Historical Introduction.

Although there were many early contributors to the development of column chromatography, two pioneers are considered the fathers of this analytical technic. They are David Talbot Day (1859-1925) and Mikhail Tswett (1872-1919). Day was a graduate chemist who became a distinguished geologist and mining engineer. He suggested the idea of chromatography to the First International Petroleum Congress and the Geological Society of Washington in 1900 and 1903 respectively. Day passed oil through fuller's earth and fractionated it into saturated aliphatic hydrocarbons, aromatic and unsaturated substances and finally, nitrogen and sulfur compounds.

Tswett, a Russian, studied botany and physical chemistry at the University of Geneva, then returned to Russia. He demonstrated the potential use of chromatography by separating xanthophylls and chlorophyllins using pet ether as a solvent and calcium carbonate as adsorbent. Tswett's achievement was superior to Day's in two respects. First, he recognized and correctly interpreted chromatographic processes, and second, he devised a useful laboratory method. The original Tswett column is still widely used for both preparative and analytical purposes.

Basic Nomenclature.

Tswett recognized the importance of chromatography and devised the nomenclature which is most widely used today (see figure 1). The tube filled with the adsorbent was called the chromatographic column. The washing liquid was called the solvent, wash liquid or developer. The series of zones in the column was the chromatogram and the washing of the mixture to form the chromatogram was referred to as development.

Contemporary terminology has replaced adsorbent with sorbent, sorptogram for chromatogram and sorptographic analysis for chromatographic analysis (1), and has introduced terms such as elution to mean a type of development when the solutes are washed through the columns and collected. Eluate indicates the solvent fraction plus solute collected in the filtrate. It is customary to distinguish between three main types of chromatographic procedures: elution analysis, frontal analysis, and displacement analysis. Since we will be primarily concerned with elution analysis in this discussion, the other two types of chromatography will not be mentioned further.

Column Chromatography of Proteins.

The column chromatography of proteins has been treated in numerous review articles and books (2-15). The instability of proteins in the presence of the usual type of adsorbent has led to the development of three
substantially different types of adsorbents for proteins. These are calcium phosphate gels, cellulose and Sephadex. The first two have general applicability to column chromatography of proteins (2).

Calcium phosphate gels have for some time been widely used in the purification of enzymes by batch procedures, but, in column work, the extremely high resistance imposed by such material to the flow of aqueous buffers has been a serious handicap. However, higher flow rates have been made possible by mixing the gel with Super-Cel, or by forming the gel in the presence of cellulose (16 and 17). Recently, methods have been developed for the preparation of calcium phosphate in forms which permit adequate flow without the addition of filter aids. One of these consists chiefly of brushite (CaHPO$_4 \cdot 2$H$_2$O) (18). Another is hydroxylapatite Ca$_5$(PO$_4$)$_3$OH (19).

The cellulose adsorbents are available as either anion or cation exchangers, and both types have been used in the chromatography of proteins (9 and 20-23). The most commonly employed cellulose ion-exchangers are diethylaminoethyl cellulose, an anion exchanger and carboxymethyl cellulose, a cation exchanger. Many others have also been used (Table I).

Recently ion-exchange Sephadex, the cross linked dextran, has been used in the separation of proteins and polypeptides. Undoubtedly both ion-exchange and gel filtration phenomena are involved in this type of separation (24-27).

Elution of Proteins from Ion-Exchangers.

Although basic studies of the adsorption of proteins on ion-exchange surfaces are lacking, the formation of multiple electrostatic bonds between the protein and the adsorbent is undoubtedly involved. Both the protein and the ion-exchanger are polyelectrolytes, and they are, therefore, capable of interacting at several points, provided interchange distances are favorable. Moreover, experience shows that the adsorbed protein is more lightly bound than a singly charged substance under the same conditions, but can be eluted from a suitable ion-exchanger if the pH is changed to reduce the number of charges on the protein or adsorbent, or if the salt concentration is raised to compete for the existing charges.

The eluting buffer is usually selected for its effectiveness in controlling pH in the region in which chromatography is to be conducted.

Two types of elution are used for the separation of proteins on ion-exchange columns.

