INHIBITION OF THE ACETONE-BUTYL ALCOHOL FERMENTATION BY ACIDS

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INTRODUCTION

During the course of a study of the effects of acids on the metabolism of the anaerobic organism *Clostridium acetobutylicum* (Weizmann) certain observations were made having to do with the more purely physico-chemical relationships of the acid association.[1] It is with some of these that this paper deals. Reference will be made to acid concentrations causing inhibition of the fermentation with considerations of the mechanism of the inhibitory effects.

An enormous literature has accumulated during the last forty years pertaining to the behaviour of microörganisms in acid media; since the early work of Kitasato (1888) and of Paul and Krönig (1896) the subject has been one of more or less continuous interest. With the general adoption, some fifteen years ago, of precise methods for the measurement of hydrogen ion concentration, microbiologists were enabled to characterize, much more definitely than was hitherto possible, the effects of many acids in terms of CH_+. But, as is frequently the case at a time when an easily applicable method becomes available for the accurate estimation of a factor formerly capable of only approximate determination, one discerns in some of the literature of the period immediately

[1] Until recently it has been the custom in this laboratory to name the organism *Bacillus granulobacter-pectinovorum*; but, in order to minimize confusion, the name suggested by McCoy, Fred, Peterson and Hastings (1926) has been adopted in this paper. The strain of the organism employed throughout all of our investigations is the original strain which was utilized industrially in Toronto during the war.

following the general introduction of pH measurements a tendency to emphasize unduly the significance of the information provided by the new technique. It seems to be generally recognized today, however, that the toxicity of acids for bacteria is not always merely a function of hydrogen-ion concentration, though in many cases this factor does, in reality, exert a predominant influence. Other factors involving, on the one hand, the anion, the undissociated acid and specific groups and, on the other, the nature of the organism and its substrate, must enter into any appraisal of the facts observed in studies of acid tolerance.

It is not proposed to review the extensive literature relating to the limits of acid concentration tolerated by microörganisms and to the general problems of the effects of acids on living cells. Summaries of investigations in these fields have been presented by numerous workers in the past. Attention is directed to certain papers which, in addition to contributing to the development of the subject, provide the key to the literature: e.g., Foster (1921), Wolf and Shunk (1921), Hall and Fraser (1922), Evans (1922), Berridge (1924), Pratt (1924), Falk and Harrison (1926), Katagiri (1926), Eggerth (1927). Investigations of a closely related nature, in the field of general physiology, have been reported by numerous workers, including Paine (1911), Harvey (1915), Crozier (1916, 1918), Collett (1919), Pantin (1923), Smith (1925), Lillie (1926, 1927), Velluz (1927), Taylor (1928), Bodansky (1928), Borissovsky and Wwedensky (1930). Some aspects of the problems of toxicity are presented by Miller (1920) and by Falk and Winslow (1926) while more extended reviews of related subjects are to be found in the works of Overton (1901), Traube (1913), Loeb (1913), Lillie (1923), Jacobs (1924), Henderson (1930).

The literature of the acetone-butyl alcohol fermentation reveals only a few references to studies of the growth of the related organisms in acidified media. None of the occasional studies which are encountered appears to represent a systematic investigation of the effect of any considerable range of acids. As early as 1876 Fitz observed that the growth of his butyl alcohol producing organism, *Bacillus butylicus*, in a medium otherwise favourable was impeded by 0.1 per cent (1.1×10^{-2} M) butyric acid. This

is an isolated observation and is of historical interest only, especially because the organism with which Fitz worked, though related to *Clostridium acetobutylicum*, was probably quite a distinct species, exhibiting a different degree of tolerance to acids and yielding different end-products. According to Fitz, his organism was quite acid-sensitive and it was necessary to culture it in the presence of $CaCO_3$. In Beijerinck's classical paper (1893) it is stated that "butyl bacteria are very sensitive to acids, 2 to 3 cc. of normal acid in 100 cc. being sufficient to stop the butyl fermentation completely." Here again the species studied were probably *not* identical with our organism. More recently Speakman (1920), working with *Cl. acetobutylicum*, observed that the fermentation proceeded to completion in cultures initially acidified with appreciable quantities of acetic, propionic and butyric acids. Limiting concentrations of these acids were not determined. At about the same time, Reilly *et al.* (1920) investigated the possible conversion of acetic acid to acetone when the acid was added to fermenting maize mash, the organism in this case in all probability being identical with our own. These workers however made no attempt to define the inhibiting concentrations of the acid. Fred, Peterson and Mulvania (1926) studied the influence of varying amounts of inorganic and organic acids on "*Granulobacter pectinovorum*," an organism which is probably identical with *Clostridium acetobutylicum*. Qualitative observations were made on the influence of lactic, acetic, butyric, sulphuric, hydrochloric and phosphoric acids on growth, on gas production and on the formation of the characteristic "head" in 5 per cent maize mash. The general conclusion of these authors was that "it is not the percentage of acid, but the pH value which determines its inhibiting effect on the butyl alcohol fermentation." In the case of the six acids studied, observations indicated that growth and fermentation were severely retarded in flasks in which the initial acidity corresponds to pH values of 4.7 to 4.8 and that by neutralizing the acid this inhibiting property was removed. A preliminary abstract report of some of the results included in the present paper was made by the author some time ago (Wynne 1929).

These few papers appear to constitute the immediate literature

of the subject. It is here proposed to attempt to define more accurately than heretofore some of the physico-chemical relationships of the acid association and to discuss the mechanism of the inhibitory effects of acids.

EXPERIMENTAL

In all work of this type distinction must be made between effects on growth and on fermentation. The two processes are, of course, not identical. There can be no appreciable fermentation without growth unless a relatively large number of organisms is added as inoculum. With maize as substrate it is very difficult to arrive at any quantitative estimate of growth. Ordinary anaerobic plating methods for determining numbers of cells are unreliable with this organism; direct counts have equally doubtful value, particularly in the case of maize cultures. Even with "liquid" cultures direct counts of this organism are frequently nearly valueless, owing chiefly to two factors: (1) the particularly slimy nature of the culture, at certain periods of its development, renders accurately representative sampling very difficult; (2) the organism characteristically exhibits peculiar aggregations the disintegration of which for purposes of direct counting is nearly impossible. Growth in liquid media such as glucose-peptone can, with a reasonable degree of accuracy, be defined positively or negatively upon careful observation of the culture. Quantitative estimates of numbers of organisms are best made, in such cases, with the assistance of nephelometric determinations of turbidity and comparison with known suspensions, or by estimation of the organisms precipitated by centrifuging a known volume of medium. Our observations on the growth of the organism in glucose-peptone solutions lead to the conclusion that the degree of fermentation runs parallel with the multiplication of the organism. But, though growth is often difficult to estimate accurately, degree of fermentation, on the other hand, can much more readily be put on a quantitative basis.

