secutive fractions) was titrated with standard silver nitrate after the addition of 1 drop of 1 $M$ potassium chromate.

The capacity and interstitial volume, $V$, of the column were determined as follows: hydrochloric acid, 0.60 $M$, was passed through the column until the eluate had the same composition as the eluant. After draining the liquid in the tube to the level of the resin, 0.60 $M$ sodium nitrate was passed through the column until all the hydrogen and chloride ions were removed. The total quantity of hydrogen ion in the eluate expressed in millimoles (determined by titration with sodium hydroxide) was divided by 0.60 to find the interstitial volume. It was 35.5 ml. The total quantity of chloride ion (determined by titration with silver nitrate) minus the quantity of hydrogen ion represents the chloride originally present as RCl—i.e., the total capacity of the column. It was 78.0 me. Since the column contained 27.1 grams of oven-dried resin, the capacity was 2.88 me. per gram of dry, chloride-form resin.

### RESULTS

When 1.447 mmol. of chloride and 1.744 mmol. of bromide were taken, no halide was found in the first five fractions of 7.85 ml. each. Successive fractions contained the following amounts of halide: 2, 16, 68, 176, 325, 389, 290, 124, 44, 10, 4, 0, and 0 micromoles. The total is 1.448 mmol. and represents the chloride content of the mixture. Thereafter, fractions of two pipetfuls (15.70 ml.) were titrated. The quantities of halide found in successive fractions were 22, 98, 246, 368, 386, 303, 189, 90, 36, 10, 4, 0, and 0. This total is 1.752 mmol. and represents the bromide content of the sample.

A plot of $M$, the molarity of the halide in a fraction, against $U$, the total volume of eluate, reveals a fairly close conformity to Equation 1

$$\log M = \log M_m = 0.217p\left(\frac{C}{C+1}\right)\left(\frac{U-U_m}{U_m}\right)^2 \qquad (1)$$

with the following values for the parameters:

$$C_{Cl} = 2.27,\ p_{Cl} = 74,\ C_{Br} = 5.91,\ p_{Br} = 65$$

Other similar elutions yielded errors of +0.017, +0.004, and +0.008 mmol. for chloride and +0.010, −0.039, and +0.010 for bromide. Three analyses by this method of a sample of reagent-grade potassium bromide yielded 0.24, 0.23, and 0.26% chloride. The label indicated that the reagent contained not over 0.3% chloride. A correction was applied for the chloride content of this reagent when it was used in halide mixtures.

The major source of inaccuracy in the foregoing determinations is undoubtedly the cumulative error in the titration of ten or more fractions for the determination of each halide. Seven mixtures of chloride, bromide, and iodide were analyzed by a slightly different technique in which all the eluate fractions containing any one halide were combined before the titration, which was done potentiometrically. The errors for chloride ranged from +0.002 to +0.012 mmol., for bromide from 0.001 to −0.008 mmol. The presence of iodide in these mixtures does not interfere with the determination of chloride and bromide, but 1800 ml. of 0.6 $M$ sodium nitrate must be passed through the column in order to remove the iodide before the column can be used again. The iodide is contained in so large a fraction of the eluate that its determination by this method is not recommended. The research is being continued in order to develop a good ion-exchange procedure for the determination of each halide in a mixture of all three.

### SUMMARY AND CONCLUSIONS

The method described for the determination of chloride and bromide in halide mixtures by ion-exchange chromatography requires less than 3 hours. The error in a single determination of any one halide seldom exceeds 0.2 mole % of the total halide in the sample.

### ACKNOWLEDGMENT

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# Derivative Polarographic Titration of Glucose

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THE direct potentiometric titration of alkaline ferricyanide with glucose was investigated by Britton and Phillips in 1940 (*3*). Although this method seems to afford a rapid and precise determination of reducing sugars, it appears to have found little application. This is probably due in part to the cumbersome experimental requirements of potentiometric titrations at high temperatures.

The method of derivative polarographic titration recently developed in this laboratory is ideally suited to high temperature titrations (*8*). This method eliminates the reference half-cell and the connecting salt bridge, which are sources of experimental difficulty in high temperature titrations, provides continuous indication, and eliminates plotting of end points. Complete details of the theory and experimental technique have been published (*8*).

