PHYSICAL PROPERTIES OF THE STEROID HORMONES

Edited by
LEWIS L. ENGEL

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FOREWORD

THIS is an important volume written by an outstanding group of investigators who have given generously of their time so that the research efforts of their colleagues may be aided. It is important that this work has been done by practising scientists so that the information has been assured an efficient presentation. The individual contributors are congratulated on the excellency of their efforts.

The creation of a volume such as this is a task far beyond the abilities and energies of any single individual. It was imperative, therefore, to have an initiator, an inducer, a persuader, and a correlator. These four functions have been executed successfully and cheerfully by the Editor, Dr. Lewis Engel, who performed his duties in his usual scholarly manner. I can report that the co-authors still refer to the Editor with affection and respect.

This volume clearly illustrates the efficiency of modern methodology for the solution of problems of isolation, identification and quantitative determination of steroids. We have not only entered the era of " machine " chemistry but automation is essentially with us. I have not heard of an instrument which analyzes a mixture of steroids in a few milliseconds and presents the operator with a printed record of their structures and their quantitative abundance. The data so orderly presented in this volume will aid in the designing and executing of such valuable tools for the steroid chemist.

Steroid chemistry and all its subdivisions and interrelated fields have been enriched because this book was written. All who so profitably will use this volume will be grateful to those who labored so well.

RALPH I. DORFMAN.

EDITORIAL NOTE

IT has not been found possible, without delaying publication and expanding editorial labor beyond reasonable limits, to eliminate from this book individual variation in some details of nomenclature and symbols. In the absence of agreed — and universally *accepted* — international conventions it is hoped that, although complete consistency has not been attained, any ambiguity has been avoided.

Examples of the variation in question are the use of the equivalent names allopregnane or 5α-pregnane, and of Δ^5-pregnene or 5-pregnene. Again, in the presentation of absorption data there are used the abbreviation "O.D." for optical density and "m." for mole, as in concentrations expressed in the form 2.1 mg (7.0×10^{-6} m.)/100 ml.

This volume is not an exhaustive compilation of physical data. Attention is directed to *Pouvoir Rotatoire Naturel. I. Steroides* by J.-P. Mathieu and A. Petit, Pergamon Press, as well as *Infrared Absorption Spectra of Steroids* by K. Dobriner, E. R. Katzenellenbogen and R. Norman Jones, Interscience Publishers, New York, 1953, and Volume II of the same work by G. Roberts, B. S. Gallagher and R. Norman Jones, Interscience Publishers, New York, 1958. Important data are also presented in *The Chromatography of Steroids* by I. E. Bush, Pergamon Press, Oxford, 1961, and in *Optical Rotatory Dispersion* by C. Djerassi, McGraw-Hill Book Company, Inc., New York, 1960.

CONTENTS

PARTITION COEFFICIENTS*

LEWIS L. ENGEL† AND PRISCILLA CARTER

From the John Collins Warren Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts

IN the processing of tissues and body fluids for the isolation and identification of steroid hormones and their metabolites, partitions between two liquid phases play a predominant role. They are employed in the preliminary separation of these substances from other classes of compounds and also in the more delicate procedures involved in the resolution of mixtures of closely related substances by countercurrent distribution and liquid–liquid partition chromatography. For these reasons, increasing attention has been given to the measurement of partition coefficients of steroid hormones and their derivatives and metabolites in varous two-phase liquid–liquid systems. With the aid of these physical constants, it is possible to establish

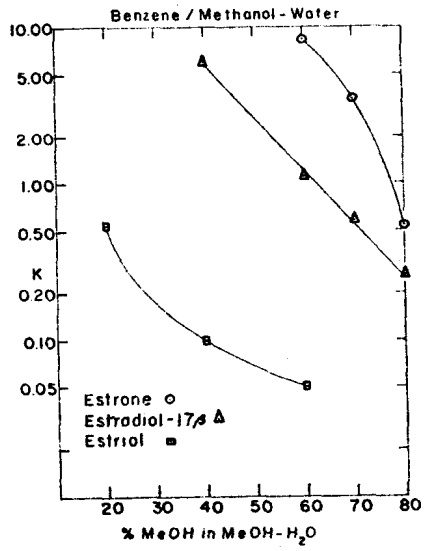

FIG. 1

Relation between phase composition and partition coefficient, *K*, for Estrone, Estradiol-17β and Estriol in the system Benzene/Methanol : Water

