Some experiments in the use of Antiseptics in the manufacture of Glue and Gelatine*.

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In the manufacture of glue and gelatine, an important point for consideration is the action of putrefactive and liquefying bacteria on the dilute liquors obtained during the course of manufacture. Since these contain besides gelatin the peptones and proteoses which are its degradation products, the liquors constitute admirable nutrient media for the growth of most organisms. Necessity often arises during work on a large scale to store these dilute solutions for some considerable time and if no preventive steps are taken, the solutions become highly putrid and completely lose their power of setting.

That the reaction of the nutrient medium has considerable influence on the growth and development of bacteria is a fact well recognised in biochemistry and the course that most naturally suggested itself was to keep the gelatine liquors at a reaction least favourable for the propogation of bacteria.

The problem however is complicated by the fact, noted early during the course of the work, that not only different acids had their own specific influence, but also that admixtures of most chemicals with gelatine lowered its 'setting' power. Work had therefore to be directed towards the discovery of possible optimum points at which with the aid of a minimum quantity of acid or alkali the effects of bacterial action might be counteracted.

It is not well known what happens, when owing to the influence of bacteria or chemicals, gelatine refuses to gel. Bacterial liquefaction of gelatine invariably leads to ultimate putrefaction, but the converse is not true, since a solid gelatine jelly may turn putrid without liquefaction. It is common knowledge that the liquefaction of gelatine is due to the action of proteolytic enzymes secreted by certain bacteria which split up the gelatin molecule into its ultimate products of disintegration, amines and amino-acids and very often ammonia. To the manufacturer of glue and gelatine the percentage of gelatin in his product is a matter of vital importance and he has to pay the best attention to the conservation of the gelatin molecule as such.

*The word 'gelatine' is used when reference is made to the commercial substance and the word 'gelatin' is employed when the protein of that name is under discussion.

The rate of disintegration of the gelatine by acids or by bacteria was determined.

A. By comparison of the precipitate formed by tannic acid before and after treatment.

B. By comparative viscosity determinations.

C. By determination of the melting point of gels.

These experiments are described in detail in the following paragraphs :—

A. Precipitation Experiments.

Experiment I. 250 cc of a 20 % solution of leaf gelatine (Gold Label Brand—Coignet) were tubed in quantities of 10 cc and sterilised by the discontinuous method (for 15 minutes on three consecutive days).

Six tubes were kept as controls. To each of six others 2 cc of a liquefied gelatine solution which was turning putrid was added. To each of another six tubes 2 cc of 12 N. Hcl. was added. The contents of each tube were well shaken together and kept in the hot incubator.

A set of these tubes was examined every 24 hours in the following manner. 1 cc of the well stirred solution was pipetted out into a 10 cc centrifuge tube drawn into a capillary at one end and graduated. A little distilled water was added and then enough of a freshly made solution of 2% tannic acid until there was no more precipitate. In the case of the tubes containing acid 1 cc of a N solution of Na OH was added before precipitation by means of tannic acid. The solution was then made up finally with water to the 10 cc mark in the centrifuge tube. The centrifuge was worked for exactly 20 minutes in each case and care was taken to see that the handle was turned round at the same regular speed in all experiments. The precipitate settled to the bottom as a hard cake and its quantity was read off on the graduated scale. Since the conditions of experiment in each case were identical, the figures obtained indicate roughly the progress of the change undergone by the gelatin present in the various solutions.

The above method appears to give relatively more reliable comparative results than the more laborious method of precipitating the protein with magnesium or zinc sulphate and determining the same quantitatively by means of a nitrogen estimation by Kjeldahl's method.

The results may be tabulated as below :—

Expt.	Time. Hrs.	Control.	Bacteria.	Acid.
1	0	22	21	23
2	24	23	18	18
3	48	22	16	19
4	72	22	9	17
5	96	24	10	13
6	120	22	8	14

Though only roughly quantitative the results show clearly the relative progress in the rupture of the gelatin molecule by acids and by bacteria.

Fig. 1 indicates graphically the progress of the decomposition in comparison with the controls.

B. Viscosity Method.

The degradation of the protein in glue and gelatine liquors was also studied in the following manner.

