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Stability Studies on Human Pituitary Prolactin

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ABSTRACT

Differently charged isomers of human pituitary prolactin were subjected to stability studies under various conditions. The study was guided by analytical electrophoresis and radioimmunoassay. In alkaline medium (pH 10) it was found that each isohormone was converted into faster-migrating components, probably due to deamidation. A quantitative study of this alteration was made by measuring the rate constant for this conversion assuming first-order kinetics. The result indicated that the rate constant decreased with increased acidity of the examined prolactin components. Furthermore, this alteration was also found to be paralleled by a decreased immunoactivity of the components. At neutral pH the conversion reaction was studied both in 0.9 % NaCl and in human serum. For the least acidic isohormone the rate constant in serum was calculated to be about four times higher than that in the saline solution which in turn was comparatively low. It was concluded that the alteration observed at pH 10 might be attributed to non-enzymic deamidation. The conversion of prolactin that occurred in human serum indicated that this reaction could be caused by enzymes.

A study of the immunoactivity of human prolactin as a function of time at various storage conditions is also included in this work. The stability of the hormone was found to be strictly concentration dependent and also affected by buffer ionic strength. In the presence of ethylene glycol (50%, v/v) at $-20^{\circ}C$ and at a protein concentration in the range of 0.1–2 mg/ml the hormone was found to be both immunologically and electrophoretically stable for several years.

INTRODUCTION

The presence of electrophoretically separable forms of human prolactin has been demonstrated in highly purified preparations from pituitary glands (5, 7, 11, 18) but also in amniotic fluid (1, 6, 16), cerebrospinal fluid (14), serum samples (21) as well as in urine from pregnant women (14). The cause of this type of heterogeneity has not yet been clearly established,

although evidence for the involvement of deamidation has been accumulated (3, 8-12). Thus, alkali treatment of ovine prolactin increased the relative amounts of faster-migrating components (8, 10).

The present paper describes a quantitative study of the stability in alkaline medium, in saline and in human serum of prolactin isohormones recovered from the human pituitary. The study was accomplished by measuring the first-order rate constant for the transformation of the components into more acidic forms.

The long-term storage stability of the hormone under different conditions are also included.

MATERIALS AND METHODS

Isolation of individual prolactin components

Differently charged isoforms of human pituitary prolactin (molecular weight 22000) were recovered from a highly purified preparation (18) of the hormone by preparative electrophoresis in agarose suspension as described in a preceeding paper (12). In the present runs, samples of 7-8 mg of prolactin were applied.

Procedures for incubation

The isolated isohormones were dissolved in 0.5 M ammonium sulphate (pH adjusted to 10) to give a final concentration of 100 ug/ml. Incubations were performed at 37°C for 28 h and at time zero, as well as all subsequent times, 0.1 ml-aliquots were removed, desalted on Sephadex G-25 columns (PD-10, volume 9 ml, Pharmacia Fine Chemicals) and lyophilized.

In some additional experiments the least acidic component (prolactin I, see Fig. 1) was incubated (at 37°C for 54 h) in fresh serum (from a lactating woman) and in 0.9% (w/v) NaCl buffered at pH 7 by 0.01 M potassium phosphate. During incubation aliquots were removed and treated as before.

Analytical electrophoresis

Analytical runs were carried out in polyacrylamide gel (18) and in agarose suspension. In the latter case samples of 10 ug were applied and the electrophoreses were performed at a voltage of 820 V for 18 h as described previously (12).

After completed runs and subsequent staining the polyacrylamide gels were scanned at 560 nm, which permitted estimations of the relative amounts of unaltered and converted prolactin isohormones. A similar estimation of

the hormone distribution following runs in agarose suspension was made from the prolactin activity pattern obtained by radioimmunoassays.

The aliquots withdrawn from the pH 10 incubates were run in agarose suspension and polyacrylamide gel, as well, whereas those from the incubations at neutral pH were run in agarose suspension.

