GRADUAL RELEASE OF SPERMINE AND RNA FROM RAT LIVER MICROSOMES TREATED WITH EDTA

Jahangir A. KHAWAJA
Department of Medical Chemistry, University of Helsinki, SF-00170 Helsinki 17, Finland

Received 20 December 1971

1. Introduction

It has been previously shown that chelating agents such as EDTA [1–3] and pyrophosphate [4] can release between 50–60% RNA from hepatic microsomes. Sabatini et al. [3] have shown that the addition of increasing amounts of EDTA dissociates microsomes in a gradual fashion: the small subunits being the first to be released from the microsomal membranes.

The polyamines, spermine and spermidine, are associated with the isolated ribosomes of both bacterial and animal origin [5–9]. Previous work from this laboratory has shown that polyamines such as spermine may be involved in the attachment of ribosomes to membranes of the endoplasmic reticulum [9, 10]. In our preliminary experiments reported earlier [11], no polyamines could be detected after treatment of microsomes with 15 mM EDTA, a condition which should release most of the small subunits from the microsomes. As this finding was apparently at variance with our more recent observations [9, 10], it was decided to re-investigate the release of the polyamine spermine from the microsomes, under conditions that would gradually remove ribosomal subunits from the microsomal membranes. The present results show that when ribosomal subunits are gradually removed from the microsomes by treatment with EDTA, there is a concomitant release of endogenous spermine as well as of ^{14}C-spermine administered *in vivo* to the rat. The release of spermine closely followed that of RNA from the microsomes, both reaching a limiting value of about 60% at 50 mM EDTA.

2. Materials and methods

^{14}C-spermine tetrahydrochloride (specific activity 7.4 mCi/mmole) was supplied by the New England Nuclear Corp. EDTA (disodium salt) was a product of E. Merck, Darmstadt. Source of the rest of the material has been previously described [9, 10].

2.1. *Preparation and purification of microsomes*

Male rats of the Wistar strain (200–250 g) were used. They were starved for 16–20 hr prior to sacrifice. The livers were rinsed in 0.25 M sucrose made in 25 mM KCl–2 mM $MgCl_2$–50 mM Tris-HCl, pH 7.5 (medium M), and homogenized in 3 vol of medium M in a glass homogenizer of Potter-Elvehjem type. The homogenate was centrifuged at 10,000 *g* in a Sorvall centrifuge for 10 min at 4°. The supernatant fraction was removed and centrifuged at 149,000 *g* for 30 min. The resulting microsomal pellet was resuspended in medium M (3–5 ml/g tissue equivalent). Such a microsomal preparation may contain free ribosomes in addition to those bound to membranes [12]. For further purification, 5 ml portions of the microsomal suspension were layered on a discontinuous sucrose gradient (3 ml of 0.5 M sucrose overlaid on 4 ml of 2 M sucrose, all made in 25 mM KCl–2 mM $MgCl_2$–50 mM Tris-HCl, pH 7.5 (buffer M). The gradient was centrifuged in a 50 Ti rotor of a Spinco L50 centrifuge at 149,000 *g* for 210 min. Free ribosomes were sedimented in the form of a pellet. The interphase layer was removed, diluted with buffer M and centrifuged at 149,000 *g* for 30 min to obtain the purified microsomes. The ratio of RNA to protein in this preparation averaged 0.1.

North-Holland Publishing Company – Amsterdam

Table 1
Release of endogenous spermine from unlabelled microsomes treated with increasing amounts of EDTA.

EDTA (mM)	RNA released (%)	Spermine released (%)
6	15 (9–23)	4 (0–13)
8	30 (27–35)	19 (14–24)
10	38 (35–40)	27 (16–36)
15	43 (40–45)	34 (26–36)
20	48 (47–49)	40 (28–49)
30	50 (44–54)	44 (42–47)
50	55 (50–58)	54 (51–57)

The microsomes were treated with EDTA as described in the Materials and methods section. The suspension was then loaded onto a discontinuous gradient (14 ml of 5% sucrose overlaid on 14 ml of 20% sucrose made in 50 mM KCl–10 mM Tris-HCl, pH 7.5) and centrifuged at 68,000 *g* for 2 hr. RNA and spermine were estimated in the pellet. The results are expressed as the mean of 3 experiments on different microsomal preparations (range in parenthesis).

