PROTEASE AND AMYLASE OF *ASPERGILLUS ORYZAE*

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Contents.

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I. Introduction.

There are at least four large industries in Japan, in the manufacturing processes of which *Aspergillus oryzae* is of the first importance.

According to Lafar[1] the characters of the mould are as follows:—

Aspergillus oryzae, (AHLBUNG) COHN (=*Eurotium oryzae*, AHLBURG) was first identified (as *Eurotium oryzae*) by AHLBUNG in 1876, and was renamed *Aspergillus oryzae* by COHN in 1883, after which it was examined by BÜSGEN, though the full morphological description—by WEHMER—was not given untill 1895. The species produces a luxuriant mould vegetation, which is usually yellow-green (rarely yellow), with large, closely set, tough conidiophores about 2 mm. high. It grows rapidly on a large variety of liquid and solid media, and is easily cultivated, even at room temperature, the optimum temperature being above 30°C. Sometimes after several weeks, or even longer periods according to the environment, the colour gradually turns brown. The peculiarities of the conidiophores, sterigmata and conidia enable the species to be distinguished with comparative ease from most other moulds. This species secretes a very active diastase.

The four industries mentioned above are the manufacture of "sake" (rice wine); "shoyu" (soy sauce); "miso" (soy cheese); and "shochu" (distilled alcoholic liquor). The total yearly quantities of these products are approximately as follows:—

"Sake" (rice wine) 4,500,000 "koku" (=812,000 kilolitres)
"Shoyu" (soy sauce) 5,000,000 "koku" (=902,000 kilolitres)
"Miso" (soy cheese) 450,000,000 "koku" (=1,690,000 kilograms)
"Shochu" (distilled alcoholic liquor calculated as 95 % alcohol) 220,000 "koku" (=39,700 kilolitres)

"Sake" (rice wine) is made from rice only. Steamed rice (80 %) is mixed with rice "koji" (*Aspergillus oryzae* cultured on steamed rice) (20 %) in water containing yeast culture. The principal actions, the saccharification

1) F. Lafar: Technical mycology, (1911), p. 227

of rice starch and the alcoholic fermentation of the saccharified rice, take place simultaneously. The filtered liquid is "sake" generally of the following composition:—

Alcohol (by volume)	18.00 %
Extract	4.55 "
Sugars (as glucose)	0.68 "
Dextrin	0.26 "
Volatile acids (as acetic acid)	0.02 "
Non-volatile acids (as succinic acid)	0.26 "
Glycerol	0.56 "
Ash	0.06 "
Total nitrogen	0.19 "
Specific gravity	0.9945

Owing to the high price of rice, considerable efforts are being made to find a cheaper starch substitute or to reduce the quantity of rice used by adding alcohol during the brewing of "sake".

"Shoyu" (soy sauce) is the most important food seasoning used by the Japanese, in place of vegetable or meat extract and salt, as a relish or condiment to increase the flavour and palatability of the diet. In the manufacture of this product, cooked soy beans are mixed with roasted and pulverized wheat, and *Aspergillus oryzae* is cultivated on them. This mixture is kept in brine water, to be decomposed by the protease and other enzymes produced from the mould and other microbes. After about one year the mash is filtered and the dark brown liquid called "shoyu" results.

The average composition of this sauce is as follows:—

Solid matter	35.24 %
Total nitrogen	1.24 "
Protein nitrogen	0.16 "
Sugars (as glucose)	4.08 "
Dextrin	0.88 "
Volatile acids (as acetic acid)	0.08 "
Non-volatile acids (as lactic acid)	0.93 "
Ash	16.92 "
NaCl	16.19 "
Specific gravity	1.22

"Miso" (soy cheese) is also an important seasoning and a nutritious food. To manufacture it, cooked soy beans are mixed with rice "koji" and salt. This mixture is kept about one year to ripen particularly by

the action of the enyzmes of *Aspergillus oryzae*. The whole of the pasty substance produced is used for cooking.

The average composition of "miso" is as follows:—

Moisture	48.84 %
Crude protein	13.41 "
Crude fat	5.00 "
Sugars (as glucose)	10.48 "
Other nitrogen-free soluble organic matter	4.42 "
Crude fibre	3.27 "
Ash	14.58 "
NaCl	10.71 "

"Shochu" (distilled alcoholic liquor) is made in two ways. One method consists of the distillation of the residue of "sake", the manufacture of which has already been described. The distillate so produced contains about 30 % alcohol and is used as a beverage. The second method is to distil a fermented mash of sweet potato or other suitable starchy substances previously saccharified, usually by the amylase of *Aspergillus oryzae* cultivated on steamed wheat bran or rice bran.

From the foregoing, the importance of the mould *Aspergillus oryzae* in Japanese industries and national life is easily recognized.

The use of *Aspergillus oryzae* as a saccharifying agent in many of the industries of Japan, dates back several centuries. The methods of manufacture are mostly traditional, however, and little is understood of the enzymic properties and the mould growth or of the relations between enzymic production and the mould growth or strains. For instance, for "miso" or "shoyu" manufacture, a mould producing a very active protease is preferred to a strong amylase producer, while the latter is better for "sake" or alcohol manufacture. However, there is no exact method to distinguish between these two, selection being dependent on long experience. The ripening or fermentation of "shoyu" and "miso" takes about one year, and appears to be unduly lengthy for the results obtained.

The enzymes of the mould have still further uses, as digestives in medicine, as desizing agents in the textile industry, and as clarifying agents in the manufacture of fruit jellies, and development of other uses may be reasonably expected. The replacement of malt by the mould culture in the alcohol industries of foreign countries is quite possible, as the amylolytic activity of the bran "koji" (*Aspergillus oryzae* cultivated on steamed wheat bran or rice bran) much resembles that of barley malt, when the same

quantities of raw materials are compared. The cost of bran so treated is much less than that of malted barley; and a mould growth of only two days is sufficient, in comparison with one week necessary for malting barley.

The purpose of the experiments described in this paper was to ascertain some of the principal conditions regulating the formation of protease and amylase by *Aspergillus oryzae*, and to study the properties of both enzymes with a view to their industrial application.

ACKNOWLEDGEMENT.

These experiments were carried out by the author over a period of several years—during 1918–1920 in the Takamine Laboratory, Inc., Clifton, New Jersey, U. S. A.; 1920–1921, in the laboratories of the Bureau of Animal Industry and the Microbiological Laboratory of the Bureau of Chemistry, U. S. Department of Agriculture; 1922, in the Laboratory of Applied Mycology, Hokkaido Imperial University; and, 1922–1925, in the Chemical Laboratory of the School of Fishery, in the same University.

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II. Methods used for the Quantitative Estimation of Amylase and Protease, a Formula for determining Digestibility, and Precautions in the Use of Van Slyke's Method.

In this investigation several new methods and precautions have been adopted for the purpose of solving many conspicuous problems with the greatest possible accuracy, ease and rapidity.

The three methods for estimating starch-saccharifying, starch-liquefy-

ing and proteolytic enzymes, the formula for the determination of digestibility and the precautions found necessary in the use of Van Slyke's method are first described to save unnecessary repetition in each experiment.

A. A New Method for the Quantitative Estimation of Starch-saccharifying Enzyme.

1. Earlier methods.

The most practical method for estimating starch-saccharifying enzyme, is that of LINTNER (48). The principle of this method is based upon the colour change of FEHLING's solution by the reducing sugar produced from soluble starch by amylase. This method was criticized as inaccurate by SHERMAN and his associates (85), firstly because there are only ten test tubes, with which an accurate determination can be made in the first operation; and secondly because the probable error of the method increases rapidly with the amylolytic power of the sample. Thus if the end point falls between the last two tubes (0.9 cc. and 1.0 cc.) the amylolytic power will be between 10 and 11.1, but if it falls between the first two tubes (0.1 cc. and 0.2 cc.) the amylolytic power may be anywhere between 50 and 100. LINTNER's method can be further criticized as being neither convenient nor quick enough for practical tests with many samples. For example, to test ten samples, one hundred test tubes are necessary, and the various quantities of the amylase solutions have to be measured a hundred times at definite intervals of time.

LING (47) modified LINTNER's method making it easier, but even in the modified form, it remained slow and inexact.

To obviate these difficulties, SHERMAN and his associates (85) proposed a method which depends upon the gravimetric estimation of copper oxide. This process is also, however, too laborious for general tests.

EULER and SVANBERG's method (19), which is probably the most scientific, consists in estimating the sugar produced several times during the digestion and comparing the velocity constant of the amylolytic action. But this method is not entirely satisfactory, being even more troublesome and slower than SHERMAN's.

The author's new method has been found very convenient and useful. It eliminates the defects above described, and was used in most of the experiments described in this paper. This method was proposed six years ago and has been published in the Journal of Ind. and Eng. Chem. 1920 (66), and subsequently in other journals (67) in Japan. Some corrections

based on the experience gained in more exact tests have been added.

2. Proposed Method.

a. Apparatus.

1. ERLENMEYER's flasks of about 200 cc. capacity.
2. A constant water bath of 40°C. (± 0.5°C.)
3. Test tubes of various sizes.
4. A test tube rack holding at least ten tubes.
5. A water bath in which to immerse the above rack.

b. Solution.

1. A two per cent aqueous solution of soluble starch. This solution should be practically free from reducing sugar. The reaction must be adjusted with NaOH or HCl to make it neutral or within the range of 1 cc. of 0.01 normal NaOH or HCl per 100 cc. of the solution, rosolic acid being the indicator.
2. Solution of 0.2 N NaOH.
3. FEHLING's solution.
4. Enzymic sample. In case of solid sample, it must be first extracted with distilled water for one hour if from a mould culture, and for three hours if from ground malt, 1 to 10 grams of the sample being usually extracted with 100 cc. of water and 1 cc. of toluol. If the enzyme is too strong, the solution is further diluted with a known quantity of distilled water.

c. Procedure.

1. Into a flask containing 100 cc. of the 2 % soluble starch solution, which has been placed in a water bath at 40°C., 10 cc. of the enzymic extract is introduced and the flask is allowed to remain in the bath for 30 min. At the end of that period, 10 cc. of 0.2 N NaOH is added to stop enzymic action at once. If evaporation of the digested liquid is prevented, the rest of the experiment may be postponed several days.
2. Graduated quantities (for example 5, 7, 10, 14 and 20 cc.) of the digest are then poured into a series of test tubes kept in a rack, each containing 5 cc. of FEHLING's solution, and the tubes in the rack are placed in a boiling water bath for 10 min., the tubes

being shaken once during that period. By observation of colour change – blue to red – the smallest quantity of the digest which just reduces 5 cc. FEHLING's solution is determined. It may be more convenient to use at first only 1–3 tubes containing widely varying quantities of the digest, so as to determine the approximate concentration of the digest to be used. The reduction of FEHLING's solution is then carried out within the narrower limits of this approximate value.

3. Knowing the amount of the digest which will just reduce 5 cc. of FEHLING's solution, the value of starch-saccharifying activity can be calculated, as will be shown later. The exact value is obtained by dividing the value found in the scale by the percentage of the solid or liquid sample in the diluted enzymic solution.

d. Scale of starch-saccharifying value.

As LINTNER's is the most commonly used scale, it seemed convenient to express the starch-saccharifying activity by LINTNER's unit.

It was found that with a definite concentration (i. e., the standard solution) of *Aspergillus* enzyme solution tested by LINTNER's method at 40°C. for one hour, 0.365 cc. (average of 0.36 and 0.37 cc.) of the solution was necessary for the reduction of 5 cc. of FEHLING'S solution. By LINTNER's scale the starch-saccharifying value is 100 when 0.1 cc. of enzymic solution is used. In this case:

0.365 : 0.1 = 100 : starch-saccharifying value of the solution.

When the concentration of the enzymic solution is not 5 % as used by LINTNER but Y %, we find that

$$\text{starch-sacchrifying value of the original sample} = \frac{100 \times 0.1}{0.365} \times \frac{5}{Y} = \frac{137}{Y}$$

By the author's new method, 9 cc. of the digested soluble starch solution were required to reduce 5 cc. of FEHLING's solution. Consequently the starch-saccharifying value of any enzymic[1] solution is also $\frac{137}{Y}$, if 9 cc. of the digest reduce 5 cc. of FEHLING's solution by the new method.

This standard enzymic solution was diluted twice with water and tested by the new method, when 15 cc. of the digest was required. The calculation of the starch-saccharifying value for the diluted solution is as follows:—

Starch-saccharifying value × Y/2 = 137/2 = 69.

In the same way the standard enzymic solution was diluted or concentrated to various strengths, and the quantity of digest required to reduce

5 cc. of FEHLING's solution by the new method was determined, the results being compared with the concentration of the enzymic solutions used. The saccharifying values falling between the experimental values were calculated proportionally and the following table was compiled.

The experiments in the following may also serve as examples to show how the calculations were made.

Table 1. Scale of starch-saccharifying values for amylase of *Aspergillus oryzae* and related moulds (40°C.)

Cc. of digested starch solution required	Starch-saccharifying value × Y	Cc. of digested starch solution required	Starch-saccharifying value × Y
3.50	576	10.5	110
x 3.60	548	x 11.0	103
3.75	507	11.5	96
x 4.00	438	12.0	92
4.25	397	12.5	88
4.50	356	x 13.0	84
4.75	329	13.5	80
5.00	302	14.0	76
x 5.25	274	14.5	72
5.50	260	15.0	69
5.75	246	16.0	64
6.00	232	17.0	59
x 6.25	219	x 18.0	55
6.50	210	19.0	52
6.75	202	20.0	49
7.00	194	x 22.0	44
7.25	186	24.0	38
x 7.50	178	x 25.0	36
7.75	171	26.0	33
8.00	164	28.0	29
8.25	157	29.0	27
8.50	150	30.0	25
8.75	143	32.0	22
9.00	137	34.0	20
9.25	132	x 36.0	18
9.50	127	40.0	16
9.75	123	x 43.0	14
10.00	119	50.0	11
		60.0	8

N. B. 1. The unit of value is the same as that of LINTNER.
2. Y = % of tested solution with respect to the original sample.
3. x = Experimental value.

This table can be used for every form of amylase obtained from *Aspergillus oryzae* or related mould, as will be shown in section IV. If some reducing sugar is present in the sample, it is necessary to correct the value obtained by a blank test.

If the sample of amylase is of different origin, the scale may have to be changed somewhat, since the course of digestion may differ according to the source of the enzyme. For example the final product of the decomposition of starch by *Aspergillus oryzae* is glucose, but with malt amylase the final product is maltose.

The same procedure was followed in developing a scale for the amylase of barley malt and that of similar cereals, using an aqueous solution of the enzyme obtained from the malt extract by precipitation with alcohol.

Table 2. Scale of starch-saccharifying values for the amylase from barley malt and from similar sources (40°C.)

Cc. of digested starch solution required	Starch-saccharifying value × Y	Cc. of digested starch solution required	Starch-saccharifying value × Y
x 3.25	680	7.75	157
x 3.5	540	8.0	153
x 3.75	440	8.25	148
x 4.0	340	8.5	144
4.25	305	8.75	139
x 4.5	270	x 9.0	135
4.75	262	9.25	132
5.0	254	9.5	129
5 25	245	9.75	126
x 5.5	237	10.0	124
5.75	229	10.5	118
6.0	212	x 11.0	113
6.25	201	11.5	107
6.5	191	12.0	102
6.75	180	12.5	96
x 7.0	170	x 13.0	91
7.25	166	13.5	88
7.5	162	14.0	85

Cc. of digested starch solution required	Starch-saccharifying value × Y	Cc. of digested starch solution required	Starch-saccharifying value × Y
14.5	82	x 30.0	34
15.0	79	32.0	31
16.0	73	34.0	28
17.0	68	36.0	25
18.0	63	38.0	21
19.0	58	40.0	18
x 20.0	54	45.0	16
22.0	50	50.0	14
x 25.0	44	60.0	10
27.0	38	70.0	7

N. B. 1. The unit of these values is that of LINTNER.
2. Y = % of tested solution with respect to the original sample.
3. x = experimental value.
4. The scale of saccharifying values has been improved several times. The above scale, which is now published for the third time, is based on experiments made with enzyme obtained from malt extract by precipitation with alcohol, and with buffer salts in the starch solution. The scale as first published (66), was the result of experiment with malt extract but without buffer salts. The experiments on which the second publication of the scale (67) was based, were made with malt extract and with buffer salts.

This table may be used for the malt amylase of barley and of other cereals. Accurate results are obtained by the use of from 5 to 30 cc. of the digested starch solution which exactly reduce 5 cc. of FEHLING's solution. If the digest does not fall within the limits (5–30 cc.), it is better to repeat the test with another concentration of the amylase solution. By practice the number of test tubes required to determine the quantity of digest necessary for the reduction of exactly 5 cc. of FEHLING's solution can be lessened, and thereby an economy of both time and labour is effected.

This new method for estimation of starch-saccharifying activity, the author believes to be the simplest, easiest, and most accurate, compared with other methods already published. It is applicable to various cases of amylase tests, as shown at various points in this dissertation.

Some defects of this method may be cited as follows:

1. Difficulty of judging the shade in the limiting tube which reduces just 5 cc. of FEHLING's solution.
2. Arithmetical proportion was adopted to decide saccharifying values falling between the experimental values found instead of mathematical interpolation.

3. A new scale must be made to determine saccharifying values of amylases of different origin, although no large differences may occur experimentally.

The following experiments will increase the value of the new method for many purposes.

a. Time Effect on the Scale.

The new method was employed in the following experiment always with a definite concentration of *Aspergillus* amylase but with different digesting periods. The following relations were obtained:—

Digestion period (hours)	Digest required (cc.)	Starch-saccharifying value	Time factor	Starch-saccharifying value × time factor
1/4	35.0	19	2/1	38
1/2	23.0	41	1/1	41
2/3	16.0	63	2/3	42
1/1	13.0	84	1/2	41
5/2	9.75	123	1/3	41
2/1	8.0	164	1/4	41

These results show that the saccharifying value is proportional to the digestion period. Therefore, if in order to test one enzymic sample it is digested for $1\frac{1}{2}$ hours, then the saccharifying value should be divided by three to obtain the general saccharifying value, which can be obtained by 30 minutes digestion. This correction is quite convenient for comparing results obtained with different digestion periods.

b. Reaction of Starch Solution.

In determining enzymic action the reaction of the digesting medium must be carefully ascertained, as enzymic activity varies very widely with the reaction of the medium. Moreover, as shown later (VII, appendix, A), the hydrogen ion concentration of starch solution is easily changed by addition of acid or base. Thus the determination of hydrogen ion concentration has become universally recognized as the most satisfactory method of obtaining this value.

To ascertain the optimum reaction for *Aspergillus* amylase the following experiment was made:—

The soluble starch solution used had an acidity equivalent to 2.2 cc. of 0.01 N NaOH per 100 cc. of 2 % soluble starch solution, with rosolic acid as indicator. The *Aspergillus* amylase used was a 0.2 % aqueous solution of a purified sample. The regular procedure of the new method was adopted, except that 5 cc. of the enzymic solution and 5 cc. of 0.01 N NaOH and H_2O were used instead of 10 cc. of enzymic solution.

Table. Starch-saccharifying values at different reactions of starch solution.

	2% sol. starch (cc.)	0.01 N NaOH (cc.)	H_2O (cc.)	0.2% amylase (cc.)	Digest required (cc.)	Starch-saccharifying value × Y	Starch-saccharifying value
1	100	0.0	5.0	5.0	9.0	137	1,370
2	„	1.0	4.0	„	8.5	150	1,500
3	„	2.0	3.0	„	10.0	119	1,190
4	„	3.0	2.0	„	14.0	76	760
5	„	4.0	1.0	„	17.0	56	590

N. B. The starch-saccharifying value was calculated with Y/2 % instead of Y %, because 5 cc. of enzymic solution were used here.

The above results show that the optimum reaction occurs when 1 cc. of 0.01 N NaOH is added to 100 cc. of 2 % sol. starch, i.e., when the acidity of the starch solution requires 1.2 cc. of 0.01 N NaOH to neutralize 100 cc. of the solution.

An exact method for determining the optimum hydrogen ion concentration is described later (VI, A, 1, a), and the result shows pH 4.5–5.2 as generally the optimum for *Aspergillus*, malt and related amylases. Na-citrate or phosphate may be used as regulating salts, when a stronger (0.5 N) NaOH is necessary to stop the digestion. By this method, the scale of amylo-saccharifying values was made. However, for routine work, it was found after many experiments, that the regulation of the acidity of the starch solution by the use of NaOH alone was sufficient.

B. A New Method proposed for the Quantitative Estimation of Starch-liquefying Enzyme.

Amylase or diastase is not considered as one simple enzyme. Amylases of different origins often show different velocities of action when their starch-liquefying, dextrinizing and saccharifying activities are compared. Therefore many stages of starch decomposition must be examined if an exact investigation of amylases to be made.

To test starch-liquefying enzymes, the following methods may be found most valuable.

WOHLGEMUTH (107) tested the enzymic action after digesting soluble starch, by means of iodine, which is really a method for the test of decomposition of soluble starch into dextrin, but not of starch-liquefaction.

TAKAMINE (69) ten years earlier than WOHLGEMUTH also applied the same principle but used an easier though less accurate method.

OLSSON's (64) method is to estimate the ascending velocity of a light glass ball in digested starch paste. This method seems comparatively

accurate, but the apparatus is somewhat difficult to make.

EFFRONT's (16) method is based on the insolubility of starch paste in iodine solution in which the digested product dissolves. This method though simple is not quite accurate.

Many experiments were tried for the purposs of obtaining a better method. Finally, as the result of experiments made by the author with the assistance of Mr. SHINICHI ITAYA, the following method, which has already been published in two Japanese journals (70), is proposed.

Proposed method.

Make 450 cc. of starch paste with 7.5 grams (as dry matter) of purified potato starch. To 18 cc. of this paste add 2 cc. of a regulating mixture of M/6 citric acid and M/6 Na_2HPO_4 to keep a certain hydrogen ion concentration, and digest with 2 cc. of enzymic solution for 30 min. at 40°C. Then add 2 cc. of N/2 NaOH to stop the digestion. Add further 2 cc. of M/6 citric acid and M/6 Na_2HPO_4 mixture, making the total, 2 cc. M/6 citric acid and 2 cc. Na_2HPO_4. Determine the viscosity of digested liquid at 18°C. with OSTWALD's viscosimeter of 8 cc. capacity in which distilled water flows down in 15.0 seconds. With this calculate the enzmyic strength from the table shown below.

This table was made experimentally with different concentrations of an enzymic preparation from *Aspergillus oryzae*. When 1 % solution of an enzymic sample digests 1.5 % starch paste at 40°C. in 30 min. and the time of flow is 47 seconds (which is the same viscocity as for 1 % unconverted paste) then the starch-liquefying value is 10.

Table 3. Scale of starch-liquefying values.