1. Stepwise elution
2. Gradient elution

In the former, arbitrary stepwise changes in the composition of the eluent at short volume intervals can be used to elute the protein rapidly and in relatively high concentration. However, resolution can generally be expected to be less than that attainable by gradient elution.
An example of stepwise elution of protein applied to the meats field is some recent work of Fujimaki and Deatherage (28), who are interested in the chromatographic fractionation of sarcoplasmic proteins of beef skeletal muscle on ion-exchange cellulose. One hundred gram samples of freshly slaughtered beef were extracted with distilled water and 900 mg of the extract chromatographed on cellulose phosphate. Fourteen fractions were separated on the column (2 x 40 cm); they were dialyzed, centrifuged and freeze dried. The samples were finally analyzed for enzymic activity. The results are shown in Table II. Fraction I was considered to be nucleoprotein. This was determined by the identification of nucleotides following hydrolysis and chromatography on Dowex-1, X-8 (formate form).

Fraction IV and VI were found to be oxymyoglobin and metmyoglobin from their absorption spectra. Aldolase activity was associated with fractions III through IX. Phosphopyruvic kinase, activities localized in fractions VI, VII and IX and ATPase and Cathepsin activities found in Fraction IX. Since essentially all activities were found in Fraction VI, protein in this fraction was rechromatographed on DEAE-cellulose.

Similar studies involving the fractionation of protein in rabbit muscle were reported at this conference last year (29).

Another example of stepwise elution involved a very simple method similar to that of Mitz and Yanari (30). This procedure was used to study pork cathepsins (31). The apparatus used for this study is shown in figure 2. Twenty-five grams of DEAE-cellulose were used in a column (2.5 x 25 cm). The exchanger was washed with carbon dioxide-free water and 100 mg of a sample previously fractionated with ammonium sulfate (50-60% cut). The initial solvent was CO₂ free deionized water followed by CO₂-saturated deionized water. Figure 3 shows the type of separation obtained with protein from pork.

A similar method has been used by Sliwinski and co-workers (32) in studies of beef cathepsins.

Numerous studies of proteins utilizing column chromatography have involved gradient elution. A commonly employed hydro-dynamic apparatus is pictured in figure 4, taken from Yaguchi et al. (33). Gradient elution involves either a change in ionic strength or pH of the eluting solution. Chamber I contains starting buffer and is connected to one or more chambers in series containing solutions of different pH or ionic strength.

Figure 5 is a gradient elution chromatographic apparatus used at Missouri; it includes a gradient system similar to that shown in the previous figure and a rather large chromatographic column (12 x 4.5 cm) of DEAE-cellulose. The effluent is passed through a cell and the absorbancy is automatically recorded by a Vanguard (figure 6).

An example of this type of chromatography involves the separation of cathepsins B and C by Landmann (34) who used CM-cellulose and a gradient of phosphate buffer as eluent. Sephadex G-25 was used to separate protein from a beef extract which was further purified by adsorption on DEAE-cellulose in the absence of carbon dioxide. The active proteins were extracted by lowering the pH slightly with carbon dioxide-saturated water. The extracted protein was then chromatographed on a CM-cellulose column using a phosphate
gradient from 0 to 0.05 M. Results are shown in figure 7. The first peak contained cathepsin C and a small amount of cathepsin B. The second peak had an activity which resembled cathepsin B, but this peak did not appear in a muscle preparation treated according to the usual procedures for the purification of cathepsin B. Peak 3 is cathepsin B and corresponds to the peak of highest activity in a regular cathepsin B preparation.

**Molecular Sieve Filtration (Sephadex)**

The three-dimensional network of a gel has a decisive influence on the diffusion of dissolved substances. This fact is utilized to obtain separations where the size of the solute is an important parameter. In a column packed with small swollen gel particles, solutes of a large molecular size are excluded from the gel and emerge from the column without retardation while solutes capable of diffusing into the interior of the particles are retarded. The method is called gel filtration or molecular sieve filtration. Such a gel can be made with Sephadex, small grains of hydrophilic insoluble substance made by cross-linking the polysaccharide dextran. The network has a non-ionic character and the polar properties are almost entirely due to the high content of hydroxyl groups.

This process is excellent for separation of low molecular weight materials from proteins or for the separation of proteins of differing molecular weights. It has had many uses in the study of proteins (35-42) and some use in the meats field. Ann DuFresne (personal communication), AMIF, is using Sephadex G-75 to separate hemoglobin from myoglobin. The myoglobins are further separated in CM-cellulose into three components.

**Column Chromatography of Lipoproteins.**

Column chromatography is not a very popular analytical procedure for separating lipoproteins because so many classical methods are available. These include: differential salt precipitation (43), moving boundary electrophoresis (44), ultracentrifugation (45), zone electrophoresis (46), alcohol fractionation (47), complex formation with dextrin (48), as well as the other analytical technics that will be described by speakers to follow.

