A CRITICAL ANALYSIS OF THE BUTYL ALCOHOL EXTRACTION TECHNIQUE USED IN THE QUANTITATIVE DETERMINATION OF THYROXINE

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A CRITICAL ANALYSIS OF THE BUTYL ALCOHOL EXTRACTION TECHNIQUE USED IN THE QUANTITATIVE DETERMINATION OF THYROXINE

By

Wallace Friedberg

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INTRODUCTION

Numerous instances are cited in the literature in which the biological potencies of compounds with a thyroxine moiety are not proportional to that anticipated on the basis of chemical assays. In a situation of this type both the chemical and biological assays may be unreliable. Previous investigators have demonstrated that insofar as biological thyroxine determinations are concerned, consideration must be given to certain "obstacles" which might prevent the active substance from producing its maximum response. In this respect oral administration involves possible differences in absorption from the digestive tract. The possibility of preferential destruction of one thyroidally active substance over another also complicates the picture. Once absorbed, the various active preparations are not necessarily utilized to the same degree. Different doses of the same preparation administered by the same route could give disproportionate responses. Furthermore, pure thyroxine exists in two optically active forms which must be considered separately. When thyroxine in protein combination is under observation the possible presence of other compounds with chemical and biological similarities to thyroxine should not be overlooked.

The present study deals primarily with a critical analysis of the most widely used chemical method for assaying thyroidally active proteins, the butyl alcohol extraction technique. A review of related aspects of the entire problem has been included in order that the limited findings may be properly evaluated in terms of the overall problem.

Specifically the review will consider:

1. Absorption and Utilization of Thyroxine
2. Optical activity of Thyroxine
3. Iodinated Casein
4. The Butyl alcohol Extraction of Thyroxine

The butyl alcohol extraction technique for the quantitative determination of thyroxine in protein combination was originally proposed by Leland and Foster (1932) and revised by Blau (1933, 1935), Reineke et al (1943) and others. Since this method depends on a final determination of the iodine content it is presupposed that all of the iodine in the washed extract represents thyroxine. To date no satisfactory proof has been offered that this supposition is correct; in fact considerable evidence is now available to indicate that non-thyroxine iodinated compounds occur in this extract.

In the present investigation the reliability of the butyl alcohol extraction technique as a quantitative assay for thyroxine in iodinated casein has been evaluated. The chemical aspects of the problem may be divided into several classifications as follows:

1. Iodination of casein with radioactive iodine to facilitate comparative iodine determinations.
2. Controlled purification of the butyl alcohol extract to provide quantitative information as to the percentage of iodine removed.
3. Paper partition chromatography of the extract to establish the presence of any unreported compounds.

4. Radioautograms of the chromatograms in order to locate the iodinated compounds and determine relative amounts of iodine in them.

In view of a report by Hird and Trikojus (1948) that diiodothyronine, a compound with a structure similar to that of thyroxine, was found in iodinated casein, a biological assay was carried out to establish the extent of its thyroidal activity. Earlier workers, using methods based on different principles than employed in the present investigation, reported activities ranging from one-twentieth to one-fortieth that of racemic thyroxine (Rapport et al (1931) and Gaddum (1927).

It is hoped that the results presented in this paper will contribute to a better understanding of some of the complex problems involved in the study of "thyroxine activity".

REVIEW OF THE LITERATURE

Absorption and Utilization of Thyroxine

After developing a biological test for thyroxine and thyroid preparations, Cameron and Carmichael (1921) carried on a series of experiments in which they administered thyroid and thyroxine orally to white rats and made comparisons by observing decrease in total body weight as well as relative hypertrophy of liver and spleen. On the basis of equal iodine content in the two preparations they observed that thyroid produced from two to four times the effect of thyroxine. These authors suggested that the differences in effectiveness between the two preparations might be due to bacterial decomposition of the free thyroxine, the thyroxine in combination with protein being more resistant.

In view of the work of Kendall (1915), showing that only a part of the thyroid gland contains material with thyroidal activity and the more recent report of Taurog and Chaikoff (1946a), that only 25 per cent of the iodine is thyroxine iodine, the data of Cameron et al (1921) can be reinterpreted to indicate that thyroid "thyroxine" has eight to sixteen times the effect of d,l-thyroxine. It is assumed that these investigators used racemic thyroxine inasmuch as an optically active isomer of thyroxine had not yet been isolated, Harington and Salter (1930).

During the same year, Plummer (1921) made similar observations in human beings. He reported that absorption of thyroxine from the intestinal tract apparently was erratic, although the physiological reaction

following its administration was identical to that obtained with fresh or desiccated thyroid glands.

Plummer's conclusion conforms somewhat more to present day concepts than the suggestion of Cameron et al (1921) that bacterial decomposition is responsible for the poor effect of orally administered thyroxine. However, his experimental subjects, human beings, are notoriously poor for carefully controlled experiments.

Harington and Salter (1930), working with a peptide product of tryptic digestion of thyroid, containing no iodine compound other than thyroxine, found that oral administration of it caused a pronounced increase in the oxygen consumption of rats, while l-thyroxine similarly administered had practically no effect. According to these investigators, the wide range of solubility of the digestion product compared to the relative insolubility of thyroxine made it highly probable that the absorption of the latter would be insignificant while the peptide would be easily absorbed, thus accounting for its high degree of effectiveness.

Quoting experiments by J. H. Means of Boston, who found that the digestion product is fully as effective as an equivalent dose of thyroid (oral administration to patients with subnormal basal metabolic rate), Harington et al concluded that the increased activity of thyroid thyroxine, when compared to free thyroxine, is probably due to the linkage and the optical activity of the natural thyroxine (optical activity will be discussed below).

Using thiouracil-treated chicks after the method of Dempsey et al (1943), Monroe and Turner (1949) found crystalline d,l-thyroxine to be

20 per cent as effective when administered orally as when given by subcutaneous injection as the disodium salt. On the other hand, desiccated thyroid was about equally effective perorally as parenterally. In a separate experiment on the d,l-thyroxine equivalents of the feed and the feces, it was calculated that iodinated casein was about 83 per cent absorbed.

These experiments show rather conclusively that thyroid is more readily absorbed than crystalline thyroxine. Apparently, thyroid is also absorbed better than iodinated casein.

Thompson and co-workers (1933), reporting on the effects of various compounds when administered orally to myxedema patients, observed that the disodium salt of thyroxine is more effective than the monosodium salt and the latter more effective than pure thyroxine. These authors suggested that solubility is the important factor in absorption, since thyroxine is the least soluble of the three and the disodium salt the most soluble. They also cited the work of Barnes who obtained evidence that thyroxine may be destroyed to the extent of about 11 per cent, in vitro, by the action of pancreatic enzymes.

It is significant that Monroe and Turner (1949) did not find an appreciable difference between the monosodium and disodium salts of thyroxine when the crystalline salts were administered orally. The apparent discrepancy between the two reports might, however, be accounted for in that Thompson and co-workers administered the disodium salt in alkaline solution. There also may be species differences.

