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TOXIC ACTION OF CARBONIC AND OTHER WEAK ACIDS ON
THE MENINGOCOCCUS.

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IN attempting to discover an effective method of direct antiseptic treatment of cerebrospinal meningitis, it would seem advisable, when one considers the delicate nature and fundamental physiological importance of the nervous structures involved, to employ, as the basis of the antiseptic fluid in view, a solution as uninjurious as possible to mammalian living tissues. Ringer showed long ago that the tissues of cold-blooded animals are capable of long survival, if instead of perfusing or bathing them with 0.6 per cent NaCl solution, regarded up till then as the physiological solution *par excellence*, a solution containing in addition to the sodium chloride, physiological amounts of calcium and potassium salts and a trace of sodium bicarbonate were employed. Many years afterwards Locke [1] succeeded in extending this line of work to mammalian tissues, and showed that a Ringer's solution of modified composition and containing in addition sufficient oxygen and a physiological percentage of glucose was capable of sustaining the activity of the excised mammalian heart for long periods. The power of conserving the vital activities of the mammalian tissues in contact with the Ringer-Locke fluid has since been abundantly confirmed by various workers in the case of many different organs. It has been pointed out, too, by Professor Halliburton [2] that the cerebrospinal fluid itself is in its composition to all intents and purposes nothing but a Ringer-Locke fluid of physiological origin. We have, therefore, abundant grounds for taking as the basis of any antiseptic solution with which it is proposed to treat the nervous system the Ringer-Locke fluid, in the hope that at any rate this will have no special deleterious effect of its own, and that we shall have only to fear such from the antiseptic agent we add to it. It

2 *Toxic Action of Carbonic Acid on the Meningococcus*

will be better too, if in all probability, instead of an antiseptic foreign to the body, we can find a physiological antiseptic agent, produced by an exaggeration or diminution of the physiological factors involved. From this general point of view the work described in the following has been undertaken.

I was led to this inquiry by my previous work on the toxic action of copper compounds of amino-acids and in particular copper-alanine on protozoa [3]. For, in the early part of last year, in some preliminary experiments, the opportunity for which was kindly afforded me by Professor R. T. Hewlett in the Bacteriological Laboratory, King's College, a toxic action of copper-alanine (one part in 100,000 of water) was found on the meningococcus also, on thirty minutes' exposure.

In further experiments carried out in association with Lieutenant-Colonel Mervyn Gordon and Major Hine, at the Central Cerebrospinal Laboratory, Royal Army Medical College, it was shown that the meningococcus did not survive in concentrations of copper-alanine 1 in 1,000 in sodium chloride solution, on twenty minutes' exposure, but in less concentration or in broth or serum, no toxic effect was observed in this time limit. Experiments were also made with the copper-alanine in Ringer-Locke solution. A somewhat more toxic effect was witnessed, as in this case the meningococcus did not survive in 1 in 10,000 copper-alanine on twenty minutes' exposure. No toxic effect was observed on five or ten minutes' exposure, and as for purposes of local treatment, intrathecal or naso-pharyngeal, a short time limit is obviously essential, the above results did not indicate any special advantage in the employment of copper-alanine as a bactericide in the treatment of cerebrospinal meningitis. Nor could the solutions of 1 in 10,000 in Ringer-Locke fluid be regarded as suitable for intrathecal use without fear of injury to the delicate tissues of the central nervous system.

In these experiments, however, it was noticed that in two out of three controls in 0.85 per cent sodium chloride solution alone, the meningococcus survived. On the other hand, the meningococcus had not survived in all three controls in Ringer-Locke solution alone. In all these cases the control solutions had been inoculated at the commencement of the series, and planted out in the usual way at the end—in each case the exposure having been forty-five minutes at 37° C.

This unexpected observation in the case of Ringer-Locke solution opened up therefore a further line of inquiry. For, if it held good that Locke's modification of Ringer's fluid, corresponding in its salt constituents to the natural fluids of the body, possessed in addition bactericidal properties towards meningococcus, obviously the value of this solution for irrigation purposes and intrathecal use would be still further evident.

Investigation has therefore been carried out by me in this direction in the Bacteriological Laboratory, King's College, with the assistance of Mr. F. Welch.

TECHNIQUE.

