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Rostov-on-Don
State University

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ION-EXCHANGE CELLULOSES AND THEIR USE IN CHROMATOGRAPHY

E.G.Davidova and V.V.Rachinskii

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I. INTRODUCTION

The successful development of sorption technique and chromatography is to a considerable extent dependent on the discovery and utilisation of new types of synthetic sorbents. The production and introduction into sorption technology, particularly chromatography, of a new type of ion-exchange sorbent, the ion-exchange celluloses, is an outstanding achievement of modern chemistry and biochemistry.

The number of papers dealing with the chromatographic use of ion-exchange celluloses has been rapidly growing in recent years, and about 500 have already been published. The use of ion-exchange celluloses in chromatography and other varieties of sorption technology has enabled considerable progress to be made in the resolution of some of the most difficult problems in biochemistry — the separation of proteins, enzymes, nucleic acids, and other high- and low-molecular-weight substances found in living matter.

It is to be expected that ion-exchange celluloses will rapidly find wider application in ever increasing numbers of chemical fields.

The object of this review is to provide an account of current knowledge of the properties and uses of ion-exchange celluloses.

II. PREPARATION OF ION-EXCHANGE CELLULOSES

We shall not concern ourselves here with the ion-exchange properties of natural and commercial (including chromatographic) celluloses, which may be found in a number of monographs, e.g. those of Rogovin[1] and Hais and Macek[2]. We note only that both natural and the various types of commercial cellulose possess ion-exchange properties mainly because of the presence of carboxyl groups, but their absorption capacity is negligibly small, being only ~ 0.05 mg-equiv. g^{-1}, which corresponds to about one carboxyl group for 90–130 glucose structural units[3,4].

The use of ion-exchange forms of cellulose for chromatographic purposes already has a history of more than 50 years. Goppelsröder[5] mentioned oxycellulose (a weakly acidic carboxyl-type cation exchanger) as a suitable material for capillary analysis. Oxycellulose is obtained by saponifying the esters present in the initial cellulose and oxidising the primary alcoholic groups present in the 6-position of the glucose structural units[1,2]. Even in this process definite limitations are found in the content of carboxyl groups: there exists a certain critical level for the introduction of these ionogenic groups into cellulose; above this level the cellulose loses the character of an insoluble sorbent. For oxycellulose it was found that the content of carboxyl groups could not exceed 1–4%; with more than 5% (every fifth glucose structural unit then being oxidised in the 6-position) the oxycellulose becomes soluble in water at pH above 9.[2]

The "pioneering" work on the production of different types of ion-exchange cellulose includes that of Lautsch and coworkers[6], Micheel and Albers[7], Sober and Peterson[8,9], and Porath[11]. Lautsch and coworkers obtained a number of ion-exchange cellulose ion exchangers: a carboxyl-type cation exchanger, the ether of glycolic acid containing the $-CH_2OCH_2COOH$ group, by the action of chloroacetic acid in alkaline solution; a strongly acidic cation exchanger containing the $-CH_2OCH_2CH_2CH(SO_3H)CH_3$ group by the action of butane sulphone; and an anion exchange containing $-CH_2\overset{+}{N}R_3X^-$ groups by esterification with *p*-toluenesulphonyl chloride and reaction of the ester with tertiary amines. In each case one ionogenic group could be introduced for each 20–30 glucose structural units; this was the limit of ion-exchange capacity for these ion exchangers, since with more ionogenic groups the cellulose decomposed.

Micheel and Albers[7] obtained a carboxyl-type cation-exchange cellulose in the form of the phthalic acid mono-

ester. This weakly acidic cation exchanger was insoluble in alkalis when containing 5% of carboxyl groups.

Sober, Peterson, and coworkers[8,9] and Porath[11] undertook systematic studies of the production of ion-exchange celluloses for chromatography.

Sober and Peterson[8,9] described the preparation of a series of new cation- and anion-exchange celluloses: CM-cellulose (carboxymethylcellulose); P-cellulose (phosphorylated cellulose); DEAE-cellulose (diethylaminoethylcellulose); and ECTEOLA-cellulose. From DEAE-cellulose, Porath[11] prepared the anion exchanger TEAE--cellulose (triethylaminoethylcellulose), and he also obtained the strongly acidic cation exchangers SE-cellulose (sulphoethylcellulose) and SM-cellulose (sulphomethylcellulose). The abbreviated and full names of some ion--exchange celluloses and their properties are given in Table 1. These are prepared from natural α-cellulose.

Sober and Peterson[8,9] used powdered cotton cellulose for the preparation of cation exchangers and powdered wood cellulose for anion exchangers. α-Cellulose was found to be the most suitable substrate for the introduction of ionogenic groups.

The absorption capacity of ion-exchange celluloses is limited on the one hand by the need for the sorbent to be insoluble, and on the other by the requirement that the sorbent should have good physical properties suitable for use both in paper and in column chromatography.

The ion-exchange celluloses obtained by the above groups of workers had absorption capacities of less than 1 mg-equiv. g^{-1}, which is considerably lower than for ion--exchange resins. However, in most cases this capacity was quite sufficient.

The ion-exchange celluloses are prepared by normal etherification of cellulose; for example, DEAE-cellulose was obtained by heating a mixture of cellulose and β-diethylaminoethyl chloride in alkaline medium[9], and SM- and SE-celluloses were prepared analogously[11].

TABLE 1. Types of ion-exchange cellulose.

Abbreviated name	Full name	Ionogenic group	Acidic or basic properties	Total absorption capacity*, mg-equiv. × g^{-1}
Cation exchangers				
SE	sulphoethylcellulose	$-C_2H_4SO_3H$	strong acid	0.4
P	phosphorylated cellulose	$-PO_3H_2$	strong acid	0.8
CE	carboxyethylcellulose	$-C_2H_4COOH$	moderately strong acid	0.7
CM	carboxymethylcellulose	$-CH_2COOH$	weak acid	0.7
C	carboxycellulose (oxycellulose)	$-COOH$	weak acid	0.7
Anion exchangers				
PAB	*p*-aminobenzylcellulose	$-CH_2C_6H_4NH_2$	weak base	0.6
ECTEOLA	reaction product of cellulose with ethylene chlorohydrin and triethylamine	not known	weak base	0.5
AE	aminoethylcellulose	$-C_2H_4NH_2$	weak base	0.7
TEAE	reaction product of DEAE-cellulose with ethyl bromide	not known	moderately strong base	1.0
DEAE	diethylaminoethylcellulose	$-C_2H_4N(C_2H_5)_2$	moderately strong base	1.0
GE	guanidoethylcellulose	$-C_2H_4NHCNH_2NH_2OH$	moderately strong base	0.7

* Approximate values are given.