Seconds of flow	Starch-liquefying value × Y	Seconds of flow	Starch-liquefying value × Y
x 20	100.0	x 28	33.3
21	87.5	29	28.0
22	75.0	30	27.0
23	62.5	31	26.0
x 24	50.0	x 32	25.0
25	48.0	33	22.5
26	46.1	x 34	20.0
x 27	40.0	35	19.4

Seconds of flow	Stareh-liquefying value × Y	Seconds of flow	Starch-liquefying value × Y
36	18.8	57	4.8
x 38	16.6	x 60	3.1
40	15.5	x 62	2.5
x 43	12.5	x 65	1.6
45	11.2	67	1.2
x 47	10.0	x 69	0.8
x 50	8.3	x 72	0.4
x 53	6.3	x 75	0.2
x 55	5.0	x 81 (no digestion)	0.0

N. B. Y=% of enzymic solution.
x=experimental value.

This table can be used for amylases from moulds and cereals, as the experiment with the amylases from *Aspergillus* and malt showed almost the same result. If a viscosimeter of different flowing velocities is used, then this table may be used by calculating the relative velocities.

Absolute accuracy cannot be claimed for the method, but at present a better one does not seem to be available. The viscosity of starch paste varies according to the starch used, its treatment and period preserved.

The optimum hydrogan ion concentration of a starch solution to secure the greatest activity of the starch-liquefying enzyme from *Aspergillus oryzae* is pH 4.5–5.2 as shown later (VI, A, 1, c), and may be recognized as the same as that of the starch-saccharifying action.

C. A New Method for the Quantitative Estimation of Protease.

For the determination of the proteolytic powers of substances containing proteases (pepsin, trypsin, etc.) many different methods have been used. Some of these may be grouped as follows:–

1. The enzyme acts upon an insoluble protein and the rate at which the latter is digested into soluble products is observed—methods of GRÜNHAGEN (32), GRÜTZNER (33), METT (53), PALLADIN (78).

2. The enzyme acts upon a solution or suspension of protein and the time required to carry the digestion to a definite stage, or the amount of protein remaining undigested at the end of a definite time, is determined —methods of ALLEN (4). EINHORN(17), FULD and LEVISON (28), FULD and GROSS (31), ROBERTSON (79), ROSE (82), WITTE (106).

3. The enzyme acts upon protein or polypeptid, and the cleavage

products are determined by chemical or physical methods—methods of ABDERHALDEN (1), ALLEN (4), HEDIN (31), KOBER (41), KOELKER (42), SCHÜTZ (84), SÖRENSEN (90), VOLHARD (100).

4. The enzyme is allowed to act on a protein solution, and the progress of the digestion is measured by increase of electrical conductivity or decrease of turbidity or viscosity of the solution—methods of BAYLISS (6), HATA (35), LIEBERMANN (46), SPRIGGS (92).

None of the above was quite satisfactory for making comparison between many samples of mould extract, as will be described later.

The author's method, as described below, was based, mainly, on FULD and GROSS's (31) method for trypsin. Prior to some small changes made in connection with these experiments, this work had already been published in American and Japanese journals (67) (68).

Short description of the method of FULD and GROSS.

Principle: Undigested casein in a solution will be precipitated by acetic acid alcohol solution, but after complete digestion no precipitation takes place.

Solutions required: 1. One tenth per cent of casein solution. 2. Acetic acid alcohol solution—One part of glacial acetic acid mixed with 49 parts of water and 50 parts of alcohol.

Procedure: Ten test tubes are prepared with the following different quantities of enzymic solution.

1. 1 cc. of original enzymic solution.
2. „ „ 1/2 dilution of original enzymic solution.
3. „ „ 1/4 „ „ „ „ „
4. „ „ 1/8 „ „ „ „ „
5. „ „ 1/16 „ „ „ „ „
6. „ „ 1/32 „ „ „ „ „
7. „ „ 1/64 „ „ „ „ „
8. „ „ 1/128 „ „ „ „ „
9. „ „ 1/256 „ „ „ „ „
10. „ „ 1/512 „ „ „ „ „

Add 2 cc. of 0.1 % casein solution to each tube and keep in a constant water bath at 30°C. for one hour. Then add 6 drops of acetic acid alcohol solution to each tube to precipitate undigested casein. Take the tube which is clear and contains the least quantity of enzyme, and calculate the proteolytic power.

Calculation: If 0.016 cc. of original enzymic solution is required to digest 2 cc. of 0.1 % casein solution, then the proteolytic power is:

$$0.016 : 2 = 1 : x \quad \therefore \quad x = \frac{2 \times 1}{0.016} = 125$$

Therefore the enzymic solution contains 125 units of protease. One unit of protease by this method is the proteolytic activity of its sample capable of digesting 1 cc. of 0.1 % casein solution in one hour at 38°C.

Proposed method.

1. Solution.

a. One tenth per cent of casein solution—0.5 gram casein (as dry matter) are mixed with 200 cc. of water and 15 cc. of 0.1 N NaOH and warmed to dissolve. After cooling, the solution is neutralized with 0.1 N HCl, using phenolphthalein as indicator on porcelain plate. About 5 cc. of 0.1 N HCl is required and 1 cc. of 0.1 N NaOH is added to make 0.002 N NaOH reaction and the whole made up to 500 cc. with water. The casein is made by HAMMERSTEN's method.

b. Mixture of magnesium sulphate and nitric acid solution– 4 parts of saturated magnesium sulphate solution mixed with 1 part of concentrated nitric acid.

2. Procedure.

To four test tubes with 5 cc. of 0.1% casein solution immersed in water bath at 40°C. add 0.2, 0.5, 1.5 and 4 cc. of protease solution respectively. Mix well and after one hour add 0.5 cc. of mixture of nitric acid and magnesium sulphate solution to each tube.

The limit between incomplete and complete digestion of the specimens can be readily seen in the tube. This limit is indicated by a gradation from an opaque appearance in the solution, where the digestion is incomplete, to a transparent appearance in the case of complete digestion. The protease solution may then be diluted in proper ratio to suit the next test.

Using the same procedure but with the following quantities, 0.2, 0.3, 0.45, 0.7 and 1.0 cc., the protease is again tested. The closer and smaller quantities permit more exact reading of the opacity of the solutions, which is desirable after the preliminary tests. The two points between which opacity changes to transparency permit the proteolytic activity of a given sample to be approximated. In order to obtain a more accurate figure, 5 cc. of 0.005% casein solution may be used as representing complete digestion.

3. Calculation.

To express the proteolytic activity, the following unit was devised. If 0.005 gram (or cc.) of an original enzymic substance digests completely 5 cc. of 0.1% casein solution (0.005 gram) in one hour at 40°C., then the proteolytic value of this substance is 100.

This method differs from that of FULD and GROSS in the following points:–

1. The reaction of casein solution was made optimum for the protease tested (see foot note).

2. A different procedure and different unit of proteolytic value were used in order to make the test simpler and quicker.

3. Undigested casein is precipitated with nitric acid and magnesium sulphate mixture instead of acetic acid alcohol solution. The latter, in some cases, seems to precipitate casein incompletely. The mixture of nitric acid and magnesium sulphate precipitates natural casein completely but peptone does not at all.

Formerly 0.5 % casein was used, but in many cases 0.1 % casein was found more convenient. Calculation of the proteolytic value is made in the same way but experiment shows the proteolytic value with 0.5 % casein is only about half that with 0.1 % casein. Therefore it is better to state the percentage of casein used, when it is other than 0.1 %.

This method is not sufficiently accurate but the quickness and ease of the procedure make it very useful.

Optimum Reaction of Casein Solution in order to obtain Maximum Activity in *Aspergillus* Protease.

It was found that there are different optimum reactions for casein digestion by *Aspergillus* protease, according to the methods of casein liquefaction and amino acid production.

The experimental data are given later (V, C, 1, c). For casein liquefaction, the optimum pH is 8.0, for amino acid production, 6.2. Actions of buffer solutions, such as borate, phosphate and NaOH were compared. With the first two, the casein liquefaction was weaker than with NaOH alone. Therefore in the experiments that follow only NaOH was used to make the casein solution optimum for protease.

To decide this quantity of NaOH, the following experiment was made:—

After the neutralization of casein solution, with phenolphthalein as indicator, to each 500 cc. of the solution containing different quantities of NaOH as shown below, 0.5, 0.6, 0.7, 0.8 and 0.9 cc. respectively of 0.05 % purified protease of *Aspergillus* were added to 5 tubes. Procedure was as described in Section II, C.

Table. Proteolytic values at different hydrogen ion concentrations.

Cc. of 0.1 N NaOH in 500 cc. 0.1 % casein	pH before digestion	Minimum quantity of enzyme sol. to digest casein completely	Proteolytic value
0.0	7.5	0.8 cc.	1,250
0.5	7.6	0.7 „	1,428
1.0	7.7	0.6 „	1,666
2.0	7.9	0.7 „	1,428
4.0	8.2	0.9 „	1,111

The optimum reaction of the casein solution was attained by addition of 1.0 cc. of 0.1 N NaOH to 500 cc. of 0.1 % neutral casein solution. If the method is used for protease of another origin, the optimum reaction should be first determined.

D. Formula for Determination of Digestibility with Protease

To express the digestibility the following formula was used:—

$$\text{Digestibility} = \frac{x}{a} \times 100$$

x = digested quantity.

a = quantity used for digestion.

To determine "x" and "a", the following data were calculated at the estimation of α-amino nitrogen by VAN SLYKE's method.

x = Nd − Nb − Ne − Np

a = Nt − Np

Nd = mgm. amino nitrogen in the digested liquid.

Nb = mgm. nitrogen of blank test with water and capryl alcohol.

Ne = mgm. amino nitrogen produced by the self digestion of enzyme solution only.

Np = mgm. amino nitrogen contained in protein solution before digestion.

Nt = mgm. amino nitrogen from same quantity of protein, digested by strong HCl for 24 hours.

E. Influence of Toluol, Amyl Alcohol, Capryl Alcohol and Di-phenyl Ether upon the Determination of α-amino Nitrogen by Van Slyke's Method.

VAN SLYKE (98) stated in his paper on the quantitative determination of aliphatic amino groups, that amyl alcohol, which in the original description of the method was recommended to prevent the foaming of viscous solutions, must be replaced for this purpose by capryl alcohol. MITCHEL and ECKSTEIN (57) recommended di-phenyl ether, as the substitute for capryl alcohol, which, however, was not imported into U. S. A. during the World War. Owing to temporary shortage of this chemical, the author made some tests with amyl alcohol and di-phenyl ether, and also used toluol as antiseptic for digesting liquids. In blank tests they showed no appreciable error. To ascertain any possible error, the following experiment was tried.

Solution required.

1. One-tenth normal leucin solution: 1.3119 grams of air-dry leucin were dissolved in 100 cc. of water containing 4 cc. of conc. HCl.

2. Digested peptone solution: 100 cc. of 3 % Witte peptone, 30 cc. of 1 % *Aspergillus* protease and 20 cc. of regulating mixture (N/1 CH_3-COOH 7 cc., N/1 CH_3COONa 30 cc. and H_2O 63 cc., which made the whole solution to pH 6.2) were mixed and digested for 4 hours at 50°C.

3. Capryl alcohol (Kahlbaum Co.).

4. Di-phenyl ether (Eastman Kodak Co.).

Procedure.

Same operation as decribed by VAN SLYKE (98).

Table 4. Amount of amino-nitrogen produced with different form inhibitors and with toluol.

Composition of samples (cc.)		(cc.)	Temperature (°C.)	Pressure (mm.)	Amino N. (cc.)	Amino N. (mgm.)
H_2O	10		23.0	767	0.44	0.25
„	10	amyl alc. 1.0	25.0	749	0.80	0.44
„	8	toloul 2.0	23.0	746	0.90	0.50
„	10	capryl alc. 0.3	24.0	775	0.51	0.29
N/10 leucin	10	— —	23.0	767	24.12	13.65
„	5	— —	„	„	12.30	6.96
„	10	amyl alc. 0.5	„	765	26.50	51.00
„	5	„ „ „	„	760	13.47	7.55
N/10 leucin	10	toluol 0.2	23.0	767	25.92	14.67
„	5	„ „	„	766	13.30	7.51
„	10	toluol 0.2 amyl alc. 1.0	22.5	„	26.48	51.00
„	5	amyl alc. 1.0	22.5	„	13.60	7.70
„	5	toluol 0.4	23.0	„	13.30	7.51
„	10	capryl alc. 0.5	23.0	774	24.02	13.75
„	10	— —	„	„	23.94	13.73
Dig. peptone	10	capryl alc. 0.3	24.0	775	21.78	12.40
„	10	di-phen. ether 1.0	„	„	22.05	12.55
„	10	amyl alcohol 0.5	„	„	22.55	12.83
„	10	toluol 0.3	„	„	23.15	13.17
„	10	cap. alc. 0.3 + toloul 0.2	„	„	22.71	12.91

The result shows that the addition of amyl alcohol and toluol distinctly increase nitrogen gas, although their effects in blank tests are almost nil. Increase of nitrogen gas by amyl alcohol is about 5-6 % and that of toluol a little less. As di-phenyl ether solidifies easily at these temperatures, its use was discontinued. As tests with capryl alcohol were found to be reliable, it was used in these experiments.

Toluol causes error in quantitative experiments, so a short digestion period of 4 hours without antiseptic was adopted instead of a period extending over a few days. It may, however, be allowed that the preliminary experiments with toluol as antiseptic have some value in showing the relative amount of amino nitrogen produced during digestion.

III. Enzymes found in *Aspergillus Oryzae*.

It is certain that an organism such as *Aspergillus oryzae* produces many kinds of enzyme. As the methods for making quantitative estimation of enzymes are not yet generally precise, it is difficult to know the quantity of these enzymes or even the strength of their enzymic activities. The detection of enzymes qualitatively is, however, comparatively easy and the more investigations that are made, the more enzymes will be discovered.

Up to the present, the following enzymes have been found to be present in *Aspergillus oryzae*.

Enzyme	Author	Date	Reference
Amylase	KORSCHELT	1876	(43)
Protease	VINE	1909	(99)
Maltase	WOHLGEMUTH	1912	(107)
Chymase	"	"	"
Lipase	"	"	"
Erepsin	"	"	"
Haemolysin	"	"	"
Invertin	MATSUYAMA and SAITO	1915	(52)
Glucosidase	"	"	"
Peroxydase	"	"	"
Katalase	"	"	"
Sulphatase (Phenol-SO_4→H_2SO_4)	KURONO	1924	(44)
Inulase (Inulin→fructose)	TAKAHASHI	"	(94)
Lactase	NEUBERG and ROSENTHAL	"	(59)
Nuclease (Nucleic acid→Phosphoric acid)	NOGUCHI	"	(61)

Not only must the different kinds of enzymes present in *Aspergillus oryzae* be known, but also if technical applications for them are to be found out, their quantity and behavior, and the influence of various conditions on their production or upon their actions must be thoroughly appreciated.

Among these enzymes, protease and amylase will probably prove the most important in industry. The detailed investigations given here were made particularly on these two enzymes with this point in view.

IV. Conditions influencing Enzyme Production.

There are many factors which influence the growth of *Aspergillus oryzae* and the enzyme production.

In many cases, improvement of growth of mould and of enzyme production are contemporaneous, but this is not absolute as will be shown later (IV, C).

Temperature, duration, aeration and other operations of culture; material, moisture and reaction of culture media; strains of the mould, different treatment of spore used; all of these may have important influence upon the growth of the fungus. In industrial practice, care is required in regard to the mixture of various strains of mould and also in regard to contamination, both as to the nature and quantity of the various microbes that may be introduced from various sourses.

Some of the problems studied to learn about conditions influencing enzyme production are as follows:—

A. Comparison of Endo- and Exo-enzymes produced during Fungous Growth.

On this subject, there are a few studies, Dox (14) with *Penicillium camemberti*, Young (109) and Funke (29) with *Aspergillus niger* being the chief writers. They came to the common conclusion that enzymes are present in the mycelium of the fungus, in the greatest amount at the period of its sporulation and that they rapidly excrete after that time. No experiment has been reported on this subject with *Aspergillus oryzae*.

EXPERIMENTAL.

Composition of the culture medium.

"Teruuchi peptone" (which contains as much amino acid as peptone)

	25 grams
Pure glucose	25 grams
5 % $MgSO_4 \cdot 7\ H_2O$	10 cc.
0.01 % $FeSO_4$	,,
5 % KCl	,,
M/3 KH_2PO_4	100 cc.

These were mixed together and made with water to one litre, heated and filtered. 20 cc. of the mixture were placed in each 150 cc. ERLENMEYER's flasks with cotton plug inserted, and sterilized in an autoclave for 10 min. at 110°C. *Aspergillus oryzae* No. 119 having previously been separated from "moyashi" (mould ferment) was used in this investigation and incubated at 30°C. It grows well with abundant spores, and produces strong amylase and protease.

The enzymes in the mycelium (=endo-enzyme) were tested after treating with acetone, as ALBERT and BUCHNER (3) tried with yeast, and as also DOX (14) for the endo-enzymes of *Penicillium*. The method of testing endo- and exo-enzymes as adopted for this particular experiment was as follows:—

The mycelium from four flasks was removed after certain hours of cultivation, washed twice with water quickly, then squeezed to remove as much as possible of the water, and torn apart or teased out with the fingers. The wet mass thus obtained was immersed for 10 min. in a large volume (about 100 cc.) of acetone with constant stirring; then filtered off with suction. It was immersed again for 2 min. in a fresh quantity of acetone and filtered off as before, then immersed for the third time in ether for 3 min., dried as much as possible, by suction and afterwards in a desiccator.

This dry mycelium was weighed, ground finely in a mortar and extracted with water of 100 times its weight for 2 hours. The extract was then filtered through filter paper and tested by the author's methods for its starch-saccharifying and proteolytic activities.

A check experiment was tried for any decrease of enzymic activity by the acetone treatment, comparing it with sample obtained by grinding with sand. No remarkable difference was noticed.

The exo- (extracellular-) enzymes were tested with the culture liquid of the same four flasks, mixed with the water used to wash the mycelium and filled up to a certain volume.

The total enzymic activity for endo-enzymes was estimated on the basis of weight of the mycelium grown, and for exo-enzymes on the basis of the volume of liquid culture medium (80 cc. from four flasks).

The growth and sporing were always remarked by appearance.

Table 5. Amount of enzymes produced during fungous growth.

Culture period	Growth	Spores	Weight of mycelium	Endo-enzymes				Exo-enzymes			
				Total quantity		For 1 gram of mycelium		Total quantity		For 1 gram of mycelium	
				Starch-sacchari. value	Proteolytic value	Starch-sacchari. value	Proteolytic value	Starch-sacchari. value	Proteolytic value	Starch-sacchari. value	Proteolytic value
42 hrs.	good	none	1.005 grams	49	31	49	31	32	24	32	24
48 „	„	few	1.184 „	82	79	69	67	72	56	61	47
54 „	„	„	1.470 „	123	147	84	100	120	64	82	44
60 „	„	„	1.415 „	146	160	103	113	264	128	187	90
66 „	„	light yellow	1.315 „	111	164	64	125	296	232	225	177
72 „	„	half yellow	1.260 „	79	156	63	124	288	384	229	305
78 „	„	almost yellow	1.215 „	56	152	46	126	312	396	258	327
4 days	„	complete yellow	0.950 gram	15	119	15	125	312	384	328	404
5 „	„	brown	0.875 „	10	58	11	66	312	304	357	348
7 „	„	dark brown	0.756 „	8	35	11	47	312	192	413	254
11 „	„	„	0.756 „	8	18	11	24	312	56	413	74

pH of the medium: before culture = 5.2
after 4 days = 8.2
after 7 days = 8.4

The supposition that *Aspergillus oryzae* produces exo- and endo-enzymes is accepted provisionally. The exo-enzymes can be obtained by percolating the mature mould with water, but the endo-enzymes can not be obtained by mere treatment with water. Previously, experiments had been made to obtain the endo-enzymes from "koji" residue, i. e., *Aspergillus oryzae* cultivated on a large scale on some solid substratum, and the whole washed free of exo-enzymes, by treating with many solvents, but the results were always negative. Experiments here show that exo- and endo-enzymes are the same in quality and that after spore formation, almost all enzymes have been excreted into the culture medium. It is also shown that the increase and decrease of enzymes in the mycelium is rapid, but in the culture medium it is slow and the maximum quantity does not decrease even after the mycelium has autolysed, if the contents are kept sterile in normal reaction and preserved properly.

The maximum production of exo-enzymes occurs about ten hours later than that of endo-enzymes.

The decrease of protease in this experiment is supposed to be due to the increase of alkalinity of the medium, which is more injurious to *Aspergillus* protease than to the amylase, as shown later (V, E.).

It is improbable that the excretion of enzyme is due to the autolysis of the mycelium, because at the time of the most rapid excretion of enzymes, the weight of mycelium is found to be increasing, and the maximum production of exo-enzyme occurs simultaneously with the maximum formation of the mycelium.

B. Quantity of Enzymes produced on Wheat Bran during different Culture Periods.

Wheat bran is regarded in general industry as the best culture medium to produce strong enzyme from *Aspergillus oryzae*.

To ascertain the relation between culture period and enzyme quantity produced on wheat bran, the following experiment was made:—

Five grams of wheat bran and 5 cc. of water were placed in a 150 cc. cylindrical bottle with cotton plug inserted and the whole sterilized for 15 min. at 115°C. in an autoclave. After cooling, it was inoculated with *Aspergillus oryzae* and kept in an incubator at 30°C. After a certain period, 95 cc. water and 1 cc. toluol were added to each of two of these cultures. The content was mixed well, and after one hour standing, filtered, and the extract was tested for proteolytic and starch-saccharifying activities by the

author's method. The enzymic values were calculated for one gram of used bran.

Table 6. Amount of enzymes produced during fungous growth.

Culture period	Growth	Spores	Starch-saccharifying value	Proteolytic value
30 hrs.	slight	none	98	70
36 „	complete	few	118	80
42 „	„	many	203	100
48 „	„	abundant	222	100
3 days	„	„	222	100
5 „	„	„	222	100
8 „	„	„	222	100

This result shows that two days is enough to produce the maximum quantity of enzymes.

The above two experiments proved also that amylase and protease are produced simultaneously in the same ratio and that their quantity is not decreased by a long period of cultivation.

C. Influence of Culture Media on Enzyme Production.

FUNKE (29), BÜSGEN (9), KATZ (38), DUCLAUX (15), GREZES (30), KYLIN (45), WENT (103) and YOUNG (109) studied this subject. Most of their results can be summarized as follows:—

1. The addition of a particular substance to the culture medium does not cause entirely new enzymes to develop, but stimulates the production of a particular enzyme that is already normally produced by the organism.

2. Substances of closely related chemical structure which can be decomposed by enzymes, appear to be more efficient in stimulating the formation of the enzymes, than those not so closely related. For example, for the production of protease, proteins are the strongest stimulants, followed by peptides, amides, ammonia and other inorganic nitrogenous substances that are progressively weaker. For amylase, starch is the strongest stimulant, followed by dextrin, maltose and glucose.

3. The result of the study of EULER and ARSANOJ (21) is also worthy of attention, i. e., that on the amylase formation of a fungus, the addition of peptone in culture media, which otherwise contains only inorganic nit-

rogen, has directly or indirectly a good influence.

The author's experiments on this subject with *Aspergillus oryzae* were performed by using synthetic and natural culture media.

