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DNA-binding protein that induces cell differentiation

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Macrophage and granulocyte-inducing (MGI) proteins regulate the growth and differentiation of myeloid hematopoietic cells. One class of these proteins (MGI-1) induces cell growth and another class (MGI-2) induces cell differentiation. Results obtained with DNA-cellulose column chromatography have shown that the differentiation-inducing protein MGI-2 can bind to double-stranded cellular DNA, but that there was no such binding under the same conditions by the growth-inducing protein MGI-1. DNA binding may thus be used to separate MGI-2 from MGI-1. The MGI-2 from mouse bound to DNA from mouse and calf. There were different elution peaks of the MGI-2 bound to DNA suggesting a heterogeneity of MGI-2 molecules, and the last peak eluted from the DNA cellulose column was enriched for one of the molecular forms of MGI-2. After one further step of purification by polyacrylamide gel electrophoresis, this molecular form of MGI-2 was active at a concentration of 6.5 x 10^{-11} M. In normal development MGI-1 induces MGI-2. This induction of a DNA-binding differentiation-inducing protein by a growth-inducing protein is an efficient mechanism for the normal coupling of growth and differentiation. It is suggested that this may also be a mechanism for the normal coupling of growth and differentiation in other types of cells.

Key words: DNA-binding protein/differentiation/growth/myeloid cells/leukemia

Introduction

A normal developmental program requires both growth and differentiation. Experiments with cells in culture have identified growth factors for different cell types. These include growth-inducing proteins for different normal hematopoietic cells including myeloid cells (see Sachs, 1978; Clarkson *et al.*, 1978), erythroid cells (Clarkson *et al.*, 1978) and T lymphocytes (Mier and Gallo, 1980); and for other cell types such as nerve cells (Yanker and Shooter, 1982), epidermal cells (Carpenter and Cohen, 1979) and fibroblasts (Sato and Ross, 1979). Studies with myeloid hematopoietic cells have also identified differentiation-inducing proteins, and shown that in normal development the myeloid growth-inducing protein induces production of the myeloid differentiation-inducing protein (Sachs, 1980; Liebermann *et al.*, 1982; Lotem and Sachs, 1982, 1983; Symonds and Sachs, 1982). This thus provides an effective mechanism for the normal coupling of growth and differentiation. The origin of myeloid leukemia involves an uncoupling of growth and differentiation (see Sachs, 1978, 1980, 1982; Lotem and Sachs, 1982, 1983).

A variety of DNA-binding proteins have been identified (Gilbert and Muller-Hill, 1966; Richardson, 1966; Ptashne, 1967; Pettijohn and Kamiya, 1967; Alberts *et al.*, 1968; Litman, 1968; Weideli *et al.*, 1978, 1980; Pfahl, 1982). The identification of these regulators of growth and differentiation raises the question as to whether they may function by interacting with cellular DNA. The methods used to identify DNA-binding proteins include DNA-cellulose chromatography (Alberts *et al.*, 1968; Litman, 1968; Weideli *et al.*, 1978), which has been used to measure DNA binding of different proteins from bacteria (Alberts *et al.*, 1968; Litman, 1968) and *Drosophila* (Weideli *et al.*, 1978, 1980), and DNA binding of the glucocorticoid-receptor complex (Pfahl, 1982). We have also used this procedure in the present experiments. The results indicate that the protein inducer of differentiation of myeloid cells binds to cellular DNA and that, under the conditions tested, there was no such DNA binding by the myeloid growth-inducing protein. This suggests that the gene activation required for differentiation may result from direct interaction of the differentiation-inducing protein with DNA.

Results

DNA-cellulose chromatography

For DNA-cellulose chromatography we have used columns of cellulose coupled to double-stranded DNA (Alberts *et al.*, 1968; Litman, 1968). The myeloid growth and differentiation-inducing proteins were obtained from serum-free conditioned medium from a clone of myeloid leukemic cells that can be induced to produce and secrete these proteins (Weiss and Sachs, 1978). The myeloid growth-inducing protein will be referred to as MGI-1 (macrophage and granulocyte inducer, type 1). This growth inducer has also been referred to as mashran gm, colony stimulating factor (CSF), colony stimulating activity (CSA) and MGI. The myeloid differentiation-inducing protein will be referred to as MGI-2. This differentiation inducer has also been referred to as MGI, D factor and DF (reviewed in Sachs, 1978, 1980, 1982). MGI-1 was assayed by its ability to induce colonies of cells from normal myeloid precursors (Pluznik and Sachs, 1965). MGI-2 was assayed by its ability to induce the synthesis of lysozyme, which is one of the stages in myeloid cell differentiation, in a clone of myeloid leukemic cells that can be induced to differentiate by MGI-2 (Krystosek and Sachs, 1976). The DNA coupled to cellulose was double-stranded DNA from calf thymus and from two clones of myeloid leukemic cells.

