THYROACTIVE IODINATED PROTEINS:
I IMMUNOLOGY,
II THYROID HORMONE ANALYSIS

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ABSTRACT

THYROACTIVE IODINATED PROTEINS: I IMMUNOLOGY, II THYROID HORMONE ANALYSIS

by Terrence Wynn Mischler

Thyroactive iodinated casein has been injected and fed to animals for increasing their thyroid activity ever since its synthesis some twenty-five years ago. Yet neither its immunological properties nor the concentration of all its thyroid hormones have ever been determined. These properties of a commercially synthesized iodinated casein, Protamone, were investigated using immunological double diffusion and thin-layer chromatography.

Protamone contains at least one antigenic component found by diffusing anti-Protamone against it in double diffusion analysis. This antigen was called the Protamone antigen. It and two others were observed when anti-Protamone was diffused against casein. Thus Protamone produced antibodies against three antigens of casein origin in Protamone. However, anti-casein diffused against Protamone would result in no precipitation and when diffused against casein no precipitation of the Protamone antigen was seen. Thus, the Protamone antigen when present in casein was unable to produce

antibodies. Iodinated casein prepared in the laboratory contained at least two antigens of casein origin. Proteins extracted from rat thyroid glands contained two thyroid specific antigens. They are believed to represent the 19S and 27S proteins known to exist in the gland.

There was, with one exception, no cross reaction between any of the anti-iodinated protein sera and their respective proteins. However, when anti-Protamone was diffused against iodinated casein the Protamone antigen was again observed. Therefore, the Protamone antigen was present in Protamone, casein and iodinated casein, yet it produced antibodies only when in Protamone. Hence it is concluded that the process of Protamone synthesis altered the antigen such that it was able to produce antibodies. This alteration was not involved with the iodination process since the iodinated casein prepared in the laboratory did not contain the altered antigen. Finally, anti-Protamone demonstrated the presence of the Protamone antigen in a deficient commercial thyroid preparation suspected of containing poorly iodinated casein.

Two-dimensional thin layer chromatography of Protamone hydrolysates demonstrated at least twenty-two components in the acidified n-butanol soluble fraction. There were only five components remaining when the extract was washed with 4N NaOH - 5% Na_2CO_3. Two of these five components were identified as thyroxine and triiodothyronine by a number of

independent criteria. The concentration of these two hormones was determined to be 0.79% thyroxine and 0.61% triiodothyronine. This is biologically equivalent to 3.28% thyroxine. The combined activity of thyroxine and triiodothyronine in Protamone and similar iodinated proteins as calculated from this analysis, is sufficient to account for thyroidal activity values obtained earlier from biological assays in mammals. Since this chemical assay is sufficient to account for all biologically determined thyroidal activity, it is unlikely that there are other thyroid active compounds in iodinated casein which have thyroid activity in mammals. This was the first time that a complete analysis of all thyroidal hormones has been accomplished on a thyroid active iodinated protein synthesized in vitro.

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By

Terrence Wynn Mischler

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INTRODUCTION

The *in vitro* synthesis of a thyroid active iodinated protein was accomplished by Dr. E. Paul Reineke some twenty-five years ago. The use of a properly buffered casein solution and incubation at relatively high temperatures, after addition of the correct amount of iodine, resulted in the thyroid active protein called iodinated casein. It was shown, after vigorous hydrolysis, that L-thyroxine could be isolated from this substance. However further research has led a few investigators to suspect that other iodinated thyronines, including triiodothyronine, may also be synthesized during the iodination of casein. Assays of iodinated casein for its thyroxine content have shown considerable variation in their values. Therefore, the true total biological activity in terms of thyroxine content is, at this time, not really known.

There is no information regarding the immunological properties of synthetic thyroid active iodinated caseins and their immunological relationships, if any, to naturally occurring thyroid proteins. These synthetic proteins are fed and/or injected into animals as a protein--yet no data exists on their antigenicity.

It was decided to concentrate this investigation on the commercial iodinated casein--Protamone since it is a

standardized product readily available and is widely used to increase thyroid activity of animals.

The purpose of the research reported herein was two-fold. First, to study the immunological properties of Protamone and compare them to those of two other iodinated proteins; namely, rat thyroid protein and a laboratory-prepared iodinated casein. The second purpose was to obtain an analysis of all thyroid active compounds that exist in Protamone. Therefore, the hydrolysates of Protamone were fractionated and the compounds known to have thyroid activity identified. At this time an attempt will be made to determine the concentration of these compounds and then if this is successful the total thyroid activity of Protamone in terms of hormone concentration will be established.

REVIEW OF THE LITERATURE

Iodinated Casein

With the isolation of thyroxine by Kendall (1915) and its synthesis by Harington and Barger (1927), there were numerous attempts to form thyroxine *in vitro* by iodination of proteins. The publications concerning these early attempts have been thoroughly reviewed by Reineke (1942a, 1946, 1949). The attempts at *in vitro* synthesis were, in general, not successful but considerable information was obtained on methods and conditions of iodination. Those claims of success were met with skepticism since assays of early products were questionable. Crystalline thyroxine was first isolated from a protein iodinated *in vitro* by Ludwig and von Mutzenbecher (1939) and verified by Harington and Pitt-Rivers (1939). There was considerable research in the 1940's by Reineke and co-workers on the conditions needed for iodination of proteins.

Reineke and Turner (1942a) found that a bicarbonate buffer, above pH 7.0, was needed to produce a thyroid active iodinated casein. Reineke, Williamson and Turner (1942b) investigated the progressive iodination of casein and found that maximum activity was obtained when 2 moles of iodine per mole of tyrosine was used for iodination. Up to this time all research on protein iodination had been conducted at 38°C,

considered optimal for thyroxine formation. But in 1943a Reineke, Williamson and Turner iodinated casein with progressive amounts of iodine at higher temperatures (70°C for 20 hours) and, in addition to confirming their earlier report regarding concentration of iodine per tyrosine, they obtained a fourfold increase in activity when incubating at 70°C instead of 38°C. This highly active iodinated casein in the tadpole assay was found to have an apparent 8-11% thyroxine content when compared to a D,L-thyroxine standard. Since Reineke and Turner (1943c) determined that D-thyroxine had no activity this value of 8-11% thyroxine should be halved and thus equals 4-5½% thyroxine.

Crystalline thyroxine was isolated from iodinated casein by Reineke and Turner (1943b) after hydrolysis with 40% barium hydroxide. It was identified by its characteristic crystalline structure, ultraviolet absorbtion curve and iodine content. The yield was 0.424%, whereas, if the acid insoluble fraction of iodinated casein was considered to be all thyroxine, then the yield increased to 2%. Thyroxine is insoluble at pH 4.5 as first determined by Kendall (1915).

Reineke and Turner (1943c) isolated L-thyroxine from iodinated casein by acid hydrolysis and found it had twice the potency of D,L-thyroxine obtained by barium hydroxide hydrolysis. This indicated that barium hydroxide hydrolysis produced racemation of thyroxine resulting in a D,L-mixture.

Reineke, Turner, Kohler, Hoover and Beezley (1945a) used both chemical and biological assays to determine the thyroxine content of iodinated casein. The foundation of the chemical assay was laid by Leland and Foster (1932) in which they extracted hydrolyzed thyroid glands with n-butanol, then 1N NaOH and considered the iodine in this extract to be thyroxine. Blau (1935) modified this method by first extracting the hydrolyzed gland with acidified n-butanol and then washing this solution with a mixture of 4N NaOH and 5% Na_2CO_3. The iodine content of the washed extract was considered to be thyroxine. Reineke et al. (1945a) applied the Blau method to iodinated casein, using 40% barium hydroxide for hydrolysis and found it contained n-butanol soluble iodine equivalent to 3.04% thyroxine. They also demonstrated that diiodotyrosine had no appreciable thyroxine activity and that a loss of only 7% thyroxine occurred during hydrolysis. There are many other chemical assay procedures and the reviews of Pitt-Rivers (1950) and Barker (1962) should be consulted for more information. Bioassays, on iodinated casein, were also conducted by these investigators using the guinea pig CO_2 production method described by Reineke and Turner (1942a). A 2.79% thyroxine content was found, which closely agreed with the chemical assay of 3.04%.

Finally, Reineke and Turner (1945b) found that the addition of any one of a number of manganese oxides, to the incubation mixture, would further increase the potency of the

iodinated casein to 3.37%, as determined by their chemical assay.

Friedberg (1951), using paper chromatography investigated the Blau extraction method when applied to hydrolysates of iodinated casein. He demonstrated that two other compounds, besides thyroxine, were present in the washed n-butanol extract of hydrolyzed iodinated casein. It was then apparent that an error was introduced into the thyroxine assay of iodinated casein using the Blau method, because two compounds besides thyroxine were included in the assay.

Reineke (1954) employed a highly specific isotope dilution technique and found a 1.04% thyroxine content in iodinated casein. This compared to a 3.24% thyroxine content obtained by the Blau chemical analysis on the same material. The 1.04% thyroxine content was considered to be a very accurate estimate of the thyroxine content of iodinated casein, but it is too laborious to be used as a routine analytical procedure.

One assay has been published on the commercial iodinated casein--Protamone, by Turner and Bauman (1962). They employed the thyroxine substitution method in rats to biologically assay this substance. These workers modified the usual procedure by daily injecting 400 μg of tapazole per 100 gm. of rat. They determined a mean thyroxine secretion rate value by subcutaneous thyroxine injections and then using the same rats determined the value obtained by injecting Protamone

instead of thyroxine. They obtained a mean of 1.40% thyroxine equivalent in Protamone, whereas the manufacturer's value was 1.00% thyroxine. However, this publication did not indicate the dosage or number of protamone injections given to the rats. Nor was any evidence presented demonstrating that a steady state equilibrium was obtained during the period of Protamone injections.

There have been few attempts to demonstrate that compounds other than thyroxine are present in iodinated casein. Hird and Trikojus (1948) employed paper chromatography to find two compounds, other than thyroxine, in iodinated casein. One of these appeared to be diiodothyronine and the other was believed to be triiodothyronine because of its position between thyroxine and diiodothyronine.

Friedberg (1951) also using paper chromotography found at least 10 iodinated components in the n-butanol soluble fraction of hydrolyzed iodinated casein. As discussed above, three compounds remained in the alkali-washed extract of hydrolyzed iodinated casein, one of which appeared to be thyroxine. This is the only published evidence that Blau's extraction is not specific for thyroxine values in Protamone.

There has been limited research on the structural and/or immunological properties of non-thyroidal proteins iodinated <u>in vitro</u>. Wormall (1930) is the only investigator of immunological properties of non-thyroidal iodinated proteins that was found in the literature. He demonstrated

by a test tube precipitation test that iodinated serum proteins of a number of mammals lost their species specificity and that a new specificity characteristic for iodoproteins was produced. He believed that 3:5 diiodotyrosine was responsible for this change.

