Some Applications of the Separation of Large Molecules and Colloidal Particles.

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To the chemist the word separation is closely associated with preparative and analytical work but there is no doubt that all of us realize that separation methods form the key to some of the greatest problems in chemistry. It is significant that the Dutch word Scheikunde—the art of separation—still means chemistry as a whole, and we have many recent examples which demonstrate how the development of new and highly specific methods of separation have opened new fields to the structural chemist. I have chosen as the subject for this lecture a field in which such methods have made great contributions to our knowledge and where, as I see it, the future holds great promise. I shall not at all limit myself to work done in my own laboratory; I shall rather try to emphasize some general aspects of the development in this highly fruitful field, particularly in biochemistry, and I shall briefly touch on some possible unexplored applications.

When discussing the methods of colloid separation it is to be remembered that some of the most valuable and interesting applications of such methods have been made with substances which barely or not at all deserve to be called colloids. Such has been the case, for example, in the extension of electrophoresis from proteins to peptides and amino-acids. Despite the title of my lecture it is very tempting to include some of this work here.

A suitable classification of colloid separation methods would be to distinguish between procedures where the material remains in one phase during the separation, and operations depending upon the transfer from one phase to another. In the first case (*e.g.*, ultra-centrifugation, electrophoresis, diffusion, thermodiffusion, ultra-filtration) differences in rates of migration through a medium are utilized; in the second, other effects come into play (salting out, partition). We have, with this classification, to let the " surface " separation methods (adsorption, flotation, adsorption chromatography) form an intermediate group, characterized by the separation occurring at the interface between phases. This classification has significance also from another point of view, especially when dealing with biocolloids, which often suffer irreversible changes if they have to be transferred from one phase to another.

Now it is not at all my intention to describe these methods one after another, but rather to point towards some recent developments and their applications.

It is interesting to note how the chemical nature of colloidal particles often played a subordinate role in the early development of colloid chemistry. The classical investigations on Brownian movement and sedimentation were made on systems with particles sometimes of gold, sometimes of mastic, or other even less-defined material. In such cases this was, of course, justified as interest lay exclusively in certain physical properties which depend only on particle size, but there are many other cases, particularly in the application of colloid chemistry to biological phenomena, where too exclusively physical considerations have led to schematic and over-simplified theories. If, thus, chemical specificity was neglected by the early colloid chemistry it is today predominant, and particularly so with biocolloids. This development is also reflected in the frequent use of the term " macromolecules " instead of " particles " in investigations dealing with an important group of substances in this field.

On the other hand, in colloid chemistry the methods of separation are mostly based on physical phenomena, and this to such an extent that they are also utilized for determination of important physical characteristics of colloid substances such as particle size, shape, and surface electric potential. Also it should be remembered that physical methods, being usually more gentle than those based on chemical reactions, have great advantages when we have to deal with substances which easily undergo irreversible changes (*e.g.*, many biocolloids).

The applications of the methods to which I shall refer thus have a dual purpose: the separation, and the measurement of a physical property on which the separation depends. Thus, in ultra-centrifugation and electrophoresis the rate of sedimentation or the electrophoretic mobility can be measured in the same apparatus, and often even in the same experiment, in which the separation of a complex system into its components is studied. There is, however,

a natural tendency towards differentiation in this respect and the construction of separation apparatus is now often modified according to whether quantitative characterization or merely separation is the chief object. It should be noted, however, that with colloids and macromolecules the quantitative characterization possible by such methods is often among the best means of identification. Thus, even if preparative separation is the chief purpose it may be desirable not to loose entirely the possibility of quantitative characterization (by rates of sedimentation or migration) just as a fractional distillation and crystallization should, if possible, be carried out so that the boiling points and solubilities of the components are under continuous control.

