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ORIGINAL PAPER

Biochemical and biological evidence of the activity of high potencies

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1. A method is described for investigating the possible action of microdoses of mercuric chloride on the hydrolysis of soluble starch with malt diastase.
2. The microdoses of the mercuric chloride used in the latest crucial series carried out in 1946, 1948, and 1952, were what are termed 'high potencies' made in accordance with the pharmaceutical method of preparation of drags ordinarily used in the practice of homceotherapy.
3. These microdoses were prepared by separate stages of dilution, the solution at each stage being subjected to mechanical shock. The solutions were, theoretically, 'dilutions' of the order of 1 in 10^{-61} and on present physical theory would not contain any molecules of the original mercuric chloride.
4. The difference in rate of hydrolysis between flasks containing starch, diastase, and distilled water (controls) and flasks containing starch, diastase and microdoses of mercuric chloride (tests) were compared colorimetrically by the Spekker absorptiometer, and the frequencies of the differences statistically analysed, as the results obtained showed biological scatter. More than 500 such comparisons were carried out. The differences of means were examined by the Fisher "t" test, the variances tested and Cochrane and Cox's test applied where indicated. All the series gave a highly significant difference in the rate of hydrolysis between controls and tests, the microdoses stimulating the process. Statistically the significance is shown by the fact that a probability of <0.001 was obtained independently in each of the three years 1946, 1948 and 1952. The control results gave an approximately normal distribution.
5. The distribution, control methods, and accessory control procedures were considered to exclude, as a cause of the effects, adsorption of the original drug and the presence of extraneous contaminants by chance solely in test flasks. The only difference between control and microdose flasks was the addition of microdose, the distilled water being common to both controls and tests.
6. It was concluded that a factor, unidentified, derived from the mercuric chloride used, was present in solutions prepared by serial dilution with mechanical shock which could affect the distilled water diluent, that this change was transferable to subsequent 'ultramolecular' stages of 'dilution', and that this factor was the source of the activity in the microdose solutions producing the acceleration of the rate of hydrolysis.
7. In an addendum there is described recent biological work which is also providing evidence of the presence of an active selective factor in 'high potencies' derived from Strophanthus sarmentosus by the same methods of dilution with mechanical shock.

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The primary purpose of a series of investigations carried out under my direction over the last fifteen years has been to determine whether microdoses of the order of 'potencies' as used in the practice of homceotherapy could have any effect on an enzymatic process. The estimate of the rate of hydrolysis of starch with diastase was adopted as a possible means of detecting any such effect.

The drug used throughout has been mercuric chloride, which for this particular purpose was first used by Persson (1932, 1933, 1934, 1935, 1936) working in the Biochemical Institute of Leningrad under Professor Ginsberg. His technique, obtained from his papers and from personal communications, gave results which on occasion were sufficiently suggestive to justify plans for a serious investigation under more carefully controlled conditions.

Our basic technique was first developed in the Trust Laboratories by Dr Pettigrew and Dr Herd, both Barbour Research Scholars in Biochemistry at Glasgow University. Later experimental work led to improvement in technique and controls following a very helpful personal communication in 1939 from Hopkins. A standard range of experimental error was arrived at and no crucial experiments were undertaken until a large number of prior control tests were found to be close to these limits. This point is emphasized because the rate of hydrolysis of starch with diastase will vary considerably unless carefully controlled conditions are present.

The amount of detail given in this paper and its appendices has been found necessary both to meet requests from interested research workers and in order to supply sufficient information regarding the control methods to make possible adequate assessment. This is especially desirable because the results are difficult to explain theoretically.

The drug was made from a good pharmacopœial brand of mercuric chloride, as the aim of the research was to study the action, if any, of a drug as it might ordinarily be used. To use a highly purified chemical was, therefore, not considered advisable for crucial tests, but the same type of result was also obtained with an Analar preparation. Whether or not the additional concomitants in the original drug entered into the effects obtained, the methods of control, including spectrographic analysis, indicated that the results were related to the nature of the added microdose, derived from the mercuric chloride.

In the experiments to be described the term microdose is used to designate a so-called 'high potency' of mercuric chloride prepared in general conformity with the homoeopathic pharmacopœial method (Leeser, 1938). This method of preparation, involving series dilution in separate bottles with succussion (mechanical shock), results in a solution which does not, from the standpoint of present physical theory, contain a molecule of the original mercuric chloride preparation, provided no adsorption from prior use is present. Calculation shows that after a dilution of approximately 10^{-22} none of the ordinary primary substances remains. The microdoses actually used termed S27 to S31 were theoretically of the order of 10^{-61} to 10^{-71} (Appendix F). This apparent contradiction in terms will be discussed later.

In estimating enzyme activity there are, broadly speaking, two main procedures available. In the one the amount of chemical change over as short an interval as possible from the onset of the reaction is measured thus ensuring nearest approximation to constant conditions. In this way the activity of the enzyme is arrived at. In the other, the length of time required to produce a given amount of chemical change is measured. The conditions of the experiment are kept as constant as possible, the enzyme requiring to be stable under the selected conditions.

In the experiments to be described, however, the primary aim was not to measure the activity of a single enzyme, but to find whether a microdose might affect the rate of chemical change of an enzyme process, in this case, the hydrolysis of starch with diastase. It was necessary to have a controlled process sufficiently constant to make possible an accurate comparison of the rate of chemical change in two flasks the one containing, as an addition, microdose, the other, distilled water.

The procedure preferred was to use a fixed time of incubation and to obtain a colorimetric comparison of the stage reached by the chemical change in each of the flasks at the end of the selected time. It was found possible, by using a less active enzyme mixture, stable under the conditions laid down, to obtain such comparisons with a longer incubation time than usual. This longer time, as will be seen, ensured very accurate timing and careful measurement of small scattered differences. It also enabled eight pairs of experiments to be carried through in one session, under identical conditions, which proved to be the best method to provide sufficient results for statistical assessment.

The colorimetric assessment of the stage of chemical change reached in the given incubation time, and thus of the overall rate of hydrolysis, was obtained by measurement, with the Spekker absorptiometer, of the absorption of green light by the test or control solutions after the addition of Lugol iodine.

A malt diastase was used with Lintner's soluble potato starch as substrate. Although malt diastase is a relatively crude substance containing, as far as is known, at least 5 enzymes, it was found that it could be adequately controlled and gave the desired slower rate of hydrolysis, while having the necessary thermostability.

Experiments with a bacterial α-amylase showed that if one used a dilution sufficient to give the longer incubation time required, the thermolability was too great. Various proportionate mixtures of this a-amylase and α-amylase and β-amylase, both obtained from U.S.A., did not indicate that any advantage was to be gained. Bacterial amylases have for some time been extracted from *Bacillus subtilis* culture on a suitable medium for industrial purposes, while Cablo and Redfern (1947) isolated α-amylase in a purified state from an industrial product. It has been obtained in crystalline form by Meyer, Fuld and Bernfeld (1947). As *B. subtilis* was cultured from the malt diastase solutions used, the possibility of any hydrolytic action being due to its presence was investigated. Under the experimental conditions of the technique described no effect was found. When necessary the *B. subtilis* could be removed by sintered glass filtration. (See Appendix D1 on bacterial investigations.)

The amylolytic enzymes which take part in the total digestion of starch are α-amylase, β-amylase, Z-enzyme, and R-enzyme, recently found in potato, with, in this case where malt diastase was being used, maltase. The part these enzymes play is discussed later. It might be argued that the use of crude diastase might result in fractions of the diastase powder having unequal activity. As will be seen the plan of the experiment allowed for small differences from day to day within set limits, but for each day the one solution was made up and was common to all flasks, both control and microdose. The greatest difficulty was to obtain malt diastase without artificial loading which was sufficiently thermostable for the period of each experimental session. A series of batches had to be tested using sampling methods until a suitable one was obtained.

The technique required to deal adequately with known factors which might profoundly modify such a sensitive reaction, and also to take care of any unknown factors by adequate control methods. The known factors requiring careful consideration were the constancy of the pH, the time from preparation of the diastase solution, the time of incubation, the temperature of the bath, the temperature of the solutions in the flasks, the accuracy of the method of the estimation of the colour reaction obtained, and the effect of CO_2 concentration on the solution in the flasks.

Previously in these laboratories, microdoses of mercuric chloride of molar concentration of the order 10^{-6}, with the quantities of starch and diastase used, were, as is well-known, found to inhibit the reaction on all occasions. On the other hand in concentrations of the order of from 10^{-9} to 10^{-14} molar, a much more scattered effect was obtained, but statistically in a series of experiments some stages of dilution, prepared by serial dilution with mechanical shock, were found to give results suggesting stimulation of the rate of the starch-diastase reaction (Boyd, 1941, 1942, 1946). In one of these series (1946), evidence was obtained statistically ($P = 0.001$) that the procedure of mechanical shock with dilution had relationship to a stimulative effect obtained with microdoses of the stage 10^{-9} molar, as tests with a comparable set of dilutions prepared without mechanical shock showed the usual fade out of inhibition at 10^{-8} and no stage giving stimulation. This supported the use of this procedure in the preparation of 'high potency' microdoses, apart from the necessity to follow the pharmaceutical method under examination.

During one of the series, carried through by Dr Pettigrew in 1940, a few tentative sets of experiments were included, somewhat skeptically, using microdoses of the order of 'high potency' solutions. To our surprise one set of these solutions made in twenty-eight stages, now termed S28 (nominally 10^{-64}) over a series of 27 pairs of test flasks showed stimulative effects with a statistical significance ($P = 0.04$) by the *t*-test. The matter was left in abeyance for further investigation at a later date when increased experience and improved equipment might make it possible to retest for a 'high potency' effect with more detailed controls.

A series of tests using the nominal range S26 and S30 were run in 1946. The technician carrying out these runs had increasing experience of the evolution of the technique over a prior period of four years and was a meticulous and careful worker. The results were statistically highly significant for stimulation. In 1948, after a series of further experiments over two years to test the technique still more carefully, and to introduce semi-automatic suction pipetting, the same technician was again put on to a new series of S27. Again the result was statistically highly significant.

It was felt, however, that until these results could be repeated by another independent technician, trained separately in the technique, publication would not be justified. Plans for further controls were made to include even greater timing accuracy extending to every part of the technique, including the time of day when solutions were made and heated before mixing, and additional checks for bacterial contamination, CO_2 concentration etc. Attempts were made to train several technicians without success, none of these maintaining the necessary meticulous care and sense of rigid attention to detail over long periods of time necessary to attain sufficiently constant control results within the permitted experimental error. It is to be understood that 'training' does not refer merely to the use of the absorptiometer and of suction pipetting, but to continued practice in timing and in the general technique including the details of cleaning, sterilization, and handling of microdose solutions found necessary to prevent the appearance of aberrant inhibition in odd flasks at scattered intervals which might have seriously complicated the assessment of crucial tests.

In 1950, however, a new technician was found, whose previous training had been along lines requiring the same qualities of precision and conscientious attention to detail as were required, and she was finally trained over about eighteen months. While not trained for as long as the earlier skilled technician, her control results over some months fell satisfactorily within the range of experimental error, and it was decided to proceed to crucial tests. Delay was experienced due to inability to obtain a fresh supply of a sufficiently thermostable diastase. In 1952 a new batch of malt diastase was obtained which was stable and the conditions were felt to be sufficiently satisfactory for crucial tests to be attempted.

The technique and methods described are those used in the 1952 series of tests, and are recorded as the most accurate that could be devised for this particular method. Later in 1952 a physical chemist joined the staff for additional investigations bearing on the technique and the possible nature of the microdoses. The 1946 and 1948 results are included in the statistical section, while any distinctive differences in the methods then used are described in Appendix E.

