THE TRYPTIC ACTIVITY OF PANCREATIN
A CRITICAL STUDY OF SOME ASSAY PROCESSES AND STANDARDS

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INTRODUCTION

THE official process for the assay of trypsin in pancreatin suffers from certain serious defects. The chief defect, which in fact renders the results obtained by the present process almost valueless as a quantitative estimate of tryptic activity, is the presence of active enzyme in the blank. Experiments were therefore undertaken with a view to devising an amended process, really a rationalisation of the present process. These experiments indicated that the standard for tryptic activity required by the present British Pharmacopœia is several times higher than that required by the B.P. 1932. This led to the examination of a number of commercial samples of pancreatin with a view to deciding which standard is most suitable. The position was found to be complicated by the fact that manufacturers of weaker pancreatins are apparently in the habit of adding anything up to 80 per cent. of lactose before selling the material as pancreatin B.P.

The work described in the present paper may thus be classified under three headings.

1. A critical examination of the B.P. 1948 assay process with suggested amendments.

2. A comparison of the results obtained by assaying several samples of pancreatin for tryptic activity by the 1932 process, the 1948 process and the amended 1948 process.

3. An examination of the strength of commercial samples of pancreatin and an estimate of the strength which they would have possessed if they had not received an addition of lactose. The assay process for amylase is the same in the 1932 and 1948 Pharmacopœias. In a previous paper[1] it was shown that it is tryptic activity which limits the degree to which a sample of pancreatin may be diluted and still comply with the official standards for the three enzymes. This was so using the 1948 B.P. standard for lipase and the 1932 standard for trypsin. It is much more so if the 1948 or amended 1948 standard for trypsin is employed. For these reasons the following work is confined to the assay of trypsin and all methods, results, standards, etc., refer to that enzyme unless otherwise mentioned.

When trypsin is determined by methods involving formol titration three titration figures may be recorded. For purposes of clarity these three types of result will be given the following names in this paper. I. The quantity of alkali required to adjust the mixture to pH 8·7

(*p*H 7·0, or pink to phenolphthalein as the case may be) after the digestion period and immediately before addition of the formaldehyde will be called the "neutralising titration." II. The quantity of alkali required to bring the mixture back to *p*H 8·7 (or pink to phenolphthalein) after addition of the formaldehyde will be called the "formol titration." III. The difference between the formol titration of the test and that of the blank will be called the "assay titration."

Experimental—Choice of Methods of Assay

Many methods have been used for the determination of tryptic activity. They may be classified on the basis of two considerations:—

1. The nature of the protein used as substrate. 2. The choice of the method for estimating the degree of digestion. The quantity of unchanged protein may be determined by precipitation and either matching of the resultant turbidity or filtering off and weighing, or filtering and estimating total nitrogen in the precipitate. Alternatively the -COOH groups or $-NH_2$ groups liberated during the digestion may be estimated chemically or the concentration of one particular amino acid liberated may be estimated, for example, colorimetrically.

The choice of casein as substrate in the official assay is a good one. It is easy to obtain and pancreatin is often used to "peptonise" milk.

The U.S.P. gives conditions so that at the end of digestion there should be no precipitation with acid; all the casein must have been changed. This is a vague end point. Turbidity methods also are relatively difficult and of low accuracy. Northrop[2] estimated either the weight or total nitrogen content of unchanged casein precipitated by acid. These methods are essential where the kinetics of the digestion are being studied but are lengthy tedious processes for simple assay work.

Of the colorimetric processes for determining a liberated amino acid that of Anson[3] has been widely used and highly praised. The difficulty is that the preparation of the substrate, hæmoglobin, is involved, and the product has to be carefully preserved so that the method is not very suitable for use as an official process.

Willstätter's[4] well-known method of determining -COOH groups liberated during digestion uses casein as substrate and necessitates the use of considerable quantities of alcohol. It does not seem to have any outstanding advantages over the formol titration method using casein as substrate and estimating the acidity released by blocking with formaldehyde the $-NH_2$ groups liberated by digestion. For these reasons only assay processes using this last method have been investigated in the work described in this paper.

Materials Used

Pancreatin. The various commercial samples of pancreatin examined were labelled as follows:—A1, A2, A3, from the same firm, Pancreatin; B. Pancreatin Triple B.P.; C. Pancreatinum B.P.; D. Pancreatinum B.P.; E. Triple Pancreatin B.P.; F. Pancreatin Triple U. S. P.

Casein. Of several samples of commercial casein examined none complied with the requirements of the 1948 Pharmacopœia. A sample of casein complying with the B.P. specification prepared by a slight modification of the method described in Biochemical Preparations[5] was very kindly supplied by Messrs. Benger's Food, Ltd., but it is at present not available on the market. Under these circumstances most of the work was carried out with B.D.H. "light white soluble casein." It is difficult to adjust the slightly turbid blank to *p*H 7·0 in the B.P. 1948 process but in the ammended process, using only phenolphthalein as indicator, B.D.H. casein gave perfectly satisfactory results. These results agreed exactly with those obtained by using the above specially prepared soluble casein over a wide range of pancreatin strengths. It seems a pity to specify a type of casein requiring special preparation when an available commercial brand is satisfactory.

