METHODS IN MEDICAL RESEARCH

METHODS IN
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Volume 5

A. C. CORCORAN, *Editor-in-Chief*

METHODS FOR SEPARATION OF COMPLEX MIXTURES AND HIGHER MOLECULAR WEIGHT SUBSTANCES, *Lyman C. Craig, Editor*

METHODS OF RENAL STUDY, *A. C. Corcoran, Editor*

IMMUNOCHEMICAL METHODS OF DETERMINING HOMOGENEITY OF PROTEINS AND POLYSACCHARIDES, *Melvin Cohn, Editor*

1952

PREFACE

THIS SERIES of volumes has now, we feel, become known and valued to the extent that it is no longer necessary either to defend its institution or to explain its purpose and plan. Dr. A. C. Corcoran, who has been informally associated with the project from the beginning, now appears both as an Associate Editor and as Editor-in-Chief, and has furnished this volume with all the preface required. To him, to the other Associate Editors and the many contributors, we wish to express warmest thanks both on our own account and on behalf of the readers and users of the work. We are also indebted to readers of earlier volumes, and to other friends, for many helpful suggestions.

It is with regret that the Governing Board accepts the resignation of Dr. C. M. MacLeod, whose other duties have pressed too heavily upon him.

Irvine H. Page
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Colin M. MacLeod
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EDITOR'S PREFACE

THE EDITORSHIP of a volume of *Methods* carries a somewhat equivocal status. The only firmly established tradition is that the editor should furnish an introduction, even when it is his impression that it will not be read by most who use the book. The introduction has two major purposes: one, it should provide some background on the collation of the volume and, two, it should enable the Editor to justify his share of the task.

At the outset, the Editor is guided in the selection of topics and the choice of Associate Editors by the Governing Board; the Associate Editors then order, arrange and edit their sections more or less autonomously. As a result, the Editor's task and responsibilities are far from overwhelming. Dr. Cohn's section on immunochemistry illustrates this principle of desuetude. For various reasons it was so delayed in transit that the Editor's task was wholly undertaken by Dr. MacLeod, a member of the Governing Board. To him our thanks and praise, as also the reader's, for the material lies within his special competence and the outcome therefore more fortunate than the plan.

Dr. Cohn's section also demonstrates a major function of the *Methods* series. Those who seek acquaintance with immunologic methods will appreciate the scope and importance of its content; others, more familiar with the field, will recognize that this body of information could only otherwise be obtained by visits to the laboratories of the contributors or by tedious and often unsatisfying searches of sources not readily available.

Dr. Craig's section illustrates other aspects of modern scientific technology. The methods apply to a wide variety of molecular species and indissolubly embody both physical and chemical principles. It can be fairly presumed that the techniques described will be as useful in industrial as they are in medical and biologic research. Thus, it seems that both the disciplines and the orientations of science have begun to converge.

The section brought together by the Editor's alter ego, Associate Editor Corcoran, is certainly the most heterogeneous and least penetrating of the three. The procedures listed range from ultramicroscopic anatomy to routine clinical testing. This variety is imposed by the topic, which is substantively the kidney as an organ for study rather than as an isolatable aspect of humoral

biology. Since the procedures of renal research are usually adapted or designed for purposes of a particular experiment, many of those described will serve more as exemplars than as models.

Lastly, the Editor wishes to thank all who have contributed in any way and to join with Associate Editors and contributors in the hope that this volume—which is a tool, not a text—will find its place in laboratories rather than libraries.

—A. C. CORCORAN.

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SECTION I

Methods for Separation of Complex Mixtures and Higher Molecular Weight Substances

ASSOCIATE EDITOR—*Lyman C. Craig*

INTRODUCTION

MEDICAL RESEARCH has always profited from advances in the understanding of the complex physicochemical systems found in living matter. Among the techniques needed, not the least important are those which permit the separation and recovery of crucial substances from the biochemical matrix, occurring as they often do in relatively small amounts.

