COMPREHENSIVE BIOCHEMISTRY

EDITED BY

MARCEL FLORKIN

AND

ELMER H. STOTZ

VOLUME 4

SEPARATION METHODS

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MARCEL FLORKIN

Professor of Biochemistry, University of Liège (Belgium)

AND

ELMER H. STOTZ

Professor of Biochemistry, University of Rochester, School of Medicine and Dentistry, Rochester, N.Y. (U.S.A.)

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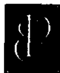

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CONTRIBUTORS TO THIS VOLUME

PROFESSOR P. CHOVIN, D. Sc.

Assistant Director of the Town Laboratory for Explosives, Radioactivity, Electricity, Chemistry, Public and Industrial Health, Scientific Research, 39 bis, Rue de Dantzig, Paris 15 (France)

L. C. CRAIG, B. S., PH. D.

The Rockefeller Institute, New York 21, N.Y. (U.S.A.)

EDGAR LEDERER, PH. D., D. Sc.

Professor of Biochemistry, Laboratoire de Chimie Biologique, Faculté des Sciences, Paris and Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, Seine et Oise (France)

MICHAEL LEDERER, PH. D.

Laboratorio di Chromatografia dell C. N. R., Istituto di Chimica Generale ed Inorganica, Piazzale delle Scienze 5, Roma (Italia)

GENERAL PREFACE

The Editors are keenly aware that the literature of Biochemistry is already very large, in fact so widespread that it is increasingly difficult to assemble the most pertinent material in a given area. Beyond the ordinary textbook the subject matter of the rapidly expanding knowledge of biochemistry is spread among innumerable journals, monographs, and series of reviews. The Editors believe that there is a real place for an advanced treatise in biochemistry which assembles the principal areas of the subject in a single set of books.

It would be ideal if an individual or small group of biochemists could produce such an advanced treatise, and within the time to keep reasonably abreast of rapid advances, but this is at least difficult if not impossible. Instead, the Editors with the advice of the Advisory Board, have assembled what they consider the best possible sequence of chapters written by competent authors; they must take the responsibility for inevitable gaps of subject matter and duplication which may result from this procedure.

Most evident to the modern biochemist, apart from the body of knowledge of the chemistry and metabolism of biological substances, is the extent to which he must draw from recent concepts of physical and organic chemistry, and in turn project into the vast field of biology. Thus in the organization of Comprehensive Biochemistry, the middle three sections, Chemistry of Biological Compounds, Biochemical Reaction Mechanisms, and Metabolism may be considered classical biochemistry, while the first and last sections provide selected material on the origins and projections of the subject.

It is hoped that sub-division of sections into volumes will not only be convenient, but will find favour among students concerned with specialized areas, and will permit easier future revisions of the individual volumes. Toward the latter end particularly, the Editors will welcome all comments in their effort to produce a useful and efficient source of biochemical knowledge.

Liège/Rochester
March 1962

M. Florkin
E. H. Stotz

PREFACE TO SECTION I

(Volumes 1–4)

Students and teachers of Biochemistry would not deny the importance of a sound understanding of at least certain areas of organic and physical chemistry in the comprehension of modern biochemistry. Toward this end the Editors have constituted the first section of Comprehensive Biochemistry. This section is intended neither as a textbook of organic nor of physical chemistry, but rather as a collection of chapters which seem generally pertinent in the interpretation of biochemical techniques and in the understanding of the chemistry of biological compounds and reaction mechanisms. Certain areas of organic and physical chemistry have been reserved for later presentation in context with specific biochemical topics, but the material of Section I seems to the authors to underlie all of modern biochemistry. The choice of material for Section I may well not agree with that of individual readers, and comments toward the construction of future volumes will be appreciated.

Section I has been subdivided into groups of topics designated as Atomic and Molecular Structure (Volume 1), Organic and Physical Chemistry (Volume 2), Methods for the Study of Molecules (Volume 3) and Separation Methods (Volume 4). It is hoped that all may find general favour, and that the individual volumes will find a special place on the shelf of the specialist.

Liège/Rochester
August 1962

M. FLORKIN
E. H. STOTZ

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Chapter I

Countercurrent Distribution

LYMAN C. CRAIG

The Rockefeller Institute, New York

1. Introduction

Countercurrent distribution is a relatively simple extraction process which is specifically designed for the purpose of separating relatively small amounts of mixtures of solutes under the mildest conditions in the laboratory. It is a logical extension of one of the oldest known separation methods; simple partition between two immiscible solvents. Since it is a multiple partition process, entirely discontinuous and stepwise in nature, it permits a rigid systematization with division and subdivision of the solute exactly according to the binomial theorem. This permits a useful mathematical interpretation, more exact than that possible for any other known countercurrent separation process. It is, therefore, ideally suited for analysis and for the establishment of the purity of a given preparation, particularly one of interest in biological chemistry where the experimenter can ill afford to lose any of a very valuable sample.