As applied to the experimental results which follow, the expression "inhibiting concentration" defines the degree of acidity, total and dissociated, which, when asociated with an otherwise favour-

able culture medium, is just sufficient to prevent completely the fermentation of 3 per cent maize by the organism at 37 to 38°C. To determine the acid concentrations which are capable of such effect we have employed a method involving the estimation of the total gas evolved during a given period of incubation. By this method the gas produced during the fermentation of small cultures containing, for example, 5 to 7 grams of carbohydrate in 3 per cent concentration was estimated by measuring the loss in weight of the cultures when the evolved gases were made to pass through concentrated H_2SO_4 contained in Alwood valves attached to the flasks. It was assumed that CO_2 and H_2 are the only gaseous or volatile products of the fermentation which are not absorbed by the acid. Total acidity was defined by titration; hydrogen-ion concentration was measured electrometrically, the readings being corrected to 25°C. for both the hydrogen and saturated KCl-calomel electrodes.

The substrate employed throughout the investigation was 3 per cent maize mash, prepared in the following manner. Portions of ground whole corn weighing 6 grams were suspended in 150 cc. distilled water in 300 cc. Erlenmeyer flasks and steamed at 100°C. for forty minutes, after which 50 cc. water were added and the flasks plugged and autoclaved at 120°C. for one and one-half hours.

The experiments which immediately follow refer to studies of the inhibition of the fermentation of maize. In each experiment flasks containing 6 grams of maize in 3 per cent concentration were acidified with varying amounts of the acids whose inhibiting concentrations were under investigation. After standing for only such time as was required for the solution of the acid, each flask was inoculated with approximately 0.5 cc. of well-shaken maize culture of the organism, about twenty-four hours old. Usually the inoculum was neutralized just before being used. The flasks were fitted with Alwood fermentation valves containing concentrated H_2SO_4 and were then weighed at room temperature and incubated at 37 to 38°. Initial molar concentrations of the added acids were calculated from data obtained by titrating accurately measured quantities of the various acids in aqueous

solution, with N/10 NaOH. Initial pH values were obtained by direct measurement on samples removed aseptically from flasks of an exactly similar duplicate series. The latter were also incubated and served to provide qualitative confirmation of the inhibiting concentrations revealed by the experimental series. At the end of the incubation period the experimental flasks were weighed at room temperature. From a consideration of the losses in weight, conclusions were drawn as to the initial inhibiting concentrations of the acids added. In tables 1 to 5, inclusive, are recorded data which pertain to the influence of thirty representative acids. An ideal experiment would have been one in which the effects of these acids were investigated simultaneously under identical conditions; this was impossible, so that one had to be content with studies of smaller groups of acids. The effects of acids grouped together in any single experiment were studied simultaneously except where otherwise stated; it will be observed that in the case of many of the acids two or more experiments are reported; the significance of the variations will be discussed later. In table 5 condensed data referring to Experiments 5 to 10 are recorded, the figures having been derived from findings of the sort detailed for the first four experiments, but which, for the sake of brevity, have been omitted in the case of the later experiments.

Experiment 1

The data relating to this experiment are summarized in table 1. If, in the case of each acid, one plots degree of fermentation against initial pH one obtains a curve with the aid of which it is possible to estimate accurately the inhibiting pH for that acid. When this is done the values recorded in table 2 are obtained. It is observed that, in the case of most of the acids, flasks having an initial reaction of pH 3.90 to 3.70 failed completely to ferment. Pyruvic acid was a notable exception, the inhibiting reaction in this case being between pH 3.51 and 3.25. Caproic acid also stood apart from the others, inhibiting the fermentation at a reaction of pH 4.4. The same general zoning of the acids can be observed if one employs, not complete inhibition of fermentation but rather 50 per cent fermentation as an index of the degree of acid influence.

TABLE 1

Experiment 1. Fermentation of 3 per cent maize as affected by added acid

ACID	CONCENTRATION OF ADDED ACID	FLASK 1			FLASK 2			FLASK 3			FLASK 4			FLASK 5			FLASK 6		
		a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
Sulphuric	1.98 N	0.05	5.45	2.17	0.10	5.04	2.07	0.20	4.40	1.43	0.30	3.91	0.32	0.40	3.52	−0.03	0.50	3.21	No fermentation
Hydrochloric	2.04 N	0.05	5.54	2.30	0.10	5.14	2.21	0.20	4.38	2.02	0.30	3.82	0.21	0.40	3.42	−0.04	0.50	3.10	No fermentation
Nitric	2.00 N	0.05	5.55	2.19	0.10	5.12	2.09	0.20	4.39	2.17	0.30	3.91	0.19	0.40	3.40	0.00	0.50	3.09	No fermentation
Phosphoric	3 N	0.30	4.72	2.05	0.40	4.37	1.99	0.50	4.06	0.80	0.60	3.78	0.03	0.70	3.57	0.02	0.80	3.41	No fermentation
Acetic	Pure acid	0.05	4.62	2.18	0.10	4.30	2.15	0.20	3.93	2.18	0.30	3.73	1.18	0.40	3.64	−0.04	0.50	3.58	No fermentation
Butyric	Pure acid	0.30	4.03	2.14	0.40	3.92	2.16	0.50	3.84	0.01	0.60	3.79	0.73	0.70	3.75	0.15	—	—	—
Caproic	Pure acid	0.05	4.98	2.28	0.10	4.75	2.24	0.15	4.58	2.30	0.20	4.46	0.59	0.25	4.38	0.09	0.30	4.30	No fermentation
Succinic	1.00 N	0.50	4.98	2.19	1.00	4.46	2.19	2.00	4.10	2.14	3.00	3.92	0.01	4.00	3.80	−0.02	5.50	3.65	No fermentation
Tartaric	2.00 N	0.25	4.67	2.14	0.50	3.78	1.93	0.75	3.53	1.03	1.00	3.39	0.19	1.25	3.28	0.14	1.50	3.19	No fermentation
Maleic	2.00 N	0.25	4.98	2.27	0.50	4.18	2.07	0.75	3.62	−0.02	1.00	3.20	−0.05	1.25	2.98	0.00	1.50	2.85	No fermentation
Crotonic	0.50 N	3.00	4.13	2.24	4.00	4.02	2.29	5.00	3.95	2.32	6.00	3.90	2.19	8.00	3.78	−0.02	10.00	3.66	No fermentation
Pyruvic	20% aqueous solution	0.10	4.72	2.29	0.20	3.93	2.29	0.30	3.51	2.32	0.40	3.25	−0.02	0.50	3.08	0.05	0.60	2.96	No fermentation
Levulinic	Pure acid	0.10	4.45	2.10	0.20	4.13	2.04	0.30	3.95	0.56	0.40	3.85	−0.01	0.50	3.75	0.00	0.60	3.67	No fermentation
Control flask		—	6.02	2.30															

a = cubic centimeters of acid added to substrate containing 6 grams maize in 200 cc. water.

b = initial pH after addition of acid and inoculum.

c = grams loss in weight after six days' incubation at 37 to 38°.

