A STUDY OF THE EFFECT OF VISCOSITY ON THE RESOLUTION OF BANDS IN THE PREPARATIVE ULTRACENTRIFUGE

Thesis by

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ABSTRACT

Band velocity experiments in the preparative ultracentrifuge are usually performed in a linear density gradient established by mixing a 5% and a 20% sucrose solution. This stabilizing density gradient introduces a significant viscosity gradient which in turn may effect the resolving power of the sedimentation velocity experiment. The fast components travel on the average through more viscous solutions than do the slow components.

In this investigation the effect of viscosity gradients was examined by experiments in which density stability was achieved by using a cesium chloride density gradient and the viscosity was independently controlled by introducing a uniform sucrose concentration or a positive or negative sucrose gradient in the cesium chloride gradient.

Experiments performed with a mixture of tritiated thymidine labelled polyoma DNA components I, II, and III showed that the separation of band maxima increased as the direction of the viscosity gradient was changed from positive to zero to negative with respect to field. On the other hand the band widths increased in the same order. The resolution in terms of the separation between the bands divided by the sum of the band widths was clearly lowest with the negative viscosity gradient and was approximately the same for the positive and zero viscosity gradients.

TABLE OF CONTENTS

Section — Page

I. Introduction 1

II. Materials, Equipment, and Methods 2

III. Results 17

IV. Treatment of Data and Conclusions 38

V. Appendix 47

VI. Bibliography 59

INTRODUCTION

Preparative ultracentrifugation is constantly used as a method of separation by people in Biology, Chemistry, and the ill-defined area in between. The sedimentation coefficient is a number of great significance in the isolation of small organisms or large molecules. In the field today both sucrose and cesium chloride preformed density gradients are used as tools of separation. In sucrose gradients the resolution is substantially lower than in band-centrifugation in the analytical ultracentrifuge due to the significant viscosity gradient present. Although both cesium chloride and sucrose gradients are used daily no one has determined whether one method is actually superior to another or if it is possible to get an even better separation by some combination of the two. No adequate theoretical or experimental investigation has been done on the resolution of bands in the preparative ultracentrifuge.

A study was proposed with mixed gradients of sucrose and cesium chloride to study the effect of viscosity on resolution. It was hoped that an inverted viscosity gradient would enhance the band resolution. A necessary positive density gradient could be achieved with cesium chloride while the viscosity could be varied with the amount of sucrose. This study was a comparison of the three cases of viscosity gradients, positive, negative, and none at all.

MATERIALS, EQUIPMENT, AND METHODS

Reagent grade sucrose was used to make up solutions of 5% and 20% sucrose. Preliminary gradients were made with these solutions to check the linearity of the gradients and to get a feel for the technique. The major problem with the initial gradients stemmed from the lack of hydrostatic balance when equal volumes of the two solutions were used. Once this problem was removed the system behaved much better. There was a problem with "back-flow" because the valve hole was too large, but this was corrected by simply leaving the valve partially closed. (The correct opening was marked for reproducibility.)

These preliminary gradients were made singly from a specially made lucite mixing block, Fig. 1. Mixing was done with a reshaped paper clip in a cork, Fig. 2, attached to a stirring motor, as per Roger Radloff in the same laboratory. The gradients were pumped into the centrifuge tubes, Beckman ½" x 2" cellulose nitrate tubes, with a Technicon proportioning pump.

In making the density gradients the lighter material is pumped into the tubes first and heavier material is pumped underneath it through a capillary tube. The reason for this procedure is because highly viscous material does not flow well. Instead of a smooth and constant flow large drops tend to form and splash into the gradient below.

After sampling the gradients with a Buchler fractionat-

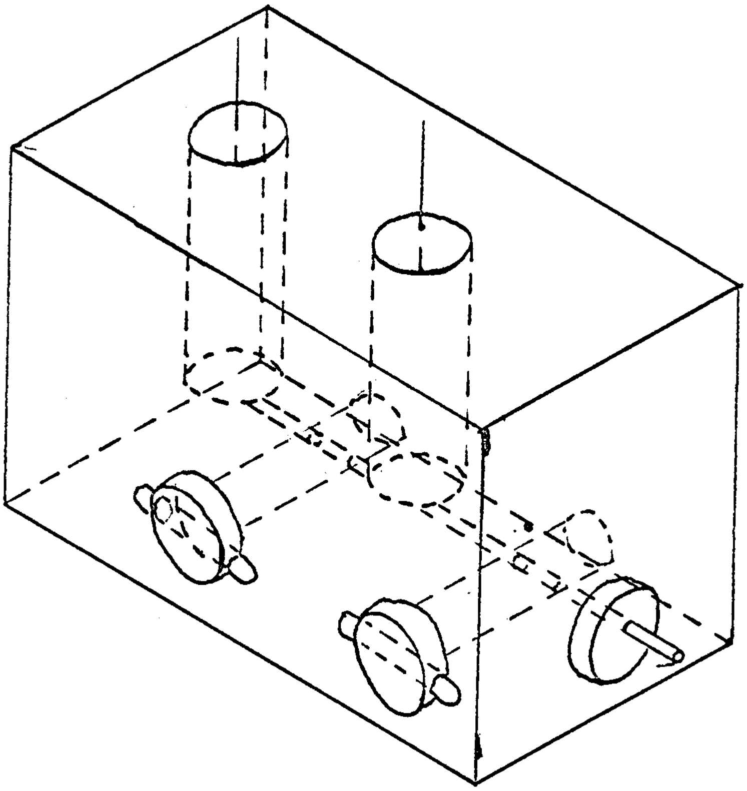


FIG. 1

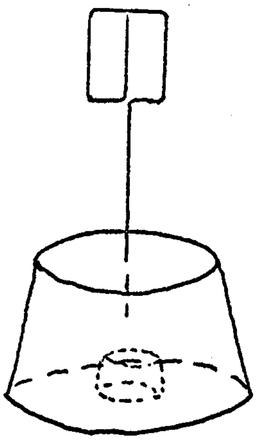


FIG. 2

ing apparatus into ½ dram shell vials the refractive index of each sample was read with a Carl Zeiss Refractometer. These values were compared to the table of refractive index vs. per cent sucrose, with temperature corrections, in the Handbook of Chemistry and Physics[1]. This data was then graphed and examined for linearity.

Since refractive index readings are insufficient for determining the densities of solutions containing more than one solute an alternate method was necessary for measuring the densities of the sucrose-cesium chloride solutions. The method of Linderstrom-Lang[2] was adapted and bromobenzene-kerosene density gradients were made for the measurement of the double solute solutions. The gradient was pumped into burette for an easy measurement of position in the gradient. The total range of the gradients made was approximately 1.05 gm./ml. to 1.40 gm./ml. Standard density solutions of cesium chloride were made up as markers and were used each time a density measurement was made. The standard solutions were made up by calculating the approximate densities desired and weighing out amounts of cesium chloride sufficient to give these values. The cesium chloride was dissolved and diluted to close to the correct volume, at which point the refractive index was taken and a more accurate density value was obtained.

The densities of the cesium chloride standards were determined by the relation between the refractive index and the density:

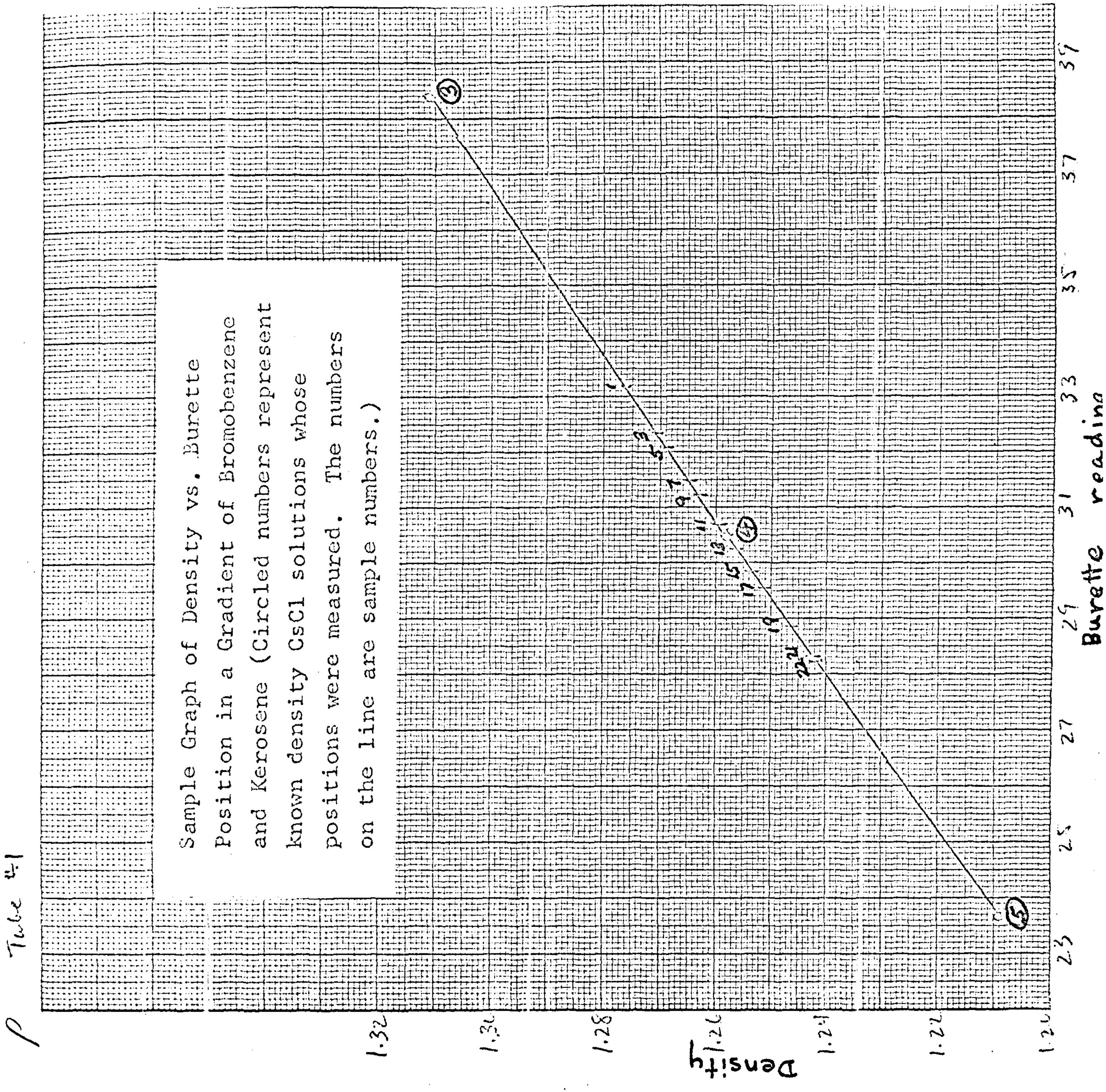
$$\rho = (10.2402)\, n_D^{25} - 12.6483 \qquad \rho < 1.38$$

Rather than making a gradient whose range ran between the density of kerosene and the density of bromobenzene, two solutions were mixed prior to the making of the gradient. These calculations may be found in Appendix I.

There was a problem at first with putting the drops of samples into the column. The small drops had a tendency to stay at the meniscus. They were not heavy enough to break through. The first answer to this was to put kerosene on top of the gradient. The less dense kerosene should then present less of a barrier to the drops. A still more satisfactory procedure was to place the drop in the column below the meniscus level.

It was found that 1 microliter samples were best suited for this column after testing 1, 2, 5, and 10 microliter drops. In order to get drops of this size Drummond Microcaps were used. After drawing up the appropriate amount into the glass capillary and drying the outside of the glass, the end of the capillary was inserted below the meniscus of the column and the drop was forced out with the Drummond bulb apparatus and the capillary was simultaneously drawn back through the meniscus and out of the column. To determine the density of a solution a graph was made of density vs. burette reading and the markers of given density were plotted. From the line between these marker values the density of the unknown solution could be determined from its position. (Figs. 3, 4)

In order to distinguish the markers from the unknowns the idea of coloring the markers was considered. This creat-



Sample Graph of Density vs. Burette Position in a Gradient of Bromobenzene and Kerosene (Circled numbers represent known density CsCl solutions whose positions were measured. The numbers on the line are sample numbers.)

Fig. 3

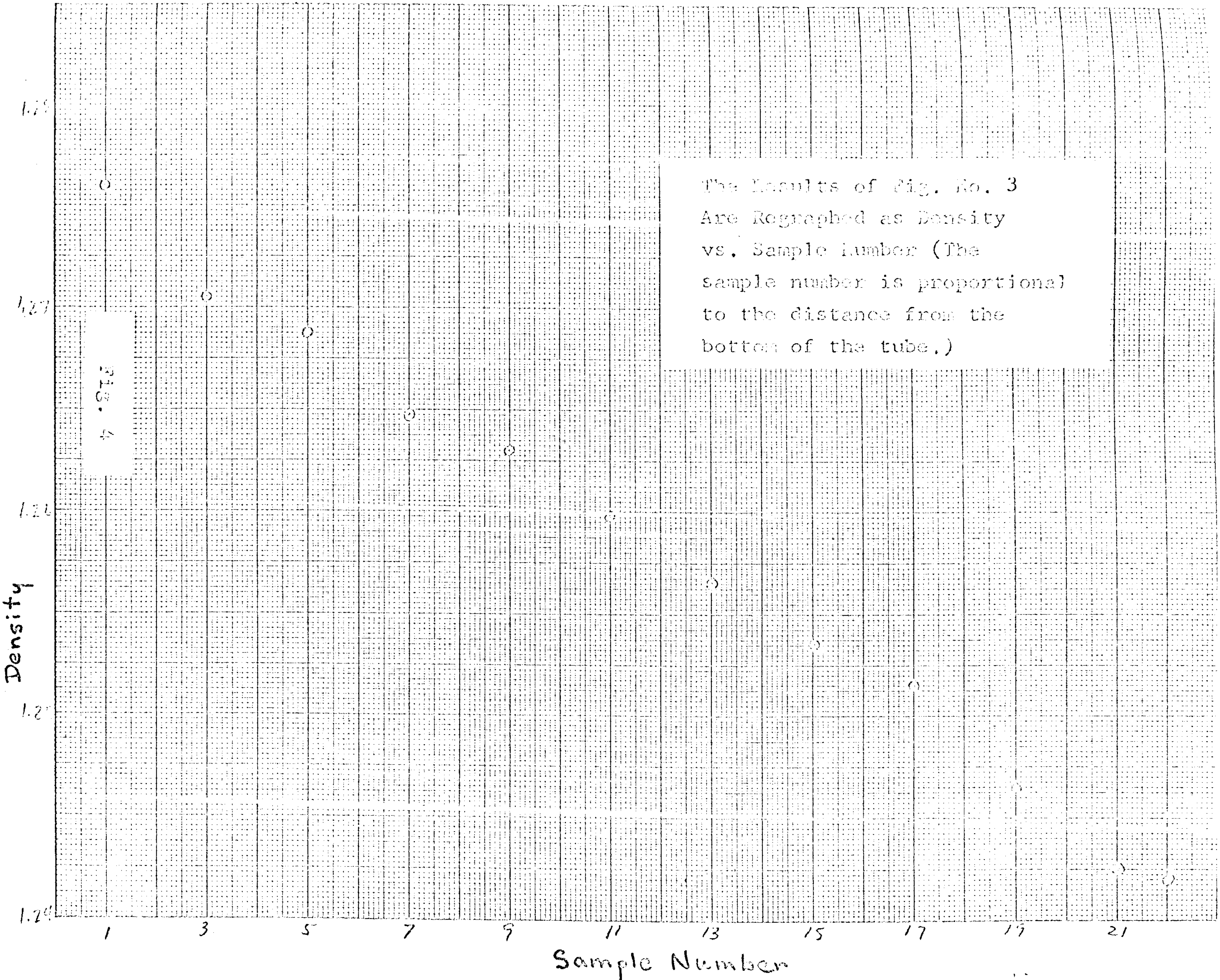


Fig. 4

The Results of Fig. No. 3 Are Regraphed as Density vs. Sample Number (The sample number is proportional to the distance from the bottom of the tube.)

ed more trouble than it was worth and the idea was discarded. Too little dye was indistinguishable and too much dye created a problem in the determination of the densities of the standards. In place of the dye an ordered procedure was used in which the standards were placed in the column and read first. Since there is a downward drift of drops over a length of time as noted experimentally and discussed by Miller and Gasek[3], it was decided that an arbitrary time must be used to consider as the buoyant position. If the standards are read in the same way there should be no problem. The calibration of the gradient in this manner gave densities to the third decimal place. The procedure for reading and recording the position of drops was not to read the location until the succeeding drop had been entered into the column. A steady consistent procedure of entering the drops and recording the position of the previous one gave good results. After the positions were determined in the column the column was cleared with sand.

