A NOTE ON THE B.P.1948 ASSAY PROCESS FOR TRYPSIN IN PANCREATIN

By G. E. FOSTER and W. SMITH

From the Control Laboratories, The Wellcome Chemical Works, Dartford, and the Analytical Laboratories, Allen and Hanburys Ltd., Ware

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THE British Pharmacopœia 1948 monograph on pancreatin includes an assay process for proteolytic activity which is different from the process used in the 1932 pharmacopœia. The latter depended upon the digestion of skimmed milk by pancreatin under standard conditions and a subsequent formol titration of the amino-acids formed, while the former employs casein as the substrate. The official process is based upon the work of N. Evers and W. Smith[1], who modified a process described by A. R. Smith[2] before this Conference in 1912. Since the publication of the B.P.1948 some disquieting results have been obtained which suggest that the standard of proteolytic activity, required by the new assay, is higher than that demanded by the B.P. 1932. It was the purpose of the present work to investigate this alleged difference.

For our purpose it was decided to assay a series of pancreatin samples (I) by the B.P.1932 process and (II) by the B.P.1948 process and make a critical comparison of the results. Work was carried out in our two independent laboratories, using as far as practicable the same materials. While the B.P.1932 process requires skimmed milk, easily prepared in the laboratory, it was found that casein of the quality required for the B.P.1948 process was unobtainable in this country. Evers and Smith used Hammarsten's casein, obtainable in Germany before the war, but only a small pre-war stock was available for the present work. Accordingly, it was decided to carry out experiments using two makes of purified light casein, representing the best quality of casein on the market. Control experiments were performed using Hammarsten's casein.

EXPERIMENTS USING THE B.P.1948 PROCESS

Assays were carried out strictly in accordance with the directions of the B.P.1948, to which reference should be made for experimental details. Preliminary work revealed that with some samples of pancreatin it is necessary to grind the preparation in a mortar under the chloroform water when preparing standard solutions. If this is not done low figures for the proteolytic activity result. Table I summarises the results obtained and for purposes of comparison these have been stated as (I) difference in 0·1N sodium hydroxide titration, as required by the B.P. and (II) difference in N sodium hydroxide titration calculated for 1 g. as suggested by Evers and Smith. The figures stated are in most cases the average of several determinations.

It will be seen that the results are influenced by the quality of casein

10 per cent. acetic acid added with constant stirring to make the excess acid about 1 per cent. over that required to coagulate the casein. After settling, the clear liquid is removed by siphoning and the curd thoroughly washed several times with cold water. The casein is dissolved in the minimum amount of dilute ammonia solution, the solution filtered and the casein reprecipitated with acetic acid. This process of dissolving and reprecipitating is repeated twice. After washing with water and alcohol the casein is extracted with ether in a Soxhlet apparatus and dried in a vacuum at 60° to 70°C.

It was found that a simpler procedure of precipitating casein gave a product which, though inferior to Hammarsten's casein, would satisfy the requirements of the B.P. Appendix I, p. 641. Skimmed milk is centrifuged to remove the extraneous matter and the milk heated to 37°C. The casein is precipitated by the cautious dropwise addition of dilute hydrochloric acid with constant stirring until the *p*H is 4·0. The curd is thoroughly washed by decantation first with hot water acidified with hydrochloric acid, then with several lots of hot water, followed by alcohol and finally with ether. The curd is dried by exposure on a stainless steel tray and finally sieved and powdered.

Samples of pancreatin X, Y and Z, were assayed using casein prepared as described above and the results were very similar to those obtained using Hammarsten's casein.

Discussion

The results obtained during this investigation leave no doubt that the B.P.1932 requires a much less stringent standard for the proteolytic activity of pancreatin than does the B.P.1948. Some estimate of the quantitative difference between the two standards may be arrived at by consideration of the data on sample Y. This specimen of pancreatin is one which only just complies with the requirements of the B.P.1932, which requires the "B" titration to be not less than 9 ml. of 0·05N sodium hydroxide. But according to the B.P.1948 method 1 g. of pancreatin (Y) is equivalent to 6·6 ml. (purified light casein(1)) or 8 ml. (Hammarsten's casein). As the B.P.1948 standard requires 1 g. of pancreatin to be equivalent to 18 ml. of N sodium hydroxide it would appear that the official standard is about 2½ times as stringent as that of the B.P.1932.