General principles

The actual tests consisted simply of comparing, after a given time of incubation, members of pairs of flasks, the first member containing starch, diastase, and distilled water, the second containing starch, diastase, and microdose prepared in the same distilled water. The comparison depended on the Spekker absorptiometer value obtained with the coloured solution after the addition of Lugol iodine to each flask. This value, with OG1 green filters, is designated in these experiments

absorptiometer green value, (A.G.V.). The 'test results' were in terms of the differences in A.G.V. between the first control flask and the second test flask of each separate pair, with the Spekker set at 0.30 for standard with the first flask. In addition pairs of control blanks with starch, diastase and distilled water in both flasks were similarly measured and the difference values between the first and second flasks of each pair of blanks provided the 'control results' for statistical analysis. These results thus gave a measure of any small control differences due to experimental bias in the measurement of second flasks.

In addition, the pairs of flasks were also compared with a distilled water standard with the absorptiometer set at 1. This provided another set of differences for comparison and gave the range of variation of the first control flasks of all the pairs, and also provided a check that the stage of hydrolysis was within the limited region most suitable for the use of the green filters.

For supplementary information, by using a sampling procedure with suitable quantities of the reagents in one large flask, serial absorptiometer values relative to time could be obtained. In this way hydrolysis curves with the green filter could be compiled for various purposes of control, and also to assess the activity of any batch of diastase and the concentration of enzyme and substrate required to give the best range of A.G.V. for a given time of incubation.

In order to appreciate the nature of the reaction and the interpretation of results, the hydrolysis of soluble starch with malt diastase, the starch–iodine complex, and possible factors involved in stimulation require to be considered.

The constitution of the starch substrate and content of the malt diastase made up of enzymes and accessory substances make the interaction of the two, leading to hydrolysis, a complex process. A summary of recent knowledge and theory about them is given in Appendix A and will help those unfamiliar with the process to picture the nature of the experiments. For the immediate purpose of the methods to be described it is only necessary to appreciate that the action of the diastase on the starch leads to a breakdown of the amylose and amylo-pectin in it. The slightly colloidal starch solution first loses its viscosity, then there is a change in the iodine reaction colour from blue through purple to red, then the red colour fades till only the iodine colour remains. Absorption peaks are at approximately 580 mμ. 560 mμ and 520 mμ respectively at the three colour stages mentioned (Swanson, 1948). Amylose forms a stable blue complex with iodine, while amylopectin forms a less stable red complex. The structure of the blue complex is a helical amylose chain of glucosidic units within which the iodine molecules are arranged parallel to the helix axis (Rundle and French 1943). Degradation by heating, acids, or enzymes progressively breaks down amylopectin to produce smaller fragments—only the largest of which still give a red colour with iodine. The hydrolytic products of starch form highly-coloured addition products with iodine, the achromic point coming at about six glucose units, i.e. dextrins of six or less glucose units show no intensification of the colour of an iodine solution. This is attributed to the inability of the dextrins to form a complete turn in the helix (Hanes, 1937). The blue colour is given by chains over 33 units while a red colour is produced by chains of 7–13 units. Intermediate colours are given by chain lengths of 13–33 units (Swanson, 1948). The degree of colour change produced in this way on addition of Lugol iodine is differentiated by the Spekker absorptiometer more clearly if the absorption measurement is carried out with a suitable spectral band instead of with white light. The OG1 green filters were found to be the most suitable.

In practice it was also found that the absorption of the green light in the Spekker decreased with a resulting increase in the A.G.V., due to the change of density of the iodine colour reaction as hydrolysis increased. Where such curves are shown with Spekker readings on the abscissae measured against distilled water at 1–0, the absorption = 1 – x where x = Spekker reading. While no exact quantitative relationship between light absorbed with this filter and the degree of hydrolysis can be deduced with its use, it is possible to obtain a curve of rate of hydrolysis in terms of the A.G.Y. This form of curve is obtainable from day to day, subject to such factors as might modify the overall rate on different days. Similar curves can be obtained between two independent runs of sample tests measured accurately, providing common original solutions are used. It is necessary to explain that such curves are never identical and are not sufficiently constant to provide more than general information. Figs 1 and 2 give examples of such double sampling flask runs, using a Merck diastase.

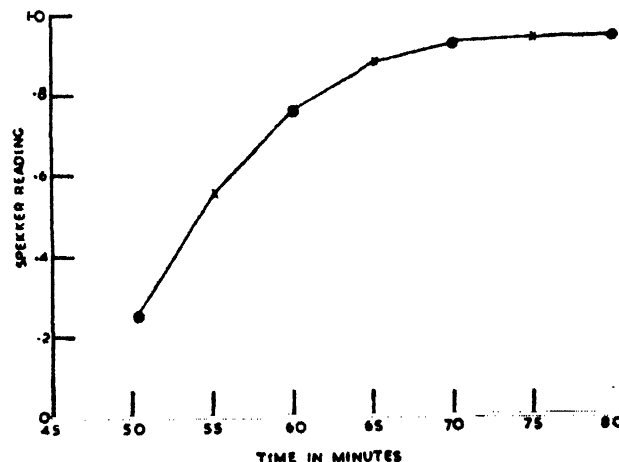

Fig. 1 Two normal sampling tests run simultaneously. Soluble starch 5 g. in 500 ml. distilled water. Merck diastase 0.045 g in 100 ml distilled water, filtered Whatman 40, kept cold while being filtered. The two curves are very similar. First test ●. Second test *x*. These were tests to assess quantities for a 60-minute incubation period for paired flask tests. Each sampling flask contained 240 ml starch, 48 ml diastase, and 48 ml distilled water.

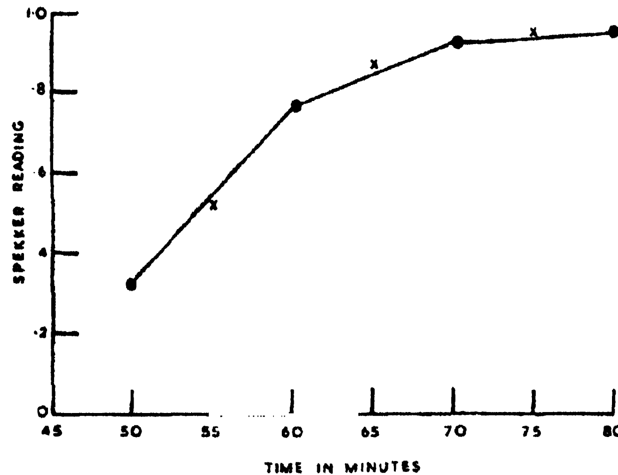

Fig. 2 Two normal sampling tests run on separate days. Soluble starch 5 g in 500 ml distilled water. Merck diastase 0.045 g in 100 ml distilled water, filtered Whatman 40, kept cold while being filtered. The two curves are closely similar. These were tests to assess quantities for a 60-minute incubation time for paired flask tests. Each sampling flask contained 240 ml starch, 48 ml diastase, and 48 ml distilled water.

The transmission curve for the particular filter used has a peak giving 41.2 per cent, transmission in the region 520 mμ to 540 mμ. When this peak was compared with spectrophotometric measurements at stages in an experiment where Spekker readings were 0.64 and 0.77 respectively against distilled water at 1.0, one solution, giving a Spekker reading with this filter of 0.64, gave a maximum absorption of 47 per cent, at approximately 520 mμ, while another solution with a Spekker reading of 0.77 gave a maximum absorption of 36 per cent, also at approximately 520 mμ. This filter should give theoretically within this range the widest differentiation.

In practice as against others of the H455 set it gave the widest variation in Spekker reading for a given difference in optical density between two samples near this range. If there is an increase or diminution of the rate of hydrolysis in a test flask when compared with the control flask of the pair, the difference will show as a greater or lesser Spekker reading respectively with reference to the green light. The reading, as pointed out above, is comparative. Thus comparative readings between pairs of control flasks can also provide a measure of experimental error, and provide control differences for statistical assessment.

The term stimulation is used where an increase of rate of hydrolysis is found in test flasks as against control and refers to the reaction as a whole.

The relative concentration of an enzyme and its substrate plays its part in varying the rate of reaction. Activation of the substrate is caused by adsorption or chemical union of enzyme and substrate. This combination is thought to take place at certain specific points on the surface of the molecules. At such points the enzyme and substrate are said to 'fit', and if this fit is even slightly imperfect the substrate molecules may be under strain, altering slightly the stereo-chemical pattern, thus making the molecule more chemically reactive. In this way hydrolysis may occur more readily. The activation of the substrate does not necessarily result in a chemical modification of substrate such as hydrolysis, as other substances and processes enter in. These necessary substances may be part of the activating system or part of the reaction system subsequent to the activation of the substrate. There may be activators of the enzyme itself, which may enter in by removal of material that inhibits by blocking the reactive centres in the enzyme. Traces of heavy metals may play a part in this activation or inhibition, by effects depending on polarity producing alteration in the stereo-chemical pattern of specific centres. In hydrolysis, water molecules are involved in the reaction subsequent to the activation by the enzyme (Baldwin, 1952). It is thus evident that stimulation of a hydrolytic process with increased rate might be due to several combinations of factors, or to some single factor, including the part played by the trace elements such as sodium, calcium or magnesium, which are present even in carefully distilled water.

In the experiments, described, however, these latter elements were present in the starch, diastase, and to a lesser extent, in the distilled water and were, therefore, common to all solutions whether control or test.

pH and the use of buffers

Normally solutions in such experiments are buffered. In the crucial experiments, after careful consideration and experiment, it was decided to omit any buffer. In view of the fact that we were testing for possible action of either chance trace quantities of mercuric chloride, or for an unknown 'potency effect' derived from mercuric chloride, it was essential to avoid, if possible, the introduction of any additional chemical substances which might contain inhibitory contaminants.

The method required suitable quantities of diastase and starch to give a rate of hydrolysis to produce after 90 min a control A.G.V. within a moderate range of difference on the Spekker in the region of 0.60—0.80 against distilled water at 1.0. This provided sufficient time to examine sixteen flasks at each main test while limiting as far as possible the time of the diastase in the incubating bath at 37.5°, including the preheating period before addition to starch.

The use of a sodium acetate—sodium veronal buffer adjusted by addition of 0.1 N H_2SO_4 definitely slowed this rate for the same quantities of starch and diastase as used without the buffer. This was clearly introducing inhibitory action, as the pH at

which the actual unbuffered experiments were carried out was of the same order as that used with the veronal buffer adjusted to suit. As the effect looked for was one of slight stimulation it was likely to be masked by the inhibitory action of the ingredients or contaminants in the buffer. It was, therefore, desirable to use the diastase without buffer, provided there was found evidence that the solutions were remaining at each main test at a sufficiently constant pH level. Experimental findings without buffer provided the necessary evidence that this was the case.

It was found that the A.G.V. variations obtained in 16 flask tests both of controls and test flasks and in samples from big flask runs at 90 min incubation, were not relative to any small differences of pH, while a 16 flask test run in pairs as normally, except for two minutes between each member of each pair, showed no pH difference greater than 0.06 between the members of any pairs of flasks. The initial pH of the mixture of starch, diastase and water of a series of eight runs of large flask sampling tests was in the range of 5.23–5.5. The range of pH drop from start to finish in these eight runs averaged 0.09, the A.G.V. continuing to rise as hydrolysis proceeded (Table 1).

As the statistical examination depended on comparative difference in Spekker readings between members of pairs of two control flasks and between members of pairs of control and microdose flasks, the results of the series did not depend on day-to-day identity of initial pH of the starch–diastase solution provided the control A.G.V. always fell within the permitted range. All solutions on any one day were made with the one weighing of starch and diastase and with the same distilled water and were thus common to all flasks. With the quantities used the initial pH was always found to be within the desired limits.

In a series of experiments with sets of small flasks, each flask of a set was individually adjusted to a different pH level, using suitable veronal buffer solutions (Hobson and Macpherson, 1952). An increased quantity of diastase was used to compensate for the slowing of the reaction from the use of the buffer. The A.G.V. of each flask was taken after ninety min incubation. It was found that differences of pH in the range 5.0–5.5 gave rise to a difference between flasks in A.G.V. of 0.02 at most. Two such sets are shown in Fig. 3.

This was within the permitted experimental variation about the mean of the controls of normal paired flask tests on any day.

