THE DETERMINATION OF PROPYLTHIOURACIL IN URINE WITH 2:6-DICHLOROQUINONE- CHLOROIMIDE

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(RECEIVED FOR PUBLICATION APRIL 16, 1951)

Previous colorimetric methods for the determination of antithyroid compounds of the thiouracil type have been based upon the bluish-green colour which is given when compounds containing the C=S group react with Grote's (1931) reagent. Methods for the determination of thiouracil levels in urine using this reaction have been described by Anderson (1944), Williams, Jandorf, and Kay (1944), Chesley (1944), Christensen (1945), Paschkis, Cantarow, Rakoff, and Tillson (1945), and others. With the exception of Williams *et al.* (1944), all of these workers have reported on the instability of the stock Grote reagent, which necessitates not only the frequent preparation of fresh reagent, but also the inclusion of standards with each set of unknowns. The method of Williams and his colleagues, however, has the disadvantage of a 12-hour digestion procedure and the inconvenient adjustment of the reaction with a strong base.

Recently Olson, Ely, and Reineke (1947) have claimed to have produced a stable Grote reagent by the application of a suitable ageing process.

Since the method described here for the colorimetric determination of propylthiouracil involves a new colour reaction, it is not necessary to review further previous work.

A new colour reaction for antithyroid drugs of the thiouracil type has already been described (McAllister, 1950). This is based upon the reaction of the compound in a buffer at pH 8.0 with 2:6-dichloroquinone-chloroimide to give a yellow chloroform-soluble complex. This colour reaction has also been shown to be specific for the newer antithyroid compounds of the mercaptoimidazole type (McAllister, 1951a), and has been applied on a quantitative basis to the determination of methylthiouracil in urine (McAllister, 1951b). In applying this colour reaction to the determination of propylthiouracil in urine, certain improvements have been introduced, the most important of these being a preliminary ether extraction of the compound from the urine at pH 6.0.

Experimental

Gibbs (1927a, b) has shown that 2:6-dichloroquinone-chloroimide reacts with phenols, unsubstituted in the para-position, with the formation of blue or violet

indophenol pigments. The reagent has also been used for the detection of thiamine by Raybin (1938) and by Scudi (1941) for the estimation of pyridoxin. Fearon (1944) has described a method for the determination of uric acid in urine which is based upon the yellow, the intensity of which is given when this compound reacts with the chloroimide reagent at pH 9.2. In this paper Fearon also noted the behaviour of various amino compounds and urinary solutes in the reaction at this pH .

The colour reaction for other compounds has been found to be dependent upon the pH of the reaction mixture; it is more dependent indeed than the reaction for the thiouracils, and by using a pH of 8.0 the reaction with most amino-acids is considerably slowed down. Furthermore, uric acid, the most reactive urinary constituent in the test, gives a slower reaction with the reagent at this pH .

Scope of the Chloroimide Reaction.—Solutions of the various compounds listed in Table I were dissolved in distilled water, and where necessary the pH of their solutions raised to 8.0. Five ml. of buffer-chloride solution, pH 8.0, was then added, followed by 0.1 ml. of a 0.4% solution of 2 : 6-dichloroquinone-chloroimide in aldehyde-free absolute ethanol. Colour reactions were then allowed to proceed for one hour at room temperature.

Five ml. of chloroform was then added to each and the mixtures vigorously shaken. Results are given in Table I.

Results

With the exception of thiourea, none of the compounds tested gave colorations which were removable from the aqueous phase by means of chloroform.

Uric acid was the most reactive of the group tested. At pH 8.0 the colour given by this compound tends to be orange, whereas at higher pH values it is pure yellow.

The results obtained with the amino-acids in the test were in agreement with those reported by Fearon (1944), although his reactions were carried out at pH 9.2. Amino compounds in general react very slowly with the chloroimide reagent and give colorations which are weak in comparison to the amount (2 mg.) under test.

Colour reactions with the chloroimide reagent are very dependent upon the pH of the reaction mixture. At pH values below 8.0, practically no colorations are given by amino-acids, and the reaction with other compounds is slowed down considerably. At pH values of 10 and above, the reagent tends to undergo a spontaneous decomposition, with the formation of a reddish mixture of pigments. Gibbs (1927a, b) found that this reagent decomposition was accelerated by exposure to light, and Fearon found that it could be repressed by the inclusion of sodium chloride in the buffer mixture. In this work, due mainly to the lower pH value used, reagent decomposition has not been encountered.