Experiment II. A 20 per cent solution of Gold Label gelatine in water was sterilised by the discontinuous method, allowed to cool to room temperature, which was noted, and the time taken for the clear solution to run through a 25 cc pipette from the top calibration mark to one made below the bulb was noted with the help of a stop-watch.

To 100 cc of the sterile gelatine solution contained in a flask, a loopful of a liquefied solution of gelatine, (containing liquefying bacteria from septic tank effluent) was added and the flask was well shaken and kept at room temperature for six hours. The time taken by this solution to run between the two marks on the same pipette was then carefully noted, the temperature of the solution being kept at the same point as in the previous experiment.

The results were as under :—

Temperature of experiment :—31°C

	Expt.	Control solution.	Infected solution after six hours.
Time taken to run	1	173	72
through pipette in	2	172	64
seconds.	3	175	67
		173 sec.	68 sec.

Experiment III. An attempt was made to determine the influence of there action of the gelatine solution on the liquefying power of proteolytic bodies by using the above method.

5 per cent solutions of French gelatine in dilute sulphuric acid of varying normalities were exposed to the atmosphere for about 24 hours and their viscosities determined as indicated.

The following results were obtained.

TABLE I.

Reaction.	I	II	III	Rough Mean.
N/1000 soln.	30·6	30·0	30·4	30 seconds.
N/500	30·7	30·4	30·8	31
N/250	31·2	31·0		31
N/100	31·0	30·7	30·7	31
N/50	31·0	31·0		31
N/20	34·6	34·7	34·7	35
N/10	32·6	33·0	32·6	33
N/5	31·2	31·6		32
N./	28·7	28·8		29
2N./	28·1	28·1		28
4N./	28·0	28·0		28
Water	26·7	27·1	27·4	27

It is clear from the above that:—

(1) From N/1000 to N/20 it is the bacterial action that is chiefly taking place.

(2) At N/20 the bacterial action is inhibited by the acid and higher viscosity results.

(3) From N/20 to 4N the chief action is due to the acid which gradually reduces the viscosity with increase of concentration.

(4) At 4N increase in the concentration of the acid has ceased to have any effect on the viscosity of the gelatine solution.

Certain difficulties were however met with in this method of procedure which tended to vitiate the accuracy of the results. It was difficult to maintain the temperature constant during the experiments, solid impurities in the solutions tended to block up the exit orifice of the viscosimeter thus introducing

serious errors, and the actual differences in the viscosity were not sufficiently marked for small differences in the reaction of the solutions to be studied. Evaporation at the exit orifice also introduced errors. It was therefore found advantageous to adopt the method detailed below under C. for finding the optimum points referred to previously.

C. Melting Point Method.

Experiment IV. Nutrient gelatine was made in the following manner. 200 gms of Nelson's leaf gelatine were dissolved in about 500 cc of distilled water in a sterile flask. A watery paste was made of 10 grms of Witte's peptone, 5 grms sodium chloride and 5 gms fresh Lemco and stirred up with the gelatine solution. The mixture was then made neutral to phenolphthalein by adding the estimated quantity of a N solution of NaOH, boiled to precipitate phosphates, allowed to cool, made up to a litre and clarified in the usual manner with the whites of two eggs.

The bright clear liquor obtained on filtration was tubed in quantities of 5 cc and the tubes were sterilised by the discontinous method for three days. Stored in the cold incubator overnight, the tubes were found to have set to a clear transparent jelly in the morning.

To investigate the inhibitive action of chemicals on the liquefaction of gelatine by bacteria, cultures were made use of which were presumed to be the most active liquefiers likely to be met with, *viz.*, strains isolated from a septic tank effluent, and also two pure cultures actually obtained in a glue factory.

The two latter may be more fully referred to in case they should be met with in practice.

During the course of large scale work it was found that despite the greatest care, several successive draws of glue would not gel, and even if they did, would run through the nets of the drying frames. Bacteriological examination of samples of the draws revealed the existence of two different species of bacteria.

They were distinguished respectively as A7 and B1, and their characteristics may be summarised as follows :

	A7.	B1.
Colonies.	White transparent with fern-like edges.	Pearly white with fine rounded edges.
Appearance.	Long stout sporing bacilli.	Minute non-sporing bacilli.
Staining properties.	Gram-positive.	Gram-negative.

