THE COMPLEXING OF LYSOZYME WITH POLY C AND OTHER HOMOPOLYMERS

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ABSTRACT Lysozyme forms very large complexes with poly C, in acetate buffer solutions (pH 5.4), when the ratio of lysozyme to poly C concentration is 3/2. When this is less than 3/8 there is virtually no complexing, as evidenced by the low light-scattering power of such mixtures. At such relatively high poly C concentrations, the addition of pancreatic ribonuclease causes both the intensity and dissymmetry of scattering to rise to very high values after which time the intensity falls exponentially with time and with very little change in dissymmetry. Other homopolymers also form largest complexes with lysozyme at characteristic concentration ratios.

INTRODUCTION

Recent experiments (1) involved the simultaneous addiiton of lysozyme and very small quantities of pancreatic ribonuclease to acetate buffer (pH 5.4) solutions of poly C. The light-scattering power of the resulting mixtures increased to a very high value and then fell exponentially with time. This behavior was compared with the rate at which acid-soluble hydrolysis products appeared in the same mixtures. However, good hydrolysis data were not obtained unless the poly C concentration was greater than about 70 μg/ml. Under these conditions lysozyme (with no pancreatic ribonuclease present) showed no tendency to complex with poly C; the light-scattering power of the mixtures was hardly greater than that of the buffer itself. Consequently, it was suggested that the intensity and dissymmetry of the light scattered from such mixtures rose, upon addition of ribonuclease, because the enzyme produced oligonucleotides that fell within a range of sizes favorable for complexing with lysozyme. The scattering eventually dropped because these oligonucleotides eventually become too small for complexing.

However, it is not a critical range of oligonucleotide sizes that is important in this phenomenon; rather it is a critical concentration of oligonucleotides greater than some minimal size. Experimental results described in the present report confirm this. Lysozyme complexes directly with poly C of high molecular weight provided that the concentration of the latter is low enough (the condition deliberately avoided earlier (1) in order to get good hydrolysis data). At pH 5.4 the largest complexes are formed when lysozyme concentration/poly C concentration is 3/2.

Other such ratios exist for poly G, poly A, and poly U. They are much more critical for polypyrimidines than for polypurines.

MATERIALS AND METHODS

Specifications, suppliers, and notations used for all compounds are listed in Table I.

Light-Scattering Measurements

9 ml of 0.1 M acetate buffer solution (pH 5.4) containing up to 200 μg of polyribonucleotide per ml was deposited in 25 mm diameter light-scattering cells. Solutions of pancreatic ribonuclease and lysozyme were made with the same buffer, the respective concentrations depending on the type of experiment desired. In some experiments these solutions were mixed and 3 ml of the mixture was added to the contents of the light-scattering cells (bringing the volume up to 12 ml). In others the proteins were added separately, but always as 1.8 ml of lysozyme and 1.2 ml of ribonuclease solution.

TABLE I
MATERIALS

Compound	Specifications and supplier
Polycytidylic acid (poly C)	Potassium salt, control numbers 27622 and 21724 from Miles Laboratories, Inc., Elkhart, Indiana.
Polyguanylic acid (poly G)	Sodium salt, control number 5272 from Miles Laboratories, Inc.
Polyadenylic acid (poly A)	Potassium salt, control number 110638 from Miles Laboratories, Inc.
Polyuridylic acid (poly U)	Ammonium salt, control number 41855 from Miles Laboratories, Inc.
Ribonuclease	Pancreatic, 5× crystallized, lot 59616 from General Biochemicals, Chagrin Falls, Ohio.
Lysozyme	3× crystallized, lot 50433, from General Biochemicals.

A Brice-Phoenix light-scattering photometer was used to obtain all measurements. Its sensitivity was kept constant at the same value used in obtaining earlier data (1). The angular distribution of scattered-light intensity was obtained as previously described (1). Only the measurements at 45° and 135° with the transmitted beam are shown on the figures since they are sufficient to obtain the information desired here. The ordinate (G) on the figures is the galvanometer deflection, corrected as previously described (1). It is numerically equal to about $2.5 \times 10^5 R$, in which R is the Rayleigh ratio (2). The 4358-A Hg line was used in all experiments.

Measurement of Hydrolysis Products

These data were obtained when homopolymer concentration was high enough as previously described (1). They again showed that hydrolysis by ribonuclease was complete just beyond the time when the scattered-light intensity was highest.

