MICRO-DETERMINATION OF DNA IN BIOLOGICAL MATERIALS BY GAS-CHROMATOGRAPHIC AND ISOTOPE DILUTION ANALYSIS OF THYMINE CONTENT

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1. Introduction

Methods for determination of nucleic acids must depend on their component sugars, bases or phosphorus. Measurement of specific base concentrations - the most reliable procedure - usually calls for prior separation of DNA and/or RNA, followed by hydrolysis and final determination of individual bases by UV-spectroscopy, by fluorescence assay or by gas chromatography. Fluorescence and spectrophotometric methods have been widely used on account of their high sensitivity, despite their low specifity. Gas chromatography on the other hand proves to be more specific and even more sensitive [1, 2]. Up to now for RNA or DNA investigations amounts of the order of 10 mg have been required [2, 3]. The nucleic acids were hydrolyzed, their bases were silylated and small aliquots (nanograms) were used for gas chromatography.

This paper gives a brief outline of a procedure for the thymine determination in 10–50 mg samples of fresh tissue. After tissue decompostition by heating with formic acid, extraction of lipids, drying, sublimation of thymine, applying it to a thin-layer plate, chromatography and elution of the isolated thymine spot followed by subsequent silylation for gas chromatography, the thymine content is determined, yields being controlled by isotope dilution techniques.

This procedure increases the sensitivity by about two orders of magnitude over the previous ones. Its general applicability is demonstrated by thymine determinations in various tissue types. These are compared to the values of UV-spectroscopy. These results are shown to be in good agreement with each other and with data from literature.

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2. Materials

Anaesthetized albino rats were bled, and the weights of the organs were taken. These were subsequently frozen and kept at −20°C. Hexamethyldisilazane HMDS) and trimethylchlorosilane (TMCS), specially purified, were purchased from Pierce Co., (Rockford, Ill, USA). Thymine (2-^{14}C-labeled) specific activity 50 Ci/mole came from C.F.Boehringer, (Mannheim, Gy.). Its purity, checked by TLC, was found to be higher than 99%. Thin-layer plates coated with silica gel with fluorescent indicator dye (1500 LS 254) came from Schleicher u. Schüll, (Dassel, Gy.).

3. Methods

Tissue samples are lyophilized, ground in a mortar and ^{14}C-thymine is admixed. To this material 98% formic acid has to be added (1 ml/20 mg dry material) in an ampoule. After sealing, it is exposed to 175°C for 45 min, cooled down, opened, the formic acid evaporated off and the lipids extracted with petroleum ether b.p. 40–60°C. The residue is subjected to an acetone-water extraction (98/2 v/v) to remove most of the thymine, the clear extract being evaporated and dissolved in 0.2 ml of 0.01 N HCl. This is transferred to a microscope slide, dried and the residue sublimated (90 min, 170°C) to another cooler slide, leaving behind nonvolatile impurities. The sublimate is taken up in acetone/water (98/2 v/v), applied to a TLC plate and carefully dried (70°C, 30 min). The chromatograms are developed with acetone, thymine running at R_f 0.61.

Table 1.
Thymine and DNA content of several tissues, as determined by gas chromatography and UV spectrophotometry.

Tissue	Micrograms thymine per 100 mg fresh tissue		Micrograms DNA per 100 mg fresh tissue		Micrograms DNA per 100 mg fresh tissue
	by UV	by GC	by UV	by GC	according to literature
liver	28	30	233	250	168– 277 [4, 5]
brain	16	13	133	108	94– 200 [5]
kidney	49	33	408	274	267– 418 [4, 5]
spleen	170	183	1420	1520	770–1650 [5]
yeast [a]	13	12	99	92	98– 114 [4]

[a] Average of five determinations, standard deviation of thymine content: SD = ± 1.7 μg/100 mg fresh tissue (UV), SD = ± 1.6 μg/100 mg fresh tissue (GC). Relative standard deviation ($\overline{SD}_{rel} = \frac{s.\,100}{average}$ %) : SD_{rel} = ± 13% (UV, GC).

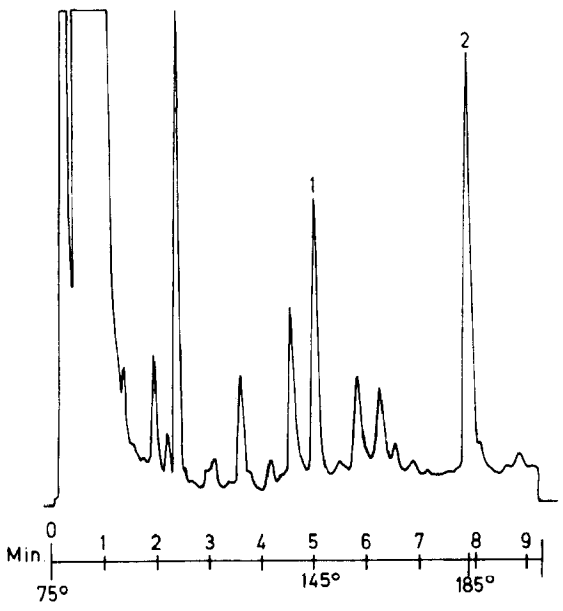

Fig. 1. Gas chromatogram of silylated products. Peak marked 1: thymine. Peak marked 2: phenanthrene as internal standard. Starting material was about 10 mg of lyophilized rat liver tissue.

