NITROCELLULOSE MEMBRANE FILTER BINDING ASSAY OF 16S.23S RNA COMPLEX FORMATION

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ABSTRACT

A simple and quick method of assay of the formation of the bimolecular complex between 16S and 23S RNAs has been developed. The method depends on the binding of 23S RNA to the nitrocellulose filter with the help of the pentameric protein complex $(L7/L12)_4L10$ and the consequent association of radioactive 16S RNA to the filter through 23S RNA. The optimum conditions and stoichiometry of the complex formation, the IF3-induced dissociation of 16S . 23S RNA complex and the binding of poly U to the complex have been demonstrated by this technique.

INTRODUCTION

It has been demonstrated in this laboratory that 16S and 23S RNAs form a bimolecular complex under two specific ionic conditions: (i) high salt, high Mg^{++} concentrations and (ii) low salt and high Mg^{++} concentrations in presence of 1 M ethanol[1-7]. Light scattering was used to detect such complex formation and the technique of density gradient centrifugation was employed to characterise the complex. It was also possible to demonstrate the complex formation by polyacrylamide gel electrophoresis but in presence of ethanol only. This technique could not be used under high salt condition due to flow of heavy current. All the three methods are laborious and time consuming. In the search of a simple and quick method, the nitrocellulose filter binding assay has been developed.

The assay is based on the fact that 23S RNA binds to nitrocellulose filter in the presence of 23S RNA binding proteins of 50S ribosomes[8,9]. Among the 23S RNA binding proteins, L7, L12 and L10 were chosen as it is easy to isolate these in the form of a pentameric complex[10] $(L7/L12)_4L10$. L10 binds directly to 23S RNA and is responsible for the binding[11,12] of L7/L12. Normally 16S RNA does not bind to nitrocellulose filter but when it is complexed with 23S RNA which in turn is associated with $(L7/L12)_4L10$, the binding of the former can be effected. Unfortunately, the reverse method of binding of 23S RNA through 16S RNA associated with 30S ribosomal proteins can not be followed as these proteins are known not to bind 16S RNA to nitrocellulose filter[13]. This has also been confirmed in this laboratory.

MATERIALS AND METHODS

The 30S and 50S ribosomes of *E. coli* were prepared by the ultracentrifugation method. 16S and 23S RNAs were isolated from 30S and 50S ribosomes respectively by the phenolization procedure. 5S RNA was removed from 23S RNA by gel filtration[14]. For the preparation of ^{32}P-labelled RNAs *E. coli* was grown in ^{32}P-containing medium. The isolation procedure was the same as mentioned above.

The pentameric protein complex $(L7/L12)_4L10$ was isolated from 50S ribosomes by treatment with 1 M NH_4Cl at 37°C as described by Hamel *et al*[10]. For the preparation of ^{35}S-labeled proteins, *E. coli* was grown in ^{35}S-containing medium and the isolation procedure was the same as mentioned above.

IF3 was prepared from 1 M NH_4Cl wash of 50S ribosomes as described by Schiff *et al*[15]. [^{3}H]-Poly(U) was obtained as gift from Dr S. Mitra of Oak Ridge National laboratory, Oak Ridge, Tennessee, U.S.A.

Binding of $(L7/L12)_4L10$ *to* 23*S* RNA *as studied by gel filtration*

The mixture containing 23S RNA and $(L7/L12)_4L10$ in reconstitution buffer (20 mM Tris-HCl, pH 7.5, 400 mM KCl, 20 mM magnesium acetate and 6 mM β-mercaptoethanol) was passed through Sephadex G-100 column (1.2 × 10 cm) pre-equilibrated with reconstitution buffer. The elution was done with the same buffer. Fractions (0.36 ml) were collected, dried on Whatman No. 3 MM filter paper pieces which were counted in Rack Beta liquid scintillation counter of LKB.

Nitrocellulose filter binding assay

In all nitrocellulose filter binding experiments 23S RNA was mixed with 2.5 times molar excess of $(L7/L12)_4L10$ and equimolar amount of ^{32}P-16S RNA in 1 ml reconstitution buffer (composition mentioned above) or alcohol containing mixture (1 M ethanol, 60 mM ammonium acetate and 20 mM magnesium acetate). The incubation was done at 40°C for 60 min and then the mixture was filtered through nitrocellulose filter (0.45 μ, of Millipore Corporation, U.S.A.) which was dried and counted in liquid scintillation counter of LKB.

