STERILE PHARMACEUTICAL PREPARATIONS OF PROTEOLYTIC ENZYMES FOR CLINICAL USE IN DENTAL PRACTICE

DRY POWDERS AND SOLUTIONS CONTAINING A BACTERICIDE

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INTRODUCTION

THE reports of previous workers concerning the preparation of sterile solutions of proteolytic enzymes suitable for injection are not very helpful since they are so old as to be unrelated to modern practice. Injections of trypsin and amylase, or more simply injections of the mixed pancreatic enzymes, were at one time thought to be effective in the treatment and cure of cancerous growths and tuberculous nodules. These ideas were fully discussed and adversely criticised in the British Medical Journal[1] in 1909. Apparently the pancreatic solutions for injection had been considered to be sterile on account of the use of 60 per cent. glycerin, as vehicle. Thus we read " the medium, 60 per cent. glycerin, had already been found to meet the requirements, containing the enzymes of the fresh pancreas-extract in an active sterile condition." Thymol, toluene and chloroform in saturated aqueous solutions have, for many years, been used to prevent bacterial multiplication in prolonged enzyme experiments. It appears that while they probably inhibit to some extent weak solutions of trypsin, their deleterious action is negligible when relatively strong solutions of the enzymes are under consideration[2,3]. The acridine colouring matters are said to be strongly inhibitory for trypsin[4] while gentian violet is weakly inhibitory[5].

The question as to whether, on injection, proteolytic enzymes can give rise to antibodies which may neutralise the digestive action of the enzymes or cause anaphylactic reactions when subsequent injections are given has not been completely answered. A good summary of the evidence is given by C. Oppenheimer[6]. The antigenic nature of the enzymes, even if established does not preclude their use in some branches of dental practice. The preparations described below were elaborated for experimental use in the Dental Department of this University.

As far back as 1929 Wilkinson[7] suggested that, prior to the operation of the permanent root canal filling of teeth, the necrotic pulp tissue might be effectively removed by deliberate infection with a non-pathogenic organism having marked proteolytic properties. The " Reading " bacillus of Donaldson[8] was selected for the purpose but was found to be not entirely satisfactory. Other proteolytic enzymes of non-bacterial origin were naturally sought for and preliminary investigations with pepsin, trypsin and papain were carried out[7].

The enzyme preparations used in these earlier experiments had, natur-

ally, not been prepared in accordance with modern pharmaceutical practice. For example the technique used to ensure sterility of the papain would not now be regarded as adequate. There was thus some doubt as to whether the digestion of the necrotic tissue was due to the papain or to proteolytic bacteria living in the papain solution as substrate.

The object of the present investigation was, therefore, to obtain highly active preparations of pancreatin and papain, sterile, and containing such a quantity of bactericide as would be capable of preventing the multiplication of bacteria in the root canals of the teeth. It was hoped that such preparations would liquefy the necrotic tissue which could be drained away leaving a sterile, clean cavity for the subsequent operation of root filling. Although the subject is complex it is generally agreed that non-living and denatured proteins are more readily attacked by proteolytic enzymes than are living and native proteins respectively[6]. Thus undamaged living tissue at the apex of the tooth should suffer relatively little from attack by the active enzymes.

Although only papain had so far given promising results it was decided to include pancreatin in the present investigation because a pancreatin of known very high tryptic activity was available[9].

Experimental

Methods and Materials. The assay processes used were those previously described for pancreatin[9] and for papain[10].

The *pancreatin* employed was that previously described as A[9]; while the papain was that referred to as sample E[10].

The *filter papers* used throughout the work were Whatman Number 1. The *cysteine hydrochloride* was of commercial quality. Solutions of 0·5 per cent. strength were made by dissolving 0·5 g. of cysteine hydrochloride in about 10 ml. of water, neutralising with N sodium hydroxide to *p*H 7·0 using bromothymol blue and making up to volume. For convenience such solutions are usually referred to as being 0·5 per cent. solutions of cysteine. Other strengths were prepared similarly.

