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Specific Immunoprecipitation of Mouse Thyrotropins in Plasma and Thyrotropic Tumor Extracts.* (29329)

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Attempted immunoassay of thyrotropic hormone (TtH) of mice, begun by us many years ago, was unsuccessful until 2.3 mg of a highly purified TtH, free from detectable serum protein, was obtained from R. W. Bates (Nat. Inst. Arth. Met. Disease). An antiserum (a-mouse TtH) produced with this purified TtH gave specific precipitation with as small a quantity as 0.002 ml of serum of highly functional thyrotropic tumor (TtT)-bearing mice. This is estimated to contain somewhat less than 1 mu of TtH. This a-mouse TtH gave no reaction with 0.2 ml serum specimens of mice bearing adrenotropic (AtT) and mammo-somatotropic (MStT) tumors and with normal sera. In immunoelectrophoresis of the serum, the TtH was localized in the globulin region.

Materials and methods. Preparation of a-mouse TtH. The mouse TtH of Bates contained 12.5 u/mg. It was prepared by successive percolation and chromatographic fractionation(1,2). Two units, incorporated in complete Freund adjuvant (Difco), were injected into each of 2 rabbits intramuscularly, and 4 weeks later, 3 units in saline were injected intraperitoneally; 2 weeks later, a third injection of 2 units was given subcutaneously. The rabbits were exsanguinated 12 days after the last injection. Serum 120, which gave a strong and specific reaction with TtH and was used in the present study, will be referred to as "a-mouse TtH." The second antiserum was poor.

Antiserum against bovine TtH (Thytropar, Armour), to be referred to as "a-beef TtH," was produced in the same manner. Each rabbit was given 14 units of Thytropar during a period of 3 months. Antisera prepared against plasma of hypophysectomized mice (a-mouse serum) were prepared in the same manner.

TtT. In mice, TtT was induced by radiothyroidectomy with I^{131}(3) and was carried in successive passages in isologous LAF_1 mice. A functional, transplantable thyrotropic pituitary tumor was induced in rats by Astatine^{211} (Sinha, Kunii and Furth, to be published). Bioassays for TtH were made with McKenzie's assay(4).

Agar-gel precipitation (Ouchterlony) test. The plates were prepared with Noble agar (Difco), phosphate buffer, and merthiolate by standard procedure. Petri dishes with wells

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TABLE I. Agar-Gel Precipitation Tests of Sera of Pituitary Tumor-Bearing Mice with a-Mouse TtH Serum.

Serum in well, ml	Number of plasma samples* examined															
	TtT 6113		Other TtT strains		AtT		MStT		Normal		Hypex		Thyroidex	
	+	—	+	—	+	—	+	—	+	—	+	—	+	—
.2					0	1	0	1	0	3	0	1		
.1	4	0	5	0	0	3	0	5	0	6	0	4	2	8
.05	8	0	3	3					0	1				
.02	14	0	0	3	0	1	0	1	0	2	0	3		
.01	4	0	0	2										
.006	3	1	0	2										
.003	1	0	0	1										
.0025	3	2												
.0012	1	0												

* Mostly from different mice.

of various sizes were used, our favorite plate containing 6 wells. The plates were kept in a moist chamber for 4-6 days at room temperature and were read daily.

Immunoelectrophoresis. Immunoelectrophoresis was performed essentially as described by Grabar and Williams(5). The glass plate used measured 50 × 75 mm. The 1% Noble agar (Difco) was made up with Veronal buffer of pH 8.6 and ionic strength 0.05. Electrophoresis was carried out at constant 60 v. Usually, 0.01 ml of plasma was electrophoresed. After 1 hour and 45 minutes to 2 hours of electrophoresis of the antigen, the grooves were filled with 0.2 ml of antiserum. Occasionally, the antiserum was refilled after 30-45 minutes. The plates were kept in a moist chamber, at room temperature, for about 48 hours. Photographs were taken at this stage. For permanent preparation, the plates were washed in saline for 2-3 days and were then rinsed with distilled water and kept at 37°C wrapped in a moist filter paper for drying without cracking. Amido black 10B was used for staining.