RESULTS

Binding of $(L7/L12)_4L10$ *to* 23*S* RNA *as well as* 16*S*, 23*S* RNA *Complex.*

In order to check the binding of $(L7/L12)_4L10$ to 23S RNA, ^{35}S-labeled $(L7/L12)_4L10$ complex was used. The gel filtration through Sephadex G-100 was employed to detect the complex formation (figure 1). In this experiment reconstitution condition (composition of the buffer mentioned under Materials and Methods) was maintained. This was necessary not only for the binding of the proteins to 23S RNA but also the complex formation between 16S and 23S RNAs. As expected, both 16S and 23S RNAs are eluted in the void volume. $(L7/L12)_4L10$, in absence of any RNA, is eluted in later fractions (figure 1). In presence of 23S RNA, however, the protein complex is eluted in the void volume along with 23S RNA. That the binding is specific is shown by the fact that the

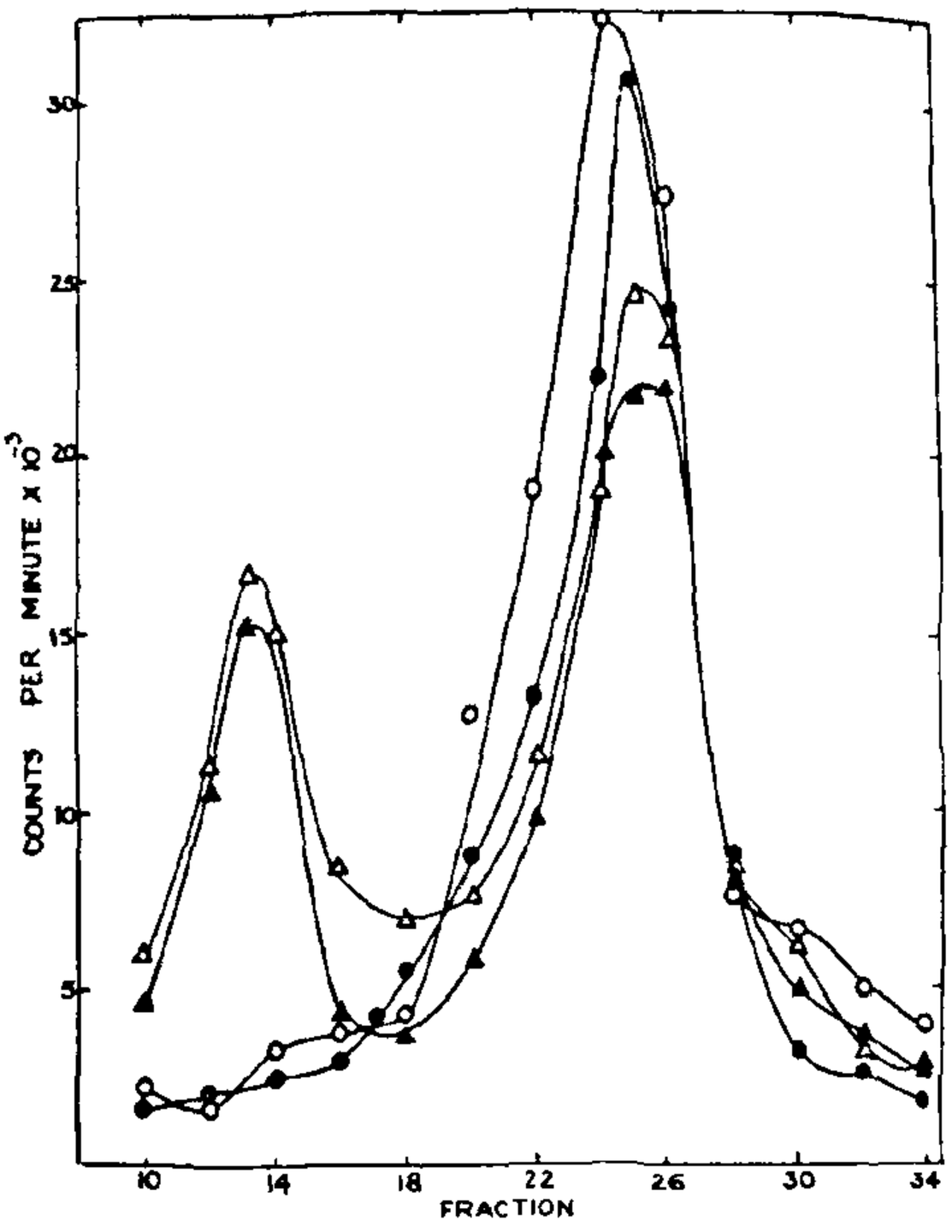

Figure 1. Binding of $(L7/L12)_4L10$ proteins to 23S RNA as studied by gel filtration. Either 23S RNA (60 pmole) or 16S RNA (60 pmole) or a mixture of the same amounts of both and 300 pmole of ^{35}S-labeled $(L7/L12)_4L10$ proteins (115 counts/min/pmole complex) were incubated in 0.2 ml of reconstitution buffer containing 20 mM Tris-HCl, pH 7.5, 400 mM KCl, 20 mM Mg^{++} and 6 mM β-merceptoethanol. The mixture was incubated for 60 min at 40°C to allow the complex (if any) to be formed and then cooled on ice. It was then passed through sephadex G-100 column as described under 'Materials and Methods'. As a control 300 pmole of ^{35}S-labeled $(L7/L12)_4L10$ were also passed through the column. The counting data of the fractions have been presented in the figure.
○ $(L7/L12)_4L10$, △ 23S RNA + $(L7/L12)_4L10$, ● 16S RNA + $(L7/L12)_4L10$, ▲ 16S.23S RNA complex + $(L7/L12)_4L10$

elution of the proteins is not affected by the addition of 16S RNA. However, when both 16S and 23S RNAs are present under this condition (reconstitution condition) when the 16S.23S RNA complex is expected to be formed, the protein complex is also eluted in the void volume.