Calculation of kinetic data for the conversion reaction

According to the method described by Lewis et al. (10), the percentage amount of the unaltered component in the sample was calculated. The percentage at zero time was taken as its initial concentration (a), while the percentage at each subsequent time was used as a measure of the amount of unaltered component remaining. This value was designated (a-x), where x represents the amount of converted products. The logarithm of a/(a-x) was plotted against the corresponding incubation time used; the slope represented the rate constant (k_1) for the conversion of each isohormone to more acidic forms.

Radioimmunoassay

The prolactin activity was assayed by a radioimmunosorbent technique (22, 23) using a highly purified human prolactin preparation (18) both for labelling with ^{125}I and for raising antibodies. The relative activity was expressed in percentage using the prolactin preparation (18) as a provisional standard.

Growth hormone activity was determined by a similar radioimmunoassay as described elsewhere (18).

Radioreceptor assay

The prolactin isohormones were assayed for their ability to bind to receptor homogenates prepared from pigeon crop sac mucosa cells (2) and from rabbit and rat adrenals (15). ^{125}I-prolactin was supplied by AB KABI Diagnostica, Stockholm, Sweden. In the assay approximately 15000 (crop sac receptor) or 50 000 (adrenal receptors) cpm were added to each tube containing the sample to be assayed and 0.3 mg of membrane protein in a final volume of 0.4 ml assay buffer (25 mM Tris-HCl, 10 mM $MgCl_2$, 0.5% bovine serum albumin, pH 7.6). The incubation was carried out for 20 h at $4^{o}C$ and was terminated by dilution with 1 ml cold assay buffer (2). Bound and free ^{125}I-prolactin were separated by centrigation in a Beckman Microfuge B for 10 min. The supernatant was decanted and the membrane pellet was counted for radioactivity in a Packard auto-gamma counter. The relative activity was expressed in percentage using a heterogenous prolactin preparation (18) as a provisional standard.

Storage stability of prolactin

Highly purified human prolactin (3) was stored under the following conditions:

1. as freeze-dried powder in sealed Ellerman tubes at 5°C.
2. in 0.02 M Tris-HCl buffer (pH 9.0) at -20°C and -80°C.
3. in 0.02 M Tris-HCl buffer (pH 9.0) containing 0.5 KCl at -20°C.
4. in 0.02 M Tris-HCl buffer (pH 9.0) containing 50% (v/v) ethylene glycol.
5. in 0.01 M ammonium bicarbonate (pH 7.8).

