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# FRACTIONATION OF BOVINE THYROTROPHIN AND LUTEINIZING HORMONE ON CELLULOSE ION EXCHANGE COLUMNS


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## SUMMARY

Chromatographic studies of the behavior of TSH on DEAE-C and on CM-C have shown that crude TSH can be purified approximately 20 to 40 times with recovery of 30 to 50% of the starting activity. The potency of the most active fractions is 20 to 50 U.S.P. units/mg. LH can be separated from TSH by chromatography on DEAE-C at pH 9.5. The TSH is retained on the column while the LH passes through unadsorbed.


## INTRODUCTION

Early attempts to purify pituitary thyrotrophin resulted in the preparation of fractions which were about 100 times more potent than the bovine anterior pituitary

Abbreviations: TSH, thyroid-stimulating hormone; LH, luteinizing hormone; DEAE-C, diethyl amino ethyl cellulose; CM-C, carboxymethylcellulose.

*References p. 438.*

powder used as starting material[1,2]. Subsequently, STEELMAN showed that thyroid-stimulating hormone (TSH) behaved as a basic protein when it was purified by an electrical transport method[3]. Following the demonstration by HEIDEMANN that bovine TSH could be adsorbed on the weak cation exchanger IRC-50[4], several groups of investigators employed this resin to prepare fractions having potencies of 2 to 5 U.S.P. units/mg[5–7].

CM-C has been used to prepare fractions which have potencies of 5 to 10 units/mg[7]. When examined by paper electrophoresis and in the ultracentrifuge, these preparations appeared to be homogeneous*. The sedimentation constant of these preparations is 2.5 S, which is close to the value reported by PIERCE AND NYC for similar material[6,7]. Despite the apparent homogeneity of this 5 to 10 units/mg material, column chromatography on DEAE-C showed that it contains several components and that TSH activity was associated with no more than 30% of the protein placed on the column[8].

We wish to report here experiments in which TSH having a potency of 20 to 50 units/mg was prepared by solvent extraction according to the procedure of BATES[9], followed by chromatography on CM-C and DEAE-C. In the course of chromatography on DEAE-C, it is possible to separate TSH from LH, which is the most common biologically active contaminant of TSH.

## MATERIALS AND METHODS

### *Pituitary powder*

The starting material for these experiments consisted of powders of desiccated anterior lobes of bovine pituitaries. One batch was an acetone dried powder No. 128,300* containing 0.03 U.S.P. unit of TSH/mg. A second batch was a lyophilized powder which contained 0.05 to 0.1 units/mg. The TSH fractions isolated from each batch did not differ in their chromatographic properties.

### *Preparation of crude TSH*

The early part of this work was carried out with a fraction prepared from bovine anterior pituitary powder by the method of CIERESZKO[2]. This fraction usually has a potency of about 1 unit/mg. It contains no prolactin or growth hormone and less than 0.1 U.S.P. unit of corticotrophic activity/mg. Later in these studies, partially purified TSH fractions having a potency of 1 to 2 units/mg were prepared by the percolation procedure of BATES[9] and were used as the starting material for chromatographic studies.

### *Bioassays*

Thyroid-stimulating activity was determined by depletion of $^{131}I$ in the baby chick, according to the procedure of BATES AND CORNFIELD[10]. All potencies are expressed in terms of U.S.P. units of TSH. Luteinizing hormone was estimated in laying hens by the procedure of FRAPS**. Luteinizing hormone activity is expressed as the amount of protein in $\mu g$ which will produce ovulation in 50% of the hens tested ($OD_{50}$). The relative potency of various preparations is given in Table III as 100 times the reciprocal of $OD_{50}$.

* This material was obtained through the courtesy of the Endocrine Study Section, National Institutes of Health, Bethesda, Md.

** R. M. FRAPS, unpublished data.

*References p. 438.*

*Chemical analyses*

Protein concentration was estimated either by the absorption at 276 mμ or by the modified biuret-Folin reaction described by LOWRY *et al.*[11].

*Preparation and operation of columns*

CM-C was prepared from Whatman powdered cellulose by the procedure of PETERSON AND SOBER[12]. Different batches, which contained 0.7 to 0.9 mequiv. of carboxyl groups/g of CM-C, all behaved in the same manner during chromatography of TSH. DEAE-C was either prepared from Solka Floc BW-200* by the procedure of PETERSON AND SOBER[12] or was obtained through commercial channels**. TSH behaved in the same way on all lots of DEAE-C used in these experiments.