The methods for the production of ion-exchange celluloses have given good reproducibility in the physical properties of the sorbents and in their physicochemical characteristics (absorption capacity, etc.). A considerable part of the work on the uses of ion-exchange celluloses has been carried out on materials produced by the actual investigators. At the present time ion-exchange celluloses are produced by a number of foreign companies, such as Whatman (UK) and Serva (W. Germany), as well as at the Institute of Antibiotics, Academy of Sciences of the USSR (Leningrad) and the Textile Institute (Moscow). Table 1 lists the main known types of ion-exchange cellulose used in chromatography, the cation exchangers in order of decreasing acidity, and the anion exchangers in order of increasing basicity. A whole range of cellulose-based ion exchangers is available, and workers can select those with the required physicochemical properties.

III. PROPERTIES OF ION-EXCHANGE CELLULOSES

Structure

In their chemical structure the ion-exchange celluloses are linear polymers comprising long macromolecular chains of β-glucopyranose units joined by 1–4 β-glucosidic bonds. At intervals of the order of 50 Å the hydrogen of the cellulose hydroxyls is replaced by the various ionogenic groups. The individual linear macromolecules interact by molecular-type bonds (mainly polar) and by hydrogen bonding[1], the difference in the relative proportions of the two forms of interaction producing the differences in solubility and reactivity between the cellulose and its derivatives. Although the linear cellulose chains are cross--linked, the cross-linking is very weak.

The introduction of groups which cause partial rupture of the hydrogen bonds can increase the solubility. It should also be borne in mind that bonds retained in the ion-exchange celluloses may be broken during chromatography of some materials; thus it has been shown[11] that in chromatography of proteins on ion-exchange celluloses the protein molecules are adsorbed by hydrogen bonding as well as by the formation of ionic and polar bonds. A kind of competition arises for hydrogen bonding between the cellulose macromolecules on the one hand, and between cellulose and protein molecules on the other, which explains the high absorption capacity of ion-exchange celluloses towards proteins and other high-molecular-weight substances. The cellulose macromolecules can also combine with each other by ordinary valence bonds (glucosidic and ester linkages).

Physical Properties

Because of the irregular thread-like shape of the cellulose particles, it is impossible to obtain an accurate evaluation of their dispersity and dimensions. Direct microscopic measurement of particle lengths has produced distribution curves which show the maximum length to be 350–500 μm; the maxima on the distribution curves lie in the range 50–100 μm. The diameter of the particles is 15–20 μm. The moisture content of air-dried ion-exchange celluloses is 10–15%. The density is 0.1–0.2 g ml^{-1}, i.e. like commercial cellulose they are fairly "loose". The

specific volume of ion-exchange celluloses in the swollen state is 10–15 $cm^3\ g^{-1}$, and the swelling factor is ~ 2.

Ion-exchange celluloses possess good filtration properties; when they are used in column chromatography, the flow rate of solvent can be controlled within the limits 5–25 ml $cm^{-2}\ h^{-1}$. No data exist on their combined water content.

Physicochemical Properties

The extent of dissociation of the ionogenic groups of ion-exchange celluloses and their acidity and basicity can be estimated from their titration curves [19]. Table 1 includes a qualitative indication of the acidic and basic properties of ion-exchange celluloses, and their approximate total absorption capacities.

The total absorption capacity of an ion exchanger, is a sorption constant and is determined by the number of ionogenic groups which have exchangeable ions; in other words, the total absorption capacity as a constant is independent of the composition of the solution in contact with the ion exchanger. However, the utilisation of this total absorption capacity by different ions varies with the energy of the bond produced by the sorbed ions. If the sorption of one ion is studied in the presence of other ions, the distribution follows the ion-exchange isotherm and the law of equivalence of exchange.

The sorbed ions compete for the sorption centres of the ion exchanger in accordance with their bond energies, and of course hydrogen ions (with cation exchangers) or hydroxide ions (with anion exchangers) sorbed by the ion exchangers block part of the total exchange capacity and so also compete; as a result, the real absorption capacity of the ion exchanger depends on the pH of the solution.

The sorption of dissociable high-molecular-weight compounds (polyelectrolytes) and many low-molecular-weight organic compounds by ion exchangers is a complex process, and has been little investigated theoretically or experimentally. Dissociable organic compounds are generally amphoteric substances the ionogenic groups of which differ in their degree of dissociation, and the number of free ionic bonds will vary with the pH of the solution and the presence and concentration of other substances. In addition to polar ionic sorption, organic molecules may also be attached by other types of bond.

In view of what has been said, the laws of ion exchange established for the sorption of inorganic ions will not apply to the sorption of dissociable organic compounds. The absorption capacity of ion-exchange celluloses towards organic compounds cannot be expressed as milliequivalents per gramme of sorbent, nor, in the case of high-molecular-weight compounds of indefinite molecular weight, even as millimoles per gramme of sorbent. In such cases ion-exchange celluloses will have a specific absorption capacity for a specific substance under specific conditions, and this must be expressed as milligrammes of substance per gramme of sorbent. If a more rigorous approach is desired, the universal standard unit of mass of ion exchanger must be taken as 1 g of the ion-exchanger matrix (the mass of the ion-exchanger less the mass of sorbed ions or compounds). At the present time, however, this unit of measurement of the mass of an ion-exchanger has not found widespread use, and the absorption capacity is usually measured per gramme of air-dried (or dry) ion exchanger in the ionic form taken for the experiment.

The sorption properties of ion-exchange celluloses have as yet been very little studied, and there have been no systematic studies of absorption capacity, sorption isotherms, or the kinetics and dynamics of sorption.

