Onderstepoort J. vet. Res. 39(3), 125–132 (1972)

INTERFERON INDUCTION BY BLUETONGUE VIRUS AND BLUETONGUE VIRUS RIBONUCLEIC ACID

P. A. L. EKSTEEN and H. HUISMANS, Veterinary Research Institute, Onderstepoort

ABSTRACT

EKSTEEN, P. A. L. & HUISMANS, H. Interferon induction by bluetongue virus and bluetongue virus ribonucleic acid. *Onderstepoort J. vet. Res.* 39(3), 125–132 (1972).

The stimulation of interferon synthesis by bluetongue virus and by bluetongue virus ribonucleic acid was investigated in order to determine if there is a difference in the mechanism of induction. The molecular mass of the interferon formed after the two induction processes was determined using Sephadex gel filtration. A value of 24 000 was found in both cases. These results suggest that the two induction processes are basically similar and that double-stranded ribonucleic acid is the active inducing principle in both stimulating processes.

INTRODUCTION

Bluetongue virus (BTV) and isolated BTV double-stranded ribonucleic acid (RNA) are both active inducers of interferon synthesis in mice (Huismans, 1969). The mechanism of interferon stimulation has not yet been clarified. Several reports, however, indicate that at least two distinct stimulating processes can be identified, the first being induction of interferon synthesis by virus infection and the second induction by bacterial endotoxin (Youngner, Stinebring & Taube, 1965). The endotoxin type of stimulation does not, in contrast to virus stimulation, require RNA or protein synthesis (Finter, 1966). It has therefore been suggested that endotoxin stimulates the release of preformed or precursor interferon (Youngner & Hallum, 1968).

A very important discovery, with regard to the interferon induction process, was the finding that interferon synthesis is specifically stimulated by natural or synthetic double-stranded RNA (Field, Tytell, Lampson & Hilleman, 1967; Tytell, Lampson, Field & Hilleman, 1967). This work immediately posed the question as to whether this type of stimulation is of the endotoxin type or the virus type. Youngner (1970) has provided evidence that stimulation of interferon synthesis by a synthetic double-stranded polynucleotide is not inhibited by cycloheximide. Therefore new protein synthesis does not seem to be required and he suggested that stimulation of interferon synthesis by double-stranded RNA is of the endotoxin type. This result is disputed by Ho & Ke (1970), who have shown that in their *in vitro* system the stimulation of interferon synthesis by poly I:C does require protein synthesis. They conclude that the idea of stimulation of "preformed interferon" by double-stranded RNA is probably invalid. De Clercq & Merigan (1970) have furthermore shown that all inducers of interferon require new protein synthesis for both the initiation and the termination of interferon synthesis. They suggest that there is no fundamental difference in the mechanism of interferon production between different inducers.

A marked disparity of the kinetics of interferon synthesis was, however, observed after induction by BTV and BTV double-stranded RNA respectively (Huismans, 1969). This raised some doubt as to whether the two induction mechanisms are identical. The only way in which the "endotoxin stimulated" type can be distinguished from virus-induced interferon is by the difference in their molecular masses (Youngner & Stinebring, 1966), that of interferon stimulated by endotoxin being about 90 000 (Hallum, Youngner & Stinebring, 1965) whereas the molecular mass of virus-induced interferon is in the order of 26 000 to 38 000 dalton (Merigan, 1964; Carter, 1970). No molecular mass determination of the interferon induced by either BTV or BTV double-stranded RNA has previously been carried out. In this paper such a determination is reported and it was found that the molecular mass of interferon induced by BTV double-stranded RNA is indistinguishable from that of the virus-induced interferon.

MATERIALS AND METHODS

Virus

BTV serotype 10 was used and methods for the production, titration and purification of this virus have been described by Verwoerd (1969).

Assay of interferon activity

The plaque reduction method used for titration of interferon activity has been described by Huismans (1969). Mouse fibroblast L-cells were incubated overnight with different dilutions of interferon and subsequently challenged with ecbovirus SA-1 as an indicator virus. The reciprocal of the dilution at which the plaque count was 50% of that of the controls was taken as the interferon titre and was expressed in interferon units (IU).

Interferon production in mice

Mice were injected intravenously with either purified BTV or BTV double-stranded RNA. Blood was collected by cardiac puncture at various intervals after injection as indicated under Results. Blood samples were prepared for titration and molecular mass determination as follows: Blood plasma was obtained after centrifugation for 10 min at 1 500 *g*. The plasma was acidified to pH 2,5 with HCl and then centrifuged for 2 h at 100 000 *g* at 4°C. The precipitate of denatured protein was discarded. The supernatant was then diluted with PBS to obtain a constant pH of 6,8.