One of the most important developments in colloid separation during recent years is the construction of high-speed ultra-centrifuges of large capacity, for purposes of separation. There are commercially available machines with rotors taking up to 1100 ml. at 20,000 r.p.m. (42,000 g) or 162 ml. at 40,000 r.p.m. (105,000 g) which have found extensive use in biochemical and virus laboratories, and thus have facilitated, among other things, also investigation of the nature of submicroscopic particular matter other than virus, occurring in biological material. The isolation of materials such as mitochondria, microsomes, and similar particles of electron-microscopic dimensions in quantity has permitted investigation of their chemical nature and particularly their enzymic function. This is an extremely interesting field and I shall return to these problems later.

The extension of methods of separating colloids to crystalloid substances which are in the molecular-weight region of, let us say, 1000—10,000 appears to be particularly important and fruitful, especially as there is a lack of methods for dealing with substances of this range. Thus it is of great value that molecular-weight determinations can now be made even in this range by ultra-centrifugal methods, thereby avoiding some of the difficulties due to impurities which disturb such determinations by other procedures (freezing point, vapour pressure, osmotic pressure). This development is largely due to the method of interpreting sedimentation diagrams developed by Archibald, and now used as routine in several laboratories. It makes it possible to evaluate sedimentation diagrams in which the boundary does not leave the meniscus in the ultra-centrifuge cell completely, owing to sedimentation being too slow and diffusion too fast.[1]

In this way Pedersen and his colleagues [2] in Uppsala obtained good values for the molecular weight even of sucrose (M, 342), and the procedure has been very useful in determining molecular weights of polypeptides. As an example I may mention that Porath in my laboratory determined the molecular weight of the antibiotically active polypeptide bacitracin as 1460, in good agreement with the minimum (1496) calculated from the sulphur content and also with the value found by Craig who used a method based on the influence of substitution on partition coefficients.

The same problem has recently been attacked in a somewhat different way by Kegeles [3] and by Pickels, Harrington, and Schachman [4] by arranging a boundary starting in the middle of the cell (a so-called synthetic boundary) instead of letting it be formed spontaneously at the top as usual. In this way even a very slowly moving boundary will migrate sufficiently before it is too much blurred by diffusion, to make it possible to determine directly the rate of sedimentation. Thus these authors have studied the sedimentation of, *e.g.*, dextrins, salmine, clupein, and vitamin B_{12}. No doubt recent developments of diffusion methods employing interferometric observation will also be useful in this field.

Returning now to the colloids and macromolecules proper, it is to be noted that ultra-centrifugation—whether it is made for particle-size or molecular-weight determinations or for separations—is almost always performed as a " boundary " separation. This means that the separation is never complete : the centrifuge cell contains a number of partially overlapping layers of the different components. This corresponds to what is also the usual procedure in electrophoresis and in the so-called frontal analysis in chromatography. If the rates of sedimentation are sufficiently different such experiments may be utilized for complete separation of substances (the " up-and-down centrifugation " used in virus purification), but with smaller

[1] W. J. Archibald, *J. Phys. Colloid Chem.*, 1947, **51**, 1204; see also D. F. Waugh and D. A. Yphantis, *J. Phys. Chem.*, 1953, **57**, 312.

[2] C. H. Li, A. Tiselius, K. O. Pedersen, L. Hagdahl, and H. Carstensen, *J. Biol. Chem.*, 1951, **190**, 326.

[3] G. Kegeles, *J. Amer. Chem. Soc.*, 1952, **74**, 5532.

[4] E. G. Pickels, W. F. Harrington, and H. K. Schachman, *Proc. Nat. Acad. Sci.*, 1952, **38**, 943; see also H. K. Schachman and W. F. Harrington (*J. Polymer Sci.*, 1954, **12**, 379).

differences this results in incomplete separation unless the experiment is repeated many times. It would be highly desirable to be able to perform " zone " separations in the ultra-centrifuge, that is, to obtain the fractions completely separated in layers, in analogy with chromatography or zone electrophoresis. Obviously the technical obstacle to be overcome is the stabilization of the zones and this is no doubt a difficult problem, especially if one wants to be able to remove the separated fractions from the cell. Use of density gradients to stabilize such " zone sedimentation " has been attempted, for example, by Brakke [5] who has separated virus preparations into zones in an ultra-centrifuge with a gradient of sucrose carrying the zones. This seems to me to be a particularly promising development. Similar experiments on cell fragments have recently been described by Holter.[6]