Kamal and Turner (1951) investigated the electrophoretic properties of iodinated casein using the Tiselius apparatus. They demonstrated that non-iodinated casein consisted of an alpha and beta component, whereas iodinated casein had a single component with a mobility slightly faster than either alpha or beta casein. Casein incubated at 70°C, without added iodine, also showed the same electrophoretic pattern. Thus it appeared that incubation and not iodination produced this change in electrophoretic patterns.

Finally, Williams, Meister, Faircloth and Florsheim (1964) have suggested the possibility that a poorly iodinated casein was added to defective foreign commercial desiccated thyroid preparations that were deficient in thyroidal activity. This could increase the organically bound iodine content enough to meet the only U.S.P. requirement; namely, 0.2% organically bound iodine, and consequently decrease the thyroid activity to even less than that of the original preparation. These conclusions were based on P/N and iodotyrosine/iodothyronine ratios which were higher than in a standard active thyroid preparation. These high ratios were found both in a mixture of laboratory-prepared poorly iodinated casein with a

known active thyroid preparation and the defective thyroid preparations. These workers also found by ion-exchange chromatography that 20% of the total iodine in the commercial iodinated casein, Protamone, was in the triiodothyronine and thyroxine fraction.

Properties of Rat Thyroid Proteins

There has been considerable research on the biochemical and immunological properties of thyroid proteins. The biochemical characteristics have been reviewed by Robbins and Rall (1960), Edelhoch (1965), Edelhoch and Rall (1964) and the immunological properties have been reviewed by Belyavin (1964). However, almost all the published biochemical investigations have used bovine, porcine or human thyroid proteins and the immunological investigations have been directed almost exclusively toward research on the problem of autoimmune diseases in the human.

The proteins of the rat's thyroid have received little attention. Lachiver, Fontaine and Martin (1965) labeled thyroid proteins *in vitro* with I^{131}, then waited varying lengths of time before removing the glands and extracting the proteins. They separated the proteins by sucrose gradient centrifugation and found three protein fractions with sedimentation coefficient of 12S, 19S and 27S; the 19S fraction corresponded to thyroglobulin. The I^{131} associated with the 12S fraction reached 4-5% of total protein-bound I^{131} after 4-5 hours and then decreased with time. On the other hand,

I^{131} associated with the 27S fraction increased progressively with time and contained about 10% of total protein bound I^{131} after 32 hours. The remainder of the I^{131} was bound to the 19S fraction.

Robbins, Salvatore, Vecchio and Vi (1966) have investigated the time course of iodination of rat thyroid protein by "equilibrium" and "pulse" labeling. Thyroglobulin (19S) was labeled at a faster rate than the 27S iodoprotein, but the 27S protein contained a higher concentration of labeled constituents. Thus the 27S protein was found to be a major storage site of thyroid hormone even though it may only constitute 10% of the total protein. Also shown by these authors was an ultracentrifugal heterogeneity of thyroglobulin, in which a slower-sedimenting fraction, containing less iodine, was separated from a more highly iodinated faster-sedimenting fraction. It was believed that the slower component was "newly synthesized" thyroglobulin whereas the faster component might be "old" thyroglobulin. The only immunological investigations of thyroid saline extracts where auto-immunity was not being studied, was carried out on beef and hog thyroids by Perelmutter and Stephenson (1964). They observed that 19S (thyroglobulin) and 27S components gave rise to two precipitation bands when diffused against anti-thyroid serum by the immunodiffusion techniques. Immunoelectrophoresis indicated that these antigens had mobilities characteristic of $alpha_2$ globulins. They concluded that the 19S and 27S components appear to have different immunochemical properties.

Thin-Layer Chromatography (TLC) of Iodinated Compounds

West, Wayne and Chavre (1965) were the first to separate iodinated tyrosine and thyronines using TLC. They had limited success employing silica gel as a supporting medium for separation and identification of L-thyroxine and its metabolites in both plasma and bile.

Milstien and Thomas (1965) separated the thyroid hormones and iodide using cellulose as their medium. They described a method for analyzing the iodide content of the cellulose area containing the separated compounds.

The method of Faircloth, Williams and Florsheim (1965) was able to separate all naturally occurring thyroid compounds, iodide and 3:5 diiodothyronine, using two dimensional development on a cellulose medium. One solvent was formic acid/H_2O 1:5 and the other was T-butanol/2N NH_3OH/chloroform 376:70:60. They also reported a procedure for iodine analysis of the chromatographed compounds.

Relative Potencies of Thyroxine to Its Analogues

The literature abounds with publications and reviews regarding the *in vitro* and *in vivo* synthesis, isolation, metabolism, biochemistry and biological properties of thyroxine and its analogues. The text edited by Pitt-Rivers and Trotter (1964) and the reviews by Roche and Michel (1956), Mayo Clinic Proceedings Vol. 39, No. 8 and DeGroot (1965) should be consulted for this information. Assays of thyroxine have already

been discussed above. The concern of the review presented in this thesis is the relative potencies of thyroxine to its analogues, since the bioassay of iodinated casein was based on metabolic responses resulting from injections of iodinated casein, with thyroxine used as a reference compound.

Roche and Michel (1956) have reported that four iodinated thyronines were found in the thyroid gland: (1) L-thyroxine, (2) L-3:5:3' triiodothyronine, (3) L-3,3',5' triiodothyronine and (4) L-3,3' diiodothyronine. These authors state, that number 4 was only slightly less active than thyroxine. However, Stasilli, Kroc and Meltzer (1959) were unable to confirm this report and believe that the L-3:3' diiodothyronine of Roche et al. (1956) was contaminated with L-3:5:3' triiodothyronine. Stasilli et al. (1959) also reported no appreciable calorigenic activity in 38 of 40 thyroxine analogues tested in rats. The only two having any appreciable activity were the naturally occurring thyroid hormones, thyroxine and L-3:5:3' triiodothyronine. It therefore appears that triiodothyronine and thyroxine are the only iodinated compounds which have any significant biological activity, at least when tested in the rat. Finally no publication besides that of Roche et al. (1956) was found which describe any iodinated thyronines in the thyroid gland other than thyroxine and triiodothyronine (see Pitt-Rivers and Rall, 1961).

There is considerable difficulty in the determination of relative potencies of thyroxine versus triiodothyronine because of the differences in plasma protein binding and biological half-lives of these two hormones. Gross and Pitt-Rivers (1953) found that triiodothyronine goiter prevention activity was five times greater than thyroxine using a molar basis of comparison. Heming and Holtkamp (1953) used thyroidectomized rats to compare the calorigenic activities of triiodothyronine and thyroxine. They found that triiodothyronine was 3.5 times more potent than thyroxine using a molar basis of comparison. Heming and Holtkamp also found this same ratio when the goiter prevention potencies of these two hormones were determined. Stasilli et al. (1959) injected triiodothyronine and thyroxine for 14 days in rats and at the same time determined their metabolic rates from day zero until return to control level. These workers found that triiodothyronine had 8 times the calorigenic and goiter prevention potencies of thyroxine using a weight basis for comparison. Finally Reineke and Lorscheider (1967) found that triiodothyronine had 4.09 times more activity per unit weight than thyroxine, as shown by the thyroxine substitution method of Reineke and Singh (1955).

MATERIALS AND METHODS

Starting Materials

A. Protamone

The commercially synthesized iodinated casein Protamone (Lot No. 1114)* was supplied by Agri-Tech, Inc. of Kansas City, Missouri and used throughout these investigations, except for one occasion, where Lot No. 1676 was obtained for comparison with Lot No. 1114. A 2.5% solution of each sample was prepared by dissolving the required amount in $NaHCO_3$ solution at pH 8.0 (700 mg $NaHCO_3$ in 100 ml of distilled H_2O) and then stored at -20°C until used. The commercial procedure of preparing Protamone is unpublished; however, the principal difference between the laboratory method used for synthesis of iodinated casein and the method for Protamone synthesis was the handling of the acid precipitated iodinated protein. Protamone is placed in a vacuum rotary dryer at 15-20 pounds per square inch steam pressure at 25 inches of vacuum until dried. The laboratory prepared iodinated casein was lyophilized and stored at -20°C.

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*Thyroxine content of Lot No. 1114 was stated to be 1.07% in the certificate of analysis issued by the manufacturer Hoffman-Taff, Inc., Springfield, Missouri.

B. Bovine Casein

Bovine casein was obtained by acidifying skimmed non-pasteurized bovine milk to pH 4.6 by the addition of 1N HCl. The precipitated casein was washed four times with acidified distilled water, lyophilized and stored at -20°C. Commercial casein, used to synthesize Protamone was obtained for comparison with the laboratory-recovered casein. A 3.0% solution of each protein was prepared as previously described for solutions of Protamone.

C. Iodinated Casein

Iodinated casein was synthesized in the laboratory according to the method of Reineke et al. (1945a). Five gms of $NaHCO_3$ was added to 700 ml of distilled water, then 20 gms of laboratory prepared casein was mixed into this buffered solution. This casein solution was heated to 40-45°C in a water bath and 3.7 gms of powdered iodine was added slowly with constant stirring. This iodine-casein solution was mixed for 1.5 hours, then incubated at 65-70°C for 20 hours with vigorous stirring. The iodinated casein was precipitated at pH 4.5 with 1N HCl, washed 4 times with acidified water, lyophilized and stored at -20°C. A 3% solution was prepared as previously described for Protamone. Chemical assay of this compound by Hoffman-Taff, Inc., indicated a 0.43% thyroxine content. Also this compound, when placed in the food of 7 Holtzman rats at a level of 0.075%, resulted in a twofold increase in the O_2 consumption of these rats over 7 control rats.

This difference was significant at the P level of 0.01 using a one-sided paired T-test. These two assays indicated that the compound had sufficient thyroidal activity for use in subsequent investigations.

D. Rat Thyroid Protein

The thyroids from 120 rats were removed, trimmed and frozen at -20°C. These thyroids were homogenized in cold 0.85% NaCl at one gland per 0.5 ml of saline. The extracted proteins were centrifuged and the total volume of supernatant was recovered. This supernatant was lyophilized and stored at -20°C. The protein concentration of supernatant was calculated to be 1.16% and was maintained whenever the protein was redissolved in distilled water.

