HYDRODYNAMIC SHEAR BREAKAGE OF DEOXYRIBONUCLEIC ACID

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ABSTRACT

Deoxyribonucleic acid (DNA) was broken by hydrodynamic shear in a capillary. The effects of parameters associated with chemical reactions, temperature, catalysts, and ionic strength, were examined to determine the possible effect of a chemical reaction upon the breakage. No catalysts or ionic strength effects were found, but there is a large temperature effect. The approximate shear stress required to break the molecules is about one sixth of the theoretical force. The temperature and stress results are evidence for the importance of hydrolysis in the breakage mechanism.

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Large polymers, like DNA, are broken under the influence of sufficiently high mechanical shear. The shear may be applied in many ways, by sonication, in a syringe, in a high speed blendor or in a capillary tube. Aside from some biological considerations of how gently nucleic acids should be handled,[1] the main problem has been the determination of the nature of the breakage reaction, mechanical versus chemical, and interwoven with this the force necessary to break the chemical bonds involved.

The problem of determining the force necessary to break one of these molecules is complicated by most of the apparatus used to acheive the breakage. In these cases the shear stress applied to a polymer can only be crudely estimated. In capillary tubing the flow is well understood in the absence of large molecules, this provides a better basis for estimating the forces present when a long chain-like molecule, like DNA, is added. One theoretical attempt to explain the properties of a three dimensional chain in solution is made by Zimm.[2] In a capillary, with flow rates that do not produce turbulence, the most accurate estimates of forces can be made.

The breakage of the DNA is not affected by the presence of free radical trapping agents.[3] There seems to be an assumption that both strands are broken at once.

This is reasonable if the force is applied along the length of the chain since the force necessary to rupture one bond of the double chain molecule will certainly be enough to break the other immediately. Harrington and Zimm[4] believe the mechanical stress is the primary cause of breakage of polystyrene in various solvents. One of the major factors in their rejection of the hypothesis of mechanical stress aiding thermal bond breaking was the lack of temperature dependence. A strong temperature dependence is found in the breakage of DNA in my work and others. (Harrington and Zimm used DNA but seemed nore interested in polystyrene and perhaps did not check temperature dependence of DNA breakage.) Their other arguments relate to the potential function of the carbon carbon bond they are breaking. In DNA a phosphorus oxygen bond is broken. The mechanisms of breakage of different long chain polymers need not be the same.

Harrington and Zimm did notice that solvent affected the breakage. This supports the hypothesis that the mechanical deformation lowers the activation energy of a chemical reaction. In the case of DNA, the streching of a bond to a certain point facilitates hydrolysis. If this were the case one would expect that there might be temperature dependence, catalysts or dependence upon other chemical conditions.

The purpose of this work was to investigate the effects of different chemical environments upon the breakage of DNA by hydrodynamic shear in a capillary tube. DNA is useful in investigating shear breakage because the molecules are initially all the same size. The variables considered were temperature, ionic strength and the possibility of using imidazole as a catalyst. The temperature experiments allowed me to compare my results, using an electron microscope to determine breakage length distributions, with those of Ray Bowman, who used ultracentrifugation to determine distributions. The finding of a chemical catalyst would help determine the nature of the reaction, the degree to which it is chemical or mechanical. The results of the temperature investigations did compare favorably with Bowman's, the other tests were not as conclusive. There is conflicting evidence about the effects of imidazole, but any large catalytic activity is ruled out. Changing the ionic strength by one order of magnitude had no effect.

Experimental

DNA Preparation. DNA was prepared by extraction from a λ phage deletion mutant b_2b_5c by Ray Bowman in September 1966. Control experiments subjecting this preparation to all of the procedures except the capillary shearing process resulted in total broken DNA less than 5% by weight. This mutant DNA is known to be $13.2 \pm 1.5\ \mu$ long.[5]

Electron Microscopy. The DNA samples were prepared by the method of Wetmur, Davidson, and Scaletti[6], uranyl acetate staining of a Kleinschmidt mounting. To increase contrast, the grids were then rotary shadowed by evaporation of a 80% platinum-20% palladium foil at an angle of 7°-8°. Enlarged projections of negatives taken in a Phillips EM 200 electron microscope were traced and measured with a map measurer (accuracy ± 3%). Total magnification was 50,400. These methods resulted in a measurement of 10.4 ± 0.5 μ for the length of whole λ $b_2 b_5$c DNA. The discrepancy is probably due to variation in Kleinschmidt technique which effects the observed length of DNA. The important point is that my measurements are self consistent.

