Significance of Phosphorus–Nitrogen Ratio in U.S.P. Thyroid

By ALBERT D. WILLIAMS, LESTER MEISTER, MARJORIE FAIRCLOTH, and WARNER H. FLORSHEIM

Biologically defective U S.P. thyroid tablets which have appeared recently on the American market have been prepared from triple strength U.S.P. desiccated thyroid powder supplied by a European distributor. This material, with an unnaturally high iodotyrosine and low iodothyronine content, also has a phosphorus–nitrogen ratio greater than that found in genuine U.S.P. thyroid. Chemical evidence suggests that suboptimally iodinated casein was added to thyroid powder to increase the organic iodine content of the blend. Artificial mixtures have been prepared which closely resemble the physical, chemical, and biological characteristics of known defective U.S.P. thyroid.

A CHEMICAL METHOD for the detection of biologically defective but pharmacopeial thyroid preparations recently has been described (1). Since the organic iodine content in these samples met U.S.P. requirements, even though the thyronine iodine content was reduced, the balance of the organic iodine in the defective preparations was thought to consist of nonhormonal iodinated organic compounds. Twenty-two of 24 biologically defective thyroid preparations studied have been traced to a single European distributor of triple strength U.S.P. desiccated thyroid powder.

The possibility existed that an unnatural iodinated protein had been added to genuine desiccated U.S.P. thyroid, resulting in a mixture with an organic iodine content of approximately 0.6%; the product had characteristics capable, on dilution, of satisfying all current U.S.P. requirements. Several iodinated proteins which could have been used for this purpose were iodinated casein, iodinated peanut protein, or artificially iodinated thyroid powder.

More than 30 years ago, Gutman *et al.* (2) suggested the feasibility of adulterating desiccated thyroid with iodinated casein and pointed out that such adulteration would be undetectable by existing pharmacopeial methods. Although it is possible to identify such substandard thyroid preparations by biological and chemical means (1), the adulterant has not yet been directly identified. Evidence suggesting the presence of iodinated casein in defective U.S.P. thyroid is reported here based on established knowledge that casein is inexpensive and easily iodinated and may be the only known phosphoprotein having such characteristics.

PROCEDURE

Iodine Assay.—Methods for the determination of total iodine in thyroid, iodoproteins, and iodinated compounds have been described (1).

Phosphorus Assay.—Powdered samples of thyroid or iodoprotein were extracted twice with 10% trichloroacetic acid (TCA) to remove inorganic phosphorus. The residue was extracted with dimethoxymethane. The phosphorus content of the lipid-free, TCA-insoluble residue was determined by King's modification (3) of the Fiske and Subbarow method (4).

Nitrogen Assay.—Nitrogen analysis of the samples was done by a micro-Kjeldahl digestion, followed by steam distillation of the ammonia into boric acid for titrimetric measurement (5).

Column Chromatography.—Dowex-1 anion-exchange resin columns and eluting solutions were prepared as described by Galton and Pitt-Rivers (6). Following alkaline hydrolysis of 20–60-mg. samples of iodinated protein, desiccated thyroid or pure iodinated compounds (1), the filtered hydrolysates were neutralized and diluted to known volumes. Suitable portions were washed into Dowex-1 columns previously equilibrated with pH 5.6 acetate buffer. The columns were developed with aqueous acetic acid of progressively decreasing pH, and the pH 1.8 and pH 1.4 effluents were collected separately. These effluents were analyzed for total iodine. The fraction of the total iodine of the original sample which appeared in the pH 1.8 and in the pH 1.4 effluent then was calculated.

Preparation of Poorly Iodinated Casein.—Casein was iodinated by a modification of a procedure of Reineke and Turner (7). To a solution of 1 Gm. $NaHCO_3$ in 700 ml. of water at 70°, 20 Gm. of casein was added slowly with constant stirring. After the casein had dissolved, 0.5 Gm. of finely ground iodine was added slowly over a period of 4 hours with constant stirring, and the mixture was stirred at 70° for 20 hours. After dialyzing against flowing tap water overnight, the iodinated casein was precipitated by slow addition, with constant stirring, of 3 *N* HCl to pH 3.9. The precipitate was recovered by filtration and washed with acidified (pH 4.0) water. This iodocasein (sample 115) was dried at 60°, ground to pass through an 80-mesh screen, and had an iodine content of approximately 1%. Another iodocasein (sample 119) was prepared as described above, except that iodination and incubation temperatures were 40°.