Chromatography on DNA-cellulose columns with DNA from calf thymus or the myeloid leukemic cells showed that MGI-2, but not MGI-1, bound to the column. Attempts to elute the MGI-2 bound to the DNA-cellulose column with NaCl (Alberts *et al.*, 1968) showed (Table I) that at most 2.3% of the bound MGI-2 was eluted with high salt. Since the salt did not remove most of the MGI-2, this indicates that not only ion exchange is involved and therefore elution of the bound MGI-2 with 0.1% SDS (Weideli *et al.*, 1978) was used. SDS eluted the bound MGI-2 and an SDS elution profile from a column with calf thymus DNA is shown in Figure 1.

As can be seen in Figure 1, no MGI-1 activity was bound to the DNA-cellulose and it was all recovered in the unbound

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Table I. Lack of elution of MGI-2 from a DNA-cellulose column by high salt

Type of DNA	% MGI-2 eluted by NaCl at:		Total MGI-2 eluted by high salt (%)
	0.55 M	0.70 M	
Calf thymus	1.4	0.9	2.3
Myeloid leukemic clone 11	0.03	0.0	0.03

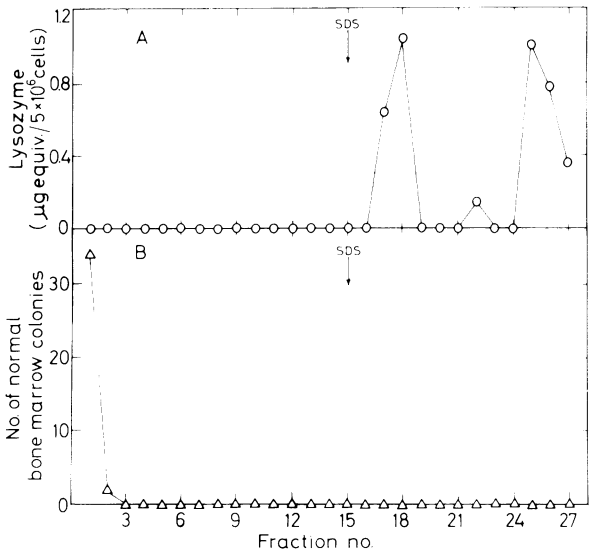

Fig. 1. Elution profile of **(A)** MGI-2 and **(B)** MGI-1 from a DNA-cellulose column with calf thymus DNA. There was no significant loss of the MGI-1 and MGI-2 activity loaded on the column.

Table II. Chromatography of MGI-1 and MGI-2 on DNA-cellulose columns with DNA from different sources

Source of DNA	% MGI-1		% MGI-2		% Distribution of bound MGI-2 in peaks:		
	unbound	bound	unbound	bound	Early	Middle	Late
Calf thymus	100	0	0	100.0	39.6	3.0	57.4
Myeloid leukemia							
Clone 1	100	0	7.5	92.1	47.3	32.8	12.4
Clone 11	100	0	4.0	96.0	58.2	22.8	19.0

The peaks referred to as early, middle and late are shown in Figure 1. With clone 1 DNA, 8.5% of the bound MGI-2 was eluted in the first fraction after the addition of SDS. Protein recovery from the columns was measured by the method of Stein and Moschera (1981) and there was no significant loss of the loaded protein.

front. In contrast, MGI-2 activity was not eluted until after the addition of SDS. The results with MGI-2 indicate (Figure 1A) that there was more than one peak of activity eluted after adding SDS. Experiments with DNA from the two clones of myeloid leukemic cells also showed, like those with calf thymus DNA, no binding of MGI-1, binding of MGI-2 and different MGI-2 elution peaks after adding SDS (Table II). The percentage of bound MGI-2 in the different peaks (Figure 1) indicates that more MGI-2 was bound in the middle peak with DNA from the myeloid leukemic clones than with DNA from calf thymus (Table II).

