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An introduction to the study of chromatography of aureomycin ...

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AN INTRODUCTION TO THE STUDY OF CHROMATOGRAPHY OF AUREOMYCIN

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CHAPTER I

INTRODUCTION

PURPOSE OF THE STUDY

Part of a current research program undertaken by the faculty in chemistry at the College of the Pacific is concerned with studying chemical compounds which are favorably absorbed by malignant tissue. A compound which is preferably absorbed is Aureomycin. The reason for this preference of absorption is still not known. In order to determine the cause of this absorption, it is necessary to establish a method of recovering tetracycline and its derivatives from malignant tissue. When working with recovery of tetracycline and its derivatives, the small scale of operations immediately suggests the use of chromatographic techniques of separation, identification, and estimation.

The literature contains only one reference to the chromatography of tetracycline. In Antibiotics, June, 1957, Selzer and Wright[5] described a procedure for paper chromatography of Aureomycin and its derivatives using a pyridine containing resolving system. While we were able to repeat their procedure, the sharpness of the chromatograms left much to be desired. For this reason alone it seemed desirable to attempt to develop a superior procedure. There is also a further objection to using the Selzer and Wright method;

namely, a solvent containing pyridine would prohibit the testing of absorption of tetracycline on malignant tissue directly on a chromatogram. Thus it was the purpose of this project to devise chromatographic procedures capable of yielding well-defined chromatographic spots and using solvent systems that would not combine strongly with proteinaceous material.

The use of paper chromatography is not a new development in chemistry. Pliny mentions the use of papyrus in the detection of ferrous sulphate, but the great popularity of present-day paper chromatography is due to Martin and Synge.[4]

PARTITION CHROMATOGRAPHY

The phenomenon of adsorption takes place in chromatography. Partition, or paper, chromatography is a method of separating substances by distributing them between two liquid phases, one of which is mobile and the other essentially fixed by sorption to a support. The support itself may or may not be active in the separation process. Partition chromatography is thus essentially a differential countercurrent application of the liquid-liquid distribution. It is, however, sometimes complicated by other types of distribution. The method originally developed by Martin and Synge[4] for the purpose of separating mixtures of acetylated amino acids has since been widely applied to analytical problems.

Two methods of partition chromatography may be employed: the method using columns, the "bulk" application, and that using strips or sheets of paper, the "one-" or "two-dimensional"

application. The former employs a column of silica gel or starch, to which is sorbed one fluid phase, over which the second fluid phase passes. The latter employs strips or sheets of filter paper to support the one phase, the second passing over this, driven by capillary forces. The types of apparatus used in these two methods are different, but the principle is the same for both.

The theory proposed by Martin and Synge[4] for the partition chromatogram was developed on analogy to that of the packed distilling column. Using the assumptions that diffusion upward and downward in the column was negligible, and that the distribution constant of a solute between the two phases was not sensitive to concentration or to the presence of other solutes, they derived and defined the R_f value, in which the movement of the zone is relative to that of the advancing front of the developer liquid, the mobile phase:

$$R_f = \frac{\text{Movement of zone}}{\text{Movement of advancing front of mobile phase}}.$$

The measurement of the R_f value is relatively simple: the center of the original spot is taken as an origin and the distance to the center of the developed spot, or zone, and that to the front of the developer are measured.

In general, the procedure for paper chromatography is to place a small amount of the mixture to be separated near the end of a strip of filter paper which is then arranged so that the adsorbent solvent can pass over the spot, carrying it along in the direction of flow. The filter paper retains

an aqueous phase. The end of the paper is then dipped into the solvent, which flows through the spot. The flow is caused by capillarity. The chamber should be sealed.

After the solvent has moved sufficiently far, the chamber is opened, the strip or sheet is removed and examined. It is obvious that the apparatus used with paper strips and sheets must be different from that used for column chromatography. The solvent can be run upward or downward, but the paper must be kept in an atmosphere saturated with the solvents used.

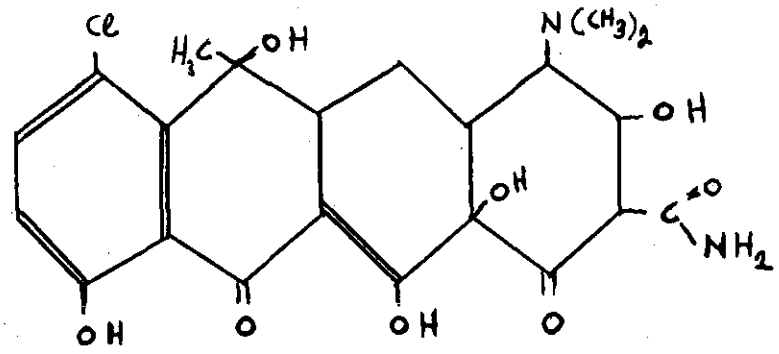
A problem which occurs often when working with paper chromatograms is streaking. Consden[1] and colleagues have called attention to the effects of metal-ions in paper. Filter paper is made in many grades, and these may differ widely in their behavior. Even the best filter paper may contain soluble material. Paper usually contains three forms of cellulose, classified on the basis of behavior. -cellulose is insoluble in strong NaOH, and forms are soluble, but the is precipitated by mineral acid from the alkaline solution, while the remains soluble. A good filter paper should also be low in reducing substances.

We may conclude that filter paper cannot be considered an inert material, even toward water.

CHAPTER II

HISTORICAL AND THEORETICAL BACKGROUND

Aureomycin was discovered by Dr. B. M. Duggar[2] in 1948 in his work for the Lederle Laboratories. In 1952, the structure was found by Hochstein[3] to be:



Aureomycin belongs to the tetracyclines. It is an antibiotic which is used to fight diseases caused by bacteria and rickettsia. This may be one reason why it is favorably absorbed by malignant tissue. Due to the poor blood supply occurring in many advanced malignancies in humans, it is not uncommon for the malignancy to become bacterially infected. For this reason, it has become common medical practice to give antibiotics to these persons. In these cases, Aureomycin is preferably absorbed by the malignancy and by bones. In order to further the studies of this phenomenon by our group, it is highly desirable that effective chromatographic procedures for the examination of tetracycline compounds be developed.