Binding of 23S RNA to nitrocellulose filter

In this experiment various amounts of $(L7/L12)_4L10$ complex were added to ^{32}P-labeled 23S RNA. It is evident from the results presented in figure 2 that there is no binding of 23S RNA to the nitrocellulose filter in absence of proteins. The binding was small when half the equivalent amount of complex was added but it was about 60 per cent when an equivalent amount of protein complex was present. About 75% of 23S RNA could be bound to the filter in presence of 2.5 times excess of the pentameric complex. Even the addition of 5 times excess did not increase the binding any further.

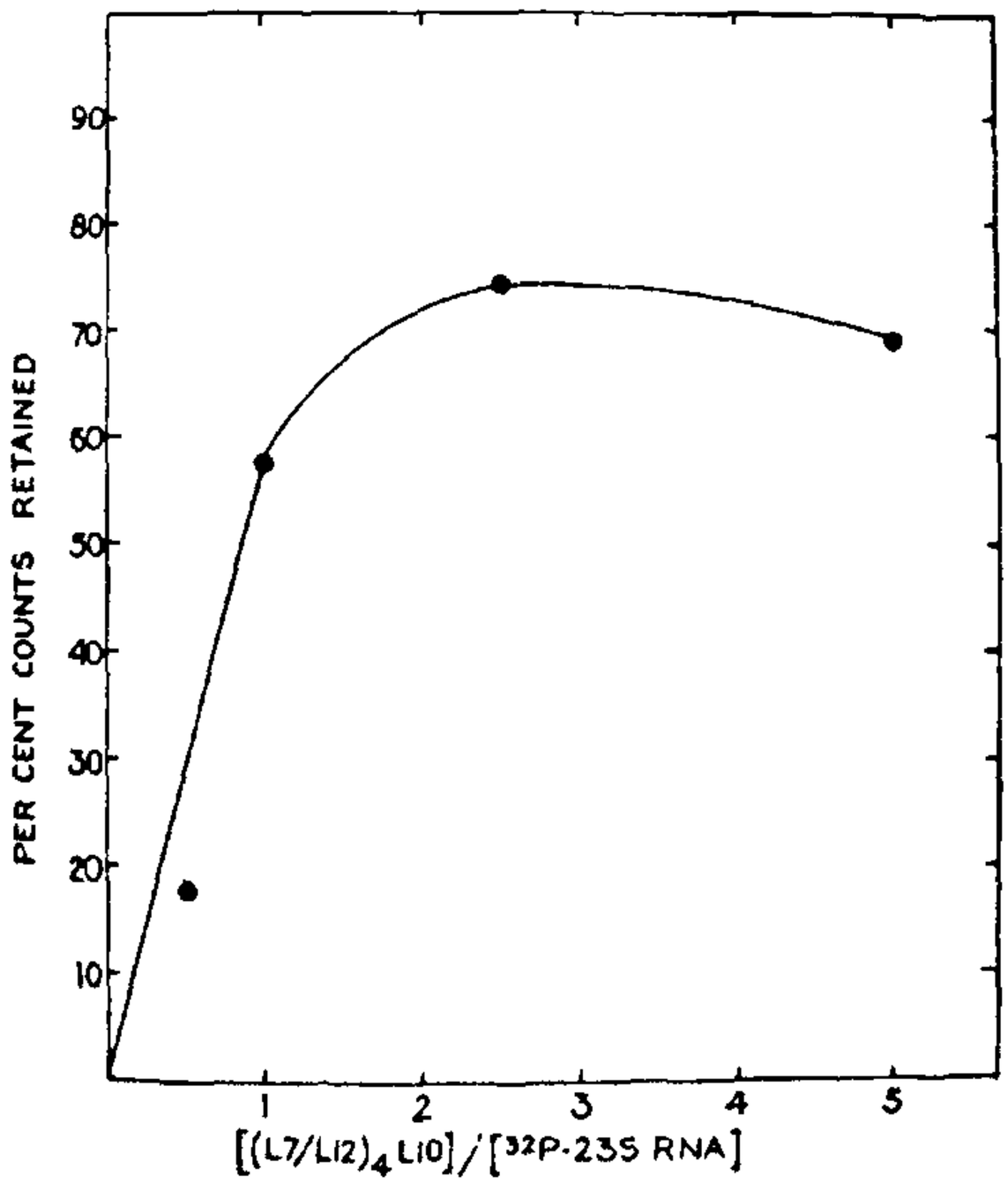

Figure 2. Binding of 23S RNA to nitrocellulose filters with the help of $(L7/L12)_4L10$ proteins. ^{32}P-23S RNA (3.9 pmole having 10^4 counts/min) was incubated at 40°C for 60 min with varying amounts of $(L7/L12)_4L10$ proteins in 1 ml of reconstitution buffer as described under 'Materials and methods'. The mixtures were filtered through the nitrocellulose filters which were dried and counted in liquid scintillation counter, as described under 'Materials and Methods'.