RESULTS AND DISCUSSION

Effects of Varying Poly C Concentration in the Absence of Ribonuclease

3 ml of buffer solution containing 60 μg of lysozyme per ml was added to 9 ml of the buffer containing various weights of poly C per ml. Results are shown in Fig. 1. After lysozyme addition the solutions contained 15 μg of lysozyme and the designated weights of poly C per ml. Before protein addition G_{45} was always less than 20.

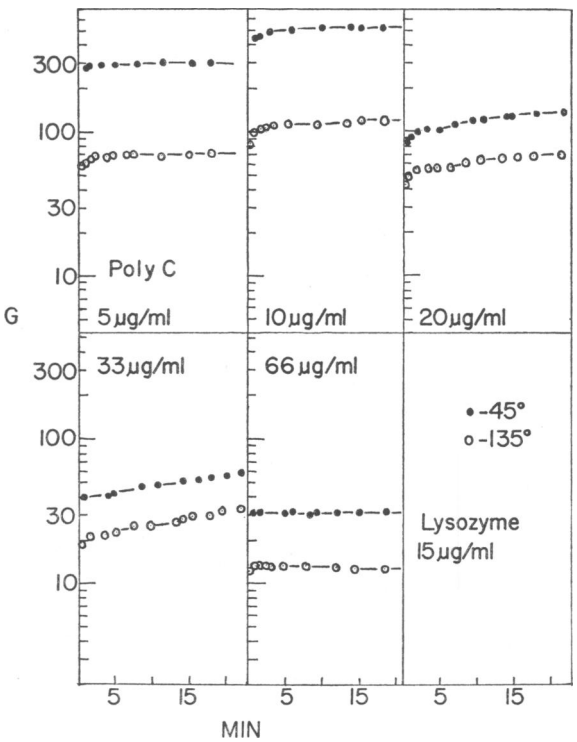

FIGURE 1 Curves of scattered-light intensity vs. time, obtained by adding lysozyme to solutions containing various amounts of poly C. Final concentrations are shown on the figure. The ordinate (G) on this and all following figures is numerically equal to about 2.5×10^5 R, in which R is the Rayleigh ratio. Before lysozyme addition, G_{45} was never greater than 15 at these concentrations.

The data show that the largest complexes are formed at about 10 μg of poly C per ml. The dissymmetry (G_{45}/G_{135}) is highest at this poly C concentration, approaching five. Thus, the scattering centers are very large and probably more round than long (1, 2). As poly C concentration is increased, both the intensity and the dissymmetry of scattering decrease until at greater than 70 μg of poly C per ml, the light-scattering power of the mixture is not much greater than that of the buffer itself.

Similar experiments were carried out in which poly C concentration was varied in the presence of other fixed lysozyme concentrations. Some such results are shown in Fig. 2. Scattered-light intensities, 15 min after lysozyme addition, are plotted as a function of poly C concentration. The dissymmetry is always highest just before

G_{45} is greatest. Arrows on the figure indicate the points of maximum dissymmetry. They always occur when lysozyme concentration/poly C concentration is $\frac{3}{2}$. The G's are necessarily lower at 7.5 μg of lysozyme per ml, because there are fewer scattering centers per unit volume of solution; however, the dissymmetry shows that they are just as large as they are at higher lysozyme (and poly C) concentrations. The proper concentration ratio determines their size. This is not a unique property of poly C. Experiments like those shown in Fig. 1 were carried out with poly G,

