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IODINATION AND PURIFICATION OF OXYTOCIN

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SUMMARY

Oxytocin was iodinated using the thallium chloride method. Purification of iodination reaction mixture on Sephadex G-10 and G-15 was compared. Sephadex G-10 effected the separation of iodo-oxytocin from excess free radioiodide. Sephadex G-15 succeeded in separating iodinated from non-iodinated oxytocin as well as from radioiodide. It was found that the storage of unfractionated iodination mixture resulted in an apparent increase in the molecular size of iodinated hormone and that this process was accelerated at temperatures of −20° in comparison with 4°.

INTRODUCTION

The metabisulfite reduction step at the end of the chloramine-T iodination procedure[1] has been considered deleterious to the integrity of neurohypophyseal hormones[2,3]. In order to explore alternative methods of iodination we adapted the thallium chloride procedure for the iodination of nucleic acids[4,5] to the iodination of oxytocin. Literature reports indicate that iodinated neurohypophyseal peptides are retarded on Sephadex gels[6] in comparison with the elution of native hormones. Our own previous experience was that radioiodide appears retarded on highly crosslinked Sephadex gels in comparison with NaI[7]. On the basis of this information, we defined a purification system for iodinated oxytocin which succeeded in separating iodinated hormone from excess radioiodide on one hand, and unreacted hormone on the other.

MATERIALS AND METHODS

Oxytocin (0-3251), arginine vasopressin (V-0377) and lysine vasopressin (V-3000) were purchased from Sigma (St. Louis, Mo., U.S.A.). Syntocinon-10 was purchased from Sandoz (Basle, Switzerland). Arginine vasotocin was the kind gift of Dr. S. Guttman (Sandoz). 4-Ser-8-Ileu-oxytocin was prepared by us from salmon pituitary glands as previously described[8]. Angiotensin I (Schwarz/Mann, Orangeburg, N.Y., U.S.A.) was a gift of Dr. G. E. Wilkins (St. Paul's Hospital, Vancouver, B.C., Canada). The C-terminal tripeptide of oxytocin (MSH release inhibiting hormone) was purchased from Beckman (Palo Alto, Calif., U.S.A.). Glycine amide,

L-prolyl-L-leucyl amide, and L-leucyl-L-glycyl amide were purchased from Fox (Los Angeles, Calif., U.S.A.). All reagents were of analytical grade.

Antibodies were prepared in guinea pigs according to the procedure of Goodfriend *et al.*[9]. 1-Ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride (Calbiochem, La Jolla, Calif., U.S.A.) was the coupling reagent, and human serum albumin (Sylvana Fr. V) was the carrier molecule. The antiserum used in this work (GP-4) was characterized for cross-reactivity with related peptides. Cross-reactivity was compared on a molar basis[10].

The thallium chloride iodination procedure employed by Commerford for iodination of DNA[4] and by Getz, Altenburg and Saunders for RNA[5] was adapted for oxytocin. The adaptation was based on the concentration of reactants described by Commerford[4] and the number of cytosine residues in calf thymus DNA from literature values[11]. The reaction mixture contained: 100 μl ($\approx$ 2.2 nmoles) oxytocin (Syntocinon-10), 5 μl (1 mCi per 5 μl) of $Na^{125}I$ (carrier free; New England Nuclear, Boston, Mass., U.S.A.; Cat. No. NEZ-033), 100 μl ($\approx$ 4 nmoles) KI, 100 μl (24 nmoles) thallium chloride, and 100 μl (16 nmoles) of Na_2SO_3. Iodination reaction was carried out as described by Commerford[4], with the omission of the final step (addition of ammonium acetate to raise the pH of reaction mixture to 9).

In our earlier experiments the entire iodination mixture was subjected to gel filtration (Fig. 1). Subsequently, the mixture was divided into 50-μl aliquots, and one such aliquot put on Sephadex G-15 immediately after iodination (Fig. 2). The remainder was stored at 4° or —20°. Prior to Sephadex chromatography, the volume of the 50-μl aliquot was made up to 400 μl with the column buffer.

Sephadex G-10 (Pharmacia, Piscataway, N.J., U.S.A.; Lot No. 1032), column 9 $\times$ 120 mm, was equilibrated in 0.1 *M* sodium acetate–0.04 *M* acetic acid buffer (pH 5)[4,12] with specific conductivity 3.2 $m\Omega^{-1}$. Chromatography was performed at 2°. The flow-rate ranged from 40–45 ml/h. Hundred fractions, 0.4 ml each, were collected.