RESULTS

Column Chromatography.—Recovery of the following pure compounds from Dowex-1 anion-exchange columns was evaluated: sodium L-thyroxine (T-4), sodium L-3,3′,5-triiodothyronine (T-3), L-3,5-diiodothyronine (T-2), L-3,5-diiodotyrosine (DIT), and L-3-monoiodotyrosine (MIT).[1] Alkaline hydrolysates of duplicate samples of each pure compound were neutralized and filtered. Portions of the diluted filtrates were chromato-

Received April 9, 1964, from the Radioisotope and Medical Services, Veterans Administration Hospital, Long Beach, Calif., and the University of California Medical School, Los Angeles.

Accepted for publication June 1, 1964.

Supported in part by research grant A-4455 from the U. S. Public Health Service, Bethesda, Md.

Presented in part to the Federation of American Societies for Experimental Biology, Chicago meeting, April 1964.

The authors thank Miss Audrey Kam and Mr. David J. Myers for technical assistance in certain phases of this work. We are also indebted to the many individuals and pharmaceutical companies who provided information and samples for our use.

[1] These compounds were purchased from California Corporation for Biochemical Research, Los Angeles, Calif.

TABLE I.—DOWEX-1 COLUMN RECOVERY OF HYDROLYZED PURE COMPOUNDS

Compd.	% of Total Iodine in Compd. Iodine in pH 1.8 Effluent	Iodine in pH 1.4 Effluent
Sodium L-thyroxine	1.2	92.7
Sodium L-3,3′,5-triiodothyronine	2.0	75.2
L-3,5-Diiodothyronine	0.6	100.0
L-3,5-Diiodotyrosine	95.4	2.3
L-3-Monoiodotyrosine	94.7	1.2

graphed on Dowex-1 columns, collecting the pH 1.8 and the pH 1.4 effluents separately.

The values for iodine recovered in the pH 1.8 and in the pH 1.4 effluents (Table I), expressed as per cent of total iodine in each compound, are the averages of duplicate experiments, which agreed closely. These results show a 95% recovery of MIT and DIT iodine in the pH 1.8 effluent with only 1–2% appearing in the pH 1.4 effluent. In contrast, 93% of the T-4, 75% of the T-3, and the bulk of the T-2 iodine were recovered in the pH 1.4 effluent with only 1–2% appearing in the pH 1.8 effluent. These results are comparable to those reported by Galton and Pitt-Rivers (6), who separated I^{131}-labeled iodinated tyrosines and thyronines by Dowex-1 anion-exchange column chromatography.

In the results reported here, the iodine in the pH 1.8 effluent from the Dowex-1 columns is considered to represent DIT + MIT (iodotyrosine) iodine, while the iodine in the pH 1.4 effluent represents T-4 + T-3 (iodothyronine) iodine. No attempt was made to separate MIT from DIT or T-4 from T-3.

The mean values for DIT + MIT and T-4 + T-3 iodine of several different iodocaseins, an iodoardein sample,[2] and a number of effective and defective U.S.P. thyroid samples appear in Table II. The organic iodine content of each preparation was determined after two extractions with TCA to remove inorganic iodine known to be present in some of the synthetic iodoproteins. TCA-soluble iodine was not found in the U.S.P. thyroid samples or in iodocasein samples 86, 115, and 119. However, TCA-soluble iodine constituted 10–30% of the total in iodoardein and in iodocasein samples 70 and 46.

The iodothyronine iodine contents of samples 86 and 70, unlike those of iodotyrosine iodine, were similar (Table II). Both were samples of iodinated casein[3]; sample 86 was supplied as pharmaceutical grade, *i.e.*, free of inorganic iodine. Sample 70 contained appreciable inorganic iodine, and the possibility that the alkaline hydrolysis procedure resulted in some iodination of tyrosine residues was not investigated.

The mean iodothyronine iodine content of the effective thyroid samples is significantly higher than that of the defective samples ($p < 0.001$). Conversely, the mean iodotyrosine iodine content of the defective samples is higher ($p < 0.001$) than that of the effective samples (Table II).

[2] Iodinated peanut protein. Marketed as Ardein by Imperial Chemical Industries, Ltd., England. Kindly supplied by Professor A. S. Parkes, Cambridge, England.

[3] Marketed as Protamone by Agri-Tech, Inc., Kansas City, Mo.

Phosphorus Content of Iodoproteins.—The content of phosphorus and nitrogen in thyroglobulin, iodocasein, iodoardein, and effective and defective U.S.P. thyroid samples was measured and the mean values, expressed as milligrams of phosphorus per gram of nitrogen, are given in Table III. The phosphorus–nitrogen (P/N) ratio in defective U.S.P. thyroid is significantly higher ($p < 0.001$) than in effective thyroid. Iodinated casein has the highest P/N ratio.