Isolation of double-stranded RNA

Double-stranded RNA was isolated from BTV infected L-cells as described previously (Verwoerd, Louw & Oellermann, 1970). A cytoplasmic extract was prepared from infected cells and deproteinized by phenol extraction, followed by ether extraction to remove phenol residues. The RNA was precipitated by 2 volumes of ethanol. Single-stranded ribosomal RNA and messenger RNA were then removed by a salt precipitation with 1,0 M NaCl. The supernatant contains mainly transfer RNA and double-stranded RNA which were separated on a column of methylated-albumin-

Received 16 August 1972 - Editor

kieselguhr. The purified double-stranded RNA was stored in a PBS solution at —20°C.

Polyacrylamide gel electrophoresis

Conditions for the electrophoresis of BTV double-stranded RNA have been described (Verwoerd *et al.* 1970). This procedure was modified in that sodium dodecyl sulphate (SDS) was omitted from both the gels and the buffer solution. Electrophoretic separation took place for 4½ h at 100 V and 8 mA/gel column. After electrophoresis the gels were fixed with 1 M acetic acid for 15 min and then stained with 0,08% methylene blue in a 0,2 M sodium acetate buffer, pH 4,7. After 1 hour the gels were destained by repeated changes of distilled water. The background cleared within 24 hour. The destained gel column was scanned by a Vitatron flying spot densitometer.

Sephadex gel filtration

A column of Sephadex G-100 (Pharmacia), 2,5 × 45 cm in size, was exhaustively washed with an 0,02 M sodium phosphate buffer, pH 7,4 containing 0,1 NaCl. The flow rate of the column was 6 ml/hour. The void volume of the column was determined with dextran blue 2 000. The column was calibrated with known molecular mass markers. Serum samples with known interferon concentration were applied to the calibrated column in a volume not larger than 2 ml. Fractions were collected by an Ultrorac fraction collector. Optical density measurements were made and recorded by a Uvicord.

Results

It was important in this investigation to establish that the material that was to be used for stimulation of interferon synthesis was of a highly purified and well defined nature.

The method used for purification of BTV has been described previously (Verwoerd, 1969). The purity of the BTV preparation was confirmed by the following experiments. The virus sedimented as a single sharp band in sucrose density gradients. Electronmicroscopy of the purified virus indicated the presence of a homogeneous population of virus particles, free from cellular or non-virus material. Furthermore, gel electrophoresis of the solubilized polypeptides in the virus solution, as described by Verwoerd, Els, de Villiers & Huismans (1972), indicated the presence of virus specific polypeptides only.

The double-stranded RNA was isolated as described under Methods. Criteria for the purity of the double-stranded RNA isolated in this way were as follows. The thermal denaturation curve of the double-stranded RNA was identical to the single-step curve obtained with RNA isolated from highly purified BTV (Verwoerd *et al.* 1970). Gel electrophoretic fractionation of the RNA on polyacrylamide gels indicated the presence of only ten RNA components. A scan of the fractionation pattern is shown in Fig. 1. The ten virus components are numbered from 1 to 10 in order of decreasing size. The homogeneity of these ten components was confirmed by calculating their molar ratio, as described by Fujii-Kawata & Miuru (1970).