The stability of the Linderstrom-Lang gradient was such that the middle range of the gradient, the range necessary for this study, was still useful in a gradient made six months previous.

With this column the linearity of cesium chloride-sucrose gradients was tested. In one case, the first η+ gradient, the results seemed to indicate a lack of precision of the gradient from tube to tube. Using the Linderstrom-Lang column and testing a hypothesis the cause was found to be the lack of plumbness which created an unequal sampling from the mixing chamber.

For these experiments the centrifuge runs were done on a Beckman-Spinco Model L Preparative Ultracentrifuge. A black SW-50 rotor was used. The centrifuge was run at a speed of 45,000 revolutions per minute. The times for these runs varied with the viscosity gradients used.

Tritium labelled Polyoma DNA was obtained from Robert Watson. Later a second supply of labelled DNA was personally isolated. In both cases the virus was isolated by the methods of Winocour[4] and Murakami[5] while the DNA was extracted by a modified version of the Weil[6] phenol extraction. The phenol used was distilled under an argon atmosphere.

After the gradients were made in the centrifuge tubes a small amount of Bayol was layered on top. A thin lamella of DNA was placed on top of the Bayol and being denser immediately went to the interface of the gradient and the Bayol.

For counting the tritiated DNA two types of counting solutions were used. Bray's solution was used at first because of its ability to be mixed with a small quantity of water. Bray's solution is made up with 60 gm. naphthalene, 4 gm. PPO (Packard's primary scintillator), 200 mg. POPOP (Packard's secondary scintillator), 100 ml. methanol, 20 ml. ethylene glycol, and diluted to 1 liter with p-dioxane. The second solution was a toluene solution. The composition was 4 gm. PPO, 0.05 gm. POPOP, and diluted to 1 liter with toluene.

When the Bray's solution was used the centrifuge tubes were dripped directly into polyethylene scintillation vials. This method was not useful because there was an immediate precipitation of all solutes. The counting was extremely erratic and thoroughly unuseful. Attempts were made to break up the precipitate and either to dissolve it in the Bray's solution or suspend it. The vials were mixed vigorously with a Vortex mixer but the material all reprecipitated once the vials were replaced in the counter. For this reason the Bray's solution was eliminated and the toluene solution was used.

For counting the distribution throughout the tube a Buchler fractionating apparatus was used and six drop samples were dripped onto Whatman GF/A 2.4 cm. filters. The filters were skewered with pins and elevated above an aluminum foil covered styrofoam board which acted as a pin cushion. The filters were dried with a 250 watt Reflector Infra-Red Industrial bulb for fifteen minutes. The dried filters were placed in Packard glass scintillation vials containing the toluene counting solution. Ten min. counting periods were used on a Packard Tri-carb Liquid Scintillation Spectrometer, Model 527.

The sucrose-cesium chloride solutions were made up by mixing equal volumes of double concentration sucrose and double concentration cesium chloride.

If the density of a solution of sucrose is ρ_1 and the density of a solution of cesium chloride is ρ_2 the density of a solution which is the same concentration in each may be approximated by the following equation:

$$\rho_1 + \rho_2 - 1 = \rho_{12} \qquad (1\text{-}1)$$

This is an approximation for the density when there is no interaction between water and the solute. The "1" being subtracted is merely the density of water which is being counted in the densities of both solutions.

If solution #1 has a concentration of sucrose of x and solution #2 has a concentration of cesium chloride of y, a solution of concentration x in sucrose and y in cesium chloride may be made by mixing equal volumes of 2x concentration of sucrose and 2y concentration cesium chloride since the volume is doubling.

Too shallow a density gradient is unstable. A sucrose gradient is known to be stable. Its range is from 1.02 gm./ml. to 1.08 gm./ml. This is the range picked for the mixed gradients. It was desired to minimize the effect of the density gradient to better observe the effect of the viscosity. A range 1.22 gm./ml. to 1.28 gm./ml. was picked. This was sufficiently far from the buoyant density to avoid the buoyancy effect (slowing down as buoyancy is neared).

For the commonly used viscosity range the solutions were to range from that of 5% sucrose to 20% sucrose. For ease of mixing solutions the constant viscosity solutions were chosen to be 10% in sucrose. The calculations for the bulk solutions used in making the sucrose-cesium chloride solutions may be found in Appendix II.

Originally there was an attempt to derive an equation which would give a numerical value for the separation of two types of macromolecules. A separation factor was defined as the difference between two peaks divided by the average of the distance travelled by the two peaks. This method was discarded because the important factor, spreading, was neglected and it could not be put into the equations. The effect of the spreading would have to be seen experimentally.

An equation was derived for the movement of a macromolecule of known s-value and may be found in Appendix III. The equation was solved for time. A graph of time vs. distance for two s-values would give the separation of the peaks at any time by simply looking at the correct time coordinate. This may also be used for calculating the running time given a desired position and the other parameters, buoyant density, initial viscosity, initial density, density gradient, viscosity gradient, and the running speed.

Experimentally it was found that the data did not agree with the results from the equation. The original viscosity values were questioned since they were merely approximated at first by the viscosity of strictly the sucrose and later by the equation:

$$\eta_s + \eta_c - \eta_{H_2O} = \eta_{sc} \tag{1-2}$$

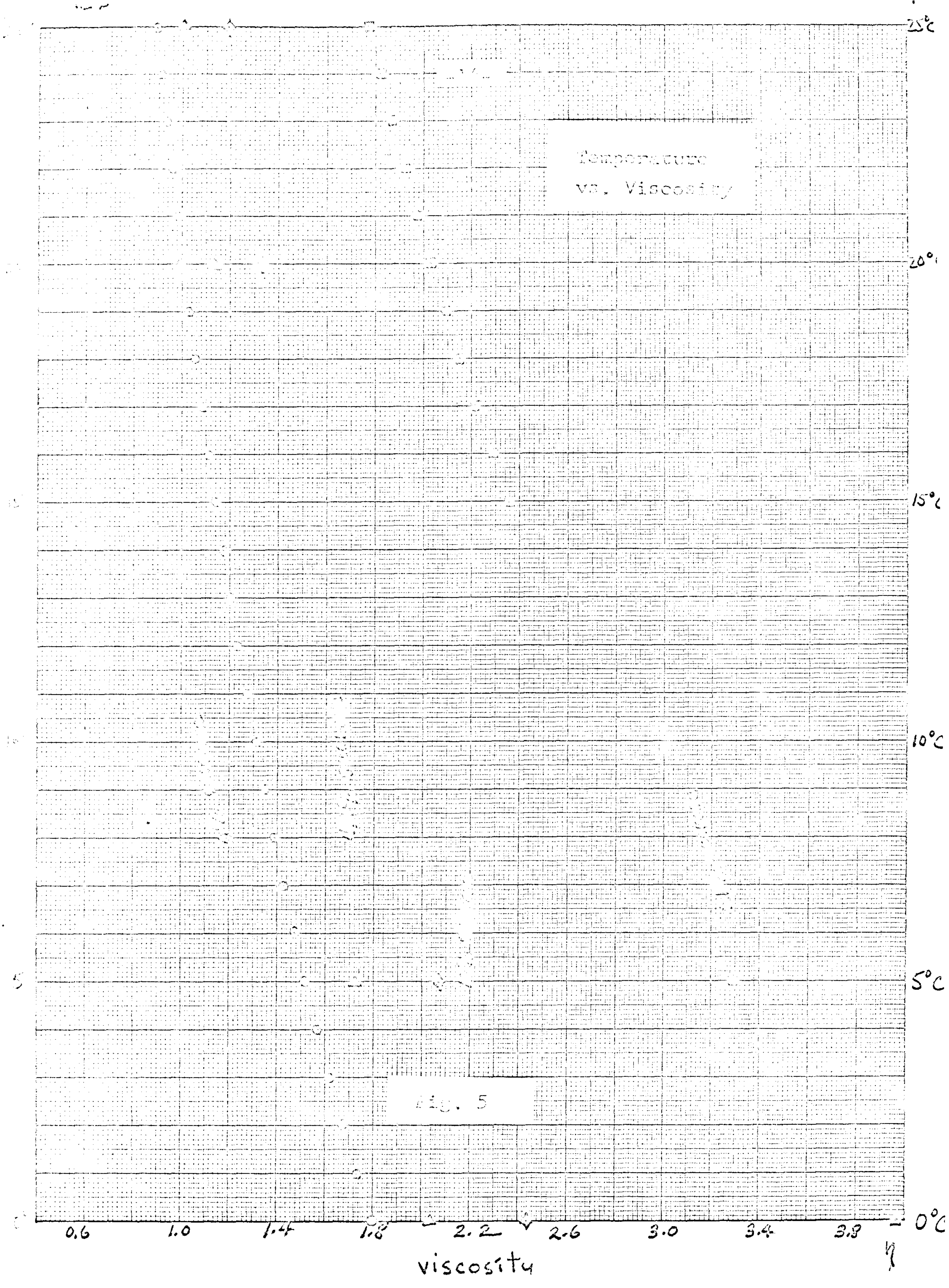
where η_s, η_c, η_{H_2O}, and η_{sc} are the viscosities of sucrose, cesium chloride solution, water, and sucrose-cesium chloride respectively. This approximation still gave values for running time which were too high. Measurements of viscosities were finally done with the mixed solute solutions. Viscometer measurements were done with an Ubbelohde type viscometer, a Cannon 50 A280.

The viscosity values, see Fig. 6 , when used in the equation still gave times which did not agree with those obtained experimentally.

Experiments were done to check the temperature of the solutions in the centrifuge. It had been assumed that the temperature of the solutions was the same as the rotor temperature. A small deviation from this can have a large effect on the viscosity. This is known to be true for sucrose. In the graph of viscosity versus temperature, Fig. 5, it may be seen that there is a twenty per cent difference between the viscosity of 21% sucrose at 20 deg. C and at 25 deg. C. It is assumed that temperature would therefore affect the sucrose-cesium chloride mixture in a like manner if not by the same amount.

The temperature data was taken by measuring the temperature of a solution before placing the centrifuge tube in the bucket and immediately after the rotor had come to a stop and had been removed from the chamber. During the run there was a thermometer suspended just over the axis of rotation of the rotor and in close proximity. This temperature was noted to be certain that the conditions of the run were as close to the actual separation run conditions as possible.

One could consider that the temperature decreased at a constant rate over the period of the run and through the range of the initial and final temperatures, however, since no constant record of the temperature was possible this assumption



Temperature vs. Viscosity

Fig. 5

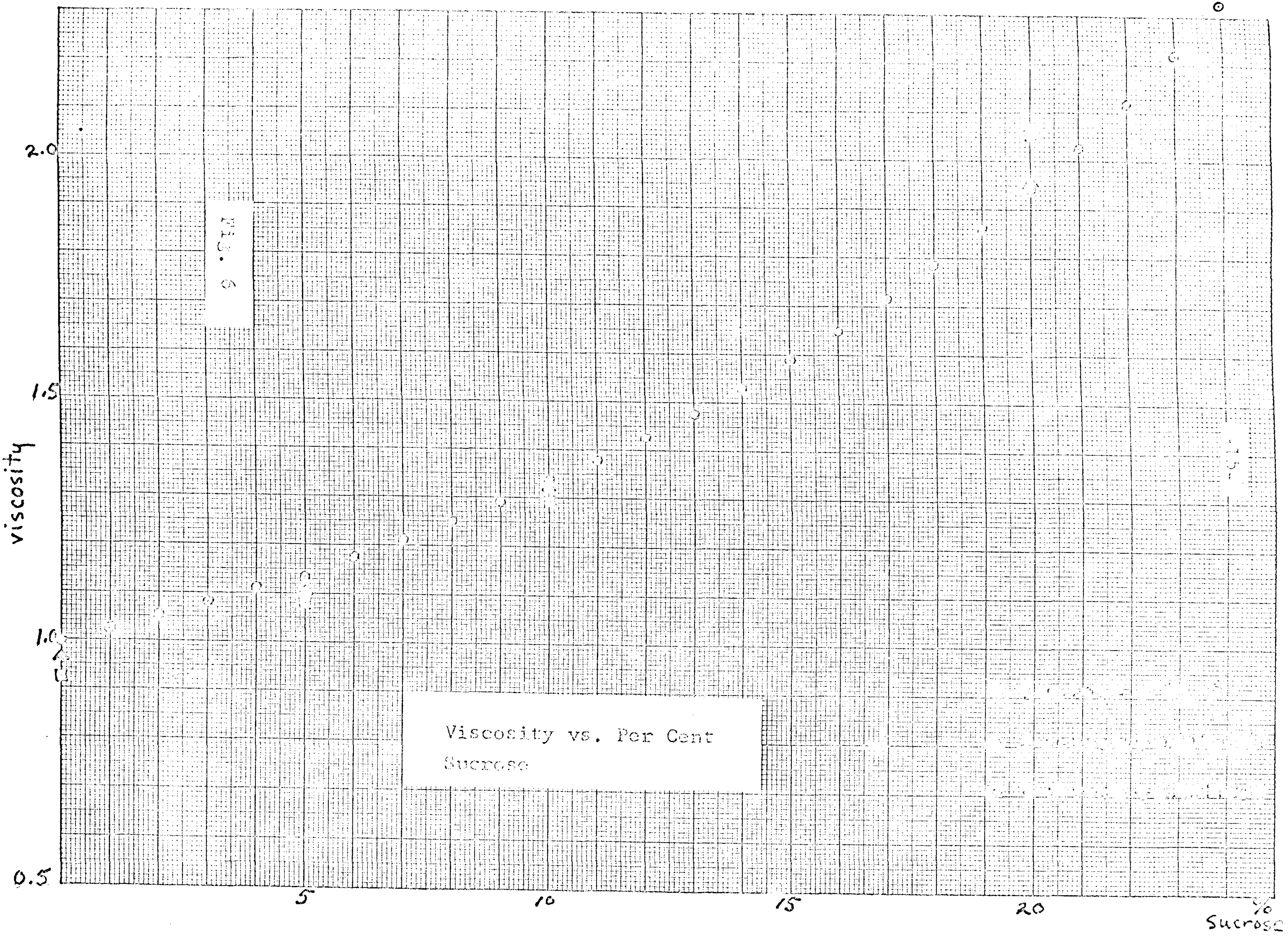


Fig. 6

Viscosity vs. Per Cent Sucrose

may not be valid. It is also possible that the solution cooled down rapidly and merely reequilibrated at a rapid rate once the rotor was slowing down and stopping. Although this is not highly likely the chamber temperature does warm to room temperature rapidly once the door to the chamber is opened. The temperature of the solution could also come to rest at somewhere other than that recorded by the chamber thermometer. If this is so than there is doubt as to how long the temperature took to equilibrate and therefore how long it took to cool from the initial to the final temperature. These are merely speculations and no conclusions may be drawn from them. The temperature was an uncontrolled variable and therefore the viscosity was not controlled. The derived equation lacks usefulness since the viscosity is not known during a run. The experimental data becomes more qualitative in nature and may be used to state only very generally the relative merits of different types of viscosity gradients.

RESULTS

The data for the experiments at constant viscosity (10% sucrose throughout) may be found in Figures 7-13. In general the sedimentation of the polyoma DNA components was examined in three gradients in each centrifuge run. Figures 7-9 represent the results for an experiment performed in triplicate for 170 minutes. Qualitatively the results were in agreement with each other. Figures 10 and 11 represent the results for another experiment in which the time for sedimentation was decreased to 160 minutes so as to be able to detect fast materials that might have sedimented ahead of polyoma I. Comparison of the results of these two runs indicates the absence of any homogeneous components with a sedimentation coefficient higher than that for polyoma I. It is apparent in Figures 7-13 that the polyoma DNA samples contain an unidentified inhomogeneous material, presumably mouse DNA, that gives rise to an elevated baseline.

Figures 12 and 13 represent the results for another preparation of polyoma DNA at 160 minutes. This preparation contains a larger relative amount of polyoma DNA I than the preparation used in the earlier experiments.

