Chromatography of Arbovirus Ribonucleic Acid Forms on Columns of Benzoylated- Diethylaminoethyl Cellulose

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Received for publication 20 May 1969

Benzoylated-diethylaminoethyl cellulose (BD-cellulose) column chromatography was found to be useful in resolving most of the ribonucleic acid (RNA) forms from the replicative cycle of group A arbovirus Semliki Forest virus (SFV). The elution patterns were independent of molecular weight and appeared to be related to the degree of secondary structure in the molecule. Fractions of RNA were taken from a sucrose density gradient of cytoplasmic extracts of SFV-infected chick cells pretreated with actinomycin D. In a linear salt gradient, 16S material cochromatographed with the rapidly eluted ribonuclease resistant core of the double-stranded SFV-RNA and with the homopolymer duplex polyinosinic acid: polycytidylic acid. This fraction, therefore, probably contains an SFV-RNA form similar to the completely double stranded replicative form (RF) of several RNA viruses and bacteriophages. Faster moving ($>20S$) sucrose gradient fractions eluted more slowly, suggesting a decreasing proportion of secondary structure with increasing sedimentation value. The fractions, therefore, seemed to contain replicative intermediate (RI) structures. The two single stranded forms of SFV-RNA (42S and 26S) could only be eluted from BD-cellulose in the presence of urea or dimethyl sulfoxide, suggesting the presence of minimal secondary structure. Under these conditions, the single-stranded viral RNA forms could not be resolved. Molecular sieve chromatography of the single-stranded RNA forms, performed by passage through an agarose column, also failed to resolve these forms. The viral RNA forms containing a high degree of secondary structure, probably the RF and the RI, could, therefore, be rapidly separated from each other and from the single-stranded forms.

It has been suggested by Gillam et al. (7) and demonstrated by Kelly and Sinsheimer (9) that a property of the BD-cellulose column, in addition to its lipophylic anion exchange effect (11, 16), is the ability to separate species of ribonucleic acid (RNA) by their degree of secondary structure. Gillam et al. postulated that bases of the polynucleotide chains not involved in secondary structure or in complementary base pairing become intercalated between the stacked aromatic groups of the resin. The greater the secondary structure in an RNA chain, the smaller the proportion of bases available for interaction with the resin, and the more easily such an RNA can be eluted from the column. Double-stranded bacteriophage RNA forms, either the replicative form (RF) or the ribonuclease-resistant core of viral RNA, which have no bases available for interaction with the column, are the first RNA

forms to be eluted, coeluting with native deoxyribonucleic acid (DNA). Replicative intermediate (RI) forms, containing a double-stranded RNA duplex and a nascent tail of a single-stranded RNA are next eluted by the salt gradient, probably because of their decreased proportion of secondary structure as compared to the RF (9).

These results suggested that the benzoylated-diethylaminoethyl (BD)-cellulose column might be useful in analyzing the RNA forms produced during the replication of an animal RNA virus. Semliki Forest virus (SFV), arbovirus group A, was used in this study. Forms of SFV-RNA with varying degrees of secondary structure were resolved by the BD-column. The properties of the viral RNA in the fractions most easily eluted by a salt gradient suggested that it contained the completely double-stranded viral RF. Fractions of RNA were also eluted more slowly than the

RF by the salt gradient; these would, therefore, appear to contain RNA forms with less secondary structure than the RF, probably RI forms of SFV-RNA. Single-stranded forms of the virus could not, however, be eluted by the salt gradient, but came off the column only upon addition of solutions containing urea or dimethyl sulfoxide (DMSO). Under such conditions, these RNA forms were not resolved. The results suggested that, as in the case of other RNA viruses, RF and RI forms of SFV are produced during replication. These forms may be partially resolved from each other and completely separated from single-stranded RNA forms of the virus on the BD cellulose column. BD-cellulose chromatography was particularly useful in separating single stranded 26S viral RNA from partially double-stranded RNA forms (RI) with similar sedimentation values (4, 6, 13, 14).