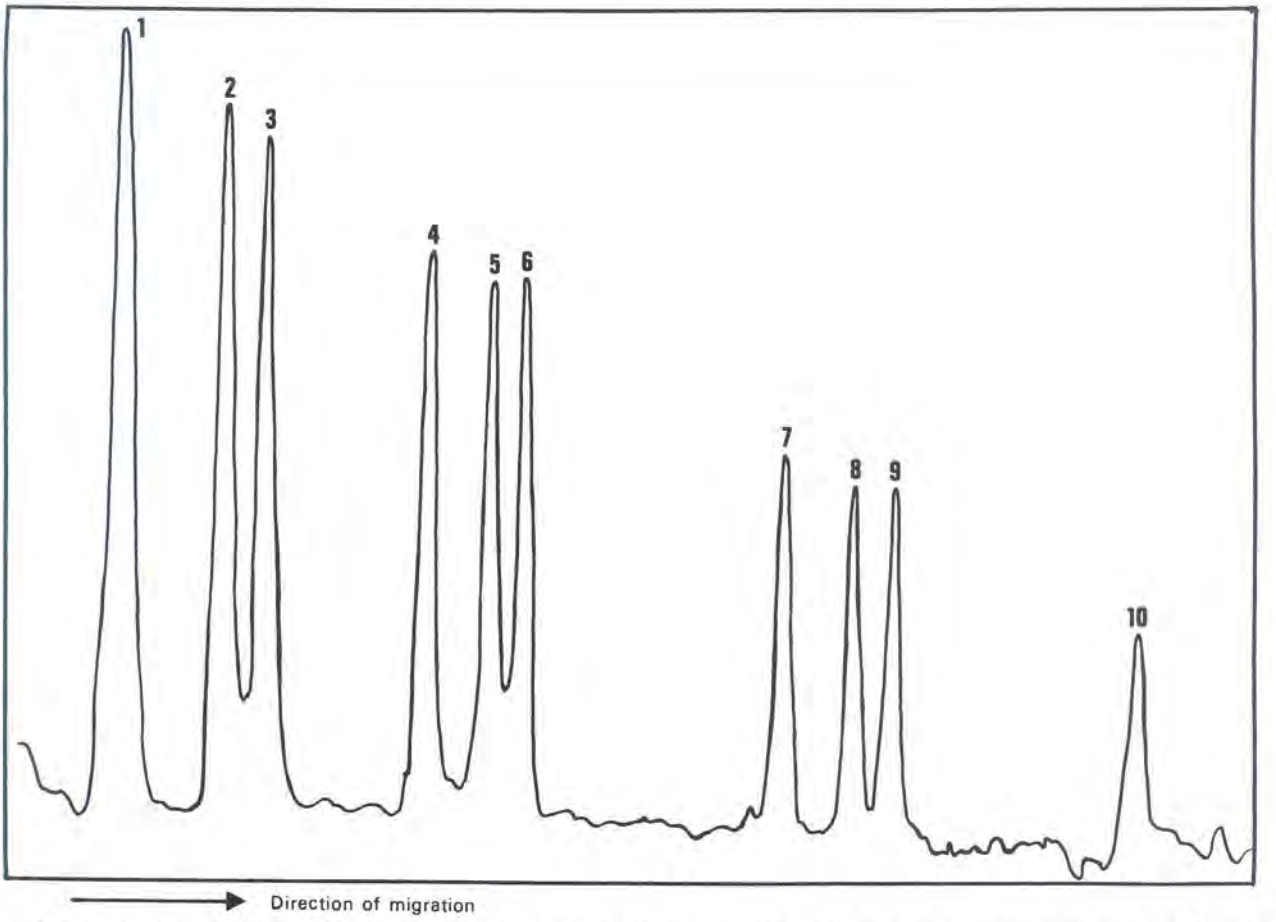

FIG. 1 Scan of polyacrylamide gel after separation of bluetongue double-stranded RNA. The ten virus components are numbered in order of decreasing size

TABLE 1 Normalized ratios of respective peak areas in a scan such as shown in Fig. 1 to the molecular mass of the relevant RNA component

Genome segment	Normalized ratio peak area/molecular mass
1.	1,03
2.	1,00
3.	0,98
4.	0,96
5.	0,91
6.	1,00
7.	0,94
8.	0,99
9.	1,06
10.	1,10

The result is shown in Table 1. The ratio of the respective peak areas, shown in Fig. 1, to the molecular mass of the relevant RNA component is approximately constant for all ten RNA species. This result confirms, by a different method, the earlier results of Verwoerd *et al.* (1970) that the double-stranded RNA components are present in equimolar amounts. The molecular mass of the RNA components used for the calculation were as reported by Verwoerd *et al.* (1972).

Stimulation of interferon synthesis

(a) *Kinetics of interferon synthesis*

If the mechanism of interferon stimulation by double-stranded RNA is different from that of virus it is logical to assume that such a difference could be reflected by a difference in the kinetics of interferon synthesis. Earlier results (Huismans, 1969) have indicated such a difference and these results were confirmed by the following experiment. A number of mice were injected intravenously either with a dose of 3 optical density units of purified BTV each or with 75 μg of BTV double-stranded RNA. Mice from both groups were bled in groups of six at various intervals between 2 and 24 hours after injection and the serum assayed for interferon activity. The result is shown in Fig. 2. The kinetics of interferon stimulation by the two methods are obviously different. The double-stranded RNA induces maximum titres of interferon approximately 4 to 5 hours earlier than BTV itself.

