ORGANIC MATTER IN THE EXPIRED BREATH WITH ESPECIAL REFERENCE TO ITS INHIBITING POWER ON OXIDIZING FERMENTS.*

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In 1895 Billings, Mitchell, and Bergey (1) published the results of a large number of experiments showing that specific poisons of an organic nature are not present in expired air. These results reversed the generally prevailing opinion of sanitarians, who now began to account for the ill effects of overcrowding by physical agents rather than by specific poisons in the exhaled breath. Recently Weichardt (2) published the results of experiments which he claims prove the presence of poisons of an organic nature in the expired breath. His experiments on the effect upon mice of products collected from the expired breath of elderly persons have been criticized by Inaba (3) who believes Weichardt's results to be due rather to the heterotonic nature of the fluids injected than to toxins or protein poisons.

Weichardt also believes that he has detected the presence of split proteins by experiments *in vitro*. The vapor from the expired breath of a fatigued man sixty-three years old was filtered through cotton, passed through ten cubic centimeters of distilled water, and then concentrated *in vacuo* to three cubic centimeters. The solution containing the products of the expired breath left a residue weighing nine milligrams. This residue turned brown when heated on platinum foil and gave off odoriferous fumes. Weichardt also noted that a characteristic odor was produced when the solution of the respiratory products was acidified with hydrochloric acid or neutralized with sodium hydroxide.

Further evidence of the presence of organic matter in the expired breath is presented by Weichardt who found that the oxidation of guaiacum by blood is inhibited by the substances in the expired

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breath, just as it is prevented by diphtheria toxin. In the experiment designed to demonstrate this fact, one cubic centimeter of the fluid, containing the products from the expired air, was placed in each of a series of tubes. To each tube was then added a convenient amount of a very dilute solution of fresh blood, 0.1 of a cubic centimeter of the guaiacum being used as indicator.

Weichardt claims that this substance in expired air, which is capable of inhibiting the oxidation of guaiacum by blood, may also be found in the air of a closed room in which several persons have slept. He exposed 0.25 of a gram of finely powdered calcium chloride in a platinum dish covered with filter paper to keep out gross particles. On the following morning the calcium chloride was dissolved in water and the protein split products were demonstrated in the solution by means of the inhibition of the guaiacum reaction.

It is interesting to note that Weichardt claims to have obtained the toxic substances by allowing the expired breath to pass through normal hydrochloric acid, while Merkel (4) claims to have found a base which is poisonous when free, but innocuous after contact with an acid.

Rosenau and Amoss[1] (5) filtered the expired breath through two glass bulbs firmly packed with glass-wool and collected the moisture by condensing it in a Drechsel bottle set in a freezing mixture. This liquid when injected into guinea pigs exhibited no toxic effects, but sensitized the animals so that a subsequent injection of normal human serum produced the characteristic symptoms of anaphylaxis. By this method they have demonstrated the presence of a protein-like substance in the expired breath. This substance may or may not be toxic. This work has recently been corroborated by Jordan and Hixson,[2] who also obtained similar results with filtered saliva as the sensitizing agent. In view of the fact that filtered saliva sensitizes animals against a subsequent injection of normal blood serum, it may be argued that the experiments of Rosenau and Amoss are inconclusive.

[1] A brief review of the literature on the organic matter in the expired breath is given by Rosenau and Amoss (5). A complete digest of the work on the composition of expired air and its effect on animal life is given by Billings, Mitchell, and Bergey (1).

[2] Personal communication from Dr. E. O. Jordan.

These observers, however, carefully guarded against the contamination of the condensed liquid by saliva, and are certain that no contamination occurred. The liquid condensed was uniformly sterile. If any saliva solution had passed the two glass-wool filters, it is almost certain that this liquid would not have been free from bacteria.

The method employed in the following experiments for collecting the products of the expired breath was essentially the same as that used in the experiments of Rosenau and Amoss (5).

EXPERIMENTS WITH THE GUAIACUM REACTION.

It is well known that a 5 per cent. solution of gum guaiacum in alcohol takes on a blue color in the presence of blood and a small amount of oxygenated turpentine or hydrogen peroxide, that the delicacy of this test is variable, and that different samples of the commercial gum do not react with equal sensitiveness. Various modifications of the reagent have been proposed in order to make the tests more uniform and to increase the delicacy of the reaction as a test for blood. Among these modifications we find the method of Weichardt who purifies the resin by precipitating it from a solution in equal parts of acetic acid and alcohol by the addition of water. A purified product, called guaiacin, can be obtained on the market. A 5 per cent. emulsion of guaiacin in 70 per cent. alcohol was used in the following experiments.

Experiment 1.—The concentrated and dialyzed liquid condensed during five hours from the expired breath of S., plus dilute blood solution, plus two drops of ozonized turpentine, plus guaiacin indicator, gave a very slight blue color. Five c.c. of a weak solution of calcium chloride plus blood solution with ozonized turpentine gave a strong blue color with guaiacin. With the guaiacin indicator, a control of 5 c.c. of water and dilute blood solution showed about the same amount of blue color as the liquid condensed from the expired breath.

