weight is 9.3 mg in Vit. D deficient cortisol fed rat in comparison with 5.9 mg in comparable control rats. Following Vit. D administration this value is 12.9 mg in cortisol fed rats and 8.1 mg in controls. Suppression of body weight increment by cortisol is thus not accompanied by proportionate decrease of bone salt mass. Histological sections indicate that cartilage cell proliferation is inhibited by cortisol, and also that osteoblasts and osteoclasts are less prominent. The cellular activity of bone tissue seems to have been checked so that resorption as well as formation of bone is depressed. In bones of Vit. D treated rats not only has calcification of proliferative cartilage and osteoid been initiated but also the inhibitory effect of cortisol on bone cell activity appears to have been overcome.

Summary. Correlation of the effect of Vit. D in augmenting concentrations of citrate in plasma and bone with its antirachitic action has been studied. Rats were made rachitic by feeding a Vit. D deficient diet which was also low in available phosphorus. Addition of cortisol to the diet of such rats reduced concentration of citrate in serum and also blocked the effect of Vit. D in increasing serum and bone citrate levels. Antirachitic action of Vit. D as measured by rise of serum phosphorus concentrations and by histological evidences of calcification of rachitic cartilage and osteoid was not suppressed by cortisol. The antirachitic action of Vit. D and its effect upon citrate metabolism can, therefore, be separated. The tibias of Vit. D deficient cortisol fed rats show evidences of increased calcification in comparison with rachitic controls which might in part be due to inhibition of bone resorption as well as retardation of cartilage growth by cortisol. This increased calcification in cortisol fed rats is associated with extreme depletion of extracellular phosphate.

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Rapidly Sedimenting Properties of Specifically Precipitating Component of a Hashimoto's Disease Serum. (23604)

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Evidence has been presented that chronic thyroiditis in humans may be the result of an autoimmunization process in which an individual produces antibodies against tissue components of his own thyroid. Thus, Rose and Witebsky have shown that thyroiditis similar to that in humans can be produced in dogs and rabbits by injecting the animal with a thyroid extract, either of other thyroids of the same species or even a portion of the individual's own thyroid(1,2). Roitt, Doniach, Campbell and Hudson have reported, moreover, that the serum of patients with Hashimoto's disease contains materials capable of

precipitating with human thyroid extract(3). Witebsky, Rose, Terplan, Paine and Egan have shown that the serum of patients with chronic thyroiditis agglutinates tanned sheep red cells coated with human thyroid extract (4). Presumably, these effects are due to antibodies produced against the patient's own thyroid. That patients with Hashimoto's disease have a high gamma globulin has also been reported(5), and this fits in with the thesis of autoantibody production.

We have had occasion to confirm the observation that serum from a patient with chronic thyroiditis diagnosed as Hashimoto's disease gives a precipitin reaction with an extract of normal human thyroid. Further studies of this serum were made which involved quantitative precipitation reactions, Ouchterlony double diffusion of serum and thyroid extract on agar plates, paper electrophoresis and ultracentrifugation studies. Our most significant finding has been that the antibodies reside in the heavy globulin fraction rather than the light globulin.

