J. Indian Chem. Soc.,
Vol. LX, December 1983, pp. 1152-1155

Light Scattering as a Monitoring Device in the Studies Involving Macromolecules*

B. NAG and D. P. BURMA

Molecular Biology Unit, Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221 005

Light scattering has been used as a monitoring device in the study of the macromolecules like proteins, nucleic acids and nucleoprotein particles like ribosomes and viruses, using a spectrofluorometer for the purpose. The molecular weight of a globular protein can be determined by measuring the scattering by a 10-20 micromolar solution and comparing that with the standard proteins of known molecular weights. In case of polyribonucleotides the scattering is dependent not only on the chain length but also the higher order structures. Scattering by ribosomes or viruses is proportional to their amounts within certain range. Light scattering measurements also reflect the differences in the structural features of viruses. The decrease in light scattering due to the degradation of the substrate has been utilized for following the action of enzymes like RNase I on polyribonucleotides.

LIGHT scattering has been extensively used for various biological investigations[1,2]. The basic principles have been elucidated by Steiner and Beers[3]. This technique has been specially useful for the study of the macromolecules[4,5]; for example, it has been successfully used in the study of dissociation and association of ribosomal subunits[6-9]. Specially designed apparatus (for example, described by Gorisch *et al*[9]) has been built for such measurements and in recent years laser beam is being used for these studies. Simple spectrofluorometers have also been used for studying static equilibrium at 90° scattering. In this laboratory light scattering is being routinely used for monitoring various processes involving macromolecules. With the help of this technique it has recently been possible to understand the mechanism of association and dissociation of ribosomal subunits[10-14], a phenomenon which is known for 25 years but the mechanism of which was not known. Some of our studies with proteins, RNAs and nucleoproteins like viruses as well as the use of this technique in enzyme assays will be described here.

Experimental

Materials and methods :

Bovine serum albumin, ovalbumin, trypsin, lysozyme, ribonuclease I and 5'-AMP were obtained from Sigma Chemical Co., U.S.A. Deoxyribonuclease I was procured from Worthington Biochemicals, U.S.A. Various synthetic polynucleotides as poly I, poly C, poly A, poly U and poly I:C were procured from the Miles Laboratory, U.S.A.

Bacterial viruses P22 and MB78 purified by isopycnic centrifugation were obtained as gifts from M. Chakravorty of this Department MB78 is a new virus isolated in her laboratory[15].

Preparation of RNase I : The enzyme was purified from the extract of *Salmonella typhimurium* according to the procedure described by Datta and Burma[16].

Preparation of 70S ribosome and its subunits : *Escherichia coli* 70S ribosome and its subunits were prepared from the alumina extract of *E. coli* according to the procedure of Datta and Burma[16].

Preparation of 23S and 16S ribosomal RNAs and transfer RNAs : 23S and 16S RNAs were isolated from 50S and 30S ribosomal subunits by treatment with phenol in presence of sodium dodecyl sulphate and cold ethanol precipitation at −20° according to Amils *et al*[17]. 5S RNA was prepared from intact 70S ribosome and purified by passing through Sephadex G-100 column. Transfer RNA was prepared from 100,000 g supernatant of the extract of *E. coli* according to the method of Zubay[18].

Light scattering measurements :

Light scattering measurements were carried out in an Aminco-Bowman spectrofluorometer. The excitation and emission monochromators were set to 400 nm. A sample volume of 1 ml was used in a 1 ml cuvette and readings were taken at an angle of 90°.

Scattering by proteins, rRNAs, tRNAs and synthetic polynucleotides : The scattering at 400 nm by proteins, different ribosomal RNAs, tRNAs and synthetic polynucleotides of various molecular sizes was measured in 1 ml containing 20 m*M* Tris-HCl, *p*H 7.6 and indicated amounts of polynucleotides as shown in Figs. 1 and 2. The scattering by bacteriophages as well as by ribosomes and their subunits was measured in 1 ml containing 20 m*M* Tris-HCl, *p*H 7.6, 30 m*M* NH_4Cl and 0.1 m*M* $MgAc_2$.

*Dedicated to the memory of Prof. J. N. Mukherjee, my (D.P.B) first teacher in Physical Chemistry, although for a brief period, at the University College of Science, Calcutta.