MATERIALS AND METHODS

Materials. Diethylaminoethyl (DEAE)-cellulose (capacity, 0.83 meq/g) was purchased from Schleicher & Schuell; pyridine, reagent grade, from J. T. Baker Chemical Company; benzoyl chloride, reagent grade, from Eastman Organic Chemicals, Rochester, N.Y. The latter was not redistilled. Actinomycin D was donated by Merck, Sharp & Dohme; uridine-5-³H and *Escherichia coli* B transfer RNA (tRNA) were obtained from Schwarz BioResearch Inc., Orangeburg, N.Y.; pancreatic ribonuclease, from Worthington Biochemical Corp.; and carrier free ³²P-H₃PO₄ from New England Nuclear Corp., Boston, Mass. Pronase, grade B, was the product of the California Corporation for Biochemical Research; DMSO was purchased from Fisher Chemical Corporation. Urea, from Baker Chemical Company, was deionized with a mixed ion exchange resin (Rexyn I-300, Fischer Corp.) treated with acid-washed Norit and passed through a filter of diatomaceous earth (Celite 545, Johns-Manville Corp., New York, N.Y.).

Filters (HA, 25 mm, 0.45 μ m) were obtained from the Millipore Corp., New Bedford, Mass.

The synthetic homopolymer duplex polyinosinic acid:polycytidylic acid (poly rI:rC) was obtained from the Miles Laboratories, Inc., Elkhart, Ind. Agarose beads (Bio gel, A-50m, 2%) were the product of the Bio-Rad Laboratories, Richmond, Calif.

Preparation of the BD-cellulose. The BD-cellulose was made according to the procedure of Gillam et al. (7). Benzoyl group content was 2.7 moles per mole of anhydroglucose.

Chromatographic methods. BD-cellulose chromatography was performed in column (5 by 1.4 cm, inside diameter) at room temperature. RNA was dissolved in 10 ml of the initial buffer and eluted sequentially with gradients of NaCl and DMSO (gradients 1 and 2, Table 1), or gradients of NaCl and urea (gradients 1 and 3, Table 1). At the completion of each run, the column was washed with a solution of 20% ethyl alcohol and 2 M NaCl and then with 100 ml of initial buffer to prepare it for reuse.

Cell cultures and sucrose-density gradient analysis. The methods used in preparing pools of SFV and the monolayers of primary chick fibroblasts have been described previously (6). Sucrose-density gradient analysis was also performed by previously described methods (6) as was the preparation of viral RNA (4).

Ribonuclease treatment of RNA. RNA was treated with 2 μ g of ribonuclease per ml at 37 C for 10 min. Thereafter, to hydrolyse the ribonuclease, 50 μ g of Pronase per ml was added, and the mixture was incubated at 37 C for 10 min. Preincubation of the Pronase at 37 C for 2 hr in 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride buffer at pH 7.4 was used to decrease any endogenous ribonuclease activity of the Pronase preparation (8).

Measurements of acid-precipitable radioactive material. Solutions to be counted were chilled to 0 C and 1/10 volume of 50% trichloroacetic acid was added. This was incubated for 10 min at 0 C; it was then poured onto a Millipore filter and suction was applied. The filter was washed three times with 10-ml samples of 2.5% trichloroacetic acid at 0 C. The filter was allowed to dry in an oven at 55 C for 30 min;

TABLE 1. Three gradients used in elution of RNA from columns of BD-cellulose

Gradient no.	Initial buffer ^a	Terminal buffer ^a
1	0.2 M NaCl 0.025 M K phosphate (pH 6.8) 0.001 M MgCl ₂	1.8 M NaCl 0.025 M K phosphate (pH 6.8) 0.001 M MgCl ₂
2	— 0.2 M NaCl 0.025 M K phosphate (pH 6.8)	18% (v/v) DMSO 1.6 M NaCl 0.025 M K phosphate (pH 6.8)
3	— 2.2 M NaCl 0.025 M K phosphate (pH 6.8)	8 M urea 2.2 M NaCl 0.10 M K acetate (pH 3.5) 1 M NH ₄ Cl

^a Volume, 180 ml.

it was then placed in counting vials containing 8 ml of toluene with 4% Liquifluor (Nuclear Chicago Corp.) and counted in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc.).

