SEPARATION OF PURIFIED HUMAN CHORIONIC GONADOTROPIN INTO SINGLE BANDS BY ISOELECTRIC FOCUSING AND THEIR CHARACTERIZATION*

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Received 7 August 1971

1. Introduction

During the past two years several investigators have reported further success in purification and characterization of human chorionic gonadotropin (HCG) [1–3]. Recently we described a chromatographic method for the purification of HCG [4], which is similar to the procedure described by Van Hell [5]. We found however, that the highly purified hormone is still inhomogeneous in starch gel electrophoresis using discontinuous buffer systems and in disc electrophoresis in presence of 6 M urea. Furthermore, subfractions of native [6] and modified [7] HCG have been isolated after treatment with 8 M urea.

This paper reports the separation of purified HCG into 6 single bands by preparative isoelectric focusing with thin layer electrophoresis in Sephadex. Up to now the bands have been characterized by their IP, content of *N*-acetyl neuraminic acid (NANA) and biological activity. Moreover the results obtained by refocusing and disc electrophoresis provides additional information about the subunit pattern of HCG.

2. Materials and methods

Chromatography: The starting material with a biological activity of 2660 I.U./mg was purchased from Schering AG, Berlin. Sephadex types G-25, G-75 and CM C-25 were obtained from Pharmacia, Uppsala. The crude HCG was purified by one chromatography as previously described [4].

* Part VIII of a series of human chorionic gonadotropin. For part VI and VII see [4].

Isoelectric focusing: Ampholine was obtained from LKB, Bromma, Sweden. Analytical and preparative isoelectric focusing was performed on a Sephadex G-75sf thin layer as described by Radola [8], using a pH gradient 3–10. The separated bands were refocused both in Sephadex G-75sf and in a 12.5% polyacrylamide gel with and without 6 M urea according to Awdeh et al. [9] using several modifications.

Disc electrophoresis in a 12.5% polyacrylamide gel was carried out in half micro tubes (diameter 1.6 mm)†, in a buffer system described by Reisfeld and Small [10] at pH 9.3 with 6 M urea. The samples were dissolved in 10 M urea.

NANA was detected after hydrolysis with 0.1 N H_2SO_4 at 80° for 80 min according to Warren [11]. NANA and *N*-acetylneuraminyl lactose, prepared as described by Kuhn and Brossmer [12] were used as standard.

Bioassay of HCG was performed by a modification of the rat ventral prostate assay as previously described [4].

3. Results

One time chromatography of crude HCG with a starting activity of 2660 I.U./mg on CM-Sephadex C-25 at pH 5.0 with an ammoniumacetate buffer gave a purified hormone with a biological activity of 4500 I.U./mg. Preparative isoelectric focusing on Sephadex G-75sf with a gradient pH 3–10 caused a separation into 6 sharp bands (see fig. 1), which were freed from Ampholine by gel filtration (Sephadex

† Experimental details will be published elsewhere.

North-Holland Publishing Company – Amsterdam

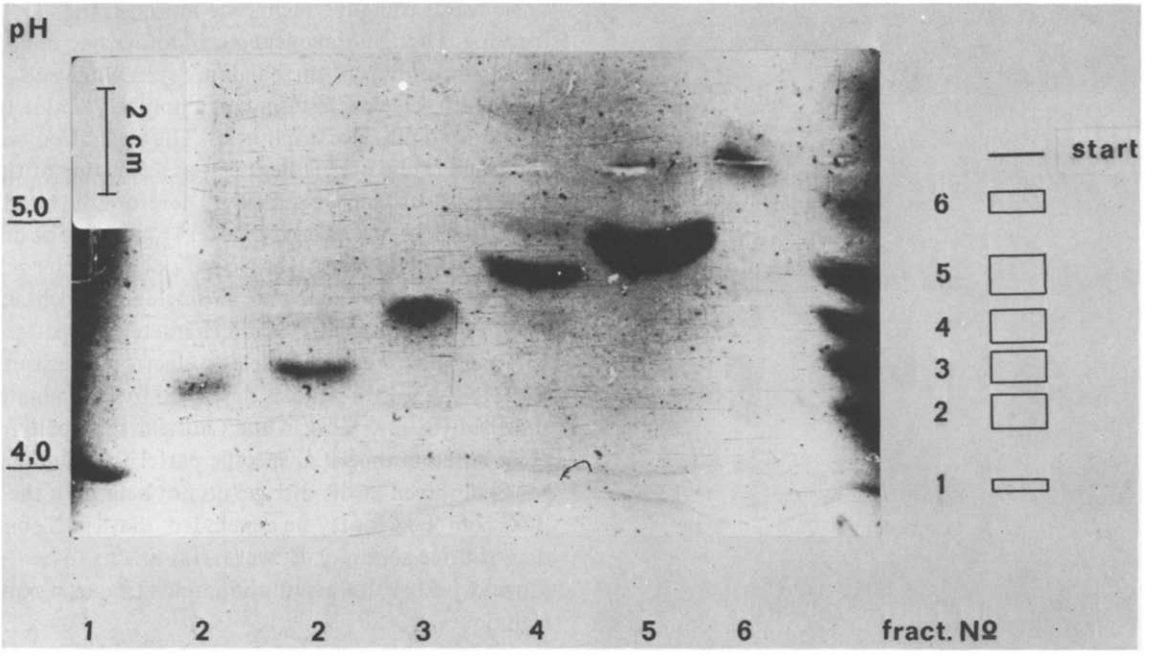

Fig. 1. Refocusing of 6 isolated bands, obtained by isoelectric focusing (right side) on a Sephadex thin layer with a gradient pH 3–10.