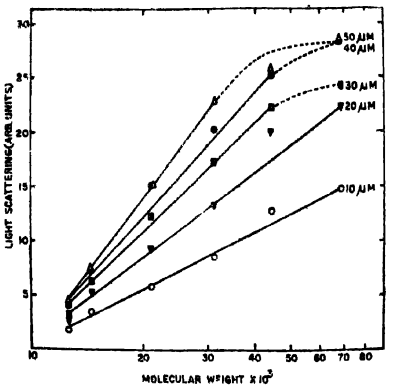

Fig. 1. Light scattering by globular proteins of various sizes. The scattering by the following proteins at 400 nm was measured as described under materials and methods:
RNase I (M.W. 12,600), Lysozyme (M.W. 14,500), Trypsin (M.W. 21,000), DNase I (M.W. 31,000), Ovalbumin (M.W. 45,000), Bovine Serum albumin (M.W. 68,000). The concentrations of the proteins were as follows:
○ $1 \times 10^{-5} M$
● $2 \times 10^{-5} M$
▽ $3 \times 10^{-5} M$
□ $4 \times 10^{-5} M$
▲ $5 \times 10^{-5} M$

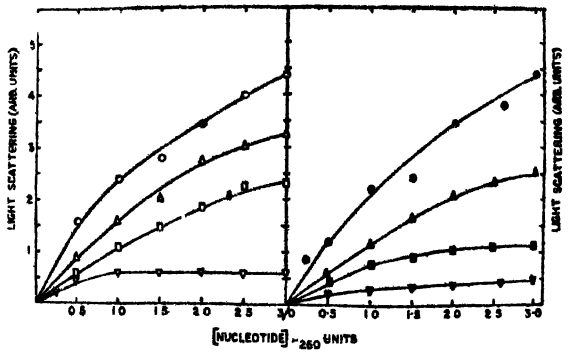

Fig. 2. Light scattering by different polynucleotides. Scattering was measured as described under materials and methods:
A. ○ 23S RNA, △ 16S RNA, □ 5S RNA, ▽ 4S RNA
B. ● poly I:C, ▲ poly I, ■ poly A, ▼ 5'-AMP

Measurement of ribonuclease I activity by light scattering: The nucleolytic action of ribonuclease I on different ribosomal RNAs, tRNAs and various polyribonucleotides was measured in 1 ml containing 100 mM Tris-HCl, pH 7.0, 0.1 mM $MgAc_2$, 3 A_{260} units of RNA (or synthetic polynucleotide) and 6 units of RNase I. The decrease in scattering at 400 nm was followed.

Results and Discussion

Light scattering by proteins :

For standardisation of the procedure, a number of proteins of various molecular weights (12,600-68,000) were chosen and used at different concentrations. Scattering measurement was done at 400 nm and scattered light (arbitrary units) was plotted against logarithm of molecular weight (Fig. 1). It is clear that at low concentrations (10 μM and 20 μM) the light scattering linearly increases with the logarithm of the molecular weight. At higher concentrations (30, 40 and 50 μM) there is deviation from linearity at higher molecular weights.

Light scattering by ribonucleic acids :

In this case both synthetic polynucleotides like poly A, poly I and poly I:C as well as naturally occurring RNAs like 23S, 16S, 5S and 4S RNAs were used (Fig. 2). These polynucleotides are of widely varying molecular weight and various sizes. Since there was no correlation between scattering and molecular weight, light scattering (arbitrary units) was plotted against the amount of polyribonucleotides (A_{260} units). Due to widely varying molecular weights and heterogeneity in some cases the comparison was possible by this way as the same amount of nucleotide (A_{260} units) was present for each set of measurements. Under this condition, 23S RNA (2904 nucleotides) scattered maximally and in the case of tRNAs which were of the smallest size (~80 nucleotides) the scattering was minimum. Scattering by 16S RNA (1541 nucleotides) was less than that by 23S RNA. The scattering by 5S RNA (120 nucleotides) was still lower no doubt but much more than 4S RNAs.

Among the synthetic polynucleotides, poly I:C scattered maximally, poly I was next and poly A scattered still less. 5'-AMP which was used as control scattered to small extent which may be considered as blank value.