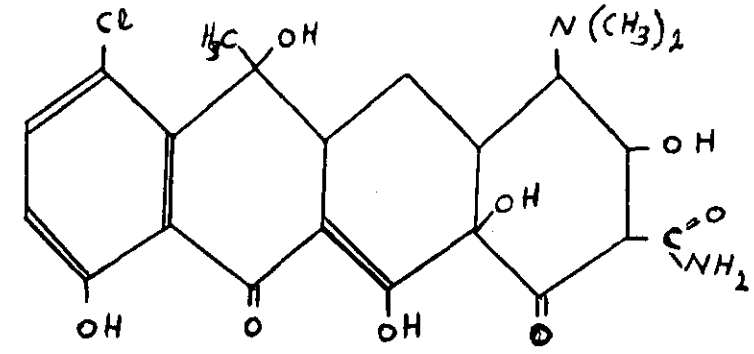
Historically, little research has been done in

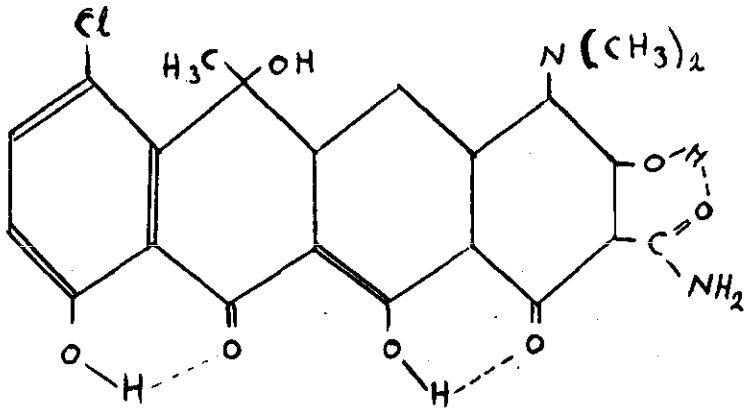
applying chromatographic methods to the identification and recovery of these compounds. The only reference to the chromatography of tetracyclines is the work of Selzer and Wright.[5]

The filter paper used was Whatman No. 1 and the resolving agent was chloroform, nitro-methane, pyridine (10:20:3). Buffered paper of pH 3.5 was used, and the compound was applied as a methanolic solution. The positions of the spots were identified by U. V. light, after exposure of the paper to ammonia vapor.

In running a compound of this nature, the same difficulties must be anticipated which occur with similar compounds such as amino acids. One of such difficulties is streaking. One of the similarities between Aureomycin and the amino acids lies in the amphoteric behavior, which includes a definite iso-electric point, being soluble in acids and bases and undergoing ionization. Another similarity is the zwitter-ion formation.

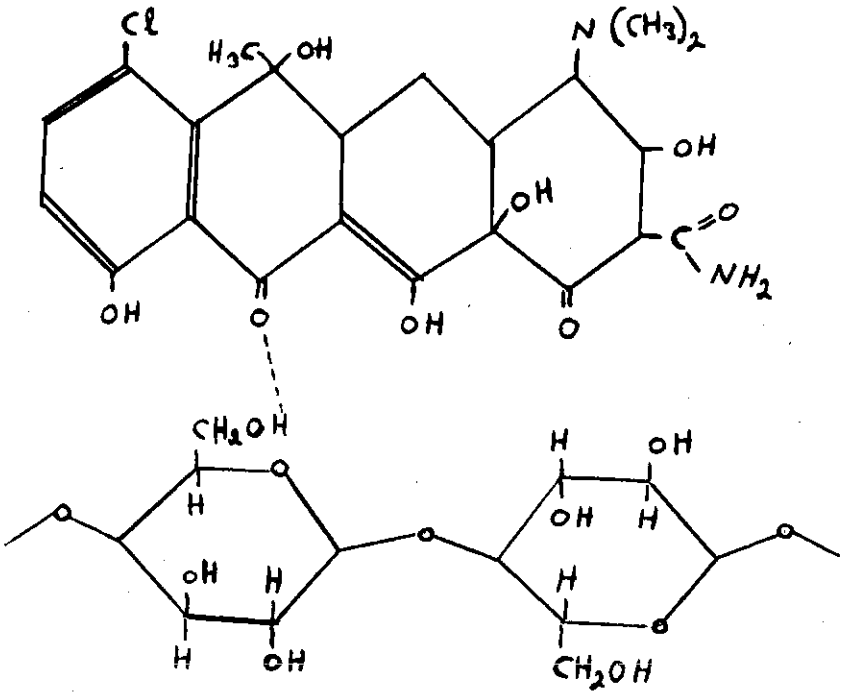
In looking upon the compound the possibilities of internal proton-bonding is easily seen.



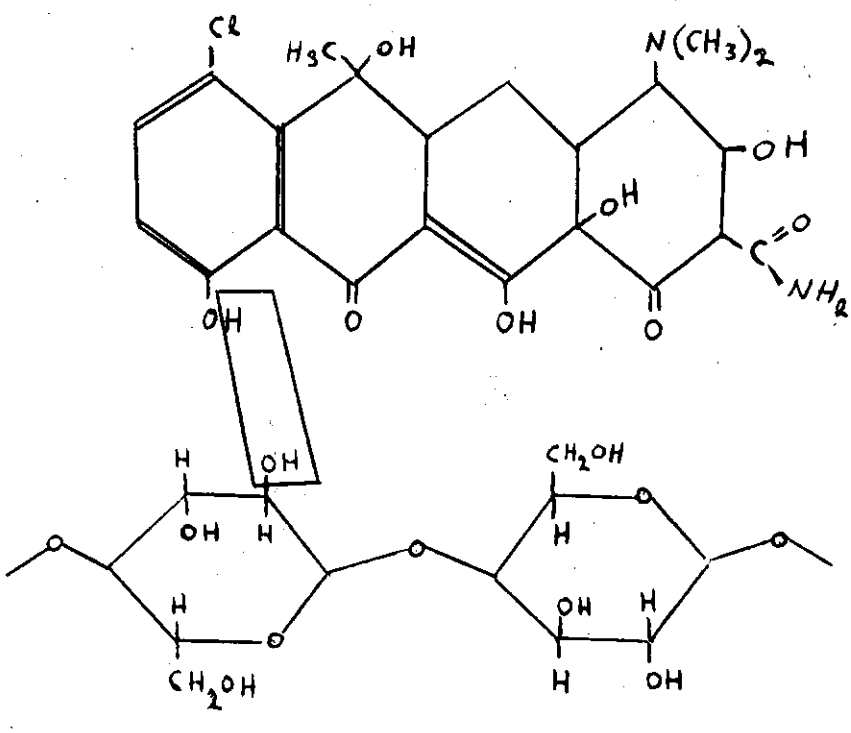


This internal proton-bonding minimizes ionization and this state has its maximum at the iso-electric point. In a solution with a pH different from the iso-electric point, the internal proton-bonding is disturbed. This disturbance represents an increase in the polarity and thus an increase in reactivity of the compound. This increase in reactivity can now lead to various reactions which can cause streaking. In the opinion of the investigator, the following reactions can occur between Aureomycin, cellulose, and the metal-ions present in the filter paper used:

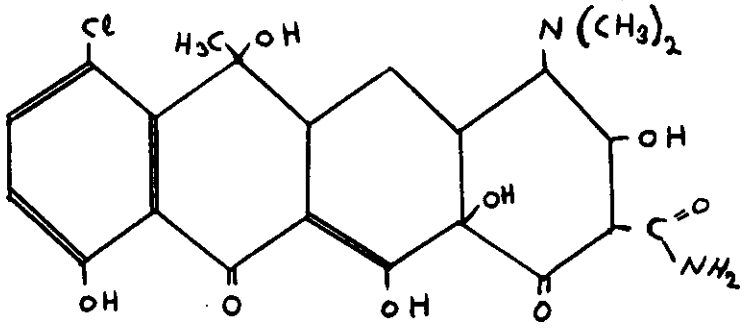
1. Proton-bonding.