Batches of DEAE-C and CM-C were prepared for columns by washing with a solution which was 0.5 *N* NaOH and 0.5 *N* NaCl, followed by a thorough washing with water until the pH was about 6 and the absorbance of the wash water was less than 0.020 at 276 mμ. The batch was then suspended in the appropriate buffer and stirred at room temperature for 1 h. The cellulose was allowed to settle for a half hour and the supernatant solution was decanted. It was then resuspended in buffer and again stirred slowly to free the suspension of fine bubbles which tend to slow the flow rate. The suspension was then poured into a column of the type employed by STEIN AND MOORE for starch chromatography[13]. Each portion settled under gravity and the liquid standing above the column was allowed to run down to the top of the cellulose. Additional portions of cellulose suspension were added until the desired height was reached. Dimensions of the columns used are indicated in the tables and figures. The settled column was washed with the initial buffer until the pH of the influent and effluent were the same.

Weighed samples were usually dissolved in a small volume of buffer which was then equilibrated against the initial buffer by dialysis at 2°. Samples which were rechromatographed without intervening lyophilization, were concentrated to the desired volume by flash evaporation at less than 10° and then equilibrated at 2°.

Gradients were applied as indicated in the figures by Roman numerals. Usually a mixing device was used which was similar to that suggested by ALM *et al.*[14]. In some experiments linear gradients were obtained by use of a device like that of PARR[15].

## RESULTS

*Experiments with diethylaminoethyl cellulose*

Preliminary experiments were carried out using the conditions employed by SOBER *et al.*[16] for the anion exchange chromatography of plasma proteins. At pH 7.0 in 0.005 *M* sodium phosphate buffer, TSH was not completely retained on the column and iodine-depleting activity was found in most of the fractions that were eluted. Similar results were obtained when 0.01 *M* tris(hydroxymethyl)aminomethane buffer was used over a pH range of 7.5 to 8.5. Retention of the hormone was finally achieved on DEAE-C columns at pH 9.5 in sodium glycinate buffer having an ionic strength of 0.005, as shown in Fig. 1.

* Purchased from Brown Co., Berlin, New Hampshire.
** Purchased from Eastman Kodak, Rochester, New York.

*References p. 438.*

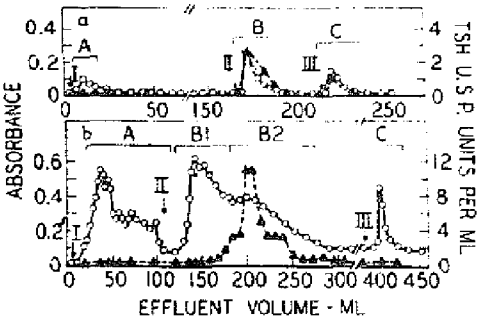


Fig. 1. Column chromatography of TSH on DEAE-C. Column size 0.9 × 15 cm, temperature 2°, fraction size 3 ml. ○, u.v. absorption at 276 mμ; ▲, TSH activity in U.S.P. units/ml of effluent. Fractions pooled as indicated by brackets. a, 10 mg TSH (5 U.S.P. units/mg); solvents I, pH 9.5, $\Gamma/2 = 0.005$, sodium glycinate; II, stepwise change to pH 9.5, $\Gamma/2 = 0.10$, sodium glycinate; III, stepwise change to 0.20 *M* $NaH_2PO_4$ (Expt. 6-25, Table II). b, 140 mg TSH (4 U.S.P. units/mg); solvents I, pH 9.5, $\Gamma/2 = 0.005$, sodium glycinate; II, gradient to pH 9.5, $\Gamma/2 = 0.10$ sodium glycinate; III, stepwise change to 0.20 *M* $NaH_2PO_4$ (Expt. 6-61, Table II).

Fig. 1 illustrates a pair of experiments in which the conditions of adsorption were the same but the manner of elution was different. Fig. 1a (Expt. 6-25, Table II) shows that when elution was carried out by the stepwise change of solvents indicated, the TSH, having a potency of 5 units/mg, can be resolved into the 3 components labelled A, B, and C in the figure. Peak B, which was eluted by raising the ionic strength to 0.1, contained 80% of the TSH and it was estimated that the highest potency at 181 effluent ml, was about 20 units/mg. When gradient elution was carried out, as shown in Fig. 1b, peak B was partly resolved into two components. The highest potency at 207 effluent ml was again estimated to be about 20 units/mg. When the peaks were pooled and recovered by dialysis and lyophilization, Fraction B2 (Table II, Expt. 6-61) was found to have a potency of 15 units/mg or over three times the potency of the starting material.

