

## TOXICITY AND CHEMICAL POTENTIAL

BY W. LASH MILLER

In 1895 Scheurlen<sup>1</sup> discovered that the addition of sodium chloride to solutions of phenol increased their toxicity towards anthrax spores and towards staphylococcus; he explained his discovery by a hydrate theory. The discovery was confirmed and the theory rejected by Beckman;<sup>2</sup> and in 1897 Scheurlen<sup>3</sup> discarded his theory in favor of another one, viz., that the degree of dissociation of phenol in solution is affected by the addition of salt. In the meantime, Paul and Krönig<sup>4</sup> had published the results of an extensive series of experiments in which they showed that a number of other salts shared the power of sodium chloride to affect the toxicity of phenol; their conclusions were summed up in the words "In general, organic salts seem to have less influence than inorganic salts, and sodium salts are more effective than those of potassium, but we are not able to offer a satisfactory explanation."

While writing a paper "On the Second Differential Coefficients of Gibbs' Function  $\zeta$ ,"<sup>5</sup> at about the time this toxicological work was coming out, it occurred to me that the increase in the chemical potential of the phenol due to addition of salt to its aqueous solution might well account for its increased action on bacteria; if this were so, a solution of phenol to which salt had been added would have the same toxic effect as the (more concentrated) solution of phenol in pure water which has the same phenol potential; that is, two phenol solutions, with or without salt, which were in equilibrium with the same solution of phenol in some immiscible solvent, would prove to be isotoxic.

This conclusion was supported by the observations of

<sup>1</sup> Arch. f. exp. Path. u. Pharm., **37**, 74 (1895).

<sup>2</sup> Centrbl. f. Bakteriologie, **20**, Abt. I, 577 (1896).

<sup>3</sup> Münch. Med. Wochenschr., **44**, 81 (1897).

<sup>4</sup> Zeit. phys. Chem., **21**, 414 (1896).

<sup>5</sup> Jour. Phys. Chem., **1**, 633 (1897).

Paul and Krönig that a 4 percent solution of phenol in absolute alcohol (with which phenol is miscible in all proportions), and a 4 percent solution of phenol to which an equivalent amount of sodium hydrate has been added, are neither of them toxic to anthrax; while "acid. carbol. liquef.," containing about 90 percent phenol, is if anything, less toxic than a 5 percent solution of phenol in water.

### **The "Biological Lift" and the "Machine"**

No opportunity to test this theorem further presented itself until the winter of 1902-3, when Prof. J. J. Mackenzie undertook the necessary bacteriological experiments. It was decided to work with anthrax, but instead of using spores dried on threads or garnets, as did Paul and Krönig, it seemed better to infect the phenol solutions from a suspension of the spores by means of a platinum loop.

Experiments with 30 percent sulphuric acid, however, showed that the volume of liquid lifted by a given loop, as determined by titration, was a very variable quantity. When the plane of the loop was kept parallel with the surface of the liquid, and lifted out with a sharp turn of the wrist (the "biological lift"), the volume of  $\frac{1}{26}$  normal potassium hydrate needed to neutralize the loopful of acid varied from 1.14 to 1.61 cc. When the plane of the loop was kept perpendicular to the surface, the amount fell to 0.29-0.42 cc if taken from the middle of the vessel, and to 0.16 if taken from the edge of the meniscus. A quick lift, moreover, might remove 40 percent more acid than a slow one. A platinum tube was then made by winding fine platinum wire around a pin, heating, hammering, and dissolving out the pin with nitric acid; this was lifted perpendicularly, it removed acid equivalent to 1.34-1.40 cc potash if lifted quickly, 0.94-0.97 if lifted slowly.

The factors on which uniformity depends having thus been discovered, a "machine" was constructed, consisting of a balanced beam to one end of which the platinum wire with loop or tube was attached by a set screw (binding post), the

other end being somewhat overweighted by a piece of chain, whose end hung down to the table.<sup>1</sup> In using this apparatus, the loop was immersed in the acid till just covered, and a trigger was sprung; the weight of the chain lifted the tube perpendicularly through the centre of the meniscus at a fixed speed; and then as the loop rose and the length of chain on the table increased, the whole came gently to rest, without jolting out the drop. With this device the volume of acid lifted depended only on the depth to which the tube was immersed; if attention were paid to this matter uniform results were attained, the deviation between greatest and least of six successive determinations never exceeding five percent of a total volume of about 0.005 cc.

This machine was used in all the work with anthrax and staphylococcus referred to below, and gave good satisfaction; but it soon became apparent that equal volumes of the same suspension of spores or cocci were far from containing an equal number of cells; even when the suspension had been thoroughly centrifuged to remove clumps, two 10 cc portions of agar infected by two successive loopfuls would often differ by 20 percent in the number of colonies they would produce.