Breakage of DNA. The standard breakage solution was 0.00025 M EDTA, 0.1 M Na^+, 0.005 M phosphate buffer at pH 7.0, and DNA at an optical density of 0.05 OD or about 2.5 μg/ml, which is a smaller concentration than that at which self-protection occurs.[7] The solutions were heated at 75° C for five minutes and quenched in an ice bath for fifteen minutes to separate the "sticky ends." In order to remove small particles that might create turbulence in the capillary, the solutions were filtered through a 8 μ millipore filter by gravity into the breakage apparatus. The DNA was sheared inside a capillary of 0.038 cm. inside diameter and 24.5 cm. in length. The size of a breakage solution

was 10 ml. and 2.5 ml. of that did not pass through the capillary each pass (dead space). Presumably there is some mixing so that over a large number of passes all of the DNA has spent an equal amount of time in the capillary. The solutions were passed through this capillary by nitrogen gas at 34 psi. The apparatus sat in a water bath for temperature control. Samples (0.3-0.6 ml.) were taken after various numbers of passes. These samples had to be heated to 75°C, quenched again before nounting on electron microscope grids. The percentage broken was the weight percentage of halves measured out of the total of halves and wholes. Intermediate (3/4) sizes and pieces smaller than 3,5 μ were not considered. Other procedures found unacceptable were estimating the size (whole, half or quarter) by direct observation in the electron microscope and printing the microscope negatives and then measuring. The estimation was grossly inaccurate and the printing was more inconvenient than tracing.

Results

The first effect investigated was that of temperature. Three temperatures were used 26°, 46°, and 7°C. and the results were significant. The agreement with Ray Bowman's work is good. Results are in figures 1,2, and3. At 46°C the probability of breakage was about p= 0.05. This means that 5% of the whole molecules are broken into halves on each pass. At 26°C, the first three points follow the

theoretical breakage at p = 0.01, but the amount broken at 300 passes corresponds to p =0.003. At 7° C essentially none of the material is broken.

The effect of imidazole on the shear breakage of DNA was investigated. The 0.005 M phosphate buffer in the standard solution was replaced with 0.05 M imidazole/ imidazolium buffer at pH 7.0. The results are shown in Figures 1 and 4. Two experiments were done, supposedly identical conditions. The results of the first were very close to those of phosphate buffered solutions; the second followed a p = 0.01 breakage curve all the way to 300 passes. The 90% broken point in this experimental curve is based on finding one whole molecule.

The low ionic strength experiment was done with total Na^+ concentration 0.01 M. The results are in Figures 1 and 3. Keeping DNA in the cold for a week at this ionic strength did not affect the percentage of wholes.

Statistical Significance

Most of these percentages are based on samples of about size 20. Assuming that each molecule photographed is either a half or a whole, then Figure 5 gives 95% confidence limits of accuracy of estimating the percentage of half molecules[8]. This is generally around $\pm$ 20 %.

Discussion and Conclusions

The first problem is the poor fit between the experimental curves and the theoretical curves except in two experiments. The most likely reason is that after three samples have been taken, the dead volume composes almost one third of the solution. Since DNA is not very mobile in solution, the mixing is probably not perfect and some DNA never passes theough the capillary. Any other reason must involve postulating a decrease in the probability of breakage.

From many previous experiments the breakage pattern expected is bimodal. The parabolic distribution of stress causes molecules to brak in the middle, producing a set of molecules one half the original length. This is nicely demonstrated in the standard conditions breakage pattern (Figure 2). In Figure 3a one can see the rise of a third mode, the quarter nolecules. At 100 passes there were many quarters. This is the only experiment in which high enough shear and temperature were used to produce quarters. The ionic strength experiment breakage patterns do not show bimodality and must be placed in doubt for this reason. The imidazole breakage patterns do exhibit bemodality, with one exception discussed later. It must be remembered that we are dealing with very small statistical samples, so the peaks are not expected to be sharp.

The two imidazole experiments are contradictory. The first one, which resembles closely the reults in phosphate buffered solution, has a strange distribution at 300 passes. There appear to be quarters present which also appear in the second experiment; this would argue in favor of catalytic activity. There are also apparently 3/4 molecules which would suggest random breakage or, more likely, an experimental error inthis sample. The second experiment indicates activity by its value at 300 passes. The percentage broken is just that predicted at p = 0.01, the breakage probability calculated from up to 100 passes in both phosphate and imidazole solutions. Perhaps this one run gave "proper mixing". In any case it is shaped differently than the others, suggesting an anomaly somewhere.

It is quite clear that imidazole does not exert large effects upon the rate of breakage of DNA. To determine with more certainity if it has a positive effect on the rate larger statistical samples in further experiments would be needed. Double stranded DNA was allowed to set in imidazole buffer for a week with no effects.

The change of an order of magnitude in salt concentration didnot obviously affect the rate of breakage of DNA. However there was a large amount of material of a length which was intermediate in length between whole and half molecules. This was not reproduced due to lack of time, and there are many possible sources of error, such as

improper quenching, turbulence caused by ~~causing~~ particles in the breakage solution, or the effect of sodium concentration on the Kleinschmidt technique.