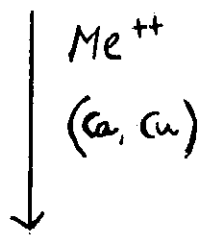


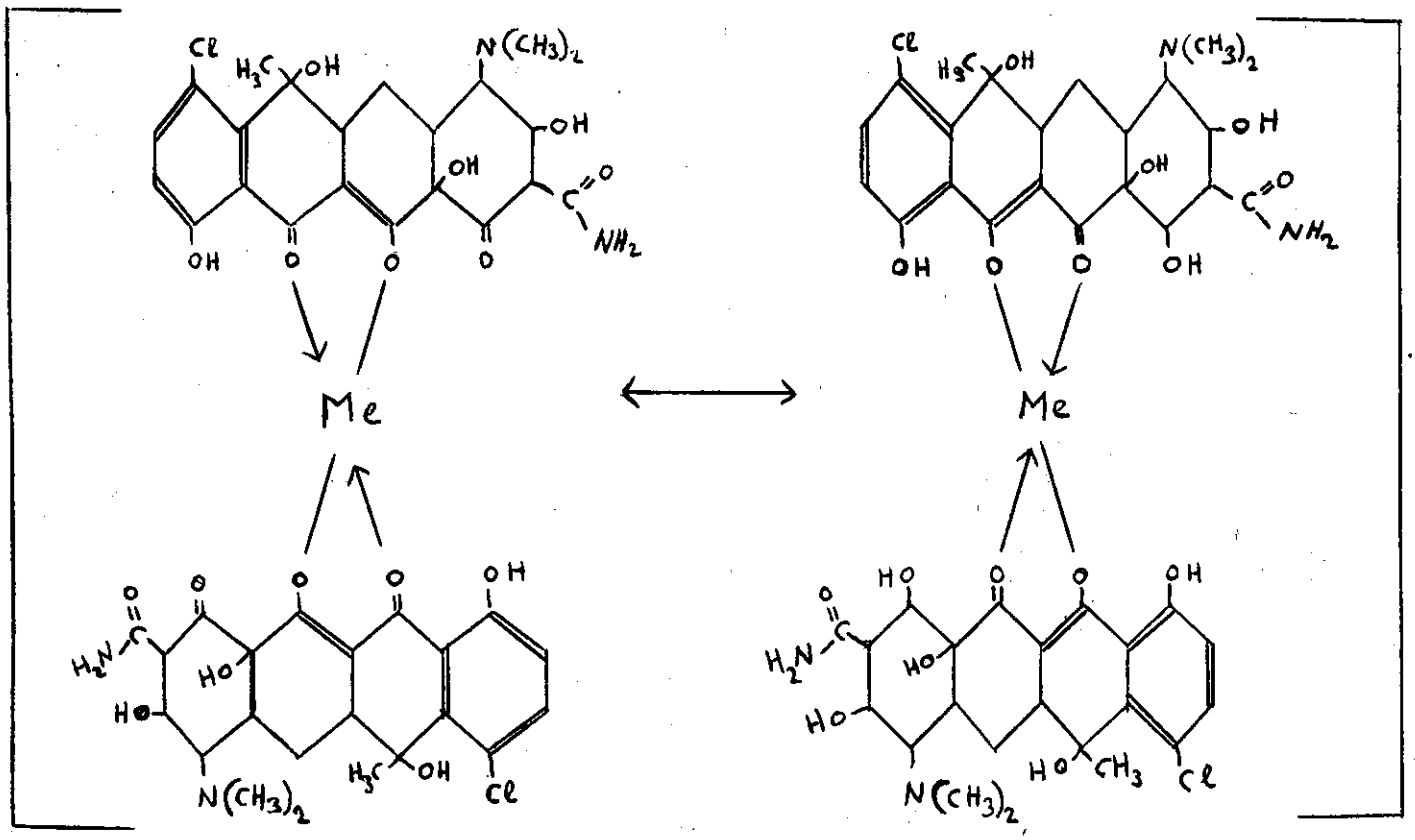
2. One mol water split off.