E. Commercial Thyroid Preparation Possibly Containing Iodinated Casein

The Review of Literature indicated that Dr. L. Meister has evidence which suggests that some commercial thyroid preparations may have been adulterated with an iodinated casein compound. Seven coded preparations supplied by Dr. L. Meister were tested by an immunological procedure to determine if evidence of adulteration could be found. They were dissolved in a $NaHCO_3$ solution at pH 8.0 at a starting concentration of 2%. A considerable amount of each sample failed to go into solution; therefore centrifugation was used to clarify the solutions. The supernatant was frozen at -20°C until used.

Preparation of Antisera

Two different methods were used for the formation of antiserum against the different compounds. One was the use of the alum precipitin technique, the other the use of Freund's complete adjuvant. The preparation of the antigen-adjuvant mixtures was, with one exception, the same for formation of all antisera. Therefore the general procedure for preparing these mixtures will be described below.

A. Freund's Complete Adjuvant Method

Equal volumes of Freund's complete adjuvant (Difco Laboratories) was emulsified with the antigen solution in a Waring Blender. The water-in-oil emulsion was considered to be stable when a drop of it placed on cold tap water remained intact. The emulsions were stored at 10°C while being used.

B. Alum Precipitin Method

The proportions of constituents used to prepare this antigen-adjuvant mixture are described below and can be adjusted to give the desired total volume. One ml of the antigen solution was mixed with 3.2 ml of distilled water. Then 3.6 ml of 10% alum (Potassium aluminum sulphate - $KAl(SO_4)_2 \cdot 12H_2O$) was added and pH adjusted to 6.5 with 5N NaOH. The sediment which resulted was washed 3 times with isotonic saline (containing Merthiolate 1:10,000) and made up to a volume of 4 ml with this solution. The one exception was the preparation of alum-precipitated rat thyroid protein, where

because of the low concentration of protein (1.16%), equal volumes of all constituents were employed. All of the antigen-adjuvant mixtures were stored at 10°C prior to being injected.

Dutch black-belted rabbits were used for antisera production. Control bleedings were taken prior to immunization, and at completion of the injection schedule the animals were bled again--both times by cardiac puncture. The blood was allowed to clot at room temperature for one hour and then stored overnight at 10°C. The serum was then decanted, centrifuged and frozen at -20°C until used. The following are the compounds to which antisera were prepared, the adjuvants employed and the immunization schedule used for each antiserum.

A. Rabbit anti-Protamone (abbreviated anti-Protamone)

1. Freund's complete adjuvant method

One ml of the emulsified antigen was injected subcutaneously in five abdominal sites on the first and third week. The second week one ml was injected intramuscularly in the thigh. The fourth week one ml was injected intraperitoneally and the fifth week the animals were bled.

2. Alum precipitin method

Two ml of the alum-precipitated antigen was injected intramuscularly once a week for five weeks in the thigh of each rabbit. On the third week one ml was injected into each of two foot pads. The rabbits were bled on the sixth week.

B. Rabbit anti-Lyophilized bovine casein. (The casein prepared in the laboratory as described earlier was used as antigen.)

1. Freund's complete adjuvant method

One ml of the emulsified antigen was injected subcutaneously in five abdominal sites in each rabbit on the first and second week. The third week one ml was injected intraperitoneally and the rabbits were bled on the fifth week.

2. Alum precipitin method

Two ml of the alum-precipitated antigen was injected intramuscularly in the thighs of each rabbit once a week for seven weeks. Also one ml was injected into each of two foot pads on the third week. Rabbits were bled on the eighth week.

C. Rabbit anti-iodinated casein. (The antigen was Lyophilized iodinated bovine casein prepared as described earlier.)

Anti-iodinated casein was prepared by the alum precipitin and Freund's complete adjuvant method in the same way as described for formation of anti-casein.

D. Rabbit anti-rat thyroid protein (abbreviated anti-thyroid protein)

1. Alum precipitin method

Two ml of the alum precipitated antigen was injected intramuscularly into the thighs of rabbits once a week for three weeks. The rabbits were bled on the fourth week.

Antibodies directed against any rat serum proteins contaminating the thyroid protein preparation were removed by the mixing of anti-thyroid protein with rat serum. This resulted in absorbed anti-thyroid protein.

E. Rabbit anti-bovine blood serum
(abbreviated anti-bovine serum)

1. Alum precipitin method

Two ml of the alum precipitated antigen was injected intramuscularly into the thighs of rabbits once a week for four weeks. The rabbits were bled on the fifth week.

F. Rabbit anti-rat blood serum
(abbreviated anti-rat serum)

1. Alum precipitin method

The immunization schedule was the same as that used to produce anti-thyroid protein.

Immunological Double Diffusion

The basic theory of double diffusion was developed by Ouchterlony (1958). A layer of buffered agar, usually in a petri dish, has most often a series of 4 wells (holes) arranged in a circle around a center well. The antiserum is deposited in the center well and the antigens in the outer wells. This can of course be reversed as the need arises. There is a diffusion of antigen and antibody toward each other in the agar with a meeting of the two diffusion fronts which result in a zone of optimum proportions somewhere in the overlapping area. Antigen-antibody precipitation occurs

and a white precipitin line appears in the agar, thus indicating that at least one antigen-antibody system is present. Crowle (1961) gives a complete review of all types of immunodiffusion methods and should be consulted for further details.

The following procedure was developed for double diffusion. Five ml of 1% Oxoid Ion agar No. 2 (Colab No. L12) prepared in phosphate buffer at pH 7.4, ionicity 0.15 (containing Merthiolate 1:10,000), was pipetted onto a 2 x 3 inch glass slide (see Appendix 1 for buffer formula). After the agar gelled the slide was laid on a piece of paper, which had drawn on it the appropriate well pattern, and the wells were cut with a 10 mm cork borer. The agar was removed using curved forceps, the wells charged with suitable reactants, and left to diffuse and precipitate in a humid atmosphere for four days at 25°C. The agar was washed in frequent changes of distilled water for 3 days to remove the non-precipitated proteins. Then a moistened piece of Whatman No. 40 filter paper was laid on the agar and the agar was dried to a film over night at room temperature. Staining was done with Amido Schwarz (see Appendix 2 for formula) and destaining accomplished with 2% acetic acid.

Two different well patterns were employed. The first consisting of four wells arranged in a circle 5 mm from the center well, was used for most of the immunological experiments. Second, when the purpose was to compare two antigenic solutions to one antiserum or one antigenic solution to two

antiserums, a three well pattern was used. This consisted of 3 wells in a triangular arrangement each 5 mm apart (see Results for illustrations of these patterns). Whenever there was fusion of precipitin lines it was concluded that the antigens involved were serologically identical (figures 2, 3, 4, 5, 6 and 7).

Thin-Layer Chromatography (TLC)

TLC employs a thin layer of supporting medium, usually spread on a glass plate, for separation of compounds. The compounds to be separated, are deposited on the layer and the solvent is allowed to migrate up the medium resulting in separation. This technique is, in many ways, a modification of paper chromatography. However TLC has the advantage that many different supporting media can be used in conjunction with a variety of solvent mixtures. Absorption, partition and ion-exchange chromatography can be employed separately or in combination, resulting in a more versatile technique than paper chromatography. TLC also has the advantage of faster development time, greater sensitivity and simplicity. The primary disadvantage of TLC is that large amounts of compounds are at times difficult to separate because of the low capacity of the thin layer of supporting medium. The books of Stahl (1965) and Randerath (1966) should be consulted for more information regarding thin layer chromatography.

The separation of iodinated compounds was accomplished by a thin layer composed of cellulose powder. Fifteen gms of MN 300 HP cellulose (Macherey, Nagel and Co.) was homogenized with 90 ml of distilled water in a Waring Blender. This slurry was spread onto glass plates, using a spreader and spreading board manufactured by Research Specialties Co., forming a wet layer 0.250 mm thick. The plates were allowed to dry until they could be handled and then heated at 110°C for 15 minutes, resulting in a dry mat of cellulose powder on a glass plate.

Two solvent systems were employed for separation of iodinated compounds isolated from thyroactive proteins (modification from Faircloth, et al. (1965)).

1. Formic Acid/water 3:5
2. n-Butanol/2N NH_4OH/chloroform 37:7:6

The developing chambers were lined with filter paper that was then saturated with the solvent, leaving about 1.5 cm of solvent on the bottom of the tank and finally sealed with a cover. It is important that the tanks are saturated at all times so that the solvent front migrates uniformly up the cellulose layer.

The iodinated compounds, listed below and followed by their abbreviations were used for reference standards. They were dissolved in acidified n-butanol and stored at 10°C.

1. thyroxine (T_4)
2. 3:5:3' triiodothyronine (T_3)

3. 3:5 diiodothyronine (T_2)
4. diiodotyrosine (DIT)
5. monoiodotyrosine (MIT)
6. KI (I-)

The concentration of these compounds was 0.04 mg/ml, except for KI, where it was 0.4 mg/ml. Mixtures of these compounds were made on a one-to-one basis and used where appropriate. The solutions to be separated were deposited using Drummond micro-pipettes (1 micro-liter for one dimensional and 10 micro-liters for two dimensional TLC). The sample was spotted on the cellulose 2.0 cm from the edge and the solvent evaporated to dryness with hot air from a portable hair dryer. If iodine analysis was to be performed on any of the chromotographed solutions, the pipette was rinsed 2 times with n-butanol and the rinsings were added to the spot. This edge was then immersed in the solvent and the tank resealed.

When the solvent front reached at least 14 cm, the plate was removed, solvent front marked and the cellulose dried. The location of iodinated compounds was determined by the FFCA iodine-specific spray of Gemlin and Virtanen (1959). The area containing iodine appeared as a deep blue spot on a light green background (see Appendix 3 for procedure). However these spots fade rapidly in the light so they must be marked soon after spraying. This spray follows the Beer-Lambert law and darkness of spots is proportional to amount of iodine present. A spray specific for benzene rings,

Pauly's reagent described by Boock (1952), was used on a limited basis (see Appendix 4 for procedure). The FFCA spray was very sensitive, as spots with a concentration of only 0.001 microgram of hormonal iodine could be detected. However Pauly's spray could detect concentrations only in the range of 0.10 microgram of hormonal iodine. RF values on one dimensional chromatographs were calculated in the usual manner by dividing the distance the compound migrated from its origin by the distance the solvent front migrated from the origin.

