N^1 -Diethylsulphanilamide: a Reagent for the Colorimetric Estimation of Thyroxine

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The estimation of thyroxine in thyroid substance can be performed by biological assay, or by the determination of 'thyroxine iodine' in the hydrolyzed material. In the latter case the iodine content of the precipitate at pH 4.5, or of its butanol extract after treatment with a strongly alkaline aqueous phase, which retains the diiodotyrosine, can be expressed as 'thyroxine'. The methods have been reviewed by Harington (1933) and Salter (1940). On adapting the original butanol-extraction procedure of Leland & Foster (1932), as modified by Blau (1935), to artificially iodinated proteins, Reineke, Turner, Kohler, Hoover & Beezley (1945) compared their results with those of biological assay in the guinea pig and were led to conclude that the chemical method reliably accounted for the thyroxine present; the properties of any non-thyroxine component of the butanol layer would need to parallel closely those of thyroxine itself. From an examination of thyroid-gland preparations from different species, Parkes (1946) concluded that a fairly close relationship exists between acid-insoluble iodine (and total iodine) and the biological activity as determined in the *Xenopus* tadpole. On the other hand, Deanesly & Parkes (1945) found a very marked divergence from this correlation in the case of the acid-insoluble-iodine fractions from artificially iodinated proteins.

It would seem desirable to have an alternative method for the chemical assay of thyroxine, and we have been engaged in investigating the possibility of developing a specific colour reaction which could be quantitatively applied. Thyroxine gives a characteristic, though non-specific, red colour with nitrous acid, under special conditions, while the ninhydrin test is weakly positive (Harington, 1933). Recently Roche & Michel (1946) have reported a method for the quantitative estimation of thyroxine and diiodotyrosine based on the nitrous acid reaction. A further qualitative test was described by Komant (1930) in which a red or purple colour, transient in dilute solution, was produced by thyroxine in the presence of diazotized sulphanilic acid in alkaline solution. A colour, though much less intense, was also given by diiodotyrosine. Since both

the 3' and 5' positions in thyroxine are occupied, the reaction mechanism, as inferred but not proved by Komant, presumably involves the replacement of the iodine at one of these points by the arylazo grouping. Thyroxine *O*-methyl ether does not react under these conditions (Loeser, Ruland & Trikojus, 1938). In examining this reaction further we have selected as reagent N^1 -diethylsulphanilamide from a number of diazotizable amines using sensitivity, stability of the colour formed and the preference of the reaction product for the butanol phase in *n*-butanol-water mixtures as criteria. This last property was taken into account since, by transferring to butanol, thyroxine may be conveniently separated from contaminants, such as diiodotyrosine, prior to assay. The conditions of the reaction have also been studied with the object of increasing the thyroxine specificity and in order to standardize a procedure suitable for quantitative analysis.

EXPERIMENTAL

Preliminary studies

Selection of amine

Sulphanilic acid was chosen by Komant (1930) rather than its arsenic analogue, *p*-nitraniline or *p*-aminobenzoic acid, all of which gave, after diazotization, coloured reaction products with thyroxine in an alkaline medium (optimum pH 9-10). In Table 1 is listed a number of amines together with the results of our attempts to couple them with thyroxine, at pH 9-10, the test solutions being constituted as follows:

Thyroxine. A 0.1% (w/v) solution in 0.004*N*-NaOH.

Alkaline solution. NaOH (20 g.) and NaHCO₃ (28.8 g.) were dissolved in water and diluted to 1 l.

Diazo solution. In most cases the direct method of diazotization was used. The test amine (0.005 mol.) was dissolved or suspended in 9 ml. of 10*N*-HCl, water was added to a volume of 100 ml. and 5 ml. of this solution or suspension was mixed with 25 ml. of 4.5% (w/v) aqueous NaNO₂ at 0° and allowed to stand for 10 min. Diazotization was checked by coupling a sample of the solution with α - or β -naphthol under conditions comparable to those used for the reaction with thyroxine. Only those amines whose diazotized solutions gave a strong positive reaction with

Table 1. *Reactions between thyroxine and various diazotized amines in aqueous solution (pH 9-10) and the solubilities, relative to the aqueous phase, of the coloured products in n-butanol*

