is no peculiarity in the distribution of other nitrogen factors and no reason is apparent why there should be. In the case of the creatinine we have the fact of lower muscular structure and lower muscular tone to possibly account for the observed findings.

This series of observations was made at the suggestion of Prof. J. H. Long in the summer of 1913.

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A COMPARISON OF METHODS FOR THE DETERMINATION OF THE PROTEOLYTIC ACTIVITY OF PANCREAS PREPARATIONS.

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In recent years many suggestions have been made as to methods for finding the tryptic power of various pancreas preparations, but these still leave the problem of a generally definite method far from solution.

For a long time the classic Kuehne method was held in esteem as a standard for the comparison of other procedures and it still has its uses. Often egg albumin has been employed instead, but its digestion, as compared with the fibrin of the Kuehne scheme, is so slow that practically it is much less suitable as a substratum. The mistake of employing raw egg has often been made, although it is known that trypsin has but little action on other than denatured proteins. It was recently pointed out by one of us[1] that many of the results reported by Wroblewski, Bednarski and Wojczynski[2] are rendered meaningless by this use of raw egg in the estimation of tryptic activity. In some cases this resistance to digestion seems to be due to the presence of the so-called antitrypsin in the native protein solution. That this is the case with serum has been shown by several authors, for example by Oppenheimer and Aron,[3] but the structure of the native protein itself is a strong factor, and possibly the strongest one, in the case of raw egg. In any event uncooked egg can not be well used to show tryptic activity.

While shreds of fibrin are well adapted for the purpose of a qualitative test of the proteolytic power of pancreas extracts, there are many difficulties in the way of successful use in quantitative comparisons. Some of these difficulties are inherent in the use of a solid substance which can not be acted on uniformly by the digesting medium. The rapidity of digestion will depend on the degree of comminution of the fibrin and on the frequency of shaking the test vessels.

An equally important objection to fibrin is usually overlooked and it is not clearly stated in the literature. It is this, that even after the most

[1] Long and Johnson, THIS JOURNAL, **35**, 1194 (1913).

[2] *Beitr. chem. Physiol. und Path.*, 1, 288 (1902).

[3] *Ibid.*, 4, 279 (1904).

careful purification fibrin retains the peculiar ferment which brings about, more or less rapidly, the phenomenon described as fibrinolysis, resulting in the gradual solution of the fibrin. This fact, long known, does not appear to have been sufficiently considered where fibrin has been used. Fibrin kept in glycerol, as often recommended, changes in a few days at the ordinary temperature; indeed, a weight of a hundred grams or more in glycerol may become quite liquid in ten days or two weeks. Even where the water is removed as far as possible by glycerol and the chopped mass is pressed dry and treated with toluene, autolysis still takes place in time, as pointed out by one of us[1] before. For satisfactory quantitative comparisons it is essential that the fibrin be practically fresh.

The uncertainty in the employment of solid substance is avoided by the use of normal milk, as originally suggested by Roberts[2] in his studies on pancreatic ferments. The Roberts method is based on the observation of the so-called metacasein reaction. Ordinary milk does not coagulate on boiling, but if its casein be modified in a certain way, coagulation on boiling follows readily. The modified product was called metacasein by Roberts and is similar to the caseogen of Arthus and Pagès, and the paracasein of other authors, in the first stages of their production. In a given small quantity of milk the coagulation on boiling is not observed until the whole of the casein has been converted to this stage. The value of the test depends on this point. By longer digestion the reaction disappears, because the casein is converted further into true derivatives. The unit of measurement in the test is the number of milligrams of milk converted to give the metacasein reaction in five minutes by 1 milligram of the ferment.

It will be recognized at once that there are certain weighty objections to this as a quantitative test. Roberts seemed to regard the composition of market milk as sufficiently uniform for the purpose, but this can not be the case. The casein content of milk is, of course, variable where the product of individual cows is considered, but the value for mixed market milk has been assumed to be pretty constant, with the casein about 3%. We are inclined to think that the average casein content of the mixed milk delivered in our cities is lower than it was some years ago. Under city ordinances relating to milk the fat is made the most important constituent and in consequence cows are selected and fed for fat production. In our experiments the average casein content of the milk used was about 2.5% and a change in this would naturally make a change in the amount of ferment required to complete the test. This will be discussed below.