The use of cellulose columns without DNA (Figure 2)

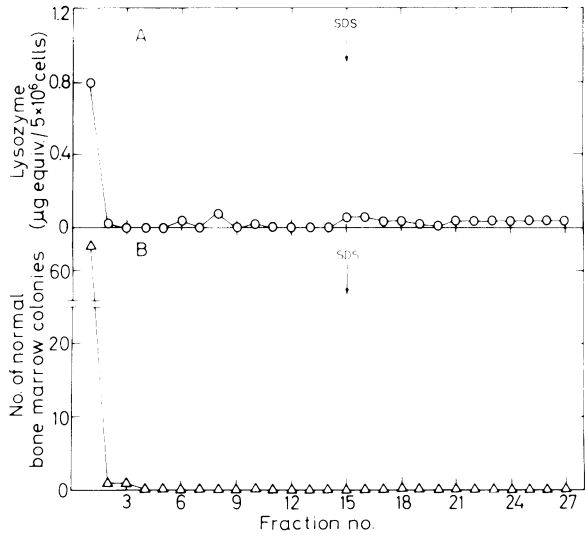

Fig. 2. Elution profile of **(A)** MGI-2 and **(B)** MGI-1 from a cellulose column without DNA. There was no significant loss of the MGI-1 and MGI-2 activity loaded on the column.

Table III. Lack of binding to DNA-cellulose column by lysozyme, chymotrypsinogen and bovine serum albumin

Protein	Isoelectric point (pI)	% Protein	
		Unbound	Bound
Lysozyme	10.9	100	0
Chymotrypsinogen A	9.5	100	0
Bovine serum albumin	4.7	100	0
MGI-2, late peak	6.8	0	100

The isoelectric points of egg white lysozyme, chymotrypsinogen A and bovine serum albumin were taken from Sophionapoules (1973), Long *et al.* (1962) and Magdoff (1960), respectively.

showed elution of both the MGI-1 and MGI-2 activity in the unbound front. To check further the specificity of the DNA binding, three proteins which are not known as DNA-binding proteins, egg white lysozyme (Miles Labs) with a pI of 10.9, chymotrypsinogen A (Pharmacia) with a pI of 9.5 and bovine serum albumin (Pharmacia) with a pI of 4.7, were chromatographed on DNA-cellulose columns. In the fractions from the column, chymotrypsinogen and bovine serum albumin were identified by silver staining (Sammons *et al.*, 1981) after electrophoresis on a 7–17% gradient SDS-polyacrylamide slab gel (Maizel, 1971) and lysozyme was identified using a turbidometric assay with *Micrococcus lysodeikticus* as described (Krystosek and Sachs, 1976). In contrast to MGI-2, all these proteins were eluted only in the unbound front (Table III).

The late peak of MGI-2 bound to the DNA cellulose column had a pI of 6.8 determined as described in Materials and methods. This MGI-2 thus had a lower pI than lysozyme and chymotrypsinogen which did not bind to the DNA-cellulose column (Table III) This MGI-2 also did not bind to a Mono S cation exchange column pH 7.0. These results provide further evidence that the binding of MGI-2 to the DNA cellulose column did not only involve ion exchange.

Fractionation of MGI-2 bound to DNA on a polyacrylamide gel

Fractions 25–27 in the late peak of MGI-2 activity eluted

by SDS from the cellulose column with calf thymus DNA (Figure 1A) were combined, concentrated and then electrophoresed on a 7% polyacrylamide gel. Electrophoresis of the original MGI-2 activity which had not been chromatographed on DNA-cellulose showed three main peaks of MGI-2 activity after elution from the polyacrylamide gel (Figure 3A). However, the late peak eluted from the DNA-cellulose column showed an enrichment for one of the molecular forms of MGI-2, so that there was only one main peak of MGI-2 activity after elution from the polyacrylamide gel (Figure 3B). The slight shift of this peak in Figure 3B compared with Figure 3A is presumably due to the SDS bound during elution with SDS from the DNA-cellulose column.