A large number of special methods are available for the determination or isolation of specific substances, but among the general methods of organic chemistry, such as distillation or crystallization, many are not suitable, either because of the unstable nature of the biochemically interesting compounds or because of the initial complexity of the mixtures.

Extraction with solvents has always been one of the most useful general procedures. As countercurrent distribution, it has now been advanced to the position of a highly powerful and specific preparative and analytical approach which can be adapted to a wide variety of substances, especially those requiring mild conditions.

Similarly, adsorption techniques have long been among the most useful tools for separating substances of biochemical interest.

Paper chromatography is a new development in this field which has already firmly established itself as one of the outstanding advances in the biochemical methods of recent times.

When larger molecular species are involved electrophoresis and the ultracentrifuge have been accepted without question for more than a decade as necessary tools. Nonetheless, recent years have brought forth improvements in technique for both methods.

—J. Delafield Gregory and Lyman C. Craig.

COUNTERCURRENT DISTRIBUTION

LYMAN C. CRAIG, *The Rockefeller Institute for Medical Research*

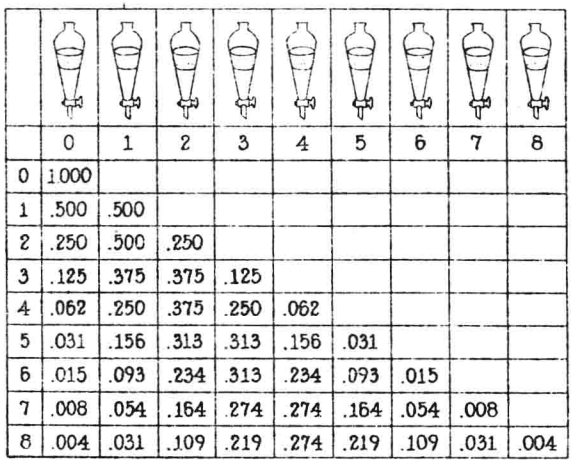
NEARLY EVERYONE working in the biological sciences as well as in chemistry is familiar with simple extraction as a tool generally applied in the first preliminary investigations of a new problem. It has been used this way for the separation of substances with widely differing properties since the beginning of chemistry. Only in recent years have its possibilities as well for the final characterization of a wide variety of closely related substances been really appreciated. Naturally realization of the fullest potentialities can be accomplished only through its integration with the many useful and specialized analytical techniques now available, and by rather extensive equipment. However, even with the techniques and equipment of most laboratories, far more can be accomplished than has been done in the past.

In dealing with an unknown preparation one of the first considerations should be that of fractionation. A mixture requires fractionation for isolation of the component of possible interest. A supposedly pure substance also requires a fractionation attempt in order to prove that it is not a mixture.

All methods of fractionation, in the broadest sense, have a common theoretical basis in that they deal with the manipulation of fractional parts. To be sure, in certain cases, the fraction removed or transferred is so large that for practical purposes separation is complete. The majority of separations, however, are not accomplished so easily and losses occur at each step, losses in labor as well as in material. Proper systematization minimizes such losses. This can be understood perhaps better by stepwise fractional extraction than by any other approach.

Let us take the case of a single solute, A, which can be distributed between 2 immiscible solvents. If at equilibrium with equal volumes of the 2 phases, half of the solute is found in each phase, then the partition ratio of the solute is 1. If the upper phase is transferred to a new equilibration tube (separatory funnel) containing an equal volume of fresh lower phase, and an equal volume of fresh upper phase is added to the first tube, the state of affairs represented by line 1, Table, of Figure 1, will be reached. Half the total solute will be in each of the 2 units. Now if both are equilibrated and the upper from 1 transferred to tube 2, that from tube 0 transferred to tube 1, fresh upper phase added to 0 and fresh lower

phase added to tube 2, the state of affairs shown by line 2 will be reached: 0.25 of the original will be in tube 2, 0.5 will be in tube 1 and 0.25 will be in tube 0. If this process of alternate equilibration and transfer is continued until a total of 9 units is in the series, then the distribution of solute in each unit will be shown by the bottom line of the table. The number of stages applied is given by the