One and two dimensional thin layer chromatography was used for separation of iodinated compounds. Figure 1 illustrates both one and two dimensional thin layer chromatography of all standards listed above. A mixture of all standards was placed at point 1 and appropriate combinations of standards deposited at points 2-7. The chromatogram was developed first in the formic acid/H_2O solvent, thus separating the mixture along the edge and also separating the standards 2-4. The plate was dried and then developed at a right angle to the first run with the n-butanol/2N NH_4OH/chloroform solvent, thus separating this mixture a second time and also separating standards 5-7. The plate was dried again and sprayed with the FFCA reagent. This results, as seen in Figure 1, in a two dimensional separation of the mixture in both solvents and a one way separation of the standards in each of the solvents. As illustrated for thyroxine a triangulation method (Randerath, 1966) was then used for

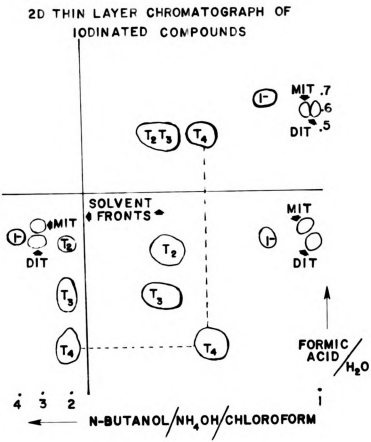
Figure 1. Two dimensional thin layer chromatograph of iodinated compounds.

Column 1: Mixture of all iodinated compounds chromatographed with both solvents.

Columns 2-4: Reference standards chromatographed only with the formic acid/water solvent.

Columns 5-7: Reference standards chromatographed only with the butanol/NH_4OH/chloroform solvent.

The triangulation method was used to identify a compound chromatographed two-dimensionally. Two lines were drawn, parallel to the sides of the plate, through the known reference standard chromatographed in each solvent. These two lines will intersect at the location of the same substance on the two dimensional part the chromatograph. The identification of thyroxine using this method is seen in Figure 1.


2D THIN LAYER CHROMATOGRAPH OF
IODINATED COMPOUNDS
MIT .7
.6
DIT .5
T2 T3
T4
SOLVENT
FRONTS
MIT
DIT
T2
T3
T4
MIT
DIT
FORMIC
ACID
H2O
4
3
2
1
N-BUTANOL/NH4OH/CHLOROFORM

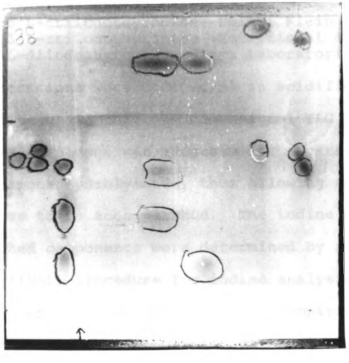


Figure 1

identification of a compound chromatographed two-dimensionally. This method involved the drawing of two lines, parallel to the sides of the plate, through the known reference standard chromatographed in each solvent. These two lines will intersect at the location of the same substance on the two-dimensional part of the chromatogram. If one-dimensional TLC was used for separation of mixtures, standards were chromatographed in parellel columns.

Commercially synthesized iodinated thyronines were chromatographed, using the formic acid/water solvent in combination with the highly sensitive FFCA spray, to determine their purity. These compounds, along with their source, are listed below.

1. L-thyroxine--Glaxo Laboratories, England
2. L-thyroxine--Merck, Sharp and Dohme
3. L-thyroxine--Baxter Laboratories
4. 3,5,3'L-triiodothyronine--Smith, Klein and French
5. 3,5,3'L-triiodothyronine--Nutritional Biochemicals
6. 3,5 L-diiodothyronine--Glaxo Laboratories, England

Their concentrations were 0.04 mg/ml in acidified n-butanol.

It will be shown that one dimensional TLC using the formic acid/H_2O solvent was adequate for separation of T_4 and T_3 from Protamone hydrolysates, thus allowing an assay of these hormones to be accomplished. The iodine contents of chromatographed components were determined by a modification of Barker's (1951) procedure for iodine analysis. The location of the compounds was determined by comparisons to reference standards chromatographed parallel to the mixture of unknowns. The cellulose with the unknowns could not be

sprayed with the FFCA reagent as it interfered with the analysis. A 1.5 cm square of cellulose, corresponding to the location of the component, was scraped off the plate and placed into a 40 ml centrifuge tube. At least two blanks of similar size per individual hormone analysis were removed at the same time. Two ml each of 2N HCl and 7N H_2SO_4 were added to all tubes. Tubes containing thyroxine had 5 ml of water added, whereas tubes with triiodothyronine had just 2 ml of water added because of the lower T_3 concentration. The solutions were mixed, centrifuged and 5 ml of the supernatant was placed in matched photometric tubes for iodine analysis. The solutions were equilibrated at 50°C in a water bath whereupon 0.5 ml of arsenious acid (Hycel Arsenious Acid Reagent--0.25% As_2O_3 in 2.3% H_2SO_4) plus 0.5 ml of ceric ammonium sulfate (Hycel Ceric Ammonium Sulfate Reagent--0.95% $Ce(NH_4)_4(SO_4)_4$) were added. The tubes were incubated at 50°C for exactly 20 minutes and then 0.5 ml of 1% brucine sulfate was added. The percent transmittance was read at 480 mμ with a Coleman Junior II Spectrophotometer and the results recorded as percent transmittance (%T) minus %T for the cellulose blank. Thyroxine and triiodothyronine standard curves were constructed by first placing a graduated series of known amounts of these two hormones on cellulose plates, spraying the plates with the formic acid/water solvent and then analyzing the cellulose as described above. The percent transmittance minus the blank versus the <u>total hormonal iodine per spot</u> was plotted

on linear graph paper. A different standard curve for thyroxine and triiodothyronone was obtained and the reason appeared to be that the cellulose was more concentrated in the T_4 tubes and affected the analysis. However, if T_3 was analyzed the same as the T_4 (i.e. addition of 5 ml water instead of 2 ml) a curve identical to that for thyroxine was obtained (Appendix 5). The iodine values of unknowns were read from the appropriate standard curve. The percentage of each hormone in Protamone was determined by dividing the hormonal iodine in each spot by a conversion factor (0.6533 for T_4 and 0.5856 for T_3) to obtain amounts of total hormone per spot, then multiplying by a dilution factor of 25,000 to determine the total amount of hormone per hydrolysate. Finally this value was divided by the original weight of the Protamone hydrolyzed and multiplied by one hundred to give the percent of the two hormones in Protamone, calculated on a weight basis.

Hydrolysis of Iodinated Proteins

The method of Reineke et al. (1945a) was used for hydrolysis of Protamone. One-tenth gm of Protamone was placed in 15 x 150 mm test tubes along with 0.32 gm of barium hydroxide ($Ba(OH)_2 \cdot 8H_2O$) and 0.64 ml of distilled water. An air reflux condenser was attached and the mixture refluxed for 20 hours in a boiling water bath. At this time 2.5 ml of distilled water was added and the supernatant decanted into a 60 ml separatory funnel. The remaining precipitate of barium salts

was decomposed by adding 0.2 ml of n-butanol, 0.5 ml of 3.5N HCl and warming. When decomposed the salts were transferred into the separatory funnel and this combination made up to a volume of 10 ml with distilled water. The pH of this solution was adjusted to 4.0 with 3.5N HCl. It was then extracted with an equal volume of n-butanol. At this time some hydrolysates were washed with equal and then half volumes of 4N NaOH containing 5% Na_2CO_3. The final volume of the extracted hydrolysates was made up to 25 ml in either case with n-butanol. The acid n-butanol extract is referred to as unwashed Protamone hydrolysate, whereas it is termed washed Protamone hydrolysate when extracted with the 4N NaOH - 5% Na_2CO_3 solution.

The acid insoluble fraction of Protamone hydrolysates was obtained by the method of Reineke and Turner (1943b). The hydrolysis was performed exactly the same as above, except that just 10 ml of water was added to the barium hydroxide solution after completion of hydrolysis. The pH was then adjusted to 4.5 with 3.5N HCl. The resulting precipitate was washed 2 times with acidified distilled water and dissolved in acidified n-butanol to a volume of 25 ml.

Rat thyroid protein was hydrolyzed by the method of Lemieux and Talmage (1966) using 8% barium hydroxide. Ten mg of lyophilized thyroid protein was placed in a 15 x 150 mm test tube and extracted with ether 3 times. One ml of 8% barium hydroxide was added, an air reflux condensor was

attached and this mixture was refluxed for 6 hours in a boiling water bath. The pH was then adjusted to 1 with 3.5N HCl and the solution was extracted with 1 ml of n-butanol. This extracted hydrolysate was made up to a final volume of 2 ml with n-butanol.

RESULTS

Double Diffusion Analysis of Iodinated Proteins

The results of the immunological investigations of thyroactive iodinated proteins are summarized in Table 1 and presented in more detail in Figures 2-7.

Antiserums produced against bovine and rat serum demonstrated at least 8 antigenic components in their corresponding serums.

Each anti-serum against Protamone, produced by either of the two immunization methods, demonstrated at least one antigenic component in Protamone. These two components were shown to be serologically identical; thus Protamone contained at least one antigenic component that was referred to as the Protamone antigen (Figure 2). The antiserum prepared by the alum precipitin method also revealed another Protamone component which appeared as a faint precipitin line (Figure 2) and was found to be of bovine serum origin. The anti-Protamone produced by Freund's adjuvant method gave stronger precipitations and was used for the remainder of the research.

Dilutions of 1/10, 1/100 and 1/1000 of the 2.5% Protamone solution resulted in no change in the number of Protamone antigens; only a loss of the one component at 1/1000 Protamone dilution. Protamone Lot No. 1676 reacted the same as Lot No. 1114 when diffused against anti-Protamone, showing that the

same serological component was present in both preparations (Figure 3).

Each immunization method demonstrated that laboratory prepared casein had at least four antigenic components and they were serologically identical. Again the anti-casein produced using Freund's adjuvant gave the strongest precipitin reaction and was used for the remainder of these investigations (Figure 4). The same four components, detected in the laboratory prepared casein, were also found in the commercial casein (Figure 4). Dilutions of 1/10 or 1/100 casein resulted in no change in the number of serological components, only a loss of them at a dilution of 1/100.

Anti-Protamone diffused against either laboratory or commercially prepared casein produced a single precipitin line that was serologically identical to the Protamone component. Also it was observed that Anti-Protamone contained antibodies directed against two other casein antigens (Figure 5). It must be made clear that the Protamone antigen, demonstrated to exist in casein by anti-Protamone, was not detected when anti-casein was diffused against casein. When anti-casein was diffused against Protamone no precipitation occurred, nor did it occur if the 2.5% Protamone, diluted 1/10, 1/100 or 1/1000, was diffused against anti-casein.

It appears now that Protamone does not have a completely compound-specific antigen since anti-Protamone will react with casein. However anti-casein will not react with

Protamone. A possible explanation will be presented later in this thesis.