Experiment 2.—Pure calcium chloride was exposed in Petri dishes in a cabinet of 500 cubic feet capacity in which twelve persons remained for one hour. The persons came out quickly and the room was closed for the next twenty-four hours, during which time the five Petri dishes remained exposed to the vitiated air. The calcium chloride absorbed the moisture and the saturated solution of the salt thus obtained was added to dilute blood solution and tested with the guaiacin indicator. All tubes gave a blue color. This experiment was repeated with the same results. These results do not confirm those of Weichardt who found that calcium chloride in a Petri dish covered with a filter to keep

out particulate matter and exposed in a bedroom over night absorbed from the expired breath of the person sleeping there a substance which prevented the bluing of the guaiacin indicator by blood.

Experiment 3.—Calcium chloride was placed in sterile Petri dishes and breathed upon directly until a saturated solution of calcium chloride was obtained (two hours). A mixture of equal parts of this solution and blood solution were tested with the guaiacin, a blue color resulting immediately.

Experiment 4.—In order to determine the effect upon the guaiacin indicator of calcium chloride alone the following tubes were tested. The results are given below.

Solutions of calcium chloride.	Result of guaiacum.
1 per cent. solution	Slight bluing.
25 per cent. solution	Blue immediately.
1 per cent. solution made alkaline with sodium hydroxide	Yellow.
25 per cent. solution made alkaline with sodium hydroxide	Yellow.
1 per cent. solution made acid by acetic acid	Tan.
25 per cent. solution made acid by acetic acid	Tan.

These results are in accordance with those obtained by Alsberg (6), who found that various chlorides, with and without the aid of hydrogen peroxide, could bring about the bluing of guaiacum. Among the metals tried were cobalt, nickel, aluminum, copper, and iron; even sodium and potassium chlorides gave a positive test in the presence of hydrogen peroxide. "In all cases the reaction was most marked in neutral or weakly acid solution. Noticeable amounts of alkali or mineral acids retarded the reaction."[3] Calcium salts were not tested.

Smith and MacDonald (7) have shown that guaiacum gives a positive reaction with each of the mineral constituents of human saliva. It would seem, therefore, from these facts that the guaiacum test is unreliable. Alsberg, in fact, as the result of his experiments comes to the conclusion that the guaiacin reaction is extremely unreliable.[4] Are we therefore justified in assuming that an organic body exists in the expired breath when we obtain a negative test for blood with guaiacum in a solution known to contain blood and to which there has been added the fluid obtained in Weichardt's experiment? To answer this question a series of three tubes was prepared.

Experiment 5.—Tube 1 contained 5 c.c. of dilute blood solution; tube 2, 5 c.c. of dilute blood solution plus 2 drops of N/10 sodium hydroxide, and tube 3,

[3] Alsberg, C. L., *loc. cit.*, p. 43.

[4] Alsberg, C. L., *loc. cit.*, p. 52.

5 c.c. of blood solution plus 2 drops of N/10 hydrochloric acid. On testing these tubes with guaiacin indicator and oxygenated turpentine the following results were obtained:

Tube 1	Blue immediately.
Tube 2	Yellow,—later green.
Tube 3	Very light blue.

This inhibiting effect of small amounts of acids and alkalis on the guaiacum reaction for blood is more sharply and definitely shown by using 2.5 cubic centimeters of a 25 per cent. solution of calcium chloride with an equal amount of dilute blood solution. Thus with tubes 1, 2, and 3 (neutral, alkaline, and acid, as indicated above), the following results were obtained with the guaiacin indicator after three hours:

Tube 1	Deep blue.
Tube 2	Yellow.
Tube 3	Tan.

If sodium chloride was used instead of calcium chloride, the results were slightly different. For example, when the blood was diluted with physiological normal salt solution, the following results were obtained:

Tube 1	Deep blue.
Tube 2	Blue after 3 hrs.
Tube 3	Brown after 3 hrs.

As the result of the above experiments it is plain that factors other than some specific organic compound in the expired breath are able to account for the inhibition of the guaiacum test for blood as reported by Weichardt. It should be noted that this observer collected some of his material by allowing the expired breath to pass through normal hydrochloric acid and then concentrating and neutralizing the resulting solution. From these experiments it will be seen that if he did not add sufficient alkali for neutralization, or if he added too much, a negative result would be obtained with the guaiacum test.

RESIDUE ON EVAPORATION.

The expired breath of S. (May 5) was allowed to bubble through N/10 hydrochloric acid for five hours. The resulting liquid, amounting to 55 c.c., was evaporated to dryness on a water bath in a weighed platinum dish leaving a

residue of 0.0021 gm. On heating, dense white fumes were given off. There was no odor, blackening, or charring, and no weighable residue.

Experiment 6.—In a similar experiment, lasting four hours (S.), only 0.0004 gm. of residue was found in the 25 c.c. of liquid collected, but in this experiment the delivery tube inside the Drechsel bottle did not dip into the acid solution until some condensation had taken place. This residue was volatile at dull red heat.

Experiment 7.—Still another experiment of this kind was carried out under the same conditions as the first, with the result that in the 35 c.c. of liquid, 0.0019 gm. of residue was found and this was entirely volatilized with dense fumes. There was no odor, charring, or blackening.