3. Chelate formation with metal-ions.

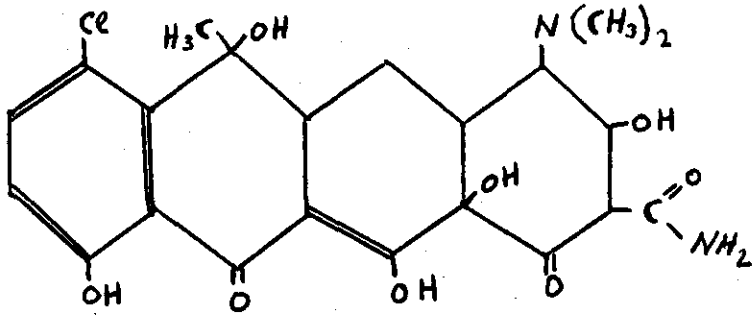




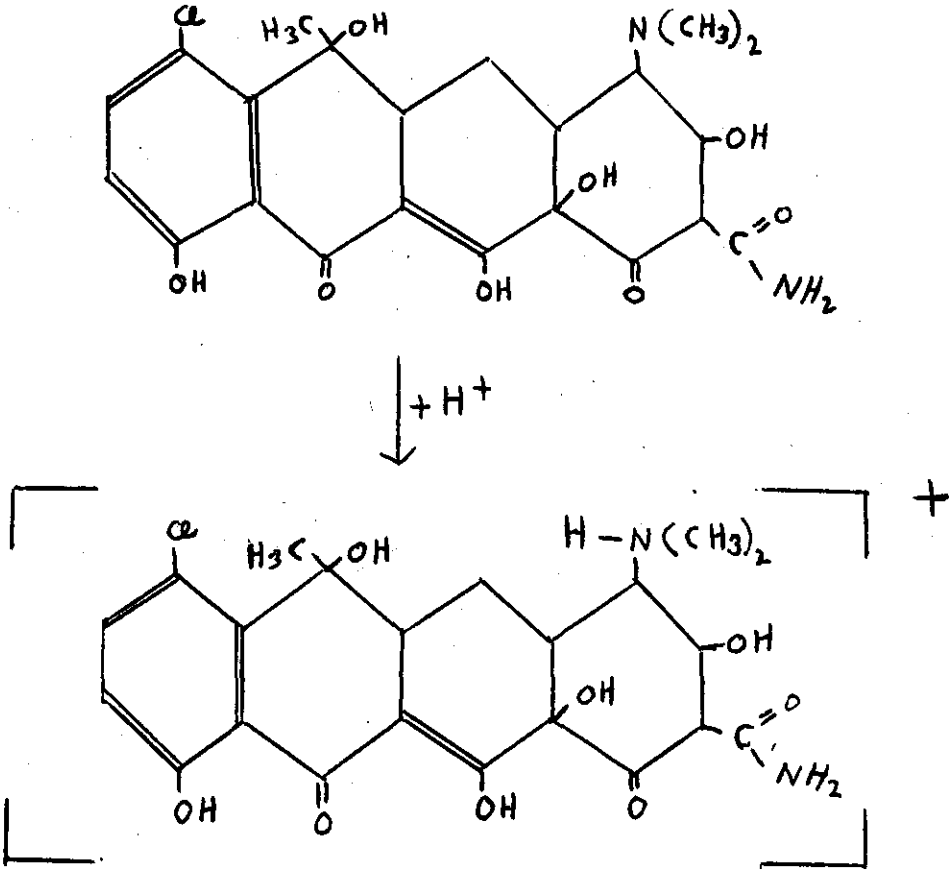


The resonance between the two structures shown above will tremendously stabilize the metal complexes. Further, the inner complex salts generally have a low solubility. It is to be expected that this type of chelate formation will cause heavy streaking in the running of paper chromatograms.

Looking upon the formula of Aureomycin,



we can see it is a base, since it is able to accept a proton and thus ionize.



Since Aureomycin is a base, it will easily ionize in acid. As the solvent systems used have more or less acidity, there will be a higher or lower ionization of the Aureomycin. It is well known that there are difficulties involved in running ionized compounds on paper chromatography. This excludes strongly acidic solutions. It was observed that with increasing acidity there was increased streaking. Aureomycin easily ionizes in an acid because the pH of the solution is different from that of the iso-electric point. The possibility of running Aureomycin in a basic solution is, of course, present. But the acidic solution is to be chosen. All solvent systems used were acidic.

CHAPTER III

EXPERIMENTAL

TECHNIQUES

The Aureomycin was used in its base rather than salt form. It was dissolved as 0.001% solution in pure methanol and stored in darkness. During the work the methanolic solutions showed decomposition, which required new working solutions made up every second month. The filter paper used was Whatman No. 1. The time of running the spots varied from 10 to 150 minutes.

Because of anticipated streaking, precautions were taken to eliminate all the usual causes of this phenomenon. Thus glass tanks were used which were sealed with plate glass lids. Before running the spots, the atmosphere in the tanks was saturated with the resolving solution for twenty-four hours. Fresh resolving solutions were prepared for each group of chromatograms. All the solutions had a 20% water saturation.

In the search for a suitable solvent system for Aureomycin, three different classes of resolving mixtures were used:

1. Methanol, acetic acid, 20% water saturation. The acetic acid differed in percentage from 1 to 10.

2. Butyl-alcohol, 10% Pyridine, 20% water saturation. The acetic acid differed in percentage from 1 to 5.

3. Nitro-ethane, Pyridine, Chloroform (6:1:3). The acetic acid differed in percentage from 1 to 5.

To identify the spots, the same method used by Selzer and Wright was applied.

RESULTS

Firstly, the behavior of Aureomycin using pure methanol as solvent was studied. As was to be expected, the spots moved with the front, and the problem was to retard this movement. Traces of water and acetic acid had no influence, but by increasing the acidity and by saturating the solution with 20% water, it was possible to slow the movement of the spots. However, by thus increasing the acidity, the streaking of the spots was also increased, presumably due to increased salt formation.

Acidities from 1 to 10 percent were used, and by running over a long period of time, some R_f values, which may be usable, were obtained. But they might prove to be too low for separation of derivatives of Aureomycin because of the very low magnitude of the R_f values obtained.

Figures 2,3,4,5,6, indicate that by increasing the acidity the R_f values increased. But as has been mentioned, another phenomenon took place: streaking.

Using 1, 2, 3 and 4% of acetic acid, no streaking occurred, and the spot applied moved as one unit. But with an increase above 4% of the acid, streaking began. Thus, while the R_f values became better, the streaking increased

to an extent which negated any practical use of these higher acidities.

The streaking probably occurred because the applied compound, Aureomycin, chelated with the paper and thus decreased in concentration, so that it made the identification of the applied compound almost impossible.

Another phenomenon observed with acidity above 4% was the decrease in rapidity of movement. It then took a longer time to establish a constant R_f value. This was caused by the decrease of the concentration, which was caused by the chelation.

TABLE I

METHANOL BASED SOLVENT SYSTEM

Time (minutes)	R_f	Time (minutes)	R_f
1% acetic acid		6% acetic acid	
10	.2	30	.48
20	.16	40	.36
30	.15	50	.35
40	.13	60	.33
60	.13	120	.33
120	.13		
2% acetic acid		7% acetic acid	
10	.23	30	.6
20	.19	40	.46
30	.16	50	.4
40	.16	60	.39
60	.16	90	.36
120	.16	120	.36
3% acetic acid		8% acetic acid	
10	.26	40	.6
20	.21	50	.5
30	.21	60	.45
40	.2	90	.4
60	.2	120	.4
120	.2		
4% acetic acid		9% acetic acid	
10	.3	60	.53
20	.24	90	.45
30	.22	120	.43
40	.22	150	.43
60	.22		
120	.22		
5% acetic acid		10% acetic acid	
20	.4	60	.63
30	.33	90	.5
40	.32	120	.46
60	.3	150	.46
120	.3		