When this MGI-2 peak eluted from the 7% polyacrylamide gel was concentrated and electrophoresed on a 7 – 17% SDS gradient polyacrylamide slab gel, it showed one band by silver staining with an apparent mol. wt. of 55 000. The MGI-2 which showed this one band had been purified 3570-fold in two steps (Table IV) and was active at a concentration of 6.5 x 10^{-11} M.

Discussion

The results indicate that myeloid differentiation-inducing protein MGI-2 can bind to double-stranded cellular DNA, but that there was no such binding under the same conditions by the myeloid growth-inducing protein MGI-1. DNA binding may thus be used to separate MGI-1 from MGI-2. At most 2.3% of the bound MGI-2 was eluted by high salt from the DNA-cellulose columns which were used to measure DNA binding. This indicates that not only ion exchange was involved and therefore SDS (Weideli *et al.*, 1978) was used for elution. SDS removed the protein indicating that hydrophobic interactions may also be involved. Since SDS is a detergent and thus usually denatures proteins, this requirement for denaturing conditions to elute the protein indicates that specific interactions are involved with strong binding.

It has been suggested (Alberts *et al.*, 1968) that the stronger the affinity of a protein for DNA under conditions similar to those used in our experiments, the longer it will be retained by the DNA during the elution procedure. The existence of different elution peaks of MGI-2 indicates a heterogeneity of MGI-2 molecules which differ in their affinity for DNA. The last peak of MGI-2 eluted from the DNA-cellulose column showed an enrichment for one of the molecular forms of MGI-2. This molecular form of MGI-2 had a pI of 6.8, which is lower than the pI of lysozyme (10.9) and chymotrypsinogen (9.5), two proteins that did not bind to the DNA-cellulose column. This again indicates that the DNA binding of MGI-2 did not only involve ion exchange.

After one further step of purification by electrophoresis on a polyacrylamide gel, this molecular form of MGI-2 was active at a concentration of 6.5 x 10^{-11} M. The MGI-2 purified by a two-step procedure, binding to DNA-cellulose and polyacrylamide gel electrophoresis, thus showed an activity within the range of activities obtained by other procedures that have been used to purify MGI-2 (Lipton and Sachs, 1981), different forms of MGI-1 (CSF) (Landau and Sachs, 1971; Burgess *et al.*, 1977; Stanley and Heard, 1977; Lipton and Sachs, 1981) and interleukin 3 (Ihle *et al.*, 1982).

Two clones of mouse myeloid leukemic cells were used in the present experiments. One clone (no. 11) (Fibach *et al.*, 1973) can be induced to differentiate to mature cells by MGI-2 and was also the source of MGI-1 and MGI-2. The other clone (no. 1) (Lotem and Sachs, 1974) can be induced by MGI-2 for some differentiation-associated properties (Symonds and Sachs, 1983). MGI-2 bound to DNA from both these clones and to DNA from calf thymus. This indicates that DNA regions that bind MGI-2 from mouse cells are also present in calf. The larger size of one of the MGI-2 peaks in chromatography with DNA from the mouse myeloid leukemic clones compared with DNA from calf thymus, may reflect differences in binding to particular DNA sequences. It will be interesting to determine if there are different forms of MGI-2 for differentiation to macrophages or granulocytes

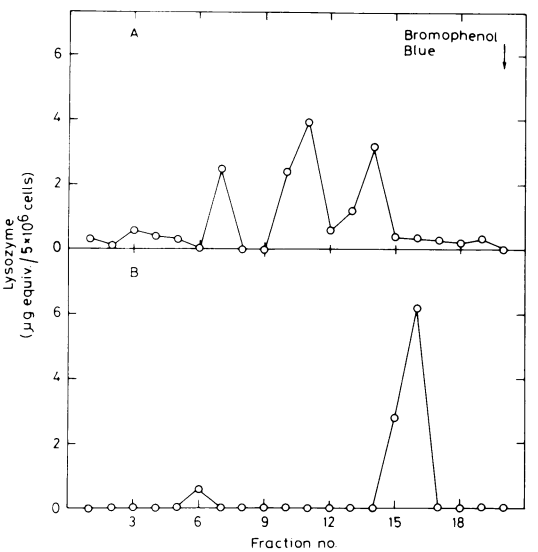

Fig. 3. Elution profile of MGI-2 from a 7% polyacrylamide gel. **(A)** MGI-2 that has not been chromatographed on a DNA cellulose column. **(B)** MGI-2 from the late peak (fractions of 25 – 27 in Figure 1A) eluted by SDS from a cellulose column with calf thymus DNA. The recovery of the loaded MGI-2 activity was 56% of the 273 units of MGI-2 loaded in **(A)** and 19% of the 37 units of MGI-2 loaded in **(B)**. One unit of MGI-2 activity equals 1 μg equivalent lysozyme secreted per 5 x 10^6 clone 11 cells.