Attempted Sterilisation by Heat. Since enzymes are protein in nature and proteins are denatured by heat it was not to be expected that enzyme solutions could be sterilised by heat. As shown previously, when trypsin solutions are heated at 55°C. there is a loss of more than half the activity in 40 minutes[9]. Over 55°C. heat inactivation becomes rapid being complete in 1 minute at 100°C. Papain is notably a heat-resisting enzyme but, as previously shown, heat destruction became rapid when solutions were heated at over 80°C.[10], being complete in a few minutes at 100°C.

Although enzymes have been shown to possess much greater heat resistance in the dry state it has not been found possible to sterilise the powders by dry heat. As previously shown, lipase[11] even in the form of dry powder is destroyed in an hour between 120°C. and 130°C. One hour at over 130°C. is necessary to kill spores of *Bacillus subtilis*[12]. Further experiments have shown that, although heating the dry powder at 80°C. for one hour has no effect on the tryptic activity of pancreatin

one hour at 120°C. results in the destruction of over 50 per cent. of the activity. One hour at 150°C. causes charring of the pancreatin. Although papain is said to be a very heat-resistant enzyme, heating for one hour at 130°C. reduced the assay titration from 6·0 to 4·4 corresponding to approximately 60 per cent. reduction in activity.

Many years ago Schmidt[13] stated that if trypsin powder were suspended in water-free glycerol it could be heated to 292°C. without being destroyed or indeed much affected. De Souza[14] in 1911 and Ohta[15] in 1912 both failed to confirm this observation. To investigate the possibility of heat sterilisation of a glycerin suspension, pancreatin in very dry powder was suspended in glycerin which had been purified by fractionation under reduced pressure. As the result of several experiments it was concluded that heating for one hour at 70°C. completely inactivated the suspension.

For the above reasons it was decided to abandon heat treatment as a means of sterilisation for proteolytic enzyme preparations.

Sterilisation by Filtration

The Doulton Filter. The effects of filtering a 0·3 per cent. solution of pancreatin in chloroform water through a Doulton filter are shown in Table I.

TABLE I

EFFECT OF FILTERING A 0·3 PER CENT. SOLUTION OF PANCREATIN THROUGH A DOULTON FILTER

	Assay titration. 0·1 N sodium hydroxide
	ml.
Before filtration	5·1
After filtration	1·7

In conjunction with a suitable graph relating assay titration with enzyme quantity the results indicate that only about 1/7 the original activity remained in the filtrate.

In the case of papain equal parts of a 1 per cent. solution of the enzyme and a previously neutralised 1 per cent. solution of cysteine hydrochloride were mixed. After clarification by filtration through paper the solution, now containing 0·5 per cent. of papain and 0·5 per

TABLE II

EFFECTS OF FILTERING CYSTEINE SOLUTIONS AND PAPAIN SOLUTIONS AND MIXTURES OF THE TWO

Solution assayed	Assay titration. 0·1 N sodium hydroxide
	ml.
Papain-cysteine solution before filtration	6·0
Papain-cycteine solution after filtration through paper	6·0
Papain-cysteine solution after Doulton filtration	0·3
Unfiltered cysteine solution plus Doulton-filtered papain solution	0·6
Unfiltered papain solution plus Doulton filtered cysteine solution	5·6

cent. of cysteine, was passed through a clean sterile Doulton filter. The first 3 results of Table II show that the activity was mainly lost in the process.

The remaining figures in Table II show clearly that the loss of activity was due to retention of the enzyme, and not the cysteine, by the filter. In these experiments either the 1 per cent. papain or the 1 per cent. cysteine solution was filtered prior to being mixed with an equal volume of the complementary unfiltered solution.

The Seitz Filter. The results recorded in Table III show that filtration of a 0·3 per cent. solution of pancreatin in chloroform water through a Seitz filter causes little more loss of activity than filtration through an ordinary filter paper.

TABLE III

EFFECTS OF FILTERING A 0·3 PER CENT. SOLUTION OF PANCREATIN THROUGH A SEITZ FILTER

Pancreatin solution	Assay titration. 0·1N sodium hydroxide
	ml.
Before filtration	5·1
After filtration through paper	4·9
After filtration through Seitz filter	4·8

Repeated sterility tests have confirmed the fact that such pancreatin solutions can be satisfactorily sterilised by Seitz filtration.