Materials and Methods. The serum used was that obtained from one patient at thyroidectomy. Her thyroid was reported by the pathology laboratory to be a typical example of Hashimoto's disease. A second portion of serum was obtained six months postoperatively. The patient was of blood group A. Clinical laboratory studies revealed a radioactive iodine uptake of 9%, B.M.R. of 10, thymol turbidity of 17 units; clinical gamma globulin determination of 2.7 units (normal 1.1). Thyroid extract was prepared from normal gland obtained at autopsy by homogenizing one part of wet tissue in five parts of 0.9% saline. The homogenate was permitted to stand overnight at 5° and was then centrifuged. The supernate was cleared by filtration and the extract was used in most of these experiments. In a few experiments involving agar diffusion, more concentrated extracts were prepared using one part wet tissue to either equal or four parts of saline. Borate buffer used was of pH 8.0 and ionic strength 0.16 M. It was made by dissolving 10.3 g boric acid, 7.8 g sodium chloride, and 1.1 g sodium hydroxide in water to 1 liter volume. Protein concentrations of the solutions used were determined by micro-Kjeldahl analysis. The protein in the precipitates obtained in the precipitin reaction was determined as follows: Precipitates were washed once with 3 ml borate buffer and twice with 3 ml saline and then dissolved in 0.02 N NaOH. The solutions were transferred to the calibrated micro Beckman cells and diluted to 0.4 ml with additional sodium hydroxide. Readings were made in a Beckman DU spectrophotometer at 280 mμ(6). A conversion factor of 1 optical density unit = 0.775 mg of protein per ml based on the nitrogen content of human gamma globulin solutions times 6.25 as obtained by Kjeldahl analyses was applied. The conversion factor for thyroid extract was the same as for globulin in borate buffer and in 0.02 N NaOH. Ouchterlony double diffusion plates were prepared according to the modification described by Korngold(7). Four stainless steel penicillin assay cylinders were arranged at the corners of a square around the fifth central cylinder at a distance from the central cylinder of 2.5 cm. Merthiolate (Lilly) 1 part/10,000 was used in the agar as a bacteriostatic agent. The reservoirs were filled with 0.2 ml of serum or extract and incubated at 25°.

Results. Precipitin reaction. When portions of the Hashimoto's disease serum were added to successive dilutions of thyroid extract, a typical precipitin curve was observed. The amounts of precipitate formed at optimum were of the order of 3 mg/ml of serum. The amount of precipitate obtained passed through an optimum and when the supernates were tested with additional antibody or antigen, additional precipitates were observed in the classical antigen excess or antibody excess regions. Serum taken six months postoperatively gave no precipitate with thyroid extract. Neither did normal serum give precipitate with extract.

Ultracentrifuge studies. The sera obtained at thyroidectomy and six months later were analyzed ultracentrifugally in an analytical Spinco ultracentrifuge (Type E). Pictures were taken at 4 minute intervals and showed a large number of components with negative s-values (rapidly rising components) in the Hashimoto's disease serum, presumably lipo-

TABLE I. Protein Precipitated from Hashimoto's Disease Serum and Serum Fractions by Thyroid Extracts. 0.2 ml serum fraction and 0.2 ml of thyroid extract used.

Serum fraction	Serum protein used, μg	Thyroid extract added, μg protein: 34	69	137	274
		Protein precipitated, μg*			
Upper layer	3000	4	1	6	4
Lower "	3600	12	28	60	49
Pellet	1800	20	41	59	22
Hashimoto serum ⅓ conc.	5000	23	60	126	175

* Results are avg of duplicate tubes. Values are amt of precipitate above control values of 11, 12, 8, and 10 for serum fractions and saline as listed.

protein, even though the patient had not eaten for 12 hours. Serum taken six months post-operatively did not show these negative s-value components.

To determine sedimentation property of the precipitating component, three 1 ml portions of serum were centrifuged at 60,000 rpm for 53 min in a Spinco analytical ultracentrifuge with a preparatory rotor and partition cell until the heavy globulin peak had just crossed the partition. This insured that all heavy globulin components were in the lower compartment. The light globulin boundary had just become visible at the surface. The upper and lower compartment solutions were removed and the pellet taken up in a minimum amount of saline. The corresponding fractions of the three runs were pooled and made up to 3 ml. These three fractions, upper compartment, lower compartment, and pellet along with a dilution of the original serum (⅓ original concentration) were tested for precipitation with thyroid extract. Precipitin tests were set up in duplicate with 0.2 ml of two-fold dilutions of thyroid extract and an equal volume of serum or serum fraction. Dilutions of antigen were selected on the basis of a preliminary precipitin test. Tubes were incubated at 36° for 1 hr. and then at 5° for 2 days. Protein analyses were run on precipitates (see experimental). The results are given in Table I. It can be seen that the bulk of the precipitate was obtained with the pellet and with the bottom compartment contents. These two fractions contained the rapidly sedimenting components. The upper phase which showed only a trace of precipitin contained essentially the same concentration of globulin as the bottom phase, but did not contain any of the heavy globulin. Therefore, the bulk of the precipitin activity must have been associated with the heavy components rather than with the normal antibody of molecular weight 160,000 and s value of about 7.0 S.