There are other possible methods of separating colloidal substances according to their particle size, even though they may be based upon less fundamental physical properties. This should not be a serious limitation, however, if the separation is more important than the quantitative characterisation of the separated fractions, which after all may be accomplished subsequently by other suitable methods. Thus ultra-filtration is one of the oldest methods of colloid chemistry, and, particularly since through the pioneer work of Elford we learned how to prepare membranes of homogeneous and well-defined particle size, it has proved its usefulness in many applications, particularly in the field of viruses.

A filtration process has of course an " all-or-none " character, that is, a given material will either be held back or pass through. It would appear possible in principle to develop a somewhat

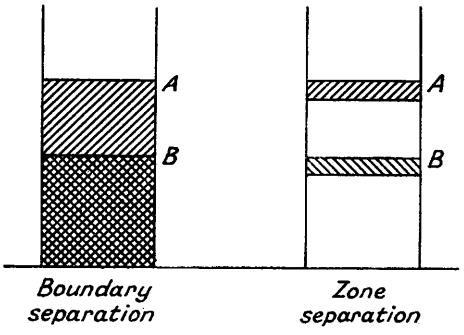


FIG. 1. *The difference between " boundary " and zone separations in electrophoresis, chromatography, sedimentation, and ultra-filtration.*

generalized ultra-filtration procedure in which one would utilize differences in molecular frictional coefficients when a mixture of different substances is forced through a medium offering resistance to their migration. This would, of course, in many cases resemble chromatography, although the retardation of the substances relative to the solvent would depend on friction rather than on adsorption or partition. This possibility was discussed some years ago between Martin, Synge, and myself and Synge and I in 1950 [7] reported some experiments which showed that such effects can be utilized for separation according to molecular size, but it is difficult to obtain pure filtration effects as adsorption also interferes. Another difficulty is to prevent the gel from becoming violently deformed or from collapsing, as rather high pressures have to be used to force the liquid through the thick ultra-filter. It appears that electro-osmotic transport of the medium would be of the best way of avoiding this difficulty, as then the deforming effect of the medium is counterbalanced by the effect of the electric field. As a matter of fact separations which appear to depend in part on filtration have been observed in zone electrophoresis in gels. Mould and Synge [8] have been able to work out a procedure which they call electrokinetic ultra-filtration; this seems however to depend to a considerable extent on adsorption separation according to molecular size. They have applied it with success to the zone separation of polysaccharides related to starch, and of other neutral substances.

I shall turn now to *electrophoretic methods of separation.* which in recent years have had many new applications, particularly in biochemistry. The methods referred to above all depend on particle or molecular *size*, and as electrophoretic migration properties ultimately depend on charge or surface potential it is perhaps not superfluous to point out how necessary

[5] M. G. Brakke, *Arch. Biochem.*, 1953, **45**, 275.
[6] H. Holter, *Experientia*, 1953, **9**, 346, where reference are given to earlier work on sedimentation in gradients.
[7] R. L. M. Synge and A. Tiselius, *Biochem. J.*, 1950, **46**, xli.
[8] D. L. Mould and R. L. M. Synge, unpublished work.

it is in applying separation methods for studying homogeneity to try several different methods in parallel. There are many examples in protein chemistry of substances having almost identical molecular weights but differing widely in electrophoretic properties, and *vice versa*. It is particularly to be remembered that a change in particle size which does not markedly affect the chemical properties has little if any effect on electrophoresis. The most valuable applications of electrophoresis are in biochemistry, particularly among proteins, enzymes, viruses, and similar polar substances of high molecular weight. In recent years, however, the method has found extensive use also for polypeptides, and even simple peptides and amino-acids, and now also for saccharides, and for nucleotides and their constituents. Thus, here again we find that methods which were originally developed for application to colloids and large molecules have gradually and by suitable modifications found important use also with substances of intermediate and low molecular weight. I must admit that I have often been surprised at the very pronounced specificity in electrophoretic properties among biocolloids, which sometimes reflect even species or strain differences (for example, with certain viruses), and recent experiences with peptides in my own and other laboratories also indicate a rather striking specificity in this respect. It is also interesting how frequently mixtures which cannot be resolved by other methods lend themselves to electrophoretic separation. This may often be explained by the fact that components with pronounced electrochemical differences tend to be co-precipitated (especially if they are of opposite charge) in many of the usual fractionation methods, whereas in the electrophoretic separation they remain in solution and thus are allowed to display their differing individual characters.

