

wherein R, R<sup>1</sup> and R<sup>2</sup> = H or alkyl group, H<sup>2</sup> = H, OH, alkyl or alkyloxy, and H<sup>1</sup> and H<sup>3</sup> = H or OH. Four derivatives of indoethylamine, three those of phenyl-piperidyl-carbinols, and one that of phenyl-pyrazolone complete the list of substances investigated.

With an increase in the number of C atoms in R, R<sup>1</sup> and R<sup>2</sup>, the hyperglycemic action increases.

There is little correlation between the pressor action and hyperglycemic action as the chemical structure varies. They are often diametrically opposite.

When the structure of a compound approaches that of epinephrine, a small amount will be necessary to cause a distinct response of hyperglycemia. The epinephrine homologs also raise blood sugar by subcutaneous injection.

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LILLY RESEARCH LABORATORIES, INDIANAPOLIS.

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#### PANCREATIN AND ITS ASSAY.\*

BY F. E. WILLSON.

The U. S. P. X has definite standards for the trypsin and amylase content of pancreatin. The B. P. 1932, also, has definite standards for these two enzymes in pancreatin and in addition has a standard for lipase content. The methods of determining tryptic and amylase content differ materially in the two pharmacopœias and also the standards set do not compare very closely. For this reason it is interesting to compare the different methods and observe some of the difficulties met with.

*The U. S. P. Trypsin Method.*—According to the U. S. P. X trypsin test, pancreatin should convert not less than twenty-five times its weight of casein into soluble proteoses. Therefore in this particular assay casein is used as the substrate. The actual method employed is essentially that known as the Fuld-Gross (1), (2). A 0.2% solution of casein is prepared by the use of sodium hydroxide. A definite quantity of a solution of the pancreatin to be tested is added to a definite quantity of the casein solution. Digestion is allowed to proceed for one hour's time, after

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which the degree of digestion of the casein is determined by the addition of an alcohol-acetic acid mixture. (U. S. P. X, page 275.)

This particular method has been the cause of considerable criticism and controversy among those concerned with the evaluation of pancreatin. The cause of variations in results obtained in carrying out this test is chiefly accounted for by the indefiniteness of the end-point. This particular difficulty was pointed out in an earlier paper (3).

Another source of variance in results may be found in the sample of powdered casein used for the substrate. There is a considerable difference in the various caseins on the market. A number of samples we have tested failed to give a clear solution in an alkaline solution. They are therefore unsuitable for use. Also several samples of casein examined have had a decided alkalinity which also would make them unsuitable for the U. S. P. test, since a more definite hydrogen-ion concentration is necessary. Furthermore with the casein supply there is a possibility of variance in moisture content of the different lots of casein.

Still another point that should be observed in carrying out trypsin assays is that the pancreatin to be tested should be left in contact with water for at least one hour before test if the full tryptic activity is to be obtained.

From these difficulties pointed out it will be observed that the present U. S. P. method is not an entirely satisfactory method.

*The B. P. Trypsin Method.*—The B. P. 1932 employs a modification of the Smith-Sorenson method (4) for determining the tryptic activity. Fresh milk from which the cream has been removed is used as the substrate. In other words, fresh milk furnishes the casein supply. To ensure a constant amount of casein being present, directions are given for adjustment. The milk solution is also adjusted to a definite  $p_H$  after which the pancreatin solution is added and the digestion allowed to proceed for one and a half hours at 38° to 40° C. At the end of this time the digestion mixture is rapidly cooled to 20° C. and a formol titration carried out by which the amino acids formed during the digestion are titrated. A blank is run on the milk and a boiled sample of pancreatin for correction purposes. Definite limits have been adopted in terms of  $N/20$  sodium hydroxide solution for pancreatin to meet this tryptic standard.

The method, in general, seems quite satisfactory for use. Many factors which enter into the U. S. P. method are eliminated in this method. Furthermore, a definite end-point is obtained which cannot be interpreted differently by various operators.