Experiment 8.—Another experiment of four hours' duration, with the same person, resulted in the collection of 30 c.c. of liquid. This was evaporated to dryness on a water bath leaving a residue weighing 0.0014 gm. This residue was taken up in ammonia-free water and Nesslerized. Calculated as ammonium chloride, 0.001365 gm. was found. In each case there was noticed the characteristic odor mentioned by Weichardt, but his observations on the blackening by ignition were not corroborated.

The results in these experiments seem to indicate that the organic matter in the expired breath retained by hydrochloric acid consists, within the limits of experimental error, of ammonia. Lehmann and Jessen (8) found that more ammonia was present in the fluid collected from the breath of a person with decayed teeth than in that obtained from a person whose teeth were sound. Bergey (1) also found variable amounts of ammonia in the condensations of the expired breath.

THE EFFECT OF THE PRODUCTS OF THE EXPIRED BREATH ON THE OXIDATION OF PHENOLPHTHALIN BY BLOOD.

From the experiments already cited, the guaiacum test for blood seems so unreliable and so readily affected by many agents that no satisfactory conclusions can be drawn from the results of the inhibition of this test by products of the expired breath. The use of the phenolphthalin test for blood naturally suggests itself as a means of testing the correctness of Weichardt's findings. This reagent was first proposed as a test for plant oxidases by Kastle (9) and was later employed by Kastle and Amoss (10) to determine the variations in the oxidase and peroxidase activity of human blood in health and disease. This test depends upon the fact that phenolphthalin, which is the leuco-compound of phenolphthalein, is

colorless in alkaline solution and is readily oxidized to phenolphthalein, which in alkaline solution exhibits a characteristic deep purplish red color. This reaction is quantitative and is very delicate. Kastle and Amoss made quantitative determinations using blood in a dilution of one part in 25,000, and by employing this reagent Kastle (11) was able to detect one part of blood in 80,000,000 parts of water. By this method, moreover, the processes of oxidation by blood can be studied quantitatively and even the variations in the rate of reaction can be investigated.

THE PHENOLPHTHALIN REAGENT.

The oxidase reagent consists of 0.032 of a gram of phenolphthalin plus twenty-one cubic centimeters of N/10 sodium hydroxide made up to 100 cubic centimeters with water.

The peroxidase reagent consists of the same substances as the oxidase reagent plus ten cubic centimeters of 0.034 per cent. hydrogen peroxide, the whole being made up to 100 cubic centimeters with distilled water.

THE BLOOD SOLUTIONS.

For the oxidase tests, 10 c. mm. of blood of A., taken with a Zeiss red blood corpuscle pipette, were diluted to 100 c.c. with distilled water, making a dilution of 1:10,000. Two c.c. of the fresh blood solution were measured in a pipette, by means of the apparatus designed by Rosenau (12), into a glass stoppered bottle. There were then added 5 c.c. of the solution to be tested for the inhibitive action plus 5 c.c. of the oxidase reagent, all in a glass stoppered bottle which was then set aside for twenty-four hours at room temperature. The peroxidase tests were made in the same manner except that the 10 c. mm. of blood were diluted to 250 c.c., making a dilution of 1:25,000. For the sake of convenience and for comparison of the oxidase and peroxidase tests, the oxidation was allowed to proceed at room temperature for about twenty-four hours. As a matter of fact, the percentage of oxidation in the peroxidase tests is apparently greater at the end of one hour than at the end of twenty-four hours, due to the fading in color of the phenolphthalein in alkali, to the presence of hydrogen peroxide, and to another influence, for Kastle and Amoss (10) found the fading to vary with the character of the blood, "being more rapid in the case of diseased bloods, poor in hemoglobin." The greatest depth of color in the oxidase tests was found after twenty-four hours.

The percentage of oxidation in each case was determined by comparing the colored solution with a known solution of phenolphthalein in alkali by means of a Duboscq colorimeter. This standard solution differed slightly from the solution usually employed, being made up of 0.0318 gm. of phenolphthalein in 50 per

cent. alcohol (dissolved first in 50 c.c. of absolute alcohol and then diluted with water to 100 c.c.), and 1 c.c. of this solution plus 1 c.c. of N/10 alkali plus 10 c.c. of water, making a total dilution of 12 c.c. to correspond with the total volume of the tests.

It was found that a permanent standard could be made with a dilute aqueous solution of basic fuchsin and a very small amount of Loeffler's methylene-blue. In a glass stoppered bottle this standard keeps well and without change for a month. It is evident that such a standard saves the time necessary to measure out carefully the phenolphthalein solution and the N/10 alkali.

The standard cell in the colorimeter is set at a convenient number, generally at 5 or 10 mm. It will be seen by calculating the amounts of phenolphthalin oxidized and of phenolphthalein formed, and the percentage in each case, that a simple formula can be obtained whereby the end results may be secured by a single calculation. If x is the reading, and the standard set is at 10 mm., then 100/x is the percentage of phenolphthalin oxidized. If the standard is set at 5, then 200/x is the percentage of phenolphthalin oxidized.