Peterson and Sober [9] determined the absorption capacity of some ion-exchange celluloses towards proteins, obtaining the following values:

DEAE 0.498 g of blood serum protein per gramme of sorbent,

ECTEOLA 0.05 g of blood serum protein per gramme of sorbent,

CM 0.98 g of CO-haemoglobin per gramme of sorbent,

P 0.93 g of CO-haemoglobin per gramme of sorbent.

O'Donnell and Thompson [13] investigated the variation of the absorption capacity of ion-exchange celluloses with temperature; for example, a decrease of temperature from 25° to 1° greatly reduces the absorption capacity of DEAE-cellulose towards insulin.

The rate of sorption is known to be determined by the rates of the successive stages of the sorption process, i.e. of diffusion and of actual sorption. The part played by these steps in establishing sorption equilibrium on ion-exchange celluloses has not been investigated. Consideration of the structural features of ion-exchange celluloses suggests that the sorption of various substances on them will be extramicellar in nature; the intradiffusional stage of sorption is practically excluded here.

The extramicellar nature of the sorption and the high degree of dispersion of ion-exchange celluloses (particle diameters of the order of 10–20 μm) should theoretically produce a more rapid sorption than on ion-exchange resins. With inorganic ions and low-molecular-weight organic substances the time required for establishment of sorption equilibrium is probably limited by the time of the extradiffusional stage of sorption. It may well be, however, that with high-molecular-weight substances the time required for the establishment of sorption equilibrium will also depend significantly on the time required for the actual act of sorption.

The literature contains extremely scanty information on the kinetics of sorption of substances on ion-exchange celluloses. When the sorbent is stirred with a solution of calcium ions, the equilibrium for the sorption of Ca^{2+} by SE- and P-celluloses in various ionic forms is attained in 10 s, which corresponds to the time required for the external diffusion of the inorganic ions being exchanged [14]. With DEAE-cellulose equilibrium is established in the exchange of chloride and iodide ions in slightly more than ~ 30 s, i.e. as with ion-exchange resins, sorption by anion exchangers is slower than by cation exchangers. This difference may reflect the influence of the rate of the chemical stage of sorption, which is not always instantaneous. Gusev [15] studied the kinetics of sorption of Na^+ and Fe^{3+} ions under somewhat unusual conditions, with sorption of the ions by a dense layer of CM-cellulose, and found the sorption by the surface of the dense cellulose layer to be very rapid, while further sorption inside the layer was very slow, sorption equilibrium being established in 3 h. This result is only comprehensible if, under the conditions indicated, the time required for establishment of the equilibrium is determined by diffusion inside the very dense

layer of sorbent (internal diffusion). However, such conditions do not apply in chromatographic experiments. Gusev also found the rate of sorption to be greater in acid than in neutral media; this effect is also observed with ion-exchange resins, and is associated with the greater swelling of the ion exchangers in acidic media and the greater mobility of the hydrogen ions which participate in the exchange.

Nikolaev[16] has shown that equilibrium is established in 5 min in the sorption of proteins and enzymes on ion-exchange celluloses.

There is very little information available on the sorption isotherms for various compounds on ion-exchange celluloses. Nikolaev[16] investigated the statics of the sorption of asparaginase from 0.08 M NaCl and from buffer solutions of pH 7.0 and 6.5; he obtained a sorption isotherm of convex shape.

There is a little information available on sorption series for some ion-exchange celluloses; for example, Head and coworkers[17] obtained the following sorption series for P-cellulose: $U^{4+} > UO_2^{2+}$; $Th^{4+} > Fe^{3+} > La^{3+} > H^+ > Cu^{2+}$; and $Ca^{2+} > Na^+$. These agree with results obtained for phosphate resins. Veder and coworkers[18] established the following sorption series for anions with ECTEOLA-cellulose:

citrate > formate > HPO_4^{2-} > SO_4^{2-} > phthalate > > salicylate > succinate > glutamate > NO_3^- > > benzoate > Br^- > propionate > tartrate > > caproate > Cl^- > lactate > acetate > I^- > > ascorbate > glycerate.

In investigating the sorption of compounds on ion-exchange celluloses, due account must be taken of the possibility of complex-formation between the ions and the ionogenic groups or other ions in the solution[17,19].

It is evident from this review that the properties of ion-exchange celluloses have been very little studied. Practically no information is available on the mechanisms of the sorption processes or the quantitative laws of sorption of inorganic and organic compounds on cellulose ion exchangers, but this gap in our knowledge will obviously be filled by appropriate studies in the near future.

IV. TECHNIQUE OF THE APPLICATION OF ION-EXCHANGE CELLULOSES

Choice of Sorbent

Ion-exchange celluloses are selected on the basis of their properties and the composition of the mixtures to be separated. DEAE-cellulose is usually employed for the separation and analysis of proteins. About 80% of published work on the use of ion-exchange celluloses relates to the chromatographic analysis of proteins, and 75% of this is concerned with the use of DEAE-cellulose, ~ 20% with CM-cellulose, and ~ 3% with TEAE-cellulose; this information is taken from Serva's catalogue[12], and is illustrated in Table 2, which is based on the catalogue but which we have supplemented.

DEAE-cellulose is used for the separation of enzymes and proteins having isoelectric points at a neutral or acid pH, and for the purification of viruses and chromatography of polysaccharides, peptides, and nucleotides; TEAE-cellulose is used similarly, and in some cases gives cleaner separations of proteins and other high-molecular-weight compounds. AE- and GE-celluloses have not yet found wide application. Good separations of nucleic acids and viruses are attained with ECTEOLA-cellulose. CM-cellulose is used for the analysis of neutral and basic proteins.

The strongly acidic celluloses such as SE and P are generally employed for the chromatography of low-molecular-weight substances. PAB-cellulose is in a class of its own. In its primary form it can be used to separate nucleic acids, but it has one important specific property: the amino-group is readily diazotised, and then couples with proteins, enzymes, antigens, and other materials. Because of this, PAB-cellulose can act as a selective sorbent; for example, a PAB-azoantigen selectively absorbs antibodies, PAB-azohistone and PAB-azoguanine selectively absorb nucleotides, and PAB-azotyrosine selectively absorbs tyrosinase. Enzymes attached to PAB-cellulose lose their activity. The discovery of this sorbent opens up new possibilities in chemistry and biochemistry. The production of specific sorbents is extremely important for the development of sorption technology and chromatography.