A Critical Study of the 1948 B.P. Assay Process

1. *The Blank.* In the B.P. 1948, process there is no indication whatever that the blank should be titrated at once, on the contrary, by inference, it is kept at "laboratory temperature" for 20 minutes while the digest is being kept at 55°C. It soon became apparent that considerable digestion was occurring during the 20 minutes at laboratory temperature. To determine the extent of this digestion 5 experiments were carried out. One was at room temperature with previously boiled enzyme solution. In the other four, unboiled enzyme solution was used and the digests were kept for 20 minutes at 0°C., 17°C. (winter room temperature), 24°C. (summer room temperature) and 55°C. Table I shows the results obtained.

TABLE I

Digestion occurring in the test compared with that in the blank kept under varying conditions. Each digest and blank contained either 8 or 25 mg. of pancreatin A. All figures are ml. of 0·1N sodium hydroxide

		8 mg.			25 mg.		
Temperature °C.	Enzyme solution	Neutralising titration	Formol titration	Assay titration	Neutralising titration	Formol titration	Assay* titration
0	Unboiled	0·3	2·75	0·15	0·9	3·6	0·9
17	,,	1·0	3·1	0·5	2·2	5·1	2·4
24	,,	1·4	3·65	1·05	2·6	5·7	3·0
55	,,	3·5	7·4	4·8	4·6	9·3	6·6
20	Boiled	0·0	2·6	0·0	0·0	2·7	0·0

* Calculated using the boiled enzyme experiment as blank.

Unless the enzyme solution has previously been boiled, some digestion occurs during the 20 minute period even at 0°C. This degree of digestion increases with increasing temperature and is, of course, greater with

stronger pancreatins. The extent of digestion in the blank at room temperature may be more than one third that of the true assay titration and more than one half of the assay titration using as blank the mixture kept at 17°C. for 20 minutes. The difference in the blank at winter and at summer temperatures may be equivalent to 10 per cent. of the true assay titration or 16 per cent. of the result in the B.P. 1948 assay process. Since the formol titration of the casein solution alone, used in each digest, amounts to 2·2 or 2·3 ml. of 0·1N sodium hydroxide these difficulties can all be overcome by using previously boiled enzyme solution in the blank. If this is done no digestion occurs in the blank so that the conditions of storage are unimportant.

To stress the importance of this it may be mentioned that on one occasion 15 mg. of a pancreatin gave an assay titration of 5·0 while 30 mg. gave an assay titration of 5·2 ml. although the corresponding neutralising titrations were 8·5 and 12·0 and the formol titrations 13·5 and 17·2 respectively. This illustrates how very misleading results by the B.P. 1948 method can be.

2. *The use of neutral formaldehyde solution.* In the B.P. 1932 the solution of formaldehyde used was directed to be "previously neutralised to phenolphthalein." In the 1948 process this direction is omitted, presumably because the digest and blank are now adjusted before the formol titration to *p*H 7·0 and the official solution of formaldehyde is required to be "neutral or slightly acid to litmus." To bring 10 ml. of a commercial sample of Liquor Formaldehydi to *p*H 8·7 was found to require 0·8 ml. of 0·1N sodium hydroxide. Since this quantity is variable and increases the blank unnecessarily it was decided that in the modified process the requirement that the formaldehyde should be previously neutralised should be reintroduced.

3. *Variation in the degree of digestion with time and quantity of enzyme.* Schütz's[6] Law states that $x = k \sqrt{E}\, t$, where E is the quantity of enzyme present, at the time of digestion and x the resultant degree of digestion in that time. Schütz's Law was first shown to apply to tryptic digestion by Borissov[7]. Northrop[2] verified that the Schütz Law applies to most of the range of the digestion of casein by trypsin. Northrop[2] and Moelwyn Hughes[8] have discussed the derivation of Schütz's Law from the first order reaction equation provided that the following assumptions are made. 1. That the enzyme has considerable affinity for the products of digestion. 2. That the concentration of the products of digestion is large with respect to the quantity of enzyme. 3. That during the period under consideration the concentration of the substrate does not appreciably change. It is obvious that during the early part of the reaction condition 2 is not fulfilled while in the later stages of the reaction condition 3 is not fulfilled. Hence Schütz's constant k tends to fall off at the beginning and end of the reaction. It will be shown in the next section that a fall in *p*H accompanies digestion in the official process and this also accounts for some deviations from Schütz's Law.

The time course of the reaction is plotted in Figure 1. The fact that a plot of the assay titration against the square root of the time is almost a straight line shows how closely the modified form of Schütz's Law $x = k\sqrt{t}$ is followed.