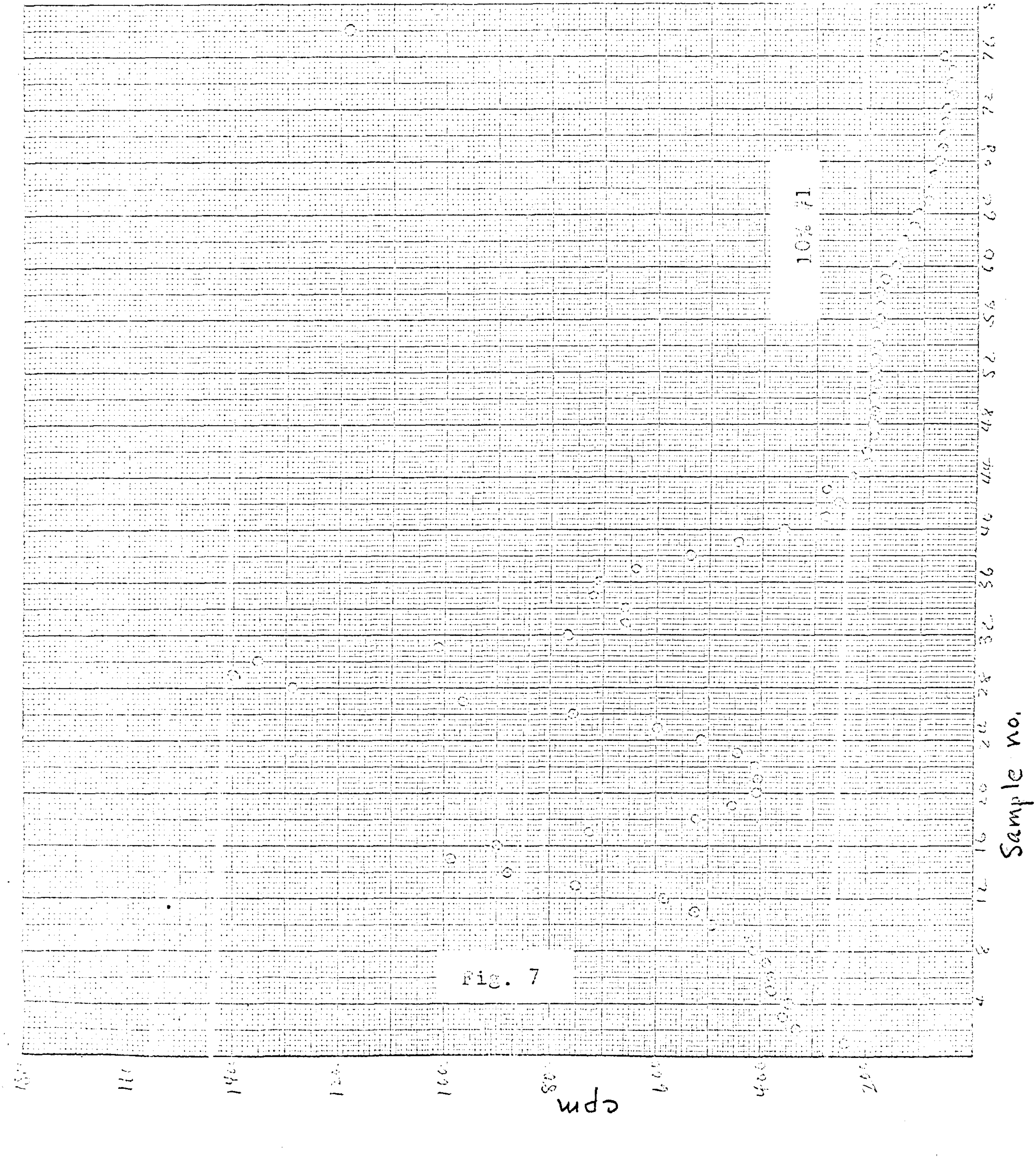

10% #1
Sample no.
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76
cpm
1800
1600
1400
1200
1000
800
600
400
200

Fig. 7

10% #2

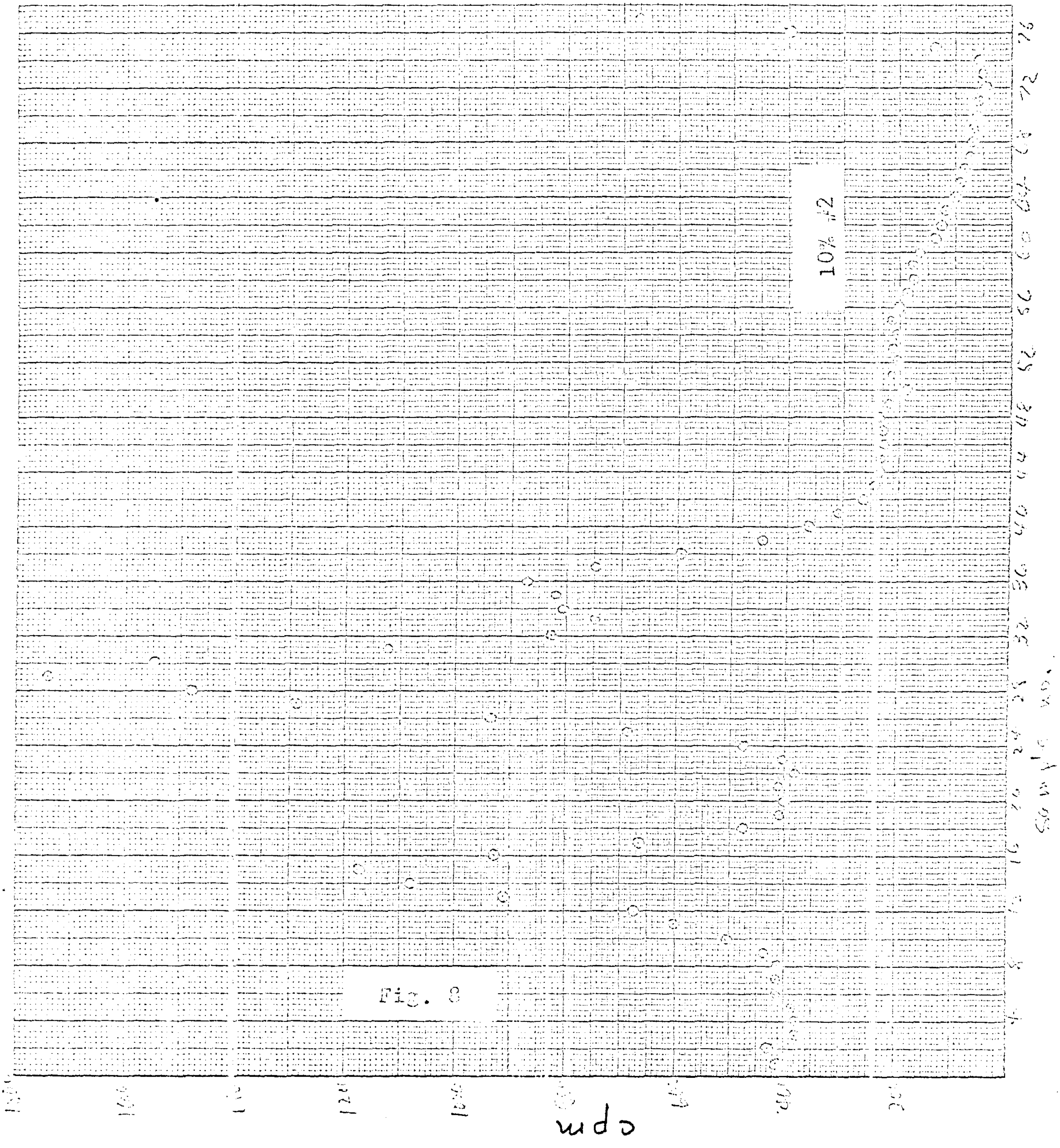

10% #2
cpm
180
160
140
120
100
80
60
40
20
Sample no.
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76

Fig. 8

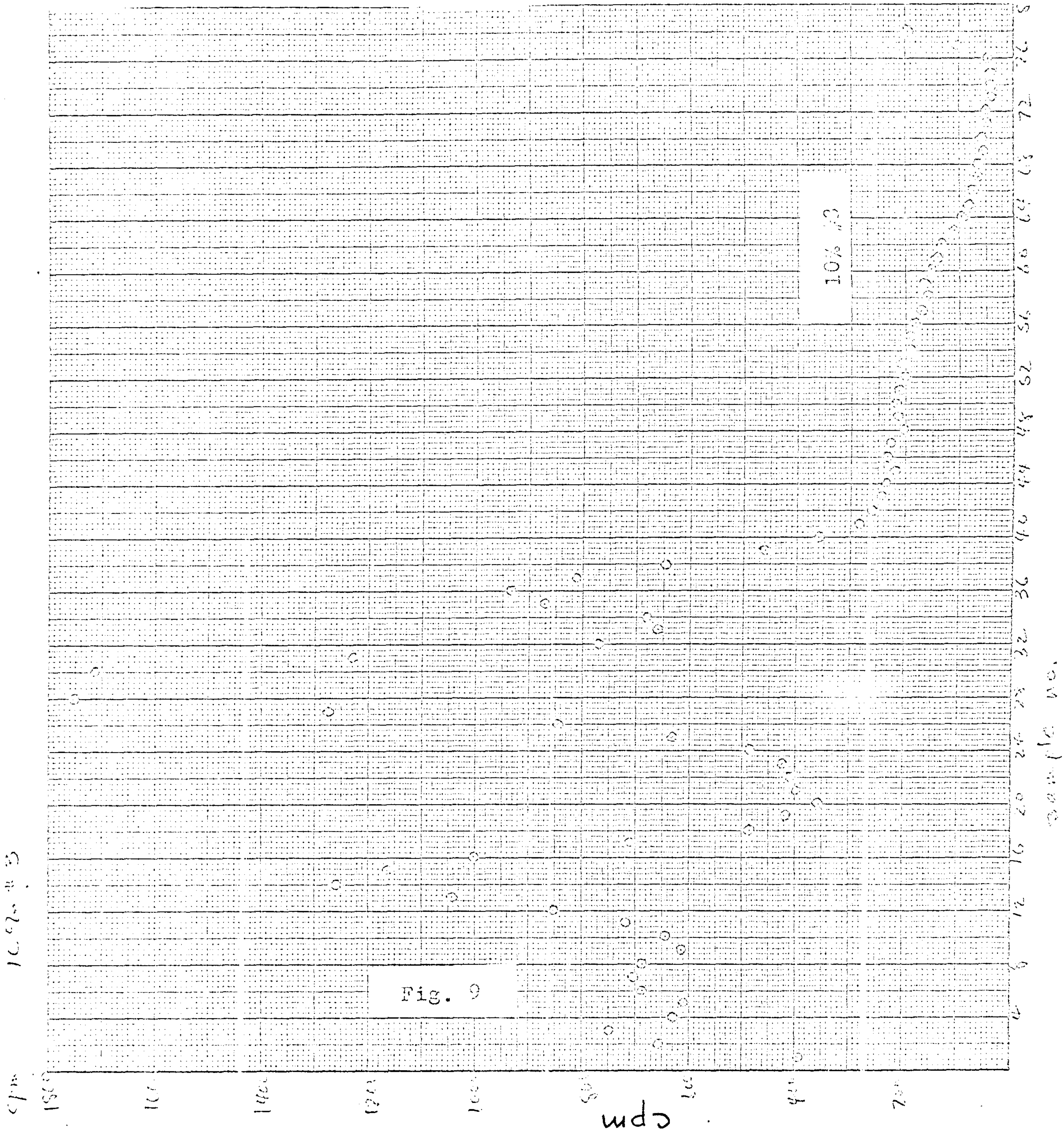

10% #3
cpm
1800
1600
1400
1200
1000
800
600
400
200
cpm
sample no.
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76
80
10% #3

Fig. 9

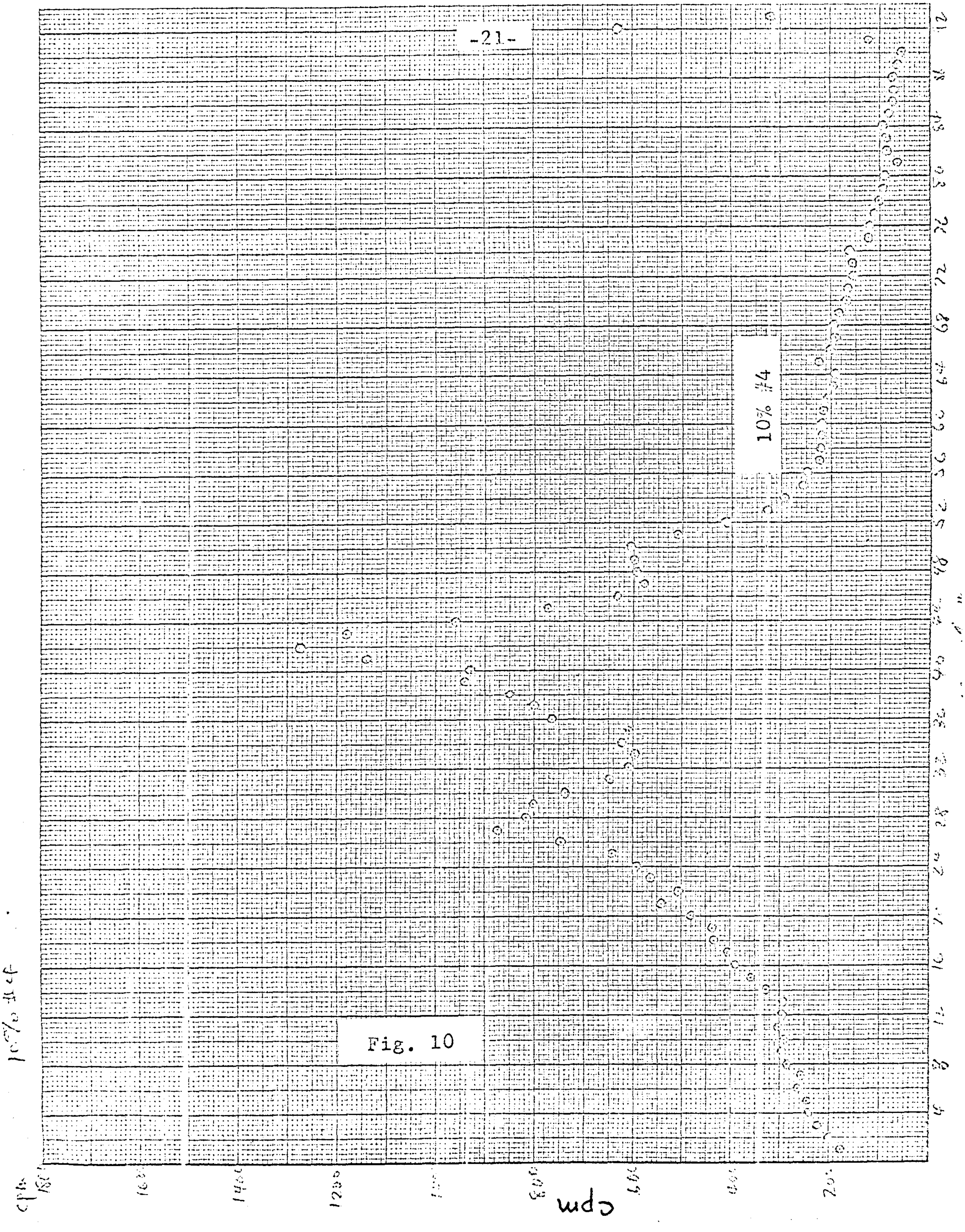

10% #4
10% HCF
cpm
1800
1600
1400
1200
1000
800
600
400
200
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76
80
84
88
92

Fig. 10

10% #6

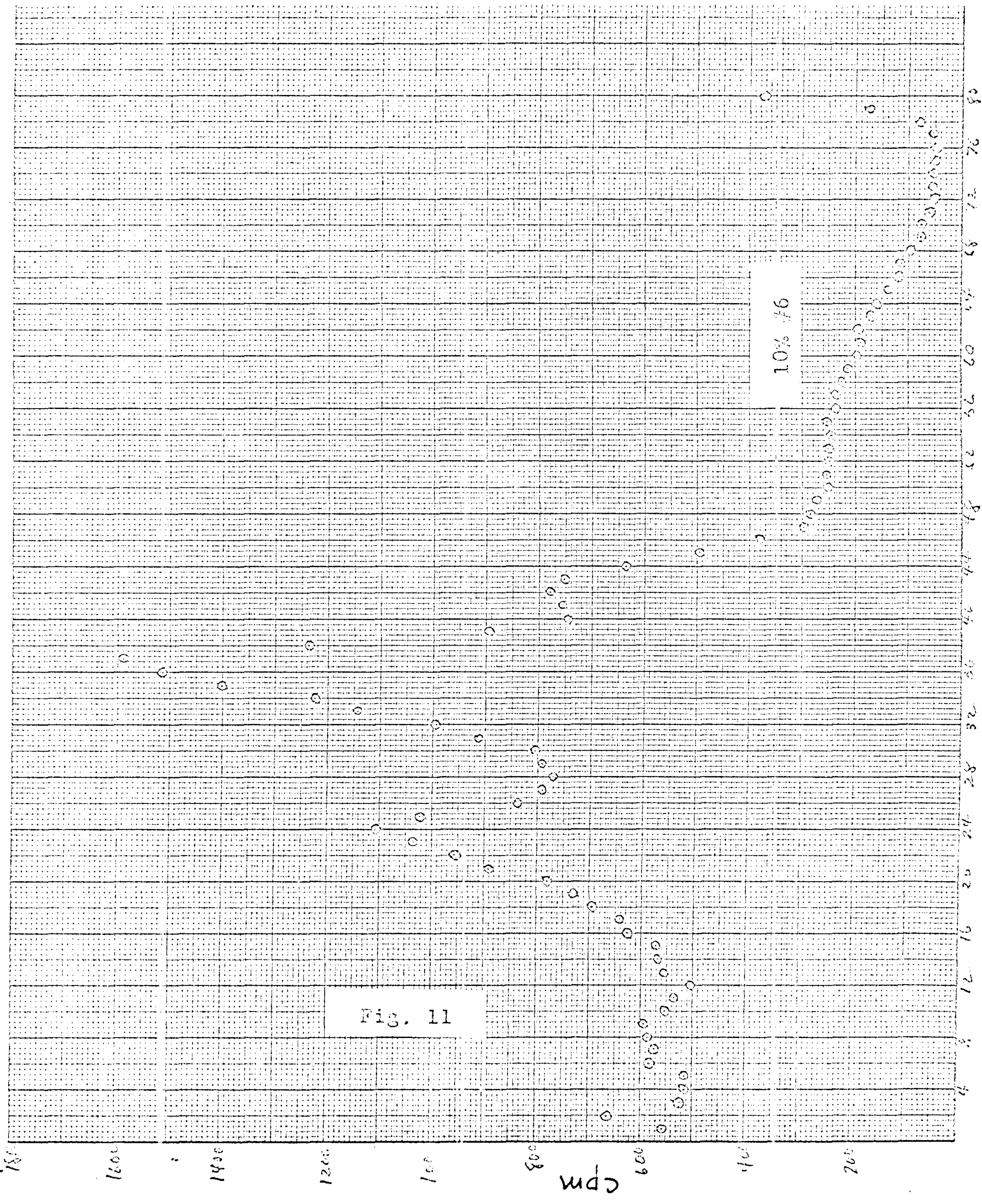


Fig. 11

KEUFFEL & ESSER CO.