#### Determination of the Equivalent Solutions

Toluene was selected as immiscible solvent, and 50 cc of aqueous solutions of phenol of various known concentrations, with or without salt, were placed with 10 cc toluene in stoppered bottles in a thermostat at 25° C and shaken repeatedly. When the two layers had finally separated, a glass tube with a thin bulb blown on the lower end was passed through the upper layer, the bulb crushed against the bottom of the bottle, and a portion of the lower (aqueous) layer pipetted out for analysis. The concentration of phenol in the upper layer was calculated by difference.

In analyzing the solutions, at first Koppeschaar's method<sup>1</sup> was used (it depends on the action of bromine on phenol); but his procedure was found most unsatisfactory, duplicate

<sup>1</sup> Zeit. anal. Chem., 15, 233 (1876).

analyses often differing by as much as two percent. The trouble was found to lie in the formation of tribrom-phenol-brom; Mr. S. J. Lloyd studied the rate at which this interfering chemical was produced,<sup>1</sup> and established conditions under which accurate determinations could be made. Lloyd's method of analysis<sup>2</sup> was used in all subsequent work.

The following are the results of the determinations with solutions of phenol in water, and with solutions containing 2 percent of salt as well as phenol. By "percent" is to be understood number of grams phenol (or salt) in 100 cc solution.

No Salt

Aqueous layer

0.21 0.45 0.76 0.97 1.13 1.34 1.42 1.70 1.88% phenol

Toluene layer

0.34 0.88 1.69 2.22 2.65 3.24 3.80 4.93 5.73% phenol

2.0% Salt

Aqueous layer 0.20 0.44 0.58 0.79 1.00 1.25 1.49 2.00% phenol

Toluene layer 0.38 0.98 1.37 1.98 2.75 3.75 5.38 9.02% phenol

These results are plotted in the accompanying figure, in which the abscissas of points on the two curves which have the same ordinate give the percentage of phenol in "chemically equivalent" solutions, one with no salt, the other containing 2.0 percent salt in addition to the phenol. Thus, solutions containing 0.6, 0.7, 0.8 and 1.0 percent phenol without salt are chemically equivalent to, i. e., have the same chemical potential of phenol as, solutions containing 2.0 percent

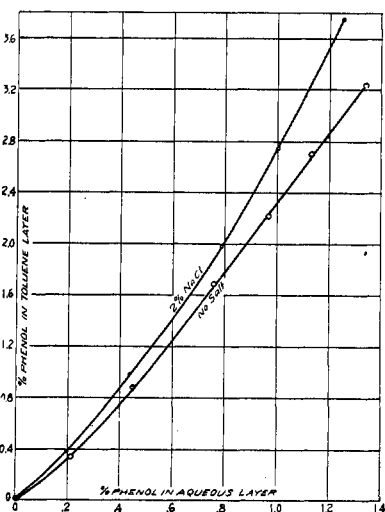


Fig. 1

<sup>1</sup> Jour. Am. Chem. Soc., 27, 7 (1905).

<sup>2</sup> Ibid., 27, 16 (1905).

salt and 0.54, 0.63, 0.72 and 0.88 percent phenol, respectively; and on the assumption made, these solutions should be isotoxic each to each. The same numerical results can, of course, be obtained without the graph, by arithmetical interpolation from the experimental results given above.

A large number of determinations were also made with higher concentrations of phenol and of salt; but for the purpose in hand toluene proved a very unsuitable solvent, as the ratio between the concentration of phenol in toluene and that in the aqueous phase increases rapidly with the concentration. The hydrocarbon known as coal-oil, or kerosene, is freer from this objection; Mr. J. S. Laird, and after him Mr. C. G. Fraser made use of it for the determination of equivalent solutions, and Mr. E. I. Fulmer extended the experiments with toluene; the results obtained with the two solvents are in good agreement.

#### **Experiments with Anthrax Spores and with Staphylococcus**

Prof. Mackenzie's first experiments with anthrax and solutions of phenol and salt showed that in general the order of toxicity of the solutions is that of the concentration of their chemically equivalent phenol solutions, and a note to that effect was published.<sup>1</sup> They were continued in 1905-6 under his supervision by Dr. J. S. Lemon, and extended to include experiments with staphylococcus as well. No further work was done with anthrax, and that with staphylococcus was brought to a conclusion by Mr. Laird, working in the chemical laboratory through the winter of 1909-10, and by Mr. Burgess in 1913-14.