Fearon (1944) has shown that the colour reaction for thiourea is catalysed by copper. Although the colour reaction for this compound is rendered specific by removing the complex from the aqueous phase by means of chloroform, even with catalysis of the reaction with the metal it is not sensitive enough to be used on a quantitative basis. Fearon has also shown that the colour given by uric acid in the reaction is turned red by the addition of silver salts. In this work no such catalysis of the colour reaction for the thiouracils by metals has been observed, nor any silver binding NH group effect.

TABLE I
COLOUR REACTIONS AT pH 8.0 OF VARIOUS COMPOUNDS WITH 2:6-DICHLOROQUINONE-
CHLOROIMIDE

Compound (2 mg. of each or amount noted)	Colour Reaction	Solubility of Coloured Complex in Chloroform
<i>Pyrimidines</i>		
Uracil	No reaction	—
Thymine	„ „	—
<i>Purines</i>		
Uric acid	Rapid orange	Insoluble
Guanine HCl	No reaction	—
Adenine HCl	„ „	—
Xanthine	Rapid yellow	Insoluble
<i>Amino Compounds</i>		
Ammonia	No reaction	—
Methylamine	Slow violet	Insoluble
Glycine	„ „	—
Alanine	Faint pink	„
Serine	„ „	„
Valine	No reaction	—
Cystine	„ „	—
Tyrosine	„ „	—
Tryptophane	„ „	—
Histidine	Slow; faint violet	Insoluble
Arginine	No reaction	—
Histamine	„ „	—
<i>Water-soluble Vitamins</i>		
Ascorbic acid	No reaction	—
Aneurin	Yellow, turns brown	Insoluble
Pantothenic acid	No reaction	—
Pyridoxin	Blue, decomposes	Insoluble
Riboflavin	No reaction	—
<i>p</i> -Amino-benzoic acid	„ „	—
Biotin (100 µg.)	„ „	—
<i>Urinary Solutes</i>		
Urea	No reaction	—
Creatine	„ „	—
Creatinine	Faint pink	Insoluble
Allantoin	No reaction	—
Hippuric acid	„ „	—
<i>Miscellaneous</i>		
Alloxan	No reaction	—
Dialuric acid	Rapid yellow	Insoluble
Barbituric acid	Rapid violet	„
Barbitones	No reaction	—
Sulphonamides	„ „	—
Glucose	„ „	—
Lactose	„ „	—
Fructose	„ „	—
Thiourea	Slow violet	Soluble
Methylamine	„ „	Insoluble

The Colour Reaction for Propylthiouracil

The colour reaction for 2-thiouracil and its 4-methyl derivative has already been described (McAllister, 1950, 1951b). A similar reaction is given by 6-n-propylthiouracil, but the yellow product is more soluble in chloroform.

Reagents.—The following reagents were used in the test :

Solution of 0.4% 2:6-Dichloroquinone-chloroimide.—A 0.4% solution of the compound in aldehyde-free absolute ethanol is stored in a brown bottle ; the reagent will keep for about six weeks.

Buffer-Chloride Solution pH 8.0.—To 50 ml. of a 0.2 M. solution of boric acid in 0.2 M. potassium chloride was added 3.97 ml. of 0.2N. sodium hydroxide solution. Then 100 ml. of a 20% aqueous solution of pure sodium chloride was added, and the volume of the mixture made up to 200 ml. with distilled water.

Since the sodium chloride depresses the pH of the mixture the pH should be checked and then adjusted to 8.0 by the addition of decinormal soda.

Standard Propylthiouracil Solution.—This was prepared by dissolving 25 mg. of 6-n-propylthiouracil in 5 ml. of aldehyde-free absolute ethanol and then making the volume to 250 ml. with distilled water. The solution will keep for about one week. It contains 100 μ g. per ml.

Procedures.—The following procedures were used in the development of the colour reaction :

Chloroform/Water Partition of the Coloured Complex.—To 1 ml. (100 μ g.) of the standard propylthiouracil solution was added 4 ml. of water and 5 ml. of the borate buffer at pH 8.0. Then 0.1 ml. of the 0.4% chloroimide reagent was added, and the colour allowed to develop for 45 minutes. Then 10 ml. of chloroform was added to the mixture and the tube well shaken. The distribution of the coloured complex in the solvent and aqueous layers was examined in this and in another series containing 200 μ g. of propylthiouracil treated similarly.