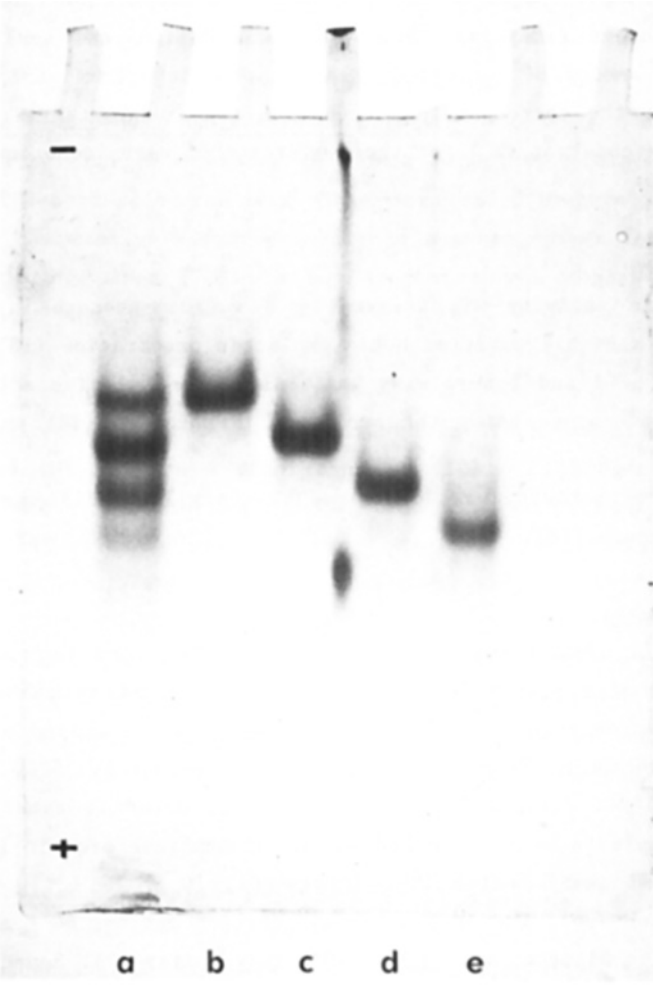

Fig. 1. Polyacrylamide gel electrophoresis. Runs at pH 9.0 in 0.05 M Tris-HCl buffer of heterogeneous prolactin (a) and the isolated components (b: prolactin I, c: prolactin II, d: prolactin III, e: prolactin IV).

In 2.-5. the hormone was kept at three different protein concentrations; 100, 10, and 1 ug/ml. Aliquots of 30 ul stored in sealed Ellerman tubes were subsequently thawed (one at a time) and diluted to a final concentration of 50-100 ng/ml in 50 mM potassium phosphate buffer (pH 7.0) containing 0.2% bovine serum albumin. The hormone was known to be very stable in the diluent buffer at very low concentrations and the diluted samples were frozen and stored until assayed.

RESULTS

Isolation of individual prolactin components

The preparative runs in agarose suspension yielded the prolactin isohormones in separate fractions of high purity, which agreed with our earlier findings (12).

In the present study the amount of material applied was markedly increased in comparison with that in previous runs and we were able to identify and recover a fourth prolactin component, which appeared still more acidic than the other isoforms. This isohormone was designated prolactin IV on the analogy of our preeceding denotation (12). It represented about 5% of the total amount of prolactin recovered. Fig. 1 illustrates the electrophoretic pattern obtained after runs in polyacrylamide gel of the heterogenous prolactin preparation and the isolated components. As shown each isohormone stained as a single band indicating high purity.

Table 1

Immunoactivity of prolactin isohormones recovered by eletrophoresis in agarose suspension[1]

Prolactin fraction	Prolactin immunoactivity (%)	Growth hormone immunoactivity (%)
I	130 ± 5 (3)	1.3 ± 0.2 (3)
II	135 ± 6 (3)	1.2 ± 0.2 (3)
III	111 ± 2 (3)	1.4 ± 0.2 (3)
IV	85 ± 4 (3)	0.7 ± 0.2 (3)

[1] Values are given as means ± S.E.M. with the number of preparations in parenthesis.

Radioimmunoassay data (Table 1) showing a decreased immunoactivity with increasing acidity among the different isohormones thereby confirming results published previously (12). The fastest migrating component (prolactin IV) thus exhibited an activity which was about 65% of that of the most active forms. Table 1 also indicates that the growth hormone contamination of the different components (including prolactin IV) was low.

Results from the binding assays (Table 2) also indicated a low potency of prolactin IV, whereas no significant differences were measured among the other isohormones (prolactin I-III), which were approximately equipotent.

Table 2

Receptor binding of prolactin isohormones recovered by electrophoresis in agarose suspension.

Prolactin fraction	Receptor binding activity (%)[1]		
	pigeon crop sac	rat adrenal	rabbit adrenal
I	101	-	-
II	94	98	105
III	108	89	118
IV	46	41	32

[1] Values are given as means of triplicate determination.

Conversion of the prolactin isohormones

Fig. 2 shows the pattern obtained by agarose suspension electrophoresis of prolactin I and prolactin IV before and after incubation in ammonium sulphate at pH 10 and 37°C (28 h). Apparently, following incubation both isohormones showed an extensive alteration in electrophoretic behaviour, being converted into more acidic forms. It can further be seen that the alteration of prolactin I is more pronounced than that of prolactin IV. This was confirmed by the values for the rate constant, k_1 (Table 3). The

