STUDIES ON TOXICITY.

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I. *Criteria of Toxicity toward Microorganisms.*

Extensive studies of the effect of toxic agents have been made in recent years upon both unicellular organisms and multicellular tissues. There have been many criteria of injury and death suggested and used, but terms have not always been employed by various writers in the same sense. The present paper is an attempt to indicate those criteria which have been found helpful when dealing with microorganisms, and particularly to point out the usefulness of staining reactions of cells in securing quantitative results on studies of toxicities.

In general any agency or material which produces an injurious effect upon the normal functions of a cell may be characterized as toxic. If two or more such substances produce equal harmful effects in equal lengths of time under otherwise uniform conditions they may be termed isotoxic. Commonly in such studies the observations are continued until all of the cells have undergone the maximum injury detectable by the criterion used.

Several methods of detecting cell injury on the one hand or toxicity of materials on the other have been proposed. Among those which are apparently most useful for studies of microorganisms in that results may be followed quantitatively are the following: changes in rapidity of multiplication or power of reproduction, changes in the electrical conductivity of the cells, changes in the permeability of the cell membranes, plasmolysis, variations in rate of metabolism, and changes in the ability of the cell to stain with various dyes.

In work with microorganisms, particularly with bacteria, the criterion of injury most frequently used has been the death of the cell as indicated by the complete loss of reproductive power. Antiseptics,

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germicides, and disinfectants are toxic substances which more or less promptly kill cells as evidenced by their loss of reproductive capacity when replaced in a favorable environment. For representing graphically the relationships between time and the death of cells three types of graphs are in common use. The number or percentage of cells still living after varying intervals of contact with the toxic agent may be plotted against time, the curve thus secured is commonly termed the survivor's curve. Or the number of dead cells may be plotted against time of exposure, the resultant curve being termed the death curve. Or the number of cells which succumb during each equal interval of time may be plotted against time to constitute a rate curve, or distribution curve. The area under the rate curve is proportional to the total number of cells, the area to the left of any point on the x axis is proportional to the total number of cells dead, the area to the right is proportional to the total number of cells still living. It is possible, therefore, to derive each curve from either of the others. Much study has been devoted by microbiologists to the forms which these curves assume, and to the development of simple equations to represent them.

It is apparent that curves of the same general types as those used for survival or death of microorganisms may be used for the illustration of effects of toxic agents when some criterion of injury other than failure to reproduce is used. A curve of the type of a survivor's curve may be plotted to show the relationship between time and cells not exhibiting the particular effect of the toxic agent. Possibly the term survivor's curve may well be used for all curves of this type provided it is clearly understood in each case that the criterion of a "survivor" is not necessarily ability to reproduce. A rate or distribution curve may also be plotted. There is some danger of confusion, however, if the term death curve is applied to a plot of time against the number of cells showing the change to be observed regardless of the criterion used. Possibly such a curve may be termed a toxicity curve.

Many quantitative studies have been made of the effect of toxic agents in which changes in the electrical conductivity of cells has been the criterion of injury. The studies of Osterhout (1922), of Shearer (1919), and of Brooks (1923), are particularly interesting in their quantitative approach. The graph of electrical conductivity and

time may properly be developed as a toxicity curve, and solutions producing equal changes in electrical conductivity (or more usually the maximum change in conductivity) under otherwise identical conditions in equal lengths of time may be termed isotoxic with reference to this particular criterion. A similar toxicity curve might be constructed as a graph of permeability against time.

Of particular interest for the securing of quantitative data on toxicity are the methods which have been set forth in a series of papers from the University of Toronto on the general subject of chemical potential and toxicity. The detailed technique may be found in the papers by Miller (1920), Burgess (1920), Fraser (1920, 1921) and Fulmer (1921). In this series is sought an explanation of such phenomena as the increase of toxicity of phenol in aqueous solution when sodium chloride, alcohol or other substances are added. It was found that the addition of NaCl or alcohol to a solution of phenol in contact with an immiscible solvent such as toluene or kerosene causes the phenol to become less concentrated in the water and pass in part to the other solvent. This fact may be expressed in one of several ways: the chemical potential of the phenol is increased; the fugacity or tendency of the phenol to escape is increased; the solubility of phenol in the aqueous phase is decreased; or the phenol is driven into the non-aqueous phase. For these studies yeast cells were used generally, the criterion of injury first employed was the loss of the power of the cell to reproduce; *i.e.*, death rates were determined by plating. Later the acquisition of the ability of the cells to stain with an aqueous solution of methylene blue was taken as a second criterion of toxic action.