Administering an alkaline solution of thyroxine (disodium salt) directly into an isolated intestinal loop, Schittenhelm and Eisler (1932) found that 90 per cent of the thyroxine is absorbed.

Harington and Salter (1930) reported their tryptic digestion product of thyroid, containing only thyroxine iodine in peptide linkage, to be slightly more active (oxygen consumption of rats) than an equivalent dose of l-thyroxine when both were injected subcutaneously. In the case of thyroxine in peptide linkage, as it is believed to be in the thyroid gland, it seemed quite likely to them that the active principle is liberated slowly, and therefore is utilized more efficiently. They also suggested that thyroxine, when injected in large doses, is partially lost by excretion before it is utilized by the organism.

These considerations should be taken into account when physiological rather than pharmacological properties of the hormone are being studied. The best assays are conducted with small doses over a period of several weeks.

More recently, Frieden and Winsler (1948), using the goiter prevention method (Dempsey, 1943), reported results which indicate that the biological activity of thyroxine, combined as thyroid protein, is about four times as great as d,l-thyroxine. These investigators determined the "thyroxine" content of the thyroid material by the chemical methods of Reineke et al (1943) and Blau (1935). Even assuming the dextrorotatory isomer of thyroxine to have no activity, the thyroid material was found to be twice as active as l-thyroxine.

Feng (1950), comparing the calorigenic effects of intraperitoneally injected thyroid and d,l-thyroxine also found thyroid to be more active when comparisons were made on the basis of their l-thyroxine content. The thyroxine iodine of the thyroid was estimated as being 25 per cent of the total iodine (Taurog and Chaikoff, 1946a) and one-half of the d,l-thyroxine was assumed to be biologically active (Reineke and Turner, 1945). Apparently, thyroxine when administered as a protein moiety is enhanced in activity. An alternate conclusion would be that other constituents with thyroidal activity and low iodine content are present in thyroid and iodinated casein.

Optical Activity of Thyroxine

Foster, Palmer and Leland (1936) found that thyroxine administered parenterally as the pure l-thyroxine or given orally in combination as thyroid was equivalent insofar as its calorigenic effect on guinea pigs was concerned. This was in accord with the finding of Harington and Salter (1930) that thyroxine, isolated by enzymatic hydrolysis of the thyroid gland, is in the form of its levorotatory isomer.

Palmer et al (1935) reported that in guinea pigs, thyroxine administered orally in the form of thyroid gland produced twice the calorigenic effect of d,l-thyroxine. Apparently therefore, the dextrorotatory isomer has no measurable activity under the conditions of these experiments. In more direct comparisons, Reineke and Turner (1943) found the activity of l-thyroxine, isolated from iodinated casein, to be approximately twice that of an equivalent amount of d,l-thyroxine, also derived

from casein. These investigators used per cent increase of the basal metabolic rate of guinea pigs as a criterion of activity. Again in 1945 the same investigators obtained similar results using other assay methods: l-, and d,l-thyroxine were compared in their ability to reduce the thyroid weight of thiouracil-treated chicks and rats and to stimulate metamorphosis in tadpoles.

On the other hand Gaddum (1929), measuring the oxygen consumption of rats, found that the dextrorotatory isomer possessed a small amount of activity; the numerical ratio varying between 1.5 and 3. When he tested the l-thyroxine on tadpoles by an earlier method (Gaddum, 1927) it was similarly more effective, but the ratio varied between 1.2 and 2. The possibility exists that the activity reported for d-thyroxine by Gaddum was due to incomplete resolution of the sample.

Salter et al (1935) when comparing the calorigenic effect of d-, and l-thyroxine in patients with spontaneous myxedema found the two forms to have essentially identical potencies. Pitt-Rivers and Lerman (1948) also reported that d-thyroxine possessed physiological activity when tested in myxedema patients, but only one-eighth to one-tenth that of l-thyroxine. Observing the effect on the basophils of the pituitary gland in thiouracil-treated rats Griesbach and co-workers (1949) reported d-thyroxine to have 0.3 the activity of l-thyroxine; both by direct comparison of the dextro- and levorotatory isomers and by calculating the results of an assay of d,l-thyroxine.

These investigators believe their method to be more sensitive than the thyroid weight or calorigenic techniques.

The question of d-thyroxine activity is not closed. However, in most practical considerations no significant error will result if the dextrorotatory isomer is assumed to be without activity.

Iodinated Casein

With the conditions for preparing iodinated casein with high biological activity established (Reineke et al 1943), it was desirable to know the relative activity of this material.

Reineke and co-workers (1945) reported close agreement between biological and chemical assays of iodinated casein, when the apparent thyroxine content of the iodinated protein was determined by their method (Reineke et al 1943).

Frieden and Winzler (1948) comparing the parenteral thyroidal activity of natural and synthetic thyroproteins by the goiter prevention method (Dempsey, 1943), reported synthetic thyroprotein to have less activity than expected from its apparent l-thyroxine content. The chemical assay technique of Reineke et al (1945) as well as the method of Blau (1935) were used to determine apparent thyroxine. Two normal sodium hydroxide was used as the hydrolytic agent in the latter method (no significant difference was found between the two methods).

In 1949, Reineke and co-workers reported an isotope dilution technique for the determination of thyroxine. Applying it to iodinated casein, they reported thyroxine values which were about 25 per cent of those obtained by the butyl alcohol extraction method of Reineke et al (1943).

When the data of Frieden and Winzler (1948) are corrected for true thyroxine, on the basis of these findings, the biological activity is found to be almost twice that expected from the chemical assay.

Peng (1950) found no significant difference between the calorigenic effects of thyroid and iodinated casein when preparations of equal thyroxine content were administered to rats. The thyroxine content of iodinated casein was determined by isotope dilution while 25 per cent of the total thyroid iodine was assumed to be thyroxine iodine (Taurog and Chaikoff, 1948).

In 1948 Bird and Trikojus established, by chromatographic analysis, the presence of two compounds besides thyroxine, with similar solubility characteristics, in a butyl alcohol extract of the hydrolysate of iodinated casein. One was shown to be diiodothyronine by its similarity to a reference compound and the other was believed to be triiodothyronine because of its position on the chromatogram, between thyroxine and diiodothyronine. This information helps explain the high chemical assay reported by Frieden, et al (1948) for iodinated protein. In other words the chemical assay is apparently not specific for thyroxine.