It is clear, therefore, that within the range of A.G.V. found in the tests the influence of small changes of pH within 5–0 to 5–5 is negligible. Fig. 4 is an example of a sampling test run by two technicians working together so as to obtain a series of practically simultaneous pH and Spekker values. The arrows in the figure indicate the times at which pH estimations were made. The pH values were 5–46 ± 0–01, 30 min incubation onwards.

Further, owing to cross checks in the technique such as pipette interchange, change in position in the incubation bath etc., there was no reason for properly cleaned microdose test flasks alone to develop, by chance, pH differences sufficiently large to alter significantly the rate of hydrolysis. Any such chance difference, if present at all, would be scattered between control and test pairs of flasks and would be taken care of by the statistical examination (see also Appendix D4).

It would appear, therefore, that variations of pH of the order found in unbuffered tests were insufficient to affect materially the comparative differences obtained between members of test pairs of flasks.

Method

Equipment. The necessary details of the laboratory ventilation with the temperature control and of the apparatus available for tests are given in Appendix B. In this appendix details are given of the glassware and of the pipettes with especial reference to weighing tests of output and range of error (Figs. 5, 6, 7).

Pipetting. The 3 ml. vitreosil pipettes for diastase, water and microdose had, on ten weighings, an average output variation of not more than ±0.002 ml. for any pipette, while the 1 ml. iodine pipette had a maximum variation of 0.002 ml, thus giving the required accuracy. The semi-automatic suction pipetting is also described.

Table 1 Changes in pH and Spekker Readings During Two Sampling Test Runs

Time after mixing	Test 1		Test 2	
	Spekker	pH	Spekker	pH
10 minutes		5.35		5.47
20 minutes		5.35–6		5.45
30 minutes		5.33–4		5.44
40 minutes		5.32		5.43
50 minutes		5.32		5.425
60 minutes		5.31–2		5.43
65 minutes	0.38		0.38	
70 minutes		5.31		5.43
75 minutes	0.56		0.57	
80 minutes		5.31		5.43
85 minutes	0.69		0.68	
90 minutes		5.31		5.43
95 minutes	0.79		0.79	
100 minutes		5.31		5.43
105 minutes	0.83		0.84	
110 minutes		5.31		5.43

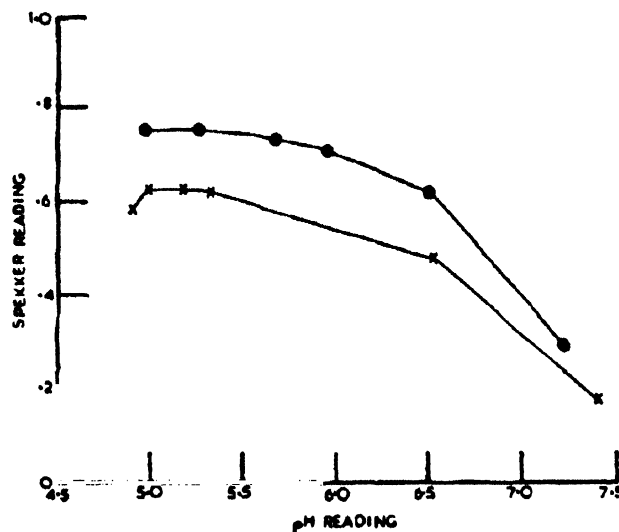

Fig. 3 Two runs with small flasks containing starch–diastase solution with in addition veronal buffer solutions added to give a different pH in each flask. Incubation time 90 min for each flask. The change in velocity of the reaction with change in pH is seen, but it will be noted that in the range of pH found normally with unbuffered solutions (5.23–5.5) there is no difference in Spekker readings greater than 0.02. Soluble starch 5 g in 500 ml distilled water; ●, B.D.H. diastase 0.17 g in 100 ml distilled water; *x*, B.D.H. diastase 0.16 g in 100 ml distilled water. Both filtered Whatman 40.

Incubation. The incubating bath, also described, had a temperature control accuracy of ±0.005 about the required temperature, which, for crucial tests, was 37.5°C. This gave on test a temperature difference of approximately 0.015°C between the solutions in different flasks during incubation time (Appendix B).

Cleaning of glassware. The nature of the experiments requires special cleaning methods. Many usual methods have been tried and discarded. From 1946 to 1951 concentrated HNO_3 was used for all glassware. Flasks, etc., are now cleaned by Lissapol solutions followed by thorough rinsing and boiling in double distilled water, and by baking in dry heat at 150°C. for 2½ hours. Stills are cleaned with concentrated HNO_3. Pipettes are thoroughly washed, boiled and then baked similarly. Microdose and control bottles are treated separately on similar lines. The present method requires that no glassware is allowed to dry between stages of cleaning and that the only water coming in contact with them after Lissapol treatment is distilled. Surgical rubber gloves, free of dusting powder, are used. Full details of the methods of cleaning are given in Appendix C. Apart from insufficient practice in the technique, lack of attention to these details is the chief cause of irregular controls.

Preparation and analysis of reagents

Starch. Exactly 5 g Lintner's soluble starch (Hopkin and Williams) is weighed on to a previously weighed powder paper. This is made into a paste in a small beaker with cold distilled water. About 100 ml of boiling distilled water is added to the

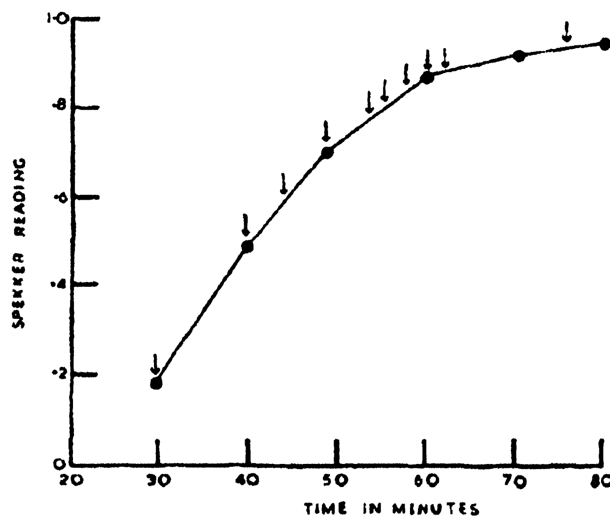

Fig. 4 Sampling test carried out by two technicians to obtain simultaneous pH and A.G.V. readings. Soluble starch 5 g in 500 ml distilled water. B.D.H. diastase 0.43 g in 100 ml distilled water, filtered Whatman 40, and sintered glass No. 5. Solutions not buffered. Starch 240 ml, diastase 48 ml, and distilled water 48 ml ↓, = time at which pH measured. pH values 5.46 ± 0–01 throughout.

Fig. 5 The Biochemical Laboratory.

paste from a larger beaker containing 250 ml boiling water. The starch paste and boiling water are thoroughly stirred and transferred to the larger beaker. The small beaker is rinsed with the solution which is then poured back into the larger beaker where it boils for three minutes. The beaker is then removed from the bunsen flame and about 200 ml cold water is added. The solution is poured into a 500 ml graduated flask and the volume is made up to 500 ml when cold. Examination of the starch solution with the dark ground microscope showed it to be a colloidal solution. This solution which had pH = *c*. 5.3 gave, with Lugol iodine, a maximum absorption in the region of 600 mμ on the Spectrophotometer. Culture from starch powder or solution gave an atypical *Balantidium coli* (see Appendix D1 re sterilization).

Diastase. Malt diastase (B.D.H., 1952) is kept in a refrigerator. Of this, exactly 0.15 g. is weighed on to a previously weighed powder paper, and put into a 100 ml graduated flask through a filter funnel. Cold distilled water is added through the funnel to the flask up to the graduated mark. The flask is shaken until the diastase is dissolved and the solution is filtered through Whatman No. 40 filter paper into a stoppered bottle. This solution has pH = *c*. 5.9. The quantity of diastase powder and the time of incubation suitable to the technique (90 min in latest series) has to be selected for each new supply batch of diastase and starch using a Spekker standard to give an A.G.V. of approximately 0.70 against distilled water at 1.0 on addition of 1 ml. Lugol iodine (1 in 100). The thermostability, under the particular experimental conditions, of the diastase to be used is of first importance and the method for testing this is described under procedure. Culture from solution gave *B. subtilis* with various batches (see Appendix D1 re sterilization).

Iodine. One ml Lugol iodine is made up to 100 ml with cold distilled water and a fresh solution is made up for each test. The Lugol iodine was made specially with spectrographically tested distilled water.

Water. The double distilled water (pyrogen free) for making all experimental solutions is obtained from a Baraglass still followed by a vitreosil still and is the same as that used for the final stages of cleaning (Appendix C). Water samples are periodically cultured for bacterial or fungal growth and should be sterile. Frequent conductivity tests are made to check the purity and a standard of specific conductivity of approximately 1.5×10^{-6} mhos after standing in a flask is considered suitable. On delivery from the still this water has a pH of over 5.8 and sp. conductivity of approximately 0.7×10^{-6} mhos (Fig. 8).

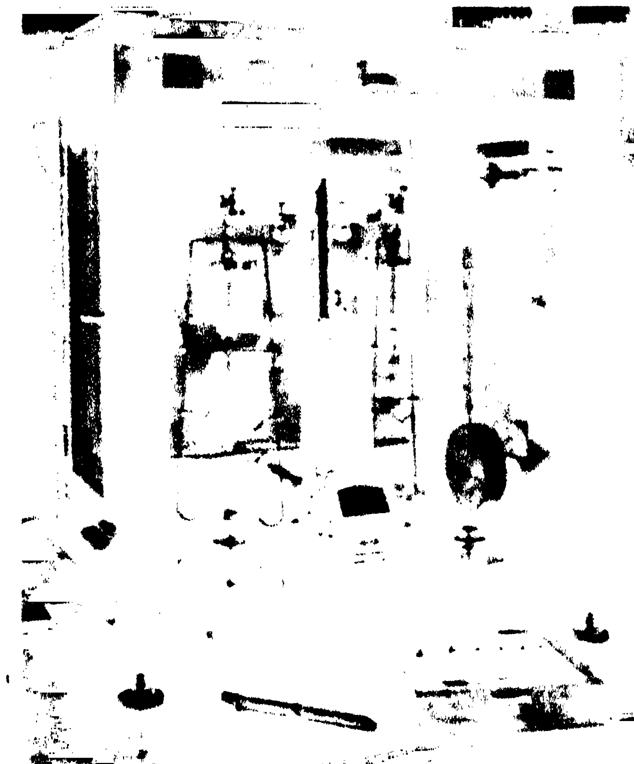

Fig. 6 The Nivoc Aperiodic Balance.

Microdoses. The microdoses are carefully prepared and are handled in accordance with certain assumptions discussed in detail in Appendix F(B). Twenty-six numbered pyrex weighing bottles with ground glass stoppers chosen at random from a pool are placed in numbered holes on a rack. The first one is filled with 10 ml of double distilled water, while the remaining 25 are filled with 5 ml double distilled water, using a vitreosil pipette. The initial solution is made by dissolving 0.1 g mercuric chloride (Hydrarg. perchlor. obtained from Nelson) in 10 ml double distilled water in the first weighing bottle giving a solution of 1 in 100 termed SI. From this primary solution one drop is transferred by capillary tube to the second weighing bottle already containing 5 c.c. double distilled water, the first bottle being replaced in the rack. The second bottle is succussed 10 times after which one drop of this solution termed S2 is transferred to the third bottle from the rack containing 5 ml. double

Fig. 7 The Spekker Absorptiometer.

Fig. 8 The Stills.

distilled water. The second bottle is then returned to its correct place in the rack and the third bottle is succussed ten times giving a solution termed S3. The same procedure is continued up to solution S26. This solution normally provides the common solution for all the separate microdoses used during any one set of experiments, and because succussion is used, the solution is assumed to be sufficiently homogeneous. The question of homogeneity and dilution is discussed later (Appendix F).