Laboratory prepared iodinated casein was observed to contain at least one antigenic component when diffused against anti-iodinated casein serum prepared by the Alum precipitation method and it was found to be of casein origin. The Freund's adjuvant method of immunization did not produce any antibodies to iodinated casein in rabbits. When anti-casein was diffused against iodinated casein two-antigenic components were found to exist in iodinated casein. A bovine serum antigen was found in iodinated casein, but it was not identical to any of the components found using anti-iodinated casein or anti-casein. Thus it appeared that laboratory prepared iodinated casein could produce no antibodies which were compound specific, and that all antigenic components found in iodinated casein were either of casein or bovine serum origin.

Rat thyroid protein was found to contain three antigenic components when diffused against anti-thyroid protein. A rat serum component was found in rat thyroid protein since anti-rat serum could cause one precipitin line. Absorbed anti-thyroid protein was diffused against rat serum and rat thyroid protein at the same time. Two antigenic components specific to thyroid proteins were observed and also one specific to rat serum was present (Figure 6). It can be seen in Figure 6, that even though the absorption of all antibodies to rat

serum protein was not complete, there was sufficient separation of precipitin lines to justify this conclusion.

The immunological interrelations, if any, between the thyroactive iodinated proteins were studied using the double diffusion technique. There was no cross reaction of the anti-iodinated casein with Protamone nor with rat thyroid protein. The same was shown when anti-thyroid protein was diffused against iodinated casein and Protamone. There were no precipitation lines formed when anti-Protamone was diffused against rat thyroid protein. However when anti-Protamone was diffused against iodinated casein one precipitation line was formed and it was serologically identical with the Protamone antigen (Figure 7). Therefore this one Protamone antigen was found to exist in Protamone (Figure 2), in casein (Figure 5) and in iodinated casein (Figure 7). However, only anti-Protamone could demonstrate its presence in any of these three substances.

Immunological analyses of unknown commercial thyroid preparation submitted by Dr. L. Meister revealed that two of them contained the Protamone antigen and that anti-casein produced no reaction in any of the preparations. When the identities of the compounds were obtained from Dr. Meister it was learned that one of the preparations which reacted, when diffused against anti-Protamone, contained poorly iodinated casein prepared in his laboratory that was mixed with U.S.P. thyroid powder. The other preparation which contained

Table 1

SUMMARY OF SEROLOGICAL COMPONENTS IN ANALYZED PROTEINS

ANTIGENS → / ANTISERA ↓	BOVINE SERUM	BOVINE CASEIN	PROTAMONE 2.5%	PROTAMONE .25%	PROTAMONE .025%	IODINATED CASEIN	RAT SERUM	THYROID PROTEIN
ANTI-BOVINE SERUM	8	1	1			1		
ANTI-CASEIN		4	0	0	0	2		
ANTI-PROTAMONE		3	1	1	0	1		0
ANTI-IODINATED CASEIN		2	0			1		0
ANTI-RAT SERUM							8	1
ANTI-THYROID PROTEIN			0			0		3
ABSORBED ANTI-THYROID PROTEIN							1	2

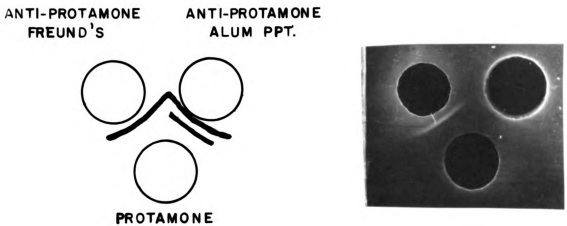


Figure 2

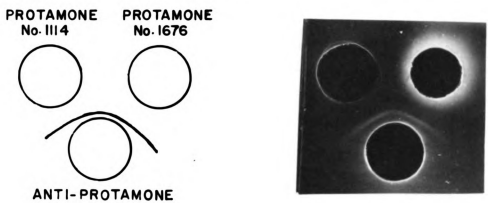


Figure 3

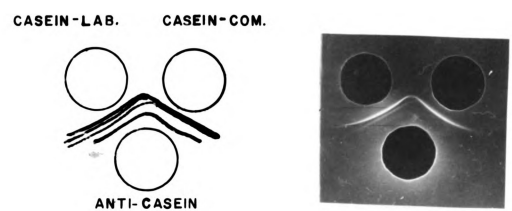


Figure 4

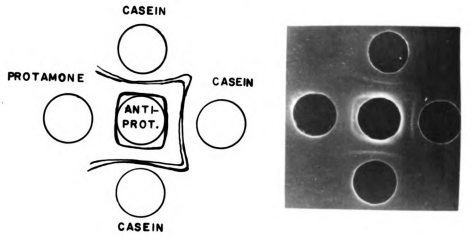


Figure 5

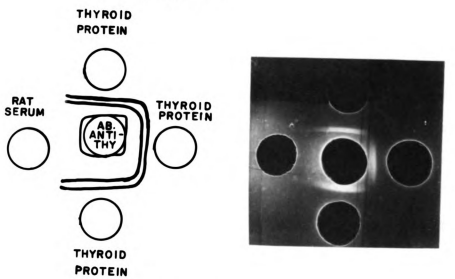


Figure 6

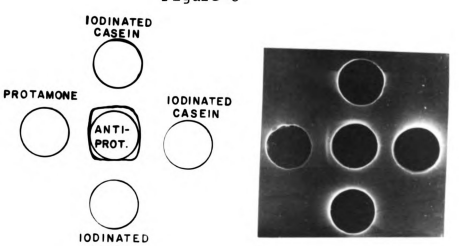


Figure 7

the Protamone antigen, was a defective foreign commercial preparation suspected of containing iodinated casein. There were three other defective foreign preparations which, along with two normal U.S.P. thyroid powders, gave no immunological reaction.

Purity of Commercial Iodinated Thyronines

The average Rf values of chromatographed reference compounds are presented in Table 2. It can be observed that only the formic acid-water solvent was able to separate the iodinated thyronines tested. However, the other solvent had the advantage of being able to separate DIT from T_2. The method of separation and identification of these standards was discussed and illustrated in an earlier section (Figure 1).

Because of the extreme sensitivity of the TLC method the purity of commercial iodinated thyronines was determined employing the formic acid/water solvent. The L-thyroxine synthesized by Glaxo Laboratories contained not only thyroxine but also triiodothyronine as a contaminant. The L-thyroxine supplied by Merck, Sharp and Dohme contained not only thyroxine, but also two other compounds, one which migrated slower than thyroxine and another with the same Rf as triiodothyronine.

The Baxter Laboratory L-thyroxine was completely pure as no other iodinated compound than thyroxine could be found. The two preparations of L-triiodothyronine contained impurities. The Smith, Klein and French L-triiodothyronine was contaminated with a small amount of thyroxine. The Nutritional

Table 2

AVERAGE RF VALUES OF IODINATED COMPOUNDS USING TLC ON CELLULOSE

	FORMIC ACID/H_2O	N-BUTANOL/NH_4OH/CHLOROFORM
T_4	.30	.54
T_3	.50	.70
T_2	.70	.70
DIT	.70	.04
MIT	.78	.08
I^-	.74	.22

Biochemicals Co. L-triiodothyronine was contaminated with thyroxine and with a component which mitrated slightly faster than triiodothyronine. The one sample of diiodothyronine tested contained no other iodinated compounds.

Iodinated Compounds in Protamone Hydrolysates

One dimensional TLC of unwashed Protamone hydrolysate revealed at least 8 iodinated compounds labeled 0-7 in order of increasing migration rates (Figure 8). The washed hydrolysate showed only 7 compounds since number 1 disappeared (Figure 8). However, there were quantitative differences between the concentrations of compounds 6 and 7. These two compounds were present in large amounts in the unwashed hydrolysate, but in the washed hydrolysate they were almost absent.

Two dimensional TLC of the unwashed Protamone hydrolysate increased the number of iodinated compounds separated from 8 to at least 22 (Figure 9). MIT and DIT were identified and compounds which corresponded with those labeled 0, 2, 3, 4 and 5 in Figure 8 were also observed. Compounds 6 and 7 listed in Figure 8 were composed primarily of MIT and DIT as seen in Figure 9. Compounds 2 and 4 were identified as thyroxine and triiodothyronine. The evidence for this identification will be presented at a later time.

The TLC of the washed Protamone hydrolysate is seen in Figure 10. Almost all iodinated compounds have been removed

Figure 8. One dimensional thin layer chromatography of Protamone hydrolysate.

Column 1: reference compounds

Column 2: unwashed Protamone hydrolysate

Column 3: washed Protamone hydrolysate

1D THIN LAYER CHROMATOGRAPH OF PROTAMONE HYDROLYSATE

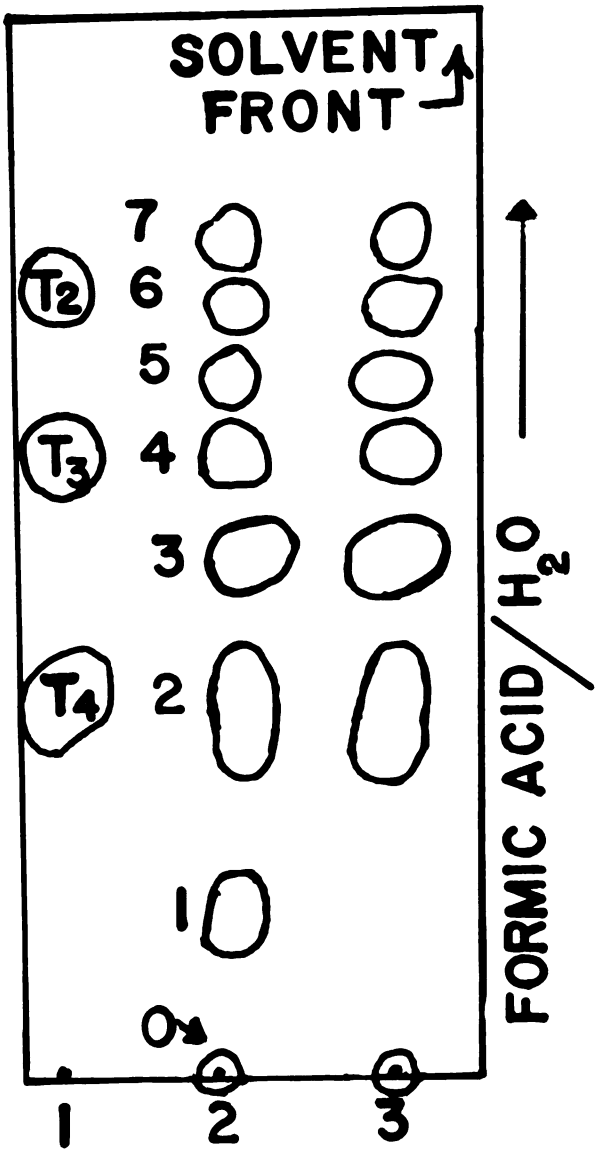


Figure 8

Figure 9. Two dimensional thin layer chromatograph of unwashed Protamone hydrolysate.