The Butyl Alcohol Extraction of Thyroxine

The foundation for thyroxine determinations, based on a quantitative isolation of the compound, was laid by E. C. Kendall. He was the first to demonstrate the application of alkali for the hydrolysis of thyroid protein as an essential step in concentrating the iodine-containing constituents. After treating the thyroid protein with NaOH and subsequent

dialysis of the hydrolysate, Kendall (1913) separated the products into two main groups: one containing 60 per cent of the iodine and 9 per cent of the nitrogen and the other 40 per cent of the iodine and 91 per cent of the nitrogen. In 1915 he reported that upon hydrolysis in alcoholic NaOH, desiccated thyroid was altered such that the iodine was in two forms of organic combination. Approximately half of the total iodine was soluble in acid while the other half was acid-insoluble. Furthermore, the acid-insoluble fraction was capable of producing the symptoms of hyperthyroidism as well as relieving the symptoms of hypothyroidism. With these two fundamental principles firmly established: (1) hydrolysis of the thyroid protein and (2) association of the physiologically active material with the acid-insoluble portion of the hydrolysate, Kendall continued his investigations and succeeded in isolating the physiologically active compound in a crystalline state (1915, 1919).

Harington and Randall (1929) devised a chemical assay for thyroxine at the request of the British Pharmacopea. Thyroid protein was hydrolyzed by boiling it in N NaOH solution for four hours. The filtrate was adjusted to pH 5 with 50 per cent H_2SO_4 and the acid-insoluble iodine was considered thyroxine iodine.

Leland and Foster (1932) contended that Harington and Randall's analysis for thyroxine, based on its insolubility in acid, although extremely simple technically, did not completely exclude diiodotyrosine from the "thyroxine" fraction. Their conclusion was based on an experiment in which they rehydrolyzed the acid-insoluble fraction, prepared by the previous method, in 2N NaOH for 18 hours, extracted it with normal

butyl alcohol and washed the alcoholic extract with N NaOH. They observed a strong nitrous acid test on the wash solution which was assumed to be due to a large diiodotyrosine fraction. An analysis for inorganic iodide, according to the method of Foster and Gutman (1930), indicated only a small amount and convinced them that there was no serious destruction of thyroxine. Furthermore, on comparing per cent thyroxine iodine by their butanol extraction technique with the method of Harington, et al (1929) on the same sample of desiccated thyroid, Leland and Foster found the former technique to indicate a thyroxine content about half that determined by the acid-insoluble iodine analysis. In their method for thyroxine determination the latter authors hydrolyzed desiccated thyroid for 18 hours with 2N NaOH, extracted the aqueous mixture with normal butyl alcohol and washed the alcoholic extract with N NaOH to remove the remaining diiodotyrosine. The hydrolysis with 2N NaOH was recommended to increase the yield of thyroxine. As judged by recovery experiments, unavoidable destruction of thyroxine during alkaline hydrolysis was about 15 per cent.

Blau (1933) modified the extraction procedure of Leland, et al by carrying out the initial step in an acid medium. The thyroxine values obtained by this modification were about 11 per cent higher than by the former method. He also introduced an alkaline washing solution which afforded a more desirable distribution between the thyroxine and non-thyroxine iodine fractions.

In 1935 Blau reported that a shorter period of hydrolysis was necessary when $Ba(OH)_2 \cdot 8H_2O$ was the hydrolytic agent. In recovery experiments,

comparing the stability of thyroxine when boiled for six hours in eight per cent $Ba(OH)_2 \cdot 8H_2O$ and 2N NaOH, the recovery of the thyroxine iodine was on the average six per cent higher by the former method. Blau's technique for quantitative thyroxine isolation consisted in hydrolyzing desiccated thyroid (or fresh thyroid) in eight per cent $Ba(OH)_2 \cdot 8H_2O$ solution for six hours, adjusting the pH of the hydrolysate to about 3.7 with HCl and extracting with normal butyl alcohol. He then washed the extract with a solution consisting of 4N NaCH and five per cent Na_2CO_3. The recoveries of thyroxine from mixtures of thyroxine and diiodotyrosine dissolved in an eight per cent $Ba(OH)_2 \cdot 8H_2O$ hydrolysate of testicular powder averaged close to 100 per cent and the effect of inorganic iodine was invariably too small to measure.

Comparing the apparent thyroxine content of iodinated casein by the method of Blau and by a tadpole assay technique, Reineke and coworkers (1945) found that results by the former method were considerably higher than the latter biological assay. This led them to the supposition that the high chemical assay might be due to incomplete hydrolysis of the protein, with the result that some non-thyroxine compounds were soluble in normal butyl alcohol. In comparison with the former method, the results obtained by a 20 hour hydrolysis with 40 per cent $Ba(OH)_2 \cdot 8H_2O$ showed far better agreement with the biological assay. In view of the fact that the apparent thyroxine content did not change significantly between periods of hydrolysis from eight to twenty-eight hours, 20 hours was recommended.

Investigating the thyroid content of rat thyroid glands, Taurog and Chaikoff (1946) found that values obtained with $Ba(OH)_2 \cdot 8H_2O$, using Blau's technique (1935), were more variable than those obtained with 2N NaOH as the hydrolytic agent. However, they did confirm Blau's finding that NaOH caused greater destruction of thyroxine than $Ba(OH)_2 \cdot 8H_2O$. It should be pointed out that Blau (1935) also reported his technique to give variable results.

Recent reports, reviewed in other sections of this paper, suggest that the butanol extraction technique, even under the most accepted conditions, is not specific for thyroxine.

EXPERIMENTAL PROCEDURE

Preparation of Radioactive Thyroxine from Casein

The method described by Reineke and co-workers (1943) was used with only minor modifications.

Iodination

Twenty grams of casein (purified, vitamin-free), nine gms. of sodium bicarbonate (C.P.), and 670 ml. of distilled water were mixed in a 29 cm. by 10 cm. glass cylinder. Through a rubber stopper, fitted snugly into the mouth of the cylinder, a short length of metal tubing was inserted to permit the passage of an electrically operated glass stirring rod, bent into a triangle at the end to facilitate stirring.

Radioactive iodine-131 solution was prepared from a radioactive iodide solution in a 50 ml. test tube, by the addition of a small crystal of potassium iodate and one drop of concentrated phosphoric acid to about 1 ml. of the iodide.* A small sodium iodide crystal was added and produced a color change, indicating that iodine had been released from the iodide. Five ml. of distilled water were then added to the solution.

The glass cylinder was placed in a constant temperature thermostatically controlled water bath and the heating unit and stirring apparatus were turned on, the latter being set for rapid stirring. When the temperature of the bath had reached 40 degrees centigrade, stirring was stopped and the radioactive iodine solution, previously prepared, was

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* Purchased from Oak Ridge National Laboratory, Carbide and Carbon Chemicals Co., Oak Ridge, Tenn., and used in concentrations of approximately 10 mc. per ml.

added to the mixture in the cylinder. The tube was rinsed with five ml. of water which was also added to the mixture. Stirring was resumed for several minutes during which time 3.6 gms. of iodine crystals were weighed out on a filter paper. Stirring was interrupted while the iodine crystals were added to the mixture in the cylinder, and then resumed and continued for 18 hours at a temperature of 70 degrees centigrade.