No.	Diazotized amine	Colour		Transferred to <i>n</i> -butanol phase
		Thyroxine (0.1%)	Control (no thyroxine)	
1	Sulphanilic acid	Deep red	Yellow	-
2	Sulphanilamide	Red	Yellow	-
3	<i>N</i> ¹ -Ethylsulphanilamide	Rose-red	Pale yellow	+
4	<i>N</i> ¹ -Diethylsulphanilamide	Rose-red	Pale yellow	+
5	Sulphaguanidine	Red	Yellow	+
6	<i>m</i> -Sulphaguanidine	Red	Deep yellow	Partly
7	Sulphapyridine	Orange	Yellow	+
8	Sulphadiazine	Deep red	Yellow	-
9	Sulphathiazole	Red	Orange-yellow	-
10	Sulphamerazine	Yellow-red	Orange-yellow	-
11	1-Aminonaphthalene-4-sulphonamide	Brownish yellow	Brownish yellow	-
12	1-Aminonaphthalene-4-sulphonic acid	Brownish yellow	Brownish yellow	-
13	2-Aminonaphthalene-4:8-disulphonic acid	Red	Orange-yellow	-
14	4-Aminobenzoic acid	Red	Yellow	-
15	Ethyl 4-aminobenzoate	Red	Yellow	+
16	3:5-Dibromo-2-aminobenzoic acid	Yellow	Yellow	-
17	4-Aminoacetophenone	Brown-red	Yellow	+
18	<i>p</i> -Nitraniline	Red (purplish)	Deep yellow	+
19	2:4-Dichloroaniline	Red	Pale yellow	+
20	2:5-Dichloroaniline	Deep pink	Pale yellow	+
21	4-Diethylaminoaniline	Yellow	Yellow	-
22	<i>o</i> -Toluidine	Yellow	Yellow	-
23	<i>m</i> -Toluidine	Deep orange	Yellow	+
24	<i>p</i> -Toluidine	Orange	Yellow	+
25	2- <i>p</i> -Xylidine	Yellow	Yellow	-
26	4- <i>m</i> -Xylidine	Yellow	Yellow	-
27	<i>p</i> -Anisidine	Yellow	Yellow	-
28	4-Aminodiphenyl ether	Brownish yellow	Brownish yellow	-
29	4-Aminodiphenylamine	Brownish yellow	Brownish yellow	-

either of these two phenols are included in the table. The large excess of nitrite is as used by Komant (in our later studies with *N*¹-diethylsulphanilamide this reagent was appreciably reduced in quantity without affecting the reaction).

Procedure. The thyroxine solution (1 ml.) was mixed with the alkaline solution (1 ml.) and the solution of the diazotized amine (1 ml.) then added. A colour, when produced, was formed almost immediately. Redistilled *n*-butanol (1 ml.) was added after 3 min. and the tube was well shaken. A control (1 ml. of 0.004*N*-NaOH) was treated in the same way.

Comments on Table 1. Although a systematic study of the relationship between amine structure and reactivity with thyroxine was not made, the presence of certain substituents in the position *para* to the amino group precluded reaction with the amino-acid but not with naphthol under the conditions used. If the colours in the control tube and in that containing thyroxine were comparable a negative reaction was recorded. This occurred when the *p*-substituent was $-\text{N}(\text{CH}_3)_2$, $-\text{OCH}_3$, $-\text{OC}_6\text{H}_5$ and $-\text{CH}_3$. Unless the substituent is halogen (amines 19, 20) it would seem that both positions *ortho* to the amino group should be unoccupied (amines 22, 25, 26). This may explain the apparent anomalies of

negative reactions with the naphthalene analogues of sulphanilic acid and its amide (amines 11, 12). With the amino group in the 2-position in naphthalene (amine 13) the reaction was strongly positive.

Derivatives of aniline substituted in the *para* position by $-\text{NO}_2$, $-\text{COCH}_3$, $-\text{COOH}$, $-\text{COOC}_2\text{H}_5$, $-\text{SO}_3\text{H}$, $-\text{SO}_2\text{NH}_2$, $-\text{SO}_2\text{NHR}$, and $-\text{SO}_2\text{NRR}'$ all gave a positive reaction although not all of the pigments formed were of equal intensity of colour, and they varied in their degree of preference for the aqueous or *n*-butanol phase. The group of sulpha drugs and 2:4-dichloroaniline gave the best results. *N*¹-Diethylsulphanilamide was made the subject of further study owing to the stability of its diazonium salt, the readiness with which the pigment transferred to the *n*-butanol phase and the light colour of the control. This amine was also more easily diazotized than 2:4-dichloroaniline and had the further advantage of freedom from halogen. There is, of course, the possibility that other amines may prove even more suitable, particularly since, in our subsequent studies with *N*¹-diethylsulphanilamide, the conditions for diazotization and colour development eventually adopted differed materially from those used in the above comparative tests.

