Extraction and Characterization of a Thyrotropic Material from the Human Placenta

JEROME M. HERSHMAN and WILLARD R. STARNES

From the Metabolic Research Laboratory, Veterans Administration Hospital and the Department of Medicine, University of Alabama Medical Center, Birmingham, Alabama 35233

ABSTRACT A material with thyrotropic activity was extracted from fresh human placentas. After chromatography of the extract on carboxymethyl cellulose, thyroid-stimulating activity ranged from 0.12 to 1.06 mU of thyroid-stimulating hormone (TSH) per mg of protein in bioassays of eight preparations. The amount of TSH per placenta varied from 113 to 2200 mU and approximated the content of the pituitary gland. Additional purification by gel filtration on Sephadex G-100 gave a maximum activity of 8 mU/mg. The most active portion was eluted in the same position as ^{125}I-labeled bovine or human TSH, a fact suggesting that the molecular size of this thyrotropic substance was similar to that of pituitary TSH. Another placental fraction with weaker activity was eluted earlier indicating that the placental material was heterogeneous.

In the McKenzie mouse bioassy, the response of the placental thyrotropin paralleled that of the beef TSH standard. There was no long-acting thyroid stimulator effect. Antibodies to both human and bovine pituitary TSH neutralized the biologic activity of the placental TSH. Placental thyrotropin cross-reacted very weakly in a sensitive radioimmunoassay for human pituitary TSH; it cross-reacted completely in a radioimmunoassay for bovine pituitary TSH, and this assay was used for following the purification. The role of this thyrotropic material as a possible cause of thyroid hypertrophy and hyperfunction in pregnancy and in patients with trophoblastic tumors remains to be investigated.

INTRODUCTION

The human placenta produces at least two polypeptide hormones which have a close structural and biologic relationship to hormones of the anterior pituitary gland: chorionic gonadotropin resembles luteinizing hormone (1, 2), and placental lactogen resembles growth hormone (3, 4). Several observations suggested that the placenta might also produce a thyroid-stimulating hormone (TSH). In 1963, Odell, Bates, Rivlin, Lipsett, and Hertz found TSH activity on bioassay of choriocarcinoma tissue from two patients with this trophoblastic tumor who also had increased thyroid function (5). In 1965, Hennen reported the extraction of a thyrotropic substance from normal human placentas (6). In 1967, Burger found TSH activity in crude preparations of chorionic gonadotropin (7). Here we shall report our experience with the extraction and biologic characterization of a placental thyrotropin.

METHODS

Extraction procedure. Placentas were obtained immediately after delivery from mothers who were clinically euthyroid. The placentas were packed in ice, brought to the laboratory, and processed immediately in a room at −15°C. Pieces weighing 10–20 g were rinsed in 0.1 M NaCl; 100–200 g was placed in a Waring blendor; 5 volumes (v/w) of acetone was added; and the tissue was homogenized for 5 min. It was filtered at room temperature (21–23°C) through a Buchner funnel; the cake was rinsed with acetone, rehomogenized in cold acetone for 5 min, filtered, washed again with acetone, and dried in air. The resulting powder was 13.5 ±0.3% (mean ±SE of 27 runs) of the wet weight of the placenta.

5-g aliquots of eight different placental powders were combined; the 40 g powder was mixed and extracted by the method of Reisfeld, Lewis, Brink, and Steelman (8) for the fractionation of human pituitaries. The powder was extracted for 2 hr with 0.3 M KCl (20 ml/g of powder), pH 5.5, at 5°C with a magnetic stirrer. The suspension was centrifuged for 30 min (20,000 *g*) at 0°C; the residue was reextracted at pH 5.5 for 1 hr with 10 ml of 0.3 M KCl/g of powder; the supernatants were combined, and the residue was discarded. The combined supernatants were adjusted to pH 8.5 and chilled to 0°C. Ethanol at −15°C was added slowly to a final concentration of 30% while the solution was stirred and its temperature maintained at 0°C. The resulting suspension was centrifuged at 20,000 *g*, 0°C. The supernatant

This work was presented in part at the Third International Congress of Endocrinology, Mexico City, July 1968.

Received for publication 25 October 1968.

was adjusted to pH 5.0 and centrifuged again. Then it was dialyzed against demineralized water. The dialyzed supernatant was lyophilized and called fraction E. This fraction represented 2.5% of the dry placental powder, or 0.34% of the wet weight of the placenta, and constituted 16.3% of the soluble protein in the acetone-precipitated placental powder.

