SUMMARY

1. The Michaelis constants of fumarase for its two substrates vary with pH in the same way as the initial velocities of enzyme reaction.

2. The effects of temperature on the rates of the hydration and dehydration reactions of fumarase at various pH values are described and activation energies are calculated. The activation energies of both reactions vary with pH, and for the hydration reaction vary markedly with temperature. At pH values above the optimum, the activation energy increases at a critical temperature, which varies with the pH, as the temperature is increased. At pH values around the optimum there is no variation of activation energy over the temperature range investigated. At pH values lower than the optimum the activation energy decreases at a critical temperature of 18°, which is independent of the pH, as the temperature is increased.

3. The effect of temperature on the Michaelis constants of fumarase at various pH values is

described, and the heats of formation of the enzyme-substrate complexes are calculated. The formation of the fumarase-malate complex is exothermic, while the formation of the fumarase-fumarate complex is endothermic. Moreover, the heats of formation of the fumarase-fumarate complex at low pH values change at 18° in such a way as to compensate for the changes in activation energies that occur under similar conditions at this temperature.

4. The variation of equilibrium constant with temperature is described.

5. An overall picture of the heat changes associated with the action of fumarase is proposed.

6. The significance of these results is discussed.

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The Electrophoresis of Acid Mucopolysaccharides on Filter Paper

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Electrophoresis, using strips of filter paper as a matrix to support the buffer solutions, has frequently been used to separate individual proteins from mixtures: e.g. serum (Durrum, 1950; Cremer & Tiselius, 1950; Wieland & Fischer, 1948; Grassman, Hannig & Knedel, 1951); enzymes (Wallenfels & Pechman, 1951) and artificial mixtures of purified proteins (Kunkel & Tiselius, 1951). Nucleotides have also been separated by means of a modification of this method (Smellie & Davidson, 1951).

The present paper deals with the application of the method to the separation of some acid mucopolysaccharides. It was hoped that it would enable

relatively small amounts of material to be examined and that it might give an indication of the amount and nature of any impurities. Blix (1940) showed that, in free solution at pH 8.0, hyaluronic acid from synovial fluid had an electrophoretic mobility of $-12 \text{ cm}^2 \text{ V}^{-1} \text{ sec}^{-1} \times 10^{-5}$ and that chondroitin sulphate from cartilage had a mobility of $-17 \text{ cm}^2 \text{ V}^{-1} \text{ sec}^{-1} \times 10^{-5}$. According to Jorpes (1946) the mobility of heparin preparations was -17 to $-19 \text{ cm}^2 \text{ V}^{-1} \text{ sec}^{-1} \times 10^{-5}$ over the pH range 2-8, and that of chondroitin sulphate was $-16 \text{ cm}^2 \text{ V}^{-1} \text{ sec}^{-1} \times 10^{-5}$. These values suggest that using electrophoretic methods chondroitin

sulphate could be separated from hyaluronic acid but not from heparin. Recently, Gardell, Gordon & Åqvist (1950), using Hyflo Super-Cel slabs moistened with buffer, achieved the electrophoretic separation of hyaluronic acid from chondroitin sulphate.

METHODS AND MATERIALS

Apparatus. Initially apparatus of the type described by Durrum (1950) was used, the length of the filter-paper strips not immersed in buffer being about 24 cm. and the height of the apex of the filter paper above the buffer level being 11 cm. Later experiments with extracts of skin showed that this apparatus was not big enough to allow migrations of sufficient distance to effect good separations. An apparatus in which the filter paper was supported in a horizontal position was then made. It was similar to that described by Grassman *et al.* (1951) except that owing to the greater length of the filter-paper strips (54 cm.) a number of lateral supports of Perspex were incorporated in the box containing the filter paper, and the filter paper was stretched as much as possible so as to prevent undue sagging between the supports. Large vessels were used to hold the buffer solutions, the levels being accurately equalized by means of a siphon which was removed while the current was running. No precautions were taken to protect the ends of the filter paper from pH changes at the electrodes. All the experiments were carried out at room temperature.

