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Physical Properties of DNA from Synchronously Grown Mesophilic and Thermophilic Bacteria

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PHYSICAL PROPERTIES OF DNA FROM SYNCHRONOUSLY GROWN MESOPHILIC AND
THERMOPHILIC BACTERIA

by

CAROL A. COX

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Master of Arts
Department of Chemistry

Western Michigan University
Kalamazoo, Michigan
December 1980

ACKNOWLEDGEMENTS

The author wishes to express her deepest gratitude to Dr. Jochanan Stenesh for his guidance and patience during the course of her graduate studies. Sincere appreciation goes also to Dr. Robert H. Anderson for his computer programs; to David B. Cox and R. Kevin Bannow for their support and aid in the completion of this work.

Finally, acknowledgement is due the Department of Chemistry at Western Michigan University for financial support and intellectual training.

Carol A. Cox

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PHYSICAL PROPERTIES OF DNA FROM SYNCHRONOUSLY
GROWN MESOPHILIC AND THERMOPHILIC BACTERIA.

WESTERN MICHIGAN UNIVERSITY, M.A., 1980

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INTRODUCTION

The first thermophilic bacterium was isolated by Miquel in 1879 (Allen, 1953). Thermophiles are bacteria which preferentially grow at elevated temperatures (50-80°C), at which many macromolecules necessary for life are ordinarily destroyed. Mesophiles, on the other hand, grow at more moderate temperatures (20-45°C) where macromolecular denaturation is no longer a problem.

Many thermophilic organisms have been isolated from a variety of sources (Allen, 1953). Work done in this laboratory utilizes two species of bacteria from the genus Bacillus. The thermophile is B. stearothermophilus and the mesophile is B. licheniformis. The present work was performed to try and learn more about the phenomenon known as thermophily, particularly as to the possible role played by the relative physical properties of DNA.

To date, three major theories have been presented to explain the phenomenon of thermophily. The first of these suggests that thermophily results from the presence of heat stable lipids (Gaughran, 1947). The second accounts for the phenomenon by postulating a system involving rapid rates of protein synthesis and degradation (Allen, 1953). The third and most popular theory, views thermophily as the result of physical-chemical differences between important macromolecules of the thermophile and those of the mesophile (Campbell, 1968).

The third theory has received considerable experimental support thus far. Most of the supporting evidence comes from comparative studies of proteins and nucleic acids from thermophilic and mesophilic bacteria. The proteins glyceraldehyde-3-phosphate dehydrogenase (Amelunxen, 1966 & 1969), aspartokinase (Kuranutsu, 1968) and peptidase (Matheson, 1967) isolated from B. stearothermophilus have all shown a higher degree of thermal stability than corresponding proteins from mesophiles. The α -amylase isolated from B. coagulans grown at 55°C was found to be more heat stable than the same enzyme isolated from the organism grown at 37°C (Koffler, 1957).

Previous work from this laboratory, in support of the third theory, has shown that thermophilic deoxyribonucleic acid (DNA; Stenesh, 1968) and thermophilic ribosomal ribonucleic acid (Stenesh, 1967a) are more heat stable than the corresponding mesophilic macromolecules. Additional work has dealt with differences in fatty acids (Shen, 1970), ribosomes (Stenesh, 1967b), ribosomal proteins (Pickett, 1973) as well as a comparison of the fidelity of DNA replication under in vivo conditions (Gupta, 1978).

The latter study indicated some differences in the apparent fidelity of DNA replication for mesophiles and thermophiles as a function of growth temperatures. That study involved a chemical analysis of the DNA based on the incorporation of labeled nucleosides.

The present study represents a follow-up of this work in which

the DNA is subjected to physical analysis. Specifically, heat denaturation profiles and viscosities were determined for DNA isolated from synchronously grown cultures of B. licheniformis and B. stearothermophilus, each grown at two temperatures. The purpose of this study was to determine whether the differences in the apparent fidelity of replication are such that they lead to measurable differences in the physical properties of the DNA.

MATERIALS AND METHODS

Chemicals

Cells:

Bacillus licheniformis (NRS 243)
Bacillus stearothermophilus 10

Nutrient medium:

Bactoagar - Difco
Trypticase - BBL
Yeast extract - Difco

DNA isolation:

Ethylenediaminetetraacetic Acid (EDTA) - Aldrich
Sodium Chloride - Mallinckrodt
Sodium Citrate - Merck
Sodium Hydroxide - Mallinckrodt
Lysozyme (No. L6876) - Sigma
Sodium Lauryl Sulfate - Sigma
Sodium Perchlorate - Fisher Scientific
IsoAmyl Alcohol - Sigma
Chloroform - Mallinckrodt
Ribonuclease-A (No. R-5000) - Sigma
Ethanol (95%) - Mallinckrodt

Viscosity:

Acetone - Mallinckrodt

Reagents

DNA Isolation:

Buffer I (Saline-EDTA):	0.15 M NaCl 0.1 M EDTA adjusted to pH 8 with conc. NaOH
Buffer II (Saline-Citrate):	0.15 M NaCl 0.015 M Sodium Citrate adjusted to pH 7 with conc. NaOH
Buffer III (dilute Saline- Citrate):	0.015 M NaCl 0.0015 M Sodium Citrate adjusted to pH 7 with conc. NaOH
Buffer IV (conc. Saline- Citrate):	1.5 M NaCl 0.15 M Sodium Citrate adjusted to pH 7 with conc. NaOH
Chloroform - IsoAmyl alcohol	24:1 (v/v)
Sodium Perchlorate:	5 M
Ribonuclease	50 μ g/ml
Sodium Lauryl Sulfate	25% (w/v)
Lysozyme	5 mg/gm wet packed cells

Note: DNA dissolved in Buffer II for all tests.

Viscosity:

Cleaning Solutions:	Triply distilled acetone
	Chromic acid:
	10 g Sodium Dichromate
	20 ml Hot H ₂ O
	175 ml Conc. H ₂ SO ₄

Equipment

Spectrophotometers:	Bausch & Lomb, model Spectronic 20 Carl Zeiss, model PMQ II
Shaker:	Controlled Environment Incubator Shaker, New Brunswick Scientific Co., Inc. Burrell, model BB (wrist-action)
Lyophilizer:	Virtis, model 10-117
Centrifuge:	Sorvall, model RC-2 refrigerated centrifuge
Constant Temperature Circulator:	HAAKE, series F
Viscometer:	Cannon Inst. Co., #50-5-328 Cannon-Ubbelohde dilution type
Timers:	GCA/Precision Scientific, TIME-IT

Organisms and Growth Conditions

The bacteria used for these experiments were Bacillus licheniformis (NRS 243) and Bacillus stearothermophilus 10.

The bacteria were grown in flasks containing 150 ml of nutrient liquid medium. The nutrient medium consisted of 1% Trypticase and 0.2% yeast extract. The cultures were incubated in an incubator shaker. The mesophile, B. licheniformis, was grown at 37°C and 45°C and the thermophile, B. stearothermophilus, was grown at 45°C and 55°C. Cell growth was followed spectrophotometrically at 540 nm (Baush & Lomb, Spectronic 20). The absorbance values corresponding to the midlog and late log phase were obtained from Gupta (1978) and Madison (1977). The growth temperatures of the organisms were chosen so as to have essentially one optimum growth temperature for each organism (37°C for B. licheniformis and 55°C for B. stearothermophilus) as well as one common temperature (45°C).

Synchronous Cell Cultures

Synchronous cultures were obtained by the procedure outlined by Gupta (1978).