	A7.	B1.
Liquefying power.	Liquefies gelatine after 40 hours.	Liquefies gelatine within 24 hours.
Behaviour with sugars.	Produces acid and gas within 24 hrs. with glucose, galactose, maltose, lactose and mannite and does not act upon dextrin, saccharose, inulin and dulcite even after 6 days.	Produces acid and no gas with glucose, galactose maltose, lactose, dextrin, saccharose and mannite and no action at all with inulin and dulcite.

In every experiment, 0·5 c.c. of this culture was added to the5 c.c. of gelatine in the test tube after allowing the latter to melt and the tube was then well stirred. To make up a normal HCl solution, say, 0·5. c.c. of a 12 Normal solution of HCl was then added and the whole again stirred up. One pipette was used for the culture and a separate one for the acid and great care was taken to see that the pipettes were used exactly in the same manner in each case. Each experiment was repeated thrice and the results of each were separately noted.

Three tubes each of N/1, N/2, N/4, N/8, N/16, N/32, N/64, N/128, and N/256 HCl were thus made. Each tube contained 0·5. c.c. HCl of suitable strength, 0·5.c.c. of the culture liquid and 5. c.c. of the nutrient gelatine medium and was kept during the day at room temperatures (30°C—32°C) and left to set overnight in the cold incubator at 20°C. The tubes were examined at the same time next morning, and the several characteristics of the jelly or of the liquid in the tubes were carefully noted, sufficient attention being paid towards expressing the results comparatively. The results of experiments with HCl are to be found in TABLE II while Fig. II expresses the same graphically.

Attempts to determine accurately the melting points of the jellies for a comparative study failed in all cases, and the following method alone was found capable of giving reliable results. (All the bacteriological tubes used were of the same dimensions). The tubes, as soon as they were taken out of the cold incubator were placed in a slanting position under a fan. The tube to melt first was noted and the intervals at which the other tubes melted were carefully ascertained. All the tubes being kept at the same slope, the melting point was taken to be the time at which the jelly assumed the new horizontal. The results obtained plotted graphically as in Fig. III, bring out the quality of the jelly more forcibly than either the Lipowitz test or the accurate determination of melting points.

