STUDIES ON THE PROLACTIN-LIKE SUBSTANCE IN HUMAN PLACENTA II

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The existence of prolactin-like substance in the animal placentas of various species has been reported by some authors (Ehrhardt, 1936; Ray *et al.*, 1955), but only a few informations have been obtained on the human placenta. Ehrhardt (1936) found that a prolactin-like substance was contained in the human and animal placentas and that in the human placenta this substance was mainly contained in the early stage of the pregnancy. Fujii (1938) has reported, however, that the human placenta does not contain such a substance.

In the previous publication, the authors (Ito and Higashi, 1953) have reported that a material obtained from the human placenta by the "acid-acetone" method of Lyons (1936-37) possesses the activities of proliferating the mucous membrane of the crop-glands and producing the crop milk in pigeons. This method, however, is not always suitable for the extraction of the active substance from the human placenta because of its low yield.

The present paper describes an improved method for the preparation of the prolactin-like substance in human placenta. A precipitate obtained by this method causes a marked proliferation of the mucous membrane of the crop-glands, a secretion of the crop milk, and an increase of the number of the fatty granules in the crop milk of pigeons.

These activities are similar to those of the lactogenic hormone of anterior pituitary.

MATERIALS AND METHODS

The human placenta of normal delivery was used as the starting material. The placenta was frozen with dry ice to avoid the denaturation or autolysis and was carried back to the laboratory.

The biological activity of the prolactin-like substance in the human placenta was assayed by the observation of the appearance of the fatty granules in the crop milk and the proliferation of the mucous membrane of the crop-glands in pigeons. Namely, the "local" intradermal method for the assay of prolactin (Lyons and Page, 1935; Lyons, 1936-37) was employed. The male and female pigeons having never nested and weighing about 300 g were used. Forty eight hrs. after a single injection with a test solution over the crop-glands, the pigeons were sacrificed and the crop-glands were removed, weighed and provided for the histological studies. The fatty

granules in the crop milk were examined by staining with the solutions of saturated Sudan III in 70% ethanol and hematoxylin, and the tissues of the crop-glands were prepared with the usual hematoxylin-eosin staining method.

EXPERIMENTS AND RESULTS

1) *Separation of the pH 5.4-precipitate from the human placenta*

The human placenta was minced and placed immediately in 1.5 vols. of weekly alkaline water (pH 8.6~8.8). Extraction was carried out for 6 hrs. at 0~5°C under constant stirring. The residual tissue was extracted twice with 2 vols. of water. The solution was adjusted to pH 5.4 with *N* HCl. After being stood over night in an ice box, the precipitate was separated by centrifugation and lyophilized.

2) *Freezing followed by thawing and filtration in acid or alkaline medium*

The pH 5.4-precipitate was dissolved in the 100 vols. of water at pH 1.8~2.0 or pH 8.6~8.8. The pH of the solution was maintained to be constant with *N* HCl or *N* NaOH. The turbid solutions were frozen in refrigerator, followed by thawing at 5~10°C, being wrapped up the frozen mass in a gauze and stiffed with absorbent cotton and each of the filtrates was lyophilized.

	Yield	The colour of the solutions	The colour of the lyophilized solutions
pH 8.6~8.8 filtrate	60.4%	reddish brown (turbid)	light red
pH 1.8~2.0 filtrate	64.0%	brownish yellow (clear)	gray

Of each fraction the biological activity was assayed. The results are shown in Table 1.

As shown in Table 1, the proliferation of the stratum epithelium and the cells of the germinativum was remarkable in the pigeons administered with the acid filtrate. The amounts of the crop milk and the fatty granules in the crop milk in the group injected with the acid filtrate were larger than those with the alkaline filtrate. Moreover, this tendency was similarly observed on the increase of the crop-gland weight. From these results, it was supposed that the acid filtrate is more effective than the alkaline filtrate.

3) *Fractional precipitation with ammonium sulfate of the acid filtrate*

The yield of the precipitate at the concentration of 10% of ammonium sulfate (the 10%-precipitate) was 38.5%, and the yields of the precipitates at other concentrations were low (Table 2). These fractions were divided into 3 groups; the <10%-, 10~25%- and >25%-precipitate, and the biological activity of each fraction was assayed. The results are shown in Table 3.