Ion-exchange celluloses are used in column, paper, and thin-layer chromatography and in drop sorption techniques. The experimental procedure in each of these methods is different, and must be dealt with separately.

Column Chromatography

Columns of ion-exchange cellulose with a length to diameter ratio of 10 : 1 are usually employed; the size of the column and hence the amount of cellulose are selected in accordance with the amount of mixture to be separated, the separating ability of the column, and the required yields of the separated components of the mixture.

Organic substances are generally separated by displacement or elutive column chromatography, with buffer solutions as eluting solvents. Experience has shown that best results are obtained if the sorbent is first treated with the buffer solution which is to be used as eluent, thus becoming saturated with the ions and other components in equilibrium proportions.

Work with ion-exchange celluloses has shown[20] that in some cases, particularly with concentrated eluate solutions, the chromatography is accompanied by partial dissolution of the cellulose, so that dissolved cellulose fragments. polysaccharides with ionogenic groups, pass into solution; this may lead to errors. This effect can be detected or tested for by analysis of the eluate for polysaccharides[21,22]. The eluate can be readily freed from such impurities, for instance by passage through small columns of ion-exchange resins[23].

Account should also be taken of the possibility of decomposition of the cellulose by the enzyme cellulase[24,25]. Some ion-exchange celluloses are stable towards the action of this enzyme, but some are not[24]; the former group includes P-, SM-, and CM-celluloses, and the latter DEAE-cellulose and the cellulose prepared from Whatman paper.

The cellulose ion exchangers employed in chromatographic analysis can be regenerated by the usual method[26].

Ion-exchange celluloses withstand the not unduly prolonged action of strong acids and alkalis[17].

TABLE 2. Bibliographic index of the applications of ion-exchange celluloses.