TABLE II

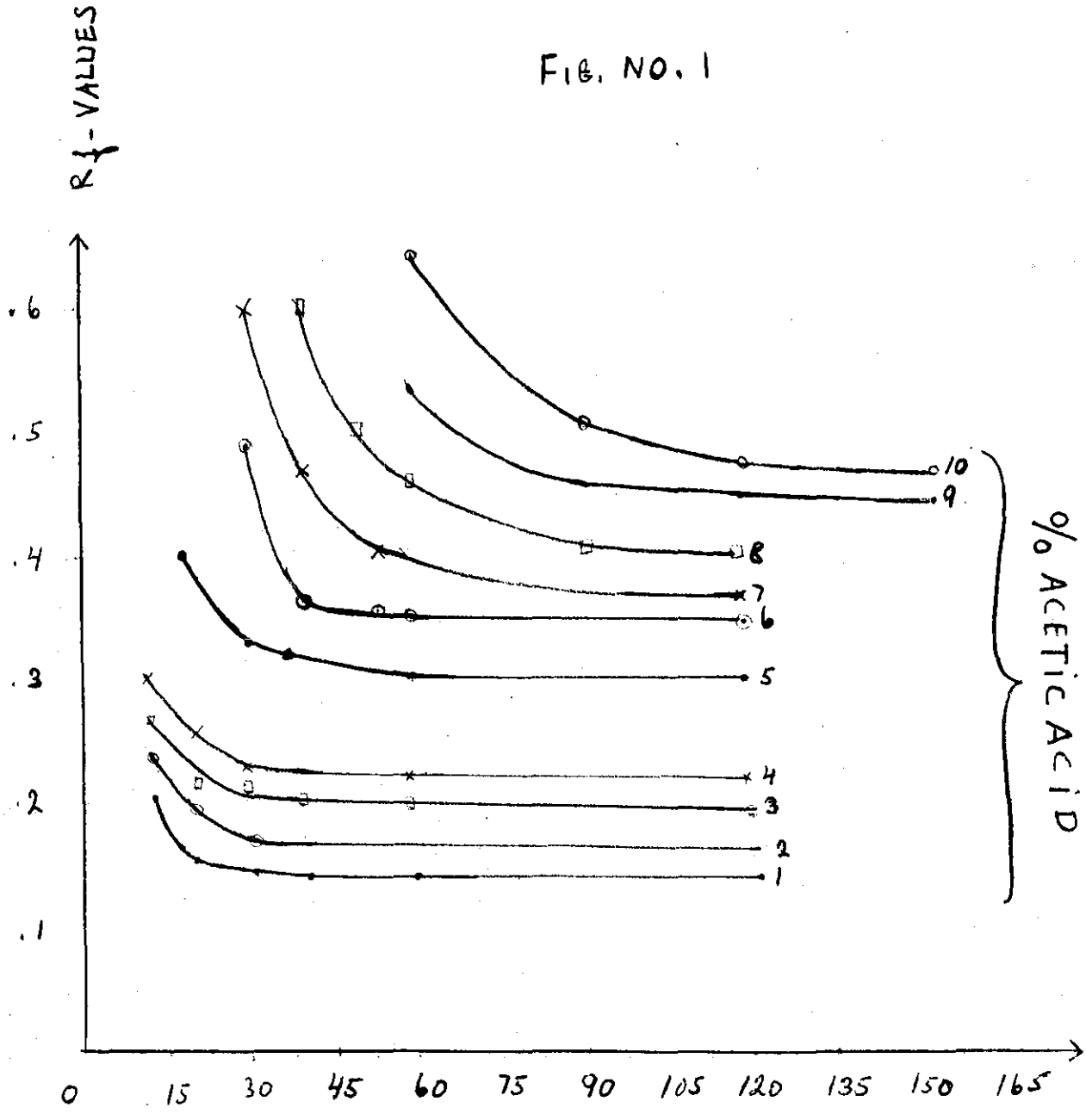
R_f VALUES OBTAINED USING METHANOL BASED SOLVENT SYSTEM

% Acetic acid	R_f
1	.13
2	.16
3	.2
4	.23
5	.3
6	.33
7	.36
8	.4
9	.43
10	.46

NOTE: The last six values are imprecise due to heavy streaking.

After this series of experiments, the filter paper was buffered with a buffer pH 3.5 (McElvaine's Buffer). It soon became evident that this had only one influence: increased streaking. In this case, streaking was not now limited to a percentage of acetic acid above 4, but also for the lower values. Even a solution of pure methanol produced streaking.