The responses of the increase of the amounts of the crop milk and of the granules of the fat in the crop milk were striking in the groups to which the <10%- or 10~25%-precipitate was injected. The same tendency was also seen on the increase of crop-gland weight. In the histological observation, the proliferation was observed in a limited area of the stratum epithelium in the group

Table 1. Effects of alkaline and acid filtrates on the pigeon's crop-glands

Pigeons No.	1	2	3	4	5	6	7	8
Fractions	Control 1		pH 8.6~8.8 filtrate		Control 2		pH 1.8~2.0 filtrate	
Dose (per pigeon)	pH 8.6~8.8 distilled water (0.5 ml)		20 mg (0.5 ml)	20 mg (0.5 ml)	pH 1.8~2.0 distilled water 0.5 ml		20 mg (0.5 ml)	20 mg (0.5 ml)
Macroscopic observations	Cong. ^{*1} of muc. memb.	±	—	+	±	+	±	+
	Enlarg. ^{*2} of muc. memb.	±	+	+	—	+	+	±
	Quantity of crop milk	±	—	—	—	+	+	±
Changes of weight of crop-glands	Body wt. (g)	440	390	370	310	250	390	375
	Crop-glands wt. (g)	1.510	1.739	1.381	1.373	0.790	1.580	2.001
	Crop-glands wt. / Body wt.	0.0034	0.0045	0.0037	0.0044	0.0032	0.0041	0.0053
Amount of the fatty granules in the crop milk	±	+	+	+	±	+	+	±
Histological observations	Prolif. ^{*3} of cells of strat. germin.	—	+	±	±	±	+	±
	Prolif. ^{*4} of strat. epit.	—	+	—	+	±	±	±
	Disjun. ^{*5} of strat. corn.	+	+	+	±	+	+	+

* 1 Congestion of mucous membrane

* 2 Enlargement of mucous membrane

* 3 Proliferation of cells of stratum germinativum

* 4 Proliferation of stratum epithelium

* 5 Disjunction of stratum corneum

These abbreviations are used also in Tables 3 and 4.

Table 2.

Fractions	Dry weight (g)	Yield (%)
<10%-precipitate	0.592	38.5
10~20%-precipitate	0.104	7.0
20~25%-precipitate	0.020	1.3
25~30%-precipitate	0.028	1.9
>30%-precipitate	0.058	3.9

injected with the <10%-precipitate, but in the group injected with the 10~25%-precipitate the proliferation in whole area of the stratum epithelium and the cells of the stratum germinativum was shown remarkably. The proliferation of the stratum epithelium and the cells of the stratum germinativum of the group injected with the >25%-precipitate was inferior to those of the group injected with the 10~25%-precipitate. It may be obvious from these results that the active principle in the pH 5.4-precipitate is concentrated in the 10~25%-precipitate.

4) *The separation of the >25%-(NH₄)₂SO₄-precipitate from the pH 5.4-supernatant*

By the previous experiments, it was proved that the 10~25%-precipitate in the pH 5.4-precipitate was effective. Therefore, the >25%-precipitate was separated from the pH 5.4-supernatant by the fractional precipitation with ammonium sulfate, and the activity of this fraction was assayed.

Comparing the activity of the 10~25%-(NH₄)₂SO₄-precipitate from the pH 5.4-precipitate to that of the >25%-(NH₄)₂SO₄-precipitate from the pH 5.4-supernatant, the former was generally stronger than the latter. The secretion of crop milk and increase of the number of the fatty granules in the crop milk in the group injected with the former were stronger than those in the group injected with the latter. But the proliferation of the stratum epithelium and of the cells of the stratum germinativum was alike as that of the latter (Table 4 and Fig. 1).

DISCUSSION

The authors (Ito and Higashi, 1953) isolated the material from the human placenta by the "acid-acetone" method of Lyons which possesses the activities similar to the anterior pituitary lactogenic hormone. But, this preparative method is not suitable in this case, since the concentration of the acetone added is so high that it consumes very long time to obtain the complete precipitation. A part of the active protein was denatured and the yield was considerably low.