The applicability of the derivative polarographic method to the ferricyanide-glucose titration was predicted from a study of typical polarograms of the ferricyanide-glucose system during the course of the titration. Such polarograms were realized experimentally using stationary platinum wire electrodes under conditions similar to the various stages in the derivative titration.

Figure 1, *A*, shows the polarogram of "pure" ferricyanide at the beginning of the titration. The slope of the polarographic wave, $di/dE$, as it crosses the zero current axis is very small and thus the experimentally measured derivative voltage, $dE/di$, is high. The lower dotted portion of this curve indicates a point early in the titration, where a small amount of glucose has been added. Ferrocyanide formation is indicated by the anodic portion of the wave. A sharp drop in derivative voltage is produced. In Figure 1, *B*, about midway through the titration, the polarogram has the form shown, where the slope of the wave as it crosses the zero current line is very large and the measured $dE/di$ is close to zero. Figure 1, *C*, indicates the polarogram at the equivalence point. The dotted line represents either the reduction of unknown electroactive species of the glucose system or the reduction of hydrogen ions. In either case the derivative voltage is large and remains so after the equivalence point due to the irreversible nature of the glucose system. The expected course of the derivative voltage during the titration is shown in Figure 1, *D*. These predictions were verified experimentally.

In Figure 2 are plotted the data for a typical titration followed under potentiometric control and by the derivative method. In all cases studied the derivative end point was coincident with the ordinary potentiometric equivalence point.

### APPARATUS AND EXPERIMENTAL

The apparatus used for derivative polarographic titration has been fully described (*8*). A 150-ml. beaker served as titration

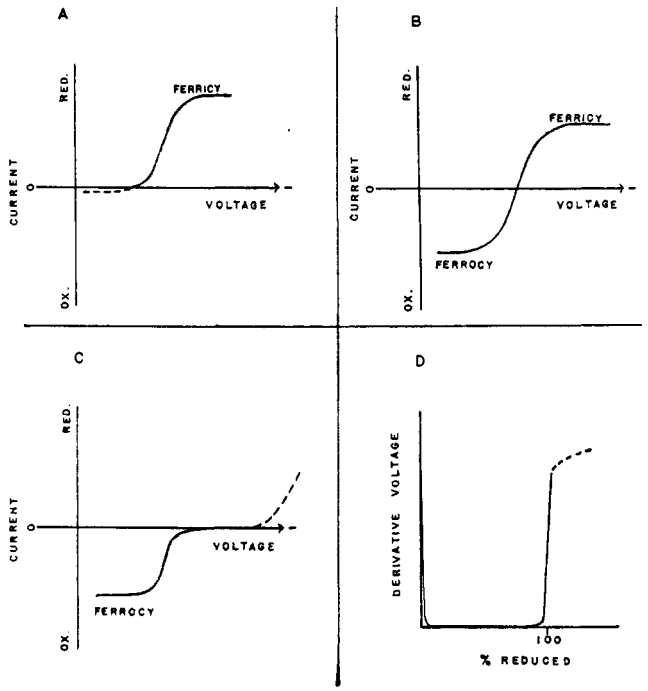


**Figure 1. Typical Polarograms of Ferricyanide-Glucose System**

*A.* **Polarogram of solution at beginning of titration**
*B.* **Polarogram of solution half-titrated**
*C.* **Polarogram of solution at equivalence point**
*D.* **Variation of derivative voltage during titration**

vessel for the milligram-scale determinations. For the microgram titrations, a flat-bottomed 15-ml. vial was used. The temperature was simply and effectively controlled by wrapping a length of flexible heating tape (Scientific Glass Apparatus Co., Bloomfield, N. J.) around the titration vessel and using a Variac to control the voltage to the heating tape. This arrangement was used to keep the contents of the titration vessel at the boiling point. The Variac was adjusted occasionally to prevent overheating. With this apparatus magnetic stirring could be provided for the titration. The derivative voltage was followed with a Beckman Model G pH meter. For those titrations where potentiometric measurements were made concurrently, a platinum–saturated calomel electrode system with an extended salt bridge of saturated potassium chloride was also inserted.