RESULTS

Chromatography on BD columns using salt gradients for elution. RNA intermediates in the replicative cycle of an RNA virus such as SFV can be specifically labeled in the presence of actinomycin D, which inhibits only the RNA synthesis of the host cell. The viral RNA forms for this study were obtained from chick fibroblasts infected with SFV at a virus to cell multiplicity of 40 to 1 in the presence of 2 μ g of actinomycin D per ml. Labeled RNA was obtained 1 hr after infection of chick fibroblasts by addition of 100 μ Ci of uridine-5- 3 H and incubation for 20 additional min. At this time, no single-stranded viral RNA is produced (4). RNA was extracted with sodium dodecylsulfate (SDS) and phenol at 25 C, and 0.1 ml of the extract was layered over a 6 to 30% sucrose gradient and sedimented at 300,000 $\times$ g for 100 min. To test ribonuclease sensitivity, 32 P-labeled RNA prepared in the same way, was treated with ribonuclease (2 μ g/ml, 37 C for 10 min) and layered on the same gradient. Gradient fractions were collected and analyzed for acid-precipitable 3 H and 32 P counts (Fig. 1). The tritium-labeled RNA was mainly polydisperse, ranging from 14S to 30S with a sharp peak at 16S. After ribonuclease treatment, 32 P-labeled material sedimenting more rapidly than 16S was entirely absent. A peak of RNA persisting at 16S corresponded to the ribonuclease-resistant core RNA.

A mixture of the 3 H-labeled RNA and 32 P-labeled ribonuclease-resistant core RNA was applied to a BD-cellulose column. The material was suspended in 10 ml of 0.025 M potassium phosphate buffer at pH 6.8, containing 0.2 M NaCl and 10^{-3} M MgAc₂ (initial buffer); chromatography was carried out as described in Materials and Methods. The profile of 3 H-labeled SFV-RNA and 32 P-labeled core on BD-cellulose chromatography (Fig. 2) can be compared directly with the profile of the same mixture of RNA forms analyzed by sucrose density gradient centrifugation (Fig. 1). The 32 P-labeled 16S ribonuclease-resistant core RNA emerged with a discrete peak of the 3 H-labeled RNA in each case. This peak was eluted earliest from the BD-cellulose column; however, it was nearer the top of the sucrose gradient profile than any other discrete 3 H-labeled RNA form.

Sucrose gradient centrifugation of the 3 H-labeled RNA was repeated in the absence of

FIG. 1. Sucrose-density gradient centrifugation of RNA from SFV-infected chick embryo fibroblasts. There are two RNA preparations, one with and one without ribonuclease pretreatment. Chick embryo fibroblasts (10^8) were infected with SFV at a virus cell multiplicity of 40 to 1 in the presence of 2 μ g of actinomycin D per ml. After 1 hr, 100 μ Ci of uridine-5- 3 H was added for 20 min. RNA was extracted by the phenol-SDS method; a 0.1-ml amount of the extract was sedimented in a 6 to 30% sucrose gradient for 100 min. at 300,000 $\times$ g. An additional 0.1 ml sample from RNA labeled with 32 P-orthophosphate was treated with ribonuclease before sedimentation as described in Materials and Methods. Fractions were collected and analyzed for optical density at 260 nm and samples were removed from each fraction for assays of acid-precipitable radioactivity. The bottom of the gradient is to the left. Fractions indicated by the arrows (tube no. 5, 10, and 15) were selected for fractionation by BD-cellulose chromatography in a similarly run gradient with 32 P-labeled RNA omitted (Fig. 3-5). Symbols: (○) 3 H counts/min, no pretreatment with ribonuclease; (●) 32 P counts/min, SFV RNA with ribonuclease pretreatment.

ribonuclease pretreated core 32 P-RNA. This was done to obtain fractions of RI for subsequent chromatography on BD-cellulose columns. A profile of 3 H-labeled RNA similar to that seen in Fig. 1 was obtained. The contents of tubes indicated by the arrows (Fig. 1, fraction no. 5, 10, and 15) were analyzed on columns of BD-cellulose together with samples of gradient-purified, ribonuclease-treated 32 P-labeled core. A sucrose gradient fraction taken from the lower part of the tube (fraction no. 5, 25S region, Fig. 1 and 3) when chromatographed on BD-cellulose showed clear separation of the major peak of the 3 H-labeled RNA from the ribonuclease-resistant core. Sucrose gradient fraction 10 (20S region, Fig. 1

and 4) had on BD-cellulose chromatography a major peak of ^3H -labeled RNA co-chromatographing with the ^{32}P -labeled core and several apparently discrete forms of more slowly eluted RNA. Finally, fraction 15 (16S region, Fig. 1 and 5) contained a still more predominant peak of RNA co-chromatographing with the ribonuclease-resistant core. Some more slowly eluting ^3H -labeled RNA was, however, also present in this fraction.