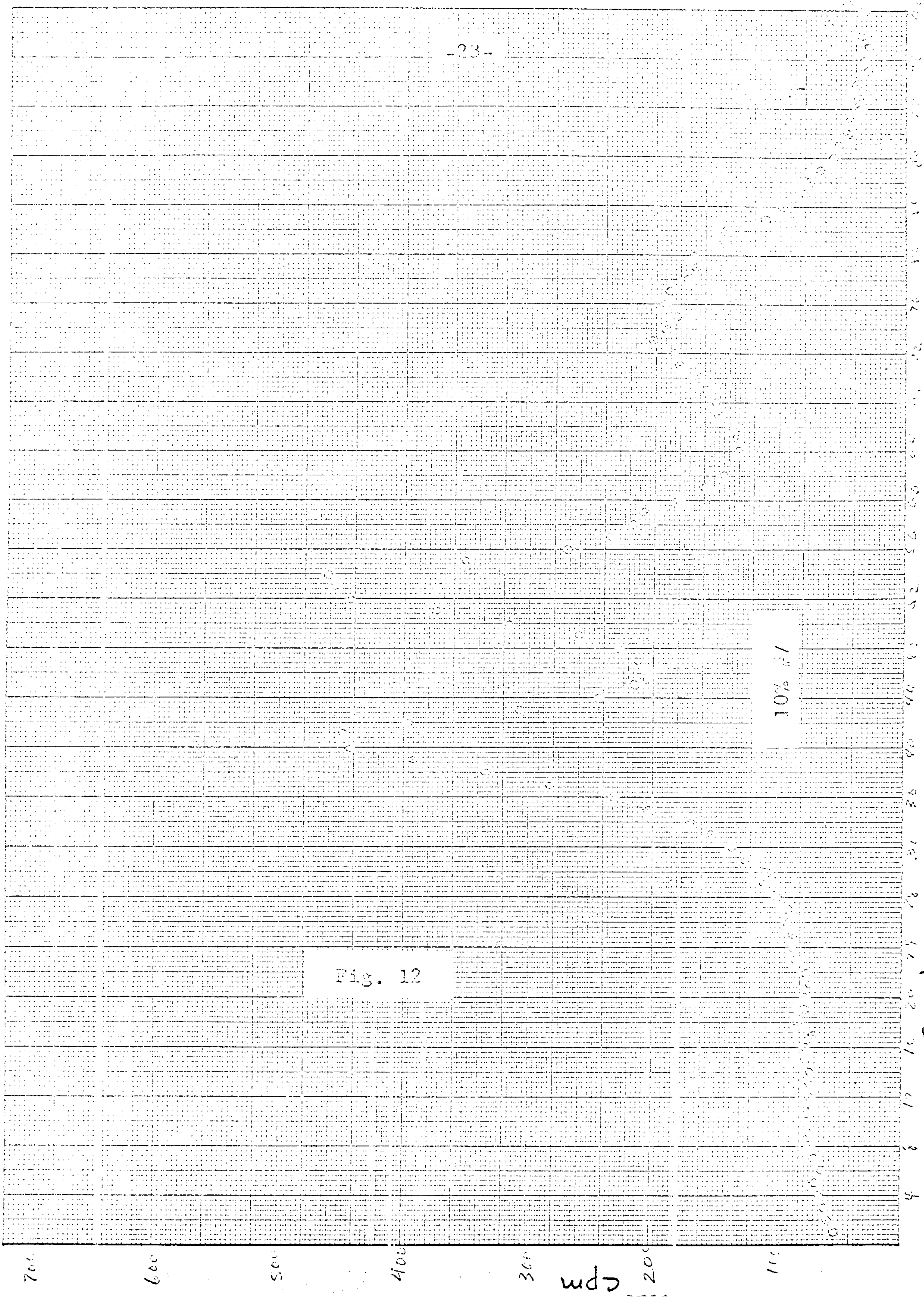


Fig. 12

10% B-1 (10 ml)

KEUFFEL & ESSER CO.

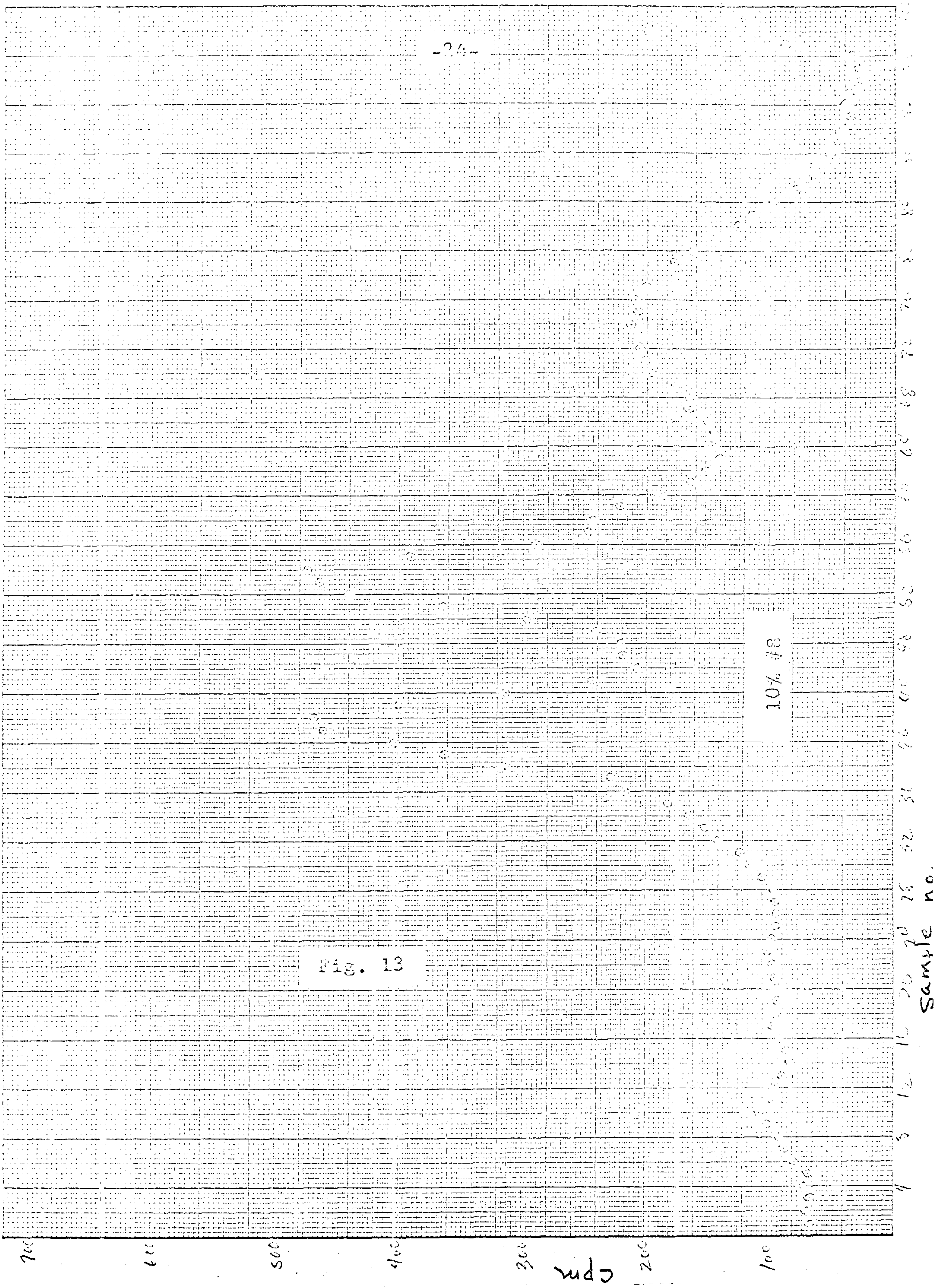

10% #8
cpm
100
200
300
400
500
600
700
Sample no.
1
5
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76
80
84
88
92

Fig. 13

The data from the positive viscosity gradients may be found in Figures 14-19. Two separate centrifuge runs were made. The first run, 230 minutes, (Figures 14-16) was the run which created the uneven sampling problem as mentioned previously. The bands were at different positions in supposedly equal gradients. After correcting the cause for the unequal gradients the experiments given in Figures 17-19 were performed at 200 minutes.

It can not be decided by merely looking at the data whether this type of gradient gives better or worse results than the constant viscosity system. For this reason a more quantitative analysis was used. (See the section on Treatment of Data and Conclusions.)

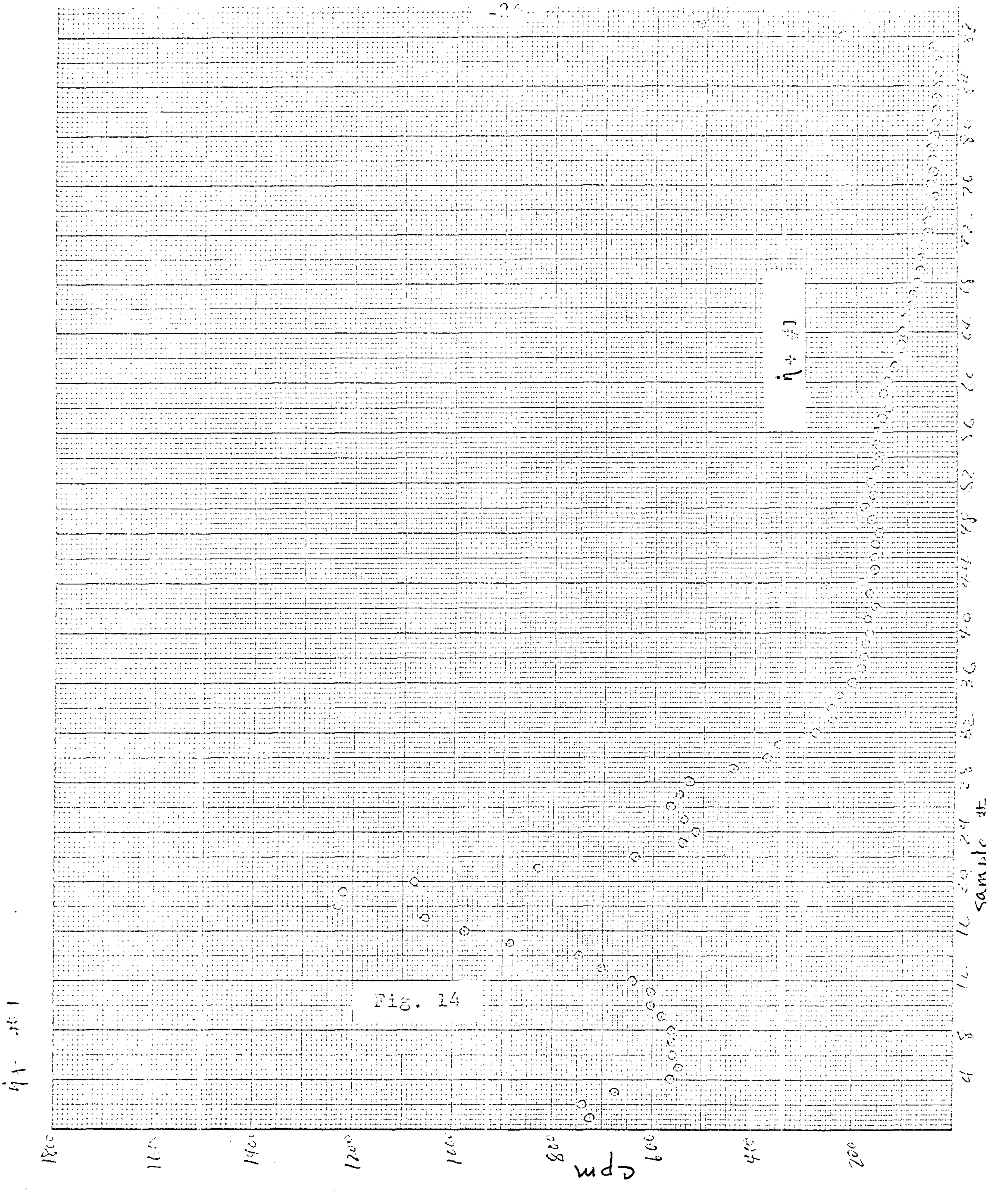

Rt #1
Rt #1
cpm
1800
1600
1400
1200
1000
800
600
400
200
sample #
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76
80
84
88

Fig. 14

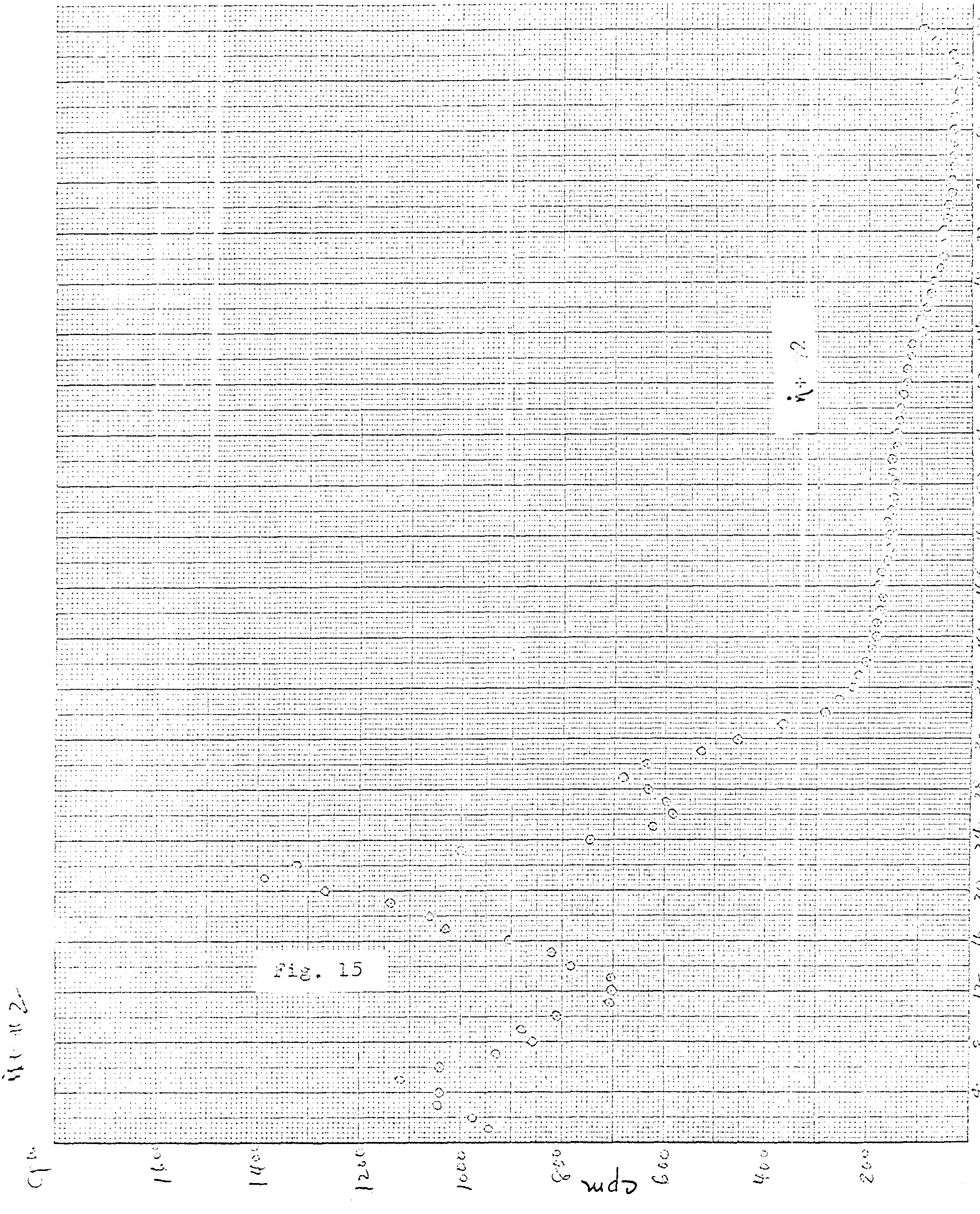

Ni+ #2
cpm
200
400
600
800
1000
1200
1400
1600
Sample #
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76
80
Ni+ #2