Results. Precipitation tests with a-mouse TtH. Table I is a summary of precipitation tests of sera of mice bearing transplanted TtT and of other murine sera. It shows that a-mouse TtH gives precipitation lines with plasma of TtT-bearing mice (Fig. 1) but not with plasma of normal or hypophysectomized mice. The most recently isolated TtT strain (6113) gave the strongest reaction. Other, less functional TtT strains (4183, 17, 82, and 3052) had lower titers. Plasma of

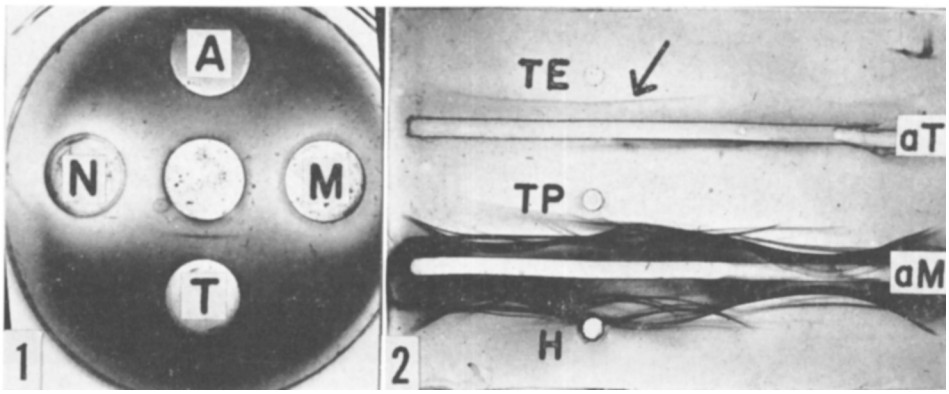

FIG. 1. Agar-gel precipitation reactions of a-mouse TtH (in central well). The peripheral wells contain undiluted serum of thyrotropic (T), mammotropic (M), adrenotropic (A) tumor bearing and normal (N) mice.

FIG. 2. Immunoelectrophoresis of TtT extract (TE), TtT plasma (TP) and hypex mouse plasma (H) precipitated with a-mouse TtH (aT) and a-hypex mouse serum (aM). The antisera were in the grooves. The arrow points to the hormone line consistently found in preparations in which the gel diffusion plates indicated strong precipitation with a-mouse TtH.

TtT mice lyophilized over 5 years ago and rehydrated (not listed in Table I) gave titers similar to those of 6113 sera. Precipitation tests with sera of thyroidectomized mice were negative with two exceptions. Tests with normal sera concentrated up to 5 times gave no precipitation with a-mouse TtH.

Precipitation reaction of rat TtT plasma with a-mouse TtH. Plasma of rats bearing TtT smaller than 1 cm across gave no reaction with a-mouse TtH. Tests with plasma from rats bearing larger tumors gave a faint but distinct precipitation line.

Precipitation with tumor extracts. Saline extracts were prepared from various types of pituitary tumors. Lyophilized TtT were extracted following the percolation technique of Bates *et al*(1). The extracts were tested with a-mouse TtH and a-mouse sera. Protein concentration was estimated by optical density at 276 mu. TtH preparations containing 0.01 mg protein, reacted with a-mouse TtH but 0.06 mg reacted also with a-mouse sera. Correspondingly, antisera prepared against such preparations were not as specific as the a-mouse TtH serum prepared with Bates purified preparation but could be rendered specific by absorption tests.

Precipitation with Thytropar. The a-mouse TtH gave no precipitation line with Thytropar in the concentration 0.1 u and 0.01 u.

Immunoelectrophoresis with a-mouse TtH. The a-mouse TtH serum gave a single, though weak, line with TtT extracts and with sera of TtT mice (Fig. 2). The 2 types of antisera (a-mouse TtH and a-mouse serum) served as a good guide in the course of purification procedures for estimation of TtH and contaminating serum proteins. Immunoelectrophoresis as used was not sensitive enough to detect as small concentrations of protein as the agar-gel precipitation technique did.