It would seem that this criterion of injury is worthy of more attention in toxicity studies. It has been shown that all cells which take up the stain are dead, that is, they no longer can reproduce. However, it was found that the ability to reproduce was lost *before* the cell acquired the ability to stain. In other words, cells which stain are dead, but a dead cell does not of necessity immediately acquire the ability to stain. The two phenomena are closely related, so that cell staining is a satisfactory criterion of toxicity.

Data are so much more rapidly obtained by observations on the staining reactions of yeast cells than by other methods of securing quantitative results on toxicity that it has been thought advisable to

secure and plot the data secured by this means. It is believed that results of value may be secured by a study of the shapes of survivor's and rate curves obtained.

Summary.—The acquisition of the ability of yeast cells to stain with methylene blue as an index to the toxicity of the solution in which the cells are placed is an important addition to the criteria which have been suggested for the detection of toxicity, and may be adapted to a wide variety of such studies.

Solutions which are isotoxic when one criterion of toxicity is used may not be isotoxic when some other criterion is used.

It is desirable that comparative studies should be made of the results secured by the use of various criteria of toxicity that have been suggested.

II. On Conformity of Yeast Survivor Curves to the Monomolecular Reaction Law.

Differences in opinion as to the interpretation to be attached to the form of the curve secured in the investigation of death rates of microorganisms by plotting the number of surviving organisms against time has led to two groups among students of the problem. The one group holds that experimental data thus plotted will give a logarithmic curve, and attempts to explain this on the basis of the laws of physical chemistry, even suggesting in some cases that the form of this curve is evidence of the monomolecular nature of the reaction. The second group holds that any resemblance between the curves such as has been described is superficial and fortuitous and that we must look to variations in resistance of individual cells as well as to the distribution of such variations to account for the form assumed by the curve.

Paul and Krönig (1897) were apparently the first to develop a satisfactory method of determining death rates of microorganisms and used it to secure data on the numbers of bacteria surviving after varying intervals of time in the presence of disinfectants. They showed that disinfection, that is, killing of the organisms present, did not occur at the same instant, but they did not deduce any exact relationships between number of survivors and time. Ikeda (1897) attempted to do this, and concluded that the ratio between the products of time and survivors was a constant. Renewed interest was taken in the problem when Madsen

and Nyman (1907) plotted data which showed excellent agreement between the curves secured and the curve of a monomolecular reaction. Chick (1908) repeated and extended these experiments and concluded: "An ideal case of disinfection, such as that of anthrax spores, may therefore be supposed, with experimental support, to proceed in accordance with the equation— $\dfrac{dn}{dt} = Kn$, an equation exactly similar in form to that expressing the course of a unimolecular reaction, or reaction of the first order." However, she noted discrepant results when a non-sporing organism (*Bact. paratyphosum*) was used, "the reaction velocity diminishing during disinfection more rapidly than is accounted for by the fall in number of the surviving bacteria." "This divergence is due to differences in resistance between individuals of the various ages contained in such cultures." Phelps (1911) on theoretical grounds, using the evidence presented by earlier writers, states: "The rate of dying, whether under the influence of heat, cold, or chemical poison, is unfailingly found to follow the logarithmic curve of the velocity law, if the temperature be constant. This curve never reaches a zero value, although approaching it indefinitely." Lee and Gilbert (1918) clearly differentiate the two schools of thought with reference to the disinfection process under the terms "vitalistic" and "mechanistic." The vitalistic concept is that "the progressive nature of the disinfection process is accounted for as being due to a type of biological variation, namely, *permanent differences* in the degree of resistance possessed by the various individual microorganisms of a 'pure culture.' This is commonly known as the theory of variable permanent resistance. The mechanistic conception of the process of disinfection is that it is an orderly time-process presenting a close analogy to a chemical reaction, the micro-organism and the disinfectant being regarded as the respective agents." They conclude as a result of data which they present: "The explanation why disinfection is not sudden, but gradual, and proceeds in close agreement with the well defined logarithmic law, known as the Mass Law, is best found in those chemical processes which proceed as 'uni-molecular' reactions." Recently Cohen (1922) has contributed a careful study of the effect of temperature and hydrogen ion concentration upon the viability of *Bact. coli* and *Bact. typhosum* in water. He concludes that with the exception of a more or less prolonged "induction period," "the mortality of bacteria whether by strong disinfectants or by milder agents follows the laws of logarithmic decline. It is shown that the course of the disinfection process can be expressed by mathematical relations comparable to those used in dealing with monomolecular chemical reactions." He also emphasizes that "The chance distribution of variable resistances assumed for bacteria is paralleled by the chance distribution of variable energy quotas in the molecules of a substance. Furthermore, the logarithmic rate observed, whether in chemical phenomena or in disinfection, is actually a statistical resultant of like significance in both."

