Quantitative Methods in Histology and Microscopic Histochemistry

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In statistical collaboration with

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PREFACE

> "I often say that when you can measure what you are speaking about and express it in numbers you know something about it; but when you cannot express it in numbers, your knowledge is of meager and unsatisfactory kind: it may be the beginning of knowledge, but you have scarcely, in your thoughts, advanced to the stage of *science*."
>
> LORD KELVIN: Popular Lectures and Addresses. 1883.

Consultation of recent histological and histochemical literature shows that the microscopical specimens are still mostly described in purely qualitative terms. This is undoubtedly due mainly to the descriptive nature of these branches of biology, and in many cases there is no necessity of using more objective and accurate terms. However, there are also many problems in histology and histochemistry which are difficult to study without a quantitative approach. This is particularly true in experimental studies, where many minor changes may be overlooked in purely qualitative examination or in which subjective bias may badly distort the results.

At present, no systematic introduction to the quantitative methods in histology and microscopic histochemistry is available, although much valuable information is scattered in recent literature. This little book is a humble effort to fill the need of such a guide. It is hoped that it will prove useful in the hands of histologists and histochemists who are not familiar with the possibilities for quantitative work in their field and also to the students of various branches of experimental biology who would welcome the addition of quantitative microscopical methods to their armament.

It must be made quite clear that only methods with which the writer has had at least some personal experience are discussed and that some important quantitative techniques are therefore excluded. No priority is claimed over the methods presented, many of which have long been in use, if not in histology or histochemistry, perhaps in geography or chemistry.

As basic knowledge of statistics is essential in all kinds of quantitative work, I am very glad to have been able to persuade Mr.

Jaakko Kihlberg to collaborate in the writing of this volume. He is fully responsible for chapters I and XI, and he has essentially contributed to chapters VI and VII. It has been a pleasure to work with him.

The idea of compiling this volume ripened in the course of the academic year I spent in the University of Edinburgh, and a considerable proportion of actual writing was done during that period. I am very grateful to Professor *J. H. Gaddum* for the time I had the opportunity of spending in his department, and to Dr. *M. Vogt*, under whose guidance I had the pleasure of working. I also owe my best thanks to Dr. *R. Barer*, of the University of Oxford, who kindly demonstrated his ingenious methods and equipment during my visit to Oxford. He has further given me valuable advice both orally and in numerous letters as well as helped in the construction of a photometer. I am much indebted to many other British colleagues, particularly to Dr. *A. G. E. Pearse*, of the Postgraduate Medical School of London University, as well as to Dr. *K. W. Metcalf*, of the University of Bristol, and to Drs. *F. Jacoby* and *B. F. Martin*, of the University of Cardiff, for stimulating discussions on histological and histochemical problems related to the subject of this volume. My journey to Great Britain was made possible by a grant given by the *British Council*, which is gratefully acknowledged. Dr. *M. J. Karvonen* has given valuable suggestions and assisted in the proof-reading, for which I am very grateful to him. Thanks are due to Dr. *H. R. Mitchell* for correcting the English and to Mrs. *T. Rytilä* for typing the manuscript.

I should also like to thank Dr. *R. C. Mellors* and *the American Association for the Advancement of Science* for permission to reproduce Fig. 21, Messrs. *Kodak Ltd.*, London, for Figs. 35 and 36, and Messrs. *Mullard Ltd.*, London, for Fig. 42. A number of figures from various sources have been reproduced slightly modified; the corresponding references are given in the text.

For excellent co-operation and friendly dealings I wish to thank Dr. *H. Karger* and Messrs. *S. Karger AG.*, Medical Publishers.

Autumn 1954. O. E.

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INTRODUCTION

All histological and a great proportion of histochemical observations are done by examining tissues under the microscope. To illustrate the great importance of subjective judgment in this respect, let us consider a blood smear under a high magnification. A haematologist might describe the view shortly as follows: «There are strongly coloured, large erythrocytes, some of which are oval. Probably pernicious anaemia.»

Let us then show the same visual field to a layman, say to an artist, who can be expected to have experience in description of forms and colours. The artist might think that the corpuscles are pale, small rather than large, and remarkably circular, i.e. his opinion would be the exact opposite to that of the haematologist.

Both opinions can be motivated. The haematologist has seen thousands of blood smears and the cells now concerned seem to him large, strongly coloured and aspherical in comparison to his idea of normal erythrocytes. The artist, on the other hand, compares the colour of the erythrocytes to the strong red hues used by expressionists, and the monotonous sphericity of these cells to the rich forms of non-figurative sculptures. That the cells are small is obvious to him because a microscope is necessary to make them visible.