(b) *Optimum dose for stimulation*

In order to obtain interferon of a sufficiently high specific activity for the molecular mass determination it was necessary to determine the optimal conditions of interferon stimulation in both cases. For this reason the optimum dose of BTV and BTV double-stranded RNA used for induction was determined. Mice were injected with varying amounts of the two inducers. In the case of stimulation with double-stranded RNA the mice were bled 3 hours after injection and in the case of induction with BTV itself at 8 hours. The interferon activities were determined and the results are shown in Fig. 3.

An increase in the amount of material used for induction in both cases results in a corresponding increase in interferon titre. However, an optimum dose is reached, after which further increase has very little effect. In the case of stimulation with double-stranded RNA the optimum dose is approximately 120 μg RNA per mouse whereas in the case of stimulation with BTV the optimum is in the order of 3 optical density units of virus per mouse. Injection of mice with a larger amount of virus markedly increases their mortality rate.

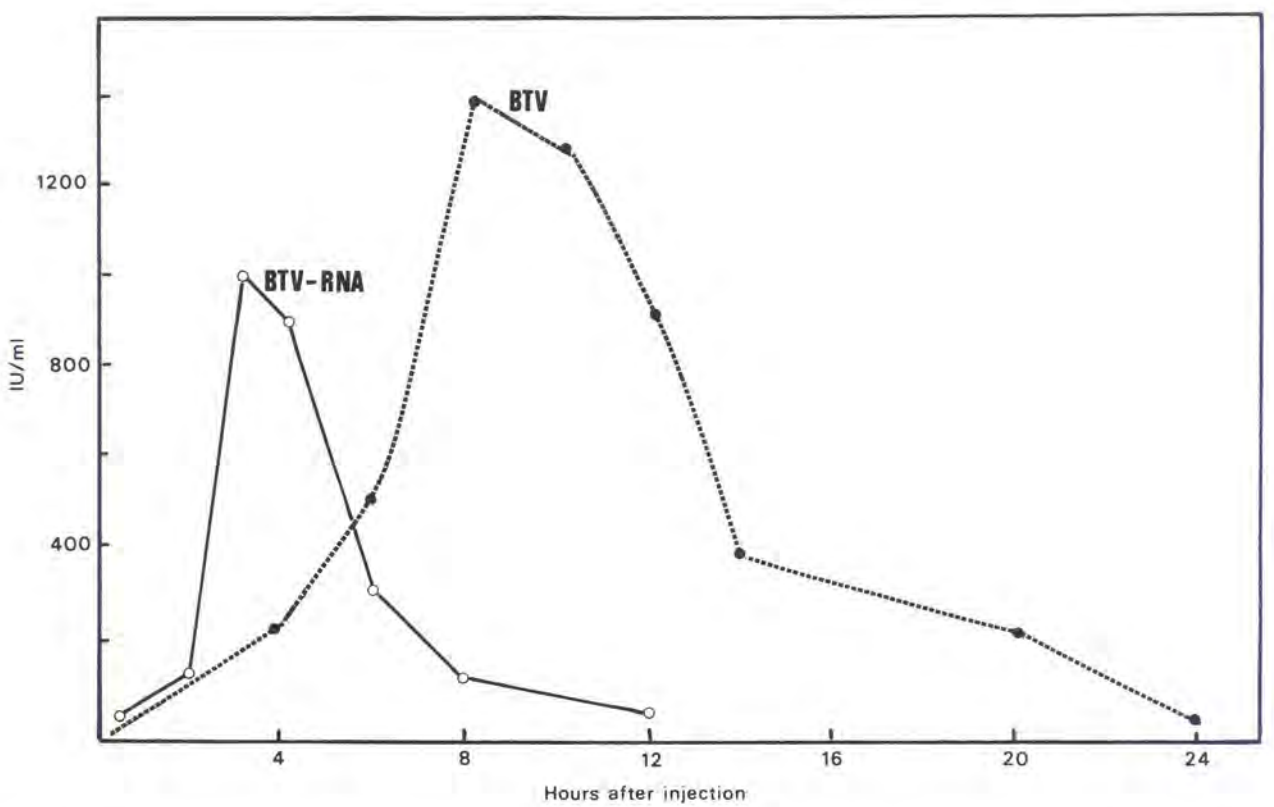

FIG. 2 Kinetics of interferon stimulation by bluetongue virus (.....●.....) and by isolated bluetongue virus double-stranded RNA (——o——)

