A precise investigation of some micro-organisms and soluble ferments, their chemical history and relation to disease : including also a practical study of the disinfecting value of 'Sanitas' fluids / by C.T. Kingzett.

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PREFACE.

I have conducted the investigation, the results of which are incorporated in this memoir, as an answer to some charges which have been brought against "Sanitas" as a disinfectant. These charges have been made by microscopists who have been content with mere appearances, and they have not been substantiated by a single chemical experiment the result of which can be weighed or measured, nor by one test the fairness or accuracy of which is acceptable to a trained scientific mind. It is not worth while to argue about conclusions drawn exclusively from mere microscopic appearances which are in themselves so terribly misleading. I am content to insist upon the accuracy of my views regarding the relations of micro-organisms to disease—views which are entertained by the leading investigators in this department of science and by the clearest thinkers, and which are sustained in their entirety by the chemical experiments that I have made and which afford in themselves the weight and measure of their correctness.

It is to be feared that there will always be the hurried and undignified, because unscientific, search for new remedies, attending every great advancement in scientific knowledge, such as that which has been made in recent years in relation to micro-organisms and the propagation of disease. There will always be found, too, the little thinkers and the inaccurate observers; those who miss the real truth—as those who grasp it; in consequence, it is requisite from time to time to dissipate the clouds of uncertainty and error that gather round the truth, and thus to reveal the facts to the observation of those who otherwise do not know what to believe.

C. T. KINGZETT.

A PRECISE INVESTIGATION

OF

SOME MICRO-ORGANISMS AND SOLUBLE FERMENTS;

THEIR CHEMICAL HISTORY AND RELATION TO DISEASE,

ETC.

I.—INTRODUCTORY REMARKS.

In a previous paper,* treating of the precise relations of micro-organisms to disease, and the science of disinfection, I attempted to prove that micro-organisms are rather the indirect than the immediate causes of certain diseases; indeed, it is not going too far to say that at the present time there is a consensus of opinion amongst those who are best entitled to pronounce upon the subject, to the effect that so-called germ diseases are really due to the physiological effects of chemical substances elaborated by or in micro-organisms. I take credit as a chemist for having all along advocated these views, which are now very generally adopted. I have, however, gone further, and pointed out that if these views be correct, and are to be accepted, it necessarily follows that current views respecting disinfection must be amended in consequence. If micro-organisms do not in themselves constitute the *viri* of disease, it is not binding upon us to adopt as measures of disinfection those which aim at nothing less than their destruction; and it is well for humanity that something less than this suffices, for it is practically impossible to annihilate the thousand forms of

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* British Medical Journal, May 16th, 1885, p. 980.

microscopic life, all of which may, under certain circumstances, initiate disease in man. These living things are co-eternal and co-extensive with all other things in this life, and their origin and relationships are far too ill understood by man to fit him to be their conqueror. To banish such forms of life from our midst is impossible, and it is the idlest sham to deny the fact. Our power to control the life of these microbes is limited to the matter of conditions, so far as we have made ourselves acquainted with their life history, and that mainly from a chemical point of view. We know or can ascertain that they find life possible in certain numerous media, and we also know, or can find out by actual experiment, to what extent their life is interfered with by certain chemical substances. To suspend their life actions by means of suitable chemical substances, until that part of the body which has been diseased through their influence has recovered its normal strength and state ; similarly, so to influence, or to interfere with, the life of these micro-organisms that the particular poisons which they are capable of producing shall be no longer formed, or if allowed to be formed, shall be instantly destroyed in a chemical sense: these are the legitimate objects of science. To exterminate orders in creation; to aim at killing the prey by means which are necessarily fatal at the same time to the host: these objects are unreasonable and therefore unattainable.

We must begin, then, upon certain broad lines, believing that if we discover a chemical substance which exhibits the general property of arresting the functions, in various known media, of whole classes of micro-organisms, as indicated by a cessation in the production of what are regarded as normal products, we have in that said substance a reliable antiseptic, a true disinfectant.

From general lines, the investigation will in due course lead to a better knowledge of the individual micro-organisms and their specific life histories.

The germ theory of disease, as followed by many, concerns itself alone with the parasite and utterly ignores the host. Bacteria are observed in diseased parts and the various tissues, but in what relation to the disease and the tissues they stand—how the tissues (or rather their cells) are affected by the bacteria (or rather their chemical products)—are subjects which at present receive no

attention. Virchow has, also, recently indicated this defect in current investigations,* and it may be safely predicted that after a time there will be a more or less sudden devotion of scientific attention to the life history of the cells which build up the human body, and parasitic micro-organisms will then for a while be the subject of neglect. It seems to be a pity that both subjects should not be dealt with simultaneously.

Until we know more of cell life it appears to me that progress depends upon investigation conducted in the chemical laboratory; for chemists can construct many mixtures of known composition upon which micro-organisms will grow, multiply and flourish, and similarly they can both qualitatively and quantitatively ascertain in what ways the chemical molecules are broken up and re-arranged by the life agencies of the micro-organisms, and thus they can also place a quantitative value upon the action of so-called antiseptics and disinfectants.

It is in this sense that I have lately conducted an investigation of which I shall next proceed to describe the details and results so far as it has been carried. It is desirable, however, to precede this description by a few remarks, intended to anticipate an objection which will surely be raised against this investigation. It will be said that the value of such a research is diminished, since it only deals with zymases and with micro-organisms not known to be specifically associated with diseases. My answer is, that all we know respecting the nature and habits of pathogenic micro-organisms, warrants us in reasoning from analogy that they would be found to be similarly influenced could they be studied; but, so far, we know next to nothing of their behaviour to chemical substances, and so they cannot be studied in the same way. I may even go further and point out that the particular forms of life which are concerned in inducing the process of putrefaction are undoubtedly associated with and responsible for certain diseased processes, if not for so-called specific diseases; at least, they are responsible for the whole of those evils for the successful combat with which the practice of Antiseptic Surgery has been designed, and is now almost universally employed.

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* Archiv. der Path. Anat. Bd. 101, Heft. 1.

II.—THE CHEMICAL HISTORY OF SOME MICRO-ORGANISMS.

Yeast and the Fermentation of Glucose.

Experiment I.—A solution of glucose dissolved in water was prepared, containing, as was afterwards ascertained, 1·670 grms. of glucose in each 100cc.