	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

$(x+y)^n$

Let x = Fraction in upper layer

Let y = Fraction in lower layer

$N = n\left(\frac{K}{K+1}\right)$

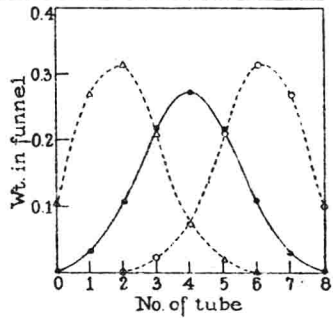


FIG. 1.—Distribution series; 8 stages.

vertical column on the left while the number of the unit is given by the top horizontal column.

If the fraction of the original in a unit is plotted against the consecutive number of the unit as given in the table, a distribution curve can be obtained with a maximum at tube 4. Had the partition ratio been 3, then the distribution would have been the curve on the right for 8 stages, while for a ratio of 0.333, it would have been that on the left. If 2 solutes had been present in equal amount

and the K's had been 3 and 0.333, respectively, then the separation in each tube would be plainly evident from the 2 curves.

Mathematically, the Table of Figure 1 is represented by the binomial expansion, $(X + Y)^n = 1$, where n is the number of stages or *transfers*, X is the fraction in the upper phase and Y is the fraction in the lower phase. If X is the fraction in the upper phase of a single tube, then $1 - X$ is the fraction in the lower phase. By definition, the partition ratio K is $X/(1 - X)$. Thus X is equal to $K/(K + 1)$ and the fraction in the lower phase is $1 - [K/(K + 1)]$ or $1/(1 + K)$. The binomial expansion in terms of the partition ratio for equal volumes becomes

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

Obviously it soon becomes laborious to perform such multiple extractions individually in separatory funnels as represented. Nonetheless many laboratories have performed separations involving up to 20 or more separatory funnels in the series. Fortunately, mechanical equipment has been developed (10, 12, 20, 30, 16, 29) for the purpose and the number of units in the series can now be expanded almost indefinitely if the particular fractionation problem requires it. One extractor in use at the Rockefeller Institute (10) contains 220 equilibration units. It is fully automatic and is equipped with electric motors, time clock, etc. From 100,000 to 200,000 individual extractions can be performed in a 24 hr period. This equipment is commercially available.*

Such mechanical equipment, however, is valueless unless suitable systems can be found. The term system as used here means any mixture of liquids or solids which will yield 2 clear liquid phases when they are brought together and thoroughly mixed. The 2 phases are always equilibrated at the temperature of the experiment just prior to their use. To be suitable, a system must meet the following main requirements.

1. It must be capable of dissolving the solute of interest and should furnish a partition ratio within a practical range. If equal volumes of each phase are to be used in the mechanically operated equipment, the range would be from 0.01 to 100. With hand-operated equipment the range should be much narrower, from 0.2 to 5. In the latter case, however, compensation can be made easily for a K too high or too low by using a larger or smaller volume in either the upper or lower phase. Thus where r_v is the ratio of the 2 volumes, the product Kr_v should be within the limits of 0.2–5.

To reach the best separation possible with the least number of

* Obtainable from O. Post, 6822-40th Road, Maspeth, N. Y.

transfers, with a mixture of 2 components, the geometric mean of the 2 products, Kr_v and $K'r_v$ should be 1 (6). Here K and K' are the respective partition ratios of the 2 components.