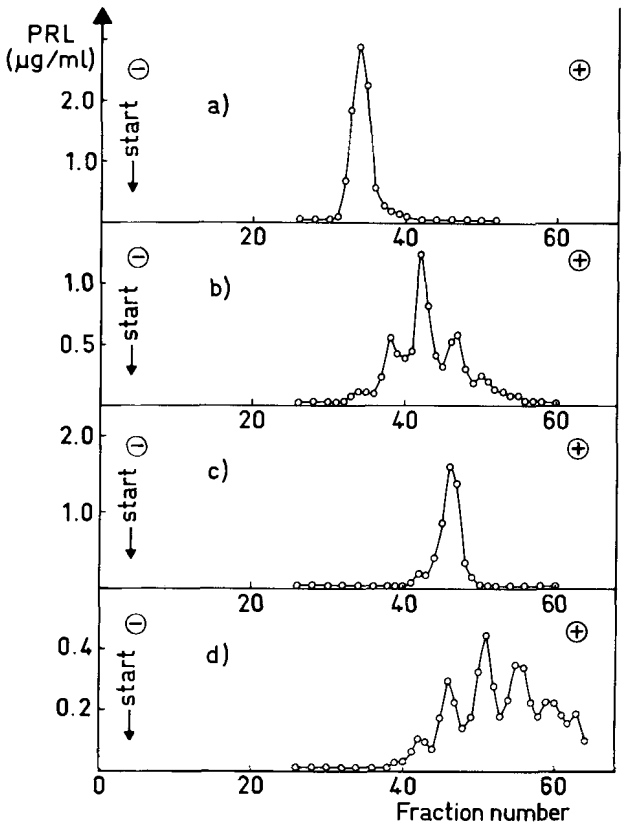

Fig. 2. Electrophoresis in agarose suspension of unmodified forms (a and c) and 28 h incubates (0.5 M ammonium sulphate, pH 10 and 37°C) (b and d) of prolactin I and prolactin IV. Buffer: 0.03 M veronal (pH 8.6). Voltage: 820 V. Duration: 18 h.

value of the constant for prolactin I was about two times larger than those for the other isohormones, which were rather similar. Furthermore, it was shown that the immunopotency of the components decreased by increased conversion rate. Thus, the isohormone with the larges k_1 value (prolactin I) also showed the greatest loss in immunoactivity.

Fig. 3 shows the results obtained following electrophoresis in agarose suspension of prolactin I before and after incubation (54 h) in 0.9% NaCl and human serum. Obviously the conversion reaction was more far-reaching in the latter case, yielding a k_1 value about 4 times larger than that obtained after incubation in saline (Table 4). According to radioimmunoassay data, also given in Table 4, about 80% of the immunoactivity was preserved after the incubation at neutral pH.

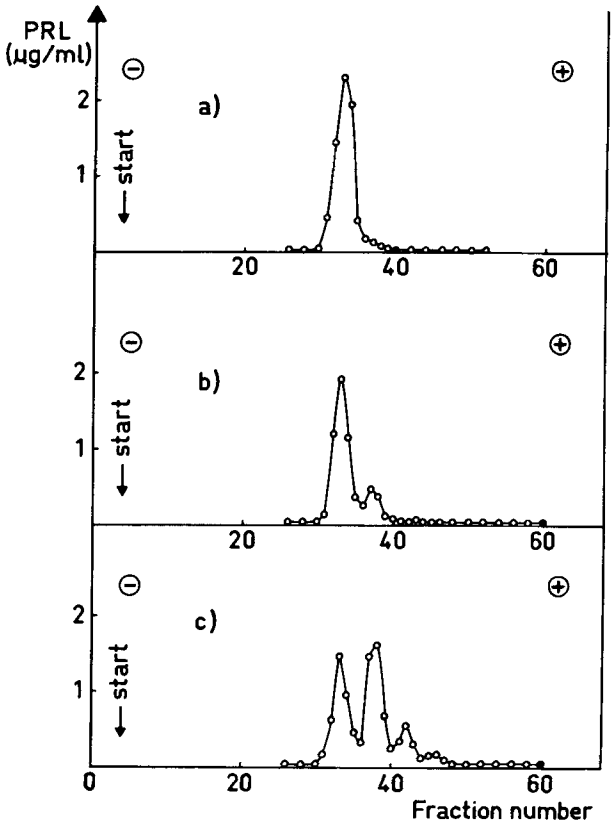

Fig. 3. Electrophoresis in agarose suspension. Runs of prolactin (PRL) I before (a) and after incubation (54 h at37°C) in saline (b) and in human serum (c). Buffer: 0.03 M veronal (pH 8.6). Voltage: 820 V. Duration: 18 h.