Table 2
Release of ^{14}C-spermine from microsomes treated with increasing amounts of EDTA.

EDTA (mM)	RNA released (%)	^{14}C-spermine released (%)
6	26	7
8	36	22
10	38	22
15	48	32
20	50	38
30	57	53
50	59	59

Microsomes were labelled *in vivo* with ^{14}C-spermine as described in Materials and methods section. The values represent the mean of 2 experiments and are based on the radioactivity and RNA determined in the pellet.

For the preparation of labelled microsomes, the rats were treated intraperitoneally with 10 μCi of ^{14}C-spermine in 1 ml of 0.9% NaCl, and sacrificed 24 hr later.

2.2. *EDTA treatment of microsomes*

EDTA was added to portions of microsomes (1.2 mg RNA) suspended in 2 ml of 50 mM KCl–2 mM $MgCl_2$–10 mM Tris-HCl, pH 7.6 (buffer A), and the suspension was allowed to stand for 10 min at 0°. It was then loaded onto 28 ml of 5–20% linear sucrose gradient made in 50 mM KCl–10 mM Tris-HCl, pH 7.6, and centrifuged at 63,000 *g* for 7 hr in the SW 25.1 rotor at 4°. After centrifugation, 1 ml fractions were collected for analysis for absorbance at 260 nm and for the determination of radioactivity. The pellet obtained after centrifugation was suspended in 1 ml of H_2O and RNA and radioactivity were determined.

2.3. *Determination of spermine radioactivity*

The fractions from the sucrose gradients were precipitated with 0.5 N (final) cold perchloric acid using bovine serum albumin as a carrier. After centrifugation, the pellet was used for the determination of RNA. The supernatant was made alkaline by the addition of NaOH and was further processed for the determination of radioactivity as described previously [9].

RNA was determined by the orcinol method [13] and protein by the method of Lowry et al. [14]. Polyamines were estimated according to the method of Raina and Cohen [15].

3. Results

3.1. *The release of RNA and of endogenous spermine from the microsomes*

If microsomes are exposed to a medium containing a high Mg^{2+} concentration, substantial amounts of polyamines are lost from the microsomes [6, 7]. In a preliminary study [11], EDTA treatment of microsomes prepared in this way resulted in the release of polyamines so that the polyamines were difficult to detect by the method used [11]. Therefore, the microsomes used in the experiments to be described were isolated in a medium of low ionic strength. Determination of RNA and spermine in the pellets, obtained after centrifugation, showed that RNA was gradually released from the microsomal pellet as the EDTA concentration was increased (table 1). The released RNA reached a limiting value of 55% at an EDTA concentration of 50 mM. Increasing the concentration of EDTA to 100 mM did not result in a further release of RNA to any considerable extent. These observations are in agreement with the previous studies on guinea pig liver microsomes [3] and membrane-bound ribosomes from HeLa cells [16].