## REAGENTS

Stock 0.05 *M* potassium ferricyanide solution was prepared from the reagent grade salt. The 0.0005 *M* ferricyanide used in the microgram titrations was prepared by proper dilution of the stock. These ferricyanide solutions were stored in black painted bottles and withdrawn only as needed. Ferricyanide solutions prepared with distilled water only were found to be more stable than those containing 2% sodium carbonate. The ferricyanide should be standardized every few days against freshly prepared standard glucose solution. Loss of oxidizing strength is indicated by a lowering of the initial derivative voltage of the titration mixture.

Standard 0.01 *M* glucose solutions were prepared from Bureau of Standards, sample 41, glucose, and were used only on the day they were prepared.

Stock 1 *M* sodium carbonate solution was prepared from the C.P. anhydrous salt.

## PROCEDURE

For the milligram-scale titrations, 10.0 ml. of 0.05 *M* ferricyanide, 10.0 ml. of *M* sodium carbonate, and 30 ml. of water were placed in the titration beaker, heated to boiling, and titrated with the 0.01 *M* glucose. The ferricyanide and sodium carbonate solutions were run in from a buret, and the water was added from a graduated cylinder. The effect of variations of these quantities is discussed below. The rate of addition of glucose influences the total time of oxidation. Therefore, the glucose was added in 1- to 2-ml. increments; the next increment was not added until the solution again came to boiling. This was done to within about 0.5 ml. of the end point and then the titration was continued dropwise with about 30-second to 1-minute intervals between drops. This procedure roughly standardizes the total titration time for samples of the same general size. The end point was taken as the first drop that produced a 200- to 300-mv. rise in derivative voltage.

For the titration of about 20 micrograms of glucose, 1.0 ml. of 0.0005 *M* ferricyanide, 1.0 ml. of 1 *M* sodium carbonate, and 3.0 ml. of water were used. All quantities were measured from burets. Using 0.0001 *M* glucose (prepared by dilution of 0.01 *M* stock), the titration was performed as before. In this case, where addition of the small quantities of titrant does not cause the solution to cease boiling, it is best to use timed increments. The break at the end point is not so large as in the milligram titrations. The first drop causing a rise in derivative voltage of 100 to 200 mv. was taken as the end point.

## DISCUSSION AND RESULTS

Like all reducing sugar methods except complete wet combustions, alkaline ferricyanide procedures are empirical. However, ferricyanide methods possess certain advantages over other volumetric sugar methods (*1*) and according to Kirk (*6*) are the most adaptable to microgram-scale work. Official methods of the Association of Official Agricultural Chemists now include ferricyanide procedures for the determination of reducing sugars in flour (*2*). The original Whitmoyer method (*11*) as modified by Miller and Van Slyke (*7*) and Kirk and associates (*5, 9*) has found considerable application in blood sugar determinations. Hassid applied modifications of the Whitmoyer procedure to plant sugar analysis (*4*). While the derivative polarographic method offers a simple and direct titration, the results of this method are controlled completely by the factors affecting the empiricism of general ferricyanide procedures. These variables were first studied by Wood (*12*). The object of the present investigation was not primarily to establish exact reduction equivalents (R.E.) for the titration, but rather to examine completely all variables affecting the accuracy and precision of the method. A critical study reveals that there are four general factors to be considered.

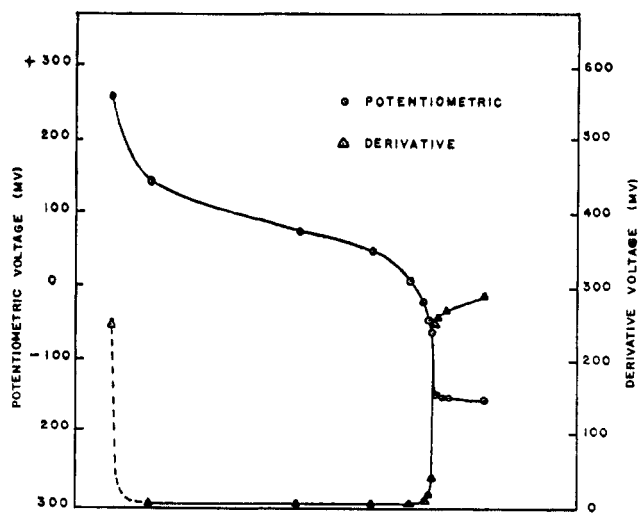


**Figure 2. Comparison of Derivative and Potentiometric End Points**

**1. pH Control.** Effective control of pH is most easily obtained using sodium carbonate. In the titration of about 10 mg. of glucose, measurement of the pH before and after showed a decrease of only about 0.5 pH unit. The carbonate-bicarbonate ion system provides adequate buffering. Titration mixtures diluted twofold show no appreciable change in pH. Part of the pH effect is probably explained by the length of time required for complete oxidation of glucose with changing pH. Van Slyke and Hawkins (*10*) and Wood (*12*) noted that lowering the pH caused a slowing of the reaction. This should lead to a lowering of the

