part of this cushion was crushed when the gel was pushed out and it was therefore important that no proteins migrated into the cushion.

To study the quality of the concentration gradients, the polyacrylamide gels were stained with 0.5% Amido Black in 7.5% acetic acid. The gels were then de-stained for various times, depending on the size of the gel. When the low-concentration part of a gel was almost completely de-stained, the amount of stain in the gel was recorded directly in a previously described micro-densitometer[5].

The gels were prepared for electrophoresis by removal of the water above the gel. A 20% sucrose solution in phosphate-buffered saline (PBS), pH 6.8, was layered on the top of the gel and the meniscus between the sucrose solution and the upper surface of the gel as well as the migration distance were indicated on the outside of the electrophoresis capillary.

On acrylamide gradients prepared at pH 8.8, a 3% stacking gel at pH 6.8 was layered. PBS-buffered test samples in 5% sucrose were layered on the top of the gradients or on the stacking gel.

The electrophoreses were run with a square wave (500 Hz, 75 V), layered on an off-set potential of 25 V (IB Electronic, Gothenburg, Sweden). The square wave had a 25% or 50% duty cycle. The electrophoresis was indicated with Bromophenol Blue and the run was interrupted when the tracking dye had migrated 80% of the gradient length. This corresponded to 5.6, 10.0 and 12.0 mm in the 5-, 50- and 100-μl gradients, respectively. Standard gels with various concentrations were prepared and run according to a detailed description given in a previous paper[5]. After electrophoresis, the gels were pushed out from the electrophoresis capillaries, fixed in 80% ethanol and stained with 0.5% Amido Black in 7.5% acetic acid. As a result of swelling of the gel

during this preparation, the length was increased by approximately 20%. The protein patterns were recorded directly from the gels by micro-densitometry[5].

Samples of soluble thyroid proteins were prepared from the supernatant of rat thyroid homogenates centrifuged at 105,000 × *g* for 1 h. All test samples were prepared in 5% sucrose in PBS, pH 6.8. For comparison, the samples were run on both standard gels of varying concentrations and on the gradient gels.

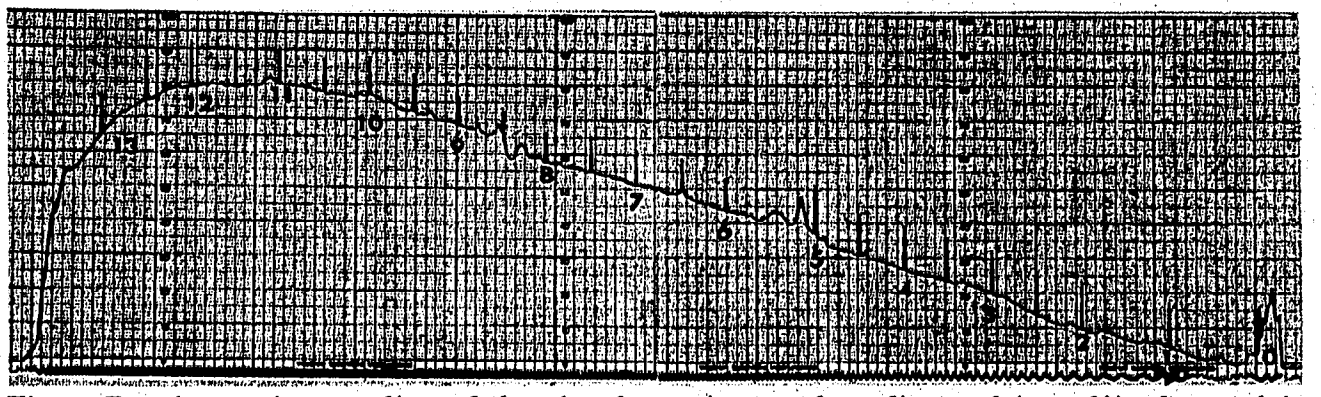

Fig. 3. Densitometric recording of the absorbance in a 5-μl gradient gel (5–25%) after staining (Amido Black) and partial de-staining. O = origin. The figures represent the length of the gel in millimetres.

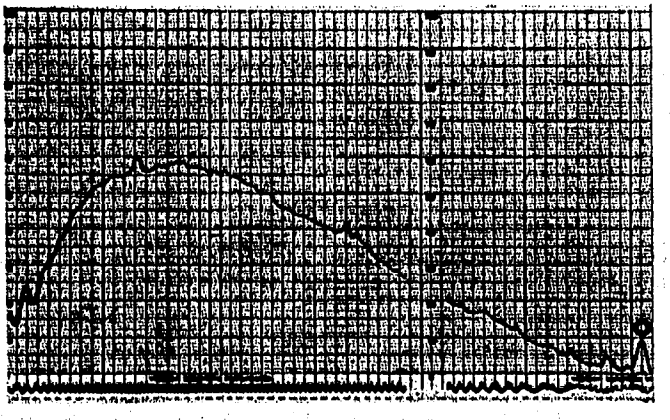

Fig. 4. Densitometric recording of the absorbance in a 50-μl gradient gel (5–25%). This recording was obtained with slower paper speed than in Fig. 3. O = origin.