A pasty solution of ordinary yeast was then made, containing about 12·10 per cent. by weight in volume. A microscopic examination of the yeast solution revealed the presence of numerous yeast cells of ordinary appearance and many other sorts of living micro-organisms.

A quantity (995 cubic centimetres) of the glucose solution was inoculated with 5cc. of the yeast solution, and the total solid contents of the mixture was determined and found to be 1·690 per cent.

A mixture (A) of 75cc. glucose and yeast solution with 25cc. of pure water was placed in one flask, fitted with a cork and bent glass delivery tube, while in a second similar apparatus was placed a mixture (B) of 75cc. glucose and yeast solution, 20cc. of "Sanitas" fluid (as sold)* and 5cc. water.

After standing at the ordinary temperature of the laboratory during 31 days a chemical examination of the several mixtures was made. That is to say—the total solid contents of each solution was determined, and the amount of alcohol produced by fermentation was ascertained by distilling a known quantity of each mixture, and taking the specific gravities of the distillates. The results are tabulated as follows :—

	Original Total Solids.	Final Total Solids.		Loss in Weight.	Percentage of loss.	Alcohol produced.	Alcohol calculated.
A.	1·2675	0·486	=	0·7815	61·6	·4576	·4000
B.	1·7295	1·700	=	0·0295	2·3	None.	—

The alcohol is calculated upon the basis of the following equation, which represents the fermentation of glucose into alcohol by the agency, as it is supposed, of a soluble zymase, formed by the yeast cells.

$C_6 H_{12} O_6 = 2C_2 H_6 O$ (Alcohol) $+ 2CO_2$ (Carbonic Anhydride).

* This solution upon evaporation to dryness left a residue=to 2·31 per cent.

Experiment II.—A fresh solution of glucose was prepared and inoculated with some yeast; its total solid contents=8·11 per cent.

A. 75cc. of this inoculated solution was mixed with 25cc. water.

B. 75cc. of the inoculated solution was mixed with 25cc. of "Sanitas" Fluid (the total solid residue of which dry at 100°C.= 2·694 per cent.).

The two mixtures were placed in flasks as before, fitted with corks and tubes for the delivery of any gas that might be evolved, and kept during seven days. Mixture A gave off a great deal of gas; it was not accurately measured, but over 375cc. was roughly measured as collected. Mixture B did not give off even a trace of gas.

The chemical examination of the solutions at the termination of the experiment gave results which are here tabulated as follows:—

	Original total solids.	Final total solids.	Loss in weight.	Percentage of loss.	Alcohol produced.	Alcohol calculated.
A.	6·0825	3·4950	2·5875	42·5	1·44	1·32
B.	6·7560	6·7560	None.	—	None.	—

A microscopical examination of mixture A showed the presence of many living yeast cells and a great number of other micro-organisms (many of which were in active motion), including single cells, association of cells in rods or chains and the *bacterium termo*. A similar examination of mixture B revealed the presence of much fewer yeast cells (not growing), and no moving micro-organisms were to be seen.

One third of the mixture A was now taken while in active fermentation, and to it was added 10cc. of "Sanitas" Fluid, the mixture being replaced in the original flask. The addition of the "Sanitas" absolutely put an end to the fermentation; not a trace of gas was evolved, and an examination, which was made after seven days, showed that practically no further loss of glucose had occurred, and consequently, also, no further alcohol was produced.

Original. total solids.	Final total solids.	Alcohol at start.	Alcohol at end.
1·4344	1·424	·500	·525

The trivial change that is noticeable undoubtedly occurred in the interval between the original examination of mixture A and the time when the "Sanitas" was added.

This result is interesting because it proves that in addition to its property of preventing the development of yeast cells in glucose solution, together with the resulting chemical changes, "Sanitas" fluid also arrests their development instantly, even when in the most active stage of growth.

EXPERIMENT III.—A further solution of glucose, impregnated with yeast, was prepared, yielding a total solid residue of 8·016 per cent. dry at 100° C. Three mixtures of this solution were prepared as follows :—

A. 75cc. of inoculated glucose solution + 25cc. water.

B. 75cc. „ „ „ + 5cc. „ +20cc. "Sanitas" fluid.

C. 75cc. of inoculated glucose solution + 25cc. of a 20 % solution of "Sanitas" emulsion.*

The "Sanitas" fluid which was used in mixture B yielded 4·758 per cent. of solid matters, dry at 100° C.

After standing seven days the experiments were brought to a conclusion by a chemical examination of each mixture and the following tabulated results were obtained :—

	Original total solid contents.	Final solid contents.	Loss in grms.	Per Cent. of loss.	Alcohol formed.	Alcohol calculated.
A.	6·012	4·2855	1·7265	28·55	0·870	0·882
B.	6·9636	6·9585	0·0051	0·073	None	—
C.	7·3355	7·287	0·0485	0·633	None	—

These results are confirmative of those observed in the earlier experiments, and at the same time show that very small quantities of "Sanitas" emulsion (and "Sanitas" oil, from which it is made) act like "Sanitas" fluid in preventing alcoholic fermentation dependent upon the growth of yeast.

YEAST AND THE FERMENTATION OF STARCH.

I thought it would be of some interest and importance to ascertain, if, by the living presence of yeast in a solution of starch, that chemical substance is resolved into alcohol. Such a result, if established by experiment, would be of importance, not alone from a chemical and technical point of view, but also as bearing upon the

*=5cc. of "Sanitas" emulsion in the undiluted state, and consequently about 2·5cc. of "Sanitas" oil.

germ theory of disease. It is too often assumed without ascertained foundation in fact, that each particular micro-organism exhibits only one mode of life so to say; in other words, that each micro-organism has the property of initiating one chemical change only. Thus, by a habit of mind, we identify the yeast plant strictly with alcoholic fermentation, the *bacterium lactis* only with the souring of milk, and so on. If, however, it can be shown that the yeast plant can split up other chemical substances besides sugar, such for example as starch and gum, and produce other products besides alcohol, say for instance, acetic acid, the field of life-action of minute forms of life is seen to be much larger than was before thought, and we are thereby enabled to understand that, whereas under one set of circumstances, a micro-organism may give rise to the formation of chemical products which cause disease, under other circumstances merely innocuous products may result, and thus no injury to health will ensue.

Experiment.—A solution of starch containing about 2½ per cent. was made, and to 145cc. of it there was added 5 cc. of a thick mixture of yeast suspended in water. The total solid contents of the inoculated mixture, dry at 100°C., were now estimated and found to be 2·300 per cent.