There are several points in the B. P. method which may give a considerable variance in results. The first and most serious objection to the test is the use of fresh milk as the substrate. The opinion is expressed that milk does not furnish a substrate that is constant enough in composition to give perfectly satisfactory results. This is true even though the adjustments directed by the B. P. are carried out. The difference in the milk supply in different parts of the country was demonstrated during certain coöperative work carried out on rennin. Quite different results were obtained depending upon the section of the country the tests were carried out in and also in the source and processing the milk had gone through. This apparently was due to differences in the composition of the milk. The same conditions, it would seem, will apply to the B. P. test. Carrying out the test in

different parts of the country or even different seasons of the year are liable to give quite different results. This could be avoided by the use of powdered casein of specified purity which would have supplied a substrate of constant composition and would not have required the adjustment that the milk does before use.

*The Modified Smith-Sorensen Test.*—It has been pointed out that the present U. S. P. method of determining trypsin is far from satisfactory. The question then arises, how can the present method be improved or what methods are available for substitution? In our opinion the present method should be discarded and a new one substituted. In a previous paper (5) a modification of the Smith-Sorensen test was suggested. Since that time considerable work has been done on it in this laboratory as well as other laboratories. The results so far obtained indicate the method is satisfactory and at the same time not liable to the various sources of error that the present U. S. P. method contains. The method previously suggested has not been changed with the exception of a few minor points.

The adjustment of the  $p_H$  of the casein solution has been a troublesome problem in working out this method. At first a combination of phenolphthalein and bromthymol blue was used. This was not entirely satisfactory so after considerable experimentation an aqueous solution of cresol red seemed most suitable. To give some idea of the different results obtained with different indicators two solutions of casein were prepared. One was prepared to have a  $p_H$  of not more than 8.0 while the other was prepared to have a  $p_H$  greater than 8.0. The following table gives the results obtained with various indicators.

Indicator.	Solution I.	Solution II.
Cresol Red (aqueous)	7.6	8.8
Brom-Thymol Blue (alcoholic)	6.9	7.7
Phenol Red (alcoholic)	No comparison possible	
Phenol Red B. P. (alcoholic)	..	...
Phenol Red (aqueous)	8.0+	8.4+
Thymol Blue	..	8.40
By potentiometer (quinhydrone electrode)	7.54	8.65

In determining the  $p_H$  with the various indicators the Hellige Hydrogen-Ion Comparator with the standard discs supplied for each indicator was used.

From the above table it will be observed that with both solutions the  $p_H$  obtained using an aqueous solution of cresol red approximated very closely the  $p_H$  obtained by potentiometric means.

Phenol red proved to be entirely unsatisfactory. The difficulty seemed to lie in the fact that the casein solution apparently absorbs some of the color of the indicator which gives a resulting color that does not compare with the comparison standard. This color change seems to be more pronounced with alcoholic solutions of phenol red than aqueous solutions of the indicator.

A change is suggested in the strength of the alkali to be used for titration in the Modified Smith-Sorensen method. A  $N/10$  sodium hydroxide was previously suggested. Since then a  $N/20$  sodium hydroxide solution has been used during certain coöperative work carried on. Lately, we have used  $N/50$  sodium hydroxide which has the advantage of giving a greater volume required for neutralization and in this way reducing the experimental error. Another advantage of including  $N/50$  alkali would be that the method would be more available for U. S. P. inclusion

if it were looked upon favorably. A *N*/50 sodium hydroxide is official while a *N*/20 sodium hydroxide is not.

Another point in which the proposed method could be improved upon for inclusion in the U. S. P. would be in regard to the temperature of digestion. The present method suggests a temperature of 55° C. This is the temperature used in the original Smith-Sorensen method. A temperature of 52° C. would be more suitable if the method were to be included in the next U. S. P. The reason for this is that most laboratories carrying out enzyme assays according to the present U. S. P. directions have a water-bath regulated at 52° C. for pepsin assays. Therefore if the temperature of digestion for this tryptic activity method were changed to 52° C. the same bath would be available for carrying out the digestion. This, however, would be a matter for further investigation.