Experiment 1.—The vapor from the expired breath of M. (female, age 23 years) was condensed for a period of three hours in the Drechsel bottle already described, and set in a freezing mixture. The total amount was 20 c.c. (solution 1).

The vapor in the expired breath of L. (male, age 26 years) was also collected in the same manner in a separate apparatus for a period of two hours. The total volume was 15 c.c. (solution 2). The expired breath of R. (male, age 19 years) was passed for three hours through 10 c.c. of N/10 hydrogen chloride, using a 16 oz. Drechsel bottle set in water at 20° C. This solution was neutralized with N/10 sodium hydroxide (solution 3). Its total volume was 30 c.c. The solutions were brought to room temperature and tested for inhibiting power on the peroxidase reaction of the diluted blood of L. (1:25,000) in the manner already described. At the end of one hour, and of thirty hours, they were compared in the tintometer against the standard set at 5 mm., with the following results:

Solution No.	Reading after 1 hr.	Per cent. of phenolphthalin oxidized.	Reading after 30 hrs.	Per cent. oxidized.
1	3.2	31.2	3.2	31.2
2	3.0	33.3	3.0	33.3
3	3.0	33.3	3.0	33.3
Control	2.9	34.5	2.9	34.5

These figures show slightly less oxidation in the case of the condensed liquids than in the control. All, however, agree within the limits of experimental error.

Experiment 2.—Vapor from the expired breath of M. was collected for three hours in an apparatus placed in a freezing mixture (solution 1). The solution was kept cold until used (twenty-four hours). The vapor from the expired breath of M. was collected for two hours by passing it through a Drechsel bottle containing 10 c.c. of N/10 hydrochloric acid. During the collection, the

bottle was kept in a freezing mixture. The fluid was concentrated *in vacuo* to one third its volume and 10 c.c. of N/10 sodium hydroxide were added (solution 2). The solution was kept in a cold room until used (twenty-two hours). As in solution 2, vapor from the expired breath of M. was passed through N/10 hydrochloric acid in the cold. The acid was neutralized and the fluid was then placed in a tube for concentrating colloids by means of a direct current. A direct current of 110 volts and about 0.1 ampere was passed through this solution for three hours. The liquid in the cathodal end of the tube was marked solution 3.

These solutions were tested in duplicate for any inhibitory effects on the oxidation by peroxidases and oxidases, and the readings were compared at the end of twenty-four hours. The standard was set at 10 mm.

Solution No.	Peroxidase test.		Oxidase test.	
	Reading on tintometer when compared with standard set at 10 mm.	Per cent. of phenolphthalin oxidized.	Reading on tintometer when compared with standard set at 10 mm.	Per cent. of phenolphthalin oxidized
1	6.3	31.5	3.1	64.5
1	6.3	31.5	3.1	64.5
2	6.0	33.3	2.4	83.3
2	6.0	33.3	2.4	83.3
3	6.4	31.2	4.1	48.8
3	6.4	31.2	4.1	48.8

The results in this experiment show practically no difference in the amount of oxidation in the case of the peroxidases. However, in the oxidase test the simple condensations in the cold (solution 1) showed some inhibiting power (20 per cent.) over the acid collections (solution 2), and the greatest diminution was in the case of the concentrated solution. It was thought that if organic matter were in the expired breath in the form of colloids, they would travel in the direction of the current and collect in the cathodal end of the tube.[5] Of course the presence of sodium chloride in the solution should be considered as the first possible cause of any inhibition of the oxidase reaction. The ends of the tubes were closed by celloidin membranes, but there was hardly sufficient area to allow complete dialysis. In fact, chlorides were found in the liquid after the passage of the current was discontinued.

Experiment 3.—Another attempt at concentration by the direct current was made on March 18. For two hours on three successive days M. breathed into

[5] Dr. C. S. Hudson in a personal communication states that he has been able to concentrate invertase by this method even to the extent of obtaining this ferment free from nitrogen.

10 c.c. of N/10 hydrochloric acid in the same apparatus, placed in water at 20° C. At the end of each period the liquid was neutralized with N/10 sodium hydroxide and immediately placed in the concentrating tube. The current was allowed to flow over night. When new liquid was added each day, that in the cathodal end was kept and the remainder replaced by new material. It was planned to concentrate in this manner the expired breath for six hours, allowing the minimum time for any possible decomposition. The fully concentrated liquid was dialyzed for two hours in a collodion sac (solution 1). The liquid in the anodal end of the tube constituted solution 2.

Five gm. of calcium chloride were exposed for forty-eight hours to the air of a closed cabinet of 300 cubic feet capacity in which eight persons had been for one hour. The carbon dioxide content went up to 6.5 per cent. The calcium chloride absorbed water to about 15 c.c. of the total volume, and the solution thus formed was dialyzed in a celloidin sac for two hours (solution 3). Five gm. of calcium chloride were dissolved in 15 c.c. of water (solution 3 B).