Before making the particular microdoses for each day's test, 5 ml of the control solutions of double distilled water are pipetted into separate weighing bottles which are placed in separate holes at the back of the rack. The bottles for the fresh microdoses are then filled with 5 ml water from the same flask using the same pipette and placed in a separate row in the rack. This procedure is carried out before any of the twenty-six previously prepared microdose solution bottles are touched. The use of a numbered rack with replacement of each bottle after use is essential to avoid unconscious error in the sequence. The further microdoses for each test are then prepared with these additional bottles. Each numbered microdose bottle with ground glass stopper is protected from dust by a glass cover.

The S26 source for test microdoses although made in distilled water, if prepared with care, kept cool and protected in a stoppered pyrex weighing bottle with a glass cover will remain 'active' for some time. Should, however, the trend towards stimulation appear to cease, an extremely rare occurrence except with old solutions, then, after two or three days of negative sixteen flask tests, it is advisable to remake the microdose series. This difference of activity was well illustrated in the 'blindfold' series where one set of microdoses (S31) were made from an S26 stage which was three months old and two other sets (S27) from new S26 stages of two freshly made sets of serial dilutions. The activity of the old microdoses was very much less than that of the fresh microdoses (Table 7). Any rise of sp. conductivity of a microdose sample above 3 μmhos at 18°C also indicates the advisability of making a fresh microdose series.

Table 2 Spectrographic Comparison of Reagents

Element	Mercuric Chloride	Distilled Water p.p.m.	Batch No. 313410/511213 Diastase p.p.m.	Soluble Starch p.p.m.
Sodium	Present	0.1 to 0.2	400	200
Potassium	N.D.	N.D.	2000	80
Phosphorus	N.T.	N.T.	200	100
Magnesium	Present	0.2	400	150
Calcium	Present	0.5	300	100
Aluminium	N.D.	<0.05	100	60
Silicon	Present	0.2	40	60
Iron	Present	0.1 to 0.2	20	40
Tin	N.D.	N.D.	<0.04	20
Manganese	N.D.	N.D.	2	0.4
Copper	Present	0.1	1	0.4
Strontium	N.D.	N.T.	0.4	0.4
Barium	N.D.	N.D.	<0.2	<0.2
Lead	Present	N.D.	<0.2	0.2
Silver	N.D.	N.D.	<0.1	<01
Mercury	(Chemically present 74.1 %)	N.D.	N.D.	N.D.
Lithium	N.T.	N.D.	4	1 to 2
Rubidium	N.D.	N.T.	<20	N.D.
Boron*	Present	<2	<2	N.T.

Aluminium trace in water doubtful.
N.D. = Not detected.
N.T. = Not tested for.
Elements tested for but not detected: Mercuric Chloride, Diastase, Soluble Starch: Bi, Cd, Co, Cr, As, Au, Be, Ga, Ge, In, Ni, Te, V, Sb, Zr, W, Zn.
Distilled Water: Bi, Cd, Co, Cr, Ni, Sb, Zn.
Note that Mercury is the only ingredient in Mercuric chloride which is not present in one of the other reagents. The quantity of Mercury found chemically indicates content of over 99 per cent. Mercuric chloride.
* It is difficult to estimate the amounts as Boron is present in the graphite electrodes.

Analysis of reagents. Table 2 gives the spectrograph analysis of the reagents used in the crucial tests, including the mercuric chloride used for the microdoses. Cross comparison shows that, with the exception of Hg, all other elements tested for in the mercuric chloride and detected were also present in one or other of the reagents used in the control tests, and were thus excluded as a cause of effect in the microdose solutions. The gas analysis of the water is given in Appendix D5.

pH and Solutions. When mixed, the solution of starch, diastase and water, or microdose in water, should have a pH in the region of 5.2–5.5 without buffer.

Experimental procedure

Crucial tests. The exact timing of events is vitally important for accurate results.

The starch solution is made first. The diastase solution is made at the same time each day, i.e. 12.30 p.m. It is then filtered and divided into eight small weighing bottles.

Sixteen numbered 50 ml conical flasks are placed on the stainless steel bench at even temperature, and the numbers noted. This number includes the flasks to be used for control blanks. 15 ml of the starch solution are pipetted into each flask using a single 15 ml vitreosil pipette.

Beginning at exactly 12.45 p.m. the flasks are quickly placed in order in the incubating bath already at 37.5°C followed by the eight small bottles of diastase. Thus the starch and diastase solutions are warmed in the bath for a similar length of time each day before the pipetting begins. They remain in the bath during the pipetting (Fig. 9).

Test flasks. When the separate starch and diastase solutions have reached bath temperature 75 min being found necessary for this, 3 ml of water are added to the first flask, the water being taken from a flask of the same water as that used for the microdoses. Exactly one minute later 3 ml of microdose are added to the second flask from a microdose bottle on the bench. The same pipette is used for water and microdose and it is then discarded into a vitreosil pot of distilled water. The numbers of all pipettes are also noted.

Exactly two minutes from the time the second flask received its microdose the first flask receives 3 ml of diastase from one of the eight bottles of diastase in the bath, and one minute later the second flask receives the same quantity from the same bottle delivered from the same pipette which is then discarded into the same vitreosil pot, being considered contaminated by the microdose already in the second flask.

Four minutes from the time the second flask begins to receive its diastase the third and fourth flasks receive their water, microdose and diastase in the same way as the first and second, and so on. The whole operation is timed so that the first of each pair of flasks receives its diastase seven minutes after the second of each preceding pair.

Fig. 9 Semi-automatic pipetting with suction from a water pump. The rubber tube ends in a rubber cap fitting over the pipette stem and having a small side-opening which permits control of the degree of suction by the technician's finger.

Control blanks. Along with the test pairs of flasks and included in the time sequence are either two or four pairs of control blanks, the first flask receiving water as above and the second also receiving the same water delivered from the same pipette but taken from a small bottle similar to the microdose bottles.

The pipettes are always wet with the solution before use. After the solution is run into the flask the surface of the liquid is touched with the tip of the pipette.

A separate small bottle of diastase is used for pipetting for each pair of flasks instead of drawing from one large flask in order to prevent pipettes which have touched the surface of the liquid in the first flask from transferring starch-diastase mixture into a common solution.

After each flask has received its three solutions it is gently shaken to mix them, while all flasks are shaken gently from time to time during incubation, without removing them from the bath.

Exactly 90 min from the time at which it begins to receive its diastase the first flask is removed from the bath and is immediately cooled in a tank with running tap water. Exactly one minute later the second flask of the pair is removed from the bath and put in the cold tank. The first flask is allowed to cool for exactly 2 ½ min, when 1 ml of the Lugol iodine solution is pipetted in and the flask is shaken.

The absorptiometer cell (1 cm) of the Spekker is rinsed out with the solution from the first flask and is then filled with the solution and put into the Spekker. The Spekker is set at 0.30 on the drum and adjusted to balance with the galvanometer reading zero. By the time this has been done the second flask has been cooling for 2½ min and exactly at the required time 1 ml of iodine solution is added to it and the flask is shaken. The second cell is rinsed and filled with the solution and the A.G.V. of the solution with the starch–iodine colour is compared with the A.G.V. of the previous solution of the pair. The first method gives a comparative difference between each second flask and a predetermined standard zero of 0.30 for the first flask. These results provide the main data for statistical analysis.

The absorption of the two solutions is then compared with that of distilled water as a check on technique. Another absorptiometer cell is filled with distilled water and the Spekker adjusted with this cell to give a galvanometer reading of zero with a drum reading of 1.0: then the two cells used for the test are again examined in sequence and the reading noted. This is referred to as the second method.

The temperatures of the solutions in the cells are then taken as a check against any relative change in absorption being due to temperature change during the particular examination of each pair.

Timing is kept accurately throughout the whole procedure. An electric wall clock with a large second hand is used for reference.

The test is continued thus, each flask having exactly 90 min incubation time and exactly 2½ min cooling before iodine is added.

The laboratory temperature, humidity and barometric pressure are noted each day. The technician wears a head cover and a gauze mask over the nose and mouth, when pipetting for crucial tests (Fig. 10).

Fig. 10 Comparison of microdose and control on Spekker Absorptiometer.

From day to day the pipettes for control blanks or microdose tests are interchanged. Flasks are chosen at random for test or control pairs of flasks, and the position of these latter in the incubation bath is changed from day to day, the sequence of control and test pairs being altered each day. About 90 numbered weighing bottles with ground glass stoppers, indiscriminately mixed, are kept protected from dust in a rack. The bottles are picked at random, for either microdose or control water (see reagents). Later it was found that a satisfactory proportion of the bottles had been used during the tests for both microdose and control (Appendix D4). One set of eight pipettes was kept for diastase only and a second set of eight for either water or microdose. This method of interchange was necessary to exclude adsorption of mercuric chloride on the glass as an explanation of the results.

It will be seen from this procedure that the only time variable is the progressively longer time of preliminary heating of the diastase before its addition to the starch as the tests proceed. This amounts to seven minutes between each successive pair of flasks and one minute between each member of a pair. It is, therefore, of importance that the thermostability at 37.5°C. of the diastase used should be good for the duration of the experiment.

The seven-minute periods may, therefore, cause slight variation in values as against distilled water for separate pairs of flasks. This does not affect the results, as this time period is the same for both members of each pair, and results are concerned solely with differences between the individual members of each pair. The A.G.V. variation of all the control flasks, as referred to distilled water at 1.0, should not be greater than ±0.02 distributed about the mean reading of the first control flasks of all the pairs for that particular day. Any first control flask showing a greater deviation makes the result of that pair suspect.

The first method whereby the first flask is set at 0.30 automatically neglects these small changes between the pairs which do not affect the statistics, while the second method acts as a check against an aberrant first flask. Such an aberrant value, if low,

might make the second flask relatively higher and suggest a stimulation. Such an occurrence was very rare and only occurred with the first flasks of two test pairs out of 296, and these two results were accordingly excluded.

With the second method, an alteration in the level of mean values of all control flasks against distilled water at 1.0 may be found on different days. This alteration, which is due to various experimental factors affecting the overall rate of reaction, such as humidity affecting the apparent weight of starch or diastase, was of no statistical consequence, as all flasks were involved for the particular day.

Within this day to day range of from about 0.60 to 0.80, owing to the design of the absorptiometer, it was to be expected that a given difference in A.G.V. on different parts of the scale would be equivalent as, for example, that between 0.60 and 0.62 and between 0.78 and 0.80. In practice both methods were used for all tests and the measured differences between individual members of pairs of flasks as given by the first method where 0.30 was used as standard for the first flask of each pair and the second method, were practically identical within 0.005 on the Spekker (Appendix B). These daily variations in rate of reaction within control limits do not, therefore, affect the summation of Spekker differences for statistical analysis. Where the readings fell between scale markings, it was possible to read to approximately 0.005, and any other indeterminate readings were allotted in the normal manner. In the statistical analysis such readings as fell between the group interval used were redistributed in conformity with normal statistical procedure.

The preliminary bath heating time also results in a one minute difference between the heating time of the diastase for the first flask and the diastase for the second of each pair, any effect on the diastase occurring before the diastase is added to the second flask, as the actual incubation time for each flask after the addition is exactly the same. The method of comparison for statistical purposes and the procedure adopted provided for this difference between the first and second flasks by using the first as the relative standard. The statistical results in the series were, therefore, always dependent on the relative reading of the second flasks whether of control or test pairs, all the pairs being subject to the same one minute difference. The recommended control range of differences of ± 0.02 between members of control pairs distributed about the setting of 0.30 adjusted for the first flask of the pair included any small difference due to this one minute difference in preheating.

An example is given of a working record of a single session using this procedure, the table showing the scattered nature of the differences obtained (Table 3).

Special tests for unconscious bias

The procedure for these tests was exactly the same, except that the microdose and control bottles were first noted and the result put in an envelope, then the bottle numbers were covered by similar opaque labels. The technician then left the laboratory and another member of staff who had no knowledge of the correct numbers came in, numbered the fresh labels and mixed the position of the bottles, after which she left the laboratory without further contact with the technician. In the experiment the technician followed the procedure as if all the bottles with their new numbers contained microdoses, the control water for the first flasks of each pair being taken from a common flask.