The relationship between assay titration and quantity of enzyme in the digest for a 20-minute period of digestion is shown in Figure 2. Again Schütz's Law in the form $x = k\sqrt{E}$ is verified for all but the lower and higher assay figures since the plot of x against $\sqrt{E}$ is practically a straight line.

4. *The Effect of pH on Digestion by Trypsin.* The optimum *p*H for trypsin acting on casein has been shown by Northrop[9] to be in the neighbourhood of 8·6. The problem is complex for several reasons. Trypsin is probably not a single enzyme. Further the *p*H has at least two, in fact opposing, effects. As already mentioned Pace[10] has shown that the stability of trypsin decreases rapidly with increase of *p*H so that it becomes markedly unstable at *p*H values over 10. On the other hand, Northrop[11] has shown that trypsin reacts with negatively charged protein ions. If therefore the experiments are designed so that the alkali destruction of trypsin is not

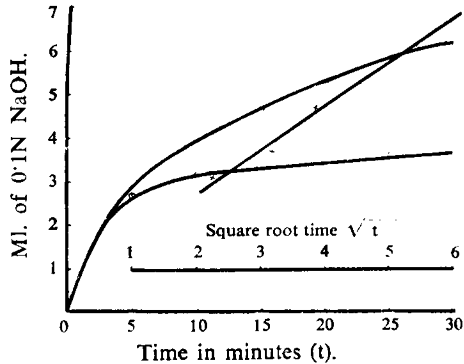

Fig. 1. Titration-time Curves of the Digestion of Casein by Trypsin. Top curve, assay titration against time; middle curve, assay titration against square root of time; lowest curve, neutralising titration against time.

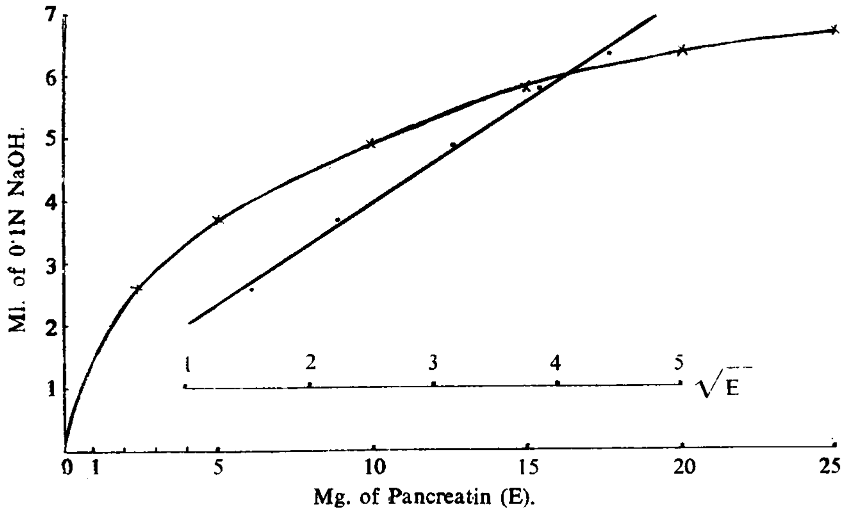

Fig. 2. Empirical Curve for Comparing Strength of Pancreatin.

a limiting factor the rate of digestion will increase with increasing *p*H and indeed will follow closely the titration curve of the casein. To find out the effects of these various factors on the process under consideration the *p*H of a number of solutions was determined with the glass electrode.

Most of the figures refer to experiments using the high strength pancreatin A. 5 ml. of a 0·16 per cent. solution, i.e., 8 mg. of pancreatin, was used per digest as this gave an assay titration of about 5 ml., approximately the upper limit to be recommended. From the results shown in Table II it is clear that in the digest there is a rapid fall from the initial *p*H of 8·7, which becomes more gradual as digestion proceeds, reaching *p*H 7·02 or lower with large degrees of digestion. According to graphs given by Northrop[9] a fall of *p*H from 8·6 to 7·0 corresponds to a loss of 25 per cent. of the activity of trypsin acting on casein. This increase in acidity is reflected in the increasing "neutralising titration" as digestion proceeds.

TABLE II

*p*H OF SOLUTIONS AND DIGESTS (GLASS ELECTRODE)

Solution	*p*H
Pancreatin solution (0·16 Pancreatin A)	6·77
" " (0·5 per cent. Pancreatin D)	5·41
" " (0·5 per cent. Pancreatin C)	6·03
4 per cent. Casein solution (adjusted to *p*H 8·7 using phenolphthalein)...	8·82
Digest (Pancreatin A) kept at room temperature for 0·0 minutes	8·72
" " " " " 1 "	8·50
" " " " " 10 "	7·87
" " " " " 20 "	7·59
" (Pancreatin D) " " " 20 "	8·25
" (Pancreatin C) " " " 20 "	8·18
" (Pancreatin A) kept at 55°C. for ... 10 "	7·12
" " " " ... 20 "	7·02
" (Pancreatin A) 0·5 per cent. kept at 55°C. for 20 "	6·02

That the increase in acidity is not directly proportional to the extent of digestion as measured by the assay titration is clearly shown in Figure 1. The change of *p*H during digestion results in the higher potency pancreatins giving lower assay titrations than would be the case if the digest could be maintained at constant *p*H. It also accounts for some of the deviations from Schütz's Law. It is clear that pancreatin can be compared in strength only by constructing an empirical curve as in Figure 2.