Together with the solutions described above a control of distilled water was tested for its effect on the peroxidase and oxidase reactions, and gave the following results:

Solution No.	Peroxidase test.		Oxidase test.	
	Reading on tintometer when compared with standard set at 10 mm. Average of 5 readings.	Per cent. of phenolphthalin oxidized.	Reading on tintometer when compared with standard set at 10 mm. Average of 5 readings.	Per cent. of phenolphthalin oxidized.
1	6.1	32.8	18.4	10.9
1	6.2	32.3	18.4	10.9
2	15.0	13.3	33.0[6]	6.6
2	15.5	13.0	33.0[6]	6.6
3	8.1	24.7	60.0[7]	3.5
3	8.1	24.7	60.0[7]	3.5
3 B	12.3	16.3	Very faint pink	—
3 B	12.3	16.3		—
Control	6.2	32.3	2.5	80.0
Control	6.2	32.3	2.5	80.0

In this series we find that the concentrated products of the expired breath of one person during a period of six hours (solution 1) had no effect on the peroxidase reaction, but the oxidase reaction was greatly inhibited. Strangely enough the liquid left over from such a

[6] In this case the standard was set at 3 mm. and the reading was 9.9. When this is calculated in terms of the standard set at 10 mm., we get 33.

[7] Estimated, but too faint for exact comparison.

concentration (solution 2) showed decided inhibition in the peroxidase tests and a greater inhibitory action on the oxidase test than the concentrated liquid. The calcium chloride that had been exposed in the cabinet, and the control showed marked effect on the peroxidase and the oxidase. It is to be noted that the control (solution 3 B) was not dialyzed.

The results in this series direct our attention to the effect of the salts present on the reactions. The mixtures were accordingly titrated by Volhard's method with N/10 silver nitrate.

Solution.	Salt present.	N/10 silver nitrate required in c.c.	Amount of salt present in gm.
Control	None	0.0	———
1	NaCl	0.6	0.0029 NaCl.
2	NaCl	1.4	0.0055 NaCl.
3	$CaCl_2$	47.0	0.2608 $CaCl_2$.
3 B	$CaCl_2$	48.0	0.2664 $CaCl_2$.

These results show that the inhibiting effect is roughly proportional to the amount of inorganic matter present. In order to determine the effects of the salts which might be found in the various fluids in the attempt to absorb any organic matter that might be present in the expired breath, a series of experiments with sodium chloride, calcium chloride, and ammonium chloride were set up in both the peroxidase and the oxidase tests.

Experiment 4.—Two c.c. of blood were added to varying amounts of the salts and water to make a series of concentrations, in each case with a total volume of 12 c.c. from N/5 to N/100.

Amount of N/1 taken.	Amount of water added.	Solution No.
2.4 c.c. to make 12 c.c. of N/5	2.6 c.c.	1
1.2 c.c. to make 12 c.c. of N/10	3.8 c.c.	2
0.6 c.c. to make 12 c.c. of N/20	4.4 c.c.	3
0.24 c.c. to make 12 c.c. of N/50	4.76 c.c.	4
0.12 c.c. to make 12 c.c. of N/100	4.88 c.c.	5

These tests were allowed to stand at room temperature for twenty-four hours and were then compared with the standard with the following results:

Solution containing blood plus reagent.	Peroxidase reaction.		Oxidase reaction.		N/10 HCl in c.c. required to discharge pink color.
	Reading on tintometer when compared with standard set at 5 mm. Average of 5 readings.	Per cent. of phenolphthalin oxidized.	Reading on tintometer when compared with standard set at 5 mm. Average of 5 readings.	Per cent. of phenolphthalin oxidized.	
N/5 NaCl.....	6.6	15.1	2.0	50.0	0.9
N/5 NaCl.....	6.6	15.1	2.0	50.0	0.9
N/10 NaCl.....	6.5	15.4	1.7	58.8	0.9
N/10 NaCl.....	6.5	15.4	1.7	58.8	0.9
N/20 NaCl.....	6.6	15.1	1.8	55.5	0.9
N/20 NaCl.....	6.6	15.1	1.8	55.5	0.9
N/50 NaCl.....	4.2	23.8	1.8	55.5	0.9
N/50 NaCl.....	6.5	15.4	1.8	55.5	0.9
N/100 NaCl.....	4.2	23.8	1.8	55.5	0.9
N/100 NaCl.....	6.5	15.4	1.8	55.5	0.9
N/5 $CaCl_2$.....	10.6	9.5	40.0	2.5	0.9
N/5 $CaCl_2$.....	10.6	9.5	40.0	2.5	0.9
N/10 $CaCl_2$.....	8.0	12.5	30.0	3.3	0.9
N/10 $CaCl_2$.....	8.0	12.5	30.0	3.3	0.9
N/20 $CaCl_2$.....	6.2	16.0	18.0	5.5	0.9
N/20 $CaCl_2$.....	6.2	16.0	18.0	5.5	0.9
N/50 $CaCl_2$.....	6.0	16.7	3.2	31.2	0.9
N/50 $CaCl_2$.....	6.0	16.7	3.2	31.2	0.9
N/100 $CaCl_2$.....	5.5	18.1	5.0	20.0	0.9
N/100 $CaCl_2$.....	6.0	16.7	5.0	20.0	0.9
Control.....	6.8	14.7	1.6	62.5	0.9
Control.....	7.5	13.3	1.6	62.5	0.9