Acid mucopolysaccharide preparations. Except where otherwise stated, the acid mucopolysaccharide preparations were as follows. Hyaluronic acid, as supplied, had been prepared from acetone-dried human umbilical cords by extracting with water and precipitating from the extract with ethanol saturated with potassium acetate. The water-soluble material from this precipitate was reprecipitated with ethanol saturated with potassium acetate. It contained 4.30% N; 31.9% hexosamine and 1.6% S. Chondroitin sulphate, prepared from bovine trachea by the method of Bray, Gregory & Stacey (1944), contained 4.72% N and 23.2% hexosamine. Heparin, prepared commercially from ox lung tissue as described in the British Pharmacopoeia monograph, contained 4.01% N and 23.8% hexosamine.

Filter paper and its preparation. Either Whatman no. 2 or 3 MM filter paper was used. Being the more absorbent of the two, 3 MM paper was used when dealing with large amounts of dilute acid mucopolysaccharide solutions. The width of the filter-paper strips varied in accordance with the amount of material used and the method followed in detecting the acid mucopolysaccharides. When these were detected by staining, strips of 2 cm. width were employed; when the detection was by elution and subsequent colorimetric determination, strips 5 cm. wide or more were used.

The acid mucopolysaccharides were usually dissolved in water and put on the filter paper as a thin band at the origin, which was marked with pencil, so as to come about 5 cm. inside the cathode end of the box. Where more than one application was needed, the solution on the paper was dried off, using gentle heat or, in the case of degraded samples, in an electric oven at about 100°. Durrum's (1950) own procedure was followed when using his apparatus. When the horizontal apparatus was used the anode end of the paper was first dipped in the buffer solution so as to wet it up to the point where the acid mucopolysaccharides had been applied. The excess buffer was then blotted off, and the paper placed in the apparatus.

The anode end of the paper was next dipped into the buffer in the anode compartment. After 10–15 min. the buffer had flowed along the paper sufficiently to moisten it up to and just past the origin. The cathode end was then dipped into the cathode compartment buffer. Equilibrium was established in about 15 min. The movement of the acid mucopolysaccharides due to buffer flow was followed by duplicating the procedure on an extra strip of paper using a drop of a 4% acid fuchsin solution instead of the acid mucopolysaccharide solution. The acid mucopolysaccharides could be moved back to the origin by temporarily raising one end of the box to produce a slight buffer flow (Kunkel & Tiselius, 1951). The acid fuchsin also served to indicate approximately the distance of migration of the chondroitin sulphate during electrophoresis (Gardell *et al.* 1950).

In the Durrum apparatus a potential gradient of approximately 5.0 V./cm. was applied for 3–4 hr., and in the horizontal apparatus 3.5–4.0 V./cm. for about 16 hr. After separation had been effected the papers were removed and dried at 110°.

Detection and estimation of the acid mucopolysaccharides. A number of staining procedures were tried in order to show the position of the acid mucopolysaccharides after migration on the filter paper but no really satisfactory method was devised. Either the filter paper adsorbed the dye used (e.g. toluidine blue and mucicarmine) or, being a polysaccharide, reacted in the same manner as the acid mucopolysaccharides (e.g. the periodic acid fuchsin stain). The metachromatic stain with toluidine blue or azure A was the least unsatisfactory. The dried papers were dipped into the toluidine blue solution (0.125%, w/v) or azure A (0.05%, w/v), quickly withdrawn, and blotted. The sulphated acid mucopolysaccharides were easily seen as reddish bands on a blue background. Hyaluronic acid was less easily distinguished. With highly polymerized specimens a reddish colour could be seen, but depolymerized specimens showed only a deepening of the blue background and in small amounts were invisible. Whatman no. 2 filter paper gave better results than 3 MM. As little as 25 µg. chondroitin sulphate and heparin could be seen on strips 2 cm. wide.

The most satisfactory method of detecting the positions of acid mucopolysaccharides was to elute them quantitatively from 1 cm. wide segments cut from the dried filter paper and then to determine the hexosamine or hexuronic acid content of each eluate.