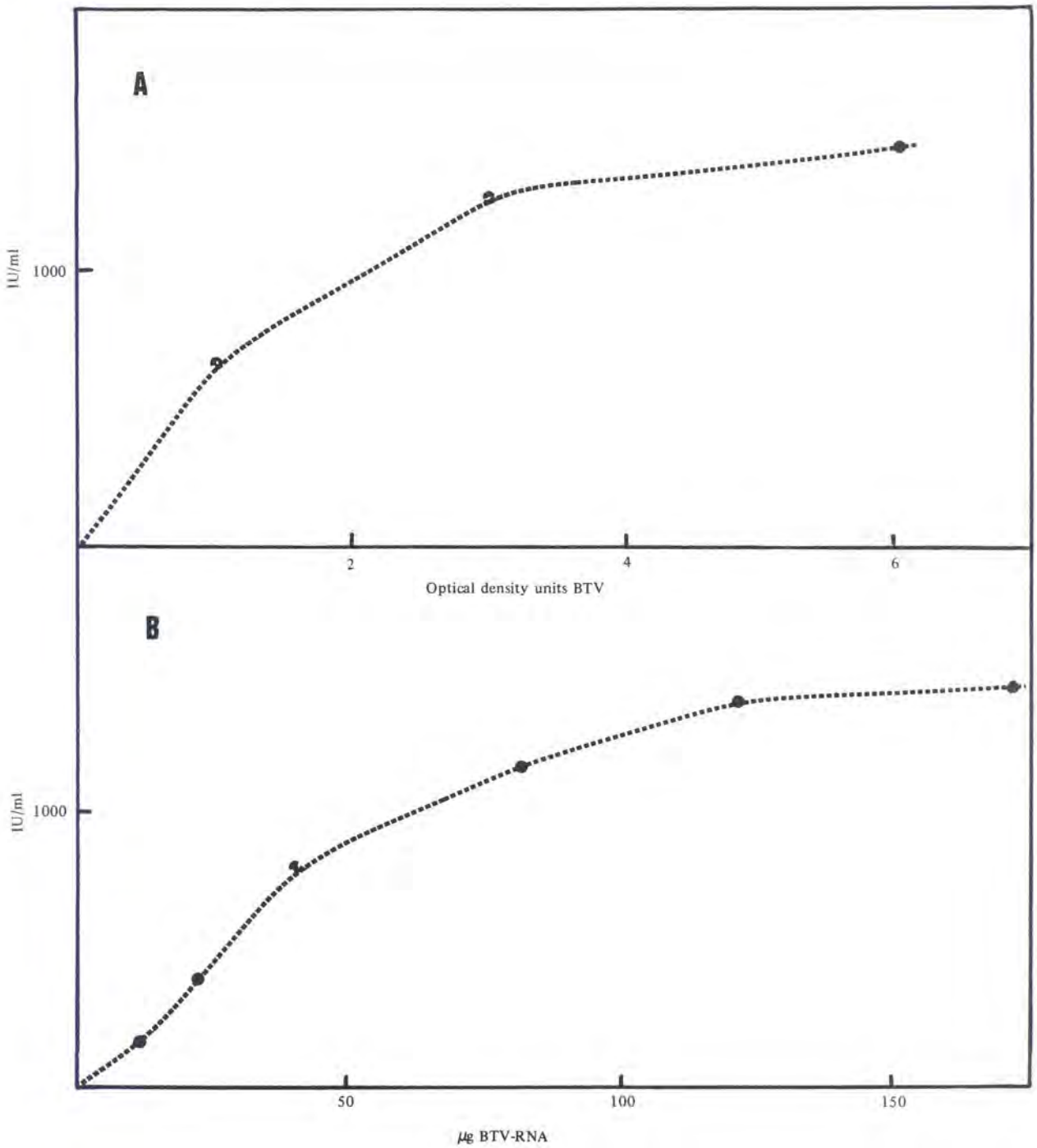

FIG. 3 Determination of the optimum dose of bluetongue virus (A) and bluetongue virus double-stranded RNA (B) required for interferon stimulation

Molecular mass determination

Sephadex gel filtration was used to determine the molecular mass of the interferon. A column of Sephadex G 100 was packed and tested as described under Methods. Bovine albumin, chymotripsinogen, myoglobin and cytochrome C, with known molecular masses ranging from 67 000 to 12 400, were used for calibrating the column. A plot of the molecular masses of these markers versus elution volume is depicted in Fig. 4.