During the development of the mechanistic theory, its opponents have not been idle. Reichel (1909) insisted that the results secured by Madsen and Nyman must

arise either from unequal cell resistance or from an unequal distribution of the organisms in the liquid. Loeb and Northrop (1917) comment on Chick's assumption that "'bacteria are killed by disinfectants at a rate corresponding to that of a monomolecular chemical reaction; *i.e.*, that in each interval of time the same percentage of individuals alive at this time is killed.' She was probably led to such an assumption by the fact that the ascending branch of the mortality curve in her experiments was generally very steep. The agencies used by her for killing the bacteria were so powerful that the ascending branch became almost a vertical line, thus escaping detection. Hence she noticed usually only the less steep descending branch which could be interpreted as a monomolecular curve for the reason that her experiments lasted only a short time." For the fruit flies with which they worked they conclude: "The fact that the frequency curve of deaths is that of a probability curve shows that the difference in the duration of life of different individuals for the same temperature is due to individual variation." Brooks (1919) after a review of the data concludes: "the course of such processes as hemolysis is very largely dependent upon variations in resistance among the different individuals, and secondarily upon the course of the fundamental reaction," and further "Unnatural assumptions would be requisite for the explanation of a resemblance between the course of such processes in general and that of a monomolecular reaction.

"The supposition that such a general resemblance exists is not supported by the available evidence." Peters (1920) studied the effect of mercury upon *Colpidium*, and concluded that death occurs in "such a way that there is a straight line relationship between the time and the logarithm of the percentage of survivors for a large part of the death process. The apparent logarithmic law can be interpreted in terms of the variation in the resistance of individuals of the protozoon culture to the toxic agent." Smith (1921) studied the survivor curve of *Botrytis* spores treated with phenol. He definitely shows the characteristic curve for these to be sigmoid in many cases, but as the quantity of phenol is increased there is a tendency for the maximum death rate to occur proportionately earlier and the curve approaches more nearly the logarithmic type. He states: "Both types of curves are shown to be explicable on the assumption that the individual spores differ in resistance and that a frequency curve showing the distribution in the resistance grades approaches the normal curve." Recently Reichenbach (1922) has reviewed the theoretical basis of disinfection. He concludes that it is quite impossible to imagine how two bacteria having equal resistances should die after different periods of time when subjected to the same unfavorable environment. While in many cases the survivor's curve is approximately logarithmic, this cannot be taken for granted under any given set of conditions until an actual test has been made.

It is apparent that it is a matter of unusual interest both from a theoretical and a practical point of view to determine whether or not

there is such substantial conformity to the logarithmic law as would permit of its utilization for estimation and comparison of the value of disinfectants.

EXPERIMENTAL.

It was pointed out by Fulmer (1921) that yeast cells taken from a wort culture in which the alcohol has reached a certain concentration, are much more resistant to phenol than cells from a culture which contains little alcohol. This fact was taken advantage of by using cells from a young culture for weak poisons and old cells with their greater resistance for the stronger poisons. Clark (1922) made a further study of the effect of the accumulation of alcohol upon the yeast.

The yeast was filtered, washed, and then suspended in the poison in the "rocker" tube at 25°C. At intervals a 1 cc. sample was placed in 1 cc. of 0.01 per cent methylene blue. The percentage of the cells stained by the dye was determined microscopically.

Survivor's curves were plotted using as an index of toxicity the per cent of cells stainable by methylene blue after a given time in contact with the poison. Twenty-seven curves were chosen at random for analysis in this paper. The tabular data are too voluminous to publish in this connection.

In each case the numbers of surviving organisms have been calculated in percentages. To make the various curves comparable the lengths of the abscissæ used to represent complete disinfection have been made equal, that is equal distances for the x axis represent equal percentages of the total disinfection time. A frequency curve showing the distribution of resistances was then plotted. Resistance is here interpreted as the time required under the conditions of the experiment for a cell to acquire stainability with the methylene blue. The logarithms of the numbers representing the survivors were also plotted.

A study of the twenty-seven groups of data thus plotted shows decided differences in the distribution of resistances. These are illustrated by solid blocks in the accompanying figures. It was found that the shape of the frequency curves secured by joining the tops of these blocks varied widely. The types of these curves may be included under several classes and subclasses. These groups together with the number of cases belonging to each are listed below.