FIG. NO. 1



TIME IN MINUTES

METHANOL BASED SOLVENT SYSTEM

FIG. NO. 2

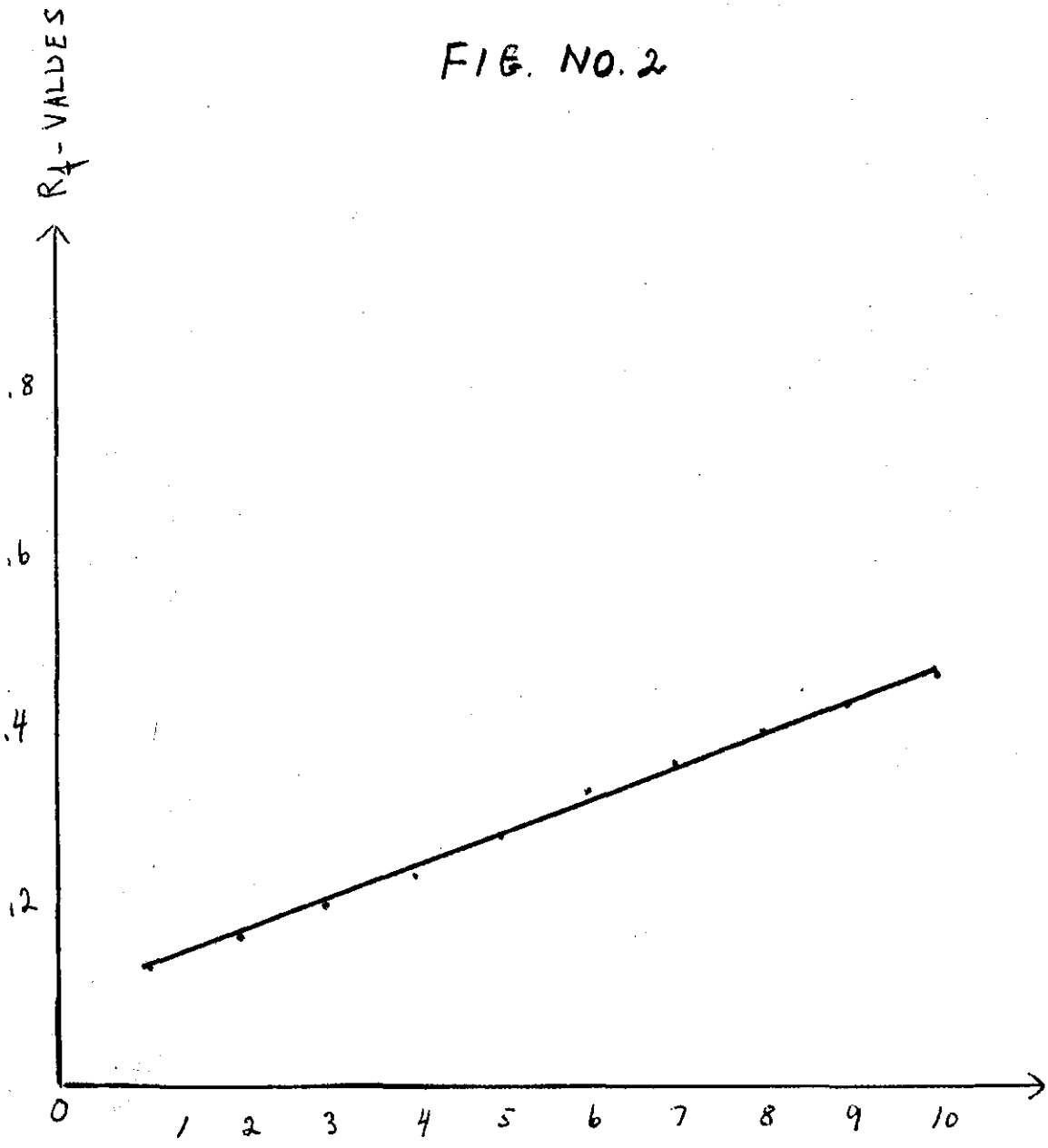

R_f - VALUES
.8
.6
.4
.2
0
1
2
3
4
5
6
7
8
9
10
ACIDITY A PERCENTAGE ACETIC ACID

TABLE III

BUTANOL BASED SOLVENT SYSTEM

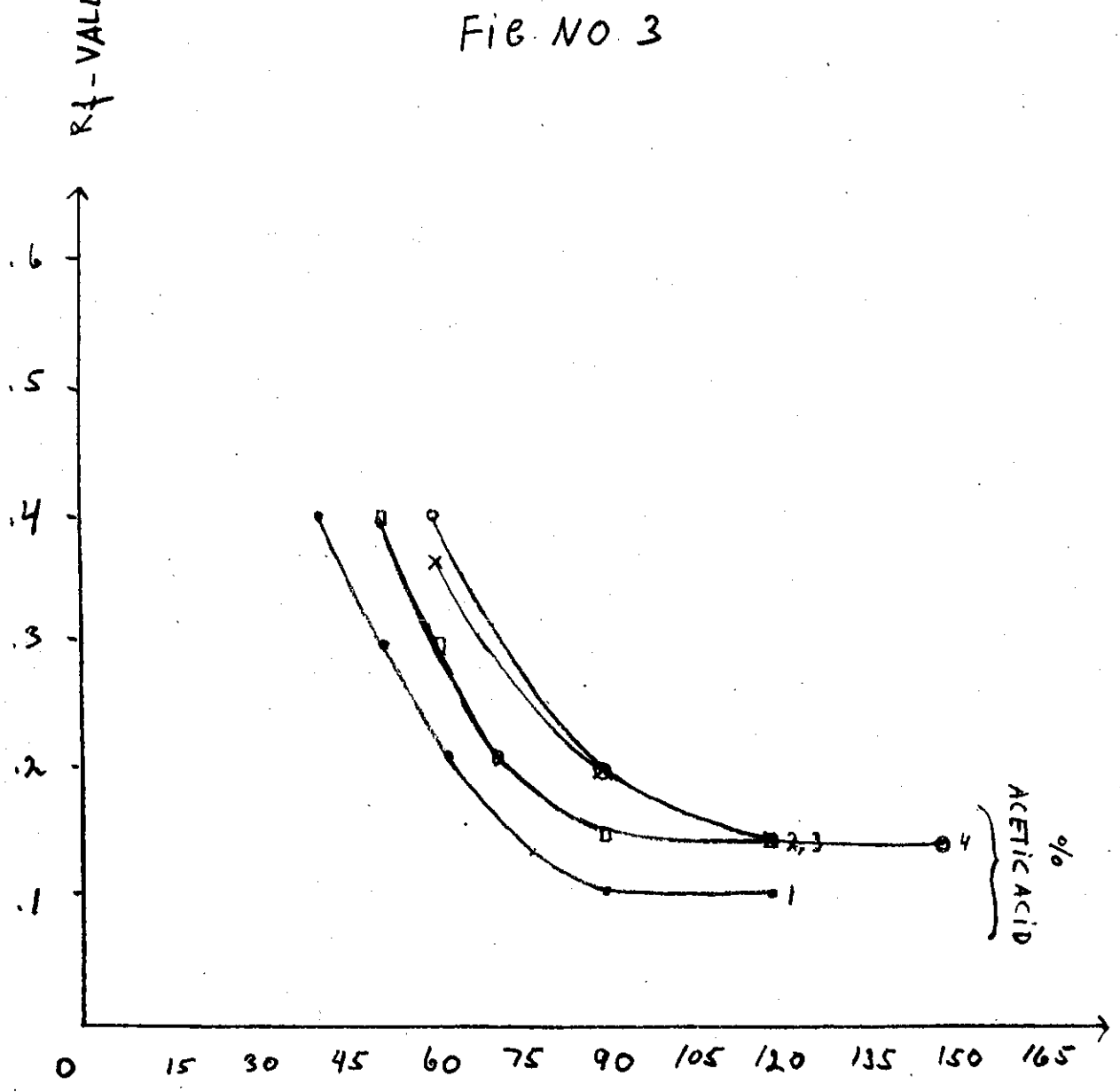
Time (minutes)	R_f
1% acetic acid	
40	.4
50	.3
60	.2
90	.1
120	.1
2% acetic acid	
50	.4
60	.3
70	.2
90	.14
120	.14
3% acetic acid	
60	.36
90	.20
120	.14
4% acetic acid	
60	.43
90	.2
120	.14
150	.14