Countercurrent distribution can be applied to any solute which can be partitioned between two immiscible phases. It is, therefore, not restricted to molecules of a certain size or type. The immiscible phases can be mixtures of solvents, buffers, salts and various complexing agents. This makes it have a very wide applicability. Although more often used as a small-scale preparative or analytical method, it can be used for micro separations or can be modified for large-scale preparative work.

With these advantages and others not mentioned it is only fair to ask why this form of multiple extraction has not been more widely used when indeed many of its advantages were fully realized by Jantzen[1] thirty years ago. Two considerations are probably responsible for the fact that it did not begin to be popular until about ten years ago. One was the labor involved together with the view that the same or a superior separation could be achieved by a continuous extraction process with much less labor. The

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equipment available today makes this view questionable and greatly reduces the labor of the discontinuous process. The second point deals with the problem of suitable systems. Great improvement along this line now has resulted from the publication of partition data for the separation of nearly every type of solute known. A discussion of equipment and systems will form a considerable part of this chapter.

The literature dealing with extraction is enormous and widely scattered among many papers dealing with all kinds of chemical subjects. All too often an observation of interest to partition forms only a small part of a long paper. Partly for this reason and partly because an attempt at complete coverage would be too unwieldy for this chapter, no claim for completeness will be made. For a more complete coverage, see Craig and Craig[2], Hecker[3] or Weisiger[4].

In countercurrent distribution nearly all separations will be made using conditions which give complete equilibrium between the two phases. Thus the simple partition law is satisfied. As used in this work, C_1 is the concentration

$$K = C_1/C_2 \tag{1}$$

of the solute in terms of weight, or something proportional to it, in the upper phase; C_2 is the concentration in the lower phase. This designation will hold whether or not the polar phase is the heavier. C_1 and C_2 include all the solute of interest in the respective phases whether or not it exists in more than one readily interconvertible form.

It is interesting to think of simple partition in terms of a certain probability since this naturally leads to the mathematics to be used later in the interpretation of the results. K for a given concentration level and temperature will be very constant because of the very high number of molecules involved but as the population density of the molecules increases it will show a certain shift. The effect of this in countercurrent distribution will be discussed later in connection with non-ideal distributions.

In spite of this latter complication slight differences in the probability, here expressed as K, can be exploited better if repetitive extractions are made so that the mathematics of the binomial theorem or its approximation can be directly applied.

The systematization which will permit this is given schematically in Fig. 1. Here each contacting phase is represented by a rectangle and numbered serially starting with 0. The stages or transfer numbers are on the left. The top row gives the state of affairs after equilibration at 0 transfers. Unit quantity of solute is assumed in this scheme and a K of one. The phase volumes are all equal. At transfer 1 after equilibration, the fraction of the solute in each of the four phases in contact is represented by the decimal

inside the appropriate rectangle. Similarly, the distribution of fractional parts at transfer 2, 3 and 4 is shown.

It is obvious from Fig. 1 that the solute will spread equally to the right and to the left if the upper and lower phases are moved alternately, equilibrating at each stage. It is equally apparent that the same distribution of

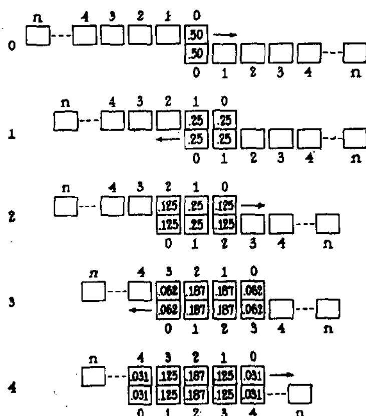

Fig. 1. A systematic extraction scheme. (Taken from *Techniques of Organic Chemistry*, Vol. III, A. WEISSBERGER (Ed.), Interscience, New York, 1950.)

fractional parts will result if only one phase is moved at each equilibration. Here the solute will migrate as it spreads. In the development to follow, the lower phase will remain stationary.

A further simplification results if the total fraction in each unit in both upper and lower phase is combined. When the scheme of Fig. 1 is treated in this way, the figures given in Table I are obtained. Here the scheme is expanded to 8 transfers.