The E fraction was dissolved in 0.01 M phosphate buffer, pH 6.2, and placed on a 2.5 × 30 cm column of carboxymethyl cellulose (Whatman CM-52). Highly purified human[1] or beef[2] TSH labeled with ^{125}I by the procedure of Greenwood, Hunter, and Glover (9) was added to the E fraction; the ^{125}I was monitored in the eluates as a marker for the location of thyrotropic activity. The 10,000–30,000 cpm ^{125}I (50% counting efficiency) added to the column did not interfere with subsequent assays for thyrotropin. Thyrotropic activity was eluted from the column with 1.0 M NaCl. Some of the active fractions from the carboxymethyl cellulose column were fractionated by gel filtration on a 2.5 × 90 cm column of Sephadex G-100 with 0.005 M sodium glycinate buffer, pH 9.5, as described by Condliffe for the purification of human pituitary TSH (10).

Bioassay. The bioassay of TSH was performed by a modification of the McKenzie mouse assay (11). The determination of relative potency and the statistical analysis of the assay data were calculated on an IBM computer using the EXBIOL program kindly provided by Dr. E. Sakiz and Dr. R. Guillemin. The index of precision, λ, was usually in the range of 0.15–0.40. The 95% confidence limits for relative potency in either four or six point assays varied from ±20–55% of the mean relative potency.

Antibody neutralization. Neutralization of the biologic effect of TSH was carried out by incubation of TSH with antibody for 22–24 hr at 5°C. The amount of antibody used was selected to obtain neutralization of approximately one-half the TSH activity in the bioassay. Antibody to beef TSH (B-TSH) was prepared by repeatedly injecting rabbits with National Institutes of Health (NIH) B-TSH B2 (4 U/mg) for a total of 8–16 mg; antibody to human TSH (H-TSH) was prepared in a rabbit immunized with crude H-TSH (approximately 0.1 U/mg) or was that supplied by the National Pituitary Agency.

Radioimmunoassay. In the radioimmunoassay of B-TSH by the double antibody method, solutions were made up in a buffer containing 0.25% human serum albumin 0.01 M phosphate–0.15 M NaCl, pH 7.5. 200 μl of unknown or standard and 100 μl of a 1/10,000 dilution of rabbit antibody to B-TSH in 0.05 M ethylenediamine tetraacetate (EDTA) were placed in a 10 × 75 mm disposable glass tube. After 24 hr of incubation at 5°C, 100 μl of ^{125}I-labeled B-TSH (10,000 cpm) was added and incubation was continued for 4 days. Then 50 μl of 1/500 rabbit serum and 50 μl of a goat antibody to rabbit globulin were added and incubation carried out for 20 hr at 5°C. The tubes were centrifuged in the cold, the supernatant decanted, the precipitate washed once with the albumin-phosphate-saline buffer, recentrifuged, and the supernatant decanted. The tubes containing the precipitates were counted

[1] Obtained from the National Pituitary Agency, Baltimore, Md.

[2] Obtained from Dr. John G. Pierce.