Table IV. Purification of MGI-2 bound to DNA

Fraction	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Conditioned medium	0.5	584	7.5	77.9	1	100
DNA-cellulose (late peak)	9.7	282	0.0056	52 140	669	50
Polyacrylamide gel electrophoresis	3.5	56.1	0.000202	278 000	3570	9.6

One unit of MGI-2 activity (U) equals 1 μg equivalent lysozyme secreted per 5 x 10^6 clone 11 cells. Protein concentrations were determined by the method of Stein and Moschera (1981), except for the most purified sample whose protein concentration was determined using silver protein staining (Sammons *et al.*, 1981) as described in Materials and methods. Polyacrylamide gel electrophoresis was on a 7% polyacrylamide gel as described in Materials and methods.

(Liebermann *et al.*, 1982).

The binding of MGI-2 to DNA indicates that differentiation may be induced by this protein by direct interaction with DNA and it will be interesting to characterise the sequences involved in this interaction. In normal development MGI-1 induces MGI-2 (Sachs, 1980; Liebermann *et al.*, 1982; Lotem and Sachs, 1982, 1983; Symonds and Sachs, 1982). This induction of a DNA-binding protein by the growth-inducing protein is an efficient mechanism for the normal coupling of growth and differentiation in development, which may also exist in other types of cells.

Materials and methods

Cells, cell culture, source and assays for MGI-1 and MGI-2

Myeloid leukemia clone 11 was derived from a spontaneous myeloid leukemia (Fibach *et al.*, 1973), and myeloid leukemia clone 1 from an X irradiation-induced myeloid leukemia (Lotem and Sachs, 1974). Cells were cultured in Eagle's medium with a 4-fold concentration of amino acids and vitamins (H-21, Grand Island Biological Co., NY) (EM) with 10% horse serum. MGI-1 and MGI-2 were obtained from serum free conditioned media from myeloid leukemic clone 11 cells, induced to secrete those proteins (Weiss and Sachs, 1978) by 1 μg/ml lipopolysaccharide B from *Salmonella typhimurium* (Difco Labs, Detroit, MI). Two days after seeding 8 x 10^5 cells/ml in EM with 10% horse serum and lipopolysaccharide, the medium was removed, the attached cells washed, serum-free EM added and the conditioned medium collected 1 day later. This medium containing MGI-1 and MGI-2 was then concentrated 100-fold with polyethylene glycol (40 000, Serva Feinbiochemica, Heidelberg) (Lotem and Sachs, 1981). MGI-2 was assayed by its ability to induce the synthesis of lysozyme (Krystosek and Sachs, 1976) in clone 11 myeloid leukemic cells. The secreted lysozyme was measured as described (Krystosek and Sachs, 1976) and the results expressed as μg equivalents lysozyme secreted per 5 x 10^6 cells in 4 days. MGI-1 was assayed by induction of myeloid cell colonies in agar (Pluznik and Sachs, 1965) from 5 x 10^4 nucleated normal mouse bone marrow cells.

DNA-cellulose chromatography

DNA-cellulose was prepared by coupling double-stranded DNA to acid-washed Whatman CF11 cellulose (Alberts *et al.*, 1968; Litman, 1968). The double-stranded DNA from the leukemic clones was extracted from the cells by the method of Hirt (1967) and the double-stranded DNA from calf thymus was obtained from Sigma Chemical Co., St. Louis, MO. The double-stranded nature of the DNA preparations used for binding to the cellulose was checked by electrophoresis on a 1% agarose gel (ME; Seakem, Rockland, ME) followed by staining with acridine orange (McMaster and Carmichael, 1977).