Retaining a casein solution as the substratum for digestion, and aiming

[1] Long and Johnson, *loc. cit.*

[2] Sir William Roberts, M.D., F.R.S., *Proc. Roy. Soc.* (*London*), **32**, 145 (1881).

at greater precision to be secured by employing a definite substance, Gross[1] recommended a weak solution of sodium caseinate. Small constant volumes of such a solution are mixed with gradually diminishing volumes of the dilute tryptic solution and incubated an hour at 38° (or 40°). At the end of the time the test tubes containing the mixtures are removed from the bath and treated with 3 drops of a very weak acid solution, which produces a precipitate in the tubes in which the digestion has not reached the proper stage. As the reaction is sharp it is possible to recognize at once the tube in which the digestion just fails to be complete. A convenient number of steps in the dilutions must be chosen to give close results. The object of the test is to determine how many cubic centimeters of the casein solution may be digested by 1 cc. of the ferment solution in the unit of time, here 1 hour. A better measure, when dealing with solid ferments, is to find the number of milligrams of casein changed by 1 mg. of the ferment to the point where no precipitate is formed, in the unit of time. The tryptic activity varies inversely as the weight of ferment required and the time.

In place of making the dilutions with water, which has a destructive action on the strongest ferments free from excess of salts, we have experimented with the use of a phosphate mixture to preserve a constant low hydroxyl content but little above neutrality, as will be explained below.

In addition to these three general schemes we have carried out, with the six ferments employed, a series of tests with the formaldehyde titration as worked out by Sörensen and colleagues. Here again we employed casein dissolved in the right amount of sodium hydroxide as the substratum and after digestion through definite periods titrated, after the addition of the formaldehyde, to measure the amino acids formed. For the purposes of quantitative comparisons we have not thought it desirable to follow out the optical method, in which the measure of digestion is preferably the change in rotation in some artificial polypeptide, or certain other methods in which the liberation of tyrosine from one of its simple polypeptides is the measure of the digestive activity. For the practical purpose in view, the methods referred to give the most direct and easily followed results.

Trypsin is employed at the present time not only in the course of scientific research but also as an agent of protein digestion on the large scale, and as a remedy in the experimental study of disease, or in the routine treatment of various disorders. It is produced as a commercial product by many firms and some of the products obtained are stronger than any which the ordinary laboratory worker is likely to be able to prepare himself. Some standards for the valuation or comparison of these "trypsins"

[1] *Arch. exp. Path. und Pharm.*, 58, 57 (1906). This work is based on the method of pepsin digestion suggested by Fuld, where edestin was used as the substratum.

or "pancreatins" is desirable, as each manufacturer makes his own statement of value in terms which are sometimes vague and not translatable into other terms.

In the investigations of the Council on Pharmacy and Chemistry of the American Medical Association the question of the comparison of these standards has come up more than once and the present study is the result of an effort to throw some light on these points.[1] The plan of our study has been this: We have made tests with six well-known American commercial brands of trypsins, representing strong as well as relatively weak preparations, and have compared their activity by the four methods outlined above. The important details of the tests will now be given.

The Roberts Metacasein Reaction.

It is not a difficult matter to secure uniform and consistent results by this test, which is carried out practically as follows: 50 cc. of fresh milk are diluted to 100 cc. with water, the ferment solution to be added later making part of the final volume. A number of flasks are prepared with the milk and the right amount of water added. The smaller ferment volumes are placed in test tubes, and flasks and tubes are warmed to 40° in a thermostat. When the temperatures are constant the contents of the tubes are poured into the proper flasks and the liquids mixed by shaking. At the end of each minute a 5 cc. portion is withdrawn from each flask and quickly boiled. If a coagulation occurs at the end of four to six minutes with one of the dilutions, the result with this flask is regarded as a close approximation to the proper value and a second test is made with dilutions which should give the end point in an interval very near to 5 minutes. Roberts showed that for time intervals nearly the same the inverse relation between time and strength holds, as in many similar reactions. In other words, if it is found that 5.1 mg. of the ferment is sufficient to bring about the reaction in 4.5 minutes, 4.41 mg. will do it in 5 minutes. The dilution to 100 cc. was always observed.

Milks from several dairies were used in the course of the investigation and in different trials the casein content was found to be about 2.5% by the Van Slyke-Bosworth titration. The following figures show some typical results obtained on different days with milk from one dealer:

Four samples of milk of 50 cc. each gave 2.5, 2.5, 2.4, 2.6% casein and 4.1, 4.1, 3.9, 4.2 mg. of ferment, respectively.

In the mean, a trifle over 4 mg. of the active ferment was required to bring about the reaction. The results are not always as close as the above

[1] This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry of the American Medical Association.

and it is almost always necessary to have two persons to make the tests so as to make the boilings at the right instant of time.

In a preliminary series of tests with a product which we will call Trypsin A, made up to a dilution of 2 mg. to 1 cc., we found these results, which are illustrative of many others:

Mg. of ferment added:	6.0	5.0	4.2	4.1	4.0	
Time of onset of reaction (min.):	3	4	4.5	5.0	5.5	
Mg. for 5 minutes calculated:	3.6	4.0	3.8	4.1	4.4	Mean, 4.0

In general, values like the first are rejected and new tests made. A large number of tests were made with this same trypsin on milks obtained on different days from the same dairy. From 3.8 to 4.1 mg. of the ferment were required to produce the metacasein reaction in 5 minutes, the mean value being 4.0 mg. This will be used in the comparisons below. It was found that with other milks more of the ferment was required as the content of casein ran up to about 2.8%. This is a point which every one working with the test will have to observe.