Column 1: unwashed Protamone hydrolysate

Columns 2-5: regerence compounds

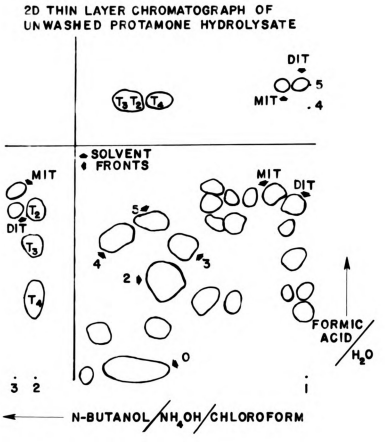
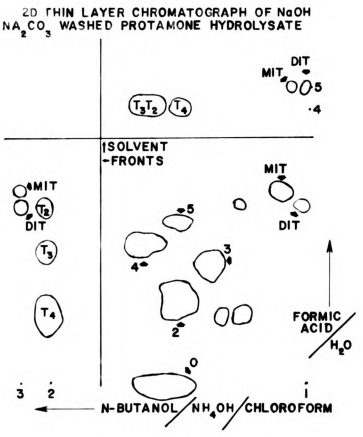

2D THIN LAYER CHROMATOGRAPH OF
UNWASHED PROTAMONE HYDROLYSATE
DIT
MIT
.5
.4
T3 T2 T4
SOLVENT
FRONTS
MIT
MIT
DIT
DIT
T2
T3
T4
5
4
3
2
0
FORMIC
ACID
H2O
3 2
1
N-BUTANOL/NH4OH/CHLOROFORM

Figure 9

Figure 10. Two dimensional thin layer chromatograph of Na_2CO_3-NaOH washed Protamone hydrolysate.

Column 1: washed Protamone hydrolysate

Columns 2-5: reference compounds


2D THIN LAYER CHROMATOGRAPH OF NaOH
NA_2CO_3 WASHED PROTAMONE HYDROLYSATE
DIT
MIT
.5
.4
T_3T_2
T_4
SOLVENT
FRONTS
MIT
MIT
T_2
DIT
DIT
5
3
4
T_3
T_4
2
FORMIC
ACID
H_2O
0
3
2
1
N-BUTANOL
NH_4OH
CHLOROFORM

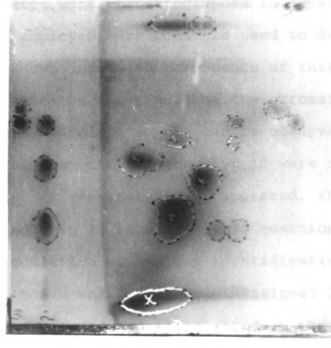

X

Figure 10

by the washing process except for the five labeled 0, 2(T_4), 3, 4(T_3), and 5. A trace of MIT, DIT and four others can be seen. It is apparent from Figures 9 and 10 that compounds labeled 0, 2(T_4), 3, 4(T_3), and 5 are identical in both the washed and unwashed hydrolysates of Protamone.

The acid insoluble fraction of the Protamone hydrolysate still contained compounds 0, 2(T_4), and 3 along with a trace of number 4(T_3) (Figure 11). There also were traces of MIT and DIT. However it can be observed from comparison of Figures 9, 10 and 11 that compound 0, 3 and 4(T_3) were less concentrated in the acid-insoluble fraction of Protamone hydrolysate than in the butanol extract.

The number and identity of iodinated compounds in thyroid proteins have already been clearly established. Thyroid protein hydrolysates were chromatographed in order to ascertain if the method employed here could be used to detect and identify all known iodinated components of this gland. Figure 12 illustrates the result of the chromatography of a thyroid protein hydrolysate. It can be observed that all known iodinated compounds of the thyroid were found and identified correctly. A very faint spot appeared, that could not be identified, and this is labeled with a question mark.

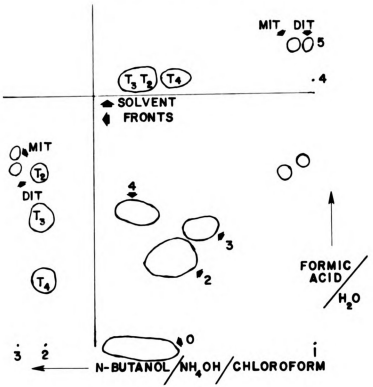
The triangulation method for identification of unknowns by comparison to knowns, when two dimensional TLC has been employed, was discussed and illustrated in the Materials and Methods. This procedure identified compounds 2 and 4 as

Figure 11. Two dimensional thin layer chromatograph of the acid-insoluble fraction of Protamone hydrolysate.

Column 1: acid insoluble fraction of Protamone hydrolysate.

Columns 2-5: reference compounds.

2D THIN LAYER CHROMATOGRAPH OF
THE ACID INSOLUBLE FRACTION OF
PROTAMONE HYDROLYSATE



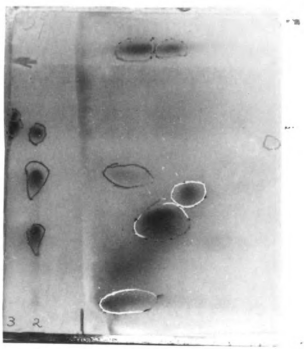


Figure 11

Figure 12. Two dimensional thin layer chromatograph of Thyroid Protein hydrolysate.

Column 1: rat thyroid hydrolysate.

Columns 2-5: reference compounds.

2D THIN LAYER CHROMATOGRAPH OF
THYROID PROTEIN HYDROLYSATE

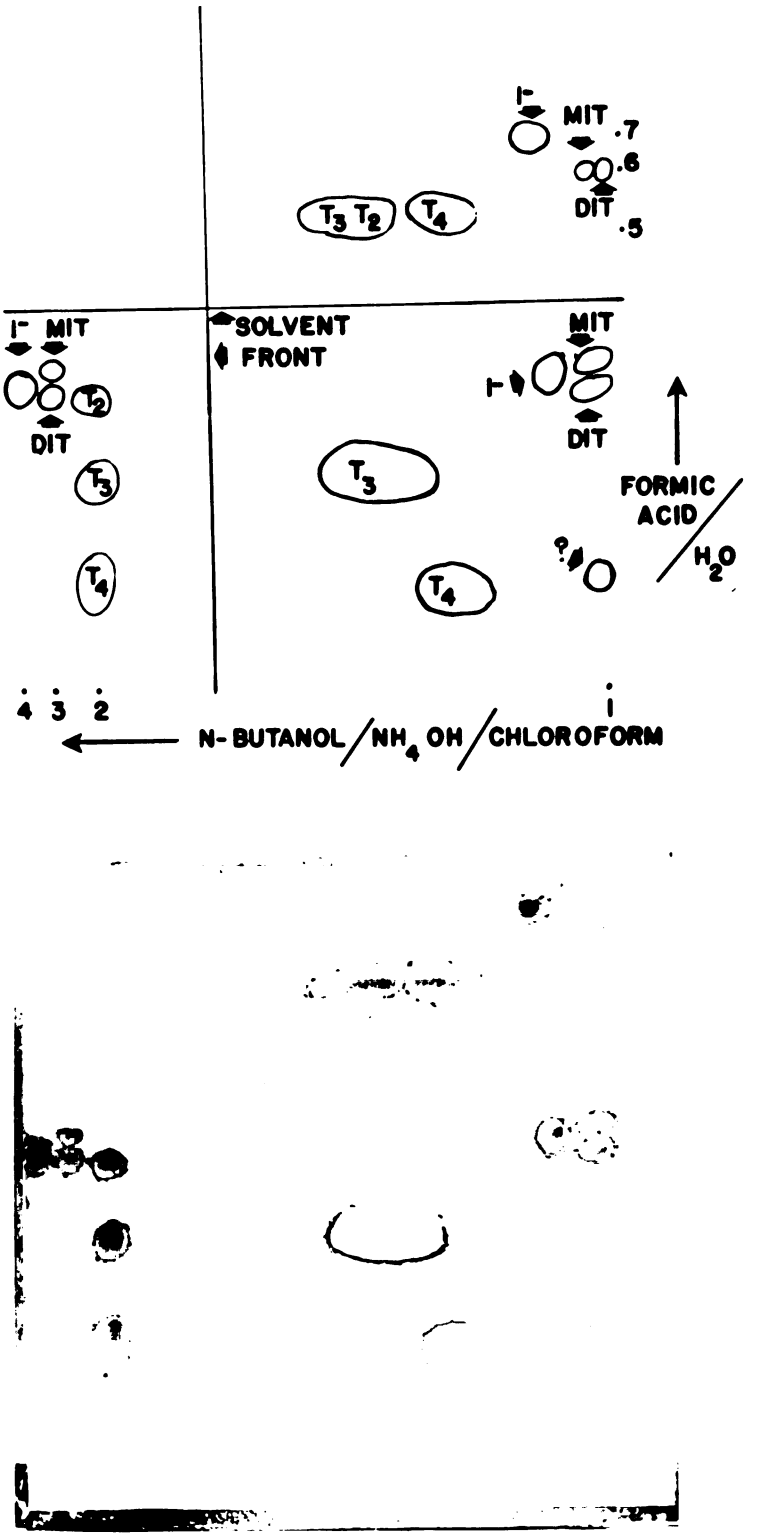


Figure 12

thyroxine and triiodothyronine, respectively (Figures 9, 10, and 11). However other evidence was obtained to confirm this conclusion. The washed Protamone hydrolysate was separately diluted with 1/2 volume of 0.4 mg/ml thyroxine and triiodothyronine and then chromatographed. The chromatogram of these two mixtures resulted in an increase of spot density, as shown by the FFCA spray, in the areas which corresponded to T_4 and T_3 as identified by the triangulation method. Pauly's reagent also reacted with compounds 0, 2(T_4), 3 and 4(T_3) indicating the presence of a benzene ring structure. The chromatographed position of the only known thyroidal iodinated thyronines, thyroxine and triiodothyronine, from thyroid hydrolysates was identical to compounds identified as thyroxine and triiodothyronine in Protamone hydrolysates. Finally, the FFCA spray obviously demonstrated that these compounds contained iodine.

Therefore based on the evidence presented, the identity of compounds 2 and 4 was determined to be thyroxine and triiodothyronine, respectively. The identity of the other compounds was not determined; however, it can be stated that they are iodinated compounds and that numbers 0 and 3 contain a benzene ring.

The only other compounds identified were MIT and DIT. Finally no evidence of free iodide could be found in Protamone hydrolysates.