FIG. II

* To get the Correct Normality multiply all Normalities by 0.6

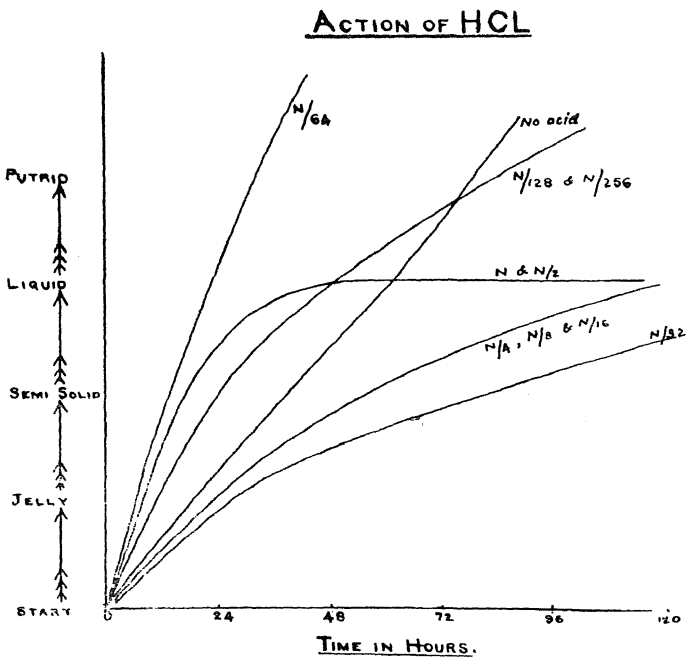

FIG III

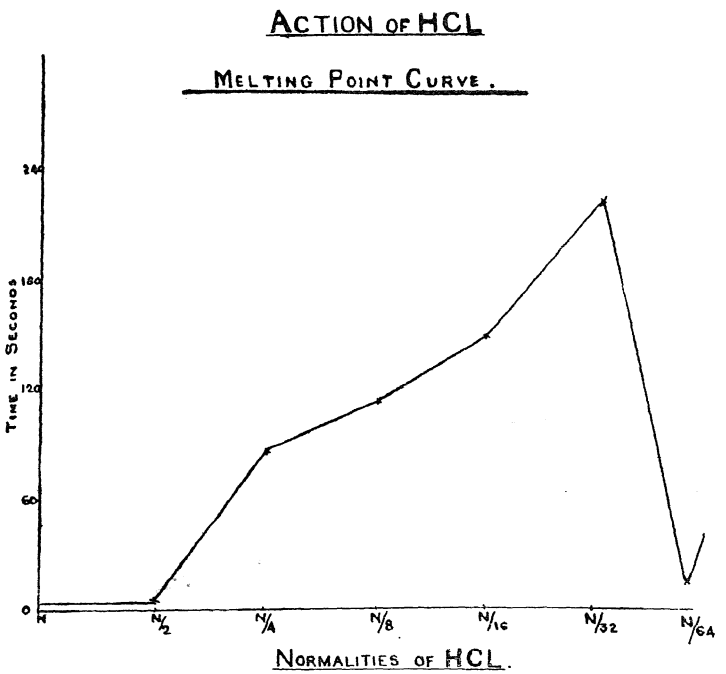

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TABLE II.

Action of HCl.

Normality. 0·6 × (—)	24 hrs. A B C	48 hrs. A B C	72 hrs. A B C	96 hrs. A B C	120 hrs. A B C	Meaning of symbols used.
N	/- /- /-	L L L	L L L	L L L	L L L	1- ... clear.
N/2	/- /- /-	L L L	L L L	L L L	L L L	- ... cloudy.
N/4	/- /- /-	$ $ $	$ $ $	$ $ $	$ $ $	S ... set.
N/8	do	do	do	do	do	$ ... set but less firm than S.
N/16	do	do	do	do	do	
N/32	do	S S S	S S S	S S S	do	L ... liquid.
N/64	do	L L S	L L L putrid	L L L putrid	L L L putrid	L/S ... liquid on top and semi-solid at bottom.
N/128	- - -	S L/S L/S	L/S L/S L/S	L L L putrid	L L L putrid	
N/256	- - -	L/S L/S L	L/S L/S L	do	do	
No acid	- - -	L/S L/S L/S	L L L	L L L	L L L	

N tubes contain :—
0·5 cc sewage
0·5 cc 12 N × 0·6 Hcl.
5·0 cc 20% of gelatine.
6 cc.

It can be inferred from the results obtained.

(1) That while both HCl and bacteria liquefy gelatine, the action of the latter alone is attended by final putrefaction.

(2) *That a normality of the solution of 0·6 × N/32, HCl, * phenolphthalein being the indicator, exerts the greatest inhibitive action on the growth of the bacteria* and that the combined effect of the bacteria and the acid is least at this reaction.

(3) That a normality of 0·6 × N/64 appears to be an optimum for the growth of liquefying organisms. Hence, if the gelatine or glue liquors are to have an acid reaction using HCl they are best obtained at about a normality of about 0·6 × N/32, which on calculation gives 4 parts by weight of HCl to every 1000 parts of gelatine finally obtained.

Experiment V. The above experiments were repeatedd using sulphuric acid instead of hydrochloric acid and the results

*Phenolphthalein is the indicator used throughout these experiments unless the contrary is started

obtained are tabulated in a similar manner in TABLE III and graphically represented in Figs. IV and V.

TABLE III.

The influence of H_2SO_4 on the setting etc. of gelatine.

Normality 0·4 × (—)	After 24 hrs.	After 48 hrs.	After 72 hrs.	After 96 hrs.	After 120 hrs.	Meaning of symbols used.
	A B C	A B C	A B C	A B C	A B C.	
N	/- /- /-	$ $ S	L L L	L L L	L L L	/- ... clear
N/2	do	s s s	1 1 1	1 1 1	1 1 1	— ... cloudy
N/4	do	do	do	do	do	S ... solid
N/8	do	$ $ $	$ $ $	s s s	s s s	$ ... solid but looser than S
N/16	do	S S S	do	do	do	s ... solid but looser than $
N/32	do	do	bo	do	L/s s s	L ... Liquid
N/64	do	do	S S S	S S S	S S S	1 do at slightly higher temperature
N/128	- - -	P P P solid	L/s L/s L/s putrid	L/s L/s L/s putrid	L L L putrid	
N/256	do	s s s putrid	L/s L/s L/s putrid	L/s L/s L/s putrid	L L S putrid	L/s ... liquid on top and semi-solid at bottom
No acid	do	L/s L/s L/s putrid	L L L putrid	L L L putrid and cloudy	L L L putrid and cloudy	P ... putrid

It will be inferred from the results obtained that in the case of sulphuric acid

(1) *A normality of 0·4 × N/64 has the greatest inhibitive action on the growth of the liquefying bacteria* and that.

