Rapid Papers

(Pages 977–995)

Biochem. J. (1979) **177**, 977–979
Printed in Great Britain

A Rapid and Sensitive Method for Measuring Ribonucleic Acid in Ribosomal Preparations

By CAROLINE MACKIE, T. H. E. BRYANT* and JOHN MOWBRAY
Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, and **Department of Physics, Middlesex Hospital Medical School, Cleveland Street, London W.1, U.K.*

(*Received* 12 *October* 1978)

By using the fluorescence enhancement of ethidium bromide when it binds to RNA, a very rapid, simple and sensitive assay for the concentration of ribosomal RNA in complex mixtures has been devised.

Yields of ribosomes from eukaryotic tissues are commonly assessed either by measuring the A_{260} of the ribosomal preparation and applying an empirically derived coefficient, or by the method of Fleck & Munro (1962). The former gives very variable results from preparation to preparation, and the latter, although reproducible, is a time-consuming procedure that may involve losses of material during the extraction process, and requires a tissue-specific conversion factor for contaminating peptides and carbohydrate.

Under certain conditions it may be necessary or desirable to prepare ribosomes contaminated with protein. For instance, the preparation of pure ribosomes (assessed by the ratio $A_{260}/A_{280} > 1.7$) from heart muscle results in low yields; in order to obtain high yields of active ribosomes, contamination with protein must be accepted (C. Mackie, & K. D. Thomas, unpublished work). This contamination may be an advantage where differences in ribosomal activity are under investigation, as proteins important in the control of such activity may otherwise be removed during the purification procedure. Measurement of the absorbance of a ribosomal preparation in the presence of contaminating proteins then becomes an extremely inaccurate measure of ribosomal yield, owing to the considerable absorption of some proteins at 260nm as well as at 280nm.

Le Pecq & Paoletti (1966) have reported that ethidium bromide binds to RNA, resulting in an increase in the fluorescence intensity at 690nm. They suggested that this binding could be used as the basis of an assay for RNA, and Beers & Wittliff (1975) have shown that using ethidium bromide it is possible to assay RNA (mainly ribosomal) along with DNA, although their extraction of RNA is inefficient and the method is as slow as and less sensitive than current methods for measuring RNA. The fluorescent enhancement of ethidium bromide on binding has also been used to investigate ribosomal structure (Bollen *et al.*, 1970; Gatti *et al.*, 1977). We now report that ethidium bromide may be used to provide a very rapid, reproducible and technically straightforward assay for ribosomal RNA in complex mixtures, which thus allows immediate adjustment of the concentration of fresh active ribosomes.

Materials and Methods

Ethidium bromide was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., pancreatic deoxyribonuclease 1 (grade 1) was from the Boehringer (London) Corp., yeast RNA (type XI), bovine pancreatic ribonuclease A (type 1A) and sucrose (grade 1) were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K., and pure KCl and $MgCl_2$ were from E. Merck, Darmstadt, Germany. All other reagents were of analytical quality. Buffer A contained 0.5M-KCl and 0.05M-Tris/HCl, pH9.0; buffer B contained 0.25M-KCl, 0.005M-$MgCl_2$, 0.001M-dithiothreitol, 10% (v/v) glycerol, 1% (v/v) Triton X-100 and 0.02M-Tris/HCl, pH7.4; buffer C comprised 0.1M-KCl, 0.005M-$MgCl_2$, 0.25M-sucrose, 0.001M-dithiothreitol and 0.05M-Tris/HCl, pH7.4. Ethidium bromide stock solution was 6mg/ml in buffer A and was stable for at least 2 months in the dark. Standard RNA solution contained 3mg of yeast RNA/ml of buffer A and was stored at −20°C in 20μl portions.