Initially, slants containing 0.2% yeast extract, 1.0% Trypticase, and 2.0% Bactoagar, were inoculated with cells from stock cultures and incubated at the appropriate temperature overnight. The next morning the cells from two slants were washed, with 10 ml sterile water, into a one liter flask containing 150 ml sterile medium. Exponential growth was maintained for 12 generations; the late log phase was not exceeded at any time. The late log phase corresponds to an absorbance at 540 nm of approximately 0.7 and 0.65 for the mesophile at 37°C and 45°C, respectively; for the thermophile it corresponds to 0.7 and 0.65 at 45°C and 55°C, respectively. In order to maintain exponential growth for 12 generations, cells were transferred three times to fresh sterile medium. Enough cells were transferred each time to insure an initial absorbance of approximately 0.1 at 540 nm.

The bacterial culture, after 12 generations of growth, was filtered through a sterile filtration unit as described by Gupta (1978). The time of filtration was not to exceed one minute, using suction from a water aspirator, in order to insure synchrony. One hundred and fifty ml of bacterial culture, in the midlog phase, were filtered at one time. The midlog phase corresponds to an absorbance

at 540 nm of 0.55 and 0.45 for the mesophile at 37°C and 45°C, respectively; for the thermophile it corresponds to an absorbance of 0.40 and 0.50 at 45°C and 55°C, respectively. The filtrate containing the smallest cells was then returned to the incubator shaker and the cells allowed to grow for one generation. The entire filtration process and transfer to the incubator required less than two minutes and was performed at room temperature with the exception of the filter flask itself, which was submerged in a water bath at the incubation temperature. Cell growth was monitored throughout the incubation period by checking the absorbance every half hour using 5 ml aliquots of bacterial culture each time. After exactly one generation, the cells were harvested by centrifugation at 10,000 x g for 5 minutes, washed by centrifugation with Buffer I, weighed, and stored at -20°C.

Lysis of Cells

Lysis of all bacterial samples (B. licheniformis grown at 37° and 45°C; B. stearothermophilus grown at 45° and 55°C) was performed using identical procedures. The conditions were more drastic than those used previously (Marmur, 1961) because of the fact that difficulties were encountered in lysing the B. licheniformis culture grown at 45°C using the original milder conditions. In order to avoid unequal treatment, all of the samples were lysed using the more drastic, but identical, conditions. The degree of lysis was determined by visually noting the increase in solution viscosity.

Approximately 1 gram of frozen cells was suspended in 12 ml of Buffer I. Lysis was effected in two steps. First, 5 mg of lysozyme were added to the cell suspension. The mixture was then incubated at 37°C with occasional shaking for four hours. This procedure was followed by the addition of 1.0 ml of sodium lauryl sulfate and incubation for 10 minutes in a 60°C water bath. The lysed cell suspension was cooled to room temperature and the DNA was then isolated.

DNA Isolation

DNA was isolated by a modification of the procedure used by Marmur (1961).

After the DNA was precipitated with ethanol the first time, it was transferred to 5 ml of Buffer III and gently dispersed. The solution was then adjusted to standard saline-citrate concentration by the addition of Buffer IV. Ribonuclease was added to a final concentration of 50 μ g/ml and the mixture was incubated for 30 minutes at 37°C. Following the digestion of the RNA, the deproteinization procedure was repeated, but only once. After this final deproteinization, the DNA was again precipitated using two volumes of ice cold ethanol. The spooled out DNA was then dispersed in 3-5 ml of distilled water. This DNA solution was dialyzed against 1 liter of distilled water for 48 hours with four changes. The dialyzed DNA solution was lyophilized and stored at -20°C.

Thermal Denaturation

Thermal denaturation profiles were determined for each of the DNA samples. The previously isolated and lyophilized DNA was dispersed overnight in 10 ml of distilled water at 4°C. Buffer IV was added to bring the concentration up to that of standard saline-citrate. From these stock solutions appropriate dilutions were made, using Buffer II, to obtain 3 ml of a 20 μ g/ml solution. These diluted solutions were then used for the thermal denaturation studies.

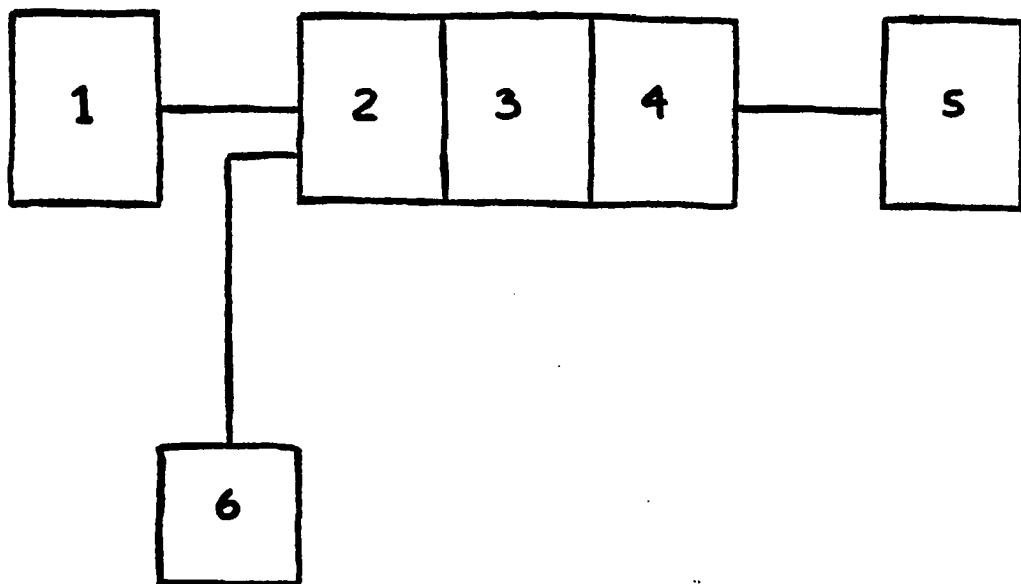
The approximate instrumental set-up is shown in Figure 1. Initially, the temperature inside the cuvette was calibrated against the temperature in the water bath. This was done by placing 2.4 ml of distilled water in a cuvette and immersing a thermometer in it. The temperature of the water bath, starting at 25°C, was varied by increments of 5°C. After each increment, the temperature was allowed to equilibrate for 20 minutes. Temperature readings were then taken of both the bath and the cuvette. In this way, the unit was calibrated for external heat loss. The bath temperature was plotted versus the cuvette temperature and this calibration curve was used to obtain the corrected cuvette temperature.

For determination of the actual thermal denaturation profiles, 3 ml of the 20 μ g/ml DNA solution were placed in the experimental cuvette and 3 ml of Buffer II were placed in the blank cuvette. The starting temperature was again 25°C and the temperature was increased

Figure 1

Thermal Denaturation Set-up
(Zeiss, model PMQ II)

1. Indicator and mechanical zero point corrector
2. Detector unit
3. Sample changer
4. Monochromator
5. Lamp unit
6. Constant temperature circulator (HAAKE)