TABLE IV

R_f VALUES OBTAINED USING BUTANOL BASED SOLVENT SYSTEM

% Acetic acid	R_f
1	.1
2	.14
3	.14
4	.14

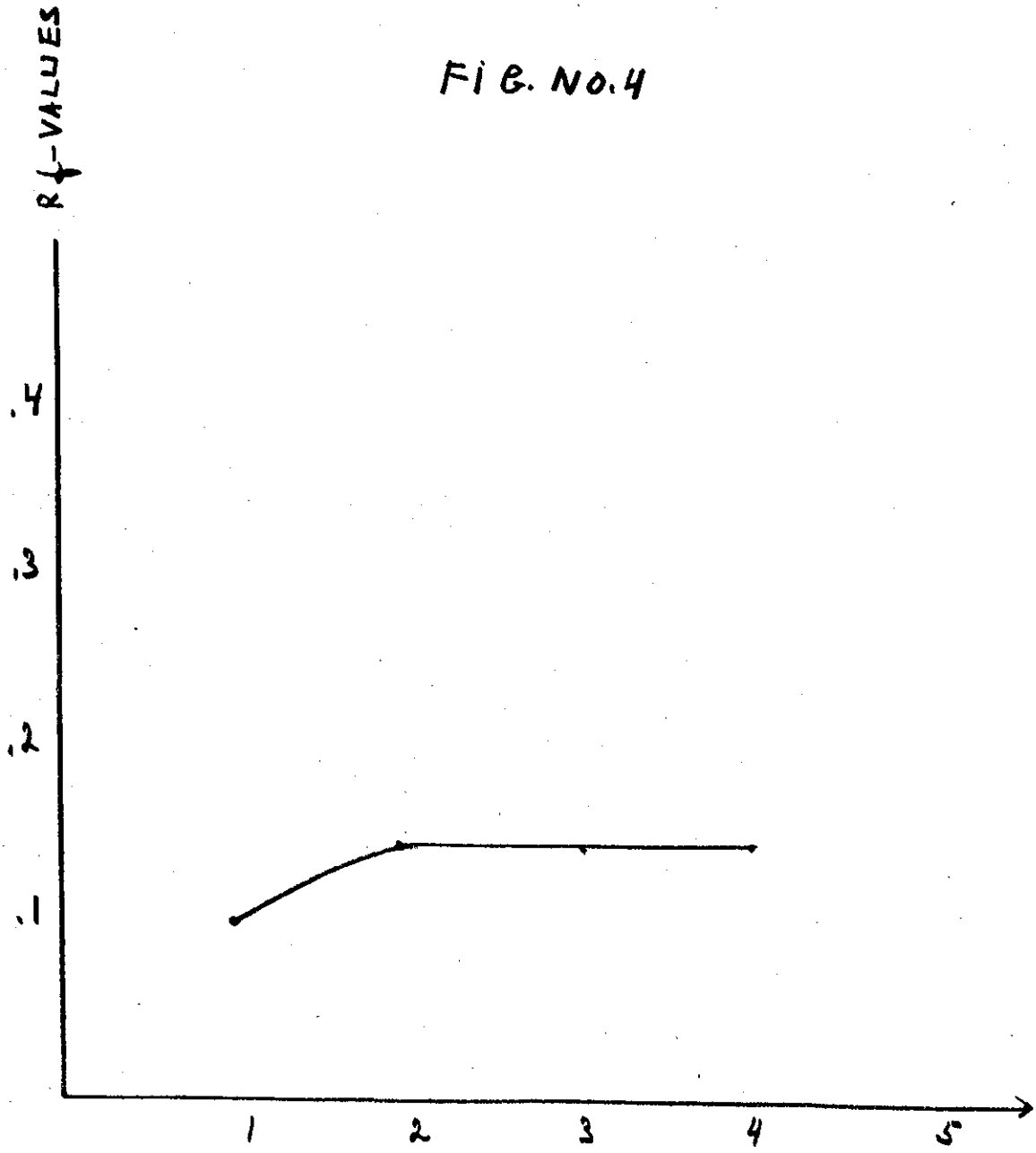
Fig. NO 3



TIME IN MINUTES

BUTANOL BASED SOLVENT SYSTEM

FIG. NO. 4



ACIDITY A PERCENTAGE ACETIC ACID

BUTANOL BASED SOLVEN SYSTEM

The last series was with a solvent consisting of nitro-ethane, pyridine, chloroform (6:1:3), acetic acid. The paper was buffered with the previously used buffer.

Again, the lowest percentages of the acid gave fairly sharp R_f values. But above 5% acid the streaking became so heavy that it was impossible to obtain precise R_f values at all. Figures 5 and 6 represent this series.

TABLE V

NITRO-ETHANE BASED SOLVENT SYSTEM

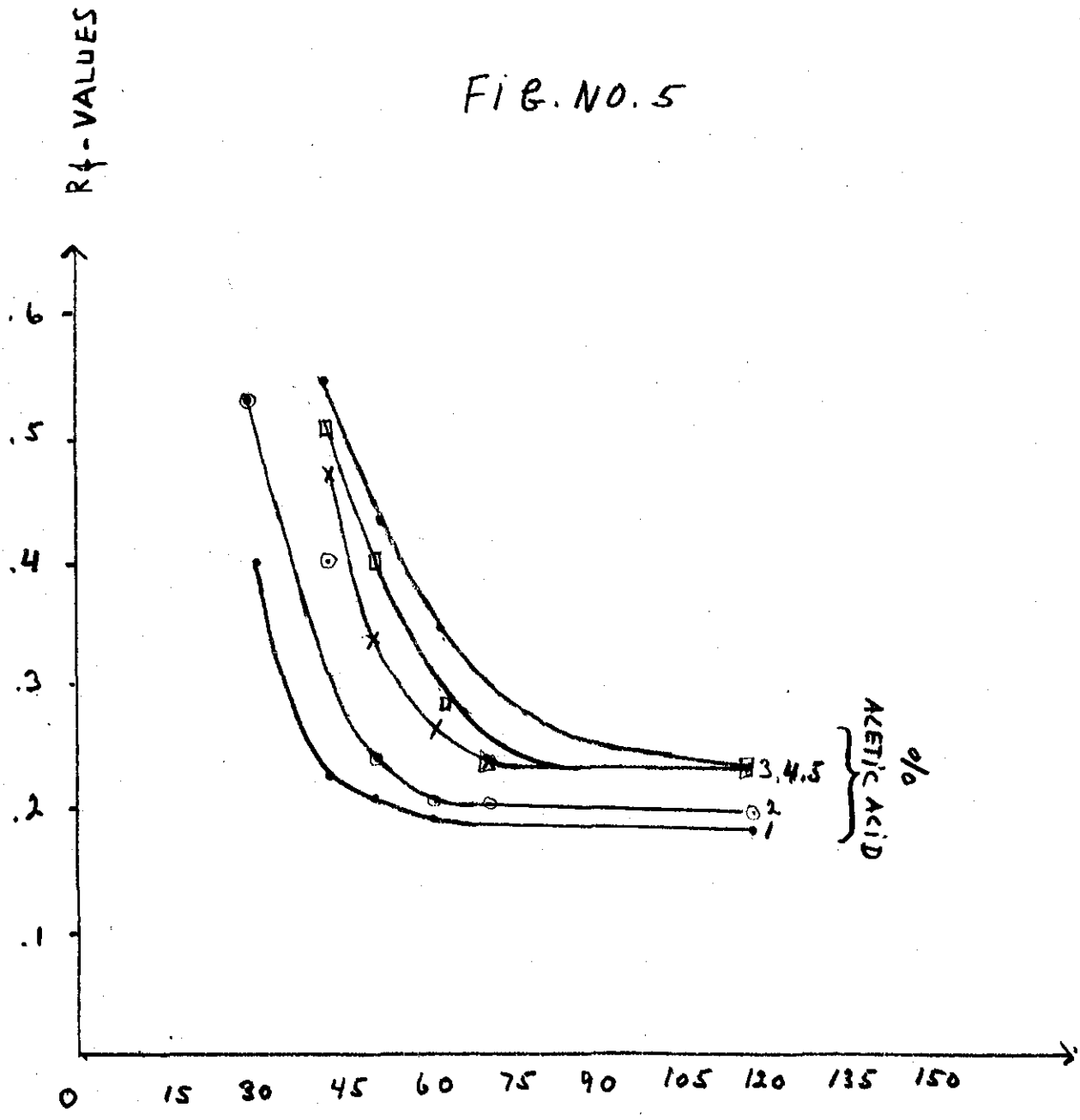
Time (minutes)	R_f
1% acetic acid	
30	.4
40	.23
50	.2
60	.18
120	.18
2% acetic acid	
30	.53
40	.4
50	.23
60	.2
70	.2
120	.2
3% acetic acid	
40	.46
50	.33
60	.25
70	.22
120	.22
4% acetic acid	
40	.5
50	.39
60	.26
70	.22
120	.22
5% acetic acid	
40	.53
50	.43
60	.33
120	.22