In the ordinary free " boundary " electrophoresis, taking place in a U-tube with optical observation of the movement of the boundaries, little new in principle has been added to the type of apparatus now in common use in biochemical laboratories. The introduction of interference methods, instead of observation by the curvature of light passing the refractive-index gradients at the boundaries, is of considerable interest and will probably allow investigation of more dilute solutions. Svensson [9] has worked out an arrangement by which a record is obtained of the concentration (interferometry) and the gradient (refraction) on the same plate. As many disturbing factors influence electrophoretic separations at high concentration, it is essential to work at the lowest possible concentrations if clear-cut results are to be obtained.

Perhaps the most important new development in electrophoresis during the last few years is the introduction of zone methods, especially from the point of view of separation. I have already referred to some obvious advantages of zone methods over boundary methods when discussing ultra-centrifugation, and in the case of electrophoresis some further arguments in favour of zone methods may be added. Low concentrations are necessary to eliminate some of the " boundary anomalies " which often interfere seriously in boundary electrophoresis separations. This appears to be achieved more easily in zone electrophoresis, probably because the front and the rear of a zone can be brought much closer together in zone experiments than in the usual U-tube apparatus for boundary electrophoresis. Thus the changes in ionic composition at the boundaries (which always have opposite signs at the front and at the rear) have a chance to neutralize each other by diffusion. This is probably the main reason why in zone electrophoresis one has been able to study a number of substances of low molecular weight (*e.g.*, amino-acids and peptides) which owing to the boundary anomalies it has been difficult or impossible to study by the usual forms of boundary methods.[10]

The fundamental problem in zone electrophoresis—which otherwise requires only a very simple apparatus as no optical observation needs to be made—is to find a suitable supporting medium, without which the zones would be upset by gravitational convections. The most popular medium has been filter paper, no doubt under the stimulating influence of filter-paper chromatography. Paper offers the advantage of easy localization of zones by suitable staining. It is also quite striking how much one can achieve with very simple equipment by zone electrophoresis in filter paper. It has found extensive use in the study of serum and plasma for clinical purposes, and also as a valuable supplement to chromatography in the separation of amino-acids and peptides and for the separation of many other important substances of interest to the biochemist, for example, proteins, enzymes, nucleotides, and other fission products of nucleic acids. Filter paper, however, was not originally made for filter-paper chromatography or electrophoresis and it has some disadvantages which are more evident with proteins than with, lor example, peptides and depend on its marked adsorption of many substances, particularly

[9] H. Svensson, *Acta Chem. Scand.*, 1949, **3**, 1170; 1951, **5**, 1301.

[10] For a review of zone electrophoresis, see, *e.g.*, P. Flodin and A. Tiselius, *Adv. Protein Chem.*, 1953, **8**, 1.

those of basic character. Attempts are now being made in the author's laboratory, and elsewhere I believe, to modify chemically the surface properties of the cellulose in the paper to diminish its adsorption, *e.g.*, by esterification or other suitable pre-treatment. I shall not go into detail about the various forms of zone-electrophoresis apparatus but will refer only to an arrangement which has been worked out in Uppsala by Haglund and myself [11] and considerably improved by Flodin and Porath.[12] The electrophoretic separation takes place in a column packed with a suitable filling medium (we use starch or cellulose powder) which forms one limb of a U-tube in an electrophoresis apparatus similar to the common type. After the zones have been electrophoretically separated on the column, this is removed and placed in a fraction collector of the type commonly used in chromatography, so that the zones can be washed out one after the other. The same column can thus be used repeatedly. The apparatus has considerable capacity and has found extensive use in my laboratory also for preparative separation of proteins and other substances.