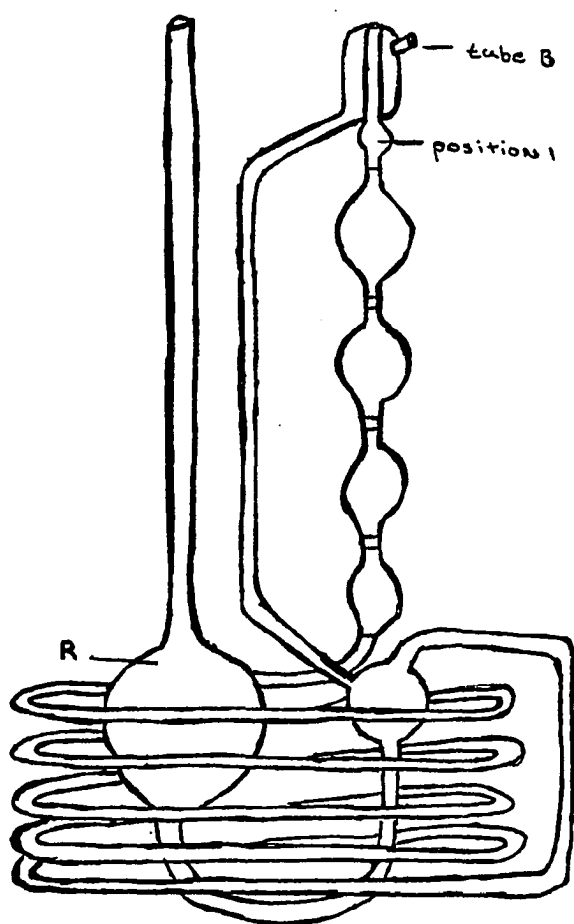
by 3-5°C with a 20 minute equilibration period. The absorbance of the DNA solution was measured at 260 nm, using Buffer II as a blank, as a function of increasing temperature up to a bath temperature of approximately 98°C. The observed absorbance was corrected for the thermal expansion of the sample. The thermal denaturation profiles were computer drawn using a Digital Equipment Corporation (Model, DECsystem-10), a Time Sharing Peripherals plotter (Model, 212), and the SPLINE subroutine program. The T_m was obtained from these curves and represents the midpoint of the transition between 25°C and 93°C. The points plotted in Figure 3 are computer generated and smooth curves were drawn through them; these curves were not used for calculating the T_m values except for that of Figure 3d.

Viscosity

Viscosity was determined using a dilution type Cannon-Ubbelohde viscometer. The viscometer was vertically aligned and submerged securely in a 25°C glass water bath. The viscometer was then thoroughly cleaned with chromic acid followed by copious amounts of distilled water and finally rinsed thoroughly with fresh triply distilled acetone.

Ten ml of Buffer II was pipetted into reservoir R (Figure 2). Using suction, the solution was drawn up to position 1. The outflow time was measured by allowing the liquid sample to flow freely down past the upper etch mark, measuring the time for the meniscus to pass from the upper etch mark to the lower etch mark of each bulb. The time was measured to the nearest 0.1 second. This procedure was repeated with Buffer II until reproducible results were obtained. From these data, t_0 , the outflow time for the solvent was determined. The viscometer was again cleaned, as described above, without changing its position. Ten ml of stock solution was then pipetted into the reservoir and after a 20 minute equilibration period the outflow time of the stock solution was measured repeatedly, as described above. The sample was diluted while in the viscometer by addition of 5 ml of Buffer II to the reservoir. The solution was mixed by repeatedly applying slight pressure to tube B. Four such dilutions were made, adding a total of 20 ml of Buffer II to the original

Figure 2
Dilution Type Viscometer



sample in the viscometer. Outflow times were measured after each dilution. The viscometer was cleaned thoroughly after each series of viscosity determinations. Because of the differences in bulb volumes and in the height of the bulbs, the measurements of capillary flow through the four bulbs represented flow at varying rates of shear. The average rate of shear for each bulb was calculated from data supplied by the manufacturer. The lines in Figures 4-8 are computer drawn (except line ~~~M~~~ in Figure 8) using the least squares method.

RESULTS AND DISCUSSION

Thermal Denaturation Profiles

The thermal denaturation profiles for the DNA's are shown in Figure 3. The T_m , or melting out temperature, was taken as the midpoint of the transition between 25°C and 93°C. The melting out temperature is that temperature at which 50% of the hyperchromic effect is observed. In all but one case the DNA's melted out over a narrow range of temperature. The one case being that of B. stearothermophilus grown at 55°C (Figure 3d). The scattered results may have been due to formation of an air bubble in the cuvette during the experiment. The T_m , in this case, was estimated as indicated by the dotted line. The T_m values are given in Table 1. The T_m for B. licheniformis grown at 37°C and that for B. stearothermophilus grown at 55°C agree well with those reported previously, 88.6°C and 91.0°C, respectively (Stenesh, 1968). Moreover, at the common growth temperature of 45°C, the thermophilic DNA was more stable (higher T_m) than the mesophilic one, as would be expected. For each organism, within the accuracy of the T_m determination, an increase in growth temperature led to a lower T_m value, indicative of a less stable DNA. These changes in DNA stability must be a reflection of the changes in the apparent fidelity of replication observed as a function of increasing growth temperature. It must be noted, however, that the hyperchromic effects for B. licheniformis at 37°C (23%) and

Figure 3

Thermal Denaturation Profiles
for DNA from:

- (a) B. licheniformis, 37⁰C
- (b) B. licheniformis, 45⁰C
- (c) B. stearothermophilus, 45⁰C
- (d) B. stearothermophilus, 55⁰C