Figure 10 demonstrates that the washed Protamone hydrolysate could be separated one dimensionally, employing the formic acid/water solvent because no contaminating substances appeared when the hydrolysate was chromatographed the second time using the n-butanol/2N NH_4OH/chloroform solvent. The iodine analysis was performed according to the method previously described. The percent concentration of thyroxine and triiodothyronine, calculated on a weight basis, is presented in Table 3. The thyroxine equivalent of 3.28% was determined by multiplying the percent triiodothyronine content by its thyroxine potency (4.09 as determined by Reineke and Lorscheider, 1967), resulting in a value of 2.49% and then adding the 0.79% of thyroxine to yield a final value of 3.28% thyroxine equivalent. The value of 4.09 for triiodothyronine potency versus thyroxine was used because it is in the mid-range of those published, except for the report of Stasilli et al. (1959) which gave about twice this value.

Table 3

PERCENT CONCENTRATIONS OF THYROXINE AND TRIIODOTHYRONINE IN PROTAMONE

	GRAMS PERCENT	STANDARD ERROR
THYROXINE	.79	±.023 n=12
TRIIODOTHYRONINE	.61	±.010 n=6
THYROXINE* EQUIVALENT	3.28	

*Thyroxine equivalent was determined by multiplying the percent T_3 by its thyroxine potency (4.09), resulting in a value of 2.49% and then adding the 0.79% of T_4 to yield a final value of 3.28% thyroxine equivalent.

DISCUSSION

The immunological research on iodinated protein has revealed a number of interesting properties. Protamone was shown to contain at least three different antigens since anti-Protamone could precipitate three antigens in casein. One of these antigens could also be precipitated in two different lots of Protamone and was called the Protamone antigen. Since the dilutions of 2.5% Protamone (1/10, 1/100, 1/1000), when diffused against anti-Protamone, resulted in no change in number of Protamone antigens, it can be concluded that within the concentrations of Protamone tested no antigen-antibody imbalance prevented the precipitation of the remaining two Protamone antigens. They probably were not concentrated enough in the original solution to react in the double diffusion analysis, but were in sufficient concentration for antibody formation in the rabbits. However these antigens were in high enough concentration in the casein for reaction with their antibodies. Finally a trace of another serological Protamone component was seen and it was determined to be of bovine serum origin.

The laboratory and commercially prepared casein, analyzed with the anti-casein, revealed that the same four antigens were present in both solutions. The Protamone antigen was

not found in casein by the anti-casein, even though its presence was demonstrated to exist by use of the anti-Protamone. Nor could the Protamone antigen be found in either casein, using anti-casein, when the caseins were diluted 1/10 or 1/100. Also when anti-casein was diffused against the dilutions of Protamone no evidence of precipitation could be seen. Therefore it was concluded that anti-casein contains no antibody specific for the Protamone antigen, since dilutions listed above had eliminated any antigen-antibody imbalance, at least within the concentrations tested. The other two antigens known to be present in Protamone were probably in too low concentration to react when diffused against the anti-casein, assuming that there were antibodies against these antigens present in the casein.

Double diffusion analysis of laboratory prepared iodinated casein revealed that this substance contained only antigens which were serologically identical with antigens either in casein or bovine serum. Therefore, it was concluded, based on this evidence, that the iodination processes employing lyophilized proteins do not qualitatively alter the immunological properties of iodinated casein. The only change was a loss in the total number of antigens found.

Rat thyroid proteins contained at least two thyroid specific antigens and another one common to both the thyroid protein and rat serum. Perelmutter and Stephenson (1964) reported that the 19S (thyroglobulin) and 27S fractions of

beef and hog thyroid had their own specific immunological properties. Lachiver et al. (1965) and Robbins et al. (1966) have both demonstrated the presence of 19S and 27S protein fractions in the rat thyroid gland. Based on these reports, the two thyroid specific antigens were considered to represent the 27S and 19S proteins reported to be present in the rat thyroid.

There was, with one exception, no cross reactivity between any of the anti-iodinated protein sera and their respective iodinated proteins. There was however an immunological reaction between anti-Protamone and iodinated casein demonstrating the presence of the Protamone antigen in the iodinated casein. Since this same antigen was also found to exist in non-iodinated casein, it can be concluded that its presence could not directly be a consequence of the processes of iodination. These results are at variance with the report of Wormall (1930) since no common antigenicity specific to iodinated proteins was found. It would be reasonable to expect that iodinated thyroid protein would not have an antigenicity common to in vitro iodinated proteins. But if Wormall's results were to apply to all iodinated proteins synthesized in vitro, then Protamone and iodinated casein should have a common antigenic factor.

It was apparent that a common antigen--the Protamone antigen--was present in Protamone, iodinated casein and casein. Yet, only when it was present in the Protamone did it

appear to have the ability to produce an immune reaction in rabbits. A possible explanation for this unusual situation is that at some point during Protamone synthesis this antigen becomes somehow altered such that it can now produce antibodies. The antibodies produced are, however, able to react with both the altered antigen in the Protamone and the unaltered antigen in iodinated casein and casein.

The question of what causes this alteration to occur in Protamone cannot be completely answered. As already mentioned, it appears not to be involved with iodination since neither laboratory prepared iodinated casein nor casein itself contained the altered antigen. The only factor that could cause the alteration was the handling of the acid-precipitated iodinated protein. The Protamone is heated to dryness, as described in the Materials and Methods, whereas both iodinated casein and casein itself were lyophilized. This heating process might alter the protein such that the Protamone antigen could then produce antibodies.

It seems possible that the commercial casein, used to synthesize Protamone, may have contained the altered antigen. This is considered unlikely since commercial casein reacted to the anti-casein the same as the laboratory prepared casein. Therefore, a tentative conclusion is that the method of drying Protamone resulted in the immunological alteration in the Protamone antigen so that it can produce antibodies. To fully answer the question of when and how this change occurs is a

problem in itself and was not considered to be a part of this research; consequently, no further study on the problem was attempted.

The results of the immunological analysis of Dr. L. Meister's unknown compounds illustrates a practical use of anti-Protamone. The antiserum reacted with a poorly-iodinated casein mixed with U.S.P. thyroid and also with a defective commercial thyroid preparation suspected of containing poorly iodinated casein. Anti-casein did not react with any of the compounds. The failure of anti-casein to react indicated that non-iodinated casein is not present, but instead an iodinated casein which has undergone an iodination process similar to that used in the synthesis of Protamone. These results suggest the possibility that immunological procedures can be used to detect the presence of iodinated casein, in other formulations such as feed mixtures.

The question as to whether Protamone has antigenicity has been answered. Protamone definitely has antigenicity and its origin is that of the casein from which it was synthesized. One of its antigens can form antibodies only when subjected to the processes of Protamone synthesis. There is no evidence equating the alteration of the Protamone antigen to Protamone thyroid activity since iodinated casein which has thyroid activity does not show this alteration. Finally, and most importantly, it must be remembered that when Protamone is injected or even fed to animals it is a foreign

protein with antigenic properties and therefore the possibility of an immune response always exists.

An extremely sensitive technique for separation and identification of iodinated compounds in hydrolysates of iodinated proteins was described. This method was employed to determine the purity of certain chemically synthesized iodinated thyronines. It was determined that not all of these preparations were completely pure, although the amount of impurities is not great, this contamination would be a source of error when these compounds are used as standards in experimentation. A method was presented for determining the iodine content of iodinated compounds separated by thin layer chromatography.

A considerable number of different iodinated compounds, at least 22, were shown to exist in the unwashed hydrolysate of Protamone. This result confirms and extends the report of Friedberg (1951). The use of TLC instead of paper chromatography increased the number from 10, found by Friedberg, to 22. It is obvious that the iodination of casein is an extremely complex chemical process which results in the synthesis of a variety of iodinated compounds. There were at least 5 iodinated compounds remaining in the washed Protamone hydrolysate. This follows the reports of Hird and Trikojus (1948) and Friedberg (1951) in which they indicated the presence of three compounds in the washed hydrolysates of Protamone. The results reported herein confirm their observations but with the

addition of 2 more components, resulting in a total of 5. It is difficult to determine which of the compounds in this report correspond to the three reported by the other authors. It is probable that the thyroxine identified by both methods is the same. But compound 0 found by TLC does not appear to have been seen by either Hird and Trikojus or Friedberg. These authors believed that they demonstrated 3,5-diiodothyronine, but nothing corresponding to T_2 was found using TLC. It is quite possible that compound 5 could have been mistaken for T_2 on paper chromatographs. The compound thought to be triiodothyronine by these authors could have been a combination of compound 3 and triiodothyronine (compound 4).

The acid insoluble fraction of the Protamone hydrolysate demonstrates the original observation of Kendall (1915) that thyroxine is insoluble in acid, but also that there are at least 3 other compounds that are to some extent also acid-insoluble. It was apparent from comparison of Figures 9, 10 and 11 that these compounds were present in lower concentrations in the acid-insoluble fraction than in either the washed or unwashed butanol extracts.

The identification of compounds 2 and 4 as thyroxine and triiodothyronine was based on several independent criteria. The comparison of their migration rates in two completely different solvents with those of known standards is considered in itself to be a very reliable means for identification. But additional evidence was obtained to substantiate this

identification. It was found that by adding separately thyroxine and triiodothyronine to the hydrolysate and then chromatographing, there was an increase in the amount of compounds in the positions corresponding to T_4 and T_3. Therefore thyroxine and triiodothyronine when added to the hydrolysate, chromatographed identically with material thought to be T_4 and T_3. The use of Pauly's reagent and the FFCA spray confirmed the presence of a benzene ring and of course iodine. Finally, all iodinated compounds known to exist in rat thyroid gland hydrolysates were identifiable. The identity of compounds removed by the alkali wash was not determined since reference standards were not available. However some of them are probably one or more of the 3 possible iodinated histidines or the one iodinated tryptophan cited in the review by Reineke and Turner (1942a).

These results confirm the conclusion of Friedberg (1951) that the Blau extraction procedure is not specific for thyroxine and in fact shows that at least 4 other compounds were included in the alkali-washed n-butanol extract of Protamone hydrolysate. The chemical analysis of Reineke et al. (1945a) showing a 3.0% thyroxine content in iodinated casein, based on the assumption that T_4 was the only compound in the washed fraction, is therefore in error.

Reineke and Turner (1943b) believed that the acid-insoluble fraction of an iodinated casein hydrolysate was not an accurate estimation of thyroxine content since its iodine

content was 40.1%, whereas if it were all thyroxine its iodine concentration should be 65.3%. Therefore this fraction must contain impurities in the form of other iodinated compounds.

These results confirm their belief by showing that 3 compounds in addition to thyroxine occur in the acid-insoluble fraction of an iodinated casein hydrolysate.