Experiment 2

This experiment is similar to the first, except that nitric and hydrochloric acids have been omitted, the first experiment having indicated that the three mineral acids behave similarly, as one would expect. Heptylic acid was included in experiment 2 and the range of initial concentration of all acids was considerably narrowed. Figure 1 illustrates the inhibiting relationships of the acids in terms of initial pH. Here again, can be observed the fact that the inhibiting effect of certain of the acids appears to be associated definitely with the factor of hydrogen ion concentration, the initial inhibiting concentrations of these acids falling within

TABLE 2

Experiments 1 and 2. Initial pH associated with inhibiting concentrations of various acids added to 3 per cent maize

	SULPHURIC	HYDROCHLORIC	NITRIC	PHOSPHORIC	ACETIC	BUTYRIC	CAPROIC	
pH	3.78	3.78	3.79	3.72	3.68	3.73	4.37	Experiment 1 (Control = pH 6.02)
	3.74	—	—	—	3.70	3.77	4.39	Experiment 2 (Control = pH 5.32)
	SUCCINIC	TARTARIC	MALEIC	CROTONIC	PYRUVIC	LEVULINIC	HEPTYLIC	
pH	3.92	3.38	3.62	3.78	3.50–3.25	3.88	—	Experiment 1
	3.97	3.56	3.67	3.77	3.18	3.77	4.65	Experiment 2

the range of pH 3.80 to 3.65, approximately. In both experiments the initial inhibiting reaction in the case of succinic acid appears to be shifted slightly to the more alkaline side of this zone. The divergence, however, is not great. Experiment 2 reveals again an apparently detached position of pyruvic acid as well as an unmistakable difference in the behavior of caproic and heptylic acids on the one hand and the remaining acids on the other.

There seems to be a legitimate conclusion to be drawn from these two experiments, namely that certain of the acids so far studied—sulphuric, nitric, hydrochloric, acetic, butyric, crotonic, levulinic, maleic, phosphoric and possibly succinic—inhibit the

acetone-butyl alcohol fermentation when present initially in such amount as to establish a hydrogen ion concentration which falls definitely within a narrow zone. In other words, in the case of these acids, one is concerned primarily with a pH effect; although other factors probably exert secondary influences, we are reasonably justified in concluding that hydrogen ion concentration takes first place among the factors associated with the inhibitory influence of any of the acids just mentioned. The so-called inhibiting zone of initial pH to which reference has been made is

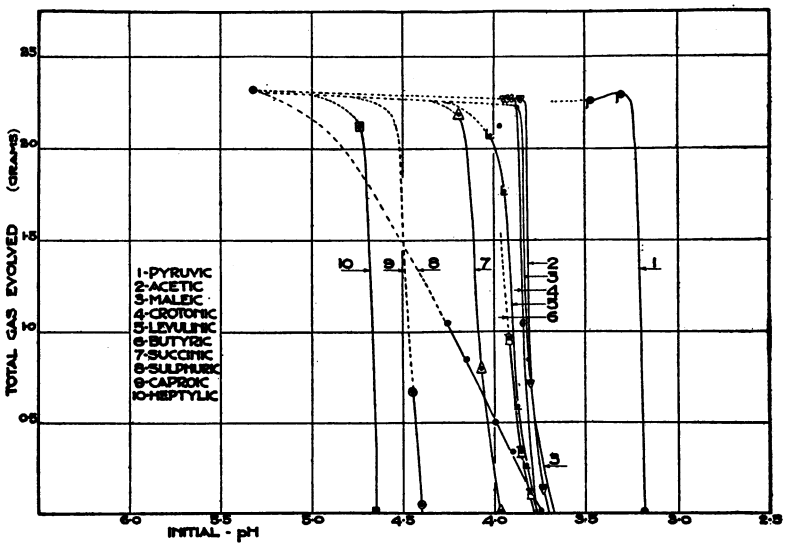

FIG. 1. DEGREE OF FERMENTATION IN RELATION TO INITIAL HYDROGEN ION CONCENTRATION ESTABLISHED BY DIFFERENT ACIDS (EXPERIMENT 2)

apparently not a fixed region. The two experiments whose results have just been recorded indicate that this is true, and many other similar experiments have yielded results which fail to establish evidence of an unchanging and invariable region of initial inhibiting hydrogen ion concentration. Summarized data derived from such experiments and recorded in table 5 make this point clear. Several factors are involved in this variability; they relate both to the nature of the substrate and to the physico-chemical behaviour of the cells introduced as inoculum. It is extremely

unlikely that throughout its life history the organism exhibits a fixed physico-chemical nature; investigations of the life cycles and the dissociation of numerous bacterial species (Löhnis, 1921), Hadley (1927), Cunningham (1931) reveal the improbability of such stability. Variations are, therefore, to be expected in the reactions of cells which, though of similar origin and age, are presumed frequently to manifest profound differences of structure and behaviour, although it is not always possible accurately to discern and measure these.

Experiment 3

In view of the suggestion in the previous experiment that certain of the lower fatty acids behave differently from other representative organic acids and the mineral acids, a more extensive study of inhibition by the lower fatty acids was made. In figure 2 data derived from such a study are presented graphically. For each acid it is observed that there is a certain critical initial reaction, acidification beyond which causes an immediate and very marked diminution in the degree of fermentation. Moreover, the inhibiting initial pH levels are obviously not the same for all the acids. Formic, acetic, propionic, butyric and isobutyric acids appear to bring about inhibition at initial pH values which fall within a narrow zone, approximately pH 3.75 to 3.65. But, with valeric and isovaleric acids, one observes a slightly greater toxicity which becomes progressively more apparent in the higher homologues, caproic, heptylic, caprylic and nonylic acids. Inhibiting concentrations of these last four acids are associated in this experiment with initial pH values of approximately 4.35, 4.75, 5.00 and 5.10 respectively, as compared with pH 3.90 for valeric acid and the zone previously mentioned for the lower homologues. The experiment was repeated several times and in every case the same general pH relationships were observed for these acids. All of the pH values which have just been recorded refer to conditions which bring about complete inhibition. The homologous acids bear similar relations to each other when 50 or 75 per cent fermentation is arbitrarily chosen as the basis of comparison. Experiment 3 is strictly comparable with Experiment I since the initial

reactions of the control flasks in the experiments were practically identical, pH 6.02 in experiment 1 and pH 6.07 in experiment 3. It is observed that the initial inhibiting reactions are very nearly identical in both experiments in the case of the acids which are common to both, namely acetic, butyric and caproic.