Hydrolysis

After the 18 hour incubation period the cylinder was removed from the water bath and allowed to cool at room temperature for about one-half hour. Sufficient 3.5N HCl was added to precipitate the protein, and caprylic alcohol was added dropwise to reduce foaming. The mixture was then filtered through a Buchner funnel by means of negative pressure. The protein in the funnel was washed by adding 3.5N HCl in sufficient quantity to cover the precipitate. Washing was enhanced by gentle stirring and the liquid was removed by suction. The precipitate was washed twice in this manner after which the damp precipitate was put into a 500 ml. reaction flask and 128 ml. of distilled water, 64 gms. of $Ba(OH)_2 \cdot 8H_2O$ and about one-half ml. of caprylic alcohol were added. The mixture was boiled gently under a Liebig reflux condenser for 20 hours, the flask being agitated frequently by shaking until it was apparent that there was no further danger of foaming.

Extraction

After the 20 hour hydrolysis, heating was discontinued and the hydrolysate was permitted to cool until the barium salts had settled

and the flask could be handled easily. The supernatant liquid was decanted and saved. A mixture of N butyl alcohol and 3.5 N HCl was added to the barium salts in amounts of approximately two and five ml. respectively. The barium salts were then vigorously stirred in this butyl alcohol-acid mixture to aid in their solution and suspension. The mixture of barium salts was combined with the supernatant liquid and 3.5 N HCl was carefully added to the mixture, from a burette, until it was acid to congo red paper. Approximately 40 ml. of the mixture were removed to a separatory funnel for extraction of "thyroxine", and about 20 ml. of N butyl alcohol were added. The aqueous and organic phases were mixed by swirling the mixture around in the funnel. After the two phases had separated the butyl alcohol (upper) layer was removed and washed with an equal amount of a solution prepared by dissolving 480 gms. of NaCH and 150 gms. of Na_2CO_3 in water and making up to a volume of 3000 ml. Separation of the wash solution and the butyl alcohol extract was permitted to take place over a period of not less than one hour. The washing and separation procedure was repeated with one-half the volume of wash solution used the first time.

Chromatographic Analysis

The "ascending" technique for chromatographic analysis, developed by Williams and Kirby (1948), was adapted to the study of the characteristics of the purified butyl alcohol extract of the hydrolysate of iodinated casein. Solvent mixtures used by Hird and Trikojus (1948), in a study of thyroxine and its analogues, were also found to be satisfactory for the purposes of this experiment.

The apparatus consisted of a 43.5 cm. by 20 cm. glass cylinder in the bottom of which was placed a nine cm. Petri dish. The cylinder was fitted with a plate glass cover and the seal was made air-tight with desiccator grease. A 40 cm. by 28 cm. sheet of Whatman No. 1 filter paper was marked with a pencil line parallel to the shortest axis and three cm. from the end of the sheet. The filter paper was rolled into a tube and the edges were stapled together. Solutions of the samples to be analyzed were placed in spots at intervals along the pencil line encircling the bottom. For purposes of comparison the paper was also spotted with thyroxine, diiodothyronine, and diiodotyrosine. A dropping pipette, the tapered end of which had been heated in a flame until the lumen was reduced in size, was originally used to apply the solution, in later studies the flow of liquid was found to be more easily controlled with a 0.5 ml. pipette. Small spots were found to give the most reproducible results. After being spotted, the paper was dried at room temperature for at least 15 minutes.

The solvent mixtures were prepared by shaking together in a separatory funnel, a mixture of 120 ml. of N amyl alcohol, 120 ml. of N butyl alcohol and 240 ml. of 2N NH_4OH.* The organic and aqueous phases were then allowed to separate for a period of time not less than one hour. The aqueous (lower) layer was drawn off and an amount was placed in the glass cylinder just sufficient to cover the bottom. The Petri dish was placed in the center of the cylinder and the paper tube was placed, penciled end down, in

* In the earlier experiments, in which the radioautograms were prepared, the solvents did not contain amyl alcohol. Instead, twice the amount of butyl alcohol was used.

the dry Petri dish. The cylinder was sealed with the plate glass and the tube was allowed to stand in the Petri dish for approximately three hours to enable the paper to become impregnated with the aqueous phase. The paper tube was then removed from the cylinder and 32 ml. of the organic phase were disponsed from the separatory funnel into the Petri dish. The tube was again placed in the same manner as before and the cylinder was sealed with the plate glass.

The solvent front was allowed to move up the paper for varying periods of time, up to 30 hours. In all cases, the paper tube was removed from the cylinder before the solvent had reached the top. The paper was then dried at room temperature for several hours. By means of an atomizer attached to an airline, the dried paper was sprayed, until saturated, with a 0.4 per cent solution of triketohydrindene hydrate (ninhydrin) in N butyl alcohol, in order to render the amino acids visible. The soaked paper was then allowed to stand in a drying oven at about 75 degrees centigrade for approximately 15 minutes. Blue spots of various shades appeared, representing the compounds in the mixture. The Rf values were determined by dividing the distance the front of the spot in question moved by the distance the solvent front moved, the origin in both cases being the point of application of the spot.

Preparation of the Radioautograms

Radioautograms were prepared by placing Kodak No Screen X-ray film in direct contact with the chromatogram, between two flat pieces of glass. The procedure was carried out in complete darkness. Exposure