Studies with Artificial Mixtures.—Sufficient suboptimally iodinated casein (approximately 1% organic iodine) was blended with effective U.S.P. thyroid powder to produce mixtures with a total organic iodine content of approximately 0.6%. The components in Table IV used for the preparation of two artificial mixtures containing no detectable inorganic iodine are listed together with the total iodine content of each. The iodotyrosine and iodothyronine iodine content and the P/N ratios of each mixture in Table IV can be compared to corresponding values in Tables II and III for effective and defective U.S.P. thyroid samples. The biological activity of one of the artificial mixtures reported in Table IV was estimated to be less than 60% of that of a standard thyroid preparation; the other artificial mixture was inert.

DISCUSSION

In this extension of an earlier report of chemically detectable differences between biologically effective and defective U.S.P. thyroid (1), a high iodotyrosine and a low iodothyronine iodine content of defective U.S.P. thyroid has been demonstrated (Table II). Estimates of the MIT + DIT and T-4 + T-3 iodine content of effective desiccated thyroid are similar to those reported for hydrolysates of fresh thyroid glands from several species (8, 9). A low iodothyronine and a high iodotyrosine content in suboptimally iodinated casein having a total organic iodine content of about 1% has been observed by Roche and Michel (10).

The mean P/N ratio of the iodocasein samples (Table III) agrees well with published data for casein, a phosphoprotein (11). In accordance with others (12), thyroglobulin has a low P/N ratio. The results (Table III), an indication of a P/N ratio significantly higher in defective than in effective U.S.P. thyroid, seem notably relevant since they strongly suggest the presence of iodinated phosphoprotein in defective thyroid. The increased P/N ratio in the defective preparations is explained most readily by assuming that an iodocasein was blended with desiccated thyroid powder. This would raise the organic iodine content of the mixture to triple strength U.S.P. but with a concomitant elevation of phosphorus.

Determination of the P/N ratio in thyroid offers a secondary procedure for detection of nongenuine thyroid. Although the P/N ratio of commercial thyroid preparations seems at present a reliable indicator of adulteration by iodinated casein, it does not necessarily predict the hormonal content of these preparations. The hormonal content of iodocasein is chiefly related to its organic iodine content, for which there is an optimal level approximating 6% (7, 10). Optimally iodinated casein

TABLE II.—FRACTIONATION OF HYDROLYSATES OF IODINATED PROTEINS AND DESICCATED THYROID ON DOWEX-1 COLUMNS

Sample	Organic Iodine, %	% of Organic Iodine: DIT + MIT Iodine (pH 1.8 Effluent)	% of Organic Iodine: T-4 + T-3 Iodine (pH 1.4 Effluent)
Iodocasein (No. 86)[a]	5.92	29.9	19.7
Iodocasein (No. 70)[a]	5.10	48.5	20.9
Iodocasein (No. 46)[b]	6.15	54.9	14.1
Iodocasein (No. 115)	0.96	80.4	11.3
Iodocasein (No. 119)	0.89	86.2	4.5
Iodoardein	0.70	24.5	8.4
Effective U.S.P. thyroid (11 samples):		44.4 ± 1.2[c] (38.7–47.6)	14.4 ± 0.7[c] (11.6–19.6)
Defective U.S.P. thyroid (11 samples):		61.5 ± 1.7 (53.1–67.5)	7.7 ± 0.4 (6.7–10.3)

[a] Kindly supplied as Protamone by Agri-Tech, Inc., Kansas City, Mo. [b] Produced by Agri-Tech, Inc., by a process since modified. [c] Mean, standard error, and range of values.

TABLE III.—PHOSPHORUS CONTENT OF IODINATED PROTEINS AND DESICCATED THYROID

Sample	Samples, No.	mg. P per Gm. N: Mean	S.E.	Range
Effective U.S.P. thyroid	9	14.0	0.71	11.1–17.6
Thyroglobulin	3	4.4[a]	0.57	3.7– 5.6
Defective U.S.P. thyroid	10	30.2[a]	3.17	18.8–52.0
Iodocasein	5	56.8[a]	4.10	46.6–67.7
Iodoardein	1	4.6		

[a] Significantly different from effective U.S.P. thyroid ($p < 0.001$).

may have a thyroxine content of 1% in addition to small but biologically significant amounts of triiodothyronine (13)—although compared to desiccated thyroid, the hormone in iodocasein is biologically less available perorally in the human (14) and in the rat (15).