As a further illustration of the degree of uniformity which may be expected in the working of the test the following series is given, a product which we mark Trypsin B being used. Two dilutions were employed, in one 200 mg. to 100 cc. and in the second, 600 mg. to 100 cc. The following table shows the results of the trials:

Mg. of ferment added:	12	12	15	15.8	15.8	16	16	17
Time to onset of react. (min.):	6.0	6.25	4.75	5.0	5.0	4.5	4.5	4.0
Mg. for 5 minutes, calc.:	14.4	15.0	14.3	15.8	15.8	14.4	14.4	13.6

Mean, 14.7

It will not be necessary to give the details of the other experiments as they showed about the same variations as above. By repeating them and taking mean values the results given below were obtained. The means for all of the samples are here tabulated:

Name of sample: Trypsin A,	B,	C_I,	C_{II}; Pancreatin D,		E,	F.
Mg. for 5 minutes calc.: 4.0	14.7	27.3	24.3	32.9	33.8	47.3

Trypsins C_I and C_{II} were different preparations from the same manufacturer of which C_{II} was the fresher. In the comparisons below, it alone will be used. In this preparation dilutions of 700 mg. to 100 cc. were made, while with numbers D, E and F the favorable dilutions were found to be 0.870, 1.5 and 1.7 g. in 100 cc., respectively. These dilutions were not found at once but only after a number of trials with different strengths and with different samples of milk. It will be seen that the weakest product has less than one-tenth the strength of the strongest.

The values given present a fair picture of the behavior of the better grades of such preparations available in this country. All these samples have some amylolytic as well as proteolytic activity but are practically devoid of action on fats. A considerable number of other preparations

examined were found to be weaker than these, but need not be discussed here because no new principle is involved in the experience gained with them.

The probable reason for the variations in the individual series is found in the character of the products used. These are not soluble extracts, but more or less insoluble powders which are brought into solution as well as possible by rubbing up in a mortar with successive portions of water. Even with this treatment we can not be certain that all of the true enzyme has left the solid and gone into solution. Heat can not be applied in the making of the solutions and they must be made fresh, as loss of strength always follows in dilute aqueous solution on standing. The variations from possible changes in casein content of the milk must not be lost sight of.

But this variation in the casein content has not the effect calculated if there is a variation in the dilution at the same time. A change in the dilution changes the concentration of the enzyme with reference to the casein, and this is an important point. Changes in the dilution give somewhat perplexing results. All the above values are based on trials with milk containing almost exactly 3% of fat as well as the uniform casein content mentioned. It has been recently shown that increase in the fat of milk increases the time required in the ordinary rennet coagulation,[1] and it is probable that the above reaction would be affected in the same way. Roberts found that the time of digestion to produce the metacasein reaction is increased greatly by dilution, and stated that if the reaction is secured with 50 cc. of milk, undiluted, in three minutes, the same milk diluted with an equal volume of water would require six minutes. In other words, the rapidity of formation of metacasein is proportional to the concentration of the ferment.

In a long series of tests we found this relation to hold only approximately, but as the point was not a necessary part of the present enquiry we have not followed it to a definite conclusion. For our purpose it was thought better to make all the comparisons under the constant dilutions, as given.

It must be kept in mind that the time given, in the practical application of the test, is the very beginning of the reaction, or onset, as Roberts called it. By long digestion the phenomenon disappears because of the formation of soluble products without the coagulating property of casein. Some little uncertainty naturally obtains in detecting just the right point of the onset of the coagulation, and this uncertainty can be overcome only by considerable practice with the method.

The following table shows the mean results of the above experiments, arranged in the order of ferment strength and with the use of the Roberts

[1] Kreidel and Lenk, *Biochem. Z.*, **63**, 151 (1914).

unit, the parts of milk converted by one part of the enzyme. The gram and cubic centimeter are here taken as equivalent.

Number:	A,	B,	C,	D,	E,	F.
Mg. of ferment for 50 cc. of milk:	4.0	14.7	24.3	32.9	33.8	47.3
Digestion units:	12500	3400	2050	1520	1480	1060

The values in the last line will be used for the comparisons to follow.

The Sodium Caseinate Digestion, Fuld-Gross Method.

The strength of casein solution usually recommended for this method is 1 mg. to 1 cc., that is, 0.1%. Of this solution 2 cc. or 2 mg. of casein are to be used in each test, that is, this amount is to be added to each of a series of test tubes along with the various dilutions of the ferment solution to be incubated for each trial. While this weak casein solution is satisfactory for very weak enzymes it is not as convenient for strong solutions, and the practice of this laboratory has been to employ a solution of double the strength with three times the volume, that is to use 12 mg. of casein in 6 cc. in place of 2 mg. The ferment is made up in solutions of various strengths, in some cases as low as 0.05 mg. to 1 cc.

