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PREFACE

The papers in this volume are those delivered at the 1966 Laurentian Hormone Conference which was held at Mont Tremblant, Quebec, during the period August 28 to September 2. Four major areas of research were explored during this meeting. These were concerned with thyroid physiology, a number of studies with thyroid hormones, contemporary developments in peptide hormone chemistry and biochemistry, and special studies of insulin physiology, particularly in man. Factors controlling thyroid stimulation are dealt with in the first two chapters and special studies of the mode of action of thyroid hormones, including antithyroid drugs, are the subjects of the next two chapters. The mode of action of steroid hormones and hormonal steroids are problems of great contemporary interest, and these are discussed on the basis of effects on normal and neoplastic tissues. A definitive segment of research in endocrinology has been concerned with the metabolism of the steroid hormones *in vivo* and *in vitro*. We are pleased to be able to present two chapters containing an account of contemporary developments in this field. The discussion of an important clinical condition involving adrenocortical steroid production is given in the chapter concerned with congenital adrenal hyperplasia. With the development of modern methods for the production of complex molecules having biological activity, the synthesis of peptide hormones and of insulin represents a triumph of chemical imagination. The chapters concerned with these developments are therefore particularly timely. The accompanying discussion of gastrointestinal hormone biochemistry illustrates the broadening field of interest in hormones of these types. Not so long ago the assay of insulin in blood plasma was considered a nearly insoluble problem. Now we are able to present investigations on at least two insulinlike activities accurately measurable in human serum. The particular control of insulin activity in man is dealt with in the final chapter.

The organization and program of the Laurentian Hormone Conference are made possible by contributions from pharmaceutical companies whose continuing interest and support are gratefully acknowledged. Sponsors of the 1966 meeting were Abbott Laboratories; Armour Pharmaceutical Company; Ayerst Laboratories Division of American Home Products Corp.; Ayerst International; Ayerst Laboratories Division of Ayerst; McKenna and Harrison, Ltd.; Baxter Laboratories, Inc.; Berlin Laboratories, Inc.; Ciba Company, Limited; Ciba

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Their contributions made it possible for us to have among our speakers as special guests from abroad Drs. G. Morreale and F. Escobar of the Instituto G. Marañon, Madrid, Dr. V. Mutt of the Karolinska Institute, Stockholm, and Dr. E. R. Froesch of the University of Zurich.

As in the past, the Committee on Arrangements has had the kind cooperation of a group of skillful chairmen for the individual sessions. We are indebted to Drs. J. Robbins, L. Engel, T. Gallagher, E. Baulieu, G. Aurbach, H. Fevold, and R. Levine for their competent handling of the discussions which are such a stimulating feature of the Laurentian Conference meetings. As secretaries to the conference we thank also Miss Pauline Illsley and Mrs. Mina Rano. Miss Joanne Sanford assisted the committee as its executive secretary.

GREGORY PINCUS

Shrewsbury, Massachusetts
May, 1967

CONTENTS

I. THYROID PHYSIOLOGY

The Long-Acting Thyroid Stimulator: Its Role in Graves' Disease

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I. Introduction

Thyrotropin was established as a pituitary entity almost forty years ago; until just ten years ago, stimulation of the thyroid, and possibly other tissues, by this hormone had prominence in medical texts as the pathogenetic mechanism at work in Graves' disease. The theory was held despite general lack of substantiation by results of bioassays which sought to prove increased levels of thyrotropin in the blood in the clinical syndrome.

In 1956 (1) Adams and Purves, using their method of assay of thyrotropin, described a phenomenon, the cause of which has come to be known as "the long-acting thyroid stimulator," commonly referred to as LATS. The phenomenon was a prolonged effect on the release of radioiodine from the thyroid of guinea pigs following injection of serum from a patient with Graves' disease. Having, at that time, developed a thyrotropin bioassay method using mice (30), adapted from the Adams and Purves procedure (2), I was readily able to confirm their observations (31). In the intervening eight years the phenomenon of the long-acting thyroid stimulator has become widely recognized and generally accepted as important in the pathogenesis of Graves' disease.