The composition of the Ringer-Locke solution used was NaCl, 0.9 per cent; KCl, 0.042 per cent; CaCl₂ (anhydrous), 0.024 per cent; dextrose, 0.1 per cent; NaHCO₃, 0.02 per cent. The water employed was distilled in glass. The NaHCO₃ is added last to the remaining constituents after their previous sterilization. The NaHCO₃ itself cannot be heated to ensure sterilization, but in these experiments practically this has proved negligible. Five cubic centimetres of distilled water was added to a twenty-four-hour culture on tryptagar slope of an isolated strain of meningococcus (Foster II) used throughout, forming a milky suspension (approximately 5,000 million meningococci to one cubic centimetre). Of this, in earlier experiments, 0.1 cubic centimetre was added to 10 cubic centimetres of the respective test solutions in sterile test tubes, and also to the same amounts of water, 0.85 to 0.9 per cent sodium chloride solution, and of trypsin broth; these latter were for control purposes; in subsequent experiments, in order to ensure greater uniformity in results, 0.2 cubic centimetre of the meningococcal suspension was taken as the inoculating dose. After five, twenty, and sixty minutes' exposure of the meningococcus in suspension to the various solutions kept at 37° C., the test tubes were well shaken and a three-millimetre loopful of each solution was planted out on tryptagar slopes. These were then incubated at 37° C., and the results read off in twenty-four and forty-eight hours. The forty-eight hours' incubation is necessary as in many instances the twenty-four hours' incubation proved insufficient, and the result, therefore, at that period is unreliable. Either the subcultures of meningococcus showed growth or not, and the result was charted as + or —; even single colonies were marked +.

THE BEHAVIOUR OF THE MENINGOCOCCUS IN RINGER-LOCKE SOLUTION,
AND THE ACTION OF ITS INDIVIDUAL CONSTITUENTS.

At first, using 0.1 cubic centimetre of meningococcal suspension as the inoculating dose, as previously employed, two out of three separate experiments showed that the meningococcus did not survive on sixty minutes' exposure to Ringer-Locke solution, thus appearing to confirm the original results. Controls in water and in 0.85 and 0.9 per cent sodium chloride solution survived. The action therefore of the individual constituents of Ringer and of Ringer-Locke solutions was investigated.

Water.—In water distilled in glass, as well as in ordinary distilled water and in tap water, the meningococcus was shown to survive on five, twenty, and sixty minutes' exposure.

Pure Sodium Chloride 0.85 and 0.9 per cent solutions. The survival of the meningococcus in these solutions has almost invariably been evident on five, twenty and sixty minutes' exposure at 37° C. and planting out, with incubation for twenty-four and forty-eight hours at 37° C. Since the

4 *Toxic Action of Carbonic Acid on the Meningococcus*

completion of my experiments my attention has been directed to the work of Flexner [4] and of Shearer [5], both of whom conclude that physiological solutions of sodium chloride have a destructive or toxic action on the meningococcus. The discrepancy between their and my results may be explained in part by the different conditions of experiment. In my experiments, I have confined myself to a short time limit of exposure, whereas Flexner's results apparently refer to comparatively long periods of exposure, and Shearer's to an exposure of seventy-five minutes. In a few experiments which I have since made, survival of the meningococcus has been evident on exposures for two hours, followed by forty-eight hours' incubation. Shearer notes, however, that freshly isolated meningococci are more vulnerable to the action of sodium chloride than old laboratory cultures; the former seldom resist the action of 0.85 per cent pure sodium chloride for more than twenty minutes, though the latter could sometimes resist the action for three to four hours. It may be that a difference in results is due to the salt itself employed. Throughout my experiments I was using "pure sodium chloride," but quite recently coming towards the end of this particular stock bottle, I started on another. The meningococcal controls in this sodium chloride solution did not survive as usual (and the results in several sets of experiments, in consequence, were discarded as valueless). The experiments had been carried out precisely in the same way as before except in the alteration of the salt employed. On reverting to the first stock bottle, the meningococcus again survived. Both specimens of the sodium chloride were Kahlbaum's "guaranteed pure for analysis."

Calcium Chloride, Potassium Chloride, and Dextrose respectively in 0.85 and 0.9 per cent sodium chloride solution have each been favourable to the survival of the meningococcus in my experiments.

