Studies on the purification of thyrotropin

Preparations of thyroid-stimulating hormone with an activity of 5 U.S.P. units/mg have been obtained by the use of cation-exchange chromatography[1,2]. CONDLIFFE AND BATES have followed cation-exchange chromatography with chromatography on an anion-exchange column of diethylaminoethylcellulose (DEAE-cellulose) which has resulted in preparation of material of 15–20 U.S.P. units/mg[3]. Use of the exchangers in reverse order has yielded similar results[4]. In this laboratory the use of an extraction based on countercurrent-distribution studies has allowed the separation of a pepsin inhibitor from the thyrotropic hormone[5,6]. It was noteworthy that during the extraction procedure the major portion of the thyrotropic activity was found to be concentrated at the interphase in a layer of insoluble material which had not lost specific activity.

It has now been found that with the extraction procedure one can concentrate thyrotropic activity to a stage of 5 units/mg without the necessity of chromatography, thus making possible the ready preparation of such material in gram quantities by batch procedures. When the extraction is followed by the anion-exchange chromatography of CONDLIFFE AND BATES[3], material of 20 U.S.P. units/mg can be readily obtained. Rechromatography of this material yields a fraction which assays 40 U.S.P. units/mg. Further evidence of purification by these steps has been obtained by the use of starch-gel electrophoresis[7].

The method of preparation has been carried out as follows. The thyrotropic activity from 2 kg of frozen beef-anterior pituitaries was concentrated to the stage previously used as starting material for chromatography (0.8 units/mg, 4–5 g yield). This material was shaken in a separatory funnel with *n*-butanol – 0.05 *M* *p*-toluenesulfonic acid exactly as described for removal of the pepsin inhibitor[5]. 165 ml of each phase were used per g of material. 1- or 2-g quantities were extracted with a yield of 100 mg of 5 units/mg material from each g. 200–300 mg batches of the 5 units/mg material were chromatographed on columns of DEAE-cellulose (1.9 × 20 cm) using 0.005 *M* glycine buffer, pH 9.5, and gradient elution[3]. Results similar to those of CONDLIFFE AND BATES were obtained except that little or no protein emerged beginning with the solvent front. After 300–400 ml of the starting buffer had passed through the column, a gradient to 0.1 *M* glycine was applied over 250 ml, followed by 250 ml of 0.1 *M* glycine, pH 9.5. Inactive material emerged first (with two or more components indicated), followed by the active fraction which appeared as a broad peak partially overlapping the peak representing inactive material. After dialysis and freeze-drying, 20–25 mg of material assaying approximately 20 U.S.P. units/mg were obtained from 200 mg of 5 unit/mg material. Further elution with 1.0 *M* NaH_2PO_4 yielded a small amount of material which assayed 5–10 U.S.P. units/mg. Fig. 1 shows typical results of rechromatography of 20 units/mg material under the same conditions. Following dialysis and freeze-drying, material indicated as fraction 1 assayed 5–10 U.S.P. units/mg, fraction 2 about 30 U.S.P. units/mg and fraction 3 about 40 units/mg. The overall yield of fraction 3 from 1 kg of pituitaries was 5–6 mg. When examined by starch-gel electrophoresis[7], the 5 unit/mg material was shown to contain at least six components, a result in marked contrast to that found with moving-boundary electrophoresis[5] and paper electrophoresis. Fig. 1 also shows the results with the starch-gel method of the 20 units/mg material and of fractions 1 and 3 obtained by rechromatography. In the case of the 40 units/mg material (fraction 3) one of the two principal bands seen in the 20 units/mg material has disappeared and one principal band remains, which, however, on close examination appears to consist of two components.

Biological activity was recovered[7] from the areas indicated by "a" in Fig. 1 and from an area comparable to "a" after electrophoresis of 20 units/mg material at pH 8.6 (borate buffer, ionic strength ($I/2$), 0.012). Experiments were carried out in which the two components indicated to be in fraction 3 were bioassayed separately. Within the experimental error of the resolution and removal of the two bands in the starch gel, both components appeared to be active. The band nearer the origin yielded 3–4 times more activity than the other (about 40–50% of the total activity applied to the gel was recovered). In contrast, electrophoresis of the middle fraction (fraction 2) showed only one band in the area comparable to "a". This band was also shown to contain about 40% of the total units placed in the gel. The mobility of this active band, however, appeared to be greater than that of the two active bands of fraction 3. It is not yet possible to determine whether the differences in mobility between fractions 2 and 3 and the two closely related active bands from fraction 3 represent slightly different or slightly degraded forms of thyrotropin or whether the active principle is still bound to several closely related proteins.