I. No well marked maximum, distribution of resistances
    relatively uniform............................... 4 cases.
II. With one maximum well marked.................... 19 cases.
    1. With maximum to the left of median............ 14 cases.
        1st place, *i.e.*, the largest percentage of cells died
            in the first tenth of the time.............. 9 cases.
        2nd place.................................. 3 cases.
        3rd place.................................. 2 cases.
    2. With maximum median........................ 2 cases.
    3. With maximum to the right of median.......... 3 cases.
III. With two maxima................................ 4 cases.

One or more typical illustrations will be given for each group, and
the character of the curves noted. In each case the poison used is
indicated in parenthesis following the number of the curve, the first
number expressing the number of grams of phenol and the second the
number of grams of alcohol per 100 cc. of solution.

Fig. 1, *a* (0.650–0) illustrates an instance in which the distribu-
tion of resistance is quite uniform, that is, nearly the same num-
bers of organisms die in each equal interval of time. The survivor's
curve in this case approximates a straight line, and the plot of the
logarithms of the survivors is curved throughout its length. Appar-
ently this is not the most common of distribution of resistances. It is
evident that a distribution of this type is entirely out of harmony
with the monomolecular concept. Fig. 1, *b* (0.580–4.75) illustrates
a case in which the survivor plot is somewhat curved, but is far
from logarithmic. Fig. 1, *c* (0.65–0) is interesting in that during
the first two periods of time comparatively small numbers of cells
stained, in later periods a larger and quite uniform number stained.
The survivor's curve is therefore practically two straight lines. ˙ It is
evident that in certain cases (4 among 27 studied) the yeast cells
acquire the ability to stain in such a fashion·that the survivor's
curve approximates a straight line.

In Fig. 2 is given a series of three curves in which there is a distinct
maximum during the first time period. Nine of the total of twenty-
seven distributions are of this general type. Fig. 2, *a* (0.650–0)
illustrates a survivor's curve which is an excellent approach to the
logarithmic type; *i.e.*, the logarithms of the numbers (except at the
end) plot as a straight line. In some cases, as shown in Fig. 2, *b*

(0.570–4.75) the survivor's curve is logarithmic for more than half its length, and practically linear for the remainder.   Fig. 2, c (0.500–0) shows a survivor's curve with a double inflection; in this case there is no resemblance to a logarithmic curve.   It is evident even in cases in which the maximum number die in the first time period the survivor's curve plotted may or may not be logarithmic.
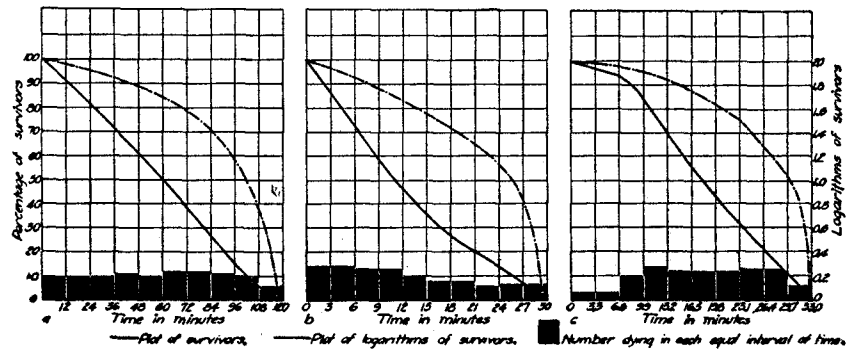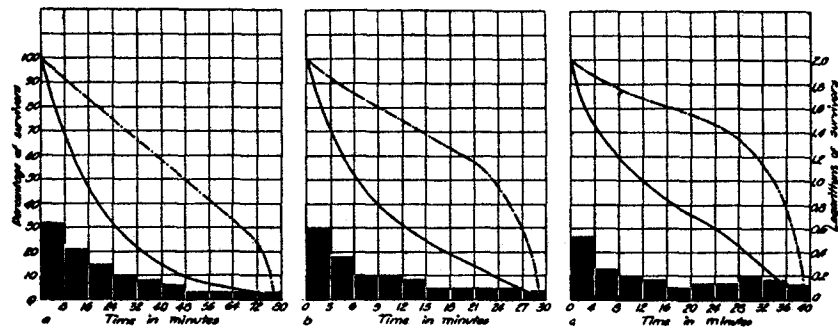

FIG. 1.

FIG. 2.

The two curves in Fig. 3 show distributions in which the maxima are reached in the second time period.   It will be noted that the survivor's curves are sigmoid in shape and are not strictly logarithmic. In Fig. 3, a (0.650–0) a portion of the curve approximates a logarithmic curve.   In Fig. 3, b (0.620–4.75) however, the approximation is even less evident.

