amino acids is not required, and preliminary experiments or routine analyses of special collagen features such as the glycine or imino acid contents may thus be performed in only 115 min. Only 3-Hyp, homoserine and the Met-S-oxides are not accounted for by program B.

Fig. 1 shows a typical elution curve of collagen amino acids obtained by using program A. 3-Hyp, the two Met-S-oxides, 4-Hyp and Asp appear as well-separated peaks if the pH of the first elution buffer is lowered to 2.84. Also at the flow rates used (105 ml/h), there are no difficulties in resolving Ser and Thr if the first buffer contains 4% (v/v) of methanol. The other three citrate buffers are free of methanol. The figure also shows that the conditions used give a good separation of Glu–Pro and Gly–Ala, in spite of the amounts present, as well as Ile–Leu and Tyr–Val.

At present the basic amino acids are eluted from a second short column (14 cm; Aminex A-5) at 50° in 85 min. The pH of the citrate buffer used is 5.28. Hyl, Lys, His, ammonia and Arg are well separated.

Approximately 0.05 micromole of an amino acid is required for an accurate analysis. This figure has to be multiplied by a factor of 2 and 4, respectively, for the determination of the imino acids Pro and Hyp. The amount of protein necessary for a total analysis is approximately 1.2 mg and may be scaled down to 1 mg if experienced workers operate the instrument. The reproducibility of the results is very satisfactory.

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Received November 6th, 1968

J. Chromatog., 39 (1969) 326–328

CHROM. 3868

The separation of maleic and fumaric acids by gel filtration on Sephadex G10

Columns of Sephadex* gels will fractionate substances according to their molecular dimensions[1]. In addition to acting as molecular sieves, these gels reversibly adsorb certain types of molecules[2]. Adsorption effects, which may be positive or negative, are particularly marked for the highly cross-linked G10 and G15 gels. Molecules with π-electron systems are adsorbed positively. Negative adsorption is shown by anions. A possible explanation for this partial ion-exclusion is that solvation makes

* Sephadex gels are cross-linked polysaccharides manufactured by Pharmacia, Uppsala, Sweden.

the actual ionic size considerably greater than would be expected from simple considerations of molecular weight[2].

For gel chromatography, the elution characteristics of a solute can be expressed as a K_D value[2], where K_D is the distribution coefficient of the solute between the internal and external aqueous phases of the gel. The K_D value of a carboxylic acid which is small enough to diffuse into the gel should be dependent on pH. This is because in buffers of low pH, the acid will have a high K_D since it can diffuse freely through the gel. In buffers of high pH, the acid will exist as the anion which will have a low K_D due to ion-exclusion. Consequently, in a buffer of given pH, K_D should be proportional to the % ionisation of the acid. Hence a plot of K_D against pH will have the same shape as a plot of % ionisation against pH. It should therefore be possible to predict the optimum pH for the separation of two or more carboxylic acids if their dissociation constants are known.

Fig. 1 is a plot of % ionisation against pH calculated[3] for two similar hypothetical carboxylic acids, R_1 and R_2, having pKa values of 3 and 4 respectively. If % ionisation is proportional to K_D, the optimum value of buffer pH for separating the two acids can be predicted from Fig. 1 by finding the pH at which the difference in % ionisation of the two acids, ΔI, and hence ΔK_D, is a maximum. ΔI is plotted against pH in Fig. 2. The maximum value of ΔI and therefore the optimum pH for separation is 3.5.

Fumaric and maleic acids were chosen to test the above hypothesis as they are similar carboxylic acids (geometrical isomers) and have dissociation constants which are well separated. [The second pKa of fumaric acid is 4.38 and of maleic acid is 6.23 (ref. 3)].

Materials and methods

Column preparation and operation. The column, of diameter 1.5 cm, was packed

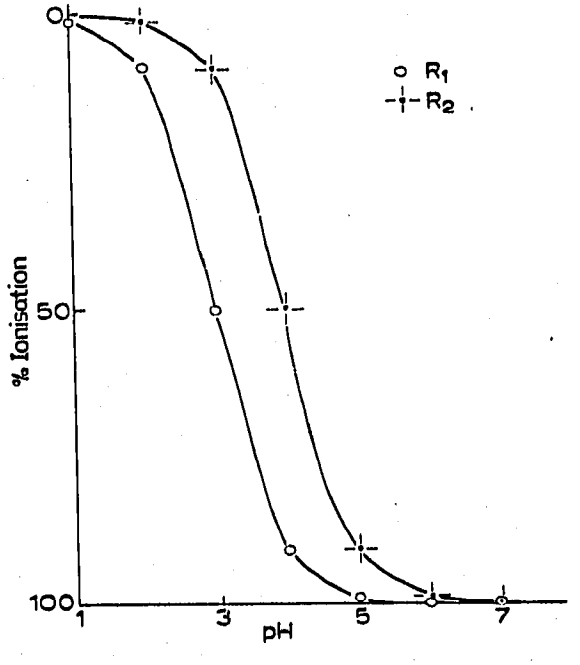

Fig. 1. The effect of pH on the % ionisation of two weak acids of pKa 3 and 4 respectively.