Although "large" to a haematologist is something else than "large" to an artist, his description may nevertheless convey the right meaning to another haematologist, whose subjective criteria are largely similar. However, even every haematologist is different from any other haematologist as is every histologist from any other histologist, and discrepancies may appear. These discrepancies can be eliminated by using objective instead of subjective criteria, i.e. by measuring instead of assessing.

The use of quantitative instead of qualitative terms is always combined with extra trouble. If one wants to answer the question "how large?" instead of just saying "large", measurements must be done. However, measuring every erythrocyte in the blood of a man would keep the whole population of London busy 8 hours a day for

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six months, supposing that 1 second is given for measuring each cell. This creates a new question "how many cells are to be measured?" Suppose that this problem is solved and the measurements are carried out. A number of figures is obtained and with them new questions: "what to do with these figures?", "how to compare this data with that obtained from another person?", etc.

To solve these questions, the investigator must be familiar with statistical principles. Though there still are scientists who think that statistics is a kind of scientific snobbery by which right things are proved wrong and vice versa, an effort to understand the statistical method will prove to anybody the value and necessity of statistical considerations in design of experiments and in interpretation of quantitative data.

In many biological papers either inaccuracy of the method or the limited number of observations has been said to prevent the use of statistical methods. However, the main basis of statistics is the necessity of using quantitative methods which are not faultless, and of limiting the number of observations. Results obtained with a small material particularly need statistical analysis and may then be found sufficiently reliable. Creation of pertinent testing methods for small samples is indeed one of the most important achievements of modern statistics.

It has frequently been claimed that, e.g., absorption measurements on stained tissue sections are useless unless it can be shown that reliable information is thus obtained regarding absolute amounts of some specific substances in the tissue. This is equivalent to throwing the child away with the washing. A change in the absorption properties of a stained tissue may, it is true, be due to increase, decrease, or redistribution of stainable materials, to loss or gain in the stainability of these materials, to appearance of new materials reacting with the dye, to an altered physical state or indeed nearly anything else. Still, if the change in the absorption has been proved to depend on some experimental procedure it is completely justifiable to use absorption measurements as an empirical means of obtaining information of the state of the tissue. Generally, any quantitative method can be used, although many variables would simultaneously influence the property to be measured.

This volume describes means by which figures can be extracted from tissue sections and tries to show the critical ways of handling the figures thus obtained. No effort has been made to include all quantitative methods hitherto published. Many valuable techniques, e.g. polarization microscopy, have not been discussed at all. The two aspects mainly concerned are the quantitative topography of and light absorption measurements in tissue sections. Even these subjects are broadly outlined and the emphasis has rather been on the presentation of basic facts in an understandable way than in thorough review of the knowledge accumulated. The result is a mixture of histology, physics, chemistry, and statistics. It has been tried to make the mixture palatable, by using simple words and no mathematics above the high-school level; and nourishing, by combining materials which complete each other's value. Key references are given to modern literature, frequently without regard to historical or priority aspects.

Many methods of quantitative histology and histochemistry require complicated equipment both expensive and difficult to use. As such apparatus can anyway be reached by a few laboratories only, and as the writer's experience with them is negligible or zero, they are not dealt with in detail. Practically all methods to be presented are possible with equipment either already available in most histological laboratories or easy to construct without excessive funds or technical facilities. It is felt that there is an unnecessarily big gap between highly specialized quantitative studies done with complicated and ingenious devices and studies in which no attempt has been done to quantitate the data. Obviously, as has frequently been pointed out by masters of the advanced methods of quantitative histochemistry, there are many sources of error in the quantitative methods. This fact does nevertheless not eliminate the use of simple techniques as long as their limitations are kept in mind and the results obtained with them are correctly interpreted. This volume tries to point out the numerous sources of error and give hints to their control. Basic knowledge of the various aspects of quantitative methods is believed to be useful not only to the histologist doing quantitative work in practice but also to his colleague who does not like bias although he prefers to express his observations in qualitative terms.

CHAPTER I

Variability and its Measures

Distributions.