After the complete test had been carried through, the labels were scraped off the bottles and the original code looked up, and the results thus arrived at.

Extra cleaning of the outside of the bottles was required to eliminate any traces of gum. Otherwise the interchange of pipettes, flasks, bottles and bath positions was carried out as usual.

Examples of this interchange in 1952 are given in Appendix D4 along with examples from 1946. A similar form of interchange was used for all tests in the 1952 series, and also in 1946 and 1948 except that in these earlier years both the diastase and the microdose or water pipettes were also mixed.

Sampling tests

The starch and diastase solutions are made up in bulk as in the crucial test procedure. The relative quantities of diastase to starch are normally the same, but for special experiments such as testing the comparative activity of samples of diastase, quantities were suitably adjusted. The starch and diastase solutions are measured into separate flasks, 240 ml starch solution into the one and 48 ml diastase plus 48 ml distilled water into the other and both are then placed in the incubating bath at 37.5°C. at 12.45 p.m. At 2.30 p.m. the diastase and water solution is added to the 500 ml starch flask and the solutions mixed by gentle shaking.

After 60 min or other suitable time samples of 21 ml, the amount normally used in small flask tests, are withdrawn at intervals, usually of 10 min, transferred to a conical flask which is then cooled for 2½ min and 1 ml Lugol iodine (1:100) added. A Spekker cell is then rinsed with the solution and filled, the A.G.V. being compared against a zero for distilled water at 1.0 (Fig. 1).

Test for thermostability of diastase during experiment

A test of sixteen pairs of control blanks is carried out normally and the A.G.V. against distilled water at 1.0 is noted for both flasks of each pair. Owing to the seven minutes between each pair and the fixed incubation time, it is possible to calculate the length of time during which the diastase for each flask of the pairs has been in solution and subjected to the particular circumstances required for the test, the second of each pair being one minute longer than the first. Normally

Table 3 Record or 16 Flask Run From Series tor Unconscious Bias Tests ('Blindfold')
July 9th, 1952 (*a*)

Solution	Bottles		Flasks	Pipettes		Time D.W. or M. Added	Time Diastase Added	Time Out	Time After Cooling	Spekker I	Spekker II Against 1.0	Temp. Cells
	Code	Actual		D.W. or M.	Diastase							
Control	Flask (100 ml.)		82	20	33	2.27 p.m.	2.30 p.m.	4.00 p.m.	4.02 p.m.	0.3	0.645	23.0° C.
S27	5	93	72	20	33	2.28 p.m.	2.31 p.m.	4.01 p.m.	4.03 p.m.	0.34	0.685	22.9° C.
Control	Flask		68	39	18	2.35 p.m.	2.38 p.m.	4.08 p.m.	4.10 p.m.	0.3	0.66	22.9° C.
D.W.	2	82	74	39	18	2.36 p.m.	2.39 p.m.	4.09 p.m.	4.11 p.m.	0.3	0.66	22.8° C.
Control	Flask		87	32	38	2.43 p.m.	2.46 p.m.	4.16 p.m.	4.18 p.m.	0.3	0.665	22.8° C.
S27	1	31	69	32	38	2.44 p.m.	2.47 p.m.	4.17 p.m.	4.19 p.m.	0.31	0.675	22.8° C.
Control	Flask		84	36	26	2.51 p.m.	2.54 p.m.	4.24 p.m.	4.26 p.m.	0.3	0.66	23.0° C.
S27	3	13	80	36	26	2.52 p.m.	2.55 p.m.	4.25 p.m.	4.27 p.m.	0.285	0.645	23.0° C.
Control	Flask		66	43	24	2.59 p.m.	3.02 p.m.	4.23 p.m.	4.34 P.m.	0.3	0.655	23.0° C.
D.W.	6	18	88	43	24	3.00 p.m.	3.03 p.m.	4.33 p.m.	4.35 p.m.	0.285	0.645	22.8° C.
Control	Flask		95	21	23	3.07 p.m.	3.10 p.m.	4.40 p.m.	4.42 P.m.	0.3	0.64	23.0° C.
S27	4	68	96	21	23	3.08 p.m.	3.11 p.m.	4.41 p.m.	4.43 p.m.	0.35	0.69	23.0° C.
Control	Flask		92	27	42	3.15 p.m.	3.18 p.m.	4.48 p.m.	4.50 P.m.	0.3	0.64	22.7° C.
D.W.	8	113	63	27	42	3.16 p.m.	3.19 p.m.	4.49 p.m.	4.51 p.m.	0.3	0.64	22.8° C.
Control	Flask		64	10	14	3.23 p.m.	3.26 p.m.	4.56 p.m.	4.58 p.m.	0.3	0.65	23.0° C.
D.W.	7	134	79	10	14	3.24 p.m.	3.27 p.m.	4.57 p.m.	4.59 p.m.	0.32	0.67	22.9° C.

(b) Analysis of Spekker Readings, Second Method (II) Differences between flasks against mean of all first flasks (controls) = 0.652

Controls (First Flask)	Control Pairs (Second Flask D.W.)	Test Pairs (Second Flask microdose)
−0.007	+0.008	+0.033
+0.008	-0.007	+0.023
+0.013	-0.012	−0.007
+0.008	+0.018	+0.038
+0.003		
−0.012		
−0.012		
−0.002		

Reagents: Starch – 5 gm. Lintner soluble in 500 ml distilled water.
Diastase: 0–15 gm. B.D.H. in 100 ml Distilled water.
Filtered Whatman 40.
Diastase solution made 12.30 p.m. Eight bottles into incubating bath 12.45 p.m. Starch in flasks into bath 12.45 p.m.
Distilled water: same for controls and microdoses. Laboratory
Temperature, 72°F.
Humidity, 65%
Barometer, 30.2 in.
Average temp.
Spekker cells, 22.9°C.

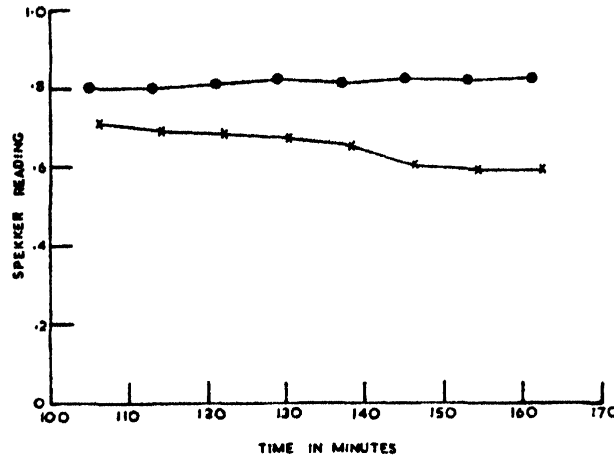

Fig. 11 Comparative 16 flask tests of batches of Merck and B.D.H. diastase for thermostability. Quantities of Merck and B.D.H. diastase were adjusted to comparable A.G.V. readings for each flask at 90 min incubation. Soluble starch 5 g in 500 ml distilled water, x, Merck diastase 0.05 g; • B.D.H. diastase 0.15 g, both in 100 ml distilled water, filtered Whatman 40. The Spekker reading of the first flask of each pair of flasks is given. The time indicates the period during which the diastase was heated in the incubation bath at 37.5°C. prior to being pipetted into flasks with starch solution, after which incubation time for all flasks was identical (90 min). The thermostability of the 1952 batch of B.D.H. diastase is better than the particular batch of Merck diastase. The smaller quantity of Merck required shows a greater activity with greater thermolability.

this is the time of preheating at 37.5° added to the time before the diastase is added to a particular pair of flasks. This preheating period may be at an altered temperature or be substituted by a cold bath, according to the information required.

Fig. 11 shows the partial deactivation with preheating time at 37.5° of one batch of Merck, compared with the better thermostability of the diastase used for the 1952 tests. The partial deactivation of the a-amylase is also shown (Fig. 12). The reading of the first flask of each pair is given. This was much more rapid and increased with dilution of the a-amylase. This is a test, not for activity against time, but to ascertain the effect of preheating on the diastase when in solution. For this, comparison requires to be made between solutions of starch–diastase incubated for the same time, but with different time of preheating.

Accessory control procedures

A number of additional control procedures were carried out to check or eliminate factors which from a critical viewpoint might be thought to cause chance variation in the comparative readings of test pairs. Each is separately dealt with in the Appendix D.

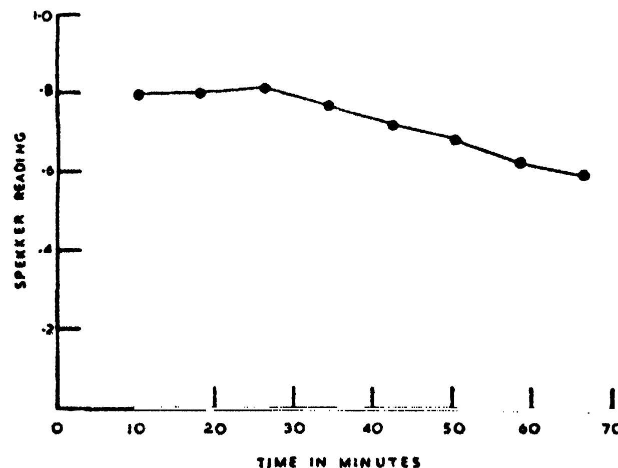

Fig. 12 Comparative 16 flask test of α-amylase for thermostability. Soluble starch 5 g in 500 ml distilled water, filtered Whatman 42. α-amylase 0.06 g in 500 ml distilled water, filtered Whatman 40. The time amylase was kept in cold bath (14° C.) until pipetted into starch solution in separate flasks is shown. Incubation time 60 min thereafter for all flasks. The degree of dilution of the a-amylase required was much greater than with the other diastases tested, showing high activity, while in spite of preliminary cooling and short time before pipetting marked thermolability was found. First flask results.

1. Bacteriological factors in starch and diastase. Possible effect of *B. subtilis*.
2. Variable factors in the starch—iodine complex, such as temperature of solution in Spekker cells, continuation of hydrolysis during Spekker measurement.
3. Effect of mercuric chloride on starch—iodine colour.
4. Interchange of glassware. Control exclusion of adsorption from prior molecular solutions of mercuric chloride as an explanation of results.
5. Possible contamination of prepared microdoses. Effect of Succussion.
6. Effect of CO_2 concentration. Conductivity measurements.
7. Use of Lissapol N in cleaning.

Results

The results obtained were very suitable for statistical analysis of the differences by methods widely used in biological experiments where effects are of the scattered type.

I would like to acknowledge to Dr Robb, M.A., F.R.A.S., Lecturer in Statistics, Glasgow University, my gratitude for his help and advice on the statistical problems over the many years of the research work. By visiting the laboratories and considering the technique used, he was able to advise statisticians on the best methods for the analysis of the results. The chief method used throughout has been the Fisher *t*-test. In addition, the significances of the variances were tested and where these differed significantly Cochran and Cox's test was applied as well.

From the statistical point of view, after studying the technique, Dr Robb in a personal communication commented "actually your technique, with all the variables which have beset you, follows the method I have outlined and you have done everything possible to reduce to a minimum these causes of experimental error, such as room temperature, pipettes, fatigue etc.".

The statistical results of the tests carried out in 1946 and 1948 are shown in Table 4. The analysis was made by Mr Gunn, M.A., Fellow of St. John's College, Cambridge, now Professor of Natural Philosophy (Cargill Chair), Glasgow University, to whom I would express my sincere thanks for the statistical work he so kindly undertook for the Trust from time to time (Boyd, 1942, 1946). Professor Gunn reported on the 1946 results: "significant difference is shown from the controls by every set of the series. The probabilities are very strong indeed. This means that there is certainly a difference between your solutions and the controls", and in 1948 "I have completed the calculations of statistics for the data you sent me. The 't' value comes out at 4.63 which with n = 62 (number of microdoses and controls −2) is, of course, highly significant."

