Preliminary fractionation of parathyroid extract with ammonium sulfate*

A simple method for concentration of the hormonal activity of a hot-dilute-acid extract of bovine-parathyroid tissue is reported in this communication. By this method, which involves step-wise precipitation with ammonium sulfate, a considerable portion of protein with low activity can be removed from the extract, and material with a 3- to 4-fold increase in specific activity has been obtained in moderately good yield.

The initial extracts were prepared by a modification of the classical hot-dilute-HCl treatment[1,2]. A mixture of 1 part by weight of bovine parathyroid tissue,** finely ground in the frozen state, and 10 parts of 0.1 *N* HCl containing 0.32 g disodium ethylenediaminetetraacetate (EDTA)***/l was warmed for 5 min in a hot water bath at 80°–90°, then heated in a boiling water bath for 15 min with very vigorous mechanical stirring. It was immediately centrifuged at 2000 rev/min in a refrigerated centrifuge set at 4°. The disk of solid lipid that formed at the top of each centrifuge bottle was carefully pried loose at the edges so that it remained entire, and the turbid brown fluid underneath was decanted away from the sedimented residual tissue. It was filtered with suction through Whatman No. 40 paper on a Büchner funnel in a cold room at 4°, the paper being changed after the addition of each 50–100 ml of extract.

The remaining operations were conducted at 4° in a cold room or in a refrigerated centrifuge. An amount of powdered $(NH_4)_2SO_4$ (recrystallized with EDTA) sufficient to bring the solution to 0.1 saturation was added slowly from a mechanical hopper[3] to a known volume of the filtrate, with mechanical stirring, which was continued for 1–2 h after addition of the $(NH_4)_2SO_4$, and the mixture was then centrifuged at 2000 rev/min for 20 min. The process of precipitation of the supernatant with $(NH_4)_2SO_4$ was continued step-wise, collecting precipitates at 0.2, 0.3, 0.4 and 0.6 saturation. The fraction that precipitated between 0.3 and 0.4 saturation was allowed to stand 24 h before centrifugation. The precipitates were transferred with a small volume of water to cellophane bags and dialyzed with internal and external stirring for 16 h against 3–4 l water. The contents of the bags, largely suspended precipitates, were brought into solution by the drop-wise addition of 1.5 *N* HCl to pH 3. The solutions were stored in a freezer without apparent loss of biological activity.

The fractions were tested for biological activity by determination of the serum Ca 6 h after subcutaneous injection into acutely parathyroidectomized rats[4,5] at a dose level of 1 mg N per rat. Table I shows the distribution of N and of biological activity for two repetitions of the procedure. Although all the fractions possessed some biological activity, a major portion of the relatively inert material had been precipitated at 0.2 saturation. In prep. *a*, Fraction 4, precipitated between

TABLE I

CONCENTRATION OF HORMONAL ACTIVITY BY FRACTIONATION WITH $(NH_4)_2SO_4$

Fractionation procedure and biological test as described in text. Fractions 1–5 represent the precipitates obtained at the $(NH_4)_2SO_4$ concentration indicated. The values for serum Ca represent the means of groups of 4–5 rats. The mean serum Ca of control rats was 5.6 mg%. N was determined by the method of LOWRY *et al.*,[8] using Armour protein standard. The amount of parathyroid tissue extracted was 60 g in *a* and 30 g in *b*.

Preparation	*$(NH_4)_2SO_4$ saturation*	*Serum Ca (mg %)*		*% of total N in extract*	
		a	*b*	*a*	*b*
Initial hot-acid extract		8.6	7.8	(100)	(100)
Fraction 1	0–0.1	7.6	7.6	31	48
Fraction 2	0.1–0.2	8.1	9.5	22	6
Fraction 3	0.2–0.3	7.7	9.1	19	17
Fraction 4	0.3–0.4	9.8	10.5	4	4
Fraction 5	0.4–0.6	—	7.2	—	10

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*** This concentration of EDTA was found to be neither essential nor deleterious to the activity of the extract.

0.3 and 0.4 saturation, was significantly ($P<0.05$) more potent than all the others. This finding was essentially confirmed in prep. *b*, although here the superiority of Fraction 4 was not so clearly demonstrated ($P = 0.2$). As a preparative procedure, the material precipitating before the collection of this active fraction was removed in two rather than three steps, at 0.1 and 0.3 saturation. Seven such batches of Fraction 4, each prepared from 60 g of ground glands, were tested in rats independently. At a uniform dose of 1 mg N, the average serum calcium was 9.8 mg %, tending to confirm the relatively high potency of Fraction 4. A pool (PFP-1) of five other such products* was biologically assayed more extensively in parathyroidectomized rats, according to the method of MUNSON *et al.*[4,5], using as standard a commercial extract** containing 100 USP units/ml. From the combined data of three assays, the potency was estimated[6] to be 122 units/mg N, representing an increase in specific activity over the initial extract of approximately 3–4 fold, and a yield of about 30%. (The standard error of the estimate was + 34 or — 27 units.)

TABLE II

DISTRIBUTION OF BIOLOGICAL ACTIVITY BETWEEN PRECIPITATE AND SOLUBLE FRACTION AT pH 6.5

5 to 7 ml of each preparation in solution at pH 2.0 was brought to pH 6.5 by the dropwise addition of 0.2 *N* NaOH, then allowed to stand for 2 h at 4°. The tube was centrifuged at 4° and 4000 rev/min for 20 min. The precipitate was dissolved in the original volume of 0.1 *N* HCl. The doses and conditions of biological test were the same as in Table I.

	Serum Ca, mg %		
	Prep. I	*Prep. II*	*Prep. III*
Soluble fraction	8.2	9.2	8.6
Precipitate	10.7	11.3	10.4

A limited amount of additional information about Fraction 4 has been accumulated. It has been lyophilized without apparent loss in specific activity. On gradual addition of base to a solution of the preparation containing 1.2–1.4 mg N/ml, a precipitate began to form at pH 5, and remained largely undissolved to pH 10. Nevertheless, a measurable proportion of activity was in solution at pH 6.5. The specific activity of the precipitate was significantly ($P<0.01$) greater than that of the soluble portion (Table II). When centrifuged at pH 3.5 in a Spinco Model L ultracentrifuge at 100,000 × *g* for 17 h, only 7% of the N was sedimented, and the sediment showed no increase in specific activity. On paper electrophoresis for 17 h in 0.1 *M* phthalate buffer at pH 3.5, using a potential of 150 V, three fractions with low mobility moved toward the cathode, the bulk of the material remaining stationary. Fraction 4 contained phosphaturic activity[7] as well as Ca-mobilizing activity. The further purification of Fraction 4 is in progress.

Biological Research Laboratories and Department of Pharmacology, School of Dental Medicine and Medical School, Harvard University, Boston, Mass. (U.S.A.)

SAMUEL FRIEDMAN
PAUL L. MUNSON

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