One adsorbent used is powdered glass. This material has been used (49) as adsorbent in a column for the separation of lipoproteins of blood serum. A typical chromatogram is shown in figure 8. There are two fractions of the alpha_1-lipoprotein type, i.e., with a cholesterol, phospholipid ratio below 1. The first one is not adsorbed at pH 8.8 and the second is eluted at pH 9.4. Above pH 9.4, lipoproteins of the beta-type with a cholesterol, phospholipid ratio above 1, are eluted.

**Column Chromatography of Lipids.**

The last 10 years have seen great strides in lipid research, largely as a result of the development or adaptation of various forms of chromatography. Many reviews of the application of chromatography to the lipid field have recently appeared (see reference 3, page 451), and the subject is well treated in two text books on the subject (50-51).

A major portion of the developments in the column chromatography of lipids involves adsorption technics, and the elution technics described
for proteins also apply to lipids. Adsorbents as cellulose (52), charcoal (53 and 54), alumina (55) and florisil (56) have been used successfully in the past, but silicic acid has become the most effective and popular adsorbent for the separation of lipids.

An extensive review of the chromatography of lipids on silicic acid and a detailed discussion of the preparation and properties of these adsorbents have been given by Wren (57). By far the most important application of silicic acid adsorption chromatography has been the separation of classes of lipids and, in particular, to the further subfractionation of phospholipids.

Since Trappe's introduction to silicic acid chromatography for lipid separations in 1940 (58), considerable experience has been gained by this procedure. Because of the great potential use of silicic acid chromatography and because of its apparent unreliability on occasions, Hirsch and Ahrens (59) made a systematic study of the many variables and suggested a standardized method of preparing the adsorbent, and packing and conditioning the columns and eluting lipid mixtures. These investigators used both stepwise and gradient elution technics.

Figures 9 and 10 are pictures of the chromatographic equipment used at the Missouri station for lipid fractionations. Hirsch and Ahrens found that the most successful elution of lipids into classes was carried out with a solvent mixture of increasing polarity. The solvent pair used was ethyl ether and pet ether (b.p. 60-70). Samples were applied in amounts up to 300 mg (but containing less than 50 mg of any one component) to a column of 18 g of silicic acid. Reproducible separations of neutral lipid classes from one another and from phospholipids were achieved with fixed amounts of mixtures of petroleum ether and diethyl ether by increasing the proportion of diethyl ether stepwise and by final elution with methanol. The method proved reliable in the chromatography of various synthetic and natural lipid mixtures.

An elution diagram of the lipid components of human plasma is shown in figure 11. Lipid content was determined gravimetrically, cholesterol esters and cholesterol was identified by the Libermann-Burchard reaction, non-esterified fatty acids were determined by reaction to bromcresol green. Phospholipids were determined by phosphorous analysis. The last three peaks contained cephalin, lecithin and sphingomyelin respectively.

After separation, other methods may be used for further separation and study of the lipid classes. Silicic acid has been used in more thorough study of the lipoproteins and phospholipids (60) and paper chromatography seems to be particularly adaptable to the analysis of phospholipid (61). Gas and thin layer chromatography are being used to study many classes of lipids that might first be isolated using silicic acid column chromatography. Infra red spectroscopy is also used to identify and study chromatographic fractions (62).

Hornstein and co-workers (63) used silicic acid columns to separate phospholipid from neutral fat. The columns were developed with successive 300 ml portions of chloroform-methanol 20:1, chloroform-methanol 1:1 and methanol. They were also concerned with the nature and concentration
of free fatty acids present in cured and cooked meats and with their possible effects on flavor (64). They found that naturally occurring free fatty acids in meat could be determined in the presence of large amounts of unsaponified fat by adsorbing the free fatty acids on a strong anion base exchange resin (amberlite IRA-400) and washing the resin free of fat with pet ether and converting the free fatty acids to their methyl esters directly on the resin with anhydrous methanol-HCl. The nature and concentration of the fatty acids were then determined by gas chromatography.

Recovery data was presented for free fatty acid mixtures added to 10 ml. of 5% fat emulsions and was based on the recovery of n-heptadecanoic acid as an internal standard. The average recovery for 7, C-18 fatty acids (lauric, myristic, palmitic, stearic, oleic, linoleic, and linolenic) was 102.2%. Absolute recoveries ranged from 60-95%.