RESULTS AND DISCUSSION

Recordings of the stained and partially de-stained gradient gels are shown in Figs. 3 and 4. It is evident that the recorded absorbance in the gel increases almost linearly from the origin towards the end of the gradient gel. This is due to the fact that the rate of disappearance of stain from the gel during de-staining is correlated with the polyacrylamide concentration, and a high-concentration gel retains the stain longer than does a low-concentration gel. Hence the absorbance pattern indicates that the polyacrylamide concentration increases almost linearly from the origin towards the end of the gradient gel. The border between the gradient and the cushion gel is not sharp but forms a smoothly curved region. This is probably partly due to diffusion of the gel constituents during the slow polymerization process and partly to diffusion

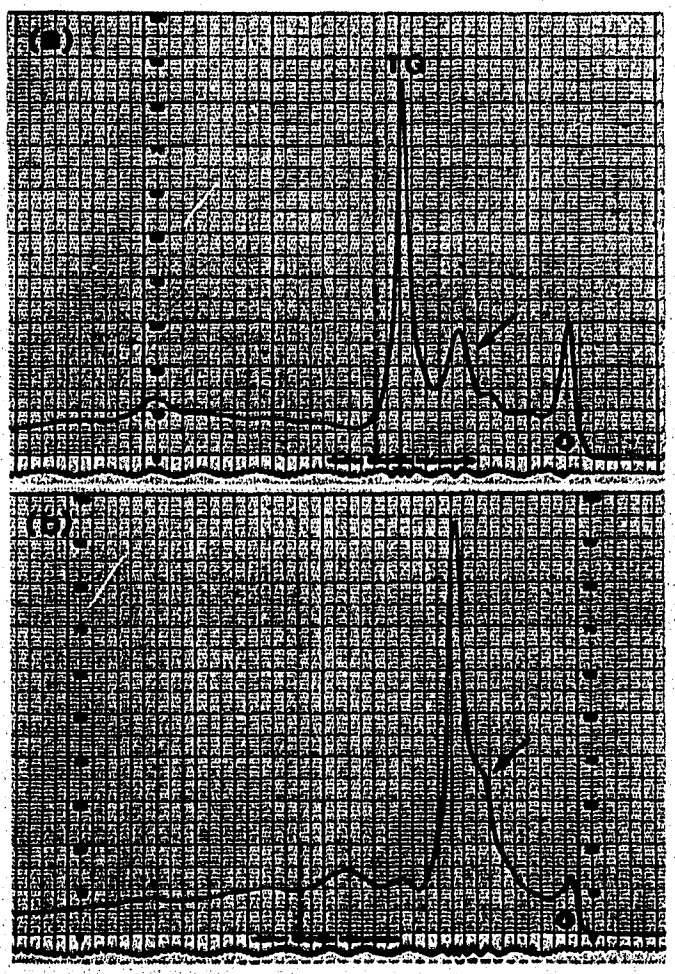

Fig. 5. Densitometric recordings of the first part of (a) 10% and (b) 20% separation gels (5 μl). Identical test samples of rat thyroid soluble proteins were separated on the gels. O = origin; TG = thyroglobulin. Slowly migrating proteins (indicated by arrows) did not separate from thyroglobulin in the 20% gel.

of stain in the gel. The diffusion of stain out of the gel is faster at the ends, which results in the steep decline of the absorption at the high-concentration end of the gel (Figs. 3 and 4).

Separation patterns of the larger soluble thyroid proteins on a 10% gel and a 20% gel from 5-μl capillaries are shown in Figs. 5a and 5b. The thyroglobulin band (TG, mol. wt. 670,000) is well separated from the slower migrating components in the 10% gel but not in the 20% gel. In a gradient gel, Fig. 6a, the TG band migrates a greater distance than in a 10 or 20% gel and the separation from slower migrating components is maintained or improved.

In a 10% separation gel, albumin migrates near the front[5]. In the gradient gel, the albumin fraction is retarded but migrates a greater distance than in the 20% separation gel (Figs. 6a and 6b). The densitometric recording of a 50-μl gradient gel (Fig. 7) demonstrates that the extended prealbumin region contains a large number of fractions. In this gel, several proteins are also recorded in the region between thyroglobulin and albumin.