With papain the results were not quite so satisfactory. The results shown in Table IV were obtained by filtering a solution containing 0·5 per cent. of papain and 0·2 per cent. of cysteine through a Seitz filter. Much of the activity was removed by the filtration, but an appreciable amount could be restored by the addition of a further quantity of cysteine.

TABLE IV

EFFECTS OF SEITZ FILTRATION ON THE ACTIVITY OF 0·5 PER CENT. PAPAIN, 0·2 PER CENT. CYSTEINE SOLUTIONS

Solution assayed	Assay titration. 0·1N sodium hydroxide
	ml.
Unfiltered solution	5·1
Solution after filtration through paper	5·0
Solution after Seitz filtration	0·3
Seitz filtration and additional cysteine to a final concentration of 0·5 per cent.	3·0

The percentage of cysteine was, therefore, increased to 0·5 per cent. and an attempt was made to see if the filter could be caused to become saturated with active material. The results are shown in Table V.

The improved results are partly due to the increased percentage of activator, but it is clear that a more active filtrate is obtained when the filter pad has become saturated with the enzyme.

TABLE V

EFFECTS OF SEITZ FILTRATION ON THE ACTIVITY OF A SOLUTION OF 0·5 PER CENT. OF PAPAIN AND 0·5 PER CENT. OF CYSTEINE

Solution assayed	Assay titration. 0·1N sodium hydroxide
	ml.
Unfiltered solution	6·0
Filtered solution	
1st 25ml. filtrate	2·5
2nd ,, ...	4·1
3rd ,, ...	5·0

The 5/3 Sintered Glass Filter. The results of filtering 0·15 per cent. solution of pancreatin and 0·5 per cent. papain—0·5 per cent. cysteine solution through a 5/3 sintered glass filter are shown in Table VI.

TABLE VI

THE RESULTS OF FILTRATION THROUGH A 5/3 SINTERED GLASS FILTER

	Assay titration. 0·1N sodium hydroxide
	ml.
0·15 per cent. pancreatin solution :—	
Before filtration	4·9
After filtration through paper	4·8
After filtration through 5/3 sintered glass filter	4·7
0·5 per cent. papain—0·5 per cysteine :—	
Before filtration	6·0
After filtration	5·9

The chief drawback of this method is the very slow rate of filtration. It is also difficult to clean the glass filter at the end of the process.

THE ACTION OF BACTERICIDES ON THE PROTEOLYTIC ACTIVITY OF PANCREATIN AND PAPAIN

When in use in the pulp cavity, enzyme preparations will be at body temperature and, under such conditions, must retain their proteolytic activity for at least several hours, preferably for several days. At the same time the multiplication of bacteria must be inhibited and if possible a condition of sterility attained. It was necessary, therefore, to ascertain what bactericide at concentrations sufficient to control bacterial multiplication, would be without a deleterious effect on the enzymes at 37°C.

Chlorocresol. The resistance of pancreatin and papain to 0·2 per cent. of chlorocresol is shown in Table VII.

Phenylmercuric Nitrate. 10-ml. quantities of a 3 per cent. solution of pancreatin containing 0·002 per cent. of phenylmercuric nitrate were stored at 37°C. and 60°C. for various times. At the conclusion of storage each 10-ml. quantity was diluted to 100 ml. with water and the resultant solution assayed for proteolytic activity. The results are shown in Table VIII.

TABLE VII

EFFECTS OF 0·2 PER CENT. CHLOROCRESOL ON THE ACTIVITY OF SOLUTIONS OF PANCREATIN AND ACTIVATED PAPAIN AT 37°C.