The laminar flow of fluid in a capillary follows Poiseuille's Law,

$$v = \frac{2V}{\pi a^2}\left(1 - \frac{r^2}{a^2}\right)$$

a = radius of capillary
r = 0 at center of capillary
v = fluid velocity at r
V = volume rate of flow

This parabolic flow pattern sets up a linear shear gradient ,G

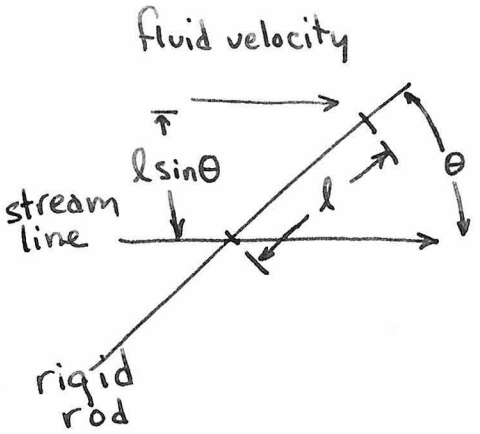
$$G(r) = \frac{4V}{\pi a^2}\,\frac{r}{a^2}$$

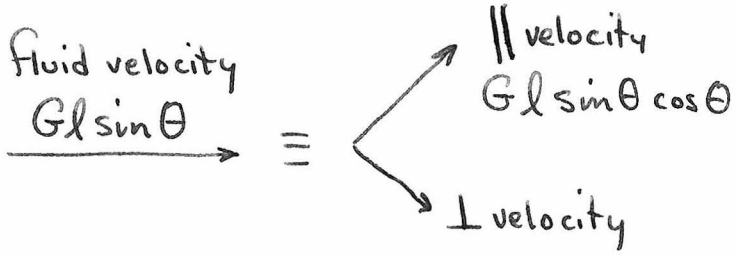
The maximum shear occurs at maximum r which is a, that is, at the wall of the capillary. So the maximum shear in laminar flow in a capillary is

$$G_{MAX} = \frac{4V}{\pi a^3}$$

From the volume flow rates of 0.93, 0.64, and 0.38 cm^3/sec, we can calculate the maximum shear in the solutions at 46°, 26°, and 7°C. These shears are 173,000, 120,000, and 71,000 sec^{-1}.

Approximating DNA with a rigid rod we can calculate the shearing force which is applied by the interaction of the solvent with the molecule, Translating the origin to the center of the molecule, the (relative) velocity of the fluid passing a point 1 on the rod, tilted at an angle θ to the streamlines, is the linear gradient times the distance fromthe origin, $G \cdot 1 \cdot \sin\theta$.





This velocity may be resolved into a parallel velocity and a perpendicular one which will not be considered,. Assuming a frictional coefficient, f, the frictional force per unit length at a distance, l from the origin is $f \cdot G \cdot l \times \sin\theta \cos\theta$ away from the origin. The tension, τ, at some point x is

$$\tau = \int_{L/2}^{X} -f G l \sin\theta \cos\theta \, dl$$

This is greatest at x = 0 or the middle of the molecule. The tension is a parabolic function of distance from the origin.

$$\tau(0) = \frac{f G L^2}{8} \sin\theta \cos\theta$$

L = length of molecule

The maximum tension exerted on the molecule is when $\theta = 45^\circ$ and it is at r = a. The tension is

$$\tau_{MAX} = \frac{f G_{MAX} L^2}{16}$$

From these considerations, we can see that the molecule should break in the middle and near the wall of the capillary. With an understanding of the frictional force, we could compute the breaking force, Davison[7] gives $f = 3\pi\eta/(\log L/\rho)$.

Letting L = 10μ, ρ = 20 Å, we arrive at the following values for τ, using the viscosity of water which is realistic.

Temp.	η	τ
46° C	0.588 poise	0.0150 poise
26°	0.873	0.0223
7°	1.428	0.0363

The tension at the center of the molecule subjected to the maximum forces is 1.7×10^{-4} dyne. This is one sixth the value Davison calculated for breaking DNA. Since Harrington and Zimm[4] suggested that this is probably an upper limit, applicable in the case of pure mechanical breakage, this agreement is encouraging. A chemical assist could account for the extra energy necessary to break the bond. The gross assumptions in this account include the rigid rod in place of a very flexible coiled chain, laminar flow in place of a locally turbulent, complicated flow around the DNA, and the calculation of the frictional coefficient.

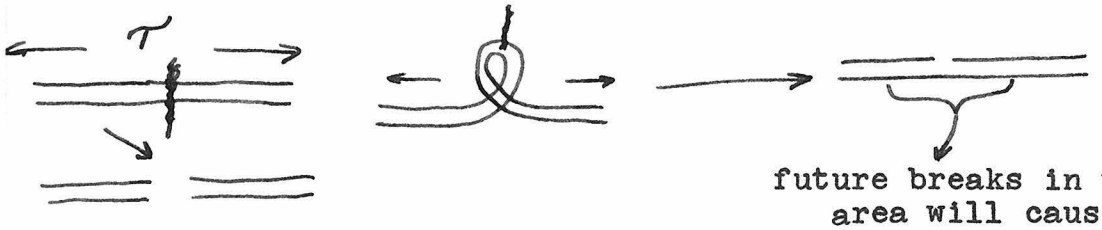
In calculations of the shear kinetics it is important to know what percentage of the material travels outside a certain radius. Most of the material flows through the middle.