The method of bioassay used was the measurement of ^{32}P uptake into the thyroids of chicks[8]; 0.1% bovine plasma albumin was used in making dilutions for bioassay[9]. Within the limits of accuracy of the assay method, material which originally assayed 40 units/mg lost approximately half of its potency when stored as a solid in the cold for 4 weeks. Fraction 3 also gave the expected stimulation of the uptake of ^{131}I in chick thyroids[10]. Samples of fractions 1, 2 and 3, when assayed

on the basis of the weight response of beef-thyroid slices[11], had potencies of approximately 5, 40 and 50 units/mg, respectively*.

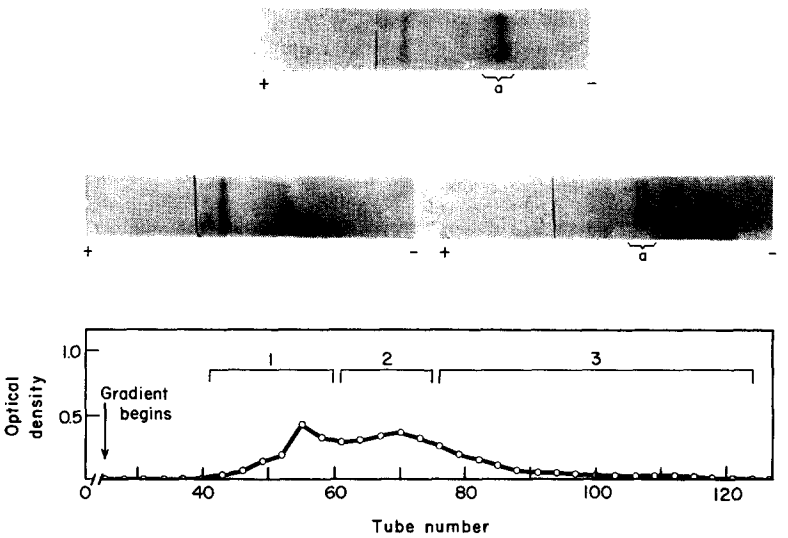

Fig. 1. Bottom: Rechromatography of 80 mg of a thyrotropin preparation (20 U.S.P. units/mg) on DEAE-cellulose (1.9 × 20 cm); 5 ml effluent collected per tube at 3°. Effluent curves were determined as previously described[1]. Yield of fraction 1, 20 mg; fraction 2, 27 mg; fraction 3, 15 mg. Top: Starch-gel electrophoresis of thyrotropin preparations in acetate buffer, pH 5.0, $\Gamma/2$ 0.012. Gels 2.5 cm wide, 0.5 cm deep. Above – 0.75 mg of starting material for rechromatography, 20 U.S.P. units/mg; left – 0.75 mg of fraction 1, 5–10 U.S.P. units/mg; right – 0.75 mg of fraction 3, 40 U.S.P. units/mg. Constant current of 11 mA, with approximately 260 V across the gel, was applied for 3–4 h.

The authors wish to acknowledge the support of the United States Public Health Service (grant No. C-2290).

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1 J. G. PIERCE AND J. F. NYC, *J. Biol. Chem.*, 222 (1956) 777.
2 P. G. CONDLIFFE AND R. W. BATES, *J. Biol. Chem.*, 223 (1956) 893.
3 P. G. CONDLIFFE AND R. W. BATES, *Arch. Biochem. Biophys.*, 68 (1957) 229.
4 P. G. CONDLIFFE, R. W. BATES AND R. M. FRAPS, *Abstract Am. Chem. Soc. Meeting*, Sept. 1957, No. 192, p. 800.
5 M. E. CARSTEN AND J. G. PIERCE, *J. Biol. Chem.*, 229 (1957) 61.
6 J. HILLIARD AND P. M. WEST, *Endocrinology*, 60 (1957) 797.
7 O. SMITHIES, *Biochem. J.*, 61 (1955) 629.
8 F. S. GREENSPAN, J. P. KRISS, L. E. MOSES AND W. LEW, *Endocrinology*, 58 (1956) 767.
9 R. W. BATES AND P. G. CONDLIFFE, personal communication.
10 J. P. KRISS, F. S. GREENSPAN, W. H. GARNES AND W. LEW, *Endocrinology*, 59 (1956) 555.
11 J. L. BAKKE, M. L. HEIDEMAN, JR., N. L. LAWRENCE AND C. WIBERG, *Endocrinology*, 61 (1957) 352.

Received January 8th, 1958

* We wish to thank Dr. J. L. BAKKE and Mrs. NANCY LAWRENCE for the assay by the weight response of bovine thyroid slices.
** Fellow in Cancer Research of the American Cancer Society.