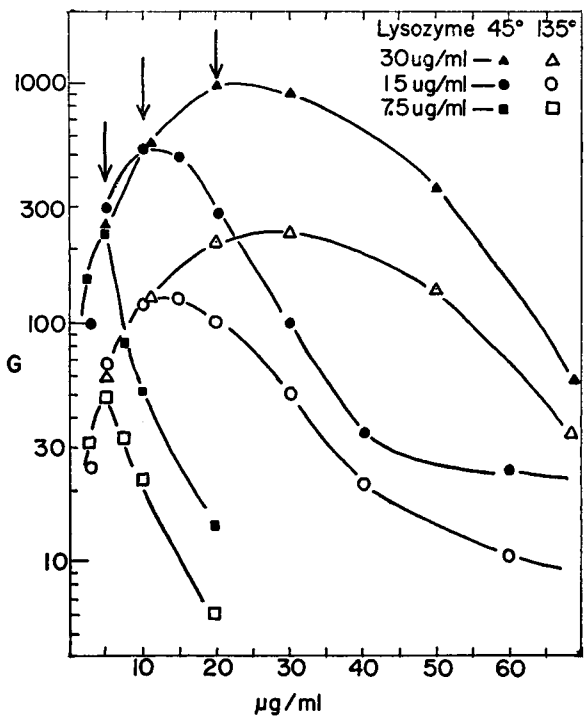

FIGURE 2 Curves of scattered-light intensity vs. poly C concentration 15 min after lysozyme addition in experiments such as those shown in Fig. 1. Note that the points of highest dissymmetry (arrows) occur just to the left of the peaks when the concentration ratio is 3 to 2 in favor of lysozyme.

poly A, and poly U. Scattered-light intensities 15 min after lysozyme addition are plotted as a function of homopolymer concentration in Fig. 3.

The dissymmetry is always highest just to the left of the G_{45} peaks. This region is much more sharply defined for polypyrimidines; in fact there is hardly a dissymmetry change worth noting near the broad poly A maximum. Lysozyme concentration/homopolymer concentration is 15/10 (poly C), 15/9 (poly A), 15/6 (poly U), and 15/22 (poly G). These ratios are preserved at other lysozyme concentrations. At about 11 μg of lysozyme per ml, the poly U curve looks like the poly C curve of Fig. 3, the only difference being that the peak occurs at a much lower poly U concentration.

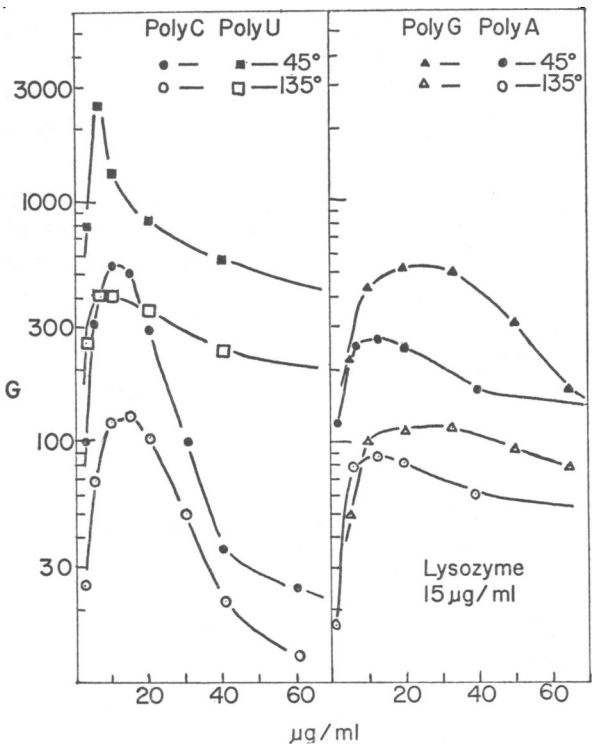

FIGURE 3 Curves like the 15 μg/ml curve of Fig. 2, obtained with all four homopolymers. Note that polypyrimidine curves have much sharper peaks.

Effects of Including Pancreatic Ribonuclease in the Mixtures

This enzyme has little or no effect on lysozyme-polypurine complexing (see Fig. 5 of reference 1). However, it drastically modifies the complexing of lysozyme with polypyrimidines because it catalyzes complete hydrolysis of the latter.

3 ml of buffer solution containing 2 μg of ribonuclease and various weights of lysozyme per ml was added to 9 ml of the same buffer containing 200 μg of poly C per ml. After protein addition the mixtures contained 0.5 μg of ribonuclease, 150 μg of poly C, and the designated weights of lysozyme per ml. Results are shown in Fig. 4.

The light-scattering power of the mixtures rises more slowly at the lower lysozyme concentrations. At 12.5 and 15 μg of lysozyme per ml there is a region of low dissymmetry clearly visible to the left of the peaks. The peaks shift toward lower times with increasing lysozyme concentration. These observations may be compared with those results shown in Fig. 2, which do not involve ribonuclease.

