lysis than with fibrinogen lysis and to be especially applicable in the diagnosis of defibrination syndrome.

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Purification of Bovine Thyroid-Stimulating Hormone by Solubility Fractionation with Ammonium Sulfate*

(33999)

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Ammonium sulfate fractionation has been a useful method for purifying proteins but has achieved only limited success when applied to TSH. Fraenkel-Conrat and co-workers (1) as well as Albert (2) incorporated ammonium sulfate precipitation of TSH in early procedures which provided low yields of activity. Bergman and co-workers (3, 4) also employed ammonium sulfate for the recovery of TSH from solution. However, none of these techniques provided a preparation suitably high in biological activity. Heideman's introduction of ion exchange chromatography for TSH purification (5) has led to preparations by Bates and Condliffe (6) and by Pierce and Carsten (7) with potencies of 20–60 U/mg of protein. Since substantial losses of biological activity were encountered during ion exchange chromatography, these investigators utilized simplified methods for preliminary purification of TSH. Bates and Condliffe used the alcohol percolation technique developed by Bates *et al.* (8) while Pierce and Carsten (7) employed an interphase precipitation technique. The most spectacular achievement with respect to TSH purification was recently reported by Condliffe and Jakoby (9) who crystallized bovine TSH by extracting a highly purified preparation with decreasing concentrations of ammonium sulfate. This suggested to us that the use of ammonium sulfate techniques might also be valuable in the preparation of highly purified TSH. The present paper gives the results of a study conducted on solubility fractionation of TSH with ammonium sulfate as a means of purifying the hormone.

Materials and Methods. General. Two batches of crude bovine TSH (0.49 and 0.84 μ/mg), prepared by the method of Ciereszko (10), were purchased from Princeton Laboratory Products Company, Princeton, New

* An abstract of part of this study appeared in Clin. Res. 16, 37 (1968).

Jersey and used as starting material for further purification. Ammonium sulfate, Baker and Adamson reagent grade, was obtained from Allied Chemical, diethylaminoethyl cellulose (DEAE-cellulose) in the microgranular form (Whatman DE-52) from Reeve Angel, Clifton, New Jersey and tris (hydroxymethyl)aminomethane (Tris) from Calbiochem.

Purification by fractional precipitation. Usually 1 g of Ciereszko TSH was dissolved in 8 ml of 0.5 *M* Tris, pH 9.2, at room temperature with the aid of a magnetic stirrer; and 12 ml of 50% ammonium sulfate (w/v) in 0.5 *M* Tris, pH 9.2, were added slowly to give a final ammonium sulfate concentration of 30%. Then the suspension was stirred for 30 min at 24°, centrifuged at 0° for 30 min at 15,000*g*, and the supernatant was decanted. The residue was dissolved in buffer and 50% ammonium sulfate was added to produce a final ammonium sulfate concentration of 25% in a volume of 20 ml. The precipitate and supernatant were recovered as described above. This procedure was repeated with progressively lower ammonium sulfate concentrations until supernatants were obtained after treatment of the dissolved residues with 30, 25, 15, 10, and 0% ammonium sulfate. Sufficient crystalline ammonium sulfate was then added to each supernatant to a final concentration of 50%, the mixture was stirred for 10 min, and the precipitated protein was recovered after centrifuging at 0° for 30 min at 15,000*g*. The ammonium sulfate was carefully drained from the tubes and the protein was reserved for future use.

Purification by ammonium sulfate extraction. One g of crude Ciereszko TSH was dissolved in 0.5 *M* Tris buffer, pH 9.2, and 50% ammonium sulfate (w/v) in buffer was added to achieve a 30% concentration in 25.0 ml. The mixture was stirred with a magnetic stirrer at room temperature for 30 min and centrifuged in the cold at 15,000*g*. The residue was then extracted with 25 ml of 30% ammonium sulfate. Sequential extraction of the undissolved residue was carried out with 30, 25, 15, 10, and 0% ammonium sulfate and each extract was brought to a 50% concentration with crystalline ammonium sulfate. The protein from each fraction was recovered by centrifugation.

Purification from DEAE-cellulose with ammonium sulfate. Crude TSH was suspended in 30% (w/v) ammonium sulfate in Tris buffer and applied to a 1.5 × 20-cm column of DEAE-cellulose previously equilibrated with the ammonium sulfate–Tris buffer. The column was eluted sequentially with 25-ml volumes of solutions containing decreasing concentrations of ammonium sulfate in Tris buffer. The eluates were brought to a 50% ammonium sulfate concentration, and the protein in each fraction was recovered and assayed. In a modification of this procedure, the column was eluted with a linear gradient of ammonium sulfate in Tris buffer decreasing from 50 to 0%.