Fig. 15

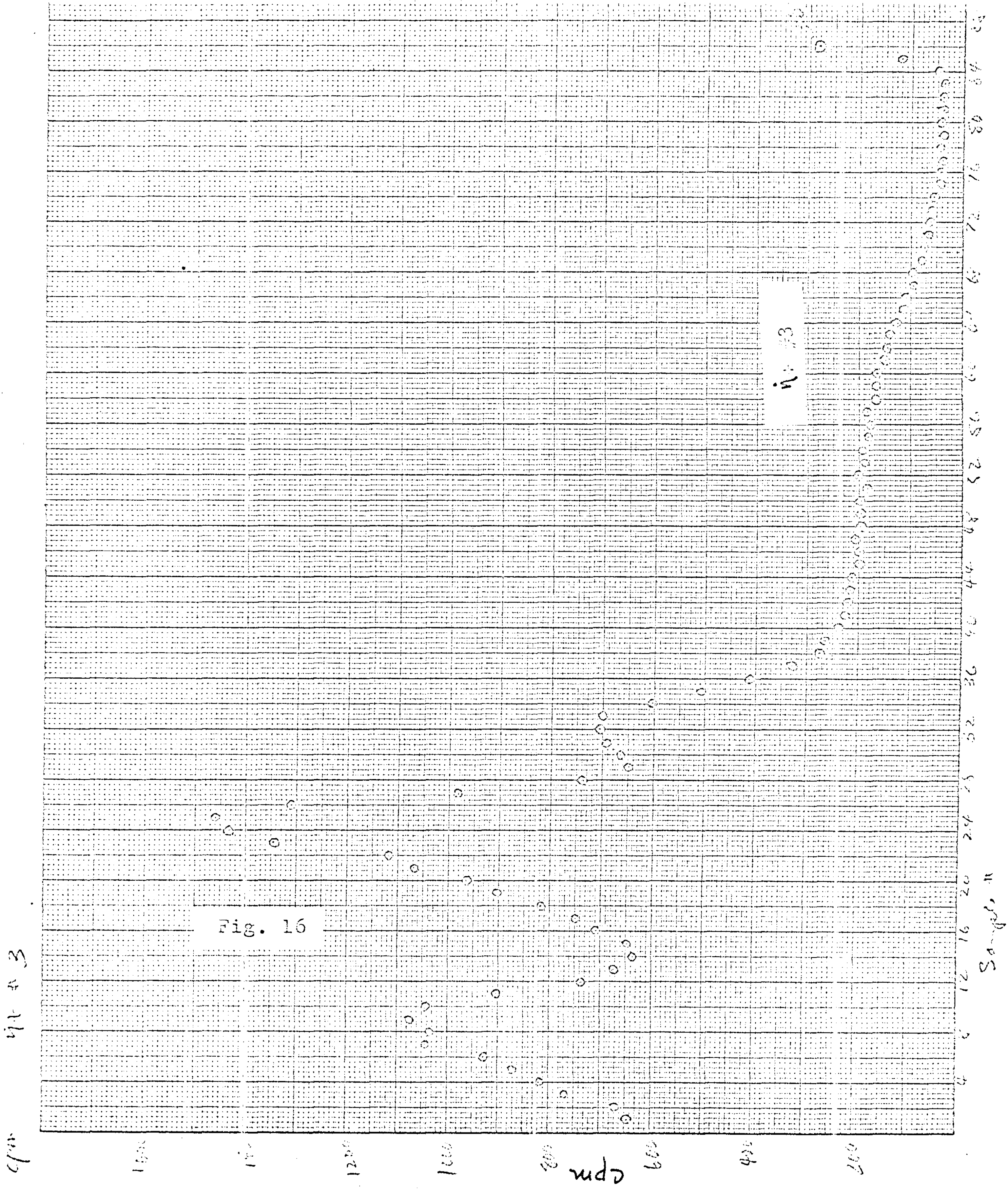

Vial #3
vial # 3
cpm
1600
1400
1200
1000
800
600
400
200
Sample #
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76
80
84
88

Fig. 16

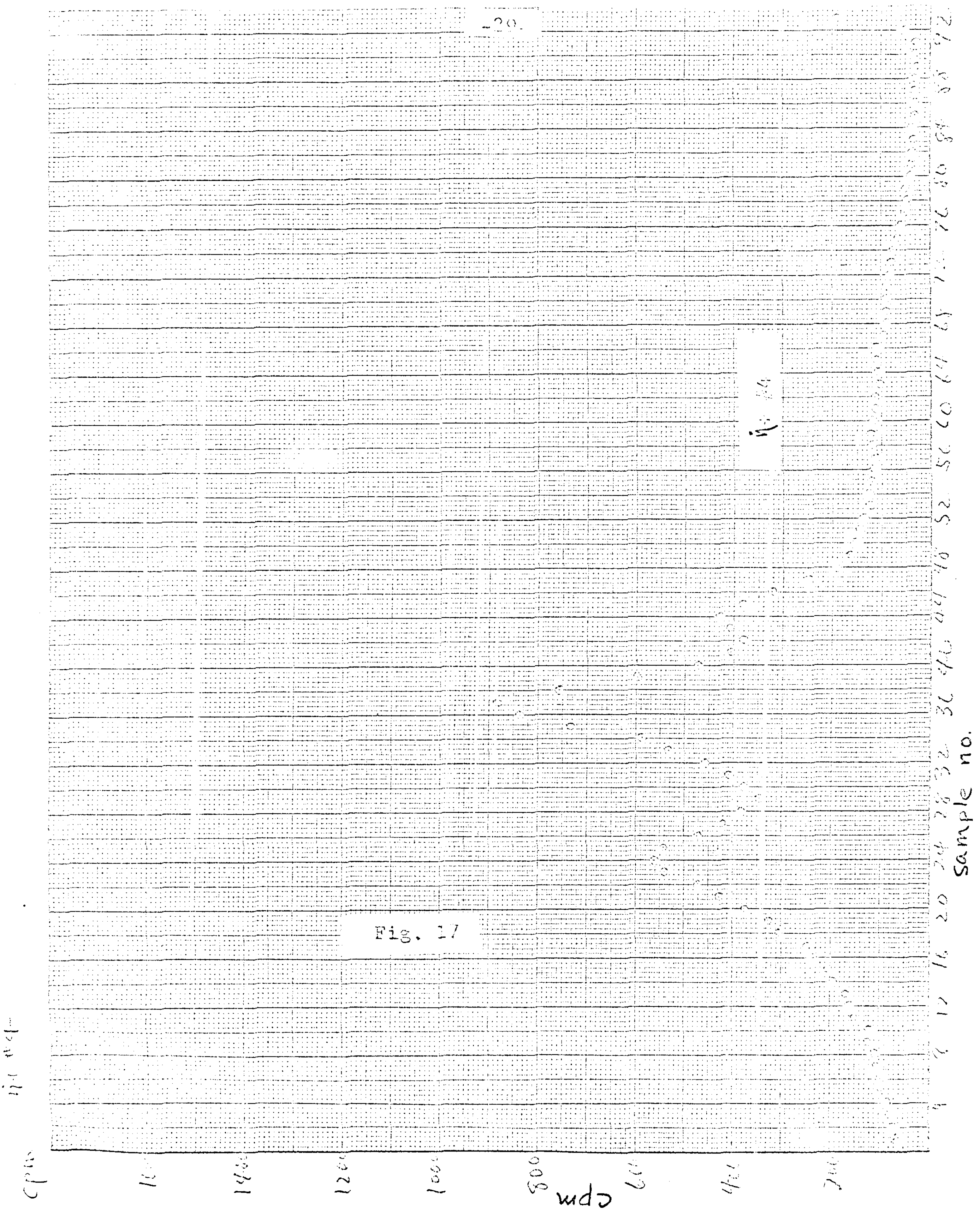

cpm
200
400
600
800
1000
1200
1400
1600
cpm
Sample no.
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76
80
84
88
92

Fig. 17

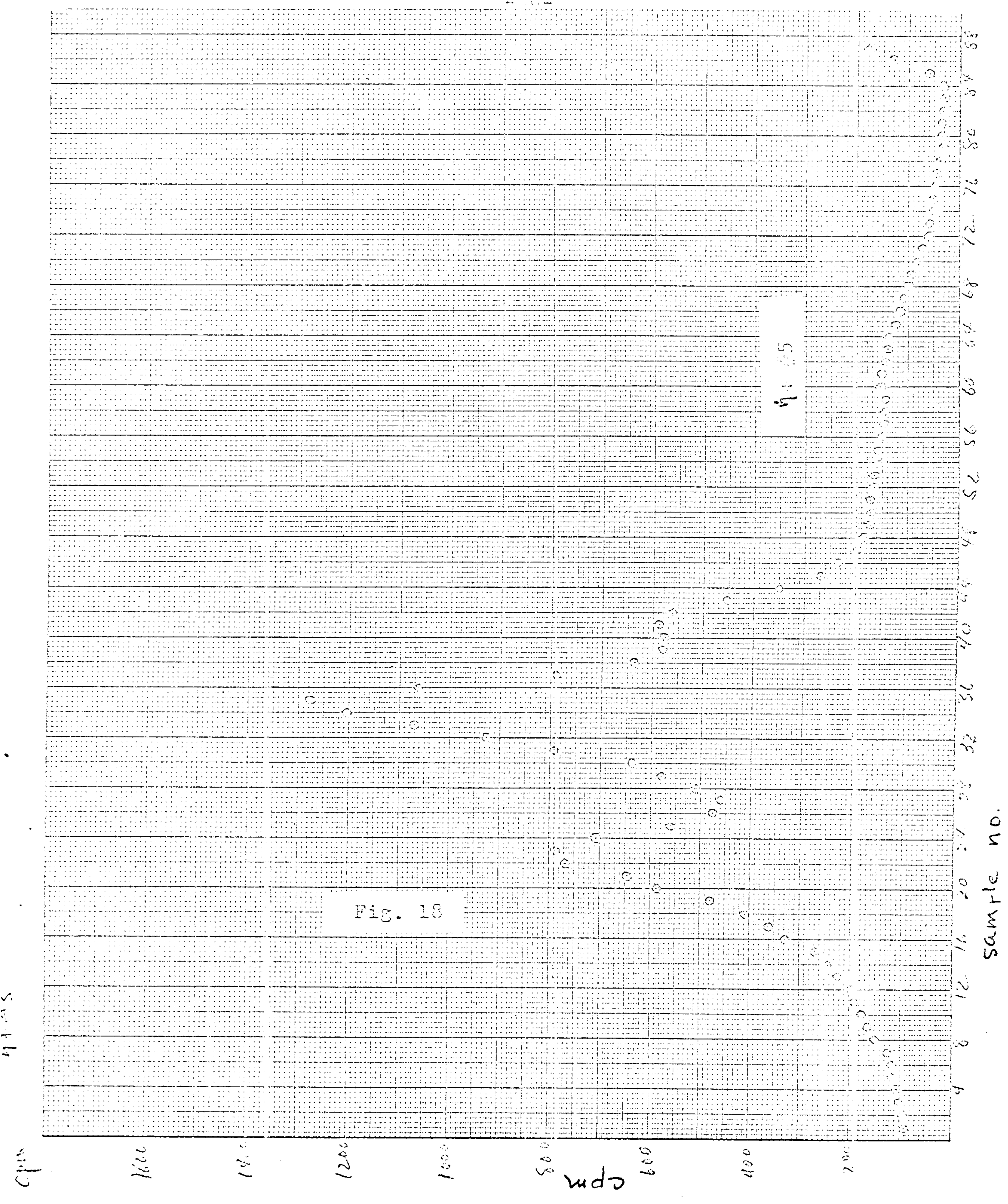

cpm
1600
1400
1200
1000
800
600
400
200
cpm
sample no.
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76
80
84
88
H-35

Fig. 18

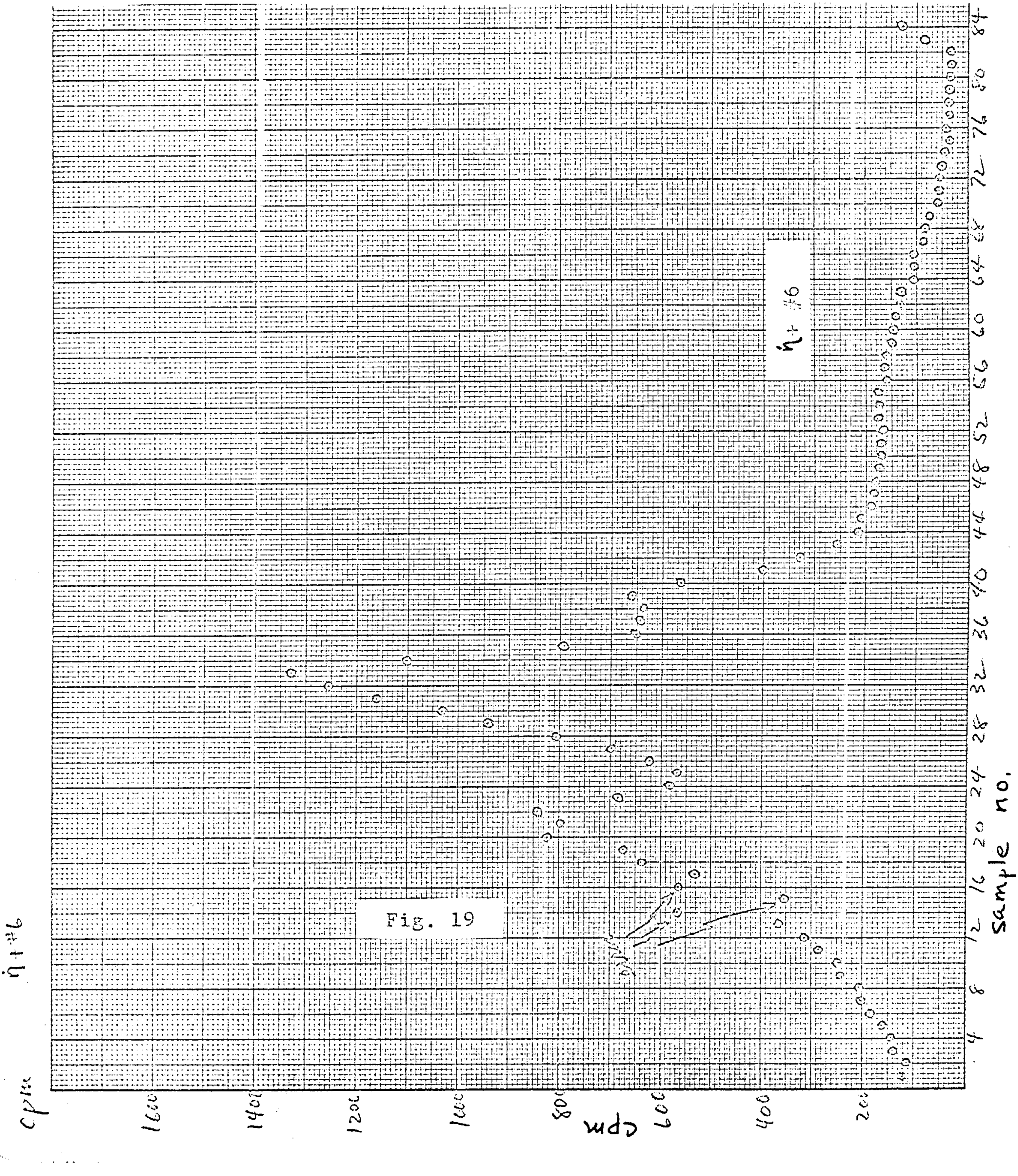


Fig. 19

ή+ #6
sample no.
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76
80
84
cpm
200
400
600
800
1000
1200
1400
1600
cpm
ή+ #6

The data for the negative viscosity gradient may be found in Figures 20-22. This experiment was the third run in a series. In the earlier runs shorter times were used and very poor separations were observed.