reduction equivalent—i.e., overtitration in the derivative polarographic method.

**2. Temperature Control.** Using a boiling titration mixture provides the simplest temperature control. This procedure gives good results in the Lane and Eynon volumetric method (*2*). Its use in the derivative polarographic technique leads to much larger voltage breaks at the end point than are obtained at about 95° C. Small variations in the boiling point lead to unavoidable errors, but for routine analysis this procedure is satisfactory.

**3. Length of Oxidation Period.** This factor is the most difficult to control in a direct titration method. The problem is best solved by the incremental method of titration discussed above. The addition of 90% of the volume of glucose required and heating for a specific length of time, followed by dropwise titration to the end point, gave poor results in this study.

The glucose concentration affects the reduction equivalent and its influence is largely explained by the change in time necessary for complete oxidation of the varying amounts of glucose. In analysis of a completely unknown sample it is best to run a careful titration of the sample, followed by a titration using a standard glucose solution of about the same molarity as the unknown. With all other factors held fixed, this method serves to eliminate the above error.

**4. Ionic Strength Effect.** The most puzzling variation in ferricyanide oxidations is the increase in reduction equivalent with increasing initial concentration of ferricyanide. This effect has been noted in all types of ferricyanide procedures, but no general explanation has appeared.

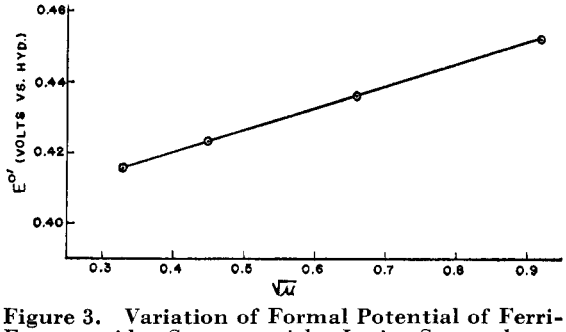


**Figure 3. Variation of Formal Potential of Ferri-Ferrocyanide System with Ionic Strength at Constant pH**

Preliminary studies showed that the formal potential of the ferricyanide-ferrocyanide system in alkaline media was a linear function of the square root of the ionic strength at constant pH. Figure 3 shows the data obtained from e.m.f. measurements of the system platinum-ferricyanide-ferrocyanide—sodium carbonate–saturated calomel electrode. Ionic strength variation was produced by changing only the over-all concentration of the 1 to 1 ratio of ferri-ferrocyanide, keeping the pH constant with a fixed increment of sodium carbonate. Ionic strengths were calculated in the usual manner, assuming complete ionization.

The increase in reduction equivalent caused by increasing the initial ferricyanide concentration was then investigated as a function of the resulting change in ionic strength. Over a limited range the reduction equivalent was found to increase linearly with the square root of ionic strength. This effect is shown by the solid line of Figure 4. A similar, though far less pronounced, increase is produced by increasing the sodium carbonate concentration (maintaining constant pH). The dotted line of Figure 4 illustrates this factor, which was qualitatively observed by Wood (*12*). The explanation of the marked increase in the case of the ferricyanide can be given only in a qualitative manner. The increase in ionic strength gives an effective oxidizing power higher than that expected. Moreover, during the course of the titration, the conversion of ferricyanide to ferrocyanide further increases the ionic strength by a factor of about 1.5, resulting in a higher potential throughout the later stages of the oxidation. The oxidation of glucose may be considered to proceed as:

$$\text{Glucose} + \text{Fe(CN)}_6^{---} \longrightarrow G_1 + G_2 + G_i \quad (1)$$