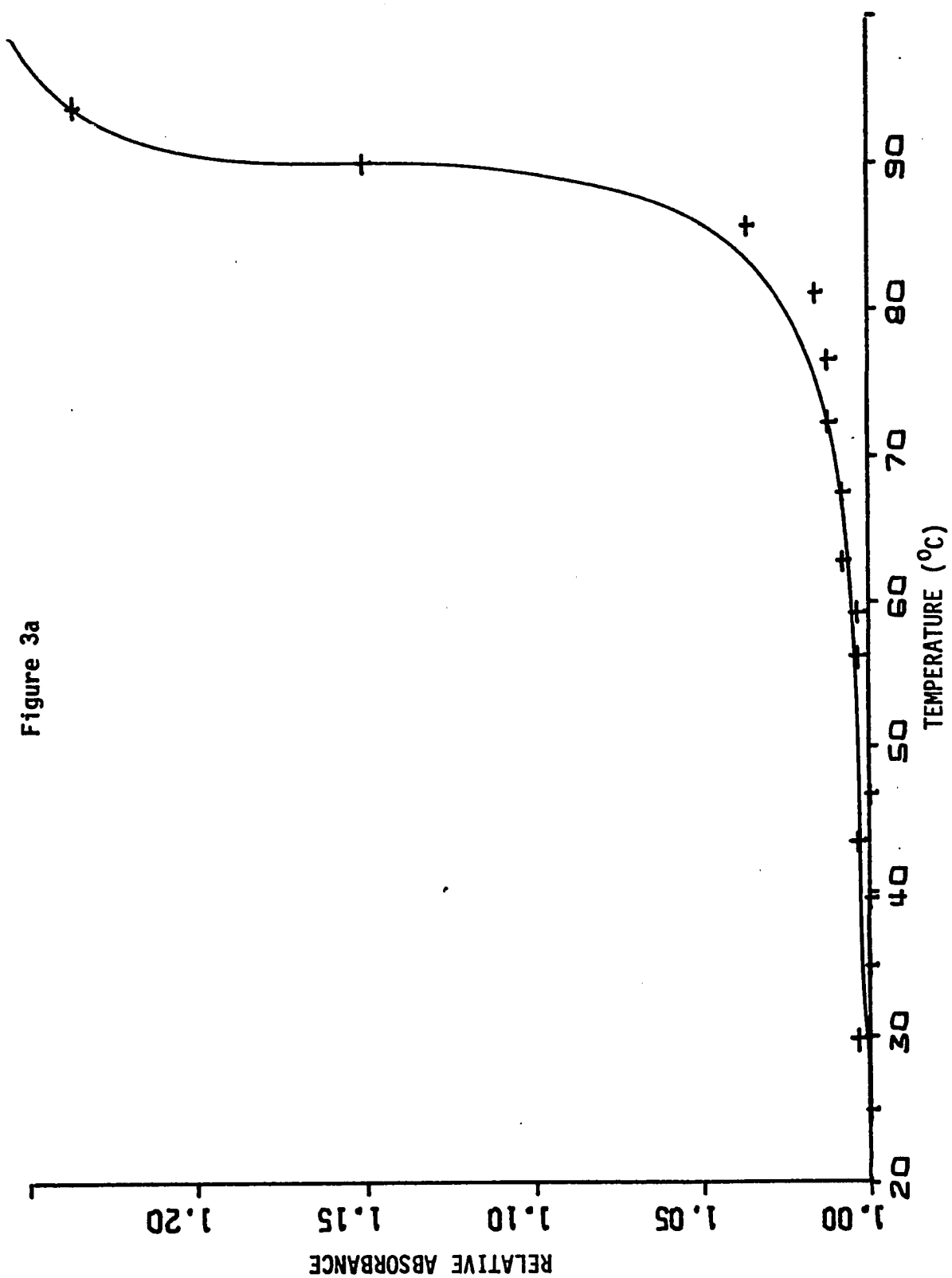


Figure 3b

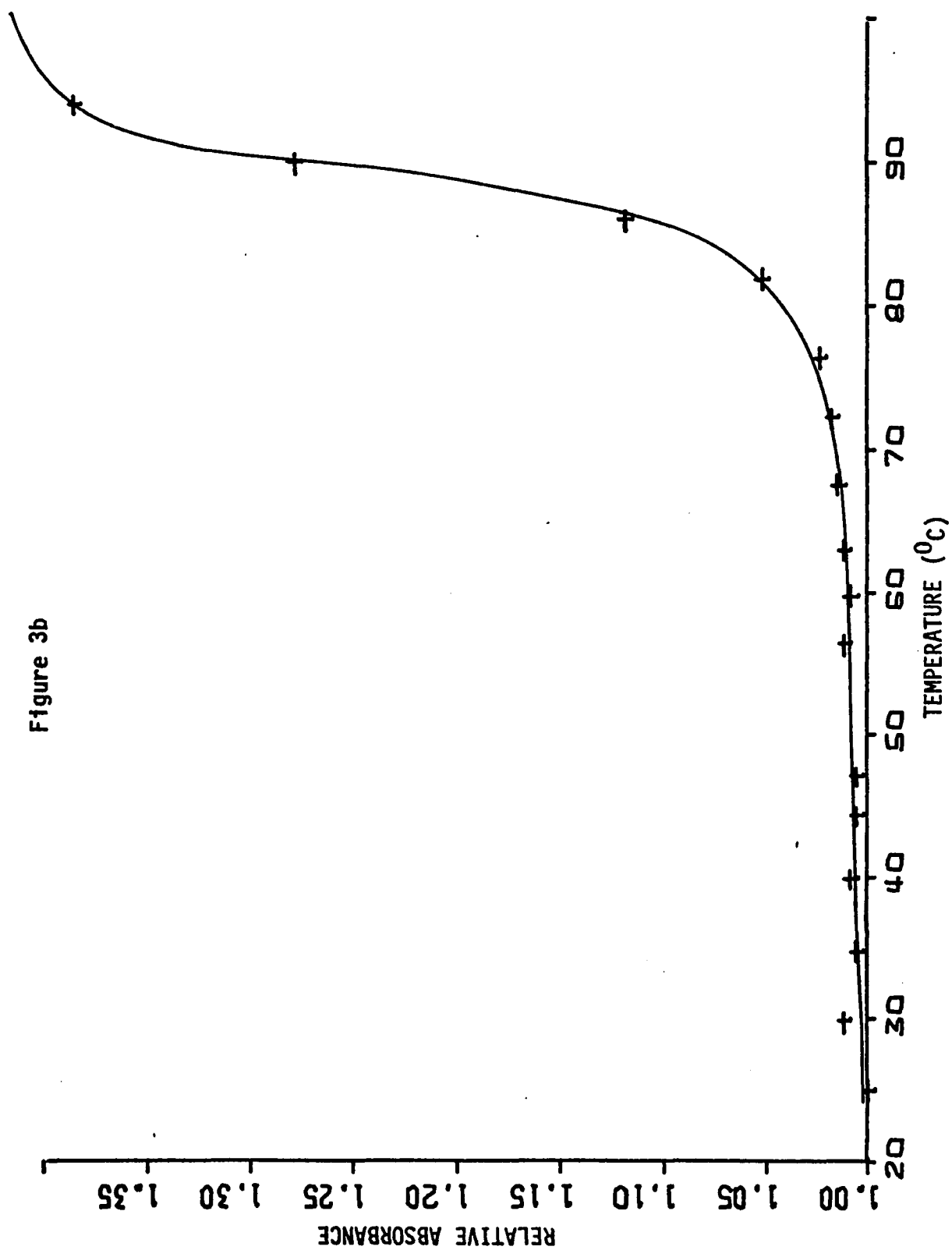


Figure 3c

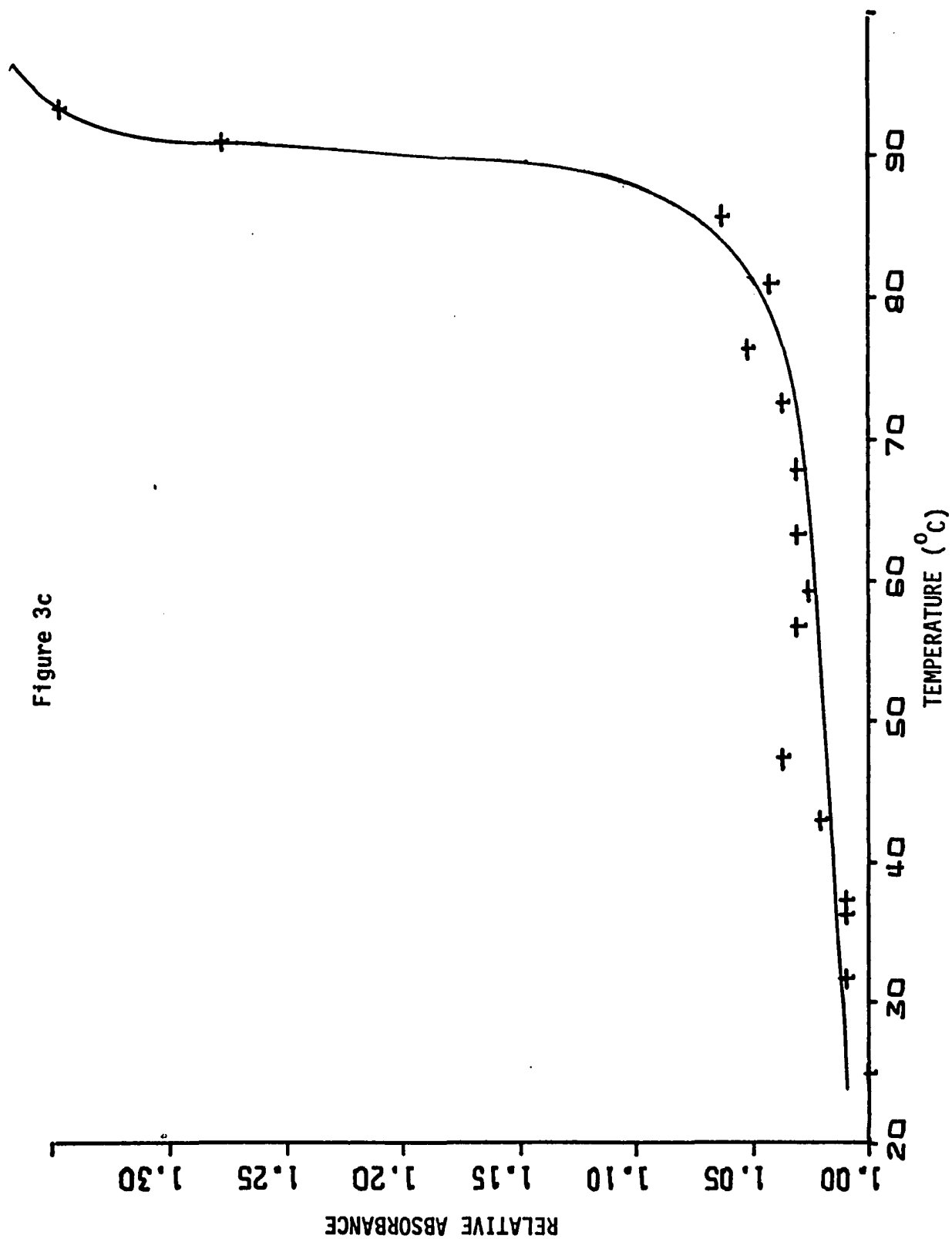


Figure 3d

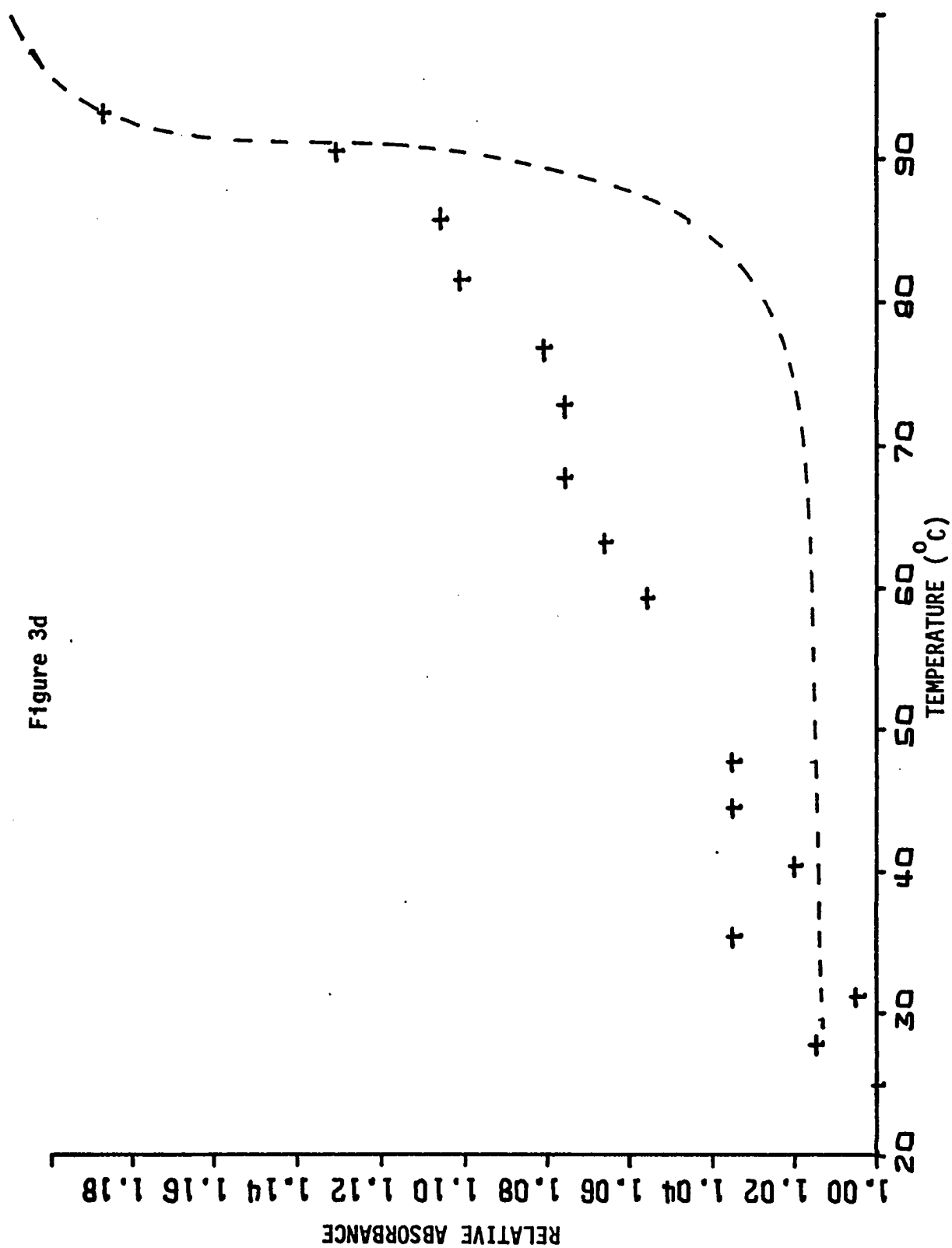


Table 1

Thermal Denaturation of DNA

Organism	Growth Temperature($^{\circ}\text{C}$)	T_m ($^{\circ}\text{C}$)
<u>B. licheniformis</u>	37	88.5
<u>B. licheniformis</u>	45	87.8
<u>B. stearothermophilus</u>	45	89.7
<u>B. stearothermophilus</u>	55	88.3*

* estimated value

B. stearothermophilus at 55°C (19%) was less than that observed previously (41% and 40%, respectively; Stenesh, 1968). This would indicate that these DNA's were partially denatured and/or degraded, presumably due to the more drastic lysis conditions required in the present study. It is likely that the DNA's from B. licheniformis at 45°C and B. stearothermophilus at 45°C , even though they showed a higher hyperchromic effect, were also denatured and/or degraded to some extent.

Viscosity Measurements

Viscosity measurements were carried out in order to determine whether the DNA from the thermophile differed from that of the mesophile in some physical parameter other than that reflected in the thermal denaturation profiles. The viscosities were measured as a function of rate of shear and also as a function of concentration.