It may be seen that these graphs indicate both a greater separation of the peaks and a much greater degree of spreading than observed in either of the other types of gradients. These experiments were performed with the DNA used in the experiment at constant viscosity presented in Figures 12 and 13.

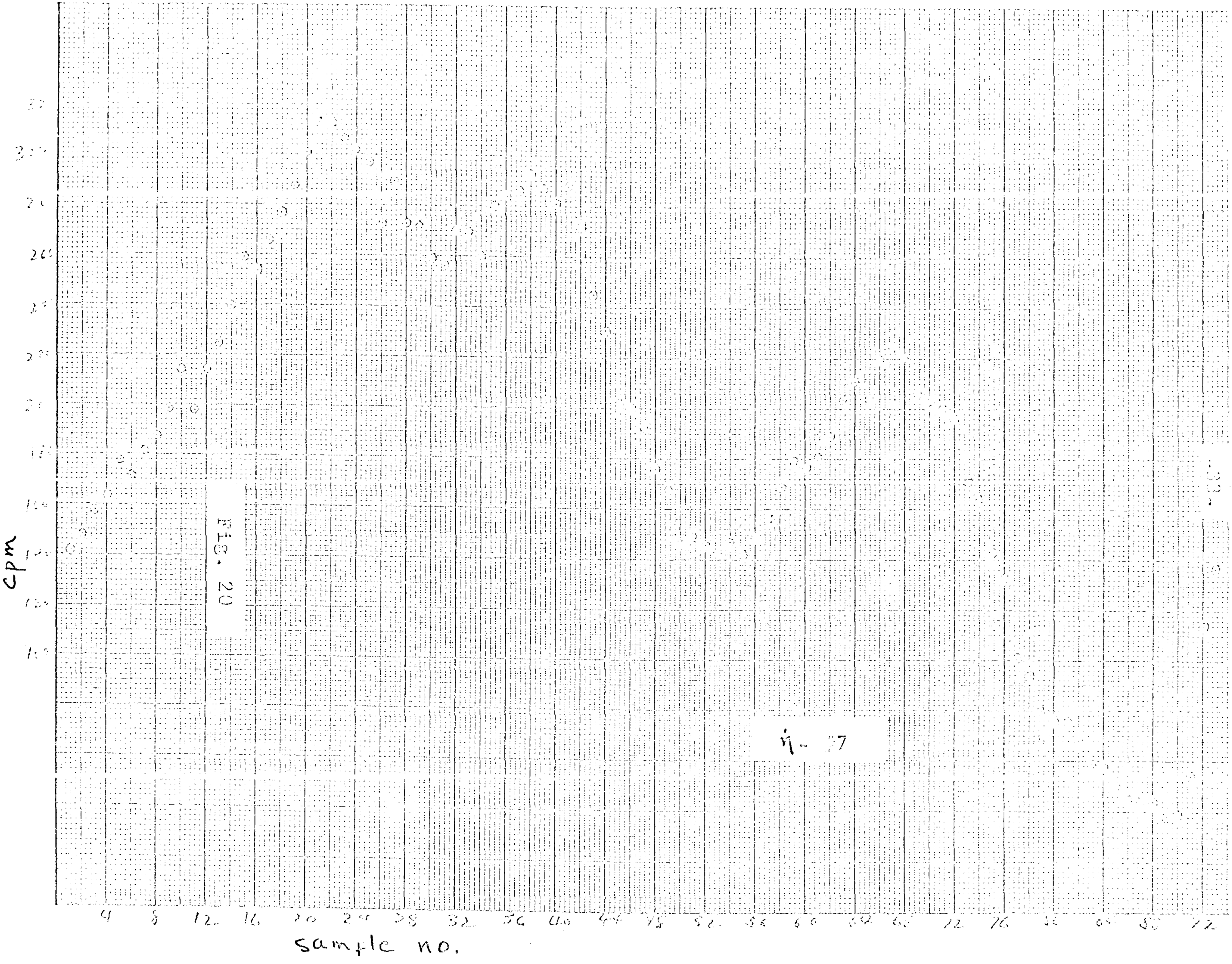


Fig. 20

230 min

η- #8

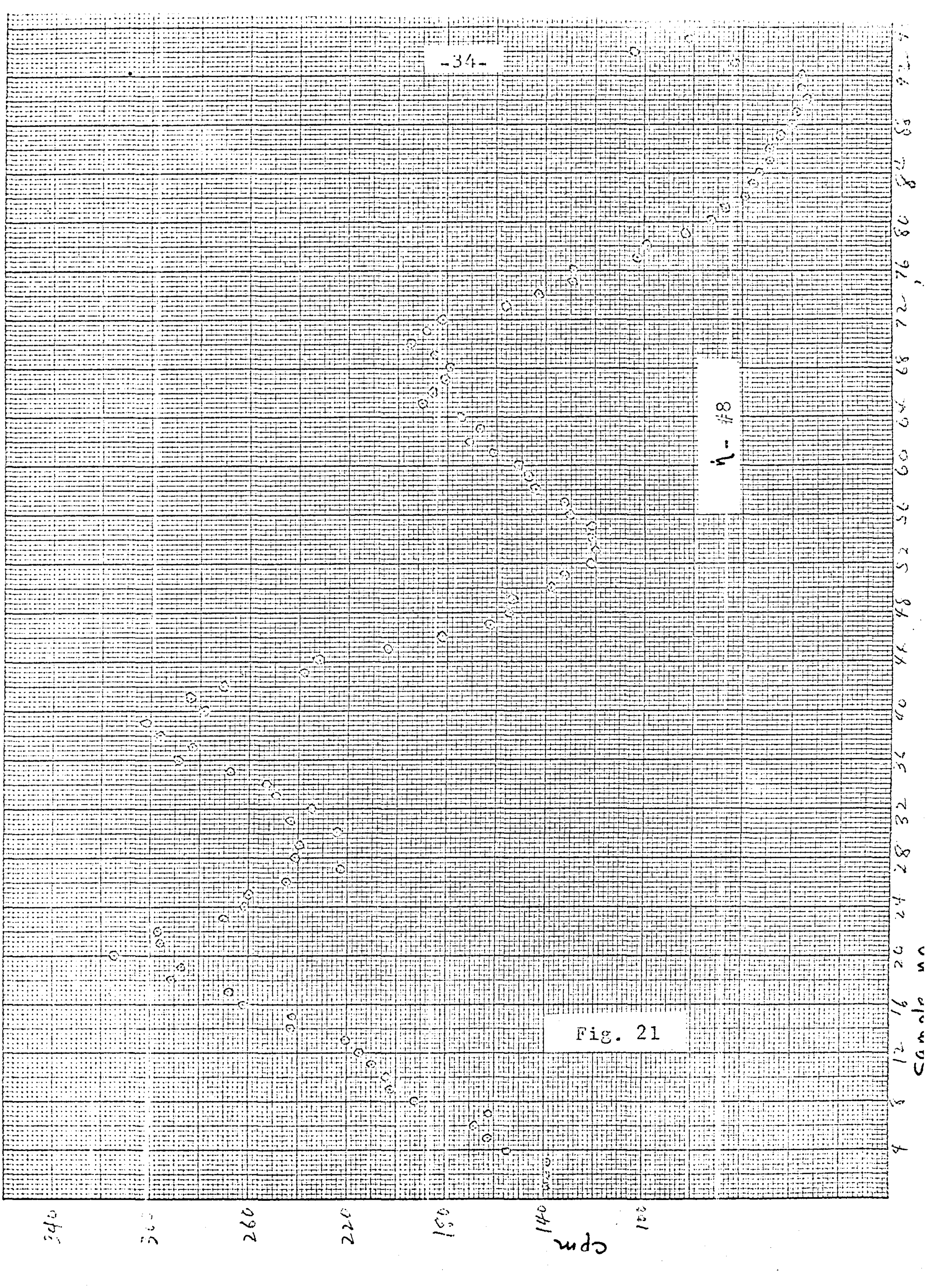


Fig. 21

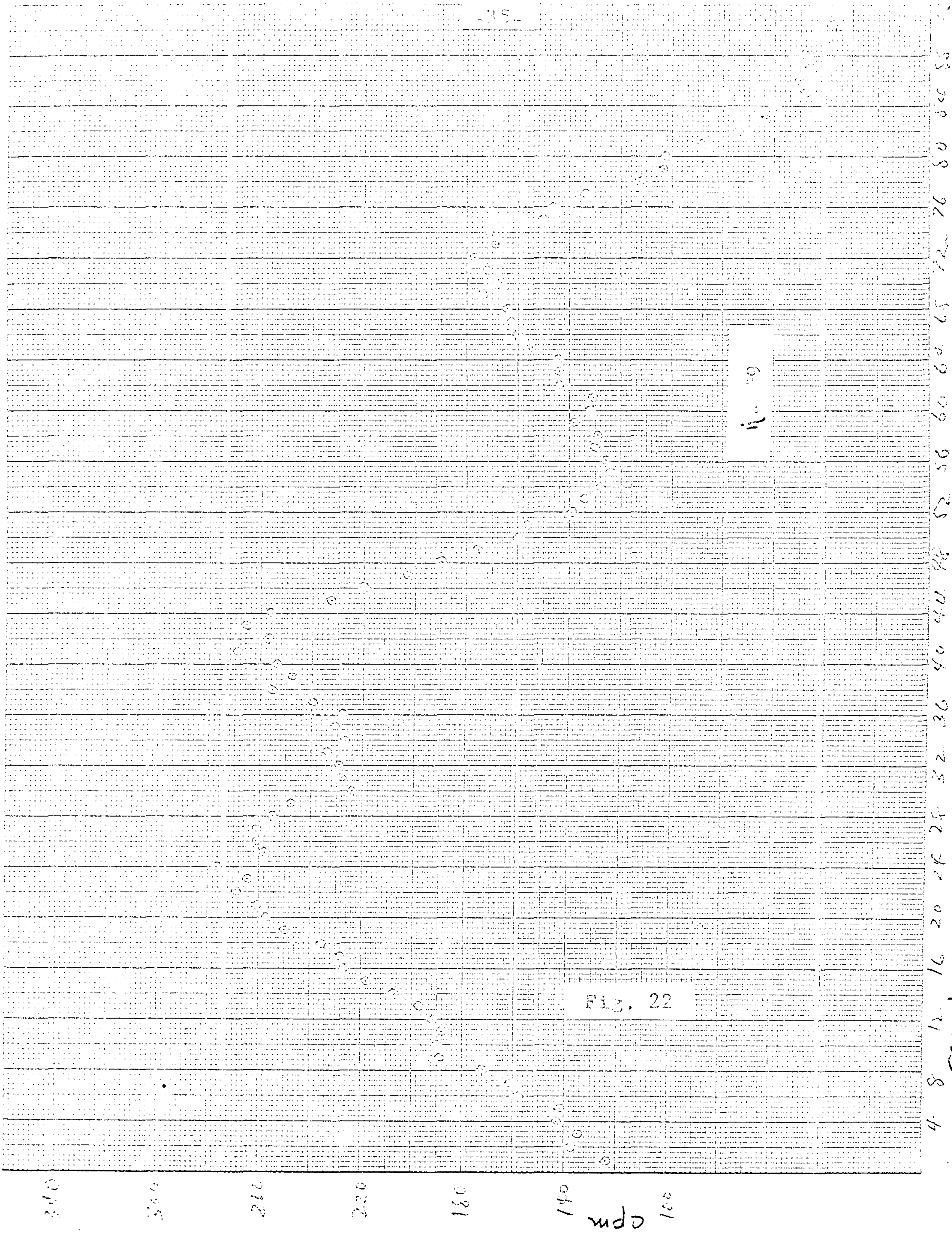

340
300
260
220
180
140
100
cpm
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76
80
84
88
Sample no.

Fig. 22

TABLE I

SUMMARY OF EXPERIMENTAL CONDITIONS FOR SEDIMENTATION VELOCITY EXPERIMENTS REPORTED IN FIGURES 1-22*

Figure	Type of Viscosity Gradient	Tube Number	Time at 45K rpm
Fig. 1-9	none	#1-#3	170 min.
Fig. 10,11	none	#4,#6	160 min.
Fig. 12,13	none	#7,#8	160 min.
Fig. 14-16	positive	#1-#3	230 min.
Fig. 17-19	positive	#4-#6	200 min.
Fig. 20-22	negative	#7-#9	230 min.

*All runs were performed in a black anodized SW-50 rotor. The temperature indicated by the thermometer during the experiments was 20°.

TABLE II

TEMPERATURE MEASUREMENTS

Time	Chamber	Bucket	Room Temperature
		26.75°C	
12:19	17.6°C		
3:20	19.5°C		
		22.75°C	
		28.0°C	
11:00	20.8°C		
12:41	20.2°C		21.38°C
		22.0°C	
		28.45°C	28.40°C
1:11	25.0°C		
2:13	21.9°C		
		25.00°C	23.38°C
			24.4°C
3:25	22.0°C		
4:35	21.3°C		
		24.5°C	

TREATMENT OF DATA AND CONCLUSIONS

It was necessary to define some mathematical representation of the resolution of bands taking into account the separation between the modes and the spreading of the band in order to be able to state which gradients gave the best resolution. A term was used which was the distance between the modes divided by the sum of the standard deviations of the two bands. In order to obtain the standard deviations from the experimental curves in a very simple way it was necessary to approximate. The curves were considered to be Gaussians in which case the standard deviation may be obtained by taking half the width of the band at a height of 60.7% of the peak height.

This technique for obtaining the standard deviations was not entirely applicable because in some cases where there was a great deal of overlap the portion of the curve at a height of 60.7% could not be measured.

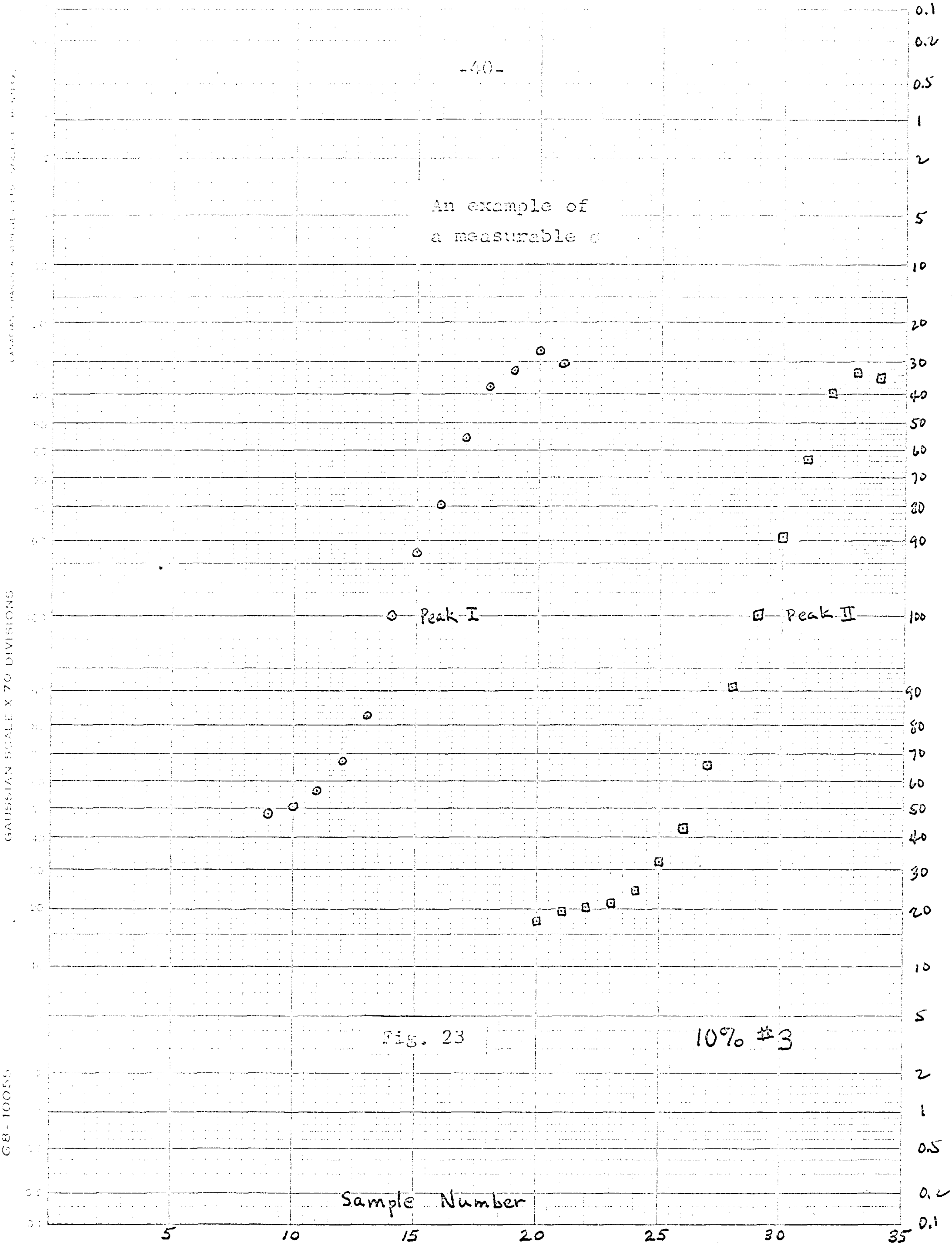
The height of the curve relative to the peak was calculated for all the experimental curves at each of the data points and graphed on Gaussian paper. At first it was thought that the standard deviation should be calculated for the Gaussian which was approximated by the curve. In that case the curve obtained on the Gaussian paper should be approximated by a straight line. This would give an easily measurable value for the standard deviation.