First, a series of tubes are charged with 6 cc. of the casein solution, made as described below, and warmed to 40° in the bath. In other tubes the dilutions of the ferment are poured and each of these small volumes is made up to 4 cc. and also warmed to 40°. Then the dilutions in series are poured into the properly labeled casein tubes, which are shaken and digested for 1 hr. at 40°. At the end of the time the tubes are taken from the bath and immediately treated with 3 drops of the dilute acid, made by mixing 1 volume of glacial acetic acid with 50 volumes of alcohol and 49 volumes of water. Where the required digestion is complete no precipitate or cloudiness appears, but if digestion is not complete the presence of the unaltered casein is shown by the formation of a cloud, or even a heavy precipitate. In the records to follow, complete digestion is indicated by —, and a precipitate by +, or ++ if heavy. A faint cloud indicating nearly complete digestion is shown by ±. The true end point can be estimated by the appearance of this cloud or opalescence.

We shall not describe the experiments made with the weak solutions referred to, but give only those where we used our stronger solution of casein. In some preliminary experiments we dissolved 800 mg. of anhydrous casein in 50 cc. of water plus 5 cc. of 0.1 *N* sodium hydroxide. The solution was made to 400 cc., of which a volume of 6 cc., representing 12 mg., was taken for each test. The ferments were made up in water solution with 0.05 mg. to 1 cc. The following results of Table I were obtained in the trials:

TABLE I.

Ferment A. Cc.	Ferment B. Cc.	Ferment C. Cc.	Ferment D. Cc.	Ferment E. Cc.	Ferment F. Cc.
1.00++	1.00++	1.00++	1.00++	1.00++	1.00++
1.50+	1.50++	2.00++	1.50++	2.00++	2.00++
1.75±	2.00+	3.00+	2.00++	3.00++	3.00++
2.00—	2.50+	3.50±	3.00++	5.00+	4.00++
2.50—	3.00±	4.00—	4.00+	6.00±	4.50+
3.00—	3.50—		4.50±	7.00—	5.00±
			5.00±	8.00—	5.50—
			5.50—		
Activity, 1 : 120–137	Activity, 1 : 70–80	Activity, 1 : 70	Activity, 1 : 43–48	Activity, 1 : 34–40	Activity, 1 : 43–48

In another series of tests the same casein solution was used but the ferments were made up in the strength of 0.25 mg. to 1 cc. Results, Table II.

TABLE II.

Ferment A. Cc.	Ferment B. Cc.	Ferment C. Cc.	Ferment D. Cc.	Ferment E. Cc.	Ferment F. Cc.
0.10+	0.10++	0.10++	0.30++	0.30++	0.20++
0.20+	0.20++	0.20++	0.50++	0.50++	0.30++
0.25±	0.30+	0.30++	0.80++	0.80++	0.50++
0.30±	0.40+	0.40++	1.00±	1.00++	0.80+
0.35±	0.50±	0.50+	1.20±	1.50+	1.00±
0.40—	0.60—	0.60±	1.20—	2.00+	1.20—
0.50—	0.70—	0.70—		2.50—	1.50—
				3.00—	
Activity, 1 : 120–140	Activity, 1 : 80	Activity, 1 : 80	Activity, 1 : 40	Activity, 1 : 24	Activity, 1 : 40

These preliminary trials suggested a better system of dilution which was arranged in this way. The ferments were dissolved to make solutions of 50 mg. to 100 cc. Of these solutions, 20 cc. were, in each case, diluted to make 100 cc., giving as the final strength 0.1 mg. to 1 cc.

The casein solution was prepared by dissolving 500 mg. of pure, dry casein in 50 cc. of water plus 4.5 cc. of 0.1 *N* sodium hydroxide. When solution was complete about 100 cc. more of water was added and then 22.5 cc. of 0.01 *N* hydrochloric acid to neutralize the excess of alkali and leave the casein as acid salt.[1] The volume was then diluted to 250 cc., 6 cc. of this casein solution was always taken, equivalent to 12 mg.

The following series of ferment dilutions was followed and for the several ferments the parts of the series indicated were used.

No.....	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
Vol. (cc.)....	4.0	3.6	3.2	2.8	2.4	2.0	1.6	1.2	1.0	0.8	0.6	0.4

For Ferment A, numbers 6 to 11 or 12; for B, 4 to 10; C, 1 to 8 or 9; D, 1 to 8; E, 1 to 8; F, 1 to 8 were used with a few variations, as shown.

The incubations were made as before.

[1] Long, THIS JOURNAL, 28, 372 (1908).

TABLE III.