Elution with NaCl. 4 ml DNA-cellulose columns (in 1 x 15 cm tubes) were loaded with 8 mg of protein from the 100-fold concentrated conditioned medium. After loading, the column was washed (10 – 15 bed volumes) with 10 mM phosphate 50 mM NaCl, pH 7.4 followed by a 50 mM to 1.0 M NaCl elution gradient (Alberts *et al.*, 1968). Chromatography was carried out at 4°C, 1 ml fractions were collected and the ionic concentration of each fraction was determined. The eluted fractions were dialysed against EM overnight at 4°C, filtered through a 0.45 micron millipore filter and assayed for MGI-2 activity at 5% and 10%.

SDS elution. 4 ml DNA-cellulose columns (in 1 x 15 cm tubes) were loaded with 10 mg of protein from the 100-fold concentrated conditioned medium. After loading, the sample was left at 4°C with the matrix for 15 min before extensive washing (20 – 25 volumes of the column) with Buffer A (10 mM Tris-HCl, pH 7.4, 25 mM NaCl, 5 mM Mg acetate, 5 mM β-mercaptoethanol, 0.1 mM EDTA and 10% glycerol) at 4°C (8 ml/h). The bound proteins were eluted in one step with 0.1% SDS in Buffer A at room temperature (Weideli *et al.*, 1978). The fractions were dialysed against EM at 30°C overnight, filtered through a 0.45 micron millipore filter and assayed for MGI-1 and MGI-2 activity at 2.5%. A column without DNA was prepared by u.v. irradiation (Litman, 1968) of Whatman CF11 cellulose without adding DNA. This column was eluted in the same way as the column with DNA.

Ion exchange chromatography

A Mono S (HR 5/5, Pharmacia Fine Chemicals, Uppsala) cation exchange column designed for use on h.p.l.c. for the separation of proteins was used. 1 mg protein was applied to the column in 1 ml of 10 mM phosphate, 20% ethylene glycol, pH 7.0. The column was washed with the same buffer for 30 min, and a 10 mM to 1 M NaCl gradient was then applied for 60 min at a flow rate of 1.5 ml per min. Fractions of 1.5 ml were collected, dialysed against EM at 30°C overnight, filtered through a 0.45 micron millipore filter and assayed for MGI-2 at 7.5% and 10%.

Polyacrylamide gel electrophoresis

Eluted MGI-2 fractions from a DNA-cellulose column were combined, concentrated 10-fold with polyethylene glycol (40 000 Serva Feinbiochemica, Heidelberg), and then electrophoresed on a preparative 7% polyacrylamide slab gel (Maizel, 1971). Electrophoresis of MGI-2 which had not been chromatographed on DNA-cellulose was run under similar conditions. The 7% polyacrylamide gel (13 cm x 15 cm x 1.5 mm) was run at 150 V until the bromophenol blue front had travelled 10 cm into the resolving gel. The gel was cut into 20 x 0.5 cm slices, minced by forcing through a 3 ml syringe and resuspended in 4 ml EM. The samples were shaken overnight at room temperture, the supernatants filtered through a 0.45 micron millipore filter, and assayed for MGI-2 activity at 10 or 15%. Samples from the preparative 7% polyacrylamide gel were analysed on a 7 – 17% SDS gradient polyacrylamide slab gel (Maizel, 1971) and the proteins visualised by silver staining (Sammons *et al.*, 1981).

Isoelectric focusing was carried out as described by Maizel (1971). The protein was applied to pre-equilibrated 2.5 ml disc isoelectric focusing urea gels. The gels were run for 700 volt-hours in parallel with protein-free gels (for pH determination). After completion of the electrophoresis, the gels were cut into 0.5 cm slices, minced by forcing through a 1 ml syringe and resuspended in 3 ml EM. The protein-free minced slices were resuspended in 2 ml double-distilled water. The samples were shaken overnight at room temperature, the supernatants filtered through a 0.45 micron millipore filter, and assayed for MGI-2 activity at 5% and 10%. The hydrogen ion concentration of the protein-free supernatants was determined on a calibrated pH meter.

Protein determination using silver staining

The purified protein was electrophoresed with five different concentrations of bovine serum albumin (Pharmacia Fine Chemicals, Uppsala) (1, 2.5, 5, 10 and 20 ng) on a 7 – 17% SDS gradient polyacrylamide slab gel. Electrophoresis was carried out as described by Maizel (1971), the protein visualised in the gel by the silver staining technique of Sammons *et al.* (1981), and the amount of silver staining estimated by comparison with the staining of bovine serum albumin.

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