McCarthy and Duthie (65) found that this method could be used quantitatively provided the Amberlite resin IRA-400 was used and recharged four or five times. They found, however, that best results could be obtained by using a column procedure employing silicic acid treated with isopropanol-KOH. After adsorption of the lipid mixture on the column, the neutral lipids were eluted with ethyl ether. The fatty acids could then be removed from the column with 50 ml of 2% formic acid in ethyl ether followed by 75-100 ml of ethyl ether. Phospho-lipids were retained on the column. The results of 26 analyses of fatty acids mixed with various other lipids showed an average recovery of 98.3% for free fatty acids. The range was from 95.5 to 103.8%.

Table III shows the distribution and recovery of C¹⁴ labeled lipids after separation on KOH-treated silicic acid. These investigators also used infrared spectrophotometry to show that the glyceride fraction was free of carboxyl absorption while the free fatty acid fractions were free of esters.

It is extremely informative and nearly obligatory to examine all column fractions during the separation of lipids by some other analytical method in order to determine their purity. The speakers to follow will outline some of these procedures for you.

In conclusion, I would like to say that the use of column chromatography is in its infancy in regard to its use as a tool in separating tissue components of meat. I hope that this paper and its bibliography will act as a crutch to speed the use of this important analytical technic to maturity in studies of the many ramifications of our ever growing field.
BIBLIOGRAPHY


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34. Landmann, W. A. Campbell soup symposium on meat tenderness. (1962).


figure 1. The chromatographic column. The tube filled with adsorbent is called the chromatographic column. The washing liquid is called the solvent or developer. The series of zones (solutes) is called the chromatogram.
Table I
Cellulose Ion Exchangers

<table>
<thead>
<tr>
<th>Anion Exchangers</th>
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</thead>
<tbody>
<tr>
<td>DEAE-Cellulose</td>
<td>(strongly basic tertiary amine,</td>
</tr>
<tr>
<td></td>
<td>(-\text{O-C}_2\text{H}_4\text{-NH}_3\text{-H(C}_2\text{H}_5\text{)}\text{Cl}^+)</td>
</tr>
<tr>
<td>TEAE-Cellulose</td>
<td>(strongly basic guaternary amine,</td>
</tr>
<tr>
<td></td>
<td>(-\text{O-}(\text{C}_2\text{H}_4\text{-N}^+(\text{C}_2\text{H}_5\text{)}\text{)}\text{Cl}^-)</td>
</tr>
<tr>
<td>AE-Cellulose</td>
<td>(slightly basic amine,</td>
</tr>
<tr>
<td></td>
<td>(-\text{O-C}_2\text{H}_4\text{.NH}_2)</td>
</tr>
<tr>
<td>GE-Cellulose</td>
<td>(strongly basic,</td>
</tr>
<tr>
<td></td>
<td>(-\text{O-C}_2\text{H}_4\text{.NH}_2\text{.NH}_2\text{.Cl}^+)</td>
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<tr>
<td>ECTEOLA-Cellulose</td>
<td>(mixed amines, (\text{Cl}^-))</td>
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</table>

<table>
<thead>
<tr>
<th>Cation Exchangers</th>
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</thead>
<tbody>
<tr>
<td>CM-Cellulose</td>
<td>(high capacity for basic and neutral</td>
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<tr>
<td></td>
<td>proteins, (-\text{O-CH}_2\text{-COOH})</td>
</tr>
<tr>
<td>P-Cellulose</td>
<td>(strongly acidic, (-\text{O-PO}_3\text{H}_2))</td>
</tr>
<tr>
<td>SE-Cellulose</td>
<td>(strongly acidic,</td>
</tr>
<tr>
<td></td>
<td>(-\text{O-C}_2\text{H}_4\text{SO}_3^-\text{H}^+)</td>
</tr>
<tr>
<td>PAB-Cellulose</td>
<td>(diazotization + coupling,</td>
</tr>
<tr>
<td></td>
<td>(-\text{O-CH}_2\text{-C}_6\text{H}_4\text{-NH}_2)</td>
</tr>
</tbody>
</table>


Table II
Chemical and enzymic properties of freeze-dried, fractionated sarcoplasmic protein from beef Longissimus dorsi from Fujimaki and Deatherage (28)