*Observations on the conditions
of Komant's test*

Komant described two sets of conditions leading to the formation of coloured products between thyroxine and diazotized sulphanilic acid: (a) in which equal volumes (1 ml.) of solutions of thyroxine (0.1 %), the diazonium chloride and Na_2CO_3 (10 %) were mixed to give a red colour (the colour tone changing gradually to brown although in more dilute solutions of thyroxine the colour was found to fade in a few minutes), and (b) in which 5 vol. of the diazonium chloride solution were added. Under the latter conditions Komant observed the production of a violet colour which rapidly faded on passing a current of oxygen through the solution. Qualitative observations have shown that these subsequent changes of colour are largely dependent upon pH. Thus, if under conditions (a) (pH 9–10) 2N-NaOH (1 ml.) is added immediately after colour development, the initial colour quality is retained for several days, even with dilute (0.005 %) solutions of thyroxine, while that of the control (no thyroxine) is straw rather than dark yellow. Similarly, under conditions (b) (pH about 8.5) the initial red-purple colour does not change to violet and then to brown, but persists for several days if alkali (1 ml. 10N-NaOH) is immediately added. Moreover, if NaOH is not added, the subsequent decompositions appear to be independent of reactions with oxygen, since in controlled tests the bubbling of oxygen, air or nitrogen effected the changes (without, however, the development of an intermediate violet coloration) at similar rates; even mechanical agitation of the solution in a closed tube caused the initial red-purple colour to pass rapidly to reddish orange, whereas that of the contents of a similar stationary tube changed gradually *through violet* to brown. The colour changes are probably mainly due to side reactions of diazotized sulphanilic acid, these being inhibited at high pH.

Investigations with N¹-diethylsulphanilamide. Effects of pH and other factors on the production, stability and specificity of the colour

Reaction at pH 11–12. Under the conditions used for the comparative test (pH 9–10, Table 1) diazotized N¹-diethylsulphanilamide also produced a colour with diiodotyrosine (orange red) and tyrosine (deep yellow) while the control was yellow. Although diiodotyrosine can be separated from thyroxine by partitioning between aqueous NaOH and *n*-butanol, tyrosine is more evenly distributed between the two phases. A differentiation was made possible by carrying out the reaction at a higher pH as described below. It may be noted that colour development also depends upon the order of mixing of the reactant solutions, thyroxine, diazonium salt and alkali and *n*-butanol and that the addition of a further amount of alkali both intensifies and stabilizes the colour initially produced (as mentioned above for the colour formed with diazotized sulphanilic acid).

Thyroxine solution (0.004 %; 1 ml.) was mixed with *n*-butanol (1 ml.) and then 1 ml. of aqueous NaOH of selected concentration was run in. The mixture was shaken and the solution of the diazonium chloride (1 ml.) was then added and shaking continued for 30 sec. 2N-NaOH (1 ml.) was then immediately added. The results of these tests and of those carried out at the same time with diiodothyronine, diiodotyrosine, tyrosine and control solutions are given in Table 2. It can be seen that the range 0.2–0.3N-NaOH provides optimum conditions (pH 11–12) for the production of an immediate colour of good intensity with a pale control and negative results with tyrosine and diiodotyrosine. The alkali initially added, if below 0.15N, produced only yellow or brown colours even after 24 hr., whereas if above 0.5N, a colour developed only on standing. In order to check the optimum pH for the development of an immediate colour, a series of tests was carried out with buffer solutions over the pH range 10–12.8. The buffer was formed from glycine, NaOH and NaCl (Walbaum, 1920). The solution of thyroxine (0.1 %; 1 ml.) in buffer at the test pH was shaken with butanol (1 ml.), and 1 ml. of the diazo solution (previously brought to pH 7) was added. The results confirmed the pH range 11–12 as optimal; the conditions of the buffer medium were not, however, suitable for general adoption, as the control was yellow (due to glycine) with a corresponding yellow tint in the test colour.