It was found that 100 ml. of 0·16 per cent. pancreatin A required 1·75 ml. of 0·1N sodium hydroxide to raise the *p*H from 6·77 to 8·7, but when duplicate assays were carried out with the two solutions no difference was observed in the results.

Some difficulty was experienced in adjusting the 4 per cent. B.D.H. soluble casein solution to *p*H 8·7 colorimetrically. The resultant solutions varied from *p*H 8·62 to 8·84 by the glass electrode, but again duplicate assays with the two substrate solutions resulted in identical results.

5. *Adjusting to pH 7·0 or pH 8·7 before the formol titration.* In the B.P. 1932 process the formol titration was carried out in the usual way the digest being neutralised to phenolphthalein, the neutral formaldehyde solution added and the digest again titrated to phenolphthalein, the second titration being the formol titration. In the B.P. 1948 process this procedure was varied, by Evers and Smith[12] presumably as a result of a paper by Northrop[13]. The digest and blank are brought to *p*H 7·0 and the formaldehyde solution added. The formol titration is the alkali then required to bring the *p*H to 8·7. This means that the buffer value between 7·0 and 8·7 is added to the formol titration. The buffer value of the test differs from that of the blank.

An examination of Northrop's[13] paper shows that the variation was introduced owing to the difficulty of determining the exact end-point in the case of small quantities of aminoacids. To find the effect of the variation in the assay process the following experiment was carried out. An assay was set up using a double quantity of test and blank. At the end of the digestion period each 100 ml. quantity was divided into 2 parts resulting in 2 exactly equal pairs of test and blank. The members of one pair were brought to *p*H 7·0 before addition of formaldehyde, while those of the second pair were adjusted to *p*H 8·7 before addition of the formaldehyde. All were finally titrated to *p*H 8·7. The results are shown in Table III.

TABLE III

DIFFERENCES IN ADJUSTING TO *p*H 7·0 OR 8·7 BEFORE ADDITION OF FORMALDEHYDE

		Ml. of 0·1N Sodium Hydroxide		
		Neutralising titration	Formol titration	Neutralising* formol
Adjusted to *p*H 7·0	Test	*−0·05	12·1	12·05
	Blank	−1·0	3·7	2·7
Adjusted to *p*H 8·7	Test	4·95	7·0	11·95
	Blank	0·05	2·6	2·65

* A minus sign denotes the addition of 0·1N hydrochloric acid.

It will be seen that the formol titration of the B.P. 1948 is equal to the true formol titration plus the neutralising titration, i.e., it includes the acidity developed during digestion with two absolutely arbitrary alterations, (1) if the blank or digest has a *p*H above 7·0 alkali equivalent to the acid necessary to bring it down to 7·0 is added, (2) if the digest should be below *p*H 7·0 then the alkali required to bring it up to *p*H 7·0 is deducted. Either the neutralising titration should be excluded or it should be added, on the grounds that the acidity is in fact produced by trypsin, without alteration.

It may be mentioned that the quantity of alkali, 3·5 ml., required to change the digest from *p*H 7·0 to 8·7 was exactly the same in the presence

and in the absence of the formaldehyde, indicating that the formol titration at *p*H 7·0 does not include any free-NH_2 groups missed by a formol titration starting at 8·7.

The neutralising titration may, of course, include alkali required to neutralise acid produced by the action of lipase upon fat if the casein and pancreatin are not absolutely fat free. Some commercial caseins do contain up to 1 per cent. of fat.

There are also theoretical considerations opposed to the inclusion of the neutralising titration in the formol titrations. The formol titration was especially designed to measure acidity liberated by neutralising the basicity of the free amino groups by addition of formaldehyde and to exclude acidity or alkalinity of the digest which might result from other causes.

Further, an examination of Figure I shows that the neutralising titration falls off with the degree of digestion at an even greater rate than does the assay titration. To include it in the assay titration would therefore emphasise the deviations from Schütz's Law and render the assay titration still less proportional to the quantity of trypsin present.