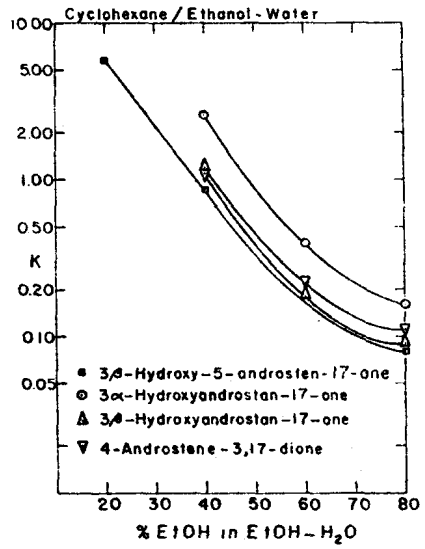

FIG. 2

Relation between phase composition and partition coefficient, *K*, for 3β-Hydroxy-5-androsten-17-one, 3α-Hydroxyandrostan-17-one, 3β-Hydroxyandrostan-17-one, and 4-Androstene-3,17-dione in the system Cyclohexane/Ethanol : Water

* This work was supported by grants from the National Cancer Institute, United States Public Health Service, a grant from the American Cancer Society, Inc., and a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

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† Permanent Faculty Fellow of the American Cancer Society.

more rational methods of procedure for isolation and identification. Apparatus, some solvent systems and mathematical treatment of three-phase countercurrent distribution have been described,[34] but this procedure has not yet been applied to the separation of mixtures of steroids.

A knowledge of the value of the partition coefficient can be extremely useful in determining the type of process required to achieve a given separation. It can, for example, help to decide whether the best procedure in a given instance is continuous extraction in one of the many types of apparatus available for this purpose,[14] hand extraction or a short countercurrent distribution. In order to make these choices it is also necessary to know something of the properties of the substances which accompany the material whose isolation is sought. In these preliminary processes,

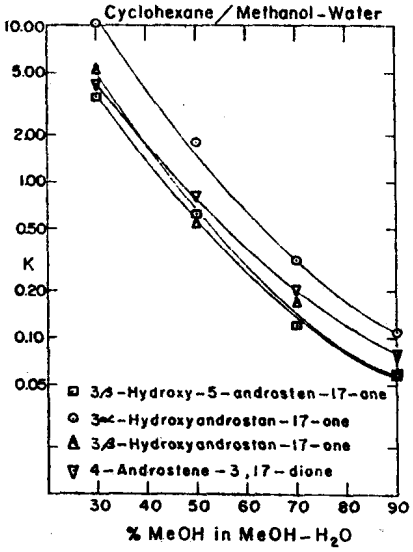

FIG. 3

Relation between phase composition and partition coefficient, K, for 3β-Hydroxy-5-androsten-17-one, 3α-Hydroxyandrostan-17-one, 3β-Hydroxyandrostan-17-one, and 4-Androstene-3,17-dione in the system Cyclohexane/Methanol : Water

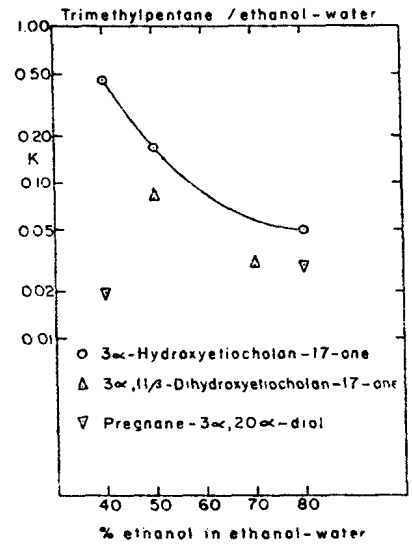

FIG. 4

Relation between phase composition and partition coefficient, K, for 3α-Hydroxyetiocholan-17-one, 3α-11β-Dihydroxyetiocholan-17-one, and Pregnane-3α,20α-diol in the system 2,2,4-Trimethylpentane/Ethanol : Water

employed for the concentration of the desired component, it is important to use partition coefficients that are either very large or very small; hopefully the corresponding constants for the contaminants are such that they will not be concentrated in the same phase as the steroids. These ends are only approximately possible, and for this reason finer fractionation becomes necessary.