To estimate the role of molecular weight on separation on the BD-cellulose column, chromatography was performed on two preparations of double-stranded RNA which differed widely in *S* value. Ribonuclease-resistant core RNA from SFV, which is 16S (4), and (poly rI:rC), a synthetic double-stranded nucleotide homopolymer, which was 4S, were eluted from the column in

FIG. 2. BD-cellulose column chromatography of RNA extracts of SFV infected and chick embryo fibroblasts treated with actinomycin D. There are two RNA preparations identical to the preparations described in the legend to Fig. 1. ^3H -uridine-labeled RNA without ribonuclease treatment and ^{32}P -orthophosphate-labeled RNA with ribonuclease treatment were added to 10 ml of initial buffer and placed on a BD-cellulose column. Fractionation was performed as described in Materials and Methods. Fractions were assayed for acid-precipitable radioactivity. (○) ^3H counts/min, no ribonuclease treatment; (●) ^{32}P counts/min, ribonuclease treatment. Gradients no. 1 and 2 (Table I) were passed through the column. Only the results of gradient 1, which eluted 85% of the acid-precipitable RNA, are shown. In this and in all subsequent figures, points which appear to be omitted before and after the peaks of labeled materials indicate zero counts/min.

FIG. 3. Chromatography on a BD-cellulose column of RNA from sucrose gradient functions. Fraction no. 5 was used from a sucrose gradient similar to the one shown in Fig. 1, but only ^3H -labeled RNA was present. The fraction was added to a sample of ^{32}P -orthophosphate-labeled 16S core RNA which had been treated with ribonuclease as described in Materials and Methods and purified in a sucrose gradient. The mixture was adsorbed to a BD-cellulose column in 10 ml of initial buffer. Chromatography was carried out as described in Materials and Methods using gradients no. 1 and 2 (Table I) and the fractions were assayed for acid-precipitable radioactivity. Symbols: (○) ^3H counts/min; (●) ^{32}P counts/min. Only the results using gradient 1 are shown.

the same position (Fig. 6). Upon sedimentation after passage through the BD-cellulose, the viral core RNA still sedimented at 16S. It appears unlikely, therefore, that molecular weight plays an important role in resolution of double-stranded RNAs on the BD-cellulose column during sodium chloride elution.

Single stranded RNA forms. Two single-stranded forms of SFV-RNA have been previously described. Both are ribonuclease-sensitive and have the same base composition (5, 6, 13). They are, however, easily separated from one another in a sucrose density gradient, one form sedimenting at 42S, the other, at 26S. The 42S form is found in the mature virion and in SFV-infected cells; the 26S form is found only in SFV-infected cells. Only the 42S form is infectious

FIG. 4. Chromatography on a BD-cellulose column of RNA from sucrose gradient fractions. This was run as described in the legend to Fig. 3, except that a fraction similar to no. 10 (Fig. 1) was used. Symbols: (○) ^3H counts/min; (●) ^{32}P counts/min.

FIG. 5. Chromatography on a BD-cellulose column of RNA from sucrose gradient fractions. This was run as described in the legend to Fig. 3, except that a fraction similar to no. 15 (Fig. 2) was employed. Symbols: (○) ^3H counts/min; (●) ^{32}P counts/min.

in an assay for infectious viral RNA. Conversion of the 42S form to the 26S form has been shown in the case of the closely related Western equine encephalitis virus by heat or DMSO treatment (14). It was of interest to determine how these RNA forms behaved on the BD-cellulose column.

The 42S RNA used in this study was prepared from ^{32}P -labeled purified SFV (4), and the ^3H -labeled 26S RNA from chick cells treated with actinomycin D, which had been infected with SFV (5). Both forms, when applied to a BD-cellulose column, failed to be eluted by 1.8M sodium chloride. However a gradient of DMSO eluted both RNA forms quantitatively in a single major peak of acid-precipitable radioactive material (Fig. 7a). The two forms of single-stranded SFV were also eluted together by a linear gradient of urea (Fig. 7b). Neither of these gradients was able to separate the two RNA forms from each other.