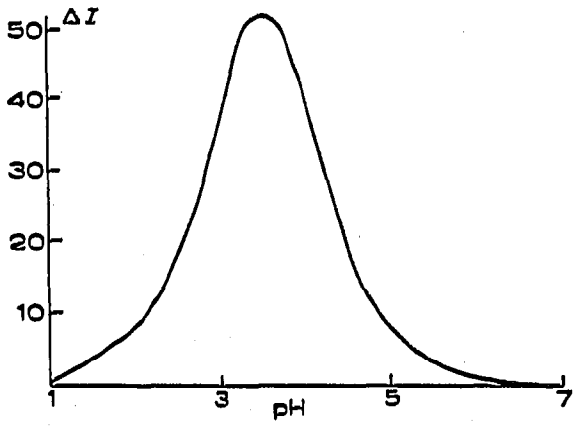

Fig. 2. The effect of pH on the difference in % ionisation of two acids of pKa 3 and 4 respectively.

with Sephadex G10 gel to a height of 56 cm as described by DETERMANN[4]. A water-jacket maintained the temperature of the column at 25°. Upward-flow elution was adopted to facilitate deoxygenation of the sample and buffer since the column effluent was to be monitored polarographically. The buffer and sample were deoxygenated with a stream of oxygen-free nitrogen. The buffer was passed up the column by an L.K.B. "Perpex" peristaltic pump, giving a flow-rate of 92 ml/h. The sample, 0.6 ml containing 0.4 mg of carboxylic acid, was introduced into the buffer-stream through an L.K.B. "ReCyChrom" selector valve. The same column was used throughout the whole series of experiments.

Polarographic monitoring of the column. A continuous-flow polarographic cell, shown in Fig. 3, monitored the effluent from the column. The cell was connected to a Cambridge polarograph and Leeds & Northrup "Speedomax H" strip-chart recorder so that chromatograms were recorded automatically. The polarograph was set at a constant potential, usually −1.4 V, which was just greater (negatively) than the half-wave potential of fumaric and maleic acids. The buffer, in addition to its role as eluent for the chromatograph, served as the polarographic background electrolyte[5]. The buffer also neutralised any ion-exchange properties that the gel might possess[2].

With this chromatographic arrangement, elution volumes were reproducible to ± 1 ml and the acids were eluted as symmetrical peaks.

Calculation of K_D. A substance, on elution from the column, can be characterised by K_D, the distribution coefficient between the internal aqueous phase and the aqueous phase external to the gel. K_D is given by

$$K_D = \frac{V_e - V_0}{V_i} \qquad \text{(i)}$$

where V_e is the elution volume of the substance, V_0 the void volume of the column and V_i the internal aqueous volume of the gel (ref. 2).

V_e was measured with the recorder chart and a measuring cylinder. V_0, determined as the elution volume of Blue Dextran 2000 (Pharmacia), was 34 ml. V_i, calculated from the water-regain (manufacturer's value) and the dry weight of the gel, was 39 ml.

Buffers. Since the polarographic estimation of maleic and fumaric acids requires background electrolytes of high ionic strength[6], McIlvain buffers with ionic strength