It is evident, however, that some influence other than hydrogen-ion concentration is at work in the case of the higher members of the series. If, in experiment 3, the toxic properties of the fatty

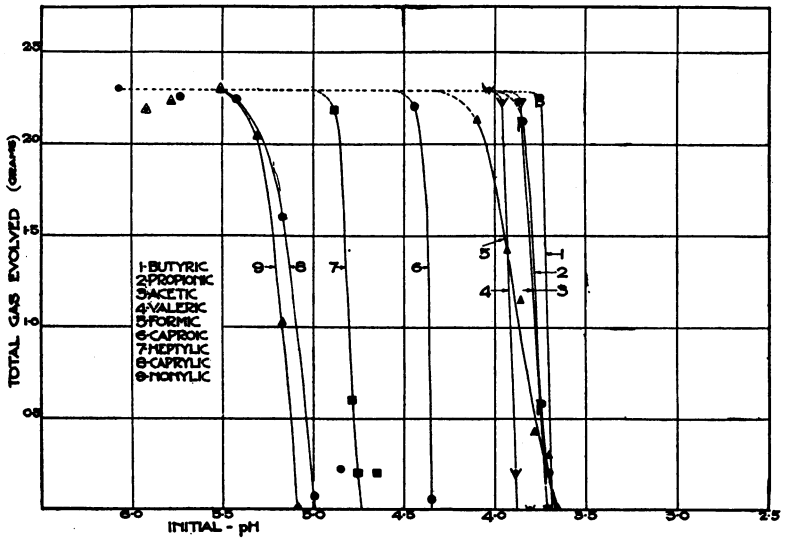

FIG. 2. DEGREE OF FERMENTATION IN RELATION TO INITIAL HYDROGEN ION CONCENTRATION ESTABLISHED BY THE LOWER FATTY ACIDS (EXPERIMENT 3)

acids are examined from the point of view of initial molar concentration rather than CH_+ the inhibiting concentrations detailed in table 3 are observed. We have here a suggestion of an interesting relationship among the intermediate acids, butyric, valeric, caproic, heptylic and caprylic, but the range of variation of acid concentration in this experiment is somewhat too great to furnish an adequate basis on which to interpret the action of the acids.

Experiment 4

The experiment was therefore repeated with the same acids, omitting formic and isobutyric, but with the range of concentration of each considerably narrowed in order to define more accurately the inhibiting molar concentrations. The data per-

TABLE 3

Experiment 3. Concentrations of fatty acids causing inhibition of the fermentation of 3 per cent maize

ACID	MOLAR CONCENTRATIONS $\times 10^{-2}$	pH
Formic	Between 0.40 and 0.45	3.66
Acetic	Between 1.72 and 2.58	3.71
Propionic	Between 3.01 and 3.35	3.67
Butyric	Between 2.16 and 2.70	3.68
Isobutyric	Between 2.16 and 2.70	3.76
Valeric	Between 1.47 and 1.68	3.88
Isovaleric	Between 1.68 and 1.89	3.85
Caproic	Between 0.56 and 0.68	4.35
Isocaproic	Between 0.56 and 0.68	4.34
Heptylic	Between 0.27 and 0.34	4.73
Caprylic	Between 0.17 and 0.23	5.00
Nonylic	Between 0.22 and 0.27	5.10

TABLE 4

Experiment 4. Concentrations of fatty acids causing inhibition of the fermentation of 3 per cent maize at 37 to 38°

	ACETIC	PROPIONIC	BUTYRIC	VALERIC	ISO-VALERIC
Molar concentration $\times 10^{-2}$	3.32	3.20	2.80	1.42	1.45
	CAPROIC	ISO-CAPROIC	HEPTYLIC	CAPRYLIC	NONYLIC
Molar concentration $\times 10^{-2}$	0.80	0.82	0.35	0.22	0.22

taining to this experiment are plotted in figure 3, the charts correlating initial concentration and degree of fermentation. The inhibiting concentrations derived from this figure are recorded in table 4; their significance is discussed later.

The inhibiting molar concentrations of the various acids are

not absolutely invariable from experiment to experiment although the acids occupy the same relative positions with respect to their toxicities. The greatest variations are exhibited by the three lowest members of the series, acetic, propionic and butyric, omitting, for the moment, formic acid which, in many of its properties, seems to stand apart from the others. The evidence suggests that these three acids to a greater extent than the higher homologues owe their toxic influence to purely hydrogen-ion effects;

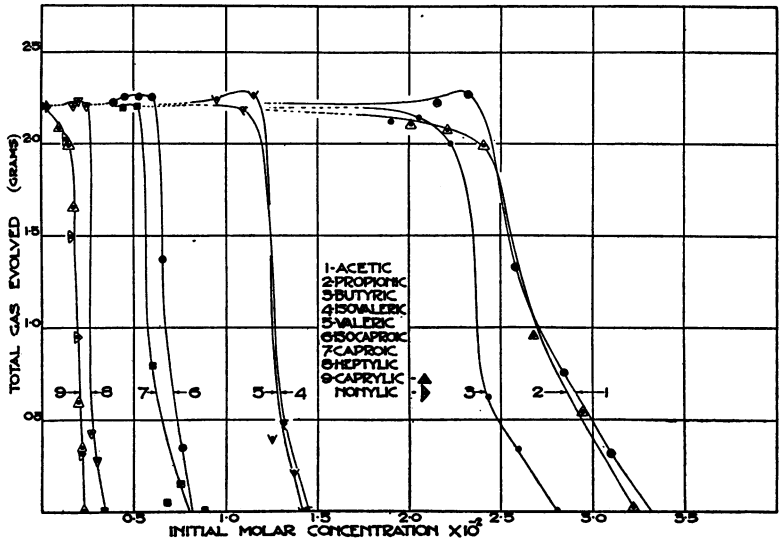

FIG. 3. DEGREE OF FERMENTATION IN RELATION TO THE INITIAL MOLAR CONCENTRATION OF THE LOWER FATTY ACIDS (EXPERIMENT 4)

any factors which cause variation in the dissociation of the acid naturally affect the total amount of acid necessary to bring the culture medium to a given level of CH_+. And, since the dissociation constants of these acids are very low—of the order of magnitude 1.5×10^{-5}—small variations of initial CH_+ involve relatively large differences of total acid. We find therefore upon examining the results of other experiments similar to experiments 3 and 4 that, particularly in the case of acetic, propionic and butyric acids, inhibiting molar concentrations vary quite appreciably. The variations in the case of the higher acids are not

TABLE 5

Summary of further experiments on the determination of the initial concentrations of acids which inhibit the fermentation at 37 to 38°C.