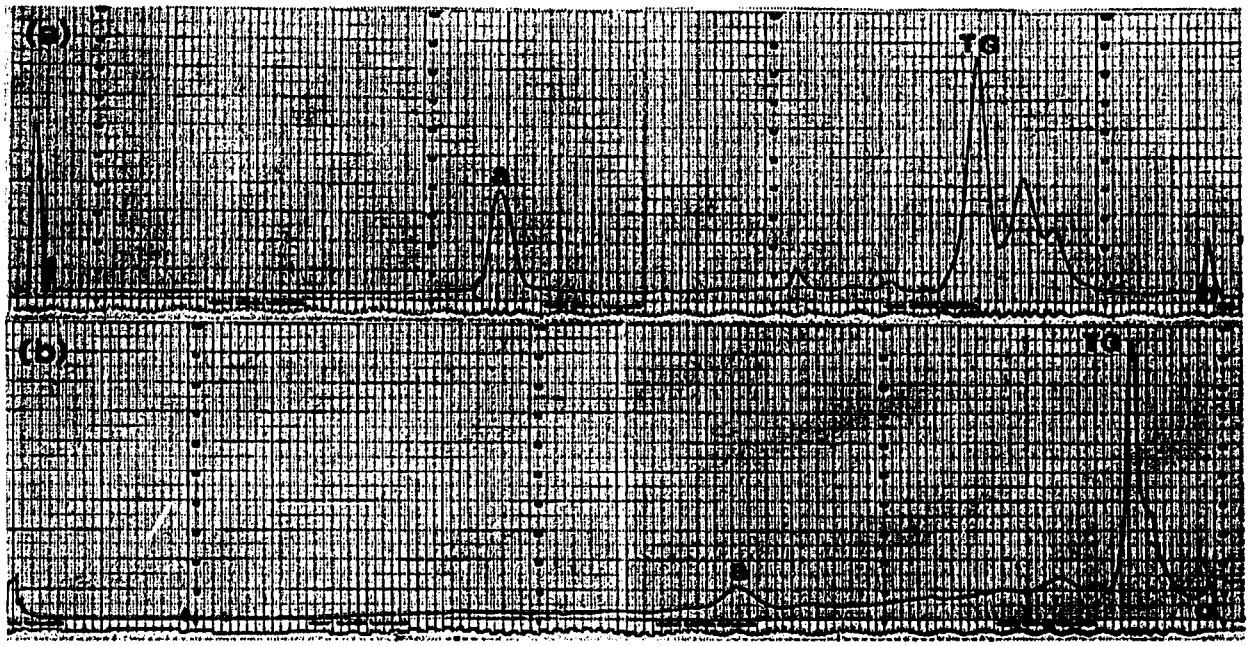

Fig. 6. Densitometric recordings of the separation of thyroid proteins on (a) a 5–25% gradient gel (5 μl) and (b) on a 20% separating gel. O = origin; TG = thyroglobulin; a = albumin. Albumin migrates a greater distance in the gradient gel than in a 20% gel during otherwise identical separation conditions.

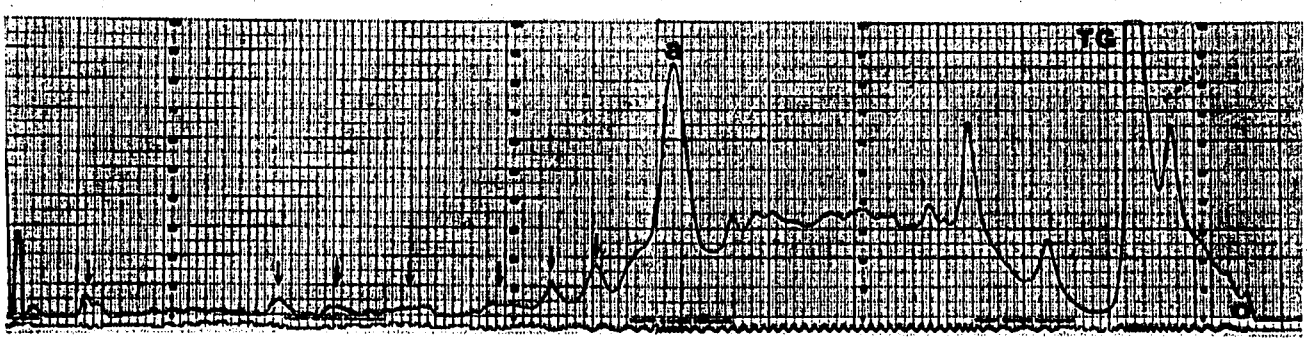

Fig. 7. Densitometric recording of the separation pattern of thyroid proteins in a 5–25% gradient gel (50 μl), pH 8.8, 3% stacking gel, pH 6.8. O = origin; TG = thyroglobulin; a = albumin. Seven fractions (indicated by arrows) were observed in the prealbumin region of the gel.

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