<table>
<thead>
<tr>
<th>pH of elution buffer</th>
<th>Ppt. Solub. with in TCA&lt;sup&gt;1&lt;/sup&gt; water</th>
<th>O.D.(3mg/5 ml 280mms 260mms Aldolase LDH PFK Myok. ATPase Cathep.</th>
<th>Enzymic activity</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>† +</td>
<td>0.200 0.340</td>
<td>- - - -</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>+ +</td>
<td>0.321 0.265</td>
<td>- + - -</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>+ +</td>
<td>0.268 0.202</td>
<td>++ + - -</td>
<td>-</td>
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<td>6</td>
<td>+ +</td>
<td>0.398 0.398</td>
<td>+ ++ - -</td>
<td>-</td>
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<tr>
<td>7</td>
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<td>- - - +</td>
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<td>-</td>
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<td>+ +</td>
<td>0.459 0.293</td>
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<td>+ +</td>
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<td>-</td>
</tr>
<tr>
<td>8</td>
<td>+ †</td>
<td>0.370 0.321</td>
<td>++ ++ - -</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>+ †</td>
<td>0.306 0.236</td>
<td>++ + + + † t;</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>+ †</td>
<td>- - - -</td>
<td>- - - -</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>1</sup>TCA = Trichloracetic acid
figure 2. Equipment used in study of pork cathepsins. Twenty-five grams, DEAE-Cellulose in a column (2.5 x 25 cm).

figure 3. Elution diagram of ammonium sulfate (50-60%) soluble pork sarcoplasmic proteins. Elution with CO₂-free water and CO₂-saturated water. Largest peak contained greatest amount of cathepsin activity. From Parrish (31).
figure 4. Hydrodynamic apparatus for gradient elution column chromatography. Chamber I contains starting buffer and is connected to one or more chambers in series containing solutions of different pH or ionic strength. From Yaguchi et al. (33).

figure 5. Gradient elution chromatographic apparatus used to separate proteins. DEAE-Cellulose column (4.5 x 12 cm).
figure 6. Vanguard automatic ultraviolet analyzer.

figure 7. CM-Cellulose separation of beef cathepsins previously purified by DEAE-Cellulose adsorption and elution with CO$_2$-saturated water. Phosphate gradient between 0 and 0.05 M. Peak at 100 ml contained cathepsin C and small amount of cathepsin B. Peak at 300 ml had activity resembling cathepsin B. Peak at 1100 ml had cathepsin C activity. From Landmann (34).
figure 8. Chromatographic separation of serum lipoproteins on powdered glass. First two peaks contain alpha-lipoprotein (cholesterol/phospholipid < 1). Above pH 9.4 beta-lipoproteins (cholesterol/phospholipid > 1) are eluted.
From Carlson (49).

figure 9. Elution apparatus and silicic acid column for lipid analysis.
figure 10. Column chromatographic apparatus for lipid analysis.

figure 11. Stepwise elution diagram of blood plasma lipids on 18 gm silicic acid column. The abscissa shows the tube number collected fractionally and the various solvents used to effect elution. From Hirsch and Ahrens (59).
### Table III

Distribution and Recovery of C\(^{14}\)-Labeled Lipids After Separation on KOH-Treated Silicic Acid

McCarthy and Duthie (65)

<table>
<thead>
<tr>
<th>Labeled Substance Added*</th>
<th>Activity Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control activity</td>
</tr>
<tr>
<td>Tripalmitin-1-C(^{14})</td>
<td>171,400 cpm</td>
</tr>
<tr>
<td>Cholesterol-4-C(^{14})</td>
<td>369,280 cpm</td>
</tr>
<tr>
<td>Cholesteryl palmitate-1-C(^{14})</td>
<td>657,360 cpm</td>
</tr>
<tr>
<td>Palmitic acid-1-C(^{14})</td>
<td>185,600 cpm</td>
</tr>
<tr>
<td>Linoleic acid-1-C(^{14})</td>
<td>100,490 cpm</td>
</tr>
<tr>
<td>Butyric acid-1-C(^{14})</td>
<td>233,250 cpm</td>
</tr>
</tbody>
</table>

*Mixed with glycerides and FFA prior to separation

\(^{1}\)An aliquot equivalent to that separated

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MR. PEARSON: Thank you, Dr. Bailey, for a very informative talk.

Following right along in the line of Column Chromatography there is another technique that is being extensively used in research, particularly in the field of lipids and to some extent on other derivatives affecting proteins, that is Gas Chromatography. This morning we have Dr. H. B. Craig of North Carolina State College who is going to discuss this topic for us.