(2) A solution of the above reaction sets to the hardest jelly. Hence if sulphuric acid is to be used, the glue liquors are best kept at about a normality of 0·4 × N/64 which means 1·8 parts by weight of sulphuric acid to 1000 parts of solid gelatine.

Experiment VI. In experiments in which a concentrated solution, of sulphur dioxide in water was used instead of sulphuric acid, the following results were obtained.

FIG. IV

⊗ To get correct Normality multiply by 0.4.

ACTION OF H_2SO_4.

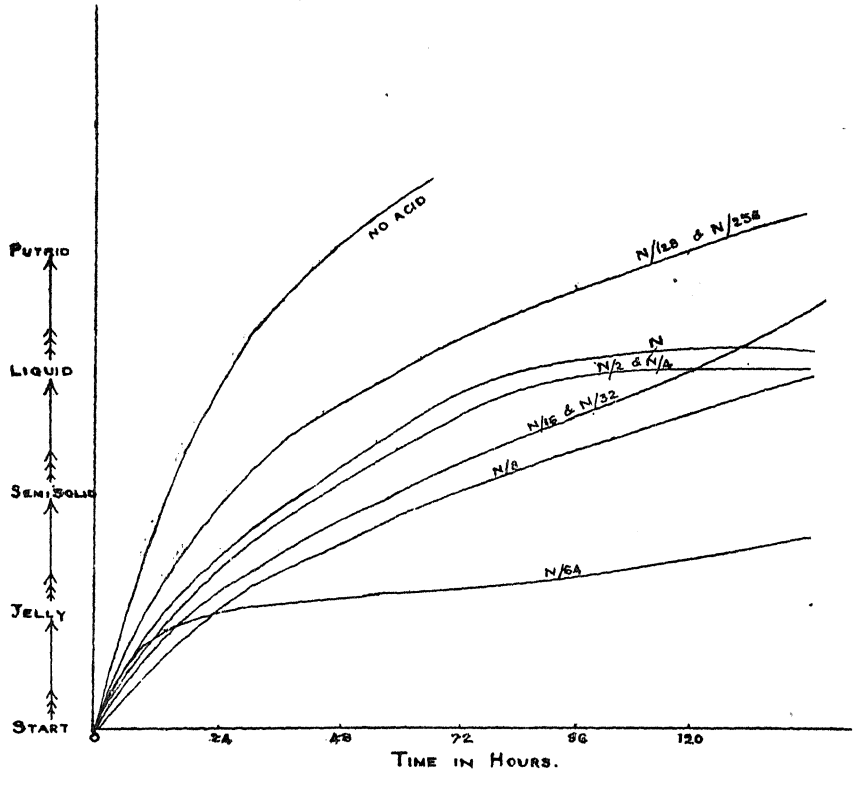

FIG V

MELTING POINT CURVE.

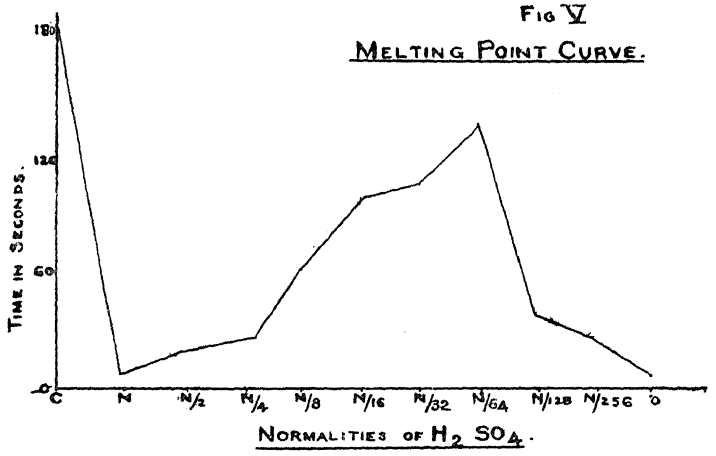

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Fig. VI

Action of SO_2

[* To get correct Normality multiply by 0·03]