Lysozyme does not complex with cytidylic acid but does with poly C of high molecular weight. Therefore, there must exist some hydrolysis fragments of some minimal length that are able to form such complexes. Pancreatic ribonuclease can-

not avoid reducing the concentration of these poly C hydrolysis fragments. When this concentration reaches some critical value (near ⅔ that of the lysozyme present according to Fig. 2) the largest complexes are formed. Hence, the scattering peaks move to the left with increasing lysozyme concentration, because the critical concentration ratio is reached in shorter times.

Other experiments were carried out in order to further clarify the role played by pancreatic ribonuclease. 1.2 ml of the buffer containing 5 μg of ribonuclease per ml was added to 9 ml of the buffer containing 200 μg of poly C per ml. 1.8 ml of the

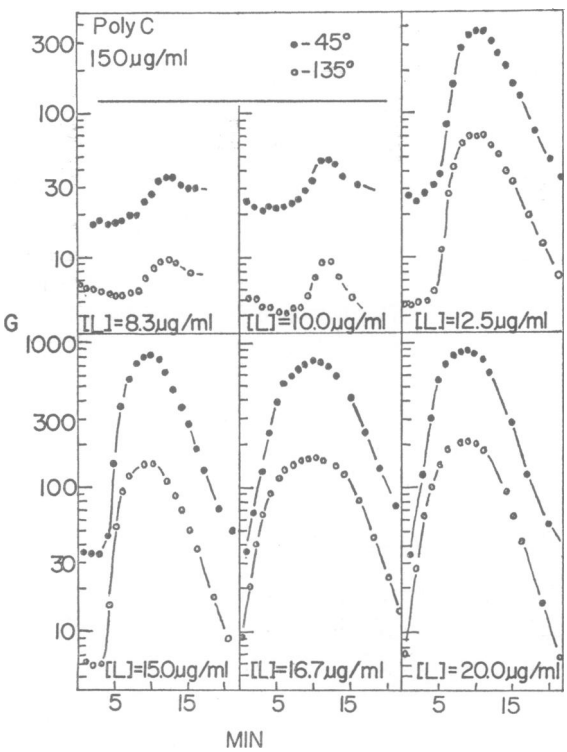

FIGURE 4 Curves of scattered-light intensity vs. time obtained by the simultaneous addition of ribonuclease and various amounts of lysozyme to poly C solutions. After addition the solutions contained 0.5 μg of ribonuclease, 150 μg of poly C, and the designated weights of lysozyme (L) per ml.

same buffer, containing 93 μg of lysozyme per ml, was added at some later time. The data (Fig. 5) show how the scattered-light intensity changes with time after all three components are present. The negative numbers are the delay times in minutes between ribonuclease and lysozyme additions. With all components present the concentrations were 0.5 μg of ribonuclease, 14 μg of lysozyme, and 150 μg of poly C hydrolysis products per ml.

The time (t_m) of maximum scattering at 45° is related to the delay time (t_d) by the simple formula

$$t_m + t_d = 11. \quad (1)$$

When $t_d > 11$ there is no significant rise in turbidity. The solution is a mixture of the proteins and cytidylic acid, i.e., there is nothing left for lysozyme to complex with.

The scattered-light intensity decays exponentially with time beyond the maxima. The rate of decay is not markedly influenced by the time (t_d) separating protein additions. Nor was it influenced markedly by lysozyme concentration changes (Fig. 4). However, it is strongly dependent on ribonuclease concentration as shown by the following experiment.