1. Experiments with Liquid Synthetic Culture Media.

Culture media were used beside the addition of 5% of organic substances as special ingredients which are described in each case,—CZAPEK's mixture (i. e. 10 cc. each of 5 % $MgSO_4 \cdot 7\ H_2O$, 5 % KH_2PO_4, 20 % $NaNO_3$ and 0.01 % $FeSO_4$ in one litre of culture medium). If organic nitrogenous substance was used, $NaNO_3$ was eliminated. Glucose was used, the pure preparation of E. Merck Co. Soluble starch and casein were prepared by the author. WITTE peptone was of Kahlbaum Co. TERUUCHI peptone was of Goto Fuundo Co. made by tryptic digestion, dissolved completely in water, and no coagulation or precipitation occured with heat, acid, or alkali.

Twenty cubic centimetres of the medium were placed in each 150 cc. ERLENMEYER's flask with cotton plug inserted and sterilized for 10 min. at 110°C. in autoclave. *Aspergillus oryzae* No. 119 was used. After incubation at 30°C. for 4–7 days, two flasks each were taken and by the same method as above (IV, B), tested for amylase and protease of exo-enzymic nature (which would be practically the same as total enzymic quantity for such a long culture period).

Table 7. I Series. Amount of enzymes produced on different culture media.

Special ingredients	Growth	Spores	Starch-sacchari. value	Proteolytic value
	After 4 days			
5% soluble starch	4	none	1.0	0
„ glucose	3	few, yellow	1.7	0
„ casein	6 (least)	none	0.0	0
2.5% sol. starch + 2.5% casein	2	few	3.0	0
„ „ „ + „ glucose	5	„	1.1	0
„ casein + „ „	1 (best)	„	2.3	0.2
	After 6 days			
5% soluble starch	good	yellow	0.6	0
„ glucose	slight	green	0.9	0
„ casein	„	none	0.5	0

Special ingredients	After 6 days			
	Growth	Spores	Starch-sacchari. value	Proteolytic value
2.5% sol. starch + 2.5% casein	good	none	4.9	0.3
„ „ „ + „ glucose	„	light yellow	0.7	0
„ casein + „ „	„	„	4.9	4.9
	After 9 days			
5% soluble starch	good	green	1.2	1.2
„ glucose	„	„	0.6	0.8
„ casein	„	light yellow	0	0
2.5% sol. starch + 2.5% casein	„	„	4.9	1.2
„ „ „ + „ glucose	„	„	1.5	1.2
„ casein + „ „	„	„	2.3	1.2

Table 8. II Series. Amount of enzymes produced on different culture media.

Special ingredients	After 4 days		
	Weight of mycelium	Starch-sacchari. value	Proteolytic value
2.5% WITTE peptone + 2.5% sol. starch	714.7 mgm.	5.5	less than 0.3
„ TERUUCHI peptone + „ „ „	714.9 „	6.5	0.7
	After 6 days		
2.5% WITTE peptone + 2.5% sol. starch	704.8 mgm.	14.6	0.3
„ TERUUCHI peptone + „ „ „	501.6 „	8.9	0.8

N. B. The mycelium weight was that in 40 cc. culture medium.

Table 9. III Series. Amount of enzymes produced on different culture media after 6 days.

Special ingredients	M/3K-H_2PO_4 (cc.)	Growth	Spores	Starch-sacchari. value	Proteolytic value
2.5% soluble starch + 2.5% TERUUCHI peptone	0.2	good	many	7.7	0.8
„ „ „ „ „ „	0.5	„	„	8.9	0.8
„ „ „ „ „ „	1.0	„	„	7.7	0.8
„ „ „ „ „ „	2.0	„	„	7.7	2.5
„ glucose „ „ „ „	0.2	„	„	4.4	0.6
„ „ „ „ „ „	0.5	„	„	4.4	0.8
„ „ „ „ „ „	1.0	„	„	4.4	0.8
„ „ „ „ „ „	2.0	„	„	4.4	2.5

N. B. In this series, nitrate and phosphate from CZAPEK's mixture were eliminated and the above quantities of phosphate added.

The results obtained, may be summarized as follows:—

1. Growth of the mould and production of both enzymes are good in a mixture of casein or peptone and soluble starch or glucose with CZAPEK's mixture, the inorganic ingredients being always contained in the medium.

2. On the medium containing only soluble starch or glucose (as the source of nitrogen an inorganic salt is added), the growth is good but the enzymes, especially protease are weak.

3. On casein medium the mould grows poorly, and the production of enzymes is accordingly poor.

4. On starch medium the mould produces more amylase than on glucose medium.

5. The greater the addition of primary potassium phosphate the better the production of protease.

It should be remembered that these results are deduced from experiments with the *Aspergillus oryzae* No. 119, which can produce strong amylase and protease. As shown later (IV, D) there are many strains of *Aspergillus oryzae*, some of which produce weak amylase and strong protease, or strong amylase and weak protease. If protease production is weak, the natural protein is with difficulty utilized by the mould, and growth is poor. The amylase produced is then poorer too than that from a nitrogen source, such as TERUUCHI peptone, which is easily assimilated. Accordingly, in order to produce a mould strong in protease, but weak in amylase, glucose is a better nutrient than starch.

2. Experiments with Solid Natural Culture Media.

In industries, solid natural media are generally used upon which to cultivate the mould. For example, steamed rice for "sake" brewing, cooked soy beans and crushed roasted wheat mixture for "shoyu" brewing, and steamed wheat bran for the alcohol industry are used.

In the following experiments a comparison of enzyme production on various solid media was made.

As preliminary experiments, the relation of enzyme production to capacity of culture bottle, quantity of medium and water, duration and temperature of sterilization and cultivation were studied, with wheat bran as culture medium, and the best conditions for successful growth were adopted.

Five grams of culture media described below, were placed with 5 cc. of water in 150 cc. capacity cylindrical bottle. The medium was mixed well, the bottle plugged with cotton, and the whole sterilized for 15 min.

at 115°C. in an autoclave. After cooling, the medium was inoculated with *Aspergillus oryzae*, well shaken and kept in an incubator at 30°C. for 4 and 6 days. Then 95 cc. of water and 1 cc. of toluol were addded, and the whole well mixed together. After standing for one hour at room temperature, the mixture was filtered and the filtrate tested for starch-saccharifying and proteolytic enzymes by the author's methods.

Table 10. Amount of enzymes produced on different culture media.

Culture media	After 4 days			After 6 days		
	Growth	Proteolytic value	Starch-saccharifying value	Growth	Starch-saccharifying value	Proteolytic value
Rice (polished and crushed)	good	27.4	16.6	good	68.0	40.0
Waste of potato starch manufacture (crushed)	„	16.0	3.3	„	13.6	3.3
Koryang (*Andropogon Sorghum Broth.* var. *vulgaris* subsp. *japonicus* HACK.) (crushed)	„	16.0	3.3	„	16.0	3.3
Soy beans (crushed)	„	72.0	45.0	„	68.0	45.0
Oats (crushed)	„	30.0	6.4	„	46.4	6.0
Wheat (crushed)	„	119.0	80.0	„	164.0	135.0
Herring cake (crushed)	slight	18.4	20.0	slight	20 4	33.2
Dried sweet potatoes (crushed)	good	18.4	4.0	good	18.4	4.0
Rice bran	„	52.0	56.0	„	92.0	83.0
Wheat bran	„	184.0	166.0	„	184 0	166.0
Soy bean cake (crushed)	„	65.0	75.0	„	95.0	105.0

It might be supposed that the enzyme production as well as growth of the mould, would be changed to some extent by the physical conditions of the culture media. A peculiar fact was noted in this experiment for natural culture media, viz. that their starch and protein contents have little influence on the ratio of amylase and protease produced. For example, even on soy beans, which contain practically no starch or dextrin, strong amylase is produced, and on polished rice, which contains much starch but less protein, the mould gives rise to strong protease.

D. Activities of Enzymes obtained from *Aspergillus* Species.

For technical purposes, when mould is used only for its enzymes, its enzymic activity and the nature of those enzymes are the most important considerations. For mould classification both the activity and the nature of enzyme found may also be useful for the identification of species. This problem has hitherto been studied very little.

TAKAHASHI and YAMAMOTO (95) found that generally, *Aspergillus oryzae* taken during the "sake" brewing process, produced strong amylase, but that strong protease was produced from the *Aspergillus oryzae* taken during the "shoyu" brewing process.

The author showed in another paper (69) that the enzymic activity and the nature of enzyme produced by *Aspergillus oryzae* are not changed by previous cultivation on special media.

From the many investigations already referred to, the author thought it is possible to determine the activity and nature of enzymes obtained from different moulds, and therefore undertook the following experiments

1. Kind of Enzymes Studied.

Starch-saccharifying enzyme and protease were selected, on account of their importance and possibility of making relatively accurate measurements.

2. Collection of *Aspergillus* Species.

Only *Aspergillus* species were studied. Many pure cultures were obtained from the Takamine Laboratory; the Mycrobiological Laboratory of the Bureau of Chemistry, U. S.. Department of Agriculture; CRAL's Bacteriological Museum, Wien; Laboratory of Applied Mycology, Hokkaido Imperial University; Tokyo Brewing Institute and the Central Laboratory of the South Manchuria Railway Co.

The purity and correctness of classification of these cultures were kindly examined and confirmed by Prof. HANZAWA and Mr. NAGANISHI.

Some of the cultures were separated by the author from many kinds of "moyashi" (mould ferment) and identified by Drs. THOM and CHURCH. In accordance with their opinions, some strains which are usually recognized in Japan as *Aspergillus oryzae*, were classified as *Aspergillus flavus* or the intermediate form of *Aspergillus oryzae* and *flavus*.

It is possible that some of them are entirely one and the same kind, but is very difficult to determine this fact, as even morphologically similar kinds often prove enzymically different.

3. Methods of Cultivation.

All cultures were kept on "koji"-agar[1] medium. As substrata for enzyme production, wheat bran and peptone-glucose were used. These media were found, by the above experiments, to be the best for this purpose. The preparation of these media and test of enzymes were the same as described in a previous section (IV, A and B). The hydrogen ion concentration of the starch solution used, was about pH 5.2 and of casein solution about pH 7.9, which were determined as the optimum of enzymic activity for these moulds as shown later (V, C, 1 and VI, A, 1).

Table 11. *Aspergillus* species collected.

Culture No.	Former mark	Name of species	Former owner	Origin
1		*(A.) niger* VAN TIEGHEM	Lab. of Appl. Mycology, Hok. Imp. Univ.	
3		"	"	
5		*Awamori* NAKAZAWA	"	
7		"	"	
8		"	"	
14		"	"	
15		"	Brewing Inst., Japan	
16		*melleus* YUKAWA	"	
20		*Awamori* NAKAZAWA	"	
21		"	"	
22		"	"	
23		"	"	
25		"	"	
26		*oryzae* (AHLB.) COHN	"	
31		*nidulans* FISCHER	"	

1) "Koji" is steamed rice on which *Aspergillus oryzae* has been cultivated. "Koji"-agar is a culture medium of agar with the "koji" extract.

Culture No.	Former mark	Name of species	Former owner	Origin
36		*oligosporus*	Brewing Inst., Japan.	
39		*Awamori* NAKAZAWA	"	
45		*giganteus* WEHMER	"	
49		*Aureus* NAKAZAWA	"	
51		*Awamori* NAKAZAWA	"	
55	A	*niger* VAN TIEGHEM	"	
56	B	"	"	
57	A	*Ochraceus* WILHELM	"	
61		*Oryzae* (AHLB.) COHN	"	"Koji"
62	Higuchi blue	"	"	
63	Higeta	"	"	"Sake koji"
64	Sake A	"	"	"
65	"	"	"	"
66	Sake B	"	"	"
67	Sake C	"	"	"
68	Sake D	"	"	"
69	Sake E	"	"	"Shoyu koji"
70	Sake F	"	"	"
71	Soya G	"	"	"
72	Soya H	"	"	"
73	Soya I	"	"	"
74	Soya J	"	"	"
75	Tamari K	"	"	"Tamari koji"
76	Tamari L	"	"	"
77	Tamari M	"	"	"
78	Tamari N	"	"	"
79	Tamari H	"	"	"
80	Tamari P	"	"	"
81		"	"	(NISHIMURA) "Shoyu koji"
82	A113	"	"	(NISHIMURA) "
83	A123	"	"	(NISHIMURA) "
84		*Ostianus* WEHMER	"	
85		*repens* CORD	"	
87		*Wentii* WEHMER	"	
90	ST3	*oryzae* (AHLB.) COHN.	Lab. of Appl. Mycol. Hok. Imp. Univ.	"Moyashi"
91	ST4	"	"	"
92	ST5	"	"	"
93	ST6	"	"	"
94	ST7	"	"	"
95	ST8	"	"	"
96	ST9	"	"	"

Culture No.	Former mark	Name of species	Former owner	Origin
97	ST10	*oryzae* (AHLB.) COHN	Lab. of Appl. Mycol. Hok. Imp. Univ.	"Moyashi"
98	ST11	"	"	"
101	AO6	"	Author	"
102	3509	*parasiticus* SPEARE	C. THOM	"
103	4145×1	*tamari* KITA	"	
106	3565	"	"	
107	189	*flavus* LINK	"	
108	108	"	"	
109	AO3a	Intermediate form of *oryzae* & *flavus*	Author	
110	APa	"	Takamine Lab.	Tamura's "Moyashi"
112	AO1	"	Author	
113	AO2a	"	"	Hishiroku's "moyashi"
114	AO2b	*oryzae* (AHLB.) Chon	"	Tsuboi's "moyashi"
115	AO c	*flavus* LINK	"	
116	AO3b	*parasiticus* type	"	Nippon Jokosha's "moyashi"
117	AO4a	Intermediate form of *flavus* & *oryzae*	"	"Moyashi"
119	AO01d	*oryzae* (AHLB.) COHN	Takamine Lab.	"
120	4328	*flavus* LINK	C. THOM	
124	AOAb	Intermediate form of *flavus* & *oryzae*	Author	
125	AON	"	Author (Brewing Inst. Japan)	"Moyashi
126	A	"	Takamine Lab.	
128	AOP	"	Author (Brewing Inst. Japan)	
129	4272×2	*flavus* LINK	C. THOM	
132		*ochraceus* WILHELM	Author	
134		*flavus* LINK	KRAL's Museum	
135		*minimus* WEHMER	"	
138		*cellulosae* HOPFE	"	
141		*clavatus* DESMAZIERES	"	
149	141	*terreus* THOM	C. THOM	
150	4235—2	*tamari* KITA	"	
159		sp. green (new?)	Central Lab.	
161		*Rehmii* ZUKAL	"	
162		*ochraceus* WILHELM	"	
163		*japonicus* SAITO	"	
167		*flavus* LINK	"	
169		*sulfureus* FRESENIUS	"	
170		*varians* WEHMER	"	
172		*ostianus* WEHMER	"	
183	4474×1	*cellulosae* HOPFE	C. THOM	
184		*Awamori* NAKAZAWA	Central Lab. (NISHIMURA)	
185		*aureus* NAKAZAWA	"	

Culture No.	Former mark	Name of species	Former owner	Origin
186		*oryzae* (?) greenish brown	Central Lab. (NISHIMURA)	
187		*oryzae* (AHLB.) COHN	„	
188		„	„	
189		*ochraceus* (yellowish green)		
190		sp. 1 (white)	Central Lab.	Chinese "moyashi"
191	M2	*ochraceus* WILHELM	Author	Dry bonito
192	S2	„	„	„
193	M3	*glaucus* (LINK)	„	„

N. B. Cultures Nos. 5, 7, 8 and 51 were given as *Aspergillus luchuensis* INUI but as the result of their study by Mr. NAGANISHI (34), they were corrected as *Aspergillus Awamori* NAKAZAWA. Also No. 49 which was formerly named *Aspergillus luchuensis* INUI, was changed to *Aspergillus aureus* NAKAZAWA.

Table 12. Comparison of productivities of amylase and protease.

Culture No.	On wheat bran				On peptone-glucose			
	Duration of culture (days)	Growth	Starch-sacchari. value	Proteolytic value	Duration of culture (days)	Growth	Starch-sacchari. value	Proteolytic value
1	6	good	9(IV)	5(IV)	7	good	c(IV)	0.3(III)
3	3	„	8(IV)	5(IV)	10	„	o(IV)	o(IV)
5	6	„	116(II)	4(IV)	10	„	5.8(I)	c(IV)
7	6	„	90(II)	3(IV)	7	slight	5.8(I)	c(IV)
8	6	„	52(II)	6(IV)	10	„	5.8(I)	o(IV)
14	6	„	30(III)	7(IV)	10	good	5.8(I)	o(IV)
15	6	„	27(III)	c(IV)	10	„	6.5(I)	o(IV)
16	3	„	10(IV)	100(II)	7	„	c(IV)	15.6(I)
20	3	„	60(II)	3(IV)	10	„	5.8(I)	0.3(III)
	3	„	46(III)	3(IV)	10	„	5.8(I)	0.2(III)
22	6	„	139(II)	5(IV)	4	„	5.8(I)	0.1(IV)
23	12	„	18(IV)	5(IV)	10	„	3.4(I)	c(IV)
25	10	„	116(II)	o(IV)	10	„	6.5(I)	o(IV)
26	4	„	25(III)	50(II)	10	slight	c(IV)	o(IV)
31	6	„	3(IV)	7(III)	6	„	o(IV)	0.6(III)
36	6	„	116(II)	100(II)	10	slight, no spore	0.4(IV)	1.2(III)
39	6	„	116(II)	3(IV)	16	good	8.9(I)	o(IV)
45	5	„	260(I)	250(I)	4	„	2.7(II)	12.5(I)
49	5	„	33(III)	6(IV)	4	„	0.8(II)	0.5(IV)
51	4	„	55(II)	6(IV)	6	„	2.6(IV)	o(IV)
55	4	„	46(III)	8(IV)	6	„	2.8(IV)	c(IV)

Culture No.	On wheat bran				On peptone-glucose			
	Duration of culture (days)	Growth	Starch-sacchari. value	Proteolytic value	Duration of culture (days)	Growth	Starch-sacchari. value	Proteolytic value
56	5	good	46(III)	3(IV)	6	good	2.9(II)	0(IV)
57	5	,,	7(IV)	200(I)	6	,,	0(IV)	7.3(II)
61	3	,,	210(I)	200(I)	4	,,	1.0(II)	4.2(II)
62	3	,,	119(II)	100(II)	4	,,	2.3(II)	10.3(I)
63	3	,,	122(II)	80(II)	4	,,	3.4(I)	3.7(II)
64	3	,,	129(II)	80(II)	4	,,	3.4(I)	4.2(II)
65	5	,,	232(I)	125(II)	4	,,	3.4(I)	4.2(II)
66	3	,,	260(I)	125(II)	4	,,	4.9(I)	12.5(I)
67	5	,,	210(I)	50(II)	6	,,	0.6(III)	0.4(III)
68	3	,,	84(II)	125(II)	4	,,	0.6(III)	4.2(II)
69	3	,,	194(II)	166(II)	4	good, few spores	1.7(II)	8.5(II)
70	3	,,	194(II)	125(II)	4	good	2.8(II)	3.1(II)
71	3	,,	194(II)	125(II)	4	,,	2.6(II)	1.3(III)
72	3	,,	42(III)	100(II)	4	,,	0(IV)	1.1(III)
73	3	,,	27(III)	63(II)	6	,,	0(IV)	0(IV)
74	3	,,	46(III)	125(II)	6	,,	0(IV)	1.3(III)
75	5	,,	24(III)	40(III)	4	,,	0(IV)	6.5(II)
76	3	,,	33(III)	100(II)	6	,,	0(IV)	2.5(II)
77	3	,,	48(III)	168(II)	4	,,	0(IV)	10.3(I)
78	3	,,	18(III)	40(III)	4	,,	0(IV)	1.3(III)
79	3	,,	39(III)	100(II)	4	,,	0(IV)	2.5(II)
80	3	,,	38(III)	125(II)	4	,,	0(IV)	1.8(III)
81	3	,,	36(III)	83(II)	4	,,	0(IV)	7.5(I)
82	3	,,	24(III)	25(III)	6	,,	0(IV)	0.7(III)
83	3	,,	52(II)	58(II)	6	,,	0 6(III)	0.8(III)
84	3	good, few spores	8(IV)	67(II)	4	,,	0(1V)	4.2(II)
85	3	good	36(III)	67(II)	4	,,	0(IV)	2.0(III)
87	3	good, few spores	9(IV)	4(III)	6	,,	0(IV)	0(IV)
90	3	good	232(I)	200(I)	4	,,	4.1(II)	5.0(II)
91	3	,,	178(II)	200(I)	4	,,	2.8(II)	2.5(II)
92	3	,,	211(I)	200(I)	6	,,	2.5(II)	1.5(II)
93	3	,,	119(II)	168(II)	6	,,	2.2(II)	4.0(II)
94	3	,,	137(II)	100(II)	4	,,	1.1(II)	5.0(II)
95	3	,,	221(I)	250(I)	4	,,	2.6(II)	3.1(II)
96	3	,,	156(II)	83(II)	4	good, few spores	1.9(II)	2.5(II)
97	3	,,	201(I)	200(I)	4	good	3.0(I)	8.9(II)
98	3	,,	150(II)	83(II)	4	,,	0(IV)	0.6(III)
101	3	,,	40(III)	150(II)	5	,,	0(IV)	2.1(III)
102	3	,,	8(IV)	200(I)	5	,,	0(IV)	20.8(I)

Culture No.	On wheat bran				On peptone-glucose			
	Duration of culture (days)	Growth	Starch-sac-chari. value	Proteolytic value	Duration of culture (days)	Growth	Starch-sac-chari. value	Proteolytic value
103	3	good	18(III)	183(II)	5	good	c(IV)	12.5(I)
106	3	,,	14(III)	83(II)	6	,,	o(IV)	12.5(I)
107	5	,,	35(III)	100(II)	4	,,	o(IV)	2.5(II)
108	3	,,	18(III)	200(I)	5	,,	o(IV)	2.5(II)
109	5	,,	188(II)	200(I)	6	,,	o(IV)	3.2(II)
110	3	,,	25(III)	135(II)	–		—	—
112	3	,,	210(I)	250(I)	6	,,	3.0(I)	16.7(I)
113	3	,,	178(II)	200(I)	6	,,	0.8(II)	16.7(I)
114	5	,,	250(I)	42(III)	–		—	—
115	5	,,	194(II)	200(I)	4	,,	0.7(III)	32.3(I)
116	3	,,	9(IV)	250(I)	4	,,	o(IV)	31.8(I)
117	5	,,	137(II)	168(II)	5	,,	2.1(II)	4.0(II)
119	3	,,	184(II)	166(II)	5	,,	3.9(I)	2.5(II)
120	5	,,	25(III)	92(II)	7	,,	0.6(III)	16.1(II)
124	3	,,	68(II)	63(II)	5	,,	o(IV)	3.1(II)
125	3	,,	16(III)	46(III)	5	slight	o(IV)	0.8(III)
126	3	,,	68(II)	92(II)	7	good	0.6(III)	1.0(III)
128	3	,,	36(III)	92(II)	7	,,	0.5(III)	2.0(III)
129	5	,,	66(II)	125(II)	4	,,	c(IV)	2.8(II)
132	3	,,	16(III)	130(II)	5	,,	o(IV)	16.1(I)
134	5	,,	30(III)	100(II)	4	,,	o(IV)	3.0(II)
135	5	slight	2(IV)	20(III)	6	slight	o(IV)	o(IV)
138	5	good	7(IV)	50(II)	4	good	c(IV)	20.7(I)
141	5	,,	22(III)	25(III)	4	,,	o(IV)	12.5(I)
149	3	,,	8(IV)	125(II)	4	,,	o(IV)	5.6(II)
150	5	,,	33(III)	100(II)	4	,,	o(IV)	8.3(II)
159	3	slight	o(IV)	35(III)	6	slight	o(IV)	o(IV)
161	3	good	5(IV)	92(II)	4	good	o(IV)	11.4(I)
162	3	,,	7(IV)	259(I)	6	,,	o(IV)	62.0(I)
163	5	,,	2(IV)	25(III)	4	,,	o(IV)	0.4(III)
167	3	,,	18(III)	147(II)	4	,,	o(IV)	3.0(II)
168	5	slight	33(III)	25(III)	6	slight, no spore	o(IV)	o(IV)
169	3	,,	6(IV)	25(III)	6	good	o(IV)	2.1(III)
170	5	,,	o(IV)	o(IV)	4	slight	0.7(III)	o(IV)
172	5	,,	7(IV)	125(II)	4	good	o(IV)	4.0(II)
183	5	good	o(IV)	71(II)	4	,,	o(IV)	3.1(II)
184	5	,,	60(II)	o(IV)	5	,,	8.5(I)	0.6(III)
185	5	,,	60(II)	5(IV)	5	,,	4.6(I)	o(IV)
186	3	,,	21(III)	167(II)	6	,,	c(IV)	20.7(I)