Present analytical procedures (1, 16) overestimate the iodothyronine content in iodocasein. Therefore, it is theoretically possible to adulterate U.S.P. thyroid with an optimally iodinated casein yielding a product with substandard clinical thyroactivity yet having an acceptable P/N ratio and an apparently satisfactory iodothyronine content. However, commercial adulteration of thyroid with optimally iodinated casein probably would be precluded due to the effort and expense involved.

The mixtures of suboptimally iodinated caseins with U.S.P. thyroid closely mimic triple strength U.S.P. thyroid powder physically but are low in biological activity. Although the total organic iodine content is similar in these mixtures (Table IV), the iodothyronine content varies by a factor of 2, being dependent on the iodocasein moiety (Tables II and IV).

Since Ardein may be commercially available and has been studied in its iodinated forms for thyroactivity (17), the possibility existed that iodoardein had been used to adulterate desiccated thyroid. However, the low P/N ratio and the relatively low iodotyrosine content of the single iodoardein sample available for study (Tables II and III), in contrast to high corresponding values in defective U.S.P. thyroid, make this possibility most unlikely.

It seemed possible that desiccated thyroid powder could have been artificially iodinated to increase the organic iodine content of the material. Such treatment would not change the P/N ratio of the product; in view of the high P/N ratios of the defective thyroid samples, it is improbable that thyroid powder was iodinated directly.

SUMMARY AND CONCLUSIONS

Appropriate amounts of two noncommercial iodinated casein preparations have been mixed with effective U.S.P. thyroid to obtained two products, each having a total iodine content equivalent to triple strength U.S.P. thyroid and containing no inorganic iodine. These mixtures have biological activities varying from inert to 60% of a standard commercial thyroid preparation and have a P/N ratio higher than genuine U.S.P. thyroid. Chemical characteristics (P/N ratios and iodothyronine and iodotyrosine content) of the biologically less active artificial mixture resemble corresponding values for a group of biologically defective commercial thyroid preparations. Clinically defective commercial U.S.P. thyroid preparations differ strikingly from effective samples in relative iodothyronine and iodotyrosine content and in P/N ratios.

The evidence presented supports the hypothesis that suboptimally iodinated casein, free of inorganic iodine, has been added to desiccated thyroid, raising the total iodine content of the mixture to a level equivalent to triple strength U.S.P. thyroid. Such material, satisfying all current

TABLE IV.—CHARACTERISTICS OF ARTIFICIAL THYROID MIXTURES

Mixture No.	Components	Organic Iodine[a] %	% of Organic Iodine: DIT + MIT Iodine	% of Organic Iodine: T-4 + T-3 Iodine	Mg. P / Gm. N	Bioassay[b]
1	Iodocasein[c] Desiccated thyroid[d]	0.620	42.1	13.1	45.8	<60%
2	Iodocasein[e] Desiccated thyroid[d]	0.665	51.2	6.3	46.7	Inert

[a] The artificial mixtures contained no detectable inorganic iodine. [b] The authors thank the Wilson Laboratories, Chicago, Ill., for bioassay of these samples by a rat goiter-prevention method. [c] Sample 115, Table II (iodinated at 70°). [d] Effective U.S.P. desiccated thyroid powder (0.20% iodine). [e] Sample 119, Table II (iodinated at 40°).

U.S.P. chemical requirements for desiccated thyroid —although biologically substandard—may thus have been used for the manufacture of defective U.S.P. thyroid tablets. It has not been possible to ascertain with certainity the primary source of the nongenuine thyroid material.

REFERENCES

(1) Williams, A. D., Meister, L., and Florsheim, W. H., THIS JOURNAL, 52, 833(1963).
(2) Gutman, A. B., Benedict, E. M., and Palmer, W. W., *Proc. Soc. Exptl. Biol. Med.*, 29, 1198(1932).
(3) King, E. J., *Biochem. J.*, 26, 292(1932).
(4) Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 66, 375(1925).
(5) Hawk, P. B., Oser, B. L., and Summerson, W. H., "Practical Physiological Chemistry," 13th ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1954, p. 881.
(6) Galton, V. A., and Pitt-Rivers, R., *Biochem. J.*, 72, 310(1959).
(7) Reineke, E. P., and Turner, C. W., *Res. Bull. Agri. Expt. Station*, Coll. Agri., Univ. Missouri, No. 355, 1942, pp. 36–41.
(8) Lerner, S. R., *Arch. Biochem. Biophys.*, 103, 36(1963).
(9) Kennedy, T. H., and Purves, H. D., *Australian J. Biol. Sci.*, 9, 586(1956).
(10) Roche, J., and Michel, R., *Ann. Endocrinol.*, 12, 317(1951).
(11) Cohn, E. J., and Edsall, J. T., "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 341.
(12) Derrien, Y., Michel, R., and Roche, J., *Biochim. Biophys. Acta*, 2, 454(1948).
(13) Turner, C. W., and Bauman, T. R., *J. Dairy Sci.*, 45, 1256(1962).
(14) Hamilton, C. F., Albert, A., and Power, M. H., *Endocrinol.*, 43, 406(1948).
(15) Frieden, E., Tuckich, E. B., and Winzler, R. J., *ibid.*, 45, 82(1949).
(16) Pitt-Rivers, R., in "Hormone Assay," Emmens, C. W., ed., Academic Press Inc., New York, N. Y., 1950, pp. 513–542.
(17) Pitt-Rivers, R., and Randall, S. S., *J. Endocrinol.*, 4, 221(1945).