Table I summarizes the results of experiments in which crude fractions, most of which had potencies of about 1 unit/mg, were used as the starting material. In most

TABLE I

RECOVERY OF TSH FROM CRUDE FRACTIONS BY CHROMATOGRAPHY ON DEAE-C

| *Experiment No. and elution fraction* | *Column size cm* | *Starting material* | | *TSH eluted* | | *Yield %* | *Purification* |
|---|---|---|---|---|---|---|---|
| | | *Potency USP u/mg* | *Total TSH USP units* | *Potency of peak USP u/mg* | *Total TSH USP units* | | |
| 6-69* | 0.9 × 10 | 1.2 | 480 | 10 | 400 | 83 | 8 × |
| 6-95B1* | 1.9 × 25 | 1.1 | 7,500 | 2.9 | 880 | 12 | 2.6 × |
| B2 | | | | 0.9 | 3,800 | 51 | 0.3 × |
| B3 | | | | 3.2 | 600 | 9 | 2.9 × |
| 7-3B1* | 3.4 × 15 | 1.1 | 2,700 | 1.5 | 230 | 8 | — |
| B2 | | | | 5.0 | 1,500 | 56 | 4.5 × |
| 7-121* | 3.4 × 15 | 1.0 | 3,000 | 4.0 | 2,360 | 79 | 4.0 × |
| 8-13** | 3.4 × 15 | 0.4 | 1,100 | 3.0 | 1,070 | 92 | 7.5 × |
| 8-35* | 3.4 × 15 | 1.0 | 5,000 | 5.0 | 6,400 | 128 | 5 × |
| 8-43* | 10 × 12 | 0.58 | 5,200 | 6.0 | 6,000 | 115 | 10 × |
| 8-101B2** | 10 × 12 | 1.1 | 12,000 | 6.0 | 2,600 | 22 | 5.5 × |
| B3 | | | | 2.0 | 4,000 | 33 | 1.8 × |
| B4 | | | | 1.0 | 1,300 | 11 | — |

* Starting material prepared by acetone fractionation.
** Starting material prepared by percolation.

*References p. 438.*

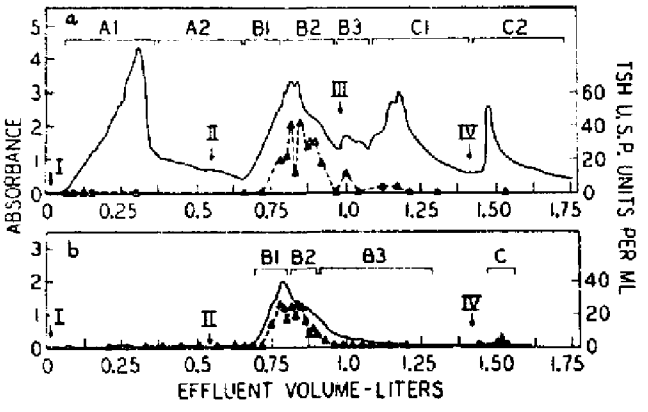


Fig. 2. Column chromatography of TSH on DEAE-C. Column size 1.9 × 25 cm, temperature 2°, fraction size 5 ml. Solid line, u.v. absorption of the effluent at 276 mμ; ▲ TSH activity in U.S.P. units/ml of effluent. Gradient elution according to PARR[15]. Fractions pooled as indicated by brackets. Solvents: I, pH 9.5, $\Gamma/2 = 0.005$, sodium glycinate; II, Linear gradient to pH 9.5, $\Gamma/2 = 0.10$, sodium glycinate; III, Linear gradient to pH 9.5, $\Gamma/2 = 0.20$, sodium glycinate; IV, Stepwise change to 0.20 $M$ $NaH_2PO_4$. a. 6.8 g TSH (1.1 U.S.P. units/mg) applied to the column in 100 ml of the initial buffer. (Expt. 6-95, Table I.) b. Rechromatogram of fraction 6-95B2 (550 mg, 6.9 units/mg). The TSH was applied to the column in 63 ml of the initial buffer. (Expt. 6-107, Table II.)

experiments about 50% of the TSH activity was recovered in fractions such as 6-95B2 (Fig. 2a) in which there was at least a 5-fold increase in potency. Most of the remaining activity could be accounted for in adjacent fractions of lower potency.