For purposes of comparison, the results obtained with thiouracil and methylthiouracil treated likewise are also included (Table II).

TABLE II
CHLOROFORM/WATER PARTITION OF COLOURED COMPLEXES AT pH 8.0

Compound (100 μ g. each)	Colour Reaction	Approximate Percentage of Colour Distributed after Shaking*	
		Aqueous (10 ml.)	Chloroform (10 ml.)
2-Thiouracil	Yellow	50	50
Methylthiouracil	„	—	100
Propylthiouracil	„	—	100

* A similarly treated series containing 200 μ g. of each of the above compounds gave: thiouracil, approximately 25% removed by 10 ml. solvent; methylthiouracil, approximately 50% removed by 10 ml. solvent; propylthiouracil, 100% removed by 10 ml. solvent.

Results

As will be seen from the results given in Table II considerable differences in the solubility of the reaction products are exhibited. Absorptiometer measurements of the colours given by equal weights of thiouracil, methylthiouracil, and propyl-

thiouracil were found to be approximately the same. This would appear to show that the type of substituent on the thiouracil ring does not influence the colour reaction, but does have a marked effect on the solubility of the coloured reaction products in chloroform.

In previous work it was found that the colour reaction for the thiouracils could be accelerated by shaking during the development period, but once the latter had been completed this effect was vitiated. A similar aeration effect has been noted by Fearon (1944) in the chloroimide reaction for glycine, thiourea, and indole.

Effect of pH on the Solubility of the Coloured Complexes.—The pH of the reaction mixture has a marked effect on the solubility of the coloured products in the solvent. As the pH rises, the solubility of the coloured products in chloroform decreases, until at pH 10 none of the colour given by 100- μ g. amounts of thiouracil and methylthiouracil is removable from the aqueous phase by means of the solvent. Propylthiouracil, on the other hand, gives, in amounts up to 100 μ g., colorations which can be completely removed from the reaction mixture at pH 10 by 10 ml. chloroform.

Quantitative Aspects of the Colour Reaction.—This has already been reported for thiouracil and its 4-methyl derivative (McAllister, 1951b). For propylthiouracil the sensitivity of the reaction is about 20 μ g. In the aqueous phase, the colour has been found to be stable for at least 60 minutes, and in chloroform for at least 30 minutes, as determined by absorptiometer measurements.

The colour system obeys Beer's law with concentrations of propylthiouracil up to 150 μ g., and the graphs obtained by plotting concentrations against absorptiometer readings are reproducible. Amounts of propylthiouracil in excess of 150 μ g. give colours which can be diluted with further amounts of chloroform.

Determination of Propylthiouracil in Urine

When the colour reaction is applied direct to dilute samples of urine satisfactory recovery values can be obtained in the case of methylthiouracil (McAllister, 1951b). Further experience of this method, when applied to the determination of propylthiouracil in urine, has shown that when the urinary level of the drug is low there is a tendency for the reagent to be taken up by other urinary solutes before the drug reaction can be completed. This may be overcome to a certain extent by the use of a more concentrated reagent—for example, 0.8%—but under such conditions the colours given by the higher amounts of propylthiouracil (200 μ g.) are partially destroyed by aeration during the extraction process. During attempts to overcome this difficulty it was found that propylthiouracil could be quantitatively removed from urine at pH 6.0 by shaking with ether.

Ether Extraction of Propylthiouracil.—The reagents which were employed for the colour reaction have already been described. Anaesthetic ether was employed for the extraction.

Procedure.—Complete 24-hour samples of the urine were collected in bottles containing 10 ml. of chloroform. Analysis had to be carried out without delay.

Urine, 100 ml., was measured out and the pH adjusted to 6.0 by the usual methods. Of this, 50 ml., or an aliquot containing up to 5 mg. of propylthiouracil, should be taken, and transferred to a small separating funnel. The urine was then extracted three times

DETERMINATION OF PROPYLTHIOURACIL IN URINE 437

with 100-ml. amounts of peroxide-free ether. The ether extracts were then pooled and washed once with 100 ml. of distilled water. The combined ether extracts were then taken to dryness on a steam bath. To the dry residue was then added 2 ml. of aldehyde-free absolute ethanol, and the mixture shaken until solution was effected. The solution was then washed into a 100-ml. volumetric flask and made to the mark with distilled water.