The results of the three series in 1952 were analysed by Miss Carruthers, B.Sc., now of the Mathematical Department, Glasgow University, and by MrGiles, B.Sc., of the actuarial staff of a large insurance company. The methods used are described in the report of Mr Giles. To them I make grateful acknowledgment. These results are given in the following tables. The statistical method used tests for the positive, i.e. stimulative, effect, in the test flasks as against controls. Tables 5 and 6 are shown separately in order to give the statistical assessment of the action of two sets of microdoses of different 'potencies', i.e. stages of shock dilution, with controls carried out at the same time. In the first series of experiments analysed in Table 5 the S27 stage of shock dilution only was used.

The results of two different methods of procedure used in this series for measurement of A.G.V. differences were examined separately, the close similarity of the results indicating the accuracy of Spekker measurement which was, for each method, carried out on a different part of the Spekker scale. It also provided strong support for the technical reliability of the control and test results. The results were very significant (see Section B, Mr Giles report).

Microdoses of four different stages, S28, S29, S30 and S31, were used in the second series analysed in Table 6.

Table 4 Analysis by Professor J. C. Gunn of Results of Experiments Made in January to April, 1946, and May to July, 1948

Solution	*No. of Tests*	*Mean*	*Corresponding Mean of Absorptiometer Differences*	*Sum of Squares*	t	P	*Remarks*
1946							
Control	53	0.038	-.00481	43.92			
S26	26	2.538	+.00769	80.46	8.2	<.001	Highly significant
S27	26	2.115	+.00557	60.65	7.4	<.001	Highly significant
S28	26	2.077	+.00538	63.85	7.2	<.001	Highly significant
S29	25	2.160	+.00580	59.36	7.5	<.001	Highly significant
S30	25	1.960	+.00480	46.96	7.2	<.001	Highly significant
Mixed	128	2.172	+.00586	316.4	9.2	<.001	Highly significant
1948							
Control	32	0.688	+.00344	248.9			
S27	32	4.906	+.02453	572.7	4.6	<.001	Highly significant

Control: both flasks contained distilled water, starch and diastase.
Test: one flask contained distilled water, starch and diastase. The other flask contained microdose in distilled water, starch and diastase.
The results were based on an absorptiometer reading for the first flask of .200 in 1946 and .300 in 1948. The group interval was .005.
For information, the corresponding mean of the absorptiometer differences for each set of results has been added to the table.

Table 5 Analysis by Miss Carruthers and Mr Giles of Results of Experiments Made in March, 1952, the Readings of Flask Differences Having Been Obtained by Two Methods of Absorptiometer Measurement

	Solution	*No. of Tests*	*Mean of Absorptiometer Differences*	*Sum of Squares*	t	P	*Remarks*
A	Control	30	.00367	.00330			
	S27	34	.01103	.00409	2.7	.005	Very significant
B	Control	30	.00300	.00353			
	S27	34	.01015	.00397	2.6	.006	Very significant

A. Analysis by Miss Carruthers. The absorptiometer readings of differences between members of each pair of flasks were obtained by adjusting the absorptiometer to give .300 with the first flask, and then comparing this with the reading obtained with the second flask. The group interval was .005. Green filters were used.

B. Analysis by Mr Giles. The absorptiometer reading for each member of each pair of flasks was matched against a basic standard obtained with distilled water and an absorptiometer setting at 1, and the differences between members of each pair of flasks thus arrived at. The group interval was .01. Green filters were used.

In these particular experiments, four different solutions were tested at each session along with controls, but in varying sequence, the results being combined for analysis when the series had been completed. These results were very significant. The individual stages showed some variation in level of significance.

Table 7 gives the results of a third series, undertaken as a very strict control on the technique. One set of microdoses of stage S31 derived from an S26 three months old microdose and two sets of S27 derived from two independent sets of microdoses freshly made were tested. In this third series the technician was unaware of the nature of the solutions being tested, whether control or test. Results were not checked and allotted to the relevant solutions until all the tests for each day had been run. Particulars of the precautions taken are given under the section on Procedure. The statistical results of the two freshly made sets of microdoses were very significant, the old microdoses were not significant, while the total results of all the tests of this series taken together were very significant.

The final Table 8 gives the statistical analysis of all the 1952 results taken together, in order to provide the data for a histogram. The three series were run practically consecutively between January and July, and could for this purpose be summated. The S27 (Analar) significant results included in Table 7 were excluded from Table 8 as the mercuric chloride was of different origin from that used originally.

Report by Mr Giles on statistical analysis of 1952 results

A. In each experiment we have two samples; firstly the observed differences between first and second control blanks, and secondly the differences between control flasks and microdose flasks. Thus a set of n_1 'control' tests provides n_1 differences x_1, $i = 1, 2, —n_1$, with an arithmetic mean $\bar{x} = 1/n_1 S(x)$, and a set of n_2 microdose tests provides n_2 differences $\bar{x'} = 1, 2, —n_2$, with an arithmetic mean $\bar{x'} = 1/n_2 S(x')$. The significance of the difference $\bar{x'} - \bar{x}$ is tested by means of Fisher's t-test where

$$t = \frac{\bar{x}' - \bar{x}}{S}\sqrt{\left(\frac{n_1 n_2}{n_1 + n_2}\right)}$$

$$S^2 = \frac{S(x - \bar{x})^2 + S(x' - \bar{x}')^2}{n_1 + n_2 - 2}$$

Number of degrees of freedom $= n_1 + n_2 - 2$.

The tables show the mean values $\bar{x}, x'$ together with the sums of the squares $S(x - \bar{x})^2$, $S(x' - \bar{x}')^2$. Because the technique and measurement are identical for both control and test pairs an experimental bias in the measurement of second flasks will affect $\bar{x'}$ and $\bar{x}$ equally, and so there will be no bias in $\bar{x}' - \bar{x}$.

The assumption of normality inherent in the application of the t-test was tested by comparing actual group frequencies and those expected from a normal distribution with the same mean and variance as the sample. The difference

Table 6 Analysis by Miss Carruthers, Checked by Mr Giles, of Results of Experiments Made in April—May, 1952

Solutions	*No. of Tests*	*Mean of Absorptiometer Differences*	*Sum of Squares*	t	P	*Remarks*
Control	47	.00298	.00428			
Mixed S28, S29, S30, S31	78	.00859	.01509	2.4	.009	Very significant

The absorptiometer readings of differences between members of each pair of flasks were obtained by adjusting the absorptiometer to give .300 with the first flask and then comparing this with the reading obtained with the second flask. The group interval was .005. Green filters were used.

Table 7 Analysis Partly by Miss Carruthers and by Mr Giles of Results of 'Blindfold' Experiments to Exclude Unconscious Bias, May to July, 1952

Solution	*No. of Tests*		*Mean of absorptiometer differences*	*Sum of Squares*	t	P	*Remarks*
Control		59	.00008	.01105			
S31 (3 months old)	27	72					
S27 (Fresh)	27		.00736	.02310	2.5	.006	Very significant
S27 (Analar—fresh)	18						

The absorptiometer readings of differences between members of each pair of flasks were obtained by adjusting the absorptiometer to give .300 with the first flask and then comparing this with the reading obtained with the second flask. The group interval was .01. Green filters were used. The S31, an old microdose was statistically inactive (P = .15). It is included in the results. Both freshly made microdoses from different sources were significantly active.

between actual and expected was found to be always less than twice the standard deviation—a very satisfactory result considering the size of the samples. This test was applied to the data of Table 5, for both the sample of controls and the sample of tests. The examination of the data for Tables 6–8 showed that they were also suitable for the application of the *t*-test.

A second method of analysis has also been applied. The significance of the variances has been tested and where these variances have differed significantly Cochran and Cox's test has been applied to find whether there is a significant difference between means.

The results obtained have been found in all cases to be as significant as those shown in the tables for the *t*-test. For example, in Table 6 the results were found to be significant on the 0.001 significance level used there for the *t*-test.

B. For those who are unaccustomed to statistical tests the following may be of interest. The *t*-test begins by assuming that all the samples, i.e. results obtained, come from one normal group or population, in which case there will be no significant difference between the control results and the microdose results. If, however, we find that the differences between the mean of the control results termed $\bar{x}$ and the mean of the microdose results $\bar{x}'$ is large enough to be significant we cannot retain the assumption that the results are all part of the one group. We, therefore, conclude that the control results and the microdose results belong to different groups, i.e. that some factor in the experiment, in this case the microdose solutions, has produced the difference. The level of significance is termed P or probability. The smaller the value of *P*, the greater is the significance of the result. In biological experiments a value of P = <0.05 is considered significant, P = <0.01 may be termed very significant, and P = <0.001 or less, highly significant. This latter figure means in ordinary language that it is 1000 to 1 against these results having been obtained by chance.

A histogram of the data for Table 8 is shown (Fig. 13). This is a diagrammatic method for giving a pictorial representation of differences between the sets of results, in this case controls and microdoses.

In the actual experiments in 1952, it was found that, in the complete series of 130 control pairs of blanks and 166 test flask pairs, as shown in the histogram, no control results gave an A.G.V. greater than 0.32 while an appreciable number of test flasks, considered separately from the statistical assessment of the frequencies of test and control results as a whole, gave an A.G.V. greater than 0.32.

It would appear that, statistically, the results obtained in these experiments can be stated to be highly significant and indicate that a difference of a definite 'stimulative' character has been demonstrated between the action of 'high potency' microdose solutions and control solutions of distilled water.

Discussion

To assist in the consideration of the experiments, brief extracts from two personal communications are given which state clearly the problems presented by the results.

In the first, after careful consideration of the MSS. of earlier work published in 1942, Hopkins commented: "If the results obtained involve some intellectual puzzlement.......one must not look to errors in manipulative technique for an explanation......we ought first to be quite sure of the adequacy......at these dilutions......of the method employed. There is no doubt that the precautions taken in its application seem quite perfect, but is there nothing inherent in the method itself which in such a region might lead to uncertainties in spite of these precautions?" The difficulties he then presented were the problem of

Table 8 Analysis by Giles of all 1952 Results Taken Together

Solutions	*No. of Tests*	*Mean of absorptiometer differences*	*Sum of Squares*	t	P	*Remarks*
All Controls	130	.00192	.0176			
All microdoses	166	.00843	.0358	4.4	$<10^{-4}$	Very highly significant

The data for this table include all the 1952 results, except the result obtained with S27 Analar and included in Table 7 (see text). The group interval was .01.

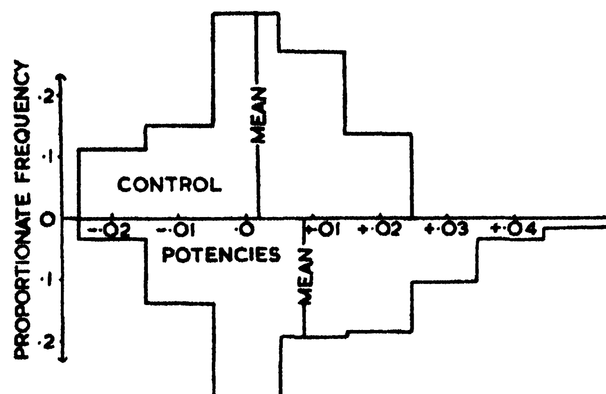

Fig. 13 Histogram of 1952 results used for Table 8, showing the frequency distribution of differences. Note with area to the right that the +.02 block is larger with 'potencies' than with controls while blocks further to right are only present with 'potencies'. The increase to right indicates stimulative effects in the microdose flasks. The shift of the mean is also seen.

controlling conditions governing the intensity of the starch-iodine colour, the possibility, with unbuffered solutions, of the irregular distribution of CO_2 from the chemist's respiration affecting the pH and thus the velocity of the reaction, and the variation in size of colloidal particles with irregular adsorption of the metal leading to a non-uniform distribution. After developing these points the writer then added "such suggestions would be hypercritical were it not that such extreme dilutions were under study, yielding such a puzzling result" (Hopkins, 1939). Although these comments referred to experiments dealing with dilutions which, though extreme, were yet in the molecular range, apart from reference to presence of the metal Hg, they still apply. In the present paper these difficulties have been discussed and the control procedures described which eliminate uncertainties of the kind he mentions. Details are to be found under Procedure, pH and use of Buffers, and especially under Accessory Control Procedures in Appendix D.