Scattering by ribosomes and bacterial viruses :

The ribosomes of *Escherichia coli* and viruses of *Salmonella typhimurium* which are of very large size and both nucleoproteins were also used for scattering studies. Although the scattering plotted against the logarithm of concentration was not perfectly linear yet it increased with the increasing amount of the ribosome or virus added (Fig. 3). It

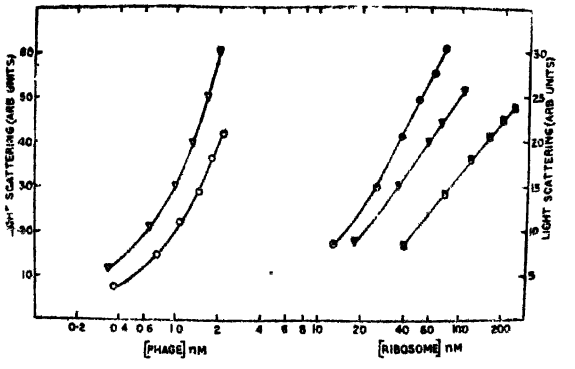

Fig. 3. Light scattering by bacterial viruses and ribosomes. The scattering was measured as described for the Fig. 1 with the only difference that 30 mM NH_4Cl and 0.1 mM $MgAc_2$ were also present in these cases.
▽ MB78 phage, ○ P22 phage
● 70S ribosome, ▼ 50S ribosome, ■ 30S ribosome

is interesting to note that MB78 scatters light somewhat more than P22 although they are of same size (molecular weight $\sim 27 \times 10^6$). It may be due to the fact that MB78 has a tail which is missing in P22 (reference 15). The scattering by 70S, 50S and 30S ribosomes (molecular weights, 2.7×10^6, 1.85×10^6 and 0.85×10^6, respectively) is proportional to their sizes and practically linear with the amount of ribosome present in the solution. Due to their smaller size than viruses, 60 to 200 fold excess of the ribosomes produce the same amount of scattering as the viruses.

Light scattering as a tool for measurement of enzyme activity :

The scattering by the macromolecules has been taken advantage of in following enzyme activity. Fig. 4 shows the decrease in light scattering of the polyribonucleotides due to the action of RNase I. The details have been described in materials and methods. The 23S and 5S RNAs are found to be degraded at very fast rates. The rate of degradation of 16S RNA is comparatively slow whereas there is hardly any degradation of 4S RNAs. Similarly, poly A is found to be a good substrate whereas poly I is found to be a poor one. Poly I:C, a double stranded polynucleotide, remains practically uneffected by RNase I. The results are in agreement with those obtained by direct spectrophotometric measurements[19,20].

As mentioned in the introduction, light scattering is extensively used in various investigations specially in the study of the macromolecules. Sophisticated instruments are available for measurement of light scattering ; laser beam is also used for such purposes. One of the advantages of this technique is that it does not produce much of perturbance in the structure of the macromolecules that are being investigated.

It is clear from the results presented here that light scattering can be used for several purposes, for example, the determination of the molecular weight of a globular protein. It can be used like the gel filtration technique by making a standard curve with a few proteins of known molecular weight (Fig. 1). A concentration of 10-20 μM is most suitable for such measurement. Even the concentrations of organelles like ribosomes and complex living systems like viruses can be determined by such measurement (Fig. 3). The scattering is naturally dependent on the size, for example, the extent of scattering by 70S, 50S and 30S ribosomes is in the order of their molecular weights. Viruses scatter light to the same extent at 1/100th concentration of the ribosome. One of the interesting observations in this connection is the difference in scattering by P22 and MB78 (Fig. 3) which are practically of the same size. As already mentioned, it is quite likely that MB78 scatters more light than P22 as it has a long tail[15]. As expected, there are anomalies in case of polyribonucleotides which have extended structures unlike globular proteins. Poly I:C which is of small molecular weight in comparison to 23S RNA scatters to the same extent. For comparative purposes equivalent amounts of nucleotide units were used irrespective of their molecular weights. Another interesting observation made in this connection is that although 5S and 4S RNAs are of comparable size, 5S RNA scatters much more than 4S RNAs. This may be due to the differences in the three-dimensional structural organisation. Unlike the structure of tRNAs that of 5S RNA may not be as tightly folded, as predicted from the enzymatic studies[19,20].

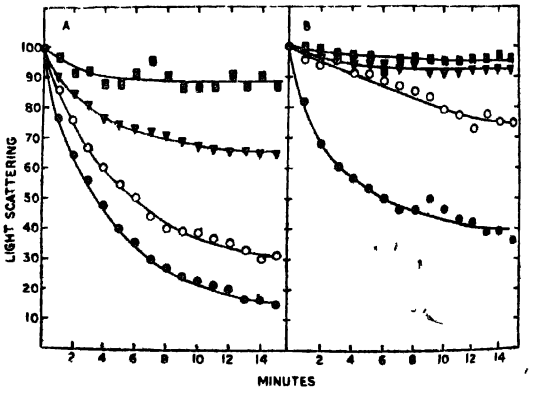

Fig. 4. Measurement of ribonuclease I activity by light scattering. The activity of ribonuclease I on different ribosomal RNAs, tRNAs and various polynucleotides was followed by measuring the decrease in scattering at 400 nm. Details described under materials and methods.