Column chromatography. TSH-rich protein obtained by ammonium sulfate precipitation was dialyzed 18 hr at 5° against 0.005 *M* sodium glycinate buffer, pH 9.5. Approximately 20,000 cpm of highly purified bovine TSH labeled with ^{125}I (32 U/mg containing 100 μCi/μg) was added to the dialyzed protein (about 23 ml) as a marker. The mixture was applied to a 2.4 × 30-cm column of DEAE-cellulose previously precycled and equilibrated with 0.005 *M* glycinate buffer, pH 9.5. The column was eluted at 5° with 150 ml of 0.005 *M* glycinate buffer followed by 126 ml of a linear gradient of 0 to 0.02 *M* NaCl in 0.005 *M* glycinate buffer, pH 9.5, then 325 ml of a linear gradient of 0.02 to 0.5 *M* NaCl in 0.005 *M* glycinate buffer.

TSH. The bioassay of TSH was performed by a modification of the McKenzie mouse assay (11). For determinations of specific activity, the protein content of each fraction was measured by the method of Lowry *et al.* (12) using rabbit gamma globulin as a standard. Highly purified beef TSH (kindly provided by Dr. John G. Pierce) was labeled with ^{125}I by the procedure of Greenwood *et al.* (13) and purified by passage through a 1 × 50-cm column of Sephadex G-100 before each use.

Results. The TSH precipitated with 30% ammonium sulfate was placed on a DEAE-

TABLE I. Purification of TSH by Eluting DEAE-cellulose with Decreasing Concentrations of Ammonium Sulfate.[a]

Concentration of $(NH_4)_2SO_4$ (w/v)[b]	TSH activity (U/mg of protein)	TSH recovered (U/fraction)
30%	NR	—
25	2.0	382.8
22	2.3	139.5
20	4.6	99.2
18	4.3	66.5
15	2.0	31.8
10	1.4	8.3
0	0.4	4.3
Total		732.4

[a] A total of 629.4 units of TSH, with a specific activity of 0.63 U/mg was applied to the column.

[b] Volumes of 25 ml of each concentration were passed through the column and collected.

cellulose column and batch eluted with 25 ml of each of several ammonium sulfate solutions progressively lower in salt concentration. The results are presented in Table I and are representative of those obtained in 8 experiments. TSH activity appeared in all of the eluates but the highest specific activity TSH was extracted in the 18 and 20% ammonium sulfate fractions. The increase in specific activity in these fractions over the starting material represents approximately a 7-fold purification. Recovery of biological activity in the 6 fractions was 116.4% of that applied with approximately 25% representing the more highly purified TSH. Similar results were achieved with smaller amounts of protein on 1 × 5-cm columns eluting with 10.0-ml volumes of ammonium sulfate. A modification of the extraction technique was investigated in which elution of the column was carried out with a linear gradient of ammonium sulfate of decreasing salt concentration. Purification of TSH was inferior to the batch elution method with the added problem of locating and assaying TSH in many fractions.

Batch extraction of TSH in a centrifuge tube was performed with decreasing concentrations of ammonium sulfate (Table II). All of the original biological activity (111%) was recovered in the extracts of the 30% ammonium sulfate precipitate. The 15% extract, containing the protein not soluble in 25% ammonium sulfate but soluble in 15%, contained protein with the greatest TSH activity. The increase from 0.84 to 4.7 U/mg represents a purification of approximately 5-fold and was consistently reproduced in numerous experiments. This high activity TSH accounted for 81% of the original activity in the preparation. Total recoveries in numerous experiments ranged from 90 to 100% with the TSH-rich fraction accounting for 60–80% of the total biological activity.

In a critical examination of solubility fractionation of proteins by solvents of decreasing concentration, Zittle and DellaMonica (14) found that the ammonium sulfate concentration, at which each protein passed into solution corresponded very closely to the concentration precipitating the protein in batch precipitation experiments. A modified batch precipitation method was explored as a possible purification procedure with the results shown in Table III. Tracer amounts of ^{125}I-labeled TSH were added to this preparation to provide a second means of determining TSH-rich fractions and total recovery. The protein precipitated with 30% ammonium sulfate contained all of the TSH as demonstrated by the total recovery of TSH activity and ^{125}I-labeled TSH. The most potent TSH was obtained in the 15% ammonium sulfate su-

TABLE II. Purification of TSH by Ammonium Sulfate Extraction.[a]

Concentration of $(NH_4)_2SO_4$ (w/v)[b]	TSH activity (U/mg of protein)	TSH recovered (U/fraction)
30%	0.6	102.2
25	0.4	11.0
15	4.7	668.7
10	1.9	138.0
0	NR	—
Total		919.9

[a] For extraction 985 mg of 0.84 U/mg of TSH (827.4 U) were used.