The results of these experiments, which are published in detail in the following papers, show that in general the toxicity of the solutions studied, (viz., phenol with various concentrations of sodium chloride, phenol with a fixed concentration of each of ten other salts) is in each case the same as that of the chemically equivalent solution of phenol in water; one or two exceptions were met with that may be

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<sup>1</sup> Trans. Roy. Soc. Canada, Sec. III, 51 (1903).

ascribed to toxicity of the salt itself, and one (viz., acetic acid and phenol) that needs further study. In dilute solutions, osmotic pressure less than 1.75 atmos., it was shown that staphylococci were killed by plasmolysis, without regard to the toxicity of the solutions employed; and that the resistance of the cocci to this attack is variable, and depends on their previous history.

The experience gained in this work brought out the weak points in the technique employed; apart from the difficulty of infecting the solutions with a constant number of cells—a minor matter—the trouble lies in the variability of the microbes employed as test objects. It proved quite useless to compare the death-rate of a given culture in a phenol solution with that of another culture in the equivalent phenol-salt solution; comparisons could be made only between two solutions infected at the same time from the same suspension. It was, therefore, impossible to cut down the work by determining once for all the toxicity of a set of phenol solutions and using the results as a standard.

This same variability of the microbes was the cause of another and even greater loss of time in the laboratory. It was never safe to assume, from the result of previous experiments, that the cells would all be killed after the poison had acted for a certain number of minutes, or that action of the poison for less than another (shorter) time would have a negligible effect; so in order to guard against the danger of having to repeat the whole series, it was always necessary to prepare and incubate many more plates than in the end proved useful, and each wasted plate took just as much time to prepare, and occupied just as much space in the incubator, as one that in the end proved worth while.

#### **Experiments with *Saccharomyces*: a Convenient Criterion of Death**

In view of the necessarily slow progress of the work under such circumstances, a search was made for some criterion of death that could be applied quickly and that would obviate

the three or four days' delay involved in waiting for the surviving cells to grow into countable colonies on agar; and it was also decided to select some one organism for the subsequent work and try to find conditions under which a more or less standard strain could be developed. A couple of almost adventures with anthrax and staphylococcus emphasized the desirability of working with a non-pathogenic form, and in the end the choice fell upon yeast.

Mr. C. G. Fraser, after experimenting with a number of other dyes, found that under certain conditions methylene blue was without action on living yeast cells, but rapidly stained dead cells, whether they had died a natural death or had been killed by heat or by poison. Comparison with the results of plating experiments showed that while the two criteria of death are not identical—a cell may be poisoned enough to lose its power of reproduction, without being dead enough to stain—yet they are so nearly alike that a few minutes with the microscope enables one to decide whether it is or is not worth while to pour a plate. This aid has proved of great assistance in all our subsequent work; the difference between the two criteria is being made the subject of further study.

He then compared the toxicity of phenol solutions towards yeast with that of the equivalent solutions containing phenol and salt, both by staining and by plating; and paid particular attention to the differences between the results obtained by the two methods. With low concentrations of salt the equivalent solutions are slightly more toxic, this difference increases with increase in the concentration of the salt; concentrated salt solutions themselves proved toxic. Similar results with phenol and alcohol were obtained by Mr. Fulmer.

#### **Standard Conditions for Yeast Culture**

This problem was taken up by Mr. E. I. Fulmer, who studied the resistance towards phenol exhibited by yeast cells taken from a culture in wort at varying intervals after inoculation. He found conditions under which yeast cells comparable from a toxicological point of view could be grown;

and traced the abnormal behavior of older cells to the influence of the alcohol generated in the wort. A number of experiments showing the regular rate of increase in the number of cells, and in the evolution of carbon dioxide under these standard conditions, were carried out by Mr. N. Clark.

As an interesting outcome of this work on variability, Mr. Fulmer was led to study the adaptability of yeast to ammonium fluoride, and its loss of resistance when grown in solutions free from that salt. Details of all these experiments will shortly be published.

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The results of this work, carried out at intervals over many years as opportunity offered, may be summed up in the statement that the toxicity of a solution containing phenol and an indifferent salt depends primarily on the chemical potential of the phenol in the solution; two solutions have the same toxicity if they are such as to be in equilibrium with the same solution of phenol in an immiscible solvent (toluene or kerosene). Complications may arise from the toxicity of the salts themselves, or in dilute solutions from plasmolysis of the cell independent of the toxicity of the solutions employed. One or two individual cases which do not seem to fall under either of these two heads deserve further study.

The observation of Paul and Krönig, that solutions of mercuric chloride in aqueous alcohol show a maximum of toxicity when the ratio of alcohol to water in the solution is as 1 to 3, affords another illustration of the same principle; for Laird has shown that the solubility of mercuric chloride in aqueous alcohol passes through a minimum at the same ratio.

*The University of Toronto*  
*June, 1920*