The second communication presented another aspect of the problem more nearly related to the 'high potency' microdoses (Gunn, 1946). "In considering possible sources of error one can make a well-marked division into two questions (a) are the solution and control samples presented for comparison genuinely what they purport to be? and (b) does the instrumental set up and process of observation provide a quite unbiased measurement of the properties of the two kinds of objects, 'control—control' and 'control—solution' pairs? The second question involves simply that the process of measurement should be the same in the two cases. The question has really nothing to do with instrumental errors or with any sort of error which is equally applicable to both sets of measurements. In your experimental technique you have obviously succeeded in reducing such experimental error to a completely acceptable level, so they need not be further considered". Gunn then pointed out the value of a blindfold test where the observer was unaware of the actual type of solution, control or microdose, being measured. He also recommended that a microdose solution biochemically active should be spectroscopically examined, as, if the real degree of 'dilution' was as stated, no trace of the original substance would be detected.

These valuable suggestions have been followed. In this paper there has been described a carefully planned 'blindfold' series under Procedure with a table recording the result (Table 7). This 'blindfold' series provided a rigid test for unconscious bias as it included a set of tests using S31 microdoses derived from the S26 stage of a three months old set of microdoses and two sets using S27 microdoses derived from two freshly made sets of microdoses, one derived from the mercuric chloride normally used and one from an Analar preparation of the same drug. The old microdoses were found to be statistically inactive while the fresh microdoses were found to be very significantly active. All results were included. The spectrographic analysis of the reagents is given in Table 3 under Reagents, while the further spectrographic analysis of 'high potency' solutions found to produce stimulation is given in Appendix D5. They show no distinctive difference from the control solutions as was expected. The detailed method of preparing the microdoses in separate stages, thus ensuring the degree of dilution recorded, is described under Reagents.

The dominant question that remains is the control of the process of dilution. It is to be expected that mercuric chloride is adsorbed to Pyrex glass. It was, therefore, necessary to determine whether the stimulation produced by the microdoses might have been due to solutions which were not so dilute as calculation would appear to indicate. While this in no way affects the main investigations into the possible activity of microdoses prepared by the particular technique, it is of considerable importance for any conclusion as to the nature of the solutions showing activity. Could there be introduced into the solutions in the bottles used, traces of mercuric chloride due to adsorption to the glass on some previous occasion when more concentrated microdoses were used, or could some flasks or pipettes be affected similarly?

The best method to eliminate the chance of effects due to prior adsorption appearing only in test microdose flasks or bottles and nowhere else is by cross control and careful interchange of glassware. Pipettes are continuously checked as one pipette was always used for both members of each pair of flasks. A detailed analysis of the interchange of glassware has been made

and crucial examples have been given in Appendix D4. This excludes adsorption to the glassware of mercuric chloride as an explanation of the results.

The efficiency of the cleaning methods was investigated at the Isotope School, Harwell, by our physical chemist using radio-isotope methods (Appendix G). Using the radio-isotope Hg^{203}, a microdose bottle which had contained a relatively strong solution of 1:400 $Hg^{203}Cl_2$ for twenty-four hours was cleaned in accordance with the cleaning procedure (Appendix C). Samples of water shaken and left standing in it showed no radioactivity with methods capable of detecting 10^{-9} g. The cleaning methods were, therefore, capable of removing any $HgCl_2$ loosely adsorbed to Pyrex glass. Further, spectrographic analysis of active S27 microdoses showed no evidence of Hg in the solutions down to 10^{-7}.

The normal primary mental reaction that the results are due to contaminants has been met by methods of cleaning (Appendix C) and interchange of glassware and within the limits of modern analytical procedure, by conductivity measurements, chemical analysis, and by spectrographic analysis, as described in the text and in Appendix D5. Appendix D6 deals especially with the problem of CO_2 contamination.

There remains the suggestion that an unsuspected difference has been introduced between the controls and the dilutions during the process of dilution. Apart from straight extraneous contaminants the possibilities would appear to be that the shock method of dilution increases the gaseous atmospheric contamination in the microdoses, or that it either affects the distilled water or disturbs the homogeneity of the dilutions. A further suggestion has been made that surface tension changes due to the solute cause local concentration on the surface layer or in the bulk of the liquid (Weiser, 1949). This lack of homogeneity might lead to a transference of a higher concentration of the drug at each dilution than is calculated. Thus the dilutions might still be within the molecular range though outside the range of physical detection.

The suggestion that the results are due to the effect of shock on the distilled water used for microdoses, and not to the microdose originally present, has been excluded by a block of highly significant tests in which the second flask of the control blanks was given, in place of microdose, 3 ml. distilled water from a bottle to which had been added a drop of distilled water, the bottle being then successed. This was the identical process followed in preparing the microdoses apart from the presence of the microdose itself. These tests are described in Appendix D6. Confirmatory tests of the same character were also carried out in 1952.

The degree of atmospheric contamination was checked by conductivity measurements and chemical analysis as described in Appendices D5 and 6, while any effect on pH is discussed under pH and use of Buffers, in the text.

The question of adequate homogeneity of the solutions and the possibility of transference of unexpected concentrations from one stage to another is scarcely likely to permit molecular transfer to 27 stages of 1–200 dilution, but this question is also discussed in Appendix F. It would certainly be a much simpler explanation if for any reason a sufficiently high concentration transfer took place, but there is no evidence that this is so, while analysis of earlier stages of dilution contra-indicates this theory (Appendix F), as do radio-isotope experiments (Appendix G).

It is, therefore, considered that, as controls have excluded other alternatives if we allow for limitation of modern analysis including radio-isotope methods to detection within the limits of 10^{-9}, we are left with a conclusion which may be defined thus:

1. Microdoses derived from mercuric chloride and prepared by 27–31 stages of serial dilution with mechanical shock, nominally of the order of 10^{-61} to 10^{-71}, were found to accelerate the hydrolysis of starch with diastase.
2. There being no material trace of mercuric chloride nor of contaminants in the microdoses additional to those normally present in the same distilled water as is used for controls, and there being highly significant effects obtained by these microdose solutions, it is concluded that an active factor derived from the mercuric chloride accelerates the hydrolysis of starch with diastase. This stimulative factor is capable of affecting the microdose distilled water, and of thus transferring its activity to subsequent ultra molecular stages of the 'high potency' microdose preparations. The nature of this factor is unknown.

In the five crucial test series reported, 326 pairs of control–microdose flasks and 215 pairs of control–control flasks were compared, i.e. over 1000 solutions, excluding those used for accessory controls and sampling procedures.

Addendum

This unknown "high potency" factor has also recently been demonstrated with a biological heart rate recorder (Boyd and Eadie, 1952) by means of a technique previously developed to test microdoses of the molecular range down to 10^{-11} (Boyd, 1953). Figs. 14, 15, 16 and 17 show the lay-out of the recording apparatus. This technique is now being applied to testing of "high potency" of *Strophanthus sarmentosus* and other drugs on the frog heart. In the earlier 1953 paper details are given of the primary solution of *Strophanthus* from which the "high potency" were derived.

In these latter experiments, to be published in full later, definite evidence has been obtained of the action of microdoses in distilled water of S27 stages derived from *S. sarmentosus* as against controls of the same distilled water. These have been prepared from the primary solution by the method described under Reagents with all the precautions against contamination as detailed in this present paper and appendices. Figure 18 gives some examples of the change in heart rate and ECG which may be produced by this type of dose, along with the negative result of previous application of

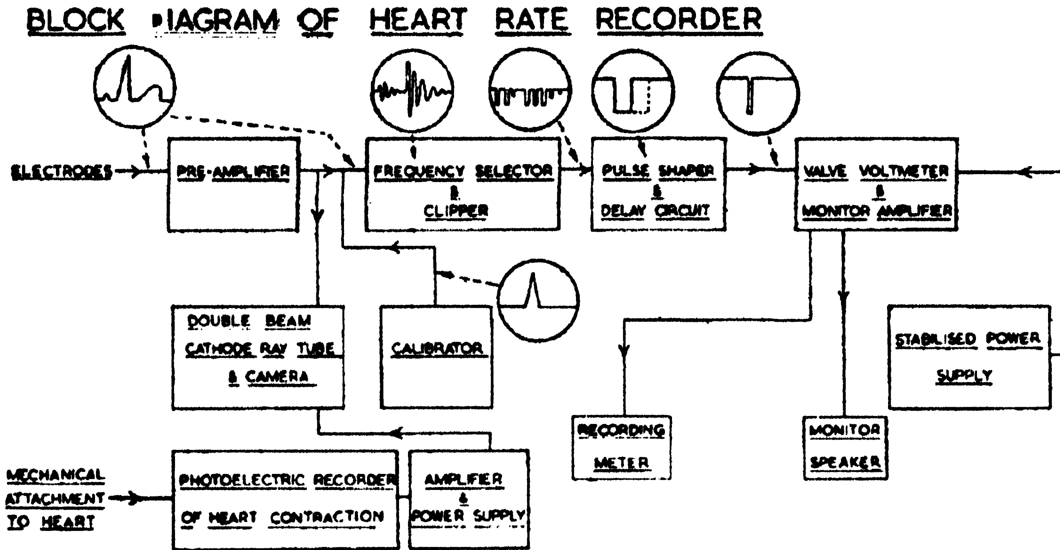

Fig. 14 Block diagram of circuits. Insets show the selection shaping of the 15 c/s frequency component of the ECG.

controls of distilled water. The factor is not easily destroyed and in Figure 19 examples are shown of its activity using ordinary S27 microdoses and microdoses previously exposed to both boiling and freezing and then brought to room temperature, along with controls of distilled water submitted to the same procedure. Boiling to the extent shown does not destroy the factor but may impair its activity. The action of this factor is selective, depending on the individual frog sensitivity to the microdose at the time of application.

Table 9 shows the frequency of heart rate responses to control applications of 0.06 ml distilled water, and to applications of 0.06 ml S27 *S. sarmentosus*, recorded up to the date of presentation of this paper (March, 1954). More

[*By kind permission of Electronic Engineering*

Fig. 15 General view of apparatus.

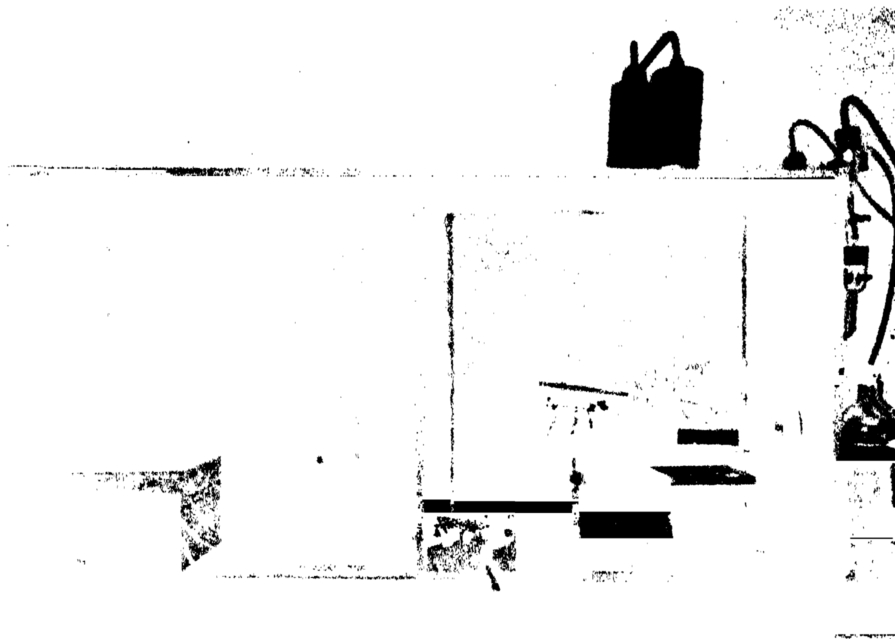

Fig. 16 View of the mechanical recorder attached to the apex of the heart. The set-up 011 the frog of the Ringer-platinum electrodes and the Ringer drip supply is seen. The screening cage, pre-amplifier, calibrator and marking trigger are also shown.