Material	Sorbent	Ref.
Acetylenemonocarboxylate hydrolase	DEAE	147
Acetyltrypsin	DEAE	148
Acetyltrypsinogen	DEAE	149
Adenosine 5'-phosphate	CM, DEAE	131
Alanine-activating pH 5 enzymes	DEAE	132, 133
Albumen (human blood serum)	CM, DEAE	134–136
Alcohol dehydrogenase	CM, DEAE	139, 140
Aldehyde dehydrogenase	CM, DEAE	137
Aldolase	DEAE	138
L-Amino-acid oxidase	DEAE	142
Amino-acids	DEAE	120
Aminoacyl-RNA enzymes from ribosomes	DEAE	144
Aminoacyl-RNA synthetase	DEAE	143
γ-Aminobutyraldehyde dehydrogenase	DEAE	145
Aminoglucosidase	DEAE	141
Aminooxidase	AE	84
Antiserum (diphtheria)	SE, SM	146
Asparaginase	DEAE	16
Carbamoylphosphate synthetase	DEAE, P-cellulose	235
Carbonic anhydrase	DEAE	236
Carboxypeptidase	DEAE	237
Catalase	DEAE	238, 239
Cathepsin	DEAE	239
	DEAE, CM, SM	240
Cell proteins	DEAE	241
Cell stimulating hormones	CM	242
Ceruloplasmin	DEAE	244, 245
Chloroperoxidase	DEAE	369, 370
Chymotrypsinogen	CM, DEAE	371
α-Chymotryptsinogen derivatives	CM	372
Cobalamine	DEAE	243
Collagen	CM	127
Collagenase	DEAE	246
Corticotropin	DEAE	247, 248
Creatinine deaminase	DEAE	249
Creatinine transaminase	DEAE	249
Cytochrome c	CM	82, 373
DNA	ECTEOLA	96, 98, 99, 212–218
DNA polymerase	DEAE	219, 220
Deoxyribonuclease	DEAE	225, 226
Deoxyribonucleotides	CM	171, 227
Diaphorase	DEAE, ECTEOLA	221–224
Dihydroxydehydrase	DEAE	229
Diphosphogalactose-4-epimerase	DEAE	228
Elastase	CM	230
		60, 374, 375
	DEAE	233, 375
Enolase	SM	376
Enzymes of calf thymus	DEAE	351
Ferritin	DEAE	352
Fibrinogen	DEAE	353
Flavin nucleotide	DEAE	300
Flavone	CM	354
Folic acid derivatives	TEAE	299
β-Galactosidase	DEAE	167
	TEAE	168
Gelatin	CM	231
γ-Globulin	CM	154, 186
Glucopeptides	DEAE	202
α-Glucoprotein	DEAE	203
Glucose-6-phosphate	DEAE	190
Glucose-6-phosphate dehydrogenase	DEAE	187–189
	P-cellulose	188
Glucosidase	DEAE	191
α-Glucosidase	DEAE	192
β-Glucosidase	DEAE	193
	TEAE	194
Glutamine decarboxylase	DEAE	195
Glutamine dehydrogenase	DEAE	196, 197
Glutamine transamidase	CM	198
γ-Glutamylcysteine synthetase	DEAE	199
Glutathione peroxidase	DEAE	81
Glutathione synthetase	DEAE	200
Glutin (wheat)	CM	201
Glyceraldehydephosphate dehydrogenase	DEAE	183
α-Glycerophosphate dehydrogenase	DEAE	184
Glycolate oxidase	DEAE	182
Glycylglycine dipeptidase	DEAE	185
Gonadotropin	DEAE	204, 205
Haemoglobin	CM	51, 56–58
Haemoglobin (CO, A and F)	CM	55
Haptoglobin	DEAE	169
Heparin	ECTEOLA	171, 172
Hexokinase	DEAE	170
Histogen	P-cellulose	180
	CM	181
Histone	CM	158, 179
Hormones	CM	206–209, 120
	DEAE	89, 90, 211
Hydrogenase	DEAE	173, 174
α-Hydroxy-β-ketoreductoisomerase	DEAE	175
β-Hydroxypropionate dehydrogenase	DEAE	176
20-β-Hydroxysulphatase	DEAE	177
Hypoxanthine dehydrogenase	DEAE	178
Inorganic ions	P-cellulose	17, 127
Insulin	DEAE	62
Insulinase	DEAE	232, 233
Invertase	TEAE	234
2-Keto-3-deoxy-D-glucokinase	DEAE	250
Lactate dehydrogenase	DEAE	43, 251–255
Lactoaldehyde dehydrogenase	DEAE	85
Lactoaldehyde reductase	DEAE	85
Leucine aminopeptidase	DEAE	256
Lipase	DEAE	257, 258
Malate synthetase	DEAE	259
Malic dehydrogenase	DEAE	260
Malic enzyme	DEAE	261
Malonyl Co-A dehydrogenase	DEAE	262
Malonyl Co-A oxidase	DEAE	263
Maltase	TEAE	234
Meromyosin	DEAE	60, 264
β-Methylaspartase	DEAE	265
Methyl malonate isomerase	DEAE	266
Microsomal extract	DEAE	267
Mucopolysaccharide sulphotransferase	DEAE	268
Myoglobin	DEAE	95, 269
	CM	270, 271
Myosin	DEAE	61, 225, 272
NAD	DEAE	190
NAD-L-gulonate dehydrogenase	DEAE	80
NADP	DEAE	190
Nucleopeptides	ECTEOLA	273
Nucleoproteins	ECTEOLA	274, 275
	DEAE	276
5-Nucleotidase	DEAE	277
Nucleotides	TEAE	278
	ECTEOLA	279
Nucleotide transphosphorylase	CM	79
Ornithine carbamoyltransamidase	DEAE	280
Ovomucoid	TEAE	281
Pancrease	CM, DEAE	282, 283
Parapepsinogen	DEAE	284
Pepsinogen	DEAE	285
Peroxidase	TEAE	286
	CM	287
Phenol oxidase	DEAE	350
Phosphatase, acid	DEAE	355, 356
Phosphatase, alkaline	ECTEOLA	357
	CM, DEAE	87
Phosphodiesterase	DEAE	358–361
Phosphogluconate	DEAE	190
Phosphomonoesterase	TEAE	286, 362
	DEAE	363
Phosphooxidase	DEAE	366
Phosphoprotein	ECTEOLA, TEAE	364
Phosphoprotein phosphatase	TEAE	365
Phosphorylase (potato)	DEAE	367
Phosphotransbutyrylase	DEAE	368
Plasminogen	CM, DEAE	88, 289
Polygalacturonase	CM	290
	DEAE	291
Polyol dehydrogenase	DEAE	58
Polysaccharides	DEAE	117, 292
Procarboxypeptidase	DEAE	293
Procarboxypeptidase A	DEAE	215
Procarboxypeptidase B	DEAE	294
Proteins:		
Acetyltrypsin	DEAE	152
Albumin	DEAE	151
Diglycine, triglycine	DEAE, ECTEOLA	150
CO-Fibrin	DEAE	153
γ-Clobulin	CM	154–157
Ovalbumin	DEAE	158
Proteinase	DEAE	295
Protein phosphokinase	DEAE	296
Proteolytic inhibitor	DEAE	297
Prothrombin	DEAE	298
Pyridoxine phosphate dehydrogenase	DEAE	288
Riboflavin-5'-phosphate	DEAE	30
Ribonuclease	CM	74, 301–305
	DEAE	306, 307
Ribonuclease inhibitor	DEAE	308
Ribonucleotides:		
Adeninemononucleotides	DEAE	101
Adenine oligonucleotides	DEAE	108
	ECTEOLA	318
Oligonucleotides	DEAE	101, 102
Polynucleotide coenzyme	ECTEOLA	320
RNA hydrolysate	ECTEOLA	319
RNA	ECTEOLA	103, 107, 309–312
RNA, "soluble"	ECTEOLA	313–316
	DEAE	106, 317
Serum, chicken blood	DEAE	325
Serum, human blood	DEAE	10, 26, 47, 64, 326–328
Succinate diaminotransamidase	DEAE	321
Sulphate nucleotide	ECTEOLA	322
Sulphate oxidase	DEAE	324
Sulphate reducing enzyme	DEAE	323
Thyrotropin	CM	90, 329, 330
	DEAE	331
Transaldolase	DEAE	189, 332
Transamidase	AE, DE	101
Transketolase	DEAE	189
Trypsin	CM	333–335
Trypsin inhibitor	CM, DEAE	336–338
Trypsinogen	CM	335
Tryptophan pyrrolase	DEAE	339
Tyrosinase	DEAE	340–342
	PAB-isophenol	343
Tyrosine transaminase	DEAE	344
Uridine diphosphate galactose-4-epimerase	DEAE	345
Uridine diphosphate glucose-pyrophosphatase	DEAE	346
Uridine diphosphate glucose-pyrophosphorylase	DEAE	347
Uridine kinase	CM, DEAE	348
Urokinase	DEAE	349
Uropepsinogen	DEAE	349
Virus (bacteriophage T2)	ECTEOLA	110, 159
Virus (ECHO)	ECTEOLA	160
Virus (potato X)	DEAE	162
Virus (tobacco mosaic)	CM	161
	DEAE	162
	ECTEOLA	109, 113
Virus (vaccinia)	ECTEOLA	163
Vitamin B_{12}	P-cellulose	119
Vitamin K_1 reductase	DEAE	164–166

In the chromatographic separation of mixtures of substances on ion-exchange celluloses by displacement chromatography with buffer solutions, the distribution constants or sorption factors of the components, the differences in which determine the success of the chromatographic separation, depend on the pH of the solution and the concentration of salts present in it. Optimum conditions for the separation can be found by proper selection and control of the pH and the concentration of salts and other components in the eluent. Experience with various sorbents, including ion exchangers, has shown that the effective separation of mixtures of organic substances by displacement column chromatography requires the use not of a single eluent, but of several eluents in succession, or an eluent of continu-

ously varying composition. This has brought into use such methods of chromatography as single-stage, multistage, and continuous gradient elution.

Several devices exist for setting up concentration gradients [12], differing in how the eluent concentration varies with time.

Elution gradients, their theoretical prerequisites, and practical methods for obtaining them have been actively discussed in the literature, particularly in connection with the use of ion-exchange celluloses [20,27-34].