Sephadex G-15 (Pharmacia; Lot No. 268) was equilibrated with the same buffer as Sephadex G-10. A Pharmacia K 15/30 column (15 $\times$ 246 mm) was used and eighty 1.0-ml fractions collected. Chromatography was performed at 2°.

Elution of non-iodinated oxytocin for the purpose of column standardization was monitored by radioimmunoassay. Results were compared with a standard curve and expressed in terms of hormone weight. Radioimmunoassay method described by Robertson *et al.*[10] for antidiuretic hormone was followed with minor modifications:

(1) EDTA and L-cystine were omitted from the buffer and powdered BSA (Armour Fr. V) was substituted;

(2) shorter (3-day) incubation time was used:

(3) approx. 4000 cpm were added to each tube and the assays conducted in triplicate.

Elution of ^{125}I-oxytocin from Sephadex columns was followed by radioactivity measurements and by the competence of column fractions to bind to antibody[13]. The incubation mixture in this type of immunoassay consisted only of buffer, antibody and an aliquot of the column fraction. The column fraction aliquots were taken and diluted in such a manner as to yield an approximately equal number of counts ($\pm$ 10%). In contrast to the conventional competitive binding radioimmunoassay neither cold oxytocin or exogenous tracer were added to the incubation mixture. For each column

fraction assayed in this manner a blank, consisting of identical column fraction aliquot and buffer, was prepared. Blanks served to determine the proportion of iodinated material which, at the end of the incubation period, appeared in the "antibody-bound" fraction in the absence of antibody. The column fraction(s) with the highest B/F ratio and the lowest blank value were considered as the best tracer preparations[13] and were used in radioimmunoassays. Incubation time for column immunoassay was 16–24 h at 2°.

RESULTS

The antiserum employed throughout this study (GP-4) demonstrated considerable specificity for oxytocin. It did not cross-react with arginine or lysine vasopressin and angiotensin I in the range up to 100 ng (100 pmoles) per ml. Its cross-reactivity with arginine vasotocin was less than 1/200th of that with oxytocin. The antiserum showed partial cross-reactivity with oxidized (performic acid) oxytocin and with the C-terminal tripeptide: 1/8–1/9th of that with oxytocin. Glycine amide, Pro-Leu-NH_2 and Leu-Gly-NH_2 showed no cross-reactivity in the assay system. 4-Ser-8-Ileu-oxytocin cross-reacted with our GP-4 antiserum at approx. 1/50th level of oxytocin.

The radioactivity pattern obtained following the passage of ^{125}I-oxytocin through Sephadex G-10 is illustrated in Fig. 1. Two major peaks of radioactivity are found to emerge: one in the V_0 (void volume) region of the column and the second corresponding to the elution volume of Na^{125}I.

Radioimmunoassay of the column fractions by measurement of binding to antibody demonstrated that the elution of iodooxytocin was confined to the first major peak of radioactivity (Fig. 1). Thus this system separated the iodinated hormone from the excess free radioiodide. In several experiments, binding to antibody was compared between the ^{125}I-oxytocin in the iodination mixture prior to gel filtration to that obtained after gel filtration. In all cases, the B/F ratio for purified hormone was found to be approximately twice as high as that of the unpurified preparation. The blank (*i.e.* the co-precipitate) was also reduced from $B/F = 0.10$ to 0.05.

Elution of ^{125}I-oxytocin from Sephadex G-10 was also monitored using four other antisera. The maximum binding of iodinated hormone to all four antisera corresponded to the same fraction as was shown with GP-4 antiserum (Fig. 1). Gel filtration on Sephadex G-10 of iodo-oxytocin prepared by other methods[2,12] showed that the immunoreactive region corresponded to that of the ^{125}I-oxytocin prepared by the thallium chloride method. However, the binding obtained with the lactoperoxidase method of preparation[12] was very low.

Although chromatography on Sephadex G-10 does result in some purification of ^{125}I-oxytocin, the separation from non-iodinated hormone is not accomplished. This is evidenced by the finding of non-iodinated oxytocin in the V_0 region (Fig. 1), and by the retention of approx. 20% of uterotonic activity[14] in fraction 10 of ^{125}I-oxytocin.