When the RNA forms eluted by DMSO were precipitated with salt-ethyl alcohol, resuspended in a small volume, and sedimented for a second time on a sucrose gradient, the 26S and 42S RNA forms were again separated from each other. This demonstrated that the RNA forms had remained intact during the course of BD-

cellulose column chromatography, and that the 42S form had not been degraded to a lower molecular weight form.

It is not known whether the 42S and 26S forms of viral RNA differ in molecular weight or in secondary structure. The inability to separate the two RNA forms is not sufficient to support the conclusion that single-stranded RNA forms elute from the BD-cellulose column independently of their molecular weight. Additional attempts to separate the two single-stranded RNA forms on the basis of size by molecular sieve chromatography were unsuccessful (Fig. 8).

DISCUSSION

Among the forms of SFV-RNA which are synthesized during infection are a ribonuclease-resistant form sedimenting with a sharp peak at 16S before ribonuclease treatment, presumably the completely base paired RF, and a form of RNA which is polydisperse on sucrose-density gradient analysis. The latter has many of the properties of the RI form of the RNA bacteriophages (4). Models of the RI suggest that it is a double-stranded RNA template with nascent, partially completed single-stranded viral RNA

FIG. 6. BD-cellulose chromatography of two double-stranded RNA forms of different size, 16S double-stranded SFV-RNA core and 4S synthetic polynucleotide copolymer, poly rI:rC. A mixture of ^3H -uridine-labeled ribonuclease-resistant core of SFV-RNA and 0.5 mg of the synthetic copolymer in 10 ml of initial buffer were placed on a BD-cellulose column. Chromatography was carried out as described in Materials and Methods using gradients no. 1 and 3 (Table I). Absorbancy at 260 nm was read and each fraction was assayed for total acid-precipitable radioactivity. Symbols: (●) ^3H counts/min, core of the SFV; (○) absorbance at 260 nm, poly rI:rC synthetic polynucleotide copolymer.

chains (3). The chains appear to grow by displacement of a previously completed RNA chain (semiconservative replication) or by production on a complete duplex (conservative replication). The data of Kelly and Sinsheimer (10) suggest that both forms of replication may occur in infected bacteria. It is possible that the structure of the purified RI form is not the same as that of the replicating system functioning *in vivo* (1).

The present results indicate that RF and RI forms are produced during SFV replication. The first RNA forms to be eluted from the BD-cellulose column co-chromatographed with the ribonuclease-resistant core of SFV-RNA and with poly rI:rC. If SFV replicates by the conservative method, the RF and the core would be the same structure; if, however, the replication is by the semiconservative method, then the core would be composed of a duplex with one chain intact and the other containing breaks. In either case, the coelution of poly rI:rC and virus core with a fraction of the SFV-RNA suggests that this frac-

FIG. 7. BD-cellulose chromatography of single-stranded SFV-RNA forms. Two single-stranded forms of SFV (26S and 42S) were placed in 10 ml of initial buffer. A gradient of sodium chloride (gradient no. 1, Table I) was passed through the column, but no acid-precipitable radioactive material was eluted. A second gradient was passed through the column: (a) a gradient of DMSO (gradient no. 2, Table I); (b) the experiment was repeated, except that the second gradient passed through the column was of urea (gradient no. 3, Table I). Fractions were assayed for total acid-precipitable radioactivity. Symbols: (●) counts/min of ^3H -labeled 26S SFV single-stranded RNA; (○) counts/min of ^{32}P -labeled 42S SFV single-stranded RNA. Only the urea or DMSO gradient is shown in each case.

tion contained a completely double-stranded RF. The proportion of viral RNA with a high degree of secondary structure which eluted together with the ribonuclease-resistant core of the RI on the BD-cellulose column was determined by planimetry from Fig. 3-5. Tube no. 5

Fig. 8. Agarose (A-50m, 2%) column chromatography of single-stranded RNA forms. 26S and 42S SFV RNA forms were placed in 0.5 ml of a solution containing 0.1 M sodium chloride and .001 M magnesium acetate buffer. The column (90 by 1.6 cm, inside diameter) was washed with the same buffer. Fractions (5 ml) were collected and assayed for acid-precipitable radioactivity. Symbols: (●) counts/min of ^3H -labeled 26S RNA; (○) counts/min of ^{32}P -labeled 42S RNA.

from the sucrose gradient (Fig. 1) on the BD-cellulose column contained 20.8% RF (Fig. 3). This tube was the furthest removed from the 16S core area of the sucrose gradient. Tube no. 10 contained 34% (Fig. 4) and tube no. 15, the closest to the core, 50% (Fig. 5).