EXPERIMENT NUMBER	ACID	MOLAR CONCENTRATION $\times 10^{-2}$	pH	SUBSTRATE (MAIZE)	INITIAL pH OF CONTROL
				per cent	
5	Acetic	6.24	3.80	3	6.78
	Propionic	5.17	4.00		
	Butyric	5.20	3.92		
	Valeric	1.60	4.54		
	Caproic	0.85	5.30		
6	Valeric	Between 1.47 and 1.68	Between 4.13 and 4.04	3	6.00
	Isovaleric	Between 1.26 and 1.47	Between 4.17 and 4.07		
	Caproic	Between 0.68 and 0.80	Between 4.60 and 4.51		
	Heptylic	0.28	4.97		
7	Acetic	6.0	3.55	3	6.10
	Propionic	Between 5.3 and 6.0	3.63		
	Butyric	Between 5.4 and 5.9	3.65		
	Valeric	Between 1.5 and 1.9	3.95		
	Isovaleric	Between 1.5 and 2.0	3.96		
	Caproic	Between 0.60 and 0.80	4.62		
	Isocaproic	Between 0.60 and 0.85	4.65		
	Heptylic	Between 0.25 and 0.37	4.95		
	Caprylic	Between 0.12 and 0.20	5.14		
8	Formic	0.38	3.67	3	5.36
	Acetic	3.44	3.47		
	Propionic	3.45	3.50		
	Butyric	3.80	3.47		
	Isobutyric	3.85	3.48		
	Caprylic	0.21	4.52		
	Nonylic	0.14	4.80		
	Glycollic	0.45	3.62		
	Lactic	1.25	3.25		
	Glyceric	0.90	3.14		
	β-hydroxy-butyric	1.90	3.60		
9	Tartaric	1.0	3.43	3	6.69
	Malonic	1.3	3.87		
	Maleic	0.65	4.04		
	Sulphuric	—	3.95		

TABLE 5—*Concluded*

EXPERIMENT NUMBER	ACID	MOLAR CONCENTRATION × 10^{-2}	pH	SUBSTRATE (MAIZE)	INITIAL pH OF CONTROL
				per cent	
10	Acetic	5.2	3.40	3	5.73
	Monochloracetic	Between 0.005 and 0.01	Between 5.55 and 5.45		
	Dichloracetic	About 0.025	About 5.40		
	Trichloracetic	Between 0.008 and 0.018	Between 5.50 and 5.40		

nearly so pronounced, suggesting again that their effect is bound up more intimately with the undissociated molecule. In table 5 are summarized data derived from other experiments illustrating the variations of which we have spoken.

DISCUSSION

The data summarized in tables 1 to 5 indicate at once a lack of complete uniformity in the results of different experiments. There seems to be no justification for the assertion that any given concentration of hydrogen ions is at all times completely inhibitive, or capable of causing any given degree of inhibition. We have observed for example that with pyruvic acid, under certain conditions, a concentration equivalent to pH 3.2 was necessary to effect complete inhibition, whereas with nonylic acid under similar conditions a much lower concentration of hydrogen ions was associated with an equal inhibitory effect, namely pH 5.1. Similarly, whereas in one instance acetic acid at a level of pH 3.4 prevented the fermentation, monochloracetic acid was so much more toxic that a similar result was brought about by a concentration corresponding to a reaction of pH 5.4. We must conclude therefore that factors other than the hydrogen ion, introduced with certain acids, exert profound influences on the physiological activity of the organism. On the other hand, in the case of many acids the evidence points to a preponderating hydrogen ion effect. This

seems to be true for hydrochloric, sulphuric, nitric, phosphoric and many representative organic acids including the lower fatty acids, formic, acetic, propionic and possibly butyric and isobutyric as well as hydroxy derivatives of acetic propionic and butyric acids, crotonic, levulinic, succinic, tartaric, malonic and maleic acids. Among these acids there is not always complete uniformity of toxicity expressed in terms of hydrogen ion concentration; but the evidence which we have accumulated suggests that, if it were possible completely to control all other variable factors, hydrogen ion concentration would be revealed as the predominant inhibiting factor associated with the effects of these acids. Evidence of the type provided by experiments 1, 2, 8 and 9 lends support to this conclusion.

Reference has already been made to the apparent relatively high tolerance of the organism for pyruvic acid. On one occasion, not hitherto cited in this paper, a maize flask fermented to completion, whose initial reaction, established by the addition of pyruvic acid, was pH 2.95, quite an abnormal level. This phenomenon of high tolerance has been observed also in isolated instances with other acids, such as lactic and glyceric in experiment 8. The fact that these three acids are 3-carbon atom compounds bearing close relationships one to another and to intermediate compounds which have been postulated for inclusion in the metabolic progression of the organism is of possible significance in this connection. Further investigation of this point is, however, desirable before attempting to formulate a definite conclusion.

The introduction of chlorine into the acetic acid molecule renders this acid very much more toxic. The dissociation constants of the three chloracetic acids are much higher than the acetic acid constant, and very much smaller amounts of these acids are necessary to cause complete inhibition of the fermentation, as illustrated in table 5, experiment 10. However, from a consideration of the inhibiting concentrations it is evident that some factor other than hydrogen ion concentration is here involved. It would seem unlikely that speed of penetration is a factor of primary importance since the induction period through which the organisms normally retain their viability should provide more than sufficient time for

the penetrating acid to establish an equilibrium between intracellular and extracellular concentrations. Rosenblatt and Rozenband (1909) observed a similarly greater toxic effect of the chloracetic acids as compared with acetic in the case of alcoholic fermentation by yeast. Recently Luundsgaard (1930) has observed that monoiodoacetic acid in concentration of 0.001 M inhibits the fermentation of sugar by yeast when the latter is present in relatively high concentration. It is evident that halogen substitution derivatives of acetic acid have a marked influence on the fermenting mechanism of microörganisms.