Sodium Bicarbonate.—Repeated experiments showed that the meningococcus did not survive on sixty minutes' exposure to sodium bicarbonate 0.02 to 0.04 per cent in sodium chloride solution. From this it was inferred that the injurious effects on the meningococcus in Ringer-Locke solution were due to the sodium bicarbonate. Repetition, however, of my previous experiments with Ringer-Locke solution, using 0.1 cubic centimetre of the suspension as the inoculating dose, failed to confirm the earlier results, and no difference was observed in the Ringer-Locke solution with or without the sodium bicarbonate. It is difficult to explain these contradictory results. It may, however, be mentioned that the Ringer-Locke solution employed in the earlier experiments was stock solution in

¹ Dr. Locke informs me that a similar variation in the behaviour of "chemically pure" sodium chloride from different sources in physiological solutions, was observed by him in conjunction with Dr. Rosenheim in 1908. Certain specimens give solutions which fail to keep the mammalian heart alive beyond two or three hours, and prevent it showing any improvement with dextrose.

which a commercial sodium chloride had been used which is no longer obtainable. It is possible this was of a toxic nature. It became of interest to ascertain the effect of increasing the percentage amount of sodium bicarbonate; but with one per cent and two per cent in Ringer-Locke solution, the meningococcus continued to survive. Not only this, but when sodium chloride solution was used, the addition of one per cent sodium bicarbonate exerted no greater effect than that of 0.02 per cent had done, while even in the two per cent solution the meningococcus survived. Repetition of the experiment on several occasions confirmed this, at first sight, paradoxical result. That increased percentages of NaHCO_3 had less toxic effect could hardly be explained on the supposition that it acted by virtue of its alkalinity or the relative concentration of the hydroxyl ions. Considering the well-known facts so important for the theory of respiration, of the dissociation of NaHCO_3 in solution into Na_2CO_3 and CO_2 , it seemed not impossible that in dilute solutions with more complete dissociation of the salt, the free CO_2 , especially in the case of micro-organisms, might be the active factor, and it became worth while to investigate the action in physiological solution of CO_2 and other weak acids on the meningococcus. In order to get rapidly a definite idea of the effect of weak acids, experiments were made first with acetic acid.

ACTION OF ACIDS.

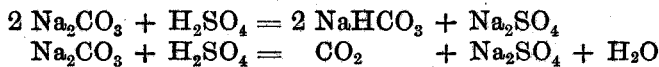
Acetic Acid.—After exposure of the meningococcus 0.2 cubic centimetre suspension to ten cubic centimetres solutions of acetic acid of strength respectively one per cent, 1 in 1,000, and 1 in 10,000 in 0.9 per cent sodium chloride solution, a toxic effect was definitely shown. The meningococcus survived only on five minutes' exposure in the 1 in 10,000 solution. Controls in sodium chloride solution and in water survived as usual on the sixty minutes' exposure. Further experiments were made with M/2500 acetic acid in 0.9 per cent sodium chloride solution (corresponding roughly to 1 in 50,000). Survival of the meningococcus was noted only on the five minutes' exposure; longer than this proved fatal.

Carbonic Acid.—Greater interest would seem to attach to the investigation of the effect of carbonic acid on the meningococcus. This is the weakest physiological acid. It is constantly present in greater or less amount in the blood and other body fluids. Its effects on living tissues when not pushed to their limit are reversible. A concentration too great to be borne by the central nervous system when perfused through its blood-vessels would probably be successfully resisted if the solution was introduced intrathecally, the persistence of the normal blood current in the central nervous system ensuring its survival.

Solutions of free carbonic acid were obtained by passing the washed gas from a Kipp apparatus through the fluid used, for periods varying from

6 Toxic Action of Carbonic Acid on the Meningococcus

one to two minutes, some approximation to saturation being thus obtained. In order, however, to obtain solutions containing known percentages of free carbonic acid, the method employed was one suggested to me by Dr. F. S. Locke which he had already made use of in order to prepare perfusion fluids for the mammalian heart, of known free CO₂ content. It possesses also the advantage of giving a solution that can be sterilized by boiling. The method depends on the conversion of sodium carbonate by sulphuric acid into sodium sulphate, sodium bicarbonate and (in accordance with the relative amounts of Na₂CO₃ and H₂SO₄) the percentage of CO₂ required. Thus:—



It is obvious, therefore, that by the mixture of suitable amounts of equivalent solutions of Na₂CO₃ and H₂SO₄ we can readily obtain within wide limits the required percentages of NaHCO₃ and CO₂. The Na₂SO₄ formed is for our purposes negligible. Ideal quantitative accuracy is not required in the use of this method, as the small percentage of NaHCO₃ always left in the final solutions acts as a "buffer" preventing the presence of free H₂SO₄.