After consideration it was decided that a more accurate representation of the actual spread could be obtained by taking the actual band width at a height of 60./% of the peak and dividing by two. This would consider the true width rather than the possible narrower Gaussian which was approximated and taking half of the total width would compensate at least partially for a skewing the band.

In Fig. 24 it may be seen that a difficulty arose in the estimation of the band width. Since there are no data points which go as low as 60.7% on one or both halves of the peak it was necessary in some cases to try to extrapolate the curve to some value. This of course causes a lower degree of accuracy since it is not a measurable value.

In order to use some measurable quantity in all cases so that the results might be compared directly between data, another resolution term was defined. In this case it was decided to use the fact that in good separations of bands the overlap is small and therefore the height of the lowest point between peaks is lower. The farther from the mode of a band the smaller the height of the curve becomes. Also the ratio of the height of the curve to the peak height may be related to the number of standard deviation units distance the point is from the mode. This ratio is therefore a measure of the spread of the band. An approximation of the contribution of one band to the total curve is that the band will contribute one half of the total height at the lowest point between the two modes.

In order to measure the resolution the peak heights were measured along with the minimum height between the peaks. The ratio of half the minimum height to each of the peak heights



An example of
a measurable σ

Fig. 23

10% #3

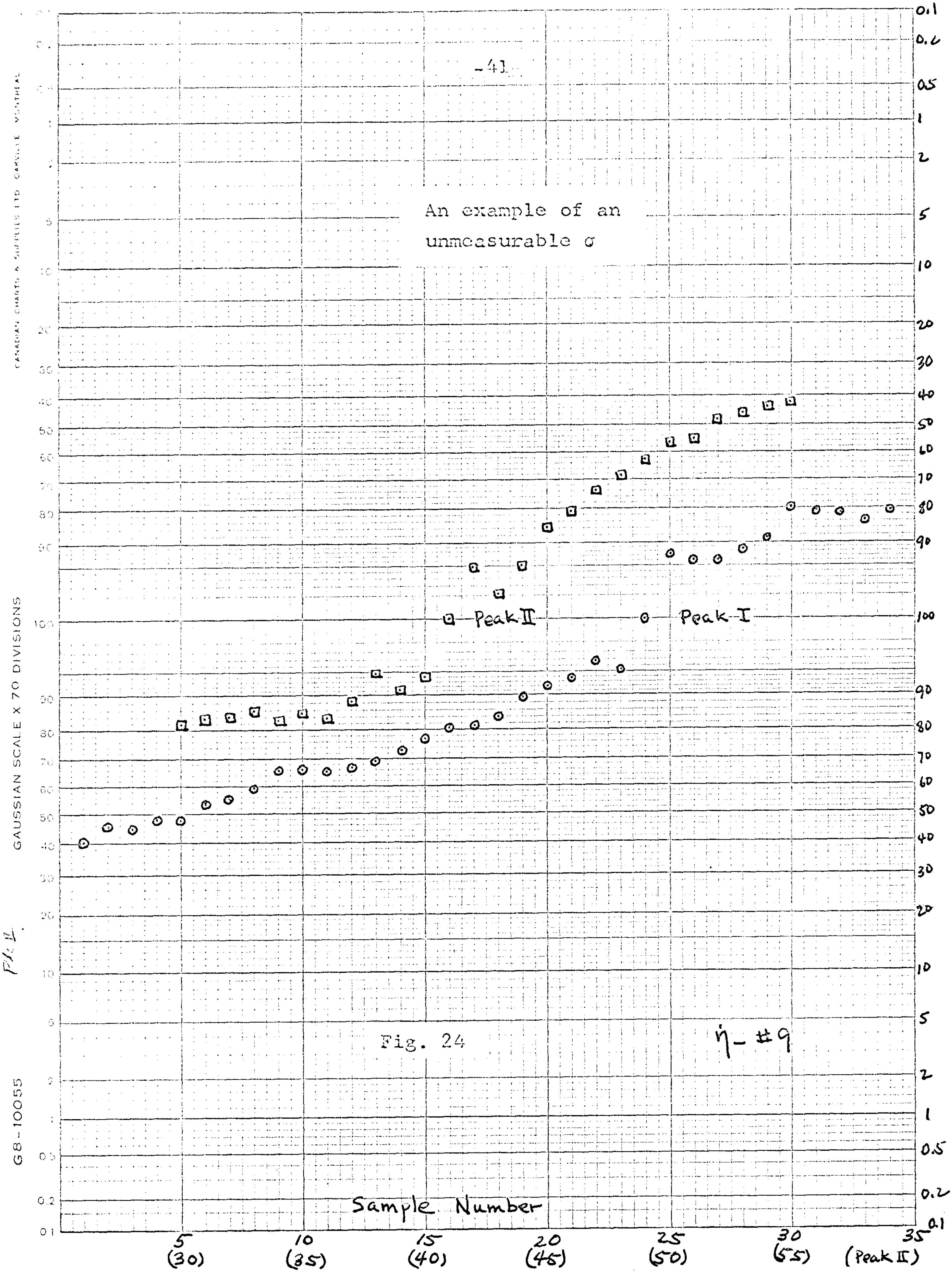

An example of an unmeasurable σ
□ Peak II
○ Peak I
η - #9
Sample Number
GAUSSIAN SCALE X 70 DIVISIONS
G8-10055
0.1
0.2
0.5
1
2
5
10
20
30
40
50
60
70
80
90
100
90
80
70
60
50
40
30
20
10
5
2
1
0.5
0.2
0.1
5 (30)
10 (35)
15 (40)
20 (45)
25 (50)
30 (55)
35 (Peak II)

Fig. 24

TABLE III

Resolution From
Gaussian Graphs

	Tube	σ_I	σ_{II}	$\sigma_I + \sigma_{II}$	Δy	Resolution	Average for Run
10%	#1	$\frac{5.3}{2}$	$\frac{5.3}{2}$	5.3	14	2.6	
	#2	$\frac{4.5}{2}$	$\frac{4.8}{2}$	4.6	14	3.0	2.9
	#3	$\frac{5.3}{2}$	$\frac{4.4}{2}$	4.8	15	3.1	
	#4	$\frac{9.9}{2}$	$\frac{9.5}{2}$	9.7	15	1.6	1.6
	#6	$\frac{9.0}{2}$	$\frac{7.0}{2}$	8.0	13	1.6	
	#7	$\frac{6.5}{2}$	$\frac{6.2}{2}$	6.4	13	2.0	2.0
	#8	$\frac{6.5}{2}$	$\frac{6.0}{2}$	6.2	12	1.9	
					Average for #1-#8		2.3
π^+	#1	$\frac{7.1}{2}$	$\frac{7.3}{2}$	7.2	16	2.2	
	#2	$\frac{13.7}{2}$	$\frac{8.3}{2}$	11.0	16	1.5	1.8
	#3	$\frac{11.5}{2}$	$\frac{8.4}{2}$	10.0	16	1.6	
	#4	$\frac{9.5}{2}$	$\frac{6.2}{2}$	7.8	13	1.7	
	#5	$\frac{6.7}{2}$	$\frac{6.0}{2}$	6.4	12	1.9	1.7
	#6	$\frac{8.3}{2}$	$\frac{6.7}{2}$	7.5	11	1.5	
					Average for #1-#6		1.7

	Tube	σ_I	σ_{II}	$\sigma_I + \sigma_{II}$	Δy	Resolution	Average for Run
ή-	#7	$\frac{26.5}{2}$	$\frac{21.0}{2}$	23.8	16.5	0.69	
	#8	$\frac{20.7}{2}$	$\frac{17.6}{2}$	19.2	19	0.99	0.78
	#9	$\frac{30.0}{2}$	$\frac{22.8}{2}$	26.4	17	0.65	

Average for #7-#9 0.78

Δy is the distance between peaks.

10% refers to gradients with 10% sucrose throughout.

ή+ refers to gradients with increasing viscosity.

ή- refers to gradients with decreasing viscosity.

Resolution is defined as Δy divided by $\sigma_I + \sigma_{II}$ with larger values indicating better resolution.

TABLE IV

Resolution in Terms of Standard Deviation Units

a	c	b	c/a	c/b	fc/a	t_I	$\bar{t}_I$	fc/b	t_{II}	$\bar{t}_{II}$	$\Sigma\bar{t}$	tube
970	193	1383	0.199	0.140	0.0775	1.81	1.89	0.0545	2.00	2.09	3.98	10% #1
1153	196	1723	0.170	0.114	0.0662	1.90		0.0444	2.10			#2
1240	188	1890	0.152	0.099	0.0591	1.95		0.0386	2.16			#3
852	289	1255	0.339	0.230	0.1319	1.49	1.48	0.0896	1.73	1.72	3.20	#4
1085	375	1570	0.346	0.239	0.1347	1.47		0.0925	1.71			#6
429	92	442	0.214	0.208	0.0833	1.77	1.78	0.0810	1.79	1.79	3.57	#7
448	94	454	0.210	0.207	0.0818	1.78		0.0806	1.79			#8
720	270	1210	0.375	0.223	0.1459	1.42	1.52	0.0868	1.75	1.73	3.25	$\dot{\eta}+$ #1
1100	342	1365	0.311	0.251	0.1210	1.55		0.0977	1.68			#2
1055	309	1442	0.293	0.214	0.1140	1.58		0.0833	1.77			#3
545	164	865	0.301	0.190	0.1171	1.57	1.55	0.0740	1.84	1.83	3.38	#4
765	220	1258	0.288	0.175	0.1121	1.59		0.0681	1.88			#5
825	275	1310	0.333	0.210	0.1295	1.50		0.0818	1.78			#6
207	78	236	0.377	0.331	0.1467	1.41	1.42	0.1289	1.50	1.48	2.90	$\dot{\eta}-$ #4
211	79	225	0.374	0.351	0.1455	1.42		0.1365	1.46			#5
291	120	273	0.412	0.440	0.1602	1.35	1.38	0.1712	1.30	1.34	2.72	#7
295	110	282	0.373	0.390	0.1451	1.42		0.1518	1.39			#8
257	104	249	0.404	0.418	0.1572	1.37		0.1628	1.34			#9

a is the height of Peak I; b is the height of Peak II; c is ½ the height at the minimum between the peaks; c/a and c/b are the relative heights of ½ the minumum to each of the peaks; fc/a and fc/b are conversions to heights of a Gaussian curve; t_I and t_{II} are distances in standard deviation units of the minimum from the peak; $\bar{t}_I$ and $\bar{t}_{II}$ are the average values for a particular run; $\Sigma\bar{t}$ is the sum of the averages for a particular run.

Average values for $\Sigma\bar{t}$ for each type gradient:

10%-----3.58
$\dot{\eta}+$-----3.32
$\dot{\eta}-$ ---2.81

was taken. Using the Normal Error Curve Tables from the C.R.C. Standard Math Tables[7] and relating the peak height to the Normal Curve height at the peak the ratios were converted to heights on a Gaussian and the number of standard deviation units from the peak were noted. The larger the value in standard deviations the greater relative distance from the peak. If the peak heights are comparable then the total standard deviations from both of the bands may be summed to give an indication of the degree of separation. The greater the separation the larger this sum becomes until there is no overlap area and the relative peak height goes to zero.

The results according to both sets of calculations of the resolution tend to indicate that the best results were achieved using the 10% sucrose solution (constant viscosity). This is followed by the positive viscosity gradient. The worst results were achieved by the negative viscosity gradient in which the graphs of data show a much larger degree of spreading. If conclusions are to be drawn from this data they are that the best resolution may be achieved with uniform viscosity.

The weakness of this conclusion from the presented data lies in the uncertainty of the temperature throughout the run which creates an uncertainty in the viscosity and the fact that this study was done with only a single density gradient. Possibly with better control of the temperature agreement of calculated distances and times with experimental results could be achieved. For a larger density gradient it is possible the results would shift from the no-viscosity-gradient preference to a preference of a negative viscosity gradient. A larger density gradient would tend to decrease the spreading of the bands.

Since it does not appear that temperature and therefore viscosity can be easily controlled no further experiments are suggested at this time. Without further experimentation and considering just this available data it is suggested that one will achieve the best resolution of bands in a preparative ultracentrifuge when no viscosity gradient is present.

APPENDIX

I. Calculations for the starting solutions of the Linderstrom-Lang column

Let K= kerosene in ml.
B= bromobenzene in ml.
H= "heavy" solution in ml.
L= "light" solution in ml.

The density of kerosene is approximately equal to 0.79 gm./ml.[2] The density of bromobenzene is 1.49 gm./ml.

$$H + L = 54 \text{ ml. (burette volume)} \qquad (1)$$

Let the desired starting solutions have densities ρ_H = 1.44 gm./ml. and ρ_L = 1.07 gm./ml.

To balance hydrostatically:

$$1.44\ H = 1.07\ L = 1.07\ (54 - H) \qquad (2)$$

Solving the equation for H and substituting back into (1) also gives L.

$$H = 23.0 \text{ ml.} \qquad L = 31.0 \text{ ml.}$$

For H:

$$K + B = 23.0 \text{ ml.} \qquad (3)$$

The conservation of mass equation gives:

$$23.0 \text{ ml.} \times 1.44 \text{ gm./ml.}$$
$$= K \times 0.79 \text{ gm./ml.} + B \times 1.49 \text{ gm./ml.} \qquad (4)$$

The solution to the simultaneous equations is:

$$K = 1.64 \text{ ml.} \qquad B = 21.4 \text{ ml.}$$

For L:

$$K + B = 31.0 \text{ ml.} \qquad (5)$$

The conservation of mass equation gives:

$$\begin{aligned}&31.0 \text{ ml.} \times 1.07 \text{ gm./ml.}\\ &= K \times 0.79 \text{ gm./ml.} + B \times 1.49 \text{ gm./ml.} \qquad (6)\end{aligned}$$

The solution to the simultaneous equations is:

$K = 18.6$ ml. $\quad B = 12.4$ ml.

APPENDIX

II. Calculations for the starting solutions for the sucrose-cesium chloride gradients

The solutions were to be:

	density
(a) 5% sucrose	1.02
(b) 20% sucrose	1.08
(c) 10% sucrose	1.04

Using equation (1-1):

(1) $1.02 + y_1 - 1 = 1.22$
$y_1 = 1.02$ a + d

(2) $1.08 + y_2 - 1 = 1.28$
$y_2 = 1.20$ b + d

(3) $1.08 + y_3 - 1 = 1.22$
$y_3 = 1.14$ b + e

(4) $1.02 + y_4 - 1 = 1.28$
$y_4 = 1.26$ a + f

(5) $1.04 + y_5 - 1 = 1.22$
$y_5 = 1.18$ c + g

(6) $1.04 + y_6 - 1 = 1.28$
$y_6 = 1.24$ c + h

The solutions should have the following cesium chloride concentrations:

Solution	density	to be used in
(d) 1.61 M	1.20	(1) and (2)
(e) 1.15 M	1.14	(3)
(f) 2.10 M	1.26	(4)
(g) 1.45 M	1.18	(5)
(h) 1.95 M	1.24	(6)

For mixing double concentrations were used.

(a) 10% sucrose
(b) 40% sucrose
(c) 20% sucrose
(d) 3.22 M CsCl
(e) 2.30 M CsCl
(f) 4.20 M CsCl
(g) 2.90 M CsCl
(h) 3.90 M CsCl

APPENDIX

III. Derivation of a centrifuge equation

Let the subscript "a" represent the top of the centrifuge tube and the conditions there. This is the initial position of the macromolecule. The subscript "x" will then represent a position in the tube and its conditions. "ρ" will represent density and "η_r" will represent the relative viscosity of the bulk solution. "ρ_o" will represent the buoyant density of the material. "s" is the sedimentation coefficient. "$\dot{\rho}$" and "$\dot{\eta}$" represent change with respect to distance.