A knowledge of the values of the partition coefficients of at least some of the components of a mixture is required in the application of countercurrent distribution. This technique has been discussed at length in numerous reviews,[14, 43] and it is only necessary to comment here that it is applicable for the separation of mixtures, for the characterization and quantitative estimation of individual components of such mixtures, and for the study of radiochemical purity.[4, 39a, 39b]

I. THE MEASUREMENT OF PARTITION COEFFICIENTS

The partition coefficient in this chapter is defined as follows:

$$K = \frac{C_u}{C_l} = \frac{W_u}{W_l} \cdot \frac{V_l}{V_u} \qquad \text{(A)}$$

where C, W and V are concentration of solute, weight of solute, and volumes of phases. u and l refer to the upper, less dense phase and lower, denser phase respectively. In principle, this constant can be determined by measurement of solute concentrations in the two phases at equilibrium after a single equilibration or after multiple equilibrations as in countercurrent distribution. In the latter case the calculation can be made from the analysis of the distribution.[14, 43] Such values are known with greater precision than those obtained by simple equilibration. They are, however, subject to certain errors inherent in the distribution process and the apparatus employed.

When the constant is measured by simple equilibration, the operation is carried out as follows:

Measured portions of a solution of the substance to be examined are transferred to glass stoppered tubes using a suitable solvent which is then removed under conditions which will not cause decomposition of the solute. Then measured volumes (usually equal) of the two mutually saturated phases are added, and the tubes are inverted gently twenty to thirty times to establish equilibrium. After the layers have separated, samples of the two phases are withdrawn from each tube and

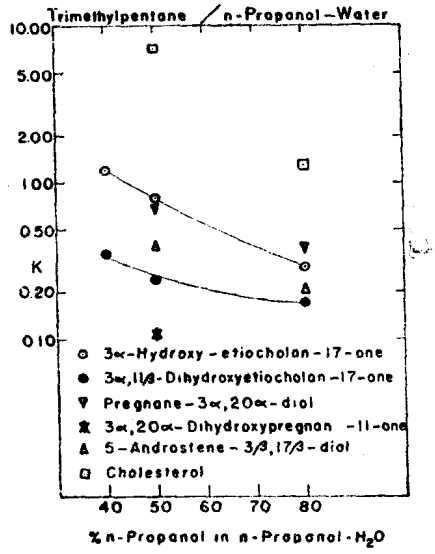

FIG. 5

Relation between phase composition and partition coefficient, K, for 3α-Hydroxyetiocholan-17-one, 3α,11β-Dihydroxyetiocholan-17-one, Pregnane-3α,20α-diol, 3α,20α-Dihydroxypregnan-11-one, 5-Androstene-3β,17β-diol, and Cholesterol in the system 2,2,4-Trimethylpentane/n-Propanol : Water

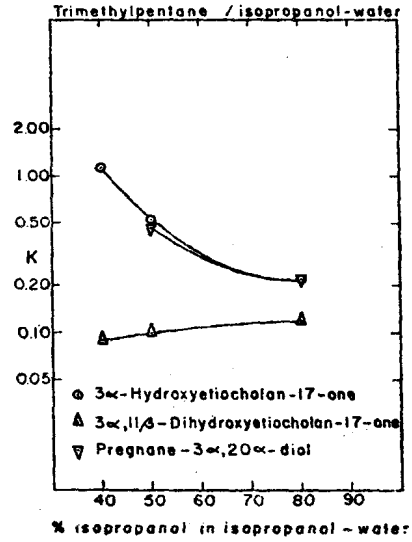

FIG. 6

Relation between phase composition and partition coefficient, K, for 3α-Hydroxyetiocholan-17-one, 3α,11β-Dihydroxyetiocholan-17-one, and Pregnane-3α,20α-diol in the system 2,2,4-Trimethylpentane/iso-Propanol : Water