A. ■ 4S RNA ▼ 16S RNA ○ 5S RNA ● 23S RNA
B. ■ poly U ▲ poly I:C ○ poly I ● poly A

Finally, the use of the technique in enzyme assays has been demonstrated (Fig. 4). The decrease in light scattering properly reflects the action of RNase I on its substrates. Two RNAs, 23S and 5S are degraded at fast rates[19,20] and this is reflected in the rate of decrease of light scattering. The decrease is slow in the case of 16S RNA and negligible in the case of tRNA, truly reflecting the rates determined by hyperchromicity. This is also true for the synthetic polynucleotides like poly A, poly I, poly I:C except in the case of poly U. The rate of degradation of poly U can not be conveniently measured by hyperchromicity due to its unordered structure but it is a good substrate for RNase I as determined by trichloroacetic acid precipitation method. May be, due to the unordered structure the difference in scattering between poly U and oligo U (or UMP) is negligible. The same method has also been successfully used for the assay of proteolytic enzymes (results not presented).

Acknowledgements

The authors sincerely thank Dr. S. K. Sengupta of the Department of Chemistry of this University for critical discussion and helpful suggestions, the Indian Council of Medical Research for the award of a Junior Research Fellowship to one of them (B.N.) and the Department of Science and Technology, Government of India for financial support.

References

1. P. DOTY and J. T. EDSALL, *Advances in Protein Chem.*, 1951, 6, 35.
2. E. P. GEIDUSCHEK and A. HOLZER, *Advances in Biol. and Med. Physics*, 1958, 6, 431.
3. R. F. STEINER and R. F. BEERS, JR. in "Polynucleotides", Elsevier Publishing Company, Amsterdam, 1961, p. 174.
4. R. L. SINSHEIMER, *J. Mol. Biol.*, 1959, 1, 43.
5. C. TANFORD, "Physical Chemistry of Macromolecules", John Wiley & Sons, Inc., New York, 1961, p. 275.
6. R. S. ZITOMER and J. G. FLAKS, *J. Mol. Biol.*, 1972, 71, 263.
7. A. WISHNIA, M. A. BOUSSERT, M. GRAFFE, P. H. DESSEN and M. GRUNBERG-MANAGO, *J. Mol. Biol.*, 1975, 93, 499.
8. V. FAVANDON and F. POCHON, *Biochemistry*, 1976, 15, 3903.
9. H. GORISCH, D. J. GOSS and L. J. PARKHURST, *Biochemistry*, 1976, 15, 5743.
10. B. NAG and D. P. BURMA, *Current Sci. (India)*, 1982, 51, 1158.
11. D. P. BURMA, B. NAG and D. S. TEWARI, *Proc. Natl. Acad. Sci., U.S.A.*, 1983, 80, 4875.
12. D. P. BURMA, Proceedings of the Symposium held on the occasion of completion of 60 years of Saha Institute of Nuclear Physics, Calcutta, 1983 (in press).
13. B. NAG and D. P. BURMA, Proc. Indo-Soviet Binational Symposium held at Madurai, 1983, (in press).
14. D. S. TEWARI and D. P. BURMA, *Biochem. Biophys. Res. Comm.*, 1983, 114, 348.
15. A. JOSHI, J. Z. SIDDIQI, G. R. K. RAO and M. CHAKRAVORTY, *J. Virol.*, 1982, 41, 1038.
16. A. K. DATTA and D. P. BURMA, *J. Biol. Chem.*, 1972, 247, 6795.
17. R. AMILS, E. A. MATHEWS and C. R. CANTOR, *Nucleic Acids Res.*, 1978, 5, 2455.
18. G. ZUBAY in "Procedures in Nucleic Acid Research", (Eds.) G. L. CANTONI and D. R. DAVIES, Harper & Row, New York, 1967, p. 455.
19. D. P. BURMA, *Current Sci. (India)*, 1982, 51, 723.
20. D. S. TEWARI and D. P. BURMA, *Biochem. Biophys. Res. Comm.*, 1982, 109, 256.