Amount flowing outside R	R
1%	0.95a
0.1%	0.984a
0.01%	0.995a

Under experimental conditions at least 90% of the material broken is inside .98a, but a great deal of it might be expected to be outside .95a. This means that the shearing

force on the molecules broken is within five percent of the maximum shear, the quantity used in calculations.

Two types of breakage can be envisioned, one in which the end to end tension pulls two bonds apart simultaneously, the other involving extreme bending and sequential breaking.



DNA with single strand nicks could be broken to determine the relative difficulty of breaking one and two strands. Levinthal and Davison[7] indicate that P^{32} labeled DNA becomes easier to break upon sitting presumably because of single strand breaks caused by radioactive disintegration. If breaking nicked DNA required about half the shearing force needed to break unnicked DNA, this would support the idea that both strands are broken simultaneously, the critical force being twice that needed to break one strand. If in order to complete breakage of the double chain, the second chain has to be broken somewhere near the first break and in a similar manner, then one would expect nicked DNA to require the same shear stress as the unnicked. Unfortunately nicking is a random process, but radioactive DNA could be hybridized with cold DNA to put nicks on only one strand. If single strand breaks could be found to be formed in capillary passage then this hypothesis would be furthered.*

However, lacking direct evidence, the extreme kinking that must be necessary(after all DNA supercoils) makes this breakage mechanism less likely.

The dramatic effect of temperature upon the rate of breakage under constant shear stress seems to imply that a chemical reaction, probably hydrolysis is very important in the final breakage. If, however, the breakage is related to sharp bends of the DNA it is possible that the slower flow rates at low temperatures do not "tighten kinks" rapidly enough to cause breakage. What is needed is further evidence of a mechanically assisted chemical reaction, which I did not find, and studies of the same shear rate at different flow rates(which requires a different solvent).

From the calculations of shear it is not easy to see why the DNA breaks more readily at 46°C and does not break at all at 7°C. This change in volume rate of flow under the same pressure is cancelled by a change in viscosity with temperature. The shear stress, ηG(η is found in f), is the significant quantity in calculating breakage.

The overall reliability of this work is low, because of experimental difficulties and statistical considerations of the data. The negative conclusions drawn from it, limits of possible effect of imidazole and ionic strength are valid. The advantage to using the electron microscope in place of the centrifuge is that the length distributions can be determined very accurately at any concentration, if the Kleinschmidt procedure is properly standardized.

A large temperature effect was found which agreed with other, previous, work. This could be caused by an increase of the rate of a mechanically assisted chemical reaction or by increased bending (but not shear stress) due to an increased flow rate. Imidazole in high concentration is suspected of increasing the rate of breakage. This increase cannot be great, at most twofold. No change inrate was observed accompanying a change in ionic strength. The comparison of theoretical and demonstrated forces necessary to break DNA, and the large temperature effect imply that a chemical reaction is an integral part of the breakage mechanism. The shear can be regarded as lowering the activation energy of the reaction. This work does not indicate whether a concerted two strand break or a sequential process of single strand breaks are involved. Both are compatible with a chemical reaction.

Acknowledgements

I would like to acknowledge the generous help, both practical and theoretical given me by Dr. Davidson, Dr. Callis and Ray Bowman.

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* Single strand breaks are part of Ray Bowman's current research.