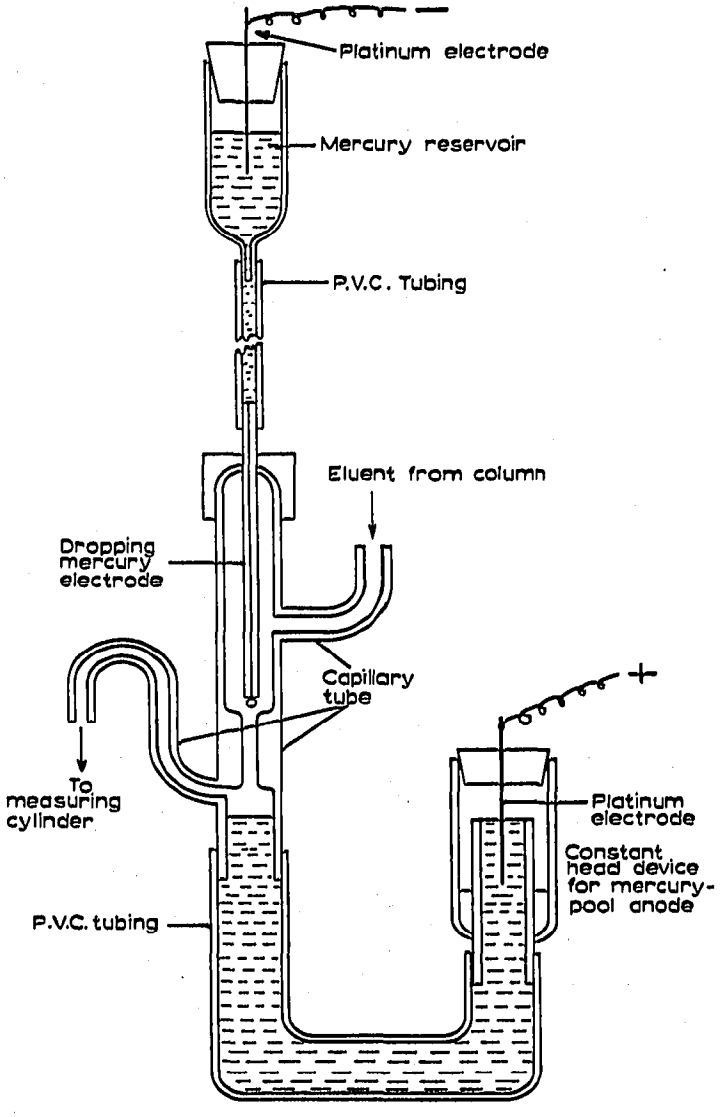

Fig. 3. The continuous-flow polarographic cell.

constant[7] at 0.5 M and 1.0 M over the pH range 2–8 were used. Additionally, the acids were eluted with formate, acetate, phosphate and tris(hydroxymethyl)aminomethane buffers covering the same pH range but of ionic strength 0.2 M.

Results and discussion

A plot of K_D against pH for maleic and fumaric acids is shown in Fig. 4. The ionic strength of the buffers was 1 M. The K_D values in buffers of high pH, where the acids are in the form of the dianions, are, as expected, low, indicating partial ion-exclusion. The K_D values for total ion-exclusion would be 0 (Eqn. (i)).

As the pH of the buffer decreases, the K_D values increase. For maleic acid, a plateau is reached at about pH 4 because, under these conditions, maleic acid exists as the half-acid (HOOC—CH = CH—COO^-).

Ideally, a substance which diffuses freely through the gel will have a K_D value of 1 (Eqn. (i)). Maleic acid, in solutions more acid than pH 4, has a K_D value of about

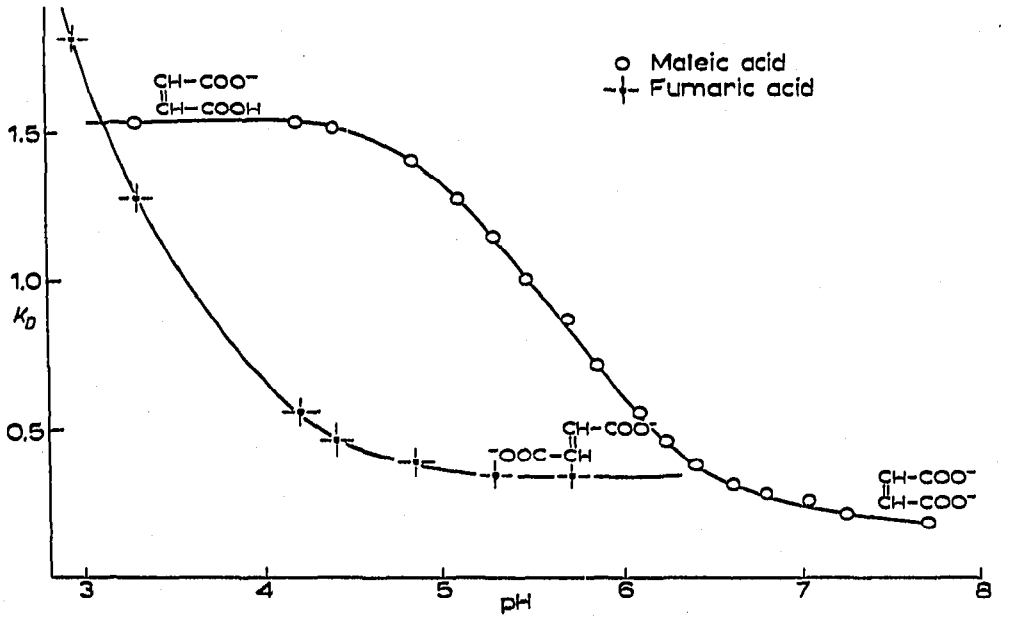

Fig. 4. The effect of pH on the distribution coefficients (K_D) of maleic and fumaric acids.