Solution stored at 37°C.	Time of Storage	Assay titration 0·1 N sodium hydroxide	
		Before storage	After storage
		ml.	ml.
0·15 per cent. of pancreatin in distilled water	24 hours	4·9	3·9
0·15 per cent. of pancreatin in chloroform water	24 „	4·9	3·2
0·15 per cent. of pancreatin and 0·2 per cent. of chlorocresol...	24 „	4·0	2·4
0·5 per cent. of papain and 0·5 per cent. of cysteine	24 „	5·6	5·6
	48 „	5·6	5·6
	5 days	5·6	5·6
0·5 per cent. of papain and 0·5 per cent. of cysteine and 0·2 per cent. of chlorocresol	24 hours	5·6	5·6
	48 „	5·6	5·6
	5 days	5·6	5·1

TABLE VIII

EFFECTS OF 0·002 PER CENT. OF PHENYLMERCURIC NITRATE ON THE TRYPTIC ACTIVITY OF A 3 PER CENT. SOLUTION OF PANCREATIN

Phenylmercuric nitrate	Time of heating	Temperature	Assay titration 0·1N sodium hydroxide
per cent.		°C.	ml.
Nil	Nil	—	5·1
0·002	24 hours	37	5·1
Nil	1 hour	60	3·7
0·002	1 „	60	3·7

The action of phenylmercuric nitrate on papain was not investigated.

Creosote and Cresol. These two bactericides are widely used in dental practice, and it was considered necessary to determine whether, in saturated aqueous solution, they had any deleterious effect on the proteolytic enzymes. In the earlier experiments with pancreatin, creosote was used, while in the later papain experiments the action of the perhaps more popular cresol was examined. The effect of incubating at 37°C. a

TABLE IX

EFFECTS OF A SATURATED AQUEOUS SOLUTION OF CREOSOTE ON THE TRYPTIC ACTIVITY OF PANCREATIN IN 3 PER CENT. SOLUTION AT 37°C.

Solution	Time of storage	Assay titration 0·1N sodium hydroxide
		ml.
3 per cent. of pancreatin without creosote...	0 hour	5·0
	24 hours	4·4
3 per cent. of pancreatin in water saturated with creosote	1 hour	5·0
	24 hours	4·3

3 per cent. pancreatin solution made with water saturated with creosote at 37°C. for varying periods of time as shown in Table IX. Before the assay the solution was diluted 10 times.

In a similar experiment a saturated solution of cresol in water was shown to have no significant deleterious effect on 0·5 per cent. solution of papain in the presence of 0·5 per cent. of cysteine in 24 hours at 37°C.

The Preparation of Sterile Enzyme Solutions for Clinical Use

In view of the experiments described above it was decided to sterilise the pancreatin solution by filtration through a Seitz filter, but to employ the 5/3 sintered glass filter for the papain-cysteine solutions.

In both cases trouble was caused by frothing and by slow filtration. To some extent these difficulties were overcome by using the apparatus shown in Figure 1, which is an adaptation of that described by Abrahamson[16]. Either a Seitz filter or a sintered glass filter can be fitted to the adapter. Frothing is reduced, and such froth as is formed does not tend to be drawn out of the receiver by the pump. Further, when the pressure in the receiver has been sufficiently reduced the suction may be cut off suddenly when the valve will close. Provided that the valve is efficient, the filtration system may then be disconnected from the pump when filtration will continue satisfactorily for several hours without any attention.

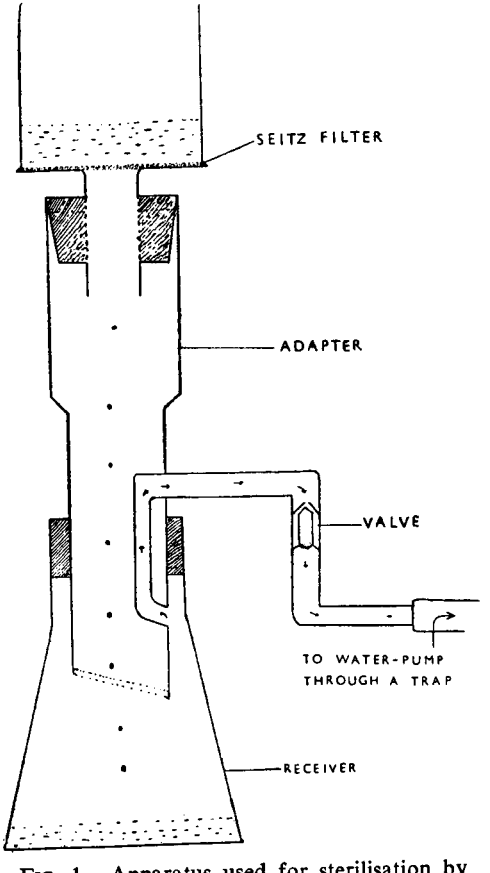

FIG. 1. Apparatus used for sterilisation by filtration.