Ferment A. Cc.	Ferment B. Cc.	Ferment C. Cc.	Ferment D. Cc.	Ferment E. Cc.	Ferment F. Cc.
0.60+	0.80++	1.00++	1.20++	1.20++	1.20++
0.80±	1.00++	1.20++	1.60+	1.60++	1.60+
1.00—	1.20+	1.60±	2.00±	2.00+	2.00±
1.20—	1.60±	2.00—	2.40—	2.40+	2.40—
1.60—	2.00—	2.40—	2.80—	2.80±	2.80—
2.00—	2.40—	2.80—	3.20—	3.20—	3.20—
	2.80—	3.20—	3.60—	3.60—	3.60—
Actvity,	Activity,	Activity,	Activity,	Activity,	Activity,
1 : 120	1 : 80	1 : 75	1 : 60	1 : 40	1 : 65

These results, given in Table III, are slightly different from the preliminary ones, but because of the more careful preparation of the ferment solutions, and the better dilutions, they are taken to represent pretty closely the right relations.

The ferment solutions were allowed to stand in a cool place over night, protected by toluene. The digesting values were found to be considerably diminished in five cases by the 24 hours delay and were now as follows:

A, 1 to 60; B, 1 to 60; C, 1 to 50; D, 1 to 66; E, 1 to 35; F, 1 to 30.

The greatest change comes in the most active solution, which is an experience we have frequently observed in earlier work. With the weaker ferments the change is relatively less and in one case there even appears to be a slight gain in activity. This ferment contains the largest amount of foreign matter, especially sodium chloride and phosphate, while Ferment A is a practically soluble powder, free from tissue remains and inorganic substances.

To test the behavior of phosphate addition, and with the hope of securing a solvent which would give more uniform results than we found with water, we made up both the casein solution and the ferment dilutions in a mixture of 4 volumes of molar/100 HNa_2PO_4, and 1 volume of molar/100 H_2KPO_4, of which the hydrogen concentration is about $(H) = 0.45 \times 10^{-7}$, and the hydroxyl, therefore about $(OH) = 1.4 \times 10^{-7}$. Tests made in the same manner with this mixture, using a little more acid in the precipitation, gave the results shown in Table IV.

TABLE IV.

Ferment A. Cc.	Ferment B. Cc.	Ferment C. Cc.	Ferment D. Cc.	Ferment E. Cc.	Ferment F. Cc.
0.40+	0.60+	0.80+	0.80+	1.20+	1.20+
0.60±	0.80±	1.00+	1.00+	1.60+	1.60+
0.80—	1.00—	1.20±	1.20±	2.00+	2.00±
1.00—	1.20—	1.40—	1.60—	2.40±	2.40—
1.20—	1.60—	1.60—	2.00—	2.80—	2.80—
				3.20—	3.20—
Activity,	Activity,	Activity,	Activity,	Activity,	Activity,
1 : 200	1 : 120	1 : 100	1 : 100	1 : 50	1 : 60

It will be seen that the phosphate stimulates the stronger and purer enzyme in marked degree but has much less action on the others. This

is probably due to the fact that some of the products, especially C, E and F, contain much tissue phosphate. These products resemble pancreas powders, rather than tryptic extracts. This behavior suggests a modification of the usual process when it comes to the examination of purified extracts of the pancreas which have been prepared in such a manner as to remove the larger part of the salts. On being allowed to stand over night, however, all the solutions were apparently weakened, but in uneven degree. This behavior would call for detailed study which can not be taken up here. For the present purpose we shall make use of the results from the water solutions only.

Digestion and Formaldehyde Titration.

The formaldehyde titration was carried out on the products obtained by the action of the ferments on relatively strong solutions of sodium caseinate, by the general process developed and improved by Sörensen and colleagues.[1] Casein solutions were made by dissolving 20 g. of dry casein in 170 cc. of 0.1 *N* sodium hydroxide plus 100 cc. of water. When solution was complete the volume was diluted to 500 cc. Portions of 25 cc. plus the amount of ferment to be used later were treated with 10 cc. of neutralized formaldehyde and titrated with 0.1 *N* sodium hydroxide and phenolphthalein, running to sharp rose red. These results were used in correction of the actual titration results later. Portions of 100 cc. of the caseinate solution were warmed to 40° in flasks. The amount of ferment to be used was rubbed up with water and the solution made to 5 cc. in each case. After warming to 40° each ferment solution was poured into a volume of the casein solution and the mixture incubated through 2 hrs. at 40°. At the end of each 30 minutes one-fourth of each volume, 26.25 cc., was withdrawn, treated with 10 cc. of the formaldehyde and phenolphthalein and titrated with the alkali. Of Ferment A 50 mg. were added to the 4 g. of casein, while of the others 200 mg. were taken. But in the tabulated results the titration values for A are multiplied by four to bring all to the same basis for comparison, as shown below. The figures are already corrected for the amount of alkali required in the blank titration, and, as given, measure the amount of amino acids liberated or developed by the digestion of 1 g. of casein contained in each 25 cc. of original solution through the added ferments.