Figure 1

Apparatus for Paper Partition Chromatography

FIGURE 1

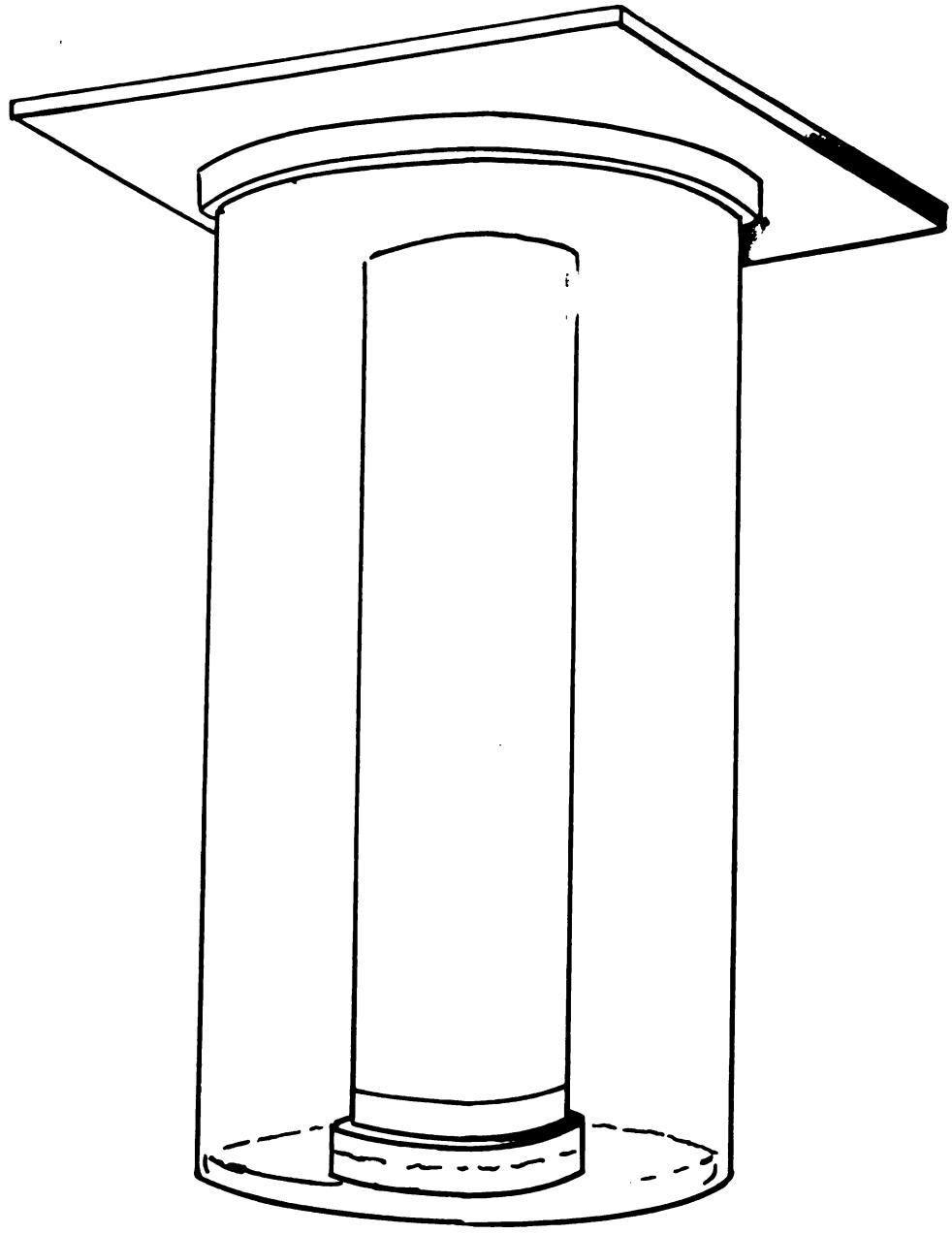


FIG.1 APPARATUS FOR PAPER PARTITION CHROMATOGRAPHY

time was determined by the Beta count per minute, of the portion of the chromatogram to be recorded. An unshielded mica window Geiger Mueller tube was placed directly over the chromatogram. A 24 hour exposure was sufficient for a Beta count of 5000 per minute. The autogram in Figure 2 was exposed for six days. The exposed autograms were developed and fixed with Kodak X-ray developer and Hypo, at full strength.

Densitometer readings of various areas on the developed radioautograms were taken by means of an Ansco-Sweet Densitometer.

The Efficiency of the Sodium Hydroxide-Sodium Carbonate Wash Solution

Radio activity determinations were made of samples of the butanol extract of the acid-insoluble portion of the hydrolysate of iodinated protein with a scaling unit in conjunction with a Geiger Mueller tube. The samples were taken from: unwashed extract, extract washed once, and twice-washed extract. The wash solution consisted of a 4N NaOH and five per cent Na_2CO_3 mixture as originally described by Blau (1935). An amount of wash solution equal to the volume of butyl alcohol extract was used in the first washing while one-half of that volume was used for the second washing. The samples were evenly distributed on an aluminum disc and dried at room temperature before radioactivity determinations were made.

Biological Assay of Diiodothyronine

The thiouracil technique described by Dempsey and Astwood (1943) was used to assay purified diiodothyronine. Groups of six male albino

rats purchased from Rockland Farms, New York, were used throughout the experiment. The rats were fed ad libitum with a standard diet devised by Dr. C. A. Hoppert, Department of Chemistry, Michigan State College, (see appendix) and drinking water containing 0.2 per cent thiouracil was available at all times. The thiouracil was put into the drinking water one day before the injections were begun, and for 14 days the rats were given daily subcutaneous injections of the appropriate amount of d,l-thyroxine or d,l-diiodothyronine. On the tenth day one rat in group two was found dead. The test compounds were dissolved in 0.1N NaOH and the pH was adjusted to about eight with dilute HCl. Six rats were kept in each cage and the room temperature was 24±1 degrees centigrade. Artificial lights were turned on from approximately 8:00 A.M. to 5:00 P.M.

After the 14 day experimental period all the rats were sacrificed and the thyroid glands were dissected out and trimmed free of fat. The rats were weighed to 0.1 gm. on a triple beam balance and the thyroid glands to 0.1 mg. on a Roller Smith balance.

The thyroxine was supplied by Dr. E. P. Reineke of the Department of Physiology and Pharmacology and the diiodothyronine was purchased from S. A. F. Hoffmann-La Roche and Company, Basel, Switzerland, and further purified by the author (see appendix).

Results

Experiment I

Inasmuch as recent evidence indicates that several non-thyroxine compounds are carried along with thyroxine when iodinated casein is

assayed by the butyl alcohol extraction technique, it was of interest to determine the number of these compounds.

Paper partition chromatography was utilized to separate the components of the twice-washed butyl alcohol extract of iodinated casein. The rsults indicate the presence of three compounds other than thyroxine, triiodothyronine, and diiodothyronine (these last three compounds were reported to be in an unpurified butyl alcohol extract, by Hird and Trikojus, 1948*.

An intense blue spot appeared when compound III, Rf 0.20, was treated with Ninhydrin reagent (an amino acid indicator). Compound I, Rf 0.03, and compound II, Rf 0.10, gave comparatively weak ninhydrin tests. Control runs with d,l-thyroxine indicated an average Rf of 0.41. Individual results are listed in table I.

Experiment II

Since the butyl alcohol extraction technique depends on an iodine determination, the non-thyroxine iodinated compounds on the chromatogram were located and their concentrations, in a relative manner, were indicated by the preparation of radioautograms. An autogram prepared from a chromatogrammed extract, after purification with a sodium hydroxide-sodium carbonate solution,* revealed a spot, 7, just below a thyroxine control. Although the control, RF 0.53, was abnormally high when compared to the average of thyroxine controls (0.41) run with only slightly different solvents and listed in Table I, the spot in question bears the

* In this particular experiment the butyl alcohol extract was washed twice with equal amounts of each solution.

same relation to the thyroxine as the intense blue spot Rf 0.20, described as compound III in the results of the preceding experiment, i.e., it would seem that spot 7 is correspondingly high. An examination of the radioautogram prepared from unwashed butyl alcohol extract reveals six spots below spot 7.