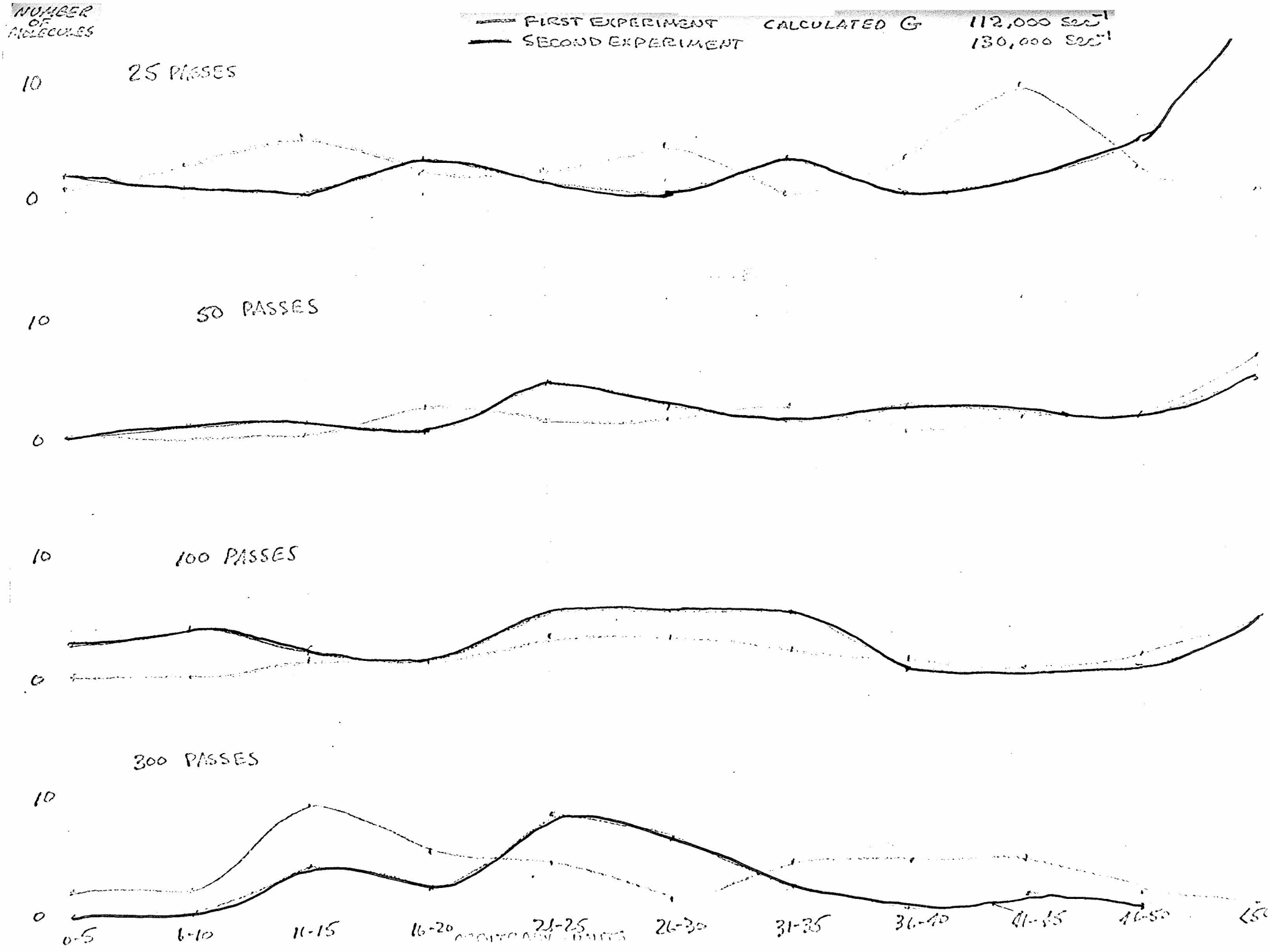
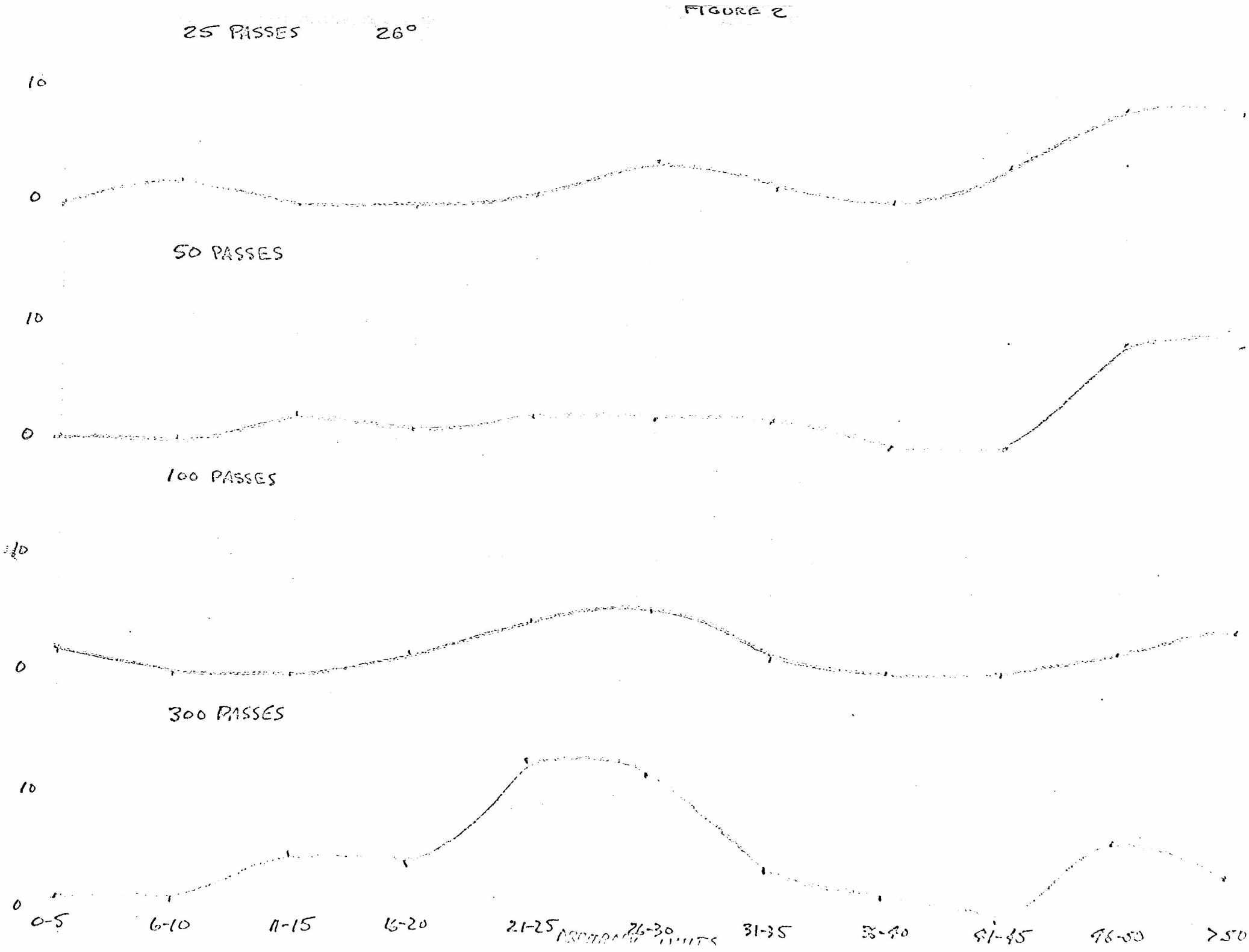