1.5. The half-acid must therefore be adsorbed onto the gel-matrix to some extent, due either to the π-electron system associated with the carbon-carbon double bond or, more probably to hydrogen-bonding of the hydroxyl group. It would be interesting to study the elution of maleic acid in buffers of pH less than 3 where further protonation of the acid with a corresponding increase in K_D would be expected. However, the glucosidic linkages of Sephadex gels are hydrolysed in solutions of high acidity.

Fumaric acid, on the other hand, does not give a plateau in the pH range investigated, probably because the first and second dissociation constants [p*Ka* 2.02 and 4.38 (ref. 3)] are so close together that the half-acid has no separate existence. At a pH lower than about 1, fumaric acid is fully protonated and the K_D–pH curve will presumably level off. As with maleic acid, at low pH, fumaric acid is adsorbed onto the gel-matrix.

Elution of the acids with buffers of ionic strengths 0.2 *M* and 0.5 *M* gave K_D–pH plots of the same shape as 1 *M* buffers. However, in the case of maleic acid, the curves were displaced progressively to higher values of pH with decreasing ionic strength, due to the effect of ionic strength on p*Ka*. If K_D is proportional to % ionisation of the acid, then the approximate p*Ka* of maleic acid can be deduced from the K_D–pH curves since the p*Ka* is given by the pH at the value of K_D which lies half-way between the K_D of the dianion ($^-$OOC—CH = CH—COO$^-$) and that of

TABLE I

p*Ka* VALUES FOR MALEIC ACID AT VARYING IONIC STRENGTHS

pKa	*Ionic strength*
5.7	1.0
6.1	0.5
6.3	0.2

the half-acid (HOOC—CH = CH—COO^-). The pKa values for maleic acid at varying ionic strengths obtained from K_D–pH plots are shown in Table I.

The literature value (measured at low ionic strength) for the pKa of maleic acid is 6.23 (ref. 3) which is in reasonable agreement with Table I.

The K_D–pH plots at 0.5 M and 0.2 M ionic strength had small, but reproducible inflections at pH 5.6.

Since the K_D–pH plot for the 0.2 M range of buffers is similar in shape to the curves for McIlvain buffers, change of buffering-ion does not affect the values of K_D.

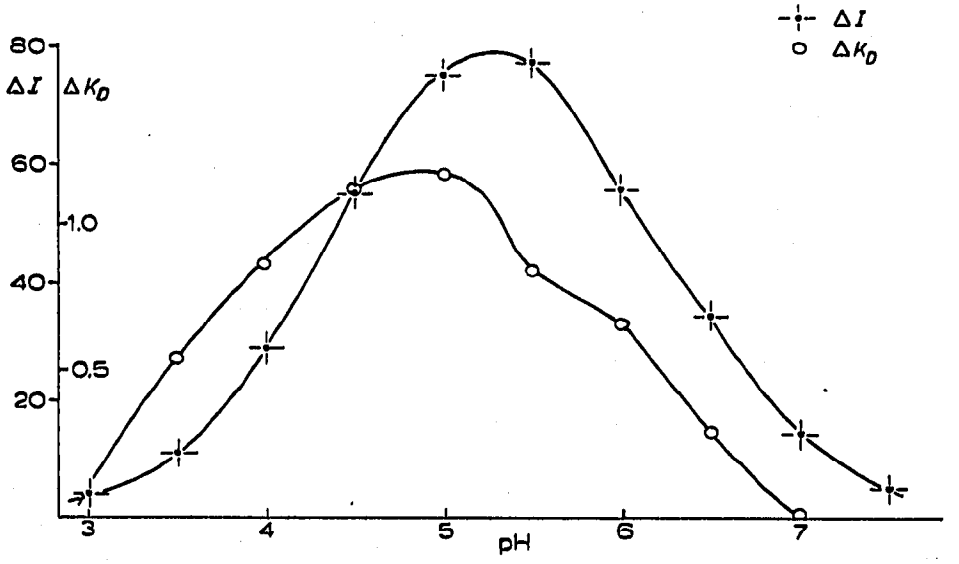

Fig. 5. The effect of pH on the difference in % ionisation (ΔI) and the difference in distribution coefficients (ΔK_D) of maleic and fumaric acids.

ΔI–pH and ΔK_D–pH curves are shown in Fig. 5. The K_D values used to calculate ΔK_D were those obtained with buffers of 0.2 M ionic strength. The theoretical (ΔI) and experimental (ΔK_D) buffers for optimum separation, which were pH 5.3 and 5.0 respectively, are in reasonable agreement.

Hence, the dissociation constants of maleic and fumaric acid can be used to predict the optimum conditions for the separation of a mixture of the two acids.

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Received November 8th, 1968