Time. Min.	Cc. of 0.1 *N* NaOH required for 25 cc. of					
	A.	B.	C.	D.	E.	F.
30	32.0	10.0	13.2	12.2	11.7	10.0
60	38.8	16.2	15.8	15.1	14.7	13.2
90	45.6	17.7	17.2	17.0	16.6	14.4
120	48.8	18.7	18.8	18.7	17.7	15.9

[1] Sörensen, *Biochem. Z.*, **7**, 45 (1907); **25**, 1 (1909); Henriques, *Z. physiol. Chem.*, **60**, 1 (1909); Henriques and Sörensen, *Ibid.*, **63**, 27 (1910).

It will be seen that approximately the same order is observed here as in the casein digestion, with Ferment A much stronger than the others, and B, C and D nearly the same. In the case of A the amino acid liberation indicates a rather advanced degree of digestion. For the purpose of comparison, putting the result for A as 100, we have these values in series:

A 100, B 38, C 38, D 38, E 36, and F 33.

For this comparison we have taken the alkali requirement at the end of the 2-hour digestion period. The relations for other times are nearly the same.

The Fibrin Digestion.

Coming finally to the oldest method for comparing tryptic values we have the following observations to record: For the reasons already suggested it is much more difficult to secure comparable results in this way than in any of the later methods suggested. As originally described, by the digestion of fibrin the simple solution was usually understood. But the first stage of this solution yields a globulin-like body which represents no very advanced condition of digestion. The choice of the right digestion period here, along with the proper weight of substance to be digested are important elements in this test. By making the period long enough even the weakest products appear to have some value and many of the results found in the literature regarding the activity of tryptic extracts are based on periods of many hours' duration or through the night in many cases. In our studies we have followed two general schemes. In the first we have started with some small, definite amount of prepared fibrin, usually 2 g., and have noted the time required to practically digest all of it. A few stray flakes are always left, even with the most active preparations. In the other case we begin with a larger weight, as 5 g., and determine the amount of soluble nitrogen formed in a definite time, as three hours or four hours. This nitrogen must be diminished by the amount obtained in a blank experiment where the same weight of fibrin is taken, with the same alkalinity of reaction, but where the ferment has been killed by heat. This plan has been varied by neutralizing the digested mixture with dilute acetic acid, boiling and filtering off the neutralization precipitate. The soluble nitrogen now found by the Kjeldahl determination on the filtrate is naturally less.

Comparing the trypsins by the first scheme we have this result. The preparations were taken in amount of 25 mg. in 50 cc. of water, plus 100 mg. of sodium carbonate, with 2 g. of moist fibrin added to each flask, after bringing the temperature in each case up to 40°. Incubation was continued until practically all the fibrin had been digested, which required the following times, in minutes, in different experiments:

A 8 to 15 minutes, B 13 to 25, C 50 to 70, D 75 to 100, E 160 to 180, F 140 to 160.

There is, therefore, a very great difference between the different products when measured in this way, and the findings vary with the degree of disintegration of the fibrin and the frequency with which the flasks are shaken during the digestion. Our fibrin was ground very fine in a meat chopper. With large flakes the digestion is much slower.

The same ferments were mixed in amounts of 100 mg. with 5 g. of a different fibrin in 50 cc. of water, plus 100 mg. of sodium carbonate. The digestions were continued through 3.5 hrs. at 40°. At the end of the time the contents of each flask was slightly acidified with acetic acid and boiled, then filtered. The soluble nitrogen was found in the filtrates, and was as follows, corrected in each case by the nitrogen found in a blank with dead ferment:

A 196.3, B 137.5, C 126.3, D 119.3, E 73.1, and F 119.0 mg.

Kjeldahl determinations on three samples of the fibrin gave the amount of total nitrogen in 5 g. equal to 274.4, 278.6, and 274.4 mg.

The results for fibrin A show that about 72% of the substance had been digested to an advanced stage, as the neutralization precipitate had been filtered out here and in the other cases. But these results are not comparable with those of the other digestion; they are given to show the practical difficulty in making such comparisons with fibrin.

In another set of digestions with 5 g. of a fibrin containing 216 mg. of nitrogen, and the same amounts of ferment as before, we found these weights of soluble nitrogen, not diminished by the neutralization precipitate, but otherwise corrected. The digestions were continued 4 hrs. here:

A 209.5, B 204.4, C 173.9, D 148.4, E, 100.2, and F 157.1 mg.

It appears that nearly the whole of the fibrin was made soluble by Ferments A and B. Comparing the results with those secured in the short periods with 2 g. of fibrin, it is evident that here the major part of the digestion is completed in a period much below 3 or 4 hrs. The effect of the long digestion is to make the weaker products appear *relatively* stronger and nearly as good as the others.

