THE DETERMINATION OF 4-METHYL-2-THIOURACIL IN ANIMAL TISSUE AND BLOOD

by

H. VAN GENDEREN, K. L. VAN LIER, AND J. DE BEUS
State Institute of Public Health, Utrecht (Netherlands)

INTRODUCTION

The methods for the determination of thiouracil in animal tissues by WILLIAMS *et al.*[1, 2] did not prove successful for the determination of low concentrations (± 1 mg per cent) of the 4-methylderivative. This is not due to lack of sensitivity of the colour reaction with GROTE's reagent, which we used in the modification given by MØRCH[3], but to the method of precipitation.

The recovery of added methylthiouracil was bad and difficulties were encountered by the formation of red coloured reaction products of GROTE's reagent with impurities of the extract.

With other methods of precipitation reliable results were obtained, at least for serum, with the procedure given by CHESLEY[4] (see below). For whole blood and tissue-pulp this method was not suitable. We therefore tried to extract the methylthiouracil with a mixture of organic solvents, along the lines indicated by MØRCH, who used ether-percolation for urine. The solution so obtained was purified by partition between water and another organic solvent.

Due to the acidic nature of methylthiouracil the solubility in water depends upon the p_H.

In Table I data are given for the distribution of methylthiouracil between an aqueous buffer of p_H 2.8 and three organic solvents.

TABLE I

DISTRIBUTION COEFFICIENTS OF 4-METHYL-2-THIOURACIL IN A WATER (MC ILVAINE-buffer p_H = 2.8)-organic solvent system at 18° C

buffer/chloroform*	46
buffer/methylene chloride	37
buffer/ethyl ether**	10

* purified, ethanol free ** peroxide free.

Before measuring, the organic solvents were saturated with the buffer and conversely the buffer with the organic solvents. The methylthiouracil content of both buffer and organic solvent, was determined after shaking. The sum of these contents varied between 99 % and 101 % of the quantity of methylthiouracil added to the system.

References p. 486.

If, however, a buffer of $p_H = 8$ is used, practically all the methylthiouracil remains in the aqueous phase. By addition of ethanol to the system buffer $p_H = 2.8$ — organic solvent, the solubility in the organic solvent is increased considerably. When equal volumes of buffer p_H 2.8 and chloroform are used and 10 volume per cent of ethanol is added to this system the distribution coefficient is approximately 4.

Irregular results were obtained with (peroxide free) ether extraction and purification. With chloroform-ethanol (20%) extraction of the acidified and dried blood or tissue pulp, and purification of this extract by partition between an acid aqueous phase and chloroform (without ethanol) it was possible to obtain constant high recoveries of added methylthiouracil.

With an alkaline water layer the distribution coefficient is much higher than with an acid one, but then such an amount of impurities will enter the aqueous phase together with the methylthiouracil, that a correct determination is impossible.

Purification of the extract is therefore carried out with an acid waterlayer (p_H below 4); the distribution coefficient (see Table I) is high enough to ensure complete extraction.

Later on in our work it was found that chloroform, which is liable to deterioration, could be replaced, with equal success, by the stable methylene chloride*.

The reaction with GROTE's reagent is a rather delicate process. Impurities of the extract may cause the formation of red or brown coloured products or may decrease the velocity of formation of the green coloured reaction product with methylthiouracil. In the presence of ethanol the development of the colour is much slower but at the same time the colour is remarkably stable. We have taken advantage of this in our determinations.

EXPERIMENTAL

1. *Preparation of Grote's reagent*

GROTE's reagent is prepared according to the prescription of MØRCH[3]: Dissolve 1 g of sodium nitroprusside and 1 g of hydroxylamine hydrochloride in 20 ml of water and add 2 g of sodium bicarbonate. Shake till the fizzing stops, add 0.1 ml of bromine and shake again. Filter the solution into a volumetric flask and dilute with water to 50 ml. If the reagent is kept in an ice-box it can be used for about 10 days. During this time the light extinction of the colour developed with a standard solution of methylthiouracil will decrease slightly. Differences between one batch of reagent and another one are difficult to avoid. For these reasons it is necessary to run an experiment with a standard solution of methylthiouracil once a day.