An assumption is made that density and viscosity are linear through the tube. In other words "$\dot{\rho}$" and "$\dot{\eta}$" are taken as constants.

$$s^{Na}_{20,w} = s^{Cs}_{20,w} \text{ (mass ratio) } = s^{Cs}_{20,w}\,(0.755)$$

$$= s_a \eta_{ra} \frac{\rho_o - \rho_{20,w}}{\rho_o - \rho_a}\,(0.755) \tag{1}$$

$$s_a = \frac{s^{Na}_{20,w}}{\eta_{ra}} \frac{\rho_o - \rho_a}{\rho_o - \rho_{20,w}} \frac{1}{(0.755)} \tag{2}$$

$$s_x = \frac{s^{Na}_{20,w}}{\eta_{rx}} \frac{\rho_o - \rho_x}{\rho_o - \rho_{20,w}} \frac{1}{(0.755)} \tag{3}$$

$$s_x = s_a \frac{\eta_{ra}}{\eta_{rx}} \frac{\rho_o - \rho_x}{\rho_o - \rho_a} \tag{4}$$

Let $\rho_x = \rho_a + \dot{\rho}\Delta x$ (5)

$$\eta_{rx} = \eta_{ra} + \dot{\eta}\Delta x \tag{6}$$

where $\Delta x = x - x_a$

substituting (5) and (6) into (4)

$$s_x = s_a \frac{\eta_{ra}}{\eta_{ra} + \dot{\eta}\Delta x} \frac{\rho_o - \rho_a - \dot{\rho}\Delta x}{\rho_o - \rho_a} \tag{7}$$

$$s_x = s_a \frac{1}{1 + (\dot{\eta}/\eta_{ra})\Delta x} \left(1 - \frac{\dot{\rho}\Delta x}{\rho_o - \rho_a} \right) \tag{8}$$

$$\frac{dx}{dt} = s_x \omega^2 x \tag{9}$$

Substituting (8) into (9)

$$\frac{dx}{dt} = s_a \omega^2 \frac{x}{1 + (\dot{\eta}/\eta_{ra})\Delta x} \left(1 - \frac{\dot{\rho}\Delta x}{\rho_o - \rho_a} \right) \tag{10}$$

Let $y = \Delta x = x - x_a$ or $x = y + x_a$

then $dy = dx$

Let $\delta = \rho_o - \rho_a$

Equation (10) becomes

$$\frac{dy}{dt} = s_a\omega^2 \frac{y + x_a}{1 + (\dot{\eta}/\eta_{ra})\, y} \left(1 - \frac{\dot{\rho}y}{\delta}\right) \tag{11}$$

Separating variables

$$s_a\omega^2 dt = \frac{dy}{y + x_a} \, \frac{1 + (\dot{\eta}/\eta_{ra})y}{1 - (\dot{\rho}/\delta)y} \tag{12}$$

$$\int_0^t s_a\omega^2 dt = \int_0^y \frac{dy}{(y + x_a)(\,1 - (\dot{\rho}/\delta)y)}$$

$$+ \frac{\dot{\eta}}{\eta_{ra}} \int_0^y \frac{y\,dy}{(y + x_a)(\,1 - (\dot{\rho}/\delta)y)} \tag{13}$$

$$\int_0^t s_a\omega^2 dt = \int_0^y \frac{dy}{x_a + y - (\dot{\rho}x_a/\delta)y - (\dot{\rho}/\delta)y^2}$$

$$+ \frac{\dot{\eta}}{\eta_{ra}} \int_0^y \frac{y\,dy}{x_a + y - (\dot{\rho}x_a/\delta)y - (\dot{\rho}/\delta)y^2} \tag{14}$$

The right hand integrals are of the form

$\int \frac{dy}{Y}$ and $\int \frac{y\,dy}{Y}$ where Y is a quadratic.

The integrals are:

$$\int \frac{dy}{Y} = \frac{1}{\sqrt{-q}} \ln \frac{2cy + b - \sqrt{-q}}{2cy + b + \sqrt{-q}} \tag{15}$$

$$\int \frac{y\,dy}{Y} = \frac{1}{2c} \ln Y - \frac{b}{2c} \int \frac{dy}{Y} \tag{16}$$

where $Y = a + by + cy^2$ and $q = 4ac - b^2$

For equation (14) the constants are defined as follows:

$$a = x_a; \quad b = 1 - (\dot{\rho}/\delta)x_a; \quad c = -(\dot{\rho}/\delta) \qquad (17)(18)(19)$$

$$\therefore q = -4x_a(\dot{\rho}/\delta) - (\,1 - (\dot{\rho}/\delta)x_a)^2 \tag{20}$$

$$q = -\left(1 + (\hat{\rho}/\delta)x_a \right)^2 \tag{21}$$

$$s_a\omega^2 t\Big|_0^t = \int_0^y \frac{dy}{Y} + \frac{\hat{\eta}}{\eta_{ra}}\left(\frac{1}{2c}\ln Y - \frac{b}{2c}\int \frac{dy}{Y}\right)\Big|_0^y \tag{22}$$

$$s_a\omega^2 t = \left(1 - \frac{\hat{\eta}}{\eta_{ra}}\frac{b}{2c}\right)\int_0^y \frac{dy}{Y} + \frac{\hat{\eta}}{\eta_{ra}}\frac{1}{2c}\ln Y\Big|_0^y \tag{23}$$

$$s_a\omega^2 t = \left(1 - \frac{\hat{\eta}}{\eta_{ra}}\frac{b}{2c}\right)\frac{1}{\sqrt{-q}}\ln\frac{2cy + b - \sqrt{-q}}{2cy + b + \sqrt{-q}}\Bigg|_0^y$$

$$+ \frac{\hat{\eta}}{\eta_{ra}}\frac{1}{2c}\ln Y\Big|_0^y \tag{24}$$

$$s_a\omega^2 t = \left(1 - \frac{\hat{\eta}}{\eta_{ra}}\frac{1 - (\hat{\rho}/\delta)x_a}{-2(\hat{\rho}/\delta)}\right)\frac{1}{1 + (\hat{\rho}/\delta)x_a}$$

$$\ln\frac{-2(\hat{\rho}/\delta)y + 1 - (\hat{\rho}/\delta)x_a - \left(1 + (\hat{\rho}/\delta)x_a\right)}{-2(\hat{\rho}/\delta)y + 1 - (\hat{\rho}/\delta)x_a + \left(1 + (\hat{\rho}/\delta)x_a\right)}\Bigg|_0^y$$

$$+ \frac{\hat{\eta}}{\eta_{ra}}\frac{1}{-2(\hat{\rho}/\delta)}$$

$$\ln\left(x_a + \left(1 - (\hat{\rho}/\delta)x_a\right)y - (\hat{\rho}/\delta)y^2\right)\Big|_0^y \tag{25}$$

$$s_a\omega^2 t = \left(1 - \frac{\hat{\eta}}{\eta_{ra}}\frac{1 - (\hat{\rho}/\delta)x_a}{-2(\hat{\rho}/\delta)}\right)\frac{1}{1 + (\hat{\rho}/\delta)x_a}$$

$$\ln\frac{-2(\hat{\rho}/\delta)\left(y + x_a\right)}{2\left(-(\hat{\rho}/\delta)y + 1\right)}\Bigg|_0^y$$

$$+ \frac{\hat{\eta}}{\eta_{ra}}\frac{1}{-2(\hat{\rho}/\delta)}\ln\left((x_a + y)\left(1 - (\hat{\rho}/\delta)y\right)\right)\Big|_0^y \tag{26}$$

$$s_a\omega^2 t = \left(1 - \frac{\dot{\eta}}{\eta_{ra}} \frac{1 - (\dot{\rho}/\delta)x_a}{-2(\dot{\rho}/\delta)}\right) \frac{1}{1 + (\dot{\rho}/\delta)x_a}$$

$$\left(\ln\left(\frac{-(\dot{\rho}/\delta)(y + x_a)}{1 - (\dot{\rho}/\delta)y}\right) - \ln(-(\dot{\rho}/\delta)x_a)\right)$$

$$+ \frac{\dot{\eta}}{\eta_{ra}} \frac{1}{-2(\dot{\rho}/\delta)}$$

$$(\ln((x_a + y)(1 - (\dot{\rho}/\delta)y)) - \ln x_a) \tag{27}$$

$$s_a\omega^2 t = \left(1 - \frac{\dot{\eta}}{\eta_{ra}} \frac{1 -(\dot{\rho}/\delta)x_a}{-2(\dot{\rho}/\delta)}\right) \frac{1}{1 + (\dot{\rho}/\delta)x_a}$$

$$\left(\ln\left(\frac{y + x_a}{x_a}\right) - \ln(1 - (\dot{\rho}/\delta)y)\right)$$

$$+ \frac{\dot{\eta}}{\eta_{ra}} \frac{1}{-2(\dot{\rho}/\delta)}$$

$$\left(\ln\left(\frac{y + x_a}{x_a}\right) + \ln(1 - (\dot{\rho}/\delta)y)\right) \tag{28}$$

$$s_a\omega^2 t = \left(1 - \frac{\dot{\eta}}{\eta_{ra}} \frac{1 - (\dot{\rho}/\delta)x_a}{-2(\dot{\rho}/\delta)}\right) \frac{1}{1 + (\dot{\rho}/\delta)x_a}$$

$$+ \frac{\dot{\eta}}{\eta_{ra}} \frac{1}{-2(\dot{\rho}/\delta)}) \ln\left(\frac{y + x_a}{x_a}\right)$$

$$+ \left(\frac{\dot{\eta}}{\eta_{ra}} \frac{1}{-2(\dot{\rho}/\delta)} - \left(1 - \frac{\dot{\eta}}{\eta_{ra}} \frac{1 - (\dot{\rho}/\delta)x_a}{-2(\dot{\rho}/\delta)}\right)\right.$$

$$\left.\frac{1}{1 + (\dot{\rho}/\delta)x_a}\right) \ln(1 - (\dot{\rho}/\delta)y) \tag{29}$$

$$s_a\omega^2 t \;=\; (\frac{1}{-2(\dot{\rho}/\delta)} (-2(\dot{\rho}/\delta) - \frac{\dot{\eta}}{\eta_{ra}}(1 - (\rho/\delta)))$$

$$\frac{1}{1 + (\dot{\rho}/\delta)x_a} + \frac{1}{-2(\dot{\rho}/\delta)}(\frac{\dot{\eta}}{\eta_{ra}}$$

$$(1 + (\dot{\rho}/\delta)x_a) \frac{1}{1 + (\dot{\rho}/\delta)x_a}) \ln(\frac{y + x_a}{x_a})$$

$$(\frac{1}{-2(\dot{\rho}/\delta)} (\frac{\dot{\eta}}{\eta_{ra}}(1 + (\dot{\rho}/\delta)x_a))$$

$$\frac{1}{1 + (\dot{\rho}/\delta)x_a} - \frac{1}{-2(\dot{\rho}/\delta)} (-2(\dot{\rho}/\delta)$$

$$- \frac{\dot{\eta}}{\eta_{ra}}(1 - (\dot{\rho}/\delta)x_a)) \frac{1}{1 + (\dot{\rho}/\delta)x_a})$$

$$\ln(1 - (\dot{\rho}/\delta)y) \tag{30}$$

$$s_a\omega^2 t \;=\; \frac{1}{-2(\dot{\rho}/\delta)} \frac{1}{1 + (\dot{\rho}/\delta)x_a} ((-2(\dot{\rho}/\delta) + 2\frac{\dot{\eta}}{\eta_{ra}}$$

$$(\dot{\rho}/\delta)x_a) \ln(\frac{y + x_a}{x_a}) + (2(\dot{\rho}/\delta) + 2\frac{\dot{\eta}}{\eta_{ra}})$$

$$\ln(1 - (\dot{\rho}/\delta)y)) \tag{31}$$

$$s_a\omega^2 t \;=\; \frac{1}{2(\dot{\rho}/\delta)(1 + (\dot{\rho}/\delta)x_a)} ((2(\dot{\rho}/\delta))(1 - \frac{\dot{\eta}}{\eta_{ra}}x_a)$$

$$\ln(\frac{y + x_a}{x_a}) + (2(\dot{\rho}/\delta)(1 + \frac{\dot{\eta}}{\eta_{ra}} \frac{1}{(\dot{\rho}/\delta)})$$

$$\ln(\ 1 - (\dot{\rho}/\delta)y)) \qquad (32)$$

$$s_a\omega^2 t \ = \frac{1}{1 + (\dot{\rho}/\delta)x_a}\left(\left(\ 1 - \frac{\dot{\eta}}{\eta_{ra}}x_a\right)\ \ln\left(\frac{y + x_a}{x_a}\right)\right.$$

$$\left. - \left(\ 1 + \frac{\dot{\eta}}{\eta_{ra}}\ \frac{1}{(\dot{\rho}/\delta)}\right)\ \ln(\ 1 - (\dot{\rho}/\delta)y)\right) \qquad (33)$$

$$t \ = \frac{1}{s_a\omega^2}\left(\frac{1}{1 + (\dot{\rho}/\delta)x_a}\left(\left(\ 1 - \frac{\dot{\eta}}{\eta_{ra}}\ x_a\right)\ \ln\left(\frac{y + x_a}{x_a}\right)\right.\right.$$

$$\left.\left. - (\ 1 + \frac{\dot{\eta}}{\eta_{ra}}\ \frac{1}{(\dot{\rho}/\delta)}\ \ln(\ 1 - (\dot{\rho}/\delta)y)\right)\right) \qquad (34)$$

Substituting from equation (2)

$$t \ = \frac{\eta_{ra}(\ \rho_o - \rho_{20,w})(0.755)}{s^{Na}_{20,w}\ \delta\,\omega^2}$$

$$\frac{1}{1 + (\dot{\rho}/\delta)x_a}\left(\left(\ 1 - \frac{\dot{\eta}}{\eta_{ra}}x_a\right)\ \ln\left(\frac{y + x_a}{x_a}\right)\right.$$

$$\left. - \left(\ 1 + \frac{\dot{\eta}}{\eta_{ra}}\ \frac{1}{(\dot{\rho}/\delta)}\right)\ \ln(\ 1 - (\dot{\rho}/\delta)y)\right)$$

$$(35)$$

EXAMPLE

$$s_{20,w}^{Na} = 19.9 \times 10^{-13} \text{ sec.}; \; \omega^2 = 1.33 \times 10^9 \frac{\text{radian}^2}{\text{sec.-min.}};$$

$$\rho_o = 1.702 \frac{\text{gm.}}{\text{cc.}}; \; \rho_{20,w} = 0.998 \frac{\text{gm.}}{\text{cc.}}; \; \rho_a = 1.228 \frac{\text{gm.}}{\text{cc.}};$$

$$\eta_{ra} = 1.10; \; \delta = 0.474 \frac{\text{gm.}}{\text{cc.}}; \; \dot{\rho} = 0.0149 \frac{\text{gm.}}{\text{cc.-cm.}};$$

$$\dot{\eta} = 0.200 \text{ cm.}^{-1}; \; x_a = 5.5 \text{ cm.}; \; (\dot{\rho}/\delta) = 0.0314 \text{ cm.}^{-1};$$

$$\frac{\dot{\eta}}{\eta_{ra}} = 0.182 \text{ cm.}^{-1}$$

$$t = \frac{1.10(0.704)0.755}{19;9 \times 10^{-13}(0.474)1.33 \times 10^9} \; \frac{2.303}{1 + 0.0314(5.5)}$$

$$((1 - 0.182(5.5))\log(\frac{y + 5.5}{5.5})$$

$$-(1 + \frac{0.182}{0.0314})\log(1 - 0.0314y))$$

$$t = 907(-0.001 \log(1 + \frac{y}{5.5})$$

$$- 6.80 \log(1 - 0.0314y))$$

At a time t_1 the macromolecule has travelled a distance $y = 1$.

$$t_1 = 907(-0.001 \log(1 + \frac{1}{5.5})$$

$$- 6.80 \log(1 - 0.0314))$$

$$t_1 = 907(-0.001 \log(1.182) - 6.80 \log(0.9686))$$

$$t_1 = 907(-0.001(0.07262) - 6.80(-0.01386))$$

$$t_1 = 907(0.0941) = 85.3 \text{ min.}$$

A comparison of the theoretical results with the experimental data follows.

$$t_{3.1} = 907\left(-0.001 \log\left(1 + \frac{3.1}{5.5}\right) - 6.80 \log(1 - 0.0314(3.1))\right)$$

$$= 907(-0.001 \log(1.563) - 6.80 \log(0.902))$$

$$= 907(-0.0002 + 0.3026) = 274.3 \text{ min.}$$

$t_{3.1} = 274.3$ min.; experimental $t_{3.1} = 200$ min.

$t_{3.7} = 330$ min.; experimental $t_{3.7} = 230$ min.

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