The standard given for computing the tryptic activity of pancreatin samples using this method is a more or less arbitrary one based to some extent on the present U. S. P. standard. A sample that digests 25 times its own weight of casein into soluble proteoses should require on the Modified Smith-Sorensen method approximately 5.0 cc. of *N*/50 sodium hydroxide.

The feeling is expressed that using the proposed method for assay of tryptic activity a more satisfactory method of determination would be available with less divergent results being obtained by different workers.

#### COMPARISON OF RESULTS BY THE U. S. P., B. P. AND MODIFIED SMITH-SORENSEN METHODS.

Having considered these three methods it is of interest to see how they compare as far as actual results are concerned. Five samples of pancreatin labeled U. S. P. were procured on the market at the same time. They were all carefully assayed according to each method and the results expressed in terms of percentage of the standard adopted for each test. The results are as follows:

T.	U. S. P. Method.	B. P. Method.	Smith-Sorensen Method.
Sample A	150%	256%	160%
Sample B	220%	296%	226%
Sample C	210%	231%	234%
Sample D	105%	244%	122%
Sample E	220%	343%	234%

From these results it will be observed that with the exception of two samples the others are at least twice as strong as the U. S. P. requirements. This is not entirely surprising since the method of manufacture and the resulting activity will be controlling factors. Since there are two standards to be met by the U. S. P., the samples of pancreatin must be put on the market according to the lowest of the two activities shown to meet the U. S. P. standard. This is to say, it would be almost impossible to market a sample of pancreatin assaying exactly the U. S. P. requirements by both the tryptic standard and the amylase standard.

The B. P. standard seems a little lower than the present U. S. P. standard. From the results on the above samples no satisfactory conclusions can be obtained as to the ratio. In some cases the ratio is relatively large, whereas with Sample C the ratio is very small. The one conclusion that can be drawn, however, is that

pancreatin samples meeting the U. S. P. standard for trypsin will also meet the B. P. standard for this same activity.

*The U. S. P. Amylase Assay.*—The U. S. P. X states that pancreatin should convert not less than twenty-five times its weight of starch into soluble carbohydrates. In carrying out this particular assay a 3.75% starch paste is used and digestion is allowed to proceed 5 minutes with the pancreatin solution. The completeness of digestion is determined by adding a definite amount of the digestion mixture to a dilute iodine solution. If no color is produced the pancreatin meets the U. S. P. specification for amylase.

This U. S. P. method is for the most part satisfactory but there are a few points in it that may cause differences in results. One of the most important of these points concerns the preparation of the starch to be used for test. Detailed instructions are given by the U. S. P. for the starch to be used. It amounts to this, that ordinary potato starch is thoroughly washed with distilled water and dried. The moisture content is then determined and the starch used on the dry basis for the preparation of the 3.75% paste. If the U. S. P. method of washing is carried out and the washed starch dried slowly by a gradual raising of temperature of 50° C., there seems to be less difference in results than if ordinary purified potato starch is used. However, if the washed starch is immediately subjected to a temperature of 50° C. and maintained at this temperature until the greater part of the moisture is driven off, there seems to be a hardening action of some sort on the starch granules. Using starch dried in this manner invariably gives lower results of amylolytic power than when starch dried gradually or not subjected at all to washing and drying is used. There seems very little necessity of going through this washing with subsequent drying if the starch to be used has a neutral reaction. Most of the potato starch of commerce, to-day, is a highly purified product.

Another point in regard to the starch is the method of determining the moisture content. The moisture content is directed to be carried out by heating four hours at a temperature of 120° C. In determining the moisture content in this manner, with many samples of starch it has been a subject of some doubt as to whether the actual moisture content was being determined. With a number of samples, some caramelization has taken place when subjected to this temperature. It would appear that a more satisfactory method would be to dry at 100° C. possibly for a considerably greater length of time. Investigation has shown that caramelization does not take place at this temperature.

