Ion Exchange Chromatography of Prolactin in Urea-containing Buffers*

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Moore and Stein (1) have reviewed the work of Boardman and Partridge and others on problems encountered in attempting the chromatography of nonbasic proteins on Amberlite IRC-50 by elution analysis with the use of buffers of constant composition for the elution. Boardman and Partridge (2) suggested that the difficulty in achieving reversible equilibrium binding was due to hydrogen bonding between the protein and the resin. For this reason recent attempts to subject nonbasic proteins to this type of elution analysis on this resin have involved the addition of urea to the buffers used for elution. In this way successful chromatography was achieved with insulin (3) and glucagon (4).

The present report deals with the extension of elution analysis with the use of urea-containing buffers of constant composition to the chromatography of prolactin, a larger protein than insulin or glucagon. In addition to providing a technique for the fractionation and analysis of prolactin, this system provides an opportunity to test the effect of protein molecular weight on the chromatographic system by comparing the behavior of prolactin with that of insulin as reported earlier (3).

EXPERIMENTAL PROCEDURE

The prolactin used was a partially purified sample of ovine prolactin which corresponded to Fraction A in the preparation of Cole and Li (5). The crop sac-stimulating potency of the fractions of prolactin was assayed by the method of Riddle, Bates, and Dykshorn (6), and the luteotropic activity was assayed as described by Lyons et al. (7). Prolactin was recovered from the urea-phosphate buffers by dialysis and lyophilization.

The preparation and operation of the columns has been described before (3). Unless stated otherwise, chromatograms were prepared as described by Lyons et al. (7). Prolactin was recovered from the urea-phosphate buffers by dialysis and lyophilization.

RESULTS

Most of the studies of the chromatographic behavior of prolactin were carried out with a preparation of the hormone which was 20 to 25 i.u. per mg (i.e. about two-thirds of the potency of the purest preparations available), in the hope that the hormone and its contaminants would respond differently to changes in conditions, and that an improved isolation procedure could be developed. When this partially purified preparation of the hormone was submitted to chromatography in 0.13 M phosphate-8 M urea, the elution pattern obtained showed four main peaks (Fig. 1), and assay for the crop sac-stimulating potency of various fractions indicated that the least retained peak was about 15 i.u. per mg, whereas the next two peaks were 30 to 35 i.u. per mg, and the fourth peak was essentially inactive. A similar distribution of crop sac-stimulating activity was obtained on submitting to chromatography a sample of the hormone which had been purified further. This latter sample was prepared by submitting the previously mentioned preparation of the hormone to isoelectric fractionation, discarding precipitates which formed above pH 6.2 and below pH 4.5, and retaining the protein which precipitated between pH 4.5 and pH 6.2. This purified fraction gave a chromatogram which showed three peaks (Fig. 2). Prolactin has already been shown to contain three biologically active components when examined by electrophoresis (8) or by extended countercurrent distribution (9). Nevertheless, it was necessary to demonstrate that the three chromatographic peaks were not artifacts of the chromatographic system, and therefore, fractions were isolated from several chromatograms and then individually submitted to chromatography a second time in the original chromatographic system. The results of these experiments are presented in Fig. 3 and indicate that the three peaks are discrete, stable components appearing in reproducible positions on the chromatogram.

All three components represented by the three chromatographic patterns shown in Fig. 3 were found to contain a crop sac-stimulating potency of 30 to 35 i.u. per mg. The three fractions were also assayed for luteotropic activity (7), and although quantitative values cannot be given for the activities of the fractions, there was some luteotropic activity found in each fraction.

The partially purified hormone was submitted to chromatography in 8 M urea buffers of different pH values. Chromatograms obtained at many pH values are represented by those shown in Fig. 4 and indicate a gradual increase in the elution volumes of the prolactins as the pH is lowered.

The effect of changing the concentration of urea was studied next. This series of experiments is represented by the two chromatograms shown in Fig. 5, in which it may be seen that lowering the urea concentration from 8 M to 7 M results in essentially complete retention of the prolactin. This is observed not only as a loss of the main peaks from their former positions on the chromatogram, but also as a low recovery (30%) of protein compared to the quantitative recovery when 8 M urea was used.