the amount of solute in each phase measured by an appropriate method. The partition coefficient is then calculated using Equation (A). It is important that analyses be performed on both phases and the recovery calculated, since this provides the only means of knowing whether the solute has been completely dissolved. Analysis of both phases also increases the accuracy and precision in situations where the value of the partition coefficient differs greatly from unity. In such cases it is the ratio of a large to a small number (or the reciprocal) and a small error in the value of the small number produces a large effect upon the value of the ratio. If the partition coefficient in these situations is calculated from analysis of one phase only, a large error may be introduced.

It is difficult to assign limits of errors to the measurement of this constant, since they depend upon both the analytical procedure employed and the numerical value of the partition coefficient. As pointed out above, values differing greatly from one may be subject to quite considerable error.

The partition coefficient may also be estimated from countercurrent distribution curves. In this situation, both the precision and the accuracy of the value obtained

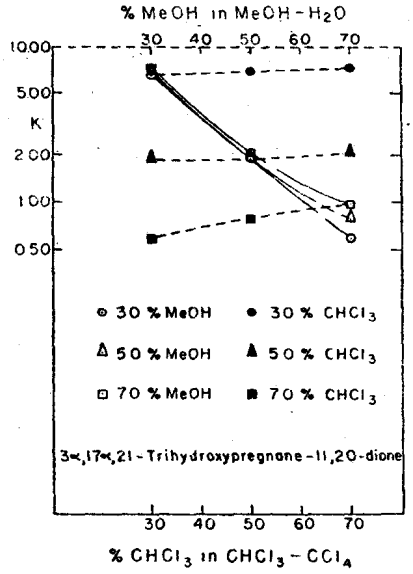

FIG. 7

Relation between phase composition and partition coefficient, K, for 3α,17,21-Trihydroxypregnane-11,20-dione in the system Methanol : Water/Chloroform : Carbon tetrachloride. The ordinate is the partition coefficient (K) plotted on a logarithmic scale. The lower (solid) abscissa gives the composition of the lower phase and the upper (dashed) abscissa gives the composition of the upper phase. —— Variation in K with changing lower phase composition at three fixed upper phase compositions. – – – – – – Variation in K with changing upper phase composition at three fixed lower phase compositions

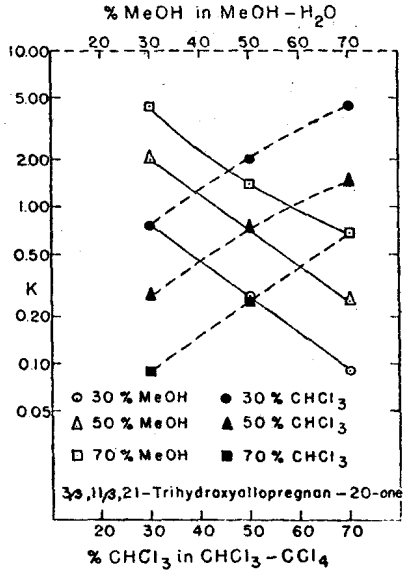

FIG. 8

Relation between phase composition and partition coefficient, K, for 3β,11β,21-Trihydroxyallopregnan-20-one in the system Methanol : Water/Chloroform : Carbon tetrachloride. The ordinate is the partition coefficient (K) plotted on a logarithmic scale. The lower (solid) abscissa gives the composition of the lower phase and the upper (dashed) abscissa gives the composition of the upper phase. —— Variation in K with changing lower phase composition at three fixed upper phase compositions. – – – – – – Variation in K with changing upper phase composition at three fixed lower phase compositions

depend upon the variations in the exact phase compositions of the mixtures used in different runs and the variations in ambient temperature. However, since such a partition coefficient is calculated from essentially replicate determinations, it is generally a more reliable value that that determined by single equilibration procedures.