Experiment 5.—In testing the effect of sodium and ammonium chlorides the following results were obtained after twenty-four hours:

Solution containing blood plus reagent.	Peroxidase reaction.		Oxidase reaction.	
	Reading on[1] tintometer when compared with standard set at 5 mm. Average of 5 readings.	Per cent. of phenolphthalin oxidized.	Reading on tintometer when compared with standard set at 5 mm. Average of 5 readings.	Per cent. of phenolphthalin oxidized.
0.85% NaCl.....	6.4	15.6	7.6	12.0
0.85% NaCl.....	8.5	18.1	8.0	12.5
N/5 NaCl.....	5.5	18.1	5.0	20.0
N/5 NaCl.....	4.8	21.2	6.0	16.7
N/10 NaCl.....	4.5	22.2	5.0	20.0
N/10 NaCl.....	4.5	22.2	5.0	20.0
N/20 NaCl.....	5.0	20.0	5.0	20.0
N/20 NaCl.....	5.5	18.1	5.0	20.0
N/5 NH_4Cl.....	26.0	3.8	Very slight	——
N/5 NH_4Cl.....	30.0	3.3	pink.[8]	——
N/10 NH_4Cl.....	10.0	10.0	29.0	3.4
N/10 NH_4Cl.....	12.0	8.3	29.0	3.4
N/20 NH_4Cl.....	5.5	18.1	8.2	12.2
N/20 NH_4Cl.....	5.5	18.1	8.2	12.2
N/50 NH_4Cl.....	5.5	18.1	5.5	18.1
N/50 NH_4Cl.....	5.5	18.1	5.5	18.1
N/100 NH_4Cl.....	5.0	20.0	5.5	18.1
N/100 NH_4Cl.....	5.0	20.0	5.5	18.1
Control.....	4.8	21.2	5.2	19.2
Control.....	5.0	20.0	5.0	20.0

Sodium chloride solutions up to N/5 seem to have no effect on the blood peroxidase and oxidase reactions with phenolphthalin in alkali. When the whole solution is made isotonic there is slight diminution in the peroxidase activity and more in the oxidase reaction. This is in harmony with the fact recently brought out that the catalase activity of the blood is decreased by preventing the laking of blood. Calcium chloride and ammonium chloride in higher concentrations show an inhibiting power on the peroxidase reaction. Calcium chloride in N/100 solution affects the oxidase reaction, and this effect is increased with the concentration of the calcium. That the diminution was not due to any acidity of the salts is seen from the fact that the amount of N/10 hydrochloric acid necessary to

[8] Deepened slightly on addition of more alkali.

discharge the color in every case was the same. Ammonium chloride in N/20 solution also decreases the oxidase reaction as the concentration of the salt increases; in N/5 solution there is very little oxidation. It is to be noted that in the latter case there was a slight deepening of the color on the addition of more alkali, showing that there was some oxidation. This oxidation, though slight, was hidden, probably on account of the decrease in alkalinity due to a loss of ammonia.

The results are such as one would expect. Theoretically the stronger base and acid would unite leaving the weaker base to form a salt with the phenolphthalin. In the calcium chloride experiment theoretically we should have the sodium of the phenolphthalin salt in the reagent and any excess as a free base unite with the chlorine of the calcium chloride leaving the calcium to form the organic salt and to furnish the alkalinity. Ammonium chloride acts in this way, so our experiment shows that the calcium and ammonium salts of phenolphthalin do not serve well as indicators for the peroxidases and especially the oxidases.

Experiment 6.—Since guaiacum has been shown to give a positive reaction with certain salts it was decided to test the conduct of the phenolphthalin reagent towards the salts alone. To that end 2 c.c. of water were used instead of dilute blood solution. The solutions therefore contained 5 c.c. of the reagent, 7 c.c. of water, and sufficient salt to make the total solution N/5.

At the end of twenty-four hours the tests showed the following results:

Peroxidase reagent.	Oxidase reagent.
Control—faint pink.	Control—very faint pink.
N/5 calcium chloride—very faint pink.	N/5 calcium chloride—colorless.
N/5 sodium chloride—3.3 per cent. oxidation.	N/5 sodium chloride—5.5 per cent. oxidation.

Sodium chloride in N/5 solution seems to effect a slight oxidation of phenolphthalin in twenty-four hours with and without the aid of hydrogen peroxide. Kastle reports a faint pink color in a similar experiment.

THE EFFECT OF SALTS ON THE COLOR OF PHENOLPHTHALEIN IN ALKALI

Experiment 7.—It is to be expected that the presence of salts of a weak base should decrease the depth of color of phenolphthalein in alkali. Accordingly

[9] The addition of alkali gave no color.