In order to overcome these difficulties, the following methods were applied to the extraction of the active protein; the extraction with glacial acetic acid (Payne *et al.*, 1950), with a mixture of chloroform and water (Schwenk *et al.*, 1943), with 70% acetone following fixation of tissue with picric acid (Dudlay, 1924), and with alkaline distilled water of pH 8.6~8.8. Considering the results of these experiments, it was supposed that the extraction with alkaline water of pH 8.6~8.8 followed by the fractional precipitation with ammonium sulfate was the most suitable method for the extraction of the substance in the human placenta.

The process of successive freezing and thawing permitted to obtain a clear

Fig. 1. The histological pictures of the crop-glands of pigeons

A and B: The crop-gland of pigeons at 48 hrs. after a single injection of the extract of alkaline water of pH 8.6~8.8

C: The crop-gland of pigeons at 48 hrs. after a single injection of 10 mg of the 10~25%-precipitate from the pH 5.4-precipitate (Table 4). The proliferation of the cells of the stratum germinativum is remarkable.

D: The crop-gland of pigeons at 48 hrs. after a single injection of 10 mg of the 10~25%-precipitate from the pH 5.4-precipitate (Table 4). The disjunction of the stratum corneum and the proliferation of the cells of the stratum germinativum is remarkable.

E: The crop-gland of pigeons at 48 hrs. after a single injection of 10 mg of the 10~25%-precipitate from the pH 5.4-precipitate (Table 4). The proliferation of the stratum epithelium is remarkable.

G and H: The crop-gland of pigeons at 48 hrs. after a single injection of 10 mg of the 25%-precipitate from the pH 5.4-supernatant (Table 4). The proliferation of the stratum epithelium alone is remarkable.

and decolorized solution. This filtrate is adjusted to pH 8.6~8.8 with *N* NaOH, and after the centrifugation, the supernatant was submitted to the fractional precipitation with ammonium sulfate. The each fraction was assayed by the pigeon's crop-gland method and the precipitate at concentrations of 10~25% of ammonium sulfate from the pH 5.4-precipitate was shown to be effective (Table 3).

The assay method employed is the "local" method introduced by Lyons and Page (1935). In this assay, it must be paid attention to use the young pigeons weighing about 300 g, which has never nested. Because, the nesting pigeons cause proliferation of the mucous membrane of the crop-gland and production of the fatty granules in the cells of the stratum epithelium of the crop-gland without any treatment, and this condition continues until the breeding settles.

In these experiments, the histological changes of the tissue of the crop-gland and the increases of number of the granules of the fat in the gland were employed mainly as the indices of the activity instead of the increase of the weight, because a large amount of the material was required to induce the significant change in the weight of the gland. The histological changes do not coincide with the change in the weight of the crop-gland; for instance, in the response of the weight of the crop-glands, the group injected with the <10%-precipitate was stronger than the group with the >25%-precipitate, but in the histological response the reverse is the case (Table 3). This phenomenon may be caused by the fact that the proliferation of the stratum epithelium is remarkable in the group injected with the 10~25%-precipitate from the pH 5.4-precipitate, but in the group injected with the <10%-precipitate the proliferation occurs rather on the tissue of the stratum proprium (*Tunica propria mucosae*).

As shown in Table 4 and Figure 1, the injection of the >25%-precipitate from the pH 5.4-supernatant also produces proliferation of the mucous membrane of the crop-gland. In this case, however, the proliferation of the cells of the stratum germinativum is different from that produced by the 10~25%-precipitate from the pH 5.4-precipitate, and also the amount of crop milk is small. From these reasons, the 10~25%-precipitate from the pH 5.4-precipitate and > 25%-precipitate from

Table 4. Effects of the 10~25%- and >25%-(NH₄)₂SO₄-precipitate from the pH 5.4-precipitate and >25%-(NH₄)₂SO₄-precipitate from the pH 5.4-supernatant on the pigeon's crop-glands