The use of buffer salts to establish different levels of hydrogen ion concentration has been deliberately avoided for the reason that their addition to bacterial cultures has often, undoubtedly, a more profound effect than that involved in the mere regulation of the reaction.

On the alkaline side of neutrality, as on the acid side, maize mash, beer-wort and glucose-peptone are fermented by the organism over a wide pH range. The establishment of unchanging pH levels by the addition of NaOH to carbohydrate substrates is impossible and therefore, where buffers are not employed, the alkaline tolerance of the organism and its fermenting mechanism can be only approximately defined. Experiments have shown that the organism grows and functions relatively normally in a medium whose initial reaction is pH 11.0. The processes of fermentation of course immediately produce a more acid reaction.

In their toxic effects on the fermentation the lower fatty acids exhibit certain interesting relationships. Among these acids it is observed that there is a noteworthy lack of general uniformity of inhibiting power at equivalent concentrations. It has already been pointed out that our evidence indicates that formic, acetic, propionic and possibly butyric and isobutyric acids owe their toxicity primarily to the influence of a certain "critical" concentration of hydrogen ions. With the exception of formic acid these acids have very low dissociation constants and, therefore, one might be led to suspect that their toxic effects must be dependent largely upon the concentration of undissociated acid rather than of hydrogen ions. But the fact that the inhibiting concentrations

of these acids fall within the pH zone which includes a considerable number of other representative organic and inorganic acids indicates that there is probably no essential difference in the cause of the inhibition in all these cases: a limiting hydrogen ion concentration appears to be the important factor. Further experiments on the effects of the lower fatty acids in the presence of their sodium salts may assist in establishing the validity of this statement in so far as it refers to these acids. It must be remembered that the acids which cause inhibition at equivalent CH_+ levels exhibit very marked differences in the speeds with which they penetrate into living cells. But speed of penetration is probably not an important limiting factor under conditions such as those which prevail in our experiments. When one examines the effects of acids higher in the series than butyric, one finds that CH_+ as a controlling factor must take a secondary position. In experiment 3 (fig. 2) for example it is observed that butyric, valeric, caproic, heptylic, caprylic and nonylic acids inhibit the fermentation at initial pH levels of 3.68, 3.88, 4.35, 4.73, 5.00 and 5.10 respectively: it is impossible to escape the conclusion that CH_+ has here only secondary significance and that the real cause of the paralysis of the normal physiological functioning of the organism is associated with some other factors or phenomena.

From the point of view of molar concentration it is observed that acetic, propionic, butyric and isobutyric acids are equally toxic at approximately equivalent concentrations. This uniformity is probably merely a reflection of the close resemblance of the dissociation constants of these acids; the variations in the inhibiting molar concentrations of these acids from experiment to experiment are due, no doubt, to factors which influence the ionization of the acid, to the general nature of the culture medium and to variation in the vitality of the inoculum. The higher homologues, from valeric to nonylic, are quite definitely tolerated to a much smaller extent than are the lower acids and there is evidence of a regularly decreasing tolerance as we ascend the series. Though our experiments do not permit us to state specifically the concentrations of the higher acids which at all times effect inhibition of the fermentation, nevertheless the variation, from experi-

ment to experiment, in these effective concentrations is relatively small. Data pertaining to butyric, valeric, caproic, heptylic, caprylic and nonylic acids point to the conclusion that to obtain the same degree of inhibition the required amount of each successive higher homologue must be about one-half to one-third of the concentration of the previous lower homologue (cf. tables 3, 4, 5). Nonylic acid and, to a smaller extent, caprylic acid display irregularities which can be explained on the basis of their very low solubility in the culture medium at 38° and which therefore do not seriously affect conclusions which it is possible to deduce from the behaviour of the other acids.

TABLE 6

Relative capillary values of the lower fatty acids (Traube "rule")

	FORMIC	ACETIC	PROPIONIC	BUTYRIC	VALERIC
Capillary value (c)	1.38	0.352	0.112	0.051	0.0146
	ISO-VALERIC	CAPROIC	HEPTYLIC	CAPRYLIC	NONYLIC
Capillary value (c)	0.0158	0.0043	0.0018	0.00045	0.00014

"c" is a constant for each acid, representing the concentration which causes a lowering of about 14 per cent in the surface tension of water. The above data are reproduced from Freundlich "Colloid and Capillary Chemistry," 1926, p. 65.

This general relationship at once suggests Traube's rule as it applies to the lower fatty acids in aqueous solution. In effect, this rule states "that the surface activity increases strongly and regularly as we ascend the series. Thus, in order to get the same lowering of surface tension of water we need of each successive higher homologue about one third of the concentration of the previous member which is smaller by one CH_2 group" (Freundlich p. 64). These relations are illustrated in table 6. Furthermore, our experiments indicate also that isomeric homologues are approximately equally toxic at equal concentrations. As Freundlich points out, isomeric substances have almost equal capillary values and therefore lower the surface tension of water about equally. Related to this property of capillary activity is that property of

many substances which affects their adsorbability by materials presenting a large surface, such as blood-charcoal. It is generally true that the adsorption of organic substances from solution in water and other polar liquids by non-polar solids increases regularly as we ascend an homologous series. The application of these considerations, however, to systems involving bacterial suspensions may be misleading. Bacterial cells in suspension undoubtedly present large surfaces suitable for the adsorption of accessible substances but it is doubtful if we are yet in a position to conclude that a relationship which has been shown to exist for the adsorption of organic substances by systems represented by blood-charcoal necessarily always holds equally well when the adsorbing surfaces are those of bacterial cells. The very properties which we associate with the living cell imply a comparative instability of the molecular structure with consequent variation in the physico-chemical nature and behaviour of the organism. That such variation might manifest itself in a changing capacity for the adsorption of the sort of compounds we have been considering would seem to be a possibility. Therefore, one hesitates to postulate a strict parallelism between the data derived from these inhibition studies and the data of Freundlich and others which refer to much more completely understood physical systems. But is not this apparent adsorbing faculty of the cell merely related to the possible lipoid nature of the cell membrane which adsorbs the capillary active substance by simple solution? Warburg has demonstrated (Michaelis, 1925, p. 62) that erythrocytes freed of all lipoid substances are able to adsorb capillary active substances in a manner identical with that shown by inanimate charcoal models. It is probable that bacterial cells, if it were possible to free them of lipoids, would behave similarly. Conclusions as to the significance of lipoid solubility in this connection are governed by definitions of adsorption. The adsorbed fatty acid is attracted by both the aqueous phase and the solid (probably lipoid) phase, and according to the generally accepted view it constitutes a monomolecular layer on the cell surfaces, with the molecule so oriented that the non-polar end of the chain is attached to the surface of the cell whilst the polar -COOH group extends into