DISCUSSION

Prolactin has already been examined by electrophoresis (8) and countercurrent distribution (9) and three biologically active

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components were detected in fully active (35 i.u. per mg) preparations of the hormone. The chromatographic separation of the prolactins, although less impressive than the resolution of the insulins (3, 10), is comparable to that secured by extensive electrophoresis and countercurrent distribution. This resolving power combined with its rapidity suggests the chromatographic method as a suitable procedure for the isolation of the individual prolactins.

The chromatographic fractionation of prolactin furnishes a further test of the identity of luteotropin and prolactin. The latter is generally recognized for its role in the development and function of the mammary gland (11), and is commonly assayed by measuring its stimulation of the growth of the pigeon's crop sac (6). The most potent preparations of prolactin, as determined in this manner, are also the most potent in stimulating the secretion of progesterone by the corpus luteum (12). For this reason, and because the two activities have never been dissociated, prolactin and luteotropin have been considered to be identical (13). The present chromatographic technique provides further evidence in favor of this supposition. Both the crop sac and luteotropic activities were found in all three chromatographic peaks. This would be extremely unlikely to occur unless luteotropic activity were an inherent property of the same molecule.
The present study permits a comparison of the chromatographic behavior of insulin and prolactin, two proteins which, although similar in isoelectric point, stability, and solubility in various solvents, differ greatly in size. Prolactin exists in its monomeric form (14) with a particle weight of 25,000 under a variety of conditions (15-17), whereas the insulin monomer has a molecular weight of 5733. Although insulin aggregates on either side of its isoelectric point, guanidine has been shown to cause disaggregation (18), and the urea employed in the chromatographic system should do the same. In fact, the chromatographic behavior of insulin in urea-containing buffers was unchanged when the load applied to the column was varied from 1 to 100 mg (3), and since, as noted below, a change in the particle weight would probably affect the chromatography, it is probably the monomer that is undergoing ion exchange.

The difference in molecular weight seems a likely explanation of the greater sensitivity of the chromatography of prolactin to changes in urea concentration. The elution volume of insulin under the same chromatographic conditions used for prolactin increases gradually as the urea concentration is lowered (3). This is in contrast to the abrupt onset of extreme binding of prolactin when the urea concentration is lowered from 8 M to 7 M. This extreme binding is probably due to multipoint attachment (through hydrogen bonds (3)) between protein and resin, which would be expected to result in essentially irreversible binding (2). That prolactin is severely bound to the resin at higher concentrations of urea than in the case of insulin is expected, since the larger molecule would have more chance to form multipoint attachments than the smaller one. Furthermore, this general picture is supported by the finding (4) that the small molecule of glucagon is retained by the resin less than the somewhat larger insulin, in spite of its more basic isoelectric point which would be expected on the basis of simple ion exchange to cause glucagon to be retained more, rather than less. The observations just discussed support the notion that hydrogen bonding is occurring in these ion exchange systems, as postulated by Boardman and Partridge (2), and that although moderate concentrations of urea are adequate to control severe hydrogen bonding when molecules the size of insulin are to be chromatographed, 8 M urea is the minimal concentration which is sufficient to control the problem in the chromatography of a molecule as large as prolactin.

Prolactin has been successfully chromatographed on columns of Amberlite IRC-50 ion exchange resin with 0.13 M phosphate 8 M urea buffers at pH 6.00. This system resolves three components in the fully active hormone. These three components were found to be equally active in crop sac-stimulating potency, and each was found to contain lutectropic activity.

Although ion exchange appeared to predominate in the chromatography of prolactin in 8 M urea, a nonequilibrium type of binding, presumably multipoint hydrogen bonding, occurred in 7 M urea and resulted in essentially irreversible binding of the prolactin to the resin. Thus, a higher concentration of urea is required to overcome severe hydrogen bonding in the case of prolactin than in the case of the smaller protein, insulin.

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