The plot is obviously linear within the range investigated and could therefore be used for the determination of the molecular mass of interferon.

Interferon synthesis was induced in mice with both BTV and BTV double-stranded RNA under optimal conditions. The serum was collected, treated as described and assayed for interferon activity, before application to the Sephadex column. The column was then eluted with buffer and the different fractions of the eluate assayed for interferon activity. The result obtained with

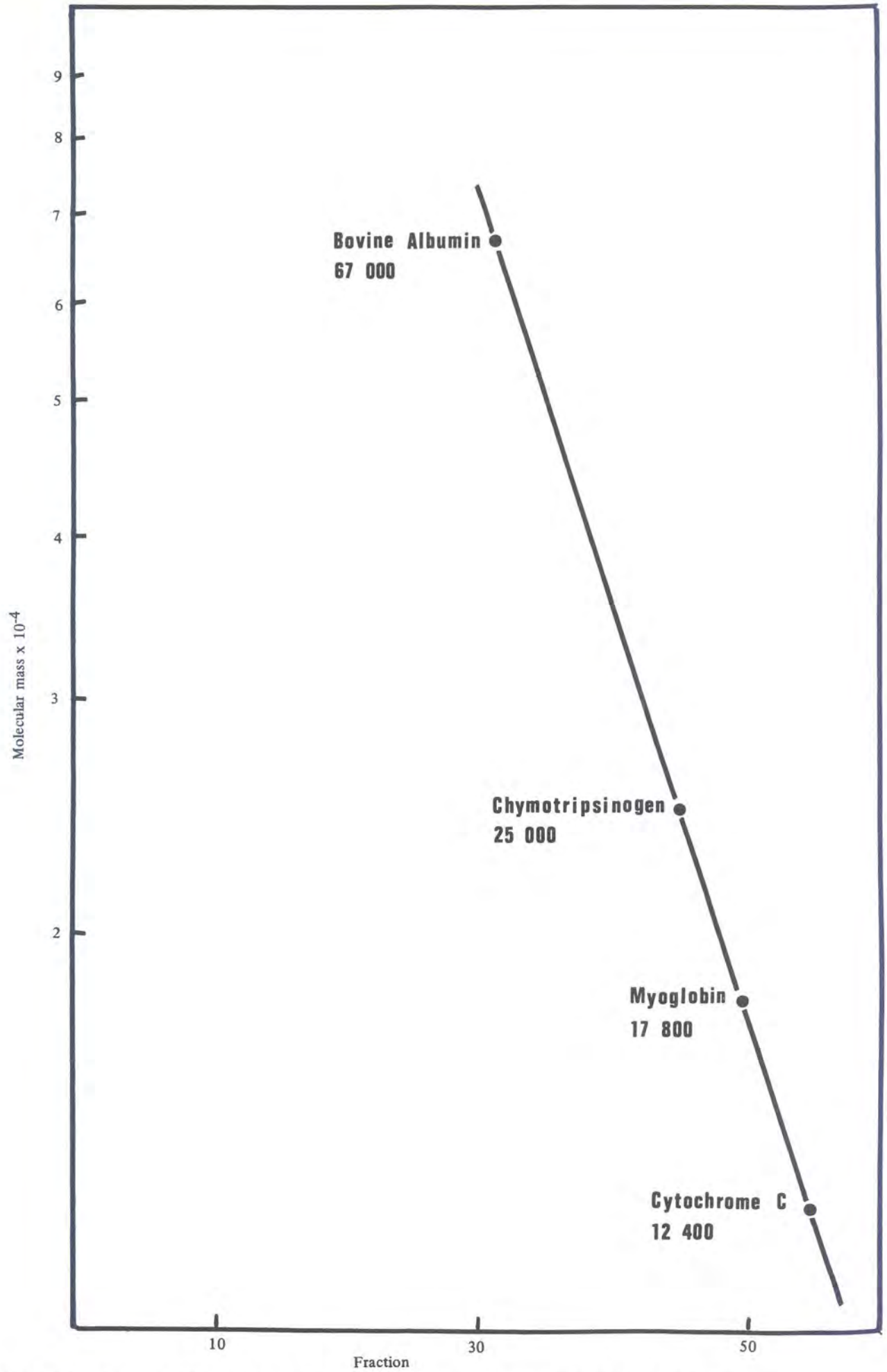

FIG. 4 Calibration curve of different molecular mass markers versus eluation volume on a Sephadex G100 column