Development of Colour.—Aliquots, of 2 ml. and 1 ml., were taken and the volume of each adjusted to 5 ml. with water. Then 5 ml. of the borate-buffer, pH 8.0, was added to each, followed by 0.1 ml. of the 0.4% chloroimide reagent. After mixing, the tubes were allowed to stand for 45 minutes, when 10 ml. of chloroform was added to each, and the mixtures well shaken until all of the yellow had been extracted by the solvent. The chloroform extracts were allowed to settle, and the aqueous supernatant in each removed by suction. They were then filtered through a small No. 42 Whatman filter paper, and read in a Spekker absorptiometer, readings being taken against chloroform and using the Spekker violet filter.

Standard Reference Graph.—The colour reaction which has been described was applied to 10, 20, 30, 40, 50, and 100 μ g. of propylthiouracil each in a volume of 5 ml. water, and the colours given by each amount extracted with 10 ml. chloroform. No colour was given by the reagent blank.

Results

Recovery at Various pH Values.—Various amounts of propylthiouracil dissolved in aldehyde-free absolute ethanol were added to normal samples of urine. Aliquots, 100 ml., of each of these were taken, and the pH of each adjusted to 6.0, 7.0, and 8.0 by the usual methods. Then 50-ml. aliquots of each were removed and extracted with three 100-ml. amounts of ether. The propylthiouracil content of each was determined by the method which has been described. Results are given in Table III.

TABLE III
RECOVERY OF PROPYLTHIOURACIL FROM URINE AT VARIOUS pH VALUES

Urine pH	Propylthiouracil Added (mg.%)	Propylthiouracil Found (mg.%)	Recovery (%)
1. 6.0	10.0	8.49	84.9
2. 7.0	10.0	6.43	64.3
3. 8.0	10.0	5.30	53.0

The best recovery values were obtained with urine at pH 6.0. Thiouracil types of compounds are readily broken down at pH 8–9, and this may account for the low values obtained at pH 8.0, although some other factor may be involved.

Material at Ether/Water Interface.—The ether extraction of urine often results in the separation of an indeterminate layer at the ether/water interface. Since this represents a potential loss of material it has been examined. At pH 6.0, the ether extracts usually separate well. In a typical experiment with urine at pH 7.0 it was found that the indeterminate layer contained 10 μ g. propylthiouracil together with some uric acid.

In such instances, where an indeterminate layer forms at the interface, the best method is to combine it with the ether extracts, and then remove it with the water used to wash them.

Treatment of Residue.—The residue left after removal of the ether should be as dry as possible in order to effect its solution in absolute ethanol. The concentration of the latter should not exceed 2 ml. per 100 ml. of water used to make the solution to volume; 2 ml. of absolute ethanol will dissolve up to 5 mg. of propylthiouracil. Amounts of the latter in excess of this may be dissolved in larger amounts of ethanol, and a proportionate increase made in the water.

Recovery of Propylthiouracil.—With various amounts of propylthiouracil added to urine, the recovery values obtained with the method averaged 85%.

Interfering Substances.—A large number of normal and pathological urines containing no propylthiouracil have been examined by the method and no interfering compounds have been detected. Gibbs (1927a, b) has shown that the chloroimide reagent is a general test for phenols unsubstituted in the para-position, with which it forms blue or violet indophenol pigments. Whether phenols of this type give coloured products, which are removable from the reaction mixture at pH 8.0 by means of chloroform, has not as yet been determined, but, as Fearon (1944) has pointed out, p-cresol, the commonest urinary phenol, does not give a colour with the reagent, and other urinary phenols are almost entirely present in fresh urine in an esterified non-reacting form.

The commoner pathological constituents of urine such as proteins, acetone, acetoacetic acid, do not interfere in the reaction.

Excretion of Propylthiouracil.—In patients receiving 100 mg. of propylthiouracil a day, urinary levels of the drug obtained by the method averaged 52 mg. a day.

Summary

A specific colour reaction for antithyroid compounds of the thiouracil type has been described, and a method for the determination of propylthiouracil in urine given.

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