Mathematically the development of the fractions in each contacting unit, each fraction in Table I, is given by the simple binomial in eqn. 2 where

$$(x + y)^n = 1 \tag{2}$$

x is the concentration in the lower phase and y that in the upper. The exponent n is the number of transfers. Since the fraction in each phase at equilibrium is fixed by K, it follows that eqn. 2 in terms of K and n is given in eqn. 3.

$$\left(\frac{1}{K + 1} + \frac{K}{K + 1}\right)^n = 1 \tag{3}$$

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TABLE I

DISTRIBUTION OF FRACTIONAL PARTS FOR A SOLUTE WITH A K OF 1

Transfer No.	Tube No. 0	1	2	3	4	5	6	7	8
0	1.000								
1	.500	.500							
2	.250	.500	.250						
3	.125	.375	.375	.125					
4	.062	.250	.375	.250	.062				
5	.031	.156	.313	.313	.156	.031			
6	.015	.093	.234	.313	.234	.093	.015		
7	.008	.054	.164	.274	.274	.164	.054	.008	
8	.004	.031	.109	.219	.274	.219	.109	.031	.004

(Reprinted with permission from *Analytical Methods of Protein Chemistry*, Pergamon, London, 1960.)

The most practical way of showing the distribution of fractions along a train of contacting units (separatory funnels or tubes) is by a plot of the fraction or of something proportional to the amount in each unit against the serial number of the unit. Fig. 2 is such a plot at 8 transfers. The central curve would be that obtained with a K of 1. The right dashed curve would be that obtained with a K of 3 while the left would result from a K of 0.30.

If a mixture of two solutes with K's of 3 and 0.30 had been taken, the sum of the two curves would have resulted and the chart would give an excellent overall analytical picture with a certain amount of overlap in tubes 3 to 5. With the application of higher numbers of transfers, this overlap area would decrease but never completely disappear in the absolute sense. It is entirely practical to perform 10 to 12 transfers with individual contacting units but becomes progressively less practical for higher numbers unless a train of contacting units is used which enables many simultaneous extractions to be made. Since equipment for accomplishing this is now

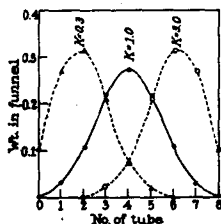

Fig. 2. Countercurrent distribution patterns with partition ratios of 0.3, 1.0 and 3.0. (Taken from *Techniques of Organic Chemistry*, Vol. III, A. WEISSBERGER (Ed.), Interscience, New York, 1950.)

readily available, the mathematics and reasoning will be further developed.

Let us assume that the scheme of Fig. 1 had been continued until 25 units filled with both phases were in the contacting train. Thus 24 transfers would have been applied. With a mixture of two solutes with K's of 0.707 and 1.414, the binomial of eqn. 3 would permit calculation[5] of the respective

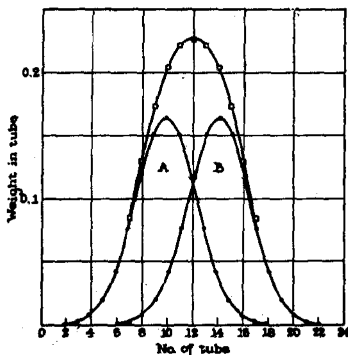

Fig. 3. Countercurrent distribution patterns at 24 transfers for two solutes with K's of 0.707 and 1.414. (Taken from *Techniques of Organic Chemistry*, Vol. III, A. WEISSBERGER (Ed.), Interscience, New York, 1950.)

two lower curves shown in Fig. 3. The sum of these two curves would give the upper curve which would be the curve experimentally found with such a mixture if the two solutes behaved ideally.

In order to calculate a curve such as A or B or for smaller numbers of transfers, eqn. 4 is very convenient. It is arranged to calculate each

$$T_{n,r} = [n!/r!\,(n-r)!]\,[1/(K+1)]^n\,K^r \tag{4}$$

term of the binomial directly[5]. In this equation r is the tube number and $T_{n,r}$ is the fraction for a given number of transfers and tube number.

Even this becomes laborious when n, the number of transfers, becomes much higher than 20. However, in this case an approximation can be used which gives values well within the experimental error. The form of this approximation in terms of the partition ratio, K, and of the numbers of transfers, n, is given in eqn. 5. Here y is the total fraction of solute in the

$$y = 1/\sqrt{2\pi nK/(K+1)^2}\cdot\exp - x^2/[2nK/(K+1)^2] \tag{5}$$

xth tube either to the right or left from the maximum of the bell-shaped curve. This equation is not as complicated as it appears to be and can be