TABLE IV

Process	Ml. of 0·1N Sodium Hydroxide		
	Neutralising titration	Formol titration	Assay titration
B.P. 1948 process. 15 ml. of casein solution 30 ml. of water 5 ml. of enzyme solution. Heated rapidly to 55°C. (1 minute taken) 20 minutes at 55°C. ...	3·2	7·2	4·6
15 ml. of casein solution 30 ml. of water 5 ml. of enzyme solution. Placed in bath at 55°C. 20 minutes (5 minutes required to attain 55°C.)	3·4	7·2	4·6
15 ml. of casein solution 30 ml. of water heated to 55°C. Added 5 ml. of enzyme solution and keep in bath at 55°C. 20 minutes. (Temp. fell to 52°C. on addition of enzyme solution, regained 55°C. in 3 minutes) ...	3·5	7·8	5·2

6. *Adjusting the Digest to 55°C.* The B.P. 1948 process directs that the digest shall be rapidly heated to 55°C. Rapid heating may be variously interpreted. If the process is relatively slow then a variable amount of digestion occurs during the heating, if it is really rapid, for example, by heating over a gauze directly heated by a bunsen, it appears likely, as suggested by the results in Table IV that some enzyme is destroyed in the thin layer of liquid in contact with the hot glass.

From Figure 3 it can be calculated that a drop of temperature of the digest from 55°C. to 52°C. for 3 minutes would cause a drop in the assay titration of just less than 0·1 ml. On the other hand, as shown in Table V, a solution of trypsin maintained at 55°C. in the absence of substrate loses strength relatively rapidly, more than corresponds to 0·1 ml. in the assay titration in 3 minutes.

It is advisable, therefore, to preheat the casein solution, but not the enzyme solution, to 55°C. before mixing.

7. *Heat Inactivation of Trypsin in the Absence of Substrate.* It has been shown by Pace[9] that at 50°C. trypsin-kinase is heat-inactivated at a rate in fair agreement with the unimolecular reaction equation, and that the rate of inactivation varies, not only with temperature, but also with *p*H. The time of half-decomposition in minutes at 50°C. was found to be 277 at *p*H 6·0, 300 at *p*H 7·0 and 128 at *p*H 8·5.

A 0·16 per cent. solution of pancreatin A was kept at 55°C. and 5 ml. quantities assayed after 20 and 40 minutes. The results in Table V show a quite rapid rate of inactivation. Even in 3 minutes the destruction would correspond to a drop of approximately 0·15 ml. of 0·1N sodium hydroxide in the assay titration.

TABLE V

HEAT INACTIVATION OF TRYPSIN IN PANCREATIN SOLUTION. BLANK IN ALL CASES 2·6 ML. OF 0·1N SODIUM HYDROXIDE

	Neutralising titration	Formol titration	Assay titration
Time of storage of pancreatin solution at 55°C. prior to assay :—			
0 minutes	3·4	7·2	4·6
20 ,, ...	2·2	6·1	3·5
40 ,, ...	2·0	5·1	2·5
Time of storage of pancreatin solution at 20°C. prior to assay :—			
0 minutes	3·6	7·5	4·7
20 ,, ...	3·6	7·5	4·7
60 ,, ...	3·6	7·5	4·7
120 ,, ...	3·6	7·5	4·7
Overnight in refrigerator	3·6	7·5	4·7
3 days in refrigerator	3·7	7·2	4·6

The relative stability of the solution of pancreatin at 20°C. and *p*H 6·8 may be compared with the instability of pure solutions of trypsin at *p*H 7·8 and 25°C. reported by Schwert[14].

8. *The Upper Limit to the Assay Titration.* It is generally agreed that enzymes are most accurately assayed by determining only the initial rate of reaction. For this purpose not more than 25 per cent. or at most 50 per cent. of the substrate should be used up; or with a complex process such as the one under consideration not more than 25 per cent. or at most 50 per cent. of the possible reaction, measured in this case by the assay titration, should occur. To determine this upper limit digestions

TABLE VI

DIGESTION BROUGHT ABOUT BY 25 MG. OF PANCREATIN A PER DIGEST

Time of digestion	Neutralising titration	Formol titration	Assay titration
0 minutes	0·0	2·6	0·0
5·5 hrs. at 55°C. plus 16·5 hrs. at 37° C.	5·8	14·0	11·4
3·5 hrs. at 55°C. plus 16·5 hrs. at 37° C.	5·4	12·6	10·0
Room temperature (20°C.) 19 hours	5·2	12·4	9·8

using the strong pancreatin A were carried out for several hours at 55° C. and then overnight in the incubator at 37° C. The results are shown in Table VI.

It is clear that the practical upper limit to the assay titration is not much above 12 ml. of 0·1N sodium hydroxide. This means that in assay work the assay titration should not be much above 3 or 4 and certainly not above 6 ml. To assay a pancreatin giving an assay titration over 6 the assay should be repeated using a smaller amount of pancreatin. The extensive degree of digestion occurring during the assay was confirmed by precipitating unchanged casein at *p*H 4·8 and weighing. For assay titrations of 3·0 and 5·0 only 10·6 per cent. and 5·2 per cent. respectively of the total casein remained unchanged at the end of the assay.