The first set of graphs are plots of specific viscosity (η_{sp}) versus the average rate of shear (\bar{G}). The specific viscosity is calculated from the following equation: $\eta_{sp} = (t/t_0) - 1$ where t_0 is the outflow time of the solvent and t the outflow time of the sample. The average rate of shear is calculated from the equation: $\bar{G} = 2/3(\text{shear rate at wall})$ where the shear rate at the wall is given by A/t_0 where A is a different constant for each bulb, supplied by the manufacturer, and t_0 is the outflow time of the solvent. The specific viscosity and the average rate of shear were calculated for each bulb.

Instead of plotting η_{sp} versus \bar{G} , which at times displays upward curvatures (Eigner, 1962 & 1965), it has been suggested to plot instead the reciprocal, $1/\eta_{sp}$, versus \bar{G} (Eigner, 1962). This plot allowed for satisfactory extrapolations to $\bar{G}=0$. These plots are shown in Figures 4-7. It can be seen that the dependence of the viscosity on the rate of shear is similar in all cases. This indicates that in overall molecular asymmetry the DNA's do not differ

Figure 4

Viscosity of DNA as a function
of rate of shear

B. licheniformis, 37°C

(+)	40 $\mu\text{g/ml}$
(x)	27 $\mu\text{g/ml}$
(◇)	20 $\mu\text{g/ml}$
(⋈)	16 $\mu\text{g/ml}$
(⋈)	13 $\mu\text{g/ml}$