Pigeons No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Fractions	Control 1	Control 2	10~25%-(NH ₄) ₂ SO ₄ precipitate from pH5.4-precipitate		10 mg 10 mg (0.5 ml) (0.5 ml)		>25%-(NH ₄) ₂ SO ₄ precipitate from pH5.4-precipitate		>25%-(NH ₄) ₂ SO ₄ precipitate from pH5.4-supernatant		>25%-(NH ₄) ₂ SO ₄ precipitate from pH5.4-supernatant		>25%-(NH ₄) ₂ SO ₄ precipitate from pH5.4-supernatant	
Dose (per pigeon)	Un- injected	pH8.6~8.8 distilled water (0.5ml)	10 mg (0.5ml)	10 mg (0.5ml)	10 mg (0.5ml)	10 mg (0.5ml)	10 mg (0.5ml)	10 mg (0.5ml)	10 mg (0.5ml)	10 mg (0.5ml)	10 mg (0.5ml)	20 mg (0.5 ml)	20 mg (0.5 ml)	20 mg (0.5 ml)
Cong. of *1 muc. memb.	+	+	+	±	+	±	+	+	+	+	+	+	+	±
Enlarg. of *2 muc. memb.	+	+	±	+	+	+	+	+	+	+	±	±	±	+
Quantity of crop milk	±	—	±	+	+	+	±	+	+	—	+	+	+	±
Body wt. (g)	420	380	400	350	410	400	350	425	400	390	425	400	385	435
Crop-glands wt. (g)	1.725	1.874	2.666	1.673	2.079	2.025	1.641	1.579	1.981	1.679	2.144	2.331	2.051	2.427
Crop-glands Body wt.	0.0041	0.0049	0.0067	0.0048	0.0051	0.0051	0.0047	0.0037	0.0050	0.0043	0.0051	0.0058	0.0053	0.0056
Amount of the fatty granules in the crop milk	±	±	±	±	±	±	±	±	±	+	±	±	±	±
Prolif. of cells *3 of strat. germin.	±	±	±	±	±	±	±	+	±	±	±	±	±	±
Prolif. of *4 strat. epit.	+	+	±	+	±	±	+	+	±	+	±	+	+	+
Disjun. of *5 strat. corn.	—	—	±	±	±	±	+	+	+	—	±	±	±	±

the pH 5.4-supernatant may be quite different.

It is assumed, therefore, that the factor which acts on the secretion of the crop milk and which acts on the proliferation of the crop-gland tissues may be different and might be separated by the further purification process.

SUMMARY

A prolactin-like substance was isolated from the human placenta by the method of extraction with alkaline water of pH 8.6~8.8 and the fractional precipitation with ammonium sulfate. The substance possesses the crop-gland-stimulating activity similar to the anterior pituitary lactogenic hormone. The activity is concentrated in a precipitate at the concentration of 10~25% of ammonium sulfate from the pH 5.4-precipitate. This fraction causes proliferation of the mucous membrane of the crop-gland, production of the crop milk and increase of the granules of the fat in the crop milk.

REFERENCES

- Dudlay, H.W. (1924). *Biochem. J.* **18**, 665.
Ehrhardt, K. (1936). *Münch. med. Wochschr.* **29**, 1163.
Fujii, K. (1938). *J. Japan. Obstet. Gynecol.* **33**, 1. (In Japanese)
Ito, Y. and K. Higashi (1953). *J. Pharm. Soc. Japan.* **73**, 89. (In Japanese)
Lyons, W.R. and E. Page (1935). *Proc. Soc. Exptl. Biol. Med.* **32**, 1049.
Lyons, W.R. (1936-37). *Ibid.* **35**, 645.
Payne, R.W., N.S. Raben and E.B. Astwood (1950). *J. Biol. Chem.* **187**, 719.
Ray, E.W., S.C. Averill, W.R. Lyons and R.E. Johnson (1955). *Endocrinology* **56**, 4, 359.
Schwenk, E., G.A. Fisher and S. Tolkshorf (1943). *J. Biol. Chem.* **147**, 535.