Ultracentrifuge study of the pellet and lower compartment fraction revealed appreciable quantities of rapidly sedimenting globulin in both fractions.

Relative gamma globulin contents of the various solutions used in the precipitin reaction (Table I) were determined by simultaneous paper electrophoresis analysis of these solutions and comparing the areas of the gamma globulin peaks. The areas showed that the upper, lower and pellet solutions as used contained 42, 57, and 53%, respectively, of the amount of gamma globulin present in the original serum of ⅓ concentration as used. This emphasizes that the differences in amounts of precipitate obtained with these fractions cannot be due to difference in gamma globulin contents.

Paper electrophoresis studies were also made on the serum obtained 6 months post-operatively and on normal control sera. It can be seen from the results in Table II that the gamma globulin/albumin ratio of the top, bottom and pellet fractions increase in that order. Moreover, the gamma globulin/albumin ratio of the original Hashimoto's disease serum was greater than that of the 6-month post-operative serum and of several normal human sera run simultaneously. This is in line with observations made by others(8).

Ouchterlony double diffusion technic. A line of precipitate between reservoirs containing serum and undiluted extract appeared after 12 days when 1.5% agar was used. The line formed was closer to the antibody than the antigen reservoir, indicating a slower diffusion of antibody than antigen. The slower diffusion was not due to a low antibody concentration (see precipitin reaction). This is further indication of the high molecular weight of the antibody. Reservoirs with con-

centrations of ½, ¼ and ⅛ of the original thyroid extract had been set up on the same plate and all these dilutions showed lines by the 17th day. In order to speed up diffusion rate, the agar concentration was dropped to 1.2%. Lines formed in 6 days and were essentially equidistant from the reservoirs. The rate of formation of lines in this system was much slower than in the case of the ovalbumin-rabbit anti-ovalbumin system where lines formed in three days on similar plates. This again is evidence for the slow diffusion of the precipitating component of human serum.

Single lines were the rule, although in a few cases double lines appeared. These may have been due to artifact. More concentrated thyroid extracts (1 part tissue to 4 parts saline or to 2 parts saline) were also tested. Both reacted similarly with Hashimoto's serum and gave only one precipitation line.

Ouchterlony test of the sera obtained 6 months post-operatively gave a faint line in one run but no lines were observed in other attempts. Precipitin reactions using whole serum were negative.

To test the specificity of the reaction against other human sera, plates were prepared with undiluted thyroid extract in the center reservoir and a Hashimoto's disease serum and samples of normal sera of blood groups O, A, B and AB in the other reservoirs. Precipitation did not occur with any of the sera tested except that from the Hashimoto's disease patient.

TABLE II. Relative γ-Globulin/Albumin Ratios* for Hashimoto's Disease Serum as Determined by Paper Electrophoresis.

	γ-globulin / Albumin
Upper layer	.88
Lower "	1.18
Pellet	2.57
Original whole serum	1.09
Serum after six mo	.70
Normal serum No. 1	.78
" " " 2	.60
" " " 3	.36

* These ratios were calculated from relative areas of the peaks of the densitometer tracing of dyed paper electrophoresis strip and are not absolute values due to trailing errors inherent in paper electrophoresis. Correction has been made for the more intense staining of albumin by the dye on an equal weight basis. Human albumin is stained to an intensity of 1.4 times that of human globulin. A Spinco paper electrophoresis apparatus was used. Veronal buffer pH 8.6; $\Gamma/2 = 0.075$; 75 V; 16 hr at room temp.; stained with bromophenol blue and scanned with an Analytrol photoelectric scanner.