II. FACTORS WHICH INFLUENCE THE PARTITION COEFFICIENT

Although the partition coefficient is temperature dependent, in most of the systems with which experience has been obtained in this laboratory, where a constant temperature room is not available, this factor has not been disturbing in countercurrent distributions which last as long as three or four days. However, it can be expected that in multi-component systems, the compositions of which are such that the two phases are approaching miscibility, the temperature dependence would be great. Even in two component systems in which there is great mutual solubility marked temperature dependence of partition coefficients may be encountered.

Secondly, variations in the composition of the two phases may result in different values for partition coefficients, particularly in multi-component systems.

III. PREPARATION OF SOLVENT SYSTEMS

At any given temperature and pressure the composition of a two-component, two-phase system is fixed.[30] When more components are added, additional degrees of freedom are added and it becomes necessary to specify precisely how the system is made up. Common practice is to indicate the volumes of the individual components that are mixed and allowed to equilibrate. Finally, for measurement of the partition coefficient, equal volumes of the two phases are transferred into glass stoppered tubes as described above. This procedure has been employed in the preparation of all systems employed in this laboratory. The final equilibrium compositions of the two phases cannot be determined readily.*

IV. SOLVENT SYSTEMS

Certain general considerations dictate the choice of solvent systems for the countercurrent distribution of steroids. A primary consideration is that the solvents must neither react irreversibly with the substances present as solutes, nor must they interfere with any analytical procedure to be employed for the analysis of the distribution. The stability of the solvents is important. Since water is almost inevitably one component of a solvent system, care must be taken to employ substances which are not attacked rapidly by water. Esters, for example, present a risk in acidic or basic media, since hydrolysis of the ester with liberation of acid and alcohol would produce a changing phase composition, pH of the aqueous phase, and partition coefficient during the course of the distribution. Under some conditions, too, solvolysis of a solute may be a disturbing factor.[7]

It is generally true that the maximum resolution of two substances is achieved if the two phases in a countercurrent distribution have widely differing dielectric constants. Thus, a system consisting of an aliphatic hydrocarbon and water would be expected to give efficient separations. However, limitations of solubility may make such systems impractical. The addition of solvents in which the steroids

* Vapor phase chromatography may provide a solution to this analytical problem.

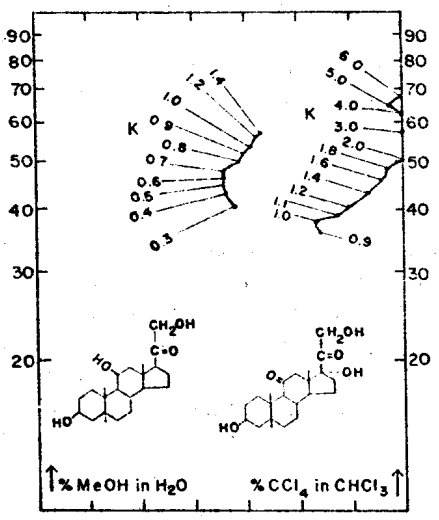

FIG. 9

Nomogram for the determination of the partition coefficients, K, of 3β,11β,21-Trihydroxyallopregnan-20-one and 3α,-17α,21-Trihydroxypregnane-11,20-dione in the system Methanol : Water/Chloroform : Carbon tetrachloride

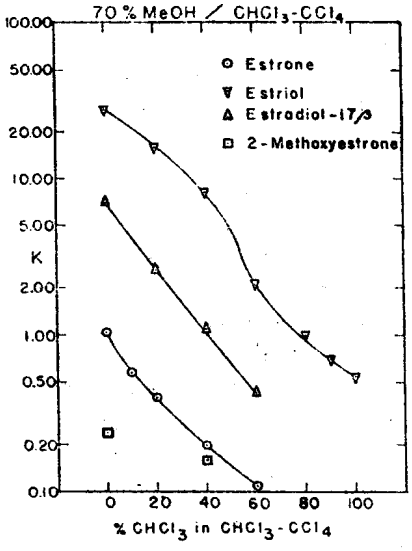

FIG. 10

Relation between phase composition and partition coefficient, K, for Estrone, Estradiol-17β, Estriol and 2-Methoxyestrone in the system 70% Methanol/Chloroform : Carbon tetrachloride

have more favorable solubilities will improve the capacity of the system at the expense of resolution. With increasing addition of the second type of solvent, capacity is increased, but resolution diminishes as the compositions of the two phases approach one another. In the limiting case, i.e. miscibility, when the phase compositions approach identity, all partition coefficients approach the same limiting value.[25]