Culture No.	On wheat bran				On peptone-glucose			
	Duration of culture (days)	Growth	Starch-saccharl. value	Proteolytic value	Duration of culture (days)	Growth	Starch-saccharl. value	Proteolytic value
187	3	good	21(III)	125(II)	5	good	0(IV)	15.6(I)
188	5	„	164(II)	7(IV)	6	„	0.5(III)	0.5(III)
189	3	slight	8(IV)	125(II)	6	„	0(IV)	3.1(II)
190	3	„	5(IV)	25(III)	6	slight, fewspores	0(IV)	2.1(III)
191	3	good	34(III)	250(I)	4	good	0.6(III)	40.0(I)
192	3	„	30(III)	200(I)	4	„	0(IV)	31.2(I)
193	3	„	5(IV)	3(IV)	6	slight	0.3(III)	0(IV)

N. B. The Roman letters next to the enzymic value indicate the groups of enzmyic activities according to the following classification:

Group	On wheat bran		On peptone-glucose	
	Starch-saccharl. value	Proteolytic value	Starch-saccharl. value	Proteolytic value
I	over 200	over 200	over 3.0	over 10.0
II	200–50	200–50	3.0–0.8	10.0–2.5
III	50–12	50–12	0.8–0.2	2.5–0.6
IV	12–0	12–0	0.2–0	0.6–0

From the above results, the *Aspergillus* species may be classified as follows, according to their enzymic activities:—

1. Strong amylase and strong protease producers:—
   A. oryzae, Nos. 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 78, 90, 91, 92, 93, 94, 95, 96, 97, 112, 113, 119.
   A. intermediate form of *flavus* & *oryzae*, No. 117.
   A. flavus, No. 115.
   A. oligosporus, No. 36.
   A. giganteus, No. 45.
2. Strong amylase and weak protease producers:—
   A. Awamori, Nos. 5, 7, 8, 14, 15, 20, 21, 22, 25, 184.
   A. oryzae, Nos. 114, 188.
   A. niger, No. 56.
   A. aureus, No. 185.
3. Weak amylase and strong protease producers:—
   A. oryzae, Nos. 72, 74, 75, 76, 77, 79, 80, 81, 85, 98, 101, 186, 187.
   A. flavus, Nos. 107, 108, 120, 129, 134, 167.
   A. intermediate form of *flavus* & *oryzae*, Nos. 109, 110, 124, 126, 128.
   A. ochraceus, Nos. 132, 162, 189, 191, 192.

A. tamari, Nos. 103, 106, 150.
A. Ostianus, Nos. 84, 172.
A. cellulosae, Nos. 138, 183.
A. parasiticus, Nos. 102, 116.
A. Rehmii, No. 161.
A. niger, No. 57.
A. repens, No. 85.
A. melleus, No. 16.
A. clavatus, No. 141.
A. terreus, No. 149.

4. Weak amylase and weak protease producers:—
A. oryzae, Nos. 26, 73, 78, 82, 83.
A. niger, Nos. 1, 3, 55.
A. Awamori, Nos. 23, 51.
A. nidulans, No. 31.
A. aureus, No. 49.
A. varians, No. 170.
A. Wentii, No. 87.
A. intermediate form of *flavus* & *oryzae*, No. 125.
A. minimus, No. 135.
A. sulfureus, No. 169.
A. glaucus, No. 193.
A. sp. green, No. 159.
A. japonicus, Ne. 163.
A. sp., No. 190.

V. Properties of Protease from *Aspergillus Oryzae*.

A. Literature and Preparation for Experiments.

1. Historical.

In 1895, WEHMER (102) found that various species of *Penicillium* and *Aspergillus* liquefied the gelatin of culture media to a considerable distance beyond the colony itself or beyond the reach of mycelium. BOURQUELOT (8) studied the proteolytic enzyme of *Aspergillus niger* after it had been cultivated on RAULIN's fluid. An extract of the mycelium was prepared by triturating with sand and chloroform water. This extract could digest fibrin, coagulate egg white, and produce peptone in neutral solution, while in 0.2 % hydrochloric acid solution no peptone was found. He concluded

therefore that the extract contained trypsin, but that the presence of pepsin was very doubtful. MALFITANO (51) made a more detailed study of the protease of *Aspergillus niger*. He found that the enzyme was active only in acid solution, and then digested gelatin readily. The production of protogelatose, deuterogelatose, and gelatin peptone was demonstrated. The extract would not digest METT's tubes of coagulated egg albumin nor boiled fibrin. Fresh fibrin, on the other hand, was digested in the presence of 0.01 N acid, while the fibrin in the control test merely swelled. Milk was first curdled and then digested and the presence of caseose and peptone was demonstrated in the digested solution. DOX (14) concluded from his experiments that the intracellular protease of *Penicillium camemberti* readily digested casein, gelatin and WITTE peptone, but had no action on fibrin, ovalbumin, or other natural proteins. In this respect he thought it closely analogous to erepsin, discovered by COHNHEIM in the intestines of mammals.

VINES (99) reported on the protease in "Taka-diastase" an enzymic medicine made from *Aspergillus oryzae*. He examined the existence of protease by the liquifaction of fibrin, biuret and tryptophane reactions with peptone. All proofs were positive and especially in an acid medium, but the reaction change was not studied further. WOHLGEMUTH (108) by using Taka-diastase, tested for proteolytic enzymes by FULD-GROSS' method (31), i. e., by addition of acetic acid to digested casein, found the action to be stronger in weak alkaline or neutral than in a weak acid solution. He also found with FULD's edestin method (28) for pepsin that, even in such a strong acid solution, Taka-diastase could readily digest edestin. SZANTO (93) studied also the proteolytic action of Taka-diastase by FULD-GROSS' method and concluded that neutral reaction is best and that all additions of acids or alkalies decrease its action. Here it should be noticed that the FULD-GROSS method uses phenolphthalein as indicator to neutralize the casein solution and that, therefore, the supposed neutral solution has really an alkaline reaction of about pH 8. The experiments of WOHLGEMUTH and SZANTO agree then in regard to the optimum reaction of the proteolytic action of Taka-diastase in weak alkaline solution. On the other hand OKADA (63) found the optimum reaction for the proteolytic action of Taka-diastase to be pH 5.07, i. e., weak acid reaction, by measuring free amino nitrogen in digested peptone.

For comparison, the optimum reactions of various animal and vegetable proteases as already published are described below.

Protease	Origin	Optimum pH	Remarks	Authors	Reference
Pepsin		1 4	37°C., edestin	MICHAELIS and MENDELSOHN	(55)
„		1.5-1.6	37°C., acid albumin	SÖRENSEN	(91)
„		1.4	egg albumin	OKADA	(63)
„	yeast	4.0-4.5		LUNDÉN	(50)
„	barley malt	3.7-4 3		„	„
„	pig stomach	1.6	40°C., 1 hr., edestin	OSHIMA and SASAKI	(72)
„	fish (silver trout) stomach	3.0	„	„	„
„	fish (*Seriola quinqueradiata*) stomach	3.0	„	„	(75)
„	fish (*Tunnus orientalis*) stomach	2.8	„	„	„
Trypsin	pancreas	8.0	37°C,, peptone	SHERMAN and NEUN	(86)
„	„	8.3	casein	PALISACH	(77)
„	„	9.7	37°C., gelatin	DERNBY	(12)
„	crab; fish (*Paralithodes camtschatica*) liver	8.0	40°C., 4 hrs., WITTE pepton→amino acid	OSHIMA and KONDO	(76)
„	„	7.2-8.05	40°C., 4 hrs., Casein liquefaction	„	„
„	„	7.2	40°C., 4 hrs., Casein→amino acid	„	„
„	yeast	7.0	peptone	LUNDÉN	(13)
„	animal tissues	7.8	„	DERNBY	(50)
„	barley malt	6 3	„	„	„
Erepsin	pig intestine	7.7	albumose	RONA and ARNHEIM	(81)
„	animal tissues	7.8	glycyl-glycin	DERNBY	(13)
Papain		5.0	egg albumin and gelatin	FRANKEL	(26)
Protease	Taka-diastase (*Aspergillus oryzae*)	5.07	37°C., WITTE peptone	OKADA	(63)
„	animal kidney	7.8	peptone	HEDIN	(36)
„	animal tissues	7.0-7.5	casein and peptone	FALK, NOYES and SUGIURA	(23)
„	*Aspergillus ochraceus*	5.1	40°C., 4 hrs., WITTE peptone→amino acid	OSHIMA and HOSHI	(74)
„	„	6.1	40°C., 4 hrs., casein→amino acid	„	„
„	„	7.4	40°C., 2 hrs., casein liquefaction	„	„
„	*Bacillus Natto*	5.9	„	OSHIMA	(73)
„	„	7.8-8.4	40°C., 4 hrs., casein→amino acid	„	„
„	„	6.0	40°C., 4 hrs., edestin liquefaction	„	„
„	„	8.0-8.8	40°C., 4 hrs, edestin→amino acid	„	„
„	„	9.1	40°C., 4 hrs., WITTE peptone→amino acid	„	„

From the above, it is known that *Aspergillus oryzae* produces some kind of protease, but it is uncertain, what kind of proteins can be digested by it. Further, influences of the temperature and hydrogen ion concentration of the digesting liquid on the action, the velocity of the action, destructive effects of temperature, reaction of the enzymic solution on the enzyme, and also, the influence of treatment of proteins to be digested, have not yet been studied. The author has investigated and endeavoured to settle these problems.

2. Preparation of Enzyme Sample.

To obtain a relatively strong and pure enzyme sample, the following method was adopted. Wheat bran, moistened with water in a flask closed with a cotton plug, was sterilized in an autoclave, and after cooling, inoculated with pure culture of mould, and kept a few days in a incubator at 30°C. After the formation of spores, this cultivated substance was extracted with water and filtered through paper pulp. To the filtrate, 4 times its bulk of strong alcohol was added; the precipitate was collected and dried at low temperature. This enzymic preparation contains, of course, many kinds of enzymes besides protease.

Throughout the experiment, *Aspergillus oryzae* No. 119 was used. It grows well on usual culture media and produces strong amylase and protease.

3. Preparations of Glycinin, Edestin and Casein.

a. Glycinin. Yellow Mammoth soy beans were used. To obtain pure glycinin from them, OSBORNE's method (65) was followed principally. According to OSBORNE and CAMBELL (56), glycinin is a globulin, soluble in over 2 % NaCl solution, and uncoagulable by heat in that solution. Given glycinin the main part of soy bean proteins, the digestion of protein in "shoyu moromi" may be best studied by considering the enzymic action in this protein.

b. Edestin. Hemp seed freed from oil was treated with five times its weight of 10 % NaCl solution, then after a few hours, warmed to 65–75°C., and filtered through hot paper pulp. After this the filtrate was cooled, the edestin crystallized out and filtered again, and the same process repeated. The precipitate on the filter paper was washed with alcohol and ether, and dried at room temperature.

Edestin is also a vegetable globulin, soluble in hot salt solution, and

in acid or alkaline solutions without any kind of salt.

c. Casein. The common procedure with acetic acid and caustic soda was followed.

B. Determination of Proteolytic Process.

Aspergillus oryzae is known to produce some kind of protease, but it is uncertain how the proteolytic action takes place during digestion. To determine the process precisely, different kinds of protein, i. e., Witte peptone, casein, egg white and beef protein were taken as substrata, digested and the process compared with action of trypsin and pepsin.

It is now quite generally believed that, if an enzyme is not destroyed, its activity is maintained until the chemical equilibrium of the digesting material is reached. On this principle the experiments were continued for a relatively long period of digestion and a large quantity of enzyme was used. After digestion the remaining enzyme was tested. As toluol was used as an antiseptic, the result may not be considered exact, but sufficient for the purpose of comparison. The influence of toluol, amyl alcohol etc. in amino nitrogen determination by VAN SLYKE's method has already been described (II, E.).

1. Casein as Substratum.

It was intended to ascertain how the proteolytic action of *Aspergillus* protease in the digestion of casein differs from pepsin and trypsin, and the following experiment was undertaken.

a. Digestion with Aspergillus Protease, Pepsin and Trypsin.

Ten grams of anhydrous casein, 300 cc. H_2O and 20 cc. N/1 NaOH were mixed, slightly warmed, and neutralized with about 25 cc. N/1 HCl, phenolphthalein being the indicator. In flasks of 200 cc. capacity with rubber stoppers, the following liquids were mixed and kept in a water bath at 40°C.

Table 13. Composition of digesting liquids.

10 cc. of	100 cc. of	10 cc. of	1.5 cc. of
2% *Aspergillus* protease	2% Casein	H_2O	toluol
2% Pepsin	„	N/25 HCl	„
2% Pancreatin	„	2% Na_2CO_3	„

After definite periods, 10 cc. from each flask were taken, and the amino nitrogen determined by VAN SLYKE's method, using capryl alcohol as foam inhibitor. Calculation of the amino nitrogen was made by correction with water blank test. For comparison, the total amino nitrogen was determined by digestin with 20 % HCl for 24 hrs. boiling. The total nitrogen was determined by KJELDAHL's method.

Table 14. Amino nitrogen (mgm.) in 1 gram of digested casein.

Dig. period / Enzyme	At beginning	24 hrs.	(Difference)	3 days	(Difference)	6 days	(Difference)	After complete hydrolysis with HCl	(Difference)
Aspergillus protease	5.56	72.68	67.12	83.54	10.86	84.34	0.80	115.23	30.89
Pepsin	„	17.18	12.62	20.05	2.87	23.55	3.50	„	91.68
Pancreatin	„	46.44	40.88	48.87	2.43	50.27	1.40	„	64.96

N. B. Total nitrogen in 1 gram of casein = 155.77 mgm.

b. Remaining Proteolytic Activity in the digested Liquids.

Before drawing any conclusion from the above experimental data, it is necessary to examine whether these proteases were destroyed or not during the digestion. If proteolytic activity was destroyed soon after the mixing with casein solution, either by its reaction or temperature, nothing can be said with any exactness concerning the production of amino acids from casein.

The method of the test was as follows: 1 cc. of digested liquid of enzymic solutions which had been kept for 6 days at 40°C. and showed no precipitate after the addition of $MgSO_4$ and HNO_3 was mixed with 5 cc. of 0.2 % casein solution. The mixture was kept for 1–5 hours at 40°C. after which 0.5 cc. of a mixture of $MgSO_4$ and HNO_3 was added and precipitation was observed. The clear liquid proved positively that some proteolytic activity remained, but the precipitate was negative or showed only a weak action.

Table 15. Digestion of casein by digested liquids.

Digested liquid or enzyme solution	Regulator of reaction	By adding $MgSO_4+HNO_3$	
		After 1 hr.	After 5 hrs
Aspergillus protease+casein	—	clear	clear
Aspergillus protease	—	"	"
Pepsin+casein	0.5 cc. N/5 HCl	"	"
Pepsin	"	"	"
Pancreatin+casein	0.25 cc. 2% Na_2CO_3	a little pptn.	"
Pancreatin	"	much pptn.	less pptn.

c. Amino Nitrogen produced from Preparations of Aspergillus Enzyme, Pepsin and Trypsin.

To control the above experimental data, the following test was made to determine amino nitrogen which results from autolysis of enzymic preparations.

To 50 cc. of 2 % enzymic solution, 1 cc. toluol was added in flask with tight rubber stopper, and kept in water bath at 40°C. After definite periods, the amino nitrogen was determined by Van Slyke's method and the results calculated after a control or blank test had been made.

Table 16. Amino nitrogen (mgm.) in 1 gram enzyme.

Dig. period / Enzyme	Before digestion	After 1 day	2 days	3 days	6 days
Aspergillus protease	16.20	23.25	—	26.60	29.23
Pepsin	22.00	23.25	—	25.50	—
Pancreatin	5.55	10.60	—	10.30	—
Trypsin (in 0.2 % Na_2CO_3)	—	—	60.60	—	—
Trypsin	—	—	60.25	—	—

The trypsin used was Fairchild's and had a much stronger proteolytic activity than Park, Davis & Co.'s Pancreatin.

d. Ammonia in Digested Liquids.

FOLIN and DENIS' method (9) was followed. For estimating the ammonia, 5 cc. of digested liquids (6 days' digestion at 40°C.) were taken,

and 2 cc. of 10 % NaOH and 15 cc. Nessler's reagent were added and the whole made up to 200 cc. with water. For contrast, pure ammonia solution was taken and compared with Duboscq's colorimeter.

Table 17. Ammonia in digested liquids.

Ammonia N in 1 gram casein	+0.1 gram *Aspergillus* protease	4.36 mgm.
„	+0.1 gram Pancreatin	4.56 „
„	+0.1 gram pepsin	0.60 „
„	digested by 20% HCl for 24 hrs.	8.68 „
Total N in 1 gram casein	+0.1 gram *Aspergillus* protease	192.00 „

e. Tryptophane in digested Liquids.

The same digested liquids as above were used. To 10 cc. of these, 0.5 cc. of saturated bromine water was added and the colours compared with control test.

Table 18. Colour reaction of tryptophane in digested liquids.

Digested liquids	Colour reaction
Water only	yellow
Casein solution	colourless
Casein solution digested by pepsin	colourless
„ Pancreatin	pink
„ *Aspergillus* protease	deeper pink

The results obtained in all the above experiments may be expressed as follows:—

1. The protease from *Aspergillus* produced much amino acid from casein as did also trypsin. Pepsin produced very little.

2. By long digestion at 40°C., *Aspergillus* protease retained its activity well, whereas that of trypsin was much weakened. RÖDER (80) proved too that a temperature of 40°C. destroyed trypsin.

3. The production of ammonia by *Aspergillus* protease was very

small. As this quantity may correspond with the quantity of ammonia from casein by HCl digestion, no special ammonia forming enzyme can be recognized.

4. Tryptophane reaction was clearly proved with *Aspergillus* protease as in the case of trypsin, although there was no confirmation of the reaction by pepsin digestion.

2. Egg White aud Beef Protein as Substrata.

Fresh eggs were cooked for 15 minutes in boiling water, the shell, egg membrane and all of the yolk removed and the coagulated albumen rubbed through a sieve of No. 40 meshes. The beef protein was prepared by Dr. BERG by treating fresh meat with alcohol and ether. Detailed description of its preparation can be found in his report (7). Amino nitrogen content of these mixtures was not determined at the beginning, as the determination was difficult with these solid substances.

Table 19. Composition of digesting mixtures.

No.	5 cc. of 2% enzyme	Protein	H_2O (cc.)	N/5 HCl (cc.)	2% Na_2CO_3 (cc.)	Toluol (cc.)
1	Pepsin	5 grams egg white	35	10	—	1
2	Trypsin	„	40	—	5	„
3	„	„	45	—	—	„
4	*Aspergillus* protease	„	45	—	—	„
5	Pepsin	1 gram beef protein	35	10	—	„
6	Trypsin	„	40	—	5	„
7	„	„	45	—	—	„
8	*Aspergillus* protease	„	45	—	—	„

Table 20. Appearance of the digesting mixtures (digestion at 38°C.).

No.	Before digestion	After 44 hrs.	After 5 days
1	Precipitation	turbid	clear
2	„	clear	„
3	„	clear with a slight pptn.	„
4	„	maximum pptn.	„
5	„	turbid with a slight pptn.	pptn.
6	„	clear, with much pptn.	„
7	„	„	„
8	„	„	„

Remaining Proteolytic Activity in the digested Liquids.

After 5 days tested as usual and found positive existence of protease in all samples.

Table 21. Amino-N (mgm.) in 1 gram egg white (not dried).

No.	After 44 hrs. digestion	After 5 days digestion
1	2.91	3.05
2	6.78	7.18
3	8.74	9.68
4	7.22	11.39
	Amino-N by complete hydrolysis with HCl	14.90
	Total N	18.81

Table 22. Amino-N (mgm.) in 1 gram beef protein (air dry).

No.	After 44 hrs. digestion	After 5 days digestion
5	18.35	20.30
6	54.29	59.97
7	52.60	58.61
8	33.97	78.84
	Amino-N by complete hydrolysis with HCl	116.70
	Total N	146.96

Here also the protease of *Aspergillus* showed unmistakably strong digesting properties on these proteins and produced much amino acid. The addition of sodium carbonate for trypsin digestion appeared favorable for beef protein, but unfavorable for egg white.

3. Conclusions.

1. The protease preparation from *Aspergillus oryzae* digests casein, beef protein and egg white, and produces amino acids in large quantity, the results being similar to trypsin.

2. At 40°C. this enzyme solution at about neutral reaction, will keep its activity for at least 9 days, whereas trypsin loses its activity.

3. Tryptophane reaction was proved to be positive in casein digestion. This suggests that all other cases might be similar. In this respect, the protease from *Aspergillus* more closely resembles trypsin than pepsin.

4. The quantity of ammonia produced was very small, no special ammonia-forming enzyme being recognized.

5. The protease preparation contained originally 1.62 % of α-amino nitrogen, but after autolysis of 6 days at 40°C. this increased to 2.92 %.

C. Factors Influencing the Action of *Aspergillus* Protease.

1. Influence of Hydrogen Ion Concentration.

a. Witte Peptone as Substratum.

Solution.

1. 3 % Witte peptone solution. In about 250 cc. of water, 7.5 grams of air-dry Witte peptone were dissolved, kept for 5 minutes in boiling water, then cooled down, made up exactly to 250 cc. with water and then filtered through filter paper.

2. 1 % *Aspergillus* protease. Water solution of protease preparation from *Aspergillus oryzae*. The enzymic solution was clear enough without filtration.

3. Regulating mixtures (buffer solution). To keep the digesting liquids as near as possible at constant hydrogen ion concentration, the following salt solutions were made and used.