1 c.c. of phenolphthalein standard plus 7.6 c.c. of water plus 1 c.c. of N/10 sodium hydroxide plus 2.4 c.c. of N/1 ammonium chloride solution (making a total of 12 c.c. of N/5 ammonium chloride solution) was compared with 1 c.c. of standard phenolphthalein solution plus 10 c.c. of water plus 1 c.c. of N/10 sodium hydroxide. The result was that a layer of 5 mm. of the solution without ammonium chloride was equal in intensity to a layer 22 mm. thick of the solution plus ammonium chloride. In other words, the presence of ammonium chloride to the extent of N/5 solution decreases the color of the standard phenolphthalein reagent by 80 per cent.

FURTHER EXPERIMENTS WITH EXPIRED AIR.

Experiment 7.—For three hours a day for six successive days the expired breath of M. was blown through the same sterile Drechsel bottle in which there were 10 c.c. of N/10 hydrochloric acid. The bottle was set in water at 20° C. and was not placed in the cold during the intervals between the blowing periods. The two glass-wool filters were kept warm by the heat from an electric light and the first filter was replaced by a dry sterile filter when there was visible condensation or contamination with saliva.

At the end of the last breathing period 11 c.c. of N/10 sodium hydroxide were added making the total volume 75 c.c. (solution 1). This liquid reacted slightly alkaline to litmus. Some of solution 1 was placed in an Arnold sterilizer (temperature 100° C.) for twenty minutes, taken out, and allowed to come to room temperature (solution 2).

With a similar apparatus and with the same technique that was used for solution 1, the expired breath of R. for twelve hours, of M. for two hours, and of L. for seven hours, all in the same apparatus, was passed through 10 c.c. of N/10 hydrochloric acid. This solution was kept at 44° F. for six hours before it was brought to room temperature and 11 c.c. of sodium hydroxide were added (solution 3). The total volume was 90 c.c. Some of solution 3 was heated in an Arnold sterilizer at 100° C. for twenty minutes and was then allowed to cool (solution 4).

Ten c.c. of N/10 hydrochloric acid plus 55 c.c. of redistilled water were placed in a sterile Drechsel bottle, and carbon dioxide from a cylinder of the compressed gas was allowed to bubble through for five minutes. Eleven c.c. of N/10 sodium hydroxide were added (solution 5). Some of solution 5 was heated in an Arnold sterilizer (solution 6).

The control consisted of 10 c.c. of N/10 hydrochloric acid plus 11 c.c. of N/10 sodium hydrate.

These solutions were tested for their effect on the peroxidase and oxidase reactions in the usual manner excepting that the blood solutions were in contact with the various solutions to be tested for thirty minutes before the reagents were added. At the end of twenty hours the following results were obtained:

Solution No.	Peroxidase reaction.		Oxidase reaction.	
	Reading of tintometer when compared with standard set at 5 mm.	Per cent. of phenolphthalin oxidized.	Reading of tintometer when compared with standard set at 5 mm.	Per cent. of phenolphthalin oxidized.
1	6.1	16.4	1.4	71.4
1	6.1	16.4	1.4	71.4
2	8.0	12.5	2.5	40.0
2	5.8	17.2	2.5	40.0
3	7.5	13.3	1.4	71.4
3	6.1	16.4	1.4	71.4
4	5.9	16.9	1.9	52.6
4	5.9	16.9	1.9	52.6
5	No color	0	No color	0
5	No color	0	No color	0
6	5.8	17.2	2.8	15.7
6	5.8	17.2	2.8	15.7
Control	5.5	18.1	1.9	52.6
Control	5.5	18.1	1.9	52.6

These results show that practically no effect is produced on the peroxidase reaction by the products of expired breath retained by hydrochloric acid. The effect of an excess of carbon dioxide in preventing the reaction absolutely is what might be expected for the reason that carbon dioxide reacts more strongly acid than the phenolphthalin. The carbon dioxide, therefore, combines with the sodium hydroxide and prevents the formation of the sodium salt of the phenolphthalin so that the oxidation to phenolphthalein is not accomplished. The results in the oxidase test show some interesting variations. It cannot be said now what factor is able to influence the process as shown in solutions 1 and 3 and solutions 2 and 4. The higher percentages over the control in solutions 1 and 3 are perhaps due to the slight excess of alkalinity, as an excess of one cubic centimeter of N/10 sodium hydroxide was added, in one case accidentally, and in the other cases to make the tests uniform. It will be remembered that the volumes in solutions 1 and 3 were less, and the alkalinity for a given quantity would, therefore, be increased. The decrease after heating is not understood, but it may be due to the decrease in dissolved oxygen in the solution. In the case of solution 6, the acid carbonate remaining after drawing off

the carbon dioxide did not favor the reaction in the same manner that the carbonate does.

Experiment 8.—In still another experiment the expired breath was forced through 10 c.c. of N/10 hydrochloric acid for nineteen hours (S., seven hours, R., four hours, and L., eight hours) in the manner already described. The liquid was kept in the cold for five hours before testing. Ten c.c. of N/10 sodium hydroxide were added after the solution had been brought to room temperature (solution 1). The total volume was 90 c.c.

One c.c. of saliva was diluted to 80 c.c. with distilled water, 10 c.c. of hydrochloric acid were added and the whole solution was well shaken. After the solution had stood for thirty minutes, 10 c.c. of N/10 sodium hydroxide were added (solution 2). The control consisted of 10 c.c. of N/10 hydrochloric acid plus 70 c.c. of distilled water plus 10 c.c. of N/10 sodium hydroxide.