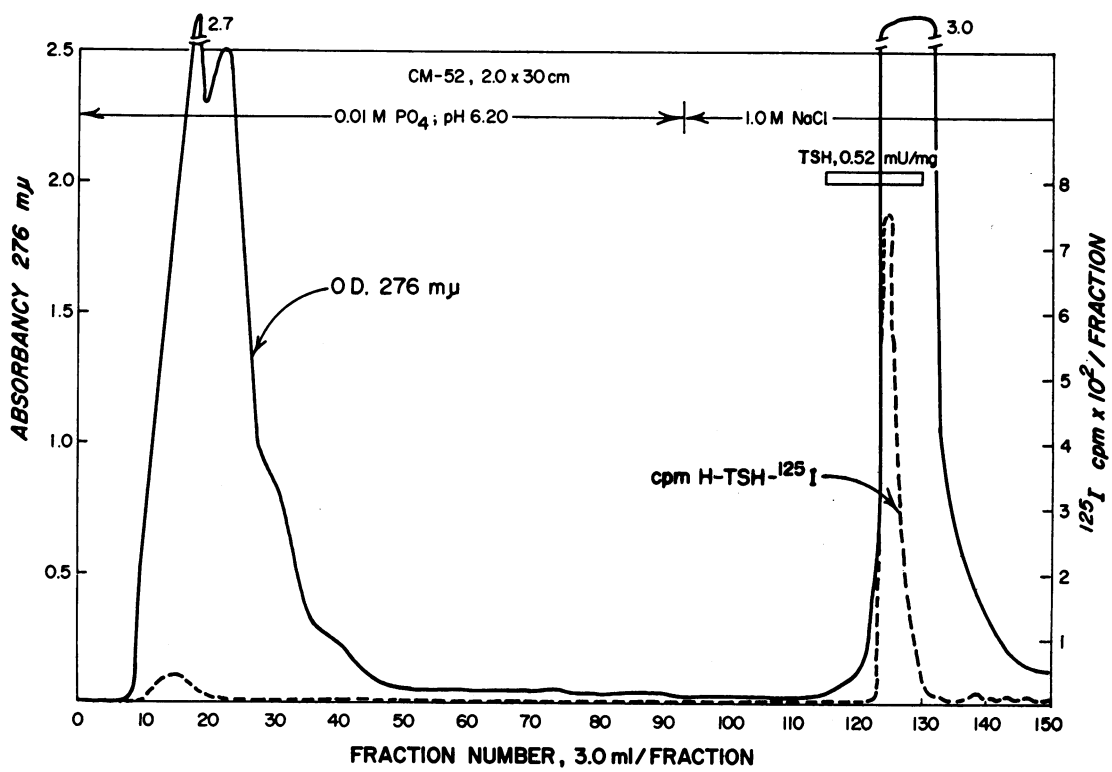

FIGURE 1 Chromatography of 350 mg of fraction E on a 2 × 30 cm column of carboxymethyl cellulose (CM-52); Human TSH-^{125}I (H-TSH-^{125}I) was added as a marker. The active fraction contained 0.52 mU of TSH per mg of protein.

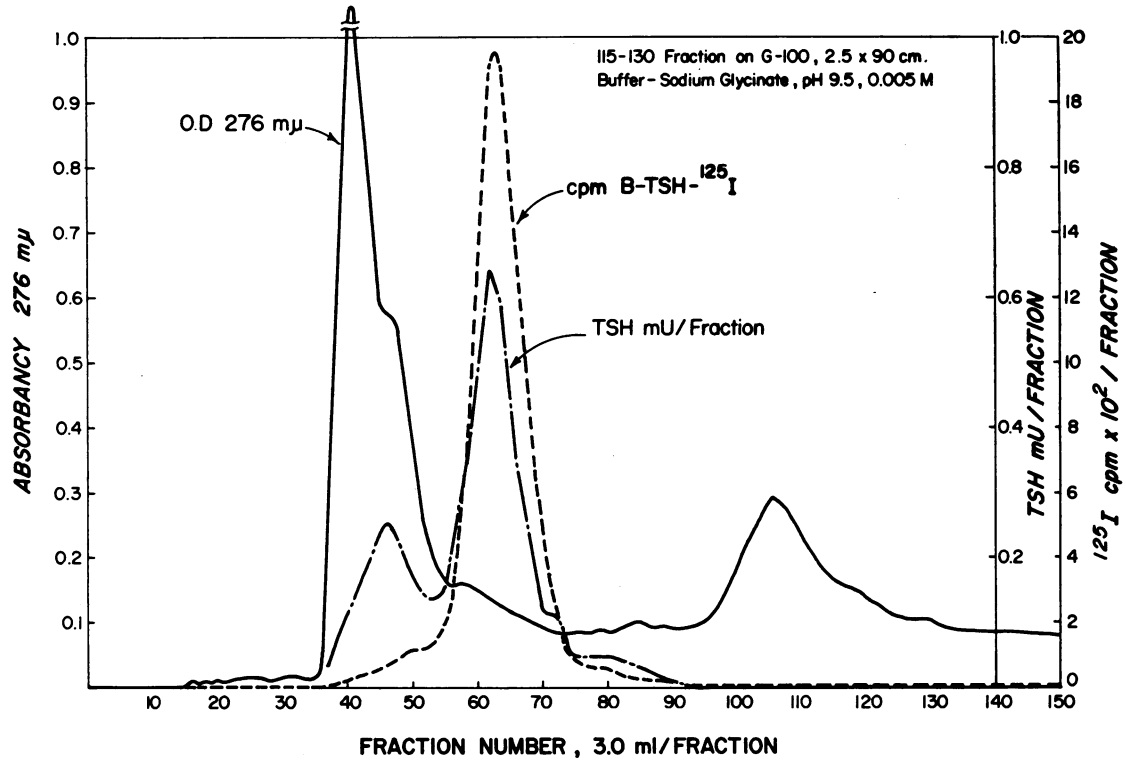

FIGURE 2 Gel filtration of the placental thyrotropin from CM-52 on Sephadex G-100. Bovine TSH-^{125}I (B-TSH-^{125}I) was added as a marker. TSH activity in each fraction was estimated by radioimmunoassay in the beef system.

in an automatic well-type scintillation counter and the results expressed as the per cent of the added ^{125}I which was precipitated.. The usable range of the standard curve was 0.5–40 μU of TSH (in terms of the International Beef TSH Standard[3]), the precipitation varying from approximately 65–5% respectively in this range of the standards. 90–95% of the ^{125}I-label was precipitable with excess antibody.