Densitometer readings of radioautograms made from chromatogrammed extracts (Table II), before and after washing, indicated that the sodium hydroxide-sodium carbonate wash solution has a specific action in removing compounds below spot 7. Readings at 7 were 0.61 for the unwashed and 0.37 for the washed extracts. Moving up the autogram from spot 7, readings taken at points directly opposite one another had values of 1.49 and 1.49, 0.92 and 0.82, 0.63 and 0.62 for the unwashed and washed extracts respectively. A visual inspection and densitometer readings of the area below spot 7 indicated almost a complete removal of iodinated compounds by the wash solution.

Experiment III

Radioactivity measurements of aliquot samples of a butyl alcohol extract were carried out in order to determine the actual change in iodine concentration before and after purifying with the previously described wash solution. This would give some indication of the efficiency of this part of the butyl alcohol extraction technique. Results are recorded in Table III. The average count of an aliquot before purification was 16.2 and after the first and second washings it was 7.9 and 7.2 respectively. The per cent change in counts was 52 after the first washing and 8.4 after washing a second time.

TABLE I

Rf VALUES OF SPOTS BELOW THYROXINE (ASCENDING) ON CHROMATOGRAMS PREPARED FROM THE PURIFIED HYDROLYSATE OF IODINATED CASEIN. THE SPOTS WERE MADE VISIBLE WITH NINHYDRIN REAGENT. RACEMIC THYROXINE CONTROL VALUES ARE INCLUDED.

Preparation No.	Spot Number			Thyroxine Control
	I	II	III	
1	0.06	0.12	0.24	---
2	---	---	0.20	0.40
3a	0.03	0.12	---	---
3b	0.03	---	0.21	0.45
3c	0.01	0.08	0.20	---
3d	0.04	---	0.18	0.41
3e	0.03	0.07	0.20	0.36
3f	---	0.10	0.20	0.41

TABLE II

DENSITOMETER READINGS OF RADIOAUTOGRAMS PREPARED FROM CHROMATOGRAPHED BUTYL ALCOHOL EXTRACT OF THE HYDROLYSATE OF IODINATED CASEIN, BEFORE AND AFTER PURIFYING WITH A SODIUM HYDROXIDE-SODIUM CARBONATE SOLUTION

Point On Radioautogram	Densitometer Reading*	
	Unwashed	Twice Washed
10	0.63	0.62
9	0.92	0.82
8	1.49	1.49
7	0.61	0.37
6	0.52	0.28
5	0.66	0.22
4	1.06	0.22
3	1.0	0.20
2	0.80	0.22
1	1.12	0.28

Background reading 0.06

* Densitometer readings are on a logrithmic scale. Thus, readings of 1 and 2 would indicate 10 per cent and 1 per cent transmission of light respectively.

FIGURE 2

Figure 2

Radioautograms of a chromatogrammed butanol extract of the hydrolysate of iodinated protein, before and after purifying the extract with a NaCl-Na_2CO_3 solution.

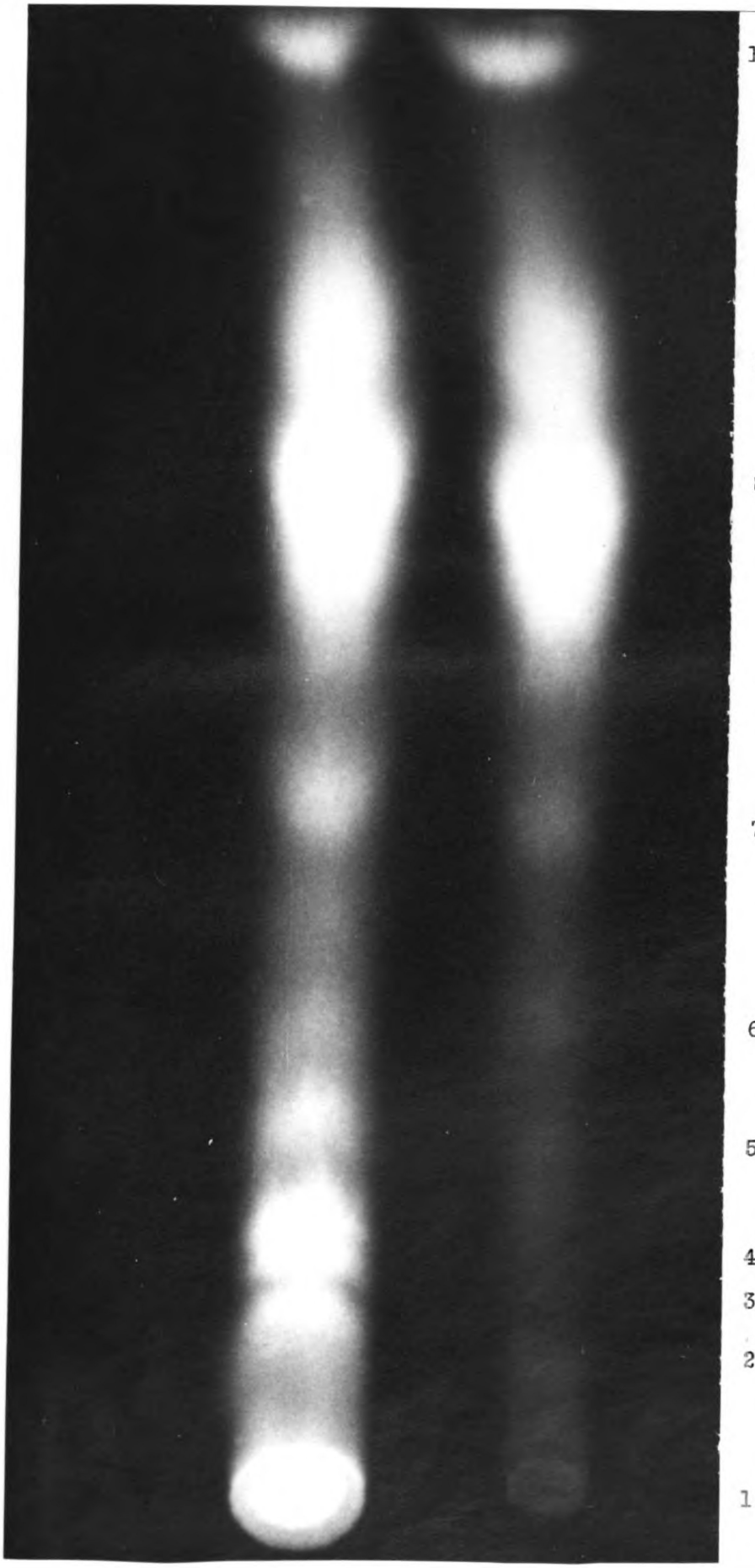

10
9
8
7
6
5
4
3
2
1

TABLE III

RADIOACTIVITY DETERMINATIONS IN COUNTS PER SECOND BEFORE AND AFTER PURIFYING AN HYDROLYSATE OF IODINATED PROTEIN WITH A SODIUM HYDROXIDE-SODIUM CARBONATE SOLUTION. COUNTS ARE CORRECTED FOR BACKGROUND.