II. Method of Assay of the Long-Acting Thyroid Stimulator

An outline of our current bioassay procedure (38), for both thyrotropin and the long-acting thyroid stimulator, is listed in Table I. After 10 days on a low-iodine diet, mice are injected with 15 μc ^{125}I and 10 μg sodium L-thyroxine, and used for the assay procedure after a further 4 days. Blood (0.1 ml) is obtained by retro-orbital puncture im-

TABLE I
Method of Assay of Thyrotropin and the Long-Acting Thyroid Stimulator

Animal:	Mouse	
Preparation:	Days 1–10	Remington-type diet
	Day 10	15 μc ^{125}I intraperitoneally
		10 μg L-thyroxine subcutaneously
		0.066% thyroid USP in diet
	Day 14	Assay
Route:	Intravenous	
Response:	Increase in blood ^{125}I at 2 and 9 hours	

mediately before the intravenous injection of test material and 2 and 9 hours later; radioactivity in the blood samples is measured in a well-type scintillation counter with an automatic sample changer. An increase in radioactivity which is maximal at 2 hours is indicative of thyrotropin, whereas the long-acting thyroid stimulator causes an increase which is greater at 9 hours.

In the studies to be reported the following routine was adopted: all samples were injected in a volume of 0.2 ml or 0.5 ml, 6 mice per sample. Between 120 and 150 mice were used in any one assay, and the first 4 groups (i.e., 24 mice) were given a control solution (0.2 ml of 1% human serum albumin in 0.9% sodium chloride solution–1% HSA) and 3 doses of USP standard thyrotropin—0.05 milliunits (mU), 0.2 mU, and 0.8 mU—each in 0.2 ml of 1% HSA. When serum was to be assayed, usually as 0.5-ml volume, 0.5 ml of 5% HSA was given to another group of 6 mice as an additional control.

TABLE II
Statistical Analysis in the Long-Acting Thyroid Stimulator Assay

1[a] Logarithmic transformation of counts per minute (cpm)
2[a] Covariance analysis which gives "adjusted data"
3[a] Duncan's multiple range test (38); i.e., differentiation of responses from the control, and from each other, for P 0.05 or 0.01
4 Antilogarithm—"adjusted cpm"

[a] By IBM computer.

Table II summarizes the statistical methods used to analyze assay results; they were developed following reports by Guillemin and his colleagues concerning similar analyses applied to data obtained in the bioassay of luteinizing hormone (48) and thyrotropin (49). The validity and worth of the analysis in the assay of the long-acting thyroid stimulator were detailed recently (38). A further point brought

out in that report (38) was the marked variability from week to week which is seen in the bioassay. Responses to standard doses of thyrotropin or aliquots of one serum containing the long-acting thyroid stimulator, and even "responses" to control solutions, varied so markedly that it was recommended that material should be assayed in a single batch of mice if the results were to be compared one with the other. In the present paper, comparison is made only between responses so obtained.

Representative assay slopes for increasing doses of standard thyrotropin and for a serum containing the long-acting stimulator are shown in Fig. 1. The downward slope from 2 to 9 hours with smaller doses of

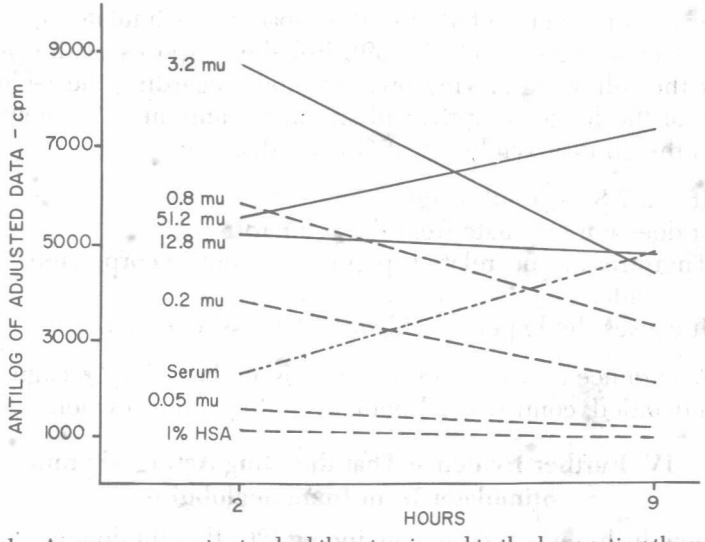

FIG. 1. Assay responses to standard thyrotropin and to the long-acting thyroid stimulator. – – –, Responses to the routine standard or control solutions used in every assay; ———, responses to progressive (fourfold) increases of the dose of standard thyrotropin; — — — — —, responses to the injection of serum containing the long-acting thyroid stimulator. The points are the mean of 6 observations in every instance. Reproduced from McKenzie and Williamson (38).

thyrotropin (0.05–3.2 mU) is seen in comparison with the upward slope following injection of the serum. Two further features are delineated in the figure; with a large dose of thyrotropin, 50.2 mU, the slope is upward from 2 to 9 hours and, moreover, the response at 2 hours is significantly less than the 2-hour response to 3.2 mU. These phenomena may be germane to the mode of thyroid stimulation, as will be brought out later (Section V).