Since the solvents are employed in relatively large volumes for countercurrent distributions, substances should be chosen which can be purified readily. The importance of trace contaminants in solvents should not be underestimated, since the solutes in low concentrations are exposed to them for relatively long periods of time. The role of traces of copper in the destruction of corticosteroids has been noted.[33] Furthermore, if infrared spectroscopy of residues from the tubes of the distribution is contemplated, the presence of residues from unpurified solvents often makes it impossible to obtain satisfactory spectra. A compendium of information concerning properties and purification of organic solvents has been published.[41]

Another factor which may contribute to the successful selection of solvent systems for countercurrent distribution is relative densities of the two phases. Solvents should be chosen which give the maximum difference in density so as to accelerate separation of the phases. This point should be borne in mind particularly when three- and four-component solvent systems are designed.

High boiling, essentially non-volatile liquids such as the glycols, which are employed so effectively in paper chromatography, are not convenient for countercurrent distribution because of the difficulty in removing them for analysis of the solute. Similarly, ether, pentane and similar low boiling solvents are unsatisfactory.

since their rapid volatilization leads to changes in phase composition even during the course of a relatively short countercurrent distribution.

The toxicity of the solvent should also be considered and adequate ventilation provided and precautions taken when benzene, chlorinated hydrocarbons, or other toxic agents are employed.

A further factor is the tendency of the system to form emulsions, which not only slow down the distribution but also may lead to asymmetry of countercurrent distribution curves. In our experience benzene, toluene and cyclohexane are offenders, particularly in the presence of lipids derived from tissue and urinary extracts.

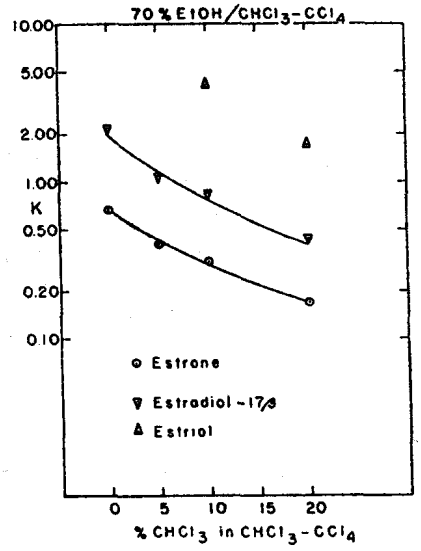

FIG. 11

Relation between phase composition and partition coefficient, K, for Estrone, Estradiol-17β, and Estriol in the system 70% Ethanol / Chloroform : Carbon tetrachloride

V. TYPES OF SOLVENT SYSTEMS

Two-Component Systems

Two-component, two-phase systems offer a wide choice and variety but in general have not provided very useful systems for countercurrent distribution, although preliminary separations into groups of compounds of similar polarity may be accomplished effectively with such systems.

Three-Component Systems

Systems composed of two organic solvents in addition to water have been extremely useful in countercurrent distributions. The possibilities of variation in phase composition over a relatively wide range make it possible to have a large choice of partition coefficients in related systems.[24, 31] In addition, this procedure has the advantage that the determination of partition coefficient in several related solvent systems is a more precise means of characterizing a solute than the determination of partition coefficient in a single system.