Elution was carried out in the apparatus shown in Fig. 1. The strip of filter paper to be eluted was suspended by copper or nichrome wire at the lower end of the condenser. The acid mucopolysaccharides on the filter paper were completely extracted after refluxing for about 2.5 hr. An oil bath fitted with a test-tube rack enabled twenty extractions to be made simultaneously. The eluate was then evaporated to dryness under reduced pressure and the residue extracted once with 10 ml. of ether to remove any paraffin oil that might have entered the apparatus. Subsequently either hexosamine determinations were made using the Morgan and Elson reaction as modified by Johnston, Ogston & Stanier (1951), or hexuronic acid determinations using the carbazole method of Dische (1947). The former method was the more satisfactory, but filter paper which contained no acid mucopolysaccharides gave an eluate which evoked a slight response on the colorimeter due to a non-specific yellow colour. With the Dische method this non-specific effect was more marked, and in addition charring occurred if any trace of filter paper was washed into the test tube as a result of the

elution. The final colours that were obtained with both methods were read in a Unicam D.G. spectrophotometer, and the optical densities obtained were used as the ordinates in Figs. 3-5.

RESULTS

Experiments using the Durrum apparatus

Fig. 2 illustrates the appearance of the filter-paper strips after electrophoresis and staining in 0.125% (w/v) toluidine blue. Approximately 50 μ g. of each acid mucopolysaccharide were subjected to electrophoresis for 4 hr. with a potential gradient of 5 V./cm. on 2 cm. wide strips of no. 2 Whatman filter

Fig. 1. Apparatus for elution of acid mucopolysaccharides from filter-paper strips.

paper. Sodium phosphate, 0.1 M (pH 6.7), was used as the buffer. The strip of filter paper on which acid fuchsin was placed is illustrated to show that the heavy red band migrated at approximately the same rate as chondroitin sulphate. From these results it would seem that the hyaluronic acid contains an impurity which migrates at about half the rate of chondroitin sulphate, and that the chondroitin sulphate and heparin contained no gross impurities. Although the heparin migrated at a slightly faster rate than the chondroitin sulphate they were not resolved when mixed.

In Fig. 3 are shown the results obtained by eluting the filter-paper strips after electrophoresis and determining the hexuronic acid content of the eluates. The amounts of acid mucopolysaccharides used and conditions of electrophoresis were the same as those for Fig. 2. Here the impurity in the hyal-

uronic acid does not appear to be separated from the hyaluronic acid, but the assymmetric form of the curve suggests an impurity with a greater mobility than the hyaluronic acid. On the other hand, chondroitin sulphate and heparin both have slower-moving components which did not appear in the strips stained with toluidine blue.

Fig. 2. The appearance of the filter-paper strips after electrophoresis of acid mucopolysaccharides in 0.1 M-sodium phosphate buffer at pH 6.7 and staining with 0.125% (w/v) toluidine blue.

Fig. 3. The positions of the acid mucopolysaccharides as shown by elution of the filter paper and hexuronic acid determination (ordinate, optical densities at 530 $m\mu$). The experimental details were the same as for Fig. 2.

These results indicate that the technique of filter-paper electrophoresis can resolve mixtures of acid mucopolysaccharides with largely differing mobilities (e.g. hyaluronic acid and chondroitin sulphate) but that the method is not sensitive

enough to separate a substance from another of slightly different mobility (e.g. heparin and chondroitin sulphate were not separated). It can be

obtaining satisfactory results from extracts of skin even with increased electrophoresis time.

Experiments using the horizontal apparatus

In Fig. 4 are shown the results obtained using the horizontal apparatus. Approximately 1 mg. quantities of each of the acid mucopolysaccharides were placed on 5 cm. wide strips of no. 3 MM filter paper and electrophoresis carried out for 16 hr., in a 0.1 M-sodium acetate-acetic acid buffer, pH 5.0, with a potential gradient of 3.8 V./cm. The positions of migrating substances were shown by eluting strips and making hexosamine determinations on each eluate. There are impurities in all three substances, although the small peak between 5 and 10 cm. from the origin in the chondroitin sulphate diagram was not due to the presence of hexosamine but to a non-specific yellow colour. Chondroitin sulphate was separated from hyaluronic acid. The rate of migration of chondroitin sulphate was not affected by the presence of hyaluronic acid and vice versa. There was no separation of heparin from chondroitin sulphate.