III. Assessment of the Clinical Role and Chemical Identity of the Long-Acting Thyroid Stimulator

Despite, or perhaps in part due to, the many publications on the subject (reviewed up to December 1964 in reference 35 and further augmented in reference 10), the precise role of the long-acting thyroid stimulator in the clinical syndrome remains a matter of controversy. In particular, whether it is primarily associated with hyperthyroidism or with ophthalmopathy, presumably in a causal role, is debated. That it is a 7 S γ-globulin (IgG) was conclusively established by Kriss and his co-workers (26). However, its relationship to pituitary thyrotropin is still a matter of controversy, and evidence regarding the precise molecular requirements entailed in the capacity to stimulate the thyroid gland is controversial (11, 26, 39). For the purposes of the present report the following are my own opinions regarding the γ-globulin nature of the long-acting thyroid stimulator and an assessment of its role in the clinical syndrome of Graves' disease:

1. It is a 7 S γ-globulin (IgG).
2. It does not originate from the pituitary.
3. Thyrotropin, or related peptide, is not incorporated in the molecule.
4. It causes the hyperthyroidism of Graves' disease.

The evidence on which these opinions are based and recognition of the undecided, controversial points is published elsewhere (36).

IV. Further Evidence That the Long-Acting Thyroid Stimulator Is an Immunoglobulin

Although the evidence is convincing (26) that the long-acting thyroid stimulator is an IgG, there is no proof that it is an antibody. Among other criteria (62), this would require the formation of the long-acting thyroid stimulator under conditions recognized as appropriate for antibody production; as reported below, this was attempted both *in vitro* and *in vivo*.

A. Formation of the Long-Acting Thyroid Stimulator *in Vitro*

In view of the failure to extract the stimulator from a number of tissues obtained at the necropsy of appropriate subjects (37), Dr. Julius Gordon of the Department of Experimental Surgery, McGill University, and I turned to the circulating lymphocyte as a potential

source of the long-acting stimulator. Bach and Hirschhorn (4) reported that white blood cells cultured in the presence of phytohemagglutinin synthesized γ-globulin. Although there is controversy regarding this finding (15), it now seems likely that the lymphocyte *in vitro* can indeed synthesize IgG when appropriately stimulated (57). While a specific antigen may be a superior stimulant, phytohemagglutinin is effective in this regard as a nonspecific stimulant.

The method of cell culture was that of Bain, Vas, and Lowenstein (5), modified as follows. The harvested leukocytes were washed 3 times with 5% fetal calf serum in 0.9% sodium chloride solution. The cells were resuspended in 3–10% calf serum in culture medium 199 and incubated in either 4- or 40-ml aliquots. Phytohemagglutinin "P" was prepared according to the method of Rigas and Osgood (46) and was included in the culture medium at a final concentration of 20 μg/ml; one experiment was carried out without the inclusion of phytohemagglutinin. Cells for culture were obtained on 5 occasions from a subject, E.M., who had Graves' disease; her serum contained the long-acting thyroid stimulator in high concentration (Table III). Leukocytes from 120–150 ml of blood (and 200 ml, Assay 1, Table III) were cultured in a ratio of 2 million cells per milliliter of medium for periods up to 7 days, with change of medium every 1–3 days. Each collection of medium was filtered on Sephadex G-25 with 0.05 *M* ammonium bicarbonate as solvent. The protein so obtained was either assayed directly (listed in Table III as whole protein) or filtered on Sephadex G-200, to obtain a fraction of predominantly 7 S protein, which, on the basis of studies with human serum (33), was expected to contain the long-acting thyroid stimulator. The whole protein was dissolved in 3.5 ml and the 7 S protein fraction in 1.5 ml of 0.9% sodium chloride solution for assay in a dosage of 0.5 ml and 0.2 ml, respectively.

Blood was obtained, 100–150 ml, from each of 5 normal volunteers; white cells were harvested and treated exactly as described for E. M. cells, with incubation for the periods listed in Table III.