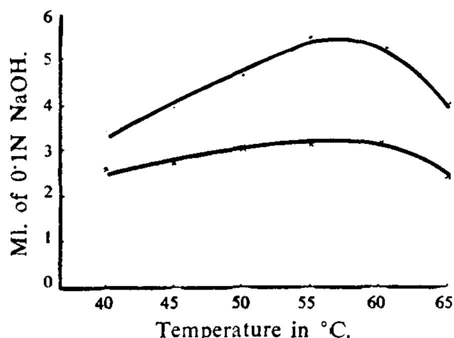

FIG. 3. Effect of temperature in the rate of heat inactivation of pancreatin. Upper curve, assay titration; lower curve, neutralising titration.

9. *Optimum temperature.* Owing to the complex nature of trypsin and the fact that a rise of temperature increases the rate of heat inactivation of the enzyme, the latter rate being also dependent on the *p*H, there is some confusion in the literature as to the optimum when using such crude preparations as pancreatin. The results shown in Figure 3 indicate that 55° C. is near the optimum for the modified official process with its 20 minutes digestion period.

MODIFIED ASSAY PROCESS FOR TRYPSIN IN PANCREATIN

Taking all the above findings into consideration the following modification of the B.P. 1948 process is recommended. Dissolve 4 g. accurately weighed, or purified casein in 90 ml. of water containing 1 ml. of 1·0N sodium hydroxide, adjust the *p*H of the solution to 8·7 and make up the volume to 100 ml. with water. In each of two flasks place 15 ml. of casein solution and 30 ml. of chloroform water, adjust the temperature to 55° C. and place in a water-bath at 55° C. To one flask add 5 ml. of a freshly prepared 0·15 per cent. solution of pancreatin unfiltered in chloroform water. To the other add 5 ml. of a portion of the same enzyme solution previously boiled and cooled. Maintain both flasks at 55° C. for 20 minutes. Cool rapidly to room temperature and to each flask add 0·75 ml. of solution of phenolphthalein 0·1 per cent. w/v. Bring both solutions to *p*H 8·7 by addition of 0·1N sodium hydroxide. Add 10 ml. of solution of formaldehyde previously neutralised to phenolphthalein to each flask and titrate both liquids with 0·1N sodium

hydroxide until the *p*H is again 8·7. The difference between the two titrations should not be less than 3·0 ml. and not more than 5·0 ml.

An Examination of Commercial Pancreatins by the B.P. 1932 Assay Process

In the first place a graph, Figure 4. was prepared relating quantity of enzyme with corresponding assay titration. This curve can now be used to compare the strengths of pancreatins with the B.P. limits or with each other.

To understand how the figures in column 4 of Table VII were obtained, suppose that x mg. of a given pancreatin gave an assay titration of 5·2; from the graph of Figure 4, this corresponds to 2·88 mg. An assay titration of 4 corresponds to 1·68 mg. Therefore the quantity of the given pancreatin which should give a titration figure of 4 is $x \times 8/2{\cdot}88$; this is the figure in column 4. The figures in column 5 were obtained by dividing 50 by the figures in column 4.

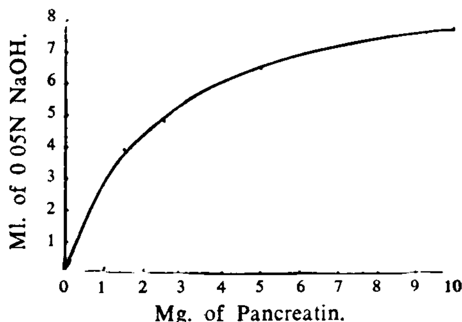

Fig. 4. Relation of the quantity of enzyme with the assay titration.

It will be seen in Figure 4 that an assay titration of 8 is on the flat part of the curve. Approximately 8·8 times as strong a pancreatin is required to comply with the upper limit of the B.P. as compared with the lower limit. Only pancreatins A1 and A2 exceeded this upper limit although F is about equal to it.

These limits appear to be very wide.

TABLE VII

Results of assaying 6 samples of commercial pancreatin. B.P. 1932 requires 50 mg. of pancreatin to give an assay titration between 4·0 and 8·0 ml. of 0·05N alkali. All results as ml. 0·05N sodium hydroxide

Pancreatin	Mg. in digest	Assay titration	Mg. pancreatin equivalent 4·0 ml. assay titration	Strength in terms B.P. limit = 1·0
A 1	1·5	3·9	1·54	32·5
A 2	5	5·2	2·8	17·9
B	20	4·2	18·3	3·1
C	50	6·1	20·0	2·5
D	50	4·7	36·5	1·4
E	20	4·7	14·5	3·45
F	5	3·3	7·12	7·0
F	10	5·2	5·85	8·5

THE ASSAY OF COMMERCIAL SAMPLES OF PANCREATIN BY THE B.P. 1948 PROCESS

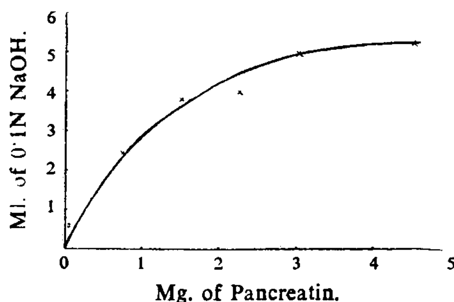

FIG. 5. Curve showing relation between the amount of pancreatin and the corresponding assay titration.