the aqueous phase. Recent studies of Trillat (1929) demonstrate very clearly this type of arrangement. Using a method involving spectrographic examination of x-rays diffracted by fatty acids on the surface of mercury, he was able to demonstrate that the acid molecule is so oriented that its -COOH group is attached to the mercury whilst the carbon chain extends into the gaseous phase. With increasing number of C atoms there was observed for each additional atom a regular increase in the lattice-spacing. This type of experiment has led to the view that similar orientations prevail when fatty acids in aqueous solution are adsorbed by living cells. The phenomenon in such cases appears to represent a state of equilibrium involving attractions of the polar and non-polar portions of the molecule by two different phases, a conception which implies a certain degree of solution in each phase. Distinction between the adsorption by bacterial cells of fatty acids in aqueous solution and the solubility of the acids in the two phases would seem merely to emphasize differences in the equilibrium position of which we have spoken. Whatever may be one's precise definition of adsorption the phenomenon in every case involves concentration at an interface, and it is this accumulation of adsorbed substance which seems to exert some profound influence on the metabolic processes of the cell. Merely to state, however, that the inhibitory effects of caproic acid are due to the adsorption of this acid at the cell surfaces does not explain the mechanism of the inhibitory action. One can only speculate as to what constitutes the essential physiological effect of this concentrated layer of adsorbed substance, resulting in the failure of the organism to function normally.

Adsorption of certain capillary active substances, such as the saturated paraffins, by bacteria is frequently possible without any noticeable effect on metabolism: the influence of adsorbed fatty acids is therefore due to something more than the mere physical presence of a foreign substance. Recent investigations and speculations of Quastel and his collaborators (1926, 1927) are interesting in this connection. They believe that the enzymic activities of microörganisms are due to the presence, on the cell surfaces, of electric fields of varying intensity, some of which are strong

enough to bring about activation of certain substrate molecules and others of which are not so powerful. In other words cell surfaces can be compared with those of other heterogeneous catalysts which exhibit regions of graded activity. It is conceivable that adsorbed fatty acids, through the influence of their polar groups, may, by causing electronic disturbances, bring about changes in the ability of certain of the active centres to accomplish the function for which they are normally responsible, and that both the degree of adsorption and the distance of the polar group of the acid from the cell surface may govern the magnitude of the effect. Furthermore, the presence of the adsorbed layer may affect the accessibility of the substrate molecules to the active areas which are normally concerned with their degradation. One must not lose sight of the possibility that at least part of the inhibitory effect of the capillary active acids is due to their adsorption by the colloidal substrate particles, rendering the latter immune to enzymic attack. Recent studies of Borissovsky and Wwedensky (1930) on the inhibition of the action of salivary diastase on starch by butyric, valeric and heptylic acids suggest such an explanation.

Somewhat similar in a general way to the results which we have obtained with the fatty acids are the results, of experiments on the activation of starfish eggs by acids recorded by Lillie (1926). Lillie was concerned with phenomena of activation rather than of inhibition, but it is not unlikely that the essential cause of the two effects can be ascribed to the same general influences. Lillie found that, among the fatty acids, acetic, propionic and butyric were closely similar in their activating powers; that is, the molar concentrations at which these acids produced complete activation in the same time and at the same temperature were almost identical, namely 0.25, 0.24 and 0.22 $\times 10^{-2}$ M respectively (ten minutes at 20°). Valeric and caproic acids in concentrations of 0.18 and 0.14 $\times 10^{-2}$ M accomplished a similar effect in the same time. This definite increase in activating power on passing from butyric to valeric Lillie interprets as indicating "that adsorption as a factor in the action of the acid first becomes relatively important with valeric acid" (p. 345).

As to the site and mechanism of activation Lillie believes the

process to be an effect of the undissociated molecules in the external solution, a conclusion based on his observations that acetate ions and hydrogen ions acting by themselves in concentrations much higher than those of the solutions used had no activating effect. Accordingly Lillie believes that the undissociated molecules penetrate into the cell interior where they are partially dissociated, the rate of activation being determined by the CH_+ at the site of the activation reaction within the cell. This conception is based, to some extent, on the belief that the undissociated molecules penetrate much more rapidly than the ions and that therefore the latter, entering as such from the exterior, have a relatively negligible activating effect. This would appear to be a logical explanation of the observed results in experiments where *rates* of action are studied; in studies such as our own, however, where speed of penetration can be said to be relatively unimportant as a controlling factor and where one is concerned with effects related to acid concentrations which are presumed to have reached conditions of equilibrium between the interior and exterior of the cells, it would seem that a somewhat different interpretation is desirable.

Leaving out of consideration for the moment the fatty acids from valeric to nonylic which, it appears, must be considered apart from other acids, it has been shown that a wide range of organic and inorganic acids brings about complete inhibition at concentrations corresponding to practically equivalent CH_+ values. That this should be the case for such markedly different acids as formic, acetic, propionic and butyric on the one hand and the mineral acids on the other, is significant. There are at least three possible explanations of the general mechanism of inhibition by acids: (1) it is the result of the influence of hydrogen ions at the outer surface of the cell, (2) it is dependent upon the CH_+ within the cell, (3) it is brought about in some manner by undissociated acid which enters the cell or which possibly exerts its effect without passing into the interior.

In aqueous solution sulphuric acid is approximately 96 to 98 per cent ionized in the dilutions in which we have used it; therefore its effect is almost entirely due to an inhibiting concentration of its