Table II summarizes the experience with rechromatography on DEAE-C of fractions previously purified by chromatography on CM-C or DEAE-C. The experi-

TABLE II

CHROMATOGRAPHY OF PARTIALLY PURIFIED TSH ON DEAE-C

| *Expt. No. and elution fraction* | *Column size cm* | *Starting material* | | *TSH eluted* | | *Yield %* | *Purification* |
|---|---|---|---|---|---|---|---|
| | | *Potency USP u/mg* | *Total TSH USP units* | *Potency of peak USP u/mg* | *Total TSH USP units* | | |
| 6-25B*,** | 0.9·10 | 5.0 | 48 | 8 | 35 | 73 | 1.6 × |
| C | | | | 4 | 9 | 19 | — |
| 6-47B1** | 0.9·10 | 2.5 | 175 | 2.5 | 31 | 18 | — |
| B2 | | | | 14 | 85 | 50 | 3.7 × |
| 6-61B1** | 0.9·15 | 4.0 | 560 | 1 | 22 | 4 | — |
| B2 | | | | 15 | 510 | 91 | 3.75 × |
| 6-107B1*** | 1.9·25 | 6.9 | 3,800 | 4.9 | 1,300 | 34 | — |
| B2 | | | | 10.0 | 2,100 | 55 | 1.5 |
| B3 | | | | 3.2 | 310 | 8 | — |
| 8-87B1§ | 1.9·15 | 10.0 | 3,900 | 9 | 465 | 12 | — |
| B2 | | | | 21§§§ | 1,850 | 47 | 2 × |
| B3 | | | | 13§§§ | 455 | 12 | — |
| 8-145B§ | 0.9·8 | 21 | 200 | 40§§ | 180 | 90 | 2 × |

* Stepwise elution.

** Starting material prepared by acetone fractionation and CM-C chromatography.

*** Starting material prepared by acetone fractionation and DEAE-C chromatography.

§ Starting material prepared by acetone fractionation and chromatography on DEAE-C and CM-C.

§§ Potency estimated by assay of pooled peak and spectrophotometric determination of the protein concentration.

§§§ Minimum value for the potency. Higher values were also obtained depending on the dose injected into the chicks.

*References p. 438.*

ment designated 6-107 shows the distribution of TSH when fraction 6-95B2 (Table I) was rechromatographed on DEAE-C under the same conditions. This experiment is illustrated in Fig. 2b to show that the position of the TSH activity is the same in the original and in the rechromatogram. The fractions recovered in experiment 6-107 were subsequently placed on CM-C columns (Expts. 7-37 and 7-63 in Table IV and Fig. 3), with results which are described later in this paper. There appears to be more than one component which possesses TSH activity. However, it must be remembered that individual values of the TSH concentration may be in error by approx. 30%, which could account for some of the irregularities in the TSH activity curves of the effluent in these and in other experiments.

*Separation of TSH and LH on DEAE-C*

Crude TSH prepared by acetone fractionation contains considerable amounts of LH as well as TSH. Fractionation on CM-C columns fails to effect a complete separation of the two hormones. For example, preparation 5-47 (Table III), which was prepared from 1-95F by chromatography on CM-C, had about the same LH potency as 1-95F, although the ratio of TSH to LH went up from 0.47 to 2.3. When a fraction similar to 1-95F was chromatographed on DEAE-C as shown in Fig. 2a, it was found that LH activity was located in Fraction A1 (Table III, 6-95A1) with little TSH activity. The TSH/LH ratio was reduced to 0.01. The later TSH-containing fraction 6-95B2 was rechromatographed on DEAE-C as shown in Fig. 2b and the fractions 6-107B1, B2 and B3 were cut as indicated. Upon assay the TSH/LH ratio was found to have increased from 2.3 in preparation 5-47, to 80 in the case of 6-107B2, indicating a reduction of 40 times in the contamination of TSH by LH. Thus, a good separation of the LH and TSH activities (ratio of 0.01 for 6-95A1 and of 80 for 6-107B2) was obtained. Assuming the $OD_{50}$ of pure LH to be no more than 4 $\mu$g, the amount of LH present in a TSH preparation such as 6-107B2 would be 0.5% (w/w), or less. The most