Four-Component Systems

Even greater latitude is permitted when four components are employed and a nomographic approach to systems of this type has already been reported.[25]

Simplifications of the nomographic technique have recently been made which avoid some of the laborious computations required in the earlier method. If, in the initial graphs, phase composition is plotted against partition coefficient on three cycle semilogarithmic paper, transformation of the measured partition coefficient values to the logarithms is avoided. If now the vertical axes of the solvent composition ratios of the two phases are plotted on the logarithmic scale of single cycle semilogarithmic paper, a second transformation to logarithms is avoided. The remainder of the construction of the nomograms is similar to that originally described.

Systems Containing Non-volatile Components

Systems with non-volatile components are used primarily for initial fractionations and separations when it is desired to separate compounds into groups. Such systems include those incorporating acids, bases or buffers. Their applications are numerous. For countercurrent distribution such systems are less convenient because of the difficulties involved in isolation of the steroid solutes. It is sometimes possible to circumvent this difficulty in the case of acidic or basic solutes by driving the solute into the organic phase at the end of the distribution by adjustment of pH of the aqueous phase.

VI. COMMENTS ON THE TABLES

Criteria for Acceptance of Data

The criteria for acceptance of a partition coefficient of a substance in the tables which follow are:

(1) that the constant must have been measured either on the pure crystalline compound by a single equilibration or by countercurrent distribution,

(2) that if it were measured by countercurrent distribution, only those cases in which a Gaussian curve is obtained are acceptable, and

(3) that the constant be measured by chemical and physical (including radiochemical) methods. Partition coefficients measured by biological assay have not been included.

Tabulation of Compounds

Compounds are listed in the order of their empirical formulae. Alphabetical order prevails within groups of compounds having the same empirical formulae.

Systematic names are used except for those trivial names listed in Table I.

VII. INDEX OF SYSTEMS

Table No.

Table No.	
VI	Benzene / Methanol : Water (Fig. 1)
VII	Benzene : Petroleum ether / Ethanol : Water
VIII	Benzene : n-Hexane / Ethanol : Water
IX	Petroleum ether / Methanol : Water
X	Petroleum ether / Ethanol : Water
XI	n-Hexane / Methanol : Water
XII	sec-Butanol : n-Hexane / Water
XIII	Ethyl acetate : n-Hexane / Ethanol : Water
XIV A	Ethyl acetate : Cyclohexane / Ethanol : Water
B	Ethyl acetate : Cyclohexane / Ethanol : Water (Fig. 2)
C	Ethyl acetate : Cyclohexane / Ethanol : Water
D	Ethyl acetate : Cyclohexane / Ethanol : Water
XV	Cyclohexane / Methanol : Water (Fig. 3)
XVI	2,2,4-Trimethylpentane / Ethanol : Water (Fig. 4)
XVII	2,2,4-Trimethylpentane / n-Propanol : Water (Fig. 5)
XVIII	2,2,4-Trimethylpentane / iso-Propanol : Water (Fig. 6)
XIX	2,2,4-Trimethylpentane / Acetone : Water
XX	Methanol : Water / Chloroform : Carbon tetrachloride (Fig. 7, Fig. 8, Fig. 9, Fig. 10)
XXI	Ethanol : Water / Chloroform : Carbon tetrachloride (Fig. 11)
XXII A	Ethanol : Water / n-Hexane : Chloroform
B	Ethanol : Water / n-Hexane : Carbon tetrachloride
XXIII A	2,2,4-Trimethylpentane / sec-Butanol : Water
B	2,2,4-Trimethylpentane / Aqueous alcohols (Steroid acetates)
XXIV	Methanol : Water / Chloroform : Carbon tetrachloride (Steroid derivatives)
XXV	Miscellaneous Systems
XXVI	Miscellaneous Systems (Steroid acetates)
XXVII	Miscellaneous Systems (Estrogens)
XXVIII A	Miscellaneous Systems (Estrogen derivatives)
B	Miscellaneous Systems (Estrogen derivatives)
C	Miscellaneous Systems (Estrogen derivatives)
XXIX	Miscellaneous Systems (Estrogen sulfates)
XXX	Miscellaneous Systems (Glucosiduronates)
XXXI	Miscellaneous Systems (Bile acids)
XXXII	Miscellaneous Systems (Conjugated bile acids)

Acknowledgements — The authors wish to acknowledge their thanks to the following for making available to us unpublished measurements of partition coefficients: Drs. B. Baggett R. H. Purdy, R. Walker and I. Weliky ; Misses I. Bjerkedal, A. Dimoline, L. L. Fielding, M. Halla, O. Klein, G. Lanman, M. Witkos and M. Zalkans ; and Mrs. N. Trofimow and Mr. A. Mahoney.