The ^{125}I-labeled TSH was purified by passage through a 1 × 50 cm column of Sephadex G-100 before each assay. The B-TSH-^{125}I from a single iodination was kept at −20°C and could be used for 2–3 months as long as it was repeatedly purified by gel filtration to remove damaged TSH and iodide-^{125}I arising from spontaneous deiodination.

The radioimmunoassay of H-TSH by the double antibody method was performed with the materials supplied by the National Pituitary Agency using a minor modification of the method described by Odell, Rayford, and Ross (12).

Protein was measured by the method of Lowry, Rosebrough, Farr, and Randall (13).

RESULTS

Preparation. In a typical preparation, 42.6 g of acetone-dried powder yielded 820 mg of E fraction. Fig. 1 shows the results of chromatography of 350 mg of this material on carboxymethyl cellulose. An unretarded, usually amber-colored fraction was devoid of any thyrotropic activity. A fraction eluted with 1.0 M NaCl contained the H-TSH-^{125}I. The eluate indicated by the bar in the figure contained 0.52 mU of TSH per mg of protein, based on the bioassay, with recovery of 192 mU of TSH in this zone. TSH was not detected in other zones of the eluate. The total amount of TSH in the E fraction was equivalent to 447 mU or 10.5 mU/g of acetone-precipitated powder. This calculation ignores possible losses in the extraction procedure.

An acetone-precipitated powder was prepared from 100 ml of pooled serum from women in labor; this was extracted in the same way as the placental powder. TSH could not be detected in bioassays of the eluate from the carboxymethyl cellulose column of E fraction from this serum. Liver and kidney tissues obtained at autopsy from hospitalized patients were processed in the same manner and did not contain detectable quantities of TSH in the bioassay of the appropriate fractions.

In eight different placental preparations, there was considerable variability of recovery of thyrotropic activity. The specific activity of the active fraction eluted from the carboxymethylcellulose column ranged from 0.12–1.06 mU/mg of protein (0.52 ±0.11 mU/mg, mean ±SE). The recovery of TSH per gram of placental powder ranged from 1.7–32.4 mU/g. Assuming the aver-

[3] Obtained from the Division of Biological Standards, National Institute for Medical Research, London, England.

age weight of the placenta to be 500 g, equivalent to 67.5 g of the powder (13.5 g of powder per 100 g of placenta), the amount of TSH in a placenta was calculated and found to vary from 113–2200 mU/placenta (645 ±235 mU/placenta).

Additional purification of the active material from the carboxymethyl cellulose column was obtained by gel filtration on Sephadex G-100, as shown in Fig. 2. Several protein peaks were obtained. ^{125}I-labeled B-TSH added as a marker was found on the descending limb of the small second protein peak and corresponds with the maximum TSH activity measured by both bioassay and radioimmunoassay (*vide infra*). ^{125}I-labeled H-TSH was located in the same position in other Sephadex G-100 columns. The highest specific activity of the most active fraction in this column was 8 mU/mg in the bioassay. In Fig. 2, there is a second smaller peak of TSH activity which coincided with the major protein peak. This indicates that the placental material is heterogeneous compared with purified pituitary H-TSH or B-TSH.

Characterization. Fig. 3 shows that the response of the placental thyrotropin in the bioassay was parallel to that of the Bovine International Standard for TSH. In contrast, the Human Thyrotropin Research Standard A[8] showed a slope that was flatter as in Fig. 3. In assays for long-acting thyroid stimulator (LATS), the response of the placental thyrotropin paralleled that of the bovine pituitary standard (Fig. 4); a potent LATS serum was also analyzed for comparison. The response of the placental thyrotropin was less at 8 hr than at 2 hr. In five assays for LATS activity, the ratio of the 8 hr response to the 2 hr response was 0.70 ±0.03 (SE) for the 0.5 mU B-TSH standard and 0.76 ±0.05 (SE) for the preparations of placental TSH (PL-TSH), there being no significant difference between these means.