Effect of degree of polymerization on migration of hyaluronic acid

It was noticed that hyaluronic acid, prepared by extraction with alkali from pig skin and rat skin, migrated faster than the umbilical cord hyaluronic acid used in the previous experiments. Owing to differences in the method of preparation of the hyaluronic acid, the samples prepared from skin were greatly depolymerized whilst those prepared from umbilical cord were highly polymerized. To determine whether the degree of polymerization influences the rate of migration of hyaluronic acid, samples of hyaluronic acid from several sources treated in the ways indicated in Table 1, were simultaneously subjected to electrophoresis on separate strips of filter paper in the same apparatus.

Fig. 4. The electrophoresis of the acid mucopolysaccharides in the horizontal type apparatus in 0.1 M-sodium acetate buffer at pH 5.0. Ordinates represent optical densities at 526 m μ . found in hexosamine determinations carried out on the eluates.

seen that elution of strips and chemical determination of a component of the acid mucopolysaccharides in the eluate was superior to the staining technique. The Durrum apparatus was discarded for the horizontal apparatus owing to the difficulty in

Table 1. *The distances of migration of hyaluronic acid from various sources before and after treatment with N-NaOH*

(The electrophoresis was conducted on 3MM Whatman filter paper in 0.1 M-sodium acetate buffer, pH 5.0, for 17 hr. with a potential gradient of 3.8 V./cm.)

Source of hyaluronic acid	Treatment	State of solution	Distance of migration (cm.)
Bovine synovial fluid	Protein removed with trypsin and adsorption on Lloyd's reagent	Highly viscous	3-9
Bovine synovial fluid	As above and then incubated in N-NaOH at 37° for 48 hr.	Non-viscous	23-28
Umbilical cord	Umbilical cords extracted with cold water and hyaluronic acid precipitated with ethanol	Viscous	9-13
Umbilical cord	As above and then incubated in N-NaOH	Non-viscous	24-29
Pig skin	Prepared by method of Meyer & Chaffee (1941) i.e. initial extraction with N-NaOH at 37° for 48 hr.	Non-viscous	23-27

The results indicated that treatment with alkali increased the rate of migration markedly.

There seem to be two possible explanations for this increased rate of migration. Meyer (1951) showed that undegraded samples of hyaluronic acid possessed fewer acidic groups than degraded samples, as judged by the ash content of their salts. This indicates that degraded samples have a greater charge/weight ratio than undegraded samples, and degraded samples would consequently be expected to have a greater electrophoretic mobility than undegraded samples of hyaluronic acid. Unfortunately the effect of polymerization on the electrophoretic mobility in free solution does not seem to have been studied.

The sample of hyaluronic acid that had not been degraded by alkali formed viscous solutions. The migration of these viscous solutions may have been mechanically obstructed by the filter paper. On two occasions the filter-paper strips were allowed to touch the lid of the apparatus so that a layer of buffer was held between the paper and the lid. The results obtained by elution and glucosamine determination showed very diffuse peaks. These would have been unsatisfactory for most purposes, but they implied that in these circumstances, the rates of migration of hyaluronic acid from umbilical cord in the undegraded and degraded states did not differ greatly. Thus on one occasion the undegraded sample showed a peak at 19–24 cm. from the origin, whereas the alkali-treated samples had a peak at 26–33 cm. (cf. Table 1 where values of 9–13 cm. and 24–29 cm. respectively are given). Presumably the hyaluronic acid was able to migrate in the buffer solution between the paper and the lid of the box at a rate approaching that in free solution. Nevertheless, the degraded sample still migrated faster than the undegraded sample. It is concluded that both the factors mentioned above contribute to the increase in velocity of migration of hyaluronic acid on depolymerization.

Examination of glucosamine-containing compounds obtained from skin

Meyer & Chaffee (1941) prepared both hyaluronic acid and chondroitin sulphuric acid from pig skin. The hyaluronic acid was said to be sulphate-free whereas the chondroitin sulphate fraction was contaminated with some material, presumably hyaluronic acid, which was removed by incubation with pneumococcus hyaluronidase. Meyer & Chaffee's method was used to prepare similar fractions which were examined by electrophoresis in the horizontal apparatus. Amounts equivalent to approximately 10 g. of skin were placed on 5 cm. wide strips of no. 3MM Whatman filter paper and electrophoresis was carried out for 16 hr. at a potential gradient of

4.0 V./cm. in 0.1M-sodium acetate-acetic acid buffer, pH 5.0. The positions of acid mucopolysaccharides were determined by elution of the strips and hexosamine determination. The results obtained are presented in Fig. 5.