Table 23. Composition of regulating mixtures.

No.									
1	M/3 Na_2HPO_4	40 cc.	+	N/1 NaOH	20 cc.	+	H_2O	30 cc.
2	„	50 „	+	„	5 „	+	„	45 „
3	„	70 „		—	— „	+	„	30 „
4	„	40 „	+	M/3 NaH_2PO_4	40 „	+	„	20 „
5	—			„	70 „	+	„	30 „
6	N/1 CH_3COOH	7 „	+	N/1 CH_2COONa	30 „	+	„	63 „
7	„	10 „	+	„	30 „	+	„	60 „
8	„	25 „	+	„	10 „	+	„	65 „
9	M/3 NaH_2PO_4	50 „	+	N/1 HCl	20 „	+	„	30 „
10	„	20 „	+	„	40 „	+	„	40 „

Procedure.

In each test tube of 15 cc. capacity, 5 cc. of the peptone solution, 1 cc. of regulating mixture and 1.5 cc. of 1 % *Aspergillus* protease were mixed together. The tubes were closed tight with cork stoppers, and immersed in a water bath at 40°C. (± 0.5°C.). All tubes in the water bath were shaken at intervals of 1 hour. After exactly four hours digestion, the test tubes were put into boiling water for 10 min. to stop digestion, then cooled down in tap water. Four series of the same experiment were generally made, and, with two of them, the hydrogen ion concentration was tested by CLARK's colorimetric method (10) just before digestion and with the others after digestion[1]. Some change in hydrogen ion concentration was observed to take place during digestion, especially in the tubes on the alkaline side.

One series of digested liquids, to which no indicator was added, was used for the determination of amino nitrogen.

The VAN SLYKE's apparatus used, was that described by the inventor (98). The deaminization was continued in each case for 6 minutes and the absorption for four minutes.

To show the digestibility of the medium, the following calculation was performed.

$$\text{Digestibility} = \frac{Nd - Nb - Ne - Np}{Nt - Np} \times 100$$

Nd=mgm. amino nitrogen in the digested liquid.

Nb=mgm. nitrogen of blank test with 10 cc. H_2O+0.3 cc. capryl alcohol. 0.3 mgm. was taken always for this correction which corresponds to about 0.5 cc. gas.

Ne=mgm. amino nitrogen produced by the self digestion of enzyme solution only. For the enzyme preparation of *Aspergillus oryzae* 0.3 mgm. was taken. This experiment is described later.

Np=mgm. amino nitrogen contained in peptone solution before digestion. 2.38 mgm. were measured.

Nt=mgm. amino nitrogen from same quantity of peptone digested by strong HCl for 24 hrs. 17.23 mgm. were measured.

The results obtained, were as follows:—

1) All solutions for colorimetric determination of hydrogen ion concentration were kindly loaned by Dr. BERG.

Table 24. Digestion of WITTE peptone at different pH and 40°C. for 4 hrs.

No. of reg. mixture	pH			Digestibility (%)
	Before digestion	After digestion	Mean	
1	9.8	8.8	9.3	19.1
2	8.2	7.4	7.7	33.3
3	7.4	7.0	7.2	35.6
4	7.0	6.8	6.9	38.3
5	6.6	6.6	6.6	41.2
6	6.4	6.2	6.3	42.0
7	5.6	5.6	5.6	42.6
8	5.0	5.0	5.0	36.8
9	4.0	4.1	4.1	26.5
10	3.4	3.4	3.4	3.6

Digestion at 37.5°C. for 20 hrs.

Operation as above, except that 1.5 cc. of 1/12% enzyme solution (therefore, Ne=0.06 mgm.) were used and an incubator of 37.5°C. was employed.

After the digestion period, to stop further action, the tubes were plunged into ice water – instead of being boiled – and the amino nitrogen was immediately determined.

Table 25. Digestion of WITTE peptone at different pH at 37.5°C. for 20 hours.

No. of reg. mixture	pH			Digestibility (%)
	Before digestion	After digestion	Mean	
1	9.8	9.1	9.5	3.8
2	8.0	7.3	7.6	23.8
3	7.4	6.8	7.1	29.4
4	7.0	6.6	6.8	30.6
5	6.5	6.4	6.5	38.9
6	6.0	5.9	6.0	43.3
7	5.6	5.6	5.6	44.8
8	4.8	4.9	4.9	32.0
9	4.3	4.2	4.3	19.1

Both results show exact agreement as to the optimum pH value, i. e. pH 5.6, but with a sharper curve for 20 hrs. digestion. This probably was due to the difference of enzyme concentration used.

b. Edestin as Substratum.

As edestin is insoluble in plain water but soluble in acid solution, the procedure was as follows: To 15 grams of air-dry edestin were added 260 cc. water and 75 cc. N/5 HCl. The mixture was kept for 5 minutes in boiling water, cooled down and made up to 400 cc. Although almost clear, it was filtered through cotton. For digestion tests: 4 cc. of this solution, 1 cc. regulating mixture, 1 cc. N/10 NaOH and 1.5 cc. 1 % protease solution from *Aspergillus oryzae* were mixed together. Digestion for 4 hours at 40°C. To determine the digestibility of edestin in acid solution according to FULD's method (28) for pepsin, one experiment (No. 0) was carried out without neutralization, or addition of regulating mixture, the solution being the same as FULD's edestin solution. To prove also the liquefaction of edestin for another series of similar experiments, 1 cc. of HNO_3 and $MgSO_4$ mixture (4 parts of saturated $MgSO_4$ + 1 part conc. HNO_3) was added. To avoid heavy precipitation in amino nitrogen determination, 0.5 cc. N/1 HCl was added to each sample before VAN SLYKE's operation. In this case, Ne=0.54, Nt=17.54.

Table 26. Digestion of edestin at different pH at 40°C. for 4 hrs.

No. of reg. mixture	Appearance			pH		Digestibility (%)
	Before dig.	After dig.	With $MgSO_4$ + HNO_3	Before dig.	After dig.	
1	Clear	clear	apparent maximum pptn.	9.2	9.0	2.4
2	Pptn.	much pptn.	pptn.	7.8	7.0	21.8
3	„	pptn.	„	6.6	6.7	26.1
4	„	„	„	6.2	6.2	29.7
5	„	slight pptn.	„	5.4	5.4	40.0
7	„	„	slight pptn.	5.2	5.2	44.0
8	Slightly turbid	clear	„	4.8	4.8	43.8
9	Clear	slightly turbid	much pptn.	3.6	4.0	17.1
10	„	clear	apparent maximum pptn.	3.0	3.0	0.2
0	„	„	slight pptn.	4.3	4.2	37.3

This result shows the optimum to be about pH 5.2. At pH 9.0 the

activity was very slight and at pH 3.0 there was no action at all. It is true that even in the acidity of FULD's method (28) for pepsin, this protease has quite a strong action, which fact was also recognized by WOHLGEMUTH (108). Contrary to his suggestion, this appears to be due not to the existence of pepsin, but to the fact that the pH range of the digesting liquid, in which the protease acts, is wide enough to include the acidity in FULD's method. The liquefaction of edestin seems to run parallel with amino nitrogen production.

c. Casein as Substratum.

From the above experiments, it was proved that the optimum reaction of the amino acid production from WITTE peptone or edestin, and the liquefaction of edestin agreed at about pH 5.2, i. e., a weak acid reaction whereas the optimum reaction of casein liquefaction by the same protease by the author's method (II, C) was distinctly alkaline, even with phenolphthalein as indicator. The same conclusions were arrived at by WOHLGEMUTH (108) and SZANTO (93) for casein liquefaction by Taka-diastase with FULD-GROSS' method (31).

The question arises as to whether the optimum reaction of casein digestion is exceptional. To solve this question, the following experiment was performed:

Solutions.

1. Three per cent casein solution. 7.5 grams of air-dry casein were mixed with 100 cc. of H_2O and 75 cc. of N/5 NaOH. This was dissolved by warming and N/5 HCl was added to neutralize, phenolphthalein being the indicator. It was then made up to 250 cc. with water.

2. *Aspergillus* protease solutions. 1%, 0.3% and 0.1% water solutions of *Aspergillus* protease were used.

3. HNO_3 and $MgSO_4$ mixture, regulating mixture, and CLARK's colorimeter for the estimation of hydrogen ion concentration were used as before.

Procedure.

5 cc. of 3% casein were mixed with 1 cc. of *Aspergillus* protease and 1 cc. of regulating mixture in test tubes, kept for 4 hours at 40°C. Then 1 cc. of HNO_3 and $MgSO_4$ mixture or acetic acid alcohol solution was

added to precipitate the undigested casein. Care was taken that the acetic acid alcohol solution was added after the digested liquid was acidified with HCl to pH 5. Both methods of precipitation gave exactly the same results. The limiting concentrations of protease solution for casein liquefaction were estimated by observation. The proteolytic values were calculated from the definition by the author's method (II C.), i. e., if 0.005 gram of original enzymic substance completely dissolves 0.005 gram casein in one hour at 40°C., the proteolytic value of this substance is 100.

Table 27. The pH and appearance of the digesting casein solution.

No. of reg. mixture	Before digestion			After digestion		
	1% protease	0.3% protease	0.1% protease	1% protease	0.3% protease	0.1% protease
1	10.0 (clear)	—	—	10.0 (clear)	—	—
2	9.2 („)	9.4 (clear)	9.8 (clear)	7.7 („)	8.2 (clear)	9.4 (clear)
3	8.2 („)	8.3 („)	8.6 („)	7.3 („)	7.5 („)	8.0 (turbid)
4	7.3 („)	7.4 („)	7.5 („)	7.0 („)	7.1 („)	7.2 (pptn.)
5	6.9 („)	6.9 („)	7.0 („)	6.7 („)	6.9 (sl. tur.)	6.7 („)
6	6.6 (sl. tur.)	6.6 („)	6.6 („)	6.6 („)	6.7 („)	6.6 („)
7	6.2 (turbid)	6.2 (turbid)	6.2 (turbid)	6.2 („)	5.8 („)	6.0 („)
8	5.3 (pptn.)	—	—	5.3 (sl. tur.)	—	—
9	4.5 („)	—	—	4.4 (pptn.)	—	—
10	3.4 („)	—	—	3.4 („)	—	—

Table 28. Results of casein liquefaction.

No. of reg. mixture	Appearance of digested liquids on addition of HNO_3+$MgSO_4$ or acetic acid			Limiting concentration of protease (%)	Proteolytic value
	1% protease	0.3% protease	0.1% protease		
1	Much pptn.	much pptn.	much pptn.	?	?
2	Clear	pptn.	„	0.8	200
3	„	slight pptn.	pptn.	0.4	400
4	„	turbid	„	0.6	260
5	„	„	„	1.0	160
6	Slightly turbid	very turbid	„	2.0	80
7	Turbid	„	„	3.0	53
8	„	„	„	?	?
9	Pptn.	pptn.	„	?	?
19	„	„	„	?	?

The results show that the optimum reaction of casein liquefaction is pH 8.0 as the mean value before and after digestion. With other experiments of 1 hour and 20 hours digestion, the same results were obtained.

By way of comparison a further experiment was made. To each tube of the digested liquid with 1% protease, 0.5 cc. N/1 NaOH was added to stop digestion and to keep undigested casein in solution. The amino nitrogen content was then determined. The calculation of digestibility was the same as in the case of WITTE peptone or edestin digestion, except Ne=1.07 and Nt=16.65.

Table 29. Digestibilities by estimating the amino-N produced.

No. of. regulating mixture	Digestibility (%)
1	1.2
2	29.3
3	33.0
4	36.2
5	38.9
6	40.8
7	42.6
8	24.4
9	15.0
10	0.3

The figures show that the optimum reaction for amino acid production from casein by *Aspergillus* protease was about pH 6.2, which is a distinctly acid reaction, and a little weaker than that with WITTE peptone or edestin. A test by another method which is described later (V, D, 2) yielded the same result.

Why is the optimum reaction of casein liquefaction alone, so alkaline? It is unlikely that the optimum reaction of protein liquefaction and amino acid production by *Aspergillus* protease is different. It is supposed that casein has its iso-electric point at pH 4.7, when it precipitates best, and therefore it is harder to digest in weak acid than in weak alkaline solutions. Whatever the reason, this difference is quite important in connection with the test of proteolytic action of enzymes by casein liquefaction, either by the author's or FULD-GROSS' method.

d. Glycinin as Substratum.

As glycinin cannot be dissolved in pure water, a weak alkaline solution of glycinin was first made, placed in tubes and then neutralized with an acid. 15 grams of air dry glycinin were added to 200 cc. water and 30 cc. N/5 NaOH. This almost clear solution was made up to 400 cc. without heating, and filtered through cotton. In each tube, 4 cc. of this glycinin solution, 1 cc. regulating mixture, 1 cc. N/16.6 HCl and 1.5 cc. 1% *Aspergillus* protease were mixed and digested at 40°C. for 4 hours. The observation of the pH value was difficult, due to the heavy turbidity and precipitation of the contents. Ng=0.63 Nt=16.65.

Table 30. Digestion of glycinin at different pH.

No. of reg. mixture	Appearance		pH		Digestibility (%)
	Before dig.	After dig.	Before dig.	After dig.	
1	Clear	clear	9.4	9.4	2.6
2	Turbid	pptn.	8.0	7.4	31.6
3	Very turbid	„	7.2	6.8	32.8
4	Much pptn.	slight pptn.	6.2	6.6	30.1
5	„	„	5.6	5.8	27.6
6	„	„	5.2	5.6	27.7
7	„	„	5.0	5.4	29.3
8	Pptn.	pptn.	4.4	4.0	29.9
9	Clear	„	3.8	3.7	17.4
10	„	„	3.2	3.2	0.4

The figures show that the change of digestibility compared with the change of reaction was irregular. The reason for this may be inferred from observation of the appearance of the digesting liquids (except at the extremes when pH=over 9 or less than 4). Best digestion took place at such reaction when glycinin was not precipitated but remained mostly in solution. In this state, the digestion proceeded much more easily than the digestion of solid protein. At the reaction of pH 5–6.2, when, usually, during the digestion of many other proteins, the maximum proteolytic activity of *Aspergillus* protease is shown, decidedly less digestion took place in this experiment, because the precipitation of glycinin was heaviest.

(Appendix) Autolysis of Enzymic Preparation from Aspergillus Oryzae.

In former experiments (V, B, C), it was proved that the preparations of pepsin, trypsin and *Aspergillus* protease themselves produce amino nitrogen to quite a measurable degree during digestion. The exact correction for this amino nitrogen in digestion experiments is a very difficult problem, as it seems impossible to ascertain the quantity of amino nitrogen derived from self-digestion, when it is mixed with other digesting substances. To determine approximately the quantity of amino nitrogen, produced from the enzyme itself during digestion, the water solution of 2% enzyme was kept for 4 hours at 40°C. and the amino nitrogen produced was measured by VAN SLYKE's method.

Amino-N (mgm.) produced by autolysis of 1 grm. *Aspergillus* protease.

Before digestion	14.85 mgm.
After „	20.55 „

2. Influence of Common Salt.

a. WITTE Peptone as Substratum.

"Shoyu moromi" (soy sauce mash) contains 16–20% (=3–3.5 M) NaCl. To decide its influence upon proteolytic digestion, WITTE peptone was digested with protease from *Aspergillus oryzae* as follows:—

Three per cent WITTE peptone solution was prepared with 4 and 5 M NaCl solutions instead of water. The peptone solution with 5 M NaCl showed a considerable undissolved portion but that with 4 M NaCl appeared the same as with water solution. By mixing the peptone solution with 4 M NaCl and peptone water solution, in certain ratios, the salt concentration of the 3% peptone solution was varied. Regulating mixture No. 6 was used throughout.

Table 31. Digestion of WITTE peptone containing different quantities of NaCl.

Concentration of NaCl		pH		Digestibility (%)
In peptone soln. (M)	In digesting liquid (%)	Before dig.	After dig.	
5.00	about 20.0	5.6	5.5	25.8
4.00	„ 16.0	5.8	5.8	34.7

Concentration of NaCl		pH		Digestibility (%)
In peptone soln. (M)	In digesting liquid (%)	Before dig.	After dig.	
2.00	about 8.0	5.9	6.0	39.1
1.00	„ 4.0	6.0	6.0	42.2
0.50	„ 2.0	6,0	6.1	43.1
0.30	„ 1.0	6.1	6.1	43.4
—	—	6.4	6.1	44.6

As peptone does not dissolve well in 5 M NaCl solution, the presence of this quantity of salt causes the results to be unreliable. It may be said that peptone digestion will decrease from 44.6% to 34.7% of digestibility in the presence of 16% NaCl, with a gradual decrease of digestibility upon the gradual increase of the salt content. Here the pH value of the digestion mixture changes a little, owing to the buffer action of NaCl. To make sure of the pH influence with peptone solution containing 4 M NaCl, the following experiment was made:—

Table 32. Digestion of WITTE peptone containing NaCl at different pH.

No. of regulating mixture	pH		Digestibility (%)
	Before dig.	After dig.	
3	7.5	7.3	27.7
4	6.7	6.7	30.4
5	6.2	6.2	32.2
6	6.0	6.0	34.9
7	5.4	5.4	34.0

It was noticed that a slight change of pH value had not much influence upon digestibility.

b. Glycinin as Substratum.

Comparison of NaCl Solution and NaOH Solution as Solvent of Glycinin.

Glycinin was dissolved in NaOH solution and neutralized with HCl. By the addition of "$MgSO_4 + HNO_3$" (4 parts of saturated $MgSO_4$ solu-

tion and 1 part of conc. HNO_3), the undigested protein was always precipitated, but when the glycinin was completely digested no preciptation took place.

Table 33. Composition of the digesting liquids.

No.	Glycinin (gram)	N/5 NaOH (cc.)	20% NaCl (cc.)	N/5 HCl (cc.)	H_2O (cc.)	2% *Asp.* protease (cc.)	Toluol (cc.)
1	0.5	1	25	1	23	5	2
2	0.5	—	25	—	25	5	2
3	0.5	1	—	1	48	5	2

Table 34. Appearance of the digesting liquids.

No.	At beginning	After 2 days	After 4 days	After 9 days
1	Almost clear	clear	clear	clear
2	Clear with much pptn.	„	„	„
3	Very turbid	pptn.	„	„

Table 35. By adding $MgSO_4 + HNO_3$ to the liquids.

No.	At beginning	After 2 days	After 4 days	After 9 days
1	Pptn.	pptn.	pptn.	pptn.
2	„	„	more pptn.	„
3	„	clear	clear	clear

Table 36. Amino N (mgm.) in 1 gram digested glycinin.

No.	At beginning	After 2 days	(Diff.)	After 4 days	(Diff.)	After 9 days	(Diff.)	After complete hydrolysis	(Diff.)
1	6.20	53.88	47.68	60.28	6.40	66.18	5.90	111.0	44.82
2	„	51.18	44.98	57.78	6.60	64.73	6.95	„	46.27
3	„	82.38	76.18	95.68	13.30	108.63	12.95	„	2.37

After the above experiments, the remaining proteolytic activity in the digested liquids was examined, was described in section V, B, 1, b, in the following way: 5 cc. of 0.2% casein solution were mixed with liquids which had already been digested for 9 days, and after 3 days renewed digestion at 40°C., the undigested casein precipitated with $MgSO_4 + HNO_3$. The results obtained were as follows:

1. Pptn. It could not be determined whether this was due to undigested glycinin or to destruction of protease.
2. ,, ,,
3. Clear. Positive proof of strong proteolytic activity.

Influence of Common Salt upon Digestion.

Table 37. Composition of the digesting liquids.

	Glycinin (cc.)	N/5 NaOH (cc.)	20% NaCl (cc.) (final concn.)	N/5 HCl (cc.)	H_2O (cc.)	2% *Aspergillus* protease (cc.)	Toluol (cc.)
1	0.5	1	5.0 (2%)	1	43.0	5	2
2	,,	,,	12.5 (15%)	,,	35.5	,,	,,
3	,,	,,	25.0 (10%)	,,	23.0	,,	,,
4	,,	,,	— (0)	,,	50.0	,,	,,
5	,,	—	25.0 (10%)	—	30.0	—	,,

Table 38. Appearance of the liquids.

	Before digestion	After 42 hrs.	After 6 days
1	Almost clear	clear	clear
2	,,	,,	,,
3	,,	,,	,,
4	Much pptn.	much pptn.	much pptn.
5	,,	very turbid	very turbid

Table 39. By addition of $MgSO_4 + HNO_3$ to the liquids.

	Before digestion	After 42 hrs.	After 6 days
1	Pptn.	clear	clear
2	,,	much pptn.	almost clear

	Before digestion	After 42 hrs.	After 6 days
3	Pptn.	much pptn.	slight pptn.
4	„	clear	clearest
5	„	maximum pptn.	much pptn.

Residual Proteolytic Activity in digested Liquids after 6 Days.

Same operation as before (V, B, 1.).

1. Clear.
2. Almost clear.
3. Slight pptn.
4. Clearest.
5. Pptn.

Table 40. Amino-N (mgm.) in 1 gram digested glycinin.

	Before digestion	After 48 hrs.	(Diff.)	After 6 days	(Diff.)	After complete hydrolysis with HCl	(Diff.)
1	4.50	73.88	69.38	89.68	15.80	111.00	21.32
2	„	53.78	49.28	75.88	22.10	„	35.12
3	„	46.78	42.28	58.48	11.70	„	52.52
4	„	34.98	30.48	78.18	43.20	„	32.82
5	„	4.50	0.0	4.29	-0.21	„	106.71

N. B. Total nitrogen in 1 gram of glycinin = 146.0 mgm.

These experiments with glycinin show the following results:—

1. A 2% NaCl solution causes more digestion than water alone during the first period, but later vice versa.

2. A solution containing over 2% NaCl has an increasing inhibiting effect upon the proteolytic action of *Aspergillus* protease.

3. It is clear that to a great extent *Aspergillus* enzyme splits glycinin into amino acid.

c. Edestin as Substratum.

To most of the following liquid was added first HCl to dissolve edestin and then NaOH to neutralize.

Composition of Digesting Liquids.

One gram air dry edestin, 85 cc. H_2O and 15 cc. N/5 HCl were mixed and dissolved until the solution became clear. With this clear edestin solution, the results obtained, were as follows:—

Table 41. Composition of digesting liquids.

	Edestin soln. (cc.)	H_2O (cc.)	N/5 NaOH (cc.)	20% NaCl (cc.) (final conc.)	2% *Aspergillus* protease (cc.)	Toluol (cc.)
1	20	20	—	—	5	1
2	"	17	30	—	"	"
3	"	—	"	17 (8%)	"	"
4	"	13	"	4 (2%)	"	"
5	"	20	"	20 (10%)	"	"

Table 42. Appearance of the liquids.

	Before digestion	After 44 hrs.	After 5 days
1	Clear	clear	clear
2	Pptn.	"	"
3	"	"	"
4	"	"	"
5	Turbid	"	"

Table 43. Appearance after addition of $MgSO_4$ + HNO_3.

	Before digestion	After 44 hrs.	After 5 days
1	Pptn.	clear	clear
2	"	"	"

	Before digestion	After 44 hrs.	After 5 days
3	Pptn.	clear	clear
4	„	„	„
5	„	pptn.	pptn.