NUMBER OF MOLECULES
FIRST EXPERIMENT
SECOND EXPERIMENT
CALCULATED G 112,000 sec⁻¹
130,000 sec⁻¹
25 PASSES
10
0
50 PASSES
10
0
100 PASSES
10
0
300 PASSES
10
0
0-5
6-10
11-15
16-20
21-25
26-30
31-35
36-40
41-45
46-50

FIGURE 2


25 PASSES 26°
10
0
50 PASSES
10
0
100 PASSES
10
0
300 PASSES
10
0
0-5
6-10
11-15
16-20
21-25
26-30
31-35
36-40
41-45
46-50
>50

FIGURES

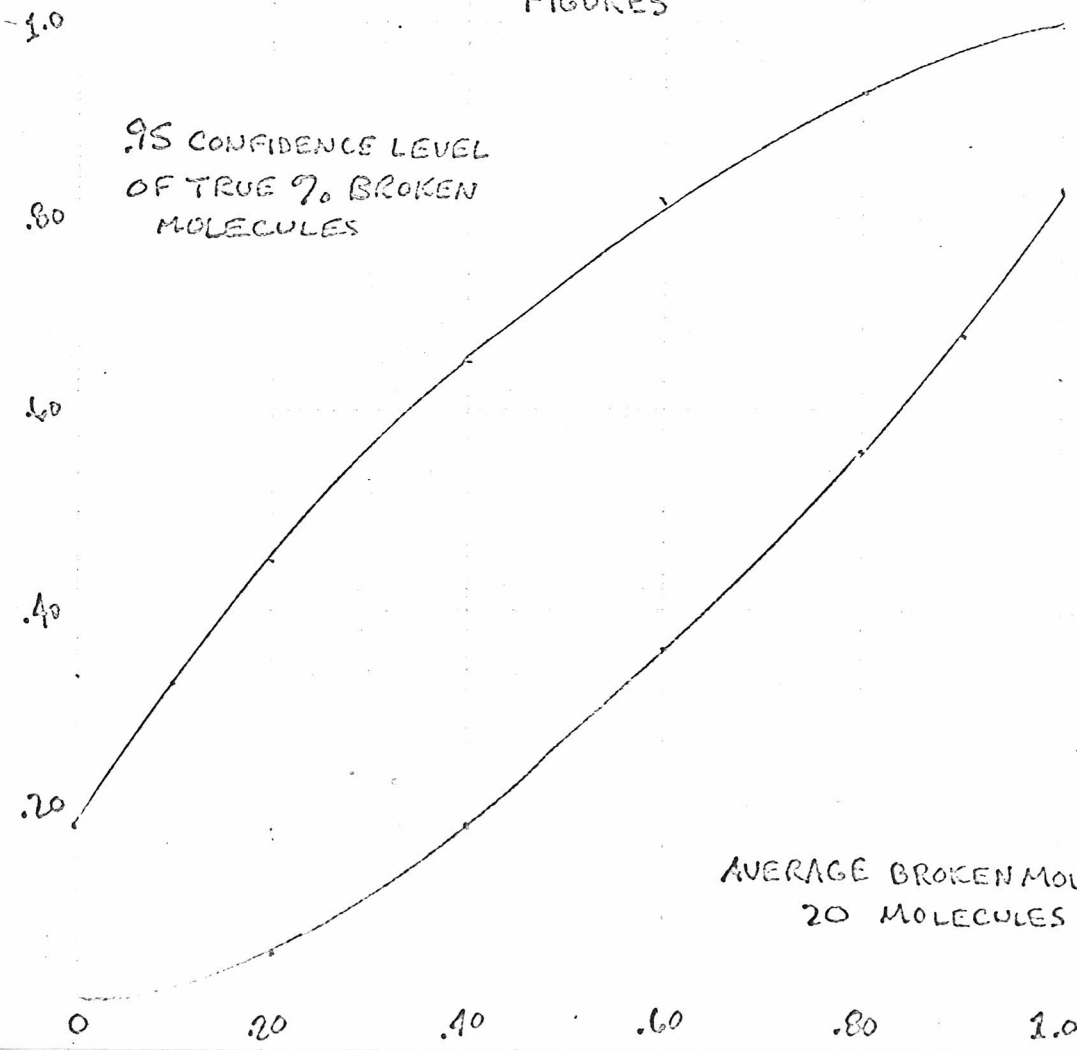

1.0
.80
.60
.40
.20
95 CONFIDENCE LEVEL OF TRUE % BROKEN MOLECULES
AVERAGE BROKEN MOLECULES 20 MOLECULES
0
.20
.40
.60
.80
1.0