Fig. 17 Addition of solutions to the auriculo-ventricular junction of the frog heart using a calibrated capillary tube.

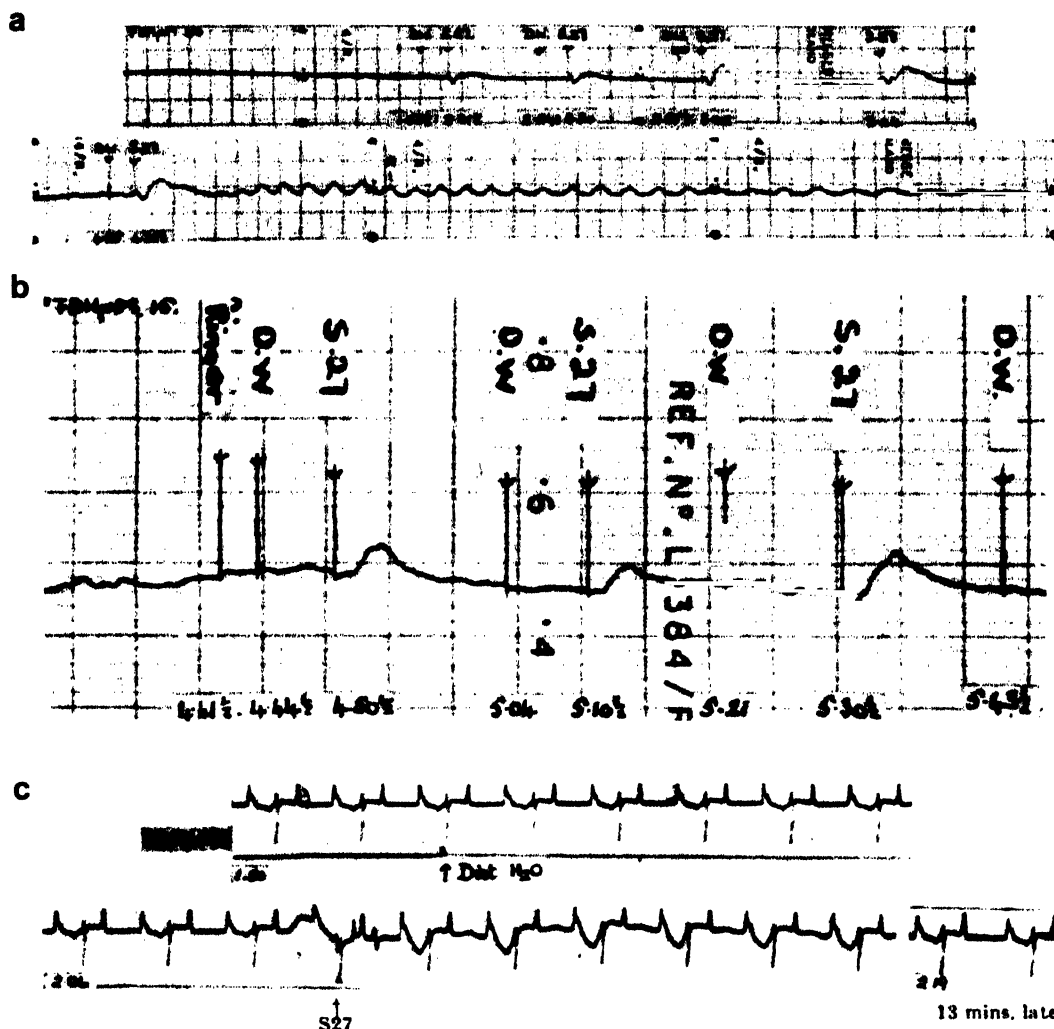

Fig. 18 Effect of direct applications, on the exposed hearts of three sensitive frogs, of 0.06 ml of S27 microdoses derived from *Strophanthus sarmentosus*. S27 microdoses were prepared as in the biochemical experiments, with sterile precautions, in separate bottles by serial dilution and mechanical shock, the diluent being from the same flask of distilled water as that used for control applications. The rate recorder vertical timing lines were five minutes apart. The horizontal lines 0.4 = 40 beats per minute etc. The moment of drug application in (a) and (b) is indicated by a vertical excursion of the pen produced by a hand trigger which also marks a simultaneous ECG. This marking is also seen in Figure 19. (*a*) Continuous record of the heart rate showing the rate changes following five applications of the S27 with control applications of 0–06 ml of the distilled water. ECG omitted. (*b*) Another frog showing heart rate change following each of three applications of 0.06 ml S27 of the same drug. ECG omitted. (*c*) The ECG response of another frog heart to, (1) application of 0.06 ml of distilled water, and (2) effect following application of 0.06 ml of S27 microdose of *Strophanthus sarmentosus*. Note the marked depression of P–Q with rise of S–T. (3) Later recovery. The change in the complex at moment of application was not counted as an effect. Rate record omitted.

detailed analyses and supplementary information will be published later. Of seventy-one frogs tested with S27 'high potency' microdoses 49.2 per cent, showed rate responses compared with 2.8 per cent, showing rate change after distilled water. All distilled water controls in this series were given mechanical shock in the same way as the S27 drug solutions.

The total number of applications of distilled water to the 71 frogs, including the 36 insensitive frogs, was 274, while the S27 microdoses were also applied on 262 occasions. The drug applications produced 88 rate changes (33.5 per cent) compared with two rate changes occurring after distilled water (0.73 per cent). An immediate distinctive reaction on application was obtained in 19 per cent, of the drug applications, while no immediate response was found after any distilled water applications. The total number of drug applications to the 35 frogs which showed a rate reaction was found to have been 156 of which 56 per cent, produced an effect. The proportion of frog preparations found unsuitable for testing owing to persistent irregularity of heart rate was found to vary, increasing in warmer weather, and at spawning time, and diminishing during colder spells.

It would appear, therefore, that the preparation of solutions derived from a primary substance by the pharmaceutical method of serial dilution in small quantities with mechanical shock results in the presence of a selective derived factor which is capable of influencing biochemical activity under suitable experimental conditions.

Further investigation of this factor is being carried out.

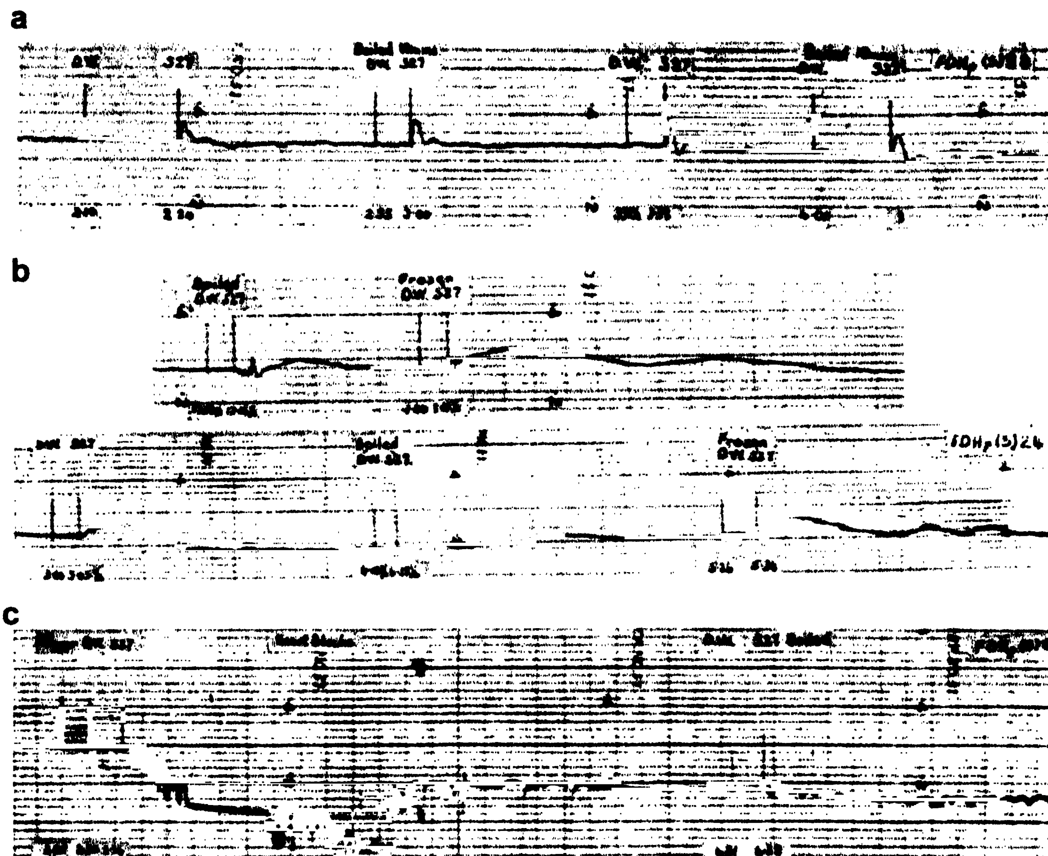

Fig. 19 Effects of potencies which have been boiled and frozen. (a) Response to S27 *Strophanthus sarmentosus* where an ordinary microdose was alternated with a boiled microdose. The control distilled water in the latter case was also boiled. Note the microdose was active in spite of being boiled for 10 min and allowed to cool to room temperature. (b) The effect of five doses of microdose S27 *Strophanthus sarmentosus*, two microdoses having been boiled, the control distilled water also having been boiled. Both were allowed to cool to room temperature. The two other microdoses were frozen along with the distilled water control and were then allowed to melt and come to room temperature. It can be seen that both frozen microdoses were active while one boiled microdose was active and the other only slightly active. One ordinary S27 microdose effect was included with its distilled water control for comparison. (c) The effect of two doses of microdose S27 *Strophanthus sarmentosus*. The first microdose, an ordinary one, gave a sudden marked aggravation lasting for a considerable time. The second microdose, boiled for ten seconds and allowed to cool to room temperature, still gave a definite effect, but in this frog to a lesser degree.

Table 9 Total Frogs—132. Unsuitable Frogs (rate persistently irregular)—not tested—61. Suitable Frogs (rate regular prior to drug tests)—tested—71.

Tested Frogs	*Distilled water*		*S27* Strophanthus sarmentosus	
*Frogs reacting**	*No.*	*%*	*No.*	*%*
	2	*2.8*	*35*	*49.2*
Total applications (all frogs tested)	274		262	
Applications (immediate reaction)	Nil	0	50†	19.0
Applications (reaction within 2 min)	2	0.73	38	14.5
Total applications producing reaction	2	0.73	88	33.5

Note: S27 equivalent to homoeopathic 32*c* high potency.
The comparative heart rate response of exposed hearts of suitable frogs (Rana Temperaria) to direct applications on the auriculo-ventricular junction of 0" 06 ml. double distilled water control and of microdoses of 0.06 ml. S27 *Strophanthus sarmentosus* in the same distilled water. Suitable frogs were those with an approximately regular heart rate recording after recovery from initial shock and prior to control or drug application.
* One frog reacted after distilled water only. The other which reacted also responded to microdose.
† Confirmed by rate measurement on simultaneous e.c.g. films except in one case.

Acknowledgments

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who developed the technique in the early years, and who has carefully studied and advised on the text of this report on the completion of the fifteen year's work. Financial support has been received from the Beit Research Committee of the British Homoeopathic Association, from the Scottish Homoeopathic Research and Educational Trust, and from private subscribers through the Trustees of the Boyd Medical Research Trust, to all of whom grateful acknowledgment is made. In addition support is being given by the National Health Service Management Board of the Glasgow Homoeopathic Hospital for the biological investigation.

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