To compare these digestion results on fibrin with the milk and casein tests we shall take the first series and reduce the results to the basis of milligrams of fibrin, with about 25% of dry protein, made soluble in 10 minutes by 1 mg. of ferment. We use the last figures in each case, that is, those representing the longest digestion times:

A gave 53.2, B 32.0, C 11.4, D 8.0, E 4.4, and F 5.0 mg. of fibrin for 1 mg. of ferment.

The same general order appears here as in the other methods of valuation, but the steps are not separated by the same distances.

The general relations for all the methods are shown in Table V, as follows, in which the units of comparison are:

(1) For the metacasein reaction: parts of milk brought to the onset stage by 1 part of ferment in 5 minutes;

(2) Sodium caseinate digestion, Fuld-Gross method: milligrams of casein digested in 1 hr. by 1 mg. of ferment to the point where no precipitate is given by the acetic acid reagent;

(3) The formaldehyde titration: the relative amounts of alkali required to neutralize the amino acids from 1 g. of casein in 2 hrs. digestion;

(4) The fibrin digestion: milligrams of fibrin digested to the soluble stage by 1 mg. of ferment in 10 minutes.

TABLE V.

Ferment.	Metacasein reaction.	Fuld-Cross test.	Formaldehyde titration.	Fibrin digestion.
A	12500	120	100	53.2
B	3400	80	38	32.0
C	2050	75	38	11.4
D	1520	60	38	8.0
E	1480	40	36	4.4
F	1060	55	33	5.0

This tabulation brings out clearly the lack of uniformity in the behavior of the six ferments in the four reactions. While the tests agree in showing approximately the position which each ferment holds in the series they do not agree as to relative quantitative value. For example, Ferments A and B are far apart in the first test but not so far in the last, and Ferment F actually appears stronger than E by two of the tests, while by the others it is weaker.

What is the explanation of this? We believe this must be sought in two directions. These pancreas preparations are mixtures of several ferments and among them there are probably at least two which have proteolytic properties. It is the common experience of manufacturers of ferments that the pancreases themselves present very different degrees of activity, and investigators have noted the same fact.[1] Further differences are developed by methods of extraction and activation employed. Some manufacturers hold that activation by the addition of parts of the intestine is necessary while others do not make this addition at all. The proferments become activated in very different degrees in this manner and possibly some do not become activated at all. It is plain that variations creep in from the very beginning of the manufacturing treatment, and it is inevitable that the finished products should differ, not only quantitatively, but also qualitatively. We have no definite scientific definition of what trypsin really is, and we do not usually take into account that apparently fundamental differences in behavior sometimes result from the presence of quantities of inorganic salts in the finished products of the market. The process followed by each manufacturer is a secret carefully

[1] Gulewitsch, *Z. physiol. Chem.*, **27**, 540 (1899).

guarded, which provides a situation that obliges the investigator of such products to work in the dark.

The differences in behavior toward acids and toward temperature elevation which have been noted by different workers[1] may probably be traced to the salt content of the different preparations and especially to the relations between the phosphates present. We find that in heating these trypsins their behavior changes in marked manner. In aqueous solution they were all very sensitive to heat and an elevation of temperature to 60° for even 5 minutes brought about great alterations in some respects. For the metacasein reaction 55° is an excessive temperature, as illustrated by the following results where the weights of ferments required to bring about the onset are given:

A 180, B 80, C 120, D 80, E 160 and F 160 mg.

While the ferments are all enormously weakened the interesting fact is shown that they do not suffer at all in the same manner. Some are much more thermostable than others.

In the Fuld-Gross reaction, using all quantities as employed in the regular test, we find these digestive values for 1 hr. incubation, the ferments having been heated previously to 55°:

A 1 to 33, B 1 to 24, C 1 to 22, D 1 to 23, E 1 to 11 and F 1 to 15.

Here, also, there is exhibited a weakening effect, but in far less marked degree than before. While for A the value in the Fuld-Gross reaction is reduced fourfold, in the metacasein reaction it is reduced forty-fivefold. For the other ferment the changes are in very different degrees. This peculiar behavior suggests that different ferments are concerned in the two reactions, the one which is active in digesting the casein being relatively thermostable. There are other observations which suggest the presence of a mixture of ferments in the pancreas preparations and in earlier work we have noticed this with reference to the behavior toward acids. That a mixture of ferments must be concerned in some of the phenomena observed has been suggested by Sörensen[2] and Edie.[3]

It was hoped at the outset of these investigations that it might be found possible to translate tryptic activity as expressed in terms of one standard into terms of another but this does not appear likely, with our present knowledge, because of the presence of mixtures of bodies with unknown properties in the various preparations as practically dealt with. The translation of standards is possible in a general way only, and the tables above gave some idea of the extent to which this is possible. But, while this hope of finding uniform relations can not be realized at present, the studies permit us to make some positive suggestions as to the necessity

[1] Long and Johnson, THIS JOURNAL, **35**, 1188 (1913); Edie, *Biochem. J.*, 8, 84 (1914).