TABLE III

SEPARATION OF TSH AND LH ON DEAE-C

| *Preparation* | *TSH potency USP u/mg* | *Luteinizing hormone* | | *TSH/LH* |
|---|---|---|---|---|
| | | *$OD_{50}$ µg* | *Relative potency* | |
| 1-95F | 0.7 | 68 | 1.5 | 0.47 |
| 5-47 | 5.0 | 45 | 2.2 | 2.3 |
| 6-95A1 | 0.05 | 25 | 4.0 | 0.013 |
| 6-107B1 | 4.9 | 400 | 0.25 | 20 |
| B2 | 10.0 | 800 | 0.125 | 80 |
| B3 | 3.3 | 400 | 0.25 | 13 |
| 6-61A | 0.05 | 8 | 12.5 | 0.004 |
| 61B1 | 0.9 | 45 | 2.2 | 0.41 |
| 61B2 | 15 | 130 | 0.77 | 20 |
| 7-49A | 0.05 | 50 | 2.0 | 0.025 |
| 49B1 | 1.1 | 45 | 2.2 | 0.5 |
| 49B2 | 5 | 500 | 0.2 | 25 |
| 49B3 | 2 | 500 | 0.2 | 10 |
| 49C | 1 | 650 | 0.15 | 6.7 |
| Armour 227-80* | 0.36 | 4 | 25 | 0.014 |

* This preparation has a potency of 200 Weaver Finch Units/mg[17].

*References p. 438.*

potent LH preparations described in this report appear to be about half as potent as Armour preparation 227-80, but they contain considerably less TSH. The high TSH content of this potent Armour LH preparation indicates that further purification can be expected. Additional studies on the chromatography of LH will be reported in a later publication.

*Experiments with carboxymethylcellulose*

Fractions from DEAE-C chromatograms can be further purified on carboxymethylcellulose columns. Fig. 3 illustrates the rechromatography of the Fractions B1 and B2 in Fig. 2b (Table II: 6-107B1, 6-107B2) on CM-C columns. The conditions previously reported[7] of absorption at pH 6 at low ionic strength followed by gradient elution with 1 *M* NaCl were used in both experiments. Similar patterns were obtained in each case and the potency of the material recovered in the main peak was at least

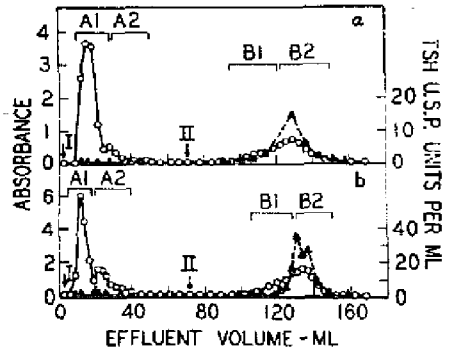


Fig. 3. Rechromatography of TSH on CM-C. Column size 0.9 + 20 cm, temperature 2°, fraction size 3 ml. ○, u.v. absorption at 276 mμ; ▲, TSH activity in U.S.P. units/ml of effluent. Fractions pooled as indicated by brackets. Gradient elution according to ALM *et al.*[14]. Solvents: I, pH 6.3, 0.01 *M*, sodium phosphate; II, gradient to 1 *M* NaCl. a, 237 mg TSH (4.9 units/mg, Expt. 7-37 in Table IV) applied to the column in 2 ml of the initial buffer. b, 100 mg TSH, (10 units/mg, Expt. 7-63 in Table IV) applied to the column in 2 ml of the initial buffer.

TABLE IV

CHROMATOGRAPHY OF PARTIALLY PURIFIED* TSH ON CM-C

| *Expt. No. and elution fraction* | *Column size cm* | *Starting material* | | *TSH eluted*** | | *Yield %* | *Purification* |
|---|---|---|---|---|---|---|---|
| | | *Potency USP u/mg* | *Total TSH USP units* | *Potency of peak USP u/mg* | *Total TSH USP units* | | |
| 7-37B1 | 0.9·20 | 4.9 | 1,160 | 2 | 30 | 3 | — |
| B2 | | | | 21*** | 370 | 32 | 4.3 × |
| 7-63B2 | 0.9·15 | 10 | 1,000 | 18*** | 300 | 30 | 1.8 × |
| 7-103 | 0.9·15 | 2.9 | 885 | 10 | 400 | 45 | 3.5 × |
| 7-117 | 0.9·15 | 3.2 | 1,000 | 10.5*** | 256 | 26 | 3.3 × |
| 7-141 | 1.9·15 | 4 | 3,000 | 15 | 2,180 | 73 | 3.7 × |
| 8-57 | 3.4·15 | 5 | 15,000 | 10*** | 3,900 | 26 | 2 × |
| 8-113B1 | 1.9·15 | 3 | 2,300 | 5 | 200 | 9 | 1.7 × |
| B2 | | | | 23*** | 1,700 | 74 | 7.7 × |
| B3 | | | | 15 | 340 | 15 | 5 × |