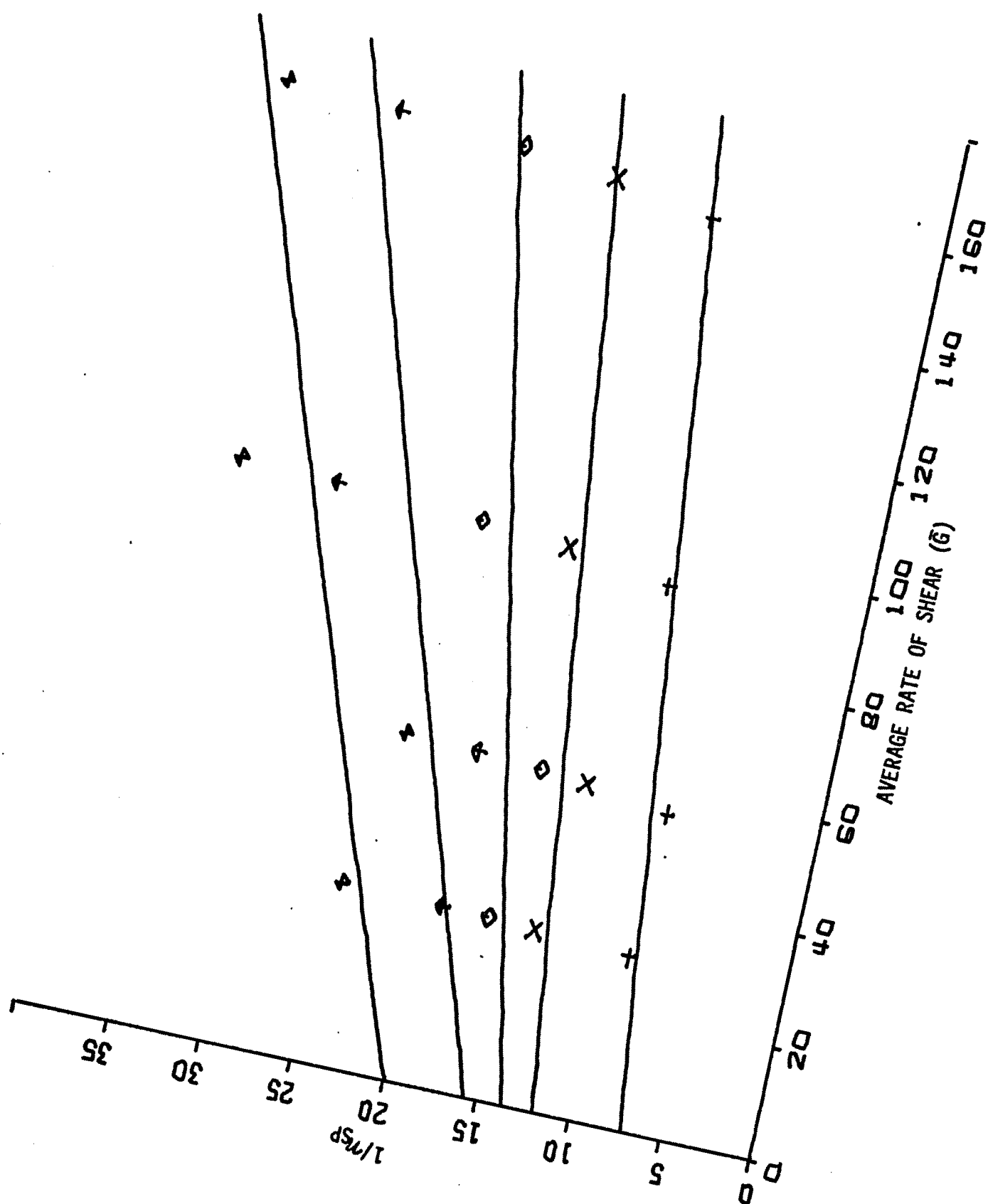


Figure 5

Viscosity of DNA as a function
of rate of shear

B. licheniformis, 45°C

(+) 86 $\mu\text{g/ml}$
(x) 57 $\mu\text{g/ml}$
(◇) 43 $\mu\text{g/ml}$
(⊕) 34 $\mu\text{g/ml}$
(∞) 29 $\mu\text{g/ml}$

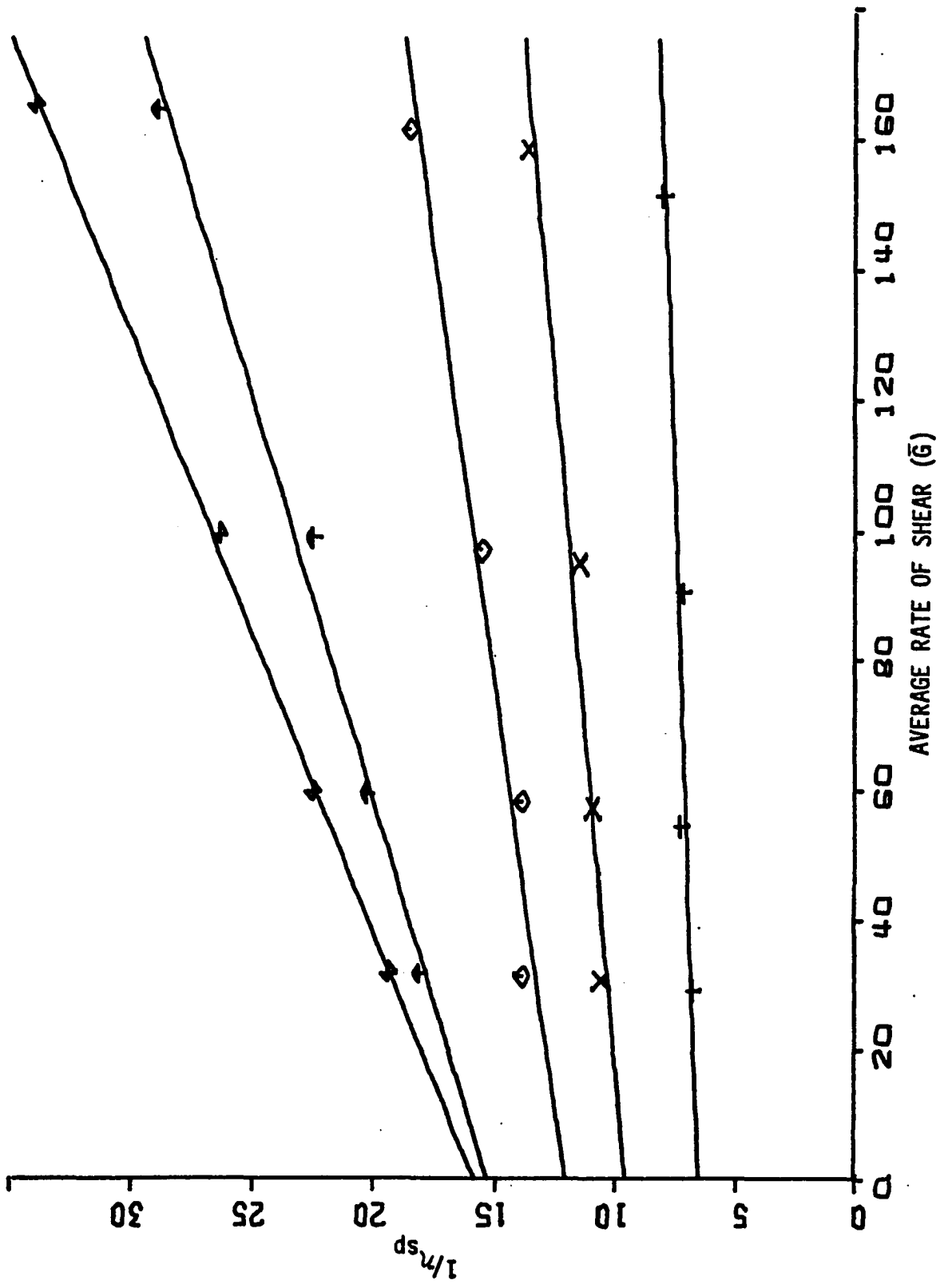


Figure 6

Viscosity of DNA as a function
of rate of shear

B. stearothermophilus, 45°C

(+)	54 $\mu\text{g/ml}$
(x)	36 $\mu\text{g/ml}$
(\diamond)	27 $\mu\text{g/ml}$
(Φ)	22 $\mu\text{g/ml}$
(Δ)	18 $\mu\text{g/ml}$