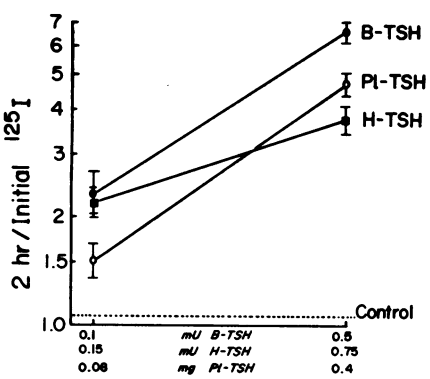

FIGURE 3 Multiple four point bioassay of beef pituitary TSH (B-TSH) (International Standard), placental TSH (Pl-TSH), and human pituitary TSH (H-TSH) (International Reference Standard A). Responses are the log $\frac{\text{Blood } ^{125}\text{I at 2 hr}}{\text{Blood } ^{125}\text{I at 0 time}} \pm$ SE. The dashed line shows the response of mice injected intravenously with a control solution.

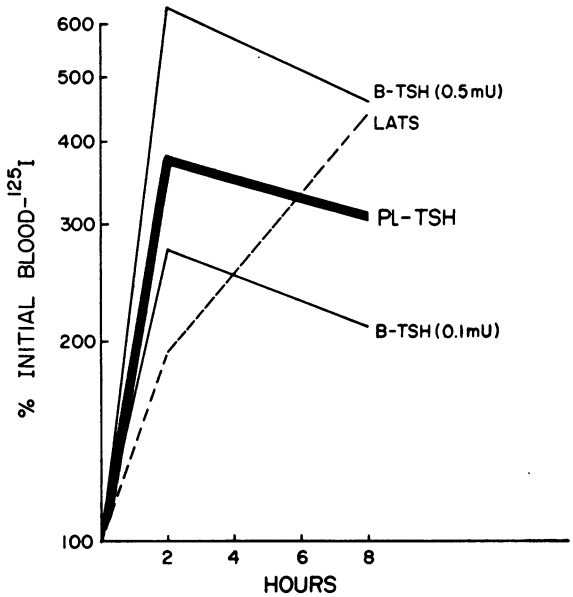

FIGURE 4 Bioassay of bovine TSH (B-TSH), placental TSH (PL-TSH, 0.2 mU), and a potent long-acting thyroid stimulator (LATS) serum for LATS. Mice were bled at 0, 2, and 8 hr after injection of test substances intravenously.

Preparations of pituitary B-TSH and H-TSH and PL-TSH were reacted with an antibody to either pituitary B-TSH or H-TSH and then bioassayed. As shown in Fig. 5 in the third set of bars, the antibody to B-TSH

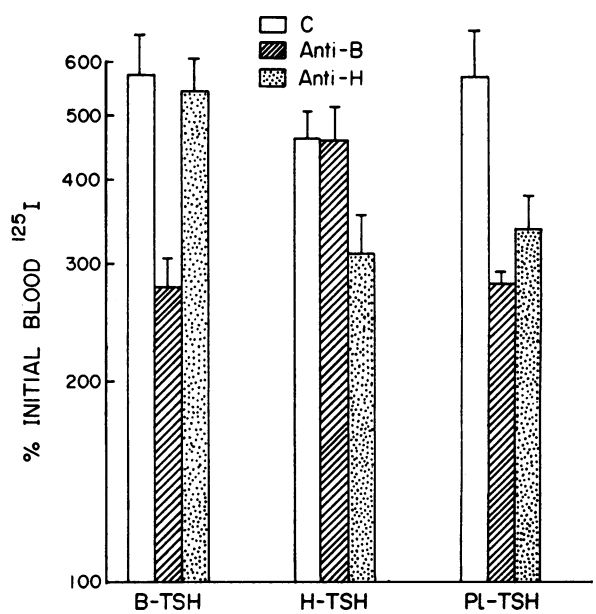

FIGURE 5 Bioassay response of bovine TSH (B-TSH), human TSH (H-TSH), and placental TSH (PL-TSH) after reaction with antibodies to B-TSH (anti-B) or to H-TSH (anti-H). Open bars are control (*C*, no antibody) responses. Vertical lines above the bars represent SE.