Determination of Iron Content in Mice

By THEODORE H. EICKHOLT* and WALLACE F. WHITE

A colorimetric method was employed in the estimation of the normal iron content of mice. An estimate of the total amount of iron present per mouse was 1.457 ± 0.152 mg. This represented an average of 52 ± 6 mcg. per Gm. body weight of mouse with approximately 11.5 per cent of the total iron per mouse present in the gastrointestinal tract and 88.5 per cent in the remainder of the carcass.

AN ESTIMATION of normal iron content of mice, the total amount contained therein compared with the amount contained in the separated entire gastrointestinal tract, is indicated in studies of iron absorption following oral administration.

There are numerous methods available for estimating iron content; *o*-phenanthroline, α,α'-dipyridyl, and benzidine methods (1–13). A sodium sulfocyanate or potassium thiocyanate method was described by Kennedy (14), improved and simplified by Farrar (15), Andes and Northup (16), and Wong (17), resulting in the method for the determination of iron in blood and hemoglobin published by the Fisher Scientific Company (18).

Employing a modification of the Fisher method, it was the purpose of this investigation to determine an estimate of the normal amount of iron present in a whole mouse carcass and that present in the separated gastrointestinal tract. This latter method of separation of carcass and gastrointestinal tract is essentially the method described by Cori *et al.* (19–21).

EXPERIMENTAL

The method employed was a colorimetric analysis with the Fisher model AC electrophotometer. The procedure as published (18) was followed except for the elimination of the tungstate solution. Since the tungstate solution was only involved in precipitating protein, it was unnecessary in the procedures employed in this investigation.

The iron, after being liberated and oxidized with sulfuric acid and potassium persulfate, was treated with potassium thiocyanate. The electrophotometer scale reading of the resulting colored solution was determined, and the iron concentration was calculated from this using a calibration curve.

Following the determination and calculation of the standard iron concentration curve, as described in the Fisher method, iron determinations were done on whole mice fasted at least 20 hours but not longer than 24 hours. Six B4BC male mice, 5 weighing 27 Gm. and one weighing 26 Gm., were sacrificed and digested in a Kjeldahl flask using concentrated sulfuric and nitric acids. The complete digestion resulted in a clear solution of sulfuric acid with or without a small amount of white precipitate (calcium salts) in the bottom of the flask.

The sulfuric acid solution was cooled and slowly diluted to 100 ml. with distilled water and was used as the sample solution for iron determination using the modified Fisher method.

At all times, precautions were taken to use glass distilled water and carefully cleaned glassware.

A separate determination on the gastrointestinal tract and on the remainder of the mouse carcass was done on three 32.5-Gm. B4BC male mice employing the same procedure.

In experiments done to observe the method and obtain more preliminary control figures for the amount of iron present, mice in groups of 6 were fasted, sacrificed, separated as to carcass and entire gastrointestinal tract, digested, and an aliquot amount obtained for sample determination. In a number of animals equaling that of the controls, the same procedure was employed, except that 1 hour prior to sacrificing the animals received a total oral dose of 1, 2, or 4 mg. of ferrous sulfate in solution, the concentration of which was varied to provide near equal volumes orally administered.

Ordinarily, the feces had not been dealt with. In two of the above groups of animals, one a con-

Received April 6, 1964, from the Department of Pharmacology, University of Minnesota, Minneapolis.
Accepted for publication May 5, 1964.
Abstracted in part from a thesis submitted by Theodore H. Eickholt to the Graduate School, University of Minnesota, in partial fulfillment of Doctor of Philosophy degree requirements.
* Present address: School of Pharmacy, Northeast Louisiana State College, Monroe.