Ribosome preparation

A perfused rat heart (Mowbray & Ottaway, 1973) was homogenized (VirTis 45 homogenizer; 3×10s at 36000rev./min) in 10ml of buffer B at 4°C. The homogenate was centrifuged for 10min at 8000*g* (MSE 18; 8500rev./min; 8×50ml head; 4°C) and the supernatant stored on ice for 1h before being layered over 3ml of 1M-sucrose/20mM-Tris/HCl/150mM-KCl/5mM-$MgCl_2$ (pH7.4) and re-centrifuged for 2h at 100000*g* (Spinco L2; 40000rev./min; 50 Ti head; 4°C). The ribosomal pellet was resuspended in approx. 400μl of buffer C.

Digestion with nuclease

Whole homogenates were incubated for 1h at 37°C with deoxyribonuclease (0.3mg/ml) and, after cooling, for a further 10min at 0°C with ribonuclease A (1mg/ml). Ribosomes were incubated for 10min at 0°C with ribonuclease A (1mg/ml).

Assay procedure

A 5μl portion of ethidium bromide stock solution was added to 3ml of buffer A in a 1cm-path-length spectrofluorimeter cuvette. Fluorescence intensity (I_0) was measured at an emission wavelength of 590nm and excitation wavelength 540nm in an Aminco–Bowman 4-8202 instrument with a photomultiplier microphotometer attachment. Standard RNA solution (10μl) was added to give a total RNA concentration of 9.95μg/ml, and the fluorescent intensity (I_S) of this solution measured. A sample of ribosomal suspension containing 1–65μg of RNA was then added and the fluorescent intensity (I_R) measured. Since there is a linear relationship between RNA concentration and fluorescence intensity (see below) the concentration of RNA (c_{RNA}) in μg/ml is given by:

$$c_{RNA} = \frac{9.95\,(I_R - I_S)}{F\,(I_S - I_0)}$$

where

$$F = \frac{\text{Fluorescence enhancement given by unit weight of the RNA type to be assayed}}{\text{Fluorescence enhancement given by unit weight of the reference RNA used as standard}}$$

Results and Discussion

It is thought that the quantum efficiency of fluorescence of ethidium bromide is only increased when the dye is intercalated in double-stranded regions of nucleic acids; hence a 2.2-fold greater increase in quantum efficiency of fluorescence is observed when ethidium bromide binds to calf thymus DNA than when it binds to an equal concentration of yeast RNA (Le Pecq & Paoletti, 1966). It is to be expected, therefore, that the interaction of the dye will vary with different species of RNA. Thus strictly it is necessary to compare the fluorescence enhancement given by a specific type of RNA with some absolute measure of the same RNA or, at least, some approximation to this, such as that obtained after purification and digestion followed by assay of the free nucleotides by the method of Fleck & Munro (1962). However, in practice it is possible to express routinely the concentration of an unknown RNA relative to a convenient stable RNA solution. This reference RNA solution can be standardized at some suitable time against an absolute estimate of the RNA species to be assayed. A correction factor (F; see above) is then introduced to accommodate the difference in fluorescence enhancement given by the reference RNA and the assayed species.

By using the same reference yeast RNA over about 2 years, we have found no significant variation in the value of F for ribosomes from cardiac muscle.

Batches of ribosomes prepared from rat heart muscle have been assayed by this technique and also subjected to extraction and assay by the method of Fleck & Munro (1962). The fluorescence assay gave a value, measured against Sigma yeast RNA as standard, which was 2.7 ± 0.2 ($n = 6$) times greater than the value obtained by the conventional method (i.e. $F = 2.7$). Two factors may contribute to this difference. First, RNA may be lost during the extraction procedure of Fleck & Munro (1962), although it seems unlikely that this could account for even a major part of the discrepancy. Secondly, yeast RNA may have fewer sites capable of enhancing ethidium bromide fluorescence per unit weight than has the more highly organized RNA in the ribosomes.

A linear relationship has been found between fluorescence intensity and RNA concentration at least up to 100μg/ml (Fig. 1). The conditions used are suitable for measuring down to 1μg of ribosomal RNA (Fig. 1, inset). With heart ribosomes, fluorescence intensity was found to be linear up to about 65μg of ribosomal RNA per assay, but additions in excess of this resulted in very turbid conditions and decreased specific intensity.