The choice of eluent and the forms of gradient are greatly dependent on the mechanism of sorption of the dissociable organic substances on the ion exchangers. Unfortunately, this has been very little investigated, either theoretically or practically. In this Review we can only touch upon some of the general theories relating to this type of sorption.

Dissociable organic compounds are usually amphoteric, i.e. according to the pH of the solution they dissociate predominantly by scission of acidic (hydrogen ions) or basic (hydroxide ions) from ionogenic radicals. The organic molecules will exist as neutral molecules and anions in the former case, and as neutral molecules and cations in the latter, the relative amounts of neutral and ionic molecules depending on the degree of ionisation of the ionogenic radicals in the molecule. At a certain pH — the isoelectric point — the dissociation of both types of ionogenic group is equally probable, and the dissociating molecule takes on the properties of a dipolar ion (zwitterion).

The sorption of dissociable organic compounds on ion exchangers is a complex process which may involve all types of bonding.

Since ion exchangers are polar sorbents containing ionogenic groups having ionic bonds, one of the main mechanisms of sorption of dissociable substances on ion exchangers is that of ion-exchange sorption. As in the case of inorganic substances, ion-exchange columns can be used to find the nature of organic compounds, their ionic constitution, etc.; for example, at a given pH "basic" compounds will be firmly sorbed on cation exchangers and loosely held on anion exchangers.

The sorption of dissociable organic compounds is greatly influenced by the nature of other ions present in the solution; these include hydrogen and hydroxide ions, the concentrations of which define the pH of the solution. The ions will combine both with the sorbent and with the dissociating organic ions in the solution.

The distribution of other ions present between the sorbent and the organic molecules in solution depends on the corresponding bond energies and the ionic concentrations. These ions and the ions of the organic compound will compete for the sorption sites in the ion exchanger, and changing the concentration of the former ions will therefore alter the distribution coefficients for the organic material being sorbed. One general feature is observed in this process: the sorption of the dissociating organic compound will decrease as the concentration of other ions is increased, because the latter will utilise more and more of the available ionic bonds in the ion exchanger and in the dissociating organic molecules. The other ions present thus act as a barrier which hinders the sorption of the dissociating organic compound. For this reason, solutions of electrolytes, such as buffer solutions, are used to elute the sorbed organic substances. Experience with the chromatography of organic substances has shown that some are not eluted, after introduction into the column of sorbent (or onto the paper). This means that such organic materials do not dissolve in the eluent employed. However, if the concentration of salts in the eluent is slowly raised, a certain "critical" concentration is reached when the organic material becomes almost completely soluble and begins to move at the flow rate of the eluent. Because each component has its own critical elution concentration, gradient elution can achieve an efficient separation of a mixture of organic substances. This method of chromatographic separation was discovered by Tiselius [35,36] in the chromatographic analysis of proteins, and has sometimes been called the "all or nothing" method or "salting-out" chromatography.

One further application of gradient elution must be mentioned. One of the negative effects in the chromatographic separation of mixtures of substances is the spreading of the fronts of the chromatographic zones, and gradient elution is one of the methods available for overcoming this. In gradient elution the forward and rear boundaries of the chromatographic zones occur at different eluent concentrations, lower at the forward edges and higher at the rear edges, so that at the rear boundary regions the conditions are such that the sorption of the substance is less than at the forward boundary. This means that desorption will be greater at the rear than at the front, and the chromatographic zones will become narrower and form sharp peaks, so improving the efficiency of the chromatographic separation.

Paper Chromatography

Ion-exchange chromatographic paper is also used analytically. A detailed bibliography and description of working with ion-exchange paper can be found in a Czech monograph [2]. Several firms produce ion-exchange paper for chromatography in addition to powdered ion-exchange cellulose.

Two methods are used for chromatography on ion-exchange paper [37]: with a "dry" and with a "wet" start. In the former, which is generally used in paper chromatography, the eluting solvent is absorbed by the dry paper, and in the latter the paper is first impregnated with the buffer solution and is then used similarly to an ion-exchange column.

Knight has shown that the "dry" start method gives a better separation of amino-acids [38].

Thin-Layer Chromatography

In recent years much success has been achieved by the use of the so called thin-layer chromatography, first suggested by Izmailov and Shraiber [39] and developed by Stahl [40,41]. Nikolaev [42] has reviewed this field in detail.

The essence of the method is that the sorbent is mixed with some cementing or binding material (such as gypsum), and the resulting paste is then deposited in a thin layer (~ 0.5 mm) on a glass plate. This thin layer is the analogue of the column or sheet of chromatographic paper. One- or two-dimensional chromatograms can be obtained. The main advantage of thin-layer chromatography is that, because of the fine dispersion (< 25 μm) of the sorbing material deposited on the glass plate, there is very rapid

capillary uptake of the solvent, and the chromatography takes very little time. Clean separations of mixtures can be achieved on 10 × 10 cm^2 or 15 × 15 cm^2 plates. Ion-exchange celluloses can be used as sorbents in thin-layer chromatography, and the Serva company provides prepared mixtures of ion-exchange cellulose powders with cementing material, specially designed for thin-layer chromatography [12].

The following procedure is recommended for the preparation of the plates. 10 g of the sorbent is mixed with 60–80 ml of water, and the paste is spread in a thin even layer on the plate with a special frame or other device. The success of the chromatography depends very much on the preparation of the thin layer, and it is very important to have an even thickness. The glass plate must have a good plane and even surface. The deposited sorbent layer is dried in the air at room temperature. The sample is introduced on to the sorbent layer with a micropipette, and the plate is placed vertically with its edge in the eluting solvent. The eluent front moves at a rate of 10 cm in 15 min.

As in paper chromatography, a very convenient method for developing the chromatograms, in addition to the use of indicator reagents, is to use ultraviolet light; for example, nucleotides show up as dark spots in ultraviolet light (λ = 253 mμm), and proteins can be detected in ultraviolet light after heating the moist chromatogram for several minutes at 150°.

Fractional Sorption

Hess and Walter have shown [43] that selective separation of individual components from the test material can in some cases be achieved simply by adding the ion-exchange cellulose to the substrate; for example, certain dehydrogenases have been selectively extracted from plasma proteins. This procedure has been called fractional sorption, and has found application as a rapid method in clinical diagnostics, specially standardised DEAE- and CM-cellulose being available for this purpose [12].