The methods I have discussed so far have originally been developed for colloidal substances but their use has been, or is being, extended to substances of lower molecular weight. I shall now also mention chromatography where the development has been rather the opposite—from small to larger molecules. It would lead me altogether too far to try to discuss this method here; it must suffice to emphasize how much of the recent development in this field we owe to British scientists. Also I would like to stress that, even though chromatography has its most successful applications to small or medium-sized molecules, it is of the utmost importance in the study of large complicated molecules because of the scope afforded for analysis of complicated mixtures of breakdown products. The separation of colloidal substances by chromatography is a very intriguing problem which so far has met with only partial success. Adsorption chromatography must be based upon an easily reversible adsorption–desorption equilibrium—if not, the process is impracticably slow. Here most proteins fail and in many cases they even become denatured on the surface. With some particularly stable proteins of rather low molecular weight (cytochrome, lysozyme, ribonuclease) ionic-exchange columns have been used successfully by Paleus and Neilands,[13] Hirs, Moore, and Stein,[14] and Tallan and Stein; [15] also hæmoglobin has been chromatographed on such columns.[16] Partition chromatography of proteins has been performed by Herbert and Pinsent,[17] by Martin and Porter,[18] and by Porter.[19] Porter *et al.* have studied extensively the physicochemical background of such separations. The difficulty here is, of course, to find a suitable solvent pair, and we know since the work of Brönsted that it is characteristic of colloids and substances of large molecular weight to collect almost completely in one of the two phases. Brönsted studied the distribution of colloids between two phases a few degrees below their critical point of mixing, where even in such cases the phases are sufficiently similar to give a clear partition. In the author's laboratory it has been found that some proteins lend themselves to chromatography by adsorption promoted by salts added in high concentrations to the solution—this "salting out" adsorption is, however, often not quite reversible. With amino-acids, many peptides, and dyes, salting-out chromatography often works quite nicely. It has also yielded interesting results with very large particles (*e.g.*, Rous sarcoma virus; see Riley [20]). This can perhaps be explained by the greater stability towards denaturation which often characterizes such material, compared with normal proteins.

Adsorbents such as aluminium hydroxide gel, calcium phosphate, and talcum, are often used in preparation of enzymes and other proteins in "batch" operations. They can also be packed into columns when mixed with a suitable filter aid (*e.g.*, Celite). It appears difficult, however, to elute proteins from such columns with sufficient differentiation, and separations are possible only with substances which differ greatly in adsorption affinity. In order to elute a protein zone from such adsorbents it appears necessary to apply an excessively strong eluting agent, *e.g.*, a higher pH or phosphate concentration than would correspond to equilibrium. This is to be expected if the establishment of equilibrium is too slow. The excessive elution

[11] H. Haglund and A. Tiselius, *Acta Chem. Scand.*, 1950, **4**, 957.
[12] P. Flodin and J. Porath, *Biochim. Biophys. Acta*, 1954, **13**, 175.
[13] S. Paleus and J. B. Neilands, *Acta Chem. Scand.*, 1950, **4**, 1204.
[14] C. H. W. Hirs, S. Moore, and W. H. Stein, *J. Biol. Chem.*, 1953, **200**, 493.
[15] H. H. Tallan and W. H. Stein, *ibid.*, 1953, **200**, 507.
[16] N. K. Boardman and S. M. Partridge, *Nature*, 1953, **171**, 208.
[17] D. Herbert and J. Pinsent, *Biochem. J.*, 1948, **43**, 193.
[18] A. J. P. Martin and R. Porter, *ibid.*, 1951, **49**, 215.
[19] R. Porter, *ibid.*, 1953, **53**, 320.
[20] V. T. Riley, *Science*, 1948, **107**, 573; 1949, **109**, 361; *Proc. Soc. Exp. Biol. Med.*, 1950, **73**, 92; *J. Nat. Cancer Inst.*, 1950, **11**, 199, 215.

tends to wipe out the differences in rates of migration of the zones on the column and usually results in bad separations.