Table 2. Colours developed between diazotized N¹-diethylsulphanilamide and thyroxine and related compounds at varying pH (strength of alkali added as indicated). Reaction in water—*n*-butanol. 30 sec. after mixing the reagents additional alkali (2N-NaOH; 1 ml.) was added to the tubes and the colours of the butanol phases noted; after standing for 24 hr. the colours were again observed

NaOH (1 ml.) ...	0.05 N		0.1 N		0.2 N		0.3 N		0.5 N		N	
	30 sec.	24 hr.	30 sec.	24 hr.	30 sec.	24 hr.	30 sec.	24 hr.	30 sec.	24 hr.	30 sec.	24 hr.
Thyroxine (0.004 %; 1 ml.)	Yellow	Orange	Brown	Brown	Light purple	Light purple	Deep pink	Light purple	Pale pink	Deep pink	No colour	Pink
3.5-Diiodothyronine (0.004 %; 1 ml.)	Yellow	Brown	Brownish pink	Orange brown	Pink	Pink	Pale pink	Light pink	Straw	Pale pink	No colour	Straw
3.5-Diiodotyrosine (0.004 %; 1 ml.)	Yellow	Yellow	Deep yellow	Yellow	Straw	Light yellow	No colour	Straw	No colour	Straw	No colour	Straw
Tyrosine (0.004 %; 1 ml.)	Light yellow	Yellow	Deep yellow	Deep yellow	No colour	Straw	No colour	Straw	No colour	Straw	No colour	Straw
Control (no amino-acid)	Yellow	Yellow	Yellow	Yellow	No colour	Straw	No colour	Straw	No colour	Straw	No colour	Straw

Reaction in butanol phase with excess alkali. Although the depth of colour even in dilute solutions was quite satisfactory when the reaction was carried out at pH 11-12 followed by the addition of 2N-NaOH, duplicate values did not show sufficiently close agreement when the reaction was examined quantitatively in the Spekker Absorptiometer (Adam Hilger) under these conditions. After various additional trial experiments it was found that replication of tests could be achieved by allowing the reaction to proceed slowly in the butanol phase, excess alkali being added initially. Under the conditions described below in *Procedure*, it was found by titration that about 5% of the excess alkali in the reaction mixture was in the butanol phase. Since thyroxine is completely transferred to the butanol layer in aqueous alkali-butanol mixtures, this procedure also permitted a maximum reaction to take place while enhancing the specificity. The solution of the diazo compound as well as that of the dye possesses marked stability at high pH. After the addition of the alkaline diazo solution, the reaction can be interrupted at any stage by simply stoppering the tubes, and then started again by allowing access to the atmosphere. Neither light nor temperature has a deteriorating effect upon the reaction provided the pH is maintained at a high level. Thus, after the diazonium hydroxide was added in one test, the tubes were placed in a boiling water bath for 0.5 hr., but neither the control nor thyroxine tubes showed any apparent change. However, on standing overnight at room temperature, the normal reaction took place in the thyroxine tube. On the other hand, elevating the temperature during the reaction by placing the tubes in a thermostat at 25° reduced the time for full colour development. Continual gentle agitation, while not essential, was found to assist the reaction by

equalizing the fall in pH in the butanol layer. Violent shaking, on the other hand, facilitated diffusion of alkali from the aqueous phase, so that the pH did not fall to a level for colour development.

The mechanism of the reaction probably depends on the slow absorption of CO₂ from the atmosphere by the NaOH contained in the butanol phase, as no colour develops when the tubes are stoppered, or when CO₂-free air is passed over the butanol surface. Various attempts were made to speed up the reaction by the addition of minimal amounts of dilute acid, buffer solutions or salts, or by passing CO₂ over the butanol surface; however, neither duplicates nor colour quality were as satisfactory as by the slow process. The reaction was also tried unsuccessfully in *n*-butanol with all solutions prepared in this solvent, the diazotization being carried out with *n*-butyl nitrite.

Specificity. Table 3 gives a list of phenols, diiodo-phenols and amino-acids tested under the procedure ultimately adopted and described below. Thyroxine and thyroxamine produce about the same depth of colour, while that from diiodothyronine is very much weaker. Thyronine behaves like diiodotyrosine and tyrosine. The peptide, glutamylthyroxine, also gives a negative reaction under these conditions; on the other hand, thyroxyl diiodotyrosine, owing to its preference for the butanol phase, gives a stronger reaction, at equivalent dilution, than thyroxine.