The U. S. P. directs that the equivalent amount of starch to make a 3.75% paste is mixed with boiling water and boiled for approximately 5 minutes. In preparing a starch paste in this manner, many times a satisfactory paste is not obtained. The paste produced is invariably quite thick in consistency and often very "lumpy." This increases the chance of using a paste that is not exactly 3.75% starch. This can be overcome and avoided by heating water to between 50° and 60° C. and then adding a water suspension of the starch with constant stirring. The starch mixture is then brought to boiling and boiled for 5 minutes and finally adjusted to weight. In this way a perfectly homogeneous starch paste can be prepared which is much thinner in consistency than one prepared according to U. S. P. directions. This difference in consistency makes it much easier to handle and weigh out for test.

The end-point obtained also is often a matter of doubt. This phase of the amylase test was, however, quite fully dealt with in an earlier paper (3).

*The B. P. Amylase Assay.*—The B. P. amylase test differs somewhat from the U. S. P. test. Instead of using ordinary potato starch, soluble starch is employed. A 1% solution of this soluble starch with 5% of sodium chloride is used in place of 3.75% starch paste. The test is carried out on a small quantity of this starch solution as compared to the U. S. P. method of using 200 Gm. of the paste. The digestion is carried out for one hour at 40° C. instead of the U. S. P. time of five minutes at 40° C. At the end of the digestion period the tubes are rapidly cooled to 20° C. and *N/50* iodine solution added directly to the digestion tubes instead of withdrawing a definite amount and adding it to iodine solution as is the case with the U. S. P. method.

There is one serious objection to this B. P. method. The quantities of pancreatin used for tests are too small. For instance, a series ranging from 0.35 cc. to 0.65 cc. using 0.05-cc. intervals are directed to be used for tests. These quantities are much too small to warrant accurate results, especially when dealing with enzymes.

The use of soluble starch as the substrate appears to be a good one. The chance that a homogeneous mixture will not be obtained is very remote. However, no mention of moisture content of the soluble starch is made which may cause some variance in results.

The use of sodium chloride in making the starch solution is probably an unwise choice in one respect. It has been proven that sodium chloride is an activator for pancreatic amylase. Therefore the results obtained may be higher than the absolute activity of the enzyme.

Another interesting point in the B. P. directions is that on the addition of the iodine solution to the digested mixtures, the end-point in the series is that tube showing no blue color. In other words, they take as their end-point simply the complete conversion of the starch present. In the U. S. P. method the starch must be converted down through the various dextrans to the sugars. It is the dextrans that give the red and purple colors with iodine solution that the U. S. P. directs must not be present.

#### COMPARISON OF U. S. P. AND B. P. AMYLASE ASSAYS.

Some idea of how the U. S. P. and B. P. assay results compare can be gained from results obtained on the five samples of U. S. P. samples which were mentioned in dealing with trypsin activity. The results are as follows:

	U. S. P. Assay.	B. P. Assay.
Sample A	100%	900%
Sample B	45%	500%
Sample C	8%	110%
Sample D	80%	800%
Sample E	75%	720%

In expressing the above results a pancreatin converting 25 times its weight of starch into soluble carbohydrates was expressed as being 100%.

A similar method was used for expressing the B. P. results. Therefore in stating a sample is 900% by the B. P. what is meant is that the sample is 9 times the strength allowed by the B. P.

In looking over the above results it is interesting to note that though all five samples were labeled U. S. P. there is only one sample that actually meets the U. S. P. specifications. This does not, however, mean that the above samples were put on the market below the U. S. P. specifications. It is simply a case of dealing with an enzyme of unstable activity. The instability of pancreatic amylase is a point to which very little attention has been paid up to the present time. Whether this particular instability applies to the liquefying power as well as the starch conversion power of pancreatic amylase is an open question, it seems, at the present time. From observations made, the liquefying power does not necessarily parallel the starch conversion power. This particular point is being investigated, further, with pancreatic amylase and amylases from other sources at the present time.