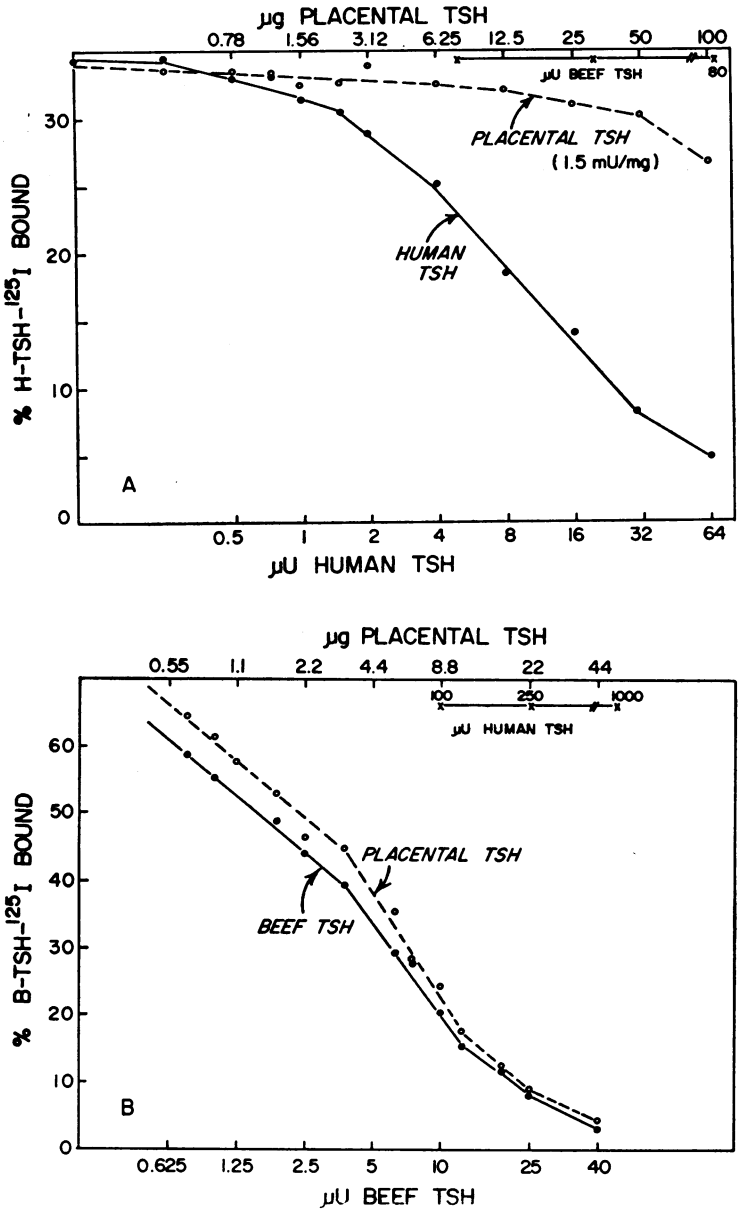

FIGURE 6 (A) Response of placental TSH in radioimmunoassay for human TSH (H-TSH) using antibody to human TSH. Beef TSH does not cross-react. (B) Response of placental TSH in a radioimmunoassay for beef TSH (B-TSH) using antibody to beef TSH. Human TSH does not cross-react.

partially neutralized the PL-TSH ($P < 0.01$ for comparison with control); the antibody to pituitary H-TSH neutralized the activity of the PL-TSH to a lesser extent ($P = 0.05$ for comparison with control). This quantity of antibody to B-TSH partially neutralized B-TSH ($P < 0.01$) but did not neutralize H-TSH; this amount of antibody to H-TSH partially neutralized H-TSH ($P < 0.05$) but not B-TSH.

The PL-TSH was tested for cross-reaction in a sensitive radioimmunoassay for pituitary H-TSH shown in Fig. 6, panel *A*. There was only a small degree of cross-reaction; 100 μg of purified placental thyrotropin with a potency of 1.5 mU/mg on bioassay was equivalent to only 3 μU of H-TSH in the immunoassay. B-TSH did not cross-react in this system. Panel *B* shows the cross-reaction of the same PL-TSH in a radioimmuno-

assay for pituitary B-TSH. Pituitary H-TSH and human chorionic gonadotropin did not cross-react in this assay. The placental material paralleled the beef standard very closely. The activity of the PL-TSH based on this immunoassay was 0.9 mU/mg. Repeated bioassays of PL-TSH preparations gave results very similar to those obtained by use of the radioimmunoassay of B-TSH. The latter assay could be performed with as little as 5 μU in contrast with 5 mU for the bioassay. The B-TSH immunoassay was used for assaying eluates from columns as shown in Fig. 2.