Let us first return to the example of red blood cells mentioned in the introduction. Suppose that the size of the erythrocytes is under investigation. The investigator may have measured diameters of 423 erythrocytes. This series of numbers may be further studied in different ways. One way, which has proved to be of great value, is to put the figures into the order of magnitude. The number of erythrocytes of each size is then counted (this were naturally impossible, if the diameters had been measured so precisely that all

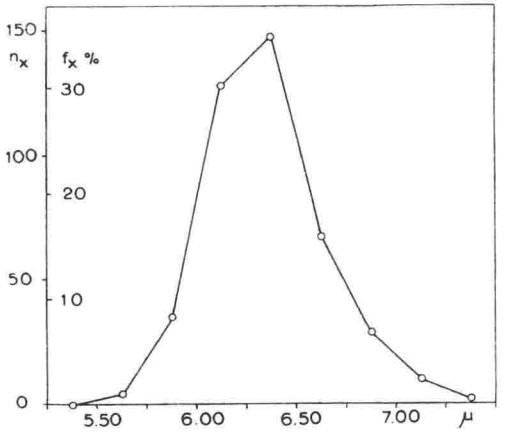

Fig. 1. Diameter-frequency distribution curve of red blood cells.

cells had different diameters; here we suppose that the diameters have been measured to the nearest half of a μ). The number of cells of some size x is then the *frequency* n_x of this size. By putting the diameters on the abscissa and the corresponding frequencies on the ordinate a *frequency distribution curve* is obtained. The frequency can as well be expressed as per cent of the total number of ob-

servations (relative frequency f_x) which in the present example is simply done by dividing the absolute frequency by 4.23. A curve like this is well known amongst clinicians as a Price-Jones curve. An actual curve is illustrated in Fig. 1.

The distribution of some other property of the erythrocyte, such as the haemoglobin content, can be examined in the same way. In general, the empirical distribution of any observed variable is similarly obtained.

In many applications it is useful to form a series of *cumulative* frequencies by adding step by step the frequencies upwards from down (i.e. from the smallest size-class) (Fig. 2). As the frequency f_x

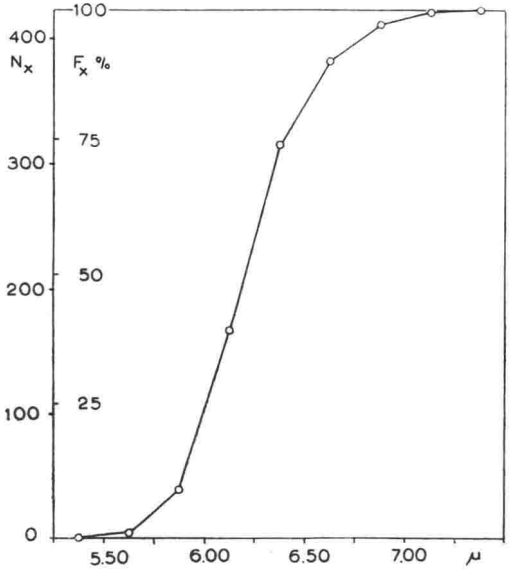

Fig. 2. Cumulative distribution curve prepared from the same data as Fig. 1.

tells how great a proportion of the observations belong to a given size-class, the cumulative frequency F_x tells how great a proportion belongs to this class or to classes of smaller size.

In grouping observations the number of classes should not be made unduly large. A practical rule is that the number of classes should be approximately equal to the cube root of the number of

observations. For example, some 30 observations should be grouped in 3 or 4 classes, some 100 observations in 4 or 5 classes, 1000 observations in about 10 classes and so on. Generally, the number of observations in a single class should not remain smaller than 5.

If the distribution is skew, i.e. if the frequency curve shows a "tail" in one or another direction, it may be useful to apply unequal class intervals, whereas the intervals should be kept constant in all ordinary classes. In particular, if the logarithms of the observed values are of interest (see page 15), the class boundaries can be chosen in geometric progression, for example forming the classes (in arbitrary units) 1, 2 ... 3, 4 ... 7, 8 ... 15, 16 ... 31, etc., in this case the lower boundary of the class rising in geometric progression. Note well that a) the cumulative frequency curve is unaffected by the grouping, but b) the frequency curve must be adjusted for unequal class intervals. For example, if the per cent frequencies are

25 % in size class 2 ... 3
15 % in size class 4 ... 7,

the interval is two units in the first class, the frequency being thus $\frac{25}{2} = 12.5\%$ *per unit*, whereas the interval in the second class is 4 units, the frequency per unit being $\frac{15}{4} = 3.75\%$. The frequency curves should always be drawn adjusted as described here.

Two main types of distributions can be distinguished on the basis of the nature of the variable in question, viz. a) continuous and b) discrete distributions. All size distributions, for example, are continuous, as the variable (the size) can obtain any value on the continuous size axis. All distributions based on counting, on the other hand, are discrete, as the result of counting can obtain integral values only.

Special Distributions.

In building mathematical models so as to aid the treatment of statistical data, the *distribution functions* play an important role. A distribution is said to be completely known if there is a mathematical equation with one or more *parameters* by which one can calculate

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