* Starting material prepared by acetone fractionation and chromatography on DEAE-C.
** Elution carried out at pH 6 with a gradient to 1 *M* NaCl.
*** Minimum values for the potency. Higher values were also obtained, depending on the dose injected into the chicks.

*References p. 438.*

18 units/mg. This figure is a minimum value since in the case of preparation 7-63 (Table IV) a potency of 18-30 units/mg was found, depending upon the dose injected. The yields are calculated from the minimum values. Table IV summarizes a number of experiments of this type in which the yields were found to be quite variable due to differences between the slopes of the dose-response curves for the standard and the unknown. These bioassay difficulties are not encountered with less potent fractions that have not been submitted to chromatography on both DEAE-C and CM-C. Similar results are indicated in Table II, Expt. 8-87B2, in which a minimum value of 21 units/mg was found, but the maximum value was 36 units/mg.

## DISCUSSION

TSH and LH appear to be similar proteins. They are both adsorbed to IRC-50 or to CM-C at pH 6 and until the development of DEAE-C and its application to this problem, they could not be separated without using conditions where the risk of destroying TSH is considerable. ELLIS[18], for example, has employed metaphosphoric acid as a precipitating agent for recovering TSH from solution, leaving LH in the supernatant. However, in view of the inactivation or alteration of TSH in acid solution[7a], it seems preferable to separate the two hormones under as mild conditions as possible.

The elution patterns obtained in column chromatography on DEAE-C suggest strongly that TSH activity may be associated with two or more chemical components[8]. PIERCE, WYNSTON AND CARSTEN have prepared high-potency TSH by a combination of counter-current distribution and chromatography on DEAE-C, which displays two bands in starch gel electrophoresis[19],* both of which were active. ELLIS has reported that sheep TSH preparations contain two active components when placed on IRC-50 columns. It would appear that TSH, like other pituitary hormones, exists in more than one chemical form. Preparations 8-87B2 (Table II) and 7-37B2 (Table IV), which had potencies of over 20 units/mg, were examined for N-terminal amino acids by the technique of SANGER[20]. DNP-phenylalanine and DNP-threonine were found, together with traces of DNP-serine, when a 24-h hydrolyzate of the DNP-protein was examined by 2-dimensional paper chromatography. These same two preparations also displayed 2 or 3 bands in starch gel electrophoresis.

The TSH potency of the most active fractions recovered from DEAE-C and CM-C columns is at least 20 units/mg. Values as high as 50 units/mg have been obtained in the case of one fraction (8-113B2, Table IV). Several factors have to be taken into account, which affect the specific biological activity. The lower limit of response is about 2 milliunits total dose as shown by BATES AND CORNFIELD[10]. If the specific activity of a fraction is 20 units/mg, this corresponds to 0.1 $\mu$g of protein, which under the conditions of the assay is injected in a volume of 0.2 ml. At this level of protein concentration it has been found that in a 48-h assay, the solution will have lost its iodine-depleting ability by the second 24-h period unless an inert protein solution such as 0.1% bovine serum albumin is used to make up dilutions for assay. Another factor affecting the estimate of potency is that the slope of the dose response curve of the

* We wish to thank Dr. J. G. PIERCE for comparing two of our preparations with one of his potent fractions, by starch gel electrophoresis. In this comparison the two bands which he has found to be associated with TSH activity were observed in preparations from both laboratories.

*References p. 438.*

highly purified TSH which has been chromatographed on DEAE-C and CM-C is less than that of the standard preparation. Thus, at a dose level of 0.1 μg/day, the calculated potency may be 50 units/mg, but at a higher dose level of 0.5 μg/day, the calculated potency will be only 20 units/mg. Since variation of this sort is not encountered in fractions that have not been subjected to extensive purification by chromatography, it is clear that the highly purified material is less stable and has a different quantitative effect on iodine depletion in the thyroid than do the cruder fractions. Similar variation in the apparent potency of other pituitary hormones has been observed in a number of laboratories.

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