The curve relating mg. of pancreatin and the corresponding assay titration is shown in Figure 5. Using this graph as explained in the previous section the results of assaying 6 commercial samples of pancreatin are shown in Table VIII.

TABLE VIII

Pancreatin	Pancreatin in digest	Assay titration N/10 sodium hydroxide	Pancreatin equivalent assay titration 4·5 ml.	Strength in terms B.P. limit = 1·0
	mg.		mg.	
A 3	15	4·6	14·4	1·73
A 2	15	5·06	11·0	2·27
A 1	15	5·1	11·0	2·27
B	25	4·8	20·3	1·23
C	25	4·47	26·7	0·94
D	25	3·1	51·1	0·49

THE ASSAY OF COMMERCIAL SAMPLES OF PANCREATIN BY THE PROPOSED MODIFICATION OF THE B.P. 1948 PROCESS

The graph relating mg. of pancreatin per digest with the resulting assay titrations, using varying quantities of Pancreatin A only, is shown in Figure 6. A plot of the square roots of the quantities against the assay titrations is also shown and is approximately a straight line for assay titrations of 2·5 and over.

Lactose content and the true strength of commercial samples of Pancreatin. The Pharmacopœia at present allows the addition of lactose to commercial pancreatin in the preparation of pancreatin B.P. In discussing the quality of commercial pancreatins therefore two points are of interest, namely the strength of the pancreatins as manufactured and the strength of the pancreatins as sold. That the latter differ widely can clearly be seen from the first part of Table IX. There is, however, much less difference in the strengths of the pancreatins as manufactured. Table X shows the percentage of lactose in the various pancreatins

examined and also the results of assaying the samples so that the quantity of real pancreatin in each digest was 7·5 mg. (i.e.) an allowance has been made for the lactose present. From the second part of Table IX it will be seen that all except two of the pancreatins examined would have passed the proposed test provided that they had not received additions of up to 87·6 per cent. of lactose.

The Keeping Properties of Pancreatin. Three of the samples of pancreatin (A1, C and D) were retested after 12 months' storage in the manufacturer's container on a shelf in the laboratory. Three other samples (B, E and F) were retested after 6 months' similar storage. In all cases any deterioration was within the experimental limits

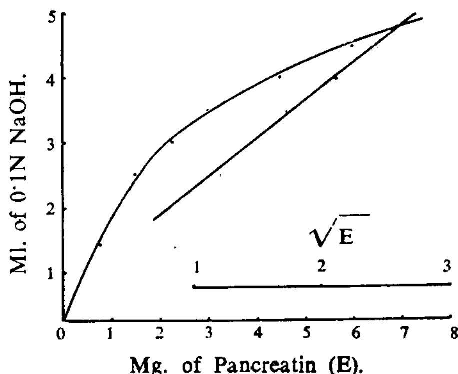

FIG. 6. Relation of the quantity and the square root of the quantity of pancreatin to the assay titration. Lower graph represents $\sqrt{E}$

TABLE IX

RESULTS OF ASSAYING COMMERCIAL SAMPLES OF PANCREATIN BY THE PROPOSED MODIFICATION OF THE B.P. 1948 ASSAY PROCESS

Sample No.	Pancreatin in digest	Assay titration	Pancreatin equivalent to assay titration of 3·0	Strength in terms of lower limit =1·00
	mg.		mg.	
A1	7·5	5·0	2·19	3·42
B	7·5	1·5	24·2	0·31
C	7·5	0·8	56·2	0·13
D	7·5	0·6	1·30	0·06
E	7·5	1·8	16·1	0·47
F	7·5	2·7	9·95	0·75

Sample No.	True pancreatin per digest	Assay titration	Pancreatin equivalent to titration of 3·0	Strength in terms of lower limit (3·0)=1·00
	mg.		mg.	
A1	7·5	5·0	2·19	3·42
B	7·5	3·5	5·1	1·47
C	7·5	2·5	10·05	0·71
D	7·5	3·1	7·25	1·03
E	7·5	3·4	5·63	1·33
F	7·5	2·7	9·95	0·75

of error of the method. It may be mentioned that the results recorded in Table IX were all obtained with the samples after storage.