Evers and Smith suggested that a reasonable limit for pancreatin would be that 1 g., when assayed by their method, should give a titration of not less than 15 ml. of N sodium hydroxide. This they stated was approximately equivalent to the B.P.1932 standard. The present work does not support the latter statement, though this appears to have been justified on the six samples tested at the time.

When Evers and Smith published their paper, there is no doubt that pancreatin complying with their suggested standard was readily available. At the present time much pancreatin is of lower activity. It therefore seems to us that if it is desirable that the official standard for proteolytic activity of pancreatin should be amended to bring it more in harmony

with the material at present available, this could be achieved by halving the required proteolytic activity and could best be brought about by doubling the amount of pancreatin employed in the assay. When proteolytic activities are to be compared the amount of samples taken should, of course, be adjusted to give titrations of 4·0 to 4·5 ml. of 0·1 N sodium hydroxide by the B.P.1948 method.

Our experience in this work has again emphasised the necessity to use casein, similar in quality to Hammarsten's, for assay purposes and it is hoped that the details, here published for preparing suitable material, will be of value to other workers in this field.

SUMMARY

1. Samples of pancreatin have been assayed for proteolytic power using the B.P.1948 method and it has been found that the pancreatin, at present available, often fails to comply with the official standard.

2. Experiments using different samples of casein as substrate in the assay process established that the quality of the casein influences the results of the assays.

3. For satisfactory results to be obtained casein, similar in quality to Hammarsten's casein, is required. A process for the preparation of suitable casein is described.

4. Three samples of pancreatin have been assayed by the methods of the B.P.1948 and B.P.1932 respectively. It has been shown that the B.P.1948 standard for proteolytic activity is more stringent than that of the B.P.1932.

5. A suggestion is made for amending the official standard in order to bring it into harmony with the quality of the pancreatin at present available.

REFERENCES

1. Evers and Smith, *Quart. J. Pharm. Pharmacol.*, 1936, **9**, 392.
2. Smith, *yearb. Pharm.*, 1912, 525.
3. Hammarsten, *Hoppe Seyl. Z.*, 1883, **7**, 227; 1885, **9**, 273.

DISCUSSION

The two papers dealing with pancreatin were discussed together, the first being presented by Dr. Bullock and the second by Dr. Foster.

THE CHAIRMAN said that it was felt that the B.P.1948 method for the assay of pancreatin was an improvement on the 1932 method, but he could confirm Dr. Foster's statement that manufacturers found it difficult to comply with the B.P. requirements. He agreed with Dr. Bullock's suggestion of reducing the amount of lactose but a certain proportion was desirable in any preparation of this kind.

DR. NORMAN EVERS (Ware) said that the most important observation in Dr. Bullock's paper concerned the digestion of the blank. It was to be

hoped that as a result of these two papers, a more satisfactory process would be obtained. The reason for adjusting the *p*H to 7·0 was that if one took a pure amino acid, such as aminoacetic acid, adjusted the *p*H to 8·7, and then added formaldehyde and titrated, one did not get 100 per cent. results. He did not think that Dr. Bullock and Mr. Sen had taken sufficient notice of the type of casein used, as Foster and Smith had shown that it was important. Certainly it led to greater accuracy to have a clear solution for the titration. From experience he could confirm that the quality of pancreatin was very much poorer than it had been before the war. He thought that the work showed the need for the B.P. to state limits of accuracy of analytical methods rather than the standard figure as at present.

DR. G. E. FOSTER, in reply, said that he was inclined to agree with Dr. Evers that this was an analytical process for which great accuracy could not be expected. It was not known whether pancreatin was one enzyme or many, and obviously any assay process must be empirical. What had to be decided was which method, in all circumstances, was the best. One of the merits of the paper by Bullock and Sen was the fact that the blank solution was boiled, and in this respect the B.P.1948 process should be amended. However, he was inclined to believe that the suggested difference in the titration technique was something which might, if adopted, lead in time to further trouble.