At this stage the experiments with pancreatin were discontinued because it had been found that solutions of varying pancreatin between 1 per cent. and 10 per cent. did not, in the presence of a bactericide, prove capable of digesting the necrotic pulp tissue of split extracted teeth. This failure of *in vitro* experiments indicated the futility of preparing pancreatin solutions suitable for clinical trial.

On the contrary, the *in vitro* experiments with papain were much more promising, and so attempts were made to prepare ampoules containing sterile papain solutions of high proteolytic activity. It is difficult to obtain clear

solutions of papain of high strength. Solutions of 3 per cent. strength are cloudy and can be obtained clear only after filtration through several filter papers.

Cysteine presents a further difficulty. By careful filtration through several filter papers a clear solution containing 4 per cent. of papain and 2 per cent. of cysteine was obtained but on standing for a few hours a precipitate separated out which could be traced to the cysteine. Filtration through a 5/3 sintered glass filter proved impossible owing to practically complete blockage of the filter. On standing overnight even a 0·5 per cent. solution of cysteine will deposit. Possibly the oxidation of the cysteine to cystine plays a part in the process. Fortunately, the tendency of cysteine solutions to deposit appears to be reduced a little by the presence of papain, but it has not proved possible to obtain stable solutions containing more than 4 per cent. of papain and 0·5 per cent. of cysteine.

For clinical trial, solutions containing 2 or 3 per cent. of papain and 0·4 or 0·5 per cent. of cysteine respectively were employed. In practice such solutions contained 0·2 per cent. of chlorocresol as well. They were clarified by filtration through several filter papers and sterilised by filtration through a 5/3 sintered glass filter in the apparatus described above. Finally the sterile solution was distributed aseptically into 2 ml. sterile glass ampoules in the usual way. The ampoules were finally sealed.

The resultant preparation was repeatedly shown to possess high proteolytic activity which in a number of cases was proved to remain undiminished on storage for at least 2 months in a refrigerator. Repeated tests confirmed the sterility of the contents of the ampoules prepared in this way.

The Preparation of Sterile Active Powders of Pancreatin and Papain

The procedure to be employed in order to obtain a sterile powder from a sterile solution by spray drying has been fully described by Bullock and Lightbown[17]. By spray-drying and with aseptic precautions a sterile solution of pancreatin, prepared as described above, a powder was obtained. This was proved to be sterile, and to possess a tryptic activity identical with that of the original powder, no detectable loss of activity having occurred during the drying process.

In the case of papain a solution containing 3 per cent. of papain and 0·5 per cent. of cysteine was sterilised by filtration through a 5/3 sintered glass filter. The filtrate was spray dried using aseptic precautions and with an air inlet temperature to the dryer of 85° C. The resultant powder was proved to be sterile and to contain the full activity of the original papain. Some of the powder was supplied to the Dental Department for use as a paste.

Discussion

The sterilisation of enzyme solutions or even the maintenance of sterility in such solutions is always a problem of theoretical as well as practical interest. The metabolic processes of bacteria are mainly brought

about by the mediation of enzyme systems. Both the metabolic enzymes and the enzymes to be sterilised are protein in nature and the problem is so to damage the former that life is impossible and yet to leave the latter practically unaltered.

The first section of this paper indicated that neither moist nor dry heat is a suitable means of attaining this differential inactivation. It has been abundantly confirmed that 1 hour at 150° C. is necessary for sterilisation by dry heat; as shown above, 1 hour at 130°C. is sufficient to inactivate to a considerable extent both trypsin and papain even when in the form of dry powders. Table VIII indicates that " heating with a bactericide " could not be used as a method of sterilisation. Although at 37° C. 0·002 per cent. of phenylmercuric nitrate had no effect on trypsin in 24 hours; at 60° C. the assay titration fell from 5·1 to 3·7, indicating about 50 per cent. destruction in 1 hour. This loss in activity was in fact due to the higher temperature; being the same in the absence of the bactericide. 0·2 per cent. of chlorocresol at 60° C. reduced in 30 minutes the assay titration from 5·1 to 0·9.