100cc. of the inoculated mixture was placed in a flask fitted with an india-rubber cork and bent glass tube, the end of which dipped into a bath of mercury. The fermentation of the starch commenced immediately, gas being freely evolved from the 19th of May until June 5th, when, although it had slackened, it was still proceeding. On June 16th, although the fermentation had not ceased, the experiment was brought to a conclusion, and the total solid contents and alcohol present in the fermented mixture were determined. The total solid contents had fallen from 2·300 to 1·326 per cent., showing a loss of 42·34 per cent., and an actual loss of 0·974 grm. in weight. The alcohol present in the fermented mixture amounted to 0·542 grm., as ascertained by the specific gravity of the distillate of an aliquot part.

Now, if we assume the fermentation of starch experienced in this experiment to have taken place in accordance with the following equation:—

$$C_6 H_{10} O_5 + H_2 O = 2C_2 H_6 O + 2CO_2$$

then a loss of 0·974 grm. starch should result in the production of 0·552 grm., alcohol, and there was practically obtained 0.542 grm., which is a closely approximating amount.

It is thus established that yeast cells can split up the molecule of starch into alcohol and carbonic anhydride, just as it can also split up the molecule of sugar into the same products. Whether or not the starch molecule is first of all converted into glucose by the yeast or a soluble ferment produced from it, in this process of fermentation into alcohol and carbonic anhydride, is unascertained.

Yeast and the fermentation of Gum.

Experiment.—A solution of East Indian gum in water was prepared and inoculated with 5cc of a pasty mixture of yeast suspended in water. The amount of total solid contents dried at 100°C. was now determined and found to be 5·460 per cent.

On May 19th, 100cc. of this prepared mixture was placed in a flask, which was fitted as described in the preceding section dealing with the fermentation of starch. Until May 28th the fermentation as judged by the evolution of carbonic anhydride, proceeded but slowly; then there was a somewhat sudden change and fermentation proceeded rapidly. On June 16th the experiment was brought to a conclusion, and the fermented mixture was chemically examined. with the results now to be described.

The total solid contents dry at 100 C. had diminished from 5·460 grm. to 4·7355 grm. indicating a loss of 0·7245 grm. or 13·26 per cent.

The fermented solution contained no alcohol, but it was strongly acid in character, and the acidity of the distillate obtained from 130 cc. of the fermented solution (which had been made up to 150cc. by addition of water) was determined, and calculating from the result thus obtained, the total acidity of the whole quantity was equivalent to 54·23cc. of decinormal caustic soda solution. This acidity is equal to 0·3253 grm. of pure acetic acid, and the identity of the acid was subsequently established by well known chemical methods. The chemical constitution of gum is, unfortunately, not yet ascertained, and therefore, it is not possible to express with any degree of certainty the stages of decomposition of its molecule by

the yeast. If, however, we assume the formula of gum to be $C_6H_{10}O_5$ and the chemical change to be as follows :—

$$C_6H_{10}O_5 + H_2O + O_4 = 2\,CO_2 + 2\,H_2O + 2\,C_2H_4O_2$$

then 0·7245 grm. gum should yield 0·536 grm. acetic acid, whereas there was only found 0·3253 grm. It is possible, of course, that oxidation by other agencies (micro-organisms) had proceeded further, and that part of the acetic acid had been resolved into carbonic anhydride and water.

As the product in question contained no alcohol at all, I see no reason for rejecting the idea that the fermentation was of a direct character, and that the immediate products were acetic acid and carbonic anhydride.

In any case, the results are interesting, and while they open up a wide field for new investigation, they accentuate the ideas to which I have called attention in earlier pages of this paper.

Bacterium Lactis and the Fermentation of Milk and Milk Sugar.

Experiment I.—A quantity of fresh milk was taken and the milk sugar present in it was determined by first of all greatly diluting it with water (10cc. with 90cc. water), and then estimating the sugar by the well known process which makes use of Fehling's solution volumetrically. It contained 4·422 grm. milk sugar in each 100cc. It was also experimentally proved to be quite free from lactic acid.

Quantities of the milk were now mixed with water and Sanitas Fluid respectively, as follows, and placed in flasks loosely covered with filter paper, on July 7th.

A. 50cc. milk + 50cc. water.
B. 50cc. „ + 30cc. „ + 20cc. "Sanitas."
C. 50cc. „ + 25cc. „ + 25cc. „
D. 50cc. „ + .. 50cc. „

On July 20th, the perfect coagulation of each mixture was effected by warming, after which each was filtered, the filtrate was then made up to a known volume by washing the coagulum with warm water and the sugar present in each filtrate, and its acidity were determined.

In order to understand the table that follows, it must be stated that "Sanitas" Fluid reduces Fehling's solution like sugar, and this

capacity was estimated before-hand and calculated into terms of milk sugar (100cc. of this particular sample reduced 540·5cc. Fehling's solution=to 3·62 grms. milk sugar). Similarly, the acidity of the Sanitas fluid was determined volumetrically (100cc. of this particular sample required 90cc. N/10 Na HO solution.

	Sugar originally present.	Sugar present at end.	Difference due to fermentation.	Original acidity.	Final acidity.
A.	2.211	None	2.211 grms.	—	28cc. N/10Na HO
B.	2.935	2.935	—	18cc.	18cc. „
C.	3.116	3.116	—	22.5cc.	22.5cc. „
D.	4.021	4.021	—	45.0cc.	45.0cc. „

The first conclusion to be drawn in connection with these results, is that "Sanitas" Fluid is competent when present in sufficient amount (and that is not a large one) to wholly prevent the conversion of milk sugar into lactic acid by the agency of the *bacterium lactis*. A microscopical examination of the fermented mixture A, revealed the presence of a great number of the *bacteria* in question, but none could be observed in the mixtures B, C, D; similarly the *bacterium termo* was seen to be present in A in a very active state, but it was not present at all in B, C and D.

Now, to look at the results from a quantitative and chemical point of view; the lactic fermentation is expressed by the following equation:—

$$C_6\ H_{12}\ O_6 = 2\ C_3\ H_6\ O_3,$$

which indicates the splitting up of 1 molecule of milk sugar into 2 molecules of lactic acid.