[b] Extraction was carried out with 25.0 ml of each concentration of ammonium sulfate in 0.5 *M* Tris buffer, pH 9.2.

TABLE III. Distribution of ^{125}I-Labeled Hormone and Biological Activity of TSH Purified by Fractional Precipitation with Ammonium Sulfate.[a]

Concentration of $(NH_4)_2SO_4$ (w/v)[b]	TSH in supernatant		^{125}I-TSH recovered
	(U/mg of protein)	(U/fraction)	(cpm/fraction)
30%	0.2	57.0	1352
25	2.1	460.0	4121
15	6.0	1680.0	31,341
10	2.5	198.8	2525
0	0.6	1.9	644
Total		2397.7	39,983

[a] The starting material consisted of 2.47 g of TSH with an activity of 0.84 U/mg (total, 2075) with 40,200 cpm of ^{125}I-TSH (100 μCi/μg) added.

[b] Supernatants were obtained from 20.0 ml of each concentration of ammonium sulfate in 0.5 *M* Tris buffer, pH 9.2.

pernatant indicating that TSH was predominately precipitated by 25% ammonium sulfate but was not precipitated at 15%. This TSH-rich fraction contained 81.0% of the total biological activity (78.4% of the added ^{125}I-TSH) representing slightly more than a 7-fold increase in specific activity. Total recovery of TSH as indicated by biological activity and by ^{125}I-TSH was essentially 100%. These results are similar to those obtained with the batch extraction procedure with the degree of purification and high recoveries being essentially the same.

Attempts to further purify 2–4 U/mg TSH by subjecting the protein to a second series of extraction or precipitation steps have met with very limited success. In most instances no increase in biological activity was observed; however, in some experiments an additional 2-to 3-fold purification could be obtained. Although relatively large amounts of starting material were used in the experiments presented, purification with the extraction and with the modified precipitation techniques was equally as successful with 60–200 mg of protein using reduced volumes.

Chromatography of ammonium sulfate-purified TSH on DEAE-cellulose resulted in further separation of protein and biological activity as presented in Fig. 1. Tracer amounts of ^{125}I-TSH were used as an aid in locating the TSH peak. The peak of biological activity was slightly sharper than the radioactive peak and was composed of protein with a potency of 12–13.5 U/mg. This represents an additional 5-fold increase over the 2.58 U/mg TSH applied to the column.

Discussion. The results of this investigation demonstrate that solubility fractionation of Ciereszko TSH with ammonium sulfate is a valuable technique for obtaining a 5-to 7-fold purification of the hormone. The potency of this material usually exceeds that which is available from the Endocrinology Study Section, NIH and is comparable to

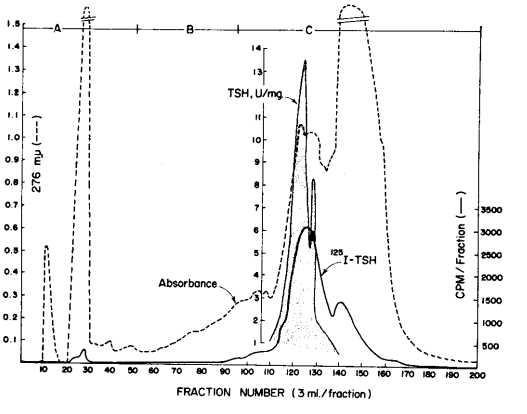

FIG. 1. Purification of TSH by chromatography on DEAE-cellulose: TSH (428.2 mg of 2.58 U/mg) was applied to a 2.4 × 30-cm column of microgranular DEAE-cellulose. The column was eluted at 5° with (A) 150 ml of 0.005 glycinate buffer, pH 9.5, followed by (B) 126 ml of a linear gradient of 0 to 0.02 *M* NaCl in 0.005 *M* glycinate buffer, pH 9.5, then (C) 325 ml of a linear gradient of 0.02–0.5 *M* NaCl in 0.005 *M* glycinate buffer, pH 9.5.

that achieved with the Pierce interphase or Bates' percolation procedure. The results obtained with three variations in solubility fractionation techniques indicated that approximately the same degree of purification was accomplished by each but that lowest recoveries of the higher potency TSH occurred when extraction was carried out on a DEAE-cellulose column. The precipitation and extraction techniques carried out in test tubes resulted in a similar degree of purification and in recovery of from 60 to 80% of the total TSH in the high potency fraction.

The solubility fractionation techniques investigated met with very limited success in producing a highly purified TSH (10 U/mg or higher). Only an occasional small scale purification yielded TSH with a specific activity greater than 8 U/mg. In addition, repeated fractionation never produced protein with biological activity exceeding 8.0 U/mg. The primary importance of the solubility fraction techniques lies in the simplicity of the methods, the speed with which they may be done and the excellent recoveries of higher potency TSH. The TSH obtained in this way is then subject to further purification by DEAE-cellulose chromatography yielding highly purified hormone.