Discussion. In normal human serum ultracentrifugation schlieren patterns(9), there are generally 2 well resolved globulin peaks. The larger peak corresponds to gamma globulin with an s value of about 7.0 S and the smaller peak to gamma globulin of higher molecular weight with an s value of approximately 19-20 S. In Hashimoto's disease serum, we have found that the serum components, which are precipitated by thyroid extract and which are considered to be auto-antibodies, are associated with the heavy globulin fraction rather than the light globulin fraction and these are therefore in the class of the complete Rh antibodies(10) and some of the antibodies of the Wasserman type(11).

That they are of the heavy fraction is evidenced by two types of experiments. Ultracentrifuge studies showed that the precipitating protein sedimented with the components of value 20 S. Ouchterlony double diffusion experiments also indicated that the antibody was of a high molecular weight and slow in diffusing since the lines in this system were slow in forming.

Summary. Serum was obtained from a patient with Hashimoto's disease at thyroidectomy and six months post-operatively. The first serum was found to form a precipitate on the addition of thyroid extract as has been described by others. On ultracentrifugation, it was found that the precipitating components were in the heavy globulin fraction ($s = 20$ S) rather than in the light globulin fraction ($s = 7$ S). Double diffusion experiments by the Ouchterlony method also indicated that the antibody was of high molecular weight. The antibody seemed to have disappeared from the serum within six months after surgery.

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Stimulation of ACTH-Release in Humans by Non-Pressor Fraction from Commercial Extracts of Posterior Pituitary.* (23605)

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Isolation of a purified material of hypothalamic origin which stimulates release of ACTH *in vitro* was recently reported from this laboratory(1). Similar activity was found in the corresponding fraction when commercial extracts of posterior lobe of the pituitary were subjected to the same fractionation procedures. Evidence has been obtained that the active material is a small peptide different from vasopressin and oxytocin (1). The physiological significance of these findings as well as the composition and structure of the active factor are still to be elucidated. Even at this stage of fragmentary knowledge, it was of interest to investigate whether or not the hypophysiotropic fraction would stimulate the release of endogenous ACTH in humans. Stimulation of ACTH-release in the human has never been reported in the absence of stressing conditions such as fever, pain, discomfort, surgical procedures, electroshock, insulin shock, forced muscular exercise or emotional upset. The active hypophysiotropic material appeared devoid of any side effects on the basis of pharmacological studies(1) and produced increases of the plasma 17 hydroxycorticoids when administered to dogs(2,†). It was therefore decided to study its possible ACTH-releasing activity in humans without regard for the explanatory mechanism, *i.e.*, directly hypophyseal (specific) or trans-hypothalamic (non-specific), of any positive result as long as it would have been obtained in the absence of stress or general discomfort.

Material and methods. Two types of clinical investigations were carried out in a total of 36 human subjects. a. 5 to 10 mg of a material obtained by chromatographic fractionation of oxycellulose-washed Protopituitrin‡ and designated as fraction D(1), were administered by I.V. perfusion in 150 to 200 ml of saline over 4 hours to 14 hospitalized children (age 4 to 16 years) convalescent from various conditions. Determinations of the plasma "free" 17OH-corticoids (17-OHC) were done by a modification of the method of Nelson and Samuels(3) at times 0, 2, 4, 6 hours as a test of stimulation of ACTH-release. In some of these studies measurements of the urinary excretion of 17OHC by the method of Glenn and Nelson(4) were

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† Unpublished observations.

‡ Protopituitrin (Parke, Davis & Co.) is a crude acetic acid extract of post. pituitary (fraction C in Waring & Landgrebe's outline of Kamm fractionation procedure(7)). It is the commercial starting material for Pitressin® and Pitocin®.