Remaining Proteolytic Activity in digested Liquids after 5 Days.

Same procedure as before described was followed.

1. Pptn. Protease was destroyed by HCl.
2. Clear.
3. „
4. „
5. Pptn. Probably due to the undigested casein.

Table 44. Amino N (mgm.) in 1 gram digested edestin.

	Before dig.	After 44 hrs.	(Diff.)	After 5 days	(Diff.)	After complete hydrolysis with HCl	(Diff.)
1	2.0	78.2	76.2	89.4	11.2	117.0	27.6
2	„	98.2	96.2	103.7	5.5	„	13.3
3	„	90.7	88.7	101.1	10.4	„	15.9
4	„	104.5	102.5	116.0	11.5	„	1.0
5	„	30.5	28.5	33.0	2.5	„	84.0

The results of the above experiments with edestin may be summarized as follows:—

1. *Aspergillus* protease can digest edestin in an acid solution as strong even as that used in FULD's pepsin method.

2. In such strong acid solution, however, this enzyme is destroyed rather quickly.

3. Previous treatment with acid influences digestion very favourably.

4. Addition of NaCl seems to have the same effect on edestin as in the case of glycinin.

(Appendix) Total Nitrogen of Proteins and WITTE *Peptone, and their Amino Nitrogen Contents before and after Complete Hydrolysis.*

As mentioned before, the amino nitrogen contents of proteins and WITTE peptone used as substrata had great importance in controlling the results of these experiments. Total nitrogen and amino nitrogen before and after their complete hydrolysis were, therefore, determined as follows:

Proteins were completely hydrolyzed by the usual method with strong HCl, and amino nitrogen was determined by VAN SLYKE's method. Total nitrogen was determined by KJELDAHL's method. Amino nitrogen before hydrolysis was determined with protein solution. The quantities of air-dry casein and edestin, and filtered solution for WITTE peptone and glycinin used, were the same as in the digestion expriments.

Table 45. Nirogen contents in proteins.

Proteins	Total nitrogen (%)	Amino nitrogen (%)
Casein	14.45	11.10
WITTE peptone	14.70	11.50
Edestin	17.16	11.70
Glycinin	14.60	11.10

3. Influence of Hydrogen Ion Concentration upon Glycinin Digestion in Common Salt Solution.

To avoid, if possible, any interruption of precipitation of glycinin, sodium chloride solution was used as solvent. To 15 grams air-dry glycinin were added 100 cc. of water and 30 cc. of 0.2 N NaOH which, when dissolved, was made up to 400 cc. with NaCl solution, regulated so as to make one mole NaCl concentration, and, without heating, filtered through cotton. 4 cc. of this solution were placed in each tube with 1 cc. N/22 HCl, 1 cc. regulating mixture and 1.5 cc. of 1 % *Aspergillus* protease.

In connection with these experiments, it was noted that pecipitation in the digesting liquids was much less than in the experiments with pure water solutions.

Table 46. Digestibility of glycinin at different pH.

No. of reg. mixture	Appearance		pH		Digestibility (%)
	Before dig.	After dig.	Before dig.	After dig.	
1	Clear	clear	—	9.2	0.6
2	Slightly turbid	pptn.	—	7.4	23.9
3	"	"	—	6.4	27.3
4	"	"	—	6.2	28.2
5	"	"	—	5.8	30.0
6	"	"	5.6	5.5	30.0
7	"	"	—	5.2	30.0
8	Pptn.	"	—	4.9	27.1
9	"	"	—	3.6	4.8
10	"	"	—	3.0	0.3

A fairly regular relation is seen between the change of digestibility and reaction, although the maximum digestibility was reduced, due to the inhibiting action of salt. The optimum reaction was between pH 5 and 6.

These two experiments "2" and "3" offer good examples of protein digestion with a protease, where the solubility of the protein, as well as the reaction of the digesting liquid, has a great influence upon digestibility.

4. Influence of Common Salt and Cooking upon Glycinin Digestion.

In "shoyu" brewing, soy beans are cooked generally for 2–3 hours in an open utensil. Cooking in autoclave at a pressure of 15 lbs. for 1.5–2 hours, however, is gradually obtaining appreciation among the brewers. Autoclaving has several advantages. It makes the texture of the soy beans softer, which may give a better opportunity for growth of mould, and also makes digestion mechanically easier. It is useful to know what influence the cooking has upon protein digestion by enzyme.

WATERMAN (101) recently made an interesting experiment on protein from navy beans *(Phaseolus vulgaris)*. He used phaseolin from the beans and digested it with pepsin, and then with trypsin. The digestibility was measured by the amino nitrogen produced. His experiments indicate that phaseolin is rendered more readily digestible by boiling with distilled water. Cooking for 5 minutes gave a noticeable increase in digestibility, while cooking for 40 minutes was apparently sufficient to produce the

maximum effect. Cooking for 4 hours gave a slight decrease in digestibility.

Such digestibility may be altered by the chemical or physical change of the protein substratum. Such change sometimes takes place through simple mechanical treatment. To eliminate if possible such a factor, glycinin was used suspended in water and also in sodium chloride solution, which keeps glycinin in solution.

Preparation of glycinin solutions.

1. In water: As in "2" of this section. In each tube, 4 cc. of this solution were taken and mixed with 1 cc. of N/16.6 HCl, loosely corked, then cooked. Much precipitate was observed before cooking.

2. In 1 M NaCl solution: As in "3" of this section. In each tube, 4 cc. of this solution were added to 1 cc. N/16.6 HCl and cooked. Before cooking, the solution was almost clear.

3. In 3 M NaCl solution: Fifteen grams of air dry glycinin, 100 cc. of water and 30 cc. of N/5 NaOH were mixed and made up to 400 cc. with NaCl solution, and regulated to make the whole solution of 3 M NaCl content. After filtration through cotton, 4 cc. of this solution and 1 cc. of N/16.6 HCl, were placed in each tube and then cooked. In appearance, the solution before cooking was almost clear.

Procedure.

For cooking, both a boiling water bath and an autoclave were used to maintain the temperature for the various periods required. After cooking, the tubes were cooled down in cold water, and to each tube was added 1 cc. of regulating mixture No. 6 and 1.5 cc. of 1% protease solution from *Aspergillus oryzae*. Digestion in the usual manner at 40°C. for 4 hours was then completed.

Table 47. Digestibility of glycinin after cooking.

No. of dig. liquid	Glycinin in	pH before cooking	Method of cooking	Appearance after cooking
1	Water	6.4	no cooking	coagulated
2	„	„	10 min. in boiling water	more coagulated
3	„	„	30 min. „	„
4	1 M NaCl	6.0	no cooking	almost clear

No. of dig. liquid	Glycinin in	pH before cooking	Method of cooking	Appearance after cooking
5	1 M NaCl	6.0	10 min. in boiling water	slightly turbid
6	„	„	30 min. „	more turbid
7	„	„	30 min. in 15 lbs. pressure	coagulated
8	3 M NaCl	5.2	no cooking	almost clear
9	„	„	10 min. in boiling water	slightly turbid
10	„	„	30 min. „	more turbid
11	„	„	30 min. at 10 lbs. pressure	coagulated

Table 48. Digestibility of glycinin after cooking (continued)

No. of dig. mixture	pH		Appearance after digestion	Digestibility (%)
	Before digestion	After digestion		
1	5.6	5.6	some pptn.	28.4
2	—	—	„	36.9
3	—	—	„	34.8
4	5.5	5.5	„	30.0
5	—	5.5	„	35.3
6	—	5.5	„	36.2
7	—	5.5	„	34.9
8	—	—	„	17.2
9	—	—	„	18.7
10	—	—	„	18.0
11	—	—	„	20.8

From the above results of experiments, the following influences of cooking may be deduced.

1. There is a very distinguishable difference between the digestibility of cooked and uncooked proteins, no matter whether the concentration of the salt solution, or the period and temperature of cooking is varied. It is possible that this better digestibility of cooked protein is due to the destruction of antiprotease in protein by heating.

2. In the case of water solution, the digestibility of glycinin cooked for 10 minutes in boiling water is better than in that cooked for 30 minutes. In the case of NaCl solutions of 1 and 3 M concentrations, not much difference between them can be observed. By cooking at high pressure, the digestibility of glycinin in 1 M NaCl solution, compared with the glycinin cooked in boiling water, is decreased.

3. This experiment shows also the influence of sodium chloride upon digestion and that 1 M NaCl solution (about 0.5 M NaCl solution in digesting liquids of 7.5 cc.) is rather better than the pure water solution. Three mole solutions (about 1.5 M NaCl solution in digesting liquid) inhibit digestibility to a great degree. It should be considered here, that in a former experiment (V, C, 2, b), the glycinin digestion in 2% NaCl solution was also better than when pure water only was used during the first period, but that later it was the opposite. This phenomena may be due to the fact that, although NaCl keeps glycinin in solution and makes digestion easier, it checks the proteolytic action of the enzyme itself.

5. Influence of Enzyme Quantity upon WITTE Peptone Digestion.

Different quantities of *Aspergillus* protease solution were used for the digestion of WITTE peptone. Here of course, "Ne" gives different mgm. of nitrogen in each case.

Regulating mixture used, was No. 6.

Table 49. Digestion at 40°C. for 4 hrs.

% of enzyme solution	pH		Digestibility (%)
	Before digestion	After digestion	
2.0	6.2	6.2	56.3
1.0	6.1	6.1	45.2
1/2	6.0	6.0	35.4
1/4	6.0	6.0	24.0
1/8	6.0	6.0	14.5

So far as this range of enzyme quantity is concerned, it may be generally stated that a 100% difference in enzyme quantity causes 10% difference in digestibility.

The results of similar experiments, but with 20 hours and 4 days digestion at 37.5°C., will be described. Volume of enzymic solution used was 0.4 cc.

Table 50. Digestion at 37.5°C. for 20 hrs. and 4 days.

% of enzyme solution	pH		Digestibility (%)	
	Before dig.	After dig.	After 20 hrs.	After 4 days
2.0	6.0	6.0	64.8	77.5
1.0	„	„	52.7	67.2
1/2	„	„	38.4	57.0
1/4	„	„	26.2	49.2
1/8	„	„	18.4	36.1
1/16	5.8	5.8	10.9	27.9

From these results it may be said that this digestion process follows SCHÜTZ's law, i. e., the quantity of digestion product is proportional to the square root of the quantity of protease used, but not directly proportional to the quantity of protease. More exact experiments and calculations are described later (V, D, 2, c).

D. Examination of *Aspergillus* Protease in Relation to its Reaction Velocity.

1. WITTE Peptone as Substratum.

a. Influence of Hydrogen Ion Concentration on Reaction Velocity.

As preparation for the detailed examination of reaction velocity of *Aspergillus* protease, the influence of hydrogen ion concentration upon the action was determined.

Solution.

1. Three per cent WITTE peptone solution.
2. One per cent *Aspergillus* protease solution.
3. Regulating mixtures. Composed as follows:—

Table 51. Composition of regulating mixtures.

No.						
1	M/3 H_3PO_4	20 cc.	+	M/3 KH_2PO_4	40 cc.
2	„	80 „	+	„	80 „
3	„	60 „	+	„	100 „
4	„	40 „	+	„	120 „
5	„	30 „	+	„	130 „
6	„	20 „	+	„	140 „
7	„	10 „	+	„	150 „
8	M/3 Na_2HPO_4	30 „	+	„	130 „
9	„	100 „	+	„	60 „
10	„	140 „	+	„	20 „
11	„	155 „	+	N/1 NaOH	5 „
12	„	145 „	+	„	15 „

Procedure.

In hard glass test tubes of 25 cc. capacity were placed 5 cc. of 3% WITTE peptone and 1 cc. of regulating mixture. These were warmed for 10 minutes in a constant water bath at 40°C., then 1.5 cc. of 1% *Aspergillus* protease were added. After exactly 4 hours digestion, 1 cc. of N/1 NaOH was added to stop enzymic action. With 2 cc. of this digested liquid, the amino nitrogen was estimated. VAN SLYKE's micro apparatus was shaken in every case, 4 minutes for deaminization and 3 minutes for absorption.

The results were calculated as follows:—

Digestibility = x/a × 100.

a = Nt − Np = 3.49 mgm. Total amino nitrogen capable of being produced from WITTE peptone in 2 cc. digesting liquid.

x = Nd − Nb − Ne − Np. Amino nitrogen (mgm.) in 2 cc. digested liquid produced by digestion.

Nd = Amino nitrogen (mgm.) in 2 cc. digested liquid.

Nb = 0.1 mgm. nitrogen of blank test with 2 cc. of water only.

Ne = 0.08 mgm. Amino nitrogen produced by the autlolysis of enzyme solution.

Nt = 4.05 mgm. Amino nitrogen from same quantity of peptone digested by strong HCl.

Np = 0.56 mgm. Amino nitrogen contained in peptone solution before

digestion.

Hydrogen ion concentration was determined by the colorimetric methods of MICHAELIS (56) and CLARK (10), and with K type electric apparatus of Leeds and Northrup Co., before and after digestion, using a larger quantity of digesting liquid of the same composition.

Table 52. Digestion of WITTE peptone at different pH.

No. of reg. mixture	pH			x	Digestibility $=x/a \times 100$	Enzymic activity $E \times 10^3 = x^2 \times 10^3$
	Before dig.	After dig.	Mean			
1	3.2	3.2	3.2	0.620	17.77	384
2	3.6	3.6	3.6	0.880	25.21	774
3	4.4	4.4	4.4	1.000	28.65	1,000
4	4.8	4.8	4.8	1.175	33.67	1,380
5	5.1	5.1	5.1	1.330	38.11	1,768
6	5.3	5.3	5.3	1.440	41.26	2,073
7	5.8	5.8	5.8	1.395	39.97	1,946
8	6.1	6.0	6.0	1.175	33.67	1,380
9	6.5	6.5	6.5	1.123	32.18	1,261
10	6.8	6.8	6.8	1.033	29.60	1,067
11	7.6	7.4	7.5	0.936	26.82	876
12	8.2	7.7	7.9	0.820	23.50	672

The reason why enzymic activity is expressed by x^2, is explained later (V. D, 1, b).

The optimum reaction is here pH 5.3. In another experiment (V, C, 1, a), it was pH 5.6. The value pH 5.3 found here believed to be more accurate, as it was determined electrometrically, and was confirmed in the next experiment.

b. Determination of Formula applicable to the Reaction Velocity.

The reaction velocity of many enzymes, i. e., amylase, invertin, esterase, catalase and erepsin, is changed by the digesting period, or enzyme quantity, according to the following monomolecular formula:—

$$K = \frac{1}{t} \times \log \frac{a}{a-x}$$

K = velocity constant.
t = digesting period.

a=initial concentration of digesting substratum.
x=digested product after time "t".

In this case, we compare the enzymic activity with the velocity constant "K". In the case of the digestion of protein with pepsin in stomach juice the reaction does not follow the above formula, but proceeds proportionally to the square root of the quantity of enzyme or digesting period, therefore:—

$$x=K\sqrt{E} \quad \text{or} \quad x=K\sqrt{t}$$

E=enzymic activity or enzyme quantity.

This is known as SCHÜTZ's formula and originated by EMIL SCHÜTZ (85) in 1885. Though there has been much discussion, it is concluded that it is correct, at least for the first one third of peptic digestion. Tryptic digestion of pancreatic juice agrees also with this formula.

With SCHÜTZ's formula, the enzymic activity should compare with x^2, when all other conditions are equal, because $E=(x/K)^2$, then $E \propto x^2$.

The reaction velocity of the amylase from *Aspergillus oryzae* corresponds to a monomolecular formula as shown in section VI, B. What then is the reaction velocity of the protease from the same mould? This subject was studied by digesting WITTE peptone and casein.

Procedure: The same operation was carried out as described in section V, D, 1, a, with regulating mixtures Nos. 5, 6 and 7, but with digesting periods of 1 to 6 hours. The results were calculated with monomolecular and SCHÜTZ's formulae.

a=3.490 mgm. Amino nitrogen capable of being produced from the sample.
Km=constant of monomolecular formula.
Ks=constant of SCHÜTZ's formula.
x=amino nitrogen (mgm.) in 2 cc. sample produced by digestion.

Table 53. Digestion of WITTE peptone at 3 kinds of pH for different hours.

	Digesting hours (t)	x	Digestibility $=x/a\times 100$	$Km\times 10^5 = 1/t \log\frac{a}{a-x}\times 10^5$	$Ks\times 10^4 = x/\sqrt{t}\times 10^4$
Regulating mixture No. 5. pH=5.1	1.0	0.580	16.62	7,893	5,800
	2.0	0.955	27.36	6,943	6,754
	3.0	1.190	34.10	6,037	6,871
	4.0	1.330	38.11	5,210	6,650
	6.0	1.605	45.99	4,459	6,554
Regulating mixture No. 6. pH=5.3	1.0	0.616	17.65	8,434	6,169
	2.0	0.975	27.94	7,115	6,895
	3.0	1.297	37.16	6,726	7,488
	4.0	1.440	41.26	5,777	7,200
	6.0	1.686	48.31	4,777	6,884
Regulating mixture No. 7. pH=6.0	1.0	0.623	17.85	8,540	6,230
	2.0	0.940	26.93	6,815	6,648
	3.0	1.160	33.24	5,849	6,697
	4.0	1.395	39.97	5,541	6,915
	6.0	1.620	46.42	4,517	6,615

1. The constants of the monomolecular formula are seen to be smaller with longer digestion, so the reaction does not follow this formula.

2. As the results obtained by using SCHÜTZ's formula kept fairly constant, the reaction velocity of *Aspergillus* protease can be assumed to follow SCHÜTZ's law, i. e., the digestion product of WITTE peptone increases proportionally to the square root of the digestion period. As shown in section V, C, 1, the digestibility of WITTE peptone by *Aspergillus* protease, decreased 10% for 100% increase of protease quantity.

3. The constant was always the largest with the regulating mixture No. 6, where pH was 5.3. Therefore this experiment proved that the reaction pH 5.3 is also the optimum for this enzymic action.

c. Influence of Temperature on Reaction Velocity.

Enzymic action, just as other chemical actions is influenced by temperature, and as enzymes are destroyed by heat, too high a temperature weakens their activity. The author showed in another paper (97) that the amylase of *Aspergillus* has 50°C. as its optimum temperature at 0.5–2.0 hours digestion, but 40°C. is the optimum for 24 hours digestion.

Here the temperature influence was studied with only four hours digestion using WITTE peptone liquid, regulated to pH 5.3. The operation was similar to that described in section V, D, 1, a, but with different digesting temperatures.

Table 54. Digestion of WITTE peptone at different tempertures.

Digesting temperature (°C.)	x	Digestibility $=x/a \times 100$	Enzymic activity $E \times 10^3 = x^2 \times 10^3$
10	0.355	10.17	126
20	0.580	16.62	336
30	1.010	28.94	1,020
35	1.220	34.96	1,488
40	1.390	39.83	1,932
45	1.440	41.25	2,073
50	1.545	44.26	2,387
55	1.520	43.55	2,310
60	1.450	41.55	2,102
70	0.520	14.90	270

It is seen that the optimum temperature is 50°C., and that at 70°C. the activity decreases to only 1/9 and at 10°C. to 1/16.

2. Casein as Substratum.

a. Influence of Hydrogen Ion Concentration on Reaction Velocity.

Solution.

1. Casein solution. Ten grams of water-free casein were prepared by the method described in section V, A, 3, and mixed with 300 cc. of water and 14 cc. N/1 NaOH. The mixture was heated until it became clear, neutralized with N/10 HCl, phenolphthalein being the indicator and made

up to 400 cc. with water. 40 cc. of this solution and 10 cc. water make 2% casein solution.

2. 0.1% *Aspergillus* protease.

3. Regulating mixtures. Same as described in section V, D, 1, together with the following mixtures:

No. of regulating mixture	
1 a	M/3 H_3PO_4
10 a	7 cc. of no. 10 + 3 cc. of no. 11.
10 a	5 „ „ 5 „ „
10 a	3 „ „ 7 „ „

4. 20% Na_2SO_4, acidified slightly with H_2SO_4.

Procedure.

Erlenmeyer's flasks of 150 cc. capacity with 40 cc. casein solution and 10 cc. of regulating mixture were placed in a constant water bath at 40°C. To this mixture was added 2 cc. of 0.1% *Aspergillus* protease and the whole digested for 2 hours. After this N/1 HCl was added, to dissolve undigested casein, and 20 cc. of 20% Na_2SO_4, to precipitate all undigested casein. The precipitate was collected on a filter paper, washed with 20% Na_2SO_4, and the nitrogen was estimated by KJELDAHL's method. The difference between total N in casein before digestion (=a=0.1610 gram) and N in undigested casein, is N in digested casein (=x). For convenience of calculation, two hours and 0.1% protease were taken as unit.

Table 55. Digestion of casein at different pH.

No. of reg. mixture	Appearance of dig. liquid.		pH before dig.	N in undig. casein (mgm.)	N. in dig. casein (=x)(mgm.)	Digestibility =x/a x 100	Enzymic activity $E=x^2$
	Before dig.	After dig.					
1a	Clear	clear	3.2	114.00	47.00	29.19	2,209
4	„	pptn.	4.6	93.05	67.95	42.20	4,617
8	Turbid	„	6.2	82.65	78.35	48.66	6,138
9	Clear	turbid	6.8	79.80	81.20	50.43	6,593
10	„	sl. turbid	7.4	75.53	85 47	53.09	7,305
10a	„	turbid	7.8	79.80	81.20	50 43	6,593
10b	„	clear	8.1	81.23	79.77	49.55	6,363
10c	„	„	8.8	84.08	76.92	47.78	5,616

The optimum reaction according to this experiment is pH 7.4, which

nearly coincides with the result of complete liquefaction of casein described in section V, C, 1, c. It is much more alkaline than the optimum reaction of amino acid production.

b. Determination of Formula applicable to the Reaction Velocity.

Digestion at 40°C. with different durations was carried out by the following procedure:— Same as above experiment with digesting liquid containing only No. 10 regulating mixture (pH 7.4), digesting periods being different. The results were calculated with monomolecular and SCHÜTZ's formulae. As the unit of digesting period, 2 hours were taken.

Table 56. Digestion of casein for different hours.

Hours digested	N in undig. casein (mgm.)	Digestibility $=x/a \times 100$	$Km \times 10^4$ $=1/t \log a/(a-x) \times 10^4$	$Ks \times 10$ $=x/\sqrt{t} \times 10$
1	118.27	26.54	2,679	604
2	98.33	38.93	2,141	648
3	53.36	48.22	1,907	631
5	59.14	63.27	1,740	644
20	9.98	93.80	1,208	478

The results show the constant of SCHÜTZ's formula to be much more uniform than that of the monomolecular formula. This conclusion coincides with the experiment of WITTE peptone digestion described in section V, C, 1, a.

c. Reaction Velocity with Different Quantities of Enzyme.

Instead of different digesting periods, the different concentrations of enzyme were tried. The digesting period was always 2 hours. For calculation, enzyme concentratian (=e) was put in the place of digesting period (=t) and the unit of the concentration was 0.1%.

Table 57. Digestion of casein with different quantities of enzyme.