Table 3. *Reactions between diazotized N¹-diethylsulphanilamide and various hydroxy compounds and amino-acids, compared with thyroxine*

Compound	Conc. (%)	Colour produced	
		Immediately	After 24 hr.
Thyroxine	0.005	No colour	Light purple
Thyroxamine	0.005	No colour	Light purple
3:5-Diiodothyronine	0.005	No colour	Pale pink
Thyronine	0.005	No colour	Straw
3:5-Diiodotyrosine	0.02	No colour	Straw
Tyrosine	0.02	No colour	Straw
Control	—	No colour	Straw
Glutamylthyroxine	0.005	No colour	Very light yellow
Thyroxyl diiodotyrosine	0.005	No colour	Purple
3:5-Diiodotyrosine methyl ester	0.005	No colour	Straw
3:5-Diiodo-4-hydroxybenzoic acid	0.005	Straw	Yellow
3:5-Diiodo-4-hydroxybenzaldehyde	0.005	No colour	Straw
*3:5-Diiodo-4-hydroxyphenyllactic acid	0.02	No colour	Yellow
3:5-Diiodo- <i>p</i> -cresol	0.005	Pink	Deep red
<i>p</i> -Cresol	0.02	No colour	Deep pink
<i>p</i> -Hydroxyphenyllactic acid	0.02	No colour	Yellow
<i>p</i> -Hydroxyphenylpyruvic acid	0.02	No colour	Yellow
<i>p</i> -Hydroxybenzoic acid	0.02	No colour	Yellow
Salicylic acid	0.02	No colour	Yellow
D-Glutamic acid	0.02	No colour	Light yellow
DL-β-Phenylalanine	0.02	No colour	Light yellow
L-Histidine	0.02	Yellow	Deep yellow
L-Hydroxyproline	0.02	No colour	Light yellow
L-Tryptophan	0.02	No colour	Yellow
Glycine	0.02	No colour	Light yellow

* Saul & Trikojus (1948) reported the value of diazotized N¹-diethylsulphanilamide in enabling them to follow the course of the conversion of 3:5-diiodo-4-hydroxyphenyllactic acid into the corresponding analogue of thyroxine.

Both components of this dipeptide presumably engage in reaction with the diazo compound. Of the monocyclic types tested, only *p*-cresol and its diiodo derivative gave a positive result.

It should be pointed out that some of the substances tested have not been shown to occur naturally; others could be removed by procedures which would not affect thyroxine. Thus *p*-cresol and its diiodo derivative are readily soluble in ether, and mixtures of these phenols and thyroxine have been shown after ether treatment to match the colour given by the same amount of thyroxine treated similarly. Complete hydrolysis should eliminate irregularities due to contamination by a peptide such as thyroxyl diiodotyrosine. This would also be necessary in a method depending upon the estimation of the iodine content of a butanolic extract.

METHOD

Reagents

Preparation of N¹-diethylsulphanilamide. 4-Acetamidobenzenesulphonyl chloride (40 g.) was dissolved in acetone (200 ml.) and added to a cold aqueous solution of diethylamine, prepared from diethylamine hydrochloride (44 g.) and 2*N*-NaOH (200 ml.). The mixture was chilled, powdered NaHCO₃ (16 g.) added and the reaction allowed to proceed at room temperature for some hours with shaking at frequent intervals. Acetone was removed on the water bath under reduced pressure, the residue was chilled and water (200 ml.) added. The precipitate of *N*¹-diethyl-4-acetamidobenzenesulphonamide which separated was hydrolyzed by boiling for a few minutes with 6*N*-HCl (40 ml.). Following the addition of water, heating was continued for a further few minutes. The hot filtered solution was cooled and made alkaline with 2.5*N*-NaOH. The resulting precipitate was washed with water (crude yield, 32 g.) and recrystallized, first, from 60% (v/v) ethanol with the aid of charcoal, and then from a mixture of ethanol and light petroleum (b.p. 60–80°). It separated as colourless prisms, m.p. 105–106°. (Mangini (1940) records 105–106°.) Found: N, 12.5%. (Calc. for C₁₀H₁₆O₂N₂S: N, 12.3%.)

Diazotization. Preliminary tests showed that the large excess of NaNO₂ used by Komant was not necessary for the production of a stable solution of the diazonium salt of this amine. Accordingly, the diazotization was carried out as follows: the amine (1.14 g., 0.005 mol.) was dissolved in 10*N*-HCl (9 ml.) and the solution diluted to 100 ml. with distilled water. 5 ml. of this solution was diazotized at 0° with 5 ml. of 4.5% (w/v) NaNO₂ (A.R.); ice-cold water (20 ml.) was then added and the resulting solution used after 10 min. Reduction of the quantity of nitrite did not affect the rate of colour development with thyroxine, while the control was somewhat paler.

n-Butanol. This was purified by distillation over solid NaOH; the fraction boiling at 116° was used in this work.