Sephadex G-15 achieved separation of non-iodinated oxytocin, iodinated hormone and unreacted Na^{125}I, which were eluted from the column in the above order (Fig. 2). When gel filtration was conducted immediately following the iodination reaction, the radioactivity of the effluent fractions showed two poorly resolved peaks. The more retarded of these corresponded in its elution volume to Na^{125}I. In our

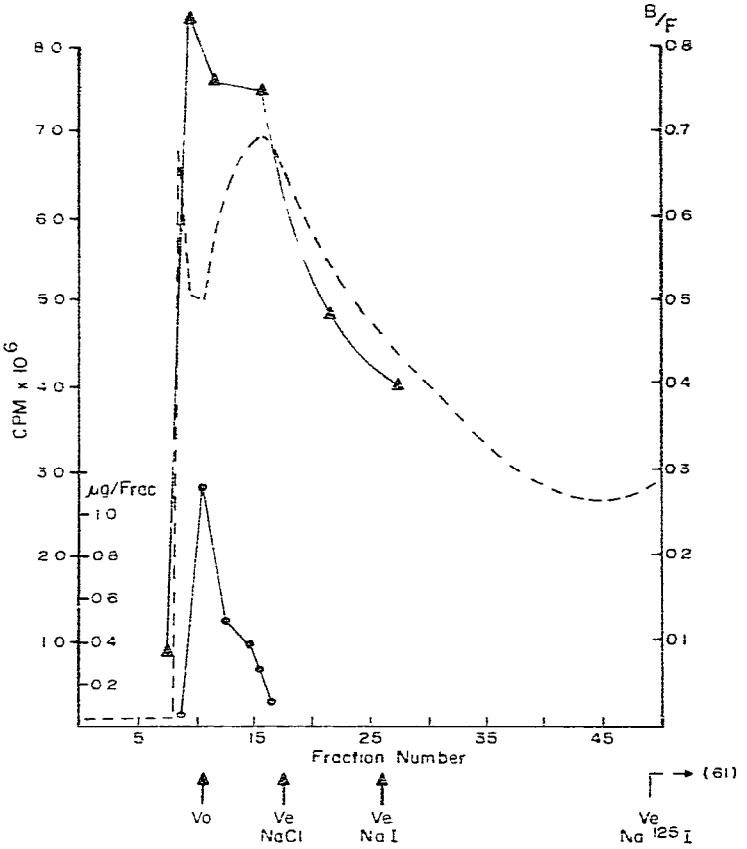

Fig. 1. Chromatography of iodinated oxytocin on Sephadex G-10 (9 × 120 mm). Loading sample, 0.4 ml of iodination reaction mixture; fractions, 0.4 ml. – – –, Radioactivity (cpm); ▲—▲, immunological activity (B/F); ●—●, non-iodinated oxytocin (μg per fraction; load, 2.2 μg). V_0, void volume of the column; V_e, elution maximum. Column buffer, sodium acetate (pH 5; 3.2 mΩ^{-1}). Temp., 2°.

experience, the best tracer oxytocin for use in radioimmunoassay systems was derived from fractions 40–43. Non-iodinated hormone was eluted early in this system (Fig. 2) and did not present a contamination hazard. Sodium iodide, which is known to interfere with antigen–antibody binding[15] had a smaller elution volume (fraction 36) than that of the major oxytocin immunoreactive peak.

Iodinated oxytocin presented an interesting phenomenon following storage. When the Sephadex G-15 chromatography was carried out immediately following iodination, the results represented in Fig. 2 were obtained repeatedly. Remaining aliquots of the same iodination mixture were stored at 4° and at —20°, respectively. Material stored at 4° for 5 days and then chromatographed on Sephadex G-15 showed the same elution pattern as the freshly chromatographed iodination mixture (Fig. 2) with the best immunoreactive iodo-oxytocin eluting in the region of fraction 40. However, when storage for the same period of time took place at —20°, the region of highest binding to the antibody was found in the V_0 region of the column (Fig. 3).