It was found convenient to use $\frac{1}{4}$ normal solutions for the additions necessary for the formation of the small amount of NaHCO₃ used. For the further equal amounts of H₂SO₄ and of Na₂CO₃ forming the required percentage of CO₂, $\frac{1}{2}$ normal solutions were made use of. The following solutions were investigated:—

Solution No.	Ringer-Locke (without alkali or dextrose)	N/4H ₂ SO ₄	N/4Na ₂ CO ₃	N/2H ₂ SO ₄	N/2Na ₂ CO ₃	Volume per cent CO ₂
1	c.c. 50	c.c. 0.25	c.c. 0.5	c.c. 1	c.c. 1	11
2	50	0.25	0.5	2	2	22
3	50	0.25	0.5	4	4	44

The necessary amounts of H₂SO₄ can be added to the non-alkaline Ringer-Locke fluid, and the mixture sterilized. The Na₂CO₃ solutions can be separately sterilized, and added in the cold to the H₂SO₄ mixture. Toxic effects on the meningococcus were found with Solutions 2 and 3, but not with Solution 1.

Similar solutions were investigated also in which fifty cubic centimetres sodium chloride solution were used in place of the Ringer-Locke solution. With these a toxic effect was found with Solution 1.

The results, together with toxic effects obtained at the same time with sterilized solutions of 0.9 per cent sodium chloride through which the CO₂ gas itself had been passed,¹ are given in the following tables of two experiments.

¹ (The supposed saturation with CO₂ thus obtained may be assumed to have been anything between sixty to eighty volumes per cent).

A third experiment was made with similar results: 0.2 cubic centimetre meningococcal suspension was taken as usual, as the inoculating dose to 10 cubic centimetres of each solution; results with Ringer-Locke solution (0.02 per cent NaHCO₃) and with NaHCO₃ (0.02 per cent) in sodium chloride solution, obtained at the same time and under the same conditions of experiment are given also, and in these 0.1 cubic centimetre suspension was taken as the inoculating dose as in the earlier experiments.

(1)	Forty-eight hours' incubation at 37° C.		
	5	20	60
Exposure in minutes at 37° C.	5	20	60
Ringer-Locke solution CO ₂ (Solution 1)	+	+	+
" " " (Solution 2)	+	+	-
" " " (Solution 3)	+	-	-
NaCl 0.9 per cent solution CO ₂ (Solution 1)	+	-	-
CO ₂ gas in 0.9 per cent NaCl solution	+	-	-
NaCl 0.9 per cent solution alone	+	+	+
Water (glass, distilled)	+	+	+
Broth	+	+	+
Meningococcal suspension at room temperature	+	+	+
Subculture +			

(2)	Forty-eight hours' incubation at 37° C.		
	5	20	60
Exposure in minutes at 37° C.	5	20	60
Ringer-Locke solution CO ₂ (Solution 2)	+	+	-
" " " (Solution 3)	-	-	-
NaCl 0.9 per cent solution CO ₂ (Solution 1)	+	+	-
CO ₂ gas in 0.9 per cent NaCl solution	+	-	-
Ringer-Locke solution (0.02 per cent NaHCO ₃)	+	+	+
NaHCO ₃ (0.02 per cent) in 0.9 per cent NaCl solution	+	+	-
NaCl 0.9 per cent solution alone	+	+	+
Water (glass, distilled)	+	+	+
Broth	+	+	+
Meningococcal suspension at room temperature	+	+	+
Subculture +			

It will be seen from the above that Ringer-Locke CO₂ solution, sodium chloride CO₂ solution, and sodium chloride solution, through which CO₂ gas has been passed, respectively, exert a definite toxic effect on the meningococcus. On the other hand, it will be seen that the meningococcus survived in Ringer-Locke solution (0.02 per cent NaHCO₃), but did not survive exposure of sixty minutes to NaHCO₃ (0.02 per cent) in sodium chloride solution. Controls in sodium chloride solution alone, water, and in broth, survived as usual.