In one experiment (assay 10, Table III) the white blood cells from 130 ml of E.M.'s blood were disrupted by sonication. Direct count of aliquots in the cell suspension revealed that only 3% of the white cells remained intact. The preparation was centrifuged after sonication, and the supernatant fluid was filtered in Sephadex G-25 to obtain proteins for assay; as identified by polyacrylamide gel electrophoresis, the predominant stainable protein in the extract was hemoglobin from contaminating red blood cells.

Table III lists 10 separate assays, the results of which may be sum-

TABLE III

Long-Acting Thyroid Stimulator Assay of Protein from Incubation of White Blood Cells[a]

Assay	Source of cells	Days of incubation	Test material	Assay response (cpm) Test	Control	Duncan's test of significance
1	E.M. 1	2	Whole protein	1445	412	+
	E.M. 1	3	Whole protein	1198	412	+
	—	—	Fetal calf serum	462	412	−
2	E.M. 2	3rd + 6th	7 S protein	2010	1300	+
3	E.M. 3	3	Whole protein	1010	460	+
	—	—	Fetal calf serum + phytohemagglutinin	573	460	−
	E.M. 3	—	Protein from preincubation wash	555	460	−
4	N.S. 1	2nd	7 S protein	356	284	−
5	E.M. 4	2nd	7 S protein	1414	693	+
	E.M. 4	3rd + 6th	7 S protein	1474	693	+
	E.M. 4	7th	Whole protein (protein-deficient medium; cells lysed)	831	693	−
	N.S. 1	4th + 5th	7 S protein	705	693	−
6	N.S. 1	3rd + 6th	7 S protein	1073	961	−
7	N.S. 2	2	7 S protein	475	313	−
	N.S. 3	2	7 S protein	433	313	−
	N.S. 4	3	Whole protein	351	313	−
8	N.S. 5	2	7 S protein	1442	1054	−
9	E.M. 5	—	Protein from preincubation wash	1124	1133	−
	E.M. 5	2	7 S protein from white blood cells incubated without phytohemagglutinin	1591	1133	−
	E.M. 5	—	0.2 ml serum	4825	1133	+
10	E.M. 6	—	Protein from sonicated white cells	506	454	−
	—	—	0.2 ml of E.M.'s serum	1335	454	+

[a] The figures after initials E.M. indicate the six separate occasions of obtaining blood from this patient, and those after N.S. (normal subject) identify five volunteers who

marized as follows. When phytohemagglutinin was included, the protein in the culture medium of E.M.'s white blood cells contained the long-acting thyroid stimulator after 2 days (assay 1) or 3 days (assays 1 and 3) of incubation. With daily changes of medium, the stimulator was identified in the medium of the second day of incubation (assay 5) and in combined extracts from days 3 and 6 (assays 2 and 5). When phytohemagglutinin was not included in the incubation, no long-acting thyroid stimulator was found (assay 9); gel filtration showed the stimulator to be in the 7 S fraction of the protein. Negative assays were obtained with the following preparations: (a) the preincubation wash of the white cells (assays 3 and 9); (b) fetal calf serum with added phytohemagglutinin (assays 1 and 3); (c) the protein extracted from E.M. cells either without incubation (assay 10) or by their being incubated in a protein-deficient medium, which causes death of the cells (assay 5). Protein from the incubation of white blood cells obtained from normal subjects did not contain the long-acting thyroid stimulator (assays 5–8).

To test further the possibility of adsorption of the stimulator to white blood cells, and subsequent elution into incubation medium, cells from a normal subject were incubated for 48 hours in medium containing E.M.'s serum instead of fetal calf serum. After this, the medium was changed daily for 2 days, using fetal calf serum and phytohemagglutinin in place of E.M.'s serum, and the media were collected for assay. The 7 S protein of these media did not give responses indicative of the presence of the stimulator.

When white blood cells from E.M. or from normal subjects were incubated in the presence of phytohemagglutinin, typical "blast" cells, as described by Nowell (41), were visible microscopically at the end of incubation.

Similar studies were carried out with cells from 5 other patients. Positive assays were found with the protein from the white cell incubation, stimulated with phytohemagglutinin, from 2 subjects who had the long-acting thyroid stimulator in the blood. The white cells of one patient with Graves' disease, who did not have the long-acting thyroid

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donated blood. Days of incubation indicate continuous incubation (e.g., 2) or daily change of medium (e.g., 2nd). The terms whole protein and 7 S protein, and the mode of expressing the assay response and Duncan's test of significance ($P < 0.05$), are explained in the text. The control for each assay was the response to the injection of a solution of human albumin—1, 5, or 10%—to approximate the concentration of protein in the test solutions.

stimulator in the blood on assay of the unconcentrated serum, did not give a positive result in this procedure. Two subjects who had thyroid adenoma, with hyperthyroidism, had their white cells tested in this way; negative results were obtained here also.