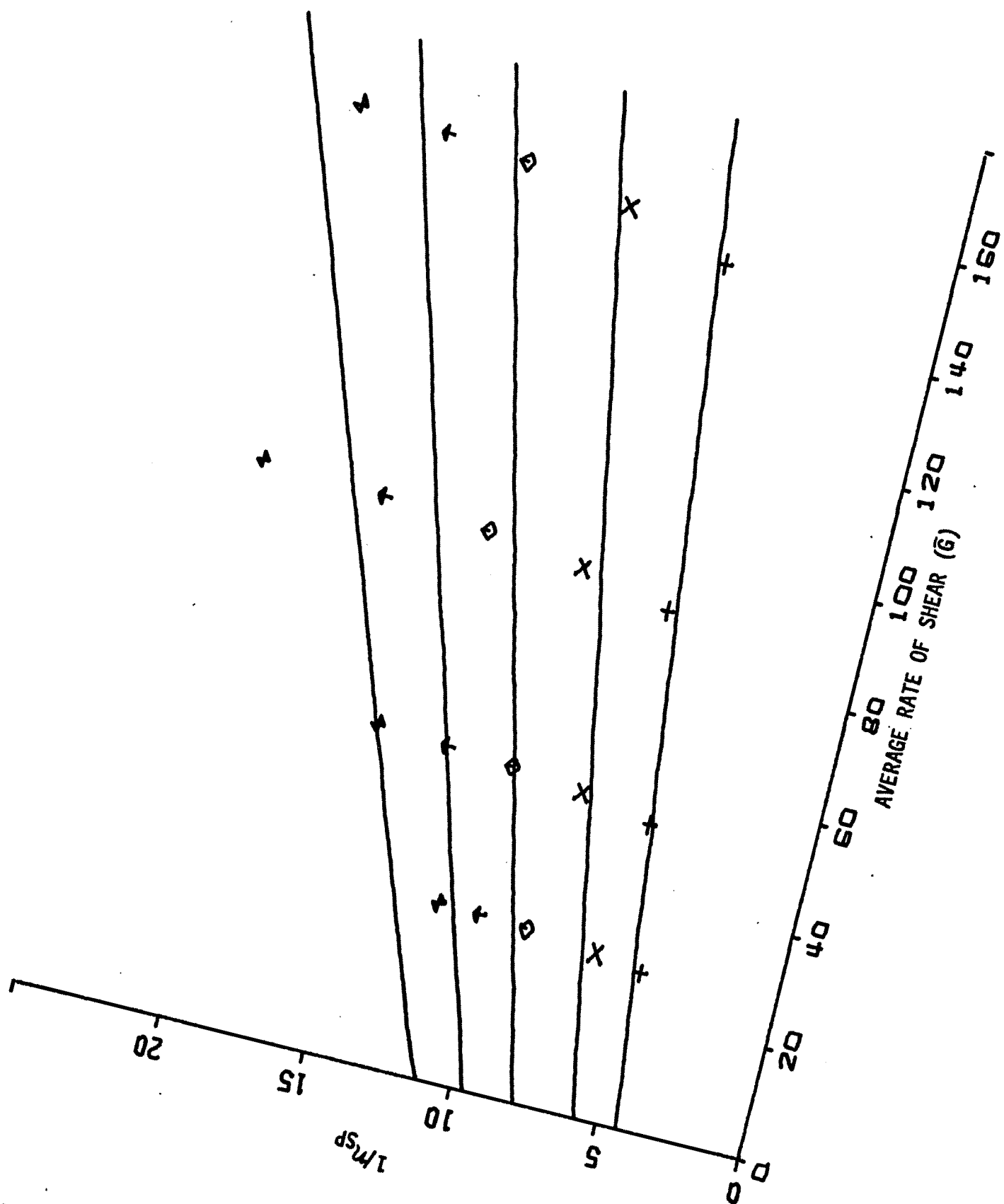
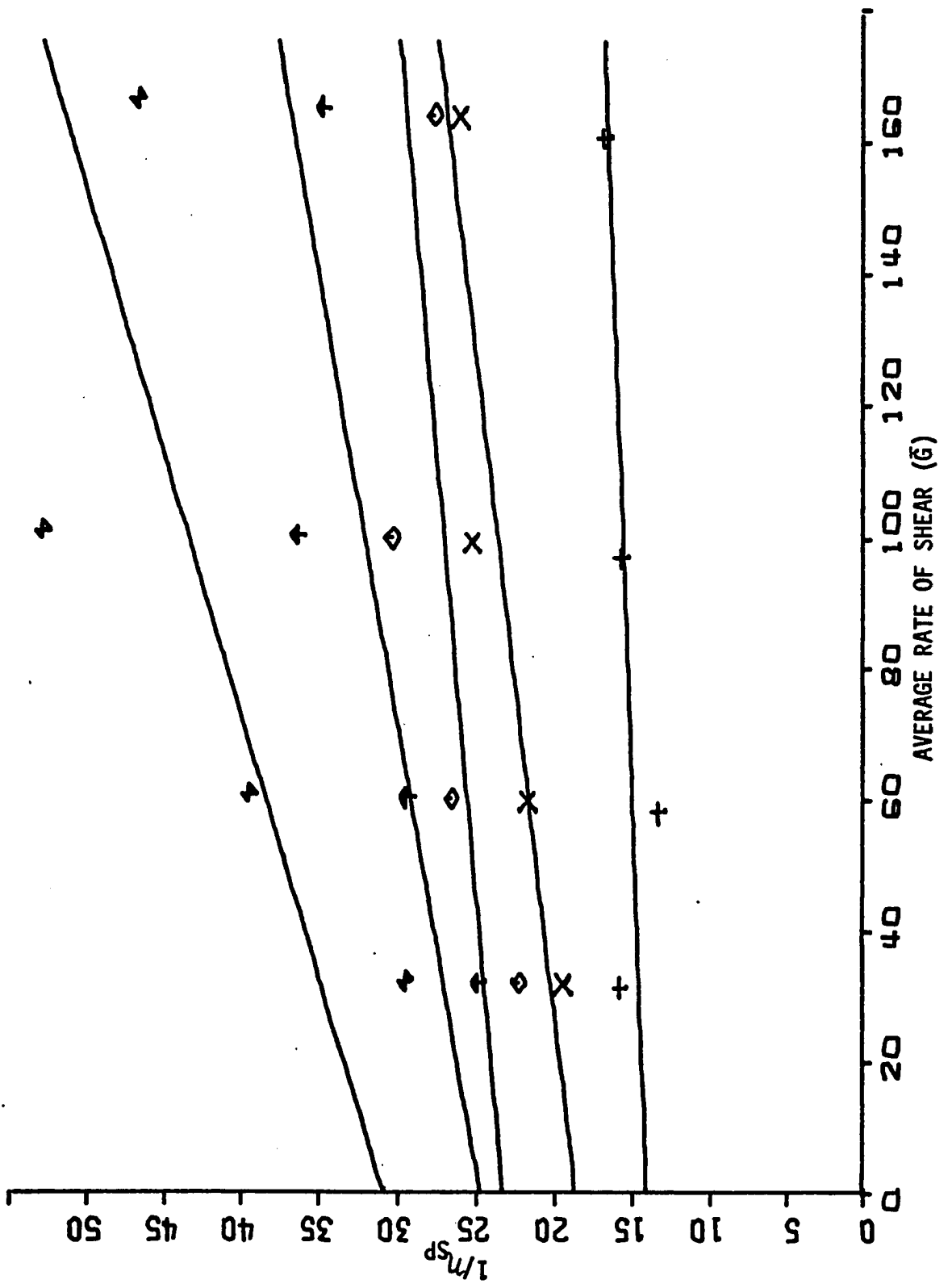


Figure 7

Viscosity of DNA as a function
of rate of shear

B. stearothermophilus, 55°C

(+) 22 $\mu\text{g/ml}$
(x) 15 $\mu\text{g/ml}$
(\diamond) 11 $\mu\text{g/ml}$
(\oplus) 8.8 $\mu\text{g/ml}$
(\sphericalangle) 7.3 $\mu\text{g/ml}$



significantly.