Enzyme concn. (%)	N in undig. casein (mgm.)	Digestibility $=x/a \times 100$	$Km \times 10^4$ $=1/e \log a/(a-x) \times 10^4$	$Ks \times 10$ $=x/\sqrt{e} \times 10$
0.05	123.98	23.20	2,270	527
0.1	102.60	36.28	1,956	584

Enzyme concn. (%)	N in undig. casein (mgm.)	Digestibility $=x/a \times 100$	$Km \times 10^4$ $=1/e \log a/(a-x) \times 10^4$	$Ks \times 10$ $=x/\sqrt{e} \times 10$
0.20	74.10	53.97	1,685	615
0.40	47.74	70.35	1,320	567

Although the results are somewhat irregular, there is closer agreement with SCHÜTZ's than with the monomolecular formula.

d. Reaction Velocity with different digesting Temperatures.

The operation was the same as in "a" excepting that one regulating mixture only, no. 10 (pH 7.4), was used, and the digestion period was 2 hours at different digesting temperatures.

Table 58. Digestion of casein at different temperatures.

Digesting temperature (°C)	N in undigested casein (mgm.)	N in digested casein (mgm.)	Enzymic activity $E=x^2$
20	133.24	27.76	770
30	118.28	42.72	1,824
40	104.03	56.97	3.245
45	96.90	64.10	4,108
50	108.30	52.70	2,814
55	125.38	35.62	1,268
60	136.80	24.20	585
70	148.92	12.08	145

The optimum temperature of this experiment was 45°C. At 50°C., which is the optimum of WITTE peptone digestion, the casein digestion was decreased to about half of that at 45°C. It is possible that this difference is due to the difference in the hydrogen ion concentrations of the two digesting liquids.

3. Optimum Hydrogen Ion Concentration for Albumin Digestion.

In the experiments of section V, C, 1, c and V, D, 2, a, the optimum reaction of casein digestion with *Aspergillus* protease was much more basic than with that of other proteins. This was especially true in the experiments of casein liquefaction. The cause is supposed to be the nature of

casein and not the different optimum reactions between protein liquefaction and amino acid production. To confirm this supposition, egg albumin was tried for digestion. Albumin of Merck Co. was dissolved in water, made to 2% solution, and filtered through filter paper. A faintly milky solution was obtained.

a. By Amino Acid Production.

Procedure.

Five cubic centimeters of 2% albumin, 1 cc. of regulating mixture, and 2 cc. of 0.5% *Aspergillus* protease were mixed and kept in a hard glass test tube for 4 hours at 40°C., then placed for 10 minutes in a boiling water bath to stop digestion, after which amino nitrogen was determined by VAN SLYKE's method. Digestibility and enzymic activity were calculated by SCHÜTZ's formula. With the same digesting liquids, hydrogen ion concentration was determined by the electric method, before and after digestion. No obvious difference between the reactions before and after digestion was obtained.

a—Nt—Na=2.084 mgm. Total amino nitrogen (mgm.) capable of being produced from albumin in 2 cc. digesting liquid.

x=amino nitrogen (mgm.) in 2 cc. digested liquid.

Nb=nitrogen of blank test. =0.1 mgm.

Ne=0.06 mgm. Amino nitrogen produced by autolysis of enzyme solution only.

Nt=2.204 mgm. Amino nitrogen from the same quantity of albumin digested by strong HCl.

Na=0.094 mgm. Amino nitrogen contained in the albumin solution before digestion.

Table 59. Amino acid production of albumin at different pH.

No. of reg. mixture	pH	Appearance of liquid	x	Digestibility $=x/a \times 100$	$E \times 10^4 = x^2 \times 10^4$
1	3.6	clear	0.000	0.00	0
3	4.0	sl. turbid	0.167	8.01	278
5	4.3	„	0.374	17.94	1,399
6	4.4	„	0.339	16.27	1,149

No. of reg. mixture	pH	Appearance of liquid	x	Digestibility $=x/a \times 10$	$E \times 10^4 = x^2 \times 10^4$
7	5.0	clear	0.203	9.79	412
8	5.8	„	0.145	6.96	210
10	7.6	„	0.070	3.36	49
10 & 11 (0.5 cc. each)	8.1	„	0.041	1.97	17

b. By Albumin Liquefaction.

Procedure.

Four times the amount of digesting liquid of the same composition as before was used. After 4 hours digestion the reaction was regulated to pH 5.0 with N/1 HCl or N/1 NaOH, and the undigested albumin was coagulated by keeping it in a boiling water bath for 10 minutes.

After filtration the nitrogen content in the coagulant was determined by KJELDAHL's method. a=39.9 mgm.

Table 60. Liquefaction of albumin at different pH.

No. of reg. mixture	pH	N in undig. albumin (mgm.)	N in dig. albumin (mgm.)	Digestibility $=x/a \times 100$	$E \times 10 = x^2 \times 10$
1	3.6	35.6	4.3	10.85	185
3	4.0	31.4	8.5	21.30	723
5	4.3	27.7	12.2	30.58	1,488
6	4.4	29.9	10.0	25.06	1,000
7	5.0	31.4	8.5	21.30	723
8	5.8	34.2	5.7	14.28	324
10	7.6	35.6	4.3	10.77	185
10 & 11 (2 cc. each)	8.1	37.1	2.8	7.20	78
5		39.9	(without digestion =a)		

The results of the above two experiments show that the optimum reaction of albumin digestion is pH 4.3, either by estimation of amino acid production or albumin liquefaction. Therefore, it may be concluded that the optimum reaction of protein liquefaction or amino acid production is fundamentally the same. The author determined also the optimum reaction for the digestion of coagulated fresh egg white, which was pH 5.0 at both estimations.

E. Destructive Influences of Temperature and Hydrogen Ion Concentration upon *Aspergillus* Protease above and below Optima.

1. Influence of Temperature.

Procedure.

Tubes containing 20 cc. each of 0.2% *Aspergillus* protease solution (pH 7.29) were kept for one hour in a water bath at different temperatures, then put into cold water. The proteolytic (casein liquefying) activities were determined by the author's method (II, C).

Table 61. Proteolytic values of *Aspergillus* protease after one hour at different temperatures.

Temperature (°C.)	Proteolytic value
70	less than 1.0
65	„ 1.0
60	1.0
55	13.8
50	28.2
45	36.0
40	36.0
35	36.0

These results show that a temperature lower than 45°C. has no influence upon *Aspergillus* protease. At 50°C., there is a slight decrease, at 55°C. about half, and at temperatures over 60°C. all of the enzymic activity is lost. It must be remembered that the destructive action will be greater when the heating period exceeds one hour.

2. Influence of Hydrogen Ion Concentration.

Procedure.

To each 20 cc. of 0.2% *Aspergillus* protease solution were added 5 cc. of phosphate mixture to obtain various hydrogen ion concentrations. After keeping one hour at 15°C., accurate quantities of Na_3PO_4 and H_3PO_4 were added to make total phosphates always 3.7 cc. M/3 Na_3PO_4 and

6.3 cc. M/3 H_3PO_4, of which the pH is 5.2. The proteolytic activities were tested by the author's method. The hydrogen ion concentrations were determined electrometrically.

Table 62. Proteolytic values of *Aspergillus* protease after one hour at different pH.

0.2 % protease + M/3 Na_3PO_4 + M/3 H_3PO_4			pH	Proteolytic value
20 cc.	1.5 cc.	3.5 cc.	2.42	less than 2.8
„	1.8 „	3.2 „	4.14	19.3
„	1.85 „	3.15 „	5.25	37.4
„	2.0 „	3.0 „	5.96	„
„	2.5 „	2.5 „	6.41	„
„	3.0 „	2.0 „	7.04	„
„	3.3 „	1.7 „	8.42	26.3
„	3.5 „	1.5 „	9.58	8.7
„	4.0 „	1.0 „	11.90	less than 2.8

1. On the acid side, from pH 4.5–5.0 the protease begins to be destroyed and at pH 2.5 it is almost completely destroyed.

2. On the basic side, at pH 8.4 its activity begins to weaken and at pH 10 it is almost all lost.

3. Influence of Hydrogen Ion Concentrations at a High Temperature.

EULER and LAURIN (20) found that yeast saccharase is most stable against heat at the optimum reaction (=pH 4–5) of its action, whereas SJÖRBERG (88) asserts, in a recent publication, that the amylase of germinated *Phaseolus vulgaris* is most stable at pH 6.5–7.0, but the optimum reaction of its action is pH 5.0. This same question is here investigated with the protease of *Aspergillus oryzae.*

Procedure.

Same operation as above, except that the tubes were kept in a constant water bath at 55°C. for one hour.

Table 63. Proteolytic values of *Aspergillus* protease.

pH	Proteolytic value
2.42	less than 2.8
4.74	2.8
5.25	9.8
5.96	11.2
6.41	12.5
7.04	11.5
8.42	7.5
9.58	less than 2.8

The result shows that pH 6.4 is the most stable reaction, but that the optimum reaction of its action is pH 5.2 as already shown.

VI. Properties of Amylase from *Aspergillus Oryzae*.

Among the enzymes of *Aspergillus oryzae*, amylase is the best known. For instance, the famous medicine "Taka-diastase" invented by Dr. TAKAMINE, is distinguished as amylase, although it is really an alcohol precipitate of a culture extract of *Aspergillus oryzae*, and consequently contains almost every kind of enzyme produced by the mould.

In general, *Aspergillus oryzae* produces strong amylase and its existence is easy to test.

Many studies on the enzyme have been reported, but there are many contradictory results, owing to the inexactness of experimental methods, especially as few have, up to the present, made a particular study of hydrogen ion concentration of the digesting starch solution.

It is very well known that malt amylase produces maltose as the final decomposition product of starch. *Aspergillus* amylase produces glucose instead of maltose, which has been proved by ATKINSON (5), KELLNER (39) and NISHIZAKI (60). As an intermediate product it is likely to produce maltose. KITA (40) considered that some part of starch will be directly converted into glucose by this amylase.

A. Influence of Hydrogen Ion Concentration, Temperature, Sodium Chloride and Alcohol upon the Action of *Aspergillus* Amylase.

1. Influence of Hydrogen Ion Concentration on the Action.

The optimum hydrogen ion concentration of various amylases of different origins, which have been reported, are as follows:—

Amylase	Origins	Optimum pH	Remarks	Author	Reference
Amylase	saliva	6.4	40°C., 30 min., sol. starch→sugar	NORRIS	(62)
„	„	6.7	sol. starch→sugar	MICHAELIS and PECHSTEIN	(54)
Chloride-amylase	„	6.7	„	„	(„)
Nitrate-amylase	„	6.9	„	„	(„)
Phosphate-, sulphate- and acetate-amylases	„	6.1–6.2	„	„	(„)
Amylase	pancreas	7.0	40°C., 30 min., sol. starch→sugar	SHERMAN, THOMAS and BALDWIN	(87)
„	„	6.8		WILLSTÄTTER, WALDSCHMIDT-LEITZ and HESSE	(104)
„	„	7.1		HAHN, HARPUDER and MICHALIK	(34)
„	liver	6.9		HOLMBERG	(37)
„	crab fish (*Paralithodes camtschatica*)	6.2	40°C., 20 hrs., sol. starch→sugar	OSHIMA and KONDO	(76)
„	malt	4.4	40°C., 30 min., sol. starch→sugar	SHERMAN, THOMAS and BALDWIN	(87)
„	„	5.0	37°C., 20–60 min., sol. starch→sugar	EULER and SVANBERG	(19)
„	„	4.9	sol. starch→sugar	ADLER	(2)
„	„	4.6–5.2	„	LÜERS and WASMUND	(49)
„	„	3.9–4.7	starch→dextrin	WINDISCH	(105)
„	„	5.2	starch liquefaction	„	(„)
„	„	4.3	25°C., sol. starch→sugar	FINE	(24)
„	„	6.0	60°C., sol. starch→sugar	„	(„)
„	„	4.5	40°C., 30 min., starch liquefaction	OSHIMA and ITAYA	(71)
„	„	4.5	40°C., 30 min., sol. starch→dextrin	„	(„)
„	„	4.5–6.2	40°C., 30 min., sol. starch→sugar	„	(„)
„	cabbage, carrot and white turnip	6.0	„	FALK, MCGUIRE and BLOUNT	(22)
„	yellow turnip	4.0–7.0	„	„	(„)
„	*Phaseolus vulgaris* (leaf juice)	5.0–5.4	37°C., 20–60 min., sol. starch→sugar	SJÖRBERG	(89)
„	*Fraxinus excelsior* („)	5.0	„	„	(„)
„	*Picea abies* („)	5.0	„	„	(„)
„	*Pinus silvestris* („)	5.0	„	„	(„)
„	*Phaseolus multiflorus* („)	5.3	„	„	(„)

„	soy bean	5.2	40°C., 30 min., starch liquefaction	OSHIMA and ITAYA	(71)
„	„	5.2	40°C., 30 min., sol. starch→dextrine	„	(„)
„	„	4.8	40°C., 30 min., sol. starch→sugar	„	(„)
„	rye	4.5	40°C., 30 min., starch liquefaction	„	(„)
„	„	4.5	40°C., 30 min., sol. starch→dextrine	„	(„)
„	„	4.5	40°C., 30 min., sol. starch→sugar	„	(„)
„	wheat	6.5	40°C., 30 min., starch liquefaction	„	(„)
„	„	5.2	40°C., 30 min., sol. starch→sugar	„	(„)
„	germinated barn yard grass	4.5–5.2	40°C., 30 min., starch liquefaction	„	(„)
„	„	5.2	40°C., 30 min., sol. starch→dextrine	„	(„)
„	„	4.5–5.8	40°C., 30 min., sol. starch→sugar	„	(„)
„	germinated wheat	6.5	40°C., 30 min., starch liquefaction	„	(„)
„	„	5.2–5.8	40°C., 30 min., sol. starch→sugar	„	(„)
„	„	5.2–6 5	40°C., 30 min., starch liquefaction	„	(„)
„	„	5.2	40°C., 30 min., sol. starch→dextrine	„	(„)
„	„	5.2	40°C., 30 min., sol. starch→sugar	„	(„)
„	Taka-diastase (*Aspergillus oryzae*)	4.8	„	SHERMAN, THOMAS and BALDWIN	(87)
„	*Aspergillus niger*	3.5–5.5	starch liquefaction	FUNKE	(29)
„	*Rhizopus Delemar*	4.8	40°C., 30 min., starch liquefaction	OSHIMA and ITAYA	(71)
„	„	4.8	40°C., 30 min., sol. starch→dextrine	„	(„)
„	„	4,8	40°C., 30 min., sol. starch→sugar	„	(„)
„	*Rhizopus Japonicus*	5.2	40°C., 30 min., starch liquefaction	„	(„)
„	„	5.2	40°C., 30 min., sol. starch→dextrine	„	(„)
„	„	5.2	40°C., 30 min., sol. starch→sugar	„	(„)

It should be kept in mind that even with the same amylase, some deviation of optimum reaction is inevitable, owing to different digesting temperatures, duration of digestion, composition of regulating mixture in digest and impurity of enzymic preparation, etc.

The author has made many experiments with many kinds of amylase as already reported (67) (69) (71).

The essential results of these studies and some new experiments, in which a few corrections of former results are contained, will be described in this and in a later section.

a. Influence of Hydrogen Ion Concentration on the Starch-saccharifying Action at 40°C.

For test sample, 0.04% purified enzymic solution of *Aspergillus oryzae* was used. The mould was cultivated on steamed wheat bran in a sterilized flask, extracted with water and precipitated by 40% $(NH_4)_2SO_4$ solution. It was then dissolved in water, dialyzed a little, and reprecipitated by 70% alcohol. The precipitate was washed with alcohol and ether.

Procedure.

The author's method (II, A, 2), slightly modified, was used, 10 cc. of M/6 Na_2HPO_4 and M/6 citric acid being added to 100 cc. of 2% soluble starch solution to produce constant hydrogen ion concentrations. 10 cc. of 0.5 N NaOH solution were used to stop the digestion. The hydrogen ion concentrations were determined with MICHAELIS' colour standard (56) and the K type electric apparatus of Leeds and Northrup Co. (10).

Table 64. Starch-saccharifying values at 40°C. and different pH.

pH	Starch-saccharifying value
2.6	275
3.1	625
3.4	1,900
3.7	2,980
4.0	4,100
4.5	4,850
4.8	5,250
5.8	5,250

pH	Starch-saccharifying value
5.8	5,050
6.2	4,850
6.5	3,725
6.7	2,980
7.2	2,100
7.8	275

b. Influence of Hydrogen Ion Concentratian on the Starch-saccharifying Action at 20°C.

For test sample, 0.05% purified enzymic solution of *Aspergillus oryzae* was used. This preparation was different from that used in the previous experiment (VI, A, 1.), the $(NH_4)_2SO_4$ treatment being omitted.

Procedure was the same as the above excepting that the digesting temperature was 20°C.

Table 65. Starch-saccarifying value at 20°C. and different pH.

pH	Starch-saccharifying value
3.7	1,520
4.0	1,700
4.2	1,760
4.5	1,800
4.8	1,840
5.0	1,840
5.2	1,840
5.4	1,800
5.8	1,760
6.2	1,680

c. Influence of Hydrogen Ion Concentration on the Starch-liquefying Action.

Sample.

0.000625% purified enzymic solution from *Aspergillus oryzae* was used, the preparation being the same as last described.

Procedure.

New starch-liquefying method (H. B.) was followed.

Table 66. Starch-liquefying value (40°C.) at different pH.

pH	Starch-liquefying value
3.1	16,000
3.7	32,000
4.5	36,000
5.2	36,000
6.2	30,080
6.7	22,400
7.2	16,000

a. The optimum reaction of the starch-sacharifying action at 40°C. of *Aspergillus* enzyme is pH 4.8–5.2.

b. There is no remarkable difference of optimum reaction between digestions at 20°C. and 40°C. for the starch-saccharifying enzyme.

c. *Beyond the limit of pH 2.5–7.8, the starch-saccharifying enzyme* has almost no action.

d. Optimum reaction of the starch-liquefying action at 40°C. is pH 4.5–5.2.

c. Influence of Temperature on the Action.

Sample.

a. Crude *Aspergillus* amylase. *Aspergillus oryzae* cultivated on wheat bran, was extracted with toluol water, and the filtrate was used for the sample.

b. Purified *Aspergillus* amylase. The above filtrate was precipitated with four times its volume of strong alcohol, and the precipitate was washed with alcohol and ether. The dried powder was dissolved again in distilled water for use as the sample.

Procedure.

The author's method for starch-saccharifying enzyme, with a buffer solution of citric acid and phosphate mixture, to keep the digest as pH

5.0 was employed. Many water baths at different temperatures were used for the digestion.

Calculation of amylolytic value was based on one gram of dry precipitate for the purified amylase and one gram of original wheat bran for the crude amylase.

Table 67. Amylo-saccharifying value of purified and crude amylase at different temperatures.

Digesting temperature (°C.)	Starch-saccharifying value	
	Purified enzyme	Crude enzyme
20	1,700	44
30	2,975	84
35	3,750	103
40	4,100	127
45	4,450	164
50	4,450	178
55	3,425	178
60	2,100	119
65	900	49
70	330	19

1. There is a marked difference in the optimum temperatures of digestion between crude and purified *Aspergillus amylase*. That of the former is 50–55°C. but that of latter is 45–50°C. This may be due to the protecting action of impurities in the crude enzyme against the destructive influence of high temperature upon the amylase.

2. At the digestion of 20°C. for 30 min., both forms of amylase exhibited about one-third of the maximum activity, at 65°C. one-fourth and at 70°C. only a slight activity.

3. Influence of Alcohol on the Action.

"Sake" (rice wine) brewing is somewhat different from beer brewing, due to the simultaneous operation of saccharification and alcohol fermentation. In "shochu" (distilled alcoholic beverage) brewing with *Aspergillus oryzae*, the simultaneous method is preferable in practice to the method with malt. To determine the inhibiting effect of alcohol on amylase action, the following experiment was made.

Enzymic sample from *Aspergillus oryzae* was the same as section VI, A. 2, and the test was made by the author's method (II, A.), excepting that three digesting temperatures and various percentages of absolute alcohol were used with 2% soluble starch solution. Hydrogen ion concentration in the digests was kept always constant at pH 5.0 by a regulating mixture.

Table 68. Starch-saccharifying values at various contents of alcohol.

Alcohol (%)	At 40°C.	At 30°C.	At 20°C.
0	4,200	3,280	1,780
5	4,040	3,000	1,780
10	3,880	2,740	1,640
15	3,280	2,540	1,500
20	3,000	2,380	1,400
30	2,540	2,060	1,190
40	1,840	1,680	920
50	1,360	1,100	680

The addition of alcohol decreases the action of *Aspergillus* amylase at a rate almost equal in proportion to the concentration of added alcohol at 20–40°C.

4. Influence of Common Salt on the Action.

Influence of NaCl on the action of *Aspergillus* protease was examined in V, C, 2. The influence on amylase of *Aspergillus oryzae* is given here.

Procedure was as in section VI, A, 3, excepting that various per centages of NaCl were used without alcohol. The hydrogen ion concentration was kept constant at pH 5.0 with a regulating mixture.

Table 69. Starch-saccharifying values in different concentrations of NaCl.

NaCl (%)	At 40°C.	At 20°C.
0	4,200	1,840
2	3,880	1,680
5	3,280	1,520
10	2,740	1,360
15	2,380	1,180
20	1,840	980

Addition of NaCl weakens the action of *Aspergillus* amylase almost proportionately to the amount of NaCl added.

B. Examination of *Aspergillus* Amylase in Relation to its Reaction Velocity.

As described in the section dealing with the reaction velocity of *Aspergillus* protease (V, D, 1.) the reaction velocities of amylase of different origin agree well with the monomolecular formula. This was proved by EULAR and SVANBERG (19) with malt amylase and by SJÖRBERG (88) with the leaf juice of *Picea abies, Pinus silvestris Phaseolus vulgaris.*

The experiment with *Aspergillus* amylase shows agreement with the results referred to above:—

The method of EULER and SVANBERG (19) was followed. Three different concentrations of the enzymic solution from *Aspergillus oryzae* were used with phosphate mixture to keep the reaction of the liquid at pH 5.0 and digestion proceeded at 40°C. After 20, 40 and 60 minutes, the sugar formed was determined with 10 cc. of digest, and calculated by the monomolecular formula.

$$1/t \log \frac{a}{a-x} = K$$

Table 70. Reaction velocity of *Aspergillus* amylase.

No. of enzymic sample	After 20 min.		After 40 min.		After 60 min.	
	sugar (mgm.)	K x 10^5	sugar (mgm.)	K x 10^5	sugar (mgm.)	K x 10^5
1	20.8	837	36.0	877	45.2	860
2	29.8	1,332	44.2	1,237	53.6	1,260
3	24.2	1,011	39.8	988	49.0	1,013

N. B. t=period of digestion.
a=initial concentration of digesting substance.
x=quantity digested at the period "t".
K=constant of the formula.
For "a" 65.0 mgm. were taken. This was obtained by the digestion of the same solution as used in the experimet with the strongest amylase (No. 2) for 4 hours. The sugar formed was calculated as glucose.