In some recent experiments on protein chromatography on calcium phosphate–Celite columns (not yet published) the author has found that strongly adsorbed proteins show a pronounced mutual displacement which gives fairly good separation even at the top of the column, before development has been attempted. Each zone will tail off somewhat into the zone on top of it, but if only a small volume of the solvent is added a typical displacement " procession " of pure zones will result. This is due to the fact that the tailing material in each case is surrounded by material which on account of its higher adsorption affinity acts as a displacer and therefore gives a much higher R_F to the tailing portion than the R_F characteristic of the main zone itself. The tailing material will therefore easily catch up with the zone to which it belongs. Development should not be carried beyond this point, but the column is then cut into segments corresponding to the zones and each segment is eluted with a strong eluting agent. The process can easily be demonstrated with coloured proteins, *e.g.*, with a mixture of serum albumin and carbon monoxide hæmoglobin.

So far, no general method of protein chromatography has been worked out which in any way compares in usefulness with the manifold applications of this highly specific method to substances of lower molecular weight. There is no doubt, however, that the importance of the problem will stimulate many more attempts in this direction. The specificity of adsorption may nevertheless be utilized in other ways for separation purposes, and the use of adsorbents in preparative work, particularly in biochemistry, offers many examples but is of course largely based on empirical principles. Foam analysis and methods analogous to flotation in the fractionation of ground ores and minerals seem to offer interesting possibilities, but with biocolloids again the risk of surface denaturation is appreciable.

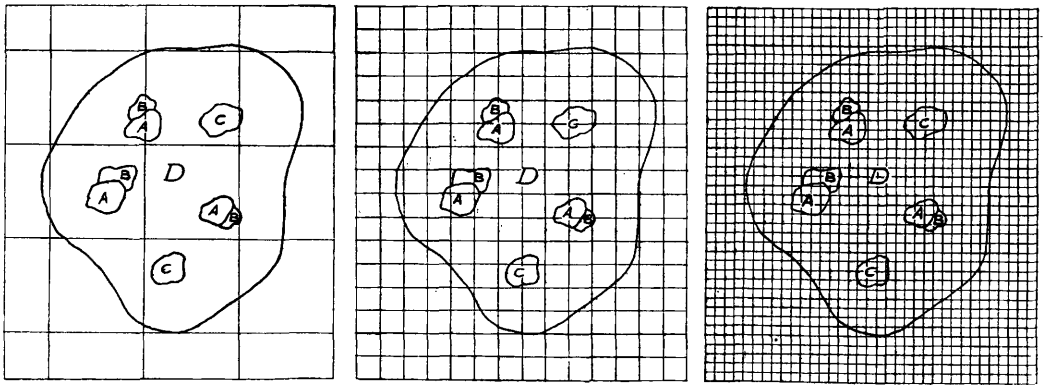
I would like to spend the last part of this lecture in a discussion of some aspects of separation methods which appear to me to be particularly significant in the field of colloids and substances of considerable particle size or molecular weight. The aim of separation is usually first preparative, and even if preparative work is sometimes regarded as inferior to other lines of chemical research we all know that in many fields of chemistry, and perhaps particularly in biochemistry, it may be the crucial problem which requires great skill and highly developed techniques. The goal would thus be to isolate a pure substance from a crude raw material, which, if it arises from biological sources, often consists of an extremely complicated mixture. It is a common experience in such work, especially towards the end of the preparation, that certain substances cling together, either because they are too similar or for other reasons, of which mutual association by formation of molecular compounds or adsorption complexes appears to be common particularly with material of high molecular weight. Such tenaciously held impurities are a great source of annoyance on the road to a pure substance, but one may look at this from a somewhat different point of view.[21] Living matter is highly organized and this organization is a condition for the proper interplay of all those chemical processes which determine life. From a chemical point of view, this organization means that certain substances are localized together, sometimes even linked together, in one way or another in the cell. It is difficult to conceive a mechanism for the most important biochemical reactions in living matter, *e.g.*, the coupling of essential enzymic reactions, without assuming some direct or indirect specific association. Many of the substances which we extract from biological material and which we use, for example, as pharmaceuticals with specific action on the human organism would seem to serve an important function also in the organism from which they are derived, but of this we often know very little. It would be a great help if we knew at least the immediate surrounding of such substances in the living cell. This is, of course, in the first place a problem for the cytochemist and much has been achieved in studies of the distribution of substances in the cell by microscopy, electron-microscopy, and microdissection in combination with ultramicrochemical methods applied to individual cells. However, it is clearly desirable to be able to do such work on a larger scale, obtaining structural information by averaging a large number of identical or nearly identical cells and at the same time isolating the structural fragments in quantities necessary for a detailed study of their chemistry both from a structural and a functional point of view. Naturally such procedures would often have value only as a supplement to the methods based on direct microscopic observation, but to my mind there is no doubt that methods which would allow us to undertake detailed analysis of structural fragments derived from cells or tissue would be of immense importance for the progress of biochemistry. The isolation of particulate elements from cells such as cell nuclei, mitochondria, microsomes,