Nitrocellulose membrane filter binding assay

The typical binding assay with ^{32}P-labeled 16S RNA is shown in table 1; 16S RNA alone, as expected, does not practically bind to the filter. This is also true when $(L7/L12)_4L10$ complex is added to 16S RNA. Similarly, when a mixture of 16S and 23S RNAs in the form of complex (under reconstitution condition) was used very little binding was observed. The small amount of binding (about 10%) may not be highly significant. However, when the mixture of the two was incubated with $(L7/L12)_4L10$, about 70% of radioactive 16S RNA is retained on the filter. This is in agreement with the data presented in figure 2. Approximately 75% of 23S RNA was bound under such condition. It is also evident (table 1) that under ordinary condition *i.e.*, in TMA buffer (when no complex formation between 16S and 23S RNAs takes place), there is only a small

Table 1 *Nitrocellulose filter binding assay of 16S.23S RNA complex formation*

Components	Incubation medium	Counts retained on the filter
16S	Reconstitution buffer	693
16S + 23S	" "	995
16S + $(L7/L12)_4L10$	" "	1,088
16S + 23S + $(L7/L12)_4L10$	" "	7,026
16S + 23S + $(L7/L12)_4L10$	Ethanol containing mixture	6,818
16S + 23S + $(L7/L12)_4L10$	Tris-Magnesium acetate-NH_4Cl (TMA)	1,586

The incubation was carried out at 40° C, for 60 min in 1 ml of reconstitution buffer (or ethanol containing mixture or TMA buffer vide 'Materials and Methods') containing 8 pmole of 16S RNA (1.25×10^3 counts/min/pmole), equivalent amount of 23S RNA (where indicated) and 2.5 times molar excess of $(L7/L12)_4L10$ (where indicated). The remaining procedure was the same as described under 'Materials and Methods'.

amount of binding of 16S RNA even when 23S RNA and $(L7/L12)_4L10$ proteins are present. However, when R buffer is substituted with 1 M ethanol containing mixture there is retention of about 70% of the radioactivity. It is known that the complex is formed under ethanol condition as well[2, 6]. These data clearly show that the nitrocellulose filter binding can be employed as an assay method for the complex formation.

Optimum conditions for the formation of the complex under reconstitution as well as ethanol condition

In order to find out the validity of the millipore filter binding assay for the complex formation, the effects of various factors on the complex formation have been studied by this method under reconstitution (figure 3) as well as ethanol condition (figure 4). It is evident from the results presented in figures 3A & B that 20 mM Mg^{++} and 400 mM KCl are optimum for the complex

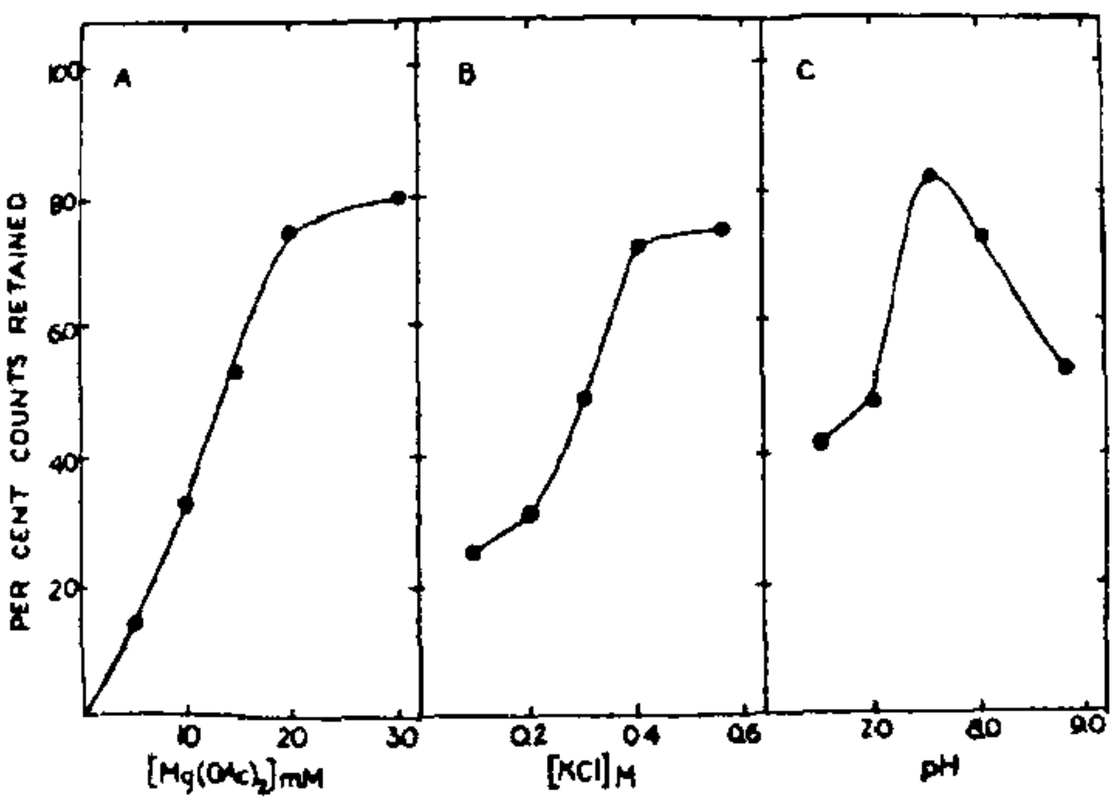

Figure 3. Dependency of the association of 16S and 23S RNAs on Mg^{++}, KCl and pH as measured by nitrocellulose filter binding assay. Equimolar amounts of ^{32}P-16S RNA (10^4 counts/min) and non-radioactive 23S RNA were mixed with 2.5 fold molar excess of $(L7/L12)_4L10$ proteins in 1 ml of reconstitution buffer.