2. The partition ratio must be reasonably independent of the concentration. All solutes and systems show more or less deviation of K with concentration, particularly at higher concentrations. The adherence to ideality required will vary with the number of transfers applied. Thus a variation of 10 per cent over a 100-fold concentration range is not serious if no more than 20–30 transfers

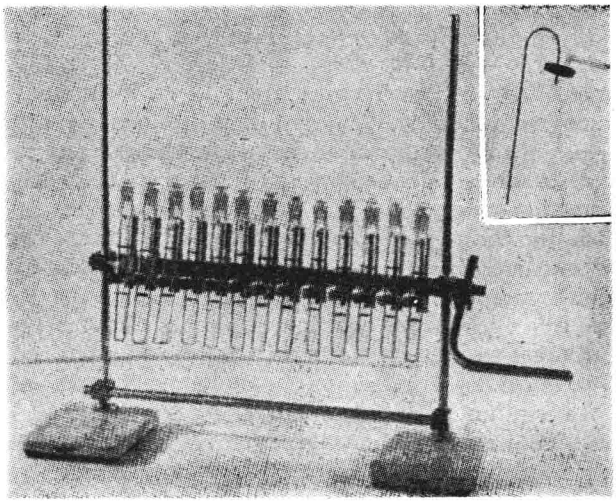


FIG. 2.—Test tube rack for preliminary countercurrent distribution.

are to be applied. But, when hundreds of transfers are involved, closer adherence is desirable.

In dealing with a new solute it is always worth taking the time, before launching forth on a countercurrent distribution run, to determine the K at the maximum concentration initially to be used in the run and at $^1/_{10}$ this concentration.

3. The system must permit equilibrium to be reached rapidly. This point is easy to test experimentally (3) when the partition ratio is determined. In general, partially miscible solvents cause little trouble in this respect.

4. The phases must separate clearly within a few minutes after equilibration and must not form stable emulsions. If emulsions do tend to form, lowering the pH probably will help. Addition of a

trace of a salt often is helpful. Increasing the volume of the organic phase often promotes separation. On close examination it may be found that a small amount of a solid film is separating at the interface. Removal of this will promote separation.

5. The solute must not be unstable in the system.

6. The system must lend itself readily to quantitative solute analysis, preferably by direct weight determination and to convenient recovery of the solute after the distribution is finished.

7. The system should not be expensive, extremely toxic or corrosive to the equipment.

Other less important points could also be mentioned. A system deficient in 1 requisite may be so favorable in the others that it is worth using in spite of disadvantages.

APPARATUS

When a suitable system has been chosen a preliminary run can be made. It may be made in the distribution apparatus or preferably in a row of glass-stoppered test tubes supported on a rack (12) as shown in Figure 2. The apparatus of Figure 2 can be assembled from standard laboratory equipment.

The tubes are held by spring clips attached by a screw to a short piece of Flexaframe rod. The stainless steel Gee clips, available from scientific supply houses, may be conveniently adapted. The rods attached to the clips are in turn attached to a longer rod which extends through 2 bearings on each end of the rod. The bearings are simply Flexaframe clamps in which the screw remains loose. The bearings are supported by 2 stands. A crank is attached to 1 end of the rod for tumbling the tubes.

In this design each tube receives its equal portion of stationary phase initially. The sample to be distributed is placed in tube 0. An arbitrary portion of the other phase, previously equilibrated with the first, is then introduced and equilibrium is reached by inverting the tubes with the crank. Twenty-five inversions are usually sufficient (3). The glass stopper will stick sufficiently not to fall out, if it is given a slight turn when it is placed tightly in position. Small interchangeable stoppers are therefore more reliable than larger ones.

After the layers have separated, the phase to be moved is transferred to the adjoining tube, 1, by a small siphon (Fig. 2, inset) preferably made from stainless steel tubing. Pressing the rubber collar, through which the siphon extends, against the mouth of the tube permits actuation by either air pressure or vacuum. The glass tube to which the pressure or vacuum line is attached also passes through the rubber collar and has a hole at the bend. The operator's finger