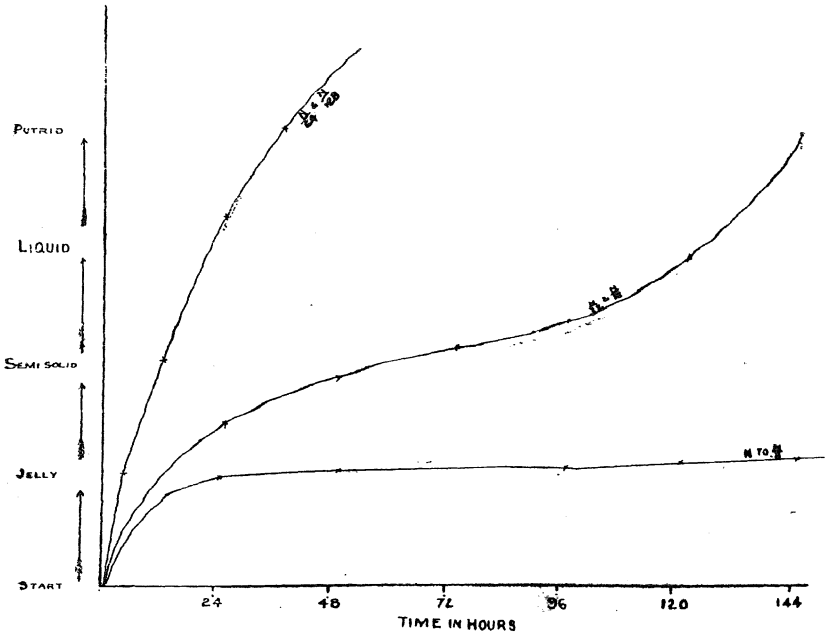

Fig. VII

*[To get correct Normality multiply by 0·03]

Melting Point Curve

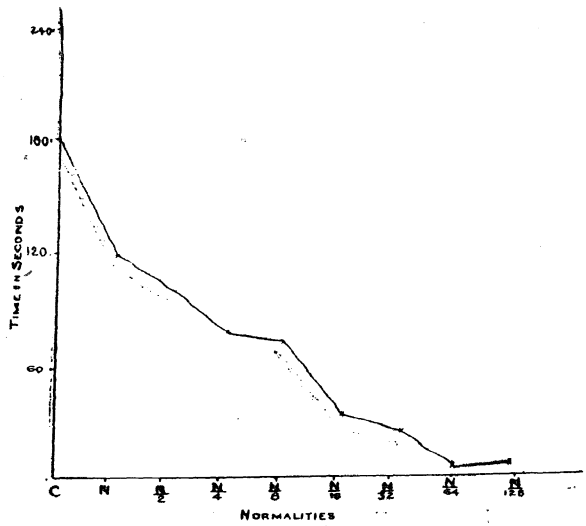

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TABLE IV.—SULPHUROUS ACID.

	Normality.	After 24 hrs.	After 48 hrs.	After 96.	After 120 hrs.
1	0·03. N.	Set.	Set.	Set.	Set.
2	0·03. N/2.	Set.	Set.	Set.	Set.
3	0·03. N/4.	Set.	Set.	Set.	Loose jelly.
4	0·03. N/8.	Set.	Set.	Set.	Loose jelly.
5	0·03. N/16.	Set but cloudy.	Set, not putrid.	Loose jelly putrid.	Liquid putrid.
6	0·03. N/32.	Set but cloudy.	Do.	Do.	Do.
7	0·03. N/64.	Set but very cloudy.	Set, turning putrid.	Liquid and putrid.	Liquid and putrid.
8	0·03. N/128.	Do.	Liquid and putrid.	Do.	Do.

The results have been plotted as in the other cases in Figures VI and VII. It will be evident from a consideration of the results obtained that the action of sulphurous acid (H_2SO_3) at the dilutions used differs considerably from that of either HCl or H_2SO_4 so far at least as there is no evidence of the liquefying action of the acid itself. Solutions from N to N/8 × (0·03) inhibit the growth of bacteria while even with N/16 × (0·03) liquefaction due to bacterial action commences only after 96 hours.