Group No.	Unwashed	Washed Once	Washed Twice
I	16.97	7.85	7.10
II	16.41	7.86	7.44
III	15.32	7.96	7.06
Average	16.23	7.89	7.20
Per cent change in radioactivity after purification		52	8.4

Experiment IV

A biological assay of diiodothyronine was carried out inasmuch as this compound is believed to be present in the purified butyl alcohol extract of the hydrolysate of iodinated casein and since it is available in a pure state. Possibly, a biological assay would be more specific for thyroxine than the butyl alcohol extraction technique.

Results of the assay, tabulated in Table IV, and depicted graphically in Figure 3, indicate a normal thyroxine response with four groups of 1, 2, 3, and 4 mcg. doses, respectively, of d,l-thyroxine. On the other hand, groups administered 10, 15, 20, and 25 mcg. doses of d,l-diiodothyronine showed a wide range of activity within each group and none of the doses administered to thiouracil-treated rats showed any significantly greater ability to reduce the thyroid weight than a 1 mcg. dose of d,l-thyroxine; at the 10 and 25 mcg. levels of diiodothyronine there was very little, if any, reduction of thyroid weight. At the 15 and 20 mcg. levels of diiodothyronine the average thyroid weights were very similar and about the same as those in the group administered 1 mcg. of d,l-thyroxine.

TABLE IV

THYROID WEIGHTS PER 100 GMS. RAT WEIGHT OF THIOURACIL-TREATED RATS ADMINISTERED THYROXINE AND DIIODOTHYRONINE

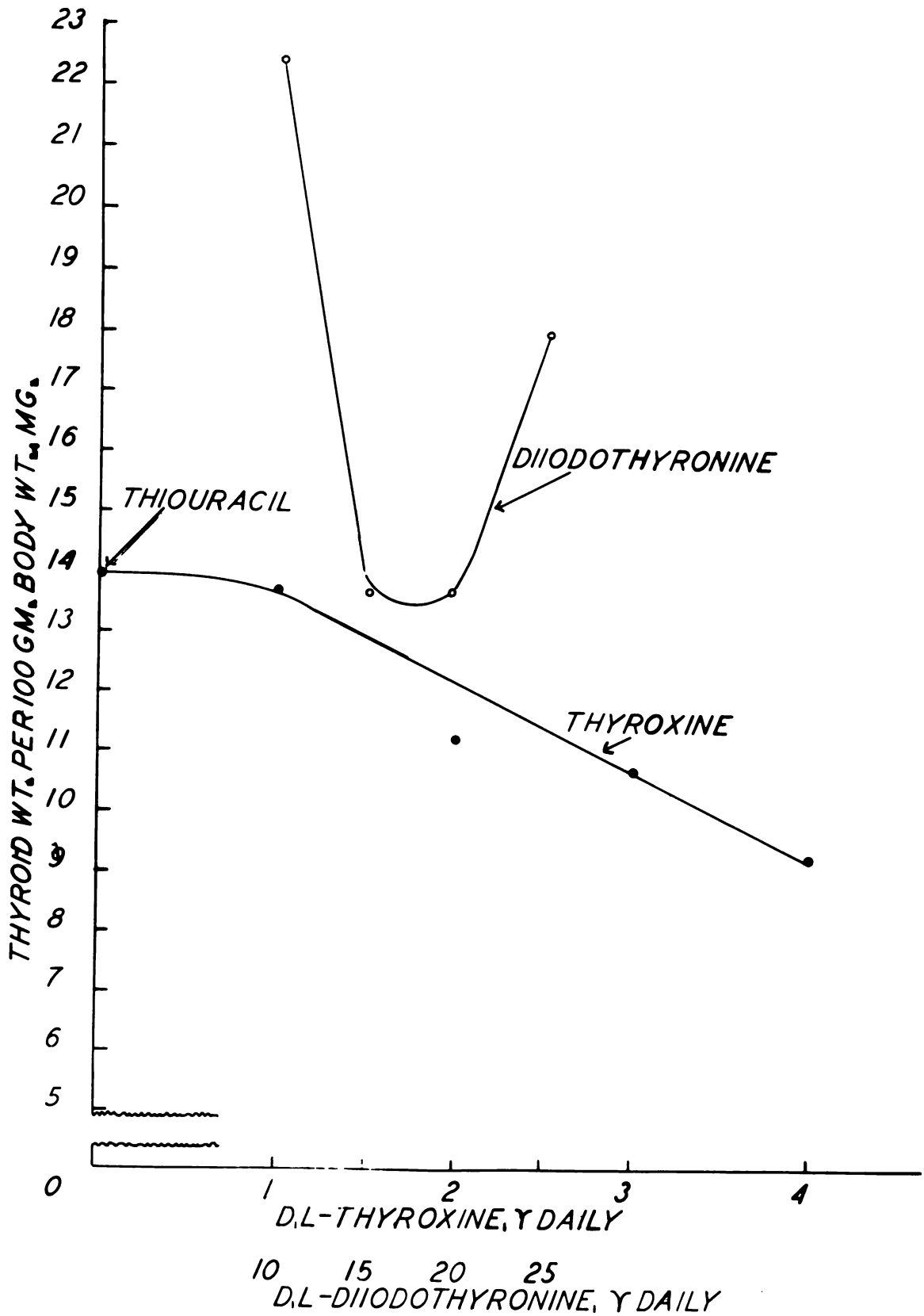
	d,l-thyroxine			
Group	I	II	III	IV
Dose	1 mcg.	2 mcg.	3 mcg.	4 mcg.
	12.47	11.13	12.26	10.34
	10.42	12.77	11.78	19.04*
	15.48	10.62	8.20	8.17
	14.71	14.65	10.20	9.84
	15.31	9.20	12.60	7.32
	13.86	8.89	9.40	10.50
Average	13.70	11.21	10.73	9.23
	d,l-diiodothyronine			
Group	I	II	III	IV
Dose	10 mcg.	15 mcg.	20 mcg.	25 mcg.
	19.20	18.47	11.14	19.22
	33.88	12.28	10.98	18.43
	27.61	16.01	13.12	20.12
	21.96	11.75	15.40	16.37
	15.93	9.85	13.12	19.25
	16.25		18.27	14.59
Average	22.47	13.67	13.67	17.99
	Control (0.2 per cent thiouracil)			
	14.68			
	17.61			
	10.27			
	11.98			
	17.15			
	12.06			
Average	13.95			

* Excluded from average.

FIGURE 3

Figure 3

The relation between dosage and thyroid weight per 100 gms. body weight of thiouracil-treated rats administered di-iodothyronine and thyroxine.