Storage stability of prolactin

The immunoactivity of prolactin was studied as a function of time during a period of 20 weeks. At a protein concentration of 100 ug/ml no decrease in prolactin activity was detected during any of the different conditions tried.

Fig. 4 illustrates the result obtained at a protein concentration of 10 ug/ml. As shown the prolactin immunoactivity appears comparatively stable

Table 3

Rate constant for conversion of prolactin isohormones at pH 10 and 37°C in 0.5 M ammonium sulphate

Prolactin fraction	Rate constant $k_1 \times 10^6$ (sec^{-1})	Preserved immunoactivity after 28 h[1] (%)
I	15	52 ± 1 (5)
II	8.3	59 ± 2 (5)
III	7.5	62 ± 4 (5)
IV	7.5	64 ± 5 (5)

[1] Values are given as means ± S.E.M. with the number of determinations in parenthesis.

Table 4

Rate constant for conversion of prolactin I in human serum and 0.9% (w/v) NaCl

Incubation media	Rate constant $k_1 \times 10^6$ (sec^{-1})	Preserved immunoactivity after 54 h[1] (%)
Human serum	2.5	81 ± 6 (3)
0.9% NaCl (pH 7)	0.6	82 ± 5 (3)

[1] Values are given as means ± S.E.M. with the number of determinations in parenthesis.

in 0.02 M Tris-HCl buffer (pH 9.0) at -80°C. Increased ionic strength of the Tris-HCl buffer seems to cause losses in prolactin activity and the use of ammonium bicarbonate is also seen to affect the immunoactivity. High stability of the freeze-dried prolactin is also indicated in Fig. 4.

At protein concentrations of 1 ug/ml the immunoactivity preserved did not exceed 20% in any of the sample assayed.

Prolonged storage (2-3 years) at a protein concentration in the range 0.1-2 mg/ml in 0.02 M Tris-HCl buffer (pH 9) at -80°C or in the presence of ethylene glycol at -20°C neither affected the immunopotency nor altered the electrophoretic pattern of prolactin.

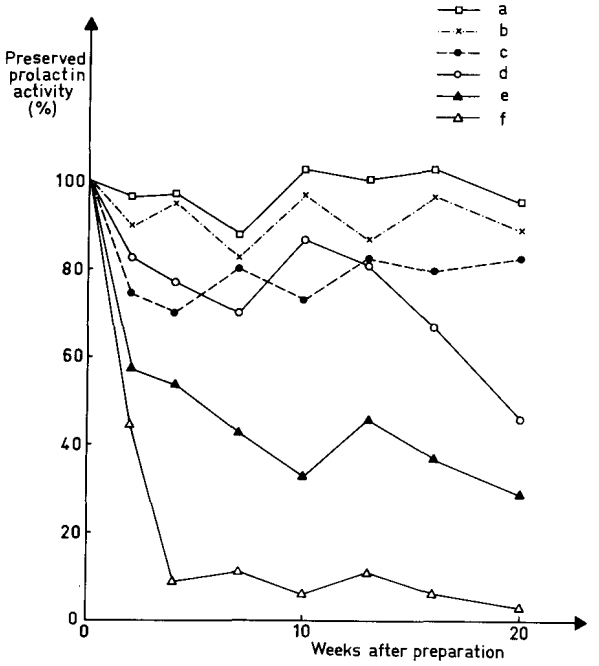

Fig. 4. Storage stability of prolactin. The immunoactivity preserved after storage of the hormone

(a) as a freeze-dried powder at +5°C and at a protein concentration of 10 ug/ml

(b) in 0.02 M Tris-HCl buffer (pH 9.0) containing ethylene glycol (50%, v/v) at -20°C.