[2] *Biochem. Z.*, 21, 300 (1909).

[3] *Loc. cit.*

of having a better understanding as to the nature of the processes employed in making our commercial preparations. Trypsin must ultimately be defined as a product made in some definite manner and as containing enzymes having definite effects. The trypsin of the physiologist is not much more clearly defined than is the product of the manufacturer. In the statement of properties and behavior put out by the latter there must be some information concerning the content of inorganic substances present, which are doubtless essential factors from certain points of view. A statement of the [H] (or [OH]) concentration in aqueous solution with a certain percentage strength would be of value here and would assist greatly in understanding the reactions referred to by different authors. An illustration of this is found in the interesting experiments of Edie, referred to above, where the substratum was a liquid in some of the cases of ferments described. The ferments themselves made up but a small portion of the liquids used, but there is no information as to the rest of the substance present. Without such information regarding reaction or salts present it is not possible to account for some of the singular results obtained with reference to the behavior toward heat or acids. In our work we have avoided some of this uncertainty by employing relatively strong solid products, where quantities of milligrams only came into play, but we have recognized the need of more information even here. Below, a suggestion will be made as to how this more definite information may be secured.

Resume.

(1) In this paper a comparison of the proteolytic value of six pancreas preparations has been made by four distinct methods, the metacasein reaction, a modification of the Fuld-Gross reaction, the formaldehyde titration of amino acids liberated in digestion, and the fibrin digestion. It was hoped to find such relations as would permit the translation of activity as expressed on a given standard in terms of another.

(2) By the four methods the activities of the six preparations are arranged in the same general order, that is, the strongest ferment by the first method is found to be the strongest by the others. For the weakest preparations the order is about the same.

(3) But the relative rank, quantitatively, of the different ferments is very different as measured by the different methods. While the strongest ferment by the metacasein reaction appears to be about 12 times the strength of the weakest, and about 10 times as strong by the digestion of fibrin, by the other tests the relation is as 2 or 3 to 1. Even greater irregularities appear in comparing some of the other ferments.

(4) It is not possible at the present time to translate the proteolytic value of a tryptic ferment from the terms of one standard to the terms of another, with the products as at present furnished by chemical or phar-

maceutical dealers, because these preparations are made by very different processes of extraction, concentration or activation, which leave, probably, mixtures of ferments in widely different proportions in the finished products, and unknown amounts of inorganic salts.

(5) There is evidence to suggest that the products sold as trypsins or pancreatins contain at least two different enzymes reacting in different ways with proteins. The effects observed in any case are mixed effects depending on the proportions in which the enzymes are present. These enzymes possess different degrees of thermostability.

(6) The desirability of a more rational definition of trypsin is pointed out. The definition should include a statement of the essential points of manufacture and should be authorized by some responsible body such as a pharmacopoeial revision committee. Since what is called trypsin is prepared for the use of medical men, these users are entitled to the fullest knowledge concerning the composition and properties of the product. There is no excuse for secrecy here and products should be made to conform to interchangeable standards.

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STUDIES ON ENZYME ACTION. XI. SOME EXPERIMENTS WITH CASTOR BEAN UREASE.

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Takeuchi,[1] in 1909, found in soy beans an enzyme, urease, capable of hydrolyzing urea into ammonia and carbon dioxide. Keisel[2] and Zemplen[3] then showed that ureases are present in a number of plants. The action of the soy bean urease under various conditions was studied by Takeuchi, Armstrong and Horton,[4] Armstrong, Benjamin and Horton,[5] and more recently by Van Slyke, Zacharias, and Cullen.[6] The application of the soy bean urease to the quantitative determination of urea was first proposed by E. K. Marshall, Jr.[7]

In view of the interest which has been developed in connection with the soy bean urease and its application to analytical work, some experiments

[1] *J. Coll. Agric., Tokyo*, 1, 1 (1909).

[2] *Z. physiol. Chem.*, **75**, 169 (1911).

[3] *Ibid.*, **79**, 229 (1912).

[4] *Proc. Roy. Soc. Lond.*, (*B*) **85**, 109 (1912).

[5] *Ibid.*, **86**, 328 (1913).

[6] *Proc. Soc. Exp. Biol. Med.*, 11, 155 (1914).

[7] *J. Biol. Chem.*, 14, 283 (1913); **15**, 487, 495 (1913); **17**, 351 (1914); 18, 53 (1914) (with D. M. Davis). For the quantitative estimation of urea by urease, cf. also Plimmer and Skelton, *Biochem. J.*, 8, 70 (1914).