MR. J. K. SEN said that Dr. Foster did not seem able to find the actual defect in the B.P.1948 process, and his suggestion of doubling the amount of pancreatin to be taken to bring the weakest pancreatin to the B.P. standard, or halving the strength of the alkali to do so, seemed unscientific, to say the least. Six or seven different types of casein had been tried and it was found that "light white soluble casein" gave about the same results as the others. Several experiments had been carried out on adjusting the *p*H to 7·0 before titration, and it was found that adjustment to 8·7 after digestion did not give variable results, whilst the adjustment to *p*H 7 was difficult because it was done colorimetrically by matching against some standard buffer and was less accurate. Boiling the blank kept it constant, and made it easy to test different pancreatins and compare their strength. Experiments at different times had shown the accuracy to be $\pm 1{\cdot}0$ per cent. which he thought was sufficient.

DR. K. BULLOCK said that if Dr. Foster would try out their process he would get repeatable results without difficulty. They agreed, of course, that the new B.P. standard was higher than previously, but it was impossible to say anything about it in one sense because the results would always depend on the temperature of the laboratory, and on a number of other factors. That undoubtedly explained some of Dr. Foster's difficulties in getting comparisons between the two laboratories. Boiling the blank was most important, and he could not conceive of an enzyme chemist who would use a blank with active enzyme in it which might amount to 30 per cent. or more of the total digestion in the actual test. The blank

must either be boiled or kept at a low temperature so that no digestion went on, and some did occur even at 0°C. On the question of the kind of casein, if one were going to adjust to *p*H 7, at the end before adding the formaldehyde one would require purified casein. If one were going to do the titration to phenolphthalein only, then, although it was of some advantage to have a clear solution, it was by no means a necessity, and it was possible to get repeatable and accurate results with ordinary light white casein. The suggestion of adjusting to *p*H 7 apparently originated in a paper by Northrop in which he estimated accurately certain quantities of free amino acids. The present B.P. process, however, was empirical and did not estimate accurately the amount of free amino acid. If the adjustment was made in a sample containing fat, then in the assay for trypsin one might well estimate some of the fatty acids which had been liberated. Secondly, one would also include acidity liberated by the digestion but not due to the removal of NH_2 groups; i.e., acidity which was not part of the formol titration at all. From Figure 1 of the paper, the lower curve, it would be seen that the acidity fell off much more rapidly with increasing quantities of pancreatin or with increasing time. It confused the issue, therefore, to include this acidity in the formol titration. Not only did the B.P. do that, but there was another factor which was introduced. If the blank was adjusted to *p*H 7 one would be starting the blank and the test differently, because during the digestion the *p*H fell so that the blank was not at *p*H 7 and had not the same buffer value as the test. By adjusting to *p*H 7, therefore, one was really adding some but not all of this acidity other than formol titration acidity. The amount of this added acidity was purely arbitrary.

He would be pleased if Dr. Foster would see whether the presence of lactose was not capable of explaining almost entirely the low quality of present day pancreatin. They themselves had found that when lactose-free, almost all the samples would comply with the standard which they suggested. It might be right to have some lactose in pancreatin, but the present situation was anomalous. There was a standard given by the B.P. and yet manufacturers were selling triple B.P. strength. The correct position would be for true pancreatin to be sold as such and then, if there was a demand for a lower strength, it could be sold as " half-strength " B.P., etc. The pure substance should be called pancreatin and the dilution should be called a dilution.

DR. G. E. FOSTER said that his paper was only intended to be in the nature of a note. They had been asked by one of the B.P. Committees to find out whether there was any substance in the complaints which had been received. There was a discrepancy between results obtained by the B.P.1932 process and those obtained by the 1948 process. The pharmacopœial assay process laid down a test and if one carried out the test exactly as directed and got the result given there, the substance passed the test and could be referred to as B.P. It did not specify the estimation of a particular substance.