[21] A. Tiselius, *Chem. Eng. News*, 1949, **27**, 1041.

etc., is of course a step in this direction and has already led to very interesting results. Here we deal with preformed fragments which are comparatively easy to isolate by the methods now at our disposal.

The point I wanted to make here is that the systematic study of particles down to colloidal dimensions, obtained by *successive* disintegration of a large number of cells of other particles, all of a similar structure, is in principle capable of giving us a fairly detailed picture of the original structure at the same time as it involves isolation of sizable quantities of the structural elements in general. To illustrate this I shall give a very simple example, which is entirely schematic. Suppose we have a large number of identical cells consisting of a medium *D* in which we have form elements of substances *A*, *B*, and *C*, which are different and distinguishable (Fig. 2). Let us suppose that all these cells are disintegrated successively and that the resulting particles can be fractionated into classes each containing particles of approximately equal size by some suitable method. By comparing the three Figures you will see that only with the finest disintegration the majority of the particles will be pure *A*, *B*, *C*, or *D*, whereas in the first and second case most of the particles will be of mixed composition (in the second case *A* and *B* will occur together in many particles but never together with *C*, whereas in the first case *A*, *B*, and *C* may occur together and, of course, with *D*). Roughly speaking the particle size, at which two substances segregate, *i.e.*, cease to occur together in the same particle, gives a measure of the distance between these substances in the original structure. If we are able to fractionate