From the above table, it is clear that the velocity constant "K" is

almost uniform throughout the digesting period.

C. Destructive Influence of Temperature and Hydrogen Ion Concentration upon *Aspergillus* Amylase.

1. Influence of Temperature.

Procedure.

In water baths of different temperatures, tubes containing 20 cc. each of 0.2% *Apergillus* enzyme (pH 7.29) were kept for one hour, then put into cold water and their amylolytic (soluble starch-saccharifying) activities determined by the author's method (II, A.).

Table 71. Starch-saccharifying values of *Aspergillus* amylase after 1 hour at different temperatures.

Temperature (°C.)	Starch-saccharifying value
70	less than 55
65	„ „ 55
60	119
55	2,975
50	3,750
45	4,100
40	4,100
35	4,100

This result shows that by heating for 1 hour in almost neutral solution, a temperature lower than 45°C. has no influence upon *Aspergillus* amylase. At 50°C. a slight decrease occurs, at 55°C. nearly half, and at over 65°C. all of the enzymic activity was lost. Longer heating has stronger destructive action upon the amylase, but at a temperature lower than 40°C. at about neutral reaction, it can keep its activity almost constant for years as shown by the following experiment:—

One barrel of "Polyzyme" (a commercial preparation of *Aspergillus* enzyme disinfected with phenol, with almost neutral reaction) was kept in a storehouse of the Takamine Laboratory Inc., Clifton N. J., U. S. A., from Feb. 1919 to May 1921. During Summer the temperature in the store house rose sometimes as high as 38°C. The starch-saccharifying value

of the "Polyzyme" in the barrel was tested three times with the following results:—

When stored	After 9 months	After 27 months
102	98	98

2. Influence of Hydrogen Ion Concentration.

Procedure.

To 20 cc. of 0.2% *Aspergillus* enzyme were added 5 cc. of phosphate mixture to produce various hydrogen ion concentrations. After standing at 15°C. for 1 hour, to each tube were added 3.7 cc. M/3 Na_3PO_4 and 6.3 cc. M/3 H_3PO_4, which made pH 5.2. The amylolytic activities were tested by the author's method. Precaution was taken to retain enough phosphates in 2% soluble starch solution to keep pH 5.2 exactly, and N/2 NaOH was used to stop digestion. The hydrogen ion concentrations were determined electrometrically.

Table 72. Starch-saccharifying value of *Aspergillus* amylase after 1 hour at different pH.

0.2% *Aspergillus* enzyme	M/3 Na_3PO_4 (cc.)	M/3 H_3PO_4 (cc.)	pH	Starch-saccharifying value
20 cc.	1.5	3.5	2.42	830
„	1.8	3.2	4.14	4,370
„	1.85	3.15	5.25	5,620
„	2.0	3.0	5.96	„
„	2.5	2.5	6.41	„
„	3.0	2.0	7.04	„
„	3.3	1.7	8.42	3,850
„	3.5	1.5	9 58	680
„	4.0	1.0	11.90	less than 495

The results show that by keeping one hour at pH 4.5–5.0 at room temperature, *Aspergillus* amylase begins to be destroyed and at pH 2.5 it is almost entirely destroyed. At pH 8.4 its activity begins to weaken and at pH 10 it is almost nil.

3. Influence of Hydrogen Ion Concentration at a High Temperature.

Procedure.

The same operation as above was carried out except that the digesting mixtures were kept in a constant water bath at 55°C. for one hour.

Table 73. Starch-saccharifying values of *Aspergillus* amylase after 1 hour at 55°C. and different pH.

pH	Starch-saccharifying value
2.42	less than 248
4.74	248
5.25	584
5.96	1,538
6.41	2,206
7.04	2,014
8.42	420
9.58	less than 248

From the above results, it is indicated that at pH 7.4 the amylase is most stable, but that pH 4.5–5.2 is the optimum reaction for its activity.

N. B. The above experiments on *Aspergillus* amylase were worked out before the end of 1925. Later S. SUGIYAMA and K. MATSUSHITA[1] reported a study on the reaction velocity of starch-saccharifying action of Taka-diastase with the following principal results:

1. The optimum temperature zone for the action of the amylase was between 30°C. and 60°C. with the optimum point near 55°C.

2. The optimum zone of hydrogen ion concentration for the action was between pH 3.5 and pH 6.0 with the optimum point near pH 4.8.

K. MATSUMOTO and K. KUBOTA[2] also obtained pH 5.0 as the optimum hydrogen ion concentration of starch-saccharifying action of Taka-diastase and pH 4.0–4.4 as that of "koji" for "sake" brewing. They made these digestions at room temperature.

Quite recently H. L. MASLOW and W. C. DAVISON[3] reported their study on *Asper-*

1.) S. SUGIYAMA and K. MATSUSHITA: On the reaction velocity of saccharifying action of Taka-diastase (in Japanese). Report of Brewing Institute, Japan, No. 93, March (1925).

2.) K. MATSUMOTO and K.K UBOTA: The optimum hydrogen ion concentration of "koji" diastase (in Japanese). Report of Brewing Institute, Japan, No. 94, March (1926).

3.) H. L. MASLOW and W. C. DAVISON: A comparison of the viscometric, copper reduction, polariscopic and iodometric methods for measuring the rate of hydrolysis of starch and dextrin by *Aspergillus oryzae*. The effect of the hydrogen ion concentration upon the starch-liquefying activity of the amylase of *Aspergillus oryzae*. The effect of the hydrogen ion concentration upon the dextrin-liquefying activity of the dextrinase of *Aspergillus oryzae*. J. Biol. Chem., 68, p. 75-99 (1026).

gillus amylase (using Taka-diastase). Their principal conclusions were as follows:

1. The optimum reaction for the starch-liquefying activity of *Aspergillus* amylase was pH 3.0 and the limits of activity were between pH 2.0 and pH 9.0 when determined with unbuffered starch solutions by the viscometric method at 54°C. The optimum was at pH 4.0 when the starch solutions were buffered with 0.5 N universal buffer.

2. The amylase was completely destroyed at pH 1.0 and 2.0 with unbuffered starch and pH 2.0 and 3.0 with starch buffered with 0.5 N universal buffer.

3. The optimum reaction for the dextrin-liquefying activity of the dextrinase of *Aspergillus oryzae* was pH 4.0 when determined by the viscometric method at 34°C.

4. The dextrinase was completely inhibited at pH 2.0.

Some of the above results coincide with those of the author, and no material contradictory conclusion was noted.

VII. Disinfectants for preserving the Amylase Soluton of *Aspergillus Oryzae*.

Preservation of an enzymic solution is always a difficult problem. For instance, the activities of solutions of malt amylase, pepsin or trypsin are gradually weakened by keeping at room temperature, although no putrefaction has taken place. The enzymic solution from *Aspergillus oryzae* is fortunately quite stable. It can be preserved for years without any weakening if kept free from bacterial infection under 40°C., at about neutral reaction. Without antiseptics, however, bacterial infection is inevitable. Moreover the usual antiseptics are mostly destructive of enzymes, toluol being the only reliable preservative in laboratory experiments, but, unfortunately, on a large scale it is not suitable, as it floats on the surface, and is therefore not sufficient to keep the whole solution sterile. It is also expensive.

For desizing textiles and in many other industrial processes, it is often convenient and economical, to use an enzymic solution which is easily obtained by extraction from cultures of *Aspergillus oryzae*. For malt amylase, success may be obtained only by concentration of malt extract in a vacuum pan, but for the enzymic solution from *Aspergillus oryzae* this method is not so satisfactory, owing to its small content of sugar, which probably acts as a preservative in malt amylase. Formaldehyde has also been used as a preservative, but this weakens enzymic activity, and the disinfecting action is not complete.

No systematic study of this problem has been reported, but, for the development of the technical application of *Aspergillus* enzyme, such study is most important. The following investigation can be said to be almost a complete settlement of this problem.

Experimental.

Enzymic solution.

Aspergillus oryzae was cultivated on steamed wheat bran for two days at 30°C., then extracted with water. The filtrate had approximately the following composition:—

Total dry matter	12.5%
Ash	1.5%

Acidity, with rosolic acid as indicator: 10 cc. of the solution was neutralized with 5 cc. 0.1 N NaOH.

Total nitrogen	0.5%
Reducing sugar (as glucose)	2.5%
Starch-saccharifying value (author's method)	120

Anticeptics.

Chemicals of U. S. Pharmacopoeia used, as from their use in connection with other work, there seemed some probability that they would prove suitable preservatives for these experiments.

Procedure.

To 50 cc. of the enzymic solution were added 10 cc. of the antiseptic solution (or 10 cc. water with antiseptic) of a strength graduated to give the per centage of the whole solution as required and as indicated in the table below. This was put in brown coloured bottles of 125 cc. capacity, corked loosely and kept at room temperature. Starch-saccharifying activity alone was tested to determine the change of enzymic activities. The author's method (II, A) was followed, and calculation of the amylolytic value was based on 1 cc. of the solution.

Table 74. Starch-saccharifying values of the enzymic solution with antiseptics after 24 hours.

Antiseptics	Per cent in enzymic solution							
	4.0 %	2.0 %	1.0 %	0.5 %	0.25 %	0.1 %	0.05 %	0.025 %
Chloroform	—	—	110	110	110	110	—	—
Chloral	—	—	,,	,,	,,	,,	—	—

Antiseptics	Per cent in enzymic solution							
	4.0 %	2.0 %	1.0 %	0.5 %	0.25 %	0.1 %	0.05 %	0.025 %
Clove oil	—	—	110	110	110	110	—	—
Cresol	—	—	,,	,,	,,	,,	—	—
Formalin (40%)	85	94	105	,,	,,	,,	—	—
KCN	—	—	—	,,	,,	,,	—	—
$HgCl_2$	—	—	25	33	69	81	91	110
Lysol	—	110	110	110	—	—	—	—
NaF	—	,,	,,	,,	110	110	—	—
Na-benzoate	—	,,	,,	,,	,,	,,	—	—
Phenol	34	100	,,	,,	,,	,,	—	—
Salicylic acid	—	—	33	100	100	100	—	—
Thymol	—	—	—	110	110	110	110	110
Toluol	—	105	110	,,	,,	,,	—	—
Xylol	—	—	,,	,,	,,	,,	—	—

The above results indicate the destructive effect of antiseptics.

After this test, 5 cc. of putrefied (with many microbes) and neutralized enzymic solution of *Aspergillus oryzae* were added to each bottle. The putrefied enzymic solution was made by exposing a normal sample to the open air at room temperature. This was kept again at room temperature and after one week the enzymic strength was tested. This would indicate the disinfecting and destructive effects of antiseptics.

Table 75. Starch-saccharifying values of the enzymic solutions with antiseptics after one week.

	4.0 %	2.0 %	1.0 %	0.5 %	0.25 %	0.1 %	0.05 %	0.025 %
Chloral	—	—	105	81	69	55	—	—
Chloroform	—	—	,,	105	105	95	—	—
Clove oil	—	—	,,	,,	,,	105	—	—
Cresol	—	—	,,	,,	,,	60	—	—
Formalin (40%)	42	64	81	85	70	,,	—	—
KCN	—	—	—	95	85	51	—	—
$HgCl_2$	—	—	20	28	46	60	51	21
Lysol	—	105	105	105	—	—	—	—
NaF	—	,,	85	73	68	60	—	—
Na-benzoate	—	—	100	95	81	,,	—	—
Phenol	4	100	105	105	,,	,,	—	—

	4.0 %	2.0 %	1.0 %	0.5 %	0.25 %	0.1 %	0.05 %	0.025 %
Salicylic acid	—	—	25	81	100	51	—	—
Thymol	—	—	—	91	102	105	105	13
Toluol	—	—	105	105	64	50	—	—
Xylol	—	—	„	„	51	46	—	—

One test without antiseptic=69.

From the above two experiments, cresol, chloroform, clove oil, phenol, lysol, thymol and toluol were found to be useful as disinfectants, but in other experiments on a larger scale, toluol, chloroform and clove oil were shown to be less effective owing to their slight solubility.

Experiments were continued with 4 other chemicals, and the effect of mixtures of phenol and cresol was examined.

The procedure was the same as before.

Table 76. Change of starch-saccharifying value of enzymic solution in the presence of phenol and cresol.

Phenol + cresol	After 24 hours	After 2 days	After 14 days
0.5% 0.25%	105	100	100
0.5 „ 0.5 „	„	„	98
1.0 „ 0.25 „	„	105	95
1.0 „ 0.5 „	100	95	87
1% toluol (contrast)	105	105	105

The results above described indicate the following kinds and quantities of antiseptics as suitable for preserving this enzymic solution:—

Antiseptic	Suitable concentration in the enzyme solution
Cresol	0.15-0.40%
Lysol	0.5 - 2.0%
Phenol	0.4 - 1.5%
Thymol	0.05- 0.20%
Phenol + cresol	0.3% P.+0.1% C.—1.0% P.+0.2% C.

These methods of preservation are now used at the Takamine Labratory Inc., Clifton, N. J., U. S. A., and prove very effective.

(Appendix) Relation between Hydrogon Ion Concentration and Quantity of NaOH or HCl in Soluble Starch and WITTE Peptone Solutions.

A. Experiment with soluble starch.

To 21.60 grams of soluble starch (20 grams as dry matter) from Eimer & Amend Co. and about 100 cc. water were added to 700 cc. boiling water. This was kept for ten minutes in a boiling water bath, cooled down and made up to one litre. The distilled water used proved of a very high purity. The pH values of the starch solutions, containing varying quantities of alkaline solution, were determined.

The standard solutions for CLARK's colorimetric method (10) were loaned by Dr. BERG of the Bureau of Animal Industry in Washington, D. C. For comparison, Dr. ZOLA of the same Bureau kindly determined some of these values with his delicate electric apparatus. The results by the two methods showed slight differences due probably to the use of starch solutions made at different times.

Table 77. Hydrogen ion concentrations of soluble starch solution with different quantities of NaOH.

N/20 NaOH in 100 cc. of 3% soluble starch solution (cc.)	pH	
	By electric method (30°C.)	By colorimetric method
0.0	3.91	3.8
0.1	4.16	4.1
0.2	4.43	4.3
0.4	—	5.2
0.8	6.54	6.5
1.2	—	7.6

Such high acidity of soluble starch is not extraordinary, when made by HCl treatment.

In the next experiment, 1,000 cc. of 2% soluble starch solution were made, which contained 12 cc. N/20 NaOH.

The results were:—

Table 78. Hydrogen ion concentrations of soluble starch solution with different quantities of HCl.

N/20 HCl in 100 cc. of 2% soluble starch solution (cc.)	pH by colorimetric method
0.0	7.5
0.4	6.4
0.8	5.2
1.2	3.7

B. Experiment with WITTE Peptone.

To 10 grams of air-dry WITTE peptone 450 cc. water were added and kept in a boilling water bath for 5 minutes, cooled down, made up to 500 cc. and filtered through filter paper. Another WITTE peptone solution was made with 3 mole NaCl solution, instead of using distilled water. The pH values were determined by CLARK's colorimetric method.

Table 79. Hydrogen ion concentrations of WITTE peptone solution with different quantities of HCl.

N/20 HCl for 100 cc. of 2% WITTE peptone solution (cc.)	pH	
	Of water solution	Of 3 M NaCl solution
0.0	6.9	7.5
4.0	6.7	7.0
8.0	6.4	6.5
12.0	5.9	5.9
16.0	5.3	5.5

This result shows that there was no great difference of pH value between WITTE peptone solutions in distilled water and in 3 mole salt solution.

A remarkable difference of pH value was, however, observed between WITTE peptone and soluble starch solution. For instance, to reduce the

pH value from 7 to 9, 0.4 cc. of N/20 HCl was sufficient for 100 cc. of 2% soluble starch solution, but 30 times this quantity was necessary for 100 cc. of 2% WITTE peptone in water solution.

This fact may be of much use in the study of the influence of acid or alkali in starch or peptone solution on enzymic digestion.

VIII. General Summary.

A. Methods used for quantitative estimation of amylase and protease, and precaution in use of VAN SLYK's method.

1. Starch-saccharifying enzyme can be estimated easily, quickly and accurately by the new method, which consists in testing reduction of FEHLING's solution and finding the enzymic activity from a prepared scale.

2. To estimate starch-liquefying enzyme, the viscosity change of starch paste is measured with OSTWALD's viscosimeter and the enzymic activity is determined from a prepared scale.

3. A protease, which acts near neutral reaction, is estimated by casein liquefaction. The undigested casein is found as a precipitate by using a mixture of nitric acid and magnesium sulphate.

4. VAN SLYKE's apparatus of α-amino nitrogen determination causes remarkable errors, due to the use of toluol as antiseptic.

B. Conditions influencing the enzyme production of *Aspergillus oryzae*.

1. The exo- (extra-cellular) and endo- (intra-cellular) enzymes of *Aspergillus oryzae* are fundamentally the same. After spore formation almost all endo-enzyme is excreted into the culture. The maximum amount of endo-enzyme is much less than that of exo-enzyme. The increase and decrease in quantity of endo-enzyme is quite rapid but of exo-enzyme is slow.

2. Amylase and protease of *Aspergillus oryzae* are produced simultaneously on one medium in the same ratio, and there is no decrease in quantity when kept for a long period, if other injurious conditions are avoided.

3. Enzyme production from *Aspergillus oryzae* on artificial culture media is stimulated more efficiently by substances of closely related chemical structure, which can be decomposed by enzyme, than by substances not

closely related. For natural culture media this conclusion is, however, not generally true, as even on soy beans which contain no starch or dextrin, the mould produces strong amylase.

4. Amylase and protease from about one hundred *Aspergillus* species and strains were compared by cultivating on two standard media. There are great variations in the activities of enzymes according to the mould and the classification of strains is often difficult from the morphological side, but may be possible by the determination of the amylolytic and proteolytic values.

C. Properties of *Aspergillus* protease.

1. Proteolytic enzyme produced from *Aspergillus oryzae* can digest natural proteins, such as beef protein, glycinin, edestin and casein, as well as WITTE peptone, and produce as much amino acid as trypsin.

2. The production of tryptophane was proved during casein digestion. In this and the properties mentioned in the preceding paragaph the protease more closely resembles trypsin than pepsin.

3. Little ammonia was produced, and it is considered that *Aspergillus oryzae* does not secrete any ammonia forming enzyme.

4. At 40°C. this enzymic solution at about natural reaction will keep its activity for at least 9 days, whereas trypsin loses its activity.

5. Optimum hydrogen ion concentrations of protein digestion by this protease are as follows:—

Protein	*Product*	*Optimum pH*	*Remarks*
Edestin	amino acid	5.2	digestion at 40°C., 4 hrs.
Casein	„	6.2	„
„	liquefaction	7.4–8.0	digestion at 40°C., 2–4 hrs.
Glycinin	amino acid	uncertain	dig. at 40°C., 4 hrs. water soln.
„	„	5.6	„ in M/1 NaCl
Albumin	„	4.3	digestion at 40°C., 3 hrs.
„	liquefaction	4.3	„
WITTE peptone	amino acid	5.3	„

These optimum reactions are the combined results of (a) optimum reaction of the proteolytic action itself and (b) the easiest reaction of digestion of used protein. For example, casein is precipitated considerably at pH 5.0 and is hard to digest, but at pH 7–8 it dissolves easily. Therefore the optimum reaction of the liquefaction is pH 7.8–8.0, whereas that of amino acid production is pH 6.2. Eliminating any exceptional influence

of individual proteins on the digesting reaction, pH 5.2–5.3 may be considered the optimum reaction for the protease action. Almost the same hydrogen ion concentration is the optimum for amylase action of the same origin.

6. The protease shows no digesting action in a liquid having a pH value of less than 3.5 or over 9.0.

7. The reaction velocity of this protease, in the experiments with WITTE peptone and casein, follows approximately SCHÜTZ's formula, but not the monomolecular formula, i. e., the quantity of digestion products is proportional to the square root of the digesting period or enzyme quantity used. The saccharification by amylase of the same origin has, however, the reaction velocity of the monomolecular formula.

8. The optimum temperature of the enzymic action is at 50°C. Above 60°C., it shows very weak action in the digestion of casein or WITTE peptone for 2–4 hours.

9. The destructive effect of heat on the enzyme is demonstrated. At one hour's heating at temperatures below 45°C. there is no effect, but at 50°C. the destruction is 50%, and at more than 60°C. the destruction is almost complete.

10. As to the destructive effect of hydrogen ion concentration on enzymic activity—after one hour at 15°C. at pH 4.1 destruction commences; at pH 2.4 it is almost complete on the acid side. On the basic side, destruction begins at pH 8.4 and is nearly complete at pH 9.5.

11. The stability of the protease against the destructive influence of high temperature is the greatest at pH 6.4.

12. The protease compared with the amylase of the same origin appears to be a little more sensitive to the destructive effect of hydrogen ion concentration.

13. The addition of common salt to the digesting liquid always decreases the digestibility of WITTE peptone proportionally to the concentration of common salt, but the digestibility of glycinin is increased by a small quantity of common salt, as the salt keeps glycinin in solution.

14. The digestion of cooked glycinin is always better than of uncooked glycinin, and cooking for 10 minutes at one atmospheric pressure is better than longer cooking at higher pressure.

D. Properties of *Aspergillus* amylase.

1. *Aspergillus* amylase is a little more resistant than the protease of

the same mould to the destructive effects of hydrogen ion concentration and of temperature.

2. One hour's heating of the enzyme in about neutral solution at a temperature below 45°C. has no effect upon the amylase. At 50°C. the activity is a little reduced, at 55°C. it is reduced nearly half and at more than 65°C. all the enzymic activity is lost. More prolonged heating has greater destructive action, but at a temperature lower than 40°C. at almost neutral reaction, the amylase undergoes no change, even after one year, provided it is protected from other injurious action.

3. At pH 4.5–5.0 for one hour temperature, destruction of the amylase begins, and at pH 2.5 it is almost entirely destroyed; at pH 8.4 its activity also begins to decrease and at pH 10 almost all is lost.

4. For heating the amylase solution, pH 6.4 is the most stable reaction, but the optimum reaction for its activity is pH 4.8–5.2.

5. The optimum reaction for the amylo-saccharifying action is pH 4.8–5.2 and that for the amylo-liquefying action is almost equal.

6. The reaction velocity of the amylase nearly follows the monomolecular formula.

7. The inhibiting effect of alcohol and common salt on the amylase action is almost proportional to their concentration.

8. Between crude and purified *Aspergillus* amylases remarkable differences of optimal temperatures for starch digestion are observed. The optimum temperature for 30 minutes digestion lies between 50 and 55°C. for crude amylase and for the purified between 45 and 50°C.

9. As disinfectants to preserve the extracted solution of *Aspergillus oryzae*, the following chemicals and concentrations are selected as the best.

Antiseptic	*Suitable contents in the enzymic soln.*
Cresol	0.15–0.40%
Lysol	0.5 –2.0 %
Phenol	0.4 –1.5 %
Thymol	0.05–0.20%
Phenol+cresol	0.3% P.+0.1% C.—1.0% P.+0.2% C.

10. Although the protease and amylase from *Aspergillus oryzae* act best in the digesting liquid at the same hdyrogen ion concentration, there is a very considerable difference between protein and starch solution without regulating salts in the quantity of acid and base.

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