Serum.—A destructive action by serum (guinea-pig) on meningococcus has been shown by Flexner. In the following preliminary experiment a rapid or increased toxic effect of serum through which CO₂ has been passed is shown. For this purpose fresh sterile serum (rabbit) was used. The serum was slightly blood-stained; 2.5 cubic centimetres of the serum so treated and 2.5 cubic centimetres of the normal serum were inoculated with 0.05 cubic centimetre of meningococcal suspension; the technique being otherwise the same as described in previous experiments. A toxic action

8 Toxic Action of Carbonic Acid on the Meningococcus

of the serum (rabbit) control is not evident under the conditions of a short time limit of exposure in my experiment. The effect of CO₂ gas in sodium chloride solution was again examined (0.2 cubic centimetre suspension to ten cubic centimetres solution), and the toxic action confirmed.

	Forty-eight hours' incubation at 37° C.			
	5	20	60	
Exposure in minutes at 37° C	5	20	60	
Serum CO ₂ gas	+	-	-	
Serum alone	+	+	+	
CO ₂ gas in 0.9 per cent NaCl solution	+	-	-	
NaCl 0.9 per cent solution alone	+	+	+	
Water (glass, distilled)	+	+	+	
Meningococcal suspension at room temperature	+
Subculture +				

Lactic Acid.—Next to carbonic the most important acid katabolite is lactic acid. It is produced by many organisms, and it seemed of interest to investigate its effect on the meningococcus which is known to ferment dextrose. Sarcocollactic acid has not so far been investigated. The pharmacopœial lactic acid was made use of. In M/2500 and M/5000 (in 0.9 per cent sodium chloride solution) it was found to exert a marked toxic effect, showing survival of the meningococcus only on the five minutes' exposure.

On the suggestion of Dr. O. Rosenheim, an attempt has been made by me to investigate the relationship which might exist between the toxic effect of the above acid solutions and their hydrogen ion concentration. A hydrogen ion concentration of PH 7.02 represents absolute neutrality, and PH 7.35 near that of the blood [6]; which reaction for cultural purposes on nutrient media is also near the optimum for the growth of most pathogenic organisms [7]. The hydrogen ion concentration of cerebrospinal fluid (man) has been represented as PH 8.1 when fresh, but, on standing, it soon reaches PH 9.25, attributed to the loss of carbonic acid [8]. According to Hurwitz and Tranter [9] the PH varies from 8.15 to 8.3, and cerebrospinal fluid is thus regarded by them as more alkaline than blood. Milroy [10] has recently confirmed this, and points out further that the PH at low CO₂ pressure is higher, or, in other words, the alkalinity is greater than blood plasma.

For the above-mentioned purpose a series of Sørensen's standard mixtures of primary potassium phosphate and secondary sodium phosphate were prepared. It was found that in a mixture of 9.75 cubic centimetres M/15 primary phosphate and 0.25 cubic centimetre M/15 secondary phosphate, corresponding to a hydrogen ion concentration of PH 5.3, the meningococcus did not survive on sixty minutes' exposure. In primary phosphate solution alone, corresponding to a hydrogen ion concentration of PH 4.5, the meningococcus failed to survive on twenty minutes' exposure. I append a typical experiment, the technique employed being the same as before.

Phosphate mixture		Methyl red 5 drops, 10 c.c. colouration (before inoculation)	PH	Toxic action—minutes		
Primary c.c.	Secondary c.c.			5	20	60
3.0	7.0	Yellow ..	7.2	+	+	+
7.0	3.0	" ..	6.4	+	+	+
9.75	0.25	Faint pink ..	5.3	+	+	—
10.0	0.0	Pink ..	4.5	+	—	—

The toxic lactic and acetic acid solutions gave similar reactions, the PH in these cases ranging also between 4.5 and 5.3. The toxic concentration of CO₂ in Ringer-Locke solution gave the same result. The much weaker concentration of CO₂ (11 vols. per cent) (Solution 1) however, which had been found toxic to the meningococcus in pure sodium chloride solution, gave only a yellow colouration with the indicator indistinguishable from that given by pure sodium chloride solution alone. In pure sodium chloride solution therefore a much weaker hydrogen ion concentration due to CO₂ is toxic to meningococcus than corresponds to PH 4.5 to 5.3. The indicators at my disposal did not permit of a more exact result than this. It is, moreover, obvious that the hydrogen ion concentration of the standard and other solutions is considerably lowered by the added meningococcal suspension which by itself possesses a distinct alkaline reaction. Further experiments in which the micro-organism itself is exposed directly to the solutions, or cultured in nutrient media of known PH, will be necessary to determine the point at which a toxic action is exerted. It will be necessary also to ascertain the hydrogen ion concentration of the cerebrospinal fluid itself in cerebrospinal meningitis.