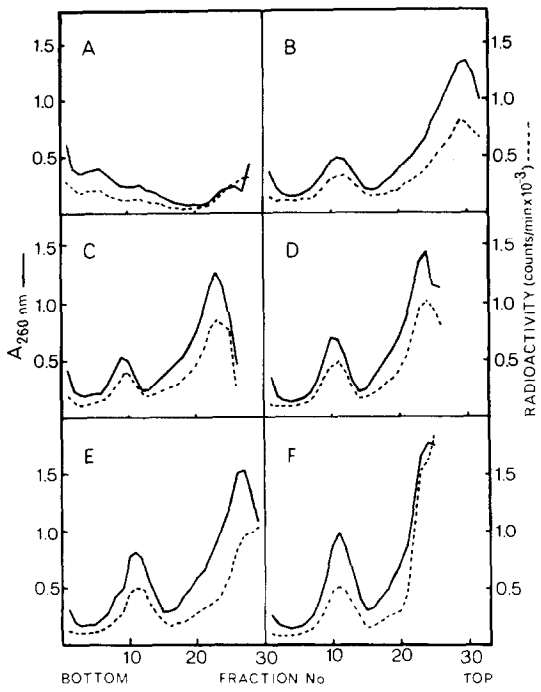

Fig. 1. Sucrose density-gradient analysis of ^{14}C-spermine-labelled microsomes treated with increasing amounts of EDTA. The conditions of centrifugation are described in Materials and methods section. The final concentration of EDTA in each case was: A) Control (no EDTA); B) 10 mM; C) 15 mM; D) 20 mM; E) 30 mM; and F) 50 mM.

The release of spermine closely followed that of RNA although, at low concentrations of EDTA, it lagged behind the RNA release. Like RNA, released spermine reached a value of 54% of the original at 50 mM EDTA. Addition of more EDTA did not result in a further release of spermine to any appreciable extent. Spermidine was difficult to detect in the amount of microsomes used in the experiment reported here. However, if the amount of microsomes was increased (equivalent to 3 mg RNA), spermidine could easily be estimated, the ratio of spermidine to spermine being about 1:7.

When the experiment described in table 1 was performed using ^{14}C-spermine-labelled microsomes, essentially similar results were obtained, which are shown in table 2. Again at low concentration of EDTA, release of ^{14}C-spermine closely followed the release of RNA from the pellet.

It is notable that the release of labelled spermine from the microsomes was very similar to that of endogenous spermine.

3.2. *Release of labelled spermine into the linear gradient*

Sucrose density-gradient analysis of the material released from the labelled microsomes upon treatment with increasing amounts of EDTA showed that ^{14}C-spermine released from the microsomes closely followed the profile of the ribosomal subunits in the gradient. Fig. 1 describes the results of an experiment performed on the same microsomal preparation. When microsomes were centrifuged without treatment with EDTA (control), 85–90% RNA was recovered in the pellet. At an EDTA concentration of 5 mM, only the small subparticles were present in the gradient; there being no peak corresponding to the large subparticles (results not shown). At 10 mM EDTA most of the small subparticles, calculated on the basis of released RNA, were present in the gradient and a small peak corresponding to large subparticles appeared (cf. Sabatini et al. [3]). Labelled spermine followed both subparticles. At this stage about 40% RNA was released from the pellet. If the EDTA concentration was further increased, there was no appreciable increase in the peak of the small subparticles whereas the large subparticles continued to be released. It was observed that when EDTA was present at concentrations greater than 20 mM, there was a considerable degradation of small subparticles as seen from the gradient profiles. At 50 mM EDTA, the amount of large subparticles reached a limiting value as there was no further increase in the peak due to the large subparticles.

Between 90–100% spermine radioactivity released from the pellets was recovered in the gradients.

In order to study whether exogenous spermine had any effect on the release of RNA and ribosomal subparticles, an experiment similar to that described in fig. 1 was performed with microsomes preincubated in 0.5 mM spermine at 0° for 5 min. The density gradient profiles failed to show any definite peak in the region of small subparticles. This was possibly due to the aggregation of these particles forming dimers and higher aggregates. The release of total RNA from the pellets was similar to that observed in the absence of added spermine.

The average ratio of absorbance at 260 nm to absorbance at 280 nm in the small and the large subparticles was 1.93 and 1.94, respectively.