DISCUSSION

We have shown that the placenta contains a thyroid-stimulating material. With the application of similar extraction techniques to pooled plasma from pregnant women and to grossly normal liver and kidney from autopsied patients, we failed to detect thyroid-stimulating activity. The content of TSH extracted from different batches of placentas varied considerably, the mean being 645 mU/placenta. This estimate agrees well with that of Hennen who calculated, on the basis of less extensive data, that there was 124 mU/100 g of placenta (6). Our estimate is probably an underestimate because it is likely that significant losses occurred during the extraction procedures. In several preparations, ^{125}I-labeled TSH was added before extraction with KCl; approximately 20–50% of the label was recovered in the E fraction. A smaller loss occurred during chromatography on carboxymethyl cellulose. The TSH content of human pituitary glands is approximately 200–1000 mU/gland (14, 15), so that the TSH content of the placenta approximates that of the pituitary. The concentration of pituitary H-TSH in the serum of pregnant women in labor is 4 μU/ml when measured by radioimmunoassay in our laboratory; this is in our normal range for men and nonpregnant women. The concentration of TSH in the placenta, 1 mU/g, is approximately 250 times the serum concentration. The PL-TSH was bioassayed against the International Beef TSH Standard. The radioimmunoassay of human serum is based on the International Human Reference Standard A. In the McKenzie bioassay in our laboratory, the Human Reference Standard A has only two-fifths the potency assigned to it when it is assayed against the International Beef Standard. In most assays, the slope of this preparation was different from that of the beef standard (as in Fig. 3), so that a valid estimate of its relative potency in terms of the beef standard cannot be obtained. This agrees with the findings of McKenzie (16), but the point remains to be settled definitely.

The response of the placental thyrotropin in the bioassay was parallel to that of the standard preparation and clearly exceeded the nonspecific responses of a variety of peptides which may cause as much as a twofold increase in radioiodine concentration in the blood of the mice (17). The assays for LATS did not show any difference between pituitary TSH and the PL-TSH in the time course of the response. This contrasts with Hennen's report that the placental material had a more prolonged effect with the ratio 9 hr response: 2 hr response of 1.0 (6). The thyroid-stimulating factor found in crude preparations of chorionic gonadotropin did not have a LATS effect (7).

The most potent preparation we obtained had a specific activity of 8 mU/mg which is only 1/1000 that of purified pituitary H-TSH. The mobility of this material on Sephadex G-100 was identical with that of ^{125}I-labeled H-TSH or B-TSH, a finding suggesting that it has the same molecular weight, approximately 25,000 (18). Another small TSH peak with much lower specific activity eluted earlier suggests that there is a second placental protein with TSH activity. This might represent an aggregate of the species with molecular weight 25,000, or binding to a carrier protein, or a less active precursor in the tissue, akin to proinsulin (19).

The biologic neutralization of PL-TSH with antibodies to both pituitary H-TSH and B-TSH is not surprising. Antibodies to pituitary B-TSH have neutralized the biologic activity of pituitary H-TSH (20). The cross-reactivity of the PL-TSH in the immunoassay for B-TSH and the paucity of reactivity in the immunoassay for pituitary H-TSH is most interesting. Clearly, the placental H-TSH has a closer immunologic resemblance to the corresponding pituitary hormone from another species. By analogy, recently Friesen, Suwa, and Pare found that monkey placental lactogen was more closely related immunologically to human growth hormone than to human placental lactogen (21).

The physiologic role of the placental thyrotropin remains speculative. Several studies showed that the activity of the thyroid gland increases in pregnancy (22–24). The frequency of thyroid enlargement in the gravid state has not been adequately explained (25). Could secretion of placental thyrotropin cause these changes? We have not yet obtained evidence that the placental material is secreted into the maternal circulation. Using the sensitive radioimmunoassay for B-TSH, we have not detected this material in the serum of women in labor. Uterine vein blood from six women obtained at cesarean section did not react in the radioimmunoassay. However, Burger's report that crude urinary preparations of human chorionic gonadotropin contain a thyrotropic factor suggests that placental thyrotropin circulates (7). Several reports on thyroid function in patients with choriocarcinoma or mole suggest that the abnormal trophoblast secretes a thyroid stimulator (5, 26–28). It is also possible that the placental material is secreted into the fetal circulation and stimulates the fetal thyroid.

Subsequent studies may clarify the role of the placental thyrotropin.

ACKNOWLEDGMENTS

We are grateful to Ann Bailey, Etherine Pearson, and Alfred Rhodes for their technical assistance.