ions either outside or inside the cell. It is extremely unlikely that the effect can be attributed to the anion; therefore inhibition of normal cell activity is due to a critical CH_+ either inside or outside the cell. If outside, difficulty arises in attempting to explain effects which, cytologically, are presumed to have their origin in the interior of the cell. If inside, then in the case of completely ionized acids we must conclude that hydrogen ions penetrate into the interior. There has been in the past some expression of doubt as to the possibility of the penetration of such ions into living cells. Recently also, for example, it has been shown by Chase and Glaser (1930) that valeric and carbonic acids differ quite distinctly from sulphuric and hydrochloric acids in their ability to affect the forward movement of paramecia. The speed of movement of these organisms in media adjusted to various pH values by the addition of HCl or H_2SO_4 was identical, even after four hours, with the speed observed in the medium at pH 7.0. With valeric and carbonic acids which are known to penetrate quickly, the speed of movement after three or four hours was proportional to the CH_+ of the external medium. These results were regarded by Chase and Glaser as evidence that, within physiological limits, valeric acid brings about an increase in the CH_+ in some parts of the interior whilst H_2SO_4 and HCl are unable to do this. On the other hand, Pantin (1923) using a related organism, the amoeba, studied the velocity of pseudopodial movement in the presence of varying concentrations of hydrochloric, acetic, butyric, lactic, sulphuric and oxalic acids; the same velocity: pH curve was obtained for all of these acids, and therefore it was concluded that inhibition of amoeboid movement depends on the hydrogen ion concentration. That H_+ ions are able to penetrate into much more highly organized cells than those of the protozoa is apparent from the simple fact that sulphuric and hydrochloric acids taste sour. As to the mechanism of the penetration of H_+ ions Taylor (1928) suggests an explanation according to which the entry of a H_+ ion, by adsorption or otherwise, into the cell membrane develops an electric charge which attracts the anion of the acid into the membrane. As Taylor points out, this method of penetration is very similar to the passage of undissociated molecules since such a pair of ions is neutral.

CONCLUSION

Since acetic and sulphuric acids inhibit at the same level of external pH, and in view of the very low dissociation constant of the former as compared with that of the latter, and, furthermore, in consideration of the probable equilibrium between internal and external concentrations of undissociated acetic acid, it is not unlikely that the mechanism of inhibition involves an effect of a concentration of H-ions in the interior which closely approximates that observed in the external medium under inhibiting conditions.

In conclusion, therefore, it can be said that, except in the case of certain acids, inhibition of the acetone-butyl alcohol fermenta-

TABLE 7

Physiological effectiveness of the lower fatty acids

Loeb (1909); membrane formation, sea-urchin eggs; nonylic > caprylic > butyric > propionic > acetic > formic
Crozier (1918); sensory activation of earthworm; caprylic > caproic > formic > valeric > butyric > propionic > acetic
Lillie (1926); activation of unfertilized starfish eggs; formic > caproic > valeric > butyric $\lesseqgtr$ propionic = acetic
Bodansky (1928); hemolysis of red blood cells; capric > nonylic > caprylic > heptylic > caproic > isocaproic > valeric > isovaleric > isobutyric = butyric > propionic > acetic
Inhibition of the acetone-butyl alcohol fermentation; nonylic $\geqq$ caprylic > heptylic > formic > isocaproic = caproic > valeric = isovaleric > isobutyric = butyric $\lesseqgtr$ propionic = acetic

tion by the acids investigated is associated with a "critical" CH_+ in the cell interior. The precise relation between the internal and external CH_+ corresponding to the inhibitory concentration of each acid is controlled by a variety of factors, but general considerations indicate that the "critical" internal CH_+ is not greatly different, except in the case of acids with specifically toxic groups and the capillary active fatty acids, from the external value which is associated with the inhibitory effect.

When the results of our study of the effects of the lower fatty acids on the fermentation are compared with results obtained by other workers in related fields, as summarized in table 7, it is observed that the acids arrange themselves in practically the same

order in all cases: the acids higher in the series are correspondingly more effective physiologically. This order, as Crozier's work demonstrates, is essentially the order of the speed of penetration of the acids into living cells, and it is to the differences in speed of penetration that Loeb (1913) and Bodansky (1928), for example, attribute the differences of effect which they observed. But it is true, also, that the lower fatty acids are adsorbed by non-polar solids in precisely the same order as that of their relative penetrabilities. And, since speed of penetration is probably not a limiting factor in our studies, we must conclude that differences in the effective inhibiting concentrations of these acids are related primarily to the degree to which they are adsorbed by the living cell. This involves the assumption that bacterial surfaces, as adsorbents, behave in a measure like those of charcoal: reference has already been made to the possible difficulties involved in such an assumption. Küster and Bojakowsky (1912), demonstrated that the partition of phenol between anthrax spores and water followed the general adsorption isotherm of Freundlich. Whether bacteria in general behave in a similar manner is not known, but our experiments suggest that the cells of *Clostridium acetobutylicum* react toward the capillary active fatty acids in a manner which is in approximate accord with the Traube rule.

SUMMARY

1. A study has been made of the inhibition of the fermentation of maize mash, under the influence of *Clostridium acetobutylicum* (Weizmann), as effected by 30 representative inorganic and organic acids.

2. With several acids, complete inhibition was effected in those flasks whose initial reaction fell within a narrow zone the limits of which varied from experiment to experiment but which, approximately, extended from pH 3.90 to 3.65. The following acids are included in this group: hydrochloric, nitric, sulphuric, orthophosphoric, succinic, malonic, maleic, levulinic, crotonic, glycollic, β-hydroxybutyric, formic, acetic, propionic, butyric and isobutyric.

3. The toxic effects of these acids are probably associated with

a "critical" CH_+ in the cell interior, closely approximating the observed extra-cellular hydrogen ion concentration associated with the inhibitory effect.

4. The three chloracetic acids are much more toxic than acetic acid. Their effect is not one of CH_+ but is probably due to the specific influence of the chlorine atom.

5. Hydroxy derivatives of the lower fatty acids are not more toxic than the normal acids at equivalent CH_+ levels. The evidence suggests that, in the case of the 3-carbon acids, the reverse may be true.

6. Pyruvic, lactic and glyceric acids were tolerated by the organism at CH_+ levels higher than for any other acids.

7. In the lower fatty acid series, formic, acetic, propionic, butyric and isobutyric acids inhibited the fermentation at nearly equivalent CH_+ levels, but with each successive higher homologue the inhibiting CH_+ was appreciably lower: e.g., pH values of 3.65–3.75 for the first five members including isobutyric, and pH values of 3.90, 4.35, 4.75, 5.00 and 5.10, respectively, for valeric, caproic, heptylic, capyrlic and nonylic acids.

8. On the basis of molar concentration, the order of the inhibiting effectiveness of the fatty acids is as follows: nonylic $\geqq$ caprylic > heptylic > formic > caproic = isocaproic > valeric = isovaleric > isobutyric = butyric $\lesseqgtr$ propionic = acetic.

9. Capillary activity has relatively little effect in the case of formic, acetic, propionic and butyric acids, but with the higher homologues its influence is very marked. The inhibiting molar concentrations of the higher homologues suggest an approximate agreement with the Traube rule as it applies to the fatty acids in aqueous solution. Adsorption of the capillary active acids is probably the chief reason for the regularly increasing toxicity of these homologues.

10. The manner in which the adsorbed acids affect the physiological behaviour of the organism is discussed.

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