(A) containing varying amounts of magnesium acetate, (B) containing varying amounts of KCl and (C) of various pH's

The mixtures were incubated at 40°C for 60 min, filtered through nitrocellulose filters which were dried and counted in liquid scintillation counter as described under Materials and Methods.

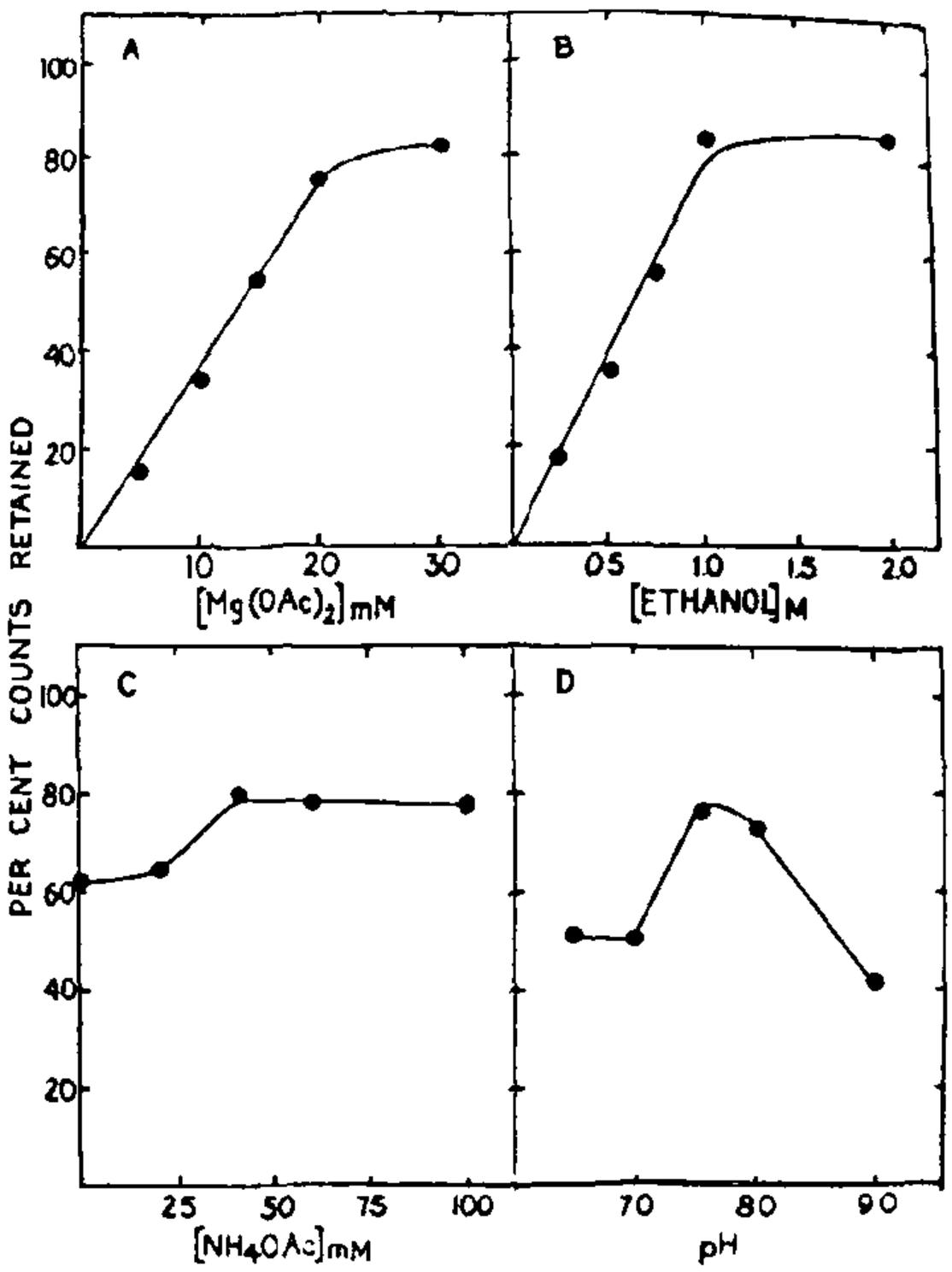

Figure 4. Dependency of the association of 16S and 23S RNAs on Mg^{++}, ethanol, ammonium acetate and pH, as measured by nitrocellulose filter binding assay. The incubation was carried out in ethanol containing mixture. The remaining procedure was the same as described in legend to figure 3. The ethanol mixture contained varying amounts of magnesium acetate (A), varying amounts of ethanol (B), varying amounts of ammonium acetate (C) and was of different pH values adjusted with NH_4OH (D).

formation under reconstitution condition. The optimum pH was found to be 7.5 (figure 3C). These results are in agreement with the data obtained by light scattering and density gradient centrifugation methods[2]. Similarly the presence of 1 M ethanol (figure 4B) was found to be optimum while magnesium acetate and ammonium acetate concentrations required under ethanol condition were 20–30 mM and 40–50 mM respectively (figures 4A and C). These are close to the values reported earlier[2]. The optimum pH (pH 7.5) determined by nitrocellulose filter binding assay (figure 4D) is also in agreement with the reported value.