(c) in 0.02 M Tris-HCl buffer (pH 9.0) at -80°C.

(d) in 0.02 M Tris-HCl buffer (pH 9.0) at -20°C.

(e) in 0.01 M ammonium bicarbonate (pH 7.8) at -20°C.

(f) in 0.02 M Tris-HCl buffer (pH 9.0) containing 0.5 M KCl.

DISCUSSION

High stability of prolactin has been shown in the pH range 7-9 at temperatures below 20°C (10 ,12), which is also confirmed by Fig. 1. Significant conversion into faster-migrating components observed when growth hormone and prolactin are exposed to alkaline media (at 37°C) has been alleged, by many investigators (3, 8-12), to be a result of deamidation.

This type of reaction may also be the cause of the transformation observed among the human isohormones of prolactin, following treatment in the different media. The faster-migrating components derived from prolactin I and prolactin IV (Fig. 2) may thus represent deamidated forms of these isohormones. Moreover, prolactin II-IV of the heterogenous preparation (Fig. 1) may all result from a deamidation of prolactin I.

On the other hand, other types of reactions have been described to occur at alkaline pH. Among these are the rupture of disulphide bonds and subsequent reactions, which may lead to an altered electrophoretic behaviour (19). In the present study it was found, however, that the conversion of the isohormones remained after reduction and alkylation, indicating that no disulphide bonds were involved in the reaction studied.

The decreased rate of conversion found in connection with increased acidity of the isohormones was suggested to depend on a limited number of amide groups being easily deamidated. This finding agreed with the observation by Lewis et al. (10), who reported a limited conversion of a heterogeneous ovine prolactin preparation. Further it was reported that the presence of enzyme inhibitors did not affect the rate of transformation. Considering the high degree of purity of the human isohormone preparations utilized we concluded that the alteration observed at pH 10 was also non-enzymic.

However, the experiments performed at neutral pH indicated that other factors than alkali, ionic strength, and temperature can influence the rate of alteration. Thus, the transformation seen in human serum (at a rate of about four times higher than that in saline) is likely to be the result of enzymatic action. It should be added that following incubation in serum (pH 7) or in buffer (pH 10) the electrophoretic migration rates of the resulting components were identical.

The demonstration of charge heterogeneity of prolactin in human cerebrospinal fluid and urine (14), in human amniotic fluid (6), and in extracts of human pituitaries (12) provides further supporting evidence of natural occurring deamidation reactions. Furthermore, deamidated forms of model peptides have been shown (17) to be more susceptible to degradation and it

was suggested that under physiological conditions deamidation could serve as a timer of development and aging. This statement is supported by the indications of an increased bioactivity with increased deamidation obtained for human prolactin (12) growth hormone (20) and placental lactogen (4). Additionally, by receptor assays it was found that the binding-capacity of prolactin decreased after extensive deamidation (Table 2). For example, prolactin IV thus showed a lower activity than prolactin III in th crop-sac radioreceptor assay, which was considered to indicate that with a continuously increased deamidation the activity reaches a maximum after which it declines.

In contrast, the immunoactivity showed a distinct decrease with increased deamidation, which was suggested to be due to a more specific binding site of the antigen-antibody complex.

In conclusion, we assumed that the cause of the charge heterogeneity of prolactin was a difference in amide content between the isohormones studied. We propose that it has a physiologic significance in being involved in the turnover process of the hormone.

Studies on the storage stability of prolactin showed that the immunoactivity was preserved at sufficiently high hormone concentrations, irrespective of the other parameters being varied. The decreased activity found in connection with the inclusion of KCl was believed to be due to formation of aggregates, which have been found to affect the immunopotency (13). Increased ionic strength known to favour hydrophobic attachments might well cause aggregation of prolactin. Finally, as no alteration of the electrophoretic pattern was observed after prolonged storage (at pH 9, with or without ethylene glycol) the hormone was concluded to be electrophoretically stable at the storage condition employed.

ACKNOWLEDGEMENT

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