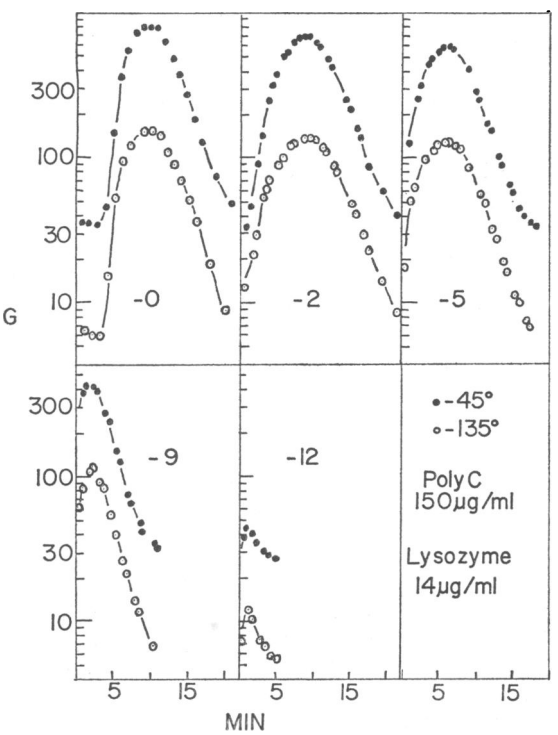

FIGURE 5 Results obtained by adding ribonuclease first and lysozyme at some later time. The negative numbers are the delay times (in minutes) between these steps. With all components present the solutions contained 0.5 μg of ribonuclease and the designated weights of lysozyme and poly C fragments per ml.

3 ml of the buffer, containing 60 μg of lysozyme and various weights of ribonuclease per ml was added to 9 ml of the buffer containing 111 μg of poly C per ml. Results are shown in Fig. 6. After protein addition the solutions contained 15 μg of lysozyme, 83 μg of poly C, and the designated weights of ribonuclease per ml. At this poly C concentration there is very little complexing without ribonuclease (see Fig. 1).

The rates at which scattering both rises and decays are strong functions of ribonuclease concentration. Insignificant quantities of ribonuclease bring about a rise in scattering; in fact, experiments like these showed that some care had to be exercised to make sure that no trace of this enzyme got into solutions where its nominal

concentration was supposed to be zero. Note that the intensity (and the dissymmetry) of scattering is about the same at the peaks even though these occur at widely different times.

All of the above results suggest experiments in which lysozyme-poly C complexes are first allowed to form and the ribonuclease is added after the initial complexing has virtually ceased. In these experiments 1.8 ml of buffer containing 100 μg of lysozyme per ml was added to 9 ml of buffer containing various weights of poly C per ml. The intensity of the light scattered at 45° and 135° was then determined as a

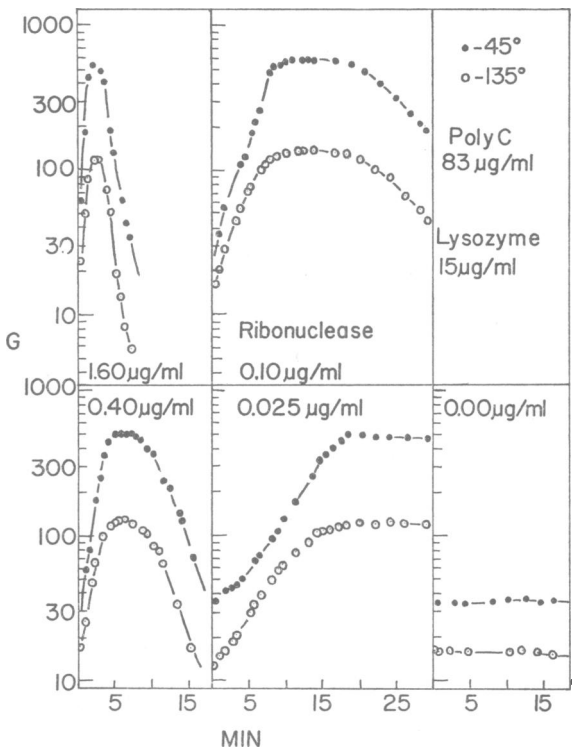

FIGURE 6 Results obtained by varying ribonuclease concentration. Final concentrations are shown on the figure.

function of time. At 15 min, 1.2 ml of buffer containing 1 μg of ribonuclease per ml was added and the scattered-light intensity was again determined as a function of time. Some of these results are shown in Fig. 7. After both proteins had been added the solutions contained 0.1 μg of ribonuclease, 15 μg of lysozyme, and the designated weights of poly C per ml. At this lysozyme concentration, the critical poly C concentration is 10 μg/ml (see Fig. 2).

In the region below 10 μg of poly C per ml the scattered-light intensity always falls at both angles upon addition of ribonuclease. The relatively high dissymmetry shows that the complexes are relatively large, even at only 3 μg of poly C per ml.

This indicates that all available poly C is bound in the complexes, that the immediate effect of ribonuclease is the destruction of this bound poly C, and hence the integrity of the complexes. Furthermore, the weight concentration of poly C fragments large enough to complex with lysozyme was already below the critical value before ribonuclease addition, after which it was reduced more.