Precipitation tests with a-beef TtH. The a-beef TtH reacted strongly with its homologous antigen, giving 3 precipitation lines in concentration of 0.1 u, 2 lines with 0.02 u and 1 line with 0.004 u of Thytropar. This a-beef TtH gave a single faint precipitation line with 5 mg of mouse TtH preparation, which reacted strongly with a-mouse TtH and in a much smaller concentration (about 0.2 mg).

Discussion. Earlier findings of immunoassay for TtH have been well reviewed elsewhere(6,7). TtH content of functional TtT was bioassayed by several investigators(8). Bates *et al*(9) estimated the blood levels of thyrotropin in tumor hosts to be about 2000 times normal. In mice bearing fully dependent tumors, the blood levels are directly related to the tumor size and hormonal activity of the tumor. The latter is greatest in primary tumors and gradually decreases in the course of transplantation(9,10). Bates found 0.1-0.3 u/mg dry weight of TtH in both normal pituitary and primary pituitary tumors. McKenzie's bioassay(4) can detect somewhat smaller quantities of hormone than do precipitation tests, but it is much more time-consuming, more expensive, and no more precise than immunoprecipitation tests. Detection of TtH by agar-gel immunoprecipitation can be improved by concentrating blood TtH about 30-fold utilizing Bates' technique (11). The most sensitive immunoradio assay, worked out for growth hormone(6), is also feasible for TtH assay. The latter promises to be an excellent guide for characterization of thyroid tumors and gauging their hormonal control.

That the specific precipitation with the a-mouse TtH serum used is due to the reaction with TtH and not with cellular components of TtT is indicated by the following: a) The hormone used for immunization was prepared by up-to-date procedures for hormone isolation, had a high specific activity and gave a single line with its antiserum; b) In the course of purification of TtH from TtT, following Bates' procedure, the TtH activity, as ascertained in bioassays, paralleled the precipitability with our a-mouse TtH; c) A similar correlation was found between bioassays and precipitability of sera of various thyrotropic tumor-bearing mice (Table I); d) The procedure used for isolation of the antigen (TtH) denatures normal serum proteins. Denaturation may modify antigenicity, but does not render serum proteins non-antigenic. Were denatured non-hormonal antigens present in the material

used for immunization, corresponding precipitation lines would be expected. But electrophoresis disclosed only a single line reactive with a-mouse TtH serum.

It is conceivable that antigens derived from the tumor induced and grafted in an isologous strain of mice circulate in the blood. They may react with antibodies prepared against native tumor proteins, but probably not with the a-mouse TtH prepared with highly purified hormone. It is noteworthy that the titer of the presumed antigen (TtH) was highest in athyroid hosts in which the TtT gave no gross or histologic evidence of autoimmunization. The titer dropped precipitously when the tumors acquired ability to grow in the normal host in which TtH production is restrained by the host's thyroid (TH). It is unlikely that such a change is paralleled with loss of specific tumor antigens.

For these reasons, we believe that the assays here reported are hormone specific. The alternate assumption that the antigen detected is tumor antigen other than hormone lacks supporting evidence. Tumor specific substances may be present in the blood. Their existence is of sufficient interest to suggest experiments aimed at their detection.

Summary. An antiserum prepared against purified murine thyrotropin precipitated specifically sera of thyrotropic tumor-bearing mice and extracts of thyrotropic tumors. The titer of precipitation reaction paralleled the bioassay titers for TtH. The test can detect TtH in 0.002 ml of serum of TtT mice but not in 0.2 ml of normal sera. In immunoelectrophoresis, a single TtH line was obtained in the globulin region, yet to be precisely localized. Slight or no group reactions were obtained among rat, beef, and mouse TtH preparations.

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Effects of Parathyroid Extract, Calciferol, Hytakerol and Crystalline Dihydrotachysterol on Feed Consumption in Normal Lactating Rats.* (29330)

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The normal mean and variation in feed consumption of female Sprague-Dawley-Rolfsmeyer rats has been reported as a preliminary to a study of the effect of food restriction on thyroxine secretion rates(1). In addition, it was observed that the feed intake of the rat increased with the advance of lactation to the 20th day, then declined rapidly

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