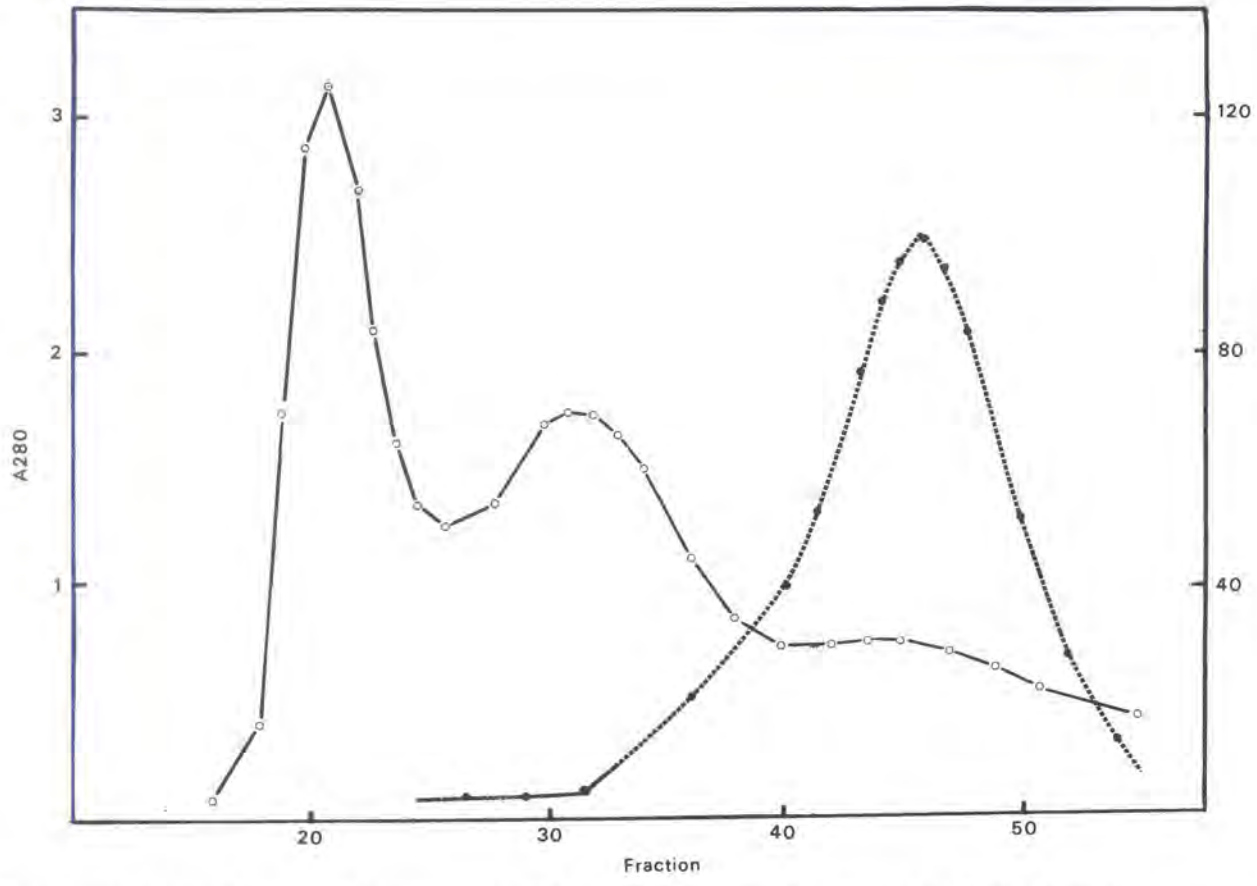

FIG. 5 Elution pattern of a serum sample containing interferon activity on a Sephadex G100 column. Optical density (———o———); interferon activity (- - - - ● - - - -)

interferon stimulated by BTV double-stranded RNA is shown in Fig. 5. Both the distribution of interferon activity in the fractions of the eluate and the optical density profile are depicted. Two fairly large optical density peaks are observed followed by a much smaller third peak which contains the interferon activity. Using the calibration curve in Fig. 4 it is possible to calculate that the interferon elutes at a position expected of a protein with a molecular mass of 24 000 dalton.

The result obtained with interferon stimulated by BTV was identical. It is therefore impossible to differentiate with respect to molecular mass between interferon induced by BTV or by BTV double-stranded RNA.