2. *Colorimetric reaction*

The extract of serum, blood or tissue was adjusted to a $p_H = 7.9$–8.1, with the help of a boric acid-borax buffer of $p_H = 8.0$ and a p_H-meter with glass electrode. 10% of 96% ethanol was added to the extracts, before addition of the reagent, to stabilize the green reaction product. Per 10 ml of extract 0.4 ml of GROTE's reagent was added. The light extinction was measured, after standing at room temperature, for 30 minutes in a spectrophotometer at 6650 Å in a 3 cm cell. The extinction-concentration diagram is in accordance with the law of BEER-LAMBERT over a wide range.

[1] We are indebted to Dr H. J. PRINS for suggesting the use of methylene chloride instead of chloroform.

References p. 486.

3. CHESLEY's *precipitation method for serum*[4]

To 5 ml of blood serum in a centrifuge tube 5 ml of a freshly prepared mixture of equal parts of 10% sodium tungstate and 2/3 N sulphuric acid is added. The tube is shaken vigorously and centrifuged. 5 ml of the clear supernatant fluid is taken, 2 ml of borate buffer $p_H = 8.0$ and 1 ml of ethanol are added and the sample is adjusted to $p_H = 8.0$ and brought to a volume of 10 ml.

Of 1 mg per cent methylthiouracil added to the serum 82–88% was recovered.

4. *Extraction procedure for blood and tissue*

10 g of tissue are ground in a mortar with sand and 7 to 10 ml of a 0.5 N alcoholic hydrochloric acid solution are added. Sufficient hydrochloric acid must be added to bring the p_H of the material below 4 (as indicated by congo-paper). The material is transferred to a distilling flask with the aid of a few ml of ethanol and evaporated to dryness under reduced pressure on a water-bath of 70° C. The evaporation is repeated once after addition of 10 ml of ethanol.

In the case of blood 10 ml of blood are pipetted directly into the distilling flask, 10 ml of 0.5 N alcoholic hydrochloric acid are added and after mixing the evaporation procedure described for tissue is followed.

The dried tissue- or blood cake is dispersed with 10 ml of ethanol and 50 ml of chloroform or methylene chloride are added. After refluxing during 15 minutes on the water-bath the liquid is decanted through a filter into a second distilling flask. This extraction with chloroform-ethanol is repeated twice and the three combined and filtered extracts are evaporated to dryness in vacuo on the water-bath.

With successively 5 ml, 3 ml and 2 ml of chloroform (or methylene chloride) and 3 ml of distilled water the residue is transferred to a centrifuge tube, shaken and centrifuged. Acidification of the water layer is superfluous because some acid of the tissue pulp has entered the extract. Most of the clear water layer is transferred to a tube and the chloroform is extracted with two more portions of 3 ml of water. The three watery extracts, which are nearly colourless, are combined, adjusted to $p_H = 8$ with the help of borate buffer and sodium hydroxide solution and filtered through a hard filter if necessary, after addition of 1.5 ml of ethanol. It is then made up to a volume of 15 ml with the buffer, 0.6 ml of reagent is added, etc.

As a rule blank determinations of tissue or blood without methylthiouracil give extinction readings slightly higher than those of aqueous blanks. The colour is yellow. Sometimes, however, light red or brown discolorations may appear, which do not necessarily interfere with the measurement of the green reaction product of methylthiouracil.

The extinction of the extract without reagent is negligibly small.

5. *Recovery of added methylthiouracil in the extraction procedure*

A few examples of determinations are given in Table II.

6. *Accuracy of results*

The accuracy is limited by the recovery and by the impossibility in actual practice, *e.g.*, feeding experiments, to run a blank with the same tissue. In our experience the difference between the blanks of various tissues (muscle, liver, kidney and blood of cow, sheep and chicken) is small. The contribution of the tissue blank to the total

References p. 486.