From this, it is calculated that a loss of 2.211 grms. milk sugar should yield 2.211 grms. of lactic acid, whereas the acidity of mixture A was only equal to 0.252 grm. lactic acid. How is this difference to be accounted for? In two ways: in the first place, unless the lactic fermentation is stopped precisely when all the milk sugar has disappeared, the lactic acid is itself resolved by subsequent changes into acetic, butyric, and propionic acids, and finally these acids are split up by oxidation into ultimate products (carbonic anhydride and water); in the second place, the lactic fermentation as thus carried out may not be pure in character; that is to say, other ferments are present besides the *bacterium*

lactis, and other products are thus produced, perhaps, alcohol amongst them.

EXPERIMENT II.—The experiment which has just been described, was repeated with another sample of milk containing 3.88 grms. milk sugar in each 100cc., but using the same sample of "Sanitas" Fluid. The mixtures were made up as before, on August 4th, and the experiments were brought to an end on August 13th.

	Sugar originally present.	Sugar present at end.	Difference due to fermentation.	Original acidity.	Final acidity.
A.	1.940	0.980	.960	—	37.5
B.	2.664	2.664	—	18cc.	18cc.
C.	2.845	2.845	—	22.5cc.	22.5cc.
D.	3.750	3.750	—	45.0	45.0cc.

Here, again, it is seen that fermentation occurred only in the unprotected milk, and inasmuch as the mixtures were not allowed to stand so long as before, the quantity of lactic acid found in A mixture, in comparison with the loss of sugar, is much greater, as was to be expected from the conclusions drawn in respect of the preceding experiment. If the whole of the 0.96 grm. of milk sugar had been changed into lactic acid, and none of this had been destroyed by other chemical changes, there would have been obtained 0.96 grm. lactic acid instead of 0.3375 grm. actually observed to be present.

EXPERIMENT III.—Some 245cc. of fresh milk was inoculated with 5cc. of very sour milk; then the milk sugar and acidity of the inoculated mixture were carefully determined, and the following mixtures were then made and placed in glasses as before. On this occasion, however, another sample of "Sanitas" Fluid was used. It contained reducing matter equal to 0.87 grms. of milk sugar per 100cc., and the acidity of the same quantity was equal to 115cc. N/10 Na HO solution.

MIXTURES.

A. 50cc. inoculated milk +50cc. water
B. 50cc. „ „ +50cc. „
C. 50cc. „ „ +25cc. „+25cc. Sanitas Fluid.

The mixtures were made on August 20th, and were examined on August 24th.

	Sugar originally present.	Sugar present at end.	Difference due to fermentation.	Original acidity.	Acidity at end.
A.	2·185	1·675	·510	0·5cc.	44·5
B.	2·185	1·654	·530	0·5cc.	45·7
C.	2·4025	2·4025	—	29·25	29·25

The results of the examination of mixtures A and B are seen to be thoroughly and mutually confirmatory in character. Taking A, the loss of the milk sugar could produce a maximum of ·510 grm. lactic acid, whereas the acidity of the ultimate mixture was equal to ·4005 grm., showing only ·1095 grm. to be accounted for. It is, however, to be noted that the approximation of the theoretical and actual quantities of lactic acid is much greater than in either of the previous experiments, and doubtless the reason is that the mixtures were only allowed to stand over four days. In order to test the supposition that alcohol might also be found amongst the products, thus accounting for the difference, one half of each mixture A and B was subjected to distillation and the specific gravity of each distillate after being made up to the original volume was determined and found to be respectively ·99977 and ·99973. Alcohol was thus proved to be present in each fermented mixture in more than sufficient amount to fully account for the deficiency in lactic acid produced. Thus, the deficiency of lactose or lactic acid to be accounted for in A is 0·0995 and in B=0·0887 grm., whereas the alcohol found in A=0·27 grm., and in B=0·28 grm. Now, 0·0995 grm. lactose would only furnish 0·0508 grm. alcohol. The explanation is this : the alcohol which was found present, was all derived from the fermentation of lactose; whereas a small proportion of the total acidity of mixtures A and B was derived from butyric and other soluble fatty acids which resulted from the rancidity of the cream or fat contained in the milk, thus swelling the amount of acid which is, as explained, not derived solely from the lactose.

Either then, the *bacterium lactis* performs the double chemical change whereby lactose is resolved into alcohol and lactic acid, or it was accompanied in these experiments with other forms of microscopic life which fermented a part of the lactose into alcohol and carbonic anhydride.

Before passing on to other experiments it should be mentioned that "Sanitas," by its acidity, determines more or less the coagulation

of milk when mixed therewith, whereas the coagulation of the unprotected milk is of course brought about by the lactic acid produced in the fermentation.

I have ascertained that just as "Sanitas" protects the milk sugar which is contained in milk from undergoing the lactic fermentation, so also it acts towards pure milk sugar. In the unprotected samples the milk sugar solution, when inoculated, steadily decreased in amount, while lactic acid and the other products were formed; whereas when "Sanitas" was present (even after 37 days in one case), there was no diminution whatever in the milk sugar, and no products of fermentation were obtained.

Similarly, while the *bacterium lactis* was richly developed in the unprotected mixtures, a microscopic examination failed to detect the presence of the ferment in the mixtures protected by "Sanitas."

Ammoniacal Fermentation.

Experiment I.—Some urine was placed in a warm situation until it had commenced to ferment and formed a deposit. A quantity of freshly voided urine was then inoculated with some of the deposit from the fermented specimen. The total solid contents of the mixture was then determined and found to be 4·474 per cent. dry at 100°C. The mixture was neutral in character.

On July 15th the following mixtures were made—

A. 50cc. inoculated urine + 50cc. Water.
B. 50cc. „ „ + 25cc. „ + 25cc. "Sanitas."
C. 50cc. „ „ + 50cc. „

The mixtures were placed in loosely corked bottles, and allowed to stand until July 27th, when each was made up to a known volume, and the total solid contents, and the alkalinity or acidity determined.

Mixture A was dark in colour, and very cloudy, while B and C, were perfectly brilliant and sweet.

	Original total. solid contents.	Total solid contents at end.	Original acidity.	Final acidity.	Final alkalinity.
A.	2·237	1·782	—	—	21·0cc. N/10 H_2SO_4
B.	3·712	3·712	22·50	22·50	
C.	5·188	5·188	45·00	45·00	

From these results, it is apparent that in B and C the urea present in the urine was entirely protected from ammoniacal fermentation, while in the unprotected (A) mixture there was a loss of 0.455 grm. in solid contents and ammoniacal carbonate was formed in consequence.