4. Discussion

The present results show that when ribosomal subparticles are gradually removed from the microsomal membranes by the addition of increasing amounts of EDTA, labelled spermine is also gradually released and is found to be associated with the released ribosomal subparticles. Also, the release of RNA from the pellets, as a result of the addition of EDTA, is closely followed by the release of endogenous as well as ^{14}C-spermine. A close correlation between the accumulation of RNA and polyamines, both in bacterial and animal systems, has been well documented [15, 17]. Our earlier results [9, 10] have indicated that spermine or Mg^{2+} is necessary for the attachment of ribosomes and ribosomal subparticles to endoplasmic reticulum membranes *in vito*. The present results suggest that polyamines may function in the ribosome–membrane interaction by associating with ribosomes. Spermine could perform this function: by linking the negatively charged ribosomes to the negatively charged membranes through ionic bridges, by neutralizing the charge on RNA phosphate groups [18] thus conferring stability and correct conformation on ribosomes or by a conbination of both possibilities. The close association of spermine with ribosomes is indicated by the concomitant release of spermine when ribosomal subparticles were released by the action of EDTA, and by the observation that the released spermine closely followed the subparticles in the gradient. It is notable that about 60% of spermine originally present in the microsomes was still associated with the pellet when all of the small subunits were dissociated from the microsomes. This indicates that polyamines may be involved not only in the association of the 2 ribosomal subunits [18] but also may be involved in the attachment of ribosomes to membranes of the endoplasmic reticulum.

Even the highest concentration of EDTA tested (250 mM) failed to release more than 60–70% RNA, in agreement with the published studies [2-4, 16]. The unreleased material has been shown to be large subunits (comprising about half of the total number of large subunits) which are tightly bound to membranes [3, 16], and are refractory to the chelating agents [16]. At this stage about 45% of spermine originally associated with the microsomes was still bound to the pellet. Further experiments are in progress to localize polyamines in the large subparticles–membrane complex.

Acknowledgements

The present study was started at the Department of Biochemistry, University of Aberdeen. I thank Prof. A. Raina of the Department of Medical Chemistry, University of Turku, for helpful discussions. Skillful technical assistance by Mrs. S. Kanerva is gratefully acknowledged. This work was supported by grants from the Sigrid Jusélius Foundation and from the National Research Council for Medical Sciences, Finland.

References

[1] G.E. Palade and P. Siekevitz. J. Biophys. Biochem. Cytol. 2 (1956) 171.
[2] H. Sachs, J. Biol. Chem. 233 (1958) 643.
[3] D.D. Sabatini, Y. Tashiro and G.E. Palade, J. Mol. Biol. 19 (1966) 503.
[4] H. Sachs, J. Biol. Chem. 233 (1958) 650.
[5] L. Stevens, Biol. Rev. 45 (1970) 1.
[6] A. Raina and T. Telaranta, Biochim. Biophys. Acta 138 (1967) 200.
[7] J.A. Khawaja and L. Stevens, Biochem. J. 104 (1967) 43 P.
[8] S.S. Cohen and J. Lichtenstein, J. Biol. Chem. 235 (1960) 2112.
[9] J.A. Khawaja, Biochim. Biophys. Acta, 254 (1971) 117.
[10] J.A. Khawaja and A. Raina, Biochem. Biophys. Res. Commun. 41 (1970) 512.
[11] J.A. Khawaja and L. Stevens, Scand. J. Clin. Lab. Invest. 23 (1969) suppl. 108, 30.
[12] E.C. Henshaw, T.B. Bojarski and H.H. Hiatt, J. Mol. Biol. 7 (1963) 122.
[13] G. Ashwell, in: Methods in Enzymology, Vol. 3., eds. S.P. Colowick and N.O. Kaplan (Academic Press, New York, 1957) p. 73.
[14] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[15] A. Raina and S.S. Cohen, Proc. Natl. Acad. Sci. U.S. 55 (1966) 1587.
[16] M. Rosbash and S. Penman, J. Mol. Biol. 59 (1971) 227.
[17] A. Raina and J. Jänne, Federation Proc. 29 (1970) 1568.
[18] J.D. Watson, Bull. Soc. Chim. Biol. 46 (1964) 1399.