From the table of results given it will also be observed that the U. S. P. standard is about 10 times that of the B. P. standard. This is not surprising since it has already been pointed out that the B. P. specifications do not require the digestion to proceed down through the dextrin stage. Therefore much higher results could be expected in relation to the U. S. P. method.

The difficulties experienced with the amylase test of the present U. S. P. is a problem to which very little attention has been paid up to the present time. How to improve upon or change the method is a matter of opinion. In the first place we feel that very few pancreatin samples on the market for any length of time will meet the U. S. P. specifications. As already pointed out this is a matter of loss of activity.

In regard to the end-point used in the present U. S. P. method it is quite liable to variance. Possibly a modification of the Fehling's solution method would prove more satisfactory as an end-point and at the same time be more accurate for the specification desired by the U. S. P., *i. e.*, conversion into "soluble carbohydrates."

*The B. P. Lipase Assay.*—The B. P., as already stated, has a definite standard for lipase content. The U. S. P. has up to the present time made no attempt to standardize pancreatin on this basis. The B. P. method for determination of lipase, utilizing the cream from fresh milk, is a very ingenious one and from the brief experience with it seems quite satisfactory. It is much simpler than any of the other lipase methods available and at the same time gives a very definite end-point. The results obtained on the five samples of U. S. P. Pancreatin used throughout the comparison of these different methods give the following results by the B. P. method.

	B. P. Lipase.
Sample A	129%
Sample B	107%
Sample C	63%
Sample D	100%
Sample E	83%

From these above results it will be observed that three meet the B. P. specifications while two are below the specifications.

#### SUMMARY.

1. The U. S. P., B. P. and Modified Smith-Sorensen assay methods for trypsin are discussed and the results by each method compared.

2. Certain changes are suggested for improving the Modified Smith-Sorensen method.
3. The U. S. P. and B. P. Amylase assay methods are discussed and compared.
4. Results of lipase content by the B. P. method are given for five samples of Pancreatin U. S. P. X examined.

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ANALYTICAL DEPT.,  
PARKE, DAVIS & CO.,  
DETROIT, MICH.

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THE DETERMINATION OF STRYCHNINE AND BRUCINE AS HYDRO-FERROCYANIDES AND THEIR SEPARATION BY MEANS OF FERROCYANIDE.\*

BY I. M. KOLTHOFF AND J. J. LINGANE.

The usual procedure for the separation of strychnine from brucine is based on the fact that brucine is fairly easily destroyed by treatment with nitric acid. In the present work it was found that this method yields approximate results only; either the brucine is not completely destroyed or on more drastic treatment part of the strychnine is decomposed also. Therefore a more exact method would be of value. It is known that ferrocyanide in acid medium gives slightly soluble crystalline hydroferrocyanides with strychnine and brucine; the former crystallizes much faster and is less soluble than the latter. Beckurts and Holtz<sup>1</sup> made use of this difference in behavior and titrated a strongly acid solution of the mixed alkaloids with standard potassium ferrocyanide, using ferric chloride test paper as an outside indicator. The success of the method depends on the slowness with which brucine is precipitated by the slight excess of ferrocyanide after the precipitation of strychnine is complete. The detection of the end-point, however, is not very sharp; after much practice an accuracy of about 5% could be obtained.

Gadreau<sup>2</sup> proposed a much more complicated method. He precipitates the strychnine and part of the brucine by addition of a large excess of ferrocyanide in weakly acid medium. The precipitate is treated with an excess of ammonia and the free alkaloids are extracted with chloroform. After evaporation of the solvent the alkaloids are dissolved in 0.1*N* hydrochloric acid and the precipitation with ferrocyanide repeated as before. The process is repeated three times and the final precipitate of strychnine hydroferrocyanide weighed after drying over sulphuric acid and finally in an oven.

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<sup>1</sup> Beckurts and Holtz, *Pharm. Zentralhalle*, 28 (1887), 119.

<sup>2</sup> Gadreau, *J. pharm. chim.* [6], 4 (1927), 145.