Fig. 5. The electrophoresis in 0.1M-sodium acetate buffer at pH 5.0 of various skin preparations, each representing material obtained from approximately 10 g. skin. The acid mucopolysaccharides were detected by elution of the strips followed by hexosamine determination. (Ordinate, optical densities at 526 m μ .)

The hyaluronic acid appears to be free of any contaminant that has a different mobility and contains hexosamine, whereas the chondroitin sulphate contains an appreciable amount of an impurity with a mobility similar to that of hyaluronic acid. No attempts were made to remove this impurity by incubation with pneumococcus hyaluronidase.

In Fig. 5 are also shown the data obtained under the same experimental conditions from an alkaline extract of approximately 10 g. pig skin, the proteins having been removed by treatment with Sevag's reagent and adsorption on Lloyd's reagent at pH 5.0. After incubation of the extract with pneumococcus hyaluronidase peaks A and B were no longer present. Peaks C and D can each be split into two fractions on the basis of solubility in 10% (w/v) barium acetate. The barium acetate-soluble fraction of peak D is chondroitin sulphate. A detailed presentation of these results will be given later.

DISCUSSION

Electrophoresis of acid mucopolysaccharides can be carried out on filter-paper strips in a manner similar to that used for proteins, amino-acids and nucleotides. Unfortunately, the detection of the positions of the acid mucopolysaccharides on the filter paper after electrophoresis is not satisfactory. There is no staining technique that will show the positions of all the acid mucopolysaccharides on the paper as distinctly and sensitively as for proteins and amino-acids. The stains which are used to demonstrate the presence of acid mucopolysaccharides in histological tissue sections either react with, or are adsorbed on to, filter paper. Thus the acid mucopolysaccharides have to be detected on a coloured background. Unsuccessful attempts were made to use substitutes for filter paper or to modify it so that the stains were not adsorbed etc., for example, by using woven glass fabric and acetylated filter paper.

The acid mucopolysaccharides could be detected after electrophoresis by cutting the filter paper into strips, eluting them and determining the hexosamine or hexuronic acid contents of the eluates. It was possible with this method to demonstrate even small amounts of substances with mobilities different from the main components. The exact amount of such impurities could not be determined owing to the presence of small amounts of substances which were extracted from the filter paper and reacted with reagents used to determine hexosamine or hexuronic acids. For example the first peak in the electrophoresis diagram for chondroitin sulphate in Fig. 4 was not due to a hexosamine-containing substance and was not always present. The second peak was always present. A comparison of Fig. 3 with Fig. 4 shows that the contaminant in the hyaluronic acid preparation contained both hexosamine and hexuronic acid. As it stained metachromatically with toluidine blue it could possibly be sulphated and account for the 1.6% sulphur in the hyaluronic acid preparation.

All the substances that could be present in acid mucopolysaccharide preparations were not detected since the tests used only detected substances containing either hexosamine or hexuronic acid. Consequently, even if an acid mucopolysaccharide preparation appears to migrate as a single peak in filter-paper electrophoresis, substances for which no tests were made, such as proteins or glycogen, might still be present. The protein content of any acid mucopolysaccharide preparation can be determined by total nitrogen and hexosamine analyses prior to electrophoresis. As many proteins contain hexosamine (up to 2–3%), acid mucopolysaccharide preparations, containing appreciable amounts of protein, may show on electrophoresis a number of peaks if hexosamine analyses are used to

detect the positions of the migrating substances. In such preparations hexuronic acid analyses would show the positions of the acid mucopolysaccharides with greater specificity.

Asymmetric peaks were obtained from mixtures of substances with differing electrophoretic mobilities which did not separate from one another in the course of electrophoretic migration. This asymmetry was more marked when the substances were not present in equal amounts. For example, a highly asymmetric hyaluronic acid peak is shown in Fig. 3, but Fig. 4, which embodies results obtained from a longer migration, shows the main hyaluronic acid peak separated from two smaller peaks. Similar results were obtained from the chondroitin sulphuric acid fraction, prepared from skin by the method of Meyer & Chaffee (1941).