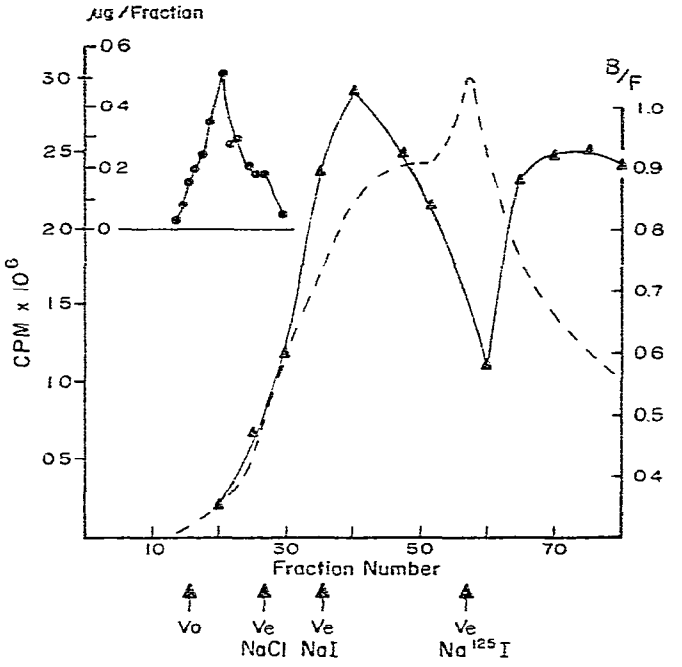

Fig. 2. Chromatography of iodinated oxytocin on Sephadex G-15 (15 × 246 mm) immediately after iodination. Loading sample, 0.05 ml of iodination mixture + 0.35 ml column buffer. Fractions, 1.0 ml. – – –, Radioactivity (cpm); ▲—▲, immunological activity (B/F); ●—●, non-iodinated oxytocin (µg per fraction; load, 2.2 µg). V_0, void volume of the column; V_e, elution maximum. Column buffer, sodium acetate (pH 5; 3.2 mΩ^{-1}). Temp., 2°.

When aliquots were stored for 15 days at 4° and at −20°, respectively, and then subjected to purification on Sephadex G-15, the highest antibody binding was found in the V_0 region for both aliquots (fraction 14). However, the binding to antibody was three times higher ($B/F = 1.0$ *vs.* $B/F = 0.3$) in the preparation that was stored at 4°. In spite of the anomalous elution pattern of the stored (up to 34 days at −20°) material, fraction 14 could be used as a tracer hormone in oxytocin radioimmunoassay, but the resulting standard curves were of decreased sensitivity.

The aging of the iodination mixture was accompanied by gradual emergence of three clearly separated peaks of radioactivity in the eluent of Sephadex G-15 (Fig. 3). Of these, the first corresponded to the V_0 region of the column, and the last to the elution maximum of radioiodide. After 15 days of storage (4° or −20°), those peaks were approximately equal in height. As the storage progressed, the middle (unidentified) peak increased at the expense of the other two peaks. However, the major immunoreactivity remained in the V_0 region.

DISCUSSION

The thallium chloride iodination method has been the method of choice in our laboratory in the past year. It could be easily performed by a novice and only required a word of caution regarding the toxicity of thallium. We found the method