In the case of the RI, the nascent RNA chain would retard the elution of the molecule from the BD-cellulose column and the greater the length of this chain, the higher the *S* value of the molecule should be. Comparison of the elution patterns from the BD-cellulose column (Fig. 2-5) with the sucrose gradient centrifugation profile (Fig. 1) supported this hypothesis. As the *S* value (or the proportion of single-stranded RNA) increased, and the percentage of double-stranded RNA in the molecule decreased, the proportion of RNA which was retarded in elution from the BD-cellulose column was greater. These results are similar to those of Kelly and Sinsheimer (9), who demonstrated that the buoyant density of RI fractions from MS2-infected *E. coli* increased linearly in progressive fractions taken from the benzoylated naphthoylated DEAE-cellulose column.

On the basis of elution patterns of the RI from the BD-cellulose column, it might be concluded that elution was an inverse function of molecular weight; however, the coelution of the two completely double-stranded RNA forms with different molecular weights is in contrast to this possibility (Fig. 6). In addition, it has been demonstrated that MS2 RNA (molecular weight = 1.1×10^6) elutes ahead of the heavier 16S (molecular weight = 0.5×10^6) bacterial ribosomal RNA (12).

The two single-stranded RNA forms of the SFV, namely the infectious 42S RNA found in the mature virion and the noninfectious 26S RNA found in the cytoplasm of infected cells, have the same base composition (5, 13). These two RNA forms were completely separated on BD-cellulose from the other partially or completely base-paired RNA forms of SFV (Fig. 7a and b); however, these single-stranded RNA forms, although well separated by sucrose-density gradient sedimentation (6), could not be resolved from each other on the BD-cellulose column (Fig. 7a and b). Also, the two single-stranded RNA forms could not be separated by molecular sieve chromatography on an agarose bead column (Fig. 8). Chromatography of RNA forms from a similar RNA virus, Western equine encephalomyelitis virus, on a methylated albumin Kieselguhr column also fails to resolve them (14). On the basis of these results, it is still not possible to determine whether the 42S and 26S forms differ in molecular weight or in degree of secondary structure.

A major difference was observed between our elution patterns on the BD-cellulose column and the patterns obtained by other workers on the benzoylated naphthoylated DEAE column (9, 12). Single-stranded MS2-RNA has been shown to elute from benzoylated naphthoylated DEAE-cellulose columns at 0.75 M sodium chloride, but the two single-stranded forms of the SFV in the present experiments could not be eluted with 1.8 M sodium chloride; they required addition of DMSO or urea for elution. The two RNA forms were eluted by 12% DMSO (v/v) plus 1.0 M sodium chloride, and also by 2.2 M urea and 0.25 M ammonium chloride. The role of sodium chloride in this system was to disrupt ionic interactions, whereas that of DMSO or urea was to disrupt base-stacking interactions with the benzoyl groups (7). In RNA forms which are partially double-stranded or which have a high degree of secondary structure, bases interact with each other and are not available for interaction with the benzoyl groups of the resin. It has been demonstrated that single-stranded MS2 RNA has extensive secondary structure (15). This may explain the difference between the behavior of single-stranded MS2 and SFV-RNA forms.

Therefore, RI and EF forms appear to be produced during SFV infection. These forms may be separated from each other and from single-stranded RNA forms on a BD-cellulose column. In sucrose gradients, the 26S form cannot be separated from the RI forms, nor can the RF be separated from the RI forms. The BD-cellulose column is a useful technique in separating these RNA forms in the case of arboviruses and,

possibly, in other RNA viruses in which intermediates in the replicative cycle have similar sedimentation values.

ACKNOWLEDGMENTS

We wish to thank Marlene Gaunard and Dorothy Hughes for their excellent technical assistance.

We also thank Herbert Cooper for many useful discussions and for criticism of the manuscript.

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