Discussion

Circulating interferon is induced in mice by both the bluetongue virion and isolated BTV RNA. Stimulation by RNA is the more rapid of the two processes and the titre reaches a maximum 4 h after infection. Stimulation by BTV is slower and the titre does not reach a maximum until 10 h after injection. This result suggested that different types of interferon might be involved.

One way of differentiating between interferon of the "endotoxin type" and virus induced interferon is by a comparison of the molecular masses of the two types.

Molecular mass determinations of the BTV and the BTV RNA-induced interferon were carried out, using Sephadex gel filtration. No difference was observed and in both cases a value of 24 000 dalton was obtained. This compares very well with earlier values for mouse interferon (Merigan, 1964). Although this result cannot be taken as conclusive proof that the two types of interferon are in fact identical it does support the conclusion of Ho & Ke (1970), that there is no fundamental difference between the two stimulation processes. The only difference is in the kinetics of stimulation.

This difference can however be explained equally well if it is assumed that in both cases the double-stranded RNA is the active inducing principle. When BTV itself is used for stimulation, a prerequisite for induction would be either uncoating of the parental virus genome or the formation of new viral double-stranded RNA. This would require a time interval, which is not needed in the case of stimulation with isolated double-stranded RNA. The lag phase of the latter process would therefore be much shorter and the maximum titre reached sooner.

Acknowledgement

The authors are indebted to Dr. D. W. Verwoerd for advice during the course of the investigation.

References

Carter, W. A., 1970. Interferon: Evidence for subunit structure. *Proc. natn. Acad. Sci., U.S.A.* 67, 620–628.

De Clercq, H. & Merigan, T. C., 1970. Stimulation or inhibition of interferon production depending on time of cycloheximide administration. *Virology*, 4, 799–802.

Field, A. K., Tytell, A. A., Lampson, G. P. & Hilleman, M. R., 1967. Inducer of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. *Proc. natn. Acad. Sci., U.S.A.*, 58, 1004–1010.

Finter, N. B., 1966. Interferons. Amsterdam: North-Holland Publishing Company.

Fujii-Kawata, I. & Miura, K. I., 1970. Segments of genome viruses containing double-stranded ribonucleic acid. *J. molec. Biol.*, 51, 247–253.

HALLUM, J. V., YOUNGNER, J. S. & STINEBRING, W. R., 1965. Interferon activity associated with high molecular weight proteins in the circulation of mice injected with endotoxin or bacteria. *Virology*, 27, 429–431.

HO, M. & KE, H., 1970. The mechanism of stimulation of interferon production by a complexed polyribonucleotide. *Virology*, 40, 693–702.

HUISMANS, H., 1969. Bluetongue virus induced interferon synthesis. *Onderstepoort J. vet. Res.*, 36, 181–185.

MERIGAN, T. C., 1964. Purified interferons: Physical properties and species specificity. *Science*, 145, 811–813.

TYTELL, A. A., LAMPSON, G. P., FIELD, A. K. & HILLEMAN, M. R., 1967. Inducers of interferon and host resistance. 3. Double-stranded RNA from reovirus type 3 virions (Reo 3-RNA). *Proc. natn. Acad. Sci., U.S.A.*, 58, 1719–1722.

VERWOERD, D. W., 1969. Purification and characterization of bluetongue virus. *Virology*, 38, 203–212.

VERWOERD, D. W., LOUW, H. & OELLERMANN, R. A., 1970. Characterization of bluetongue virus ribonucleic acid. *J. Virol.*, 5, 1–7.

VERWOERD, D. W., ELS, H., DE VILLIERS, E. M. & HUISMANS, H., 1972. Structure of the bluetongue virus capsid. *J. Virol.* in press.

YOUNGNER, J. S., 1970. Influence of inhibitors of protein synthesis on interferon formation in mice. 2. Comparison of effects of glutarimide antibiotics and tenuazonic acid. *Virology*, 40, 335–343.

YOUNGNER, J. S., & HALLUM, J. V., 1968. Interferon production in mice by double-stranded synthetic polynucleotides: induction or release? *Virology*, 35, 177–179.

YOUNGNER, J. S. & STINEBRING, W. R., 1966. Comparison of interferon production in mice by bacterial endotoxin and statolon. *Virology*, 29, 310–316.

YOUNGNER, J. S., STINEBRING, W. R. & TAUBE, S. E., 1965. Influence of inhibitors of protein synthesis on interferon formation in mice. *Virology*, 27, 541–550.

Printed in the Republic of South Africa for the Government Printer, Pretoria, by Heer Printing Co. (Pty) Ltd., Pretoria.