According to the equation :—

CH_4N_2O (Urea) $+ 2\,H_2O$. (water) $= (NH_4)_2CO_3$ (carb. ammon.),

the ammonium carbonate actually produced in A was only equivalent to ·0634 grm. urea, as compared with the loss of 0.455 grm. experienced in the solid contents.

Doubtless the *torulaceous* ferment was accompanied with other ferments, and thus the fermentation was mixed in character; but it is evident that mixed, or unmixed, the presence of "Sanitas" prohibits the fermentation, and thus there is afforded reasonable hope that this re-agent may be used with great advantage in the treatment of those diseases in which the urine is passed from the bladder in an ammoniacal condition.

Experiment II.—In the next experiment the urine was not inoculated as before.

The following mixtures were made on August 5th and examined on August 17th.

A. 50cc. Urine + 50cc. water.
B. 50cc. Urine + 25cc. „ + 25cc. "Sanitas."
C. 50cc. Urine + 50cc. „

The urine used in this experiment contained 4.147 per cent. matter dry at 100° C., and was acid in character, 100cc. requiring 10cc. N/10 NaHO.

	Original total solid contents.	Total solid contents at end.	Difference due to fermentation.	Original acidity.	Final acidity.	Final alkalinity.
A.	2.0735	1.5075	0.5660	5cc.		112.05
B.	3.5490	3.5490	—	27.5	27.5cc.	—
C.	5.0245	5.0245	—	50.0	50.0cc.	—

These figures fully confirm the results of the earlier experiment and the protective character of "Sanitas," while they show a better accord with a theoretically pure *torulaceous* fermentation.

Thus, in mixture A, the $(NH_4)_2\,CO_3$ which was produced, not only neutralised the acid urine, but also required further 112·05cc.

N/10 solution of sulphuric acid, the total alkalinity therefore being equal to 117·35cc. N/10 H_2SO_4.

Assuming the loss of solid contents to be entirely due to urea, it is expressed as 0·566 grm., whereas the ammonium carbonate which was found present required the conversion of 0·351 grm., thus accounting for 62·01 per cent. of the total quantity.

Acetic Fermentation of Alcohol.

Although I have made a great number of experiments, using both chemically prepared alcohol, and also white and red wines, upon the general plan already indicated, altogether they may be stated to have resulted in failure. That is to say, the alcohol was not oxidised into acetic acid, nor did the sugar or the total solid contents (of the wines) diminish appreciably in amount. In some few cases the acidity increased somewhat, but in these it was rather at the expense of some other constituent than sugar or alcohol.

I hope some day to continue this branch of the investigation to a successful issue.

Putrefaction.

Experiment I.—One pound of beef steak was extracted with water during two hours at a gentle heat, after which the extract was cooled, filtered and made up to 250cc. The total solid contents of this extract amounted to 2·764 grm. in 100cc.

The following mixtures were now prepared and then placed in clean stoppered bottles :—

A. 75cc. Extract of Meat + 25cc. water.
B. 75cc. „ „ + 25cc. "Sanitas" Fluid.
C. 75cc. „ „ + 20cc. water + 1cc. "Sanitas" Emulsion.

After standing 20 days, the total solid contents of each mixture was again determined and the results are given below :—

	Original total solid contents.	Total solid contents after 20 days in closed bottles.	Loss due to putrefaction.
A.	2.073	1.862	0.211grm. or 10.18 per cent.
B.	2.7465	2.7465	——
C.	2.9975	2·9975	——

A microscopical examination revealed in A the presence of a large number of the micro-organism, *bacterium termo*, in active motion, but none were visible in B and C.

EXPERIMENT II.—In the next experiment, the "Sanitas" which was used gave upon examination the following analytical results:—

Total solid residue dry at 100°C.=5.902 per cent.
Acidity of 100cc.=to 90cc. N/10 NaHO.
Peroxide of hydrogen in 100cc.=to 238cc. N/10 $Na_2 S_2 O_3$

An extract of meat was prepared showing upon examination the presence in 100cc. of 2.084 grms. dry at 100°C.

The following mixtures were placed in bottles loosely covered with filter paper:—

A. 50cc. Meat Extract + 50cc. water
B. 50cc. „ + 50cc. „
C. 50cc. „ + 50cc. "Sanitas."

Mixtures A and B became putrid upon the second day, and then revealed under microscopic examination the presence of a large number of the *bacterium termo* in most active motion. The mixture C then and thereafter remained quite undecomposed, and it contained no micro-organisms in motion.

After standing ten days, an analytical examination of the mixtures was made, and the following are the results which were obtained:—

	Original total solid residue, dry at 100° C.	Total solid residue at end.	Loss due to putrefaction.	Original Acidity.	Final Acidity.
A.	1·0425	·738	·3045	....	Neutral
B.	1·0425	·735	·3075	....	Neutral
C.	3·9935	3·9555	·0380	45cc. N/10 NaHO	45cc. N/10 NaHO

I regard the insignificant loss of matter experienced by mixture C to be due entirely to the oxidising action of the peroxide of hydrogen present in the "Sanitas," and it may be therefore disregarded. The unprotected mixtures lost respectively 29·20 per cent. and 29·49 per cent. of the dry meat extract by putrefaction, that is to say, by hydrolysis and oxidation effected

by the life action of micro-organisms, among which the *bacterium termo* is one of principal importance.

EXPERIMENT III.—Now, before the chemical examination which has just been reported was made upon the several mixtures A, B and C, they were each made up to 150cc. by the addition of water. Of these diluted mixtures there were now taken respectively 100cc. (A), 100cc. (B) and 90cc. (C), and these portions were allowed to stand further twelve days, after which they were again chemically examined. The results are expressed in such a way that the change is at once apparent.

	Total solid residue at commencement.	Total solid residue at end.	Loss due to putrefaction.	Alkalinity or acidity at start.	Alkalinity or acidity at end.
A.	0·492	0·390	0·102	Neutral.	7·77cc. N/10 acid
B.	0·490	0·375	0·115	Neutral.	8·49cc. N/10 acid
C.	2·3733	2·3733	Nil.	27cc. N/10 NaHO	27cc. N/10 NaHO

Expressing these results in words, if may be said that while the mixtures A and B during this further period of exposure, lost respectively 20·7 per cent. and 23·4 per cent. of their total solid contents by putrefaction, the mixture C lost no part of its solid contents. Further, it is important to note that, whereas in the earlier stage of the observations, no ammoniacal product was formed in A and B (unless indeed an acid product able to exactly neutralise it was also formed) in this later stage we have an alkaline product of putrefactive decomposition in mixtures A and B, which required respectively 7·77 and 8·49cc. of N/10 sulphuric acid to neutralise.