On several occasions white blood cells from the patient E.M. were incubated with or without phytohemagglutinin, in the presence of ^{14}C-labeled amino acids (*Chlorella bacillus* hydrolyzate). The γ-globulin from the incubation medium, with the addition of a carrier serum

TABLE IV

^{14}C-Amino Acid Incorporation into γ-Globulin[a]

	Phytohemagglutinin		Control	
Preparation	Cpm	Per cent	Cpm	Per cent
Total medium	159×10^6	100	36.8×10^5	100
Ammonium sulfate precipitate	27×10^6	17	4.95×10^5	13.5
Dialyzed precipitate	48×10^4	0.32	681	0.018
γ-Globulin from DEAE-Sephadex column	12.7×10^3	0.008 = 2.5% dialyzed precipitate	Not significant	—

[a] The incubation of white blood cells with ^{14}C-labeled amino acids with phytohemagglutinin is described in the text. Data headed "Control" indicate results from an incubation without phytohemagglutinin, but with an aliquot of the same white blood cells. After incubation, the media had serum that contained the long-acting thyroid stimulator added; recovery of this biological activity was approximately 50%. Precipitation was achieved by adding saturated ammonium sulfate to a concentration of 30% and then 40%; dialysis against 0.05 *M* Tris HCl, pH 8.0, buffer was for 48 hours and was followed by chromatography on a column of diethylaminoethyl (DEAE) Sephadex, using the same buffer, to isolate the γ-globulin.

containing the long-acting thyroid stimulator, was extracted as shown in Table IV, with monitoring of the ^{14}C-amino acid content of the various steps of extraction. As shown in the table, the degree of incorporation of ^{14}C-amino acid into γ-globulin was small, but significant in comparison with the lack of incorporation into γ-globulin when cells were incubated without phytohemagglutinin. The ^{14}C-labeled γ-globulin containing the long-acting thyroid stimulator was used in further experiments to test affinity of the long-acting thyroid stimulator for human thyroid tissue.

Beall and Solomon recently reported (7) an apparent affinity of the long-acting thyroid stimulator for the microsomal component of hu-

man thyroid; the affinity was both organ-specific and relatively fraction-specific, i.e., other subcellular components of the thyroid were much less effective in adsorbing the stimulator. After confirming their observations, we tested the affinity of thyroid microsomes for the ^{14}C-labeled γ-globulin-long-acting thyroid stimulator preparation, human liver microsomes being used as control material. The results of one experiment are shown in Table V, where the greater adsorption of the ^{14}C-labeled γ-globulin by the thyroid microsomes is listed.

TABLE V

^{14}C-Labeled γ-Globulin from White Blood Cell Incubation: Effect of Microsome Fraction from Thyroid and Liver[a]

Preparation	^{14}C (cpm)	Per cent
Globulin + thyroid microsomes	74,782	100
105,000 *g* supernatant fluid	50,760	68
1st week of microsomes	2,150	2.9
2nd week of microsomes	188	0.25
Globulin + liver microsomes	59,200	100
105,000 *g* supernatant fluid	55,300	93
Wash of microsomes	3,705	6.2

[a] The microsome pellets were prepared from the 0.25 *M* sucrose homogenate of tissues obtained at necropsy; they were the 105,000 *g* sediment of supernatant fluid remaining after centrifugation for 10 minutes at 8000 *g*. Washing of the microsome pellets was carried out with 0.9% sodium chloride solution.

The conclusion from these experiments is that the long-acting thyroid stimulator is indeed a product of antibody-forming lymphoid tissue; this does not, of course, establish that the long-acting thyroid stimulator so formed is an antibody. Strong evidence that it is an antibody would be obtained if its *in vitro* formation by lymphocytes were specifically stimulated by a thyroid subcellular component, but this proof has not been obtained.

B. Experimental Production of the Long-Acting Thyroid Stimulator *in Vivo*

If the long-acting thyroid stimulator is an antibody corresponding to a thyroid antigen, then immunization with thyroid subcellular components should lead to the production of an antibody which would act as a long-acting thyroid stimulator in the bioassay. To test this