Stoichiometry of the complex formation

It was demonstrated earlier by both light scattering as well as density gradient centrifugation methods that equivalent amounts of 16S and 23S RNAs are involved in the complex formation[2]. This is found to be true by nitrocellulose filter binding assay as well (figure 5). Under both reconstitution (figure 5A) and ethanol conditions (figure 5B) there is a sharp break at the point of equivalence, in the linear increase of the radioactivity associated with the filter, with the addition of increasing amount of 16S RNA. These results show that the two RNAs form a binary complex, as demonstrated earlier by other methods.

Initiation factor 3-induced dissociation of 16S.23S RNA complex

It is well established that IF3 which was known initially as 'dissociation factor' and later on renamed as 'antiassociation factor' regulates the association of the ribosomal subunits[16-18]. It has been shown in this laboratory by density gradient centrifugation method that IF3 induces the dissociation of 16S.23S RNA complex but the dissociation is partial[5]. Similar situation is observed by nitrocellulose filter binding assay (figure 6). The assay was carried out at 20 mM Mg^{++}. There is an increasing extent of dissociation of the complex with the addition of increasing amounts of IF3 but the dissociation is never complete. The reason for this partial dissociation is not known but the results indicate that the nitrocellulose filter binding assay can conveniently be used for such studies.

Binding of poly (U) to the complex

The binding of poly (U) to the 16S.23S RNA complex can also be shown by nitrocellulose filter binding assay. In order to prevent the binding of poly (U) as such to the filter, the alkali-treated membrane filters were used. It is evident from the data presented in table 2 that [^{3}H]-Poly (U) is not bound to 23S RNA alone. The binding to 16S RNA, if any, is not expected to be detected by this