Procedure

The procedure ultimately used for quantitative tests was as follows: thyroxine (2 ml. of a solution containing 1–5 mg./100 ml. of 0.1*N*-NaOH) was

placed in a glass-stoppered round-bottomed Pyrex centrifuge tube of approx. 15 ml. capacity, followed by *n*-butanol (2 ml.) and then 2*N*-NaOH (1 ml.). The contents were then thoroughly mixed by shaking the tube sideways (liquid should not pass to the neck of the tube). An alkaline solution of the diazo derivative of *N*¹-diethylsulphanilamide (3 ml., prepared by adding 1 ml. of 2*N*-NaOH to 2 ml. of the solution of the diazonium chloride was then added, the tube stoppered and the contents well shaken for 15 sec. After standing for 5 min. the stopper was removed and the tube gently agitated in a rack for 24 hr. in a water thermostat maintained at 25°. The rack, the base of which was suspended at a distance of 17 cm., was swung by a geared electric motor about 60 times/min. through approx. 3 cm. (the dimensions given are not critical). The control tube contained 2 ml. of 0.1*N*-NaOH plus reagents. After the completion of the reaction, the tubes were stoppered and centrifuged for 30 min. at 2500 r.p.m. The butanol phase was then transferred as far as possible with a Pasteur pipette to a small dry tube. To 0.75 ml. of this solution 0.25 ml. *n*-ethanolic NaOH was then added and the tubes containing the mixed solutions stoppered pending examination in the absorptiometer using Ilford spectrum green filters. Tests showed that almost identical readings could be obtained after a further 24 hr., providing precautions were taken to prevent evaporation of solvent and access of CO₂. (Note. If ethanolic NaOH is not added, the readings are irregular due to the development of a turbidity. Other measures to counteract this, such as more prolonged centrifuging, temperature stabilization or desiccation with anhydrous Na₂SO₄, were ineffective.)

Absorption characteristics. Fig. 1 shows the result of an investigation of the spectral transmittance of the solution of the dye in *n*-butanol (plus ethanolic NaOH). Comparison was with a control solution prepared under identical conditions, thyroxine only being excluded. The instrument used, the Coleman Spectrophotometer, was kindly made available by Mr A. T. Dick (Animal Health Laboratory, Council for Scientific and Industrial Research, Melbourne). Maximum absorption occurred at approx. 540 mμ. at which point the blank control transmitted 93.6% compared with pure *n*-butanol.

Standard curve. A typical calibration curve of the colour intensity produced by varying concentrations of pure thyroxine is illustrated in Fig. 2. Within the range 1–4 mg./100 ml. consistent results have been obtained provided fresh solutions of thyroxine were used for the standardization. In applying the method to the estimation of thyroxine in biological material, such as thyroid powder, some difficulty was experienced in obtaining suitable butanol extracts of the thyroxine fraction prior to assay. This has now been overcome by preliminary treatment

with ether before extracting the acid-insoluble thyroxine precipitate with butanol or by forcing the thyroxine into an aqueous alkaline phase from a

Fig. 1. Spectral transmittance of the colour produced by the reaction of thyroxine (0.01%) and diazotized N^1 -diethylsulphanilamide. Coleman spectrophotometer.

butanol extract by the addition of ether or, preferably, light petroleum. Re-extraction of the aqueous phase with butanol has provided a colourless solution suitable for colorimetric analysis. A report of the results obtained in these studies will form the basis of a further communication.

Note added 10 October 1947.

Since this paper was submitted for publication we have had access to a preliminary report by Moser (1947), in which he has shown that by the addition of alkali to the reaction product, Komant's reagent may be used for the rapid

estimation of thyroxine in pure aqueous solutions. Our observations indicate that N^1 -diethylsulphanilamide is a more stable reagent for this purpose and that the procedure outlined by us greatly enhances the specificity of the reaction.

Fig. 2. Calibration curve for the reaction: thyroxine + diazotized N^1 -diethylsulphanilamide. Hilger Spekker Absorptiometer with Ilford spectrum green filters.

SUMMARY

A colour test for thyroxine is described based upon its reaction with diazotized N^1 -diethylsulphanilamide in alkaline solution. The conditions of the reaction have been studied and a procedure has been suggested whereby thyroxine can be colorimetrically estimated.

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