Tables I and II show that filtration through a Doulton filter is not a satisfactory means of sterilising the enzyme solutions, most of the papain or trypsin being retained by the filter. Pancreatin solution may be sterilised by filtration through either a Seitz or a 5/3 sintered glass filter (Tables III and IV) little loss in activity resulting in either case. Seitz filtration was preferred because of the more rapid rate of filtration and the difficulties involved in cleaning the glass filter. With papain the Seitz filter is not so satisfactory (Tables IV and V). Much of the enzyme is retained until the Seitz pad becomes saturated with enzyme when, of course, the rate of filtration is much reduced. Filtration through a 5/3 sintered glass filter was better (Table VI) and this method was chosen for the larger scale work. It should be mentioned that the filtrates from the Seitz and 5/3 sintered glass filters were repeatedly found to be sterile when submitted to the sterility tests of the British Pharmacopoeia.

It appears from Table VII that 0·2 per cent. of chlorocresol has a considerable tendency to inactivate trypsin. Papain is much more resistant and the papain-cysteine solution had lost little activity after 5 days storage at body temperature. 0·002 per cent. of phenylmercuric nitrate was without effect on trypsin in 24 hours at 37°C. while cresol in saturated aqueous solution was without effect on papain under similar conditions. The inability of the pancreatin preparations, although of high tryptic activity, to digest the necrotic tissue even in *in vitro* experiments was confirmed. This difference in the digestive power of pancreatin and papain might be worthy of further investigation since it may indicate a fundamental difference in the affinities of the enzymes involved.

The papain and cysteine solution can be successfully sterilised in bulk without loss of activity by filtration through a 5/3 sintered glass filter and will retain the activity when distributed in sealed ampoules and stored in the refrigerator for at least 2 months. A sterile powder of papain and cysteine may be obtained by spray drying the solution. The dry powder

retained its activity for several months even when stored at room temperature.

The papain preparations were very effective *in vitro*. In clinical trials there has been some difficulty with gas formation in the temporarily sealed tooth cavity. The trials are still in progress. Meanwhile, it was thought that it would be of interest to pharmacists and others concerned with the behaviour of enzymes and micro-organisms to record the information obtained in preparing the sterile enzyme solutions containing a bactericide.

It is with great pleasure that we acknowledge the help and collaboration which we have received from Professor F. C. Wilkinson, Dr. J. K. Holt, and other members of the Dental Department of this University, particularly Miss Barbara Clough, now Mrs. Torr, who spent much time in the pharmacy department doing preliminary experiments on the assay and purification of papain.

SUMMARY

1. The effects of heat on the proteolytic activity of solutions and powders containing trypsin or papain are discussed.

2. The effects of various antiseptics and filtration through various types of filters on the proteolytic activity of solutions containing pancreatin and papain are recorded.

3. A description is given of a method of preparing ampoules containing a sterile solution of papain activated by cysteine and containing a bactericide.

4. The preparation of sterile powders of pancreatin, and of papain and cysteine is described.

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DISCUSSION

The paper was presented by DR. BULLOCK.

MISS V. W. BURRELL (Greenford) asked for details of the filter candle used.

MR. A. R. G. CHAMINGS (Horsham) asked whether the author con-

sidered that a preparation containing iodochloroxyquinoline, which had been found of use in dentistry, would be useful for its bacteriostatic properties.

DR. G. FOSTER (Dartford) suggested that, as the enzyme was heat labile, it might be preferable to use a freeze-dried rather than a spray-dried preparation.

DR. BULLOCK, in reply, said he was unable to furnish any special information about the filter candles; they were the normal Doulton type. He had no knowledge about iodochloroxyquinoline. Urea could be used with advantage to bring about the denaturation of the proteins. Freeze drying had not been used and offered little advantage, since loss of enzyme potency from spray drying was almost nil.