These facts accord with what was previously known regarding the chemistry of putrefaction, and they also assist in forming an accurate mental appreciation of the stages in which that process is completed.* They further establish the antiseptic properties of "Sanitas" and its germ-destroying character, for all the mixtures were equally exposed to the attacks of micro-organisms, which flourished in the unprotected mixtures, but were destroyed by the "Sanitas" present in the protected mixtures.

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* See also my Paper entitled "Contributions to the History of Putrefaction."—*Journ. Chem. Soc.* (Trans.) 1880, p. 15.

These experiments will, I hope, suffice to induce whatever surgeons have hitherto hesitated to avail themselves freely of "Sanitas" Fluid in the practice of Antiseptic Surgery, to take up its unrestricted use in future, for it is pre-eminently qualified above all other chemical re-agents for use in that field of science and is perfectly innocuous in itself.

III.—CHEMICAL ACTIONS OF SOLUBLE FERMENTS.

The question arises, to what extent are the various processes of fermentation with which we are acquainted, the consequences of vital functions of micro-organisms? To this question no satisfactory answer has yet been aecorded. That the processes are not due to the mere mechanical presence of the living agents is obvious, although realised by few writers upon this subject. The very essence of a process of fermentation is a change of chemical systems, and this necessitates an acting chemical agency; that is to say, fermentation is the result of a chemical impulse communicated to the fermentable substance by chemical contact with another substance secreted by or resulting otherwise from living organisms, and yet perfectly independent of them. That the real ferments are independent of the living organisms which produce them is assured by the fact that the organisms themselves do not disappear in the chemical changes which constitute the fermentation proper. It is by such reasoning as this that we arrive at the inference, for example, that before ordinary cane sugar becomes transformed by fermentation into alcohol, it is split up into glucose and lævulose by a chemical act of hydration, which is initiated by a soluble zymase which, in its turn, is excreted or secreted by yeast.

Similarly, emulsion is known to transform amygdaline into essence of bitter almonds; and barley is known to yield by germination and extraction, a soluble principle which converts starch into sugar. Well known, also, are the transformations of starch into sugar by the action of ptyalin (the ferment of saliva), and of albumenoids into peptones by the action of pepsin. It is more particularly with these last-named subjects that I shall now occupy some attention.

Saliva and the fermentation of Starch.

Experiment I.—A solution of starch was made and then inoculated with a small quantity of saliva, after which the total solid contents were determined and found to amount to 1.732 per cent. dry at 100° C.

The following mixtures were then prepared and placed in open test glasses on July 13th :—

A. 50cc. inoculated starch solution + 50cc. water.
B. 50cc. „ „ „ +25cc. „ +25cc. "Sanitas."
C. 50cc. „ „ „ +50cc. "Sanitas."

The "Sanitas" Fluid which was used in this experiment gave 5·902 per cent. total solid residue dry at 100° C., and contained matter which reduced Fehling's copper solution equal in amount to 2·7025 grm. grape sugar per 100cc.

On July 24th the solutions were made up to a known volume and chemically examined, with the results which are shown in the following synopsis :—

	Sugar originally present.	Sugar present at end.	Difference due to action of ptyalin.	Percentage of starch converted.
A.	None	0·577 grm.	0·577	60
B.	0·6756	0·700	—	—
C.	1·44	1·3512	—	—

It is seen that in the absence of "Sanitas" the starch in the unprotected mixture was converted to the extent of 60 per cent. by the chemical change :—

$$C_6H_{10}O_5 + H_2O = C_6H_{12}O_6,$$

whereas in the mixtures containing "Sanitas" practically no change occurred, the slight differences in the quantity of sugar which were found present before and after, being within the limits of error of experiment.

Experiment II. A further experiment was made with an inoculated starch solution, containing 4·472 per cent. matter dry at 100° C., and with another sample of "Sanitas" Fluid containing matter fixed at 100° C.=to 5·975 per cent., and reducing substance, which, calculated as grape sugar, amounted to 0·6493 per cent.; the total acidity of the "Sanitas" being per 100cc. = to 115cc. N/10 caustic soda solution.

Mixtures were prepared as follows on July 30th:—

A. 50cc. inoculated starch + 50cc. water.
B. 50cc. „ „ + 50cc. „
C. 50cc. „ „ + 25cc. "Sanitas" and 25cc. „
D. 50cc. „ „ + 50cc „

On August 19th a chemical examination was made of the mixtures, and the results are here appended:—

	Sugar found present.	Sugar originally present.	Gain.	Percentage of starch converted.
A.	0·9025	none	0·9025	18 per cent.
B.	0·8808	none	0·8808	„
C.	0·2284	0·1623	0·0661	trace.
D.	0.3304	0·3247	0·0057	none.

From mixture B a minute drop had been taken each morning for testing purposes, thus accounting for the slight difference between the result as compared with that of A.

Generally, the results fully confirm those of the earlier experiment.

Experiment III.—As "Sanitas" Fluid contains peroxide of hydrogen, and is, in terms of this substance, of from one to two volumes in strength, it became of interest to ascertain to what extent, if at all, the anti-zymotic character of the "Sanitas" is due to that constituent. Accordingly the following experiment was now made.

A freshly prepared and inoculated (with saliva) solution of starch was prepared, containing in each 100cc., 3·564 per cent. of substance dry at 100°C.

A solution of pure peroxide of hydrogen was diluted with water so that it should be in strength about equal to that of "Sanitas"; it contained peroxide ($H_2 O_2$) in each 100cc. equal to 213cc. N/10 solution of hyposulphite of sodium.

Mixtures were prepared on August 5th, as follows:—

A. 50cc. inoculated starch + 50cc. water.
B. 50cc. „ „ + 50cc. water.
C. 50cc. „ „ + 25cc. water + 25cc. peroxide solution.
D. 50cc. „ „ + 50cc. peroxide solution.