The viscosity at zero rate of shear was then plotted as a function of concentration. The plot of η_{sp}/c at $\bar{G}=0$ versus concentration is shown in Figure 8. This type of plot usually yields a positive slope (Stenesh, 1968). The plots from the present experiment have a slope that is almost zero (slightly negative). The line for B. stearothermophilus at 55°C has been drawn as shown in figure 8 by analogy with the lines for the other DNA's. Moreover, since the yield of this DNA was very low, only a much smaller concentration range could be covered in the experiment. Drawing a line directly through the points and having a strong negative slope would, therefore, appear to be an incorrect interpretation of the data.

The fact that the lines in figure 8 have a slope of approximately zero suggest that the DNA may have been partially denatured and/or degraded and hence did not show any significant dependence of the viscosity on the concentration. This is in agreement with the interpretation of the thermal denaturation profiles and again presumably due to the more drastic lysis conditions required in this study. Some of the effect might also have been due to an aggregation caused by contaminating protein since the DNA had not been that highly purified. Repeated purification steps were not feasible due to the very low yield of DNA from the synchronous cultures.

The extrapolation of the plots in figure 8 yields values for the intrinsic viscosity, $[\eta]$, which are listed in Table 2. The molecular

Figure 8

Viscosity of DNA as a function
of concentration

- (+) B. licheniformis, 37°C
- (x) B. licheniformis, 45°C
- (◇) B. stearothermophilus, 45°C
- (∇) B. stearothermophilus, 55°C

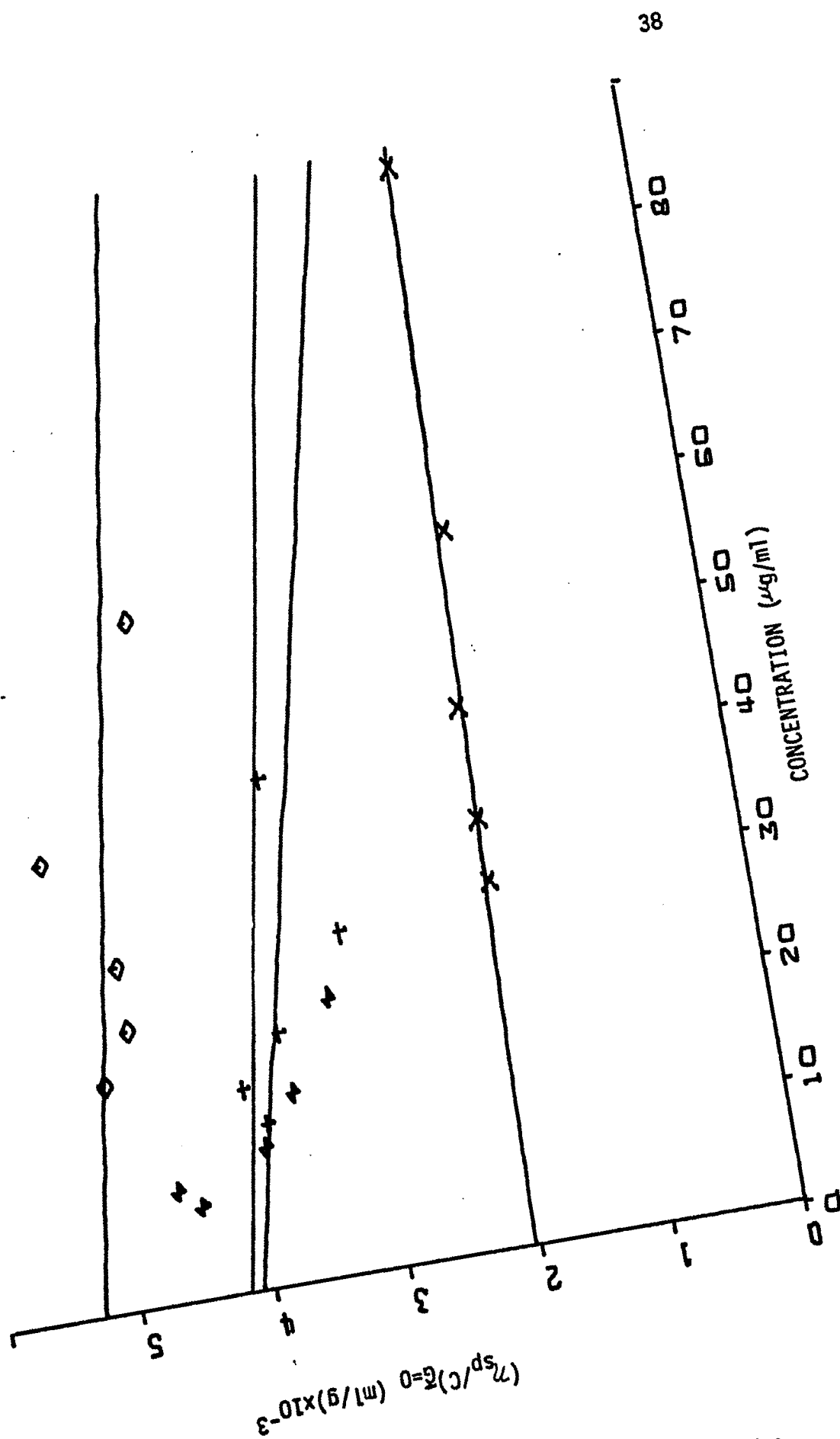


Table 2

Physical parameters of DNA

Organism	Growth Temperature ($^{\circ}\text{C}$)	$[\eta]$ (ml/g)	Mol. wt. $\times 10^{-6}$
<u>B. licheniformis</u>	37	4.1	6.4
<u>B. licheniformis</u>	45	2.3	2.9
<u>B. stearothermophilus</u>	45	5.3	9.5
<u>B. stearothermophilus</u>	55	4.2	6.8

weight can be calculated from the intrinsic viscosity by the following relationship (Eigner, 1965): $[\eta] = 6.9 \times 10^{-4} M^{0.70}$.

The calculated molecular weights (M) are also listed in Table 2. It can be seen from Table 2 that the intrinsic viscosity and molecular weights for all samples are comparable but in each case the values for the higher temperature are lower. It should be noted that a lower value for the intrinsic viscosity indicates a more flexible, a less rigid and less asymmetric DNA molecule. This is in agreement with the thermal denaturation profiles which indicated lower T_m values at the higher growth temperatures. A lower T_m value is due to less extensive hydrogen bonding and/or partial denaturation. This, in turn, would allow the DNA molecule to be more flexible, less rigid and less asymmetric.

The molecular weight values shown in Table 2 are lower than those reported previously for B. licheniformis at 37°C and B. stearothermophilus at 55°C (8.0×10^6 and 11.8×10^6 , respectively; Stenesh, 1968). This would be expected based on the conclusion reached above that the prolonged lysis led to some denaturation and/or degradation of the DNA. The lower molecular weight values for each organism at the higher temperature must reflect more extensive degradation of the DNA which would be expected if that DNA is, in fact, inherently less stable. Such DNA is likely to be degraded more readily by either enzymatic action or physical shear.

Based on the results shown in Tables 1 & 2 one can tentatively

conclude that an increase in growth temperature (and hence the corresponding changes in the apparent fidelity of replication) leads to measurable differences in the T_m , viscosity, and molecular weight of the isolated DNA. These changes are in line with the concept that the DNA, for each organism, is less stable, less rigid and less asymmetric as the growth temperature is increased. However, before this tentative conclusion can be accepted, it must be verified by repeating the same experiments using a modified isolation procedure so that the partial denaturation and/or degradation of the DNA, observed in the present study, can be avoided.

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