$$(G_1, G_2, G_i) + \text{Fe(CN)}_6^{---} \longrightarrow G_f \quad (2)$$

where $G_1$, $G_2$, and $G_i$ represent possible partial oxidation products and $G_f$ the combined final oxidation products of the glucose. These secondary reactions can be dependent on the concentration of ferricyanide and the formal oxidation potential, since they probably represent oxidations more difficult than that of glucose itself. Were the process purely an ionic strength effect, the change should be the same as that occasioned by any increase in electrolyte content—i.e., sodium carbonate. It is evident that concentration factors are operating. This explanation simply states that increasing the ferricyanide concentration furnishes the required energetics to give new paths for the consecutive reactions involved, resulting in an over-all greater consumption of oxidant per mole of glucose.

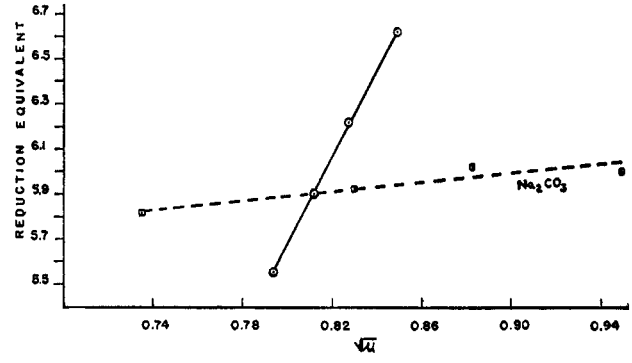


**Figure 4. Variation of Ferricyanide Reduction Equivalent with Ionic Strength**

A practical application of this effect is that the reduction equivalent can be set at some convenient value by adjusting the ionic strength through the initial ferricyanide concentration. Titrations at several ferricyanide concentrations establish the linear plot of Figure 4 and then the ionic strength necessary for the re-

**Table I. Determination of Glucose**

| Glucose Present, Mg. | Glucose Found, Mg. | Error, % |
|---|---|---|
| 8.91 | 8.95 | +0.4 |
| | 8.91 | 0.0 |
| 16.78 | 16.73 | −0.3 |
| | 16.79 | +0.1 |
| | 16.83 | +0.3 |
| 17.03 | 17.07 | +0.2 |
| | 17.05 | +0.1 |
| | 17.02 | −0.1 |
| 22.27 | 22.27 | 0.0 |
| 29.90 | 30.03 | +0.4 |
| γ 15.7 | 15.5 | −1.1 |
| | 16.2 | +3.2 |
| | 15.3 | −2.6 |
| | 16.4 | +4.3 |
| | 15.9 | +1.3 |

**Table II. Parallel Standardization Procedure**

| Glucose Present, γ/Ml. | Glucose Found | Error, % |
|---|---|---|
| 2.005 mg./ml. | 1.998 | −0.4 |
| | 2.016 | +0.5 |
| 160.4 | 160.1 | −0.2 |
| | 159.5 | −0.6 |
| 30.1 | 30.0 | −0.3 |
| | 30.3 | +0.6 |

quired reduction equivalent is read off the graph. The volume of ferricyanide required for a given ionic strength is given by:

$$\text{Ml. of ferricyanide} = \left(\frac{\mu - 0.6}{6}\right)\left(\frac{V_T}{M}\right)$$

where $\mu$ is the ionic strength, $V_T$, the initial titration volume, and $M$, the molarity of the ferricyanide reagent. The reduction equivalent was set at a value of 6.0 by this method. For routine control work this procedure may be useful. Readjustments of conditions are required as the ferricyanide solution ages. The linear dependence of the reduction equivalent on ionic strength is limited by the time factor involved in oxidizing increasing quantities of glucose, and the dilution factor with the larger titration volumes. It was possible to vary the ferricyanide concentration from 5 to 20 ml. of the 0.05 $M$ stock. For best results a fixed value of ferricyanide should be maintained. In the microgram titrations no attempt was made to vary the ferricyanide concentration.

The linear plot of Figure 4 holds only for a fixed glucose concentration (0.01 $M$). Variations in glucose concentration change the reduction equivalent (at a fixed ferricyanide concentration) and also because of a dilution effect. In the microgram region the glucose could be varied from 0.0012 to 0.00008 $M$ without changing the reduction equivalent. A wider limit should be possible in the milligram-scale titrations, where a larger titration volume is involved. However, the recommended procedure is to run a parallel standardization titration if the glucose concentration differs appreciably from the standard glucose used in establishing the reduction equivalent. An alternative procedure is to run a trial titration, calculate the glucose molarity, and accordingly dilute the titrant until its titer more nearly matches the standard.