The degree of polymerization of hyaluronic acid affects its rate of migration, and Blix & Snellman' (1945) showed that hyaluronic acid was polydisperse. Nevertheless, it does not seem likely that the spread of polymer size in any sample of hyaluronic acid would be sufficient to cause migration rates to differ enough to account for an asymmetric peak. The hyaluronic acid samples listed in Table 1 were either highly polymerized or greatly depolymerized. They represent differences in degree of polymerization far greater than would be expected in any one hyaluronic acid sample.

With the horizontal apparatus described here it does not seem possible to determine electrophoretic mobilities accurately. Evaporation and subsequent alterations in buffer concentration along the filter-paper strips would render any estimations of mobility made in this apparatus of doubtful value.

SUMMARY

1. A horizontal type of apparatus for carrying out paper electrophoresis is described.
2. The electrophoretic migration of three acid polysaccharides, hyaluronic acid, chondroitin sulphate and heparin were examined on filter-paper strips.
3. The positions of the acid mucopolysaccharides after electrophoresis were ascertained by staining with toluidine blue or by quantitative elution from segments of filter paper followed by determination of either the hexosamine or hexuronic acid content of each eluate. An apparatus for the quantitative elution of the acid mucopolysaccharides is described.
4. Extracts of skin have been examined by this method.

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Studies in the Bile Acids

1. A METHOD OF SEPARATION AND IDENTIFICATION

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In order to study the occurrence and metabolism of the bile acids, it is necessary to have methods of identifying the constituents of a mixture. Mixtures of bile acids are difficult to handle by classical techniques, which depend on the formation of characteristic crystalline derivatives. Frequently, even relatively pure compounds are not easy to crystallize, and large amounts of material are necessary if minor constituents are to be identified. As pointed out by Haslewood & Wootton (1950), much previous work is not based on acceptable experimental evidence.

In recent studies of steroid hormones and their metabolites, considerable use has been made of chromatographic separation combined with infrared spectrometry (Dobriner, Lieberman & Rhoads, 1948*a*; Dobriner *et al.* 1948*b*; Lieberman & Dobriner, 1948; Jones & Dobriner, 1949). In combination, these techniques have provided a powerful method for separating and identifying the individual non-acidic steroids of complex mixture. It therefore seemed probable that a similar method could be applied to the bile acids.

A successful procedure is described here. Initial trials with pure substances showed that the methyl esters of the common bile acids are separable on silica gel. These esters are soluble enough to enable their specific infrared spectra to be recorded in carbon disulphide solution.

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EXPERIMENTAL

Solvents. All solvents except ether were of 'C.P.' quality and were redistilled before use. Absolute ether was passed through a column of Al_2O_3 . Care was taken to use the purest solvents available, otherwise traces of unknown impurities tended to obscure parts of the spectrum. For this reason, a mixture of equal parts of pentane and hexane ('P.H.') (American Mineral Spirits Co., New York) was used as a solvent, with properties similar to those of light petroleum. Proportions of all solvent mixtures are v/v.

Chromatography. Columns of dry silica gel were used (Dobriner, personal communication). A column was prepared by filling a suitable tube with P.H. The appropriate quantity (usually 200 times the weight of the esters) of silica gel (grade 923, through 200, Davison Chemical Corporation, Baltimore) was then added in a fine stream and the solvent allowed to flow until a compact column was formed. The esters were applied as a solution in a solvent of relatively low polarity (e.g. P.H., 90:ether, 10), and development proceeded with solvent mixtures of increasing polarity. In a typical chromatograph, the solvent mixtures were: (1) P.H., 99:ether, 1 to dissolve the esters. (2) Mixtures of P.H. with ether containing 5, 10, 15, 20, 30, 40, 60 and 80% ether. (3) Pure ether. (4) Mixtures of ether and acetone, containing 5, 10 and 50% acetone. (5) Pure acetone. (6) Acetone, 50; methanol, 50. (7) Pure methanol.

Amounts of 100–250 ml. of each solvent mixture were applied, the eluate being collected in 50 ml. fractions at a rate of 2–3 drops/sec. If necessary, N_2 under pressure was admitted to the solvent reservoirs to maintain this rate of flow. Each fraction was taken to dryness at once and, if eluted material was present, the same solvent mixture was continued until no significant residue was found. The next solvent mixture was then applied.