TABLE VI

R_f VALUES OBTAINED USING NITRO-ETHANE BASED SOLVENT SYSTEM

% Acetic acid	R_f
1	.18
2	.2
3	.22
4	.22
5	.22

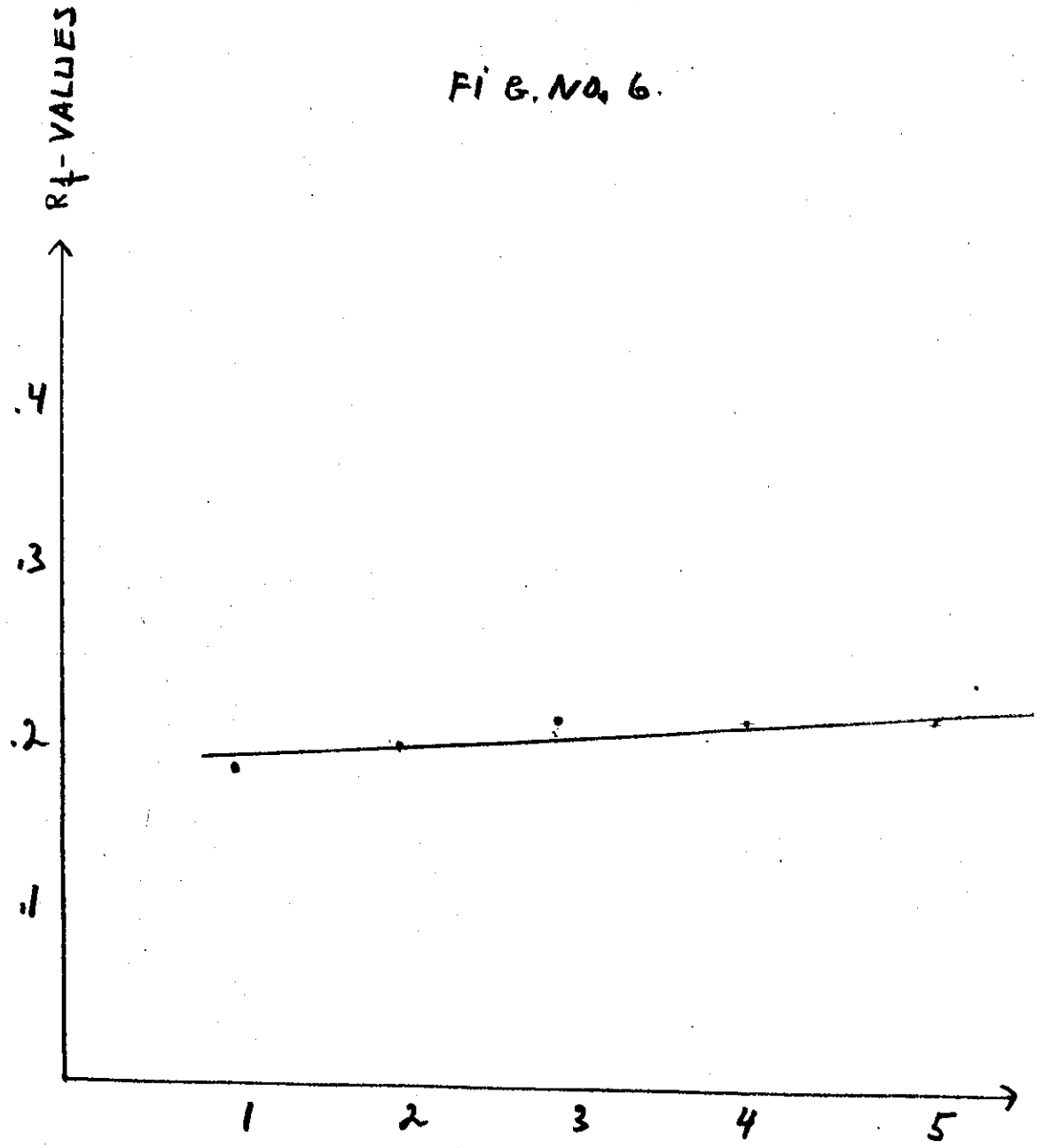
FIG. NO. 5



TIME IN MINUTES

NITRO-ETHAN BASED SOLVENT SYSTEM

FIG. NO. 6.



ACIDITY A PERCENTAG ACETI ACID

NITRO-ETHANE BASED SOLVENT SYSTEM

CHAPTER IV

SUMMARY

It seems obvious that the R_f values obtained are too low to have any practical use, especially if there should be closely related derivatives of Aureomycin present.

The greatest problem in this work was the streaking. This phenomenon caused difficulties. The streaking is probably due to chelation or precipitates of inner-complex salts. The chelation could be caused by hydrogen-bonding between the cellulose and the Aureomycin, the inner-complex salts by Cu or Ca ions. The first reaction could be catalyzed by hydrogen-ions. An increase in acid always led to an increase in chelation. The chelation was the greatest hindrance in obtaining relatively good R_f values. It is possible that Selzer and Wright[5] know a method of avoiding chelation in their work with this compound, but this investigator was not able to reproduce their results without heavy streaking; therefore, the sharpness of the spots was not good. The values obtained by methanol-based solvent were better in sharpness than those obtained by using the method of Selzer and Wright.

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