TABLE X

LACTOSE CONTENT OF PANCREATINS AND TRUE STRENGTH OF COMMERCIAL PANCREATINS

Sample No.	Lactose	Pancreatin per digest	True pancreatin per digest	Assay titration
	per cent.	mg.	mg.	
A1	0·0	7·5	7·5	5·0
B	78·6	35·1	7·5	3·5
C	67·1	22·8	7·5	2·5
D	87·6	60·5	7·5	3·1
E	59·91	18·8	7·5	3·4
F	0·0	7·5	7·5	2·7

Lactose was estimated volumetrically using the method of Lane and Eynon in which methylene blue is used as indicator.

DISCUSSION

The B.P. 1932 assay process for trypsin in pancreatin had several faults. The digestion period of 1½ hours was unnecessarily long. More important, the use of fresh milk as a substrate was objectionable, not only because of the tedious nature of the preparative process but also on account of its notable variability in constitution. It might have been expected that variable results would be obtained from time to time and in different laboratories.

The B.P. 1948 process amended as described in this paper is a definite improvement.

It has, however, been shown that during a typical digestion the *p*H of the digest falls from *p*H 8·7 to anything down to *p*H 6. This results in a progressive reduction in the activity of any given quantity of trypsin. At the optimum temperature of 55°C. there must be a steady even, if slight, destruction of the enzyme during the digestion period, again resulting in a progressive reduction in tryptic activity. Further, it has been shown that at the end of a typical digestion considerably more than 50 per cent. of the substrate has been digested. Indeed, for a satisfactory formol titration to be obtained there must be, of necessity, an accumulation of end products of digestion. It is not therefore surprising to find that, as shown in Figures 1, 2, 5 and 6, neither the graph of the time course of the digestion nor the graph of the relationship between quantity of enzyme and resultant assay titration is a straight line. Instead Schütz's Law is approximately, but only approximately, followed. Thus there is no simple mathematical relationship between the assay titration and the strength of a pancreatin. From Figure 6 it can be seen that while 2·25 mg. of pancreatin A1 give an assay titration of 3·0 ml., 7·5 mg., 3·3 times as much, is required to give a result of 5·0 ml.

The first part of Table IX shows that there is an undue variation in the strength of commercial pancreatins, one sample is approximately 50 times as active as another. This difference is shown by the second part of the table to be due mainly to the practice of adding lactose before sale. It appears to be the manufacturers of the weakest pancreatins who add the largest quantities of lactose. Indeed, if dilution with lactose had not been practised the strongest pancreatin would have been not quite 5 times as strong as the weakest.

There would appear to be two valid reasons for permitting the addition of lactose to pancreatin. In the first place the diluted preparation might be more suitable on account of its texture or other properties or because the larger quantities per dose were easier to handle. In that case it would be advisable to fix a not very high upper limit to prevent the dispensing of the presumably too strong material. Secondly, it might be necessary to permit the addition of lactose if variations in the strength of pancreatin as manufactured were large and unavoidable. In this case all batches could be diluted to a uniform standard within narrow limits. There might be something to be said for this but, in practice, as shown in the second part of Table IX there is not a very great difference in the strength of pancreatins as manufacturerd by the different firms but instead of being reduced, the difference is very greatly emphasised by the addition of lactose to the weaker materials.

If no lactose were added the lower limit of 3·0 suggested in the amended B.P. 1948 test would exclude only the two worst pancreatins.

From a comparison of the last columns in Tables VII, VIII and IX several conclusions can be drawn. The standard of the B.P. 1932 was so low that all the samples of commercial pancreatin complied with it while the best sample was 32 times stronger than the minimum and 3 or 4 times as strong as the maximum.

The products of 2 makers out of 4 complied with the requirement of the B.P. 1948, the best sample being just over twice as strong as required.

With regard to the amended test, which is really a rationalisation of the B.P. 1948 process, only one commercial pancreatin complied, being about 3·5 times as active as the minimum and equal to the maximum. If, however, the other samples had not been mixed with lactose only the two worst would have failed to comply and even these would have possessed over 70 per cent. of the required activity.

The minimum standard of the amended process appears to be about 10 times as high as the minimum of the B.P. 1932 when pancreatins of reasonable strength (A1 and F) are used.

If we assume that the purchasers of pancreatin B.P. should receive mainly pancreatin and not mainly lactose, and that the standard fixed should be one with which manufacturers could readily comply, and that it is desirable that the product should not vary from sample to sample to an undue degree, then the standards of the amended process are better than those of either the B.P. 1932 or B.P. 1948.

SUMMARY

1. Certain faults in the B.P. 1948 assay process for trypsin are pointed out. The chief is the presence of active enzyme in the blank.

2. An amended process, actually a rationalisation of the present process, is suggested.

3. A comparison of the standards of the B.P. 1932, the B.P. 1948 and the amended process is made.

4. It is found that commercial samples of pancreatin would be mainly of high quality if the weaker samples had not been further reduced in strength by addition of up to 80 per cent. of lactose.

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