The situation is somewhat more complicated above 10 μg of poly C per ml. At these higher poly C concentrations, fewer and smaller lysozyme-poly C complexes produce the scattering because both G_{45} and the dissymmetry are smaller

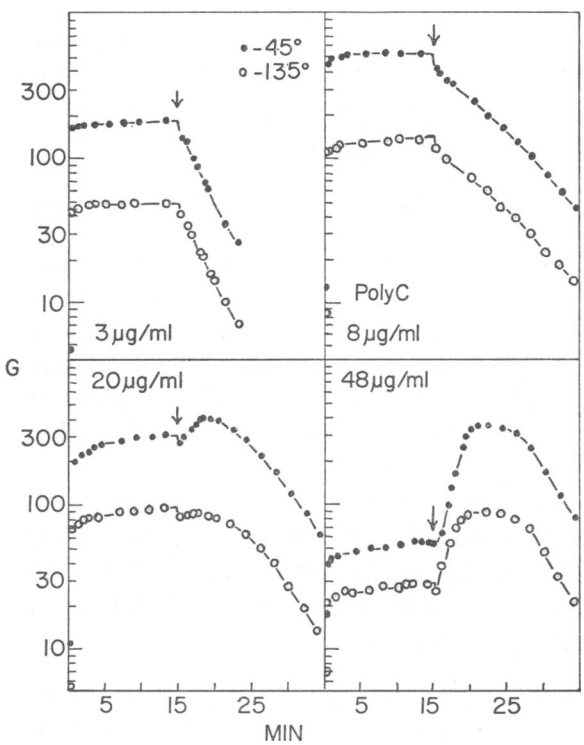

FIGURE 7 Results obtained by adding ribonuclease (arrows) 15 min after lysozyme addition. After this step the mixtures contained 0.1 μg of ribonuclease, 15 μg of lysozyme, and the given weights of poly C fragments per ml.

than at 10 μg of poly C per ml. Therefore, all the poly C is not bound in complexes. It is this excess weight concentration of poly C that inhibits the formation of large lysozyme-poly C complexes (see Fig. 1). The added ribonuclease attacks both bound and unbound poly C; therefore, it is (*a*) destroying the relatively small complexes already formed and (*b*) reducing the weight concentration of poly C chains long enough to form the largest complexes with lysozyme. As the concentration of such poly C chains approaches 10 μg/ml (from the high side) larger complexes are formed with lysozyme and the number and size of these are greatest at 10 μg/ml (see the two lower curves in Fig. 7). Eventually, the exponential decay of scattering

takes over when the ribonuclease only has poly C chains, bound in complexes, left to deal with.

Exponential Decay of Scattered-Light Intensity

This decay, always observed when ribonuclease is present, follows

$$I = I_0 e^{-\alpha t} \tag{2}$$

in which I_0 is the initial intensity at any point on the straight portion of the decay curves, t is time, and α is a constant for any given mixture, the value of which only depends on ribonuclease concentration. Since dissymmetry changes little during decay of scattering, equation 2 holds at both scattering angles. This means that particle size changes little during the decay. A sensible alternative is that the number (N) of scatterers per unit volume of buffer is decreasing; i.e.,

$$N = N_0 e^{-\alpha t} \tag{3}$$

in which the decay constant is the same as in equation 2. As ribonuclease digests poly C fragments bound in complexes, the complexes maintain their general shape and size until they reach some unstable state at which time they disintegrate. The complexes reach this unstable state more or less randomly in time. The decay rate is not influenced by events occurring during the history of the solution that lead to the formation of the largest complexes. It is only determined by the concentration of ribonuclease during the decay.

Other experiments were carried out to find if the final product of hydrolysis by ribonuclease, cytidylic acid, caused the dissociation of complexes formed either in the presence or absence of ribonuclease. Up to 150 μg of cytidylic acid per ml were included in the solutions during experiments like those shown in Figs. 1 and 5. There were no changes in the formation or breakdown of the complexes.

This work was supported by grant CA-06837 from the National Cancer Institute of the U.S. Public Health Service. The author thanks Dr. Robert Hill of the School of Physics, The University, Newcastle-upon-Tyne, England for stimulating discussions concerning complex breakdown, and a referee for sound reasoning.

Received for publication 19 March 1968 and in revised form 5 July 1968.

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