TABLE I. KEY TO NOMENCLATURE

Systematic Name	Trivial Name
C_{18}	
3-Hydroxy-1,3,5(10),6,8-estrapentaen-17-one	Equilenin
1,3,5(10),6,8,-Estrapentaene-3,17β-diol	Dihydroequilenin
3-Hydroxy-1,3,5(10),7-estratetraen-17-one	Equilin
3-Hydroxy-1,3,5(10)-estratriene-6-17-dione	6-Ketoestrone
3-Hydroxy-1,3,5(10)-estratriene-7,17-dione	7-Ketoestrone
3-Hydroxy-1,3,5(10)-estratriene-16-17-dione	16-Ketoestrone
3-Hydroxy-1,3,5(10)-estratrien-17-one	Estrone
3,17β-Dihydroxy-1,3,5(10)-estratrien-6-one	6-Ketoestradiol
3,17β-Dihydroxy-1,3,5(10)-estratrien-16-one	16-Ketoestradiol
1,3,5(10)-Estratrien-3-ol	Deoxyestrone
1,3,5(10)-Estratriene-3,17α-diol	Estradiol-17α
1,3,5(10)-Estratriene-3,17β-diol	Estradiol-17β
1,3,5(10)-Estratriene-3,16β,17β-triol	Epiestriol
1,3,5(10)-Estratriene-3,16α,17β-triol	Estriol
C_{19}	
3-Hydroxy,1-3,5(10)-Estratrien-17-one 2-methyl ether	2-Methoxyestrone
3-Hydroxy-1,3,5(10)-Estratrien-17-one 3-methyl ether	Estrone methyl ether
1,3,5(10)-Estratriene-3,17β-diol 3-methyl ether	Estradiol methyl ether
1,3,5(10)-Estratriene-2,3,17β-triol 2-methyl ether	2-Methoxyestradiol
1,3,5(10)-Estratriene-3,16α,17β-triol 3-methyl ether	Estriol methyl ether
1,3,5(10)-Estratriene-2,3,16α,17β-tetrol 2-methyl ether...	2-Methoxyestriol
3β-Hydroxy-5-androsten-17-one	Dehydroepiandrosterone
17β-Hydroxy-4-androsten-3-one	Testosterone
3α-Hydroxy-5α-androstan-17-one	Androsterone
C_{20}	
17α-Ethinyl-1,3,5(10)-estratriene-3,17β-diol	Ethinyl estradiol
1,3,5(10)-Estratriene-2,3,17β-triol 2,3-dimethyl ether	2-Methoxyestradiol methyl ether
$C_{21}H_{28}$	
1,3,5(10)-Estratriene-3,17β-diol 3-methyl ether 17-acetate	Estradiol methyl ether acetate
21-Hydroxy 4-pregnene-3,11,20-trione	Dehydrocorticosterone
11,21-Dihydroxy-18-aldo-4-pregnene-3,20-dione	Aldosterone
17,21-Dihydroxy-4-pregnene-3,11,20-trione	Cortisone
$C_{21}H_{30}$	
4-Pregnene-3,20-dione	Progesterone
21-Hydroxy-4-pregnene-3,20-dione	Cortexone
11β,21-Dihydroxy-4-pregnene-3,20-dione	Corticosterone
17,21-Dihydroxy-4-pregnene-3,20-dione	Cortexolone
11β,17,21-Trihydroxy-4-pregnene-3,20-dione	Cortisol
$C_{21}H_{34}$	
3α,17,21-Trihydroxy-5β-pregnane-11,20-dione	Tetrahydrocortisone
$C_{21}H_{36}$	
3α,11β,17,21-Tetrahydroxy-5β-pregnan-20-one	Tetrahydrocortisol