V. USES OF ION-EXCHANGE CELLULOSES

Chromatography of Proteins

The first systematic studies in the development of the chromatography of proteins on ion-exchange celluloses were made by Sober, Peterson, and their coworkers [8–10,44], who used column chromatography to analyse the proteins of human and horse blood plasma on the anion-exchange DEAE-cellulose, by means of the gradient elution method. The protein content of the individual fractions was found spectroscopically. In addition the fractions were further subjected to electrophoretic separation. It was found that with a decreasing pH gradient and an increasing salt concentration the proteins generally left the column in the order of decreasing isoelectric points. The column of anion exchanger was saturated to the extent of 170 mg of protein per gramme of sorbent. The new method gave a considerably better separation than electrophoresis. The Sober–Peterson method was later applied to the separation of blood serum proteins by many other workers [45–50], using gradient elution with DEAE-cellulose [10,46,49] or CM-cellulose [45,47,50].

Kogent and Broda [46] evolved a chromatographic separation of the high-molecular-weight compounds contained in blood serum and not precipitated by sulphosalicylic acid, using DEAE-cellulose in a phosphate buffer. In their study of normal and pathological blood serum they found these compounds to be mucopolysaccharides.

Morrison and Cook [45] showed that good results in the separation of proteins can be obtained with CM-cellulose. Hess and Walter [43] used ion-exchange celluloses for the analysis of blood proteins in clinical diagnostics. Sober and coworkers [51] used chromatography on ion-exchange celluloses to show that human and horse CO-haemoglobin is less homogeneous in protein composition than dog haemoglobin.

CO-haemoglobin has been analysed chromatographically several times, using CM-cellulose the eluent being 0.01 M phosphate buffer with a "convex" pH gradient from 6 to 8. [52–54] In addition to the chromatographic separation of the proteins, pure globulin was obtained in preparative quantities. Blood haemoglobin has also been analysed by chromatography on ion-exchange celluloses by many other workers [55–58], and the method has been used for the chromatographic analysis of various other proteins: meromyosin [59,60], *l*-myosin [61], insulin [62], β- and γ-casein [63], γ-globulins [64], β-lactoglobulin [65], and others.

Complex biological materials such as egg protein [66,67] and milk proteins [68,69] have also been analysed chromatographically. Previously unknown proteins have been discovered by chromatography on ion-exchange celluloses, for example in egg protein by Rhodes and coworkers [67] using elutive chromatography on CM-cellulose.

Several papers have been published on the separation of plant proteins [70,72]. As yet unidentified protein fractions have been found in soya bean proteins [71], and six protein fractions have been detected in barley proteins [70].

Chromatography of Enzymes

Chromatography on ion-exchange celluloses has been successfully used for the separation and isolation of enzymes. Arne [73] used a column of TEAE-cellulose to analyse gastric invertase and maltase; the latter consists of three components, while the former is a single enzyme.

Taborsky [74] separated ribonuclease into four components by chromatography on CM-cellulose.

Ispolatovskaya et al. [75,76] used DEAE- and ECTEOLA-cellulose for the separation and preparative isolation of leucitinase, pologenase, and hyaluronidase of bacterial toxin.

Others have also analysed enzymes by chromatography [77,78].

Many papers have been published on the purification of enzymes by means of ion-exchange celluloses [79–88]; for instance, the dehydrogenase isolated from rat liver was purified with DEAE-cellulose, its activity increasing 250 times [86], and the alkaline phosphatase activity of the enzyme isolated from human intestines and liver was increased 15–140 times [87].

A 50-fold purification of yeast carboxylase on a column of CM-cellulose has been described; the resulting enzyme retained its activity at 5° for a prolonged period. Ion-exchange celluloses have been used to purify hormones [89–91], antigens [92,93], and plasmogen [94].

Brown[95] has developed a procedure for the preparative isolation of pure myoglobin from crude muscle extracts using large columns of ion-exchange cellulose, and Nikolaev has shown that proteins may be concentrated by means of columns of DEAE-cellulose[16].

Chromatography of Nucleic Acids and Nucleotides

Bendich and coworkers[96] were the first to use ion-exchange celluloses for the separation and analysis of nucleic acids; they tested a number of celluloses, and obtained best results with ECTEOLA-cellulose. They achieved chromatographic fractionation of DNA differing in the composition of the heterocyclic bases. Other workers have also carried out chromatographic fractionation of DNA with ECTEOLA-cellulose[97-99] or DEAE-cellulose[100].

Staehelin and coworkers[101,102] used DEAE-cellulose in their investigation of the nucleotide sequence in RNA. By chromatography of the hydrolysate of yeast RNA treated with pancreatic RNA-ase, they observed a whole range of different mono- and poly-nucleotides: the mononucleotides uridylic and cytidylic acids; the dinucleotides AC, AU, GC, and GU; the trinucleotides AAC, AAU, GAC, AGC, GAU, AGU, GGU, and GGC; and some tetranucleotides. Even the nucleotide isomers GAC and AGC were readily separated.

Mihalyi and coworkers[103] have used ECTEOLA-cellulose for the removal of RNA from muscle myosin; the chromatography separates the RNA from the protein, the RNA/protein ratio being 1.3 before and 0.02 after. In some experiments the protein content of the RNA fraction was reduced by a factor of 50–60. Ward and Putch[104] used ECTEOLA-cellulose to separate a mixture of DNA and RNA.

Reference may also be made to other work in which ion-exchange celluloses have been used for the separation of nucleic acids and nucleotides[105-108].

ECTEOLA- and DEAE-celluloses have also been used for separating and purifying viruses[109-113]. Thus Hayer and Bolton[111] separated and purified certain animal viruses in this way. The prospects of this method for virology and in particular for the production of vaccines are obvious.

Randerath[114] separated a mixture of nucleotides by thin-layer chromatography in 5 minutes using ECTEOLA- and DEAE-cellulose.

Chromatography of Low-Molecular-Weight Organic Substances

Rouser and coworkers[115] used columns of DEAE-cellulose to separate the lipids isolated from whole beef brain and found the composition to be cholesterol 20.3%, ceramide 0.3%, cerebroside 16.8%, cerebroside sulphate 3.5%, lecithin 11%, phosphatidylethanolamine 17%, and other constituents.