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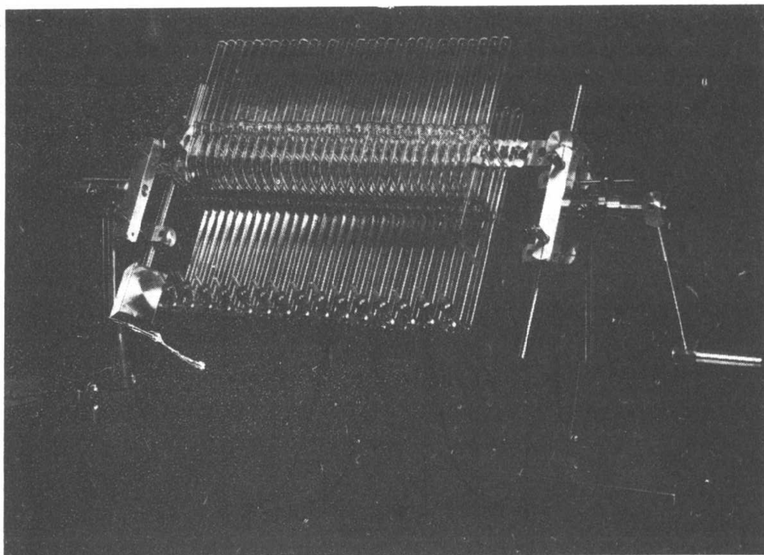

Fig. 4. A 30-tube countercurrent distribution train.

rearranged so that a theoretical distribution can be calculated in about 10 minutes with the aid of a slide rule. In countercurrent distribution work, this equation is very much used, but a discussion of its application will be postponed until the experimental procedures of countercurrent have been described. These will now be considered.

A simple distribution train for accomplishing the scheme depicted in Fig. 1 is the 30-tube hand operated train shown in Fig. 4. The glass cells fitted closely together are held in place by an aluminium frame supported at each end by bearings so that all the cells can be tipped forward and back as a unit.

The design of each cell is shown schematically in Fig. 5. The dimensions given in cm are for 10-ml lower-phase volumes and up to 15-ml upper-phase volumes. Each cell carries a flat butt joint held in place by a spring so that liquid can be added or removed at will, usually with a syringe to be described later. These joints are designed for the greatest speed in removal and will not leak. The solute is brought to equilibrium with the two phases in each tube by rocking the train forward and back as from A to B in Fig. 5 about six times. This will nearly always be sufficient for the purpose. For a theoretical discussion of the reason a longer time is not needed (see the Chapter in Weissberger by Craig and Craig[2]). The phases are permitted to separate with the cells in position B and the train then tipped to C. Here the upper phase decants through tube c into d leaving behind the lower phase.

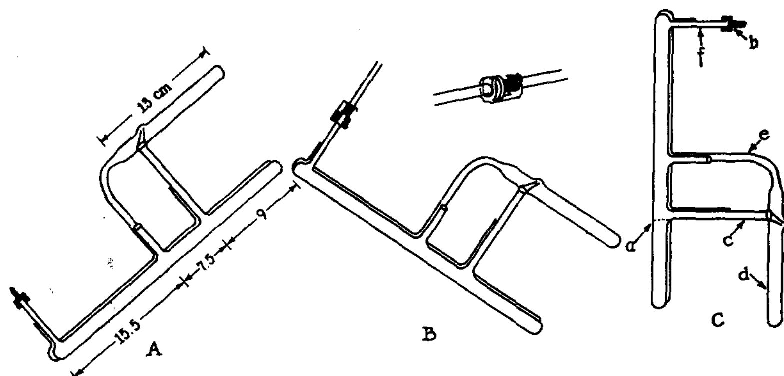

Fig. 5. Schematic drawing of individual units of a countercurrent distribution train. (Taken from *Techniques of Organic Chemistry*, Vol. III, A. WEISSBERGER (Ed.), Interscience, New York, 1950.)

On tipping back to A, the upper phase in d cannot flow back into the equilibration cell from which it has come because of the extension of the tube c but instead runs through e to the next cell of the series. Thus a single tipping operation causes each of the upper phases to advance one tube in the train where they are again equilibrated. Each time the tubes are tipped to C, a transfer is accomplished. The whole operation of equilibration, settling and transfer, usually will require 1 to 2 minutes.

The first step in starting a distribution after a suitable system has been selected, as discussed later on, is the equilibration at the temperature to be used of a quantity of the two phases sufficient for the whole run. A large separatory funnel is most convenient for this purpose. Sufficient of the lower phase is then placed in the train so that each cell will have the required amount. This operation can be accomplished rapidly by tipping the train from position B of Fig. 5 almost to C. A small funnel supplied with the apparatus is fitted with a flat joint so that it can be attached in place of b to tube f. Approximately 170 ml of the lower phase can be run into the train through this funnel at tube 5 and another 170-ml portion at tube 15. After replacing the closures, the phases are rapidly distributed through the train by tipping from A to C the required number of times.

With each transfer during the run, the upper phase in tube 0 moves to tube 1 and must be replaced by a fresh phase. This can be done by hand or more easily with a simple filling device shown in Fig. 6. A standard Erlenmeyer flask with a 24/40 joint is fitted with a laboratory dispensing head which can be purchased in 15, 10, 5, etc., ml sizes from apparatus supply houses such as Scientific Glass Inc., Bloomfield, N.J. The Erlenmeyer can

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