The total hormonal activity in terms of thyroxine equivalent was calculated in the present work to be 3.28%. This value could be subdivided into 0.79% thyroxine and 0.61% triiodothyronine. These values may be somewhat low since Reineke et al. (1945a) showed a 7% loss of thyroxine resulting from hydrolysis. The value obtained by Reineke et al. (1945a) of 2.79% thyroxine, using a biological assay, compares favorably with the value of 3.28% reported herein. The biological assay was determined on iodinated casein produced in precisely the same manner as Protamone except for one detail. A catalyst was used in Protamone synthesis as suggested by Reineke et al. (1945b) and may increase total activity enough to account for the difference. Therefore no other compound(s) having thyroid activity in mammals can be present in thyroactive iodinated casein since the chemical assay reported herein accounts for all mammalian biological activity of this substance. The value of 0.79% thyroxine is very similar to that reported by Reineke (1954) of 1.04% and also to that reported by the manufacturer of Protamone which is 1.07%.

The value of 3.28% is still lower than that reported by Reineke et al. (1943a) of 4-5.5% using the tadpole assay. Two factors may have a bearing on this high value. First, the potency of triiodothyronine may be greater in stimulating tadpole metamorphosis than in increasing metabolic rates or preventing goiters in mammals. Second, it is possible that compounds other than the two mammalian hormones, present in iodinated casein, may be biologically active in tadpoles.

The results reported herein have finally shown a value of the total thyroidal activity of an iodinated casein in terms of percent thyroxine. This is the first time a complete analysis of all thyroid hormones has been accomplished on a thyroactive iodinated protein synthesized in vitro.

SUMMARY AND CONCLUSIONS

The immunological properties of thyroactive iodinated proteins were investigated using the double diffusion technique. The primary emphasis was directed toward the antigenic properties of the commercially produced iodinated casein-Protamone--since it is a standardized product fed and injected into animals to increase thyroid activity. Antisera were produced in rabbits against (1) Protamine, (2) a laboratory prepared iodinated casein, and (3) rat thyroid proteins. The immunological research supports the following results and conclusions.

1. Protamone contained at least three antigenic components, since anti-Protamone precipitated three antigens in casein. Only one of these, the Protamone antigen, could be precipitated in Protamone by anti-Protamone.

2. Anti-casein produced no precipitation when diffused against Protamone. Therefore the anti-casein contained no antibodies against the Protamone antigen.

3. Laboratory prepared iodinated casein contained at least two antigens which were of casein origin.

4. Rat thyroid protein contained two antigens believed to represent the 19S and 27S thyroid proteins.

5. There was, with one exception, no cross reaction between any of the anti-iodinated sera and their respective iodinated proteins. When anti-Protamone was diffused against iodinated casein the Protamone antigen was precipitated. Since this same antigen was present in non-iodinated casein, it can be concluded that its presence could not be a consequence of the iodination process.

6. The Protamone antigen was demonstrated in a deficient commercial thyroid preparation suspected of having been adulterated with poorly iodinated casein.

7. These results have established that both commercial and laboratory prepared iodinated casein are antigenic and their antigenicity originates from casein. There is a common antigen present in Protamone, casein, and iodinated casein, but only when it is present in Protamone does it have the ability to produce antibodies. There must be some alteration of this antigen during the Protamone synthesis such that it can now produce an immune response. This alteration may be due to the drying of the acid-precipitated Protamone with heat, whereas the casein and iodinated casein were lyophilized.

8. The iodination of casein does not confer a new antigenic specificity since all antigens present in Protamone and iodinated casein were of casein origin.

The iodinated amino acids of Protamone were separated using thin-layer chromatography. The commercial preparation was used because it is a standardized product in wide use in

animal industries to increase thyroid activity. The primary aim was to fractionate, identify and quantify the thyroid hormones in Protamone hydrolysates. This research produced the following results and conclusions.

1. Two-dimensional TLC demonstrated the presence of at least 22 iodinated components in the initial n-butanol extract of Protamone hydrolysate. This shows that an iodinated casein contains a very complex mixture of iodinated compounds. These included thyroxine, triiodothyonine, monoiodotyrosine and diiodothyrosine.

2. Two-dimensional TLC of the alkali-washed hydrolysate demonstrated that 5 of the 22 compounds were still present.

3. Two-dimensional TLC of the acid-insoluble fraction of Protamone showed that 4 of the above 5 components were still present.

4. Two of these five compounds were identified as thyroxine and triiodothyronine. They were present in all preparations described above. The other 3 compounds are believed to be iodinated thyronines or their analogues.

5. The chemical assay, based on the n-butanol-soluble iodine, was shown to be in error since 4 other compounds besides thyroxine were present. It was also shown that the acid-insoluble fraction contained 3 other compounds besides thyroxine. Thus both of these assay procedures were not specific for thyroxine assay of Protamone.

6. The concentrations of thyroxine and triiodothyronine in Protamone were determined to be 0.79% and 0.61% respectively. A combination of these two values is biologically equivalent to 3.28% thyroxine. This value agrees rather closely with the biological assay of 2.79% thyroxine equivalent published earlier for a similar compound employing the quinea pig CO_2 production method. The combined T_3 and T_4 content of Protamone is sufficient, therefore, to account for its determined thyroidal activity in mammals. Thus it is unlikely that other unidentified thyroidally active compounds occur in this product.

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APPENDICES

1. Immunological Double Diffusion Buffer

Phosphate, ionicity 0.15 pH 7.4

$Na_2 HPO_4$	12.8 gm.
$NaH_2PO_4 \cdot H_2O$	2.62 gms.
H_2O	make up to 1000 ml.

2. Protein Stain

Amido Schwarz

Amido Schwarz	0.5 gm.
12% Acetic Acid add	225.0.ml.
1.6% Sodium Acetate add	225.0 ml.
Glycerol add	50.0 ml.

3. FFCA Iodine Specific Spray

Solution A 2.7 gm $FeCl_3 \cdot 6H_2O$ in 100 ml 2N HCl

Solution B 3.5 gm $K_3Fe(CN)_6$ in 100 ml H_2O

Solution C 5.0 gm $NaAsO_2$ in 30 ml of cold 1N NaOH. When dissolved, add 65 ml 2N HCl with vigorous stirring.

Prior to use: 5 parts A, 5 parts B, and 1 part C is mixed and sprayed in dim light.

4. Pauly's Reagent

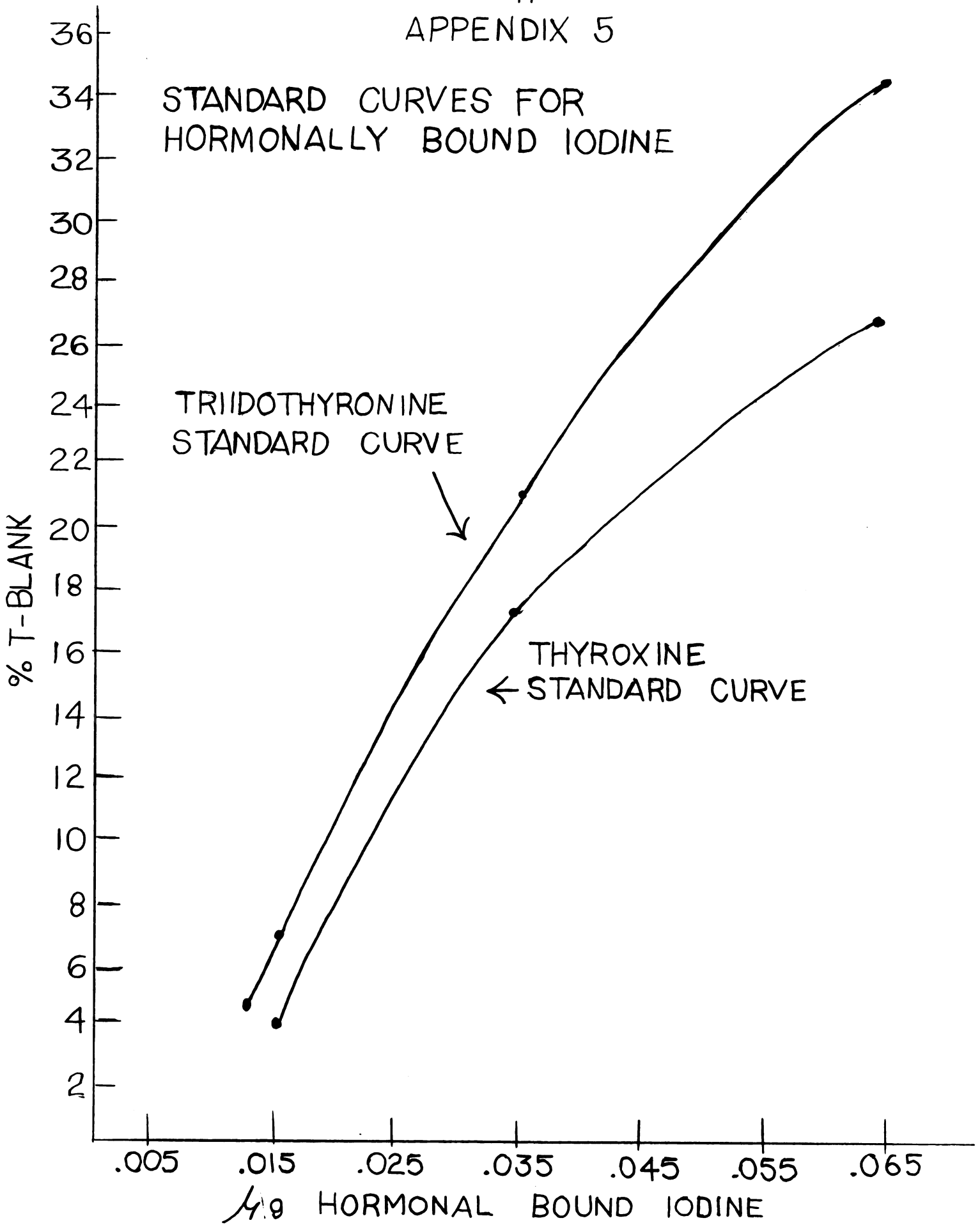
Solution A 1% sulfanilamide in 10% HCl

Solution B 5% $NaNO_2$

Solution C 1/2 saturated Na_2CO_3

Spray consists of 5 ml of solution A, and 5 ml of solution B. This mixture is extracted with 50 ml of n-butanol. The n-butanol extract is sprayed on the plate and allowed to dry; then the plate is sprayed with solution C.

APPENDIX 5


STANDARD CURVES FOR
HORMONALLY BOUND IODINE
TRIIDOTHYRONINE
STANDARD CURVE
THYROXINE
STANDARD CURVE
% T-BLANK
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.025
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