Hence, if sulphur dioxide is used, it will be enough for the manufacturer if he raises the reaction of the liquor to a normality of 0·03 × N/8 which means merely *0·36 gm of sulphur to be burnet into sulphur dioxide for every 1000 gms of the final product.*

D. THE ACTION OF ORGANIC ANTISEPTICS.

Experiments VII and VIII.—The chances of bacterial infection may be minimised and bacterial action itself checked not only by adjusting the reaction of the liquid medium but also by the use of organic antiseptics. While in the case of gelatine, it may be advantageous to investigate the influence of several antiseptics without much attention being paid to their cost, the case is different with glue which being a cheaper commodity precludes the use of costly preservatives. The action of phenol and formaline as antiseptics has been investigated, the experiments being conducted in precisely the same manner as in the case of the mineral acids already dealt with.

TABLES V and VI contain the results obtained with phenol and formaline respectively while the melting points of the jelly and their relation to the proportion of antiseptic are indicated graphically in Figures VIII and IX.

Phenol. TABLE V.

N is equal to 1 c. c. melted phenol at 40°C in 100 c. c. H_2O.

Strength.	After 24 hours.	After 48 hours.	After 96 hours.
N.	Hard jelly	Hard jelly.	Hard jelly.
N/2.	Do.	Do.	Do.
N/4.	Do.	Do.	Loose jelly.
N/8.	Opaque putrid jelly.	Opaque putrid jelly.	Opaque putrid jelly.
N/16.	Do.	Do.	Do.
N/32.	Opaque loose putrid jelly.	Loose putrid jelly	Loose putrid jelly.
N/64.	Do.	Do.	Do
N/128.	Do.	Do.	Do.

In the case of phenol, therefore, a concentration of at least *15 parts of phenol per 1000 parts of final product* is necessary to prevent infection, since every 6 c. c. of solution in which is dissolved 1·9 gm of gelatine contains 6/400 c. c. of molten phenol, the specific gravity of which may be taken roughly as unity.

Formaline. TABLE VI.

N is equal to 1 part in 1000 parts.

Strength.	After 24 hours.	After 48 hours.	After 96 hours.
N	Clear hard jelly	Clear hard jelly	Clear hard jelly
N/2	Do	Do	Do
N/4	Do	Do	Do
N/8	Cloudy putrid jelly	Cloudy jelly putrid	Cloudy jelly putrid
N/16	Do	Do	Do
N/32	Do	Putrid loose jelly	Putrid loose jelly
N/64 & N/128	Do	Do	Do