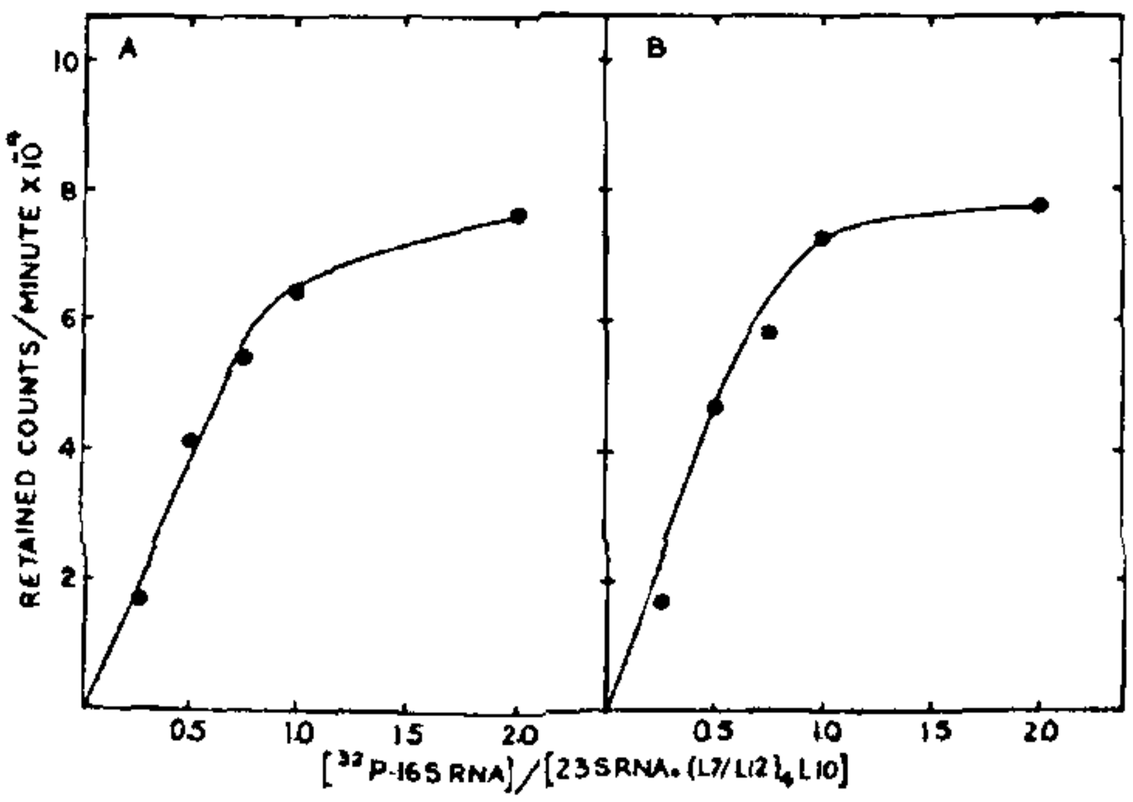

Figure 5. Stoichiometry of the association of 16S and 23S RNAs measured by nitrocellulose filter binding assay. Various amounts of ^{32}P-16S RNA of same specific activity 1.62×10^4 counts/min/pmole were added to a fixed amount (0.6 pmole) of nonradioactive 23S RNA (preincubated with 2.5 molar excess of $(L7/L12)_4L10$ proteins in 1 ml (A) reconstitution buffer or (B) 1 M ethanol containing mixture, as described under Materials and Methods. The remaining procedure was the same as described under 'Materials and Methods'. The values were corrected for the blank ones (with ^{32}P-16S RNA alone).

Table 2 *Binding of poly(U) to 16S.23S RNA complex (nitrocellulose filter binding assay)*

RNA	$(L7/L12)_4L10$	Incubation condition	[^{3}H]-Poly (U) bound (counts/min)
—	+	reconstitution	422
16S	+	"	515
23S	+	"	747
16S.23S	−	"	835
16S.23S	+	"	3,158
16S.23S	−	ethanol	1,199
16S.23S	+	"	3,567
16S.23S	−	TMA	355
16S.23S	+	"	687

16S RNA, 23S RNA or 16S.23S RNA complex (0.41 nmole) and [^{3}H]-poly(U) (0.4 nmole, 50 counts/min/pmole) were incubated along with 1 nmole of $(L7/L12)_4L10$ at 37°C for 60 min in 1 ml of the reconstitution buffer (20 mM Tris-HCl, pH 7.6, 400 mM KCl, 20 mM $MgAc_2$ and 6 mM β-mercaptoethanol) or TMA (10 mM Tris-HCl, pH 7.6, 30 mM NH_4Cl, 10 mM $MgAc_2$ and 6 mM β-mercaptoethanol) or Vasiliev's mixture (1 M ethanol, 60 mM ammonium acetate and 20 mM $MgAc_2$). After incubation the solutions were chilled in ice for 5 min, diluted with 5 ml of binding solutions and passed through alkali-treated nitrocellulose filters. The filters were washed three times with 5 ml portions of the same solution and dried. Radioactivity was measured in Rack Beta Liquid scintillation Counter of LKB

method. However, there is a very small but significant amount of binding of poly (U) to the 16S.23S RNA complex under reconstitution condition. The amount of poly (U) bound depends on the amount of poly (U) added and also that of the complex (results not presented). Similar binding was observed in the presence of ethanol containing mixture under which the complex formation takes place[2]. However, in TMA under which no complex formation takes place, there is very little binding of poly(U). Due to the heterogeneous nature of the commercial preparation of poly (U) used in these experiments it was not possible to calculate the number of moles bound to the complex. However, assuming that poly (U) has chain length of 25–50 bases, 0.16 mole of poly (U) was bound per mole of 16S.23S RNA complex.