After thirty hours the following results were obtained:

Solution No.	Peroxidase reaction.		Oxidase reaction.	
	Reading of tintometer when compared with standard set at 5 mm.	Per cent. of phenolphthalin oxidized.	Reading of tintometer when compared with standard set at 5 mm	Per cent. of phenolphthalin oxidized.
1	5.8	17.2	1.5	66.6
1	5.8	17.2	1.5	66.6
2	6.5	15.4	1.5	66.6
2	3.5	28.6	1.5	66.6
Control	3.5	28.6	1.5	66.6
Control	3.5	28.6	1.5	66.6

In spite of careful manipulations the duplicate in the peroxidase series shows a variation of over 10 per cent. However, we have proved that a contamination of one cubic centimeter of saliva (more than could possibly have gotten through the filters) shows no effect on the oxidase reaction. Using the same apparatus for the same length of time, Rosenau and Amoss (5) found no bacterial contamination where care was exercised in the breathing experiments. The test with solution 1 shows that we were unable to obtain an inhibitory agent by continuously passing the expired breath through hydrochloric acid for a period of nineteen hours.

SUMMARY AND CONCLUSIONS.

Weichardt claims to have demonstrated the presence of an organic body in the expired air. He allowed the exhaled breath to pass through hydrochloric acid solution, evaporated the resulting

solution to dryness on a water bath, and obtained a weighable residue which charred on ignition. If he neutralized the acid solution and concentrated it under reduced pressure, he obtained a solution which inhibited the bluing of the guaiacum indicator by blood. By exposing calcium chloride in a room in which the air had been vitiated, he claimed also to have obtained a substance from the air which prevented the bluing of the guaiacum indicator by blood.

The experiments here recorded show that a variable amount of matter is retained by weak hydrochloric solution when exhaled breath is passed through it, and that this matter is volatile on ignition. Contrary to the findings of Weichardt, there is no charring or blackening. Nesslerization shows the residue to consist mainly, if not wholly, of ammonium chloride. This ammonia is believed to have come from the decomposition of food particles about the teeth. In one case the person (S.) had smoked just before the experiment, so that a small amount of the ammonia from the tobacco smoke may have been held temporarily by the saliva and food particles in the mouth and been given off gradually during the experiment. Weichardt's experiments on the inhibition of the guaiacum test for blood by means of the substances retained when exhaled breath is passed through hydrochloric acid or over calcium chloride crystals are not corroborated. It is further shown that the guaiacum indicator is unreliable for these tests in view of the fact that a small amount of free acid or free alkali will inhibit the guaiacum test for blood. This fact is offered as a probable explanation of Weichardt's results.

Calcium chloride alone gives a deep blue color with the guaiacin indicator. Weichardt used this salt to collect from the expired breath certain unknown substances which he claims inhibit the oxidation of guaiacum by blood. His results are therefore inconclusive.

The phenolphthalin test for blood has been studied in this connection and further light has been thrown on this reaction. The sodium salt of phenolphthalin is colorless in alkaline solution, and is readily oxidized by minute quantities of blood to phenolphthalein which gives a characteristic deep purplish red color in alkaline solution.

It has been found that the presence of calcium chloride and ammonium chloride in small amounts retards and, in large amounts, prevents this reaction. It is believed that any salt composed of a weak base combined with a strong acid will have the same effect. This is discussed in the text. It has also been shown that the presence of calcium chloride or ammonium chloride decreases the depth of color of phenolphthalein in sodium hydroxide solution.

Carbon dioxide also prevents the oxidation of phenolphthalin by blood. Of course this does not mean that carbon dioxide prevents the action of the oxidizing ferments generally. In this particular case the substance to be oxidized, namely phenolphthalin, was not allowed by reason of the presence of the carbon dioxide to combine with the alkali and thereby assume a state in which it could be easily oxidized.

The results of one experiment seem to indicate a relation between the amount of dissolved oxygen in the solutions and the percentage of oxidation. Sodium chloride either alone or with the aid of hydrogen peroxide is able to bring about the oxidation of phenolphthalin in alkali to a very slight extent (3.5 to 5 per cent. in twenty-four hours). Therefore phenolphthalin as a test for oxidizing ferments should not be used in the presence of an appreciable amount of inorganic salts or carbon dioxide. Complete dialysis is recommended in these cases. It is also to be noted that the great delicacy of the test allows considerable dilution.

Liquids were obtained from the expired breath by passing this through weak hydrochloric acid or by condensing the moisture in it by conducting it through cooled Drechsel bottles. Attempts were then made to prove the presence in these liquids of some substance which inhibits the oxidation of phenolphthalin by blood, but all were unsuccessful. Moreover attempts to concentrate these liquids by evaporation under reduced pressure or by the passage of a direct current (colloidal travel) were also unsuccessful.

It is planned to improve upon the apparatus used to concentrate colloids by the passage of a direct current, and to test the effect of expired breath products on the rate of oxidation of phenolphthalin by blood.

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