FIG VIII

PHENOL

MELTING POINT CURVE

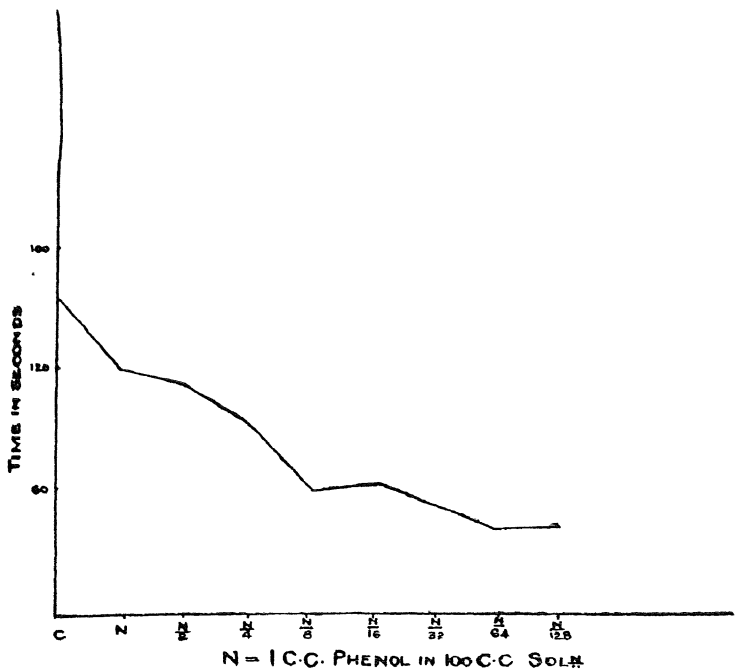
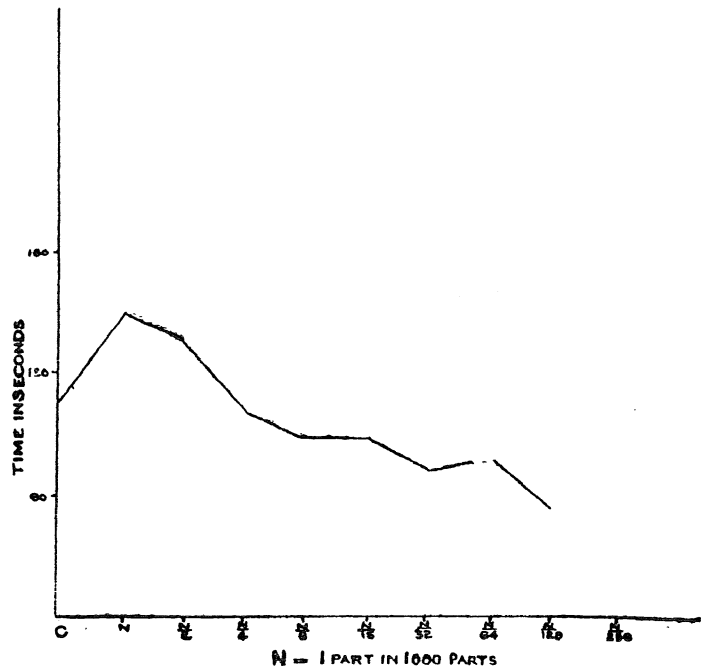


FIG IX

FORMALINE

MELTING POINT CURVE



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From TABLE VI, it will be noticed that *1·5 parts of a 40 per cent solution of formaldehyde for every 1000 parts of gelatine* will be the minimum quantity capable of exercising the necessary beneficial protective action against bacteria. It will be also seen that with insufficient quantities of phenol or carbolic acid, the gelatine becomes putrid without being liquefied. Figure IX shows how the melting points of jellies containing N and N/2 of formaline are higher than that of the control.

E. EFFECT OF CONCENTRATION ON THE GROWTH OF MOULDS.

The drying of glue and gelatine jellies takes some considerable time when done on a large scale and the use of chemicals and antiseptics does not appear to prevent the growth of higher forms of life like the moulds. An attempt was made to find out the factors which decided the growth of these bodies.

It was noticed early in the course of the work that glue and gelatine jellies of high concentration (low moisture content) were invariably free from moulds. While those of low concentration, which were loose in their texture (high moisture content) were almost always attacked. Work was therefore directed towards finding out the critical concentration beyond which moulds could not grow vigorously.

Glue solutions of concentration ranging from 5 per cent to 60 per cent were exposed to the atmosphere for several days and examined every morning. It was found that by the 4th day, the jellies ranging from 5 per cent to 30 per cent had grown moulds, while even after seven days, the stronger jellies had grown no moulds.

The critical concentration at which moulds can no longer grow differs slightly for different glues and gelatines but it has been found that a 40 per cent jelly (*i. e.* 40 gm of glue or gelatine in 100 gm of jelly) may be kept invariably free from mould, in a moist atmosphere, provided the temperature is between 28°C and 32°C.

CONCLUSION.

During the course of the manufacture of glue and gelatine there rarely arises the necessity of stocking cold dilute (say 20 per cent) gelatine liquors for periods extending to more than 48 hours. It has been shown in the preceding pages that it is not impossible by the adoption of suitable means to main-

tain liquors at the necessary stage of sterility for periods extending to even 120 hours, at a room temperature of about 30°C to 32°C, without seriously affecting the gelatin content and setting power.

Considered from a point of comparative cost, the use of sulphur dioxide may be strongly advocated especially as, besides its effect on the reaction of the liquor, it has not only a distinctive antiseptic effect but also a well recognised clarifying and decolourising effect The advantages of phenol and formaline consist chiefly in the ease of their application which however are counterbalanced by their present prohibitive cost and also by their being poisons.

Certain of the experiments above recorded were carried out by one of us at the King Institute of Preventive Medicine, Guindy to whose Director our thanks are due.

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