On August 18th they were examined as before :—

	Sugar found present.	Originally present.	Gain.
A.	0.7874	None	0.7874
B.	0.6345	None	0.6345
C.	0.7132	None	0.7132
D.	0.6564	None	0.6564

It is apparent from these results, that a dilute solution of peroxide of hydrogen is without influence upon ptyalin. This result is not new to science in a general sense, for M.M. Bert and Regnard have pointed out that while a very dilute solution of oxygenated water arrests and prevents fermentations which depend apparently upon the presence of living organisms such as *yeast*, *bacterium lactis*, *bacterium termo*, *mycodermi aceti*, etc., it altogether fails to arrest the changes which are induced by soluble ferments or zymases such as diastase. This point is now confirmed, and the experiments therefore, which have been described in this section, prove that "Sanitas" is double-barrelled, so to say—in the sense that it not only acts upon organised or living ferments, but also upon soluble or non-organised ferments. The constituent of "Sanitas" which acts upon soluble ferments is that which resembles sugar in its reducing properties and extract of hops in its bitterness, and which is designated by the formula $C_{10} H_{18} O_3$ so far as I have ascertained its nature.* It forms, indeed, the bulk of the residue which is left upon evaporation of "Sanitas" to dryness, as this evaporation causes the loss of all the thymol, camphor, peroxide of hydrogen, and acetic acid which are present in the original fluid.

Pepsin and its action upon Egg Albumen.

Experiment I.—A fresh egg was boiled to coagulate the albumen, and then three portions of the white of the egg, each weighing ·324 grms. were cut into thin slices and placed respectively in 2-oz. wide-mouthed stoppered bottles.

A. To this portion was added 50cc. of a solution containing one per cent. hydrochloric acid.

B. To this portion was added 25cc. of water acidulated to the extent of one per cent. with HCl., and 25cc. of "Sanitas," acidulated similarly with HCl.

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* Chem. News, vol. 39, p. 279.

C. This portion was placed in 50cc. of "Sanitas" containing one per cent. added HCl.

After standing overnight there was added to each portion 0·05 grm. of the prepared pepsin, sold by Messrs. Bullock & Co., and the mixtures were then placed in an oven maintained at 38°c.

After 3 hours and 50 minutes the albumen in A had all disappeared, but some remained in B, and a large quantity was left in C.

Experiment II.—In the next experiment the proceedings were identical, except that just double the quantity of white of egg was taken in each case. In A all the albumen had disappeared in 4¾ hours; in B some remained even after 6 hours, while in C most of the albumen apparently remained.

Experiment III.—Being desirous of expressing the results in a more exact quantitative manner, the following proceedings were adopted: four portions of coagulated white of egg, each weighing 1 grm., were taken, and the first portion was dried at 100° C. and the residue weighed: it amounted to .1312 grm., so that .8688 grm. of the albumen consisted of water lost during the drying. The other portions of albumen were treated as before and kept in an oven at 38°C. during 3¾ hours, after which the mixtures were filtered through tarred papers, and the undissolved portions of white of egg were thus collected and weighed after drying at 100°C.

	Undried albumen at start.	Undried albumen at end.	Quantity dissolved.	Per centage dissolved.
A.	1.000 grm.	0.114	0.886	88.6
B.	1.000 „	0.166	0.834	83.4
C.	1.000 „	0.722	0.278	27.8

or, taking the albumen dry at 100° C., the following were the results:—

	Dry albumen at the start.	Amount undissolved.	Quantity dissolved.
A.	.1312	0.0150	0.1162
B.	.1312	0.0218	0.1094
C.	.1312	0.0948	0.0364

Experiment IV.—This experiment was made exactly after the same manner as the last, upon a specimen of coagulated white

of egg containing 87·06 per cent. water, and yielding 12·94 per cent. of albumen dry at 100°C.

In the case of A, 50cc. water acidulated with pure hydrochloric acid was employed, and of such a strength that 59·75cc. N/10 NaHO was required to neutralise the whole quantity. In the case of B, there was used a mixture of 25cc. water acidulated with the same percentage of acid as A, with 25cc. "Sanitas" Fluid, to which a corresponding amount of acid had been added, although its acidity was practically double by virtue of its own acid character (25cc. = 58cc. N/10 NaHO solution). Mixture C consisted of 50cc. "Sanitas," to which had been added 1 per cent. of pure hydrochloric acid, and the total acidity of the solution was therefore equivalant to 116cc. N/10 NaHO solution.

After the mixtures, containing each 1 grm. of the white of egg, had stood over night, there was added to each 0·05 grm. pepsin. and they were all placed in a water-oven maintained at 38°C, during three hours and fifty minutes. The mixtures were then thrown upon previously tarred filters, and the undissolved portions were washed, dried, and weighed.

	Dry albumen left=to original albumen.		Quantity digested=percentage.	
A.	0·0166	0·128	0·872	87·2
B.	0·0372	0·286	0·714	71·4
C.	0·1146	0·885	0·115	11·5

Experiment V.—Although, from the results which have been described, it is obvious that "Sanitas" when present in large amount seriously interferes with the action of pepsin upon albumen in an acid medium, there is a loss in weight of the original albumen employed in the experiments, and to ascertain the nature of this loss a further investigation was necessary.

In this experiment, therefore, using similar quantities of albumen (containing 14.08 per cent. albumen dry at 100°C. and 85.92 per cent. water) different solutions were used.

A. consisted of 50cc. dilute acetic acid; total acidity=57·5cc. N/10 [NaHO.

B. „ 50cc. „ „ „ „

C. „ 50cc. "Sanitas" Fluid unmixed; total acidity the [same.

D. „ 50cc. „ „ „ „

The portions of albumen were allowed, as before, to stand in the several solutions over night, and the next morning 0.05 grm. pepsin was added to A and C only. Then all four mixtures were digested at 38°C. during 5½ hours, after which the undissolved quantities of albumen were ascertained as before.

	Dry albumen left.	= Original albumen.	Quantity dissolved.	= Percentage dissolved.
A.	0.1310	0.9304	0.0696	6.96
B.	0.1160	0.8238	0.1762	17.62
C.	0.2040	—	—	—
D.	0.1828	—	—	—

These results were unexpected, and are remarkable as affording proof that in the presence of dilute acetic acid, pepsin is robbed of its digestive function almost entirely. The loss of 17.62 per cent. in B is entirely due to the solvent action of the fluid in contra-distinction to digestive action of the pepsin, and is nearly three times as great as the loss experienced in A, where pepsin was present. It almost looks as if the dilute acetic acid in A had precipitated the pepsin into an insoluble condition. The same precipitation evidently occurred in C, but here, as also in D, the albumen evidently entered into combination with some constituent of the "Sanitas," thus leading to results which do not lend themselves to the object of the present enquiry.