Fig. 4 embodies results of the type secured when the frequency curve approaches a normal distribution curve. The best approximation is in Fig. 4, a (0.570–4.75). In this as in Fig. 4, b (0.650–0) the sigmoid character of the survivor's curve and lack of logarithmic character throughout its length are evident.
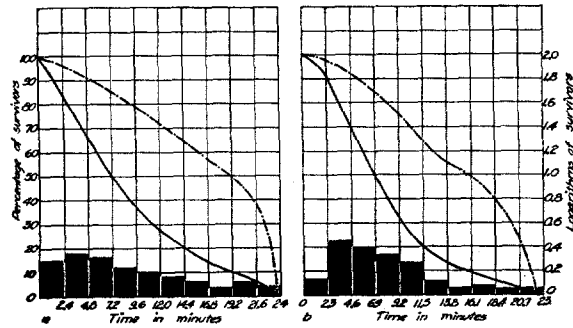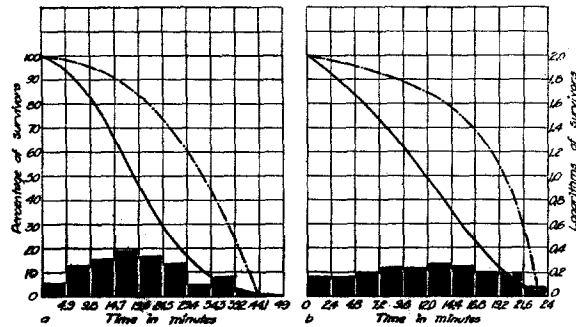

FIG. 3.

FIG. 4.

In three instances the single maximum was found to be well to the right of the middle of the rate curve. In Fig. 5 a (0.650–0) the part of the survivor's curve to the right of the maximum is approximately logarithmic, but the sigmoid character of the curve as a whole is evident in this as well as in Fig. 5, b (0.710–4.75).

In four instances two distinct maxima were to be noted; i.e., the frequency curve was bimodal. Fig. 6, a (0.650–0) illustrates a case

in which the maxima were found in the first and seventh time periods. The survivor's curve here has three points of inflection. Fig. 6, *b* (0.650–0) shows a bizarre arrangement, the survivor's curve showing four points of inflection. Fig. 6, *c* (0.465–4.75) is interesting in that the maxima occur in the second and last periods and the survivor's shows two inflections.
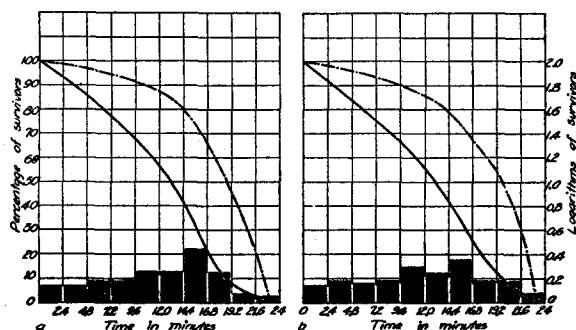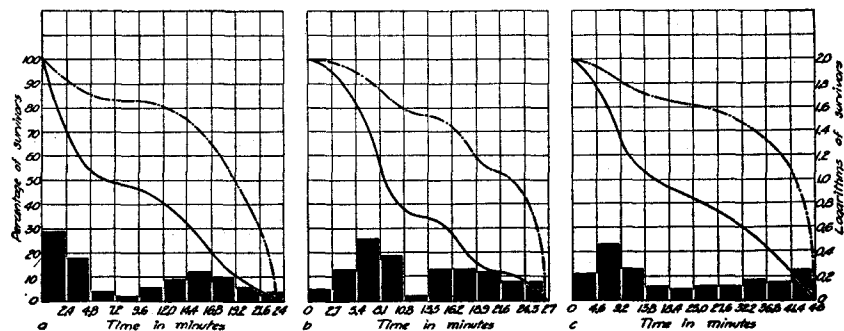

FIG. 5.

FIG. 6.

There is no obvious relationship between the type of distribution curve and the kind or concentration of poison used and the age of the culture. The concentration of yeast cells was the same in each case; *i.e.*, about 0.10 gm. of yeast per 100 cc.

SUMMARY AND CONCLUSIONS.

With yeast subjected to the action of phenol or of phenol and alcohol the curves of survivors as measured by resistance to staining with methylene blue when plotted against time do not in general prove to be logarithmic.

It is believed that such resemblances as have been found between such curves and monomolecular reaction or logarithmic curves are superficial and fortuitous. Any method therefore of evaluating disinfecting power based upon such a concept must prove misleading.

Variations in resistance of individual cells and the distribution of such variations must be regarded as of fundamental importance in accounting for rates of death of microorganisms.

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