G-75) and lyophilized. The second fraction showed the highest biological activity (see table 1). By refocusing of the isolated fractions under the same conditions the bands did not show any further splitting (see fig. 1). However refocusing on a thin layer of polyacrylamide gel with a pH gradient 3–10 with and without 6 M urea gave a further separation. Without urea the most active fraction (No. 2) showed 2 bands, whereas 6 main bands appeared in the presence of 6 M urea.

Table 1

Fraction no.	IP	NANA content (%)	Biol. activity (I.U./mg)	l.o.c.* (I.U./mg)
1	4.00	4.4	2,632	2,062– 3,312
2	4.20	8.3	7,604	5,610–10,509
3	4.45	5.1	4,014	3,000– 5,484
4	4.66	8.8	2,000	1,581– 2,498
5	4.92	7.4	1,365	1,090– 1,685
6	5.22	4.9	3,191	2,389– 4,244
start. material		6.4	4,549	1,918– 8,030

Analytical results of the 6 bands, obtained by isoelectric focusing on a Sephadex thin layer and a gradient pH 3–10.

* l.o.c. = limits of confidence.

In the disc electrophoresis, using 6 M urea, the same fractions moved with several bands to the anode. Within a certain range of the band pattern the isolated fractions No. 2–6 showed 3 main, adjacent and obviously correlated bands, which differed from each other by their intensity ratio. A characteristic example is shown in fig. 2. Fraction No. 1 contained only 2 bands with a comparatively higher mobility. A striking similarity between the band pattern, obtained by isoelectric focusing and disc electrophoresis, both in polyacrylamide gel and 6 M urea, can be observed. The results of some analytical properties are summarized in table 1. The NANA content of the separated fractions differed from each other, no direct correlation between the NANA content and the biological activity being observable.

Nevertheless the fraction No. 2 with a high NANA content showed the highest biological activity. Its isoelectric point was at pH 4.2. The two bands of this

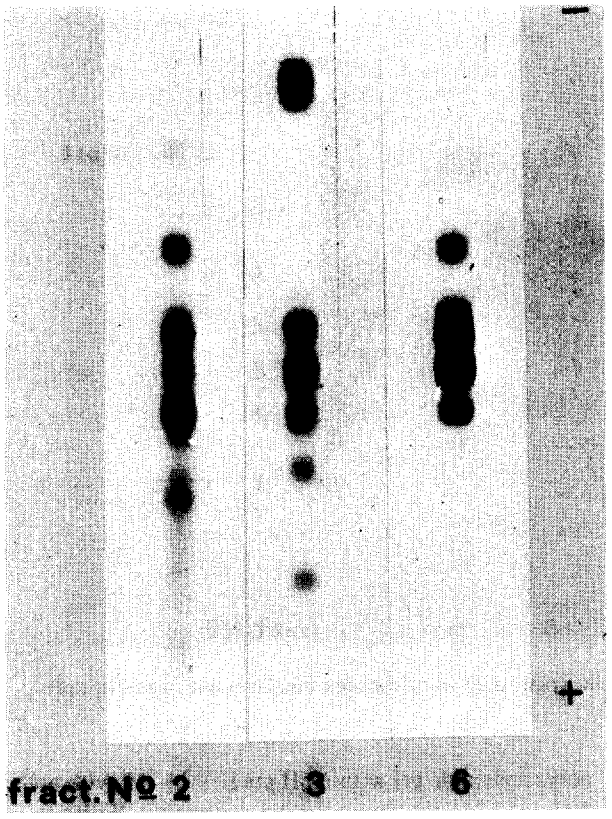

Fig. 2. Half micro disc electrophoresis of fraction No. 2, 3 and 6 in a 12.5% polyacrylamide gel, pH 9.3, in presence of 6 M urea.

fraction, obtained in analytical isoelectric focusing with polyacrylamide gel, as described above, had isoelectric points at pH 4.26 and 4.29. In comparison to the other fractions, fraction 2 again showed highest immunological activity using electroimmunodiffusion according to Laurell [13].

It is of interest, that isoelectric focusing according to Awdeh [9] with and without urea as well as disc electrophoresis in urea, though starting from three different hormone preparations (2660 I.U./mg, 4500 I.U./mg and 6700 I.U./mg) nevertheless gave the same pattern in the principal area. In each case the most active fraction showed comparable biological activity.

4. Discussion

The results show, that HCG may be separated into single bands using the technique of isoelectric focusing. Though homogeneous in refocusing under the same conditions, the bands are split when subjected to isoelectric focusing on a polyacrylamide thin layer and to disc electrophoresis. The separation on Sephadex in the pH gradient causes a splitting of the bands on the basis of their IP's. Therefore the bands may consist of several glycoprotein molecules of different molecular weight, as we have observed by gel chromatography. Exept one, all single bands, obtained by isoelectric focusing, show a characteristic pattern of 3 main bands each, in the disc electrophoresis using 6 M urea. A fourth band as described by Swaminathan and Bahl [6] and Morgan and Canfield [7] could not be detected in this HCG specific pattern. The other bands observed in the disc gel do not belong to the HCG complex. It may be concluded, that HCG consists of three subunits. However, it remains to be shown whether this assumption holds true using other techniques.

Acknowledgement

This work was generously supported by the Deutsche Forschungsgemeinschaft, Bad Godesberg, Germany. We thank Miss P. Basting for excellent technical assistance.

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