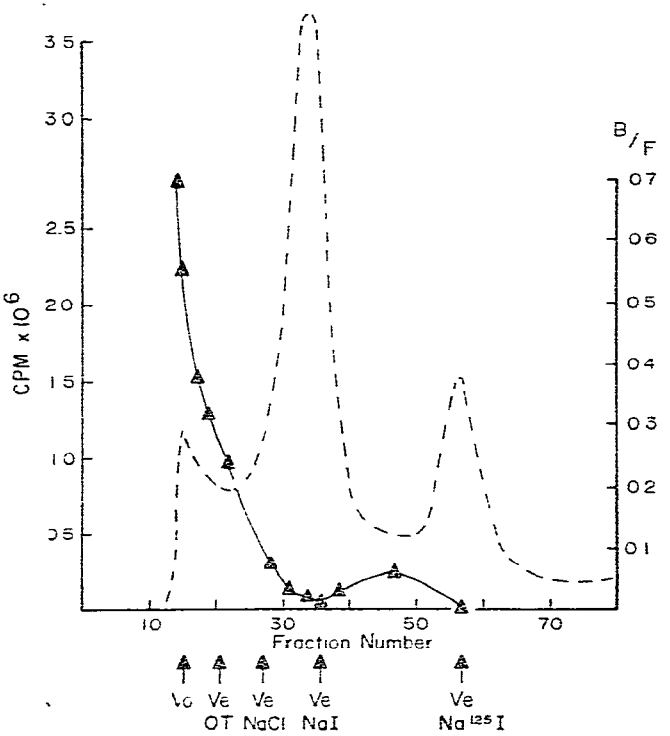

Fig. 3. Chromatography of iodinated oxytocin on Sephadex G-15 (15 × 246 mm) following storage of iodination mixture for 34 days at 4°. Loading sample, 0.05 ml iodination reaction mixture + 0.35 ml column buffer. Fractions, 1.0 ml. – – –, Radioactivity (cpm); ▲—▲, immunological activity (B/F); V_0, void volume of the column; V_e, elution maximum; OT = oxytocin. Column buffer, sodium acetate, (pH 5; 3.2 mΩ^{-1}). Temp., 2°.

equally applicable to the iodination of lysine vasopressin and arginine vasotocin. Sephadex G-15 was used for purification of all these iodinated hormones. Smaller Sephadex G-15 columns (9 × 120 mm) were found adequate for purification of iodinated vasopressin and vasotocin. The size of the Sephadex G-15 column (15 × 246 mm) reported in the present study for purification of iodo-oxytocin is excessive for the purification of 50-μl aliquots which are presently in use. However, the column has been retained in use in order to study the elution volume of stored (aged) iodination mixtures.

Retardation of iodinated neurohypophyseal hormones on Sephadex columns was observed by a number of authors[6,16–18]. Two reports[6,18] observed the elution of iodo-oxytocin after the iodide peak. In our experience, iodo-oxytocin was also eluted after the iodide peak (NaI) but before the peak of radioiodide ($Na^{125}I$). As the elution of these iodide isotopes shows an apparent difference[7], we find it difficult to compare our data with the literature reports. We have examined the material (Fig. 2) found eluted after the radioiodide on our Sephadex G-15 column. We found that tracer obtained from the region of fraction 40 is far superior to that in fraction 70.

The elution pattern of iodinated vasopressin which is illustrated in the literature[17] resembles the results obtained by us. K_{av} values[19] for oxytocin and iodinated

oxytocin calculated from Fig 2 are 0.16 and 0.78, respectively. These values are comparable to those obtained by Legros and Franchimont[17]. It could be speculated that the same forces which are responsible for the retardation of iodide[7] may account for the retardation of the iodinated neurohypophyseal hormones on Sephadex gels.

Our experiments with Sephadex G-10 demonstrate that the newer G-10 gels can be successfully used for the desalting of neurohypophyseal peptides, as was not the case in our earlier experience with Sephadex G-10[8]. We have also found that by careful selection of the G-10 fraction (the descending limb of the major radioactive peak; tube 16 or 17) an adequate tracer hormone for use in radioimmunoassay may be obtained. It was also of interest to observe that the retardation of iodinated hormone did not take place on Sephadex G-10, while radioiodide itself tends to be more retarded by the more highly crosslinked gel[7].

The immunoreactivity which was found in the V_0 region of Sephadex G-15 after storage, and which was never detected when freshly prepared iodination mixture was chromatographed, presented an interesting phenomenon. From its elution volume it resembled a polymerization product of iodo-oxytocin. However, some structural integrity of the original oxytocin molecule was retained, since fraction 14 was able to participate in a competitive binding radioimmunoassay system as a tracer hormone. Our experiments indicate that storage at $-20°$ was more detrimental to the iodination mixture than that at 4°, and we presently store all our iodinated material (fractionated and unfractionated) in the refrigerator. Our experience with the storage of iodinated lysine vasopressin (^{125}I-LVP) was more limited, but it indicates a similar trend. We found that when lysine vasopressin antisera were tested against an unfractionated iodination mixture (^{125}I-LVP), the antibody binding was much higher for the ^{125}I-LVP stored at 4° than for that stored at $-20°$.

Although forces governing the retardation of iodinated peptides, iodides, and radioiodide in particular[7] are not well understood at present, the peculiarities of elution of these substances from Sephadex gels can be exploited in order to obtain high-quality tracer hormone for radioimmunoassay of neurohypophyseal hormones.

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