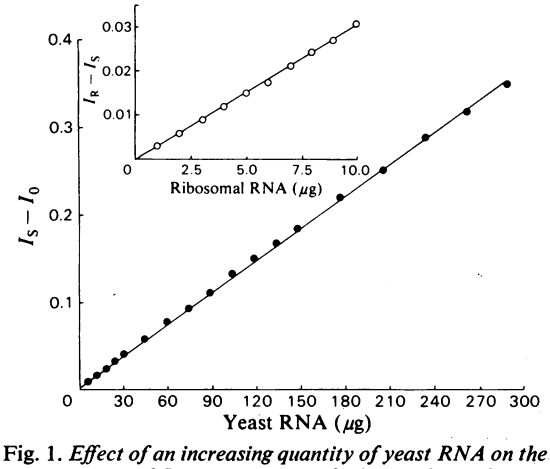

Fig. 1. *Effect of an increasing quantity of yeast RNA on the intensity of fluorescence (I_R) of ethidium bromide*
The assay procedure given in the Materials and Methods section was used. The value of I_0 in arbitrary units was 0.0357. The inset shows the effect on I_R of small quantities of heart ribosomes whose RNA content was independently determined by the method of Fleck & Munro (1962). The correlation coefficient (r) was 0.992 ($P < 0.001$, $n = 11$).

Pancreatic ribonuclease-A digestion under conditions (see the Materials and Methods section) that cleave polyribosomes (C. Mackie & K. D. Thomas, unpublished work) showed that the assay is independent of whether ribosomes are present as polyribosomes or as monomer units. Addition of an equal volume of 5% (w/v) defatted albumin to the ribosomal suspension also had no effect on the fluorescence intensity. This bears out the results of Le Pecq & Paoletti (1966), who claim that if the pH in the cuvette is high, the proteins are negatively charged and therefore do not interfere with the binding of ethidium bromide to RNA.

Whole homogenates of heart tissue were incubated with ribonuclease (as before) in order to remove non-ribosomal species of RNA. Extraction by the method of Fleck & Munro (1962) then gave a value for total ribosomal RNA. Portions of the same homogenates were treated with pancreatic deoxyribonuclease 1 followed by ribonuclease digestion as above. Ribosomal RNA was then measured by the fluorescence method. This gave a result 2.9 ± 0.2 ($n = 4$) times greater than the value obtained by using the method of Fleck & Munro (1962). This ratio is not significantly different ($t = 0.626$; $P > 0.5$) from the ratio obtained for ribosomal preparations, indicating that the fluorescence method may be used in whole homogenates to measure total ribosomal RNA. Hence the amount of ribosomes in whole tissue may be measured by this rapid and straightforward technique, without interference from contaminating peptides or proteins.

The advantages of the present assay for measuring eukaryotic ribosomal RNA are that: (1) it is extremely rapid and simple; (2) it gives very consistent values against a suitable RNA reference; (3) there are no purification steps at which material may be lost; (4) it requires about 50 times less material than does the method in common use (Fleck & Munro, 1962); (5) the content of ribosomes in complex mixtures may be assayed within 5 min of preparation, and the inevitable variability in ribosome concentration found from preparation to preparation eliminated during subsequent investigation; (6) after a simple digestion with deoxyribonuclease and ribonuclease it can be used to measure ribosome content in muscle homogenates.

We thank Mr. A. J. Bishop for valuable technical assistance.

References

Beers, P. C. & Wittliff, J. L. (1975) *Anal. Biochem.* **63**, 433–441

Bollen, A., Herzog, A., Favre, A., Thibault, J. & Gros, F. (1970) *FEBS Lett.* **11**, 49–54

Fleck, A. & Munro, H. N. (1962) *Biochim. Biophys. Acta* **55**, 571–583

Gatti, C., Houssier, C. & Fredericq, E. (1977) *Biochim. Biophys. Acta* **476**, 65–75

Le Pecq, J. B. & Paoletti, C. (1966) *Anal. Biochem.* **17**, 100–107

Mowbray, J. & Ottaway, J. H. (1973) *Eur. J. Biochem.* **36**, 362–368