It can be traced in the light of a low pressure mercury lamp.

The thymine spots are carefully scratched off the TLC plate, extracted from the sorption material with acetone-water, evaporated and taken up in 0.01 N HCl.

This solution is divided into aliquots. One is used for determination of the optical density in the UV region at pH 2. In a second aliquot the radioactivity is measured in a liquid scintillation counter. A third aliquot is evaporated to near dryness in a capillary (1 × 30 mm) at 50°C, 100 mm Hg, followed by 50°C, 0.01 mm Hg, 1 hr. Then 5 μl of silylation mixture (pyridine 7 vol., HMDS 2 vol., TMCS 1 vol. and 5 μg of phenanthrene to serve as internal GC standard) are added and the capillary is sealed and heated (150°C, 30 min.).

From this mixture aliquots (1 μl) are injected into a gas chromatograph (Varian, model 200) with a 1 × 1/8 in (3 mm) stainless steel column, packed with 5% SE 30 on Chromosorb W, temperature programmed for Δt = 14°C/min, injection port at 250°C and the detector (FID) at 275°C. The thymine eluted at 140–145°C with a relative elution volume of 0.62 (phenanthrene = 1.00). A calibration curve was obtained by plotting the peak area ratios of thymine to phenanthrene against their concentration (mg/ml) ratios for known material injected. The slope of this curve came out as 1.06, running practically linear over a range of 1 to 10 μg of thymine.

4. Results

Fig. 1 gives an example of a gas chromatogram using rat liver tissue showing several peaks, the thymine peak (marked 1) and the internal standard peak (marked 2) clearly protruding.

Quantitative data are listed in table 1 for compa-

rison with literature. Sample sizes used were in the case of yeast, liver, brain, kidney about 10 mg lyophilized material and 2 mg for spleen. Overall yield was 21 to 28%.

5. Discussion

The number of peaks in fig. 1 points out the fact that there are many volatile components besides silylated thymine, which is a major constituent. No impurities that interfere with the thymine determination have been observed.

The GC procedure proves to be more specific than UV determination, since the values of the latter tend to come out higher due to additional UV absorbing non-thymine material. This finds its expression for instance in the fact that the ratio of OD in the maximum range (264–260 nm) to OD in the minimum range (233–238 nm) varies from 4.2 in the case of pure thymine to 1.2 for material isolated from yeast.

Our results presented in table 1 compare well within reasonable limits to DNA determination on yeast and on rat tissues published in the literature [4, 5]. The values from both our methods agree within the limits of standard deviation.

This has been achieved by taking special advantage of some steps. As it is rather important to avoid acid in the silylation step, preference had to be given to formic acid hydrolysis, since elimination of excess acid is quite easy. The application of our procedure to tissues of widely differing composition is enabled by using acetone as extractant discriminating against many other low molecular weight constituents and by use of the sublimation technique, taking advantage of the relative volatility of thymine. Finally, application of microtechnique for silylation allows us to profit from the high sensitivity of gas chromatography.

There are some assumptions to be considered in our procedure which makes thymine the basis for calculation of DNA content.

1) Decomposition by formic acid goes to completion, without altering thymine content [6, 7]. This seems to be the case [8].

2) Practically all thymine originates from DNA. Thymine as free nucleoside or nucleotide amounts to less than 1% of total cellular thymine content [9, 10, 11]. In tissues of higher organisms practically no free thymine has been found [12]. Yet widely varying amounts may be found in tRNA; e.g. in rat liver tRNA there is practically no thymine [13], whereas in bakers yeast out of 100 moles of thymine found in total nucleic acids, 6 derive from tRNA [14, 15], the ratio of RNA-P/DNA-P being about 13 [5]. Generalizing the results from *E. coli* [16], the ratio of thymine from rRNA to thymine from tRNA may be calculated as 0.6. This high amount of RNA-derived thymine causes far less error in our method because under conditions of acid hydrolysis, the riboside linkage will not be cleaved quantitatively in contrast to the deoxyriboside linkage. Jervell et al. hydrolysed UMP in 98% formic acid at 165°C for 2 hr in the presence of small amounts of protein, and found 80–85% of the uracil still in the form of uridine. On the other hand deoxythymidine or deoxythymidylate added to the hydrolytic system could be completely recovered as thymine [8].

3) For exact DNA calculations the mythymine content of DNA in a given species must be known. It must be constant in all the tissues of this species. In most higher animals the base composition of DNA does not in fact vary much. For determination of DNA content, measurement of thymine concentration has already been used successfully [8, 17]. Furthermore efforts will be directed towards an increase in sensitivity and in yield.

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