REFERENCES

1. Goss, D. A., and J. Lewis, Jr. 1964. Immunologic differentiation of luteinizing hormone and human chorionic gonadotropin in compounds of high purity. *Endocrinology.* **74:** 83.
2. Bagshawe, K. D., C. E. Wilde, and A. H. Orr. 1966. Radioimmunoassay for human chorionic gonadotrophin and luteinising hormone. *Lancet.* **i:** 1118.
3. Josimovich, J. B., and J. A. MacLaren. 1962. Presence in the human placenta and term serum of a highly lactogenic substance immunologically related to pituitary growth hormone. *Endocrinology.* **71:** 209.
4. Friesen, H. 1965. Purification of a placental factor with immunological and chemical similarity to human growth hormone. *Endocrinology.* **76:** 369.
5. Odell, W. D., R. W. Bates, R. S. Rivlin, M. B. Lipsett, and R. Hertz. 1963. Increased thyroid function without clinical hyperthyroidism in patients with choriocarcinoma. *J. Clin. Endocrinol. Metab.* **23:** 658.
6. Hennen, G. 1965. Detection and study of a human-chorionic-thyroid-stimulating factor. *Arch. Int. Physiol. Biochem.* **73:** 689.
7. Burger, A. 1967. Studies on a thyroid stimulating factor in urinary chorionic gonadotrophin preparations. *Acta Endocrinol.* **55:** 587.
8. Reisfeld, R. A., U. J. Lewis, N. G. Brink, and S. L. Steelman. 1962. Human growth hormone: preparation from acetone powder. *Endocrinology.* **71:** 559.
9. Greenwood, F. C., W. M. Hunter, and J. J. Glover. 1963. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89:** 114.
10. Condliffe, P. G. 1963. Purification of human thyrotropin. *Endocrinology.* **72:** 893.
11. Hershman, J. M. 1967. Stability of thyrotropin in the rat pituitary gland after death. *Endocrinology.* **80:** 302.
12. Odell, W. D., P. L. Rayford, and G. T. Ross. 1967. Simplified, partially automated method for radioimmunoassay of human thyroid-stimulating, growth, luteinizing, and follicle stimulating hormones. *J. Lab. Clin. Med.* **70:** 973.
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265.
14. Bakke, J. L., N. Lawrence, K. P. Knudtson, S. Roy, and G. H. Needham. 1964. A correlative study of the content of thyroid stimulating hormone (TSH) and cell morphology of the human adenohypophysis. *Amer. J. Clin. Pathol.* **41:** 576.
15. Lepp, A., and L. Oliner. 1967. Dependence of hormonal yield on the collection method for human pituitary glands. I. Thyrotropin. *Endocrinology.* **81:** 299.
16. Odell, W. D., J. F. Wilber, and R. D. Utiger. 1967. Studies of thyrotropin physiology by means of radioimmunoassay. *Recent Progr. Hormone Res.* **23:** 47.
17. Werner, S. C., J. Tierney, and T. Tallberg. 1964. Thyrotropic and "long-acting thyroid stimulator" effects from certain polypeptides. *J. Clin. Endocrinol. Metab.* **24:** 339.
18. Shome, B., D. M. Brown, S. M. Howard, and J. G. Pierce. 1968. Bovine, human, and porcine thyrotropins: molecular weights, amino- and carboxyl-terminal studies. *Arch. Biochem. Biophys.* **126:** 456.
19. Steiner, D. F., D. Cunningham, L. Spigelman, and B. Aten. 1967. Insulin biosynthesis: evidence for a precursor. *Science.* **157:** 697.
20. Werner, S. C., B. C. Seegal, and E. F. Osserman. 1961. Immunologic and biologic characterization of antisera to beef thyrotropic preparations. *J. Clin. Invest.* **40:** 92.
21. Friesen, H., S. Suwa, and P. Pare. 1969. Synthesis and secretion of placental lactogen and other placental proteins. *Recent Progr. Hormone Res.* **25.** In press.
22. Pochin, E. E. 1952. The iodine uptake of the human thyroid throughout the menstrual cycle and in pregnancy. *Clin. Sci.* **11:** 441.
23. Halnan, K. E. 1958. The radioiodine uptake of the human thyroid in pregnancy. *Clin. Sci.* **17:** 281.
24. Aboul-Khair, S. A., J. Crooks, A. C. Turnbull, and F. E. Hytten. 1964. The physiological changes in thyroid function during pregnancy. *Clin. Sci.* **27:** 195.
25. Freedberg, I. M., M. W. Hamolsky, and A. S. Freedberg. 1957. The thyroid gland in pregnancy. *N. Engl. J. Med.* **256:** 505.
26. Dowling, J. T., S. H. Ingbar, and N. Freinkel. 1960. Iodine metabolism in hydatidiform mole and choriocarcinoma. *J. Clin. Endocrinol. Metab.* **20:** 1.
27. Kock, H., H. von Kessel, L. Stolte, and H. von Leusden. 1966. Thyroid function in molar pregnancy. *J. Clin. Endocrinol. Metab.* **26:** 1128.
28. Galton, V. A. 1968. Abnormalities of thyroid hormone economy in patients with hydatidiform mole. Program of the Annual Meeting of the American Thyroid Association. 35.