I dwell upon two facts, however, which are proved by these results, viz.: first the interference of acetic acid with the digestive action of pepsin, and secondly, that albumen digested in an acid solution, where no pepsin is present, loses weight by being partly dissolved.

Experiment VI.—As hydrochloric acid had been employed in the earlier experiments the results of which required complete elucidation, I now reverted to the use of that acid, using a fresh specimen of white of egg. It contained 86·32 per cent. water, and yielded 13·68 per cent. albumen dry at 100°C.

A. consisted of 50cc dilute HCl. (1 per cent. in strength).
B. „ 50cc „ „ (1 „ „).
C. „ 50cc "Sanitas" Fluid (contg. added HCl. as above).
D. „ 50cc „ („ „ „).

After standing all night with the albumen in the several solutions, 0·05 grm. of pepsin was added to A and C only, and all four mixtures were then digested at 38°C. during 3 hours.

The undissolved albumen was determined in the usual manner—

	Dry albumen left	= Original albumen.	Loss	= Percentage.
A.	0·0100	0·0730	0·9270	92·70
B.	0·1112	0·8128	0·1872	18·72
C.	0·0874	0·6530	0·3470	34·70
D.	0·2024	..	..	..

The loss of weight in albumen shown in B proves that of the total loss in weight experienced in A, viz., ·9270 grm., the proportion due to mere solution in the dilute acid is 0·1872 grm., the difference of 0·7398 grm. (73·98 per cent. upon original white of egg used) alone, is due to the true digestive action of the pepsin.

The loss in weight of albumen in C shows that pepsin loses most of its digestive capacity when present in a solution of "Sanitas," but not entirely.

The gain in weight of undissolved matter in D proves that white of egg is insoluble in "Sanitas" even when that solution is acidulated with hydrochloric acid, and that the albumen enters into combination with some constituent of the "Sanitas," forming an insoluble compound.

The same experience was made in Experiment V., by which it was also proved that in pure "Sanitas" Fluid (containing, that is to say, no added hydrochloric acid) pepsin is entirely without action upon white of egg. The addition of hydrochloric acid to "Sanitas," as provedby the result of mixture C in Experiment VI., permits of the limited action of the pepsin.

"Sanitas" then prevents, in its pure state, the fermentative change which pepsin can work upon albumen in its absence, and this antizymotic power is probably distributed over the acetic acid, the soluble camphor ($C_{10}H_{18}O_3$) the thymol and other substances which are also present in the fluid.

IV. CONCLUSIONS.

In the first part of this memoir, I have dealt generally with the collective knowledge of the relations of micro-organisms to

disease, and have pointed out that in those cases in which it can be reasonably proved that micro-organisms are in some way or the other responsible by their presence for the existence of disease, they probably act by giving rise to the production of poisonous products in the nature of zymases, but which are strictly chemical in character.

In the second part, I have proved experimentally that *Yeast* is destroyed by "Sanitas" and is prevented thereby from exercising its ordinary functions when contained either in a solution of glucose, or starch, or gum. Similarly, it has been experimentally demonstrated, in a strictly quantitative manner, that "Sanitas" destroys and prevents the functions of other micro-organisms, including the *bacterium lactis*, the *torulaceous* ferment which changes urea into ammonium carbonate, the *bacterium termo* and the collective microscopic agencies of the putrefactive process.

In the third part of this investigation, I have proved also experimentally and quantitatively that "Sanitas" Fluid prohibits the action of soluble ferments or zymases such as ptyalin (in saliva) and pepsin.

Dr. Miller, of Dundee, has described four experiments in which "Sanitas" Fluid was mixed with lymph in equal proportions, in all of which, after standing eight hours, the "disinfection" was complete.

Viewing these new results of mine, and weighing them with the results now generally known to attend the use of "Sanitas" in the treatment of wounds and as a disinfectant generally by medical men, I do not hesitate to say that all the claims which I originally made, some years ago, in respect of this agent, have been fully justified, and that by the discovery and introduction of "Sanitas" I have provided for a long-felt want. It is a typically excellent disinfectant in every true sense of that word, alike destructive of micro-organisms and of the soluble zymases to which they and other organisms give rise. Moreover, it alone, of all substances available for use, possesses those other properties which the highest authorities say are necessary for the general use of a disinfectant. In the Thirteenth Annual Report of the Local Government Board, 1883-1884, Professor Burdon Sanderson writes as follows:—

"Nor must it be forgotten that, even after the labour of discovery has been got through, and we have joyfully cried ευρηκα, what are called practical difficulties are sure to come in of such a kind as to render our achievements in a utilitarian sense fruitless. *For an antidote against infection to be of real value, it must be readily procurable, free from poisonous action, and have such physiological relations to the organism that it is capable of remaining in it sufficiently long to exercise its restraining influence on the process which it is intended to counteract. To discover such an agent is indeed a problem of difficulty.*"

I have italicised Professor Burdon Sanderson's words as specially worthy of attention, but I venture to assert that the discovery for which he looks has been already made. While he and others are seeking amongst the very products of putrefaction itself, for a reliable disinfectant, there exists in "Sanitas" the agent possessing all the characters they regard as essential to an antidote to infection for general use. Not only is "Sanitas" a natural disinfectant and a mild oxidising agent capable of readily giving off oxygen when and where required, but it is also as well qualified by its non-poisonous nature for internal administration as for external application, and so it can be used for the treatment of all infectious diseases which are located in the human body, such as cholera, typhoid fever, dysentery, ulcerated bowels and throat complaints, as also for the other contagious diseases which affect more particularly the outside surfaces of the body.

With further reference to the internal use of "Sanitas" Fluid, it may be pointed out that, although, by the experiments described in this paper, it has been proved that "Sanitas" interferes with the action of pepsin and presumably other digestive ferments, yet the quantity required to wholly arrest their action is considerable in itself, and much greater than that which is competent to arrest the action of micro-organisms. That is to say—the amount of "Sanitas" which could be given in doses for the treatment of such diseases as Cholera and Enteric Fever would not suffice to cause serious interference with the digestive processes.

In conclusion, I have pleasure in making mention of the care and assiduity observed by my assistant, Mr. H. C. Williams, during the conduct of this research.