23
22
21
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
0
THYROID WT. PER 100 GM. BODY WT. MG.
THIOURACIL
DIIODOTHYRONINE
THYROXINE
1
2
3
4
D,L-THYROXINE, γ DAILY
10
15
20
25
D,L-DIIODOTHYRONINE, γ DAILY

DISCUSSION

A study of the washing technique, originally described by Blau (1935) for the purification of the butanol extract of iodinated casein hydrolysate, has more completely defined its action in removing non-thyroxine compounds. Radioautograms of chromatogrammed butanol extract, before and after purification, have revealed that sodium hydroxide-sodium carbonate wash solution specifically removes all but traces of butanol-soluble iodinated compounds with Rf* values below that of spot 7 (approximate Rf 0.20); the compound represented by spot 7 is only partially removed by the wash solution. Three distinct spots, presumably representing at least three different iodinated compounds, have been observed which were not significantly affected by the purification procedure. Inasmuch as the concentration of thyroxine dissolved in acid butanol is known to be uneffected by a sodium hydroxide-sodium carbonate wash solution (Blau, 1935), it is presumed to be one of the 3 spots. A thyroxine control run adjacent to the unknown mixture suggests that spot 8 represents thyroxine. The other compounds with higher Rf's have solubility properties similar to thyroxine, at least with respect to the particular solutions used in the experiment.

These findings indicate that the main sources of error in the standard technique for determining thyroxine in iodinated casein are iodinated compounds similar in solubility to thyroxine. It seems possible that a similar situation exists with respect to thyroid gland. Bird and

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* Ratio between rate of movement of compound and rate of movement of solvent front.

Trikojus (1948) first reported the presence of amino acids in the butyl alcohol extract of iodinated casein hydrolysate which they tentatively identified as thyroxine, triiodothyronine, and diiodothyronine. Their conclusions were based on Rf values and ninhydrin tests. However, their work gives no indication of the number of compounds in the butyl alcohol extract after the purification procedure suggested by Blau. Furthermore, their report offers no direct evidence of the presence of iodinated compounds.

The finding of Frieden and Winzler (1948) that synthetic thyroprotein showed less thyroidal activity than expected from its apparent l-thyroxine content can now be explained by the presence of several thyroxine-related but relatively inert iodinated compounds.

Support is given to the reliability of the isotope dilution technique used by Reineke and co-workers (1949) for thyroxine determinations. Their technique gives thyroxine values which are much lower than those obtained by the butyl alcohol extraction method. Preliminary results of direct radioactivity determinations of spots on paper chromatograms reveals thyroxine-iodine activity to occupy only a small percentage of the total activity.

The data presented in this paper indicates that the slowly moving compounds, including diiodothyronine, are removed in the purification procedure. The Rf of diiodotyrosine is 0.00-0.01 (Bird and Trikojus, 1948). This is not in accord with the views of Borrows and co-workers (1949). These workers made their analysis polarographically and reported considerable amounts of diiodotyrosine in the purified extract. In their wash solution they used sodium bicarbonate whereas Blau's method calls

for sodium carbonate. This could be a misprint or, if not, may account for the apparent discrepancy between the two sets of data.

The chromatograms and radioautograms indicate the presence in the twice-washed butyl alcohol extract of a hitherto unreported iodinated amino acid with an Rf of 0.20. The amount of blackening of the X-ray plate used in preparing the autograms and the intensity of the ninhydrin test reveals the compound to be present in more than trace amounts.

It is concluded from the number of spots on a radioautogram that six compounds, present in the unwashed extract, have been greatly reduced in concentration by the purification procedure. A two dimensional chromatogram of the unwashed hydrolysate, prepared as a preliminary experiment to a future investigation, indicated the presence of more than 11 compounds giving a positive ninhydrin test.

Further evidence of the specificity of the sodium hydroxide-sodium carbonate wash solution was supplied by a controlled washing experiment; radioactivity measurements indicated that 52 per cent of the iodine was removed by the first washing while a second washing succeeded in removing only eight per cent more.

A preliminary study of the biological action of diiodothyronine, a compound in the butyl alcohol extract (Hird and Trikojus, 1948) with a structure similar to thyroxine, suggests that it has a qualitatively different action on the pituitary gland; the administration of progressively increasing doses, exceeding those reported to have an effect on the metabolism of rats (Gaddum, 1929), did not show proportional effects, if any at all, in reducing the thyroid weight of thiouracil-treated rats.

It is not unlikely that thyroxine is the only compound derived from iodinated casein with its unique effect on the thyroid-pituitary axis; increased thyroxine administration causes a decreased secretion of thyrotropic hormone by the pituitary gland (Aron et al, 1931). Formerly it was believed, on the basis of metabolism assays, that there was only a quantitative difference between thyroxine and diiodothyronine. Of practical interest is the availability of a biological assay which these preliminary experiments indicate to be specific for thyroxine.

SUMMARY

(1) Casein was iodinated with a mixture of iodine-127 and radioactive iodine-131. The iodinated protein was hydrolyzed with barium hydroxide and the acid-insoluble portion of the hydrolysate extracted with normal butyl alcohol.

(2) The alcoholic extract was washed twice with a mixture consisting of five per cent Na_2CO_3 and 4N NaOH. Radioactivity measurements indicated a 52 per cent reduction in iodine content after the first washing and an eight per cent further reduction after the second washing.

(3) The purified extract was chromatogrammed and amino acids were located by treating with ninhydrin reagent. A hitherto unreported amino acid of Rf 0.20 was found.

(4) Radioautograms of chromatogrammed, purified and unpurified extracts were prepared and densitometer readings were taken of appropriate areas. The purification procedure reduced considerably the concentration of compounds with Rf values below 0.20. An exposed area on the autogram just below a thyroxine control suggested that the amino acid with Rf 0.20 is iodinated. Several iodinated compounds were found in the butyl alcohol extract which were not appreciably altered in concentration by the sodium hydroxide-sodium carbonate wash solution used in the purification procedure.

(5) A biological assay of diiodothyronine was carried out on thiouracil-treated rats. Results suggest that this compound has no effect on the thyroid-pituitary axis.

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APPENDIX

Purification of Diiodothyronine

The diiodothyronine obtained from Hoffmann-La Roche and Company, was dissolved in hot ethyl alcohol with the aid of 1.5N HCl and precipitated by the addition of one-half saturated sodium acetate solution. The hot mixture was centrifuged and the white crystalline product washed with acetone. A second partial precipitation of the diiodothyronine was carried out by the same method. The purified preparation was stored under refrigeration in a desiccator jar until ready for use.

4 Kilo "Hoppert" Stock Ration

Yellow corn meal (Thoman)	1400 gm.
Ground whole wheat (Thoman)	1000 gm.
Whole milk powder (Borden)	800 gm.
Linseed oil meal (Thoman)	400 gm.
Alfalfa leaf meal (Thoman)	240 gm.
Brewer's Yeast (Strain G) (A. Busch)	120 gm.
Table Salt (iodized)	40 gm.

A critical analysis of the butyl alcohol extraction technique used in the quantitative determination of thyroxine.

3-3-52