Heri and Neukom[116] carried out a successful separation of the pectins isolated from beet and apples on DEAE-cellulose, obtaining fractions differing in their content of arabinose and galactose and in the degree of etherification and polymerisation. The greater the degree of polymerisation and the lower the degree of etherification, the more firmly were the pectins retained on the DEAE-cellulose.

Elutive chromatography on a column of DEAE-cellulose was used by Muller and coworkers[117] to separate five polysaccharide fractions, differing in their content of uronic acids, from soil. Ion-exchange celluloses have been employed for the isolation of seromucoids from blood serum[118].

Pawelkiewicz[119] used an ion-exchange cellulose for the isolation and purification of vitamin B_{12}.

The use of ion-exchange celluloses for the separation of amino-acids has been investigated[38,120,121]. Ion-exchange celluloses have no particular advantages over ion-exchange resins in the column chromatography of amino-acids[120].

Ion-exchange celluloses have been successfully employed for the separation of AMP, ADP, and ATP, [101] peptides[122] and sugarphosphates[17].

Chromatography of Inorganic Ions

Head's work on the analysis of inorganic substances by chromatography on ion-exchange celluloses has already been mentioned. He found that Fe^{3+}, Ce^{4+}, UO_2^{2+}, U^{4+}, Th^{4+}, and ZrO^{2+} ions are strongly absorbed from acid solutions by P-cellulose, which is not observed with resins of the sulphonic acid type. These ions can be eluted with reagents which form stable complexes with them, for example Th^{4+} and UO_2^{2+} are quantitatively eluted with 10% $(NH_4)_2CO_3$ solution, Fe^{3+} with chloroacetic acid, ZrO^{2+} with ammonium fluoride, etc.

Head and coworkers also successfully separated mixtures of Bi and Pb, Cu and Pd, Cu and Co, and Cu and Ni; for example, Bi and Pb were separated by eluting Pb with 1 *N* HNO_3 and Bi with 1 *N* HCl, in which it forms anionic complexes; and Cu was separated from Cd, Co, and Ni by elution of these with 1 M $MgCl_2$, followed by desorption of the copper remaining on the column by passing HCl.

Cerrai and Testa[19] prepared a new type of anion-exchange cellulose, TOAC (tri-n-octylaminocellulose), with the very high capacity of ~ 1 mg-equiv. g^{-1}; with this they separated a mixture of Fe^{3+}, Co^{2+}, and Ni^{3+}, using 0.8 M HCl as eluent. Iron and cobalt form anionic complexes in HCl and are sorbed by the anion exchanger, while nickel does not form complexes and moves with the eluent. The Co and Fe are separated by elution with 3 M HCl, which desorbs Co but not Fe. Differences in ion-exchange sorption and complex-forming ability have also been used to separate a mixture of Th, Zr, and U, and such mixtures as La and Th, Zr and La, and Zr and Hf have also been successfully separated.

Use of Ion-Exchange Paper and Thin-Layer Chromatography

Ion-exchange paper chromatography has been successfully used to separate amino-acids[38], barbiturates[123,124], organic acids[125], proteins[126], inorganic ions[127-129], and other materials.

Curtain[126] separated blood serum proteins on DEAE-cellulose of ion-exchange capacity ~ 0.003 mg-equiv. cm^{-2}, using gradient elution with pH and ionic strength gradients, established with a programmed photoelectric device. Albumin and β- and γ-globulins were separated on a paper strip.

Street[123,124] used DEAE-paper to separate a mixture of 5,5-substituted barbiturates, with t-$C_5H_{11}OH$–0.1 M EDTA disodium salt (1 : 1) as eluent, by circular chromatography. He was able to separate a mixture of phenobarbitone, barbitone, butanobarbitone, and quinobarbitone. The chromatographic zones were inspected in ultraviolet light.

Myhre[125] has described the separation of organic acids on CM-paper, using butanol–ethanol–water (4 : 1 : 5) as the mobile phase. Gluconic and glucuronic acids are better separated on a more acid cellulose.

Kember and coworkers[127] used phosphorylated paper for the separation of cations, and paper treated with 2-aminoethanesulphonic acid for the separation of anions.

Cerrai and Testa[128] have investigated the behaviour of inorganic cations on ICBL1 paper treated with 0.02 *N* tri-n-octylphosphine oxide in cyclohexane. As the mobile phase they employed HCl, HNO_3, and H_2SO_4 solutions of various concentrations, and they indicated the dependence of the R_F value on the acid concentration for 50 metal cations. Mixtures such as Sr–Y, Bi–Hg–Pt, U–Mo–Y–La, Th–Y–La–Ca, and U–Tl–Fe–Ge were successfully separated.

An anion-exchange paper prepared by treating Whatman paper with tri-n-octylamine has been used to separate the mixtures Fe–Co–Ni and Zn–Co–Mn–Ni, making use of the ability of these metals to form anionic complexes in HCl[22].

By thin-layer chromatography on ion-exchange celluloses, Randerath and Struck[37] were able to separate complex mixtures of heterocyclic bases and nucleotides. They used 10 g of ECTEOLA, CM-, or DEAE-cellulose in a thin-layer on a plate, with ~ 2–5 μg of material taken for the chromatography. Water was used as the mobile phase, the front moving at a rate of 10 cm in 3 min.

The following R_F values were obtained for a number of bases: adenine 0.30, adenosine 0.53, hypoxanthine 0.55, inosine 0.70, guanine 0.37, guanosine 0.58, uracil 0.72, and uridine 0.81. Nucleotides were separated with butanol–acetone–acetic acid–5% NH_4OH–water (4.5 : 1.5 : 1 : 1 : 2); the rate of movement of the front was ~ 10 cm in 1 min. The following R_F values were obtained: AMP 0.38, CDP 0.22, UTP 0.13, ADP 0.26, and ATP 0.16.

Randerath has published a fuller review of the uses of thin-layer chromatography on ion-exchange cellulose[130].

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Timiryazev Agricultural
Academy, Moscow

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MONODISPERSE AEROSOLS

N. A. Fuks and A. G. Sutugin

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