TABLE II

RECOVERY OF ADDED METHYLTHIOURACIL

E is extinction $= \log \frac{100}{T}$ in which T is the intensity of the light transmitted through the sample in per cent of the original intensity. m.t.u. = methylthiouracil.

	E	E—blank	Recovery
A. Muscle tissue (beef) blank	0.056		
Muscle tissue (beef) + 50 γ m.t.u.	0.201	0.145	91 %
Muscle tissue (beef) + 100 γ m.t.u.	0.347	0.291	91 %
Blank of reagent	0.046		
Standard solution = 100 γ m.t.u.	0.367	0.321	
B. Defibrinated sheep's blood, blank	0.071		
Blood + 100 γ m.t.u.	0.420	0.349	96 %
Blank of reagent	0.056		
Standard solution = 100 γ m.t.u.	0.420	0.364	
C. Oxalated sheep's blood, blank	0.061		
Blood + 50 γ m.t.u.	0.194	0.133	98 %
Blank of reagent	0.051		
Standard solution = 50 γ m.t.u.	0.187	0.136	
D. Ox liver, blank	0.066		
Ox liver + 50 γ m.t.u.	0.190	0.124	98 %
Blank of reagent	0.061		
Standard solution = 50 γ m.t.u.	0.187	0.126	

extinction (difference of tissue blank and reagent blank) ranged from 0.005 to 0.015 in a number of experiments, with a mean of 0.010. We have taken this mean value as a basis for calculation in the determinations in animals, which were treated with methylthiouracil. This gives an expected uncertainty of about $\pm$ 2 γ per 10 g of tissue in the result.

If the results are corrected for a constant recovery of 95%, an additional uncertainty of about $\pm$ 4% is introduced. For a single determination in the range of 100 γ per 10 g of tissue the result is not expected to deviate more than about 6 γ from the true content.

This study was made in cooperation with the "Landbouworganisatie T.N.O." and the "Rijks Instituut voor Pluimveeteelt" at Beekbergen. Results of the application of this method will be published in due time together with other methylthiouracil studies.

We are indebted to the Scientific Department of Brocades, Stheeman & Pharmacia for placing their experience at our disposal.

SUMMARY

A description is given of a method for the determination of 4-methyl-2-thiouracil in animal tissue and blood. Extraction of the material with chlorcform or methylene chloride containing 20% ethanol is coupled with partition between acid water and chloroform or methylene chloride. For the colorimetric determination GROTE's reagent is used.

RÉSUMÉ

Description d'une méthode pour le dosage du 4-méthyl-2-thiouracile dans les tissus animaux

References p. 486.

et dans le sang. L'extraction du matériel biologique par le chloroforme ou le chlorure de méthylène contenant 20 % d'éthanol est suivie d'une séparation par partage entre de l'eau acide et le chloroforme ou le chlorure de méthylène. Le dosage colorimétrique est fait au moyen du réactif de GROTE.

ZUSAMMENFASSUNG

Eine Methode zur Bestimmung von 4-Methyl-2-Thiouracil in Tiergeweben und Blut wird gegeben. Ausziehen des Materials mit Chloroform oder Methylenchlorid, das 20 % Aethanol enthält, wird mit der Scheidung zwischen saurem Wasser und Chloroform oder Methylenchlorid kombiniert. Für die kolorimetrische Bestimmung wird GROTE's Reagens benutzt.

REFERENCES

1 R. H. WILLIAMS, G. A. KAY, AND B. J. JANDORF, *J. Clin. Invest.*, 23 (1944) 613.
2 R. H. WILLIAMS, B. J. JANDORF, AND G. A. KAY, *J. Lab. Clin. Med.*, 29 (1944) 329.
3 P. MØRCH, *Acta Pharmacol Toxicol.*, 1 (1945) 106.
4 L. C. CHESLEY, *J. Biol. Chem.*, 152 (1944) 571.

Received July 8th, 1948