Contamination of the ammonium sulfate purified fractions by other pituitary hormones was not investigated; thus no information is available concerning the presence or potency of other biologically active polypeptides.

One of the problems encountered in solubility fractionation and column chromatography of TSH is the location of the fractions containing the biologically active material. The addition of tracer amounts of ^{125}I-TSH to the preparation undergoing purification provides a convenient method for the location of TSH-rich fractions. The amount of radioactive TSH added (approx 2×10^{-4} μg containing 6 μU) was far below the limits of detection by the bioassay and did not interfere with immunoassay of appropriately diluted fractions. The elution characteristics of the labeled and unlabeled TSH from DEAE-cellulose, however, indicate that the peaks, although very similar, are not identical.

Summary. Solubility fractionation of relatively crude TSH with ammonium sulfate was investigated as a means of purifying the hormone. The results obtained with three variations in solubility fractionation techniques using decreasing concentrations of ammonium sulfate demonstrated that each method yielded a 5- to 7-fold purification of the 0.49 to 0.84 U/mg starting material. Total recovery of TSH was 90–100%. Recoveries in the high potency fractions were 60–80% when TSH was extracted with ammonium sulfate or recovered by fractional precipitation and 25–35% when batch eluted from diethylaminoethyl (DEAE) cellulose. Solubility fractionation appears to be a simple and rapid method for the purification of TSH to 4–6 U/mg which may be adequate for most purposes. Highly purified TSH may then be obtained by standard column chromatography with DEAE-cellulose.

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In Vitro Incorporation of Formate-^{14}C, Glycerol-1,3-^{14}C, Choline-1,2-^{14}C, Serine-1-^{14}C, and Ethanolamine-1,2-^{14}C into the Major Phospholipids of Human Peripheral Arteries* (34000)

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Results of investigations into the synthesis of phospholipids from acetate-^{14}C and phosphate-^{32}P precursors in rat and rabbit aortas have been summarized in a previous publication (1). Synthesis of total phospholipids by the human arterial wall has been demonstrated *in vivo* from phosphate-^{32}P (2) and *in vitro* from acetate-^{14}C (3, 4), but thus far the incorporation of other precursors into the individual phospholipid classes of human arteries has not been studied.

Materials and Methods. Above the knee amputated legs were obtained immediately after surgery, and the distal femoral artery, and popliteal and tibial arteries were dissected free. Twenty specimens were obtained, all of which were from male patients, ages 60–80 and all of which showed advanced occlusive atherosclerotic changes. The arteries were freed of adventitia, placed in an isotonic Krebs–Ringer solution, and after addition of 20 μC of either formate-^{14}C, glycerol-1, 3-^{14}C, choline-1,2-^{14}C, serine-1-^{14}C, or ethanolamine-1, 2-^{14}C (Nuclear Chicago, adjusted to specific activity of 1.03 mC/mM) to each, the flasks were incubated for 4 hr in an air atmosphere at 37.5° in a Dubnoff metabolic shaking incubator. A total of four incubations were done with each ^{14}C labeled precursor. Carbon dioxide was collected in a center well containing 20% NaOH, and the $^{14}CO_2$ radioactivity was determined as previously described (5).

Washing the tissues free of radioactive precursor, extraction of the lipids, and separation of the phospholipids by thin-layer chromatography were all done as previously described (5, 6). Because of the small amounts and impurity of the phosphatidyl inositol fraction, this phospholipid was not included in the present study. After elution of the phospholipid fractions from the silica gel (6), aliquots were analyzed for phosphorous content (7). Other aliquots were assayed for radioactivity using a PPO–POPOP in toulene scintillator and a Packard model 3314 liquid scintillation spectrometer (5).

Results and Discussion. The amounts of the individual phospholipids (mg/g of dry wt of artery ± SD) were as follows: lysophosphatidyl choline, 0.4 ± 0.1; sphingomyelin, 2.6 ± 0.8; phosphatidyl choline, 3.4 ± 0.7; phosphatidyl serine, 1.1 ± 0.3; and phosphatidyl ethanolamine, 1.8 ± 0.4. Incubation with the different labeled precursors did not significantly affect the amounts of any of the phospholipid classes. Sphingomyelin was high in amount but showed the lowest degree of incorporation with all the precursor compounds.

The $^{14}CO_2$ evolved (dpm/mg of dry wt of artery) was as follows: formate-^{14}C, 1730 ± 370; glycerol-1, 3-^{14}C, 860 ± 115; choline-1, 2-^{14}C, 122 ± 25; serine-1-^{14}C, 145 ± 38; and ethanolamine-1, 2-^{14}C, 36 ± 12.

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