FIG. 2. *Structural analysis by successive disintegration (schematic).*

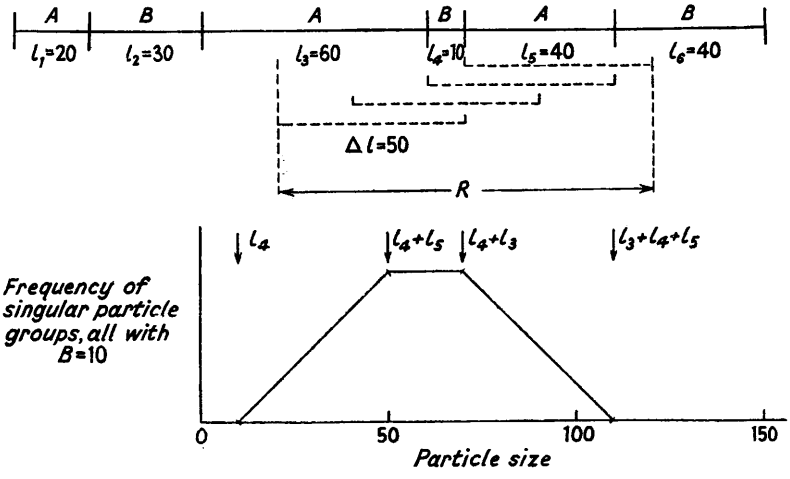


the particles in each size class according to their chemical composition (*e.g.*, by their density) we can deduce not only a rough sketch of the structure derived from the observations just indicated, but, in fact, a complete average picture of the original structure. A simple statistical treatment based upon a knowledge of the frequency distribution of the chemical composition with reference to two substances (say *A* and *B*) in each of a number of size classes of successively smaller size would generally be capable of providing us with almost complete information as to how *A* and *B* were distributed in the original structure, measured from some easily defined point of origin. It would lead too far to discuss this " puzzle analysis " in detail here. Some simple linear models may serve to illustrate the essential points. A line consisting of segments of substances *A*, *B*, *C*, *D*, *E* . . . of different lengths, none of them occurring more than once, is the simplest case. By sufficiently fine grinding we should obtain chiefly homogeneous particles consisting of pure *A*, *B*, *C*, *D*, *E*, etc., the relative amounts giving the relative lengths of the segments. Somewhat courser grinding will give us particles containing *A* and *B*, *B* and *C*, etc., from which the sequence is deduced. A linear model of the type *ABABABABA* requires a more detailed investigation, as shown in the example given in Fig. 3. A random disintegration of such a model does not give a random distribution of particle composition, but also here singular frequencies of constant composition occur in each particle-size class. Again the smallest particle sizes will contain only *A* or only *B*, but also in the larger sizes appreciable quantities of particles have constant composition with respect to *A* and *B*. This is seen in Fig. 3 if we let the linear element $\Delta 1$, representing a particle size of 50, move along the model to a number of different positions, as indicated by broken lines. All of these will give the same percentage of *A* and *B* in the particle. Any segment *B* which on each side has *A*, and any segment *A* which on each side has *B* will give rise to such singularities in the frequency

of composition. If we plot all singular frequencies containing $B = 10$ as a function of particle size we obtain the curve shown in the Figure. The position of this curve on the abscissa is entirely determined by the size of the two immediate neighbours of l_4 and thus these can be read from the curve, as indicated. By a similar treatment of the other singular frequencies the entire structure of the original model is derived. Thus if it were possible to fractionate the disintegrated material into particle-size classes covering a suitable range, and to sub-fractionate some of these further, *e.g.*, according to density (if A and B are different in this respect), the singular frequencies should stand out as large groups of particles having the same density. We only need to know the composition of a limited number of such singularities to deduce the whole structure, as each of them will contain one complete segment of either A or B, and parts of the neighbouring segments, which is all we need to complete the puzzle.

The singular frequencies are also highly significant in the analysis of two- or three-dimensional models and seem to offer the simplest approach to a solution of the problem, based upon data

FIG. 3. *Puzzle analysis of a linear model.*



which it may be possible to obtain in actual experiments. I have hesitated somewhat to enter upon such speculations in this lecture as we have far too little experimental evidence as yet to show that such an analysis is practicable. Nevertheless it seems to me extremely interesting that, in principle at least, the systematic fractionation of colloids according to particle size and chemical or physical properties of the material of which the particles consist may form the basis for a detailed knowledge of structures of a type too irregular to lend themselves to analysis by X-rays or similar methods. At the same time the colloid methods can provide us with structural elements in sizable quantities for further study of their chemical properties. To a certain extent such a " puzzle " procedure is analogous to methods used in the elucidation of the structure of proteins, as, for example, when Sanger from an identification of the products of partial hydrolysis was able to deduce the sequence of amino-acids in the polypeptide chains of insulin.

These speculations had their origin in some difficulties in biochemical preparations, of which I shall mention only the purification of protein hormones like ACTH. Here we encounter some of the most difficult separations so far experienced in protein chemistry and it seems that to a certain extent this may be due to formation of complexes by several different hormones with each other. If this occurs also in the original material, it may be of significance from a biochemical and physiological point of view.

I do not know if it is quite polite to the Society to conclude this lecture with a plea for an increased interest among chemists in the *impure* substances, but those of you who agree with my argument will admit, I hope, that many obstacles may be turned to advantage if this is kept in mind in work on the separation of colloids of biological origin.