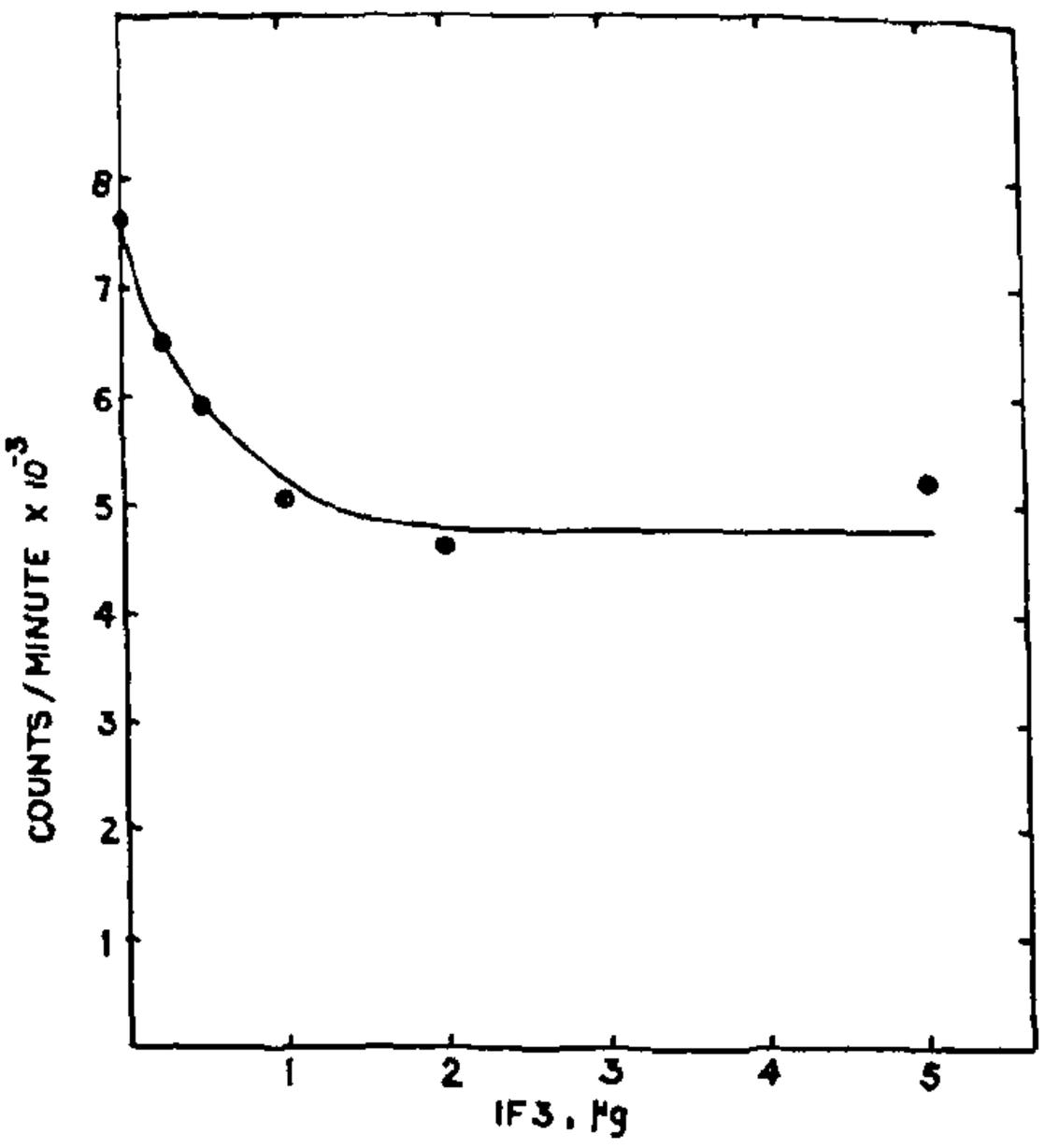

Figure 6. Effect of IF3 on the dissociation of 16S.23S RNA complex as measured by nitrocellulose filter binding assay. 23S RNA (12 pmole) was mixed with $(L7/L12)_4L10$ proteins (30 pmole) in reconstitution buffer containing 20 mM magnesium acetate. ^{32}P-16S RNA (12 pmole; 10^4 counts/min) was preincubated with varying amounts of IF3 (as indicated in the figure) and then added to 23S RNA complexed with $(L7/L12)_4L10$ and the mixture was incubated at 40°C for 60 min. It was then filtered through nitrocellulose filter which was dried and counted as described under Materials and Methods.

DISCUSSION

The results presented above clearly indicate that the nitrocellulose membrane filter binding assay could be used as efficiently as light scattering, density gradient centrifugation and polyacrylamide gel electrophoresis for carrying out various types of experiments with the bimolecular complex of 16S and 23S RNAs. The results obtained by the various methods are in complete agreement with each other. Of all the methods, the filter binding assay is the simplest and the quickest. Therefore it is being expected that this method will be more routinely used than the others. It should be pointed out in this connection that approximately 75% of radioactive 23S RNA, complexed with $(L7/L12)_4L10$ is retained on the filter (results not presented). Therefore in all experiments the binding of 16S RNA to the filter through 23S RNA is also to the same extent. Further, quite a large amount of the complex may be bound to the filter (for example, results shown in figure 6).

In some cases nitrocellulose filter binding assay method is the only convenient method available. We have demonstrated the binding of radioactive poly(U) to the complex by this method using alkali-treated membrane filters (table 2). Similarly, we have shown the binding of ^{14}C-phenylalanyl tRNA as well as N-acetyl ^{14}C-phenylalanyl tRNA to 16S.23S RNA complex by the same technique by binding the complex to the filter through $(L7/L12)_4L10$ proteins[7].

It is important to note that 16S RNA can not be bound to the filter with the help of its binding proteins[13]. Otherwise, the scope of this assay would have increased still further. In all our studies on both structural and functional aspects of the bimolecular complex we use the introcellulose filter binding assay for preliminary investigations and then switch over to other sophisticated methods. The preliminary data obtained by the binding assay are thus of great help in future investigations.

ACKNOWLEDGEMENTS

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NEWS

PHOTOGRAPHING HALLEY'S COMET

The staff of the new observatory in the Tiens Shan mountains, Kazakhstan, have photographed Halley's comet.

The scientific world lives in anticipation of the encounter of the Soviet space stations, Vega-1 and Vega-2, with the comet, expected in the spring of the next year. Kazakhstan astronomers working within the frame of the international project are in charge of coordination of the studies of processes occurring in the zone of the nucleus of Halley's comet. Its "head" is one of the most interesting and difficult objects of observation. It is in that zone that the most explosive, dynamic changes occur when the comet approaches the Sun.

While the comet is far away from the Sun, nothing in particular happens in it under the star's influence. That is why it is essential to obtain some early photographs of the nucleus, so that in future scientists could register and analyse the series of changes occurring in the nucleus zone.

The photographs are also of great practical value, as they will enable scientists to correct the progress of the interplanetary stations on their way to the space "rendezvous".

Kazakhstan scientists are preparing for extensive studies aimed at determining the temperature and density of the comet's substance and its chemical composition.

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