Using the derivative polarographic titration, small quantities of glucose were titrated using a total titration volume of 5 ml. Results indicate that about 20 micrograms of glucose can be titrated with an accuracy of about ±5%. A summary of some determinations is given in Table I. In Table II are results of several determinations run by the parallel standardization procedure discussed above. These titrations were run with a Leeds and Northrup pH meter.

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# Paper Chromatography of Nucleic Acid Derivatives

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THIS paper describes the separation by paper chromatography of the common bases, nucleosides, and nucleotides employing several single-phase mixed solvents in which 1-propanol, isoamyl, and tetrahydrafurfuryl alcohols, and an aqueous solution of a buffer salt are used to control the pH at desired levels during the operation.

Vischer and Chargaff (*6*) seem to have been the first to apply paper chromatography to the separation of nucleic acid derivatives; they located the purin and pyrimidine bases on the paper by forming insoluble mercury salts on guide strips and converting these salts to black sulfides. Reguera and Asimov (*4*) pointed out that purin bases may be located by forming insoluble silver salts on strips and converting them to brown chromates. Nucleosides or nucleotides of these bases do not give the mercury or silver tests. Hotchkiss (*2*) employed 1-butanol and 2.5% ammonium hydroxide (1 to 1) as solvent for developing the chromatogram and the stepwise elution of the separated components for identification by absorption spectrum. Carter (*1*) pointed out that ribonucleotides do not move in the solvent systems previously reported. He noted that resolution of nucleotides could be achieved by a two-phase solvent system composed of isoamyl alcohol and an aqueous solution of buffer salt such as citrate or phosphate (butanol–aqueous urea was also used). The spot was located by the quenching of ultraviolet fluorescence, as noted by Markham and Smith (*3*).

It is obvious that two-phase solvents cannot be employed in column chromatography and the authors have been interested in developing a buffered single-phase solvent that could be employed to handle much more material than on paper sheets, when new substances must be separated and characterized. Isoamyl solvents travel a great deal faster on the chromatograph, giving a run in about 24 hours, while butanol solvents require around 40 hours. 1-Propanol has been employed as well as isoamyl alcohol and tetrahydrofurfuryl alcohol has been added to the propanol (or isoamyl)–aqueous solvent mixture in the ratios propanol–tetrahydrofurfuryl alcohol–buffer (2:1:1), and amyl alcohol–buffer (1:1:1). These mixtures are about saturated with respect to 0.08 $M$ citrate, and maintain the pH at 3.02, 5.66, and 7.92, respectively. A series with 0.1 $M$ acetate buffer at pH 3.03 is also included.

### PREPARATION OF MATERIAL

The buffer salts were from reagent grade material and the organic solvents employed were redistilled before use. The nucleic acid derivatives were commercial samples which had been recrystallized and which showed the presence of a single component on the chromatogram. It was difficult to convert the commercial samples of the barium salts of adenosinediphosphate and triphosphate to their sodium salts by dissolving the barium salt in 0.2 $N$ nitric acid (*5*) and removing barium as sulfate by adding the calculated amount of sodium sulfate. Such samples are always less homogeneous than those prepared by base exchange with the calculated amount of sodium sulfate alone. Even brief contact with nitric acid causes hydrolysis.

### EXPERIMENTAL

Solutions of the various nucleic acid derivatives were made by weighing out 5-mg. samples of each, dissolving in 1 ml. of 0.08 $M$ citrate buffer, and diluting with water to 5 ml. in a volumetric flask. All solutions were protected by toluene. Spots were made in duplicate of a number of compounds simultaneously on large sheets of Whatman No. 1 filter paper, using ultramicropipets and employing 0.010- and 0.025-mg. samples of nucleic acid derivative for each spot.

The large prepared sheets were hung in pairs from the stainless steel trough in the chromatograph box, and after the air in the box had been saturated with the solvent, the experiment was started by adding solvent to the trough. In the experiments reported the direction of motion of the solvent was descending.