FIGURE 1

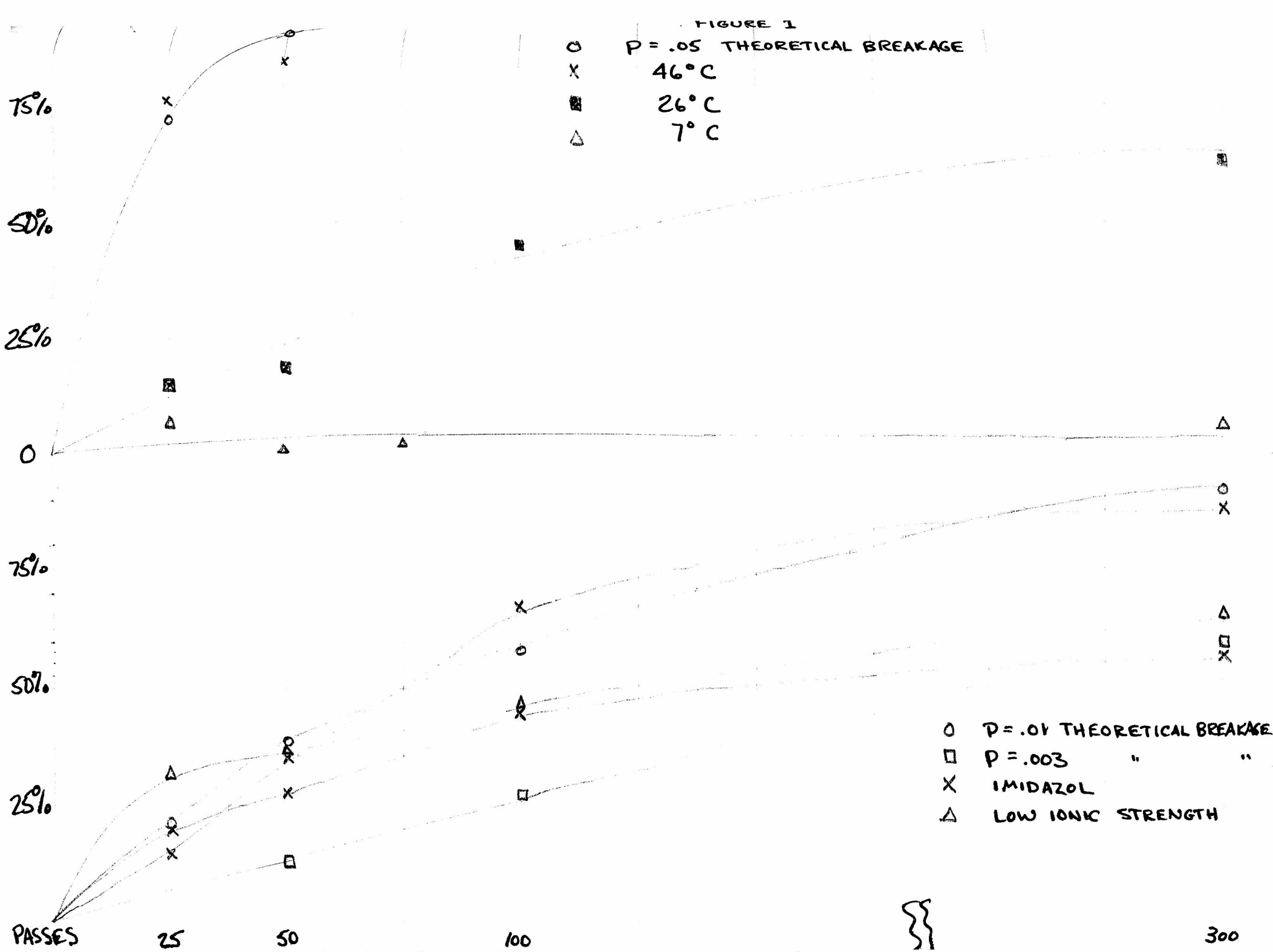
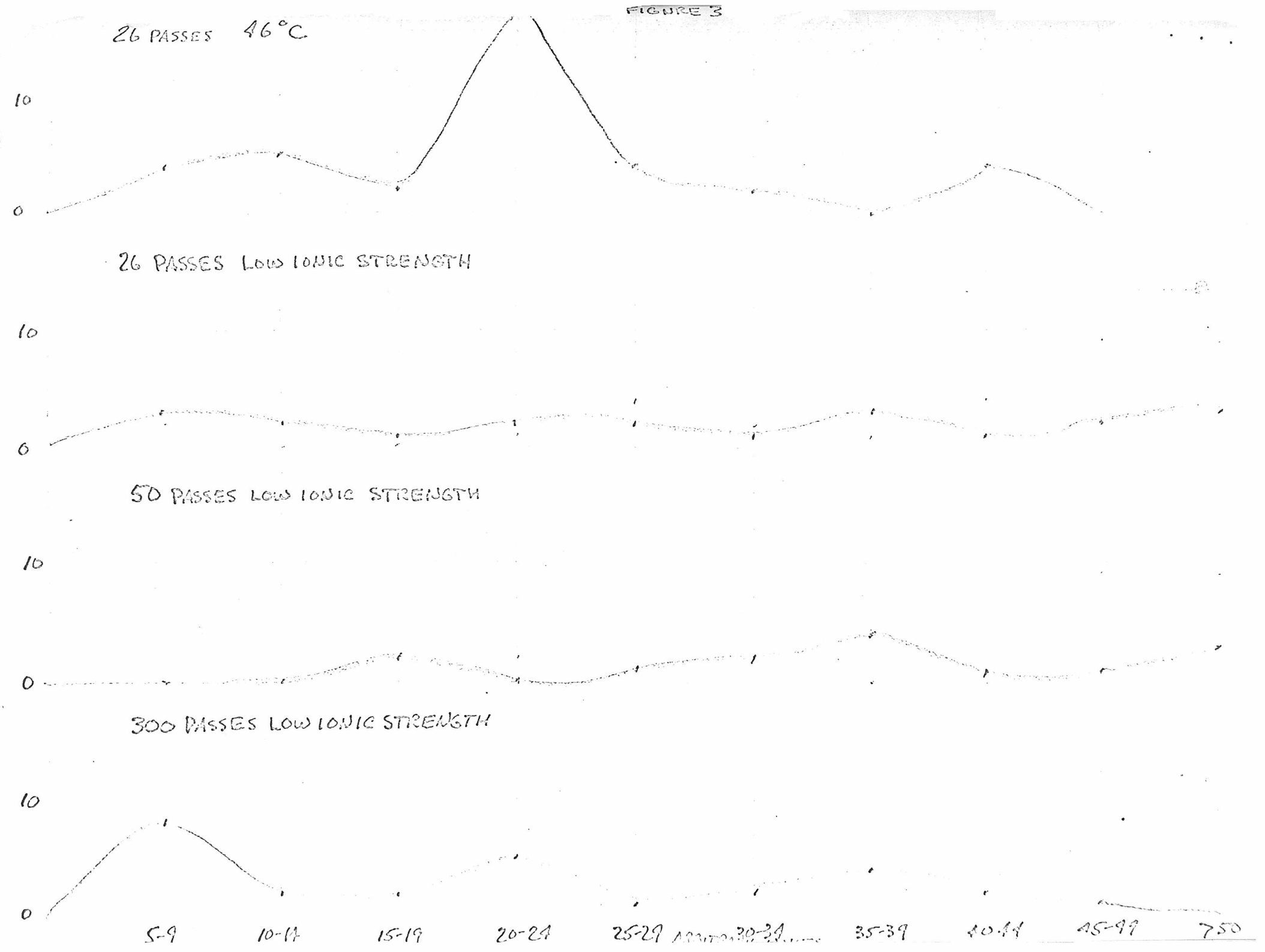

75%
50%
25%
0
75%
50%
25%
PASSES
25
50
100
300
○ P = .05 THEORETICAL BREAKAGE
X 46° C
■ 26° C
△ 7° C
○ P = .01 THEORETICAL BREAKAGE
□ P = .003 " "
X IMIDAZOL
△ LOW IONIC STRENGTH

FIGURE 3


26 PASSES 46°C
10
0
26 PASSES LOW IONIC STRENGTH
10
0
50 PASSES LOW IONIC STRENGTH
10
0
300 PASSES LOW IONIC STRENGTH
10
0
5-9
10-14
15-19
20-24
25-29
30-34
35-39
40-44
45-99
750