In interpreting the results obtained with CO₂ regard must not be lost of the fact that CO₂ as an acid occupies quite a special physiological position as a general end product of vital chemical reactions. Increased percentages of it, therefore, in physiological fluids might exert a specific inhibitory effect on the vital activity of micro-organisms also, in addition to its effect as an acid in increasing hydrogen ion concentration.

It would appear from the results of the investigation described above, that local treatment of areas, intrathecal and naso-pharyngeal, infected with meningococcus might be tried with Ringer-Locke fluid, or with sodium chloride solutions, containing, as physiological antiseptics, physiologically excessive amounts of carbonic or lactic acids.

Slater [11], Rideal [12], and others have described the bactericidal action of CO₂ in solution on various pathogenic bacteria; these, however, differ considerably in their resistance to CO₂. The local application of the gas itself has also been described with sedative and beneficial effects in the treatment of open wounds and ulcerations, as well as by rectal introduction in cases of dysentery.

In extending this investigation I have found, in preliminary experi-

10 Toxic Action of Carbonic Acid on the Meningococcus

ments, that carbonic acid in sodium chloride solution exerts a rapid toxic effect on the protozoon opalina; also on *Spirochæta pallida*. The possible use of carbonic acid in syphilitic disease of the central nervous system, and in the treatment of diseases due to protozoa, is thus suggested.

In conclusion, I desire to express my best thanks to Professor Halliburton and to Professor Hewlett for the opportunity kindly afforded me in their laboratories, and for much kind assistance throughout this work. To Lieutenant-Colonel Gordon also my best thanks are due for his kind assistance throughout, and supply of the necessary cultures and nutrient media.

CONCLUSIONS.

- (1) The meningococcus survives exposure to Ringer-Locke solution.
- (2) Sodium bicarbonate (0.02 per cent and 1 per cent) in sodium chloride solution exerts a toxic effect on exposures of sixty minutes; a 2 per cent solution has no toxic effect.
- (3) CO_2 and other acids in small concentration exert a toxic effect.
- (4) In Ringer-Locke solution containing free CO_2 twenty-two vols. per cent and upwards, the meningococcus does not survive exposure of twenty minutes. In sodium chloride solution the toxic effect is more marked.
- (5) The toxic action of serum through which CO_2 gas has been passed is pronounced.
- (6) Lactic and acetic acid in M/5000 and M/2500 respectively (in 0.9 per cent sodium chloride solution) have a similar toxic action on the meningococcus.
- (7) The preliminary experiments on the hydrogen ion concentration have not yielded sufficiently definite results to determine at what point the toxic action is exerted, and do not exclude a specific action of its own on the part of CO_2 .¹
- (8) It is suggested that normal solutions containing increased amounts of CO_2 or lactic acid may, as physiological antiseptics, be employed in the local treatment of areas, intrathecal and naso-pharyngeal, infected with meningococci, and that even the CO_2 normally occurring in the plasma and body fluids may form part of the protective processes of the body.
- (9) Preliminary experiments show that CO_2 in sodium chloride solution

¹ Attention may here be drawn to the results of K. Taylor (*Lancet*, i, p. 294, 1917), which I only became acquainted with after my own work was completed. He has investigated the concentration of various acids inhibitory to bacterial growth. He does not mention CO_2 , and regards his results as proving a specific action of acids without making any reference to hydrogen ion concentration. It is worth while, however, pointing out that if their correctness be assumed it is impossible to explain them in terms of the hydrogen ion. The ratio of the concentration toxic for one organism (even when recalculated molecularly, taking, e.g., acetic and propionic acids) is inverted in the case of another organism. This would necessitate the assumption of an inverse relation between molecular concentration and hydrogen ion concentration in either one or other of the two acids.

exerts a rapid toxic effect on the protozoon opalina; and on *S. pallida*. It is not impossible that CO₂ might also be made use of in syphilis of the central nervous system, and in diseases due to protozoa.

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